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THESIS

SEARCHING FOR A NEW ENVIRONMENTALLY-FRIENDLY BOTANICAL INSECTICIDE FROM THE RHIZOMES OF *ALPINIA GALANGA*

ANUPAP PUANGSOMCHIT

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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Anupap Puangsomchit 2014: Searching for a New Environmentally-Friendly Botanical Insecticide from the Rhizomes of *Alpinia galanga*. Master of Science (Chemistry), Major Field: Chemistry, Department of Chemistry. Thesis Advisor: Assistant Professor Wanchai Pluempanupat, Ph.D. 85 pages.

The objective of this study was to develop an alternative strategy for the control of *Spodoptera litura*. The dried rhizomes of *Alpinia galanga* were extracted with sequential polarity solvent; hexane, dichloromethane, ethyl acetate and methanol, respectively by soaking at room temperature for seven days. The topical application was used to examine the toxicity of the extracts against second instar *Spodoptera litura* larvae. Dichloromethane crude extract exhibited the most toxicity as LD_{50}= 3177 ppm and 2099 ppm after 24 and 48 hours post-treatment, respectively. Furthermore, two active ingredients as [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate were successfully isolated from dichloromethane crude extract. In addition, mode of action of insect enzyme activity was found that the both isolated compounds could inhibit acetylcholinesterase and glutathione-*S*-transferase after exposed 24 hours.

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Student's signature Thesis Advisor's signature

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Anupap Puangsomchit

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF ABBREVIATIONS

α	=	alpha
β	=	beta
δ	=	chemical shift (ppm)
d	=	doublet
dd	=	doublet of doublets
ddd	=	doublet of doublet of doublets
dt	=	doublet of triplets
g	=	gram
h	=	hour
Hz	=	Hertz
J	=	coupling constant
m	=	multiplet
mg	=	milligram
p	=	para

SEARCHING FOR A NEW ENVIRONMENTALLY-FRIENDLY BOTANICAL INSECTICIDE FROM THE RHIZOMES OF *ALPINIA GALANGA*

INTRODUCTION

Thailand is an agricultural country with a major role in food production in Southeast Asia. However one of the most serious problems in the quality and quantity of agricultural production resulting from attack of pests, especially insects in the field such as tropical armyworm *(Spodoptera litura*) (Ferry *et al*., 2004), beet armyworm (*Spodoptera exigua*) (Bullangpoti *et al*., 2011) and diamondback *(Plutella xylostella)* (Saeed *et al*., 2010).

Pesticide is a major problem in Thailand that is widely known as agricultural country. This causes the drop in agricultural efficiency. Synthetic insecticides are employed for ceasing this situation. Although they can completely vanish the pesticide, they also damage the environment because they are difficult to be biological decomposed.

Chemical control agents have been the backbone of pest managements and contributed in protecting crops from insect damages. Most of synthetic insecticides certainly have promised a great deal of human foods obtained from synthetic factories because of their convenience, fast and easy in handling, whereas they have been great concern about toxicity to non-target organisms in human and natural enemies or pollinators in environmental residue and bioaccumulation through food chain. From these dramatic problems, we need to look a new environmentally-friendly botanical insecticide from natural source.

Alpinia galanga is medicinal plant mostly found in Thailand. This plant is wildly cultivated in China, India and Southeast Asian countries such as Indonesia,

Philippines and Thailand. The rhizomes of *Alpinia galanga* are extensively used as spice or ginger substitutes for flavoring foods, and also used in traditional medicine for several purposes, such as stomachic in China, or for carminative, antiflatulent, antifungal, and anti-itching in Thailand.

There are many researchers publishing the extracts of Galangals including molecular structure and biological activities, especially, insecticidal activity. For example, we have recently reported the efficacy of (*E*)-*p*-acetoxycinnamyl alcohol, and (*E*)-*p*-coumaryl alcohol ethyl ether, which were isolated from the hexane crude extract of the rhizomes of *Alpinia galanga*, against *Bactrocera dorsalisour* (Sukhirun, 2011).

Spodoptera litura is found in Asia, with some specific problematic pest population reports occurring in Thailand, Cambodia, Hong Kong, India, the Pacific islands, Guam, American Samoa, and Hawaii. *Spodoptera litura* is an insect pest of several crops, such as soybean, mango and papaya. Consequently, tropical plants in Thailand are still a major and essential source for searching new insecticides.

OBJECTIVES

1. To search a new environmentally-friendly botanical insecticide from the crude extracts of the rhizomes of *Alpinia galanga.*
2. To isolate active ingredients from the most efficiency crude extract.

LITERATURE REVIEW

Alpinia galanga

Alpinia galanga, a plant in the ginger family, is an herb used in cooking. The Latin generic name "Alpinia" was given to commemorate Prospero Alpini, an Italian botanist who catalogued and described exotic plants. The common name "Galangal" is derived from the Arabic Khalanjan, perhaps a perversion or an adaptation of the Chinese Liangtiang (meaning 'mild ginger'). The drug has been known in Europe for seven centuries longer than its botanical origin, for it was only recognized in 1870, when specimens were examined that had been found near Tung-sai, in the extreme south of China, and later, on the island of Hainan.

Descriptions

Alpinia galanga belongs to family "Zingiberaceae". The herb grows to a height of about 5 feet, the leaves being long, rather narrow blades, and the flowers, of curious formation, growing in a simple, terminal spike, the petals white, with deep-red veining distinguishing the lippetal. The branched pieces of rhizome are from 1.5 to 3 inches in length, and seldom more than 0.75 inch thick. They are cut while fresh, and the pieces are usually cylindrical, marked at short intervals by narrow, whitish, somewhat raised rings, which are the scars left by former leaves. They are dark raddish-brown externally, and the section shows a dark center surrounded by a wider, paler layer, which becomes darker in drying. Their odour is aromatic, and their taste pungent and spicy.

Figure 1 *Alpinia galanga*

Classification of *Alpinia galanga*

Kingdom	Plantae
Division	Magnoliophtta
Class	Liliopsida
Order	Zingiberales
Family	Zingiberaceae
Subfamily	Alpinioideae
Tribe	Alpiniaiea
Genus	*Alpinia*
Species	*Alpinia galanga*

Traditional medicinal

For different countries, galangal is used distinctly (Chudiwal *et al.*, 2010). Most of the applications used in Southeast Asia countries. Dried galangal is employed only in the absence of fresh galangal whereas in Indonesia slices or powder of the fresh or dried rhizome are used frequently.

The rhizome is used against rheumatism, bronchial catarrh, bad breath, and ulcers whooping colds in children, throat infections, to control incontinence, fever and dyspepsia.

The root has been used in Europe as a spice for over a thousand years, having probably been introduced by Arabian or Greek physicians, but it has now largely gone out of use except in Russia and India.

The rhizomes have been used as flavors in native dishes and ingredients in many traditional medicines to treat various ailments, such as stomach disorders and skin diseases.

In India, the rhizomes have many applications in traditional medicines such as for skin diseases, indigestion, colic, dysentery, enlarged spleen, respiratory diseases, mouth and stomach cancer.

The rhizomes showed antibacterial, antifungal, antiprotozoal, and expectorant activities. It is used as a body deodorizer and halitosis remedy.

Allergic contact dermatitis

Alpinia galanga is also a popular spice for flavoring foods in Southeast Asian cuisines. One previous study reported a case of localized contact dermatitis (Javier *et al.*, 2006) and subsequently generalized erythema multiform like eruptions after topical application of herbal remedies. After tests showed there was an allergen in fresh and dried *Alpinia galanga*.

Antiallergic activity

The rhizomes of *Alpinia galanga* were extracted by 80% aqueous acetone that found to inhibit release of betahexosaminidase, as a marker of antigen-lgE-

mediated degranulation in RBL-2H3 cells. Moreover, nine known phenylpropanoids and *p*-hydroxybenzaldehyde were isolated from the extract. Among them, [1'S]-1'-acetoxychavicol acetate and [1'S]-1'-acetoxyeugenol acetate exhibited potent inhibitory activity with IC_{50} values of 15 and 19 mM (Matsuda *et el.*, 2003).

From the effects of various related compounds, both the 1'-abd 4-acetoxyl groups of [1'S]-1'-acetoxychavicol acetate and [1'S]-1'-acetoxyeugenol acetate were essential for their strong activity, and the 2'-3' double bond enhanced the activity. In addition, [1'S]-1'-acetoxychavicol acetate and [1'S]-1'-acetoxyeugenol acetate inhibited ear passive cutaneous anaphylaxis reactions in mice and the antigen-lgE-mediated TNF-alpha and IL-4 production, both of which participate in the late phase of type l allergic reactions, in RBL-2H3 cells.

Antidermatophytic activity

Ethanol crude extracts of Piper beetle leaves (Piperaceae), *Alpinia galanga* rhizomes (Zingiberaceae) and *Allium ascalonicum* bulbs (Lilliaceae) were studied against selected zoonotic dermatophytes (*Microsporum canis, Microsporum gypseum and Trichophyton mentagrophyte)* and the yeast-like *Candida albicans* (Trakranrungsie *et al.,* 2008).

A broth dilution method was employed to determine the inhibitory effect of the extracts and compared to those of ketoconazole and griseofulvin.

All extracts suppressed the growth of the fungi in a concentration dependent manner. Among the extracts tested, all exhibited effective antifungal properties.

Antifungal activity

An antimicrobial diterpene was isolated from *Alpinia galanga* in the screening for potentiators of phytochemical antibiotic action. The structure was

elucidated by spectral data and identified as (*E*)-8 beta, 17-epoxylabd-12-ene-15, 16-dial. This diterpene synergistically enhanced the antifungal activity of quercetin and chalcone against *Candida albicans*. Antifungal activity of (*E*)-8 beta, 17-epoxylabd-12-ene-15, 16-dial was reversed by unsaturated fatty acids. Protoplasts of *Candida albicans* were lysed by (*E*)-8 beta, 17-epoxylabd-12-ene-15, 16-dial. These results suggested that antifungal activity of (*E*)-8 beta, 17-epoxylabd-12-ene-15, 16-dial is due to a change of membrane permeability arising from membrane lipid alternation (Haraguchi *et al.*, 1996).

Furthermore, thirty-six extracts derived from ten plant species were selected to screen for their antifungal activity against clinical isolates of *Candida albicans*, *Cryptococcus neoformans* and *Microsporum gypseum*. Selection was based on their use by traditional Thai healers or their reported antimicrobial activities in an attempt to find bioactive medicines for use in the treatment of opportunistic fungal infections in AIDS patients. The disc diffusion and hyphal extension-inhibition assays were primarily used to test for inhibition of growth. Minimum inhibitory concentration was determined by dilution methods.

The chloroform extracts of *Alpinia galanga* and *Boesenbergia pandurata* had pronounced antifungal activity against *C. neoformans* and *M. gypseum*, but exhibited weak activity against *C. albicans*. Thus, *Alpinia galanga* and *B. pandurata* were excellent candidates for the development of a remedy for opportunistic fungal infections in AIDS patients.

The essential oils from fresh and dried rhizomes of *Alpinia galanga* showed antimicrobial activity against gram-positive bacteria, a yeast and some dermatophytes, using the agar overlay technique. Terpinen-4-ol was found to be the most active ingredient. An *n*-pentane/diethyl ether extract of dried rhizomes was active against *Trichophyton mentagrophytes.* 1'-acetoxychavicol acetate, 1'-acetoxyeugenol acetate and 1'-hydroxychavicol acetate were found in the antifungal active fractions obtained by LSC. Acetoxychavicol acetate was active against the

seven fungi tested and its MIC value for dermatophytes ranged from 50 to 250 µg/mL. Dried sliced rhizomes contained 1.5% of this compound.

Anti-giardial activity

One study evaluated the antigiardial activity of chloroform, methanol and water extracts of 12 medicinal plants (39 extracts), commonly used as self-medication by AIDS patients in southern Thailand. The plant extracts and a standard drug, metronidazole, were incubated with 2×10(5) trophozoites of Giardia intestinalis per milliliter of growth medium in 96-well tissue culture plates under anaerobic conditions for 24 hours.

The cultures were examined with an inverted microscope and the minimum inhibitory concentration and the IC_{50} value for each extract was determined. The chloroform extracts from *Alpinia galanga*, *Boesenbergia pandurata*, *Eclipta prostrate*, *Piper betle*, *Piper chaba*, *Zingiber zerumbet*, and the methanol extracts from *B. pandurata* and *E. prostrate* were classified as "active", i.e. with an IC_{50} of <100 micro g/mL, whereas the chloroform extract from *Murraya paniculata* was classified as being "moderately active". This study shows that extracts from some medicinal plants have potential for use as therapeutic agents against *G. intestinalis* infections (Sawangjaroen *et al.*, 2005).

Anti-HIV activity

In order to identify novel lead compounds with antiviral effect, methanol and aqueous extracts of eight medicinal plants in the Zingiberaceae family were screened for inhibition of proteases from human immunodeficiency virus type 1 (HIV-1), hepatitis C virus (HCV) and human cytomegalovirus (HCMV). In general, the methanol extracts inhibited the enzymes more effectively than the aqueous extracts. HIV-1 protease was strongly inhibited by the methanol extract of *Alpinia galanga.*

AIDS remains a major global health concern. Despite a number of therapeutic advancements, there is still an urgent need to develop a new class of therapy for human immunodeficiency virus (HIV). Here, it was shown that [1'S]-1'-acetoxychavicol acetate (ACA), a small molecular compound isolated from the rhizomes of *Alpinia galangal*, inhibited Rev transport at a low concentration by binding to chromosomal region maintenance a and accumulating full-length HIV-1 RNA in the nucleus, resulting in a block in HIV-1 replication in peripheral blood mononuclear cells. Additionally, ACA and did anosine acted synergistically to inhibit HIV-1 replication. Thus, ACA may represent a novel treatment for HIV-1 infection, especially in combination with other anti-HIV drugs (Watanabe *et al.*, 2005).

Anti-inflammatory activity

Anti-inflammatory and analgesic activity of the topical preparation of *Alpinia galanga* wild from methanol extract was studied. The anti-inflammatory activity was evaluated against Carrageenan-induced oedema in rats and in a formalin test. Piroxicam gel and methyl salicylate ointment were studied as positive controls for anti-inflammatory and analgesic activities, respectively. The degree of inhibition of oedema by preparations containing the extract at 1-5% w/w significantly varied from that of the control. The anti-inflammatory effect of SN at 4-5% was similar to the effect of Piroxicam gel at 3 h after Carrageenan injection (Nagashekhar and Shivaprasad, 2006).

