UNIVERSITI PUTRA MALAYSIA

GENETIC DIVERSITY OF TUBA PLANTS, AND TOXICITY OF THEIR ROTENOIDS FORMULATED AS NANO-EMULSION AGAINST Plutella xylostella L.

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Thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

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Chairman : Professor Dzolkhifli Omar, PhD.
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Rotenone found in the crude extract from the roots of Tuba plants is commonly used as a bioinsecticide to control insect pests of horticultural crops. The control obtained on insect pests varies greatly and this could probably be due to the source and process of extraction, toxicant preparation and application in the field. The need to improve the quality of the rotenone as botanical insecticide and to obtain consistent control of insect pest led to the studies with following objectives; 1) to identify the diversity of Tubaplants based on Random Amplified Polymorphic DNA (RAPD) analysis, Internal Transcribe Spacer (ITS) marker and their morphological characteristics, 2) to extract, and characterize bioactive compound rotenone from Tuba plants, 3) to prepare the bio-based emulsion formulation of rotenone extract and 4) to evaluate the insecticidal properties of the emulsion formulation of rotenone against Diamondback moth (DBM). Nine accession were collected from Peninsular Malaysia and subjected for identification by the Random Amplified Polymorphic DNA. The presence and absence of amplified allele was analysed using NTSYS to obtain dendrogram and coefficient of similarity index. The results obtained on the number of polymorphics loci, the percentage of polymorphics loci, Shannon's Information index and the effective number of alleles were 318, 98.45%, 0.4571 and 1.4533 respectively indicating the diversity of accessions studied. ITS sequences were used to identify the species in which double stranded DNA of the ITS region from nine accessions was amplified with the universal primer pairs ITS 4 and ITS 5. The mean number substitution per site and the number of nucleotides changes obtained for each accession plants indicated that there were 4 different species within the accessions. Basedon , morphological data of leaves examined analyzed using multivariate analysis to obtain the Principal components, dendrogram and coefficient of similarity index showed that all accessions could be clearly separated from each other based on leaf radians technique even though most of them have very similar shape.The rotenone from nine Tuba accessions was extracted using acetone. The extracts were then quantified using the High Performance Liquid Chromatography with Photodiode Array detector against the analytical standard of rotenone. All accessions contained rotenone with the concentration varies from 40 to 1500 µg/mL. The chemical elucidation of rotenone from nine accessions was conducted using Liquid Chromatography Mass Spectrometer. Rotenone and deguelin

