Analysis of Phenolics and Other Phytochemicals in Selected Malaysian Traditional Vegetables and Their Activities *In Vitro*

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ABSTRACT

A fruit and vegetable-rich diet has been associated with decreased risk of developing chronic diseases such as cardiovascular disease and cancer in humans. These protective effects have been attributed in part, to the presence of phytochemicals in fruit and vegetables, in particular flavonoids and phenolic compounds. Some plants have been used in traditional medicine for healing, ritual ceremonies and as health tonics or food supplements. Recent interest in the health-promoting properties of Malaysian traditional vegetables has been based on claims about their uses in health and medicine. However, scientific information to support these claims is largely unexplored. The overall objectives of the present study were to investigate, determine and quantify the phytochemicals, particularly phenolic compounds, in the seven samples from five species of selected Malaysian traditional vegetables (*Anacardium occidentale, Centella asiatica, Colubrina asiatica, Pluchea indica* and *Premna cordifolia*) and to evaluate their activities *in vitro,* including antioxidant and antibacterial activities of extracts of these plants and individual phytochemicals. In the first section of this project, discussed in *Chapter 3*, Malaysian traditional vegetable extracts were screened for phenolic compounds using several complimentary techniques, namely high performance liquid chromatography (HPLC) and HPLC-tandem mass spectrometry and the total phenolic content determined using the Folin-Ciocalteu assay. Flavonol glycosides were predominant in most of the species, particularly *A. occidentale* with levels ranging from 6434 to 12420 µg/g fresh weight. Chlorogenic acids were the main components identified and quantified in *C. asiatica* and *P. indica*. The total phenolic content of the vegetables were between 100 ± 7.8 and 415 ± 20 mg/ kg gallic acid equivalent (GAE) in batch 1 but lower in batch 2 ranging from 62 ± 2.5 to 386 ± 41 mg/ kg GAE. The total phenolic content of plant extracts was positively correlated with total antioxidant capacity, determined by 2, 2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and ferric reducing antioxidant potential (FRAP) assays.

A. occidentale exhibited the highest total phenolic content and total antioxidant activity, whereas *Colubrina asiatica,* which had the lowest total phenolic content, also had low antioxidant activity *in vitro*. Phenolic content and antioxidant activity were significantly ($p<0.05$) influenced by environmental factors, as in this study, plant materials in batch 1

which was harvested in rainy season, had a higher total phenolic and antioxidant content than batch 2, which was harvested in the dry season.

Based on the hypothesis that other components in addition to phenolics also contributed to the total antioxidant activities in the plants, the next objective, which was presented in *Chapter 4,* was to investigate the occurrence of phytochemicals such as triterpenes, carotenoids, α-tocopherol and vitamin C. The level of total triterpenes, biomarkers of *C. asiatica* was not significantly different between batches. The main component was madecassoside with 91 ± 4.8 µg/g fresh weight in batch 1 and 77 ± 3.4 µg/g fresh weight in batch 2. The level of carotenoids and vitamin C were low compared to previous reports. This was almost certainly due to dried samples being used in the present study, as some of the compounds would have broken down during drying process. This would have particularly affected the levels of vitamin C, which contributed only 0.9 to 5.5% to the total antioxidant activity of the plants under study.

Total antioxidant activities of plant essential oils were determined using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and the result was in agreement to the total antioxidant activities of plant extracts, which *A. occidentale* having the highest amount. The highest antioxidant activity exhibited by *A. occidentale* oil was attributed to the presence of high amounts of γ-terpinene (28%) and terpinen-4-ol (4.2%), both of which were shown to have strong radical scavenging activity.

