ESSENTIAL OILS FROM FIVE *ETLINGERA* SPECIES OF SARAWAK

Nur Anwariah Binti Marsal

This report is submitted in partial fulfilment of the requirements for the degree of Bachelor of Sciences with Honor in Resource Chemistry

Program of Resource Chemistry
Department of Chemistry
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
May 2009
DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree qualification of this or other university or institution of higher learning.

______________________________
Nur Anwariah Binti Marsal

Programme of Resource Chemistry

Faculty of Science and Technology

Universiti Malaysia Sarawak
ACKNOWLEDGMENT

Alhamdulillah, I am grateful and thankful to The Almighty Allah that I have finished my final year project.

I would like to express my utmost gratitude and appreciation to my supervisor Assoc. Prof. Dr Zaini B. Assim and my co-supervisor, Prof. Dr Fasihuddin B. Ahmad for giving me supports and guidance in this study.

I also want to thank laboratory assistants, postgraduate students and staff of Faculty of Resource Science and Technology UNIMAS for helping and supporting me while completing this project. My appreciation to all my lectures and my course mates for their help and support.

My warmest thank to all my family members especially my mother, Madam Rayah Tunu, my father, Mr. Marsal Kipli, my siblings, Nur Saltiyah Marsal, Muhammad Zulhilmi Marsal and Muhammad Zulfadli Marsal for always helping and supporting me during the completion of this project.
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 4.1: Gas chromatogram from GC/FID analysis for mixture n-alkane standard</td>
<td>17</td>
</tr>
<tr>
<td>Figure 4.2: Gas chromatogram from GC/FID analysis for leaf oil of <em>E. kenyalang</em></td>
<td>20</td>
</tr>
<tr>
<td>Figure 4.3: Gas chromatogram from GC/FID analysis for stem oil of <em>E. kenyalang</em></td>
<td>20</td>
</tr>
<tr>
<td>Figure 4.4: Gas chromatogram from GC/FID analysis for rhizome oil of <em>E. kenyalang</em></td>
<td>20</td>
</tr>
<tr>
<td>Figure 4.5: Gas chromatogram from GC/FID analysis for leaf oil of <em>E. nasuta</em></td>
<td>24</td>
</tr>
<tr>
<td>Figure 4.6: Gas chromatogram from GC/FID analysis for stem oil of <em>E. nasuta</em></td>
<td>24</td>
</tr>
<tr>
<td>Figure 4.7: Gas chromatogram from GC/FID analysis for rhizome oil of <em>E. nasuta</em></td>
<td>24</td>
</tr>
<tr>
<td>Figure 4.8: Gas chromatogram from GC/FID analysis for leaf oil of <em>E. brevilabrum</em></td>
<td>27</td>
</tr>
<tr>
<td>Figure 4.9: Gas chromatogram from GC/FID analysis for stem oil of <em>E. brevilabrum</em></td>
<td>27</td>
</tr>
<tr>
<td>Figure 4.10: Gas chromatogram from GC/FID analysis for rhizome oil of <em>E. brevilabrum</em></td>
<td>27</td>
</tr>
<tr>
<td>Figure 4.11: Gas chromatogram from GC/FID analysis for leaf oil of <em>E. coccinea</em></td>
<td>31</td>
</tr>
<tr>
<td>Figure 4.12: Gas chromatogram from GC/FID analysis for stem oil of <em>E. coccinea</em></td>
<td>31</td>
</tr>
<tr>
<td>Figure 4.13: Gas chromatogram from GC/FID analysis for rhizome oil of <em>E. coccinea</em></td>
<td>31</td>
</tr>
<tr>
<td>Figure 4.14: Gas chromatogram from GC/FID analysis for rhizome oil of <em>E. fimbriobractaeta</em></td>
<td>35</td>
</tr>
<tr>
<td>Figure 4.15: Dendogram from cluster analysis for rhizome oil of <em>Etlingera</em></td>
<td>48</td>
</tr>
<tr>
<td>Figure 4.16: Dendogram from cluster analysis for leaf oil of <em>Etlingera</em></td>
<td>49</td>
</tr>
<tr>
<td>Figure 4.17: Dendogram from cluster analysis for stem oil of <em>Etlingera</em></td>
<td>50</td>
</tr>
<tr>
<td>Figure 4.18: Filter paper spike with (a) 10% (b) 1.0% (c) 0.1% of rhizome oils from <em>E. nasuta</em> (d) DCM only</td>
<td>52</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Tables</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1: Location for Sample collection</td>
<td>8</td>
</tr>
<tr>
<td>Table 4.1: Percentage of yield and colour of the essential oils extracted from Five Species of <em>Etlingera</em>.</td>
<td>15</td>
</tr>
<tr>
<td>Table 4.2: Retention times of the n-alkanes standard.</td>
<td>16</td>
</tr>
<tr>
<td>Table 4.3: Chemical composition of essential oils extracted from <em>E. kenyalang</em></td>
<td>21</td>
</tr>
<tr>
<td>Table 4.4: Chemical composition of essential oils extracted from <em>E. nasuta</em></td>
<td>25</td>
</tr>
<tr>
<td>Table 4.5: Chemical composition of essential oils extracted from <em>E. brevilabrum</em></td>
<td>28</td>
</tr>
<tr>
<td>Table 4.6: Chemical composition of essential oils extracted from <em>E. coccinea</em></td>
<td>32</td>
</tr>
<tr>
<td>Table 4.7: Chemical composition of essential oils extracted from <em>E. fimbriobractaeta</em></td>
<td>36</td>
</tr>
<tr>
<td>Table 4.8: Chemical Composition in Rhizome Oil in Five <em>Etlingera</em> species</td>
<td>38</td>
</tr>
<tr>
<td>Table 4.9: Chemical Composition in Leaf Oils in Four <em>Etlingera</em> species</td>
<td>42</td>
</tr>
<tr>
<td>Table 4.10: Chemical Composition in Stem Oil in <em>Etlingera</em> species</td>
<td>45</td>
</tr>
</tbody>
</table>
Essential Oils from Five *Etlingera* Species of Sarawak

