BIOACTIVITIES AND CHEMICAL CONSTITUENTS OF LEAVES OF SOME *ETLINGERA* SPECIES (ZINGIBERACEAE) IN PENINSULAR MALAYSIA

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DECLARATIONS

In accordance with Monash University Doctorate Regulation 17 for Doctor of Philosophy (PhD) and Master of Philosophy (MPhil), the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes six original papers published in peer-reviewed journals. The core theme of the thesis is Bioactivity and Chemical Constituents of Wild and Cultivated Ginger Species (Zingiberaceae). The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Natural Product Chemistry Laboratory of Monash University Sunway Campus (MUSC) Malaysia under the supervision of Assoc Prof Dr LIM YAU YAN. The candidate is the lead author for all six publications.

The inclusion of co-authors in the publications reflects the fact that the work was based on team-based research and came from active collaboration between researchers of MUSC Malaysia and the Forest Research Institute Malaysia (FRIM).

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ABSTRACT

Based on results of preliminary screening of leaves and rhizomes of ginger species, leaves of *Etlingera* species were selected for study. Leaves of five *Etlingera* species were assessed for total phenolic content (TPC), and for antioxidant, antibacterial and tyrosinase inhibition activities. Highest TPC, ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP) were found in leaves of *E. elatior*. Leaves of *E. maingayi*, with the lowest TPC, AEAC and FRP, had the highest ferrous ion chelating (FIC) ability and lipid peroxidation inhibition (LPI) activity. FIC ability of *E. maingayi* and *E. fulgens* was much higher than that of young leaves of *Camellia sinensis*. All *Etlingera* species studied showed high LPI activity superior to that of young leaves of *C. sinensis.*

TPC and AEAC of leaves of *E. elatior* and *E. maingayi* were 7–8 times higher than those of rhizomes. Ranking of TPC and antioxidant activity of the different plant parts of *E. elatior* was in the order: leaves > inflorescences > rhizomes. Leaves of highland populations of *Etlingera* species displayed higher values of TPC and AEAC than those of lowland counterparts. Leaves of *Etlingera* species exhibited antibacterial activity against Gram-positive bacteria. Three out of five species displayed strong tyrosinase inhibition activity. Leaves of *Etlingera* were found to be non-cytotoxic to normal liver and kidney cells. The overall score and ranking were of the order: *E. elatior* > *E. rubrostriata* > *E. fulgens* > *E. littoralis* > *E. maingayi*.

Leaves of 21 other ginger species belonging to eight genera and three tribes were screened for TPC and AEAC for comparison with those of the five *Etlingera* species. Compared to *Etlingera* of the tribe Alpineae, the other ginger species of the same tribe such as *Alpinia* and *Elettariopsis* had lower values. Species of *Boesenbergia*, *Curcuma*, *Hedychium*, *Kaempferia* and *Scaphochlamys* (tribe Hedychieae) and species of *Zingiber* (tribe Zingibereae) had much lower values.

Effects of five different drying methods on the phenolic content and antioxidant properties of leaves of *Alpinia zerumbet*, *Etlingera elatior*, *Curcuma longa* and *Kaempferia galanga* were assessed. Thermal drying methods resulted in drastic declines in TPC, AEAC and FRP with minimal effects on FIC ability and LPI activity. Of the non-thermal drying methods, significant losses were observed in air-dried leaves. Freeze-drying resulted in significant gains in TPC, AEAC and FRP for *A. zerumbet* and *E. elatior* leaves.

Six compounds were isolated from *E. elatior* leaves and identified as 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid), 5-*O*-caffeoylquinic acid methyl ester, isoquercitrin, quercitrin and (+)-catechin. This is the first report of caffeoylquinic acids (CQA) including chlorogenic acid (CGA) in Zingiberaceae. CGA, isoquercitrin and quercitrin, the major compounds, showed DPPH radical scavenging ability but no antibacterial and tyrosinase inhibition activities.

Content of CQA of *E. elatior*, *E. fulgens* and *E. rubrostriata* leaves was significantly higher than leaves of *Ipomoea batatas*, and comparable to flowers of *Lonicera japonica.* CGA found only in leaves of *E. elatior* and *E. fulgens* was significantly higher in content than *L. japonica*, the commercial source.

From leaves of four *Etlingera* species, highest diversity of essential oil was found in *E. rubrostriata.* Composition of essential oils in *E. elatior* and *E. fulgens* were very different despite having very similar aroma and morphology. Leaves of *E. maingayi* had the highest yield of essential oils comprising mainly fatty acids that inhibited Gram-positive bacteria.

A protocol to produce a CGA standardized extract from leaves of *E. elatior* has been optimized. Freeze-drying of leaves followed by extraction with ethanol, and fractionation using Diaion HP-20 and Sephadex LH-20 yielded an extract with ~40% w/w purity.

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ABBREVIATIONS

AA	ascorbic acid
ACA	1'-acetoxychavicol acetate
AEAC	ascorbic acid equivalent antioxidant capacity
ANOVA	analysis of variance
APCI	atmospheric pressure chemical ionization
asl	above sea level
BCB	β-carotene bleaching
BHT	butylated hydroxytoluene
BR	bleaching rate
CA	caffeic acid
CD_{50}	cytotoxic dose at 50%
CD_3OD	deuterated methanol
CGA	chlorogenic acid
CGAC	chlorogenic acid content
$CHCl_3$	trichloromethane
CHI	chalcone isomerase
CHS	chalcone synthase
CQA	caffeoylquinic acids
CQAC	caffeoylquinic acid content
CV	cell viability
DCM	dichloromethane
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
DR	degradation rate
DW	dry weight
EBV	Epstein-Barr virus
ECG	epicatechin gallate
EGC	epigallocatechin

EGCG	epigallocatechin-3-gallate
EDTA	ethylenediamine tetraacetic acid
EI	electron ionization
ESI	electrospray ionization
EtOH	ethanol
eV	electron volts
FC	Folin-Ciocalteu
Fe^{2+}	ferrous
Fe^{3+}	ferric
$FeSO_4$	ferrous sulphate
F3H	flavanone 3-hydroxylase
F3’H	flavonoid 3’-hydroxylase
F3’5’H	flavonoid 3’5’-hydroxylase
FIC	ferrous ion chelating
FID	flame ionization detector
FLS	flavone synthase
FRAP	ferric reducing antioxidant power
FRIM	Forest Research Institute Malaysia
FRP	ferric reducing power
FTC	ferric thiocyanate
FW	fresh weight
GAE	gallic acid equivalent
GC	gas chromatography
HBA	hydroxybenzoic acid
HCA	hydroxycinnamic acid
HeLa	human cervical carcinoma
H_2O_2	hydrogen peroxide
HPLC	high performance liquid chromatography
HSD	honestly significant difference
IC_{50}	inhibition concentration at 50%

ID_{50}	inhibition dose at 50%
IE	inhibitory effect
IUPAC	International Union of Pure and Applied Chemistry
LC	liquid chromatography
LDL	low-density lipoprotein
L-DOPA	L-3,4-dihydroxyphenylalanine
LPI	lipid peroxidation inhibition
mAU	milli-absorption unit
MHz	mega hertz
MIC	minimum inhibitory concentration
MID	minimum inhibition dose
MS	mass spectrometry
MTT	microtetrazolium
MeOH	methanol
MRSA	methicillin-resistant *Staphylococcus aureus*
MUSC	Monash University Sunway Campus
NMR	nuclear magnetic resonance
$^1O_2$	singlet oxygen
OD	optical density
ODS	octadecyl derivatised silica
-OH	hydroxyl
ORAC	oxygen radical absorbance capacity
PAL	phenylalanine ammonia lyase
ppm	parts per million
PPO	polyphenol oxidase
RNS	reactive nitrogen species
ROS	reactive oxygen species
RP	reverse-phase
SD	standard deviation
SIRIM	Standards and Industrial Research Institute of Malaysia

SRB	sulforhodamine B
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TE	trolox equivalent
TI	tyrosinase inhibition
TLC	thin-layer chromatography
TMS	tetramethylsilane
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TPC	total phenolic content
TPTZ	tripyridyltriazine
Tris	hydroxymethyl aminomethane
UPM	Universiti Putra Malaysia
UV	ultraviolet
w/w	weight for weight

SCIENTIFIC NAMES

Gingers

Achasma megalocheilos
Aframomum arundinaceum
Aframomum giganteum
Aframomum letestuianum
Aframomum zambesiacum
Alpinia allughas
Alpinia blepharocalyx
Alpinia chinensis
Alpinia conchigera
Alpinia flabellata
Alpinia formosana
Alpinia galanga
Alpinia hookeriana
Alpinia javanica
Alpinia katsumadai
Alpinia luteocarpa
Alpinia malaccensis
Alpinia mutica
Alpinia officinarum
Alpinia oxymitra
Alpinia oxyphylla
Alpinia pinnanensis
Alpinia purpurata
Alpinia rafflesiana
Alpinia tonkinensis
Alpinia vitata
Alpinia zerumbet
Alpinia zerumbet 'Variegata'
Amomum maingayi
Amomum muricarpum
Amomum officinale
Amomum xanthioides
Boesenbergia rotunda
Curcuma aeruginosa
Curcuma alismatifolia
Curcuma aurantiaca
Curcuma domestica
Curcuma longa
Curcuma malabarica
Curcuma mangga
Curcuma parviflora
Curcuma petiolata
Curcuma roscoeana
Curcuma zanthorrhiza
Curcuma zedoaria
Elettaria cardamomum
Elettaria speciosa
Elettariopsis curtisii
Elettariopsis elan
Elettariopsis latiflora
Elettariopsis slahmong
Elettariopsis smithiae
Etlingera elatior
Etlingera fulgens
Etlingera hemisphaerica

Etlingera littoralis
Etlingera maingayi
Etlingera metriocheilos
Etlingera rubrolutea
Etlingera rubrostriata
Etlingera venusta
Hedychium coccineum
Hedychium coronarium
Hedychium longicornutum
Hornstedia fulgens
Hornstedtia maingayi
Hornstedia scyphifera
Kaempferia galanga
Kaempferia longa
Kaempferia parviflora
Kaempferia pulchra
Kaempferia rotunda
Languas galanga
Languas malaccensis
Nicolaia elatior
Nicolaia fulgens
Nicolaia maingayi
Nicolaia speciosa
Phaeomeria fulgens
Phaeomeria maingayi
Phaeomeria speciosa
Scaphochlamys kunstleri
Vanoverberghia sasakiana
Zingiber griffithii
Zingiber malaysianum
Zingiber mioga
Zingiber montanum
Zingiber officinale
Zingiber ottensii
Zingiber puberulum
Zingiber purpureum
Zingiber spectabile
Zingiber wrayi
Zingiber zerumbet

Other plants

Arnica montana
Camellia sinensis
Castanea crenata
Chrysanthemum morifolium
Chrysophyllum cainito
Embelia schimperi
Hibiscus tiliaceus
Hypericum perforatum
Ipomoea batatas
Lonicera japonica
Mangifera indica
Piliostigma reticulatum
Polygonum paleaceum
Portulaca oleracea

Microbes

Aspergillus fumigatus
Aspergillus niger
Bacillus cereus

Bacillus megaterium
Bacillus subtilis
Burkholderia cepacia
Campylobacter jejuni
Candida albicans
Cryptococcus neoformans
Enterobacter aerogenes
Escherichia coli
Klebsiella pneumoniae
Listeria monocytogenes
Micrococcus luteus
Pseudomonas aeruginosa
Pseudomonas cepacia
Saccharomyces cerevisiae
Salmonella choleraesuis
Salmonella enterica
Salmonella enteritidis
Staphylococcus aureus
Staphylococcus epidermidis
Staphylococcus mutans
Streptococcus lactis

Others

Drosophila melanogaster
Spodoptera littoralis

Chapter 1

INTRODUCTION

1.1 OVERVIEW

1.1.1 Zingiberaceae

Plants of the family Zingiberaceae are commonly known as gingers. They are perennial herbs that are variable in height and size (Larsen *et al*., 1999). Species such as *Etlingera elatior* have large leafy shoots growing up to 6 m in height while others such as *Kaempferia galanga* are almost prostrate near the ground. They produce aromatic rhizomes that are subterranean or above ground. Each rhizome can turn upwards, transforming into an erect leafy shoot. Inflorescences are terminal, either on leafy shoots or on special erect shoots near the base of the plant (Soepadmo, 1976).

For centuries, their aromatic rhizomes have been cultivated for consumption as spice or condiments and used in herbal medicine for various ailments. The three cultivated species of major commercial importance are *Zingiber officinale* (ginger) *Curcuma longa* (turmeric) and *Elettaria cardamomum* (cardamom).

In recent years, ginger plants are gaining popularity as ornamentals as their inflorescences and foliage are colourful and attractive. Species commonly planted in gardens include *Alpinia purpurata*, *A. mutica*, *A. zerumbet* 'Variegata', *Etlingera fulgens*, *Hedychium coronarium*, *Kaempferia pulchra* and *Zingiber spectabile*.

1.1.2 Ginger rhizomes

The antioxidant and other bioactive properties of major commercial ginger species used as spice and as traditional medicine e.g. *C. longa* (Araujo & Leon, 2001; Jayaprakasha *et al.*, 2005) and *Z. officinale* (Wilkinson, 1999; Vernin & Parkanyi, 2005; Remadevi *et al.*, 2005) have been fairly well studied. However, there are only a few studies done on wild ginger species, most of which are confined to rhizomes. Jitoe *et al.* (1992) and Habsah *et al.* (2000) reported that rhizomes of various wild and cultivated ginger species have antioxidant properties that are comparable to α-tocopherol. Recently, Chen *et al.* (2008) reported the antioxidant and antimicrobial properties of rhizomes of six genera belonging to 25 species of Zingiberaceae native to Taiwan. Studies have therefore focused on rhizomes of ginger species.

In Zingiberaceae, there is abundance of literature on the chemical constituents of rhizomes of species such as *Alpinia*, *Curcuma*, *Etlingera*, *Hedychium*, *Kaempferia* and *Zingiber.* Classes of compounds found in rhizomes include diterpenes, labdane-type diterpenes, sesquiterpenoids, phenylpropanoids, diaryl-heptanoids, phenylbutenoids, phenylbutanoids, sterols, phenolic acids, flavonoids, kava pyrones, vanilloids, coumarins, glycosides, glucosides and xanthones.

Recently, cosmeceutical products have been developed from rhizomes of ginger species. An example is XANWHITE developed by the Standards and Industrial Research Institute of Malaysia (SIRIM) (Rozanida *et al*., 2006). Comprising a cleanser, toner, moisturiser with sun filters and treatment cream, the skin-lightening products contain a blend from rhizomes of *Curcuma zanthorrhiza* and *Zingiber zerumbet* with bioactive compounds having tyrosinase inhibiting, antioxidant and anti-inflammatory properties.

1.1.3 Ginger leaves

Hardly any work has been done on leaves of gingers although they have been used for food flavouring and as traditional medicine. In Peninsular Malaysia, the aromatic leaves of *C. longa* are used for wrapping fish before steaming or baking (Larsen *et al.*, 1999). Leaves of *K. galanga* are used as spice for local fish dishes. Leaves of *Elettariopsis slahmong* are used to flavour native cuisine especially wild meat and fish (Lim, 2003). In Thailand, leaf infusions of *Z. spectabile* are used to abate inflamed eyelids (Sirirugsa, 1999). In Okinawa, Japan, the leaves of *Alpinia zerumbet* are traditionally used to wrap rice cakes, to flavour noodles and are commercially sold as herbal tea.

Information on the phytochemistry of leaves is limited to a few species of *Alpinia*. Compounds found in ginger leaves include flavonoids, phenolic acids, labdane-type diterpenes, diarylheptanoids, phenylbutanoids and kava pyrones. Hardly any information is available on the antioxidant and other bioactivities of leaves of ginger species.

Therefore, there are both scientific merit and commercial justifications to study the bioactivities and chemical constituents of leaves of wild and cultivated ginger species. This study focuses on analysing the phenolic content, and antioxidant, antibacterial and tyrosinase inhibition properties of leaves of selected *Etlingera* species. Preliminary screening showed that they have the highest phenolic content and strongest antioxidant activity. Comparisons were made with leaves and rhizomes of other ginger species. The effects of different thermal and non-thermal drying treatments on leaves of ginger species were also studied.

Chemical constituents of extracts and essential oils of *E. elatior* leaves were analyzed as they displayed outstanding antioxidant, tyrosinase inhibition and antibacterial activity. The species is widely cultivated for its inflorescences. The leaves are available in large quantities and are currently of no commercial value.

Of particular interest was optimizing the protocols for producing a standardized extract with strong bioactivity from leaves of *E. elatior* which would have commercial potentials for the development of herbal products.

1.2 OBJECTIVES

Objectives of the thesis were:

1. To find out the genus of Zingiberaceae with the highest phenolic content and strongest antioxidant activity through preliminary screening of leaves and rhizomes

2. To determine the extraction efficiency of different solvents for leaves of ginger species

3. To screen for phenolic content and bioactivity (antioxidant, tyrosinase inhibition and antibacterial properties) of leaves of selected wild and cultivated species of *Etlingera* with comparisons between species, between different plant parts and on altitudinal variations in populations

4. To compare *Etlingera* species with other ginger species based on phenolic content and antioxidant activity of leaves and rhizomes

5. To determine major chemical constituents with bioactive properties of extracts and essential oils from leaves of *E. elatior*

6. To develop protocols for producing a standardized extract from leaves of *E. elatior* that have commercial potentials for the herbal industries

1.3 PUBLICATIONS

During the course of this project, six papers have been published in international refereed journals. They were jointly authored with supervisors and with third year or honours students working on Zingiberaceae. Reprints of these publications are enclosed in Appendix III [pages 259–305].

Chapter 2

LITERATURE REVIEW

2.1 ZINGIBERACEAE

2.1.1 Introduction

Gingers are perennial herbs belonging to the family Zingiberaceae. Their rhizomes are commonly consumed as spice or condiments and used in traditional herbal medicine. In recent years, gingers are gaining popularity as ornamental plants as their inflorescences and foliage are colourful and attractive.

In Peninsular Malaysia, Zingiberaceae is divided into three tribes: Zingibereae, Alpinieaea and Hedychieae (Larsen *et al.*, 1999). *Zingiber* with 19 species is the only genus of the tribe Zingibereae. The tribe Alpinieae consists of nine genera with 84 species. The genera are *Alpinia, Etlingera, Hornstedtia, Amomum, Elettariopsis, Elettaria, Geocharis, Plagiostachys* and *Geostachys.* The tribe Hedychieae has seven genera with 52 species. Genera include *Boesenbergia*, *Curcuma, Hedychium, Camptandra, Scaphochlamys, Kaempferia* and *Haniffia*.

The three cultivated species of Zingiberaceae, which are of major commercial importance, are *Zingiber officinale* (ginger) *Curcuma longa* (turmeric) and *Elettaria cardamomum* (cardamom) (Larsen *et al.*, 1999). Rhizomes of *Z. officinale* are typically used as additives and flavouring in the food and beverage industry. Rhizomes of *C. longa* are popular as a spice used in curries both for flavour and colour. *Alpinia galanga* rhizomes are used as spice for certain meat dishes.

In Peninsular Malaysia, gingers are used as traditional cures, many of which are apparently associated with women-related ailments or illnesses (Larsen *et al.*, 1999). Species such as *Curcuma zedoaria*, *C. mangga*, *C. aeruginosa* and *Zingiber montanum* are used in food preparations for women in confinement after birth (Khaw, 2001). Other species such as *Curcuma zanthorrhiza*, *Zingiber ottensii* and *Z. zerumbet* are consumed either on their own or with other plant species in the form of decoctions, tonics or fresh rhizomes.

2.1.2 Uses

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* (Larsen *et al.*, 1999). In Malaysia, at least 30 or more ginger species have been cultivated for their use as spices, condiments, flavours, vegetables, traditional medicine, ornamentals and recently as cosmetics [Table 2.1].

Food

In Peninsular Malaysia, leaves of some ginger species are used for food flavouring (Larsen *et al.*, 1999). Leaves of *Kaempferia galanga* are used to prepare local fish dishes. Young inflorescences of *Etlingera elatior* are an essential ingredient of curries and rice dishes.

Young rhizomes of *Curcuma mangga, Boesenbergia rotunda* and *Zingiber zerumbet*, and young inflorescences of *Curcuma longa* and *Alpinia galanga* are also consumed as fresh vegetables by village folks (Ibrahim, 1992). The palatable young rhizomes and shoots of *C. mangga* are consumed raw with rice (Abas *et al.*, 2005). Rhizomes of *A. galanga, Z. officinale, C. longa* and *B. rotunda* have been extensively used as condiment for flavoring food (Oonmetta-aree *et al.*, 2006).

Table 2.1
Uses of ginger species in Malaysia

Ginger species	Tribe	Uses			
		Food	Medicinal	Ornamental	Cosmetic
Alpinia conchigera	Alpinieaea		+		
A. galanga		+			
A. luteocarpa				+	
A. malaccensis				+	
A. mutica				+	
A. purpurata				+	
A. vitata				+	
A. zerumbet			+	+	
A. zerumbet 'Variegata'				+	
Boesenbergia rotunda	Hedychieae	+	+		
Curcuma aeruginosa	Hedychieae	+	+		
C. longa		+	+		
C. mangga		+	+		
C. zanthorrhiza		+	+		+
C. zedoaria		+	+		
Elettariopsis latiflora	Alpinieaea			+	
E. smithiae				+	
E. slahmong		+			
Etlingera elatior	Alpinieaea	+	+		
E. fulgens				+	
E. littoralis		+			
E. maingayi				+	
E. rubrolutea		+			
E. rubrostriata				+	
E. venusta				+	
Hedychium coronarium	Hedychieae		+	+	
Kaempferia galanga	Hedychieae	+			
K. pulchra				+	
K. rotunda			+		
Scaphochlamys kunstleri	Hedychieae			+	
Zingiber griffithii	Zingibereae			+	
Z. malaysianum				+	
Z. montanum		+	+		
Z. officinale		+	+		
Z. ottensii		+	+		
Z. spectabile			+	+	
Z. zerumbet		+	+		+

The whole plant of *Elettariopsis slahmong* has a strong repulsive stinkbug odour. Considered delicacy, the aborigines in Peninsular Malaysia use the leaves of this plant to flavour their native cuisines of wild meat and fish (Lim, 2003). In Southern Thailand, the leaves are eaten raw.

In Sabah, several species of wild gingers are consumed by indigenous communities (Noweg *et al.*, 2003). Hearts of young shoots, inflorescences and fruits of *Etlingera elatior*, *E. littoralis*, *E. rubrolutea* and *Hornstedia* sp. are used as condiment, consumed raw or cooked as vegetable. In Thailand, the fruits of *E. littoralis* are edible and the young stem, after removing the outer parts, yields an aromatic tender core that is eaten raw or cooked (Sirirugsa, 1999). Flowers of *Etlingera maingayi* are edible.

In Japan, young inflorescences of *Zingiber mioga* (myoga) are eaten raw or pickled for their pleasant pungent flavour (Abe *et al.*, 2004). Leaves of myoga have been used to wrap traditional buns with sweetened bean paste. Young shoots and inflorescence of *Z. zerumbet* are used as condiments (Nakamura *et al.*, 2004).

Traditional medicine

In Peninsular Malaysia, gingers are used as traditional cures. Many of the uses are associated with women-related ailments or illnesses (Larsen *et al.*, 1999). These include *Curcuma zedoaria*, *C. mangga*, *C. aeruginosa* and *Zingiber montanum* that are used in food preparations for women in confinement after birth (Khaw, 1995). Rhizomes of *C. mangga* are used in postpartum care for womb healing (Abas *et al.*, 2005). Other species such as *Curcuma zanthorrhiza*, *Zingiber ottensii* and *Z. zerumbet* are consumed either on their own or in mixtures with other plant species in the form of decoctions, tonics or fresh rhizomes (Larsen *et al.*, 1999).

In Negeri Sembilan, Peninsular Malaysia, cut pieces of *A. galanga* rhizomes dipped in kerosene are rubbed onto skin to treat fungal infection (Ong & Norzalina, 1999). Juice from *C. longa* rhizomes is taken as post-partum medicine to get rid of flatulence. The juice is rubbed onto the skin to relieve pain and to treat ringworm infection. Decoction of *Z. officinale* rhizome is used to treat stomachache, fever and flatulence. Decoction of rhizomes of the red variety of *Z. officinale* is taken to treat high blood pressure, jaundice and diabetes. Decoction of *Z. zerumbet* rhizomes or juice is taken to treat jaundice.

In Kelantan, Peninsular Malaysia, paste from *Alpinia conchigera* rhizomes is applied to treat aches and pains (Ong & Nordiana, 1999). A decoction of leaves of *A. galanga* is consumed to treat diarrhoea. A paste of *C. longa* rhizomes, betle leaves and rice is applied to treat stomachache and abdominal swellings. Young rhizomes of *C. longa* are eaten to improve complexion. Juice obtained from *C. zanthorrhiza* rhizomes is applied onto the face to cure pimples. A decoction of *Z. officinale* rhizomes is consumed to treat abdominal swellings and stomachache, and applied to treat skin fungal infection. Young rhizomes of *Z. officinale* are eaten raw for healthier skin.

In Thailand, rhizomes of *A. galanga*, *Z. officinale*, *C. longa* and *B. rotunda* have been used as local medicines for treating stomachache and diarrhea and used as a carminative (Oonmetta-aree *et al.*, 2006). Rhizomes of *Z. zerumbet* have been used in local traditional medicine as a cure for swelling, sores and loss of appetite, and for de-worming children (Somchit & Shukriyah, 2003).

Leaves of *Zingiber spectabile* are used in the preparation of traditional medicine (Theilade, 1996). Leaves are pounded into a paste and applied onto the body to alleviate swellings. The infusion is also used to abate inflamed eyelids (Sirirugsa, 1999). The aborigines also use its leaves to treat headache and backache (Wolff *et al.*, 1999).

In Malaysia, a decoction of fruits of *E. elatior* is used to treat earache while a decoction of leaves is applied for cleaning wounds (Ibrahim & Setyowati, 1999). The leaves are also used by post-partum women and mixed with other aromatic herbs in water for bathing to remove body odour.

Ornamentals

In recent years, gingers are gaining popularity as ornamental plants as their inflorescences and foliage are colourful, attractive and long-lasting. Plants of *E. elatior* are cultivated throughout the tropics (Larsen *et al.*, 1999). Inflorescences are large and attractive with showy bracts. Farms in Australia and Hawaii are cultivating the species and selling its inflorescences as cut flowers on a commercial basis (Ibrahim & Setyowati, 1999). In Malaysia, the attractive and long-lasting inflorescences of *Z. spectabile* are sold as cut flowers (Larsen *et al.*, 1999). *Alpinia zerumbet* 'Variegata', *A. vittata*, *Scaphochlamys kunstleri* and *Hedychium coronarium* are planted for their attractive leaves and/or flowers. In Thailand, species of *Curcuma* have been cultivated for the aesthetics of their flowers and for export (Sirirugsa, 1999). They include *Curcuma alismatifolia*, *C. aurantiaca*, *C. petiolata* and *C. roscoeana.*

Cosmetics

Recently, cosmeceutical products have been developed from Zingiberaceae in Malaysia. An example is XANWHITE developed by the Standards and Industrial Research Institute of Malaysia (SIRIM) (Rozanida *et al*., 2006). Comprising a cleanser, toner, moisturiser with sun filters and treatment cream, the skin-lightening products contain a blend from the rhizomes of *Curcuma zanthorrhiza* and *Zingiber zerumbet* with bioactive phytochemicals having tyrosinase inhibition, antioxidant and anti-inflammatory properties.

A proprietary extraction from the rhizomes of *K. galanga* has been found to inhibit tyrosinase with IC_{50} values more than five fold lower than that for conventional extract (Majeed & Prakash, 2004). Tetrahydrocurcuminoids extracted from *C. longa* rhizomes have been found to inhibit tyrosinase activity. Lack of a yellow colour in tetrahydrocurcuminoids renders them useful for cosmetic applications.

2.1.3 Phytochemistry

Species of Zingiberaceae continue to attract much phytochemical interest due to their culinary uses, and to their biological and pharmaceutical activities. An excellent review of the phytochemistry of Zingiberaceae was made by Pancharoen *et al.* (2000) based on eight genera, namely, *Aframomum*, *Alpinia*, *Amomum*, *Boesenbergia*, *Curcuma*, *Hedychium*, *Kaempferia* and *Zingiber.* This updated review is based on classes of compounds isolated and identified. Interest is focused on the chemical constituents of rhizomes of species such as *Alpinia*, *Boesenbergia*, *Curcuma*, *Etlingera*, *Hedychium*, *Kaempferia* and *Zingiber.* With regard to the phytochemistry of leaves, information is restricted to a few species of *Alpinia*.

Compounds of rhizomes

In Zingiberaceae, there is much literature on the chemical constituents of rhizomes of *Alpinia*, *Curcuma*, *Etlingera*, *Hedychium*, *Kaempferia* and *Zingiber* species.

Classes of compounds found in rhizomes include diterpenes (Uehara *et al.*, 1992), labdane-type diterpenes (Itokawa *et al.*, 1981 & 1988; Nakatani *et al.*, 1994; Kong *et al.*, 2000; Zhang *et al.*, 2003; Zhang & Kong, 2004; Abas *et al.*, 2005), sesquiterpenoids (Itokawa *et al.*, 1981 & 1988; Morita & Itokawa, 1986; Toume *et al.*, 2005), sterols (Habsah *et al.*, 2004a; Ma *et al.*, 2004), phenyl-propanoids (Athamprasangsa *et al.*, 1994; Ly *et al.*, 2002 & 2003), diaryl-

heptanoids (Ravindranath & Satyanarayana, 1980; Kikuzaki *et al.*, 1991a & 1991b; Claeson *et al.*, 1993 & 1996; Athamprasangsa *et al.*, 1994; Shin *et al.*, 2002; Habsah *et al.*, 2004a; Ma *et al.*, 2004; Giang *et al.*, 2005 & 2006), phenylbutenoids (Jitoe *et al.*, 1993; Masuda & Jitoe, 1995; Masuda *et al.*, 1999a; Han *et al.*, 2005), phenylbutanoids (Tuntiwachwuttikul *et al.*, 1981), phenolic acids (Masuda *et al.*, 2000), flavonoids (Jaipetch *et al.*, 1983; Masuda *et al.*, 1991; Athamprasangsa *et al.*, 1994; Shin *et al.*, 2002; Zhang *et al.*, 2003; Zhang & Kong, 2004; Yenjai *et al.*, 2004; Jang *et al.*, 2004), kava pyrones (Habsah *et al.*, 2003), vanilloids (Ma *et al.*, 2004), coumarins (Kong *et al.*, 2000), glycosides (Ly *et al.*, 2002), glucosides (Sekiwa *et al.*, 1999 & 2000) and xanthones (Carvalho *et al.*, 2003).

Compounds of leaves

Information on the phytochemistry of leaves is limited to a few species of *Alpinia*. Classes of compounds found in leaves of Zingiberaceae include flavonoids (Williams & Harborne, 1977; Mpalantinos *et al.*, 1998), phenolic acids (Elzaawely *et al.*, 2007), labdane-type diterpenes (Sy & Brown, 1997; Zhou *et al.*, 1997), diarylheptanoids (Ngo & Brown, 1998), phenylbutanoids (Kikuzaki *et al.*, 2001) and kava pyrones (Fujita *et al.*, 1994; Mpalantinos *et al.*, 1998; Kuster *et al.*, 1999).

From the leaves of *Alpinia zerumbet*, flavonoids (rutin, kaempferol 3-*O*-rutinoside, kaempferol 3-*O*-glucuronide, (+)-catechin and (-)-epicatechin), and kava pyrones (dihydro-5,6-dehydrokawain and 5,6-dehydrokawain) have been identified (Mpalantinos *et al.*, 1998). Dihydro-5,6-dehydrokawain has been earlier reported by Fujita *et al.* (1994). Recently, Elzaawely *et al.* (2007) reported the presence of ferulic acid and *p*-hydroxybenzoic acid as major phenolic acids in ethyl acetate leaf extracts of *A. zerumbet.*

From the leaves and stems of *Alpinia katsumadai*, the presence of stilbenes, monoterpenes, diarylheptanoids, labdanes and chalcones have been reported of which six compounds were novel (Ngo & Brown, 1998). From the leaves of *Alpinia flabellata*, three phenylbutanoid dimers have been isolated (Kikuzaki *et al.*, 2001).

The most comprehensive work on the leaf chemistry of Zingiberaceae is by Williams and Harborne (1977) where leaves of *Alpinia*, *Etlingera*, *Hedychium*, *Kaempferia* and *Zingiber* were screened for flavonoids. Leaves of *Alpinia* are characterised by the frequent occurrence of glycosides of kaempferol and quercetin. Leaf flavonoids of *Zingiber* species have myricetin and quercetin glycosides. Flavonoids identified in the leaves of *E. elatior* are kaempferol 3-glucuronide, quercetin 3-glucuronide, quercetin 3-glucoside and quercetin 3-rhamnoside.

Classes of compounds

Terpenoids

Terpenoids are a class of natural products with biological activity (Demetzos & Dimas, 2001; Bakkali *et al.*, 2008). They are formed from linear arrangements of isoprene units which are derived from acetate metabolism through the mevalonic acid pathway. They are divided into classes according to the number of C_5 isoprene units they contain. The main terpenes are monoterpenes (C10) and sesquiterpenes (C15), but hemiterpenes (C5), diterpenes (C20), triterpenes (C30) and tetraterpenes (C40) also exist. A terpene containing oxygen is called a terpenoid. The monoterpenes are formed from the coupling of two isoprene units (C10). They constitute 90% of the essential oils and have a great variety of structures.

Zaeoung *et al.* (2005) analysed the volatile oils from rhizomes of *A. galanga, B. rotunda, C. longa, K. galanga* and *Z. officinale* using GC-MS. For the five species, the main compounds were *trans*-3-acetoxy-1,8-cineole, camphor, arturmerone, ethyl cinnamate and geranial (*E*-citral), respectively.

Major constituents of essential oils extracted from fresh leaves of *C. longa* were *p*-cymene and 1,8-cineole followed by *cis*-sabinol and *β*-pinene (Garg *et al.*, 2002). In *C. zedoaria* leaves, α-terpinyl acetate, dehydrocurdione, iso-borneol and selina-4(15),7(11)-dien-8-one were the major oils.

Essential oils of *A. galanga* leaves and stems are rich in 1,8-cineole and camphor while root oils are rich in α-fenchyl acetate (Jirovetz *et al.*, 2003). Four isomers of acetoxycineoles, (*trans* and *cis*)-2- and -3-acetoxy-1,8-cineoles (Kubota *et al.*, 1998) and *trans*-2- and *trans*-3-cineole glucosides (Someya *et al.*, 2001) have been isolated from the rhizomes of *A. galanga*.

Phytochemical screening of 14 species of Zingiberaceae in Malaysia showed that terpenoids were detected in leaves, stems and rhizomes (Zakaria & Ibrahim, 1986). Sirat *et al.* (1994) screened for labdane diterpenes from rhizomes of 30 species of Zingiberaceae from Malaysia. Labda-8(17),12-diene-15,16-dial was found in *Alpinia javanica, A. oxymitra, A. purpurata, A. zerumbet, Etlingera littoralis, E. rubrolutea, Hedychium longicornutum, Hornstedia scyphifera, Zingiber griffithii, Z. ottensii, Z. puberulum, Z. spectabile* and *Z. zerumbet*. Coronarin E was found only in *Alpinia javanica* and *Hedychium coronarium.*

Labdane-type diterpenes have been isolated from rhizomes of *Alpinia formosana* (Itokawa *et al.*, 1988), *A. zerumbet* (Itokawa *et al.*, 1981; Xu *et al.*, 1996), *A. calcarata* (Kong *et al.*, 2000), *Curcuma mangga* (Abas *et al.*, 2005), *Hedychium coronarium* (Nakatani *et al.*, 1994) and *Zingiber mioga* (Abe *et al.*, 2002). Nine new labdane diterpenes and two known diterpenes have been isolated from the

aerial parts of *Alpinia chinensis* (Sy & Brown, 1997). Sesquiterpenes have been reported in rhizomes of *A. zerumbet* (Itokawa *et al.*, 1981), *Curcuma parviflora* (Toume *et al.*, 2005), *C. zedoaria* (Matsuda *et al.*, 2001) and *Z. zerumbet* (Huang *et al.*, 2005).

In Zingiberaceae, stigmasterol and β-sitosterol were isolated from rhizomes of *A. zerumbet* (Habsah *et al.*, 2003). From rhizomes of *E. elatior,* stigmast-4-en-3-one, stigmast-4-ene-3,6-dione, stigmast-4-en-6*β*-ol-3-one and 5*α*,8*α*-epidioxy-ergosta-6,22-dien-3*β*-ol were isolated (Habsah *et al.*, 2004a). Sharma *et al.* (1975) isolated sitosterol and sitosterol-*β*-D-glucoside from the aerial parts of *H. coronarium.* From rhizomes of *Z. officinale*, β-sitosterol and 6β-hydroxystigmast-4-en-3-one have been isolated (Ma *et al.*, 2004).

From seeds of *Aframomum zambesiacum*, five labdane diterpenoids including zambesiacolactone A and zambesiacolactone B have been isolated along with five known labdanes and a linear sesquiterpene, nerolidol (Kenmogne *et al.*, 2006). Diterpenoids and sesquiterpenoids isolated from seeds of *Aframomum arundinaceum* were methyl 14,15-epoxy-8(17),12(*E*)-labdadiene-16-oate, 6,7-epoxy-3(15)-caryophyllene, aframodial, (-)-α-bisabolol and galanolactone (Wabo *et al.*, 2006).

Alkaloids

On screening 14 species of gingers in Malaysia, Zakaria and Ibrahim (1986) detected alkaloids in stems of *A. galanga*, and in rhizomes of *H. coronarium* and *Z. officinale.*

Phenylpropanoids

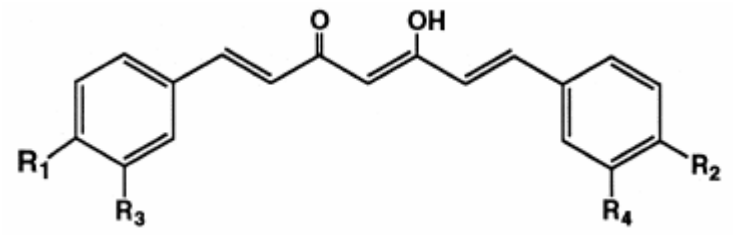
In the rhizomes of *Alpinia officinarum,* seven phenylpropanoids have been isolated by reverse-phase HPLC and their structures elucidated by MS and NMR analyses (Ly *et al.*, 2003).

Diarylheptanoids

Diarylheptanoids constitute a distinct group of natural plant metabolites characterized by two aromatic rings linked by a linear seven-carbon aliphatic chain (Keseru & Nogradi, 1995).

In Zingiberaceae, diarylheptanoids are common chemical constituents of rhizomes. They have been isolated from rhizomes of *Alpinia blepharocalyx* (Kadota *et al.*, 2003), *A. officinarum* (Uehara *et al.*, 1987; Shin *et al.*, 2002; An *et al.*, 2006), *A. conchigera* (Athamprasangsa *et al.*, 1994), *A. pinnanensis* (Giang *et al.*, 2005), *A. zerumbet* (Habsah *et al.*, 2003), *Amomum muricarpum* (Giang *et al.*, 2006), *Curcuma zanthorrhiza* (Uehara *et al.*, 1987 & 1992; Claeson *et al.*, 1993 & 1996; Masuda *et al.*, 1992; Suksamrarn *et al.*, 1994), *C. zedoaria* (Syu *et al.*, 1998), *C. longa* (Ravindranath & Satyanarayana, 1980), *E. elatior* (Habsah *et al.*, 2004a), *Z. montanum* (Masuda & Jitoe, 1994; Jitoe *et al.*, 1994) and *Z. officinale* (Kikuzaki *et al.*, 1991a & 1991b; Kikuzaki & Nakatani, 1996; Ma *et al.*, 2004).

Diaryheptanoids from *Curcuma* rhizomes are curcumin, demethoxycurcumin and bisdemethoxycurcumin (Surh, 1999; Lin & Lee, 2006). Together, they are referred to as curcuminoids [Fig. 2.1]. Curcumin has a unique conjugated structure including two methoxylated phenols and the enol form of a heptadiene-3,5-diketone linking the two phenols, giving a bright yellow colour.



Diarylheptanoid	R_1	R_2	R_3	R_4
Curcumin	OH	OH	OCH_3	OCH_3
Demethoxycurcumin	OH	OH	OCH_3	H
Bisdemethoxycurcumin	OH	OH	H	H

Fig. 2.1
Diarylheptanoids from rhizomes of *Curcuma* species

Diarylheptanoids yakuchinones A and B have been isolated from rhizomes of *Alpinia oxyphylla* (Lee *et al.*, 2006). The molecular structures of yakuchinones A and B are shown in Fig. 2.2. Diarylheptanoids from rhizomes of *Zingiber officinale* are gingerones A, B and C, and of *Z. montanum* are cassumins and cassumunarins A, B and C (Keseru & Nogradi, 1995).

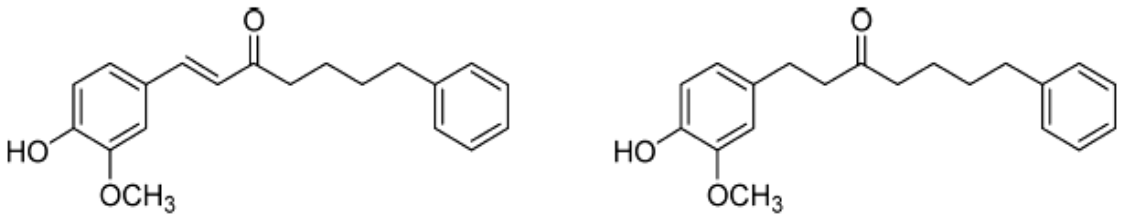


Fig. 2.2
Yakuchinone A (left) and yakuchinone B (right) from *Alpinia oxyphylla* rhizomes

Diarylheptanoids are also found in seeds, fruits and leaves of Zingiberaceae. They have been isolated from fruits of *Aframomum letestuianum* (Kamnaing *et al.*, 2003), *Alpinia blepharocalyx* (Prasain *et al.*, 1998; Tezuka *et al.*, 2001; Ali *et al.*, 2001a & 2001b; Kadota *et al.*, 2003), *A. katsumadai* (Yang *et al.*, 1999) and *A. oxyphylla* (Itokawa *et al.*, 1982), *A. rafflesiana* (Habsah *et al.*, 2004b) and from leaves of *A. katsumadai* (Ngo & Brown, 1998).

Vanilloids

In Zingiberaceae, gingerol and paradol are vanilloids derived from the rhizomes of *Z. officinal*e (Lee & Surh, 1998; Surh, 1999). The molecular structures of (6)-gingerol and (6)-paradol are shown in Fig. 2.3.

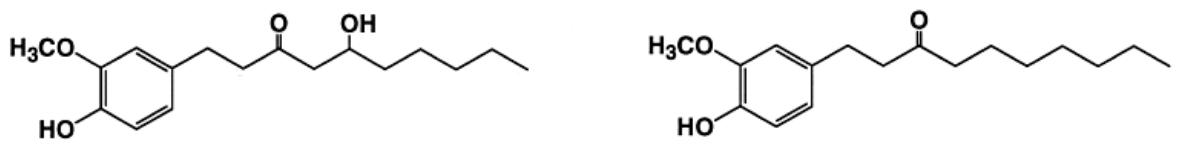


Fig. 2.3
(6)-gingerol (left) and (6)-paradol (right) from rhizomes of *Zingiber officinale*

Phenylbutenoids

Phenylbutenoids are a class of natural products found in Zingiberaceae. Various non-polar monomeric and dimeric phenylbutenoids are found in the rhizomes (Masuda & Jitoe, 1995). From rhizomes of *A. flabellata,* phenylbutenoid dimers have been isolated (Masuda *et al.*, 1999a). Rhizomes of *Z. montanum* yielded phenylbutenoids (Han *et al.*, 2005), phenylbutenoid dimers (Jitoe *et al.*, 1993) and phenylbutenoid monomers (Masuda & Jitoe, 1995).

Phenylbutanoids

From the rhizomes of *Z. montanum*, five phenylbutanoids have been isolated (Tuntiwachwuttikul *et al.*, 1981) and from the leaves of *A. flabellata*, three phenylbutanoid dimers have been identified (Kikuzaki *et al.*, 2001).

Kava pyrones

Rhizomes of *A. zerumbet* and fruits of *A. rafflesiana* yielded 5,6-dehydrokawain and flavokawin B (Habsah *et al.* 2003; Habsah *et al.* 2004b). From leaves of *A. zerumbet,* kava pyrones of dihydro-5,6-dehydrokawain and 5,6-dehydrokawain have been isolated (Fujita *et al.*, 1994; Mpalantinos *et al.*, 1998).

Chalcones

Cyclohexenyl chalcone derivatives, 4-hydroxypanduratin A and panduratin A have been isolated from the rhizomes of *B. rotunda* (Tuchinda *et al.*, 2002).

Flavonoids

In Zingiberaceae, flavonoids are known to occur in the leaves and occasionally in the rhizomes. Screening by Williams and Harborne (1977) showed that leaves of Zingiberaceae are rich in flavonoids. Three constituents, kaempferol, quercetin and proanthocyanidins were found in 22, 72 and 71% of species respectively. Three flavonols, namely, myricetin, isorhamnetin and syringetin were also found in 26, 10 and 3% of the species, respectively. Glycosides of flavonols reported in leaves of ginger species are shown in Table 2.2.

Leaves of *Alpinia* are characterised by the frequent occurrence of kaempferol and quercetin glycosides (Williams & Harborne, 1977). Flavonoids identified in the leaves of *E. elatior* are kaempferol 3-glucuronide, quercetin 3-glucuronide,

quercetin 3-glucoside and quercetin 3-rhamnoside. Leaf flavonoids of *Zingiber* species have myricetin and quercetin glycosides.

Table 2.2
Glycosides of flavonols in rhizomes of ginger species (Williams & Harborne, 1977)

Ginger species	Glycoside of flavonol
Alpinia malaccensis, A. mutica, A. zerumbet, Etlingera elatior and *Kaempferia pulchra*	Kaempferol and quercetin
Hedychium coronarium	Myricetin and quercetin
Alpinia purpurata	Quercetin
Zingiber zerumbet	Myricetin

Studying the flavonoid content of 62 edible plants in Malaysia, Koo and Suhaila (2001) reported that inflorescences of *E. elatior* contained 286 and 21 mg/kg dry weight of kaempferol and quercetin, respectively. Rhizomes of *C. longa* had only 93 mg/kg dry weight of quercetin.

In Zingiberaceae, flavonoids have been isolated from rhizomes of *Alpinia officinarum* (Shin *et al.*, 2002), *A. tonkinensis* (Zhang *et al.*, 2003), *Kaempferia parviflora* (Yenjai *et al.,* 2004) and *Zingiber zerumbet* (Masuda *et al.*, 1991; Jang *et al.*, 2004), seeds of *Aframomum letestuianum* (Kamnaing *et al.*, 2003), and leaves of *A. zerumbet* (Mpalantinos *et al.*, 1998).

From rhizomes of *Alpinia zerumbet, A. officinarum* and *Z. zerumbet*, cardamomin and alpinetin (Itokawa *et al.* 1981), galangin and kaempferide (Shin *et al.* 2002), and six kaempferol derivatives (Jang *et al.* 2004) have been isolated respectively. From leaves of *A. zerumbet,* rutin, kaempferol-3-*O*-rutinoside,

kaempferol-3-*O*-glucuronide, (+)-catechin and (-)-epicatechin have been isolated (Mpalantinos *et al.* 1998). Yun *et al.* (2005) reported the isolation of panduratin A from rhizomes of *B. rotunda.*

Most of the flavonoids found in rhizomes and seeds of Zingiberaceae are unique in that they have no free hydroxyl groups on the B-ring e.g. kaempferide, the 4'-methyl ether of kaempferol, which occurs in the rhizome of *Alpinia officinarum* and alpinetin, which occurs in the seeds of *A. chinensis* and rhizomes of *B. rotunda* (Williams & Harborne, 1977). Unlike rhizomes and seeds, flavonoids in leaves of gingers are of the 'normal' type with B-ring hydroxylation.

2.1.4 Bioactivities

Antioxidant activity

Rhizomes of Zingiberaceae are known to have antioxidant activity. Zaeoung *et al* (2005) assessed the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of methanol and water extracts of rhizomes of *A. galanga, B. rotunda, C. longa, K. galanga* and *Z. officinale.* Methanol extracts of *C. longa* exhibited the most pronounced activity. Jitoe *et al.* (1992) studied the antioxidant activity of acetone extracts of rhizomes of nine ginger species using the thiobarbituric acid (TBA) assay. Strong antioxidant activity was found in *C. longa*, *A. galanga* and *C. zanthorrhiza.* Kikuzaki and Nakatani (1993) isolated five gingerol related compounds and eight diarylheptanoids from dichloromethane (DCM) extracts of *Z. officinale* rhizomes. Using the ferric thiocyanate (FTC) assay, 12 of these compounds were found to exhibit higher antioxidant activity than α-tocopherol. The activity was probably dependent upon side chain structures and substitution patterns of the benzene ring.

Using the FTC assay, Habsah *et al.* (2000) screened DCM and methanol extracts of six species of *Alpinia* and three species of *Zingiber* for antioxidant activities. All the extracts showed strong antioxidant activity comparable with or higher than that of α-tocopherol. Both DCM and methanol extracts of rhizomes of *Alpinia malaccensis* showed the strongest antioxidant activity among the *Alpinia* species studied. DCM extracts of rhizomes of *Z. montanum* showed the strongest antioxidant activity among the *Zingiber* species studied.

Vankar *et al.* (2006) reported that extracts from six species of Zingiberaceae, namely, *Alpinia allughas, A. galanga, Hedychium coccineum, H. coronarium, Kaempferia galanga* and *Zingiber montanum* showed moderate to good anti-oxidant properties. Methanol extracts of the rhizomes showed better results for DPPH analysis than DCM extracts. Sharma *et al.* (2007) reported that rhizomes of *Z. montanum* had strong radical scavenging activity at low extract concentration using the DPPH, superoxide and hydroxyl assays. Using the FTC assay, Masuda and Jitoe (1994) reported that cassumunins A, B and C isolated from rhizomes of *Z. montanum* showed antioxidant activity that is stronger than that of curcumin.

Sreejayan and Rao (1996) compared the ability of three natural curcuminoids (curcumin, demethoxycurcumin and bisdemethoxycurcumin) isolated from rhizomes of *C. longa* to scavenge superoxide and DPPH radicals. Results showed that curcumin is the most potent scavenger of superoxide radicals followed by demethoxycurcumin and bisdemethoxycurcumin. A similar order of ranking: curcumin > demethoxycurcumin > bisdemethoxycurcumin was reported by Song *et al.* (2001) in terms of DPPH radical scavenging activity.

Chen *et al.* (2008) screened the rhizomes of 18 Zingiberaceae species belonging to five genera from Taiwan for antioxidant activity. Highest phenolic content was recorded in *Vanoverberghia sasakiana*, *C. longa* and *C. zedoaria*. Based on

antioxidant capacity, radical scavenging and reducing power, best performers were rhizomes of *C. longa*, *H. coronarium* and *V. sasakiana*.

Tyrosinase inhibition

Species of Zingiberaceae have tyrosinase inhibition properties. Zingerone and dehydrozingerone isolated from rhizomes of *Z. officinale* have been reported to exhibit potent tyrosinase inhibition activity (Kuo *et al.*, 2005). Fruits of *Elettaria cardamomum* and seeds of *Amomum xanthioides* showed tyrosinase inhibition activity (Lee *et al.*, 1997). Tyrosinase inhibition activity (IC_{50}) of a proprietary extract of *K. galanga* was over 5-fold lower than that for the conventional extract (Majeed & Prakash, 2004). Tetrahydrocurcuminoids extracted from rhizomes of *C. longa* showed effective tyrosinase inhibition activity.

Skin-lightening products have been developed from rhizomes of *C. zanthorrhiza* and *Z. zerumbet* (Rozanida *et al.*, 2006). The rhizomes have bioactive chemicals with tyrosinase inhibition, antioxidant and anti-inflammatory properties.

Antimicrobial activity

There is extensive literature on the antimicrobial activity in ginger species. Ether and ethyl acetate extracts of rhizomes of *A. galanga, C. zedoaria*, *C. longa* and *Kaempferia rotunda* were found to have significant antimicrobial effects on *Staphylococcus aureus* and *Klebsiella pneumoniae* (Thomas *et al.*, 1996). Ethanol extracts of inflorescences of *E. elatior* yielded clear inhibition zones for *Pseudomonas aeruginosa*, *Bacillus megaterium*, *Escherichia coli* and *Crypto-coccus neoformans* (Mackeen *et al.*, 1997). Methanol and DCM rhizome extracts of five out of six *Alpinia* and all three *Zingiber* species showed antimicrobial activity against *Bacillus subtilis* and *P. aeruginosa* (Habsah *et al.*, 2000). DCM

extracts appeared to be more effective than methanol extracts. All extracts were inactive against *E. coli* and *Candida albicans*.

Petroleum ether, hexane, chloroform, acetone and ethanol extracts of *Curcuma zedoaria* and *C. malabarica* rhizomes showed antimicrobial activity against all six bacteria except *E. coli* (Wilson *et al.*, 2005). Extracts of *C. zedoaria* were ineffective against *S. aureus.* All extracts were effective against *C. albicans* while petroleum ether extracts of both species and acetone extract of *C. zedoaria* were effective against *Aspergillus niger.*

Ethanol extracts of *A. galanga* showed high antimicrobial activity against *Staphylococcus epidermidis* and *Saccharomyces cerevisiae*; moderate activity against *Bacillus cereus* and *B. megaterium*; weak activity against *Streptococcus lactis*; and no activity against *Enterobacter aerogenes, P. aeruginosa* and *Salmonella* sp. and *E. coli* (Oonmetta-aree *et al.*, 2006). Extracts of *A. galanga* exhibited the strongest inhibitory activity against *S. aureus* when compared to extracts of *B. rotunda, C. longa* and *Z. officinale.* With the exception of *C. longa*, extracts of all species had no effect against *E. coli.*

Petroleum ether and DCM extracts of *Zingiber wrayi* rhizomes exhibited antibacterial activity against *B. subtilis*, *E. coli*, *S. aureus* and *Sarcina* sp. but not extracts of *P. aeruginosa* (Chairgulprasert *et al.* 2005). Ethanol extracts of rhizomes of *A. galanga*, *C. zedoaria* and *Z. purpureum* were found to inhibit against human pathogenic fungi (Ficker *et al.*, 2003). Rhizomes of *E. elatior* did not show any antifungal activity but those of *E. littoralis* were effective against *Aspergillus fumigatus*.

Jantan *et al.* (2003a) investigated the antifungal activity of rhizome oils of nine Zingiberaceae species against five dermatophytes, three filamentous fungi and five strains of yeast. Rhizomes of *B. rotunda* were effective against all the fungi.

Rhizomes of *K. galanga* showed selective toxicity against *A. fumigatus* while those of *Z. officinale* and *Z. montanum* exhibited high activity against the yeasts. Rhizomes of 18 species belonging to five genera of Zingiberaceae native in Taiwan were screened for antibacterial activity using the disc diffusion method (Chen *et al*., 2008). Results showed that all species inhibited the Gram-positive *S. aureus* with the exception of *C. longa*, and inhibited the Gram-negative *E. coli* and/or *Salmonella enterica*.

Larvicidal activity

Essential oils from rhizomes of seven species of Zingiberaceae in Malaysia were investigated for their larvicidal activity against *Aedes aegypti* (Jantan *et al.*, 2003b). Ranking was in the following order: *C. longa* > *C. zanthorrhiza* > *Z. zerumbet* > *C. aeruginosa* > *B. rotunda* > *Z. officinale* > *Z. montanum*.

Insecticidal activity

Zanthorrhizol and furanodienone (sesquiterpenoids) isolated from rhizomes of *C. zanthorrhiza*, *C. zedoaria, K. galanga* and *K. rotunda* showed pronounced toxicity against neonate larvae of *Spodoptera littoralis* in a contact residue bioassay (Pandji *et al*., 1993). None of the compounds caused significant mortality of neonate larvae of *S. littoralis* when incorporated into artificial diet, suggesting inactivation of the compounds in the larval gut. Methanol extracts of *A. oxyphylla* had insecticidal activity against larvae of *Drosophila melanogaster* (Miyazawa *et al.*, 2001). The active compound was identified as yakuchinone A.

Cytotoxicity

There is much literature indicating that rhizome extracts of many ginger species have cytotoxic activity. Ethanol extracts of rhizomes of *K. galanga* and *E. elatior* displayed cytotoxic activity against HeLa cells (Mackeen *et al.*, 1997). Rhizome

extracts of *C. longa*, *C. zanthorrhiza*, *K. galanga*, *Z. montanum*, *Z. officinale* and *Z. zerumbet* possess inhibitory activity towards Epstein-Barr virus (EBV) activation of Raji cells induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Vimala *et al.*, 1999).

Methanol rhizome extracts of *B. rotunda*, *A. galanga*, *Z. montanum* and *Z. zerumbet* showed strongly active inhibitory effect toward EBV activation in Raji cells induced by TPA (Murakami *et al.*, 1994). Rhizome and leaf extracts of *C. mangga* and *E. elatior* were moderately active, respectively. Screening of in vitro anti-tumour promoting activities of edibles plants from Thailand and Malaysia by Murakami *et al.* (1995 & 2000) showed that rhizomes of commercial ginger species of *A. galanga*, *B. rotunda*, *C. longa* and *Z. officinale* from Thailand and Malaysia strongly inhibited EBV activation.

Habsah *et al.* (2001) studied the anti-tumour promoting activity of methanol and DCM extracts of rhizomes of 12 species of gingers using the EBV activation in Raji cells assay. Methanol extracts of *Alpinia hookeriana* and *A. zerumbet* showed the highest inhibition rates. Generally, methanol extracts of *Alpinia* were more active that DCM extracts.

Ethanol extracts of rhizomes of all eight ginger species studied showed inhibitory effect on the growth of HT-29 and MCF-7 cancer cells (Kirana *et al.*, 2003). Extracts of *C. longa*, *B. rotunda* and *Z. zerumbet* had very strong inhibitory activity.

Zaeoung *et al* (2005) assessed the cytotoxic activity of methanol extracts, water extracts and volatile oils of rhizomes of *A. galanga*, *B. rotunda*, *C. longa*, *K. galanga* and *Z. officinale* against MCF7 and LS174T cancer cell lines. All volatile oils and the methanol extract of *C. longa* showed strong cytotoxic activity.

Curcumin, demethoxycurcumin and bisdemethoxycurcumin isolated from *C. zedoaria* rhizomes demonstrated cytotoxicity against OVCAR-3 cancer cells (Syu *et al.*, 1998). [6]-gingerol and [6]-paradol from rhizomes of *Z. officinale* induced apoptosis in HL-60 cells (Lee & Surh, 1998). Zerumbone from rhizomes of *Z. zerumbet* inhibited the growth of HL-60 cells (Huang *et al.* 2005) and EBV activation (Murakami *et al.* 2002). Of the six compounds isolated, 1'-acetoxy-chavicol acetate from rhizomes of *A. galanga* inhibited COR L23 and MCF7 cancer cells (Lee & Houghton, 2005).

Other bioactivities

Rhizomes of six selected ginger species were investigated for their anti-allergic activities using a RBL-2H3 cell line (Tewtrakul & Subhadhirasakul, 2007). Rhizomes of *K. parviflora* had the most potent anti-allergic effect followed by *Z. montanum* and *C. mangga*. Compounds isolated from rhizomes of ginger species have shown to inhibit human platelet aggregation (Jantan *et al*., 2008). Curcumin from *C. longa*, cardamonin, pinocembrine and 5,6-dehydrokawain from *A. mutica*, and 3-deacetylcrotepoxide from *K. rotunda* showed strong inhibition activity.

2.2 GINGER SPECIES STUDIED

The following are descriptions of genera and species of gingers studied in this project:

2.2.1 *Alpinia*

Alpina Roxb. is the largest genus of Zingiberaceae (Larsen *et al.*, 1999). They are medium-sized to large forest plants, with some species reaching a height of over 3 m. It is the only genus in the tribe Alpinieae that has a terminal inflorescence on the leafy shoot. Flowers are yellowish-green, creamy or red in

colour and are usually conspicuous. Within the genus, *A. galanga* is an economically important species that is used as spice and medicine. This widely cultivated species is not native to Peninsular Malaysia.

In the leaves of nine species of *Alpinia* screened by Williams and Harborne (1977), quercetin glycoside occurred in all species while kaempferol glycoside was found in seven species.

Alpinia galanga

Synonym: *Languas galanga*

Alpinia galanga (L.) Sw. plants can grow up to 3.5 m tall (Scheffer & Jansen, 1999). Rhizomes (2–4 cm in diameter) are branched, light red or yellow in colour, fibrous and fragrant. Inflorescences are terminal and producing many white fragrant flowers. Commonly known as the greater galanga, *A. galanga* is widely cultivated in Southeast Asia.

In Malaysia, its rhizomes are much used in the spicy meat dish (Larsen *et al.*, 1999). Young shoots and inflorescences are also consumed as fresh vegetables or *ulam* (salad) by village folk and fruits are used locally as a substitute for cardamom (Scheffer & Jansen, 1999). Rhizomes are widely used in traditional medicine e.g. skin diseases, indigestions, colic, dysentery, enlarged spleen, respiratory diseases, cholera, cancers of mouth and stomach.

In Thailand, rhizomes of *A. galanga* are used for indigestion, colic, dysentery and cancer of the stomach (Sirirugsa, 1999). It is a remedy for food poisoning. The grated rhizome is prescribed for spleen trouble and herpes. An infusion of the leaves is for stimulant and for mitigating rheumatism. Fresh rhizomes are used as a spice for flavouring food.

In Malaysian traditional medicine, rubbing fresh rhizomes of *A. galanga* onto the skin is believed to be effective in curing fungal infection (Ibrahim & Zakaria, 1987). Main components of essential oils from rhizome extracts are α-terpeneol and 1,8-cineol.

Jitoe *et al.* (1992) reported that acetone extracts of the rhizomes of nine ginger species belonging to five genera studied showed antioxidant activity. Using the TBA assay, the antioxidant activity of *A. galanga* was second in ranking, second to only *C. longa.*

Of the ether and ethyl acetate extracts of 17 ginger plant species screened for their antibacterial activity against different multi-resistant Gram-positive and Gram-negative bacteria isolated from hospitalised patients, Thomas *et al.* (1996) found that *A. galanga* performed the best. Its ether extract was more potent than the ethyl acetate extract. Both types of extracts were found to have a significant effect on *S. aureus* and *K. pneumoniae*.

Rhizomes of *A. galanga* showed strongly active inhibition effect on EBV activation in Raji cells (Murakami *et al.*, 1995) and cytotoxicity activity on human U937 human cells (Jiang *et al.*, 2006a).

Essential oils of *A. galanga* leaves are rich in 1,8-cineole, camphor, β-pinene, (*E*)-methyl cinnamate, bornyl acetate and guaiol (Jirovetz *et al.*, 2003). Stem oils contain 1,8-cineole, camphor, (*E*)-methyl cinnamate, guaiol, bornyl acetate, β-pinene and α-terpineol. Rhizome oils are rich in 1,8-cineole, α-fenchyl acetate, camphor, (*E*)-methyl cinnamate and guaiol.

Chemical investigation of the chloroform extract of *A. galanga* rhizomes yielded *p*-hydroxycinnamaldehyde and [di-(*p*-hydroxy-cis-styryl)]methane (Barik *et al.*, 1987). The former is isolated for the first time in nature and the latter is a new chemical component.

Murakami *et al.* (1994) reported that 1'-acetoxychavicol acetate (ACA) isolated from rhizome of *A. galanga* was found to be a major inhibitor toward EBV activation in Raji cells. The inhibitory activity may very well be evaluated to be one of the most potent among the active compounds from edible sources. The quantity of ACA in fresh rhizome was estimated to be 0.09% using HPLC analysis.

Alpinia malaccensis

Synonym: *Languas malaccensis*

Alpinia malaccensis (Burm.) Rose is a robust plant growing up to 4 m in height (Ibrahim, 2001a). Leaves are lanceolate and pubescent below or at margins and along midribs. When crushed, leaves emit an aromatic fragrance. Inflorescences are erect bearing white flowers. The species is commonly found in primary forests and shaded rocky outcrops at low and medium altitudes.

In Java, rhizomes of *A. malaccensis* are used to cure wounds and sores (Ibrahim, 2001a). In the Philippines, a decoction of rhizomes is used for reducing fever. In India, rhizomes are consumed as spice. Rhizomes are chewed with betel nut in Moluccas to make the voice strong and clear.

Habsah *et al.* (2000) reported that methanol and DCM extracts of rhizomes of *A. malaccensis* showed the strongest antioxidant activity among six species of *Alpinia* studied. Using the FTC and TBA assays, methanol and DCM extracts of *A. malaccensis* rhizomes showed antioxidant activity that is higher than α-tocopherol (Habsah *et al.*, 2001).

Anti-tumour promoting activity using EBV activation in Raji cells showed that methanol and DCM extracts of *A. malaccensis* rhizomes at 20 µg/ml had strong (78%) and moderate (53%) inhibition rates, respectively (Habsah *et al.*, 2001).

2.2.2 *Curcuma*

Species of *Curcuma* L. are usually small to medium-sized herbs with fleshy rhizomes (Larsen *et al.* 1999). Inflorescences consist of broad bracts joined together to form closed pouches in which small partial inflorescences develop. The bracts may be green or coloured. It is a large genus with only few indigenous species in Peninsular Malaysia. About 5–6 species are cultivated for medicinal use and for flavouring food.

Curcuma longa

Synonym: *Curcuma domestica*

Curcuma longa L. is a perennial herb with branched bright orange rhizomes. Leaves are oblong or ovate lanceolate and aromatic (Wardini & Prakoso, 1999). Inflorescences are terminal on leafy shoots, with pale green bracts and white flowers.

The species is extensively cultivated in the tropical Asia. It is the source of the spice turmeric, which is derived from the dried, ground rhizome. Turmeric has a long tradition of medicinal use (Luper, 1999). Traditional applications include the treatment of gastrointestinal colic, flatulence, hemorrhage, hematuria, menstrual difficulties and jaundice. Anti-inflammatory and hepato protective characteristics of turmeric and its constituents have been widely researched.

In Peninsular Malaysia, rhizomes of *C. longa* are a popular spice used in curries (Larsen *et al.*, 1999). They are also used as food flavouring and colouring. The aromatic leaves of *C. longa* are used for wrapping fish before steaming or baking.

A great variety of pharmacological activities of *C. longa* are available in the literature. Araujo and Leon (2001) reviewed its biological activities, which include anti-inflammatory, anti-human immunodeficiency virus, anti-bacteria, antioxidant effects and nematocidal activities. Curcumin is a major component in *C. longa*, being responsible for its biological actions. Other extracts of this plant have been showing potency too. *In vitro*, curcumin exhibits anti-parasitic, anti-spasmodic, anti-inflammatory and gastrointestinal effects; and also inhibits carcinogenesis and cancer growth. *In vivo*, experiments showed anti-parasitic and anti-inflammatory potency of curcumin and extracts in animal models.

Major constituents of essential oils extracted from fresh leaves of *C. longa* were *p*-cymene and 1,8-cineole followed by *cis*-sabinol and *β*-pinene (Garg *et al.*, 2002). Diarylheptanoids and sesquiterpenoids in *C. longa* rhizomes are important compounds believed to be responsible for producing many biological and medicinal activities of turmeric (Jiang *et al.*, 2006a). Curcuminoids such as demethoxycurcumin and bisdemethoxycurcumin are the major diarylheptanoids of *C. longa* and have been shown to contribute many of the medicinal properties of this species (Lin & Lee, 2006).

2.2.3 *Elettariopsis*

The genus *Elettariopsis* Bak. belongs to the tribe Alpinieae (Larsen *et al.*, 1999). Taxonomically revised by Kam (1982), the genus has only four or five species. Lim (2003) in his recent revision reported at least nine species with the possibility of other new taxa. Species of *Elettariopsis* are small forest plants with creeping

and slender rhizomes (Larsen *et al.*, 1999). The leafy shoots, arising 10–20 cm apart, are 1–2 m tall with 1–8 leaves. Leaves are often fairly large. Inflorescences are produced by a separate side shoot at the base of the plant. Lim (2003) classified the genus into two categories based on inflorescence morphology (clustered or single flowers) and scent parameters (strongly aromatic or aroma not discernable).

Analysis of methanol and DCM extracts of rhizomes of *Elettariopsis* sp. by Habsah *et al.* (2001) using the FTC assay showed antioxidant activity that is higher than α-tocopherol. Wong *et al.* (2006a) analysed the essential oils of leaves, rhizomes and roots of *Elettariopsis elan.* In the leaves, the most abundant component was geraniol and this may explain the geranium scent of leaves when crushed. The rhizomes and roots yielded a distinctly different odour, with camphene, fenchyl acetate and α-phellandrene as major components.

Elettariopsis slahmong

Synonym: *Elettariopsis curtisii*

Elettariopsis slahmong C.K. Lim is found throughout Peninsular Malaysia (Lim, 2003). The plant, 0.5–1.5 m tall, produces creeping rhizomes, 2–6 leaves with extended tips and clustered flowers.

The whole plant has a strong repulsive stinkbug odour. Considered delicacy, the aborigines use the leaves of this plant to flavour their native cuisine especially wild meat and fish (Lim, 2003). Old Malay kampong folks in Negeri Sembilan, Kelantan and Terengganu use the leaves for flavouring curry and other spicy dishes. In Southern Thailand, the leaves are eaten as salad.

Wong *et al.* (2006b) reported that essential oils in leaves of *E. slahmong* producing the strong stink bug odour have been analysed as aldehydes with (*E*)-2-octenal and (*E*)-2-decenal as major components. Rhizomes and roots yielded oil also rich in aldehydes with a stink bug odour similar to the leaves, but with (*E*)-2-decenal as the main constituent.

