STUDY OF SECONDARY METABOLITE(S) PRODUCED BY *NOMURAEA RILEYI* AN ENTOMOPATHOGENIC FUNGUS

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STUDY OF SECONDARY METABOLITE(S) PRODUCED BY *NOMURAEA RILEYI* AN ENTOMOPATHOGENIC FUNGUS

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ABSTRACT

This study searches for potential cytotoxic substances produced by *Nomuraea rileyi,* an active compound was isolated from mycosed *Spodoptera litura* through an activity guided fractionation process. The compound, cytotoxic against the Sf9 insect cell line, was identified to be ergosterol peroxide (5α, 8α-epidioxy-24(R)-methylcholesta-6, 22-dien-3β-ol) using nuclear magnetic resonance techniques, infrared spectrometry, and mass spectroscopy. Anticancer screens demonstrated that ergosterol peroxide inhibited the growth of hormone-dependent breast cancer cell line (T47D), hormone-independent breast cancer cell line (MDA-MB-231), human epidermoid carcinoma in mouth cell line (KB), human cervical carcinoma cell line (HeLa), lung cancer cell line (H69AR) and human cholangiocarcinoma cell line (HuCCA-1) at IC_{50} of 5.8, 28.0, 46.7, 58.4, 65.4 and 105.1 μM, respectively. Anti HIV-1 activity of ergosterol peroxide was determined by syncytium reduction assay. The HIV-1 inhibitory activity and cytotoxicity toward host cells were evaluated as EC_{50} of 15.96 μg/ml and IC_{50} of 150.77 μg/ml, respectively. The therapeutic index (IC_{50}/EC_{50}) of 9.45 was calculated for ergosterol peroxide.

In respect of *in vivo* toxic activity, ergosterol peroxide showed moderate effects against *S. litura* larvae-46.7 % mortality via topical application after 7 day post-treatment whereas the *per os* application had no effect on insect survival. No significant mortality was observed by injecting ergosterol peroxide into *Heliothis virescens*. The amounts of ergosterol peroxide produced by *N. rileyi* cultures under *in vitro* and *in vivo* were quantified. The physiological levels of ergosterol peroxide detected in mycosed and mummified cadavers were at 0.40 and 5.94 μg/ g dried weight, respectively. The mycelia and hyphal bodies cultures contained ergosterol peroxide at levels of 3.93 and 5.14 μg/g dried weight, respectively. These amounts were less than those needed to cause cytotoxicity response.

KEY WORDS: ENTOMOPATHOGENIC FUNGUS/ ERGOSTEROL PEROXIDE/ *NOMURAEA RILEYI*/ *SPODOPTERA LITURA*

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ศึกษาสารทุติยภูมิที่สร้างจากเชื้อราก่อโรคในแมลง *NOMURAEA RILEYI*
(STUDY OF SECONDARY METABOLITE(S) PRODUCED BY *NOMURAEA RILEYI* AN ENTOMOPATHOGENIC FUNGUS)

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บทคัดย่อ

จากการศึกษาการสร้างสารพิษของเชื้อราก่อโรคในแมลง *Nomuraea rileyi* ที่เป็นสายพันธุ์ที่พบได้ในประเทศไทยโดย พบว่าสารสกัดจากหนอนกระทู้ผัก(*Spodoptera litura*)ที่ตายโดยเชื้อราดังกล่าว มีความเป็นพิษต่อเซลล์ของแมลง *S. frugiperda* (Sf9) จากนั้นได้ทำการแยกบริสุทธิ์สารออกฤทธิ์โดยอาศัยกระบวนการactivity guided fractionation และพิสูจน์สูตรโครงสร้างโดยอาศัยเทคนิคของนิวเคลียร์แมกเนติกเรโซแนนซ์, อินฟราเรทสเปคโตรเมตรี และแมสสเปคโตรสโคปี พบว่าคือสาร ergosterol peroxide (5α, 8α-epidioxy-24(R)-methylcholesta-6, 22-dien-3β-ol) จากการทดสอบกับเซลล์มะเร็งพบการออกฤทธิ์ในการต้านการเจริญเติบโตต่อเซลล์มะเร็งเต้านมชนิดที่ขึ้นกับฮอร์โมน, เซลล์มะเร็งเต้านมชนิดที่ไม่ขึ้นกับฮอร์โมน, เซลล์มะเร็งเยื่อบุช่องปาก, เซลล์มะเร็งปากมดลูก, เซลล์มะเร็งปอดและเซลล์มะเร็งท่อน้ำดีในตับที่ค่า IC_{50} เท่ากับ 5.8, 28.0, 46.7, 58.4, 65.4 และ 105.1 μM ตามลำดับ นอกจากนั้นยังได้ทำการทดสอบผลกระทบต่อการต้านเชื้อเอชไอวีโดยอาศัยวิธี syncytium reduction assayตรวจสอบ พบการออกฤทธิ์ต้านเชื้อเอชไอวีที่ค่า EC_{50} เท่ากับ 15.96 μg/ml โดยมีผลกระทบต่อชนิดชองเซลล์ที่ใช้ในการทดสอบต่ำที่ค่า IC_{50} เท่ากับ 150.77 μg/mlจากการทดสอบดังกล่าวมีค่า therapeutic index เท่ากับ 9.45

ผลทดสอบการออกฤทธิ์ของ ergosterol peroxide ต่อหนอนแมลง พบว่ามีฤทธิ์ในการฆ่าหนอนปานกลางคือสามารถฆ่าหนอนกระทู้ผักได้ 46.7 %โดยวิธีทาบนตัวหนอนแต่ไม่ออกฤทธิ์เมื่อทดสอบให้หนอนกิน และไม่สามารถทำให้หนอนตายได้เมื่อฉีดเข้าลำตัวของหนอนชนิด *Heliothis virescens* นอกจากนั้นได้ทำการหาปริมาณของ ergosterol peroxide ต่อน้ำหนักแห้ง พบ 0.40 μg และ5.94 μgในหนอนที่ตายด้วยเชื้อราระยะแรกและระยะหลัง อีกทั้งสามารถพบสารดังกล่าวได้ในเส้นใยและhypahl bodyของเชื้อรา *N. rileyi* อีกโดยมีน้ำหนัก 3.93 และ 5.14 μg ต่อน้ำหนักแห้งของตัวอย่างเชื้อราหนึ่งกรัม
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LIST OF ABBREVIATIONS

°C	degree Celsius
cm	centimeter
COSY	correlated spectroscopy
DEPT	distorsionless enhancement by polarization transfer
DMSO	dimethylsulfoxide
ED_{50}	the effective concentration that exhibited reduction of syncytium formation toward 50% of control
EI-MS	electron impact mass spectrum
FAB-MS	fast atomic bombardment mass spectrometry
Fig.	figure
FT-IR	Fourier transformed infrared spectrometer
et al.	et alli (Latin), and others
etc.	ec cetera (Latin), and other things, and so on
g	gram
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum correlation
hr	hour(s)
HIV	human immunodeficiency virus
IC_{50}	the concentration that inhibited metabolic activities of 50% of cells
ID_{50}	the median inhibitory dose required to produce cell death in 50% of control level
i.e.	id est (Latin), that is
IR	infrared
Kg	kilogram(s)
L, l	litre(s)

LIST OF ABBREVIATIONS (continued)

LC_{50}	the median lethal concentration produces death in 50% of the infected larvae within a certain time
LT_{50}	the median time that required to obtain death 50% of infected larvae at a certain concentration
λ	wavelength
mg	milligram
ml	milliltre
mM	millimolar
M	molar
min	minute
MTT	3-(4,5-dimethylthaiazol-2-yl)-2,5-diphenyltetrazolium bromide
MW	molecular weight
nm	nanometer
NMR	nuclear magnetic resonance
$NaHCO_3$	Sodiumbicarbonate
No.	number
#	number
OD	optical density
rpm	rovolutions per minute
R_f	the retention factor
RT	room temperature
SE	Standard error of estimation
SD	Standard deviation
Sf9	insect cell line derived from pupal ovarian tissue of *Spodoptera frugiperda*
SMAY	Sabouraud's maltose agar medium supplemented with 1-% yeast extract

LIST OF ABBREVIATIONS (continued)

SMBY	Sabouraud's maltose broth medium supplemented with 1-% yeast extract
TEF	toluene-ethyl acetate-90%formic acid
TLC	thin layer chromatography
U	unit
UV	ultraviolet
v	volume
wt	weight
µg	microgram(s)
µl	microlitre(s)
µmol	micromole
%	percentage

CHAPTER I
INTRODUCTION

Many countries, including Thailand, are facing serious problems with the overuse and misuse of chemical pesticides. According to the report of the Office of Agricultural Economics (Thailand), Thailand during the year 2005 imported pesticide 18,529 tons. The import cost was 3,322 million baths which represents a 100% increase from year 1995. A number of persistent organochlorines (DDT) and highly toxic organophosphates (parathion) are still used to control household insects and agricultural pest. Pesticide contamination has been found in agricultural products, soil and water. The wide-spread and uncontrolled use of pesticide has led also to human health and environmental problems and to the emergence of resistant varieties in many pest populations. Thus interest has increased in developing alternative strategies for pest management.

Biocontrol is a promising strategy for pest management. The use of biological pest control agents based on entomopathogens such as viruses, bacteria, nematodes and fungi possess the advantage of high specificity and are thus harmless to non-target insects, the environment, and also users [1]. However, some of these bioinsecticides are perceived to perform poorly when compared with chemical pesticides. Increased efforts are therefore needed to develop and search for new biologically-based insecticidal agents from natural bioresources. Naturally occurring entomopathogenic fungi can be seen as an one group of organisms which may be a source of novel microbial products [2].

Nomuraea rileyi was isolated and recorded to cause epizootics on insect pests in northern Thailand [3]. It is one of entomopathogenic fungi that has been used as a biological agent for several noctuid pests [4]. The majority of researches on *N. rileyi* has been concerned recently with understanding its impact on host populations, stability and persistence in nature. In order develop optimal effectiveness, the local isolates of *N. rileyi* has been subjected to an in depth study [3, 5, 6]. However, there

has been very few studies that address possible toxic metabolites produced by *N. rileyi* [7, 8].

The overall aim of this study was isolate and identify metabolite(s) produced by a local isolate of *N. rileyi* and determine its bioactivities Moreover, the *in vivo* effects of the isolated compound to host insects were also evaluated.

CHAPTER II
LITERATURE REVIEW

1. Microbial insecticides

Insecticide resistance and the demand for reduced chemical products used in agriculture have provided an impetus to the development of alternative forms of pest control. One promising approach has been the use of microbial biological pest control agents based on entomopathogens that possess the advantages of high specificity and that are harmlessness to non-target insects, the environment and also users. Burgess and Hussey (1971) described properties of entomopathogen suitable for use as microbial insecticide, namely virulence, predictability of control, ease of application, ease of production, low cost, good storage properties, safe and acceptable, and able to reduce pest populations . The entomopathogens include viruses, bacteria, nematode, and fungi used in insect pest management.

1.1 Entomopathogenic viruses

The insect pathogenic viruses belong to seventeen families. Only viruses from Families, Baculoviridae and Reoviridae have been considered as control agents; selected virus strains have high virulence and are specific only to insects. In addition, viruses in the family Baculoviridae (BV) include two genera *Nulleoplyhedrovirus* (NPV) and *Granulovirus* (BV). The majority of the baculovirues is infectious for insect species within order Lepidoptera and exhibit a narrow host range. The displayed specificity favors them as good candidates for use in integrate pest management systems.

1.2 Entomopathogenic bacteria

The well-studied bacterial insecticides that have been investigated in detail belong to genus *Bacillus.* Four species have been used as insecticides commercially namely *B. thuringiensis, B. popilliae, B. moritai* and *B. sphaericus. B. thuringiensis* is the most successful biological control agent producing insecticidal, α- and β-exotoxins and the δ-endotoxins. The δ-endotoxin is an insecticidal crystal protein which is synthesized during sporulation [9]. *B. thuringiensis* var. *israelensis* (Bti) was first

discovered in 1976 and has demonstrated to be highly pathogenic for many aquatic Diptera. Bti has been successful utilized for controlling of mosquitoes and black flies worldwide. Resistant of insects to Bti has not been reported due to its complex mode of action involving synergistic interaction between up to four proteins. *B. thuringiensis* var. *kurstaki* (Btk) has been used for suppression of pest Lepidoptera. Btk produces both exotoxin and δ-endotoxin such as hemolysin and Cry1&2 protoxins, respectively [10]. Researchers found a mutant strain of Btk, called an HD-1 type, in a sample and genetically modified it for maximum activity against caterpillars. Btk (HD-1) is an isolate that does not produce β-exotoxin which is a toxin that mammal-biting lice are susceptible. Additionally several toxin genes from *B. thuringiensis* have been used in transgenic crops such as cotton, corn and soybean [11].

1.3 Entomopathogenic nematodes

Entomopathogenic nematodes of the families *Steinernematidae* and *Heterorhabditidae* together with their symbiotic bacteria, *Xenorhabdus* and *Photorhabdus* respectively, represent a unique biological control agent. The *Steinernematidae* enter host via the mouth, anus or spiracles while *Heterorhabditidae* have ability to penetrate the host cuticle. Inside the host hemocoel symbiotic bacteria are released from nematode and kill the host by septicemia within 24 to 48 hr [12]. A wide range of toxins and hydrolytic exoenzymes, responsible for larval death, are produced by bacteria. Later the cadaver becomes to be nutrient supply for bacterial feeding nematodes reproduce and develop into infectious juveniles. As the resources are depleted the nematodes leave the cadaver in search for new host. Entomopathogenic nematodes have a wide host range, and are capable of infecting a spectrum of insects including black vine weevil, cranberry girdler, citrus root weevils, billbugs, white grubs and mole crickets [13]. Using entomopathogenic nematodes has been considered to be safe approach to control pests and have no harmful effects human and other vertebrates. Moreover, mass-production of nematodes is may be on obtained by conventional fermentation technology. Therefore, several formulations of entomopathogenic nematodes are available on the market for controlling the soil and cryptic pest control in North America, Asia and Australia [12].

1.4 Entomopathogenic fungi

More than 700 species of fungi from around 90 genera are pathogenic to insects. Most of entomopathogenic fungi are found within Deuteromycetes and Entomophthorales. Some entomopathogenic fungi have a restricted host range, for example *Aschersonia aleyrodis* infects only scale insects and whiteflies, while others have a wide host range for example *Metarhizium anisopliae* and *Beauveria bassiana*. The commercial products of entomopathogenic fungi and their target hosted are shown in **Table 1**.

Table 1 Entomopathogenic fungi in commercial production. (modified from [14])

Fungus	***Targets***	***Products***
Hyphomycetes		
Beauveria bassiana	Colorado potato beetle, coding moth, european corn borer, pine caterpillar (Lepidoptera, Diptera, Homoptera, Coleoptera, Orthoptera)	Botanigard®, Betel® Mycotrol®, Naturalis®, Boverin®
Metarhizium anisopliae	Spittle bugs, sugarcane frog hopper (Lepidoptera, Coleoptera, Orthoptera, Hemiptera, Hymenoptera)	Metaquino®, Meta-Sin®, Green Muscle®
Paecilomyces fumosoroseus	White flies, thrips, aphids, spider mites (Lepidoptera, Diptera, Homoptera, Coleoptera, Hymenoptera, Arachnida)	PreFeRal®, PFR-97™, Ago Biocontrol Paecilomyces 50®
Verticillium lecanii	Aphids, scale insects, coffee green bug, Greenhouse whiteflies, Thrips	Vertelec®, Mycotal®
Hirsutella thompsonii	Citrus rust mites (Arachnida)	Mycar®
Nomuraea rileyi	Larvae and pupae of Lepidoptera, Coleoptera	Ago Biocontrol *Nomuraea* 50®
Zygomycetes		
Entomophthora muscae	Flies	No commercial available
Erynia neoaphidis	Aphids	No commercial available

Note: Lepidoptera (butterflies and moths); Diptera (flies); Homoptera (bugs); Coleoptera (beetles); Hymenoptera (wasps and bees); Orthoptera (grasshoppers and locusts); Hemiptera (sucking bugs); Arachnida (spiders and mites).

2. The entomopathogenic *Nomuraea rileyi*

Farlow (1883) described initially *N. rileyi* as *Botrytis rileyi*. Later *Botrytis rileyi* was transferred to *Spicaria prasina* by Charles in 1936. Brown and Smith (1957) transferred most of the species in *Spicaria* to the genus *Paecilomyces*, except *S. rileyi*, due to the differences in conidiogenous the green colored conidia and the phialides shape [15]. For these differnces, the retention of the genus *Nomuraea* was proposed to accommodate *S. rileyi* and related *Isaria atypicola* by Kish *et al.* (1974) [4]. The first suggestion of using *N. rileyi* as a biological control agent was made by Johnston (1915) in Puerto Rico. Until 1955, attempt to use *N. rileyi* experimentally for biological control against *Heliothis virescens* was made in southern USA [16]. In 1958, *S. rileyi* was observed to cause an epizootic in population of the cabbage loopers, *Trichoplusia ni* (Hubner). This stimulated a series of in the laboratory and field tests [17]. *N. rileyi* has been examined for its impact on specific hosts [18-21]. Indeed, it is well known that *N. rileyi* can induce extensive epizootics in pest caterpillars in cabbage, clover, soybeans, and velvet beans. Recent study by Tong and Hou has suggested that *N. rileyi* has potential to be a microbial control agent for the corn earworm, *Helicoverpa armigerma*, which is regarded as a serious pest in Taiwan. The possibility to use *N. rileyi* in combination with various pesticides was examined by the ability to germinate in presence with insecticides and herbicides commonly used [22]. Later studies addressed the development of cost effective protocols for conidial production [23] and its field use for the management of *S. litura* [24].

The filamentous fungus *N. rileyi* belongs to class of insect pathogenic deuteromycete (imperfect fungus). *N. rileyi* has been isolated from a numbers agricultural important Lepidopteran pests [21, 25-35]. Also *Hypera punctata* and *Leptinotarsa decemlineata* in Coleopteran are susceptible to *N.rileyi*. Reports on instar susceptibility to *N. rilyi* showed that larval age affects the susceptibility of insect. Younger *Trichoplusia ni* larvae were more susceptible whereas 3rd to 5th instar *H. zea* were more susceptible than those in 1st and 2nd instar [36]. Alternatively Tang *et al.* suggested that higher virulence of *N. rileyi* to older instars *Helicoverpa armigera*, was due to the positive correlation between their larger body surfaces and number of conidial loading [37]. The susceptibility was also increased at temperature of 20-25 °C but found to be decreased at temperature above 30 °C [36].

According to the morphological descriptions of *N. rileyi* made by Ignoffo [11], Samson [15] and Kish *et al*. [38], *N. rileyi* conidia, produce in dry divergent chains, are smooth, ellipsoidal and pale green in color, and measure 3.4-4.5 by 2-3.1 μm. Septate vegetative hyphae are smooth and slightly pigmented and have a diameter of 2-3 μm. Conidiophores growing from submerged hyphae are erect and septate. Branches, formed near a septum, develop in whorls, each giving rise to 2-4 phialides. The branches are usually cylindrical and occasionally possess a swollen base. Colony grows slowly on malt agar, attaining a diameter of 0.7-1.2 cm within one month at 25 ^{o}C. Initial growth is by yeast-like budding from the germ-tube of conidium. After a few days, yeast-like hyphal bodies produce a cream colored and sticky growth on media surface. The sporulation is initially observed within approximately 10 day before spreading throughout colony. The color of the colony progresses from white to pale green and malachite green.

Growth requirements for *N. rileyi*

Research on production and pathogenicity of *N. rileyi* has been reported by many investigators. Malt agar was recommended as the medium to grow *N. rileyi.* The fungus grows very slowly on this medium [39] about 0.7-1.2 cm within one month. Subsequently, Bell [40] found that a complex medium of Sabouround's maltose agar fortified with 1% yeast extract (SMAY) gave better growth for *N. rileyi* and sporulated within approximately 10 days. Culture temperature also has been reported to effect growth of *N. rileyi*. The optimum temperature for rapid mycelial growth and sporulation on SMAY is 25^{o}C. Cultivation of *N. rileyi* at 5, 35, 37, or 40^{o}C leads to inhibition of conidial germination and vegetative growth [41].

During the past several years, there have been several published articles reporting the nutritional requirements for *N. rileyi* [23, 40, 42-45]. In general, the growth requirements appear to be more fastidious than reported for other entomogenous fungi. In 1984, Boucias and Pendland [46] demonstrated that cuticular lipids prepared from *Anticarsia gemmatalis* larvae had potential for activation of conidial germination. Specifically, the sterol, diacylglycerol, and/or polar lipid classes were shown to stimulate conidial germination, but hydrocarbons and monoacylcerol lipids derived from the cuticular extract had little effects on the germination [42, 47]. Addition of larval cuticle from *Heliothis zea* and/or yeast extract induced germination

of conidia. Mycelial yield and protein content increased with increasing concentration of yeast extract, whereas mycelial yield was significantly decreased in the presence of cuticle, corresponding to some inherent constituent(s) of the cuticle (e.g. phenols) [43].

The standard medium commonly used for *N. rileyi* cultivation is complex and contains ingredients that one either unnecessary for growth or complicate the study of enzyme production. A semi-defined medium was developed by El-Sayed *et al.* [48]. They have demonstrated that several natural and mutant isolates of *N. rileyi* can be cultured successfully in a medium containing only two complex ingredients at very low levels (Saubouraud maltose broth fortified with yeast extract). The semi-defined medium consists of the following, a) a mixture of salts and trace elements, containing: $CaCl_2$, KCl, KH_2PO_4, $MgCl_2$:$6H_2O$, $MgSO_4$:7H2O, NaCl, Na_2HPO_4:$7H_2O$, $ZnSO_4$:$7H_2O$ and FeSo4: $7H_2O$, b) a vitamin solution containing, p-aminobenzoic acid, choline chloride, folic acid, cyanocobalamin, pyridoxine-HCl, thiamine monophosphate, and riboflavin-5-phosphate sodium, and c) D-glucose, Sabouraud maltose broth and yeast extract. They also suggested that this formulation provided good growth of *N. rileyi* and also facilitates biochemical, physiological and genetic studies with *N. rileyi.*

The growth of *N. rileyi* depends on carbohydrate (carbon) and amino acid (nitrogen) sources. Morrow and Boucias (1988) [49] suggested that utilization of carbon source during a stationary phase led to hyphal bodies' growth inhibition. In the contrast nitrogen source was uptake by hyphal bodies immediately after lag phase and completed by 30 h postinoculation. *N. rileyi* mycelia initiated to uptake nitrogen source at 30 h postinoculation and also capable reduce amino acid concentration to level that less than hyphal body culture did.

Several attempts have been made [23, 50-55], to reduce the cost of culture media. *N. rileyi* growth was reported on a inexpensive culture medium agar made of brewer’s yeast, yeast hydrolysate, skim milk powder, and whole milk powder [51]. However, the results were not consistent. The fungus could be produced also on polished rice grains [55] and crushed sorghum [56]. Devi [56] determined that maltose (carbon source) and peptone (nitrogen source) in SMAY, are required for sporulation and mycelial growth, but could be effectively be replaced with 2% barley extract and

1% soybean extract substitutes respectively with a high spore yield of 2.8 x 10^9 conidia/ g of sunstrate [23, 56].

3. The infection process of *N. rileyi*

In contrast to bacteria and viruses that pass through insect gut wall from contaminated food, fungi have a unique mode of infection. They reach the haemocoel by penetrating through the cuticle. Pathogenicity of *N. rileyi* to susceptible insects is initiated by conidial attachment to the larval cuticle. The entire infection process generally takes approximately 8 to 12 days [41]. *N. rileyi* conidia do not have muciod layer to assist the conidia attachment to the cuticle surface such as those found in *V. lecanii* and *H. thompsonii.* Instead the dry conidia of *N. rileyi* possess an outer layer compose of interwoven of hydrophobic rodlets. The rodlet layer appears to be unique to the conidial stage and has not been detected on the vegetative cells e.g. yeast-like hyphal body and mycelium. These hydrophobic conidia allow the attachment to the hydrophobic larval cuticle of the host under non-specific hydrophobic forces [57].

After the pathogen attaches to and consolidates on to the host epicuticle, it proceeds with rapid germination and growth which are influenced by the availability of nutrients, water, pH, temperature and by the effects of toxic host-surface compound. *N. rileyi* conidia which are specific to host range require diacylglycerols and polar lipids nutrients on the cuticle surface for germination [18]. In the contrast, entomopathogens with wide host ranges such as *B. bassiana* and *M. anisopliae* possess non-specific nutrients requirement for conidial germination. Ability to utilize nutrients and tolerate toxic substances on the epicuticle was suggested to be a fundamental to pathogenesis. The cuticle has two layers, the outer epicuticle and the procuticle as shown in **Figure 1**. The epicuticle is very complex thin structure without chitin. It contains phenol-stabilized proteins and is covered by a waxy layer containing fatty acid, lipids and sterols [58]. The procuticle forms the majority of cuticle and contains chitin fibrils embedded into a protein matrix together with lipid and quinines. Protein may account for up to 70% of cuticle. In many areas of cuticle the chitin is organized helically giving rise to laminate structure.

N. rileyi conidia germinate and form penetrant germ tubes from the polar ends that directly penetrate the cuticle of the larvae without forming an appressorium which

is an infection structure found in other entomopathogenic fungi. The method of penetration has been suggested to result from a combination of enzymatic degradation and mechanical force. The outer epicuticle is resistant to degradation but susceptible to mechanical force. A range of extracellular enzymes that degrade the major components of insect cuticle including chitinases, lipases, esterases and at least four different classes of proteases, have been suggested to function during fungal pathogenesis. Mohamed *et al.* (1978) indicated that *N. rileyi* produces chitinase, protease and lipase implicated in the penetration processes. The *N. rileyi* chitinase belongs to class V of glycosyl hydrolase family 18 [6]. El-Sayed *et al.* indicated that chitinolytic activity occurring at the germination stage determined the virulence of *N. rileyi*. High levels of endo- and exo-chitinase activity have been detected in virulent isolates but not in avirulent isolates [59]. Although the penetration requires the synergistic action of several different enzymes due to the complex structure of insect cuticle.

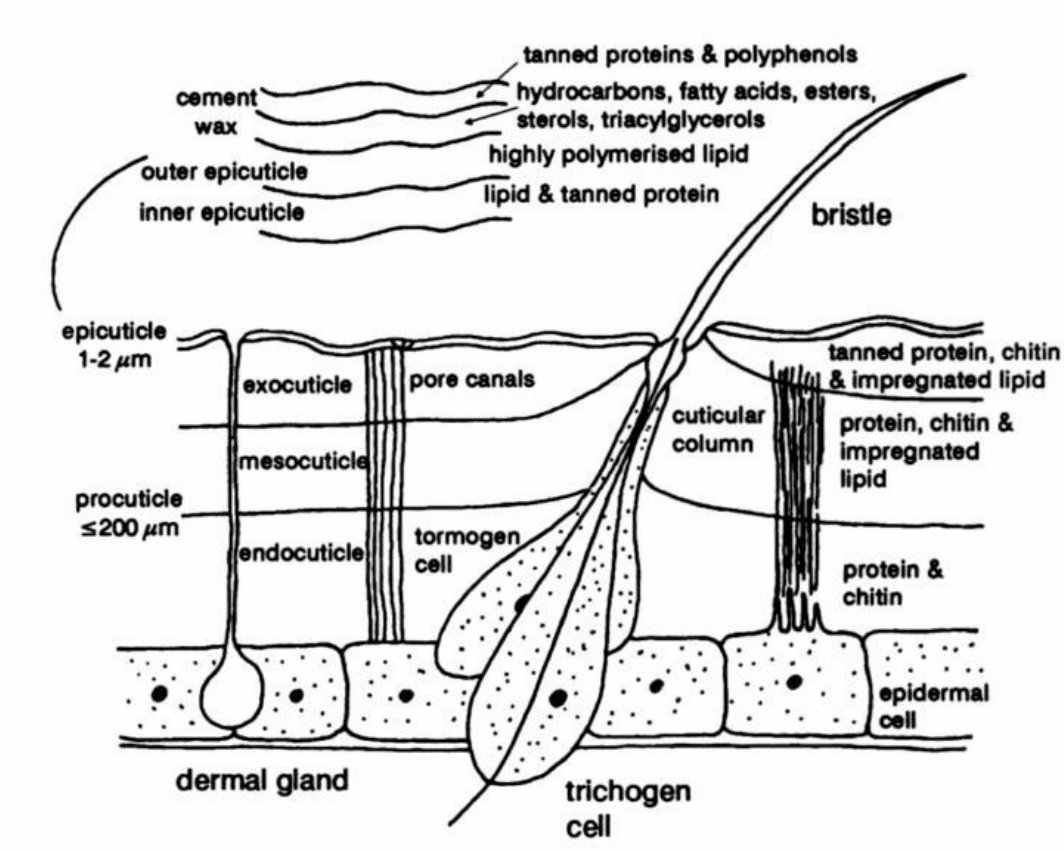


Figure 1 Structure of insect cuticle (from Clarnley 1990 [58]).

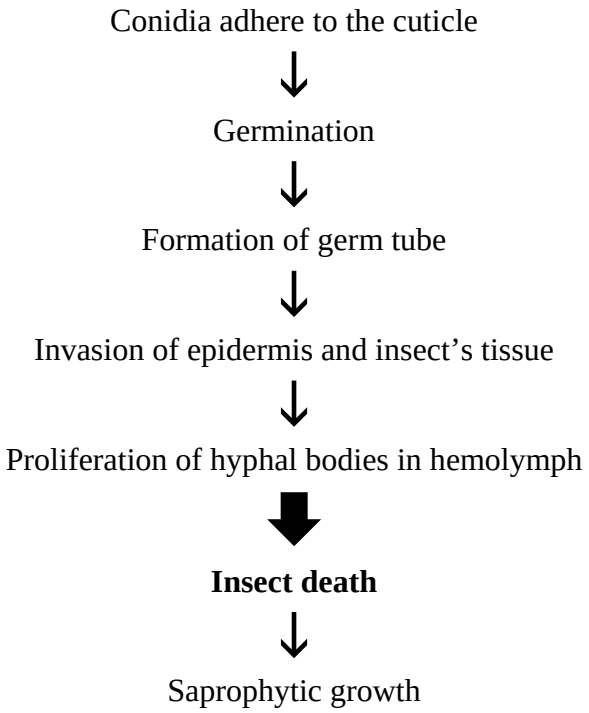


Figure 2 Diagram of infection process of *N. rileyi*

Small yellow to brown spots on the integument may represent the natural (1-2 days post-exposure) signs of infection in larvae. Reduced larval feeding, less movement, paling of the body color, and a slight swelling of the posterior abdominal segments are symptoms that may be encountered 2 to 5 days post-exposure. Growth of the fungus after it reaches the haemocoel is by budding and septum formation, which produces yeast-like hyphal bodies of the vegetative assimilative stage of *N. rileyi*. These exocellular elements are transported throughout the haemocoel. Subsequently, the noninvasive hyphal bodies, which fill the host haemocoel, synchronously convert to an invasive mycelial stage. The mycelia grow and ramify throughout host tissue and within 24 h after conversion, the insect dies [60]. After death, the larval body is completely mummified and covered by a dense white mycelial mat. Within 1 to 2 days later, pale green conidia are produced under suitable conditions (essentially high humidity). Conidia are easily dislodged and distributed by wind. The intensive sporulation observed on *N. rileyi* -cadavers subsequently facilitates the spreading of conidia to healthy insect host in the surrounding environment and allows to form epizootic in next season [41, 60-63]. Three type of resting structures have been reported in *N. rileyi*, namely intrahyphal hyphae in cadaver tissue and external fungal

mat, thick walled hyphae, and chlamydospores in the mycelium over the cadaver surface [19]. All these structures are importance to establish a long-term control of insect pest of *N. rileyi*.

4. Toxin production in *N. rileyi*

Most species of entomopathogenic fungi overcome their host by invasion of the haemolymph finally leading to physical disruption and digestion of vital organs. However, reports of fungi having the ability to kill their hosts after only limited growth suggests the intriguing possibility of insecticidal toxins [64]. There are several principle objectives for studies on toxin production in entomopathogenic fungi. These include 1) elucidation of the mode of action of fungi pathogenic to insects, 2) search for new chemicals for insect control, 3) evaluation of the safety of fungi proposed for use in pest control, or 4) study of basic natural product chemistry. In addition, the information gained in these studies can be helpful in providing markers for selecting strains of entomopathogenic fungi for insect control [65].

In 1978, a study by Wasti and Hartmann demonstrated the *in vitro* production of a substance toxic to gypsy moth larvae and showed that larvae succumbed very rapidly after exposure to a large number of *N. rileyi* spores leading to 50% and 100% mortality in 24 and 48 h, respectively. Unidentified compounds extracted from the mycelium caused 83 and 63 percent mortality of treated larvae with topical application and intra-haemocoelic injection at 13 days [7]. However, the toxicity of mycelial extracts tested also against 3rd instar larvae of *H. zea* and *H. virescens* showed that topical application resulted in no toxic activity to either species. Similarly, *per os* (feeding) treatments, gave low mortalities. However, larvae of both species were more susceptible to the extract by injection. The injected larvae became immobile and paralyzed 9-14 h following injection while control larvae are temporarily immobilized but recovered completely in 30 to 45 min. The same study indicated that larvae of *Heliothis* sp. are more sensitive to the unidentified toxin by injection than by topical and *per os* application. It is not known whether the active compound in infected haemolymph is produced by the fungus, the host, or the combined action of both [8].

5. Insect defense systems against fungal infection

In order to prevent invasion by fungal entomopathogen, insects have evolved various defense mechanisms. The defensive of insects contains both passive structural barriers, such as the peritrophic membrane and cuticle, and a cascade of active response to pathogens that reach to the haemocoel. This response consists of both cellular and humoral components. They are more accurately described as defense responses since specific immunologic memory has not been demonstrated in insect [66].

5.1 Humoral defensive response

Humoral encapsulation involves the cell-free deposition of a melanin sheath around particles causing by activation of the phenoloxidase pathway. The presence of microbial surface components, such as peptidoglycan, β-1,3-glucans and lipopolysaccharide, stimulate Ca^{+2}-dependent serine protease cleavage of pro-phenoloxidase resulting in its activation to phenoloxidase (PO) enzyme acts on tyrosine derivatives, converting them to toxic quinones that interact with proteins to form an insoluble sclerotin called melanin which is a common feature of the response to fungal infection. Melanin may partially shield cuticle from enzymatic attack or may be toxic to fungi. St Leger *et al.* [67, 68] indicated that the melanization is primarily an effective defense against weak or slow growing pathogens but ineffective against more virulent fungi.

In the absence of cells, insect with an open circulatory system sequester entomopathogens and toxins by combination of coagulation and melanization reactions, involving adhesive and covalent cross-linking of plasma component, melanin synthesis and reactive oxygen production. Recent report on the cell-free defense reaction found that lipophorin particles and phenoloxidases are involved in coagulation [69] and aggregation and inactivation of toxin.

