BIOLOGICAL CONTROL OF RICE WEEVILS (*Sitophilus oryzae* L.) IN STORED MILLED RICE BY THE EXTRACTS OF MINTWEED, KITCHEN MINT AND KAFFIR LIME

SAOWANEE BUATONE

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การควบคุมโดยชีววิธีแมลงมอดข้าวสารในข้าวสารด้วยสารสกัดจากแมงลักคา สะระแหน่ และมะกรูด

นางสาวเสาวณีย์ บัวโทน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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เสาวณีย์ บัวโทน : การควบคุมโดยชีววิธีแมลงมอดข้าวสารในข้าวสารด้วยสารสกัดจากแมงลักคา สะระแหน่ และมะกรูด (BIOLOGICAL CONTROL OF RICE WEEVILS (*Sitophilus oryzae* L.) IN STORED MILLED RICE BY THE EXTRACTS OF MINTWEED, KITCHEN MINT AND KAFFIR LIME). อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.กรกช อินทราพิเชฐ, 146 หน้า.

มอดข้าวสาร (*Sitophilus oryzae* L.) เป็นแมลงศัตรูพืชที่สำคัญที่เข้าทำลายเมล็ดข้าว ซึ่งเป็นสาเหตุทำให้เกิดความเสียหายอย่างรุนแรงต่อเมล็ดธัญพืชทั่วโลก โดยเฉพาะข้าวสาร การใช้ยาฆ่าแมลงและการรมควันด้วยสารเคมีในโรงเก็บข้าว เป็นการเพิ่มความเสี่ยงต่อความปลอดภัยในสุขภาพและสิ่งแวดล้อม ส่งผลต่อไปยังผู้บริโภค ส่วนใหญ่สารเหล่านี้ จะสัมผัสกับข้าวสาร โดยตรง ทำให้เกิดอันตรายต่อสุขภาพในระยะยาว ดังนั้นจึงได้มีการศึกษาศักยภาพของการใช้พืชที่หาได้ง่ายในประเทศไทย ได้แก่ แมงลักคา สะระแหน่ ใบมะกรูด นำมาควบคุมแมลงมอดข้าวสาร โดยใช้พืชบดและสารสกัด การควบคุมประเมินโดยการขับไล่ การฆ่า การรม การสัมผัสโดยตรง การป้องกันการสูญเสียน้ำหนักของเมล็ดข้าว และการเคลือบผิวภาชนะ และทดสอบสาเหตุการตายโดยศึกษาไซโทโครม ซี ออกซิเดส การศึกษาทดสอบคุณสมบัติบางประการของสารสกัดหยาบเหล่านี้ พบว่าสารสกัดด้วยเอทานอล มีจำนวนสารฟีนอลิก มากกว่าสารที่สกัดด้วยน้ำ สารสกัดสะระแหน่ด้วยแอลกอฮอลล์ มีสารประกอบ ฟีนอลิก สูงที่สุด 26 mg GAE/g และตรวจสารหลักในสารสกัดด้วย วิธีทินเลเยอร์โครมาโทกราฟฟี พบว่า สารส่วนใหญ่อยู่ในกลุ่ม เทอร์ปีนอยด์ การตรวจสอบพิษของสารด้วยวิธีบายชิมพ์ ลีทัลลิตี พบว่า สารสกัดจากใบพืช มีค่า ความเข้มข้นของสารที่ทำให้สัตว์ทดลองตายไปครึ่งหนึ่ง ต่ำกว่า 1,000 μg/ml อาจเป็นพืชที่มีศักยภาพขององค์ประกอบสารฆ่าแมลง พบว่าใบมะกรูดสกัดด้วยแอลกอฮอล์ ความเข้มข้น 6.4% สามารถขับไล่แมลงได้สูงสุด 80% ใน 24 ชั่วโมง ส่วนพืชบดพบว่า ใบสะระแหน่บดความเข้มข้น 1.25% ขับไล่แมลงได้ 100% ในเวลา 24 ชั่วโมง การทดสอบการตายของแมลงพบว่าจะเพิ่มขึ้นตามระดับความเข้มข้นที่สูงขึ้นซึ่งเป็นความสัมพันธ์ที่ขึ้นอยู่กับความเข้มข้นและเวลา ซึ่งสะระแหน่ที่สกัดด้วยแอลกอฮอล์มีผลต่อการตายมากที่สุด ส่วนพืชบดพบว่าใบสะระแหน่บด ความเข้มข้นต่ำสุด 0.25-0.50% มีผลต่ออัตราการตายของมอดข้าวสาร การยับยั้งการเจริญเติบโตของมอดพบว่าสะระแหน่ที่สกัดด้วยแอลกอฮอล์สามารถยับยั้งการเจริญเติบโตของมอดข้าวสารได้ 70-89% ในพืชบดพบว่า สะระแหน่สามารถยับยั้งการเจริญเติบโตของมอดข้าวสารได้ 42-74% การรมและการสัมผัสโดยตรงพบว่าสารสกัดสะระแหน่มีฤทธิ์สูงสุด รองลงมาคือ มะกรูด และแมงลักคา พืชสกัดด้วยแอลกอฮอล์มีประสิทธิภาพดีกว่าสกัดด้วยน้ำ

การควบคุมการสูญเสียน้ำหนักของเมล็ดข้าวสารพบว่า สารสกัดจากพืชสามารถควบคุมแมลงทำให้ลดการสูญเสียน้ำหนักของเมล็ดข้าวได้ 2-16% และยับยั้งการเกิดใหม่ของมอดข้าวสาร และ

การเคลือบผิวภาชนะบรรจุข้าวสารด้วยสารสกัดของพืช พบว่าสามารถยับยั้งการเจริญเติบโตของมอดข้าวสารได้ 55-88% สาเหตุการตายของมอดศึกษาจากการยับยั้งไซโทโครม ซี ออกซิเดส ซึ่งอยู่ในผนังด้านในไมโตคอนเดรีย พบว่าสารสกัดจากพืชทั้งหมดมีฤทธิ์ยับยั้งเอนไซม์ได้ดี เป็นที่ชัดเจนว่าใบพืชทั้งสามชนิดมีสารกำจัดแมลงที่มีประสิทธิภาพแต่มีพิษน้อยต่อสัตว์เลี้ยงลูกด้วยนมและสิ่งแวดล้อม จึงสามารถสรุปได้ว่าพืชทั้งสามชนิดน่าจะเป็นแหล่งของสารพฤกษเคมีสำหรับใช้เป็นทางเลือกในการควบคุมโดยชีววิธีมอดข้าวสารในการเก็บข้าวสาร

สาขาวิชาชีววิทยา	ลายมือชื่อนักศึกษา________________________
ปีการศึกษา 2553	ลายมือชื่ออาจารย์ที่ปรึกษา__________________
	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม_______________

SAOWANEE BUATONE : BIOLOGICAL CONTROL OF RICE WEEVILS (*Sitophilus oryzae* L.) IN STORED MILLED RICE BY THE EXTRACTS OF MINTWEED, KITCHEN MINT AND KAFFIR LIME. THESIS ADVISOR : ASSOC. PROF. KORAKOD INDRAPICHATE, Ph.D. 146 PP.

SITOPHILUS ORYZAE/BOTANICAL INSECTICIDE

The rice weevil (*Sitophilus oryzae* L.) is the important rice grain insect pest and causes severe damages to cereal grain throughout the world, especially damaging in stored milled rice grains. Using pesticides and fumigants on store milled rice imposes additional risks to health and environment due to the relative closeness of stored product to end user consumption. This study emphasized on toxic activities of crude extracts and dried powders of mintweed (*Hyptis suaveolens* (L.) Poit), kitchen mint (*Mentha cordifolia* Opiz) and kaffir lime (*Citrus hystrix* DC) leaves on adults and larvae of *S. oryzae*. The observations had approached the aspects of repellence, insecticide, growth inhibition, fumigation, direct contact toxicity, and grain weight loss protection**.** Surface coated protection and inhibition of mitochondrial cytochrome c oxidase activity were also investigated. The amount of total phenolic compounds of the extracts was measured. It was found that the ethanol extracts contained more phenolic compounds than the others. The kitchen mint leaf ethanol extract (MLE/e) contained highest phenolic compounds of 26 milligrame gallic acid equivalents per grame (mg GAE/g). The main phytochemicals in the extracts were identified by thin layer chromatography, which were terpenoids. The cytotoxicity of the extracts were tested by brine shrimp lethality assay to indicate the extracts were harmful to mammals. The high LC_{50} values of cytotoxicity of the extracts implied that the extracts could be applicable for biological control of insect pests. The kaffir lime leaf ethanol extract at 6.4% showed the highest repellency of 80% at 24 h the dried leaf powders of kitchen mint at 1.25% showed highest repellency of 100% at 24 h. The mortality effects were significantly dose and time dependent manner ($P<0.05$). The MLE/e processed the most potent insecticide activities. The kitchen mint leaf powder could cause total death of rice weevils at a very low concentration of 0.25%-0.50%. The MLE/e reduced F_1 progeny of rice weevils by 70%-89%. The MLP inhibited F_1 growth and development by 42%-74%. The extract fumigation and direct toxicity caused death of *S. oryzae* ranged as kitchen mint>kaffir lime>mintweed and the ethanol extracts were more potent than the water extracts.

The surface coated with the extracts was able to protect rice weight loss and grain texture by 2%-16%. It also inhibited the growth of rice weevil F_1 progeny by 88%-55%. The extracts inhibited the cytochrome c oxidase, the important enzyme for electron transport chain in cellular respiration, which is located in the inner membrane of mitochondria. The results revealed that all plant extracts inhibited cytochrome c oxidase. It is obviously the phytochemicals from the leaves of these three plants are effectively insecticides, but less harmful to mammals and environment. Therefore, it can be concluded that the three plant leaves are promising biochemical resources for the alternative biological control of irice weevils in milled rice grain storage.

School of Biology — Student's Signature ___________________

Academic Year 2010 — Advisor's Signature ___________________

Co-advisor's Signature_________________

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LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
CLP	=	*Citrus hystrix* DC leaf powders
CLE/e	=	*Citrus hystrix* DC leaf ethanol extract
CLE/w	=	*Citrus hystrix* DC leaf water extract
COX	=	Cytochrome c oxidase
CYT	=	Cytochrome c
DMSO	=	Dimethyl sulfoxide
DPPH	=	1, 1, 3, 3-tetramethoxypropane and 1, 1-diphenyl-2 picryl-hydrazyl
EC_{50}	=	Median Effective Concentration
HLP	=	*Hyptis suaveolens* (L.) Poit leaf powders
HLE/e	=	*Hyptis suaveolens* (L.) Poit leaf ethanol extract
HLE/w	=	*Hyptis suaveolens* (L.) Poit leaf water extract
IC_{50}	=	50% inhibition concentration
LC_{50}	=	Median Lethal Concentration
MLP	=	*Mentha cordifolia* Opiz leaf powders
MLE/e	=	*Mentha cordifolia* Opiz leaf ethanol extract
MLE/w	=	*Mentha cordifolia* Opiz leaf water extract
PBS	=	Phosphate-buffered saline
R_f	=	Retention factor
S.E.	=	Standard Error

LIST OF ABBREVIATIONS (Continued)

TLC	=	Thin Layer Chromatography
v/v	=	volume by volume
v/w	=	volume by weight
w/w	=	weight by weight
WHO	=	World Health Organization

CHAPTER I

INTRODUCTION

1.1 Background

1.1.1 Occurrence of insects associated with stored grain

Rice (*Oryza sativa* Linn.) is an economically important crop and the most important staple foods for the world's population. More than 90% of the world's rice is produced and consumed in Asia. Thailand is one of the major rice exporter countries of the world, which is 70% of export good and values over 114,077 million baths a year (Ago-economic, 2007). The rice plant is vulnerable to various kinds of pests, from the seeds to the stored grains. The rice losses occur when the milled grains are attacked by stored product insects, the important one is rice weevil, *Sitophilus oryzae* L. (Coleoptera: curculionidae). Damaging rice by the weevils is seriously affected the availability of food for a large number of people worldwide. Without protection, the weevils rapidly grow, develop, and damage the stored rice grains. The quality of rice grains is so poor that they do not meet the requirement for normal consumption, exportation, and industrial purposes. Chemical control of rice weevils has been uses, such as rice fumigation with insecticides (Dal Bello, Padin, Lastra, and Fabrizio, 2001). Using synthetic chemicals as insect pest control has given rise to a number of problems, including adverse effects on the environment and human health (Anonymous, 2004). Recently, there has been considerable pressure from consumers opposed the use of synthetic insecticides in foods (Padin, Dal Bello, and Fabrizio, 2002). Alternative means for rice weevil control are considerably needed to be developed, especially from natural plant products which are from the secondary metabolites of plants.

Plant secondary metabolites are chemicals those plants make for their vital function such as protection from pest infestation and Ultraviolet radiation, attraction for pollinating insects, coloring, scent, waste or plant hormones (Odeyemi, Masika, and Afolayan, 2008). Many plant products have been evaluated for their toxic properties against different stored grain pests, especially from crude extracts (Isman, 2006). However, they are largely accessible and non-toxicity to humans and the environment.

This study aims to research on the possible utilization of indigenous plants, which are mintweed (*Hyptis suaveolens* (L.) Poit), kitchen mint *(Mentha cordifolia* Opiz*)* and kaffir lime (*Citrus hystrix* DC), as the insect control agents protect milled rice grains in storage against rice weevil (*Sitophilus oryzae*). The leaves of these plants were used and their crude extracts were evaluated for the biological control efficacy in some approaches.

1.1.2 Insect pests of stored grains

Stored grain insect pests can cause reductions in weight, quality, commercial value and seed viability of the grains (Anonymous, 2004). The insect pests of stored products mainly are Coleopterans, such as *Callosobruchus* spp., *Sitophilus* spp., *Tribolium* spp., and *Prostephanus truncatus,* and followed by lepidopterans, such as *Sitotroga cerealella, Ephestia* spp. and *Plodia interpunctella* (Proctor, 1994). Visarathanonth and Sukprakarn (1988) reported that 72 species of insect pests have been recorded in association with stored products in Thailand.

Among them, *Sitotroga cerealella* and *Rhyzopertha dominica* (Fabricius) are the major pests of paddy (unhulled) rice, and *Sitophilus* spp., *Tribolium castaneum* (Herbst) are of milled (polished) rice. In this study, rice weevil (*Sitophilus oryzae*) the important pest in milled rice, was selected as the animal model for biological control by natural plant products.

Sitophilus spp. are primary Coleopteran pests of stored rice and wheat (Longstaff, 1981). It is also found to attack other crop grains, such as cereals, nut, split peas (Kern and Koehler, 1994). Grain damages are caused by larvae and adults. Without any control, the grains can be destroyed up to 100% of the stored commodity (Ofuya and Credland, 1995). There are three species of *Sitophilus* which are *S. oryzae* (rice weevil), *S. zeamais* (maize weevil) and the *S. granarius* (granary weevil). All of them are the most important pests of stored grain in the world.

1.1.3 Rice weevils (*Sitophilus oryzae*)

S. oryzae belongs to the order Coleoptera and the family Curculionidae. It grows and distributes in worm and tropical parts of the world. Its taxonomic hierarchy is as the followings: Source : http://www.itis.gov/servlet/SingleRpt/SingleRpt/search_topic=TSN&search_value =188080.

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Coleoptera
Family	Curculionidae
Genus	Sitophilus
Species	*Sitophilus oryzae*

1.1.3.1 Distribution and habitat of *Sitophilus oryzae*

S. oryzae was first described by Linnaeus in 1763. The species name of oryzae was given because it was found in rice. The adult rice weevil is attracted by lights. The rice weevil is a small snout beetle which varies in size, about 3-4 mm long. Its morphology varies from a dull reddish-brown to black with round or irregular pits on the thorax. There are four light reddish or yellow spots on the elytra (hardened forewings). The head of many adult weevils has a characteristic snout. Male rice weevils have shorter snout, wider and more distinct punctures than females. The larvae of *S. oryzae* are legless, white to creamy with a small tan head. Jadhav (2006) described *S. oryzae* differs from *S. granarius*. The adult *S. granarius* is shiny reddish brown with elongated pit on thorax, whereas the adult *S. oryzae* is dull reddish brown with irregular shaped pit on the thorax and four light spots on the wing covers. These deep round punctures and light spots are lacking in *S. granarius*. In addition, *S. granarius* cannot fly, while *S. oryzae* can fly. Larvae of both weevils are legless, humpbacked, white to creamy white with a small and tan head. The pupae have snouts like the adults. *S. zeamais* is similar to *S. oryzae*, but larger and darker (Sukprakarn, 1985).

1.1.3.2 Life cycle of *Sitophilus oryzae*

S. oryzae grows and develops inside infested rice grains. Both adults and larvae feed on the grains. Adult females lay egg within 3 days after emerging from their pupa cocoons. They can lay 300-400 eggs in its lifetime and up to 7-8 eggs per day. They will avoid damaged grains for oviposition. The female inserts her ovipositor into the hole in a grain kernel to deposit eggs and then plugs the hole with gelatinous secretion. The larvae hatch after 3-4 days at temperature of 25 ^{o}C and

70% relative humidity. The larvae develop from the 3rd to the 4th instars in about 18-22 days, after which they pupate for about 6 days. The adults stay in the grain kernels for 3-4 days until they are hardened and mature. The complete life cycle of *S. oryzae* from an egg to an adult takes 28 to 32 days under optimum conditions (Figure 1.1). *S. oryzae* adults are quite long-lived and can live from 3 to 6 months (Longstaff, 1981).

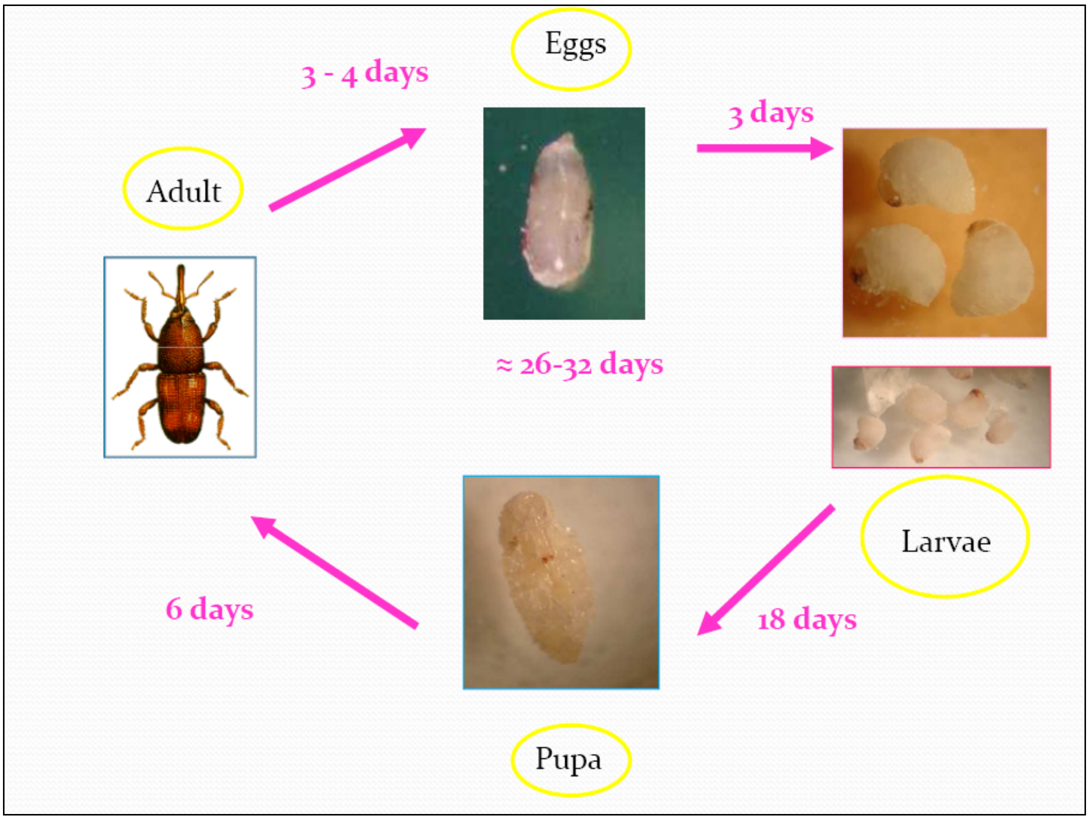

Figure 1.1 Life cycle of rice weevils, *Sitophilus oryzae* (Linnaeus).

1.1.3.3 Damaging rice grains

S. oryzae can infest many kinds of agricultural crop stores such as rice, wheat, corn, oat, barley, bean, nut, wild bird seed, rye, buckwheat, macaroni, starch, cereal products and all types of stored grains. *S. oryzae* is found in warm and tropical parts of the world. The adults fly from stored grains to infest the new grains in the filed and continue infestation throughout that storage. *S. oryzae*, in particularly, develops inside the kernel, while other weevil species do not. It hides inside the cracked grain, which is very difficult to be detected. The adults feed mainly on the endosperms and reduce the carbohydrate content of the grains. The larvae preferentially feed on the germ of the grains and reduce the protein content and vitamins of the grains (Dal Bello, Padin, Lastra, and Fabrizio, 2001). Damages caused by this insect are mainly the reduction of dry mass, the contamination of food with live or dead insects, and the commercial reduction of product values. This strongly opposes the rice industrial standard which is strictly not allowed the presence of live insects and their fragments in all kinds of foods. Therefore, rice protection from *S. oryzae* damages is very essential for rice grains in household and commercial stores.

1.1.4 Control of rice weevil

1.1.4.1 Traditional control

Traditionally, rural farmers protect their crops and commodities by proper drying of grains, cleaning containers, and bans so that they are not infested or contaminated with any insect pests. Store aeration and mechanical remove of insects are practiced as well (Mbata, 1992). However, these methods are insufficient to guarantee that the commodities are fully prevented from insect infestation. Other physically traditional methods are wood ash mix, clay, lime stone sand or silica to asphyxiate or desiccate insects (Golob and Webley, 1980). High concentrations of sand or ash are required to control insects in grain effectively, but diatomaceous earths can be used at much lower concentrations and nor preferable. However, suitable dispose of these used materials is limit to small-scale farms (Golob and Gudrups, 1999). Therefore, new and safe materials for controlling pest in infestation of grains are needed to be explored.

1.1.4.2 Chemical control

1) Fumigation

Fumigation by synthetic chemicals plays a major role in the control of insect pest stored products. Currently, phosphine and methyl bromide are commonly used as fumigants to protect stored crop worldwide. However, the insects develop resistance to these chemicals causing the fumigation failure (Evans, 1985). The use of methyl bromide is highly restricted because of its potential in the ozone depletion, which leads to harmfully affect the earth atmosphere and the radiation on the organisms. In 2005, the Montreal protocol was agreed to restrict the use of chemical fumigation on commodities in developed countries by the year 2010 (World Meteorological Organization, 1995). Recently, the uses of new fumigants such as carbonyl sulphide ethane dinitrile and ethyl formate (Haritos and Dojchinov, 2003) have been investigated as alternatives in protecting food and non-food commodities.

Natural products from plants such as essential oils and other products, ought to be investigated in order to replace the chemically synthetic fumigants, since they are rapidly degraded, locally available and low toxic to mammals and environments.

2) Insecticides

Most insecticidal products belong to four main classes of organochlorines, organophosphates, carbamates and pyrethroids (DARP, 2003). The major ones are organophosphates and pyrethroids which are the most frequently encountered classes of insecticides used in storages in developing countries. Although, very restricted availability,chlorinated hydrocarbons, Dichlorodiphenyl trichloroethane (DDT), lindane are still used. These insecticides which have been widely used in grain protection and other stored foodstuffs caused insects developed resistance (Proctor, 1994). Besides producing insecticidal resistance, synthetically chemical insecticides are dangerous to both human beings and our environments. They induce health problems those are harmful to non target organisms and pollute to water, soil, air as well as to our agricultural products. These problems have highlighted the need for the development of new types of selective pest control products for stored grains.