Biological effects

The ethanol extracts of *Curcuma longa* and *Alpinia galanga* exhibited phytotoxic activity against *Lemma minor*. These extracts were also found to possess good antifungal activities against *Trichophytn longifusus* while in the brine shrimp lethality bioassay were found to be toxic with LD_{50} of 33 and 109 micro/mL, respectively. These extracts were found quite inert in antibacterial bioassay. While

the extract from *C. longa* tested for insecticidal activity, was also found to be devoid of any activity (Somia *et al.*, 2005).

Cytotoxicity activity

Bioflavonoids are the major pigments in plants with multitude of biological activities including inhibition of proliferation or induction of apoptosis in tumor cells. Even though the safety records of most flavonoids are exceptional, its therapeutic use is still in its infancy. Pinocembrin (5,7- dihydroxyflavanone), which was isolated from *Alpinia galanga* displayed cytotoxicity against a variety of cancer cells including normal lung fibroblasts with relative nontoxicity to human umbilical cord endothelial cells.

The compound induced loss of mitochondrial membrane potential with subsequent release of cytochrome c and processing of caspase-9 and -3 in colon cancer cell line HCT 116. Processing of caspase-8 was minimal. The initial trigger for mitochondrial apoptosis appears to be by the translocation of cyctoslic Bax protein to mitochondria. Overexpression of proapopotic Baz protein sensitized the colon cancer cells to pinocembrin-induced apoptosis and Bax knockout cells were resistant to pinocembrin-induced apoptosis. Antiapoptotic protein Bcl-X(L) only partially prevented apoptosis induced by this compound .

The Bax–dependent cell death involving classical cytochrome c release and processing of caspase-9and-3 suggests that pinocembrin is a classical mitochondrial apoptosis inducer. But the failure of Bcl-X(L) overexpression to completely prevent apoptosis induced by this compound suggests that pinocermbrin is capable of triggering mitochondrial-independent cell death that needs to be clarified. The existence of cell death upon Bcl-X (L)

Overexpression is a promising feature at this compound that can be exploited against drug resistant forms of cancer cells either alone or in combination with other

drugs.

The SRB cytotoxicity assay was used to screen extracts and isolated constituent of some traditional medicinal plants from Malaysia and Thailand against two human cancer cells lines, COR L23 lung cancer cell line and MCF7 breast cancer cell line and the non-cancer MCF5 cell line. Five out of the seven species tested, i.e. Thai *Alpinia galanga, Alpinia officinarum, Cayaratia japonica, Physalis minima, Tabernaemontana divaricate,* exhibited interesting cytotoxicity activity . Following bioassay–guided fractionation, 1'-acetoxychavicol acetate (48h exposure against COR L23 cells, IC_{50} (7.8 µM against MCF7 cells, IC_{50} 23.9 µM) was isolated as the major cytotoxic component of the *Alpinia* species, physalis F as the major cyctotoxic component of *Physalis minima* (48 h expose against COR L23 cells IC_{50} 0.59 µM).

Evidence from nrDNA ITS sequence variation

The fruits of *Alpinia galanga* are used as traditional Chinese medicines; but the dry fruits of *A. conchigera, A. suishaensis, A. maclurei and A. polyantha* are also used as the medicine in local areas. Because dry fruits of these related plants are similar to those of *Alpinia galanga* in odor, morphological characters and chemical components and even anatomical characters, it is difficult to identify the medicine (Zhao *et al.*, 2001).

Nuclear ribosomal DNA internal transcribed spacer (ITS) regions of the five taxa were directly sequenced using an automated sequencer. Sequence analysis showed that the ITS 1 ranges from 177 to 178 base pair (bp), and the ITS 2 from 225 to 234 bp. The size of the 5.8S coding region is 164bp for all species. Also the pairwise sequence divergence is higher and some molecular markers were determined. According to these molecular markers, *Alpinia galanga* and the related species can easily be distinguished from each other. Therefore, evidences from nrDNA ITS sequences variation can identify the medicines at the DNA level.

Gastroprotective effect

The effects of [1'S]-1'-acetoxychavicol acetate and related phenylpropanoids isolated from the rhizomes of *Alpinia galanga* on ethanol-induced gastic lesions in rats were examined. Among them [1'S]-1'-acetoxychavicol acetate and [1'S]-1'-acetoxychavicol acetate markedly inhibited the ethanol–induced gastic mucosal lesions (ED_{50} = 0.61 and ca. 0.90mg/kg). In addition [1'S]-1'-acetoxychavicol acetate inhibited the ethanol-induced gastric mucosal lesions (ED_{50} = 0.61 and ca.0.90mg/kg). In addition, [1'S]-1'-acetoxychavicol acetate inhibited the lesions induced by 0.6M HCI (ED_{50} = 0.73 mg/kg and aspirin ED_{50} = 0.69 mg/kg) but it did not show a significant effects on indomethacin–induced gastric lesions and acid output in pylorusligated rats at doses of 05-5.0 mg/kg.

From the gastroprotective effects of various related compounds the 1'-acetoxyl group of 1'S-1'-acetoxychavicol acetate and 1'S-1'acetoxyeugenol acetate was found to be essential for their strong activity. With regard to the mode of action, the gastroprotective effects of 1'S-1'-acetoxychavicol acetate were attenuated by pretreatment with indomethacin and *N*-ethyl; malemide and, 1'S-1'-acetoxychavicol acetate significantly increased the glutathione levels of gastric mucosa in rats . These findings suggest that endogenous prostaglandins and sulfhydryl compounds are involved in the protective effects of 1'S-1'-acetoxychavicol acetate (Matsuda *et al.,* 2003).

Hypoglycemic activity

This investigation was carried out to study effects of *Alpinia galanga* rhizome on blood glucose levels. In normal rabbits, powdered rhizome and its methanol and aqueous extracts significantly lowered the blood glucose. Gliciazide also produced a significant decrease in blood glucose in the rabbits.

In alloxan-diadetic rabbits, a galangal and its methanol and aqueous extracts did not produce significant reduction in blood glucose. The hypoglycemic effects of *Alpinia galanga* in normal rabbits was comparable to gliclizide. The rhizome was found to contain high levels of certain minerals. Acute toxicity and behavioral studies revealed no visible signs of toxicity and any abnormal behavior in rabbits even at high doses. It was concluded that *Alpinia galanga* produces fall in blood glucose levels in normal rabbits and the principles, both organic and inorganic, are extractable in methanol and water (Akhtar *et al.*, 2002).

Immunostimulating activity

Hot water polysaccharide extracts of *Anacyclus pyrethru, Citrullus colocynthis* and *Alpinia galanga* were tested for their immunostimulating activity in mice. The fractions from *Anacyclus pyrethrum* and *Alpinia galanga* showed a marked stimulating effect on the reticulo-endothelial system (RES) and increased the number of peritoneal exudcate cells (PEC), and spleen cells of mice. In this case, the optimum doses were 50 and 25mg/kg for the two fractions, respectively (Bendjeddou *et al.*, 2003).

On the other hand the polysaccharide extracts of both *Anacyculus pyrethrum* and *Alpinia galanga* markedly enhanced the proliferation of the murine spleen cells in vitro using two tests (in vitro and in vivo effects). The results of then in vivo effects at a doses of 50 and 35 mg/kg, showed a stimulation index better than obtained with the in vitro effects at 50 and 25 µg/mL for *Anacyclus pyrethrum* and *Alpinia galanga*, respectively. While the extract of *Citrullus colcynthis* showed much weaker and variable immunostimulating activity.

Inhibitors of nitric oxide production

The 80% aqueous acetone extract from the rhizomes of *Alpinia galanga* showed nitric oxide (NO) production inhibitory activates in mouse peritoneal

macrophages. The structures of new neoliganans were determined on the basis of physicochemical and chemical evidences. In addition, the inhibitory effects of the constituents from rhizomes of *Alpinia galanga* on NO production induced by lipopolysaccharide in mouse pertioneol macrophages were examined. Among them, *Alpinia galanga* (IC_{50} = 68 µM), *Galanganols B* (88 µM[1'S]-1'-acetoxychavicol acetate (2.3 µM), [1'S]-1'-acetoxycinnamaldehyde (11 µM), *tran-p*-hydroxycinnamaldehyde (ca.20 µM), *trans-p*-coumaryl diacetate (19 µM) were found inhibitory activity (Morikawa *et al.*, 2005).

Inhibitory effects

The [1'S]-1'-acetoxychavicol acetate from the rhizomes of *Alpinia galanga* was found to exhibit potent inhibitory effect on the production of nitric oxide (NO) in lipopolysaccharide-activated mouse peritoneal macrophages. To clarify its mechanism of action, the effects of [1'S]-1'-acetoxychavicol acetate on the expression of interferon-beta (IFN-beta) mRNA and activation of nuclear factor-kappaB (NF-kappaB), both of which participate in the induction of inducible NO synthase, were examined in lipopolysaccharide-activated macrophages. The results were compared with those of two inhibitors of the NF-kappeB activation, constunolide and caffeic acid phenyl ethyl ester. [1'S]-1'-acetoxychavicol acetate inhibited IFN-beta mRNA expression as well as NF-kappaB activation, and two related compounds, (±)-1-acetoxy-1-(2-acetoxyphenyl)-2-propene and (±)-1-acetoxy-1-(4-acetoxyphenol)-3-butene also inhibited IFN-beta mRNA expression. In addition 1'S-1'-acetoxychavicol acetate inhibited the production of NO stimulated of NO stimulated by poly (I;C) via Toll-like receptor (Ando *et al.*, 2005).

Receptor-binding antagonist activity

Forty-nine methanol extracts of 37 species of Malaysian medicinal plants were investigated for their inhibitory effects on platelet-activating factor (PAF) binding to rabbit platelets, using 3H-PAFas a ligand. Among them, the extracts of

three Zingiberacease species (*Alpinia galanga, Boesenbergia pandurata* and *Curcuma ochorrhiza*), *Goniothalamus malayanus. Momordica charantia and Piper aduncum* are potential sources of new PAF antagonists as they showed significant inhibitory effects with IC_{50} values ranging from 1.2 to 18.4 µg/mL (Jantan *et al.,* 2005).

Toxicity

Acute (24 h) and chronic (90 days) oral toxicity studies on the ethanolic extracts of the rhizomes of *Alpinia galanga* and *Curcuma longa* were carried out in mice. Acute dosages were 0.5, 1.0 and 3/kg body weight while the chronic dosage was 100 mg/kg/day as the extracts. All external morphological, hematological, and spermatogenic changes, in addition to body weight and vital organ weight were recorded. During this investigation no significant mortality as compared to the controls was observed (Qureshi *et al.,* 1992).

The weight gain in the *Alpinia galanga* treated animals was significant as in the control group while the *Curcuma longa*-treated animals gained no significant weight after chronic treatment. *Curcuma longa* treatment induced changes in heart and lungs weight upon chronic treatment. Hematological studies revealed a significant rise in the RBC level of *Alpinia galanga*-treated animals and a significant fall in the WBC and RBC levels of the *Curcuma longa*-treated animals as compared to the controls. The gain in weight of sexual organs and increased sperm motility and sperm counts were observed in both groups of extracts-treated male mice, however, these changes were highly significant in the *Alpinia galanga*-treated group. Both extracts failed to show any spermatotoxic effects

Spodoptera litura

Spodoptera litura is also known as the Oriental leafworm moth, Cluster caterpillar, Cotton leafworm, Tobacco cutworm, Tropical armyworm, Taro caterpillar, Tobacco budworm, Rice cutworm, and Cotton Cutworm. This moth is found in Asia, with some specific problematic pest population reports occurring in Cambodia, Hong Kong, India, the Pacific islands, Guam, American Samoa, and Hawaii. In Australia, it is found in northern two thirds of the country. It is not established in the United States; however, it is a pest of national, regulatory concern.

Figure 2 Larva of *Spodoptera litura*

The Armyworm is classified as

Phylum	Arthopoda
Class	Insecta
Order	Lepidoptera
Family	Noctuidae
Genus	*Spodoptera*
Species	*Spodoptera litura*

Hosts

Spodoptera litura attacks over 300 cultivated and wild fruits including *Annona sp.* (cherimoya, atemoya, sugar apple), avocado, banana, bitter melon, citrus, coffee, guava, macadamia, mango, papaya, passion fruit, peppers, persimmon, and tomato. This pest will apparently breed in all fleshy fruits. On Oahu it is estimated that 95% of *Spodoptera litura* develop on guava, *Psidium guajava* L. (Newell and Haramoto, 1968). It does not attack cucurbit crops such as cucumber and squash.

Distribution

Spodoptera litura originally described from Taiwan is one of the most destructive fruit fly pests of East Asia and the Pacific. It is second only to the Mediterranean fruit fly. Its distribution range includes Pakistan and India to southern Japan, Indonesia to Micronesia, and the Mariana Islands and Hawaii. Recent outbreaks have occurred in southern California and Florida. Accidentally introduced into Hawaii in 1944 or 1945, this pest is currently present on all major Hawaiian Islands. It is primarily found in the lowland areas of Hawaii.

Damage

The damage to crops caused by *Spodoptera litura* result from

1) Oviposition in fruit and soft tissues of vegetative parts of certain plants.
2) Feeding by the larvae.
3) Decomposition of plant tissue by invading secondary microorganisms.

Larval feeding in fruits is the most damaging. Damage usually consists of breakdown of tissues and internal rotting associated with maggot infestation, but this varies with the type of fruit attacked. Infested young fruit becomes distorted, callused and usually drop; mature attacked fruits develop a water soaked appearance. The larval tunnels provide entry points for bacteria and fungi that cause the fruit to rot.

When only a few larvae develop, damage consists of an unsightly appearance and reduced marketability because of the egg laying punctures or tissue break down due to the decay (Steiner, 1957).

On papaya, *Spodoptera litura* is the primary pest in Hawaii. *Spodoptera litura*, the Mediterranean fruit fly and the melon fly, are infrequently found in papaya. The solanaceous fruit fly, *Dacus latifrons* (Hendel), does not attack papaya (Liquido and Cunningham, 1990). Infestation rates in papaya by *Spodoptera litura* increases with ripeness of the fruits (Liquido and Cunningham, 1990). Although the actual injury on papaya by fruit flies is relatively low, these flies are considered a major pest of papaya in terms of exporting from Hawaii to the US Mainland and Japan. It is necessary to treat the papaya fruits with post-harvest treatments to meet phytosanitary regulations.

On banana cultivars 'Brazilian', 'Valery' and 'William's', *Spodoptera litura* eggs and larvae develop in fruit at the later stages of ripeness only. Banana is not a host for the *Spodoptera litura* when the bananas are unripe and attached to the banana plant. Unripe bananas up to 3 to 4 days post harvest are also free of fruit flies (Armstrong, 1983).

The economic importance of the *Spodoptera litura* cannot be evaluated entirely from the standpoint of the actual damage to the various crops affected. It must also be considered from the standpoint of quarantine.