were found from the elucidation of each accession at retention time of 5.7 and 6.6 minute, respectively. Both have similar mass of 395.1489 with the empirical formula of $C_{23}H_{21}O_6$. Three accessions i.e. *Paraderris lianoides* KJ579433 (Tuba Tualang) from Tasik Bera, Pahang, *Derris elliptica* KJ579428 (Tuba Derris) from Universiti Putra Malaysia and *D. elliptica* KJ579429 (Tuba Merah) from Jerangau, Terengganu were chosen based on the highest concentration (> 1%) of rotenone obtained for the preparation of emulsion formulation. The emulsion system was developed for formulating the extract by constructing ternary phase diagrams with 70% (w/w) emulsion system constituted of non-ionic surfactant(s), carrier and water, and 30% (w/w) rotenone extract as active ingredient. A total of thirteen formulations comprising four of Tuba Merah (F1TM7B, F2TM14B, F3TM21B and F4TM14B), five of Tuba Tualang (F5TT7B, F6TT14B, F7TT21B, F8TT21S and F9TT14S) and four of Tuba Derris (F10TD7S, F11TD21Sil, F12TD7Sil and F13TD7Sil) were made. All formulations showed particle size of less than 100nm and surface tension value of below 30 mN/m. All formulations were also stable at 25°C for two months, the agglomeration was observed for all formulations and stable at temperature 54°C except for formulation F13TD7Sil.The toxicity of rotenone was obtained from nine Tuba accessions evaluated through dose mortality and antifeedant bioassays against DBM. The leaf-dip technique was used to obtain dose-mortality response of rotenone in the nano-emulsion formulations. All rotenone formulations gave 100% mortality 72 hours after treatment at concentration of 273 µg/mL. The F7TT21B formulation gave the lowest LC_{50} value of 0.02 µg/mL while F1TM7B, F5TT7B, F6TT14B and F4TM14B gave LC_{50} value of 10.016, 10.298, 6.741 and 5.044 µg/mL, respectively indicating influence of formulation on the toxicity of rotenone. Surfactant mixture of Agnique MBL 510H and Agnique MBL 530B,and carrier dimethylamide (Agnique AMD810) in the formulations played important role in increasing the mortality. In the antifeedant study, the formulation with mixed surfactant differed significantly in Feeding Deterrence Index compared with the control.Dietary utilization by the DBM was severely affected when fed on mustard leaves treated with all formulations of rotenone. The F6TT14B showed significantly lower consumption intake (0.07 mg/mg/day) compared to other formulations. The relative consumption rate was significantly reduced for F1TM7B, F3TM21B, F6TT14B, F9TT14S, F11TD21Sil and F12TD7Sil compared with the other formulations and control.The F6TT14B showed the lowest RGR compared to the other formulations (0.25 mg/mg/day). The percentage of efficiency of conversion of ingested food of F8TT21S (27.81%) and F9TT14S (28.53%) was significantly better compared to the control (55.42%) and other formulations. The efficiency of conversion of digested (ECD) showed that all the formulations differed significantly compared with the control. The ECD values of F2TM14B, F3TM21B, F4TM14B, F7TT21B, F11TD21Sil, F12TD7Sil and F13TD7Sil ranged from 3.95% to 5.37% and differed significantly compared with the control. The reduction in dietary utilization that reduced the growth may result from both behavioural and physiological (post-ingestive) effects.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KEPELBAGAIAN GENETIK TUMBUHAN TUBA, DAN KETOKSIKAN ROTENOIDNYA DIFORMULASIKAN SEBAGAI NANO EMULSI MENENTANG *Plutella xylostella* L.

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Rotenone terdapat dalam ekstrak mentah dari akar tumbuhan Tuba. biasanya digunakan sebagai racun serangga bio untuk mengawal serangga perosak tanaman hortikultur. Kawalan diperolehi pada serangga perosak sangat berbeza dan ini boleh mungkin disebabkan oleh sumber dan proses pengekstrakan, penyediaan yg menyebabkan keracunan dan aplikasi di lapangan. Keperluan untuk meningkatkan kualiti rotenone sebagai racun serangga botani dan untuk mendapatkan kawalan konsisten serangga perosak utama kepada kajian dengan objektif berikut; 1) untuk mengenal pasti kepelbagaian tumbuhan Tuba berdasarkan analisis RAPD, penanda ITS dan ciri-ciri morfologi mereka, 2) untuk mengambil, dan mencirikan sebatian bioaktif rotenone daripada tumbuhan Tuba, 3) untuk menyediakan rumusan berasaskan bio emulsi ekstrak rotenon dan 4) untuk menilai ciri-ciri serangga formulasi emulsi daripada rotenon terhadap larva ulat kobis (DBM). Sembilan sampel dikumpulkan dari Semenanjung Malaysia dan tertakluk untuk mengenal pasti oleh RAPD. Kehadiran dan ketiadaan alel dikuatkan dianalisis menggunakan NTSYS untuk mendapatkan dendrogram dan pekali persamaan indeks. Keputusan yang diperolehi pada bilangan polymorfiks lokus, peratusan polymorphics lokus, indeks Shannon Maklumat dan keberkesanan bilangan alel 318, 98.45%, 0,4571 dan 1,4533 masing-masing menunjukkan kepelbagaian aksesi dikaji. Urutan ITS telah digunakan untuk mengenal pasti spesies di mana dua terkandas DNA kawasan ITS dari sembilan sampel telah dikuatkan dengan pasangan buku asas universal ITS 4 dan 5 ITS Bilangan penggantian min bagi setiap tapak dan jumlah nukleotida perubahan diperolehi bagi setiap tumbuhan kesertaan menunjukkan bahawa adalah 4 spesies yang berbeza dalam aksesi. Berdasarkan, data morfologi daun yang dianalisis menggunakan analisis multivariat untuk mendapatkan komponen Prinsip, dendrogram dan pekali persamaan indeks menunjukkan bahawa semua aksesi boleh dipisahkan dengan jelas berdasarkan teknik radian daun walaupun kebanyakan daripada mereka mempunyai bentuk yang sama. Rotenon dari sembilan aksesi Tuba telah diekstrak menggunakan aseton. Ekstrak kemudiannya diukur menggunakan Kromatografi cecair prestasi tinggi dengan fotodiod pengesan tatasusunan terhadap standard analisis daripada rotenone. Semua aksesi terkandung rotenone dengan kepekatan yang berbeza-beza 40-1500 μg / mL. Ini penerangan kimia rotenone dari sembilan aksesi telah dijalankan menggunakan Kromotografi cecair spektrometer jisim. Rotenon dan deguelin didapati dari