2.2.4 *Etlingera*

Etlingera Giseke is another genus belonging to the tribe Alpinieae. *Etlingera* species are tall forest plants with larger species growing up to 6 m in height (Larsen *et al.*, 1999; Khaw, 2001). Inflorescences are raised above the ground (*Phaeomeria* group) or just appearing at soil level (*Achasma* group) (Lim, 2000 & 2001a). Flower colours range from pink, blood red to somewhat orange. Among the species, *E. elatior* is widely cultivated for its inflorescences as spice for food flavouring (Larsen *et al.*, 1999).

Etlingera elatior

Synonyms: *Alpinia elatior, Elettaria speciosa, Nicolaia elatior, Nicolaia speciosa* and *Phaeomeria speciosa*

Etlingera elatior (Jack) Smith or torch ginger grows up to 5–6 m tall forming clumps (Khaw, 2001). Rhizomes are stout (3–4 cm in diameter) and found just below ground level. Crushed leaf sheaths emit a pleasant sour fragrance. Leaves are entirely green with young leaves sometimes flushed pink. Petioles are 2.5–3.5 cm in length. The species is native to Peninsular Malaysia and Indonesia.

It is cultivated throughout the tropics (Larsen *et al.*, 1999). Inflorescences are large and attractive with showy bracts. Farms in Australia and Hawaii are cultivating the species and selling its inflorescences as cut flowers on a commercial basis (Ibrahim & Setyowati, 1999).

Young inflorescences of *E. elatior* are a compulsory ingredient in sour fish curry dishes in Peninsular Malaysia (Larsen *et al.*, 1999). It is also used in local rice dishes in the east coast. In Malaysia, a decoction of the fruit of *E. elatior* is used traditionally to treat earache while a decoction of leaves is applied for cleaning wounds (Ibrahim & Setyowati, 1999). Leaves are also used by post-partum women and mixed with other aromatic herbs in water for bathing to remove body odour.

Analysis of methanol and DCM extracts of *E. elatior* rhizomes by Habsah *et al.* (2001) using the FTC and TBA assays showed antioxidant activity that is higher than α-tocopherol. Aqueous ethanol extract from flower shoots of *E. elatior* was reported to possess antimicrobial and cytotoxicity activity against HeLa cells (Mackeen *et al.*, 1997). MIC values were 200 µg/ml against *P. aeruginosa*; 400 µg/ml against *B. megaterium.* Cytotoxic activity against the HeLa cells showed CD_{50} values in the range of 10-30 µg/ml.

Phytochemical studies on *E. elatior* rhizomes by Habsah *et al.* (2004a) resulted in the isolation of two new and six known compounds. The new compounds were identified as 1,7-bis(4-hydroxyphenyl)-2,4,6-heptatrienone and 16-hydroxylabda-8(17),11,13-trien-15,16-olide. The known compounds were 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. Using the FTC assay, antioxidant activity of 1,7-bis(4-hydroxyphenyl)-2,4,6-heptatrienone, demethoxycurcumin and 1,7-bis(4-hydroxyphenyl)-1,4,6-heptatrien-3-one showed stronger lipid peroxidation inhibition than α-tocopherol.

Wong *et al.* (1993) isolated the essential oils from the young flower shoots of *E. elatior*. Among the 49 compounds identified, major chemical classes were aliphatic alcohols and aldehydes, while terpenoid compounds constituted 18% of the oils. A similar study with inflorescences of *E. elatior* in the Amazon revealed that the main alcohol, aldehyde and terpenoid compounds to be dodecanol, dodecanal and α-pinene, respectively (Zoghbi & Andrade, 2005).

Composition of essential oils varied with different parts of *E. elatior* (Mohd Jaafar *et al*., 2007). Major components of leaves, stems, flowers and rhizomes were (*E*)-*β*-farnesene, *β*–pinene, 1,1-dodecanediol diacetate, cyclododecane and (*E*)-5-dodecane.

Koo and Suhaila (2001) conducted studies on the flavonoid content of 62 edible tropical plants. Inflorescences of *E. elatior* had flavonoid content of 307 mg/kg consisting of 286 and 21 mg/kg of kaempferol and quercetin, respectively. Flavonoids identified in the leaves of *E. elatior* are kaempferol 3-glucuronide, quercetin 3-glucuronide, quercetin 3-glucoside and quercetin 3-rhamnoside (Williams & Harborne, 1977).

Etlingera fulgens

Synonyms: *Hornstedia fulgens, Phaeomeria fulgens, Nicolaia fulgens* and *Etlingera hemisphaerica*

Etlingera fulgens (Ridl.) C.K. Lim can be recognised by its shiny undulate leaves that are dark green in colour (Lim, 2000; Khaw, 2001). When young, the underside leaves are bright red in colour, turning greenish on maturing. In older leaves, only the petiole and midrib are red (Khaw, 2001). Petioles are 1.5–2 cm in length. Rhizomes, 3 cm in diameter, occur just below the ground. The plant can grow up to 4–5 m tall. Crushed leaf sheaths emit a pleasant sour fragrance similar to that of *E. elatior*. Inflorescences are raised above the ground and

infructescences are globular in shape. Distributed in Southern Thailand and Peninsular Malaysia, the species occurs in the lowlands and at higher altitudes (Lim, 2000).

Because of its attractive red colour of young leaves and its blood red flowers, the plant is gaining popularity as an ornamental in landscape gardens (Lim, 2000). Fruit and rhizome oils of *E. fulgens* are mainly aliphatic hydrocarbons with cyclododecane, dedecanol and cyclotetradecane as main constituents (Chua *et al.*, 2005). The culinary or medicinal values of its leaves and rhizomes have yet to be studied.

Etlingera littoralis

Synonym: *Achasma megalocheilos*

Etlingera littoralis (Koenig) Giseke produces subterranean rhizomes up to 10 cm depth (Khaw, 2001). They are thick, 3–3.5 cm in diameter. The plant varies from 3–6 m tall. Crushed leaves do not have any scent. Leaves are green with young leaves sometimes flushed pink beneath. Petioles are 1.5–4.5 cm in length. Flowers are bright red entirely or with a yellow margin and occur at ground level. Lim (2001a) noted variations in the colour and shape of flowers of *E. littoralis*. They can range from entirely red or orange red with orange or yellow margins. The lips may be narrow and elongate or broad and spatulate.

The species is common in lowland forests throughout Peninsular Malaysia (Khaw, 2001) and found growing together with other *Etlingera* species (Lim, 2001a). It also occurs in Southern Thailand and Indonesia (Sirirugsa, 1999). In Sabah, several species of wild gingers are consumed by indigenous communities (Noweg *et al.*, 2003). Hearts of young shoots, inflorescences and fruits of *E. littoralis* are used as condiment, consumed raw or cooked as vegetable. In Thailand, the fruits are edible and the young stem, after removing the outer parts, yields an aromatic tender core that is eaten raw or cooked (Sirirugsa, 1999).

Using the FTC assay, methanol and DCM extracts of *E. littoralis* rhizomes showed higher antioxidant activity than α-tocopherol (Habsah *et al.*, 2001). Anti-tumour promoting activity using EBV activation in Raji cells showed that DCM extracts of *E. littoralis* at 20 μg/ml concentration had an excellent inhibition rate of 100%.

Etlingera maingayi

Synonyms: *Amomum maingayi, Hornstedtia maingayi, Phaeomeria maingayi* and *Nicolaia maingayi*

Etlingera maingayi (Bak.) Smith is usually not more than 2 m in height (Lim, 2000). Leaves are variable with undulate fringes. When crushed, leaves emit an unpleasant sour scent. Young leaves are translucent and reddish on both sides. The species occurs in Southern Thailand and Malaysia (Sirirugsa, 1999). In Peninsular Malaysia, it is widely distributed in lowland forests (Khaw, 2001). In Thailand, flowers are eaten as vegetables (Sirirugsa, 1999).

Etlingera rubrostriata

Synonym: *Etlingera metriocheilos* var. *rubrostriata*

Etlingera rubrostriata (Holtt.) C.K. Lim can easily be recognised by their oblique brown-red bars at the upper surface and green lower surface of leaves (Lim, 2001a). The leaves have a distinct dark red petiole of about 1 cm in length. The species is quite widespread and occurs in higher altitudes. Wild populations can be observed in the forests of Ulu Gombak, Selangor and Janda Baik, Pahang. Leaves have an aromatic fragrance when crushed. With striking oblique brown-red bars at the upper surface of leaves, the species has ornamental potentials.

2.2.5 *Hedychium*

The genus *Hedychium* J. Konig, of the tribe Hedychieae, occurs also throughout tropical and subtropical Asia including Malaysia, Indonesia and the Pacific Islands (Ibrahim, 2001b). Several species have been cultivated as ornamentals and some have become naturalised.

Hedychium coronarium

Synonym: *Hedychium spicatum*

Hedychium coronarium J. Konig or ginger lily is a stout herb of 1–2.5 m tall (Larsen *et al.*, 1999; Ibrahim, 2001b). Rhizomes are fleshy and strongly aromatic. Leaves are large and oblong to lanceolate in shape. Flowers are showy, fragrant and white or pale yellow in colour.

In Peninsular Malaysia, boiled leaves of *Hedychium* species are eaten for indigestion (Ibrahim, 2001b). Leaves are sometimes eaten with betel nut to ease abdominal pain. In Thailand, boiled leaves of *H. coronarium* are applied to relieve stiff and sore joints.

Diterpene constituents from the rhizomes of *H. coronarium* showed inhibitory effects on the increase in vascular permeability, nitric oxide production and nitric oxide synthase induction (Nakatani *et al.*, 1994). Methanolic extracts from the rhizomes were found to inhibit the increase in vascular permeability induced by acetic acid in mice and nitric oxide production in lipopolysaccharide activated mouse peritoneal macrophages (Masuda *et al.*, 2002).

From methanolic extract of rhizomes of *H. coronarium*, three new labdane-type diterpenes, together with six known diterpenes have been isolated (Nakatani *et al.*, 1994). Three new labdane diterpenes were isolated together with four known diterpenes from the rhizomes (Masuda *et al.*, 2002).

2.2.6 *Kaempferia*

The genus *Kaempferia* L. belongs to the tribe Hedychieae. *Kaempferia* species are small perennial herbs with short rhizome and tuberous roots (Larsen *et al*., 1999). Rhizomes and leaves are used for food flavouring and as traditional medicine. Leaves and rhizomes of *K. galanga* are used in traditional medicine, perfumery and food flavouring (Ibrahim, 1999). Rhizomes are used as expectorant and carminative. They are also used as ingredient for preparing 'Jamu', a local health tonic consumed by the Malays. Its mildly spicy leaves are ingredients for savoury dishes. Its leaves and rhizomes are eaten fresh or cooked as vegetable, and used in cosmetic powder and as food flavouring agent.

Kaempferia rotunda

Synonym: *Kaempferia longa*

Kaempferia rotunda L. is a small herb with erect leaves that have a distinctive dark red underside. Probably native to Indo-China, it is cultivated throughout tropical Asia mainly as ornamental and medicinal plants (Ibrahim, 1999).

Rhizomes of *K. rotunda* are used to treat abdominal and gastric pains (Ibrahim, 1999). Leaves and rhizomes are eaten fresh or cooked as vegetable, and used in cosmetic powder and as food flavouring agent.

Extracts from rhizomes of *K. rotunda* displayed insecticidal activity against neonate larvae of *S. littoralis* (Nugroho *et al*., 1996). Polyoxygenated cyclo-hexane derivative compounds in rhizomes had anti-feedant activity against larvae of *S. littoralis* (Stevenson *et al*., 2007).

The most abundant volatile constituents of rhizomes of *K. rotunda* were benzyl benzoate and *n*-pentadecane (Woerdenbag *et al*., 2004). Three new cyclo-hexane diepoxides together with crotepoxide and (-)-zeylenol, were isolated from rhizomes of *K. rotunda* (Pancharoen *et al*., 1996). Methanol extracts of rhizomes yielded six polyoxygenated cyclohexane derivatives (Stevenson *et al*., 2007).

2.2.7 *Scaphochlamys*

The genus *Scaphochlamys* Bak., of the tribe Hedychieae, comprises small forest plants with creeping thin rhizomes (Holttum, 1950; Larsen *et al.*, 1999). Plants in some species are supported by stilt roots above the ground. Leaves are few, elliptic and sometimes purplish beneath but more usually green. Inflorescences are terminal and bracts are spirally arranged. Flowers are white or creamy in colour, often with a median yellow band bordered with pink-purplish streaks or spots. The genus is polymorphic with about 20 species found in Peninsular Malaysia, many of which are local endemics.

Scaphochlamys kunstleri

Scaphochlamys kunstleri (Bak.) Holtt. produces fleshy rhizomes (Holttum, 1950). Leaves are large and ovate or oblong in shape with pinkish purple under-surface (Lim, 2001b). Leaf petioles are fleshy and deeply channelled. Flowers are yellow or red, or white with yellow and pink markings. This species appears to be common in some parts of Perak (Holttum, 1950). Leaves when crushed emit a mild aromatic fragrance. The plant, with its attractive leaves and flowers, is

ornamentally attractive (Lim, 2001b). The culinary or medicinal uses of its leaves and rhizomes have yet to be studied.

Rhizome oils of *S. kunstleri* have been identified (Chua *et al.*, 2005). Essential oils are rich in terpinolene (23%). Other oils found are p-cymene, linalool, (*Z*)-β-ocimene and terpinen-4-ol.

2.2.8 *Zingiber*

Zingiber G.R. Boehmer is the only genus belonging to the tribe Zingibereae. In Peninsular Malaysia, 19 species have been recorded (Larsen *et al.*, 1999). They are medium-sized forest plants ranging from 0.5–3.5 m tall with tuberous aromatic rhizomes (Theilade, 1996). The inflorescence stalk may be short or long. Inflorescences often have a cone-like appearance. Bracts are green when young, turning red in the fruiting stage. The flowers are usually very ephemeral, only lasting a few hours. The true ginger *Z. officinale* which is not native to Malaysia belongs to this genus. *Zingiber* species are found growing in damp primary, secondary and disturbed forest sites (Theilade, 1996). In Peninsular Malaysia, they occur mainly in lowland and hill forests with a few species in montane forests. Leaf flavonoids of *Zingiber* species include myricetin and quercetin glycosides (Williams & Harborne, 1977).

Zingiber officinale

Synonym: *Amomum officinale*

Zingiber officinale Roscoe is a slender perennial herb that grows up to 1.3 m in height (Sutarno *et al.*, 1999). Planted as an annual, the species produces fleshy rhizomes up to 2 cm thick and with pale yellow and aromatic flesh.

In Peninsular Malaysia, rhizomes of the true ginger *Z. officinale* are commonly used as food flavouring while young rhizomes are eaten raw or pickled (Larsen *et al.*, 1999). Many Chinese dishes are cooked with ginger rhizomes either in slices, strips or grated. The medicinal uses including properties of ginger have been reviewed by Remadevi *et al.* (2005).

Rhizomes of *Z. officinale* have many medicinal applications. They have been considered to have diaphoretic, stomachic, carminative and diuretic properties (Theilade, 1996). It has been prescribed for colds, coughs and congestion of the chest. It has also been used as a remedy for diarrhoea and dysentery. When crushed, the rhizome is applied on the head for headache. The juice of the rhizome has been used to treat migraine and to relieve menstrual cramps. Rhizomes of ginger have been used in traditional herbal medicine for the management of symptoms such as common cold, digestive disorders, rheumatism, neurologia, colic and motion sickness (Surh, 1999).

6-gingerol isolated from *Z. officinale* rhizomes possesses substantial antioxidant properties as determined by inhibition of phospholipid peroxidation and xanthine oxidase activity (Aeschbach *et al.*, 1994).

Antitumor promoter activity was demonstrated for petroleum ether, chloroform and ethanol extracts of *Z. officinale* (Vimala *et al.*, 1999), and for 6-gingerol and 6-paradol (Lee & Surh, 1998; Keum *et al.*, 2002). The cytotoxic and anti-proliferative effects of 6-gingerol and 6-paradol were associated with apoptotic cell death.

Murakami *et al.* (1995) screened the methanol extracts of 112 species of edible plants from Thailand for anti-tumour promoting activity using the inhibition test of EBV activation in Raji cells induced by TPA. It was found that young and mature

Z. officinale rhizomes showed moderately active and strongly active inhibitory effects ≥ 70% at 200 µg/ml concentration, respectively.

Ethanol extracts of *Z. officinale* exhibited potent antibacterial activity against Gram-positive and Gram-negative bacteria (Mascolo *et al.*, 1989). Treatment of ginger rhizomes with boiling water removed the antibacterial effect (Chrubasika *et al.*, 2005). This indicates that the antibacterial principle is heat labile. 10-gingerol and 6-gingerdiol had potent antifungal activity against 13 human pathogens at concentrations less than 1 mg/ml (Ficker *et al.*, 2003).

Jantan *et al.* (2003a) investigated the antifungal activities of rhizome oils of nine Zingiberaceae species using the broth microdilution and the disc gel diffusion methods. Essential oils of *Z. officinale* exhibited high activity against the yeasts with zone of inhibition ranging from 12–14 mm.

In *Z. officinale*, terpenoids were detected in leaves, stems and rhizomes, while alkaloids were detected in rhizomes and flavonoids in leaves (Zakaria & Ibrahim, 1986). Major essential oils found in the rhizomes are geranial, geranyl acetate, ar-curcumene and neral (Jantan *et al.*, 2003a).

Gong *et al.* (2004) reported that the main essential oils found in dried and fresh ginger rhizomes of *Z. officinale* are camphene (6.4 and 5.9%), *p*-cineole (6.0 and 5.9%), geranyl isobutyrate (4.9 and 3.8%) and zingiberene (4.2 and 4.1%), respectively. Results showed that most components of essential oils are similar for dried and fresh rhizomes.

The pungent principle of ginger rhizomes consists mainly of gingerols (Chrubasika *et al.* 2005). 6-gingerol, the main gingerol, is more pungent than 8-gingerol or 10-gingerol. Other gingerols include methylgingerol, gingerdiol, dehydrogingerdione, 10-dehydrogingerdione and gingerdiones.

The chemistry of ginger has been reviewed by Vernin and Parkanyi (2005). The review included extraction and analytical methods for non-volatile and volatile compounds. Properties, processing and formulations and uses of ginger were also described.

Zingiber ottensii

Zingiber ottensii Val. is a perennial herb with leafy shoots up to 1.5 m tall (Jansen, 1999). The species is distributed in Indonesia, Peninsular Malaysia and Thailand. Rhizomes are purplish inside and with a strong pungent smell. Leaves are elliptical with cream to yellow flowers. It is only known from cultivation and can easily be distinguished by its purplish rhizome flesh. It is a popular medicinal plant in home gardens.

In traditional medicine, rhizomes are pounded into a poultice and used by women after childbirth (Jansen, 1999). They are also consumed fresh or mixed with other plant species in the form of decoctions and tonics (Larsen *et al.*, 1999). Four new terpenoids and a diarylheptanoid were isolated together with 16 known compounds from rhizomes of *Z. ottensii* (Akiyama *et al*., 2006).

Zingiber spectabile

Zingiber spectabile Griff. or beehive ginger produces pale green leaves with a swollen base (Theilade, 1996). The species can be found throughout Peninsular Malaysia (Larsen *et al.*, 1999). Its leafy shoot is usually 2–3 m tall. The species produces large inflorescences up to 50 cm in height. The yellow bracts, which turn red when mature, form pouches of mucilage in which the flowers develop. Leaves when crushed emit an aromatic fragrance.

Only leaves of *Z. spectabile* are used in the preparation of traditional medicine (Theilade, 1996). Leaves are pounded into a paste and applied onto the body to bring down swellings. The infusion is also used to abate inflamed eyelids (Sirirugsa, 1999). The aborigines also use it to treat headache and backache (Wolff *et al.*, 1999). The Malays sometimes use its leaves for flavouring food (Theilade, 1996). The attractive and long-lasting inflorescences of the species are sold as cut flowers (Larsen *et al.*, 1999). It is also used in flavouring food but is mainly grown as an ornamental plant (Holttum, 1950).

Rhizomes of *Z. spectabile* were found to have cytotoxic activity on human U937 cell lines using the microtetrazolium (MTT) assay (Jiang *et al.*, 2006b). Main components of essential oils of *Z. spectabile* rhizomes are trans-d-bega motene, β-elemene, isobutyl benzoate, β-copaene and α-terpeneol (Ibrahim & Zakaria, 1987). Inflorescences of plants in the Amazon were found to be rich in β-phellandrene, α-pinene and β-pinene (Zoghbi & Andrade, 2005). Sirat *et al.* (1994) reported the presence of labda-8(17),12-diene-15,16-dial, a labdane diterpene, in the rhizomes.

2.3 FLAVONOIDS

Flavonoids are phenolic compounds of plants that are widely recognised as antioxidants with health-promoting properties in human diets. They also exhibit a wide spectrum of biological functions and play important roles in the interaction between plants and their environment (Koes *et al.* 1994, Pourcel *et al.* 2007). However, the antioxidant functions of flavonoids in plants remain unclear and are being debated by the scientific community (Hernandez *et al.* 2009).

Flavonoids are widely distributed in plants and they constitute the largest group of plant phenolics. More than 6,000 flavonoids have been identified (Harborne & Williams, 2000). They are important in contributing to the flavor and colour of many fruits, vegetables and plants products such as wine and tea (Croft, 1998;

Bravo, 1998). They occur in plants as glycosides although they occasionally occur as aglycones which lack the sugar moiety (Erlund, 2004). The glycosides are usually *O*-glycosides, with the sugar moiety bound to the hydroxyl group at the C-3 or C-7 position. They are potent antioxidants *in vitro* and are of particular interest in protection against diseases associated with oxidative stress. Epidemiological studies showed an inverse relationship between consumption of plant foods rich in flavonoids and incidence of certain diseases (Bravo & Mateos, 2008).

2.3.1 Chemistry

Flavonoids are the largest class of polyphenols. They are 15-carbon compounds with a C6-C3-C6 carbon skeleton (Croft, 1998; Ross & Kasum, 2002). Sometimes referred to as the phenylbenzopyran functionality (Marais *et al.*, 2006), the structure consists of two benzene rings (A and B), which are connected by an oxygen-containing pyrene ring (C) [Fig. 2.4].

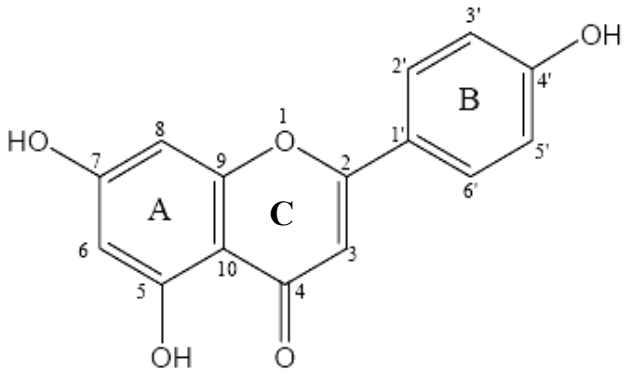


Fig. 2.4
Generic structure of the flavonoid molecule (Vermerris & Nicholson, 2006a)

Flavonoids containing a hydroxyl group in position C-3 of the C-ring are classified as 3-hydroxyflavonoids (flavonols, anthocyanidins and catechins), and those lacking the hydroxyl group are classified as 3-deoxyflavonoids (flavanones and

flavones) (Erlund, 2004). The term 4-oxo-flavonoids is often used to describe flavonoids as they carry a carbonyl group on C-4 of ring C (Aherne & O'Brien, 2002).

The chemical nature of flavonoids depends on structural class, degree of hydroxylation, other substitutions and conjugations, and degree of polymerization (Ross & Kasum, 2002). Flavonoids can be further subdivided into six major sub-classes which include flavones, flavonols, flavanones, catechins, anthocyanidins and isoflavones [Fig. 2.5]. Types and substitution patterns of flavonoids are shown in Table 2.3.

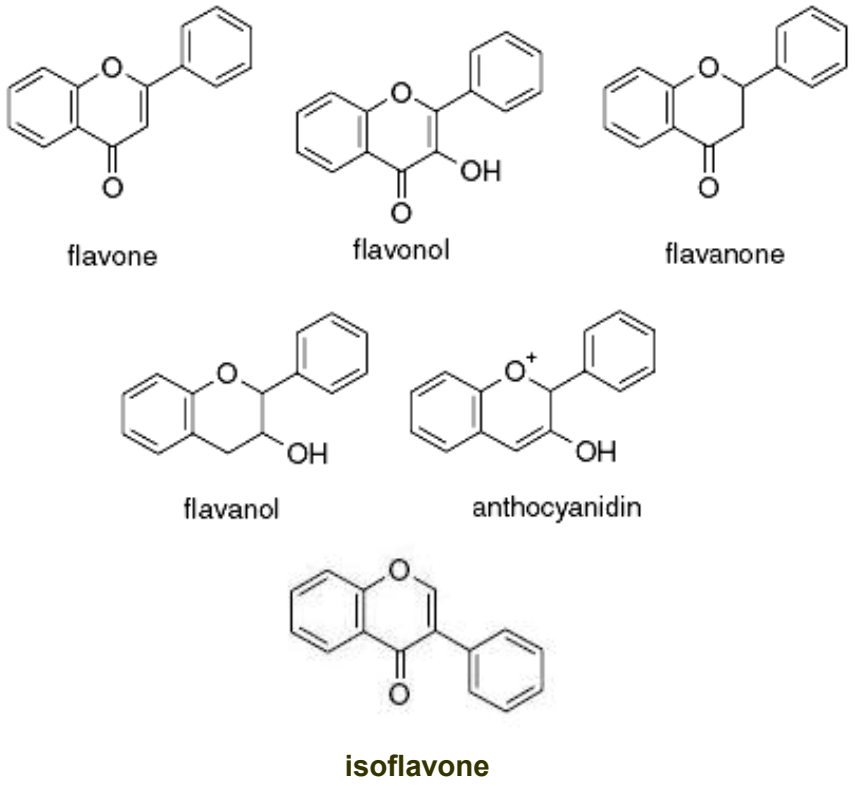


Fig. 2.5
Generic structures of the different sub-classes of flavonoids (Balasundram *et al*., 2006)

Table 2.3
Structure and hydroxylation pattern of various sub-classes of flavonoids (Formica & Regelson, 1995; Cook & Samman, 1996).

Structure	Flavonoid	Substitution pattern					
		3'	4'	5'	3	5	7
Flavone	Luteolin	OH	OH			OH	OH
	Apigenin		OH			OH	OH
Flavonol	Quercetin	OH	OH		OH	OH	OH
	Quercetrin	OH	OH		Rh	OH	OH
	Isoquercetrin	OH	OH		Gl	OH	OH
	Myricetin	OH	OH	OH	OH	OH	OH
	Fisetin	OH	OH		OH		OH
	Kaempferol		OH		OH	OH	OH
	Rutin	OH	OH		Ru	OH	
Flavanone	Taxifolin	OH	OH		OH	OH	OH
	Naringenin		OH			OH	OH
Flavanol	Catechins	OH	OH		OH	OH	OH
Anthocyanidin	Cyanidin	OH	OH		OH	OH	OH
	Delphinidin	OH	OH	OH	OH	OH	OH
	Malvidin	Me	OH	Me	OH	OH	OH
Isoflavone	Genistein		OH			OH	OH
	Diadzein		OH				OH

OH: Hydroxyl; Me: Methoxy; Rh: Rhamnose; Gl: Glucoside; Ru: Rutinose

Flavonols

Flavonols are characterized by a non-saturated 3-C chain, a double bond between C-2 and C-3, and a hydroxyl group in the 3-position. Flavonols occur mainly as *O*-glycosides (Pietta *et al*., 2003). They are commonly found in leaves with quercetin and kaempferol the most common ones. Quercetin and its glycoside are ubiquitous in fruits and vegetables.

Flavones

Flavones are characterized by a non-saturated 3-C chain, a double bond between C-2 and C-3, and an absence of the hydroxyl group in the 3-position. They are widely distributed among higher plants in the form of aglycones or glycosides. The most common flavones are luteolin, apigenin and diosmetin (Pietta *et al*., 2003).

Flavanones

Flavanones arise from flavones after reduction of the double bond in the heterocycle (position C2-C3) (Pietta *et al*., 2003). Among aglycones, the best known are naringenin and hesperidin. Citrus fruits are a major dietary source of flavanones.

Flavanols

Flavanols or catechins are a major sub-class of flavonoids. Also called proanthocyanidins or flavan-3-ols, they are characterized by a lack of the double bond at the 2-3 position and of the 4-oxo-group (Pietta *et al*., 2003; Erlund, 2004; Aron & Kennedy, 2008). They lack the carbonyl group on C-4. Major flavanols are (+)-catechin, (-)-epicatechin, epicatechin gallate (ECG) and epigallocatechin-3-gallate (EGCG). Widely distributed in plants, they are found in wine, tea, fruits and vegetables (Yilmaz, 2006; Yao *et al*., 2004a). Red wine, teas (green, black and oolong) fruits (such as plum, apple, peach, strawberry and cherry), and vegetables (broad bean and lentil) are rich in catechins. Gallates of epicatechin, epigallocatechin (EGC), ECG and EGCG are the major catechins in green, oolong and black tea. Red wines has much higher flavonoid, anthocyanin and catechin content than white wine (Katalinic *et al*., 2004).

Anthocyanidins

Anthocyanidins are characterized by hydroxylation in positions 3, 5, and 7 in the A ring and 3' and 5' in the B ring (Pietta *et al*., 2003; Erlund, 2004). Methoxyl groups can be attached to 3' and 5'. They lack the carbonyl group on C-4. Anthocyanidins commonly found in plants are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin. Anthocyanidins are found in berries, flowers and wines are rich in anthocyanidins (Yao *et al*., 2004a).

Isoflavones

Isoflavones are structurally different from the other sub-classes by having the 3-phenylchroman skeleton i.e. the B-ring is bound to carbon 3 of C-ring instead of carbon 2 (Erlund, 2004; Marais *et al.*, 2006). They are present in plant foods either as aglycones (genistein or daidzein) or as glycosides (Pietta *et al*., 2003). They are almost exclusively found in soy and its food products (Yao *et al*., 2004a).

2.3.2 Health benefits

There is considerable evidence suggesting that plant flavonoids may be health-promoting and disease-preventing dietary compounds (Middleton, 1996). Of current interest is the presence of potent antioxidant flavonoids in red wine, the cause of the so-called French paradox (Sun *et al*., 2002). Despite intake of a high-fat diet, the low incidence of coronary heart disease among the French people has been attributed to the consumption of red wine with their meals.

Flavonoids have been shown in both *in vitro* and *in vivo* experimental systems over several decades to possess anti-allergic, anti-inflammatory, antiviral, anti-cancer and anti-carcinogenic properties (Middleton, 1996; Ren *et al*., 2003).

The role of dietary flavonoids in cancer prevention has been widely discussed (Yao *et al*., 2004a). There is growing evidence that flavonoids have important effects in inhibiting carcinogenesis. Several epidemiological studies suggest that a high consumption of flavonoids is inversely related to the risk of cardiovascular diseases (Cos *et al.*, 2000).

Epidemiologic studies seem to indicate that drinking either green or black tea lowers blood cholesterol concentrations and blood pressure, thereby providing some protection against cardiovascular disease (Craig, 1999). Consuming high levels of flavonoids lowers mortality rate from heart diseases by up to 60% and lowers the risk of stroke by up to 70%. The Zutphen study of elderly men in the Netherlands conducted over a period of five years found that flavonoid intake from fruits, vegetables and tea was inversely associated with heart disease mortality and incidence of heart attack and stroke.

Knekt *et al.* (2002) conducted an epidemiological study on the effects of specific flavonoids against several chronic diseases. Results showed that quercetin intake had lower mortality from ischaemic heart disease while the occurrence of cerebrovascular disease was lower by high kaempferol, naringenin and hesperetin intake. Quercetin also lowers the risk of lung cancer in men while myricetin lowers the risk of prostrate cancer. Asthma incidence was inversely correlated with quercetin, naringenin and hesperetin intakes. A trend toward a reduction in risk of type-2 diabetes was also observed with higher quercetin and myricetin intakes.

Studies on cancer prevention demonstrated that flavonoids inhibit carcinogenesis *in vitro* and *in vivo* (Caltagirone *et al*., 2000). Flavonoids may inhibit carcinogenesis by affecting the molecular events during the initiation, promotion and progression stages. Animal studies and investigations using different cellular models suggested that certain flavonoids could inhibit tumor initiation as well as tumor progression (Makita *et al.*, 1996; Tanaka *et al.*, 1997 & 1999). Certain

flavonoids exert anticarcinogenic activity by induction of enzymes affecting carcinogen metabolism, by adduct formation between carcinogens and DNA, and by inhibiting *in vivo* carcinogenesis (Middleton, 1996).

Tea polyphenols exhibit a very broad spectrum of biological activities (Shahidi & Naczk, 2004a). Increased consumption of green tea is responsible for lowering the risk for breast cancer and recurrence among Japanese women (Imai *et al*., 1997; Nakachi *et al.*, 1998). Epigallocatechin-3-gallate (EGCG) in green tea has been found to reduce the incidence of spontaneously and chemically induced tumors in experimental animals (Huang *et al*., 1992).

2.3.3 Biosynthesis

Flavonoids are polyphenolic compounds formed as secondary plant metabolites. They are derived from two biosynthetic pathways, namely, the shikimate and the acetate pathways (Bravo & Mateos, 2008). Shikimic acid, formed from carbo-hydrates in the Calvin cycle, gives rise to phenylpropanoids (C6-C3 compounds), that combine with simple phenols such as resorcinol or phloroglucinol (C6) originated in the acetate pathway resulting in the formation of chalcones and ultimately flavonoids.

The flavonoid biosynthesis pathway [Fig. 2.6] has been often described in the literature (e.g. Shirley, 1996; Weaver & Herrmann, 1997; Dixon & Steele, 1999; Vermerris & Nicholson, 2006b).

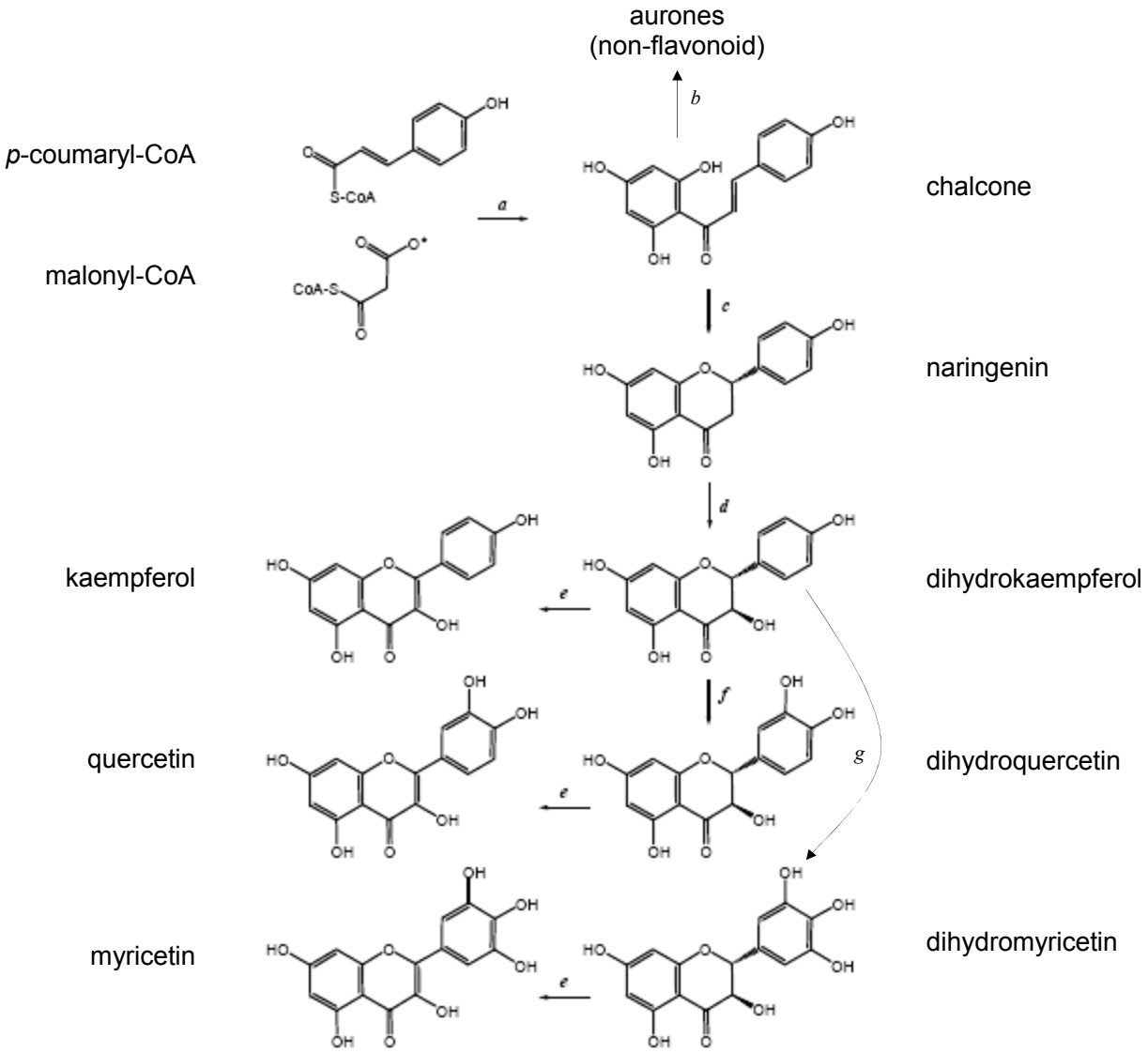


Fig. 2.6
The flavonoid biosynthesis pathway (Vermerris & Nicholson, 2006b). Chalcone synthase CHS (*a*), chalcone isomerase CHI (*c*), flavanone 3-hydroxylase F3H (*d*), flavone synthase FLS (*e*), flavonoid 3'-hydroxylase F3'H (*f*) and flavonoid 3'5'-hydroxylase (*g*) are enzymes involved. Aureusidin synthase (*b*) is responsible for synthesis of aurones (non-flavonoid).

Flavonoid biosynthesis is initiated from the condensation of *p*-coumaroyl-CoA with three molecules of malonyl-CoA, which is catalyzed by the enzyme chalcone synthase (CHS) to give rise to 4,2',4',6' tetrahydroxychalcone (Delgado-Vargas & Paredes-Lopez, 2003; Vermerris & Nicholson, 2006b). From 4,2',4',6' tetra-hydroxychalcone, formation of the six-member ring is catalyzed by chalcone isomerase (CHI) resulting in the formation of naringenin. Naringenin is then converted by flavanone 3-hydroxylase (F3H) to yield dihydrokaempferol. This compound can be converted by flavone synthase (FLS) to kaempferol. Alternatively, the B-ring of dihydrokaempferol can be substituted with additional hydroxyl groups by flavonoid 3'-hydroxylase (F3'H) or flavonoid 3',5'-hydroxylase (F3'5'H) to produce dihydroquercetin and dihydromyricetin, respectively. These two compounds can subsequently be converted by FLS to their corresponding flavones, quercetin and myricetin.

2.3.4 Antioxidant activity

The antioxidant mechanisms of flavonoids can be categorized as indirect and direct (Disilvestro, 2001). Indirect mechanisms include down-regulation of the production of radicals e.g. superoxide; elimination of radical precursors e.g. hydrogen peroxide; metal chelation; inhibition of pro-oxidant enzymes e.g. xanthine oxidase; and elevation of endogenous antioxidants e.g. superoxide dismutase which eliminates superoxide radicals inside cells.

Direct radical scavenging could involve more than one type of reaction. An example is radical mediated oxidation of membrane lipids which involves three different stages (Cook & Samman, 1996):

1. Initiation (free radicals remove hydrogen from a polyunsaturated fatty acid to form a lipid radical)
2. Propagation (lipid radical plus molecular oxygen forms lipid peroxy radical which then breaks down to more radicals)
3. Termination (new radicals react together or with antioxidants to eliminate radicals)

The antioxidant activity of flavonoids has been considered to be *via* two possible modes of action, free radical scavenging and metal chelating (Sugihara *et al.* 1999). Free radical scavengers are compounds that can neutralize free radicals to decrease their ability to cause oxidation (Decker, 2004). Metal chelators are compounds that can bind metals and decrease their reactivity in Fenton reaction.

Main structural features of flavonoids required for efficient radical scavenging [Fig. 2.7] have been summarised by Croft (1999) as:

a) An ortho-dihydroxy (catechol) structure in the B-ring for electron delocalisation
b) 2,3 double bond in conjugation with a 4-keto function, provides electron delocalisation from the B-ring
c) Hydroxyl groups at positions 3 and 5, provide hydrogen bonding to the keto group

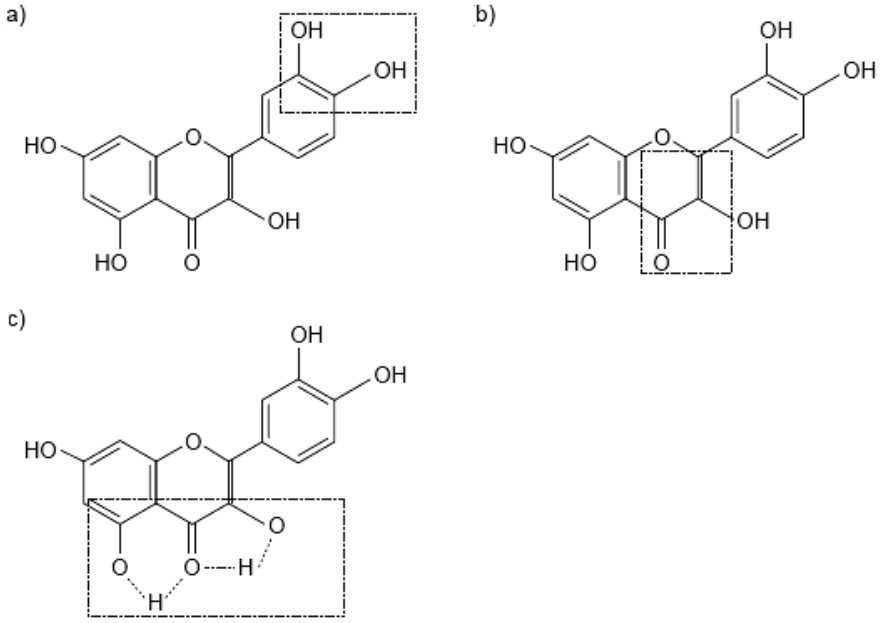


Fig. 2.7
Structural features responsible for radical scavenging properties of flavonoids (Croft, 1999)

During direct scavenging of free radicals, flavonoids stabilise the reactive oxygen species by reacting with the reactive compounds (Nijveldt *et al.*, 2001). Because of the high reactivity of the hydroxyl group of flavonoids, radicals are made inactive. The scavenging activity of flavonoids increases with the number of hydroxyl groups in rings A and B (Husain *et al.*, 1987; Frankel, 1999).

Flavonoids can act as effective chelators because of their carbonyl and hydroxyl groups (Pietta, 2000). The three structural components responsible for the metal chelating ability of flavonoids are the 3',4'-*O*-dihydroxycatechol on the B-ring; the 3-hydroxyl in conjugation with a 4-oxo function on the C-ring; and the 5-hydroxyl on the A-ring (Frankel, 1999; Sugihara *et al.*, 1999). The most efficient chelators are flavonoids with multiple hydroxyl or carbonyl group located in close proximity and thus chelating ability is related to the number of substitutions.

Mira *et al.* (2002) pointed out that flavonoids showed higher reducing capacity for copper ions than for iron ions. The copper reducing activity appeared to depend largely on the number of hydroxyl groups. Flavonoids with better Fe (III) reducing activity are those with a 2,3-double bond and those with both the catechol group on the B-ring and the 3-hydroxyl group.

Another antioxidant mechanism applicable to flavonoids is quenching of singlet oxygen (Cos *et al*., 2000). Flavonoids can interact with $^1O_2$ in two different ways. They can combine chemically with $^1O_2$ or they can transfer the excitation energy to the flavonoid molecule, which then enters an excited state.

Studies have shown that the scavenging ability of flavonoids, when complexed with metal ions, is significantly higher than that of parent flavonoids. Afanas'ev *et al.* (2001) suggested that chelation by flavonoids inhibited the pro-oxidant activity of metals whilst enhancing their antioxidant activity. Kostyuk *et al.* (2004) reported that flavonoids bound to metal ions were much less subjected to

oxidation. These flavonoid-metal complexes scavenge superoxide radicals by mimicking superoxide dismutase activity i.e. oxidising superoxide to oxygen or reducing it to hydrogen peroxide.

Flavonoids can have pro-oxidant effects (Scalbert *et al*., 2005). They can reduce metal ions and the reduced form can generate hydroxyl radicals through the Fenton reaction. In the presence of metal ions, flavonoids can act as pro-oxidants. For example, at 100 μM, flavonols (quercetin and myricetin) greatly accelerated the generation of hydroxyl radicals from hydrogen peroxide in the presence of Fe^{3+}-EDTA at pH 7.4 (Laughton *et al*., 1989).

In general, the more hydroxyl substitutions, the stronger is the pro-oxidant effect (Hanasaki *et al.* 1994; Cao *et al.* 1997). The ability to generate hydroxyl radicals is of the order: myricetin > quercetin > catechin > morin > kaempferol (Puppo, 1992). Flavones do not have such ability. It is important to note that a compound can be an antioxidant in one system but becomes a pro-oxidant in another system (Cos *et al*., 2000). However, such pro-oxidant effects have never been demonstrated *in vivo* (Scalbert *et al*., 2005). This pro-oxidant mechanism may not be significant *in vivo* as metal ions are largely sequestered.

Some flavonoids are able to interact with and inhibit the functions of proteins (Pietta, 2000). Flavonoids inhibit certain enzymes that produce superoxide radicals, such as xanthine oxidase and protein kinase C. Flavonoids are also known to inhibit cyclooxygenase, lipoxygenase, microsomal monooxygenase, glutathione S-transferase, mitochondrial succinoxidase and NADH oxidase, which are involved in the generation of reactive oxygen species. Such protein-flavonoid interactions have therapeutic implications e.g. the inhibition of cyclooxygenase and lipoxygenase has anti-inflammatory effects (Ong & Khoo, 1997).

2.3.5 Other bioactivities

Flavonols and flavones exhibited inhibitory activity against methicillin-resistant *S. aureus* (MRSA) (Xu & Lee, 2001). Myricetin inhibited the growth of multi-drug resistant *Burkholderia cepacia*, vancomycin-resistant enterococci and other medically important organisms such as *K. pneumoniae* and *S. epidermidis*.

Kim and Uyama (2005) reviewed the flavonoids with tyrosinase inhibition properties. They include flavanols, flavonols and flavones. Some flavonols possessing a 3-hydroxy-4-keto moiety, such as kaempferol and quercetin, competitively inhibit tyrosinase activity by chelating with copper in the enzyme and then irreversibly inactivate tyrosinase (Kubo *et al.*, 2000). Flavonoids with the hydroxyl group in the B-ring are more potent inhibitors than those without the hydroxyl group (Xie *et al.*, 2003).

Antiviral properties have been demonstrated in selected flavonoids (Kaul *et al.*, 1985; Middleton, 1996). Quercetin showed both anti-infective and anti-replicative activity. Hesperetin (a flavanone) showed anti-replicative activity while catechin showed anti-infective activity. Naringin (a flavanone glycoside) exhibited neither of these activities.

Flavonoids have been reported to have anti-allergic activity (Middleton, 1996). They can inhibit the stimulated release of pro-inflammatory mast cell, basophil and eosinophil granular constituents involved in the pathogenesis of diseases such as asthma. Quercetin is an active inhibitor but not taxifolin.

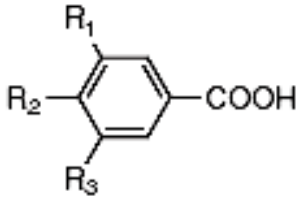
2.4 PHENOLIC ACIDS

2.4.1 Chemistry

Phenolic acids are aromatic phenols with one carboxylic acid functionality (Robbins, 2003; Stalikas, 2007). They consist of two groups, namely, derivatives of hydroxybenzoic and hydroxycinnamic acids (Fleuriet & Macheix, 2003; Lafay & Gil-Izquierdo, 2008; Mattila & Hellstrom, 2007). They differ in the pattern of hydroxylation and methoxylation of their aromatic rings (Murkovic, 2003; Mattila & Hellstrom, 2007).

Hydroxybenzoic acids

Hydroxybenzoic acids (HBA) with a C6-C1 structure [Fig. 2.8] are derived from benzoic acid. Major HBA are *p*-hydroxybenzoic, protocatechuic, vanillic, syringic and gallic acids, and are mainly present in plant food in the form of glucosides (Fleuriet & Macheix, 2003; Shahidi & Naczk, 2004b; Mattila & Hellstrom, 2007).



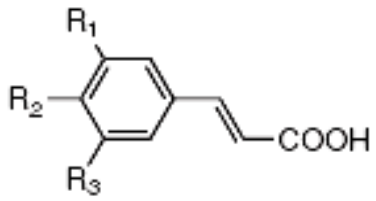
Benzoic acid	IUPAC name	R_1	R_2	R_3
p-Hydroxybenzoic	4-hydroxybenzoic	H	OH	H
Protocatechuic	3,4-dihydroxybenzoic	OH	OH	H
Vanillic	4-hydroxy-3-methoxybenzoic	OCH_3	OH	H
Syringic	3,5-dimethoxybenzoic	OCH_3	OH	OCH_3
Gallic	3,4,5-trihydroxybenzoin	OH	OH	OH

Fig. 2.8
Hydroxybenzoic acids commonly found in food and nutraceuticals (Shahidi & Naczk, 2004b)

The content of HBA is generally low in most food except for some berries such as blackberry, blackcurrant, raspberry and strawberry, and vegetables such as onions and horseradish (Murkovic, 2003; Lafay & Gil-Izquierdo, 2007). Because HBA are found in only a few plants eaten by humans, they have not been extensively studied and are not considered to be of great nutritional interest (Manach *et al.*, 2004).

Hydroxycinnamic acids

Hydroxycinnamic acids (HCA) with a C6-C3 structure [Fig. 2.9] are ubiquitous in plants. Among phenolics of fruits and vegetables, HCA play important roles due to their abundance and diversity (Fleuriet & Macheix, 2003). Derivatives of HCA commonly found in fruits, vegetables, grains and coffee are *p*-coumaric acid, caffeic acid, ferulic acid and sinapic acid (Rechner, 2003). Caffeic acid is the most common HCA in fruits while ferulic acid is most abundant in cereal grains (Lafay & Gil-Izquierdo, 2008). HCA frequently occur in foods as simple esters with quinic acid or glucose (Mattila & Hellstrom, 2007). The most extensively occurring HCA derivatives are esters of caffeic acid with quinic acid or caffeoylquinic acids, predominantly chlorogenic acid (IUPAC name: 5-caffeoyl-quinic acid) (Rechner, 2003; Lafay & Gil-Izquierdo, 2008). It is the main constituent in coffee, apple juice, artichoke, egg plant and peach (Rechner, 2003). Neochlorogenic acid (3-caffeoylquinic acid) is a major component of fruits e.g. cherry, plum, elderberry and apricot. Crytochlorogenic acid (4-caffeoylquinic) acid together with various dicaffeoylquinic acids are minor constituents of some fruits and vegetables. The absorption, metabolism, and bioactivities of caffeoyl-quinic acids have been reviewed by Morishita and Ohnishi (2001).



Cinnamic acid	IUPAC name	R_1	R_2	R_3
p-Coumaric	4-hydroxycinnamic	H	OH	H
Caffeic	3,4-dihydroxycinnamic	OH	OH	H
Ferulic	4-hydroxy-3-methoxycinnamic	OCH_3	OH	H
Sinapic	4-hydroxy-3,5-dimethoxycinnamic	OCH_3	OH	OCH_3

Fig. 2.9
Hydroxycinnamic acids commonly found in food and nutraceuticals (Shahidi & Naczk, 2004b)

2.4.2 Health benefits

Phenolic acids have attracted considerable interest in recent years due to their many potential health benefits (Mattila & Hellstrom, 2007). There is convincing evidence linking high intake of fruits and vegetables to reduced risk of chronic disease. The potential health effects of ferulic and caffeic acids have been demonstrated in many animal models and *in vitro* assays (Clifford, 2000).

The bioavailability of phenolic acids has been reviewed (Manach *et al.*, 2004; Lafay & Gil-Izquierdo, 2008). They are the main polyphenols in the human diet. Even as aglycones, they are generally absorbed in the upper part of the gastro-intestinal tract. It has been shown that the stomach constitutes an active absorption site of phenolic acids including gallic, caffeic, ferulic, coumaric and chlorogenic acids. The bioavailability of phenolic acids includes direct absorption and metabolism from food consumption. Their bioavailability is enhanced after cleavage of the main skeleton ring of flavonoids to phenolic acids by the gut microflora.

2.4.3 Biosynthesis

Phenolic acids in plants such as benzoic and cinnamic acid derivatives are synthesized through the shikimic acid pathway [Fig. 2.10] (Hakkinen, 2000; Shahidi & Naczk, 2004b; Vermerris & Nicholson, 2006b).

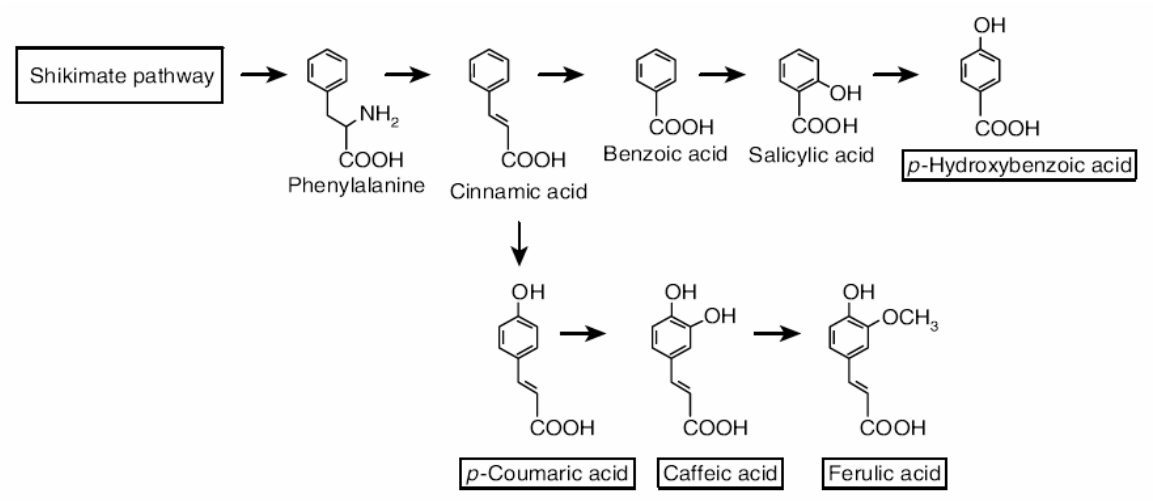


Fig. 2.10
Biosynthesis pathway of phenolic acids (Wojciak-Kosior & Oniszczuk, 2008)

In the biosynthesis of hydroxybenzoic acids, phenylalanine is converted to cinnamic acid by the enzyme phenylalanine ammonia lyase (PAL). Cinnamic acid is converted to benzoic acid probably through a process similar to β-oxidation of fatty acids. The hydroxylation of benzoic acid, catalyzed by the enzyme benzoic acid 2-hydroxylase, results in salicylic acid. Knowledge of mechanisms and enzymes involved in the biosynthesis of hydroxybenzoic acids and their derivatives is limited. Hydroxylation and possibly methylation of hydroxybenzoic acid lead to the formation of dihydroxybenzoic acid (protocatechuic acid), vanillic acid, syringic acid and gallic acid.

In the biosynthesis of hydroxycinnamic acids, hydroxylation and methylation of cinnamic acid lead to the formation of *p*-coumaric acid. The introduction of a second hydroxyl group into *p*-coumaric acid gives caffeic acid. Methylation of caffeic acid leads to the formation of ferulic acid.

2.4.4 Antoxidant activity

In many fruits and vegetables, HCA conjugates act as free radical scavengers and show strong antioxidant properties (Rice-Evans *et al.*, 1996; Chen & Ho, 1997; Natella *et al*., 1999; Kroon & Williamson, 1999). The relationships between their chemical structures and their antioxidant and free radical scavenging activity have been reported. The antioxidant activity of phenolic acids e.g. ferulic and caffeic acids based on TEAC and ABTS assays is less than flavonoids e.g. quercetin and catechin (Rice-Evans *et al*., 1997).

The antioxidant activity of phenolic acids and their esters depends on the number and position of hydroxy groups in the molecule (Shahidi & Naczk, 2004c; Andjelkovic *et al*., 2006). Hydroxycinnamic acids have been found to be more effective than their benzoic acid counterparts. The higher antioxidant activity of the hydroxycinnamic acid could be due to the CH=CH–COOH group, which ensures greater H-donating ability and radical stabilisation than the –COOH group in the hydroxybenzoic acids (Rice-Evans *et al*., 1996).

Caffeic acid and related hydroxycinnamates inhibit radical-mediated process at the following levels (Laranjinha, 2001):

1) Preventing initiating process through scavenging of •OH, ONOO− and ferryl myoglobin through reactions involving electron transfer and possibly by chelating transition metals
2) Breaking peroxidative chain reactions by stabilizing peroxyl radicals in the form of peroxides through H atom donation
3) Regenerating *α*-tocopherol by reducing *α*-tocopheroxyl radical

2.4.5 Other bioactivities

Phenolic acids such as cinnamic acid, 3-coumaric acid, caffeic acid and ferulic acid showed weak antibacterial activity against Gram-negative bacteria (Puupponen-Pimia *et al.*, 2005). The antimicrobial properties of phenolic acids might be derived from oxidation or hydrolysis (Fleuriet & Macheix, 2003). Phenolic acids are known to exhibit antimicrobial activity against a variety of microorganisms. Certain naturally occurring phenolic compounds including chlorogenic and gallic acids possess antimicrobial properties against human pathogens such as *E. coli* and *Campylobacter jejuni* (Friedman & Jurgens, 2000).

Kampa *et al.* (2004) reported the antiproliferative and apoptotic effects of phenolic acids (caffeic acid, syringic acid, sinapic acid, protocatechuic acid, ferulic acid and 3,4-dihydroxyphenylacetic acid) on T47D human breast cancer cells.

2.5 BIOACTIVITIES

2.5.1 Antioxidant activity

The oxygen paradox

All aerobic life encounters the oxygen paradox. Oxidative metabolism is essential for the survival of cells. A side effect of this dependence is the production of free radicals and other reactive oxygen species (ROS) that cause oxidative damage. Under normal conditions, the toxicity of ROS is kept in check by a highly regulated antioxidant defense system (Astley, 2003). When the rate of ROS production overwhelms antioxidant defense mechanisms, oxidative damage can lead to the onset of many chronic and degenerative diseases in humans (Antolovich *et al.*, 2002). Oxidation can also affect foods, where it is one of the major causes of chemical spoilage, resulting in rancidity and/or deterioration of food quality (Shahidi, 2000).

Antioxidants and free radicals

An antioxidant has been defined as "any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate" (Halliwell, 1995). Antioxidants are compounds that inhibit or delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions initiated by free radicals (Velioglu *et al.*, 1998). They can therefore prevent biological and chemical substances from oxidative damage induced by free radicals.

Free radicals are unstable, highly reactive and energized molecules due to the presence of one or more unpaired electrons (Kaur & Kapoor, 2001; Boots *et al.*, 2008). They react quickly with other compounds capturing their electrons needed to gain stability. They attack the nearest stable molecules. Molecules without unpaired electrons themselves become free radicals and the beginning of a chain reaction. A free radical can either obtain an electron by removing it from another molecule; or it can bind itself to another molecule forming adduct; or it can also be donated with a hydrogen atom to stabilize the delocalization of the radical (Andjelkovic *et al.*, 2006).

Examples of free radicals include reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are products of normal aerobic metabolic processes (Cao & Prior, 2002). ROS include superoxide (O_2^-), hydroxyl ($OH^\bullet$), peroxyl ($ROO^\bullet$) radicals and hydrogen peroxide (H_2O_2). RNS include nitric oxide ($NO^\bullet$) and nitrogen dioxide ($NO^\bullet_2$). There is considerable evidence that ROS and RNS can be damaging to cells leading to cellular dysfunction and diseases. Examples of typical ROS and RNS are listed in Table 2.4.

Table 2.4
Typical reactive oxygen and nitrogen species (Boots *et al*., 2008)

Radical	Non-radical
Reactive oxygen species (ROS)	
Superoxide, O_2^-	Hydrogen peroxide, H_2O_2
Hydroxyl, $HO^\bullet$	Hypochlorous acid, HOCl
Peroxyl, $RO^\bullet_2$	Ozone, O_3
Alkoxyl, $RO^\bullet$	Singlet oxygen, $^1O_2$
Reactive nitrogen species (RNS)	
Nitric oxide, $NO^\bullet$	Nitrous acid, HNO_2
Nitrogen dioxide, $NO^\bullet_2$	Nitrosyl cation, NO^+
	Nitroxyl anion, NO^-
	Peroxynitrite, $ONOO^-$
	Alkyl peroxynitrites, ROONO

Natural antioxidants are divided into exogenous and endogenous antioxidants (Percival, 1996). Exogenous antioxidants include vitamin C, vitamin E and polyphenols. Endogenous antioxidants are formed in the body to relieve oxidative stress. They include antioxidant enzymes and metal binding proteins. There are three classes of natural antioxidants (Afzal & Armstrong, 2002). They are water-soluble antioxidants, fat-soluble antioxidants and antioxidant metals.

Antioxidants can also be divided into primary and secondary antioxidants, depending on their modes of action (Antolovich *et al.*, 2002). Secondary or preventative antioxidants are compounds that retard the rate of oxidation. This may be achieved in a number of ways including removal of substrate or singlet oxygen quenching. Primary or chain-breaking antioxidants may either delay or inhibit the initiation step by reacting with a lipid radical or inhibit the propagation step by reacting with peroxyl or alkoxyl radicals.

At the cellular level, antioxidants that react directly with radicals or other reactive species can be subdivided into enzymatic and non-enzymatic antioxidants (Boots *et al.*, 2008). Enzymatic antioxidants, functioning as catalysts, react with reactive species and are subsequently recycled. Important antioxidant enzymes are superoxide dismutases, catalase and glutathione peroxidases [Table 2.5]. Non-enzymatic antioxidants can be divided into hydrophilic and hydrophobic anti-oxidants. Hydrophilic antioxidants include glutathione, ascorbate and uric acid. Hydrophobic antioxidants include α-tocopherol (vitamin E), carotenoids, and ubiquinol-10 and are mostly present in lipoproteins and membranes.

Antioxidants in foods are of interest for several reasons (Halliwell, 2002). Endogenous or added, they protect food against oxidative damage. Dietary antioxidants may be absorbed into the human body with beneficial effects. There is considerable interest in screening the antioxidant properties of plants for therapeutic uses.

Table 2.5
Enzymatic and non-enzymatic antioxidants (Boots *et al.*, 2008)

Enzymatic antioxidant	Reaction
Enzyme	$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$
Superoxide dismutases	$2H_2O_2 \rightarrow O_2 + 2H_2O$
Catalases	$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$
Glutathione peroxidases	$2\ GSH + ROOH \rightarrow GSSG + ROH + H_2O$
Non-enzymatic antioxidant	
Hydrophilic	*Hydrophobic*
Glutathione	α-tocopherol (vitamin E)
Ascorbate (vitamin C)	Carotenoids
Uric acid	Ubiquinol-10

Protective mechanisms

The protective mechanisms of antioxidants occur at various levels of defense (Shi *et al.*, 2001; Zhang *et al*., 2006a). At the first level, preventive antioxidants inhibit the formation of ROS and free radicals by chelating metal ions, reducing hydrogen peroxide, and quenching superoxide and singlet oxygen. At the second level, chain-breaking antioxidants function by scavenging free radicals, inhibiting chain initiation or breaking chain propagation. In addition, some antioxidants exert their effects by raising the levels of endogenous antioxidant defenses *in vivo.*

Because these protective mechanisms are not mutually exclusive, multiple antioxidant properties can be found in a single compound (Zhang *et al*., 2006a). These multi-potent antioxidants are of great interest in treating complex diseases as they have other pharmacological effects as well as antioxidant activity

2.5.2 Antimicrobial properties

Antimicrobial activity

Antimicrobial compounds are those that hinder the growth of microorganisms (Meyer *et al.*, 2002). Despite the abundant number of studies on antimicrobial activity of natural compounds, only relatively few have investigated their mechanism of actions and detailed knowledge for many specific compounds is still lacking.

The guiding principle of all antimicrobial chemotherapy is selective toxicity i.e. to inhibit or kill the infecting organism, without harming the host (Chopra & Greenwood, 2001). Most antibacterial agents are able to exploit differences in the structure or metabolism of bacterial and mammalian cells in order to achieve their selective effect.

Antimicrobial agents may be either bactericidal, killing the target bacterium or fungus or bacteriostatic, inhibiting its growth. Bactericidal agents are more effective, but bacteriostatic agents can be beneficial since they permit the normal defenses of the host to destroy microorganisms (Neu & Gootz, 1996).

Antibacterial compounds can exert toxicity to bacteria by inhibiting of cell wall, protein and nucleic acid synthesis, by interfering with cell wall synthesis, and by disrupting membrane leading to leakage of cytoplasmic contents (Neu & Gootz, 1996; Chopra & Greenwood, 2001).

Actions of antimicrobial compounds

Flavonoids are known to be synthesized by plants in response to microbial infection (Cowan, 1999). *In vitro*, they have antimicrobial properties against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins, and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al.*, 1996).

The possible mechanism of action of flavonoids requires further investigation. Some studies showed that flavonoids lacking hydroxyl groups on the B-ring are more active against microorganisms than are those with the -OH groups (Cowan, 1999). This finding supports the idea that their microbial target is the membrane. However, other studies have also found that the more hydroxylation, the greater the antimicrobial activity (Sato *et al.*, 1996).

Xu and Lee (2001) tested 38 types of plant-derived flavonoids belonging to seven different structural groups for activities against antibiotic-resistant bacteria using the disc-diffusion assay and broth dilution assay. Among the flavonoids examined, four flavonols (myricetin, datiscetin, kaempferol and quercetin) and two flavones (flavone and luteolin) exhibited inhibitory activity against MRSA.

The antimicrobial activity of flavonoids has been reviewed by Cushnie and Lamb (2005). The review included antibacterial, antifungal and antiviral activity. The antimicrobial properties of plant volatile oils and their constituents from a wide variety of plants have been assessed and reviewed (Deans & Ritchie 1987; Rios *et al*., 1988; Dorman & Deans, 2000).

2.5.3 Tyrosinase inhibition

Tyrosinase

Tyrosinase exists widely in plants and animals, and is involved in the formation of melanin pigments (Kim & Uyama, 2005; Petit & Pierard, 2003). Tyrosinase is a copper-containing enzyme that catalyzes two distinct reactions of melanin synthesis i.e. hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and oxidation of L-DOPA to *o*-dopaquinone.

In the food industry, tyrosinase is important in controlling the quality of fruits and vegetables (Petit & Pierard, 2003). The enzyme catalyzes the oxidation of phenolic compounds to the corresponding quinones which are responsible for the enzymatic browning of fruits and vegetables. The rate of enzymatic browning depends on the concentration of active tyrosinase and phenolic compounds, oxygen availability, pH, and temperature conditions in the tissue (Seo *et al.* 2003). Current conventional techniques to stop enzymatic browning caused by tyrosinase include autoclaving, blanching and microwaving.

Tyrosinase inhibitors

Effective tyrosinase inhibitors are becoming increasingly important in the cosmetic industry for skin-whitening and for treating pigmentation disorders (Seo *et al.* 2003; Kim & Uyama, 2005). Currently, arbutin and aloesin are used in the cosmetic industry as whitening agents because they show strong tyrosinase inhibition properties and do not exhibit side effects.

The number of chemicals that can actually be used in food systems to inhibit enzymatic browning is limited due to off-flavors, off-odors, toxicity and economic feasibility (Kim & Uyama, 2005). Currently, there is a constant search for better preservatives from natural sources to control enzymatic browning without harmful side effects. The search for tyrosinase inhibitors from natural resources is becoming more relevant as there is now evidence that tyrosinase might contribute to the dopamine neurotoxicity and neurodegenerative disorders associated with Parkinson's disease (Xu *et al*., 1997; Tief *et al*., 1998).

2.6 BIOASSAYS

2.6.1 Screening

Screening of natural products serves as a tool to establish the presence and level of a target activity in a given sample (Spainhour, 2005). The process should incorporate the proper use of controls, both positive and negative; have a defined and easily interpretable endpoint; and capable of delivering quantifiable data. Bioassays used in screening should be rapid, simple to conduct, relevant, capable of being automated and cost-effective.

Bioassays can be defined as the use of a biological system to detect bioactivity of a crude extract, chromatographic fraction, mixture, or a pure compound (Sarker *et al*., 2006). They are very crucial in assessing bioactivity and pharmacological actions of plant extracts and their ethno-medical uses (Gurib-Fakim, 2006). A limitation of bioassays is that they provide no data on individual compounds (Robards, 2003). They do not distinguish between members of a class of bioactivities and provide only semi-quantitative measurements of substances detected. Nevertheless, they continue to be in use because of their simplicity, low cost and the potential to generate activity data of direct relevance.

Bioassays could involve the use of *in vivo*, *ex vivo* or *in vitro* systems (Sarker *et al*., 2006). *In vitro* bioassays are faster and small amounts of test compounds are needed, but might not be relevant to clinical conditions. The trend has now moved from *in vivo* to *in vitro*. Bioassays available today are robust, specific and more sensitive to even small amounts of test compounds. Most of the modern bioassays are microplate-based and require a small amount of extract, fraction, or compound for the assessment of activity.

