A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Identification and characterization of juvenile hormone antagonist-based insecticidal substances from *Streptomyces abikoensis*

곤충 유충호르몬 저해 활성 기반의 방선균 ***Streptomyces abikoensis*** 유래 살충 활성 물질의 동정과 특성 구명

By

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Major in Entomology

Department of Agricultural Biotechnology

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UNDER THE DIRECTION OF ADVISER YEON HO JE
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ABSTRACT

Mosquitoes transmit deadly pathogens which cause great dangers to the human population. Although neurotoxic insecticides have been used to control mosquitoes, available insecticides are decreasing due to their environmental toxicity and the development of insecticide resistance. Moreover, increasing global temperature accelerates the spread of the mosquito population.

Insect growth regulators (IGRs) with juvenile hormone agonist (JHA) activity such as pyriproxyfen and methoprene disrupt the actions of juvenile hormone and cause mortality to target insects. These are considered alternatives to conventional insecticides due to their high specificity and low toxicity. However, according to recent studies, resistant populations against pyriproxyfen and methoprene were reported. Thus, developing insecticides with a novel mode of action is needed. Actinomycetes produce numerous secondary metabolites, including insecticidal compounds, so it was thought that new IGR-active substances could be found from actinomycetes.

In this study, 232 actinomycetes were screened for their juvenile hormone antagonist (JHAN) and mosquitocidal activities. Four strains showed JHAN and mosquito larvicidal activities while exhibiting no acute toxicity against adult guppies. Among them, *Streptomyces abikoensis* IMBL-1939 strain showed the highest JHAN and mosquito larvicidal activities. Main active compounds were found in n-hexane extract of IMBL-1939 strain and isolated through bioassay-guided fractionation. It was identified as carbazomycin A by mass spectrometer and NMR spectrum data. Carbazomycin A exhibited high larvicidal activity with an LC_{50} value of 4.8 ppm after 48 h against the 3rd instar of *Aedes*

albopictus mosquitoes. In addition, carbazomycin A reduced the juvenile hormone (JH) triggered biting behavior and ovary development rate, and decreased the transcription of juvenile hormone-related genes such as *Kr-h1* and *Hairy*. In addition, carbazomycin A showed relatively low toxicity to non-target organisms such as silkworms and honeybees.

To optimize carbazomycin productivity, Plackett-Burman design was applied to evaluate the variables affecting the production of carbazomycin A and B. Several variables were identified as significant factors affecting carbazomycin production and further optimized using a central composition design. It was confirmed that the yield of carbazomycin A was increased by 20% through the optimized medium. Based on lab-scale optimization data, pilot culture and extraction processes were carried out, culture conditions for pilot culture using a fermenter were established, and elevated production of carbazomycin was observed.

In addition, the production of carbazomycin A using chemical synthesis has proceeded. Carbazomycin B, produced larger than carbazomycin A in *Streptomyces* culture, was converted to carbazomycin A through base methylation with 94% yield. Methylation could be helpful in product carbazomycin A in mass culture. Also, the reported total synthesis pathway of carbazomycin A has been improved for mass production.

Finally, n-hexane extract of culture filtrate of the IMBL-1939 strain was formulated as a wettable powder (WP) with a 1% main ingredient for practical applications. WP showed much higher mosquito larvicidal activity than the same concentration of n-hexane extract. In semi-field conditions, the WP showed about 95% mortality at a concentration of 200 ppm. These results suggested that carbazomycin A and extract of the *S. abikoensis* IMBL-1939 could be effectively developed as an eco-friendly insecticide to control mosquitoes.

Key words: actinomycetes, insect growth regulators, juvenile hormone antagonists, *Aedes albopictus*, carbazomycin A

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INTRODUCTION

Pests transmit various infectious pathogens. Especially mosquitoes are the vector of arboviruses such as chikungunya, yellow fever, dengue fever viruses, and parasites like malaria (World Health, 2020). These vector-borne diseases cause over 0.7 million death per year. In 2015, 212 million malaria infections were reported, resulting in 429,000 deaths (Organization, 2016, 2020). The number of deaths caused by malaria keeps decreasing due to various global efforts to eradicate malaria.

Vector control is the primary countermeasure to eradicate vector-borne diseases for public health (Benelli & Beier, 2017; Wilson et al., 2020). Currently used mosquito larvicides are classified into organophosphates (OPs), bacterial insecticides, insect growth regulators (IGRs), oils, and films. Among them, IGRs (methoprene, pyriproxyfen, and diflubenzuron) and bacterial insecticides (*Bacillus thuringiensis isrealensis* (*Bti*), and spinosad), and natural products with relatively low environmental toxicity are mainly used in the USA. Conversely, OPs such as temephos are gradually banned due to environmental toxicity issues in Florida, USA (Lloyd, 2018).

In Korea, the market size of mosquitocides was 23.15 billion won in 2020, of which the larvicides market was 5.025 billion won, accounting for about 22%, while diflubenzuron (IGR, 8.3%), temephos (OP, 4%), *Bti* (2.8%), dinotefuran (neonicotinoid, 0.8%), spinosad (0.7%), and methoprene (IGR, 0.5%) (KCDA, 2018). Including adulticides, the use of chemical pesticides such as temephos, which are relatively toxic to the environment, was more than 90%.

Environmental problems and bioaccumulation of chemical insecticides became serious due to their high toxicity (Md Meftaul et al., 2020). Moreover, vector resistance to mainly used insecticides, including pyrethroids, organochlorides, carbamates, and organophosphates, was detected in every region except the EU. Especially resistance populations to most used adulticides, pyrethroids are progressively emerging (Auteri et al., 2018; Benelli & Mehlhorn, 2016) (Cuervo-Parra et al., 2016).

In addition, climate anomalies and the increasing temperature increase the abundance of mosquitoes and shorten the development of pathogens and vectors. Due to higher replication rates, the evolution of pathogen and vector will accelerate (Nosrat et al., 2021; Rocklöv & Dubrow, 2020; Ryan et al., 2019; Yi et al., 2014).

Research on insect growth regulators (IGRs) has begun as an alternative to environmental toxicity and resistance problems. IGRs were considered difficult for insects to acquire resistance due to their mode of action that disrupts insect hormone and development. In addition, IGRs are considered eco-friendly insecticides because of their high specificity to target insects and low toxicity to non-target organisms (Dhadialla et al., 2009; Williams, 1956).

JH has been regarded as a target for novel pesticides because juvenile hormone regulates metamorphosis, development, reproduction, and overall physiological regulation (Riddiford, 2008). Juvenile hormone agonists (JHAs), which mimic the action of juvenile hormones, such as methoprene and pyriproxyfen, were used to control mosquito larvae due to their low environmental toxicity and high specificity to the target insects (Henrick, 2007; Invest & Lucas, 2008). However, in recent studies, pests have developed resistance to methoprene and pyriproxyfen (Su et al., 2021; Su & Trdan, 2016). In mosquitoes, resistance

populations have been reported in areas that used methoprene in the long term over ten years (Lawler, 2017). In addition, the toxicity of pyriproxyfen to non-target aquatic invertebrates and fish is also reported (Maharajan et al., 2018). Therefore, acquiring resistance to JHAs will be a matter of time, and novel pesticides need to be developed.

JH receptor methoprene-tolerant (Met) and its mode of action were reported through the studies of JH signaling. Met dimerizes with its partner protein (Tai/FISC/SRC) in a JH-dependent manner and works as a transcription factor of JH response elements (JHREs) (Charles et al., 2011; Jindra et al., 2015; Jindra et al., 2013; Lee et al., 2015).

In previous studies, *in vitro* JH screening system using the signaling pathway of JH and a yeast-two hybrid system was developed (Lee et al., 2015). Through the high throughput screening system, it has become possible to indirectly screen massive amounts of JHAs and JHANs without a direct investigation. Chemicals (Lee, Lim, et al., 2018), plant extracts (Lee et al., 2017), plant essential oils (Lee, Ha, et al., 2018; Park et al., 2020), fungi (Woo et al., 2020), and actinomycetes (Kim et al., 2020) were screened for their IGR activity based on JH action.

Actinomycetes produce 45% of discovered bioactive substances, including antibiotics, enzymes, antifungal agents, and herbicides, derived from whole microbes, during the stationary phase of their growth (Harir et al., 2018; Mahajan & Balachandran, 2012; Watve et al., 2001). Over 80 years, actinomycetes are still the primary source of various metabolites. Among them, insecticidal activity of actinomycetes has been reported, and many insecticidal substances such as avermectin, prasinons, doramectin, milbemycin, tetranectin, and antimycin have been identified (Vurukonda et al., 2018). Some showed insect antifeedant and growth inhibitory activities (Arasu et al., 2013). Antimycin

derivatives derived from *Streptomyces sp*. AN120537 showed JHAN and insecticidal activities against larvae of *Plutella xylostella* and *Aedes albopictus* (Kim et al., 2020).

Secondary metabolites of microorganisms are widely used in pharmacology, food, and agriculture industries. Therefore, mass production of secondary metabolites and process optimization have become important in the industry, and various methods are being developed through metabolic engineering (Robertsen et al., 2018). Traditionally, statistical methodologies have been used to optimize the production of secondary metabolites (Kim et al., 2016). The composition of the medium has a very significant influence on the production of secondary metabolites. However, the optimization of the medium is complicated by numerous variables and combinations of nutrients.

Many variables can be analyzed with reduced experiments through the design of experiments (DOE) approach. The Plackett-Burman design can screen key factors from multiple variables with minimum experiments. Further optimization of significant variables that affect the production of metabolites, response surface methodology (RSM) could be used for finding optimal concentration. Recently, statistical methods have been used in many optimizations to produce secondary metabolites (Gao et al., 2009; Singh & Rai, 2012).

It was expected that actinomycetes would produce IGR compounds by previous reports. Therefore, in this study, to find IGR compounds, culture filtrates of actinomycetes were screened for their IGR activities through an *in vitro* IGR screening system. IGR compounds with JHAN activity were isolated from actinomycetes and identified through structural elucidation, and their characteristics have been evaluated. In addition, production optimization of JHAN compounds by the DOE approach was carried out. Also, the

formulation proceeded, and the effects were confirmed to evaluate the applicability of IGR pesticides. These mosquitocidal compounds with JHAN activity are expected to be used as novel insecticides for resistance and environmental problems in mosquito control.

LITERATURE REVIEW

1. Juvenile hormone

Juvenile hormones (JH) are acyclic sesquiterpenoids produced at corpora allata in insects. JH has multiple effects during the life cycle of insects in metamorphosis, development, diapause, reproduction, caste determination, and polyphenism (Klowden, 2013).

The existence of JH was discovered as an 'inhibitory hormone' in blood-sucking Hemiptera, *Rhodnius prolixus* (Wigglesworth, 1936). The last instar nymphs of *R. prolixus* which implanted corpora allata of the second or third instar nymphs, failed to metamorphosis and became supernumerary nymphs. This suggested that hormones secreted from corpora allata regulated metamorphosis and molting. Later, many studies were conducted on the function of JH. Williams (1956) isolated active juvenile hormonal extracts from Cecropia silkworm, *Platysamia cecropia*. JH extracts derived from Cecropia prevented the metamorphosis of pupae of Cecropia silkworm. In addition, these extracts showed their interspecific activity against *Tenebrio molitor*, *R. prolixus,* and *Periplaneta americana*, suggesting that JH is present in various insects (Williams, 1956).

The chemical structure of JH I was elucidated from lipid extracts of silk moth by Roller et al. (1967). Soon later, other related forms of JH, JH II, and JH III were elucidated from corpora allata of *Manduca sexta* (Judy et al., 1973; Meyer et al., 1968). JH III is now considered the main active form in insects except for some Hemipterans, which use JH III skipped bisepoxide (JHSB3) (Kotaki et al., 2009). The titer of JH is regulated by the synthesis in the corpora allata and degradation of JH by JH esterase. JH synthesis is

regulated by allatostatins, allatotropin, and several neuropeptides through stimulatory and inhibitory activity in corpora allata (Gilbert et al., 2000).

The signaling pathway of JH was discovered after the development of the juvenile hormone agonist (JHA). JH receptor gene, *Methoprene-tolerant* (*Met*), was found by Wilson and Fabian (1986) through the ethyl methane sulfonate mutagenesis screening experiment against *Drosophila melanogaster*. *Met*-mutated *Drosophila* showed high resistance to the effects of methoprene and JH III. After that, many studies showed the properties of Met as a JH receptor. *In vitro* synthesized Met showed a high affinity with JH III (Miura et al., 2005)

Met protein was revealed as a basic helix-loop-helix (bHLH) – Per-Arnt-Sim (PAS) domain family (Ashok et al., 1998). Met forms homodimer or heterodimer with paralog of Met, germ-cell expressed (Gce). The presence of JH inhibited the formation of Met/Met or Met/Gce formation (Godlewski et al., 2006). Later, another partner protein with bHLH-PAS domain members was revealed. Taiman (Tai) and its homologous, Ftz-F1 interacting steroid receptor coactivator (FISC) and steroid receptor coactivator (SRC), dimerizes with Met with JH dependent manner. JH binds into the PAS-B domain of Met and forms the JH receptor (JHR) complex (Charles et al., 2011; Jindra & Bittova, 2020)

JHR complex binds to the specific sequence of JH response elements (JHREs) such as *Krüppel-homolog 1* (*Kr-h1*) and *BR-C* and works as a transcription regulator to signal the activity of JH. *Kr-h1* is the main gene in JH signaling, which acts as anti-metamorphic activity and knock-down of *Kr-h1* or *Met* caused precocious metamorphosis (Jindra et al., 2015).

2. Juvenile hormone agonist (JHA)

After Rachel Carson's silent spring, the toxicity of pesticides to the environment came to public attention. Moreover, massive uses of DDT caused resistance to pests, leading to the need for novel insecticides with low toxicity and specificity. Since the discovery of JH and its activity, Williams suggested the development of 'Third-generation pesticides' as alternative pesticides that disrupt insect hormone activity by mimicking it (Williams, 1967).

In 1965, the first botanical JHA, 'the paper factor,' was discovered from Canadian balsam fir (Slama & Williams, 1965). Nymphs of *Pyrrhocoris apterus* reared on the paper towels made from balsam fir failed to metamorphosis and continued molting, resulting in supernumerary nymphs. This phenomenon was only exhibited against the family Pyrrhocoridae. Later, JH active compound was isolated from balsam fir and identified as juvabione (Bowers et al., 1966).

Methoprene was first registered in 1975 by the Environmental Protection Agency and became the first JHA commercialized IGR insecticide. Methoprene and JH mimics have been successfully used to control mosquitoes (Henrick, 2007).

One of the most successful JHA, pyriproxyfen which contains the 4-phenoxy-phenyl group, was commercialized in 1986. Methoprene and pyriproxyfen were still used to control mosquitoes and several medical pests due to their low toxicity and high specificity (Invest & Lucas, 2008).

3. Juvenile hormone antagonist (JHAN)

After discovering JHA, it was assumed that there might be compounds with opposite

activity, JHANs. Theoretically, JHANs will shorten the larval period and accelerate metamorphosis. These were thought to be able to use controlling pests that cause damage in the larval stage (Stall, 1986).

Precocenes derived from the extract of *Ageratum houstonianum* caused precocious metamorphosis and inhibited ovary development (Amsalem et al., 2014; Miall & Mordue, 1980; Pratt & Bowers, 1977). Precocenes selectively destroyed the corpora allata of insects and prevented JH synthesis. In addition, materials like KK-22, ETB, and imidazoles also induced precocious metamorphosis and showed JHAN activity. Most of them are now known to inhibit the biosynthesis of JH.

Fluoromevalonate (FMev) showed anti-JH activity against all tested Lepidoptera. FMev decreased titer of the JH and induced precocious metamorphosis in larvae. Later, FMev showed JHAN activity as a competitive inhibitor in mevalonate diphosphate decarboxylase and cholesterol synthesis in human cells (Sánchez et al., 2015). Based on these results, FMev might work as an inhibitor of JH synthesis in Lepidoptera.

After the discovery of the juvenile hormone receptor, Met, and its mode of action, a high throughput screening system using a yeast two-hybrid system was developed (Lee et al., 2015). Through the *in vitro* screening system, several chemicals which disrupt the formation of juvenile hormone receptor complex were discovered from plant extracts, plant essential oils, fungi, chemicals, and *Streptomyces* (Kim et al., 2020; Lee, Ha, et al., 2018; Shin et al., 2018; Woo et al., 2020).

4. Actinomycetes-derived secondary metabolites

Actinomycetes are gram-positive, aerobic, filamentous bacteria that play a role in nutrient cycling in various ecosystems. They are widely distributed in soil and known to produce geosmin, which causes their earthly odor.

The value of actinomycetes is in the numerous secondary metabolites, especially antibiotics. Since the discovery of streptothricin and streptomycin in 1942 and 1944, at least 7,000 antibiotics have been discovered from actinomycetes (Amelia-Yap et al., 2022; Berdy, 2005; De Simeis & Serra, 2021). By this time, two-thirds of the currently known natural antibiotics, including antibiotics, enzymes, antifungal, antiviral, and insecticidal compounds, are derived from actinomycetes, and the genus *Streptomyces* produces 75% of them (de Lima Procópio et al., 2012; Kaur et al., 2014; Newman et al., 2003; Sun et al., 2002; Zhao et al., 2020). Among them, various secondary metabolites such as avermectin, milbemycin, spinosad, nanchangmycin, and doramectin from actinomycetes showed potent insecticidal activity, although the reasons for why they produce insecticidal compounds are unclear (Strong & Brown, 1987; Sun et al., 2002; Wang et al., 2011).

In the life cycle of *Streptomyces*, nutrient deficiency causes forming of aerial mycelium and programmed cell death (PCD) for reabsorption of energy. In general, antibiotics and secondary metabolites are produced in this transition stage to protect themselves from other bacteria.

Recent studies have revealed that actinomycetes play a crucial role in supporting the defense of host plants by contributing to stress tolerance by providing secondary metabolites (Lin et al., 2012; Ruiu, 2020). Plant hormones and exudates from host plants affect the growth and production of bioactive substances in actinomycetes. Moreover, Plant-actinomycetes relationship and association with vertebrates, fungi, and invertebrates

of actinomycetes have been found (Van der Meij et al., 2017).

5. Carbazomycin derivatives

Carbazole is a heteroaromatic tricyclic alkaloid consisting of two six-membered benzene rings fused on either side of a five-membered nitrogen-containing ring. Murrayanine was the first discovered natural carbazole alkaloid with bioactivity from the plant *Murraya koengii* Spreng (Das et al., 1965). Many novel carbazole derivatives have been found from extracts of *M. koengii* and murraya plants (Sukari et al., 2001). These plants have been used for their incredibly diverse range of pharmacological activities like antidiabetic, antifungal, anticancer, inflammatory, and antioxidant activity due to the presence of carbazole alkaloids (Arndt W Schmidt et al., 2012).

After that, enormous carbazole alkaloids were found in the higher plants, mainly of the genus Murraya, Glycosmis, and Clausena (Wu et al., 1999). Also, several carbazole alkaloids have been isolated from *Streptomyces* and fungi (TePaske et al., 1989). 3,4-dioxygenated tricyclic carbazole alkaloids were found only in *Streptomyces*.

Carbazomycin A and B have been isolated from *Streptoverticillium ehimense* H 1051-MY 10 (Sakano et al., 1980; Sakano & Nakamura, 1980). Carbazomycin B exhibited antifungal activity against phytopathogenic fungi and weak antibacterial activity. Also, carbazomycin B and C inhibited 5-lipoxygenase activity (Hook et al., 1990). Because of the most potent antibiotic activity among carbazomycin, carbazomycin B was regarded as a primary active compound.

The biosynthetic route and source of carbon framework of carbazomycin B were

described by Nakamura et al. (1980). Through time course studies of the labeled precursor, L-tryptophan was revealed as a biogenetic precursor of carbazomycin B (Kaneda et al., 1990; Yamasaki et al., 1983). Recently, several genes involving the biosynthesis of carbazole alkaloids in *Streptomyces* were identified (Kobayashi et al., 2019).

MATERIALS AND METHODS

Part 1. Screening of mosquito larvicidal *Streptomyces* strains based on IGR activities

1) Actinomycetes isolation and fermentation

One hundred thirty-eight actinomycetes (mostly *Streptomyces*) strains were deposited from the Korean agricultural culture collection (KACC). Also, actinomycetes were isolated from soil samples collected at various positions in the Korean peninsula (Fig. 1). Soil samples were collected from 5 cm depth on a sterile spoon and transferred to a sterile polythene bag. Soil samples were serial diluted in distilled water and cultured in humic acid vitamin agar (Hayakawa & Nonomura, 1987).

Isolated actinomycetes strains were cultured at M3 medium (10 g glucose, 10 g soytone, 20 g soluble starch, 0.02 g iron sulfate heptahydrate, and 3g calcium carbonate per liter distilled water) in baffled flasks with 20% of working volume. All the flasks were incubated on a rotary shaker at 150 rpm, 28 ℃, for five days. After fermentation, the culture broths of actinomycetes were centrifuged and filtered using Whatman no.1 filter paper to obtain culture filtrate. Culture filtrates were used to screen JHAN and mosquito larvicidal activity (Fig. 2).

2) Yeast two-hybrid β-galactosidase assays

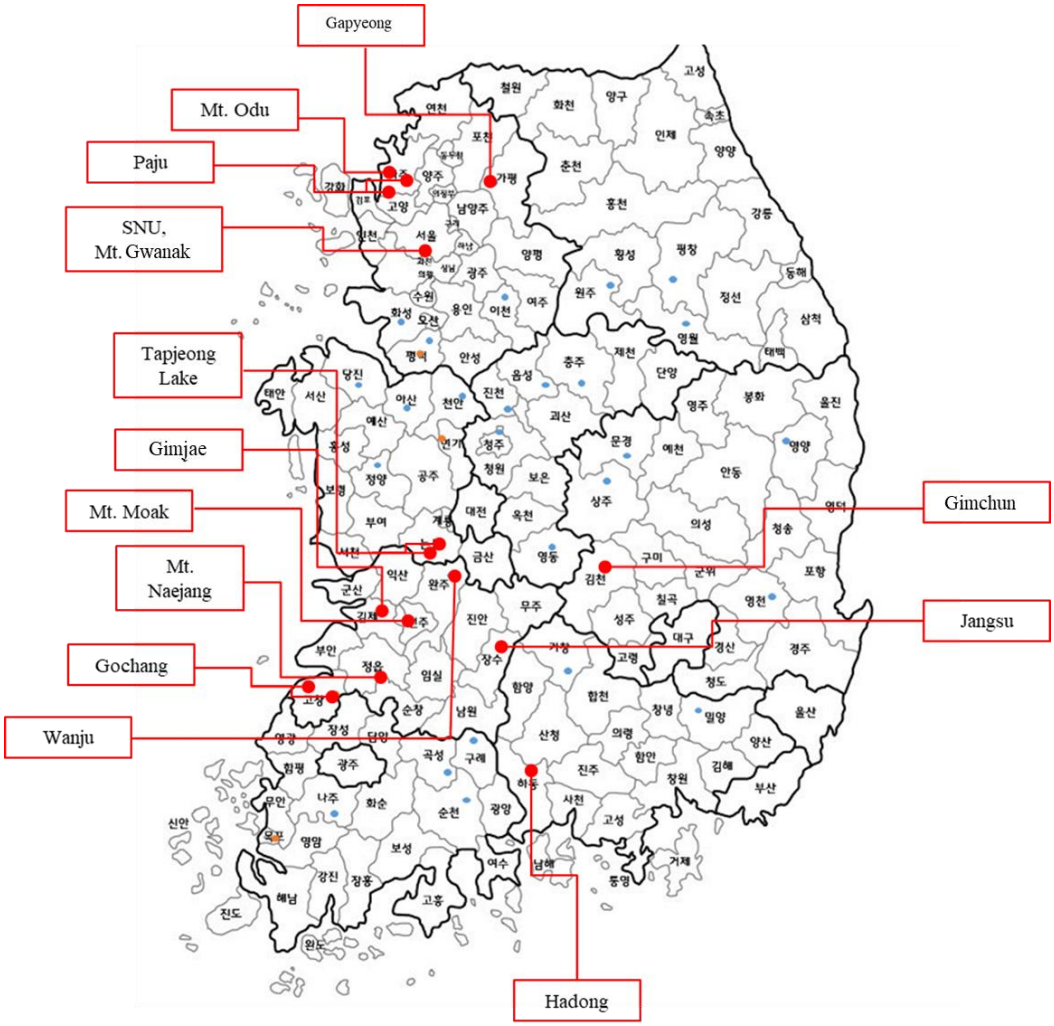

Fig. 1. Soil collected spot.

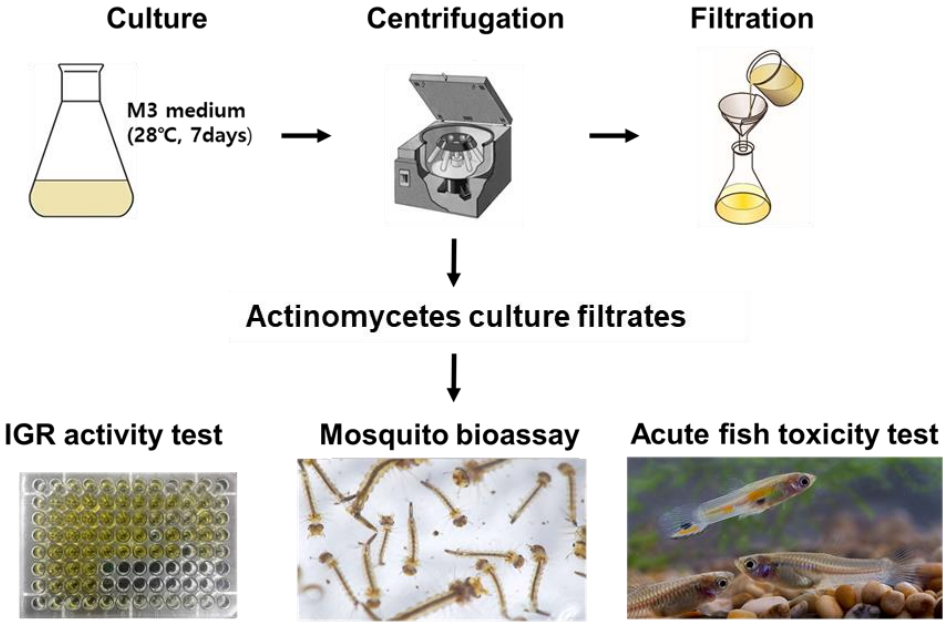

Fig. 2. Flow chart for screening of secondary metabolites with IGR activity from actinomycetes.

The yeast two-hybrid β-galactosidase assay for determination of JHA and JHAN activities using Y187 yeast cells transformed with *Met* and *FISC* of *A. aegypti* was performed (Lee, Ha, et al., 2018). To determine the JHA and JHAN activities of secondary metabolites of actinomycetes, transformed yeast cells were incubated at 30°C in DDO (SD -Leu/-Trp) media. For the JHA activity test, 100 μl of yeast cells (2.0×10^6 cells/ml) distributed in 96-well plates were treated with 1 μl of actinomycetes culture filtrates. Positive control was treated with 0.033 ppm of pyriproxyfen (Lee et al., 2015). For the JHAN activity test, 100 μl of transformed yeast cells with 0.033 ppm of pyriproxyfen were distributed in 96-well plates, and 1 μl of culture filtrates and various concentrations of crude extracts according to the amount of extracts were treated in each well. The cells for JHAN and JHA activity were incubated for 3 h and subjected to the β-galactosidase assays using the Yeast β-galactosidase Assay Kit (Thermo Scientific. USA). The optical density was measured at 420 nm, and the OD_{420} values were converted to an arbitrary unit representing JHA and JHAN activity (Park et al., 2020).

$$\text{JHA activity} = \frac{\text{OD420 of sample}}{\text{OD420 of pyriproxyfen (0.033ppm)}}$$

$$\text{JHAN activity} = \frac{\text{OD420 of pyriproxyfen (0.033ppm)} - \text{OD420 of sample}}{\text{OD420 of pyriproxyfen (0.033ppm)}}$$

3) Mosquito bioassay

Aedes albopictus (Skuse) (Diptera: Culicidae) were obtained from the Korean disease

control and prevention agency (KDCA). Mosquito larvae were reared at 28°C and 70% relative humidity under photocycle of a 14 h light: 10 h dark and fed on a diet of TetraMin fishflakes.