1.1.4.3 Alternative strategies for stored products pest management

The problems caused by pesticides and their residues have increased. The need for effective and biodegradable pesticides with greater selectivity is essence. Alternative strategies include the search for new types of insecticides and for re-evaluation and uses of traditionally botanical pest control agents. However, new insecticides will have to meet the entire standards. They must be pest specific, non-toxic to mammals, biodegradable, less prone to pest resistance, and relatively less expensive (Isman, 2006). Botanical insecticides which tend to have broad-spectrum activities are safe, relatively specific in their modes of actions and easy to process and use. They also tend to be safe for higher animals and environments. The best known and successful example of insecticide discovered from plant is the natural pyrethrin, found in *Chrysanthemum sp*., which lead to the development of the most used class of synthetic insecticides named pyrethroids. Neem, *Azadirachta indica* of Meliaceae family, is the most important botanical insecticide which is presently used throughout the world (Garcia, Grisoto, Vendramim, and Machado, 2006). However, other plants, especially from tropical regions, process insecticidal potential as well. The potential use of bioactive plant materials in storage pest management systems was economically and environmentally friend. The manipulation of natural chemicals, such as insect attractants, repellents, stimulants, antifeedants and arrestants, which are normally encountered by insects, may fulfill the friendly environment required (Shaaya, Kostjukovski, Eilberg, and Sukprakarn, 1997; Talukder, Islam, Hossain, Rahman, and Alam, 2004). Therefore presently, researchers are seeking new classes of naturally occurring pesticides that can be compatible to newer pest control approaches.

1) The use of plant-based protectants for the control of post-harvest insect pests

The use of plant materials for the protection of field crops and stored commodities against insect attack has a long history (Golob and Webley, 1980; Jilani, 1984). Botanical insecticides have been reported to have a wide range of biological activities against insects (Isman, 2006). They are secondary metabolites from which plants produced to cope with stresses and herbivores such as alkaloids, terpenoids and polyphenolics. They potentially act as repellents, feeding deterrents, poisons or hormone mimics against enemy attaction (Odeyemi, Masika, and Afolayan 2008), oviposition deterrence, and growth regulatory activities (Rozman, Kalinovic, and korunic, 2007). They also play a major role in communication, defense, symbiosis and pollination. Although, plants share similar biochemical requirements, they produce a wide variety of secondary metabolites that are involved in different interactions among organisms. Researchers have discovered many complex biological functions of plant secondary metabolites such as attractants (pigments and scents), deterrents (repellent/antifeedent) or toxins (compounds that affect the growth and development of another animal ; Liu, Goh, and Ho, 2007).

2) Classification of bio-potential plant products

On the basis of plant products on the physiological activities of insects, Jacobson (1982) conventionally classified the plant components into 6 groups, namely repellents, feeding deterrents/antifeedants, toxicants, grain protectants, chemisterilants, and attractants. However, the bio-potential of plant products may also be classified as follows:

3) Repellents

Repellents are chemicals that drive pests away to give high plant protective capacity with minimal impact on the ecosystem. They act on olfactory or other receptors of insects causing insects orient movements away from them. Repellents from plant origin are considered safe in pest control as they minimize pesticide residues; ensure safety of the people, food, environment and wildlife (Talukder, Islam, Hossain, Rahman, and Alam, 2004). Bioactive extracts, powders and essential oil from different plants were reported as repellents against different insects of economically stored products (Xie, Fields, and Isman, 1995; Nerio, Olivero-Verbel, and Stashenko, 2006)

4) Feeding deterrents/antifeedants

Antifeedants, sometimes referred to as feeding deterrents, are chemicals that inhibit feeding, although do not kill insects directly (Isman, 2006). Jilani (1984) defined antifeedants as chemicals which retard or disrupt insect feeding by rendering the treated materials unattractive or unpalatable. Antifeedants are of great value in protecting stored commodities from insects. Some naturally occurring antifeedants include glycosides of steroidal alkaloids, aromatic steroids (Talukder and Howse, 1994). The screening of several medicinal herbs showed that the root bark of *Dictamnus dasycarpus* possessed significant feeding deterrence against two stored-product insects (Liu, Xu, Wu, Goh, and Ho, 2002). Taylor, Field, and Sutherland (2007) reported that crude methanol extracts from four cultivated varieties of mature lentil seeds (*Lens culinaris* Medik.) were found to possess antifeedant and insecticidal properties in laboratory tests with the rice weevil (*Sitophilus oryzae* L.), an insect pest of stored products.

5) Toxicants/Insecticides

Toxicants are specific types of chemicals which directly kill insects. They are also referred as insecticides. There are many reports indicated that plants produced toxic chemicals to different stored product insects (Su, 1991; Weaver et al., 1994; Talukder, 1995; Xie, Fields, and Isman, 1995; Park, Lee, Choi, Park, and Ahn, 2003; Talukder, Islam, Hossain, Rahman, and Alam, 2004) listed the uses of 43 plant species which expressed toxicant effects on different species of storedproduct insects. Methyl allyl disulfide and diallyl trisulfide were the two major constituents in garlic, *Allium sativum*, essential oil those were potent as a contact toxicant, fumigant and feeding deterrent against *Sitophilus zeamais* and *Tribolium castaneum* (Huang, Lam, and Ho, 2002). Shaaya et al. (1991) found that five compounds extracted from 11 plants exhibited positive activity against three major storage pests which were *R. dominica*, *O. surinamensis*, and *S. oryzae*. Roy, Amin and Uddin (2005) established leaf extracts of Shiyalmutra (*Blumea lacera*) as botanical insecticides against lesser grain borers and rice weevils. There is a report that the extracts from fruit of melgota *(macaranga postulate)* had repellent and insecticidal activities against *S. oryzae* (Rahman et al., 2006).

6) Grain protectants

In some parts of Eastern Africa, leaves of some plants were traditionally mixed for use as grain protectants (Udo, 2005). Some parts of neem (*Azadirachta indica*) had traditionally been long used as natural protectants for stored grains by mixing with stored grains. Additionally, leaves, fruits, seeds, and oil of neem were also used to protect stored grains (Islam and Talukder, 2005). Mixing stored grains with leaf, bark, seed powder, or oil extracts of plants reduced oviposition rate and suppressed emergence of adult insects. It also reduced damage rate of seeds (Tapondjou et al., 2002). Leaf powers of *Ocimum gratissimum* and *Vernonia amygdalina* were used to manage *S. oryzae* (Law-Ogbomo and Enobakhare, 2007). Moreover, protein enrich flour extracts were promisingly observed in the protection of rice against *S. oryzae* (Pretheep-Kumar, Mohan, and Ramaraju, 2004).

7) Reproduction inhibitors

Plant products are able to inhibit growth of insects. Underground parts, extracts, oils and vapor of plants can suppress fecundity and fertility of many insects. Many researchers reported that plant parts, oils or extracts mixed with grains reduced insect oviposition, egg hatchability, post embryonic development and progeny production (Saxena, Dixit, and Harshan, 1992). It also indicated that plant derivatives, including the essential oils, caused infertility of insect eggs (Tunc, Berger, Erler, and Dagli, 2000).

8) Insect growth and development inhibitors

Plant extracts showed deleterious effects on the growth and development of insects and reduced larval, pupal and adult weight significantly. They also lengthened the larval and pupal periods and reduced pupal recovery and adult eclosion (Khanam, Talukder, and Khan, 1990). Crude extracts also retarded development and caused mortality of larvae, cuticle melanization and high mortality in adults (Jamil, Rini, and Thyagarajan, 1984). It was reported that grains coated with plant extracts completely inhibited the development of insects, such as *S. oryzae* (Roy, Amin, and Uddin, 2005). Plant derivatives also reduce the survival rates of larvae, pupae, and adult emergence (Tripathi, Prajapati, Aggarwal, Khanuja, and Kumar, 2000).

9) Metabolic inhibition

The use of insecticidal plants is especially prevalent in developing countries, where plants grow locally and are cheaper than synthetically chemical pesticides for subsistence farmers to use. The most direct mode of insecticidal activity is the measurement of mortality. Toxic chemicals in plant extracts or essential oils express insecticidal effects by contact toxicity, neuro-toxicity (Enan, 2001) stomach poisoning, or fumigation (Lee, Peterson, and Coasts, 2003). Death of insects may cause by indirect means, such as suffocation via blockage of the spiracles (Williams and Mansingh, 1993) Most insecticides contact with body surface or flow through respiration.

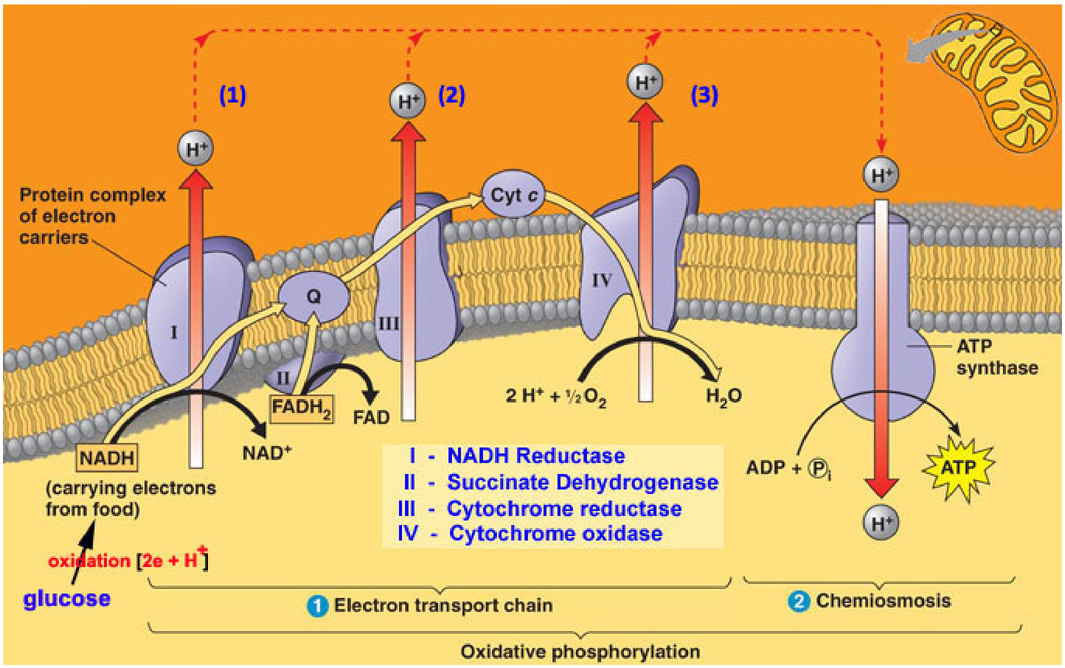

Figure 1.2 The inner mitochondrial membrane illustrating electron transport proteins (complexes I-IV) and ATP synthetase (complex V).

Source : http://www.hyperbaric-oxygen-info.com/aerobic-cellular-respiration.html

Many new insecticides appear to affect cell respiration in mitochondria. Several insecticides inhibit the electron transport, resulting in blocking the production of Adenosine triphosphate (ATP) and causing a decrease in oxygen consumption by the mitochondria. Rotinone, pyridaban and tolfenpyred inhibit Nicotinamide adenine dinucleotide (NADH) dehydrogenase (complex I) in mitochondria electron transport chain (Figure 1.2). Hydramethylnon is an inhibitor for cytochrome bc_1 (complex II). Hydrogen cyanide and phosphine are inhibitors for cytochrome c oxidase (complex VI). Some formate ester exerted their effects on mitochondria by inhibition of cytochrome c oxidase which caused mitochondrial impairment in *S. oryzae* (Haritos and Dojchinov, 2003).

1.1.5 Plants of interests

Aromatic plants mainly process monoterpenes which contain potentially biological activities including toxicity to insects (Lee, Peter, Tumalii, and Choi, 2004). Particularly, the family Labiaceae are among the most widely used for controlling insect pests, including stored product pest insects. Recently, researchers have focused on testing the biological activities of extracts of mintweed (*Hyptis suaveolens* (L.) Poit), kitchen mint (*Mentha cordifolia* Opiz) and kaffir lime (*Citrus hystrix* DC) against *S. oryzae*. The extracts of these plants and other kinds of *Hyptis* spp. and *Citrus* spp. were shown to have antifeedant, repellent, anti-oviposition and growth disruptor properties against many insect pests such as *Sitophilus zeamais*, *Callosobruchus machlatus. Rhyzopertha dominica* and *S. oryzae* (Shaaya, Kostjukovski, Eilberg, and Sukprakarn, 1997; Iloba and Ekrakene, 2006). Nowadays, researchers are seeking for new classes of naturally occurring pesticides for safer pest control approaches. Botanical insecticides can often easily be obtained by farmers and small-scale industries. Many aromatic plants which are herbs or spices and locally available may be effectively used in insect pest controls. Mintweed (Labiatae), kitchen mint (Lamiaceae), and kaffire lime (Rutaceae) are of interest to this study for investigation in the biological control of *Sitophilus oryzae* infested in milled rice grains.

1.1.5.1 *Hyptis suaveolens* (L.) Poit

Hyptis suaveolens (L.) Poit (family Lamiaceae, common name-mintweed or maeng luk kha) is well-known among Northern Thailand (Figure 1.3). Its classification is as the followings:

Kingdom Plantae

Division Magnoliophyta

Class Magnoliopsida

Order Lamiales

Family Lamiaceae

Genus Hyptis

Species *Hyptis suaveolens* (L.) Poit

(Source : http://plants.usda.gov/java/profile/symbol=HYSU3.html)

Figure 1.3 *Hyptis suaveolens* L. Poit, mintweed or maeng lukkha.

Its leaves are used for folklore remedies, such as anti-itching. The major chemical constituents in *H. suaveolens* oil are sabinene, β-caryophyllene, and

1,8-cineole (Peerzada, 1997). *H. suaveolens* is aromatic and frequently used for treatments of gastrointestinal infection, cramps and pain, and skin infections (Azevedo et al., 2001). Iloba and Ekrakene (2006) reported that *H. suaveolens* had moderate insecticidal activities against *Sitophilus zeamais* and *Callosobruchus machlatus.*

1.1.5.2 *Mentha cordifolia* Opiz

Mentha cordifolia Opiz (family- Lamiaceae, common name kitchen mint or saranae) is an aromatic and spicy plant (Figure 1.4). It is classified as the followings:

Kingdom Plantae

Division Magnoliophyta

Class Magnoliopsida

Order Lamiales

Family Laminaceae

Genus Mentha

Species *Mentha cordifolia* Opiz

(Source : http://www.homolaicus.com/scienza/erbario/utility/botanica_sistematica/hypertext/ 1248.html)

Figure 1.4 *Mentha cordifolia* Opiz, kitchen mint or saranae.

It is widely used as a food seasoning and conventional medicine for antispasmodic, antiseptic, cosmatics, toothpastes and mouthwashes (Kizil and Toncer, 2006). There are reports that basil and mint were used for pest deterrent. The essential oils of several spices like anise (*Pimpinella anisum* L.) and peppermint (*Mentha piperita* L.) had fumigant toxicity to major stored product pests, i.e., *Rhyzopertha dominica* and *S. oryzae* (Shaaya et al., 1991). *Mentha arevensis* L. was also used as fumigant against *S. oryzae* (Lee, Lee, Choi, Park, Kim, and Campbell, 2001).

1.1.5.3 *Citrus hystrix* DC

Citrus hystrix DC (family Rutaceae, common name kaffir lime or magrud) is a native plant to temperate and tropical Asia, including Thailand (Figure 1.5). It is classified as the followings:

Kingdom Plantae

Division Magnoliophyta

Class Magnoliopsida

Order Sapindales

Family Rutaceae

Genus Citrus

Species *Citrus hystrix* DC

Source : http://www.homolaicus.com/scienza/erbario/utility/botanica_sistematica/hypertext/0358.html.

Figure 1.5 *Citrus hystrix* DC, kaffir lime or magrud.

Kaffir lime is used as food and additive. *C. hystrix* is well known for the production of bioactive terpenoids (Isman, 2006). This compound is responsible for the aroma and identified as (-)-(s)-citronellal, mostly in the leaf oil up to 80%, citronellol, nerol, and limonene. (Katzer, 2002). Omar et al. (2007) reported that terpenes isolated from tropical species of the rutales significantly exhibited antifeedant activity against *S. oryzae*. Oil extracts from peels of several species of citrus are toxic to mosquitoes and can be used as fumigant against mosquito larvae (Thomas and Callaghan, 1999). Tawatsin, Wratten, Scott, Thavara, and Techadamrongsin (2001) investigated the repellent effect of essential oils of turmeric (*Curcuma longa*), kaffir lime (*Citrus hystrix*), citronella grass (*Cymbopogon winterianus*) against mosquitoes.

1.2 Research objectives

The main objective of this study was to biologically control of rice weevil (*Sitophilus oryzae*) in stored milled rice grains by extracts of aromatic plants. The research will focused on the followings:

1.2.1 To primarily investigate some phytochemical properties of crude extracts of *Hyptis suaveolens* L. Poit, *Mentha cordifolia* Opiz and *Citrus hystrix* DC.

1.2.2 To investigate the insecticidal effects of the plant extracts on larvae and adults of the rice weevils.

1.2.3 To investigate the effect of the plant extracts on insect cytochrome c oxidase.

1.3 References

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CHAPTER II

EXTRACTION AND IDENTIFICATION OF BIOLOGICALLY ACTIVE COMPONENTS FROM MINTWEED, KITCHEN MINT AND KAFFIR LIME LEAVES

2.1 Abstract

Hyptis suaveolens, Mentha cordifolia and *Citrus hyptrix* leaves were extracted in water and 80% ethanol. The yield recoveries were higher in ethanol extracts. The recovery of kitchen mint leaf ethanol extract (MLE/e) was highest of approximately 31% and of kaffir lime leaf water extract (HLE/w) was lowest of about 22%. The total phenolic compounds contents of ethanol extracts were slightly higher than of water extracts. MLE/e contained highest total phenolic compounds of 26 mg GAE/g and HLE/w contained lowest of 18 mg GAE/g. The amounts of total phenolic compounds were well correlated with the yield recovery of the extracts. TLC analysis and vanillin detection indicated that the major compounds constituted in the extracts were terpenoids group.

2.2 Introduction

Plants are favorably exploited as for the biological control of insect pests and stored grain product pests, including the management of rice weevil (*Sitophilus oryzae*) in rice grain infestation (Huang, Ho, Lee, and Yap, 2002; Lee, Peterson, and Coasts, 2003). They produce different kinds of secondary metabolites which constitute important sources of pesticides include terpenoides, phenolics, flavonoides, tannins, essential oils and alkaloids. These phytochemical compounds have different behavioral and physiological effects on insects (Isman, 2006). Different biological activities of plant derivatives were demonstrated for the control of stored grain pests (Rajendran and Srianjini, 2008). Naturally, botanical insecticides are believed to be the sources of bioactive chemicals with complicated action mechanisms, to which the insect pests hardly develop resistances. However, some plant products are toxic or less dangerous to mammals, human beings and environment (Isman, 2006). Recently, the aspects of insecticidal properties of plant products including toxicity, repellence, oviposition and growth inhibition effects have been established by some researchers in particularly, against insect pests of stored grains at laboratory scale (Odeyemi, Masika, and Afolayan, 2008). Plant extracts contain several secondary compounds such as, phenolic and terpenes which have both toxic and behavioral activities on insects (Boudet, 2007). Saljoqi, Afridi, Khan, and Rahman (2006) reported that the toxic and repellent properties of extracts from six plant extracts, bakain drupes (*Melia azdarach*), habulas leaves (*Myrtus communis*), mint leaves (*Mentha longifolia*), bakain leaves, harmal shoots and seeds (*Pegnum harmala*) and lemon grass roots (*Cymbopogon citrates*) against *S. oryzae*. Rahman et al. (2006) conducted the insect repellent assays using extracts of different plants on stored-product pests. Therefore, plant-based extracts or active compounds may have potential to control this destructive *S. oryzae*. Lee et al. (2001) showed that menthone, limonene and linalool isolated from *Mentha arevensis* had fumigant toxicity again *S. oryzae*. Iloba and Ekrakene (2006) reported that *H. suaveolens* had moderate insecticidal activities against *S. zeamais* and *Callosobruchus machlatus.* Omar et al. (2007) reported that terpenes isolated from tropical species of the Rutales significantly exhibited antifeedant activity against rice weevils. Therefore, there are several local plants species in Thailand to which are potentially used in insect pest control. For instance, the leaves of mintweed, kaffir lime and kitchen mint could significantly exhibit insecticidal activity against *S. oryzae* in stored milled rice.

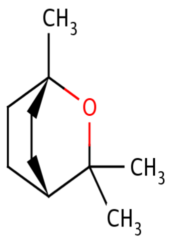
This study aimed to establish the biological control of rice weevils infest in milled rice grains by using mintweed (*Hyptis suaveolens* (L.) Poit), kitchen mint *(Mentha cordifolia* Opiz) and kaffir lime (*Citrus hystrix* DC). The finding of this study will be a better replacement for synthetically chemical insecticides in controlling *S. oryzae* in milled rice storage at laboratory scale. The major groups of phytochemicals from leaf extracts from these plants are expected to partially separate to some extent in order to correlate the plant products and their activities in relation to the biological control of rice weevils in milled rice storage. Substantial information from this study also expected for the needed bases of further investigation on the biological control of this insect at industrial scale in the future.

2.2.1 Phytochemicals constituents of selected plant species

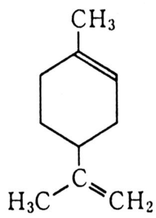
Mintweed (*H.suaveolens*), kitchen mint (*M. cordifolia*), and kaffir lime (*C. hystrix*) are aromatic plants. Mintweed is weed plant and kitchen mint and kaffir lime are vegetables or spices. The present study exploited their leaves to investigate their bioefficacy on the biological control of rice weevils (*S. oryzae*) in milled rice storage.

2.2.1.1 *Hyptis suaveolens* (L.) Poit (Laminance)

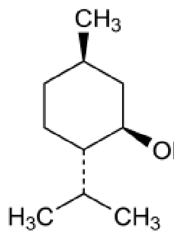
Hyptis suaveolens (L.) Poit, local name “Maeng Luk Kha”, belongs to the family Lamiaceae. This plant is known as a weed, found in open areas of any kind of soils throughout Thailand. Parts of it are used in folklore remedy. The leaves of mintweed are aroma and frequently used for the treatment of gastrointestinal infection, cramps and pain, as well as for skin infections (Azevedo et al., 2001). Some chemical compounds were identified from *H. suaveolens* extracts (Peerzada, 1997). The major chemical constituents in *H. suaveolens* oil were sabinene, limonene, eugenol, α-terpinene, α-terpineol, β-caryophyllene, menthol, bi-cyclogermacrene, β-phellandrene and 1, 8-cineole (Figure 2.1) (Peerzada, 1997; Azevedo et al., 2001).



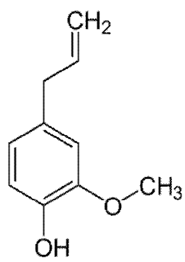
1,8 cineol



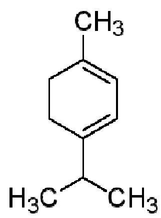
Limonene



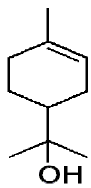
L-Menthol



Eugenol



α-Terpinene



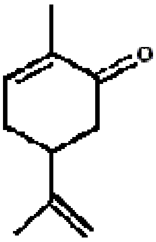
α-Terpineol

Figure 2.1 Chemical structures of some constituents isolated from *Hyptis suaveolens* (Lide and Milne, 1995) .

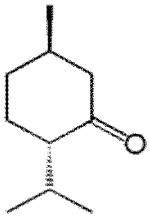
Some biological properties of *H. suaveolens* were investigated, including insecticidal and repellent activities against *Sitophilus zeamais* and *Callosobruchus machlatus* (Iloba and Ekrakene, 2006). There is no evidence about the biological control of *Sitophilus oryzae* by *H. suaveolens* products. In this study, extraction and identification of some *H. suaveolens* productions from leaves were performed to investigate their activities in controlling *rice weevils, S. oryzae*, in milled rice grain storage.

2.2.1.2 Kitchen mint (*Mentha cordifolia* Opiz) (Laminance)

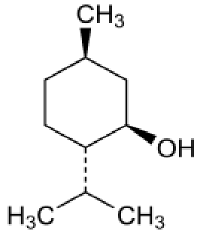
Kitchen mint (*Mentha cordifolia* Opiz), locally named "Saranae", belongs to the family Lamiaceae. It is a fast growing cultivar in Thailand .The genus *Mentha* consist of more than 25 species and are well known in containing of monoterpenes, such as menthol, menthone, carvone and pulegone. They are commonly used as vegetable and seasoning. They are also used in pharmaceutical industries as a flavor and fragrance in some formulations, such as antispasmodic, antiseptic, cosmetics, toothpastes and mouthwashes (Kizil and Toncer, 2006). Basil and mint have been used for pest deterrent. The essential oils of several spices like anise (*Pimpinella anisum*) and peppermint (*M. piperita*) are found to have fumigant toxicity to *Rhyzopertha dominica* and *S. oryzae* which are the major stored product pests (Shaaya et al., 1991). There was a report that *M. arevensis* exhibited toxicity towards rice weevils and it was rich in menthol, menthone, limonene, alpha-pinene, beta-pinene and linalool (Figure 2.2) (Lee et al., 2001). The toxicity of the essential oil from the leaves of *M. longifolia* was investigated against *S. zeamais*, the major cause of deterioration in maize stored grains (Odeyemi, Masika, and Afolayan, 2008). However, there is no evidence of *M. cordifolia* activities on the control of *S. oryzae* in milled rice grain storage .