penerangan setiap kesertaan pada masa penahanan masing-masing sebanyak 5.7 dan 6.6 minit. Kedua-dua mempunyai jisim yang sama 395.1489 formula ivmpiric $C_{23}H_{21}O_6$. Tiga aksesi iaitu *Paraderris lianoides* KJ579433 (Tuba Tualang) dari Tasik Bera, Pahang, *Derris elliptica* KJ579428 (Tuba Derris) dari Universiti Putra Malaysia dan *D. elliptica* KJ579429 (Tuba Merah) dari Jerangau, Terengganu telah dipilih berdasarkan kepekatan tertinggi rotenone yang diperolehi (> 1%) bagi penyediaan formulasi emulsi. Sistem emulsi dibangunkan untuk merumuskan ekstrak dengan membina gambar rajah fasa pertigaan dengan 70% (w / w) sistem emulsi terdiri daripada surfaktan bukan ionik (s), dan air, 30% (w / w) rotenone ekstrak aktif bahan. Sebanyak tiga belas formulasi yang mana empat daripada Tuba Merah (F1TM7B, F2TM14B, F3TM21B dan F4TM14B), lima Tuba Tualang (F5TT7B, F6TT14B, F7TT21B, F8TT21S dan F9TT14S) dan empat Tuba Derris (F10TD7S, F11TD21Sil, F12TD7Sil dan F13TD7Sil) diperolehi. Semua rumusan menunjukkan saiz zarah kurang daripada 100nm, nilai ketegangan permukaan dibawah 30 mN / m. Semua formulasi stabil pada suhu 25°C selama dua bulan dan pengaglomeratan diperhatikan untuk semua rumusan dan stabil pada suhu 54°C kecuali F13TD7Sil. Ketoksikan daripada rotenone telah dinilai melalui dos kematian dan 'antifeedant' bioesai terhadap DBM. Teknik daun celup telah digunakan untuk mendapatkan dos kematian respon rotenone dalam rumusan nano emulsi. Semua rumusan rotenone memberikan 100% kematian 72 jam selepas rawatan pada kepekatan 273 µg / mL. Formulasi F7TT21B memberikan nilai LC_{50} terendah 0.02 µg / mL manakala F1TM7B, F4TM14B, F5TT7B dan F6TT14B memberikan nilai LC_{50} daripada 10.02, 10.29, 6.74 dan 5.04 µg / mL masing-masing menunjukkan rumusan mempengaruhi ketoksikan rotenone. Surfaktan bercampur yang Agnique MBL 510H dengan Agnique MBL 530B dan pembawa dimetilamid (Agnique AMD810) dalam rumusan memainkan peranan penting dalam meningkatkan kematian. Dalam kajian antifeedant, formulasi menggunakan surfactant campuran memberikan berbeza dengan ketara berbanding dengan kawalan di makan Indeks Pencegahan. Penggunaan makanan oleh DBM yang telah terjejas teruk apabila diberi makan di atas daun dirawat dengan semua formulasi rotenone. F6TT14B yang menunjukkan pengambilan penggunaan jauh lebih rendah (0.07 mg / mg / hari) berbanding dengan rumusan lain. Kadar penggunaan relatif telah berkurangan untuk F1TM7B, F3TM21B, F6TT14B, F9TT14S, F11TD21Sil, F12TD7Sil dan berbanding dengan rumusan dan kawalan yang lain. F6TT14B menunjukkan bacaan terendah berbanding dengan rumusan lain (0.25 mg / mg / hari). Peratusan kecekapan penukaran makanan ditelan dari F8TT21S (27,81%) dan F9TT14S (28.53%) menunjukkan signifikan lebih baik berbanding dengan kawalan (55.42%) dan formulasi lain. Kecekapan penukaran dicerna (ECD) menunjukkan semua formulasi berbeza dengan ketara berbanding dengan kawalan. Nilai ECD F2TM14B, F3TM21B, F4TM14B, F7TT21B, F11TD21Sil, F12TD7Sil dan F13TD7Sil adalah di antara 3.95% hingga 5.37% dan berbeza dengan ketara berbanding dengan kawalan. Pengurangan dalam penggunaan diet yang mengurangkan pertumbuhan boleh disebabkan oleh kedua-dua tingkah laku dan fisiologi. mereka, aktiviti antifeedant, dan kesan ke atas pembangunan dan matang.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy in Entomology. The members of the supervisory committee were as follows:

