PHYTOCHEMICAL INVESTIGATION ON SOME SPECIES FROM THE GENERA *ELETTARIOPSIS* AND *ETLINGERA*

YASODHA SIVASOTHY

UNIVERSITI SAINS MALAYSIA

2008

PHYTOCHEMICAL INVESTIGATION ON SOME SPECIES FROM THE GENERA *ELETTARIOPSIS* AND *ETLINGERA*

by

YASODHA SIVASOTHY

Thesis submitted in fulfillment of the

requirements for the Degree of

Doctor of Philosophy

MAY 2008

ACKNOWLEDGEMENT

Firstly, I would like to take this opportunity to thank my supervisor, Assoc. Prof. Dr. Wong Keng Chong for his advice and guidance. I am also thankful to my co-supervisor, Prof. Dr. Boey Peng Lim for his encouragement and helpful suggestions throughout the course of this study.

Next, I would like to acknowledge the Dean of the Institute of Graduate Studies (IPS) for giving me a chance to pursue my postgraduate studies in USM. Special thanks to the Dean of the School of Chemical Sciences, Prof. Dr. Wan Ahmad Kamil Mahmood for providing me with the assistance and facilities which ensured the success of my research.

I would also like to thank Institut Pengajian Siswazah for awarding me with the Graduate Assistant Scheme which covered my allowance and my tuition fee.

I am very grateful to Datuk Lim Chong Keat from FRIM and Mr. Baharuddin Sulaiman from the School of Biological Sciences, USM for providing me with the plant materials and in the identification of them for my research. I would also like to thank Mr. Shanmugam from the School of Biological Sciences, USM for helping me prepare the voucher specimens for certain of those species.

I would like to forward my appreciation to the technical and laboratory staffs of the School of Chemical Sciences, in particular, Mr. Chow Cheng Por, Mr. Clement D'Silva, Mr. Tan Chin Tong, Mr. Megat Hasnul, Mr. Yee Chin Leng, Mr. Chee Sai Gnow, Mr. Aw Yeong and Mr. Mohd. Fahmi Mohd. Yusoff for their assistance during the duration of this study.

I would like to acknowledge Mr. Khoo Kay Hock and Mr. Hilman from the Centre for Drug Research, USM for their help with the GC-MS analyses.

Special thanks to Madam Wong Lai Kwai and Madam Han Yan Hui from the Department of Chemistry, National University of Singapore for their help with the DP-MS and the NMR analyses.

I would also like to thank my colleagues and friends for their cooperation and moral support throughout this project.

Finally, I would like to convey my deepest gratitude to my parents for their love and encouragement.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SCHEMES

LIST OF ABBREVIATIONS

GC: Gas Chromatography

GC-MS: Gas Chromatography-Mass Spectrometry

FID: Flame Ionization Detector

RI: Retention Index

R_t: Retention Time

TLC: Thin Layer Chromatography

UV: Ultra-Violet Spectroscopy

Prep-TLC: Preparative Thin Layer Chromatography

Prep-GC: Preparative Gas Chromatography

TCD: Thermal Conductivity Detector

FT-IR: Fourier Transform Infrared

DP-MS: Direct Probe-Mass Spectrometry

EI: Electron Ionization

NMR: Nuclear Magnetic Resonance

DEPT: Distortionless Enhancement by Polarization Transfer

COSY: Correlated Spectroscopy

HMQC: Heteronuclear Multiple Quantum Correlation

HMBC: Heteronuclear Multiple Bond Correlation

NOESY: Nuclear Overhauser Enhancement Spectroscopy

ROESY: Rotating frame Overhauser Effect Spectroscopy

NOE: Nuclear Overhauser Effect

ppm: parts per million

s: singlet

d: doublet

t: triplet

q: quartet

m: multiplet

dd: double doublets

br: broad

eV: electron volts

amu: atomic mass unit

ax: axial

eq: equatorial

s: strong

w: weak

LIST OF APPENDICES

KAJIAN FITOKIMIA KE ATAS BEBERAPA SPESIES DARIPADA GENERA *ELETTARIOPSIS* DAN *ETLINGERA*

ABSTRAK

Minyak pati bahagian daun dan akar enam spesies *Elettariopsis* dan tiga spesies *Etlingera* yang diperolehi dari Malaysia telah dipencilkan melalui kaedah penghidrosulingan dan dianalisiskan dengan kaedah GC kapilari dan GC-MS dengan menggunakan dua kolum dengan kekutuban yang berbeza. Kedua-dua minyak pati *Elettariopsis smithiae* didominasi oleh monoterpena, dengan komponen utama geranial (38.1%) dan neral (29.1%) dalam minyak pati daun, dan kamfena (22.9%) dan α-fenchil asetat (15.7%) dalam minyak pati akar. Minyak pati daun *E. rugosa* mempunyai kandungan tinggi seskuiterpena, terutamanya spatulenol (29.5%), sementara kebanyakan komponen dalam minyak pati akarnya adalah monoterpena dengan β-felandrena (26.2%) dan kamfena (15.2%) dua komponen utama yang hadir dalam kandungan paling tinggi. Kedua-dua minyak pati daun dan akar *E. elan* mempunyai kandungan monoterpena yang tinggi dengan geraniol (71.6%) sebagai komponen major dalam minyak pati daun manakala kamfena (28.6%), α-fenchil asetat (8.6%) dan α-felandrena (8.4%) dalam minyak pati akar. Komponen bukan terpena membentuk sebahagian besar minyak pati daun dan akar *E. slahmong* dengan aldehid alifatik, terutamanya *trans*-2-oktenal (46.3% and 8.1%, masing-masing) dan *trans*-2-dekenal (36.8% and 79.4%, masing-masing) membentuk bahagian utama. Monoterpena didapati mendominasi profil minyak pati daun dan akar *E. triloba*, dengan β-pinena (31.2%), neral (12.3%), geranil asetat (11.3%) dan geranial (10.7%) merupakan komponen utama dalam minyak pati daun sementara kamfena (29.3%) dan α-felandrena (10.4%) mencirikan minyak pati akarnya. Minyak pati daun dan akar *E. curtisii* mempunyai kandungan tinggi monoterpena dengan β-pinena (42.7% and 29.6%, masing-masing) and β-felandrena (19.9% and 17.6%, masing-masing) dua komponen utama dalam kedua-dua minyak pati tersebut.

Minyak pati daun *Etlingera littoralis* dicirikan oleh kehadiran *trans*-metil isoeugenol (37.7%), β-pinena (30.4%) dan β-felandrena (8.6%) manakala *trans*-metil isoeugenol (58.1%) dan sandarakopimara-8(14),15-diena-3β-ol (9.1%) didapati dominan dalam minyak pati rizom dan akarnya. Komponen utama dalam minyak pati daun *E. elatior* adalah mirsena (13.5%), α-humulena (11.8%), β-kariofilena (10.7%), dodekanol (9.9%) dan α-pinena (8.5%) sementara minyak pati rizom dan akarnya mengandungi kuantiti monoterpena yang tinggi dan dicirikan dengan kehadiran kamfena (18.0%), β-pinena (16.9%), bornil asetat (9.2%) dan α-pinena (8.6%). Minyak pati daun *E. elatior* var. *Thai queen* dicirikan oleh komponen bukan terpena manakala minyak pati rizom dan akarnya mempunyai kandungan monoterpena yang tinggi. α-Pinena (24.4%), dodekanol (18.9%) dan dodekanal (15.9%) merupakan komponen utama dalam daun tetapi rizom dan akarnya dicirikan dengan kehadiran kamfena (15.1%), dodekanol (12.9%), bornil asetat (10.7%) and dodekanal (10.6%).