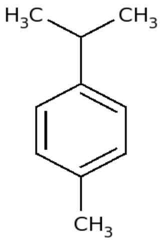
Carvone



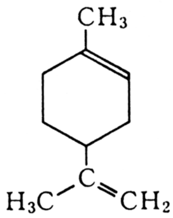
Menthone



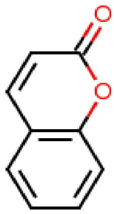
L-Menthol



P-cymene



Limonene



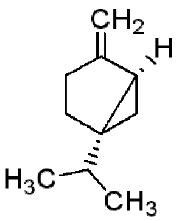
Coumarin

Figure 2.2 Chemical structures of some constituents from *M. cordifolia* Opiz (Lide and Milne, 1995).

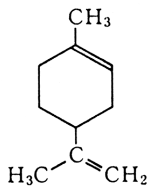
2.2.1.3 Kaffir lime (*Citrus hystrix* DC) (Rutacieae)

Kaffir lime (*Citrus hystrix*) grows in the tropical areas of the new world and is a native plant to Southeast Asia. It is in the order of Rutales. Its products are rich in bioactive terpenoids (Isman et al., 2006). The major compound responsible for the aroma characteristics is identified to be (-)-(s)-citronellal, which is found in its leaf oil up to 80% (Figure 2.3). The minor components are citronellol (10%), nerol and limonene. (Katzer, 2002). Terpenes were also found in tropical species of the Rutales and significantly exhibited antifeedant activity against rice weevils (Omar et al., 2007). However, essential oils of turmeric (*Curcuma longa*), kaffir lime (*C. hystrix*), citronella grass (*Cymbopogon winterianus*) were investigated on the repellent effect against mosquitoes (Tawatsin, Wratten, Scott, Thavara, and Techadamrongsin, 2001).

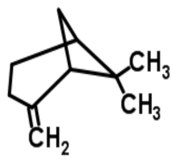
Oil extracts from citrus peels were found toxic to mosquitoes, including larvae, when used as fumigant (Thomas and Callaghan, 1999). Whole citrus peel extracts, of which d-limonene is the major ingredient, have also been tested against some insects. Lime peel oil is toxic to the cowpea weevil (*Callosobruchus maculates* F.), the maize weevil (*Sitophilus zeamais* Motschulsky), and the hide beetle (*Dermestes maculatus* DeGeer) (Don-Pedro, 1996). There no substantial information of *C. hystrix* in controlling of *S. oryzae* in milled rice grain storage.



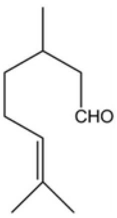
Sabinene



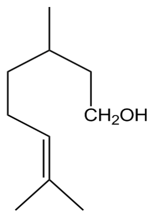
Limonene



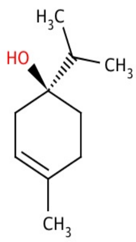
β-pinene



Citronellal



Citronellol



(+)-Terpinen-4-ol

Figure 2.3 Chemical structures of some constituents from *C. hystrix* DC (Lide and Milne, 1995).

2.3 Materials and methods

2.3.1 Materials

Folin-Ciocalteus's phenol reagent and Sodium carbonate were purchased from Merck Chemical supplies (Damstadt, Germany). Sodium carbonate was purchased from Riedel-de Haën, AG, Seelze-Hannover, Germany. Vanillin was purchased from BDH Chemicals Ltd. (Poole, England). Gallic acid was from Eastman Organic Chemicals, Kingsport, Tennessee, USA. The other chemicals are designated in the text. All chemicals used were of analytical grade.

2.3.2 Collection for plant materials and preparation for extracts.

The fresh leaves of mintweed, kaffer lime and kitchen mint were collected from the surrounding areas of Suranaree University of Technology (SUT) campus. They were completely dried in sunlight, ground into powder and kept at 4 ^{o}C before use. A hundred gram of the powder was extracted in 80% ethanol or distilled water in Soxhlet extraction apparatus. The extracts were continuously refluxed until they became colorless. The solvents were removed by evaporation using a rotary vacuum evaporator, dried by lyophilizer at -70 ^{o}C. The extracted powders were stored at 4 ^{o}C.

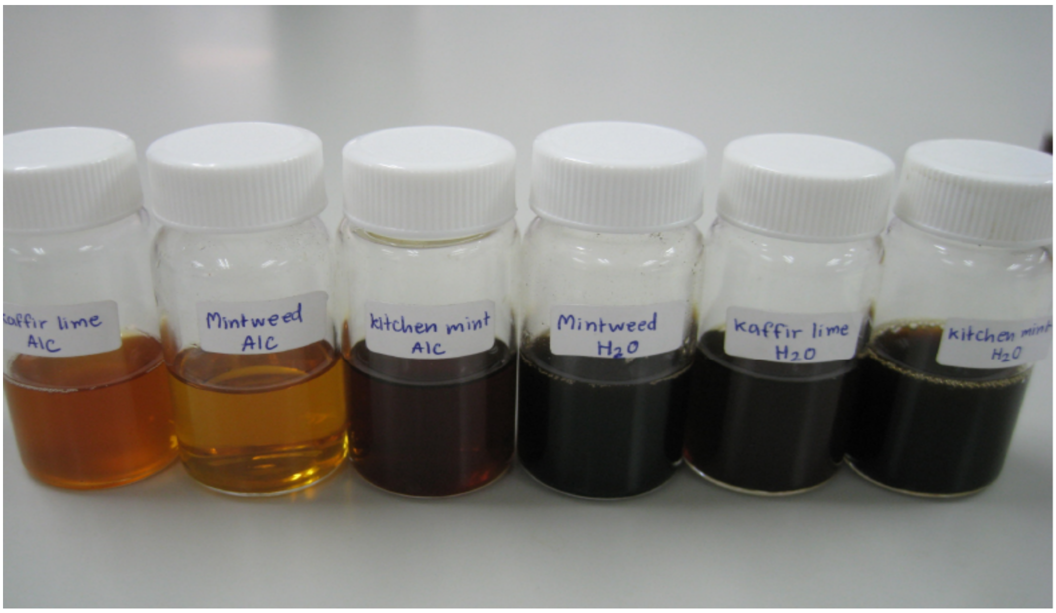


Figure 2.4 Crude product of leaf ethanol and water extracts of mintweed, kitchen mint and kaffir lime.

2.3.3 Determination of total phenolic compounds

Total phenolic compounds in the plant leaf extracts were measured by the Folin-Ciocalteau colorimetric method using gallic acid as a standard (Kyung-Hee, Rong, Yang, and Steve, 2005). A hundred and fifty micrograms of the extract was dissolved in its original solvent (Figure 2.4). The ethanol extract was dissolved in 40% ethanol. An aliquot of 100 µl extract solution was added to 2 mL of 2% Na_2CO_3 and in incubated for 2 min. A hundred microliter of Folin-Ciocalteau reagent in methanol (Folin:Methanol. 1:1 v/v) was added and further incubated for 30 min. The absorbance of the color of reaction product was measured at 750 nm. The gallic acid was as a standard and the total phenolic compounds were expressed as milligrams gallic acid equivalents (GAE) per gram extract. All analyses were performed in three replicates.

2.3.4 Thin layer chromatography (TLC) fingerprints of plant leaf extract

The major components of total phenolic compounds were determined by TLC. The silica gel 60 F_{254} 0.2-mm pre-coated plated (from E. Merck, Darmstadt, Germany). The mobile phase used for the development of the chromatograms was the mixture of toluene-chloroform-ethyl alcohol (5:3:2) and toluene-ethyl acetate-ethyl alcohol (5:4:1). Bands developed were visualized under ultraviolet light at 254 nm and in visible light at 366 nm (CAMAG UV cabinet), and finally in visible light after spraying with 1% vanillin in concentrated sulfuric acid.

2.4 Results

2.4.1 Yield recovery of crude extracts

Leaf extracts of mintweed (*H. suaveolens*), kaffir lime (*C. hystrix*) and kitchen mint (*M. cordifolia*) were recovered as shown in Table 2.1. The maximal and minimal yields were obtained from the leaf extracts of *M. cordifolia* and *C. hyptix*, respectively. The yields of mintweed leaf water extract (HLE/w), kaffir lime leaf water extract (CLE/w) and kitchen mint leaf water extract (MLE/w) were recovered 21.94, 23.58, and 27.26%, respectively. The leaf alcohol extracts of mintweed (HLE/e), kaffir lime (CLE/e), and kitchen mint (MLE/e) were 22.85, 25.86, and 31.03%, respectively. In general, the yields obtained from the plant extracts are adequate for further studies and development of biopesticides.

Table 2.1 Yield recovery of mintweed, kaffir lime and kitchen mint extracted in water and ethanol.

Plant	**Solvent**	**Leaf extract**	**Percentage yield (w/w) (Average±S.E.)**
H. saoveolens	H_2O	HLE/w	21.94±3.55
	EtOH	HLE/e	22.85±2.03
C. hyptrix	H_2O	CLE/w	23.58±3.15
	EtOH	CLE/e	25.86±1.55
M. cordifolia	H_2O	MLE/w	27.26±3.62
	EtOH	MLE/e	31.03±2.68

2.4.2 Total phenolic compounds

In this study, the total phenolic content in the ethanol and water extracts of kitchen mint, kaffir lime, and mintweed were determined spectrometrically according to the Folin-Ciocalteu method and calculated as GAE. The results were shown in Table 2.2. The total phenolic content of ethanol extract of kitchen mint 26.38±0.88 GAE (mg/g) was higher than that of the water extract 21.05±1.72 GAE (mg/g). Among these plant extracts, the total phenolic content of ethanol extracts were higher than those of water extracts.

Table 2.2 Total phenolic compound contents in water and ethanol extracts of mintweed, kaffir lime, and kitchen mint leaves.

Plant	Extracts	Total Phenolic Compounds GAE(mg/g)±S.E.
H. saoveolens	HLE/w	18.04±1.18
	HLE/e	20.58±1.71
C. hyptrix	CLE/w	21.40±0.57
	CLE/e	22.16±1.67
M. cordifolia	MLE/w	21.05±1.72
	MLE/e	26.38±0.88

2.4.3 Thin layer chromatography analysis

TLC analysis detailed information of ethanol and water extracts of kaffir lime, mintweed and kitchen mint are shown in Table 2.3. The TLC chromatographs of all extracts shown before and after spraying with the vanillin reagent are in figures 2.5-2.10. The identification of the monoterpenoids was selectively performed in the solvent system of toluene-chloroform-ethyl alcohol at the proportion of 5:3:2 (v/v/v) and toluene-ethyl acetate-ethyl alcohol at the proportion of 5:4:1. The expected bands of phenolic compounds were revealed by vanillin-sulphuric acid reagent. Several different fluorescence bands were observed under UV light at 254 nm (Figures 2.5-2.10). For every specific spot of color with R_f, we made an assignment with a type of compound.

The apparition of red spotlights certified their presence in all our samples of survey. Spary detection this relies on a color reaction between the compound on the TLC plate and a spray reagent introduce on to the plate as a fine mist from a spary consister. Vanillin/sulfuric acid was detected many terpene give red and blue colors. Natural produces with in little functionality may give poor coloration. When using spary detection in preparative TLC showed many colour especially red colour (Figures 2.5-2.10).

Table 2.3 Detailed informations on TLC analyses of kitchen mint, mintweed, and kaffir lime leaf extracts.

Plant	Solvent	Type of Extract& Solvent System	Light Source	Observations No. of Spots	R_f Values (Corresponding Colors)
M. cordifolia	Alc	toluene:chloroform: ethyl alcohol 5:3:2	Before Spraying	10	0.06 (Blue), 0.28 (pink), 0.65 (yellow),0.66 (pink), 0.68 (deep red), 0.71 (blue), 0.75 (pink) 0.76 (yellow), 0.81 (red), 0.91 (red)
			After Spraying	4	0.11 (light red), 0.16 (light red), 0.78 (deep red), 0.85 (deep red)
	H_2O	toluene:chloroform:ethyl alcohol 5:3:2	Before Spraying	2	0.68(right blue),0.71(blue)
			After Spraying	1	0.78(red)
H. saoveolens	Alc	toluene:ethyl acetate :ethyl alcohol 5:4:1	Before Spraying	10	0.23 (pink), 0.25 (yellow), 0.28(blue), 0.55 (blue), 0.6 (pink), 0.75 (blue), 0.78 (pink), 0.83 (deep red), 0.88 (pink), 0.95 (yellow)
			After Spraying	5	0.33 (red), 0.46 (deep red), 0.71 (red), 0.83 (red), 0.88 (red)
	H_2O	toluene:ethyl acetate :ethyl alcohol 5:4:1	Before Spraying	3	0.41 (green), 0.56 (green), 0.8 (blue)
			After Spraying	1	0.41(red)

Table 2.3 (Continued).

Plant	Solvent	Type of Extract& Solvent System	Light Source	Observations No. of Spots	R_f Values (Corresponding Colors)
C. hytrixs	Alc	toluene:chloroform: ethyl alcohol 5:3:2	Before Spraying	9	0.16 (green), 0.2 (violet), 0.21 (light red), 0.26 (deep blue), 0.3 (blue), 0.33 (light blue), 0.38 (red), 0.48 (blue), 0.55 (yellow)
			After Spraying	3	0.26 (red), 0.38 (red), 0.78 (red)
	H_2O	toluene:chloroform: ethyl alcohol 5:3:2	Before Spraying	3	0.5 (green), 0.56 (violet), 0.75 (light red), 0.83 (blue), 0.96 (green)
			After Spraying	1	0.8 (red)

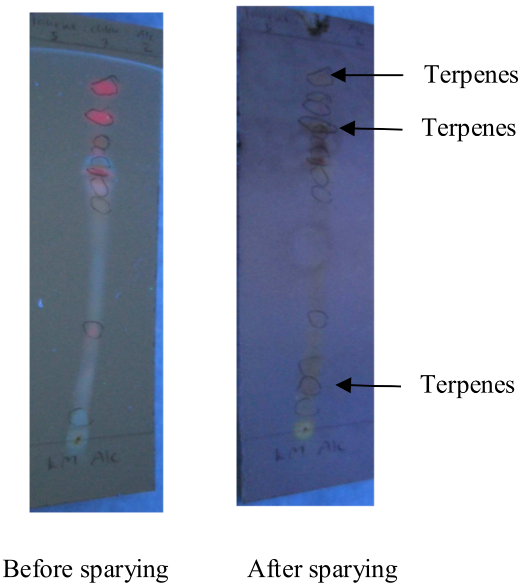


Figure 2.5 TLC chromatographs of the ethanol extract of *M. cordifolia* using a mobile phase of toluene-chloroform-ethyl alcohol at the ratio of 5:3:2 before and after spraying with vanillin-sulphuric acid reagent.

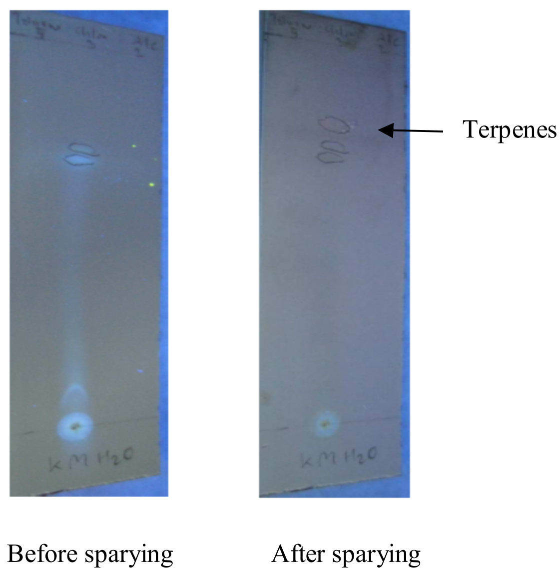
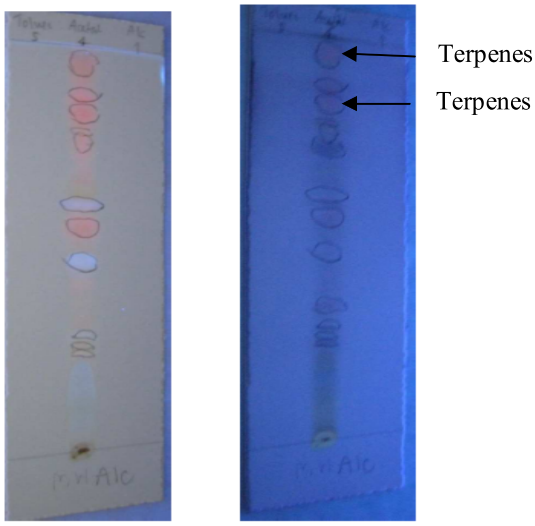
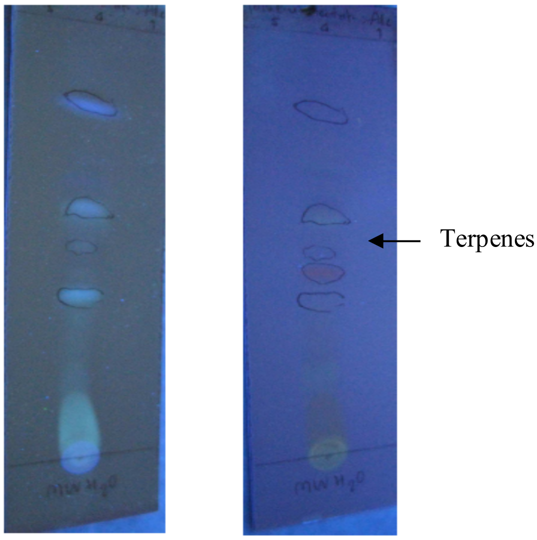


Figure 2.6 TLC chromatographs of the water extract of *M. cordifolia* using a mobile phase of Toluene-chloroform-ethyl alcohol at the ratio of 5:3:2 before and after spraying with vanillin-sulphuric acid reagent.



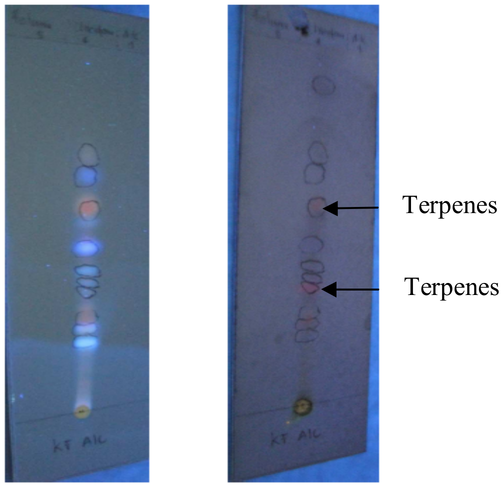
Before sparying After sparying

Figure 2.7 TLC chromatographs of the ethanol extract of *H. saoveolens* using a mobile phase of toluene-ethyl acetate-ethyl alcohol at the ratio of 5:4:1 before and after spraying with vanillin-sulphuric acid reagent.



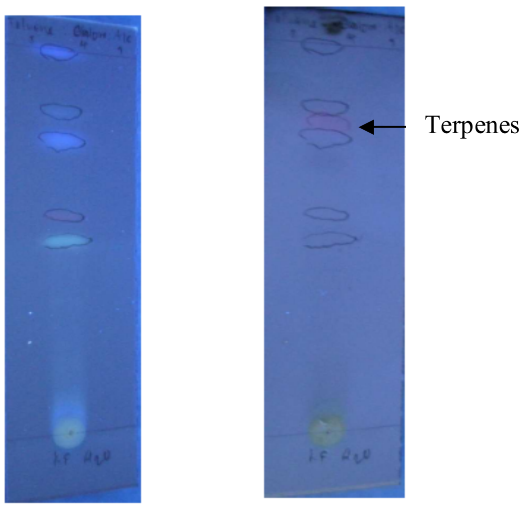
Before sparying After sparying

Figure 2.8 TLC chromatographs of the water extract of *H. saoveolens* using a mobile phase of toluene-ethyl acetate-ethyl alcohol at the ratio of 5:4:1 before and after spraying with vanillin-sulphuric acid reagent.



Before sparying After sparying

Figure 2.9 TLC chromatographs of the ethanol extract of *C. hytrix* using a mobile phase of toluene-chloroform-ethyl alcohol in the ratio of 5:4:1 before and after spraying with vanillin-sulphuric acid reagent.



Before sparying After sparying

Figure 2.10 TLC chromatographs of the water extract of *C. hytrix* using a mobile phase of toluene-chloroform-ethyl alcohol at the ratio of 5:4:1 before and after spraying with vanillin-sulphuric acid reagent.

2.5 Discussion

The phytochemical compounds contained in the extracts draw our attention to their potential roles in the biological control of insect pests, in particularly *S. oryzae* infested in milled rice storage. The phenolic compounds are considered as the major group of the secondary metabolites produced by plants. Some contributes to protection and antioxidant activity in plants. Several thousand phenolic compounds occur widely throughout the plant kingdom (Kyung-Hee, Rong, Yang, and Steve, 2005). A number of the phenols possess distinct biological activities. TLC is conventionally used for analyzing the secondary metabolites in plants. Some components like monoterpene and terpenenes (1,8 cineol and limonene) which have some activities on insects (Odeyemi, Masika, and Afolayan, 2008). Similary were also found in the crude extracts of mintweed, kaffir lime, and kitchen mint leaves. The insecticidal properties of many plant extracts and essential oils are mainly terpenoids (Regnault-Roger and Hamraoui, 1995). Monoterpenoids are typically volatile and rather lipophilic compounds that can penetrate into insects rapidly and interfere with their physiological functions and growth (Lee, Peterson, and Coasts, 2003). Jacobson (1982) pointed out that the most promising botanical insect control agents are in the families of Annonaceae, Asteraceae, Canellaceae, Lamiaceae, Meliaceae and Rutaceae. As our prescreening study, it was found that mintweed in the family of Lamiaceae and kaffir lime and kitchen mint in the family of Rutaceae present of monoterpene. Therefore, our findings are in agreement with the others.

Uses of botanical products in rice weevil control instead of chemical-derived insecticides can reduce costs and environmental pollution. The extract yield recoveries were in a good correlated with the amounts of total phenolic compounds. Ethanol was able to extract more compound contents than water. The amount of total phenolic content obtained could affect the efficacy of the extract activities in the biological control of insects.

Many species of Lamiaceae are strongly aromatic and toxic to insects, e.g. *Hyptis* and *Ocimum* spp. Other species in this genus were used to control stored product pest insects and other pest insects (Rajendran and Sriranjini, 2008). Considering toxic effects of *H. suaveolens, M. cordifolia*, and *C. hystrix* against *S. oryzae*, it is possible that the extracts of these plants can be used as natural control agents. Most of these plants are widely distributed and easily grown. Furthermore, the extraction method is simple and low cost. The techniques used could be applicable for on-farm use.

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CHAPTER III

CYTOTOXICITY AND BIOCONTROL OF RICE WEEVILS (*Sitophilus oryzaes*) BY MINTWEED *(Hyptis suaveolens* (L.) Poit), KITCHEN MINT (*Mentha cordifolia* Opiz) AND KAFFIR LIME (*Citrus hystrix* DC)

3.1 Abstract

In the present study, the botanicals powder and extracts from three common spices, mintweed *(Hyptis suaveolens* L. Poit), kitchen mint *(Mentha cordifolia* Opiz*)* and kaffir lime (*Citrus hystrix* DC) were test in controlling of rice weevils (*Sitophilus oryzae*), emphasis on repellency, insecticidal activity, and growth inhibition of rice weevil progenies in stored milled rice grains. The plant powder were mixed with 20 g milled rice as direcet admixtures at six dose rates which 0, 0.5, 2.5, 5, 7.5, and 10% (w/w) for mintweed, kaffir lime and 0.25, 0.50, 0.75, 1.00, and 1.25% (w/w) for kitchen mint. The plant extracts were applied at 20 g milled rice as direct admixtures at five dose rates which 0, 0.8, 1.6, 3.2, and 6.4% (v/w) and isolated by extracted using Soxhlet's apparatust. Adults of *S. oryzae* were exposed to the treat milled rice and mortality were assessed after 5, 10, 15, 20 days of exposure. The repellent using cup bioassay were assessed after 15, 30 minute and 1, 12, 24 hour. In the present study, plant extracts were screened for their cytotoxicity using brine shrimp lethality test. Brine shrimp lethality bioassay of ethanol and water extracts of the leaves showed that the extracts were

moderately cytotoxic at high concentration. The LC_{50} values using Probit analysis method for ethanol and water extracts were 131.08 and 59.10 µg/ml, respective. All extracts evaluated were relatively ineffective against the adult stage. It appears that the efficacy in controlling the rice weevils was ranged as kitchen mint>kaffir lime>mintweed; and of the ethanolic extracts were higher than those of water extracts. The most repellent efficacy of ethanolic extract was kaffir lime leaves with EC_{50} of 13.23 mg/ml (24 hours) or 3 fold of control, of water extracts was kitchen mint leaves with EC_{50} of 19.04 mg/ml or 3.5 fold of control.