**ABSTRACT**

Essential oils from five *Etlingera* species were studied for their chemical composition and their biological activity on *A. salina*, *Captotermes* spp. and three species of fungal (*Termetes Versicolor*, *Gloeophyllum trabeum*, *Chaetomum globosum*). Essential oils were extracted by hydrodistillation method and subsequently analyzed using gas chromatography/flame ionization detector (GC/FID). The percentage of essential oils obtained from the five *Etlingera* species ranged from 0.11% to 1.83%, with the highest percentage was obtained from the leaf of *E. nasuta* and the lowest percentage was obtained from the stem of *E. kenyalang*. The major constituent of *E. kenyalang* were (E)-p-mentha-2,8-dien-1-ol (43.3%), ethyl isohexanoate (24.5%) and octanal(23.9%). *E. nasuta* was rich in methyl laurate (26.2%), octanone (26.0%) and (-)-β-bisabolene (24.1%). *E. brevilabrum* was contain high abundance of mercaptan (20.7%), decane (21.2%) and β-farnesene (11.8%) while *E. coccinea* has methylfurfurylthiol (27.3%), acetylpyrroline (22.4%) and ethylmethyl pyrazine (18.8%) as the major compounds. Isopulegyl acetate (16.8%), perilla aldehyde (8.8%) and (+)-α-phellandrene (8.3%) were the major components in *E. fimбриобрactета*. Essential oils were subjected to three biological activity tests, i.e. brine shrimp, anti-termites and anti-fungal bioassay. No biological activity on *A. salina* and fungi was observed for all essential oils tested. However, the entire tests carried show that the *Captotermes* spp. repelled to the smell of the essential oils tested.