2.6.2 Phenolic content

Folin-Ciocalteu assay

The Folin-Ciocalteu (FC) assay is frequently used to analyse total phenolic content of plant samples (Robards, 2003; Shahidi & Naczk, 2004c). The assay is convenient, simple and produces comparable data (Singleton *et al.*, 1999). The content of phenolics is expressed as gallic acid or catechin equivalents. Results measured with this assay however give a fairly accurate estimate of the amount of antioxidants in samples and would often correlate well with antioxidant capacity measured by various other assays (Kahkonen *et al.*, 1999).

Main components of FC reagent are phosphotungstic and phosphomolybdic acids (Robards, 2003). These acids are able to oxidise phenols and are themselves reduced to give a blue complex. The chromophore produced is a blue phosphotungstic–phosphomolybdic complex of undefined structure; the underlying chemistry of this reaction is not well understood yet (Bravo & Mateos, 2008). A disadvantage of this assay is the interference of reducing substances such as ascorbic acid which reacts with FC reagent and thus overestimated results are obtained.

The FC method has the following advantages (Huang *et al*., 2005):

1) FC reagent is commercially available, and the procedure has been standardized
2) The long-wavelength absorption of the chromophore minimizes interference from the sample matrix, which is often coloured
3) It is a commonly accepted assay and routinely practiced in dietary antioxidant research laboratories throughout the world
4) A large body of comparable data has been produced

Molybdate assay

The molybdate assay is a colorimetric method developed by Clifford and Wright (1976) to determine total caffeoylquinic acid content in plant samples. The assay involves adding molybdate reagent to plant extracts and the solution is examined spectrophometrically against a reagent blank at 370 nm for caffeoylquinic acid and 353 nm for caffeic acid.

2.6.3 Antioxidant activity

DPPH radical scavenging

The DPPH assay has been widely used to evaluate free radical scavenging activity of extracts from plants, food materials and of single compounds (Scalzo, 2008). This assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$). The scavenging of DPPH radicals is measured by the decrease in absorbance at 517 nm that occurs due to reduction by the antioxidant to the corresponding hydrazine (Gordon, 2001; Sanchez-Moreno, 2002). The assay involves the following hydrogen atom transfer reaction (Huang *et al.*, 2005):

$$DPPH\bullet + \text{antioxidant-H} \rightarrow DPPH\text{-H} + \text{antioxidant}\bullet \text{ (stabilised radical)}$$

The DPPH assay is considered a valid and easy assay to evaluate scavenging activity of antioxidants, since the radical compound is stable and does not have to be generated as in other radical scavenging assays. It is rapid, simple, sensitive and does not require expensive reagents or sophisticated instruments (Brand-Williams *et al.*, 1995). Results are highly reproducible and comparable to other free radical scavenging methods.

Ferric reducing power

Two methods have been developed to measure ferric reducing ability. One is known as ferric reducing power (FRP) while the other as ferric reducing antioxidant power (FRAP). This has caused some confusion in the terminology of methodology.

The first method was developed by Oyaizu (1986) and adopted by Yen and Chen (1995), Chu *et al.* (2000), and Nakajima *et al*. (2007). It is based on the reduction potassium ferricyanide [$K_3Fe(CN)_6$] by antioxidants to potassium ferrocyanide [$K_4Fe(CN)_6$] which forms a blue complex with $FeCl_3$. Referred to as ferric reducing power (FRP), this method was used in this study.

The second method was developed by Benzie and Strain (1996). It involved the reduction of ferric tripyridyltriazine $Fe(TPTZ)^{3+}$ to ferrous tripyridyltriazine $Fe(TPTZ)^{2+}$ in acidic medium. Referred to as ferric reducing ability of plasma (FRAP) (Benzie & Strain, 1996) or ferric reducing antioxidant power (FRAP) (Benzie & Strain, 1999), the method was adopted by Pulido *et al.* (2000), Ou *et al*. (2002) and Firuzi *et al.* (2005).

Ferrous ion chelating

The ferrous ion chelating (FIC) assay measures the ability of samples in chelating Fe^{2+} ions. Addition of samples (acting as ligands) would compete with ferrozine to form a complex and decrease absorbance. The visible absorption spectrum of the ferrous complex of ferrozine exhibits maximum absorbance at 562 nm (Stookey, 1970). The absorbance rapidly increases to maximal values within 10 min and then remains stable for at least 60 min (Riemer *et al*., 2004). In the FIC assay, low absorbance indicates higher chelating ability. The assay is dependent on concentration and does not exhibit correlation with total phenolic compounds in the extracts.

β-carotene bleaching

The β-carotene bleaching (BCB) assay is a useful assay that mimics lipid peroxidation in food and biological systems (Koleva *et al.*, 2002). It is a popular method for assessing antioxidant capacity in food chemistry and appears to be more suitable for analysing less polar samples. The method is simple, sensitive, relatively rapid and reproducibility of the results is dependent on many variables. Because the method employs an emulsified lipid, the resulting haze can present some difficulty in obtaining reproducible results (Kulisic *et al.*, 2004). The method is also tedious compared to DPPH radical scavenging assay and is not sensitive to small variations in phenolic content.

The BCB assay is based on coupled oxidation of β-carotene and linoleic acid which estimates the ability of antioxidant compounds in an extract to scavenge the linoleic acid peroxide in aqueous emulsion phase at 50°C (Kaur & Kapoor, 2001; Koleva *et al.*, 2002). The presence of phenolic antioxidants reduces the depletion of the yellow colour of β-carotene by neutralizing linoleic acid free radical. BCB is measured by the decrease in the initial absorbance at 470 nm.

2.6.4 Antibacterial properties

Disc-diffusion method

The disc-diffusion method is widely used to screen for antibacterial and anti-fungal activity of plants. Crude extracts have been used to test individual species (Ibrahim & Osman, 1995) or a variety of plant species (Mackeen *et al*., 1997, Chung *et al.*, 2004, Wiart *et al.*, 2004). Essential oils extracted from plants have also been tested for antimicrobial activity (Wannissorn *et al.*, 2005).

In the disc-diffusion method, antimicrobial activity is evaluated based on the ability of the plant extracts to diffuse through agar to affect the target organisms (Chung *et al.*, 2004). When screening for antibacterial activity using the disc-diffusion method, a paper disk soaked with plant extract is laid on top of an inoculated agar plate. Using this assay, the size of the zone of inhibition can be influenced by a number of factors. They include inoculum density, volume of extract solution, thickness of agar layer and duration of the assay (Cole, 1994; Burt, 2004). This method is useful only for initial screening of antibacterial activity and comparison of published data is not feasible.

Minimum inhibition dose

Minimum inhibitory dose (MID) is another quantitative method for determination of antimicrobial activity (Habsah *et al.*, 2000; Mackeen *et al.*, 2000). MID is the minimum dose per disc to inhibit growth of the test microorganisms. Extracts are serially diluted and loaded onto paper discs. It is the minimum concentration that showed positive activity using the disc diffusion method.

2.6.5 Tyrosinase inhibition

Tyrosinase inhibition assay

The mushroom tyrosinase inhibition assay is a basic step to assess the direct effect of given skin lightener on tyrosinase activity (Petit & Pierard, 2003). The substrate of the enzyme is L-tyrosine and the reaction requires the presence of L-DOPA as co-substrate. The inhibition activity of tyrosinase is quantified following the detection of dopachrome.

The most widely used *in vitro* assay for the DOPA oxidase activity of tyrosinase is the dopachrome method (Winder & Harris, 1991). L-DOPA is used as the substrate and the formation of dopachrome was measured at 475 nm. A major disadvantage of this method is that dopachrome is unstable in aqueous solution and is further oxidised even while its accumulation is being monitored. This instability results in the assay being linear for only a short period (2-3 min). The absorption coefficient of dopachrome is relatively low and the assay is therefore of low sensitivity.

2.7 NATURAL PRODUCTS

2.7.1 Introduction

There has been a remarkable resurgence of interest in natural product research over the last decade (Sarker *et al*., 2006). With the outstanding development in separation science, spectroscopic techniques and *in vitro* assays, natural product research has gained renewed attention. Strategies for research on natural products have evolved quite significantly. There are two broad categories. They are:

1) Older strategies focus on chemistry of compounds from natural sources, but not on activity. They involve isolation and identification of compounds from natural sources followed by testing of bioactivity.
2) Modern strategies involve bioassay-guided isolation and identification of active compounds from natural sources. Focus is more on bioactivity. There is more focus on isolating target compounds (assay-guided isolation) rather than trying to isolate all compounds present in any extract.

Currently, there are a number of well-established methods available for extraction and isolation of natural products (Sarker *et al*., 2006). When choosing a method, one should weigh the advantages and disadvantages of available methods, focusing on their efficiency and the total cost involved. Continuous progress in the area of separation technology has increased the variety and variability of the extraction and isolation methods that can be successfully utilized in the extraction and isolation of natural products.

2.7.2 Extraction

The typical extraction process involves drying and grinding of plant materials or homogenizing fresh plant parts e.g. leaves and flowers or maceration of total plant parts with a solvent (Sarker *et al.*, 2006). Plant materials such as leaves, stems, roots, seeds or fruits are usually frozen and pulverized using liquid nitrogen in a mortar and a pestle (Zheng *et al*., 2004).

The choice of solvents is important to maximize the yields of the compounds of interest, while minimizing the extraction of unwanted compounds (Jones & Kinghorn, 2006). Water, ethanol and methanol are used for extraction of polar compounds; ethyl acetate and DCM for compounds of medium polarity; and n-hexane, chloroform for non-polar compounds (Sarker *et al*., 2006).

Organic solvents are much easier to evaporate than water. The use of organic solvents also prevents microbial growth, which is one of the most serious problems associated with aqueous extraction (Shimizu & Li, 2006). Solvent extraction procedures applied to plant materials include maceration, percolation, Soxhlet extraction, pressurized solvent extraction, ultrasound assisted solvent extraction, extraction under reflux and steam distillation (Seidel, 2006).

The extraction conditions applied for sample preparation can have an important influence on the type of flavonoids isolated as well as on the yield of extraction (Bravo & Mateos, 2008). The type of solvent to be used for the extraction of flavonoids from foods depends mainly on the type of compounds to be extracted, that is whether they are aglycones, glycosides, acylated or methoxylated flavonoids, etc., which might affect their polarity and thus their solubility on the selected solvent. Methanol and aqueous methanol are the most commonly used solvents. Alcohols cause instability of cell membranes, facilitating extraction of phenolic compounds; besides, they inactivate enzymes such as polyphenol oxidase and thus contribute to an increased stability of the extracted compounds (Arts & Hollman, 1998). Sequential extraction with different solvents has also been applied to ensure more complete extraction of the flavonoids (Bravo & Mateos, 2008).

2.7.3 Isolation

Crude extracts from plants contain a wide range of compounds. Fractionation is done to separate these compounds into discrete fractions based on their polarities, structure and/or molecular sizes (Sarker *et al.*, 2006). This often involves liquid-liquid extraction into solvents of varying polarities using column chromatography.

For most extracts, a single separation method is often insufficient to isolate individual compounds because many compounds could have similar properties. Generating too many fractions in the initial fractionation is not advisable as the target compound could be diluted over many fractions. This would make it hard to monitor the target compound. Often the goal of fractionation is to yield mixtures of compounds with similar characteristics. Subsequent isolation steps are required to purify compounds.

Different methods can be used to monitor fractions depending on the purpose. For isolation of individual compounds, thin-layer chromatography (TLC) is often used to detect different compounds either by illumination under 254 and 365 nm UV light or spraying the TLC plate with different stains such as Dragendorff's, Folin-Ciocalteu and ninhydrin reagents to detect alkaloids, phenols and amines, respectively (Houghton & Raman, 2003).

If producing herbal extracts is the goal, often a bioassay-guided approach is adopted. In this case, fractions are monitored based on their bioactivities. This is done by subjecting the different fractions to various bioassays to identify the most active fractions. Another approach is to pool active fractions for subsequent isolation of active components.

Chromatography

The chromatographic techniques used in the isolation of various types of natural products can be broadly classified into two categories (Sarker *et al.*, 2006). Classical chromatographic techniques include TLC, preparative TLC, open-column chromatography and flash chromatography. Modern techniques are high performance liquid chromatography, multi-flash chromatography, vacuum liquid chromatography, chromatotron and solid-phase extraction.

Chromatography is a method of separation with the use of two phases: stationary phase and mobile phase (Zheng *et al*., 2004). Analytes are introduced to the system by either directly applying it to the stationary phase or indirectly through the use of mobile phase. As the mobile phase passes through the stationary phase, the components of the mixture partition or equilibrate between the two phases, resulting in differential migration rates through the system. The differential rate of migration of the analytes is determined by their relative tendencies to interact with the stationary phase and mobile phase. Separation can be accomplished by selective adsorption on the solid phase, such as particle clay, silica gel, alumina, cellulose, or other solid support. The stationary phase is usually packed into a column. Consequently, each substance appears in the adsorbent medium eluting from the column at a different time. This time difference is called the retention time. The technique is very powerful because it allows separation of compounds of very similar physical and chemical properties, including isomers, within a given extract.

Thin-layer chromatography

One of the fastest and most widely used chromatographic techniques is thin-layer chromatography (TLC). This method employs glass or aluminium plates pre-coated with the sorbent (e.g. silica gel) to varying thickness depending on the amount to be loaded (Gurib-Fakim, 2006). The compound mixture is loaded both in the preparative or analytical plates at around 2 cm from the bottom and lowered in a tank containing the solvent. The latter migrates up the plates and separates the compound mixture according to the polarity of the components. Several reagents are available for visualization of the separated materials.

High performance liquid chromatography

High performance liquid chromatography (HPLC) is commonly used in determining phenolic compounds present in a wide range of spices, herbs, beverages, fruits and vegetables (Jandera *et al.,* 2005). Its main advantage over column chromatography is that higher pressure, applied with a pump, allows packing material with finer particle size, typically 5 microns, to be used. This provides enhanced separation of compounds.

Reverse-phase (RP) columns are almost exclusively employed with HPLC because they provide more stable performance and better recovery of compounds compared to their normal-phase counterparts (Latif, 2006). The performance of normal phase packing materials such as silica gel are less consistent because they are hard to regenerate and are often disposed after each usage. This can be costly as analytical HPLC columns have to be factory packed for precision.

Typically, an HPLC system consists of the following components: solvent reservoir, injection system, column, HPLC pump, detector, sample collector (optional), and a computer serving as a data station for the detector information as well as a way to control and automate the HPLC pump [Fig. 2.11].

The most commonly used columns are packed with C_{18} octadecyl derivatised silica (ODS). It offers good selectivity for a wide range of compounds based on polarity (Proestos *et al.*, 2006). Moreover, it allows samples to be loaded in an aqueous solvent, further improving its versatility over normal phased columns that often require pre-adsorption. Other less commonly used packing materials such as phenyl and C_8 ODS cater to more specific applications, namely, aromatic and non-polar compounds, respectively.

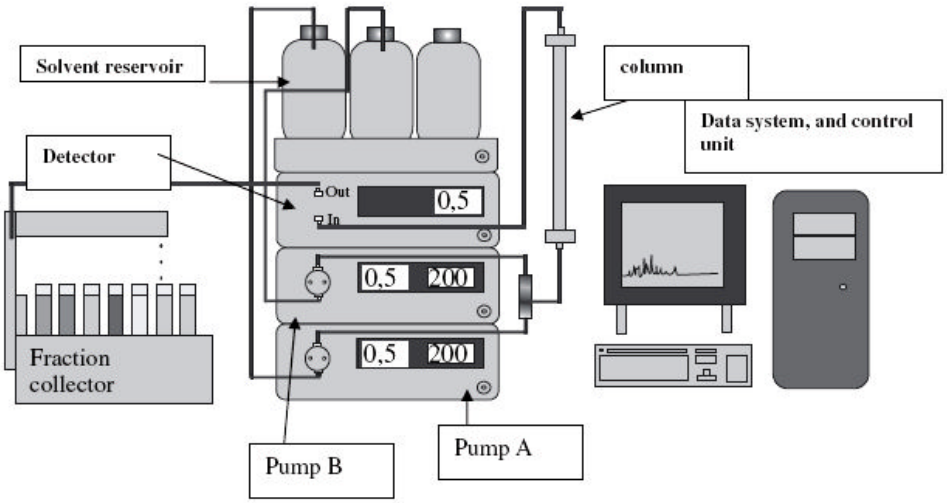


Fig. 2.11
A typical HPLC system with two pumps to allow for gradients, column, detector, fraction collector and data station (Cseke *et al*., 2006)

Most HPLC configurations often include a UV detector because it can be applied to a wide range of compounds. Monitoring absorbance at different wavelengths confer different sensitivity and selectivity to the method. Identification is based upon both retention time and absorption spectra. Most benzoic acid derivatives show an absorption maximum at 246-262 nm (Lee & Widmer, 1996). Flavonoids typically absorb at two different bands namely 240-285 and 300-550 nm (Merken & Beecher, 2000).

HPLC is the most powerful and frequently used technique for the separation of natural compounds such as flavonoids, both their aglycones and glycosides (Bravo & Mateos, 2008). It offers significant advantages in terms of simplicity, speed, cost, sensitivity, specificity, precision and sample preservation. It is versatile and adaptable for specific requirements through the use of adequate stationary phases, composition of mobile phases, and the possibility to couple with a wide range of selective detectors. In addition, HPLC has the advantage of simultaneous separation and quantification of flavonoids without preliminary derivatization. However, because of the lack of standard compounds for many

flavonoids, especially for flavonoid glycosides, and the enormous number of different flavonoids existing in nature, identification of these compounds is not a straightforward task. In the case of flavonoid glycosides, it has become an accepted practice to hydrolyze them into aglycones before HPLC analysis. For the separation of flavonoids, common chromatographic conditions include the use of almost exclusively an RP C18 column and a binary solvent system containing acidified water (solvent A) such as aqueous acetic, phosphoric, sulfuric, or formic acids, and a less polar organic solvent (solvent B) such as methanol or acetonitrile.

2.7.4 Structure elucidation

Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a well-established and the most commonly used method for natural product structure analysis (Stobiecki & Kachlicki, 2006). The studies of phenolic structures using ^{1}H-NMR along with ^{13}C-NMR have become the method of choice for the structure elucidation of these compounds. Chemical shifts and multiplicity of signals corresponding to particular atoms and their coupling with other atoms within the molecule allow for easy identification of the aglycone structure, the pattern of glycosylation and the identity of the sugar moieties present.

NMR is a powerful and theoretically complex analytical tool for structure elucidation (Friebolin, 2005; Hummer & Schreier, 2008). Basic principles are the nuclear spin and the splitting of energy levels in a magnetic field. All nuclei that contain odd numbers of protons or neutrons possess an intrinsic magnetic moment and the mechanical property of spin. The overall spin of the nucleus is determined by spin quantum number I, with the most commonly measured nuclei ^{1}H and ^{13}C having a spin of I = 1/2. Nuclei with such a spin can only adopt two discrete states according to the magnetic field, parallel and anti-parallel, also

referred to as spin up and spin down, respectively. Transitions between these states can be caused and registered *via* radiation or emission of electromagnetic waves. The magnetic field and the molecule structure determine the resonance frequency. Further structural data can be elucidated by observing spin-spin coupling, a process by which the precession frequency of a nucleus can be influenced by the magnetization transfer from nearby nuclei. A typical NMR spectrum contains information regarding what groups there are, how many there are, where they are located in the molecule, what their neighbors are and how they are related to those neighboring groups (Zheng *et al*., 2004). The method is non-destructive and the sample after analysis could be recovered and used for other purposes.

Mass spectrometry

Next to NMR, mass spectrometry (MS) is certainly the most important tool in the structural determination of organic compounds and of natural products (Vogler & Setzer, 2006). It offers outstanding sensitivity which is much better than that of NMR. However, the interpretation of MS spectra is more complex than NMR spectra. A mass spectrometer produces charged particles (ions) from the chemical substances that are to be analyzed. Subsequently, these charged particles are fragmented due to their high energy, and then electric and magnetic fields are used to measure the mass of the newly generated charged particles. There are many different techniques in mass spectrometry that can be divided according to their ion formation and according to the process of how the originally generated ions and the ions resulting from fragmentation reactions are sorted.

MS has proven to be a very powerful technique for analysis of flavonoids and other phenolic compounds because of its high sensitivity and the possibility to combine with different chromatographic techniques e.g. gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-

MS) for both qualitative and quantitative determinations (Bravo & Mateos, 2008). In this study, the compounds were identified using EI-MS and ESI-MS. Electron ionization or electron impact ionization (EI) is the oldest and best characterized of all the ionization methods (Vogler & Setzer, 2006). A beam of electrons passes through the gas-phase sample and collides with the neutral analyte molecule. This collision can knock off an electron from the analyte molecule, resulting in a positively charged radical ion. Most mass spectrometers use electrons with energy of 70 eV for EI. However, for molecules that are easily fragmented, analyses are often done at 40 eV. The EI mass spectra provide reproducible structural information searchable from libraries of mass spectra.

Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the two most widely used ionization methods for flavonoids (Prasain *et al.*, 2004). ESI is more often used to ionize polar and non-volatile molecules such as condensed tannins and anthocyanidins. Furthermore, because it is a soft ionisation technique that causes minimal fragmentation, it is ideal for mass analysis of many compounds. Modern LC-ESI-MS is efficient and easy to use, making it the most popular technique for analysis of flavonoids (Cuyckens & Claeys, 2004).

Ions from ESI-MS can be analysed in either positive or negative modes. The negative mode produces deprotonated molecular ions ($[M - H]^-$) while the positive mode results in protonated molecular ions ($[MH]^+$), molecular ions with adducts (e.g. $[M + Na]^+$ and $[M + K]^+$) and/or dimers (e.g. $[M + H + M]^+$ and $[M + Na + M]^+$) (Zagorevskii, 2004). Sodium is the most common adduct generated in positive ESI-MS analysis of medicinal plants (Pietta & Gardana, 2003). Many but not all flavonoids are capable of yielding adducts. Flavonol-3-*O*-glycosides generate sodium or potassium adducts, while flavonol-4'-*O*-glycosides and flavone glycosides do not.

Chapter 3

MATERIALS AND METHODS

3.1 CHEMICALS AND EQUIPMENT

Chemicals and equipment used in this project are listed in Appendix I [pages 254–256] and Appendix II [pages 257–258], respectively. They are classified under bioassays and natural product research.

3.2 PRELIMINARY SCREENING

3.2.1 Choice of genus and solvent

Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves and rhizomes of five ginger species belonging to five genera were screened using methanol and dichloromethane (DCM) as solvents. The species tested were *Etlingera maingayi*, *Alpinia malaccensis*, *Elettariopsis slahmong*, *Zingiber spectabile* and *Scaphochlamys kunstleri*. The objective of the preliminary screening was to determine the genus with the highest phenolic content and antioxidant activity, and the solvent suitable for extraction of leaves and rhizomes of ginger species.

3.2.2 Extraction efficiency of solvents

Further testing on the extraction efficiency of solvents was conducted using leaves of *Etlingera elatior* and *Curcuma longa*. Solvents tested were methanol, 50% aqueous methanol, ethyl acetate and dichloromethane. Leaves (1 g) were powdered with liquid nitrogen in a mortar and extracted using the different

solvents (50 ml) with continuous swirling for 1 h at room temperature using an orbital shaker. Extracts were filtered under suction and stored at –20°C for further use. This extraction method was also adopted for rhizomes.

As methanol was found to be the most efficient solvent, the efficiency of methanol extraction was tested by performing multiple extractions of 50 ml methanol. Plant material was recovered after the first extraction and re-extracted twice. The extraction efficiency of samples was expressed in terms of percentage based on their TPC values.

3.3 GINGER SPECIES STUDIED

3.3.1 *Etlingera* species

Based on the above screening, leaves of five *Etlingera* species (tribe Alpinieae) were chosen for more detailed studies on bioactivity. The species were *E. elatior*, *E. fulgens* and *E. maingayi* of the *Phaeomeria* group, and *E. rubrostriata* and *E. littoralis* of the *Achasma* group [Fig. 3.1]. In the former, inflorescences are raised above the ground while in the latter, inflorescences appear at soil level. Voucher specimens were deposited in the herbaria of Forest Research Institute Malaysia (FRIM) and of Monash University Sunway Campus (MUSC) Malaysia.

Besides TPC, bioactivity studies on antioxidant activity, antibacterial properties and tyrosinase inhibition of the five *Etlingera* species were also carried out. Having outstanding properties, leaves of *E. elatior* were analyzed for chemical constituents. Protocols for developing a standardized extract from leaves of *E. elatior* were developed.

Phaeomeria group of *Etlingera* species

E. elatior *E. fulgens* *E. maingayi*

Achasma group of *Etlingera* species

E. littoralis *E. rubrostriata*

Fig. 3.1
Phaeomeria and *Achasma* groups of *Etlingera* species studied (photographs taken by E.W.C. Chan)

3.3.2 Other ginger species

Leaves of 21 other ginger species belonging to eight genera and three tribes were screened for TPC and AEAC for comparison with those of the five *Etlingera* species. The tribes and genera of ginger species are Alpinieae (*Alpinia* and *Elettariopsis*), Hedychieae (*Boesenbergia*, *Curcuma*, *Hedychium*, *Kaempferia* and *Scaphochlamys*), and Zingibereae (*Zingiber*). For 14 of these species including those of *Etlingera*, comparisons were made between leaves and rhizomes. Names and sampling locations of these species including those of *Etlingera* are shown in Table 3.1. Photographs of some of the other ginger species studied were shown in Fig. 3.2. Voucher specimens were deposited in the herbaria of FRIM and of MUSC.

Table 3.1

Locations of leaves and rhizomes of 26 ginger species screened for phenolic content and antioxidant activity

Species	Tribe	Location of sampling
Alpinia galanga	Alpinieae	Bukit Maluri
A. malaccensis		FRIM
A. purpurata		Sunway
A. zerumbet		Janda Baik
A. zerumbet 'Variegata'		FRIM
Boesenbergia rotunda	Hedychieae	Sungai Buluh
Curcuma aeruginosa	Hedychieae	Damansara Utama
C. longa		FRIM & Bukit Maluri
C. mangga		Damansara Utama
C. zanthorrhiza		Damansara Utama
Elettariopsis latiflora	Alpinieae	FRIM
E. slahmong		FRIM
E. smithiae		Janda Baik
Etlingera elatior	Alpinieae	Janda Baik & FRIM
E. fulgens		Janda Baik & FRIM
E. littoralis		Genting Highlands
E. maingayi		Janda Baik
E. rubrostriata		Ulu Gombak & Janda Baik
Hedychium coronarium	Hedychieae	Lake Gardens
Kaempferia galanga	Hedychieae	Damansara Utama
K. pulchra		Sungai Buluh
K. rotunda		Bukit Maluri
Scaphochlamys kunstleri	Hedychieae	FRIM
Zingiber officinale	Zingibereae	Bukit Maluri
Z. ottensii		Bukit Maluri
Z. spectabile		FRIM & Janda Baik

Fig. 3.2
Some of other ginger species studied (photographs taken by E.W.C. Chan). Abbreviations: *A.* = *Alpinia*, *C.* = *Curcuma*, *E.* = *Elettariopsis*, *K.* = *Kaempferia*, *S.* = *Scaphochlamys* and *Z.* = *Zingiber.*

3.4 BIOASSAYS

3.4.1 Extraction

For the analysis of total phenolic content and antioxidant properties which included DPPH radical scavenging, ferric reducing power, ferrous ion chelating and lipid peroxidation inhibition, the same extraction protocol was employed. Fresh leaves and rhizomes (1 g) of ginger species were powdered with liquid nitrogen in a mortar and extracted using methanol (50 ml), with continuous swirling for 1 h at room temperature using an orbital shaker. Extracts were filtered under suction and stored at –20°C for further use.

For the analysis of caffeoylquinic acid content, leaves of ginger species were extracted in the same manner except that 50% aqueous methanol was used.

3.4.2 Phenolic content

Folin-Ciocalteu assay

Total phenolic content (TPC) of extracts was determined using the Folin-Ciocalteu (FC) assay (Kahkonen *et al.*, 1999). Samples (300 µl in triplicate) were introduced into test tubes followed by 1.5 ml of FC reagent (10 times dilution) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min in the dark before absorbance at 765 nm was measured. TPC was expressed as gallic acid equivalent (GAE) in mg per 100 g fresh material. The calibration equation for gallic acid was $y = 0.0111x – 0.0148$ ($R^2 = 0.9998$).

Molybdate assay

Caffeoylquinic acid content (CQAC) of extracts was determined using the molybdate assay (Clifford & Wright, 1976). Molybdate reagent was prepared by dissolving 16.5 g sodium molybdate, 8.0 g dipotassium hydrogen phosphate, and 7.9 g potassium dihydrogen phosphate in 1 L deionised water. The reagent (2.7 ml) was added to plant extracts (0.3 ml), mixed, and incubated at room temperature for 10 min. Absorbance was measured at 370 nm. CQAC was expressed as mg chlorogenic acid equivalent (CGAE)/100 g. The calibration equation for CQA was y = 8.6966x (R^2 = 0.9979).

3.4.3 Antioxidant activity

DPPH radical scavenging

Free radical scavenging (FRS) using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay (Miliauskas *et al.*, 2004) was adopted with modifications. Different dilutions of the extract (1 ml; triplicate) were added to 2 ml of DPPH (5.9 mg/100 ml methanol). Absorbance was measured using a spectrophotometer at 517 nm after 30 min. FRS was calculated as IC_{50} and expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg ascorbic acid/100 g (Leong & Shui, 2002) as follows:

$$\text{AEAC (mg AA/100 g)} = IC_{50(\text{ascorbate})}/IC_{50(\text{sample})} \times 10^5$$

The IC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/ml.

Ferric reducing power

The potassium ferricyanide assay reported by Chu *et al*. (2000) was adopted with modifications for assessing ferric reducing power (FRP). Different dilutions of the

extract (1 ml) were added to 2.5 ml phosphate buffer (0.2 M; pH 6.6) and 2.5 ml of $K_3Fe(CN)_6$ (1% w/v). The mixture was incubated at 50°C for 20 min. Trichloro-acetic acid solution (2.5 ml; 10% w/v) was added to stop the reaction. The mixture was then separated into aliquots of 2.5 ml and diluted with 2.5 ml of water. To each diluted aliquot, 500 µl of ferric chloride solution (0.1% w/v) were added. After 30 min, absorbance was measured at 700 nm. FRP was expressed as mg GAE/g. The calibration equation for gallic acid was y = 16.767x (R^2 = 0.9974).

Ferrous ion chelating

Ferrous ion chelating (FIC) ability was assessed using the ferrous-ferrozine assay reported by Singh and Rajini (2004) with modifications. Solutions of 2 mM $FeSO_4$ and 5 mM ferrozine were diluted 20 times. $FeSO_4$ (1 ml) was mixed with different dilutions of extract (1 ml), followed by ferrozine (1 ml). Absorbance was measured at 562 nm after 10 min. The ability of extracts to chelate ferrous ions was calculated as follows:

$$\text{Chelating effect \%} = (1 - A_{sample}/A_{control}) \times 100$$

β-carotene bleaching

For assessing lipid peroxidation inhibition (LPI) activity, the β-carotene bleaching (BCB) assay reported by Kumazawa *et al.* (2002) was adopted. β-carotene and linoleic acid emulsion was prepared by adding 3 ml of β-carotene (5 mg in 50 ml chloroform) to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was evaporated under vacuum and oxygenated ultra-pure water (100 ml) was added and mixed well. Initial absorbance of the emulsion was measured at 470 nm. Aliquots of the emulsion (3 ml) were mixed with 10, 50 and 100 µl of extract, and incubated in a water bath at 50°C for 1 h.

Bleaching rate of β-carotene was measured at 470 and 700 nm. Measurement at 700 nm is needed to correct for the presence of haze. LPI activity was expressed as AOA (%) and calculated as follows:

$$\text{Bleaching rate (BR) of β-carotene} = \ln(A_{initial}/A_{sample})/60$$
$$\text{AOA (\%)} = (1 - BR_{sample}/BR_{control}) \times 100$$

where $A_{initial}$ and A_{sample} are absorbance of the emulsion before and 1 h after incubation, and BR_{sample} and $BR_{control}$ are bleaching rates of the sample and negative control, respectively.

3.4.4 Antibacterial properties

Extraction

For the screening of antibacterial activity, fresh leaves of each species were cut into small pieces and 100 g were weighed and freeze-dried overnight. Dried samples were then crushed in a mortar with liquid nitrogen and extracted with 250 ml of methanol three times for 1 h each time. Samples were filtered and the solvent was removed using a rotary evaporator. Dried extracts were kept at –20°C for further analysis.

Test bacteria

Antibacterial activity of plant extracts were assessed using Gram-positive bacteria of *Bacillus cereus*, *Micrococcus luteus* and *Staphylococcus aureus*, and Gram-negative bacteria of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella choleraesuis* [Table 3.2].

Table 3.2
Properties and pathogenicity of bacteria tested

Bacteria	Properties and pathogenicity
Bacillus cereus	Gram-positive rods that cause food-borne illness characterised by vomiting, fever and/or diarrhoea
Micrococcus luteus	Gram-positive cocci that infect the mouth and upper respiratory tract
Staphylococcus aureus	Gram-positive cocci that cause food poisoning
Escherichia coli	Gram-negative rods that cause gastrointestinal and urinary tract infections
Pseudomonas aeruginosa	Gram-negative rods that cause gastrointestinal, urinary tract and respiratory system infections
Salmonella choleraesuis	Gram-negative rods that cause acute gastroentritis

Disc-diffusion method

The disc-diffusion method as described by Chung *et al*. (2004) was used to screen for antibacterial activity. Agar cultures of Gram-positive bacteria of *B. cereus*, *M. luteus* and *S. aureus*, and Gram-negative bacteria of *E. coli*, *P. aeruginosa* and *S. choleraesuis* were prepared. Suspensions of bacteria (100 µl) were spread evenly onto 20 ml Mueller-Hinton agar preset in 90 mm Petri dishes.

Paper discs (6 mm diameter) impregnated with 1 mg of plant extract dissolved in 100 µl solvent were transferred onto the inoculated agar. Streptomycin susceptibility discs (10 µg) and methanol impregnated discs were used as positive and negative controls, respectively. After incubation overnight at 37°C, inhibition zones were measured and recorded as mean diameter (mm). Anti-bacterial activity was also expressed as inhibition percentage of streptomycin and arbitrarily classified as strong for inhibition of ≥70%, moderate for inhibition 50 < 70% and weak for inhibition <50%.

EDTA method

Preliminary investigation to improve the efficacy of leaf extracts of *Etlingera* species against Gram-negative bacteria was carried out by adding 2 mM of ethylenediamine tetraacetic acid (EDTA) to the agar before culturing the bacteria. It is the first time the method has been used for testing antibacterial activity of plant extracts.

3.4.5 Tyrosinase inhibition

Extraction

Fresh leaves (10 g) were extracted three times using methanol (100 ml) each time. Methanol was removed by drying at 35°C in a rotary evaporator prior to storage at –20°C. Analysis of methanol extracts for antioxidant and tyrosinase inhibition was done in triplicate.

Dopachrome method

Tyrosinase inhibition activity of leaves of five *Etlingera* species was determined using the dopachrome method with L-3,4-dihydroxyphenylalanine (L-DOPA) as substrate (Masuda *et al*., 2005). The leaves of *Hibiscus tiliaceus* were used as positive control.

Assays were conducted in a 96-well microtiter plate and a plate reader was used to measure absorbance (A) at 475 nm with 700 nm as reference. Samples were dissolved in 50% dimethyl sulphoxide (DMSO). Each well contained 40 µl of sample with 80 µl of phosphate buffer (0.1M, pH 6.8), 40 µl of tyrosinase (31 units/ml) and 40 µl of L-DOPA (2.5 mM). Each sample was accompanied by a blank that has all the components except L-DOPA. This gave a final sample concentration of 0.5 mg/ml. Results were compared with a control consisting of

50% DMSO in place of sample. The percentage tyrosinase inhibition was calculated as $(A_{control} – A_{sample})/A_{control}$ x 100%.

3.4.6 Cytotoxicity

Extraction

Fresh leaves of *Etlingera* species (10 g) were powdered with liquid nitrogen in a mortar and extracted sequentially with dichloromethane, methanol, and water (100 ml each for 1 h). Extracts were pooled and evaporated using a rotary evaporator to obtain composite samples of polar and non-polar constituents.

Sulforhodamine B assay

WRL-68 (human liver) and Vero (African green monkey kidney) cells were seeded in a 96-well plate at 10,000 cells/well. Cells were kept overnight before incubating with plant extracts at 0.1–1,000 mg/ml. Paclitaxel was used as positive control. After 72 h, the sulforhodamine B (SRB) assay (Voigt, 2005) was conducted to determine the number of viable cells. Absorbance was measured with a microplate absorbance reader and results were expressed as cell survival (%) with IC_{50} determined from dose-response curves.

3.4.7 Summary of bioassays

Bioassays used for assessing antioxidant activity and other bioactivities are summarized in Table 3.3. They comprised the DPPH, potassium ferricyanide, ferrous-ferrozine and beta-carotene bleaching assays for antioxidant activity, dopachrome assay for tyrosinase inhibition, disc-diffusion method for anti-bacterial activity, and SRB assay for cytotoxicity.

Table 3.3
Summary of bioassays and bioactivities

Bioassay	Bioactivity
DPPH assay	Free radical scavenging (FRS)
Potassium ferricyanide assay	Ferric reducing power (FRP)
Ferrous-ferrozine assay	Ferrous ion chelating (FIC)
Beta-carotene bleaching assay	Lipid peroxidation inhibition (LPI)
Dopachrome assay	Tyrosinase inhibition (TI)
Disc-diffusion method	Antibacterial activity
Sulforhodamine B assay	Cytotoxicity

3.5 DRYING TREATMENTS

The effects of different thermal and non-thermal drying treatments on the phenolic content and antioxidant properties were tested on leaves of four ginger species, namely, *Alpinia zerumbet*, *Etlingera elatior*, *Curcuma longa* and *Kaempferia galanga*.

3.5.1 Thermal drying methods

Thermal drying methods used were microwave-, oven- and sun-drying. In microwave-drying, leaves (1 g) were dried in a microwave oven for 4 min. Oven-drying involved drying leaves (1 g) for 5 h in an oven set at 50°C. Leaves were sun-dried in the greenhouse for three days with about 27 h of daylight. Mid-day temperature in the greenhouse can reach 35°C.

3.5.2 Non-thermal drying methods

Non-thermal drying methods used were freeze- and air-drying. In air-drying, leaves were dried at room temperature ranging from 25° to 30°C for three days at a relative humidity of 33%. In freeze-drying, leaf samples were lyophilized at 0.125 mbar and at –50°C overnight in a freeze-dryer.

3.5.3 Extraction and analysis

Fresh and dried leaves were extracted and analysed for TPC and antioxidant properties as in sections 3.4.1, 3.4.2 and 3.4.3.

3.5.4 High performance liquid chromatography

Extracts of fresh and freeze-dried leaves of *E. elatior* were dissolved in 50% methanol and analysed using reverse-phase high performance liquid chromatography (HPLC) with a phenyl column. A 15-min linear gradient from 5% to 100% MeOH, was used to elute samples at 1 ml/min. Mobile phases were acidified with 0.1% trifluoroacetic acid for better resolution. Elution was monitored at 254 nm. Chromatograms of fresh and freeze-dried leaves were overlaid to display differences in constituents and their overall peak areas calculated.

3.6 CHEMICAL CONSTITUENTS

3.6.1 Phenolic compounds

Extraction of leaves

Leaves of *E. elatior* (1.5 kg) were lyophilized overnight in batches at 0.125 mbar and –50 °C in a freeze-dryer and subsequently ground using a blender. Ground leaves were extracted once with methanol to inactivate polyphenol oxidase, filtered, and extracted five times with water. The container was sonicated for 8 min, when fresh solvent was added each time. After sonication, each extraction system was allowed to stand for 1 h using 2 L of solvent in the closed container at room temperature. The methanol extract was dried in a rotary evaporator and the dried extract was suspended in water. The water-soluble component of the suspension was collected by filtration and combined with the water extract. Freeze-drying of the combined water extract yielded 80 g of dried extract.

Isolation of compounds

Column chromatography

The dried leaf extract of *E. elatior* (30 g) was fractionated with MCI gel CHP 20P using a H_2O:MeOH 0-100% step-gradient into 17 fractions.

Compound **1** (30 mg) was purified from fraction 2 (0.5 g) with Chromatorex ODS (H_2O:MeOH; 0−40%), and silica gel 60 ($CHCl_3$:MeOH:H_2O; 7:3:0.5 to 5:5:2).

Compound **2** (35 mg) was purified from fraction 8 (2.0 g) with Sephadex LH-20 (H_2O:MeOH; 0−100%) and silica gel 60 ($CHCl_3$:MeOH:H_2O; 7:3:0.5 to 5:5:2)

Compounds **3** (50 mg) and **4** (10 mg) were purified from fraction 9 (1.5 g) with Sephadex LH-20 (H_2O:MeOH; 0−100%), Chromatorex ODS (H_2O:MeOH; 0−40%), and LiChroprep® RP-18 (H_2O:MeOH; 0−40%).

Compound **5** (50 mg) was purified from fraction 15 (1.0 g) with Sephadex LH-20 (H_2O:MeOH; 0−100%), silica gel 60 ($CHCl_3$:MeOH:H_2O; 7:3:0.5 to 5:5:2) and Chromatorex ODS (H_2O:MeOH; 0−40%).

Compound **6** (100 mg) was purified from fraction 16 (2.0 g) with Sephadex LH-20 (H_2O:MeOH; 0−100%), Chromatorex ODS (H_2O:MeOH; 0−40%) and silica gel 60 ($CHCl_3$:MeOH:H_2O; 7:3:0.5 to 5:5:2).

Thin-layer chromatography

Eluents from the column chromatography were pooled into fractions based on TLC analysis using silica gel 60 F_{254} plates ($CHCl_3$:MeOH:H_2O; 8:2:0.2, 7:3:0.5 or 6:4:1). Bands were detected by UV illumination and by spraying 10% H_2SO_4 with heating.

Structural elucidation of compounds

Nuclear magnetic resonance spectroscopy

Compounds were dissolved in deuterated methanol (CD_3OD) and subjected to ^1H and ^{13}C NMR analysis using a Bruker DRX 300 MHz spectrometer operated at 300 MHz for ^1H and 75 MHz for ^{13}C. Chemical shifts were recorded in ppm (δ) using tetramethylsilane (TMS) as internal standard.

Mass spectrometry

Isolated compounds of *E. elatior* leaves were subjected to analysis using electrospray ionization mass spectrometry (ESI-MS) and electron ionization mass spectrometry (EI-MS). Analysis was conducted using a ThermoFinnigan LCQDeca and ThermoFinnigan Polaris Q mass spectrometers, respectively. Analysis with EI-MS was operated at 40 eV to minimise the fragmentation of ions. Mass spectra of ESI-MS were acquired in both positive and negative ion modes. Analytes were introduced into the mass spectrometer by direct infusion. Mass ranging up to 800 m/z was measured.

Quantitation of compounds

Ginger species

Caffeoylquinic acid content (CQAC) and chlorogenic acid content (CGAC) of *E. elatior* leaves were compared with those of four other *Etlingera* species (*E. fulgens*, *E. rubrostriata*, *E. littoralis* and *E. maingayi*), three commercial ginger species (*A. galanga*, *C. longa* and *Z. officinale*), and two important sources of CQA (flowers of *Lonicera japonica* and leaves of *Ipomoea batatas*). Contents of quercitrin and isoquercitrin were quantified in leaves of *E. elatior.*

Extraction of leaves

Fresh leaves (1 g) were powdered with liquid nitrogen in a mortar and extracted by 50% aqueous methanol (50 ml), with continuous swirling for one hour at room temperature using an orbital shaker. Extracts were filtered under suction and stored at –20°C for further use. Analysis of extracts was done in triplicate.

Caffeoylquinic acid content

Caffeoylquinic acid content (CQAC) of leaf extracts was determined using the molybdate assay (Clifford & Wright, 1976).

Chlorogenic acid content

Chlorogenic acid content (CGAC) of leaf extracts was quantified using reverse-phase HPLC (Agilent Technologies 1200 Series) with Thermo Scientific BDS Hypersil Phenyl Column (4.6 x 100 mm). A 15-min linear gradient from 5% to 100% MeOH, was used to elute samples at 1 ml/min. Mobile phases were acidified with 0.1% trifluoroacetic acid for better resolution. A 20 µl loop was used for injection and elution was monitored at 280 nm. Identity of CGA was determined by matching UV spectrum and retention times. The amount of CGA present was quantified using peak areas. The calibration equation of peak area (mAU*s) against concentration of CGA (mg/ml) was y = 7286.7x (R^2 = 0.9998). CGAC was expressed as mg CGA/100 g of fresh leaves.

Flavonoid content

Quercitrin and isoquercitrin in leaves of *E. elatior* were quantified using reverse-phase HPLC similar to the method described for chlorogenic acid. The calibration equation of peak area (mAU*s) against concentration (mg/ml) was y = 18664x (R^2 = 0.9980) for quercitrin and y = 28846x (R^2 = 0.9960) for isoquercitrin. Quercitrin and isoquercitrin content was expressed as mg/100 g of fresh leaves.

Bioactivity of compounds

Bioactivity of the three major isolated compounds was determined based on commercial standards. The methods used to determine their DPPH radical scavenging, antibacterial and tyrosinase inhibition activities have been described

in sections 3.4.3, 3.4.4 and 3.4.5, respectively. Caffeic acid and ascorbic acid were used as controls for DPPH radical scavenging.

3.6.2 Essential oils

Extraction

Leaves (500 g) of four species of *Etlingera*, namely, *E. elatior*, *E. fulgens*, *E. maingayi* and *E. rubrostriata*, were collected from Janda Baik, Pahang. Leaves were sorted, cleaned and their mid-ribs removed. They were then cut into small pieces, immersed in 1 L of deionised water and hydro-distilled for 16 h in a 5 L flask attached to an Allihn condenser with continuous cooling with ice cold water. Essential oils extracted were collected with a modified Clavenger apparatus.

Analysis

Oils of four species of *Etlingera* were sent to the Division of Biotechnology of FRIM for analyses using gas chromatography (GC) and GC-MS. The protocol is essentially the same as that described for analysing oil of *Alpinia conchigera* (Ibrahim *et al*., 2009).

GC-MS analyses, used to identify essential oils, were performed on HP 5975-7890 GC-MSD system operating in the electron ionization (EI) mode at 70 eV, equipped with HP-5MS fused silica capillary column (30 m x 0.25 mm; 0.25 µm film thickness). The column and injector temperature were the same as those for GC. The chemical constituents were identified by comparison of their retention times (RT) with literature values and their mass spectral data with those from the HPCH2205.L and NIST05a.L mass spectral database.

GC analysis, used to quantify essential oils, was carried out on a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector (FID) using fused silica capillary column CBP-5 (25 x 0.25 mm; 0.25 µm film thickness). Helium was used as carrier gas and the injector and detector temperature were set up at 220° and 280°C, respectively. The oven temperature was programmed from 60° to 230°C at 3°C/min and finally held at 230°C for 10 min whilst the volume injected was 1.0 µl. Composition of essential oils was expressed as percentage of total peak area.

Antibacterial activity

Antibacterial activity of essential oils from leaves of the four *Etlingera* species was screened using the wet disc diffusion method (Holder, 1989). Agar cultures of Gram-positive bacteria of *B. cereus*, *M. luteus* and *S. aureus*, and Gram-negative bacteria of *E. coli*, *P. aeruginosa* and *S. choleraesuis* were prepared. Suspensions of bacteria (100 µl) were spread evenly onto 20 ml Mueller-Hinton agar preset in 90 mm Petri dishes. Paper discs (6 mm diameter) were impregnated with 10 µl of essential oils serially diluted two-fold with DMSO. Impregnated discs were transferred onto inoculated agar together with streptomycin susceptibility discs (10 µg) as positive controls and DMSO discs as negative controls. After incubation overnight at 37°C, inhibition zones were measured and recorded as mean diameter (mm). Results were expressed as minimum inhibitory concentration (MIC), the minimum concentration of essential oils required to show a zone of inhibition.

3.7 STANDARDISED EXTRACT

3.7.1 Drying of leaves

The effect of two drying treatments on the TPC, CQAC and CGAC of *E. elatior* leaves were evaluated. The purpose was to determine a suitable drying treatment for storage and maceration prior to extraction. The treatments were microwave- and freeze-drying. In microwave-drying, leaves (1 g) were dried in a microwave oven for 4 min. In freeze-drying, leaf samples were lyophilized at 0.125 mbar and at –50°C overnight in a freeze-dryer.

3.7.2 Choice of solvent

Leaves of *E. elatior* (1 g, in triplicate) were extracted with 50 ml of 70%, 50% and 30% aqueous ethanol for one hour in orbital shaker. Extracts were analysed for TPC and CQAC using the Folin–Ciocalteu and molybdate assays, respectively.

3.7.3 Extraction of leaves

Leaves of *E. elatior* (50 g, in triplicate) were freeze-dried and ground in a blender. Ground leaves were extracted 4 times with 500 ml of 30% aqueous ethanol for one hour each time in orbital shaker. Crude extracts were filtered under suction and the solvent removed with a rotary evaporator at 50˚C. For each batch, residues were weighed (~4 g) and stored at –20°C for further use.

3.7.4 Separation with Diaion

The 30% ethanol crude extracts of leaves (in triplicate) were subjected to column chromatography. Each extract was dissolved in 10 ml of 20% aqueous ethanol and chromatographed over a 40 g Diaion HP-20 column. Fractions were eluted using a H_2O:EtOH 0-35% step-gradient with an increment of 5% ethanol every 100 ml. The column was flushed with 200 ml of 100% ethanol after elution of each extract. Eluents from 0-5% ethanol, 10–35% ethanol and 100% ethanol were pooled into fractions 1, 2 and 3, respectively. Fractions were dried in a rotary-evaporator at 50˚C prior to analysis. CGA was eluted in fraction 2.

3.7.5 Separation with Sephadex

Attempts were made to further refine fraction 2 (10–35% ethanol) that had the highest CGAC. The fractions were re-dissolved in 5 ml of 20% aqueous ethanol and chromatographed over a 10 g Sephadex LH-20 column. The column was eluted with 100 ml of water (fraction 2.1) followed by 200 ml of 20% aqueous ethanol (fraction 2.2) and 200 ml of ethanol (fraction 2.3). Fractions were dried in a rotary-evaporator at 50˚C prior to analysis. CGA was eluted in fraction 2.2.

3.7.6 Bioactivity of fractions

The various fractions collected after passing through Diaion HP-20 and subsequently Sephadex LH-20 were tested for radical scavenging, antibacterial and tyrosinase inhibition activities.

3.7.7 LC-MS of fraction

As fraction 2.3 collected from Sephadex-LH20 showed enhanced tyrosinase inhibition, the fraction was analysed with liquid chromatography mass spectrometry (LC-MS) to identify major components. The analysis was done using a ThermoFinnigan LCQDeca mass spectrometer. Sample components were separated with a GL-Sciences Inertsil 5 µm (2.1 x 150 mm) column eluded with a 35-min linear gradient from 20% to 100% MeOH acidified with 1% acetic acid at 0.25 ml/min. LC-MS mass spectra were acquired in negative ion mode. Mass ranging up to 2000 m/z was measured. A chromatogram was generated using a full range photo-diode array scan from 200-800 nm.

Chapter 4

RESULTS AND DISCUSSION

4.1 PRELIMINARY SCREENING

4.1.1 Choice of genus and solvent

To find out the genus with the highest phenolic content and strongest antioxidant activity, and the solvent suitable for extraction of leaves and rhizomes of ginger species, total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves and rhizomes of five ginger species belonging to five genera were screened using methanol and dichloromethane (DCM) as solvents. The species were *Alpinia malaccensis*, *Elettariopsis slahmong*, *Etlingera maingayi*, *Scaphochlamys kunstleri* and *Zingiber spectabile.*

Results showed that methanol extracts of leaves of *E. maingayi* had the highest TPC and AEAC with values of 1110 ± 93 mg GAE/100 g and 963 ± 169 mg AA/100 g followed by leaves of *A. malaccensis* with values of 744 ± 61 mg GAE/100 g and 800 ± 62 mg AA/100 g, respectively [Table 4.1]. With the exception of *A. malaccensis* and *Z. spectabile*, TPC and AEAC of leaves of *E. maingayi*, *E. slahmong* and *S. kunstleri* were significantly higher than those of rhizomes.

The efficiency of DCM extraction was based only on AEAC as the solvent does not extract phenolic compounds. Compared to methanol, DCM extraction yielded much lower values for all five species [Table 4.1]. Leaves of *E. maingayi* and *A. malaccensis* yielded AEAC values of only 21 ± 4 and 26 ± 1 mg AA/100 g, respectively. These values were 46 and 31 times lower than those of methanol extracts. In all cases, leaves had significantly higher values than rhizomes despite very low values.

Table 4.1
Screening of total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves (L) and rhizomes (R) of five ginger species using methanol and dichloromethane (DCM) as solvent (fresh weight)

Species and location	Solvent	Part	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
Etlingera maingayi Janda Baik	Methanol	L	1110 ± 93a	963 ± 169a
		R	160 ± 52b	122 ± 53b
	DCM	L		21 ± 4c
		R		2 ± 1d
Alpinia malaccensis FRIM	Methanol	L	744 ± 61a	800 ± 62a
		R	564 ± 209a	745 ± 342a
	DCM	L		26 ± 1b
		R		3 ± 2c
Elettariopsis slahmong FRIM	Methanol	L	346 ± 45a	269 ± 67a
		R	219 ± 57b	197 ± 76a
	DCM	L		17 ± 4b
		R		7 ± 3c
Zingiber spectabile FRIM	Methanol	L	242 ± 7a	121 ± 24a
		R	157 ± 100a	124 ± 109a
	DCM	L		9 ± 1b
		R		4 ± 2c
Scaphochlamys kunstleri FRIM	Methanol	L	203 ± 21a	171 ± 33a
		R	73 ± 3b	14 ± 2b
	DCM	L		23 ± 3c
		R		2 ± 1d

Values of TPC and AEAC are means ± SD (n = 3). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA compares extraction values of leaves and rhizomes of a species and does not apply between species. Abbreviations: GAE = gallic acid equivalent and AA = ascorbic acid.

From the preliminary screening of species and solvents, *Etlingera* species were selected for study and methanol was the choice of solvent for extraction. Leaves of *Etlingera* species were accorded high priority as they yielded the highest TPC and AEAC. Furthermore, the bioactivity and chemical constituents of *Etlingera* leaves have never been studied in depth.

The extraction efficiency of different solvents (methanol, 50% aqueous methanol, ethyl acetate and DCM) was also tested using leaves of *Etlingera elatior* and *Curcuma longa*. Results showed that more polar solvents i.e. methanol and 50% aqueous methanol extracted more phenolic compounds and thus yielded higher values of TPC, AEAC and ferric reducing power (FRP).

TPC, AEAC and FRP values of leaves of *E. elatior* were 2320 ± 110 mg GAE/100 g, 3260 ± 110 mg AA/100 g and 16 ± 0.7 mg GAE/g for methanol, and 2480 ± 158 mg GAE/100 g, 3390 ± 94 mg AA/100 g and 17 ± 0.1 mg GAE/g for 50% aqueous methanol, respectively [Table 4.2]. Values of leaves of *C. longa* were 289 ± 17 mg GAE/100 g, 171 ± 39 mg AA/100 g and 1.8 ± 0.1 mg GAE/g for methanol, and 284 ± 8 mg GAE/100 g, 163 ± 7 mg AA/100 g and 1.2 ± 0.1 mg GAE/g for 50% aqueous methanol, respectively.

These values of methanol and 50% aqueous methanol were significantly higher than those of ethyl acetate and DCM. As methanol gave significantly higher FRP value than 50% aqueous methanol for leaves of *C. longa*, as well as ease of evaporation, methanol was chosen as the extract for extraction. Most of the antioxidant compounds in *E. elatior* and *C. longa* leaves are phenolic in nature. DCM being a non-polar solvent extracted minimal amounts of phenolic compounds and showed low TPC, AEAC and FRP values. Ethyl acetate, being slightly more polar than DCM, extracted more phenolic compounds and yielded significantly higher values.

Table 4.2
Extraction efficiency of methanol, 50% aqueous methanol, ethyl acetate and dichloromethane (DCM) on leaves of *Etlingera elatior* and *Curcuma longa* based on total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP)

Species and solvent	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
Etlingera elatior			
Methanol	2320 ± 110a	3260 ± 110a	16 ± 0.7a
50% methanol	2480 ± 158a	3390 ± 94a	17 ± 0.1a
Ethyl acetate	665 ± 24b	614 ± 29b	5.4 ± 0.1b
DCM *	84 ± 3c	22 ± 2c	0.4 ± 0.1c
Curcuma longa			
Methanol	289 ± 17a	171 ± 39a	1.8 ± 0.1a
50% methanol	284 ± 8a	163 ± 7a	1.2 ± 0.1b
Ethyl acetate	163 ± 13b	48 ± 4b	0.8 ± 0.1c
DCM *	47 ± 5c	28 ± 6c	0.3 ± 0.1d

* Samples extracted with DCM were re-dissolved in methanol

TPC, AEAC and FRP values are means ± SD (n = 3). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA compares extraction values of a species and does not apply between species. Abbreviations: GAE = gallic acid equivalent and AA = ascorbic acid.

There are no satisfactory solvents suitable for isolation of all classes or a specific class of plant phenolic compounds (Shahidi & Naczk, 2004c). Generally, water, ethanol and methanol are used for extraction of polar compounds; ethyl acetate and DCM for compounds of medium polarity; and n-hexane, chloroform for non-polar compounds (Sarker *et al*., 2006).

Organic solvents are much easier to evaporate than water (Shimizu & Li, 2006). The use of organic solvents also prevents microbial growth, which is one of the most serious problems associated with aqueous extraction. Methanol and aqueous methanol are the most suitable solvent for extracting phenolic compounds from plant tissues due to their ability to inhibit the action of polyphenol oxidases that cause the oxidation of phenolic compounds and their ease of evaporation compared to water (Waterman & Mole, 1994; Arts & Hollman, 1998; Yao *et al*., 2004b; Bravo & Mateos, 2008).

4.2 *ETLINGERA* SPECIES

4.2.1 Extraction efficiency

Based on TPC, methanol showed high extraction efficiency of leaves of *Etlingera* species. Yields of the first extractions ranged from 83% in *E. rubrostriata* and *E. littoralis* to 88% in *E. maingayi* [Table 4.3]. Second extractions yielded values of 8–13%. Third extractions yielded values of 4% and 6%. For leaves of *E. elatior*, the main species of study in this project, the triple extractions yielded values of 84%, 12% and 4%.

TPC and AEAC of leaves of four *Etlingera* species were evaluated using methanol and 50% aqueous methanol extraction. Both extractions yielded comparable data for all species [Table 4.4]. The exception was TPC of leaves of *E. fulgens* where methanol extraction (2360 ± 134 mg GAE/100 g) was significantly higher than that of 50% methanol (1950 ± 171 mg GAE/100 g). It was decided that methanol be used for future extractions of leaves of *Etlingera* species.

Table 4.3
Methanol extraction efficiency of leaves of *Etlingera* species

Etlingera species	Extraction	TPC (mg GAE/100 g)	Yield (%)
E. elatior	First	3550 ± 304	84 ± 1.3
	Second	514 ± 48	12 ± 1.2
	Third	146 ± 6	4 ± 0.2
E. rubrostriata	First	3480 ± 390	83 ± 1.2
	Second	543 ± 83	13 ± 0.9
	Third	187 ± 26	4 ± 0.3
E. littoralis	First	2810 ± 243	83 ± 1.1
	Second	382 ± 19	11 ± 0.7
	Third	184 ± 22	6 ± 0.5
E. fulgens	First	2270 ± 31	86 ± 1.4
	Second	271 ± 34	10 ± 1.0
	Third	101 ± 13	4 ± 0.4
E. maingayi	First	1112 ± 93	88 ± 0.5
	Second	99 ± 15	8 ± 0.5
	Third	50 ± 5	4 ± 0.2

Abbreviations: TPC = total phenolic content and GAE = gallic acid equivalent

Methanol has been recommended for the extraction of phenolic compounds from fresh plant tissues (Waterman & Mole, 1994). It is the solvent most commonly employed as it produces good yields (Escribano-Bailon & Santos-Buelga, 2003). Methanol had also been reported to be the most suitable solvent for extracting phenolic compounds from fresh young shoots of tea, compared with chloroform, ethyl acetate and water (Yao *et al*., 2004b).

Table 4.4
Extraction efficiency of leaves of *Etlingera* species with 100% and 50% methanol

Etlingera species	Methanol	TPC (mg GAE /100 g)	AEAC (mg AA/100 g)
E. elatior	100%	3490 ± 409a	4580 ± 341a
	50%	3570 ± 314a	4460 ± 652a
E. rubrostriata	100%	2250 ± 113a	2290 ± 118a
	50%	2200 ± 129a	2300 ± 151a
E. fulgens	100%	2360 ± 134a	2130 ± 247a
	50%	1950 ± 171b	1870 ± 261a
E. littoralis	100%	2150 ± 94a	1990 ± 87a
	50%	2000 ± 58a	1870 ± 83a

Values of TPC and AEAC are means ± SD (n = 3). For each column, values followed by the same letter (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA compares values of 100% and 50% methanol of each species and does not apply between species. Abbreviations: GAE = gallic acid equivalent and AA = ascorbic acid.

4.2.2 Moisture content

The moisture content of leaves of five *Etlingera* species was calculated by drying of leaves in a microwave oven for 5 min. For the five species, values varied from 66% in *E. elatior* to 75% in *E. maingayi* [Table 4.5]. Leaves of *E. maingayi* and *E. fulgens* had the highest moisture content of 75% and 74%, respectively, while leaves of *E. elatior* had the lowest moisture content.

Table 4.5
Moisture content (%) of leaves of *Etlingera* species

Etlingera species	Moisture content (%)
E. maingayi	75 ± 1.2a
E. fulgens	74 ± 0.1a
E. rubrostriata	72 ± 2.8ab
E. littoralis	71 ± 0.8b
E. elatior	66 ± 2.0c

Values of moisture content are means ± SD (n = 3). Values followed by the same letter (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test.

4.2.3 Antioxidant properties

Leaves of *Etlingera* species

Of the five *Etlingera* species studied, leaves of *E. elatior* and *E. rubrostriata* had the highest TPC [Table 4.6]. Values were 3550 ± 304 and 3480 ± 390 mg GAE/100 g, respectively. Leaves of *E. fulgens* and *E. maingayi* had significantly lower TPC of 2540 ± 91 and 1110 ± 93 mg GAE/100 g, respectively.

Results showed that leaves of *E. elatior* and *E. rubrostriata* had the highest AEAC and FRP. Values were 3750 ± 555 mg AA/100 g and 20 ± 2.1 mg GAE/g for *E. elatior*, and 3540 ± 401 mg AA/100 g and 17 ± 2.4 mg GAE/g for *E. rubrostriata*, respectively. Moderately high AEAC and FRP were found in the leaves of *E. littoralis* and *E. fulgens*. Values were 2930 ± 220 mg AA/100 g and 12 ± 1.0 mg GAE/g for *E. maingayi*, and 2030 ± 126 mg AA/100 g and 9.4 ± 0.4 mg GAE/g for *E. fulgens*, respectively. Lowest values of 963 ± 169 mg AA/100 g and 4.9 ± 0.8 mg GAE/g were found in the leaves of *E. maingayi*. Among the

species of *Etlingera* studied, leaf AEAC and FRP shared the same order of ranking as leaf TPC of *E. elatior* ~ *E. rubrostriata* > *E. littoralis* > *E. fulgens* > *E. maingayi.* It is evident that *Etlingera* species with high leaf TPC also have high AEAC and FRP.

Table 4.6
Total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP) of leaves of *Etlingera* species (fresh weight)

Etlingera species	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
E. elatior	3550 ± 304a	3750 ± 555a	20 ± 2.1a
E. rubrostriata	3480 ± 390a	3540 ± 401a	16 ± 2.4a
E. littoralis	2810 ± 242b	2930 ± 220b	12 ± 1.0b
E. fulgens	2540 ± 91c	2030 ± 126c	9.4 ± 0.4c
E. maingayi	1110 ± 93d	963 ± 169d	4.9 ± 0.8d

TPC, AEAC and FRP are means ± SD (n = 3). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$, as measured by the Tukey HSD test. Abbreviations: GAE = gallic acid equivalent and AA = ascorbic acid.

In terms of ferrous ion chelating (FIC) ability, the trend was reversed with leaves of *E. maingayi* and *E. fulgens* having the highest values [Fig. 4.1]. Leaves of *E. maingayi* and *E. fulgens* were superior to the FIC ability of young leaves of *Camellia sinensis*, used as positive control. Values of leaves of *E. elatior* and *E. littoralis* were comparable. Lowest values were found in the leaves of *E. rubrostriata.*

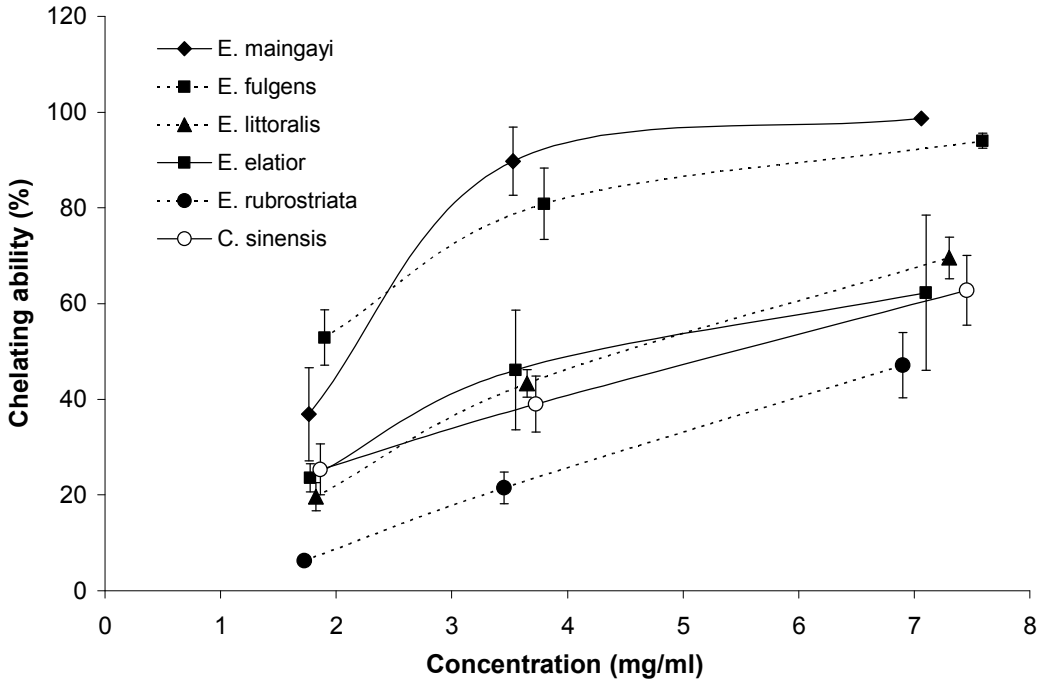


Fig. 4.1
Ferrous ion chelating (FIC) ability of leaves of *Etlingera* species (fresh weight). Young leaves of *Camellia sinensis* were used as positive control. Results are means ± SD (n = 3).

In terms of lipid peroxidation inhibition (LPI) activity based on β-carotene bleaching (BCB), leaves of *E. maingayi* had the highest values [Fig. 4.2]. LPI activity of *E. maingayi* was similar to or slightly better than that of rhizomes of *C. longa.* Leaves of *E. rubrostriata*, *E. littoralis* and *E. elatior* had slightly lower values. Although leaves of *E. fulgens* showed the lowest LPI activity, values were higher than that of young leaves of *C. sinensis* but lower than that of *Zingiber officinale* rhizomes. With the exception of *E. fulgens*, leaves of all *Etlingera* species studied showed high LPI activity, comparable with that of rhizomes of *C. longa* and superior to that of young leaves of *C. sinensis* and rhizomes of *Z. officinale*.

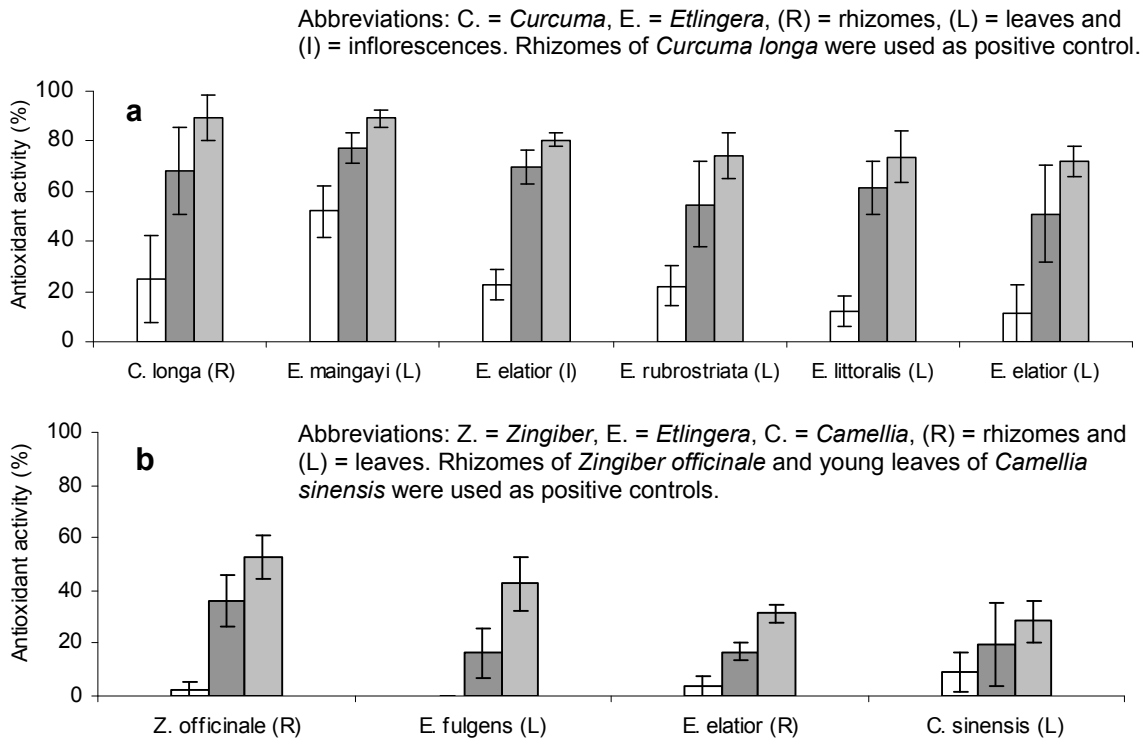


Fig. 4.2
β-carotene bleaching (BCB) activity of leaves of *Etlingera* species (fresh weight). For each species, left, middle and right bars are extract concentrations of 0.2, 1.0 and 2.0 mg in 3 ml, respectively. Results are means ± SD (*n* = 3).