The leaf powders of mintweed (HLP), kitchen mint (MLP) and kaffir lime (CLP) were able to repel the rice weevils out of the admixed rice grains. The repellent activity of them was dose- and time- dependent fashions.

3.2 Introduction

Rice (*Oryza sativa* L.) is one of the most important staple foods in the world. Stored rice seed and milled rice grains are mostly infested by rice weevils (*Sitophilus oryzae* L.) (Coleoptera: Curculionidae) causing heavily economic losses. They damage rice carbohydrate in the grains leading to weight loss and product contamination (Park, Lee, Choi, Park, and Ahn, 2003). Presently, the control of rice weevils relies on fumigation and residual grain protection by synthetic insecticides, such as DDT, lindane, pirimiphos-methyl, and aluminium phosphide (phosphine) (Daglish, 2004). However, these chemicals are costly. Some are explosive and hard to handle. Some are potentially mutagens inducing insect resistance and carcinogenesis in humans (Collins, 1998; Dal Bello, Padin, Lastra, and Fabrizio, 2001). The biological control of weevils on wheat, corn and rice storages using different plants was evidenced before and after harvest

(Kim, Roh, Kim, Lee, and Ahn, 2003; Law-ogbomo and Enobakhare, 2007). Some crude plant extracts were evaluated for their toxic properties against different stored grain pests. They also proved non-toxicity to humans and the environment (Huang, Chen, and Ho, 2000). The extracts from the families of Asteraceae, Piperaceae, Annonaceae, Laminaceae and Rutaceae were demonstrated to process various biological controls such as antifeedant, repellant, and insecticidal properties to many insect species (Park, Peterson, Zhao, and Coats, 2004; Isman, 2006). Iwu et al. (1990) demonstrated that essential oil isolated from *H. suaveolens* inhibited the growth of both gram-negative and gram-positive bacteria as well as some fungi, *Candida albican* and *Aspergillus niger*. Iloba and Ekrakene (2006) reported that *H. suaveolens* has moderate insecticidal activities against *Sitophilus zeamais* and *Callosobruchus machlatus.* The insecticidal activity of mintweed *(Hyptis suaveolens* L. Poit*),* kitchen mint (*Mentha cordifolia* Opiz) and kaffir lime (*Citrus hystrix* DC) against insect pests were reported (Keita, Umoek, and Smith, 2006). These plants are widely available and used as herbs, vegetables and flavors in tropical countries.

In Thailand, rice is one of the economically important crops and it is appeared that the stored milled-rice grains are damaged by rice weevils (*S. oryzae*) causing devalued for household consumption and export. Therefore, searching for new alternative means to protect rice grains, those are safe for humans and environments, is very essential. The leaf extracts of mint weed, kitchen mint, and kaffir lime were used to evaluate their efficacy in the control of rice weevils in milled rice grain storage.

3.3 Materials and methods

3.3.1 Rice weevil rearing

Adult rice weevils (*S. oryzae*) were obtained from naturally infested milled rice from a local market in Nakornrachsima, Thailand. The insects were reared in a plastic container (3.7 cm height × 7.5 cm diameter) under laboratory conditions at 28±2 ^{o}C, 65-70% relative humidity, 12 hour light : 12 hour dark and covered with a fine mesh cloth for ventilation. Thai jasmine rice (*O. sativa*) var. KDML 105 was used in all experiments. The insects were allowed to deposit their eggs. After 2 weeks of oviposition, they were removed through a sieve with mesh sized of 2 mm. The container was returned to the rearing room and the culture was continued for a month to allow the new adults emerged. The insects of 7-10 days old were used for the experiments (Figure 3.1).

Figure 3.1 Adult rice weevils (*Sitophilus oryzae*) feed on milled rice.

3.3.2 Plant material collection and preparation for extracts

The fresh leaves of mintweed, kaffir lime and kitchen mint were collect from surrounding on Suranaree University of Technology (SUT) campus. The leaves were clean, dried in sunlight, ground into powder, and kept at -20 ^{o}C for further studies. The leaf powder of 30 g was extracted in 100 ml ethanol or water in Soxhlet extraction apparatus at 60 ^{o}C for 48 hours. The crude extracts were filtered, evaporated under reduced pressure at 70 ^{o}C in a rotary vacuum evaporator, and then dried in -80 ^{o}C lyophilizer, the lyophilized powder was stored at 4 ^{o}C.

3.3.3 Cytotoxicity test by brine shimp lethality assay (BSLA)

The brine shrimp lethality bioassay was carried out using standard procedure (Meyer et al., 1982). Artificial seawater was prepared by dissolving 3.6 g of sea salt granules in 1 litter of distilled. The brine shrimps were cultured in a two-compartment box which divided a small and a large compartment by a pored partition. The brine shrimp (*Artemia salina*) dried eggs were sprung into the small compartment filling with the sea water. The large compartment was shined with light. The culture box was kept under the light for 48 hours to allow egg hatching. The adult brine shrimps moved through the pores towards the light into the large compartment. Ten brine shrimps were transferred into a well of a 24-well plate containing 10 ml sea water. The extract was dissolved in 0.1% DMSO and put into the well to obtain the dilutions of 1, 10, 50, 100, 500, and 1000 ug/ml. The culture was continued for 24 hours. The survived brine shrimps were counted and calculated for mortality. The experiments were performed in triplicate. The LC_{50} values were calculated using the Probit analysis method.

3.3.4 Repellent test of plant leaf extracts

The effects of crude extracts on repellence were conducted by a cone bioassay. The setting was as in Figure 3.2. Forty grams of milled rice grains were thoroughly mixed with 0.8, 1.6, 3.2, and 6.4% extracts. The mixed grains were put in a plastic cone strainer with 2 mm pore size. The cone was put on top of the mouth of a bottle (7 × 10 cm^2) which was placed on a plastic plate, coat with glycerine. The adult weevils were introduced into the middle of the mixed grains through a-long funnel. The weevils those escaped out from the cone were counted after 15, 30, and 60 minutes, 1, 12, and 24 hours. Forty percent ethanol and water were used as controls. The tests were done in triplicate and three repeats.

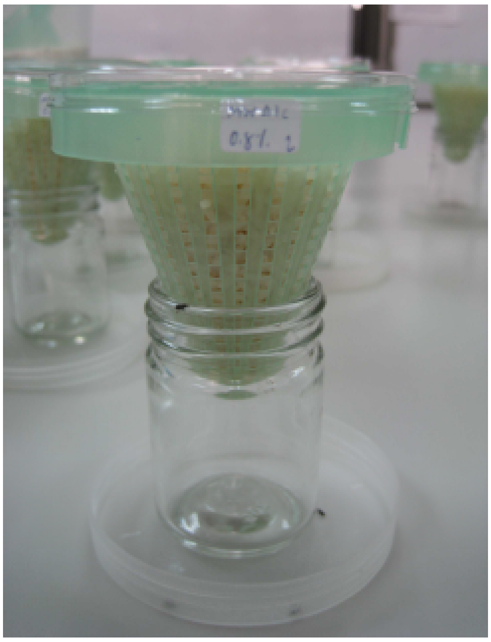

Figure 3.2 The cone bioassay used in rice weevil repellent test.

The perforated cone contained 40-gram milled rice grains which were mixed with various concentrations of extracts. The weevils were introduced into the middle of the cone. The escaped weevils would fall on the plate.

3.3.5 Repellent test of plant leaf powders

The effects of plant powders on repellence were conducted by a cone bioassay. Forty grams of milled rice were mixed with the mintweed or kaffir lime powders at 0.5, 2.5, 5.0, 7.5, and 10.0% (w/w) or with the kitchen mint powder at 0.25, 0.50, 0.75, 1.00 and 1.25%. The admixed grains were put in a perforated cone (2 mm pore size). The cone was rested on the mouth of a bottle (7 × 10 cm^2), which was placed on a plate. Twenty adult rice weevils were introduced into the middle of the admixed grains through a-long stemmed funnel. The whole setting was wrapped around with nylon cloth and tightly secured with a rubber band. After 15, 30, and 60 minutes, 1, 12, and 24 hours, the weevils those escaped from the cone were counted. Unmixed rice grains were used as control. The tests were done in triplicate and three repeats.

3.3.6 Effect of plant leaf extracts on weevil mortality

Twenty-gram milled rice was placed in a bottle (7.5 × 5.5 cm^2) and thoroughly mixed with 0.5 ml of 0.8, 1.6, 3.2, and 6.4% extracts. Twenty-adult weevils were introduced into the mixed grains. The bottle was covered with cloth sheet and secured with a rubber band. The death insects were considered by no response when poked with forceps. The dead weevils were investigated and counted after 5, 10, 15, and 20 days. Forty percent ethanol and water were used as controls. The experiments were done in triplicate and three repeats. The mortality was calculated using the Abbott formula (Abbott, 1925).

3.3.7 Effect of plant leaf powder on weevil mortality

Twenty grams of milled rice grains were mixed with the mintweed or kaffir lime powders at 0.5, 2.5, 5.0, 7.5, and 10.0% (w/w) or with the kitchen mint powder at 0.25, 0.50, 0.75, 1.00, and 1.25%. The admixed grains were put in a-200 ml container. Twenty adult rice weevils were introduced into the admixed grains. The container was covered with cloth sheet, held in place with rubber bands and kept in the culture room. After 5, 10, 15, and 20 hours, the dead weevils were sieved out and counted. The dead weevils were considered by no response when probing with forceps. The experiment was conducted in triplicate and three repeats. The mortality was calculated using Abbott's formula (Abbott, 1925).

3.3.8 Growth and development of weevil progenies by plant leaf extracts

Twenty grams of milled rice were placed in a bottle (7×9 cm^2) and 20-adult weevils were introduced into this bottle. The bottle was then covered with cloth sheet. The weevils were cultured, allowed to lay eggs inside the rice grains and removed after 2 weeks. The rice grains were then thoroughly mixed with 0.5 ml of 0.8, 1.6, 3.2, and 6.4% extracts. The treated grains were then kept until the emergence of F_1 progenies. The number of progenies (F_1) was counted at 49 days of the culture. Inhibition rate (% IR) or reduction of the adult emergence was calculated using Tapondjou, Adler, Bouda and Fontem (2002) method as follows:

$$\text{Inhibition rate (\% IR)} = (\text{Cn-Tn}) \times 100/\text{Cn}$$

where: Cn is the number of newly emerged insects in the untreated (control) sample.

Tn is the number of insects in the treated sample.

3.3.9 Growth and development of weevil progenies by leaf powders

20 g milled rice was placed in plastic container (7×9 cm^2) and 20 unsexed adult of *S. oryzae* were introduced into the containers, and the container were cover with cloth sheet. After 2 weeks, the introduce adult insects were removed from the container. Plant extract were mixed with rice treat at concentrations of 0.8, 1.6, 3.2, 6.4 w/v (1 ml solution with 20 g of treated rice). The grains were stored for 21 days. At the end of 49 days, the number of adults that emerged (F_1) were counted and The percentage of reduction of the weevils was then calculated using the following formula.

Inhibition rate (% IR) or reduction of the adult emergence was calculated using Tapondjou, Adler, Bouda and Fontem (2002) method as follows:

$$\text{Inhibition rate (\% IR)} = (Cn\text{-}Tn) \times 100/Cn$$

Where: Cn is the number of newly emerged insects in the untreated (control) sample.

Tn is the number of insects in the treated sample.

3.4 Statistical analysis

Analyses of variance for each parameter and their interactions were carried out to determine the levels of significance by applying factorial experiment in Completely Randomized Design (CRD). All experiments were performed in triplicate and data are the mean±S.E. Data were subjected to one-way analysis of variance (ANOVA). The means were separated using the Duncan multiple rang tests when ANOVA was significant ($P<0.05$).

3.5 Results

3.5.1 Cytotoxicity of plant leaf extracts

The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxicity (Krishnaraju et al., 2005). In the present study the leaf crude extracts obtained from the plants were screened for their cytotocity by this assay. The LC_{50} values were calculated using Finney computer program. The ethanol extract of kitchen mint (*Mentha cordifolia*) (MLE/e) showed most prominent activity with LC_{50} of 59.10 µg/ml. The extracts of kaffir lime (*Citrus hystrix* DC) (CLE/e) and mintweed (*Hyptis suaveolens*) (HLE/e) exhibited significant brine shrimp lethality with LC_{50} of 67.06, and 88.98 µg/ml, respectively. In addition, the water extracts of mintweed (HLE/w), kitchen mint (MLE/w) and kaffir lime (CLE/w) showed moderate lethality with LC_{50} of 88.98, 131.08, 137.15 µg (Table 3.1). The LC_{50} values those were higher than 1000 µg/ml mean the low cytotoxicity. The degree of lethality was found in direct proportion to the concentration of the extracts. This indicated that the extracts obtained from the plant leaves with LC_{50} much lower than 1000 µg/ml could be potential sources of insecticidal constituents. The significant lethality of several leaf extracts to brine shrimps could indicate the presence of the potent cytotoxic components which warranted further investigation.

Table 3.1 The cytotoxicity of the plant leaf extracts assayed by the brine shrimp mortality at 24 hours. The data represent the mean of LC_{50} values. (n = 6)

Plant Leaves	Extracts	LC_{50} (µg/ml)
Mintweed	HLE/e	88.98
(*Hyptis suaveolens (L.) Poit*)	HLE/w	144.05
Kitchen mint	MLE/e	59.10
(*Mentha cordifolia Opiz*)	MLE/w	131.08
Kaffir lime	MLE/e	67.06
(*Citrus hystrix DC*)	MLE/w	137.15

HLE/w = *Hyptis suaveolens* (L.) Poit leaf water extract

HLE/e = *Hyptis suaveolens* (L.) Poit leaf ethanol extract

MLE/w = *Mentha cordifolia* Opiz leaf water extract

MLE/e = *Mentha cordifolia* Opiz leaf ethanol extract

CLE/w = *Citrus hystrix* DC leaf water extract

CLE/e = *Citrus hystrix* DC leaf ethanol extract

3.5.2 Repellent efficacy

3.5.2.1 Repellency of plant leaf extracts

The repellent effects of mintweed (*Hyptis suaveolens* (L.) Poit), kitchen mint (*Mentha cordifolia* Opiz) and kaffir lime (*Citrus hystrix* DC) leaf extracts on the rice weevils in protecting milled rice grains at 24 hours are dose dependent manner (Tables 3.2, 3.3, and 3.4). At 24 hours of treatment, the repellent efficacy was increased as the extract concentration increased. The repellent effects of HLE/e approximately increased from 49% to 72%; of MLE/e increased from 55% to 75%; and of CLE/e increased from 53% to 80.00% (Tables 3.2). While the repellent effects of HLE/w approximately increased from 46% to 72%; of MLE/w increased from 51% to 72%; and of CLE/w increased from 51% to 71%. At the maximal dose of 6.4%, all extracts showed repellency about 3-3.5 fold of the control. The repellent efficacy of the ethanol extracts were CLE/e>MLE/e>HLE/e and those of the water extracts were MLE/w>CLE/w >HLE/w. The CLE/e showed the highest repellency of 80% at the concentration of 6.4% and 24 hours. It was approximately 3.2 fold of the control. While, the MLE/w showed the highest repellency of 72% or 3.6 fold. The EC_{50} values at 24 hours of the HLE/e, MLE/e and CLE/e were 18.95, 15.63, and 13.23 mg/ml, respectively. The EC_{50} values of the water extracts of these three plants were 27.13, 19.04, and 19.87 mg/ml, respectively.

Table 3.3 shows the repellent effects of the ethanolic extracts of three plant leaves. The CLE/e showed the highest repellency of 80% at the concentration of 6.4% and 24 hours of treatment. However, the lowest repellency of HLE/e was showed 49.17% at the concentration of 0.8% and 24 hours of treatment.

Table 3.4 shows the repellent effects of the water extracts of three plant leaves showed similar pattern as of the ethanol extracts. The MLE/w showed the highest repellency of 71.67% at the concentration of 6.4% and 24 hours of treatment.

However, the repellent effects of all ethanol extracts were slightly higher than those of the water extracts of the same plants as well as of the same concentration levels. It was concluded that the highest repellent activity of the ethanol extracts was the CLE/e and of the water extracts was the MLE/w.

Table 3.2 The dose-dependent repellent effects of mintweed, kitchen mint and kaffir lime leaf extracts on rice weevils in milled rice grain protection at 24 hours. (n= 6)

Conc. (%)	% Repellant activity (Mean±S.E.)					
	Ethanolic extract			Water extract		
	HLE/e	MLE/e	CLE/e	HLE/w	MLE/w	CLE/w
0	23.33±0.10a	23.33±0.10a	23.33±0.10a	20.00±0.57a	20.00±0.57a	20.00±0.57a
0.8	49.17±0.23b	55.00±0.29b	53.33±1.11b	45.83±0.23b	51.67±0.14b	51.33±0.1b
1.6	55.83±0.15b	56.67±0.27b	60.00±0.18c	49.17±0.15b	52.50±0.14b	55.50±0.11c
3.2	64.17±0.15c	65.00±0.41c	66.66±0.08c	57.50±0.15c	66.67±0.15c	62.50±0.10d
6.4	72.50±0.22c	75.00±0.25d	80.00±0.12d	67.50±0.20d	71.67±0.06c	70.83±0.10e
EC_{50} (mg/ml)	18.95	15.63	13.23	27.13	19.04	19.87

a-e Means within a column followed by different letters are significantly, P<0.05 Duncan multiple range test (DMRT).

Table 3.3 Comparison of repellent activities of the leaf ethanol extracts on adult rice weevils at designated concentration and time, 20-adult rice weevils in 40 grams rice grains. (n = 6)

Ethanol extract	Conc. %	% Repellance of adult rice weevils (Mean±S.E.)				
		15 Min	30 Min	1 Hour	12 Hour	24 Hour
HLE/e	0.8	21.66±0.11b	30.83±0.24b	39.17±0.19b	45.83±0.26b	49.17±0.23b
	1.6	34.17±0.13c	41.67±0.16c	48.33±0.15c	53.33±0.10bc	55.83±0.15b
	3.2	50.83±0.13d	54.17±0.13d	54.17±0.07c	59.17±0.16c	64.17±0.15c
	6.4	52.50±0.11d	55.83±0.10d	62.50±0.15d	68.33±0.23d	72.50±0.22c
MLE/e	0.8	21.67±0.26b	28.33±0.75b	40.83±0.47b	51.67±0.17b	55.00±0.29b
	1.6	35.00±0.60c	36.67±0.42c	44.17±0.15b	51.67±0.28b	56.67±0.27b
	3.2	48.33±0.18d	51.67±0.17d	55.00±0.29c	58.33±0.36c	65.00±0.41c
	6.4	52.50±0.41d	53.33±0.27d	61.67±0.44d	66.67±0.46d	75.00±0.25d
CLE/e	0.8	18.33±0.25b	39.17±0.19b	45.00±1.19b	50.83±1.10b	53.33±1.11b
	1.6	31.67±0.21c	40.83±0.12b	49.17±0.19b	54.17±1.13bc	60.00±0.18c
	3.2	34.17±0.20c	44.17±0.15b	50.00±0.09b	59.17±0.09cc	66.66±0.08c
	6.4	44.17±0.18d	53.33±0.11c	65.00±0.11c	75.00±0.16d	80.00±0.12d
Control	40	11.67±0.14a	16.67±0.12a	23.33±0.10a	23.33±0.10a	23.33±0.10a

a-e Means within a column followed by different letters are significantly, $P<0.05$ Duncan multiple range test (DMRT).

Table 3.4 Comparison of repellent activities of the water leaf extracts on adult rice weevils at designated time, 20-adult rice weevils in 40 grams rice grains. (n = 6)

Water extract	Conc. %	% Repellance of adult rice weevils (Mean±S.E.)				
		15 Min	30 Min	1 Hour	12 Hour	24 Hour
HLE/w	0.8	21.67±0.9b	28.33±0.22b	36.67±0.08b	42.50±0.11b	45.83±0.23b
	1.6	27.50±0.12c	35.83±0.13b	41.67±0.11b	45.83±0.17b	49.17±0.15b
	3.2	32.50±0.16d	42.50±0.10c	50.83±0.13c	54.17±0.16c	57.50±0.15c
	6.4	38.33±0.11e	56.67±0.09d	59.17±0.12d	67.50±0.69d	67.50±020d
MLE/w	0.8	21.67±0.18b	26.67±0.09b	35.00±0.15b	41.67±0.08b	51.33±0.14b
	1.6	34.17±0.20c	34.17±0.13c	43.33±0.08c	48.33±0.11c	55.50±0.14b
	3.2	46.67±0.12d	48.33±0.07d	55.00±0.12d	61.67±0.13d	66.67±0.15c
	6.4	51.67±1.11d	52.50±0.07d	61.67±0.21d	70.00±0.10e	71.67±0.06c
CLE/w	0.8	20.83±0.16b	38.33±0.20b	44.17±1.11b	51.67±0.07b	53.33±0.1b
	1.6	30.00±0.16c	40.00±0.18b	49.17±0.10c	54.17±0.10bc	57.50±0.11c
	3.2	32.50±0.18c	46.67±0.18c	51.67±0.11c	58.33±0.13c	62.50±0.10d
	6.4	43.33±0.12d	53.33±0.07d	63.33±0.13d	65.00±0.11d	70.83±0.10e
Control -H_2O		11.67±0.57a	16.67±1.52a	20.00±0.14a	20.00±0.14a	20.00±0.14a

a-e Means within a column followed by different letters are significantly, P<0.05, Duncan multiple range test (DMRT).

Statistical analyses presented in Table 3.5 shows the significances of percent repellent of *S. oryzae* treated with three plant extracts (P), with two solvent extracts (S), with five concentrations (C) and three storage periods (T) and interactions from plant extracts and time, concentration and time, plant extracts and solvent and concentration and time. Their had a significant effect on mortality of *S. oryzae*. While, plant extracts and solvent and concentration , solvent and time show non significant effects.

Table 3.5 Analysis of variance for percent repellance of *S. oryzae* with plant extract treatments, at two solvent, with five concentrations and five times.

Source of Variation	Degree of Freedom	Mean Square	F-value
Plant (P)	2	0.123	4.385*
Solvent (S)	1	1.509	53.790*
P × S	2	0.208	7.408*
Concentration (C)	4	58.444	2083.587*
P × C	8	0.292	10.404*
S × C	4	0.023	0.819
P × S × C	8	0.138	4.931*
Time (T)	4	18.348	654.116*
P × T	8	0.279	9.946*
S × T	4	0.024	0.839
C × T	16	0.286	10.213*
P × S × C × T	88	0.037	1.310*
Error	750	0.028	
Total	900		

* = Significant ($P<0.05$)

P = mintweed, kitchen mint, kaffir lime. S = ethanolic extract, water extract.

C= concentration of 0.8%, 1.6%, 3.2%, 6.4% T = 15 min, 30 min 1, 12, 24 hours.

3.5.2.2 Repellency of plant leaf powders

The leaf powders of mintweed, *Hyptis suaveolens* (L.) Poit (HLP), kitchen mint, *Mentha cordifolia* Opiz (MLP) and kaffir lime, *Citrus hystrix* DC (CLP) were able to repel the rice weevils out of the admixed rice grains. The repellent activity of them was dose-and time-dependent fashions. The repellency steadily increased range from 2.5 to 5.9 fold as the dose increased at 24 hours, compared to the control. Even though, the HLP and the CLP effects were only slightly different as comparing by concentration and incubation time (Table 3.6). The effects were approximately 1.5 fold at 1 hour and 24 hours as compare to 15 minutes. The repellent efficacy of the MLP was very high. As comparing at the same magnitude of concentration 0.5% (w/w) at 24 hours of incubation, the repellent activity of the MLP was approximately 1.6 fold of the others. The MLP at 1.0% which was 10 fold less concentrated still showed similar repellent activity to the other leaf powders. More importantly, MLP at 1.25% and 24 hours of incubation exhibited the absolute repellent activity, 100%, or 6 folds of the control.