**Keywords:** *Etlingera*, Gas Chromatography/Flame Ionization, essential oils.
**Minyak Pati daripada Lima Spesies Etlingera Sarawak**

**ABSTRAK**

Komposisi kimia minyak pati daripada lima spesies Etlingera dan aktiviti biologinya terhadap A. salina, Coptotermes spp. dan tiga spesies kulat (Termetes Versicolor, Gloeophyllum trabeum, Chaetomum globosum) telah dikaji. Minyak pati telah di ekstrak dengan kaedah penyulingan hidro dan seterusnya dianalisis menggunakan kromatografi gas/pengesan nyalaan ion. Peratusan minyak pati yang diperolehi daripada lima spesies Etlingera adalah dalam julat 0.11% hingga 1.83% dengan peratusan tertinggi diperolehi daripada daun E. nasuta dan peratusan terendah diperolehi daripada batang E. kenyalang. Komponen utama dalam E. kenyalang adalah (E)-p-menta-2,8-dien-1-ol (43.3%), etil isoheksanoat (24.5%) dan oktanal (23.9%). E. nasuta kaya dengan metil laurate (26.2%), oktanon (26.0%) dan (-)-β-bisabolene (24.1%). E. brevilabrum mengandungi mercaptan (20.7%), dekana (21.2%) dan β-farnesene (11.8%) dengan kelimpahan yang tinggi manakala E. coccinea mempunyai metilfurfurilthiol (27.3%), acetilpirrolina (22.4%) dan etilmetil pirazina (18.8%) sebagai komponen utama. isopulegil asetat (16.8%), perilla aldehid (8.8%) dan (+)-α-phellandrene (8.3%) adalah komponen utama dalam E. fimbriobractaeta. Minyak pati telah diuji dengan tiga ujian bioaktiviti iaitu A. salina, Coptotermes spp. dan tiga spesies kulat (Termetes Versicolor, Gloeophyllum trabeum, Chaetomum globosum) telah dijalankan. Tiada aktiviti biologi keatas A. salina dan kulat telah dicerap pada semua minyak pati yang telah diuji. Namun semua ujian menunjukan Coptotermes spp. menghindar bau minyak pati yang telah diuji.

**Kata kunci:** Etlingera, Kromatografi Gas/Pengesan Nyalaan Ion, minyak pati.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Contents</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Declaration</td>
<td>i</td>
</tr>
<tr>
<td>Acknowledgement</td>
<td>ii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>iii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>iv</td>
</tr>
<tr>
<td>Abstract</td>
<td>v</td>
</tr>
<tr>
<td>Abstrak</td>
<td>vi</td>
</tr>
</tbody>
</table>

## CHAPTER 1  INTRODUCTION

1.1 Introduction  
1.2 Objectives  

## CHAPTER 2  LITERATURE REVIEW

2.1 Family of *Zingiberaceae*  
2.1.1 Genus of *Etlingera*  
2.2 Essential Oils  
2.3 Application of Essential Oils for Chemotaxonomy Purposes  
2.4 Bioactivity Test of Essential Oils from *Zingiberaceae*  

## CHAPTER 3  MATERIALS AND METHODS

3.1 Samples Collection  
3.2 Extraction of Essential Oils  
3.3 Gas Chromatography/Flame Ionization Detector
3.4 Percentage of Essential Oils 9
3.5 Kovat’s Index 10
3.6 Percentage of individual component in Essential Oils 10
3.7 Cluster Analysis 11
3.6 Bioactivity Test
   3.6.1 Bioactivity test on Artemia salina 11
   3.6.2 Bioactivity test on fungal 12
   3.6.3 Bioactivity test on termites 13

CHAPTER 4 RESULT AND DISCUSSION

4.1 Percentage of Essential Oils 14
4.2 Compound Determination Using Kovat Index 14
4.3 Chemical Composition of Essential 18
   4.3.1 Chemical Composition of the Essential Oils of E. kenyalang 18
   4.3.2 Chemical Composition of the Essential Oils of E. nasuta 23
   4.3.3 Chemical Composition of the Essential Oils of E. brevilabrum 26
   4.3.4 Chemical Composition of the Essential Oils of E. coccinea 30
   4.3.5 Chemical Composition of the Essential Oils of E. fimbriobractaeta 34
4.4 Comparison of Chemical Composition in Rhizome Oils in Five Etlingera species 37
4.5 Comparison of Chemical Composition in Leaf Oils in Four Etlingera species 41
4.6 Comparison of Chemical Composition in Stem oils in Four Etlingera species 44
4.7 Comparison with Other Species and Genus in Zingiberaceae Family 47
CHAPTER 5  CONCLUSION

REFERENCES
CHAPTER ONE

GENERAL INTRODUCTION

1.1. Introduction

*Zingiberaceae* is one of the significant components of the herbaceous ground flora of Malaysian tropical forests. *Zingiberaceae* is one of the families from zingiberales order. This family includes some medicinally important species in particular members of genera of *Alpinia*, *Curcuma* and *Zingiber* (Ibrahim, 1998). The genus *Etingera* belongs to the *Zingiberaceae* family. There are several species in this genus, but in this study only five of the species from genus *Etingera* were analyzed. The essential oils from five species of genus *Etingera* were extracted through hydrodistillation.