To screen insecticidal actinomycetes, culture filtrates with JHAN activity were applied to 3rd instar larvae of *A. albopictus* in 6-well plates. One milliliter of culture filtrates was applied to larvae of *A. albopictus* in 4 ml aged tap water with TetraMin fish flakes, and the number of dead larvae was recorded every 24 h for three days. Selected actinomycetes culture filtrates were further examined for their mosquito larvicidal activity. Five hundred microliters of culture filtrates were applied with 4.5 ml of aged tap water with 2nd, 3rd, and 4th instar larvae of *A. albopictus*. Crude extracts and chemicals were dissolved in ethanol and applied with 1% volume in various concentrations.

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4) Acute fish toxicity test

Ten milliliters of actinomycetes culture filtrates were treated with adult guppies in 1 L tap water. A number of dead guppies were recorded every 12 h for two days, and actinomycetes culture filtrates with over 70% mortality within 48 h of treatment were considered toxic.

5) Solvent extraction

Actinomycetes culture filtrates with JHAN and mosquito larvicidal activity was solvent extracted in order of n-hexane, ethyl acetate, and n-butanol with the same volume. Each solvent extract was evaporated by a rotary evaporator (EYELA, Japan) and diluted in

ethanol to an appropriate concentration based on the amounts of extracts to examine JHAN activity and mosquito larvicidal activity.

6) Morphological and physiological characterization

Actinomycetes were cultured on various International Streptomyces Project (ISP) media at 28°C for 14 days to examine the morphological characteristics of mosquito larvicidal strains. The substrate mycelium and aerial mycelium color were recorded. At various temperatures (20–42°C), growth was determined on ISP-2 medium as the basal medium. Growth at various NaCl concentrations (0.0, 2.5, 5.0, 7.5, and 10%, w/v) and, different pH (5, 6, 7, 8, 9, 10, and 11) and temperatures (20, 27, 30, 37, and 42°C) were examined at Bennett's agar medium respectively at 28°C for 14 days. Fully grown mycelium is considered good growth, and weak or inhibited growth of aerial mycelium is considered poor growth.

7) Phylogenetic characterization

Selected actinomycetes strain with JHAN and insecticidal activities were characterized by 16s rRNA gene sequencing. 9Genomic DNA of actinomycetes was extracted by conventional phenol-chloroform extraction. PCR amplification was performed using the KOD FX neo (TOYOBO, Japan) and primer 27F (5'-AGAGRRTGARCCTGGCTCAG-3') and 1492R (5'-GGRTACCTTGRRACGACTT-3') according to the manufacturer's instruction. Multiple alignments of 16s rRNA gene sequence of selected strain with relative type strains were performed using CLUSTAL W in MEGA-X (Kumar et al., 2018).

Reference sequences were obtained from the EzBioCloud database, and the sequence of *Bacillus subtilis* ABQL01000001 was used as an out-group (Yoon et al., 2017). The phylogenetic tree was constructed by MEGA-X using the neighbor-joining method with bootstrap analysis with 1,000 replicates.

8) Statistical analysis

Statistical analysis was performed by one-way ANOVA using SPSS software (version 24, SPSS, Inc., Chicago, IL, USA). The mean values were compared using Tukey HSD and Scheffe's method, and *P*-values less than 0.05 were considered significantly different among their means.

Part 2. Identification and characterization of mosquito larvicidal compounds based on JHAN from *Streptomyces abikoensis* IMBL-1939 strain

1) Bioassay-guided fractionation

To isolate bioactive substances from an n-hexane extract of *Streptomyces abikoensis* IMBL-1939 strain, n-hexane extract was fractionated by open column chromatography. Silica gel 60 with 230~400 mesh (Sigma-Aldrich. St Louis, MO, USA) was used to pack the column. The column was equilibrated with n-hexane. The total extract was performed in an open column with a ratio of 9:1 of n-hexane/ethyl acetate. Fractions were monitored by the thin-layer chromatography for homogeneity and pooled together based on spotting pattern and retardation factor (Rf) value. Isolated fractions were concentrated and tested for their JHAN and mosquito larvicidal activity.

2) Mosquito bioassays

For bioassay-guided fractionation, 50μl of ethanol dissolved fractions were applied to 3rd instar larvae of *A. albopictus* in 4.95 ml aged tap water in 6-well plates with TetraMin fish flakes, and the number of dead larvae was recorded every 24 h for three days.

To calculate lethal concentration (LC) and inhibition of adult emergence rate (IE) of carbazomycins, 50 μl of ethanol dissolved carbazomycin A, B, and D with various concentrations (0, 1, 2.5, 5, and 10 ppm) treated on mosquitoes. A number of dead larvae were recorded every 24 h until every mosquito became an adult. All tests were performed

by the WHO guideline (WHO, 2005).

3) Structural elucidation of JHAN compounds

Isolated fractions were subjected to GC-MS analysis using ISQ-LT (Thermo Scientific, USA) with a VF-5ms column (EI-MS, electron energy, 70 eV). Electrospray ionization-mass spectrometry (ESI-MS) was performed using a high-resolution LC/MSMS spectrometer Q-TOF 5600 (AB SCIEX, Canada). The C18 column was eluted with solvent A (0.1% formic acid / H_2O) and solvent B (0.1% formic acid / Acetonitrile) at a flow rate 0.2 ml/min. The following gradient was used 10% solvent B for 2 min, followed by a linear gradient from 10% B to 100% B in 28 min.

The ^{1}H, ^{13}C, and 2D (HMBC, HSQC, COSY, and ROESY) nuclear magnetic resonance (NMR) spectrums were obtained using a high-resolution NMR spectrometer Avance 600 (600 MHz). The chemical shifts were expressed in d (ppm), and MeOD was used as a solvent.

4) RNA preparation and quantitative PCR (qPCR)

Adults female of *A. albopictus,* which had eclosed within 1 h, were treated with 0.5 μg of carbazomycin A (JHAN) and 0.5 μg of pyriproxyfen (JHA), each dissolved in 0.5 μl of acetone, respectively. Adult female mosquitoes were fed on 10% sucrose solution, and total RNA was extracted from the mosquitoes at 0, 6, 12, 18, and 24 h after treatment using Qiazol (Qiagen, Germany) according to the manufacturer's instructions. cDNA was synthesized using the ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan) according to

the manufacturer's instructions.

The qPCR was conducted with the EvaGreen qPCR Mastermix (ABM, Canada) and the CFX96™ Real-Time System (BIO-RAD, USA). The cycling profile used for qPCR was as follows: a preheating step for enzyme activation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 58°C for 15 sec, and 72°C for 30 sec. Relative transcription levels were quantified via the $2^{-\Delta Ct}$ method. The *RPS7* was used as a reference gene for gene expression normalization (Lee et al., 2015). Primers specific to *Hairy* (*Hairy*-Fw, 5′-TGACCGTGAAACATTTGGAA-3′; *Hairy*-Re, 5′-CGGTCTCCAAGGTTTGTCAT-3′), *Kr-h1* (*Kr-h1*-Fw, 5′-CCCCAAAAGTAATGTTTAGC-3′; *Kr-h1*-Re ,5′-GTTGCTGTGT TGCTGATTAT-3′) and *RPS7* (*RPS7*-Fw, 5′- TCAGTGTACAAGAAGCTGACCGGA-3′; *RPS7*-Re, 5′-TTCCGCGCGCGCGTCACTTATTAGATT-3′) were used for qPCR. All assays were independently repeated three times, and at least ten mosquitoes were used for qPCR.

5) Ovary dissection

Female adults of *A. albopictus,* which had eclosed within 1 h, were treated with 0.5 μg of carbazomycin A dissolved in 0.5 μl of acetone. Negative control mosquitoes were treated with 0.5 μl of acetone. Adult mosquitoes were fed on 10% sucrose solution. Two days after treatment lab mouse was provided 5 h for blood-feeding. Three days after blood feeding, mosquitoes were dissected to observe ovary development. Dissected ovaries were observed using a Nikon E200 microscope (Nikon, Japan).

6) Non-target bioassays

Ten milliliters of culture filtrate of actinomycetes were treated with adult guppies in 1 L tap water for screening, and 1 ml of ethanol dissolved carbazomycin A and hexane extract were treated with 10 ppm as a final concentration. A number of dead guppies were recorded every 12 h for two days.

To evaluate the acute dermal toxicity of carbazomycin A against adult honey bees, *Apis mellifera* workers were captured and anesthetized with CO_2. Fifty nanograms of carbazomycin A and n-hexane extracts of IMBL-1939 dissolved in 1 μl of acetone were applied to each thorax of a honey bee using a micropipette. Negative control honey bees were treated with the same volume of acetone, and positive control was treated with 50 ng of permethrin. Treated honey bees were transferred to plastic cups and fed on 50% sucrose solution. The number of dead honey bees was recorded every 24 h for two days after treatment.

The leaf dipping method evaluated the oral toxicity of carbazomycin A on the silkworm Bombyx mori. Mulberry leaves were immersed in 2 ppm of carbazomycin A, and permethrin (ositive control) treated water with 0.01% Triton X-100. Control leaves were dipped in distilled water with 0.01% Triton X-100. Treated leaves were dried at room temperature and were fed on the 3rd instar larvae of a silkworm. Mortality was determined at 48 h after being exposed.

M. aeruginosa FBCC-A59 strain was deposited from the Freshwater bioresources culture collection (KBCC). *M. aeruginosa* was cultured in BG11 medium, and 100 ul of cultures were distributed to each well of 96-well plates. Carbazomycin A and n-hexane extract dissolved in ethanol were treated with 1 ul with the final concentration of 0.1, 1.0,

and 10.0 ppm. After 24 h, optical density was measured at 650 nm, and the OD_{650} values were converted to an arbitrary unit to evaluate anti-cyanobacterial activity.

$$\text{cyanobacteria growth inhibition rate} = \frac{OD\ 650\ nm\ (control-sample)}{OD\ 650\ nm(control)}$$

7) Statistical analysis

Statistical analysis was performed by one-way ANOVA, and two-way ANOVA using SPSS software (version 24, SPSS, Inc., Chicago, IL, USA). A comparison of the mean values was performed using Tukey HSD, and Scheffe's method, and *P* values less than 0.05 were considered significantly different among their means. The LC and IE values were calculated using SPSS by Finney's Probit analysis method.

Part 3. Applications of carbazomycin derivatives as bio-pesticides

1) Initial medium selection

To select the initial medium for optimization in carbazomycin production, commonly used Streptomyces media were chosen to evaluate their effects on carbazomycin production. For inoculum preparation, *S. abikoensis* IMBL-1939 strain was cultured in an M3 medium at 30℃, 150 rpm in 50 ml Erlenmeyer flasks containing 10 ml for 5 days. Seeds were inoculated with 5% volume at five common media GSS, FM, Bennett, M3, and GYM for 5 days at 30℃, 150 rpm in an Erlenmeyer flask. The compositions of five common media are listed in Table 1. After the cultivation, fermented media were centrifuged and extracted with the same volume of n-hexane. The n-hexane extracts were concentrated by a rotary evaporator and dissolved with ethanol. To calculate the production of carbazomycins, the TLC pattern of carbazomycin in n-hexane extracts was monitored with a ratio of 3:1 of n-hexane/ethyl acetate. Through the TLC pattern, the amounts of carbazomycin A and B were measured by the ImageJ program. The measured value was divided by the total mean value to calculate the relative production amount. Experiments were carried out in triplicates.

2) Plackett–Burman design

Plackett-Burman design was used to select effective variables for carbazomycin A and B production. According to the composition of the GYM medium, glucose, yeast extract, malt extract, calcium carbonate, and the type of flasks (baffled or not) were selected for this experiment. For each variable, two different levels were tested (-1 and +1) (Table. 2).

Table 1. Composition of five different media used for selection of a basal medium

M3	g/L	GYM	g/L	Bennett	g/L
Glucose	10	Glucose	4	Glucose	10
Soytone	10	Yeast extract	4	Yeast extract	1
Soluble starch	20	Malt extract	10	Beef extract	1
Calcium carbonate	3	Calcium carbonate	2	Peptone	2
GSS	g/L	Fish meal	g/L		
Glucose	20	Glucose	20		
Soluble starch	10	Soluble starch	10		
Soy peptone	25	Soybean meal	12.5		
Beef extract	1	Fish meal	12.5		
Yeast extract	4	Beef extract	1		
Sodium chloride	2	Yeast extract	4		
Calcium carbonate	2	Sodium chloride	2		
		Calcium carbonate	2		
		Dipotassium phosphate	0.25		

Table 2. Variables and their levels for the Plackett-Burman design

Variable	Component	-1 value (g/L)	+1 value (g/L)
X_1	Yeast extract	2	6
X_2	Glucose	2	6
X_3	Malt extract	6	14
X_4	Calcium carbonate	1	3
X_5	Flask type	Without baffle	Baffled flask

Twelve experiments were conducted using design-expert 13 software (State-Easy Co., Minneapolis, MN, USA), and all the trials were performed in duplicate. Production of carbazomycin A and B in each run were measured by TLC using the ImageJ program. The measured value was divided by the mean value to calculate the relative production amount. Experimental data were analyzed by the design-expert 13 software. Factors under a 0.05 *P*-value were considered significant and proceeded with further optimization using response surface methodology.

3) Response surface methodology (RSM)

Central composite design was applied to determine the optimum levels of glucose and malt extract for carbazomycin production. Glucose and malt extract were experimented with at 5 levels (-1.414, -1, 0, +1, and +1.414) (Table 3). Nine runs were conducted, and carbazomycin A and B production was measured in previous methods. Response from the central composite design was subjected to multiple regression models to obtain the parameters of the statistic models. The accuracy of the polynomial model equation was judged statistically by the coefficient of determination R^2 and lack of fit tests. The significance of the regression was tested by the analysis of variance (ANOVA). Regression analysis and a three-dimension surface plot model were made using design expert 13.0.

4) Antifoam

Antifoam 204 (Sigma-Aldrich, St Louis, MO, USA) was added to the carbazomycin production medium in various concentrations (0.005%, 0.01%, 0.05%, and 0.1%) and *S.*

Table 3. Variables and their levels for the central composite design

Variable	Coded level of variable				
	−1.414	−1	0	1	+1.414
Glucose	3.2	4	6	8	8.8
Malt extract	9.2	10	12	14	14.8

abikoensis IMBL-1939 strain was cultured with the same method previously. After fermentation, the wet weight of cells and carbazomycin productivity were measured.

5) Optimization of fermentation condition in pilot culture

Inoculum preparation for pilot culture, *S. abikoensis* IMBL-1939 strain was cultured in 400 ml in 2 L flasks at carbazomycin production medium with the optimized condition for 5 days. The strain was grown in 50 L fermenters (KBT Ltd., Korea) with a working volume of 30 L with 0.05% antifoam 204 at 30℃, 1800 L/h of airflow rate, one vvm, and 150 rpm of agitation speed. The pH was adjusted to 7.5 - 8.0, and 5% volume of lab-cultured inoculum was used.

To examine the daily production of carbazomycin derivatives, samples were collected at 1, 2, 3, and 5 days after fermentation. The fermentation ran for 7 days. After fermentation, the culture broth was centrifuged using a tubular type centrifuge. n-Hexane extraction for culture filtrate proceeded three times, and production of carbazomycin derivatives was measured.

6) Conversion of carbazomycin B to A

Methylation of carbazomycin B was followed by Moody's method (Moody & Shah, 1989). To acetone dissolved carbazomycin B, potassium carbonate, and methyl iodide were added and heated for 6 h at 40℃. Solution was diluted with dichloromethane, and the organic layer was washed with DDW, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography (n-hexane: ethyl acetate =

9:1) to get carbazomycin A. Methylation was carried out from the Kwon lab at Seoul national university.

7) Chemical synthesis of carbazomycin A

Total chemical synthesis of the carbazomycin A pathway is based on the existing research of Knölker and Bauermeister (1993) and Knölker and Fröhner (1999). Chemical synthesis was carried out from the Kwon lab at Seoul national university.

1. Methylation of 2-methoxy-3,4-dimethyl-5-nitrophenol

To tetrahydrofuran (THF) dissolved 2-methoxy-3,4-dimethyl-5-nitrophenol, trimethylsilyl-diazomethane (TMS-DAM) was added into the solution. The solution was incubated at room temperature for 40 h.

2. Ortho-formylation of 2,3-dimethylphenol

To a 2,3-dimethylphenol dissolved in acetonitrile, magnesium chloride, triethylamine, and dry paraformaldehyde were added. The reaction mixture was heated to 80℃ under reflux for 5 h. Solution was diluted with ether, and the organic layer was dried over $MgSO_4$ and concentrated under reduced pressure.

3. Dakin oxidation of 2-hydroxy-3,4-dimethylbenzaldehyde

The mixture of 2-hydroxy-3,4-dimethylbenzaldehyde and 1N sodium hydroxide solution was warmed to 45℃, and 6% hydrogen peroxide was added. The mixture was incubated

for 8 h and stopped the reaction by HCl, and extracted with ether. The extract was dried over $MgSO_4$, and concentrated under reduced pressure.

4. Methylation of 3,4-dimethylbenzene-1,2-diol

3,4-Dimethylbenzene-1,2-diol dissolved in DMF was added to methyl iodide/sodium hydride. The mixture was stirred at room temperature for 1 h and diluted with dichloromethane, and the organic layer was washed with DDW, dried over Na_2SO_4 and concentrated under reduced pressure.

8) Carbazole alkaloids

Carbazole alkaloids were purchased from Sigma-Aldrich (St Louis, MO, USA) and TCI chemicals (Tokyo, Japan) and synthesized by the Kwon lab at Seoul national university. Used chemicals are listed in Table 5. JHAN activity test and mosquito bioassays proceeded as described in the previous tests.

9) Formulation

The n-hexane extract of *S. abikoensis* IMBL-1939 culture filtrate was formulated to wettable powder (WP) by Farmhannong Co., Ltd. (Korea). The composition of WP is listed in Table 4. Properties of WP were tested by following quality and efficacy test collection methods by pesticide formulation from the Ministry of Food and Drug Safety (Korea) and the National Institute of Food and Drug Safety Evaluation (Korea).

Table 4. List of carbazole alkaloids used in this study

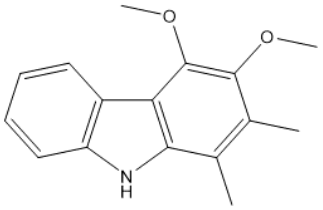
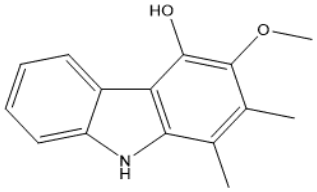
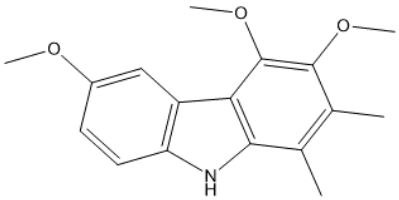
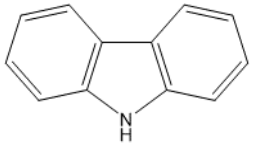
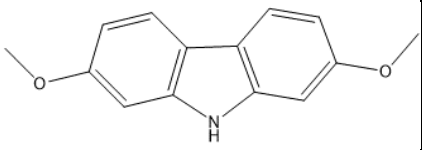
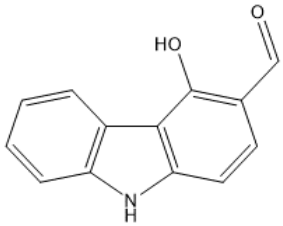
No	Name	Structure	Source	Cas No.
CA 1	Carbazomycin A		Isolated from *Streptomyces*	75139-39-8
CA 2	Carbazomycin B		Isolated from *Streptomyces*	75139-38-7
CA 3	Carbazomycin D		Isolated from *Streptomyces*	108073-63-8
CA 4	Carbazole		Sigma-Aldrich	86-74-8
CA 5	2,7-Dimethoxy-9H-carbazole		TCI chemicals	61822-18-2
CA 6	4-Hydroxy-9H-carbazole-3-carbaldehyde		Synthesized	1375487-97-0

Table 4. (Continued)

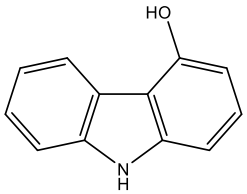
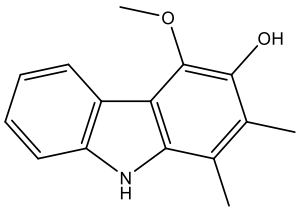
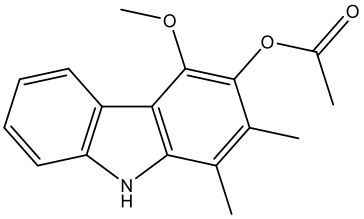
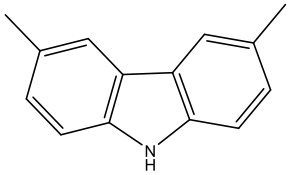
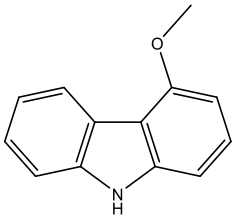
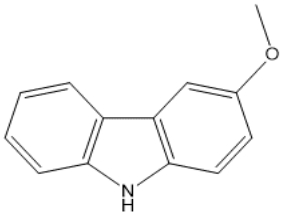
No	Name	Structure	Source	Cas No.
CA 7	9H-Carbazol-4-ol		Synthesized	52602-39-8
CA 8	3-hydroxy-4-methoxy-1,2-dimethyl-9H-carbazole		Synthesized	125771-80-4
CA 9	1,2-Dimethyl-3-methoxy-9H-carbazol-4-ol acetate		Synthesized	76306-36-0
CA 10	3,6-Dimethylcarbazole		TCI chemicals	5599-50-8
CA 11	4-methoxy-9H-carbazole		Synthesized	6933-50-2
CA 12	3-methoxy-9H-carbazole		Sigma-Aldrich	18992-85-3

Table 5. Components of the wettable powder

Component	Percentage (%)
n-Hexane extract	1
DMSO	5
White carbon	10
Sodium lignosulfonate (SLS)	10
Kaolin	64

1. Fineness

To evaluate the fineness of WP, 200 ppm of WP solution diluted in DDW was filtered by a 75 μm sieve (200 mesh) and shaken and washed with DDW. The unfiltered residue was dried at 80℃ for 4 h, and the mass of residue was measured (standard: residues within 2%).

2. Wettability

To evaluate the wettability of WP, 5 g of WP was put in a beaker with 100 ml of DDW beaker slowly and measured the time when WP was entirely wet (standard: 2 min).

3. Moisturizing property

To evaluate a moisturizing property, 5 g of WP was put in a mass cylinder with 100 ml of DDW slowly and measured the time when WP was entirely moisturized (standard: 1 min).

4. Suspensibility

To evaluate suspensibility, 5 g of WP was put in 100 ml of DDW in a 200 ml capped mass cylinder and shaken 30 times and left for 10 min, and measured the volume of sediment (standard: less than 2 ml of sediment).

5. Bulk density

The 100 ml metal container was filled with WP carefully and weighed the mass of WP.

6. Dispersibility

To evaluate dispersibility, 100 ml of WP solution with 200 ppm was put into a 200 ml capped mass cylinder and shaken 30 times, and left for 2 h, and dispersibility was observed.

10) Mosquito bioassays

To determine mosquito larvicidal activity of wettable powder (WP), various concentrations of WP were applied to thirty 3rd instar larvae of *A. albopictus* in 500 ml aged tap water in a 2 L plastic tank with TetraMin fish flakes. For control, additives, n-hexane fraction, and carbazomycin A were treated. After 48 h, the number of dead larvae was recorded.

For the semi-field test, fifty 3rd instar larvae of *A. albopictus* were treated with various concentrations of WP (100, 200, and 500 ppm at final concentration) in a 2 L plastic tank with a net at a shady place in Gwanak mountain (Seoul, Korea) followed by WHO guideline (WHO, 2005). A number of dead larvae were recorded 96 h after treatment. All Bioassays were tested independently and repeated three times. Mortality was corrected by Abbott's formula (Abbott, W.S, 2005).

11) Statistical analysis

Statistical analysis was performed by one-way ANOVA using SPSS software (version 24, SPSS, Inc., Chicago, IL, USA). A comparison of the mean values was performed using Scheffe's method, and P values less than 0.05 were considered significantly different among their means.

RESULTS

Part 1. Screening of mosquito larvicidal *Streptomyces* strains based on IGR activities

1) Screening of mosquito larvicidal actinomycetes with JHAN activity

A total of 94 actinomycetes strains was isolated from soil samples, and 138 strains of actinomycetes were deposited from KACC. Used actinomycetes strains are listed in Table 6. All actinomycetes were fermented in an M3 medium and filtrated to obtain culture filtrates. Actinomycetes culture filtrates were screened for their IGR activity. No culture filtrate showed significant JHA activity in the primary IGR activity screening test. Meanwhile, 34 culture filtrates showed over 0.3 JHAN activity (Table 6). Fourteen culture filtrates with JHAN activity showed over 70% mortality on mosquito screening bioassay (Fig. 3). Only IMBL-1939, 1952, 2005, and 2064 did not exhibit acute fish toxicity against adult guppies (Fig. 4). Four strains without exhibiting fish toxicity were chosen for further characterization. Among four actinomycetes culture filtrates, the culture filtrate of IMBL-1939 showed the highest mosquito larvicidal activity with a 100% mortality rate at 10% (v/v) against every instar larvae of *A. albopictus* (Fig. 5). In culture filtrates of IMBL-1952, 2005, and 2064 strains, the mortality rate decreased as the instar increased.