Quarantine regulations to prevent establishment of *Spodoptera litura* in areas where it does not occur are vigorously enforced. The U.S. government has strict laws regulating the movement of certain commodities to prevent the establishment of oriental fruit flies into the continental U.S. The Japanese government restricts the entry of untreated hosts of this pest into their country.

Biology

Development from egg to adult takes about 16 days in Hawaii. Developmental periods may be extended considerably by cool weather.

Eggs:

Female flies insert eggs under the skin of fruit in clusters of 10 to 50 about 1/25 to 1/8 inch below the fruit surface. The eggs measure about 1/25 by 1/250 inch and are white, elongate, and elliptical. They hatch in 1-1/2 days.

Larvae:

The white larva is legless, and resembles an elongated cone. The mouth is at the pointed end of the body. There are 3 larval stages, or instars. The third instar is about 2/5 inch long. The entire larval stage lasts for 11-15 days.

Pupae:

When mature, larvae drop to the ground and pupate in the soil. The puparium is yellowish-brown and seed-like. Adults emerge in about 10 days.

Adults:

The color of the fly is highly variable but mostly yellow with dark markings on the thorax and abdomen. Generally, the abdomen has two horizontal black stripes and a longitudinal median stripe extending from the base of the third segment to the apex of the abdomen. These markings may form a "T" shaped pattern, but the pattern varies considerably.

Females begin to lay eggs about 8 days after emergence from the puparium. Under optimum conditions, a female can lay more than 3,000 eggs during her lifetime, but under field conditions approximately 1,200 to 1,500 eggs per female is considered to be the usual production. Ripe fruit are preferred for egg laying, but immature ones may be also attacked (Steiner, 1957).

Behavior

Emerging adults crawl up through the soil, usually at an angle. Although they have been reported crawling up from greater depths, the adult usually doesn't have to emerge from a depth greater than 1 to 2 inches. Most flies emerge between 7:00 and 10:00 A.M., this period may be extended with overcast skies, rain or low temperatures but rarely goes into mid-afternoon (Christenson and Foote, 1960).

Adult flies primarily feed during the morning hours. They search for food in all types of vegetation, including low cover plants and shrubs, and may travel to areas where host plants do not occur (Christenson and Foote, 1960). Without food, flies die within three days at an average temperature of 80°F (Christenson and Foote, 1960).

***Spodoptera litura* distribution**

The larvae feed on a wide range of plants and have been recorded from over 40 mostly dicotyledonous plant families. It is a major pest of many crops.

Spodoptera litura is probably native to India and Southeast Asia; it is well distributed in the Pacific Islands, but not known from Tokelau and Tuvalu (Waterhouse and Norris, 1987).

Management studies of *Spodoptera litura*

The introductions made for the biological control of *Spodoptera litura* worldwide, and give comprehensive records of introductions for Pacific Island countries. Those accorded with success are: egg parasites - *Telenomus nawaii* (egg parasite); larval parasites - *Apanteles marginiventris, Peribaea orbata, Chelonus sp., Palexorista sp.*, and many more. The conclusion of them is that *Spodoptera litura* is probably native to the Pacific region and that many natural enemies already attack it, so it is unclear what further prospects there are for biological control.

There are, however, countries in the Pacific where severe outbreaks occur. It is thought likely that in newly cleared areas or after cyclones, it may require some time before natural enemies are established (or re-established) and for the food sources of the parasites to develop. In this connection, in many Pacific Islands, *Coleus blumei* is planted in taro gardens and this may be a source of nectar or pollen for the adult parasites.

Chemical sprays may be needed when biological control of *Spodoptera litura* is insufficient. They should be used with caution so as not to disrupt the balance generally established between natural enemies and this pest, otherwise, more harm than good might be done. In support of this, (Stechmann and Semisi, 1984) noted in Samoa more damage in fields where insecticide had been applied than in those unsprayed. Those products to be considered are those that are non-toxic to beneficial insects, such as the biologically derived pesticides, Spinosad (derived from the soil actinomycete, *Saccharopolyspora spinosa*) and Bt (from the bacterium, *Bacillus thuringiensis*). Neem extracts have been considered in India.

Resistance to insecticides is a major problem associated with the chemical control of insect pests, which is characterized by rapid evolution under strong selection of gene(s) that confers survival to insecticides (Ahmad *et al.*, 2008). This selective pressure exerted by the insecticides abruptly increases the frequency of the

genetic condition expressed as resistance within the exposed population.

The development of resistance is a result of the selection pressure exerted on sprayed populations increasing the frequency of resistant individuals (Torres-Vila *et al.,* 2002), but several factors including temperature are also involved in influencing the evolution of insecticides resistance (Raymond and Marquine, 1994).

At present, the extensive use of conventional insecticides such as organophosphate, carbamate and pyrethroids against *Spodoptera litura* has produced prevalent resistance in China (Wu *et al.,* 1995; Huang *et al.,* 2006). With high resistance to conventional insecticides, the insect growth regulators (IGRs) and newer insecticides were recently introduced to control this pest (Chen *et al.,* 2008; Su *et al.,* 2012). In the case of IGRs, flufenoxuron, chlorfluazuron, tebufenozide, and methoxyfenozide were used to control *Spodoptera litura* in Shandong and Jiangsu Provinces and had high toxicity to *Spodoptera litura*, in which resistance to flufenoxuron and methoxyfenozide was barely produced (Huang *et al.*, 2006).

In addition, the newer insecticides bearing novel modes of action such as indoxacarb, abamectin, emamectin benzoate, fipronil, and spinosad were recently introduced into Hunan Province for management of the pests.

The extensive use of these newer insecticides against *Spodoptera litura* have provided an ideal environment for its evolution of resistance and *Spodoptera litura* was found to have inherent risks for resistance to indoxacarb (Wang *et al.,* 2009). Previous exposure and selection with insecticides can confer resistance to newly introduced insecticides through cross-resistance (Bisset *et al.,* 1997; Sayyed *et al.,* 2008), reducing the effectiveness of many new insecticides. There are some data available on the newer insecticide resistance in *Spodoptera litura* from cash crops and vegetables growing countries such as Pakistan (Ahmad et al., 2008; Shad *et al,* 2012).

There are three principal cultural methods that may be used for controlling this pest.

1) Field sanitation

2) Trap crops

3) Resistant varieties.

Of utmost importance and effectiveness is field sanitation. This practice reduces re-infestation pressure. All unmarketable and infested fruits must be destroyed. Crops should be plowed and disked under as soon as harvest has been completed.

Liquido (1990) reported that papaya fruits left on the ground serve as a major breeding site and reservoir of resident melon fly populations. He also reported that the number of adults in the orchard had a higher significant correlation with the percent infestation in fruits on the ground than the percent infestation in tree fruits.

Although previous study concerned melon fly infestation, similar results would be expected for the *Spodoptera litura* since the density of *Spodoptera litura* in papaya is much greater than that of melon fly (Liquido and Cunningham, 1990). This information further supports the importance of removing fallen fruit for the management of *Spodoptera litura* populations in papaya orchards.

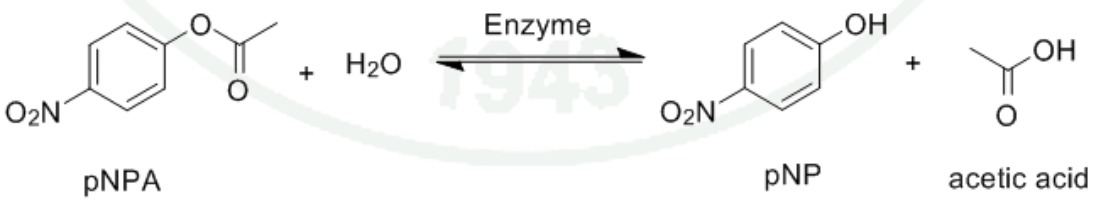
They suggested that pre-harvest control measures such as field sanitation could enhance the quality of marketable fruit by allowing the use of less damaging schedules of post harvest quarantine treatments. For example, vapor heat, dry heat, hot water double dip or a combination of these treatments) could be applied at lower kill temperatures or shorter treatment durations (Liquido, 1990; Liquido, *et. al*., 1989; Liquido and Cunnigham, 1990).

Mode of action study of insect enzyme activity

Carboxylesterase activity

The esterase reaction, defined as the hydrolysis of an ester to its component alcohol and acid, encompasses hydrolysis of a diverse range of carboxylic, thio-, phospho-, and other ester substrates. It sites within a broader set of hydrolase reactions that also include glycosylases, proteases, amidases, and many other (Webb, 1992). Even more generally it can be considered a particular case, involving water, of acylation of a nucleophile. Use of other nucleophile in otherwise similar reactions generates, for example, various dehalogenase activities (Ollis *et al.*, 1992)

Carboxylesterase (CEs) metabolism plays important role in *Spodoptera litura* resistance. It can be utilized for enhancing toxicity of insecticides and pesticides such as malathion and permethrin by using corresponding alcohol and carboxylic acid, hydrolysis (Scheme 1) of tri-acylglycerols and can *trans*-esterify fatty acids to fatty acid ethyl esters of variety of esterified drugs like meperidine, cefuroximine axetil, cefpodoxime proxetil, cocaine and heroin. CEs of insects are located in cytosol, microsomes; mitochondria and nuclei (Bullangpoti, 2007). Increase of CEs is used for devising biochemical diagnostic methods of detection of insecticide resistance (Brown and Brogdon, 1987).



Scheme 1 Reaction of pNPA assay

Source: Ganske (2009)

Glutathione-*S*-transferase (GSTs) activity

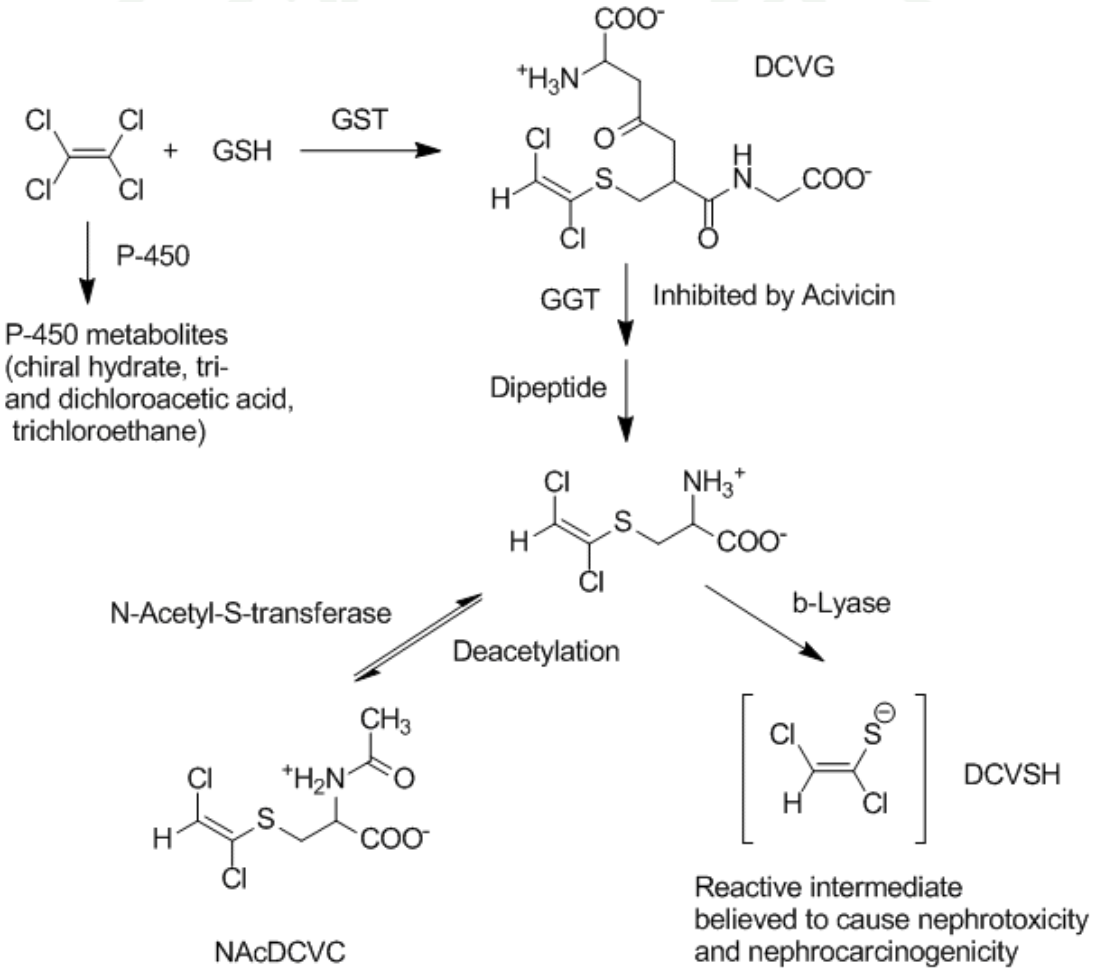
Glutathione-*S*-transferase is found ubiquitously in aerobic organisms. They were first discovered in animal in 1961 where they were postulated to play a role in the detoxification of drug (Booth *et al.*, 1961). Their central role in detoxification and drug resistance pathways in mammals has now been established but additional functions are continually being attributed to this complex enzyme family. For example, GSTs are important mediators in oxidative stress responses, are involved in the synthesis of prostaglandins, and facilitate intracellular transport of hydrophobic compounds. Mammalian GTSs are particular well studies due to their role in cancer epidemiology and treatment. Several GSTs can metabolize environmentally derived carcinogens and polymorphisms in these genes are linked to cancer risk. In addition, the over expression of GSTs in tumor cells can contribute to drug resistant (Landi, 2000).

In 1985, Glutathione-*S*-transferase had only been identified in a limited number of insect species including the grass grub, greater max moth, housefly and American cockroach (Mannervik, 1985). The importance of this enzyme family in many different types of insecticide resistance had been demonstrated and multiple forms were known to exist in individual insect. Those GSTs have now been studied in over 30 different insect species is testimony to the importance of this enzyme family.

Glutathione-*S*-transferase plays important role in detoxification of organophosphate and organochlorine by catalyzes the formation of thiol group (Scheme 2) of glutathione to electrophilic xenobiotic that provides ability to scavenge toxic compound like oxidative stress.

Therefore, the aim of the previous study was to investigate the effect of melatonin on synthetic parathyroid insecticide-induced antioxidative enzymes activity in *Spodoptera litura* larvae. In addition, an activity of enzymatic antioxidants

in glutathione-*S*-transferase (GST) was assessed. There was no significant change in GST levels in the melatonin-treated groups. In conclusion, the results of the current study revealed that *Spodoptera litura* toxicity activated oxidant systems in all antioxidant systems in some tissues of insects. Melatonin administration led to a marked increase in antioxidant activity and inhibited GST (Karthi and Shivakumar, 2014).



