Extraction and Formulation Development of *Derris elliptica* for Insect Pest Control

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ชื่อวิทยานิพนธ์ การสกัดและการพัฒนาสูตรตำรับโล่ติ๊นเพื่อควบคุมแมลงศัตรูพืช

ผู้เขียน นางสาวอรรถวดี แซ่หยุ่น

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บทคัดย่อ

โล่ติ๊นหรือหางไหลแดง (*Derris elliptica* Benth) เป็นพืชที่เกษตรกรใช้ในการควบคุมแมลงศัตรูพืชมานาน เนื่องจากสารสกัดจากโล่ติ๊นมีสาร rotenone ซึ่งมีคุณสมบัติในการฆ่าแมลงในปริมาณสูง แต่จากความยุ่งยากในการเตรียมสารสกัด และสารสกัดที่ได้มีคุณภาพต่ำ จึงส่งผลต่อประสิทธิภาพในการฆ่าแมลง และเป็นข้อจำกัดของการใช้โล่ติ๊นเพื่อควบคุมแมลงศัตรูพืช ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อเปรียบเทียบการสกัดสารจากโล่ติ๊นด้วยการหมักซึ่งเป็นวิธีดั้งเดิมและเทคนิค pressurized liquid extraction (PLE) จากนั้นนำสารสกัดที่ได้มาพัฒนาเป็นสูตรตำรับที่มีประสิทธิภาพและมีความคงตัวที่ดี

งานวิจัยนี้ได้ทำการสกัดสารจากลำต้นและรากของหางไหลแดง และลำต้นของหางไหลขาว (*Derris malaccensis* Prain) ด้วยวิธีการสกัดทั้ง 2 วิธีดังกล่าวเพื่อเปรียบเทียบประสิทธิภาพของวิธีการสกัด สำหรับการสกัดด้วยเทคนิค PLE ได้มีการศึกษาถึงผลของตัวแปรต่างๆ ได้แก่ ชนิดของตัวทำละลาย อุณหภูมิ และความดันต่อประสิทธิภาพในการสกัด พบว่า chloroform เป็นตัวทำละลายที่สามารถสกัด rotenone ออกมาได้ดีกว่า 95% ethanol และสภาวะที่เหมาะสมในการสกัดด้วยเทคนิค PLE คือ อุณหภูมิ 50 ^{o}C และความดัน 2000 psi ซึ่งการสกัดด้วย PLE ในสภาวะนี้จะได้สารสกัดจากรากหางไหลแดงที่มีปริมาณ rotenone สูงที่สุด คือ 46.1% w/w นอกจากนี้การสกัดด้วยเทคนิค PLE ยังสามารถลดปริมาณการใช้ตัวทำละลาย และลดเวลาที่ใช้ในการสกัดลงได้

สารสกัดจากโล่ติ๊นได้ถูกนำมาใช้ในการเตรียมเป็นสูตรตำรับในรูปแบบแกรนูลกระจายตัวได้ในน้ำ และรูปแบบอิมัลชันเข้มข้น โดยแกรนูลที่เตรียมได้มีความกร่อนน้อย และสามารถกระจายตัวในน้ำกลายเป็นของเหลวแขวนตะกอนได้อย่างรวดเร็ว ส่วนอิมัลชันเข้มข้นสามารถกระจายตัวได้ดีในน้ำกลายเป็นอิมัลชันได้เร็วเช่นกัน โดยทั้งของเหลวแขวนตะกอนและอิมัลชันที่ได้มีค่า pH และความหนืดที่เหมาะสมสำหรับการนำไปใช้โดยการฉีดพ่น

การศึกษาความคงตัวแบบเร่งของสารพบว่า rotenone ในสารสกัดและในสูตรตำรับมีการสลายตัวแบบปฏิกิริยาอันดับหนึ่ง โดย rotenone ในสูตรตำรับรูปแบบแกรนูลและรูปแบบอิมัลชันเข้มข้นมีความคงตัวมากกว่า rotenone ในสารสกัด นอกจากนี้ rotenone ในสูตรตำรับรูปแบบแกรนูลยังมีความคงตัวดีกว่าในรูปแบบอิมัลชันเข้มข้นทั้งการเก็บในสภาวะเร่งและในสภาวะปกติ อย่างไรก็ตามผลิตภัณฑ์ที่ได้ควรเก็บไม่ให้โดนแสงในที่แห้งและเย็นเพื่อความคงตัวของสารสำคัญ เนื่องจากปฏิกิริยาหลักที่มีผลต่อการสลายตัวของ rotenone ได้แก่ ปฏิกิริยาการสลายตัวด้วยน้ำ ปฏิกิริยาออกซิเดชัน และการสลายตัวด้วยแสง

การศึกษาการสลายตัวของ rotenone ในสูตรตำรับหลังจากฉีดพ่นบนใบพืชพบว่าประสิทธิภาพของสูตรตำรับจะยังคงอยู่เป็นเวลา 3 วัน หลังจากฉีดพ่น แต่เนื่องจากการฉีดพ่นในสภาวะการเพาะปลูกจริง การรดน้ำหรือน้ำฝนอาจชะล้างเอาสูตรตำรับออกไป ดังนั้นจึงอาจจะต้องเพิ่มความเข้มข้นของสาร หรือเพิ่มความถี่ในการฉีดพ่น เพื่อคงประสิทธิภาพการออกฤทธิ์ของสูตรตำรับไว้

การศึกษาประสิทธิภาพของสูตรตำรับในห้องทดลองพบว่า สูตรตำรับสารสกัดโล่ติ๊นรูปแบบอิมัลชันเข้มข้นมีประสิทธิภาพในการฆ่าหนอนกระทู้ผักได้ดีกว่าสูตรตำรับรูปแบบแกรนูล แต่ประสิทธิภาพของสูตรตำรับอิมัลชันเข้มข้นในการฆ่าหนอนกระทู้ผักที่ทดสอบในเรือนกระจกต่ำกว่าการทดสอบในห้องทดลอง ซึ่งอาจเป็นเพราะสารส่วนหนึ่งถูกชะออกไปจากการรดน้ำและ rotenone สลายตัวเมื่อโดนแสง ดังนั้นจึงอาจจะต้องเพิ่มความถี่ในการฉีดพ่น หรือเติมสารเสริมฤทธิ์ลงในสูตรตำรับเพื่อเพิ่มประสิทธิภาพการออกฤทธิ์ของ rotenone

สารสกัดโล่ติ๊นและ rotenone ในรูปแบบเม็ดบีด calcium alginate ถูกเตรียมขึ้นโดยการปรับสัดส่วนความเข้มข้นของ sodium alginate และ calcium chloride รวมทั้งปรับขนาดของปลายหลอดฉีดยาหรือเข็มฉีดยาที่ใช้ สำหรับการปลดปล่อยโรติโนนจากเม็ดบีดจะขึ้นอยู่กับการพองตัวของเม็ดบีด และเม็ดบีดสามารถควบคุมการปลดปล่อยสารออกมาอย่างช้าๆ ทำให้การออกฤทธิ์นานขึ้น และยังป้องกัน rotenone จากการสลายตัวด้วยแสงได้

งานวิจัยนี้สามารถใช้เป็นแนวทางในการพัฒนาสูตรตำรับสารฆ่าแมลงจากพืช โดยการเลือกวิธีการสกัด และวิธีการเตรียมสูตรตำรับที่เหมาะสม ซึ่งจะทำให้ได้สูตรตำรับที่ผลิตได้ในท้องถิ่น มีราคาถูก และมีความปลอดภัย เพื่อเป็นทางเลือกให้กับเกษตรกรในการควบคุมศัตรูพืช

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ABSTRACT

Derris elliptica Benth has previously been known as an important source for compounds with insecticidal properties. Derris extract was used as an alternative insecticide due to its high content of rotenone. However, laborious preparation process and low quality of derris extracts affect the effectiveness and limit their use in insect control. Therefore, the objectives of this study were to compare conventional maceration and pressurized liquid extraction (PLE) techniques for the efficient extraction of derris extract and develop effective and stable derris formulations.

The comparison of maceration and PLE for the efficient extraction of the stems and roots of *D. elliptica* and *Derris malaccensis* Prain was studied. The effects of experimental variables, such as solvent, temperature and pressure, on PLE efficiency have been evaluated. Chloroform was found to be a better extraction solvent compared to a commonly used solvent, 95% ethanol. PLE using chloroform as extraction solvent at 50 ^{o}C and 2000 psi was found to be an optimal conditions and gave the extract from the roots of *D. elliptica* with the highest rotenone content (46.1 %w/w). Moreover, PLE required smaller amount of extraction solvent and shorter extraction time.

Water dispersible granules and emulsifiable concentrate liquids containing derris extract (equivalent to 5% w/w of rotenone) were developed with simple techniques. The derris water dispersible granules gave short disintegration time and low friability and provided yellow suspension when diluted with water. The derris emulsifiable concentrate also rapidly provided a stable yellow emulsion when diluted with water. The obtained suspension and emulsion exhibited the appropriate pH and viscosity for spray application.

Accelerated degradation kinetics of rotenone in the derris extract, and in both formulations, indicated that its degradation followed first-order kinetics. Derris water dispersible granules and emulsifiable concentrate clearly prolonged the stability of rotenone in derris extract. The derris water dispersible granules showed better stability of rotenone than the derris emulsifiable concentrate either in accelerated conditions or in real-time storage. However, the developed formulations should be kept in cool and dry place, and protected from light in order to preserve the active component because the degradation pathways of rotenone mainly occur through hydrolysis, oxidation, and photolysis.

The study of rotenone degradation after application onto plants indicated that both formulations would be effective for up to 3 days after spraying. In addition, the formulations applied in the fields may be washed off by water or rainfall. Therefore, the effective concentrations for spraying and frequency of applications must be considered.

Preliminary efficacy testing indicated that the derris emulsifiable concentrate was clearly more effective than derris water dispersible granules in controlling *Spodoptera litura*. However, Derris emulsifiable concentrate showed lower efficacy to *S. litura* control in greenhouse than those in laboratory conditions because the formulation may be washed off from the foliar, and rotenone possibly decomposed under greenhouse conditions. Therefore, repeated application or addition of synergists may be necessary to increase the effectiveness of the formulations.

Calcium alginate beads containing rotenone and derris extract were successfully prepared by ionotropic gelation with varying the proportion of sodium alginate and calcium chloride and diameter of syringe or needle. The rotenone release of the beads is mainly related to the swelling behavior of the alginate. Calcium alginate beads could provide control release of rotenone in longer duration (1-7 days) and protect rotenone from photodecomposition.

This research can be used as a guidance for suitable selection of extraction method and preparation process in formulation development. The locally produced, cheap and safe formulated products of natural pesticides will be alternative formulations for utilization in agricultural countries.

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CHAPTER 1

INTRODUCTION

1.1 Background and rationale

Pest control in recent years has become a major problem in almost all agricultural countries. A number of pest control strategies have been developed to manage various pests under different situations. However, pesticides continue to be the single most widely used pest control due to their ease of application and rapidity of action. Unfortunately, many older, non-patented, more toxic, environmentally persistent and inexpensive conventional synthetic pesticides are used extensively in developing countries. In Thailand, large amounts of pesticides are imported to the country in order to improve the efficacy of agricultural production, resulting serious health problems and environmental contamination. The presence of residues of these pesticides in agricultural products, food commodities, and other components of the environment has proved toxic to humans, domestic animals, birds, fish and other organisms. In addition, some of pests have developed resistance to one or more groups of pesticides (Ecobichon, 2001; Thapinta and Hudak, 2000).

To overcome increasing problems encountered with the excessive use of pesticides, efforts are being made to turn to the use of alternative methods that are environmentally friendly, and are of relatively lower cost compared to the chemical pesticides. Botanical pesticides are examples of such pest control alternatives, and they are of increasing interest due to their proven efficacy and rapid decomposition in the environment after usage. A large number of plants have been reported to possess insecticidal properties. Derris, pyrethrum, tobacco, neem, stemona, ryania, sabadilla and a number of other lesser known botanical pesticides were used to protect agricultural crops from the ravages of insect and non-insect pests in different parts of world. Many of the extracts from these indigenous plant sources have been developed in the form of insecticide products (Bohmont, 2000; Brem et al., 2002; Charleston et

al., 2005; Dhaliwal and Arora, 2001; Evans, 2002; Javed et al., 2008; Komalamisra et al., 2005; Pureesatien et al., 2008; Ray, 1991; Soloway, 1976; Tyler et al., 1976).

Derris elliptica Benth (Figure 1.1) has previously been known as an important source for compounds with broad-spectrum insecticidal properties (Gupta, 2007), *Derris* is a genus of the family Papillionaceae. It is locally known in Southeast Asian countries as “Derris” or “Tuba” and in Thailand as “Lotin” or “Hang lai daeng”. Extracts from the stems and roots of this plant have been used over centuries as fish poisons and as insecticidal preparations. These properties are mainly due to the presence of rotenone (Figure 1.2), a compound that is highly toxic to cold-blooded animals, especially fish and insects (Evans, 2002; Tyler et al., 1976).

Figure 1.1 *Derris elliptica* Benth.

Rotenone is classified by the World Health Organization as a moderately hazardous Class II pesticide. There are some reports indicating that an exposure to rotenone may cause toxicity to mammals. However, rotenone is usually used in small quantities and it is rapidly broken down in soil and water. Almost all toxicity may be lost after 2-3 days of summer exposure. Rotenone is therefore good for the environment and safe for agriculturists and other users (Craig, 2001; Fukami and Nakajima, 1971; Mutsumura, 1975; Ray, 1991).

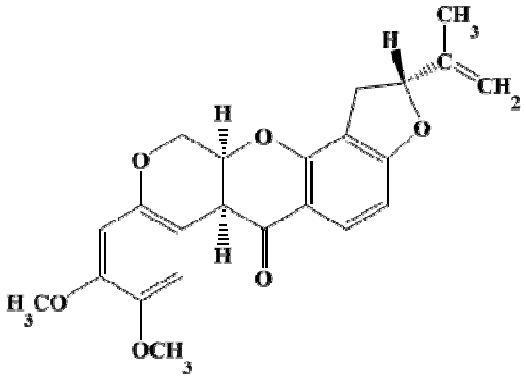


Figure 1.2 Chemical structure of rotenone

Rotenone is commonly sold as a dust containing 1 to 5% active ingredients for home and garden use, but liquid formulations used in organic agriculture can contain as much as 8% rotenone and 15% total rotenoids. These formulations are mostly marketed in the United States and Europe (Isman, 2006; Ray, 1991). However, most rotenone-containing products have relative high cost and the formulation ingredients are commercial secrets. *D. elliptica* is easy to grow and provides high root yield within 2 years (Tongma *et al.*, 2004). There is one Thai study on growth patterns of this plant and accumulation of rotenone and other rotenoids in *Derris* species harvested at different ages. The quantity of rotenoids in the roots accumulates until the age of 27 months, after which it decreases, the highest quantity being found at the age of 26 months (Srijugawan et al., 1993). Therefore, it is possible to grow this plant on a large scale with relative ease for commercialization of the dried root or its products within 2-3 years after planting. Farmers in Thailand and other developing countries usually macerate the derris roots in water and use the resulting milky suspension for spraying their crops (Takei, 1929). However, this method of preparation is inconvenient and the amount of active compound in the suspension

is low and unstable. Moreover the quality and efficacy of the extracts may vary from application to application. Therefore, there was a need for the development of formulations of derris extracts.

Water dispersible granules (WDG) were chosen as one of the formulations because they potentially offer significant advantages in packaging, ease of handling, stability and safety. Since the surface area of granules is lower than comparable volumes of powder formulations, granules are usually more stable to the effects of the atmosphere (Ansel et al., 1995; Knowles, 2008). Granules are associated with a much lower inhalation hazard compared to dusts and wettable powders. Granules can readily be dispersed in water to form fine suspensions in the spray tank, and only require gentle agitation to maintain a uniform mixture.

Emulsifiable concentrates (EC) are widely used for formulating pesticides. However, most currently used pesticide formulations require the use of organic solvents and other additives, which may present a problem in term of user and environmental safety (Knowles, 2008). In this study, emulsifiable concentrate formulations, using mixtures of mineral oil or soybean oil solution and emulsifier were prepared. The plant extract was miscible in the liquid mixture and the system formed a fine oil-in-water (o/w) emulsion when introduced into an aqueous phase under conditions of gentle agitation (Charman et al., 1992). The concentrate liquid formulation was preferred, rather than the emulsion formulation due to ease of handling and delivery.

Calcium alginate beads have been widely used as controlled release formulations of agrochemicals due to their simple preparation and biodegradability (Kenawy and Sakran, 1996; Pepperman and Kuan, 1995; Roy et al., 2009). Moreover, calcium alginate beads can be handled with relative ease and safety (Matthews, 1979). In this thesis, rotenone and derris extract were encased in small beads made of polymer. The beads were expected to control the release of rotenone and protect rotenone from UV light so it would be applied more effectively under field conditions.

There is wide variation in the quality and quantity of extracts obtained from a plant. Such variations affect the performance and shelf-life of formulated products. High quality raw plant materials were required for insecticide production. Standardized procedures need to be developed for extraction, identification, purification of active ingredients. Simple formulation technology will have to be developed so that ready-to-use pesticides can be produced at the local

level. Therefore, a lot of works need to be done for the development of effective, stable and standardized formulations of derris extracts.

1.2 Review of literature

1.2.1 Pesticides

1.2.1.1 Definition of pesticides (Hayes, 1991)

Pesticides are defined under the Federal Insecticide, Fungicide and Rodenticide Act as amended; they include

(1) Any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest (insect, rodent, nematode, fungus, weed, other forms of terrestrial or aquatic plant or animal life or viruses, bacteria, or other micro-organisms, except viruses, bacteria, or other micro-organisms on living man or other animals, which the administrator declares to be a pest).

(2) Any substance or mixture of substances intended for use as a plant regulator, defoliant or desiccant.

1.2.1.2 Classification of pesticides (Bohmont, 2000)

Pesticides are classified in several ways, each having its own value for a given purpose. One of the most common means to classify a pesticide is based on the type of organism against which they are effective (Table 1.1).

Table 1.1 Pesticides as classified by their target species (Bohmont, 2000)

Types of pesticides	**Target species**
Acaricide	Mites, ticks
Algicide	Algae
Attractant	Insects, birds, other vertebrates
Avicide	Birds
Bactericide	Bacteria
Defoliant	Unwanted plant leaves
Desiccant	Unwanted plant tops
Fungicide	Fungi
Growth regulator	Insect and plant growth
Herbicide	Weeds
Insecticide	Insects
Miticide	Mites
Molluscicide	Snails, slugs
Nematicide	Nematodes
Piscicide	Fish
Predacide	Vertebrates
Repellents	Insects
Rodenticide	Rodents
Silvicide	Trees and woody vegetation
Slimicide	Slime molds
Sterilants	Insects, vertebrates

Pesticides are also classified based on their toxicity. The toxicity category is assigned on the basis of the highest measured toxicity, oral, dermal, or inhalation; effects on the eyes and external injury to the skin are also considered. There are three systems being used in Thailand to classify the risk to human health and warn people of the danger. Table 1.2 compares the three systems (Bartlett and Bijlmakers, 2003).

Table 1.2 Toxicity classes of pesticides (Bartlett and Bijlmakers, 2003)

WHO		**EPA**		**Thai government**	**Example chemical**
Class	**Description**	**Category**	**Warning**	**Color code**	
Ia Ib	Extremely hazardous Highly hazardous	I	Danger-poison	Red label	Methyl parathion Methamidophos
II	Moderately hazardous	II	Warning	Yellow label	Paraquat
III	Slightly hazardous	III	Caution	Blue label	Malathion
IV	Unlikely to be hazardous	IV	None	Blue label	Glyphosate

The Thai Government had introduced a color code for pesticide containers. A red stripe on the label indicates that the chemical is highly poisonous, yellow indicates moderately poisonous and blue indicates slightly poisonous. These colors are used at the bottom of the pesticide label, with pictures to show the type of protective clothing that should be used.

The World Health Organization (WHO) has created a ‘toxicity classification’ with five classes. This system is used in technical documents, including those produced by the Thai Government.

The US Environmental Protection Agency (EPA) has a ‘toxicity ranking’ with four categories. The EPA warnings are often used on pesticide labels.

1.2.1.3 Botanical pesticides

Botanical pesticides are derived or extracted directly from plants (Table 1.3). More than 100 species of plants with insecticidal properties have been listed. Some plants are not necessarily toxic but contain chemicals with adversely affect the behavior and physiology of insects. The use of botanical pesticides offers several advantages over the synthetic pesticides. The naturally occurring phytochemicals exert a wide range of behavioral and physiological effects on insects and, therefore, it is difficult for insects to develop resistance easily against these pesticides. In addition, the available evidence indicates that botanical pesticides are biodegradable in contrast to persistent synthetic pesticides. However, all the plant materials or isolated components have temporary or restricted used in localized areas around the world and their utility as pest control materials has remained obscure (Bohmont, 2000; Dhaliwal and Arora, 2001).

Table 1.3 Botanical pesticides traditionally used for pest control in agricultural crops

Pesticide	Main source	References
Rotenone	*Derris elliptica*	Dhaliwal and Arora, 2001;
	Derris malaccensis	Evans, 2002;
	Derris ulignosa	Tyler et al., 1976
	Lonchocarpus urucu	
	Lonchocarpus utilis	
	Tephrosia toxicaria	
	Tephrosia virginiana	
	Tephrosia vogelii	
	Amorpha fruticosa	
Pyrethrin	*Chrysanthemum cinerariaefolium*	Dhaliwal and Arora, 2001;
	Chrysanthemum coccineum	Evans, 2002;
	Chrysanthemum marschallii	Tyler et al., 1976
Nicotine	*Nicotiana tabacum*	Dhaliwal and Arora, 2001;
	Nicotiana rustica	Evans, 2002;
	Nicotiana glauca	Tyler et al., 1976

Table 1.3 Botanical pesticides traditionally used for pest control in agricultural crops (continued)

Pesticide	Main source	References
Azadirachtin	*Azadirachta indica*	Charleston et al., 2005;
	Melia azedarach	Dhaliwal and Arora, 2001
Stemofoline	*Stemona collinsae*	Brem et al., 2002;
	Stemona curtisii	Pureesatien et al., 2008;
Veratridine	*Sabadilla officinarum*	Dhaliwal and Arora, 2001;
	Veratrum sabadilla	Evans, 2002
Ryanodine	*Ryania speciosa*	Dhaliwal and Arora, 2001;
		Evans, 2002;
		Tyler et al., 1976
Pellitorine	*Anacyclus pyrethrum*	Dhaliwal and Arora, 2001
Quassin	*Quassia amara*	Dhaliwal and Arora, 2001

1.2.1.4 Formulations of pesticides

Pesticides are biologically active in extremely small quantities, so the chemicals have to be prepared in a form that is convenient to use and to spread evenly over large areas. The preparation of the active ingredient in a form suitable for use is referred to as ‘formulation’. The science of pesticide formulation covers a very broad field, as it deals with the development, production, and storage of the formulations, as well as with the interaction of pesticides with the environment, including plants, insects, animals, soil, air, and water. Formulations make an active ingredient more convenient to handle, safer, easier and more accurate to apply and in some cases, more attractive to the pests.

The choice of formulation is determined by the type of use, the crop to be treated, the cultural methods that most commonly used in the region, and any specific requirements on the part of the user. But the wide range of formulation types that have been developed also follow from variation in the physicochemical properties of the active substances themselves. Among these, melting point, solubility and chemical stability are important factors.

The development of a formulation also involves testing further important criteria such as crop compatibility, environmental behavior, and toxicity. Moreover, it is important that the product, once sold, remains stable in storage under various conditions, retaining its activity for at least two years (Bayer CropScience, 2005; Bohmont, 2000; Martin et al., 2001; Matthews, 1979).

Formulations are classified as solids or liquids on the basis of their physical state in the container at the time of purchase. A formulation can contain more than one active ingredient, and many have to be further diluted with an appropriate carrier (e.g., water) prior to use. Solid formulations can be divided into two types: ready-to-use, and concentrates which must be mixed with water to be applied as a spray. Dusts, granules, and pellets are ready-to-use. Wettable powders, water dispersible granules, soluble powders, and microencapsulated formulations are intended to be mixed with water. Liquid formulations are mixed with a carrier follow. The carrier will generally be water, but in some instances labels may permit the use of crop oil, diesel fuel, kerosene, or some other light fuel oil as a carrier. Aerosols really refer to a delivery system that moves the active ingredient to the target site in the form of a mist of very small particles: solids or liquid drops. The particles can be released under pressure or produced by fog or smoke generators. Fumigants deliver the active ingredient to the target site in the form of a gas. Some fumigants are solids that sublime (turn into a gas) in the presence of atmospheric moisture. Others are liquids under pressure that vaporize when the pressure is released (Martin et al., 2001). The characteristics, advantages and disadvantages of each formulation are described in Table 1.4, 1.5 and 1.6.

Many of botanical pesticides are currently used in agriculture and industrialized world. Derris, pyrethrum, neem and tobacco have been utilized and developed to be well-known commercial pesticide products (Table 1.7). They are usually formulated in form of the emulsifiable concentrate or wettable powder because most of extracts or active compounds are insoluble or poorly soluble in water. Some formulations contained both of pure active ingredients and the extracts from plant such as products of rotenone and cubé extract. Occasionally, some synergists are also added to the formulations such as piperonyl butoxide was used as the synergist of pyrethrin.

Table 1.4 Solid formulations of pesticides (Bohmont, 2000; Martin et al., 2001; Matthews, 1979)

Formulation	Description	Advantage	Disadvantage
Dusts (D)	- Manufactured by the sorption of an active ingredient onto a very fine, dry inert carrier made from talc, chalk, or clay - The size of individual dust particles is variable. - Used in cracks and crevices, and for spot treatments	- Usually ready to use, with no mixing - Require simple equipment - Effective in hard-to-reach indoor areas - Provide excellent coverage	- High inhalation and drift hazard - Expensive because of low percentage of active ingredient
Granules (G)	- The coarse particles are made from an absorptive material such as clay, corn cobs, or walnut shells. - The active ingredient either coats the outside of the granules or is absorbed into them. - Usually used for soil applications	- Ready to use; no mixing - Drift hazard is low; particles settle quickly - Low hazard to applicator - Weight carries the formulation through foliage to soil target - Simple application equipment - May be more persistent than wettable powders (WPs) or emusifiable concentrates (ECs)	- Does not stick to foliage - More expensive than WPs or ECs - May need to be incorporated into soil - May need moisture to activate pesticidal action

Table 1.4 Solid formulations of pesticides (continued)

Formulation	Description	Advantage	Disadvantage
Pellets (P or PS)	- Manufactured to create a pellet of specific weight and shape - The uniformity of the particles allows them to be applied by precision applicators	- Similar to granules	- Similar to granules
Wettable powders (WP or W)	- Dry, finely ground formulations - Contain wetting and dispersing agents - Must be mixed with water for application as a spray - Upon dilution, a suspension was formed in the spray tank and required constant agitation	- Low cost - Easy to store, transport and handle - Lower phytotoxicity hazard than ECs and other liquid formulations - Easily measured and mixed - Less skin and eye absorption than ECs and other liquid formulations - Container disposal is less difficult	- Inhalation hazard to applicator while pouring and mixing the concentrated powder - Require good and constant agitation (usually mechanical) in the spray tank - Abrasive to many pumps and nozzles, causing them to wear out quickly - Residues may be visible.

Table 1.4 Solid formulations of pesticides (continued)

Formulation	Description	Advantage	Disadvantage
Water dispersible granules (WDG) or Dry flowables (DF)	- Like WP formulation, except the active ingredient is prepared as granule-sized particles	- Similar to WPs, except more easily measured and mixed - Cause less inhalation hazard to the applicator during pouring and mixing	- Require good and constant agitation (usually mechanical) in the spray tank - Abrasive to many pumps and nozzles - Residues may be visible.
Soluble powders (SP)	- Dissolve readily and form a true solution when mixed with water	- Similar to WPs	- Inhalation hazard to applicator during mixing
Microencapsulated formulations	- Particles of pesticides (either liquid or dry) surrounded by polymer coating - Must be mixed with water for application as a spray - The capsules slowly release and prolong the active life of pesticide by providing a timed release of active ingredient	- Increase safety to applicator - Easy to mix, handle, and apply	- Constant agitation necessary in tank - Some bees may pick up the capsules and carry them back to the hives where the released pesticide may poison entire hives

Table 1.5 Liquid formulations of pesticides (Bohmont, 2000; Martin et al., 2001; Matthews, 1979)

Formulation	Description	Advantage	Disadvantage
Soluble liquids (SL) or solutions (S)	- Dissolve readily in water - Contain the active ingredient and one or more additives - When mixed with water, they form a solution	- No agitation necessary	- Very few formulations of this type available
Emulsifiable concentrates (EC)	- Contained active ingredient in oil based solvent and emulsifier - Must be mixed with water for application as a spray - Upon dilution, an emulsion was formed in the spray tank	- Product is easy to handle, transport and store - Little agitation required; not abrasive; will not settle out or separate when equipment is running - Little visible residue on fresh fruits and vegetables and on finished surfaces	- Easy to overdose or underdose through mixing or calibration errors - Phytotoxicity hazard usually greater - Easily absorbed through skin of humans or animals - May be corrosive

Table 1.5 Liquid formulations of pesticides (continued)

Formulation	Description	Advantage	Disadvantage
Liquid flowables (FL)	- Finely ground active ingredients are mixed with a liquid, along with inert ingredients, to form suspension - Must be mixed with water to be applied - Upon dilution, a suspension was formed in the spray tank - Require constant agitation	- Seldom clog nozzles - Easy to handle and apply	- Require moderate agitation to prevent settling or separation - May leave a visible residue

Table 1.6 Aerosols and Fumigants (Bohmont, 2000; Martin et al., 2001; Matthews, 1979)

Formulation	Description	Advantage	Disadvantage
Aerosols (A)	- The pesticide is driven through a small opening by an inert gas under pressure, creating fine droplets - These products are used in greenhouses, in small areas inside buildings, or in localized outdoor areas.	- Ready to use - Easily stored - Convenient way of buying small amount of pesticide - Retain their potency over fairly long time	- Expensive - Practical for very limited uses - Have odor problem - Risk of inhalation injury - Hazardous if punctured, overheated, or used near an open flame - Difficult to confine to target site of pest
Fumigants	- The active ingredients are gases, volatile liquids, or solids that become gases when released during application. - Fumigants are used in soil, greenhouses, granaries, and grain bins.	- Toxic to a wide range of pests - Can penetrate cracks, crevices, wood, and tightly packed areas such as soil or grains - Single treatment will usually kill most pests in treated area.	- Target area must be enclosed or covered to prevent the gas from escaping. - Highly toxic to humans; specialized protective equipment, including respirators, must be used.