Table 6. List of actinomycetes strains used in this study

From		Lab no.	Scientific name	JHAN activity	Mosquito larvicidal activity
KACC	17211	IMBL-1938	*Actinomyces haliotis*	-0.101	
KACC	14697	IMBL-1939	*Streptomyces abikoensis*	0.446	100
KACC	21059	IMBL-1941	*Streptomyces aburaviensis*	-0.206	
KACC	14609	IMBL-1942	*Streptomyces achromogenes subsp. rubradiris*	-0.161	
KACC	20191	IMBL-1943	*Streptomyces acidiscabies*	0.021	
KACC	17197	IMBL-1946	*Streptomyces adustus*	-0.048	
KACC	14698	IMBL-1947	*Streptomyces anandii*	-0.145	
KACC	14703	IMBL-1949	*Streptomyces aureofaciens*	-0.116	
KACC	14685	IMBL-1950	*Streptomyces avellaneus*	0.144	
KACC	14696	IMBL-1951	*Streptomyces avellaneus*	0.125	
KACC	14701	IMBL-1952	*Streptomyces avellaneus, Kitasatospora aureofaciens*	0.447	90
KACC	18225	IMBL-1953	*Streptomyces bambusae*	0.214	
KACC	14763	IMBL-1954	*Streptomyces bellus*	-0.124	
KACC	14776	IMBL-1955	*Streptomyces bikiniensis*	-0.173	
KACC	21080	IMBL-1956	*Streptomyces blastmyceticus*	-0.092	
KACC	14678	IMBL-1957	*Streptomyces chattanoogensis*	-0.171	
KACC	20738	IMBL-1961	*Streptomyces chattanoogensis*	-0.117	
KACC	16599	IMBL-1962	*Streptomyces cirratus*	-0.224	
KACC	19457	IMBL-1963	*Streptomyces costaricanus*	-0.21	
KACC	20730	IMBL-1964	*Streptomyces costaricanus*	-0.179	
KACC	21072	IMBL-1966	*Streptomyces costaricanus*	0.054	
KACC	18226	IMBL-1967	*Streptomyces fabae*	0.141	
KACC	14684	IMBL-1968	*Streptomyces flavofungini*	0.372	100
KACC	14693	IMBL-1969	*Streptomyces flavofungini*	0.526	100
KACC	14681	IMBL-1970	*Streptomyces fulvissimus*	-0.133	
KACC	18248	IMBL-1971	*Streptomyces gilvifuscus*	-0.163	
KACC	20719	IMBL-1972	*Streptomyces glomeratus*	-0.088	
KACC	21076	IMBL-1973	*Streptomyces gougerotii*	-0.104	
KACC	16470	IMBL-1974	*Streptomyces gramilatus*	-0.244	

Table 6. (Continued)

From		Lab no.	Scientific name	JHAN activity	Mosquito larvicidal activity
KACC	15078	IMBL-1975	*Streptomyces gramineus*	-0.178	
KACC	15079	IMBL-1976	*Streptomyces gramineus*	-0.006	
KACC	17180	IMBL-1977	*Streptomyces graminifolii*	-0.091	
KACC	20938	IMBL-1981	*Streptomyces griseofuscus*	-0.045	
KACC	14710	IMBL-1982	*Streptomyces hygroscopicus*	0.074	
KACC	20740	IMBL-1986	*Streptomyces katrae*	0.135	
KACC	14689	IMBL-1987	*Streptomyces koyangensis*	0.168	
KACC	20561	IMBL-1988	*Streptomyces koyangensis*	0.494	90
KACC	21074	IMBL-1989	*Streptomyces lydicus*	-0.426	
KACC	14695	IMBL-1990	*Streptomyces malaysiensis*	-0.256	
KACC	14608	IMBL-1992	*Streptomyces mauvecolor*	0.603	50
KACC	16834	IMBL-1993	*Streptomyces microflavus*	-0.157	
KACC	20735	IMBL-1995	*Streptomyces misionensis*	-0.13	
KACC	14672	IMBL-1996	*Streptomyces nodosus*	-0.083	
KACC	14676	IMBL-1997	*Streptomyces nodosus*	-0.121	
KACC	20742	IMBL-1998	*Streptomyces nojiriensis*	-0.128	
KACC	20722	IMBL-1999	*Streptomyces olivaceus*	-0.109	
KACC	18227	IMBL-2000	*Streptomyces olivicoloratus*	0.053	
KACC	20725	IMBL-2001	*Streptomyces owasiensis*	0.088	
KACC	17632	IMBL-2002	*Streptomyces panaciradicis*	0.109	
KACC	21068	IMBL-2003	*Streptomyces phaeochromogenes*	0.15	
KACC	16598	IMBL-2004	*Streptomyces phaeopurpureus*	-0.08	
KACC	14682	IMBL-2005	*Streptomyces platensis*	0.564	90
KACC	14683	IMBL-2006	*Streptomyces polychromogenes*	-0.172	
KACC	18247	IMBL-2008	*Streptomyces polymachus*	-0.094	
KACC	20713	IMBL-2009	*Streptomyces pseudovenezuelae*	-0.035	
KACC	14671	IMBL-2010	*Streptomyces pulveraceus*	0.907	50
KACC	14708	IMBL-2011	*Streptomyces pulveraceus*	-0.083	
KACC	20253	IMBL-2012	*Streptomyces puniciscabiei*	-0.103	

Table 6. (Continued)

From		Lab no.	Scientific name	JHAN activity	Mosquito larvicidal activity
KACC	16580	IMBL-2013	*Streptomyces rhizophilus*	0.121	
KACC	17181	IMBL-2014	*Streptomyces rhizosphaerihabitans*	0.184	
KACC	21078	IMBL-2015	*Streptomyces rimosus*	0.518	0
KACC	14675	IMBL-2017	*Streptomyces roseochromogenus*	0.654	30
KACC	14679	IMBL-2018	*Streptomyces roseochromogenus*	0.148	
KACC	14712	IMBL-2020	*Streptomyces roseolus*	-0.261	
KACC	21067	IMBL-2021	*Streptomyces rutgersensis*	-0.146	
KACC	17182	IMBL-2023	*Streptomyces sasae*	-0.198	
KACC	20176	IMBL-2024	*Streptomyces scabiei*	-0.042	
KACC	20194	IMBL-2027	*Streptomyces scabiei*	-0.049	
KACC	20198	IMBL-2028	*Streptomyces scabiei*	-0.036	
KACC	14612	IMBL-2031	*Streptomyces sp.*	0.567	0
KACC	14673	IMBL-2032	*Streptomyces sp.*	0.04	
KACC	14674	IMBL-2033	*Streptomyces sp.*	-0.004	
KACC	14686	IMBL-2034	*Streptomyces sp.*	0.331	100
KACC	14687	IMBL-2035	*Streptomyces sp.*	0.066	
KACC	14688	IMBL-2036	*Streptomyces sp.*	0.723	100
KACC	14694	IMBL-2038	*Streptomyces sp.*	0.468	40
KACC	14699	IMBL-2039	*Streptomyces sp.*	0.368	0
KACC	14706	IMBL-2040	*Streptomyces sp.*	0.053	
KACC	14711	IMBL-2041	*Streptomyces sp.*	-0.067	
KACC	14713	IMBL-2042	*Streptomyces sp.*	-0.156	
KACC	14842	IMBL-2044	*Streptomyces sp.*	0.132	
KACC	20048	IMBL-2045	*Streptomyces sp.*	-0.04	
KACC	20109	IMBL-2046	*Streptomyces sp.*	0.476	0
KACC	20153	IMBL-2047	*Streptomyces sp.*	0.555	100
KACC	20173	IMBL-2048	*Streptomyces sp.*	0.216	
KACC	20177	IMBL-2049	*Streptomyces sp.*	0.543	0
KACC	20179	IMBL-2050	*Streptomyces sp.*	-0.074	
KACC	20201	IMBL-2052	*Streptomyces sp.*	-0.13	

Table 6. (Continued)

From		Lab no.	Scientific name	JHAN activity	Mosquito larvicidal activity
KACC	20206	IMBL-2053	*Streptomyces sp.*	0.405	0
KACC	20215	IMBL-2054	*Streptomyces sp.*	-0.1	
KACC	20217	IMBL-2055	*Streptomyces sp.*	0.064	
KACC	20232	IMBL-2056	*Streptomyces sp.*	-0.138	
KACC	20233	IMBL-2057	*Streptomyces sp.*	0.336	100
KACC	20235	IMBL-2058	*Streptomyces sp.*	-0.253	
KACC	20236	IMBL-2059	*Streptomyces sp.*	0.01	
KACC	20669	IMBL-2060	*Streptomyces sp.*	0.96	50
KACC	20701	IMBL-2061	*Streptomyces sp.*	0.053	
KACC	20702	IMBL-2062	*Streptomyces sp.*	-0.037	
KACC	20703	IMBL-2063	*Streptomyces sp.*	-0.022	
KACC	20704	IMBL-2064	*Streptomyces sp.*	0.773	100
KACC	20705	IMBL-2065	*Streptomyces sp.*	0.021	
KACC	20707	IMBL-2066	*Streptomyces sp.*	0.316	70
KACC	20708	IMBL-2067	*Streptomyces sp.*	-0.056	
KACC	20710	IMBL-2068	*Streptomyces sp.*	0.013	
KACC	20712	IMBL-2069	*Streptomyces sp.*	0.048	
KACC	20714	IMBL-2070	*Streptomyces sp.*	-0.266	
KACC	20715	IMBL-2071	*Streptomyces sp.*	0.329	0
KACC	20720	IMBL-2072	*Streptomyces sp.*	0.971	0
KACC	20727	IMBL-2074	*Streptomyces sp.*	-0.305	
KACC	20732	IMBL-2075	*Streptomyces sp.*	0.2	
KACC	20734	IMBL-2076	*Streptomyces sp.*	-0.251	
KACC	20743	IMBL-2077	*Streptomyces sp.*	0.185	
KACC	20744	IMBL-2078	*Streptomyces sp.*	0.963	30
KACC	21045	IMBL-2080	*Streptomyces sp.*	0.79	10
KACC	21046	IMBL-2081	*Streptomyces sp.*	0.887	50
KACC	21061	IMBL-2082	*Streptomyces sp.*	0.131	
KACC	21062	IMBL-2083	*Streptomyces sp.*	0.651	90
KACC	21065	IMBL-2084	*Streptomyces sp.*	-0.085	

Table 6. (Continued)

From		Lab no.	Scientific name	JHAN activity	Mosquito larvicidal activity
KACC	21066	IMBL-2085	*Streptomyces sp.*	0.445	50
KACC	21071	IMBL-2086	*Streptomyces sp.*	0.074	
KACC	21081	IMBL-2089	*Streptomyces sp.*	-0.172	
KACC	21110	IMBL-2090	*Streptomyces sp.*	-0.039	
KACC	20729	IMBL-2091	*Streptomyces spectabilis*	-0.032	
KACC	20739	IMBL-2092	*Streptomyces sporocinereus*	0.81	100
KACC	20709	IMBL-2093	*Streptomyces spororaveus*	-0.055	
KACC	21079	IMBL-2094	*Streptomyces sporoverrucosus*	0.081	
KACC	14691	IMBL-2096	*Streptomyces tanashiensis*	-0.076	
KACC	20209	IMBL-2098	*Streptomyces thermocarboxydus*	-0.034	
KACC	20143	IMBL-2100	*Streptomyces thermodiastaticus*	0.052	
KACC	20149	IMBL-2102	*Streptomyces thermoviolaceus*	0.073	
KACC	20110	IMBL-2104	*Streptomyces thermovulgaris*	0.06	
KACC	14705	IMBL-2114	*Streptomyces tricolor*	0.022	
KACC	21125	IMBL-2115	*Streptomyces tsukiyonensis*	-0.046	
KACC	20633	IMBL-2116	*Streptomyces venezuelae*	-0.094	
KACC	21060	IMBL-2118	*Streptomyces verne*	-0.114	
KACC	14677	IMBL-2119	*Streptomyces virginiae*	0.3	0
KACC	20724	IMBL-2121	*Streptomyces xanthophaeus*	-0.025	
KACC	15465	IMBL-2123	*Streptomyces yeochonensis*	0.04	
SNU	1	IMBL-2124		0.445	0
SNU	2	IMBL-2125		0.21	
SNU	3	IMBL-2126		0.274	
SNU	4	IMBL-2127		0.091	
SNU	5	IMBL-2128		0.045	
SNU	6	IMBL-2129		0.016	
SNU	7	IMBL-2130		0.081	
SNU	8	IMBL-2131		0.098	

Table 6. (Continued)

From		Lab no.	Scientific name	JHAN activity	Mosquito larvicidal activity
SNU	9	IMBL-2132		-0.006	
SNU	10	IMBL-2133		0.03	
SNU	11	IMBL-2134		-0.023	
Gochang	1	IMBL-2135		0.031	
Gochang	2	IMBL-2136		-0.004	
Gochang	3	IMBL-2137		0.025	
Gochang	4	IMBL-2138		0.036	
Gochang	5	IMBL-2139		0.023	
Gochang	6	IMBL-2140		0.051	
Gochang	7	IMBL-2141		0.042	
Gochang	8	IMBL-2142		0.073	
Gochang	9	IMBL-2143		0.114	
Gochang	10	IMBL-2144		0.013	
Gochang	11	IMBL-2145		0.065	
Gochang	12	IMBL-2146		0.01	
Gochang	13	IMBL-2147		0.078	
Gochang	14	IMBL-2148		0.172	
Gochang	15	IMBL-2149		-0.04	
Gochang	16	IMBL-2150		0.224	
Gochang	17	IMBL-2151		-0.082	
Gochang	18	IMBL-2152		-0.06	
Gochang	19	IMBL-2153		0.088	
Gochang	20	IMBL-2154		0.029	
Gochang	21	IMBL-2155		0.034	
Mt. Naejang	1	IMBL-2156		-0.054	
Mt. Naejang	2	IMBL-2157		-0.13	
Mt. Naejang	3	IMBL-2158		-0.121	

Table 6. (Continued)

From		Lab no.	Scientific name	JHAN activity	Mosquito larvicidal activity
Mt. Naejang	4	IMBL-2159		-0.101	
Mt. Naejang	5	IMBL-2160		-0.137	
Mt. Naejang	6	IMBL-2161		-0.33	
Mt. Naejang	7	IMBL-2162		-0.102	
Mt. Naejang	1	IMBL-2163		-0.043	
Lake. Tapjeong	1	IMBL-2164		-0.13	
Lake. Tapjeong	2	IMBL-2165		-0.221	
Lake. Tapjeong	3	IMBL-2166		-0.023	
Mt. Moak	1	IMBL-2167		-0.12	
Mt. Moak	2	IMBL-2168		-0.024	
Mt. Moak	3	IMBL-2169		-0.17	
Mt. Moak	4	IMBL-2170		-0.235	
Mt. Moak	5	IMBL-2171		-0.273	
Mt. Moak	6	IMBL-2172		-0.247	
Mt. Moak	7	IMBL-2173		-0.273	
Mt. Moak	8	IMBL-2174		-0.235	
Mt. Moak	9	IMBL-2175		-0.214	
Mt. Moak	10	IMBL-2176		-0.121	
Paju	1	IMBL-2177		-0.204	
Paju	2	IMBL-2178		-0.188	
Paju	3	IMBL-2179		-0.249	
Paju	4	IMBL-2180		-0.102	
Wanju	1	IMBL-2181		-0.156	
Wanju	2	IMBL-2182		-0.196	
Wanju	3	IMBL-2183		-0.089	
Wanju	4	IMBL-2184		-0.167	
Wanju	5	IMBL-2185		-0.198	

Table 6. (Continued)

From		Lab no.	Scientific name	JHAN activity	Mosquito larvicidal activity
Wanju	6	IMBL-2186		-0.121	
Hadong	1	IMBL-2187		-0.235	
Hadong	2	IMBL-2188		-0.247	
Hadong	3	IMBL-2189		-0.099	
Hadong	4	IMBL-2190		-0.169	
Hadong	5	IMBL-2191		-0.233	
Gimje	1	IMBL-2192		-0.289	
Gimje	2	IMBL-2193		-0.235	
Gimje	3	IMBL-2194		-0.221	
Gimje	4	IMBL-2195		-0.135	
Mt. Odu	1	IMBL-2196		-0.128	
Mt. Odu	2	IMBL-2197		-0.092	
Mt. Odu	3	IMBL-2198		-0.375	
Mt. Odu	4	IMBL-2199		-0.294	
Mt. Odu	5	IMBL-2200		0.361	20
Mt. Odu	6	IMBL-2201		-0.165	
Mt. Gwanak	1	IMBL-2230		-0.147	
Mt. Gwanak	2	IMBL-2231		-0.163	
Mt. Gwanak	3	IMBL-2232		-0.256	
Mt. Gwanak	4	IMBL-2233		-0.103	
Mt. Gwanak	5	IMBL-2236		-0.021	
Mt. Gwanak	6	IMBL-2237		-0.034	
Gapyeong	1	IMBL-2238		-0.111	
Gapyeong	2	IMBL-2239		-0.318	
Gapyeong	3	IMBL-2240		-0.306	
Gapyeong	4	IMBL-2241		-0.264	
Gimchun	1	IMBL-2242		-0.237	
Gimchun	2	IMBL-2243		-0.22	

Table 6. (Continued)

From		Lab no.	Scientific name	JHAN activity	Mosquito larvicidal activity
Jangsu	1	IMBL-2269		-0.111	
Jangsu	2	IMBL-2270		-0.318	
Jangsu	3	IMBL-2271		-0.306	
Jangsu	4	IMBL-2272		-0.264	

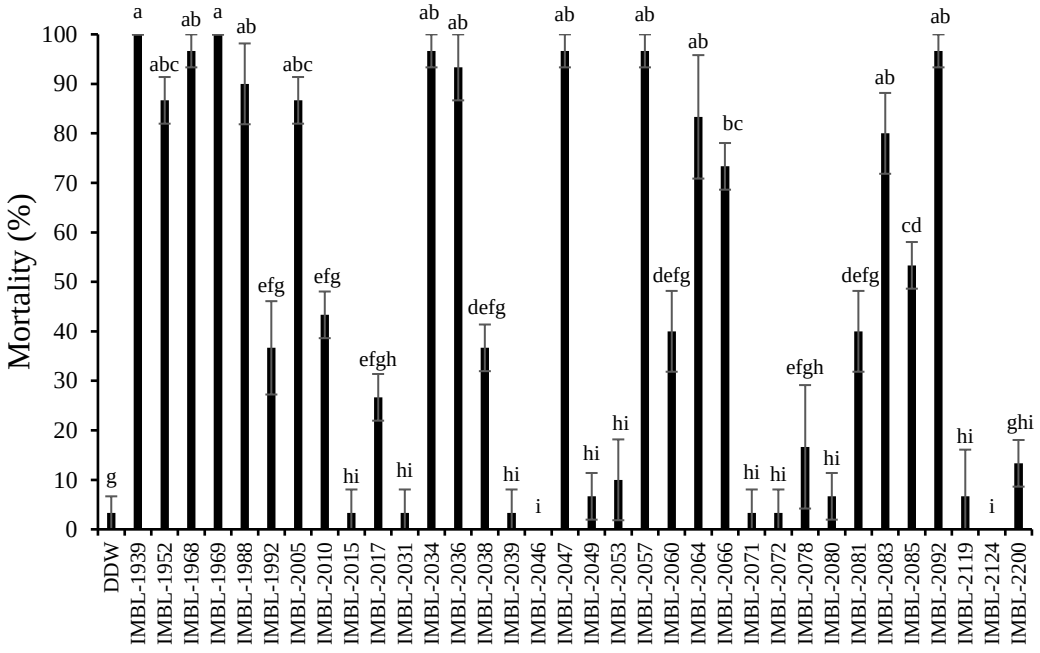


Fig. 3. Mosquito larvicidal activity of actinomycetes culture filtrates with over 0.3 JHAN activity. One milliliter of culture filtrates was applied to larvae of *A. albopictus* in 4 ml of aged tap water. Mortality was evaluated after 72 h treatment. The error bars indicate the standard error of the mean. Different letters above the error bars indicate significant differences by Tukey HSD test ($P < 0.05$).

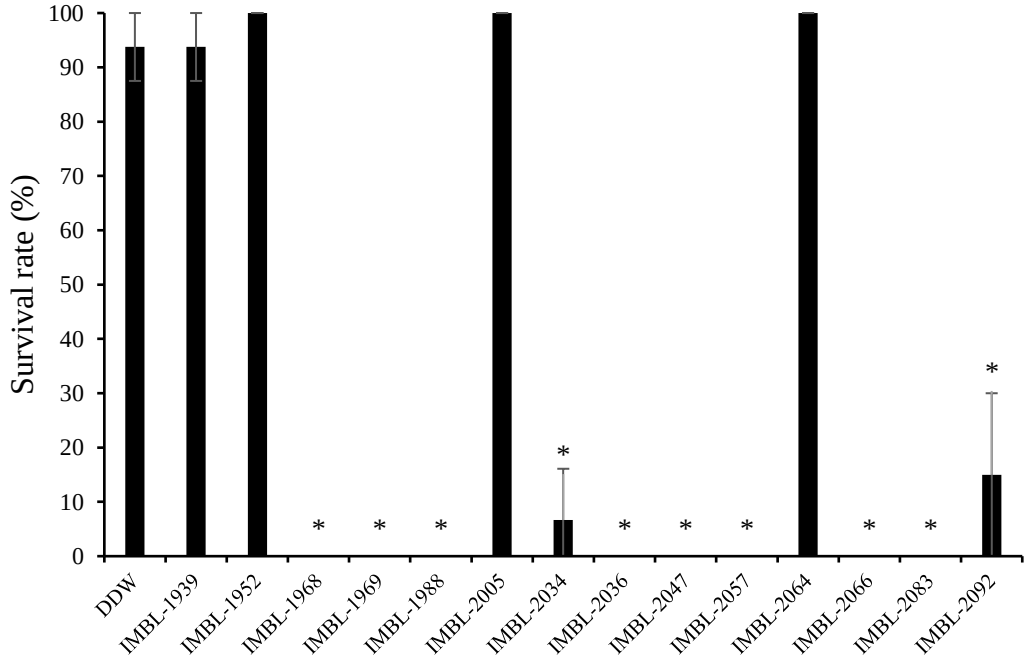


Fig. 4. Acute fish toxicity of actinomycetes culture filtrates against adult guppies. Ten milliliters of actinomycetes culture filtrates were treated with adult guppies in 1 L tap water. A number of dead guppies were recorded every 12 h for 2 days. The error bars indicate the standard error of the mean. Asterisks (*) indicate significant differences between survival rate of culture filtrates by Scheffé's test ($P < 0.05$).

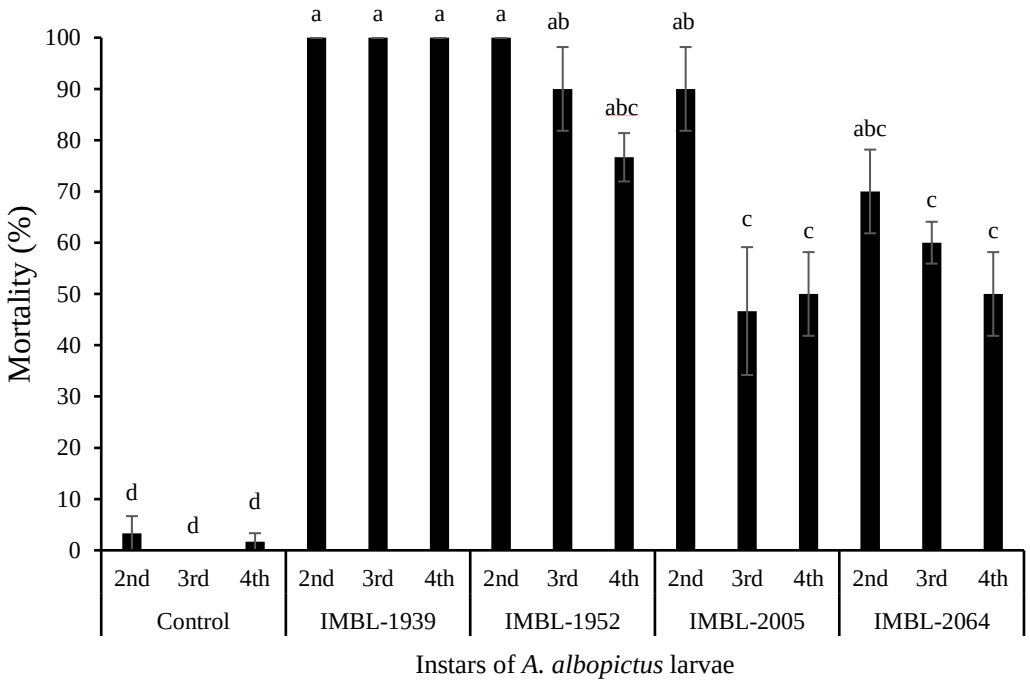


Fig. 5. Mosquito larvicidal activity of actinomycetes culture filtrates with JHAN activity against second, third and fourth instar larvae of *A. albopictus*. The mortality was calculated every 24 h for three days after treatment. The error bars indicate the standard error of the mean. Different letters above the error bars indicate significant differences by Scheffé's test ($P < 0.05$).

2) Morphological characteristics and phylogenetic analysis of mosquito larvicidal actinomycetes

Four selected strains formed a well-developed substrate and aerial mycelium in the tested media. Growth, the color of substrate mycelium, aerial mycelium, and the presence of pigmentation of selected actinomycetes strain in various ISP media were summarized in Table 7. Every actinomycete strain exhibited optimum growth at 30°C and pH at 7.0 ~ 9.0 with 0% NaCl concentration (Table 8).

Analysis of 16S rRNA sequence revealed that IMBL-1939, IMBL-1952, IMBL-2005, and IMBL-2064 were most closely related to *Streptomyces abikoensis* NBRC 13860 (99.79% similarity), *Kitasatospora aureofaciens* ATCC 10762 (99.65% similarity), *Streptomyces amphotericinius* 1H-SSA8 (99.14% similarity), and *Streptomyces resistomycificus* NRRL ISP-5133 (99.15% similarity) strain, respectively (Fig. 6). Four strains showed distinct clade with the most closely related strain.

3) JHAN and mosquito larvicidal activities of the selected actinomycetes culture extracts

To identify insecticidal JHAN compounds, the selected culture filtrates were solvent-extracted with n-hexane, ethyl acetate, and n-butanol. Each fraction was evaporated and diluted with ethanol at a higher concentration than the initial concentration upon extraction (Fig. 7). IMBL-1939 showed the highest JHAN activity in the n-hexane fraction among the selected actinomycetes strains. Ethyl acetate fractions of IMBL-1952, IMBL-2005, and IMBL-2064 showed relatively high JHAN activity, and these fractions showed JHAN

Table 7. Morphological characteristics of selected actinomycetes strain

Strain	Culture media	Growth	Aerial mycelium	substrate mycelium	Diffusible pigment
IMBL-1939	ISP-2	very good	light gray	brown	-
	ISP-4	very good	white	ivory	-
	ISP-5	very good	white	light brown	-
IMBL-1952	ISP-2	very good	gray	yellow	-
	ISP-4	very good	gray	ivory	-
	ISP-5	very good	ivory	light yellow	-
IMBL-2005	ISP-2	very good	white	yellow	-
	ISP-4	very good	white	ivory	-
	ISP-5	very good	ivory	ivory	-
IMBL-2064	ISP-2	very good	dark gray	yellow	-
	ISP-4	very good	dark gray	white	-
	ISP-5	good	dark gray	white	-

Table 8. Effect of salinity, pH, and temperature on the growth of selected actinomycetes strain

		IMBL-1939	IMBL-1952	IMBL-2005	IMBL-2064
Temperature (°C)	42	++	++	++	++
	37	++	++	++	++
	30	++	++	++	++
	23	++	++	++	++
pH	5	+	+	-	+
	6	++	++	-	+
	7	++	++	++	++
	8	++	++	++	++
	9	++	++	++	++
	10	++	++	++	+
	11	++	++	++	+
NaCl (%)	0.0	++	++	++	++
	2.5	+	+	+	-
	5.0	-	+	-	-
	7.5	-	+	-	-
	10.0	-	+	-	-

++ = good growth, + = poor growth, - = no growth

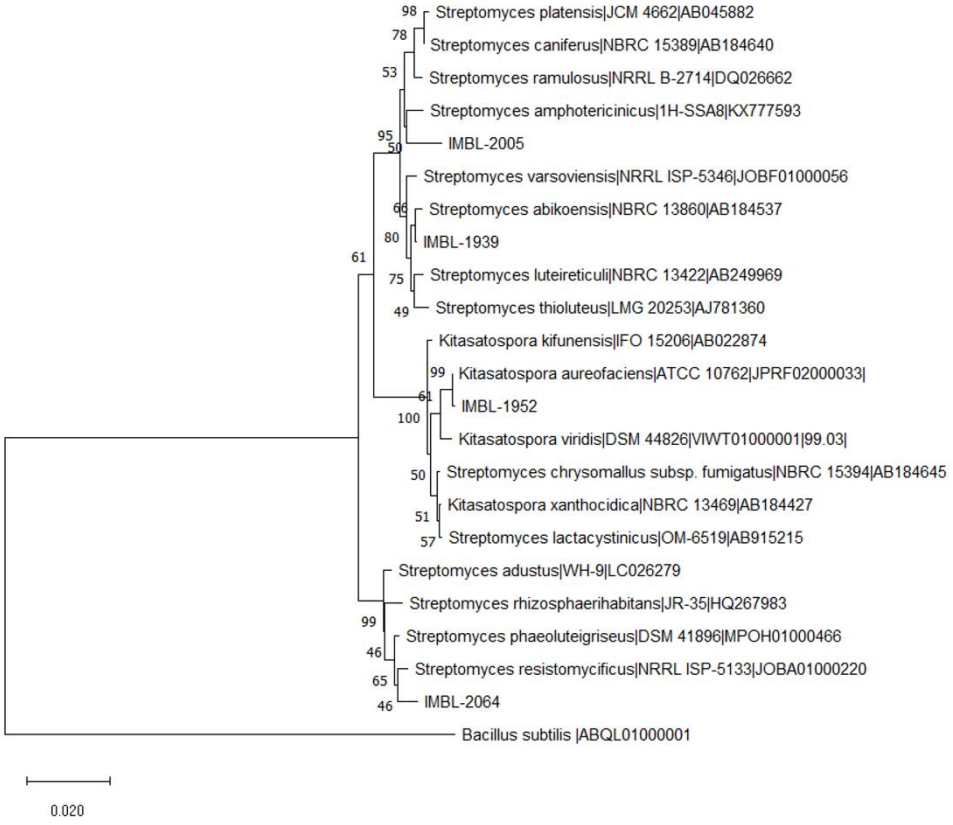


Fig. 6. Phylogenetic tree of selected actinomycetes strains based on its 16s rRNA sequence. Phylogenetic tree based on 16S rRNA gene sequences of selected actinomycetes and related taxa. The tree was constructed by the neighbor-joining method. Numbers at branch points indicate bootstrap percentages (based on 1,000 replicates). Scale bar indicates 0.02 substitution per nucleotide.

