THE YIELD AND BIOLOGICAL ACTIVITY (LC_{50}) OF ROTENONE EXTRACTED FROM *Derris elliptica*

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To my most beloved sayang and mak

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ABSTRACT

The objective of this research was to determine the effect of the processing parameters on the extraction yield of rotenoids resin, rotenone and their biological activities (LC_{50}). The research was divided into three stages: preliminary, optimization and verification phases. Preliminary study was carried out to determine the most appropriate processing parameters for the optimization study. The optimization study was carried out using a Central Composite Design (CCD) employing the Design-Expert® software version 6.0 to determine the effects of processing parameters on the three selected response variables which were the yield of rotenoids resin, yield of rotenone and biological activity (LC_{50}) of rotenoids resin. The processing parameters studied were the types of solvent (acetone, chloroform and ethanol), solvent-to-solid ratio (2.0 ml/g to 10.0 ml/g) and raw material particles size (0.5 mm to 5.0 mm in diameter). The theoretical maximum yield of rotenoids resin in dried roots obtained from the optimization phase was 12.26 % (w/w) and 5.99 % (w/w) for the rotenone. The multiple response variables analysis have consistently verified the theoretical results in the range of 3.62 ml/g to 4.72 ml/g (solvent-to-solid ratio) and 0.83 mm to 1.41 mm in diameter (raw material particles size) using the acetone extract. The biological activity (LC_{50}) value of rotenoids resin was indirectly correlated to the optimum processing parameters due to inconsistency of rotenone content (mg) and the low value of LC_{50} which was less than 100 ppm for all treatments. This is due to the presence of other constituents in the rotenoids resin (tephrosin, 12αβ-rotenolone and deguelin) which contributed to the low LC_{50} values. The optimization of the processing parameters resulted in an increase of yield of rotenoids resin but reduced yield of rotenone.

ABSTRAK

Objektif kajian ini adalah untuk menilai kesan parameter pemprosesan terhadap pengekstrakan keberhasilan resin rotenoids, rotenone dan aktiviti biologikalnya (LC_{50}). Kajian ini dibahagikan kepada tiga peringkat: fasa saringan, pengoptimuman dan penentusahan. Kajian saringan dijalankan untuk menentukan parameter pemprosesan yang paling relevan untuk kajian pengoptimuman. Kajian pengoptimuman dijalankan menggunakan analisis 'Central Composite Design (CCD)' menggunakan perisian 'Design-Expert® version 6.0' bagi menilai kesan parameter pemprosesan bagi tiga variabel respon yang dipilih iaitu keberhasilan resin rotenoids, keberhasilan rotenone dan aktiviti biologikal (LC_{50}) bagi resin rotenoids. Parameter pemprosesan yang dikaji adalah jenis pelarut (aseton, kloroform dan etanol), nisbah pelarut terhadap pepejal (2.0 ml/g hingga 10.0 ml/g) dan saiz partikel bahan mentah (0.5 mm hingga 5.0 mm dalam diameter). Keberhasilan maksimum teori resin rotenoids di dalam akar kering yang diperolehi daripada fasa pengoptimuman adalah 12.26 % (w/w) dan 5.99 % (w/w) untuk rotenone. Analisis kepelbagaian variabel respon mengesahkan secara konsisten keputusan teori di dalam julat 3.62 ml/g hingga 4.72 ml/g dan 0.83 mm hingga 1.41 mm dalam diameter menggunakan pengekstrakan aseton. Nilai aktiviti biologikal (LC_{50}) resin rotenoids tidak berkaitan secara langsung dengan parameter pemprosesan optimum disebabkan oleh kandungan rotenone (mg) yang tidak konsisten dan nilai LC_{50} yang rendah di mana kurang daripada 100 ppm bagi semua rawatan. Ini disebabkan oleh kewujudan kandungan lain di dalam resin rotenoids (tephrosin, 12αβ-rotenolone dan deguelin) di mana turut menyumbang kepada nilai LC_{50} yang rendah. Pengoptimuman parameter pemprosesan di dapati telah menyebabkan peningkatan keberhasilan resin rotenoids tetapi mengurangkan keberhasilan rotenone.