It can be seen that leaves of *Etlingera* species with high TPC, AEAC and FRP have low FIC ability and *vice versa*. This would mean that phenolic compounds in extracts responsible for antioxidant activities of scavenging free radicals and reducing ferric ions might not be directly involved in ferrous ion chelation. The compounds responsible could be nitrogen-containing compounds, which are generally better chelators than are phenols. Similar observations were made with leaves of *Alpinia*. Of four species studied, leaves of *Alpinia galanga*, with the lowest TPC, AEAC and FRP, exhibited the highest FIC ability (Wong, 2006).

High BCB activity of leaves of *Etlingera* species reflects their ability to strongly inhibit lipid peroxidation. There appears to be no correlation between BCB activity and antioxidant activity as measured by the other assays. Lim and Quah

(2007) also reported that methanolic extracts of *Portulaca oleracea* showed that TPC correlated well with AEAC and FRP but not with BCB activity.

Different plant parts

Analyses of different plant parts of *E. elatior* showed that leaves had significantly higher TPC, AEAC and FRP than inflorescences and rhizomes at $P < 0.05$ [Table 4.7]. Values were 3550 ± 304 mg GAE/100 g, 3750 ± 555 mg AA/100 g and 20 ± 2.1 mg GAE/g for leaves, 295 ± 24 mg GAE/100 g, 268 ± 45 mg AA/100 g and 1.5 ± 0.2 mg GAE/g for inflorescences, and 187 ± 46 mg GAE/100 g, 185 ± 59 mg AA/100 g and 0.9 ± 0.2 mg GAE/g for rhizomes, respectively.

Table 4.7
Total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP) of different parts of *Etlingera elatior* (fresh weight)

Plant part	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
Leaves	3550 ± 304a	3750 ± 555a	20 ± 2.1a
Inflorescences	295 ± 24b	268 ± 45b	1.5 ± 0.2b
Rhizomes	187 ± 46c	185 ± 59c	0.9 ± 0.2c

TPC, AEAC and FRP values are means ± SD (n = 3). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$, as measured by the Tukey HSD test. Abbreviations: GAE = gallic acid equivalent and AA = ascorbic acid.

Similarly, leaves of *E. elatior* showed superiority over inflorescences and rhizomes in terms of FIC ability [Fig. 4.3]. FIC ability of leaves was comparable to that of young leaves of *C. sinensis*. LPI activity of leaves was much higher than that of rhizomes but slightly lower than that of inflorescences [Fig. 4.2]. LPI

values of inflorescences and rhizomes were comparable to those of rhizomes of *C. longa* and young leaves of *C. sinensis*, respectively. Ranking of TPC, AEAC, FRP and FIC was in the order: leaves > inflorescences > rhizomes.

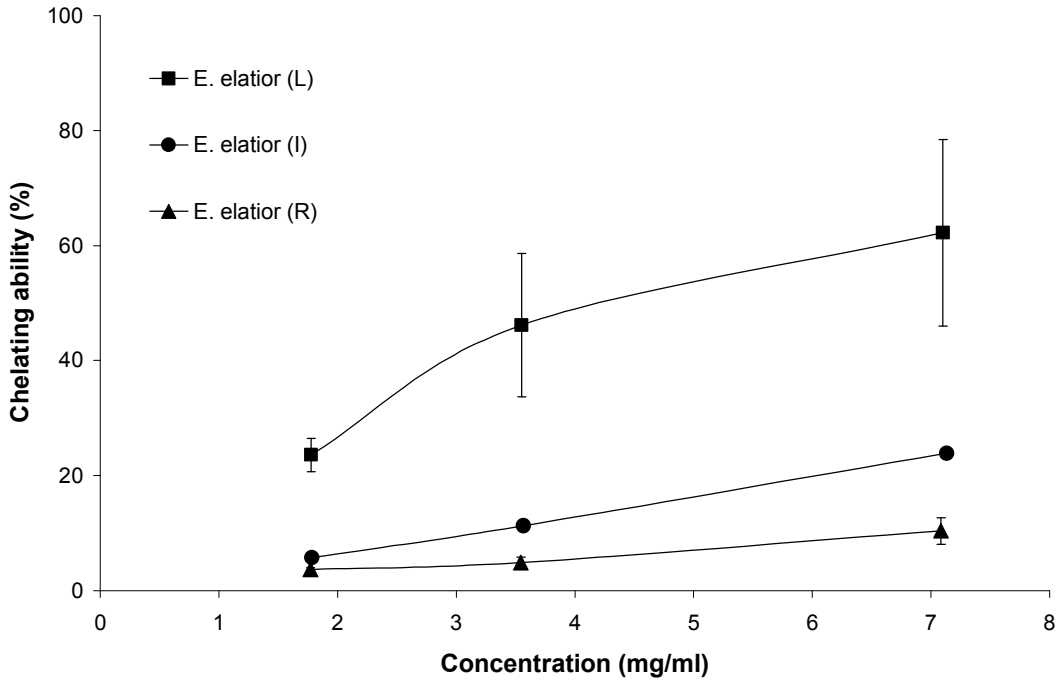


Fig. 4.3
Ferrous ion chelating (FIC) ability of leaves (L), inflorescences (I) and rhizomes (R) of *Etlingera* species (fresh weight)

Results of this study on the different plant parts of *E. elatior* reaffirmed that both TPC and antioxidant activity of leaves were significantly higher than those of inflorescences and rhizomes. TPC, AEAC and FRP of leaves were 12, 14 and 13 times higher than inflorescences, and 19, 20 and 22 times higher than rhizomes, respectively. Leaves of wild and cultivated *Etlingera* species therefore contain more antioxidants than do other plant parts. Recently, Elzaawely *et al*. (2007) reported that ethyl acetate extracts from leaves of *Alpinia zerumbet* showed higher inhibition of β-carotene oxidation and scavenging activity of free radicals than did rhizomes. This further supports our result that leaves have free radical scavengers that are more effective than those found in rhizomes.

Altitudinal variation

Leaves of all *Etlingera* species sampled from highland populations were found to have higher TPC and AEAC than those of lowland counterparts. Leaves of *E. rubrostriata*, *E. elatior* and *E. fulgens* showed significantly higher values at higher altitude, while those of *E. littoralis* were marginally higher [Table 4.8].

Table 4.8
Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves of four *Etlingera* species sampled from highland and lowland locations

Etlingera species	Location	Altitude (m asl)	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
E. elatior	Janda Baik	400	3550 ± 304a	3750 ± 555a
	FRIM	100	2390 ± 329b	2280 ± 778b
E. rubrostriata	Ulu Gombak	300	3480 ± 390a	3540 ± 401a
	FRIM	100	2430 ± 316b	2640 ± 508a
E. littoralis	*Genting Highlands*	800	2810 ± 243a	2930 ± 220a
	FRIM	100	2340 ± 386a	2220 ± 913a
E. fulgens	Janda Baik	400	2270 ± 31a	2030 ± 126a
	FRIM	100	1280 ± 143b	845 ± 158b

Values of TPC and AEAC are means ± SD (n = 3). For columns of each species, values followed by the same letter (a–b) are not significantly different at $P < 0.05$ measured by the Tukey HSD test. ANOVA does not apply between species. Abbreviations: GAE = gallic acid equivalent, AA = ascorbic acid and asl = above sea level.

Highest leaf TPC and AEAC were found in highland populations of *E. elatior*, with values of 3550 ± 304 mg GAE/100 g and 3750 ± 555 mg AA/100 g followed by leaves of *E. rubrostriata*, with values of 3480 ± 390 mg GAE/100 g and 3540 ± 401 mg AA/100 g, respectively. Lowland populations of *E. fulgens* had the lowest values of 1280 ± 143 mg GAE/100 g and 845 ± 158 mg AA/100 g, respectively.

Higher altitudes seem to trigger an adaptive response in *Etlingera* species. Higher leaf TPC and AEAC of highland populations over those of lowland counterparts could be due to environmental factors such as higher UV-B radiation and lower air temperature.

There is increasing evidence that enhanced UV-B radiation induces production of phenolic compounds in plants (Bassman, 2004). Accumulation of flavonoids is markedly increased in both the lower epidermis and underlying tissues when primary leaves of barley are exposed to UV-B (Liu *et al*., 1995). Enzymes associated with the synthesis of phenolics are produced in greater quantity with increased activity (Chalker-Scott & Scott, 2004). Phenylalanine ammonia lyase (PAL) is up-regulated, resulting in the accumulation of flavonoids and anthocyanins, which have antioxidant ability (Jansen *et al*., 1998). Low temperatures have also been shown to enhance PAL synthesis in a variety of plants, leading to increased production of flavonoids and other phenolics (Chalker-Scott & Scott, 2004). Decreased temperatures triggered greater biosynthesis of phenolics in some plant species, even in the absence of UV-B radiation (Bilger *et al.*, 2007). Recently, Albert *et al.* (2009) reported that lower air temperature rather than enhanced UV-B radiation as the key factor influencing the altitudinal variation of phenolics in *Arnica montana.*

4.2.4 Antibacterial activity

Using the disc-diffusion method, leaves of all five *Etlingera* species were found to inhibit Gram-positive *Bacillus cereus*, *Micrococcus luteus* and *Staphylococcus aureus* [Table 4.9]. Leaves of *E. elatior*, *E. fulgens* and *E. maingayi* exhibited moderate inhibition of the three bacteria. Moderate inhibition was shown by leaves of *E. rubrostriata* on *B. cereus* and *S. aureus*, and by leaves of *E. littoralis* on *S. aureus*. Mean diameter of the zone of inhibition of streptomycin was 23 mm for *M. luteus*, and 17 mm for *B. cereus* and *S. aureus*. Methanol showed no

inhibitory effect on the three bacteria. Streptomycin was used as positive control because it is an antibiotic for Gram-positive and Gram-negative bacteria.

Table 4.9
Antibacterial activity of leaves of *Etlingera* species (fresh weight)

Etlingera species	Zone of inhibition (mm)					
	Gram-positive bacteria			Gram-negative bacteria		
	B. cereus	*M. luteus*	*S. aureus*	*E. coli*	*P. aeruginosa*	*S. cholerasuis*
E. elatior	10(59)++	13(57)++	11(65)++	–	–	–
E. fulgens	11(65)++	12 (52)++	11(65)++	–	–	–
E. littoralis	8(47)+	9(39)+	9(53)++	–	–	–
E. maingayi	9(53)++	14(61)++	11(65)++	–	–	–
E. rubrostriata	9(53)++	11(48)+	10(59)++	–	–	–
Streptomycin	17	23	17	16	17	15
Methanol	–	–	–	–	–	–

Figures in parentheses are inhibition percentages compared to streptomycin. Antibacterial activity is categorized as strong +++ for inhibition ≥ 70%, moderate ++ for inhibition 50 < 70%, or weak + for inhibition < 50%. Concentration used was 1 mg extract per disc. Abbreviations: *B.* = *Bacillus*, *M.* = *Micrococcus*, *S.* = *Staphylococcus*, *E.* = *Escherichia*, *P.* = *Pseudomonas* and *S.* = *Salmonella*.

Among the Gram-positive bacteria, *S. aureus* appeared to be more sensitive. Screening for antibacterial activity of 191 plant extracts belonging to 30 families of plants from Sabah, Malaysia, showed similar results (Chung *et al*., 2004). About 52% of the extracts inhibited *S. aureus*. Leaves of *Etlingera* showed no activity on Gram-negative bacteria of *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella choleraesuis.* Antibacterial studies of extracts from various ginger species also showed no inhibition of Gram-negative bacteria (Chandarana *et al*., 2005; Wong, 2006).

Gram-negative bacteria have an outer membrane consisting of lipoprotein and lipopolysaccharide, which is selectively permeable and thus regulates access to the underlying structures (Chopra & Greenwood, 2001). This renders the Gram-negative bacteria generally less susceptible to plant extracts than the Gram-positive bacteria.

Preliminary investigation on the use of ethylenediamine tetraacetic acid (EDTA) to improve the efficacy of leaf extracts of *Etlingera* species against Gram-negative bacteria was carried out. Adding 2 mM EDTA to the agar caused *P. aeruginosa* to be susceptible to all leaf extracts of *Etlingera* species [Table 4.10]. Moderate inhibition of *P. aeruginosa* was shown by leaves of *E. elatior* and *E. fulgens*. Leaves of *E. littoralis*, *E. maingayi* and *E. rubrostriata* showed weak inhibition. EDTA, however, inhibited the growth of *E. coli* and *S. choleraesuis.*

EDTA has been reported to permeabilise the outer membrane of *P. aeruginosa*, making the bacteria susceptible to antibiotics and certain antiseptic agents (Haque & Russell, 1974). Bacteria can be either exposed to the permeabiliser, together with the antibiotic, or pre-treated with the permeabiliser prior to introduction of the antibiotic (Ayres *et al*., 1999). It is the first time the method has been used for testing antibacterial activity of plant extracts. Initial findings warrant further investigations.

Table 4.10
Antibacterial activity of leaves of *Etlingera* species on *Pseudomonas aeruginosa* after adding 2 mM of ethylenediamine tetraacetic acid (EDTA) to the agar

Etlingera species	Zone of inhibition (mm)
E. elatior	9(50)++
E. fulgens	10(56)++
E. littoralis	7(39)+
E. maingayi	8(44)+
E. rubrostriata	8(44)+
Streptomycin	18

Figures in parentheses are inhibition percentages compared to streptomycin. Antibacterial activity is categorized as strong +++ for inhibition ≥ 70%, moderate ++ for inhibition 50 < 70%, or weak + for inhibition < 50%. Concentration used was 1 mg extract per disc with 2 mM EDTA added into Mueller-Hinton agar.

4.2.5 Tyrosinase inhibition activity

With outstanding leaf TPC and AEAC, methanol extracts of leaves of five *Etlingera* species were analysed for tyrosinase inhibition activity using the dopachrome method with L-DOPA as the substrate. Leaves of *Hibiscus tiliaceus* were chosen as positive control as they displayed the highest tyrosinase inhibition among 39 tropical plant species screened (Masuda *et al.*, 2005).

Tyrosinase inhibition properties was strongest in leaves of *E. elatior* (55%), which was significantly higher than *H. tiliaceus*, the positive control (44%) [Table 4.11]. Inhibition of leaves of *E. fulgens* (49%) and *E. maingayi* (43%) was comparable. Activity of leaves of *E. rubrostriata* (30%) and *E. littoralis* (22%) was significantly lower.

Table 4.11
Tyrosinase inhibition activity of leaves of *Etlingera* species (fresh weight)

Etlingera species	Tyrosinase inhibition % (0.5 mg/ml extract)
E. elatior	55 ± 3.1a
E. fulgens	49 ± 6.5ab
E. maingayi	43 ± 4.2b
E. rubrostriata	30 ± 4.0c
E. littoralis	22 ± 5.2c
Hibiscus tiliaceus	44 ± 4.6b

Results are means ± S.D. (n = 3). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$ as measured by Tukey HSD test. Leaves of *Hibiscus tiliaceus* were used as positive control.

Etlingera species are the largest of the gingers and they grow in open forest sites that are exposed to strong sunlight. Three out of five species studied had activity values that were significantly higher or comparable to the positive control. Findings from this study agree with the observation made by Masuda *et al.* (2005) that plant species, which are exposed to full sunlight, possess strong antioxidant activity and high tyrosinase inhibition ability.

4.2.6 Cytotoxicity

All leaf extracts of *Etlingera* species did not exhibit cytotoxic effect on WRL-68 and Vero cells, which are normal human liver and African green monkey kidney cells, respectively [Table 4.12]. IC_{50} values ranged from 560 to 663 µg/ml and from 591 to 704 µg/ml, respectively. These values are much higher than IC_{50} of paclitaxel which were 0.08 and 0.01µg/ml, respectively.

Table 4.12
Cytotoxity of leaf extracts of *Etlingera* species on WRL-68 and Vero cells using the sulforhodamine B assay

Etlingera species	Cell	Cell survival (%)*			IC_{50} (µg/ml)
		0.1 µg/ml	10 µg/ml	1000 µg/ml	
E. elatior	WRL-68	98.9 ± 0.37	97.9 ± 0.33	31.0 ± 0.79	563
	Vero	100 ± 0.13	95.7 ± 1.09	25.7 ± 1.31	704
E. rubrostriata	WRL-68	101 ± 1.70	97.9 ± 1.27	27.0 ± 0.79	560
	Vero	99.6 ± 0.67	95.6 ± 0.82	24.5 ± 0.55	698
E. maingayi	WRL-68	101 ± 0.52	98.4 ± 1.37	22.3 ± 0.72	660
	Vero	119 ± 0.81	119 ± 0.58	4.12 ± 0.52	636
E. fulgens	WRL-68	99.3 ± 0.44	98.0 ± 1.30	26.3 ± 0.79	561
	Vero	97.5 ± 0.13	97.7 ± 0.20	19.5 ± 0.56	624
E. littoralis	WRL-68	98.0 ± 0.85	98.6 ± 0.82	24.9 ± 0.69	663
	Vero	97.6 ± 0.23	98.7 ± 0.48	21.8 ± 0.52	591
Positive control	Cell	0.001 µg/ml	0.1 µg/ml	10 µg/ml	IC_{50} (µg/ml)
Paclitaxel	WRL-68	95.5 ± 0.29	37.3 ± 0.35	16.9 ± 1.01	0.08
	Vero	65.6 ± 0.83	17.2 ± 0.88	6.40 ± 0.24	0.01

* Number of living cells relative to the control was expressed as cell survival (%)

4.2.7 Overall score and ranking

Overall score and ranking of *Etlingera* species studied were based on phenolic content, and on antioxidant, antibacterial and tyrosinase inhibition activities of leaves. Ranking was of the order: *E. elatior* > *E. rubrostriata* >*E. fulgens* >*E. littoralis* > *E. maingayi* [Table 4.13]. Based on this evaluation, leaves of *E. elatior* were chosen for determination of major chemical constituents with bioactive properties and for developing a standardized extract with commercial potentials.

Table 4.13
Overall score and ranking of *Etlingera* species based on phenolic content, antioxidant activity and other bioactivities of leaves

Etlingera species	Phenolic content	Antioxidant activity				Other bioactivity		Overall score	Overall ranking
	TPC	AEAC	FRP	FIC	LPI	AB	TI		
E. elatior	1	1	1	2	2	1	1	9	1
E. rubrostriata	1	1	1	3	2	2	3	13	2
E. fulgens	3	3	3	1	3	1	1	15	3
E. littoralis	2	2	2	2	2	3	3	16	4
E. maingayi	4	4	4	1	1	1	2	17	5

Abbreviations: TPC = total phenolic content, AEAC = ascorbic acid equivalent antioxidant capacity, FRP = ferric reducing power, FIC = ferrous ion chelating, LPI = lipid peroxidation inhibition, AB = antibacterial and TI = tyrosinase inhibition. The species with the lowest overall score was given the highest overall ranking and *vice versa*.

4.3 OTHER GINGER SPECIES

4.3.1 Antioxidant properties

Leaves

Leaves of 21 other ginger species screened for antioxidant properties belong to eight genera and three tribes. The tribes and genera are Alpinieae (*Alpinia* and *Elettariopsis*), Hedychieae (*Boesenbergia*, *Curcuma*, *Hedychium*, *Kaempferia* and *Scaphochlamys*), and Zingibereae (*Zingiber*).

Compared to TPC and AEAC of leaves of *Etlingera* species, the other ginger species had lower values [Table 4.14]. Among the Alpinieae species, which include *Etlingera*, leaves of *A. zerumbet* showed high TPC and AEAC with values of 1990 ± 62 mg GAE/100 g and 2180 ± 42 mg AA/100 g, respectively. Leaves of the commercially cultivated *A. galanga* had the lowest values of 392 ± 50 mg GAE/100 g and 90 ± 36 mg AA/100 g, respectively.

Although most *Etlingera* species and some *Alpinia* species displayed high phenolic content and radical scavenging activity, species of *Elettariopsis*, which also belong to Alpinieae, had much lower values ranging from 303–423 mg GAE/100 g and 147–395 mg AA/100 g, respectively.

Table 4.14
Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of methanol extracts of leaves of 26 ginger species (fresh weight)

Ginger species	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	Source
Alpinia zerumbet	1990 ± 62a	2180 ± 42a	Wong (2007)
A. purpurata	1190 ± 174b	1100 ± 113b	Wong (2006)
A. zerumbet 'Variegata'	1150 ± 41b	1250 ± 184b	Wong (2006)
A. malaccensis	744 ± 61c	800 ± 62c	PSD
A. galanga	392 ± 50d	90 ± 36d	Wong (2006)
Boesenbergia rotunda	260 ± 8	157 ± 2	Lim (2007b)
Curcuma zanthorrhiza	503 ± 57a	287 ± 39a	Joe (2006)
C. aeruginosa	282 ± 78b	140 ± 47b	Joe (2006)
C. mangga	275 ± 36b	118 ± 11b	Joe (2006)
C. longa	230 ± 19b	113 ± 18b	PSD
Elettariopsis latiflora	423 ± 26a	395 ± 27a	Lim (2007a)
E. slahmong	346 ± 45b	269 ± 67b	Lim (2007a)
E. smithiae	303 ± 18b	147 ± 21c	Lim (2007a)
Etlingera elatior	2390 ± 329a	2280 ± 778a	PSD
E. rubrostriata	2250 ± 113a	2290 ± 118a	PSD
E. littoralis	2150 ± 94a	1990 ± 87b	PSD
E. fulgens	1280 ± 144b	845 ± 158c	PSD
E. maingayi	1110 ± 93b	963 ± 169c	PSD
Hedychium coronarium	820 ± 55	814 ± 116	PSD
Kaempferia galanga	146 ± 9a	77 ± 7a	Lianto (2006)
K. rotunda	140 ± 48ab	46 ± 15b	PSD
K. pulchra	112 ± 9b	30 ± 3b	Lianto (2006)
Scaphochlamys kunstleri	203 ± 21	171 ± 33	PSD
Zingiber officinale	291 ± 18a	96 ± 7a	PSD
Z. spectabile	242 ± 7b	121 ± 24a	PSD
Z. ottensii	162 ± 13c	52 ± 6b	PSD

Values of TPC and AEAC are means ± SD (n = 3). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA compares values of leaves of species in each genus and does not apply between genera. Abbreviations: PSD = present study data, GAE = gallic acid equivalent and AA = ascorbic acid.

TPC and AEAC values of leaves of genera belonging to Hedychieae and Zingibereae were comparatively lower. They included species such as *Boesenbergia rotunda*, *Curcuma aeruginosa*, *C. longa*, *C. mangga*, *C. zanthorrhiza*, *Hedychium coronarium*, *Kaempferia galanga*, *K. rotunda* and *Zingiber ottensii* which are used in food flavouring and traditional medicine. Among these species, *H. coronarium* had the highest TPC and AEAC with values of 820 ± 55 mg GAE/100 g and 814 ± 116 mg AA/100 g, respectively. Species of *Kaempferia* had very low phenolic content and radical scavenging activity with values ranging from 112–146 mg GAE/100 g and 30–77 mg AA/100 g, respectively. Leaves of *Kaempferia pulchra* exhibited the lowest values. Alpinieae species are medium- to large-sized forest plants of which *Etlingera* is the largest (Larsen *et al.*, 1999). Zingibereae species are medium-sized plants and Hedychieae species are small- to medium-sized herbs.

Foliage of tropical forest plants produces more antioxidants when exposed to elevated light conditions (Frankel & Berenbaum, 1999). Plants growing along the seashore which receive much sunlight have efficient antioxidant properties to prevent oxidative damage (Masuda *et al*., 1999b). These observations may also apply to species of *Etlingera*, which have the highest leaf TPC and AEAC. *Etlingera* species are the largest of the ginger plants and can grow up to 6 m in height (Khaw, 2001). They grow in gaps of disturbed forest and are continually exposed to direct sunlight.

Alpinia species with high TPC and AEAC are medium-sized to large forest plants (Larsen *et al*., 1999). The other genera are small- to medium-sized herbs. Among the various tribes and genera of gingers, there appears to be a positive correlation between the phenolic content and radical scavenging activity of leaves with plant size and site conditions. Larger ginger plants growing in exposed forest sites have higher antioxidant properties than smaller plants growing in shaded sites.

Leaves and rhizomes

TPC and antioxidant activity of methanol extracts of leaves and rhizomes of 14 species from the same plant or location were also assessed for comparison purpose. Results showed that 11 out of 14 species (80%) showed significantly higher TPC and/or AEAC in leaves than in rhizomes [Table 4.15].

Species with leaves having significantly higher TPC and AEAC than rhizomes were *Curcuma aeruginosa*, *C. mangga*, *C. zanthorrhiza*, *Kaempferia galanga* and *Scaphochlamys kunstleri.* Leaves of *Etlingera elatior* and *E. maingayi* which had the highest TPC and AEAC were 7–8 times higher than those of rhizomes. Species with higher TPC or AEAC were *Alpinia galanga*, *Boesenbergia rotunda*, *Elettariopsis slahmong* and *Zingiber officinale*. Exceptions were AEAC of *Alpinia galanga*, and TPC and AEAC of *Curcuma longa* where rhizomes showed significantly higher values than leaves.

TPC and AEAC of leaves and rhizomes of *Alpinia malaccensis* and *Zingiber spectabile* were comparable. Values were generally more variable between rhizomes than between leaves of a species as evident in *A. malaccensis*, *C. longa* and *Z. spectabile*.

A comparison was made between the TPC and AEAC of rhizomes of *A. galanga*, *C. longa* and *Z. officinale* collected from the field and those purchased from the supermarket [Table 4.16]. Rhizomes of *A. galanga* and *Z. officinale* purchased from the supermarket had significantly higher TPC and AEAC than collected rhizomes. Rhizomes of *Z. officinale* from the supermarket had higher AEAC but comparable TPC. Collected rhizomes of *C. longa* had significantly higher TPC but comparable AEAC.

Table 4.15
Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of methanol extracts of leaves (L) and rhizomes (R) of 14 ginger species (fresh weight)

Species and location	Part	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	Source
Alpinia galanga Bukit Maluri	L R	392 ± 50a 214 ± 20b	90 ± 36a 168 ± 13b	Wong (2006)
A. malaccensis FRIM	L R	744 ± 61a 564 ± 209a	800 ± 62a 745 ± 342a	PSD
Boesenbergia rotunda Sungai Buluh	L R	260 ± 8a 197 ± 50a	157 ± 2a 89 ± 7b	Lim (2007b)
Curcuma aeruginosa Damansara Utama	L R	282 ± 78a 145 ± 31b	140 ± 47a 55 ± 11b	Joe (2006)
C. longa FRIM	L R	230 ± 19a 534 ± 205b	113 ± 18a 390 ± 127b	PSD
C. mangga Damansara Utama	L R	275 ± 36a 112 ± 21b	118 ± 11a 33 ± 1b	Joe (2006)
C. zanthorrhiza Damansara Utama	L R	503 ± 57a 250 ± 52b	287 ± 39a 134 ± 21b	Joe (2006)
Elettariopsis slahmong FRIM	L R	346 ± 45a 219 ± 57b	269 ± 67a 197 ± 76a	Lim (2007a)
Etlingera elatior FRIM	L R	2390 ± 329a 326 ± 76b	2280 ± 778a 295 ± 96b	PSD
E. maingayi Janda Baik	L R	1110 ± 93a 160 ± 52b	963 ± 169a 122 ± 53b	PSD
Kaempferia galanga Damansara Utama	L R	146 ± 9a 57 ± 1b	77 ± 7a 17 ± 1b	Lianto (2006)
Scaphochlamys kunstleri FRIM	L R	203 ± 21a 73 ± 3b	171 ± 33a 14 ± 2b	PSD
Zingiber officinale Bukit Maluri	L R	291 ± 18a 157 ± 18b	96 ± 7a 84 ± 3a	PSD
Z. spectabile FRIM	L R	242 ± 7a 157 ± 100a	121 ± 24a 124 ± 109a	PSD

Values of TPC and AEAC are means ± SD (n = 3). For each column, values followed by the same letter (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA compares values of leaves and rhizomes of each species and does not apply between species. Abbreviations: PSD = present study data, GAE = gallic acid equivalent and AA = ascorbic acid.

Table 4.16
Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of methanol extracts of rhizomes of three commercial ginger species collected in the field and purchased from the supermarket (fresh weight)

Ginger species	Location	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
Alpinia galanga	Bukit Maluri	150 ± 22a	96 ± 6a
	Supermarket	214 ± 20b	168 ± 13b
Zingiber officinale	Bukit Maluri	157 ± 18a	84 ± 3a
	Supermarket	184 ± 11a	107 ± 9b
Curcuma longa	FRIM	534 ± 205a	390 ± 207a
	Supermarket	386 ± 219b	275 ± 183a

Values of TPC and AEAC are means ± SD (n = 3). For each column, values followed by the same letter (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA compares values of leaves and rhizomes of a species, and does not apply between species. Abbreviations: GAE = gallic acid equivalent and AA = ascorbic acid.

Analysis of metal ion chelating properties showed that six of the eight species studied clearly displayed higher FIC ability in leaves than in rhizomes. The species were *C. longa*, *K. galanga*, *A. galanga* and *E. elatior* [Fig. 4.4], and *C. zanthorrhiza*. *S. kunstleri*, *Z. spectabile* and *E. maingayi* [Fig. 4.5].

Of particular interest is *C. longa* where TPC and AEAC were significantly higher in rhizomes, but the FIC ability was higher in leaves [Fig. 4.4]. The most outstanding was the FIC value of *A. galanga* leaves which was more than 20 times higher than that of rhizomes. FIC values of leaves and rhizomes of *C. zanthorrhiza* were comparable at higher concentrations [Fig. 4.5]. At lower extract concentration, leaves of *S. kunstleri* showed lower values but at higher concentration, values were comparable. In the case of *Z. spectabile*, although TPC and AEAC were comparable, FIC value of leaves was higher than that of rhizomes.

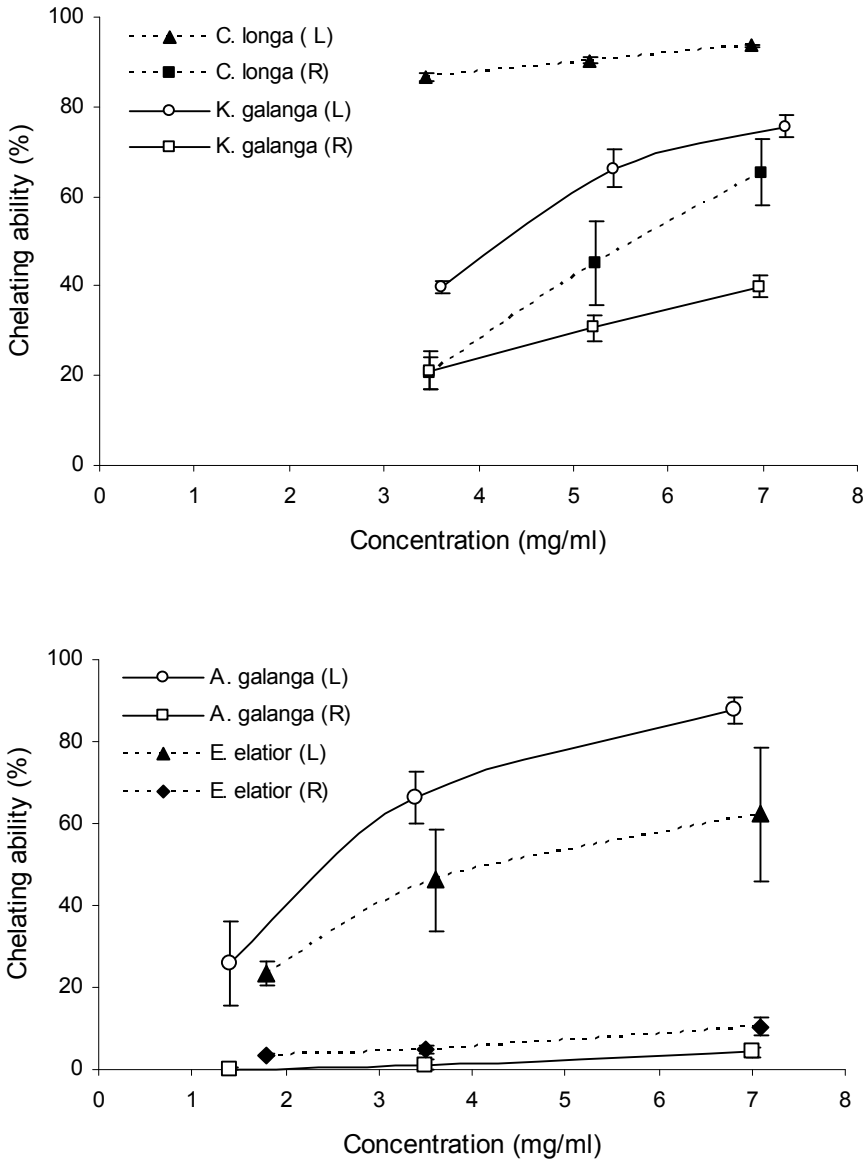


Fig. 4.4
Ferrous ion chelating (FIC) ability of leaves (L) and rhizomes (R) of *Curcuma longa*, *Kaempferia galanga*, *Alpinia galanga* and *Etlingera elatior* (fresh weight)

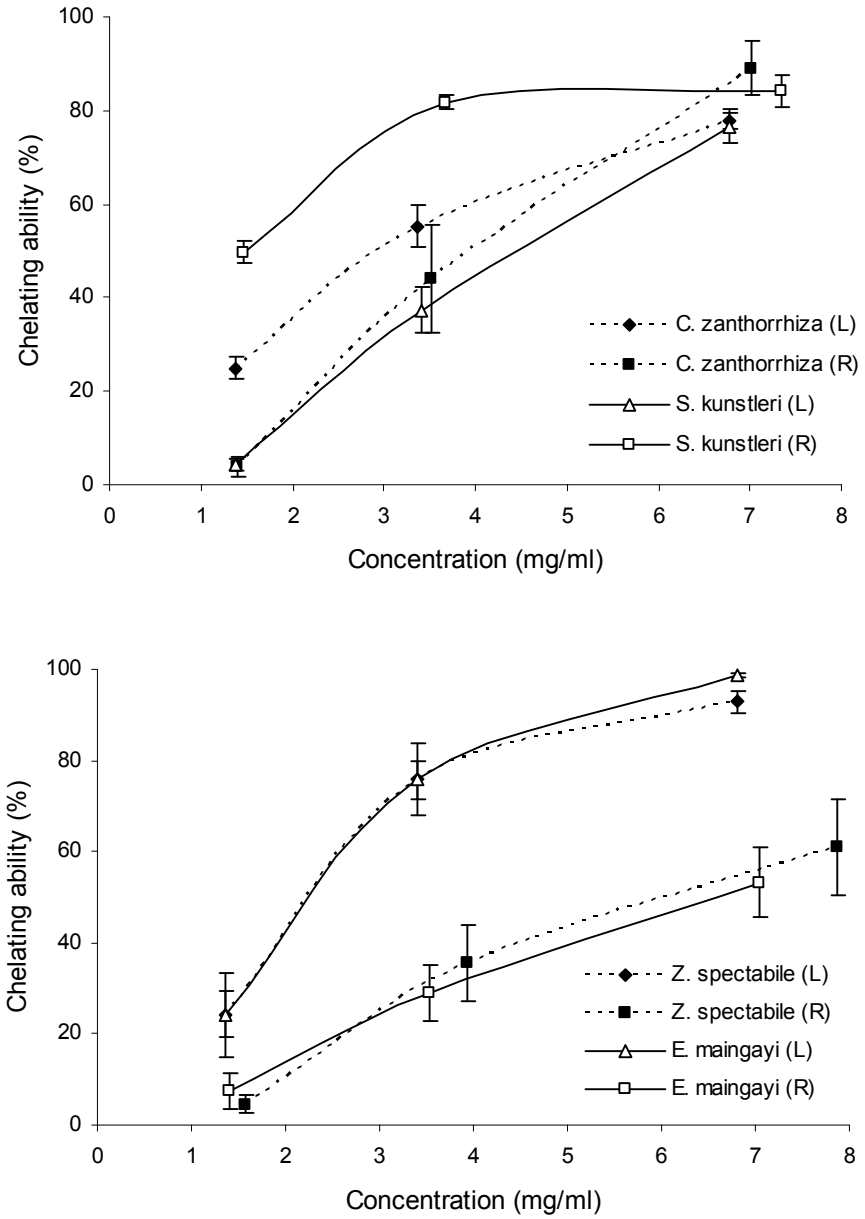


Fig. 4.5
Ferrous ion chelating (FIC) ability of leaves (L) and rhizomes (R) of *Curcuma zanthorrhiza*, *Scaphochlamys kunstleri*, *Zingiber spectabile* and *Etlingera maingayi* (fresh weight)

There are few studies comparing the antioxidant properties of leaves and rhizomes of ginger species. Essential oils from leaves of *Aframomum giganteum* had higher antioxidant activity than those from rhizomes (Agnaniet *et al*., 2004).

Leaves of *A. zerumbet* showed higher inhibition of β-carotene oxidation and radical scavenging activity than rhizomes (Elzaawely *et al*., 2007). Contrary to our results, higher phenolic content and antioxidant activity have been reported in rhizomes than in leaves of *Z. officinale* (Katsube *et al.*, 2004). These studies involved one or two ginger species and it is not known whether their comparisons were based on plant samples from the same or different locations. Our present study is probably the first where the phenolic content, radical scavenging activity and metal ion chelating ability of leaves and rhizomes of ginger species from the same plant or location were systematically compared.

Antioxidants are secondary metabolites produced by plants to protect against oxidative damage by free radicals (Larson, 1988). In Zingiberaceae, it is generally believed that antioxidants produced by the plant are transported to the rhizomes where they are accumulated. This implies that rhizomes would have higher antioxidant activity than other plant parts. However, results of this study showed that this might not be true as the majority of the species studied had significantly higher phenolic content and antioxidant activity in leaves than in rhizomes. Similar observations have been made by Herrmann (1988) who reported much greater concentrations of flavones and flavonols in leaves of vegetables which are exposed to sunlight. Only trace amounts were found in unexposed parts below the soil surface which include roots and rhizomes. This could explain why leaves have significantly higher phenolic content and antioxidant activity than rhizomes in ginger plants.

4.4 DRYING TREATMENTS

Drying food products enhances their storage stability and at the same time, reduces their volume and weight (Sablani & Rahman, 2007). This minimises the costs of packaging, transportation and storage. Removal of moisture content following drying prevents microbial growth and inhibits deteriorative enzymatic and chemical reactions (Rahman & Perera, 2007). The retention of antioxidant and functional activities in dried products are important factors that would promote their marketability as nutraceuticals (Orsat & Raghavan, 2006). Thus maintaining the nutritional and physical quality during drying is of commercial interest to food manufacturers.

Common drying treatments can be divided into thermal and non-thermal methods. They differ in operational principles and have varying effects on the quality of the resulting product. The present study focuses on the effects of different thermal and non-thermal drying methods on the phenolic content and antioxidant properties of leaves of *E. elatior*. Leaves of *A. zerumbet*, *C. longa* and *K. galanga* were also included to give a more comprehensive overview of the drying effects.

4.4.1 Thermal drying methods

Thermal drying methods of ginger leaves were microwave-, oven-, and sun-drying. For microwave-drying, leaves were dried in a microwave oven for 4 min. Oven-drying involved drying at 50°C for 5 h in an oven. Leaves were sun-dried in the greenhouse for three days.

Antioxidant properties of heat-treated leaves of *E. elatior* were adversely affected by microwave-, oven- and sun-drying. Percentage losses were 40%, 42% and 70% for TPC, 59%, 58% and 75% for AEAC, and 44%, 43% and 76% for FRP, respectively [Table 4.17]. Percentage losses in antioxidant properties of 70–76% were highest for sun-drying.

Table 4.17
Percentage loss in total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP) of leaves of *Etlingera elatior*, *Alpinia zerumbet*, *Curcuma longa* and *Kaempferia galanga* following thermal drying (fresh weight)

Species	Thermal drying	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)	Source
		Percentage loss compared to fresh leaves			
E. elatior	Microwave-drying	–40 ± 8a	–59 ± 9a	–44 ± 10a	PSD
	Oven-drying	–42 ± 13a	–58 ± 16a	–43 ±15a	
	Sun-drying	–70 ± 13b	–75 ± 13a	–76 ± 11b	
A. zerumbet	Microwave-drying	–50 ± 5a	–58 ± 2a	–55 ± 5a	Wong (2007)
	Oven-drying	–43 ± 10a	–49 ± 6a	–56 ± 8a	
	Sun-drying	–47 ± 9a	–57 ± 9a	–46 ± 10a	
C. longa	Microwave-drying	–58 ± 14a	–59 ± 9a	–71 ± 5a	Lianto (2006)
	Oven-drying	–77 ± 2b	–77 ± 1b	–81 ± 1b	
	Sun-drying	–81 ± 1c	–84 ± 2c	–86 ± 2c	
K. galanga	Microwave-drying	–36 ± 10a	–27 ± 13a	–44 ± 3a	Lianto (2006)
	Oven-drying	–66 ± 1b	–66 ± 2b	–81 ± 2b	
	Sun-drying	–91 ± 1c	–86 ± 1c	–88 ± 2c	

Values of TPC, AEAC and FRP are means ± SD (n = 3). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA applies between thermal drying methods of a species. Abbreviations: PSD = present study data, GAE = gallic acid equivalent and AA = ascorbic acid.

Heat-treated leaves of *A. zerumbet* resulted in losses in TPC, AEAC and FRP compared to those of fresh leaves. Percentage losses were 50%, 58% and 55% for microwave-drying, 43%, 49% and 56% for oven-drying, and 47%, 57% and 46% for sun-drying, respectively [Table 4.17]. Losses in antioxidant properties were similar between the three drying methods. FIC values of heat-treated leaves following microwave-, oven- and sun-drying were comparable to those of fresh leaves [Fig. 4.6].

For leaves of *C. longa* and *K. galanga*, thermal drying also resulted in declines in TPC, AEAC and FRP. Percentage losses were 58–81%, 59–84% and 71–86% for *C. longa*, and 36–91%, 27–86% and 44–88% for *K. galanga*, respectively [Table 4.17]. Percentage losses in antioxidant properties were the least for microwave-drying and the most for sun-drying. For *C. longa*, the losses were 58–71% and 81–86% and for *K. galanga*, the losses were 27–44% and 86–91%, respectively.

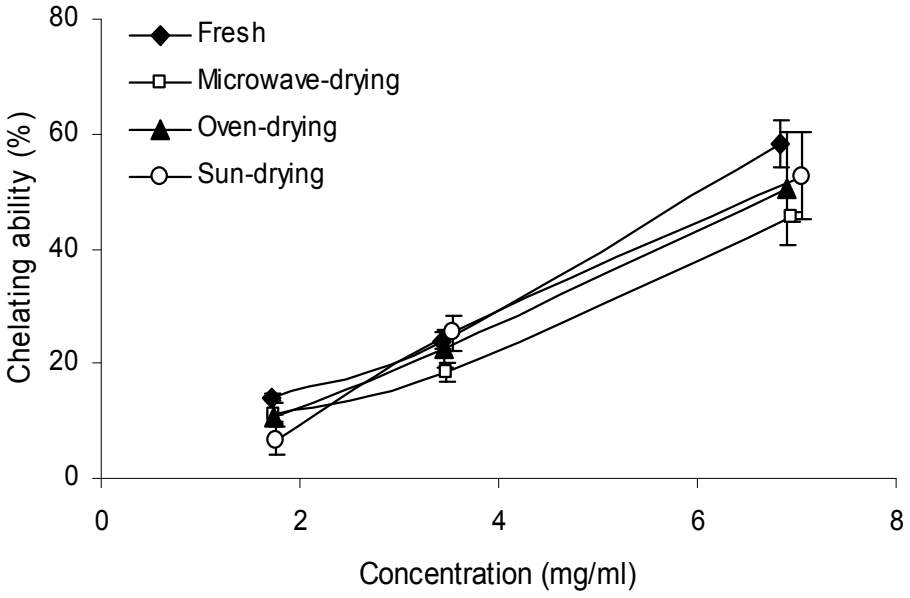


Fig. 4.6
Ferrous ion chelating (FIC) ability of heat-treated leaves of *Alpinia zerumbet* with fresh leaves as control

FIC values of fresh leaves of *E. elatior* were slightly higher than those of microwave- and oven-dried leaves [Fig. 4.7]. Heat-treated leaves yielded slightly higher values of LPI activity [Fig. 4.8]. Leaves of *E. elatior* microwave-dried for 2, 4, 6 and 8 min resulted in significant losses in TPC and antioxidant activity, but their declines were comparable between different drying durations [Table 4.18].

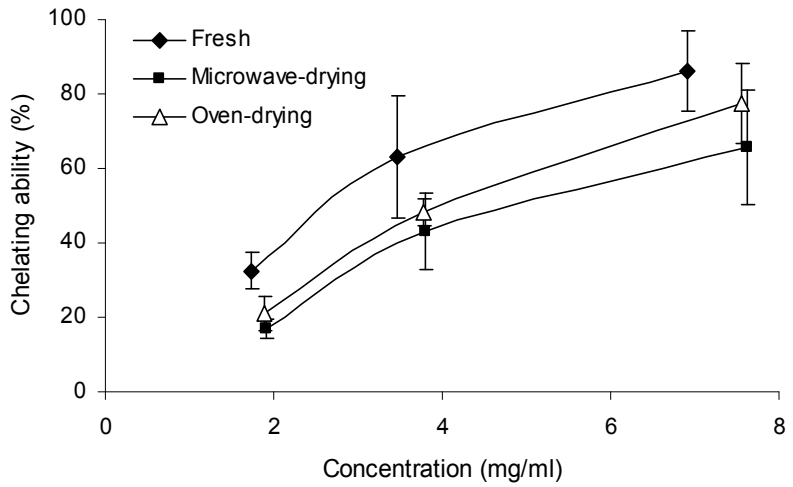


Fig. 4.7
Ferrous ion chelating (FIC) ability of heat-treated leaves of *Etlingera elatior* with fresh leaves as control

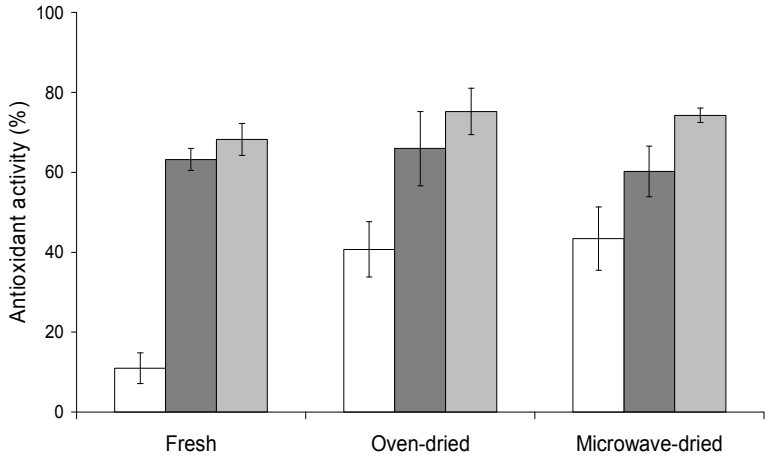


Fig. 4.8
Lipid peroxidation inhibition (LPI) activity of heat-treated leaves of *Etlingera elatior* with fresh leaves as control

Table 4.18
The effect of microwave-drying (MD) over different durations on the total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP) of leaves of *Etlingera elatior* (fresh weight)

Duration of MD	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
Fresh	1770 ± 151a	1420 ± 140a	8.5 ± 0.9a
2 min	928 ± 183b	644 ± 106b	3.7 ± 1.1b
4 min	783 ± 52b	597 ± 73b	2.6 ± 0.3b
6 min	818 ± 45b	491 ± 55b	2.5 ± 0.2b
8 min	783 ± 76b	498 ± 55b	3.1 ± 0.3b

TPC, AEAC and FRP values are means ± SD (n = 3). For each column, values followed by the same letter (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Abbreviations: GAE = gallic acid equivalent and AA = ascorbic acid.

Processing methods are known to have variable effects on the phenolic content and antioxidant activity. Effects include little or no change, significant losses, or enhancement in antioxidant properties (Nicoli *et al*., 1999). Food processing can improve the properties of naturally occurring antioxidants or induce the formation of new compounds with antioxidant properties so that the overall antioxidant activity increases or remains unchanged (Tomaino *et al*., 2005). Increase in antioxidant activity following thermal treatment is attributed to the release of bound phenolic compounds as demonstrated in tomato (Dewanto *et al*., 2002b) and sweet corn (Dewanto *et al*., 2002a).

Many studies have reported losses in TPC and antioxidant activity following thermal treatments. They were reported mainly in vegetables (Gazzani *et al*., 1998; Ismail *et al*., 2004; Zhang & Hamauzu, 2004; Toor & Savage, 2006; Roy *et al*., 2007). Losses in antioxidant properties of heat-treated samples have been attributed to thermal degradation of phenolic compounds (Maillard & Berset, 1995). Intense and/or prolonged thermal treatment may result in a significant loss of natural antioxidants (Tomaino *et al*., 2005). Factors such as degradative enzymes, heat decomposition due to slow heat transfer and degradation of phytochemicals may also cause declines in antioxidant properties (Lim & Murtijaya, 2007). Declines in TPC and antioxidant activity are often accompanied by loss of other bioactive properties (Roy *et al*., 2007).

This study showed that thermal drying methods had two major effects on the antioxidant properties of ginger leaves. TPC, AEAC and FRP were adversely affected but not FIC ability and LPI activity. Compounds such as those containing nitrogen-donating atoms could contribute substantially to the FIC ability and thus values were not affected by reduction in phenolic content. LPI depends more on the type of antioxidants present rather than their concentration. Some flavonols such as quercetin are good inhibitors of lipid peroxidation while flavanols such as catechin are poor inhibitors (Kumazawa *et al*., 2002).

It would be unrealistic to infer that cooking and other food processing resulted in gains or losses in antioxidant activity without analysing a wide range of antioxidant properties and testing a variety of samples. A single treatment applied on a given sample could have variable effects on antioxidant properties. For instance, Chang *et al*. (2006) reported gains in TPC and FIC ability, losses in FRP, but DPPH radical scavenging activity remained unchanged for hot air-dried tomatoes. Ismail *et al.* (2004) reported significant losses in TPC in all vegetables studied while LPI ability was unchanged in some vegetables after thermal treatment. Turkmen *et al*. (2005) found that TPC and DPPH radical scavenging

activity increased or remained unchanged depending on the type of vegetable but not the type of cooking. Yamaguchi *et al.* (2001) found that DPPH radical scavenging activity increased in some cooked vegetables, while in others, the activity decreased.

An interesting finding related to this study is the effect of microwave-drying on the antioxidant properties of leaves of *E. elatior*. Leaves microwave-dried for 2, 4, 6 and 8 min showed significant declines in TPC, AEAC and FRP but the declines were comparable. A likely explanation is that microwave-drying for 2 min is sufficient to decompose all heat-labile antioxidants and subsequent heating would have no incremental effect.

4.4.2 Non-thermal drying methods

Air-drying of ginger leaves resulted in significant losses in TPC and antioxidant activity for all four species. Air-drying of *E. elatior* and *A. zerumbet* leaves significantly decreased TPC, AEAC and FRP by 49%, 51% and 53%, and by 51%, 48% and 50%, respectively [Table 4.19]. It resulted in drastic declines of 80%, 84% and 83% for leaves of *C. longa*, and 70%, 77% and 71% for leaves of *K. galanga*, respectively.

Freeze-dried leaves of *E. elatior* showed significantly higher values in TPC, AEAC and FRP compared to those of fresh leaves [Table 4.19]. Percentage gains were 26%, 47% and 36%, respectively. FIC ability of freeze-dried leaves showed little change compared to that of fresh leaves [Fig. 4.9].

Table 4.19
Total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP) of fresh, air-, and freeze-dried leaves of *Elingera elatior*, *Alpinia zerumbet*, *Curcuma longa* and *Kaempferia galanga* (fresh weight)

Species	Non-thermal drying	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)	Source
E. elatior	Fresh	2500 ± 554a	2990 ± 891a	17 ± 4.2a	PSD
	Air-drying	1270 ± 341b	1460 ± 439b	8.0 ± 2.4b	
A. zerumbet	Fresh	2470 ± 240a	3020 ± 428a	11 ± 2.0a	Wong (2007)
	Air-drying	1220 ± 241b	1570 ± 358b	5.5 ± 1.2b	
C. longa	Fresh	391 ± 36a	251 ± 19a	2.9 ± 0.1a	Lianto (2006)
	Air-drying	78 ± 5b	41 ± 4b	0.5 ± 0.1b	
K. galanga	Fresh	130 ± 7a	48 ± 3a	0.7 ± 0.1a	Lianto (2006)
	Air-drying	39 ± 3b	11 ± 1b	0.2 ± 0.0b	
E. elatior	Fresh	2420 ± 210a	2960 ± 362a	14 ± 0.8a	PSD
	Freeze-drying	3050 ± 226b	4360 ± 56b	19 ± 2.0b	
A. zerumbet	Fresh	1990 ± 62a	2180 ± 42a	11 ± 0.2a	Wong (2007)
	Freeze-drying	2550 ± 55b	2530 ± 45b	12 ± 0.2b	
C. longa	Fresh	399 ± 15a	243 ± 28a	2.1 ± 0.1a	Lianto (2006)
	Freeze-drying	357 ± 20b	222 ± 12a	1.8 ± 0.1b	
K. galanga	Fresh	133 ± 5a	42 ± 3a	0.7 ± 0.1a	Lianto (2006)
	Freeze-drying	112 ± 11b	38 ± 5a	0.6 ± 0.1a	

Values of TPC, AEAC and FRP are means ± SD (n = 3). For each column, values followed by the same letter (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA does not apply between species and between drying methods. Abbreviations: PSD = present study data, GAE = gallic acid equivalent and AA = ascorbic acid.

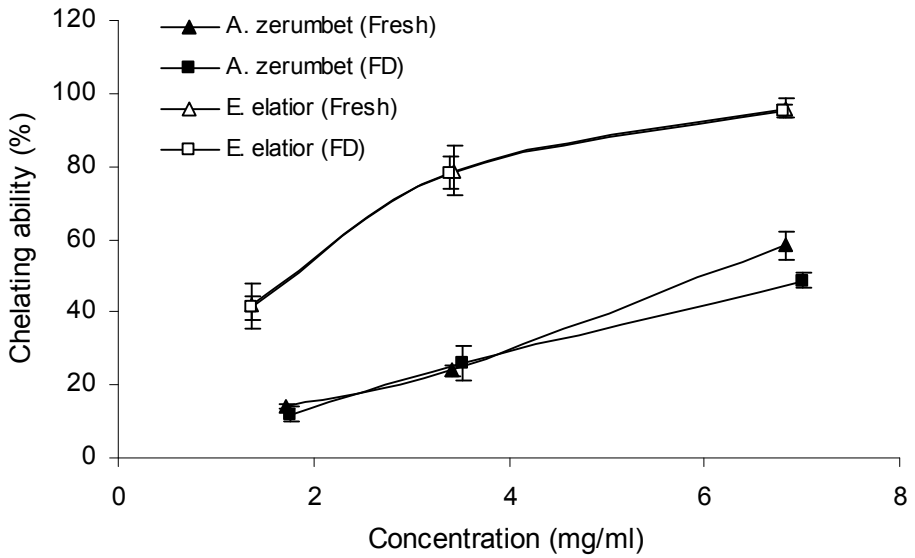


Fig. 4.9
Ferrous ion chelating (FIC) ability of freeze-dried leaves of *Alpinia zerumbet* and *Etlingera elatior* with fresh leaves as control

Similarly, freeze-drying of *A. zerumbet* leaves significantly increased TPC, AEAC and FRP compared to those of fresh leaves. Values of freeze-dried leaves were 2550 ± 554 mg GAE/100 g, 2530 ± 45 mg AA/100g and 12 ± 0.2 mg GAE/g while those of fresh leaves were 1990 ± 62 mg GAE/100 g, 2180 ± 42 mg AA/100g and 11 ± 0.2 mg GAE/g, respectively [Table 4.19]. This amounted to percentage gains of 28%, 16% and 9%, respectively. FIC values of freeze-dried leaves of *A. zerumbet* were comparable to those of fresh leaves [Fig. 4.9].

Freeze-drying of *C. longa* and *K. galanga* leaves resulted in a small decrease in TPC of 11% and 16%, respectively [Table 4.19]. There were no significant changes in AEAC of leaves of the two species following freeze drying although a small decrease in FRP of 14% was observed in leaves of *C. longa*.

HPLC chromatograms of extracts of fresh and freeze-dried leaves of *E. elatior* revealed some interesting results [Fig. 4.10]. Apices of major compounds remained relatively unchanged. The chromatograms showed greater amounts of

minor compounds in freeze-dried than fresh leaves. Between retention times of 4 and 10 min, overall peak area of freeze-dried leaves was 11,930 mAU*s compared to 9,050 mAU*s of fresh leaves. This represented an increase of 32%, which is comparable to the 26% increase in TPC.

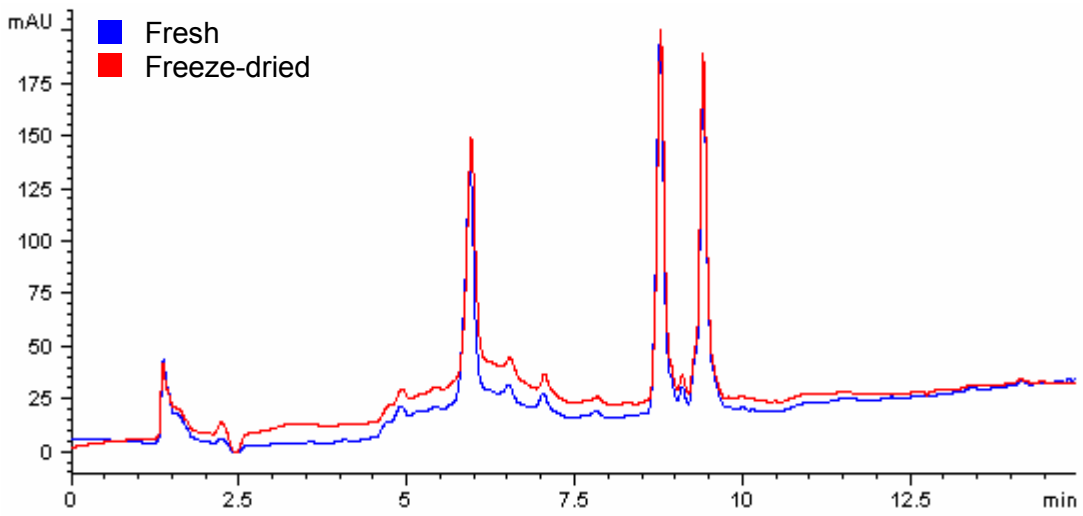


Fig. 4.10
Overlay of chromatograms (254 nm) showing greater amounts of minor compounds in freeze-dried than fresh leaves of *Etlingera elatior*

An experiment was conducted to test the stability of antioxidant properties of freeze-dried leaves of *E. elatior*. After storage in sealed Petri dishes at room temperature for a week, there was loss of 23%, 15% and 21% in TPC, AEAC and FRP for the control while freeze-dried leaves showed minimal declines of only 7%, 2% and 5%, respectively [Table 4.20]. It should be noted that the stored freeze-dried leaves with TPC, AEAC and FRP values of 2850 ± 124 mg GAE/100 g, 4210 ± 227 mg AA/100 g and 18 ± 2.5 mg GAE/g remained significantly higher than those of fresh control leaves with values of 2420 ± 210 mg GAE/100 g, 2960 ± 362 mg AA/100 g and 14 ± 0.8 mg GAE/g, respectively. Both the freeze-dried and control leaves did not show any declines in metal chelating ability after storage for a week [Fig. 4.11].

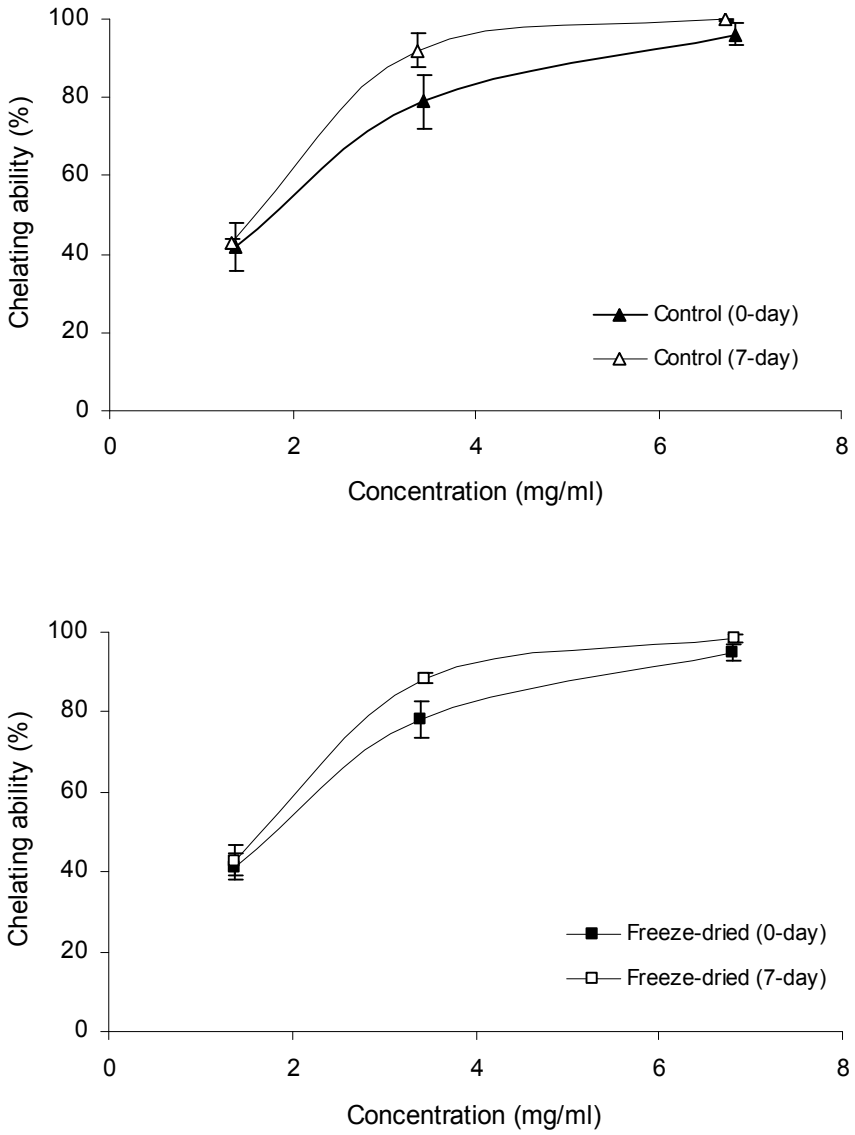


Fig. 4.11
Ferrous ion chelating (FIC) ability of freeze-dried leaves of *Etlingera elatior* after a week of storage with fresh leaves as control

Table 4.20
The effect of one-week storage on total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC) and ferric reducing power (FRP) of fresh and freeze-dried leaves of *Etlingera elatior* (fresh weight)

Species	Drying method	Storage (day)	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
E. elatior	Fresh	0	2420 ± 210a	2960 ± 362a	14 ± 0.8a
		7	1860 ± 180b	2510 ± 157b	11 ± 1.0b
	Freeze-dried	0	3050 ± 226c	4280 ± 55c	19 ± 2.0c
		7	2850 ± 124c	4210 ± 227c	18 ± 2.5c

TPC, AEAC and FRP values are means ± SD (n = 3). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Abbreviations: GAE = gallic acid equivalent and AA = ascorbic acid.

Unlike leaves of *E. elatior* and *A. zerumbet*, which showed significant gains in TPC, AEAC and FRP, freeze-drying resulted in slight declines of 11%, 9% and 14% for leaves of *C. longa*, and 16%, 10% and 14% for leaves of *K. galanga*, respectively [Table 4.19]. Declines in AEAC for *C. longa* and in AEAC and FRP for *K. galanga* were insignificant. FIC values of freeze-dried leaves of *C. longa* were comparable to those of fresh leaves [Fig. 4.12]. Freeze-dried leaves of *K. galanga* showed decline in FIC values.

Declines in antioxidants resulting from air-drying could be due to enzymatic degradation as the process was carried out at room temperature and required three days for samples to dry. Heat transfer is slow due to the low heat capacity of air during drying.

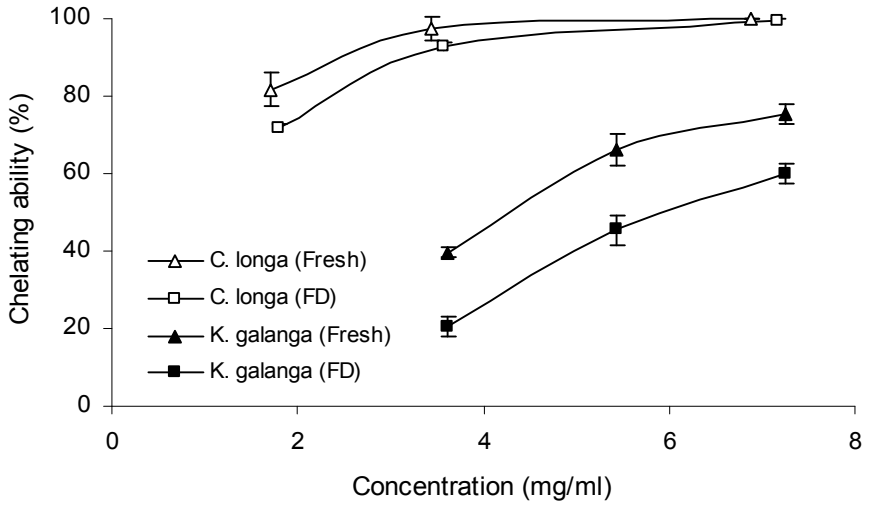


Fig. 4.12
Ferrous ion chelating (FIC) ability of freeze-dried leaves of *Curcuma longa* and *Kaempferia galanga* with fresh leaves as control

Freeze-drying of leaves of *E. elatior* and *A. zerumbet* significantly increased TPC, AEAC and FRP but had little effect on FIC ability. There is no thermal degradation in freeze-drying and neither does the process allow degradative enzymes to function. Freeze-drying is known to have high extraction efficiency because ice crystals formed within the plant matrix can rupture cell structure, which allows exit of cellular components, access of solvent and consequently better extraction (Asami *et al*., 2003; Escribano-Bailon & Santos-Buelga, 2003).

However, freeze-drying resulted in slight but significant declines in TPC and FRP for *C. longa* and in TPC for *K. galanga.* Unlike freeze-dried leaves of *E. elatior* and *A. zerumbet* which were thick, powdery and easy to extract, those of *C. longa* and *K. galanga* were thin, papery and difficult to extract. This could explain why freeze-drying has different effects on these two groups of species.

Results showed that freeze-drying had three major effects on the antioxidant properties of ginger leaves. Firstly, leaves of *E. elatior* and *A. zerumbet* showed enhancement in antioxidant properties following freeze-drying. Secondly, freeze-

dried leaves of *E. elatior* remained stable following one-week storage under room temperature. Thirdly, freeze-dried leaves of *C. longa* and *K. galanga* had the least decline in antioxidant properties compared with microwave-, oven-, sun- and air-dried leaves.

The third effect on the retention of antioxidant properties has often been reported. Hsu *et al*. (2003) reported that freeze-dried yam flours displayed the highest antioxidant activity, compared to hot air- and drum-dried flours. Asami *et al*. (2003) found that freeze-dried marionberry, strawberry and corn yielded higher TPC than air-dried samples. Bodo *et al*. (2004) reported that freeze-dried water hyacinth leaves had higher antioxidant activity than sun- and oven-dried leaves. Mao *et al*. (2006) reported higher TPC and antioxidant activity in freeze-dried than hot air-dried daylily flowers. Wong (2007) reported that tea infusion from freeze-dried leaves of *A. zerumbet* had better antioxidant properties than those of a commercial *A. zerumbet* tea.

The first effect on antioxidant properties enhancement and the second effect on the stability of antioxidant properties have seldom been reported. Nindo *et al*. (2003) reported that freeze-drying enhanced the total antioxidant activity of asparagus by about 50%. Chang *et al.* (2006) reported gains in FIC ability, but TPC, FRP and DPPH radical scavenging activity remained unchanged for freeze-dried tomatoes. Arts and Hollman (1998) reported that (-)-epicatechin level was 2.4% higher in freeze-dried apples. De Sotillo *et al.* (1994) reported that storage of freeze-dried extract of potato peel waste for 15 days showed no degradation in phenolics and antioxidant activity. Escribano-Bailon and Santos-Buelga (2003) suggested that freeze-drying would not affect phenolic compounds and allowed samples to be kept for longer periods. To our knowledge, the present study is the first to demonstrate the enhancement and stability of antioxidant properties following freeze-drying of ginger leaves.