Table 1.7 Details of the commercial botanical pesticide formulations (Duso et al., 2008; Kumar et al., 2003; PAN, 2008)

Product trade name	Active ingredient	Formulation type	Manufacturer
Agway rotenone dust	Rotenone (1.00%) and Cubé extract (1.50%)	Wettable powder or Dust	Bonide Products, Inc., USA
Arab power pak insect fogger-pyrethrin	N-octyl bicycloheptene dicarboximide (1.00%), Piperonyl butoxide (1.00%) and Pyrethrin (0.50%)	Aerosol	Waterbury Companies Inc., USA
Begick's bonide rotenone 5 organic insecticide	Rotenone (5.00%) and Cubé extract (10.0%)	Wettable powder	Bonide Products, Inc., USA
Biopiren® plus	Pyrethrin (2%)	Emulsifiable concentrate	Intrachem Bio Italia S.p.A., Italy
Clean crop pyrethrins rtu	Pyrethrin (0.02%) and Piperonyl butoxide (0.2%)	Granules	Chem-Tech, Ltd., USA
Econeem®	Azadirachtin (0.3%)	Emulsifiable concentrate	Margo Bio-control Pvt. Ltd., Bangalore
Econeem Plus®	Azadirachtin (1%)	Emulsifiable concentrate	Margo Bio-control Pvt. Ltd., Bangalore
Fortune Aza®	Azadirachtin (3%)	Emulsifiable concentrate	Fortune Bio tech Lab, Hyderabad, Andhra Pradesh

Table 1.7 Details of the commercial botanical pesticide formulations (continued)

Product trade name	Active ingredient	Formulation type	Manufacturer
Fulex nicotine fumigator	Nicotine (13.4%)	Ready-to-use solution	Fuller System, Inc., USA
Limonool®	Azadirachtin (0.03%)	Emulsifiable concentrate	Bio Multi-tech (Pvt) Ltd., Bangalore
Neem Azal™-F	Azadirachtin (5%)	Emulsifiable concentrate	EID Parry (India) Ltd., Chennai
Nimbecidine®	Azadirachtin (0.03%)	Emulsifiable concentrate	T. Stanes and Company Ltd., Coimbatore
Oikos®	Azadirachtin (3.2%)	Emulsifiable concentrate	Sipcam, Milan, Italy
Pyrethrin 101 concentrate	Pyrethrin (1.00%) and Piperonyl butoxide (10.0%)	Emulsifiable concentrate	Amvac Chemical Corporation, USA
Rotena®	Rotenone (6.2%)	Emulsifiable concentrate	Serbios, Milan, Italy
Rotenone-pyrethrins liquid spray	Pyrethrin (0.8%), Rotenone (1.10%) and Cubé extract (1.10%)	Soluble concentrate	Bonide Products, Inc., USA
Soluneem®	Azadirachtin (6.5%)	Water soluble powder	Vittal Mallya Research Foundation, Bangalore

1.2.2 Derris plants

Derris plants are evergreen lianas growing in the wild and subtropical areas of Asia. They were once widely cultivated as important natural resources for insecticidal products. It had been hundreds of years since human beings used the derris root powder for fishing. They were used by native people to treat infestations of insects and some other pests. These plants are also extensively used in cattle to control ticks and other ectoparasites. Derris plants received much attention from phytochemical viewpoint because of their plentiful production of flavonoids. There are many reports about the insecticidal activities of derris extracts (Table 1.8).

Table 1.8 Insecticidal activities of extracts from derris plants

Source	**Extract**	**Biological activity**	**References**
D. elliptica, root	Ethanolic extract with Soxhlet extraction	Insecticidal activity (3^{rd} instar larvae of *Plutella xylostella*, LD_{50} = 24.25 ppm)	Visetson and Milne, 2001
D. elliptica, root	Ethanolic extract with stirring soaking	Insecticidal activity (3^{rd} instar larvae of *P. xylostella*, LD_{50} = 89.07 ppm)	Visetson and Milne, 2001
D. elliptica, root	-	Insecticidal activity (2^{nd}-3^{rd} instar larvae of *Ostrinia furnacalis*, LC_{50} = 30.71 and 4.28 ppm at 3 and 5-day post exposure, respectively)	Sukonthabhirom na Pattalung et al., 2003
D. elliptica, root	Water extract	Larvicidal activity (mosquito larvae, LD_{50} = 5.6 g/l)	Sangmaneedet et al., 2004
D. elliptica	Ethanolic extract	Larvicidal activity (late 3^{rd}-early 4^{th} instar larvae of *Aedes aegypti*, LC_{50} = 20.49 and LC_{90} = 47.49 mg/l)	Komalamisra et al., 2005

Table 1.8 Insecticidal activities of extracts from derris plants (continued)

Source	Extract	Biological activity	References
D. elliptica	Petroleum ether extract	Larvicidal activity (late 3rd-early 4th instar larvae of *Aedes aegypti*, LC_{50} = 11.17 and LC_{90} = 27.74 mg/l)	Komalamisra et al., 2005
D. elliptica	Petroleum ether extract	Larvicidal activity (late 3rd-early 4th instar larvae of *Culex quinquefasciatus*, LC_{50} = 4.61 and LC_{90} = 15.42 mg/l)	Komalamisra et al., 2005
D. elliptica	Petroleum ether extract	Larvicidal activity (late 3rd-early 4th instar larvae of *Anopheles dirus*, LC_{50} = 8.07 and LC_{90} = 15.77 mg/l)	Komalamisra et al., 2005
D. elliptica	Petroleum ether extract	Larvicidal activity (late 3rd-early 4th instar larvae of *Mansonia uniformis*, LC_{50} = 18.84 and LC_{90} = 46.95 mg/l)	Komalamisra et al., 2005
D. elliptica	Methanolic extract	Larvicidal activity (late 3rd-early 4th instar larvae of *A. aegypti*, LC_{50} = 13.17 and LC_{90} = 32.22 mg/l)	Komalamisra et al., 2005
D. elliptica	Methanolic extract	Larvicidal activity (late 3rd-early 4th instar larvae of *C. quinquefasciatus*, LC_{50} = 18.53 and LC_{90} = 54.4 mg/l)	Komalamisra et al., 2005

Table 1.8 Insecticidal activities of extracts from derris plants (continued)

Source	Extract	Biological activity	References
D. elliptica	Methanolic extract	Larvicidal activity (late 3rd-early 4th instar larvae of *A. dirus*, LC_{50} = 16.17 and LC_{90} = 54.23 mg/l)	Komalamisra et al., 2005
D. elliptica	Methanolic extract	Larvicidal activity (late 3rd-early 4th instar larvae of *M. uniformis*, LC_{50} = 45.16 and LC_{90} = 142.75 mg/l)	Komalamisra et al., 2005
D. elliptica, root	Methanolic extract	Insecticidal activity (*Polyphagotarsonemus latus*, LC_{50} = 0.02%)	Worawong and Pimsamarn, 2006
D. elliptica, root	Water extract at 60 °C	Insecticidal activity (*Brevicoryne brassicae*, mortality = 53.47%)	Moyo et al., 2006
D. elliptica	Methanolic extract	Antioviposition activity (*P. xylostella*, EC_{50} = 48,075 mg/l)	Sombattepsut, 2005
D. elliptica	Methanolic extract	Repellent activity (*P. xylostella*, EC_{50} = 80.0 mg/l)	Sombattepsut, 2005
D. elliptica, root	Ethanolic extract	Larvicidal activity (3rd instar larvae of *Spodoptera litura*, LC_{50} = 37.67 ppm)	Rattanapan, 2007

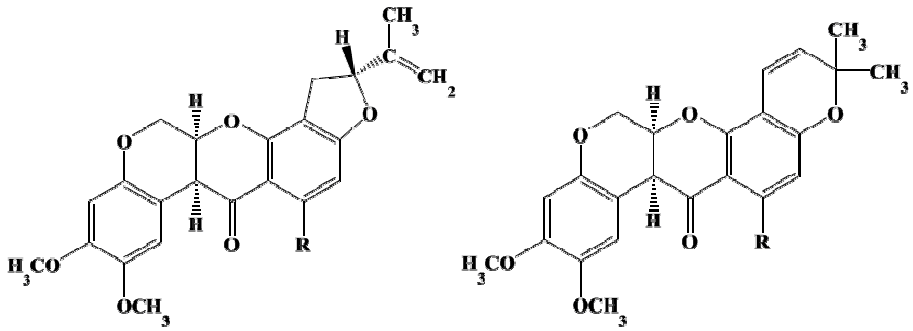
Moreover, fresh *D. elliptica* powder (FDP) and dried *D. elliptica* powder (DDP) were used to study their efficacy for treatment of cutaneous myiasis in pigs. The results showed that both FDP and DDP were effective on killing fly larvae. DDP was the most effective treatment which yielded cumulative death percentages of larvae at 38.0, 70.0, 80.0 and 88.0% after 3, 6, 9 and 12 hours of exposure, respectively. FDP yielded lower cumulative death percentages of 22.0, 48.0, 64.0 and 86.0% at the same exposure periods. In vivo, DDP was effective in a treatment of cutaneous myiasis in pigs. All experimental pigs had normal appetite and condition throughout the experiment without any signs of side effect from DDP. Larvae exposed to DDP died within 24 hours and an inflammation caused by larval migration was gradually recovered within 7 days (Sangmaneedet et al., 2005).

Beside of insecticidal activities, some of derris plant extracts displayed antibacterial activities. The ethyl acetate fraction of *D. malaccensis* methanolic extract exhibited significant inhibitory activity (IC_{50} < 1 μg/ml) against *Helicobacter pylori* (Takashima et al., 2002). Another study was investigated on *D. elliptica* leaves and stem, *Derris indica* leaves, root bark and root heart-wood, and *Derris trifoliata* leaves and root. The successive extraction with petrol (60-80 °C), dichloromethane, ethyl acetate, butanol and methanol gave fractions which demonstrated a broad spectrum of antibacterial activity against 25 pathogens. Significant activity was exhibited by the methanol fractions of leaves and root heart-wood, petrol, butanol and methanol fractions of the root bark of *D. indica* and petrol and ethyl acetate fractions of *D. trifoliata* (Khan et al., 2006).

Two species, *Derris elliptica* and *Derris malaccensis* are widely used as insecticide in China and South East Asian countries. *D. elliptica* is generally called “Lotin” or “Hang lai daeng” in Thai. It is a rambling climber, with branches covered with brown hairs. Leave are pinnate and 30-50 cm long with 11-15 leaflets. Leaflets are narrowly oblong-obovate, 9-13 cm when mature, smooth above and subglaucous and silky beneath, half as broad. Racemes are lax, 15-30 cm long, with reddish flowers in stalked clusters. Calyxes are 6-8 mm long, shallowly toothed. Petals are pink, standard 13-17 mm in diameter, with 2 auricles at base, softly ferruginous-hairy outside, wings and keel with interlocking longitudinal folds. Fruits are elliptic to oblong-elliptic, 3.5-7 cm long, flat, leathery, narrowly winged with 1-3 flat and reniform seeds (Du Puy, 1993).

D. malaccensis, locally known in Thailand as “Hang lai khao’’, is a climbing shrub with 5-7 leaflets, rachis and petioles glabrous or sparingly strigillose leave. The proximal pair of leaflets is not with deflexed petiolules. The leaflet blades are 10-15 cm long and 5-7 cm wide or sometimes smaller, elliptic, and usually gradually caudate-acuminate. Inflorescences are 10-16 cm long. Calyxes are pink and petals are white to pink, the banner glabrous Pods are 7.5 cm long and 2.7 cm wide with the wings on both suture 2-4 mm wide (Welsh, 1998).

These plant roots contain rotenone (1) as a toxic substance. In addition to rotenone, other rotenone-related compounds are sumatrol (2), deguelin (3) and toxicarol (4) and their chemical structures are shown in Figure 1.3 (Fukami and Nakajima, 1971).



(1) R = H, Rotenone (3) R = H, Deguelin

(2) R = OH, Sumatrol (4) R = OH, Toxicarol

Figure 1.3 Chemical structure of rotenone and rotenoids

1.2.3 Rotenone and rotenoids

Rotenone is one of the oldest botanical insecticides, which has been used for centuries and is still used worldwide. Rotenone is the trivial name of the main insecticidal component of certain plant of “*Derris*”, “*Lonchocarpus*”, and “*Tephrosia*” species. *D. elliptica* and *D. malaccensis* are economically important sources of rotenone from Malaya and the East Indies where the dry product is called derris or tuba, *L. utilis* and *L. urucu* from South America where it is called timbo or cubé and *Tephrosia* species from East Africa. Rotenone is used in the

form of ground roots, resins, or as a crystalline material which is extracted by solvents, such as chloroform. Commercially available extracts vary considerably in the amount of rotenoids present, depending on the locality where produced and the botanical source. Although rotenone is considered the most active ingredient, the other extractives also possess appreciable toxicity (Mutsumura, 1975; Ò Brien, 1967).

Rotenone is an isoflavonoid compound. The empirical formula is $C_{23}H_{22}O_6$, and the molecular weight is 394.42. It is colorless, crystalline solid with a melting point of 165-166 °C. Rotenone is sparingly soluble in water (15 mg/l at 100 °C) but is readily soluble in many organic solvents and oils (Ray, 1991). Rotenone is present in the form of colorless crystals, but readily oxidized by light, and becomes yellow, orange, and then deep red.

Rotenone is a selective, non-systemic insecticide used on fruit trees and vegetables to control aphids, maggots, bagworms, codling moths, Japanese beetles, leaf hoppers, Mexican bean beetles, cabbage worms, stinkbugs, flea beetles and vegetable weevils, both as stomach and contact poisons (Fukami and Nakajima, 1971; Mutsumura, 1975; Ray, 1991; Tyler et al., 1976). In general, rotenone is used in home gardens for insect control, for lice and ticks on pets, and fish eradications as part of water body management. In veterinary medicine, rotenone is used in the powder form to control parasitic mites on chickens and other fowl, and for lice and ticks on dogs, cats and horses (Gupta, 2007).

Rotenone significantly reduced hatching and affected female survival of *Tetranychus urticae* with 85.14% mortality. Oviposition rates also decreased after treatments with rotenone. Rotenone reduced hatching of *Phytoseiulus persimilis* eggs and affected female survival with 100% mortality (Duso et al., 2008). In another study, rotenone showed anthelmintic activity to larvae of *Haemonchus contortus* and *Trichostrongylus colubriformis* with LC_{50} values in larval development assays of 0.54 and 0.64 µg/ml, respectively. The compound also caused complete cessation of movement in adult *H. contortus* after 72 hours at a concentration of 20 µg/ml. Toxicity of rotenone towards the larvae both species was increased in the presence of piperonyl butoxide. This significant synergism suggests that these nematode species are able to utilize a cytochrome P450 enzyme system to detoxify rotenone and indicates that a role may exist for cytochrome P450 inhibitors to act as synergists for other anthelmintics which are susceptible to oxidative metabolism within the nematode (Kotze et al., 2006).

The acute toxicity of rotenone to insects, fish, and mammals is attributable to inhibition of NADH: ubiquinone oxidoreductase (in the electron transport chain) as the primary target, resulting in termination of the transport of electrons to O_2 and consequently inhibiting the synthesis of adenosine triphosphate (ATP), which is fatal for the organism. There are four steps in poisoning insects; (1) inactive, locomotive instability, and refusal to eat; (2) knockdown; (3) paralysis and (4) death (Fukami and Nakajima, 1971; Mutsumura, 1975).

Information on structure-activity relationships of rotenone and rotenoids is summarized. Rotenone is more active than the other principal components in derris resins. Deguelin and toxicarol differ from rotenone in having a pyran E-ring in place of dihydrofuran bearing an unsaturated side chain. In addition, toxicarol is substituted nuclearly (D-ring) by a hydroxyl group, which is likely cause of its virtual inactivity (Soloway, 1976). Not only the site of inhibitory action by rotenone in the mitochondrial respiratory chain was confirmed but the relationship between the chemical structure and the inhibitory potency of rotenoids (Table 1.9) was also established (Burgos and Redfearn, 1965).

Moreover, rotenone and rotenoids were reported to have anticancer activity in rats and mice based on three observations: (1) dietary rotenone reduces the background incidence of liver tumors in mice and mammary tumors in rats, (2) prevents cell proliferation induced by a peroxisome proliferator in mouse liver, and (3) deguelin and three of its derivatives inhibit phorbol ester-induced ornithine decarboxylase (ODC) activity. The study about the possible relationship between these two types of activity was found that inhibition of NADH: ubiquinone oxidoreductase activity lowers the level of induced ODC activity leading to the antiproliferative effect and anticancer action (Fang and Casida, 1998).

In addition, the isolated rotenoids, rotenone, rotenolone, dehydrorotenone, deguelin, tephrosin, toxicarol, dehydrodeguelin and elliptone, from *D. malaccensis* showed antibacterial activity against *H. pylori* (MIC = 0.3-9.8 mg/ml) (Takashima et al., 2002).

In the first half of the 20^{th} century, rotenone was considered a non-toxic alternative to the lead- and arsenic-based pesticides in common use. However, rotenone is classified by the World Health Organization as a moderately hazardous Class II pesticide. There are some reports indicating that acute oral toxicity of rotenone is moderate for mammals, but there is wide variation between species (Table 1.10). The oral LD_{50} of rotenone ranges from 132

to 1500 mg/kg in rats. The reported oral LD_{50} values of rats vary considerably, possibly because of differences in the plant extracts used. Studies have shown that rotenone is more toxic to female than male rats. The reported LD_{50} of rotenone in white mice is 350 mg/kg and it is highly irritating to the skin in rabbits (WHO, 1992).

Table 1.9 Relationship between structure of rotenoids and degree of $NADH_2$ dehydrogenase inhibition (Burgos and Redfearn, 1965)

Rotenoids	**Concentration needed for 50% inhibition (µmole/mg protein)**
(±0-6a,12a-Dihydrorotoxen-12(6H)-one	4000
6,6-Dimethyl-6a,12a-dihydrorotoxen-12(6H)-one	2000
Rotenone	1.8
Deguelin	3.7
Elliptone	3.6
(-)-Isorotenone	21.0
6′,7′-Dihydrorotenone	4.8
Munduserone	90.0
Sumatrol	3.2
Toxicarol	7.3
Malaccol	7.9
Rotenol	1360
Derritol	1270
Epirotenone	2200
(+)-Isorotenone	4000
Rotenone reduced with $NaBH_4$	2.0
Epirotenone reduced with $NaBH_4$	25.0
Rotenone oxime	103
Methylrotenone	8000

Table 1.10 Toxicity of rotenone (Ò Brien, 1967)

Organism	**Route**	**LD_{50} (mg/kg)**
Rat	Oral	132
Guinea pig	Oral	200
	Intraperitoneal	15
Chicken	Oral	996
Honeybee	Oral	3
Milkweed bug	Topical	25
American cockroach	Topical	2000
	Oral	1000
	Injected (males)	5
Japanese beetle	Topical	25
	Injected	40

Rotenone is believed to be moderately toxic to humans with an oral lethal dose estimated from 300 to 500 mg/kg. Human fatalities are rare, perhaps because rotenone is usually sold in low concentrations (1 to 5% formulation) and because its irritating action causes prompt vomiting. The mean particle size of the powder determines the inhalation toxicity. Rotenone may be more toxic when inhaled than when ingested, especially if the mean particle size is very small and particles can enter the deep regions of the lungs. Absorption of rotenone in the stomach and intestines is relatively slow and incomplete, although fats and oils promote its uptake. Rotenone is metabolized in the liver by nicotinamide adenine dinucleotide phosphate (NADP)-linked hepatic microsomal enzymes. Several metabolites have been identified as rotenoids, such as rotenolone I and II, hydroxyl and dihydroxyrotenones. Animal studies indicate that possible metabolites are carbon dioxide and a more water-soluble compound that can be excreted in the urine. It has been reported from the studies conducted on rats and mice that approximately 20% of the applied oral dose (and probably most of the absorbed dose) is excreted in urine within 24 hours. Unabsorbed rotenone from the GI tract excretes in feces (Extoxnet, 1996; Gupta, 2007).

Rotenone is a general use pesticide (GUP), but uses on cranberries and for fish control are restricted uses. It is EPA toxicity class I or III - highly toxic or slightly toxic, depending on formulation. Rotenone, when formulated as an emulsified concentrate, is highly toxic and carries the signal word "DANGER" on its label. Other forms are slightly toxic and require the signal word "CAUTION" instead (Extoxnet, 1996).

Rotenone is available as technical-grade solution at concentrations of 35%, 90%, or 95%, wettable powders containing 5% or 20% of active substance and 0.75 - 5% dusts. It is also available as a 5% emulsifiable concentrate. They are marketed in America and Europe. Trade names for products containing rotenone include Chem-Fish®, Cuberol®, Fish Tox®, Noxfire®, Rotacide®, Sinid® and Tox-R®. It is also marketed as Curex Flea Duster®, Derrin®, Cenol Garden Dust®, Chem-Mite®, Cibe Extract® and Green Cross Warble Powder®. The compound may be used in formulations with other pesticides such as carbaryl, lindane, thiram, piperonyl butoxide, pyrethrins and quassia (Extoxnet, 1996; Tyler et al., 1976).

For dusting purposes the commercially powdered roots is finely ground and diluted with a suitable carrier (talc, clay) to a concentration of 1%. Coated dusts, made by mixing the carrier with a liquid extract of the root and then drying, yield particles more uniform in size than are in the powdered root. For spray purposes the powdered roots may be mixed with water; however, a non-aqueous extract using ethylene dichloride, trichloroethylene, or chlorbenzene is preferable. Rotenone extracts with oil and emulsifying agents as well as extracts dissolved in paraffin oil with added perfuming agents are excellent as household and cattle sprays. Rotenone may be an ingredient in sprays and aerosols containing pyrethrins to aid in knockdown (Tyler et al., 1976).

It is of public knowledge that the juice obtained from raw root of derris is diluted with water to use it as an insecticide. It is also well-known that alcohol extracts of its dried root, is diluted with soap water to use it for the same purpose. However, the principal ingredient of derris loses their insecticidal activity when it contacts with water for a time. In addition, it is very unstable against alkali as well as water. Moreover, rotenone decomposes in the presence of light and air therefore its efficacy for controlling of many insects remained only for a short period of time after application. It is rapidly broken down in soil and water. The half-life in both of these environments is between 1 and 3 days. It does not readily leach from soil, and it is not expected to

be a groundwater pollutant. Rotenone breaks down readily by exposure to sunlight. Nearly all of the toxicity of the compound is lost in 5 to 6 days of spring sunlight or 2 to 3 days of summer sunlight. A number of photodecomposition products are formed when bean leaves are exposed to light. It is also sensitive to heat, with much of the rotenone quickly lost at high temperatures (Extoxnet, 1996; Gupta, 2007; Mutsumura, 1975).

Rotenone photodegradation in different solvent solutions and in the solid state was studied. The main photoproducts obtained after exposure to sunlight were identified (Figure 1.4). It was found that several photochemical reactions occur when solutions or residual deposits of rotenone are irradiated in the presence of oxygen. The photoreaction products are oxidized derivatives, so oxygen plays a critical role (Cheng et al., 1972).

1.2.4 Extraction techniques

Plant extracts are widely used in the pharmaceutical, cosmetic, food and agricultural industries for the development of commercial natural products. Extraction is a crucial step because it is necessary to extract the desired chemical components from plant materials and prepare sample for the qualitative and quantitative analysis of plant constituents. Traditionally, a broad spectrum of extraction procedures such as Soxhlet extraction, percolation, maceration, digestion, extraction under reflux and steam distillation is commonly used to extract the natural compounds. Most of these conventional extraction methods are relatively simple but they are increasingly becoming the bottleneck in routine analysis from disadvantages such as long extraction time, labour intensive manual procedures, unsatisfactory reproducibility and relatively high organic solvent consumption, with the associated risks for the human health and the environment. Consequently, several alternative techniques for sample preparation have been developed to solve these problems.

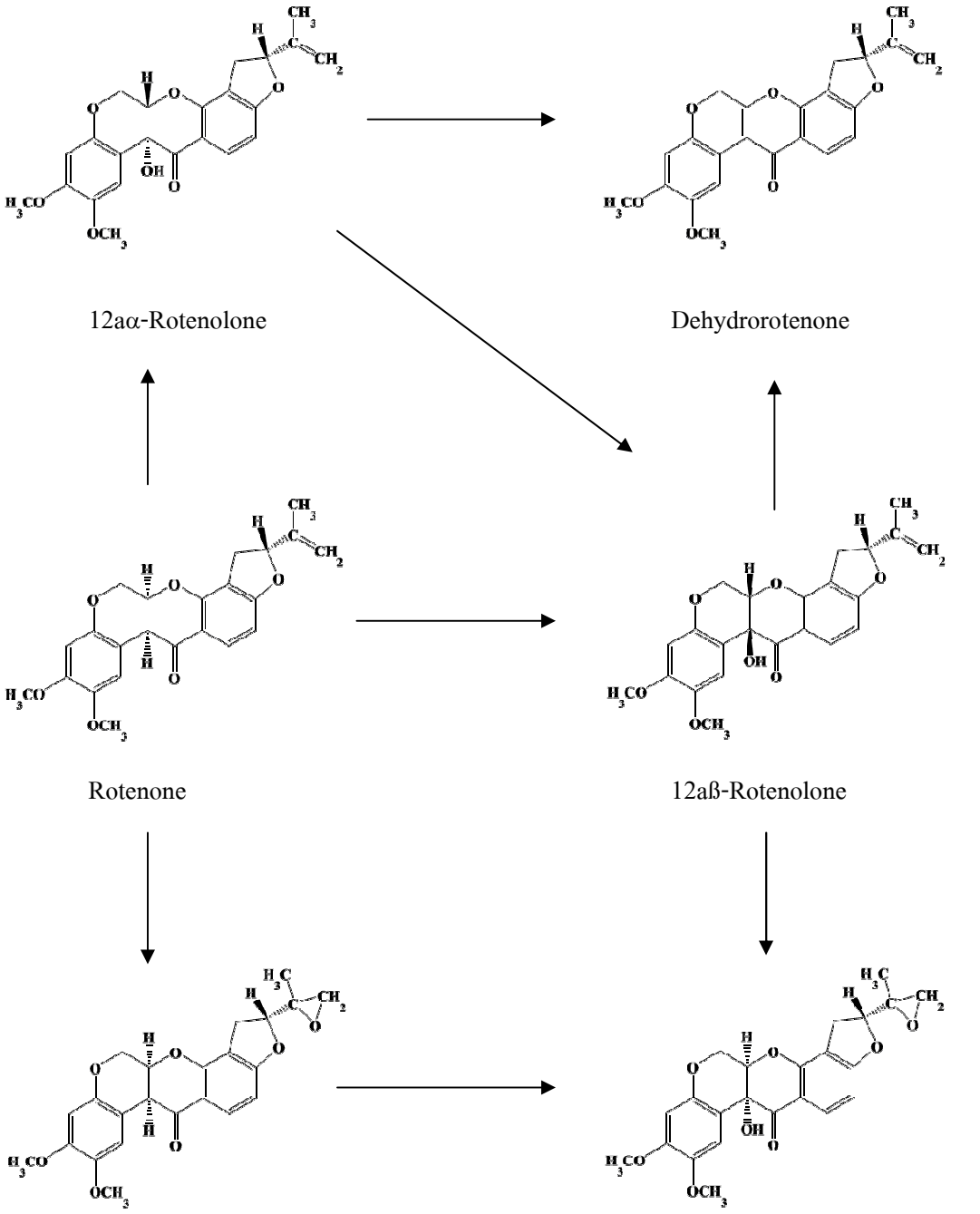


Figure 1.4 Major products of rotenone photodegradation

1.2.4.1 Microwave-assisted extraction (MAE)

Microwaves are electromagnetic radiations with a frequency from 0.3 to 300 GHz. Microwaves are transmitted as waves, which can penetrate biomaterials and interact with polar molecules such as water in the biomaterials to create heat. Consequently, microwaves can heat a whole material to penetration depth simultaneously. MAE offers a rapid delivery of energy to a total volume of solvent and solid plant matrix with subsequent heating of the solvent and solid matrix, efficiently and homogeneously (Huie, 2002; Kaufmann and Christen, 2002; Wang and Weller, 2006).

MAE has been considered as a potential alternative to traditional extraction for the extraction of metabolites from plants. It has been used for several reasons: (1) reduced extraction time (2) reduced solvent usage and (3) improved extraction yield. MAE is also comparable to other modern extraction techniques such as supercritical fluid extraction (SFE) due to its process simplicity and low cost. By considering economical and practical aspects, MAE is a strong novel extraction technique for the extraction of natural compounds. However, compared to SFE, an additional filtration or centrifugation is necessary to remove the solid residue during MAE. Furthermore, the efficiency of microwaves can be very poor when either the target compounds or the solvents are non-polar, or when they are volatile.

1.2.4.2 Pressurized liquid extraction (PLE)

Pressurised liquid extraction (PLE) has been developed as an alternative to current extraction methods such as Soxhlet, maceration, percolation or reflux, offering advantages with respect to extraction time, solvent consumption, extraction yields and reproducibility. PLE uses organic solvents at elevated pressure and temperature in order to increase the efficiency of the extraction process. Increased temperature accelerates the extraction kinetics and elevated pressure keeps the solvent in the liquid state, thus enabling safe and rapid extractions. Furthermore, high pressure forces the solvent into the matrix pores and hence should facilitate extraction of analytes. High temperatures decrease the viscosity of the liquid solvent, allowing a better penetration of the matrix and weakened solute–matrix interactions. In addition, elevated temperatures enhance diffusivity of the solvent resulting in increased extraction speed (Huie, 2002; Kaufmann and Christen, 2002; Wang and Weller, 2006).

PLE is considered as a potential alternative technique to SFE for the extraction of polar compounds (Brachet et al., 2001). Particular attention should be paid to the PLE performed with high extraction temperature, which may lead to degradation of thermolabile compounds.

1.2.4.3 Supercritical fluid extraction (SFE)

Supercritical state is achieved when the temperature and the pressure of a substance is raised over its critical value. The supercritical fluid has characteristics of both gases and liquids. Compared with liquid solvents, supercritical fluids have several major advantages: (1) the dissolving power of a supercritical fluid solvent depends on its density, which is highly adjustable by changing the pressure or/and temperature; (2) the supercritical fluid has a higher diffusion coefficient and lower viscosity and surface tension than a liquid solvent, leading to more favorable mass transfer. To develop a successful SFE, several factors must be taken into consideration. These factors include the selection of supercritical fluids, plant material preparation, modifiers and extraction conditions (Huie, 2002; Kaufmann and Christen, 2002; Wang and Weller, 2006).

SFE offers unusual possibilities for selective extractions and fractionations because the solubility of a chemical in a supercritical fluid can be manipulated by changing the pressure and/or temperature of the fluid. Furthermore, supercritical fluids have a density of a liquid and can solubilize a solid like a liquid solvent. The solubility of a solid in a supercritical fluid increases with the density of the fluid, which can be achieved at high pressures. The dissolved natural compounds can be recovered from the fluid by the reduction of the density of the supercritical fluid, which can usually be reduced by decreasing pressure (Poiana et al., 1998). Therefore, SFE can eliminate the concentration process, which usually is time-consuming. Furthermore, the solutes can be separated from a supercritical solvent without a loss of volatiles due to the extreme volatility of the supercritical fluid. Additionally, the diffusivity of a supercritical fluid is one to two orders of magnitude higher than that of other liquids, which permits rapid mass transfer, resulting in a larger extraction rate than that obtained by conventional solvent extractions (Roy et al., 1996).

Supercritical CO_2 extraction uses a moderate extraction temperature as low as 30 8 °C. The low supercritical temperature of CO_2 makes it attractive for the extraction of heat sensible compounds. As SFE uses no or only minimal organic solvent (organic modifiers) in extraction, it is a more environmentally friendly extraction process than conventional extraction. SFE can be directly coupled with a chromatographic method for simultaneously extracting and quantifying highly volatile extracted compounds. However, the economics and onerous operating conditions of the SFE processes has restricted the applications to some very specialized fields such as essential oil extraction, coffee decaffeination and to university research.

1.2.4.4 Ultrasound-assisted extraction (UAE)

Although the use of ultrasonic energy to aid the extraction of compounds from of the usefulness of ultrasonically assisted extraction are worth noting. Fundamentally, the effects of ultrasound on the cell walls of plants can be described as follows:

(1) Some plant cells occur in the form of glands (external or internal) filled with essential oil. A characteristic of external glands is that their skin is very thin and can be easily destroyed by sonication, thus facilitating release of essential oil contents into the extraction solvent; and

(2) Ultrasound can also facilitate the swelling and hydration of plant materials to cause enlargement of the pores of the cell wall. Better swelling will improve the rate of mass transfer and, occasionally, break the cell walls, thus resulting in increased extraction efficiency and/or reduced extraction time.

UAE is an inexpensive, simple and efficient alternative to conventional extraction techniques. The main benefits of use of ultrasound in extraction include the increase of extraction yield and faster kinetics. Ultrasound can also reduce the operating temperature allowing the extraction of thermolabile compounds. Compared with other novel extraction techniques such as MAE, the ultrasound apparatus is cheaper and its operation is easier. Furthermore, the UAE, like Soxhlet extraction, can be used with any solvent for extracting a wide variety of natural compounds. However, the effects of ultrasound on extraction yield and kinetics may be linked to the nature of the plant matrix. The presence of a dispersed phase contributes to the ultrasound wave attenuation and the active part of ultrasound inside the extractor is restricted

to a zone located in the vicinity of the ultrasonic emitter. Therefore, those two factors must be considered carefully in the design of ultrasound-assisted extractors (Huie, 2002; Wang and Weller, 2006).