Bahagian rizom, akar, buah dan daun *E. littoralis* diekstrakan secara berasingan dengan menggunakan pelarut petroleum eter diikuti etil asetat. Setiap ekstrak ditulenkan melalui kaedah kromatografi kolum dengan menggunakan gel silika atau Sephadex LH-20, kaedah TLC penyediaan atau kaedah GC penyediaan untuk memberi sembilan sebatian. Ketulenan setiap sebatian diuji sama ada dengan TLC atau GC dan strukturnya ditentukan dengan kaedah-kaedah spektroskopi seperti IR, GC-MS, DP-MS, 1D NMR, 2D NMR dan UV. Data putaran optik spesifik bagi sebatian-sebatian tertentu diperolehi untuk membantu dalam mengenalpasti isomer optik yang sebenar. Sandarakopimara-8(14),15-diena-3β-ol, isopimara-7,15–diena-3β-ol, 15-isopimarena-8β,19-diol, *trans*-metil isoeugenol dan metil vanilin telah diidentifikasikan dalam bahagian rizom dan akar, *ent*-katecin dan *ent*-(*E*)-labda-8(17),12-diena-15,16-dial pula dipencilkan daripada buah manakala α-tokoferol dan 19β–asetoksisandarakopimara-8(14),15-diena, sejenis diterpena baru daripada kelas pimarana, didapati hadir dalam daun.

PHYTOCHEMICAL INVESTIGATION ON SOME SPECIES FROM THE GENERA *ELETTARIOPSIS* AND *ETLINGERA*

ABSTRACT

The volatile oils of the aerial and underground parts of six Malaysian *Elettariopsis* and three *Etlingera* species were isolated by hydrodistillation and analysed by capillary GC and GC-MS, using two columns of different polarity. The oils of *Elettariopsis smithiae* were dominated by monoterpenoids, the major components being geranial (38.1%) and neral (29.1%) in the leaf oil, and camphene (22.9%) and *α*-fenchyl acetate (15.7%) in the oil from the roots. The leaf oil of *E. rugosa* contained high levels of sesquiterpenoids, particularly spathulenol (29.5%), while the root oil contained mainly monoterpenoids, with *β*-phellandrene (26.2%) and camphene (15.2%) being clearly the two most abundant components. Both the leaf and root oils of *E. elan* were overwhelmingly monoterpenoid in character, with geraniol (71.6%) accounting for the major part of the leaf oil, while camphene (28.6%), *α*-fenchyl acetate (8.6%) and *α*-phellandrene (8.4%) characterized the root oil. Non-terpenoids constituted most of the leaf and root oils of *E. slahmong*, with aliphatic aldehydes, particularly *trans*-2-octenal (46.3% and 8.1%, respectively) and *trans*-2-decenal (36.8% and 79.4%, respectively), accounting for the major part. Monoterpenoids dominated the profile of the leaf and root oils of *E. triloba*, with *β*-pinene (31.2%), neral (12.3%), geranyl acetate (11.3%) and geranial (10.7%) the principal contributors in the leaf oil, while camphene (29.3%) and *α*-phellandrene (10.4%) characterized the root oil. The leaf and root oils of *E. curtisii* were principally monoterpenoid, with *β*-pinene (42.7% and 29.6%, respectively) and *β*-phellandrene (19.9% and 17.6%, respectively) being the two most prominent components in both.

The leaf oil of *Etlingera littoralis* was characterized by the occurrence of *trans*-methyl isoeugenol (37.7%), *β*-pinene (30.4%) and *β*-phellandrene (8.6%), while in the rhizome and root oil, *trans*-methyl isoeugenol (58.1%) and sandaracopimara-8(14),15-diene-3*β*-ol (9.1%) predominated. Regarding the leaf oil of *E. elatior*, the major components were myrcene (13.5%), *α*-humulene (11.8%), *β*-caryophyllene (10.7%), dodecanol (9.9%) and *α*-pinene (8.5%). The rhizome and root oil, however, was dominated by monoterpenoids, with camphene (18.0%), *β*-pinene (16.9%), bornyl acetate (9.2%) and *α*-pinene (8.6%) clearly the most abundant. Non-terpenoids were predominant in the leaf oil of *E. elatior* var. *Thai queen* while the rhizome and root oil was dominated by monoterpenoids. *α*-Pinene (24.4%), dodecanol (18.9%) and dodecanal (15.9%) were the major constituents present in the leaves, while in the rhizome and root oil, camphene (15.1%), dodecanol (12.9%), bornyl acetate (10.7%) and dodecanal (10.6%) predominated.

The air-dried rhizomes, roots, fruits and leaves of *E. littoralis* were separately fractionated by solvent extraction using petroleum ether followed by ethyl acetate. Each extract was further fractionated by repeated column chromatography over either silica gel or Sephadex LH-20, preparative TLC or preparative GC, giving nine isolated compounds. The purity of each of the compounds was examined using either TLC or GC, and its structure elucidated by spectroscopic techniques such as IR, GC-MS, DP-MS, 1D NMR, 2D NMR and UV. Specific optical rotation data were also obtained for certain of these compounds to aid in the identification of the exact optical isomer. Sandaracopimara-8(14),15-diene-3*β*-ol, isopimara-7,15–diene-3*β*-ol, 15-isopimarene-8*β*,19-diol, *trans*-methyl isoeugenol and methyl vanillin were identified from the rhizomes and roots, *ent*-catechin and *ent*-(*E*)-labda-8(17),12-diene-15,16-dial from the fruits, *α*-tocopherol and a new pimarane type diterpene, 19*β*–acetoxysandaracopimara-8(14),15-diene, from the leaves.

CHAPTER ONE

INTRODUCTION

1.1 Natural Products Chemistry

Natural Products Chemistry is a field focused on the discovery of new compounds with biological activities which may be of medical importance and which are produced by plants, animals or other organisms. Extracts are assayed for biological activity and the active fractions are purified to find the active compound(s). Structural characterization of the compounds by spectroscopic and related analytical methods complete the preliminary investigations which may lead to new therapeutic drugs.

1.2 The Zingiberaceae Family

1.2.1 The Origin of the Word 'Ginger'

The word 'ginger' truly refers to the edible ginger of commerce known in the Malay language as *halia* and botanically as *Zingiber officinale* Roscoe, while 'gingers' is a general term for members of the ginger family. The name *Zingiber* probably originated from the Arabic word *zanjabil* and later the Sanskrit word *singabera* (meaning horn-root), which gave rise to the classical Greek name *zingiberi* and finally *zingiber* in Latin. Botanically, *Zingiber* gives its name to the whole ginger family, Zingiberaceae (Larsen *et al*., 1999).

1.2.2 Habitat

Gingers thrive in a wide range of habitats from riverine to limestone rocks and from the lowlands to the upper montane regions. Most gingers are terrestrial, growing naturally in damp, humid, shady areas with good light but several native species can tolerate the full exposure of the sun.

Gingers are generally abundant in lowland to hill forests, notably between 200 m and 500 m above sea level. Gingers are less profuse in higher altitudes and rather scarce on very high

mountains. On some isolated islands which are relatively dry, the diversity of gingers is quite low and sometimes they are even absent. Some species inhabit secondary forests, open places such as along road-sides, forest gaps, riverbanks and swampy vegetation. The climate of Peninsular Malaysia allows continuous growth of the rhizomes throughout the year although more vigorous growth may be apparent during the rainy season (Larsen *et al*., 1999).

1.2.3 Distribution

These are plants of tropical and subtropical regions distributed mainly in Asia. The Zingiberaceae comprises about 1200 species of which about 1000 occur in Tropical Asia. By far, the richest area is the Malesian region, a floristically distinct region that includes Malaysia, Indonesia, Brunei, Singapore, the Philippines and Papua New Guinea, with 24 genera and about 600 species. With the present knowledge, there are about 18 genera with more than 160 species of Zingiberaceae in Peninsular Malaysia. These include *Alpinia, Amomum, Boesenbergia, Camptandra, Curcuma, Elettaria, Elettariopsis, Etlingera, Geocharis, Geostachys, Globba, Haniffia, Hedychium, Hornstedtia, Kaempferia, Plagiostachys, Scaphochlamys* and *Zingiber*. Many of these are rare and very local in distribution and consequently highly vulnerable to endangerment (Larsen *et al*., 1999).