4.5 PHENOLIC COMPOUNDS

4.5.1 Structural elucidation

Chemical constituents of leaves of *E. elatior* were analyzed because they displayed outstanding antioxidant, tyrosinase inhibition and antibacterial activity. The species is widely cultivated in the tropics for its inflorescences as spice. Leaves are available in large quantities and are currently of no commercial value.

From this study, six natural compounds were isolated from the aqueous extract of leaves of *E. elatior*. Three were caffeoylquinic acids, one was a flavanol and the remaining two were flavonols. The compounds were numbered according to their order of elution.

Caffeoylquinic acids

Caffeoylquinic acids (CQA) isolated from leaves of *E. elatior* were identified based on spectroscopic analysis, and on comparison of ^{1}H and ^{13}C NMR data with literature values.

Compound **1** was identified as 3-*O*-caffeoylquinic acid (3-CQA) or neo chlorogenic acid (Nakatani *et al*., 2000). Compound **3** was identified as 5-*O*-caffeoylquinic acid (5-CQA) or chlorogenic acid (CGA) (Madhava Naidu *et al*., 2008). Compound **4** was identified as 5-*O*-caffeoylquinic acid methyl ester (Me 5-CQA) or methyl 5-*O*-caffeoylquinate (Lin *et al*., 2002). The ^{1}H and ^{13}C NMR spectra of 5-CQA, methyl 5-CQA and 3-CQA are shown in Figs. 4.13, 4.14 and 4.15a, respectively. This is the first time CQA have been isolated from Zingiberaceae.

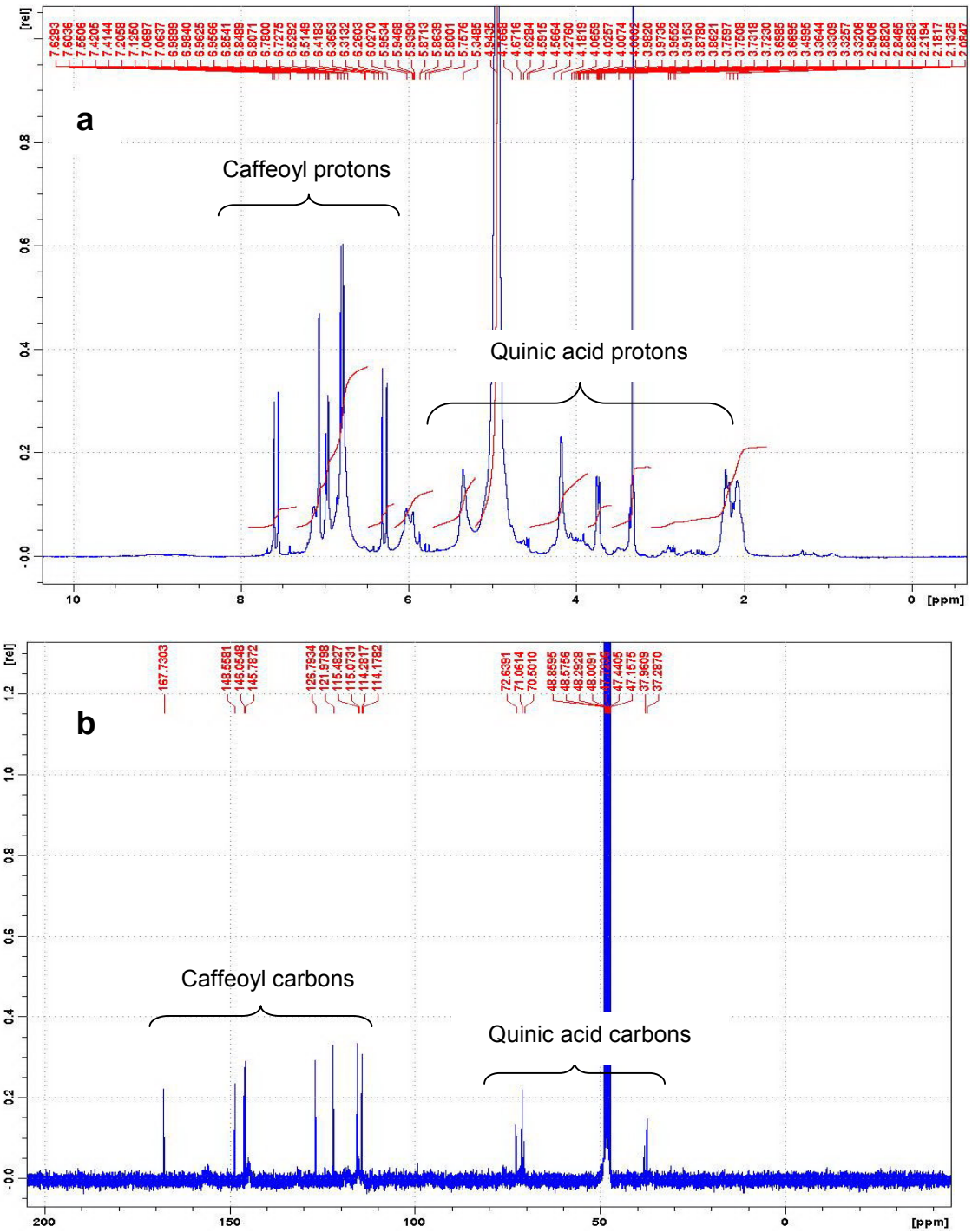


Fig. 4.13
^{1}H NMR (a) and ^{13}C NMR (b) spectra of 5-*O*-caffeoylquinic acid (5-CQA or CGA) in deuterated methanol

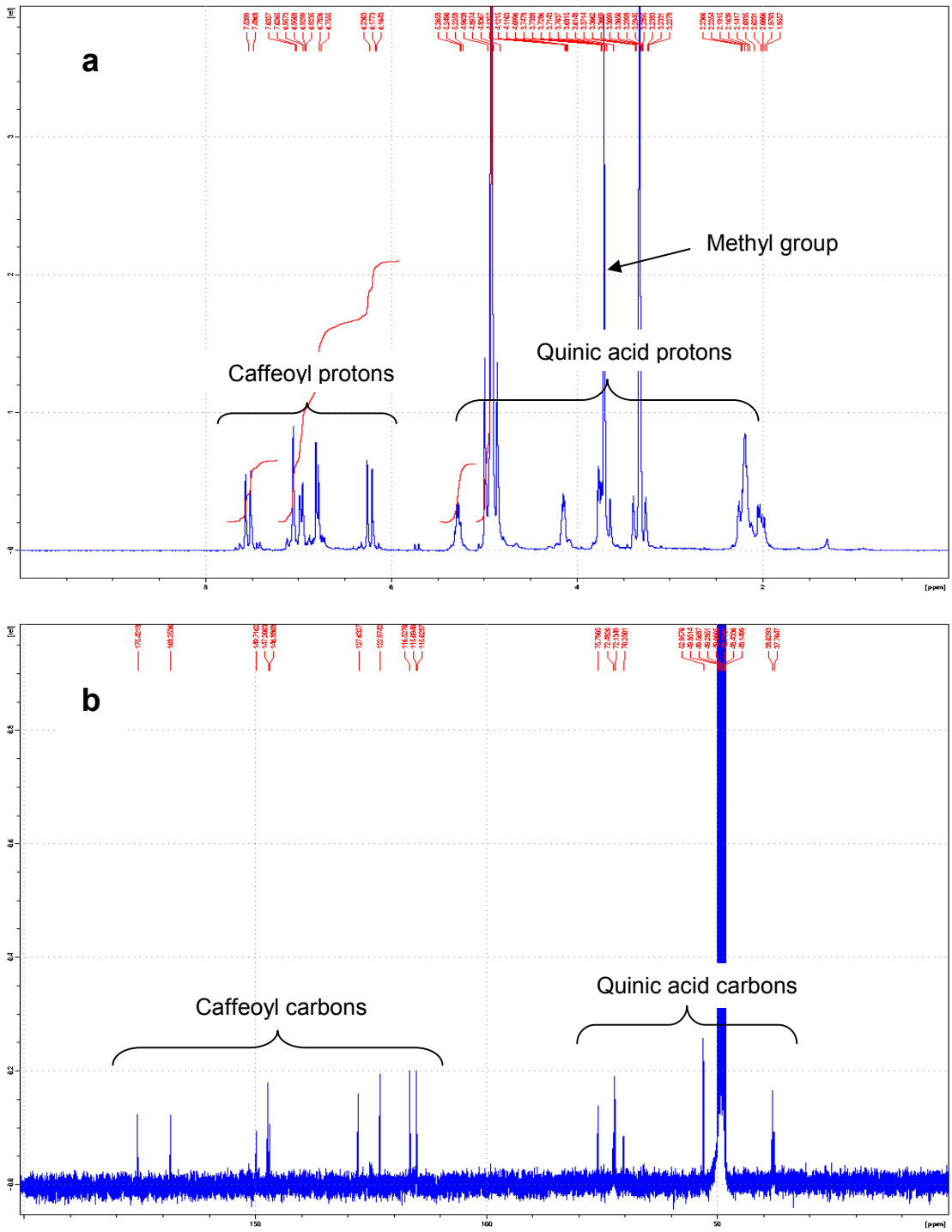
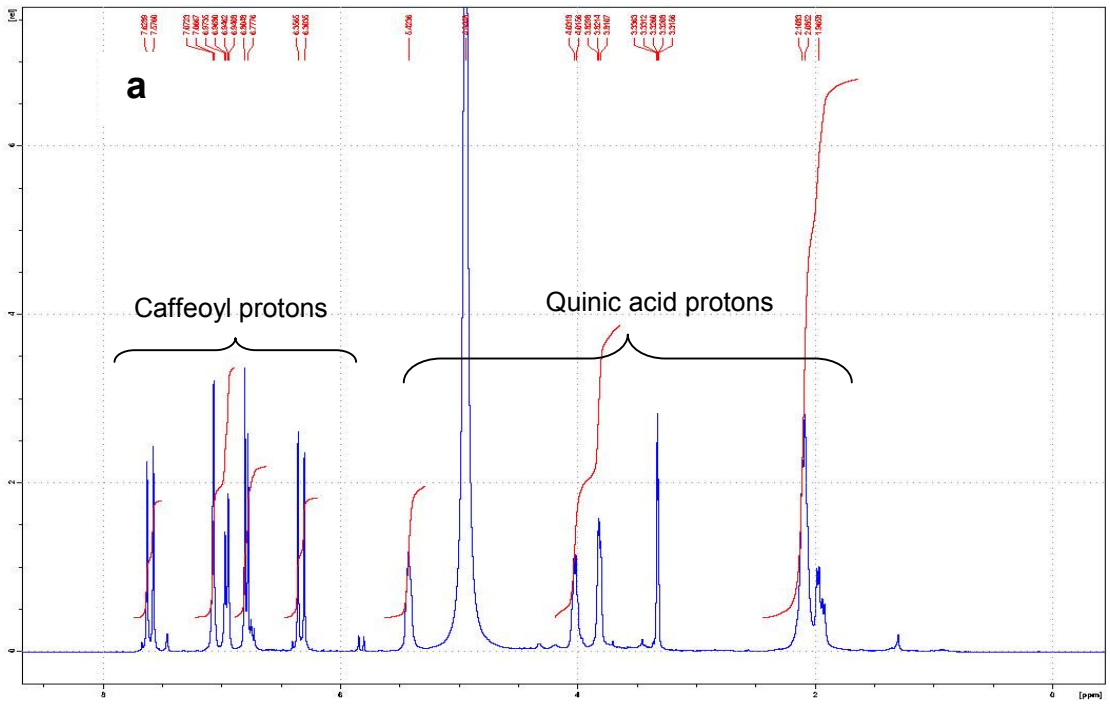


Fig. 4.14
^{1}H NMR (a) and ^{13}C NMR (b) spectra of 5-*O*-caffeoylquinic acid methyl ester (methyl 5-CQA) in deuterated methanol



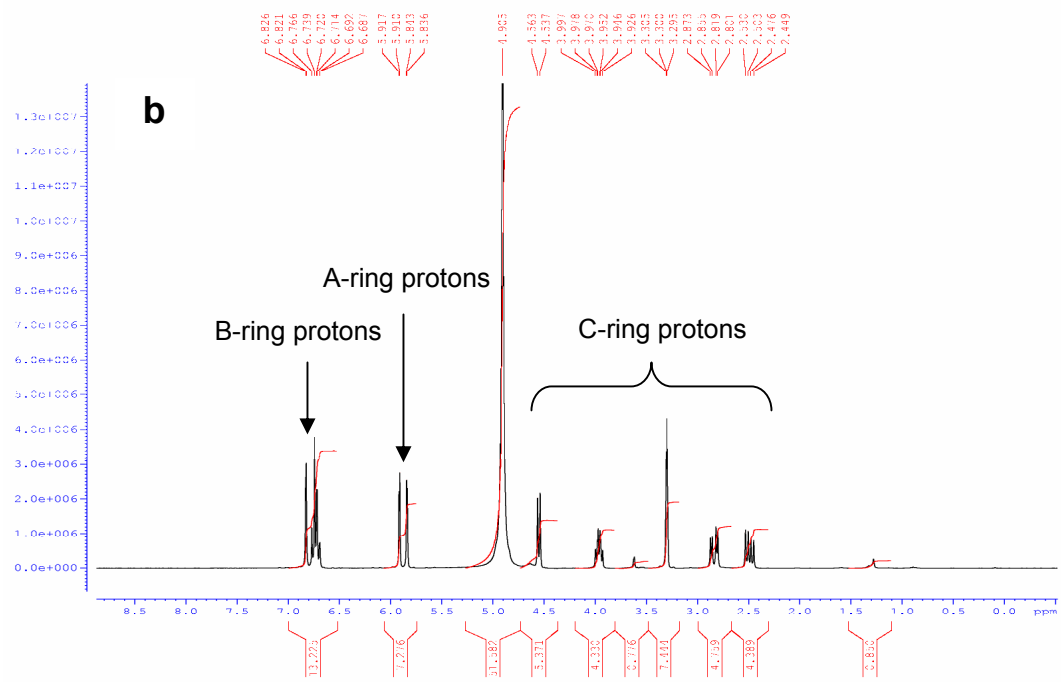
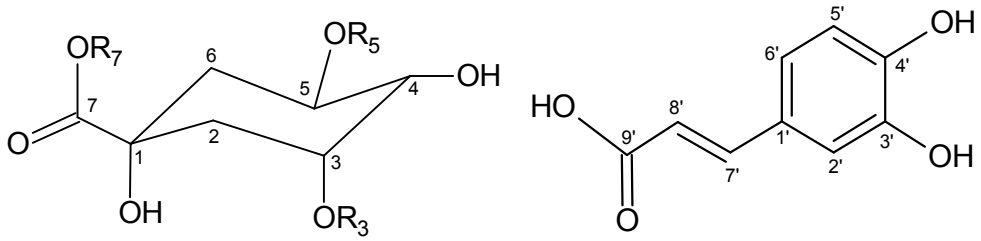


Fig. 4.15
^{1}H NMR spectra of 3-*O*-caffeoylquinic acid (a) and (+)-catechin (b) in deuterated methanol

All three compounds displayed similar ^{1}H and ^{13}C NMR spectra [Table 4.21]. Both 3-CQA and 5-CQA have a molecular formula of $C_{16}H_{18}O_9$. Their ^{1}H NMR showed 10 signals, corresponding to the 10 non-OH protons. Me 5-CQA, with a molecular formula of $C_{17}H_{20}O_9$, had an extra methoxyl signal at 3.68 ppm. The H-4 of 3-CQA was shifted downfield by 0.15 and 0.10 ppm when compared to 5-CQA and Me 5-CQA, respectively.

All three compounds had an ABX spin system at 7.05, 6.77, and 6.95 ppm, and trans-conjugation at 7.56 ppm (*J* = 15.9 Hz) and 6.27 ppm (*J* = 15.9 Hz) derived from the caffeoyl group. ^{13}C NMR of 3-CQA and 5-CQA exhibited 16 signals. Me 5-CQA had an extra methoxy signal at 53.0 ppm. Another key feature of Me 5-CQA was the upfield shift by 1.6 ppm of the quinic acid carboxyl carbon compared to the free carboxylic group.



Quinic acid Caffeic acid

Compound	Identity	R_3	R_5	R_7
1	3-CQA	caffeoyl	H	H
3	5-CQA (CGA)	H	caffeoyl	H
4	Me 5-CQA	H	caffeoyl	methyl

Fig. 4.16
Molecular structures of 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA or CGA) and 5-*O*-caffeoylquinic acid methyl ester (Me 5-CQA)

Table 4.21
^{1}H and ^{13}C NMR spectra of 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA or CGA) and 5-*O*-caffeoylquinic acid methyl ester (Me 5-CQA) from leaves of *Etlingera elatior*

Atom	3-CQA		5-CQA (CGA)		Me 5-CQA	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	76.0		76.1		75.8	
2	37.3	1.92-1.99 (*m*)	38.2	1.92-1.98 (*m*)	37.8	1.96-2.02 (*m*)
3	72.4	5.42 (*br s*)	71.3	4.10 (*m*)	70.3	4.12 (*m*)
4	73.3	3.82 (*m*)	72.0	3.67 (*m*)	72.1	3.72 (*m*)
5	70.0	4.02 (*m*)	73.5	5.36 (*m*)	72.5	5.26 (*m*)
6	39.1	2.10 (*m*)	38.8	2.11 (*m*)	38.0	2.17 (*m*)
1’	127.9		127.8		127.6	
2’	115.1	7.07 (*d* 1.66)	115.2	7.04 (*d* 1.81)	115.1	7.03 (*d* 1.80)
3’	146.7		147.1		147.2	
4’	149.4		149.6		149.7	
5’	116.5	6.79 (*d* 8.16)	116.5	6.76 (*d* 8.16)	116.5	6.77 (*d* 8.13)
6’	122.9	6.96 (*dd* 1.66, 8.17)	123.0	6.94 (*dd* 1.82, 8.12)	123.0	6.94 (*dd* 1.80, 8.22)
7’	146.8	6.33 (*d* 15.9)	146.8	6.28 (*d* 15.9)	146.9	6.21(*d* 15.9)
8’	115.7	7.60 (*d* 15.9)	115.1	7.56 (*d* 15.9)	115.0	7.51 (*d* 15.9)
9’	168.8		168.7		168.3	
COO-	182.1		177.0		175.4	
OCH_3					53.0	3.68 (*s*)

Values of ^{1}H (300 MHz) and ^{13}C (75 MHz) are in ppm (δ), and of *J* (bracketed) are in Hz.

Compound **1** (3-CQA) has the caffeoyl group attached to carbon 3 and OH groups at carbons 1, 4 and 5 [Fig. 4.16]. Compound **3** (5-CQA or CGA) has the caffeoyl group at carbon 5 and OH groups at carbons 1, 3 and 4. Compound **4** (Me 5-CQA) differs from 5-CQA in that the carboxyl group at carbon 7 is esterified with a methyl group.

Following NMR analysis, the identity of these compounds was reaffirmed using EI-MS and ESI-MS. The spectra of 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA or CGA) and 5-*O*-caffeoylquinic acid methyl ester (Me 5-CQA) are shown as Fig. 4.17. 3-CQA has a mass of 354. The mass of 353.2 was due to the removal of a proton [Fig. 4.17a]. The masses of 354 and 368 were those of 5-CQA [Fig. 4.17b] and methyl 5-CQA [Fig. 4.17c], respectively.

3-*O*-caffeoylquinic acid (3-CQA) or 1R,3R,4S,5R)-3-[(E)-3-(3,4-dihydroxyphenyl) prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid (IUPAC name) has a molecular formula of $C_{16}H_{18}O_9$ and a mass of 354.31. 3-CQA is found in stone fruits e.g. prunes, plums, cherries and apricots (Nakatani *et al*., 2000).

5-*O*-caffeoylquinic acid (5-CQA) or 1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxy phenyl) prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carboxylic acid (IUPAC name) has a molecular formula of $C_{16}H_{18}O_9$ and a mass of 354.31. It is an ester of caffeic and quinic acids with axial hydroxyls on carbons 1 and 3, and equatorial hydroxyls on carbons 4 and 5 (Clifford, 2000). It is the most common CQA and is the only one that is commercially available. Coffee beans are one of the richest dietary sources of CGA (Clifford, 1999). It is also found in pome fruits e.g. apple and pear, and in Brassica vegetables e.g. kale, cabbage and Brussel sprout.

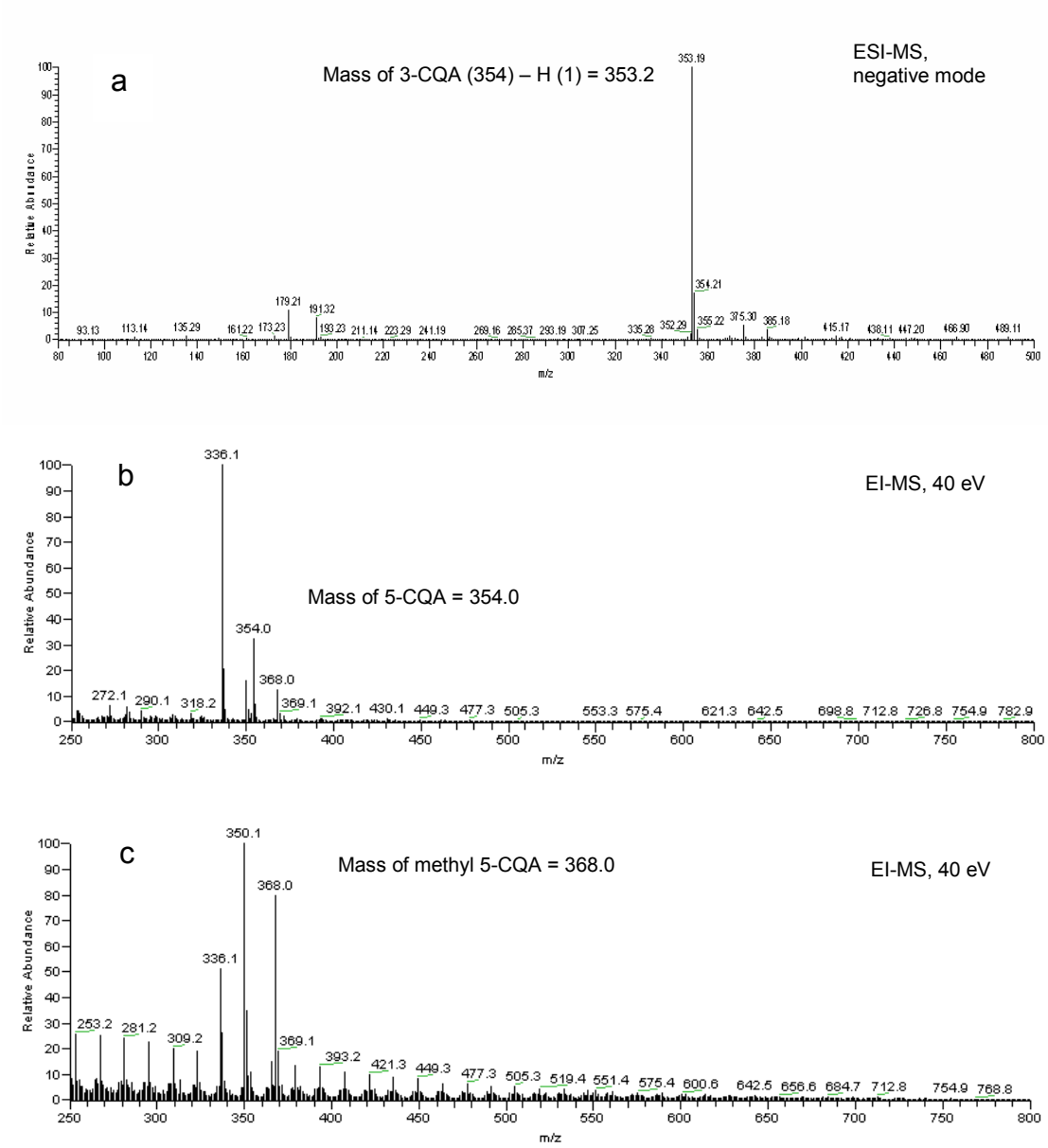


Fig. 4.17
EI-MS and ESI-MS spectra of 3-*O*-caffeoylquinic acid (a), 5-*O*-caffeoylquinic acid (b) and 5-*O*-caffeoylquinic acid methyl ester (c)

5-*O*-caffeoylquinic acid methyl ester (methyl 5-CQA) or methyl 1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]oxy-1,4,5-trihydroxycyclohexane-1-carb oxylate (IUPAC name) has a molecular formula of $C_{17}H_{20}O_9$ and a mass of 368.34. Methyl 5-CQA has been isolated from fruits of *Embelia schimperi* (Hussein *et al*., 2000) and from rhizomes of *Polygonum paleaceum* (Wang *et al*., 2005).

Flavonoids

Flavonoids isolated from leaves of *E. elatior* were elucidated by ^{1}H NMR and identified as (+)-catechin, quercetin 3-*O*-glucoside (isoquercitrin) and quercetin 3-*O*-rhamnoside (quercitrin) [Table 4.22]. Isoquercitrin and quercitrin are common glycosides of quercetin (Hopia & Heinonen, 1999).

Based on spectroscopic analysis and on comparison of ^{1}H NMR data with literature values, Compound **2** was identified as (+)-catechin (Hirose *et al.* (1990). Compound **5** was identified as quercetin 3-*O*-glucoside or isoquercitrin (Panda & Kar, 2007). Compound **6** was identified as quercetin 3-*O*-rhamnoside or quercitrin (Jeong & Shim, 2004). ^{1}H NMR spectra of (+)-catechin, isoquercitrin and quercitrin are shown in Figs. 4.15b and 4.18.

In Zingiberaceae, (+)-catechin has been isolated from leaves of *A. zerumbet* (Mpalantinos *et al.*, 1998). Isoquercitrin and quercitrin have been reported in leaves of *E. elatior* by Williams and Harborne (1977).

Table 4.22
^{1}H NMR spectra of flavonoids (+)-catechin, isoquercitin and quercitrin from leaves of *Etlingera elatior*

Atom	(+)-Catechin	Isoquercitrin	Quercitrin
Aglycone			
2	4.55 (d 7.8)		
3	3.96 (m)		
4	2.84 (dd 5.4,16.2) 2.49 (dd 8.1,16.2)		
6	5.91 (d 2.1)	6.19 (d 1.8)	6.19 (d 2.1)
8	5.84 (d 2.1)	6.40 (d 1.8)	6.36 (d 2.1)
2’	6.82 (d 1.6)	7.58 (d 2.1)	7.33 (d 2.1)
5’	6.75 (d 8.1)	6.81 (d 14.1)	6.9 (d 8.1)
6’	6.70 (dd 1.6, 8.1)	7.54 (dd 2.1, 14.1)	7.30 (dd 2.1, 8.1)
5-OH		12.63 (s)	
Glycosyl			
1”		5.46 (d 7.2)	5.34 (br s)
2”			4.21 (br s)
3”			3.74 (dd 3.5, 9.2)
4”			3.40 m
5”			3.31 (d 5.7)
CH_3			0.93 (d 6.0)

(+)-Catechin and quercitrin were measured in deuterated methanol, and isoquercitrin in deuterated DMSO. Values of ^{1}H (300 MHz) and ^{13}C (75 MHz) are in ppm (δ), and of *J* (bracketed) are in Hz.

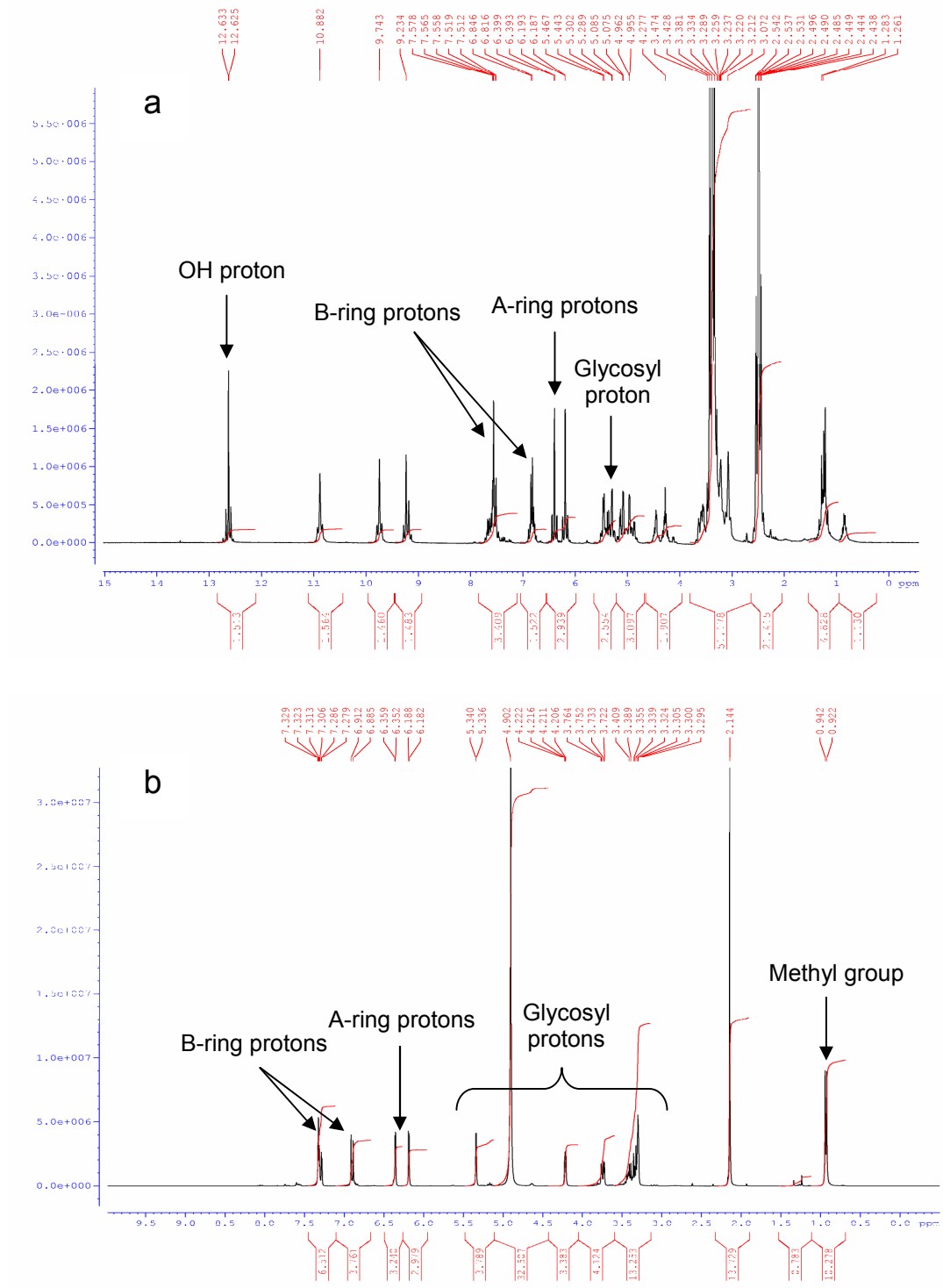


Fig. 4.18
^{1}H NMR spectra of isoquercitrin in deuterated DMSO (a) and of quercitrin in deuterated methanol (b)

All three compounds had an ABX spin system at the B-ring. For isoquercitrin and quercitrin, this was observable at around 7.46, 6.86 and 7.42 ppm. For (+)-catechin, the ABX system was slightly upfield at 6.82, 6.75 and 6.70 ppm. (+)-Catechin also had three additional signals at 4.55, 3.96 and 2.49-2.84 ppm corresponding to the non-OH protons on the C-ring. Isoquercitrin showed a signal at 5.46 ppm, corresponding to the 1'' proton of the glucoside group. Quercitrin showed six signals at 5.34, 4.21, 3.74, 3.40, 3.31 and 0.93 ppm, corresponding to protons of the rhamnoside group. Because isoquercitrin was analysed in deuterated DMSO, an OH signal was observed at 12.63 ppm.

Following NMR analysis, the identity of these compounds was reaffirmed using EI-MS, ESI-MS and ESI-MS/MS. The spectra of (+)-catechin, quercetin 3-*O*-glucoside (isoquercitrin) and quercetin 3-*O*-rhamnoside (quercitrin) are shown as Figs. 4.19, 4.20 and 4.21, respectively.

The mass of 290.1 matched that of (+)-catechin with mass of 290.3 [Fig. 4.19]. The mass of 463.3 was due to the subtraction of a proton from isoquercitrin with mass of 464.4 [Fig. 4.20a]. The mass of 301.3 matched that of a quercetin fragment (mass 302) resulting from the cleavage of a glucose group from carbon 3 [Fig. 4.20b]. When run under positive mode, the mass of 471.1 was due to the addition of a sodium atom with mass of 23.0 to quercitrin with mass of 448.4 [Fig. 4.21]. Flavonoids glycosylated at carbon 3 readily form adducts with alkali metals such as sodium (Zagorevskii, 2004).

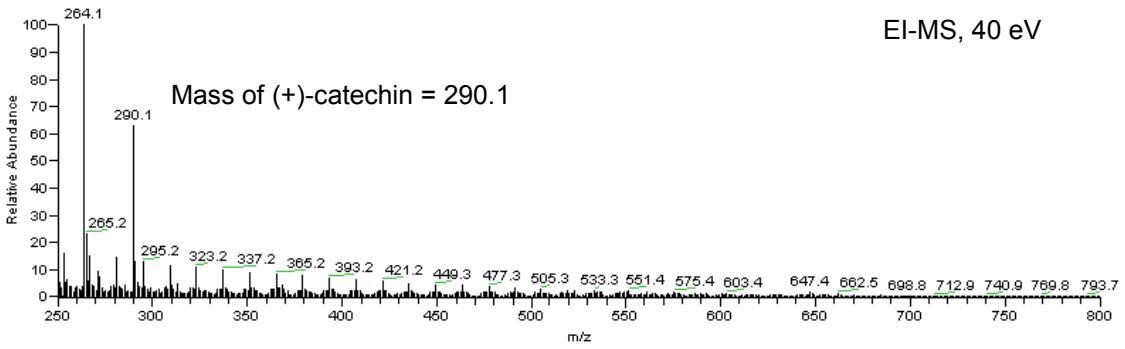
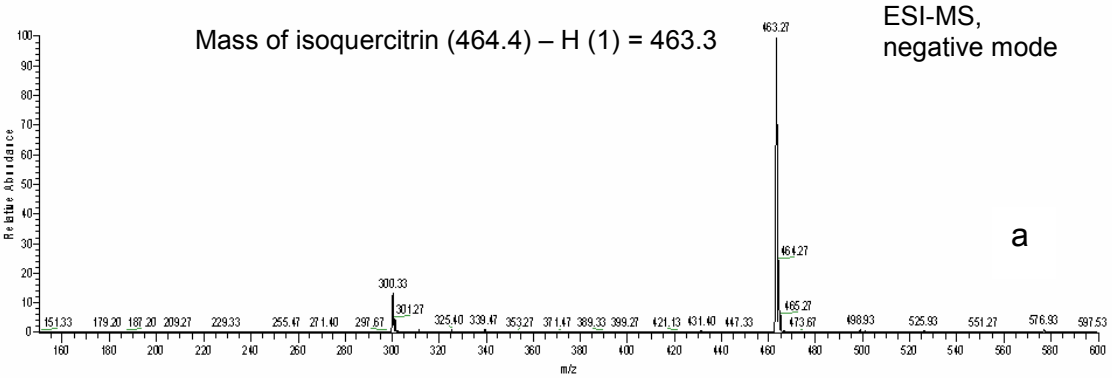


Fig. 4.19
EI-MS spectrum of (+)-catechin



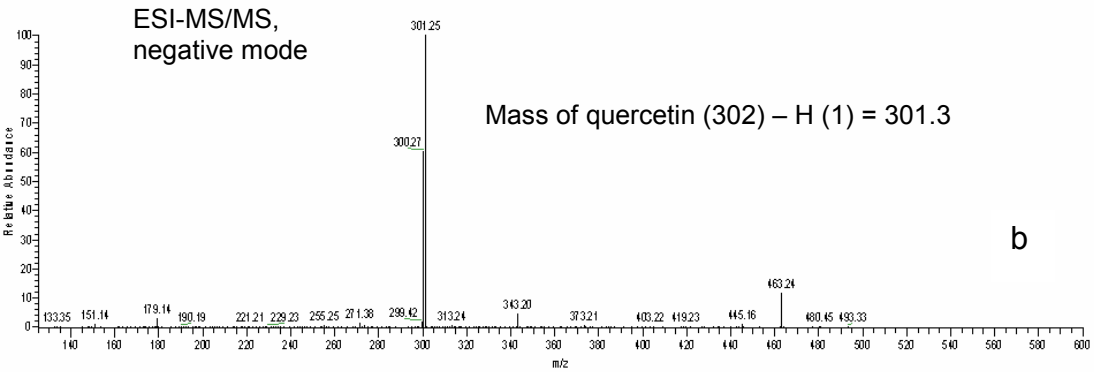


Fig. 4.20
ESI-MS (a) and ESI-MS/MS (b) spectra of isoquercitrin

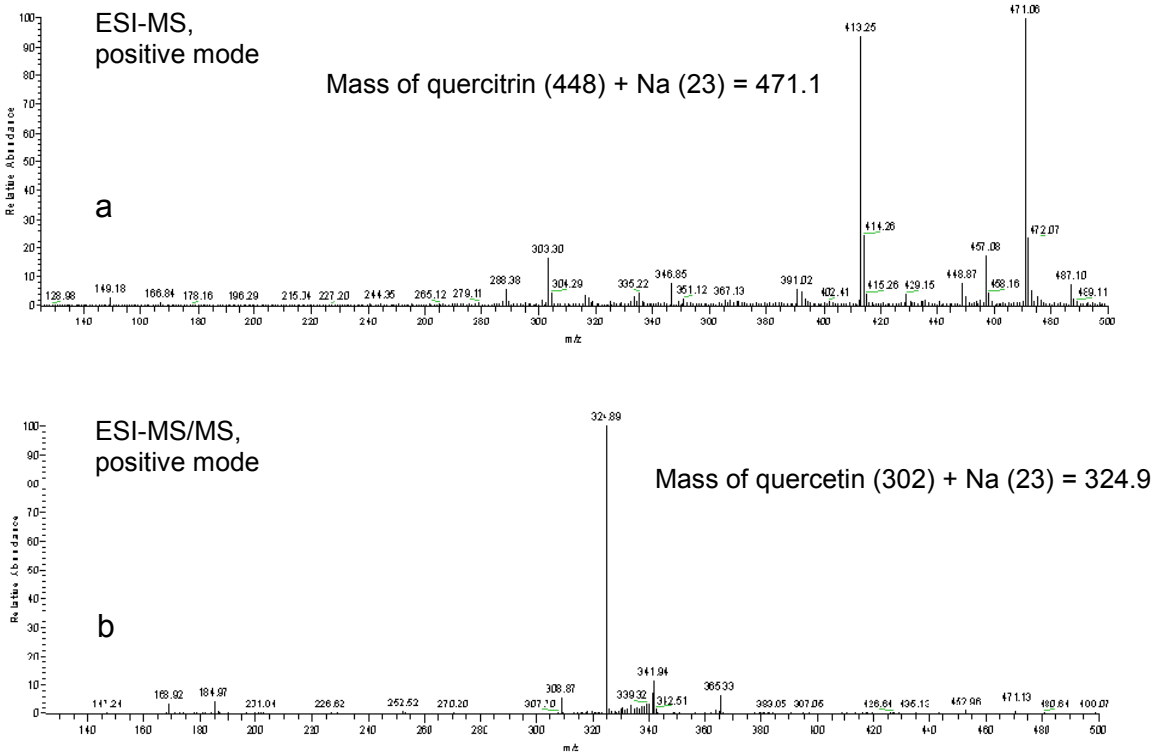


Fig. 4.21
ESI-MS (a) and ESI-MS/MS (b) spectra of quercitrin

(+)-Catechin or (2R,3R)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2H-chromene-3,5,7-triol (IUPAC name) has a molecular formula of $C_{15}H_{14}O_6$ and a mass of 290.27. It is a monomeric flavanol with an ortho-dihydroxyl group in the B-ring at carbons 3' and 4' and a hydroxyl group at carbon 3 in the C-ring [Fig. 4.22]. Red wine, teas, fruits (plum, apple, peach, strawberry and cherry), beans, grains and cocoa are rich in the flavanol (Yilmaz, 2006).

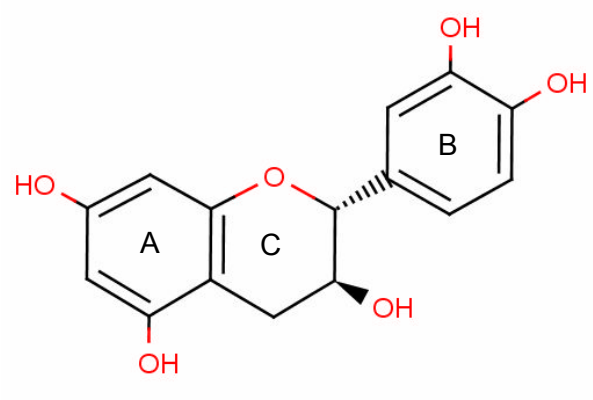


Fig. 4.22
Molecular structure of (+)-catechin

Quercetin 3-*O*-glucoside (isoquercitrin) or 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2S,3R,4S,5R,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-chromen-4-one (IUPAC name) has a molecular formula of $C_{21}H_{20}O_{12}$ and a mass of 464.38. Isoquercitrin has hydroxyl groups at carbons 3', 4', 5 and 7 with a glucoside moiety as the sugar group attached to carbon 3 [Fig. 4.23a]. It has been found in peels of apple and pear, in leaves and flowers of *Hypericum perforatum* and in tea (Pietta *et al*., 2003; Wach *et al*., 2007).

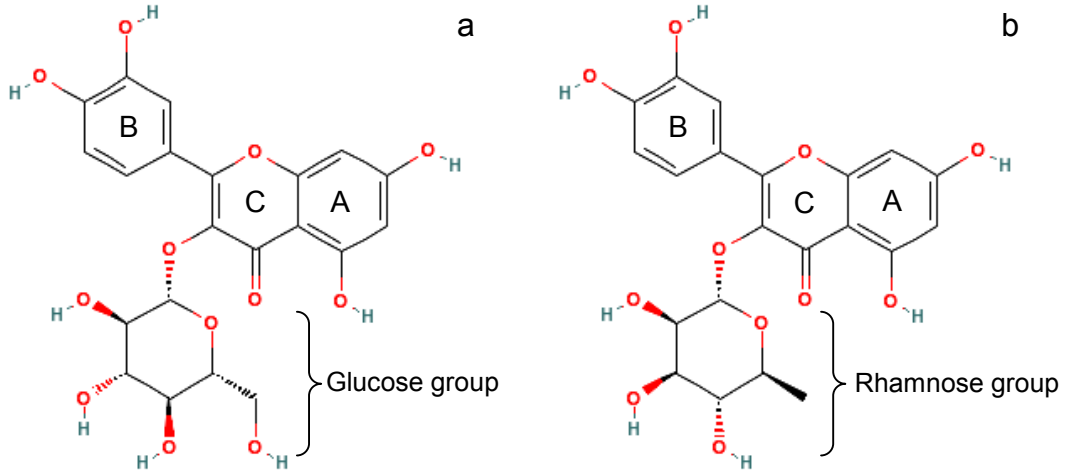


Fig. 4.23
Molecular structures of isoquercitrin (a) and quercitrin (b)

Quercetin 3-*O*-rhamnoside (quercitrin) or 2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-{[(2*S*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyl-2-tetrahydropyranyl]oxy}-4-chromenone (IUPAC name) has a molecular formula of $C_{21}H_{20}O_{11}$ and a mass of 448.38. Quercitrin shares a similar structure as isoquercitrin with hydroxyl groups at carbons 3', 4', 5 and 7 except that a rhamnose moiety as the sugar group is attached to carbon 3 [Fig. 4.23b].

4.5.2 Quantitation of compounds

Of the six compounds isolated from leaves of *E. elatior*, 5-CQA or chlorogenic acid, isoquercitrin and quercitrin were the major compounds (Fig 4.24).

Caffeoylquinic acids

HPLC analysis showed that 5-CQA with retention time (RT) of 5.74 min was the dominant CQA [Fig. 4.24]. Because of the lack of commercially available standards, the quantities of 3-CQA and Me 5-CQA were estimated using the calibration curve of 5-CQA as they have the same chromophoric groups and similar mass. The quantity of 3-CQA (RT of 3.62 min) and Me 5-CQA (RT of 6.74 min) was estimated to be 3 and 30 mg/100 g, respectively. These values were meagre compared to 294 ± 53 mg/100 g of 5-CQA.

Caffeoylquinic acid content (CQAC) and chlorogenic acid content (CGAC) of *E. elatior* leaves were compared with that of four other *Etlingera* species, three commercial ginger species, and two important sources of CQA. Leaves of *E. elatior* and *E. fulgens* displayed the highest content of CQA and were the only two ginger species with CGA [Table 4.23]. CQAC of leaves of *E. elatior*, *E. fulgens* and *E. rubrostriata* was significantly higher than leaves of *Ipomoea batatas*, and comparable to flowers of *Lonicera japonica*. CGA found only in leaves of *E. elatior* and *E. fulgens* was significantly higher in content than leaves of *I. batatas* and flowers of *L. japonica*.

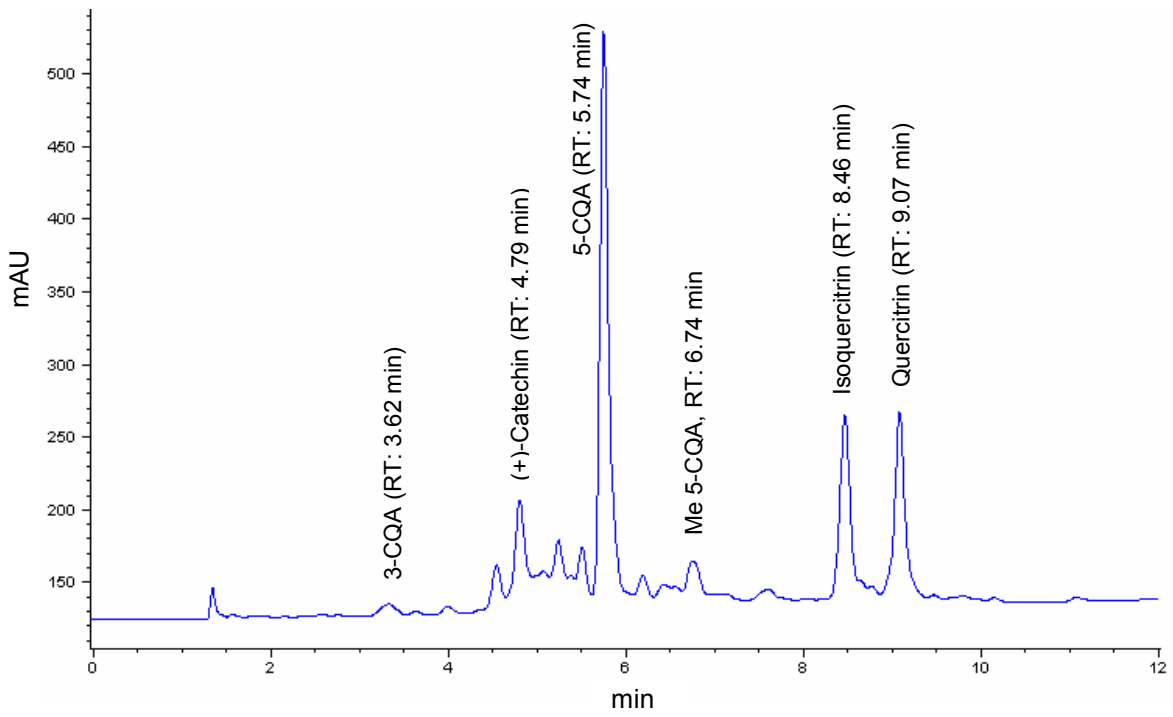


Fig. 4.24
HPLC chromatogram of *Etlingera elatior* leaf extract at 280 nm showing peaks and retention times (RT) of 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA or CGA), 5-*O*-caffeoylquinic acid methyl ester (Me 5-CQA), (+)-catechin, isoquercitrin and quercitrin

Flowers of *L. japonica* are a commercial source of CGA. Leaves of *E. elatior* and *E. fulgens*, which currently have no economic value, could serve as alternative sources of CGA. This is especially true for plants of *E. elatior* which are widely cultivated for their inflorescences as spice. Unlike flowers of *L. japonica* which are small and seasonal, leaves of *E. elatior* are large and available in abundance. Furthermore, its leaves are non-cytotoxic to normal liver and kidney cells [section 4.2.6], and the harvesting of leaves is non-destructive to the plants. However, extraction of CGA from *E. elatior* leaves has to be optimised before their commercial potentials can be realised.

Table 4.23
Total phenolic content (TPC), caffeoylquinic acid content (CQAC) and chlorogenic acid content (CGAC) of leaf extracts of five *Elingera* and three commercial ginger species (fresh weight)

Species	TPC (mg GAE/100 g)	CQAC (mg CGAE/100 g)	CGAC (mg CGA/100 g)
Etlingera elatior	2390 ± 329a	320 ± 62a	294 ± 53a
E. fulgens	1280 ± 144b	326 ± 45a	219 ± 14b
E. rubrostriata	2250 ± 113a	201 ± 23b	–
E. littoralis	2150 ± 94a	129 ± 23c	–
E. maingayi	1110 ± 93b	48 ± 13d	–
Alpinia galanga	392 ± 50c	24 ± 14de	–
Curcuma longa	230 ± 19d	44 ± 6d	–
Zingiber officinale	291 ± 18d	11 ± 8e	–
Lonicera japonica	533 ± 41e	250 ± 49ab	173 ± 13c
Ipomoea batatas	386 ± 23c	125 ± 10c	115 ± 16d

TPC, CQAC and CGAC values are means ± SD (n = 3). For each column, values followed by the same letter (a−e) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Rich in CGA, flowers of *Lonicera japonica* and leaves of *Ipomoea batatas* were included for comparison. Abbreviations: GAE = gallic acid equivalent, CGAE = chlorogenic acid equivalent and CGA = chlorogenic acid.

Flavonols

HPLC analysis showed that the content of isoquercitrin with RT of 8.46 min and quercitrin with RT of 9.07 min was found to be 117 ± 32 and 79 ± 19 mg/100 g, respectively [Fig. 4.24].

It can therefore be seen that 5-CQA or CGA at 294 ± 53 mg/100 g is the dominant compound in leaves of *E. elatior* followed by isoquercitrin at 117 ± 32 mg/100 g and quercitrin at 79 ± 19 mg/100 g. Ranking of the three major compounds is of the following order: CGA > isoquercitrin > quercitrin.

4.5.3 Bioactivity of compounds

Antibacterial activity

Commercial standards of 5-CQA or CGA, isoquercitrin and quercitrin, the three major compounds found in leaves of *E. elatior*, showed no antibacterial activity when tested using the disc- diffusion method at 1 mg/disc.

In general, findings from this study are in agreement with earlier reports on antibacterial activity of phenolic compounds. Using the broth dilution method, CGA was found to be ineffective against *Listeria monocytogenes* (Wen *et al.*, 2003) but exhibited weak minimum inhibitory concentration (MIC) against *Staphylococcus mutans* and *S. aureus* (Daglia *et al*., 2007). When tested against 12 bacterial strains, CGA and isoquercitrin showed no inhibitory activity using the agar diffusion assay (Puupponen-Pimia *et al.*, 2001). Using the paper disc assay, quercetrin showed no inhibitory activity against *Salmonella enteritidis* and *B. cereus* at 100 µg/disc but only showed weak inhibition at 400 µg/disc (Arima *et al*., 2002).

Tyrosinase inhibition activity

Commercial standards of all three major compounds found in leaves of *E. elatior* showed no tyrosinase inhibition activity when tested using the dopachrome method with L-DOPA as substrate.

Findings on CGA contradicted earlier studies which reported that CGA showed strong non-competitive tyrosinase inhibitory activity, comparable to that of arbutin (Tada *et al.*, 2001 & 2002). Findings on isoquercitrin and quercitrin are in agreement with Kubo *et al.* (2000). They found no tyrosinase inhibition activity for isoquercitrin and postulated that a sugar moiety attached to the 3-hydroxyl group as in 3-*O*-glycosides may hinder the approach of the molecule to the active site of tyrosinase. It can be inferred that quercitrin with the attached rhamnose moiety will similarly have little or no tyrosinase inhibition activity. Jeong and Shim (2004) have reported weak tyrosinase inhibition activity in quercitrin. The IC_{50} of quercitrin was > 50 µg/ml compared to 3.8 µg/ml of quercetin.

Antioxidant activity

When tested for antioxidant activity, all three compounds showed DPPH radical scavenging activity [Table 4.24]. In terms of IC_{50} in µg/ml, CGA (7.24) was weaker than caffeic acid (3.69) and ascorbic acid (3.87). Values of isoquercitrin (5.72) and quercitrin (6.20) were comparable, weaker than ascorbic acid but stronger than CGA. However, when expressed in µM, these compounds have very different strength order because of their differences in mass. On conversion to µM, the IC_{50} of CGA (20.4) was comparable to that of caffeic acid (20.5) and stronger than that of ascorbic acid (22.0).

From the data, it becomes apparent that calculating IC_{50} of DPPH radical scavenging in µg/ml or in µM would lead to totally different interpretations of the radical scavenging ability of the compounds. This could be among the reasons why data in the literature comparing the antioxidant properties of phenolic acids are often contradictory (Marinova *et al*., 2009). Furthermore, the DPPH radical scavenging ability of compounds published in the literature was expressed in many different units making comparison between different sources difficult.

Table 4.24
DPPH radical scavenging activity (IC_{50}) of major compounds found in leaves of *Etlingera elatior*

Compound	DPPH radical scavenging activity IC_{50} (µg/ml)	IC_{50} (µM)
Chlorogenic acid	7.24 ± 0.27	20.4 ± 0.77
Isoquercitrin	5.72 ± 0.60	12.8 ± 1.32
Quercitrin	6.20 ± 0.20	13.4 ± 0.42
Caffeic acid	3.69 ± 0.10	20.5 ± 0.54
Ascorbic acid	3.87 ± 0.08	22.0 ± 0.44

Caffeic acid and ascorbic acid were used as controls.

According to Cuvelier *et al.* (1992), esterification of caffeic acid (CA) by a sugar moiety decreased its antioxidative activity. This observation was supported by a number of other studies. Chen and Ho (1997) reported that CA demonstrated more effective antioxidant activity than CGA. DPPH radical scavenging activity was 51.5% for CA and 36.3% for CGA. Rosmarinic acid (dimer of CA with four hydroxyl groups) had the strongest activity of 85.6%. Similarly, Ohnishi *et al.* (1994) reported higher radical scavenging of 35% for CA and 28% for CGA, compared to 19% for both α-tocopherol and ascorbic acid.

On the other hand, Sroka and Cisowski (2003) reported stronger radical scavenging ability in CGA (93%) than CA (44%) and suggested that the binding of quinic acid strengthens the antioxidant activity of CA. Similarly, Nara *et al*. (2006) found stronger radical scavenging activity in CGA than CA from potato peel with values of 7.9 and 3.9 µmol TE/g, respectively. The radical scavenging activity (%) of CQA derivatives in sweet potato leaves was in the order: CGA > 3,4,5-triCQA > 3,4-diCQA ~ 3,5-diCQA ~ 4,5-diCQA > CA ~ *p*-coumaric acid

(Islam, 2006). Radical scavenging ability of CGA and CA from medicinal herbs yielded IC_{50} of 12.4 and 13.6 μM, respectively (Yokozawa *et al.*, 1998).

Phenolic acids are known to be chain-breaking antioxidants, acting through radical scavenging related to their hydrogen-donating capacity (Laranjinha, 2001; Siquet *et al.*, 2006). Their antioxidant activity depends on the number and position of hydroxy groups in the molecule (Shahidi & Naczk, 2004c; Andjelkovic *et al.*, 2006). Important mechanisms underlying their antioxidant activity are the catechol group and the double bond conjugated with the catechol ring, which confer greater antioxidant capacity (Rice-Evans *et al.*, 1996; Laranjinha, 2001). Nakatani *et al.* (2000) reported that the position of esterification on the quinic acid molecule with CA is unlikely to influence the antioxidant activity. This would mean that the different isomers of CQA may have similar antioxidant properties.

CGA is a natural scavenger because the one-electron oxidation product formed by the reaction with free radicals is rapidly broken down to further products that cannot generate any free radicals (Shibata *et al.*, 1999). Unlike many dietary antioxidants such as vitamins C and E which scavenge free radicals, but themselves form secondary radical species, CGA scavenges •OH and protects other endogenous antioxidants from being depleted (Zang *et al.*, 2003). Cheng *et al.* (2007) pointed out that molecules bearing *ortho*-dihydroxyl group, such as CGA, possess significantly higher antioxidant activity than those bearing no such functionalities.

Another antioxidant property of CGA is its ability to chelate iron (Kono *et al.*, 1998). The binding site of iron is the phenolic OH group of CGA. The formation of hydroxyl radicals is prevented as the iron complex cannot catalyse the Fenton-type reaction. The iron chelation ability of CGA bearing catechol or galloyl groups is greater than those without these groups (Andjelkovic *et al.*, 2006).

Isoquercitrin and quercitrin showed comparable DPPH radical scavenging activity that was much stronger than α-tocopherol (Kawaguchi *et al*., 2007). Their IC_{50} values were reported to be 8.8, 8.3 and 25.6 μM, respectively. Isoquercitrin and quercitrin isolated from leaves of *Castanea crenata* were found to have similar DPPH radical scavenging activity as α-tocopherol, all with IC_{50} of 12 μg/ml (Choi *et al*., 2000). Of three compounds isolated from flowers of *Chrysanthemum morifolium*, DPPH radical scavenging of quercitrin was stronger than CGA, with IC_{50} of 20.2 and 30.1 μg/ml, respectively (Chung, 2006). DPPH radical scavenging IC_{50} of isoquercitrin (6.39 μg/ml) and quercitrin (5.99 μg/ml) from leaves of *Piliostigma reticulatum* was weaker compared to quercetin and ascorbic acid (Aderogba *et al*., 2005). From medicinal herbs, isoquercitrin, quercitrin and quercetin yielded IC_{50} of 13.3, 11.1 and 7.9 μM, respectively (Yokozawa *et al.*, 1998). From the fruits of *Chrysophyllum cainito*, IC_{50} of isoquercitrin (74 μM) was lower than quercetin (41 μM) (Luo *et al.*, 2002).

The weaker radical scavenging ability of isoquercitrin and quercitrin than that of quercetin can be explained by their structure-activity relationship. Quercetin, the aglycone, has a 3-OH which is the active site donating a hydrogen atom for scavenging DPPH radicals (Seyoum *et al*., 2006). In the absence of the 3-OH group, the hydrogen donating ability of isoquercitrin and quercitrin is derived from the 3'4'-dihydroxyl (catechol) group of the B-ring. Compounds with this substitution have shown to have good inhibitory activity against DPPH radicals. In addition, the attachment of the sugar moiety to carbon 3 may hinder the access of free radical scavengers to DPPH radicals (Yokozawa *et al*., 1998).

Bioavailability of quercetin glycosides studied in a rat model showed that the bound sugar moiety has effect on their bioavailability and the nature of glycosylation markedly influences the efficiency of quercetin absorption (Morand *et al.*, 2000). The binding of a glucoside moiety as in isoquercitrin improves the

absorption of isoquercitrin and the binding of a rhamnose moiety as in quercitrin markedly depressed its absorption.

From the literature, some information is available on the antioxidant properties of other compounds found in leaves of *E. elatior*, namely, (+)-catechin, 3-CQA and methyl 5-CQA.

(+)-Catechin has been reported to have antioxidant properties involving radical scavenging (Nakao *et al.*, 1998; Nanjo *et al.*, 1996 & 1999; Katalinic *et al.*, 2004; Kawaguchi *et al.*, 2007) ferric reducing (Katalinic *et al.*, 2004), ferrous chelating (Morel *et al.*, 1993), lipid peroxidation inhibition (Katalinic *et al.*, 2004; Zhang *et al.*, 2006b) and LDL oxidation (Teissedre *et al.*, 1996). Important structural features for scavenging activity of (+)-catechin are the ortho-dihydroxyl group in the B-ring (Nanjo *et al.*, 1996 & 1999). In the LDL oxidation system, (+)-catechin without a keto group in the 4-position is more inhibitory than flavonols (Teissedre *et al.*, 1996).

3-CQA has been reported to have radical scavenging activity (Nakatani *et al.*, 2000) and ferric reducing ability (Moreira *et al.*, 2005). Me 5-CQA showed scavenging activity of DPPH and superoxide anion radicals (Wang *et al.*, 2005; Park *et al.*, 2007). Closely related methyl esters of 3-CQA and of 4-CQA isolated from prune fruits have high oxygen radical absorbance capacity (ORAC) values (Kayano *et al.*, 2004a & 2004b).

4.6 ESSENTIAL OILS

4.6.1 Extraction of oils

Essential oils were extracted from leaves (500 g) of four *Etlingera* species by hydro-distillation. Leaves of *E. maingayi* yielded the most oil of 1317 mg/100 g. The yield of the other three species, namely, *E. elatior*, *E. fulgens* and *E. rubrostriata* was 86, 133 and 39 mg/100 g, respectively.

4.6.2 Analysis of oils

The chemical composition of essential oils from leaves of four *Etlingera* species as determined by GC-MS is presented in Table 4.25. The quantity of individual oils in each species is shown in Table 4.26. The types of essential oils identified in *E. elatior*, *E. fulgens*, *E. maingayi* and *E. rubrostriata* were 15, 11, 4 and 23, respectively. Of these species, only leaves of *E. elatior* have been studied (Mohd Jaafar *et al.*, 2007).

Sesquiterpenes were the major constituents of leaves of *E. elatior* comprising (*E*)-farnesene (13.6%), (*E*)-caryophyllene (8.56%), isodaucene (1.84%), β-bisabolene (0.32%) and β-sesquiphellandrene (0.18%). (*E*)-farnesene and (*E*)-caryophyllene, the major constituents, have also been reported by Mohd Jaafar *et al.* (2007). Oils of *E. fulgens* comprised mainly of dodecyl acetate (21.6%), an ester, and pentadecanol (14.1%) and hexadecanol (3.60%), both long-chain alcohols. Dodecyl acetate, pentadecanol and hexadecanol were also present in *E. elatior* but in much smaller amounts. Dodecanal, an aldehyde, detected in leaves of *E. elatior* (3.09%) and *E. fulgens* (8.08%) was previously reported in inflorescences of *E. elatior* by Wong *et al.* (1993). It can be seen that essential oils from leaves of *E. elatior* and *E. fulgens* were moderately diverse. The composition of essential oils of both species was different despite having very similar aroma and morphology.

Table 4.25
Composition of essential oils from leaves of *Etlingera*

Essential oil	Type	RT	Composition (%)			
			E. elatior	*E. fulgens*	*E. maingayi*	*E. rubrostriata*
α-Thujene [cde]	Monoterpene	7.68				0.09
Sabinene [bcd]	Monoterpene	10.47				0.11
Undecane	Alkane	18.91		0.02		0.09
Linalool [bce]	Monoterpenol	19.32				1.45
Terpinen-4-ol [abe]	Monoterpenol	23.77				2.78
α-Terpineol [abc]	Monoterpenol	24.69	0.90	0.11		2.55
Decanal [bd]	Aldehyde	25.26				0.39
Octanoic acid	Fatty acid	25.28			0.40	
Geraniol [ac]	Monoterpenol	28.31				0.22
Methyl myrtenate	Monoterpenic ester	29.97	0.87			
Undecanal	Aldehyde	30.56		0.24		
Methyl decanoate	Fatty acid ester	31.38			0.19	
Myrtenyl acetate	Monoterpenol ester	31.38				0.15
Tetradecene	Alkene	34.53	0.54			
β-Elemene [b]	Sesquiterpene	34.54				0.26
Dodecanal	Aldehyde	35.51	3.09	8.08		
(*E*)-Caryophyllene [bcde]	Sesquiterpene	35.85	8.56			
Decanoic acid	Fatty acid	36.53			42.6	
(*E*)-Farnesene [bcd]	Sesquiterpene	37.57	13.6			0.36
Tridecanone	Ketone	37.35				0.41
Isodaucene	Sesquiterpene	39.39	1.84			
β-Bisabolene	Sesquiterpene	39.82	0.32			0.57
β-Sesquiphellandrene [abe]	Sesquiterpene	40.47	0.18			
Hedycaryol	Sesquiterpenol	41.88				0.25
(*E*)-Nerolidol [cd]	Sesquiterpenol	42.35		0.27		1.53
Caryophyllene oxide [a]	Sesquiterpene epoxide	43.10	5.15			
Dodecanoic acid	Fatty acid	44.03			44.6	4.41
Dodecyl acetate	Ester	44.15	6.68	21.6		6.01
γ-Eudesmol [c]	Sesquiterpenol	45.22				0.64
α-Eudesmol [c]	Sesquiterpenol	46.15				2.03
Pentadecanol	Alcohol	47.13	6.39	14.1		1.01
(*E*,*E*)-Farnesol [ac]	Sesquiterpenol	48.81				0.28
Octadecene	Alkene	50.94	1.95			
Hexadecanol	Alcohol	51.65	1.66	3.60		
Phytol	Diterpenol	56.59		0.28		1.35
Cyclohexadecanolide	Cyclic ester	56.97		0.75		
Geranyl linalool	Diterpenol	59.35				0.93
Oleic acid	Fatty acid	63.35		1.38		
Docosene	Alkene	64.32	1.24			

[abcde] Previously reported in leaves of *Elettariopsis elan* (Wong *et al*., 2006a), *Alpinia conchigera* (Ibrahim *et al*., 2009), *Alpinia galanga* (Jirovetz *et al*., 2003), *Etlingera elatior* (Mohd Jaafar *et al*., 2007) and *Curcuma longa* (Garg *et al.*, 2002), respectively. Composition of essential oils was expressed as percentage of total peak area. Abbreviation: RT = retention time (min).

Table 4.26
Composition and diversity of essential oils from leaves of *Etlingera* according to types

Type	Composition (%)			
	E. elatior	*E. fulgens*	*E. maingayi*	*E. rubrostriata*
Alcohol	8.05 (2)	17.7 (2)		1.01 (1)
Aldehyde	3.09 (1)	8.32 (2)		0.39 (1)
Cyclic ester		0.75 (1)		
Ester	6.68 (1)	21.6 (1)		6.01 (1)
Fatty acid		1.38 (1)	87.6 (3)	4.41 (1)
Fatty acid ester			0.19 (1)	
Hydrocarbon	3.73 (3)	0.02 (1)		0.09 (1)
Ketone				0.41 (1)
Monoterpene				0.2 (2)
Monoterpene derivative	1.77 (2)	0.11 (1)		7.15 (5)
Sesquiterpene	24.5 (5)			1.19 (3)
Sesquiterpene derivative	5.15 (1)	0.27(1)		4.73 (5)
Diterpene derivative		0.28 (1)		2.28 (2)
Total	53.0 (15)	50.4 (11)	87.8 (4)	27.9 (23)

Amount of essential oils was expressed as percentage of total peak area. Figures in brackets indicate the diversity of a given type.

Leaves of *E. maingayi* had the highest yield of essential oils but had the least diversity of compounds among the *Etlingera* species studied. They comprised almost entirely of three fatty acids (87.6%) and one fatty acid ester (0.19%). The two major fatty acids were dodecanoic acid $C_{12}H_{24}O_2$ (44.6%) and decanoic acid $C_{10}H_{20}O_2$ (42.6%). The unpleasant sour scent of leaves of *E. maingayi* may be attributed to their high fatty acid content.

Essential oils from leaves of *E. rubrostriata* were most diverse with 23 different types identified. However, they only represented 27.9% of the total composition, implying the presence of uncommon oils. Despite having many different types of oils, leaves of *E. rubrostriata* do not emit any scent. This is probably due to their low essential oil content (39 mg/100 g).

4.6.3 Antibacterial activity

Essential oils from leaves of all the four *Etlingera* species inhibited Gram-positive bacteria of *B. cereus*, *M. luteus* and *S. aureus* with no activity on Gram-negative bacteria of *E. coli*, *P. aeruginosa* and *S. choleraesuis* [Table 4.27]. Essential oils from leaves of *E. maingayi* showed the strongest activity with minimum inhibitory concentration (MIC) of 6.3 mg/ml for *B. cereus* and *M. luteus*, and 12.5 mg/ml for *S. aureus*. Of the Gram-positive bacteria, *M. luteus* was the most susceptible with all *Etlingera* species having MIC of 6.3 mg/ml.

There are few studies on the antibacterial activity of essential oils from leaves of ginger species. One such study was on leaves of *Alpinia conchigera* by Ibrahim *et al*. (2009) who reported weak activity against Gram-positive *Staphylococcus epidermidis* and *S. aureus*, and Gram-negative *Pseudomonas cepacia* and *P. aeruginosa*.

Table 4.27
Minimum inhibitory concentration (MIC) of essential oils from leaves of *Etlingera* species against Gram-negative bacteria

Etlingera species	MIC (mg/ml)		
	B. cereus	*M. luteus*	*S. aureus*
E. elatior	25.0	6.30	50.0
E. fulgens	25.0	6.30	100
E. maingayi	6.30	6.30	12.5
E. rubrostriata	12.5	6.30	50.0

Abbreviations: *B.* = *Bacillus*, *M.* = *Micrococcus* and *S.* = *Staphylococcus*

4.7 STANDARDISED EXTRACT

A standardised herbal extract is one that has been processed so that it contains a specified amount of certain active compound(s). Commercially, the amount is listed on the label to inform consumers that the product contains the stated amount of active compound(s).

A rapid method for producing a CGA standardised extract of ~40% w/w purity isolated from leaves of *E. elatior* was developed. Aqueous ethanol solvents were used for both extraction and isolation. The entire fractionation process was done fairly rapidly with only gravity flow.