The high phenolic content, antioxidant activity and occurrence of volatile components exhibited by *A. occidentale* has led to the final objective of this study, which is presented in *Chapter 5.* This was to screen for antimicrobial activities of *A. occidentale* extracts and essential oil against selection of Gram-positive (*Enterococcus faecalis, Staphylococcus aureus,* Meticillin-resistance S*taphylococcus aureus* (MRSA)*,* coagulase negative *Staphylococci* (CoNS) *and Lactobacillus acidophilus),* Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*) and fungi (*Candida albican*) using disc diffusion and minimum inhibitory concentration (MIC) methods. Investigation of the modes of action was determined using growth inhibition curve, scanning (SEM) and transmission (TEM) electron microscopy. *A. occidentale* was shown to have promising effects at 25 mg/ml with regard to inhibiting the growth of Gram-positive bacteria including MRSA. The essential oil and its major component, γ-terpinene at only 2.5% (v/v)

inhibited the growth of all Gram-positive and Gram-negative bacteria. None of the *A. occidentale* extracts or oil exhibited antibacterial activities against *Lactobacillus acidophilus*, an important strain of bacteria found in the human gut. This indicates selective effects of *A. occidentale*.

A. occidentale extract and oil inhibited the growth of *S. aureus* cells within a 2-hour incubation observed in time-kill experiments. SEM and TEM examination revealed that the oil and its component, γ-terpinene, inhibited the bacteria through bacteriostatic and bactericidal effects which damaged the bacterial cell wall. Testing the oil and γ-terpinene against epidemic-MRSA (EMRSA) biofilms indicated an anti-adhesive effect, which disrupted the bacterial colonies in the biofilms to produce more extracellular polysaccharides (EPS). The effects of *A. occidentale* oil were comparable with tea tree oil, a widely used topical antiseptic.

All the Malaysian traditional vegetables under study are claimed to have medicinal properties and health effects. The results in the present study have provided some information on phytochemical and nutritional properties of Malaysian traditional vegetables, and as a consequence provide a sound scientific base for promoting their consumption particularly in Malaysia.

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AUTHOR'S DECLARATION

I declare that the work contained within this thesis is original, carried out between October 2004 and December 2007. I have been solely responsible for the organisation, experimentation, analysis, data processing and thesis writing, unless otherwise cited and acknowledged.

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LIST OF ABBREVIATIONS

amu	atomic mass units
ANOVA	analysis of variance
ABTS	2, 2'-Azinobis - 3 - ethylbenzothiazoline- 6 - sulfonic acid
APCI	atmospheric pressure chemical ionisation
ATCC	American Type Culture Collection
BHI	brain heart infusion
CLSI	Clinical and Laboratory Standards Institute
CoNS	coagulase-negative staphylococci
CVD	cardiovascular disease
DMSO	Dimethyl sulfoxide
DPPH	1, 1-diphenyl-2-picrylhydrazyl
ECG	epicatechin gallate
EDTA	ethylene-diamine-tetra-acetic acid
EGCG	epigallocatechin gallate
EMRSA	epidemic meticillin-resistant *Staphylococcus aureus*
EPS	extracellular polysaccharides
FA	formic acid
FAO	Food and Agriculture Organisation
FRAP	Ferric reducing antioxidant potential
FTC	ferric thiocyanate
GAE	gallic acid equivalent
GC-MS	gas chromatography tandem mass spectrometry
GI	gastrointestinal
GSH	glutathione transferase
GSHPx	glutathione peroxidase
HCl	hydrochloric acid
HMDS	hexamethyldisilazane
HPLC	High Performance Liquid Chromatography
LDL	low density lipoprotein
M	molar
M	molecular ion (in MS analysis)
MH	Mullen Hinton agar
MIC	minimum inhibitory concentration
NIST	National Institute of Standards and Technology
MPA	metaphosphoric acid
MRSA	meticillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MS^2	Tandem mass spectrometry
m/z	mass/charge ratio
NA	nutrient agar
NCI	National Cancer Institute
NMR	nuclear magnetic resonance
NNISS	National Nosocomial Infections Surveillance System
OH	hydroxyl group
ORAC	Oxygen Radical Absorbance Capacity
PA	phosphoric acid
PAL	phenylalanine ammonia lyase
PBS	phosphate buffer saline

