CHARACTERIZATION OF EPICUTICULAR WAXES FROM ZINGIBERACEAE FAMILY

Norhafiza Md Nor

Bachelor of Science with Honours (Resource Chemistry) 2006
CHARACTERIZATION OF EPICUTICULAR WAXES FROM ZINGIBERACEAE FAMILY

NORHAFIZA MD NOR

This project is submitted in partial fulfillment of the requirement for the degree of Bachelor of Science with Honours (Resource Chemistry)

FACULTY OF RESOURCE SCIENCE AND TECHNOLOGY

UNIVERSITI MALAYSIA SARAWAK

MAY 2006
DECLARATION

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

NORHAFIZA MD NOR

Resource Chemistry Programme
Faculty of Resource Science and Technology
Universiti Malaysia Sarawak
ACKNOWLEDGEMENT

I would like to express my gratitude to my supervisor, Assoc. Prof. Dr Zaini Assim for his assistance and constructive advice throughout doing this project. Not forgetting to Mr Rajuna Tahir, for always being there to assist in any technical. Last but not least to my family for financial assistance and words of encouragement and fellow classmate for helping me in some way in completing this final year project. Thank you.
# TABLE OF CONTENT

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>iii</td>
</tr>
<tr>
<td>TABLE OF CONTENT</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLE</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF FIGURE</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td>x</td>
</tr>
<tr>
<td>CHAPTER 1 INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Research objective</td>
<td>4</td>
</tr>
<tr>
<td>CHAPTER 2 LITERATURE REVIEW</td>
<td>5</td>
</tr>
<tr>
<td>2.1. Zingiberaceae family</td>
<td>5</td>
</tr>
<tr>
<td>2.2. Epicuticular waxes</td>
<td>6</td>
</tr>
<tr>
<td>CHAPTER 3 MATERIALS AND METHODS</td>
<td>8</td>
</tr>
<tr>
<td>3.1 Sample collected</td>
<td>8</td>
</tr>
<tr>
<td>3.2 Extraction of epicuticular wax</td>
<td>8</td>
</tr>
</tbody>
</table>

iv
3.3 Instrumentation analysis.  
3.3.1. Gas chromatography

3.4 Analysis of Data

3.4.1. Leaf surface area
3.4.2. Kovat’s retention index
3.4.3. Semi quantitative analysis
3.4.4. Relative respond factor (RRF)
3.4.5. Concentration of aliphatic hydrocarbon
3.4.6. Cluster analysis.

CHAPTER 4 RESULT AND DISCUSSION

4.1. Relationship between leaves surface area and total wax extracted.
4.2. Quantitative analysis of aliphatic hydrocarbon in each genus from Zingiberaceae family.
4.3 Isoprenoid/ n-alkane ratio
4.5. Cluster analysis

CHAPTER 5 CONCLUSION AND RECOMMENDATION

REFERENCES
LIST OF TABLE

Table 4.1 percentage of wax extracted from leaves for six Ginger plants 13
Table 4.2 Retention time for aliphatic and isoprenoid (pristane and phytane) standard 15
Table 4.3 Kovat's index for genus *Elletaria* 19
Table 4.4 Kovat's index for *Elletariopsis kerbyi* 19
Table 4.5 Kovat's index for *Etlingera triorgalys* 20
Table 4.6 Kovat's index for genus *Alpinia* 20
Table 4.7 Kovat's index for genus *Zingiber* 21
Table 4.8 Kovat's index for *Hedychium coronarium* 21
Table 4.9 The concentration of individual n-alkane isoprenoids of aliphatic hydrocarbon 22
Table 4.10 The ratio of pristane/C$_{17}$ and phytane/ C$_{18}$ 23
Table 4.11 Chemical composition of epicuticular wax in genus *Elletaria* 24
Table 4.12 Chemical composition of epicuticular wax in *Etlingera triorgalys* 25
Table 4.13 Chemical composition of epicuticular wax in *Elettariopsis kerbyi*

