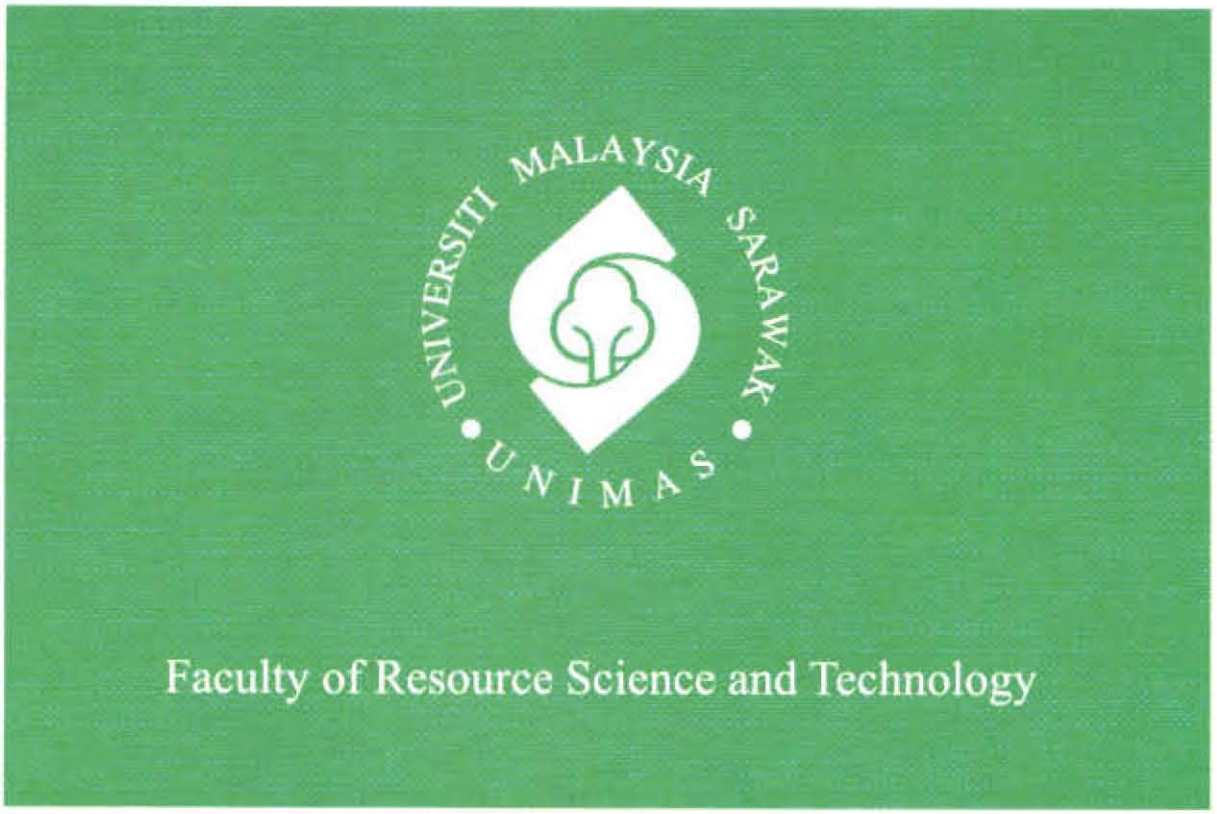

CHEMICAL STUDIES AND BIOLOGICAL ACTIVITIES OF *Alpinia Ligulata*

Farehah Binti Ismail

Bachelor of Science with Honour
(Resourse Chemistry)
2010

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Alpinia ligulata

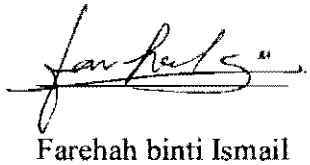
Farehah Binti Ismail

This Final Year Project is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honors
(Resource Chemistry)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2010

Declaration

I hereby declare that no portion of the work referred to in this dissertation has been submitted in support of an application for another degree or qualification to this university or any other institution of higher learning.



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ACKNOWLEDGEMENT

I am very grateful to Allah S.W.T that I have finished my Final Year Project successfully. I would like to dedicate my deep appreciation to my supervisor, Professor Dr. Fasihuddin Ahmad for his supervision and advice. Thank you so much for being such a good supervisor that committed to help student in his guidance. I would also want to thank my seniors who are postgraduate student and all lab assistance for their help and kindness. Special thank dedicated for my fellow friends who helped me in completing this Final Year Project. Thank you so much for your guidance, knowledge and experience, because of that I can finish my Final Year Project successfully. Finally, this Final Year project dedicated for my parent, who always support me. Thank you so much.

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LIST OF ABBREVIATIONS

μg	microgram
μl	microliter
μg/ μl	microgram / microliter
μg/ml	microgram / mililiter
mg/ml	miligram / mililiter
g	gram
mg	miligram
min	minutes
mL	mililiter
M	Molar
%	percent
DCM	Dichloromethane
$CHCl_3$	Chloroform
EtOAc	Ethylacetate
MeOH	Methanol
GC-MS	Gas Chromatography-Mass spectrometry
NMR	Nuclear Magnetic Resonance
FTIR	Fourier Transform Infrared Spectrometer
TLC	Thin Layer Chromatography
PTLC	Preparative thin layer chromatography

Chemical Study and Biological Activity of *Alpinia ligulata*

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ABSTRACT

Alpinia sp. is one of the largest species in Zingiberaceae family. The chemical and biological activity studies were carried out on the stems and leaves of *Alpinia ligulata*. Dichloromethane crude extract of the leaves of *A. ligulata* was further purified using extensive column chromatography. Compound 1 isolated from combined fraction 25-36 was subjected to purification process and further analyzed by using various spectroscopic techniques especially Gas Chromatography-Mass Spectrometry (GC-MS), Fourier Transform Infrared Radiation (FTIR) and Nuclear Magnetic Resonance (NMR). Compound 1 showed one peak at retention time of 40.774 min in the gas chromatogram. FTIR spectra showed the presence of hydroxyl (-OH), carbonyl (C=O), and phenyl group observed at 3312, 1660, and 1600-1450 cm^{-1} respectively. ^{1}H-NMR and ^{13}C-NMR showed the presence of –OH group at C-3 and C-5 position, methoxy group at C-7 and C-4' and aromatic group. Compound 1 gave m/z value of 314.05 corresponding to molecular formula of $C_{17}H_{14}O_6$ and melting point 179-180 °C. From all analysis data and comparison to published data, Compound 1 was characterized as 3,5-dihydroxy-7,4'-dimethoxyflavone or Kaempferol-7,4'-dimethyl ether. Termiticidal activity showed that dichloromethane, chloroform and methanol crude extracts of the stem and dichloromethane crude extract of leaves showed strong termiticidal activity with LC_{50} of 1.259, 63.09, 31.62 and 5.012 µg/mL respectively. All the extract was not toxic against *Artemia salina*.

Key words: chemical study, biological activity, Zingiberaceae, *Alpinia ligulata*

ABSTRAK

Alpinia sp. merupakan salah satu spesies terbesar dalam famili Zingiberaceae. Kajian kimia dan aktiviti biologi telah dijalankan terhadap batang dan daun Alpinia ligulata. Kromatografi turus telah dilakukan terhadap ekstrak mentah diklorometana daripada daun A. ligulata. Sebatian 1 daripada fraksi 25-36 ditulenkan dan dianalisis menggunakan Kromatografi Gas-Spektroskopi Jisim (GC-MS), Infra-merah (FTIR) dan spektroskopi resonan magnetik nuklear (NMR). Sebatian 1 menunjukkan satu puncak pada minit ke 40.774 dalam chromatogram gas. Spektra FTIR menunjukkan kehadiran kumpulan hidroksil (-OH), karbonil (C=O) dan fenil pada gelombang 3312, 1660 dan 1600-1450 cm^{-1}. Spectra ^{1}H NMR dan ^{13}C-NMR menunjukkan kehadiran kumpulan –OH pada posisi C-3 dan C-5, kumpulan metoksi pada posisi C-7 dan C-4'dan kehadiran kumpulan fenil. Sebatian 1 mempunyai berat molekul 314.05 yang merujuk kepada molecular formula $C_{17}H_{14}O_6$ *dan mempunyai takat lebur 179-180 °C. Setelah dianalisis menggunakan semua data dan perbandingan dengan data yang telah diterbitkan, Sebatian 1 adalah 3-5-dihidroksi-7,4'-dimetoksiflavon atau kaempferol-7,4'-dimetil eter. Ujian aktiviti anai-anai menunjukkan ekstrak mentah diklorometana, kloroform dan metanol daripada bahagian batang, dan ekstrak diklorometana daripada bahagian daun mempunyai aktiviti anti anai-anai yang sangat baik dengan kepekatan perencatan LC_{50} masing-masing 1.259, 63.09, 31.62 dan 5.012 µg/mL. Semua ekstrak mentah tidak memberi sebarang kesan ketoksikan terhadap Artemia salina.*

Kata kunci: kajian kimia, aktiviti biologi, Zingiberaceae, Alpinia ligulata

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

Alpinia sp. is a very common plant in the forest and Malay villages. There are more than 35 known species of *Alpinia* in Malaysia (Holtum, 1950). This includes ten endemic species from Peninsular Malaysia, ten endemic species from Borneo and 15 species are widespread in Malaysia and Borneo (Holtum, 1950; Lim, 1972; Kam, 1982; Smith, 1985, 1986, 1987, 1988, 1989; Turner, 1995; Theilade, 1998; Theilade & Mood, 1997; Sasaki & Nagamasu, 1998, 2000; Takano, 2001). *Alpinia* sp. was divided into several distinct groups according to the size and structure of the flower and the existence of inflorescence bracts. *A. galanga* and *A. conchigera* is well known widespread species in Malaysia with the local name 'lengkuas' or 'galangal' and 'lengkuas ranting' respectively. The rhizome of *A. galanga* have similar characteristic with lemon and ginger smelling and commonly used as flavoring agent and spice in food. In spite of local 'galangal', there are other *Alpinia* sp. with hardly noticeable flower such as *A. hansenii*, *A. glabra* and *A. aquatica* (Boyce, 2006).

Some of *Alpinia* spp. such as *A. aquatica* and *A. purpurata* are used for ornamentals purposes because of their flashy flowers. They are also traditionally used as stomachic, febrifuge, cough, mixture for after childbirth (Fasihuddin & Hasmah, 1993), treating colds, invigorating the circulatory system, and reducing swellings (Kong *et al.*, 2000). The decoction of *A. galanga* leaves can be used to bath woman after childbirth, while, rhizomes of *A. galanga* infusion mixed with coconut milk and honey are used to cure anemia (Fasihuddin & Hasmah, 1993).

In addition, *A. katsumadai* and *A. oxyphylla* have been used in traditional Chinese medicine as digestive stimulant and endocrine agent. A. *katsumadai* known for their aromatic characteristic and used to treated distention, nausea, vomiting, poor appetite and greasy tongue coating, while A. *oxyphylla* has the capability to threat systemic exhaustion, fear of cold, cold extremities, sore and weak lower back (Ehrman *et al.*, 2007).

Besides, *A. nigra* shoot is considered to have anthelmintic properties and the aqueous crude juice is consumed by the natives of the north-east India, Tripura in particular as a popular cure against intestinal worm infections (Roy and Swargiary, 2009). The crude extract from this plant are reported to cause destruction of surface tegument which leading to intestinal parasite paralysis and death. The plant extract is also responsible for distortion and disorganization of cytoplasmic organelles and vacuolization of tegument in parasitic worms (Roy & Swargiary, 2009).

These traditional uses and their effectiveness might be explained by the occurrence of biologically active constituents known as secondary metabolites in *Alpinia* sp. such as labdene diterpenes, sesquiterpenes, diarylheptanoid and flavonoids. The occurrence of secondary metabolite such as labdene diterpenes which is one of the terpenes derivatives are known to be insecticidal especially termiticidal (Cseke *et al.*, 2006). The presences of diarylheptanoid also lead to the new antiplatelate discoveries. The new secondary metabolite discoveries from *Alpinia* sp. with specific medicinal properties are believed to be a lead compound to the new drugs (Dong & Chen, 1998).

Zingiberaceae has a rich source of compounds of phytochemical interest. Plants from this family have been reported to have anti-inflammatory, antioxidant and anticancer

properties (Khairunnisa, 2008). Thus, this present study focus on wild *Alpinia* sp. which have not been exploited before which is *Alpinia ligulata*. The phytochemical studies and biological activity of *Alpinia ligulata* will be carried out in this research in order to isolate and characterize their secondary metabolites constituent.

1.2 OBJECTIVES

The objectives of this research are

a) to isolate, purify and characterize secondary metabolites of *Alpinia ligulata*

b) to investigate the biological activity of the crude extract, semi-pure compound and pure compound especially toxicity to Brine shrimp (*Artemia salina*) and termiticidals activity against *Coptotermes* sp.

CHAPTER 2

LITERATURE RIVIEW

2.1 ZINGIBERACEAE FAMILY

The family of Zingiberaceae is the largest family in the order of Zingiberales with 53 genera and over 1200 species (Kress *et al.*, 2002). There are 232 species of Zingiberaceae recorded in Peninsular Malaysia and Borneo including 35 species of *Alpinia sp.* (Holtum, 1950; Lim, 1972; Kam, 1982; Smith, 1985, 1986, 1987, 1988, 1989; Turner, 1995; Theilade, 1998; Theilade & Mood, 1997; Sasaki & Nagamasu, 1998, 2000; Takano, 2001). They are perennial herbs with tuberous rhizomes, rarely ephyphytic. The leaves are lanceolate, usually distinctious, sometime spirally arranged and often sheathed. The flower is irregular, bisexual, often in dense, head-like inflorescences, bracteates. Perianth-segments three plus three; outer tubular calyx-like; inner is corolla like. They consist of one fertile stamen, one petal-like labellum but sometimes more than one in rare case which is the side ones may be petal-like. Their inferior ovary consists of ovule which is capsule with a few seeds (Keng, 1987).

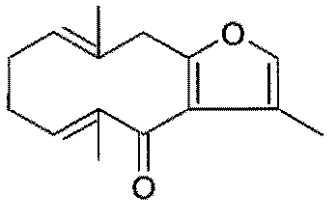
Member of Zingiberaceae are known for their role as spice, flavoring agent and as medicinal herbs. Rhizomes of several species are also used as insect repellants. Many compounds with novel structures and biologically active compound have been identified from this plants family (Pancharoen *et al.*, 2000). Table 2.1 shows some species from Zingiberaceae family and their traditional uses.

Table 2.1: Uses of some species from Zingiberaceae in traditional medicine

Species	Medicinal uses	Reference
Costus speciosus	Used to treat chicken pox, high fever, cough and fungal infection	Fasihuddin & Hasmah, 1993
Curcuma domesctica	Rhizomes used for stomach illness, fever, skin infection and cough	Fasihuddin & Hasmah, 1993
Etlingera giseke	Used to treat earache, while leaves are applied for cleaning wound	Ibrahim & Setyowati, 1999 cited in Chan *et al.*, 2008
Hedychium coronarium	A decoction of the stem near the rhizome may be used as a gargle and the juice of the chewed stem is applied to swelling	Burkill, 1966; Perry, 1980 cited in Jasril *et al.*, 2003
Kaempferia galangal	Rhizome used for malaria, stomach illness, gastric and high fever	Fasihuddin & Hasmah, 1993
Zingiber officinale	Use for women after birth, cough, stomach illness and to cure wound	Fasihuddin & Hasmah, 1993

Members of the Zingiberaceae family have attracted many continuous phytochemical studies due to their considerable importance as medicinal plants. Previous study reported the isolation of various secondary metabolites which includes furanodienone **1**, xanthorrhizol **2** from the rhizomes of *Curcuma xanthorrhiza*, dehydrocurdione **3** and zederone **4** from *Curcuma zedoaria*. Cinnamic acid derivatives such as ethyl ester cinnamate **5** and p-methoxycinnamate **6** had been isolated from *Kaemferia galangal*, while flavanones pinostrobin **7**, alpinetin **8**, pinocembrine **9** and chalcone cardamonine **10** from *Kaemferia pandurata* (Pandji *et al.*, 1993). Kaempferol derivatives, which are 7,4'-dimethoxy-3,5-

dihydroxyflavone or kaempferol 7, 4'-dimethyl ether **11**, together with 5,7,4'-trimethoxy-3-hydroxyflavone **12**, 3,7,4'-trimethoxy-5-hydroxyflavone **13**, 3,4'-dimethoxy-5,7-dihydroxyflavone **14** were isolated from the rhizome of *Hedychium thyrsiforme* (Jasril *et al.*, 2003). Previous study also reported the isolation of diarylheptenone **15**, paradol **16**, phenylpropanoid **17** and diterpenes **18** from Z. *officinale* (Ma *et al.*, 2004).