PDA	photodiode-array detector
PO	propylene oxide
TAA	total antioxidant activity
TEAC	Trolox Equivalent Antioxidant Capacity
TPTZ	ferric-2,4,6-tri-2-pyridyl-s-triazine
ROS	reactive oxygen species
R_t	retention time
SD	standard deviation
SEM	scanning electron microscopy
SGLT	sodium-dependent glucose transporter
SI	spectral index
SOD	superoxide dismutase
SMRSARL	Scottish MRSA Reference Laboratory
TBA	thobarbituric acid
TEAC	trolox equivalent antioxidant capacity
TEM	transmission electron microscopy
TIC	total ion count
TLC	thin layer chromatography
UK	United Kingdom
US	United State
UV	ultra violet
UV-VIS	ultra violet visible
VISA	vancomycin intermediate *Staphylococcus aureus*
VRE	vancomycin resistant enterococci
VRSA	vancomycin resistant *Staphylococcus aureus*
WHO	World Health Organisation
XTT	3-bis(2-methyloxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide

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AIMS OF STUDY

Malaysian traditional vegetables have played important roles in traditional medicine among local people in Malaysia especially the Malays, and have been consumed as dietary intake since ancient times. Even though a database of Malaysian medicinal plants has been documented in 1966, there are still gaps of information, research and development, on the phytochemical content and biological activities of these vegetables. Therefore, this study aimed to achieve the following objectives:

1. Screening, identification and quantification of phenolic compounds and their total antioxidant activity in seven varieties from five species of Malaysian traditional vegetables (*Anacardium occidentale, Centella asiatica, Colubrina asiatica, Pluchea indica* and *Premna cordifolia*) using HPLC, HPLC-MS and spectrometric assays.

2. Identification and quantification of other phytochemicals including terpenoids (triterpenes, carotenoids), vitamins and volatile compounds presence in Malaysian traditional vegetables and their antioxidant activities using HPLC, HPLC-MS, GC-MS and spectrometric assays.

3. Screening for antimicrobial activities from the plant extracts and essential oils of Malaysian traditional vegetables against two different microorganism environments i.e. planktonic (free floating organisms) and sessile (biofilms).

CHAPTER 1

INTRODUCTION

Plants have contributed to the stability of the earth by producing oxygen from carbon dioxide during photosynthesis. They also have provided shelters for human, animal, insects and microorganisms. In the world, it is estimated that the total number of plant species is between 300,000 and 500,000. Of these, approximately 250,000 have been identified and classified (Frusciante 2000) and nearly 35,000 to 70,000 species are used for medicinal purposes all over the world (ICS-UNIDO 2006). At least 6,500 medicinal plant species were reported in Asia, which is one of the largest biodiversity regions of the world and in Malaysia, more than 1,200 species have been identified as medicinal plants (Chang *et al.* 2000). It was also reported that only 7,000 plant species are presently or have previously been cultivated as food crops (Frusciante 2000).

1.1 Medicinal plants, herbs, traditional vegetables and exotic vegetables

'Medicinal plants' or 'herbs' are defined by Encyclopædia Britannica as dried parts of various plants cultivated for their aromatic, pungent, or otherwise desirable substances usually used for medicinal purposes (Anon 2008a). Herbs are defined as a flowering plant, in which the stem does not become woody (Anon 2008b). Apart from medicinal uses, herbs usually come under the term 'spices and herbs', plants that are valued for flavour in cooking and as fragrances. They can consist of rhizomes, bulbs, bark, flower buds, stigmas, fruits, seeds, and leaves. Examples of Western herbs are thyme, parsley, rosemary, fennel and marjoram (Anon 2008b). In Western countries in the past, the priest employed herbs in worship, incantations, magical rites and rituals. This also occurs in Malaysia and in South East Asia, where traditional physicians called *bomoh* use herbs especially citrus for traditional ceremonies to protect against bad spirits (Saidin 2000). Today, all the traditional elements are neglected by the current generation, although plants are still used for medicinal purposes. Nowadays, with the latest technology and advances in laboratory facilities, the search for novel plant compounds continues after the discovery of vinca alkaloids for cancer treatment in the 1950s (Cragg and Newman 2005).