Table 4.14 Chemical composition of epicuticular wax in genus *Alpinia*

Table 4.15 Chemical composition of epicuticular wax in genus *Zingiber*

Table 4.16 Chemical composition of epicuticular wax in *Hedychium coronarium*
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>GC/FID trace for n-alkane standard</td>
<td>14</td>
</tr>
<tr>
<td>4.2</td>
<td>GC/FID trace for extract of epicuticular wax from <em>Elleteria</em> sp</td>
<td>16</td>
</tr>
<tr>
<td>4.3</td>
<td>GC/FID trace for extract of epicuticular wax from <em>Ellenariopsis kerbyi</em></td>
<td>16</td>
</tr>
<tr>
<td>4.4</td>
<td>GC/FID trace for extract of epicuticular wax from <em>Ellingera triorgalys</em></td>
<td>17</td>
</tr>
<tr>
<td>4.5</td>
<td>GC/FID trace for extract of epicuticular wax from <em>Hedychium coronarium</em></td>
<td>17</td>
</tr>
<tr>
<td>4.6</td>
<td>GC/FID trace for extract of epicuticular wax from genus <em>Zingiber</em></td>
<td>18</td>
</tr>
<tr>
<td>4.7</td>
<td>GC/FID trace for extract of epicuticular wax from genus <em>Alpinia</em></td>
<td>18</td>
</tr>
<tr>
<td>4.8</td>
<td>Cluster analysis of chemical component present in six genera of Zingiberaceae family</td>
<td>29</td>
</tr>
</tbody>
</table>
ABSTRACT

Characterization of leaves epicuticular wax from six Zingiberaceae genera were carried out to determine the present and amount of aliphatic hydrocarbon. The extraction of epicuticular wax was done using chloroform and analyzed with Gas Chromotography/Flame Ionization Detector and GC/MS. The hydrocarbon distribution ranges from C9-C24. Chemical component present in the wax was determined by Kovat's retention indices and using cluster analysis to determine the correlation. It reveals that Alpinia, Elletaria, Elletariopsis kerbyi, Etingera triorga and Hedychium coronarium have some similarities due to the component present and its amount. The result might be useful in chemotaxonomic study for the genera.

Key words: Zingiberaceae; leaf epicuticular wax; aliphatic hydrocarbon; chemotaxonomy
ABSTRAK


Kata kunci: Zingiberaceae; lilin epikutikular daun; hidrokarbon alifatik; kemotaksonomi
CHAPTER 1
INTRODUCTION

1.1 Introduction

The Zingiberaceae is the largest family of the order Zingiberales. It consists of about 50 genera and over 1,000 species distributed throughout South-East Asia. Genus Alpinia, Ellettariopsis, Hornstedtia, Zingiber, and Elingera belong to the Zingiberaceae family. These genera are often used as ingredients in traditional medicine and have been subjected to study the characterization of epicuticular wax from Zingiberaceae family.

Cuticles provide the interface between primary plant tissues and the atmosphere (Walton, 1990). Plant cuticles contain SCL, is called 'cuticular waxes', not only embedded within the cutin (intracuticular waxes) but also on their surface epicuticular waxes (Baker, 1982; Holloway, 1982). It is widely accepted that all plant cuticles carry a thin film of epicuticular waxes on the surface of their cutin matrix (Baker, 1982; Holloway, 1982; Walton, 1990).

Studies on plant epicuticular wax can be used as supplementary information to classical taxonomy of which is the approach focuses on the chemical aspect of the wax because every plant species differ in their wax components and composition.

For many plant systems if has been shown that the chemical composition of epicuticular wax crystals differ from the composition of bulk cuticular waxes.
Evidence for this distinction largely exploited the characteristic shapes and the high mass portions of epicuticular crystals (Baker, 1982; Gulz et al., 1992; Jetter and Riederer, 1994).

To analyze and identify the differences in each *Alpinia, Elleteria, Elletariopsis kerbyi*, and *Hedychnium coronarium, Etlingera triorgalis, Zingiber* and *Etlingera* genera, extraction method with immersion in CHCl₃ was used (Sase et al., 1998a; Sase et al., 1998b).