Scheme 2 GSH-conjugation pathway

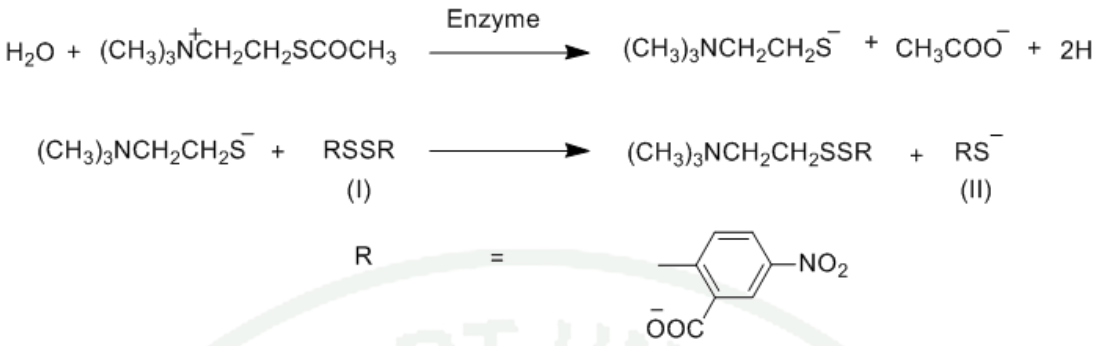
Source: Brian (2000)

Acetylcholinesterase (AchEs) activity

Acetylcholinesterase remains three major groupings. One is an ancient group of six major clades implicated in neuro/developmental functions, the only catalytically competent members of which are the acetylcholinesterase (AchEs). Many papers have been published on the molecular biology and/or biochemistry of insect AchEs over the last 10 years, the majority dealing with the molecular basis of target site resistance to organophosphate and carbamate insecticides.

Acetylcholinesterase is significant factor to resist organophosphate and carbamate compounds. This enzyme plays resistant role to insecticide by phosphorylation (Scheme 3) and decrease activity. Cause the accumulation of acetylcholine reducing sensitivity of synapses, stimulate neurotransmission of central nervous system and peripheral nervous system occurring changing behavior in insect call chemical avoidance.

Therefore, the aim of the previous study was to investigate the effect of melatonin on synthetic pyrethroid insecticide-induced antioxidative enzymes activity in *Spodoptera litura* larvae. In addition, activities of enzymatic antioxidants viz. superoxide dismutase (SOD), glutathione-*S*-transferase (GST), catalase (CAT), glutathione reductase (GR), α, β-esterase, and acetylcholine esterase (AChEs) were assessed. There was no significant change in GST levels in the melatonin-treated groups. Melatonin modulates cypermethrin-induced changes in the activities of esterase and AChEs, whereas SOD, CAT, and GR activity was significantly increased in melatonin-treated samples when compared to control. In conclusion, the results of the current study revealed that SP toxicity activated oxidant systems in all antioxidant systems in some tissues of insects. Melatonin administration led to a marked increase in antioxidant activity and inhibited GST and AChEs in most of the tissues studied (Karthi and Shivakumar, 2014).



Scheme 3 Reaction of acetylcholine assay

Source: George (1961)

MATERIALS AND METHODS

Materials

1. Plant Materials

The fresh rhizomes of *Alpinia galanga* (20 kg) were collected from Prachin Buri province, Thailand on November 2012.

Figure 3 The rhizomes of *Alpinia galanga*

2. Instrument

^{1}H NMR spectrum was recorded on Varian INOVA 400 spectrometer at the Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand.

^{13}C NMR spectrum was recorded on Bruker (300 MHz) at the Scientific and Technological Research Equipment Centre, Chulalongkorn University, Bangkok, Thailand.

Chemical shifts were given in ppm (δ) for ^{1}H and ^{13}C NMR. Deuterochloroform ($CDCl_3$) used as the solvent and internal reference (at δ 7.26 ppm). The following abbreviations were used for multiplicity: s = singlet, d = doublet, dd = doublet of doublet, dt = doublet of triplet, ddd = doublet of doublet of doublet, t = triplet, m = multiplet. Coupling constants (J) are reported in Hertz (Hz).

Rotary evaporator (IKA®RV10 basic, Thailand) was used at the Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand.

UV spectra were performed with AS ONE handy UV lamp SLUV-6 spectrometer on 254 and 365 nm at the Department of Chemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand.

Centrifuge (Hettich : Universal 16R) was performed at the Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand.

Microplate reader was used at the Department of Zoology, Faculty of Science, Kasetsart University, Bangkok, Thailand.

3. Chromatographic System

3.1 Vacuum Liquid Chromatography (VLC) or Quick Column chromatography

Vacuum Liquid Chromatography was performed on a glass column with a medium porosity sintered glass frit, packed with Merck silica gel absorbent 60G. The silica gel was added to the column (10.5×19 cm) by slurry packing method. The

height of the packed silica gel bed was about 13 cm. The chromatographic column was run under reduced pressure using water aspirator.

3.2 Column Chromatography (CC)

Column Chromatography was performed on a glass column using Merck silica gel 60 absorbent (70-230 mesh). The size of chromatographic column depended on the amount of sample. The ratio of sample to adsorbent was about 1 to 40-80 by weight.

The silica gel column chromatography was developed with various suitable solvents to obtain the best separation. Thin Layer Chromatography (TLC) monitored each fraction.

3.3 Thin Layer Chromatography (TLC)

3.3.1 Preparative Thin Layer Chromatography (PTLC): PTLC plate was prepared as follows 20 g of Merck silica gel 60 PF_{254} with a fluorescent indicator (254 and 365 nm) mixed with 40 mL distilled water. Then the suspension was transferred to 20×20 cm glass plated. The plates were left to dry at room temperature for 48 hours.

3.3.2 Thin Layer Chromatography (TLC): TLC investigation routinely used for chromatographic separations was carried out on Merck pre-coated silica gel 60 F_{254} supported on aluminum sheet.

Methods

1. Insect Rearing

Spodoptera litura populations were received from the Ministry of Agriculture, Bangkok, Thailand. Larvae were reared on an artificial diet (Silkmate 2S, Nihon Nosan Kogyo Co. Ltd) according to the modified method (Arakawa *et al*., 2008) in a controlled environment at 27°C, 70% R.H., and 16L-8D photo-period until being used in this test. These larvae were fed with kale leaves.

2. Extraction

The fresh rhizomes of *Alpinia galanga* (20 kg) were dried in the sun for three days. The dried rhizomes of *Alpinia galanga* (2 kg) were crushed into small pieces and ground to fine powder using a blender. Dried powder of the rhizomes of *Alpinia galanga* were sequentially extracted with hexane, dichloromethane, ethyl acetate and methanol by soaking at room temperature for seven days.

Each solvent crude extracted was filtered through a Buchner funnel using Whatman number 1 filter paper. The filtrate was evaporated to dryness under reduced pressure using rotary evaporator to give the extracts. All crude extracts were collected and stored at 4°Cuntil further use in the experiments.

3. Bioassay on *Spodoptera litura*

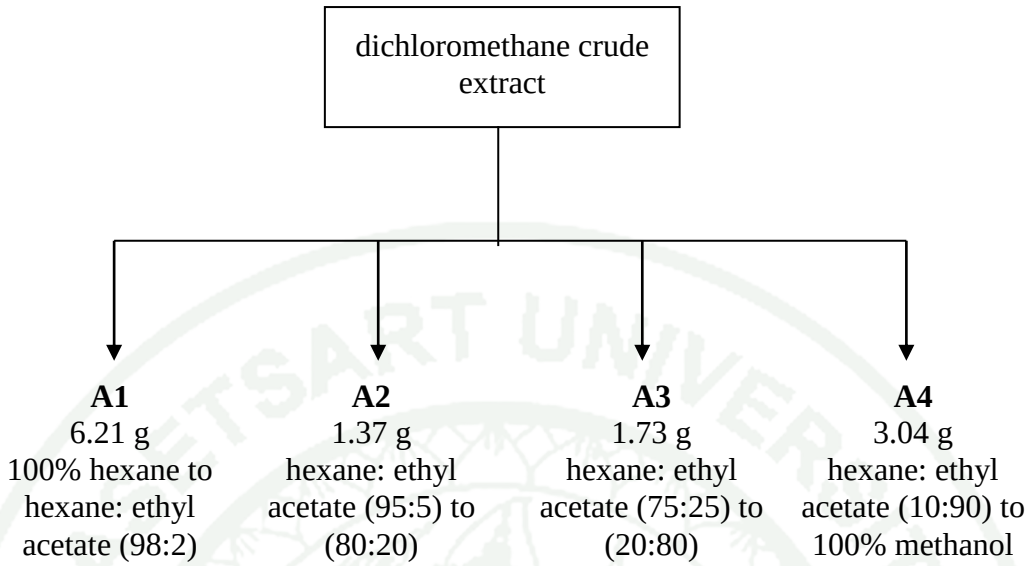
Second instar *Spodoptera litura* larvae were used in all the experiments to determine the median lethal dose (LD_{50}) by the topical method. Four concentrations of each extract were prepared in acetone (AR grade) (1250, 2500, 5000 and 10000 ppm).

The treatment was applied topically to the thorax region of second instars and 30 larvae were used in three replicates in each treatment.

In case of controls, acetone was applied topically. The treated larvae and controlled larvae were then placed in Petri dishes (diameter 100 mm) and provided kale leaves for feeding. Mortality was recorded after 24 hours and 48 hours post-treatment. The LD_{50} values were determined by Probit analysis using the StatPlus Program (2008 version). *Spodoptera litura* behavioral responses such as paralysis or knockdown were recorded.

4. Isolation and Purification of the Dichloromethane Crude Extract

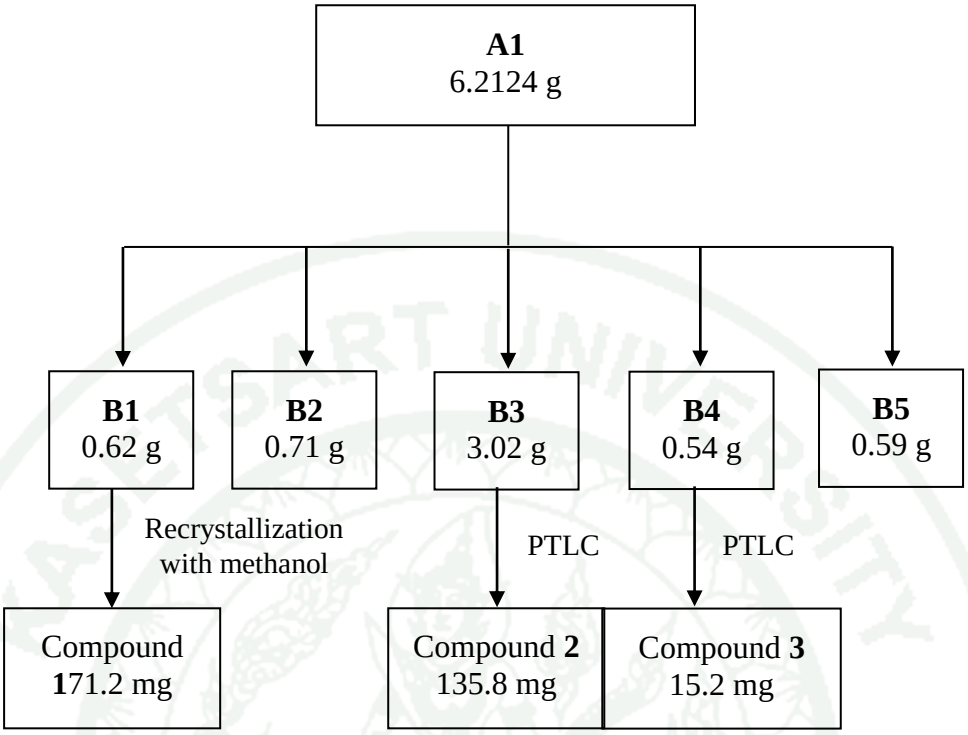
The most active crude dichloromethane extract (15.99 g) was fractionated using vacuum silica gel column chromatography (Kiesel gel 60G, Merck, Thailand) and eluted with eluted with a gradient of gradually increasing polarity (2-10% increments) of hexane-ethyl acetate and ethyl acetate-methanol, respectively. The eluted solution of each solvent system was collected that was equivalent to 250 ml. All fractions were subjected to Thin Layer Chromatography (TLC), and those with similar components were combined. Using this procedure, four fractions (**A1**, **A2**, **A3** and **A4**) were obtained (Scheme 4). Then, all fractions were further studied their toxicity against *Spodoptera litura*.



Scheme 4 Isolation of the dichloromethane crude extract.

4.1 Isolation and Purification of Fraction **A1**

Fraction **A1** (6.21 g) was subjected to silica gel column chromatography (100 g and column size 2.5×21.0 cm by eluting with 100% hexane to give fraction **B1**. Then, the use of mixture solvent as hexane and ethyl acetate (90:10) could afford fraction **B2** while hexane and ethyl acetate (70:30) and (40:60) provided fraction **B3** and **B4**, respectively**.** Finally, fraction **B5** was obtained when methanol was used as solvent (Scheme 5).



Scheme 5 Isolation of the **A1** fraction.

Fraction **B1** was purified by recrystallization using methanol to give compound **1** (71.2 mg, 0.0034%) as mixture of long chain alkenes.

Fraction **B3** was purified by preparative thin layer chromatography with 7:1 hexane:ethyl acetate to afford compound **2** (135.8 mg, 0.0068%).

[1'S]-1'-Acetoxychavicol acetate: Yellow solid; $[\alpha]_D^{20}$ -50 (*c*=0.5, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz), δ: 7.37 (2H, d, *J* = 8.7 Hz), 7.07 (2H, d, *J* = 8.7 Hz), 6.26 (1H, d, *J* = 5.7 Hz), 5.98 (1H, ddd, *J* = 17.1, 10.5, 5.8 Hz), 5.30 (1H, dt, *J* = 17.2, 1.3 Hz), 5.25 (1H, dt, *J* = 10.5, 1.2 Hz), 2.30 (3H, s), 2.11 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz), δ: 170.0, 169.4, 150.7, 136.4, 136.3, 128.6, 121.7, 117.3, 75.8, 21.1, 21.0.