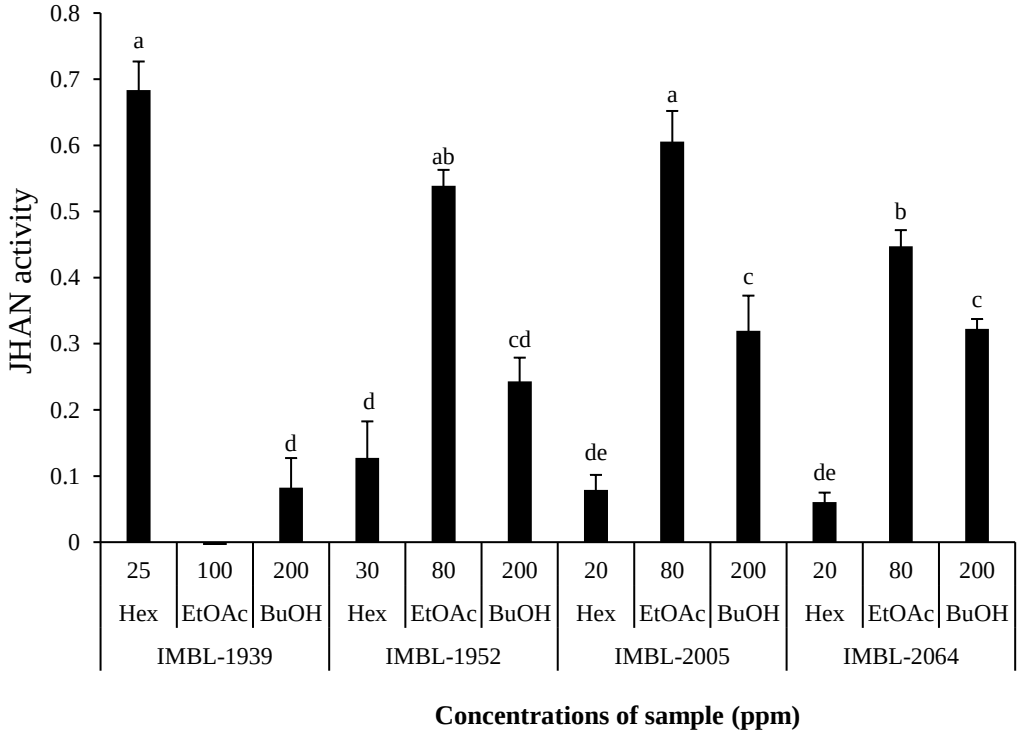


Fig. 7. JHAN activities of crude extracts from actinomycetes culture filtrates. To estimate the JHAN activity, 0.033 ppm of pyriproxyfen and crude extracts were applied to a yeast two-hybrid β-galactosidase assay. Crude extracts were dissolved with ethanol at various concentrations based on the amounts of each solvent extract. Different latter indicates significant differences by Scheffé's test ($P < 0.05$).

activity in a concentration-dependent manner (Fig. 8). The difference in the extraction solvent showing high JHAN activity suggested that JHAN compounds from IMBL-1939 and other strains could be different. In addition, a bioassay against 3rd instar larvae of *A. albopictus* was carried out with crude extracts which exhibited JHAN activity. n-Hexane fraction of IMBL-1939 showed high mosquito larvicidal activity against 3rd instar larvae of *A. albopictus* at a concentration of 100 ppm with mortality greater than 80% (Fig. 9). Ethyl acetate fractions of IMBL-1952 and IMBL-2005 also showed mortalities greater than 75% at a concentration of 200 ppm. As a result, IGR compounds from IMBL-1939 were selected for further investigation.

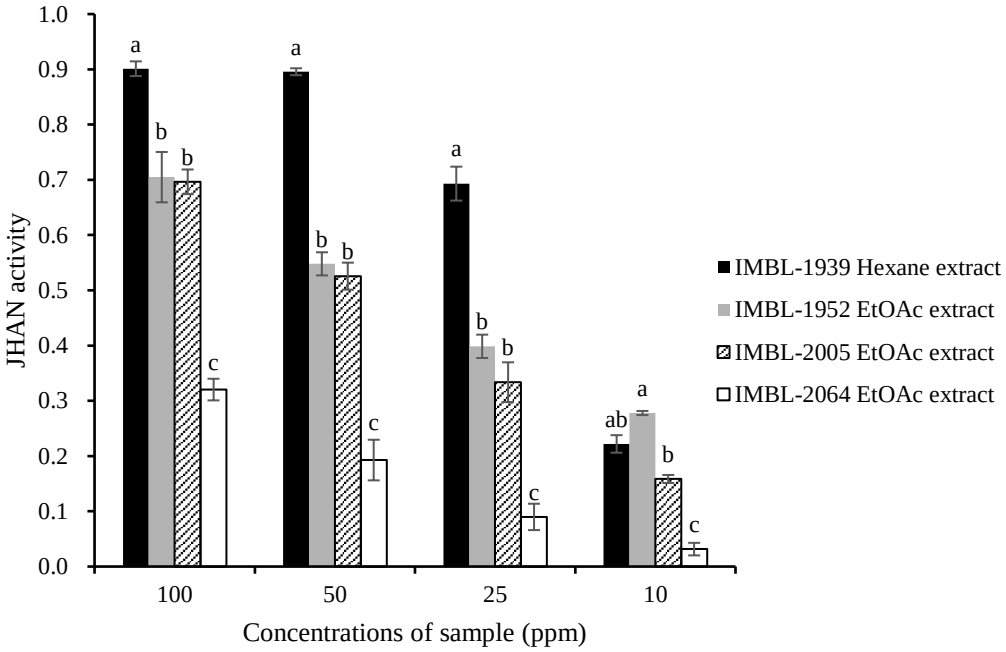


Fig. 8. Concentration-dependent JHAN activity of crude extracts from actinomycetes. To estimate the JHAN activity, 0.033 ppm of pyriproxyfen and crude extracts of actinomycetes culture filtrates were applied to a yeast two-hybrid β-galactosidase assay. The error bars indicate the standard error of the mean. Different latter indicates significant differences between each extract with same concentration by Scheffé's test ($P < 0.05$).

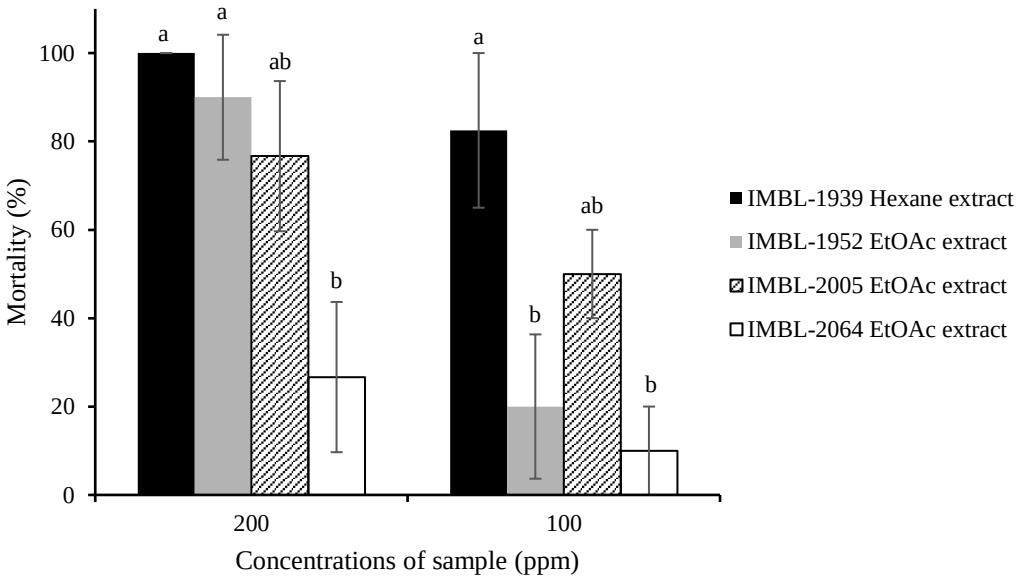


Fig. 9. Mosquito larvicidal activity of crude extracts from actinomycetes. Third instar larvae of *A. albopictus* were treated with 100 and 200 ppm of each extract diluted with ethanol. Mortality was calculated three days after treatment. The error bars indicate the standard error of the mean. Different latter indicates significant differences between each crude extract with the same concentration by Scheffé's test ($P < 0.05$).

Part 2. Identification and characterization of mosquito larvicidal compounds based on JHAN from *Streptomyces abikoensis* IMBL-1939 strain

1) Isolation and structural elucidation of JHAN and mosquito larvicidal compounds

Bioassay-guided fractionation was proceeded to isolate the main JHAN active compound from mosquito larvicidal *S. abikoensis* IMBL-1939 strain (Fig. 10). The n-hexane extract of IMBL-1939 was fractionated by open column chromatography with n-hexane: Ethyl acetate 9:1, and the fractions with the same Rf values were pooled and concentrated with a rotary evaporator. Three active compounds were isolated from n-hexane extracts (Fig. 11A). Rf values of each fraction were 0.55, 0.45, and 0.42 (n-hexane: ethyl acetate = 3: 1)

A compound in fraction 1, most nonpolar, showed the highest JHAN activity and mosquito larvicidal activity (Fig. 11B and 11C). Fraction 1 was soluble in methanol, ethanol, and DMSO. In GC-MS analysis, the molecular weight of fraction 1 was measured as 255 (Fig. 12). By HR-LC-MS analysis, fraction 1 was observed in positive ESI mode, and molecular mass was determined as $[M+H]^+$ m/z = 256.1488 (Fig. 13). The structure of fraction 1 was verified as carbazomycin A by comparing its NMR pattern and MS data (Fig. 14-20). The chemical shift of carbazomycin A in ^1H, ^{13}C NMR is summarized in Table 9.

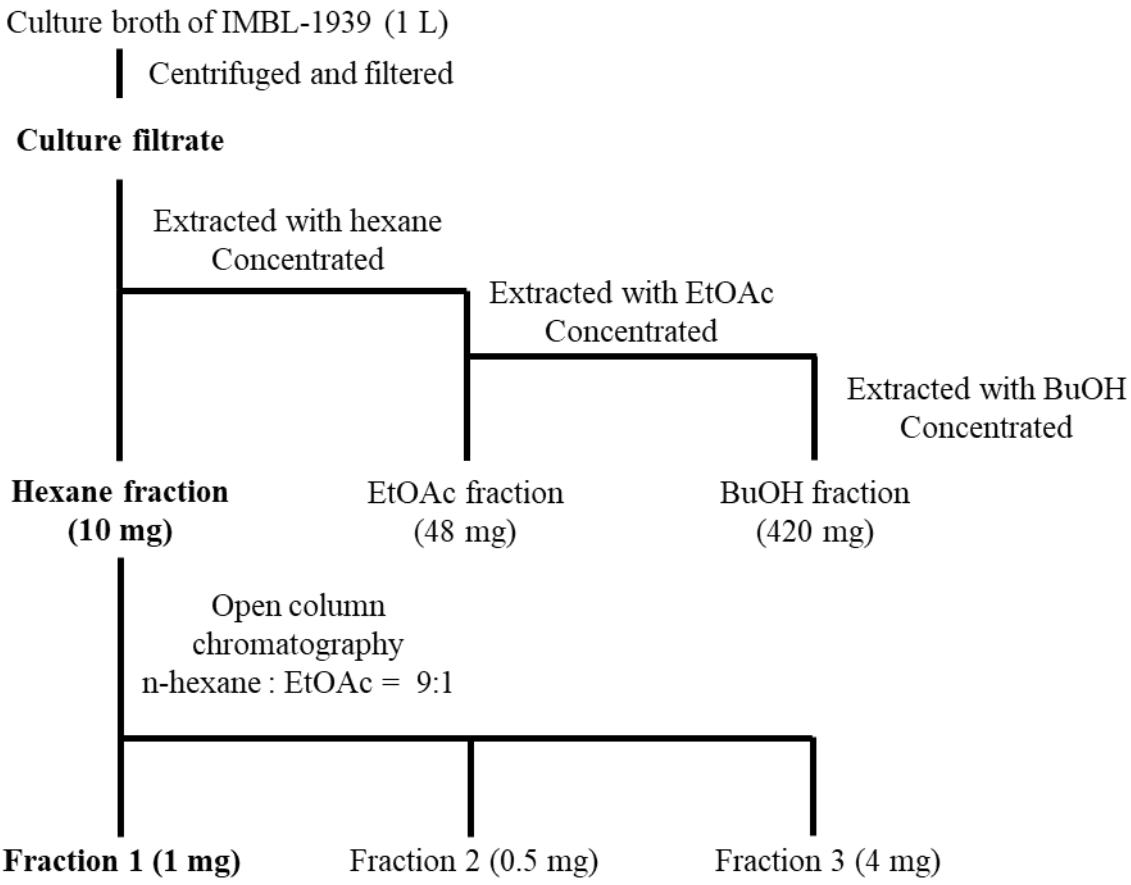


Fig. 10. Flow chart of isolation of JHAN compounds from *Streptomyces abikoensis* IMBL-1939 strain. Bold fractions exhibited the highest mosquito larvicidal activity.

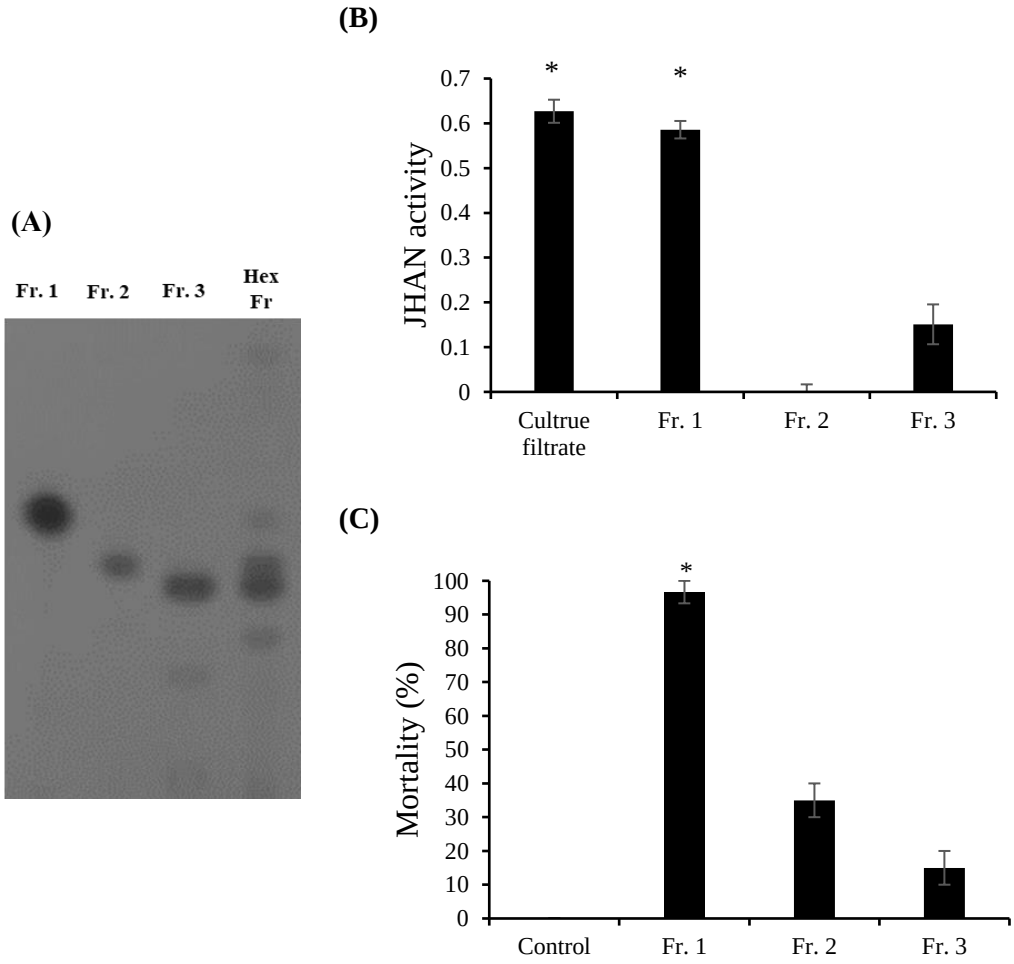


Fig. 11. Isolation of JHAN compound from *S. abikoensis* IMBL-1939 strain. (a) isolated fractions on TLC (n-hexane: ethyl acetate = 3: 1); (B) JHAN activity of isolated fractions; (C) Mosquito larvicidal activity of isolated fractions. The error bars indicate the standard error of the mean. Asterisks (*) above the error bars indicate significant differences by Scheffé's test ($P < 0.05$).

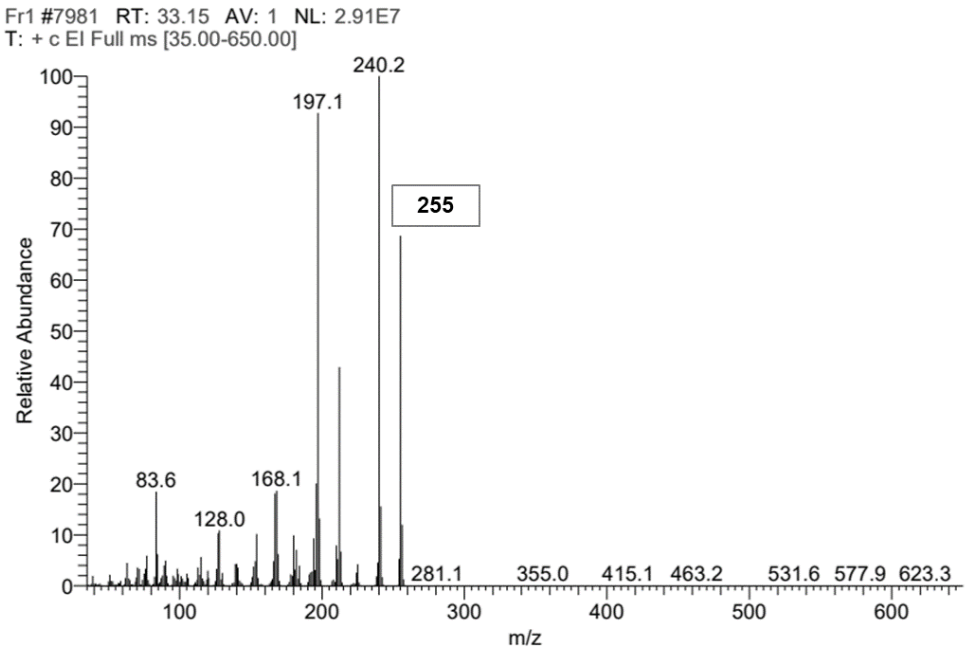


Fig. 12. GC-MS spectrum and fragmentation pattern of fraction 1.

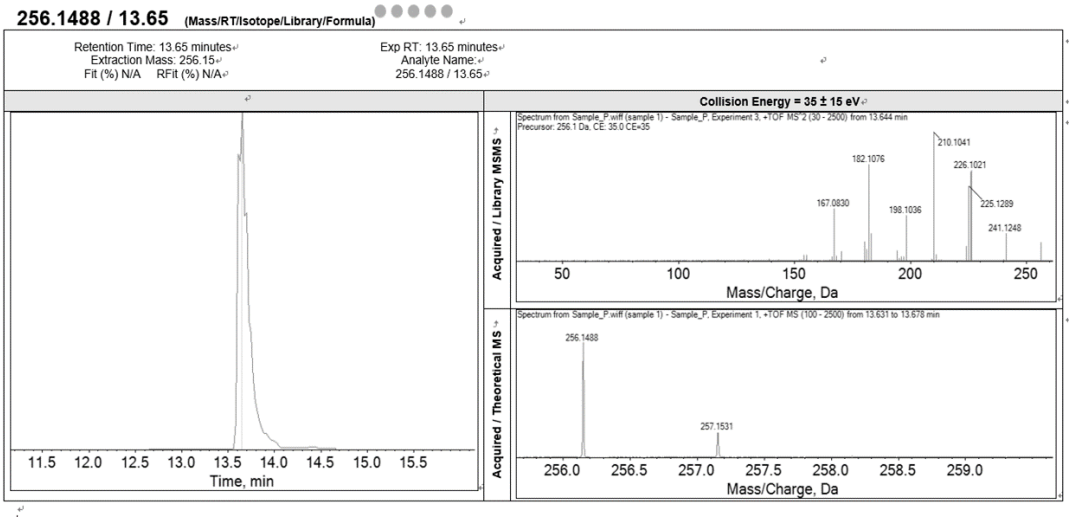


Fig. 13. LC-MSMS fragmentation pattern of fraction 1.

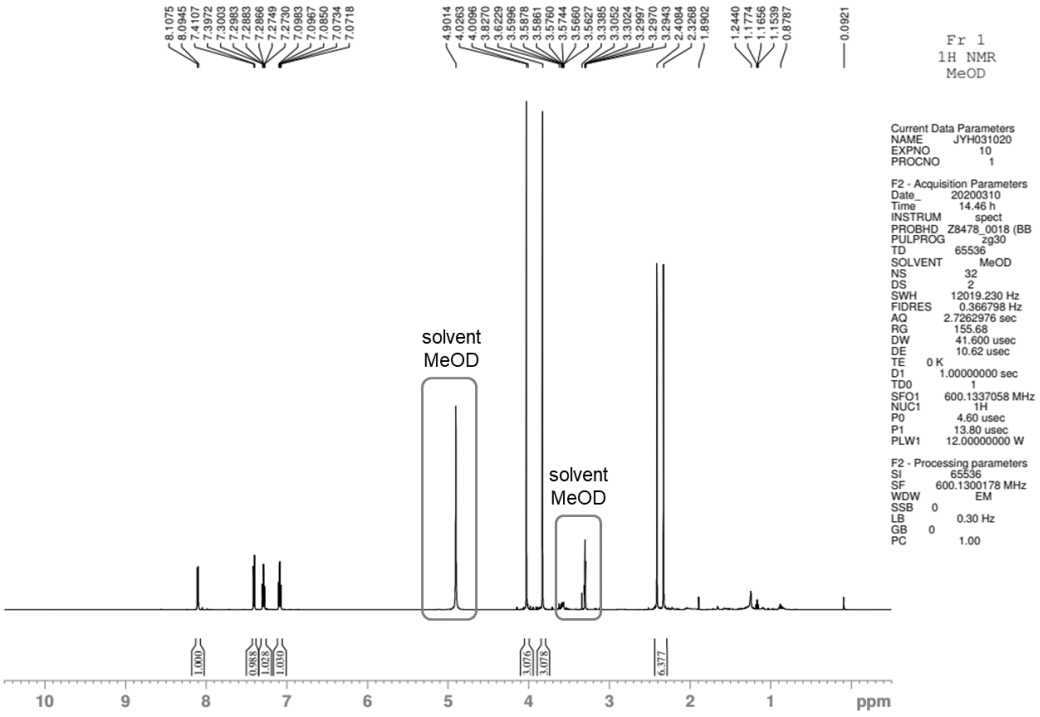


Fig. 14. ^{1}H NMR spectrum of fraction 1.

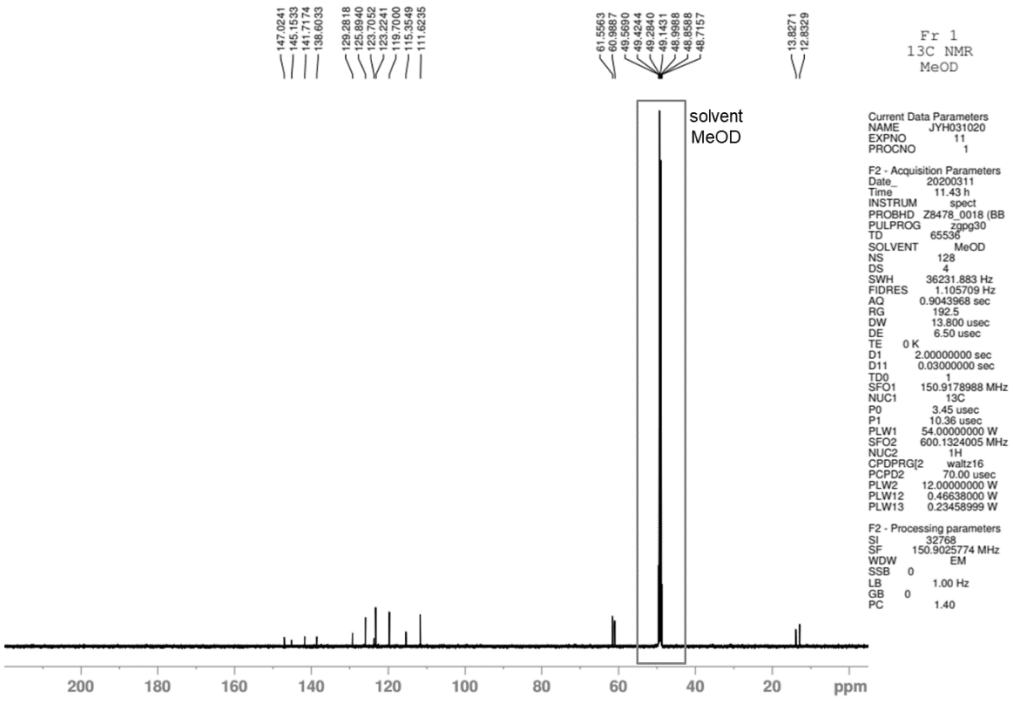


Fig. 15. ^{13}C NMR spectrum of fraction 1.

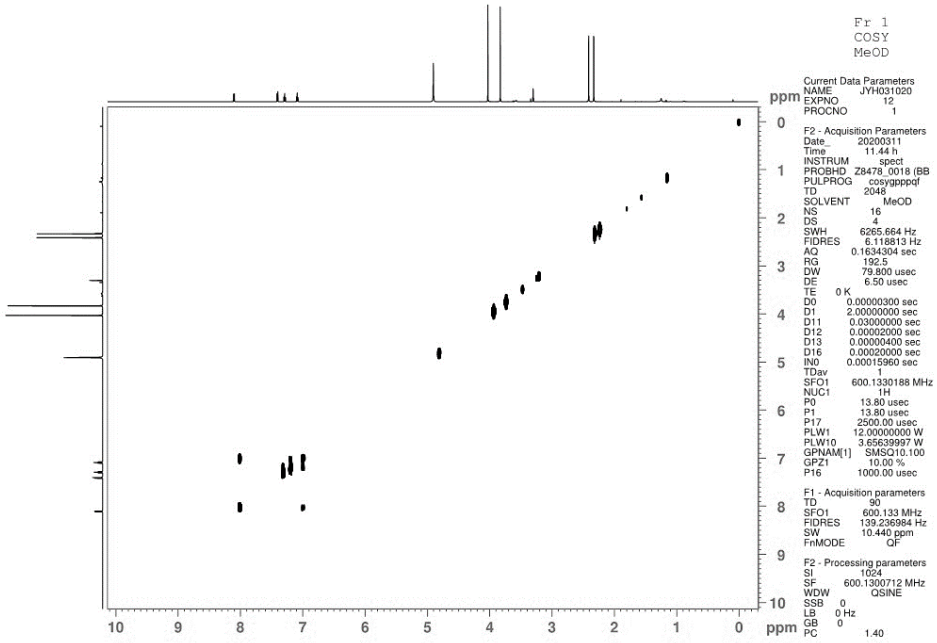


Fig. 16. COSY spectrum of fraction 1.

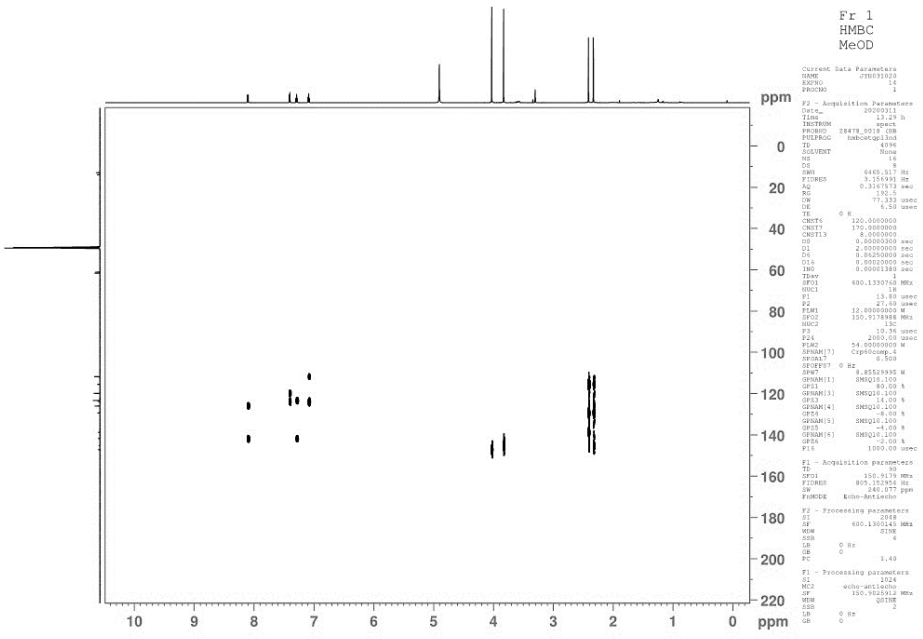


Fig. 17. HMBC spectrum of fraction 1.