1.2.5 Toxicity tests against insects (Mutsumura, 1975)

There are several ways to administer insecticides to insects. The most commonly employed method is topical application, where the insecticide is dissolved in a relatively nontoxic and volatile solvent, and is then allowed to come in contact with a particular location on the body surface. When knowledge of the exact amount of insecticide inside the body is required, the injection method is usually employed. The insecticide is commonly dissolved in carrier material, such as propylene glycol, and injected into the body cavity. These standard methods cannot be used in certain cases where the insect's mode of life or its morphological arrangement conflicts with the testing method; for instance, dipterous larvae cannot withstand the skin injury caused by the injection method, and topical application cannot deliver a sufficient quantity of insecticide.

A number of specially designed testing methods are available for many these unorthodox cases. For instance, the dipping method is used for dipterous larvae. The insects are simply picked up with a pair of forceps and dipped into the insecticide preparation, which is either a suspension or an emulsion. LC_{50} is generally used to express results with the dipping method. However, the mortality rate often does not increase because of the limitation of the insecticide's low solubility or limited amount that can be suspended.

The contact method is another way of exposing insects to an insecticide. The insecticide in a solvent is applied to the container or the panel surface where insects are to walk or rest. The solvent is evaporated by rotating the container or the panel, so that the insecticide is evenly spread over a known area.

Based on the various types of practical control measures and the insects' mode of life, there are several ways of testing the effectiveness of insecticides toward various insect species. The leaf-dipping method for testing two-spotted spider mites, the grain fumigation method for stored product pests, and the feeding method for various larvae are typical examples.

1.3 Objectives of this study

(1) To compare the pressurized liquid extraction and maceration for extraction of *Derris elliptica* and *Derris malaccensis*

(2) To develop three types of formulations containing derris extract; water dispersible granules, emulsifiable concentrate and calcium alginate beads

(3) To study the physicochemical properties and stability of rotenone in the developed formulations

(4) To carry out the efficacy test of the selected formulations against *Spodoptera litura*

CHAPTER 2

Extraction of Rotenone from *Derris elliptica* and *Derris malaccensis* by Pressurized Liquid Extraction Compared with Maceration

2.1 Introduction

Pressurized liquid extraction (PLE) has been used since 1995 as an alternative to conventional extraction methods such as Soxhlet extraction, maceration, percolation and reflux. PLE is an extraction process performed at elevated temperature, usually between 50 and 200 °C and at pressure between 500-3000 psi and allows the universal use of solvents or solvent mixtures with different polarities. It represents an effective extraction technique with the advantages of shorter extraction time and lower consumption of solvents (Ahmed, 2001; Ong, 2004; Richter et al., 1996).

Firstly, application of PLE has focused on the extraction in the area of environmental research. However, the commercial PLE instrument was successfully developed, resulting in its application to extraction of agrochemicals, pharmaceuticals and nutraceuticals from food, plants and diverse of matrices (Carabias-Martinez et al., 2005; Kaufmann and Christen, 2002; Wang and Weller, 2006).

PLE is highly dependent of variables that have a significant effect on the extraction efficiency. Therefore, it is necessary to optimize the conditions for extracting compounds from any matrices. Optimization of the extraction process generally begins with an appropriate choice of the extraction solvent. Often, the same solvent used for conventional extractions is initially tested in PLE. Optimization of the other extraction conditions, such as sample size, sample particle size, extraction time, the number of cycles, temperature and pressure, is normally accomplished using the classical one-variable-at-a-time method, in which the optimization is assessed by systematic alteration of one variable while the others are kept constant (Carabias-Martinez et al., 2005; Ong, 2004).

Several applications of PLE have been published in the field of natural product research and these are summarized in Table 2.1. An evaluation of PLE has been made for the extraction of various metabolites covering a large range of structures and polarities present in different vegetal matrices such as roots, fruits, seeds and rhizomes.

Table 2.1 Application of PLE to natural product extraction

Compounds	**Matrices**	**Extraction conditions**	**References**
Aristolochic acid I & II	*Radix aristolochiae*	Methanol; 25 ml; 120 °C; 150 bar; 15-20 min	Ong et al., 2000
Berberine	*Coptidis rhizoma*	Methanol; 30 ml; 120 °C; 15-20 min	Ong et al., 2000
Carotenoids	Green algae (*Haematococcus pluvialis* and *Dunaliella salina*)	Acetone;15 ml; 40 °C; 1500 psi; 3 cycles of 5 min	Denery et al., 2004
Catechin and epicatechin	Grape seed (*Vitis vinifera*)	Methanol; 100 °C; 100 atm; 5 min	Piñeiro et al., 2004
Curcumin	Turmeric rhizome (*Curcuma xanthorrhiza*)	Methanol; 20 ml; 50-100 °C; 140 bar; 3 cycles of 6 min	Benthin et al., 1999
Deacylsaponins (calculated as escin)	Horse chestnut seed (*Aesculus hippocastanum*)	Defatting with dichloromethane; 20 ml; 100 °C; 140 bar; 2 cycles of 5 min; followed by extraction with methanol; 20 ml; 100 °C; 140 bar; 2 cycles of 6 min	Benthin et al., 1999

Table 2.1 Application of PLE to natural product extraction (continued)

Compounds	Matrices	Extraction conditions	References
Dianthrons (calculated as hypericin)	St. John's wort herb (*Hypericum perforatum*)	Defatting with dichloromethane; 20 ml; 100 °C; 140 bar; 5 min; followed by extraction with methanol; 20 ml; 50-100 °C; 140 bar; 3 cycles of 5 min	Benthin et al., 1999
Flavanones and xanthones	Osage orange tree root bark (*Maclura pomifera*)	Dichloromethane; 15 ml; 40, 80 or 100 °C; 13.8 MPa; 3 cycles of 5 min	Da Costa et al., 1999
Ginsenosides	American ginseng root, dried powder (*Panax quinquefolius*)	Methanol or 1% Triton X-100 in water; 20 ml; 120 °C; 1500 psi; 10 min	Choi et al., 2003
Isoflavones	Soybeans, freeze-dried (*Glycine max*)	70% Ethanol; 100 °C; 100 atm; 3 cycles of 7 min	Rostagno et al., 2004
Kavalactones	Kava root (*Piper methysticum*)	Acetone;15 ml; 40 °C; 2000 psi; 3 cycles of 5 min	Denery et al., 2004
Polyphenols	Apple peel and pulp, freeze-dried (*Malus pumila*)	Methanol; 40 ml; 40 °C; 1000 psi; 5 min	Alonso-Salces et al., 2001
Proanthocyanidins	Malt (*Hordeum vulgare*)	Acetone-water (80:20); 14 ml; 60 °C; 100 MPa; 10 min	Papagiannopoulos et al., 2002

Table 2.1 Application of PLE to natural product extraction (continued)

Compounds	Matrices	Extraction conditions	References
Sesquiterpenes	Curcuma rhizomes (C. kwangsiensis, C. phaeocaulis and C. wenyujin)	Methanol; 120 °C; 1500 psi; 5 min	Yang et al., 2005
Silybin	Milk thistle fruit (*Silybum marianum*)	Defatting with hexane; 20 ml; 100 °C; 140 bar; followed by extraction with methanol; 20 ml; 100 °C; 140 bar; 5 min	Benthin et al., 1999
Thymol	Thyme herb (*Thymus vulgaris*)	Hexane; 35 ml; 50 °C; 140 bar; 5 min; followed by dichloromethane; 50 °C; 140 bar; 5 min	Benthin et al., 1999
α-tocopherol	Brazilian grape seed (*Myrciaria cauliflora*)	Hexane; 100 °C; 1500 psi; 3 cycles; 30 min	Dos Santos Freitas et al., 2008

Extraction methods, such as Soxhlet extraction, stirring soaking and maceration are commonly used to extract rotenone from *Derris* plants (Khamis et al., 2002; Visetson and Milne, 2001). To the best of our knowledge, PLE has not been applied for extraction of rotenone from *Derris* plants so far. We therefore carried out a comparative study to evaluate PLE as a possible alternative to conventional maceration for the efficient extraction of rotenone from the dried stems of *D. elliptica* and *D. malaccensis* and the dried roots of *D. elliptica* with regard to extraction time, solvent consumption and extraction yields.

2.2 Experimental methods

2.2.1 Plant samples

The stems and roots of *D. elliptica* (Figure 2.1 and 2.2) were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla Province, Thailand in December of 2004. The stems of *D. malaccensis* (Figure 2.3) were collected from Chana District, Songkhla Province, Thailand in August of 2004. Authentication of plant materials was carried out at the herbarium center of the Department of Forestry, Bangkok, Thailand where herbarium vouchers have been kept identifying the plant species. Voucher specimens of these plants were also kept in the Herbarium of Southern Center of Thai Traditional Medicine at Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla Province, Thailand. Only stems and roots with a diameter size less than 1.5 cm were used. They were washed with tap water to remove the remaining soil and other unwanted materials, cut into small pieces (5 mm cubes) and dried in hot air oven at 40 °C for 24 hours. The dried plants were then ground and stored in plastic bags and kept in the dark at room temperature (30±2 °C) until use.

Figure 2.1 Stems of *D. elliptica*

Figure 2.2 Roots of *D. elliptica*

Figure 2.3 Stems of *D. malaccensis*

2.2.2 Chemicals

HPLC grade acetonitrile, analytical grade methanol, 37% hydrochloric acid and sodium hydroxide were purchased from Labscan Asia (Bangkok, Thailand). Commercial grade chloroform and 95% ethanol were obtained from High Science distributor (Bangkok, Thailand). All other solvents were AR grade purity. Nitrogen gas (99.9% purity) was purchased from TIGT (Songkhla, Thailand). Water was purified by passing it through a Milli-Q purification system. Standard rotenone (98% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). Hydrogen peroxide solution was purchased from Siribuncha (Nonthaburi, Thailand).

2.2.3 Extraction of rotenone by maceration using organic solvents

The dried plant sample (50 g) was placed in a stopped conical flask and macerated with 500 ml of chloroform at room temperature (30±2 °C) for 3 days with occasional stirring. The solvent was then filtered and evaporated in a rotary evaporator (Eyela, Tokyo, Japan) under vacuum at 45 °C. The residue was placed in a vacuum oven (Napco, Chicago, USA) at room temperature until dry. The crude extract was stored in a glass container and protected from light and kept in a desiccator at –20 °C. The extraction was carried out on three separate occasions (n = 3). The above procedure was repeated using 95% ethanol as solvent instead of chloroform, again replicated on three occasions (n = 3).

2.2.4 Extraction of rotenone by pressurized liquid extraction (PLE)

Extractions were performed on a Dionex ASE 200 Accelerated solvent extractor (Archemica, Bangkok, Thailand) (Figure 2.4). The dried plant sample (10 g) was packed in a 22-ml stainless steel extraction cell. A cellulose filter was placed at the bottom of the extraction cell. The PLE was carried out with static time of 6 min per 1 cycle. Solvent, temperature and pressure were varied to optimize the extraction efficiency. The experimental conditions are shown in Table 2.2. Three replicate extractions for each experimental condition were performed (n = 3). The extraction procedures were as follows: (i) extraction cell and collection vial were loaded onto the automated carousel, (ii) cell was filled with solvent, (iii) heat-up time was applied, (iv) static extraction was undertaken, in which all system valves were closed, (v) cell was rinsed with 60% of cell volume with extraction solvent, (vi) solvent was purged from cell with gaseous N_2 for 60 sec and (vii) depressurization took place. About 30 ml of solvent was used in each of extraction process. The extracts were collected into glass collection vials. The solvent was evaporated to dryness by rotary evaporator under vacuum at 45 °C and the residue was placed in a vacuum oven at room temperature (30±2 °C). The crude extract was stored in a glass container and protected from light and kept in a desiccator at -20 °C.

Table 2.2 Experimental conditions of PLE

Condition	Solvent	Temperature (°C)	Pressure (psi)
1	95 % EtOH	50	1500
2	$CHCl_3$	50	1500
3	$CHCl_3$	60	1500
4	$CHCl_3$	70	1500
5	$CHCl_3$	50	1000
6	$CHCl_3$	50	1750
7	$CHCl_3$	50	2000
8	$CHCl_3$	30 (RT)	2000

Figure 2.4 Dionex ASE 200 Accelerated solvent extractor

2.2.5 HPLC analysis

2.2.5.1 Separation studies and development of stability-indicating method

Analytical determinations of rotenone are commonly carried out by HPLC (Abidi, 1984; AOAC, 1996; Bushway, 1984; Bushway and Hanks, 1977; Cabizza et al., 2004; Cabras et al., 2002; Draper et al., 1999; Holm et al., 2003; Jiménez et al., 2000; Martel and Zeggane, 2002, Moore et al., 2000). Most of the reported HPLC methods for analysis of rotenone were developed on C18 column at room temperature, using methanol or acetonitrile and water in different ratios as mobile phase at the detection wavelength of 210 nm or 294 nm. Although rotenone has a maximum absorption with the highest molar absorptivity at 210 nm but the detection wavelength at 294 nm was employed due to a more stable baseline obtained and more selective detection (Holm et al., 2003). So attempts were made to develop a simple method on a C18 column using acetonitrile-water as the mobile phase at a flow rate of 1 ml/min and detection wavelength was 294 nm.

The chromatographic system (Shimadzu, Kyoto, Japan) consisted of SCL-10A VP system controller fitted with LC-10AD VP pump, DGU-14A degasser, SPD-10A VP UV-Visible detector, SIL-10AD VP auto injector and CTO-10AS VP column oven. The column was a Waters® Spherisorb S5 ODS2 (250 x 4.6 mm, 5 µm). Firstly, HPLC studies were performed on all reaction solutions and separations were achieved using isocratic elution. Different mobile phase compositions were tried to get good separation between rotenone and degradation products. The studies were carried out by decreasing the ratio of acetonitrile from 80% to 50%. However, the peaks of rotenone and degradation products were not well separated or did not have an acceptable shape. Some studies afforded a good resolution of rotenone and degradation products with gradient mode (Cabizza et al., 2004; Cabras et al., 2002; Draper et al., 1999). The HPLC method was therefore tried in gradient mode on varying linear gradient profile. Initially, acetonitrile and water were used as the mobile phase in the ratio of 50:50 reaching 85:15 in 10 min. The ratios of initial and final concentration of mobile phase and the run times from 10-30 min were varied until satisfactory resolution was obtained. Finally, the gradient profile for the separation of rotenone was as follows: initial mobile phase acetonitrile/water 50:50 (%v/v) reaching 70:30 (%v/v) in 30 min. Before each injection, the mobile phase system had to be stabilized for 10 min with an

acetonitrile/water mobile phase 50:50 (%v/v). The flow rate was 1 ml/min. The analysis was performed at a wavelength of 294 nm.

2.2.5.2 Preparation of standard and sample solutions

The stock solution (0.10 mg/ml) of standard rotenone was prepared by dissolving 10.0 mg of rotenone in 100 ml methanol. The working solutions (1.2-20 µg/ml) were freshly prepared by suitable dilution of the stock solutions with methanol, and 20 µl of each were injected onto the HPLC column via the auto-injector three separate times (n = 3). The mean peak areas for each concentration were calculated, and standard calibration curves were constructed by plotting concentrations against peak areas. Since rotenone is known to decompose when exposed to light, the stock solution was kept in the dark at 4 °C and used within 1 month. This solution was stable over this period as determined by an HPLC assay.

The rotenone contents in the extracted samples were determined as above. Before analysis, a portion of crude extracts obtained from PLE and maceration was accurately weighed and quantitatively transferred to a 25 ml volumetric flask with methanol. The sample was sonicated until complete solubilization and the volume was made up to 25 ml with methanol. The resulting solution was then filtered and used for HPLC analysis. The injection volume was 20 µl. The analysis was performed in triplicate.

2.2.6 Forced degradation studies (modified from Bakshi et al., 2001)

Stress studies were carried out under the conditions of acid hydrolysis, base hydrolysis and oxidation. The ability of the proposed method to separate rotenone from its degradation products was evaluated for an indication of stability-indicating property.

The dichloromethane extract obtained from maceration of the dried roots of *D. elliptica* which contained 36.13% w/w of rotenone was used in this study. Approximately 7 mg of the crude extract was accurately weighed and quantitatively transferred to a 25 ml volumetric flask with methanol. The sample was sonicated until complete solubilization and the volume was adjusted to 25 ml with methanol. The resulting solution with concentration of 100 µg/ml of rotenone was used as a stock solution for degradation studies.

2.2.6.1 Acid hydrolysis

A 1 ml of 0.1 N hydrochloric acid was added to 4 ml of crude extract stock solution and kept at room temperature (30±2 °C) for 3 hours. The mixture was then neutralized by 1 ml of 0.1 N sodium hydroxide and adjusted to 10 ml with methanol. The resulting solution was then filtered and used for HPLC analysis.

2.2.6.2 Base hydrolysis

A 1 ml of 0.1 N sodium hydroxide was added to 4 ml of crude extract stock solution and kept at room temperature (30±2 °C) for 3 hours. The mixture was then neutralized by 1 ml of 0.1 N hydrochloric acid and adjusted to 10 ml with methanol. The resulting solution was then filtered and used for HPLC analysis.

2.2.6.3 Oxidation

A 3 ml of 3 %v/v hydrogen peroxide was added to 4 ml of crude extract stock solution and kept at room temperature (30±2 °C) for 24 hours. The mixture was then adjusted to 10 ml with methanol. The resulting solution was then filtered and used for HPLC analysis.

2.2.7 Validation of the developed stability-indicating method

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Typical validation characteristics which should be considered are specificity, linearity and range, accuracy, precision, detection limit and quantitation limit (ICH, 2005).

2.2.7.1 Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. The specificity of the developed HPLC method for rotenone was carried out in the presence of the other components in the crude extract by determining the peak purity of rotenone using a PDA detector. A common peak purity technique

involves normalizing and comparing spectra taken across the peak. Absorption spectra at the beginning, the apex, and at the end of the peak were normalized and overlaid.

2.2.7.2 Linearity and range

Linearity of the method was studied by analysis of 5 concentrations of standard solutions prepared in methanol in the range of 1.2-20 µg/ml (1.2, 4, 8, 12, and 20 µg/ml) using the HPLC method. The experiment was performed in triplicate. The peak areas versus concentration data were treated by least-squares linear regression analysis. The linearity test was carried out for 3 consecutive days. The minimum acceptable coefficient (r^2) to establish linearity was set at 0.999.

2.2.7.3 Accuracy

Accuracy of the developed method was tested in triplicate by fortifying a mixture of crude extract solutions with six concentrations of the standard solutions (0, 1.2, 4, 8, 12, and 20 µg/ml) and determining the recovery of added analyte. The average percentage recovery should be 95-105%.

2.2.7.4 Precision

Precision of the method was verified by repeatability and intermediate precision studies. Repeatability studies were performed by analyses of three different concentrations of the standard solutions in triplicate on the same day. Intermediate precision of the method was checked by repeating the studies on three different days. The percentage relative standard deviation (%RSD) was calculated and reported for each type of precision. The acceptable %RSD should not exceed 2%.

2.2.7.5 Detection limit

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing

the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit. In this study the signal-to-noise ratio was calculated with VP software program version 6.1 by Shimadzu.

2.2.7.6 Quantitation limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1. In this study the signal-to-noise ratio was calculated with VP software program version 6.1 by Shimadzu.

2.2.8 Statistical data analysis

Statistical data analysis was performed using the *t*-test with $p < 0.05$ as the minimal level of significance.

2.3 Results and discussion

2.3.1 Development of stability-indicating HPLC assay method

2.3.1.1 Development and optimization of the method

From the optimization of HPLC method with gradient mode, the best separation was achieved on C18 column using a mobile phase composed of acetonitrile-water with an initial concentration of 50:50 (%v/v) and a final concentration of 70:30 (%v/v) in 30 min. The flow rate was 1 ml/min and detection wavelength was 294 nm. In the selected optimal experimental conditions, rotenone exhibited a well-defined chromatographic peak with a retention time of 16.6 min. The chromatograms obtained by injection of a solution of standard rotenone (12 μg/ml), a

solution containing chloroform extract from the roots of *D. elliptica*, and a solution containing ethanolic extract from the roots of *D. elliptica* are shown in Figure 2.5A, 2.5B and 2.5C. The rotenone peak was clearly separated from the peaks of other compounds.

The HPLC chromatographic conditions for determination of rotenone and rotenoids in the extracts, formulations, and residues on olives and in olive oil have been described by Cabizza et al. (2004) and Cabras et al. (2002). The chromatographic separation was carried out on a C18 column using a gradient solvent system consisted of acetonitrile and water 50:50 reaching 85:15 (%v/v) in 15 and 10 min, respectively. In this study, the gradient mobile phase system was adjusted to be acetonitrile-water 50:50 reaching 70:30 (%v/v) in 30 min in order to achieve the best separation of the peaks of rotenone and degradation products. The mobile phase was easily prepared and gave reproducible results. However, the disadvantage of this method over earlier reported methods was the longer run time.

2.3.1.2 Stability-indicating nature of the developed method

As shown in Figure 2.6, the method was able to resolve all the components in the stress samples. The peaks of the degradation product were well-resolved from rotenone. The method thus proved to be selective and stability-indicating. Moreover, this study shows the opportunity of using these chromatographic conditions to investigate the stability of rotenone both in the plant extract and the prepared formulations. From these HPLC chromatograms indicated that rotenone is slightly unstable in acid and in the presence of oxidizing agent and strongly unstable in base.

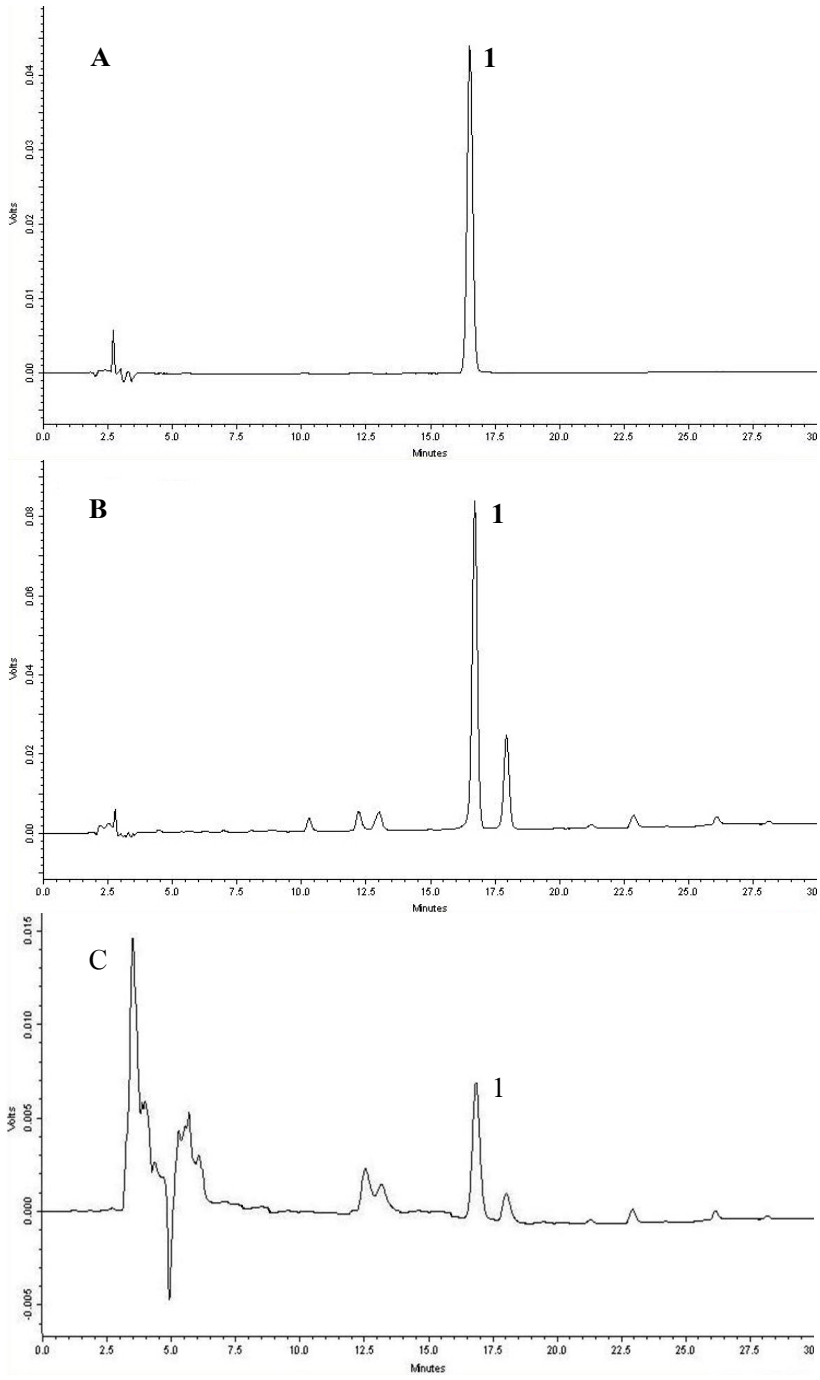


Figure 2.5 HPLC chromatograms of rotenone (1) in standard solution at 12 µg/ml (A), in a solution containing chloroform extract from the roots of *D. elliptica* (B), and in a solution containing ethanolic extract from the roots of *D. elliptica* (C)

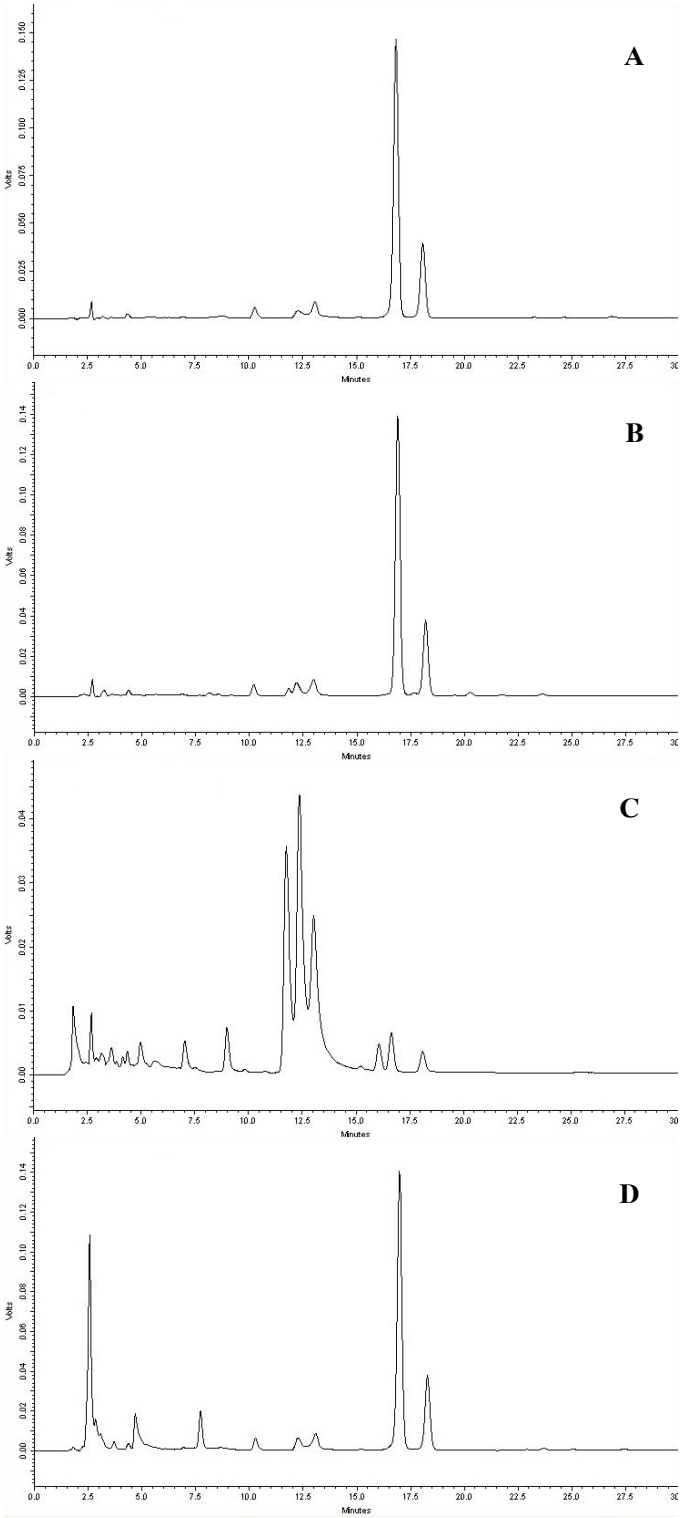


Figure 2.6 HPLC chromatograms of *D. elliptica* extract (A), and in acid hydrolysis (B), base hydrolysis (C), and oxidation (D) conditions for 24 hours (Rotenone peak exhibited at retention time of 16.6 min.)

Table 2.3 showed the percentage of rotenone remaining under different stress conditions. Slow degradation of rotenone was observed in oxidative condition. Rotenone still remained 94.6±0.1% of the starting amount after 24 h of exposure to 3% hydrogen peroxide at room temperature. In addition, rotenone was found to faster degrade under acidic condition. Rotenone was degraded to 94.5±0.2% remaining within 3 h at room temperature. In contrast, rotenone was found to decompose rapidly in alkali condition. Whole of rotenone was completely degraded within 3 h at room temperature. The results indicated that the degradation of rotenone occurs by oxidation and hydrolytic reactions. Rotenone can be broken down to dehydrorotenone by oxidation reaction. Moreover, rotenone and dehydrorotenone are readily base hydrolyzed to give derrisic acid and its analogs (Figure 2.7) (Fukami and Nakajima, 1971; Ò Brien, 1967).

Table 2.3 Rotenone remaining (%) under different stress conditions

Stress condition	**Rotenone remaining (%)**
Acid hydrolysis	94.5±0.2
Base hydrolysis	n.d.
Oxidation	94.6±0.1

n.d. = not detected

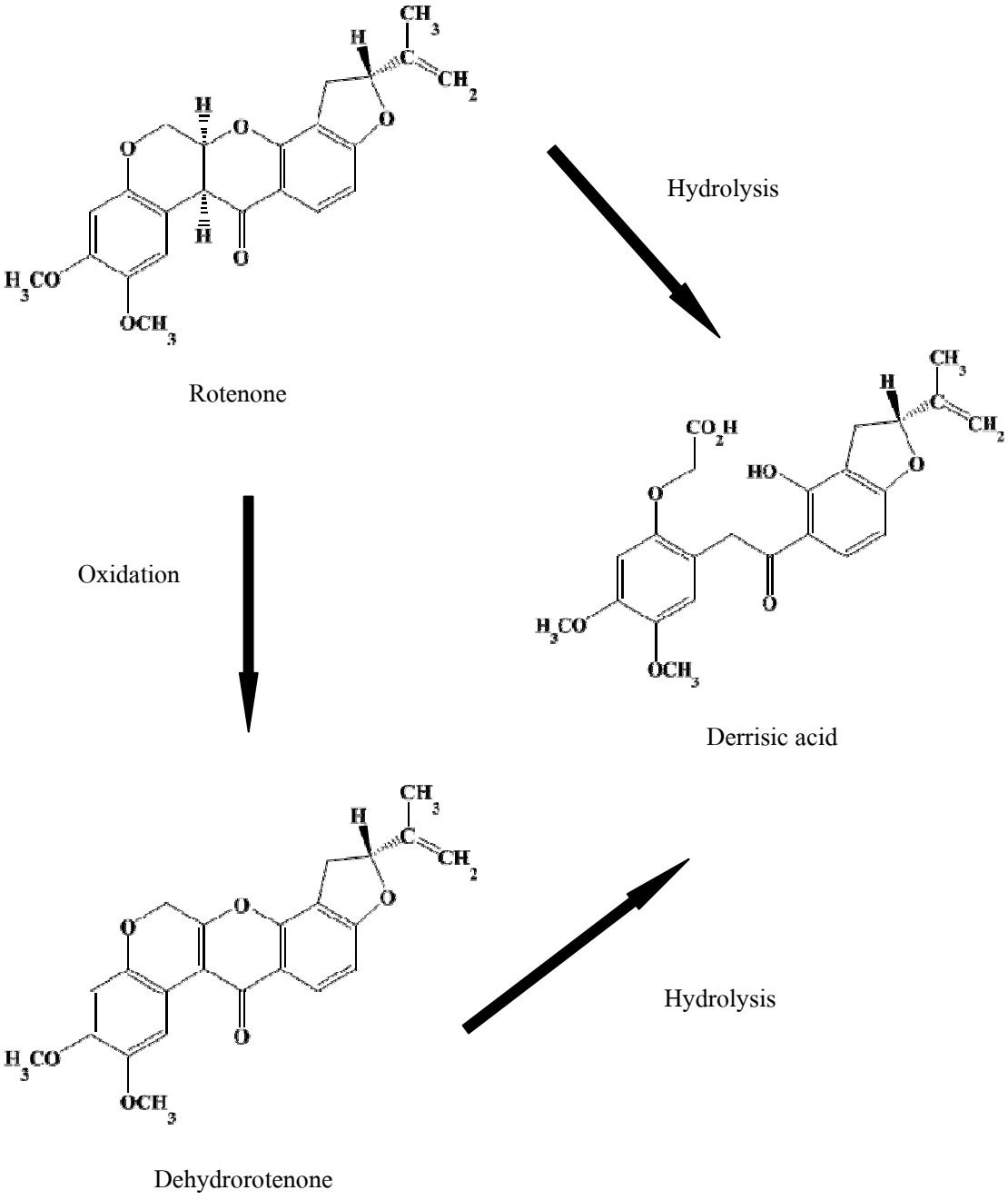


Figure 2.7 Degradation products of rotenone by oxidation and hydrolysis reactions

2.3.2 Validation of the developed stability-indicating method

For specificity, the purity of the peak was tested using the PDA detector to ensure that the compound was not co-eluting with an impurity peak. The absorption spectra confirmed that rotenone peak is homogenous and pure in all the analyzed stress samples.