1.2.4 Use and Commercial Importance

The main gingers of use come from the genera *Alpinia, Amomum, Curcuma* and *Zingiber*, and, to a lesser extent, *Boesenbergia, Kaempferia, Elettaria, Elettariopsis, Etlingera* and *Hedychium*. At least 20 or more ginger species have been cultivated for their use as spices, condiments, flavours, fresh vegetables, medicine, ornamentals and cut flowers. The presence of essential oils in many Zingiberaceae species have made some species important since the time of the ancient Greek (Larsen *et al*., 1999).

One of the earliest uses was as spices. *Zingiber officinale* (*halia* in Malay) is one of the best and oldest known spices of the Zingiberaceae. Until today, it is still in demand as one of the ingredients in food, bakeries (ginger bread, biscuits) confectionaries, beverages (ginger beer) and traditional medicine. The old rhizomes are used fresh in flavouring while its young rhizomes are eaten raw or pickled as a relish. Turmeric, known as *Curcuma domestica* Val. in Peninsular Malaysia and *Curcuma longa* L. in India and other Asian countries is popular (after ginger) as a spice used in curries. It is also used as a food flavouring and in ancient times it was even exploited as a dye. The broad aromatic leaves of *Curcuma domestica* are used for wrapping fish before steaming or baking. Turmeric has a long list of uses ranging from spice, flavour, traditional medicine and in cultural beliefs and rites. *Alpinia galanga* (L.) Sw. is a minor spice in some western countries. In Malaysia, its rhizomes are called *lengkuas* and much used in the spicy meat dish called *rendang*. In Malaysia, the leaves of *Kaempferia galanga* L. (called *cekur*) is familiar in *perut ikan*, a favourite local dish. The young rhizomes of *Curcuma mangga* Val. & Van Zijp, *Boesenbergia rotunda* (L.) Mansf. and *Zingiber zerumbet* Smith, and young inflorescence of *Curcuma domestica* and *Alpinia galanga* are also consumed as fresh vegetables or *ulam* (a term equivalent to salad) by the village folk (Ibrahim, 1992).

Many studies and surveys have shown that at least more than ten cultivated species of Zingiberaceae have been frequently used in traditional medicine. Many of the medicinal gingers are used in traditional cures which are apparently associated with women-related ailments or illness, e.g. post-partum medicines for women during confinement. Ginger species including *Curcuma zedoria* (Berg.) Rosc. (*temu kuning* in Malay), *Curcuma mangga* (*temu pauh*), *Curcuma aeruginosa* Roxb. (*temu hitam*) and *Zingiber montanum* (Koenig) Theilade (*bonglai*) are used in food preparations for women in confinement after birth. The rhizomes of *Zingiber officinale* and *Curcuma domestica* are frequently used as a carminative for relieving flatulence. The latter is also used as an anti-spasmodic in diarrhoea. Similarly, *Curcuma xanthorhiza* Roxb. (*temu lawak*), *Zingiber ottensi* Val. (*lempoyang hitam* or

bonglai hitam) and *Zingiber zerumbet* (*lempoyang*) have roles in herbal medicine (Khaw, 1995). Besides these, *Globba* species are occasionally used in traditional medicine (Ibrahim, 1995).

1.2.5 The Genus *Elettariopsis*

Baker (1892) founded the genus *Elettariopsis*, describing three species, two of which were found on Penang Hill: *E. serpentina* Bak. and *E. curtisii* Bak. collected by Curtis. The third Baker taxon, *E. exserta* was collected by Scortechini from 'Goping, Straits Settlements' in June 1885. Ridley (1907) listed seven species including the ones in Baker, adding *E. latiflora* Ridl. (which he first found in Singapore prior to 1899), *E. pubescens* Ridl. (based on *Amomum biflorum* Jack), *E. cyanescens* [now *Haniffia cyanescens* (Ridl.) Holtt.] and *E. longituba* [now *Elettaria longituba* (Ridl.) Holtt.]. Holttum (1950) dealt with Ridley's *E. pubescens*, reverting it to *Amomum biflorum* Jack. He also considered *E. latiflora* Ridl. as a synonym of *E. curtisii*.

Despite Scortechini's clear diagnosis of *E. exserta*, it is still remained unknown and uncertain to Holttum who resorted to regard it as a larger form of *E. curtisii*. According to Lim (2003), *E. curtisii* is clearly not to be confused with *E. latiflora* and *E. exserta*. He is convinced that *E. curtisii* is endemic to Penang Island while *E. exserta* and *E. latiflora* are found mainly in the south of the Peninsula, in Johor and Singapore.

Based on Lim's (2003) findings, there appear to be three forms of *E. triloba*, one scentless and the other two strongly aromatic but different. According to this authority, the 'true' *E. triloba* has yet to be ascertained in the wild within Peninsular Malaysia. The two aromatic forms which resembled *E. triloba* have been elevated in rank by Lim to two new species and are quite wide spread throughout Peninsular Thailand and Malaysia. One of these, *E. slahmong*, possesses a stink bug scent resembling that exuded by insects such as *Catacanthus incarnatus* and *Tantao ocellatus*. The largest population of this species has

been observed in Tioman. The other species, which has a very strong and sweet lemon scent, is recognized by Lim as *E. elan*. However, Holttum recognized *E. triloba* (Gagnep.) Loesen., which is a Vietnamese species, as extant in the Peninsula, ascribing it to a local species which seemed similar. According to Kam (1982), *E. triloba* is found in Perak, Selangor, Pahang and Johor. Holttum's assertion that the crushed leaves of both *E. curtisii* and *E. triloba* exude an unpleasant pungent odour somewhat similar to that emitted by various kinds of bugs, can be corrected based on the fact the *E. triloba* which he was referring to was actually *E. slahmong*, one of the two new species identified by Lim (2003).

Kam (1982) recognized two new species and a new variety, *E. burttiana* and *E. smithiae* and its variety *rugosa*. *E. burttiana* is mainly confined to central Perak (Lim, 2003). *E. smithiae* and its variety *rugosa* differ in the shape and width of the lamina and also its colour sheen. Both have a distinct lemon scent but the variety *rugosa* has a trace of stink bug odour in it. Lim (2003) elevated the variety to the status of a new species and named it *E. rugosa*.

In advance of an ongoing revision of the genus *Elettariopsis* in Peninsular Malaysia, nine species have been confirmed by Lim (2003): *E. elan* C.K. Lim, *E. slahmong* C.K. Lim, *E. triloba* (Gagnep.) Loesen., *E. curtisii* Bak., *E. smithiae* Kam, *E. rugosa* (Kam) C.K. Lim, *E. exserta* (Scort.) Bak., *E. latiflora* Ridl. and *E. burttiana* Kam.

E. elan

This is a highly aromatic herb with a lemon scent. It is 60 to 90 cm in height, arising from creeping rhizomes. The leaves, 2-5 in number, consist of leafless sheaths, which form distinct pseudostems 5 x 8 mm to 25 cm at close or distant intervals from the creeping rhizomes. Their laminas are lanceolate or ovate, with a dimension of 35 x 5-8 cm, and are dark green in colour. The inflorescence emerges from the basal shoots, with penducle 1-5 cm forming a cincinnus of 7-10 flowers exerting from the bracts. Flowers with labellum are

of typical coloration, white with red median stripe flanked by yellow streaks. The fruit is globular, green to reddish brown in colour, six-ridged with a diameter of 2 cm (Lim, 2003).