In Malaysia, 'traditional vegetables' are defined as indigenous plants either used in the daily diet or for medicinal purposes (Saidin 2000). The local term 'ulam' is used to differentiate between traditional vegetables and medicinal plants but traditional vegetables are a subset of medicinal plants (*Figure 1.1*). Western vegetables, in practice are vegetables such as carrot, potatoes, cabbage, lettuce and broccoli which are produced, sold and eaten in the daily diet.

Traditional vegetables also referred to as indigenous vegetables, though not necessarily indigenous to a country, can be associated with traditional planting and production systems, local knowledge of farmers, and history of local usage and selection (Keller *et al*. 2004). However, they are frequently under-rated in preference to introduced exotic vegetables (Rubaihayo 1995, 2002) and are widely under-utilised and neglected in research and development (FAO 1988). Hence, the potential of traditional vegetables has also not been properly evaluated. Traditional vegetables are normally perishable, low yielding and their value as commercial crops has not been explored. Typically, they are used as a regular side dish or a sauce accompanying staple foods such as rice, maize, cassava, sweet potatoes and yams (Rubaihayo, 1995, 2002). Unfortunately, the consumers have not been taught to appreciate the role of the traditional vegetables. Most of the traditional vegetables are produced throughout the developing world in small plots or are home grown. Therefore, by carrying out research on traditional vegetables, we can assess their nutritional and potential medicinal properties, and, as a consequence, provide a sound scientific base for promoting their consumption both locally and via a wider market.

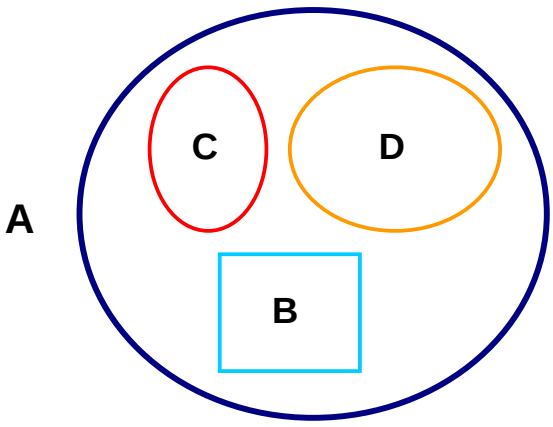


Figure 1.1 Schematic of medicinal plants (**A**), herbs (**B**), spices (**C**) and traditional vegetables (**D**) (Adapted from: Saidin 2000)

1. 2 Malaysian traditional vegetables

Malaysia is endowed with agro-climatic conditions suitable for the cultivation of a wide range of plants especially traditional vegetables. They are known locally as *ulam* and are domesticated and cultivated to some extent due to low level production by the local farmers. There has not been much research on species of traditional vegetables and little is known about their nutritional and medicinal values. Such information would help their promotion as important local commodities especially in Malaysia. In the present study, the phytochemical content and biological properties of the traditional Malaysian vegetables i.e. *Centella asiatica* (L.) Urban (pegaga), *Anacardium occidentale* L. (gajus), *Premna cordifolia* (bebuas), *Pluchea indica* (beluntas) and *Colubrina asiatica* (peria pantai) were investigated. These plants were obtained from Seberang Perai, Penang, Malaysia and the taxonomic hierarchy of each species is shown in *Table 1.1*. These vegetables have been long recognised to their healing properties and their use as a health tonic. The medicinal uses are shown in *Table 1.2.*

Table 1.1 Taxonomic hierarchy of the traditional vegetables investigated in the study (Anon 1996, Saidin 2000).