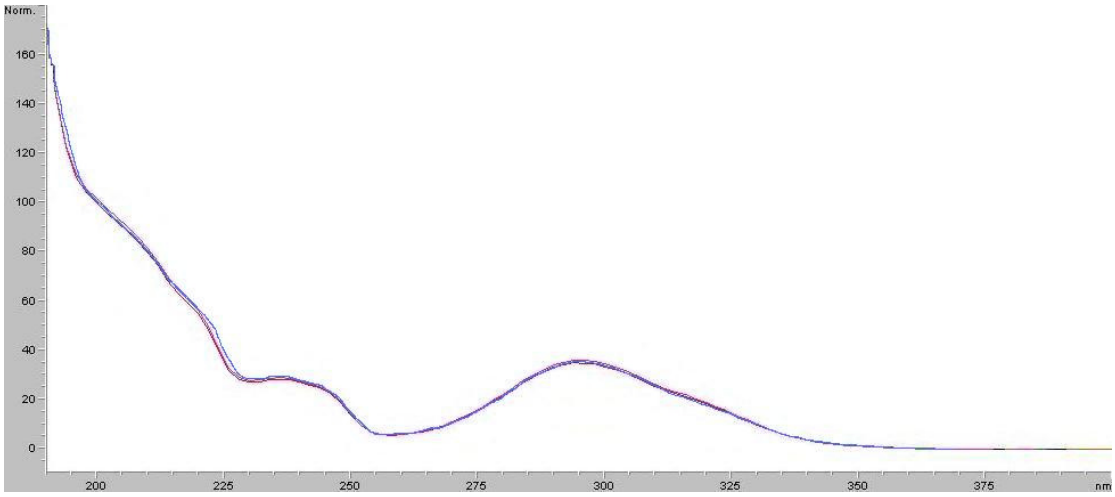


Figure 2.8 Absorption spectra at the beginning, the apex, and the end of rotenone peak

Standard calibration curves of rotenone were constructed by plotting concentrations against peak areas. The chromatographic signals show a linear dependence with the rotenone concentration enabling the use of this signal for rotenone quantification, according to the following regression equation:

$$Y = 50112X + 5620$$

$$r^2 = 0.9999 \pm 0.0002$$

(Y = peak area; X = rotenone concentration)

Good linearity was observed in the range of 1.2-20 µg/ml. A linear simple regression by the least squares method was applied and showed excellent correlation coefficient (r^2) greater than 0.999.

The precision of the method was determined by intra-day repeatability and intermediate precision studies and expressed as relative standard deviation (RSD) of a series of measurements. The experimental values obtained in the determination of rotenone in the samples are presented in Table 2.4. The %RSD values for intra-day and inter-day precision were lower than 2.0%, indicating that the method was sufficiently precise.

Table 2.4 RSD (%) of intra-day and inter-day precision studies

Concentration	RSD (%)	
(μg/ml)	Intra-day precision	Inter-day precision
1.2	0.95	1.66
8	0.92	1.37
20	0.58	0.73

Good recoveries were obtained at each concentration as shown in Table 2.5. The mean percentage recovery of rotenone in the crude extract was ranged from 99.54-100.77%.

The limit of detection (LOD) value for rotenone was 0.24 μg/ml and the limit of quantitation (LOQ) value was 0.71 μg/ml, respectively as calculated by signal-to-noise ratio.

The HPLC method validation results showed that determination of rotenone in the extract could be performed by validated HPLC method described above with acceptable accuracy and precision.

Table 2.5 Recovery (%) of rotenone spiked in crude extract at various concentrations

Sample	**Recovery (%) on each studied concentration**					**Recovery (%)**
	1.2 μg/ml	**4 μg/ml**	**8 μg/ml**	**12 μg/ml**	**20 μg/ml**	**(mean±SD)**
1	104.92	100.03	98.79	100.08	100.02	100.77±2.38
2	98.27	99.17	100.23	100.09	99.96	99.54±0.82
3	98.72	100.82	100.03	99.72	100.20	99.90±0.77
Average	**100.64**	**100.01**	**99.68**	**99.96**	**100.06**	**100.07±0.63**

2.3.3 Effect of solvent types on the yields of rotenone from dried plant

The yields of rotenone from the stems of *D. malaccensis* by PLE and maceration when using different type of solvents were calculated as absolute yields of rotenone (g) in 100 g of dried plant (%w/w) and shown in Figure 2.9. Chloroform extracts from maceration were found to contain significantly higher absolute yields of rotenone than ethanolic extracts, whereas the absolute yields of rotenone of chloroform extracts and ethanolic extracts from PLE at 50 °C, 1500 psi were not significantly different ($p > 0.05$). This may be due to the PLE conditions assisting the solubility of rotenone both in chloroform and in 95% ethanol.

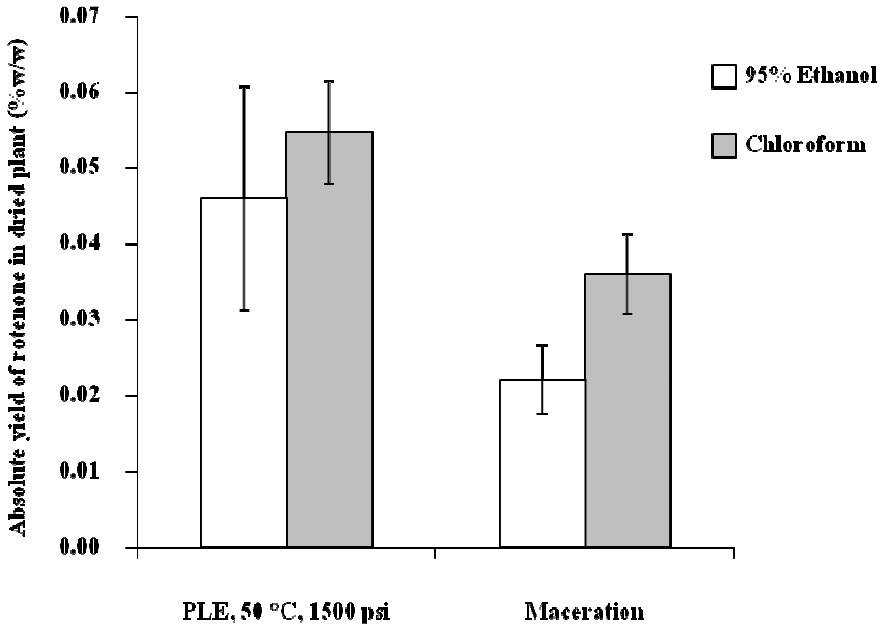


Figure 2.9 Absolute yields of rotenone from dried stems of *D. malaccensis* using 95% ethanol as extraction solvent compared with chloroform (n = 3)

2.3.4 Effect of temperature and pressure on PLE procedures

The effects of other factors such as temperature and pressure on PLE procedures on rotenone content of crude extract were also investigated and the results are displayed in Figure 2.10. By maintaining the pressure at 1500 psi, the highest rotenone content was obtained when using chloroform as extraction solvent at 50 °C (Figure 2.10A). At higher temperatures (60 °C

and 70 °C), rotenone contents of the extracts decreased. This decrease in the rotenone content could be due to a possible degradation of rotenone at temperatures above 50 °C. The main reasons for the enhanced performance when using PLE over the other conventional methods of extraction are the higher solubility of analytes in solvent and higher diffusion rate as a result of higher temperature. At higher temperature, the strong solute-matrix interaction caused by van der Waals forces, hydrogen bonding and dipole attractions between solute molecules and active sites on the matrix are disrupted (Ong, 2004). However, high temperature may promote decomposition of compounds. Pressure is used to maintain the solvent in the liquid phase during the extraction process and to ensure that the solvent remains in intimate contact with the sample (Alonso-Salces et al., 2001; Ong, 2004; Rostagno et al., 2004). It was also reported that the pressure does not significantly affect extraction (Choi et al., 2003; Papagiannopoulos et al., 2002; Rostagno et al., 2004). In this study, pressure was observed to have no effect on the extraction of rotenone. The increase in pressure from 1000 to 2000 psi using chloroform as the extraction solvent (at 50 °C) led to no significant difference in the rotenone content of the extracts ($p > 0.05$) (Figure 2.10B). However, the pressure of 2000 psi was chosen to ensure that the solvent remained in the liquid phase.

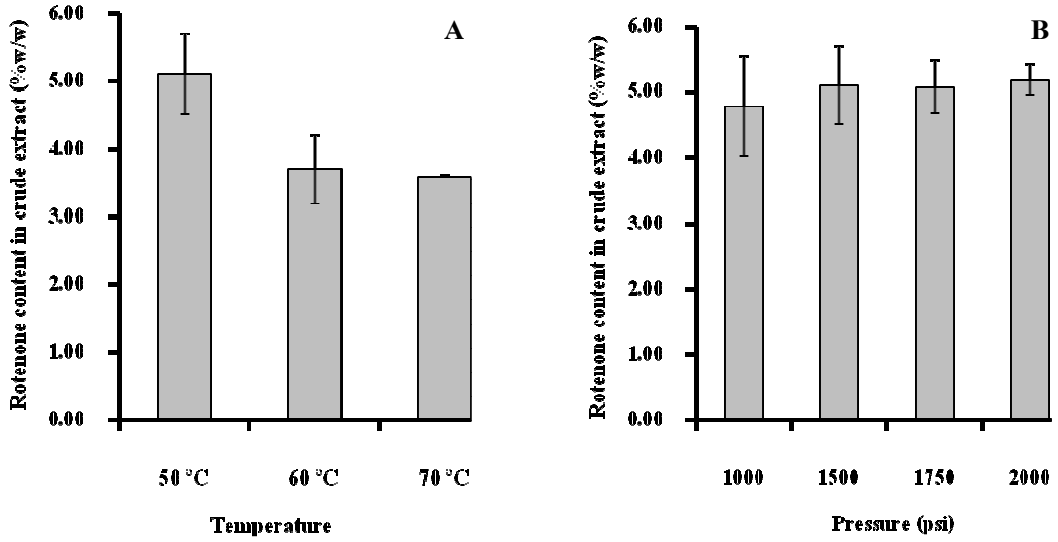


Figure 2.10 Rotenone contents of chloroform crude extracts from the stems of *D. malaccensis* by using PLE with variation of temperature at 1500 psi (A) and pressure at 50 °C (B) (n = 3)

2.3.5 Comparison of the yields of extracts and absolute yields of rotenone and rotenone contents of the extracts from the different parts of the plants and the different extraction methods

The yields of ethanolic extracts from the stems of *D. malaccensis* and the stems and roots of *D. elliptica* were significantly higher than chloroform extracts ($p < 0.05$), whereas the absolute yields of rotenone and the rotenone contents of chloroform extracts were significantly higher than ethanolic extracts ($p < 0.05$) (Table 2.6). Therefore, Chloroform displayed better solvent properties for rotenone than 95% ethanol. The appearances of chloroform extract and ethanolic extract are shown in Figure 2.11A and 2.11B.

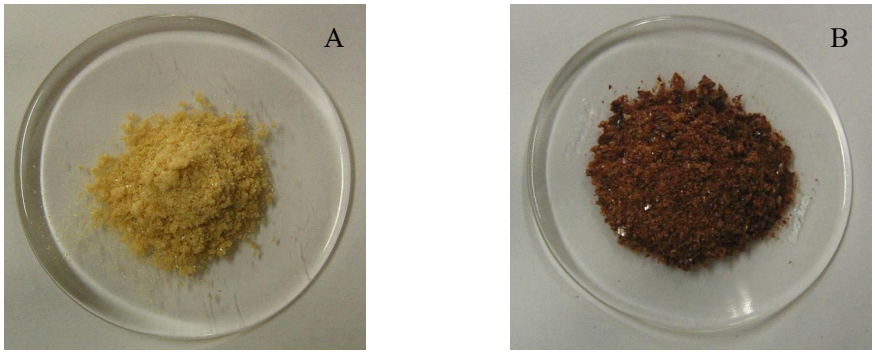


Figure 2.11 Chloroform extract (A) and ethanolic extract (B) from the roots of *D. elliptica*

Moreover, the absolute yields of rotenone from the roots of *D. elliptica* were significantly higher than those from the stems of *D. malaccensis* and the stems of *D. elliptica* ($p < 0.05$). The extracts from the stems of *D. elliptica* and *D. malaccensis* contained small amounts of rotenone (mg) in 100 mg of crude extract (0.37-9.37 %w/w) whereas the extracts from the roots of *D. elliptica* contained much higher rotenone contents (15.03-46.08 %w/w) (Table 2.6 and Figure 2.12).

In addition, it was observed that the rotenone contents from the extraction by PLE using chloroform at room temperature (30 °C) were comparable to maceration. The extraction performed by PLE at 50 °C also showed significantly higher rotenone contents than at 30 °C ($p < 0.05$). However, the chloroform extracts obtained from the stems of derris plants by

maceration and PLE showed similarities of rotenone content (Figure 2.12). This might be due to the stems originally containing small amounts of rotenone that could be completely soluble in chloroform, so there was no difference between maceration and PLE.

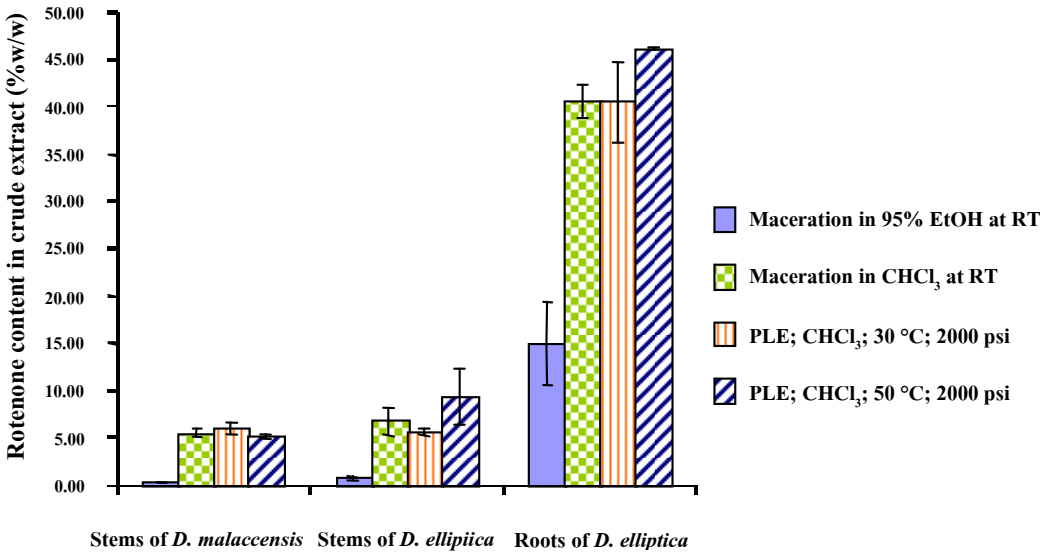


Figure 2.12 Rotenone contents of crude extracts from the stems and the roots of *D. elliptica* and the stems of *D. malaccensis*. Extractions were performed by using PLE compared with maceration (n = 3).

2.3.6 Comparison of the extraction methods

The data presented in Table 2.7 shows that using PLE to extract rotenone from plant enables a significant three-fold reduction in volume of the organic solvent used and reduction in extraction time (72 h to 30 min). In addition, the extracts obtained from PLE had a higher rotenone content than the extracts obtained from maceration. The cost of the extraction by PLE may be higher than maceration due to the relatively expensive equipment used. However, PLE is more likely to be used commercially, since ease of scale-up, possible semi-automation of the extraction process, and saving of labour and time will be attractive to commercial producers.

Table 2.6 The yield of extracts and absolute yield of rotenone from dried plant and rotenone content of the extracts

Plant	**Extraction method**	**Solvent**	**Yield of extract (%w/w) (n = 3)**	**Absolute yield of rotenone (%w/w) (n = 3)**	**Rotenone content (%w/w) (n = 3)**
Stems of *D. malaccensis*	Maceration	95 % EtOH	5.96±1.30*	0.022±0.005*	0.37±0.00*
	Maceration	$CHCl_3$	0.66±0.03	0.036±0.005	5.46±0.47
	PLE; 30 °C; 2000 psi	$CHCl_3$	0.64±0.02	0.038±0.004	5.95±0.61
	PLE; 50 °C; 2000 psi	$CHCl_3$	0.73±0.20	0.038±0.012	5.19±0.24
Stems of *D. elliptica*	Maceration	95 % EtOH	3.62±0.60*	0.026±0.001*	0.73±0.14*
	Maceration	$CHCl_3$	0.89±0.03	0.061±0.015	6.78±1.48
	PLE; 30 °C; 2000 psi	$CHCl_3$	0.93±0.15	0.052±0.009	5.56±0.37
	PLE; 50 °C; 2000 psi	$CHCl_3$	1.18±0.19	0.11±0.05	9.37±2.95
Roots of *D. elliptica*	Maceration	95 % EtOH	11.42±0.51*	1.71±0.44*	15.03±4.41*
	Maceration	$CHCl_3$	6.26±0.54	2.54±0.29	40.57±1.73
	PLE; 30 °C; 2000 psi	$CHCl_3$	8.62±0.47	3.51±0.57	40.53±4.31
	PLE; 50 °C; 2000 psi	$CHCl_3$	7.83±1.26	3.61±0.57	46.08±0.20

* Significance difference ($p < 0.05$), when compared with chloroform extracts

Table 2.7 Comparison of the extraction methods

	Maceration	**PLE (at 50 °C, 2000 psi)**
Volume of solvent/weight of dried plants	10 ml/g	3 ml/g
Extraction time	72h	30 min
Rotenone content of extract from root of *D. elliptica* (n = 3)	40.57% (w/w) (±1.73); RSD 4.26%	46.08% (w/w) (±0.20); RSD 0.43%

2.4 Conclusions

The extraction of rotenone from *D. elliptica and D. malaccensis* by PLE and maceration were compared. Two types of organic solvents were used, chloroform and 95% ethanol. The results showed that chloroform was a good solvent for extraction of rotenone. PLE was carried out with the optimized conditions (50 °C, 2000 psi, using chloroform as extraction solvent). The extracts from the roots of *D. elliptica* contained the highest rotenone content. PLE using chloroform at elevated temperature and pressure was found to give higher extraction efficiency compared to maceration. PLE also required smaller amount of extraction solvent and shorter extraction time.

In this study, it was possible to develop a selective and validated stability-indicating HPLC assay method which could separate rotenone and its degradation products formed under a variety of stress conditions. Rotenone was found to be strongly unstable under base condition and unstable under acid and oxidation conditions. The developed method is accurate, precise, specific and selective. It is proposed for analysis of rotenone and degradation products in stability samples from crude extract and formulations.

CHAPTER 3

Development and Evaluation of Granule and Emulsifiable Concentrate Formulations Containing *Derris elliptica* Extract for Insect Pest Control

3.1 Introduction

The science of pesticide formulation covers a very broad field, as it deals with the development, production, and storage of the formulations. The choice of formulation is influenced by the following factors: physical properties of the pesticide (melting point, solubility, and volatility), chemical properties of the pesticide (hydrolytic stability, thermal stability, and irradiation stability), mode of application of the formulation (soil, foliar), crop to be treated and the agricultural practices, biological properties of the pesticide (crop selectivity, transport through soil or grass cover, and LD_{50} for mammals and non-mammalian species), and economic considerations.

Pesticides are formulated for a number of different reasons. A pesticide active ingredient in a relatively pure form is rarely suitable for field application. An active ingredient usually must be formulated in a manner that increases pesticide effectiveness in the field, improves safety features and enhances handling qualities. The active ingredients in pesticide products come from many sources. Some, such as nicotine, pyrethrum, and rotenone, are extracted from plants. Others have a mineral origin, while a few are derived from microbes. However, the vast majority of active ingredients are synthesized in the laboratory. Regardless of their sources, pesticide active ingredients have different solubilities. These different solubility characteristics, coupled with the intended use of the pesticide, in large measure define the types of formulations in which the active ingredient may be delivered. Manufacturers often produce various forms of a pesticide to meet different pest control needs. They have their own particular skills in formulation, details of which are a closely guarded secret because competition from rival companies. However, formulations have usually been selected on the basis of convenience to the user (Markus and Linder, 2006; Martin et al., 2001; Matthews, 1979).

Water dispersible granules (WDG) are finely divided powders that are formulated into concentrated, dustless granules. Water dispersible granules are intended for application after disintegration and dispersion in water by conventional spraying equipment. They form a suspension in water and require some agitation to maintain a uniform spray mixture. The principal advantage of this formulation is that, although it is sold in the dry form, it is not a dust and can be handled with great ease and safety. Water dispersible granules are formulated in many different ways depending on the physicochemical properties of the active ingredient and the manufacturing equipment available. This can lead to products of differing appearances and differing particle size ranges (FAO/WHO, 2006; Martin et al., 2001).

Granulation is the process in which primary powder particles are made to adhere to form larger, multiparticle entities called granules. Granulation normally commences after initial dry mixing of the necessary powdered ingredients so that a uniform distribution of each ingredient through the mix is achieved. The reasons why granulation is often necessary are to prevent segregation of the constituents of the powder mix and to improve the flow properties of the mix. Segregation is due primarily to differences in the size or density of the components of the mix, the smaller and/or denser particles concentrating at the base of a container with the larger and/or less dense ones above them. An ideal granulation will contain all the constituents of the mix in the correct proportion in each granule, and segregation of the ingredients will not occur. There are other reasons that may need the granulation of powdered material. The granulation of toxic materials will reduce the hazard associated with the generation of toxic dust that may arise when handling powders. Thus granules should be non-friable and have a suitable mechanical strength. Materials which are slightly hygroscopic may adhere and form a cake if stored as a powder. Granule will be able to absorb some moisture and yet retain their flowability because of their size. Granules, being denser than the parent powder mix, occupy less volume per unit weight. They are therefore more convenient for storage or shipment (Summers and Aulton, 2001).

In a suitable formulation a number of different excipients will be needed in addition to the active ingredient. The common types used are diluents, to produce a unit dose weight of suitable size, and disintegrants, which are added to aid the break-up of the granule when it reaches a liquid medium, and binders, which are used to ensure particle adhesion once the granule is dry (Summers and Aulton, 2001).

Granulation methods can be divided into two types: wet granulation, which use a liquid in the process, and dry granulation in which no liquid is used. In the dry methods of granulation the primary powder particles are aggregated under high pressure. There are two main processes. Either a large tablet (known as a slug) is produced in a heavy-duty tabletting press (a process known as slugging) or the powder is squeezed between two rollers to produce a sheet of material (roller compaction). In both cases these intermediate products are broken using a suitable milling technique to produce granular material, which is usually sieved to separate the desired size fraction. This dry method may be used for drugs that are not compressed well after wet granulation, or those which are sensitive to moisture (Summers and Aulton, 2001).

Wet granulation involves the massing of a mix of dry primary powder particles using a granulating fluid. Typical liquids include water, ethanol and isopropanol, either alone or in combination. Water is commonly used for economical and ecological reasons. Its disadvantages as a solvent are that it may adversely affect stability of active compounds, and it needs a longer drying time than organic solvents. This increases the length of the process and again may affect stability because of the extended exposure to heat. Organic solvents are used when water-sensitive compounds are processed or when a rapid drying time is required. Generally, granules are prepared by moistening the desired powder or blended powder mixture and passing the moistened mass through a screen of mesh size that will produce the desired size granules. The larger particles formed are then dried by air or under heat, while they are occasionally moved about on the drying trays to prevent the adhesion of the granules. A subsequent screening stage breaks agglomerate of granules and removes the fine material (Ansel et al., 1995; Summers and Aulton, 2001).

Emulsifiable concentrate (EC) is a liquid, homogeneous preparation to be applied as an emulsion after dilution in water. Emulsifiable concentrates consist of an oil soluble active ingredient dissolved in an appropriate oil based solvent, which is added an emulsifier. Major factors influencing the choice of solvent are solubility of the active ingredients and other excipients, price and toxicity (Martin et al., 2001; Matthews, 1979).

Emulsifier is an important component in these formulations. The addition of an emulsifier enables the formation of a homogeneous and stable dispersion of small globules of the solvent in water. The small globules of suspended liquid are referred to as the disperse phase, and

the liquid in which they suspended is the continuous phase. The emulsifiers orient themselves around the droplets of oil and bind the oil-water surfaces together to prevent the oil and water from separating. A suitable emulsifier should emulsify an emulsifiable concentrate formulation spontaneously to form a stable emulsion. A freshly prepared batch of emulsion should be stable for up to 24 hours before degradation occurs. But it is preferable to use the mixture as soon as possible. The factors which affect the stability of an emulsion involve a complex dynamic equilibrium in the disperse phase-interface-continuous phase system. An unstable emulsion breaks if the disperse phase separates and forms a cream on the surface, or the globules coalesce to form a separate layer. Creaming is due to differences in specific gravity between the two phases, and can cause uneven application (Martin et al., 2001; Matthews, 1979).

Agitation of the spray mix normally prevents creaming. Breaking of an emulsion after spray droplets reach a target is partly due to evaporation of the continuous phase, usually water, and leaves the pesticide in a film which may readily penetrate the surface of the target. The stability of emulsions is affected by the hardness and pH of water used when mixing for spraying and also conditions under which the concentrate is stored. High temperatures and frost can adversely affect a formulation (Matthews, 1979).

Derris elliptica have long been used as natural insecticides due to their high content of rotenone. It is a perennial plant which is easy to grow in tropical climate of Thailand. Derris root was usually macerated and the resulting milky suspension was used to spray the crops but this method is inconvenient and the amount of rotenone in the suspension is very low and unstable. Therefore, derris extract in a form that can be easily handled and be able to increase the stability during storage is required. However, derris products have not been developed in Thailand. Derris water dispersible granules and derris emulsifiable concentrate were therefore prepared and developed due to the physicochemical properties of derris extract and the advantages of the two formulations.

In this study *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) or common cutworm, which is one of the serious pests in agricultural area and causes damage to many cultivated plants such as vegetables, field crops and many kinds of fruit trees, was used for the preliminary efficacy test. A common vegetable host of *S. litura* is Chinese kale (*Brassica alboglabra* Bailey), which is a popular leafy vegetable in Thailand. Chinese kale is one of the

most commonly grown as well as intense pest attractive crops. As Chinese kale is grown throughout the year and farmers often use highly toxic insecticides to control insect pests, the insects may develop resistance to insecticides, and in such a situation demanding the use of higher concentration of insecticides than safe recommended doses. In Thailand, the pesticide residues in vegetables including Chinese kale were found since 1962 (Areekul et al., 1962). Hence, derris formulations are alternative ways using for pest control in vegetables due to their rapid decomposition.

The objectives of this study were (1) to develop two types of pesticide formulations containing derris extract; water dispersible granules and emulsifiable concentrate, (2) to study the physicochemical properties and stability of rotenone in the developed formulations, and (3) to carry out a preliminary efficacy test of the formulations against *S. litura* in laboratory experiments, prior to future field trials.

3.2 Experimental methods

3.2.1 Plant samples

The roots of *D. elliptica* were collected from the botanical garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand in April of 2007. The roots were washed with tap water, dried in air, cut into small pieces and dried in a hot air oven at 40 °C for 24 h. The dried plants were then ground to a powder and stored in plastic bags in the dark at room temperature (30±2 °C).

3.2.2 Chemicals

HPLC grade acetonitrile and analytical grade methanol and acetone were purchased from Labscan Asia (Bangkok, Thailand). Commercial grade dichloromethane was obtained from High Science distributor (Songkhla, Thailand). Water was purified by passing it through a Milli-Q purification system. Standard rotenone (98% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). The stock solution (0.1 mg/ml) was prepared by dissolving

10 mg of rotenone in 100 ml methanol. Working solutions were freshly prepared on the day of any study by suitable dilution of the stock solution with methanol. The stock solution was kept in the dark at 4 °C and used within 1 month.

Commercial grade polyvinyl pyrrolidone K-30 (PVP K-30), sodium alginate, sorbitan monooleate (Span 80) and polyethylene sorbitan monooleate (Tween 80) were purchased from Srichand United Dispensary (Bangkok, Thailand). Pharmaceutical grade lactose monohydrate was obtained from DMV International Distributor (Thailand). Microcrystalline cellulose (Avicel® PH101) and commercial grade light mineral oil were from PC Drug Center Co., Ltd. (Bangkok, Thailand). Food grade soybean oil was purchased from Thai vegetable oil public company limited (Bangkok, Thailand) and analytical grade butylated hydroxytoluene (BHT) was purchased from Sigma (St. Louis, USA). Cypermethrin 35% w/v of emulsifiable concentrate (Starship 35®) was from Hi-tech Group Chemical Supply Co., Ltd. (Bangkok, Thailand).

3.2.3 Extraction of the dried root powder of *D. elliptica*

Dichloromethane has the same polarity index as chloroform and is less toxic and cost. Therefore, chloroform was replaced with dichloromethane for extraction in this study. The dried root powder was macerated with dichloromethane at room temperature (30±2 °C) for 3 days with occasional stirring. The solvent was then filtered and evaporated in a rotary evaporator (Eyela, Tokyo, Japan) under vacuum at 40 °C. The residue was placed in a vacuum oven (Napco, Chicago, USA) at room temperature until dry. The crude extract was stored in a well-closed container and protected from light and kept in a desiccator at 4 °C. Rotenone content in the crude extract was determined by HPLC. Approximately 1 mg of the extract was accurately weighed and quantitatively transferred to a 25 ml volumetric flask, and the volume made up to 25 ml with methanol and sonicated for 5 min. The methanolic sample was then filtered before analysis. The analyses were performed in triplicate, and calibration standards were analysed on the same day as the samples.

3.2.4 HPLC analysis

The analysis of rotenone in the derris extract and derris formulations was performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) with a Restek® C18 5 µm column (250 × 4.6 mm). A linear gradient was used for the separation of rotenone with the initial mobile phase composition of acetonitrile/water 50:50 (% v/v) reaching 70:30 (% v/v) in 30 min. Before each injection, the HPLC column had to be stabilized for 10 min with the initial mobile phase composition of acetonitrile/water mobile phase 50:50 (%v/v). The injection volume was 20 µl and the flow rate was 1 ml/min. Detection was by UV spectroscopy at a wavelength of 294 nm. In our previous study, the determination of rotenone in the crude extract could be performed by validated HPLC method described above with acceptable accuracy and precision (Sae-Yun et al., 2006).

3.2.5 Determination of solubility of derris extract (modified from British Pharmacopoeia Commission, 2001)

The extract was accurately weighed to 10 mg and placed in a test tube of at least 20 ml capacity. The test tube was placed in a constant temperature, maintained at a temperature of 25±0.2 °C. Various solvents (water, 95% ethanol, methanol, ethyl acetate, chloroform, dichloromethane, acetone, hexane, soybean oil, mineral oil, Tween 80 and Span 80) were examined by adding of the strength prescribed in the monograph by increments of 10 µl, shaking frequently and vigorously for 10 min. Record the volume of solvent added when a clear solution was obtained. If the solution becomes cloudy or undissolved, the solvent was continuously added up to 10 ml. After that if the sample or parts of it remained, the experiment had to be repeated in a 100 ml volumetric flask. At lower solubility the time required to dissolve a substance can be considerably longer, at least 24 h should be allowed. Descriptive term of solubility and approximate volume of solvents required to completely dissolve a solute (in ml/g of solute) are drawn as follow (Table 3.1).