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ANOVA	-	Analysis of Variance
A.i	-	Active ingredient
CEPP	-	Chemical Engineering Pilot Plant
CCD	-	Central Composite Design
CLCE	-	Concentrated Liquid Crude Extract
CP	-	Centre point
DAT	-	Days after treatment
DBM	-	Diamondback Moth
DF	-	Dilution factor
DIW	-	Deionized water
DOE	-	Design of Experiments
EPA	-	Environmental Protection Agency
EC	-	Emulsifiable Concentrates
IS	-	Internal standard
IPM	-	Integrated Pest Management
Kg	-	Kilogram
LC_{50}	-	Lethal Concentration of 50 % mortality
LD_{50}	-	Lethal Dose of 50 % mortality
LCE	-	Liquid Crude Extract
L	-	Litre
Ib_m	-	Pound-mass
NSE	-	Normal Soaking Extraction
NPK	-	Nitrogen, Phosphorus and Kalium
ND	-	Not determined

m.p	-	Melting point
OA	-	Oxalic acid
ppm	-	Part per millions
PDA	-	Photo Diode Array
R_f	-	Retardation factor
RSM	-	Response Surface Methodology
RP-HPLC	-	Reversed-Phase High Performance Liquid Chromatography
RCBD	-	Randomized Complete Block Design
SFE	-	Supercritical Fluid Extraction
SST	-	Total of Sum of Squares
SSR	-	Sum of Squares due to Regression
SSE	-	Sum of Squares of Residuals
SF	-	Sensitivity factor
SF	-	Safety factor
SD	-	Standard deviation
SG	-	Specific gravity
SHD	-	Safe Human Dose
TLC	-	Thin Layer Chromatography
$ThD_{0.0}$	-	Threshold Dose
UTM	-	Universiti Teknologi Malaysia
UPM	-	Universiti Putra Malaysia
UV	-	Ultra Violet
VLC	-	Vacuum Liquid Chromatography

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CHAPTER I

INTRODUCTION

1.1 Research background

One of the important issues facing approximately 6.48 billions world populations (Anonymous, 2005) is food security. The over population in developing countries and low food production exacerbated the situation. Low food production productivity is due to many factors. One of the factors is due to pest and plant diseases.

Crop protections today rely heavily on synthetic pesticides (Coats, 1994). Their uninterrupted and massive use has led to several side effects such as pesticides resistance in pests (Stoll, 1988), elimination of naturally occurring bio control agents, insect resurgence, adverse effects on non-target organisms and environment contaminations with the potential effect on the entire food chain (Copping, 1998; Harris, 1999). The growing public alarm about the hazards associated with excessive use of synthetic pesticides has revived the interest in the use of environmental-friendly crop protection products or well known as phytochemical pesticides. Phytochemical pesticides are considered environmentally benign, biodegradable (Devlin and Zettel, 1999), maintain biological diversity of predators (Grainge and Ahmad, 1998) and safer to higher animals including human beings. Thus, to help meet the food requirements of the 21st century, scientist throughout the world is looking for ecologically safe plant protection technologies emphasizing use of the botanical insecticides in the integrated pest management (IPM) programmes.

A vast number of plant species produce phytochemicals that are not directly beneficial for the growth and development of the plants. These secondary compounds are regarded as a part of the plants defence against plant-feeding insects and other herbivores (Dev and Koul, 1997). The pesticide properties of many plants have been known for a long time and natural pesticides based on plant extracts such as rotenone, nicotine and pyrethrum have been commonly used in pest control during the earlier half of this century. However, after the World War II, they lost their importance with the introduction of the synthetic organic chemicals (Suraphon and Manthana, 2001). The synthetic organic chemicals were concentrated products with a high knockdown effect on pest organisms. These chemicals could be produced in large quantities at relatively lower cost and they rapidly substituted most of the other pesticides (especially natural pesticides) in the 1950s. However, with the development of resistant insects, the threat of contaminated food and high production cost problems, natural pesticides came back again in 1995 (Suraphon and Manthana, 2001).