E. slahmong

This is an aromatic herb with all parts of the plant emitting a stink-bug odour. It is 50 to 145 cm in height, arising from creeping rhizomes. The leaves, 2-6 in number form pseudostems. Their laminas are ovate or lanceolate, with a dimension of 60 cm x 13.5 cm (70 x 8.5 cm), and are light green in colour. The inflorescence emerges from the shoot or extended base shoot and clusters in a cincinnus. The bracts are cream or light green in colour. The fruit is globular, six-ridged with a diameter of 2-2.5 cm. The herb is delectable to the orang asli and much sought after by them to be used in native cuisine for cooking fish. In Southern Thailand, the leaves of *E. slahmong* are eaten as a salad (Lim, 2003).

E. triloba

The rhizomes are slender, wide-creeping and horizontal. They bear leaf-shoots at intervals of 8-15 cm. The plants are not tuffed and the roots are not tuberous. The leaf-shoots consist of 1-5 leaves which grow on pseudostems of tightly clasping leaf-sheaths which grow up to 35 cm tall. The laminas of the leaves are lanceolate with a dimension of 30 x 5-8 cm. Both surfaces of the leaves are glabrous. The inflorescence emerges from the base of the leaf-shoot, consisting of 4-8 bracts in a compact head. All the bracts and calyx are suffused pink, each subtending 1-2 flowers. The labellum is three-lobed, the middle lobe being cream with broad yellow median band bordered by a red stripe on either side towards the throat (Kam, 1982).

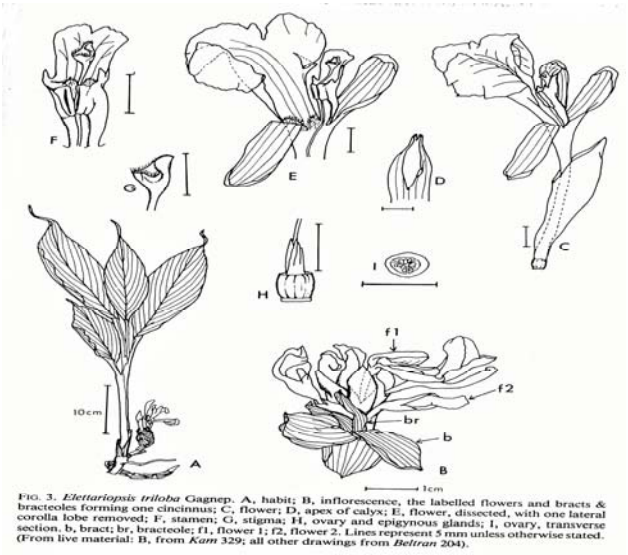

FIG. 3. *Elettariopsis triloba* Gagnep. A, habit; B, inflorescence, the labelled flowers and bracts & bracteoles forming one cincinnus; C, flower; D, apex of calyx; E, flower, dissected, with one lateral corolla lobe removed; F, stamen; G, stigma; H, ovary and epigynous glands; I, ovary, transverse section. b, bract; br, bracteole; f1, flower 1; f2, flower 2. Lines represent 5 mm unless otherwise stated. (From live material: B, from *Kam* 329; all other drawings from *Beltran* 204).

Fig. 1.1: *Elettariopsis triloba* (Gagnep.) Loesen. (Kam, 1982)

E. curtisii

This is a rather small plant with long-creeping slender rhizomes. The leaf shoots arise from them at widely spaced intervals of 6-20 cm. Each shoot consists of 1-5 leaves. The leaves are glabrous and erect, with loosely clasping sheaths of length 5-30 cm and release a strange stink bug odour when they are bruised. The leaves are used as flavours and traditional medicines by the orang asli. The laminas are elliptic, with dimensions of 24 x 4 – 68 x 10 cm, tapering to both ends. The inflorescence is long and slender, emerging from the base of the leaf-shoot, just below ground surface. The bracts are small with white calyx. The lobes are white, pale yellow with an orange centre and radiating crimson lines. The fruit is a globular capsule, with a diameter of 3 cm. It is shallowly ridged; pink speckled with dark red dots and contains white seeds (Kam, 1982; Keng *et al*., 1998).

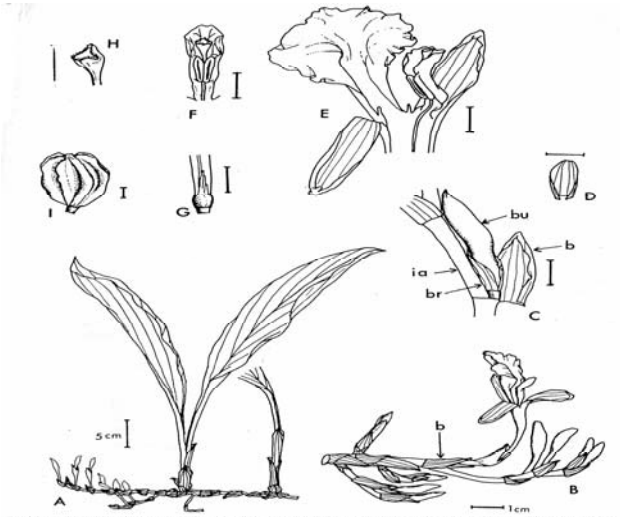

FIG. 1. *Elettariopsis curtisii* Bak. A, habit; B, partial inflorescence; C, partial inflorescence to show arrangement of bracts and bracteoles (bracts spread open); D, bracteole; E, flower, dissected, with one lateral corolla lobe removed; F, stamen; G, ovary and epigynous glands; H, stigma; I, fruit. b, bract; br, bracteole; bu, bud; ia, inflorescence axis. Lines represent 5 mm unless otherwise stated. (From live material, *Kam* 227).

Fig. 1.2: *Elettariopsis curtisii* Bak. (Kam, 1982)

E. smithiae

This is an aromatic herb which emits a lemon scent when its leaves are crushed. The leafy shoot has a distinct pseudostem with 3-8 or sometimes 9 greyish green floppy leaves which are arranged in two ranks. The flowers have white, yellow and red patterns and are produced on a long branching inflorescence. The fruit is a pale brown spherical capsule. It is slightly ridged, 3 cm in diameter, and formed along the leaf litter (Larsen *et al*., 1999).

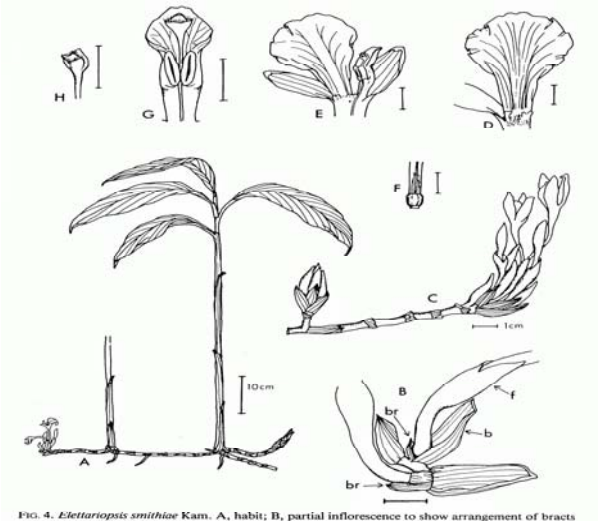

FIG. 4. *Elettariopsis smithiae* Kam. A, habit; B, partial inflorescence to show arrangement of bracts and bracteoles; C, partial inflorescence; D, labellum; E, flower, dissected, with one lateral corolla lobe removed; F, ovary and epigynous glands; G, stamen; H, stigma. b, bract; br, bracteole; f, flower. Lines represent 5 mm unless otherwise stated. (From live material: A, B, E, F, G, H from *Beltran* 110, holotype; C, D from *Kam* 183).

Fig. 1.3: *Elettariopsis smithiae* Kam (Kam, 1982)

E. rugosa

This herb is strongly aromatic. The scent of the crushed leaves incorporates a perceptible and a variable odour of the stink bug (less pungent than in *E. slahmong*) over the lemon smell. The leaves of this herb are elliptical (or ovate) and rugose (Lim, 2003).