In the past, surface extraction with organic solvents was routinely employed to probe cuticular waxes for chemical analysis (Holloway, 1984). Because solvent molecules release both intra- and epicuticular waxes together, only the overall composition of the complete wax mixture could be assessed (Walton, 1990). Meanwhile, some evidence for chemical differences showed that special wax constituents form the epicuticular crystals on diverse plant surfaces (Jeffree et al., 1975; Gulz et al., 1992; Jetter and Riederer, 1994). In these cases individual constituents are accumulated at the very plant surface (Jeffree et al., 1975; Baker, 1982), probably by diffusion and spontaneous phase separation of compounds.

Epicuticular waxes have been shown to protect plants from fungal infection. Martin et al., isolated a fungistatic component of apple leaf wax that suppressed germination of powdery mildew conidia (*Sphaerotheca pannosa*). This ether-soluble component was transferred onto susceptible broad bean leaves where it entirely suppressed formation of necrotic lesions caused by *Botrytis fabae*. Other studies have shown that cuticular waxes are associated with resistance of rose leaves to the germination of powdery
mildew conidia (Baker and Hunt, 1981), Eucalyptus camaldulensis to Phaeoseptorea eucalypti (Heather, 1967), and beetroot (Beta vulgaris L.) to Botrytis cinerea (Blakeman and Sztenjnberg, 1973). The morphology of the surface wax varies among Zingiberaceae family cultivars; morphology is believed to be dependent on the chemical composition of the cultivar (Martin and Juniper, 1970; Jeffree et al., 1975). The inherent chemical and physical properties rather than the properties of the underlying cuticular membrane influence the crystalline structure of epicuticular wax.

In a study of the epicuticular wax of Eucalyptus cinerea, Hallam (1964) postulated that wax migrates to the leaf surface through anastomosing channels between the cuticular lamellae. The pores beneath each particle of wax on the leaf cuticle of Trifolium repens, Brassica oleracea, and Poa colensoi are possible apertures for wax exudation (Hall and Donaldson, 1962). The surface waxes often show a random distribution on the leaf, although distinct patterns sometimes emerge. Hall (1967) observed different patterns of epicuticular waxes which depended on the number of pores from which the waxes were exuded, and the proximity and arrangement of the pores.

The chemical analysis was analyzed by GC-FID and GC/MS. Amount of epicuticular wax may be affected by leaf age (Cape, 1986; Cape et al., 1989; Percy et al., 1993) and by its morphology and chemical composition (Gunthardt-Goerg, 1986).
1.2 **Objective of the Research.**

The objectives of this project are:

i. To determine the organic component present in the epicuticular wax and quantitatively analyze the n-alkane in six genera.

ii. To analyze and identify the differences in each *Alpinia, Elletaria,* *Elletariopsis kerbyi,* and *Hedychium coronarium, Etingera triorgalys,* and *Zingiber* genera that would allow us to compare results from different genera.

iii. These findings are essential for chemotaxonomy of Zingiberaceae family as supplement to the classical taxonomy.
CHAPTER 2

LITERATURE REVIEW

2.1 Zingiberaceae Family

Zingiberaceae are distributed mostly in tropical and subtropical areas. The center of distribution is in South East Asia. The greatest concentration of genera and species is in the Malesian region (Indonesia, Malaysia, Singapore, Brunei, the Philippines and Papua New Guinea). The Zingiberaceae species are the ground plants of the tropical forests. They mostly grow in damp and humid shady places. They are also found infrequently in secondary forest. Some species can fully expose to the sun and grow on high elevation.

A general characteristic of the family Zingiberaceae has simple’s leaves, distichous. Flowers are delicate, ephemeral and highly modified. All parts of the plants are aromatic and fruit a capsule. Zingiberaceae is divided into four tribes such as Hedychieae, Zingibereae, Alpineae, Globbeae. It is important natural resources that provide many useful products for food, spices, medicines, dyes, perfume and aesthetics to man. Zingiber officinale, for example, has been used for many years as spices and in traditional forms of medicine to treat a variety of diseases (Srirugsa, 1995).
2.2 Epicuticular Waxes

The epicuticular waxes, which form a waxy bloom on the surfaces of many plants, are conspicuous and readily accessible to investigation, and their properties, structure and chemistry are consequently much studied (Baker, 1982). Waxes are the waterproofing component of the plant cuticle and are therefore essential for life in an aerial environment. They are embedded within cutin or Suberin polymers and continue as an amorphous layer on the outer surface of the plant.