5.2 Cellular defensive response

The fungal penetration provides direct access to the insect's haemocoel, the responses against pathogens within the haemocoel that involving to blood cells include phagocytosis, encapsulation and nodulation. Several types of circulating blood cells, or hemocytes, have been described. In larval stage Lepidoptera, two types of hemocytes were divided into adhesive and non-adhesive cells. More than 50% of

hemocytes in hemolymph are granular cells and plasmatocytes which are the only hemocytes types capable of adhering to foreign surfaces [70]. The non-adhesive hemocytes are spherule cells, oenocytoids and prohemocytes. Spherule cells have been suggested to transfort cuticular components and oenocytoids contain cytoplasmic phenoloxidase precursors importantly in melanization of hemolymph. Prohemocytes are suggested to differentiate into various hemocyte types.

The main phagocytic cells are the granulocyte cells and plasmatocytes. Phagocytosis refers to the engulfment of non-self recognition targets like bacteria and yeast. Nodulation refers to multiple hemocytes binding to aggregations of invading microbes. This is a particularly effective means of clearing the hemolymph in particular large doses of bacteria. Nodule is appeared in a sequence of granular cells trapping and followed by aggregation of plasmocytes. It has been reported that the surface carbohydrates on fungal cell wall plays a major role in phagocyte fungal recognition [71] which the galactose and mannose-specific lectins induce of hemagglutinin response in the hemolymph of insects [72]. Pendlend *et al.* (1986) demonstrated that the lack of galactose residue on *N. rileyi* hyphal bodies' wall prevents the hyphal bodies from being opsonized by a galactose specific humoral lectin [71]. Moreover the components on the surface of *N. rileyi* hyphal bodies such as mannoprotein play a role on mimic surface epitopes on insect hemocytes leading to self-recognition by hemocytes. For encapsulation is appeared in the binding of hemocytes to large targets like parasitoids and nematode.

CHAPTER III
MATERIALS AND METHODS

1. Chemicals and Media

1.1 Fungal media

Sabouraud maltose broth was obtained from Pronadisa, Spain. Yeast extract was from Merck, Germany. Agar was a product of Oxoid, Hampshire, England. And fish solubles were obtained from the tuna-canning factory of TC Union, Bangkok, Thailand.

1.2 Chemicals

Dimethylsulfoxide (DMSO), ethanol, hexane, ethyl acetate, formic acid, sulfuric acid, acetic acid, chloroform were obtained from Merck, Germany. 3-(4,5-Dimethylthaiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), *p*-anisaldehyde, griseofulvin were purchased from Sigma Chemical Co., USA. Methanol, dichloromethane were the products of Scharlau, Spain. All organic solvents were analytical or HPLC grade.

2. Fungal strain and maintenance conditions

Diseased caterpillars infected with *N. rileyi*, were collected from cabbage field from Amphor Pop-pra, Tak province, Thailand in the winter of 2002. *N. rileyi* was isolated and cultured on 3% fish-soluble extract supplemented with 1% maltose, 1% yeast extract and 1.2% agar (FMAY) at 25°C for 7 days. Conidial suspensions were prepared by extracting conidia from plates with 0.02% Tween 80 in sterile distilled water. The concentration of conidia was determined with a hemacytometer (Neubauer Improved Bright-line, Germany) using a phase contrast microscope. Flasks containing Sabouraud maltose broth plus 1% yeast extract were incubated with conidia from the agar plates and placed on a shaker for 72 h. Hyphal bodies produced in broth cultures were separated from mycelial fragments by filtering through a layer of Miracloth® (Calbiochem®, Darmstadt, Germany). Hyphal bodies were washed twice by

centrifugation in deionized water and counted with hemacytometer in preparation for insect injection.

3. Insect cultivation

Tobacco cutworm, *Spodoptera litura* (Lepidoptera: Noctuidae), larvae were collected locally and raised under laboratory condition. The larvae were confined to plastic boxes and maintained on fresh castor foliage at 25-27^{o}C. Adult females were allowed to lay eggs. The eggs were incubated at 25^{o}C until the first instar larvae emerged. Neonate larvae were fed with castor foliage until the 2nd or 3rd instar stage at which time they were infected with *N. rileyi*. The healthy pupae were selected and used as a stock to set up new colonies.

Tobacco budworm, *Heliothis virescens*(Fabricius) (Lepidoptera: Noctuidae), eggs were received from the NCSU Insectary, Raleigh, NC, US. The larvae were reared on an artificial diet at 26 ^{o}C 12 h light: 12 h dark regime according to Greene *et al.*[73]

4. Fungal cultivation.

N. rileyi was cultured in Sabouraud maltose broth supplemented with 1% yeast extract (SMYB) medium and using submerged cultivation for secondary metabolite production. Erlenmeyer flasks containing 300 ml of SMYB medium were inoculated with a conidial suspension (1x10^{8} conidia). Culture flasks were shaken at 150 rpm on a rotary shaker maintained at 25^{o}C. After incubation, the mycelial biomass and the culture broth were freeze-dried for extraction as described in the subsequent section.

5. Preparation of *N. rileyi* infected larvae

The conidial suspension was prepared with 0.02% Tween 80 in sterile distilled water plus 0.05% Triton X-1 (octylphenol ethoxylate with approximately 10 mole ethylene oxide). Five microliter aliquots of the conidial suspension (1x10^{8} conidia/ml) were applied to the cuticle of late 2nd instar larvae. . After inoculation, the larvae were fed fresh cabbage foliage and placed in growth bottles at 25^{o}C. Larval mortality was assessed daily for following stages of infection: germination of conidia after 2 days

exposure, presence of the yeast-like growth phase n the hemocoel after 5 days of exposure, the death of insect larvae occurred after 6 to 7 days exposure and mummification by white mycelia 1 day after insect death. The insect larvae at various stages of infection were collected and maintained at –20^{o}C before extraction with organic solvent as described below.

6. Extraction of fungal metabolites from *N. rileyi* cultures

Freeze-dried *N. rileyi* cultures or *N. rileyi* infected larvae were ground and extracted twice with a mixture of dichloromethane and methanol (1:1). The extracted solvent was filtered through filter paper (Whatman No.4). The organic solvent was evaporated to dryness using a rotary evaporator (Buchi, Switzerland) at 40^{o}C and the crude extract dissolved in dichloromethane and transferred into a vial and air dried. The dried crude extract was weighed and dissolved in DMSO prior to use for cytotoxic activity assays.

7. Cytotoxic activity assay

7.1 Cell culture method.

Sf9 cells obtained from the National Science and Technology Development Agency (NSTDA) derived from the pupal ovarian tissue of the fall armyworm, *Spodoptera frugiperda*, were used. The cells were maintained at 28 °C in tissue culture flasks in a non-humidified incubator. The culture medium used was TC100 medium (Gibco Laborateries, New York, USA), 0.35 g/l $NaHCO_3$, 2.25 g/l NaCl supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, USA), 2 mM L-glutamine (Gibco Laborateries, New York, USA), 100 IU/ml penicillin and streptomycin (Gibco Laborateries, New York, USA). For the assay and continuous cell propagation, adherent monolayers were harvested by gently tapping the flask and flowing medium over the adherent cells with a pipette. The cell suspension was removed from tissue culture flasks to measure cell density and viability. The cell density in the removed suspension was measured by counting the number of cells with a hemocytometer under a microscope and viability was judged by the dye exclusion method using trypan blue. For the cytotoxicity assay, cells were plated in

flat bottom 96-microtiter plates in 200 μl growth medium at the density of 4000 cells per well and allowed to attach and begin mitosis for 48 h at 28^{o}C. Wells without cells were used to blank the plate reader and, thus, eliminate media background. Controls and treatments were always examined in parallel.

Six cancer cell lines were used for anti-cancer activity test. Human cholangiocarcinoma cell line (HuCCA-1) established from tumor tissue of bile ducts was cultured in HAMF12 medium (Hyclone Laboratories) supplemented with 10% FBS (Hyclone Laboratories), 100 IU/ml penicillin. The KB cell line originally derived from epidermoid carcinoma of the floor of the oral cavity and human cervical carcinoma (HeLA, ATCC CCL-2) were cultured in DMEM (Dulbecco's modified Eagle medium, Hyclone) supplemented with 10% FBS and 2 mM L-glutamine. Hormone-independent breast cancer cells (MDA-MB-231, MD Anderson cancer center) were cultured in DMEM supplemented with 10% FBS and 2 mM L-glutamine and non-essential amino acids (SIGMA, M7145, USA). Hormone-dependent breast cancer cells (T47D, ATCC No. HTB-133) were cultured in RPMI 1640 (GIBCO) supplemented with 10% FBS 2 mM L-glutamine, 4.5 g/l glucose and 0.2 Unit/ml bovine insulin. Lung cancer multidrug resistance cell line (H69AR ATCC No. CRL-11351) was cultured in RPMI 1640 supplemented with 20% FBS, 2 mM L-glutamine, 4.5 g/l glucose and 10 mM sodium pyruvate. Cancer cell suspensions with density of 10,000 cell/well were seeded in flat bottom 96-microtiter plates and cultured at 37^{o}C under a 5% CO_2 atmosphere.

7.2 MTT assay

Crude extracts prepared from *N. rileyi* infected *S. litura* larvae dissolved in DMSO at a concentration of 20 mg/ml were diluted with TC100 medium to give a final concentration of 200 μg/ml of the test solution. In all treatments the DMSO concentration was maintained below 1% (v/v). The cell medium of each well was replaced with the test solution (200 μl volume/well) and incubated for 24 h. This time period was chosen to eliminate any phase specific toxic effect of the sample. Atotal of eight replicates were conducted for each treatment/cell combination. After exposure the culture medium containing test substance was removed and replaced with 200 μl fresh culture medium. The plate was incubated for an additional 24 h before

assessment of viability using the MTT assay. This extra 24 h allowed for sublethal damage to be repaired.

A modification of the MTT assays originally described by Mosmann [74] was used [75]. At the end of the incubation period 50 μl of MTT [3(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] solution was added to each well (final concentration 2 mg/ml) and cells were incubated at 28^{o}C for 4 h. Then medium and MTT were carefully removed by aspiration and formazan crystals were solubilized in 200 μl of DMSO and 25 μl of Sorensen's Glycine buffer, pH 10.5. The optical density (OD) of the wells was measured using a microplate reader (Molecular Devices, UK) at wavelength of 570 nm. Then cytotoxicity was calculated as the percent reduction in absorbance (minus background) relative to controls in the following equation:

$$\text{Percent cell death} = \frac{[OD_{\text{control well}} - OD_{\text{treated well}}]}{OD_{\text{control well}}} \times 100$$

7.3 Measurement of ID_{50} values

The extracts dissolved in DMSO were diluted in TC100 medium and applied in final concentrations of 100, 50, 25, 12.5, 6.3, 3.1, 1.6 or 0.8 μg/ml. Then 200 μl at each concentration was added to each well. The cells were incubated for 24 h at 28^{o}C. Then the cytotoxic effect of the extract was determined using the MTT assay. The optical density of the wells was measured at wavelength of 570 nm. The data between concentrations and optical densities were plotted and curve fitting was performed according to log-4 parameter relationship as in the equation, $y = (A-D)/(1+(x/C)^{B})+D$ using the Softmax computer program (version 2.35) (Molecular Device, UK).

ID_{50} value was estimated from this concentration-response curve and determined as the concentration of the extract or tested substance that gave 50% reduction of optical density compared with controls.

7.4 Crystal violet assay

The different cancer cell lines were seeded in flat bottom 96-microtiter plates (10^{4}cells/well). The cells were the treated with 100 μl 10-fold serial dilutions of a 50 μg extract/ml prepared in culture medium and cultured at 37^{o}C in a 5% CO_2 atmosphere. After 3 days incubation, 20 μl of 25% glutaraldehyde were added and incubated for 20 minutes. Fixed cells were stained with 0.4% crystal violet in

methanol for 30 minutes. After the plate was dried, absorbance was measured at wavelength of 590 nm.

8 Cell-based assays for anti-HIV-1

HIV-1 can infect neighboring cells and form syncytium thus anti-HIV-1 agents should be those which act not only to the infectious viruses or contain only virucidal activity but can inhibit multiplication as well [76]. In this study, the syncytium reduction assay was carried out to evaluate anti-HIV-1 activity. The syncytium reduction assay allows the virus to encounter with the test sample not only before entry into but also during replication of HIV-1 in cells. The cytotoxicity assay was perform in parallel in order to ensure that the effect was actually from the antiviral activity of test sample but not the inability of cells to support the replication of HIV-1.

8.1 Cell line and Virus

1A2 cells, a continuous suspension cell line, originated from cloned CEM-SS^{TART} transfected with *tat* and *rev* gene, and the mutated virus, Δ Tat/Rev defective HIV-1 ($^{\Delta\ Tat/Rev}$ MC99), were used in the cell-based assays for HIV-1[77-79].

1A2 cells were propagated in growth medium; RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 2 μg/ml gentamycin at 37 °C in 5% CO_2 atmosphere. Cells in log phase, i.e. they were cultured at the concentration of $2x10^5$ cells/ml and allowed to grow for 3 days, were used in the preparation of virus stock and in all assays.

Virus stock was prepared in 1A2 cells infected with the $^{\Delta\ Tat/Rev}$ MC99 virus at an approximate multiplicity of infection (MOI) of 0.1-0.5 syncytium forming unit (SFU)/cell. After 120 min. of viral absorption at 37 °C, the unabsorbed virus was removed by centrifugation. Infected cells were resuspended in RPMI 1640-FRS and incubated at 37 °C in 5% CO_2 atmosphere until more than 75% of the cell population showed cytopathic effects (CPE) which usually observed on the 7^{th} day post infection. These infected cells were than harvested, the suspension was clarified by centrifugation at 1,200 rpm for 5 min. The supernatant was collected dispensed in small aliquots into vials and stored in liquid nitrogen.

8.2 Virus assay

The virus titer was determined by syncytium assay. In brief, the bottoms of all 96-wells were scored in four quadrants and poly-L-lysine (2.5 μg/ 50μl/ well) was added to promote cell attachment to the substrate surface. The plates were incubated at room temperature for at least 30 min then, poly-L-lysine was removed. 1A2 cells ($5x10^5$ cells/ml) were treated with 1 μg/ml polybrene for 30 min at 37 °C and removed by decanting the supernatant after centrifugation. The polybrene-treated cells was suspended to the original concentration and 100 μl each was added to 96-well flat bottom plate, followed by 50 μl RPMI 1640-FBS and 50 μl serially diluted $^{\Delta Tat/Rev}$ MC99 virus, in quadruplicate. The plates were incubated in a humidified CO_2 incubator for three days and the number of syncytium was counted. The titer of virus was expressed as syncytium forming unit (SFU).

8.3 Syncytium reduction assay

The abilities of drugs or samples that could inhibit syncytium formation by HIV-1 and their cytotoxicity were determined in parallel in 96-well tissue culture plates, using serially diluted drugs or samples. Medium used for the assay was RPMI 1640-FBS without phenol red.

The syncytium reduction assay method was similar to the syncytium assay for the virus, except 50 μl of serially two-fold dilutions of the drugs or samples were added in place of RPMI-FBS. This assay detected the effect of drugs or samples that could reduce syncytium formation by $^{\Delta Tat/Rev}$ MC99 virus. Polybene-treated 1A2 cells, $5x10^4$ cells/ 100 μl/ well, were seeded into 96-well tissue culture plates followed by an addition 50 μl of 2-fold dilutions of drugs in triplicate. The mixtures were allowed to incubate for 1 hr before adding 100-200 SFU/ 50 μl of virus. Controls included cell control (contained neither the sample nor virus), cytotoxicity control (cells with the sample only) and virus control. Azidothymidine (3'-azido-2', 3'-dideoxythymidine, AZT, $C_{10}H_{13}N_5O_4$, MW 267.24) was used as a positive control. The cytotoxicity, cytostasis or normal appearance was recorded and the numbers of syncytium were counted after incubation at 37°C for three days. Result of anti-HIV-1 activity was expressed as the effective concentration of the sample exhibiting reduction of

syncytium formation toward 50% of the control (EC_{50}). The EC_{50} value was calculated using linear regression analysis (see Appendix B).

8.4 Colorimetric cytotoxicity assay

Colorimetric cytotoxicity assay was performed using the same batch of 1A2 cells. The procedure was similar to that of the syncytium reduction assay but without the virus. Control wells included those containing medium only (medium control), the samples or drugs with medium (drug control) and cells with medium (cell control). The plates were incubated at 37°C for three days and then added 50 μl of a solution of 1 mg/ml XTT tetrazolium salt (Boehringer Mannheim) and 1% phenazine methosulfate (PMS) per well. Cells that retain metabolic activities retain the ability to synthesize formazan, a yellowish-brown in color product. After incubation for three hr, the optical absorbances at A_{450} were measured with a reference at A_{650}. Results were analyzed and expressed as the concentration of the samples inhibiting metabolic activities of 50% of the cells (IC_{50}).

8.5 Interpretation of anti-HIV-1 results

Results were interpreted as follow.

Active: At least one concentration above 50% reduction of syncytium formation and cytotoxicity at the point < 50% and EC50 < 250 μg/ml and microscopic observation also suggested activity of the compound.

Inactive: Flat line reduction at all concentrations, or only one point where the cytotoxicity was < 50% resulted in about 50% reduction of syncytium formation, or microscopic observations did not suggest activity of the drug or the data were insufficient to render a valid judgment concerning the activity of the compound.

Toxic: All concentrations of the uninfected drug control resulted in less than 50% viability.

The values of the toxic and effective concentration (IC_{50} and EC_{50}) were used to calculate a therapeutic ratio or index ($TI = IC_{50}/EC_{50}$). A TI value of one or less, implicates that the sample will elicit adverse effects in addition to a beneficial response. A TI value of five or greater indicates a likely pharmacological response at dosages below those producing toxicity.

9. Chromatographic methods

9.1 Thin layer chromatography (TLC)

Qualitative analysis of compounds in samples was performed by TLC as adapted from Patersons (1986). The extracts and fractioned residues were chromatographed on silica gel 60 plates (Merck, Germany), size 10x10 cm, layer thickness 0.2 mm using the following solvent systems: toluene-ethyl acetate-90% formic acid (TEF) at the ratio of 5.75:4:0.25. The samples dissolved in dichloromethane were spotted on TLC plates about 1 cm from the bottom edge with capillary tubes and each spot was allowed to dry before applying another spot. A solution of Griseofulvin dissolved in chloroform and methanol (2:1, v/v) was used as a reference standard. The TLC plates were developed in TEF in a solvent saturated atmosphere in a TLC tank. After the solvent had traveled over most of the plate, the plate was removed from the tank and the solvent front noted. The solvent was allowed to evaporate. The presence of compound spots on TLC was detected by spraying with a solution of 0.5% (v/v) *p*-anisaldehyde in methanol-acetic acid-concentrated sulfuric acid (17:2:1, v/v/v) or sulfuric acid followed by heating at 105^{o}C for 8 min until colors for all spots were fully developed. The plates were subsequently visualized under UV light at a wavelength of 366 nm.

The position of a compound on a developed chromatogram was expressed by R_f (the retention factor) value calculated as follows.

$$R_f = \frac{\text{Distance of compound from origin}}{\text{Distance of solvent front from origin}}$$

9.2 Column chromatography

Column chromatography was carried out on silica gel 60 No. 7736 as an absorbent. In this study, there were two methods of eluting solvent systems for separation of compounds in crude extracts as follows.

9.2.1 Isocratic elution

Silica gel was suspended in TEF solvent by adding the solid adsorbent to a quantity of the solvent. The slurry was mixed carefully until the mixture was homogenous and free of entrapped air bubbles. The column (1.5 x 30 cm) (Spectra/Chrom^{TM} LC column) was filled about half-full with solvent, and slurry was added carefully, while the solvent was allowed to flow slowly from the column. As

the silica gel was added, the column was tapped gently with a stick in order to ensure that the column was packed evenly. After the absorbent settled, the crude infected larval extract (245 mg) dissolved in 1 ml dichloromethane was applied to the column and then eluted with TEF solvent (5.75:4:0.25, v/v/v).

The solvent was applied with a flow rate of 0.3 ml/min. Fractions were collected in equal volumes of 2 ml by a fraction collector (Pharmacia, Sweden). Solvent from these fractions was evaporated at room temperature by gentle air flow. Fractions were further analyzed by TLC. Then each combined fraction was solubilized in 400 μl of DMSO and tested for cytotoxic activity against Sf9 cells as described previously.

9.2.2 Stepwise gradient elution

Silica gel was suspended in toluene and packed into a column (1.5x30 cm) as described previously. Potentially active fractions obtained from a column (2.5x100 cm) by isocratic elution was further purified by chromatography on another silica gel column (1.5x30 cm) eluted by step gradient using increasing concentrations of ethyl acetate (10, 20, 30, 40 and 50%, v/v) in toluene with a flow rate of 0.1 ml/min. Fractions (1 ml) were collected and solvent evaporation, each fraction was subjected to TLC for compound separation. Selected fractions were subsequently tested for cytotoxic activity toward Sf9 cell line.

10. Identification of toxic compound

The melting point was measured using a Buchi535 melting point apparatus and reported without correction. UV spectra were recorded on a Shimadzu UV-VIS 2001S spectrophotometer. Infrared spectra (IR) were obtained on Perkin Elmer System 2000 FT-IR spectrometer. The amounts of approximately 1.0 mg of compounds in anhydrous potassium bromide (KBr) were prepared by grinding in an OKey mortar. Then the mixture was subjected to form KBr disc by press apparatus and then scanned for infrared absorption.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AM-400 instrument (operating at 400 and 100 MHz for ^{1}H and ^{13}C, respectively) using solutions in deuterochoroform ($CDCl_3$) with tetramethylsilane ($Si(CH_3)_4$) as an internal standard. Mass spectra were determined using Finnigan Mat GCQ spectrometer for electron

ionization mass spectroscopy (EIMS) and a Finnigan MAT 90 spectrometer for high-resolution fast atom bombardment mass spectrometry (HRFABMS)**.**

11. Quantization of ergosterol peroxide for fungal extracts

The standard extraction for ergosterol procedure previously described by Saxena *et al.* [80] was used with modification. Freeze-dried sample (fungus and mycosed insect) tissues were ground to fine powder. Forty ml of chloroform and 10 ml of hexane were added to each 10 g of ground sample and flasks were tightly closed and shaken (24h) in dark on a rotary shaker (120 rpm). After filtration, extracts were saponified with 3g KOH and incubated in hot water bath (60 °C) for 20 min. Five ml of water was added and mixed thoroughly. After partitioning with hexane, the hexane phase was collected and evaporated to obtained total sterols.

To prevent photo-oxidation, all samples were stored under N_2 gas in dark condition before analyzed with GC-MS. The sterol fraction was dissolved with CH_2Cl_2 to obtain a concentration of 10μg/μl and 50μl of spinasterol (1μg/μl) were added as an internal standard to 50μl of extract and then 1μl samples were analyzed by an Agilent 6890 Network gas chromatograph equipped with 5975 inert XL Mass Selective Detector operating in the chemical ionization mode with isobutane as the ionization gas. Chromatography was performed on a HP-1MS column (30m x 0.25mm id x 0.25μm) with helium as the carrier gas. The oven temperature was programmed from 35°C (1 min hold) to 300 °C at 10°C /min where it was held for 7.5 min. A run time of 35 min allowed separation of ergosterol peroxide which was detected and quantitated by correlating the peak area (extracted ion) of ergosterol peroxide with the internal standard (spinasterol).

For HPLC/UV analysis the crude extract from fungal mycelia was subjected to HPLC/UV analysis using reverse column (Hichrom, Excil 100-50ODS; 4.6 mm, L 22 cm) with UV absorption detection. To observe ergosterol peroxide and ergosterol in the sample, the UV absorption detector was set to measure at 210 nm, the lowest wavelength that can be measured, from time 0 to 25 min and 282 nm from 26 to 50 min due to the optimum UV absorption of ergosterol peroxide (λ_{max}204 nm) and ergosterol (λ_{max}282 nm), respectively.

12. Synthesis of ergosterol peroxide

Ergosterol peroxide used in the *in vivo* toxicity test was synthesized from ergosterol according to Bok *et al.*[81] with modifications. Ergosterol and different photosensitizers, eosin and methyl blue, were dissolved in 100 ml of ethanol and placed in photoreactor flask. The two photosensitizers were used to evaluate for optimum production of product. Oxygen was bubbled through the solution being irradiated with a mercury low pressure immersion lamp (Aldrich-Chemie). Water was circulated through the cooling coils of the photoreactor to maintain the reaction at RT. After 24h, the solution was concentrated under reduced pressure, with the resulting residue applied to silica gel column and eluted with ethyl acetate in hexane. Eluted ergosterol peroxide, re-crystallized as white needle crystals, was subjected to ^{1}H NMR analysis for structural confirmation.

13. *In vivo* toxicity tests of ergosterol peroxide

In order to evaluate toxicity of ergosterol peroxide toward host insect, three methods of applications were used as follows.

13.1 Topical application

Groups of the 3rd instar *S. litura* (15-20 mg) were used. Synthetic ergosterol peroxide was dissolved in mixture of tween 20 and 100% ethanol (1:1) at concentrations of 20, 25, 30, 35, 40 and 45 mg/ml. For topical application 3 μl of each concentration were applied to larval cuticle. Three replications of 10 larvae were tested for each concentration. The treated larvae were placed on diet and examined for mortality daily. An analysis of variance (ANOVA) was used to analyze the results. A post-hoc test was conducted to determine differences among treatment means using Games-Howell or Turkey HSD test following a check for homogeneity of variance by the Levene test. Statistical values of $p<0.05$ were considered significant.

13.2 *Per os* application

For *per os* treatments, 3rd instar *S. litura* larvae (15-20 mg) were starved initially for 2 h and then fed discs of artificial insect diet (3-mm thickness) pretreated with ergosterol peroxide dilutions. Diet discs containing mixture of tween 20 and 100% ethanol (1:1) alone served as a control. Each treatment was replicated three times. Insect weight and mortality were recorded daily.

13.3 Injection method

Forth to fifth-instar *Heliothis virescens* (80-100 mg) were used in this study. The 2.5 µl of ergosterol peroxide preparations were injected into the hemocoel of *H. virescens* larvae. Controls were injected with solvents used in ergosterol peroxide preparation. Treated and control larvae were placed on artificial diet and incubated at 25 °C. Mortality was recorded daily.

Due to the limited solubility of ergosterol peroxide in water, ergosterol peroxide was subjected to formulate into several preparations in order to use as aqueous solution for insect injection.

13.3.1 Ergosterol peroxide: PVP (Polyvinylpyrrolidone) formulation

PVP was used to formulate in order to increase solubility of ergosterol peroxide. Two mg of ergosterol peroxide was dissolved in chloroform. Then the ergosterol peroxide solution was added to the PVP (18 mg) in ethanol. The solution was mixed thoroughly before subjected to lyophilization. The preparation of ergosterol peroxide:PVP (1:9) was further tested for toxicity.

13.3.2 Liposome preparation of ergosterol peroxide

The MVLs (multilamellar vesicles) liposomes containing ergosterol peroxide were prepared with suggestion of Dr Jeffrey Hughes, Department of Pharmaceutics, University of Florida College of Pharmacy. A solution of ergosterol peroxide (10 mg/ml) in chloroform was mixed with 2 ml of chloroform solution of distearoyl phosphatidyl choline (DSPC, 20 mg/ml) and 250 µl of dipalmitoylphosphatidylglycerol (DPPG, 20 mg/ml) solution. The solution was mixed thoroughly with vortex and the chloroform was removed by rotary evaporator until dryness. One ml of phosphate buffered saline (PBS) was added to get liposome preparation (10 mg/ml).

14. Study effect of *N. rileyi* and *Paecilomyces fumosoroseus* on *Heliothis virescens*

Hyphal bodies of *N. rileyi* and blastospores of *P. fumosoroseus* were produced by inoculating conidia into SMBY broth. Cultures were incubated at 25 °C on shaker for 3 days, then filtrated through sterile Miracloth® (Calbiocheme) to remove mycelia.

The hyphal bodies were collected from filtrates by centrifugation and washed with 0.85% saline. The fungal cells were counted on hemacytometer and diluted in sterile saline. Five μl were injected into hemocoels of *H. virescens* larvae. Control larvae were injected with 5 μl of sterile saline. Injected and control were placed on artificial diet and incubated at 25 °C. The number of hyphal bodies, number of hemocytes and percent spreading of hemocytes were examined daily.

14.1 Insect hemocytes and fungal cells counting

At 1, 2, 3, 4, 5, 6 and 7 days, 10 μl of hemolymph were sampled from treated larvae and diluted in 90 μl of Hepes-buffered saline to obtain 10-fold dilution. The hemolymph suspension 10 μl was applied to hemacytometer and examine under microscope. The total numbers of hemocytes, *N. rilyi* hyphal bodies, *P. fumosoroseus* blastospores were counted.

14.2 Spreading hemocytes counting

Injected insects hemolymph 10 μl was diluted with 90 μl of Hepes-buffered saline containing a few crystals of phenylthiourea (PTU) to prevent melanization. Fifty μl of the hemolymph suspension was applied to a well of ring slide and allowed to incubate within moisture container at room temperature. After 1 hr, unattached cells were washed out with Hepes-buffered saline and hemocyte monolayers were examined with phase contrast microscope. The total number of spreading hemocytes in 5 fields was recorded. Five larvae were used in each treatment.

CHAPTER IV
RESULTS

1. Growth characteristic of *N. rileyi*

In this study, *N. rileyi* was grown on FMAY plate at 25°C. After inoculation with conidia, conidia germinated and converted to yeast-like hyphal bodies that grew by budding. In this growth phase, the hyphal bodies produced creamy mucoid colonies which converted within 4 to 6 days to white mycelial colony. The white mycelia completely covered the plate in 8 to 10 days followed by production of green conidia within approximately 12 to 14 days.

After injection the *H. virescens* larvae with *N. rilyi*, development of hyphal bodies of *N. rilyi* in insect host hemolymph was observed under phase contrast microscopy as shown in **Figure 3.** Hyphal bodies underwent extensive budding and circulated freely throughout the hemolymph (**Fig.3A** and **3B**). Interestingly, evidence of phagocytosis of hyphal bodies by hemocytes cell was not observed. **Figure 3C** depicts the transition of hyphal bodies to mycelia in hemolymph of dead insect at 3 to 4 days postinjection. Fungal mycelia grew throughout insect's tissue and penetrated through the insect's cuticle. The fungus mummified cadaver with white mycelia within 24 h postmortem as shown in **Figure 4B**. Under high humidity green conidia were produced in 1 to 2 days on external conidiophores post mummification (**Fig. 4C**).

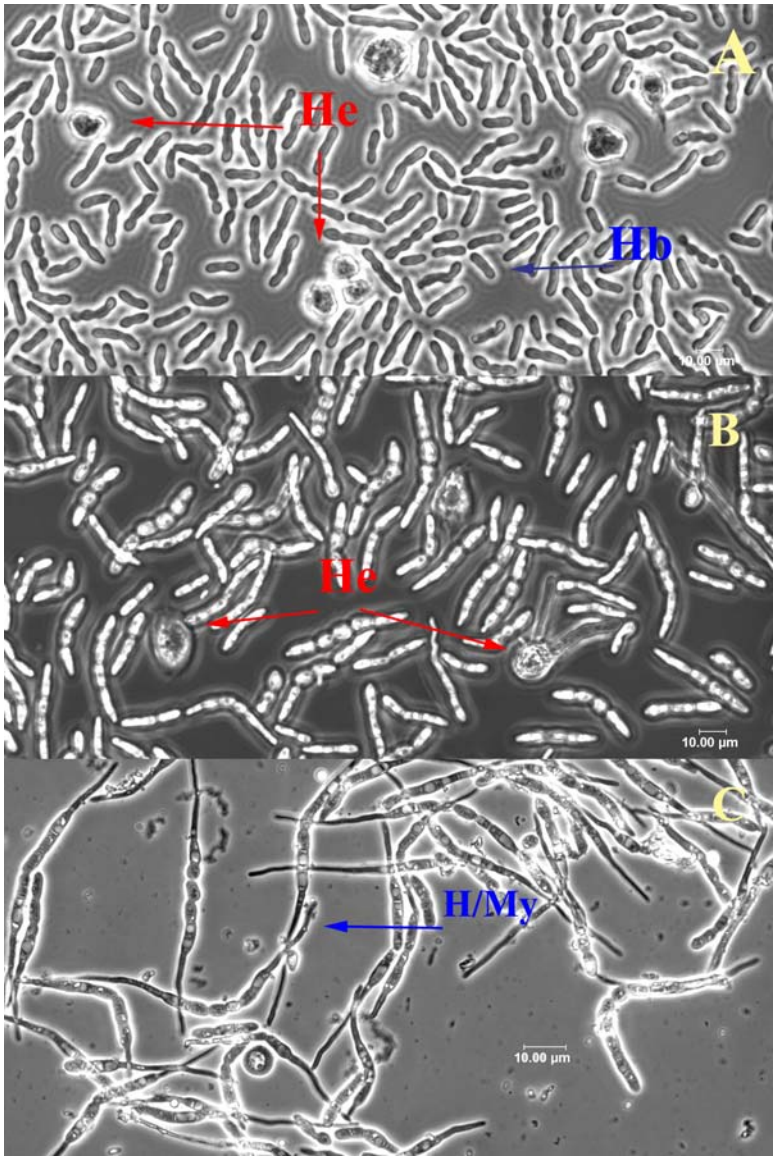


Figure 3 Development of *N. rileyi* in the hemolymph of *H. virescens. H. virescens* larvae were injected with *N. rileyi* hyphal bodies. And, after 24 hr **(A)**, 48 hr **(B)** and 96 hr **(C)**, the hemolymph were collected from the infected larvae and observed with a phase contrast microscope with 630x magnification. The red arrow indicated “He” as insect hemocytes. The blue arrows indicated “Hb” as hyphal bodies and “H/My” as transition stage of hyphal body to mycelium.