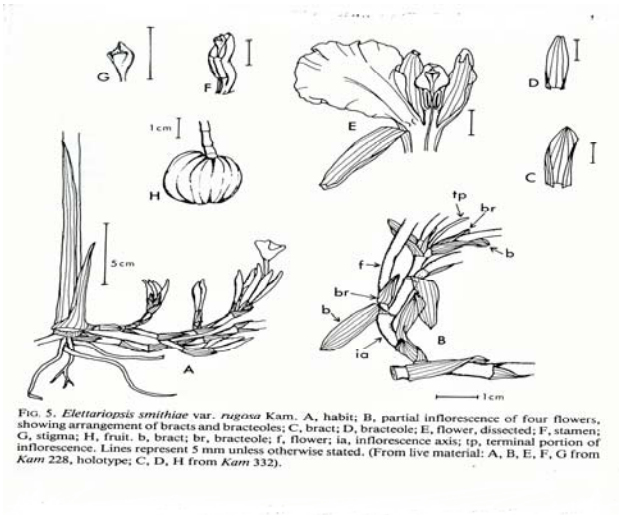

FIG. 5. *Elettariopsis smithiae* var. *rugosa* Kam. A, habit; B, partial inflorescence of four flowers, showing arrangement of bracts and bracteoles; C, bract; D, bracteole; E, flower, dissected; F, stamen; G, stigma; H, fruit. b, bract; br, bracteole; f, flower; ia, inflorescence axis; tp, terminal portion of inflorescence. Lines represent 5 mm unless otherwise stated. (From live material: A, B, E, F, G from *Kam* 228, holotype; C, D, H from *Kam* 332).

Fig. 1.4: *Elettariopsis rugosa* (Kam) C.K. Lim (Kam, 1982)

1.2.6 The Genus *Etlingera*

In 'The Zingiberaceae of the Malay Peninsula', Holttum (1950) listed four species under *Phaeomeria* Lindely. He later realized that Lindely's *Phaeomeria* (1836) was invalid, despite Schumann's later adoption and (in his 1974 paper) endorsed the generic change to *Nicolaia*, which was named by Horaninow in 1862 to honour the late Emperor of Russia (Lim, 2000).

Prompted by a suggestion by Holttum, Burtt and Smith (1986) proposed the combination of the three genera *Achasma* Griff., *Geanthus* Val. (not found in Malaysia) and *Nicolaia* Horan. under *Etlingera*, which was first used in 1972 by Giseke (Lim, 2000).

Following the change of genus name, Smith (1986) embarked on a sweeping exercise in combinations, covering some 60 species over the whole regional distribution range, and

largely on the basis of assiduous herbarium research, much of it by interpretation of literature and without being able to see type specimens (Lim, 2000).

E. elatior

E. elatior (Jack) R.M. Smith or the Torch Ginger is synonymous with *Phaeomeria speciosa* (Blume) Merr., *Phaeomeria magnifica* (Roscoe) K. Schum. or *Nicolaia speciosa* (Blume) Horan. A native to Asia, it is one of the most beautiful of all tropical flowering plants. It is a robust, coarse and large herb growing in clumps of 3-6 m tall. The leaf blades are lanceolate, with a dimension of 85 x 15 cm, and are arranged in two alternate rows upon the stem. The lower surface of the leaf is often purplish when young while the top surface is glossy green. Its inflorescence comes out of the ground instead of the terminal spike. The flower heads are broadly cone-shaped, 7-10 cm long, subtended by large crimson or pink (with white edge) bracts and seated on a stalk of about 1 m long. The corolla is pink, lip is crimson with a narrow white or yellow margin, and the anther is red. The fruit, hairy and green to reddish in colour, is a globose, with a diameter of 2.5 cm and contains many black seeds (Henderson, 1954; Keng *et al*., 1998). The plant is grown in tropical locations for both the extravagant flowers and as food. In Malaysia, where it is called *kantan*, the flower shoot is a compulsory ingredient of *laksa asam*, a favourite noodle dish in Peninsular Malaysia. The herb is also used in local dishes such as *nasi kerabu*, *nasi ulam* and *tom-yam* (Larsen *et al*., 1999).

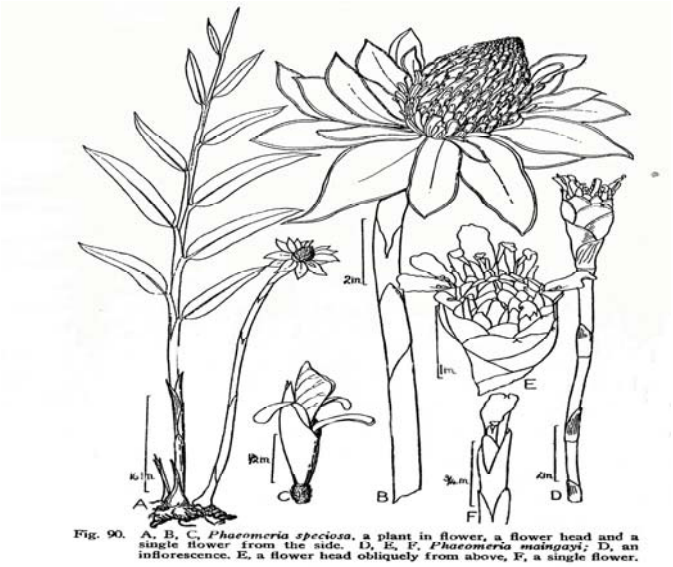

Fig. 90. A, B, C, *Phaeomeria speciosa*, a plant in flower, a flower head and a single flower from the side. D, E, F, *Phaeomeria maingayi*; D, an inflorescence. E, a flower head obliquely from above, F, a single flower.

Fig. 1.5: *Etlingera elatior* (Jack) R.M. Smith (Henderson, 1954)

E. elatior* var. *Thai queen

This herb is a variety of *E. elatior*. It is morphologically similar to *E. elatior*, the only difference is the colour of the inflorescence which is white.

E. littoralis

The synonyms of *E. littoralis* (Koenig) Giseke include *Achasma megalocheilos* Griff. and *Hornstedtia megalocheilos* Ridl. The herb has stout stems which are 4-5 m tall. The leaves which are glabrous are broadly oblong, 0.5-1 m long, with a short stalk of length 1-1.5 cm. It has a spike like-inflorescence 5-8 cm long partly embedded in the ground. The bracts are ovate or oblong, papery, pale pink with a crimson tip, subtending 4-12 flowers which open together. The calyx is pink and tubular, 7-8 cm long. The corolla is of the same length with pink lobes. The lip is red with yellow or orange margins. The fruit is a capsule globose, 3 cm in diameter (Keng *et al.*, 1998; Sha Ren Shu, 2000).

1.2.7 Previous Phytochemical Investigations

1.2.7.1 The Genus *Elettariopsis*

Two variants of *E. triloba*, designated as variant 1 and 2, were investigated for their volatile components by Mustafa *et al*. (1996). These workers found that the two variants showed differences in their volatile constituents. Variation was also detected when different parts (leaf, rhizome, root) of the plant were compared (Larsen *et al*., 1999). Examples of the major components in the leaves of variant 1 included neral (12.5%), geranial (16.1%), 2,7-dimethyl-2,6-octadien-1-ol (14.1%) and geranyl isobutyrate (10.5%), while the rhizome oil contained limonene (11.3%) and 2-carene (9.1%). In contrast, the major components in the leaf oil of variant 2 included β-caryophyllene (20.2%) and eremophilene (22.7%). While the rhizome oil contained 1,8-cineole (15.1%) and borneol (9.0%), the roots were found to yield camphene (14.1%) and β-caryophyllene (18.9%).

1.2.7.2 The Genus *Etlingera*

Some volatile components of *E. elatior* have been identified in three previous investigations. The first, by Wong *et al*. (1993), reported on the essential oil composition obtained by hydrodistillation of the young flower shoots of the herb growing in Malaysia. They positively identified dodecanol (33.2%), dodecanal (17.2%), α-pinene (13.7%) and dodecanoic acid (7.4%) as the major components.