Fraction **B4** was purified by preparative thin layer chromatography with 15:1 hexane:ethyl acetate to give compound **3** (15.2 mg, 0.00076%).

p-Coumaryl diacetate: Colorless solid; ^{1}H NMR ($CDCl_3$, 400 MHz), δ: 7.39 (2H, d, *J* = 8.6 Hz), 7.05 (2H, d, *J* = 8.6 Hz), 6.62 (1H, d, *J* = 16.1 Hz), 6.22 (1H, dt, *J* = 15.9, 6.4 Hz), 4.75 (2H, dd, *J* = 6.4, 1.3 Hz), 2.30 (3H, s), 2.10 (3H, s); ^{13}C-NMR ($CDCl_3$, 75 MHz), δ: 170.8, 169.4, 150.4, 134.0, 133.1, 127.6, 123.4, 121.7, 64.9, 21.1, 21.0.

5. Mode of action study of second instars larvae of *Spodoptera litura* after treated with isolated compound from *Alpinia galanga*

5.1 Preparing insect for enzyme extraction

For *in-vivo* treatment assay, second instar larvae of *Spodoptera litura* were treated with isolated compound of *Alpinia galanga* at LD_{50} value concentration. Control group was treated with 95% acetone (10 larvae/ replication). After treated 24 hours, the survivals of *Spodoptera litura* were used for enzyme extraction.

5.2 Extraction of enzymes activities method

This method was modified from Feyereisen (2005). To begin with, survival *Spodoptera litura* were placed micro tube and kept on ice and grinded with homogenized buffer (0.1 M potassium phosphate buffer mixed with 1 mM EDTA at pH 8.0). Then homogenates solutions were centrifuged at 40°C, 18,000 rpm for 5 minutes. Supernatants were separated and kept on ice and immediately use enzyme activity test.

5.3 Carboxyl esterase enzyme activity

The carboxylesterase activity of *p*-nitrophenylacetate (pNPA) assay was modified from Ganske (2009). 50 mM of phosphate buffer were mixed with supernatant. Then 10 mM pNPA (*p*-nitrophenyl acetate) were added and measured by kinetic mode at λ_{max} = 410 nm, 37°C with microplate reader. The activity was described as changing of yellow color of *p*-nitrophenol from hydrolysis of *p*-nitrophenylacetate.

5.4 Acetylcholinesterase activity

Acetylcholinesterase activity method was modified from Ellman (1959). 100mM of potassium phosphate buffer were mixed with supernatant 50µl and incubated at 30°C for 30 minutes. Then Tps (Tampon substrate: (2 µL of 10 mM 5,5'-dithio-*bis*-2-nitrobenzoic acid (DTNB), 2 µL of 100 mM and 46 µL of 100 mM PBK, pH 7.2) were added and measured at λ_{max} = 412 nm by kinetic mode. The activity was described as changing of yellow color generated by reaction of DTNB by microplate reader.

5.5 Glutathione-*S*-transferase (GST) activity

Glutathione-*S*-transferase activity of 1-chloro-2,4'-dinitrobenzene (CDNB) method was followed by microplate reader. 50 mM of phosphate buffer were mixed with glutathione solution, supernatant and CDNB, and finally measured at λ_{max}= 340 nm using micro plate reader. The activity was described as changing absorbance of CDNB.

6. Statistical analysis

The data of report were expressed as mean ± SD. Homogeneity of variances were calculated by Levene's test with One-way ANOVA. Different results calculated

by using Duncan's multiple range test (DMRT) and analyses median lethal dose (LD_{50}) with StatPlus 2008 before created graph with Sigma Plot 11.0.

RESULTS AND DISCUSSION

Zingiberaceae is one of the major tropical plant families, the members of which are used as spices and as medicinal herbs. Rhizomes of several species are also used as insect repellents and many compounds with novel structures and a large number of biologically active compounds have been identified from these plants (Pancharoen *et al*., 2000). *Alpinia galanga* is one such plant of this family, rhizomes of which have medicinal properties and many compounds of this plant have various biological activities (Panchareon *et al*., 2000).

Dried powder of the rhizomes of *Alpinia galanga* (2 kg) were separately extracted with hexane, dichloromethane, ethyl acetate and methanol respectively, to provide four crude extracts as shown in Table 1.The percent yield of each crude extracted was calculated by comparing the mass of crude extracts to the amount of fresh young leaves. The percent yields of hexane, dichloromethane, ethyl acetate and methanol crude extracts were obtained in 1.21%, 0.80%, 0.95% and 3.84%, respectively.

Table 1 Characteristics and amount of crude extract of the rhizomes of *Alpinia galanga* obtained in different solvent extractions.

Extract	Weight (g)	Yield[1] (% wt:wt)	Characteristics
Hexane	24.18	1.21	Yellow oil
Dichloromethane	15.99	0.80	Yellow oil
Ethyl acetate	18.94	0.95	Dark brown gum
Methanol	76.85	3.84	Black gum

[1]Weight of crude extract / weight of dried plant x 100

All crude extracts from the rhizomes of *Alpinia galangal* were examined for insecticidal activities against *Spodoptera litura* by topical application method on four concentrations at 1,250, 2,500, 5,000 and 10,000 ppm and reported percentage of mortality after 24 hours and 48 hours post-treatment (Table 2).

There have been very scanty reports to demonstrate insecticidal activity of this plant species (Chandel *et al.*, 2011; Sukhirun *et al.,* 2011). In this research, we have for the first time shown that extract of rhizomes of *Alpinia galanga* are toxic to the second instars *Spodoptera litura* using topical application.

1. Topical Application Test Against *Spodoptera litura* Second Instar Larvae

1.1 Toxicity of hexane crude extract

The 24 hours mortality percentage of second instars *Spodoptera litura* after topical application with hexane crude extract was started 6.67% mortality at the dose 1,250 ppm. The mortality percentage values significantly increase to 23.33% and 36.67% at dose 2,500 ppm and 5,000 ppm, respectively, before hitting to its highest point of 66.67% at 10,000 ppm. The mortality percentage values showed significant increase ($P>0.05$) from Duncan's Multiple Range Test in order to dose. LD_{50} values at 24 hours of *Spodoptera litura* second instars after topical application test with hexane crude extract was 6479.35 ppm (Figure 4).

After 48 hours treated in same concentration, mortality percentage slightly increased but not significant for time dependent. The mortality percentage values began at 16.67% at dose 1,250 ppm and finished at 83.33% at the end of 10,000 ppm dose.

The mortality percentage values showed significant increase correlation with increasing concentration from 1,250 ppm to 10,000 ppm at P>0.05 from Duncan's Multiple Range Test. LD_{50} values at 48 hours of second instars *Spodoptera litura* after topical application test with hexane crude extract was 5,360.84 ppm (Table 2).

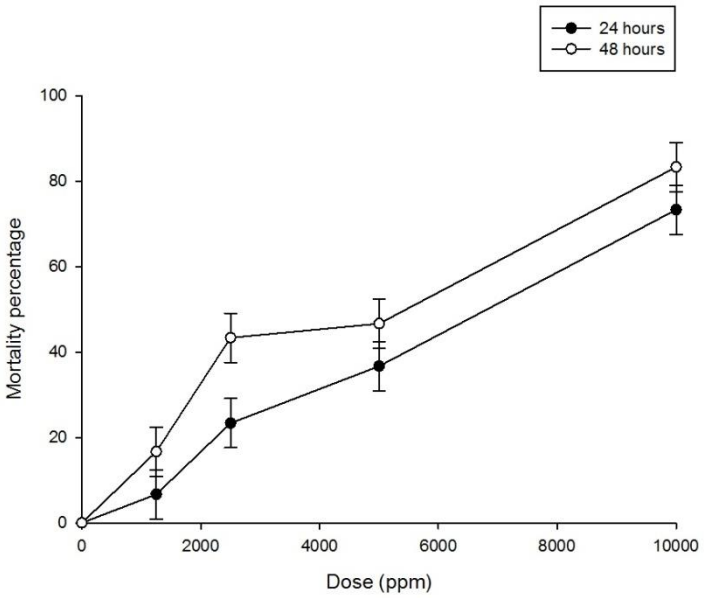


Figure 4 Mortality percentages of second instars *Spodoptera litura* after topical application test with hexane crude extract after 24 and 48 hours

1.2 Toxicity of dichloromethane crude extract

The 24 hours mortality percentage of second instars *Spodoptera litura* after topical application with dichloromethane crude extract started at 33.33% mortality at the dose 1,250 ppm before the mortality percentage values at 2,500 ppm dose showed significant increase to 36.67% and up to 73.33% at dose 5,000 ppm, respectively. Finally, the mortality percentage values gradually grew to highest point of 86.33% at 10,000 ppm. The mortality percentage values exhibited significant increase (P>0.05) from Duncan's Multiple Range Test. LD_{50} values at 24 hours of

Spodoptera litura second instars after topical application test with dichloromethane crude extract was 3,177.35 ppm (Figure 5).

After 48 hours treated in same concentration, mortality percentage was slightly increased which no significant difference for time dependent. The mortality percentage values started at 36.67% at dose 1,250 ppm and increase to highest point at 96.67% at the end of 10,000 ppm dose. The mortality percentage values showed significant increase correlation with increasing concentration from 1,250 ppm to 10,000 ppm at P>0.05 from Duncan's Multiple Range Test. LD_{50} values at 48 hours of second instars *Spodoptera litura* after topical application test with dichloromethane crude extract was 2,099.72 ppm (Table 2).

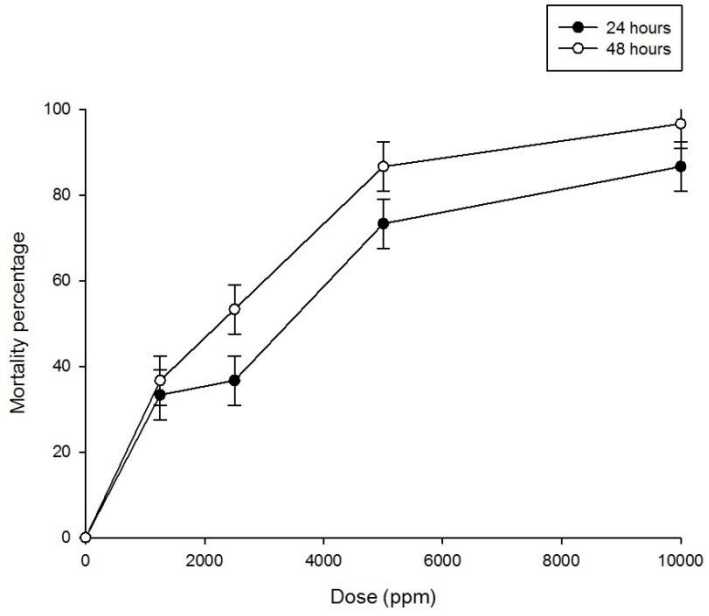


Figure 5 Mortality percentages of second instars *Spodoptera litura* after topical application test with dichloromethane crude extract after 24 and 48 hours

1.3 Toxicity of ethyl acetate crude extract

LD_{50} values at 24 hours of *Spodoptera litura* second instars after topical application test with ethyl acetate crude extracts was 5,878.99 ppm (Table 2). The 24 hours mortality percentage of second instars *Spodoptera litura* after topical application with hexane crude extracts began at 26.67% mortality at the dose 1,250 ppm. The mortality percentage values displayed significant up to 43.33% and 53.33% at dose 2,500 ppm and 5,000 ppm, respectively, before hitting to its highest point of 83.33% at 10,000 ppm. The mortality percentage values showed significant increase ($P>0.05$) from Duncan's Multiple Range Test in order to dose (Figure 6).

After 48 hours treated in same concentration, mortality percentage slightly increased but not significant for time dependent. The mortality percentage values began at 33.33% at dose 1,250 ppm and finished at 93.33% at the end of 10,000 ppm dose (Figure 8). The mortality percentage values showed significant increase correlation with increasing concentration from 1,250 ppm to 10,000 ppm at $P>0.05$ from Duncan's Multiple Range Test. LD_{50} values at 48 hours of second instars *Spodoptera litura* after topical application test with ethyl acetate crude extract was 3,950.40 ppm (Table 2).

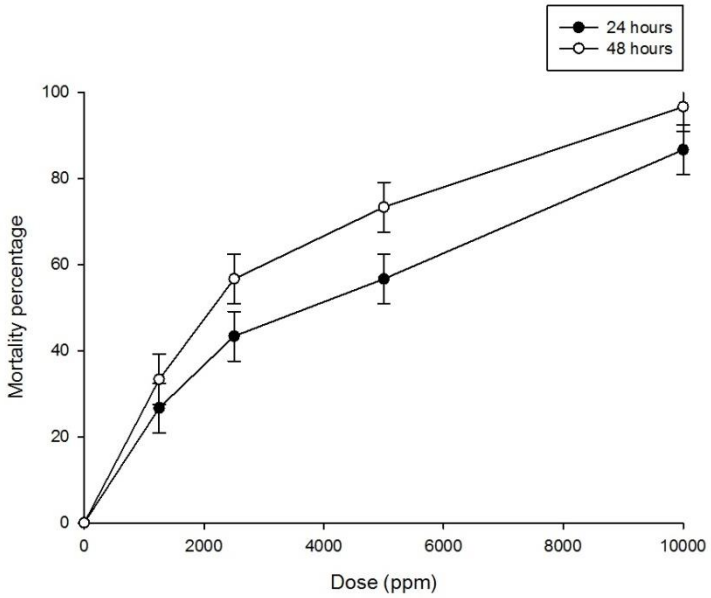


Figure 6 Mortality percentages of second instars *Spodoptera litura* after topical application test with ethyl acetate crude extract after 24 and 48 hours

1.4 Toxicity of methanol crude extract

The 24 hours mortality percentage of second instars *Spodoptera litura* after topical application with methanol crude extracts started at 33.33% mortality at the dose 1,250 ppm before the mortality percentage values at 2,500 ppm dose showed significant increase to 43.33% and up to 83.33% at dose 5,000 ppm, respectively. Finally, the mortality percentage values gradually grew to highest point of 86.67% at 10,000 ppm. The mortality percentage values showed significant increase ($P>0.05$) from Duncan's Multiple Range Test. LD_{50} values at 24 hours of *Spodoptera litura* second instars after topical application test with methanol crude extract was 6408.04 ppm (Figure 7).

LD_{50}values at 48 hours of second instars *Spodoptera litura*after topical application test with methanol crude extract was 4632.92 ppm (Table 2).

After 48 hours treated in same concentration, mortality percentage slightly increased which no significant difference for time dependent. The mortality percentage values started at 36.67% at dose 1,250 ppm and increase to highest point at 93.33% at the end of 10,000 ppm dose. The mortality percentage values showed significant increase correlation with increasing concentration from 1,250 ppm to 10,000 ppm at P>0.05 from Duncan's Multiple Range Test.