Table 3.1 Solubility criteria of the extract in various solvents

Solubility term	**Volume of solvent required to dissolve 1 g of solute (ml)**
Very soluble	less than 1
Freely soluble	from 1 to 100
Soluble	from 10 to 30
Sparingly soluble	from 30 to 100
Slightly soluble	from 100 to 1,000
Very slightly soluble	from 1,000 to 10,000
Practically insoluble	more than 10,000

3.2.6 Preparation and development of derris water dispersible granules

The compositions of water dispersible granule formulations were optimized to obtain a suitable formula with fast disintegration and low friability. Varied amounts of PVP K-30, sodium alginate, and lactose were used to prepare granules by wet granulation technique. The granules were evaluated for their physical properties such as friability and disintegration time. The formulation which gave the fastest disintegration and low friability was selected to prepare derris water dispersible granule formulation.

The derris water dispersible granules (containing 5% w/w rotenone), consisting of derris extract, PVP K-30, sodium alginate, Avicel® PH101, Tween 80 and lactose, were prepared by a wet granulation method. The derris extract, which contained 36.13% w/w of rotenone, was mixed with PVP K-30, sodium alginate, Avicel® PH101 and lactose by a geometric dilution technique to give a homogeneous powder. Tween 80 was dissolved in 5 ml of distilled water and added dropwise to the powder with continuous mixing to produce a damp mass, which was then passed through a sieve No. 14 and dried in hot air oven at 40 °C for 2 hours. After that the dried particles were screened through a sieve No. 16. The granules were stored in a glass

container at room temperature (30±2 °C) and protected from light. Water dispersible granules without derris extract were also prepared, and hereafter referred to as negative control.

3.2.7 Evaluation of the physical properties of derris water dispersible granules

3.2.7.1 Rotenone content

Approximately 10 mg of the granule sample was accurately weighed and quantitatively transferred to a 25 ml volumetric flask, and the volume made up to 25 ml with methanol and sonicated for 30 min. The methanolic sample was then filtered and rotenone content determined by HPLC as described above. The analyses were performed in triplicate, and calibration standards were analysed on the same day as the samples.

3.2.7.2 Friablity

Granules (10 g) were placed in the drum of a Roche friabilator with 25 steel-balls. The samples were rotated at 25 rev/min for 16 min, each rotation causing the granules to fall a distance of 15 cm inside the drum. Afterwards, each sample was sieved through a 45 mesh screen, and the weight of sample remaining above the screen was determined, and the percentage friability index was calculated from the following equation. Three replicates were performed for each test, and the data given as means (± SD).

$$\text{Friability index} = \frac{\text{weight of granule remaining above 45 mesh}}{\text{total weight of loaded sample}} \text{ x } 100$$

3.2.7.3 Disintegration time

Granule samples (2.5 g) were dispersed in 50 ml of distilled water (1:20 dilution) and stirred at 500 rpm at room temperature (30±2 °C). The time required for complete disintegration of the granules was recorded. Three replicates were performed for each test, and the data given as means (± SD).

3.2.7.4 Viscosity

After complete disintegration of 5% w/v granules in water, the viscosity of the resulting suspension was measured by a Brookfield Model DV III Rheometer (spindle no. 31 at 250 rpm) at 25 °C. Three replicates were performed for each test, and the data given as means (± SD).

3.2.7.5 pH

The pH of the suspension was also determined using a pH meter (Mettler-Toledo Co. Ltd., Switzerland). Three replicates were performed for each test, and the data given as means (± SD).

3.2.8 Preparation and development of derris emulsifiable concentrate

3.2.8.1 Study on determination of optimum ratios of oils and surfactants

The oils (soybean oil or light mineral oil) and surfactants (Tween 80 and/or Span 80) were mixed in different proportions in order to find the most suitable formulations, which gave a clear homogeneous liquid, and which provided stable emulsions after 1:20 dilution with water. The formulation that showed the best characteristic was selected for the preparation of the derris emulsifiable concentrate.

3.2.8.2 Preparation of derris emulsifiable concentrate

The emulsifiable concentrate containing derris extract was prepared by simple mixing. The derris extract, which contained 36.13% w/w of rotenone, was ground and mixed with Tween 80 and Span 80 using a mortar and pestle. Soybean oil and BHT were then added and mixed to give a homogeneous concentrate mixture that contains 5% w/w of rotenone. The resulting formulation was stored in a glass bottle at room temperature (30±2 °C) and protected from light. Emulsifiable concentrate without derris extract was also prepared, and hereafter referred to as the negative control.

3.2.9 Evaluation of the physical properties of derris emulsifiable concentrate

3.2.9.1 Rotenone content

Approximately 10 mg of emulsifiable concentrate sample was accurately weighed and quantitatively transferred to a 25 ml volumetric flask, and volume made up to 25 ml with methanol and sonicated for 30 min. The methanolic sample was then filtered and rotenone content determined by HPLC as described above. The analyses were performed in triplicate, and calibration standards were analysed on the same day as the samples.

3.2.9.2 Droplet size

Emulsifiable concentrate (2.5 g) was dispersed in 50 ml of distilled water (1:20 dilution) by stirring with a magnetic stirrer at 500 rpm for 5 min to complete emulsification, at room temperature (30±2 °C). The resulting emulsion was evaluated for oil droplet size by the Mastersizer E (Malvern Instrument Ltd., UK). Three replicates were performed for each test, and the data reported as means (± SD).

3.2.9.3 Viscosity

After complete dispersion of 5% w/v emulsifiable concentrate in water, the viscosity of the resulting emulsion was measured by a Brookfield Model DV III Rheometer (spindle no. 31 at 250 rpm) at 25 °C. Three replicates were performed for each test, and the data reported as means (± SD).

3.2.9.4 pH

The pH of the emulsion was also determined using a pH meter (Mettler-Toledo Co. Ltd., Switzerland). Three replicates were performed for each test, and the data reported as means (± SD).

3.2.10 Chemical stability of rotenone in derris extract and derris formulations

The stability of rotenone during storage of the derris extract and the two derris formulations was tested under accelerated conditions. Samples of each (1 g) were stored in closed glass containers at 45, 60 and 70°C with 75% relative humidity (% RH) and protected from light. The amount of rotenone in each stored sample (n = 3) were analysed at 0, 1, 3, 7, 14, 21, 28, 56, 84, 112, 140 and 168 days after storage by HPLC. Approximately 1 mg of the extract and 10 mg of the two derris formulations were accurately weighed and quantitatively transferred to a 25 ml volumetric flask, and the volume made up to 25 ml with methanol and sonicated until complete solubilization. The methanolic samples were then filtered and rotenone content determined by HPLC as described above. The analyses were performed in triplicate, and calibration standards were analysed on the same day as the samples. The amount of rotenone was plotted against the storage time in order to calculate the half-life ($t_{1/2}$) and shelf life ($t_{90\%}$). A study of the real-time stability of the derris extract and the two derris formulations was also compared to that calculated from accelerated studies. Samples of derris extract and the derris formulations (1 g) were stored in closed glass containers at room temperature (30±2 °C) and protected from light for 6 months and rotenone content remaining determined by HPLC after 6 months of storage.

3.2.11 Stability of rotenone in derris formulations after spraying onto plants

Stability of rotenone in derris formulations after spraying onto plants was studied under greenhouse conditions. Ten pots of Chinese kales were placed in a greenhouse which was exposed to sunlight. Water dispersible granule or emulsifiable concentrate samples were mixed with water (1:20 dilution) by gentle stirring to form suspension or emulsion which contained 0.25% w/v of rotenone. The prepared suspensions or emulsions were sprayed onto the leaves (50 ml/plant) of 60-day-old Chinese kales by Tango 1.5 sprayer (Osatu, Guipuzcoa, Spain) (Figure 3.1). Ten replicates were performed for each treatment. The leaves of Chinese kales were sampled at 0, 1, 3, 5, 7, 10 and 14 days after spraying. Rotenone residues on the both sides of foliage (80 cm^2) (n = 3) were determined by rinsing the leaves with acetone, and the acetone washings were

evaporated to dryness. The resulting samples were then reconstituted with methanol and quantitatively transferred to a 25 ml volumetric flask, and the volume made up to 25 ml with methanol and sonicated for 10 min. The methanolic samples were then filtered and rotenone content determined by HPLC as described above. The analyses were performed in triplicate, and calibration standards were analysed on the same day as the samples.

Figure 3.1 Application of derris formulations onto the leaves of Chinese kales

3.2.12 Preliminary efficacy testing of the two derris formulations

The efficacy of each formulation against *S. litura* was investigated under laboratory conditions (Figure 3.2). Each formulation, which contained 5% w/w of rotenone, was mixed with water (1:20 dilution) by gentle stirring to form a suspension or emulsion which contained 0.25% w/v of rotenone. The leaves of Chinese kale were dipped in the resulting suspension or emulsion (1 min), air dried (15 min) and placed in plastic boxes as feed for the larvae. Five 2nd instar larvae of *S. litura* were placed in each box. Ten replicates were performed for each treatment. Larvae mortality was recorded every 24 h for 3 days, and any growth abnormalities of surviving larvae were also investigated. The larvae in boxes containing leaves treated with control formulations (not containing derris extract) were used as the negative controls. For positive controls, the leaves were dipped into an emulsion of the pesticide cypermethrin at the concentration suggested for field applications (0.0175% w/v).

Figure 3.2 Preliminary efficacy test of derris formulations in laboratory conditions

3.2.13 Efficacy testing of derris emulsifiable concentrate in greenhouse

The efficacy of derris emulsifiable concentrate formulation against *S. litura* was investigated under greenhouse conditions (Figure 3.3). The formulation which contained 5% w/w of rotenone was mix with water (1:20 dilution) by gentle stirring to form an emulsion which contained 0.25% w/v of rotenone. The prepared emulsion was sprayed onto 60-day-old Chinese kales. Five 2nd instar larvae of *S. litura* were placed onto the Chinese kales. Each Chinese kale was then covered with a net in order to protect the larvae loss. Ten replicates were performed for each treatment. Larvae mortality was recorded every 24 h for 4 days, and any growth abnormalities of surviving larvae were also investigated. The Chinese kales treated with control formulation (not containing derris extract) were used as the negative controls. For positive controls, the emulsion of pesticide cypermethrin at the concentration suggested for field applications (0.0175% w/v) was sprayed onto the Chinese kales.

Figure 3.3 Efficacy test of derris emulsifiable concentrate in greenhouse

3.3 Results and discussion

3.3.1 Preparation of derris root extract

The extract of derris root was an orange powder (Figure 3.4) (yield 10.03% w/w relative to the weight of dried plant), and the amount of rotenone in the derris root was 3.62% w/w calculated as absolute yield of rotenone (g) in 100 g of dried plant. Since the crude extract was hygroscopic, it was kept in a desiccator at 4 °C.

Figure 3.4 Derris root extract

From the previous reports, the cultivated *D. elliptica* root contained about 13% of rotenone and the black resinous extract, which is sold in the market contained about 30% of rotenone (Evans, 2002; Tyler et al., 1976). The rotenone content of the extract from derris root (36.13% w/w) obtained in our study, from locally obtained fresh roots, is considerably higher than reported in earlier studies. It is clear that future studies need to examine how cultivation, soil conditions, geographical location, harvesting and post-harvesting process, affect rotenone formation and accumulation in derris root, particularly if there is any hope for successful commercialization of the formulations we have developed.

Although it is possible to isolate pure rotenone from derris extract, or indeed develop methods for its chemical synthesis, this would not be cost-effective for commercialization of any formulated products, since the primary aim is to provide agriculturists in developing countries with cheaper, locally produced and hopefully safer alternatives to chemically based pesticides. Additionally, it may be that the efficacy, and perhaps the overall safety, of derris extract is not only due to rotenone content, but it is due to a delicate balance of a variety of other ingredients in the derris extract. It should be relatively easy to grow *D. elliptica* on a large scale, for regular harvesting of root and preparation of standardized extracts with known rotenone content.

3.3.2 Solubility of derris extract

Solubility is commonly expressed as a maximum equilibrium amount of solute that can normally dissolve per amount of solvent or a maximum concentration of a saturated solution. These maximum concentrations are often expressed as grams of solute per 100 ml of solvent. The solubility test of derris extract is used to estimate the dissolution of the extract in various solvents. The results showed that derris extract is freely soluble in chloroform and dichloromethane, soluble in Tween 80, slightly soluble in Span 80, ethyl acetate and soybean oil. It is very slightly soluble in acetone, 95% ethanol, methanol and mineral oil and practically insoluble in hexane and water (Table 3.2). The solubility behavior of compounds remains one of the most challenging aspects in formulation development. The derris extract contains most likely

moderate non-polar compounds therefore moderate non-polar solvents and surfactants were required in the formulations.

Table 3.2 Solubility of the derris extract

Solvent	Volume of solvent in ml/g of solute	Level of solubility
Chloroform	10	Freely soluble
Dichloromethane	10	Freely soluble
Tween 80	30	Soluble
Span 80	200	Slightly soluble
Ethyl acetate	300	Slightly soluble
Soybean oil	500	Slightly soluble
Acetone	2,000	Very slightly soluble
95% Ethanol	2,000	Very slightly soluble
Methanol	10,000	Very slightly soluble
Mineral oil	10,000	Very slightly soluble
Hexane	>10,000	Practically insoluble
Water	>10,000	Practically insoluble

3.3.3 Development of derris water dispersible granules

Water dispersible granules are important delivery vehicles for active agricultural chemicals because they offer significant advantages in packaging, ease of handling and safety. This formulation is preferably used in the case of solid active substances of relatively high melting point (m.p. > 65 °C). Typically these are small granules of 0.1-3 mm in diameter and preferably of uniform size and which are free flowing, low dusting and readily disperse in water to form suspension of very small particles, which may pass through conventional spray nozzles. Derris extract, the solid pesticide, which is hard to be dissolved in water and has high melting point was therefore made as water dispersible granules.

Generally, water dispersible granules comprise active ingredient, diluent, binder, disintegrant, dispersing agent and wetting agent. The amounts of individual ingredients may vary widely, with the active ingredient generally being present in an amount from 5 to 95% w/w, the diluents generally 5 to 90% w/w, and the other ingredients generally 0.1 to 20% w/w (Lloyd and Baker, 1998; Röchling et al., 2002).

Lactose and PVP are the most extensively used pharmaceutical excipients in wet granulation. Lactose is widely used as a diluent and PVP is generally used as binder (Albertini et al., 2003; Goodhart, 1994). Sodium alginate is also used as disintegrant and suspending agent.

The compositions of water dispersible granule formulations were optimized to obtain a suitable formula with fast disintegration and low friability. Varied amounts of PVP K-30, sodium alginate, and lactose were used to prepare granules by wet granulation technique. The granules were evaluated for their physical properties such as friability and disintegration time and the result are displayed in Table 3.3.

The granule disintegration time was primarily affected by amount of sodium alginate. Increasing the proportion of sodium alginate in the formulation resulted in granules with a longer disintegration time and lower friability index value (Figure 3.5 and 3.6). High amount of sodium alginate could delay the disintegration of granules due to its slow solubility in water and slow swelling behavior. The shortest disintegration was achieved at the concentration of 5% w/w of sodium alginate and 5% w/w of PVP K-30 (Figure 3.7).

The friability of granules was influenced by the content of PVP K-30. Low friability of granules was represented in high friability index value. Increasing the PVP K-30 content gave rise to stronger granules because of its binding properties, as can be seen from Figure 3.8. The short disintegration time (less than 2 minutes) granules were obtained from formula 1, 5 and 6 and the high friability index (more than 95%) granules were obtained from formula 5, 6, 9, 10, 11 and 12. Therefore, the formula 5 and 6 were selected to evaluate for their viscosity and pH after diluted with water.

Table 3.3 Compositions of blank water dispersible granules and their physical properties

Formula	Composition of granules (% w/w)			Disintegration time* (min)	Friability index
	PVP K-30	Sodium alginate	Lactose		
1	2.5	5.0	92.5	1.45±0.05	86.8±1.2
2	2.5	10.0	87.5	2.10±0.06	78.8±0.9
3	2.5	15.0	82.5	3.14±0.11	72.2±3.0
4	2.5	20.0	77.5	3.32±0.13	66.7±2.8
5	5.0	5.0	90.0	1.42±0.10	97.3±0.6
6	5.0	10.0	85.0	1.48±0.05	95.4±0.8
7	5.0	15.0	80.0	2.03±0.01	93.2±1.8
8	5.0	20.0	75.0	2.32±0.13	91.6±1.0
9	10.0	5.0	85.0	2.41±0.14	99.0±0.6
10	10.0	10.0	80.0	3.39±0.09	98.0±0.8
11	10.0	15.0	75.0	3.47±0.05	96.0±1.4
12	10.0	20.0	70.0	4.39±0.11	96.0±1.4

* Blank water dispersible granules were dispersed in distilled water (1:100 dilution) and stirred at 200 rpm at room temperature (30±2 °C).

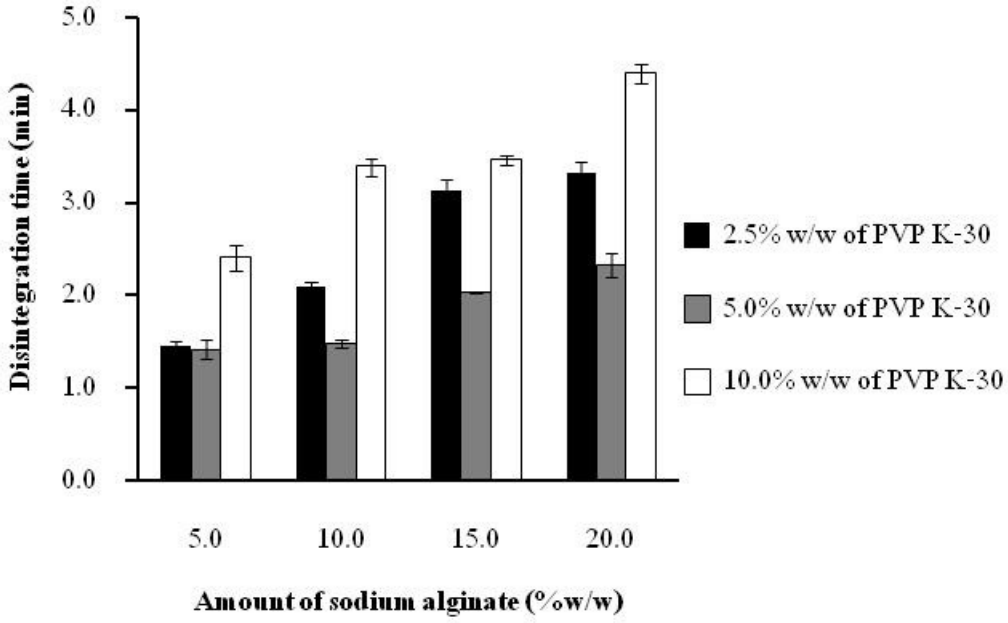


Figure 3.5 Disintegration time of water dispersible granules containing 5, 10, 15 and 20% w/w of sodium alginate

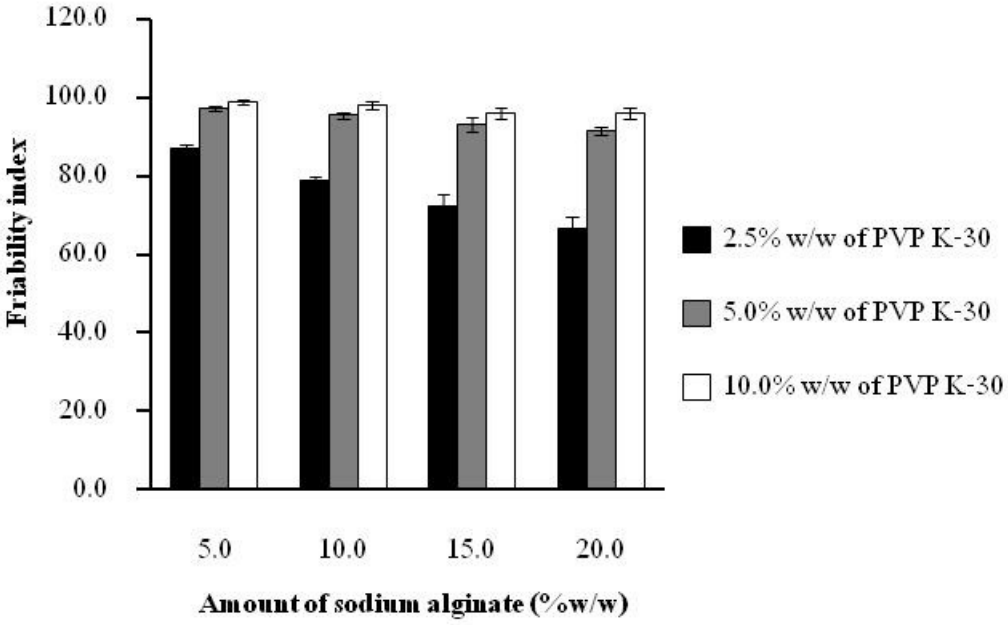


Figure 3.6 Friability index of water dispersible granules containing 5, 10, 15 and 20% w/w of sodium alginate

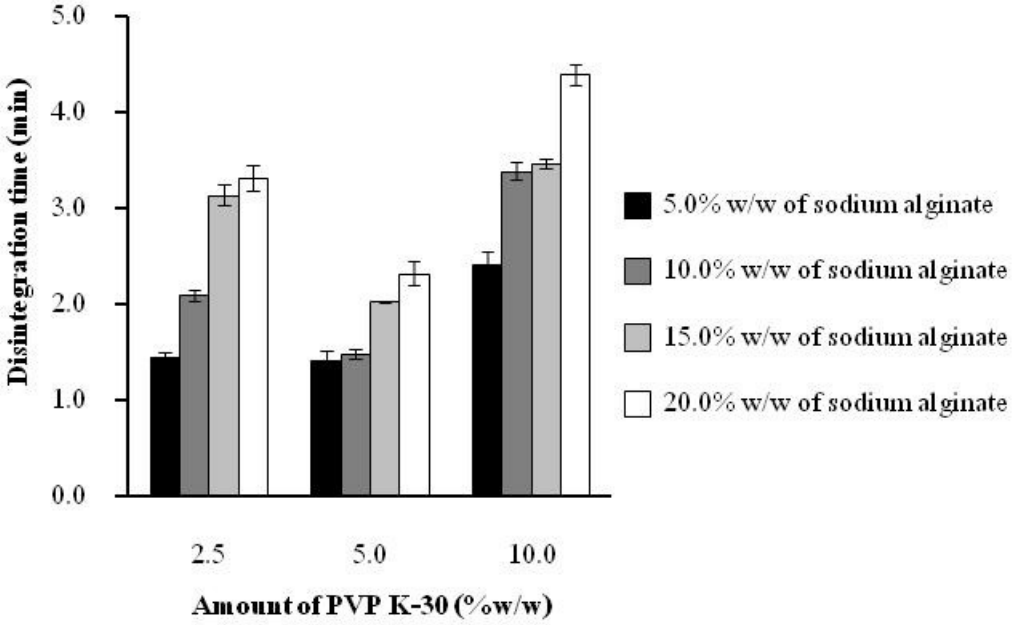


Figure 3.7 Disintegration time of water dispersible granules containing 2.5, 5, 10% w/w of PVP K-30

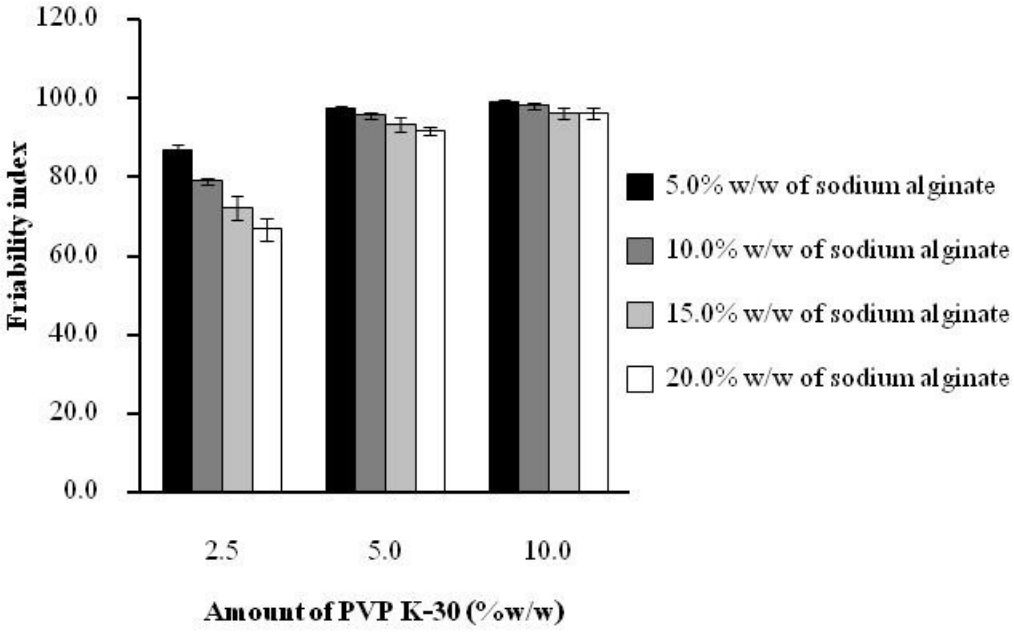


Figure 3.8 Friability index of water dispersible granules containing 2.5, 5, 10% w/w of PVP K-30

The viscosity and pH of aqueous suspensions after 1:100 and 1:20 dilution were determined and the results are listed in Table 3.4 and 3.5. The result showed that the higher proportion of sodium alginate in the formulation and higher percent of water dispersible granules in water gave more viscous suspension because of suspending and viscosity increasing properties of sodium alginate (Budavari et al., 1999; Weller, 1994). In general, more viscosity could retard the settle of particles in suspensions. However, high viscosity suspension may cause strainers and screens to plug when sprayed with a sprayer. In contrast, the pH values tended to decrease with increasing amount of sodium alginate in the formulation and increasing the percent of water dispersible granules in water. However, the pH values of the obtained suspensions were in the range of 5.90-6.61, which are not harmful to plant surface (A&L Canada laboratories, 2002).

The formula 5 was therefore chosen to formulate with derris extract and evaluated for their physical properties because it provided short disintegration time and low friability. The suspension obtained from this formula also had proper viscosity and pH for spraying.

Table 3.4 Viscosity of the obtained aqueous solution of blank water dispersible granules

Formula	**Composition of granules (% w/w)**			**Viscosity (cP)**	
	PVP K-30	**Sodium alginate**	**Lactose**	**1:100 dilution**	**1:20 dilution**
5	5.0	5.0	90.0	3.20±0.14	5.52±0.00
6	5.0	10.0	85.0	3.84±0.00	7.44±0.00
Water	-	-	-	1.54±0.09	

Table 3.5 pH of the obtained aqueous solution of blank water dispersible granules

Formula	**Composition of granules (% w/w)**			**pH**	
	PVP K-30	**Sodium alginate**	**Lactose**	**1:100 dilution**	**1:20 dilution**
5	5.0	5.0	90.0	6.61±0.03	6.02±0.03
6	5.0	10.0	85.0	6.55±0.01	5.90±0.02
Water	-	-	-	7.26±0.02	

The rotenone content of 5% w/w is usually used in commercially available products (Marderosian, 1990). Therefore, derris water dispersible granules containing 5% w/w of rotenone were prepared. Unfortunately, the production of formulation yielded derris water dispersible granules with long disintegration time (11.5±0.1min) compared to blank granules (2.63±0.17 min) after 1: 20 dilution in water and stirred at 200 rpm. This problem may be due to the incorporation of derris extract in the formulation because dried plant extracts are quite often very hygroscopic powders. In addition, formulations containing a high amount of dried extract generally showed prolonged disintegration time; therefore the release of active ingredients is affected (De Souza et al., 2001; Eder and Mehnert, 1998; Onunkwo et al., 2004; Rocksloh et al., 1999; Soares et al., 2005).

In general, the addition of disintegrant to the formulation is usually necessary to achieve or improve the disintegration. Microcrystalline cellulose is one of the generally used disintegrant. It has also been used as filler in fast disintegrating tablets containing *Rhodiola rosea* extract to reduce the hygroscopic property of the extract and enhance disintegration (Kucinskaite et al., 2007). Microcrystalline cellulose (5% w/w) was therefore added in the formulation as another disintegrant. In addition, Tween 80 was used as wetting agent to help the mixing of derris extract in the preparation process.

The derris extract containing rotenone 36.13% w/w was subjected to prepare the water dispersible granules with the components as follow:

Derris extract	13.8%
PVP K-30	5.0%
Sodium alginate	5.0%
Avicel® PH101	5.0%
Tween 80	1.0%
Lactose	q.s. to 100%

Derris water dispersible granules containing 5% w/w of rotenone were successfully developed and yellow granules obtained as shown in Figure 3.9.

Figure 3.9 Appearance of derris water dispersible granules containing 5% w/w of rotenone

3.3.4 Physical properties of derris water dispersible granules

The physical properties of the derris water dispersible granules are shown in Table 3.6. The rotenone content in the granules was determined by validated HPLC method. The resulting granules contained 5.08±0.13 %w/w rotenone. The granule friability index was high (95.79±0.82) indicated that the granules were less friable when handling or transportation.

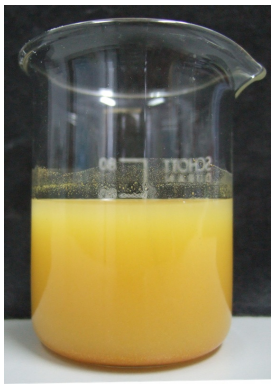


Figure 3.10 Appearance of aqueous suspension of derris water dispersible granules after 1:20 dilution

The granules completely disintegrated in water and a fine suspension obtained is shown in Figure 3.10. In fact, the type and concentration of disintegrant and the disintegration mechanisms can affect the disintegration behavior in different ways. The granules had a rapid

disintegration time of 6 min. This can be explained by the capillary action of the added microcrystalline cellulose. The viscosity of aqueous suspension was 11.04±0.07 cP. It was observed that the suspension did not cause blockage when sprayed with a hand-held sprayer. Furthermore, increasing the viscosity of suspension may result in increasing the adhesiveness of the formulation on plant foliage (Chumthong et al., 2008). The pH of aqueous suspensions was 6.87±0.15 which did not cause the damage of plant when spraying (A&L Canada laboratories, 2002).

Table 3.6 Physical properties of derris water dispersible granules

Disintegration time (min)*	5.67±0.41
pH*	6.87±0.15
Viscosity (cP)*	11.04±0.07
Friability index	95.79±0.82
Rotenone content (% w/w)	5.08±0.13

*Data refer to aqueous suspensions of granules (1:20 dilution; equivalent to 0.25% w/v rotenone).

3.3.5 Development of derris emulsifiable concentrate

Self-emulsification is a phenomenon, which has been exploited commercially for many years in formulations of emulsifiable concentrates of pesticide (Groves, 1978). Self emulsifying systems are mixtures of oils and surfactants, which are emulsified in aqueous media under conditions of gentle agitation. These formulations are used to produce concentrates of crop-sprays, which are diluted by the user, allowing very hydrophobic compounds to be transported efficiently (Pouton, 1997). The emulsifiable concentrate was chosen as a suitable formulation for derris extract owing to its insolubility in water. Furthermore, hydrolysis degradation of the active ingredient could be avoided due to the absence of water in the formulation.

The development of derris emulsifiable concentrate formulation starts with the search for a suitable solvent and the appropriate emulsifier system. The simplest products are those in which the active compounds are dissolved in oil which is commercially available, environmentally friendly, low cost, and non toxic to applicators. Soybean oil and mineral oil are commonly used in liquid agricultural products (Batta, 2003; Batta, 2004; Dezur and Pollard, 1996; Martin-Lopez et al., 2006; Wang et al., 2006). Soybean oil and mineral oil are safe food substances, classed as GRAS (generally regarded as safe) by regulatory agencies, and do not present a toxicological risk to formulators (Pouton, 2000). Therefore, soybean oil and mineral oil were used as an oil phase in this study. Tween 80 and Span 80 are nonionic surfactants, which were chosen as emulsifiers since they are generally regarded as non-toxic and non-irritant materials. They are also less affected by pH and water hardness, and help to avoid active ingredient-excipient incompatibles (Constatinides, 1995; Leyland, 1994; Neslihan Gursoy and Benita, 2004). The concentration of surfactant is usually greater than 25% in order to form stable self-emulsifying system (Neslihan Gursoy and Benita, 2004; Pouton, 1997). BHT was added as an antioxidant in the formulation since soybean oil contained high levels of polyunsaturated fatty acids which can cause oxidative degradation.