Essential oils are the highly concentrated, volatile, aromatic essences of plants. Scientists agree that essential oils may perform more than one function in living plants. In some circumstances they seem to be a part of the plant's immune system. Essential oils help protect the plants as it is the secondary metabolites of the plants. The constituents of the oils are mainly monoterpenes and sesquiterpenes which are hydrocarbons with the general formula \((C_5H_8)_n\). Oxygenated compounds derived from these hydrocarbons include alcohols, aldehydes, esters, ethers, ketones, phenols and oxides. It is estimated that there are more than 1000 monoterpenes and 3000 sesquiterpene structures. Other compounds include phenylpropenes and specific compounds containing sulphur or nitrogen. Essential oils analysis is very important in various fields. Such as chemotaxonomy, determinations of the bioactivity of the essential oils and so on.
1.2. Objectives

This project will be carried according to the following objectives:

a. To extract essential oils from various plant species of *Etlingera* of Sarawak,

b. To determine the chemical composition of essential oils from *Etlingera* of Sarawak using gas chromatography technique (GC/FID),

c. To assess the bioactivity of the essential oils from *Etlingera* of Sarawak on brine shrimps, fungus and termites and

d. To evaluate the significance of essential oils for chemotaxonomy classification.
CHAPTER TWO
LITERATURE REVIEW

2.1 Family of Zingiberaceae

_Zingiberaceae_ is one of the families from _Zingiberales_ order. _Zingiberaceae_ species are represented throughout the tropical and subtropical regions mainly Asiatic in distribution. The Indo-Malayan region is the centre of diversity for the _Zingiberaceae_. Of the 50 genera and 1500 species known in the world, at least 20 genera and 300 species are found in Malaysia (Ali _et al._, 2005). In this study, the _Etlingera_ genus from _Zingiberaceae_ family was used.

Although gingers are better known in traditional medicine, spices, condiments, or flavours, they are new as ornamentals or landscape plants. Members of ginger family, _Zingiberaceae_, can be productively grown as garden ornamental plants. Their attractive forms add variety to the garden scenery, additionally their leaves have fragrant tissues and some species have perfumed flowers (Larsen _et al._, 1999).

2.1.1 Genus of _Etlingera_

_Etlingera_ is one of the genera from the _Zingiberaceae_ family. There are 29 species of _Etlingera_ had been reported in Borneo (Chan _et al._, 2007). The inflorescence of _E. eliator_ is edible and tastes wonderful in soup. _E. elatior_ is an aromatic plant that is widely used as traditional flavouring and medicine. Leaves of _E. elatior_, mixed with other aromatic herbs in water, are used by post-partum women for bathing to remove body odour (Chan _et al._, 2007). These traditional uses may be because of the existence of biologically active volatile constituents. There were some previous studies reported that _E. elatior_ possess important biological activities (Habsah _et al._, 2005). The study of these essential oils can provide interesting raw material for the application of aromatherapy and flavour. _Etlingera_
is an important genus in Sarawak with species often dominant in lowland forest and several utilized as flavouring aromatics. Perhaps the best known is *E. elatior* which aside being a popular cut flower and landscaping ornamental has the unopened inflorescences used as a flavouring (kantan) in Sarawak laksa (Boyce, 2006). *E. elatior* is also used in the local dishes like *nasi kerabu* or *nasi ulam* and *Laksa asam*. Farms in Australia and Costa Rica are selling the inflorescences of *E. elatior*, in shades of pink, deep red and purplish black, as cut flowers (Larsen *et al*., 1999). Another species, *E. coccinea* has leaves and shoots with a strong coriander (cilantro) aroma and taste and is used in much the same way by the local Bidayuh people of western Sarawak who call it tipu. There is a vegetatively similar species, *E. triorgyalis* in which the crushed leaves smell and taste strongly of kerosene. *E. brevilabrum* with its broad oblong leaves liberally spotted deep maroon and carried on waxy-white (pruinose) culms. *E. brevilabrum* is frequently encountered on clay stream banks and have been observed that it dominating several hundred metres of streamside in Kapit Division in central Sarawak. The majority of *Etlingera* in Sarawak have flowers in shades of pink or red although exceptions include *E. brachychila* which can appear in orange and a particularly striking chrome yellow each with a contrasting marked (Boyce, 2006).