4.7.1 Drying of leaves

The effect of two drying treatments on the TPC, CQAC and CGAC of *E. elatior* leaves were evaluated [Table 4.28]. The highest amount of CQA and CGA was extracted from the freeze-dried leaves. Values of freeze-dried leaves of 3042 ±

208 mg GAE/100 g, 413 ± 33 mg CGAE/100 g and 283 ± 17 mg CGA/100 g were higher than values of fresh leaves of 2560 ± 329 mg GAE/100 g, 369 ± 26 mg CGAE/100 g and 238 ± 11 mg CGA/100 g, respectively. Microwave-dried leaves yielded a comparable amount of CQA as the fresh leaves but a higher amount of chlorogenic acid.

Table 4.28
Effect of microwave- and freeze-drying on total phenolic content (TPC), caffeoyl-quinic acid content (CQAC) and chlorogenic acid content (CGAC) of leaves of *Etlingera elatior*

Treatment	Water loss (%)	TPC (mg GAE/100 g)	CQAC (mg CGAE/100 g)	CGAC (mg CGA/100 g)
Fresh	–	2560 ± 329a	369 ± 26a	238 ± 11a
Microwave-dried	67 ± 0.6a	2079 ± 33b	357 ± 21a	264 ± 14b
Freeze-dried	69 ± 0.1b	3042 ± 208a	413 ± 33a	283 ± 17c

Values of TPC, CQAC and CGAC are means ± SD (n = 3). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Abbreviations: GAE = gallic acid equivalent, CGAE = chlorogenic acid equivalent and CGA = chlorogenic acid.

Drying of leaves enhances their storage stability and reduces their volume and weight (Sablani & Rahman, 2007). This minimises the costs of packaging, transportation and storage that may be incurred for producing the standardised extract. Furthermore, leaves can be easily ground when dry.

4.7.2 Choice of solvent

Varying concentrations of aqueous ethanol were tested for their efficiency in extracting CQA and other phenolic compounds from fresh leaves of *E. elatior*. Extraction using 70%, 50% and 30% aqueous ethanol, all yielded comparable

amounts of CQA and phenolic compounds [Table 4.29]. Values of TPC were 2246 ± 257, 2344 ± 290 and 2297 ± 408 mg GAE/100 g, and values of CQAC were 316 ± 100, 342 ± 59 and 337 ± 58 mg CGAE/100 g, respectively.

Table 4.29
Total phenolic content (TPC) and caffeoylquinic acid content (CQAC) of *Etlingera elatior* leaves using different concentrations of ethanol as solvent

Solvent	TPC (mg GAE/100 g)	CQAC (mg CGAE/100 g)
70% ethanol	2246 ± 257a	316 ± 100a
50% ethanol	2344 ± 290a	342 ± 59a
30% ethanol	2297 ± 408a	337 ± 58a

TPC and AEAC values are means ± SD (n = 3). For each column, values followed by the same letter are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Abbreviations: GAE = gallic acid equivalent and CGAE = chlorogenic acid equivalent.

4.7.3 Isolation of chlorogenic acid

As all tested solvent concentrations yielded comparable amounts of TPC and CQA [Table 4.29], 30% aqueous ethanol being the most cost-effective solvent was chosen for extraction. Leaves were freeze-dried overnight to facilitate maceration in a blender. Crude extracts were dried in a rotary evaporator at 50°C before subjected to column chromatography.

Weight, CGA content (CGAC) and CQA content (CQAC) of the crude extract were 4470 ± 240 mg, 28 ± 2 mg/g and 53 ± 2 mg/g, respectively [Table 4.30]. Yields of CGA and CQA were 234 ± 26 and 437 ± 25 mg/100 g, respectively.

Initial isolation with Diaion HP-20 yielded fractions 1, 2 and 3. Most of the CGA and CQA were eluted in fraction 2 (10–35% ethanol) with CGAC increasing from 28 ± 2 to 96 ± 4 mg/g and CQAC from 53 ± 2 to 169 ± 7 mg/g after fractionation [Table 4.30]. This represents a significant increase of 3.4 times in CGAC and 3.2 times in CQAC compared to the crude extract. Fractionation with Diaion HP-20 reduced the yield of CGA and CQA by 28.6 and 32.5%, respectively. Fractions 1 and 3 had very low CGAC (7.5 ± 0.5 and 2.2 ± 1.1 mg/g) and CQAC (73 ± 1.8 and 85 ± 17 mg/g), respectively. This implies that most of the CGA is eluted in fraction 2 with very little lost to the other fractions.

Diaion HP-20 was chosen as the column-packing material because it is capable of elution at extremely high flow rates. Gravity elution in a 20 x 230 mm column was 75 ml/min. Furthermore, Diaion HP-20 has good selectivity for aromatic hydrophobic compounds.

Table 4.30
Composition and yield of caffeoylquinic acids (CQA) and chlorogenic acid (CGA) after fractionation with Diaion HP-20 and Sephadex LH-20 columns

Column	Flow rate (ml/min)	Extract weight (mg)	CQA		CGA	
			Content (mg/g)	Yield (mg/100 g)	Content (mg/g)	Yield (mg/100 g)
Crude extract	n/a	4470 ± 240	53 ± 2a	437 ± 25a	28 ± 2a	234 ± 26a
Diaion (Fraction 2)	75	871 ± 105	169 ± 7b	295 ± 42b	96 ± 4b	167 ± 15b
Sephadex (Fraction 2.2)	3.5	201 ± 8	370 ± 17c	149 ± 13c	408 ± 52c	163 ± 15b

Values of extract weight, CQA and CGA are means ± SD (n = 3). For each column, values followed by the same letter are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Extract weight (mg) is derived from 50 g of fresh leaves. Content (mg/g) and yield (mg/100 g) are based on extract weight and fresh leaves, respectively.

Further isolation of fraction 2 using Sephadex LH-20 yielded fractions 2.1, 2.2 and 2.3. Most of the CGA and CQA were eluted in fraction 2.2 (20% ethanol) with CGAC increasing from 96 ± 4 to 408 ± 52 mg/g and CQAC from 169 ± 7 to 370 ± 17 mg/g after fractionation [Table 4.30]. This represents a significant increase of 4.3 times in CGAC and 2.2 times in CQAC compared to fraction 2. Fractionation with Sephadex LH-20 resulted in full recovery of the yield of CGA but a reduction of 49.5% in the yield of CQA, as eluents were optimised for the isolation of CGA. Fractions 2.1 and 2.3 had very low CGAC (4.5 ± 0.8 and 5.5 ± 1.2 mg/g) and CQAC (109 ± 6.7 and 71 ± 17 mg/g), respectively. This implies that most of the CGA was eluted in fraction 2.2 with very little loss to the other fractions.

Sephadex LH-20 had a much slower gravity elution than Diaion HP-20. Flow rate in a 30 x 60 mm column was only 3.5 ml/min. However, Sephadex LH-20 was able to refine CGA with a simple 3-step elution that involved the usage of minimal amounts of ethanol. It has excellent selectivity based on size exclusion and hydrophobic adsorption.

HPLC chromatograms at 280 nm showing CGA peaks of crude extract of leaves of *E. elatior*, and of standardised extract fractionated by Diaion and Sephadex are shown in Fig. 4.25. The chromatogram of the crude extract showed the presence of compounds other than CGA. Subsequently, their presence was progressively reduced through sequential fractionation with Diaion and Sephadex. Purity of the CGA standardised extract after Sephadex fractionation was reflected by the presence of a single peak.

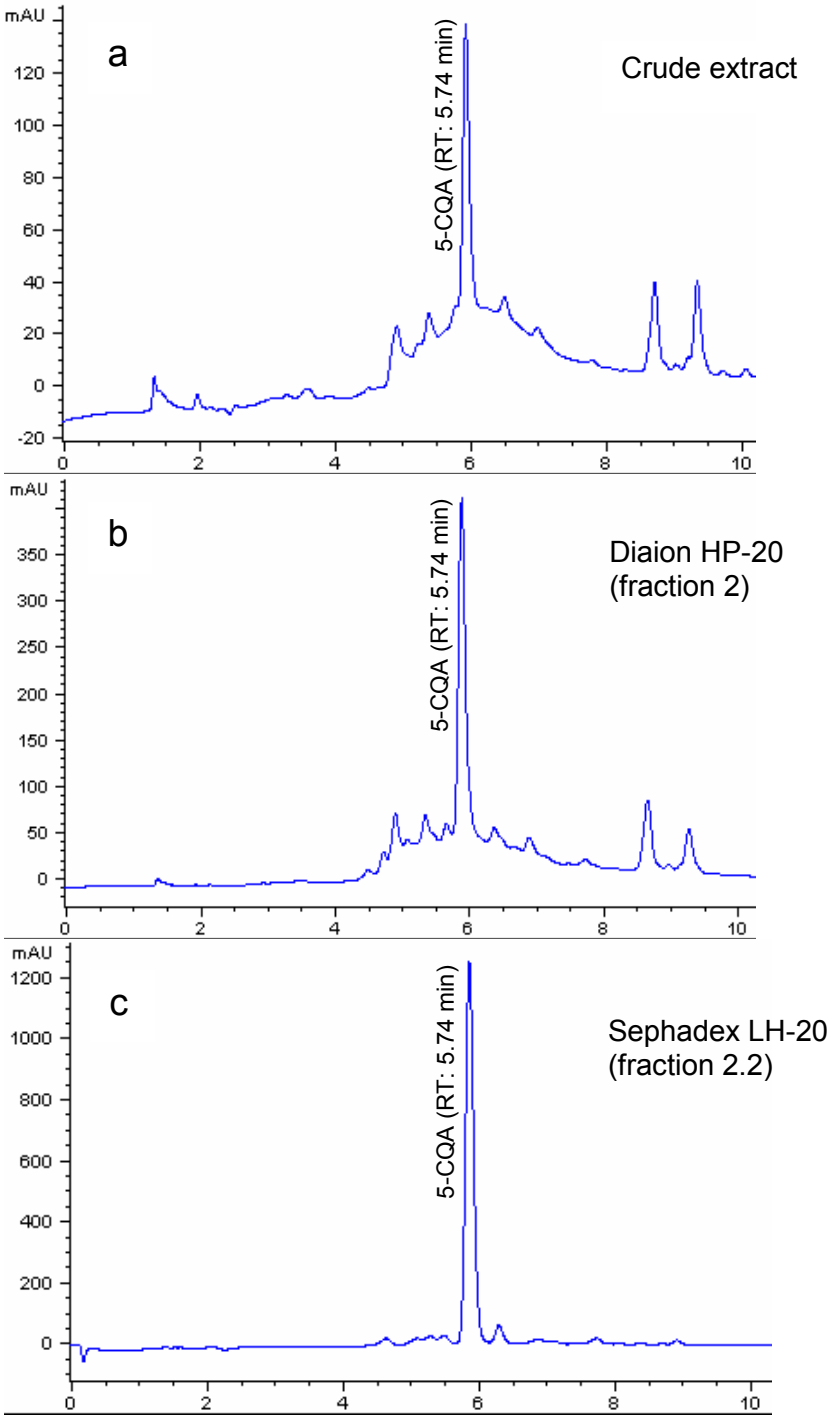


Fig. 4.25
HPLC chromatograms at 280 nm showing chlorogenic acid (CGA) peaks of crude extract (a) of leaves of *Etlingera elatior*, and of standardised extract fractionated by Diaion HP-20 (b) and Sephadex LH-20 (c)

4.7.4 Bioactivity of fractions

TPC, AEAC and tyrosinase inhibition of the crude extract were 444 ± 20 mg GAE/g, 463 ± 20 mg AA/g and 27 ± 4.1%, respectively. Antibacterial activity of the crude extract was good with minimum inhibitory dose (MID) of 0.125 mg/disc against *S. aureus*, *M. luteus* and *B. cereus*.

Table 4.31
Properties of fractions from Diaion HP-20 and Sephadex LH-20 columns based on antioxidant, tyrosinase and antibacterial properties

Fraction	TPC (mg GAE/g)	AEAC (mg AA/g)	Tyrosinase inhibition (%)	Minimum inhibitory dose (mg/disc) *S. aureus*	*M. luteus*	*B. cereus*
Diaion HP-20 column						
1	283 ± 21a	359 ± 31a	33.8 ± 4.7a	0.250	0.250	0.250
2 *	706 ± 12b	993 ± 26b	20.9 ± 4.2b	0.063	0.125	0.063
3	529 ± 14c	568 ± 69c	39.0 ± 5.0a	0.500	0.500	0.500
Sephadex LH-20 column						
2.1	533 ± 52a	750 ± 10a	38.3 ± 1.3a	0.125	0.250	0.125
2.2 *	639 ± 47b	656 ± 49b	31.9 ± 2.5b	–	–	–
2.3	748 ± 45c	870 ± 95c	80.4 ± 2.1c	0.500	0.500	1.000

* Fractions containing chlorogenic acid. Values of TPC, AEAC and tyrosinase inhibition are means ± SD (n = 3). For each column, values followed by the same letter are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA does not apply between columns. Abbreviations: TPC = total phenolic content, AEAC = ascorbic acid equivalent antioxidant capacity, *S.* = *Staphylococcus*, *M.* = *Micrococcus* and *B.* = *Bacillus.*

Fractionation of crude extract using Diaion HP-20 yielded fractions 1, 2 and 3 [Table 4.31]. Fraction 1 had the lowest phenolic content (283 ± 21 mg GAE/g) and radical scavenging activity (359 ± 31 mg AA/g), respectively. Antibacterial activity was moderate with MID of 0.250 mg/disc against *S. aureus*, *M. luteus* and *B. cereus*. Tyrosinase inhibition activity (33.8 ± 4.7%) was comparable to fraction 3 (39.0 ± 5.0%). When compared to the crude extract, fraction 2 had the most outstanding phenolic content, free radical scavenging and antibacterial activities. TPC was 706 ± 12 mg GAE/g, AEAC was 993 ± 26 mg AA/g, MID was 0.063 mg/disc against *S. aureus* and *B. cereus*, and 0.125 mg/disc against *M. luteus*. Fraction 3 showed the highest tyrosinase inhibition activity of 39.0 ± 5.0%. TPC and AEAC values were moderate, and MID values were 0.500 mg/disc against all three Gram-positive bacteria.

Further fractionation of fraction 2 using Sephadex LH-20 yielded fractions 2.1, 2.2 and 2.3 [Table 4.31]. This resulted in a reduction of the antioxidant activity of all fractions except fraction 2.3. However, there is a general decline in anti-bacterial activity and an increase in tyrosinase inhibition activity. In particular, fraction 2.3 showed enhanced tyrosinase inhibition activity from 20.9 ± 4.2% to 80.4 ± 2.1%, representing a 3.8 fold increase.

Recently, Ling *et al*. (2009) reported that a standardised extract produced from leaves of *Mangifera indica* was an ideal antioxidant. The standardised ethanolic extract had mangiferin content of 73.0 ± 0.17 mg/g dry weight, free radical scavenging activity IC_{50} of 0.17 ± 0.02 mg/ml (AEAC of 23 ± 2.7 mg/g) and TPC of 590 ± 48 mg/g. The standardised aqueous extracts had mangiferin content of 10.0 ± 0.22 mg/g dry weight, free radical scavenging activity IC_{50} of 0.49 ± 0.10 mg/ml (AEAC of 8.1 ± 1.6 mg/g) and TPC of 189 ± 109 mg/g.

4.7.5 LC-MS of fraction

Liquid chromatography mass spectrometry (LC-MS) analysis of fraction 2.3 showed two major peaks in the chromatogram [Fig. 4.26]. They were identified as isoquercitrin (RT: 15.10 min; mass: 464.4) and quercitrin (RT: 16.50 min; mass: 448.3). However, their mass spectra also showed the presence of several other co-eluting compounds. Commercial standards of isoquercitrin and quercitrin did not exhibit any tyrosinase inhibition [section 4.5.3]. It is likely that the strong tyrosinase inhibition activity of fraction 2.3 could be due to other compounds present in the fraction.

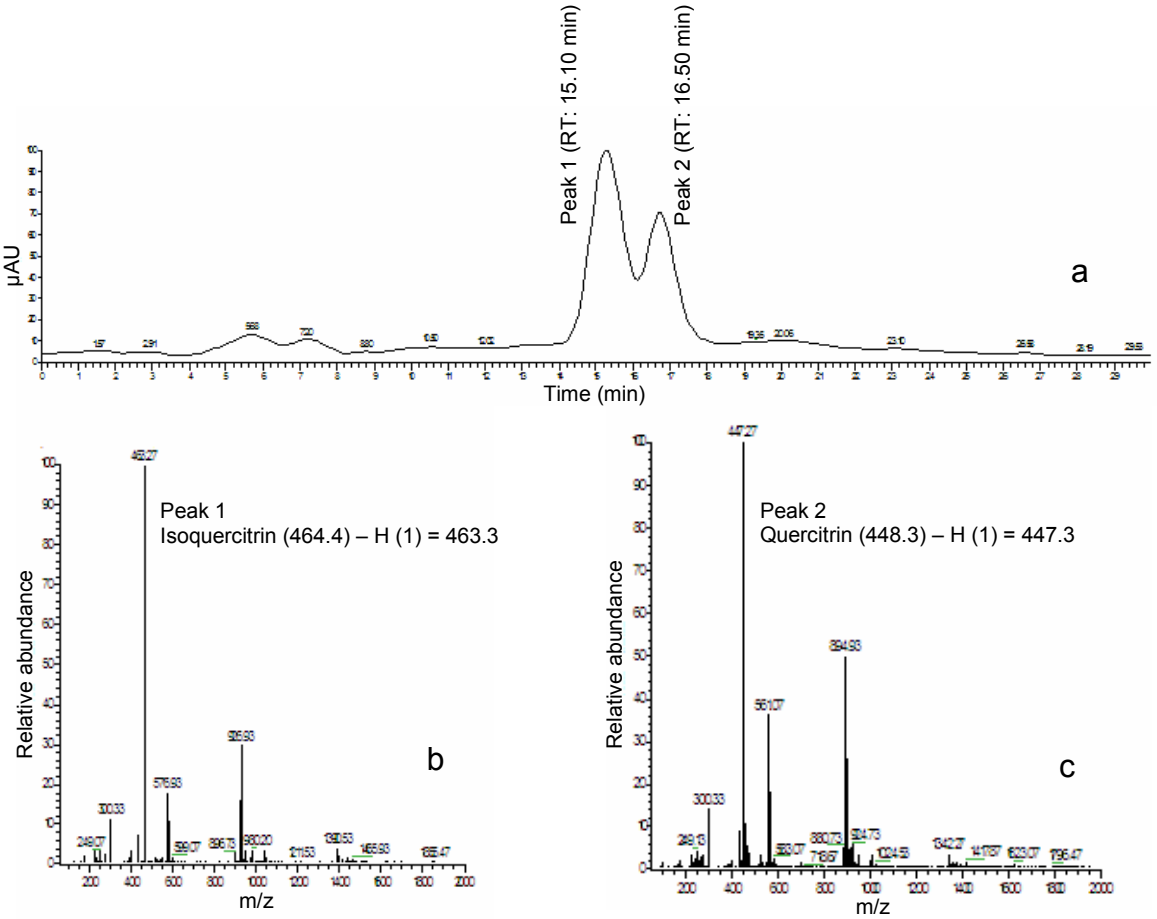


Fig. 4.26
LC-MS chromatograms of fraction 2.3 showing the peaks of isoquercitrin and quercitrin (a) with negative mode LC-MS spectra of isoquercitrin (b) and quercitrin (c)

4.7.6 Extraction protocol

The protocol for producing a CGA standardised extract of ~40% w/w purity from leaves of *E. elatior* is shown in Table 4.32.

Table 4.32
Protocol for producing chlorogenic acid (CGA) standardised extract of ~40% w/w purity from leaves of *Etlingera elatior*

Step	Protocol for CGA standardised extract	Product (duration & amount)
1	*Collect E. elatior leaves from the field* Collect 80 g leaves from the field. Sort and clean leaves and remove their mid-ribs. Cut leaves into small pieces for freeze drying.	Leaf pieces for freeze-drying (1 h, ~50 g)
2	*Freeze-dry leaf pieces* Freeze-dry leaf pieces (50 g) at –50°C overnight and grind with a blender.	Freeze-dried leaf powder (15 h, ~17 g)
3	*Extract leaf powder with 30% aqueous ethanol* Extract leaf powder 4 times with 500 ml of 30% ethanol for one hour each time in orbital shaker. Filter crude extracts under suction and remove the solvent with rotary evaporator at 50˚C. Store at –20°C for further use.	Crude leaf extract with ~3% w/w CGA (4 h, ~4 g)
4	*Fractionate crude extract over Diaion HP-20* Dissolve crude extract in 10 ml of 20% ethanol and chromatograph over a 40 g Diaion HP-20 column (20 x 230 mm). Elute the column using a H_2O:EtOH 0-35% step-gradient with an increment of 5% ethanol every 100 ml. Flush the column with 200 ml of 100% ethanol after elution of each extract. Recover eluents from 10–35% ethanol and dry in rotary-evaporator at 50˚C to obtain 10% CGA extract.	CGA extract of ~10% w/w purity (15 min, ~0.9 g)
5	*Fractionate 10% CGA extract over Sephadex LH-20* Dissolve 10% CGA extract in 5 ml of 20% ethanol and chromatograph over a 10 g Sephadex LH-20 column (30 x 60 mm). Elute the column with 100 ml of water followed by 200 ml of 20% ethanol and 200 ml of ethanol. Recover eluents from 20% ethanol and dry in rotary-evaporator at 50˚C to obtain 40% CGA extract.	CGA extract of ~40% w/w purity (2.5 h, ~0.2 g)

Chapter 5

CONCLUSION

Among 26 ginger species of *Alpinia*, *Boesenbergia*, *Curcuma*, *Elettariopsis*, *Etlingera*, *Hedychium*, *Kaempferia*, *Scaphochlamys* and *Zingiber* screened for total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC), leaves of *Etlingera* species displayed the highest values.

Eleven out of 14 ginger species showed significantly higher TPC and/or AEAC in leaves than in rhizomes. Values of leaves of *Etlingera elatior* and *E. maingayi* were 7–8 times higher than those of rhizomes. Six of the eight species clearly displayed higher ferrous ion chelating (FIC) ability in leaves than in rhizomes. This project has established that, for the first time, leaves of ginger species have significantly higher phenolic content and antioxidant properties than rhizomes.

Among five *Etlingera* species studied, highest TPC, AEAC and ferrous reducing power (FRP) were found in leaves of *E. elatior*. Leaves of *E. maingayi*, with the lowest TPC, AEAC and FRP, had the highest FIC ability and lipid peroxidation inhibition (LPI) activity. FIC ability of *E. maingayi* and *E. fulgens* was much higher than that of young tea leaves of *Camellia sinensis*. Leaves of *Etlingera* species exhibited high LPI activity, matching that of rhizomes of *Curcuma longa* and superior to that of young tea leaves.

Ranking of TPC and antioxidant activity of different plant parts of *E. elatior* was in the order: leaves > inflorescences > rhizomes. Leaves of highland populations of *Etlingera* species had higher values of TPC and AEAC than had those of lowland counterparts.

Leaves of *Etlingera* species inhibited Gram-positive but not Gram-negative bacteria. Leaves of three species of *Etlingera* displayed tyrosinase inhibition activity that was significantly higher or comparable to those of *Hibiscus tiliaceus* as positive control. With promising antioxidant activity, antibacterial properties and tyrosinase inhibition ability, leaves of *Etlingera* species have great potential to be developed into skin-lightening products and natural preservatives, applicable to nutraceutical and food industries. Unlike the commercial use of rhizomes, the harvesting of leaves does not result in destructive sampling of plants. Leaves of *Etlingera* species were found to be non-cytotoxic to normal liver and kidney cells.

Based on phenolic content and bioactivities of leaves, the overall score and ranking of *Etlingera* species were of the order: *E. elatior* > *E. rubrostriata* > *E. fulgens* > *E. littoralis* > *E. maingayi*.

This project showed that freeze-drying is superior to four other drying methods in preserving the antioxidant properties of leaves of four ginger species studied. Thermal drying (microwave-, oven-, and sun-drying) resulted in significant declines in TPC, AEAC and FRP with minimal effects on FIC ability and LPI activity. Of the two methods of non-thermal drying, air-dried leaves showed drastic losses in values for all four species. Freeze-drying had three major effects on the antioxidant properties of ginger leaves. Firstly, leaves of *E. elatior* and *A. zerumbet* showed enhancement in antioxidant properties following freeze-drying. Secondly, freeze-dried leaves of *E. elatior* remained stable following one-week storage under room temperature. Thirdly, freeze-dried leaves of *C. longa* and *K. galanga* had the least decline in antioxidant properties compared with microwave-, oven-, sun- and air-dried leaves. Freeze-drying appears to be a sound method for producing tea and other herbal products from ginger species. Due to its high operation cost, freeze-drying can be applied to produce high-value speciality tea or spice powder from ginger leaves.

Six compounds were isolated from leaves of *E. elatior*. They were identified as 3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid), 5-*O*-caffeoyl-quinic acid methyl ester, quercetin 3-*O*-glucoside (isoquercitrin), quercetin 3-*O*-rhamnoside (quercitrin) and (+)-catechin. This is the first report of caffeoylquinic acids (CQA) including chlorogenic acid (CGA) in Zingiberaceae. CGA, iso-quercitrin and quercitrin, the major compounds, exhibited DPPH radical scavenging activity but no antibacterial and tyrosinase inhibition activities.

Contents of CQA of *E. elatior*, *E. fulgens* and *E. rubrostriata* leaves were significantly higher than leaves of *Ipomoea batatas*, and comparable to flowers of *Lonicera japonica.* CGA found only in leaves of *E. elatior* and *E. fulgens* was significantly higher in content than *L. japonica*, the commercial source.

Comparing essential oils from leaves of four *Etlingera* species, *E. rubrostriata* had the highest diversity with 23 different compounds identified. Composition of oils in the leaves of *E. elatior* and *E. fulgens* were very different despite having a very similar aroma and morphology. Leaves of *E. maingayi* had the highest yield of essential oils comprising mainly fatty acids that inhibited Gram-positive bacteria.

A protocol to produce a CGA standardized extract from leaves of *E. elatior* was optimized. It involved freeze-drying of leaves and extraction with 30% ethanol. Following sequential fractionation using Diaion HP-20 and Sephadex LH-20 columns, a standardized extract of ~40% w/w purity was obtained.

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APPENDIX I: CHEMICALS

BIOASSAYS

Extraction

Liquid nitrogen (Malaysia Oxygen Sdn. Bhd., Malaysia)

Methanol CH_3OH (HmbG Chemicals, Analytical grade)

Acetone (HmbG Chemicals, Analytical grade)

Dichloromethane (HmbG Chemicals, Analytical grade)

Ethanol CH_3CH_2OH (HmbG Chemicals, Analytical grade)

Total phenolic content (TPC)

Folin-Ciocalteu's reagent (Fluka)

Gallic acid $C_7H_6O_5$ 98% (Fluka)

Sodium carbonate anhydrous Na_2CO_3 99% (Fluka)

Caffeoylquinic acid content (CQAC)

Sodium molybdate dehydrate

Free radical scavenging ability (DPPH)

L(+) ascorbic acid, $C_6H_8O_6$ 99.7% (Merck)

2,2-diphenyl-1-picrylhydrazyl (DPPH) $C_{18}H_{12}O_6N_5$ 90% (Sigma)

Ferric reducing power (FRP)

Iron (III) chloride-6-hydrate $FeCl_3.6H_2O$ 99.8% (Fischer Scientific)

Di-potassium hydrogen phosphate $K_2HPO_4.3H_2O$ (Merck)

Potassium dihydrogen phosphate KH_2PO_4 (Fischer Chemicals)

Potassium ferricyanide $K_3Fe(CN)_6$ 99% (Unilab Laboratory)

Trichloroacetic acid CCl_3COOH (Fischer Scientific)

Ferrous ion chelating (FIC)

Ferrozine 98% (Acros Organics)

Iron (II) sulphate-7-hydrate $FeSO_4.7H_2O$ (HmbG Chemicals)

β-carotene bleaching (BCB)

β-Carotene $C_{40}H_{56}$ (Sigma, Type 1: synthetic)

Chloroform (Fisher Scientific)

Linoleic acid $C_{18}H_{32}O_2$ (Fluka)

Quercetin $C_{18}H_{32}O_2$ 98% (Sigma)

Tween 40 ® (Fluka)

Antibacterial activity

Mueller-Hinton agar (OXOID, England)

Streptomycin 10 µg (OXOID, England)

Whatman 6 mm blank discs (Schleicher & Schuell, Germany)

Petri dishes 90 x 15 mm (Brandon)

Tyrosinase inhibition

3,4-dihydroxy-L-phenylalanine $C_9H_{11}NO_4$ (Sigma)

Dimethyl sulphoxide Analytical Grade (Fisher Scientific)

Tyrosinase (Sigma)

Cytotoxicity

Foetal bovine serum (PAA Laboratories)

Sulforhodamine B (Sigma)

Trichloroacetic acid (Sigma)

Dulbecco's Modified Eagle's Medium (Sigma)

Paclitaxel (Sigma)

NATURAL PRODUCT RESEARCH

Solvents

Acetone Industrial grade (HmbG Chemicals)

Chloroform Industrial grade (HmbG Chemicals)

Ethanol CH_3CH_2OH Industrial grade (HmbG Chemicals)

Methanol CH_3OH Industrial grade (HmbG Chemicals)

Isolation

Chromatorex ODS (Fuji Silysia)

Diaion HP-20 (Supelco)

LiChroprep® RP-18 (Merck)

MCI gel CHP 20P (Supelco)

Silica gel 60 (Merck)

Sephadex LH-20 (Sigma)

HPLC

Methanol HPLC grade (Fischer Scientific)

Trifluoroacetic acid HPLC grade (Fischer Scientific)

Structure elucidation

Bruker DRX 300 MHz spectrometer

Deuterated methanol (Merck)

Deuterated DMSO (Merck)

Bioactivity

Chlorogenic acid $C_{16}H_{18}O_9$ 99% (Acros Organic)

(+)-catechin $C_{15}H_{14}O_6.H_2O$ 98% (Fisher Scientific)

Quercitrin hydrate $C_{21}H_{20}O_{11}\cdot xH_2O$ ~85% (Sigma)

Quercetin 3-β-D-glucoside $C_{21}H_{20}O_{12}$ ≥ 90% (Fluka BioChemika)

APPENDIX II: EQUIPMENT

BIOASSAYS

BIO-Tek Power Wave XS Microplate Scanning Spectrophotometer
Centrigue machine (Hettich Zentrifuge, Universal 32R)
Freeze-dryer (Christ Alpha 1-4, Salm en Kipp, the Netherlands)
Heating mantle (MTOPS, Model: MSE102)
Hot plate (Favorit H50707V2)
Incubator (TS606-G/2 WTW GmbH, Germany)
Microcomputer pH meter Model HI 8424 (Hana Instruments, Portugal)
Microwave oven (SHARP Model R-248E, 230-240V, ~50Hz)
Orbital Shaker (Protech Model 719)
pH meter (Cyberscan 1100 pH)
Rotary vacuum evaporator N-N series (Eyela Tolyo Rikakikai Co. Ltd., Japan)
SECOMAM PRIM Light Visible Spectrophotometer (France)
Tecan Sunrise Microplate Absorbance Reader
Vacuum pump Aspirator A-35 (Eyela Tolyo Rikakikai Co. Ltd., Japan)
Water bath (Memmert, Germany)
Weighing balance (A&D Measurement HR-200)
Weighing balance (Scaltec SPO62)

NATURAL PRODUCT RESEARCH

Extraction

Allihn condenser
Modified Clavenger apparatus

Isolation

HPLC (Agilent Technologies 1200 Series, Germany)

Structure elucidation

Bruker DRX 300 MHz spectrometer (NMR)

ThermoFinnigan LCQDeca spectrometer (ESI-MS)

ThermoFinnigan Polaris Q mass spectrometer (EI-MS)

Shimadzu GC-2010 (GC)

Hewlett-Packard HP 5975-7890 GC-MSD (GC-MS)

APPENDIX III: REPRINTS

Reprints of publications of this project are enclosed from pages 260–305.

Publication No. 1
CHAN, E.W.C., LIM, Y.Y. & LIM, T.Y. (2007). Total phenolic content and anti-oxidant activity of leaves and rhizomes of some ginger species in Peninsular Malaysia. *Gardens' Bulletin Singapore* 59: 47–58.

Publication No. 2
CHAN, E.W.C., LIM, Y.Y. & CHEW, Y.L. (2007). Antioxidant activity of *Camellia sinensis* leaves and tea from a lowland plantation in Malaysia. *Food Chemistry* 102: 1214–1222.

Publication No. 3
CHAN, E.W.C., LIM, Y.Y. & OMAR, M. (2007). Antioxidant and antibacterial activity of leaves of *Etlingera* species (Zingiberaceae) in Peninsular Malaysia. *Food Chemistry* 104: 1586–1593.

Publication No. 4
CHAN, E.W.C. LIM, Y.Y., WONG, L.F., LIANTO, F.S., WONG, S.K., LIM, K.K., JOE, C.E. & LIM, T.Y. (2008). Antioxidant and tyrosinase inhibition properties of leaves and rhizomes of ginger species. *Food Chemistry* 109: 477–483 (Rapid Communication).

Publication No. 5
CHAN, E.W.C. LIM, Y.Y., WONG, S.K., LIM, K.K., LIANTO, F.S. & YONG, M.Y. (2009). Effects of different drying methods on the antioxidant properties of leaves and tea of ginger species. *Food Chemistry* 113: 166–172.

Publication No. 6
CHAN, E.W.C., LIM, Y.Y., LING, S.K., TAN, S.P., LIM, K.K. & KHOO, M.G.H. (2009). Caffeoylquinic acids from leaves of *Etlingera* species (Zingiberaceae). *LWT - Food Science and Technology* 42: 1026–1030.

Total Phenolic Content and Antioxidant Activity of Leaves and Rhizomes of some Ginger species in Peninsular Malaysia

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Abstract

The total phenolic content (TPC) and antioxidant activity (AOA) of leaves and rhizomes of five wild and six cultivated ginger species belonging to seven genera were compared. Altitudinal variation in leaf TPC and AOA of four *Etlingera* species was also studied. TPC was measured using the Folin-Ciocalteu method. AOA was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and expressed as ascorbic acid equivalent antioxidant capacity (AEAC). Of the 11 wild and cultivated species screened, leaves of *Etlingera* had the highest TPC and AEAC, which were seven to eight times higher than those of rhizomes. Eight species had significantly higher leaf TPC and/or AEAC than rhizomes. Leaves of highland populations of *Etlingera* species had higher values than those of lowland counterparts.

Introduction

Rhizomes of gingers (Zingiberaceae) are widely consumed as spice or condiments (Larsen *et al.* 1999; Sirirugsa 1999). Major commercially cultivated species in Peninsular Malaysia are *Zingiber officinale* Roscoe, *Curcuma longa* L. and *Alpinia galanga* (L.) Willd. As traditional medicine, rhizomes are consumed by women during ailment, illness and confinement (Larsen *et al.* 1999; Ibrahim *et al.* 2006). They are also taken as carminative for relieving flatulence.

Leaves of gingers have also been used for food flavouring. In Peninsular Malaysia, leaves of *Curcuma longa* are used to wrap fish before steaming or baking (Larsen *et al.* 1999). The leaves of *Kaempferia galanga* L. and *Curcuma longa* are ingredients of spicy fish and meat dishes. Some tribal natives use leaves of *Elettariopsis slahmong* C.K. Lim to flavour cuisine of wild meat and fish (Lim 2003). In Okinawa, Japan, leaves of *Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Sm. are traditionally used to wrap rice cakes and are commercially sold as herbal tea.

Past studies on the antioxidant activity of wild and cultivated ginger species were confined to rhizomes (Jitoe *et al.* 1992; Habsah *et al.* 2000; Zaeoung *et al.* 2005). Although their leaves have been used for food flavouring, hardly any research has been done on their antioxidant activity.

Antioxidants are molecules that are able to scavenge free radicals or prevent their generation. Phenolic compounds, in general, are able to scavenge free radicals or chelate metal ions to prevent generation of free radicals. Free radicals have been implicated in the pathogenesis of more than 50 diseases (Percival 1996). Currently, there is much interest in herbs and spices as sources of antioxidants.

In our present study, the total phenolic content (TPC) and antioxidant activity (AOA) of leaves and rhizomes of five wild and six cultivated ginger species were compared. Altitudinal variation in leaf TPC and AOA of species of *Etlingera* was also studied.

Materials and Methods

Species studied

Five wild and six cultivated ginger species were screened for TPC and AOA. Wild species studied were *Etlingera maingayi* (Bak.) R.M. Smith, *Alpinia malaccensis* (Burm.) Rose var. nobilis (Ridl.) I.M. Turner, *Elettariopsis slahmong* C.K. Lim, *Zingiber spectabile* Griff. and *Scaphochlamys kunstleri* (Bak.) Holtt. Cultivated species studied were *Etlingera elatior* (Jack) R.M. Smith, *Alpinia galanga* (L.) Sw., *Zingiber officinale* Roscoe, *Curcuma longa* L., *Curcuma zanthorrhiza* Roxb. and *Boesenbergia rotunda* (L.) Mansf. For each species, leaves and rhizomes of three plants were sampled.

For wild species, leaves and rhizomes of *Alpinia malaccensis* var. *nobilis*, *Zingiber spectabile* and *Scaphochlamys kunstleri* were sampled from Forest Research Institute Malaysia (FRIM) in Selangor, those of *Elettariopsis slahmong* from Bukit Lagong in Selangor and those of *Etlingera maingayi* from Janda Baik in Pahang. Voucher specimens of wild species studied were deposited at the FRIM herbarium (KEP).

For cultivated species, leaves and rhizomes of *Etlingera elatior* and *Curcuma longa* were sampled from FRIM, those of *Alpinia galanga* and *Zingiber officinale* from Bukit Maluri in Kepong, and those of *Curcuma zanthorrhiza* from Damansara Utama in Petaling Jaya. Plants of *Boesenbergia rotunda* were purchased from a nursery in Sungai Buluh in Selangor. Rhizomes of

Alpinia galanga, *Zingiber officinale* and *Curcuma longa* purchased from the supermarket were also screened. Voucher specimens of cultivated species studied were deposited at KEP.

TPC and AOA of leaves of lowland and highland populations of four *Etlingera* species were compared. The species studied were *Etlingera elatior* (Jack) R.M. Smith, *Etlingera fulgens* (Ridl.) C.K. Lim, *Etlingera littoralis* (Koenig) Giseke and *Etlingera rubrostriata* (Holtt.) C.K. Lim. Their identification was based on taxonomic descriptions and photographic illustrations of Lim (2000 & 2001) and Khaw (2001). Leaves of highland populations were sampled from Janda Baik and Genting Highlands in Pahang and from Ulu Gombak in Selangor, while those of lowland populations were sampled from FRIM. For each location, mature leaves were sampled from three different plants per species. Voucher specimens of *Etlingera* species studied were deposited at KEP. Altitude of locations, where the populations were sampled, was measured using a Casio altimeter (Model PRG-70-1VDR).

Extraction of samples

Fresh leaves and rhizomes (1 g) were powdered with liquid nitrogen in a mortar and extracted by methanol (50 ml), with continuous swirling for one hour at room temperature. Extracts were filtered and stored at -20°C for further use. Analysis of methanol extracts was done in triplicate for each species.

Total phenolic content

Total phenolic content (TPC) was measured using the Folin-Ciocalteu method (Kahkonen *et al.* 1999). Samples (300 µl in triplicate) were introduced into test tubes followed by 1.5 ml of Folin-Ciocalteu's reagent (10 times dilution) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min before absorption at 765 nm was measured. Total phenolic content was expressed as gallic acid equivalent (GAE) in mg/100 g material. The calibration equation for gallic acid was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$).

Antioxidant activity

Antioxidant activity (AOA) was measured using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay used by Leong and Shui (2002) and Miliauskas *et al.* (2004) with slight modifications. Defined amounts of the extract were added to 3 ml of DPPH (3.9 mg/100 ml methanol). The DPPH solution was then allowed to stand for 30 min before absorbance was measured at 517 nm. All spectrophotometric measurements were made with methanol as blank.

An appropriate dilution of the DPPH solution was used as negative control, i.e. methanol in place of the sample. Results were expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg/100 g calculated from the IC_{50} (inhibitory concentration in mg/ml of plant material necessary to reduce the absorbance of DPPH by 50%) using the following formula:

$$\text{AEAC (mg AA/100 g)} = IC_{50(ascorbate)}/IC_{50(sample)} \times 100{,}000$$

The IC_{50} of ascorbate used for calculation of AEAC was 0.00387 mg/ml.

Results and Discussion

Leaves and rhizomes of wild and cultivated species

Results from screening of five wild species showed that leaves of *Etlingera maingayi* had significantly higher TPC and AEAC than those of *Alpinia malaccensis* var. *nobilis*, *Elettariopsis slahmong*, *Zingiber spectabile* and *Scaphochlamys kunstleri* (Table 1). Rhizomes of *Alpinia malaccensis* var. *nobilis* had the highest values. Leaves of *Elettariopsis slahmong*, *Etlingera maingayi* and *Scaphochlamys kunstleri* showed significantly higher values at $P < 0.05$ than rhizomes. Leaves of other wild species were only marginally higher than rhizomes.

For six cultivated species screened, leaf and rhizome TPC and AEAC were highest in *Etlingera elatior* and *Curcuma longa*, respectively (Table 2). In five species, leaves had significantly higher TPC and/or AEAC at $P < 0.05$ than those of rhizomes. Exceptions were AEAC of *Alpinia galanga*, and TPC and AEAC of *Curcuma longa* where rhizomes showed higher values than leaves. The values of *Curcuma longa* were highly variable between rhizomes. For *Alpinia galanga*, *Curcuma longa* and *Zingiber officinale*, differences existed between collected rhizomes and those purchased from the supermarket. This implies that there is variability in TPC and AEAC between different cultivars.

In general, leaves of wild and cultivated *Etlingera* species contain the most antioxidants by having the highest TPC and AEAC. Values were 1110 mg GAE/100 g and 963 mg AA/100 g for *Etlingera maingayi* (Table 1), and 2390 mg GAE/100 g and 2280 mg AA/100 g for *Etlingera elatior* (Table 2), respectively. The outstanding leaf TPC and AEAC of both *Etlingera maingayi* and *Etlingera elatior* were seven to eight times higher than those of rhizomes.

There are very few studies comparing between the AOA of leaves and rhizomes of ginger species. Agnaniet *et al.* (2004) reported that essential oils extracted from leaves of *Aframomum giganteum* K. Schum. had higher AOA than rhizomes. Contrary to our results, Katsube *et al.*

(2004) reported higher TPC and AOA in rhizomes of *Zingiber officinale* than leaves. It is not known whether their comparisons were based on samples from same or different plants.

Table 1. Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves and rhizomes of five wild ginger species

Species and location	Voucher number	Plant part	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
Alpinia malaccensis var. *nobilis* - FRIM	EC01	Leaves Rhizomes	744 ± 61 [a] 564 ± 209 [a]	800 ± 62 [a] 745 ± 342 [a]
Elettariopsis slahmong - Bukit Lagong	EC02	Leaves Rhizomes	346 ± 45 [a] 219 ± 57 [b]	269 ± 67 [a] 197 ± 76 [a]
Etlingera maingayi - Janda Baik	EC06	Leaves Rhizomes	1110 ± 93 [a] 160 ± 52 [b]	963 ± 169 [a] 122 ± 53 [b]
Scaphochlamys kunstleri - FRIM	EC08	Leaves Rhizomes	203 ± 21 [a] 73 ± 3 [b]	171 ± 33 [a] 14 ± 2 [b]
Zingiber spectabile - FRIM	EC09	Leaves Rhizomes	242 ± 7 [a] 157 ± 100 [a]	121 ± 24 [a] 124 ± 109 [a]

Values of TPC and AEAC are means ± SD (n = 3). For column of each species, values followed by the same letter (a−b) are not significantly different at $P < 0.05$ measured by the Tukey HSD test. ANOVA does not apply between species.

This is probably the first study where TPC and AOA of leaves and rhizomes of gingers have been systematically compared. For most of the species screened, TPC and/or AEAC of leaves were significantly higher than rhizomes.

Antioxidants are secondary metabolites, which form part of the plant's protective mechanism against free radicals. In Zingiberaceae, it is generally believed that antioxidants and other secondary metabolites are transported to the rhizomes where they are accumulated. This implies that rhizomes would have higher AOA than other plant parts. However, results of this study showed that this might not be the case.

Table 2. Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves and rhizomes of six cultivated ginger species

Species and location	Voucher number	Plant part	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
Alpinia galanga - Bukit Maluri	EC10	Leaves	366 ± 15 [a]	72 ± 4 [a]
		Rhizomes	150 ± 22 [b]	96 ± 6 [b]
Supermarket		Rhizomes	214 ± 20	168 ± 13
Boesenbergia rotunda - Sungai Buluh	EC11	Leaves	260 ± 8 [a]	157 ± 2 [a]
		Rhizomes	197 ± 50 [a]	89 ± 7 [b]
Curcuma longa - FRIM	EC12	Leaves	230 ± 19 [a]	113 ± 18 [a]
		Rhizomes	534 ± 205 [b]	390 ± 127 [b]
Supermarket		Rhizomes	386 ± 219	275 ± 183
Curcuma zanthorrhiza - Damansara Utama	EC13	Leaves	503 ± 57 [a]	287 ± 39 [a]
		Rhizomes	250 ± 52 [b]	134 ± 21 [b]
Etlingera elatior - FRIM	EC14	Leaves	2390 ± 329 [a]	2280 ± 778 [a]
		Rhizomes	326 ± 76 [b]	295 ± 96 [b]
Zingiber officinale - Bukit Maluri	EC15	Leaves	291 ± 18 [a]	96 ± 7 [a]
		Rhizomes	157 ± 18 [b]	84 ± 3 [a]
Supermarket		Rhizomes	184 ± 11	107 ± 9

Values of TPC and AEAC are means ± SD (n = 3). For column of each species, values followed by the same letter (a−b) are not significantly different at $P < 0.05$ measured by the Tukey HSD test. ANOVA does not apply between species.

Photosynthesis and respiration are physiological processes comprising several free radical intermediates. Exposure to sunlight can also increase the amount of free radicals. Leaves therefore require much more free radical scavengers than other plant parts. Similarly, Frankel and Berenbaum (1999) found that foliage of tropical forest plants produced more antioxidants when exposed to elevated light conditions. This observation may also apply to species of *Etlingera*, which have the highest leaf TPC and AEAC. *Etlingera* plants grow in gaps of disturbed forest and are continually exposed to direct sunlight (Poulsen 2006). Furthermore, leaves of *Etlingera* are long lasting and do not abort. This may be due to an efficient protective mechanism delaying senescence in leaves, which is partly attributed to oxidative stress.

Altitudinal variation in leaves of Etlingera species

Leaves of all four species of *Etlingera* sampled from highland populations were found to have higher TPC and AEAC than lowland counterparts (Table 3). Leaves of *Etlingera rubrostriata*, *Etlingera elatior* and *Etlingera fulgens* showed significantly higher values at $P < 0.05$, while *Etlingera littoralis* was marginally higher. Highest TPC and AEAC were found in the leaves of highland populations of *Etlingera elatior* with values of 3550 mg GAE/100 g and 3750 mg AA/100 g, and of *Etlingera rubrostriata* with values of 3480 mg GAE/100 g and 3540 mg AA/100 g, respectively.

Table 3. Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves of four *Etlingera* species sampled from highland and lowland locations

Species and location	Voucher number	Altitude (m asl)	Moisture content (%)	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
Etlingera elatior -					
Janda Baik	EC03	400	66.1 ± 2.0	3550 ± 304 [a]	3750 ± 555 [a]
FRIM		100		2390 ± 329 [b]	2280 ± 778 [b]
Etlingera fulgens -					
Janda Baik	EC04	400	74.3 ± 0.1	2270 ± 31 [a]	2030 ± 126 [a]
FRIM		100		1280 ± 143 [b]	845 ± 158 [b]
Etlingera littoralis -					
Genting Highlands	EC05	800	71.2 ± 0.8	2810 ± 243 [a]	2930 ± 220 [a]
FRIM		100		2340 ± 386 [a]	2220 ± 913 [a]
Etlingera rubrostriata -					
Ulu Gombak	EC07	300	71.6 ± 2.8	3480 ± 390 [a]	3540 ± 401 [a]
FRIM		100		2430 ± 316 [b]	2640 ± 508 [a]

Values of TPC and AEAC are means ± SD ($n = 3$). For columns of each species, values followed by the same letter (a−b) are not significantly different at $P < 0.05$ measured by the Tukey HSD test. ANOVA does not apply between species.

Higher altitudes seem to trigger an adaptive response in the species of *Etlingera.* The higher leaf TPC and AEAC of highland populations over lowland counterparts might be due to environmental factors such as higher UV-B radiation and lower air temperature. Plants protect themselves from oxidative damage due to UV exposure by producing antioxidative compounds (Larson 1988). Enhanced UV-B radiation induces greater production of phenolic compounds (Bassman 2004). Enzymes associated with the synthesis of phenolics are produced in greater quantities or show increased activity (Chalker-Scott & Scott 2004). Phenylalanine ammonia lyase (PAL) of the phenylpropanoid pathway is up-regulated resulting in the accumulation of flavonoids and anthocyanins, which have free radical scavenging ability (Jansen *et al.* 1998). Low temperatures have also been shown to enhance PAL synthesis and activity in a variety of plants, leading to an increase in flavonoids and other phenolics (Chalker-Scott & Scott 2004).

Conclusion

Based on five wild and six cultivated ginger species belonging to seven genera, highest TPC and AOA were found in the leaves of *Etlingera.* For most species screened, leaf TPC and/or AEAC were significantly higher than those of rhizomes. Rhizomes from different cultivars showed variability in TPC and AEAC. Leaves of highland populations of *Etlingera* had higher values than lowland counterparts. Of the four species studied, highest TPC and AEAC were found in the leaves of highland populations of *Etlingera elatior* and *Etlingera rubrostriata.*

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Antioxidant activity of *Camellia sinensis* leaves and tea from a lowland plantation in Malaysia

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Abstract

Methanol extracts of fresh tea leaves from a lowland plantation in Malaysia were screened for total phenolic content (TPC) and antioxidant activity (AOA). AOA evaluation included 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical-scavenging ability, ferric-reducing antioxidant power (FRAP), and ferrous-ion chelating (FIC) ability. Ranking, based on TPC and AOA, was as follows: shoots > young leaves > mature leaves. TPC and AOA of lowland leaves were comparable to those of highland plants. A green tea produced by drying young leaves in a household microwave oven for 4 min showed significantly higher TPC and AOA than did four commercial brands of green and black tea.
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Keywords: *Camellia sinensis*; Fresh leaves; Tea; Lowland; Highland; Total phenolic content; Antioxidant activity

1. Introduction

The tea plant *Camellia sinensis* (L.) Kuntze (family Theaceae) is grown in about 30 countries worldwide (Graham, 1992). It grows best in tropical and subtropical areas with adequate rainfall, good drainage, and slightly acidic soil (Graham, 1999).

There are two varieties of tea. *C. sinensis* var. *sinensis* (China tea) is grown extensively in China, Japan, and Taiwan, while *C. sinensis* var. *assamica* (Assam tea) predominates in south and southeast Asia, including Malaysia (Adiwinata, Martosupono, & Schoorel, 1989) and, more recently, Australia (Caffin, D'Arcy, Yao, & Rintoul, 2004).

Tea is often planted in the highlands. In India and Sri Lanka, it is cultivated at elevations up to 2000 m asl (Graham, 1999). In plantations, tea is planted at a density of 5000–10,000 plants per hectare and maintained as low shrubs of 1–1.5 m in height through regular pruning during harvesting. Manual plucking of the terminal bud and two youngest leaves yields the finest quality of tea, but the high cost of labour in some countries makes mechanical harvesting an economic necessity (Caffin et al., 2004).

Fresh tea leaves are very rich in catechins, which may constitute up to 30% of dry weight (Graham, 1992). Principal catechins of young tea leaves are epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG), gallocatechin (GC), epicatechin (EC) and catechin. Content of catechins varies with climate, season, horticultural practices, leaf age and variety.

Chen et al. (2003) reported that young tea leaves were richer in caffeine, EGCG and ECG than were mature leaves. Old leaves had higher levels of theanine, EGC and EC. However, Lin, Tsai, Tsay, and Lin (2003) observed that old leaves contained less caffeine, but more EGCG, EGC, EC and catechin than did young leaves. Yao et al. (2004) reported that EGCG was the main flavanol in fresh tea shoots in Australia, constituting up to 115 mg/g dry weight of tea shoots. Bhatia and Ullah (1968) had earlier reported that the leaf bud and first leaf were richest in EGCG. Wild tea plants contained more EGCG, EGC, ECG, and total catechins than did cultivated plants (Lin et al., 2003).

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Tea is the most widely consumed beverage in the world, second only to water (Muktar & Ahmad, 2000). Of the total amount of teas produced and consumed in the world, 78% are black, 20% are green, and 2% are oolong tea. In green tea manufacture, catechin oxidation by polyphenol oxidase is prevented by steaming (Japan) or by panning (China) (Graham, 1999). The leaves retain their green colour and almost all of their original polyphenol content. Oolong tea is allowed to ferment to a limited extent and contains a mixture of catechins, theaflavins and thearubigens (Wheeler & Wheeler, 2004). Black tea is produced from fully fermented leaves and has a characteristic colour and taste.

The chemical composition of green tea is similar to that of fresh tea leaves (Chen et al., 2003). The amount of EGCG and total catechins was in the order: green tea > oolong tea > fresh tea leaves > black tea (Lin et al., 2003). Yen and Chen (1995) found the greatest amount of catechins in green tea (26.7%), followed by oolong tea (23.2%) and black tea (4.3%). Similarly, Cabrera, Gimenez, and Lopez (2003) found higher content of catechins in green tea than in oolong and black tea. Of teas sold in Australian supermarkets, the polyphenol content of green tea (25%) was much higher than that of black tea (18%) (Yao et al., 2006). Green and black teas produced from var. *assamica* had higher polyphenol contents (30%) than those from var. *sinensis* (20%) (Harbowy & Balentine, 1997).

Catechins and other polyphenols in tea exhibit powerful antioxidant activities (Dufresne & Farnworth, 2001). They act as antioxidants *in vitro* by sequestering metal ions and by scavenging reactive oxygen and nitrogen species (Frei & Higdon, 2003; Wiseman, Balentine, & Frei, 1997). They may also function indirectly as antioxidants through their effects on transcription factors and enzyme activities (Higdon & Frei, 2003).

During the processing of tea, fermentation results in the production of theaflavins and thearubigins (Lee, Lee, & Lee, 2002). Black tea comprises 2–6% of theaflavins and more than 20% of thearubigens, whereas green tea has 30–42% of catechins.

Leung et al. (2001) reported that the conversion of catechins to theaflavins during tea fermentation does not significantly alter its free-radical scavenging activity. They argued that theaflavins in black tea and catechins in green tea are equally effective antioxidants. In response to Leung et al. (2001), Lee et al. (2002) argued that green tea has a higher antioxidant capacity than black tea, regardless of whether or not fermentation affects the antioxidants in tea. This means that green tea has more antioxidant compounds than has black tea. This is in agreement with findings by Atoui, Mansouri, Boskou, and Kefalas (2005) and Yokozawa et al. (1998) that TPC of green tea was higher than that of black tea. These studies showed that the reduction of catechins during the fermentation process of tea manufacturing affects the radical-scavenging activity of tea.

Studies on the antioxidant activity of fresh leaves and tea of *C. sinensis* were carried out primarily on tea from highland plantations (Chen et al., 2003; Gulati, Rawat, Singh, & Ravindranath, 2003; Lin et al., 2003). This is the first report on TPC and AOA of *C. sinensis* var. *assamica* from a lowland tea plantation in Malaysia. Our findings would have significant implications for the quality of tea planted in the lowlands in comparison with highland tea and on the feasibility of establishing tea plantations in the lowlands. This study also investigated the possibility of using microwave drying as a rapid method for producing green tea of a quality comparable to that of commercial teas.

2. Materials and methods

2.1. Samples

Fresh shoots (leaf bud and two youngest leaves; yellowish green), young leaves (third to fifth leaves; light green) and mature leaves (sixth to eighth leaves; dark green) of *C. sinensis* var. *assamica* were collected from a lowland tea plantation in Bukit Cheeding, Selangor (altitude ~20 m asl). Fresh young leaves were also collected from a highland tea plantation in the Cameron Highlands, Pahang (altitude ~1400 m asl), for comparison. From each location, three individual plants were sampled.

Four brands of commercial *C. sinensis* tea were studied. Sea Dyke green tea, Lipton Yellow Label black tea, and Boh Cameron Highlands black tea were highland teas, while Boh Bukit Cheeding No. 53 black tea was a lowland tea. The two brands of Boh tea were produced from plantations in Malaysia. All the commercial teas were purchased from the supermarket. For each brand of commercial tea, three tea bags were sampled.

2.2. Chemicals and reagents

Chemicals used were as follows: total phenolic content (TPC) determination: Folin–Ciocalteu's phenol reagent (Fluka, 2 N), gallic acid (Fluka, 98%), anhydrous sodium carbonate (Fluka, 99%). DPPH assay: 1,1-diphenyl-2-picrylhydrazyl (Sigma, 90%), methanol (Mallinckrodt, 100%). FRAP assay: ferric chloride hexa-hydrate (Fisher, 100%), potassium ferricyanide (Unilab, 99%), trichloroacetic acid (Fisher, 99.8%), potassium dihydrogen phosphate (Bendosen, 99.5%), dipotassium hydrogen phosphate (Merck, 99%). FIC assay: ferrous sulphate hepta-hydrate (Hamburg), ferrozine iron reagent (Acros Organics, 98%). Water was purified by Elga deionizer. Absorbance was measured with an Anthelie Advanced 5 Secoman UV–vis spectrophotometer. pH was measured with a Hanna pH211 meter. Altitude of plantations was measured using a Casio altimeter (Model PRG-70-1VDR).

2.3. Microwave drying of tea leaves

Microwaved green tea was produced by drying fresh tea leaves for 4 min using a household microwave oven (Sharp Model R-248E; 800 W; 230–240 V; 50 Hz). Drying was

done in batches, of 2 g each, of leaves cut into 1 cm^2 pieces. The leaves were put into a beaker and placed in the middle of the turntable of the microwave oven. After drying, dry weights were recorded.

2.4. Preparation of extracts

2.4.1. Methanol extraction of fresh leaves

Fresh leaves (1 g) were powdered with liquid nitrogen in a mortar and extracted using 50 ml of methanol, with continuous swirling for 1 h at room temperature. Extracts were filtered and stored at −20 °C for further use.

To test the efficiency of methanol extraction, second and third extractions were conducted on some samples. After filtration, residues, along with the filter paper, were transferred back into the extraction vessel and extracted again each time with 50 ml of methanol.

2.4.2. Hot-water extraction of tea

Microwaved green tea (0.3 g dry weight, which is equivalent to 1 g fresh weight) was ground in a mortar and extracted with 50 ml of boiling water with continuous swirling for 1 h. The boiling water was allowed to cool throughout the extraction to mimic tea brewing. The same amount of microwaved green tea was extracted with 50 ml of methanol to serve as a control. Extracts were filtered and stored at 4 °C. Commercial teas were extracted in a similar manner.

2.5. Determination of total phenolic content

The amount of total phenolic content (TPC) in extracts was determined according to the Folin–Ciocalteu procedure used by Kahkonen et al. (1999). Samples (300 μl in triplicate) were introduced into test tubes, followed by 1.5 ml of Folin–Ciocalteu's reagent (diluted 10 times) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min before absorbance at 765 nm was measured. TPC was expressed as gallic acid equivalents (GAE) in mg/100 g material. The calibration equation for gallic acid was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$).

2.6. Determination of antioxidant activity

2.6.1. DPPH free-radical scavenging assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical scavenging assay was carried out in triplicate, based on the method used by Leong and Shui (2002) and Miliauskas, Venskutonis, and van Beek (2004) with slight modifications. Different dilutions of the extract, amounting to 1 ml, were added to 2 ml of DPPH (5.9 mg/100 ml methanol). The DPPH solution was then allowed to stand for 30 min before absorbance was measured at 517 nm. Spectrophotometric measurements were made using methanol as blank. An appropriate dilution of the DPPH solution was used as negative control, i.e., methanol in place of the sample. AOA was expressed as IC_{50} (inhibitory concentration in mg/ml of plant material necessary to reduce the absorbance of DPPH by 50%). The lower the IC_{50} the higher is the antioxidant activity. Results were also expressed as AEAC (ascorbic acid equivalent antioxidant capacity) in mg/100 g and calculated as follows:

$$\text{AEAC (mg AA/100 g)} = \text{IC}_{50(\text{ascorbate})}/\text{IC}_{50(\text{sample})} \times 100,000$$

The IC_{50} of ascorbate used for calculation of AEAC was 0.00387 mg/ml.

2.6.2. FRAP assay

The ferric-reducing antioxidant power (FRAP) of extracts was determined, following the method of Chu, Chang, and Hsu (2000) with modifications. Samples often have to be diluted because precipitation occurs upon colour development when the reducing power is too high. Different dilutions of extracts, amounting to 1 ml, were added to 2.5 ml phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of potassium ferricyanide (1% w/v). The mixture was incubated at 50 °C for 20 min. A total of 2.5 ml trichloroacetic acid solution (10% w/v) was added to the mixture to stop the reaction. The mixture was then separated into aliquots of 2.5 ml and each was diluted with 2.5 ml of water. To each diluted aliquot, a total of 500 μl of ferric chloride solution (0.1% w/v) was added and they were allowed to stand for 30 min for colour development. Absorbance measured at 700 nm in triplicate was used to calculate the gallic acid equivalents. Results of the FRAP assay were expressed as mg GAE/g. The calibration equation for gallic acid was $y = 16.767x$ ($R^2 = 0.9974$).

2.6.3. FIC assay

The ferrous-ion chelating (FIC) assay was adapted from Singh and Rajini (2004). Solutions of 2 mM $FeSO_4$ and 5 mM ferrozine were prepared. Each solution was diluted 20 times. Diluted $FeSO_4$ (1 ml) was mixed with 1 ml of sample, followed by 1 ml of diluted ferrozine. Assay mixtures were allowed to equilibrate for 10 min before measuring the absorbance at 562 nm. As the FIC assay is very concentration-dependent, different dilutions of each sample were assayed in triplicate. Measurements were compared with a negative control, comprising solvent in place of sample. As the sample volumes were quite large, the absorbance inherent to the sample may interfere with measurements. Furthermore, it was noted that both leaves and tea samples form a dark blue complex with ferrous ions. To correct for this occurrence, blanks containing the appropriate dilution of each sample with $FeSO_4$ were used. The ability of the sample to chelate ferrous ions was calculated relative to a negative control using the formula:

$$\text{Chelating effect } \% = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100$$

3. Results and discussion

3.1. Fresh tea leaves

3.1.1. Methanol extraction efficiency

Based on TPC, methanol showed a high extraction efficiency for young lowland tea leaves. The first extraction resulted in a yield 92.6 ± 1.4%, the second and third extractions yielding only 6.0 ± 1.4% and 1.4 ± 0.1%, respectively. Waterman and Mole (1994) had recommended methanol for the extraction of phenolic compounds from fresh plant tissues. Methanol had been reported to be the most suitable solvent for extracting phenolic compounds from fresh young shoots of tea, compared with chloroform, ethyl acetate and water (Yao et al., 2004).

3.1.2. TPC and AOA of lowland tea leaves of different ages

Phenolic compounds in tea have been found to be efficient free-radical scavengers, partly due to their one-electron reduction potential, i.e., the ability to act as hydrogen or electron donors (Higdon & Frei, 2003). A lower reduction potential indicates that less energy is required for hydrogen or electron donation that would lead to higher antioxidant activity. FRAP measures the ability of compounds to act as an electron donor while DPPH measures their ability to act as hydrogen donors.

There appear to be some discrepancies in the phenolic content of tea leaves. Chen et al. (2003) found that young tea leaves were richer in EGCG and ECG than were mature leaves, whereas Lin et al. (2003) observed that old leaves contained more EGCG, EGC, EC and catechin than did young leaves.

From this study, TPC and FRAP of shoots (7666 ± 448 mg GAE/100 g and 55.6 ± 1.8 mg GAE/g) and young leaves (7280 ± 126 mg GAE/100 g and 54.5 ± 2.8 mg GAE/g) were significantly higher than those of mature leaves (5836 ± 294 mg GAE/100 g and 21.3 ± 3.5 mg GAE/g) (Table 1). AEAC of shoots (14,470 ± 577 mg AA/100 g), young leaves (12,817 ± 537 mg AA/100 g), and mature leaves (10,219 ± 674 mg AA/100 g) were significantly different from each other. FIC ability was in the order: shoots > young leaves > mature leaves (Fig. 1).

This is the first study on FRAP and FIC ability on fresh tea leaves of different ages. No studies were made on FIC ability of tea and tea leaves. The few studies on FRAP of tea were based on evaporated extracts of old leaves (Farhoosh, Golmovahhed, & Khodaparast, 2007) and dry weights of different commercial teas (Benzie & Szeto, 1999).

Findings of significantly higher TPC, AEAC and FRAP in shoots and young leaves than mature leaves in this study support those of Bhatia and Ullah (1968) and Chen et al. (2003). EGCG and ECG, found abundantly in young leaves, lead to the higher AEAC and FRAP values observed in shoots and young leaves, compared with mature leaves, but contradict Lin et al. (2003), who had found that old leaves are rich in EGCG, EGC, EC and catechin.

The high FIC ability of shoots and young leaves (Fig. 1) suggests that they contain greater amounts of ligands that compete very well with ferrozine in chelating ferrous metal ions. This high secondary antioxidant activity acts by preventing the generation of OH radicals via the Fenton reaction. Metal ions are largely sequestered *in vivo* but high FIC ability would prevent compounds with high FRAP from aggravating certain metal overload diseases (Cao, Sofic, & Prior, 1997). Recently, Kostyuk, Potapovich, Strigunova, Kostyuk, and Afanas (2004) reported that flavonoids, bound to metal ions, were much less subject to oxidation than were the free compounds in the presence of superoxide. Flavonoids in a complex gain an additional active centre, namely, the metal ion $[M^{(n+1)+}]$ via the following reaction:

$$M^{(n+1)+} + O_2^{-}\cdot \rightarrow M^{n+} + O_2$$
$$M^{n+} + O_2^{-}\cdot + 2H^{+} \rightarrow M^{(n+1)+} + H_2O_2$$

3.1.3. TPC and AOA of lowland and highland young tea leaves

Young leaves sampled from lowland and highland plants showed comparable TPC and AOA. ANOVA was insignificant at $P < 0.05$ for TPC, AEAC and FRAP (Table 2). Based on the three separate samplings and each sampling done in triplicate, highland tea leaves showed greater variability than did lowland tea leaves. In terms of FIC ability, lowland leaves were slightly better than highland leaves (Fig. 1). This would imply that lowland leaves are slightly more effective than highland leaves in sequestering 'free' metal ions, rendering them inactive in generating free radicals.

In most countries, tea has traditionally been planted in the highlands in the belief that tea quality is improved at

Table 1
Total phenolic content (TPC) and antioxidant activity (DPPH free-radical scavenging and FRAP) of lowland tea leaves of different ages (fresh weight)

Leaf age	TPC (mg GAE/100 g)	Antioxidant activity (AOA)		
		DPPH free radical scavenging		FRAP (mg GAE/g)
		IC_{50} (mg/ml)	AEAC (mg AA/100 g)	
Shoots	7666 ± 448a	0.026 ± 0.001a	14,470 ± 577a	55.6 ± 1.8a
Young leaves	7280 ± 126a	0.030 ± 0.001a	12,817 ± 537b	54.5 ± 2.8a
Mature leaves	5836 ± 294b	0.037 ± 0.002b	10,219 ± 674c	21.3 ± 3.5b

Results are means ± SD ($n = 3$). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test.

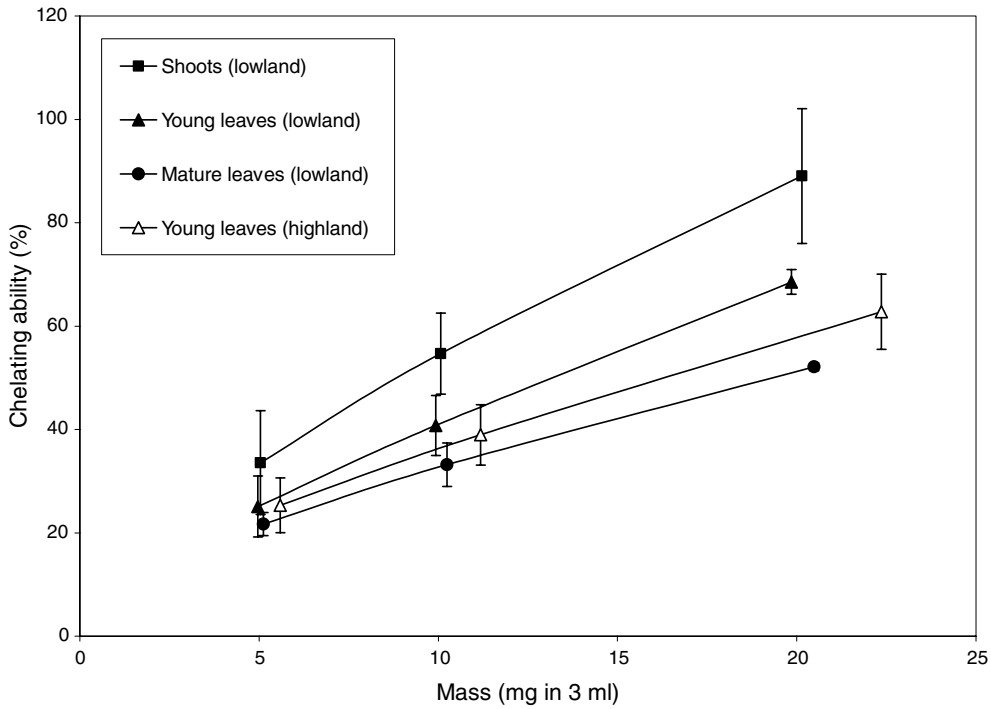

Fig. 1. Ferrous-ion chelating (FIC) ability of lowland tea leaves of different ages in comparison with highland leaves (fresh weight).