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LIST OF ABBREVIATIONS

%	Percent
°C	celcius
µg/mL	microgram per mililiter
µl	microliter
bp	Base Pair
CRD	Complete Randomize Design
DBM	Diamondback moth
DNA	Deoxyribonucleic acid
HPLC	High Performance Liquid Chromatography
hrs	Hours
ITS	Internal Transcribe Spacer
LC_{50}	Lethal concentration 50
Min	minute
ml	mililiter
MS	Mass spectra
nm	Nanometer
O/W	oil in water
PCA	Principle component Analysis
PCR	Polymerase Chain Reaction
ppm	Part per million
RAPDs	Random Amplified Polymorphic DNA
RNA	Ribonucleic Acid
rpm	revolution per minute
SE	Standard error
Sec	Second
spp	species
TABP	Tuba Akar Bukit Payung
TB	Tuba Bonsai
TD	Tuba Derris
TM	Tuba Merah
TP	Tuba Putih
TP	Tuba Putih
TS	Tuba Susu
TT	Tuba Tualang
w/w	weight/weight

CHAPTER 1

GENERAL INTRODUCTION

Nowadays, the crop protection industry is not only facing the challenge of developing better products with new compounds, but it is also required to provide good products with enhanced safety to the outside user together with less effect on the environment. Rodham (2000) said, this was to make sure the capable use of products when they were applied in the field. However, the conventional pesticides were frequently toxic to mammals, non-target pests and persist in the environment as intractable. For that reason, identifying other bioactive substances that have suitable properties, and which were effective on the target pest, cost-effective, and biodegradable were of great importance.