For emulsifiable concentrate formulations, the mixture should be a clear, monophasic liquid at ambient temperature and should have good solvent properties to allow presentation of the active compounds. From the result of Table 3.2, derris extract appeared to have higher solubility in soybean oil. The possible reason might be that soybean oil had higher polarity than mineral oil result in dissolving more isoflavone compound such as rotenone. Moreover, Tween 80 provided a higher solubility of derris extract than Span 80.

In order to find appropriate formulas, which provide clear homogeneous and stable emulsifiable concentrate, the suitable compositions of oil and emulsifiers were determined by mixing soybean oil or mineral oil with Tween 80 and Span 80 in different proportions. Four emulsifiable concentrate formulations (Figure 3.11A) which provided stable aqueous emulsions (Figure 3.11B) after 1:20 dilution are shown in Table 3.7. The viscosity and pH values of the obtained emulsions were in the range of 3.28–4.56 cP and 5.58-6.33, respectively (Table 3.7).

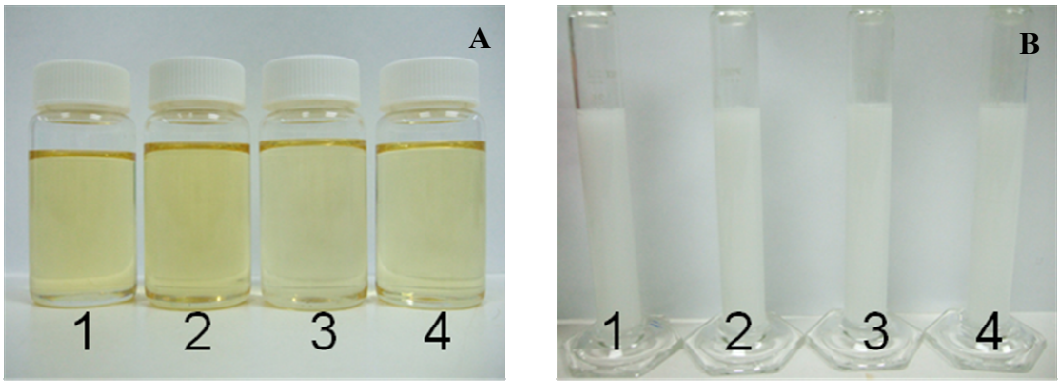


Figure 3.11 Appearance of four selected blank emulsifiable concentrates (A) and the obtained emulsions after 1:100 dilution with water (B)

Table 3.7 Emulsifiable concentrate formulations* and the physical properties of the obtained emulsion after 1:20 dilution with water

No.	Soybean oil (%)	Mineral oil (%)	Tween 80 (%)	Span 80 (%)	Viscosity (cP) (± SD)	pH (± SD)	Mean droplet sizes (µm) (± SD)
1	50	-	25	25	3.28±0.07	6.33±0.02	63.3±1.6
2	40	-	35	25	3.36±0.00	5.93±0.01	16.9±0.4
3	-	50	25	25	4.56±0.12	5.82±0.02	224±3
4	-	40	35	25	4.32±0.00	5.58±0.01	67.3±4.8

*These preliminary formulation trials do not contain derris extract.

These formulations were diluted with various volume of water in the range of 1% w/v to 3 and 5% w/v to evaluate their physical properties. Higher viscosity and lower pH values of the obtained emulsion were observed with increasing the percent of emulsifiable concentrate in water (Figure 3.12 and 3.13), which was similar to the result obtained from blank water dispersible granules.

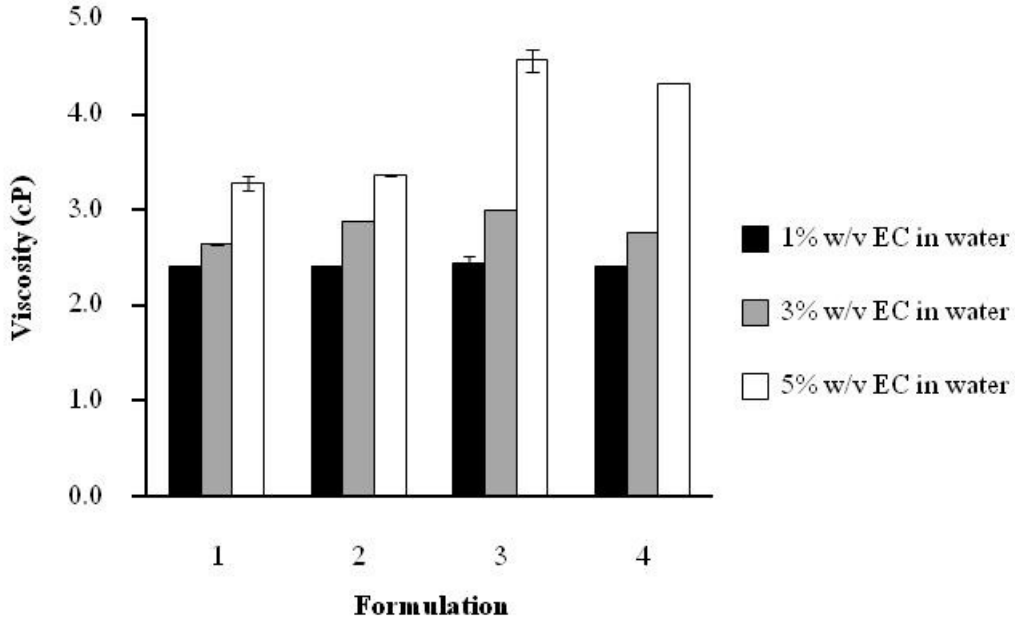


Figure 3.12 Viscosity of the obtained emulsions from emulsifiable concentrate

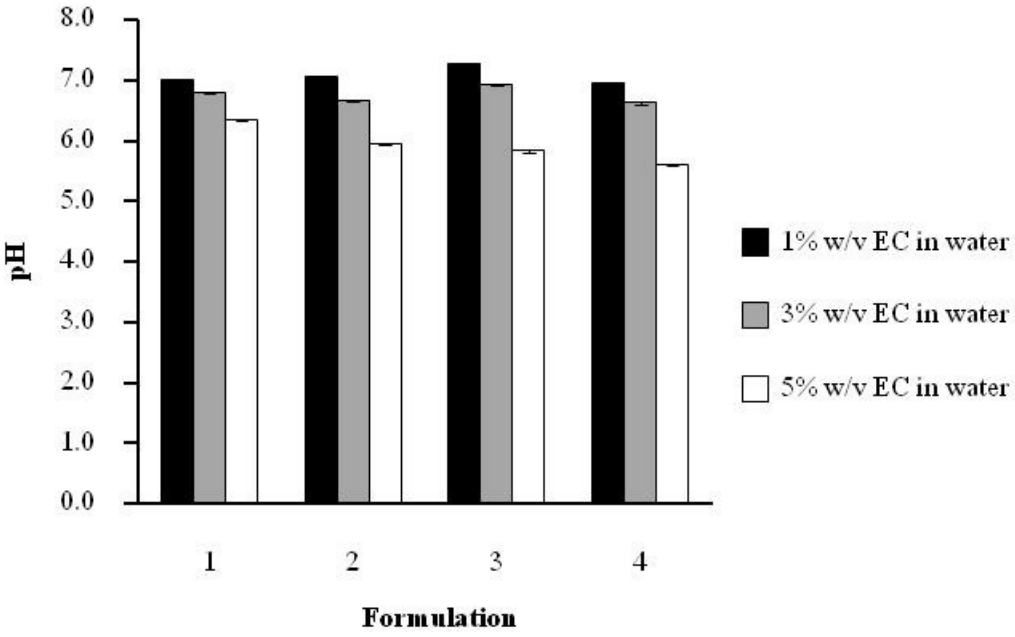


Figure 3.13 pH of the obtained emulsions from emulsifiable concentrate

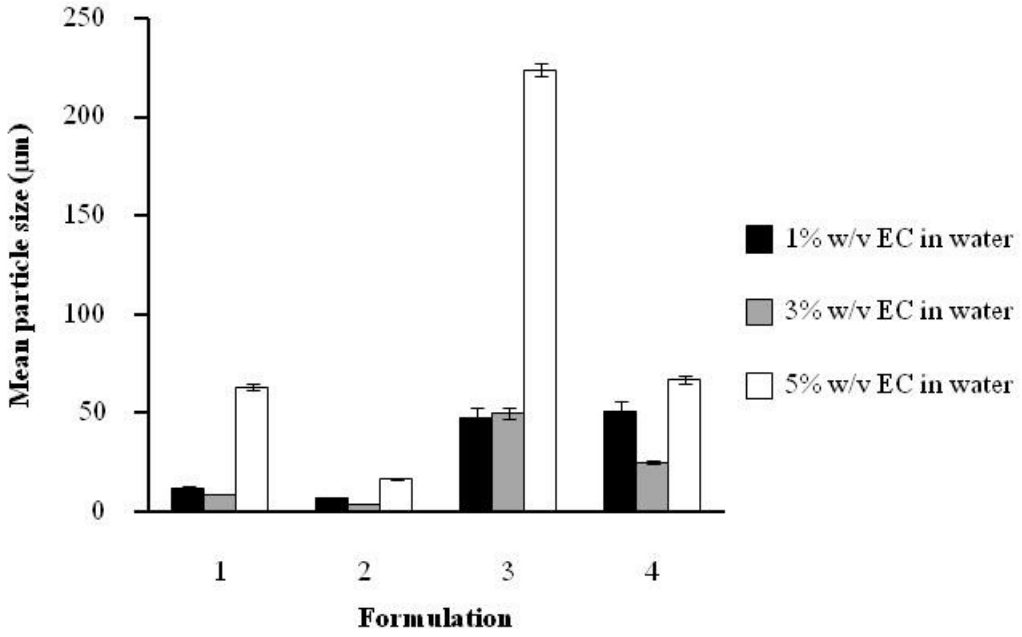


Figure 3.14 Mean droplet size of the obtained emulsions from emulsifiable concentrate

The results clearly showed that the mean droplet sizes of emulsions prepared from formulations containing soybean oil were smaller than those of formulations containing mineral oil (Table 3.7 and Figure 3.14). This may be due to the composition of soybean oil, which contains emulsifiers e.g. long-chain polyunsaturated fatty acids including, oleic acid, linoleic acid and linolenic acid (Kimura et al., 1994; Wang et al., 2006). The mean droplet sizes tended to increase with increasing the percent of emulsifiable concentrate to 5% w/v, while smaller mean droplet sizes of the emulsion obtained from the 3% w/v of emulsifiable concentrate in water (Figure 3.14).

In addition, there is a relationship between the droplet size and the concentration of the surfactant being used. Since hydrophilic surfactants with hydrophilic-lipophilic balance (HLB) > 10 are much superior at the providing fine, uniform emulsion droplets. Therefore, it seemed that the mean droplet sizes of emulsions also decreased with increasing amount of Tween 80 (Table 3.7). This could be explained by the stabilization of the oil droplets as a result of the localization of the surfactant molecules at the oil-water interface (Levy and Benita, 1990). A few studies have reported similar trends in droplet size with increases in surfactant concentration for

various self-emulsifying systems and explained that this phenomenon could be attributed to the ejection of oil droplets into the aqueous phase (Craig et al., 1995; Pouton, 1997; Quan et al., 2007).

It is known that droplet size in emulsions is one of the most important factors governing its stability; reduction of droplet size usually leads to formation of stable emulsions (Charman et al., 1992). The small droplet size may also improve overall efficacy by enhancing the spread of the formulation over the plant foliar surfaces. Therefore, formulation No. 2 (Table 3.7), which produced the smallest droplet size (16.9±0.4 μm) was selected for production of the derris emulsifiable concentrate. The suitable formula for preparation of emulsifiable concentrate containing derris extract consist of Tween 80, Span 80 and soybean oil in 35, 25, 40% w/w, respectively but some amount of soybean oil was replaced by derris extract. Although soybean oil has been higher priced than mineral oil but soybean oil is more readily available and lower in cost compared to many other vegetable oils. Additionally, the low volatility and spreadability of soybean oil may provide advantages over mineral oils (United Soybean Board, 2006).

The derris extract which contained rotenone in 36.13% w/w was subjected to prepare the emulsifiable concentrate with the components as follow:

Derris extract	13.8%
Tween 80	35%
Span 80	25%
BHT	0.1%
Soybean oil	q.s. to 100%

Dark brown viscous derris emulsifiable concentrate containing 5% w/w rotenone was successfully developed as shown in Figure 3.15A.

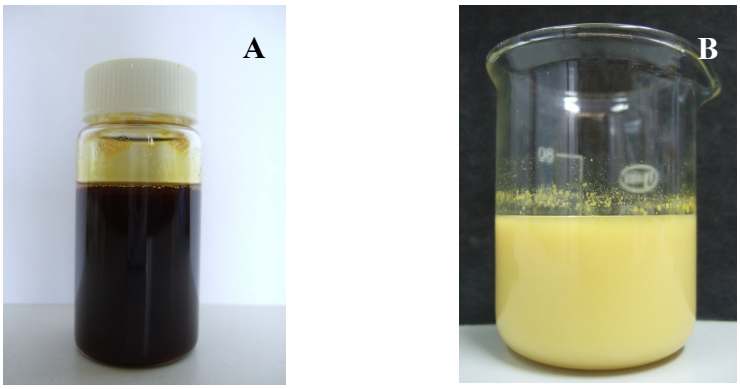


Figure 3.15 Appearance of derris emulsifiable concentrate containing 5% w/w of rotenone (A) and the obtained aqueous emulsion of derris emulsifiable concentrate after 1:20 dilution (B)

3.3.6 Physical properties of derris emulsifiable concentrate

The resulting emulsifiable concentrate contained 4.96±0.23 %w/w rotenone. The derris emulsifiable concentrate, when diluted with water (1:20), rapidly provided a stable yellow emulsion (0.25% w/v rotenone) (Figure 3.15B). Very little (< 0.1%) upward creaming layers occurred after 24 h, and any creaming that did occur could be redispersed again with gentle stirring. The physical properties of the derris emulsifiable concentrate compared to blank emulsifiable concentrate are shown in Table 3.8.

Table 3.8 Physical properties of aqueous emulsion prepared from 1:20 dilution of derris emulsifiable concentrate

Physical properties	**Blank emulsifiable concentrate**	**Derris emulsifiable concentrate**
Viscosity (cP)	3.36±0.00	4.28±0.07
pH	5.93±0.01	4.78±0.02
Mean droplet size (μm)	16.9±0.4	22.0±1.8

The viscosity of the aqueous emulsion (1:20 dilution) obtained from the emulsifiable concentrate (4.28±0.07 cP) was slightly higher than that from the corresponding aqueous control emulsifiable concentrate (3.36±0.00 cP). It was observed that the emulsion was easy to spray using portable or hand sprayers due to the low viscosity of the emulsion. The pH value of the aqueous emulsion obtained from the emulsifiable concentrate (4.78±0.02) was lower than that from the corresponding aqueous control emulsifiable concentrate (5.93±0.01). The chemical components of the extract might affect pH values of the obtained emulsion. However, this range of pH would not damage the plant because the optimal pH range of pesticide formulations for spraying to plant was 4-6 and the lower pH values gave benefit to the stability of the major compound which most labile in basic condition (A&L Canada laboratories, 2002). The formulations containing derris extract gave bigger mean droplet size of emulsion (22.0±1.8 μm) than that from control formulations (16.9±0.4 μm). The incorporation of compounds into emulsions generally increases the droplet size because of the additional drug inclusion into oil phase (Wang et al., 2006). The bigger mean inner phase diameter may be caused by the constituents in the crude extract, which consists of many components which might affect emulsion formation and droplet size therein.

3.3.7 Chemical stability of rotenone in derris extract and derris formulations

Stability is defined as the capacity of an active compound or product to remain within the established specifications to maintain its identity, quality, efficacy, safety, and purity throughout expiration dating period. Assuring adequate product stability remains one of the primary challenges in formulation development. A product, which is not of sufficient stability, can result in changes in physical and chemical characteristics.

Estimating the shelf-life of active compounds is a critical step in evaluating new formulations. The shelf-life of a product may be defined as the time that essential performance characteristics are maintained under specific handling conditions. Manufacturers' quality-assurance criteria typically require that a product must recover at least 90% of the initial value throughout its life.

3.3.7.1 Accelerated degradation of derris extract and derris formulations

Accelerated stability testing is often used in the development of active compounds to provide an early indication of product shelf-life and thereby shorten the development schedule. In accelerated stability testing, a product is stored at elevated stress conditions. Degradation at recommended storage conditions could be predicted based on the degradation at each stress condition and known relationships between the acceleration factor and the degradation rate. This information is then projected to predict product shelf-life or used to compare the relative stability of alternative formulations. Temperature is probably the most common acceleration factor used for chemicals, pharmaceuticals, and biological products since its relationship with the degradation rate is well characterized by the Arrhenius equation. This equation describes the relationship between storage temperature and degradation rate. Most accelerated testing models are based on the Arrhenius equation. The degradation rate depends on the conditions where the chemical reaction takes place. Products degrade faster when subjected to acceleration factors such as temperature, humidity, pH, and radiation (Anderson and Scott, 1991; Corradini and Peleg, 2007; Waterman and Adami, 2005).

The accelerated degradation kinetic studies of rotenone in the derris extract and were performed at three elevated temperature; 45, 60 and 70 °C (75% RH). At the end of sampling periods (1-168 days), the extent of rotenone degraded was determined and found to follow first-order kinetics in which the linearity was best met when the logarithm of the percentage remaining amount of rotenone from each temperature were plot against storage time, *t* (day) (Figure 3.16).

Integrated rate equation of first-order reaction:

$$\ln c = \ln c_0 - kt$$

$\ln c$ = logarithm of the percentage remaining amount of rotenone

$\ln c_0$ = logarithm of the percentage remaining amount of rotenone at $t = 0$

k = stability rate constant

The stability rate constant for rotenone in derris extract in each temperature was determined to be 0.0025, 0.0044 and 0.0063 day^{-1} for 45, 60 and 70 °C, respectively.

The degradation of rotenone in water dispersible granule and emulsifiable concentrate formulations also followed first-order kinetics. In case of water dispersible granule formulation, the stability rate constant (k) for each temperature was determined to be 0.0007, 0.0033 and 0.0061 day^{-1} for 45, 60 and 70 °C, respectively (Figure 3.17). In case of emulsifiable concentrate formulation, the stability rate constant (k) for each temperature was determined to be 0.0020, 0.0044 and 0.0061 day^{-1} for 45, 60 and 70 °C, respectively (Figure 3.18).

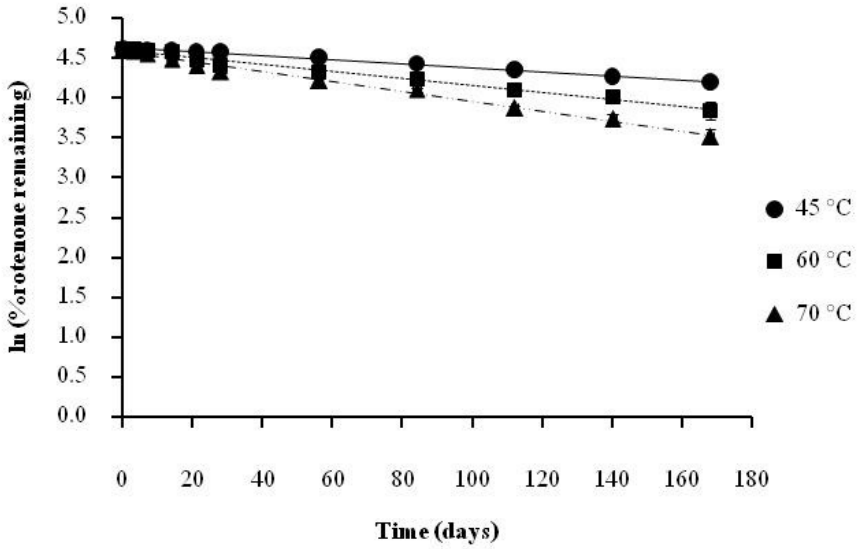


Figure 3.16 First-order plots for degradation of rotenone in derris extract at 45, 60, and 70 °C (75% RH). Rate equations for each temperature are expressed as $\ln c = 4.6203\text{-}0.0025t$, $r^2 = 0.9905$; $\ln c = 4.5969\text{-}0.0044t$, $r^2 = 0.9877$; and $\ln c = 4.5768\text{-}0.0063t$, $r^2 = 0.9912$, respectively.

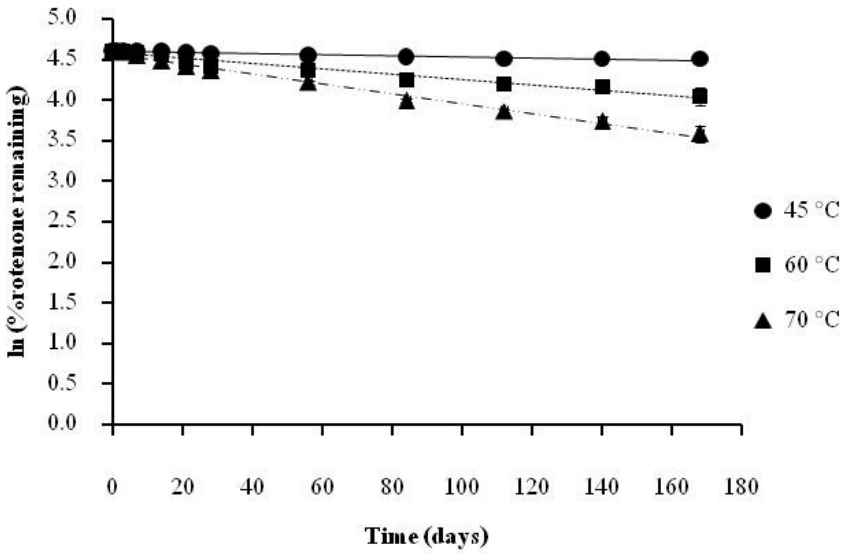


Figure 3.17 First-order plots for degradation of rotenone in water dispersible granules at 45, 60, and 70 °C (75% RH). Rate equations for each temperature are expressed as ln c = 4.5974-0.0007t, r^2 = 0.9218; ln c = 4.5808-0.0033t, r^2 = 0.9590; and ln c = 4.5702-0.0061t, r^2 = 0.9904, respectively.

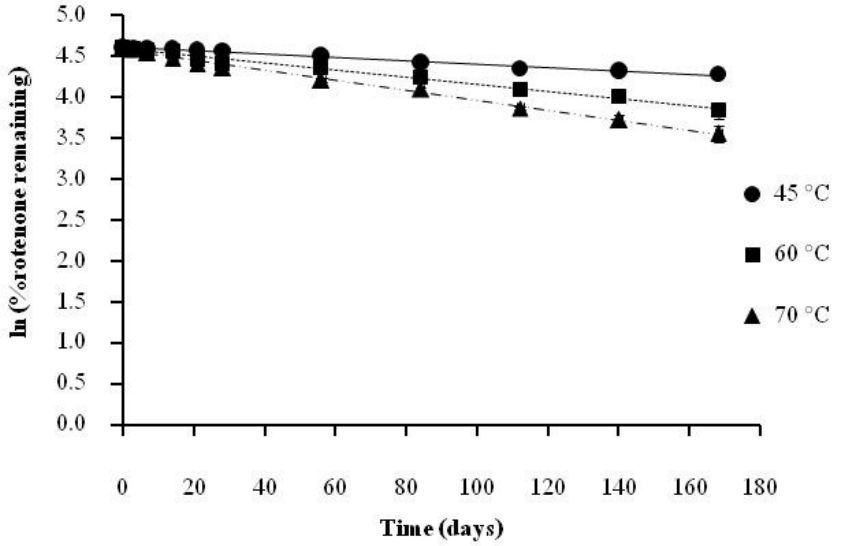


Figure 3.18 First-order plots for degradation of rotenone in emulsifiable concentrate at 45, 60, and 70 °C (75% RH). Rate equations for each temperature are expressed as ln c = 4.6111-0.0020t, r^2 = 0.9879; ln c = 4.6012-0.0044t, r^2 = 0.9904; and ln c = 4.5766-0.0061t, r^2 = 0.9937, respectively.

The stability rate constants for rotenone in derris extract, derris water dispersible granules and derris emulsifiable concentrate are shown in Tables 3.9, 3.10 and 3.11, respectively. Lower stability rate constants correlate to greater compound stability. Thus, rotenone was more stable in water dispersible granules than in emulsifiable concentrate and derris extract.

Table 3.9 Stability rate constants (*k*) for rotenone in derris extract

Storage temperature (°C), 75% RH	**ln c_0**	**Stability rate constant (day^{-1})**	**r^2**
45	4.6203	0.0025	0.9905
60	4.5969	0.0044	0.9877
70	4.5768	0.0063	0.9912

Table 3.10 Stability rate constants (*k*) for rotenone in derris water dispersible granules

Storage temperature (°C), 75% RH	**ln c_0**	**Stability rate constant (day^{-1})**	**r^2**
45	4.5974	0.0007	0.9218
60	4.5808	0.0033	0.9590
70	4.5702	0.0061	0.9904

Table 3.11 Stability rate constants (*k*) for rotenone in derris emulsifiable concentrate

Storage temperature (°C), 75% RH	**ln c_0**	**Stability rate constant (day^{-1})**	**r^2**
45	4.6111	0.0020	0.9879
60	4.6012	0.0044	0.9904
70	4.5766	0.0061	0.9937

Extrapolation of the Arrhenius plot (Figure 3.19) obtained from a relation between ln k and reciprocal of absolute temperature led to estimated k at 30 °C ($k_{30°C}$) of 1.3×10^{-3}, 0.17×10^{-3} and 0.95×10^{-3} day^{-1} for rotenone in derris extract, derris water dispersible granules and derris emulsifiable concentrate. The ln A and Ea calculated from the Arrhenius plots are shown in Table 3.12.

Logarithm form of Arrhenius equation:

$$\ln k = \ln A - \frac{E_a}{RT}$$

A = frequency factor

E_a = activation energy

R = gas constant

T = absolute temperature in Kelvins

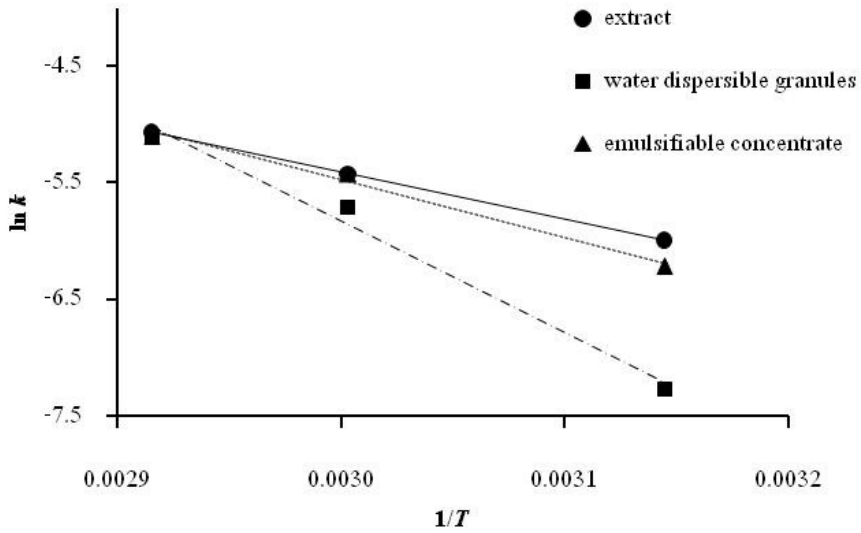


Figure 3.19 Arrhenius plots for degradation of rotenone in derris extract (●), water dispersible granule (■), and emulsifiable concentrate (▲). The Arrhenius relation for rotenone in derris extract can be expressed as ln k = 6.6754-(4028.5/T); r^2 = 0.9999. The Arrhenius relation for rotenone in water dispersible granules can be expressed as ln k = 22.943-(9589/T); r^2 = 0.9881. The Arrhenius relation for rotenone in emulsifiable concentrate can be expressed as ln k = 9.3204-(4932.2/T); r^2 = 0.9902.

Table 3.12 Frequency factor (*A*) and activation energy (*E*a) for rotenone calculated from Arrhenius equation

Sample	ln *A* (mol•day^{-1})	*E*a (kJ•mol^{-1})	r^2
Extract	6.675	33.5	0.9999
Water dispersible granules	22.94	79.7	0.9881
Emulsifiable concentrate	9.320	41.0	0.9902

The activation energy (*E*a), an independent variable in the equation, is equal to the energy barrier that must be exceeded for the degradation reaction to occur. The degradation rate depends on the activation energy for the chemical reaction and is product specific (Anderson and Scott, 1991). The order of *E*a value from the highest to the lowest was from water dispersible granules, emulsifiable concentrate and the extract, indicating that rotenone in the extract was more sensitive to start the degradation than the others.

Under the studied conditions, the predicted $t_{1/2}$ and $t_{90\%}$ at 30 °C of rotenone in extract, water dispersible granule and emulsifiable concentrate formulations are shown in Table 3.13. In case of extract, $t_{1/2}$ and $t_{90\%}$ were predicted to be 520 and 79 days, respectively. Both types of the formulations were able to clearly prolong the stability of rotenone, compared to stability in the unformulated crude extract. In this study, the stability of rotenone in solid granule formulation was compared with the liquid emulsifiable concentrate formulation, and the rotenone in granules ($t_{1/2}$ and $t_{90\%}$ were 4,176 and 633 days, respectively) showed much slower degradation rate than in emulsifiable concentrate formulation ($t_{1/2}$ and $t_{90\%}$ were 728 and 110 days, respectively). This is as expected, since compounds in solid form are usually more stable than in solutions or suspensions (Pouton, 1997).

The degradation of rotenone in derris extract and derris formulations may occur by oxidation and hydrolytic reactions (Mutsumura, 1975; Ò Brien, 1967). Surface exposure is important with degradation processes (Waterman and Adami, 2005). When derris extract is incorporate into water dispersible granules, the ingredients in the formulation are most likely able to protect rotenone from exposure to oxygen and humidity. Stability of rotenone was also improved in the derris emulsifiable concentrate because water was not used in the formulation,

and the rotenone in emulsifiable concentrates is therefore protected from exposure to humidity. In previous studies, water dispersible granule formulation has been shown to provide better stability of pyridostemin, the main component of *Stemona curtisii* extract, than in the crude extracts (Pureesatien et al., 2008). The results indicated that both types of developed formulations could prevent the degradation of rotenone during storage.

Table 3.13 Predicted $t_{1/2}$ and $t_{90\%}$ at 30 °C of derris extract and derris formulations

Sample	$t_{1/2}$ (Days)	$t_{90\%}$ (Days)
Extract	520	79
Water dispersible granules	4176	633
Emulsifiable concentrate	728	110

3.3.7.2 Real-time stability of derris extract and derris formulations

Although a product may be released based on accelerated stability data, but the real-time testing must be done in parallel to confirm the shelf-life prediction (Anderson and Scott, 1991; Aulton, 1998). In real-time stability testing, a product is stored at recommended storage conditions and monitored for a period of time. The duration of the test period should be long enough to allow significant product degradation.

In real-time stability studies, derris extract stored at room temperature (30±2 °C) and protected from light for 6 months still contained 66.7±1.4% of the starting amount of rotenone. The water dispersible granules were found to contain 83.1±2.8% rotenone, and the emulsifiable concentrates were found to contain 73.8±0.7% rotenone. These real-time values were lower than those predicted (by about 10-15%) when compared to estimated values (Table 3.14). The real-time stability data for the extract and the two formulations showed similar patterns to data from the accelerated stability. However, rotenone remaining (%) of derris water dispersible granules was lower than 90% of the initial amount after 6 months of real-time storage (Table 3.14), while the predicted shelf-life of derris water dispersible granules was longer than 6 months (21.1 months) (Table 3.13). Moreover, the results indicated that the stability of rotenone in derris

water dispersible granules and emulsifiable concentrate was less than 6 months of real-time storage (Table 3.14). However, rotenone in both types of the formulations was more stable than in the unformulated derris extract. Therefore, the real-time stability must be studied to confirm the half-life and shelf-life prediction of formulations.