Table 2
Total phenolic content (TPC) and antioxidant activity (DPPH free radical scavenging and FRAP) of lowland and highland young tea leaves (fresh weight)

Location	TPC (mg GAE/100 g)	Antioxidant activity (AOA)		
		DPPH free-radical scavenging		FRAP (mg GAE/g)
		IC_{50} (mg/ml)	AEAC (mg AA/100 g)	
Lowland	7280 ± 126	0.030 ± 0.001	12,817 ± 537	54.5 ± 2.8
Highland	7586 ± 1995	0.035 ± 0.010	11,382 ± 3355	50.4 ± 12.9

Results are means ± SD ($n = 3$).

higher altitudes (Graham, 1999). Results from this study show that tea planted in the lowlands is comparable to highland tea in terms of TPC and AOA.

Growing tea in the lowlands has a number of advantages over tea grown in the highlands. In terms of growth and yield, tea plants in the highlands have more shoots, but lower yield in terms of dry weight, than have those in the lowlands (Balasuriya, 1999). It has also been reported that leaves are smaller in the highlands and that lowland shoots develop faster. This would mean higher tea production per unit area in lowland plantations. In terms of physical features, lowland plantations with more gentle terrains are easier to manage and harvesting can be mechanized without encountering environmental problems of soil erosion and slope failure.

3.2. Microwaved green tea and commercial teas

3.2.1. Microwave drying of tea leaves

Tea leaves microwaved for 4 min shrivelled, but remained green with a faint fragrance. When ground, the green-coloured tea produced a mild-tasting yellowish infusion similar to that of commercial green tea.

This study used a one-step process of polyphenol oxidase inactivation by heating and drying using microwave energy. Batches of leaves of 2 g each were completely dry after microwaving for 4 min. Heating and drying are caused by excitation of water molecules in the leaves due to microwave absorption (Pokorný & Schmidt, 2001). Heating is reduced once the leaves are dry.

Microwave heating, using household ovens, can lead to heterogeneous heating patterns within samples (Regier & Schubert, 2001). This does not apply when microwaving leaves which were cut into 1 cm^2 pieces and placed at the centre of the oven turntable. Leaves were rapidly and evenly dried.

Gulati et al. (2003) used a two-step process, i.e., inactivation and drying. Up to 2 kg of leaves were exposed to microwave energy from 2 to 6 min, followed by a separate drying step. Drying treatments used included microwave, conventional oven, and sun drying. Although the duration of drying was not mentioned, oven and sun drying may

take hours and days, respectively. Furthermore, because microwave energy is directed from a magnetron tube as a beam in household ovens (Regier & Schubert, 2001), it would be difficult to achieve homogeneous heating and drying of 2 kg of leaves using a household microwave oven (Gulati et al., 2003).

The microwave technique used in this study can be scaled-up for industrial application. In terms of commercial feasibility, microwave ovens are more energy-efficient than are conventional ovens (Pokorný & Schmidt, 2001). Water boils much faster in a microwave oven because of efficient heat transfer. In industrial microwave ovens, even application of microwave energy allows for homogeneous heating (Regier & Schubert, 2001).

3.2.2. Water and methanol extraction of microwaved green tea

Hot-water extraction of microwaved green tea resulted in a significantly lower TPC and DPPH free-radical scavenging than did methanol extraction (Table 3). However, FRAP (Table 3) and FIC abilities (Fig. 2) were similar for both methods of extraction. Methanol appears to be a more efficient solvent than is hot water. Yao et al. (2004) also reported that hot water extracted less catechins from tea than methanol. However, after repeated extraction, both solvents yielded similar amounts of polyphenols.

The water content of fresh young leaves from Bukit Cheeding was found to be 67.0 ± 2.9%. Expressed in terms of fresh weight equivalent, TPC of methanol extract of microwaved green tea was 6784 ± 69 mg GAE/100 g. This was significantly lower ($P < 0.05$) than fresh leaves with TPC of 7280 ± 126 mg GAE/100 g, representing a 6.8% reduction.

3.2.3. TPC and AOA of commercial teas and microwaved green tea

Of the commercial highland teas, TPC, AEAC and FRAP of Sea Dyke green tea were significantly higher than Lipton Yellow Label and Boh Cameron Highlands black teas (Table 4). Lipton Yellow Label black tea had significantly higher TPC, AEAC and FRAP than had Boh Cameron Highlands black tea. However, the black teas

Table 3
Total phenolic content (TPC) and antioxidant activity (DPPH free radical scavenging and FRAP) of microwaved green tea based on methanol and hot-water extraction (dry weight)

Solvent	TPC (mg GAE/100 g)	Antioxidant activity (AOA)		
		DPPH free radical scavenging		FRAP (mg GAE/g)
		IC_{50} (mg/ml)	AEAC (mg AA/100 g)	
Methanol	20,556 ± 211a	0.013 ± 0.001a	30,000 ± 778a	126 ± 4.5a
Hot water	19,126 ± 365b	0.015 ± 0.001a	26,213 ± 923b	123 ± 10.8a

Results are means ± SD ($n = 3$). For each column, values followed by the same letter (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test.

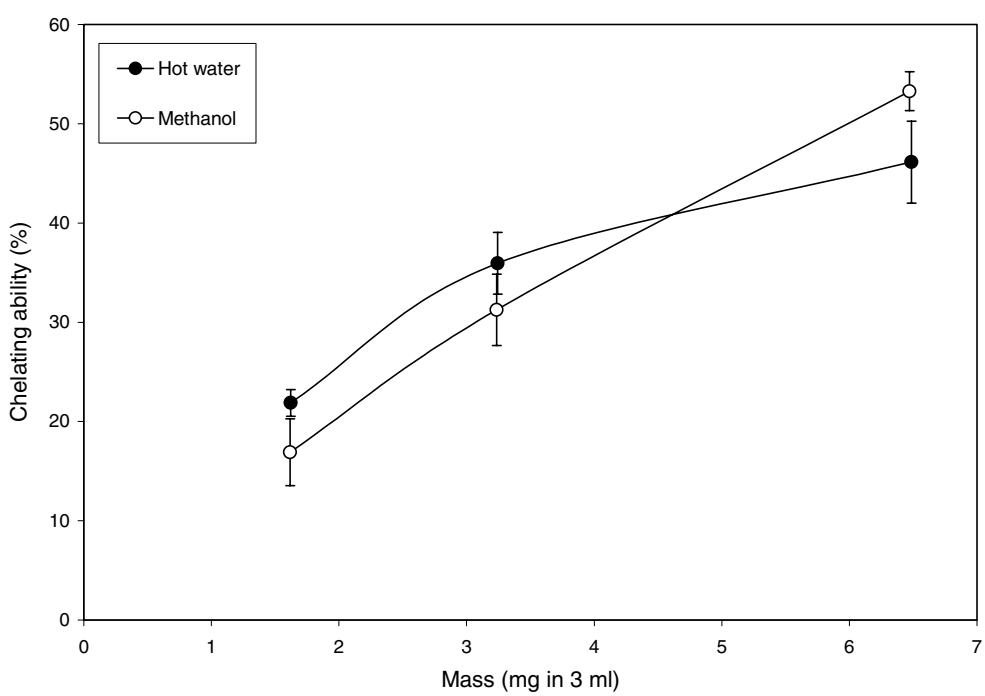

Fig. 2. Ferrous-ion chelating (FIC) ability of microwaved green tea extracted using water and methanol (dry weight).

Table 4
Total phenolic content (TPC) and antioxidant activity (DPPH free radical scavenging and FRAP) of microwaved green tea and four brands of commercial green and black tea (dry weight)

Type and brand of tea	TPC (mg GAE/100 g)	Antioxidant activity (AOA)		
		DPPH free-radical scavenging		FRAP (mg GAE/g)
		IC_{50} (mg/ml)	AEAC (mg AA/100 g)	
Lowland tea				
Microwaved green tea	19,126 ± 365a	0.015 ± 0.001a	26,213 ± 923a	123 ± 10.8a
Boh Bukit Cheeding No. 53 black tea	7409 ± 120bd	0.037 ± 0.002b	10,299 ± 563bd	44.3 ± 1.4b
Highland tea				
Sea Dyke green tea	11,367 ± 1475c	0.021 ± 0.002c	18,457 ± 1737c	83.8 ± 10.9c
Lipton Yellow Label black tea	8494 ± 803b	0.033 ± 0.003b	11,546 ± 1149b	52.5 ± 3.0b
Boh Cameron Highlands black tea	6061 ± 543d	0.051 ± 0.008d	7507 ± 1256d	36.4 ± 2.4b

Results are means ± SD ($n = 3$). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test.

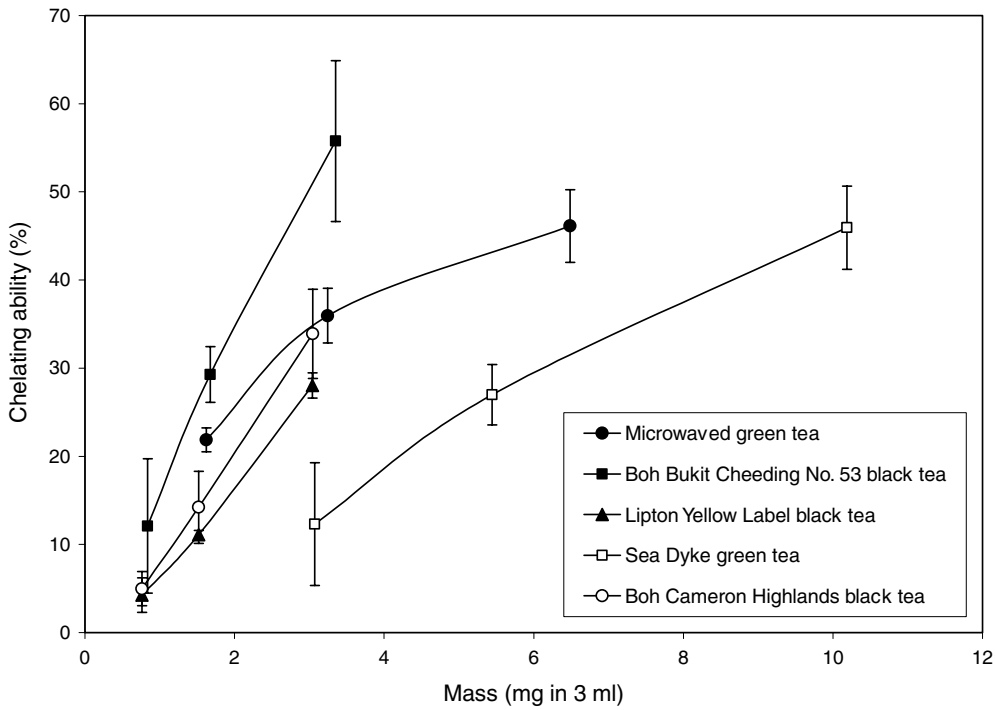

Fig. 3. Ferrous-ion chelating (FIC) ability of microwaved green tea in comparison with commercial teas (dry weight).

outperformed Sea Dyke green tea in terms of FIC ability (Fig. 3).

Comparing between the commercial lowland Boh Bukit Cheeding No. 53 black tea and the highland teas, TPC, AEAC, and FRAP values were significantly lower than those of Sea Dyke green tea (Table 4). The differences were not significant compared to Lipton Yellow Label black tea and Boh Cameron Highlands black tea. As with fresh lowland and highland leaves (Table 2), values of Boh Cameron Highlands black tea were more variable than those of Boh Bukit Cheeding No. 53 black tea.

In terms of sensory quality, there are subtle differences between the highland and lowland Boh teas. Boh Cameron Highlands black tea is characterized by its rich and invigorating aromatic flavour, and Boh Bukit Cheeding No. 53 black tea has a robust and full-bodied flavour.

In terms of FIC ability, the commercial lowland Boh Bukit Cheeding No. 53 black tea ranked the highest (Fig. 3). Ranking in FIC ability was as follows: Boh Bukit Cheeding No. 53 black tea (lowland) > microwaved green tea (lowland) ≈ Boh Cameron Highlands black tea (highland) ≈ Lipton Yellow Label black tea (highland) > Sea Dyke green tea (highland).

The microwaved green tea showed outstanding TPC, AEAC, and FRAP values (Table 4). Its values were significantly the highest compared to the four commercial brands of green and black tea. In terms of FIC ability, the microwaved green tea was better than Sea Dyke green tea (Fig. 3).

Gulati et al. (2003) dried leaf shoots using various treatments to produce green teas with TPCs ranging from 11% to 13% GAE (dry weight). This amounts to 11,000–13,000 mg GAE/100 g, which is similar to the Sea Dyke

green tea (11,367 ± 1475 mg GAE/100 g) (Table 4). The microwaved green tea, produced in this study, with TPC of 19,126 ± 365 mg GAE/100 g, was far superior. Furthermore, the 50% acetone used by Gulati et al. (2003) for extraction could have led to an over-estimation, as acetone was found to reduce the Folin–Ciocalteu reagent. Hot-water extraction yielded only 4000 mg GAE/100 g (Gulati et al., 2003).

The outstanding TPC, AEAC, and FRAP of the microwaved green tea might be caused by the release of bound phenolic compounds (Gulati et al., 2003). Microwave energy could have prevented the binding of polyphenols, including catechins, to the leaf matrix, thereby increasing their solubility. In addition, heat generated during microwaving may release additional bound phenolic compounds, brought about by the breakdown of cellular constituents (Dewanto, Wu, & Liu, 2002).

4. Conclusion

Methanol showed high extraction efficiency for fresh tea leaves. Between leaves of different ages, shoots and young leaves showed significantly higher TPC and FRAP than did mature leaves. AEAC of shoots, young leaves, and mature leaves were significantly different from each other.

TPC, AEAC and FRAP of lowland tea leaves were comparable to those of highland plants with the latter showing greater variability. In terms of FIC ability, lowland leaves were slightly better than highland leaves.

Sea Dyke green tea had significantly higher TPC, AEAC, and FRAP than had black teas of Lipton Yellow Label, Boh Cameron Highlands and Boh Bukit Cheeding No. 53 with the exception of FIC ability. The microwaved green tea had significantly higher TPC and AOA than had all the four brands of commercial green and black teas studied. Boh Bukit Cheeding No. 53 black tea showed outstanding FIC ability, surpassing that of the microwaved green tea. This study showed that tea planted in lowlands is comparable to those planted in highlands in terms of TPC and AOA.

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Antioxidant and antibacterial activity of leaves of *Etlingera* species (Zingiberaceae) in Peninsular Malaysia

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Abstract

Methanolic extracts from fresh leaves of five *Etlingera* species were screened for total phenolic content (TPC), antioxidant activity (AOA), and antibacterial activity. Analysis of TPC was done using the Folin–Ciocalteu method. Evaluations of AOA included 1,1-diphenyl-2-picrylhydrazyl free radical-scavenging ability, ferric-reducing antioxidant power (FRAP), ferrous-ion chelating (FIC) ability, and β-carotene bleaching (BCB) activity. Antibacterial activity was screened using the disc-diffusion method. Highest TPC, ascorbic acid equivalent antioxidant capacity (AEAC), and FRAP were found in leaves of *E. elatior* and *E. rubrostriata*. Leaves of *E. maingayi*, with the lowest TPC, AEAC, and FRAP, had the highest FIC ability and BCB activity. Ranking of TPC and AOA of different plant parts of *E. elatior* was in the order: leaves > inflorescences > rhizomes. Leaves of highland populations of *Etlingera* species displayed higher values of TPC and AEAC than those of lowland counterparts. Leaves of *Etlingera* species exhibited antibacterial activity against Gram-positive but not Gram-negative bacteria.
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Keywords: Etlingera; Leaves; Highland; Lowland; Total phenolic content; Antioxidant activity; Antibacterial activity

1. Introduction

Etlingera Giseke of the family Zingiberaceae are tall forest plants, with larger species reaching 6 m in height (Khaw, 2001). In the *Phaeomeria* group, inflorescences are borne on erect stalks protruding from the ground and, in the *Achasma* group, inflorescences are subterranean with flowers appearing at soil level (Lim, 2000, 2001). The varying shades of pink and red colours of bracts and flowers make *Etlingera* species very attractive plants. A total of 15 *Etlingera* species has been recorded in Peninsular Malaysia (Lim, 2001).

Plants of *Etlingera* have various traditional and commercial uses. In Sabah, Malaysia, the hearts of young shoots, flower buds, and fruits of *E. elatior*, *E. rubrolutea*, and *E. littoralis* are consumed by indigenous communities as condiment, eaten raw or cooked (Noweg, Abdullah, & Nidang, 2003). In Thailand, fruits and cores of young stems of *E. littoralis* are edible, and flowers of *E. maingayi* are eaten as vegetables (Sirirugsa, 1999). There are no reports on the use of rhizomes of *Etlingera* species.

Inflorescences of *E. elatior* are widely cultivated throughout the tropics as spices for food flavouring and as ornamentals. They are commonly used as ingredients of dishes such as *laksa asam*, *nasi kerabu*, and *nasi ulam* in Peninsular Malaysia (Larsen, Ibrahim, Khaw, & Saw, 1999). Farms in Australia and Costa Rica are cultivating the species and selling its inflorescences as cut flowers (Larsen et al., 1999). In Malaysia, fruits of *E. elatior* are used traditionally to treat earache, while leaves are applied for cleaning wounds (Ibrahim & Setyowati, 1999). Leaves of *E. elatior*, mixed with other aromatic herbs in water, are used by *post-partum* women for bathing to remove body odour.

Flavonoids in the leaves of *E. elatior* have been identified as kaempferol 3-glucuronide, quercetin 3-glucuronide,

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quercetin 3-glucoside, and quercetin 3-rhamnoside (Williams & Harborne, 1977). Flavonoid content of inflorescences of *E. elatior* has been estimated to be 286 and 21 mg of kaempferol and quercetin (per kg dry weight), respectively (Miean & Mohamed, 2001).

Phytochemical studies on rhizomes of *E. elatior* led to the isolation of two new and six known compounds of diarylheptanoids, labdane diterpenoids, and steroids (Habsah et al., 2005). Using the ferric thiocyanate method, lipid peroxidation inhibitory activity of the isolated diarylheptanoids was greater than that of α-tocopherol. Ethanolic extracts from the flower shoots of *E. elatior* have antimicrobial activity and are cytotoxic to HeLa cells (Mackeen et al., 1997).

Past studies on the antioxidant activity (AOA) of ginger species were confined to rhizomes (Jitoe et al., 1992; Habsah et al., 2000; Zaeoung, Plubrukarn, & Keawpradub, 2005). Their rhizomes have been reported to contain antioxidants comparable to α-tocopherol. Although leaves of ginger species have been used for food flavouring and as traditional medicine, hardly any research has been done on their AOA.

In our previous study (Chan, Lim, & Lim, in press), total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves and rhizomes of five wild and six cultivated ginger species, belonging to seven genera, were screened. The seven genera were *Alpinia*, *Boesenbergia*, *Curcuma*, *Elettariopsis*, *Etlingera*, *Scaphochlamys*, and *Zingiber*. Results showed that leaves of *Etlingera* had the highest TPC and AEAC. Values were significantly higher than those of rhizomes.

In this study, TPC, AOA, and antibacterial activity of leaves of five *Etlingera* species were analysed. In *E. elatior*, TPC and AOA of the different plant parts were compared. Altitudinal variation in leaf TPC and AEAC of four *Etlingera* species was also studied. This study represents the first systematic analysis of TPC, AOA, and antibacterial activity of leaves of *Etlingera* species.

2. Materials and methods

2.1. Plant materials

Five species of *Etlingera* studied were *E. elatior* (Jack) R.M. Smith, *E. fulgens* (Ridl.) C.K. Lim, and *E. maingayi* (Bak.) R.M. Smith of the *Phaeomeria* group, and *E. littoralis* (Koenig) Giseke and *E. rubrostriata* (Holtt.) C.K. Lim of the *Achasma* group. Their identification in the field was based on taxonomic descriptions and photographic illustrations of Khaw (2001) and Lim (2000, 2001). Characteristic scent of leaves when crushed was another useful cue for species identification. Voucher specimens were deposited at the herbarium of Forest Research Institute Malaysia (FRIM).

Leaves of highland populations of *Etlingera* species were sampled from Janda Baik and Genting Highlands in Pahang, and from Ulu Gombak in Selangor, while leaves of lowland populations were sampled from FRIM. For each species, mature leaves were sampled from three different clumps. Altitude of locations where populations were sampled was measured using a Casio altimeter (Model PRG-70-1VDR).

Rhizomes of *E. elatior* were collected from FRIM while its inflorescences were purchased from the supermarket. For comparison as positive controls, young leaves of the tea plant, *Camellia sinensis* (L.) Kuntze, were collected from a tea plantation in Cameron Highlands, Pahang, and rhizomes of *Curcuma longa* L. and *Zingiber officinale* Roscoe were purchased from the supermarket.

2.2. Chemicals and reagents

For TPC analysis, Folin–Ciocalteu's phenol reagent (Fluka, 2N), gallic acid (Fluka, 98%), and anhydrous sodium carbonate (Fluka, 99%) were used; for DPPH assay, 1,1-diphenyl-2-picrylhydrazyl (Sigma, 90%) was used; for FRAP assay, ferric chloride hexa-hydrate (Fisher Scientific, 100%), potassium ferricyanide (Unilab, 99%), trichloroacetic acid (HmbG Chemicals, 99.8%), potassium dihydrogen orthophosphate (Fisher Scientific, 99.5%), and dipotassium hydrogen phosphate (Merck, 99%) were used; for FIC assay, ferrozine (Acros Organics, 98%) and ferrous sulphate hepta-hydrate (HmbG Chemicals) were used; for BCB assay, β-carotene (Sigma, Type 1: synthetic), chloroform (Fisher Scientific, 100%), linoleic acid (Fluka), and Tween 40 (Fluka) were used. For disc-diffusion assay, paper discs (Oxoid, 6 mm), Muller–Hinton agar (Oxoid), nutrient broth (Oxoid), and streptomycin susceptibility discs (Oxoid, 10 μg) were used.

2.3. Preparation of extracts

For the analysis of TPC and AOA, fresh leaves (1 g) were powdered with liquid nitrogen in a mortar and extracted using 50 ml of methanol, with continuous swirling for 1 h at room temperature. Extracts were filtered and stored at −20 °C for further use. Rhizomes and inflorescences were extracted in a similar manner.

For the screening of antibacterial activity, leaves of each species were cut into small pieces and 100 g were weighed and freeze-dried. Dried samples were then crushed in a mortar with liquid nitrogen and extracted with 250 ml of methanol three times for 1 h each time. Samples were filtered and the solvent was removed using a rotary evaporator. Dried extracts were kept at −20 °C for analysis.

2.4. Methanol extraction efficiency

To test the efficiency of methanol extraction, second and third extractions were conducted. After filtration, residues, along with the filter paper, were transferred back into the extraction vessel and extracted again each time with 50 ml methanol. Measurement of extraction efficiency was based on TPC.

2.5. Determination of total phenolic content

Total phenolic content (TPC) of plant extracts was determined using the Folin–Ciocalteu assay reported by Kahkonen et al. (1999). Folin–Ciocalteu reagent (1.5 ml; diluted 10 times) and sodium carbonate (1.2 ml; 7.5% w/v) were added to the extracts (300 μl; triplicate). After 30 min, absorbance was measured at 765 nm. TPC was expressed as gallic acid equivalents (GAE) in mg per 100 g. The calibration equation for gallic acid was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$).

2.6. Determination of antioxidant activity

2.6.1. DPPH assay

The DPPH free radical-scavenging (FRS) assay used by Miliauskas, Venskutonis, and van Beek (2004) was adopted with modifications. Different dilutions of the extract (1 ml; triplicate) were added to 2 ml of DPPH (5.9 mg/100 ml methanol). Absorbance was measured at 517 nm after 30 min. FRS ability was calculated as IC_{50} and expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg AA/100 g (Leong & Shui, 2002) as follows:

$$\text{AEAC (mg AA/100g)} = IC_{50(\text{ascorbate})}/IC_{50(\text{extract})} \times 100,000$$

The IC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/ml.

2.6.2. FRAP assay

The ferric-reducing antioxidant power (FRAP) assay reported by Chu, Chang, and Hsu (2000) was adopted with modifications. Different dilutions of the extract (1 ml) were added to 2.5 ml phosphate buffer (0.2 M; pH 6.6) and 2.5 ml of potassium ferricyanide (1% w/v). The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid solution (2.5 ml; 10% w/v) was added to stop the reaction. The mixture was then separated into aliquots of 2.5 ml and diluted with 2.5 ml of water. To each diluted aliquot, 500 μl of ferric chloride solution (0.1% w/v) were added. After 30 min, absorbance was measured at 700 nm. FRAP of extracts was expressed as mg GAE/g. The calibration equation for gallic acid was $y = 16.767x$ ($R^2 = 0.9974$).

2.6.3. FIC assay

The ferrous-ion chelating (FIC) assay used by Singh and Rajini (2004) was adopted. Solutions of 2 mM $FeSO_4$ and 5 mM ferrozine were diluted 20 times. $FeSO_4$ (1 ml) was mixed with different dilutions of extract (1 ml), followed by ferrozine (1 ml). Absorbance was measured at 562 nm after 10 min. The ability of extracts to chelate ferrous ions was calculated as follows:

$$\text{Chelating effect } \% = (1 - A_{\text{extract}}/A_{\text{control}}) \times 100$$

where A_{extract} and A_{control} are absorbance of the extract and negative control, respectively.

2.6.4. BCB assay

The β-carotene bleaching (BCB) assay reported by Kumazawa et al. (2002) was adopted. β-Carotene/linoleic acid emulsion was prepared by adding 3 ml of β-carotene (5 mg/50 ml chloroform) to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was evaporated under vacuum and oxygenated ultra-pure water (100 ml) was added and mixed well. Initial absorbance of the emulsion was measured at 470 nm. Aliquots of the emulsion (3 ml) were mixed with 10, 50, and 100 μl of extract and incubated in a water bath at 50 °C for 1 h. Bleaching rate of β-carotene was measured at 470 nm and 700 nm. Measurement at 700 nm is needed to correct for the presence of haze. Bleaching rate was expressed as AOA (%) and calculated as follows:

$$\text{Bleaching rate (BR) of β-carotene} = \ln(A_{\text{initial}}/A_{\text{extract}})/60$$

$$\text{AOA } (\%) = 1 - (\text{BR}_{\text{extract}}/\text{BR}_{\text{control}}) \times 100$$

where A_{initial} and A_{extract} are absorbances of the emulsion before and 1 h after incubation, and $\text{BR}_{\text{extract}}$ and $\text{BR}_{\text{control}}$ are bleaching rates of the extract and negative control, respectively.

2.7. Screening for antibacterial activity

The disc-diffusion method described by Chung, Chung, Ngeow, Goh, and Imiyabir (2004) was used to screen for antibacterial activity. Agar cultures of Gram-positive bacteria (*Bacillus cereus*, *Micrococcus luteus*, and *Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Salmonella cholerasuis*) were prepared. Suspensions of bacteria (100 μl) were spread evenly onto 20 ml Mueller–Hinton agar preset in 90 mm Petri dishes. Paper discs (6 mm diameter) were impregnated with 1 mg of plant extract dissolved in 100 μl solvent, and transferred onto the inoculated agar.

Streptomycin susceptibility discs (10 μg) and methanol-impregnated disc were used as positive and negative controls, respectively. After incubation overnight at 37 °C, inhibition zones were measured and recorded as mean diameter (mm). Antibacterial activity was also expressed as inhibition percentage of streptomycin and arbitrarily classified as strong for inhibition of $\geqslant 70\%$, moderate for inhibition $50 < 70\%$, and weak for inhibition $<50\%$.

3. Results and discussion

3.1. Descriptions of plant specimens

Photographs of plants of the five species of *Etlingera* studied are shown in Fig. 1. Leaves of *E. elatior* are entirely green, sometimes flushed pink when young, and emit a pleasant sour scent when crushed. Leaves of *E. fulgens* are dark green, shiny, undulated, and their underside is bright red when young. They emit a pleasant sour scent similar to those of *E. elatior*. Leaves of *E. maingayi* are red-

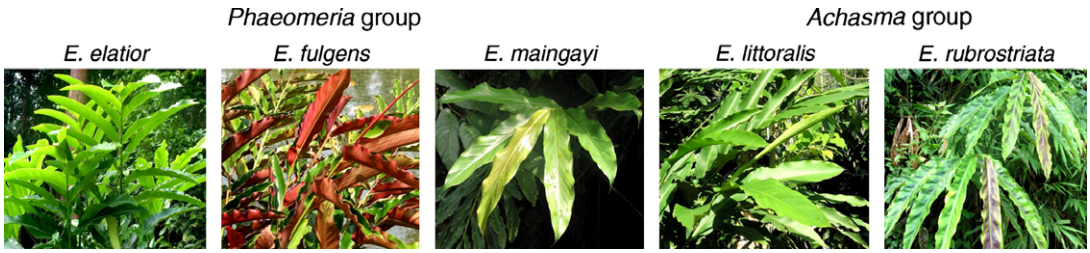

Fig. 1. Photographs of plants of five species of *Etlingera* studied.

dish below, translucent when young, and emit an unpleasant sour scent. Leaves of *E. littoralis* are entirely green, sometimes flushed pink when young, and do not have any scent. Leaves of *E. rubrostriata* are green with distinctive purplish-brown bars on upper surface and do not have any scent.

3.2. Methanol extraction efficiency of leaves

Methanol showed high extraction efficiency of leaves of *Etlingera* species. Yields of the first extraction ranged from 82.7% in *E. rubrostriata* to 88.2% in *E. maingayi*. A second extraction yielded 12.9% and 7.8%, and a third yielded 4.4% and 4.0%, respectively.

Methanol has been recommended for the extraction of phenolic compounds from fresh plant tissues. It is a suitable solvent due to its ability to inhibit polyphenol oxidase, which could alter antioxidant activity (Yao et al., 2004). High methanol extraction efficiency has been reported for leaves and flowers of *Alpinia* species (Wong, 2006), and for young leaves of *C. sinensis* (Chan, Lim, & Chew, 2007).

3.3. Total phenolic content of leaf extracts

Total phenolic content (TPC) of leaf extracts was determined using the Folin–Ciocalteu method and expressed in mg GAE/100 g. Of the *Etlingera* species analysed, leaves of *E. elatior* and *E. rubrostriata* had the highest TPC (Table 1). Values were 3550 ± 304 and 3480 ± 390 mg GAE/100 g, respectively. Leaves of *E. maingayi* and *E. fulgens* had the lowest TPC of 1110 ± 93 and 2540 ± 91 mg GAE/100 g, respectively.

Table 1
Total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and ferric-reducing antioxidant power (FRAP) of leaves of *Etlingera* species (fresh weight)

Etlingera sp.	TPC (mg GAE/100 g)	Antioxidant activity (AOA)	
		AEAC (mg AA/100 g)	FRAP (mg GAE/g)
E. elatior	3550 ± 304a	3750 ± 555a	19.6 ± 2.1a
E. rubrostriata	3480 ± 390ab	3540 ± 401a	16.6 ± 2.4a
E. littoralis	2810 ± 242bc	2930 ± 220b	11.6 ± 1.0b
E. fulgens	2540 ± 91c	2030 ± 126c	9.4 ± 0.4c
E. maingayi	1110 ± 93d	963 ± 169d	4.9 ± 0.8d

Values of TPC, AEAC, and FRAP are means ± SD ($n = 3$). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$, as measured by the Tukey HSD test.

3.4. Antioxidant activity of leaf extracts

Antioxidant activity (AOA) of leaf extracts from *Etlingera* species was evaluated using the DPPH, FRAP, FIC, and BCB assays. Activity was expressed in mg AA/100 g, mg GAE/g, chelating ability (%) and AOA (%), respectively.

Results showed that leaves of *E. elatior* and *E. rubrostriata* had high AEAC and FRAP (Table 1). Values were 3750 ± 555 mg AA/100 g and 19.6 ± 2.1 mg GAE/g for *E. elatior*, and 3540 ± 401 mg AA/100 g and 16.6 ± 2.4 mg GAE/g for *E. rubrostriata*, respectively. Moderately high AEAC and FRAP were found in the leaves of *E. littoralis* and *E. fulgens.* Values were 2930 ± 220 mg AA/100 g and 11.6 ± 1.0 mg GAE/g for *E. maingayi*, and 2030 ± 126 mg AA/100 g and 9.4 ± 0.4 mg GAE/g for *E. fulgens*, respectively. Lowest values of 963 ± 169 mg AA/100 g and 4.9 ± 0.8 mg GAE/g were found in the leaves of *E. maingayi*.

Among the species of *Etlingera* studied, leaf AEAC and FRAP shared the same order of ranking as leaf TPC i.e., *E. elatior* > *E. rubrostriata* > *E. littoralis* > *E. fulgens* > *E. maingayi*. It is evident that *Etlingera* species with high leaf TPC also have high AEAC and FRAP.

In terms of FIC ability, the trend was reversed with leaves of *E. maingayi* and *E. fulgens* having the highest values (Fig. 2). Leaves of *E. maingayi* and *E. fulgens* were superior, and leaves of *E. elatior* and *E. littoralis* were comparable to the FIC ability of young leaves of *C. sinensis* (positive control). Lowest values were found in the leaves of *E. rubrostriata.*

It can therefore be seen that leaves of *Etlingera* species with high TPC, AEAC, and FRAP have low FIC ability and vice versa. This would mean that phenolic compounds in extracts responsible for antioxidant activities of scavenging free radicals and reducing ferric ions might not be directly involved in ferrous ion chelation. The compounds responsible could be nitrogen-containing compounds, which are generally better chelators than are phenols. Similar observations were made with leaves of *Alpinia*. Of four species studied, leaves of *Alpinia galanga*, with the lowest TPC, AEAC, and FRAP, exhibited the highest FIC ability (Wong, 2006).

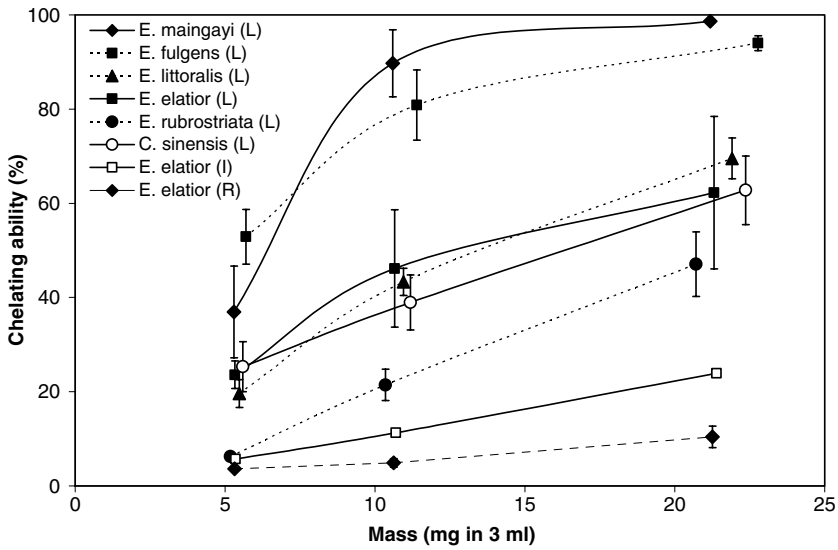

Fig. 2. Ferrous-ion chelating (FIC) ability of leaves of *Etlingera* species and different plant parts of *Etlingera elatior* (fresh weight). Young leaves of *Camellia sinensis* were used as positive control. Results are means ± SD ($n = 3$). Abbreviations: E., *Etlingera*; C., *Camellia*; R, rhizomes; L, leaves; I, inflorescences.

In terms of BCB activity, leaves of *E. maingayi* had the highest values. Its leaf BCB activity was better than that of rhizomes of *C. longa* with leaves of *E. rubrostriata*, *E. littoralis*, and *E. elatior* having slightly lower values (Fig. 3a). Although leaves of *E. fulgens* showed the lowest BCB activity, values were higher than that of young leaves of *C. sinensis* but lower than that of *Z. officinale* rhizomes (Fig. 3b).

With the exception of *E. fulgens*, leaves of all *Etlingera* species studied showed high BCB activity, comparable with that of rhizomes of *C. longa* and superior to that of young tea leaves and rhizomes of *Z. officinale*. High BCB activity of leaves of *Etlingera* species reflects their ability to strongly inhibit lipid peroxidation. There appears to be no correlation between BCB activity and AOA, as measured by the other assays. This is supported by findings of Lim and Quah (2007) that methanolic extracts of six cultivars of *Portulaca oleracea* showed that TPC correlated well with AEAC and FRAP but not with BCB activity.

3.5. TPC and AOA of extracts from different plant parts

Analyses of different plant parts of *E. elatior* showed that leaves had significantly higher TPC, AEAC, and FRAP than had inflorescences and rhizomes at $P < 0.05$ (Table 2). Values were 3550 ± 304 mg GAE/100 g, 3750 ± 555 mg AA/100 g, and 19.6 ± 2.1 mg GAE/g for leaves, 295 ± 24 mg GAE/100 g, 268 ± 45 mg AA/100 g, and 1.5 ± 0.2 mg GAE/g for inflorescences, and 187 ± 46 mg GAE/100 g, 185 ± 59 mg AA/100 g, and 0.9 ± 0.2 mg GAE/g for rhizomes, respectively.

Similarly, leaves of *E. elatior* showed superiority over inflorescences and rhizomes in terms of FIC ability (Fig. 2). FIC ability of leaves was comparable to that of young leaves of *C. sinensis*. BCB activity of leaves was much higher than that of rhizomes but slightly lower than that of inflorescences. BCB activities of inflorescences and rhizomes were comparable to those of rhizomes of *C. longa* (Fig. 3a) and young leaves of tea (Fig. 3b), respectively. Ranking of TPC and AOA (AEAC and FRAP) was in the order: leaves > inflorescences > rhizomes.

In Zingiberaceae, it is generally believed that antioxidants and other secondary metabolites are transported to the rhizomes where they are accumulated. This implies that rhizomes would have higher AOA than would other plant parts. Rhizomes of cultivated species have been reported to possess radical-scavenging compounds comparable to commercial antioxidants on a weight per weight basis. Jitoe et al. (1992) reported that AOA of extracts of *Alpinia*, *Amomum*, *Curcuma*, and *Zingiber* rhizomes were comparable to α-tocopherol. Extracts of *Z. officinale* rhizomes had better radical-scavenging ability than had butylated hydroxytoluene and quercetin (Stoilova, Krastanov, Stoyanova, Denev, & Gargova, 2007).

In our previous study (Chan et al., in press), screening of five wild and six cultivated ginger species showed that leaf TPC and AEAC were generally higher than those of rhizomes. Out of the 11 species screened, eight species had significantly higher leaf TPC and/or AEAC. Outstanding leaf TPC and AEAC of both *E. elatior* and *E. maingayi* were seven and eight times higher than those of rhizomes, respectively.

Results of this study on the different plant parts of *E. elatior* reaffirmed that TPC and AOA of leaves were significantly higher than those of rhizomes at $P < 0.05$. TPC, AEAC and FRAP were 19, 20, and 22 times higher in leaves than in rhizomes, respectively. Leaves of wild and cultivated *Etlingera* species therefore contain more antioxidants than do other plant parts.

Recently, Elzaawely, Xuan, and Tawata (2007) reported that ethyl acetate extracts from leaves of *Alpinia zerumbet*

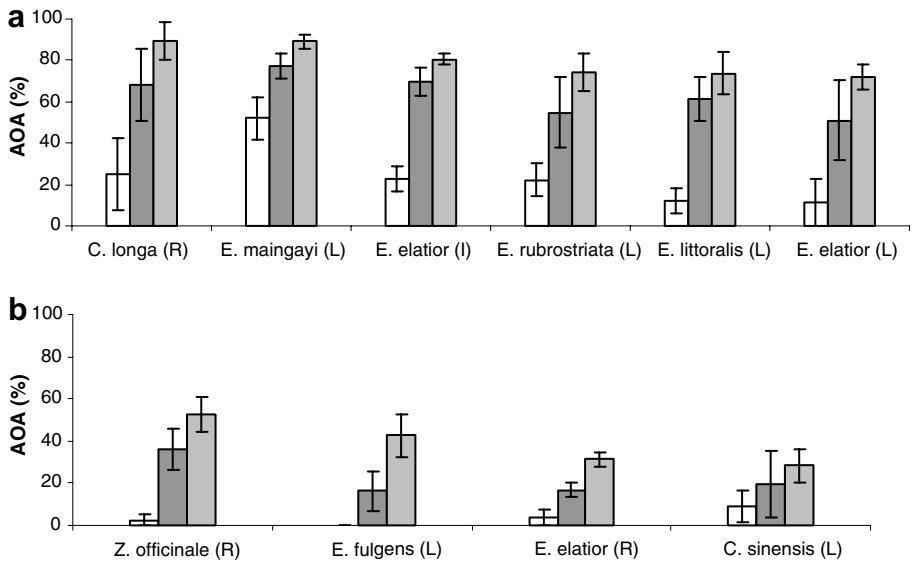

Fig. 3. β-Carotene bleaching (BCB) activity of leaves of *Etlingera* species (fresh weight). Rhizomes of *Curcuma longa* and *Zingiber officinale*, and young leaves of *Camellia sinensis* were used as positive controls. Results are means ± SD ($n = 3$). For each species, left, middle, and right bars represent extract concentrations of 0.2, 1.0, and 2.0 μg in 3 ml, respectively. Abbreviations for Fig. 3a: C., *Curcuma*; E., *Etlingera*; R, rhizomes; L, leaves; I, inflorescences. Abbreviations for Fig. 3b: Z., *Zingiber*; E., *Etlingera*; C., *Camellia*; R, rhizomes; L, leaves.

Table 2
Total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and ferric-reducing antioxidant power (FRAP) of different plant parts of *Etlingera elatior* (fresh weight)

Plant part of *E. elatior*	TPC (mg GAE/100 g)	Antioxidant activity (AOA)	
		AEAC (mg AA/100 g)	FRAP (mg GAE/g)
Leaves	3550 ± 304a	3750 ± 555a	19.6 ± 2.1a
Inflorescences	295 ± 24b	268 ± 45b	1.5 ± 0.2b
Rhizomes	187 ± 46c	185 ± 59c	0.9 ± 0.2c

Values of TPC, AEAC, and FRAP are means ± SD ($n = 3$). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$, as measured by the Tukey HSD test.

showed higher inhibition of β-carotene oxidation and scavenging activity of free radicals than did rhizomes. This further supports our result that leaves have free-radical scavengers that are more effective than those found in rhizomes.

3.6. Altitudinal variation in TPC and AEAC of leaf extracts

Leaves of all *Etlingera* species sampled from highland populations were found to have higher TPC and AEAC than those of lowland counterparts. Leaves of *E. rubrostriata*, *E. elatior*, and *E. fulgens* showed significantly higher values with greater altitude at $P < 0.05$, while *E. littoralis* was marginally higher (Table 3). Highest leaf TPC and AEAC were found in highland populations of *E. elatior*, with values of 3550 ± 304 mg GAE/100 g and 3750 ± 555 mg AA/100 g, and of *E. rubrostriata*, with values of 3480 ± 390 mg GAE/100 g and 3540 ± 401 mg AA/100 g, respectively. Lowland populations of *E. fulgens* had the lowest values of 1280 ± 143 mg GAE/100 g and 845 ± 159 mg AA/100 g, respectively.

Higher altitudes seem to trigger an adaptive response in *Etlingera* species. Higher leaf TPC and AEAC of highland populations over those of lowland counterparts might be due to environmental factors, such as higher UV-B radiation and lower air temperature. There is increasing evidence that enhanced UV-B radiation induces production of phenolic compounds in plants (Bassman, 2004). Enzymes associated with the synthesis of phenolics are produced in greater quantities or show increased activity (Chalker-Scott & Scott, 2004). Phenylalanine ammonia lyase (PAL) is up-regulated, resulting in the accumulation of flavonoids and anthocyanins, which have antioxidant ability (Jansen, Gaba, & Greenberg, 1998). Low temperatures have also been shown to enhance PAL synthesis in a variety of plants, leading to increased production of flavonoids and other phenolics (Chalker-Scott & Scott, 2004).

3.7. Antibacterial activity of leaf extracts

Using the disc-diffusion method, leaves of all five *Etlingera* species were found to inhibit Gram-positive *B. cereus*, *M. luteus*, and *S. aureus* (Table 4). 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*.

Mean diameter of the zone of inhibition of streptomycin was 23 mm for *M. luteus*, and 17 mm for *B. cereus* and *S. aureus* (Table 4). Methanol showed no inhibitory effect on the three bacteria. Streptomycin was used as positive

Table 3
Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves of *Etlingera* from highland and lowland locations (fresh weight)

Etlingera sp.	Location	Altitude (m asl)	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
E. elatior	Janda Baik	400	3550 ± 304a	3750 ± 555a
	FRIM	100	2390 ± 329b	2280 ± 778b
E. rubrostriata	Ulu Gombak	300	3480 ± 390a	3540 ± 401a
	FRIM	100	2430 ± 316b	2640 ± 508a
E. littoralis	Genting Highlands	800	2810 ± 243a	2930 ± 220a
	FRIM	100	2340 ± 386a	2220 ± 913a
E. fulgens	Janda Baik	400	2270 ± 31a	2030 ± 126a
	FRIM	100	1280 ± 143b	845 ± 158b

Values of TPC and AEAC are means ± SD ($n = 3$). For columns of each species, values followed by the same letter (a–b) are not significantly different at $P < 0.05$ measured by the Tukey HSD test. ANOVA does not apply between species.

control because it has been used as the antibiotic for Gram-positive and Gram-negative bacteria.

Among the Gram-positive bacteria, *S. aureus* appeared to be more sensitive. Screening for antibacterial activity of 191 plant extracts belonging to 30 families of plants from Sabah, Malaysia, showed similar results (Chung et al., 2004). About 52% of the extracts inhibited *S. aureus*. For all five *Etlingera* species, leaves showed stronger antibacterial activity than did rhizomes.

Leaves of *Etlingera* showed no antibacterial activity on Gram-negative bacteria of *E. coli*, *P. aeruginosa*, and *S. cholerasuis*. Antibacterial studies of extracts from various ginger species also showed no inhibition of Gram-negative bacteria (Chandarana, Baluja, & Chanda, 2005; Wong, 2006).

Gram-negative bacteria have an outer membrane consisting of lipoprotein and lipopolysaccharide, which is selectively permeable and thus regulates access to the underlying structures (Chopra & Greenwood, 2001). This renders the Gram-negative bacteria generally less susceptible to plant extracts than the Gram-positive bacteria.

Preliminary investigation on the use of ethylenediamine tetraacetic acid (EDTA) to improve the efficacy of leaf extracts of *Etlingera* species against Gram-negative bacteria was carried out. Adding 2 mM EDTA to the agar caused *P. aeruginosa* to be susceptible to all leaf extracts of *Etlingera* species but inhibited the growth of *E. coli* and *S. cholerasuis*.

EDTA has been reported to permeabilise the outer membrane of *P. aeruginosa*, making it susceptible to antibiotics and certain antiseptic agents (Haque & Russell, 1974). Bacteria can be either exposed to the permeabiliser, together with the antibiotic, or pre-treated with the permeabiliser prior to introduction of the antibiotic (Ayres, Furr, & Russell, 1999).

In this study, the pre-treatment method was not effective. This suggests a different mode of action for EDTA, possibly synergistic with plant extracts. It is the first time the method has been used for testing antibacterial activity of plant extracts. Initial findings warrant further investigations.

Table 4
Antibacterial activity of leaves of *Etlingera* species against Gram-positive bacteria using the disc-diffusion method

Etlingera sp.	Zone of inhibition in mm (inhibition %)		
	B. cereus	*M. luteus*	*S. aureus*
E. elatior	10(59)++	13(57)++	11(65)++
E. fulgens	11(65)++	12(52)++	11(65)++
E. littoralis	8(47)+	9(39)+	9(53)++
E. maingayi	9(53)++	14(61)++	11(65)++
E. rubrostriata	9(53)++	11(48)+	10(59)++
Streptomycin	17	23	17
Methanol	–	–	–

Mean diameter of the zone of inhibition is in millimetres. Figures in parentheses are inhibition percentages compared to streptomycin. Antibacterial activity is categorized as strong +++ for inhibition ⩾ 70%, moderate ++ for inhibition 50 < 70%, or weak + for inhibition < 50%. Abbreviations: *B.*, *Bacillus*; *M.*, *Micrococcus*; *S.*, *Staphylococcus*.

4. Conclusion

Results showed that methanolic extracts from fresh leaves of *Etlingera* species had high values of TPC, AEAC, and FRAP. Species with the highest leaf TPC, AEAC, and FRAP possessed the lowest leaf FIC ability and vice versa. The FIC ability of leaves of *E. maingayi* and *E. fulgens* was superior to that of young leaves of *C. sinensis*. Leaves of *Etlingera* species exhibited high BCB activity, matching that of rhizomes of *C. longa* and superior to that of young tea leaves. Ranking of TPC and AOA of different plant parts of *E. elatior* was in the order: leaves > inflorescences > rhizomes. Leaves of highland populations of *Etlingera* species had higher values of TPC and AEAC than had those of lowland counterparts. Leaves of *Etlingera* species inhibited Gram-positive but not Gram-negative bacteria. With promising antioxidant and antibacterial properties, leaves of *Etlingera* species have great potential to be developed into natural preservatives and herbal products, applicable to the food and nutraceutical industries. Unlike the commercial use of rhizomes, the harvesting of leaves does not result in destructive sampling of plants.

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Antioxidant and tyrosinase inhibition properties of leaves and rhizomes of ginger species

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Abstract

Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves of 26 ginger species belonging to nine genera and three tribes were screened. For 14 species, TPC and AEAC of rhizomes were also assessed. Ferrous ion-chelating (FIC) abilities of leaves and rhizomes of eight species were compared. Leaves of five species of *Etlingera* were analysed for tyrosinase inhibition activity. Of the 26 species, leaves of *Etlingera* species had the highest TPC and AEAC. Eleven of the 14 species had significantly higher TPC and/or AEAC in leaves than in rhizomes. Values of leaves of *Etlingera elatior* and *Etlingera maingayi* were seven to eight times higher than those of rhizomes. In terms of FIC ability, six of the eight species clearly showed higher values in leaves than in rhizomes. The most outstanding was the FIC value of *Alpinia galanga* leaves which was more than 20 times higher than that of rhizomes. Of the five species of *Etlingera*, leaves of *E. elatior* displayed the strongest tyrosinase inhibition activity, followed by leaves of *Etlingera fulgens* and *E. maingayi.* Values of their inhibition activity were significantly higher than or comparable to the positive control. Besides promising tyrosinase inhibition ability, leaves of these three *Etlingera* species also have high antioxidant activity and antibacterial properties.
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Keywords: Zingiberaceae; Leaves; Rhizomes; Total phenolic content; Antioxidant activity; Tyrosinase inhibition activity

1. Introduction

Rhizomes of ginger plants (family Zingiberaceae) have been widely used as spices or condiments (Larsen, Ibrahim, Khaw, & Saw, 1999). Rhizomes are eaten raw or cooked as vegetables and used for flavouring food. Major commercially cultivated species are *Zingiber officinale*, *Curcuma longa*, and *Alpinia galanga*. As traditional medicine, rhizomes of ginger plants are consumed by women during ailment, illness and confinement. Rhizomes are also taken as carminatives for relieving flatulence.

Leaves of ginger plants have also been used for food flavouring and in traditional medicine. In Malaysia, leaves of *C. longa* are used to wrap fish before steaming or baking (Larsen et al., 1999). Leaves of *Kaempferia galanga* and *C. longa* are ingredients of curries. Some tribal natives in Malaysia flavour their wild meat and fish dishes with leaves of *Elettariopsis slahmong* (Lim, 2003). In Thailand, its leaves are eaten as salad. Despite their repulsive stinkbug odour, leaves of *E. slahmong* are considered a delicacy. Traditionally, leaves of *Elettariopsis latiflora* have been used to relieve flatulence, to improve appetite and as an antidote to poisons. In Okinawa, Japan, leaves of *Alpinia zerumbet* are sold as herbal tea, and are commonly used to flavour noodles and to wrap rice cakes. The hypotensive, diuretic, and anti-ulcerogenic properties of tea from *A. zerumbet* leaves have been reported (Mpalantinos, de Moura, Parente, & Kuster, 1998). Leaves of *Etlingera elatior*, mixed with other aromatic herbs, are used by *post-partum* women for bathing to remove body odour (Ibrahim & Setyowati, 1999). They are also used for cleaning wounds. Leaves of

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Kaempferia rotunda and *K. galanga* are eaten fresh or cooked as vegetables, and used as cosmetic powder and as food flavouring agents (Ibrahim, 1999). In Peninsular Malaysia, boiled leaves of *Hedychium* species are eaten for indigestion (Ibrahim, 2001). Leaves are sometimes eaten with betel nut to ease abdominal pain. In Thailand, boiled leaves of *Hedychium coronarium* are applied to relieve stiff and sore joints.

Past studies on the antioxidant properties of ginger species were confined to rhizomes (Habsah et al., 2000; Jitoe et al., 1992; Zaeoung, Plubrukarn, & Keawpradub, 2005). Rhizomes of gingers have been reported to have tyrosinase inhibition properties (Lee, Kim, Kim, Heo, & Kim, 1997). Skin-lightening cosmeceutical products were recently developed from rhizomes of gingers (Rozanida, Nurul Izza, Mohd Helme, & Zanariah, 2006). Although leaves of ginger species have been used for food flavouring and in traditional medicine, little research has been done on their antioxidant and tyrosinase inhibition properties.

In our present study, phenolic contents and radical-scavenging activities of leaves of 26 ginger species were screened. For 14 species, antioxidant properties of rhizomes were assessed. For eight species, metal ion-chelating abilities of leaves and rhizomes were also compared. Leaves of five species of *Etlingera* were analysed for tyrosinase inhibition activity. This study represents the most comprehensive study, where antioxidant properties of leaves and rhizomes of ginger species were systematically compared, and tyrosinase inhibition properties of leaves of *Etlingera* species were analysed.

2. Materials and methods

2.1. Plant materials

Locations where species were sampled for leaves and rhizomes are listed in Table 1. Voucher specimens of ginger plants studied were deposited in the herbaria of the Forest Research Institute Malaysia (FRIM) and Monash University Sunway Campus (MUSC), Malaysia.

2.2. Chemicals and instruments

Folin–Ciocalteu's phenol reagent (Fluka, 2N), gallic acid (Fluka, 98%), and anhydrous sodium carbonate (Fluka, 99%) were used for TPC analysis. 1,1-Diphenyl-2-picrylhydrazyl (Sigma, 90%) was used for DPPH radical-scavenging assay. Ferrozine (Acros Organics, 98%) and ferrous sulphate heptahydrate (HmbG chemicals) were used for FIC assay. L-DOPA (Sigma), mushroom tyrosinase (Sigma), and DMSO (Fisher Scientific) were used for assessing tyrosinase inhibition. Absorbance was measured with an Anthelie Advanced 5 Secoman UV–vis spectrophotometer for TPC and antioxidant activity, and with a BIOTEK PowerWave XS Microplate scanning spectrophotometer for tyrosinase inhibition activity.

Table 1
Locations of sampling leaves and rhizomes of ginger species

Species	Tribe	Location of sampling
Alpinia galanga	Alpineae	Bukit Maluri, Kepong, KL
A. malaccensis		FRIM, Kepong, Selangor
A. purpurata		SUC, Sunway, Selangor
A. zerumbet		Janda Baik, Pahang
A. zerumbet 'Variegata'		FRIM, Kepong, Selangor
Boesenbergia rotunda	Hedychieae	Sungai Buluh, Selangor
Curcuma aeruginosa	Hedychieae	Damansara Utama, Selangor
C. longa		FRIM, Kepong, Selangor
C. mangga		Damansara Utama, Selangor
C. zanthorrhiza		Damansara Utama, Selangor
Elettariopsis latiflora	Alpineae	FRIM, Kepong, Selangor
E. slahmong		FRIM, Kepong, Selangor
E. smithiae		Janda Baik, Pahang
Etlingera elatior	Alpineae	Janda Baik, Pahang
E. fulgens		Janda Baik, Pahang
E. littoralis		Genting Highlands, Pahang
E. maingayi		Janda Baik, Pahang
E. rubrostriata		Ulu Gombak, Selangor
Hedychium coronarium	Hedychieae	Lake Gardens, KL
Kaempferia galanga	Hedychieae	Damansara Utama, Selangor
K. pulchra		Sungai Buluh, Selangor
K. rotunda		Bukit Maluri, Kepong, KL
Scaphochlamys kunstleri	Hedychieae	FRIM, Kepong, Selangor
Zingiber officinale	Zingibereae	Bukit Maluri, Kepong, KL
Z. ottensii		Bukit Maluri, Kepong, KL
Z. spectabile		FRIM, Kepong, Selangor

Abbreviations: FRIM, Forest Research Institute Malaysia; SUC, Sunway University College; KL, Kuala Lumpur.

2.3. Extraction of plant samples

For antioxidant analysis, fresh leaves and rhizomes (1 g) were powdered with liquid nitrogen in a mortar and extracted using methanol (50 ml), with continuous swirling for 1 h at room temperature using an orbital shaker. Extracts were filtered under suction and stored at −20 °C for further use. For tyrosinase inhibition, fresh leaves (10 g) were extracted three times using methanol (100 ml). Methanol was removed by drying at 35 °C in a rotary evaporator prior to storage at −20 °C. Analysis of methanol extracts for antioxidant and tyrosinase inhibition properties was done in triplicate.

2.4. Total phenolic content

Total phenolic content (TPC) of extracts was determined using the Folin-Ciocalteu assay reported by Kähkönen et al. (1999). Samples (300 μl in triplicate) were introduced into test tubes, followed by 1.5 ml of Folin–Ciocalteu's reagent (10 times dilution) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min before absorbance at 765 nm was measured. TPC was expressed as gallic acid equivalents (GAE) in mg per

100 g fresh material. The calibration equation for gallic acid was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$).

2.5. DPPH radical-scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay reported by Miliauskas, Venskutonis, and van Beek (2004) was adopted with modifications. Different dilutions of the extract (1 ml; triplicate) were added to 2 ml of DPPH$^{\cdot}$ (5.9 mg/100 ml methanol). Absorbance was measured at 517 nm after 30 min. Radical-scavenging ability was calculated as IC_{50} and expressed as ascorbic acid equivalent antioxidant capacity (AEAC) in mg ascorbic acid/100 g (Leong & Shui, 2002) as follows:

$$\text{AEAC (mg AA/100 g)} = IC_{50(\text{ascorbate})}/IC_{50(\text{sample})} \times 10^5$$

The IC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/ml.

2.6. Ferrous ion-chelating ability

The ferrous ion-chelating (FIC) assay reported by Singh and Rajini (2004) was adopted. Solutions of 2 mM $FeSO_4$ and 5 mM ferrozine were diluted 20 times. $FeSO_4$ (1 ml) was mixed with different dilutions of extract (1 ml), followed by ferrozine (1 ml). Absorbance was measured at 562 nm after 10 min. The ability of extracts to chelate ferrous ions was calculated as follows:

$$\text{Chelating effect\%} = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100$$

2.7. Tyrosinase inhibition

Tyrosinase inhibition was determined using the modified dopachrome method with L-DOPA as substrate (Masuda, Yamashita, Takeda, & Yonemori, 2005). Assays were conducted in a 96-well microtitre plate and a plate reader was used to measure absorbance at 475 nm with 700 nm as reference. Samples were dissolved in 50% DMSO. Each well contained 40 μl of sample with 80 μl of phosphate buffer (0.1 M, pH 6.8), 40 μl of tyrosinase (31 units/ml) and 40 μl of L-DOPA (2.5 mM). Each sample was accompanied by a blank that had all the components except L-DOPA. Results were compared with a control consisting of 50% DMSO in place of sample. The percentage tyrosinase inhibition was calculated as follows:

$$(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}} \times 100\%$$

3. Results and discussion

3.1. Description of plant species

Leaves of the 26 ginger species screened for antioxidant properties belong to nine genera and three tribes (Table 1). The tribes and genera are Alpineae (*Alpinia*, *Elettariopsis* and *Etlingera*), Hedychieae (*Boesenbergia*, *Curcuma*, *Hedychium*, *Kaempferia*, and *Scaphochlamys*), and Zingibereae (*Zingiber*). Alpineae species are medium- to large-sized forest plants of which *Etlingera* is the largest (Larsen et al., 1999). Zingibereae species are medium-sized plants and Hedychieae species are small- to medium-sized herbs.

3.2. Antioxidant properties of leaves

TPC and radical-scavenging activities of methanol extracts of leaves were assessed using the Folin–Ciocalteu and DPPH radical-scavenging assays, and expressed in mg GAE/100 g and mg AA/100 g, respectively.

Of the 26 ginger species screened, leaves of *Etlingera* species had the highest TPC and AEAC. Values ranged from 2390 mg GAE/100 g and 2280 mg AA/100 g in *E. elatior* to 1110 mg GAE/100 g and 963 mg AA/100 g in *Etlingera maingayi*, respectively (Table 2). Ranking was in the order: *E. elatior* ≈ *Etlingera rubrostriata* ≈ *Etlingera littoralis* > *Etlingera fulgens* ≈ *E. maingayi* in terms of TPC and *E. elatior* ≈ *E. rubrostriata* > *E. littoralis* > *E. fulgens* ≈ *E. maingayi* in terms of AEAC. Among the *Alpinia* species, leaves of *A. zerumbet* also showed high TPC and AEAC, with

Table 2
Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves of 26 ginger species (fresh weight)

Species	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
Alpinia zerumbet	1990 ± 62a	2180 ± 42a
A. purpurata	1190 ± 174b	1100 ± 113b
A. zerumbet 'Variegata'	1150 ± 41b	1250 ± 184b
A. malaccensis	744 ± 61c	800 ± 62c
A. galanga	392 ± 50d	90 ± 36d
Boesenbergia rotunda	260 ± 8	157 ± 2
Curcuma zanthorrhiza	503 ± 57a	287 ± 39a
C. aeruginosa	282 ± 78b	140 ± 47b
C. mangga	275 ± 36b	118 ± 11b
C. longa	230 ± 19b	113 ± 18b
Elettariopsis latiflora	423 ± 26a	395 ± 27a
E. slahmong	346 ± 45b	269 ± 67b
E. smithiae	303 ± 18b	147 ± 21c
Etlingera elatior	2390 ± 329a	2280 ± 778a
E. rubrostriata	2250 ± 113a	2290 ± 118a
E. littoralis	2150 ± 94a	1990 ± 87b
E. fulgens	1280 ± 144b	845 ± 158c
E. maingayi	1110 ± 93b	963 ± 169c
Hedychium coronarium	820 ± 55	814 ± 116
Kaempferia galanga	146 ± 9a	77 ± 7a
K. rotunda	140 ± 48ab	46 ± 15b
K. pulchra	112 ± 9b	30 ± 3b
Scaphochlamys kunstleri	203 ± 21	171 ± 33
Zingiber officinale	291 ± 18a	96 ± 7a
Z. spectabile	242 ± 7b	121 ± 24a
Z. ottensii	162 ± 13c	52 ± 6b

Values of TPC and AEAC are means ± SD ($n = 3$). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA compares values of leaves of species in each genus and does not apply between genera.

values of 1990 mg GAE/100 g and 2180 mg AA/100 g, respectively. Leaves of the commercially cultivated *A. galanga* had the lowest values of 392 mg GAE/100 g and 90 mg AA/100 g, respectively. Although most *Etlingera* species and some *Alpinia* species displayed high phenolic content and radical-scavenging activity, species of *Elettariopsis*, which also belong to the tribe Alpineae, had much lower values, ranging from 303 to 423 mg GAE/100 g and 147–395 mg AA/100 g, respectively.

TPC and AEAC values of leaves of genera belonging to the tribes Hedychieae and Zingibereae were comparatively lower (Table 2). They include species such as *Boesenbergia rotunda*, *Curcuma aeruginosa*, *C. longa*, *Curcuma mangga*, *Curcuma zanthorrhiza*, *H. coronarium*, *K. galanga*, *K. rotunda*, and *Zingiber ottensii* which are used in food flavouring and traditional medicine. Among these species, *H. coronarium* had the highest TPC and AEAC with values of 820 mg GAE/100 g and 814 mg AA/100 g, respectively. Species of *Kaempferia* had very low phenolic content and radical-scavenging activity with values ranging from 112 to 146 mg GAE/100 g and 30–77 mg AA/100 g, respectively. Leaves of *Kaempferia pulchra* exhibited the lowest values.

Foliage of tropical forest plants produces more antioxidants when exposed to elevated light conditions (Frankel & Berenbaum, 1999). Plants growing along the seashore, which receive much sunlight, have efficient antioxidant properties to prevent oxidative damage (Masuda et al., 1999). These observations may also apply to species of *Etlingera*, which have the highest leaf TPC and AEAC. *Etlingera* species are the largest of the ginger plants and can grow up to 6 m in height (Khaw, 2001). They grow in gaps of disturbed forest and are continually exposed to direct sunlight. *Alpinia* species with high TPC and AEAC are medium-sized to large forest plants (Larsen et al., 1999). The other genera are small- to medium-sized herbs. Among the various tribes and genera of gingers, there appears to be a positive correlation between the phenolic content and radical-scavenging activity of leaves with plant size and site conditions. Larger ginger plants growing in exposed forest sites have greater antioxidant properties than have smaller plants growing in shaded sites.

3.3. Antioxidant properties of leaves and rhizomes

TPC and antioxidant activity of methanol extracts of leaves and rhizomes of 14 species from the same plant/location were also assessed for comparison purposes. Results in Table 3 show that leaves of *E. elatior* and *E. maingayi* which had the highest TPC and AEAC were seven to eight times higher than those of rhizomes. Other species with leaves having significantly higher TPC and AEAC than rhizomes were *C. aeruginosa*, *C. mangga*, *C. zanthorrhiza*, *K. galanga*, and *Scaphochlamys kunstleri.* Species with higher TPC or AEAC were *A. galanga*, *B. rotunda*, *E. slahmong*, and *Z. officinale*. This would mean that about 80% of the species had significantly higher TPC and/or AEAC in leaves than in rhizomes. Exceptions were AEAC of *A. galanga*, and TPC and AEAC of *C. longa* where rhizomes showed significantly higher values than did leaves. TPC and AEAC of leaves and rhizomes of *Alpinia malaccensis* and *Zingiber spectabile* were comparable. Values were generally more variable between rhizomes than between leaves of a species, as evident in *A. malaccensis*, *C. longa*, and *Z. spectabile*.

Table 3
Total phenolic content (TPC) and ascorbic acid equivalent antioxidant capacity (AEAC) of leaves (L) and rhizomes (R) of 14 ginger species (fresh weight)

Species	Part	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)
Alpinia galanga	L	392 ± 50a	90 ± 36a
	R	214 ± 20b	168 ± 13b
A. malaccensis	L	744 ± 61a	800 ± 62a
	R	564 ± 209a	745 ± 342a
Boesenbergia rotunda	L	260 ± 8a	157 ± 2a
	R	197 ± 50a	89 ± 7b
Curcuma aeruginosa	L	282 ± 78a	140 ± 47a
	R	145 ± 31b	55 ± 11b
C. longa	L	230 ± 19a	113 ± 18a
	R	534 ± 205b	390 ± 127b
C. mangga	L	275 ± 36a	118 ± 11a
	R	112 ± 21b	33 ± 1b
C. zanthorrhiza	L	503 ± 57a	287 ± 39a
	R	250 ± 52b	134 ± 21b
Elettariopsis slahmong	L	346 ± 45a	269 ± 67a
	R	219 ± 57b	197 ± 76a
Etlingera elatior	L	2390 ± 329a	2280 ± 778a
	R	326 ± 76b	295 ± 96b
E. maingayi	L	1110 ± 93a	963 ± 169a
	R	160 ± 52b	122 ± 53b
Kaempferia galanga	L	146 ± 9a	77 ± 7a
	R	57 ± 1b	17 ± 1b
Scaphochlamys kunstleri	L	203 ± 21a	171 ± 33a
	R	73 ± 3b	14 ± 2b
Zingiber officinale	L	291 ± 18a	96 ± 7a
	R	157 ± 18b	84 ± 3a
Z. spectabile	L	242 ± 7a	121 ± 24a
	R	157 ± 100a	124 ± 109a

Values of TPC and AEAC are means ± SD ($n = 3$). For each column, values followed by the same letter (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA compares values of leaves and rhizomes of each species and does not apply between species.