The major components identified in the oils of inflorescence and inflorescence axis of *E. elatior* from Brazil were dodecanol (42.5%, 34.6%), dodecanal (14.5%, 21.5%) and *a*-pinene (22.2%, 6.3%), respectively (Zoghbi *et al*., 2005). There was a study to analyze the volatile constituents of essential oils of *E. elatior* (Jack) R. M. Smith from different parts of the plant that is leaves, stems, flowers and rhizomes by gas chromatography and mass spectrometry (Jaafar *et al*., 2007). In that study, the essential oils were extracted using the hydrodistillation method and analysed by GC/MS. The percentage yield of volatile constituents of the leaves, stems, flowers and rhizomes were 0.0735%, 0.0029%,
0.0334% and 0.0021%, respectively. The leaf essential oil contained \( \beta \)-pinene (19.7%), caryophyllene (15.36%) and \((E)-\beta\)-farnesene (27.90%) as major compounds whereas the stem essential oil were largely dominated by 1,1-dodecanediol diacetate (34.26%) and \((E)-5\)-dodecane (26.99%). The essential oils of the flowers and rhizomes contained the major compounds 1,1-dodecanediol diacetate (24.38% and 40.37% respectively) and cyclododecane (47.28% and 34.45% respectively).

2.2 Essential Oils

Essential oils are the highly concentrated, volatile, aromatic essences of plants. Scientists agree that essential oils may perform more than one function in living plants. In some circumstances they seem to be a part of the plant's immune system. In other situation they may simply be the end-products of metabolism. Essential oils contain hundreds of organic constituents, including hormones, vitamins and other natural elements that work on many levels. To extract essential oils in the most effective method while preserving their therapeutic benefits, they are either distilled or expressed. Pure essential oils are most commonly extracted from plants through the process of steam distillation. In this process, steam is introduced into a distillation chamber which contains the plant material. The steam breaks down the plant tissue, causing it to release its essential oil in a vaporized form. The vaporized essences, along with the steam and other substances, pass into a pipe through cooling tanks. The vapours return to liquid form and are separated from the water and captured as pure essential plant oil. There are many research on essential oils have been done by researchers. But the species that they used in their research and the way they present their works are different. For examples, there were a research to analyze the essential oil of the leaves, stems, rhizomes, and roots of the medicinal plant *Alpinia*
Galaga from southern India (Jerovetz et al., 2003). The essential oils of leaves, stems and roots from various species of Etlingera was extracted through hydrodistillation and analyzed for various analyses, such as gas chromatography analysis, anti-termites analysis, and anti-fungal analysis. Some variation in the components of the essential oils of the species may occur due to its age, geographical location and climatic conditions (Larsen et al., 1999).