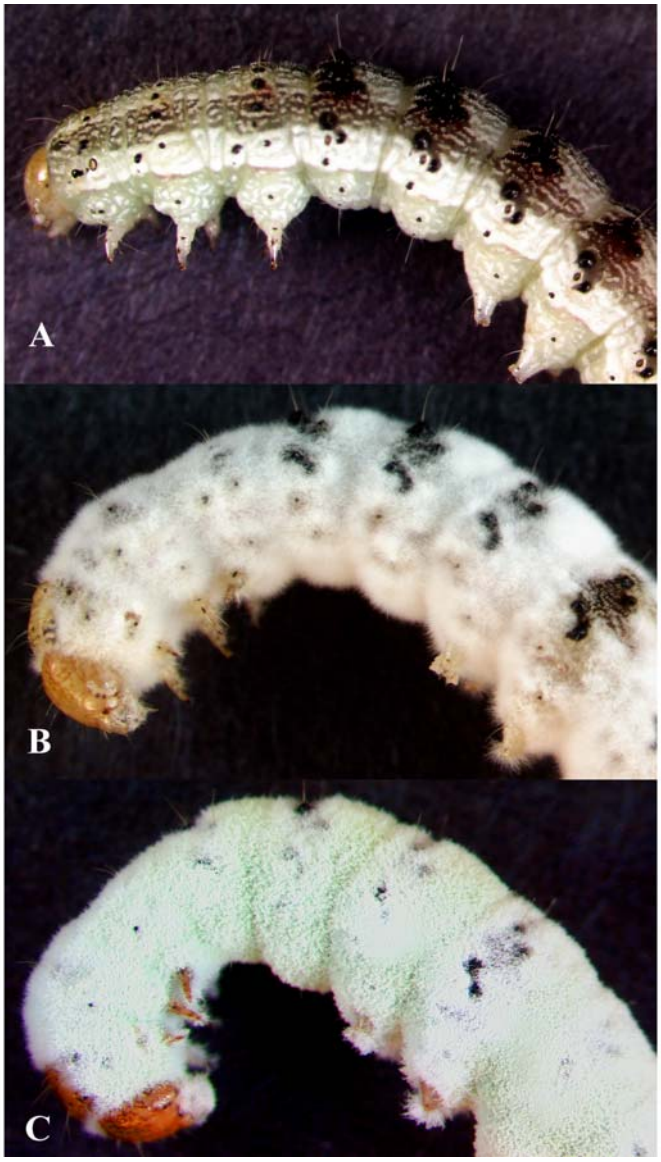


Figure 4 Photographs showing progression of sporulation of mycosed insects. The pictures showed various stages of dead *H. virescens* larvae after infection with *N. rileyi*. Recently dead larvae **(A)**, 24 hr postmortem the dead larvae appeared white **(B)**, and after 48 to 72 hr, these mycosed larvae appeared green **(C)**.

2. Cytotoxic activity of extract from *Nomuraea rileyi* infected *Spodoptera litura* toward Sf9 cells culture

The results in **Table 2** showed that the highly active crude extracts, those causing greater than 90% cell death, were those obtained at late stage of infection (7 and 8 days). Preparations from early stages of infection, i.e., 2 days and 5 days post exposure to *N. rileyi* conidia produced 44 and 41 % cell death respectively which were similar to the 46% cell death caused by preparation from healthy insects. Therefore, the extract preparations from dead insects and mummified insects were subsequently subjected to isolate and purification for active compound(s).

Table 2 The effects of various crude extract preparations from *N. rileyi* on viability of the Sf9 insect cells.

Crude extract from	**Cytotoxic activity (% cell death)***	**Remark**
Control 1**	0	
Control 2***	0	
Healthy larvae	46 ± 8.26	Alive, still feeding
N. rileyi treated larvae at 2 days post exposure	44 ± 8.49	Alive, still feeding
N. rileyi treated larvae at 5 days post exposure	41 ± 9.90	Alive, still feeding
N. rileyi treated larvae at 7 days post exposure	93 ± 1.41	Dead (see **Fig. 4A**)
N. rileyi treated larvae at 8 days post exposure	95 ± 0.71	Mummified with white mycelia (see **Fig. 4B**)

* Percent cell death after being exposed to 200 μg/ml of various extract preparations Cell viability measured by MTT assay.

** Control 1 contained extract prepared from cell culture medium.

***Control 2 contained extract from cell culture medium containing 1% DMSO.

In order to quantify toxicity of the extract, a 50% inhibitory dosage (ID_{50}) was determined for infected larvae extract. The experiment was carried out by exposing Sf9 cells to two-fold diluted crude extract in T C100 medium at final crude concentrations of 100, 50, 25, 12.5, 6.3, 3.1, 1.6 and 0.8 μg/ml. The absorbances at wavelength 570 nm for various concentrations were summarized in **Table 3** and were plotted in **Figure 5**. Concentration-response curves of Sf9 cells to crude extract analyzed with SOFTmax program in triplicate. The crude extract prepared from mummified larvae possessed ID_{50} value of 10.0 ± 1.7 μg/ml. In general, any sample that possesses an ID_{50} value of 10 μg/ml or lower is considered to contain possible toxic compound(s) [82].

Table 3 The cytotoxicity of various concentrations of crude extract preparation prepared from *N. rileyi* infected *S. litura* toward Sf9 insect cell lines.

Concentration* (μg/ml)	**Absorbance at 570 nm**		
	Replica 1	**Replica 2**	**Replica 3**
100	0.020 ± 0.017	0.010 ± 0.010	0.003 ± 0.002
50	0.013 ± 0.006	0.010 ± 0.005	0.005 ± 0.003
25	0.093 ± 0.032	0.090 ± 0.028	0.083 ± 0.027
12.50	0.382 ± 0.007	0.366 ± 0.023	0.373 ± 0.027
6.25	0.467 ± 0.029	0.482 ± 0.049	0.456 ± 0.038
3.13	0.467 ± 0.025	0.486 ± 0.061	0.482 ± 0.014
1.56	0.483 ± 0.021	0.519 ± 0.067	0.445 ± 0.037
0.78	0.500 ± 0.019	0.538 ± 0.060	0.495 ± 0.034
ID_{50} ± SE (μg/ml)**	**9.89 ± 0.02**	**8.35 ± 0.04**	**11.79 ± 0.03**
Mean value of ID_{50} ± SD (μg/ml)	**10.01 ± 1.72**		

* Final concentration of the crude extract preparation from *N. rileyi* infected *S. litura*

** ID_{50} values were calculated from concentration-response curve of Sf9 cells to crude extract *N. rileyi* infected larvae using Softmax computer program (Molecular Device, UK) to perform 4-parameters non-linear regressing line.

SE was represented "standard error" for the estimation of ID_{50} value with 4-parameters non-linear regression curve, $y = (A-D)/(1+(x/C)^B)+D$, calculated by SOFTmax program.

SD was represented "standard deviation" of mean value determination from triplicate experiments.

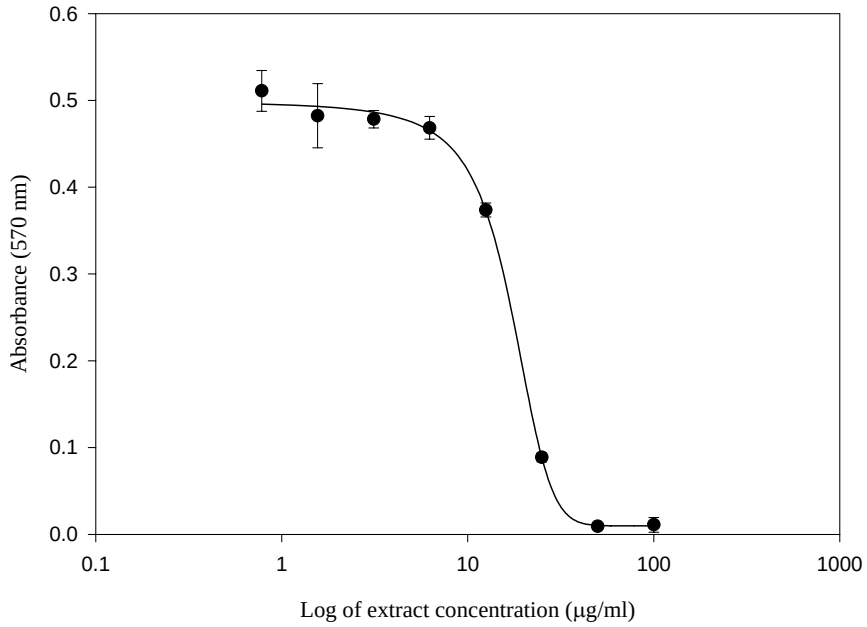


Figure 5 Concentration-response curve of Sf9 cells to crude extract preparations from *N. rileyi* infected *S. litura*. Absorbance values at 570 nm are indicative of MTT conversion to a colored product. Data points (•) represent the means and bar (I) represent standard deviation (SD) of triplicate determinations. The curve was drawn by 4-parameters non-linear regressing line.

3. The effect of heat treatment on the cytotoxic activity of crude extract from *N. rileyi* infected *S. litura* against Sf9 cells

The stability of the active compound was determined by subjecting crude preparations to various temperatures for various time periods. Data (**Table 4**) revealed that the crude extracts from infected larvae were not inactivated by heat (50, 70, 90°C, 15 and 30 min) and even retained cytotoxic activity after heating at 90°C for 60 min. This indicated the presence of a thermostable toxic substance(s).

Table 4 The effect of heat on cytotoxicity of crude extract preparations from *N. rileyi* infected *S. litura* on Sf9 cells*

Temperature (°C)	Time of exposure (min.)	Cytotoxic activity (%cell death)**
25	60	93 ± 2.08
50	15	91 ± 8.49
	30	91 ± 6.36
70	15	93 ± 8.49
	30	90 ± 3.54
90	15	93 ± 7.78
	30	88 ± 3.54
	60	90 ± 2.12

* Crude extract preparations from *N. rileyi* infected *S. litura* were subjected to various temperatures for various time periods before being tested for cytotoxicity.

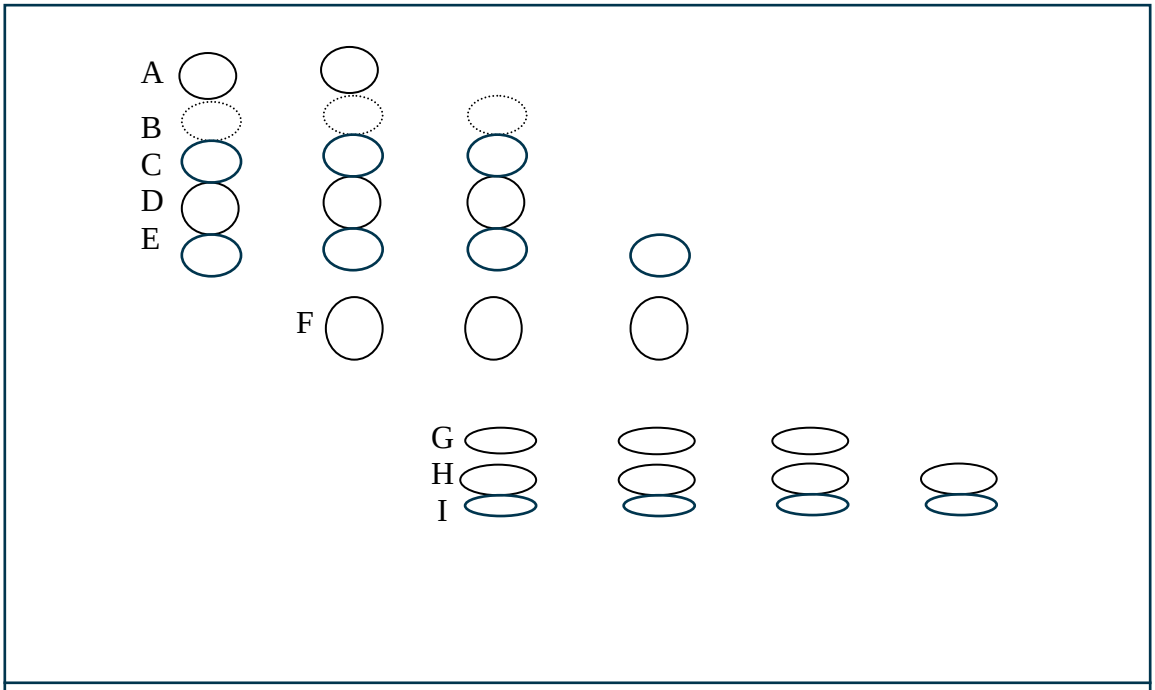
** %cell death after being exposed to 200 μg/ml of various extract preparations
Cell viability was measured by MTT assay.

4 Purification of active compound from crude extract prepared from *N. rileyi* infected *S. litura*

To separate active compound(s) from crude extract prepared from *N. rileyi* infected *S. litura*, column chromatography and TLC methods were employed. The fractions showing the same spots on TLC plate were pooled together which resulted in seven pools as shown in **Figure 6**. Each pool was then tested for toxic activity against Sf9 cell cultures. **Figure 6** showed illustration of TLC plate exhibiting different spots profile in different groups. The results from **Table 5** showed that pools 3, 4 and 5 possessed cytotoxic activities with 100 % cell death.

In order to obtain the purified active compounds, G_1-4 and G_1-5 fractions were pooled and subjected to another column chromatography using the same system. From the results in **Table 6** and **Figure 7**, group G_2-3 containing compounds G, H and I

gave 93 % cell death while group G_2-4 containing compound H and I gave only 43 % cell death. Thus compounds G and H were thought to be responsible for the majority of the cytotoxicity presented in the crude extract of *N. rileyi* infected *S. litura*. Since compound G and H were quite close to one another on TLC plate as shown in **Figure 8**, attempts were made to isolate large quantity of pure compound for further identification and determination of bioactivity. Compound G (19.6 mg) was successfully isolated from infected larvae (200 g) and was crystallized as colorless needle crystal as shown in **Fig. 9**.



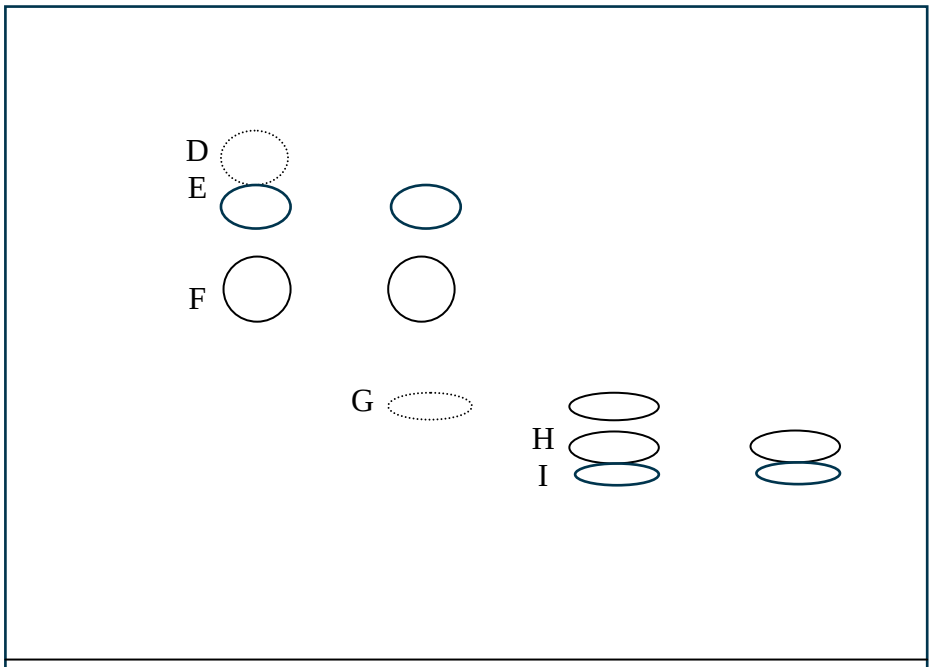
Fraction#	19-20	22-24	25-30	31-34	35-37	38-43	44-45
%Cell death	0	32	100	100	100	57	38

Figure 6 Thin layer chromatographic profiles of partially purified fractions of *N. rileyi* infected *S. litura* extract using a silica gel column and TEF (5.75:4:0.25) as the eluting solvent system.

Table 5 Cytotoxic activities of partially purified frctions of *N. rileyi* infect *S. litura* extract using a silica gel column and TEF (5.75:4:0.25) as the eluting solvent.

Group of fractions	**Fraction No.**	**Volume of eluate (ml)**	**Compound**	**% Cell death**
G_1-1*	19-21	6	A, B, C, D, E	0 ± 0.26
G_1-2	22-24	6	A, B, C, D, E, F	32 ± 2.48
G_1-3	25-30	12	B, C, D, E, F, G, H, I	100 ± 0.13
G_1-4	31-34	8	E, F, G, H, I	100 ± 0.13
G_1-5	35-37	6	G, H, I	100 ± 0.66
G_1-6	38-43	12	H, I	57 ± 7.83
G_1-7	44-55	24	R_f<0.3	38 ± 2.22

* G_1-1 was represented group number 1 obtained from the 1st column chromatography containing fractions number 19 to 21.



Fraction#	25-29	30-31	32-38	39-49
%Cell death	0	0	93	43

Figure 7 Thin layer chromatographic profiles of fractions using a silica gel column and TEF (5.75:4:0.25) as the eluting solvent system. Doted line circle represents weak spot.

Table 6 Cytotoxic activities of partially purified fractions using a silica gel column and TEF (5.75:4:0.25) as the eluting solvent.

Group of fractions	**Fraction No.**	**Volume of eluate (ml)**	**Compound**	**% Cell death**
G_2-1*	25-29	10	D′, E, F	0 ± 1.02
G_2-2	30-31	4	E, F, G′	0
G_2-3	32-38	14	G, H, I	93 ± 1.78
G_2-4	39-49	22	H, I	43 ± 5.09

* G_2-1 was represented group number 1 obtained from the 2nd column chromatography containing fractions number 25 to 29.

′ Compounds were detected as weak spots on TLC.

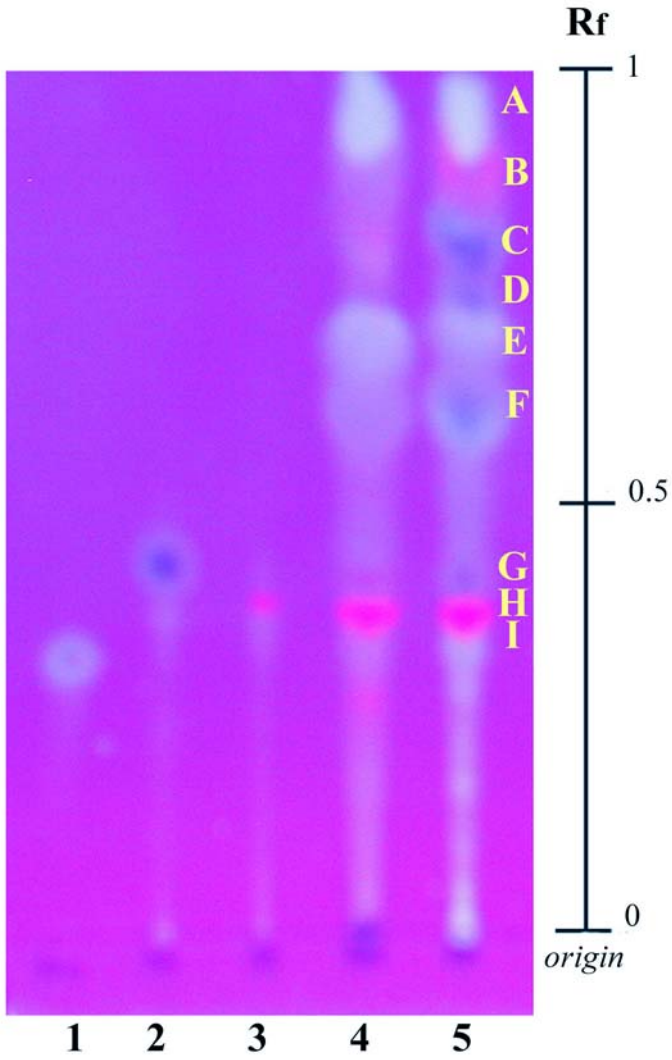


Figure 8 Chromatogram of crude extract preparation from *N. rileyi* infected *S.litura*. Crude extract preparation was dissolved in dichloromethane and approximately 10 µl portions were spotted into a silica TLC plate. After chromatography with TEF solvent, the plate was dried and spots detected by spraying sulfuric acid and examining under UV light at 366 nm. Samples were prepared from standard Griseofulvin (**lane 1**), compound G (**lane 2**), partially purified compound H (**lane 3**), crude extract from healthy larvae (**lane 4**) and crude extract from infected *S. litura* (**lane 5**). For reference, the spots were labeled A to I alphabetically.

Figure 9 The photograph showed colorless crystal of Compound G.

5. Cytotoxic activity of compound G

The ID_{50} of compound G was evaluated by cytotoxicity test against Sf9 cell line. Concentrations of compound G ranging from 0.78 to 100 μg/ml were assayed. From the results in **Table 7,** the viability of Sf9 cells' exposed to compound G was concentration dependent. It was found that ID_{50} of replica 1 to 3 were 18.77, 15.51 and 18.89 μg/ml. The mean ID_{50} value was calculated as 17.72±1.92 μg/ml.

Table 7 The cytotoxicity of compound G toward Sf9 insect cells.

Concentration* (μg/ml)	**Absorbance at 570 nm****		
	Replica 1	**Replica 2**	**Replica 3**
100	0.021±0.002	0.012±0.002	0.013±0.006
50	0.199±0.002	0.193±0.014	0.222±0.033
25	0.299±0.051	0.260±0.030	0.279±0.034
12.50	0.351±0.010	0.376±0.024	0.382±0.029
6.25	0.467±0.058	0.545±0.053	0.647±0.023
3.13	0.620±0.004	0.666±0.026	0.655±0.098
1.56	0.690±0.057	0.683±0.074	0.865±0.079
0.78	0.677±0.045	0.682±0.104	0.666±0.023
ID_{50} ± SE (μg/ml)	**18.77 ± 0.05**	**15.51 ± 0.06**	**18.89 ± 0.10**
Mean value of ID_{50} ± SD (μg/ml)	**17.72 ± 1.92**		

* Final concentration of compound G.

** Absorbance was measured spectrophotometrically at 570 nm.

SE was represented "standard error" for the estimation of ID_{50} value with 4-parameters non-linear regression curve, $y = (A-D)/(1+(x/C)^B)+D$, calculated by SOFTmax program.

SD was represented "standard deviation" of mean value determination from triplicate experiments.

6. Identification of Compound G

The cytotoxic compound G was isolated from CH_2Cl_2:MeOH extract of *N. rileyi* infected insect. Compound G was identified to be ergosterol peroxide (5α, 8α-epidioxy-24(R)-methylcholesta-6,22-dien-3β-ol) according to NMR, IR and mass spectroscopic data. The structure was elucidated by HRFABMS and NMR analysis (^{1}H, ^{13}C, DEPTs, COSY, HMQC and HMBC).

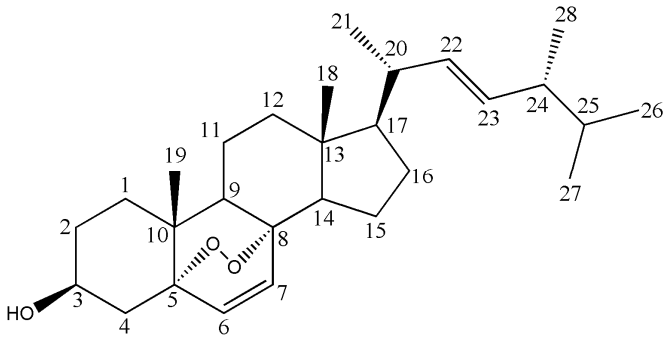
Compound G was isolated as colorless needle crystal. The melting point was found to be between 179-181 °C. The molecular formula of compound G as $C_{28}H_{44}O_3$ was determined by ^{13}C NMR and HRFABMS observing the $[M+H]^+$ ion peak at *m/z* 429.3381 (calcd. for $C_{28}H_{45}O_3$, 429.3369). The fragmentation patterns obtained by EI-MS analysis (**Fig.12**) showed molecular ion peak at m/z 410 $[M-H_2O]^+$, 396 $[M-O_2]^+$, 378 $[M-O_2-H_2O]^+$ and 363 $[M-O_2-H_2O-CH_3]^+$. The intense peak ion peak at m/z 396 showed a significant loss of 32 amu which is a characteristic feature of epidioxides. Thus the molecular mass was confirmed by ESI-TOF (Electrospray ionization time-of-flight) mass spectroscopy observing $[M+H]^+$ at m/z 429, $[M+H -H_2O]^+$ at 411 and $[M+H -O_2]^+$ at m/z 397 (**Fig. 13**). IR ν_{max}(KBr): 3366, 1653, 1457, 1378, 1075, 1045, 967 cm^{-1}.

The identification of compound G was performed using NMR data. The IR spectrum (**Fig. 11**) indicated the O-H stretching at 3366 cm^{-1}, the C-H stretching at 2956 cm^{-1} and the CH_3 bending at 1457 cm^{-1} and 1378 cm^{-1}.^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C ($CDCl_3$, 100 MHz) NMR spectral data of compound G were shown in **Table 8**. The ^{1}H- and ^{13}C NMR data indicated the presence of six methyl groups at δ 0.81, 0.88, 0.815, 0.83, 0.907, 1.0 ppm and one methane attached to a hydroxy group at δ 3.97 ppm. Four methines on double bonds were assigned at δ 6.25, 6.51, 5.22 and 5.14 ppm. The ^{1}H-NMR showed the signal of OH-absorption at δ 3.97 (1H, *m*). The absorption of olefinic protons were seen at δ 6.25 (1H, *d*, J=8.5 Hz.), δ 6.51 (1H, *d*, J=8.5 Hz.), δ 5.22 (1H, *dd*, J=7.4, 15.3 Hz.) and δ 5.14 (1H, *dd*, J=8.18, 15.3 Hz.). The methylene resonances at δ 0.88 and δ 0.81 appeared as singlets. The doublet methyl proton signals were shown at δ 1.0 (3H, *d*, J=6.59 Hz.), δ 0.907 (3H, *d*, J=6.85 Hz.), δ 0.83 (3H, *d*, J=6.7 Hz.) and δ 0.815 (3H, *d*, J=6.64 Hz.). The ^{13}C NMR spectrum showed signal for 28 C-atom. The DEPT spectra (**Fig. 18**) indicated the presence of 6

methyls (δ 12.8, 18.1, 20.8, 19.9, 19.6 and 17.5 ppm), four methines (δ 135.4, 130.7, 135.2, 132.2 ppm) corresponded to endocyclic C=C bonds between C-6 and C-7, and C-22 and C-23 respectively, and one methine (δ 66.4 ppm) attached to hydroxyl group. The signals of the olefinic carbons at δ 135.2 (C-22) and δ 132.2 (C-23) belonged to the terminal double bond at the side chain of compound G. The absorption of oxygenated methine carbon (C-3) appeared at δ 66.43. The resonances of methyl carbons were seen at δ 12.8 (C-18), δ 18.1 (C-19), δ 20.8 (C-21), δ 19.9 (C-26), δ 19.6 (C-27) and δ 17.5 (C-28).

HMQC and HMBC spectra (**Fig. 20** and **21**) showed correlation between methyl proton at 1.0 ppm (H-21) and carbon at 135.2 ppm (C-22). Additionally HMBC exhibited long range correlations between vinyl protons at 6.25 (H-6) and 6.51 (H-7) ppm with quanternary oxygenated carbons at 79.4 (C-8) and 82.1 (C-5) ppm and with methylene groups at 36.8 (C-4), 51.0 (C-14) and 51.6 (C-9) ppm. Additionally correlation between methyl proton at 1.0 (H-21) ppm with carbon at 135.2 (C-22) ppm was observed.

Table 8 ^{1}H and ^{13}C NMR spectral data of compound G



Position	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm) (multiplicity, *J* in Hz)
1	29.7	1.83 (*m*)
2	34.6	1.68 (*m*), 1.95 (*m*)
3	66.4	3.97 (*m*)
4	36.8	1.90 (*m*), 2.13 (*m*)
5	82.1	
6	135.4	6.25 (*d*, 8.5)
7	130.7	6.51 (*d*, 8.5)
8	79.4	
9	51.6	1.51 (*m*)
10	36.9	
11	20.6	
12	39.7	
13	44.5	
14	51.0	
15	23.3	
16	28.6	1.36 (*m*), 1.75(*m*)
17	56.1	
18	12.8	0.81 (*s*)
19	18.1	0.88 (*s*)
20	39.3	2.03 (*m*)
21	20.8	1.00 (*d*, 6.59)
22	135.2	5.14 (*dd*, 8.18, 15.3)

Table 8 ^{1}H and ^{13}C NMR spectral data of compound G (cont.)

Position	$\delta^{13}C$ (ppm)	$\delta^{1}H$ (ppm) (multiplicity, *J* in Hz)
23	132.2	5.22 (*dd*, 7.4, 15.3)
24	42.7	1.85 (*m*)
25	33.0	1.46 (*m*)
26	19.9	0.83 (*d*, 6.7)
27	19.6	0.815 (*d*, 6.64)
28	17.5	0.907 (*d*, 6.85)

Multiplicities are indicated as singlets (*s*), doublets (*d*), triplets (*t*), multiplets (*m*)

Table 9 Spectroscopic data of compound G

Spectroscopic data	**Compound G**
m.p. (°C)	179-181
UV λ_{max} (MeOH) nm (**Fig.10**)	204(log ε = 3.74)
IR[ν_{KBr}](cm^{-1}) (**Fig.11**)	3366, 1653, 1457, 1378, 1075, 1045, 967
^{13}C-NMR	**Table 8**
^{1}H-NMR	**Table 8**
EI-MS m/z (relative intensity) (**Fig.12**)	410, 398(6.36),397(27.38),396(100),378(11.89), 364(11.29),363(37.48),337(18.10),253(27.80), 251(14.95),211(14.15),197(10.62),159(11.94), 158(11.17),157(19.66),145(12.16),143(11.88), 91(6.78),81(6.83),79(7.61),69(8.59),67(6.24), 55(6.66),41(8.95)
TOF m/z (s/n ratio) (**Fig.13**)	429(920.8),412(345.9),411(1215.7),398(108.8), 397(650.4),396(2977.0),395(9688.6),394(179.1), 393(673.0),379(103.0),378(453.1),377(1426.9), 376(152.9),375(498.8)

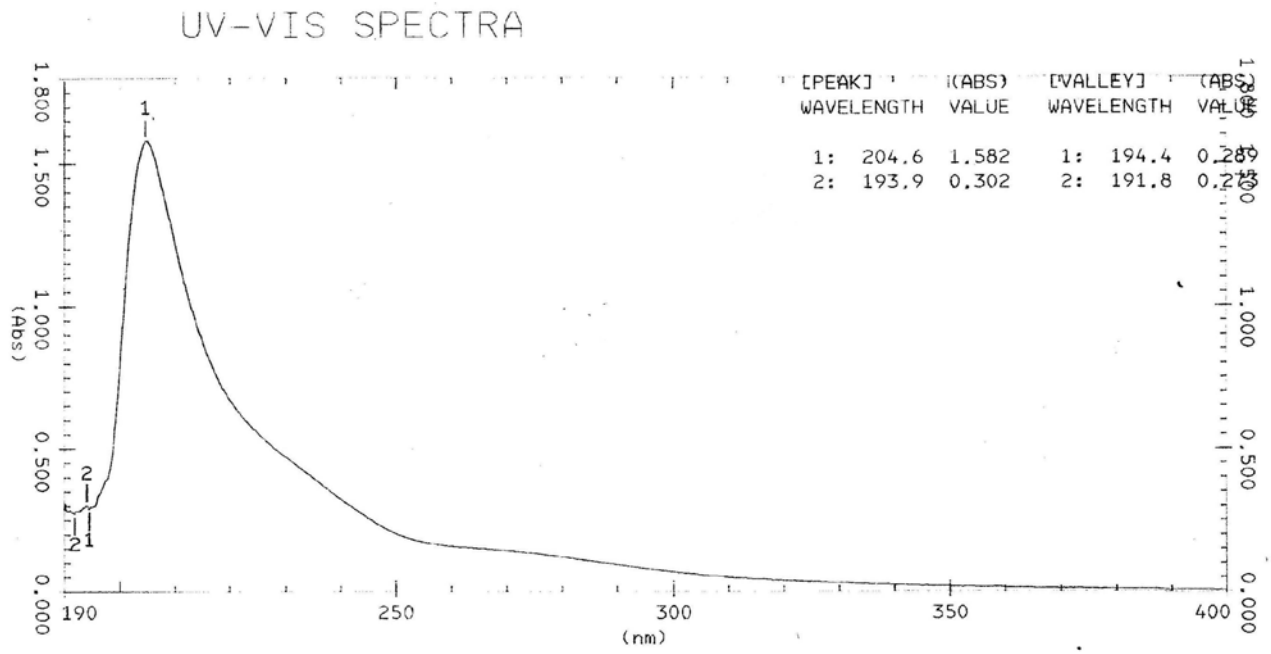


Figure 10 UV-VIS spectrum of compound G

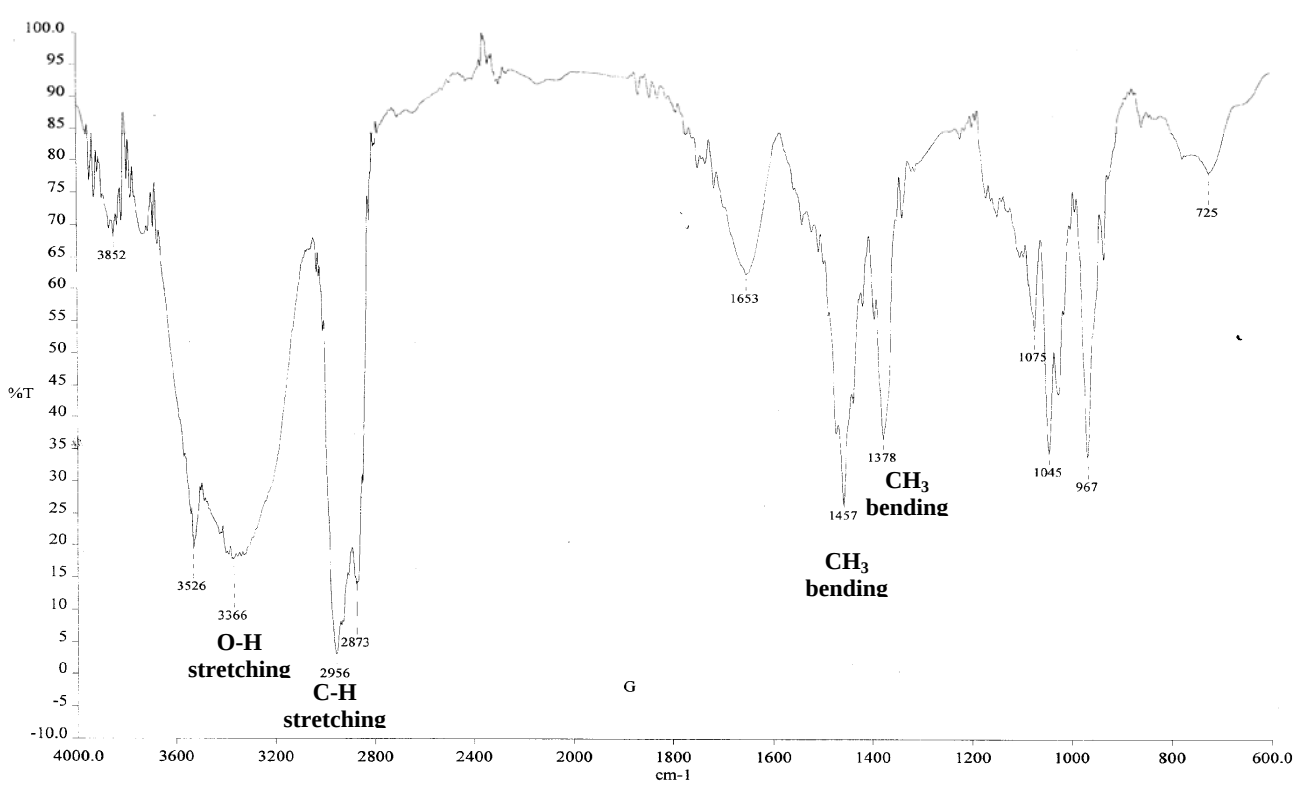


Figure 11 IR spectrum of compound G

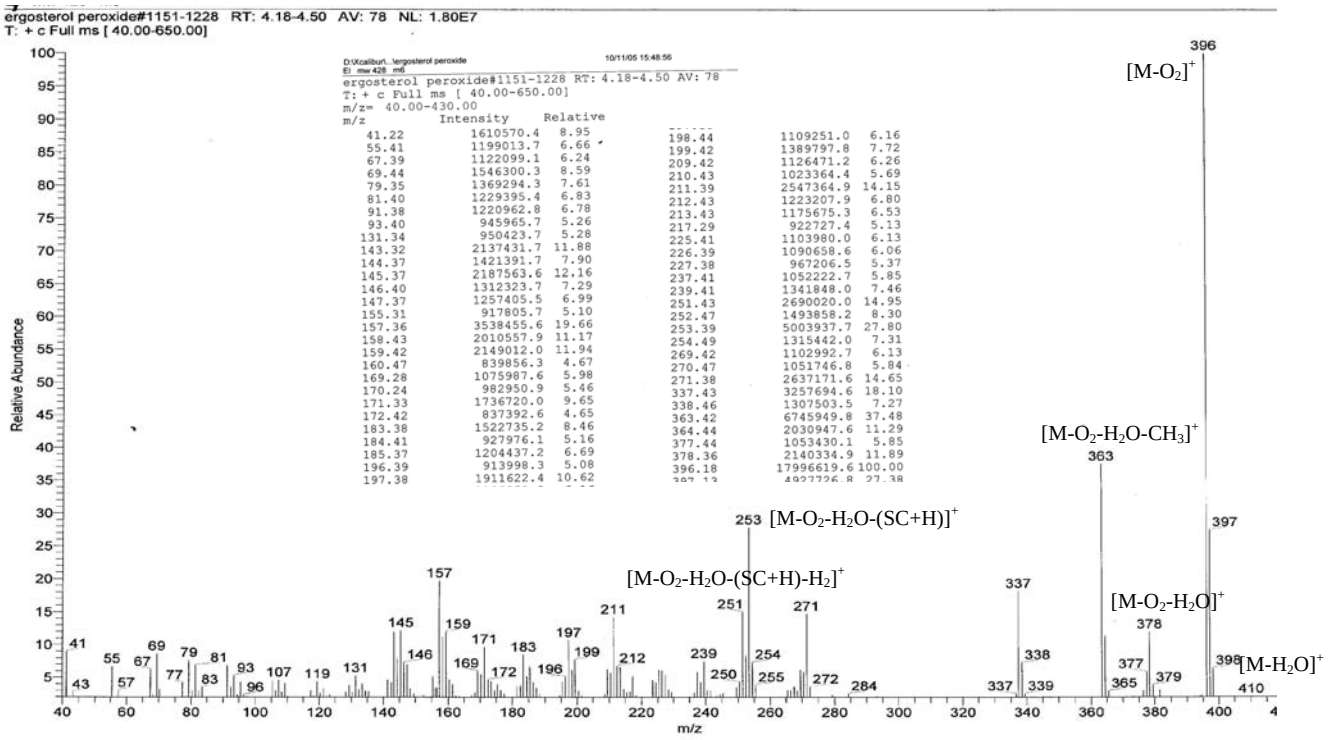


Figure 12 Mass spectrum (EI-MS) of compound G.