Furthermore, Malaysia is one of the most diverse countries to have plants and animals in the Southeast Asia region. Some of the plants show ability to adapt to and tolerate herbivores and their environment. The adapting ability derived from the production of special chemicals is called allelochemicals, which are parts of the secondary plant substances (Yu and Hsu, 1985). Plant active ingredient that shows hormonal inhibition in insects is such as azadiracthin from neem seed kernels. The other that show repellent property is such as citronella from citrus leaves (Visetson and Milne, 2001). In general, plants with pesticidal properties can be used in three ways. Initially, from the whole plant parts, in powder form or as crude extracts in water or other solvents. Secondly, as purified extracts, such as rotenone and finally as a chemical template which could be produced industrially such as pyrethrins (chemically synthesized). Moreover, natural insecticides have been used for centuries to combat insect's pests that compete for food and affect the public health (WHO, 1997). As for that reason, more than 2,000 plant species are catalogued as having insecticide properties (Heal *et al.,* 1950; Farnsworth, 1966; Sukamar *et al.,* 1991). The most commonly plant extracts such as pyrethrum, nicotine, saponin and rotenone were among the first compounds used to control insects of agricultural importance (Coasts, 1994; Grainage and Ahmed, 1998). Among the ordinary plant extracts that have been studied and commercialized is pyrethrin (which a complex of esters extracted from flowers of *Chrysanthemum cinerariefolium*) which is the one that still be used nowadays to enhance commercial preparations of the household insecticides (Bell *et al.,* 1990). Moreover, nicotine extracted from *Nicotiana glauca* and its nicotinoids derivatives are also among the choice molecules for the manufacture of new insecticides.

In addition, the one and only plant extracts that in the route of developing its niche market to be among the important insecticides is rotenone. In general, rotenone and other toxic constituents in the extract (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). Scientifically, rotenone is a bio-active compound that has a strong paralysis action (knock-down effect) on cold-blooded animals and used as an insecticide to combat pests (John, 1944; Andel 2000). Other than that, rotenoids-yielding plants have been also used for fishing based on their *itcthyotoxic* properties (Andel, 2000). For that reason, there have been reports of rotenone-containing plants used by the Indians for fishing due to its *itcthyotoxic* effect, as early as the 17^{th} century (Moretti and Grenard, 1982). Interestingly, rotenone poisoned fish is edible without any risk of food contamination to humans (Costa *et al.,* 1989). The risk of being poisoned by rotenone on mammalians can be strongly justify with the lethality tests resulted in LD_{50} (value is in milligram per kilogram (mg/kg) of body weight in mammal) range from 50 to 300 (Raws, 1986; Ellenhorn and Barceloux, 1988) and considered as a moderate hazardous substances (WHO, 1992).