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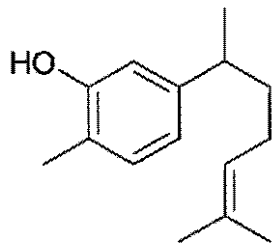

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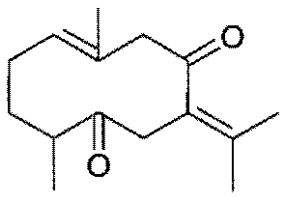

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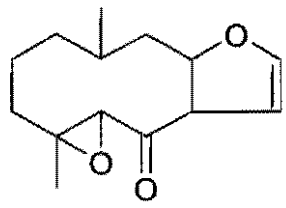

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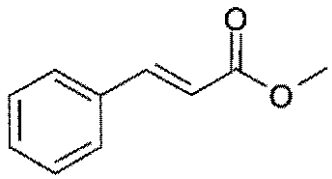

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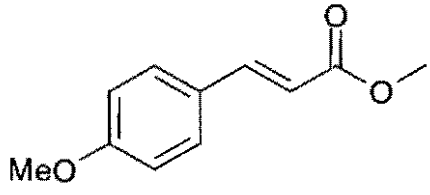

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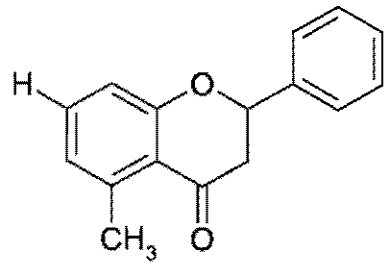
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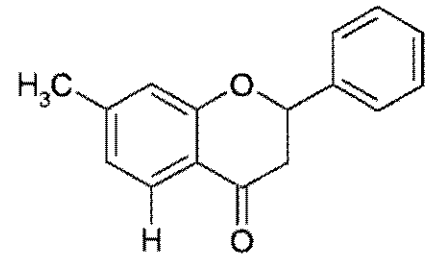
CH3
O

7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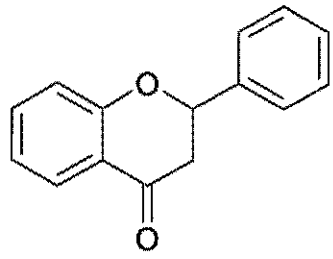
O
H
O

8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O

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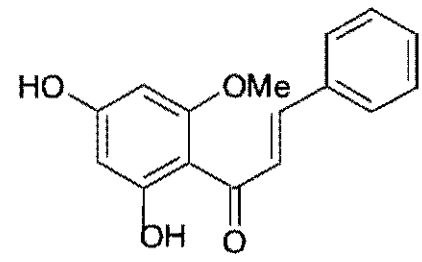
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O

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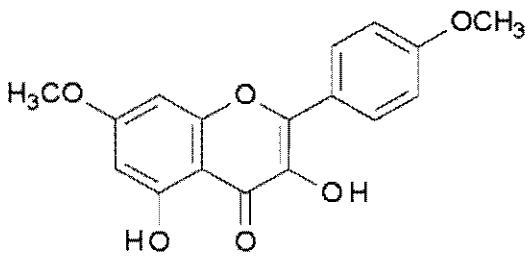
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HO
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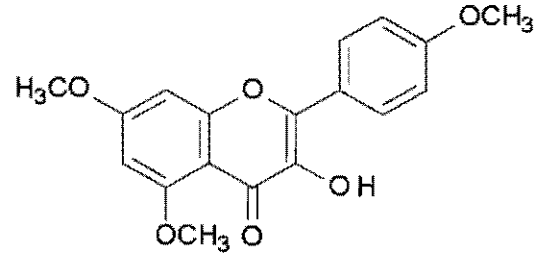
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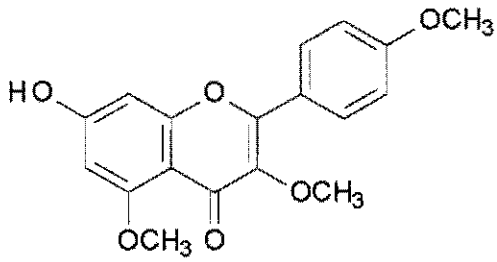
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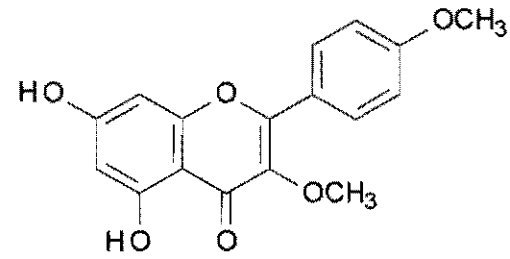
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HO
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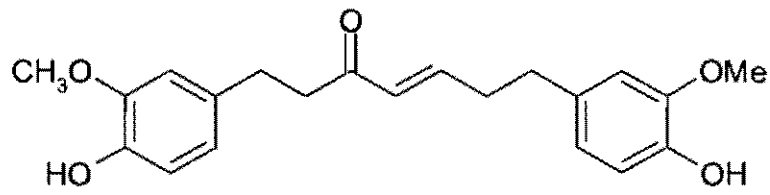
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OH

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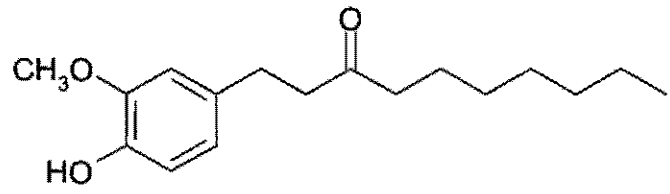
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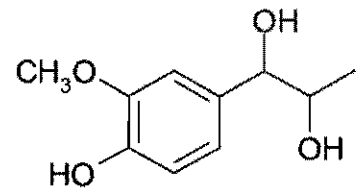
16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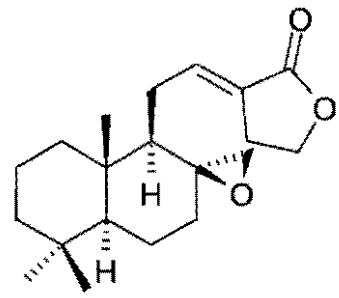
17



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2.2 *Alpinia* sp.

Member of Zingiberaceae are known for their role as spice, flavoring agent and as medicinal herbs. Rhizomes of several species are also used as insect repellants. Many compounds with novel structures and biologically active compound have been identified from this plants family (Pancharoen *et al.*, 2000). Table 2.1 shows some species from Zingiberaceae family and their traditional uses.

Most *Alpinia* species are widely used as traditional herbal medicines in South East Asia region such as China, India, Indonesia and also Malaysia. In China, *Alpinia* sp. is used for relieving stomachache, treating colds, invigorating the circulatory system, and reducing swellings (Kong *et al.*, 2000). While in India, *A. galangal* is used to treat bronchitis and heart disease (Barik *et al.*, 1986). The decoction of *A. galanga* leaves can be used to bath woman after childbirth, while infusion of rhizomes mixed with coconut milk and honey can be used to cure anemia. Their rhizomes can also be used to treat skin infection by pounding them with rice, garlic, lime juice, and vinegar. The mixture can be applied directly to the infected skin. The decoction of *A. galanga* fruits can be drink to treat stomach illness and diarrhea (Fasihuddin & Hasmah, 1993).

The essential oil of the leaf of *A. zerumbet* is used for high blood pressure and as a heart tonic in Brazilian herbal medicine. *A. zerumbet* also considered as balsamic, diuretic, and stomachic and traditionally used for colds and flu, fevers, flatulence, stomach problems and indigestion (Taylor, 2006). Decoction of *A. zerumbet* has been used for bathing to reduce fevers (Elzaawely *et al.*, 2007b). The uses of some *Alpinia* sp. are simplified in Table 2.2.

Table 2.2: Several uses of *Alpinia* sp. in traditional medicine

Species	Medicinal Uses	References
A. calcarata	Used to threat diabetis, rheumatism, fever and stomachache	Hema & Nair, 2009
A. conchigera	Treatment for fungal infection and to relieve gastro-intestinal disorder	Halijah Ibrahim *et al.*, 2009; Athamaprasanga *et al.*, 1994
A. galangal	Used to treat stomach disorders and after childbirth preparation	Fasihuddin & Hasmah, 1993
A. officinarum	Used extemally for skin infections, skin cancer and gum diseases, and internally for digestive upsets, chronic gastritis, and gastric ulceration, epigastric and rheumatic pain	Bown, 1995 cited in Yasukawa *et al.*, 2008
A. speciosa	Seeds are used as aromatic stomachic and spice	Tsu, 1988; Teng, 1990; Wang, 1972 cited in Fujita *et al.*, 1993
A. katsumadai	Treatment for stomach disorders	Ngo & Brown, 1997
A. blepharocalyx	Treatment for stomach disorders	Ali *et al.*, 2000

A. flabellate	The leaves of this plant have been used as a wrapping and flavoring material for foods on the islands of Iriomote and Ishigaki, Okinawa, Japan	Tesaki *et al.*, 2001
A. oxyphylla	Medicine for intestinal disorder and urosis	Morikawa *et al.*, 2002
A. pricei	Used to relieve symptoms of bronchitis, measles, rubella and cholera	Brown, 1995 cited in Yang *et al.*,2008
A. chinensis	Used in traditional medicine as an anti-asthmatic and analgesic	Margaros & Vassilikogiannakis, 2007

2.3 PHYTOCHEMICAL STUDY OF *Alpinia sp.*

Phytochemical investigation of *Alpinia* spp. such as *A. galangal*, *A. conchigera*, *A. calcarata*, *A. malaccensis*, *A. katsumadai* and *A. blepharocalyx* resulted in the isolation of various bioactive secondary metabolites. Labdene type diterpenoids, sesquiterpenoids, diarylheptanoids, chalcones and flavonoids are the most common bioactive compound isolated from the rhizome of *Alpinia* sp. (Hasnah *et al.*, 1994; Ngo & Brown, 1998; Ali *et al.*, 2001a; Nuntawong & Suksamran, 2008; Hema & Nair, 2009).

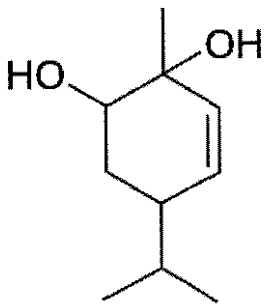
2.3.1 TERPENES

Terpenes have the basic repeating five carbon units derived from isoprene units which is $CH_2=CH(CH_2)_3$. This give rise to the structure that can be divided into isopentane units. Terpenes are well known constituent in essential oil. The biosynthesis of terpenes is through mevalonic acid (MVA) and methylerythritophosphate (MEP) pathways. Both pathways produced isopentenyl disphosphate (IPP); the main building block of terpenes. Terpenes can be classified as hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and carotenoids (C_{40}) (Fasihuddin & Hasmah, 1993; Cseke *et al.*, 2006).

Monoterpenes

Monoterpenes are major compound found in many essential oil (Cseke *et al.*, 2006). Monoterpenes have been isolated from *A. galangal* (Pooter *et al.*, 1985), *A. densibracteata* (Sy & Brown, 1996), *A. zerumbet* (Elzaawely *et al.*, 2007a) and *A. katsumadai* (Ngo & Brown, 1998). Oxygenated 3, 4-dihydroxybisaboladiene **19** has been isolated from the dichloromethane extract of *A. densibracteata* (Sy & Brown, 1996) while, linalool **20**, α-

pinene **21**, limonene **22**, borneol **23** had been isolated from *A. galangal* (Pooter *et al.*, 1985) and *A. zerumbet* (Elzaawely *et al.*, 2007a), myrcene **24** had been isolated from *A. galangal* (Pooter *et al.*, 1985) and *A. conchigera* Griff. (Halijah *et al.*, 2009).



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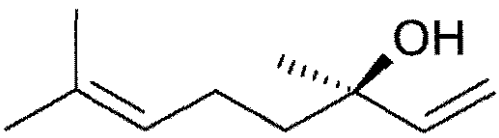

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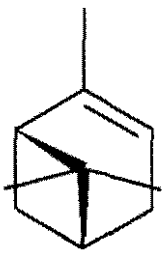

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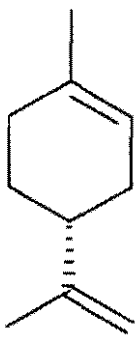

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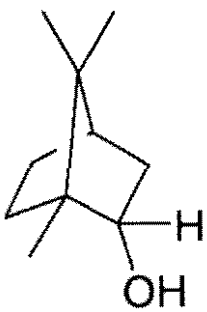

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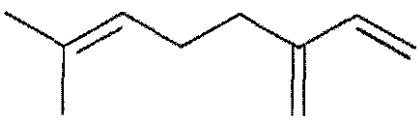
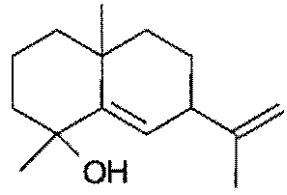


24

Sesquiterpenes

There are many novel sesquiterpenes isolated and characterized from *Alpinia* species. Generally sesquiterpenes have shown antifungal and antibacterial activities. Oxyphllol A **25**, B **26**, and C **27** isolated from *A. oxyphlla* shown antiallergic properties (Morikawa *et al.*, 2002), while furopelargone B **28** and humulene epoxide **29** have been isolated and characterize from *A. formosana* (Itokawa *et al.*, 1987). Nootkatone **30** from the essential oil of fruits of *A. oxyphylla* is a type of ketone (Xie *et al.*, 2009). Nootkatone is a flavorant used for flavoring food and tobacco (Chen *et al.*, 2006 cited in Xie *et al.*, 2009).



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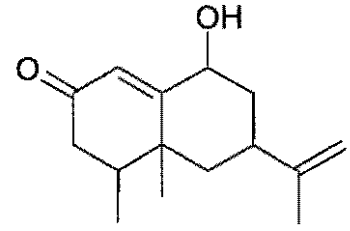

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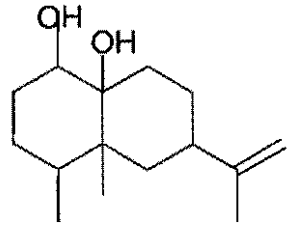

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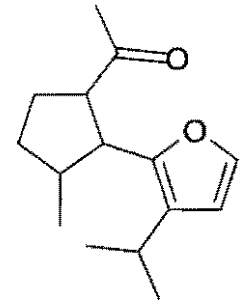

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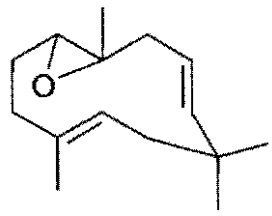

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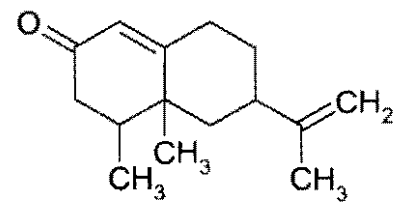
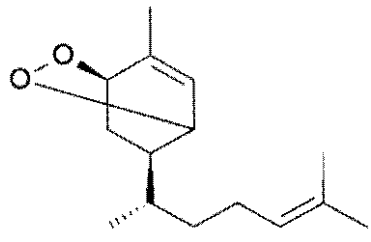


30

Besides, oxygenated bisabolane sesquiterpenes also have been isolated from *A. densibracteata* which is colorless oil compound called 1,4-Epidioxy-bisabola-2, 10-diene **31** , and gum like compound such as 3-Hydroxy,l I-hydroperoxy-bisabola-1,9-diene **32** and 3,4-Dihydroxy-bisabola-1, 10-diene **33** (Sy & Brown, 1997b). Another compound found in this class which is new calamenene sesquiterpene, (–)-(1R,4S)-8-hydroxy-13-calamenenoic acid **34** also isolated from Alpinia species which is *A. oxymitra* from Thailand (Jitsaeng *et al.*, 2009).