2.3 Application of Essential Oils for Chemotaxonomy Purposes

Chemotaxonomy is the method that used chemical information to yield useful taxonomic information. There was a study that used the essential oil of the plants to identify the species of the plants that they analyzed. They used eight of the known species of genus Angophora (Dunlop et al., 1999). A study to determine whether the terpenoid composition of the essential oil of Cannabis is useful for chemotaxonomic discrimination had also been carried out, the extracts of distillate inflorescences of 162 greenhouse grown plants of diverse origin were analyzed by gas chromatography (Karl, 2004). There was a study conducted by Hsioa & Lin (1995) where they collected 54 matured leaves samples of nine Clerodendrum taxa native to Taiwan were collected from various places on the island. The essential oils from each sample were then analyzed by gas chromatography and the relationship among taxa was analyzed by cluster analysis of the gas chromatogram data. The cluster analysis indicated similarities between morphological and chemical relationships at the intraspecific level. At the interspecific level, there were similarities and differences between morphological and chemical relationships. The volatile compounds from the aerial parts of Teucrium lepticephalum Pau and Teucrium carolipau C. Vicioso ex Pau, both belonging to the Teucrium pumilum aggregate, were analysed by capillary GC
and GC-MS. From the common and specific compounds (mono- and sesquiterpenes) of the two taxa, chemical characterization was carried out and taxonomic relationships were assessed (Isabel, 2000). Volatile constituents have been used as taxonomic characters, especially at the generic or family level (Larsen et al., 1999).

2.4 Bioactivity Test of Essential Oils from Zingiberaceae

There was a study which determined the antibacterial activity of leaf extracts of several *Etlingera* species. Leaves of *E. elatior*, *E. fulgens* and *E. maingayi* exhibited moderate inhibition of the three bacteria. Moderate inhibition was shown by the leaves of *E. rubrostriata* on *B. cereus* and *S. aureus*, and by the leaves of *E. littoralis* on *S. aureus* (Chan et al., 2007). Antimicrobial activity of *E. cardamomum* against both Grampositive and Gram-negative bacteria species was demonstrated. Its toxicity was investigated on Swiss albino’s mice. Daily, mice were treated orally with 0.003 and 0.3 mg during 7 days. Plasmatic markers and antioxidant defence systems were assessed and histological alterations were evaluated. A significant increase in creatine phosphokinase level was observed. The microscopic evaluation shows that *E. cardamomum* induce morphological perturbation in mice’s heart. The results show also an inhibitory effect of glyceraldehydes 3-phosphate dehydrogenase and an important increase in the level of thiobarbituric acid reactive substances, succinate dehydrogenase and catalase activities. Results show that *E. cardamomum* induces toxicity at 0.3mg/g mouse and affect energy metabolism and oxidative stress (Jazila, 2007). Essential oils are often fungistatic rather than fungicidal. This means that they stop the growth of the fungi while it is exposed to the oil, but once the oil is removed the fungi can continue to grow (Jobling, 2000).
CHAPTER THREE
MATERIALS AND METHODS

3.1 Samples collection

The plant samples were collected from various places in Sarawak, such as Lanjak Entimau of Ulu Katibas, Sungai Asap, Bintulu, Tanjung Batu, Lawas and also from arboretum at UNIMAS campus. The locations for sample collection of Etlingera spp. are summerized in Table 3.1. The samples were then cleaned and stored in the refrigerator in laboratory to minimize the lost of the essential oils of the plant samples.

Table 3.1: Location for Sample Collection

<table>
<thead>
<tr>
<th>Sample</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. kenyalang</td>
<td>Lanjak Entimau Wildlife Sanctuary</td>
</tr>
<tr>
<td>E. nasuta</td>
<td>Arboretum at UNIMAS campus</td>
</tr>
<tr>
<td>E. brevilabrum</td>
<td>Lanjak Entimau Wildlife Sanctuary</td>
</tr>
<tr>
<td>E. coccinea</td>
<td>Tanjung Batu, Lawas</td>
</tr>
<tr>
<td>E. fimbriobractaeta</td>
<td>Sungai Asap, Bintulu</td>
</tr>
</tbody>
</table>

3.2 Extractions of essential oils

Extraction of the essential oils from Etlingera species was performed by hydrodistillation using a Clevenger-type apparatus (Datta, 1987). The plant materials were chopped into small pieces and the essential oils from some hundred grams of leaves, stems and roots (100 g of each sample in approximately 1500mL of distilled water) was obtained by hydrodistillation using a Clevenger-type apparatus for 8 hours continuously with the
distillation rate of 1-2 drops per second. The essential oils was then transferred to 10 mL vials and dried over anhydrous sodium sulphate. The dried essential oil was then transferred to another 5 mL vials using a pasteur pipette and kept in dark place. Prior to gas chromatography analysis, 1\(\mu\)L of essential oils was dissolved with 200\(\mu\)L n-hexane. The analysis was carried out in triplicate.