Table 3.14 Predicted rotenone remaining (%) stored at 30 °C for 6 months and rotenone remaining (%) after 6 months of real-time storage at room temperature

Sample	Predicted rotenone remaining (%)	Rotenone remaining (%)
Extract	78.7	66.7 ± 1.4
Water dispersible granules	97.0	83.1 ± 2.8
Emulsifiable concentrate	84.3	73.8 ± 0.7

3.3.8 Stability of rotenone in derris formulations after spraying onto plants.

One of the advantages of natural pesticides is their rapid degradation in environment. In this study, the stability of rotenone on plants was monitored after spraying the formulations under controlled greenhouse conditions. The results showed that the degradation of rotenone from aqueous suspensions (equivalent to 0.25% w/v rotenone) of derris water dispersible granules was significantly slower than the degradation of rotenone from aqueous emulsions (equivalent to 0.25% w/v rotenone) of derris emulsifiable concentrate ($p < 0.05$) (Figure 3.20). Rotenone is decomposed by photooxidation due to the exposure to air and light (Cabras et al., 2002; Cheng et al., 1972). The presence of waxes on fruit and vegetable surfaces affects both the degradation rate and degradation pathway of rotenone. Tomato, nectarine, and plum waxes decreased the photodegradation rate of rotenone, whereas apple and pear waxes increased the photodegradation rate of rotenone compared to controls (Angioni et al., 2004).

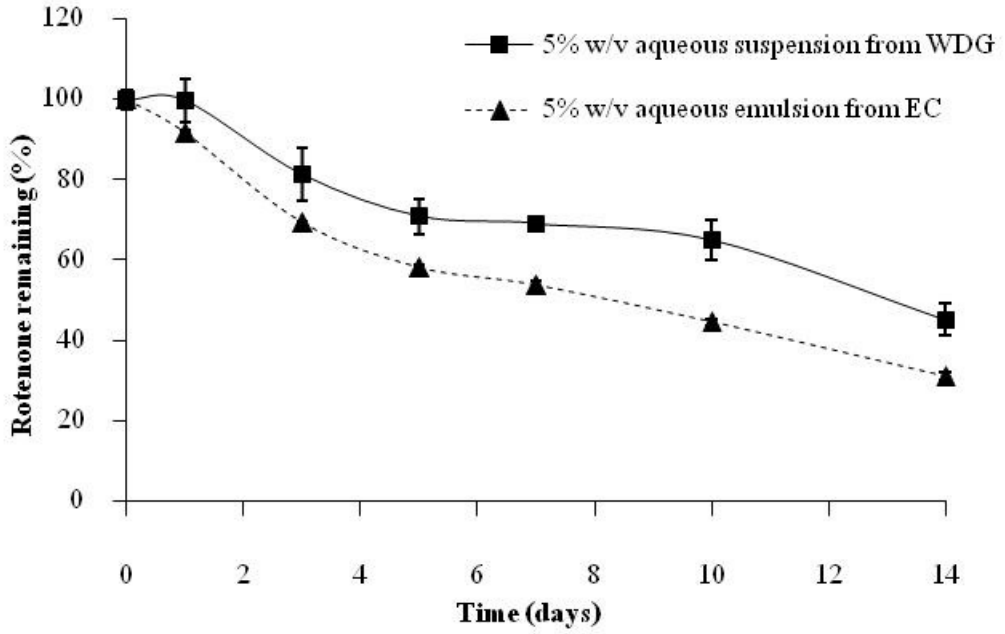


Figure 3.20 Stability of rotenone after spraying aqueous suspension of derris water dispersible granules (WDG) and emulsion of derris emulsifiable concentrate (EC) formulations on the foliage of Chinese kales. Both application sprays contained the equivalent of 0.25% w/v rotenone.

In this study, the solid particulate residues of the suspension of derris water dispersible granules on the foliage appear to protect rotenone from light and oxygen, when compared to the emulsion of derris emulsifiable concentrate. Moreover, the surfactants in the emulsion of derris emulsifiable concentrate may enhance the absorption of rotenone into the plant cuticle resulted in decreasing amount of rotenone on plant foliage. The compounds on leaf surface are easy to measure by solvent washing of the leaves, but that absorbed/transported into the leaves is not easy to assay by the HPLC methods utilized in this study, due to the large amount of endogenous components in leaf homogenates that interfere with the HPLC assay of rotenone.

As shown in Figure 3.20, at day 3, 81.3±6.5% and 69.1±0.5% of rotenone still remained on the foliage surface after spraying the suspension of the derris water dispersible granules and the emulsion of the derris emulsifiable concentrate, respectively, suggesting that the formulations would have the best efficacy up to 3 days after spraying. At day 10 and day 14, rotenone on the leaves after spraying the suspension of the derris water dispersible granules decreased to 65.0±4.9% and 45.3±3.9%, respectively. While rotenone on the leaves after 10 and

14 days of spraying the emulsion of the derris emulsifiable concentrate decreased to 44.5±0.9% and 31.1±0.9%, respectively. These results have implications for effective concentrations of rotenone and frequency of spraying, and suggest that repeated applications might be necessary when the concentration drops to 50% or below. However, if derris formulations were applied in the field, the percentage of rotenone remaining may be lower than the values from this study because rotenone could be rinse by water or rainfall. In addition, it was found that the formulations which spread on the leaves could be easily washed off by water. This may be due to consisting of water soluble ingredients in water dispersible granules and surfactants in emulsifiable concentrate. Furthermore, clearly guidelines have to be drawn on when the spraying has to stop prior to harvesting of the crop, since residues of rotenone have to be minimal in the harvested crop.

3.3.9 Preliminary efficacy testing of the two derris formulations

The efficacy of emulsions of the derris emulsifiable concentrate against 2nd instar larvae of *S. litura* was comparable to cypermethrin, while the emulsion of the control emulsifiable concentrate, as expected, did not cause death of *S. litura* in this test (Table 3.15). The suspensions of the derris water dispersible granules showed remarkably lower efficacy against the larvae of *S. litura* in the 3 day experiment, although the concentrations were equivalent to 0.25% w/v rotenone for both formulations. However, all the larvae that survived from the treatment with derris water dispersible granules exhibited abnormal growth. This may be due to the sub-lethal initial amount of rotenone on leaves dipped in aqueous suspensions of derris water dispersible granules (17.2±1.5 μg/cm^2) compared to that of rotenone on leaves dipped in aqueous emulsion of derris emulsifiable concentrate (23.9±2.5 μg/cm^2). The application of an aqueous suspension of derris water dispersible granules results in a 20% lower initial loading of rotenone, when compared to dipping into aqueous emulsions of derris emulsifiable concentrate. The initial load of an agrochemical on foliar surfaces may depend on a variety of factors, including adhesion properties, droplet size, velocity and angle of incidence of the applied spray, and plant cuticle and spray droplet interaction and extent of feeding (Baker et al., 1983; Stevens et al., 1992). Therefore, the total initial load of rotenone on foliage after application of rotenone formulations

(by dipping or spraying) may depend on a combination of amount physically adhered to the leaf cuticle surface, the amount of rotenone absorbed into the cuticle waxy layer, and the amount ingested.

Table 3.15 Efficacy of derris formulations (aqueous suspensions or emulsions, 0.25% w/v rotenone) against 2nd instar larvae of *S. litura*

Treatment	No. of deaths			Mortality	No. of survivals
	24 h	48 h	72 h	(%)	after 72 h
Derris WDG	1	0	0	2%	49*
Derris EC	48	0	1	98%	1*
Control WDG	0	0	0	0%	50
Control EC	0	0	0	0%	50
Water	0	0	0	0%	50
Cypermethrin (0.0175% w/v)**	49	1	0	100%	0

WDG: water dispersible granules

EC: emulsifiable concentrate

*The larvae that survived from treatment with derris formulations showed abnormal growth

**The concentration of cypermethrin was that commonly used for pesticidal activity

The most likely factors accounting for the higher efficacy of the emulsifiable concentrate formulation are those that affect the bioavailability of rotenone in *S. litura*. Rotenone is insoluble in water, whereas it is solubilized in the o/w emulsion foliar application. This could mean that rotenone from the o/w emulsion is transported into the leaves, whereas the insoluble rotenone in the aqueous suspensions of the water dispersible granules is not transported via the foliar cuticle. The actual bioavailability of rotenone after the application of the emulsifiable concentrate formulation might therefore be considerably higher than that for the granules, thus accounting for the lower efficacy of the latter. The remaining small particles of derris water dispersible granules on the leaves are less likely to be ingested than emulsifiable concentrates that

sometimes mix with leaf epicuticular waxes (Dent, 1991). The suspension of derris water dispersible granules may have an effect on the bioavailability of rotenone in the larvae after ingestion, may discourage feeding and account for the slow action of rotenone. In contrast, the emulsion of derris emulsifiable concentrate is in an oil soluble form that may lead to better bioavailability after leaf ingestion by the larvae; rotenone may also be absorbed through the skin of the larvae, and therefore rotenone could act effectively as both a stomach and a contact poison (Fukami and Nakajima, 1971; Mutsumura, 1975).

Additionally, the behavior of an insect affects the likelihood of contact with insecticides. *S. litura* moves 45 times faster when walking than when feeding (Salt and Ford, 1984) and this difference could account for the proportion of contacted deposit that is transferred to the insect (Ford and Salt, 1987). The feeding behavior of the insect may affect the uptake of a stomach poison applied to the food of the pest insect. The insecticide must be applied to a site on which the insect feeds, the coverage must be sufficient for a lethal dose to be ingested, and the deposit size should be small enough to be eaten by insect.

The efficacy of derris water dispersible granules may be improved by repeated application, or addition of synergists to increase the effectiveness of rotenone, or inclusion of components that might improve adhesion and initial load on the foliar surfaces. The higher mortality of 3rd instar larvae of the diamond back moth was observed by adding triphenyl phosphate (TPP) and piperonylebutoxide (PB) to derris extract (Visetson and Milne, 2001).

3.3.10 Efficacy testing of derris emulsifiable concentrate in greenhouse

From the preliminary efficacy testing of the two derris formulations, the emulsifiable concentrate exhibited higher efficacy againt *S. litura* than water dispersible granules. Hence, the emulsifiable concentrate was chosen for further efficacy study. The efficacy of derris emulsifiable concentrate formulation against *S. litura* was investigated under greenhouse conditions. The efficacy of emulsions of the derris emulsifiable concentrate against 2nd instar larvae of *S. litura* was lower than cypermethrin, while the emulsion of the control emulsifiable concentrate, as expected, did not cause death of *S. litura* (Table 3.16). It was found that half of the larvae stopped eating and moving at 24 h exposure of derris emulsifiable concentrate. Higher

percentage mortality of the larvae was observed with increasing the time of exposure for both derris emulsifiable concentrate and cypermethrin. Mortality was 100% for cypermethrin at 72 h post exposure, while only 30% mortality was found for derris emulsifiable concentrate treatment at 96 h post exposure. However, all the larvae that survived treatment with derris emulsifiable concentrate exhibited abnormal growth. The body of larvae was partly compressed because they were unable to slough off their old cuticle (Figure 3.21). In addition, the emulsion of the derris emulsifiable concentrate in greenhouse showed remarkably lower efficacy against the larvae of *S. litura* than those of laboratory condition, although the concentrations were equivalent to 0.25% w/v rotenone for both experiments. This could be explained by the less possibility in adhesion or spreading of the formulation on the foliage by spraying, resulting in lower efficacy than dipping method. In addition, the light and air in greenhouse condition caused the degradation of rotenone and some rotenone could be washed off by watering.

Table 3.16 Efficacy of derris emulsifiable concentrate (aqueous emulsion, 0.25% w/v rotenone) against 2nd instar larvae of *S. litura*

Treatment	No. of deaths				Mortality	No. of survivals
	24 h	48 h	72 h	96 h	(%)	after 96 h
Derris emulsifiable concentrate	1	3	10	15	30%	35*
Control emulsifiable concentrate	0	0	0	0	0%	50
Water	0	0	0	0	0%	50
Cypermethrin (0.0175% w/v)**	11	32	45	50	100%	0

*The larvae that survived from treatment with derris formulations showed abnormal growth

**The concentration of cypermethrin was that commonly used for pesticidal activity

Figure 3.21 Appearance of normal *S. litura* larvae (A) and abnormal *S. litura* larvae after exposure of derris formulations (B)

3.4 Conclusions

Ready-to-use stable formulations of derris extract in water dispersible granules and liquid emulsifiable concentrate using commercially feasible technology were developed. Both types of formulations were able to clearly prolong the shelf-life of rotenone during storage. The study of rotenone degradation on plants gives useful data for the consideration of use of effective concentrations for spraying and frequency of applications that might be necessary. The preliminary efficacy testing of the formulations under laboratory conditions demonstrated the higher efficacy of derris emulsifiable concentrate in controlling *S. litura*, compared to derris water dispersible granules. The efficacy of the emulsifiable concentrate in greenhouse test was lower than in laboratory conditions because of the possible degradation of rotenone. The efficacy of derris formulations may be improved by repeated application, or addition of synergists to increase the effectiveness of rotenone. However, the drawback of the derris formulations is that the stability of rotenone in real-time storage is no longer than 6 months. Hence, further studies need to be done for adjustment of the derris formulations that might increase the stability of rotenone.

CHAPTER 4

Controlled Release of Rotenone and *Derris elliptica* Extract based on Calcium Alginate Formulations

4.1 Introduction

In the last few decades, pesticides are used widely to protect plants from disease, weeds and insect damage in the agriculture and forestry. To ensure adequate pest control for a suitable period, pesticides are applied in concentrations greatly exceeding those required for control of the target organism. In addition, it is necessary to apply pesticides to the soil for controlling of soil pests because about 90% of all insects spend some or all of their life in the soil, thus increasing the likelihood of runoff or leaching and pollution of surface or ground water.

Controlled release technologies have emerged as an approach with promise to solve a diversity of problems that have in common the application of some active agents in agriculture. The advantages of controlled release formulations are longer duration of efficacy for rapidly degraded compounds, reduction in the amount of material required for pest control, decrease in the risk of environmental pollution, savings in manpower and energy by reducing the number of applications required in comparison to conventional formulations, increased safety for the farmer or pesticide applicator, and a general decrease in non-target effects. In addition, the use of controlled release formulations may reduce the losses to evaporation, co-distillation, and photolysis of pesticides (Guyot, 1994; Markus and Linder, 2006).

Many different types of controlled release system have been developed for use in agriculture including those that use a natural polymer like alginate as matrix for controlled release of pesticides. Alginate is a naturally occurring biopolymer extracted primarily from brown algae. It has a unique capacity to be used as a matrix for the entrapment and/or delivery of a variety of molecules or particles. Alginate is a linear unbranched polysaccharide composed of two monomeric units of 1,4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) varying in

composition and sequence depending of the alga origin (Figure 4.1) (Coviello et al., 2007; George and Abraham, 2006; Gombotz and Wee, 1998).

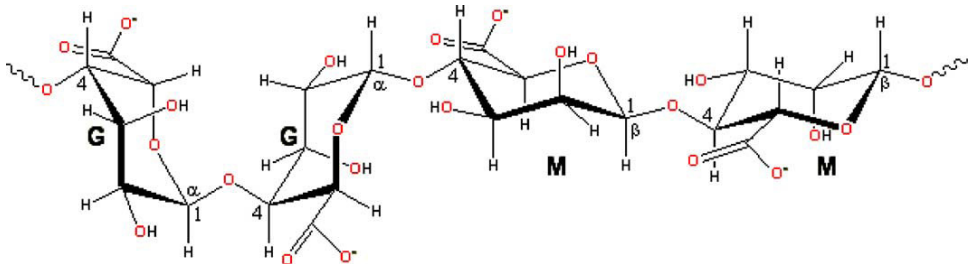


Figure 4.1 Chemical structure of alginate. G is Guluronic acid group and M is Mannuronic acid group. (from Rousseau et al., 2004)

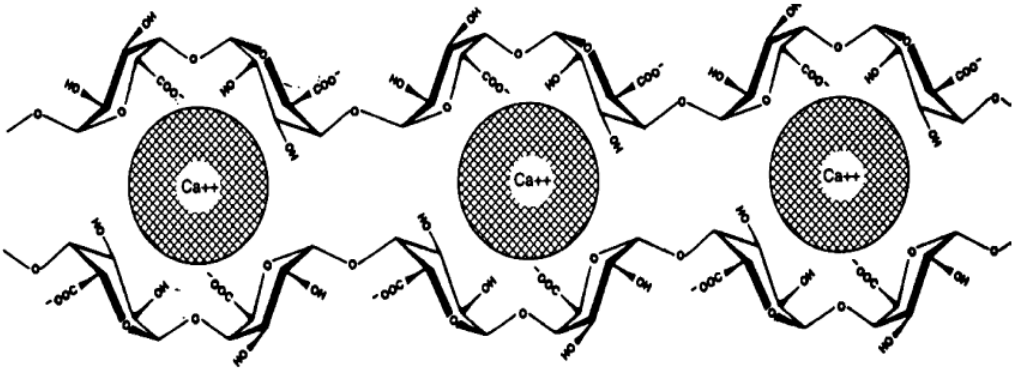


Figure 4.2 "Egg-box" model for alginate gelation with calcium ions (from Rousseau et al., 2004)

Alginate beads can be prepared by extruding a solution of sodium alginate as droplets into a divalent crosslinking solution such as Ca^{2+}, Sr^{2+}, or Ba^{2+}. Monovalent cations and Mg^{2+} ions do not induce gelation while Ba^{2+} and Sr^{2+} ions produce stronger alginate gel than Ca^{2+}. Other divalent cations such as Pb^{2+}, Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+} will also crosslink alginate gels but their use is limited due to their toxicity. The gelation and crosslinking of the polymers are mainly achieved by the exchange of sodium ions from the guluronic acids with the divalent cations (usually Ca^{2+}), and the stacking of these guluronic groups to form the characteristic egg-box structure shown in Figure 4.2. The divalent cations bind to the α-L-

guluronic acid blocks in a highly cooperative manner and the size of the cooperative unit is more than 20 monomers (George and Abraham, 2006; Gombotz and Wee, 1998).

In general, beads greater than 1.0 mm in diameter can be prepared by using a syringe with a needle or pipette. Sodium alginate solution is transferred dropwise into a gently agitated divalent crosslinking solution. The diameter of the beads formed is dependent on the size of needle used and the viscosity of the alginate solution. A larger diameter needle and higher viscosity solutions will produce larger diameter beads. The viscosity of sodium alginate can also influence the shape of the beads produced. The beads become more spherical as the concentration of the sodium alginate solution is increased. However, in general, sodium alginate solutions of greater than 5% w/v are difficult to prepare. The beads that are formed are allowed to be fully cured in the crosslinking solution for a short period of time, usually in minutes, before they are rinsed with distilled water (Gombotz and Wee, 1998).

Numerous reports have been published on the encapsulation and release agrochemicals from alginate matrices. These are summarized in Table 4.1.

Table 4.1 Summary of agrochemicals encapsulated in alginate beads

Agrochemicals	**References**
Alachlor	Gerstl et al., 1998; Pepperman and Kuan, 1995
Atrazine	Fernández-Pérez et al., 2001; Gerstl et al., 1998
Chlorpyrifos	Roy et al., 2009
1-Naphthalene acetic acid	Kenawy and Sakran, 1996
Neem seed oil	Kulkarni et al., 2000
Pentachlorophenol	Kenawy and Sakran, 1996
Thiram	Singh et al., 2009

Calcium alginate beads have been widely used in controlled drug delivery system due to their biocompatibility and biodegradability (George and Abraham, 2006). They have been also used as matrices for preparing controlled release formulations of pesticides. Calcium alginate beads can be produced by dropping a sodium alginate aqueous solution into a calcium chloride solution. However, the calcium alginate matrix formed is usually very permeable and little or no release can actually be controlled in the case of soluble compounds. Hence, a preferential use for alginate gel beads in the delivery of low solubility or macromolecular compounds has been suggested.

Rotenone and *Derris elliptica* extract have long been used to control insects on fruit trees and vegetable and also soil insects. In addition, it was found that petroleum ether extract from *D. elliptica* possessed high larvicidal activity against *Aedes aegypti* mosquitoes (Komalamisra et al., 2005). Therefore calcium alginate beads containing rotenone and derris extract were expected to develop and use for controlling soil insects in soil or controlling mosquito larvae in aquatic system. The calcium alginate beads were also expected to protect active agents from photodecomposition and minimize the inhalation hazard compared to dust formulation. In the present work, alginate was used as a matrix for preparation of the controlled release system. This formulation is based on readily available natural material and requires a simple preparation procedure. The swelling behavior, stability of rotenone in the beads under different pH and UV and release of rotenone in water have been investigated.

4.2 Experimental methods

4.2.1 Chemicals

HPLC grade acetonitrile and analytical grade methanol, 37% hydrochloric acid and sodium hydroxide were purchased from Labscan Asia (Bangkok, Thailand). Water was purified by passing it through a Milli-Q purification system. Standard rotenone (98% purity) was purchased from Sigma-Aldrich (Steinheim, Germany). The stock solution (0.1 mg/ml) was prepared by dissolving 10 mg of rotenone in 100 ml methanol. Working solutions were freshly prepared on the day of any study by suitable dilution of the stock solution with methanol. The

stock solution was kept in the dark at 4 °C and used within 1 month. Derris extract which contained 36.13% of rotenone was used in this study. Sodium alginate was purchased from Srichand United Dispensary (Bangkok, Thailand). Calcium chloride, potassium hydrogen phthalate (KHP), potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were purchased from Merck (Darmstadt, Germany).

4.2.2 Preparation of calcium alginate beads containing rotenone

Sodium alginate solution of 1, 2 and 3% w/v were prepared by dissolving sodium alginate in deionized water with gentle heating. Calcium chloride solution of 5 and 10 mM were also prepared by dissolving calcium chloride in deionized water. Rotenone-loaded calcium alginate beads were prepared by droplet extrusion of a sodium alginate aqueous suspension containing 0.5% w/v of rotenone. This suspension was prepared by mixing under magnetic stirring at 1200 rpm. Then it was added through a hypodermic syringe (2 mm diameter) or an 18-gauge needle (1 mm diameter) into calcium chloride solution with constant stirring at 250 rpm. The distance between the edge of syringe or needle and the surface of the calcium chloride solution was 10 cm. The resulting beads were allowed to cure for 15 min in calcium chloride solution under gentle magnetic stirring and then separated from the solution by filtration. The beads were washed with distilled water and placed in hot air oven at 40 °C until dry. Ten of dried beads of each formulation were then placed in 100 ml of water and swelling of the beads was investigated. The beads were stored in a glass container at room temperature (30±2 °C) and protected from light.

4.2.3 Preparation of calcium alginate beads containing derris extract

A suspension of 3% w/v sodium alginate solution containing 0.5% w/v of derris extract was prepared by mixing under magnetic stirring at 1200 rpm and it was then added dropwise into 5 mM calcium chrolide solution, using a hypodermic syringe (2 mm diameter) with a constant stirring at 250 rpm. The distance between the edge of syringe and the surface of the calcium chloride solution was 10 cm. The resulting beads were allowed to cure for 15 min in

calcium chloride solution under gentle magnetic stirring and then separated from the solution by filtration. The beads were washed with distilled water and placed in hot air oven at 40 °C until dry. The beads were stored in a glass container at room temperature (30±2 °C) and protected from light.

4.2.4 HPLC analysis

The analysis of rotenone in rotenone-loaded calcium alginate beads was performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) with a Restek® C18 5 µm column (250 × 4.6 mm). A linear gradient was used for the separation of rotenone with the initial mobile phase composition of acetonitrile/water 50:50 (% v/v) reaching 70:30 (% v/v) in 30 min. Before each injection, the HPLC column had to be stabilized for 10 min with the initial mobile phase composition of acetonitrile/water mobile phase 50:50 (%v/v). The injection volume was 20 µl and the flow rate was 1 ml/min. Detection was by UV spectroscopy at a wavelength of 294 nm.

4.2.5 Evaluation of calcium alginate beads containing rotenone or derris extract

4.2.5.1 Bead size measurement and swelling study

Ten samples of the completely dried beads from each formulation were selected and their sizes were measured using motic image stereo microscope (Olympus, Japan). Samples of dried beads (n=10) were placed in distilled water and they were taken at different intervals of time and swelling rate was determined by measuring the circumference of beads.

4.2.5.2 Rotenone content and entrapment efficiency

Approximately 5 mg of the beads sample was accurately weighed and placed in 2 ml distilled water until the beads swelled. Then the beads were crushed with methanol using mortar and pestle and quantitatively transferred to a 25 ml volumetric flask, and the volume made up to 25 ml with methanol and sonicated for 30 min. The methanolic sample was then centrifuged at 6,000 rpm for 15 min. The supernatant was used for further quantitative analysis of rotenone by HPLC as described above. The analyses were performed in triplicate, and calibration standards

were analysed on the same day as the samples. Entrapment efficiency was calculated from the following equation.

$$\text{Entrapment efficiency} = \frac{\text{rotenone loaded}}{\text{theoretical rotenone loading}} \times 100$$

4.2.5.3 Effect of pH on the stability of rotenone in alginate beads

The stability of rotenone in calcium alginate beads was carried out in 3 phosphate buffer solutions (pH 3, 7 and 10) compared to standard rotenone. Samples of each (5 mg) were placed in a glass vial and 2 ml of buffer solution was added. The beads were sampled at 0, 4, 8, 12 and 24 h. The samples were crushed with methanol using mortar and pestle and quantitatively transferred to a 25 ml volumetric flask, and the volume made up to 25 ml with methanol and sonicated for 30 min. The methanolic sample was then centrifuged at 6,000 rpm for 15 min. The supernatant was assayed for rotenone content by HPLC as described above. The analyses were performed in triplicate, and calibration standards were analysed on the same day as the samples.

4.2.5.4 Effect of UV on the stability of rotenone in alginate beads

Samples of each (5 mg) were placed in a glass vial and 2 ml of distilled water was added. Then the beads were exposed to UV light in UV cabinet (20-watt general electric UV lamp in a distance of 30 cm) at room temperature (30±2 °C) compared to standard rotenone. The beads were taken at 0, 3, 6, 12 and 24 h. The samples were crushed with methanol using mortar and pestle and quantitatively transferred to a 25 ml volumetric flask, and the volume made up to 25 ml with methanol and sonicated for 30 min. The methanolic sample was then centrifuged at 6,000 rpm for 15 min. The supernatant was assayed for rotenone content by HPLC as described above. The analyses were performed in triplicate, and calibration standards were analysed on the same day as the samples.

4.2.6 Release study of rotenone from calcium alginate beads

Calcium alginate beads containing rotenone or derris extract (50 mg) (n=3) was accurately weighed and placed into a beaker containing 200 ml of water (pH 5.5) under magnetic stirring (50 rpm) at room temperature (30±2 °C). Aliquots of 5 ml were withdrawn at 0, 2, 4, 8, 12 h and 1, 2, 3, 5 and 7 days and replaced with 5 ml of distilled water after each withdrawal to maintain sink condition. Samples were transferred to a 25 ml volumetric flask, and the volume made up to 25 ml with methanol and sonicated for 30 min. The methanolic sample was then centrifuged at 6,000 rpm for 15 min. The supernatant was assayed for rotenone content by HPLC as described above. The analyses were performed in triplicate, and calibration standards were analysed on the same day as the samples.

4.3 Results and discussion

In the present work, rotenone-loaded calcium alginate beads were prepared with various proportions and sizes. From Table 4.2, the spherical gel beads could not be produced at low concentration of sodium alginate and calcium chloride (formula 1). More spherical beads were obtained at higher concentration of the sodium alginate solution (Gombotz and Wee, 1998; Sankalia *et al*., 2005). The dried beads obtained from low concentration of sodium alginate and high concentration of calcium chloride (formula 2) could not swell in water because high proportion of calcium chloride leads to the formation of a thicker membrane that acted as a barrier for water absorption (Bladino *et al.*, 1999). Therefore, the formula 3-8 were selected for further evaluation.

4.3.1 Rotenone content and entrapment efficiency

The appearance of rotenone-loaded calcium alginate beads is shown in Figure 4.3A and B. From Table 4.3, the rotenone content of beads had range from 13.21 to 19.18% and the entrapment efficiency was varied from 57.79 to 87.52%. It was found that the high entrapment efficiency was obtained at the high concentration of sodium alginate, especially when it was

formed with the low concentration of calcium chloride. Increase in viscosity with an increase in sodium alginate concentration retarded penetration of calcium to the interior of the beads, resulted in decreased crosslinking, and increased entrapment efficiency (Jaiswal *et al.*, 2009; Sankalia *et al.*, 2005). In addition, using the smaller diameter of needle in the preparation process resulted in decrease of entrapment efficiency. The highest entrapment efficiency was achieved from formula 7, which contained 3% of sodium alginate and 5 mM of calcium chloride using 2 mm diameter of syringe. Therefore, calcium alginate beads containing derris extract were prepared by this condition. The rotenone content of the derris beads was 5.12% and the entrapment efficiency was 74.53% and the appearance of the beads is shown in Figure 4.4A and B.

Table 4.2 Concentration of sodium alginate and calcium chloride and characteristics of the beads

Formula	**Sodium alginate (% w/v)**	**Calcium chloride (mM)**	**Syringe/ needle diameter (mm)**	**Spherical shape**	**Swelling**
1	1	5	2	-	Yes
2	1	10	2	+	No
3	2	5	1	++	Yes
4	2	5	2	++	Yes
5	2	10	2	+++	Yes
6	3	5	1	+++	Yes
7	3	5	2	+++	Yes
8	3	10	2	+++	Yes

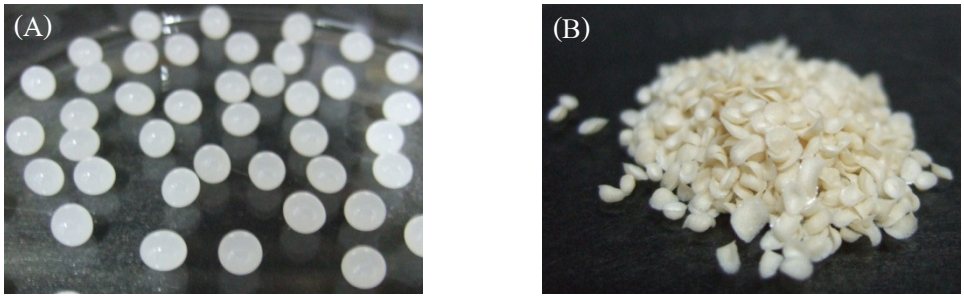


Figure 4.3 Appearance of rotenone-loaded calcium alginate beads (formula 7); wet beads (A) and dry beads (B)

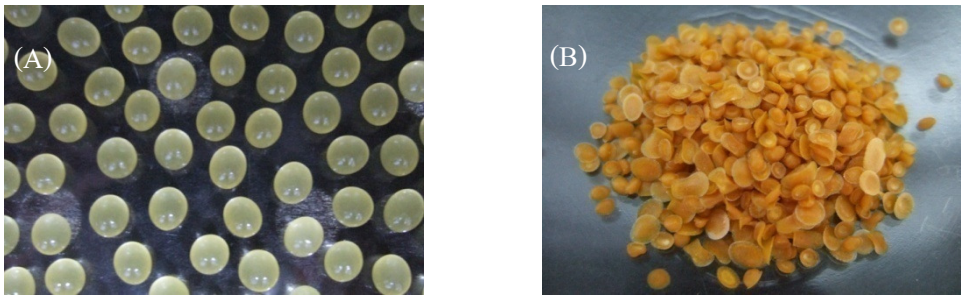


Figure 4.4 Appearance of calcium alginate beads containing derris extract; wet beads (A) and dry beads (B)

Table 4.3 Characteristics of the calcium alginate beads containing rotenone or derris extract

Formula	Sodium alginate (% w/v)	Calcium chloride (mM)	Syringe/ needle diameter (mm)	Bead size in circumference (mm)	Rotenone content (%)	Entrapment efficiency (%)
3	2	5	1	6.00±0.41	15.58±0.84	57.79±0.30
4	2	5	2	8.89±1.14	18.14±0.72	67.08±1.28
5	2	10	2	6.81±0.51	19.18±1.33	79.08±2.01
6	3	5	1	6.28±0.71	13.21±0.75	70.82±0.54
7	3	5	2	7.04±1.04	16.17±0.87	87.52±0.97
8	3	10	2	5.92±0.33	13.68±1.28	73.30±1.55
Derris	3	5	2	7.26±0.78	5.12±0.34	74.53±1.66

4.3.2 Morphology of the beads and swelling study

The size of beads is dependent on size of a droplet of sodium alginate, which is influenced by diameter of syringe or needle and viscosity of sodium alginate solution (Smrdel et al., 2008). Wet beads of rotenone-loaded calcium alginate were obtained as shown in Figure 4-3A. The spherical shape of beads in the wet state was usually lost after drying, especially for beads prepared with low sodium alginate concentration (Sankalia et al., 2005). During drying, the beads shrank significantly and their shape changed. The shape of beads changed to a spherical disk with a collapsed center (Figure 4.3B). It can be explained that calcium alginate beads usually have a heterogeneous structure with a dense surface layer and a loose core because of the heterogeneous gelation mechanism, which resulted in the collapse of beads during the drying process (Skjak-Brae et al., 1989). Furthermore, increased viscosity at a higher concentration of sodium alginate and increasing the concentration of calcium chloride resulted in larger size of beads (Table 4-3). This is in accordance with the results of Østberg et al. (1993), Sankalia et al. (2005) and Sriamornsak and Nunthanid (1999).