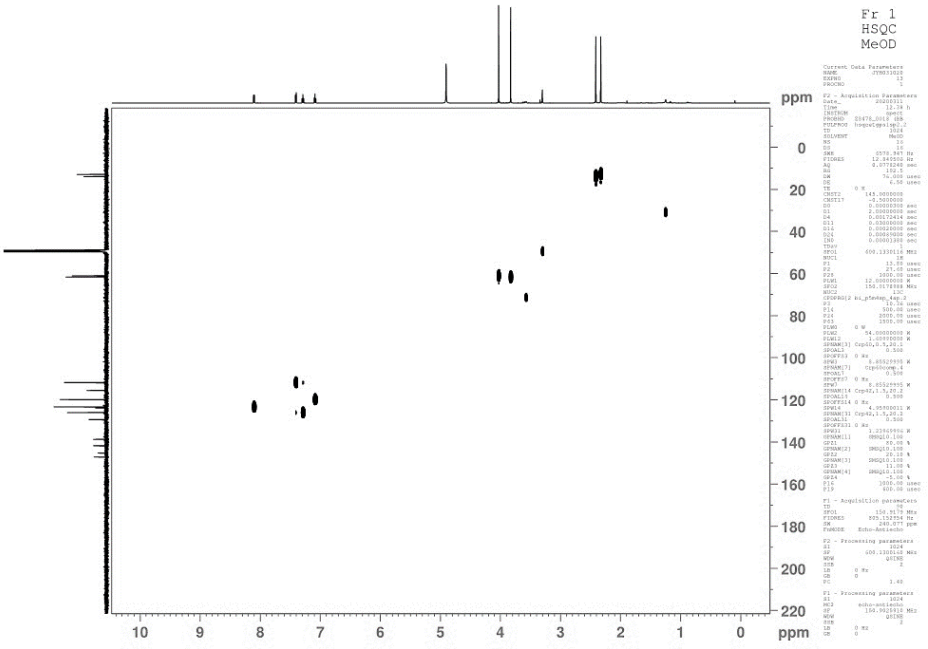


Fig. 18. HSQC spectrum of fraction 1.

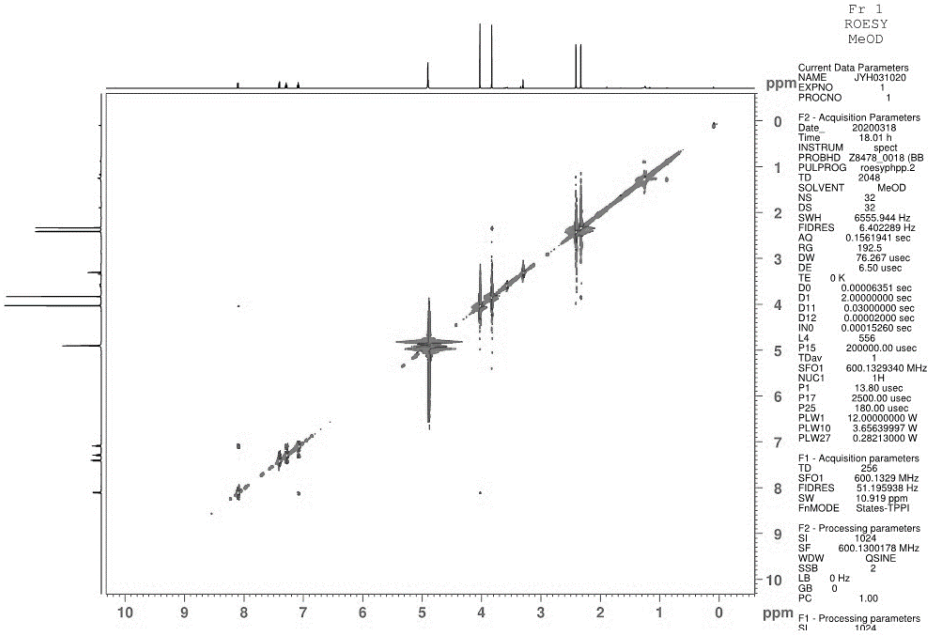


Fig. 19. ROESY spectrum of fraction 1.

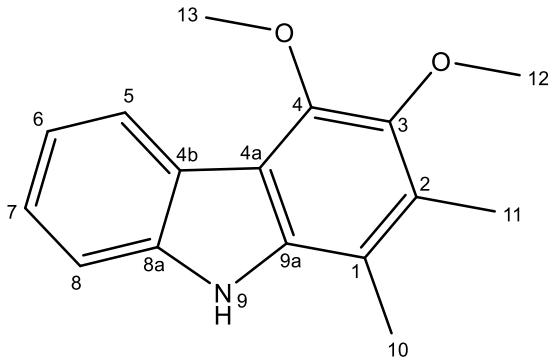


Fig. 20. Structure of carbazomycin A.

Table 9. Observed chemical shift of carbazomycin A

Position	^{1}H	^{13}C
1		115.2 s
2		129.1 s
3		145.0 s
4		146.9 s
4a		115.2 s
4b		123.6 s
5	8.10	123.1 d
6	7.09	119.6 d
7	7.28	125.7 d
8	7.40	111.5 d
8a		141.6 s
9a		138.5 s
10	2.40	13.7 q
11	2.33	12.7 q
12	3.83	61.4 q
13	4.03	61.4 q

The molecular weight of compounds in fractions 2 and 3 was revealed as 285 and 241 by GC-MS analysis. Their structures were confirmed as carbazomycin D and B by comparing the previously reported TLC pattern and molecular weight (Fig. 21, 22).

2) IGR and mosquito larvicidal activities of carbazomycin

Carbazomycin derivatives were subjected to a concentration-dependent yeast two-hybrid β-galactosidase assays to evaluate their IGR activity. Carbazomycin A demonstrated inhibition of pyriproxyfen mediated binding of juvenile hormone receptor complex Met-FISC of *Aedes aegypti* in a concentration-dependent manner without exhibiting any yeast growth inhibition (Fig. 23). Meanwhile, carbazomycin B and D showed relatively low JHAN activity than carbazomycin A.

In mosquito larvae bioassays, carbazomycin A showed strong larvicidal activity with LC_{50} values of 4.8 ppm and IE_{50} (inhibition of adult emergence) values of 2.0 ppm. Carbazomycin B and D did not show significant mosquito larvicidal activity under 20 ppm (Table 10).

3) Effects of carbazomycin A on transcription level

The relative transcription levels of juvenile hormone response elements, *Hairy* and *Kr-h1* in the adult female mosquitoes were measured by qPCR (Fig. 24). In acetone-treated mosquitoes, relative transcription levels of both genes were increased until 18 h in *Hairy*, and 6 h in *Kr-h1* and then decreased. In pyriproxyfen-treated mosquitoes, the transcription levels increased rapidly after 6 h after treatment, and the peak levels were also increased

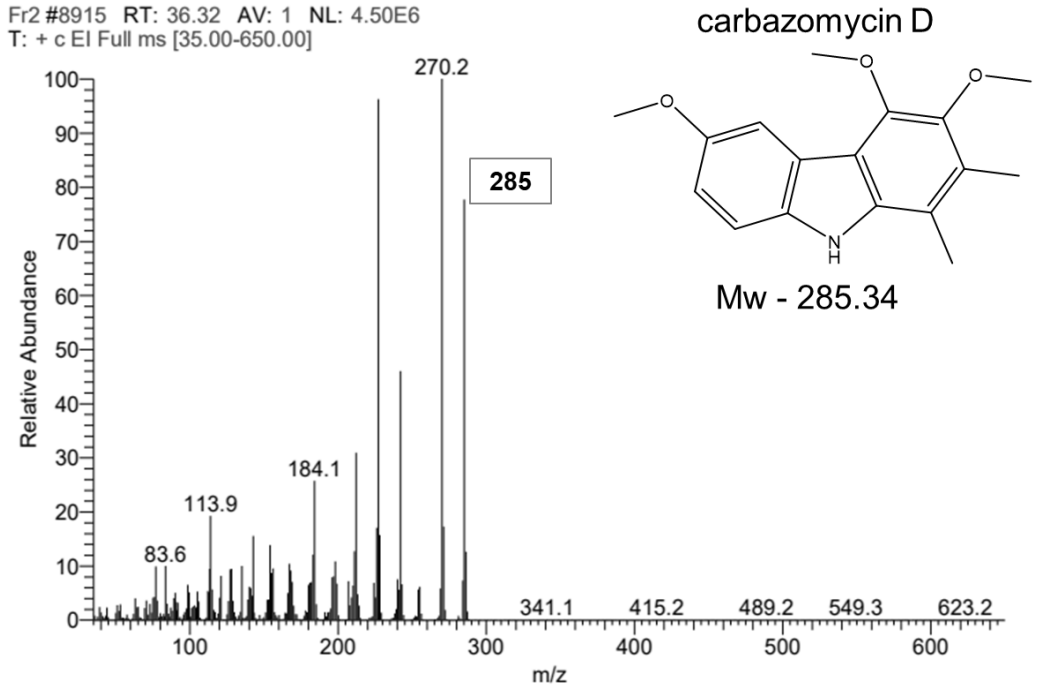


Fig. 21. GC-MS spectrum of fraction 2 and structure of carbazomycin D.

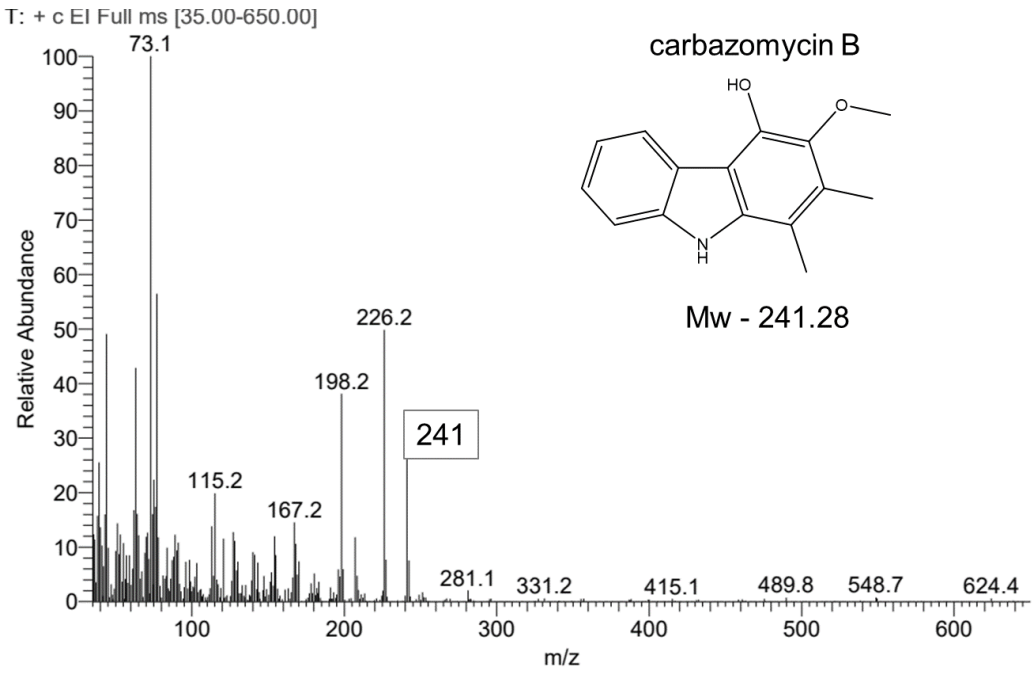


Fig. 22. GC-MS spectrum of fraction 3 and structure of carbazomycin B.

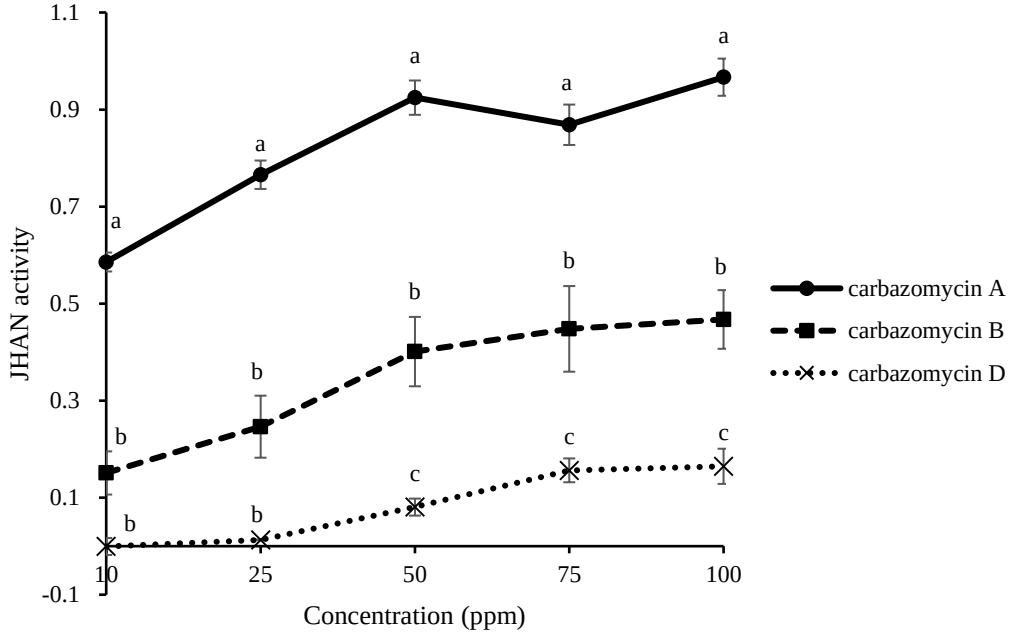


Fig. 23. Concentration-dependent JHAN activity of carbazomycin derivatives. 0.033 ppm of pyriproxyfen and various concentrations of carbazomycin derivatives were applied to a yeast two-hybrid β-galactosidase assay. The error bars indicate the standard error of the mean. Different letters above the error bars indicate significant differences of JHAN activity between carbazomycin with same concentration by Scheffé's test ($P < 0.05$).

Table 10. Lethal concentration (LC_{50}) and inhibition of adult emergence (IE) of carbazomycins against *A. albopictus* larvae

	LC_{50} (ppm) (95% Cl) [a]	LC_{90} (ppm) (95% Cl) [a]	Slope ±SE	Intercept ±SE	X^{2} [b]	Df
Carbazomycin A	4.8 (4.3 ~ 5.5)	11.4 (8.8 ~ 14.9)	3.39 ± 0.37	-2.31 ± 0.25	22.28 (0.01)	9
Carbazomycin B	> 20	> 20				
Carbazomycin D	> 20	> 20				
	IE_{50} (ppm) (95% Cl)	IE_{90} (ppm) (95% Cl)	Slope ±SE	Intercept ±SE	X^{2} [b]	Df
Carbazomycin A	2.1 (1.6 ~ 2.6)	6.1 (4.5 ~ 8.8)	2.20 ± 0.37	-0.68 ± 0.23	4.05 (0.54)	5
Carbazomycin B	> 20	> 20				
Carbazomycin D	> 20	> 20				

a. Lethal concentration after 48 h treatments
b. chi-square (*P*-value)
Cl; confidence limits, SE; standard error, DF; degree of freedom, IE; inhibition of adult emergence

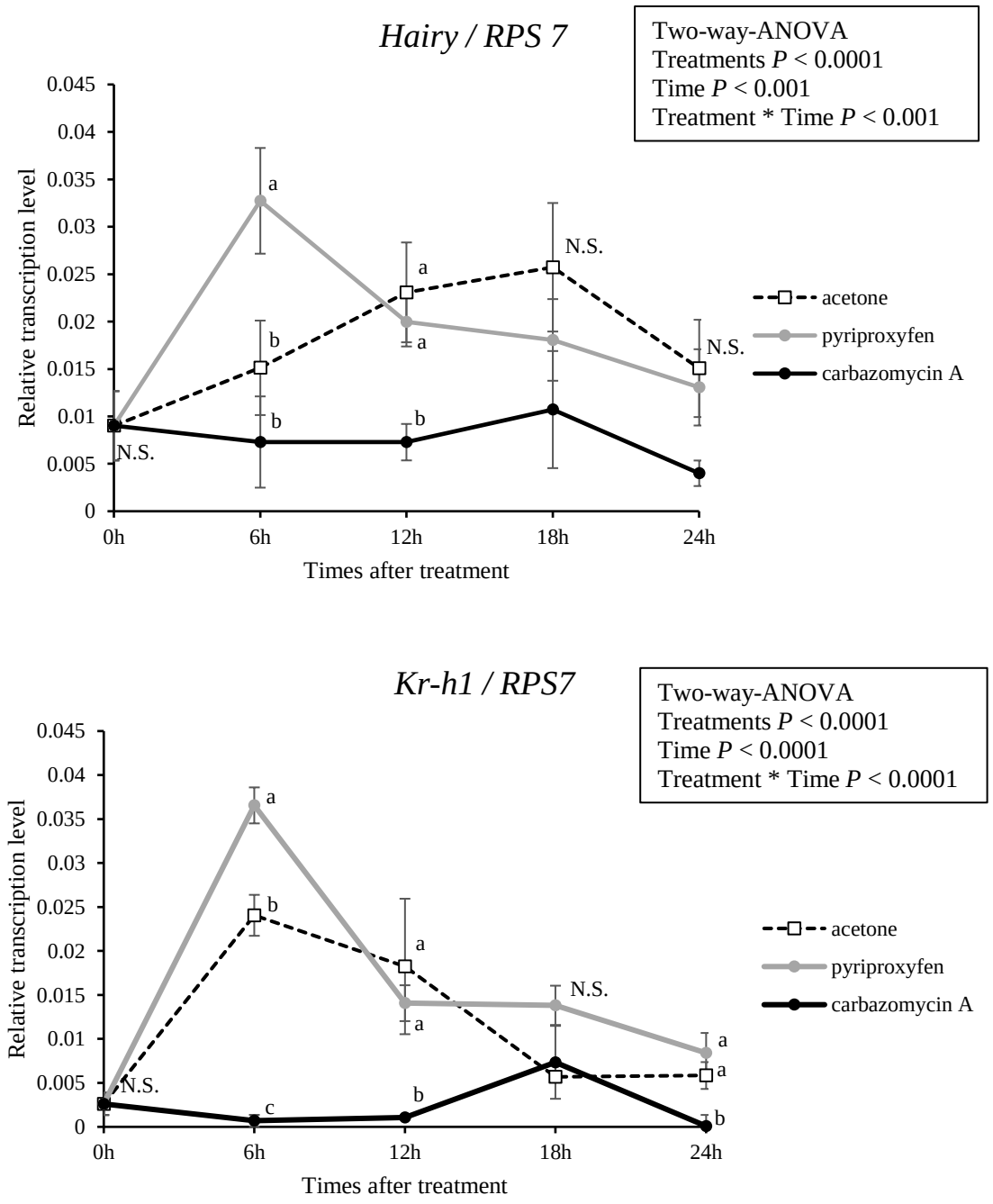


Fig. 24. Effect of carbazomycin A in the relative transcription level of juvenile hormone response elements, *Hairy* (upper), and *Kr-h1* (lower). Female *A. albopictus* adults, which eclosed within 1 h were topically treated with acetone, pyriproxyfen (JHA), and carbazomycin A (JHAN). Total RNA was extracted at various time points and subjected to qPCR to analyze the relative transcription levels. The error bars indicate the standard error of the mean. Results of two-way ANOVA are presented in the square. Different letters indicate significant differences between samples with same RNA extraction time by Scheffé's test ($P < 0.05$). N.S., not significant.

by 50% more than acetone-treated mosquitoes. In contrast, carbazomycin A treated mosquitoes exhibited significantly decreased relative transcription levels in both genes at all times.

4) Ovary development inhibition activity

Carbazomycin A caused mortality in female adult mosquitoes and decreased the blood ingestion rate by 40% (Table 11). Only 20% of mosquitoes treated with carbazomycin A showed a fully developed ovary (Fig. 25). Whereas normally developed ovaries showed an elliptical shape, undeveloped ovaries in carbazomycin A treated mosquitoes were round-shaped with undeveloped follicles.

5) Non-target toxicity of carbazomycin A

To evaluate the non-target toxicity of carbazomycin A, bioassays against *Bombyx mori* and *Apis mellifera* were carried out (Fig. 26). Carbazomycin A and n-hexane extract did not show significant toxicity against *A. mellifera*. In silkworm bioassay, n-hexane fraction, carbazomycin A, and pyriproxyfen did not show acute toxicity at a concentration of 2 ppm (Fig. 27). Also, n-hexane fraction and carbazomycin A did not show acute fish toxicity against adult guppies (Fig. 28).

In anti-cyanobacterial tests against harmful cyanobacteria *M. aeruginosa*, carbazomycin A and hexane extract exhibited a higher anti-cyanobacterial activity than ampicillin. Carbazomycin A showed similar level of anti-cyanobacterial activity with streptomycin at 10 ppm concentration (Fig. 29).

Table 11. Adult toxicity and inhibition of ovary development of carbazomycin A.

	Mortality (%)	Blood ingestion rate (%)	Ovary development rate (%)
Acetone (Control)	6.73 (5.15 – 11.88)	71.47 (65.08 – 77.87)	68.91 (63.80 – 74.02)
Carbazomycin A	40.0 (33.96 – 46.04)	30.74 (18.92 – 42.56)	20.47 (13.50 – 27.44)

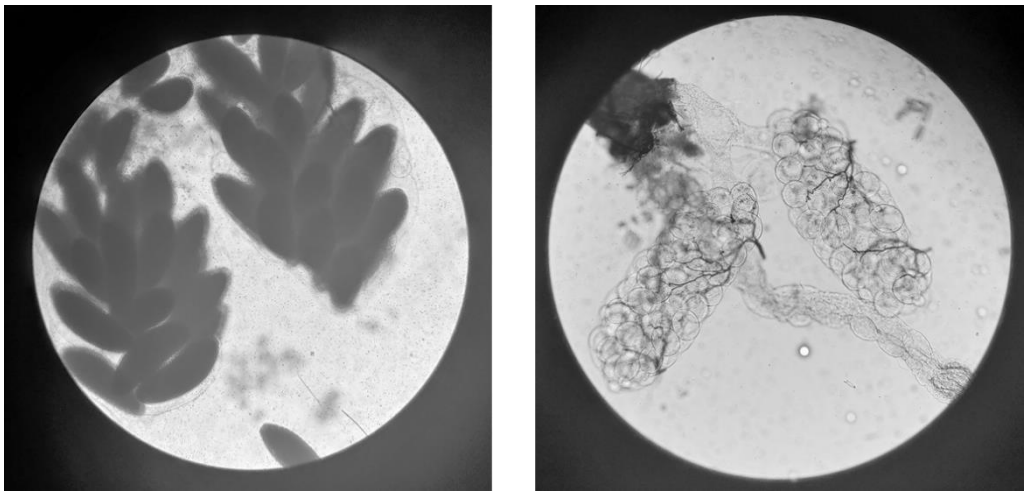


Fig. 25. Effect of carbazomycin A in ovary development. Fully developed acetone treated ovary (left); retarded carbazomycin A treated ovary (right).

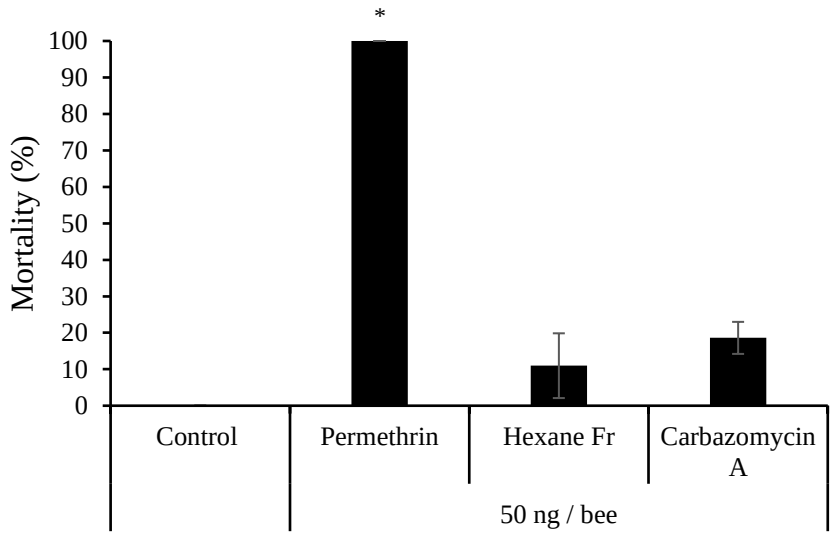


Fig. 26. Acute contact toxicity of carbazomycin A on honey bee. *Apis mellifera* workers were treated with 50 ng of acetone diluted permethrin (positive control), carbazomycin A, and n-hexane extracts of IMBL-1939. Mortality was calculated two days after treatment. The error bars indicate the standard error of the mean. Asterisk (*) above the error bars indicates significant differences by Scheffé's test ($P < 0.05$).

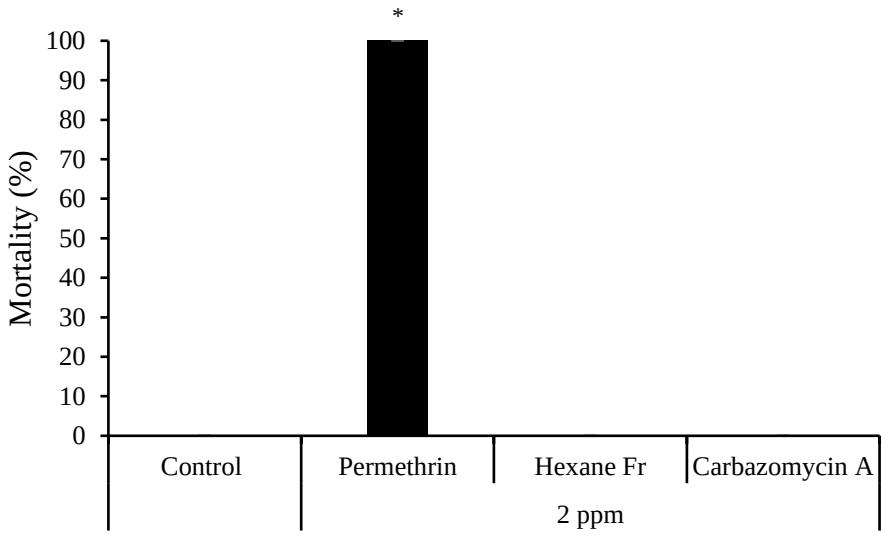


Fig. 27. Acute oral toxicity of carbazomycin A on silkworm. Mulberry leaves dipped in 2 ppm of carbazomycin A, and permethrin (positive control) treated water were fed on 3rd instar larvae of silkworm. Mortality was calculated two days after treatment. The error bars indicate the standard error of the mean. Asterisk (*) above the error bars indicates significant differences by Scheffé's test ($P < 0.05$).