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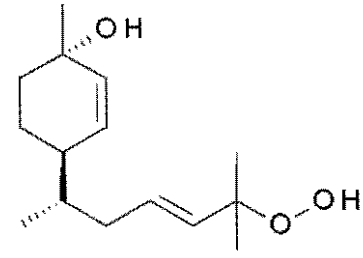

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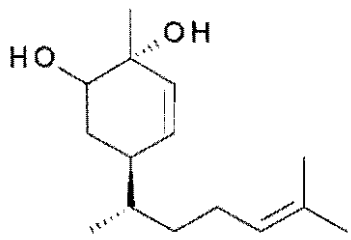

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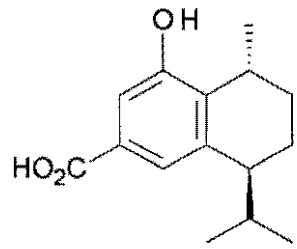


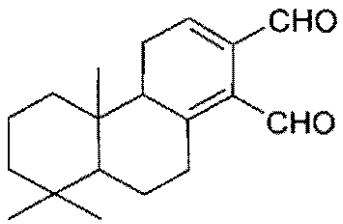
34

Diterpenes

Diterpenes consist of 20 carbon atom and can be found easily in all of the plant resin. Diterpenes are not considered as essential oil but they are considered being resins because of their higher boiling point (Cseke *et al.*, 2006). Diterpene can be considered as acyclic, monocyclic, bicyclic and many more (Fasihuddin & Hasmah, 1993).

Labdene type diterpenoids

Labdene type diterpenoids are derived from diterpenes. Several labdene diterpenes such as labda-8(17),12-diene-15,16-dial **35** and coronarin E **36** have been isolated from *Alpinia* sp. such as *A. javanica* (Hasnah *et al*, 1993), *A. malaccensis* (Nuntawong & Suksamran, 2008), *A. katsumadai* (Ngo & Brown, 1997), *A. densespicata* (Kuo *et al.*, 2009) and *A. calcarata* (Kong *et al.*, 2000; Hema & Nair, 2009). Rel-labd-12-en-15(16)-olid-7-one-8R-spiro-1'-[2S-(2,4,5-trimethoxyphenyl)-3-cyclohexene] **37** a novel labdane diterpene adducted by a phenylbutenoid was isolated from leaves of *A. flabellate* (Tesaki *et al.*, 2001).



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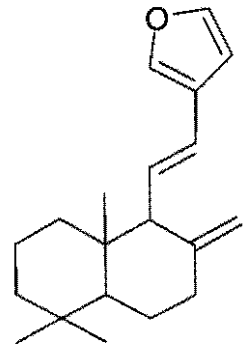

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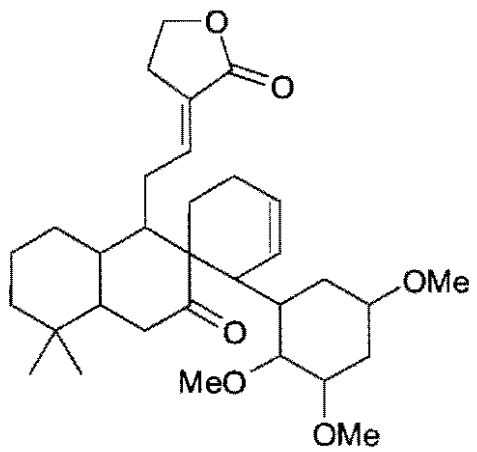
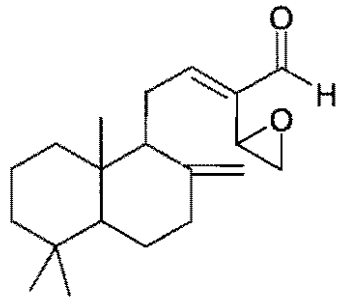


37

14ξ,15-Epoxylabda-8(17),12-dien-16-al *[E]* **38**, 15-Hydroxylabda-8(17),11,13-trien-16-al *[E,E]* **39**, 15-Hydroxylabda-8(17),11,13-trien-16,15-olide *[E]* **40**, 14ξ,15-Dihydroxylabda-8(17),12-dien-16-al *[E]* **41**, 12ξ,15-Dihydroxylabda-8(17)-13-dien-16-al *[E]* **42**, 15-Hydroxy-11ξ,14ξ-peroxylabda-8(17),12-dien-16-al **43** have been isolated from the aerial parts of the medicinal plant *A. chinensis* (Sy & Brown, 1997a).



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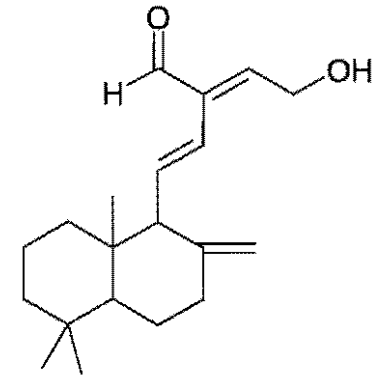

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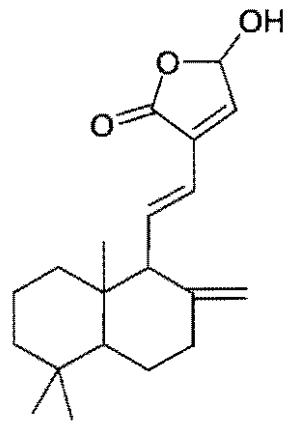

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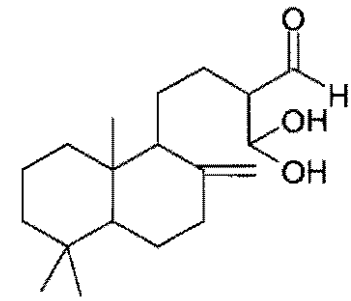

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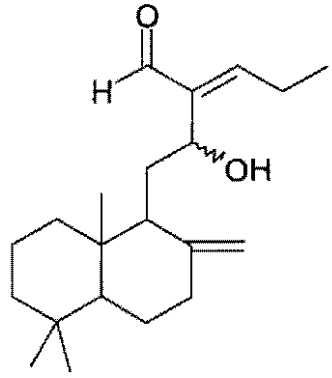

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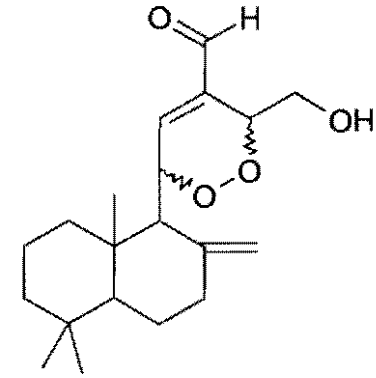


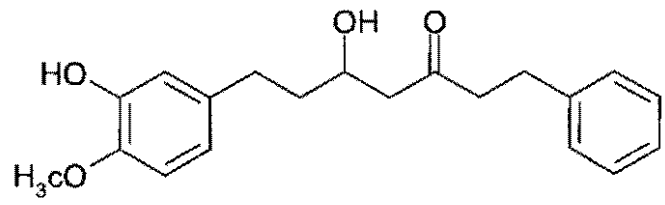
43

2.3.2 PHENOLIC COMPOUNDS

Phenols are aromatic hydrocarbon found to be the most abundance secondary metabolite in plants. Phenol is an aromatic compound which consists of –OH group attach to benzene ring. Phenolic substances are acidic and have water solubility properties. They tend to form ether linkage with carbohydrate residue (Cseke *et al.*, 2006).

Diarylheptanoids

Several diarylheptanoids have been isolated from *A. blepharocalyx* (Ali *et al.*, 2000b), *A. oxyphylla* (Itokawa *et al.*, 1980) and *A. officinarum* (Subramanian *et al.*, 2009). Diarylheptanoid is phenylpropanoid derivatives. Diarylheptanoids is a group of biologically active compound with various biological activity such as antibacterial (Subramanian *et al.*, 2009), antitumor and anti-inflammatory activity (Yasukawa *et al.*, 2008). 5-Hydroxy-7-(4"-hydroxy-3-methoxyphenyl)-1-phenyl-3-heptanone **44** have been isolated from *A. officinarum* (Subramanian et al., 2009), while 1,7-diphenyl-3,5heptanedione **45** was isolated from *A. conchigera* Griff. (Athamaprasanga, *et al.*, 1994). Deoxycalyxin **46**, epicalyxin **47**, calyxin K **48**, calyxin M **49**, calyxin J **50** and neocalyxin A **51** were isolated from *A. blepharocalyx* have biological activity as antitumor. All diarylheptanoids from *A. blepharocalyx* have chalcone and flavanone moiety (Tezuka *et al.*, 2001).



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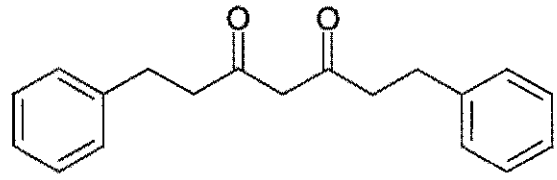

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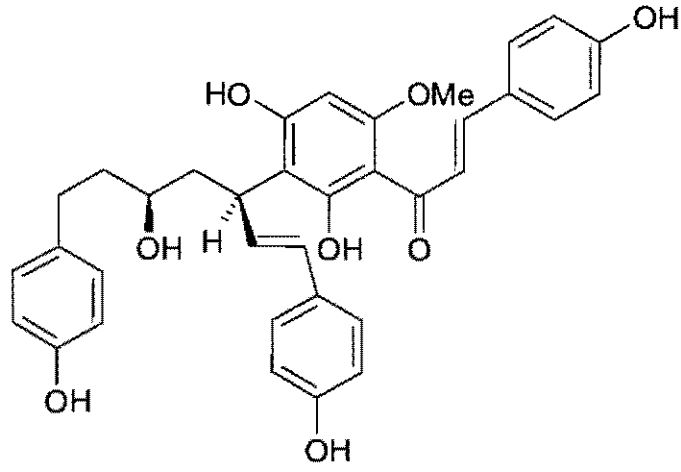

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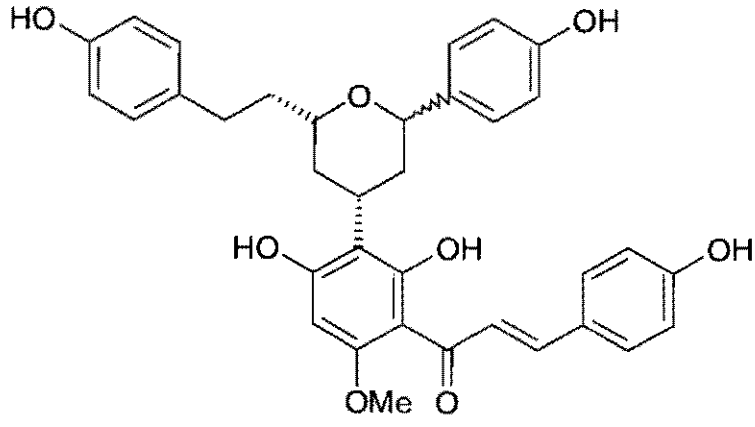

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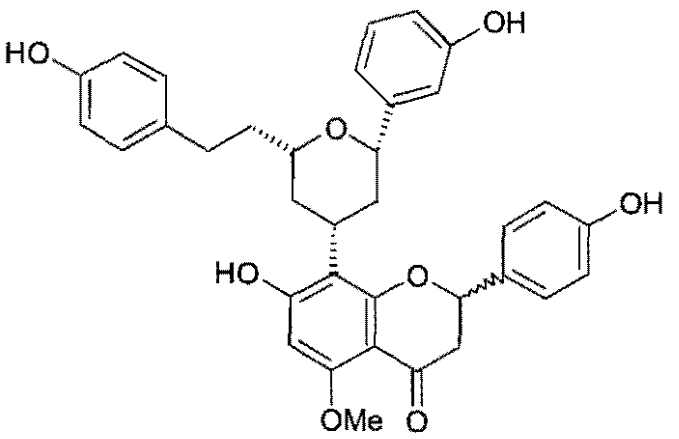

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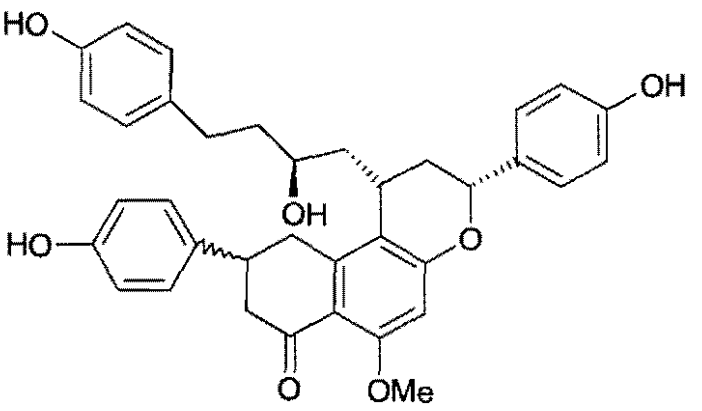

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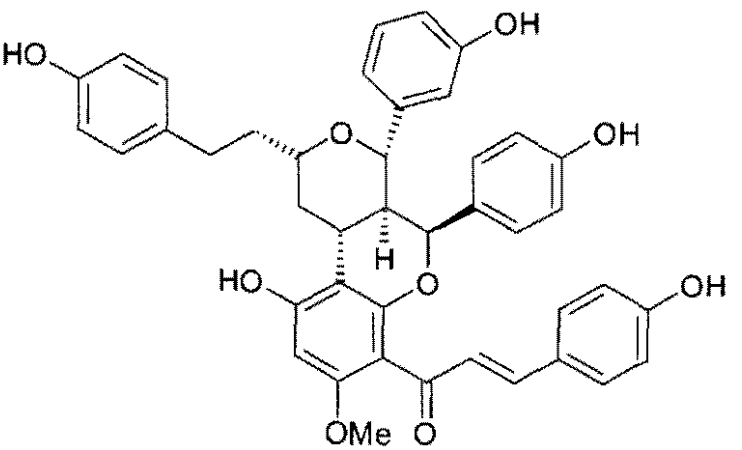

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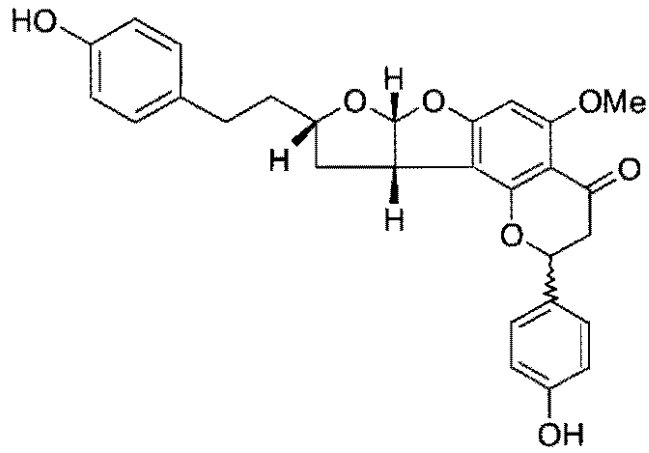
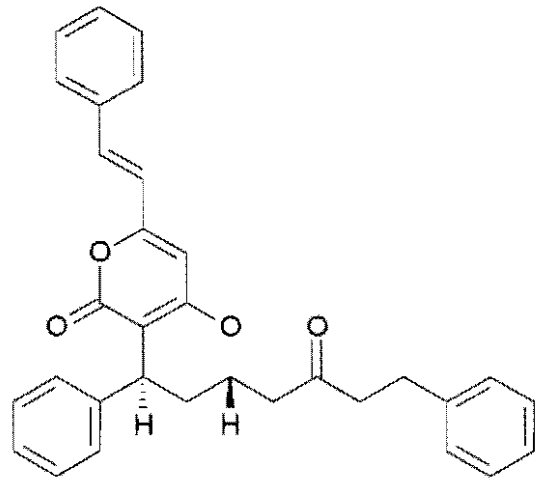


51

Two novel diarylheptanoids named katsumadain A **52** and katsumadain B **53** were isolated from the seeds of *A. katsumadai*. Both of **52** and **53** exist as yellow amorphous solid with molecular formula $C_{32}H_{28}O_4$. This diarylheptanoid was first discovered to have monocyclic α-pyrone moiety and also were investigated to have anti-emetic property (Yang *et.al*, 1999).