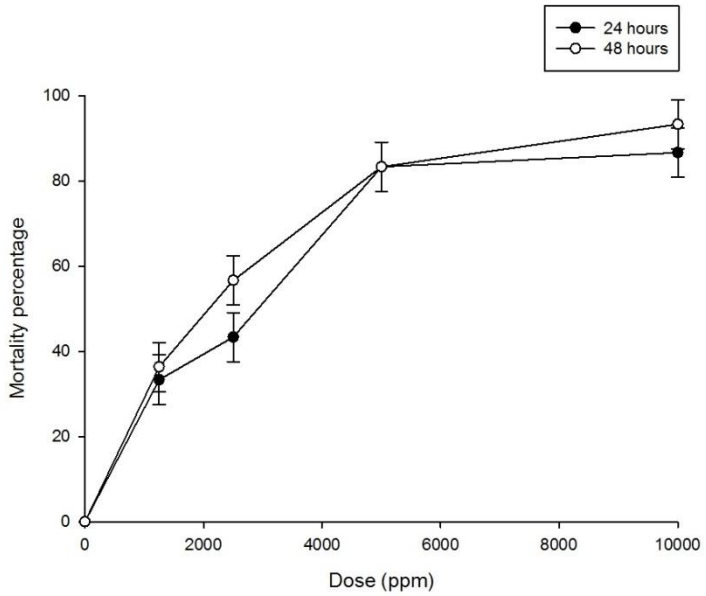


Figure 7 Mortality percentages of second instars *Spodoptera litura* after topical application test with methanol crude extract after 24 and 48 hours

Comparing LD_{50} value by topical application of the rhizomes of *Alpinia galanga* against second instar *Spodoptera litura* larvae of four crude extracts; hexane, dichloromethane, ethyl acetate and methanol at concentration 1,250, 2,500, 5,000 and 10,000 ppm. The LD_{50} values after 24 hours exposed were 6,479.35, 3,177.35, 5878.99 and 6408.40 ppm, respectively. For 48 hours after exposed, the LD_{50} values were 5,360.84, 2,099.72, 3950.40 and 4,632.92 ppm, respectively (Table 2).

Mortality occurred at a dose dependent manner and LD_{50} obtained in case of dichloromethane extract was obtained in the lowest concentration after treatment 24 hours while LD_{50} was upper 5000 ppm in case of hexane, ethyl acetate and methanol. There was no mortality in controls.

Table 2 Toxicity of *Alpinia galanga* crude extracts against second instar *Spodoptera litura* after 24 and 48 hours post-treatment.

Extract	LD_{50} ± S.D. (ppm)[2]	
	24 h	48 h
Control[1]	0 ± 0.00a	0 ± 0.00a
Hexane	6479.35 ± 178.46[d]	5360.84 ± 776.73[e]
Dichloromethane	3177.35 ± 718.73[b]	2099.72 ± 486.84[b]
Ethyl acetate	5878.99 ± 181.86[c]	3950.40 ± 283.58[c]
Methanol	6408.04 ± 158.23[d]	4632.92 ± 375.98[d]

[1]Control treatment: acetone

[2]Within each column means followed by different letter are significantly different ($P < 0.05$; Duncan's multiple range test)

After 48 hours post-treatment the efficacy was more significantly in dichloromethane with LD_{50} of 2099 ppm. Dichloromethane crude extract showed the most control efficiency against second instar *Spodoptera litura* (Table 2).

Thus, it is possible that the main active compounds may slightly polarity compound. There are other reports, which also suggest that the slightly polarity plant extract showed the insect control efficiency like *Momordica charantia, Aristolochia tagala* or *Syzygium lineare* on *Spodoptera litura* (Telang *et al.,* 2003; Baskar *et al.,* 2011; Jeyasankar *et al.,* 2011) or *Jatropha gossypifolia* are toxic to *Spodoptera exigua* (Khumrungsee et al, 2010) in a similar fashion. In addition, the toxicity is

time dependent where the higher exposure gives the higher toxicity.

1.5 Toxicity of *Alpinia galanga* four fractions from dichloromethane crude extract

The dichloromethane crude extract of the rhizomes of *Alpinia galanga* was further studied the chemical constituents and isolated by using appropriate column chromatography techniques to obtain four fractions including **A1**, **A2**, **A3** and **A4**. The percent yield of each fraction was calculated by comparing the mass of crude extracts to the amount of fresh young leaves. The percent yield of **A1**, **A2**, **A3** and **A4** fractions were 47.79%, 10.52%, 13.30% and 29.56%, respectively (Table 3).

Table 3 Characteristics and amount of four fractions from dichloromethane crude extract of the rhizomes of *Alpinia galanga*.

Extract	Weight (g)	Yield (% wt:wt)[1]	Characteristics	Solvent system
A1	6.21	47.77	Yellow oil	100% hexane to hexane: ethylacetate (98:2)
A2	1.37	10.52	Yellow oil	hexane: ethyl acetate (95:5) to (80:20)
A3	1.73	13.30	Yellow oil	hexane: ethyl acetate (75:25) to (20:80)
A4	3.04	29.56	Dark yellow oil	hexane: ethyl acetate (10:90)to 100% methanol

[1]Weight of fraction extract / weight of dichloromethane crude extract x 100

All fractions were further examined the toxicity with second instar of *Spodoptera litura* at concentration 1,250, 2,500, 5,000 and 10,000 ppm by topical application method.

The results were found that fraction **A1** showed the lowest LD_{50} as 3,063.27 at 24 hours and 2,788.82 at 48 hours (Table 4). The results implied that fraction **A1** was an active ingredient in the dichloromethane crude extract of the rhizomes of *Alpinia galanga* while mortality of fraction **A2**, **A3** and **A4** were obtained in higher LD_{50} than **A1** after 24 and 48 hours post-treatment (Table 4).

Table 4 Toxicity of *Alpinia galanga* dichloromethane crude extract against second instars *Spodoptera litura* after 24 and 48 hours post-treatment.

	LD_{50} ± S.D. (ppm)[2]	
	24 h	48 h
Control[1]	0 ± 0.00[a]	0 ± 0.00[a]
A1	3063.27 ± 199.45[b]	2788.82 ± 204.77[b]
A2	6978.83 ± 754.27[d]	6673.21 ± 811.82[d]
A3	3639.13 ± 204.86[c]	3216.76 ± 203.36[c]
A4	9018.46 ± 795.28[e]	8485.59 ± 836.33[e]

[1]Control treatment: acetone

[2]Within each column means followed by different letter are significantly different ($P < 0.05$; Duncan's multiple range test)

2. Chemical Structure Elucidation

2.1 Structure elucidation of compound **1**

Compound **1** was isolated from fraction **A1** and purified by recrystallization with methanol. This compound was determined by ^{1}H-NMR spectroscopy (Figure 19). It was found that the spectrum showed characteristic of mixture of long chain aliphatic hydrocarbons. More spectroscopic data such as GC-MS is required to determine their constitutions in the future work.

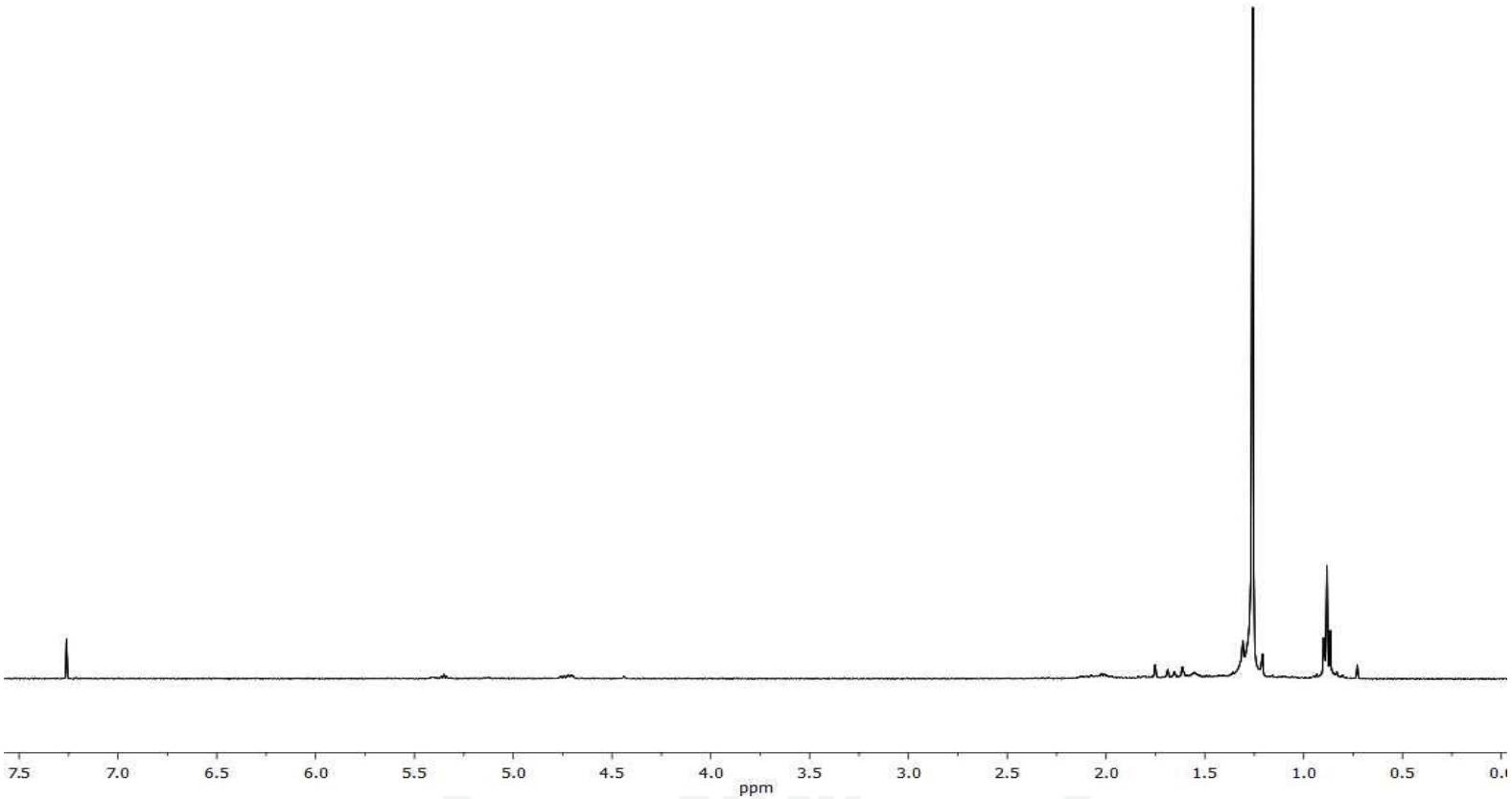


Figure 8 ^{1}H-NMR spectrum of [$CDCl_3$, 400 MHz] compound **1**

2.2 Structure elucidation of compound **2**

Compound **2** was isolated from fraction **A3** and purified by preparative thin layer chromatography using hexane and ethyl acetate (7:1). From ^{1}H and ^{13}C NMR spectra, compound **2** was [1'S]-1'-acetoxychavicol acetate (Figure 9). NMR data were consistent with the data from the previous literature (Table 5 and 6) (Naro *et al.*, 1988).

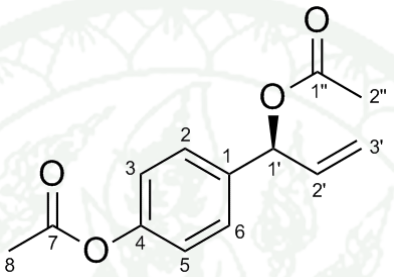


Figure 9 Structure of [1'S]-1'-acetoxychavicol acetate

Table 5 The comparison of ^{1}H NMR data between compound **2** and [1'S]-1'-acetoxychavicol acetate

Position	^{1}H NMR of compound **2** [$CDCl_3$, 400 MHz]	^{1}H NMR of [1'S]-1'-acetoxychavicol acetate [$CDCl_3$, 89.55 MHz]
2	7.37 (1H, d, *J* = 8.7 Hz)	7.36 (1H, d, *J* = 8.6 Hz)
3	7.07 (1H, d, *J* = 8.7 Hz)	7.06 (1H, d, *J* = 8.6 Hz)
5	7.07 (1H, d, *J* = 8.7 Hz)	7.06 (1H, d, *J* = 8.6 Hz)
6	7.37 (1H, d, *J* = 8.7 Hz)	7.36 (1H, d, *J* = 8.6 Hz)
8	2.30 (3H, s)	2.28 (3H, s)
1'	6.26 (1H, d, *J* = 5.7 Hz)	6.26 (1H, d, *J* = 5.9 Hz)
2'	5.98 (1H, ddd, *J* = 17.1, 10.5, 5.8 Hz)	5.99 (1H, m)
3'a	5.30 (1H, dt, *J* = 17.2, 1.3 Hz)	5.27 (1H, d, *J* = 16.3 Hz)
3'b	5.25 (1H,dt, *J* = 10.5, 1.2 Hz)	5.23 (1H, d, *J* = 9.6 Hz)
2"	2.11 (3H, s)	2.09 (3H, s)

Table 6 The comparison of ^{13}C-NMR data between compound **2** and [1'S]-1'-acetoxychavicol acetate

Position	^{13}C NMR of Compound **2** [$CDCl_3$, 75 MHz]	^{13}C NMR of [1'S]-1'-acetoxychavicol acetate [$CDCl_3$, 22.5 MHz]
1	136.3	136.2
2	128.6	128.4
3	121.7	121.6
4	150.7	150.6
5	121.7	121.6
6	128.6	128.4
7	170.0	169.7
8	21.1	21.1
1'	75.8	75.7
2'	136.4	136.2
3'	117.3	117.0
1"	169.4	169.1
2"	21.0	21.1