Abbreviation: SC = side chain of ergosterol peroxide molecule (Mw 125)

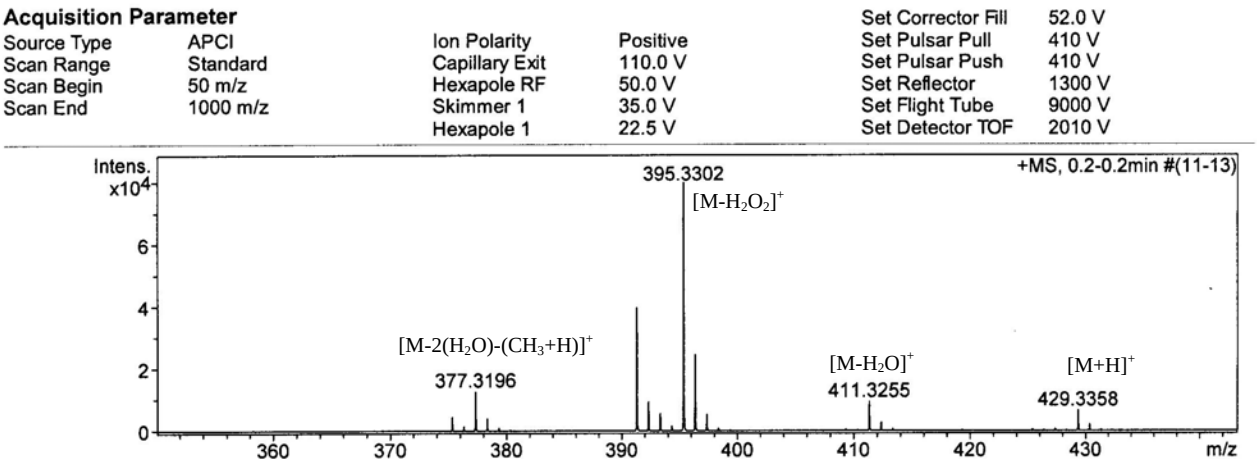


Figure 13 ESI-TOF (Electrospray ionization time-of-flight) Mass spectrum of compound G

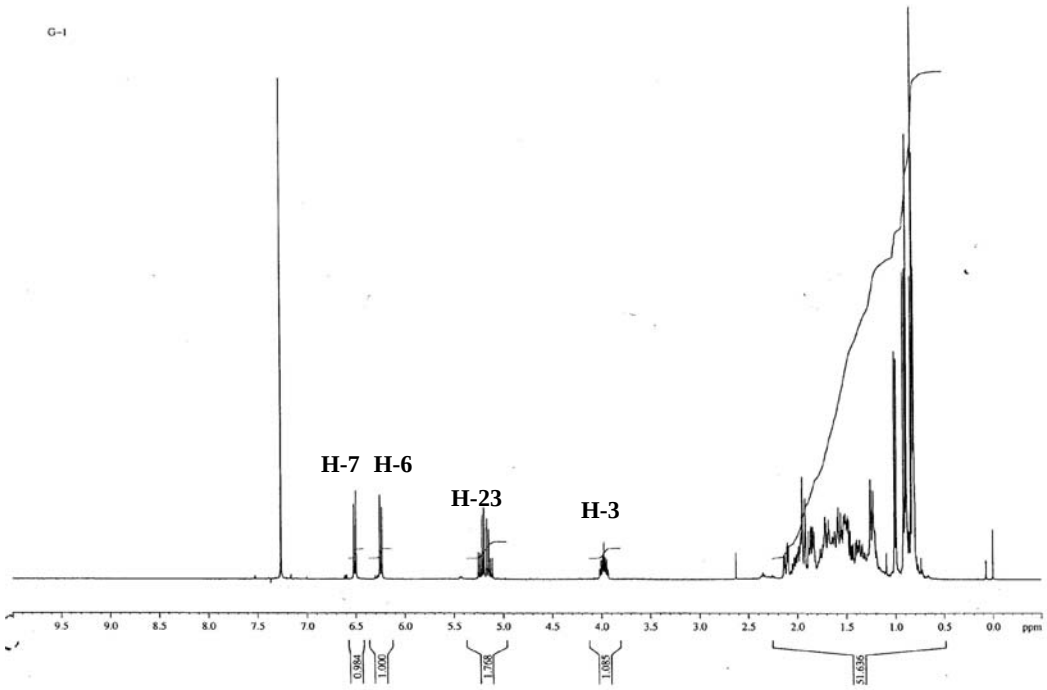


Figure 14 400 MHz ^{1}H-NMR spectrum of compound G (δ 0-10 ppm), in $CDCl_3$,showing the assignment of Compound G

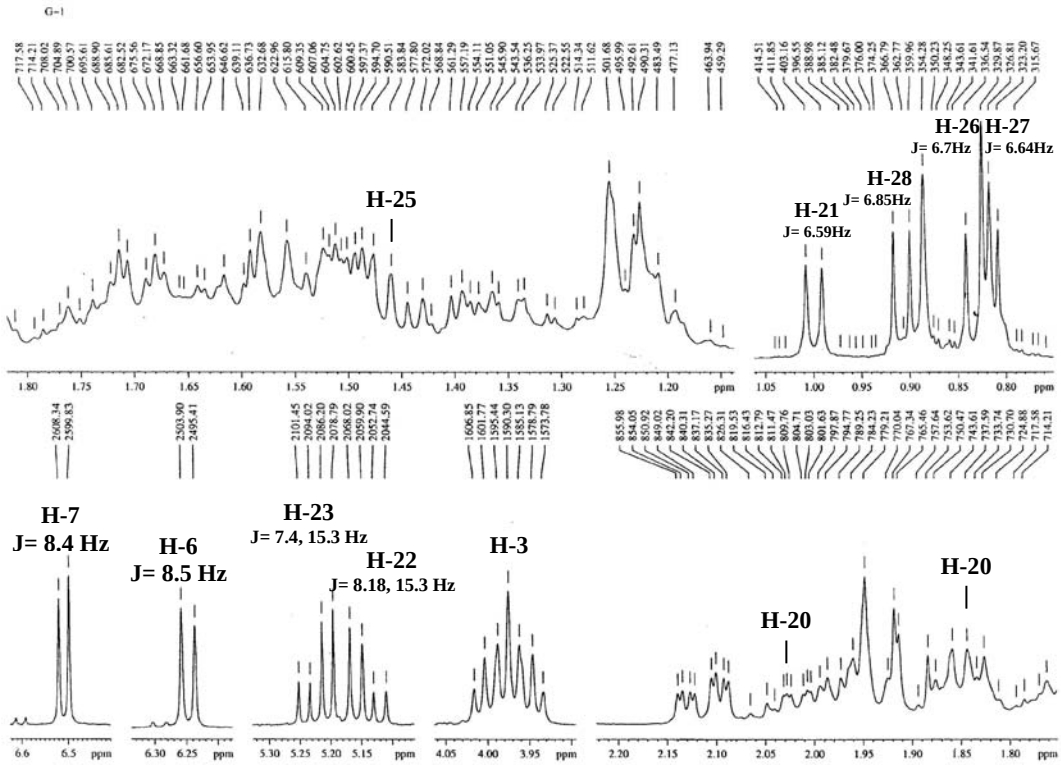


Figure 15 Expansion of 400 MHz ^{1}H-NMR spectrum of compound G, in $CDCl_3$, showing the assignment of Compound G

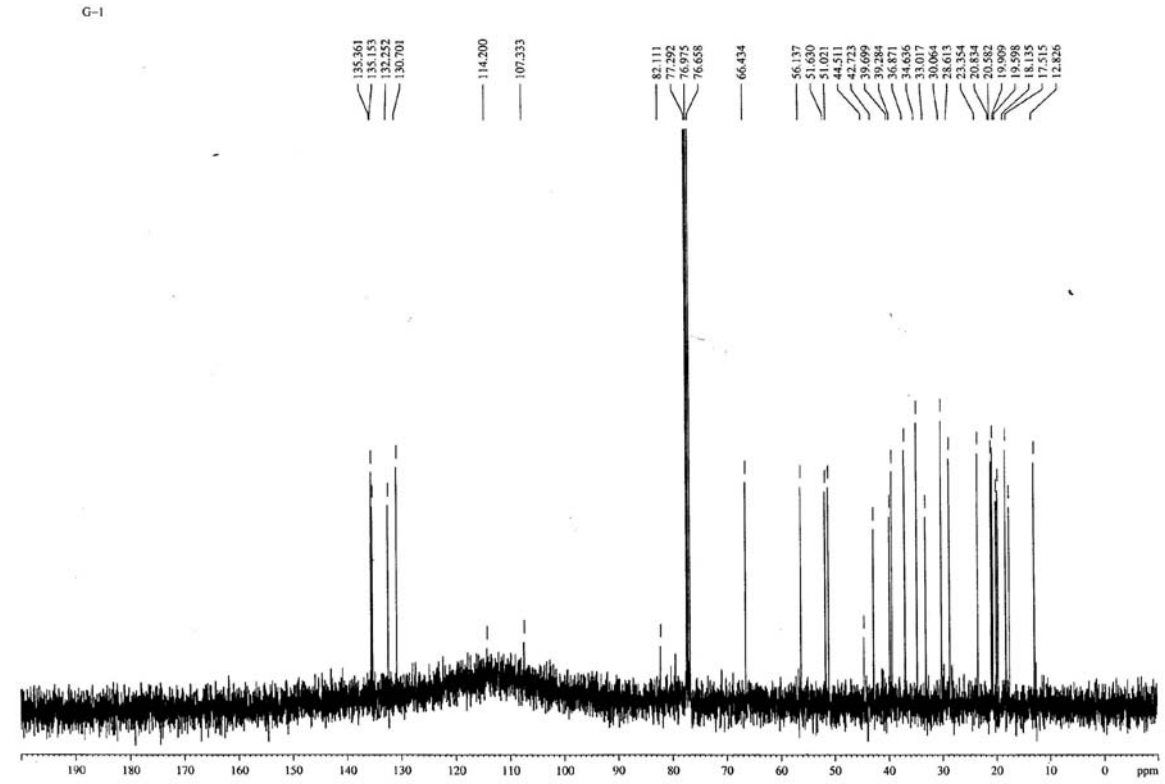


Figure 16 100 MHz ^{13}C-NMR spectrum of compound G (δ 0-200 ppm)

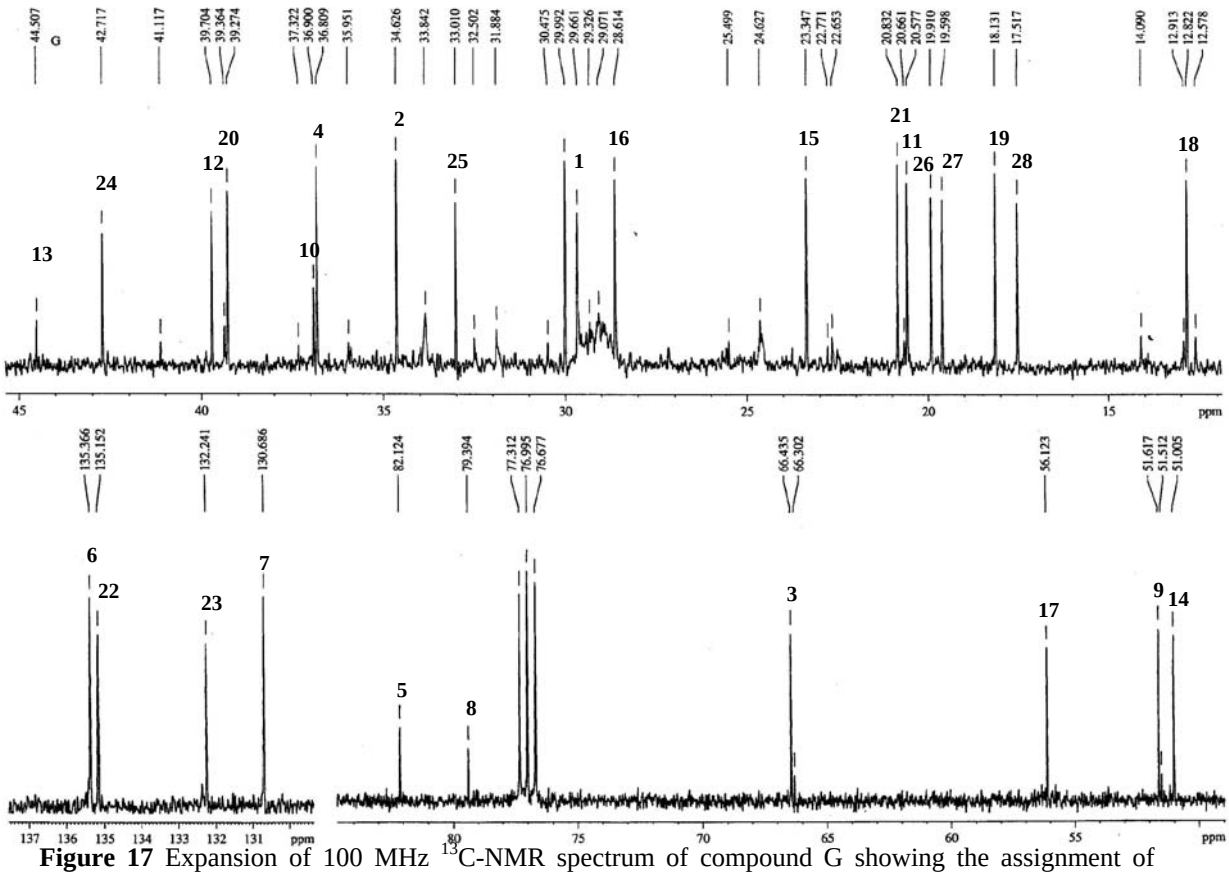


Figure 17 Expansion of 100 MHz ^{13}C-NMR spectrum of compound G showing the assignment of compound G

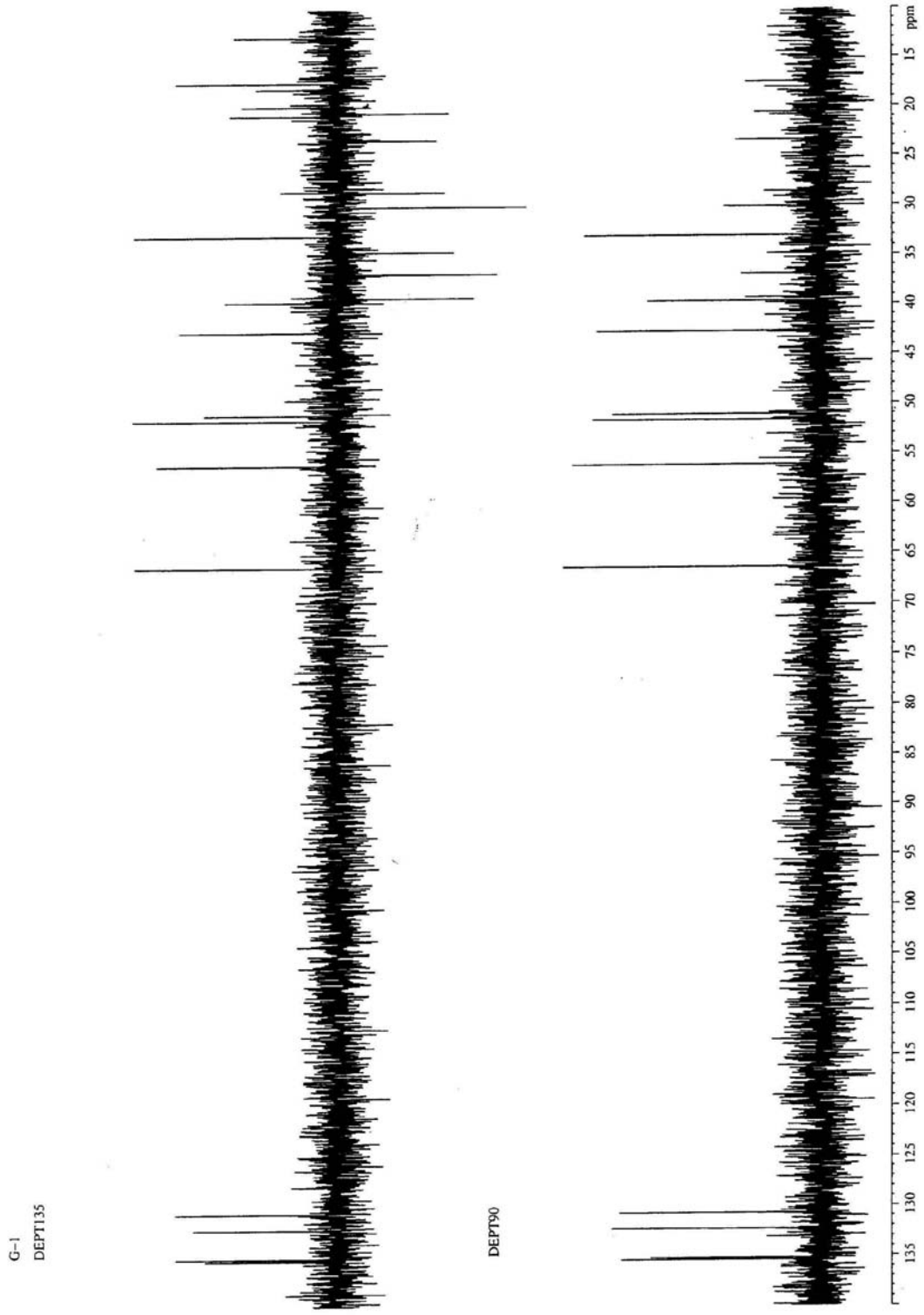


Figure 18 DEPT135 spectrum (upper) and DEPT90 (lower) of compound G (^{13}C δ 0-140ppm)

COSY

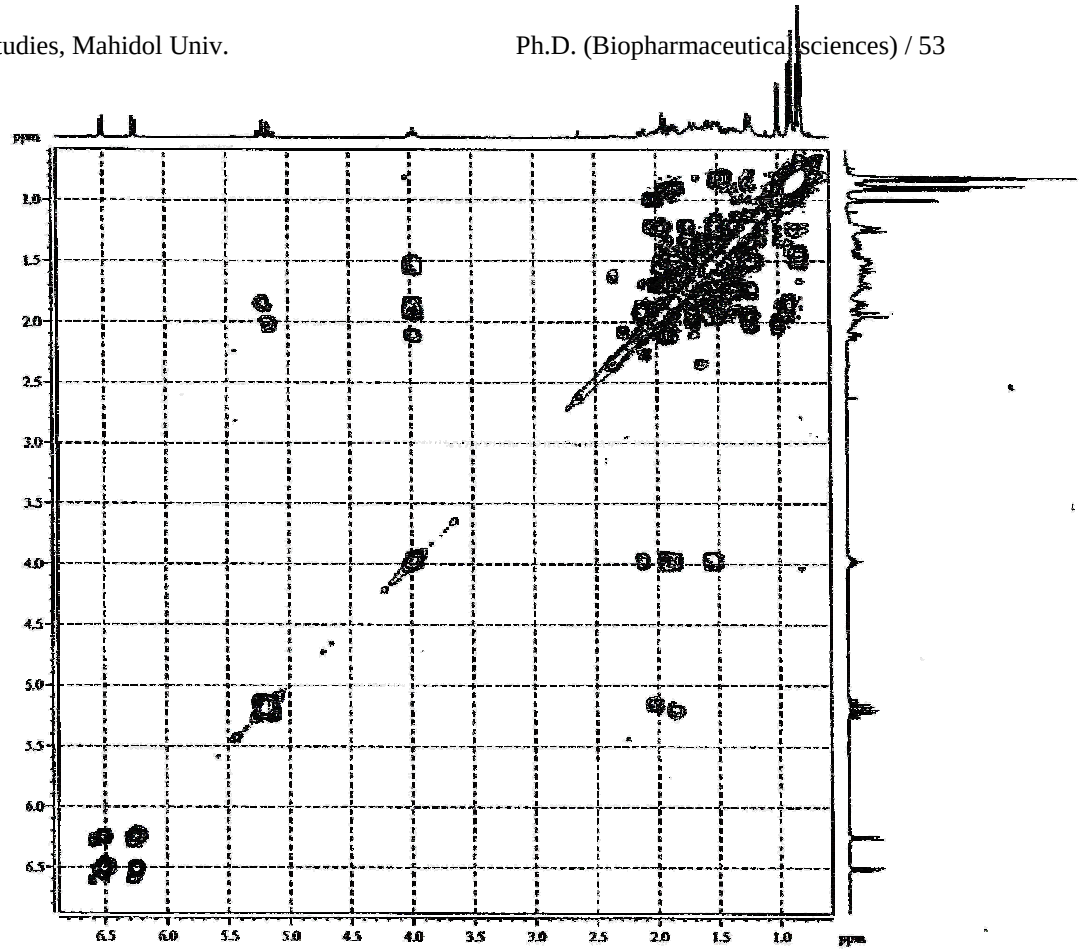


Figure 19 COSY spectrum of compound G

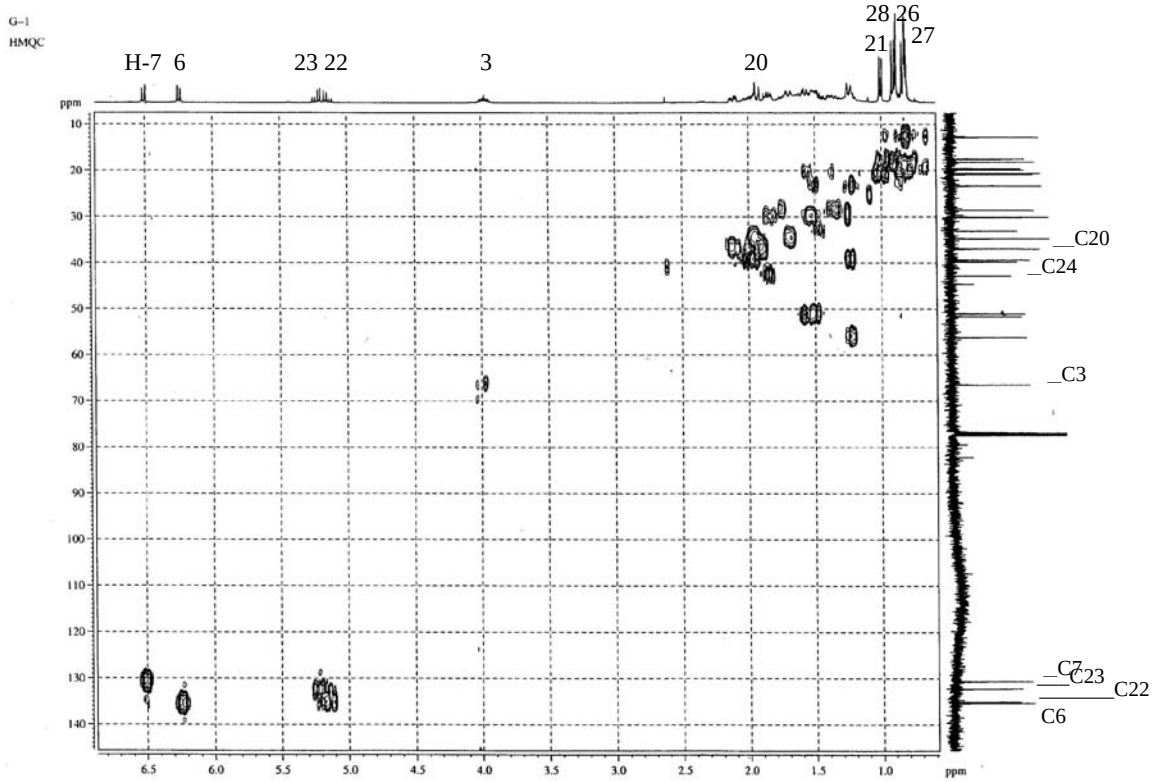


Figure 20 HMQC spectrum of compound G

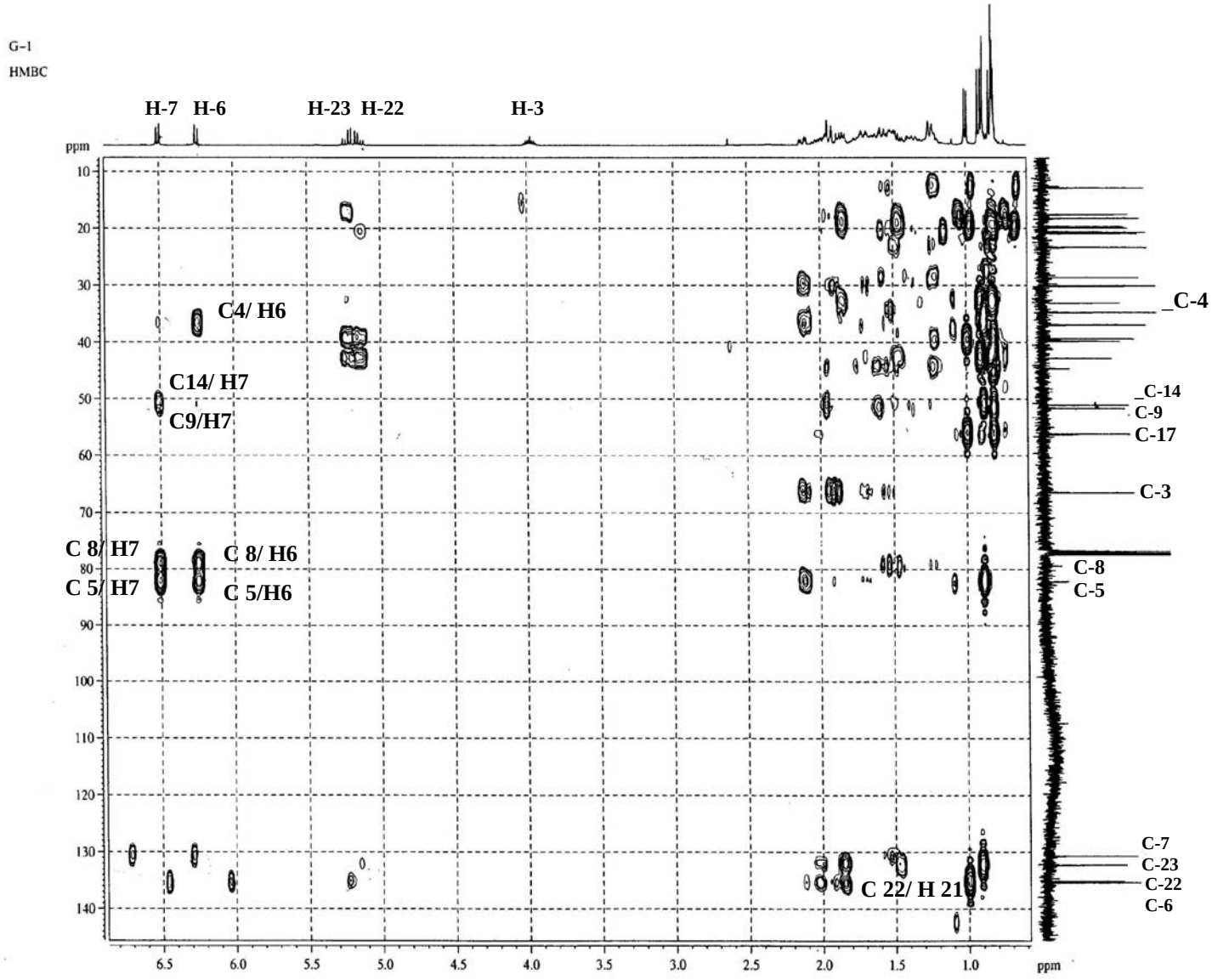
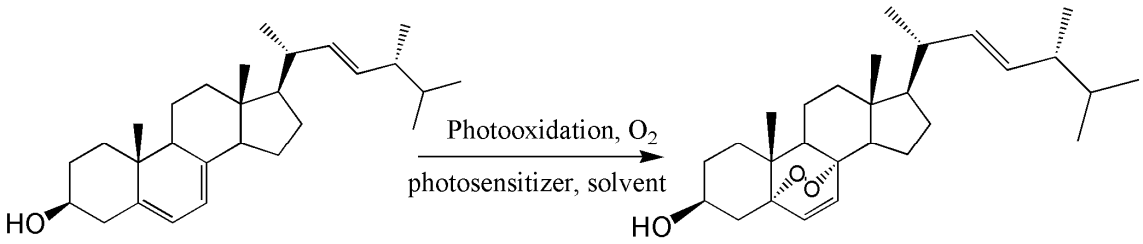


Figure 21 HMBC spectrum of compound G showing the assignment of compound G

7. Chemical synthesis of ergosterol peroxide



ergosterol ergosterol peroxide

Ergosterol peroxide was successfully synthesized by the method of photooxidation reaction. Different combinations of dyes (eosin and methylene blue) and solvents (pyridine, ethanol and chloroform) were used to synthesize ergosterol peroxide in this study (**Table 10**). The result showed that highest production yield of ergosterol peroxide (59.8 %) was obtained from reaction that occurred in ethanol with methylene blue as photosensitizer under UV light. The control reaction was conducted without photosensitizer and UV irradiation. In control reaction, continuous white light irradiation and normal air flow were provided instead of UV light and oxygen gas. Ergosterol peroxide was found to be slowly produced and detected on day 3.

Table 10 Photooxidation reactions in ergosterol peroxide synthesis

	Starter (Ergosterol)		**Photosensitizer**			**Solvent**	**Time**	**Product (Ergosterol peroxide)**		**Recovery (Ergosterol)**	
	g.	**mmole**		**g.**	**Mol%**			**g.**	**% yield**	**g.**	**% yield**
1.*	0.0396	0.1	-	-	-	EtOH	3 Days	0.0021	4.9	0.0049	12.37
2.	0.396	1	EO	0.004	0.6	EtOH	12 hr	0.0254	5.93	NR**	NR
3.	0.396	1	EO	0.040	6	EtOH	7 hr	0.0297	6.94	NR	NR
4.	0.396	1	EO	0.040	6	EtOH	12 hr	0.1625	37.97	0.0073	1.88
5.	0.396	1	EO	0.004	0.6	PY	7 hr	0.0040	0.93	NR	NR
6.	0.200	0.5	EO	0.010	2	EtOH	12 hr	0.0894	41.36	0.0071	3.55
7.	0.200	0.5	EO	0.020	6	EtOH	12 hr	0.0890	41.17	0.0107	5.35
8.	0.200	0.5	EO	0.040	12	EtOH	12 hr	0.1052	48.67	0.0203	10.15
9.	0.200	0.5	MB	0.010	6	EtOH	12 hr	0.1083	50.10	0.0064	3.20
10.	**0.200**	**0.5**	**MB**	**0.020**	**12**	**EtOH**	12 hr	**0.1293**	**59.82**	**0.0007**	**0.35**
11.	0.200	0.5	MB	0.020	12	$CHCl_3$	12 hr	0.0175	8.10	0.0111	5.55
12.	0.200	0.5	MB	0.020	12	PY	12 hr	0.0037	1.71	0.0487	24.35
13.	0.200	0.5	-	-	-	EtOH	12 hr	0.0014	0.65	0.0125	6.25

Abbreviations: EO (eosin, Mw 692), MB (methylene blue, Mw 356), PY (pyridine), EtOH (ethyl alcohol), $CHCl_3$ (Chloroform)

* Reaction no.1 was used as control. White light and normal air flow were used instead of UV light and O_2 gas.

** NR indicated not recorded

8 Determination of ergosterol peroxide in fungal extract

8.1 HPLC/UV analysis

Four elution systems in **Table 11** were tested to search for optimum elution solvent system for this HPLC/UV method. The standard authentic substances consisted of ergosterol peroxide (5 ppm) and ergosterol (5 ppm) were injected at the level of 50 μl and four elution systems were conducted to separate these compounds. From the results the optimum elution was the system of 100%isopropanol which was gradually increased from 5% to 45% in 5% H_2O in acetonitrile from 0 to 45 min and then decreased to 5% at 45 to 60 min with flow rate of 1 ml/min.