Analysis of metal ion-chelating properties showed that six of the eight species studied clearly displayed higher FIC ability in leaves than in rhizomes. The species were *C. longa*, *K. galanga*, *Alpinia galanga*, *E. elatior*, *Zingiber spectabile*, and *E. maingayi.* (Figs. 1a and b, and 2a). FIC values of leaves and rhizomes of *C. zanthorrhiza* were comparable (Fig. 2b). At lower extract concentration, leaves of *S. kunstleri* showed lower values but, at higher concentration, values were comparable. Of particular interest is *C.*

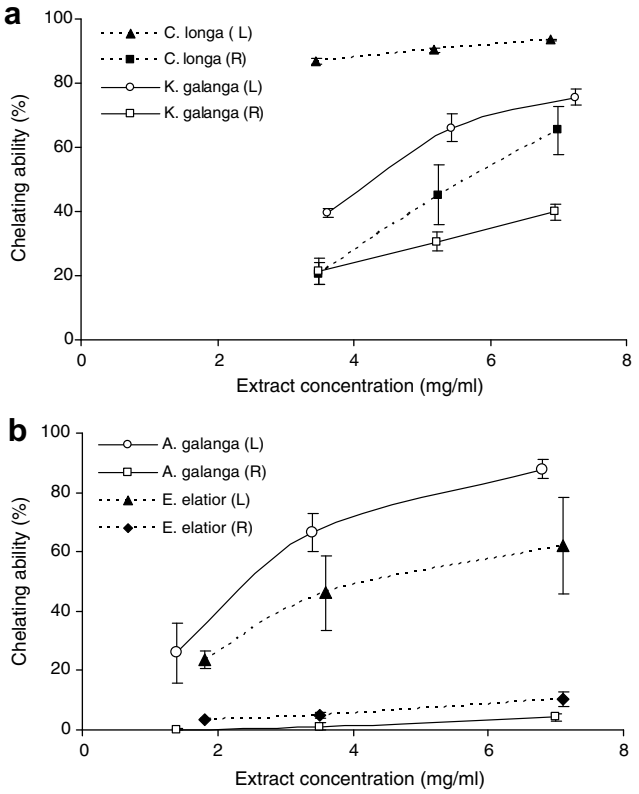

Fig. 1. Ferrous ion-chelating (FIC) ability of leaves (L) and rhizomes (R) of *Curcuma longa*, *Kaempferia galanga*, *Alpinia galanga,* and *Etlingera elatior* (fresh weight).

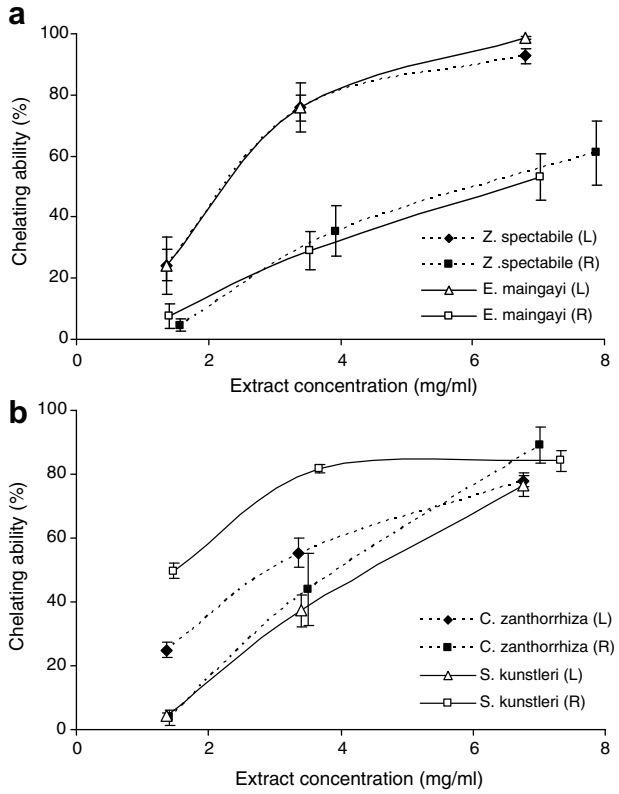

Fig. 2. Ferrous ion-chelating (FIC) ability of leaves (L) and rhizomes (R) of *Zingiber spectabile*, *Etlingera maingayi*, *Curcuma zanthorrhiza*, and *Scaphochlamys kunstleri* (fresh weight).

longa where TPC and AEAC were significantly higher in rhizomes (Table 3), but the FIC ability was higher in leaves (Fig. 1a). In the case of *Z. spectabile*, although TPC and AEAC were comparable (Table 3), FIC value of leaves was higher than that of rhizomes (Fig. 2a). The most outstanding was the FIC value of *A. galanga* leaves which was more than 20 times higher than that of rhizomes (Fig. 1b).

There are few studies comparing the antioxidant properties of leaves and rhizomes of ginger species. Essential oils from leaves of *Aframomum giganteum* had higher antioxidant activity than had those from rhizomes (Agnaniet, Menut, & Bessière, 2004). Leaves of *A. zerumbet* showed higher inhibition of β-carotene oxidation and radical-scavenging activity than did rhizomes (Elzaawely, Xuan, & Tawata, 2007). Contrary to our results, higher phenolic content and antioxidant activity have been reported in rhizomes than in leaves of *Z. officinale* (Katsube et al., 2004). These studies involved one or two ginger species and it is not known whether their comparisons were based on plant samples from the same or different locations. Our present study is probably the first where the phenolic content, radical-scavenging activities and metal ion-chelating abilities of leaves and rhizomes of ginger species from the same plant/location were systematically compared.

Antioxidants are secondary metabolites produced by plants to protect against oxidative damage by free radicals (Larson, 1988). In the family Zingiberaceae, it is generally believed that antioxidants produced by the plant are transported to the rhizomes where they are accumulated. This implies that rhizomes would have higher antioxidant activity than other plant parts. However, results of this study showed that this might not be true as the majority of the species studied had significantly higher phenolic content and antioxidant activity in leaves than in rhizomes. Similar observations have been made by Herrmann (1988), who reported much greater concentrations of flavones and flavonols in leaves of vegetables which are exposed to sunlight. Only trace amounts were found in unexposed parts below the soil surface which include roots and rhizomes. This could explain why leaves have significantly higher phenolic contents and antioxidant activities than have rhizomes in ginger plants.

3.4. Tyrosinase inhibition activity of leaves of Etlingera

With outstanding leaf TPC and AEAC, methanol extracts of leaves of five *Etlingera* species were analysed for tyrosinase inhibition activity using the modified dopachrome method with L-DOPA as the substrate. Leaves of *Hibiscus tiliaceus* were chosen as positive control as they displayed the highest tyrosinase inhibition activity among 39 tropical plant species screened by Masuda et al. (2005).

Table 4
Tyrosinase inhibition activity of leaves of five *Etlingera* species (fresh weight)

Species	Tyrosinase inhibition % (0.5 mg/ml of extract)
Etlingera elatior	55.2 ± 3.1a
E. fulgens	49.3 ± 6.5ab
E. maingayi	42.6 ± 4.2b
E. rubrostriata	29.5 ± 4.0c
E. littoralis	22.0 ± 5.2c
Hibiscus tiliaceus	43.9 ± 4.6b

Results are means ± SD ($n = 3$). Values followed by the same letter (a–c) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Leaves of *Hibiscus tiliaceus* were used as positive control.

Tyrosinase inhibition activity was strongest in leaves of *E. elatior* (55.2%), which was significantly higher than the positive control (43.9%) (Table 4). Inhibition activities of leaves of *E. fulgens* (49.3%) and *E. maingayi* (42.6%) were comparable. Activities of leaves of *E. rubrostriata* (29.5%) and *E. littoralis* (22.0%) were significantly lower. This would mean that three out of five *Etlingera* species studied had activity values that were significantly higher or comparable to the positive control.

Masuda et al. (2005) observed that seashore plant species, which are exposed to full sunlight, possess strong antioxidant activity and high tyrosinase inhibition ability. Findings from this study agree with this observation. Compared with species of other genera, *Etlingera* species had outstanding TPC and AEAC with *E. elatior* having the highest values. In our earlier study (Chan, Lim, & Omar, 2007), leaves of *E. maingayi* had the highest FIC ability and lipid peroxidation inhibition activity, and leaves of *E. fulgens* had high FIC ability. Leaves of *E. elatior*, *E. maingayi*, and *E. fulgens* also showed inhibition of all Gram-positive bacteria of *Bacillus cereus*, *Micrococcus luteus*, and *Staphylococcus aureus* tested. In our present study, leaves of these three *Etlingera* species displayed high tyrosinase inhibition activity. This would mean that, besides promising tyrosinase inhibition ability, they also have high antioxidant activity and antibacterial properties.

4. Conclusion

Of the 26 ginger species screened, leaves of *Etlingera* species had the highest TPC and AEAC. Eleven of the 14 species showed significantly higher phenolic content and/or antioxidant activities in leaves than in rhizomes. Values of leaves of *E. elatior*, and *E. maingayi* were seven to eight times higher than those of rhizomes. Six of the eight species clearly displayed higher FIC ability in leaves than in rhizomes. The FIC value of *A. galanga* leaves was more than 20 times higher than that of rhizomes. Three species of *Etlingera* displayed tyrosinase inhibition activity that was significantly higher or comparable to the positive control. With high tyrosinase inhibition, antioxidant activity, and antibacterial properties, leaves of these *Etlingera* species can be developed into skin-lightening products and natural preservatives to inhibit food spoilage.

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Effects of different drying methods on the antioxidant properties of leaves and tea of ginger species

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ABSTRACT

Effects of five different drying methods on the antioxidant properties (AOP) of leaves of *Alpinia zerumbet*, *Etlingera elatior*, *Curcuma longa*, and *Kaempferia galanga* were assessed. All methods of thermal drying (microwave-, oven-, and sun-drying) resulted in drastic declines in total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and ferric-reducing power (FRP), with minimal effects on ferrous ion-chelating ability and lipid peroxidation inhibition activity. Of the non-thermal drying methods, significant losses were observed in air-dried leaves. Freeze-drying resulted in significant gains in TPC, AEAC, and FRP for *A. zerumbet* and *E. elatior* leaves. After one week storage, AOP of freeze-dried *E. elatior* leaves remained significantly higher than those of fresh control leaves. Freeze-dried tea of *A. zerumbet* was superior to the commercial tea for all AOP studied.

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1. Introduction

Past studies on the antioxidant properties (AOP) of ginger species (Zingiberaceae) were confined to rhizomes. Although their leaves have been used for food flavouring and in traditional medicine (Larsen, Ibrahim, Khaw, & Saw, 1999), very little research has been done on their total phenolic content (TPC) and antioxidant activity (AOA).

Alpinia zerumbet, also known as Shell Ginger, is an ornamental plant with attractive fragrant flowers. In Japan, leaves of *A. zerumbet* (Getto) are sold as herbal tea, and are used to flavour noodles and wrap rice cakes. Its tea has hypotensive, diuretic, and anti-ulcerogenic properties (Mpalantinos, de Moura, Parente, & Kuster, 1998). Decoction of leaves has been used during bathing to alleviate fevers. From the leaves of *A. zerumbet*, flavonoids, kava pyrones, and phenolic acids have been isolated (Elzaawely, Xuan, & Tawata, 2007; Mpalantinos et al., 1998). Leaves of *A. zerumbet* had the highest TPC and AOA among five species of *Alpinia* studied (Chan et al., 2008). Leaves had higher inhibition of β-carotene oxidation and radical–scavenging activity than rhizomes (Elzaawely et al., 2007).

Etlingera elatior or Torch Ginger is widely cultivated throughout the tropics. Young inflorescences are commonly used as the ingredients of spicy dishes (Larsen et al., 1999). Post-partum women use *E. elatior* leaves together with other aromatic herbs for bathing to remove body odour. They are also used for cleaning wounds. Flavonoids in leaves of *E. elatior* have been identified as kaempferol 3-glucuronide, quercetin 3-glucuronide, quercetin 3-glucoside, and quercetin 3-rhamnoside (Williams & Harborne, 1977). Screening of leaves of 26 ginger species belonging to nine genera showed that species of *Etlingera* had the highest phenolic content and radical–scavenging activity (Chan et al., 2008). Leaves of *E. elatior* had the most outstanding AOP among five *Etlingera* species studied (Chan, Lim, & Omar, 2007).

Curcuma longa is a widely cultivated ginger plant with pungent rhizomes that produce turmeric, a popular spice for curries, food flavouring, and colouring. Curcumin, the active component of turmeric, is known to have a wide array of bioactivity including antioxidant, anti-inflammatory, anti-cancer, and cardio-protective properties. The aromatic leaves of *C. longa* are used for flavouring steamed and baked fish (Larsen et al., 1999). Phenolic content and radical–scavenging activity were significantly higher in rhizomes than in leaves of *C. longa*, but metal ion-chelating ability was higher in leaves (Chan et al., 2008).

Kaempferia galanga is a small, cultivated ginger plant with broadly ovate and pale green leaves. Its leaves and rhizomes are used in traditional medicine, perfumery, and food flavouring. Rhizomes of *K. galanga* are used as expectorants and carminatives. They are also used as ingredient for preparing 'Jamu', a local health tonic consumed by the Malays. Its mild spicy leaves are ingredients for savoury dishes. Its leaves and rhizomes are eaten fresh or cooked as a vegetable, and used in cosmetic powder and as a food flavouring agent. Phenolic content, radical–scavenging activity, and metal ion-chelating ability were significantly higher in leaves than in rhizomes of *K. galanga* (Chan et al., 2008).

In our present study, TPC and AOA of leaves of *A. zerumbet*, *E. elatior*, *C. longa*, and *K. galanga* as affected by three thermal drying methods (microwave-, oven-, and sun-drying) and two

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non-thermal drying methods (air- and freeze-drying) were assessed using five different antioxidant assays. These ginger species were selected for study because their leaves have been used for food flavouring and as traditional medicine. For leaves of *E. elatior*, the effects of microwave-drying for different durations and the effects of storage of freeze-dried leaves were studied. For *A. zerumbet*, AOP of tea from freeze-dried leaves were compared to those of the commercial Getto tea. This study represents the first systematic analysis of the effects of different drying methods on the AOP of ginger leaves. Aimed at developing protocols for producing herbal products with AOP comparable or superior to those of commercial ones, this study is probably the first to report that freeze-drying enhances the AOP of ginger leaves and tea.

2. Materials and methods

2.1. Chemicals and instruments

Folin–Ciocalteu's phenol reagent (Fluka, 2 N), gallic acid (Fluka, 98%), and anhydrous sodium carbonate (Fluka, 99%) were used for TPC analysis; 2,2-diphenyl-1-picrylhydrazyl (Sigma, 90%) for DPPH assay; ferric chloride hexa-hydrate (Fisher Scientific, 100%), potassium ferricyanide (Unilab, 99%), trichloroacetic acid (HmbG Chemicals, 99.8%), potassium dihydrogen orthophosphate (Fisher Scientific, 99.5%), and dipotassium hydrogen phosphate (Merck, 99%) for FRP assay; ferrozine (Acros Organics, 98%) and ferrous sulphate hepta-hydrate (HmbG Chemicals) for FIC assay; and β-carotene (Sigma, Type 1: synthetic), chloroform (Fisher Scientific, 100%), linoleic acid (Fluka), and Tween 40 (Fluka) for β-carotene bleaching (BCB) assay. Absorbance was measured with an Anthelie Advanced 5 Secoman UV–vis spectrophotometer. HPLC analysis was conducted using Agilent Technologies 1200 Series with Thermo Scientific BDS Hypersil Phenyl Column (4.6 × 100 mm).

2.2. Plant materials

Leaves of *A. zerumbet* and *E. elatior* were collected from Janda Baik in Pahang. The latter were also collected from Selayang and Kepong in Selangor. Leaves of *C. longa* were purchased from the supermarket and those of *K. galanga* were obtained from plants raised from rhizomes. Voucher specimens of these plants were deposited at the herbarium of Forest Research Institute Malaysia (FRIM). The commercial tea of *A. zerumbet* (Getto) was purchased from Okinawa, Japan.

2.3. Drying processes

Leaves were subject to five different drying methods, i.e., microwave-, oven-, sun-, freeze-, and air-drying. For each drying method, 1 g of fresh leaves was used. In microwave-drying, leaves were dried in a microwave oven (Sharp R-248E; 800 W) for 4 min. Oven-drying involved drying for 5 h in an oven (Memmert ULE 500) at 50 °C. Leaves were sun-dried in the greenhouse for three days with about 27 h of daylight. Mid-day temperature in the greenhouse can reach 35 °C. Leaves were air-dried for three days in the laboratory at ambient temperature of 25–30 °C and relative humidity of 33%. For each of the above drying methods, leaf pieces were spread out evenly on a Petri dish. In freeze-drying, leaf samples were lyophilised overnight in a vacuum flask at 0.125 mbar and −50 °C in a freeze-dryer (Christ Alpha 1–4).

2.4. Sample extraction

Extraction efficiencies of different solvents, namely, dichloromethane, ethyl acetate, methanol, and aqueous methanol (50%) were tested on *C. longa* and *E. elatior*. Leaves of the two species (1 g) were powdered with liquid nitrogen in a mortar and extracted with 50 ml of solvent, with continuous swirling for one hour at room temperature using an orbital shaker. Extracts were filtered under suction and stored at −20 °C for further use. Analysis of extracts was done in triplicate.

For subsequent analyses, fresh and dried leaves of *A. zerumbet*, *E. elatior*, *C. longa*, and *K. galanga* were extracted with methanol. For the analysis of AOP of tea extracts, 1 g of tea in powder form was extracted in 50 ml boiling water for 1 h with continuous swirling. The infusions were allowed to cool throughout extraction period. Extracts were filtered and stored at 4 °C for further analysis.

2.5. Total phenolic content

Total phenolic content (TPC) of extracts was determined using the Folin–Ciocalteu assay. Samples (300 μl) were introduced into test tubes followed by 1.5 ml of Folin–Ciocalteu's reagent (10 times dilution) and 1.2 ml of sodium carbonate (7.5% w/v). The tubes were allowed to stand for 30 min before absorbance at 765 nm was measured. TPC was expressed as gallic acid equivalent (GAE) in mg/100 g material. The calibration equation for gallic acid was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$) where y is the absorbance and x is the concentration of gallic acid in mg/l.

2.6. Determination of antioxidant activity

The methods described below were based on procedures previously described (Chan et al., 2008).

2.6.1. DPPH radical–scavenging activity

Different dilutions of extracts (1 ml) were added to 2 ml of 2,2-diphenyl-1-picrylhydrazyl (5.9 mg/100 ml methanol). Absorbance was measured at 517 nm after 30 min. Radical–scavenging ability was calculated as IC_{50} and expressed as expressed as AEAC in mg ascorbic acid/100 g as follows:

$$\text{AEAC (mg AA/100 g)} = \frac{IC_{50(\text{ascorbate})}}{IC_{50(\text{sample})}} \times 10^5$$

the IC_{50} of ascorbic acid used for calculation of AEAC was 0.00387 mg/ml.

2.6.2. Ferric-reducing power

Different dilutions of extracts (1 ml) were added to 2.5 ml phosphate buffer (0.2 M; pH 6.6) and 2.5 ml of potassium ferricyanide (1% w/v). The mixture was incubated at 50 °C for 20 min. Trichloroacetic acid solution (2.5 ml; 10% w/v) was added to stop the reaction. The mixture was then separated into aliquots of 2.5 ml and diluted with 2.5 ml of water. To each diluted aliquot, 500 ml of ferric chloride solution (0.1% w/v) were added. After 30 min, absorbance was measured at 700 nm. FRP was expressed as mg GAE/g. The calibration equation for gallic acid was $y = 16.767x$ ($R^2 = 0.9974$).

2.6.3. Ferrous ion-chelating ability

Solutions of 2 mM $FeSO_4$ and 5 mM ferrozine were diluted 20 times. $FeSO_4$ (1 ml) was mixed with different dilutions of extracts (1 ml), followed by ferrozine (1 ml). Absorbance was measured at 562 nm after 10 min. The ability of extracts to chelate ferrous ions was calculated as follows:

$$\text{Chelating effect\%} = \left(1 - \frac{A_{\text{sample}}}{A_{\text{control}}}\right) \times 100$$

where A_{sample} and A_{control} are absorbance of the sample and negative control, respectively.

2.6.4. Lipid peroxidation inhibition activity

Lipid peroxidation inhibition (LPI) activity was determined using the β-carotene bleaching (BCB) assay. β-Carotene/linoleic acid emulsion was prepared by adding 3 ml of β-carotene (5 mg in 50 ml chloroform) to 40 mg of linoleic acid and 400 mg of Tween 40. Chloroform was evaporated under reduced pressure and oxygenated ultra-pure water (100 ml) was added and mixed well. Initial absorbance of the emulsion was measured at 470 nm. Aliquots of the emulsion (3 ml) were mixed with 10 μl, 50 μl, and 100 μl of extracts and incubated in a water bath at 50 °C for 1 h. Bleaching rate of β-carotene was measured at 470 nm and 700 nm. Measurement at 700 nm is needed to correct for the presence of haze. LPI activity expressed as AOA (%) was calculated as follows:

$$\text{Bleaching rate (BR) of } \beta\text{-carotene} = \frac{\ln\left(A_{\text{initial}}/A_{\text{sample}}\right)}{60}$$

$$\text{AOA } (\%) = \left(1 - \frac{\text{BR}_{\text{sample}}}{\text{BR}_{\text{control}}}\right) \times 100$$

where A_{initial} and A_{sample} are absorbance of the emulsion before and 1 h after incubation, and $\text{BR}_{\text{sample}}$ and $\text{BR}_{\text{control}}$ are bleaching rates of the sample and negative control, respectively.

2.7. High performance liquid chromatography

Extracts of fresh and freeze-dried leaves of *E. elatior* were dissolved in 50% methanol and analysed using reverse-phase HPLC with a phenyl column. A 15-min linear gradient from 5% to 100% MeOH, was used to elute samples at 1 ml/min. Mobile phases were acidified with 0.1% trifluoroacetic acid for better resolution. Elution was monitored at 254 nm. Chromatograms of fresh and freeze-dried leaves were overlaid to display differences in constituents and their overall peak areas calculated.

3. Results and discussion

3.1. Description of plant species

Leaves of *A. zerumbet* are lanceolate, dark green, and emit an aromatic fragrance when crushed. Leaves of *E. elatior* are lanceolate, green, sometimes flushed pink when young, and have a pleasant sour scent. Leaves of *C. longa* are oblong or ovate, light green, and produce a pungent spicy aroma. The broadly ovate light green leaves of *K. galanga* have a mild spicy fragrance. *A. zerumbet* and *E. elatior* belong to the tribe Alpineae, and *C. longa* and *K. galanga* belong to the tribe Hedychieae. Alpineae species are medium- to large-sized forest plants of which *Etlingera* is the largest (Larsen et al., 1999). Hedychieae species are small- to medium-sized herbs.

3.2. Antioxidant properties of fresh leaves

3.2.1. Extraction with different solvents

TPC and AOA of leaf extracts of *C. longa* and *E. elatior* were studied using the Folin–Ciocalteu, DPPH radical–scavenging, and FRP assays, and expressed as mg GAE/100 g, mg AA/100 g, and mg GAE/g, respectively. Of the different solvents tested, methanol and 50% methanol were comparable and yielded the highest TPC and AOA. Ranking of extraction was of the following order: 50% methanol ≈ methanol > ethyl acetate > dichloromethane. The amount of antioxidative compounds extracted was reduced with solvents of decreasing polarity.

3.2.2. Extraction with methanol

Extraction efficiencies of methanol as measured by TPC values of first extraction of *A. zerumbet*, *E. elatior*, *C. longa*, and *K. galanga* leaves were comparable, being 78 ± 3%, 84 ± 1%, 83 ± 1%, and 84 ± 2%, respectively.

Of the four species, leaves of *E. elatior* had the highest TPC, AEAC, and FRP with values of 2420 mg GAE/100 g, 2960 mg AA/100 g, and 14 mg GAE/g, respectively. Leaves of *A. zerumbet* ranked second with values of 1990 mg GAE/100 g, 2180 mg AA/100 g, and 11 mg GAE/g, respectively. AOP of leaves of *E. elatior* and *A. zerumbet* had significantly higher values than those of *C. longa* and *K. galanga*. Results showed that larger plants of the tribe Alpineae growing in exposed forest sites have stronger AOP than those of smaller plants of the tribe Hedychieae growing in shaded sites (Chan et al., 2008).

3.3. Effects of thermal drying methods

Heat-treated leaves of *A. zerumbet* resulted in losses in TPC, AEAC, and FRP compared to those of fresh leaves. Losses were 50%, 58%, and 55% for microwave-drying; 43%, 49%, and 56% for oven-drying; and 47%, 57%, and 46% for sun-drying; respectively (Table 1). Losses in AOP were insignificant between the three drying methods. FIC values of heat-treated leaves were comparable or marginally lower (at higher concentration) than that of fresh leaves, with values, for example at 7 mg/ml concentration, falling in the range of 44–56% (cf, 57% for fresh sample).

AOP of heat-treated leaves of *E. elatior* were also adversely affected by microwave-, oven-, and sun-drying. Losses were 40%, 42%, and 70% for TPC; 59%, 58%, and 75% for AEAC; and 44%, 43%, and 76% for FRP; respectively (Table 1). Losses in AOP were highest

Table 1
Percentage loss in total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and ferric-reducing power (FRP) of leaves of *A. zerumbet*, *E. elatior*, *C. longa*, and *K. galanga* following thermal drying (fresh weight)[a]

Species	Drying method	Water loss (%)	Percentage loss compared to fresh leaves		
			TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
A. zerumbet	Microwave-drying	60 ± 1	−50 ± 5a	−58 ± 2a	−55 ± 5a
	Oven-drying	60 ± 2	−43 ± 10a	−49 ± 6a	−56 ± 8a
	Sun-drying	59 ± 2	−47 ± 9a	−57 ± 9a	−46 ± 10a
E. elatior	Microwave-drying	75 ± 2	−40 ± 8a	−59 ± 9a	−44 ± 10a
	Oven-drying	68 ± 5	−42 ± 13a	−58 ± 16a	−43 ± 15a
	Sun-drying	76 ± 5	−70 ± 13b	−75 ± 13a	−76 ± 11b
C. longa	Microwave-drying	83 ± 1	−58 ± 14a	−59 ± 9a	−71 ± 5a
	Oven-drying	83 ± 2	−77 ± 2b	−77 ± 1b	−81 ± 1b
	Sun-drying	81 ± 1	−81 ± 1c	−84 ± 2c	−86 ± 2c
K. galanga	Microwave-drying	93 ± 2	−36 ± 10a	−27 ± 13a	−44 ± 3a
	Oven-drying	93 ± 2	−66 ± 1b	−66 ± 2b	−81 ± 2b
	Sun-drying	84 ± 1	−91 ± 1c	−86 ± 1c	−88 ± 2c

[a] Values of TPC, AEAC, and FRP of leaves are means ± SD ($n = 3$). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA applies between thermal drying methods of a species.

for sun-drying. FIC values of fresh leaves were slightly higher than those of microwave- and oven-dried leaves. Heat-treated leaves yielded slightly higher LPI activity. TPC and AOA of leaves of *E. elatior* microwave-dried for 2 min, 4 min, 6 min, and 8 min resulted in significant losses but their declines were comparable between different drying durations.

For leaves of *C. longa* and *K. galanga*, thermal drying also resulted in declines in TPC, AEAC, and FRP. Losses ranged from 58% to 81%, 59% to 84%, and 71% to 86% for *C. longa*; and from 36% to 91%, 27% to 86%, and 44% to 88% for *K. galanga*; respectively (Table 1). Losses in AOP were the least for microwave-drying and the greatest for sun-drying.

Processing methods are known to have variable effects on TPC and AOA of plant samples. Effects include little or no change, significant losses, or enhancement in AOP (Nicoli, Anese, & Parpinel, 1999). Food processing can improve the properties of naturally occurring antioxidants or induce the formation of new compounds with AOP, so that the overall AOA increases or remains unchanged (Tomaino et al., 2005).

Increase in AOA following thermal treatment has been reported in tomato (Dewanto, Wu, Adom, & Liu, 2002a), sweet corn (Dewanto, Wu, & Liu, 2002b), Shiitake mushroom (Choi, Lee, Chun, Lee, & Lee, 2006), and ginseng (Kang, Kim, Pyo, & Yokozawa, 2006). Increase in AOA following thermal treatment has been attributed to the release of bound phenolic compounds brought about by the breakdown of cellular constituents 350: 2 min, 4 min, 6 min, and 8 min, and the formation of new compounds with enhanced AOP (Dewanto et al., 2002a, 2002b; Tomaino et al., 2005).

Many studies have reported losses in TPC and AOA of plant samples following thermal treatments. Losses were mainly reported in vegetables (Ismail, Marjan, & Foong, 2004; Roy, Takenaka, Isobe, & Tsushida, 2007; Toor & Savage, 2006; Zhang & Hamauzu, 2004). Losses in AOP of heat-treated samples have been attributed to thermal degradation of phenolic compounds (Larrauri, Rupérez, & Saura-Calixto, 1997). Declines in AOP have been attributed to degradative enzymes, thermal degradation of phytochemicals, and to loss of antioxidant enzyme activities (Lim & Murtijaya, 2007). Declines in TPC and AOA are often accompanied by loss of other bioactive properties (Roy et al., 2007).

Results of this study showed that thermal drying methods had two major effects on the AOP of ginger leaves. TPC, AEAC, and FRP were adversely affected but not FIC ability and LPI activity. Compounds containing electron-donating atoms such as nitrogen could contribute substantially to the FIC ability and thus values were not affected by reduction in phenolic content. LPI activity depends more on the type of antioxidants present rather than their concentration. Some phenolic compounds such as quercetin are good inhibitors of lipid peroxidation while others such as catechin are poor inhibitors.

It would be presumptuous to infer that cooking and other thermal processing resulted in gains or losses in AOA without analysing a wide range of AOP and testing a variety of samples. A single treatment applied on a given sample could have variable effects on AOP. Gains in TPC and FIC ability, losses in FRP, but similarity in DPPH radical–scavenging activity have been reported for hot air-dried tomatoes (Chang, Lin, Chang, & Liu, 2006). TPC and oxygen radical absorbance capacity (ORAC) declined while DPPH radical–scavenging activity increased for heat-treated purple wheat bran (Li, Pickard, & Beta, 2007). TPC significantly declined in all vegetables studied while LPI ability was unchanged in some vegetables after thermal treatment (Ismail et al., 2004). TPC and DPPH radical–scavenging activity increased or remained unchanged depending on the type of vegetable and not on the type of cooking (Turkmen, Sari, & Velioglu, 2005). DPPH radical–scavenging activity increased in some cooked vegetables, while in others, the activity decreased (Yamaguchi et al., 2001).

An interesting finding from this study is the effect of microwave-drying on the AOP of leaves of *E. elatior*. Leaves microwave-dried for 2 min, 4 min, 6 min, and 8 min, which resulted in same weight loss (75 ± 2%), showed significant but comparable declines in TPC, AEAC, and FRP. A likely explanation is that microwave-drying for 2 min is sufficient to remove the moisture content and to decompose all heat-labile antioxidants, and subsequent heating would have no effect.

3.4. Effects of non-thermal drying methods

Air-drying of ginger leaves resulted in significant losses in TPC and AOA for all four species. Air-drying of *A. zerumbet* and *E. elatior* leaves significantly decreased TPC, AEAC, and FRP by 51%, 48%, and 50%; and by 49%, 51%, and 53%; respectively (Table 2). It resulted in drastic declines of 80%, 84%, and 80% for leaves of *C. longa*; and 70%, 77%, and 71% for leaves of *K. galanga*; respectively. Declines in AOP resulting from air-drying could be due to enzymatic degradation as the process was carried out at room temperature and takes several days for samples to dry. Contrary to results of this study, air-drying at 25–32 °C for 10 days of temperate herbs of lemon balm, oregano, and peppermint had variable effects on AOP which ranged from

Table 2
Total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and ferric-reducing power (FRP) of fresh, air-, and freeze-dried leaves of *A. zerumbet*, *E. elatior*, *C. longa*, and *K. galanga* (fresh weight)[a]

Species	Drying method	Water loss (%)	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
A. zerumbet	Fresh		2470 ± 240a	3020 ± 428a	11 ± 2.0a
	Air-drying	55 ± 1	1220 ± 241b	1570 ± 358b	5.5 ± 1.2b
E. elatior	Fresh		2500 ± 554a	2990 ± 891a	17 ± 4.2a
	Air-drying	75 ± 5	1270 ± 341b	1460 ± 439b	8.0 ± 2.4b
C. longa	Fresh		391 ± 36a	251 ± 19a	2.9 ± 0.1a
	Air-drying	82 ± 2	78 ± 5b	41 ± 4b	0.5 ± 0.1b
K. galanga	Fresh		130 ± 7a	48 ± 3a	0.7 ± 0.1a
	Air-drying	81 ± 4	39 ± 3b	11 ± 1b	0.2 ± 0.0b
A. zerumbet	Fresh		1990 ± 62a	2180 ± 42a	11 ± 0.2a
	Freeze-drying	61 ± 2	2550 ± 55b	2530 ± 45b	12 ± 0.2b
E. elatior	Fresh		2420 ± 210a	2960 ± 362a	14 ± 0.7a
	Freeze-drying	76 ± 4	3050 ± 226b	4280 ± 55b	19 ± 1.3b
C. longa	Fresh		399 ± 15a	243 ± 28a	2.1 ± 0.1a
	Freeze-drying	82 ± 2	357 ± 20b	222 ± 12a	1.8 ± 0.1b
K. galanga	Fresh		133 ± 5a	42 ± 3a	0.7 ± 0.1a
	Freeze-drying	86 ± 3	112 ± 11b	38 ± 5a	0.6 ± 0.1a

[a] Values of TPC, AEAC, and FRP of leaves are means ± SD ($n = 3$). For each column, values followed by the same letter (a–b) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. ANOVA does not apply between species and between drying methods.

significant increase to significant decline (Capecka, Mareczeek, & Leja, 2005).

Freeze-drying of *A. zerumbet* leaves significantly increased TPC, AEAC, and FRP compared to those of fresh leaves. Values of freeze-dried leaves were 2550 mg GAE/100 g, 2530 mg AA/100 g, and 12 mg GAE/g while those of fresh leaves were 1990 mg GAE/100 g, 2180 mg AA/100 g, and 11 mg GAE/g, respectively (Table 2). This amounted to gains of 28%, 16%, and 9%, respectively. FIC values of freeze-dried leaves of *A. zerumbet* were comparable to those of fresh leaves.

Freeze-dried leaves of *E. elatior* similarly showed significantly higher values in TPC, AEAC, and FRP compared to those of fresh leaves. Percentage gains were 26%, 45%, and 36%, respectively (Table 2). FIC ability of freeze-dried leaves showed little change compared to that of fresh leaves.

HPLC chromatograms of extracts of fresh and freeze-dried leaves of *E. elatior* revealed some interesting results (Fig. 1). Apices of major compounds remained relatively unchanged. The chromatograms showed greater amounts of minor compounds in freeze-dried than fresh leaves. Between retention times of 4 and 10 min, the overall peak area of freeze-dried leaves was 11 930 mAU*s compared to 9050 mAU*s of fresh leaves. This represented an increase of 32%, which is comparable to the 26% increase in TPC.

An experiment was conducted to test the stability of freeze-dried leaves of *E. elatior*. Leaves were stored in sealed Petri dishes kept in the laboratory for a week at ambient temperature of 25–30 °C and relative humidity of 33%. After storage, control leaves showed a loss of 23%, 15%, and 21% in TPC, AEAC, and FRP while freeze-dried leaves showed minimal declines of only 7%, 2%, and 5%, respectively (Table 3). It should be noted that the stored freeze-dried leaves with TPC, AEAC, and FRP values of 2850 mg GAE/100 g, 4210 mg AA/100 g, and 18 mg GAE/g remained significantly higher than those of fresh control leaves with values of 2420 mg GAE/100 g, 2960 mg AA/100 g, and 14 mg GAE/g, respectively. In terms of FIC ability, control and freeze-dried leaves remained unchanged after storage for a week.

Unlike leaves of *A. zerumbet* and *E. elatior*, which showed significant gains in TPC, AEAC, and FRP, freeze-drying led to slight declines of 11%, 9%, and 14% for leaves of *C. longa*; and 16%, 10%, and 14% for leaves of *K. galanga*; respectively (Table 2). Declines in AEAC for *C. longa* and in AEAC and FRP for *K. galanga* were, however, insignificant. FIC values of freeze-dried leaves of *C. longa* were comparable to those of fresh leaves. Freeze-dried leaves of *K. galanga* showed slight decline in FIC values.

Freeze-drying of leaves of *A. zerumbet* and *E. elatior* resulted in significantly increased TPC, AEAC, and FRP but not FIC ability. There

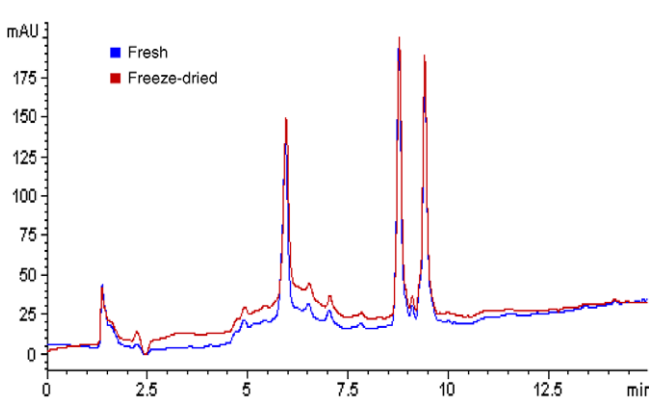

Fig. 1. Overlay of chromatograms (254 nm) showing greater amounts of minor compounds in freeze-dried than fresh leaves of *E. elatior*.

Table 3
The effect of one week of storage on total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and ferric-reducing power (FRP) of control and freeze-dried leaves of *E. elatior* (fresh weight)[a]

Drying method	Storage (day)	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
Control	0	2420 ± 210a	2960 ± 362a	14 ± 0.7a
	7	1860 ± 180b	2510 ± 157a	11 ± 1.0b
Freeze-dried	0	3050 ± 226c	4280 ± 55b	19 ± 1.3c
	7	2850 ± 124c	4210 ± 227b	18 ± 1.7c

[a] Values of TPC, AEAC, and FRP of leaves are means ± SD ($n = 3$). For each column, values followed by the same letter (a–c) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test.

is no thermal degradation in freeze-drying and neither does the process allow degradative enzymes to function. Furthermore, freeze-drying is known to have high extraction efficiency because ice crystals formed within the plant matrix can rupture cell structure, which allows exit of cellular components and access of solvent, and consequently better extraction (Asami, Hong, Barrett, & Mitchell, 2003). The HPLC chromatogram of leaves of *E. elatior*, which showed greater amounts of minor compounds following freeze-drying, supported this inference.

Freeze-drying resulted in slight but significant declines in TPC and FRP for *C. longa* and in TPC for *K. galanga*. Freeze-dried leaves of *A. zerumbet* and *E. elatior* were thick, powdery, and easy to extract while those of *C. longa* and *K. galanga* were thin, papery, and difficult to extract. Variation in the ease of extractability due to modification of the matrix could explain why freeze-drying has different effects on these two groups of species.

Results of this study showed that freeze-drying had three major effects on the AOP of ginger leaves. Firstly, freeze-dried leaves of *C. longa* and *K. galanga* had the least decline in AOP compared with leaves dried using other drying methods (microwave-, oven-, sun-, and air-drying). Secondly, leaves of *A. zerumbet* and *E. elatior* showed enhancement in AOP following freeze-drying. Thirdly, freeze-dried leaves of *E. elatior* remained stable following one week of storage under sealed conditions and room temperature.

The first effect on the retention of AOP after freeze-drying has often been reported. Freeze-dried yam flours displayed the highest AOA compared to hot air- and drum-dried flours (Hsu, Chen, Weng, & Tseng, 2003). Freeze-dried marionberry, strawberry, and corn yielded higher TPC than air-dried samples (Asami et al., 2003). Freeze-dried water hyacinth leaves had higher AOA than sun- and oven-dried leaves (Bodo, Azzouz, & Hausler, 2004). Higher TPC and AOA have been reported in freeze-dried than hot air-dried daylily flowers (Mao, Pan, Que, & Fang, 2006).

The second effect on AOP enhancement and the third effect on the stability of AOP after freeze-drying have seldom been reported. Total AOA was 50% higher in freeze-dried asparagus (Nindo, Sun, Wang, Tang, & Powers, 2003). Chang et al. (2006) reported gains in FIC ability, but TPC, FRP, and DPPH radical–scavenging activity remained unchanged for freeze-dried tomatoes. Storage of freeze-dried extracts of potato peel waste for 15 days showed no degradation in phenolics and AOA (Rodríguez de Sotillo, Hadley, & Holm, 1994). The present study is probably the first to demonstrate AOP enhancement and stability following freeze-drying of ginger leaves.

3.5. Teas of A. zerumbet

Commercial and freeze-dried teas of *A. zerumbet* were extracted with hot water and methanol. Hot-water infusion of the freeze-dried tea was light yellow in colour and produced a mild aromatic fragrance. The commercial tea infusion was more aromatic and faint yellow in colour.

Table 4
Total phenolic content (TPC), ascorbic acid equivalent antioxidant capacity (AEAC), and ferric-reducing power (FRP) of methanolic and hot-water extracts of freeze-dried and commercial teas of *A. zerumbet* (dry weight)[a]

A. zerumbet tea	Extraction	TPC (mg GAE/100 g)	AEAC (mg AA/100 g)	FRP (mg GAE/g)
Freeze-dried	Methanol	6440 ± 154a	6410 ± 111a	31 ± 0.6a
	Hot-water	3970 ± 202b	2050 ± 574b	19 ± 2.7b
Commercial	Methanol	275 ± 54c	143 ± 43c	1.1 ± 0.3c
	Hot-water	649 ± 30d	430 ± 38d	3.1 ± 0.3d

[a] Values of TPC, AEAC, and FRP of teas are means ± SD ($n = 3$). For each column, values followed by the same letter (a–d) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test.

For the commercial tea of *A. zerumbet*, TPC, AEAC, and FRP of hot-water extracts were 649 mg GAE/100 g, 430 mg AA/100 g, and 3.1 mg GAE/g, respectively (Table 4). Values of methanol extracts were 275 mg GAE/100 g, 143 mg AA/100 g, and 1.1 mg GAE/g, respectively. Hot-water extraction was more efficient than methanol extraction. For the freeze-dried tea, TPC, AEAC, and FRP of methanol extracts were 6440 mg GAE/100 g, 6410 mg AA/100 g, and 31 mg GAE/g while those of hot-water extraction were 3970 mg GAE/100 g, 2050 mg AA/100 g, and 19 mg GAE/g, respectively. Methanol extraction was more efficient than hot-water extraction.

Using the same solvent, the freeze-dried tea had stronger metal ion-chelating ability than that of the commercial tea (Fig. 2a). In terms of LPI, methanol extracts of the freeze-dried tea had the strongest activity followed by hot-water extracts of the commercial tea and hot water extracts of the freeze-dried tea, and by methanol extracts of the commercial tea (Fig. 2b). Values of both hot-water and methanol extracts of the freeze-dried tea were higher than those of the commercial tea in methanol.

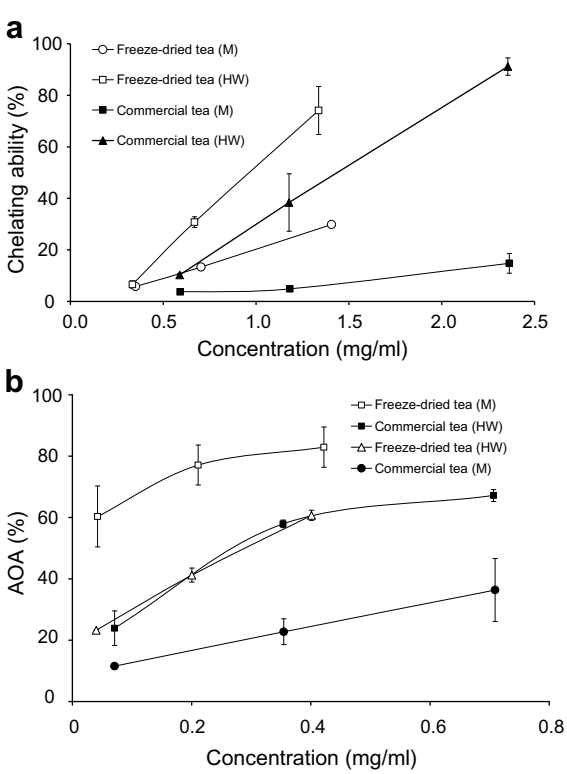

Fig. 2. Ferrous ion-chelating (FIC) ability (a) and lipid peroxidation inhibition (LPI) activity (b) of methanolic (M) and hot-water (HW) extracts of freeze-dried and commercial teas of *A. zerumbet* (dry weight).

Variability in AOP in the commercial and freeze-dried teas of *A. zerumbet* may be due to number of factors. They include drying methods, type of extraction solvents, and antioxidant assays used. The significantly lower TPC and AOA of the commercial tea could be due to the use of conventional drying methods where heat is applied during the manufacturing process. Consequently, much of the antioxidant compounds are lost through enzymatic degradation and/or heat decomposition. On the contrary, much of the AOP are retained in the freeze-dried tea as freeze-drying is non-thermal. Freeze-drying remains the best method of drying foods as the quality of freeze-dried products is comparable to fresh products (Ratti, 2001).

4. Conclusion

Results showed that freeze-drying is superior to other drying methods in preserving the AOP of ginger leaves. Thermal drying (microwave-, oven-, and sun-drying) resulted in significant declines in TPC, AEAC, and FRP with minimal effects on FIC ability and LPI activity. Microwave-drying of *E. elatior* leaves resulted in significant losses in TPC and AOA, but the declines were comparable between different drying durations. Of the two methods of non-thermal drying, air-dried leaves showed drastic losses in values for all species. Freeze-dried leaves had significant gains in TPC, AEAC, and FRP for *A. zerumbet* and *E. elatior*, but losses for *C. longa*, and *K. galanga*. Freeze-drying had minimal effect on the FIC ability of leaves of all four species. HPLC analysis showed the presence of greater amounts of minor compounds in freeze-dried than fresh *E. elatior* leaves. Values of freeze-dried leaves of *E. elatior*, after one week of storage, remained significantly higher than those of fresh control leaves. The freeze-dried tea of *A. zerumbet* was superior to the commercial tea for all AOP studied. Freeze-drying appears to be a sound method for producing tea and other herbal products from ginger species. Due to its high operation cost, freeze-drying can be applied to produce high-value speciality tea or spice powder from ginger leaves.

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Caffeoylquinic acids from leaves of *Etlingera* species (Zingiberaceae)

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ABSTRACT

3-*O*-caffeoylquinic acid, 5-*O*-caffeoylquinic acid (chlorogenic acid), and 5-*O*-caffeoylquinic acid methyl ester, as elucidated by ^{1}H and ^{13}C NMR, were isolated from leaves of *Etlingera elatior*. This is the first report of caffeoylquinic acids (CQA) including chlorogenic acid (CGA) in Zingiberaceae. Leaves of *Etlingera* species were rich in total phenols and CQA, and non-cytotoxic to normal human liver and African green monkey kidney cells. Content of CQA of *E. elatior*, *Etlingera fulgens*, and *Etlingera rubrostriata* leaves was significantly higher than leaves of *Ipomoea batatas*, and comparable to flowers of *Lonicera japonica*. CGA found only in leaves of *E. elatior* and *E. fulgens* was significantly higher in content than flowers of *L. japonica*, the commercial source.

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1. Introduction

Etlingera Giseke are tall ginger plants growing in gaps of tropical forests. Inflorescences are borne on erect stalks protruding from the ground (*Phaeomeria* group) or are subterranean with flowers appearing at soil level (*Achasma* group) (Lim, 2000, 2001). Plants of *Etlingera* have various traditional and commercial uses. Young shoots, flowers, and fruits are consumed as condiment (Noweg, Abdullah, & Nidang, 2003). Inflorescences of *Etlingera elatior* are widely cultivated as spice for curry (Ibrahim & Setyowati, 1999). Fruits are used to treat earache, while leaves are applied for healing wounds, and used by *post-partum* women for bathing to remove body odour.

Research on chemical constituents of leaves of Zingiberaceae is confined mainly to species of *Alpinia*. Classes of compounds in leaves include flavonoids (Mpalantinos, de Moura, Parente, & Kuster, 1998), phenolic acids (Elzaawely, Xuan, & Tawata, 2007), labdane-type diterpenes (Sy & Brown, 1997), diarylheptanoids (Ngo & Brown, 1998), phenylbutanoids (Kikuzaki, Tesaki, Yonemori, & Nakatani, 2001), and kava pyrones (Mpalantinos et al., 1998).

The most comprehensive work on leaf chemistry of Zingiberaceae is by Williams and Harborne (1977) where leaves of 39 species of gingers were screened for flavonoids. Leaves of *Alpinia* and *Zingiber* were found to contain kaempferol and quercetin glycosides, and myricetin and quercetin glycosides, respectively. Flavonoids in the leaves of *E. elatior* have been identified as kaempferol 3-glucuronide, quercetin 3-glucuronide, quercetin 3-glucoside, and quercetin 3-rhamnoside.

Caffeoylquinic acids (CQA) are esters of caffeic and quinic acids. The three common isomers are 5-*O*-caffeoylquinic acid (5-CQA) or chlorogenic acid, 3-*O*-caffeoylquinic acid (3-CQA) or neochlorogenic acid, and 4-*O*-caffeoylquinic acid (4-CQA) or cryptochlorogenic acid (Nakatani et al., 2000). Chlorogenic acid (CGA or 5-CQA) is the most common of the CQA and is the only one that is commercially available (Clifford, 1999). CGA is a natural antioxidant with commercial applications in medicine, food, and cosmetics (Xiang & Ning, 2008). The absorption, metabolism, and bioactivities of CQA have been reviewed by Morishita and Ohnishi (2001).

In our previous study (Chan et al., 2008), leaves of five *Etlingera* species were found to have the highest total phenolic content (TPC) and radical-scavenging activity among 26 ginger species belonging to nine genera screened. As leaves of *E. elatior* ranked first in terms of TPC and radical-scavenging activity, the species was chosen for isolation and analysis of phenolic compounds using ^{1}H and ^{13}C NMR in the present study. Subsequently, CQA and CGA of leaves of five *Etlingera* species including *E. elatior* were screened. Comparisons were made with leaves of three commercial ginger species. Flowers of *Lonicera japonica* (Japanese honeysuckle) and leaves of *Ipomoea batatas* (sweet potato) were included for comparison as they are known to have high contents of CGA (Chen, Yu, Li, Luo, & Liu, 2007; Zheng & Clifford, 2008). Leaves of *Etlingera* species were also analysed for cytotoxicity.

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2. Materials and methods

2.1. Chemicals

Folin-Ciocalteu's phenol reagent (Fluka, 2 N), gallic acid (Fluka, 98%), and anhydrous sodium carbonate (Fluka, 99%) were used for analysing total phenolic content (TPC). Molybdate reagent (Sigma, 99.5%) and chlorogenic acid (Sigma, >95%) were used for analysing contents of CQA and CGA, respectively.

2.2. Plant materials

Five species of *Etlingera* studied were *E. elatior*, *Etlingera fulgens*, and *Etlingera maingayi* of the *Phaeomeria* group, and *Etlingera littoralis* and *Etlingera rubrostriata* of the *Achasma* group. Voucher specimens were deposited at the herbarium of Forest Research Institute Malaysia (FRIM) in Kepong. Leaves of *Etlingera* species were collected from Janda Baik in Pahang. Leaves of *Alpinia galanga* were sampled from FRIM. Plants of *Zingiber officinale* were raised from rhizomes. Leaves of *I. batatas* and *Curcuma longa* were purchased from the market. Flowering plants of *L. japonica* were purchased from the nursery.

2.3. Extraction for isolation of compounds

Leaves of *E. elatior* (1.5 kg) were lyophilized overnight in batches at 0.125 mbar and −50 °C in a freeze-dryer and subsequently ground using a blender. Ground leaves were extracted once with methanol to inactivate polyphenol oxidase, filtered, and extracted five times with water. The container was sonicated for 8 min, when fresh solvent was added each time. After sonication, each extraction system was allowed to stand for 1 h using 2 l of solvent in the closed container at room temperature. The methanol extract was dried in a rotary evaporator and the dried extract was suspended in water. The water-soluble component of the suspension was collected by filtration and combined with the water extract. Freeze-drying of the combined water extract yielded 80 g of dried extract.

2.4. Column chromatography

The dried extract (30 g) was fractionated with MCI gel CHP 20P using an H_2O:MeOH 0–100% step-gradient into 17 fractions. From fraction 2 (0.5 g), compound 1 (30 mg) was purified with Chromatorex ODS (H_2O:MeOH; 0–40%) and Silica gel 60 ($CHCl_3$:MeOH:H_2O; 7:3:0.5–5:5:2). Compounds 2 (50 mg) and 3 (10 mg) were purified from fraction 9 (1.5 g) with Sephadex LH-20 (H_2O:MeOH; 0–100%), Chromatorex ODS (H_2O:MeOH; 0–40%), and LiChroprep® RP-18 (H_2O:MeOH; 0–40%).

2.5. Thin layer chromatography

Eluents from column chromatography were pooled into fractions based on TLC analysis using Silica gel 60 F_{254} plates ($CHCl_3$:MeOH:H_2O; 8:2:0.2, 7:3:0.5 or 6:4:1). Bands were detected by UV illumination and by spraying 10% H_2SO_4 with heating.

2.6. Nuclear magnetic resonance spectroscopy

Compounds were dissolved in CD_3OD and subjected to ^1H and ^{13}C NMR analysis using a Bruker DRX 300 MHz spectrometer operated at 300 MHz for ^1H and 75 MHz for ^{13}C. Chemical shifts were recorded in ppm (δ) using TMS as internal standard.

2.7. Extraction for quantitation of phenolics

Fresh leaves (1 g) were powdered with liquid nitrogen in a mortar and extracted using 50% aqueous methanol (50 ml), with continuous swirling for 1 h at room temperature on an orbital shaker. Extracts were filtered under suction and stored at −20 °C for further use. Analysis of extracts was done in triplicate.

2.8. Total phenolic content

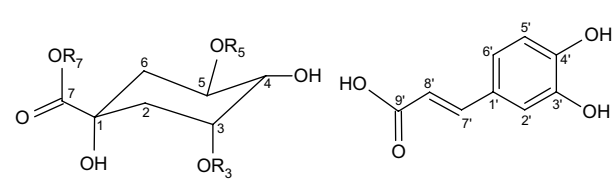
Total phenolic content (TPC) of extracts was determined using the Folin-Ciocalteu assay (Chan, Lim, & Omar, 2007). Samples (300 μl) were introduced into test tubes followed by 1.5 ml of Folin-Ciocalteu's reagent (10× dilution) and 1.2 ml of sodium carbonate (7.5%, w/v). After 30 min, absorbance at 765 nm was measured with a UV–vis spectrophotometer (Anthelie Advanced 5 Secoman). TPC was expressed as milligram gallic acid equivalent (GAE)/100 g. The calibration equation for GA was $y = 0.0111x - 0.0148$ ($R^2 = 0.9998$).

2.9. Caffeoylquinic acid content

Caffeoylquinic acid content (CQAC) of extracts was determined using the molybdate assay (Clifford & Wright, 1976). Molybdate reagent was prepared by dissolving 16.5 g sodium molybdate, 8.0 g dipotassium hydrogen phosphate, and 7.9 g potassium dihydrogen phosphate in 1 l deionised water. The reagent (2.7 ml) was added to plant extracts (0.3 ml), mixed, and incubated at room temperature for 10 min. Absorption was measured at 370 nm with a UV–vis spectrophotometer (Anthelie Advanced 5 Secoman). CQAC was expressed as milligram chlorogenic acid equivalent (CGAE)/100 g. The calibration equation for CQA was $y = 8.6966x$ ($R^2 = 0.9979$).

2.10. Chlorogenic acid content

Chlorogenic acid content (CGAC) of extracts was quantified using reverse-phase HPLC (Agilent Technologies 1200 Series) with Thermo Scientific BDS Hypersil Phenyl Column (4.6 × 100 mm). A 15-min linear gradient from 5% to 100% MeOH was used to elute samples at 1 ml/min. Mobile phases were acidified with 0.1% trifluoroacetic acid for better resolution. A 20-μl loop was used for injection and elution was monitored at 280 nm. Identity of CGA was determined by matching UV spectrum and retention times. The amount of CGA present was quantified using peak areas. The calibration equation of peak area (mAU*s) against concentration of



Compound	Identity	R_3	R_5	R_7
1	3-CQA	caffeoyl	H	H
2	5-CQA	H	caffeoyl	H
3	Me 5-CQA	H	caffeoyl	methyl

Fig. 1. Molecular structures of 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA), and 5-*O*-caffeoylquinic acid methyl ester (Me 5-CQA).

Table 1
^{1}H and ^{13}C NMR spectra of 3-*O*-caffeoylquinic acid (3-CQA), 5-*O*-caffeoylquinic acid (5-CQA), and 5-*O*-caffeoylquinic acid methyl ester (Me 5-CQA) from leaves of *Etlingera elatior*[a].

Atom	3-CQA		5-CQA		Me 5-CQA	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	76.0		76.1		75.8	
2	37.3	1.92–1.99 (m)	38.2	1.92–1.98 (m)	37.8	1.96–2.02 (m)
3	72.4	5.42 (br s)	71.3	4.10 (m)	70.3	4.12 (m)
4	73.3	3.82 (m)	72.0	3.67 (m)	72.1	3.72 (m)
5	70.0	4.02 (m)	73.5	5.36 (m)	72.5	5.26 (m)
6	39.1	2.10 (m)	38.8	2.11 (m)	38.0	2.17 (m)
1′	127.9		127.8		127.6	
2′	115.1	7.07 (d 1.66)	115.2	7.04 (d 1.81)	115.1	7.03 (d 1.80)
3′	146.7		147.1		147.2	
4′	149.4		149.6		149.7	
5′	116.5	6.79 (d 8.16)	116.5	6.76 (d 8.16)	116.5	6.77 (d 8.13)
6′	122.9	6.96 (dd 1.66, 8.17)	123.0	6.94 (dd 1.82, 8.12)	123.0	6.94 (dd 1.80, 8.22)
7′	146.8	6.33 (d 15.9)	146.8	6.28 (d 15.9)	146.9	6.21(d 15.9)
8′	115.7	7.60 (d 15.9)	115.1	7.56 (d 15.9)	115.0	7.51 (d 15.9)
9′	168.8		168.7		168.3	
COO–	182.1		177.0		175.4	
OCH_3					53.0	3.68 (s)

[a] Values of ^{1}H (300 MHz) and ^{13}C (75 MHz) are in ppm (δ), and of J (bracketed) are in Hz.

CGA (mg/ml) was $y = 7286.7x$ ($R^2 = 0.9998$). CGAC was expressed as mg CGA/100 g.

2.11. Cytotoxicity evaluation

Fresh leaves (10 g) were powdered with liquid nitrogen in a mortar and extracted sequentially with dichloromethane, methanol, and water (100 ml each for 1 h). Extracts were pooled and evaporated using a rotary evaporator to obtain composite samples of polar and non-polar constituents. WRL-68 (human liver) and Vero (African green monkey kidney) cells were seeded in 96-well plate at 10,000 cells/well. Cells were kept overnight before incubating with plant extracts at 0.1–1000 μg/ml. Paclitaxel was used as positive control. After 72 h, the sulforhodamine B assay (Voigt, 2005) was conducted to determine the number of viable cells. Absorbance was measured with a microplate absorbance reader (Tecan Sunrise) and results were expressed as cell survival (%) with IC_{50} determined from dose–response curves.

3. Results and discussion

3.1. Identification of compounds

Compounds in leaves of *E. elatior* were identified based on spectroscopic analysis, and on comparison of ^{1}H and ^{13}C NMR data with literature values. Compound 1 was identified as 3-*O*-caffeoylquinic acid (3-CQA) or neochlorogenic acid (Nakatani et al., 2000). Compound 2 was identified as 5-*O*-caffeoylquinic acid (5-CQA) or CGA (Madhava Naidu, Sulochanamma, Sampathu, & Srinivas, 2008). Compound 3 was identified as 5-*O*-caffeoylquinic acid methyl ester (Me 5-CQA) or methyl 5-*O*-caffeoylquinate (Lin, Yang, & Chou, 2002).

Compound 1 (3-CQA) has the caffeoyl group attached to carbon 3 and OH groups at carbons 1, 4, and 5 (Fig. 1). Compound 2 (5-CQA) has the caffeoyl group at carbon 5 and OH groups at carbons 1, 3, and 4. Compound 3 (Me 5-CQA) differs from 5-CQA in that the carboxyl group at carbon 7 is esterified with a methyl group.

All three compounds displayed similar ^{1}H and ^{13}C NMR spectra (Table 1). Both 3-CQA and 5-CQA have a molecular formula of $C_{16}H_{18}O_9$. Their ^{1}H NMR showed 10 signals, corresponding to the 10 non-OH protons. Me 5-CQA, with a molecular formula of $C_{17}H_{20}O_9$, had an extra methoxyl signal at 3.68 ppm. The H-4 of 3-CQA was shifted downfield by 0.15 and 0.10 ppm when compared to 5-CQA and Me 5-CQA, respectively. All three compounds had an ABX spin system at 7.05, 6.77, and 6.95 ppm, and *trans*-conjugation at 7.56 ppm ($J = 15.9$ Hz) and 6.27 ppm ($J = 15.9$ Hz) derived from the caffeoyl group. ^{13}C NMR of 3-CQA and 5-CQA exhibited 16 signals. Me 5-CQA had an extra methoxy signal at 53.0 ppm. Another key feature of Me 5-CQA was the upfield shift by 1.6 ppm of the quinic acid carboxyl carbon compared to the free carboxylic group.

Major dietary sources of CQA are coffee, vegetables, and fruits such as berries and apples (Clifford, 1999). 3-CQA and 5-CQA are dominant in prunes (Nakatani et al., 2000) and plums (Fang, Yu, & Prior, 2002). Me 5-CQA has been isolated from stems of *Ecdysanthera rosea* (Lin et al., 2002) and rhizomes of *Polygonum paleaceum* (Wang, Zhang, & Yang, 2005). Leaves and stems of *I. batatas* are rich in 5-CQA while flowers of *L. japonica* are a commercial source of CGA (Chen et al., 2007).

HPLC analysis of *E. elatior* leaves showed that 5-CQA with retention time (RT) of 5.74 min was the dominant CQA (Fig. 2). Because of the lack of commercially available standards, the quantities of 3-CQA and Me 5-CQA were estimated using the calibration curve of 5-CQA as they have the same chromophoric groups and similar molecular weights. The quantities of 3-CQA (RT of 3.62 min) and Me 5-CQA (RT of 6.74 min) were estimated to be 3 mg/100 g and 30 mg/100 g, respectively. These values were meagre compared to 294 ± 53 mg/100 g of 5-CQA (Table 2).

As mentioned earlier, flavonoids isolated from leaves of *E. elatior* are kaempferol 3-glucuronide, quercetin 3-glucuronide, quercetin 3-glucoside, and quercetin 3-rhamnoside (Williams & Harborne, 1977). CQA including CGA are the phenolic compounds first reported in Zingiberaceae. Families known to be rich in CGA are Asteraceae, Rubiaceae, and Solanaceae (Molgaard & Ravn, 1988).

CQA including CGA are widely distributed in plants and these compounds are considered to be the active principles of many medicinal plants (Morishita & Ohnishi, 2001). They are frequently taken as food and beverage, and play a role in human health. CGA acts as an antioxidant by scavenging free radicals, inhibiting lipid peroxidation, and chelating metal ions (Kono et al., 1998). It is able to scavenge free radicals at a higher rate than many phenolics and its phenolic OH group is the binding site of metals.

3.2. Quantitation of phenolics

TPC and CQAC of leaves of five *Etlingera* species including *E. elatior* were compared with those of three commercial ginger species and two important sources of CQA.

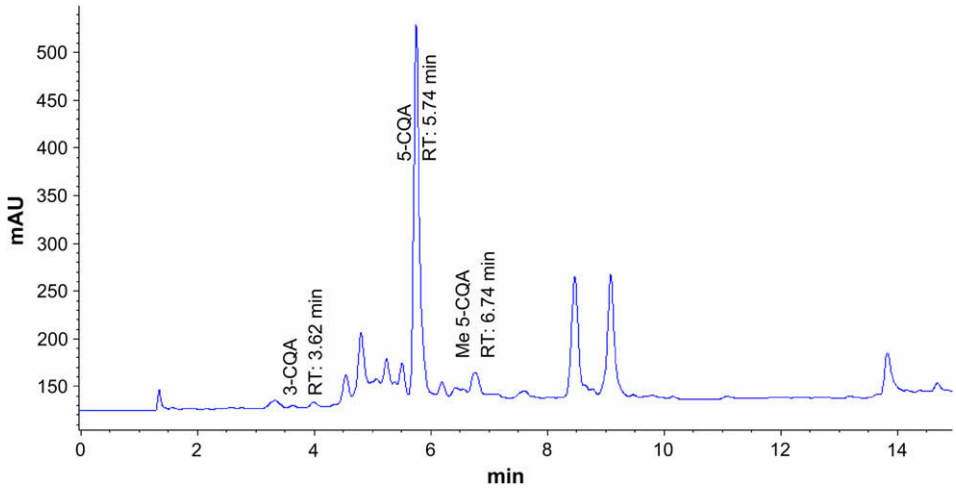


Fig. 2. Chromatogram of *Etlingera elatior* leaf extract at 280 nm showing retention times (RT) of 3-CQA, 5-CQA, and Me 5-CQA.

TPC of *Etlingera* species was significantly higher than *A. galanga*, *C. longa*, and *Z. officinale* (Table 2). Leaves of *E. elatior* and *E. fulgens* displayed the highest content of CQA and were the only two ginger species with CGA. CQAC of leaves of *E. elatior*, *E. fulgens*, and *E. rubrostriata* was significantly higher than leaves of *I. batatas*, and comparable to flowers of *L. japonica*. CGA found only in leaves of *E. elatior* and *E. fulgens* was significantly higher in content than leaves of *I. batatas* and flowers of *L. japonica*.

3.3. Cytotoxicity evaluation

All leaf extracts of *Etlingera* species did not exhibit cytotoxic effect on WRL-68 and Vero cells, which are normal human liver and African green monkey kidney cells, respectively (Table 3). IC_{50} values ranged from 560 to 663 μg/ml and from 591 to 704 μg/ml, respectively. These values are much higher than IC_{50} of paclitaxel which were 0.08 and 0.01 μg/ml, respectively.

3.4. Commercial potential

Flowers of *L. japonica* are a commercial source of CGA. Leaves of *E. elatior* and *E. fulgens*, which currently have no economic value, could serve as alternative sources of CGA as their leaves are non-cytotoxic to normal liver and kidney cells. This is especially true for plants of *E. elatior* which are widely cultivated for their inflorescences as spice. Unlike flowers of *L. japonica* which are small and seasonal, leaves of *E. elatior* are large and available in abundance. Furthermore, harvesting of leaves is non-destructive to the plants. However, extraction of CGA from *E. elatior* leaves has to be optimised before their commercial potentials can be realised.

4. Conclusion

3-CQA, 5-CQA (CGA), and Me 5-CQA were isolated from leaves of *E. elatior*. This is the first report of CQA including CGA in Zingiberaceae. Leaves of *Etlingera* species were rich in TPC and CQAC, and non-cytotoxic to normal liver and kidney cells. CQAC of leaves of *E. elatior*, *E. fulgens*, and *E. rubrostriata* was significantly higher than leaves of *I. batatas*, and comparable to flowers of *L. japonica*. Leaves of *E. elatior* and *E. fulgens*, with significantly higher CGAC than flowers of *L. japonica*, could serve as alternative sources of CGA.

Table 2
Total phenolic content (TPC), caffeoylquinic acid content (CQAC), and chlorogenic acid content (CGAC) of leaf extracts of five *Etlingera* and three commercial ginger species (fresh weight)[a].

Species	TPC[b] (mg GAE/100 g)	CQAC (mg CGAE/100 g)	CGAC (mg CGA/100 g)
Etlingera elatior	2390 ± 329a	320 ± 62a	294 ± 53a
E. fulgens	1280 ± 144b	326 ± 45a	219 ± 14b
E. rubrostriata	2250 ± 113a	201 ± 23b	–
E. littoralis	2150 ± 94a	129 ± 23c	–
E. maingayi	1110 ± 93b	48 ± 13d	–
Alpinia galanga	392 ± 50c	24 ± 14de	–
Curcuma longa	230 ± 19d	44 ± 6d	–
Zingiber officinale	291 ± 18d	11 ± 8e	–
Lonicera japonica	533 ± 41e	250 ± 49ab	173 ± 13c
Ipomoea batatas	386 ± 23c	125 ± 10c	115 ± 16d

[a] Values are means ± S.D. ($n = 3$). For each column, values followed by the same letter (a–e) are not statistically different at $P < 0.05$ as measured by the Tukey HSD test. Rich in CGA, flowers of *L. japonica* and leaves of *I. batatas* were included for comparison.
[b] TPC values are taken from Chan et al. (2008) except *L. japonica* and *I. batatas*.

Table 3
Cytotoxicity of leaf extracts of *Etlingera* species on WRL-68 and Vero cells using the sulforhodamine B assay[a].

Etlingera species	Cell	Cell survival (%)			IC_{50} (μg/ml)
		0.1 μg/ml	10 μg/ml	1000 μg/ml	
E. elatior	WRL-68	98.9 ± 0.37	97.9 ± 0.33	31.0 ± 0.79	563
	Vero	100 ± 0.13	95.7 ± 1.09	25.7 ± 1.31	704
E. rubrostriata	WRL-68	101 ± 1.70	97.9 ± 1.27	27.0 ± 0.79	560
	Vero	99.6 ± 0.67	95.6 ± 0.82	24.5 ± 0.55	698
E. maingayi	WRL-68	101 ± 0.52	98.4 ± 1.37	22.3 ± 0.72	660
	Vero	119 ± 0.81	119 ± 0.58	4.12 ± 0.52	636
E. fulgens	WRL-68	99.3 ± 0.44	98.0 ± 1.30	26.3 ± 0.79	561
	Vero	97.5 ± 0.13	97.7 ± 0.20	19.5 ± 0.56	624
E. littoralis	WRL-68	98.0 ± 0.85	98.6 ± 0.82	24.9 ± 0.69	663
	Vero	97.6 ± 0.23	98.7 ± 0.48	21.8 ± 0.52	591
Positive control	Cell	0.001 μg/ml	0.1 μg/ml	10 μg/ml	IC_{50} (μg/ml)
Paclitaxel	WRL-68	95.5 ± 0.29	37.3 ± 0.35	16.9 ± 1.01	0.08
	Vero	65.6 ± 0.83	17.2 ± 0.88	6.40 ± 0.24	0.01

[a] Number of living cells relative to the control was expressed as cell survival (%) ± S.E.M.

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