	Anacardium occidentale	*Centella asiatica*	*Colubrina asiatica*	*Pluchea indica*	*Premna cordifolia*
Kingdom	Plantae	Plantae	Plantae	Plantae	Plantae
Subkingdom	Tracheobionta	Tracheobionta	Tracheobionta	Tracheobionta	Tracheobionta
Division	Magnoliophyta	Magnoliophyta	Magnoliophyta	Magnoliophyta	Magnoliophyta
Class	Magnoliopsida	Magnoliopsida	Magnoliopsida	Magnoliopsida	Magnoliopsida
Subclass	Rosidae	Rosidae	Rosidae	Asteridae	Rosidae
Order	Sapindales	Apiales	Rhamnales	Asterales	Verbanales
Family	Anacardiaceae	Umbelliferae	Rhamnaceae	Compositae	Verbanaceae
Genus	Anacardium L.	Centella L.	Colubrina L.C. Rich. Ex Brongn	Pluchea Cass.	Premna L.

Table 1.2 Medicinal uses of selected Malaysian traditional vegetables used in the study

Botanicals	Common medicinal uses (traditionally)	Experimental studies		Part used	General references
Anacardium occidentale	folk remedy for diabetes mellitus	**Protective and hypoglycaemic effects in *in vivo*** (Kamtchouing *et al.* 1998)	*Rats* (streptozotocin-induced diabetes) Dose: 175 mg/kg – twice a day by gastric intubation Results: positive glycosuria, body weight loss, polyphagia & polydypsia	leaves	Ojewole(2003), Kögel and Zech (1985), Egwim (2005)
		Anti-inflammatory effects in rats (Olajide *et al.* 2004**)**	*Mice (*lipopolysaccharide -induced septic shock) Dose: 25-200 mg/kg Results: ↓levels of alanine and aspartat aminotransferase ($p<0.05$)	barks	
		Anti-microbial properties	Strain: *Escherichia coli*, *Pseudomonas aeruginosa,*(Dose 36 mg/ml)	leaves	Kudi *et al*. (1999)
			Showed activities against 13 bacterial strains including *Kleb. pneumoniae (*Dose 20 mg/ml)	bark	Akinpelu (2001)
Centella asiatica	o to treat leprosy in India and Madagascar o folk remedy for leucorrhoea and toxic fever o in improving memory and help to reduce mental fatigue, anxiety, and eczema	**Protective effects *in vivo*** (Hussin *et al.* 2007)	Rats (hydrogen peroxide induced) Dose: 0.3% to 5% extract for 35 weeks Results: ↓ level of MDA in blood	leaves	Kartnig (1988) Sahu *et al.* (1989) Kan (1986) Goh *et al.* (1995)
		Wound healing *in vivo* (Shukla *et al*. 1999)	Guinea pigs Dose: 0.5 to 10 mg/kg (twice a day for 7 days) Results: 56% ↑ in hydroxyproline, ↑ in tensile strength, ↑ collagen	leaves	

			content		
Colubrina asiatica	o improve digestion			leaves	Saidin (2000)
Pluchea indica	o to possess astringent and antipyretic action. o are used to reduce lumbago, leucorrhoea and dysentery o diaphoretics, nerve tonics and in poultices against atonic and gangrenous ulcer o reduces muscular pain, helps eliminate kidney stones, lowers blood sugar, promotes digestion o is a diuretic	**Anti-inflammatory activity** (Sen and Nag Chaudhuri 1991)	Rats and mice (carrageenin-, histamine-, serotonin-, hyaluronidase- and sodium mate-induced pedal inflammation. Dose: 20 mg/ ml extracts Results: inhibited protein exudation and leucocyte migration and inhibited granuloma formation	roots	Saidin (2000)
Premna cordifolia	o reduce the aroma of the fish during cooking. o to reduce fever and asthma o stimulate milk production in lactating mother.			leaves leaves and the roots shoots (young leaves)	Saidin (2000)

1.2.1 *Anacardium occidentale* L. (Anacardiaceae)