The peaks at the 210 and 282nm spectra that indicated the presence of ergosterol peroxide and ergosterol had retention times of 14.5 and 39.3 min, respectively (**Fig.22**). It was found that the absorption responded to ergosterol peroxide appeared as very small peak in chromatogram. It could be explained by the low extinction coefficient of ergosterol peroxide at 210 nm. Maximum absorption of ergosterol peroxide is obtained at 204 nm however detection at 204 nm was impossible due to limitation of UV detector.

By comparison of UV spectra of crude mycelia extract and standard compounds in **Fig. 23**, the peak at t_R 14.9 min could be identified as ergosterol peroxide however it might be marked and overlapped by impurities which eluted closely and absorbing the wavelength of 210 nm. Therefore peak area of ergosterol peroxide could not be calculated. From this result, it could be concluded that using UV absorption as method of detection was not specific enough to measure precisely the quantity of ergosterol peroxide amount due to the low level of absorption at wavelength of 210 nm.

Table 11 Solvent systems for separation of ergosterol peroxide

System	Time (min)	Solvent		Retention time (t_R)	
		Isopropanol	5% H_2O in Acetonitrile	Ergosterol peroxide	Ergosterol
1	0	5%	95%	12 min	not detected
	15	45%	55%		
	30	60%	40%		
	45	60%	40%		
	47	5%	95%		
	62	5%	95%		
2	0	5%	95%	15 min	36 min
	10	5%	95%		
	44	45%	55%		
	45	5%	95%		
	60	5%	95%		
3	0	5%	95%	14.5 min	40 min
	20	5%	95%		
	44	45%	55%		
	45	5%	95%		
	60	5%	95%		
4	0	5%	95%	14.5 min	39.3 min
	15	5%	95%		
	44	45%	55%		
	45	5%	95%		
	60	5%	95%		

Detection method: UV absorption at 0 to 25 min, at λ 210 nm and at 26 to 60 min, at λ 282 nm

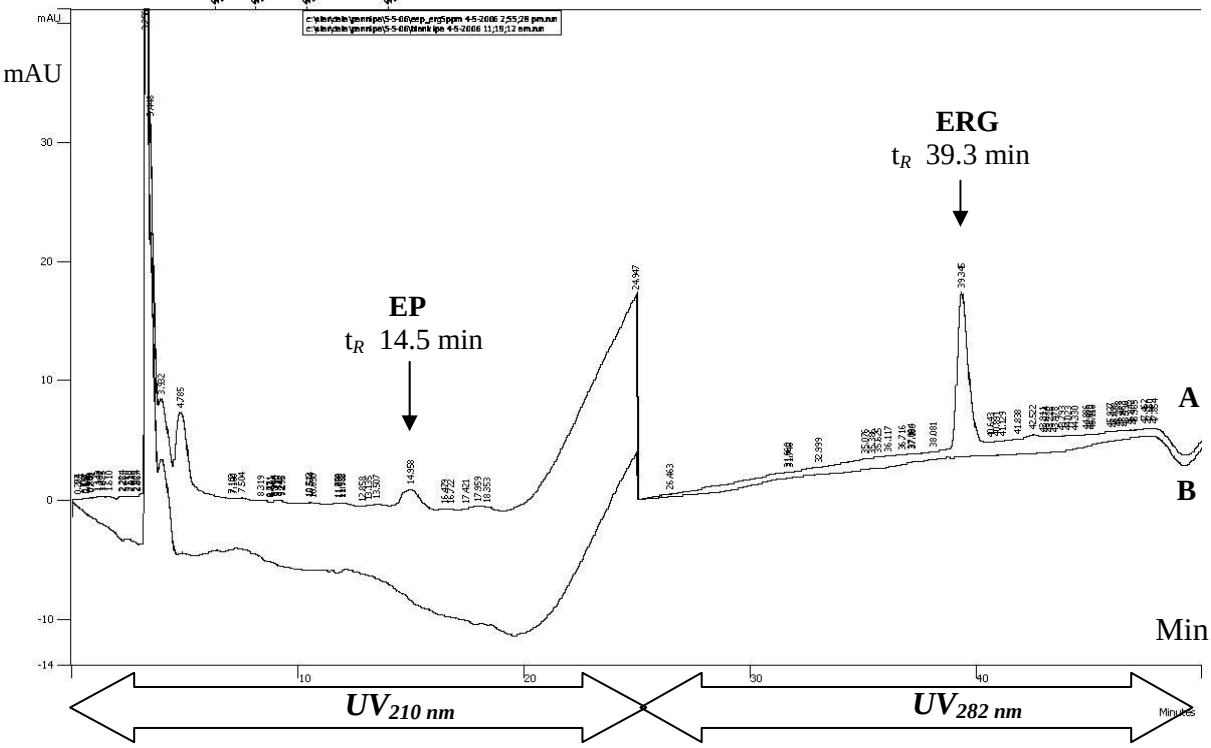


Figure 22 HPLC chromatogram of mixture of standards ergosterol peroxide (EP) and ergosterol (ERG) 5 ppm in isopropanol **(line A)** isopropanol 50 µl without EP and ERG **(line B)**. The column was eluted with solvent system #4. Compounds were detected under UV 210 nm (at 0 to 25 min) and 282 nm (at 26 to 50 min).

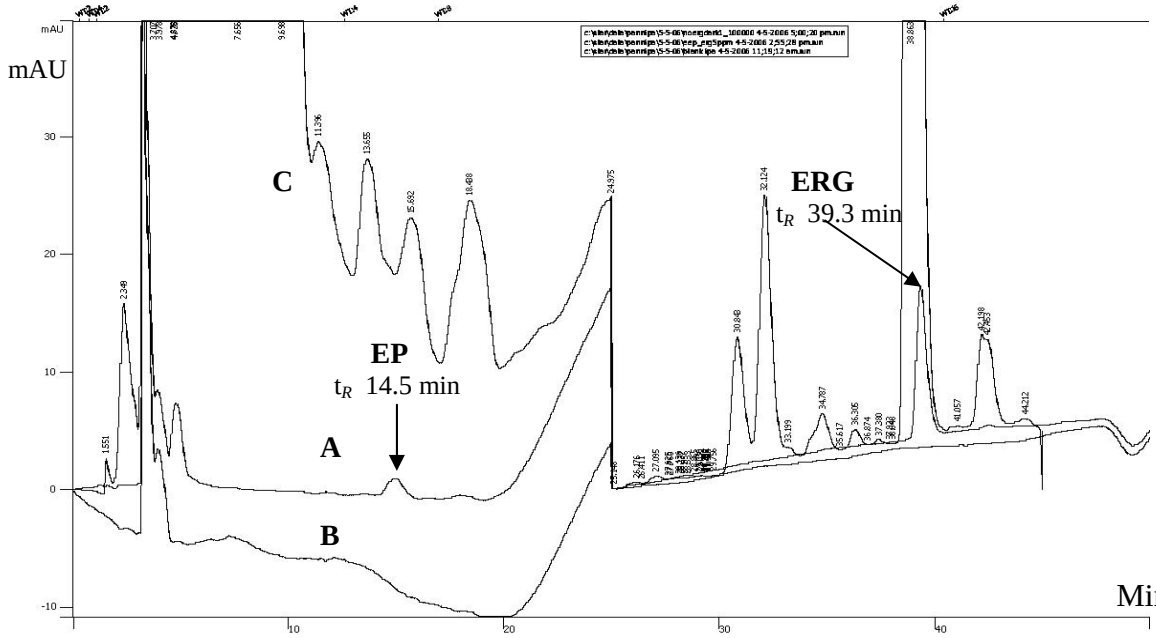


Figure 23 HPLC chromatogram of mixture of standards ergosterol peroxide (EP) and ergosterol (ERG) 5 ppm in isopropanol **(line A)** isopropanol 50 µl without EP and ERG **(line B)** and extract from *in vitro N. rileyi* mycelia **(line C)**. The column was eluted with solvent system #4. Compounds were detected under UV 210 nm (at 0 to 25 min) and 282 nm (at 26 to 50 min).

8.2 HPLC/ELSD analysis

ELSD chromatogram was presented in **Fig. 24** showing the separation of ergosterol peroxide and ergosterol at t_R 13.64 and 24.18 min respectively. It showed that peak signal that for ergosterol peroxide correlated to the injected concentration ergosterol. However, the precise amount of compounds could not be quantified due to the problem arising from noisy peaks as seen in the chromatogram.

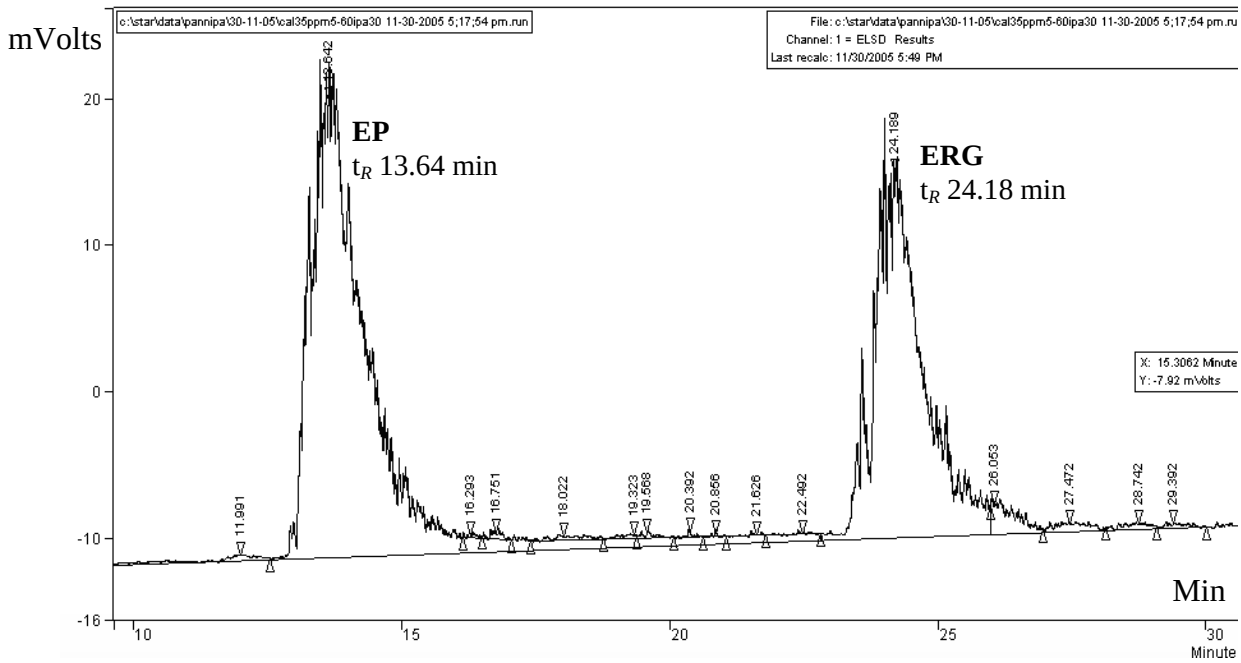


Figure 24 HPLC chromatogram of mixture of standards ergosterol peroxide (EP) and ergosterol (ERG). Compounds were detected by ELSD.

8.3 GC/MS analysis

For quantitative study by GC/MS, a selected ion monitoring (SIM) technique was employed to scan only certain ions due to presence of co-eluting compounds. Thus a small number of mass fragments unique for the particular compound were selected for comparison between suspected compound and the pure standard. The ion fragments selected for calculating the concentrations were m/z 429 and 397 for ergosterol peroxide and ergosterol respectively. The selected ion for internal standard, spinasterol, was m/z 397. The EIC (Extract Ion Chromatogram) for pure compounds were shown in **Fig. 25** and the results of standard compounds were summarized in **Table 12**. The criteria for identification of interested compound include the agreement of the retention times with those of standards and mass spectral confirmation.

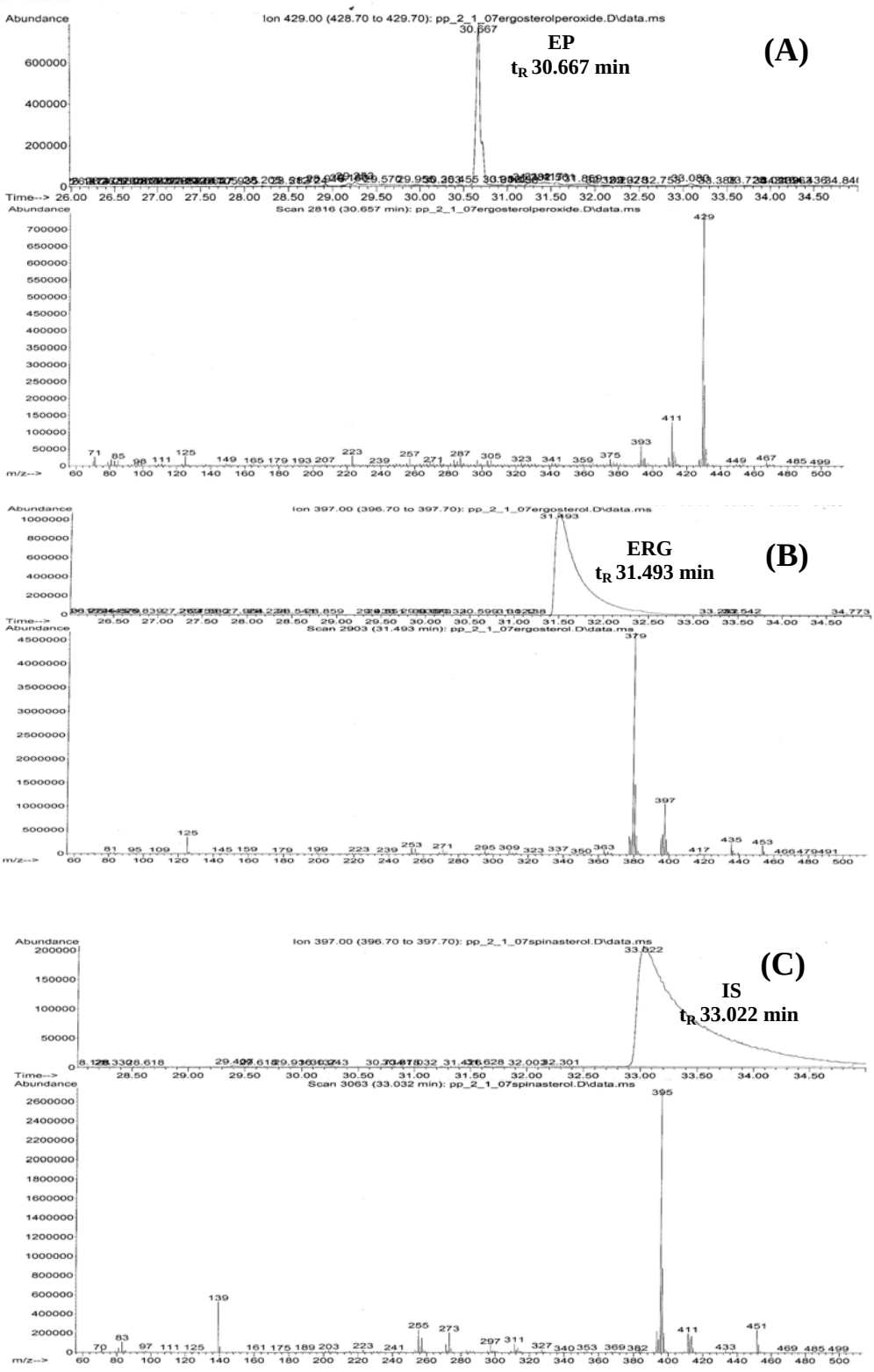


Figure 25 EIC (Extract Ion Chromatogram) of pure compounds from GC/MS showing mass spectrum of each compound Ergosterol peroxide (EP) 1 μg **(A)**, Ergosterol (ERG) 1 μg **(B)**, Spinasterol (using as internal standard, IS) 1 μg **(C)**. The mass selected was m/z 429 for detected EP and m/z 397 for detected ERG and IS.

Table 12 GC/MS analysis of pure compounds

Compound	Selected ion (m/z)	Identified ion (m/z)	Retention time (t_R, min)
Ergosterol peroxide	429	411, 429	30.67
Ergosterol	397	379, 397	31.49
Spinasterol (Internal standard)	397	395, 411	33.02

Samples of different extracts prepared from *N. rileyi* with *in vitro* and *in vivo* growth conditions (see **Table 13**) were analyzed by GC/MS to determine the amount of ergosterol peroxide and ergosterol.

8.3.1 *In vitro* fungal growth condition

The extracts of hyphal bodies and mycelia from *N. rileyi* cultured in SMBY medium were subjected to GC/MS analysis. **Fig. 26** and **Fig. 27** showed the EICs (Extract Ion Chromatogram) for the *in vitro* hyphal body extract and *in vitro* mycelia extract. It was shown that in both samples, peaks specific to ergosterol peroxide were detected at retention time of 30.65 and 30.64 min respectively but in both samples the peak due to ergosterol (31.49 min) was absent.

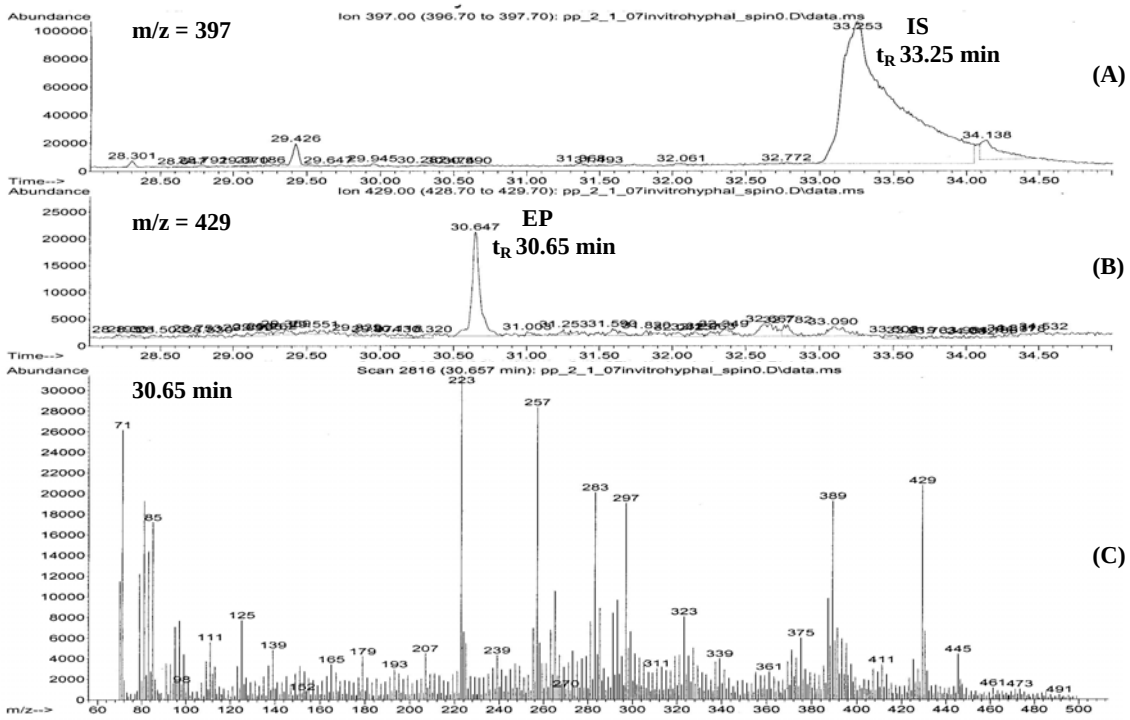


Figure 26 EICs of extract prepared from *in vitro* hyphal bodies**.** Selected ion record of m/z 397 showing internal standard's peak **(A)**, selected ion record of m/z 429 showing ergosterol peroxide's peak **(B)** and mass spectrum of compound at 30.65 min indicated ergosterol peroxide **(C)**.

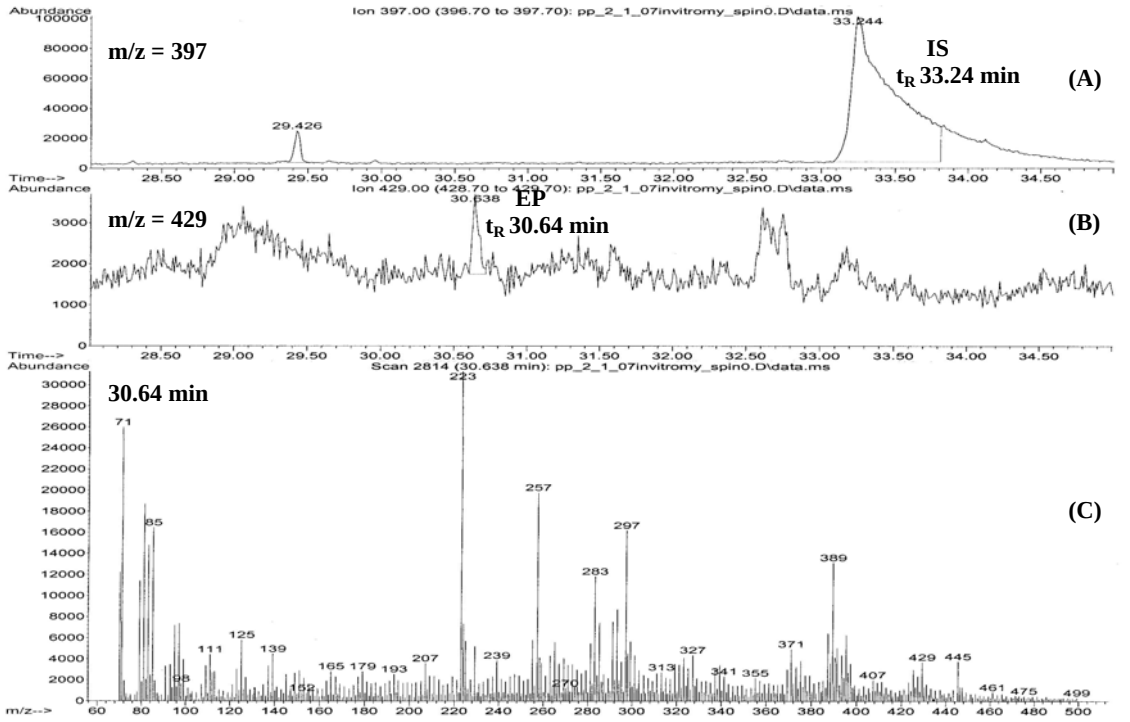


Figure 27 EICs of extract prepared from *in vitro* mycelia. Selected ion record of m/z 397 showing internal standard's peak **(A)**, selected ion record of m/z 429 showing ergosterol peroxide's peak **(B)** and mass spectrum of compound at 30.64 min indicated ergosterol peroxide **(C)**.

8.3.2 *In vivo* fungal growth condition

The extracts prepared from healthy insects, from dead insects and mycosed insects with white mycelial mat were analyzed by GC/MS to quantify amount of ergosterol peroxide. **Fig. 28** and **Fig. 29** showed the EICs (Extract Ion Chromatogram) of extracts from dead insects without and with white mycelial mat, respectively. Peaks responding to ergosterol and ergosterol peroxide were detected in both samples. In this study the extract prepared from healthy insects was subjected to GC/MS analysis and used as control. EIC of healthy insect extract was shown in **Fig. 30**. From this analysis peak of ergosterol peroxide was not be detected but peak due to ergosterol was found. The fact that ergosterol biosynthesis is unknown to occur in insects, suggests that the ergosterol detected in the extract was from components in the insect diet.

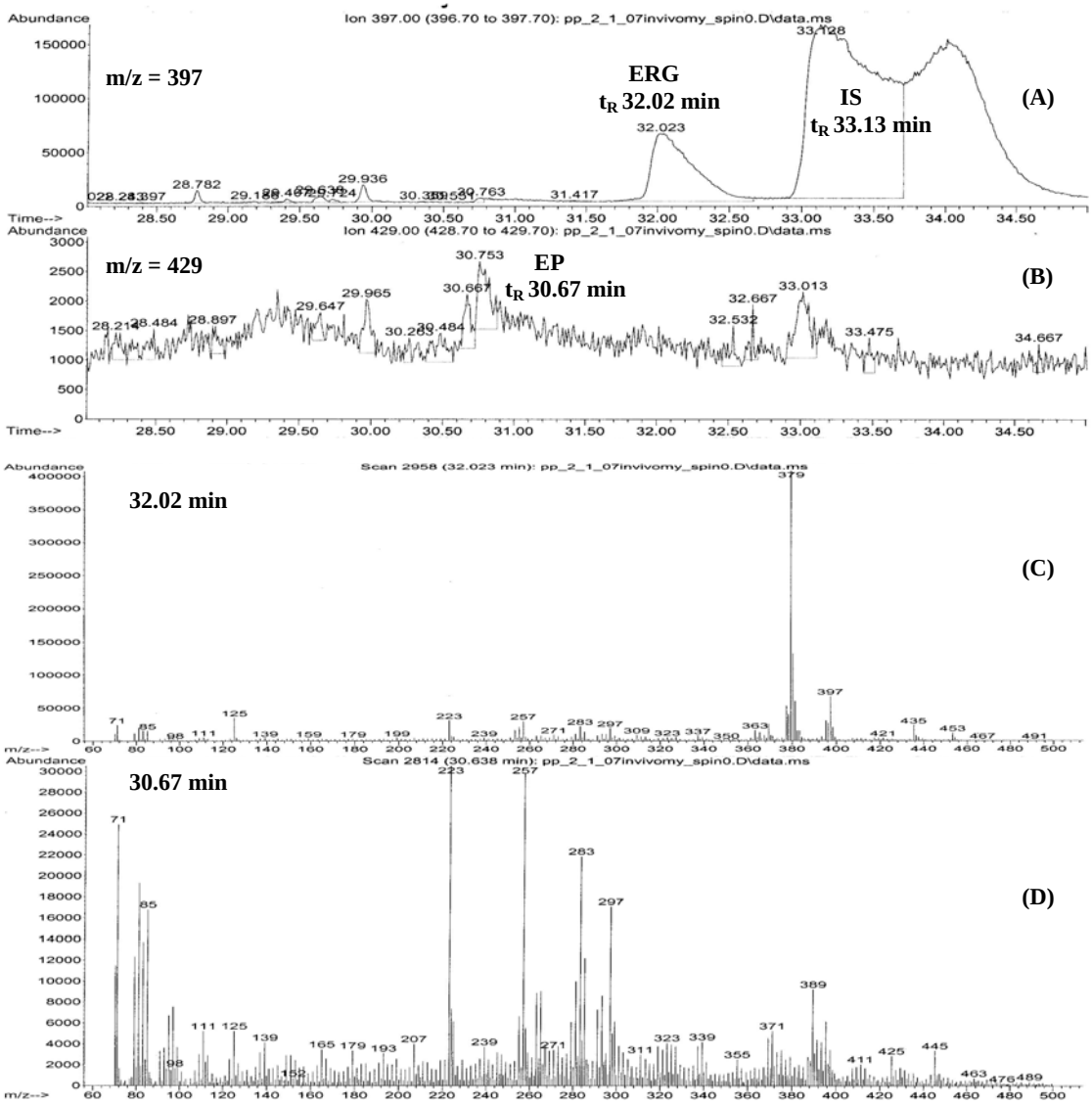


Figure 28 EICs of extract prepared from dead insects. Selected ion record of m/z 397 showing IS and ERG's peaks **(A)**, selected ion record of m/z 429 showing EP's peak **(B),** mass spectrum at 32.02 min indicated ergosterol (**C)** and mass spectrum at 30.67 min indicated ergosterol peroxide **(D)**.

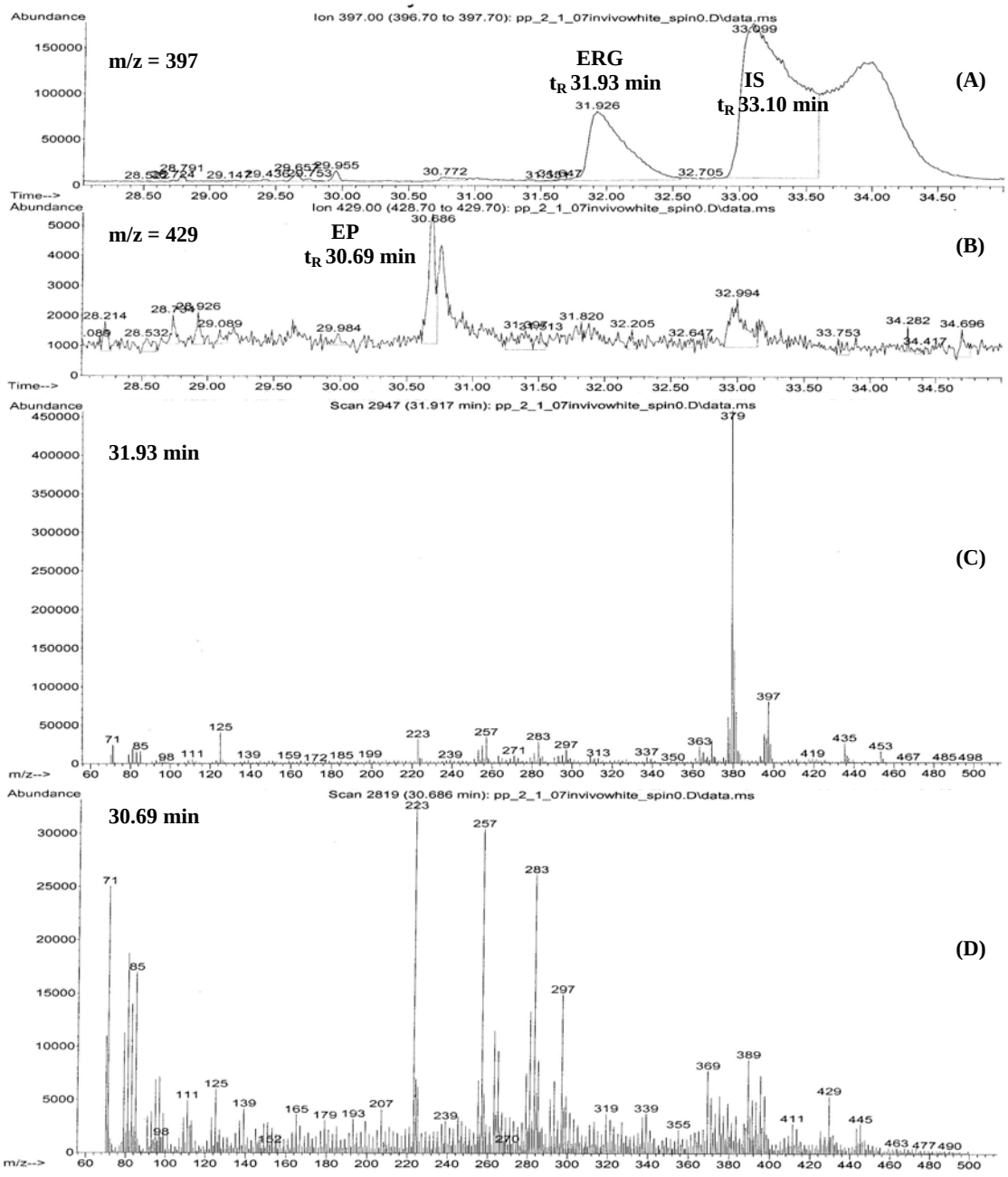


Figure 29 EICs of extract prepared from mycosed insects with white mycelial mat. Selected ion record of m/z 397 showing IS and ERG's peaks **(A),** selected ion record of m/z 429 showing EP's peak **(B)**, mass spectrum at 31.93 min indicated ergosterol **(C)** and mass spectrum at 30.69 min indicated ergosterol peroxide **(D)**.

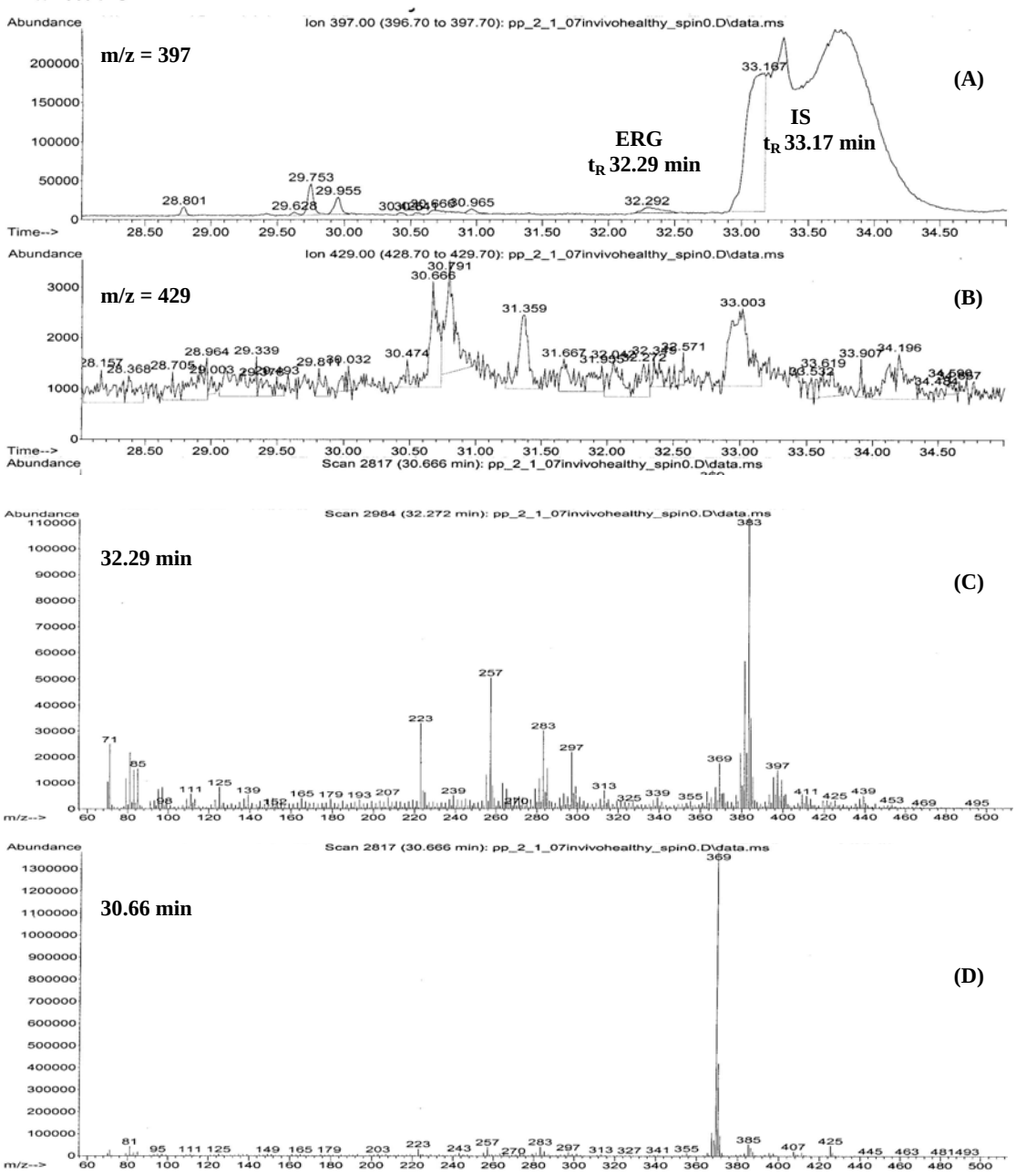


Figure 30 EICs of extract prepared from healthy insects. Selected ion record of m/z 397 showing IS and ERG's peaks **(A)**, selected ion record of m/z 429 **(B)**, mass spectrum at 32.29 min indicated ergosterol**(C)** and mass spectrum at 30.66 min indicated non-ergosterol peroxide **(D)**.