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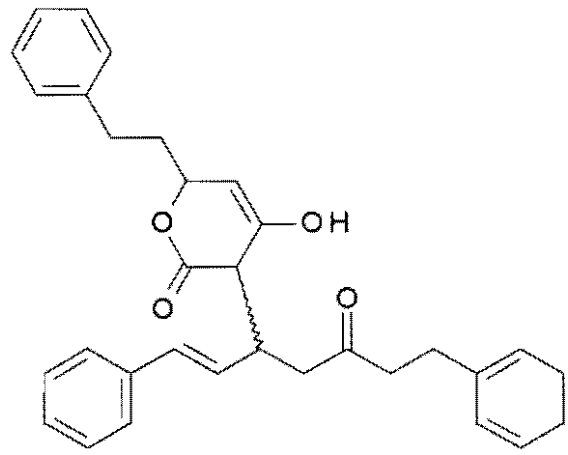
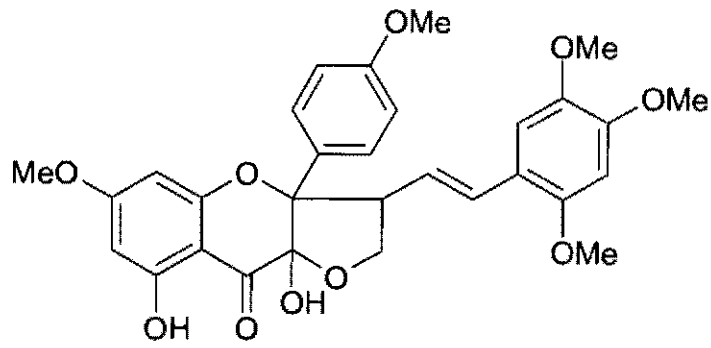


53

Flavonoids

Flavonoid is the most abundance bioactive compound in herbal plant. Previous studies on the leaves of *A. flabellate* afforded of new flavononol compound which has been identified as flavononol which are rel-5-hydroxy-7,4'-dimethoxy-3"*S*-(2,4,5-trimethoxy-*E*-styryl)tetrahydrofuro[4"*R*,5:2,3]flavononol **54** (Kikuzaki & Tesaki, 2002). Phytochemical study on *A. malaccensis* shows the presence of cardamonin **55** and flavanones which is pinocembrine **56** and alpinetin **57** (Nuntawaong & Suksamram, 2008). Galangin **58** was characterized from *A. officinarum* (Lee *et al.*, 2008), while tectochrysin **59** and izalpinin **60** have been isolated from *A. oxyphylla* (Morikawa *et al.*, 2002).



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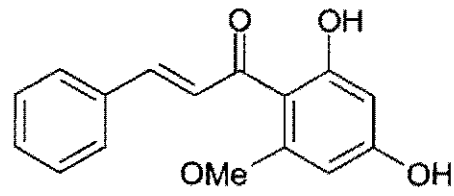

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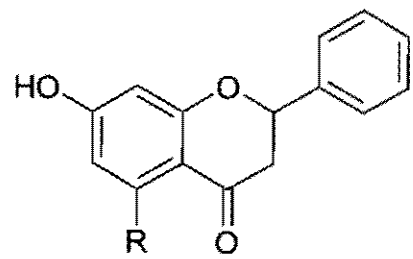
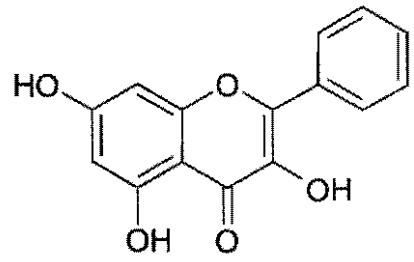


56 R= OH

57 R= OCH_3



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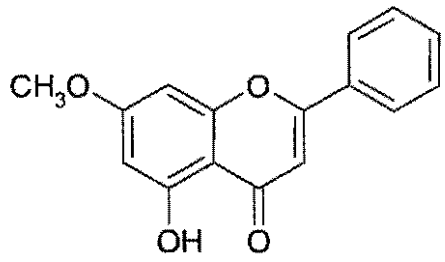
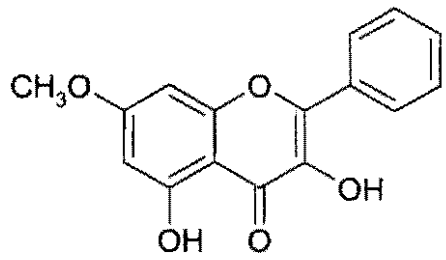


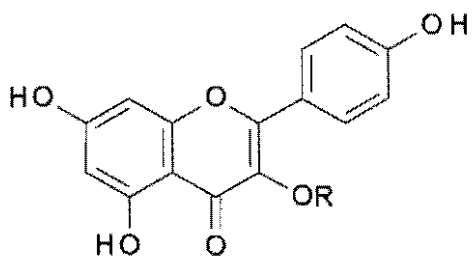
59



60

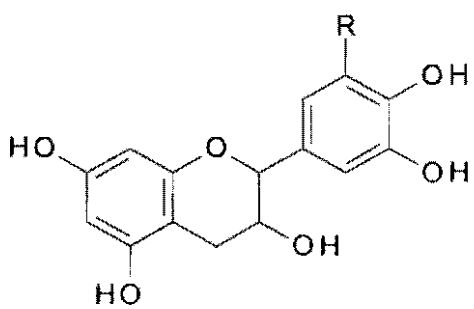
Base on previous phytochemical investigation, two flavone glycosides which are astragalin **57** and kaempferol-3- O-glucuronide **58** were isolated from the seed clusters of *A. nigra*. Many pharmacological studies have reported that 57 and 58 possess several biological activities, including antibacterial and antioxidant activities (Qiau *et al.*, 2007). In addition,

previous phytochemical studies also show that epichatechin **59** and galloepicatechin **60** were isolated from rhizomes of *A. oxymitra* (Jitsaeng *et al.*, 2009).



57 R = glc

58 R = glcUA

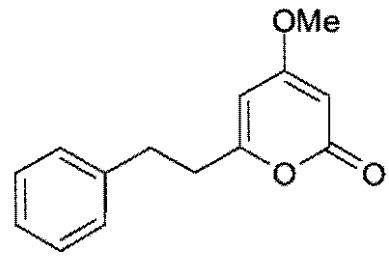


59 R = H

60 R = OH

2.3.3 STYRYL LACTONE

Kavalactone are a type of cyclic ester. The presence of kavalactone **61** was reported by Nuntawong and Suksamrarn (2008) as a major constituent in the rhizomes of *A. malaccensis*. It also presence in the leaves and rhizomes of *A. zerumbet* (Tawata *et al.*, 1996; Mpalantinos *et al.*, 1998 cited in Nuntawong & Suksamram, 2008) and rhizome of *A. speciosa* (Itokawa *et al.*, 1981).



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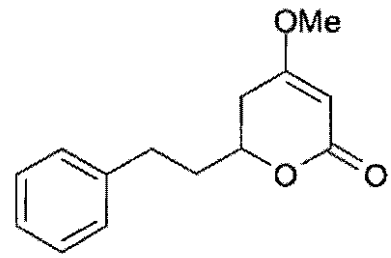
Kavapyrone, dihydro-5,6-dehydrokawain (4methoxy-6-phenethyl-2H-pyran-2-one) **62**, is a major compound in *Alpinia* leaves and it has shown plant growth inhibition against lettuce seeds (Fujita, *et al.*, 1994), insecticidal activity against *Coptotermes formosanus* and antifungal activity against *Pythium* sp. and *Corticium rolfsii* (Tawata *et al.*, 1996).



62

2.4 BIOLOGICAL ACTIVITIES OF *Alpinia* spp.

Natural products from plant have been the primary source of traditional and commercial medicines and also lead to the new drugs discoveries. Most secondary metabolite has specific biological activities that are useful for human. Previous studies shows that compound isolated from *Alpinia* sp. act as antifungal, anti-inflammatory, antimicrobial and anticancer activities. Phytochemical studies discovered many biological activities that can be lead compound. For example, flavonoids can be lead compound in order to develop anticancer drug. *A. pricei* exerts antiproliferative action and growth inhibition on human carcinoma KB cells through a mitochondria-dependent apoptotic pathway (Yang *et al.*, 2008).

The essential oils of *A. galanga* responsible for the characteristic odor as well as for their reported use in traditional medicine are responsible for their anti-amoebic activity and commonly used by AIDS patients in southern Thailand (Sawangjaroen *et al.*, 2006), while phenolic compounds from *A. zerumbet* have high content of antioxidative phytochemicals that may reduce the risk of cancer, cardiovascular disease and many other diseases (Elzaawely *et al.*, 2007b). The *A. galanga* oil also found to be more efficacious than the *Cinnamomum camphora* oil showing antiaflatoxigenic properties toward aflatoxin B_1 (toxic metabolites produce by mycotoxins) even at lower concentrations (Srivastava *et al.*, 2008). *A. galanga* also prove to be effective in lowering serum levels of lipids where as no significant effect was observed in the liver tissue. This indicates a possible role of *A. galangal* compound against various lipid disorders, a risk factor for atherosclerotic cardiovascular diseases and other arteriovascular diseases (Achuthan & Padikkala, 1997).

The plant secondary metabolites also show substantial antifungal action and have been studied in detail with a view of isolating a promising drug. For example, rhizomes of *A. officinarum* have shown to be considerable antifungal activity, especially against *Trichophyton rubrum*, the causative agent of most skin diseases (Ray & Majumdar, 1976). Galangin **58** that has been isolated from *A. officinarum* also showed antimicrobial activities toward methicillin-resistance *Staphylococcus aureus* (human pathogens that causing life-threatening systemic infections such as pneumonia, septicemia, endocarditis, and osteomyelitis) (Lee, *et al.*, 2008). The methanol extract from the rhizomes of *A. officinarum* also showed inhibitory effects on the tumor promoter (TPA) induced inflammatory ear edema in mice (Yasukawa *et al.*, 2008).

Previous study on the underground parts of 15 plant species screened, found that only the rhizomes of *A. carinata* exhibited strong antifungal activity. The minimum inhibitory concentration of it was found to be 3000 ppm. (Kishore *et al.*, 1987). Other biological active compound isolated from *Alpinia* sp. and their biological activities are simplified in Table 2.3.

Table 2.3: Example of some compound isolated from *Alpinia* sp. and their biological activities

Species	Compound (s) isolated	Biological activities	References
A. calcarata	Pinocembrine	Anticancer	Hema & Nair, 2009
A. conchigera	Phenylpropanoid derivatives Chavicol acetate Eugenol acetate	Anti-inflammatory	Yu *et al.*, 1988 cited in Athamaprasanga *et al.*, 1994

A. pricei	Desmethoxyyangonin	Anticancer	Yang *et al.*, 2008
	Cardamonin		
	Flavokawain B		
A. officinarum	Diarylheptanoids	Antitumor	Yasukawa *et al.*, 2008
		Anti-inflammation	
	Galangin	Antimicrobial	Lee *et al.*, 2008
A. oxyphlla	Sesquiterpenoids	Antiulcer	Yamahara *et al.*, 1990 cited in Xie *et al.*, 2009
A. aquatica	p-hydroxycinnamaldehyde	Antibacterial	Norazah Basar *et al.*, 2002
A. blepharocalyx	Diarylheptanoids	Antiplatelet	Dong & Chen, 1998
	Bepharocalyxin D	Antiproliferative	
A. galangal	p-hydroxycinnamaldehyde	Anti-arthritic	Phitak *et al.*, 2009
		Anti-inflammatory	
A. mutica	*Trans*-farnesol	Antibacterial	Norazah Basar *et al.*, 2002
A. zerumbet	Kavapyrone	Termiticidal	Tawata, *et al.*, 1996
		Antifungal	

CHAPTER 3

MATERIALS AND METHODS

3.1 PLANT MATERIAL

Alpinia ligulata used in this study was collected from Sematan, Sarawak. The sample was separated into leaves, stems and rhizomes. Only stems and leaves were used in this extraction purpose. The fresh sample was cut into small pieces and grounded to powder. Voucher specimen was prepared for taxonomical identification and deposited in Universiti Malaysia Sarawak Herbarium.

3.2 GENERAL EXPERIMENTAL PROCEDURES

Column chromatography, silica gel 60 (Merck, 230-400 mesh) was used in order to purify samples. Thin Layer Chromatography (TLC) was performed on SiG 60 F_{254} with 0.25 mm thickness and Preparative Thin Layer Chromatography (PTLC) on SiG 60 F_{254} with 1.0 mm thickness. Spots on TLC plate were visualized using ultraviolet light (UVG-11). Gas Chromatograms were recorded on Gas Chromatography- Flame Ionization detector using Shimadzu GC-17A. Functional groups in the isolated compound were determined using Fourier Transform Infrared spectrometer using KBr dics and recorded on Perkin Elmer GX-FTIR spectrometers. Mass spectra were recorded using Shimadzu QP 5000 Gas Chromatography-Mass Spectrometer (GC-MS). Nuclear Magnetic Resonance (NMR) model JOEL 500 MHz was used to determine the number of proton and carbon. Melting point of the pure compound was measured using electrothermal apparatus (Electrothermal 1A 1900 Operation).

3.3 EXTRACTION

The fresh sample was cut into small pieces. About 1909.0 g of stem and 2989.0 g of leaves of *Alpinia ligulata* were used for extraction. *Alpinia ligulata* leaves and stem was percolated and pulverized with methanol for three days and filtered. This process was repeated for two times. All the methanol crude extract was combined and evaporated to dryness using rotary evaporator. The residue was further partitioned using with increasing polarity in the order of hexane, dichloromethane, chloroform and ethyl acetate. This process was repeated for three times. Each extract was evaporated to dryness and the weight of each extract partition was determined (Houghton & Raman, 1998).

3.4 ISOLATION AND PURIFICATION

3.4.1 Column Chromatography

Chromatography is the primary method for isolation and purification of natural product. This process was carried out using column chromatography. The selected partition was separated using column chromatography (60 cm length and 3.2 cm in diameter). Silica gel 60 (Merck, 280-400 mesh) was used as stationary phase. The column was rinsed with suitable solvent. The column was then filled with slurry of silica gel, which was prepared by adding 220 g of the silica gel into 350 mL of hexane or suitable. Glass rod was used to push the silica gel slowly into the column and to ensure the silica gel becomes compacted and no air bubbles present in the column. Silica gel was rinsed with hexane. About 2.0 g hexane crude extract was dissolve and coated with 5.0 g silica gel before introduced to the column. A number of fractions (eluent of 25 mL each) were collected and tested using Thin Layer Chromatography (TLC). Fraction with similar retention factor, R_f were combined and

evaporated to dryness using rotary evaporator. The weight for each fraction was recorded. Similar procedures were repeated for dichloromethane, chloroform, ethyl acetate and methanol crude extract which was dissolved in dichloromethane, chloroform, ethyl acetate and methanol respectively and coated with 5.0 g silica gel before placed in the column. (Houghton & Raman, 1998).

3.4.2 Thin Layer Chromatography (TLC)

All the crude extract was observed using TLC. A portion of each crude extract from hexane, dichloromethane, chloroform, ethyl acetate and methanol were dissolved with suitable solvent and spotted on the TLC plate. The TLC plate were then developed using suitable solvent system to separate the compounds. The spots on TLC plate were observed under the ultraviolet light or sprayed with iodine indicator. The retention factor, R_f for all the spots were calculated and recorded (Houghton & Raman, 1998).

3.4.3 Preparative Thin Layer Chromatography (PTLC)

PTLC are used to further purify of crude extracts normally after column chromatography. Preparative TLC plate 60 F_{254} (1.00 mm thickness), 20 x 20 cm size are used in to separate the spots. The PTLC plate was developed with suitable solvent system to separate the fractions. Each bands separated on the PTLC were scratched into different beaker. The scratched silica gels were dissolved with suitable solvent, filtered and evaporate to dryness using rotary evaporator.