In a later study, Zoghbi and Andrade (2005) investigated the volatile constituents in the oil of the inflorescence and inflorescence axis of this herb cultivated in the state of Para, Brazil, using GC and GC-MS. The inflorescence and inflorescence axis oils yielded dodecanol (42.5% and 34.6%), dodecanal (14.5% and 21.5%) and α-pinene (22.2% and 6.3%) as the main constituents, respectively.

Recently, Jaafar *et al*. (2007) analysed the essential oils isolated from different parts (leaves, stems, flowers and rhizomes) of Malaysian *E. elatior*, using GC-MS. The leaf oil was found to contain *β*-pinene (19.7%), *β*-caryophyllene (15.4%) and *trans*-*β*-farnesene (27.1%) as the major compounds whereas the stem oil was largely dominated by 1,1-dodecanediol diacetate (34.3%) and *trans*-5-dodecene (27.0%). The oils of the flowers and rhizomes contained 1,1-dodecanediol diacetate (24.4% and 40.4%, respectively) and cyclododecane (47.3% and 34.5%, respectively) as the major compounds.

Phytochemical studies on the rhizomes of *E. elatior* (Mohamad *et al*., 2005) afforded two new compounds, 1,7-bis(4-hydroxyphenyl)-2,4,6-heptatrienone and 16-hydroxylabda-8(17),11,13-trien-15,16-olide, along with demethoxycurcumin, 1,7-bis(4-hydroxyphenyl)-1, 4,6-heptatrien-3-one, stigmast-4-en-3-one, stigmast-4-ene-3,6-dione, stigmast-4-en-6*β*-ol-3-one, and 5*α*,8*α*-epidioxyergosta-6,22-dien-3*β*–ol.

1.3 Research Objectives

1.3.1 Part 1

Since the treatments by Holttum (1950) and Kam (1982), recognition of some of the members of the *Elettariopsis* has become confused. The ongoing revision by Lim (2003) to unravel species which have been wrongly identified was based on morphology and scent. However, utilizing scent alone can be misleading, and classifying species based on this method is insufficient and unreliable. The discernibly different odour of the essential oils suggests the possible use of their principal constituents as taxonomic markers for species identification (Jantan *et al*., 1994; Salguerio, 1993). It will be interesting to see if the essential oil composition can be used as a complementary tool in the taxonomy of species of this genus.

Objectives:

1. To identify the essential oil constituents in the leaves and roots of *E. smithiae*, *E. rugosa*, *E. elan*, *E. slahmong*, *E. triloba* and *E. curtisii* using capillary GC and GC-MS techniques.
2. To carry out a comparative study between the essential oil composition of the leaves and roots of each species mentioned in (1.).
3. To carry out a comparative study of the essential oil composition of *E. smithiae*, *E. rugosa*, *E. elan*, *E. slahmong*, *E. triloba* and *E. curtisii* in an attempt to develop a taxonomic guide for species identification.
4. To confirm whether the two new species identified by Lim (2003), *E. elan* and *E. slahmong*, are identical or not to the two variants of *E. triloba* investigated by Mustafa *et al*. (1996).

1.3.2 Part 2

Objectives:

1. To identify the essential oil constituents in the leaves, rhizomes and roots *E. elatior*, *E. elatior* var. *Thai queen* and *E. littoralis* using capillary GC and GC-MS techniques.
2. To carry out a comparative study between the essential oil composition of the leaves, rhizomes and roots of of each species or variety mentioned in (1.).
3. To isolate and elucidate the structure of the major non-volatile constituents, in particular, the phenolics and terpenoids present in the leaves, fruits, rhizomes and roots of *E. littoralis*, using various spectroscopic techniques.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Collection of the Plant Materials

The leaves and roots of *Elettariopsis elan, E. slahmong, E. smithiae* and *E. rugosa* were collected from a home garden in Penang. Voucher specimens have been deposited with the herbarium of the Forestry Research Institute Malaysia (FRIM): *E. elan* C.K. Lim L6306 (KEP); *E. slahmong* C.K. Lim L6101 (KEP); *E. smithiae* Kam L3953 (KEP); *E. rugosa* (Kam) C.K. Lim L6197 (KEP). *E. triloba* was collected in the campus of Universiti Sains Malaysia, and *E. curtisii* from the Ayer Itam Dam, Penang. Voucher specimens of these two species have been deposited with the herbarium of the School of Biological Sciences, U.S.M. (10950 and 10946, respectively).

Etlingera elatior was collected from the Penang Botanical Garden, *E. elatior* var. *Thai queen* from a home garden in Penang, and *E. littoralis* from the Ayer Itam Dam, Penang. Voucher specimens of all these plants have been deposited with the herbarium of the School of Biological Sciences, U.S.M. (10615, 10765 and 10947, respectively).

2.2 Chemicals and Reagents

1. *Acetone, AR grade (Systerm, Malaysia)
2. Aluminium Chloride, $AlCl_3.6H_2O$ (Merck, Germany)
3. Boric Acid (Sigma-Aldrich, USA)
4. *Chloroform, AR grade (R&M Chemicals, UK)
5. Chloroform-*d*, with 0.03 v/v % TMS, 99.8 atom % D, stabilized with silver foil (Acros Organics, USA)
6. Diethyl Ether, AR grade (Lab-Scan, Thailand)
7. *Ethyl Acetate, AR grade (Fisher Scientific, UK)
8. *Ethanol 95%, AR grade (Systerm, Malaysia)

9. Ferric Chloride, $FeCl_3.6H_2O$ (Merck, Germany)
10. Hydrochloric Acid 37% (Fischer Chemicals, UK)
11. *Methanol, AR grade (Systerm, Malaysia)
12. Methyl-d_3 alcohol-*d*, with 0.03 v/v % TMS, 99.8 atom % D (Acros Organics, USA)
13. *Pentane, AR grade (Merck, Germany)
14. *Petroleum Ether 60-80 ^oC, AR grade (Lab-Scan, Thailand)
15. Sephadex LH-20 (Sigma-Aldrich, USA)
16. Silica Gel 60 for column chromatography, (0.040-0.063 mm) (230-400 mesh ASTM) (Merck, Germany)
17. Anhydrous Sodium Acetate (R&M Chemicals, UK)
18. Sulphuric Acid 95-98% (Systerm, Malaysia)
19. TLC Aluminium Sheets, Silica Gel 60 F_{254}, 20 cm x 20 cm (Merck, Germany)
20. Silica Gel 60 F_{254}, pre-coated glass plates 20 cm x 20 cm x 0.5 mm (Merck, Germany)

* Solvents were distilled prior to use

2.3 Isolation and Analysis of Essential Oils

2.3.1 Isolation of Essential Oils

Fresh intact leaves, chopped roots (for plants from the genus *Elettariopsis*) and rhizomes and roots (for plants from the genus *Etlingera*) were separately subjected to hydrodistillation to isolate the essential oils, carrying out by using an all-glass apparatus similar to that described in the British Pharmacopoaeia (1993) (Fig. 2.1).

The plant material (15-80 g), cleaned by rinsing under running tap water followed by distilled water to remove soil and dirt, was placed in a 0.5 L round bottom flask which was filled with distilled water (300-400 ml) to a level such that the entire plant material was immersed (Table 2.1).