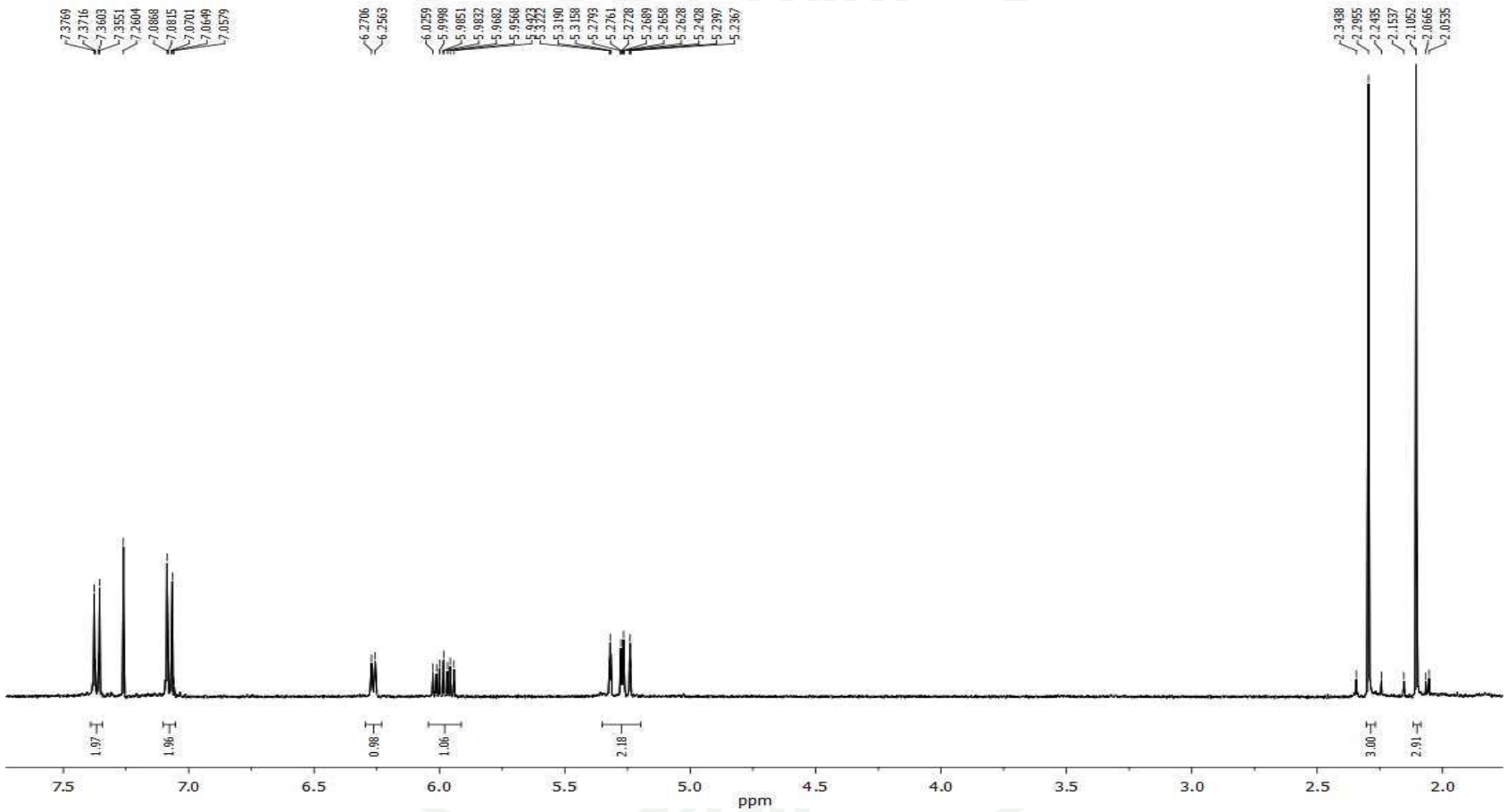


Figure 10 ^{1}H-NMR spectrum [$CDCl_3$, 400 MHz] of compound **2**

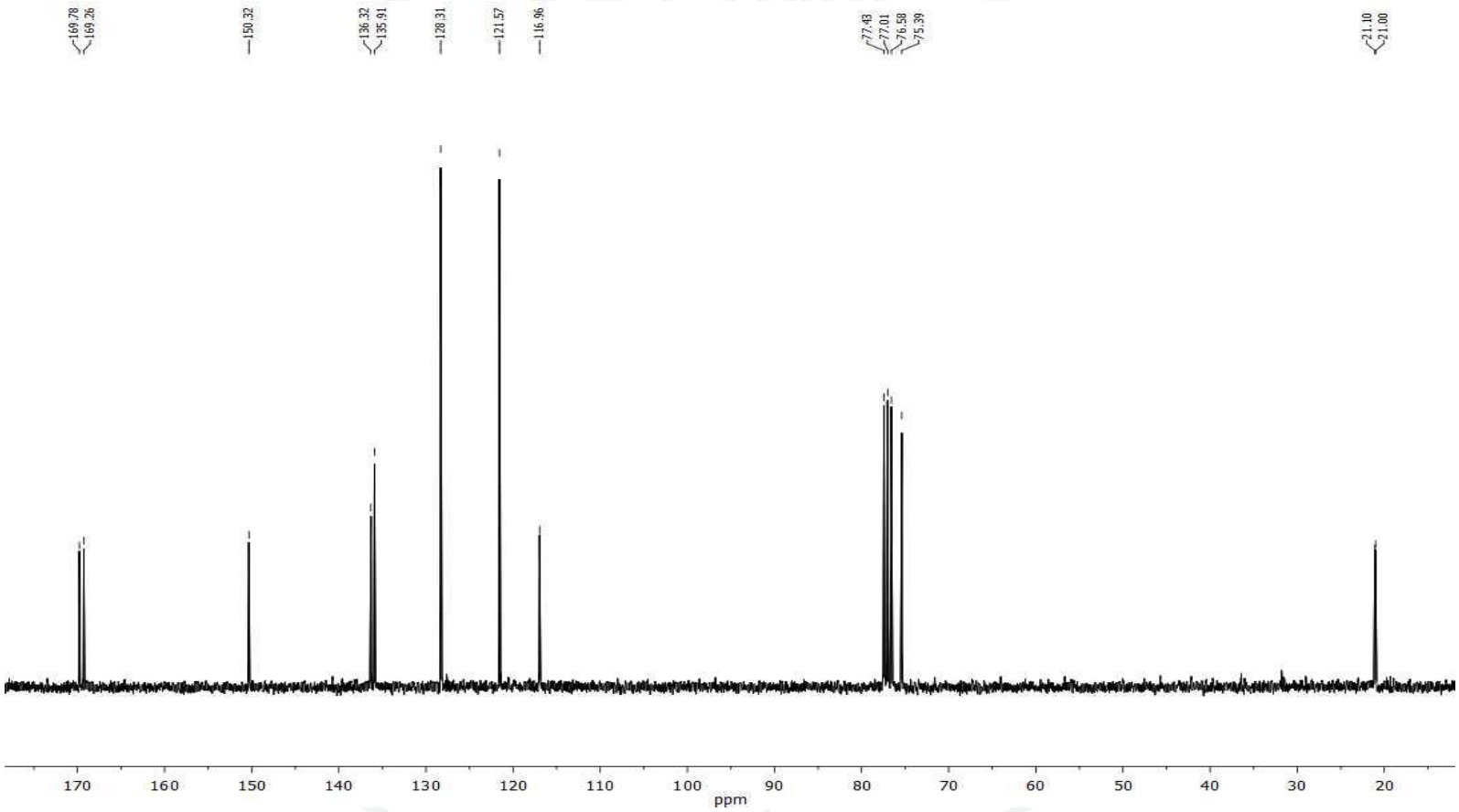


Figure 11 ^{13}C-NMR spectrum [$CDCl_3$, 75 MHz] of compound **2**

2.3 Structure elucidation of compound **3**

Fraction **A4** was purified by preparative thin layer chromatography using hexane and ethyl acetate (15:1) to afford compound **3**. The ^{1}H and ^{13}C NMR data of compound **3** were consistent with the data of *p*-coumaryl diacetate (Figure 12). The compared data were shown in Table 7 and 8 (Naro *et al.*, 1988).

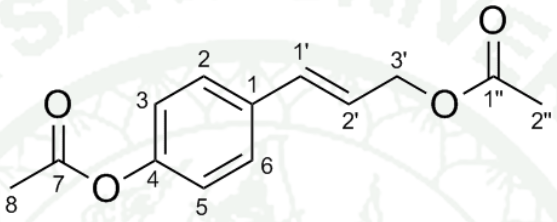


Figure 12 Structure of *p*-coumaryl diacetate

Table 7 The comparison of ^{1}H NMR data between compound **3** and *p*-coumaryl diacetate

Position	^{1}H NMR of compound **3** [$CDCl_3$, 400 MHz]	^{1}H NMR of *p*-coumaryl diacetate [$CDCl_3$, 89.55 MHz]
2	7.39 (1H, d, *J* = 8.6 Hz)	7.38 (1H, d, *J* = 8.5 Hz)
3	7.05 (1H, d, *J* = 8.6 Hz)	7.07 (1H, d, *J* = 8.5 Hz)
5	7.05 (1H, d, *J* = 8.6 Hz)	7.07 (1H, d, *J* = 8.5 Hz)
6	7.39 (1H, d, *J* = 8.6 Hz)	7.38 (1H, d, *J* = 8.5 Hz)
8	2.30 (3H, s)	2.28 (3H, s)
1'	6.62 (1H, d, *J* = 16.1 Hz)	6.64 (1H, d, *J* = 15.9 Hz)
2'	6.22 (1H, dt, *J* = 15.9, 6.4 Hz)	6.21 (1H, dt, *J* = 15.9, 5.2 Hz)
3'	4.75 (2H, dd, *J* = 6.4, 1.3 Hz)	4.71 (2H, d, *J* = 5.2 Hz)
2"	2.10 (3H, s)	2.09 (3H, s)

Table 8 The comparison of ^{13}C-NMR data between compound **3** and *p*-coumaryl diacetate

Position	^{13}C NMR of compound **3** [$CDCl_3$, 75 MHz]	^{13}C NMR of *p*-coumaryl diacetate [$CDCl_3$, 22.5 MHz]
1	134.0	134.3
2	123.4	127.7
3	121.7	121.8
4	150.4	150.4
5	121.7	121.8
6	123.4	127.7
7	170.8	170.6
8	21.1	21.0
1'	133.1	133.3
2'	127.6	127.6
3'	64.9	64.9
1"	169.4	169.0
2"	21.0	20.9