By definition, cuticular waxes are the hydrophobic compounds on the surface of the plant. Epicuticular wax is the thin film that covers the surface of leaves in higher plants (Jetter and Schaffer, 2001). No structure corresponding to a pathway for wax precursors is visible, however, and the mechanism by which the epicuticular wax is delivered to the plant surface remains unconfirmed (Pyee et al., 1994). The structure of epicuticular wax crystallites has been determined by the crystal morphology of dominant chemical constituents is well established both from correlative and genetic observations (Hallam, 1967; von Wettstein-knowles, 1974) and by experimental analysis (Jeffree, 1974; Jetter and Riederer, 1994; Lister and Thair, 1981) in many species.

However, both the morphology of the Strelitzia-type waxes found in many species (Meusel et al., 1994) and the non-random arrangement of the structures in these and other groups that show conspicuous wax patterning suggest that other as yet unidentified, mechanisms must also be involved.
The most common major components of cuticular waxes are hydrocarbons and their oxygenated derivatives such as secondary alcohols and ketones, very long-chain fatty acids, aldehydes and alcohols, and wax esters composed of very long-chain fatty alcohols and fatty acids (Kolattukudy, 1980). Each lipid class of the cuticular wax may be present as a homologous series, or one particular chain length may predominate. Cuticular wax composition varies among and within species.

The cuticle provides the first line of defense between the plant and its environment; the cuticular waxes shed rainwater from the plant surface and limit nonstomatal water loss. In addition, waxes may protect plants from bacterial and fungal pathogens (Kolattukudy, 1987) and play a role in plant-insect interactions (Eigenbrode and Espelie, 1995).

Hydrophobic makes wax a good solvent for organic pollutants and impedes the uptake of aqueous foliar sprays without the addition of surfactants. In addition, the reflective nature of waxes offers some protection against damaging UV radiation.

The same plant may show organ-to-organ differences, tissue-to-tissue differences and developmental differences. Young leaves have predominantly primary alcohols (63%), and older leaves have predominantly wax ester (42%) (Avato et al., 1990).
3.1 Sample Collected

The leaves of Zingiberaceae from Alpinia, and Elletaria genera were collected from the forest around campus UNIMAS, while Elletarioptis kerbyi, and Hedychium coronarium, Zingiber, and Etlingera genera collected from the forest around semenggok and batu 7. Sampling of leaves was carried out from 5 years old trees and fresh healthy leaves. The leaves were transferred to the frozen room and kept at 5°C overnight until analysis in the following day. There are no any microbial infections or morphological changes in the wax under these conditions (Percy et al., 1993; Sase et al., 1998a). Moreover, under these conditions, leaves remained fresh without any water loss. During transportation and keeping of leaves, care was be taken no to abrade the wax.

3.2 Extraction of Epicuticular Wax

Briefly. Leaves was weighted and leaves then immersed in chloroform for 60s to extract epicuticular wax. Crude extract obtained was transferred into 100 mL pear shape flask and 5 mL dichloromethane (DCM) will be added into it. The mixture was then evaporated under reduced pressure using rotary evaporator. The residues were dilute in 2-5 mL dichloromethane to ensure no wax will be sticking at the wall of the pear flask. The homogeneous mixture then transferred into a vial using Pasture pipette and dried by pure nitrogen gas flow (Prasad and Gulz, 1990).
3.3 **Instrumentation Analysis.**

3.3.1. **Gas Chromatography**

The analysis of epicuticular wax was performed on a SHIMADZU 17A Gas Chromatography equipped with Flame Ionization Detector. Fused silica capillary column of using DB5 fused silica column (25m long X 0.3mm internal diameter X 0.25μL film thickness). Analysis carried out with temperature programmed injection at 50°C before being raised to 30°C at the rate of 10°C / minutes. Sample that was going to be injected was diluted with 100μL of hexane. Only 1 μL of diluted sample was injected for GC/FID analysis. Variations in injection volumes accounted for by reporting wax components among samples was based on retention times (R_t) of known standards verified by GC-mass spectrometry.