A. occidentale (Figure 1.2) is popular due to its seeds (cashew apple) which are known as cashew-nuts and are eaten throughout the world. However, the young leaves and the shoots are also widely consumed particularly in Malaysia, being either half-boiled or eaten fresh with rice. The plant is an ornamental tree up to 10 m in height (Kamtchouing *et al.* 1998) and there are red or yellow varieties based on the colour of the fruits (Maia *et al.* 2000). *A. occidentale* has been the subject of research investigation especially in Asia with 117 papers retrieved under Pubmed and 99 with Sciencedirect in January 2008. However, most of the papers are reporting anti-diabetic effects of root and bark extracts (Ojewole 2003, Egwim 2005), the biological activity of bark, stem and fruit extracts (Olajide *et al.* 2004, Davis *et al.* 2007, Konan and Bacchi 2007, Luiz-Ferreira *et al.* 2007), anacardic acid from the fruits (Green *et al.* 2007, Philip *et al.* 2007), toxicity studies with the fruits (Barcelos *et al.* 2007a, 2007b) and volatile compounds of the fruits (de Lourdes *et al.* 2005, Carasek and Pawliszym 2006, Trevisan *et al.* 2006). There is not much information on the phytochemical content of the shoots of *A. occidentale*, even though there are increasing amounts of research on antioxidant levels (Vimala *et al.* 2003). Abas *et al.* (2006) reported on the high antioxidant properties of this plant by measuring the production of hydroperoxide and its degradation product (malonaldehyde) resulting from linoleic acid oxidation using ferric thiocyanate and thiobarbituric acid methods. Radical-scavenging potential was also evaluated using the 1,1-diphenyl-2-picrylhydrazyl radical.. There is no data of surveillance in Malaysia on the dietary intake of this plant and other vegetables used in this study which make it difficult to evaluate their consumption in the daily diet.

Figure 1.2 *Anacardium occidentale*

The earliest report on phytochemicals in *A. occidentale* was in 1969 and consisted of a one-page report on the analysis of polyphenols in the leaves using paper chromatography detecting the presence of β-sitosterol, ethyl and methyl gallate and hyperoside (Subramanian *et al*. 1969). There are no studies on the analysis of phenolics in the leaves of *A. occidentale* using HPLC-MS. Other phytochemical studies have been on the identification of volatile constituents of the leaves, fruits and flowers of *A. occidentale (*Maia *et al.* 2000). The major constituents from the red-variety leaves were (E)-β-ocimene, α-copaene and δ-cadinene. The major constituents of the oil from red-variety fruits were palmitic and oleic acids, while palmitic acids, furfural, 4-hydroxydodecanoic acid lactone, (E)-hex-enal, (Z)-hex-3-enol and hexadecanol were the principal components identified in the oil of yellow *A. occidentale* fruits*.* The main constituents in the flowers of red varieties are β-caryophyllene, methyl salycilate and benzyl tiglate.

1.2.2 *Centella asiatica* (L.) Urban (Umbelliferae)

C. asiatica (*Figure 1.3*) from the family of Umbelliferae (Burkill 1966, de Padua *et al*. 1999) is known locally as *pegaga* and is very popular in Malaysia normally being served with meals. It is a slender, green, creeping plant, rooting at the nodes (Vimala *et al*. 2003). It is native to a number of countries including India, Sri Lanka and South Africa and is used in the Ayurvedic system of medicine to treat various diseases. Reports have indicated that fresh extracts of *C. asiatica* have been used as internal and external agents for wound healing (Kartnig 1988). Sahu *et al*. (1989) reported that in India and Madagascar, this plant was used to treat leprosy, while the Chinese prescribe the leaves for curing leucorrhoea and toxic fever (Kan 1986). In Malaysia, the herb is typically eaten fresh as a vegetable salad, especially by the Malay and Indian communities. It is also said to have beneficial effects in improving memory and in treating mental fatigue, anxiety, and eczema (Goh *et al.* 1995, Vimala *et al*. 2003). Eighteen different varieties of *C. asiatica* can be found in Malaysia (Jaganath and Teik 1999) and approximately forty species have been identified throughout the world (de Padua *et al*. 1999). In the present study, *the* variety *nyonya* was used as it is grown widely by the farmers in Malaysia and it is reported to have a higher triterpene content than other varieties (Rosalizan 2004). *C. asiatica* tonic and tablets of crude extracts have already been produced commercially with unsubstantiated claims that consumption can improve memory, promote healing and slow down ageing processes.