3.3 Gas chromatography/flame ionization detector (GC/FID) analysis

GC/FID analysis was carried out using a Shimadzu GC-17A 5890 gas chromatograph equipped with a flame ionization detector (FID) fitted with a fused silica 25m \(\times\) 0.3mm DB-5 capillary column. Injector and detector temperatures were set at 260\(^{\circ}\)C and 280\(^{\circ}\)C, respectively. Carrier and make-up gas flow rates were 5.8mL/min He and 24.2mL/min N\(_2\). The oven temperature was programmed at 50\(^{\circ}\)C for 5 min, increased at a rate of 3.5\(^{\circ}\)C/min to 280\(^{\circ}\)C, and hold for 5 minutes at final temperature. A 1\(\mu\)L volume of diluted essential oils had been injected into the chromatograph using splitless injection mode.

3.4 Percentage of essential oils

The percentage of yield of the essential oils was calculated by the following formula:

\[
\text{Percentage of yield, } \% = \frac{\text{volume of the essential oils}}{\text{weight of the dried sample used}} \times 100
\]
3.5 Calculation for Kovat’s index

Kovat’s retention index was used in gas chromatography analysis. N-alkanes serves as the standard and the following formula was used to calculate Kovat’s index:

\[ KI = 100 \left[ \frac{(TR_x - TR_n)}{(TR_{n+1} - TR_n)} + n \right] \]

Where: \( n \) = Number of carbons

\( TR_x = \) Retention time for X analyte.

\( TR_n = \) Retention time for alkane with n carbons.

\( TR_{n+1} = \) Retention time for alkane with n+1 carbons.

In this study n-alkanes standard (C\(_{10}\) – C\(_{30}\)) was used as a reference standard. The chemical composition of the essential oils was identified by comparing the Kovat’s indices obtained in this study with the Kovat’s indices published in the literature.

3.6 Percentage of individual component in essential oils

Semi-quantitative analysis was carried out on the gas chromatographic data of the essential oils that was obtained using GC/FID to calculate the percentage of individual component in the essential oils from *Etlingera spp*. The following formula was used to calculate the percentage of individual chemical compound found in the essential oils:

\[ X\% = \frac{A_x}{A_T} \times 100 \]

Where: \( A_x = \) Peak area of compound X

\( A_T = \) Peak area of all compounds in essential oil
3.7 Cluster analysis

Cluster analysis was performed by using SPSS new version 15.0 software. This analysis was carried out to determine whether the essential oils’ components of *Etlingera spp.* are related to each other or not. If there were relationship between them, the chemical profile obtained can be used for chemotaxonomy classifications. In this analysis, the percentage of individual chemical compound calculated from semi-quantitative analysis was grouped in the multi dimensional space of the variables. The comparisons were carried out for the following:

i. Composition of rhizome oils between five species of *Etlingera*

ii. Composition of leaf oils between four species of *Etlingera*

iii. Composition of stem oils between four species of *Etlingera*

3.8 Bioactivity Tests

The biological activity test of the essential oils on *Artemia salina, Captotermes spp.* and three species of fungi (*Termetes Versicolor, Gloeophyllum trabeum, Chaetomum globosum*) was carried.

3.8.1 Bioactivity test on *Artemia salina*

The bioassay tests were carried out according to the method developed by McLaughin (1991). 20g of brine shrimp were added into 200ml of artificial seawater in a beaker for the hatching process. Continuous air was provided for successful hatching. The brine shrimp were collected after 3 days at room temperature (22°C-29°C). Exactly, 3 mg of essential oil was dissolved in 3 ml of dichloromethane. Then, 500 μL, 50 μL, 5 μL of the samples were transferred into a test tube each in 3 replicates and top up to 5ml with DCM resulting
in the final concentration of 100µg/ml, 10µg/ml and 1µg/ml respectively. After all the solvent evaporated at room temperature, 5mL of artificial seawater was added into each test tube and then approximately 2mL of the solution was added into the multi dish. Ten instar larvae of A. salina were added into each of multi dish hole and the number of surviving instar larvae was counted after 24hours. Artificial seawater and ten larvae of A. salina without samples were used as a control.