In general, rotenone and other toxic constituents in the extract tuba plant (e.g. deguelin, tephrosin and 12αβ-rotenolone) are isoflavonoids occurring in several genera of tropical leguminosae plants, such as *Derris* (Papilionaceae), *Antonia* (Loganiaceae) and *Lonchocarpus* (Fabaceae). According to John (1944) and Andel (2000), rotenone was a bioactive compound that has a knock-down effect on cold-blooded animals and could be used as an insecticide against pests. In addition, rotenoid-yielding plants have also been used for fishing based on their itcthyotoxic properties (Andel, 2000). Indeed, there have been reports of rotenone-containing plants being used by Indians for fishing due to their itcthyotoxic effect as early as the 17th century (Moretti and Grenard, 1982).

Interestingly, rotenone poisoned fish was safe to consume by humans (Costa et al., 1989). The risk of mammalians being poisoned by rotenone was supported by the lethality tests resulting in LD_{50} (value is in milligrams per kilogram (mg/kg) of body weight in mammals) ranging from 50 to 300 (Raws, 1986; Ellenhorn and Barceloux, 1988), which is considered as a moderate hazardous substance (WHO, 1992). Bradbury(1986) and Schnick(1974) reported that humans or mammals were not extremely exposed to rotenone because they were confined by inefficient gastrointestinal absorption and effective oxidizing enzyme systems. From the wide research and thorough evidence concerning its effect against targeted organisms and non-targeted organisms (especially human) rotenone was designated as one of the botanical insecticides that were exceptionally selective and environmentally friendly (Schnick, 1974; Bradbury, 1986). Other reasons concerning the safety record of rotenone as a botanical insecticide include the low concentration compared to commercial products, its high degradability and poor absorption through the gut and skin of humans.

Rotenone was a chemical with insecticidal, acaricidal and piscicidal properties (Extoxnet, 1996). It has a selective action and could be used in residence gardens for insect control and for fish eradication as could of unwanted fish species (Weier and Starr, 1950). Because of its composition, Kole et al.,(1992) reported that the extracts

could be formulated into emulsifiable concentrates (EC) and wettable powders for wide use in lakes, ponds and reservoirs to control unattractive fish as well as to control the highly resistant insect pests.

The use of agro-based materials in the formulation of pesticides has become more important because they were relatively biodegradable, have low toxicity and were renewable resources compared to those from mineral oil derived commodities. Vegetable oils, such as soybean, sunflower, and palm oil, have begun to vary in their usage over the last few years, spreading to non-food areas. Since chemical industrial firms are seeking to use ingredients that were nontoxic, biodegradable, not persistent in the environment and that originate from renewable sources, palm based-oil can now be found in adjuvants and pesticide carriers. In pesticide formulations, emulsifiable concentrates were the most common. One of the possible ways of using palm oil in the formulation was through emulsion (Chow and Grant,1992). Most studies on pesticide formulation focus on herbicides and not much information was available on insecticide formulations and their efficacy. It was important to formulate insecticide formulations that have good efficacy and that were environmentally friendly. The goals in formulating a pesticide were for simplicity of application, improved pesticide performance, stability of product in storage and during application, safety and compatibility. In recent years, there has been a trend to reduce a petroleum solvent-based formulation to formulations that are considered to be safe and environmentally friendly (Bell et al., 1998).

Consequently, research on the active ingredients, formulations, application rates and environmental impact of botanical pesticides were a requirement for the successful introduction of alternative bioactive compounds (Buss and Park-Brown, 2002). Although some studies about the nano-emulsions have been done (El-Aaser et al., 1997, Lim et al., 2013), detailed studies of insecticide formulation, particularly nano-emulsion formulation are lacking. The need to improve the quality of the rotenone as a botanical insecticide and to obtain consistent control of insect pests lead to the following objectives;

1) to identify the sources of rotenone from Tuba plants based on RAPD analysis, ITS marker and their morphological characteristics,

2) to extract, identify, quantify and structure elucidation of rotenone from Tuba plants,

3) to prepare the plant-based nano-emulsion formulation of rotenone, and

4) to evaluate the insecticidal properties of the nano-emulsion formulation of rotenone against *Plutella xylostella*.

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