Table 3.6 The repellent activity of the leaf powders of mintweed (HLP), kaffir lime (CLP), and kitchen mint (MLP) on rice weevils in stored rice milled grains. The MLP used was at the magnitude of 10 times lower. (n = 6).

Ethanolic extract	Conc. %	% Repellance of adult rice weevils (Mean ± S.E.)				
		15 Min	30 Min	1 Hour	12 Hour	24 Hour
HLP	0.5	28.33±0.88b	30.00±1.00ab	35.00±0.57b	36.66±0.33b	43.33±0.33b
	2.5	36.66±0.88bc	43.33±0.88bc	46.00±0.67c	51.66±0.33c	56.66±0.33c
	5.0	41.66±0.88bcd	53.33±1.76cd	65.00±1.15d	68.33±0.88d	71.66±0.88d
	7.5	50.00±1.15cd	60.00±1.53cd	73.33±0.88d	76.66±0.66de	81.66±0.33e
	10.0	55.00±0.58d	68.33±0.89d	73.33±0.33d	80.00±1.00e	83.33±0.88e
CLP	0.5	31.66±0.67b	35.00±0.58b	36.66±0.88b	41.66±0.88b	45.00±0.58b
	2.5	36.66±0.88bc	38.33±0.88b	45.00±0.58b	58.33±0.88c	63.33±1.45c
	5.0	43.33±0.88bcd	50.00±0.00c	55.00±0.58c	60.00±0.58c	65.00±0.58c
	7.5	46.66±0.88cd	55.00±0.58c	56.66±0.67c	66.66±0.33cd	75.00±0.58cd
	10.0	50.00±0.58d	66.66±0.33d	70.00±0.58d	76.66±0.67d	80.00±0.00d
MLP	0.25	28.33±0.67b	36.66±0.67b	43.33±0.57b	45.00±0.58b	58.33±0.57b
	0.50	30.00±0.58bc	45.00±0.58b	48.33±1.08bc	53.33±0.15bc	70.00±0.58c
	0.75	38.33±0.88cd	56.66±1.18c	60.00±0.58cd	61.66±0.57c	76.66±0.88c
	1.00	45.00±0.58d	61.66±0.57c	70.00±1.64d	73.33±1.08d	85.00±0.58d
	1.25	46.66±0.07d	65.00±0.58c	71.66±0.57c	76.66±0.57d	100.00±0.58
Control		11.66±0.00a	13.33±0.57a	13.33±0.57a	16.66±0.57a	16.66±0.57a

a-e Means within a column followed by different letters are significantly, P<0.05, Duncan multiple range test (DMRT).

3.5.3 Mortality efficacy

3.5.3.1 Mortality effect of plant leaf extracts

All plant leave extracts exhibited strongly insecticidal activity against adult rice weevils. The mortality effects were significantly dose and time dependent ($P<0.05$) (Tables 3.7 and 3.8). The MLEs processed the most potent insecticidal activities. The MLE/e at 6.4% caused nearly 50% mortality of rice weevils in one day of exposure and it was able to get rid rice weevils 100% in 15 days of exposure. Meanwhile, the HLE/e and CLE/e most equally affected about 85%. The mortality effects of the water leave extracts were less toxic than the ethanol leave extracts (Table 3.7). The mortality effect of MLE/w was slightly higher than the others, which was 76.67% at 6.4% in 10 days.

Table 3.8 is the mortality effects of the water extracts of three plant leaves showed similar pattern as of the ethanol extracts. All plant leave extracts exhibited strongly insecticidal activity against adult rice weevils. The mortality effects were significantly dose and time dependent ($P<0.05$). The CLE/w at 6.4% caused 65% mortality of rice weevils in 5 days of exposure and it was able to get rid rice weevils 70% in 10 days of exposure. While, MLE/w at 6.4% caused 63.33% mortality of rice weevils in five days of exposure and it was able to get rid rice weevils 76.67% in 10 days of exposure. The mortality effect of HLE/w was slightly lower than the others, which was 61.67% at 6.4% in 5 days and 70.00% at 6.4% in 10 days.

Table 3.7 Mortality effects of ethanolic extracts of mintweed (HLP/e), kaffir lime (CLP/e), and kitchen mint (MLP/e) on rice weevils, 20-adult rice weevils in 20-gram milled rice grains. (n = 6)

Ethanolic extract	Conc. %	% Mortality of adult rice weevils (Mean±S.E.)				
		Day 1	Day 5	Day 10	Day 15	Day 20
HLE/e	0.8	21.67±0.18b	39.17±0.30b	55.83±0.23b	61.67±0.19b	65.00±0.13b
	1.6	25.83±0.30b	41.67±0.27bc	64.17±0.15c	76.67±0.17c	79.17±0.15c
	3.2	35.00±0.19c	51.67±0.21ed	74.17±0.15d	83.33±0.11cd	85.83±0.18cd
	6.4	43.30±0.18d	60.80±0.24d	80.00±0.10d	86.70±0.05d	90.00±0.11d
MLE/e	0.8	22.50±0.22b	42.50±0.16b	63.33±0.25b	66.67±0.15b	70.00±0.10b
	1.6	30.83±0.31c	51.67±0.14c	67.50±0.17bc	81.67±0.13c	85.00±0.12c
	3.2	40.00±0.10d	55.83±0.10c	75.00±0.18c	91.67±0.08d	99.17±0.04d
	6.4	45.83±0.11d	72.50±0.18d	85.00±0.06d	95.83±0.12d	100.00±0.00d
CLE/e	0.8	17.50±0.25b	38.33±0.14b	51.67±0.09b	61.67±0.18b	65.83±0.14b
	1.6	20.83±0.28bc	51.67±0.23b	59.17±0.14bc	67.50±0.20bc	71.67±0.19c
	3.2	24.17±0.38c	58.33±0.24c	66.67±0.15c	76.67±0.15cd	83.33±0.13c
	6.4	35.00±0.15d	70.00±0.19c	79.17±0.17d	85.00±0.18d	91.67±0.18c
Control Alc 40%		0.00±0.00a	3.33±0.36a	5.00±0.34a	5.00±0.27a	5.00±0.27a

a-e Means within a column followed by different letters are significantly, P<0.05, Duncan multiple range test (DMRT).

Table 3.8 Mortality effects of water extracts of mintweed (HLP/w), kaffir lime (CLP/w), and kitchen mint (MLP/w) on rice weevils. 20-adult rice weevils in 20-grams milled rice grains. (n = 6)

Water extract	Conc. %	% Mortality of adult rice weevils (Mean±S.E.)				
		Day 1	Day 5	Day 10	Day 15	Day 20
Mintweed	0.8	8.33±0.33ab	40.00±0.57b	45.00±0.57b	53.33±0.66b	53.33±0.66b
	1.6	15.00±0.57bc	43.33±0.33bc	56.67±0.66bc	61.67±0.33b	66.67±0.88b
	3.2	20.00±1.20bc	55.00±0.57d	61.67±1.20c	71.67±0.66c	71.67±0.66c
	6.4	26.00±0.88c	61.67±1.40e	70.00±1.00c	71.67±0.66c	71.67±0.66c
Kitchen mint	0.8	13.33±0.88ab	41.67±0.66b	48.33±0.33b	61.67±1.20b	63.33±0.88b
	1.6	18.33±0.88bc	45.00±0.57bc	55.00±0.57bc	65.00±1.20b	68.33±0.66b
	3.2	21.67±0.57bc	56.67±1.20cd	63.33±0.88c	71.67±0.66b	73.33±0.88b
	6.4	30.00±0.33c	63.33±1.20d	76.67±1.20d	76.67±1.20b	76.67±1.20b
Kaffir lime	0.8	11.67±0.57b	38.33±0.66b	46.67±0.33b	60.00±1.20b	60.00±1.20b
	1.6	15.00±1.20b	46.67±0.33b	58.33±0.66c	63.33±0.88bc	63.33±0.88bc
	3.2	21.67±0.66bc	56.67±0.88c	61.67±0.66cd	71.67±0.33c	73.33±0.33c
	6.4	28.33±1.15c	65.00±0.57c	70.00±0.57d	73.33±0.33c	73.33±0.33c
Control-H_2O		0.00±0.00a	1.67±0.33a	3.33±033a	5.00±0.00a	5.00±0.00a

a-e Means within a column followed by different letters are significantly, P<0.05, Duncan multiple range test (DMRT).

Statistical analyses presented in Table 3.9 shows the significances of percent mortality of *S. oryzae* treated with three plant extracts (P), with two solvent extracts (S), with five concentrations (C) and three storage periods (T). Similary various interactions from plant extracts and solvent, plants extracts and time, plant extracts and concentration. They had a significant effect on mortality of *S. oryzae*. While, plant extracts and solvent, and concentration and time show non significant effects.

Table 3.9 Analysis of variance for percent mortality of *S. oryzae* with plant extract treatments, at two solvents, with five concentrations and five times.

Source of Variation	Degree of Freedom	Mean Square	F-value
Plant (P)	2	1.511	39.984*
Solvent (S)	1	12.052	318.847*
P × S	2	0.307	8.120*
Concentration (C)	4	212.911	5632.536*
P × C	8	0.110	2.904*
S × C	4	0.331	8.745*
P × S × C	8	0.050	1.331
Time (T)	4	58.394	1544.795*
PxT	8	0.064	1.702*
SxT	4	0.168	4.435*
C × T	16	1.694	44.806*
P × S × C × T	88	0.040	1.062
Error	750	0.038	
Total	900		

* = Significant ($P<0.05$)

P = mintweed, kitchen mint, kaffirlime S = ethanolic extract, water extract

C= concentration of 0.8%, 1.6%, 3.2%, 6.4% T = 15 min, 30 min 1, 12, 24 hour

3.5.3.2 Mortality effect of plant leaf powders

The leaf powders of mintweed, *Hyptis suaveolens* (L.) Poit (HLP), kitchen mint, *Mentha cordifolia* Opiz (MLP) and kaffir lime, *Citrus hystrix* DC (CLP) effectively cause death to rice weevils in stored rice milled grains. The mortality efficacy of them was dose- and time-dependent patterns. The HLP and the CLP of the same magnitude of concentration and incubation time showed similar mortality effects. These two leaf powders were able to totally get rid of the rice weevils at 7.50% (w/w) and 24 hours. It was evidently, the MLP was the most potent insecticide to rice weevils. The MLP could cause total death of rice weevils at a very low concentration of 0.25-0.50% within 20 days and of 0.75% within 15 days (Table 3.10). When comparing the magnitude of powder concentration at 0.5%, the MLP could cause mortality of the rice weevils approximately 2 folds.

Table 3.10 The mortality of leaves powder of mintweed, kitchen mint, and kaffir lime on adult rice weevils in admixed milled rice grains. (n = 6)

Plant powder	% Mortality of adult rice weevils (Mean±S.E.)					
	%	Day 1	Day 5	Day 10	Day 15	Day 20
Mintweed	0.50	12.50±0.31b	21.67±0.35b	40.83±0.40b	49.17±0.21b	65.83±0.14b
	2.50	15.83±0.36bc	25.83±0.27b	42.50±0.41b	63.33±0.25c	77.50±0.12c
	5.00	21.67±0.29cd	39.17±0.37c	51.67±0.31bc	72.50±0.12d	90.00±0.09d
	7.50	24.17±0.38d	43.33±0.44c	59.17±0.23c	78.33±0.13de	97.58±0.08e
	10.00	27.50±0.15d	46.67±0.41c	63.33±0.33c	83.33±0.17e	100.00±0.00e
Kaffir lime	0.50	20.00±0.30b	25.83±0.27b	31.67±0.21b	44.17±0.18b	70.83±0.09b
	2.50	20.83±0.31b	30.83±0.31bc	42.50±0.19c	63.33±0.22c	85.33±0.16c
	5.00	24.17±0.17bc	36.67±0.23cd	50.83±0.34cd	73.33±0.14d	94.17±0.12d
	7.50	26.67±0.33bc	39.13±0.26d	61.67±0.28de	79.17±023de	100.00±0.00d
	10.00	30.00±0.28c	41.74±0.18d	64.17±0.72e	83.67±0.13e	100.00±0.00d
Kitchen mint	0.25	18.33±0.29b	35.00±0.15b	71.67±0.26b	95.83±0.10a	100.00±0.00d
	0.50	26.67±0.19bc	41.67±0.13c	78.33±0.24b	99.17±0.60b	100.00±0.00b
	0.75	31.67±0.21cd	50.83±0.16d	86.67±0.08c	100.00±0.00b	100.00±0.00b
	1.00	35.00±0.19d	63.33±0.10e	92.50±0.11cd	100.00±0.00b	100.00±0.00b
	1.25	40.83±0.18e	83.33±0.17f	97.50±0.08d	100.00±0.00b	100.00±0.00b
	Control	0.83±0.27a	5.00±0.00a	5.00±0.00a	5.00±0.00a	5.83±0.00a

a-f Means within a column followed by different letters are significantly, $P<0.05$, Duncan multiple rank test (DMRT).

3.5.4 Efficacy on growth and development of rice weevil progeny

3.5.4.1 Growth and developmental effect of plant leaf extracts

The rice weevil progenies those grew and developed in the extract treated-milled rice grains were collected in one life cycle (F_1). The growth and development of the rice weevil progenies was significantly inhibited by all plant extracts in a dose dependent pattern (Table 3.11). The MLE more potently reduced the emergence of the weevil new generations than the others which showed similar reduction. The growth and developmental reduction of F_1 by MLE/e was range from 70% to 89% or 3.4-8.7 folds as compared to the control. While the reduction by water extract was range from 61% to 81% or 2.6-5.3 folds of the control.

3.5.4.2 Growth and developmental effect of plant leaf powders

The rice weevil progenies those grew and developed in the admixed-milled rice grains were collected in one life cycle (F_1). The growth and development of the rice weevil progenies was significantly reduced by all plant powders in a dose-dependent pattern (Table 3.12). The MLP more potently reduced the emergence of the weevil new generations than the others which were similar reduction. The F_1 reduction by MLP was range from 42% to 74% or 3.4-8.7 folds as compared to the control.

Table 3.11 Numbers and the inhibition of growth and development of rice weevil progenies by the leaf extracts of mintweed (HLE), kitchen mint (MLE) and kaffir lime (CLE). The emergence of the weevil progenies from the infested milled rice grains was significantly reduced (n = 6)

Plant	Conc. %	Progeny emerged			
		Ethanolic extract		Water extract	
		Average of number F_1 Progeny±S.E.	% inhibition	Average of number F_1 Progeny±S.E.	% inhibition
HLE	0.8	14.67±0.33c	64.52	19.33±0.33c	60.00
	1.6	12.33±0.88bc	70.16	15.00±0.57b	68.97
	3.2	11.33±0.66b	72.58	13.33±0.88b	72.41
	6.4	7.67±0.88a	81.45	10.67±0.66a	77.93
MLE	0.8	12.00±1.20c	70.97	18.67±0.33c	61.38
	1.6	10.67±0.33c	74.19	15.00±1.20b	68.97
	3.2	8.00±0.57b	80.65	11.33±0.88a	76.55
	6.4	4.67±0.33a	88.71	9.30±0.33a	80.69
CLE	0.8	14.33±0.88c	65.32	21.67±1.20c	55.17
	1.6	12.67±0.33bc	69.35	19.33±0.33bc	60.00
	3.2	10.33±0.88ab	75.00	17.67±0.66ab	63.45
	6.4	8.67±0.88a	79.03	15.67±0.33a	67.59
Control		41.33±0.88d	0.00	48.33±0.88d	0.00

a-c Means within a column followed by different letters are significantly, P<0.05, Duncan multiple range test (DMRT).

Table 3.12 Numbers and the inhibition of growth and development of rice weevil progenies by the leaf powders of mintweed (HLP), kitchen mint (MLP) and kaffir lime (CLP). The emergence of the weevil progenies from the infested milled rice grains was significantly reduced. (n = 6)

Plant leaf powder	Conc. %	Progeny emerged	
		Mean number of F_1 Progeny±S.E.	% inhibition
HLP (Mintweed)	0.50	36.66±1.20d	33.52
	2.50	35.33±1.25d	42.05
	5.00	28.66±0.88c	51.14
	7.50	22.66±1.20b	67.05
	10.00	19.00±0.57a	71.02
MLP (Kaffir lime)	0.50	38.33±1.20d	40.00
	2.50	28.33±0.88c	51.76
	5.00	26.33±1.20bc	54.71
	7.50	23.33±0.88b	63.53
	10.00	17.66±1.25a	68.24

a-e Means within a column followed by different letters are significantly, P<0.05, Duncan multiple range test (DMRT).

Table 3.12 Continued.

Plant leaf powder	Conc. %	Progeny emerged	
		Mean number of F_1 Progeny±S.E.	% inhibition
CLP (Kitchen mint)	0.50	28.00±0.57c	44.71
	0.75	23.33±0.88b	51.18
	1.00	17.66±0.88a	66.47
	1.25	15.66±0.88a	74.12
Control	0	61.33±0.88e	0

a-e Means within a column followed by different letters are significantly, $P<0.05$, Duncan multiple range test (DMRT).

3.6 Discussion

The biological activity of plant extracts and powders in controlling insects was reported and included toxicity to eggs, larvae and adult; repellence to adults; and reduction or inhibition of oviposition and adult emergence (Tapondjou, Adler, Bouda, and Fontem, 2002; Boeke et al., 2004). Some plants were found to contain natural toxins, which were effective against rice weevil (Lee, Peterson, and Coasts, 2003). In this study, the cytotoxic potency of *H.saovelens, M. cordifolia,* and *C. hytrix,* was pre examined by Brine shrimp lethality bioassay (BSLA) using brine shrimp as an experimental model. Brine shrimp is a crustacean whose larvae are sensitive to a variety of substances, the brine shrimp bioassay can be useful as a quick and simple test for predicting the toxicity of plant extracts and guiding their phytochemical fractionation (Soly, Wright, Anderson, Gupta, and Phillipson, 1993). In toxicity evaluation of plant extracts by brine shrimp bioassay, an LC_{50} value lower than 1000 μg/ml is considered bioactive (Meyer et al., 1982). Brine shrimp lethality bioassay of the ethanol and water leaf extracts of *H. saovelent, M. cordifolia,* and *C. hytrix* showed that the extracts were moderately cytotoxic at high concentrations.

The kaffir lime ethanolic extract exhibited high repellency to rice weevils, which was similar to the extract of neem acetone extract (Rahman and Schmidt, 1999) and to the extract of *Urtica dioica* L.and *Taraxacum officinale* (Jovanovic et al., 2007). The repellent efficacy of different plants on stored products was also evident (Rahman and Talukder, 2006; Jovanovic et al., 2007). There were some evidence supported that the strong choky odors of kitchen mint and kaffir lime exerted a toxic effect by disrupting normal respiratory activity of the weevils resulting in asphyxiation and subsequent death (Odeyemi, Masika, and Afolayan, 2008). Some plants contained irritant and foul smelling chemicals to which strongly repelled stored product insect pests (Adedire and Ajayi, 1996; Arannilewa, Ekrakene, and Akinneye, 2006). These supports the repellent activities of our studied plants to the rice weevils, which they are known naturally odor.

The insecticidal activities of mintweed, kitchen mint, and kaffir lime leaf extracts on rice weevils were dose and time dependent manner. The ethanolic extracts of all plants seemed to process stronger activities. The most potent repellent activity was obtained from the kitchen mint ethanol leaf extracts. Our findings are in agreement with the insecticidal activities of bakain drupes *(Melia azdarach)* and star anise (*Illicium verum* Hooker fil.) on *Tribolium.castaneum* infested rice grains (Saljoqi, Afridi, Khan, and Rahman, 2006). There are some reports supporting that compounds from plant extracts such as menthone, 1,8 cineol, limonene and linalool could be insecticides against insect-stored products (Peerzada, 1997; Aggarwal, Prajapati, and Sushil, 2001; Lee, Peterson, and Coasts, 2003; Lee, Annis, Tumaalii, and Choi, 2004; Isman, 2006). In this study also exhibited that the leaf extracts of mintweed, kitchen mint, and kaffir lime inhibited the growth and development of the rice weevil progeny (F_1) in stored milled-rice grains. This means that the rice grains could significantly protected earlier from the young rice weevils.

Similarly, rosemary essential oil protected against growth of *Acanthoscelides obtectus (Say) obtectus* (Tunc, Berger, Erler, and Dagli, 2000) and *Eucalyptus* essential oil completely inhibited F_1 progeny emergence of *Callosobruchus maculates* (Rahman and Talukder, 2006). Toxicity of plant powder towards insects stored product has been reported by several authors (Ashamo and Odeyemi, 2001; Lale, 2002; Ashamo and Akinneye, 2004). Udo (2005) reported that the powder of *P.guineense* can be against maize weevil (*Sitophilus zeamais* Mots) at 1% and 5% concentrations. In this study showed that plant powders and plant extracts all of treatments significantly repellency, insecticide activity and inhibited the growth and development of the rice weevil progeny

(F_1) the test insect in milled rice compare to control. The protection of the grains against rice weevil (*S. oryzae*) damage by these plant powders and plant extracts indicates that these materials, especially *M. cordifolia* could be of value in storage protection against the weevil.

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CHAPTER IV

FUMIGANT TOXIC ACTIVITY BY MINTWEED, KITCHEN MINT AND KAFFIR LIME LEAF EXTRACTS ON RICE WEEVILS (*Sitophilus oryzaes*)

4.1 Abstract

The ethanol and water leaf extracts of *H. saoveolens, M. cordifolias*, *C. hytrixs* processed insecticidal activity against *S. oryzae* adults by fumigation and dipping methods. The fumigation by *M. cordifolias* extract at 6.4% caused mortality of *S. oryzae* adults over 90% at 24 hours. The ethanol extracts by dipping test exhibited the direct contact toxicity to *S. oryzae* than the water extracts. The toxic activities of the leaf extract were ranged as *M. cordifolias* (MLE)>*C. hytrixs* (CLE)>*H. saoveolens* (HLE). The MLE/e fumigation was the most effective with LC_{50} value of 9.02 mg/ml at 24 hours. The HLE/w fumigation was the least toxicity with LC_{50} of 17.06 mg/ml at 24 hours. The direct contact of the extracts showed similar toxic effects as the fumigation, but slightly less. The LC_{50} value of MLE/e activity was 17.54 mg/ml at 24 hours. However, the lowest direct contact toxicity was from CLE/w with LC_{50} value of 27.41 mg/ml at 24 hours. The prolonged observation of the direct contact treatment, the toxicities of all extracts were increased approximately 2 folds. It is concluded that the toxic activities of *H. saoveolens, M. cordifolias* and *C. hytrixs* leaf extracts on *S. oryzae* by fumigation and direct contact are dose and time dependent manner.

4.2 Introduction

Milled rice grains in storage have often been damaged by insect infestation. The grains qualitatively and quantitatively are loose. Protecting rice grains and other dry foods from insect infestation by fumigation is one of the most effective methods, which still presently practice. Currently, there are only two fumigants widely used against stored product insect pests, i.e., methyl bromide and phosphine. The use of these fumigant chemicals may result in accumulation of toxic residues in the stored grains. The contaminate grains possibly carries the toxicity over to consumers and environment. In addition, it is very costly in the application of these chemical fumigants. However, in 2004 methyl bromide was banned in many countries because of its ozone depleting property (Hansen and Jensen, 2002). Under the Montreal protocol, the world decided to restrict the use of these fumigants in developed countries in 2005 and in developing countries in 2010 (World Meteorological Organization, 1995). Many alternatives have been tried to replace the methyl bromide fumigation in stored products. Additionally, there are reports that in many countries, insects developed resistance to phosphine (Benhalima, Chaudhry, Mills, and Price, 2004; Rahman and Talukder, 2006). There is an urgent need to develop safe alternative means that have potential to replace the toxic fumigants (Ayvaz, Albayrak, and Karaborklu, 2008). Plant extracts are considered as non-pollutant, less toxic and easily bio-degradable. Certain plants have already been reported to possess repellent, ovicidal, antifeedant, fumigants and contact toxicants against stored grain pests. (Su, 1991; Rajapakse and Emden, 1997). A large number of plant extracts from various herbs were assessed for fumigant toxicity against insects. Extracts and components from more than 75 plant species belong to different families were studied for fumigant toxicity against insect pest (Rajendran and Sriranjini, 2008). Kim, Park, Ohh, Cho, and Ahn (2003) tested methanol extracts from 30 aromatic medicinal plant species and five essential oils for their insecticide activities against adults of *Sitophilus oryzae* and *Callosobruchus chinensis* using direct contact application and fumigation methods. Recently, the determination of the insecticide activity of isolated chemical compounds from plant extracts in order to find out the most biologically active chemical components was focused (Lee, Choi, Lee, and Park, 2001). The insecticidal constituents of many plant extracts and essential oils were identified as monoterpenoid (Ahn, Lee, Lee, and Kim, 1998). It was also claimed that monoterpenoids have comparably fumigant action as methyl bromide (Shaaya, Kostjukovski, Eilberg, and Sukprakarn, 1997). Establishing the monoterpenoids as fumigants and contact toxicants on various insect pests was tried (Lee, Annis, Tumaalii, and Choi, 2004). The high volatile property of these bio-fumigants could also be of a great importance for protection of stored product from insects, including rice weevils in milled rice storage.