3.5 STRUCTURAL ELUCIDATION

3.5.1 Fourier Transform Infrared Spectrometer (FTIR)

Sample for FTIR was prepared using standard technique (Houghton & Raman, 1998). About 1.0 mg of sample was ground with 100.0 mg of potassium bromide (KBr). The mixture was then compressed to create a 1 mm thin pallet. Perkin-Elmer GX-FTIR spectrometer was used to measure the absorption in the range of 400 cm^{-1} to 4000 cm^{-1} (Pavia *et al.*, 2002).

3.5.2 Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (Shimadzu QP 5000 Series) analysis was performed using non polar column, DB-5 crosslinked (30 m long x 0.25 mm ID x 0.25 µm film thickness composed of 5 % phenyl methyl polysiloxane). The initial temperature was 50 °C. After two minutes, the temperature was increased to 300 °C with the rate of 6.5 °C/min. The temperature was then maintained for ten minutes. The temperature of the injector and detector was set to 280 °C and 300 °C respectively. Helium was used as the carrier gas. Exactly 1 µL of pure compound was diluted with dichloromethane and injected into GC-MS (Houghton & Raman, 1998; Pavia *et al.*, 2002).

3.5.3 Nuclear Magnetic Resonance (NMR)

NMR is used to identify number of proton and carbon and also to determine their environment. The ^{1}H and ^{13}C NMR spectra for pure component obtained was recorded on JEOL 500 MHz NMR using suitable solvent. The measurement for ^{1}H was performed at 500 MHz, while for ^{13}C-NMR it was performed at 125 MHz (Cseke *et al.*, 2006).

3.6 BRINE SHRIMP TOXICITY AND ANTI TERMITES TEST

3.6.1 Brine Shrimp test

Brine shrimp toxicity test developed by McLaughlin (1991) was used to determine the toxicity of crude extracts and pure compounds. About 2.0 g of *Artemia salina* egg were added into an aquarium with 1 L of seawater (salinity 22 ppt, temperature 28 °C and pH 7.5) for hatching process. An aerator was used and placed inside the container to provide air continuously. This process was carried out in 48 hours. Exactly 3.0 mg of crude extract and partitions were dissolved in 3 mL methanol. Exactly 5 µL, 50 µL and 500 µL samples were pipetted into the test tube in triplicate and the solvent was evaporated to dryness. Exactly 5 mL of seawater was added into each test tube to give final concentration of 1µg/mL, 10 µg/mL and 100 µg/mL. Exactly 2 mL of the solution were transferred into NUNC multidish and ten *Artemia salina* was added. The numbers of survivors were observed for every 1, 2, 3, 6, 12, and 24 hours of contact. After 24 hours in contact, the number of survivors larvae in each test tube were countered and the percentage of death were plotted against the concentrations of samples (on a log scales) and LC_{50} values were determined. Seawater was used as negative control for this experiment.

3.6.2 Termiticidal Activity

Termiticidal test was performed using method developed by Sakasegawa *et al.*, (2003). *Coptotermes* sp. was used for the termiticidal test. The termites were cultured for two to three days at room temperature. For the termiticidal test, 25 mm filter paper were used and placed in each six holes multidish (3 rows x 2 lines, hole diameter 25 mm). Test sample with 1 mg, 10 mg and 100 mg were diluted with 1 mL methanol to obtain concentration of 100

ppm, 10 ppm, 1 ppm. Exactly 50 mL of the diluted sample are placed on the filter paper and dried completely. Then, accurately 100 mL of distilled water were added into entire six multidish holes. Six termite (comprise of five workers and one soldier) were added in each hole. The multidish were then closed tightly and kept at 27 °C in an incubator in a dark place. The number of survivors were counted for each day. The termiticidal activity was performed in nine replicates (3 holes/multidish x 3 replicates) for each concentration. Concentration required to kill 50 % of the termites' population were calculated.

CHAPTER 4

RESULT AND DISCUSSION

4.1 EXTRACTION

Table 4.1 show the percentage yields of crude methanol extract of the *Alpinia ligulata* leaves and stem. The stem gave slightly higher yield compared to the leaves.

Table 4.1: Percentage yield of crude extract of *A. ligulata*

Parts used	Weight of fresh sample (g)	Weight of dried sample (g)	Weight of crude extract (g)	Yield of extract (%)
Leaves	2989.4	975.4	53.70	1.740
Stem	1909.0	1065.2	34.58	2.036

Based on previous data, dried leaves of *A. flabellata* yield 3.4 % of greenish residue of chloroform crude extract (Kikuzaki & Tesaki, 2001), leaves of *A. speciosa* yield 8.9 % (Fujita *et al.*, 1994) while extract from aerial part of *A. densibracteata* 1.47 % of green gum (Sy & Brown, 1997b). This indicated that the yield obtained from extraction of *A. ligulata* stem and leaves using methanol gave slightly lower yield compare to the other extraction of *Alpinia* sp.

The methanol crude extract was then subjected to solvent partition in the order of increasing polarity starting with hexane, dichloromethane, chloroform and ethyl acetate. Solvent partition is a simple method used in the initial stage to separate compounds based on their relative solubility in two different immiscible liquids and to reduce complexity. It is an extraction of a substance from one liquid phase into another liquid phase or in other word

separation of a compound from a crude extract of methanol or other polar solvent by preferentially dissolving that crude in a suitable solvent. By this process compound in the methanol crude extract can be separated to polar, semi-polar and non-polar compounds.

After the solvent partition process, the crude extract obtain from each solvent was evaporated to dryness by using rotary evaporator. The percentage yields for each crude partitioned was calculated and shown in Table 4.2.

Table 4.2: Percentage yield of crude extract of *A. ligulata* after solvent partition

Parts used	Solvent used	Color of the extractive (g)	Crude extract dried weight obtain (g)	Percentage yields (%)
Leaves	Methanol (MeOH)	Orange	1.050	0.035
	Hexane	Dark green	6.651	0.223
	Dichloromethane (DCM)	Brown green	18.89	0.632
	Chloroform ($CHCl_3$)	Brown	10.24	0.343
	Ethyl acetate (EtOAc)	Light brown	8.810	0.295
Stem	Methanol (MeOH)	Brown	14.378	0.753
	Hexane	Dark green	3.422	0.179
	Dichloromethane (DCM)	Green	2.436	0.128
	Chloroform ($CHCl_3$)	Light brown	1.101	0.058
	Ethyl acetate (EtOAc)	Dark yellow	5.003	0.262

Table 4.2 shows that the leaves of *A.ligulata* partitioned with dichloromethane gave highest yield while partitioned with hexane gave the lowest yield. Polar compound was dissolve in polar solvent while non polar compound was dissolved in non polar solvent. Based on this percentage yield information, the leaves of *A.ligulata* contain high amount of medium polarity compound because it gave highest yield of extractive with slightly polar solvent which is dichloromethane.

The stem of *A. ligulata* extracted with methanol gave highest percentage followed by ethyl acetate, hexane, dichloromethane and chloroform. The stem of *A.ligulata* contain high amount of polar component compared to the leaves because it gave highest yield of extractive with polar solvent.

4.2 ISOLATION AND PURIFICATION

4.2.1 Thin Layer Chromatography

TLC was used initially to determine the number of component of each extract and to find the best solvent for the separation process. Table 4.3 and Table 4.4 show the TLC particle of various crude extract of *A. ligulata* developed in various solvent systems and visualize with different method.

Table 4.3: TLC of Hexane, DCM, $CHCl_3$, EtOAc, and MeOH crude extract from the leaves of *A. ligulata* in various solvent system

Crude extract	Solvent system	Spots	R_f value		
			Visible	UV	Vanillin
Hexane	Hexane:DCM:Acetone (7:3:1)	1	-	0.20	0.18
		2	-	0.28	0.24
		3	-	0.38	0.30
		4	0.36	0.44	0.36
		5	0.42	0.52	0.42
		6	0.50	0.60	0.50
		7	0.56	-	0.56
		8	-	-	0.60
		9	-	-	0.64
		10	-	-	0.68
		11	-	-	0.72
		12	-	-	0.84
		13	-	-	0.82
		14	-	-	0.90
		15	-	-	0.99
Dichloromethane	Hexane:DCM:Acetone (7:5:1)	1	-	0.10	-
		2	-	0.18	0.18
		3	0.22	0.24	0.26
		4	0.30	0.30	0.34
		5	0.37	-	0.40
		6	0.44	0.42	0.46
		7	0.54	0.52	0.54

		8	-	-	0.60
		9	-	-	0.66
		10	-	-	0.74
Chloroform	EtOAc	1	-	-	0.16
		2	-	-	0.24
		3	-	-	0.40
		4	-	-	0.56
		5	0.88	-	0.70
		6	0.92	-	0.90
		7	0.96	0.96	0.96
Ethyl acetate	EtOAc	1	-	-	0.14
		2	-	-	0.24
		3	-	-	0.42
		4	-	-	0.58
		5	-	-	0.70
		6	0.88	-	0.90
		7	0.96	0.94	0.96
Methanol	$CHCl_3$: EtOAc (3:1)	1	-	-	0.06
		2	-	-	0.16
		3	-	-	0.28
		4	-	-	0.36
		5	-	-	0.44
		6	-	-	0.60
		7	-	-	0.74

	8	-	-	0.80
	9	-	-	0.92
	10	0.96	0.96	0.9

Table 4.4: TLC of Hexane, DCM, $CHCl_3$, EtOAc, and MeOH crude extract from the stem of *A. ligulata* in various solvent system

Crude extract	Developing solvent	Spots	R_f value		
			Visible	UV	Vanillin
Hexane	Hexane:DCM:Acetone (4:1:1)	1	0.10	0.10	0.10
		2	0.20	0.20	0.20
		3	0.32	0.32	0.32
		4	-	-	0.36
		5	-	-	0.44
		6	-	-	0.50
		7	-	-	0.56
		8	-	-	0.64
		9	-	-	0.76
		10	-	-	0.84
		11	-	-	0.90
		12	-	-	0.96
Dichloromethane	Hexane:DCM:Acetone	1	-	0.10	0.14
	(7:5:1)	2	0.18	0.18	0.22
		3	-	0.24	0.28
		4	0.30	0.30	0.38
		5	0.42	0.42	0.44
		6	0.52	0.50	0.50

		7	-	-	0.58
		8	-	-	0.64
		9	-	-	0.72
		10	-	-	0.80
		11	-	-	0.88
Chloroform	$CHCl_3$: DCM (4:1)	1	-	-	0.12
		2	-	0.18	0.18
		3	0.27	0.22	0.27
		4	0.32	0.32	0.32
		5	-	-	0.56
		6	0.60	-	0.60
		7	o.62	-	0.84
		8	-	-	0.94
Ethyl acetate	$CHCl_3$: EtOAc	1	0.14	0.14	0.14
	(1:4)	2	-	0.24	0.24
		3	0.32	-	0.32
		4	-	0.38	0.38
		5	-	0.44	0.44
		6	-	-	0.50
		7	0.56	-	0.56
		8	-	-	0.64
		9	-	0.82	0.92

Methanol	$CHCl_3$: EtOAc (3:2)	1	0.20	0.20	0.20
		2	-	-	0.30
		3	0.44	-	0.44
		4	-	-	0.56
		5	-	-	0.74
		6	-	-	0.84

Combination of hexane, DCM and acetone was found to give good separation for the hexane and dichloromethane crude extract for the stem and leaves of *A. ligulata*. DCM crude extract of the leaves samples gave 10 spot; 5 spot are visible, 7 spot observes under UV and 9 spot can be observes when dipped with vanillin. Most of the compounds presence in both crude extract was clearly visible when sprayed with vanillin. The chloroform extract of the leaves when develop with EtOAc reveal 3 spot that can be visualized by naked eyes, 1 spot visualize with UV light and 7 spot can be clearly visualized after sprayed with vanillin. Ethyl acetate extract from leaves, gave good separation when develop with ethyl acetate. The $CHCl_3$ extractive of the stem give best separation in the $CHCl_3$: DCM (4:1), while ethyl acetate extractive from the stem gave good separation when developed in $CHCl_3$: EtOAc (1:4) used mobile phase. However for methanol extract from both stem and leaves of *A. ligulata*, there were some difficulties in order to choose the best solvent to develop the TLC plate. A combination of chloroform and ethyl acetate was found to give the best separation.

4.2.2 Column chromatography

Based on the separation on the TLC plate, DCM and $CHCl_3$ crude extracts of *A. ligulata* leaves was used for further isolation and purification process, while for the stem, the DCM crude extractive was used. These crude extract was found to give more compound and have good separation between each other. Beside, DCM and $CHCl_3$ crude extracts of *A. ligulata* leaves also gave high percentage yield. Column chromatography is the technique that usually used in order to separate mixture from the crude extract.

4.2.2.1 Extensive column chromatography for dichloromethane crude extract of the leaves

Column (60 cm x 4 cm) and silica gel 60 (Merck, 230-400 mesh) were used to separate dichloromethane crude extract. 4.00 g sample was coated with 8.00 g of silica gel and applied into the column. Solvent system with increasing polarity was used for the elution process. The solvent used was in the order of hexane, dichloromethane, chloroform, ethyl acetate and methanol. The dichloromethane extract yields 72 fractions of 25 mL each. All of the fractions were subjected to TLC and developed using suitable solvent. Fraction 1-8 were developed in hexane, fraction 9-16 were developed in hexane: DCM (1:1), fraction 17-24 were developed in DCM, fraction 25-36 were develop in DCM: $CHCl_3$ (1:1), fraction 37-40 were developed in $CHCl_3$, fraction 41-48 were developed in CHCl3: EtOAc (3:2), fraction 49-51 and 52-60 were developed in EtOAc, fraction 61-62 and 63-64 were developed in EtOAc: MeOH (1:1) and for the fraction 65-72 were developed in MeOH. Fraction that gave similar R_f value were combined and evaporated to dryness. The combined fraction that gave good separation with several spots was subjected to Preparative Thin Layer Chromatography

(PTLC). The combined fraction that gave only single spot was subjected to Gas Chromatography-Mass Spectroscopy (GC–MS), Nuclear Magnetic Resonance (NMR) and Fourier Transform Infrared spectroscopy (FT–IR). Weight of each combined fraction was also recorded and shown in Table 4.5.

Table 4.5: Combined fractions from Column Chromatography of DCM crude extract of the *A. ligulata* leaves

Combined fractions	Weight (g)	Yield based on crude extract (%)	Physical appearance of fraction	Spots	R_f value	
					UV	Vanillin
1-8	0.000 (discarded)	0.000	Colorless	-	-	-
9-16	0.009	0.225	Colorless	1	-	0.100
				2	-	0.160
				3	-	0.220
				4	-	0.540
				5	-	0.600
				6	0.800	0.800
17-24	0.043	0.108	Yellowish	1	0.200	0.200
				2	-	0.280
				3	0.330	0.330
				4	-	0.400
				5	0.460	0.460
				6	0.560	0.560
				7	0.640	0.640
				8	-	0.700
				9	0.780	0.840
				10	-	0.900

25-36	0.118	2.95	Golden yellow (needle like crystals)	1	-	0.320
				2	0.40	0.400
				3	-	0.480
				4	0.58	0.580
				5	-	0.640
				6	-	0.740
				7	-	0.820
37-40	0.052	1.300	Golden brown	1	-	0.300
				2	-	0.440
				3	0.52	0.52
				4	0.66	0.66
				5	-	0.77
41-48	0.076	1.900	Dark green	1	0.025	0.20
				2	-	0.23
				3	-	0.44
				4	0.53	0.52
				5	-	0.58
				6	-	0.73
				7	0.81	0.81
				8	0.89	0.89
				9	-	0.96
49-51	0.576	0.144	Dark green	1	-	0.32
				2	-	0.38
				3	-	0.50

				4	-	0.60
				5	0.70	0.70
				6	-	0.82
				7	0.88	0.88
52-60	0.130	0.325	Greenish brown	1	-	0.14
				2	-	0.30
				3	0.44	0.44
				4	0.50	0.50
				5	-	0.60
				6	0.66	0.66
				7	-	0.74
				8	-	0.84
				9	0.90	0.90
61-62	0.008	0.200	Brown	1	0.20	0.20
				2	-	0.34
				3	0.40	0.40
				4	-	0.50
				5	0.80	0.80
				6	-	0.84
63-64	0.609	15.23	Dark brown	1	-	0.26
				2	-	0.34
				3	0.44	0.44
				4	-	0.66
				5	0.81	0.80
				6	0.84	0.84

65-72	1.562	39.05	Greenish brown	1	0.36	0.24
				2	0.58	-
				3	0.79	0.68

Combined fractions 25-36 gave golden brown needle like compound. This fraction was first washed with hexane and methanol since it is not soluble in both solvent in order to remove the impurity. The combined fraction was first washed with hexane and filtered by using Whatman No. 42 filter paper. The residue was collected while the filtrate was subjected to dryness. The residue was then washed with methanol in order to remove the polar compound. The filtrate from methanol was evaporated to dryness. The residue which was labeled Compound 1 gave yellow needle-like crystals.