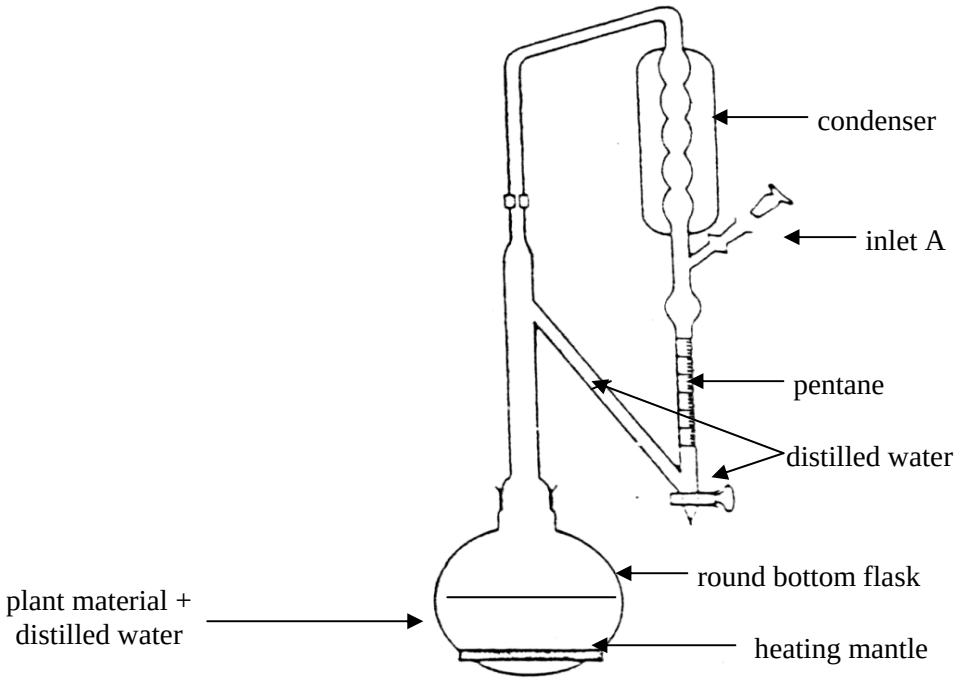

Fig. 2.1: Hydrodistillation Apparatus

Table 2.1: Weight of Plant Material and Volume of Distilled Water Used for Hydrodistillation

Species	Plant material	Weight (g)	Volume of distilled water (ml)
Elettariopsis smithiae	leaves	40	400
	roots	50	400
Elettariopsis rugosa	leaves	70	400
	roots	80	400
Elettariopsis elan	leaves	60	300
	roots	60	350
Elettariopsis slahmong	leaves	50	400
	roots	50	400
Elettariopsis triloba	leaves	50	400
	roots	50	400
Elettariopsis curtisii	leaves	45	400
	roots	20	300
Etlingera littoralis	leaves	30	300
	rhizomes and roots	45	300
Etlingera elatior	leaves	40	300
	rhizomes and roots	40	300
Etlingera elatior var. *Thai queen*	leaves	15	400
	rhizomes and roots	20	350

The entire 'V' area of the apparatus was filled with distilled water, following which a small volume (5-8 ml) of freshly distilled pentane was added through inlet A (Fig. 2.1). Inlet A was then loosely covered with a piece of aluminium foil to minimize the loss of the essential oil during the hydrodistillation. Ice-cold water was circulated through the condenser for the duration of the hydrodistillation. To minimize condensation of the vapour before it reached the condenser, the left part of the glass apparatus was wrapped with aluminium foil. Hydrodistillation was carried out for 4 hours; a small volume of pentane was added from time to time through inlet A to ensure that the volume of pentane was maintained at about 5 ml.

At the end of the hydrodistillation, the glass apparatus was left to cool and the essential oil solution in pentane was drained through the stop cock into a separatory funnel where water was removed. The resulting solution was collected into a glass vial (5 ml), concentrated to an appropriate concentration (for GC) by a gentle stream of nitrogen gas, and stored in the refrigerator until required for analysis.

2.3.2 Chromatographic Analysis of the Essential Oils

The essential oils isolated from the various plants were analysed by capillary gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS).

2.3.2.1 Gas Chromatography

GC analyses were carried out using a Hitachi G-3000 equipped with a flame ionization detector (FID). Two fused silica capillary columns of different polarity were employed: SPB-1 (30 m x 0.25 mm id, film thickness 0.25 μm) and Supelcowax 10 (30 m x 0.25 mm id, film thickness 0.25 μm). Operating conditions for both these columns were as follows: initial oven temperature, 50 ^{o}C for 1 min, then to 250 ^{o}C (for SPB-1) or 230 ^{o}C (for Supelcowax 10) at 4 ^{o}C min^{-1} then held for 10 min; injector and detector temperatures, 275

^{o}C; carrier gas, 1.0 ml min^{-1} N_2; split ratio, 50:1; injection volume, 0.4 µL. Peak areas were determined with a Hitachi D2500 Chromato-Integrator. Correction for detector response was not made.

Immediately after each GC analysis of an essential oil, a mixture containing a homologous series of *n*-alkanes ranging from C_5 to C_{32} was injected into the column under identical operating conditions. The hydrocarbons were used as standards in the calculation of retention indices (RI).

For a temperature-programmed GC, the retention index (RI) of a component in the essential oil was calculated using the following equation (Van den Dool and Kratz, 1963):

$$RI = 100i\left[\frac{t - t_{(n)}}{t_{(n+i)} - t_{(n)}}\right] + 100n \qquad (1)$$

under the condition that $t_{(n)} < t < t_{(n+i)}$.

t = retention time of the essential oil component.

$t_{(n)}$ = retention time of the alkane with n carbon atoms which is eluted just before the component.

$t_{(n+i)}$ = retention time of the alkane with (n+i) carbon atoms which is eluted just after the component.

i = difference in the number of carbon atoms between the two alkanes.

n = the number of carbon atoms in the alkanes.

The retention index values obtained based on the calculation using two neighbouring alkanes (i.e. i = 1) give best precision. When i = 1, equation (1) is simplified to:

$$RI = 100\left[\frac{t - t_{(n)}}{t_{(n+1)} - t_{(n)}}\right] + 100n \qquad (2)$$

and that was used to calculate the RI values in the present work.

2.3.2.2 Gas Chromatography - Mass Spectrometry

GC-MS analyses were performed using either a ThermoFinnigan GC 2000 coupled to a Trace MS and equipped with the NIST and MAIN Library softwares, or a Hewlett Packard 5989A GC-MS equipped with the Wiley Library software. The same capillary columns and GC operating conditions were employed as described in Section 2.3.2.1, the only difference was the carrier gas used which was helium. Significant operating parameters: ionization voltage, 70 eV; ion source temperature, 200 ^{o}C; scan mass range, 40-350 amu.

2.3.3 Identification of the Essential Oil Components

The components of each essential oil were identified by matching their mass spectra with those recorded in the MS Library and with those of authentic compounds, if available. Components were further confirmed by comparison of the experimentally calculated RI values with those of authentic standards or with values published in the literature.

2.3.4 Determination of Oil Yield

The oil containing pentane was carefully concentrated by a gentle stream of nitrogen gas until it was free from pentane (indicated by GC analysis) and then weighed. The percentage yield was determined by dividing the weight of the essential oil with the weight of the fresh plant material.

2.4 Isolation and Characterization of the Non-Volatile Constituents in the Leaves, Fruits, Rhizomes and Roots of *Etlingera littoralis*

2.4.1 Extraction Procedure

2.4.1.1 Leaves

Air-dried leaves (450 g) were ground into a fine powder and soaked in petroleum ether (4.0 L) at room temperature (28 ^{o}C) for a week. The resulting slurry was filtered, and the filtrate was evaporated at 50 ^{o}C and under reduced pressure to afford a greenish-black syrup (4.00 g).

2.4.1.2 Fruits

Air-dried fruits (30 g) were ground into a fine powder and soaked in petroleum ether (0.8 L) for a day at room temperature (28 ^{o}C). The resulting slurry was filtered, and the filtrate evaporated at 50 ^{o}C to dryness, using a rotarory evaporator, to afford a black syrup (0.35 g). The residue was next soaked in ethyl acetate (1.5 L) for a day at room temperature. The slurry was filtered, and the filtrate evaporated to dryness at 50 ^{o}C, using a rotarory evaporator, to give a reddish-brown syrup (1.00 g).