3.4 **Analysis of Data**

3.4.1. **Leaf Surface Area**

The leaf area was estimate by photocopying the leaf on graph paper and counting 1-cm squares. The result was used to correlate with the amount of wax extracted from the leaves.

3.4.2. **Kovat’s Retention Index**

Kovat’s Retention Index was done according to the result of retention time obtained from chromatogram. It is used to determine components present in the wax. The formula is as below:
Where;

\[ I_{cpd} = 100 \left( \frac{T_{cpd} - T_x}{T_{x+1} - T_x} \right) + 100x \]

Where;  
\( I_{cpd} \) = Kovats Retention Index  
\( T_{cpd} \) = Retention time for compound \( X \)  
\( T_x \) = Retention time of alkane eluted before the compound,  
\( T_{X+1} \) = Retention time of alkane eluted after the compound.  
\( x \) = number of carbon

3.4.3. Semi Quantitative Analysis

After obtaining the result for chemical composition present in the wax therefore have to determine the percentage of these compounds. Using semi quantitative analysis, data obtained from the gas chromatography were used to determine the percentage of individual compound by using normal distribution method.

\[ \% X = \frac{A_{cpd}}{\sum A_t} \times 100 \]

Where;  
\( A_{cpd} \) = Component peak area  
\( \sum A_t \) = Total peak area
3.4.4. **Relative Respond Factor (RRF)**

The quantitative data for aliphatic hydrocarbons fraction was calculated based on equation used by Simoneit (1978). Calculation of RRF was done based on standard chromatogram and calibration mixture solubility of Flame Ionization Detector using the formula shown:

\[
RRF = \frac{\text{Amount of component}}{\text{Peak area of component}} \times \frac{\text{Peak area of Internal Standard}}{\text{Amount of Internal Standard}}
\]

3.4.5. **Concentration of Aliphatic Hydrocarbon**

This part was done to determine the total amount of component present in the wax. The formula is as below:

\[
\text{Amount of Individual Hydrocarbon} = \frac{\text{Amount of Internal Standard}}{\text{Peak area of Internal Standard}} \times \text{Peak area of component} \times \text{RRF}
\]

Therefore, to obtain the concentration of component present, the formula used was:
Concentration of Selected Hydrocarbon

\[
\text{Amount of selected component} = \frac{\text{Weight of dried sample (g)}}{\text{X} \text{ Dilution factor}}
\]

3.4.6. Cluster Analysis.

Cluster analysis was done using SPSS 11.0 programmed. The percentage of component present was used as data in order relate or differentiate between these six genera.
CHAPTER 4
RESULT AND DISCUSSION

4.1 Relationship between Leaves Surface Area and Total Wax Extracted.

The percentage of wax extraction from epidermal leaf for six Ginger plants are presented in Table 4.1

<table>
<thead>
<tr>
<th>Species</th>
<th>Leaves surface area (cm²)</th>
<th>% wax extracted</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Elleteria</em></td>
<td>68.5</td>
<td>11.1</td>
</tr>
<tr>
<td><em>Etlingera triorgalys</em></td>
<td>67.5</td>
<td>4.1</td>
</tr>
<tr>
<td><em>Alpinia</em></td>
<td>54.0</td>
<td>5.7</td>
</tr>
<tr>
<td><em>Zingiber</em></td>
<td>79.0</td>
<td>53.7</td>
</tr>
<tr>
<td><em>Elletariopsis kerbyi</em></td>
<td>19.0</td>
<td>42.0</td>
</tr>
<tr>
<td><em>Hedychium coronarium</em></td>
<td>64.0</td>
<td>30.7</td>
</tr>
</tbody>
</table>

Table 4.1: The percentage of wax extracted from leaves for six Ginger plants.

4.2 Quantitative Analysis of Aliphatic Hydrocarbon in Each Genus from Zingiberaceae Family.

Quantitative analysis of aliphatic hydrocarbon was done according to gas chromatogram obtained and calculated based on the standard n-alkane chromatogram.