Compound 1 gave a single spot on the TLC plate with R_f value of 0.64 when develop in $CHCl_3$: DCM (1:1), R_f of 0.78 when developed in $CHCl_3$ and R_f of 0.85 when developed in DCM. The spot was visible when visualized under UV light and also when dipped with vanillin. Compound 1 also gave single spot when performed on 2D TLC (see Appendix 1). Compound 1 was dried, weight and subjected to various spectroscopic analysis. The weight of compound 1 obtained was 7.0 mg. The R_f value for compound 1 is given in Table 4.6.

Table 4.6: TLC for Compound 1 isolated from combined fraction 25-36

Compound	Solvent system	Ratio	Spot	Color in vanillin	R_f value	
					UV	Vanillin
Compound 1	$CHCl_3$: DCM	1:1	1	Orange	0.64	0.64
	Chloroform	100 %	1	Orange	0.78	0.78
	Dichloromethane	100 %	1	Orange	0.85	0.85

PTLC was used to further purify compound in fraction 37-40. PTLC was performed on silica gel plate 60 F_{254} (0.25 mm thickness, 20 x 20 cm size) was used. The dried fraction was dissolved in chloroform and applied as a band onto the PTLC plate. The PTLC plate was then developed in $CHCl_3$: DCM (1:1). The band on the PTLC plate was then visualized under the UV which showed a clear band that separated nicely from the other. The band was scrapped off, filtered, and dried. The yield was 2.5 mg. Solvent system and also the compound obtain gave similar characteristic to Compound 1 isolated from fraction 25-36. So the compound was combined to give total yield of 9.5 mg.

4.2.2.2 Column chromatography for the chloroform crude extract of the leaves

Column (50 cm x 4 cm) and silica gel 60 (Merck, 230-400 mesh) was used for chloroform crude extract. About 4.00 g sample was coated with 8.00 g of silica gel and applied into the column. Solvent system with increasing polarity was used for the elution process. The solvent that were used was hexane, dichloromethane, chloroform, ethyl acetate and methanol. The separation of chloroform extract gave 72 fractions of 25 mL each. All the fraction were then subjected to TLC and developed using suitable solvent. Fraction with

similar R_f value were combined and evaporate to dryness. Weight of combined fraction was recorded as shown in the Table 4.7.

Table 4.7: Combined fractions from Column Chromatography of chloroform crude extract of the *A. ligulata* leaves

Combined fraction	Weight (g)	Yield based on crude extract (%)	Physical appearance	Spot	R_f value	
					UV	Vanillin
1-8	0.00 (discarded)	0.00	Colorless	-	-	-
9-16	0.001	0.025	Colorless	1	-	0.34
				2	-	0.62
				3	0.82	0.82
17-24	0.005	0.125	Colorless	1	-	0.14
				2	-	0.24
				3	-	0.46
25-32	0.004	0.100	Colorless	1	-	0.10
				2	-	0.16
				3	0.62	0.46
33-40	0.002	0.050	Very light green	1	-	0.10
				2	0.22	0.16
				3	0.32	0.48
				4	0.58	0.58
				5	0.66	0.66
				6	-	0.74

41-48	0.049	1.225	Green	1	0.14	0.14
				2	-	0.30
				3	-	0.44
				4	0.56	0.56
				5	-	0.66
				6	-	0.74
				7	-	0.86
49-50	0.025	0.625	Dark green	1	-	0.16
				2	0.28	0.28
				3	-	0.38
				4	0.46	0.50
				5	0.60	0.70
				6	0.74	0.88
51-55	0.023	0.575	Dark green	1	-	0.16
				2	0.28	0.28
				3	0.38	0.36
				4	0.46	0.40
				5	-	0.54
				6	-	0.64
				7	0.84	0.82
				8	-	0.88
56-58	0.031	0.775	Yellowish	1	0.16	0.16
				2	-	0.20
				3	0.38	0.32

				4	-	0.40
				5	-	0.86
59-63	0.019	0.475	Yellowish brown	1	-	0.16
				2	0.22	0.24
				3	-	0.32
				4	0.42	0.40
				5	-	0.84
64-65	0.132	3.330	Brown	1	0.18	0.18
				2	0.38	0.38
				3	-	0.58
66-72	1.111	27.78	Dark brown	1	-	0.16
				2	-	0.38
				3	0.62	0.62

4.2.2.3 Column chromatography for the dichloromethane crude extract of the stem

Column (60 cm x 3 cm) and silica gel 60 (Merck, 230-400 mesh) was used for the dichloromethane crude extract. About 2.00 g sample was coated with 4.00 g of silica gel and applied into the column. Solvent system with increasing polarity was used for the elution process. The solvent was hexane, dichloromethane, chloroform, ethyl acetate and methanol. The separation of chloroform extractive gave 72 fractions of 25 mL each. All the fraction were then subjected to TLC and developed using suitable solvent. Fraction with similar R_f

value were combined and evaporated to dryness. Weight of combined fraction was recorded as shown in the Table 4.8.

Table 4.8: Combined fractions from Column Chromatography of dichloromethane crude extract of the *A. ligulata* stem

Combined fraction	Weight (g)	Yield based on crude extract (%)	Physical appearance	Spot	R_f value	
					UV	Vanillin
1-11	0.00 (discarded)	0.00	Colorless	-	-	-
12-14	0.001	0.05	Colorless	1	0.72	0.72
				2	0.84	-
15-24	0.005	0.25	Colorless	1	-	0.30
				2	0.36	0.36
				3	-	0.42
				4	0.46	0.46
				5	0.56	0.56
				6	-	0.64
				7	-	0.70
				8	-	0.84
25-40	0.017	0.85	Colorless	1	-	0.14
				2	-	0.18
				3	-	0.26
				4	0.40	0.40
				5	-	0.50
				6	0.60	0.60

				7	-	0.64
				8	0.72	0.72
				9	0.84	0.84
41-45	0.004	0.20	Colorless	1	0.40	-
				2	0.51	-
				3	0.60	-
				4	0.70	0.70
				5	0.80	-
				6	0.90	0.90
46-48	0.196	9.80	Green	1	0.14	0.20
				2	0.28	0.32
				3	0.40	0.46
				4	0.50	0.54
				5	0.60	0.60
				6	-	0.70
				7	-	0.78
				8	-	0.90
49-52	0.108	5.40	Light green	1	-	0.30
				2	-	0.44
				3	0.52	0.58
				4	0.64	0.60
				5	-	0.74
				6	-	0.80
				7	0.90	0.90

53-60	0.173	8.65	Yellow	1	-	0.20
				2	0.28	0.28
				3	0.36	0.36
				4	0.48	0.48
				5	-	0.56
				6	0.66	0.66
				7	0.80	0.80
61-63	0.026	1.30	Yellow	1	0.35	0.25
				2	0.58	0.50
				3	0.75	-
64-72	0.900	40.5	Brown	1	0.23	-
				2	0.38	0.38
				3	0.67	0.60

4.3 CHARACTERIZATION OF COMPOUND 1

4.3.1 Melting point

The melting point of Compound 1 was determined. Melting point for the yellow needle crystal of compound 1 was 179 – 180 ^{o} C. This sharp range of the melting point indicated that this compound was pure.

4.3.2 Gas Chromatography – Mass Spectrometry (GC – MS)

Compound 1 was then subjected to GC – MS. Compound 1 shows one peak at retention time of 40.774 min in the gas chromatogram. Based on the chromatogram, the Compound 1 was pure and was subjected for further spectroscopic analysis especially NMR and FTIR.

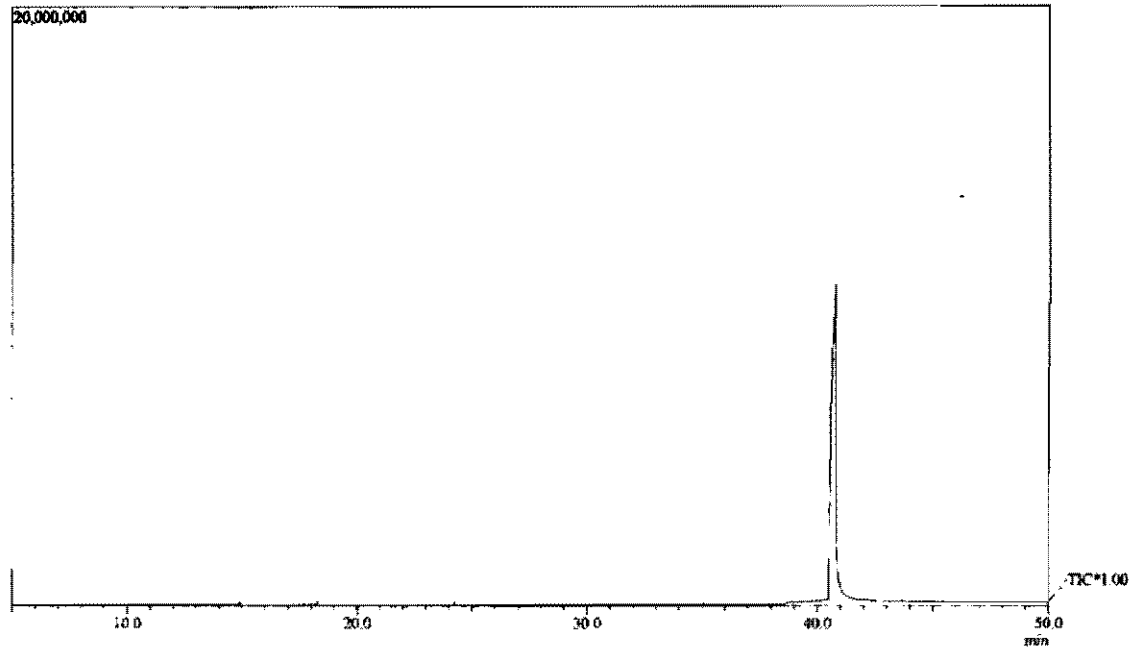


Figure 4.1: Gas chromatogram Compound 1

The molecular weight of compound 1 can be obtained by analyzing mass spectrum from GC-MS. The Mass spectra data obtained for compound 1 revealed the molecular ion peak at M^+ 314 which corresponds to the molecular formula $C_{17}H_{14}O_6$ with molecular mass of 314.05 g/mol. Other fragmentation peak was observed at m/z 299, 285, 271, 256, 228, 200, 157, 135 and 92 as shown in Figure 4.3.

This fragmentation pattern shows the similarity to the phenolic compound. Phenols typically lose carbon monoxide to give strong peak at *m/e* of *M* – 28. Phenols also can lost formyl radical (HCO·) to a give strong *M* – 29 peaks (Pavia *et.al*, 2001).

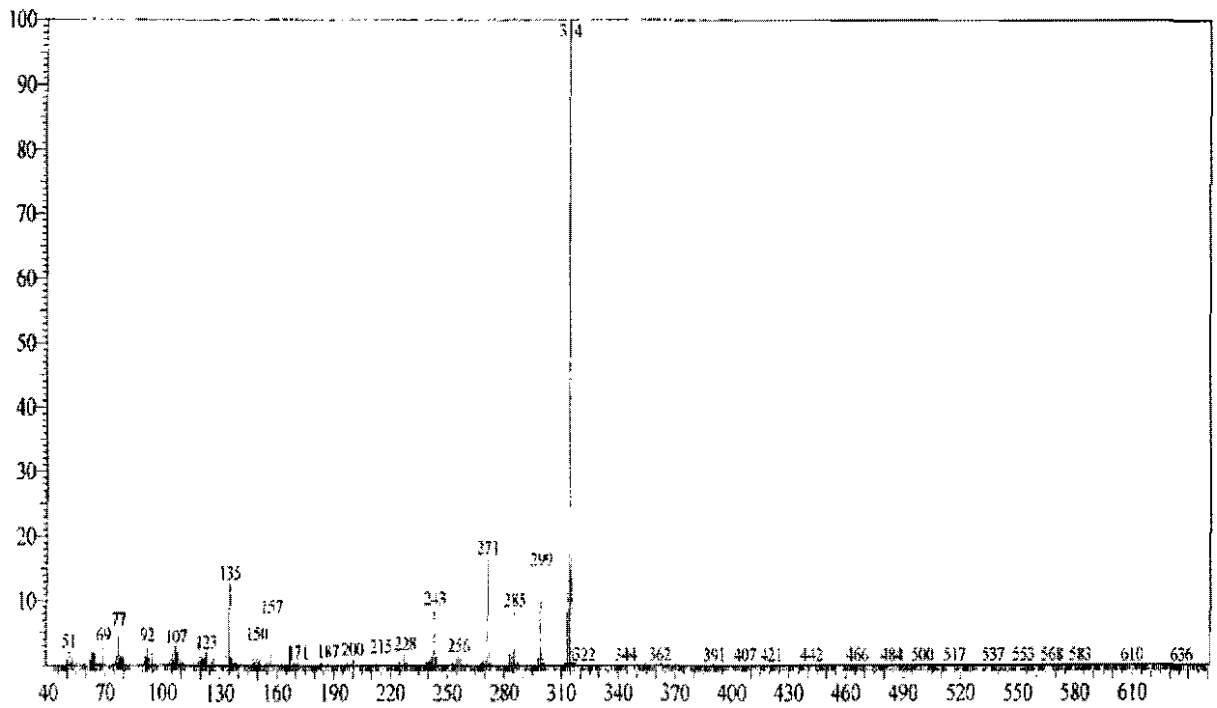


Figure 4.2: Mass spectrum for Compound 1

4.3.3 Fourier Transform Infra Red (FTIR)

FTIR was performed by using potassium bromide (KBr) pellet to determine the functional group present in Compound 1. FTIR spectra for Compound 1 is shown in Figure 4.3. The strong absorption at wavelength 3312 cm^{-1} corresponded to the hydroxyl group (O-H). The absorption band at wavelength 1660 cm^{-1} showed the presence of carbonyl group (C=O) while phenyl absorption was observed in the range of 1600-1450 cm^{-1}. Table 4.9 showed type of functional groups observed in Compound 1 while Figure 4.2 shows the FT-IR spectrum.