3.8.2 Bioactivity test on fungal

Bioactivity of essential oils from Etlingera species on three species of fungi (Termetes Versicolor, Gloeophyllum trabeum, Chaetomum globosum) were performed according to the method developed by Bauer et al. (1966). Briefly, the fungi were cultured on 2% (w/v) Malt Extract Agar (MEA). Agar media was prepared by dissolving 48 g of MEA in 1L of distilled water. The agar solution was sterilized at 121°C for 15 minutes before pouring it into the petri dishes. Each species of fungi was cultured in five petri dishes for 7 days at 25°C and 70% relative humidity.

Potato/dextrose broth (PDB) was prepared by mixing 4 g potato starch with 20 g dextrose in 1L of water. About 100 ml of prepared PDB was poured into a 500 ml erlenmeyer flask and inoculated with 10 agar plugs of about 5 mm x 5 mm from actively growing fungi culture. The inoculated PDB was incubated at 25°C for 10 days on a rotary shaker at 150 rpm.

Analytical-grade filter paper disks (13.0 mm diameter) was dipped into essential oils with concentration of 1000 µg/mL, 500 µg/mL and 250 µg/mL and dried for 30 minutes in a laminar flow. Four dries paper discs was placed equidistant from one another on the surface of the inoculated MEA plates and incubated at 25°C for 5 days. The bioassay plates
were examined for the presence of zone of inhibition surrounding the disc, which is evidence of the inhibition of germination and a measure of antifungal activity. The lowest concentration of essential oils where inhibition of fungi was observed was designated as minimum inhibitory concentrations (MICs). Inhibition zone diameters was interpreted on the basis of Barry et al. (2002), with zone diameters of ≥ 19 mm indicating susceptibility, zone diameters of 15 to 18 mm indicating susceptible-dose dependent, and zone diameters of ≤ 14 mm indicating resistance.

3.8.3 Bioactivity test on termites

Bioactivity of essential oils from *Etingera spp.* on *Coptotermes spp.* was performed according to the method developed by Sakasegawa et al. (2003). Briefly, the termite was cultured for 2-3 days in a suitable chamber maintained at room temperature. In the anti-termites test, filter paper at 25 mm diameter was placed in each hole of a six holes multi dish (3 rows × 2 lines with holes diameter 25 mm). The essential oils were diluted with DCM to give concentration of 10.0% to 0.1%. For contact anti-termites test, exactly 50 μL of the diluted essential oils was placed on the filter paper on one row. After the solvent was completely evaporated, exactly 100 μl of distilled water was added into the entire six multi dish hole. Six termites (Comprise of 5 workers and 1 soldier) were added into each hole. The multi dish were closed tightly and kept at 27°C in an incubator. The number of survivors was counted each day. The test was performed nine times [3 holes/ (multi dish × 3 times)] for each concentration. The activity of the essential oils on termites was then observed.
CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Percentage of Essential Oils

The abundance of essential oils obtained from three different parts of five species of *Etlingera* varies greatly within the range of 0.11% to 1.83% as shown in Table 4.1. Generally, the leaf of all the species obtained high percentage of yield compared to their other parts where the leaf of *E. nasuta* yield the highest amount of oil with percentage of yield 1.83%. While the lowest percentage of yield was obtained from stem *E. kenyalang* (0.11%).

4.2 Compound Determination Using Kovat Index.

Kovat’s retention index was used as quantitative test to identify the chemical component in the sample. The retention time of the n-alkanes in gas chromatogram serve as the reference for calculating the Kovat’s index of the compound detected. Only the retention times of the n-alkanes with even numbered carbon were obtained from the GC/FID analysis. Thus, for the retention times of the odd-numbered carbon n-alkanes were obtained by calculating the average retention times of the two adjacent even numbered carbons n-alkane. The retention times of the n-alkane standards obtained from GC/FID analysis are presented in the Table 4.2. Gas chromatograms from GC/FID analysis for the mixture of n-alkane standard is shown in Figure 4.1. The calculated Kovat’s indices for component detected were then compared to the Kovat’s indices compiled by Acre and Arn (2004).