As for it lethal mechanism, rotenone acts by inhibiting respiratory enzyme in the insects resulting in disruption of cellular metabolism and failure of respiratory functions (Oberg, 1961; Fukami *et al.,* 1967; Bradbury, 1986). Although rotenone is toxic to the nervous systems of insects, fish and birds, commercial rotenone products presented little hazard to humans over many decades (Schmeltz, 1971). Neither fatalities nor systemic poisonings in humans have been reported in relation to ordinary use. As for that reason, human or mammals are not highly susceptible or vulnerable to rotenone because they are protected by effective oxidizing enzyme systems (Schnick, 1974) and inefficient gastrointestinal absorption (Bradbury, 1986). This extensive research and thorough evidence on its effect against targeted organisms and non-targeted organisms (especially human) gives rotenone as one of the botanical insecticide that exceptionally selective and environmental-friendly (Schnick, 1974; Bradbury, 1986). Other reasons that accounting safety record of rotenone as a botanical insecticide are low concentration in commercial products, highly degradable and poor absorption across gut and skin of humans. Even though rotenone is a naturally occurring chemical with insecticidal, acaricidal (mite and spider killing) and piscicidal (fish-killing) properties (Extoxnet, 1996), it is a selective, non-specific insecticide and also can be used in home gardens for insect control, for lice and tick control on pets and for fish eradications as part of water body management (Weier and Starr, 1950). Because of its advantages, the extracts material can be formulated into emulsifiable concentrates (EC) and wettable powders of rotenone and extensively used in lakes, ponds and reservoirs to control undesirable fish as well as to combat the highly resistant insect pests that still posses a major threat to farmers all over the world (Kole *et al.*, 1992).

Nowadays, the production of botanical insecticide especially rotenone (from *Derris* and *Lonchocarpus* species) and pyrethrum (from *Chrysanthemum cinerariaefolium*) are dominated by the Western country such as Germany, United States (US), Canada and South America (Murray, 1997). They have the technology to extract, formulate and purify the bio-active constituents from plant material that have the insecticidal properties. One of the biggest manufacturers in Europe that produced the formulated liquid emulsion of rotenone and cube rotenoids resin is SAPHYR S. A. R. L which is based in France. According to Grinda *et al.* (1986), the used on a batch solid-liquid of Accelerated Solvent Extraction (ASE) method has made them produced as much as 14 % (w/w) yield of rotenone in dried roots and 36 % (w/w) yield of rotenoids resin in dried roots. This achievement is due to the advanced processing techniques they have implemented and the usage of strong chlorinated organic solvent that extract the bio-active constituents exhaustively. They have the technological advantages as compared to the other countries (especially in Asia) that also produced botanical insecticides product.

In Asia, only several countries are committed on developing the technology and pursuing to produce large scale of botanical insecticide such as Thailand and Vietnam. According to Hao *et al.* (1998), in Vietnam, they have conducted and set up a technical process to manufacture products from *Derris elliptica* Benth's root. They have included the emulsifiable concentrates (EC), water milk and water-soluble powder preparations. The technological protocol were established in many ways such as raw material pre-processing treatment, extraction procedures, types of solvent used, stability of rotenoids resin, biological activity, preservation and packaging. In fact, they have successfully extracted rotenone from their native *Derris* species with the yield of approximately 1.5 % (w/w) to 5.0 % (w/w) in dried roots using varies organic solvent such as acetone, chloroform and ethanol on a batch solid-liquid of Normal Soaking Extraction (NSE) method (Phan-Phuoc-Hien *et al.*, 2003). Other processing parameters (e.g.: solvent-to-solid ratio, raw material particles size, extraction temperature, extraction duration, speed of agitation and etc.) used in the process are unknown but generally ethanol is largely used as a solvent in the extraction process due to its low cost and simple process (Hien-Phan-Phuoc *et al.*, 1999). In Thailand, they have also established the technological protocol on manufacturing the botanical insecticides. They have also implemented a batch solid-liquid of Normal Soaking Extraction (NSE) method with agitation under room temperature of 26 ± 2 ^{0}C and administered for 8 hours (Suraphon and Manthana, 2001). Hence, they have managed to extract rotenone approximately 5.2 % (w/w) in dried roots. According to Pitigon and Sangwanit (1997), the most desirable solvent for the extraction of rotenone are ethanol and although chloroform is also used as a solvent, it is proven to be dangerous to human health. Therefore, ethanol is more suitable solvent for the extraction of rotenone in favour of Thailand farmers. Unfortunately, rotenone based bio-pesticide manufacturer is unavailable in Malaysia despite of its environmental-friendly effect and effectiveness to treat the persistent insect pests of Diamondback moth (*Plutella xylostella* Linn.) that always infested in the leafy vegetables farms. Thus, no rotenone based bio-pesticide listed in the Pesticide Board of Malaysia registered products until May 2006. As for that reason, Chemical Engineering Pilot Plant (CEPP) in Johor, Malaysia has initiated a research on this particular active ingredient (rotenone) since 2001 and also being the only research institute in Malaysia that undertake the research systematically by commencing the selection of *Derris* species, pre-treatment, extraction, formulation, laboratory bioassay and field trial, toxicity level and risk assessment until product registration through the Pesticide Board of Malaysia (Saiful *et al.*, 2003)