Table 4.9: Functional group presence in the Compound 1

Absorption (cm^{-1})	**Functional group presence**
3312	O – H
1660	C=O (conjugation with phenyl)
1507 and 1596	Aromatic C=C stretch
1162	C – O stretch

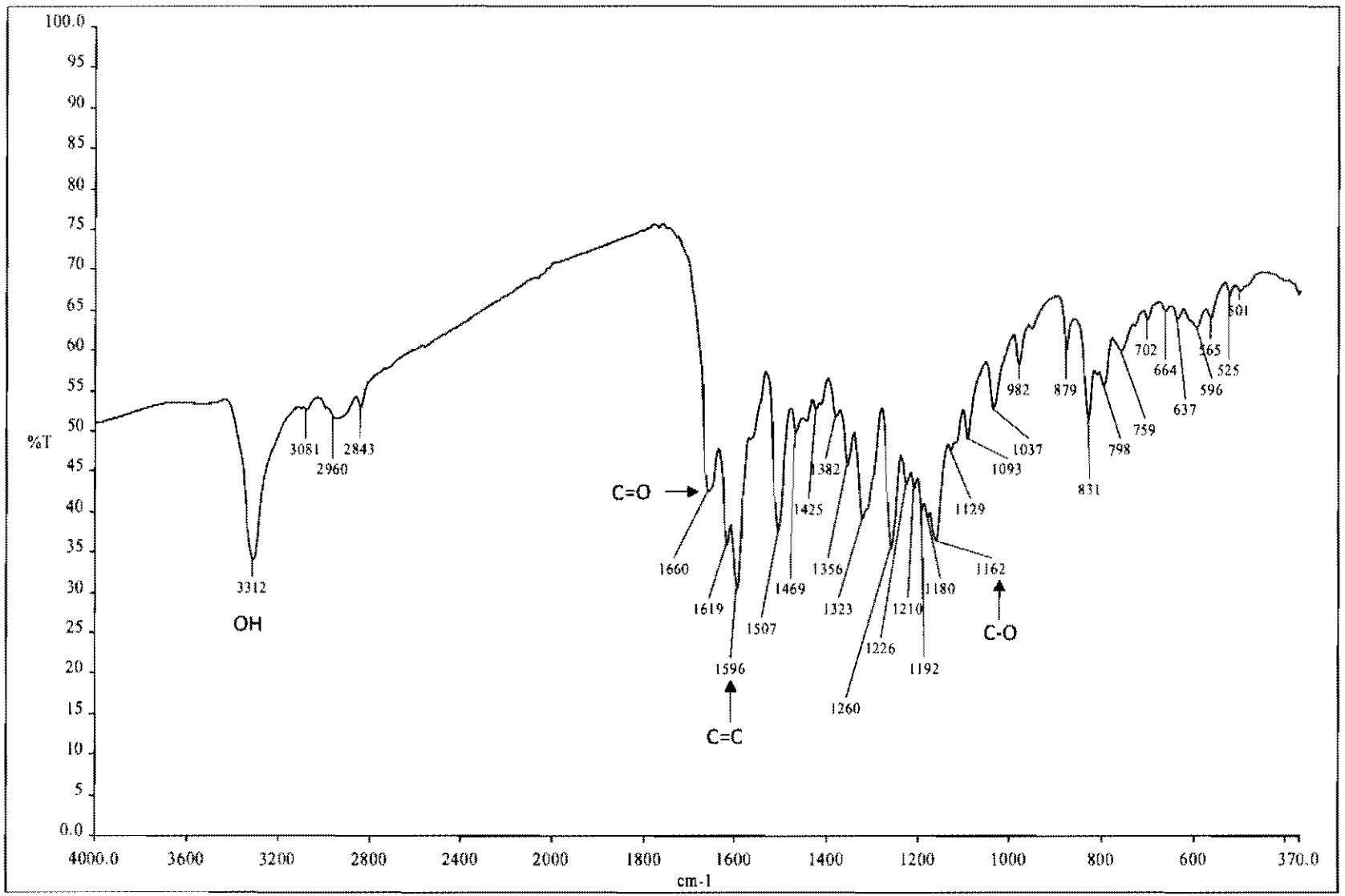


Figure 4.3: FTIR spectra for Compound 1 isolated from dichloromethane crude extract of *A. ligulata* leaves

4.3.4 Nuclear Magnetic Resonance spectrometer (NMR)

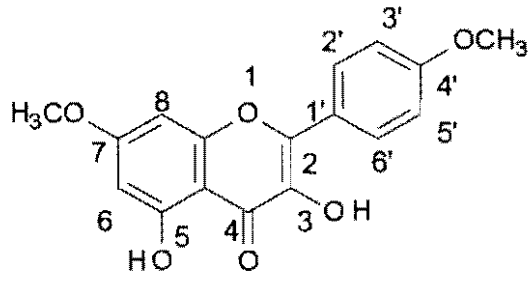
Figure 4.4 shows the ^{1}H-NMR spectra of Compound 1. The sample was dissolved in $CDCl_3$. The spectra show signal at δ 3.88 (s, 6H), δ 6.36 (d, 1H), δ 6.48 (d, 1H), δ 7.03 (d, 2H) and δ 8.17 (d, 2H) (see Appendix 6 and 7). Signal at δ 3.88 were ascribed to proton of methoxy (-OCH_3) group at C-7 and C-4' position while signal at δ 6.57 and δ 11.72 were ascribe to proton of the hydroxyl group (-OH) at C-3 and C-5 position respectively.

Figure 4.4 shows the ^{13}C-NMR spectra for Compound 1. Signal at δ 175.2 was ascribed to carbonyl (C=O) at C-4 position and supported by IR data given absorption at frequency 1660 cm^{-1}. Signal at δ 114.1 (C-3' and 5'), 123.5 (C-1'), 129.4 (C-2' and C-6') and 160.8 (C-4') ascribed to phenyl carbons with methoxy group at C-4' position and supported by 1H NMR that shows proton peak at δ 7.03 (d, 2H) and 8.17 (d, 2H) that assigned to proton of phenyl group. Table 4.10 shows the comparison of ^{1}H NMR and ^{13}C NMR data between Compound 1 and Compound **11**. The values of signal of both compounds are similar. Based on the mass spectrum, FTIR, ^{1}H NMR, ^{13}C NMR data and comparison with published data, (Mitscher *et al.*, 1985; Nawwar *et al.* 1984) compound 1 was suggested as flavone.

Table 4.10: Comparison of ^{1}H and ^{13}C NMR data of Compound 1 and Kaempferol-7,4'-dimethyl ether

	δ of ^{1}H		δ of ^{13}C	
Carbon number	Compound 1	Kaemferol-7,4' dimethy ether (Mitscher *et al.*, 1985)	Compound 1	Kaemferol-7,4' dimethyl ether (Nawwar *et al.*, 1984)
2	-	-	145.7	146.7
3	6.57 (s, 1H, OH)	6.57 (s, 1H, OH)	136.0	136.3
4	-	-	175.1	176.1
5	11.72 (s, 1H, OH)	11.71 (s, 1H, OH)	161.1	160.7
6	-	-	97.8	97.6
7	6.37 (d, 1H, ArH)	6.37 (d, 1H, ArH)	165.7	165.0
8	-	-	92.2	92.2
9	6.48 (d, 1H, ArH)	6.48 (d, 1H, ArH)	156.8	156.3
10	-	-	104.0	104.1
1'	-	-	123.2	123.2
2'/6'	7.03 (d, 2H, ArH)	7.01 (d, 2H, ArH)	129.4	129.5
3'/5'	8.17 (d, 2H, ArH)	8.16 (d, 2H, ArH)	114.1	114.2
4'	-	-	160.8	160.2
7-OCH_3	} 3.88 (s, 6H)	} 3.88 (s, 6H)	55.4	55.3
4'-OCH_3			55.8	55.9

Based on spectroscopic data, melting point and characteristic of Compound 2 was concluded as 7,4'-dimethoxy-3,5-dihydroxyflavone or kaempferol-7,4'-dimethyl ether **11**. This data is correlated with data for Compound **11** that has been isolated from leaves of *Amorpha nana* (Mitscher *et al.*, 1985), flowers of *Tamarix nilotica* (Nawwar *et al.*, 1984) and rhizomes of *Hedychium thyrsiforme* (Jasril *et al.* 2003). Previous study reported that kaemferol-7,4'-dimethyl ether inhibit strong antioxidant and anticancer activity (Jasril *et al.* 2003).



11

The ability of flavonoids to act as antioxidants, possibly through radical scavenging mechanisms, has been well documented. The flavonoids appear to have played a major role in the successful medical treatments of ancient times, and their use has persevered up to now Flavonoids have been suggested to be responsible for several biological activities, including antiallergenic, antiviral, antiinflammatory, angiogenesis inhibitor, anticarcinogenic and hepatoprotective action. (Middleton *et al.*, 2000 cited in Jasril *et al.* 2003).

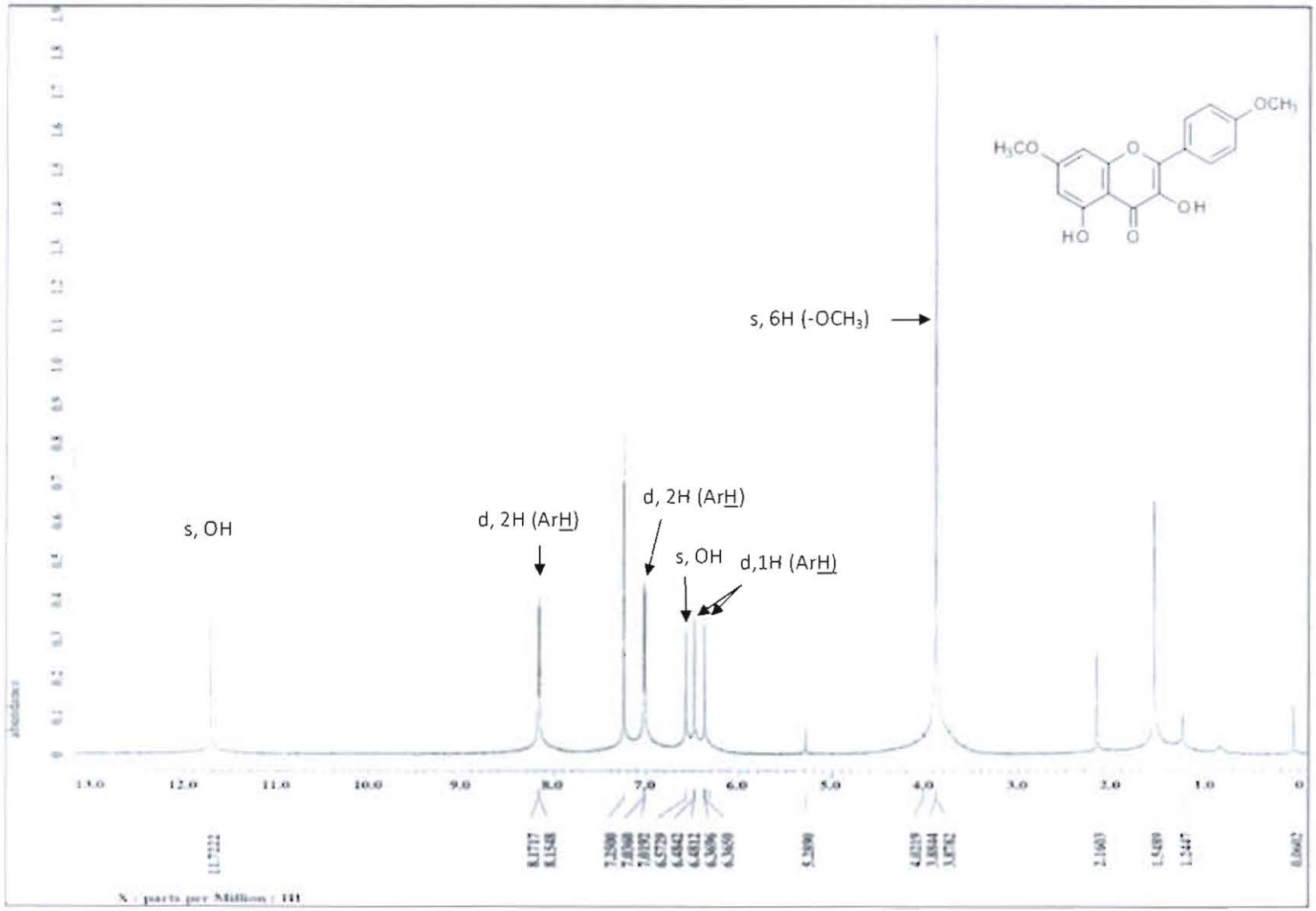


Figure 4.4: ^{1}H NMR spectra for Compound 1

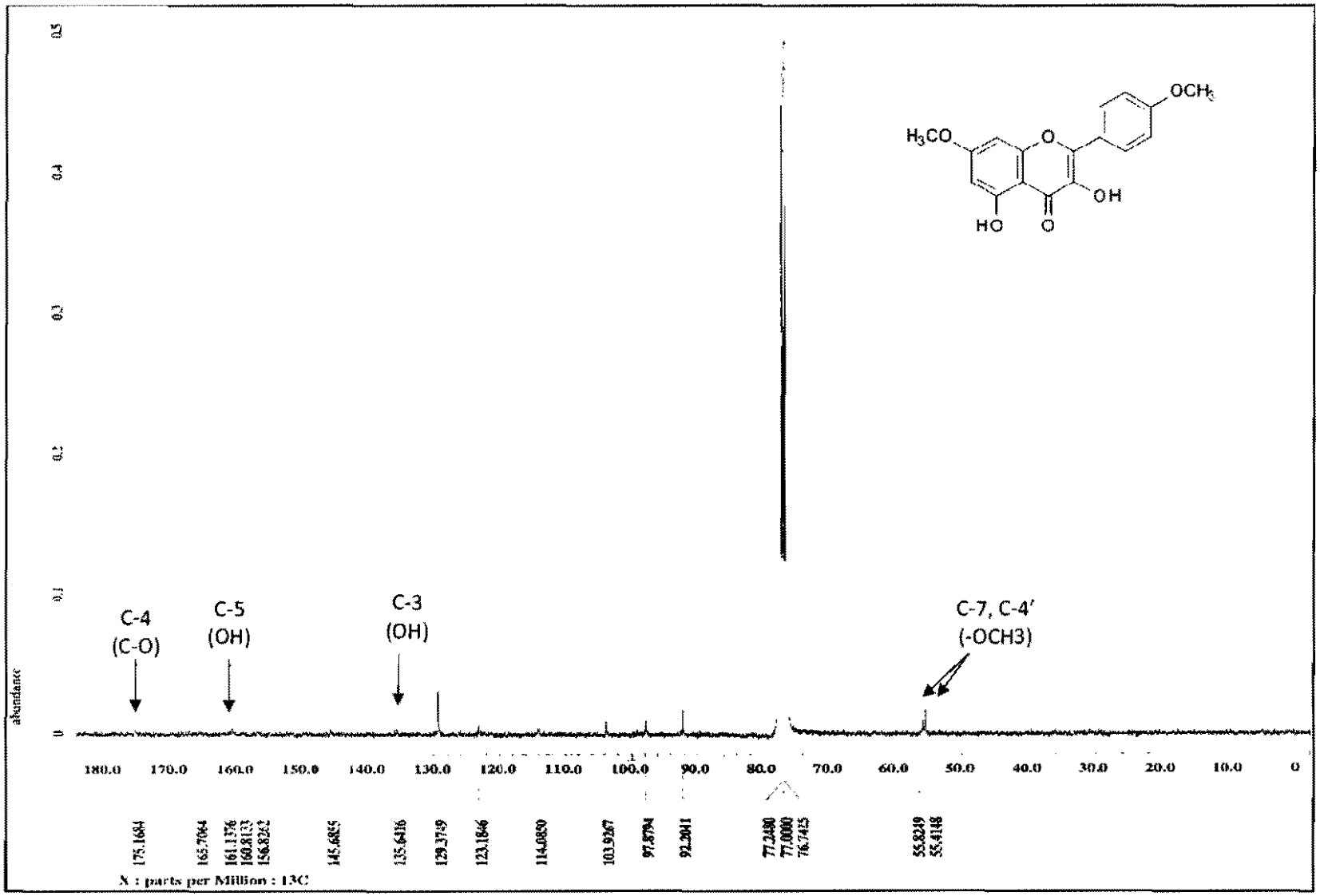


Figure 4.5: ^{13}C NMR for Compound 1

4.5 BRINE SHRIMP TOXICITY AND TERMITICIDAL ACTIVITY

4.5.1 Toxicity test against brine shrimp, *Artemia salina*

Brine shrimp *Artemia salina* toxicity test was preferred for all *Alpinia ligulata* stem and leaves crude extract. Result shows that all the crude extract was not toxic to *Artemia salina* at concentration 1, 10, 100 µg/mL (Appendix 2 and 3). Previous studies of ethanolic extract of *A. galangal* were found to be toxic to *Artemia salina* with LD_{50} of 109 µg/mL (Khattak *et al.*, 2004).

4.5.2 Termiticidal activity

This test was conducted in order to determine the termiticidal activity of crude extractive from the stem and leaves of *Alpinia ligulata*. Table 4.10 show the average (%) death of termites (*Coptotermes* sp.) in day 3 for the crude extractive of stem. Hexane, dichloromethane and methanol crude extract from stem of *A. ligulata* exhibited toxicity to *Coptotermes* sp. while for leaves only the extract of dichloromethane crude exhibited the termiticidal activity (see Appendix 4 and 5) . Figure 4.6 show the LC_{50} value for stems extractive.