8.3.3 Control ergosterol

The structure of ergosterol peroxide indicated that it may be artifact due to photo-oxidation process of ergosterol. To test this hypothesis, ergosterol was subjected to the same methods that used to extract and prepare for GC/MS of fungal samples. The result showed that the peak that assigned for ergosterol peroxide could not be detected (**Fig. 31**).

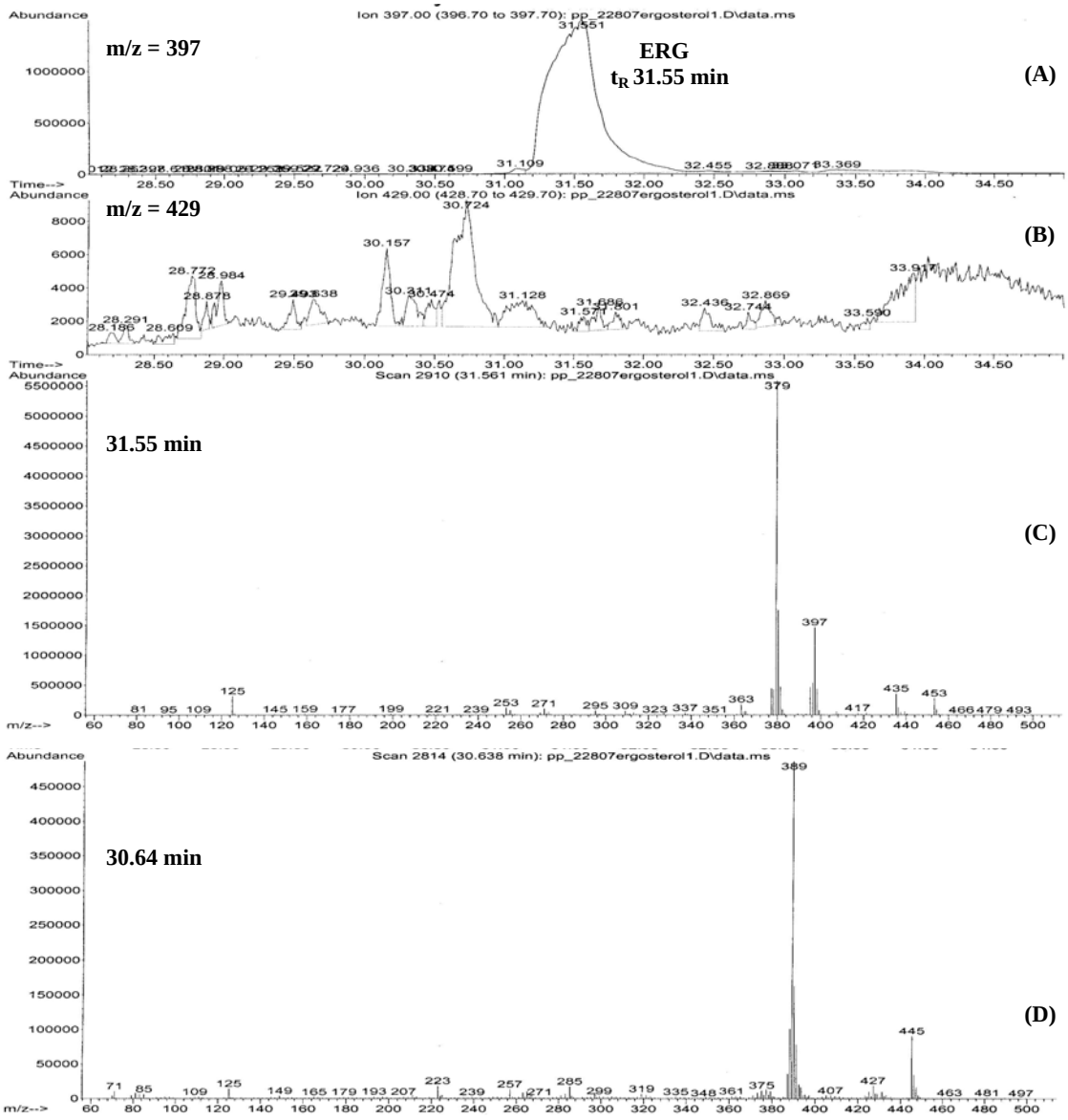


Figure 31 EICs of extract prepared from ergosterol. Selected ion record of m/z 397 showing ERG's peak **(A)** selected ion record of m/z 429 **(B)** mass spectrum at 31.55 min indicated ergosterol **(C)** and mass spectrum at 30.64 min indicated non-ergosterol peroxide **(D)**.

8.3.4 Quantitative analysis of compounds of interest

The concentration of interested compound in sample extract was obtained from the calculation of the peak area of selected ion from the following formula:

$$\text{Concentration in sample extract (ng/µl)} = [\text{Area of sample} \div \text{Area of internal std.}] \times \text{conc. of internal std.(ng/µl)}$$

Quantitative analysis of ergosterol peroxide and ergosterol determined in *in vitro* and *in vivo* fungal preparations were shown in **Table 13.** Extracts from mummified mycosed insects and from *in vitro* produced mycelia and hyphal bodies contained detectable levels of ergosterol peroxide 5.94, 3.93 and 5.14 µg/g dried weight, respectively whereas mycosed insects without external mycelia detected ergosterol peroxide at very low level (0.40 µg/g dried weight). Amounts of ergosterol peroxide detected *in vitro* cultures both hyphal bodies and mycelial phenotypes were not significantly different from *in vivo* mummified insects. Surprisingly, amounts of ergosterol detected in *in vivo* dead insect and *in vivo* mycosed insects (214.11 and 280.57 µg/ dried weight, respectively) were must higher that detected from healthy larvae (23.82 µg/ dried weight).

Table 13 Quantitative analysis of ergosterol peroxide (EP) and ergosterol (ERG) in various samples

Sample	**Compound**	**Rep. #**	**Peak area** (units)	**Conc. in extract** (ng/µl)	**Sample dried wt.** (g)	**Amount in sample** (µg)	**Amount in sample** (µg per g dried weight)	**Average** (µg per g dried weight)
1. *In vitro* hyphal bodies	IS*	1	2,504,823	500				
		2	1,462,576	500				
	EP	1	76,038	15.18	8.60	34.701	4.035	3.93
		2	91,401	31.24	8.67	33.114	3.819	
	ERG	1	ND**	ND	ND	ND	ND	ND
		2	ND	ND	ND	ND	ND	
2. *In vitro* mycelia	IS	1	1,911,758	500				
		2	1,277,911	500				
	EP	1	5,718	1.50	5.0	20.931	4.186	5.14
		2	8,836	3.46	6.489	39.513	6.089	
	ERG	1	ND	ND	ND	ND	ND	ND
		2	ND	ND	ND	ND	ND	
3. *In vivo* dead insect	IS	1	5,522,153	500				
		2	6,891,101	500				
	EP	1	3,268	0.30	10	6.007	0.601	0.40
		2	2,466	0.18	10	1.908	0.191	
	ERG	1	1,282,134	116.03	10	2,323.6	232.36	214.11
		2	2,547,535	184.70	10	1,958.6	195.86	
4. *In vivo* mycosed insect with white mycelial mat	IS	1	4,654,892	500				
		2	6,891,101	500				
	EP	1	16,410	1.76	10	45.771	4.577	5.923
		2	74,790	5.42	6.234	45.31	7.268	
	ERG	1	1,456,444	156.42	10	4,067.9	406.79	280.57
		2	1,586,315	115.10	6.234	962.24	154.35	
5. Healthy insects	IS	1	2,758,777	500				
		2	3,965,289	500				
	EP	1	ND	ND	ND	ND	ND	ND
		2	ND	ND	ND	ND	ND	
	ERG	1	127,258	23.06	10	403.92	40.392	23.82
		2	49,878	6.28	10	72.47	7.247	

***** IS indicated Internal Standard.

***** * ND indicated no responded peak was detected by this method.

9. Anticancer activity of ergosterol peroxide

The anticancer activity of ergosterol peroxide was determined against the following cancer cell lines; hormone-dependent breast cancer cell line (T47D), hormone-independent breast cancer cell line (MDA-MB-231), human epidermoid carcinoma in mouth cell line (KB), human cervical carcinoma cell line (HeLa), lung cancer cell line (H69AR) and human cholangiocarcinoma cell line (HuCCA-1). Ergosterol peroxide was found to be most active against T47D cells having an IC_{50} value of 5.8 μM. The IC_{50} values against MDA-MB-231, KB, HeLa, H69AR and HuCCA-1 cell lines were calculated to be 28.0, 46.7, 58.4, 65.4 and 105.1 μM, respectively (**Table 14**).

Table 14 Cytotoxic activity of ergosterol peroxide against cancer cell lines

IC_{50}	**Cancer cell lines***					
	T47D	**MDA-MB-231**	**KB**	**HeLA**	**H69AR**	**HuCCA-1**
μg/ml	2.5	12	20	25	28	45
μM	5.8	28.0	46.7	58.4	65.4	105.1

* T47D : hormone-dependent breast cancer cell line
MDA-MB-231 **:** hormone-independent breast cancer cell line
KB **:** human epidermoid carcinoma in mouth cell line
HeLA **:** human cervical carcinoma cell line
H69AR **:** lung cancer cell line
HuCCA-1 **:** human cholangiocarcinoma cell line

10. Anti-HIV-1 activities of ergosterol peroxide as determined by syncytium reduction assay

10.1 Enzyme inhibiting activity with Autodock computer program

Autodock program was used to evaluate the binding free energies of ergosterol peroxide into the target macromolecule such as HIV Reverse transcriptase, HIV protease and DNA-topoisomerase 1. The docking energies were calculated from a set of energy grids centered in the active site of the enzymes. The parameters for autogrid were listed in **Table 15**.

Table 15 Parameters for autogrid experiment for Autodock analysis.

Parameter	Reverse transcriptase	Protease	DNA-topoisomerase1
Macromolecule	1 vrt Resolution = 2.20	1 hsg Resolution = 2.00	1 t8i Resolution = 3.00
Num. Grid point in n, y, z	40, 40, 40	40, 40, 40	60, 50, 30
Spacing (Å)	0.375	0.375	0.375
Grid center	Center on ligand	Center on ligand	Center on ligand
Smooth	0.5	0.5	0.5

Table 16 The best docking results based on the binding free energies (see more results in **Appendix C**).

Docking for Reverse transcriptase enzyme			
Ligand	rsd	Binding energy (kcal/mol)	Docking energy (kcal/mol)
Nevirapine (validate)	0.6630	-9.14	-9.32
Ergosterol peroxide 20S, 24R		-11.2	-10.1
Ergosterol peroxide 20R, 24R		**-10.6**	**-9.74**
Docking for HIV Protease enzyme			
Ligand	rsd	Binding energy (kcal/mol)	Docking energy (kcal/mol)
Indinavir (validate)	0.4230	-14.2	-18.6
Ergosterol peroxide 20S, 24R		-12.3	-12.2
Ergosterol peroxide 20R, 24R		**-12.1**	**-12.3**
Docking for DNA-Topoisomerase 1			
Ligand	rsd	Binding energy (kcal/mol)	Docking energy (kcal/mol)
Camptothecin (validate)	0.4750	-14.2	-14.5
Ergosterol peroxide 20S, 24R		-11.0	-9.29
Ergosterol peroxide 20R, 24R		**-8.73**	**-9.45**

The validation of the docking accuracy was done by docking the ligand of nevirapine into binding site of reverse transcriptase enzyme, indinavir into binding site of HIV protease and camptothecin into binding site of DNA-topoisomerase 1. The docked ligands imposed on the nevirapine, indinavir and camptothecin to correlated enzymes with binding free energies (ΔG_b) of -9.14, -14.2 and -14.2 kcal/mol respectively.

The binding affinity was evaluated by the binding free energies (ΔG_b, kcal/mol). The enzyme, which revealed the highest binding affinities (in other words, lowest binding free energies) within ergosterol peroxide (20R, 24R) presented in **Table 16**. The docking results of enzymes reverse transcriptase and HIV protease

exhibited binding free energies of -10.6 and -12.1 kcal/mol respectively which was lower than each validating drug. Therefore, ergosterol peroxide should be able to bind at binding sites of reverse transcriptase and HIV protease and possibly correlated to anti-HIV activity.

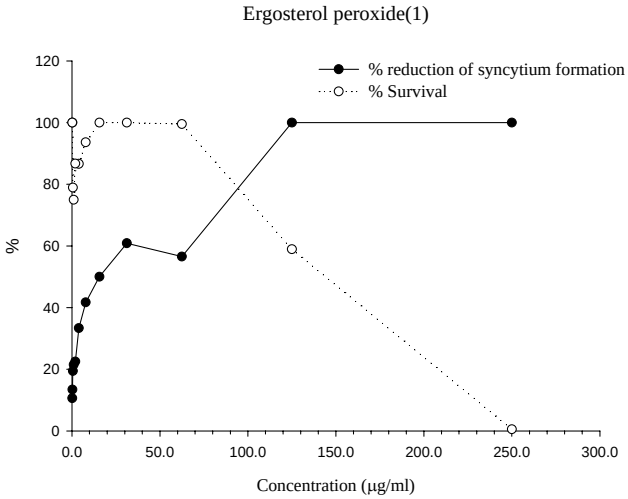
10.2 Anti-HIV-1 activity of ergosterol peroxide determined by syncytium reduction assay

This experiment was performed to determine syncytium reduction assay for detecting HIV-1 inhibitory activity of ergosterol peroxide. AZT was also tested staring at 10^{-2} μM or 2.67 ng/ml as a positive control. Data from duplicate experiments, shown in **Table 17** and **Figure 32**, indicated the ergosterol peroxide potentially possessed HIV-1 inhibitory activity with no cytotoxic effect to 1A2 cells. The average effective concentrations of ergosterol peroxide that exhibited that reduced syncytium formation (EC_{50}) and that inhibited metabolic activities (cell death, IC_{50}) were determined as 15.96 and 150.77 μg/ml. Ergosterol peroxide with a calculated therapeutic index ($TI = IC_{50}/EC_{50}$) of 9.45 is considered to be a potential active HIV-1 inhibitor compound.

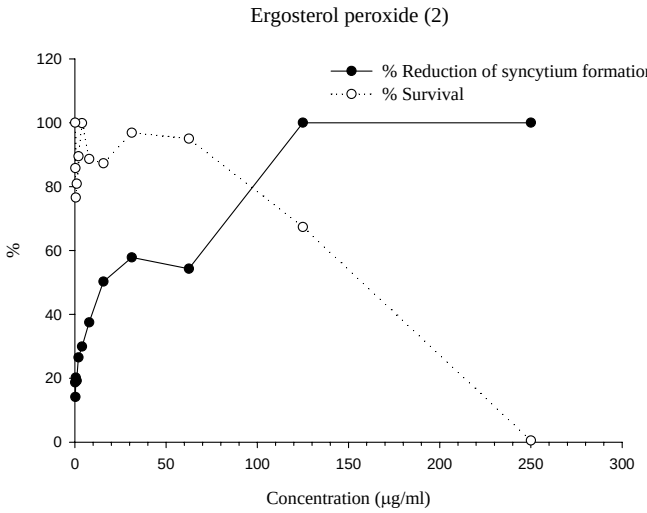
Table 17 Cytotoxic and Anti-HIV-1 activities of ergosterol peroxide as determined by syncytium reduction assay.

Compound	**Exp.**	**IC_{50} (μg/ml)**	**EC_{50} (Syncytium reduction) (μg/ml)**	**Therapeutic index (TI)**
Ergosterol peroxide	1	144.07	14.66	9.83
	2	157.46	17.25	9.13
	average	**150.77**	**15.96**	**9.45**
AZT (positive control)	1	$>2.67 \times 10^{-3}$	1.83	$>6.85 \times 10^{4}$
	2	$>2.67 \times 10^{-3}$	2.39	$>8.95 \times 10^{4}$

A.



B.



C.

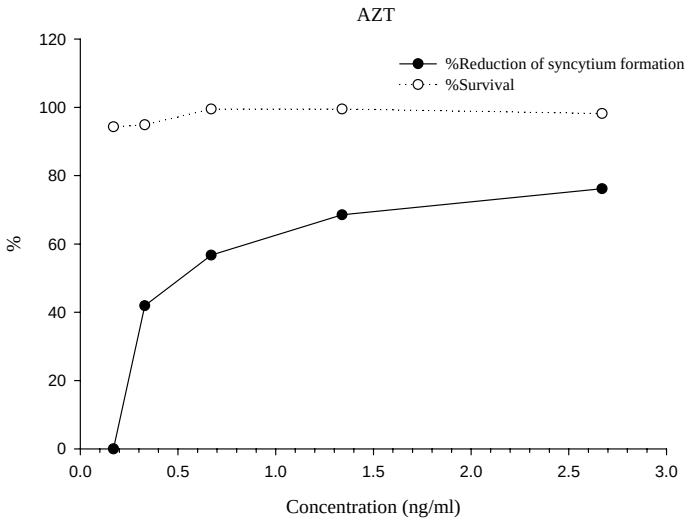


Figure 32 Effect of ergosterol peroxide(A and B) and AZT(C) on survival of uninfected cells and formation of syncytium by $^{\Delta Tat/Rev}$ MC99-infected 1A2 cells, as determined by syncytium reduction assays. Results were expressed as % survival of uninfected cells(o) and % reduction of syncytium formation by infected cells, in presence of inhibitor (•).

11. Effect of ergosterol peroxide toward *S. litura* larvae via topical and *per os* applications

Toxicity of the synthetic ergosterol peroxide compound was evaluated for its toxic activity against *S. litura* larvae via both topical and *per os* application methods. It was found that applying ergosterol peroxide (75-135 μg) on larval cuticle caused approximately 40% mortality at 7 day post-exposure which was significantly greater then control treatment (**Table 18**). Response of insect's survival to ergosterol peroxide was found to lack of dose-response correlation after topical application. The majority of the observed mortality was observed within 24 h of post treatment as shown in **Fig. 33**.

Table 18 Mean percentage mortality to *S. litura* larvae after topical application of ergosterol peroxide (EP) in Tween20:EtOH (1:1) at different concentrations

EP (μg/insect)	**Mean % mortality ± SD in days***			
	1 days	**3 days**	**5 days**	**7 days****
Control***	0	0	0	0 *a*
60	23.3 ± 15.3	30.0 ± 10.0	30.0± 10.0	30.0 ± 10.0 *b*
75	36.7 ± 5.8	36.7 ± 5.8	40.0± 0	43.3 ± 5.8 *b*
90	40.0 ± 10.0	46.7 ± 15.3	46.7± 15.3	46.7 ± 15.3 *b*
105	36.7 ± 15.3	43.3 ± 5.8	43.3± 5.8	46.7 ± 11.5 *b*
120	40.0 ± 0.0	43.3 ± 5.8	43.3 ± 5.8	46.7 ± 11.5 *b*
135	36.7 ± 11.5	36.7 ± 11.5	40.0 ± 10.0	43.3 ± 11.5 *b*

* Treatment means based on three replicates; each replicate represents 10 larvae.

** Means followed by the same letter in vertical column are not significantly different at 0.05% level of probability using Tukey HSD post-hoc test.

*** Control represented *S. litura* larvae treated with solvent (Tween20:EtOH) without ergosterol peroxide.

Figure 33 Photograph of dead *S.l itura* after topical applied with solution of ergosterol peroxide in Tween20:EtOH (1:1) on Day 1 postexposure.

Per os application of various concentrations of ergosterol peroxide caused no insect mortality. However, feeding insects ergosterol peroxide did interfere with larval development. Insects fed ergosterol peroxide at concentrations 60, 75, 120 and 135 μg/insect gained significantly less weight then control insects over the seven-day observation period (**Table 19**). The decreasing of mean weight responded to ergosterol peroxide post *per os* was not correlated to the dose. The total weight of insects treated with 60, 75, 120 and 135 μg/insect were 54-56% of the untreated controls whereas treatments of 90 and 105 μg/insect gained 83-89% of the control larvae.

Table 19 Mean *S. litura* larvae weight after treated with ergosterol peroxide in Tween20:EtOH (1:1) via *per os* application

EP (μg/insect)	Mean weight ± SD in days[*,**]			
	1	**3**	**5**	**7**
Control	16.5 ± 4.2 (100%)*a*	31.1 ± 6.1 (100%)*a*	197.9 ± 66.2 (100%)*a*	291.0 ± 122.0 (100%)*a*
60	12.9 ± 2.7 (78.2%)*b,c*	30.5 ± 7.2 (98.1%)*a*	99.7 ± 70.3 (50.4%)*b,c*	165.5 ± 127.5 (56.9%)*b,c*
75	13.5 ± 3.2 (81.8%)*b,c*	25.4 ± 9.2 (81.7%)*a*	91.7 ± 76.3 (46.3%)*b,c*	163.7 ± 137.4 (56.3%)*b,c*
90	15.0 ± 3.7 (90.9%)*a,c*	50.9 ± 27.5 (163.7%)*b*	147.6 ± 89.1 (74.6%)*a,c*	260.1 ± 183.9 (89.4%)*a,c*
105	14.7 ± 3.4 (89.1%)*a,c*	44.8 ± 22.2 (144.1%)*b*	120.42 ± 85.0 (60.8%)*b,c*	241.7± 179.0 (83.1%)*a,c*
120	15.4 ± 2.4 (93.3%)*a,c*	33.2 ± 18.1 (106.8%)*a,b*	86.9 ± 38.5 (43.9%)*b*	157.4 ± 112.5 (54.1%)*b,c*
135	13.4 ± 3.1 (81.2%)*b,c*	31.7 ± 22.3 (101.9%)*a,b*	95.5 ± 45.3 (48.3%)*b,c*	163.0 ± 101.6 (56.0%)*b,c*

***** Larval weight's means based on three replicates; each replicate represents 10 larvae. Means followed by the same letter in vertical column are not significantly different at 0.05% level of probability according to Tukey HSD and Games-Howell post-hoc tests.

** Values in parentheses shows insect weights expressed as % of control insect weight.

12. Effect of ergosterol peroxide toward *Heliothis virescens* larvae via injection

In this experiment, effect of ergosterol peroxide toward insects via injection was studied. Ergosterol peroxide was injected into the hemocoel of 3rd to 4th instar *H. virescens* larvae. To prepare ergosterol peroxide preparations solubility ergosterol peroxide in various solvents were tested as shown in **Table 20**. It was found that ergosterol peroxide at the level of 10 mg was readily dissolved in 1 ml of following solvents, 100% EtOH, 100%DMSO, ethyl acetate, sesame oil and mixture of 100% tween80 and 100% EtOH (1:1). Due to insolubility of the compound in water, formulations of ergosterol peroxide were formulated including ergosterol peroxide: PVP complex and liposomal preparation as described previously. Ergosterol peroxide's preparations were injected into 3th instar *H. virescens* and recorded insect survival daily for 7 days. Results in **Table 21** indicated that ergosterol peroxide:PVP complex and the ergosterol peroxide within entrapped liposome in aqueous solutions did not cause significant insect death when compare with controls. Ergosterol peroxide prepared in 100% EtOH, 100% DMSO, sesame oil and mixture of tween 80:100% ethanol at concentration 20 mg/ml were injected into 4th instar *H. virescens*. Results in **Table 22** shows that almost all of the solvent controls had survived rates comparable to uninjected larvae. The exception was ethyl acetate control which was highly toxic to treated larvae. No significant number of insect deaths was found after injected with ergosterol peroxide prepared in these solvents (see **Table 22**) at concentration of 50 μg/insect.

Table 20 Solubility of ergosterol peroxide in various solvents at concentration of 10 mg/ml

Solvent	**Solubility of ergosterol peroxide (10 mg/ml)**
1. 100% EtOH	dissolved
2. 100% DMSO	dissolved
3. Ethyl acetate	dissolved
4. Mineral oil	not dissolved
5. Sesame oil	dissolved
6. Tween 20	not dissolved
7. Tween 80	not dissolved
8. Tween80:100% EtOH (1:1)	dissolved

Table 21 *In vivo* toxicity of ergosterol peroxide (EP) preparations on 3th instar *H. virescens* (average insect's wt. 60-110 mg) at 7 days post injected with 2.5 μl/insect of test samples.

Sample	Solution	Dose (μg/insect)	%Mortality at day 7 (N=6)
1. EP: PVP (1:9)	1%tween 20 in H_2O	25	0
2. Ergosterol: PVP (1:9)		25	0
3. PVP (Control)		0	0
4. EP: PVP (1:9)	1%tween 80 in H_2O	25	0
5. Ergosterol: PVP (1:9)		25	0
6. PVP (Control)		0	0
7. EP-Liposome preparation	Phosphate buffer saline	0	17
8. Liposome without EP		25	17

Table 22 *In vivo* toxicity of ergosterol peroxide toward 4th instar *H. virescens* (average insect's wt.180-300 mg) at 7 days post injected with 2.5 μl/insect of test samples.

Sample		Solvent	Dose (μg/insect)	Replication	%Mortality at day 7 (N=6)
1	Ergosterol peroxide	100% EtOH	50	1	0
				2	33
	Control	100% EtOH	-		0
2	Ergosterol peroxide	100% DMSO	50	1	33
				2	0
	Control	100% DMSO	-		0
3	Ergosterol peroxide	Sesame oil	50	1	0
				2	0
	Control	Sesame oil	-		0
4	Ergosterol peroxide	Tween80: EtOH (1:1)	50	1	0
				2	0
	Control		-		0
5	Control	Ethyl acetate	-		100

13 Effect of ergosterol peroxide on cellular immune response of *H. virescens*

As the assumption that ergosterol peroxide molecules probably presented on fungal cells as a membrane bound metabolite. The inhibition of phagocytic activity from ergosterol peroxide was subjected to study. To develop method for phagocytic activity evaluation, 3rd instar *H. virescens* larvae (wt. 80-110 mg) were injected with low and high concentration of fluorescent beads, Fluoresbrite® (dia.1.79 μm), and numbers of ingested hemocyte cells were counted under fluorescence microscope. The relative degree of phagocytosis was dependent on the concentration of material being injected into the test insect. For example, insects injected with the high concentration of florescent latex beads (1.75×10^6 beads/insect) had 26% of their hemocytes recoded as being phagocytic, whereas, insects injected with a low concentration (1.75×10^5 beads/insect) had only a13% phagocytic index at 4 hr post injection.

Since ergosterol peroxide is believed to be present as a membrane-bound sterol fungal cells it was decided that injection of ergosterol peroxide-coated fluorescent latex beads would be a realistic more realistic then soluble EP preparation. Coated beads containing 0.023 ng EP/bead (5mg/ 2.15×10^8 beads) were injected into the hemocoel of test larvae. The concentration of ergosterol peroxide coated on the bead was calculated as 27.9 μg/insect. Photographs of phagocytic hemocytes were displayed in **Fig. 34**. Result in **Table 24** showed the significant differences of percentage of phagocytic hemocytes between ergosterol peroxide-coated and non-coated beads treatments. Treatments with ergosterol peroxide-coated beads were found to induce increased phagocytic activity of larval hemocytes comparing with non-coated bead treatment. At 4 hr post-injection percentage of phagocytic hemocytes in coated-beads treatment was almost 2.5 times less than control of non-coated treatment. However, clumps of phagocytic hemocytes were observed in ergosterol peroxide-coated treatment (see **Fig. 34C**). The aggregation of phagocytic cells was probably the cause for observed reduction of free phagocytic cells.

Table 23 Phagocytic activity of hemocytes after *H. virescens* larvae were injected with fluorescent latex beads after 4, 6 and 24 hr post injection.

Treatment	Concentration (beads/insect)	Time post-injected (hr)	Hemocytes count ± SD ($x10^7$ cells/ml)	Number of phagocytic cells (total 100 cells) ± SD*	% Phagocytic hemocytes
Non-coated beads (in saline)	1.75×10^5 (low)	4	2.72 ± 0.78	13.5 ± 3.5	13.5 *a,c***
		6	2.38 ± 0.61	11.0 ± 2.0	11 *a*
		24	2.86 ± 0.39	9.2 ± 3.42	9.2 *a*
	1.75×10^6 (high)	4	3.04 ±0.82	25.8 ± 23.34	25.8 *b,c*
		6	1.99 ± 0.43	34.5 ± 5.26	34.5 *b*
		24	4.23 ± 0.81	25.5 ± 11.21	25.5 *b,c*

* At least 4 larvae were used for each treatment.

** Values followed by the same letter in vertical column are not significantly different at 0.05% level of probability according to Gabriel's post hoc test.

Table 24 Phagocytic activity of hemocytes after *H. virescens* larvae were injected with ergosterol peroxide (EP)-coated fluorescent latex beads at concentration of 27.9 μg/insect after 4 and 24 hr post injection.

Treatment	Concentration (beads/insect)	Time post-injected (hr)	Hemocytes count ± SD ($x10^7$ cells/ml)	Number of phagocytic cells (total 100 cells) ± SD*	% Phagocytic hemocytes
EP-coated beads (in 1%Tween 20)	1.20×10^6	4	1.05 ± 0.64	19.6 ± 11.1	19.6 *a***
		24	1.59 ± 0.64	21.5 ± 4.93	21.5 *a*
Non-coated beads (in 1%Tween 20)	1.33×10^6	4	1.67 ± 1.03	53.5 ± 16.98	53.5 *b*
		24	1.35 ± 0.26	38.5 ± 14.27	38.5 *a,b*

Note: Cells aggregation of phagocytic hemocytes was observed in treatment of EP-coated beads injected.

* At least 4 larvae were used for each treatment.

** Values followed by the same letter in vertical column are not significantly different at 0.05% level of probability according to Gabriel's post hoc test.

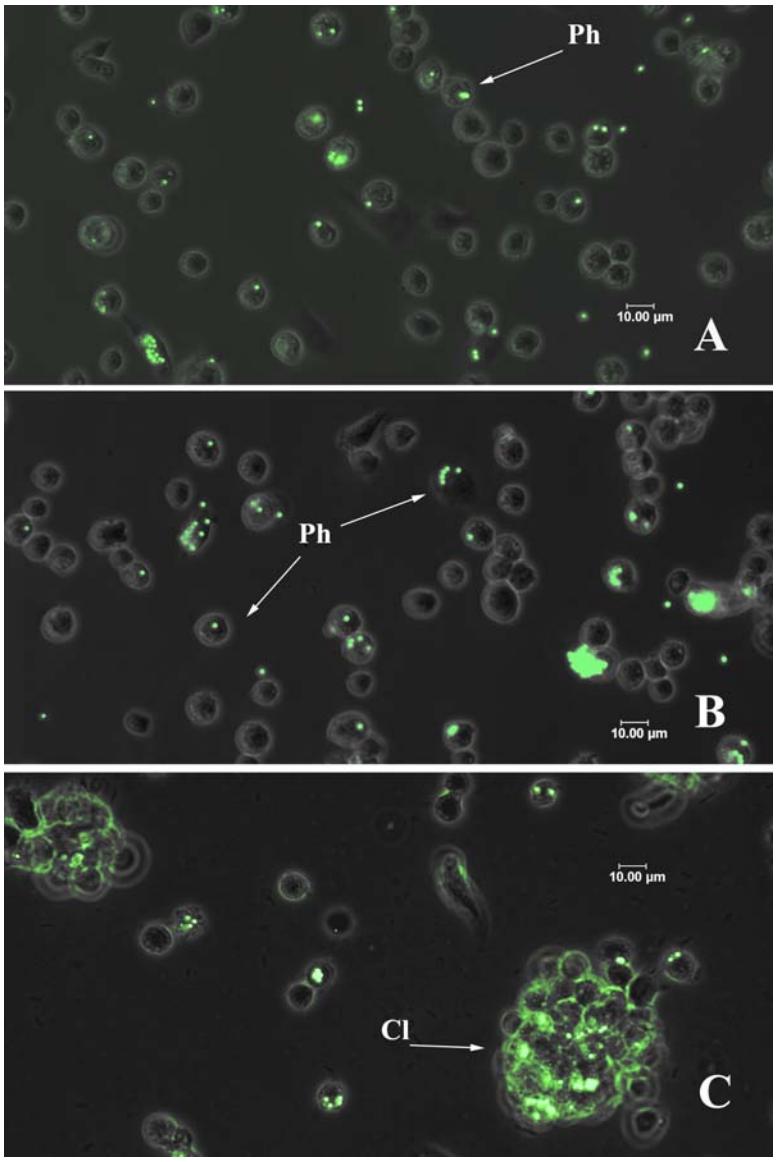


Figure 34 Phagocytosis of hemocytes at 24 hr post injection of preparation of ergosterol peroxide-coated beads **(A)**, control (non-coated beads) **(B)** and clumping of phagocytic hemocytes observed in larvae injected with ergosterol peroxide-coated beads **(C)**. The arrows indicated "Ph" as phagocytic hemocytes with ingested fluorescent beads and "Cl" as phagocytic hemocytes clumping.

14. Effects of *N. rileyi* toward *H. virescens* in comparison with entomopathogenic fungus *Paecilomyces fumosoroseus*

14.1 Virulence of *N. rileyi* and *P. fumosoroseus* toward *H. virescens*

In this experiment, hyphal bodies of *N. rileyi* and blastospores of *P. fumosoroseus* were prepared in sterile water and injected into 3rd instar *H. virescens*. Various concentrations were applied and recorded for their mortality daily for 8 days. The LC_{50} and LT_{50} values were calculated by probit analysis. Results in **Table 25** and **26** showed %mortality responses of *H. virescens* after injected with *N. rileyi* and *P. fumosoroseus.* LC_{50} and LT_{50} of were determined as 4.65×10^2 cells/ml and 4.87 days for *N. rileyi* and 1.35×10^3 cells/ml and 2.88 days for *P. fumosoroseus.*

Table 25 Concentration and mortality responses of *H. virescens* larvae to *N. rileyi* hyphal bodies and *P. fumosoroseus* blastospores. LC_{50} values were calculated by probit analysis.

Treatment	Concentration (cells/ insect)	No. Dead (N=10)	% Mortality	**LC_{50} (cells/ insect)**
N. rileyi hyphal bodies	7×10^5	10	100	**4.65×10^2**
	7×10^4	10	100	
	7×10^3	10	100	
	7×10^2	8	80	
	7×10	1	10	
	7	0	0	
P.fumosoroseus blastospores	1.13×10^7	10	100	**1.35×10^3**
	1.13×10^6	10	100	
	1.13×10^5	10	100	
	1.13×10^4	10	100	
	1.13×10^3	5	50	
	1.13×10^2	3	30	

Table 26 The mortality responses of *H. virescens* after injected with *N. rileyi* hyphal bodies and *P. fumosoroseus* blastospore. LT_{50} values were calculated by probit analysis.