Figure 1.3 *Centella asiatica*

C. asiatica has been widely studied since 1950s and in January 2008, the number of publications under *Pubmed* and *Sciencedirect* were 169 and 53 respectively. One of the major researches with this plant has been on its bioactivities, especially antioxidant and anti-aging effects. The earliest report on phytochemicals in *C. asiatica* extracts was published by Singh and Rastogi (1968), who revealed the presence of the triterpene acids, brahmic acid, iso-brahmic acid and betulic acid, along with two saponins, brahmoside and brahminoside. These saponins were shown to be tri- and tetra-glycosides of brahmic acid. Triterpenes such as asiaticoside, madecasosside, asiatic acid and madecassic acid are reported to be biomarkers of this plant and currently, research is focussed on the bioactivities of these compounds (Inamdar *et al*. 1996, Wijeweera *et al*. 2006, Hussin *et al*. 2007, Yu *et al*. 2007). The antioxidant activities in *C. asiatica* extracts have been correlated to total phenolics by Zainol *et al.* (2003). This suggests that phenolic compounds are the major contributors to the antioxidant capacity of *C. asiatica*. The leaves and roots of two of the four varieties tested contained high levels of antioxidants, broadly comparable with the activity of α-tocopherol (Zainol *et al.* 2003).

1.2.3 *Colubrina asiatica* (L.) Brongn (Rhamnaceae)

Colubrina asiatica (*Figure 1.4*) or *peria pantai* from the family of Rhamnaceae is found in Eastern Africa to India, Southeast Asia, tropical Australia and the Pacific Islands (Jones 1996). It is called lather-leaf because of its ability to produce lather in water. It is a shrub with long, climbing or drooping branches that can reach more than 6 m in length (Saidin 2000, Jones 1996). The leaves are 3.5 to 13.5 cm long; egg shaped and easily recognised by their shiny, green upper surfaces and toothed edges. They are attached to stems by

slender stalks and are arranged alternately along the branches. Flowering produces clusters of small, greenish-white flowers at the base of petioles. The fruits are small capsules, measuring less than 1.5 cm across, initially green and fleshy, the capsules become dark brown with age. Each fruit contains three tiny greyish seeds (Jones 1996).

Figure 1.4 *Colubrina asiatica*

There is a dearth of information on the medicinal properties of *Colubrina asiatica* although it is claimed to improve digestion (Saidin 2000). There is also a lack of information on the phytochemicals and biological activities of this plant. Therefore, analysis of phenolics and biological activities of *Colubrina asiatica* were among the objectives of the present study.

1.2.4 *Pluchea indica* (L.) Lees. (Compositae)

P. indica (*Figures 1.5*) is locally known as *beluntas*. It is a shrub found widely in India and Malaysia. The height is more than one metre and it normally grows in wet-sandy soil. The leaves are obovate, 3-6 cm long and 1.5-2.5 cm wide, green and with an aromatic aroma (Saidin 2000). *P. indica* leaves and roots have been reported to possess astringent and antipyretic action. Preparations of leaves and roots are used to treat lumbago, leucorrhoea and dysentery and as diaphoretics, nerve tonics and in poultices against atonic and gangrenous ulcer (Sen and Nag Chaudhuri 1991, Vimala *et al.* 2003). *P. indica* is also reported to reduce muscular pain, help eliminate kidney stones, lower blood sugar, promote digestion and is a diuretic (Saidin 2000).