2.4.1.3 Rhizomes and Roots

Air-dried rhizomes and roots (300 g) were finely ground and soaked in petroleum ether (3.0 L) at room temperature (28 ^{o}C) for a week, after which the slurry was filtered, the filtrate evaporated to dryness at 50 ^{o}C and under reduced pressure, yielding a reddish-brown syrup (1.50 g). Next, the residue was soaked in ethyl acetate (4.0 L) at room temperature (28 ^{o}C) for two weeks. The slurry was filtered; the filtrate evaporated to dryness at 50 ^{o}C and under reduced pressure to give a brown syrup (2.51 g).

2.4.2 Separation Techniques

2.4.2.1 Thin Layer Chromatography

Preliminary investigation of the chromatographic separation of the crude extracts was carried out using silica gel TLC (5 cm x 1 cm). Different solvent systems were tried to find one which could achieve the best separation for each of the mixtures. The selected solvent systems were utilized later in the column chromatographic separation of the crude extracts to isolate the components. Developed TLC plates were visualized using an UV lamp (365 nm), or dipped in reagents such as 1% $FeCl_3$ (heating not required), or 5% methanolic H_2SO_4 followed by heating at 100-105 ^oC until full development of colour has occurred to aid visualization (Jork *et al*., 1990; Harborne, 1998).

2.4.2.2 Column Chromatography

Column chromatography using either silica gel or Sephadex LH-20 was employed for component isolation or purification. When silica gel was used as the adsorbent, elution was carried out using either isocratic or gradient solvent systems. However, in the case of Sephadex LH-20, various proportions of mixtures of methanol : water (v/v) were used. Eluates were collected in 20 ml fractions and the composition of each fraction was monitored by TLC. Fractions showing similar TLC profiles were pooled, and the solvents were evaporated off. Repeated purification using the same technique was carried out until a pure compound was isolated.

2.4.2.3 Preparative Thin Layer Chromatography

This method was only employed for the final purification of F2 and L2 which could not be achieved through column chromatography. Each silica gel plate (20 cm x 20 cm x 0.5 mm) was loaded with 20 mg of sample in a narrow band. The plates were placed in a large covered glass chamber (30 cm x 30 cm x 10 cm), developed in a suitable isocratic solvent system and visualized as described in Section 2.4.2.1. The location of a component on the plate was marked, and the absorbent in the marked region was scrapped off and placed into

a conical flask and extracted repeatedly (5 x 20 ml) with chloroform. The component was recovered on evaporation of the chloroform (Zubrick, 1992).

2.4.3 Isolation and Purification

2.4.3.1 Leaves

2.4.3.1.1 Petroleum Ether Extract

The petroleum ether extract (4.00 g) (section 2.4.1.1) was chromatographed on a silica gel column (100 g). Elution was carried out using mixtures of petroleum ether : ethyl acetate in proportions of 80:20 (v/v) (300 ml), 40:60 (v/v) (300 ml) and 0:100 (v/v) (200 ml) sequentially to afford fractions LA (2.00 g), LB (0.30 g) and LC (0.80 g), respectively. LA was fractionated on a silica gel column (100 g), eluted successfully using mixtures of petroleum ether : chloroform in proportions of 100:0 (v/v) (300 ml), 80:20 (v/v) (300 ml) and 60:40 (v/v) (200 ml), followed by mixtures of chloroform : ethyl acetate in ratios of 80:20 (v/v) (200 ml) and 40:60 (v/v) (200 ml), to afford sub-fractions LA 1 (0.05 g), LA 2 (0.02 g), LA 3 (1.30 g), LA 4 (0.01 g) and LA 5 (0.02 g), respectively. Column chromatography of LA 3 over silica gel (30 g) with successive elutions using mixtures of petroleum ether : ethyl acetate in proportions of 90:10 (v/v) (150 ml) and 70:30 (v/v) (150 ml) afforded sub-fractions LA 3.1(0.60 g) and LA 3.2 (0.40 g), respectively. LA 3.1 was further separated by a 20 g silica gel column with petroleum ether : chloroform as the eluting solvent (75:25) (v/v) (300 ml) to give LA 3.1.1 (0.32 g) and LA 3.1.2 (0.18 g). Column chromatography of LA 3.1.1 over a 20 g silica gel column using an isocratic solvent system of petroleum ether : ethyl acetate (90:10) (v/v) (200 ml) provided LA 3.1.1.1 (0.10 g), LA 3.1.1.2 (0.10 g) and **L1 (30 mg)** (Appendix A1). L1, a colourless oil, $[\alpha]_D$ + 0.40^o ($CHCl_3$, *c* 2.0) gave a single brown spot with 5% H_2SO_4 on TLC [petroleum ether : ethyl acetate (95:5), R_f = 0.40; petroleum ether : acetone (95:5), R_f = 0.34; petroleum ether : chloroform (50:50), R_f = 0.66; petroleum ether : diethyl ether (90:10), R_f = 0.40; petroleum ether : chloroform : ethyl acetate (70:25:5), R_f = 0.63; petroleum ether : chloroform : acetone (80:15:5), R_f = 0.60]. Final purification of LA 3.1.1.2 to yield **L2 (25 mg)** was achieved by

chromatography over a silica gel column (15 g) with petroleum ether : chloroform (70:30) (v/v) (100 ml) as the eluting solvent, followed by preparative TLC using the same solvent system (Appendix A1). L2, isolated as a yellow oil $[\alpha]_D$ -14.4^o ($CHCl_3$, *c* 1.7), gave a single red spot with 5% H_2SO_4 on TLC [petroleum ether : ethyl acetate (95:5), R_f = 0.71; petroleum ether : acetone (98:2), R_f = 0.66; petroleum ether : chloroform (70:30), R_f = 0.63; petroleum ether : diethyl ether (95:5), R_f = 0.63; petroleum ether : chloroform : ethyl acetate (90:5:5), R_f = 0.69; petroleum ether : chloroform : acetone (95:3:2), R_f = 0.66].

2.4.3.2 Fruits

2.4.3.2.1 Ethyl Acetate Extract

The ethyl acetate extract (1.00 g) (section 2.4.1.2) was column chromatographed over Sephadex LH-20 (25 g), using methanol (300 ml) as the eluting solvent, to afford fractions FA (0.30 g) and FB (0.50 g). FB was further fractionated by passing it through a 25 g silica gel column, eluting with mixtures of chloroform : methanol in proportions of 80:20 (v/v) (200 ml), 70:30 (v/v) (300 ml), 60:40 (v/v) (300 ml) and 50:50 (v/v) (200 ml) successively to afford sub-fractions FB 1 (0.05 g), FB 2 (0.20 g), FB 3 (0.10 g) and FB 4 (0.05 g), respectively. Final purification of FB 3 to yield **F1 (48 mg)** was achieved through a small Sephadex LH-20 column (5 g), using 30 ml methanol : water (50:50) (v/v) as the eluting solvent (Appendix A2). F1 was crystallized as white needles from ethanol, mp 171 – 173 ^{o}C, $[\alpha]_D$ – 13.9^o (MeOH, *c* 3.2). F1 gave a single grayish black spot with 1% $FeCl_3$, and a reddish orange spot with 5% H_2SO_4 on TLC [chloroform : ethyl acetate (50:50), R_f = 0.11; ethyl acetate, R_f = 0.69; chloroform : acetone (50:50), R_f = 0.43; chloroform : ethyl acetate : acetone (1:1:1), R_f = 0.40; chloroform : methanol (80:20), R_f = 0.37].

2.4.3.2.2 Petroleum Ether Extract

The petroleum ether extract (0.35 g) (section 2.4.1.2) was chromatographed on a 20 g silica gel column, eluting using mixtures of petroleum ether : ethyl acetate in proportions of 100:0