Sample	Conc. (cells/ insect)	Exp. No.	% Mortality at days								**LT_{50} (days)**	**Ave. LT_{50}**
			1	2	3	4	5	6	7	8		
N. rileyi hyphal bodies	1.5 x 10^4	1 (N=30)*	0	0	0	58.6	75.9	79.3	82.8	86.2	**4.78**	**4.87**
		2 (N=30)	0	0	0	36.7	80	83.3	83.3	83.3	**4.95**	
P.fumos oroseus blasto-spores	1.5 x 10^4	1 (N=30)	0	3.3	43.3	90	90	90	90	90	**2.58**	**2.88**
		2 (N=10)	0	0	30	100	100	100	100	100	**3.18**	

* indicated total number of tested insects.

14.2 Effects of *N. rileyi* and *P. fumosoroseus* on cellular response of *H. virescens*

The numbers of hemocytes, fungal cells and spreading hemocytes post challenge with *N. rileyi* hyphal bodies and *P. fumosoroseus* blastospores were showed in **Table 27**. Number of hemocytes and *N. rileyi* hyphal bodies and *P. fumosoroseus* blastospores post challenge were plotted in **Figure 35**. At 2 day post challenge, the number of *P. fumosoroseus* blastospores was found to increase at levels higher than *N. rileyi* hyphal bodies. However after 3 days *P. fumosoroseus* treated larvae were dead. In *N. rileyi* injected larval hemolymph, the hyphal bodies replicated extensively and reached to the highest number at day 4. It was found that *N. rileyi* population in mycosed larvae was approximately 10 times higher than *P. fumosoroseus* population at day 4. While the total hemocytes were relatively constant comparing with control hemocytes.

Spreading ability of *P. fumosoroseus* infected larvae declined during infection which was correlated to the increase in the growth of *P. fumosoroseus* in hemolymph (**Fig. 36**). For *N. riley* hyphal bodies challenged larvae, suppression of hemocyte spreading was not observed until day 4 post injection when *N. rilyi* hyphal bodies were present in large numbers in hemolymph. **Figure 37** showed morphology of spreading

hemocytes dual stained with fluorescien isothiocyanate (FITC) conjugated phalloidin and 4', 6'-diamidino-2-phenylindole (DAPI). The morphology of normal hemocytes after 1 h incubation on glass slide were exhibited the attachment and spreading as shown in **Figure 37 A** and **B**. The ability of spreading of hemocytes obtained from 2 day post injection of *N. rileyi* blastospores insect was not changed (**Fig. 37C**). While the effect on hemocytes spreading and attachment activity was observed in 2 day post injection with *P. fumosoroseus* blastospores (**Fig. 37D**).

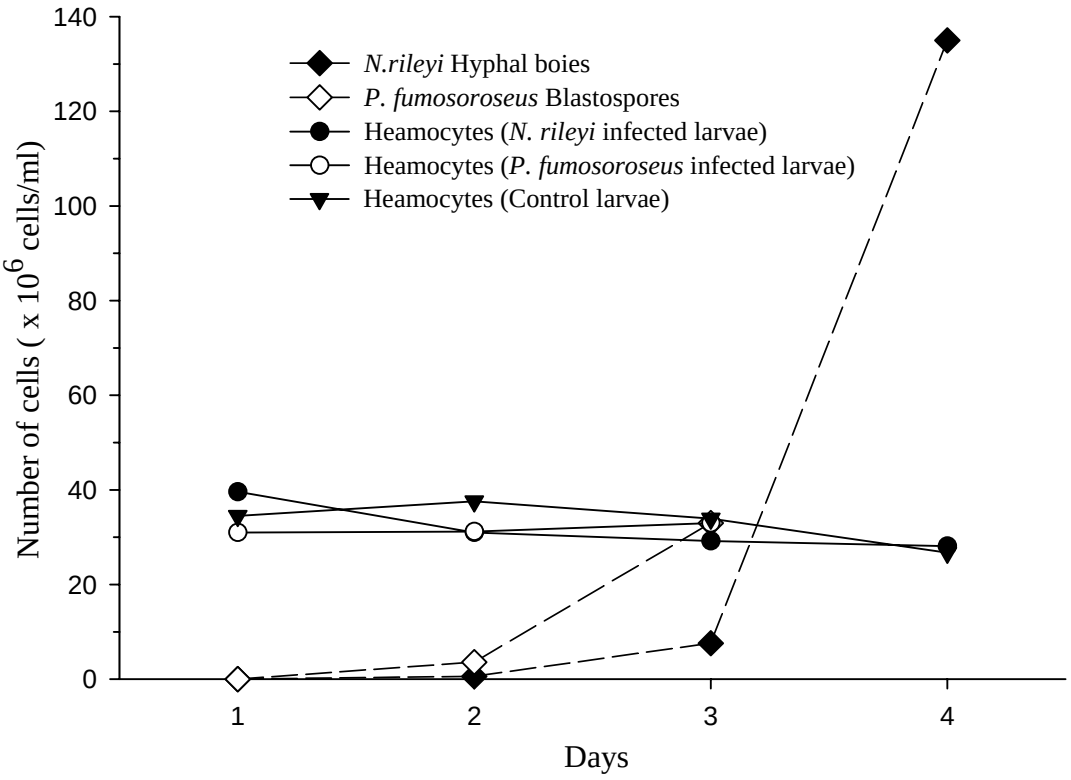


Figure 35 Growth of fungus in hemolymph post challenge with *N. rileyi* hyphal bodies (◆) and *P. fumosoroseus* blastospores (◇) at concentration of 1.5 x 10^4 cells/insect. Number of hemocytes in *N. rileyi* infected larvae (●), *P. fumosoroseus* infected larvae (○) and control larvae (▲). **Dashed lines (- - -)** were assigned for numbers of fungal cells and **solid lines (——)** were assigned for numbers of hemocytes in treated insect.

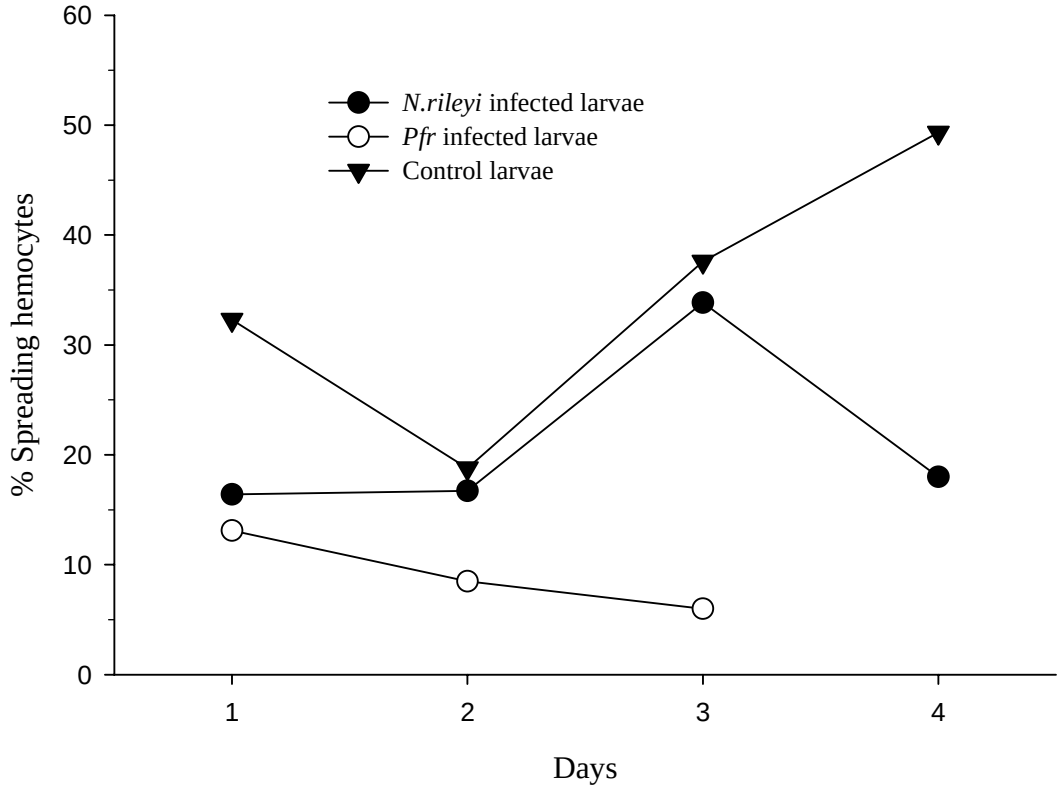


Figure 36 Percentage of spreading hemocytes from *H. virescens* larvae challenged with *N. rileyi* (●), *P. fumosoroseus* (○) and control larvae (▲).The total number of spreading hemocytes were counted in 5 fields under phase contrast microscope.

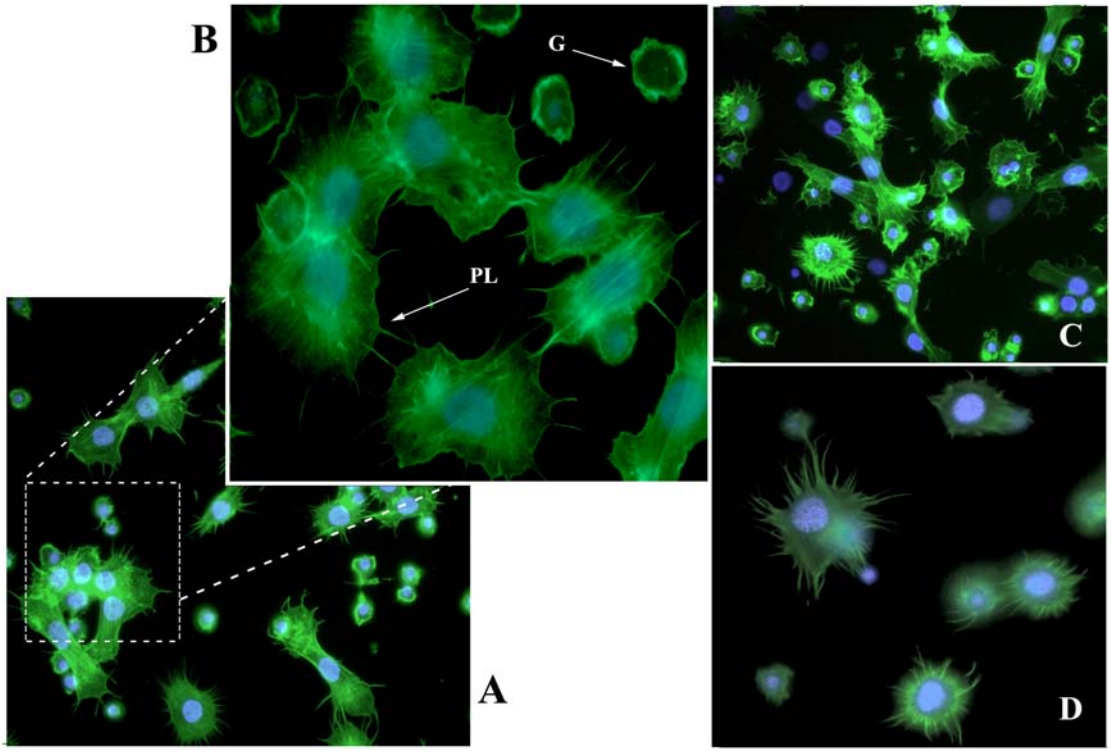


Figure 37 Morphology of spreading hemocytes after 1 h incubation on glass slide exhibited attachment and spreading. The hemocyte actin cytoskeleton was visualized with FITC-phalloidin (green) and the nucleus was stained with 4', 6'-diamidino-2-phenylindole (DAPI) (blue). Hemocyte monolayers were prepared from fungal infected larvae on day 2 post injection.

(A) Control spreading hemocytes of *H. virescens* (400x)

(B) Magnification of control spreading hemocytes of *H. virescens* (1000x) showing FITC-phalloidin labeled actin cytoskeleton in green. The arrows indicated **PL** as spreading plasmocyte exhibited filopodia formation and **G** as granular cell.

(C) Effect of *N. rileyi* infection on ability of hemocyte spreading at day 2 post injection of hyphal bodies. (400x)

(D) Effect of *P. fumosoroseus* infection on ability of hemocyte spreading at day 2 post injection of blastospores. (630x)

Table 27 Quantities of hemocytes, fungal cells and spreading hemocytes post challenge with *N. rileyi* hyphal bodies and *P. fumosoroseus* blastospores at different times.

Day	Sample	Wt (day 0)	Wt.	**Hemocytes**			**Fungal cells**			Spreading hemocytes/ Total hemocytes	**% spreading hemocytes**
				Dil.	count	Total ($x10^7$ cells/ml)	Dil.	count	Total ($x10^7$ cells/ml)		
1	**C1**	175	288	10	47	2.35	10	0	0	84/219	38.36%
	C2	114	204	10	65	3.25	10	0	0	75/266	28.20%
	C3	169	235	10	74	3.70	10	0	0	137/400	34.25%
	C4	83	156	10	62	3.10	10	0	0	113/345	32.75%
	C5	114	204	10	97	4.85	10	0	0	131/470	27.87%
	Mean ± SD					**3.45 ± 0.92**			**0**		**32.29% ± 4.39**
	N1	299	350	10	53	2.65	10	0	0	45/326	13.80%
	N2	174	228	10	79	3.95	10	0	0	170/746	22.79%
	N3	205	285	10	78	3.90	10	0	0	23/367	6.27%
	N4	201	296	10	74	3.70	10	0	0	202/995	20.30%
	N5	184	253	10	112	5.60	10	0	0	132/702	18.80%
	Mean ± SD					**3.96 ± 1.06**			**0**		**16.39% ± 6.54**
	P1	225	246	10	41	2.05	10	0	0	41/414	9.90%
	P2	215	222	10	47	2.35	10	0	0	47/172	27.33%
	P3	201	225	10	68	3.40	10	0	0	74/299	24.75%
	P4	263	267	10	75	3.75	10	0	0	8/375	2.13%
	P5	237	248	10	79	3.95	10	0	0	5/365	1.37%
	Mean ± SD					**3.10 ± 0.85**			**0**		**13.10% ± 12.31**
2	**C1**	72	220	10	73	3.65	10	0	0	106/469	22.60%
	C2	102	268	10	106	5.30	10	0	0	106/784	13.52%
	C3	95	323	10	55	2.75	10	0	0	60/448	13.39%
	C4	83	243	10	62	3.10	10	0	0	74/535	13.83%
	C5	84	244	10	80	4.00	10	0	0	140/458	30.57%
	Mean ± SD					**3.76 ± 0.99**			**0**		**18.78% ± 7.66**
	N1	132	231	10	116	5.80	10	0	0	168/1007	16.68%
	N2	239	281	10	28	1.40	10	1	0.05	16/78	20.51%
	N3	235	374	10	54	2.70	10	0	0	16/300	5.33%
	N4	188	284	10	84	4.20	10	3	0.15	157/681	23.05%
	N5	121	177	10	88	4.40	10	2	0.10	156/866	18.01%
	Mean ± SD					**3.10 ± 1.69**			**0.06 ± 0.065**		**16.72% ± 6.82**
	P1	269	305	10	72	3.60	10	2	0.10	9/462	1.95%
	P2	151	225	10	68	3.40	10	3	0.15	11/354	3.11%
	P3	198	214	10	53	2.65	10	3	0.15	26/473	5.50%
	P4	173	328	10	46	2.30	10	5	0.25	77/241	31.95%
	P5	189	239	10	73	3.65	10	23	1.15	0/189	0%
	Mean ± SD					**3.12 ± 0.61**			**0.36 ± 0.45**		**8.50% ± 13.26**

Abbreviations: C = Control larvae, N = *N. rileyi* hyphal bodies challenged larvae and P = *P. fumosoroseus* blastospores challenged larvae

Table 27 Quantities of hemocytes, fungal cells and spreading hemocytes post challenge with *N. rileyi* hyphal bodies and *P. fumosoroseus* blastospores at different times. (continued)

Day	Sample	Wt. (day 0)	Wt. (at day)	Hemocytes			Fungal cells			Spreading hemocytes/ Total hemocytes	**% spreading hemocytes**
				Dil.	count	Total ($x10^7$ cells/ml)	Dil.	count	Total ($x10^7$ cells/ml)		
3	C1	97	367	10	30	1.50	10	0	0	39/183	21.31%
	C2	92	192	10	80	4.00	10	0	0	298/552	53.99%
	C3	101	383	10	85	4.25	10	0	0	278/597	46.57%
	C4	90	269	10	72	3.60	10	0	0	90/234	38.46%
	C5	175	288	10	72	3.60	10	0	0	110/398	27.64%
	Mean ± SD					**3.39 ± 1.09**			**0**		**37.59% ± 13.36**
	N1	222	350	10	37	1.85	10	14	0.70	NR	NR
	N2	139	302	10	66	3.30	10	12	0.60	287/647	44.36%
	N3	226	319	10	57	2.85	10	12	0.60	165/482	32.23%
	N4	163	283	10	56	2.80	10	19	0.95	240/411	58.39%
	N5	134	302	10	76	3.80	10	19	0.95	3/746	0.40%
	Mean ± SD					**2.92 ± 0.72**			**0.76 ± 0.18**		**33.85% ± 24.73**
	P1	187	206	10	63	3.15	10	64	3.20	0/94	0%
	P2	192	248	10	143	7.15	10	17	0.85	21/887	2.37%
	P3	173	218	10	56	2.80	10	40	2.00	0/47	0%
	P4	95	173	10	31	1.55	10	139	6.95	10/39	25.64%
	P5	180	198	10	37	1.85	10	70	3.50	0/48	0%
	Mean ± SD					**3.30 ± 2.25**			**3.30 ± 2.29**		**6% ± 11.25**
4	C1	75	310	10	60	3.00	10	0	0	61/132	46.21%
	C2	68	393	10	66	3.30	10	0	0	84/113	74.34%
	C3	97	293	10	50	2.50	10	0	0	109/192	56.77%
	C4	109	137	10	41	2.05	10	0	0	69/181	38.12%
	C5	90	360	10	50	2.50	10	0	0	59/189	31.22%
	Mean ± SD					**2.67 ± 0.49**			**0**		**49.33% ± 16.91**
	N1	118	206	10	33	1.65	10	624	31.2	0/57	0%
	N2	190	302	10	37	1.85	10	13	0.65	21/197	10.66%
	N3	93	190	10	97	4.85	50	141	35.3	164/432	37.96%
	N4	88	147	10	56	2.80	10	2	0.10	0/28	0%
	N5	132	176	10	58	2.90	10	3	0.15	214/543	39.41%
	Mean ± SD					**2.81 ± 1.27**			**13.5 ± 18.1**		**18% ± 19.74**

Abbreviations: C = Control larvae, N = *N. rileyi* hyphal bodies challenged larvae and P = *P. fumosoroseus* blastospores challenged larvae

CHAPTER V
DISCUSSION

Over thirty years *N. rileyi* has been researched for use as microbial control agent against insect pests. Interest in this insect mycopathogen has been stimulated in part by the environmental damage caused by the over-use of chemical pesticides [38]. Although several reports of natural epizootics caused by *N. rileyi* in agricultural fields have been published since [11, 27, 31, 83], to date a commercial product of *N. rileyi* has not been introduced in practice. To develop a promising fungal mycoinsecticide for pest control several requirements are needed including cost-effective mass production of high pathogenic strains that can be formulated to provide long shelf-life without loss of infectivity and viability [14]. The optimum temperature for rapid mycelium growth and sporulation was reported at 25°C [11]. At 25°C, *N. rileyi* was able to produce spore at 8.8 days which faster than growing at 15°C and 20°C. The germination of conidia was inhibited at 5°, 35°, 37°, or 40°C [11]. Temperature has been also reported to affect infectivity of *N. rileyi* [36, 84]. The optimum temperature for infection of *N. rileyi* is about 20°C and 25°C. Temperature over 30°C was reported to reduce mortality in *H. zea* and *A. gemmatalis* [36, 85]. *N. rileyi* conidial viability was decreased at storage temperature over 30°C [86]. In addition to thermal ability the conidia are sensitive to UV inactivation and require a humid microclimate to infect host insects. Suitable formulations that provide good shelf-life and high persistence of conidia on foliage have not been successfully developed.

When compared with chemical insecticides, *N. rileyi* has a relatively long killing time, 7 to 8 days, this property often results in the crop damage by the insect's feeding. During the infection process the feeding and movement are not dramatically altered. At the time just prior to insects' death, infected larvae are found flaccid and paralyzed. The possibility that *N. rileyi* produces metabolites responsible to the insects' death from *N. rileyi* has not been examined. In fact since 1978 only 4 articles related to *N. rileyi* toxin production have been published [87-90]. Therefore focus of this research was to identify potential toxic metabolites produced by *N. rileyi* and to

elucidate their involvement in the pathogenicity of this fungus toward the insects target. The toxic effects of *N. rileyi* were reported by Wasti and Hartmann 1978. This research showed that methanolic extracts prepared from *N. rileyi* mycelia and chromatographed on a silica gel column produced 83% mortality in *Lymantria dispar* larvae after topical application at dosage of 1 μl/larvae (concentration was not reported). Intra-haemocoelic injection at dosage of 0.5 μl/larvae gave 63% mortality and the injected larvae succumbed faster then topically treated larvae [7]. By contrast, Mohamed *et al*. (1984) found that topically appliep *in vitro* methanolic extracts did not cause significant mortality to *Heliothis virescens* and *H. zea.* But the mortality rates of 37 and 45 % were observed after *per os* (feeding) treated in *H. virescens* and *H. zea* respectively. In addition, *H. virescens* larvae were more susceptible than *H. zea* (72 versus 51 % mortality, respectively) upon injection of 2.0 μl of *N. rileyi* extract into the haemocoel [8]. The differential response obtained from methanolic extract treated insects probably resulted from difference in the fungal strain, host species, and/or general protocols used to prepare extracts.

The fungal strain PP used in this study was isolated from a *N. rileyi* diseased cadaver collected in Poppra district, Tak province, Thailand. Preliminary data demonstrated that this strain could grow rapidly on artificial media and displayed high virulence against *Spodoptera litura.* This insect is a major vegetable pest in many areas of Thailand. *N. rileyi* is able to infect mostly in the family Noctuidae (Lepidoptera) that include many pests feeding on many crops such as *S. exigua, S. litoralis, S. frugiperda, H. armigera, H. virescens, H. zea,* etc [11].

The presence of toxic metabolite produced by this *N. rileyi* strain was investigated by extraction of *N. rileyi* cultures with organic solvents (methanol: dichloromethane, 1:1). In order to isolate toxic metabolite(s) from fungal extracts, bioassay guided fractionation was used in the purpose to reduce time consuming and cost on the isolation every component in the extract. The bioassay on cytotoxicity of insect cell line (Sf9) was chosen to use as a tool for screening toxic metabolite(s) within the extracts. The cell-based assay has been widely conducted in screening purpose due to its advantages over *in vivo* assay such its simplicity, selectivity for a specific type of activity, sensitivity to small amounts of active material and low cost. Comparison with an assay on *Artemia salina* (brine shrimp) showed that Sf9 cell line

was 10-fold more sensitive for fusarenon X and 3-fold more sensitive for zearalenone [91]. The main difference between *in vivo* and *in vitro* assay is that *in vitro* assay used exposure to only one type of cell. Thus, the cell line used may or may not reflect the types of cells targeted in *in vivo.* The insect cell line Sf9 is derived from pupal ovarian tissue of the fall armyworm, *S. frugiperda.* Cytotoxicity was revealed by inhibition of cell proliferation and also cell death. However there are some disadvantages to this type of assay, it is not possible to determine the mechanism involved with this assay and it only detects compounds that are capable of passing through the cell membrane leading to a chance to miss potent compounds that could be modified to improve membrane permeability.

Most entomopathogenic fungi produce toxic metabolites after multiplication of the yeast phase (hyphal bodies) in the host haemocoel [65, 92-97]. Thus, we studied cytotoxic activity at different period of times post infection with *N. rileyi*. Initial experiments by bioassay guided fractionation showed that the organic solvent extract (CH_2Cl_2: MeOH) prepared from *N. rileyi* mycosed *S. litura* at 7 and 8 days post infection possessed cytotoxic effects on Sf9 cells at concentration of 200 μg/ml (**Table 2**) whereas extracts of infected larvae at 2 and 5 day produce limit of cell death similar to that produced by extract of healthy larvae at equal concentration. In terms of fungal development, the 2 and 5 day extractions represent extracts of infected insects harboring penetrant germ tubes and hemocoelic hyphal bodies, respectively [3, 19]. The 7 and 8 day extracts contain metabolites produced by the tissue invasive and conidiophore-producing mycelial phenotypes. These findings correspond to those of Wasti *et al.* [88] and Mohamed *et al*.[89] who reported insecticidal activity from crude methanolic extracts prepared from *N. rileyi* mycelia.

It was found that heat treatment had no effect on toxic activity (i.e., treatment up to 90^oC for 60 min) of mycosed larvae extract (**Table 4**) indicated that compound(s) of interest were thermostable. Vey and Riba [98] classified toxins produced by entomopathogenic fungi into two groups as low-molecular-weight compounds such as cyclic peptides, destruxins, beauvericin, bassianolide and cytochalasin B detected in either culture filtrates or infected insects [92] and high molecular weight protein molecules such as protease from *M. anisopliae* and *B. bassiana* [99] and hirsutellins A and B from *H. thompsonii* [100].

Thin layer chromatography on silica gel plates served as a method to monitor compound(s) in the extract during purification steps. Durackova *et al.* [101] used thin layer chromatography to detect mycotoxins and suggested that this technique was suitable for initial stages of screening crude extracts. The system of toluene: ethyl acetate: 90% formic acid (TEF) was recommended by Paterson [102] and other investigators [103-105] to be used in many mycotoxins such as ochratoxin A, brevianamide A, citrinin, patulin. The TEF in ratio of 5.75:4:0.25 (v/v/v) was optimized as the best system for separation of the crude extract. TLC analysis allowed detection of nine spots that were labeled A to I alphabetically (**Fig. 8**). Thus the toxic substance(s) isolated form organic extracts obtained from dead *S. litura* and dead larvae covered with white mycelial growth were subjected to a fractionation. Samples applied to silica gel column chromatography were eluted with isocratic solvent of TEF (5.75:4:0.25). The appropriate fractions were combined according to the pattern of spots on TLC plates visualized by spraying with sulfuric acid. Each fraction was tested for toxicity toward Sf9 cell to determine the active fraction(s). The successful isolation of compounds G was obtained as a colorless crystal after applied with stepwise gradient elution system. The 50% inhibitory dose (ID_{50}) of compound G against Sf9 cells were estimated at 17.72 ± 1.92 μg/ml (data were shown in **Table 7**).

Structural elucidation of compound G was accomplished using mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopic techniques. The molecular structure was completely determined by 2-D NMR pluses COSY sequences, heteronuclear multiple quantum correlation (HMQC) and heteronuclear multiple bond correlation (HMBC). The HMQC spectrum gives one-bond H-C correlations that allows assignment of protons to respective carbons and makes it possible to analyze the HMBC spectrum. HMBC spectra provide multiple bond H-C correlations, generally two and three bond correlations. This supports information to assign carbon and proton positions in the molecule based on share correlation. These spectral data of compound G agreed with those described for ergosterol peroxide [106-108].

The quantitative study was accomplished by GC/MS analysis. Extracts from mummified mycosed insects and from *in vitro* produced mycelia and hyphal bodies contained detectable levels of ergosterol peroxide (5.94, 3.93 and 5.14 μg/g dried

weight whereas mycosed insects without external mycelia detected ergosterol peroxide at very low level (0.40 μg/g dried weight). The average dry weights of mycosed and mummified larvae were 28 and 65 mg respectively, amounts of ergosterol peroxide detected in cadaver infected by *N. rileyi* were calculated as 0.011 and 0.386 μg per larva respectively. Furthermore, amounts of ergosterol peroxide detected *in vitro* cultures both hyphal bodies and mycelial phenotypes were not significantly different from *in vivo* mummified insects. The difference in peroxide biosynthesis may simply reflect differences in fungal biomass present in the different insect samples. Alternatively, the fungal cells exposed to the atmosphere (mummified larvae) reside in an environment that is more aerobic then the hemocoel which is partially anaerobic condition. Significantly, the physiological levels detected in the mycosed insects were less then the amount that needed to cause 50% insect cell death (3.45 μg/well).

Ergosterol peroxide has been previously isolated from variety of fungi such as wood-rotting fungi [106, 109], entomopathogenic fungi [81, 107, 110, 111], *Aspergillus* sp. and *Penicillium* sp.[108, 112] , mushrooms [113-117] , marine organisms [118], lichens [119], algae [120, 121] and plants [122-124]. Comparing amounts of ergosterol peroxide in different organisms, it was found that ergosterol peroxide detected in both the *in vitro* hyphal bodies and mycelial phenotypes of *N. rileyi* at 3.93 and 5.14 μg/g fungal cell dried weight respectively, were less than previously reported for other the fungi, *Sporothrix schenckii* yeast form (360 μg/g) [125], *Paecilomyces tenuipes* (2.3 mg/g) [107] , *Armillariella mellea* (133 μg/g) [116]. Early work proposed that the ergosterol peroxide detected in biological samples was an artifact of a photo-oxidation reaction rather than a natural product [126]. However, several investigators presented the evidences that indicated ergosterol peroxide was not an artifact of the isolation process [118]. In order to prevent photooxidation, isolation in the absence of oxygen of ergosterol peroxide was performed and reported in *Fusarium moniliforme, Cantharellu cibarius, Aspergillus* sp. and *Alternoria kikuchiana.* Sheikh (1974) suggested the generation of singlet oxygen could be occurred by activation of molecular oxygen by heme proteins which are central constituents of mixed function oxygenases of animal, insect and microorganism [127]. Thus the formation of ergosterol peroxide even though carried out by $^1O_2$ would be biological process. Biosynthesis of ergosterol peroxide was studied using isotopically

labeled [^{3}H] ergosterol administered to *Penicillium rubrum* and *Gibberella fujikuroi* that led to incorporation into ergosterol peroxide by biological process [128]. In certain mycopathogens it has been proposed that ergosterol peroxide acts as a protective mechanism sequestering reactive oxygen species during phagocytosis [125]. Interestingly Keisei *et al* (2001) reported that ergosterol peroxide possessed ability to inhibit melanin biosynthesis of a mouse melanoma cell line while ergosterol did not [129]. Melanization plays important role in the insect's defense reaction against invaders generated by phenoloxidase activity. The prophenoloxidase is activated by elicitors derived from microbial cell wall components such as peptidoglycan, β-1,3-glucan, and lipopolysaccharide (LPS). As in dead *N. rileyi* larvae, most of dead *N. rileyi* infected larvae are found as non-melanized cadavers which consequently completely ramified by fungal mycelia. Therefore ergosterol peroxide probably allows the fungus avoid the defense mechanism of insect's immune by suppress or inhibit melanization.

Anticancer activity of ergosterol peroxide was examined against hormone-dependent breast cancer cell line (T47D), hormone-independent breast cancer cell line (MDA-MB-231), human epidermoid carcinoma in mouth cell line (KB), human cervical carcinoma cell line (HeLa), lung cancer cell line (H69AR) and human cholangiocarcinoma cell line (HuCCA-1) with 50% inhibitory concentration (IC_{50}) of 5.8, 28.0, 46.7, 58.4, 65.4 and 105.1 μM. Our findings on anti-cancer activity of ergosterol peroxide are in agreement with published findings on inhibiting proliferation of cancer cells property [107, 112, 130]. It was reported that ergosterol peroxide inhibited growth of HL60 human promyelocytic leukemia cells by inducing apoptosis at concentration of 25 μM [130]. Kuo *et al.* [112] also showed that the DNA topoisomerase I, which is highly expressed in tumor cells, was inhibited by ergosterol peroxide at 100 ng/μl (233.64 μM) and suggested that peroxide moiety could play important role in this action comparing with ergosterol. Activities of ergosterol peroxide toward cancer cells are different among cell types. As reported in this study, ergosterol peroxide was active against hormone-dependent breast cancer cells with IC_{50} of 5.8 μM and considered the lowest value among those previously reported [107, 112, 114].

In this study, anti HIV-1 activity of ergosterol peroxide was evaluated by determination of syncytium reduction assay. HIV-1 is capable potentially infect neighbor cells and forms syncytium. The mutated virus, ΔTat/Rev defective HIV-1 was used in this study. The syncytium reduction assay reveals both virucidal and intracellular antiviral activities since the virus has encountered the compound before entry the cells and also during replication in cells. In addition cytotoxic of ergosterol peroxide was also determined in parallel to anti-HIV-1 activity toward 1A2 cells. The EC_{50} (the effective concentration that exhibited reduction of syncytium formation toward 50% of control) and IC_{50} (the concentration that inhibited metabolic activities of 50% of cells) of ergosterol peroxide was determined as 15.96 and 150.77 μg/ml. The therapeutic index (TI = IC_{50}/EC_{50}) of ergosterol peroxide was calculated as 9.45 which considered as a potential anti-HIV-1 active compound.

Additional biological activities of ergosterol peroxide have been reported, i.e., antioxidant [116], antimycobacterial [117, 131, 132], antiplasmodial [122], leishmanicidal activity [109], anti-inflammatory [115, 121, 133], immunosuppressive [110], anti-atherosclerosis [123] and aldose-reductase inhibitory activity [134].

The insecticidal activity of ergosterol peroxide against 3rd instar *S. litura* larvae via topical and *per os* application was studied (**Table 18** and **19**). For topical application, the mortality reached the highest at 46.7% and constant for concentrations of 90-120 μg/insect. Increasing dosage did not increase effect that probably due to limitation on absorption of ergosterol peroxide through larval cuticle into insect's tissue. *Per os* application of ergosterol peroxide at concentrations of 60 to135 μg/insect caused no mortality in tested *S. litura.* In injected application (**Table 21** and **22**), various preparations of ergosterol peroxide were injected to 4th instar *H. virescens* at concentration 25 and 50 μg/insect. The results showed that no significant difference mortality between controls and injected insects. The inactive activity of ergosterol peroxide found in injected larvae probably resulted from insect cell- free immune reaction as described by Schmidt *et al* 2006. The lipophorin particles, the lipid carriers in insect hemolymph [69], may interact with ergosterol peroxide molecules leading to aggregation that could prevent the action of ergosterol peroxide at the target cells.

The toxicity of ergosterol peroxide found in *in vivo* assay indicated moderate toxicity correlated with the results from *in vitro* assay toward Sf9 cells at IC_{50} of 17.7

μg/ml (41.4 μM) compared with other toxic metabolites produced by entomopathogenic fungi such as beauvericin ($IC_{50,}$, 2.5 μM) [91] and hirsutellin A (IC_{50} , 0.5 μM) reported against Sf9 cells [135] . According to previously reports, organic solvent extract of *N. rileyi* mycelia showed insecticidal activity toward 2nd instar larvae of gypsy moth, *Lymantria dispar*, via topical application with 80% mortality [88]. Later Mohamed *et al.* reported that no toxic activity observed after topically applied crude extract to *Heliothis zea* and *H. virescens* [89]. The synthetic ergosterol peroxide caused 46.6% mortality of topical treated 3rd instar *S. litura* larvae. Possibly, ergosterol peroxide was one of compounds in the crude extract responsible for insect death.