From all manufacturers and researchers that involved on producing the rotenone as a potential botanical insecticides, they have one in common which is using a batch solid-liquid extraction method even though they have implemented different processing parameters and produced varies yield of rotenoids resin in dried roots; % (w/w) and yield of rotenone in dried roots; % (w/w). In addition, not one of them has implemented the biological activity (LC_{50}) of brine shrimp (*Artemia salina*) to acquire rapid general toxicity level in which correspond to the effect of processing parameter.

As for that reason, the objective of this research was to investigate the effect of processing parameters (raw material particles size (mm in diameter), solvent-to-solid ratio (ml/g) and types of solvent) on the yield and its biological activity (LC_{50}) of rotenone extracted from *Derris elliptica* using a batch solid-liquid extraction process.

1.2 Scopes of research

In order to achieve the objective, four scopes have been formulated in this research. The scopes were:

(1) To investigate the effect of processing parameters on the yield of rotenoids resin in dried roots; % (w/w).

(2) To investigate the effect of processing parameters on the yield of rotenone in dried roots; % (w/w).

(3) To investigate the effect of processing parameters on the biological activity (LC_{50}) of brine shrimp (*Artemia salina*).

(4) To investigate the correlation between the biological activity (LC_{50}) with the yield of rotenoids resin in dried roots; % (w/w) and yield of rotenone in dried roots; % (w/w).

The processing parameters studied were solvent-to-solid ratio (ml/g), types of solvent and raw material particles size (mm in diameter). The other relevant parameters involved in this research were fixed (control parameter) such as extraction temperature (^{0}C), weight of raw material (g) and extraction duration (hour). The experiments were design using experimental design software called Design-Expert® software version 6.0 (Stat-Ease, 2002). Each data obtained from each run of experiments was evaluated for the yield of rotenoids resin in dried roots; % (w/w), yield of rotenone in dried roots; % (w/w) and biological activity (LC_{50}) of rotenoids resin. Eventually, the correlation between these response and independent variables were analyzed and interpreted.

1.3 Contribution of the research

This study contributes new knowledge in the area of phytochemical processing and phytochemical pesticide:

(A) This research help to understand the main and interaction effects of the processing parameters towards the yield of rotenoids resin, rotenone and their biological activities (LC_{50}). The correlation determined between independent variables would further promote and enhance the usage of rotenoids resin as a phytochemical pesticide or botanical insecticide products. Understanding the effect of processing parameters against the yield of rotenoids resin, rotenone and their biological activities (LC_{50}) are essential in designing a better processing technology to maintain and preserve the bio-active constituents in the extracts and rotenoids resin effectively.

(B) Although *Derris* roots have been identified as a potential cash crop due to its abundance growth in Malaysia, no research work of its own native species has been conducted locally. The identification of appropriate processing parameters to acquire maximum yield of rotenoids resin, rotenone and their biological activities (LC_{50}) against targeted and non-targeted organism are also have not being studied. According to the local patent and industrial company database related to the botanical insecticides production, there is no rotenone-based industry listed in Malaysia until now. Therefore, the opportunities to develop an option to the synthetic pesticides and environmental-friendly natural bio-pesticide from local plant species are the main rationale why this extensive study should be completed and carried out successfully.