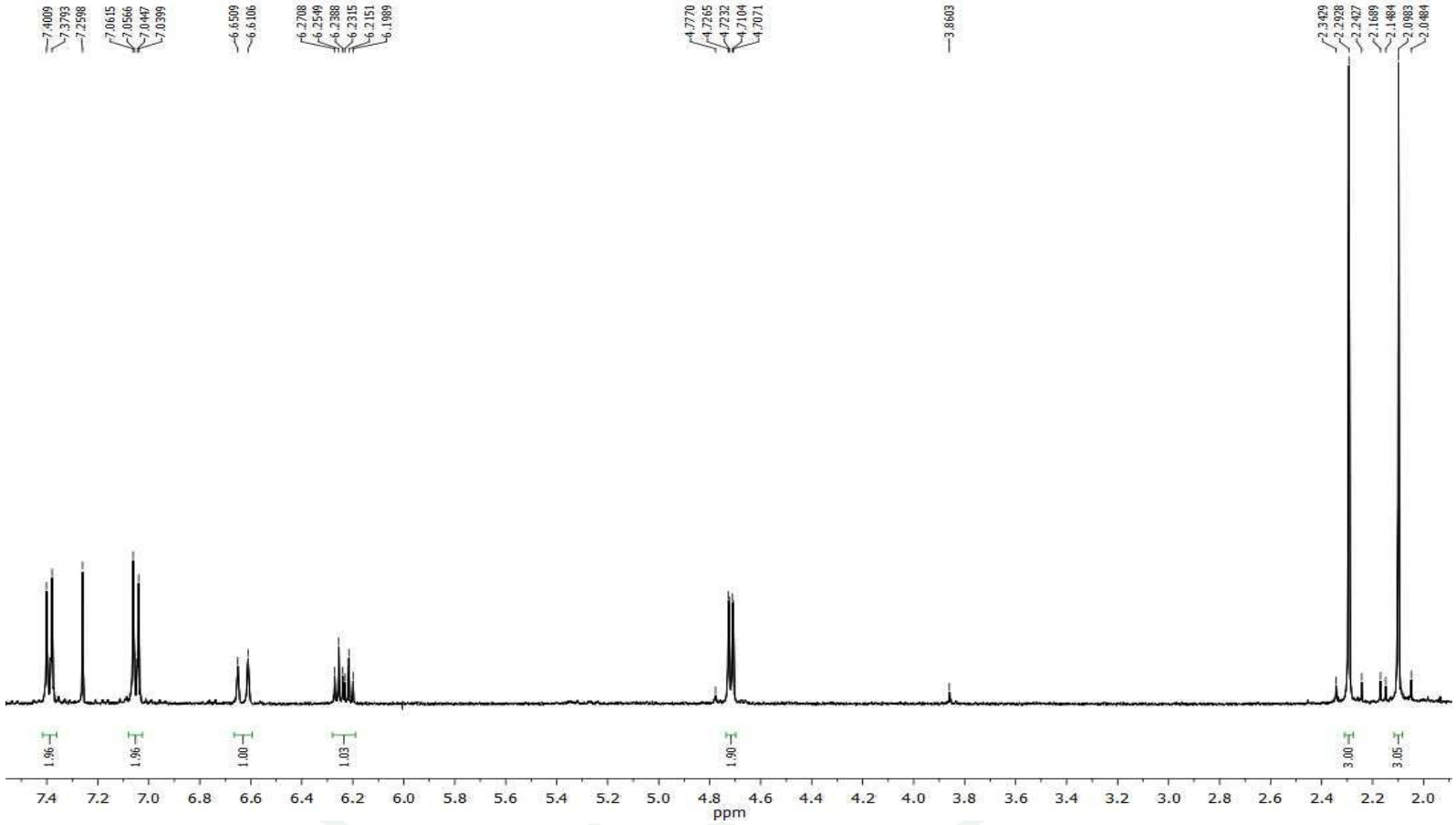


Figure 13 ^{1}H-NMR spectrum [$CDCl_3$, 400 MHz] of compound **3**

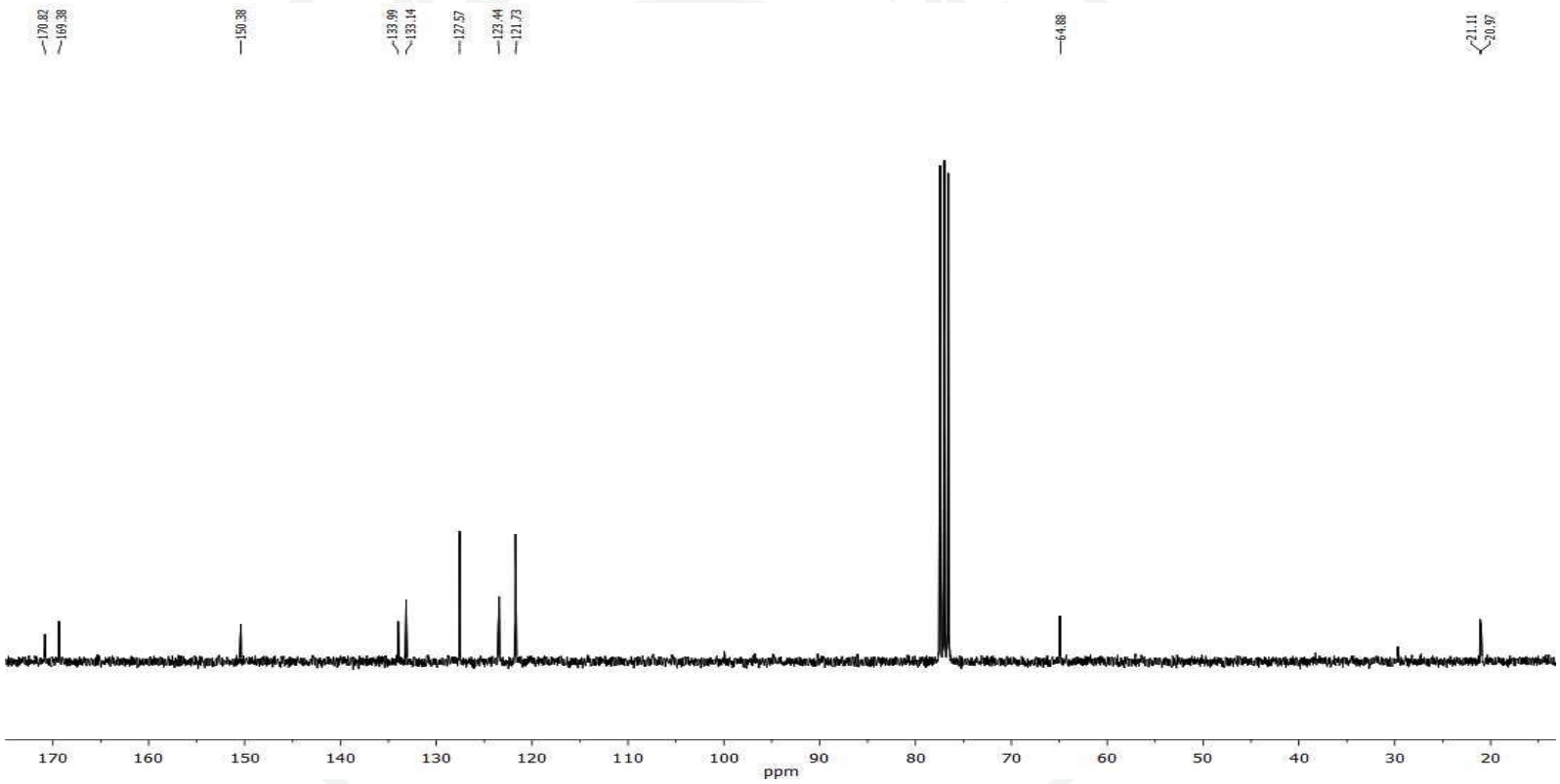


Figure 14 ^{13}C-NMR spectrum [$CDCl_3$, 75 MHz] of compound **3**

2.4 Toxicity of [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate

Toxicity of [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate were examined by topical method against second instar larvae of *Spodoptera litura* after 24 hours and 48 hours post-treatment (Table 9). The LD_{50} of [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate was obtained in good efficacy as 2,117.69 and 2,845.30 ppm after 24 hours expose, respectively.

After 48 hours, the LD_{50} was obtained in 1,451.78 ppm for [1'S]-1'-acetoxychavicol acetate and 2,379.63 ppm for *p*-coumaryl diacetate.

Table 9 Toxicity of [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate against second instars *Spodoptera litura* after 24 and 48 hours post-treatment

Compound	LD_{50} ± S.D. (ppm)[2]	
	24 h	48 h
Control[1]	0 ± 0.00[a]	0 ± 0.00[a]
[1'S]-1'-acetoxychavicol acetate	2117.69 ± 156.12[b]	1451.78 ± 138.69[b]
p-coumaryl diacetate	2845.30 ± 204.30[c]	2379.63 ± 221.41[c]

[1]Control treatment: acetone

[2]Within each column means followed by different letter are significantly different ($P < 0.05$; Duncan's multiple range test)

3. Mode of action study

However, treated insects with all crude extracts from *Alpinia galanga* showed knockdown and paralytic effect in larvae, which is indicative of action at neurotransmitter level. Phytochemicals like azadirachtin (Nathan *et al.*, 2007), rotenone and other traditional botanical insecticides (Muralidhara, 2009; Koul and Walia, 2009), α-mangostin (Bullangpoti *et al.*, 2007) and essential oil compounds (Koul *et al*., 2008) are known to affect one or the other neuron transmitters.

In this study, three methods were chosen to determine insect's enzyme activity including carboxylesterase activity (nM *p*-nitrophenol/mg protein/mL), glutathione-*S*-transferase activity (CDNB conjugated product/mg protein/mL) and acetylcholinesterase activity (acetylcholinesterase activity/mg protein/mL) by using microplate reader techniques. The assay was determined variation amount of enzyme in survival second instars *Spodoptera litura* after treated for 24 hours with 2,117 ppm for [1'S]-1'-acetoxychavicol acetate and 2,845 ppm for *p*-coumaryl diacetate by comparing with control group, which was treated with acetone (AR grade).

3.1 Carboxylesterase enzyme activity after treated with [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate

In the case of [1'S]-1'-acetoxychavicol acetate, the result was not conclusive because the value was not statistically different when compared with control. Whereas *p*-coumaryl diacetate could induce carboxylesterase activity (Table 10).

Table 10 Carboxylesterase activity of second instar larvae of *Spodoptera litura* after treated with [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate

Compound	Mean ± S.D. (nM *p*-nitrophenol/mg protein/mL)	Activity
Control[1]	12.31 ± 3.03	
[1'S]-1'-acetochavicol acetate	10.51 ± 1.38	-
p-coumaryl diacetate	21.28 ± 1.71	Induce

[1]Control treatment: acetone

3.2 Glutathione-*S*-transferase enzyme activity after treated with [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate

The results showed that glutathione-*S*-transferase enzyme could be inhibited by the use of [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate (Table 11).

Table 11 Glutathione-*S*-transferase activity of second instar larvae *Spodoptera litura* after treated with [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate

Compound	Mean ± S.D. (CDNB conjugated product/mg protein/mL)	Activity
Control[1]	$1.51\times10^{-3} \pm 0.17\times10^{-3}$	
[1'S]-1'-acetochavicol acetate	$0.00000284\times10^{-3} \pm 0.00000032\times10^{-3}$	Inhibit
p-coumaryl diacetate	$0.00208\times10^{-3} \pm 0.00024\times10^{-3}$	Inhibit

[1]Control treatment: acetone

3.3 Acetylcholinesterase enzyme activity after treated with [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate.

It was found that acetylcholinesterase was inhibited by [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate (Table 12).

Table 12 Acetylcholinesterase activity of second instar larvae *Spodoptera litura* after treated with [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate

Compound	Mean ± S.D. (acetylcholinesterase activity/mg protein/mL)	Activity
Control[1]	0.287 ± 0.006	
[1'S]-1'-acetoxychavicol acetate	0.233 ± 0.008	Inhibit
p-coumaryl diacetate	0.239 ± 0.010	Inhibit

[1]Control treatment: acetone

4. Study of Biological Activities

Moreover, all crude extracts from the rhizomes of *Alpinia galanga* were further studied for interesting biological activities such as anticancer, antibacterial and antimalarial. The results were shown in Table 13-19.

The inhibition of the crude extracts from the rhizomes of *Alpinia galanga* to anticancer (MCF7- breast cancer) was presented in Table 13. All crude extracts did not show cytotoxicity to MCF7-breast cancer.

Table 13 The inhibition of the crude extracts from the rhizomes of *Alpinia galanga* with anti-cancer (MCF7-breast cancer) by Resazurin Microplate assay (REMA).

Crude extract	% Inhibition	Activity
Hexane	-1.36	Inactive
Dichloromethane	14.14	Inactive
Ethyl acetate	40.07	Inactive
Methanol	20.43	Inactive

1) Negative control: 0.5% DMSO

2) Final concentration of sample: 50 µg/mL

3) IC_{50} of positive control: Tamoxifen = 8.55 µg/mL, Doxorubicin = 9.02 µg/mL

The crude extracts from the rhizomes of *Alpinia galanga* were studied inhibition to anti-cancer (KB-Oral cavity cancer). The results were shown in Table 14. Interestingly, the dichloromethane crude extract exhibited anti-cancer (KB-Oral cavity cancer) activity in %inhibition as 57.53%. Whereas, no activity was not observed for the other crude extracts.

Table 14 The inhibition of the crude extracts from the rhizomes of *Alpinia galanga* with anti-cancer (KB-Oral cavity cancer) by Resazurin Microplate assay (REMA).

Crude extract	% Inhibition	Activity
Hexane	12.83	Inactive
Dichloromethane	57.53	Active
Ethyl acetate	17.05	Inactive
Methanol	-4.39	Inactive

1) Negative control: 0.5% DMSO

2) Final concentration of sample: 50 µg/mL

3) IC_{50} of positive control: Ellipticine = 0.530 µg/mL, Doxorubicin = 0.421 µg/mL

For anti-cancer (NCI-H187-Small cell cancer) inhibition study, the dichloromethane and the ethyl acetate extracts from the rhizomes of *Alpinia galanga* showed inhibition to anti-cancer (NCI-H187-Small cell cancer) as 72.59% and 89.55%, respectively. While the hexane crude and the methanol crude extracts did not display the good activity. (Table 15)

Table 15 The inhibition of the crude extracts from the rhizomes of *Alpinia galanga* with anti-cancer (NCI-H187-Small cell cancer) by Resazurin Microplate assay (REMA).

Crude extract	% Inhibition	Activity
Hexane	1.50	Inactive
Dichloromethane	72.59	Active
Ethyl acetate	89.55	Active
Methanol	-15.70	Inactive

1) Negative control: 0.5% DMSO

2) Final concentration of sample: 50 µg/mL

3) IC_{50} of positive control: Ellipticine = 0.817 µg/mL, Doxorubicin = 0.110 µg/mL

In the case of Neuraminidase (NA) inhibition assay, all crude extracts from the rhizomes of *Alpinia galangal* did not show the good activity (Table 16).

Table 16 The inhibition of the crude extracts from the rhizomes of *Alpinia galanga* with Neuraminidase (NA) inhibition assay by Fluorometric determination (MUNANA-based enzyme inhibition assay).

Crude extract	% Inhibition	Activity
Hexane	8.55	Inactive
Dichloromethane	-9.40	Inactive
Ethyl acetate	-11.70	Inactive
Methanol	-14.00	Inactive

1) Negative control: 1% DMSO

2) Final concentration of sample: 100 μg/mL

3) IC_{50} of positive control: Oseltamivir carboxylare = 0.569 nM

All crude extracts from the rhizomes of *Alpinia galanga* were examined Anti-Mycobacterium tuberculosis (Anti-TB) $H_{37}Ra$ strain (Table 17). The ethyl acetate crude extract exhibited Anti-Mycobacterium tuberculosis (Anti-TB) $H_{37}Ra$ strain activity in high %inhibition as 96.90%. Whereas, no good activity was not observed for the other crude extracts.

Table 17 The inhibition of the crude extracts from the rhizomes of *Alpinia galanga* with Anti-Mycobacterium tuberculosis (Anti-TB) $H_{37}Ra$ strain by Green Fluorescent Protein Microplate assay (GFPMA).

Crude extract	% Inhibition	Activity
Hexane	-16.60	Inactive
Dichloromethane	1.35	Inactive
Ethyl acetate	96.90	Active
Methanol	-28.82	Inactive

1) Negative control: 0.5% DMSO

2) Final concentration of sample: 50 µg/mL

3) MIC of positive control: Rifampicin = 0.025 µg/mL, Streptomycin = 0.625 µg/mL, Isoniazid = 0.047 µg/mL, Ofloxacin = 0.391 µg/mL, Ethambutol = 0.938 µg/mL

The inhibition of the crude extracts from the rhizomes of *Alpinia galanga* to antibacterial against *Bacillius cereus* (Anti-*B. cereus*) was presented in Table 18. All crude extracts did not exhibit cytotoxicity to antibacterial against *Bacillius cereus* (Anti-*B. cereus*).

Table 18 The inhibition of the crude extracts from the rhizomes of *Alpinia galanga* with antibacterial against *Bacillius cereus* (Anti-*B. cereus*) by Resazurin Microplate assay (REMA).

Crude extract	% Inhibition	Activity
Hexane	0.496	Inactive
Dichloromethane	87.02	Inactive
Ethyl acetate	78.52	Inactive
Methanol	1.89	Inactive

1) Negative control: 0.5% DMSO

2) Final concentration of sample: 50 μg/mL

3) IC_{50} of positive control: Vancomycin = 2.00 μg/mL

For antimalarial (*Plasmodium falciparum,* K1 Strain) screening test (Table 19), all crude extracts from the rhizomes of *Alpinia galanga* were not observed the activity.

Table 19 The inhibition of the crude extracts from the rhizomes of *Alpinia galanga* with antimalarial (*Plasmodium falciparum*, K1 Strain) by Microculture Radioisotope technique.

Crude extract	Activity
Hexane	Inactive
Dichloromethane	Inactive
Ethyl acetate	Inactive
Methanol	Inactive

1) Negative control: 0.1% DMSO

2) Final concentration of sample: 10 µg/mL

3) IC_{50} of positive control: Dihydroartemisinine = 2.68 nM, Mefloquine = 0.0432 µM

CONCLUSION

Extraction yields of hexane, dichloromethane, ethyl acetate and methanol crude extracts from the rhizomes of *Alpinia galangal* were1.21%, 0.80%, 0.95% and 3.84%, respectively. The LD_{50}values of second instar *Spodoptera litura* larvae after topical application with hexane, dichloromethane, ethyl acetate and methanol extracts were 6,479.35, 3177.35, 5878.99 and 6408.40 ppm after exposed 24 hours and 5,360.84, 2,099.72, 3950.40 and 4,632.92 ppm after exposed 48 hours, respectively. From these results, dichloromethane extract as the most effective could be used as a choice for botanical insecticide.

Both active ingredients as [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate were isolated from dichloromethane crude extract. The LD_{50} of [1'S]-1'-acetoxychavicol acetate was obtained in 2117.69 and 1471.78 ppm after 24 hours and 48 hours post-treatment, respectively. While LD_{50} of *p*-coumaryl diacetate was 2845.30 ppm and 2379.63 ppm after 24 hours and 48 hours.

For mode of action study, acetylcholinesterase and glutathione-*S*-transferase could be inhibited by [1'S]-1'-acetoxychavicol acetate and *p*-coumaryl diacetate after exposed 24 hours. On the other hand, carboxylesterase activity was induced by *p*-coumaryl diacetate while the activity was not conclusive in the case of [1'S]-1'-acetoxychavicol acetate.

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