Swelling of the dry beads is mainly attributed to the hydration of the hydrophilic groups of alginate. Free water penetrates inside the beads in order to fill the inert pores among the polymer chains, contributing to a greater swelling degree (Hoffman, 2002; Pasparakis and Bouropoulos, 2006). The proportion of calcium chloride and sodium alginate has significant effect on swelling of beads. Calcium alginate beads containing derris extract showed the swelling pattern as same as the rotenone-loaded calcium alginate beads. As the ratio of calcium chloride to sodium alginate increased, the swelling of beads decreased (Figure 4.5). This result may be due to high proportion of calcium chloride that yielded the beads with a thicker membrane, which consequently inhibits penetration of water molecules into the bead network and cause slower swelling (Bladino et al., 1999; Roy et al., 2009). Moreover, the smaller size of the beads exhibited slower swelling because the small beads have less surface area to contact with water molecules.

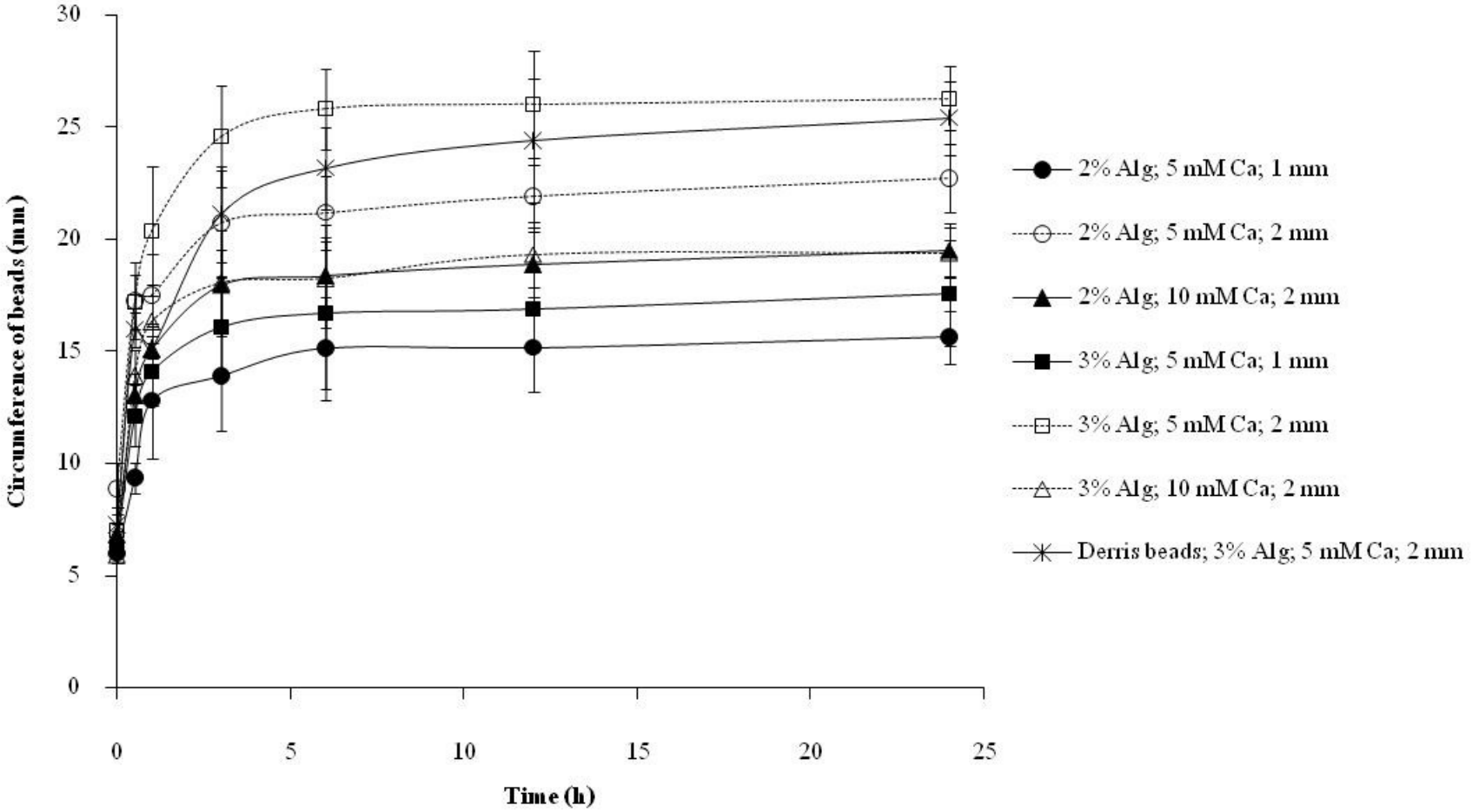


Figure 4.5 Swelling of calcium alginate beads containing rotenone and derris extract in water

4.3.3 Effect of pH on the stability of rotenone in alginate beads

The effects of different pH (3, 7 and 10) on the stability of rotenone in rotenone-loaded calcium alginate beads are given in Figure 4.6, 4.7 and 4.8. All formulations of calcium alginate beads slightly enabled to protect rotenone from the degradation caused by acid and base hydrolysis. However, after 24 h of experiment, over 80% of rotenone in calcium alginate beads and standard rotenone still remained in phosphate buffer solution pH 3. In contrast, the beads swelled and eroded in phosphate buffer solution pH 7 and 10. The amount of rotenone remaining in buffer solution pH 7 and 10 decreased to 35-40%. This could be owing to more stable alginate gel structure and more stable rotenone at acidic pH (Acartürk and Takka, 1999, Bajpai and Sharma, 2004). In addition, under acidic condition, alginate beads do not swell or erode, due mainly to alginic acid precipitation. On the other hand, under neutral and alkali conditions, the beads tend to swell and erode, influencing active agent release (Ferreira Almeida and Almeida, 2004; Yotsuyanagi et al., 1987). These results indicated that rotenone-loaded calcium alginate beads may be more suitable for application in acid soil.

4.3.4 Effect of UV on the stability of rotenone in alginate beads

The effect of UV on the stability of rotenone in rotenone-loaded calcium alginate beads in water is given in Figure 4.9. It was found that over 80% of rotenone in calcium alginate beads in water still remained after 24 h of experiment. In contrast, the amount of standard rotenone decreased to 50%. The rotenone in all formulations of calcium alginate beads was significantly more stable than standard rotenone. Therefore, calcium alginate beads enabled to protect rotenone from the photodecomposition. Consequently, these formulations could be applied with better efficiency under field conditions.

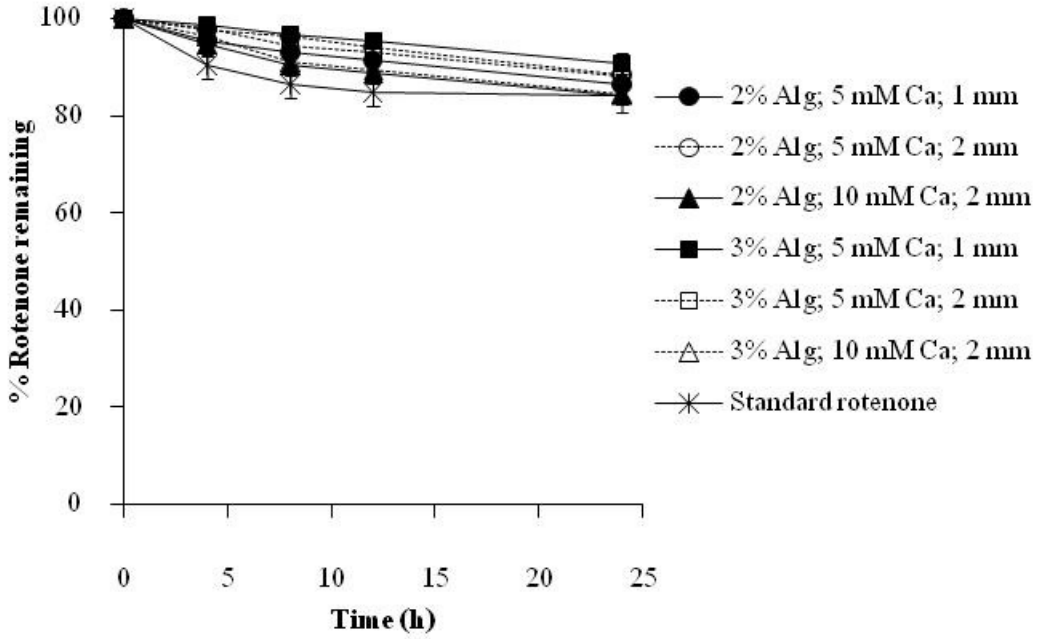


Figure 4.6 Stability of rotenone-loaded calcium alginate beads in phosphate buffer solution pH 3 compared to standard rotenone

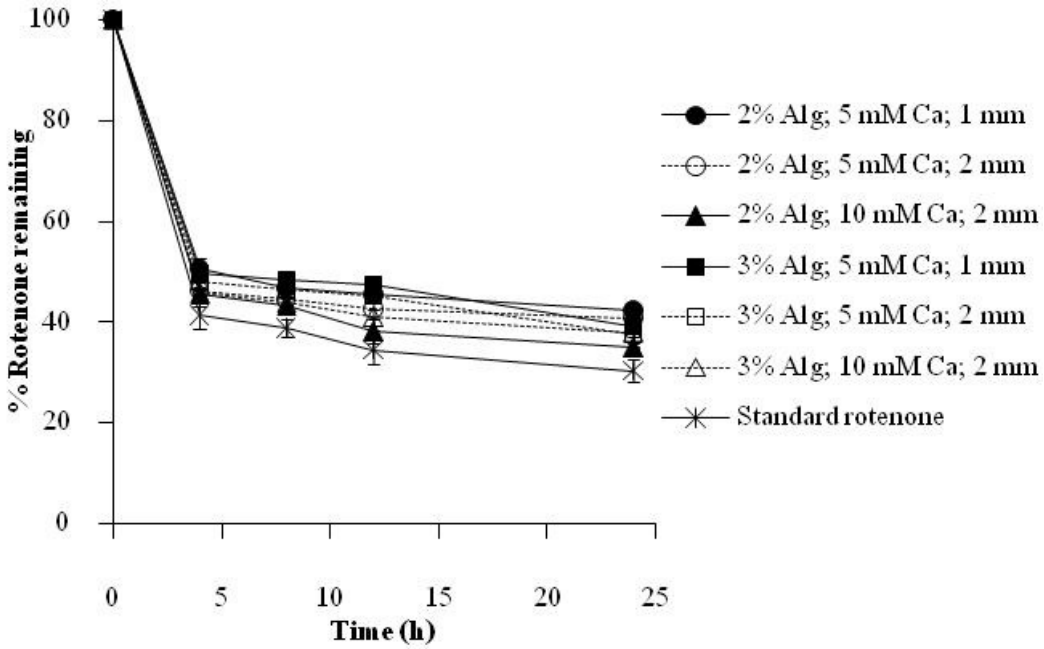


Figure 4.7 Stability of rotenone-loaded calcium alginate beads in phosphate buffer solution pH 7 compared to standard rotenone

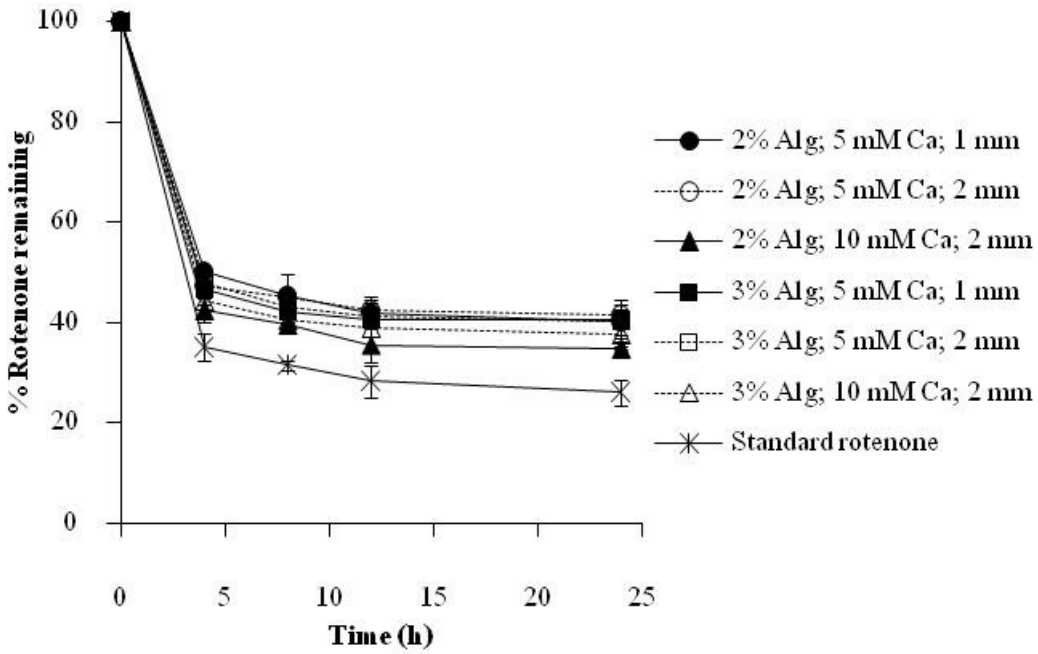


Figure 4.8 Stability of rotenone-loaded calcium alginate beads in phosphate buffer solution pH 10 compared to standard rotenone

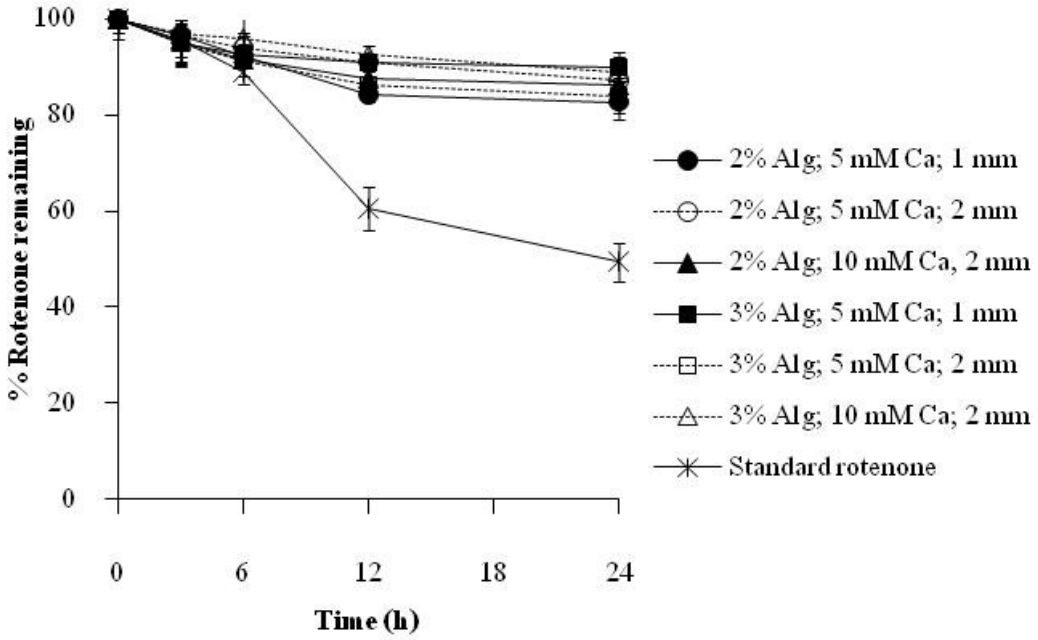


Figure 4.9 Stability of rotenone-loaded calcium alginate beads when expose to UV light compared to standard rotenone

4.3.5 Release study of rotenone from calcium alginate beads

The release of active agent from alginate matrices is usually modulated by a swelling-dissolution-erosion process (Tønnesen and Karlsen, 2002). In this study, rotenone release pattern was affected by the proportion of calcium chloride and sodium alginate. The results indicate that the rotenone release slows down with increasing sodium alginate concentration or decreasing the ratio of calcium chloride to sodium alginate (Figure 4.10). It can be explained that greater the amount of polymer, thicker the layer of polymer was formed around the rotenone and more effectively the polymer would hold the rotenone with itself (Jaiswal *et al.*, 2009). In addition, swelling behavior of beads (Figure 4.5) indicates the speed and easiness of water to penetrate the alginate matrix, as a necessary step for rotenone release. The faster swollen formulations resulted in a faster rotenone release. The smaller size of the beads also exhibited slower rate of rotenone release (Figure 4.10). The formulation which contained 3% of sodium alginate and 5 mM of calcium chloride using 2 mm diameter of syringe gave the fastest release of rotenone, more than 90% rotenone was released after 12 h. While the same formulation with smaller size, rotenone was released with a much slower release, only 29% rotenone was released after 12 h and reached to 100% after 168 h (7 days). The pH of distilled water used in this study was 5.5. The alginate bead structure and rotenone was quite stable in this condition. Since the swelling behavior and stability of calcium alginate depend on water uptake and pH, thus the humidity and pH of soil affect the release of compounds from calcium alginate beads.

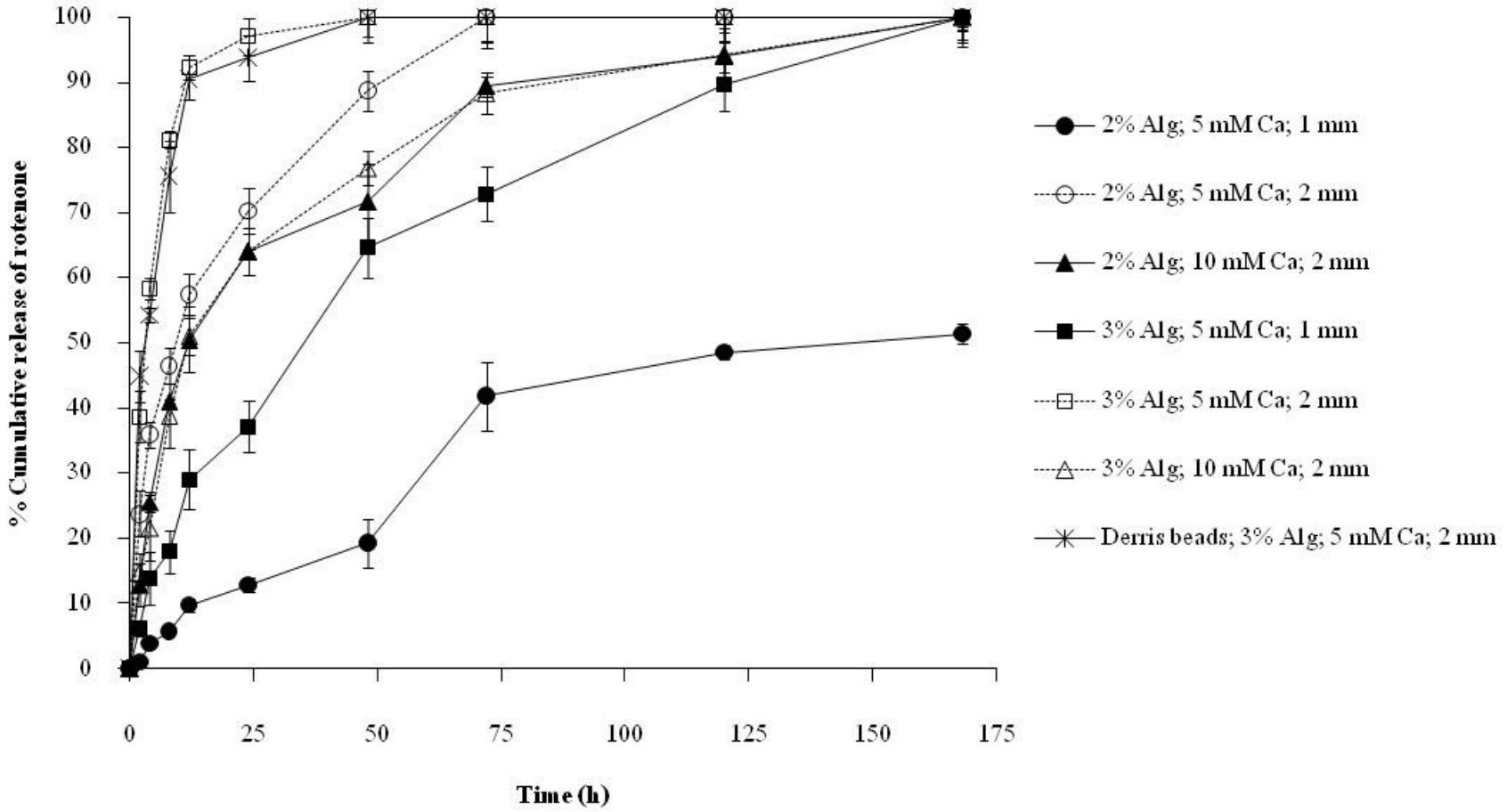


Figure 4.10 Release of rotenone from calcium alginate beads containing rotenone and derris extract in water pH 5.5

4.4 Conclusions

Several properties of alginate, like its biodegradability and ability to form gel with a variety of crosslinking agents in mild and aqueous conditions, make it a potential carrier for controlled delivery of biologically active agents. Calcium alginate gel beads containing rotenone or derris extract were prepared by ionotropic gelation. Morphology of the beads, entrapment efficiency and swelling behavior are affected by varying the proportion of sodium alginate and calcium chloride and diameter of syringe or needle used in preparation process. Rotenone-loaded calcium alginate beads were found to be more stable in acidic condition. All formulations of calcium alginate could protect rotenone from photodecomposition. The rotenone release of the beads is mainly controlled by the swelling of the polymer network. Some formulations with small size of calcium alginate beads could delay rotenone release. They might be applied together with fast release formulation to achieve longer duration of efficacy for pest control.

CHAPTER 5

CONCLUSIONS

5.1 Summary of the results of this thesis

Botanical insecticides have increasingly been used as attractive alternatives to synthetic chemical insecticides for pest management because they pose a little threat to human health and environment. The use of *Derris elliptica* extracts are well established due to the insecticidal activity of rotenone. However, commercial products of rotenone are mostly available in the United States and Europe while derris extracts are commonly used by agriculturists in China and Southeast Asian countries at the local level. Thai farmers usually macerate the derris roots in water and use the resulting milky suspension for spraying their crops. Therefore, effective, stable and standardized formulations of derris extracts are required for more convenient application.

5.1.1 Extraction of *Derris elliptica*

Extraction is the first step in the isolation of valuable natural compounds from plant materials. Traditional extraction methods are very time consuming and require large quantities of organic solvents. In an attempt to overcome these limitations, pressurized liquid extraction (PLE), which is an extraction technique that combines elevated temperature and pressure with liquid solvents was carried out as a possible alternative to conventional maceration for the fast and efficient extraction of rotenone from derris plant materials.

The extraction of rotenone from the stems and roots *D. elliptica and D. malaccensis* by PLE and maceration were compared. Chloroform was determined to be a good solvent for extraction of rotenone compared to 95% ethanol. The PLE extraction process was optimized by varying the key extraction factors of temperature (5□-7□°C) and pressure (1□□□-2□□□psi).

Extraction temperature is an important experimental factor because elevated temperature could lead to significant improvements in the capacity of extraction solvents to dissolve the analytes, in the rates of mass transport, and in the effectiveness of sample wetting and matrix penetration, all of which lead to overall improvement in the extraction and desorption of analytes from the surface and active sites of solid sample matrices. In addition, elevated pressure is needed to maintain the extraction solvents as liquids at high temperatures (usually above their boiling points). However, thermal degradation of the compounds can be a problem under PLE condition (Carabias-Martinez et al., 2 5; Huie, 2 2).

The results showed that the degradation of rotenone occurred when using temperatures higher than 5 °C and the pressure did not significantly affect to the extraction efficiency. PLE using chloroform as extraction solvent at 5 °C and 2 psi was found to be an optimal condition and gave higher extraction efficiency (rotenone content 46.1 % w/w) compared to conventional maceration (rotenone content 4 .6 % w/w). The order of rotenone content found in crude extract obtained by optimized method from the highest to the lowest were root (46.1 % w/w) and stem (9.4 % w/w) of *D. elliptica* and stem of *D. malaccensis* (5.2 % w/w), respectively. Moreover, the results from this study indicated that PLE was considerably less time and solvent consuming (3 min, 3 ml/g of dried sample) than the conventional maceration techniques (72 h, 1 ml/g of dried sample).

In summary, selection of an appropriate extraction method entails consideration of not only the recovery but also the cost, time of extraction, and the volume of solvent used. PLE showed high potential for extraction of natural compounds from plant materials. Reduces solvent consumption and shorter extraction time have been emphasized as a major advantage of PLE. The possibility of automation and coupling PLE with other steps in the analytical process is one of the most interesting aspects of this methodology. However, PLE are not always available in the average laboratory due to the high cost of equipment.

5.1.2 Development of validated stability-indicating HPLC assay method

In this study, a validated stability-indicating HPLC assay method which could separate rotenone and its degradation products formed under a variety of stress conditions (acid hydrolysis, base hydrolysis and oxidation) was successfully developed. Degradation of rotenone was found to occur in acid and alkali mediums and under oxidative stress. Separation of rotenone and the degradation products was achieved on a C18 column using a mobile phase composed of acetonitrile-water with an initial concentration of 5□:5□(%v/v) and a final concentration of 7□:3□(%v/v) in 3□min. The flow rate was 1 ml/min and detection wavelength was 294 nm. The developed method was validated with respect to specificity, selectivity, linearity, precision and accuracy. It was utilized for analysis of rotenone and degradation products in stability samples from crude extract and formulations.

5.1.3 Formulation development of *Derris elliptica* extract (water dispersible granules and emulsifiable concentrate)

Derris extracts containing rotenone have long been used as natural insecticides, but time-consuming preparation processes and the short shelf-life of the extract limit their use in pest control. In this study, ready-to-use stable water dispersible granules and emulsifiable concentrate liquids containing derris extract (equivalent to 5% w/w of rotenone) were developed with simple techniques. Non-toxic excipients were used to obtain safety and environmentally friendly formulations. The compositions of water dispersible granule and emulsifiable concentrate were optimized to obtain suitable formulations which gave proper physicochemical properties for application. These developed formulations containing derris extract could be diluted with water before application and should be occasionally shaken during application.

The developed derris water dispersible granules provided short disintegration time and low friability. The suspension obtained from this formulation also had proper viscosity and pH for spraying. In addition, water dispersible granules offer significant advantages in packaging, ease of handling and safety. The developed derris emulsifiable concentrate also provided an emulsion which had proper particle size, viscosity and pH for spraying. Emulsifiable

concentrate was suitable to formulation of derris extract because hydrolysis degradation of the active ingredient could be avoided due to the absence of water in the formulation. Simple preparation and low cost were also the advantages of this formulation.

Accelerated degradation kinetics of rotenone in the derris extract, and in both formulations, indicated that its degradation followed first-order kinetics. The degradation rate of rotenone in the water dispersible granules was slower than the emulsifiable concentrate. The predicted half-life ($t_{1/2}$) and shelf-life ($t_{90\%}$) at 30°C of rotenone in derris extract were 52 and 79 days, respectively. Derris granules and emulsifiable concentrate clearly prolong stability of rotenone in the derris extract. Both types of formulations were able to clearly prolong the shelf-life of rotenone during storage. Decomposition pathways of rotenone mainly occur through acid-base hydrolysis, oxidation, and photolysis. Therefore, formulations of derris extract should be carefully protected from moisture and light during storage to conserve the active ingredient.

The study of rotenone degradation after application onto plants indicated that both formulations would be effective up to 3 days after spraying. Therefore, this study gives useful data for the consideration of use of effective concentrations for spraying and frequency of applications. However, if derris formulations were applied in the field, the percentage of rotenone remaining may be lower than the values from this study because rotenone may be washed off by water or rainfall.

5.1.4 Efficacy of derris formulations

Preliminary efficacy testing under laboratory conditions indicated that the derris emulsifiable concentrate was clearly more effective than derris water dispersible granules in controlling of *Spodoptera litura*. The efficacy of the emulsifiable concentrate in greenhouse test was lower than in laboratory conditions due to the possible degradation of rotenone. In addition, the application method (dipping of spraying) could affect the adhesion and spreading of formulation on foliage, resulted in different efficacy. The efficacy of derris formulations may be improved by repeated application, or addition of synergists to increase the effectiveness of rotenone.

5.1.5 Calcium alginate beads containing rotenone or derris extract

Calcium alginate beads containing rotenone and derris extract were intended to use for controlling soil insects in soil or controlling mosquito larvae in aquatic system. Calcium alginate gel beads containing rotenone or derris extract were prepared by ionotropic gelation with varying the proportion of sodium alginate and calcium chloride and diameter of syringe or needle. Rotenone-loaded calcium alginate beads were found to be more stable in acidic condition. All formulations of calcium alginate could protect rotenone from photodecomposition. The rotenone release of the beads is mainly related to the swelling behavior of the alginate. Small size of calcium alginate beads could retard rotenone release. The different sizes of beads might be applied together to achieve controlled release and longer duration of efficacy for pest control.

5.2 Suggestions for future studies

To develop commercial products from the derris extracts or other botanical insecticides, several aspects have to be taken into consideration:

(1) To produce a botanical insecticide on a commercial scale, the source of plant must be obtainable. In case of *D. elliptica*, future studies need to examine how cultivation and soil conditions, and geographical location, affect rotenone formation and accumulation in derris root. It should be also grow *D. elliptica* on a large scale, for regular harvesting of root.

(2) Quality control in botanical insecticides is a major drawback in the formulation development. There is wide variation in the quality and quantity of the extracts obtained from the plants. Therefore, the performance and shelf-life of formulated product is affected, even when prepared by the same process. To provide a reliable quality of products, the rotenone content in the extracts must be standardized. This has certainly been achieved with more refined products based on rotenone.

(3) More research is needed for development of the extraction and analytical method to remove technical barriers, improve the design and scale up of the extraction systems for their industrial applications.

(4) Although derris water dispersible granules exhibited a little efficacy against *S. litura* (2%) but the result is dissatisfied compare to derris emulsifiable concentrate formulation. The strategies to improve its efficacy are still required. The efficacy of derris water dispersible granules may be improved by repeated application, or addition of synergists to increase the effectiveness of rotenone.

(5) The stability of rotenone in the both derris formulations is no longer than 6 months for real-time storage. Hence, further studies need to be done for adjustment of derris formulations that might increase the stability of rotenone.

(6) The release of rotenone from calcium alginate beads in soil should be studied. The efficacy and stability of the formulations should be also investigated.

Natural pesticidal plants in Thailand are available as alternatives to synthetic pesticide. However, a major obstacle to widespread use of natural pesticides is a lack of ready-to-use, easily applied and stable formulation. This research provides an idea for the development of cheaper, locally produced and safer alternative formulations to chemically based pesticides that encourage the natural pesticide utilization in agricultural countries.

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List of Publications and Proceedings

List of Publications:

Sae-Yun, A.; Ovatlarnporn, C.; Itharat, A.; Wiwattanapatapee, R. Extraction of rotenone from *Derris elliptica* and *Derris malaccensis* by pressurized liquid extraction compared with maceration. *J. Chromatogr. A* **2006**, *1125*, 172-176.

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