4.2.1 Plant fumigation

Biological control of insect pests by fumigation remains one of the most effective methods, in particularly for protecting grains from insect infestation. However, complications such as pest resistance and ozone depletion associated with the fumigants are great concern of human beings and for environment. This has necessitated a search on natural occurring substances for alternative of choices. It is suggested that fumigants from plant origins could have a greater potential for this purpose in the future. It is based on their efficacy, economic value and safely use in large-scale storages. Lee, Choi, Lee, and Park (2001) reported that the efficacy of 1, 8 cineole extracts from eucalyptus and benzaldehyde occurring in peach (*Prunus* *persica*) were used as fumigants for the control of rice weevils. There was an evident that the essential oil from some plants of the family Laminaceae induced 90% mortality of the adult populations of the rice weevils, maize weevils and cowpea weevils (Shaaya, Kostjukovski, Eilberg, and Sukprakarn, 1997). Prates et al. (1998) also showed that limonene expressed fumigant activity against *Tribolium confusum.* Varma and Dubey (2001) demonstrated that the essential oils from *Caesulia axillaris* and corn mint (*Mentha arvensis*) could be used as fumigants for protecting wheat grain against insect pests. Khatavkar, Walia, Srivastava, Kumar, and Parmar (2005) observed that hydro distilled neem leaf containing volatile oil showed fumigant activity against *Callosobruchus maculates* and *T. castaneum.* The oils from cinnamon (*Cinnamomum zeylandicum*), mustard (*Brassica juncea*), *Cocholeria linne* and common sweet flag were effective fumigants against *S. oryzae* (Kim, Park, Ohh, Cho, and Ahn, 2003). Recently, the essential oils of plants, mainly belong to Apiaceae, Lamiaceae, Lauraceae and Myrtaceae and their components, monoterpenoids and others, were tested for fumigant toxicity against stored insect pests including *S.oryzae* and *T. castaneum* effectively (Rajendran and Sriranjini, 2008). In addition, eucalyptus showed a broad fumigation activity because it could be most likely attributes to the presence of a wide variety of volatile compounds, especially 1, 8, cineol, aldehydes, terpenes, sequiterpenes, alcohols and phenols (Negahban and Moharramipour, 2007) This will provide further evidence that natural products could be used as fumigants for the control of stored grain insect pests. In this study, we evaluated the fumigation and contact toxic effects of plant extracts from the leaves of mintweed (*Hyptis suaveolens*), kitchen mint (*Mentha cordifolia*) and kaffir lime (*Citrus hystrix*) on the biological control of *S.oryzae* in stored milled rice. These plants are evident of having insecticidal property against insects, including stored product insect pests. Thus, this study would be of benefit for rice storage of house hold and industrial scales.

4.3 Materials and methods

4.3.1 Plant collection and preparation for extraction

Mintweed (*Hyptis suaveolen*s), kitchen mint (*Mentha cordifolia*) and kaffir lime (*Citrus hystrix*) leaves were collected, cleaned, sun dried and ground to powders. The powdered plants were extracted in 80% ethanol and water using Soxhlets extraction apparatus. The extracts were evaporated, freeze dried and kept refrigerated. The extract powders were dissolved in their original solvents as designated in the experiments.

4.3.2 Insect rearing

Rice weevils, *Sitophilus oryzae,* were collected from infested milled rice (*Oryzae sativa*) and reared in a 600-ml plastic container containing non-infested milled rice, covered with a cloth sheet and kept at 28±2 ^{o}C, 65-70% relative humidity and 12 : 12 hours/light : dark. Thai jasmine rice, KDML 105, was used for the rice weevil culture in all experiments. The rice weevils were allowed to lay eggs in the rice grains for 2 weeks. After oviposition, the parents were removed through a 2-mm mesh sieve. The container containing the same rice grains was then returned to continue the culture for one month to allow the new adults emerge. These adults were exploited in the experiments.

4.3.3 Fumigation test

The fumigation chamber was designed by using a 600-ml wide-mouth bottle with screw lid. A 3-cm diameter filter paper was blotted with 100 µl of the extracts at 0, 0.8, 1.6, 3.2, and 6.4% and then placed in the centre of a perforated cone. This cone was placed inside the fumigation chamber (Figure 4.1). Twenty adult rice weevils were put into a 20-ml vial, covered a nylon cloth and secured with elastic rubber band. The vial was then placed into the fumigation chamber. The chamber was covered with a screwed lid. The dead weevils were counted after 6, 12, 24 hours. Forty percent ethanol and water were used as controls. The tests were done in triplicate and three repeats. The LC_{50} values were calculated by Probit analysis.

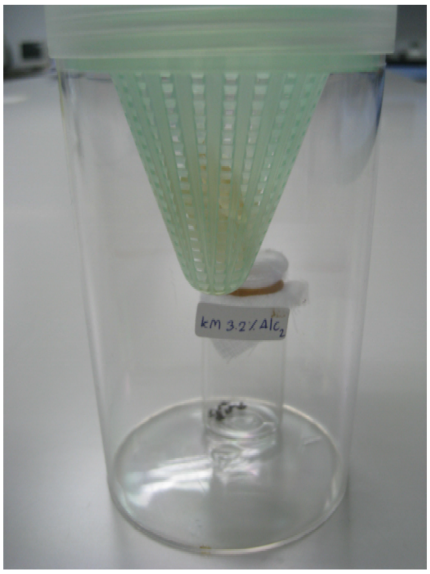

Figure 4.1 Fumigation chamber comprises of a 600-ml bottle with a screwed lid, a perforated cone containing an extract blotted filter and a 20-ml vial containing the rice weevils.

4.3.4 Direct toxicity test

Direct toxicity test was conducted by dipping method according to the method of Rahman and Talukder (2006) with some modifications. Adult rice weevils were chilled for 3 minutes in order to immobilize them. One milliliter of extract at 0, 0.8, 1.6, and 3.2% was added onto a-24 well plate. Twenty immobilized rice weevils were individually picked up and dipped into the wells for 30 seconds. The weevils were then removed, air dried, placed onto a Petri dish. The dead insects were counted at 12, 24, and 48 hours afterward. Forty percent ethanol and water were used as controls. The tests were done in triplicate and three repeats. Mortality data were corrected by Abbott formula and analyzed by Duncan's Multiple Range Test (DMRT). The LC_{50} and values were calculated by Probit analysis.

4.4 Data analysis

All data were analyzed using Analysis of Variance (ANOVA) and Completely Randomized Design (CRD). The mean values were compared using the Duncan's Multiple Range Test (DMRT) at 95% significant level. The mortality counts were corrected by Abbott's formula (Abbott, 1925). Probit analysis was used to estimate LC_{50} values.

4.5 Results

4.5.1 Fumigation effect

The direct relationship between concentration and mortality was observed. The extracts were relatively high toxic against adult rice weevils. The mortality of the adults was over 80% when insects were exposed to ethanol extracts at a concentration of 6.4% for 24 hours (Table 4.1). The fumigant efficacy of ethanol extracts and water extract were ranged as MLE (kitchen mint leaf extract)>CLE (kaffir lime leaf extract)>HLE (mintweed leaf extract). The highest mortality of the rice weevils was 94.17% obtained from MLE/e treatment with LC_{50} of 9.02 mg/ml. The lowest concentration at 0.8% of HLE/w produced 39.17% mortality of the insects and LC_{50} of 17.06 mg/ml. All extracts showed fumigation effect approximately 2-3.7 folds of the control.

The effects of the ethanol extracts and their associated interactions were significant, $P< 0.05$. The efficacy of toxicity expressed as LC_{50} was given in Table 4.2. The highest toxic efficacy at 24 hours was by MLE/e with LC_{50} of 9.02 mg/ml and by MLE/w with LC_{50} of 12.24 mg/ml. While, the lowest toxic efficacy of ethanol extract was by HLE/e with LC_{50} of 13.68 mg/ml and of water extract was by HLE/w with LC_{50} of 17.06 mg/ml.

The information shown in Table 4.3 indicates that the fumigation effect on the rice weevils obtained from the treatment of the ethanol and water extracts (S) of these three plant leaves (P), and the concentrations (C) is in a good correlation. The concentrations (C) are significantly correlated to the plants (P) and the solvents (S).

Table 4.1 Fumigation effect of the leaf extracts of mintweed, kitchen mint, and kaffir lime leaves on the rice weevils at 24 hours.

Conc. %	%Mortality (Mean±S.E.)					
	Ethanol extract			Water extract		
	HLE/e	MLE/e	CLE/e	HLE/w	MLE/w	CLE/w
control	2.50±0.36a	2.50±0.36a	2.50±0.36a	3.33±0.45a	3.33±0.45a	3.33±0.45a
0.8	42.50±0.12b	50.00±0.09b	44.17±0.22b	39.17±0.16b	46.67±0.07b	42.50±0.08b
1.6	47.50±0.15c	61.67±0.13bc	56.67±0.28b	50.00±0.07c	50.00±0.12b	50.83±0.13c
3.2	68.33±0.14d	80.83±0.15cd	71.67±0.14c	58.33±0.07c	63.33±0.13c	55.00±0.12d
6.4	80.00±0.07d	94.17±0.07d	82.50±0.09d	67.50±0.12d	78.33±0.09d	75.00±0.10d
LC_{50} (mg/ml)	13.68	9.02	10.94	17.06	12.24	15.12

a-d Mean values within a column followed by different letters are significantly, $P< 0.05$, Duncan multiple range test (DMRT).

Table 4.2 Fumigation efficacy of the plant leaf extracts on the rice weevils at 6, 12 and 24 hour treatments. The data were expressed as mean, lower and upper LC_{50} values.

Time (hours)	**Ethanol extracts**			**Water extracts**		
	LC_{50} (mg/ml)			**LC_{50} (mg/ml)**		
	H. suaveolens **(HLE/e)**	***M. cordifolia*** **(MLE/e)**	***C. hystrix*** **CLE/e)**	***H. suaveolens*** **(HLE/w)**	***M. cordifolia*** **(MLE/w)**	***C. hystrix*** **CLE/w)**
6	39.42	20.66	24.40	63.15	48.93	54.16
	(26.80,81.58)	(16.22,25.96)	(15.59,40.36)	(36.10,409.89)	(32.11,123.69)	(35.63,135.38)
12	21.00	12.46	14.77	31.32	22.26	23.87
	(14.73,29.14)	(8.24,16.36)	(9.93,19.53)	(20.27,64.20)	(15.17,31.63)	(15.62,37.74)
24	13.68	9.02	10.94	17.06	12.24	15.12
	(9.78,17.43)	(6.68,11.16)	(7.50,14.09)	(10.87,23.74)	(7.69,16.42)	(9.78,20.42)

Table 4.3 Analysis of variance shows the correlation among the plants, extracted solvents, concentrations and treatment times in fumigation of mint weed, kitchen mint, and kaffir lime leaf extracts on rice weevils.

Source of Variation	Degree of Freedom	Mean Square	F-value
Plant (P)	2	0.851	14.121*
Solvent (S)	1	4.910	81.48*
P × S	2	0.370	6.133*
Concentration (C)	4	132.789	2203.949*
P × C	8	0.119	1.978*
S × C	4	0.532	8.824*
P × S × C	8	0.088	1.454
Time (T)	2	8.498	141.039*
P × T	4	0.088	1.46
S × T	2	0.016	0.262
C × T	8	0.216	3.579*
P × S × C × T	44	0.043	0.709
Error	450	0.060	
Total	540		

* = Significant ($P<0.05$)

P = mintweed, kitchen mint, kaffir lime S = ethanol extract, water extract

C = concentration of 0 ,0.8%, 1.6 %, 3.2%, 6.4% T = 6, 12, 24 hours

However, the plants (P), the solvents (S), the concentrations (C), and the treatment times (T) are not significantly correlated. The treatment times (T) are not significant to neither the plants (P) and the solvents (S), but the concentration (C) of the extracts applied and the time incubated (T) is also well correlated, no matter what solvents (S) used. It can conclude that the fumigation effects of these three leaf plant extracts are concentration and treatment time dependent.

4.5.2 Direct toxicity

The direct toxicity as the leaf extracts directly contact the whole rice weevils was observed. It appeared that the MLEs were more toxic to the adult rice weevils than the others. The MLE/e at 6.4% and at 24 hours was able to induce mortality over 90% with LC_{50} value of 17.54 mg/ml, while the MLE/w induced 80% mortality with LC_{50} value of 23.05 mg/ml. The HLE/e and CLE/e were relatively produced same toxicity. The HLE/w and CLE/w toxicities were similar (Table 4.4).

The LC_{50} values observed at 12, 24, and 48 hours were given in Table 4.5. It is noticed that the direct toxicity of all extracts were increased when prolonged treatment. The LC_{50} values at 48 hours were reduced as the extract concentration increased proportionately, i.e., the direct toxicity was dose- and time-dependent manner.

Table 4.4 Direct toxic effects of plant leaf extracts by dipping method. The adult rice weevils were dipped in the extract at designated concentrations for 30 seconds. The dead insects were counted at 24 hours.

Conc.%	% Mortality (Mean±S.E.)					
	Ethanolic extract			**Water extract**		
	H. suaveolens (HLE/e)	*M. cordifolia* (MLE/e)	*C. hystrix* (CLE/e)	*H. suaveolens* (HLE/w)	*M. cordifolia* (MLE/w)	*C. hystrix* (CLE/w)
control	4.17±0.27a	4.17±0.27a	4.17±0.27a	3.33±0.34a	4.17±0.27a	4.17±0.27a
0.8	23.33±0.16b	26.67±0.16b	22.50±0.18b	27.50±0.10b	24.17±0.24b	22.50±0.11b
1.6	36.67±0.13c	38.33±0.22c	28.33±0.09c	38.33±0.13c	30.00±0.16c	32.50±0.14c
3.2	60.83±0.09d	69.17±0.14d	65.00±0.11d	46.67±012d	62.50±0.15d	49.17±0.19d
6.4	82.50±0.11e	92.50±0.05e	82.50±0.15e	73.33±0.20e	80.33±0.04e	76.67±0.11e
LC_{50} (mg/ml)	21.40	17.54	22.85	26.53	23.05	27.41

a-e Mean values within a column followed by different letters are significantly, P<0.05, Duncan multiple range test (DMRT).

Table 4.5 Comparison of LC_{50} values of the direct toxicity to the adult rice weevils as prolonged treatment up to 48 hours.

Time (hours)	**Ethanolic extracts**			**Water extracts**		
	LC_{50} (mg/ml)			**LC_{50} (mg/ml)**		
	H. suaveolens **(HLE/e)**	***M. cordifolia*** **(MLE/e)**	***C. hystrix*** **(CLE/e)**	***H. suaveolens*** **(HLE/w)**	***M. cordifolia*** **(MLE/w)**	***C. hystrix*** **(CLE/w)**
12	55.38	23.11	37.44	55.38	32.41	48.31
	(41.44,88.79)	(20.18,26.48)	(30.47,47.93)	(41.44,88.79)	(27.00,40.09)	(37.45,70.75)
24	21.40	17.54	22.85	26.53	23.05	27.41
	(18.20,25.04)	(15.17,20.04)	(19.85,26.32)	(20.94,33.59)	(19.82,26.86)	(23.17,33.00)
48	11.65	9.87	15.92	13.36	14.79	17.01
	(8.13-14.85)	(8.48-11.13)	(13.54-18.38)	(10.82-15.83)	(12.39-17.20)	(14.17-19.89)

The correlations of the plant extracts and the experimental conditions are illustrated in Table 4.6. The plants (P), extracted solvents (S), concentrations (C), and observing times (T) are well correlated. The concentrations (C) are also correlated observing times (T). But the times (T) are not significantly correlated with the plants (P) and the extracted solvents (S). This indicates that the mortality of rice weevils by direct contact to the extracts of these plants is dependent on the concentrations and observing times.

Table 4.6 Analysis of variance shows the correlation among the plants, extracted solvents, concentrations and treatment times in direct toxicity of mint weed, kitchen mint, and kaffir lime leaf extracts on rice weevils.

Source of Variation	Degree of Freedom	Mean Square	F-value
Plant (P)	2	1.757	49.671*
Solvent (S)	1	3.168	89.541*
P × S	2	0.261	7.363*
Concentration (C)	4	137.299	3880.794*
P × C	8	0.318	8.976*
S × C	4	0.330	9.326*
P × S × C	8	0.137	3.886*
Time (T)	2	15.915	449.836*
P × T	4	0.059	1.679
S × T	2	0.061	1.72
C × T	8	1.091	30.82*
P × S × C × T	44	0.115	3.254*
Error	450	0.035	
Total	540		

* = Significant (P<0.05)

P = mintweed, kitchen mint, kaffir lime. S = ethanolic extract,water extract.

C = concentration of 0, 0.8%, 1.6 %, 3.2%, 6.4% T = 12, 24, 48 hours.

4.6 Discussion

Plant extracts and their constituents have been studied to possess their potentials as alternative chemicals for use as insect control agents (Shaaya, Kostjukovski, Eilberg, and Sukprakarn, 1997; Huang, Ho, Lee, and Yap, 2000; Lee, Choi, Lee, and Park, 2001a; Lee et al., 2001b; Lee, Annis, Tumaalii, and Choi, 2004; Sahaf, Moharramipour, and Meshkatalsadat, 2008; Rajendran and Sriranjini, 2008;). Some plant second metabolites contain monoterpenoids, diterpenoids, sesquiterpenoids and other compounds which express toxic effects in a wide range on insects, such as ovicidal, larvicidal, repellent, deterrent, and antifeedant (Boeke et al., 2004; Pungitore, García, Gianello, Sosa, and Tonn, 2005; Isman, 2006; Liu, Goh, and Ho, 2007). Some chemicals are highly volatile, which are suitable for exploitation as fumigant agents in insect pest control in stored crop products. The present study aimed to investigate the ethanol and water extracts of mintweed (*Hyptis saoveolens*), kitchen mint (*Mantha cordifolias*), and kaffir lime (*Citrus hytrixs*) leaves on the aspects of the fumigation and the direct toxicity on the adult rice weevils. Most plant extracts and oil processing these properties belong to monoterpenes (Erler, 2005). Eessential oil of *C. citratus* was found having high fumigant activity against *S. oryzae* (Saljoqi, Afridi, Khan, and Rahman, 2006). Therefore, the plant families Laminaceae and Rutaceae those contain monoterpenes receive much attention in biological control of insect pests. It could draw an assumption that the plant leaf extracts in this study possibly consist of monoterpenes leading to the rice weevil death as results of direct contact and fumigation activities. Furthermore, there are some evidence demonstrated that the susceptibility of rice weevil to terpenoids and camphor, 1, 8 cineole, and carvocrol which process fumigant toxicity (Shaaya et al., 1991; Weaver et al., 1994).

Prates et al. (1998) found that terpenoids, 1, 8 cineole and limonene from Brazillian flora effectively controlled rice weevils by possibly interfering the respiratory and digestive system. Kim, Park, Ohh, Cho, and Ahn (2003) indicated that insecticidal mode of action of plant oils may be largely attributed to fumigant action and they may be toxic by penetrating the insect body via respiratory system. Tripathi, Prajapati, Aggarwal, Khanuja, and Kumar. (2000) showed that the adults of *T. castaneum* were more susceptible to both contact and fumigant toxicity of 1, 8 cineol than the larvae. There are evidence supported that thymol, linalool, linalyl acetate and 1, 8 cineol were tested as fumigants against *S. oryzae, T. castaneum* and *R. dominica* (Lee, Annis, Tumaalii, and Choi, 2004; Rozman, Kalinovic, and Korunic, 2007) and against adults and eggs of *T. confusum* (Erler, 2005). In this study, the fumigation of leaf extracts of *H. suaveolens*, *M. cordifolia* and *C. hytrixs* were highly effective against *S. oryzae*. Similarly, essential oil from corn mint (*Mentha arvensis*) exhibited toxicity towards the rice weevils as the results of menthol, menthone, limonene, alpha-pinene, beta-pinene and linalool (Lee, Choi, Lee, and Park, 2001). The above mentioned evidences supports our study which illustrate that the extracts of *H. suaveolens*, *M. cordifolia* and *C. hytrixs* leaves are potentially fumigant toxic against *S. oryzae*. Thus, this study will be useful in rice protection from weevil infestation at a small scale, such as rice keeping in households and small bans. Furthermore, the biological control and the management of rice weevils in milled rice storage at industrial scale will be possibly applicable. Further more, it will benefit to human beings and environments as well as producing value added to local Thai weeds and herbs.

4.7 Reference

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CHAPTER V

BIOEFFICACY OF MINTWEED, KITCHEN MINT AND KAFFIR LIME EXTRACTS ON THE MILLED RICE GRAIN PROTECTION AGAINST RICE WEEVILS

5.1 Abstract

The study was conducted to evaluate the toxicity of crude extract of mintweed *(Hyptis suaveolens* L. Poit), kitchen mint *(Mentha cordifolia* Opiz*)* and kaffir lime (*Citrus hystrix* DC) on weight losses and progeny production of rice weevil, *Sitophilus oryzae*. All extracts highly protected the rice weight loss and inhibited the growth of rice weevil progenies. The grain weight loss was approximately 2-16%. The weevil F_1 progenies emerged approximately 4-21% or 88-55% inhibition. Ethanolic and water extract of kitchen mint *(Mentha cordifolia* Opiz*)* showed strong inhibit F1 progenies emerged 86.96% and 76.16%. The results of this study show that plant extracts were significantly ($P<0.05$) reduced adult emergence, and grain weight loss in the various treatments. It can be concluded that these three plant leaves which are odorous are promising plants in developing natural agents for rice weevil control in stored milled-rice grains.

5.2 Introduction

Insect pest infestation in grains and other food produce storages is a serious problem throughout the world. This damage may cause product loss about 5 to 10% in temperate zone and 20-30% in tropical zone (Haque, Nakakita, Ikenaga and Sota, 2000). Naturally, rice weevil (*Sitophilus oryzae*) is one of the most cosmopolitan insects that severely cause losses in rice, maize, barley, wheat, and other crops (Lucas and Riudavets, 2002). *S. oryzae* can develop grow in infestated rice grains in a relatively short period so that it is able to quickly build up high population. Its larvae feed on the rice grains causing rice grains damage and reducing nutrition value, weight and market approval. However, the rice grain weight loss is seriously caused by both larval and adult rice weevils within 2 weeks. In granaries, the weevils can induce losses of the grains during storage up to 75% (Dal Bello, Padin, Lastra, and Fabrizio, 2001). Currently, the management of stored milled rice is heavily relied on the use of synthetically chemical insecticides and fumigants leading to a number of problems, including insect resistance, toxic residues in food grains, human health, environmental pollutions and increased costs of application. Some plants posses the most effectively and acceptably active ingredients. Plant-based insecticides are target specific, non toxic to human, biodegradable and inexpensive for the biological control of pests. These problems are needed to be solved by the search for varieties of biodegradable insecticides leading to the identification of new botanical insecticides which are benefit of tropical agriculture (Mekuria, Steiner, Hindorf, Frahm, and Dehme, 2005). Neem (*Azardirachta indica*) is one of the traditionally popular plants used for insect control in both small and large scale plant griculture, including postharvest stored products. Singh and Singh (2005) reported that neem kernel powder could protected wheat grains from *R. domonica*. The infested wheat was loose only 2% and still could germinate over 80%. Mishra, Mishra, and Mahapatre (1992) recorded that custard apple seed, neem seed and leaf powders produced only 3-8% wheat grain loss and 7-15% damage by *S. oryzae*.

In the present study, the insecticidal properties, such as toxicity, repellance and oviposition deterrence of mintweed (*Hyptis suaveolens*), kitchen mint (*Mentha cordifolia*) and kaffir lime (*Citrus hystrix*) leaf extracts against *S. oryzae* in stored milled rice grains were investigated for the grain weight losses and the rice texture damages.