Table 4.11: Termiticidal activity of crude extract of *A. ligulata* stem

Sample	Average death (%)			LC_{50}
	1 µg/mL	10 µg/mL	100 µg/mL	
Hexane crude extract	0	0	0	0
Dichloromethane crude extract	44.4	83.3	100	1.259
Chloroform crude extract	16.7	27.8	55.6	63.09
Ethyl acetate crude extract	0	0	0	0
Methanol crude extract	33.3	44.4	55.6	31.62

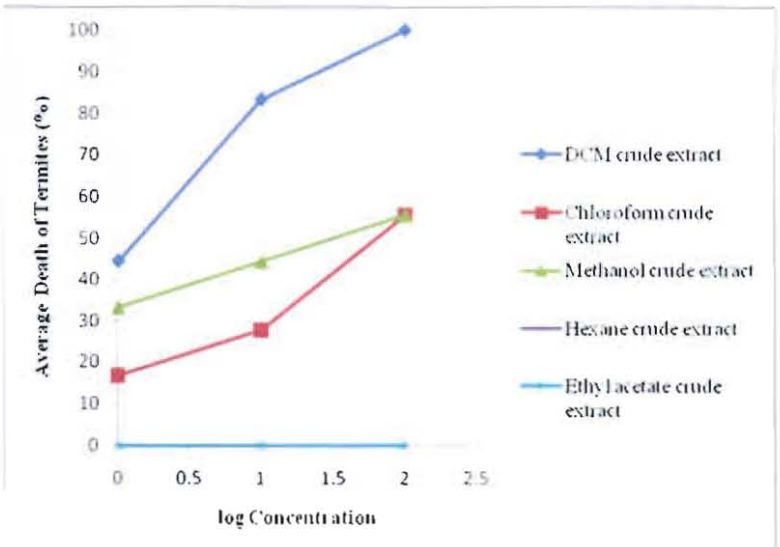


Figure 4.6: Average death of termite as a function of log concentration of the crude extract of *A. ligulata* stem

Based on the Figure 4.6 the most toxic crude from stem part was dichloromethane crude extract which gave LC_{50} 1.259 µg/mL followed by methanol with 31.62 µg/mL and chloroform crude extract which is 63.09 µg/mL. The leaves, dichloromethane crude extract of *A.ligulata* gave LC_{50} 5.012 µg/mL (Figure 4.7). Table 4.11 and Figure 4.7 shows the result of termiticidal activity of leaves crude extract of *A. ligulata*.

Table 4.12: Termiticidal activity of crude extract of *A. ligulata* leaves

Sample	Average death (%)			LC_{50}
	1 µg/mL	10 µg/mL	100 µg/mL	
Hexane crude extract	0	0	0	0
Dichloromethane crude extract	16.67	66.67	94.44	5.012
Chloroform crude extract	5.56	16.67	27.78	0
Ethyl acetate crude extract	0	0	0	0
Methanol crude extract	0	5.56	33.33	0

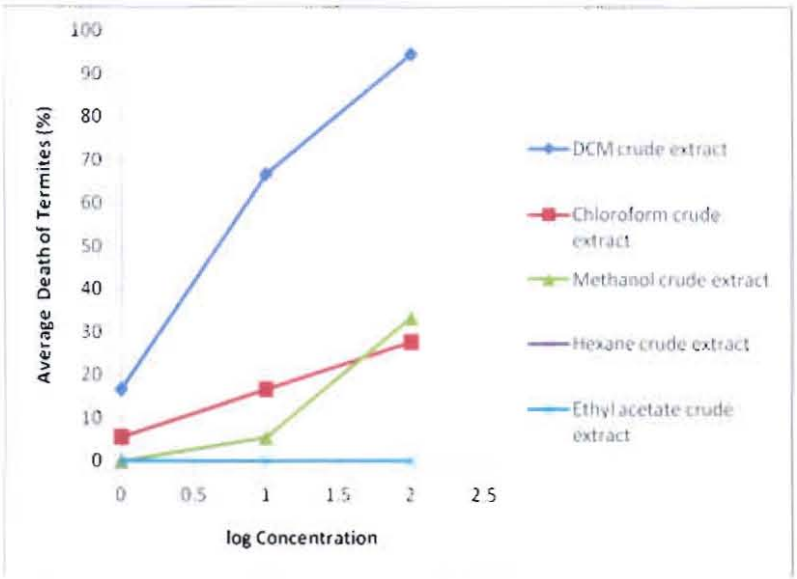


Figure 4.7: Average of death of termite as a function of log concentration of the crude extract of the *A. ligulata* leaves

The stem crude extract of Alpinia ligulata showed strong termiticidal activity against *Coptotermes* sp. compared to leaves crude extract. The stem part exhibit strong termiticidal activity even at moderate concentration of 10 µg/mL.

Previous studies show that *A. zerumbet* show moderate larvicidal activity towards *Aedes aegypti* with LC_{50} 313 ppm (Cavalcanti *et al.* 2004). Compound from rhizomes of Zingiberaceae family including *Curcuma xanthorrhiza*, *C. zedoaria*, *Kaempferia galanga* and *K. pandurata* showed toxicity against larvae of the polyphagous pest insect *Spodoptera littorali* with LC_{50} between 6.92 and 8.13 µmol kg^{-1} fresh weight irrespective of the larval stages studied (Pandji *et al.* 1993).

CHAPTER 5

CONCLUSIONS AND SUGGESTION

The research was conducted in order to extract, purify, and elucidate the structure of the pure compounds isolated from the stem and leaves of *Alpinia ligulata* and to determine the biological activity. The percentage yield of crude methanol extract of *A. ligulata* leaves and stem were 1.740 % and 2.036 % respectively. The methanol crude extract from the stem and leaves was then subjected to solvent partitioned in order of increasing polarity starting with hexane, dichloromethane, chloroform, ethyl acetate and methanol and percentage yield of each crude partition for leaves were 0.223 %, 0.632 %, 0.343 %, 0.295 % and 0.035 % respectively, while for the stem were 0.179 %, 0.128 %, 0.058 %, 0.262 % and 0.753 % respectively.

Crude extract of the dichloromethane and chloroform of *A. ligulata* leaves and crude extract of dichloromethane of the stem were subjected to column chromatography. Compound 1 was isolated from the combined fraction 25-36 and 37-40 from extensive column chromatography of dichloromethane crude extract of leaves. Compound 1 was isolated as yellow needle crystals with total yield of 9.5 mg. Compound 1 gave a single spot on the TLC plate with R_f value of 0.64 when develop in $CHCl_3$: DCM (1:1), R_f of 0.78 when developed in $CHCl_3$ and R_f of 0.85 when developed in DCM. The spot was visible when visualized under UV light and when dipped with vanillin. Compound 1 also gave single spot when performed on 2D TLC.

Compound 1 gave molecular mass of 314.05 g/mol with molecular formula of $C_{17}H_{14}O_6$. Compound 1 gave melting point of 179-180 $^{\circ}$ C. FTIR spectrum for Compound 1

showed strong absorption at wavelength 3312 cm^{-1} corresponded to the hydroxyl group (O-H). The absorption band at wavelength 1660 cm^{-1} showed the presence of carbonyl group (C=O) while phenyl absorption was observed in the range of 1600-1450 cm^{-1}.

^{1}H-NMR ($CDCl_3$) spectra show signal at δ 6.36 (d, 1H), δ 6.48 (d, 1H), δ 7.03 (d, 2H) and δ 8.17 (d, 2H). Signal at δ 3.88 were ascribed to proton of methoxy ($-OCH_3$) group at C-7 and C-4' position while signal at δ 6.57 and δ 11.72 were ascribe to proton of the hydroxyl group (-OH) at C-3 and C-5 position respectively.

^{13}C-NMR ($CDCl_3$) shows the signal at δ 114.1 (C-3' and 5'), 123.5 (C-1'), 129.4 (C-2' and C-6') and 160.8 (C-4') ascribed to phenyl carbons with methoxy group at C-4' position and supported by 1H NMR that shows proton peak at δ 7.03 (2H, *d* ArH) and 8.17 (2H, *d*, ArH) that assigned to proton of phenyl group. The signal at δ 175.2 was ascribed to carbonyl (C=O) at C-4 position and supported by IR data given absorption at frequency 1660 cm^{-1}. Based on the melting point, mass spectrum, FTIR, ^{1}H NMR, ^{13}C NMR data and comparison with publish data, the compound has been identified as 7,4'-dimethoxy-3,5-dihydroxyflavone or kaempferol 7, 4'-dimethyl ether.

The biological assay test for each crude extract from the stem and leaves was conducted in order to determine the toxicity against *Artemia salina* larvae and termiticidal activity against *Coptotermes* sp. Based on the termiticidal test, the dichloromethane crude extract of the stem showed strong activity with LC_{50} 1.259 µg/mL followed by methanol crude extract with 31.62 µg/mL and chloroform crude extract with 63.09 µg/mL. While dichloromethane crude extract of the leaves gave LC_{50} 5.012 µg/mL. The dichloromethane crude extract from both part of this plant might have potent antifeedant or repellent effect

against termites. The toxicity test against *Artemia salina* larvae, showed that none of the crude extract was toxic.

Other important biological activity such as antibacterial, antifungal and toxicity against human cancer cell should be conducted in order to evaluate the potential of this species. The phytochemical studies should be extensively carried out to characterize all the active constituent since this species has not been subjected to any chemical or biological studies earlier.

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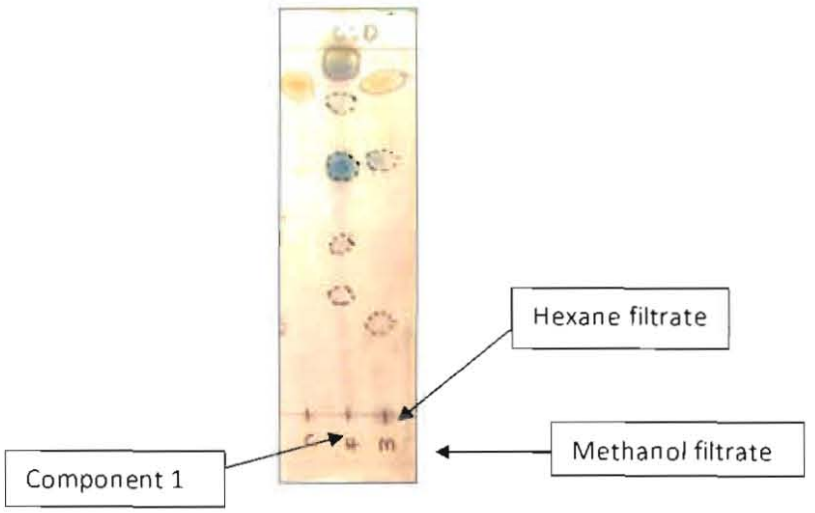
APPENDICES

Appendix 1: TLC and 2D TLC for component 1



TLC for component 1 and filtrate from fraction 25-36 developed in DCM

2D TLC for Compound 1

First solvent: DCM:$CHCl_3$ (1:1)

Second solvent: $CHCl_3$

Appendix 2: Brine Shrimp Toxicity Test result for leaves of *Alpinia ligulata*

Leaves of *A. ligulata*		**Amount of survivor of brine shrimp**			
	Concentration	Replicate 1	Replicate 2	Replicate 3	Average (%)
Hexane	1	10	10	10	100
crude extract	10	10	10	10	100
	100	9	9	8	86.7
	Control	10	10	10	100
DCM crude	1	9	9	9	90.0
extract	10	8	9	9	86.7
	100	7	8	8	76.7
	Control	10	10	10	100
$CHCl_3$ crude	1	10	10	10	100
extract	10	8	8	8	80.0
	100	7	7	7	70.0
	Control	10	10	10	100
EtOAc crude	1	10	10	10	100
extract	10	9	9	9	90.0
	100	8	9	8	83.3
	Control	10	10	10	100
MeOH crude	1	10	10	10	100
extract	10	9	10	10	96.7
	100	10	10	10	100
	Control	10	10	10	100

Appendix 3: Brine Shrimp Toxicity Test result for stem of *Alpinia ligulata*

Stems of *A. ligulata*		**Amount of survival of brine shrimp**			
	Concentration	Replicate 1	Replicate 2	Replicate 3	Average (%)
Hexane crude extract	1	9	10	10	96.7
	10	9	8	9	86.7
	100	8	8	9	83.3
	Control	10	10	10	100
DCM crude extract	1	10	10	10	100
	10	9	9	9	90.0
	100	9	9	9	90.0
	Control	10	10	10	100
$CHCl_3$ crude extract	1	10	10	10	100
	10	10	9	9	93.3
	100	9	8	9	86.7
	Control	10	10	10	100
EtOAc crude extract	1	10	10	10	100
	10	9	9	9	90.0
	100	9	9	8	86.7
	Control				
MeOH crude extract	1	9	9	9	90.0
	10	8	9	8	83.3
	100	7	7	9	76.7
	Control	10	10	10	100

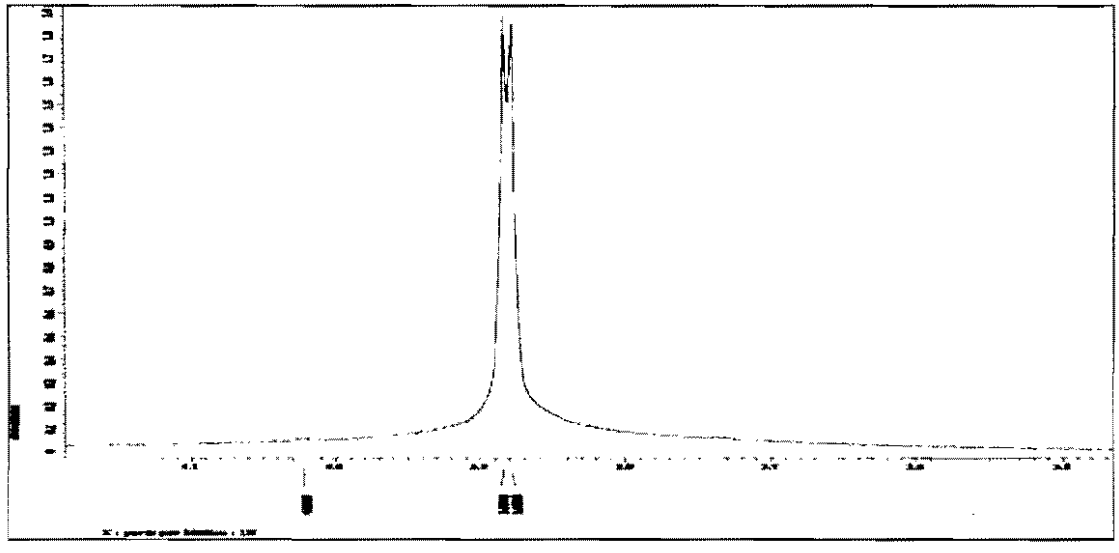
Appendix 4: Termiticidal activity test result of leaves of *A. ligulata*

Leaves of *A. ligulata*	**Amount of survivor of termites**				
	Concentration	Replicate 1	Replicate 2	Replicate 3	Average (%)
Hexane crude extract	1	6	6	6	100
	10	6	6	6	100
	100	6	6	6	100
	Control	6	6	6	100
DCM crude extract	1	5	5	5	83.33
	10	2	2	2	33.33
	100	1	0	0	5.560
	Control	6	6	6	100
$CHCl_3$ crude extract	1	6	6	5	94.44
	10	5	5	5	83.33
	100	5	4	4	72.22
	Control	6	6	6	100
EtOAc crude extract	1	6	6	6	100
	10	6	6	6	100
	100	6	6	6	100
	Control	6	6	6	100
MeOH crude extract	1	6	6	6	100
	10	6	5	6	94.44
	100	4	4	4	66.67
	Control	6	6	6	100

Appendix 5: Termiticidal activity test result of stem of *A. ligulata*

Stem of *A. ligulata*		**Average of survivor of termites after 3 days**			
	Concentration (%)	Replicate 1	Replicate 2	Replicate 3	Average (%)
Hexane crude extract	0.1	6	6	6	100
	1.0	6	6	6	100
	10.0	6	6	6	100
	Control	6	6	6	100
DCM crude extract	0.1	3	3	4	55.6
	1.0	1	1	1	16.7
	10.0	0	0	0	0.00
	Control	6	6	6	100
$CHCl_3$ crude extract	0.1	5	5	5	83.3
	1.0	4	4	5	72.2
	10.0	3	3	2	44.4
	Control	6	6	6	100
EtOAc crude extract	0.1	6	6	6	100
	1.0	6	6	6	100
	10.0	6	6	6	94.4
	Control	6	6	6	100
MeOH crude extract	0.1	4	4	4	66.7
	1.0	4	3	3	55.6
	10.0	4	2	2	44.4
	Control	6	6	6	100

Appendix 6: ^{1}H-NMR spectra for Compound 1



Appendix 7: ^{1}H-NMR spectra for Compound 1