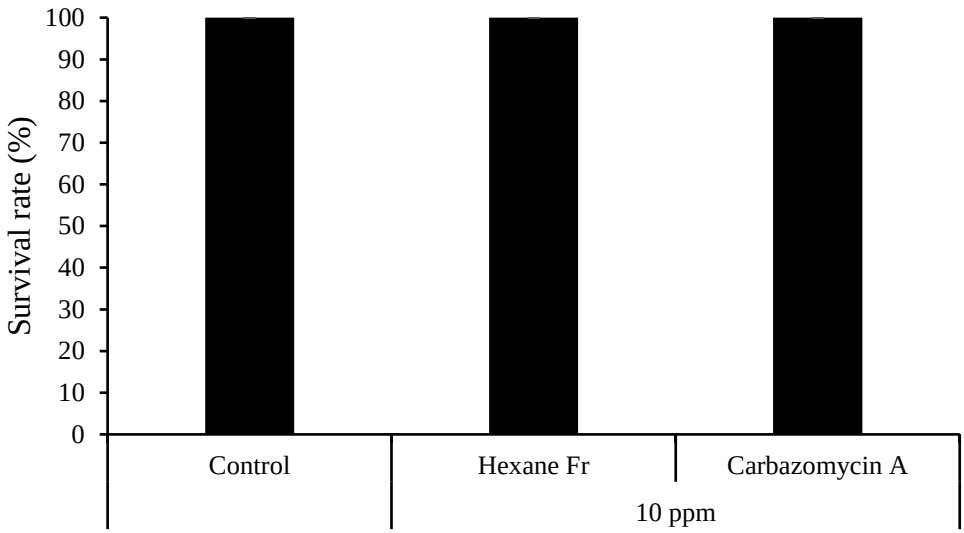


Fig. 28. Acute fish toxicity of carbazomycin A against adult guppies. Carbazomycin A and n-hexane extract were dissolved in ethanol and treated with adult guppies in 1 L tap water. Mortality was calculated two days after treatment. The error bars indicate the standard error of the mean.

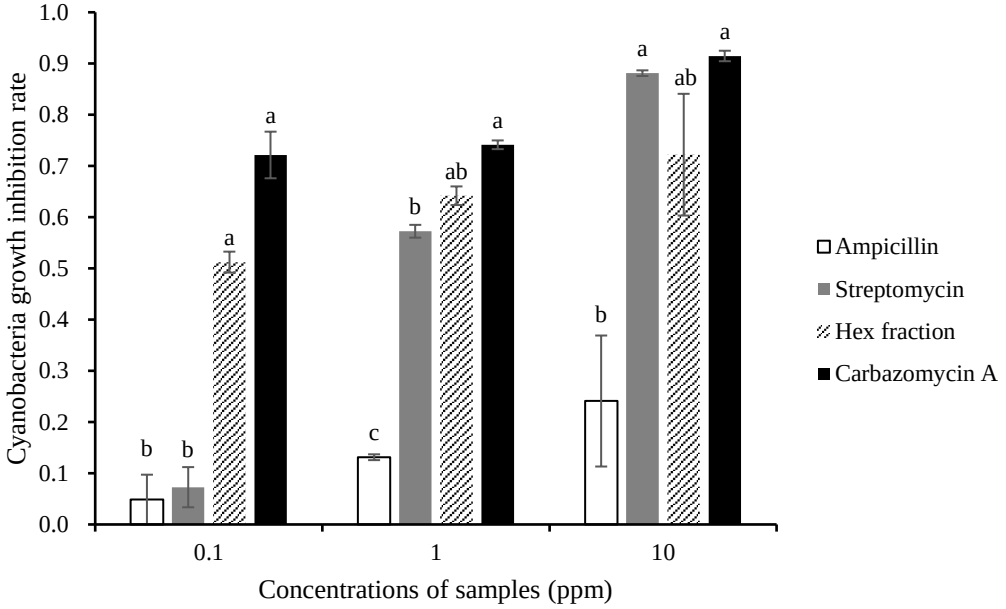


Fig. 29. Anti-cyanobacterial activity of carbazomycin derivatives on *Microcystis aeruginosa.* Carbazomycin A and n-hexane extract dissolved in ethanol were treated with 1% with the final concentration of 0.1, 1, and 10 ppm. Optical densities of 650nm were measured 24 h after treatment. The error bars indicate the standard error of the mean. Different letters above the error bars indicate significant differences between samples with the same concentration by Scheffé's test ($P < 0.05$).

Part 3. Applications of carbazomycin derivatives as bio-pesticides

6) Initial media selection

n-Hexane extracts of culture filtrates from various mediums were spotted on TLC to examine relative carbazomycins productions. As a result, GYM media showed the highest yield in both carbazomycin A and B (Fig. 30). Component of GYM was selected for carbazomycin A and B productions for further optimization,

7) Screening of factors using Plackett-Burman design

Through the Plackett-Burman design, components of the GYM medium and flask type were analyzed for their effects on carbazomycin A and B productions. The designed Plackett-Burman matrix and response on carbazomycin A and B production are shown in Table 12. Each variable was analyzed to evaluate its effects on carbazomycin production. The main effects of each variable on carbazomycin A and B production were estimated, and the significance of each variable was evaluated by calculated *P* values (Table 13).

As a result, glucose concentration significant positively affected both carbazomycin A and B productions. Meanwhile, flask type without baffle showed positive effects on carbazomycin B production. Malt extract had a negative effect with low significance (main effect: -0.194, *P*-value: 0.454) on the production of carbazomycin A. On the contrary, malt extract had a positive effect (main effect: 0.2391, *P*-value: 0.069) on the production of carbazomycin B. Effects of other variables in carbazomycin productions were insignificant, so initial concentrations were maintained.

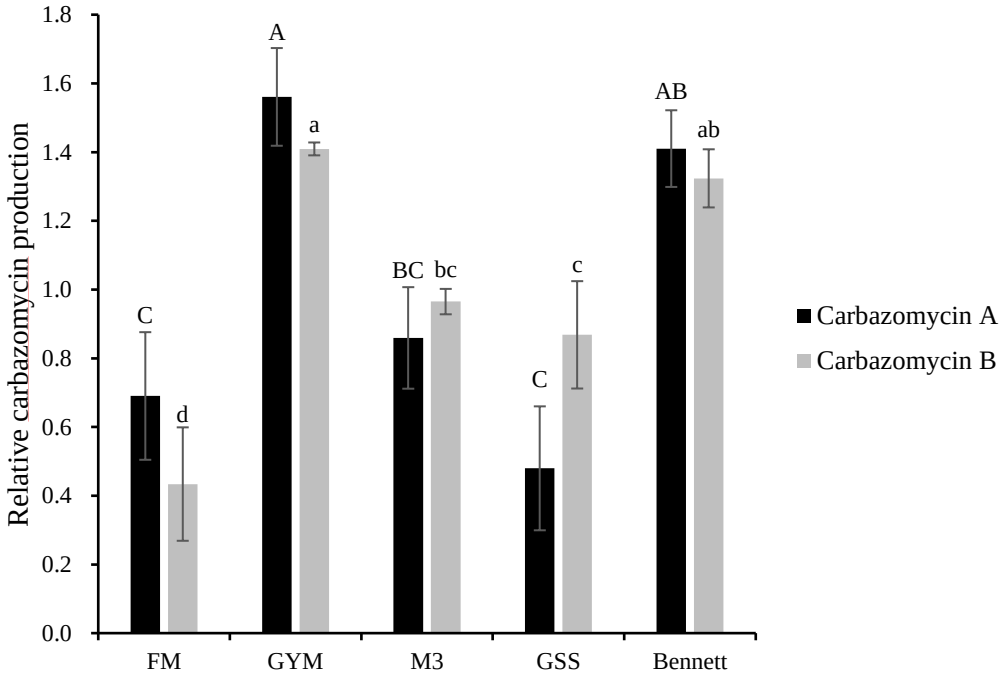


Fig. 30. Relative carbazomycins productions in various mediums for initial media selection. Relative productions were measured by the TLC pattern. The error bars indicate the standard error of the mean. Different letters above the error bars indicate significant differences by Scheffé's test ($P < 0.05$); upper-case letters refer to the production of carbazomycin A and lower-case letters refer to the production of carbazomycin B.

Table 12. Experimental design and responses using the Plackett–Burman design of 12 trials

	Variables					Relative production	
Trial. no	Yeast extract	Glucose	Malt extract	Calcium carbonate	Flask type	Carbazomycin A	Carbazomycin B
1	-1	1	-1	-1	-1	2.436	1.266
2	1	1	-1	1	-1	1.502	1.157
3	1	-1	1	-1	-1	1.020	0.986
4	1	1	-1	1	1	1.287	0.730
5	-1	1	1	1	-1	1.281	1.437
6	-1	-1	-1	1	1	0.613	0.861
7	-1	1	1	-1	1	1.110	1.285
8	1	-1	1	1	-1	0.580	1.220
9	-1	-1	1	1	1	0.655	0.615
10	1	-1	-1	-1	1	0.203	0.536
11	-1	-1	-1	-1	-1	0.544	0.732
12	1	1	1	-1	1	0.772	1.174

Table 13. Statistical analysis of Plackett-Burman design for each variable affecting the production of carbazomycin A and B

Production of carbazomycin A				
Variables	Main effect	Coefficient (± SE)	F-value	*P*-value
Intercept		1.00 (0.8786 – 1.1214)	3.13	0.098
Yeast extract	-0.0213	-0.106 (-0.2277 – 0.0151)	0.77	0.415
Glucose	0.7955	0.398 (0.277 – 0.519)	10.74	0.017
Malt extract	-0.1945	-0.097 (-0.218 – 0.024)	0.64	0.454
Calcium carbonate	-0.0278	-0.014 (-0.135 – 0.107)	0.01	0.913
Flask type	-0.4538	-0.227 (-0.348 - -0.106)	3.50	0.111

Production of carbazomycin B				
Variables	Main effect	Coefficient (± SE)	F-value	*P*-value
Intercept		0.9999 (0.9459 – 1.0539)		0
Yeast extract	-0.0655	-0.0327 (-0.0867 – 0.0213)	0.37	0.567
Glucose	0.3497	0.1749 (0.1209 – 0.2289)	10.49	0.018
Malt extract	0.2391	0.1195 (0.0655 – 0.1735)	4.90	0.069
Calcium carbonate	0.0065	0.0033 (-0.0507 – 0.0573)	0.00	0.954
Flask type	-0.2660	-0.133 (-0.187 - -0.079)	6.07	0.049

Flask type is a categorical factor, so flask without baffle was selected for optimized condition. Two continuous factors, the concentration of glucose and malt extract, were further optimized by response surface methodology (RSM).

3) Optimization of factors using response surface methodology

A central composite design was used to search for the optimum glucose and malt extract combination for carbazomycin A and B production. Table 14 shows the design matrix and responses. A more significant polynomial model was selected based on model fit statistics.

In the carbazomycin A production, the two-factor interaction model (2FI) was selected for the regression model due to its statistical values. The ANOVA of the 2FI model demonstrates the F-value (9.79), low *P*-value (0.01), and lack of fit *P*-value (0.578), which suggests that this model is significant (Table 15). The parameters of the equation were determined by 2FI regression.

$$\text{Carbazomycin A production} = 0.9884 + 0.24\ \text{glucose} + 0.04\ \text{malt extract} - 0.16\ \text{glucose} \times \text{malt extract}$$

In the carbazomycin B production, linear regression was selected for the regression model. The ANOVA for carbazomycin B was shown in Table 16, and the F-value parameter (6.43), *P*-value model (0.02), and lack of fit *P*-value (0.218) implies the model is significant and the regression equation was obtained in the same way.

Table 14. Central composite design and results

	Variables		Relative production	
Trial. no	Glucose	Malt extract	Carbazomycin A	Carbazomycin B
1	0	-1.4142	0.858	0.921
2	0	0	1.06	1.171
3	-1.4142	0	0.714	0.664
4	0	1.4142	0.836	1.171
5	0	0	0.885	1.105
6	1.4142	0	1.251	0.985
7	1	-1	1.437	1.158
8	1	1	1.271	1.37
9	-1	-1	0.544	0.792

Table 15. 2FI regression model of carbazomycin A production

	Coefficient ± SE	Df	MS	F-value	*P*-value
Model	0.9884 ± 0.0439	3	0.1883	9.79	0.0100
Glucose	0.2369 ± 0.049	1	0.4491	23.34	0.0029
Malt extract	0.0359 ± 0.049	1	0.0103	0.5346	0.4922
Glucose × malt extract	-0.1625 ± 0.0694	1	0.1056	5.49	0.0576
Residual		6	0.0192		
Lack of Fit		5	0.0200	1.31	0.5780
Pure Error		1	0.0153		

R^2= 0.8303, adj R^2=0.7455, pred R^2=0.3756
SS, sum of squares; MS, mean of square; Df, degree of freedom

Table 16. linear regression model of carbazomycin B production

	Coefficient ± SE	Df	MS	F-value	*P*-value
Model	1.01 ± 0.0474	2	0.1448	6.43	0.026
Glucose	0.1779 ± 0.0530	1	0.2531	11.24	0.0122
Malt extract	0.0676 ± 0.0530	1	0.0365	1.62	0.2434
Residual		7	0.0225		
Lack of Fit		6	0.0259	11.89	0.2184
Pure Error		1	0.0022		

R^2= 0.6476, adj R^2=0.5470 pred R^2=0.2540
SS, sum of squares; MS, mean of square; Df, degree of freedom

$$\text{Carbazomycin B production} = 1.01040 + 0.177870\ \text{glucose} + 0.067569\ \text{malt extract}$$

A three-dimensional surface model was used to assess the effect of glucose and malt extract on carbazomycin production (Fig. 31 and 32). Glucose showed a linear increase in carbazomycin A and B production at the experimented level (-1 ~ +1). In the case of malt extract, production of carbazomycin A tended to decrease as the concentration of malt extract increased. In contrast, the production of carbazomycin B tended to increase. By central composite design, the optimum composition of carbazomycin production medium (CPM) was chosen (glucose: 8 g/L, yeast extract: 4 g/L, malt extract: 8 g/L, calcium carbonate: 2 g/L).

Compared to the GYM medium, carbazomycin A produced in the CPM medium increased by about 20% to 1.76 mg/L, and carbazomycin B increased by about 5% to 6.46 mg/L (Fig. 33).

4) Effect of antifoam in carbazomycins production

Various concentrations of antifoam were added to confirm the effect of antifoam in the fermentation of *S. abikoensis* IMBL-1939 strain and carbazomycin production. 10 ml culture without antifoam showed a spherical pellet with 0.6 g of wet weight. Meanwhile, 0.1% of antifoam decreased the amount of pellet to 0.301 g, and the dispersed pellet was observed. Moreover, 0.1% of antifoam decreased 69% carbazomycin A production and

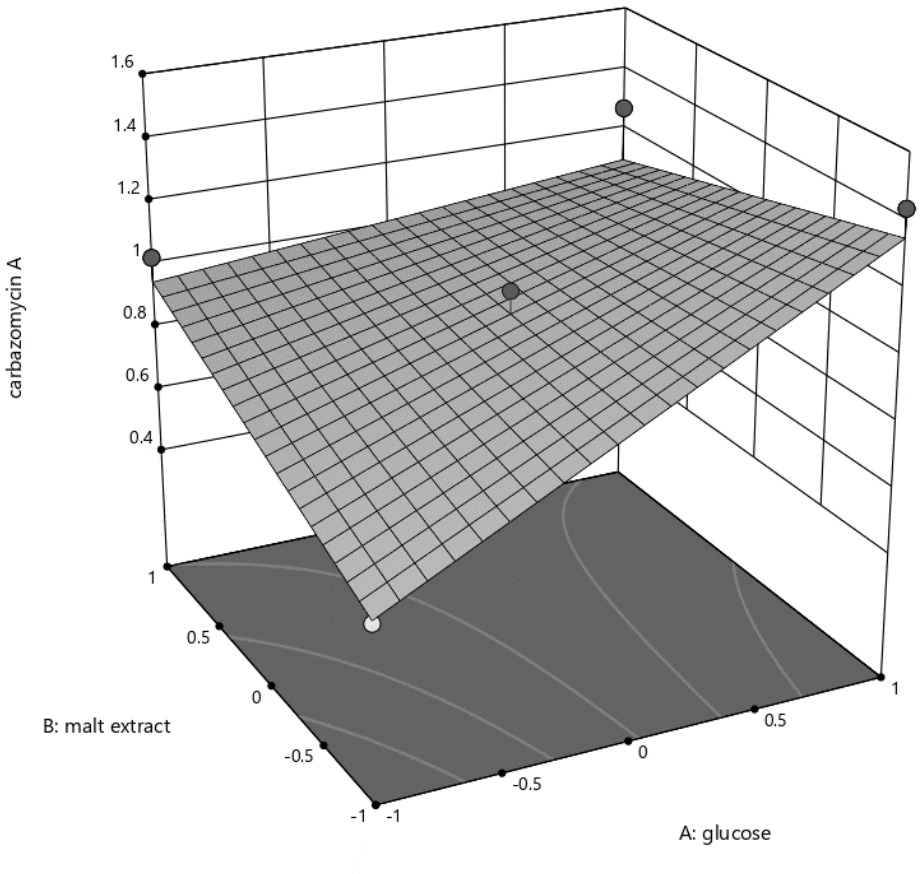


Fig. 31. 3D surface plot for glucose × malt extract in carbazomycin A production.

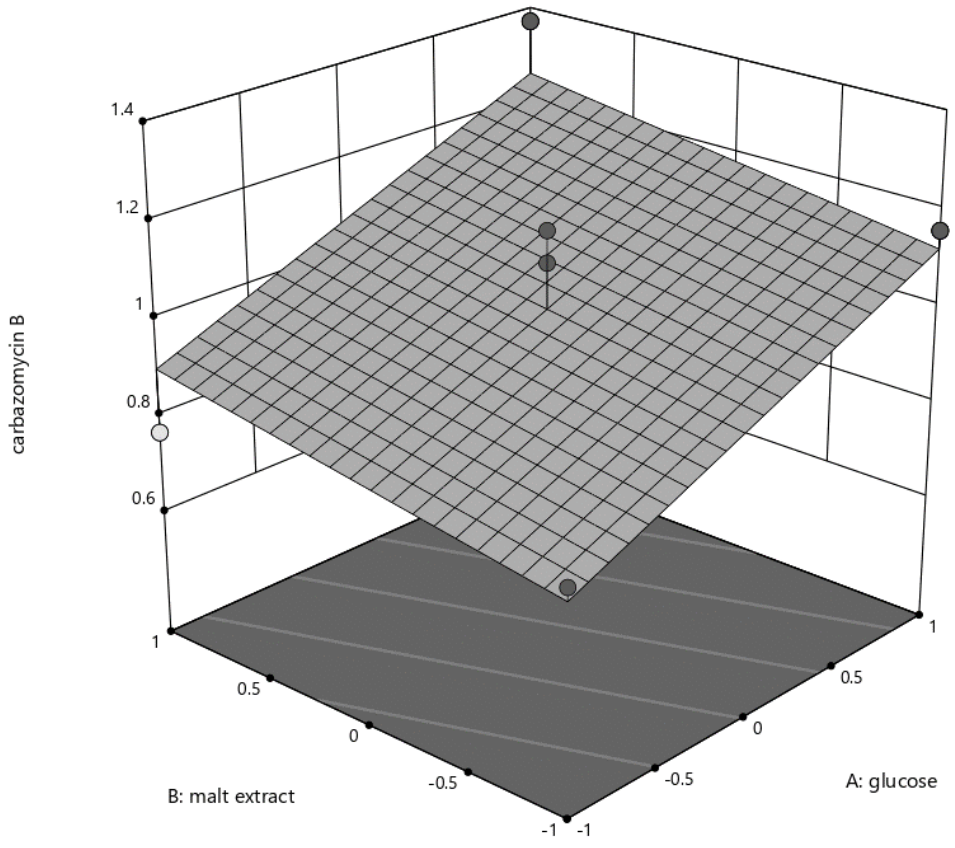


Fig. 32. 3D surface plot for glucose × malt extract in carbazomycin B production.

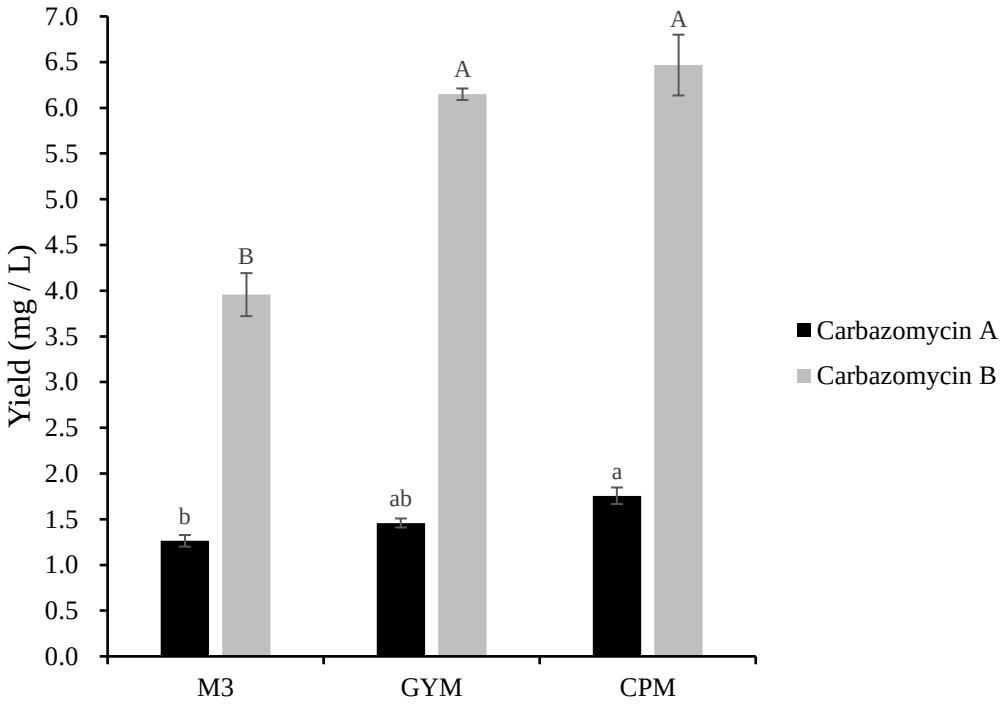


Fig. 33. The carbazomycin A and B yield in carbazomycin optimized medium (CPM). The error bars indicate the standard error of the mean. Different letters above the error bars indicate significant difference by Scheffé's test ($P < 0.05$); upper-case letters refer to production of carbazomycin A and lower-case letters refer to carbazomycin B.

51% carbazomycin B production. 0.01% and 0.005% of antifoam decreased yield by 42% and 20% of carbazomycin A. Meanwhile, 0.01% and 0.005% of antifoam did not affect the production of carbazomycin B. The essential minimum of 0.005% antifoam concentration was selected for the pilot culture (Fig. 34).

5) Carbazomycins production in pilot-scale culture

The pilot culture was carried on, and the concentration of carbazomycin produced in each culture period was evaluated by sampled culture broth. Samples were extracted by n-hexane, and carbazomycin productions were measured. As a result, it was confirmed that both carbazomycin A and B tended to increase with date except day one (Fig. 35).

Pilot cultured *S. abikoensis* IMBL-1939 strain culture broth was extracted with the same volume of n-hexane thrice, and the yield of carbazomycin A and B was listed in Table 17. Carbazomycin A and B were continuously extracted three times. The pilot-scale culture showed an increase in the yield of carbazomycin A by 3.5 times than previous yield in lab-scale culture.

6) Conversion of carbazomycin B to carbazomycin A

Carbazomycin B, which produced more than carbazomycin A could convert to carbazomycin A by methylation and provide efficient methods for mass production of carbazomycin A. Methylation mediated by potassium carbonate (K_2CO_3) converted carbazomycin B to carbazomycin A with 94.51% yield (Fig. 36).

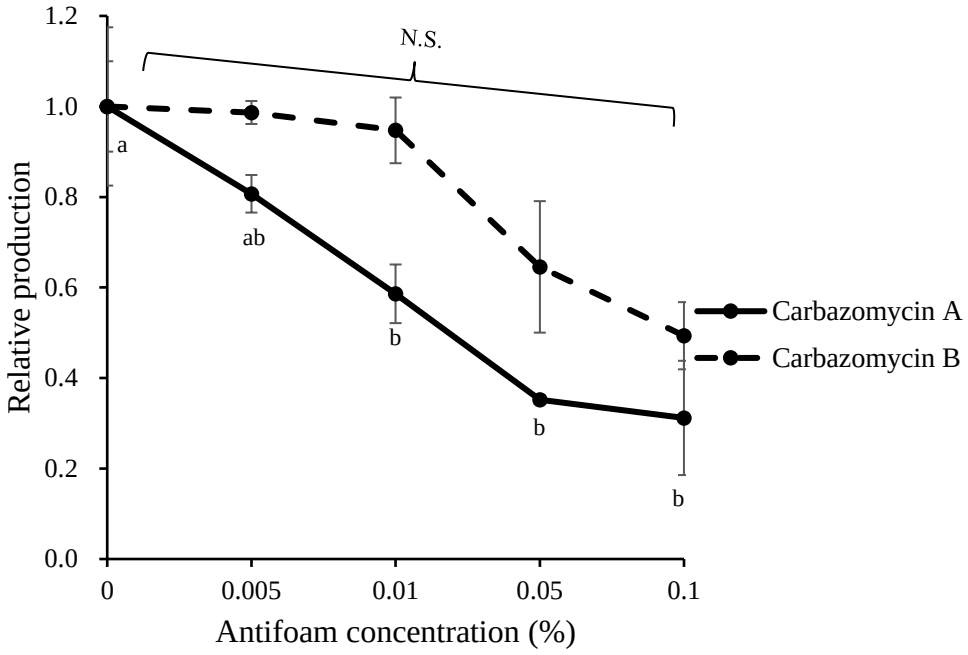


Fig. 34. Effects of antifoam in carbazomycins production. The error bars indicate the standard error of the mean. Different letters below the error bars of carbazomycin B indicate a significant difference by Scheffé's test ($P < 0.05$). N.S., not significant.

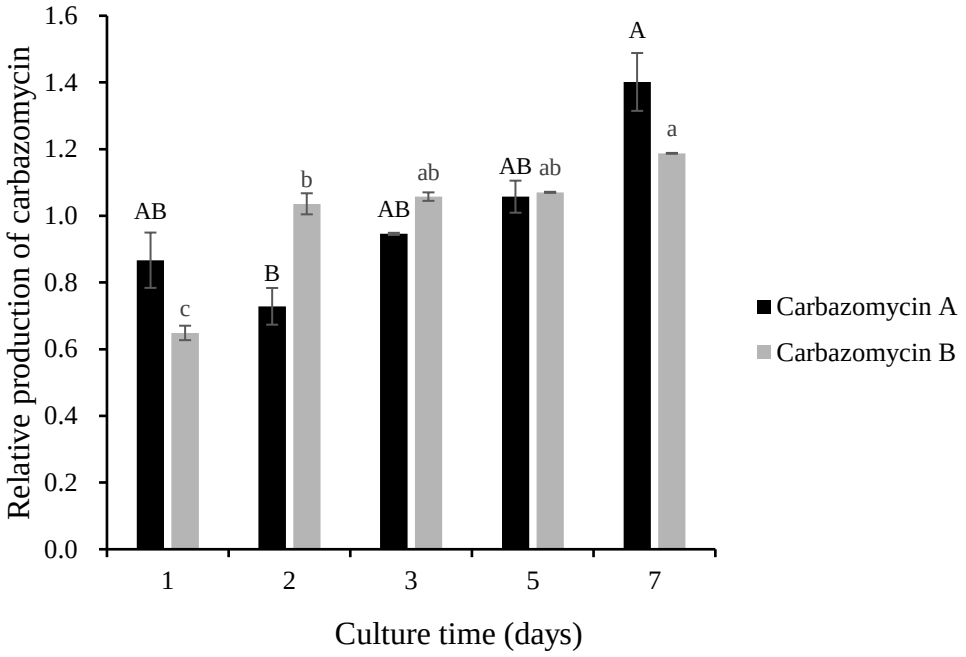


Fig. 35. Effect of culture time in pilot-scale culture on carbazomycins production. The error bars indicate the standard error of the mean. Different letters above the error bars indicate significant differences by Scheffé's test ($P < 0.05$); upper-case letters refer to the production of carbazomycin A and lower-case letters refer to carbazomycin B.