5.3 Materials and methods

5.3.1 Plant collection and extract preparation

The fresh leaves of the mintweed, kitchen mint and kaffir lime were collected, sun dried, ground into powder. The plant powder was extracted in Soxhlet extraction apparatus in 80% ethanol or distilled water. The extract was evaporated, lyophilized and stored at -20 ^{o}C.

5.3.2 Rice grain protection test

Twenty-gram milled rice was placed in a bottle (7.5 × 5.5 cm^2) and thoroughly mixed with 0.5 ml of 0.8, 1.6, 3.2, and 6.4% leaf extracts of mintweed (HLE), kitchen mint (MLE) and kaffir lime (CLE). Twenty-adult weevils were introduced into the mixed grains. The bottle was covered with cloth sheet and secured with a rubber band.

The rice weevils were cultured, allowed to lay eggs inside the rice grains and removed after 2 weeks. The same rice grains were kept to allow a new batch of weevils developed. The weevil cultures were continued for 35 and 49 days.

A hundred of sprayed grains was taken out and investigated for damages by opaque appearance and for weight loss. Forty percent ethanol and water were used as controls. The tests were done in triplicate and three repeats. The grain damage was computed using the method of FAO (1985) modified by Udo (2005) as follows:

$$\% \text{ Weight loss} = [(UNd \times DNu) / U (Nd + Nu)] \times 100$$

Where:

U = weight of undamaged grain D = weight of damaged grain

Nu = Number of undamaged grains Nd = Number of damage grains

5.3.3 Surface coated protection effect

A 500-ml plastic container with a lid was coated with 1 ml of 0.8, 1.6, 3.2 and 6.4% of plant extracts by thoroughly spraying for 10 minutes and then allowing to air dry. Forty grams of milled rice were placed in the pre-coated container. Twenty adults of *S. oryzaes* (7-10 days old), both sexes, were introduced into the container. The container was covered with lid and placed in the culture room (Owusu, 2001). After 2 weeks, the weevils were removed and the rice grains were continued for the new batch of rice weevils to develop and emerge. At day 35 and day 49, the number of a new batch of adult weevils that emerged, F_1 generations, were counted and evaluated for the reduction. The control was treated with each solvent alone. The percentage reduction in emergence of F_1 adults or the inhibition rate was calculated by using the following formula:

$$\text{Inhibition rates, } \% R = [(C_n - T_n) / C_n)] \times 100$$

Where: Cn = Number of newly emerged adults of control

Tn = Number of newly emerged adults of treatment

5.4 Data analysis

Data were expressed as mean of six replicates and were subjected to one way analysis of variance (ANOVA). Means were separated by the Duncan multiple range test using Statistics Package for the Social Sciences (SPSS). Values were considered statistically significant at $P<0.05$.

5.5 Results

5.5.1 Rice grain weight loss and damage protection

The rice grain protection by the plant leaf extracts was observed after allow the rice weevils grown and developed inside the extract-sprayed grains for more or less one life cycle. The grain weight loss was assessed at day 35 and day 49 of the weevil cultures. It was appeared that all leaf extracts well protected the rice grains from texture damage and weight loss. The texture of extract treated rice was very much similar to the control rice. Only few grains had opacity and broken. It is noticed that longer culture periods produce higher grain weight loss and damage (Figure 5.1).

Figure 5.1 Milled rice grain damage produced by rice weevils (*S. oryzae*) infestation.

In addition, the protection by MLE was slightly higher than the other extracts and very higher than the controls. At 35-day culture, 0.8% MLE/e produced 4.5% grain weight loss and 6.4% MLE/e produced 2.4% loss (Table 5.1). And 0.8% MLE/w produced 5% grain weight loss and 6.4% MLE/w produced 2.9% grain weight loss. At 49-day culture of 0.8% MLE/e, the grain weight loss was increased to 14.3% and of 6.4% MLE/e the loss was increased to 9.4%. While 0.8% MLE/w caused 16.3% grain weight loss and 6.4% MLE/w caused 10.3% loss.

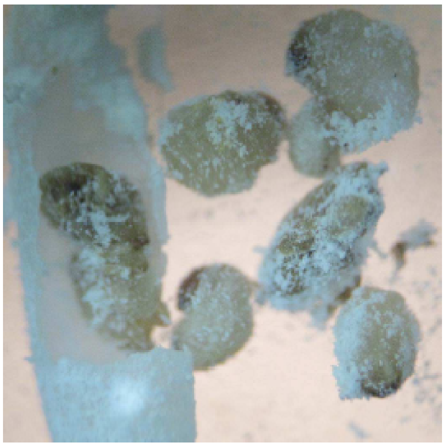
Table 5.1 Grain weight loss at 35 and 49 days of weevil infestation in milled rice grains.

Plant	Conc.%	% Weight Loss (Mean±S.E.)			
		Day 35		Day 49	
		Ethanol extract	Water extract	Ethanol extract	Water extract
HLE	0.8	4.36±0.44b	6.33±1.10b	14.45±0.51a	16.83±1.05b
	1.6	3.86±0.25ab	4.13±0.27a	13.93±0.70a	13.20±0.64a
	3.2	3.47±0.15ab	3.69±0.26a	13.10±0.24a	11.57±0.94a
	6.4	2.75±0.37a	3.12±0.24a	12.62±0.72a	11.02±0.55a
MLE	0.8	4.46±0.22bc	5.13±023b	14.26±0.68c	16.33±2.12b
	1.6	3.69±0.25ab	4.47±0.58ab	12.02±0.53b	12.64±0.67ab
	3.2	3.57±0.98ab	4.28±0.35a	11.52±0.43b	12.25±1.02a
	6.4	2.36±0.26a	2.85±0.98a	9.40±0.30a	10.32±0.63a
CLE	0.8	4.09±0.20a	4.52±0.75a	14.64±0.89b	17.68±2.30c
	1.6	3.39±0.37ab	3.64±0.39a	12.54±0.83ab	16.24±0.53bc
	3.2	2.69±0.25a	3.64±0.29a	11.82±0.74a	13.10±0.39ab
	6.4	2.62±0.19a	3.40±0.57a	10.24±0.70a	12.19±0.29a
Control		6.07±0.50c	7.20±0.82b	19.03±0.77b	24.14±0.71c

a-c Means within a column followed by different letters are significantly, $P<0.05$, Duncan multiple range test (DMRT).

5.5.2 Surface coated protection

The effects of the extracts mintweed (HLE), kitchen mint (MLE), and kaffir lime (CLE) on container surface coated which used for storing milled rice grains. The number of F_1 rice weevil adults emerged from infested milled rice in the container precoated with 0.8, 1.6, 3.2, and 6.4% of leaf water extracts shows in Table 5.2 and of ethanol leaf extract in Table 5.3. It is noticed that the F_1 progeny inhibition was dose dependent and the rice texture was heavily damaged (Figure 5.2).



A.

B.

Figure 5.2 A. Lavae rice weevils (*S.oryzae*) treated by MLE/e at 6.4%
B. Adult rice weevils (*S.oryzae*) treated by MLE/e at 6.4%

The 6.4% extract pre-coated treatment inhibited the F_1 emergence about 2-2.5 folds higher than the 0.8% treatment. The 0.8% CLE/w pre-coated treatment produced the lowest percentage of F_1 reduction of 34.78%. While the highest F_1 inhibition was from the 6.4% MLE/w treatment which was 76.18%. The 6.4% MLE/e treatment inhibited 86.96% F_1 emergence (Table 5.2).

Table 5.2 Coated surface protection effects of leaf extracts expressed as % emergence and % inhibition of the new batch (F_1) of *S. oryzae* adults after 35 days of rice grain storage in the pre-coated container.

Plant leaf extract	Conc. %	% Emergence (mean±S.E.) and % inhibition of *S. oryzae* progenies			
		Ethanol extract		Water extract	
		Mean number of F_1 Progeny±S.E.	% inhibition	Mean number of F_1 Progeny±S.E.	% inhibition
HLE	0.8	15.00±1.52b	34.78	17.33±1.20b	38.11
	1.6	12.33±0.88b	46.39	14.67±0.66b	47.61
	3.2	6.33±0.88a	72.48	9.00±1.00a	67.86
	6.4	3.67±1.20a	84.04	7.67±1.33a	72.61
MLE	0.8	14.33±1.76b	37.70	17.00±1.00b	39.29
	1.6	11.67±0.88b	49.26	14.00±1.15b	50.00
	3.2	6.00±0.57a	73.91	9.00±0.57b	67.86
	6.4	3.00±0.57a	86.96	6.67±0.88a	76.18
CLE	0.8	14.67±1.76b	36.22	17.67±0.50b	36.89
	1.6	12.33±0.57b	46.39	14.33±1.45b	48.82
	3.2	5.67±0.66a	75.35	9.33±0.66a	66.68
	6.4	4.00±1.15a	82.61	7.33±0.33a	73.82
Control		23.00±1.73c	0	28.00±1.73c	0

a-c Means within a column followed by different letters are significantly, $P<0.05$. Duncan multiple range test (DMRT).

5.6 Discussion

The leaf extracts of mintweed (*H. suaveolens*), kitchen mint (*M. cordifolia*) and kaffir lime (*C. hystrix*) processes activities on rice grain weight loss protection and larvae growth and development reduction. These effects were dose- and time dependent. This study showed that most of the leaf extracts and rice grain admix provided significant reduction in grain weight losses with normal texture of grains remained. It is suggested that that there could be some active ingredients present in these leaf extracts, which potentially interfered the feeding habit and then causing death of the larvae inside the grains. These results were supported by Srivastava and Mann (2002) who reported that the different extracts of *Peganum harmla* reduced the emergence *of Cryptocarya chinensis* and weight loss in the infested grains. Additionally, the extract pre-coated surface container well demonstrated that all extracts in this study well inhibited the rice weevil larvae growth and development. The F_1 emergence was markedly reduced. Specifically, the ethanol leaf extracts kitchen mint were found more effective than the other extracts. This finding was in agreement with the work of Khoshnoud and Khayamy (2008) who evaluated the insecticidal and progeny production effects of ethanol extract of *Verbascum cheiranthifoliun* against *S. oryzae* and *T. castineum.* Possibly, the *S. oryzae* F_1 progeny reduction was likely that immature insects were killed due to the respiration blockade. The extracts could blocked the spiracles resulting in respiration impairment. These toxic effects of the extracts may be attributed by the secondary plant metabolites. The rice weevil itself has no direct effects on the environment. However, the indirect effect is the loss of stored grain weight and the fumigation agents on the environment. When the beetles are left undisturbed in stored grain it can cause significant weight loss and in the case of seed grains it may lead to significant reduction in seed viability, i.e., reduction in seed germination.

Nonetheless, plant derived pesticides exhibit several modes of actions. Accordingly, toxicity against insects may expressed by direct killing, reproductive inhibition, repellence, interference in host finding, selection of the insect in a manner that infestation prohibition, antifeeding, and growth inhibition (Shaaya and Kostyukovysky, 2006).

The present findings indicate that the extracts of *H. suaveolens, M. cordifolia.* and *C. hystrix* leaves are highly potent for biological control of rice weevils from milled rice grain infestation and applicable for a small scale milled rice storage. However, further studies are required to investigate for a large scale milled rice storage as for commercial and industrial application and to analyze for insecticidal active biomolecules.

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CHAPTER VI

CYTOCHROME C OXIDASE INHIBITION IN THE RICE WEEVILS (*Sitophilus oryzaes*) BY PLANT EXTRACTS

6.1 Abstract

The extracts of mintweed (*Hyptis suaveolens* (L.) Poit) kitchen mint *(Mentha cordifolia* Opiz*)* and kaffir lime (*Citrus hystrix* DC) leaves contained aromatic, terpenoids and phenolic compound. In the Chapter III-V, the extracts have been used successfully for the control of rice weevils (*S. oryzae*) in milled rice. This chapter aimed to investigate the cellular mechanisms of the extracts which caused death to the rice weevils. The mitochondria of death rice weevils were isolated. Cytochrome c was reduced. Cyanide, the well known cytochrome c oxidase (COX) or complex IV inhibitor was used as the control reference. The extracts concentration of approximately 50% inhibited the rice weevils were used in the cytochrome c oxidase inhibition tests. The inhibition of cytochrome c oxidase activity in cellular respiration was ascertained as compare to cyanide. The ethanol extracts inhibited COX slightly higher than the water extracts and all inhibitions were dose dependent. MLE/e of 2.56% showed the highest inhibition of 79.54%. The COX inhibition were ranged as MLE/e/MLE/w>CLE/e/CLE/w>HLE/e/HLE/w. It indicates that the extracts of minweed, kitchen mint, and kaffir lime are applicable insecticides for the biological control of rice weevils in milled rice storage.

6.2 Introduction

The presence of secondary metabolites in plant extracts is of importance in their protection against insects in stored products, including rice weevils. However, the mechanisms at cellular and molecular levels of the plant products in preventing themselves from insect pests have not yet been known. Like synthetic insecticides, botanical insecticides can enter insects in three ways which are through ingestion, through absorption across the cuticle, and through the spiracle openings of respiratory system. In addition to playing roles in respiration and Adenosine triphosphate (ATP) synthesis, mitochondria play important energy-dependent roles in the regulation of cellular function, including intermediary metabolism, ion regulation, ion transport and cell motility. Mitochondria also serve as important targets of toxic chemicals. Previously, Haritos and Dojchinov (2003) reported that, in stored-product beetle *Sitophilu oryzae* L., formate esters exert toxicity after they are rapidly hydrolyzed to formic acid causing mitochondrial impactment. This finding is consistent with a known mode of action of formic acid that involves inhibition of mitochondria cytochrome c oxidase. The cytochrome c oxidase (COX) has been shown to be an interesting candidate for the study of cytotoxic mechanism of the secondary plant metabolites from extracts in insects. Extracts of some species of mintweed, kitchen mint and kaffir lime have been used as antioxidants, antibacterial and anti-inflammatory agents. The extracts have been successfully used in the control of stored insect pests, since these plants are rich in natural compounds including biological active components. However, scientific data on the mechanisms at cellular levels those cause insect mortality are few. Ahmed (2000) studied the efficacy of Castor-seed extract, as an insecticide against *S. oryzae*. He found that the extract inhibited cholinesterase and peroxidase enzymes in the insects and the enzyme-inhibition levels varied differently in the adult insects. It was dependent on exposure times and extracted solvents. Several researchers have also reported the inhibition of COX prevents the utilization of molecular oxygen by cell leading to loss of cell function and then to cell death (Haritos and Dojchinov, 2003). The purpose of this study was to determine the effect of the extracts mintweed, kitchen mint, and kaffir lime leaves on COX in the mitochondria of rice weevils infested in stored milled rice grains.

6.2.1 Mitochondrial activity

Mitochondria are the eukaryotic cellular organelles which contain the enzymes of citric acid cycle, the electron transport chain, and oxidative phosphorylation. This organelle is composed of inner and outer membrane (Figure 6.1). The outer membrane contains a mixture of enzymes, such as elongation of fatty acid. The enzymes for the electron transport chain and oxidative phosphorylation are embedded in the matrix side of the inner membrane. The enzyme COX is a large transmembrane protein located in the inner membrane of the mitochondria and is the terminal electron acceptor in electron transport chain. The mitochondrial activity can be measured in isolated subcellular fractions by assay for the mitochondrial specific enzymes. Cytochrome c in the reduced state has absorption wavelength at 550 nm producing a sharp band on the absorption profile. Upon oxidation, this absorption band becomes weaker and broader. This colorimetric assay is based on observation of the decrease in absorbance at 550 nm caused by the oxidation of ferricytochrome c by cytochrome c oxidase.

$$4\ Fe^{2+} \text{ cytochrome c (ferricytochrome c) } + 4H^{+} + O_2$$

$$\rightarrow\ 4Fe^{3+} \text{ cytochrome c (ferrocytochrome c)}^{+} + H_2O$$

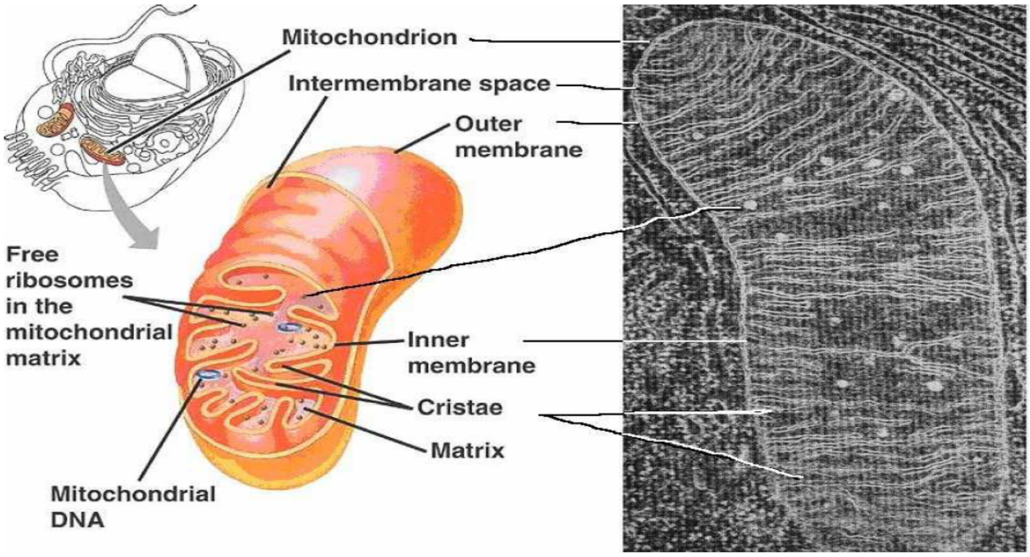

Figure 6.1 mitochondrial ultrastructure.

Source : Plant Biochemistry and Molecular Biology. Oxford University Press, Oxford.

6.2.2 Cytochrome c oxidase

COX is the terminal enzyme component (Complex IV) of the mitochondrial respiratory chain, the main energy-generating system of eukaryotic cell. The enzyme, embedded in mitochondrial inner membrane. It is a metalloprotein complex, catalyzes electrons transfer from reduce cytochrome c to a molecular oxygen, and preserves the free energy release in this exergonic reaction by maintaining the transmembrane proton gradient that is used to drive the synthesis of ATP or ion transport across the membrane.

In the simplest form of the electron transport in mitochondria (Figure 6.1), electrons (e.g. donated by Nicotinamide Adenine Dinucleotide (NADH) and Flavin Adenine Dinucleotide ($FADH_2$**)** enter the electron transport chain complex I (NADH dehydrogenase complex) (Heldt, 1997). Complex I is the first in the series of electron transport chain that are sequentially reduced and oxidized to generate a proton motive force. From complex I, electrons are passed to the ubiquinone pool. At complex I, there is potential for ATP production via generation of a proton gradient. Protons move from the matrix to the intermembrane space, during reduction/oxidation of complex I, generating a proton potential. ATP formation is driven by the proton-motive force in coupled oxidative phosphorylation.

Complex II oxidizes succinate from the Tricarboxylic acid (TCA) cycle thus contributing electrons to the ubiquinone pool. However, complex II is not corrected to translocation of protons across the inner mitochondrial membrane. Electrons from the ubiquinone pool proceed ultimately to one of two terminal oxidases in the electron transport chain cytochrome oxidase and alternative oxidase. Acceptance of electrons by complex III (cytochrome b/ct complex) of the cytochrome pathway is part of coupled oxidative phosphorylation.

Complex III is the second site in the mitochondrial electron transport chain that contributes to the generation of a proton potential. The final site contributing to generation of a proton potential is complex IV (COX) which is the terminal oxidase (Jin and Bethke, 2002). This complex accepts electrons from complex III via cytochrome c. Extrusion of protons by COX is coupled to ATP generation and reduction of molecular oxygen to water. Most of the ATP derived from respiration is produced in the mitochondria by coupled oxidative phosphorylation (Lambers, 1997). The electron transport system in insect mitochondria is similar to other animals (Heddi, Lefebvre, and Nardon, 1993).

Endogenous enzymes are known to play important roles in metabolic pathways in brain (Byers et al., 1981). Among them, COX is the terminal enzyme of the electron chain transport that reduces oxygen to water, produces ATP via oxidative phosphorylation and thus allows energy and oxygen utilization by cells. The activity of COX enzyme is linked to the metabolic demand in brain and reflects changes in neuronal activity. As a consequence, COX can be used as an endogenous marker for neuronal activities (Wong-Riley, 1989).

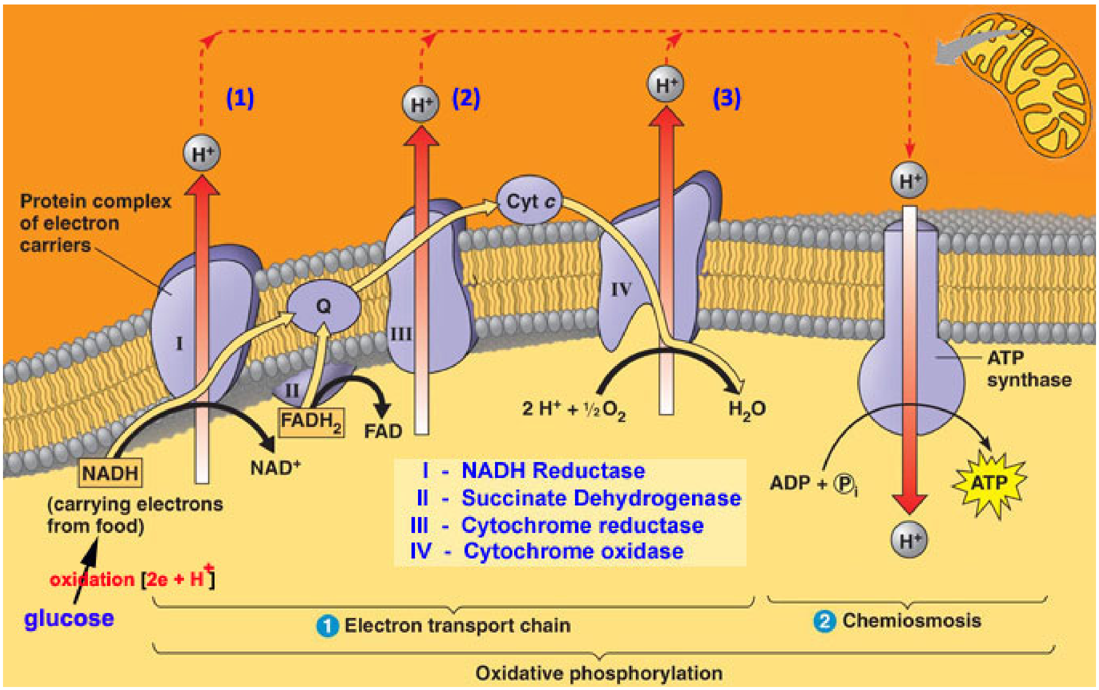

Figure 6.2 Diagrammatic structure of inner membrane of mitochondria showing electron transport chain and oxidative phosphorylation.

Source : http://www.hyperbaric-oxygen-info.com/aerobic-cellular-respiration.html.

Cytochrome c oxidase is identical to Warburg's respiratory enzyme. When it is in a reduced form it is recognizable by its clear absorption spectrum between 450 nm (minimum) and 590 nm (maximum). For oxidation of cytochrome c substance, it needs cytochrome oxidase. Way (1984) found that the activity of cytochrome c oxidase is vulnerable to be inhibited by cyanide.

6.3 Materials and methods

6.3.1 Materials

n-Dodecyl-β-D- maltoside, sodium dithionite, cytochrome c (horse heart, 85%) Tris buffer and bovine serum albumin (BSA) were purchased from Sigma Chemicals (St. Louis, MO, USA). Potassium hexacyanoferrate (III) was from Fisher Scientific.

6.3.2 Rice weevil rearing

Adult rice weevils (*S. oryza*e) were obtained from naturally infested milled rice from a local market in Nakhon Ratchasima, Thailand. The insects were reared in a plastic container (3.7 cm height × 7.5 cm diameter) under laboratory conditions at 28±2 ^{o}C, 65-70% relative humidity, 12 hours light : 12 hours dark and covered with a fine mesh cloth for ventilation. Thai jasmine rice (*O. sativa*) var. KDML 105 was used in all experiments. The insects were allowed to deposit their eggs. After 2 weeks of oviposition, they were removed through a sieve with mesh sized of 2 mm. The container was returned to the rearing room and the culture was continued for a month to allow the new adults emerged. The insects of 7-10 days old were used for all experiments.

6.3.3 Collection for plant materials and preparation for extracts.

The fresh leaves of the cultivated mintweed, kaffer lime, and kitchen mint were collected from surrounding of Suranaree University of Technology. They were completely dried in sunlight, ground, and extracted in 80% ethanol and water in Soxhlet's extraction apparatus. The crude extracts were evaporated using rotary vacuum evaporator, dried by lyophilizer at -70 °C, and stored at 20 °C. The extracts were dissolved in their original solvents for use in all experiments.