According to the isolation of ergosterol peroxide presented in this study, several possibilities can be considered such as:

1. Ergosterol peroxide was produced from *N. rileyi* as toxic metabolite during infection process. As the detection of ergosterol peroxide in mycosed and late-stage mummified cadavers only 11 ng and 0.386 μg ergosterol peroxide per larva. Whether or not these low levels can act as virulence factors is unknown. Possibly, the local concentrations are high enough to impact host functions.

2. The conversion of ergosterol to ergosterol peroxide allows the fungal cells (at the late infection stage) to evade host “non-self” recognition and represent virulence determinant due to the fact that ergosterol peroxide could act as a protective mechanism sequestering reactive oxygen species during phagocytosis.

3. The *N. rileyi* ergosterol peroxide presumably to be a membrane-bound metabolite, acts as an anti-oxidant protecting the externally-borne conidiophores.

4. The ergosterol peroxide probably involves in suppression of insect’s immune response by inhibition of melanin synthesis generated by phenoloxidase activity.

In respect to bioactivities of ergosterol peroxide including anticancer and anti HIV-1 activities, the basic structure-activity relationship may be further conducted to test the effect of analogues of ergosterol peroxide with anticancer and anti HIV-1 properties.

CHAPTER VI
CONCLUSION

In this study, ergosterol peroxide was isolated from extract of *N. rileyi* infected *S. litura* larvae by activity guided chromatographic purification. Extract of infected larvae at different time after infected with fungal conidia were tested for cytotoxicity toward Sf9 cell line. That extracts of infected larvae at day 2 and 5 possessed 44 and 41 percentage of cell death when compared with activity of the control, healthy larvae extract, of 46% cell death. The extract of dead larvae (day 7) and the extract of larvae with white mycelia mat covered (day 8) showed 93 and 95 % cell death, respectively.

Isolation of toxic compound by silica gel column chromatography from *N. rileyi* mycosed larvae was performed. Increasing polarity of eluted solvents by stepwise gradient system provided successful purification of compounds G that exhibited ID_{50} value toward Sf9 cells of 17.72 $\pm$1.92 μg/ml. The compound G was identified as ergosterol peroxide (5α,8α-epidioxy-24(R)-methylcholesta-6,22-dien-3β-ol) by methods of nuclear magnetic resonance techniques, infrared spectrometry and mass spectroscopy.

Ergosterol peroxide was synthesized from ergosterol by the method of photooxidation reaction. Using methylene blue 12 %mol as photosensitizer in photooxidation reaction gave the highest production yield of ergosterol peroxide (59.8 %). The amounts of ergosterol peroxide produced under *in vitro* and *in vivo* conditions were determined by GC-MS. The extracts from mycosed larvae with white mycelial mat and from *in vitro* hyphal bodies and mycelia contained ergosterol peroxide 5.94, 3.93 and 5.14 μg/g dried weight while the extract of mycosed larvae without external mycelia was detected at 0.40 μg/g dried weight. It was implied that ergosterol peroxide production was corresponding to the growth of *N. rileyi* both *in vitro* and *in vivo* conditions.

Ergosterol peroxide showed inhibition of proliferation on six cancer cells, hormone-dependent breast cancer cell line (T47D), hormone-independent breast cancer cell line (MDA-MB-231), human epidermoid carcinoma in mouth cell line

(KB), human cervical carcinoma cell line (HeLa), lung cancer cell line (H69AR) and human cholangiocarcinoma cell line (HuCCA-1) with IC_{50} of 5.8, 28.0, 46.7, 58.4, 65.4 and 105.1 μM, respectively.

In addition anti HIV-1 activity of ergosterol peroxide was evaluated by determination of syncytium reduction assay. The EC_{50} (the effective concentration that exhibited reduction of syncytium formation toward 50% of control) and IC_{50} (the concentration that inhibited metabolic activities of 50% of cells) of ergosterol peroxide was determined as 15.955 and 150.765 μg/ml. The therapeutic index ($TI = IC_{50}/EC_{50}$) was calculated as 9.45 considered as a potential anti-HIV-1 active compound.

The insecticidal activity of ergosterol peroxide against 3rd instar *S. litura* larvae via topical and *per os* application was studied. For topical application, the mortality reached the highest at 46.7% and constant for concentrations of 90-120 μg/insect. *Per os* application of ergosterol peroxide at concentrations of 60 to135 μg/insect caused no mortality in tested *S. litura.* No significant affect of 4th instar *H. virescens* larvae was found post injected with ergosterol peroxide at concentration 25 and 50 μg/insect.

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APPENDIXES

APPENDIX A
MEDIA, BUFFERS AND SOLUTIONS

FMAY

Maltose	10	g
Yeast extract	10	g
Fish-soluble extract	30	g
Agar	12	g
Distilled water up to	1,000	ml

TC100

TC100 medium		
Distilled water	1,000	ml
$NaHCO_3$	0.35	g
NaCl	2.25	g
Fetal bovine serum (heat-inactivated)	10%	
L-glutamine	2	mM
Penicillin and streptomycin	100	IU/ml

RPMI-1640 (1x)

RPMI medium 1640 (GIBCO)	10.4	g
Deionized distilled water	800	ml
$NaHCO_3$	2.0	g
Adjusted to pH 7.4 with 0.1 N HCl		
Deionized distilled water up to	1,000	ml
Gentamycin (2 mg/ml)	1.0	ml

RPMI-1640 (1x) without phenol red

RPMI medium 1640 without phenol red (GIBCO)	10.4	g
Deionized distilled water	800	ml

$NaHCO_3$	2.0	g
Adjusted to pH 7.4 with 0.1 N HCl		
Deionized distilled water up to	1,000	ml
Gentamycin (2 mg/ml)	1.0	ml

Tris buffer

Stock A:

Tris	2.4	g
Distilled water	100	ml

Stock B:

HCl 36-38%	1.8	ml
Distilled water	100	ml

0.05 M Tris buffer (pH7.4)

Stock A	25	ml
Stock B	42	ml
Distilled water up to	100	ml

Phosphate buffered solution, Mg^{2+} and Ca^{2+} free, pH 7.4 (PBS 10x)

NaCl	80.0	g
KCl	2.0	g
$Na_2HPO_4H_2O$	14.42	g
KH_2PO_4	2.40	g
Deionized distilled water up to	1,000	ml

Phosphate buffered solution (PBS)

NaCl	8.0	g
KCl	0.2	g
$Na_2HPO_4H_2O$	1.15	g
KH_2PO_4	0.2	g
$CaCl_2$ (anhydrous)	0.1	g
$MgCl_2.6H_2O$	0.1	g
Deionized distilled water up to	1,000	ml

HEPES

HEPES (N-2-hydroxyethyl-piperazine-N'-2-Ethanesulphonic acid)	4.76	g
NaCl	6	g
MgSO4.7H2O	180	mg
Na2HPO4.7H2O	90	mg
KH2PO4	60	mg
CaCl2	140	mg
Glucose	1	g
Deionized distilled water up to	1,000	ml

10% NaHCO3

$NaHCO_3$	10.0	g
Deionized distilled water up to	100	ml

Trypan blue dye

Physiological saline (0.85%)

NaCl	0.85	g
Distilled water	100	ml

Trypan blue (0.4%)

Trypan blue dye	0.4	g
Physiological saline	100	ml
Paper filter before use		

Fixative solution

PBS (10x)	100	µl
10% paraformaldehyde	400	µl
Distilled water	500	µl

Phalloidin stain

PBS	200	µl
Phalloidin methanolic stock solution	5	µl

DAPI stain (4',6-diamidino-2-pheylindole, dihydrochloride) (1:300)

DAPI	1	µl
PBS	300	µl

Poly-L-Lysine solution (10x)

Poly-L-Lysine	1	g
Distilled water	100	ml

Insect artificial diet

HWG (Gelacerin)	46	g
Tortula yeast	125	g
Water	3	L
(Heated at 75°C, 10-15 min)		
Pinto beans	250	g
Wheat germ	200	g
Soybean protein	100	g
Casein	75	g
Heat-sentitive mixture (added when temp. below 70 °C):		
Ascobic acid	12	g
Vitamin mix	20	g
Tetracycline	250	mg
Methy P-Hydroxybenzoate (Methyl Paraben)	10	g
Sorbic acid	6	g
40% Formalin	15	ml

APPENDIX B

Computation of 50% innibitory concentration (IC_{50}) in cell-based anti-HIV-1 assay by linear regression analysis

The toxicity of test sample against 1A2 cells was expressed as the 50% inhibitory concentration (inhibition of cellular metabolic activity; IC_{50}). IC_{50} value capable for reveal the concentration of the compound that cause toxic effect to 50% of cell population calculated by linear regressive analysis. The optical densities (OD) of test and control wells after the soluble formazan (XTT formazan) production from the XTT assay were measured and percent of cytotoxicity of each concentration of the test sample was calculated. Since, only the concentrations above and below 50% of cell viability were used to calculate IC_{50}, thus this value could implicated the estimate 50% inhibitory concentration (IC_{50}) of those sample.

From formula $\hat{y} = A + Bx$

$$B = \frac{n.\sum xy - \sum x. \sum y}{n.\sum x^2 - (\sum x)^2}$$

$$A = \frac{\sum y - B. \sum x}{n}$$

$$r = \frac{n. \sum xy - \sum x. \sum y}{\sqrt{\{n. \sum x^2 - (\sum x)^2\}\{n. \sum y^2 - (\sum y)^2\}}}.$$

Description : A = The constant term

B = The regression coefficient

x = % of cell survival compared with control

y = Concentration of the sample

r = The correlation coefficient

For example;

The sample at concentration of 125.0 and 62.5 μg/ml presented 75.0% and 40.0% of cell survival, respectively. After x and y value were input, data will be computed using the linear regression equation as above and then yielded the estimate value of IC_{50} that equal 80.35 μg/ml.

Computation of 50% effective concentration (EC_{50}) in syncytium reduction by linear regression analysis

EC_{50}, the concentration of sample that can reduce syncytium formation by 50% compared with virus control. EC_{50} also computed using linear regression equation as mentioned above, excepted x = % syncytium reduction that calculated from (1 - SFU of test/ SFU of virus control) x 100.

For example;

The sample at the concentration of 62.5 and 31.3 μg/ml presented 85.0% and 35.5% syncytium reduction, respectively. When data were input, the EC_{50} value was obtained as 40.40 μg/ml.

APPENDIX C

(Autodock program analysis)

Table 1 Parameters for autogrid

parameter	Protease	Reverse transcriptase	DNA-Topoisomerase I
Macromolecule	1hsg Resolution =2.00	1vrt Resolution =2.20	1t8i Resolution = 3.00
Num. Grid point in n,y,z,	40,40,40	40,40,40	60,50,30
Spacing (Å)	0.375	0.375	0.375
Grid center	center on ligand	center on ligand	center on ligand
Smooth	0.5	0.5	0.5

Table 2 Parameters for protease and reverse transcriptase enzyme's autodock

Number of GA runs	100
Population size	50
Maximum number of energy evaluation	1,000,000
Maximum number of generation	27000
Maximum number of top individuals that automatically survive	1
Rate of gene mutation	0.02
Rate of cross over	0.8
Mean of cauchy distribution for gene mutation	0
Variance of cauchy distribution for gene mutation	1
Number of generations for picking worst individual	10

Table 3 Parameters for DNA-Topoisomerase I enzyme's autodock

Number of GA runs	100
Population size	250
Maximum number of energy evaluation	1,000,000
Maximum number of generation	27000
Maximum number of top individuals that automatically survive	1
Rate of gene mutation	0.02
Rate of cross over	0.8
Mean of cauchy distribution for gene mutation	0
Variance of cauchy distribution for gene mutation	1
Number of generations for picking worst individual	10

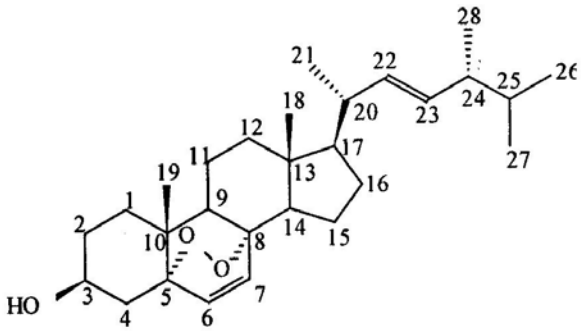
1. Reverse transcriptase enzyme :

-Pdb code =1vrt

-Ligand = nevirapine

Results from docking

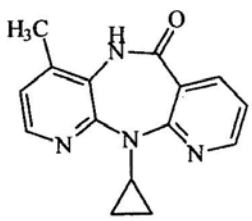
Ligand	rsd	Binding energy (kcal/mol)	Docking energy (kcal/mol)
Nevirapine (validate)	0.6630	-9.14	-9.32
Ergosterol peroxide 20S, 24R		-11.2	-10.1
Ergosterol peroxide 20R, 24R		**-10.6**	**-9.74**



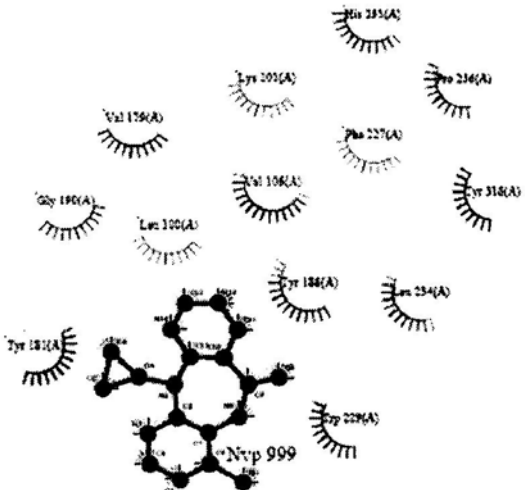
ergosterol peroxide

1.1 1vrt docking with nevirapine (redock)

1. There was no hydrogen bond between nevirapine and the binging site.



Nevirapine



The binding sites of enzyme reverse transcriptase are Pro95, Leu100, Lys 101, Lys 103, Va 1106, Tyr 181, Tyr 188 and Trp229.

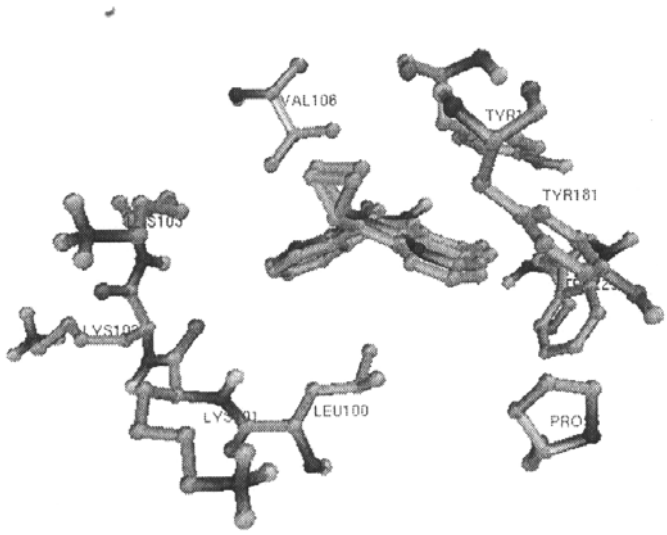


Figure1 Superimposition of nevirapine (previous conformation –green) and nevirapine post-docking

1.2 1vrt docking with ergosterol peroxide (20R, 24R)

1. H(3-OH) of ergosterol peroxide binding with N(NH) of His235 = 3.494 Å

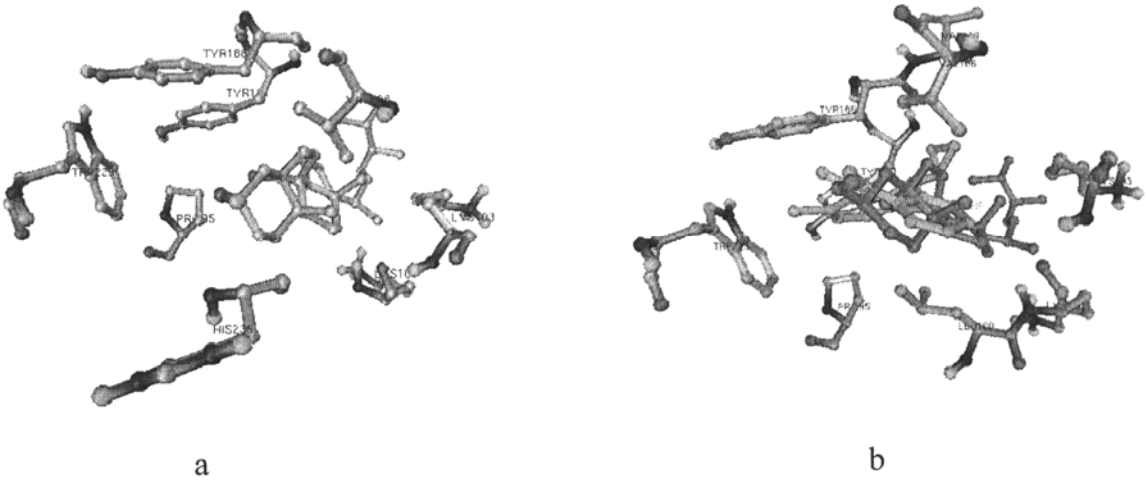
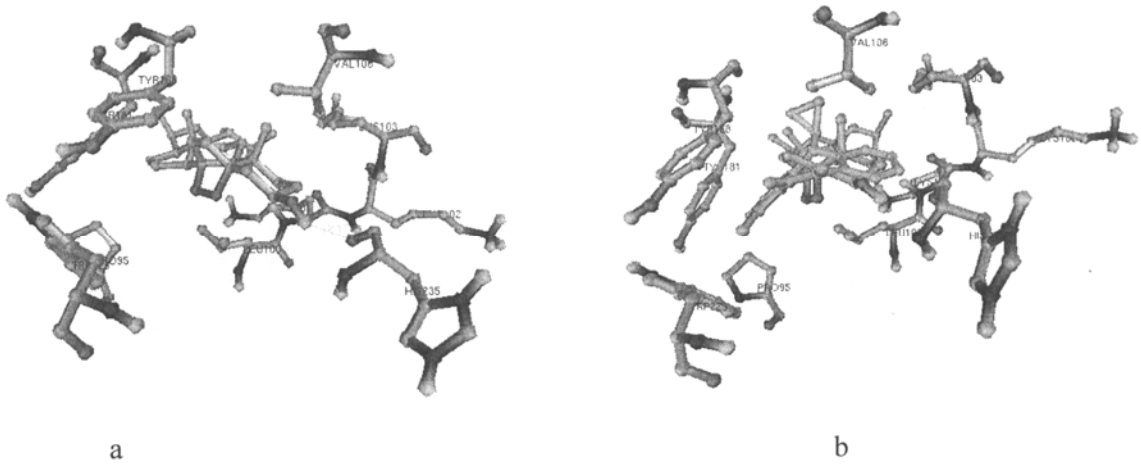


Figure 2 a) Diagram showing hydrogen bond of ergosterol peroxide (20R, 24R) and binding site b) Superimposition of ergosterol peroxide (20R, 24R) (pink) and nevirapine (green)

1.3 1vrt docking with ergosterol peroxide (20S, 24R)

1. H(3-OH) of ergosterol peroxide binding with N(NH) of His235 = 2.19 Å



a b

Figure 3 a) Diagram showing hydrogen bond of ergosterol peroxide (20S, 24R) and binding site b) Superimposition of ergosterol peroxide (20S, 24R) (pink) and nevirapine (green)

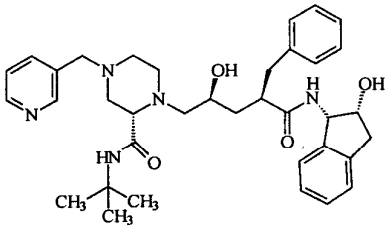
2. Protease enzyme:

-Pdb code =1hsg

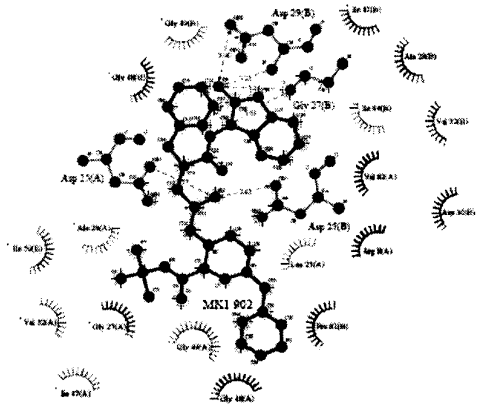
-Ligand = indinavir

Results from docking

Ligand	*rsd*	*Binding energy (kcal/mol)*	*Docking energy (kcal/mol)*
Indinavir (validate)	0.4230	-14.2	-18.6
Ergosterol peroxide 20S, 24R		-12.3	-12.2
Ergosterol peroxide 20R, 24R		**-12.1**	**-12.3**



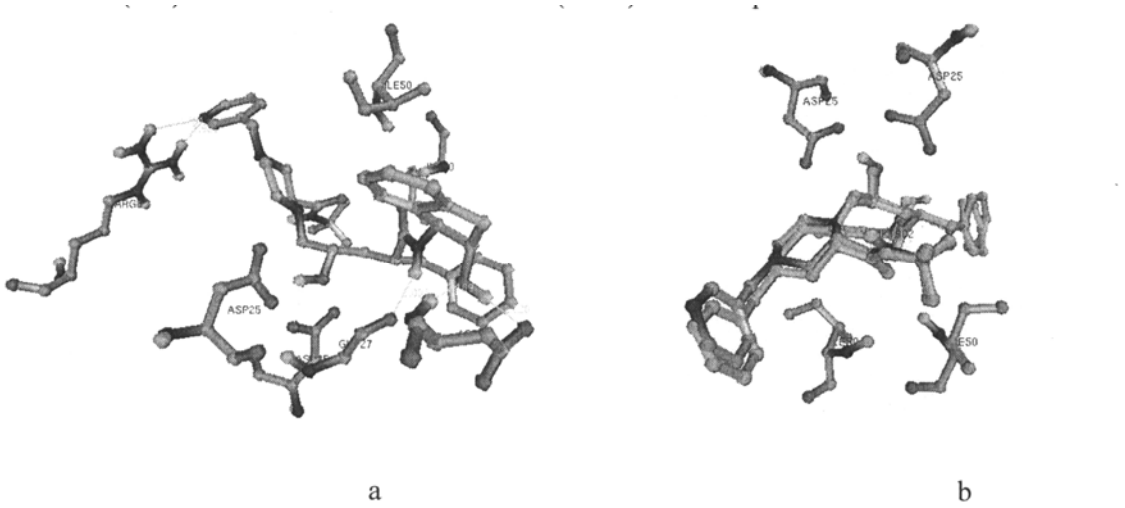
indinavir



The binding sites of protease enzyme are Asp 25, Ile 50, Asp 25’ and Ile50’.

2.1 1hsg docking with indinavir (redock)

1. H(OH) of indinavir was able to bind with O(COO) of Asp 25 and Asp 25' = 2.288 and 2.078 Å, respectively.
2. N of indinavir was able to bind with H(NH) of Arg 8 = 1.959 and 2.701 Å.
3. H(NH) of indinavir was able to bind with O(C=O) of Gly 27 = 2.033 Å.
4. O(OH) of indinavir was able to bind with H(NH) of Asp 29 = 2.169 Å.
5. H(OH) of indinavir was able to bind with O(COO) of Asp 29 = 2.228 Å.



a b

Figure 4 a) Diagram showing hydrogen bond of indinavir and binding site
b) Superimposition of indinavir (previous conformation - green) and indinavir (post docking)

2.2 1hsg docking with ergosterol peroxide (20R, 24R)

1. H(OH) of ergosterol peroxide was able to bind with O(COO) of Asp 29 = 1.835 Å.

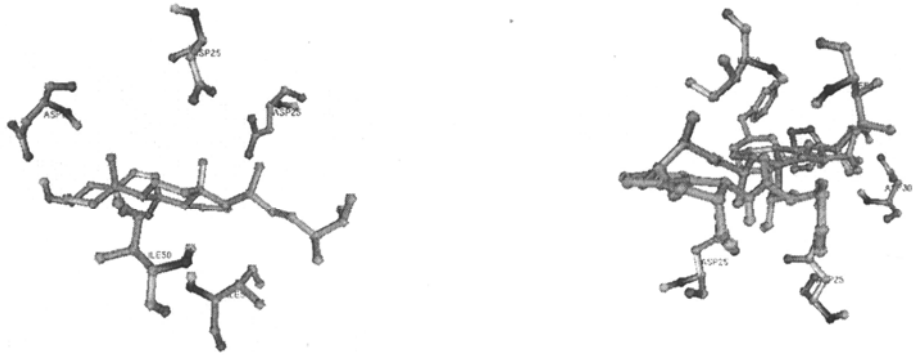


Figure 5 a) Diagram showing hydrogen bond of indinavir and binding site
b) Superimposition of indinavir (previous conformation - green) and indinavir (post docking)

2.3 1hsg docking with ergosterol peroxide (20S, 24R)

1. H(OH) of ergosterol peroxide was able to bind with O(COO) of Asp 29 = 2.123 Å.

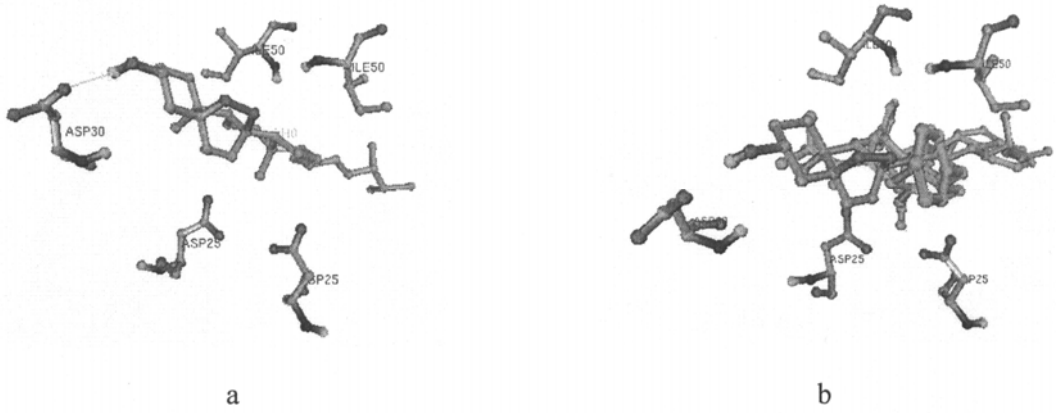


Figure 6 a) Diagram showing hydrogen bond of indinavir and binding site
b) Superimposition of indinavir (previous conformation - green) and indinavir (post docking)

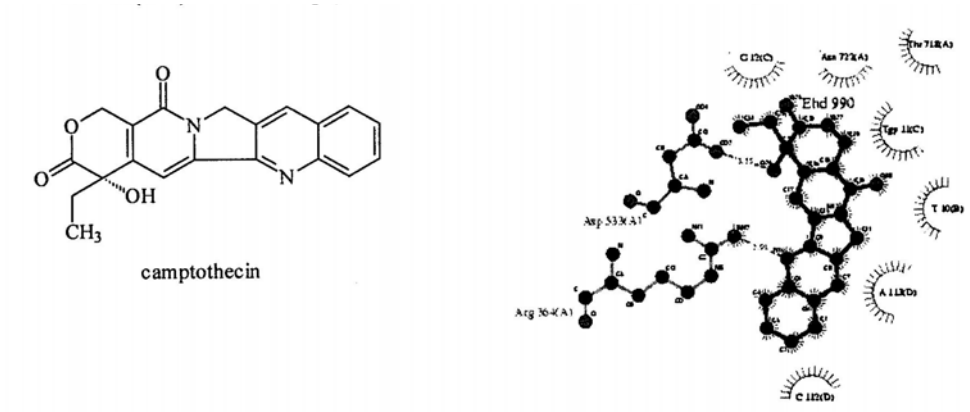
3. Protease enzyme:

-Pdb code =1t8i

-Ligand = camptothecin

Results from docking

Ligand	rsd	Binding energy (kcal/mol)	Docking energy (kcal/mol)
Camptothecin (validate)	0.4750	-14.2	-14.5
Ergosterol peroxide 20S, 24R		-11.0	-9.29
Ergosterol peroxide 20R, 24R		**-8.73**	**-9.45**



The binding sites of topoisomerase I enzyme are Asn 352, Arg 364, Lys 532, Asp 533, Asn 722 and Tyr 723.

3.1 1t8i docking with camptothecin (redock)

1. N of camptothecin was able to bind with H(NH) of Arg 364 = 2.746 and 2.164 Å.

2. H(OH) of camptothecin was able to bind with O(C=O) of Asp 533 = 2.516 Å.

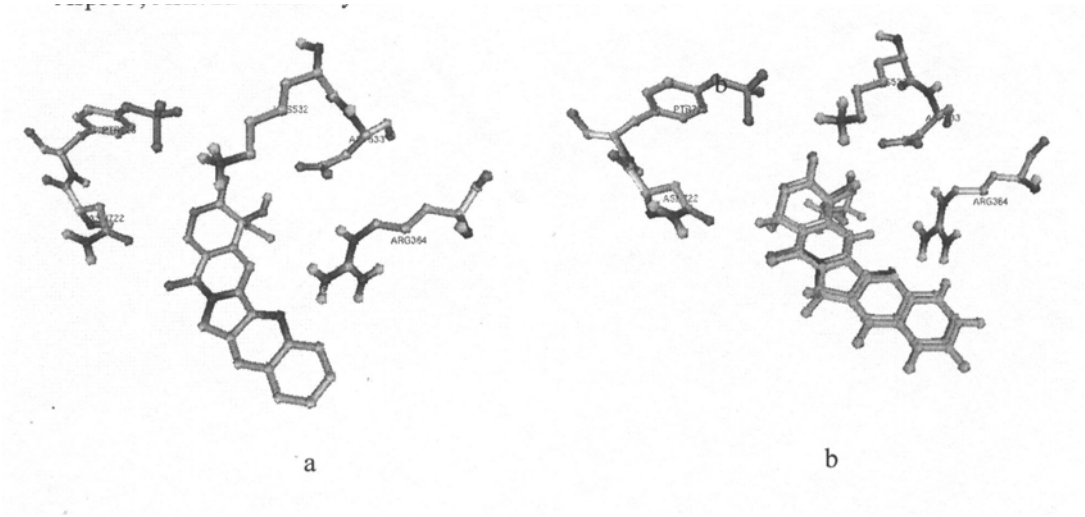


Figure 7 a) Diagram showing hydrogen bond of camptothecin and binding site
b) Superimposition of camptothecin (previous conformation - green) and camptothecin (post docking)

3.2 1t8i docking with ergosterol peroxide (20R, 24R)

1. O(3-OH) of ergosterol peroxide was able to bind with H(NH) of Lys 751 = 2.001 Å.

2. O(5-O-O) of ergosterol peroxide was able to bind with H(NH) of Asn 722 = 3.680 Å.

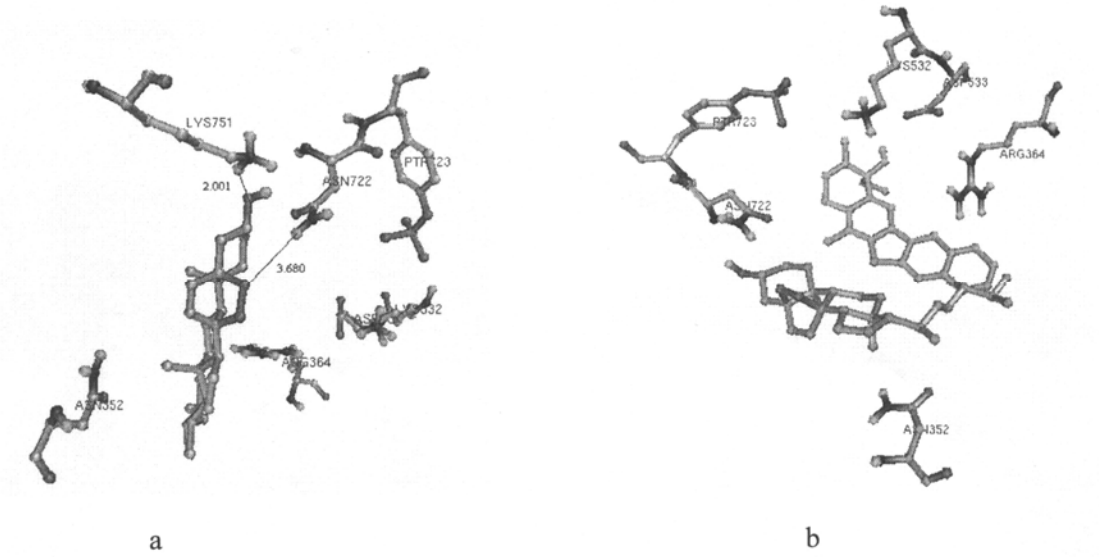


Figure 8 a) Diagram showing hydrogen bond of ergosterol peroxide (20R, 24R) and binding site b) Superimposition of ergosterol peroxide (20R, 24R) and camptothecin (green)

3.3 1hsg docking with ergosterol peroxide (20S, 24R)

No formation of H-bonding was observed.

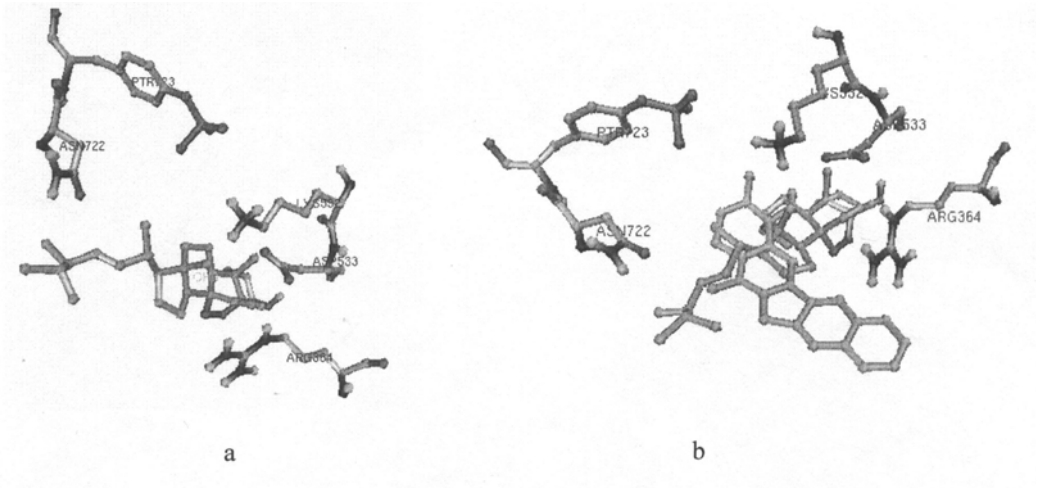


Figure 9 a) Diagram showing ergosterol peroxide (20S, 24R) and binding site b) Superimposition of ergosterol peroxide (20S, 24R) and camptothecin (green)

BIOGRAPHY

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