Table 17. Yield of carbazomycins in pilot-scale culture

Pilot-scale culture	First extraction (mg/L)	Second extraction (mg/L)	Third extraction (mg/L)
n-Hexane fraction	20.7 ± 2.2	27.2 ± 1.1	26.2 ± 1.2
Carbazomycin A	2.1 ± 0.1	2.3 ± 0.1	2.0 ± 0.1
Carbazomycin B	3.11 ± 0.2	3.01 ± 0.3	2.7 ± 0.1

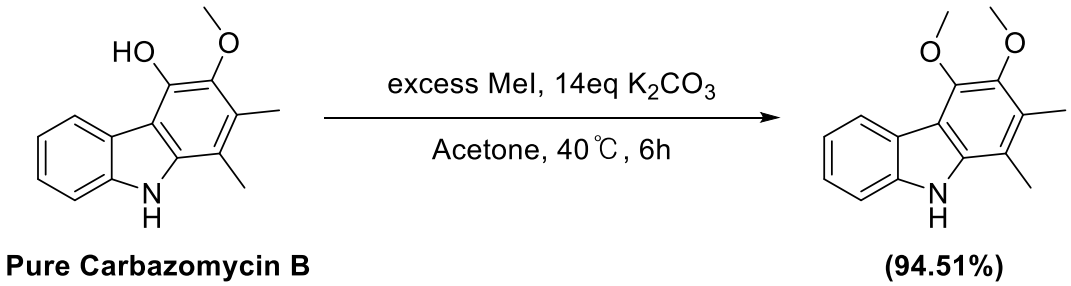


Fig. 36. Conversion of carbazomycin B to carbazomycin A.

7) Chemical synthesis of carbazomycin A

A key step in synthesizing carbazomycin A (1) is an iron-mediated reaction between cyclohexadiene (2) and arylamine (3) (Knölker & Bauermeister, 1993) (Fig. 37). Arylamine (3) was synthesized from commercially available 2,3-dimethylphenol (4) (Fig. 38). However, due to the risk of using diazomethane, to find a more secure method, trimethylsilyl diazomethane (TMS-DAM) and MeOH were used for methylation (Fig. 39). However, methylation using TMS-DAM provided 11 in only 23 % yield.

Another pathway reported by Knölker and Fröhner (1999) is using 2,3-dimethoxytoluene (12) by electrophilic bromination and halogen metal exchange which could synthesize 14 in high yield with 4 steps (Fig. 40). However, it is a problem that 2,3-dimethoxytoluene (12) is relatively more expensive than 2,3-dimethylphenol (4), and using n-BuLi which can ignite in contact with air.

The new pathway uses selective *ortho*-formylation in 2,3-dimethylphenol (4) to 2-hydroxy-3,4-dimethylbenzaldehyde (15) (Fig. 41). Aldehyde (15) was transferred by Dakin oxidation to 3,4-dimethylcatechol (16) and methylation using NaH and CH_3I to 14. The new pathway showed good yield without using dangerous or expensive compounds.

8) JHAN activity of carbazomycin derivatives

To investigate the effect and relativity of the carbazole structure on JHAN activity and mosquito insecticide activity, related substances were purchased and provided from the Kwon lab in SNU, and their activity was evaluated (Fig. 42). Among them, 4-Hydroxy-9H-

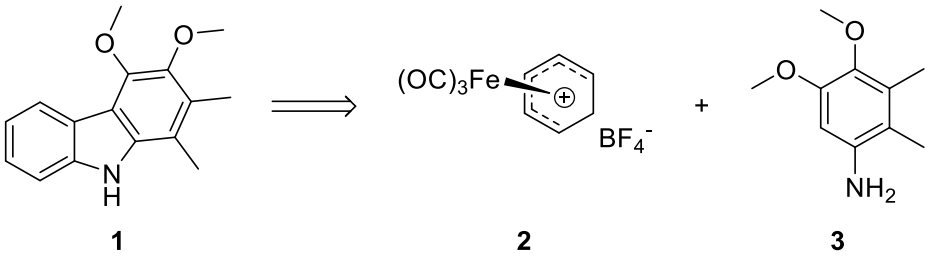


Fig. 37. Key step of carbazomycin A (1) synthesis.

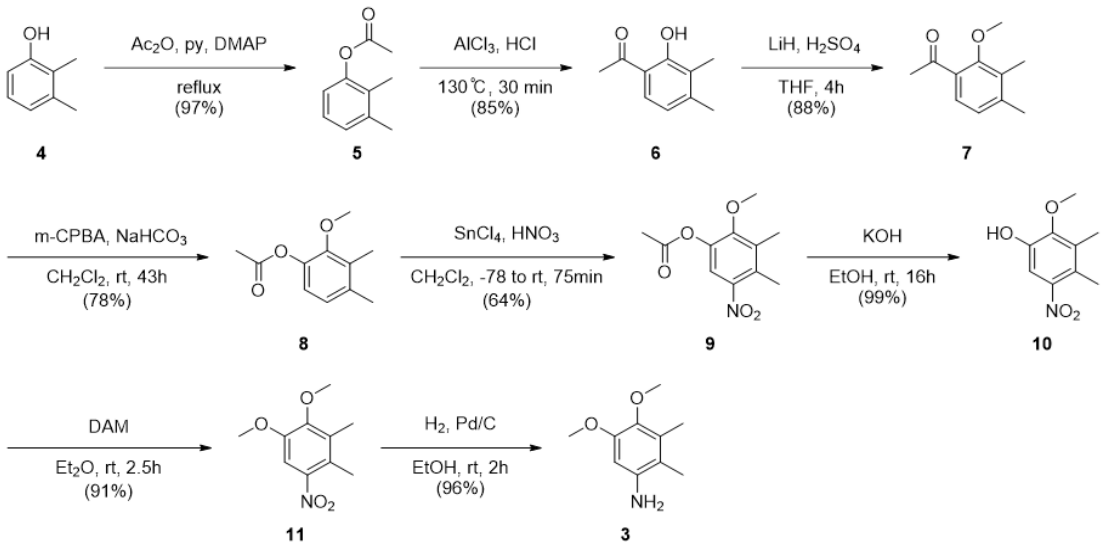


Fig. 38. Aniline (3) synthesis pathway 1.

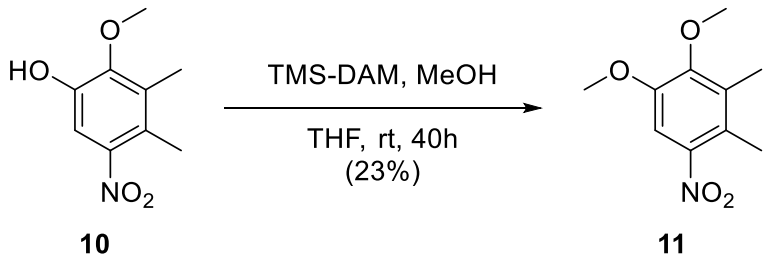


Fig. 39. OH methylation of 2-methoxy-3,4-dimethyl-5-nitrophenol.

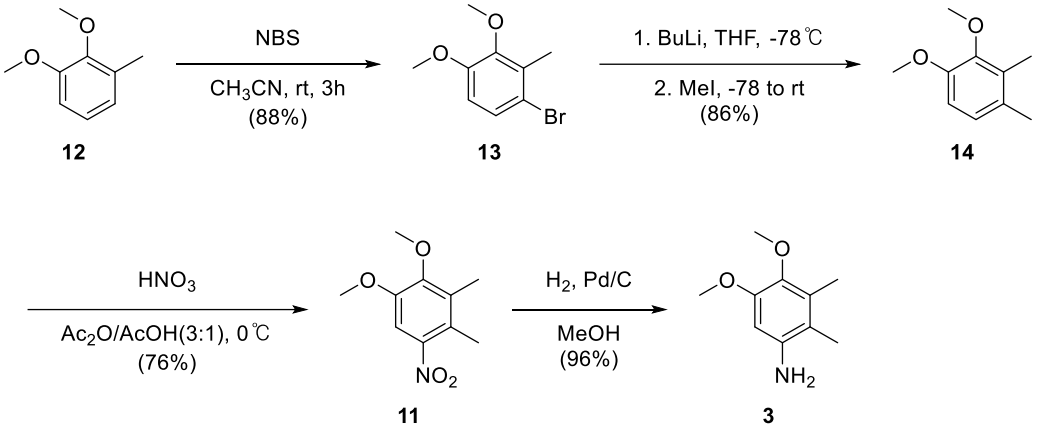


Fig. 40. Aniline synthesis pathway 2.

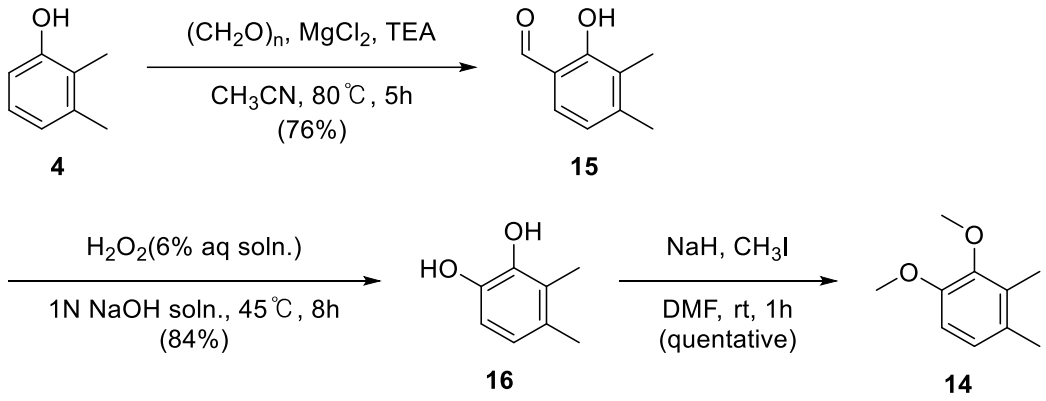


Fig. 41. Aniline synthesis pathway 3.

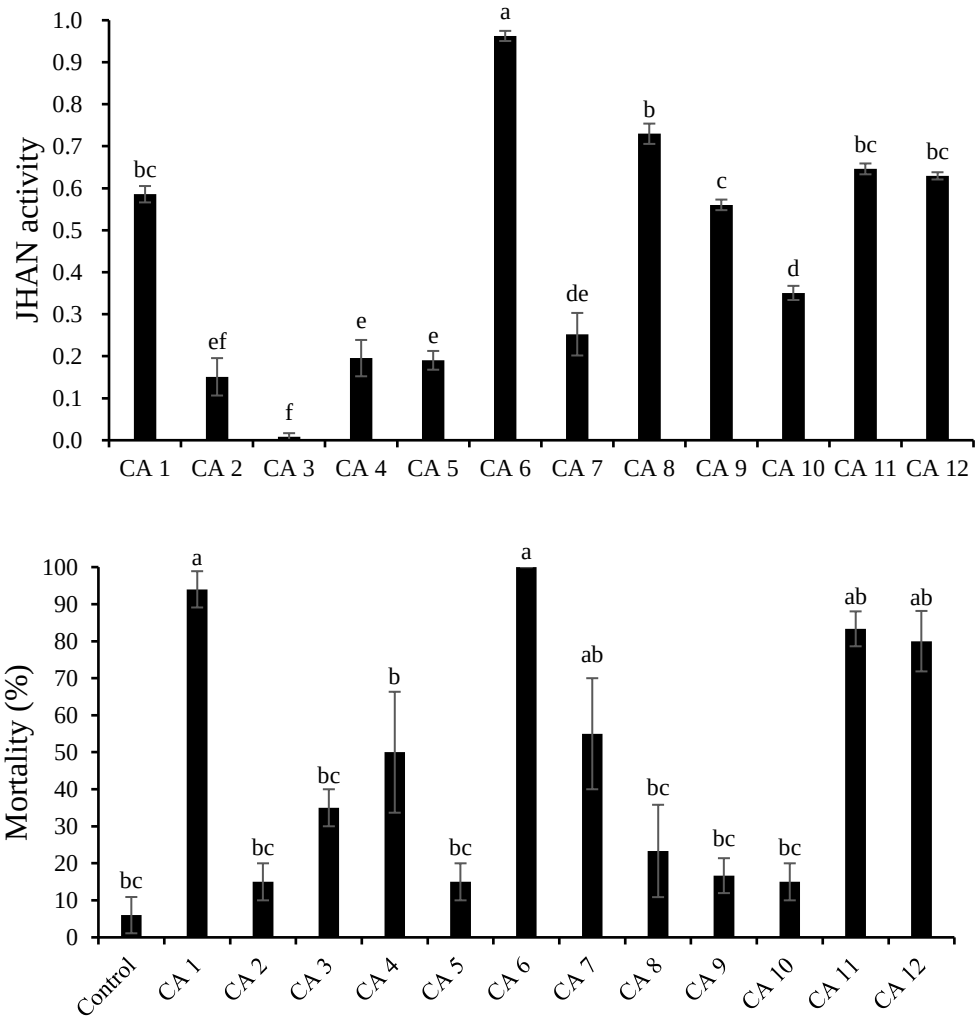


Fig. 42. JHAN activity and mosquito larvicidal activity of carbazole alkaloids. For the JHAN activity test, 0.033 ppm of pyriproxyfen and 10 ppm of carbazole alkaloids were applied to a yeast two-hybrid β-galactosidase assay (upper). For the mosquito larvicidal activity test, ethanol dissolved carbazole alkaloids were treated with 10 ppm concentration in 3rd instar larvae of *A. albopictus*. The error bars indicate the standard error of the mean. Different latter indicates significant differences by Scheffé's test ($P < 0.05$).

carbazole-3-carbaldehyde (CA 6) showed the highest JHAN activity and acute mosquito larvicidal activity within 24 h at 10 ppm. 4-methoxy-9H-carbazole (CA 11) and 3-methoxy-9H-carbazole (CA 12) showed similar levels of JHAN and mosquito larvicidal activities of carbazomycin A.

9) Mosquito larvicidal activity of wettable powder (WP)

Results of the physiological properties tests of WP are listed in Table 18. To evaluate mosquito larvicidal activity of WP, which contains 1% n-hexane extract, various concentrations of WP and n-hexane extract were treated with 3rd instar larvae of A. albopictus (Fig. 43). WP exhibited much greater larvicidal activity than n-hexane extracts with the same concentration of n-hexane extract. Over 500 ppm, WP showed 100% mortality and with 200 ppm concentration, which was similar to 100 ppm hexane extract, which has 20 times more n-hexane extract.

In a small-scale field test to evaluate the insecticidal activity of WP in mosquito habitat, WP was treated in 1 L DDW in field conditions (Fig. 44). After four days with 200 ppm of WP showed 94% of corrected mortality at 96 h after treatment and 100 ppm of WP showed 53% mortality.

Table 18. Physiological properties of wettable powder

	Properties	Standard	Results	Fitness
1	Main Ingredient		n-Hexane extract 1%	
2	Fineness	Residues within 2%	0%	Fitted
3	Wettability	Fully wetted within 2 min	33 sec	Fitted
4	Moisturizing Property	Fully moisturized within 1 min	33 sec	Fitted
5	Suspensibility	Within 2 ml of residue	0.5 ml of residue	Fitted
6	Bulk density	Bulk density was measured in 100 ml container	80 g / 100 ml	
7	Dispersibility	Not dispersed	Not dispersed	Fitted
8	pH		7.7~7.8	

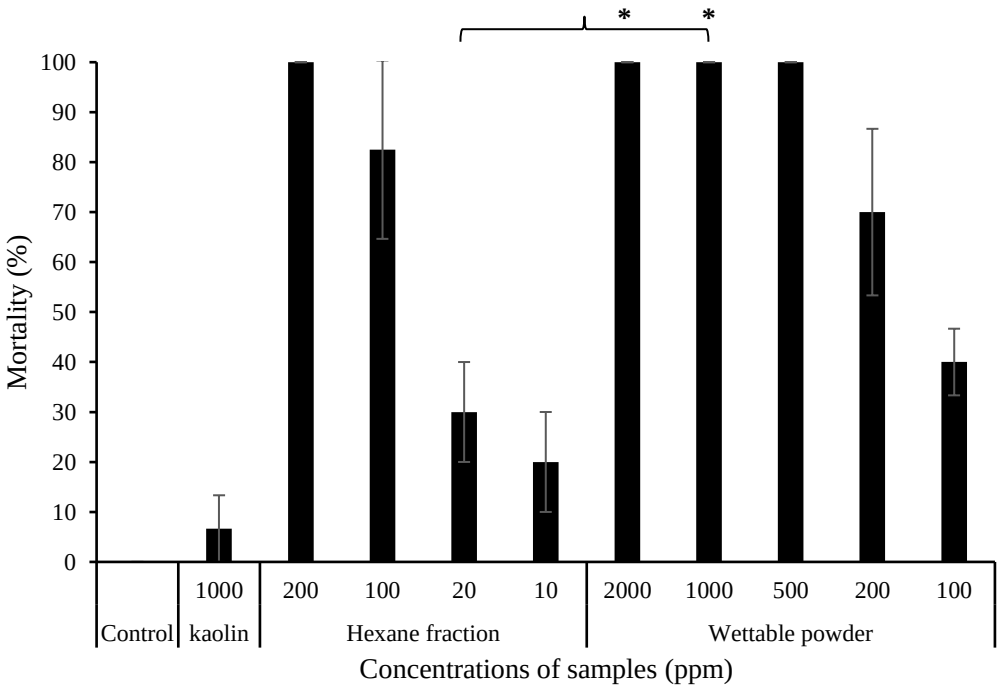


Fig. 43. Larvicidal activity of wettable powder in lab-scale bioassays. Various concentrations of WP were treated in 100 ml of DDW with 3rd instar larvae of *A. albopictus*. The mortality was calculated every 24 h for 2 days after treatment. The error bars indicate the standard error of the mean. Asterisks (*) indicate significant differences between hexane fraction and WP with same hexane extract concentration by Scheffé's test ($P < 0.05$).

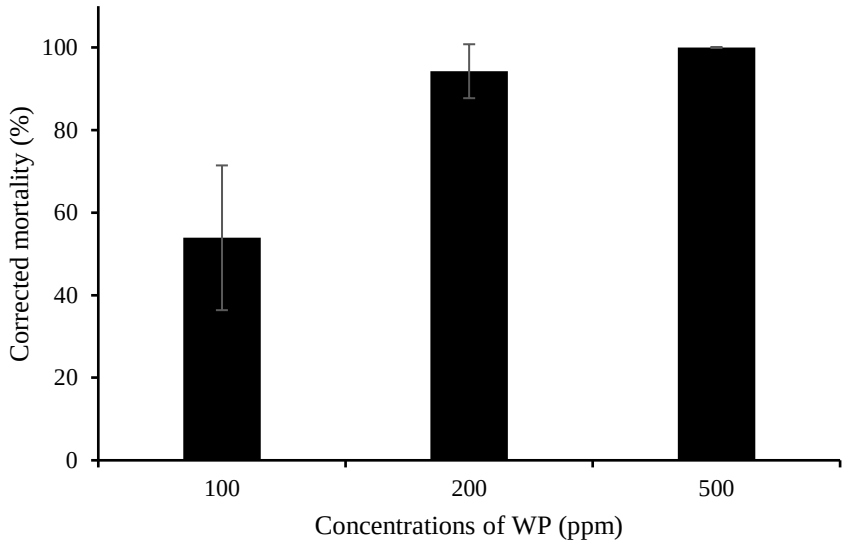


Fig. 44. Mosquito larvicidal activity of wettable powder in the semi-field test. Various concentrations of WP were treated in 1 L DDW with 3rd instar larvae of *A. albopictus*. The mortality was calculated every 24 h for 4 days after treatment and corrected by Abbott's formula. The error bars indicate the standard error of the mean.

DISCUSSION

Due to insecticide resistance and environmental toxicity, available pesticides are decreasing in pest control. Therefore, the development of novel insecticides is necessary for sustainable pest control. Large amounts of direct screening against pests have been conducted to find out novel insecticidal compounds. With an IGR screening system using transformed yeast, indirect high throughput screening without pests could be possible.

Since the discovery of streptomycin, thousands of secondary metabolites have been discovered from actinomycetes (Amelia-Yap et al., 2022). Among these metabolites, insecticidal compounds such as avermectin, spinosad, and milbemycin were widely used to control insect pests (Darriet et al., 2005). It is believed that many more IGR-active substances have not yet been found in the actinomycetes. In this study, additional IGR activity was shown in other *Streptomyces* strains, and their characteristics are different from carbazomycin A revealed in this study, so it is thought to be another substances. For this reason, the IGR screening system using yeast two hybrids will help to do with a large amount of screening.

Actinomycetes showed potent insecticidal activity. Several cases indicate that these actinomycetes-derived pesticides are produced as part of plant defense mechanisms and could have originated from co-evolution between plants and actinomycetes in the rhizosphere (van Bergeijk et al., 2020; Vurukonda et al., 2018; Zhao et al., 2020). For this reason, these insecticidal substances are considered relatively safe since they have minimal side effects on the host plant (Amelia-Yap et al., 2022). These results suggest that

actinomycete-derived compounds with juvenile hormone disruptive activity may be used as alternatives to commonly used pesticides due to their insect specificity. From the perspective of plants, juvenile hormone agonists could prolongate the larval stage, which could cause increasing damage to phytophagous insect pests (Kayukawa et al., 2020). Therefore, it seems more reasonable for actinomycetes as plant protectors to produce JHAN compounds.

In this study, carbazomycin A revealed as the main JHAN and mosquitocidal active compound. Carbazole alkaloids are usually found in higher plants, slime molds, algae, and microorganisms (A. W. Schmidt et al., 2012). Murrayanine was the first carbazole alkaloid found in the curry tree *Murraya koengii,* and dozens of additional carbazole alkaloids were later discovered from various plant parts (Das et al., 1965). Due to these carbazole alkaloids, these plants have been used for pharmacological activities like antidiabetic, antifungal, anticancer, inflammatory, and antioxidant activity (Ramsewak et al., 1999; Arndt W Schmidt et al., 2012). In addition, these carbazole alkaloids from *M. koengii* exhibited strong larvicidal activity *against A. aegypti* mosquitoes (Sukari et al., 2013).

Unlike higher plant-derived carbazole alkaloids, all known 3,4-dioxygenated carbazole alkaloids were discovered from *Streptomyces* (Arndt W Schmidt et al., 2012). Among them, carbazomycin derivatives were isolated from have been reported for their antifungal activity (Tadjuddin Naid et al., 1987; Sakano et al., 1980). Carbazomycin A and B were isolated from S*treptoverticillium ehimense* H 1051-MY 10 strain, and carbazomycin B was considered the main compound of carbazomycin due to its antifungal activity (T. Naid et al., 1987; Sakano et al., 1980). Moreover, carbazomycin B exhibited inhibitory activity against 5-lipoxygenase and lipid peroxidation. However, although the unique structure of

carbazomycin derivatives is distinct from higher plant-derived carbazole alkaloids, many activities are unknown, especially in the case of carbazomycin A.

In this study, carbazomycin A showed the most potent insecticidal activity and JHAN activity than carbazomycin B and D. Carbazomycin A decreased expression of juvenile hormone response elements, *kr-h1,* and *hairy* gene in adult female mosquitoes. Also, carbazomycin A inhibited JH triggered biting behavior, leading to reduced ovary development. JH and 20-hydroxyecdysone regulate mosquito reproduction. JH regulates the transcription of *Kr-h1*, which involves JH signaling to regulate the expression of JH-responsive genes involved in previtellogenic development (Ojani et al., 2018). It was reported that juvenile hormone deprivation caused by the removal of corpora allata caused inhibition of biting behavior in *Culex* mosquitoes (Meola & Petralia, 1980). Therefore, if carbazomycin A acts as JHAN, these results are reasonable.

In addition, in the perspective of integrated pest management (IPM), the synergistic effect of JHAN with ecdysone agonists (EAs) can be looked forward to. JHAs and EAs have not been used together because of their opposite tendencies in their mode of action. When JHA, pyriproxyfen and methoprene, and EAs are used together, the effects of each other have been decreased (Hamaidia & Soltani, 2019; Zibaee et al., 2012). Therefore, synergistic effect with carbazomycin A and EA like methoxyfenozide could be looking forward to controlling agricultural pests.

It is reported that carbazomycin A and B had non-toxic to mice at 400 mg/ kg in oral treatment (Kato et al., 1993). In this study, carbazomycin A showed low toxicity to silkworms and bees in experimented concentrations. However, mosquito control also affects a variety of aquatic organisms, including green algae. Meanwhile, LD_{50} values of

pyriproxyfen via oral and contact toxicity against honey bees are greater than 100 μg a.i / bee (Devillers & Devillers, 2020). However, pyriproxyfen may be toxic to fishes in recent studies (Maharajan et al., 2018;). Therefore, it is thought that carbazomycin A could be used as an eco-friendly insecticide through additional environmental toxicity evaluation against a variety of non-target organisms with more high concentrations.

In this study, optimization of carbazomycins production was proceeded using the design of experiments (DOE). Despite numerous studies, the metabolism of *Streptomyces* is still largely unexplored. Various environmental and physiological signals regulate and stimulate the production of secondary metabolites. It is hard to consider all of these variables in optimizing metabolite production. Statistical designs have been used for the optimization of medium and culture conditions. In this study, optimization in carbazomycin A production using Placket-Burman design and composite central design and increased carbazomycin production were observed in carbazomycin production optimized medium. However, the optimum point could not be found within the experimental level and showed concentration-dependent linear increase. Therefore, additional experiments and quantitative analysis are necessary for more detailed optimization and pilot-culture conditions. In addition, it is also thought that production can be further increased through optimization of physical factors.

Unlike previously reported, the low amount of carbazomycin B was extracted through n-hexane, which is a non-polar solvent. Therefore, focusing on the amount of carbazomycin B, the yield could be increased by extracting with a polar solvent such as ethyl acetate and *tert*-butyl methyl ether.

Through base methylation, carbazomycin B converted to carbazomycin A in 94% yield. Since carbazomycin B is produced more than A, it will be possible to generate a large

amount of carbazomycin A. Since this reaction is not a substrate-specific reaction, it is expected that a large amount of carbazomycin A can be obtained through methylation and purification on n-hexane extract.

In recent studies, many developments are being made in the mass production of discovered materials by metabolite engineering (Kim et al., 2016). Secondary metabolites produced by actinomycetes are not simply made up of a single enzyme but are made in multiple stages with various enzymes. Biosynthetic gene clusters (BGCs) have begun to be studied on how they are involved in the metabolism of primary and secondary metabolites, and in addition to the statistical analyses that have been used, these studies have recently been conducted at the gene level (Li et al., 2021; Peng et al., 2018). Spinosad, one of the actinomycetes-derived insecticidal compounds, are optimized by metabolic engineering (Lan et al., 2015). Genes involving carbazomycin biosynthesis have not been discovered. If genes and pathways are revealed in the future, it seems that optimization of the production of carbazomycin A through metabolic engineering might be possible. Moreover, through gene sequencing and analysis, the reveal of methyl transferase could make it possible to methylate carbazomycin B in platformed production. These discoveries will make it possible for efficient mass production of carbazomycin A.

Finally, pesticides are not generally used as a single component but with complex auxiliary additives. The formula plays an important role in contact, solubility, and transmission. In the case of mosquitoes present in aquatic ecosystems, the solubility of active insecticidal substances plays an important role. The role of supplements and surfactants to assist them in materials also significantly influences insecticidal activity. In this study, n-hexane fraction, including carbazomycin A was formulated to WP which is

advantageous for dilution and treatment at various concentrations. If the proper insecticide concentration is confirmed through more detailed field test results, it will be possible to make in the form of briquette and granule, mainly used as mosquito larvae insecticides and can be used conveniently.

Moreover, newly discovered carbazole alkaloids which can be produced much cheaper through chemical synthesis than carbazomycin A, showed similar levels of mosquitocidal activity to carbazomycin A. Therefore, it is expected that they can be used as alternative insecticides for resistance and toxicity problems through environmental toxicity evaluation and insecticidal characterization.

LITERATURE CITED

Abbott, W. S. (1925). A method of computing the effectiveness of an insecticide. *Journal of economic entomology*, 18(2), 265-267.

Amelia-Yap, Z. H., Azman, A. S., AbuBakar, S., & Low, V. L. (2022). *Streptomyces* derivatives as an insecticide: Current perspectives, challenges and future research needs for mosquito control. *Acta Tropica*, 106381.

Amsalem, E., Teal, P., Grozinger, C. M., & Hefetz, A. (2014). Precocene-I inhibits juvenile hormone biosynthesis, ovarian activation, aggression and alters sterility signal production in bumble bee (*Bombus terrestris*) workers. *Journal of Experimental Biology*, *217*(17), 3178-3185.

Arasu, M. V., Al-Dhabi, N. A., Saritha, V., Duraipandiyan, V., Muthukumar, C., & Kim, S.-J. (2013). Antifeedant, larvicidal and growth inhibitory bioactivities of novel polyketide metabolite isolated from *Streptomyces* sp. AP-123 against *Helicoverpa armigera* and *Spodoptera litura*. *BMC microbiology*, 13(1), 1-6.