6.3.4 Isolation for insect mitochondria

Mitochondrial isolation was performed by the procedures described by Haritos and Dojchinov (2003) and Song and Scharf (2009) with modification. Three grams of *S. oryzae* adults were manually homogenated in a tissue grinder in an ice chilled homogenizer in 10 ml isolation buffer containing 0.25 M sucrose, 0.001 M Ethylenediaminetetraacetic acid (EDTA), 0.005 M Magnesium sulfate, 0.05 M Tris buffer, pH 7.4, and 0.2% bovine serum albumin (BSA). The homogenate was filtered through double gauze nylon and rinsed with 10 ml buffer. The homogenate was centrifuged at 500×*g* at 4°C for 20 min to pellet out debris. The supernatant was transferred to a fresh tube and recentrifuged at 5,000×*g* at 4 °C for 10 min to sediment the mitochondria. The mitochondrial pellets were resuspended in 5 ml of the isolation buffer and centrifuged at 10,000×*g* at 4 °C for 10 min. The pellets were then resuspended in isolation buffer and kept at -80 °C.

6.3.5 Cytochrome c oxidase activity

COX activity was measured using the method of Storrie and Madden (1990). The cytochrome c standard from horse heart was dissolved in phosphate buffer and used for measurement of cytochrome c activity (Song and Scharf, 2009). The cytochrome c standard horse heart was reduced by Sodium dithionite. One microgram per milliliter reduced cytochrome c (36 mM final concentration, as a substrate) in the reaction buffer containing 40 mM phosphate buffer pH 6.2 containing 250 mM sucrose and 0.05% (w/v) lauryl maltoside was allowed to react with Sodium dithionite by gradually adding a few granules of it until completely dissolved. The extract sample sample concentration was arbitrarily selected base on the one that produced insecticidal activity approximately 50% which was 6.4 %. The extract sample was then diluted to obtain the final concentrations of 0.64, 1.28, 1.92 and 2.56% reaction well.

The reduced cytochrome c was put into a 96-well plate, 20 µl/well, containing 20 µl reaction buffer. The extract sample (20 µl), and 0.1% DMSO (20 µl) were added. The mitochondrial homogenate (20 µl, containing COX) was added last. The COX activity was immediately measured in microplate reader spectrophotometer (Benchmark plus Biorad Laboratory) at 550 and 565 nm for 5 minutes at the most. Cyanide (0.003%) was used as control. The inhibition was calculated as the following equation.

$$\% \text{ Inhibition} = \left[\left(\frac{\text{Absorbance of the sample}}{\text{Absorbance of reduced cytochrome c}}\right)\times 100\right]$$

6.3.6 Data analysis

Data were expressed as mean of three replicates and were subjected to one way analysis of variance (ANOVA). Values were statistically significant at P<0.05.

6.4 Results

6.4.1 Inhibition of mitochondrial cytochrome c oxidase activity

COX inhibition in *S. oryzae* mitochondrial preparations by the ethanol and water extracts of mintweed (*Hyptis suaveolens* L. Poit) kitchen mint (*Mentha cordifolia* Opiz) and kaffir lime (*Citrus hystrix* DC) leaves was first investigated. It appeared that the *M. cordifolia* leaf ethanol extract (MLE/e) expressed high potential inhibition of the COX activity of *S. oryzae* mitochondria. The MLE/e and CLE/e inhibited COX similarly and both had higher activities than HLE/e. At the maximal concentration treated, 2.56%, the COX inhibition was 79.54, 76.38, and 60.94% induced by MLE/e, CLE/e, and HLE/e, respectively (Table 6.1).

Table 6.1 Inhibition activity of COX of *S. oryzae* mitochondria by ethanol extracts of mintweed (HLE/e) kitchen mint (MLE/e) and kaffir lime (CLE/e) leaves.

Plant extracts	Conc.%	%Inhibition (Mean±S.E.)
HLE/e	0.64	31.34±3.98 a
	1.28	41.04±4.26 a
	1.92	58.70±4.41 b
	2.56	60.94±2.82 c
MLE/e	0.64	58.79±6.66 a
	1.28	66.41±3.97 ab
	1.92	70.95±5.79 abc
	2.56	79.54±3.60 bc
CLE/e	0.64	58.03±3.98 a
	1.28	62.30±4.26 ab
	1.92	71.35±4.41 bc
	2.56	76.38±2.82 cd
Cyanide	0.003	84.06±1.99

a-d Means within a column followed by different letters are significantly, $P< 0.05$, Duncan multiple range test (DMRT).

Table 6.2 Inhibition activity of COX of *S. oryzae* mitochondria by water extracts of mintweed (HLE/w) kitchen mint (MLE/w) and kaffir lime (CLE/w) leaves.

Plant extract	Conc.%	%Inhibition (Mean±S.E)
HLE/w	0.64	28.27±2.91a
	1.28	29.28±2.30a
	1.92	48.96±3.21b
	2.56	61.61±4.81c
MLE/w	0.64	38.45±3.25a
	1.28	46.92±5.31a
	1.92	66.66±3.69b
	2.56	70.25±2.62b
CLE/w	0.64	25.37±4.41a
	1.28	40.54±4.49b
	1.92	60.19±3.99c
	2.56	63.67±5.49c
Cyanide	0.003	84.06±1.99

a-c Means within a column followed by different letters are significantly, $P < 0.05$, Duncan multiple range test (DMRT).

The COX inhibition profile by the ethanol or the water extracts is similar as in Figure 6.3, which showed the COX inhibition induced by maximal concentration of 2.56%. That is inhibition potency was MLE>CLE>HLE. Therefore, it can be concluded that the MLE is best COX inhibitor for mitochondria in controlling *S. oryzae* in milled rice grain storage.

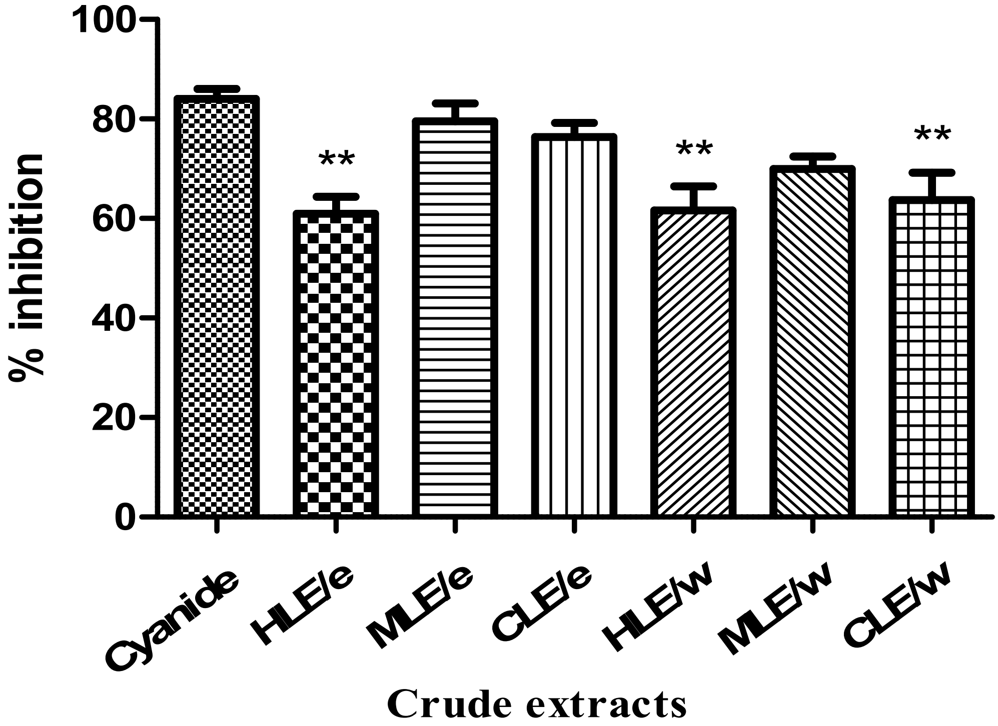

Figure 6.3 Comparison of the inhibition of COX by mintweed, kitchen mint and kaffir lime leaf extracts at the maximal concentration of 2.65%, ** = Significant (P<0.01).

The MLE/e and CLE/e inhibited COX by approximately 0.9 fold of by cyanide control, while the HLE/e inhibited 0.7 fold of cyanide. The COX inhibition by the water extracts was similar, slightly lower magnitudes. At the same concentration of 2.56% extract, the COX inhibition was 70.25, 63.67, and 61.61% reduced by MLE/e, CLE/e, and HLE/e, respectively. These three water extracts were potent about 0.7-0.8 fold of cyanide. At concentration of 2.56% of MLE/e, CLE/e, MLE/w were not significant different from the standard cyanide (P>0.01).

The spectral profile of reduced-oxidized cytochrome c standard from horse heart showing the typical absorbance at 415, 520, and 550 nm (Figure 6.4).

The action spectrum of oxidize cytochrome *c* show absorbance maximum at 409 nm and a broad feature centered at 530 nm. The spectrum of reduced-oxidized cytochrome c was shifted from 409 nm to 550 nm by the ethanol leaf extracts (Figure 6.5) and by water leaf extracts (Figure 6.6). This demonstrated that the leaf extracts inhibited COX activity in the mitochondria of rice weevils. The COX inhibition by the ethanol leaf extracts was ranged as MLE/e>CLE/e>HLE/e, and by the water leaf extracts was ranged as MLE/w>CLE/w>HLE/w.

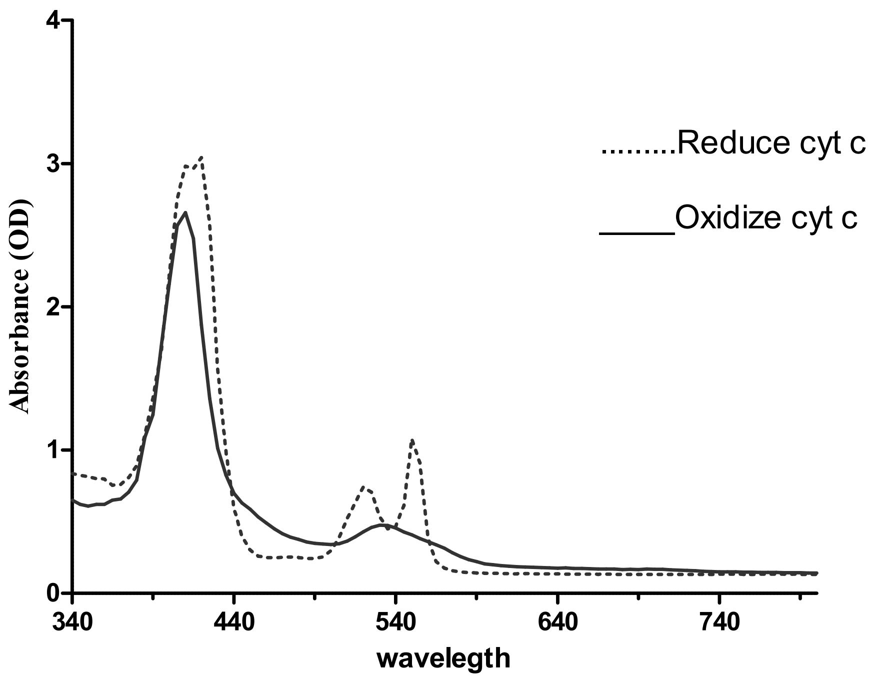

Figure 6.4 Spectral profile of reduced - oxidized cytochrome c from horse heart.

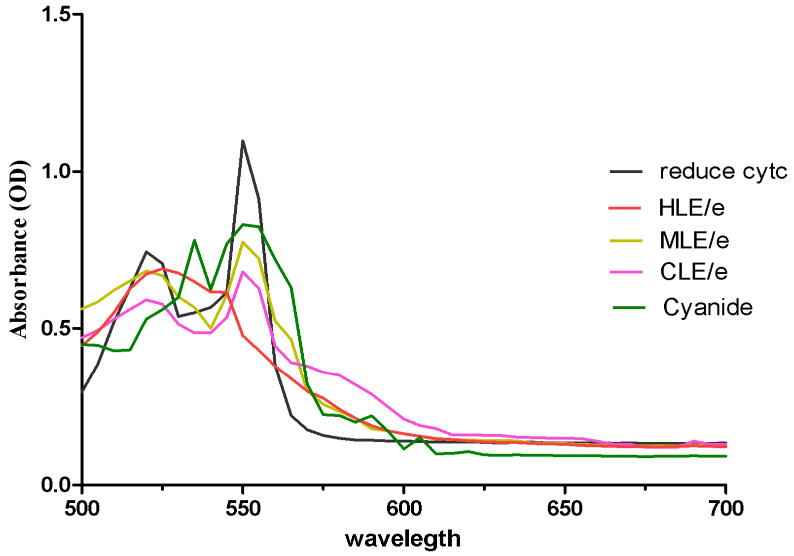

Figure 6.5 The action spectrum of reduced cytochrome c of rice weevil mitochondria in the presence of the ethanol leaf extracts of mintweed, kitchen mint, and kaffir lime.

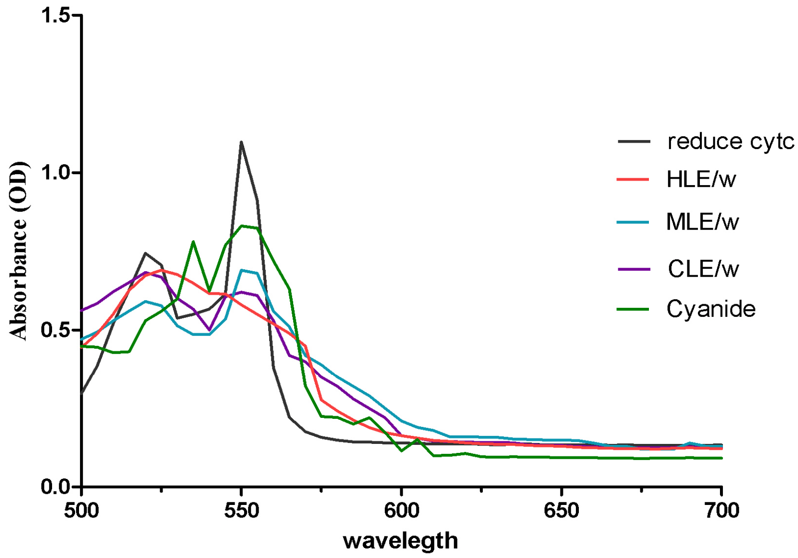

Figure 6.6 The action spectrum of reduced cytochrome c of rice weevil mitochondria in the presence of the water leaf extracts of mintweed, kitchen mint, and kaffir lime.

6.5 Discussions

A biologically active product may possess one or several modes of action need to be considered in determining efficacy. The most direct mode of insecticidal activity is a measure of mortality. Toxic chemicals, plant extracts and essential oils can have an insecticidal effect through contact toxicity, neuro-toxicity (Enan, 2001), stomach poisoning, fumigation (Choi, Lee, Park, and Ahn, 2004) or may kill by more indirect means such as suffocation via blockage of the spiracles. Since the work of Keilin (1925), the composition of cytochromes of insects has been well establish. The composition of cytochromes in isolated has been determine in the whole body of rice weevil (Haritos and Dojchinov, 2003; Song and Scharf, 2008). The findings of this study provide important confirmation of mitochondrial impacts by crude extracts. In addition, the biological activities of plant terpenoids were repellency and deterrence, reduced palatability, growth inhibition by altered protein availability, enzyme inhibition, and direct toxicity (Lee, Lee, Choi, Park, Kim, and Campbell, 2001). Lee, Peterson, and Coasts (2003) suggested that most monoterpenoids found in essential oils may interfere with basic metabolic biochemical, physiological and behavioural functions of insects. Many phenolic compounds inhibit enzyme activities in a specific, oxidative phosphorylation, ATPase and membrane transport processes (Kyung-Hee, Rong, Yang, and Steve, 2005). In this study the three plant extracts consisted of monoterpene and phenolic acid. These finding suggest that cytochrome c oxidase may be a target for secondary metabolite such as monoterpene and phenolic. However, the results revealed that all plant extract showed similar trend of cyanide and IC_{50} more than 50% inhibition. In this respects, natural insecticides may be effective and less harmful to the environment. This finding assures that the extracts of mintweed, kitchen mint, and kaffir lime cause death to rice weevils by inhibiting the cytochrome c oxidase activity in the electron transport chain of cellular respiration in mitochondria. These three plants may serve as alternatives of choices in the biological control of stored insect pests and replace the synthetically chemical insecticides.

6.6 References

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CHAPTER VII

CONCLUSION

The protection of stored products against attack by pests is essential in many countries. Novel environmentally compatible stored product control agents are urgently needed to replace synthetic pesticides that have been withdrawn for economic or regulatory reasons or are ineffective, due to the increasing difficulty of managing pesticide resistance. Control agents that are safe alternatives, and have the potential to replace these toxic fumigants, there are simple and convenient to use are required To ensure food security among the people, there is a need for control of insect pest damage on stored food. The search for safe and environmentally-friendly pest control agents has led to the exploration of natural products from plants for potential alternatives. The present study has highlighted the importance of farmers knowledge and experience of indigenous insect pest control.

The common rice weevil, *Sitophilus oryzae* Motschulsky (Coleoptera: Curculionidae), a well known pest of stored grain in most parts of the world, was identified as one of the major constraints of harvested milled rice in Thailand. The use of plants or their products is one of the recent methods being investigated for insect pest control worldwide. Therefore, the main objective of this study was to biologically control of rice weevil (*Sitophilus oryzae*) in stored milled rice grains by extracts of aromatic plants. The research was focus on the followings: 1) to primarily investigate some phytochemicals properties of crude extracts of *Hyptis suaveolens* (L.) Poit, *Mentha cordifolia* Opiz and *Citrus hystrix* DC. 2) to investigate the insecticidal effects of the plants extracts on larvae and adults of the rice weevils. 3) to investigate the effect of the plant extracts on insect cytochrome c oxidase.

7.1 Primarily investigate some phytochemicals properties of crude extracts

The total phenolic compounds contents of ethanol extracts were slightly higher than of water extracts. MLE/e contained highest total phenolic compounds of 26 mgGAE/g and HLE/w contained lowest of 18 mg GAE/g. The amounts of total phenolic compounds were well correlated with the yield recovery of the extracts. TLC analysis and vanillin detection indicated that the major compounds constituted in the extracts were terpenoid.

7.2 Investigate the insecticidal effects of the plants extracts on larvae and adults of the rice weevils

7.2.1 Cytotoxicity test by brine shimp lethality assay

The brine shrimp lethality assay represents a rapid, inexpensive and simple bioassay for testing plant extracts bioactivity which in most cases correlates reasonably well with cytotoxicity. In addition, the water extracts of mintweed (HLE/w), kitchen mint (MLE/w) and kaffir lime (CLE/w) showed moderate lethality with LC_{50} of 88. 98, 131.08, and 137.15 µg/ml. The degree of lethality was found in direct proportion to the concentration of the extracts. This indicated that the extracts obtained from the plant leaves with LC_{50} much lower than 1,000 µg/ml could be potential sources of insecticidal constituents.

7.2.2 Repellent test of plant leaf extracts and plant powder

The effects of plant leaf extracts and plant powder on repellence were conducted by a cone bioassay. The repellent efficacy of the ethanol extracts were CLE/e>MLE/e>HLE/e and those of the water extracts were MLE/w>CLE/w>HLE/w. The CLE/e showed the highest repellency of 80% at the concentration of 6.4% and 24 hours. It was approximately 3.2 folds of the control. While, the MLE/w showed the highest repellency of 72% or 3.6 folds. The EC_{50} values at 24 hours of the HLE/e, MLE/e and CLE/e were 18.95, 15.63, and 13.23 mg/ml, respectively. The EC_{50} values of the water extracts of these three plants were 27.13, 19.04, and 19.87 mg/ml, respectively.

The leaf powders of mintweed (HLP), kitchen mint (MLP) and kaffir lime (CLP) were able to repel the rice weevils out of the admixed rice grains. The repellent activity of them was dose-and time-dependent fashions. The repellency steadily increased range from 2.5 to 5.9 folds as the dose increased at 24 hours, compared to the control.

7.2.3 Effect of plant leaf extracts and plant powder on weevil mortality

All plant leave extracts exhibited strongly insecticidal activity against adult rice weevils. The mortality effects were significantly dose- and time dependent ($P \leq 0.05$). The mortality effect of MLE/w was slightly higher than the others, which was 76.67% at 6.4% in 10 days. The leaf powders of mintweed (HLP), kitchen mint (MLP) and kaffir lime (CLP) effectively cause death to rice weevils in stored rice milled grains. The mortality efficacy of them was dose- and time-dependent patterns. The MLP could cause total death of rice weevils at a very low concentration of 0.25-0.50% within 20 days and of 0.75% within 15 days. When comparing the magnitude of powder concentration at 0.5%, the MLP could cause mortality of the rice weevils approximately 2 folds.

7.2.4 Growth and development of weevil progenies by plant leaf extracts and plant powder

The growth and development of the rice weevil progenies was significantly inhibited by all plant extracts in a dose-dependent pattern and plant powder The growth and developmental reduction of F_1 by MLE/e was range from 70% to 89% or 3.4-8.7 folds as compared to the control. While the reduction by water extract was range from 61% to 81% or 2.6-5.3 folds of the control. The MLP more potently reduced the emergence of the weevil new generations than the others which were similar reduction. The F_1 reduction by MLP was range from 42% to 74% or 3.4-8.7 folds as compared to the control.

7.2.5 Fumigation test

In the fumigant test the direct relationship between concentrations and mortalities was observed. The extracts were relatively high toxic against adult rice weevils. The most fumigant efficacy of ethanolic extracts and water extract were ranged as kitchenmint (MLE)>kaffir lime (CLE)>mintweed (HLE). All extracts showed fumigation effects about 2-3.7 folds of the control.

7.2.6 Direct toxicity test

Direct toxicity test was conducted by dipping method. The mortality of adults and larvae increased with increasing extract concentrations. All the tested plant extracts had also a significant ($P < 0.05$) level of toxicity in direct contact bioassay against *S. oryzae.* It was concluded that botanical product acted as toxicity of three plant leaf extracts on rice weevil were *M. cordifolias*>*C. hytrixs*>*H. saoveolens*.

7.2.7 Rice grain protection test

Additionally, most of plant extracts admixed to the milled rice grain provided significant reduction of grain weight losses compared to the untreated check, suggesting that the presence of chemical factors that can interfere with the feeding habit of the rice weevil. Most of these substances were tested against insects attacking stored products in oder to establish new control practices with lower mammalian toxicity, low persistence in the environment and potential for commercial application.

7.2.8 Coated surface protection effect

The present study was designed to package container kept milled rice by Coated surface protection. Ethanolic and water extract of Kitchen mint (*Mentha cordifolia* Opiz) showed strong inhibit F_1 progenies emerged 86.96% and 76.16%. The results of this study show that plant extracts were significantly ($P<0.05$) reduced adult emergence. Rice weevil killed in plant extract treat milled rice appeared paralysed with their metathoracic wing unfolded.

7.3 Inhibition of mitochondrial cytochrome c oxidase activity

The synthesis of ATP, the energy of the cell, is a complex process carried out by the mitochondrial respiratory electron transport chain ivolving a series of five membrane bond complexes (I-V) Pesticides distrup many sites by binding and inhibition (I-V) to prevent oxidative phosphorylation and formulation gradient. Cyanide and phosphine block complex IV. In insect, inhibition of Inhibition of cytochrome c oxidase (complex IV) prevents the utilization of molecular O_2 by cell, leading to loss of cell function and then to cell death. The result revealed that all plant extract showed similar trend of cyanide and IC_{50} more than 50% inhibition. In this respects, natural insecticides may be effective and less harmful to the environment.

It had been well reported that extracts from a variety of plants have potent insect pest-control properties, and they have being found to affect the biology of target insects in different modes such as ovicides, repellents, antifeedants, fumigants and contact toxicants, and insecticides. In addition, plant-based pesticides are renewable in nature and cheaper. Also, some plants have more than one chemical as an active principle responsible for their biological properties. These may be either for one particular biological effect or they may have diverse ecological effects. The results of our study indicate that *M. cordifolia* has a potential for development as a commercial insecticide against *S. oryzae*. However, future research needs to be conducted to test its effects against beneficial insects and non target organisms. We would also be interested in knowing the feeding deterrent and growth inhibiting properties of this promising extract. Additionally, field studies are needed to understand the extract's effectiveness, persistence and chemical stability in field conditions.

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