Ashok, M., Turner, C., & Wilson, T. G. (1998). Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proceedings of the National Academy of Sciences*, *95*(6), 2761-2766.

Auteri, M., La Russa, F., Blanda, V., & Torina, A. (2018). Insecticide Resistance Associated with *kdr* Mutations in *Aedes albopictus*: An Update on Worldwide Evidences. *BioMed Research International*, *2018*, 3098575.

Benelli, G., & Beier, J. C. (2017). Current vector control challenges in the fight against

malaria. *Acta Tropica*, *174*, 91-96.

Benelli, G., & Mehlhorn, H. (2016). Declining malaria, rising of dengue and Zika virus: insights for mosquito vector control. *Parasitology research*, *115*(5), 1747-1754.

Berdy, J. (2005). Bioactive microbial metabolites. *The Journal of antibiotics*, *58*(1), 1-26.

Bowers, W., Fales, H., Thompson, M., & Uebel, E. (1966). Juvenile hormone: identification of an active compound from balsam fir. *Science*, *154*(3752), 1020-1021.

Charles, J.-P., Iwema, T., Epa, V. C., Takaki, K., Rynes, J., & Jindra, M. (2011). Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proceedings of the National Academy of Sciences*, *108*(52), 21128-21133.

Cuervo-Parra, J. A., Cortés, T. R., & Ramirez-Lepe, M. (2016). Mosquito-borne diseases, pesticides used for mosquito control, and development of resistance to insecticides. *Insecticides resistance. Rijeka: InTechOpen*, 111-134.

Darriet, F., Duchon, S., & Hougard, J. M. (2005). Spinosad: a new larvicide against insecticide-resistant mosquito larvae. *Journal of the American Mosquito Control Association*, *21*(4), 495-496.

Das, K., Chakraborty, D., & Bose, P. (1965). Antifungal activity of some constituents of *Murraya koenigii* spreng. *Experientia*, *21*(6), 340-340.

de Lima Procópio, R. E., da Silva, I. R., Martins, M. K., de Azevedo, J. L., & de Araújo, J. M. (2012). Antibiotics produced by *Streptomyces*. *The Brazilian Journal of Infectious Diseases*, *16*(5), 466-471.

De Simeis, D., & Serra, S. (2021). Actinomycetes: A never-ending source of bioactive compounds—An overview on antibiotics production. *Antibiotics*, *10*(5), 483.

Devillers, J., & Devillers, H. (2020). Lethal and sublethal effects of pyriproxyfen on Apis

and non-Apis bees. *Toxics*, *8*(4), 104.

Dhadialla, T. S., Retnakaran, A., & Smagghe, G. (2009). Insect growth-and development-disrupting insecticides. In *Insect development: morphogenesis, molting and metamorphosis* (pp. 679-740). Academic Press.

Gao, H., Liu, M., Liu, J., Dai, H., Zhou, X., Liu, X., Zhuo, Y., Zhang, W., & Zhang, L. (2009). Medium optimization for the production of avermectin B1a by *Streptomyces avermitilis* 14-12A using response surface methodology. *Bioresource Technology*, *100*(17), 4012-4016.

Gilbert, L. I., Granger, N. A., & Roe, R. M. (2000). The juvenile hormones: historical facts and speculations on future research directions. *Insect Biochem Mol Biol*, *30*(8-9), 617-644.

Godlewski, J., Wang, S., & Wilson, T. G. (2006). Interaction of bHLH-PAS proteins involved in juvenile hormone reception in *Drosophila*. *Biochemical and Biophysical Research Communications*, *342*(4), 1305-1311.

Hamaidia, K., & Soltani, N. (2019). Compensation of kinoprene effect on reproduction of *Culex pipiens* by methoxyfenozide, an ecdysone agonist. *Journal of Entomological Research*, *43*(2), 125-130.

Harir, M., Bendif, H., Bellahcene, M., Fortas, Z., & Pogni, R. (2018). *Streptomyces* secondary metabolites. *Basic biology and applications of actinobacteria*, *6*, 99-122.

Hayakawa, M., & Nonomura, H. (1987). Humic acid-vitamin agar, a new medium for the selective isolation of soil actinomycetes. *Journal of Fermentation Technology*, *65*(5), 501-509.

Henrick, C. A. (2007). Methoprene. *Journal of the American Mosquito Control Association*,

23(sp2), 225-239.

Hook, D. J., Yacobucci, J. J., O'Connor, S., Lee, M., Kerns, E., Krishnan, B., Matson, J., & Hesler, G. (1990). Identification of the inhibitory activity of carbazomycins B and C against 5-lipoxygenase, a new activity for these compounds. *Journal of Antibiotics (Tokyo)*, *43*(10), 1347-1348.

Invest, J., & Lucas, J. (2008). Pyriproxyfen as a mosquito larvicide. *Proceedings of the sixth international conference on urban pests*, 239-245

Jindra, M., Bellés, X., & Shinoda, T. (2015). Molecular basis of juvenile hormone signaling. *Current Opinion in Insect Science*, *11*, 39-46.

Jindra, M., & Bittova, L. (2020). The juvenile hormone receptor as a target of juvenoid "insect growth regulators". *Archives of insect biochemistry and physiology*, *103*(3), e21615.

Jindra, M., Palli, S. R., & Riddiford, L. M. (2013). The juvenile hormone signaling pathway in insect development. *Annual review of entomology*, *58*, 181-204.

Judy, K. J., Schooley, D. A., Dunham, L. L., Hall, M., Bergot, B. J., & Siddall, J. B. (1973). Isolation, structure, and absolute configuration of a new natural insect juvenile hormone from *Manduca sexta*. *Proceedings of the National Academy of Sciences*, *70*(5), 1509-1513.

Kato, S., Kawasaki, T., Urata, T., & Mochizuki, J. (1993). In vitro and ex vivo free radical scavenging activities of carazostatin, carbazomycin B and their derivatives. *The Journal of antibiotics*, *46*(12), 1859–1865.

Kaneda, M., Kitahara, T., Yamasaki, K., & Nakamura, S. (1990). Biosynthesis of Carbazomycin B II. Origin of the Whole Carbon Skeleton. *The Journal of*

Antibiotics, *43*(12), 1623-1626.

Kaur, T., Vasudev, A., Sohal, S. K., & Manhas, R. K. (2014). Insecticidal and growth inhibitory potential of *Streptomyces hydrogenans* DH16 on major pest of India, *Spodoptera litura* (Fab.) (Lepidoptera: Noctuidae). *BMC microbiology*, *14*(1), 1-9.

Kayukawa, T., Furuta, K., Nagamine, K., Shinoda, T., Yonesu, K., & Okabe, T. (2020). Identification of a juvenile-hormone signaling inhibitor via high-throughput screening of a chemical library. *Scientific Reports*, *10*(1), 18413.

Kim, H. U., Charusanti, P., Lee, S. Y., & Weber, T. (2016). Metabolic engineering with systems biology tools to optimize production of prokaryotic secondary metabolites. *Natural product reports*, *33*(8), 933-941.

Kim, J. H., Choi, J. Y., Park, D. H., Park, D.-J., Park, M. G., Kim, S. Y., Ju, Y. J., Kim, J. Y., Wang, M., & Kim, C.-J. (2020). Isolation and characterization of the insect growth regulatory substances from actinomycetes. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *228*, 108651.

Klowden, M. J. (2013). *Physiological systems in insects*. Academic press.

Knölker, H.-J., & Fröhner, W. (1999). Transition metal complexes in organic synthesis, part 54. Improved total syntheses of the antibiotic alkaloids carbazomycin A and B. *Tetrahedron Letters*, *40*(38), 6915-6918.

Knölker, H. J., & Bauermeister, M. (1993). Transition Metal-Diene Complexes in Organic Synthesis. Part 15. Iron-mediated total synthesis of carbazomycin A and B. *Helvetica chimica acta*, *76*(7), 2500-2514.

Kobayashi, M., Tomita, T., Shin-ya, K., Nishiyama, M., & Kuzuyama, T. (2019). An Unprecedented Cyclization Mechanism in the Biosynthesis of Carbazole Alkaloids

in *Streptomyces*. *Angewandte Chemie International Edition*, *58*(38), 13349-13353.

Kotaki, T., Shinada, T., Kaihara, K., Ohfune, Y., & Numata, H. (2009). Structure determination of a new juvenile hormone from a heteropteran insect. *Organic letters*, *11*(22), 5234-5237.

Korea Disease Control and Prevention Agency. (2018). Mosquito control guidelines for major infectious diseases. Ministry of Health and Welfare.

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, *35*(6), 1547.

Lan, Z., Zhao, C., Guo, W., Guan, X., & Zhang, X. (2015). Optimization of Culture Medium for Maximal Production of Spinosad Using an Artificial Neural Network - Genetic Algorithm Modeling. *Microbial Physiology*, *25*(4), 253-261.

Lawler, S. P. (2017). Environmental safety review of methoprene and bacterially-derived pesticides commonly used for sustained mosquito control. *Ecotoxicology and environmental safety*, *139*, 335-343.

Lee, S.-H., Ha, K. B., Park, D. H., Fang, Y., Kim, J. H., Park, M. G., Woo, R. M., Kim, W. J., Park, I.-K., & Choi, J. Y. (2018). Plant-derived compounds regulate formation of the insect juvenile hormone receptor complex. *Pesticide biochemistry and physiology*, *150*, 27-32.

Lee, S.-H., Lim, H. N., Choi, J. Y., Park, D. H., Ahn, B. H., Fang, Y., Kim, J. H., Park, M. G., Woo, R. M., & Lee, B. R. (2018). Mosquitocidal activity of penfluridol as juvenile hormone antagonist. *Journal of Asia-Pacific Entomology*, *21*(1), 130-133.

Lee, S.-H., Oh, H.-W., Fang, Y., An, S.-B., Park, D.-S., Song, H.-H., Oh, S.-R., Kim, S.-Y.,

Kim, S., & Kim, N. (2015). Identification of plant compounds that disrupt the insect juvenile hormone receptor complex. *Proceedings of the National Academy of Sciences*, *112*(6), 1733-1738.

Li, X., Hu, X., Sheng, Y., Wang, H., Tao, M., Ou, Y., Deng, Z., Bai, L., & Kang, Q. (2021). Adaptive Optimization Boosted the Production of Moenomycin A in the Microbial Chassis *Streptomyces albus* J1074. *ACS Synthetic Biology*, *10*(9), 2210-2221.

Lin, L., Ge, H. M., Yan, T., Qin, Y. H., & Tan, R. X. (2012). Thaxtomin A-deficient endophytic *Streptomyces* sp. enhances plant disease resistance to pathogenic *Streptomyces scabies*. *Planta*, *236*(6), 1849-1861.

Lloyd, A. M., Connelly, C. R., & Carlson, D. B. (2018). Florida coordinating council on mosquito control. Florida mosquito control: The state of the mission as defined by mosquito controllers, regulators, and environmental managers.

Mahajan, G. B., & Balachandran, L. (2012). Antibacterial agents from actinomycetes-a review. *Front. Biosci (Elite Ed)*, *4*(4), 240-253.

Maharajan, K., Muthulakshmi, S., Nataraj, B., Ramesh, M., & Kadirvelu, K. (2018). Toxicity assessment of pyriproxyfen in vertebrate model zebrafish embryos (*Danio rerio*): A multi biomarker study. *Aquatic Toxicology*, *196*, 132-145.

Md Meftaul, I., Venkateswarlu, K., Dharmarajan, R., Annamalai, P., & Megharaj, M. (2020). Pesticides in the urban environment: A potential threat that knocks at the door. *Science of The Total Environment*, *711*, 134612.

Meola, R. W., & Petralia, R. S. (1980). Juvenile Hormone Induction of Biting Behavior in *Culex* Mosquitoes. *Science*, *209*(4464), 1548-1550.

Meyer, A. S., Schneiderman, H. A., Hanzmann, E., & Ko, J. H. (1968). The two juvenile

hormones from the Cecropia silk moth. *Proceedings of the National Academy of Sciences*, *60*(3), 853-860.

Miall, R., & Mordue, W. (1980). Precocene II has juvenile-hormone effects in 5th instar Locusta migratoria. *Journal of Insect Physiology*, *26*(6), 361-364.

Miura, K., Oda, M., Makita, S., & Chinzei, Y. (2005). Characterization of the *Drosophila Methoprene-tolerant* gene product: Juvenile hormone binding and ligand-dependent gene regulation. *The FEBS journal*, *272*(5), 1169-1178.

Moody, C. J., & Shah, P. (1989). Synthesis of the carbazole alkaloids carbazomycin A and B and hyellazole. *Journal of the Chemical Society, Perkin Transactions 1*(2), 376-377.

Naid, T., Kitahara, T., Kaneda, M., & Nakamura, S. (1987). Carbazomycins C, D, E and F, minor components of the carbazomycin complex. *The Journal of antibiotics*, *40*(2), 157-164.

Newman, D. J., Cragg, G. M., & Snader, K. M. (2003). Natural products as sources of new drugs over the period 1981− 2002. *Journal of natural products*, *66*(7), 1022-1037.

Nosrat, C., Altamirano, J., Anyamba, A., Caldwell, J. M., Damoah, R., Mutuku, F., Ndenga, B., & LaBeaud, A. D. (2021). Impact of recent climate extremes on mosquito-borne disease transmission in Kenya. *PLOS Neglected Tropical Diseases*, *15*(3), e0009182.

Ojani, R., Fu, X., Ahmed, T., Liu, P., & Zhu, J. (2018). Krüppel homologue 1 acts as a repressor and an activator in the transcriptional response to juvenile hormone in adult mosquitoes. *Insect Molecular Biology*, *27*(2), 268-278.

Park, D. H., Choi, J. Y., Lee, S.-H., Kim, J. H., Park, M. G., Kim, J. Y., Wang, M., Kim, H.

J., & Je, Y. H. (2020). Mosquito larvicidal activities of farnesol and farnesyl acetate via regulation of juvenile hormone receptor complex formation in *Aedes* mosquito. *Journal of Asia-Pacific Entomology*, *23*(3), 689-693.

Peng, Q., Gao, G., Lü, J., Long, Q., Chen, X., Zhang, F., Xu, M., Liu, K., Wang, Y., Deng, Z., Li, Z., & Tao, M. (2018). Engineered *Streptomyces lividans* Strains for Optimal Identification and Expression of Cryptic Biosynthetic Gene Clusters. *Front Microbiol*, *9*, 3042.

Pratt, G., & Bowers, W. (1977). Precocene II inhibits juvenile hormone biosynthesis by cockroach corpora allata in vitro. *Nature*, *265*(5594), 548-550.

Ramsewak, R. S., Nair, M. G., Strasburg, G. M., DeWitt, D. L., & Nitiss, J. L. (1999). Biologically Active Carbazole Alkaloids from *Murraya koenigii*. *Journal of Agricultural and Food Chemistry*, *47*(2), 444-447.

Riddiford, L. M. (2008). Juvenile hormone action: a 2007 perspective. *Journal of insect physiology*, *54*(6), 895-901.

Robertsen, H. L., Weber, T., Kim, H. U., & Lee, S. Y. (2018). Toward systems metabolic engineering of Streptomycetes for secondary metabolites production. *Biotechnology Journal*, *13*(1), 1700465.

Rocklöv, J., & Dubrow, R. (2020). Climate change: an enduring challenge for vector-borne disease prevention and control. *Nature Immunology*, *21*(5), 479-483.

Ruiu, L. (2020). Plant-growth-promoting bacteria (PGPB) against insects and other agricultural pests. *Agronomy*, *10*(6), 861.

Ryan, S. J., Carlson, C. J., Mordecai, E. A., & Johnson, L. R. (2019). Global expansion and redistribution of Aedes-borne virus transmission risk with climate change. *PLOS*

Neglected Tropical Diseases, *13*(3), e0007213.

Sakano, K.-i., Ishimaru, K., & Nakamura, S. (1980). New antibiotics, Carbazomycins A and B I. Fermentation, Extraction, Purification and physico-chemical and biological properties. *The Journal of antibiotics*, *33*(7), 683-689.

Sakano, K.-i., & Nakamura, S. (1980). New antibiotics, carbazomycins A and B II. Structural elucidation. *The Journal of antibiotics*, *33*(9), 961-966.

Sánchez, C. M., Martín, J. M. P., Jin, J.-S., Davalos, A., Zhang, W., de la Pena, G., Martinez-Botas, J., Rodriguez-Acebes, S., Suarez, Y., & Hazen, M. J. (2015). Disruption of the mevalonate pathway induces dNTP depletion and DNA damage. *Biochimica et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, *1851*(9), 1240-1253.

Schmidt, A. W., Reddy, K. R., & Knölker, H.-J. (2012). Occurrence, biogenesis, and synthesis of biologically active carbazole alkaloids. *Chemical reviews*, *112*(6), 3193-3328.

Shin, S. W., Jeon, J. H., Yun, C.-S., Jeong, S. A., Kim, J., Park, D.-S., Shin, Y., & Oh, H.-W. (2018). Species-specific interactions between plant metabolites and insect juvenile hormone receptors. *Journal of chemical ecology*, *44*(11), 1022-1029.

Singh, N., & Rai, V. (2012). Improved antimicrobial compound production by a new isolate *Streptomyces hygroscopicus* MTCC 4003 using Plackett-Burman design and response surface methodology. *Bioinformation*, *8*(21), 1021.

Slama, K., & Williams, C. M. (1965). Juvenile hormone activity for the bug *Pyrrhocoris apterus*. *Proceedings of the National Academy of Sciences of the United States of America*, *54*(2), 411.

Stall, G. (1986). Anti juvenile hormone agents. *Annual review of entomology*, *31*(1), 391-429.

Strong, L., & Brown, T. (1987). Avermectins in insect control and biology: a review. *Bulletin of Entomological Research*, *77*(3), 357-389.

Su, T., Thieme, J., Cummings, R., Cheng, M. L., & Brown, M. Q. (2021). Cross Resistance in S-Methoprene-Resistant *Culex quinquefasciatus* (Diptera: Culicidae). *Journal of Medical Entomology*, *58*(1), 398-402.

Su, T., & Trdan, S. (2016). Resistance and its management to microbial and insect growth regulator larvicides in mosquitoes. *Insecticides resistance. InTech Europe, Rijeka, Croatia*, 135-154.

Sukari, M., Noor, H. M., Bakar, N. A., Ee, G., Isamail, I., Rahmani, M., & Abdul, A. (2013). Larvicidal carbazole alkaloids from *Murraya koenigii* against dengue fever mosquito *Aedes aegypti* Linnaeus. *Asian J. Chem*, *25*, 7719-7721.

Sukari, M. A., Ahmad, K., Haron, M. J., & Muse, R. (2001). Carbazole alkaloids from roots of *Murraya Koenigii* (Rutaceae). *Malaysian Journal of Analytical Sciences*, *7*(1), 263-265.

Sun, Y., Zhou, X., Liu, J., Bao, K., Zhang, G., Tu, G., Kieser, T., & Deng, Z. (2002). '*Streptomyces nanchangensis*', a producer of the insecticidal polyether antibiotic nanchangmycin and the antiparasitic macrolide meilingmycin, contains multiple polyketide gene clusters. *Microbiology*, *148*(2), 361-371.

TePaske, M. R., Gloer, J. B., Wicklow, D. T., & Dowd, P. F. (1989). Tubingensin A: an antiviral carbazole alkaloid from the sclerotia of *Aspergillus tubingensis*. *The Journal of Organic Chemistry*, *54*(20), 4743-4746.

Van Bergeijk, D. A., Terlouw, B. R., Medema, M. H., & van Wezel, G. P. (2020). Ecology and genomics of Actinobacteria: new concepts for natural product discovery. *Nature Reviews Microbiology*, *18*(10), 546-558.

Van der Meij, A., Worsley, S. F., Hutchings, M. I., & van Wezel, G. P. (2017). Chemical ecology of antibiotic production by actinomycetes. *FEMS microbiology reviews*, *41*(3), 392-416.

Vurukonda, S. S. K. P., Giovanardi, D., & Stefani, E. (2018). Plant growth promoting and biocontrol activity of *Streptomyces* spp. as endophytes. *International journal of molecular sciences*, *19*(4), 952.

Wang, X. J., Zhang, J., Wang, J. D., Huang, S. X., Chen, Y. H., Liu, C. X., & Xiang, W. S. (2011). Four new doramectin congeners with acaricidal and insecticidal activity from *Streptomyces avermitilis* NEAU1069. *Chemistry & Biodiversity*, *8*(11), 2117-2125.

Watve, M. G., Tickoo, R., Jog, M. M., & Bhole, B. D. (2001). How many antibiotics are produced by the genus *Streptomyces*? *Archives of microbiology*, *176*(5), 386-390.

Wigglesworth, V. B. (1936). Memoirs: The function of the corpus Allatum in the growth and reproduction of *Rhodnius Prolixus* (Hemiptera). *Journal of Cell Science*, *2*(313), 91-121.

Williams, C. M. (1956). The Juvenile Hormone of Insects. *Nature*, *178*(4526), 212-213.

Williams, C. M. (1967). Third-generation pesticides. *Scientific American*, *217*(1), 13-17.

Wilson, A. L., Courtenay, O., Kelly-Hope, L. A., Scott, T. W., Takken, W., Torr, S. J., & Lindsay, S. W. (2020). The importance of vector control for the control and elimination of vector-borne diseases. *PLOS Neglected Tropical Diseases*, *14*(1),

e0007831.

Wilson, T. G., & Fabian, J. (1986). A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. *Developmental Biology*, *118*(1), 190-201.

Woo, R. M., Park, M. G., Choi, J. Y., Park, D. H., Kim, J. Y., Wang, M., Kim, H. J., Woo, S. D., Kim, J. S., & Je, Y. H. (2020). Insecticidal and insect growth regulatory activities of secondary metabolites from entomopathogenic fungi, *Lecanicillium attenuatum*. *Journal of Applied Entomology*, *144*(7), 655-663.

World Health Organization. (2005). Guidelines for laboratory and field testing of mosquito larvicides.

World Health Organization. (2016). World malaria report 2015. World Health Organization.

World Health Organization. (2020). World malaria report 2020: 20 years of global progress and challenges.

World Health Organization. (2020). *Front Matter* (World malaria report 2020), Issue.

Wu, T.-S., Huang, S.-C., Wu, P.-L., & Kuoh, C.-S. (1999). Alkaloidal and other constituents from the root bark of *Clausena excavata*. *Phytochemistry*, *52*(3), 523-527.

Yamasaki, K., Kaneda, M., Watanabe, K., Ueki, Y., Ishimaru, K., Nakamura, S., Nomi, R., Yoshida, N., & Nakajima, T. (1983). New antibiotics, carbazomycins A and B III. Taxonomy and biosynthesis. *The Journal of Antibiotics*, *36*(5), 552-558.

Yi, H., Devkota, B. R., Yu, J. s., Oh, K. c., Kim, J., & Kim, H. J. (2014). Effects of global warming on mosquitoes & mosquito-borne diseases and the new strategies for mosquito control. *Entomological Research*, *44*(6), 215-235.

Yoon, S.-H., Ha, S.-M., Kwon, S., Lim, J., Kim, Y., Seo, H., & Chun, J. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and

whole-genome assemblies. *International Journal of Systematic and Evolutionary Microbiology*, *67*(5), 1613.

Zhao, H., Yang, A., Zhang, N., Li, S., Yuan, T., Ding, N., Zhang, S., Bao, S., Wang, C., & Zhang, Y. (2020). Insecticidal endostemonines A–J produced by endophytic *Streptomyces* from *Stemona sessilifolia*. *Journal of Agricultural and Food Chemistry*, *68*(6), 1588-1595.

Zibaee, A., Bandani, A. R., & Malagoli, D. (2012). Methoxyfenozide and pyriproxifen alter the cellular immune reactions of *Eurygaster integriceps* Puton (Hemiptera: Scutelleridae) against *Beauveria bassiana*. *Pesticide biochemistry and physiology*, *102*(1), 30-37.

곤충 유충호르몬 저해 활성 기반의 방선균 ***Streptomyces abikoensis*** 유래 살충 활성 물질의 동정과 특성 규명

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초록

ABSTRACT IN KOREAN

모기는 치명적인 병원균을 매개함으로써 인류에게 위협이 되고 있다. 기존까지 해충 방제를 위해 다양한 신경 작용 살충제들이 사용되었으나 저항성과 환경 독성으로 인해 사용가능한 살충제의 수는 감소하고 있으며 지구온난화로 인해 질병 매개의 범위도 넓어지고 있다.

이 중 methoprene, pyriproxyfen과 같은 유충호르몬 아고니스트 계열의 곤충 생장 조절제는 유충호르몬의 작용을 교란하여 대상 해충에 살충활성을 나타내며 높은 특이성과 상대적으로 낮은 환경 독성으로 인해 기존 신경 작용

살충제에 대한 대안으로서 사용되고 있다. 그러나 최근에는 이에 대해서도 저항성 개체가 보고되어 새로운 기작의 신규 방제제 개발이 필요해졌다. 방선균은 다양한 2차 대사산물을 만들어내는 것으로 알려져 있고, 또한 많은 수의 살충활성 물질이 보고되어 왔기에 신규 기작의 IGR 활성 물질을 찾아낼 수 있을 것으로 생각된다.

본 연구에서는 총 232 균주의 방선균으로부터 유충호르몬 안타고니스트(JHAN)을 가지는 물질을 만들어내는 방선균 균주를 선별하였고 그 결과 4개의 방선균 배양액에서 모기 살충활성과 JHAN 활성을 보이면서도 구피 성어에 대해서는 급성 어류 독성을 나타내지 않았다. 4개의 균주 중 *Streptomyces abikoensis* IMBL-1939 균주는 가장 높은 수준의 JHAN 및 모기 살충 활성 물질을 나타냈다. 용매 추출 결과, 배양액의 n-hexane 추출물로부터 살충 활성을 보여 이를 대상으로 bioassay-guided fractionation을 통해 살충 활성 물질을 분리하였으며, 질량 분석 및 NMR을 통해서 물질의 구조 동정을 진행한 결과 주요 활성 물질이 carbazomycin A로 나타났다.

Carbazomycin A는 3령 흰줄숲모기를 대상으로 48시간 이후 4.8 ppm의 LC_{50} 값을 보여 높은 살충활성을 나타내었다. 또한 모기 암컷 성충에서 *Kr-h1*이나 *Hairy*와 같은 유충호르몬 관련 유전자 (juvenile hormone response elements)들의 발현을 저해하였으며 모기 성충에서의 유충호르몬에 의해 촉진되는 흡혈 행동과 난소 발육을 저해하여 JHAN 활성을 보이는 것을 확인하였다. 이외에도 carbazomycin A의 비표적 생물에 대한 환경독성을 확인하였

으며 누에와 꿀벌에 대해 낮은 독성을 보이는 것을 확인하였다.

Carbazomycin A의 생산성을 최적화하기 위해서 실험 설계법을 사용하여 배양 조건 및 배지 조성을 선별하였다. Plackett-Burman 설계를 통해서 carbazomycin의 생성량에 영향을 끼치는 요인을 선발하고 중심합성 설계법을 이용하여 최적화를 진행하였으며 이를 통해 선발된 최적화 배지에서 carbazomycin A의 생성량을 20% 증가시켰으며 실험실 배양 결과를 바탕으로 배양기를 이용한 대량 배양을 수행하고 증가된 생성량을 확인하였다.

또한 대량으로 생성되는 carbazomycin B를 base methylation을 통해 A로 전환을 수행하여 94 %의 수율로 carbazomycin A를 얻을 수 있었으며 이를 통해서 대량 배양에서 효율적인 carbazomycin A를 생산할 수 있을 것으로 보인다. 또한 기존에 보고된 carbazomycin A의 전 합성 방법에서 개선점을 확인하여 더 안전하고 효율적인 합성 방법을 확인하였다.

마지막으로 실제 적용을 위해 hexane 추출물을 제제화를 통해 수화제(Wettable powder)를 제작하였으며, semi-field 실험에서 야외 살충활성을 검정한 결과, 제제 농도 200 ppm (주 활성물질 농도 기준 2 ppm)에서도 약 95%의 살충활성을 보이는 것을 확인하였다. 이러한 결과를 통해 carbazomycin A와 *S. abikoensis* IMBL-1939 균주의 추출물이 친환경적인 모기 방제제로써 사용될 수 있을 것이다.

검색어: Actinomycetes, *Streptomyces*, Secondary metabolites, Juvenile hormone antagonist, Insecticidal activity, Carbazomycin

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