Publications as of January 2008 revealed only eight papers retrieved under *Pubmed* and four under *Sciencedirect*. The earliest phytochemical analysis by Uchiyama *et al*. (1991) who reported the identification of five new terpenoid glycosides, namely, linaloyl glucoside, linaloyl apiosyl glucoside, 9-hydroxylinaloyl glucoside, plucheosides A and B in the aerial parts of *P. indica*. On the other hand, the roots of *P. indica* contained a new monoterpene glycoside, plucheoside C, three new eudesmane-type sesquiterpenes, plucheols A, B, plucheoside E and three new lignan glycosides, plucheosides D1, D2, D3 together with a known eudesmane-type sesquiterpene. There are no other reports on phenolics or other phytochemicals or the antioxidant activity of *P. indica* leaves.

Figure 1.5 *Pluchea indica*

1.2.5 *Premna cordifolia* Roxb. (Verbanaceae)

P. cordifolia (*Figure 1.6*) is a shrub tree with a lot of branches, silara-shaped and shady up to 2-4 metres in height. The trunk is grey in colour, the leaves are aromatic and the shapes are ovate, acuminate and sometimes chordate in about 7-15 cm long and 3-9 cm wide, yellowish green in colour and which turn a darker green when mature (Saidin 2000). Fresh young leaves are consumed with rice and are also reported to help reduce the aroma of the fish during cooking. The information regarding the phytochemical and biological activities of this plant is scarce. It has been claimed that aqueous extracts of leaves and roots of *P. cordifolia* can reduce fever and asthma, while the young leaves can stimulate milk production in lactating mother (Saidin 2000).

Figure 1.6 *Premna cordifolia*

1.3 Fruit and vegetables and health effects

Traditional vegetables are very nutritious if they are consumed fresh or cooked at a medium temperature as they contain a lot of nutrients such as vitamins, fibres and minerals. Malaysian traditional vegetables are reported to have medicinal properties providing protection against diabetes, heart disease and problems with the digestive tract (Mustafa 1994). The nutritional components of the species used in the present study have been reported by Saidin (2000) and are shown in *Table 1.3*. Nutritional elements in the diet not only play a role in contributing to human health, but fruit and vegetables which have a variety of biological properties, have been linked to disease prevention (Hollman *et al*. 1996). For instance, fruit and vegetables are documented to be high in antioxidants, which delay the oxidation of other molecules by inhibiting the initiation or propagation of oxidising chain reactions by free radical, and therefore may reduce oxidative damage to the human body (Hollman and Arts 2000, Ismail *et al*. 2004). The occurrence of such oxidative damage is believed to be a significant causative factor in the development of chronic diseases such as cancer and cardiovascular disease (CVD) (Craig 1997, Proteggente *et al*. 2002, Arts and Hollman 2005). Therefore, the risk of these diseases could be reduced by increasing daily intake of fruits and vegetables such as broccoli, spinach, shallots, potato and carrots which are rich sources of antioxidants (Hertog *et al*. 1992, Cao *et al.* 1996, Heim *et al.* 2002). Apart from the antioxidant vitamins, fruit and vegetables also contain carotenoids, polyphenols and non-antioxidant vitamins which also responsible for the protective effects against cancer and CVD (Heim *et al*. 2002, Tucker 2003, Rao and Rao 2007).

Table 1.3 Nutritional components in 100 g of selected Malaysian traditional plants used in the present study (Saidin 2000)

Species	Components	Quantity (in 100g)
A. occidentale	moisture	89 g
	protein	3.8 g
	fat	0.2 g
	carbohydrate	4.5 g
	fibre	1.5 g
	calcium	53 mg
	phosphorus	29 mg
	ferum	3.7 mg
	sodium	7.0 mg
	potassium	332 mg
	carotene	2.1 mg
	vitamin A	326 µg
	vitamin B1	90 µg
	vitamin B2	1.2 mg
	niacin	0.5 mg
	vitamin C	91 mg
C. asiatica	moisture	88 g
	protein	2.0 g
	fat	0.2 g
	carbohydrate	6.7 g
	fibre	1.6 g
	calcium	171 mg
	phosphorus	32 mg
	ferum	5.6 mg
	sodium	21 mg
	potassium	391 mg
	carotene	2.7 mg
	vitamin A	422 µg
	vitamin B1	90 µg
	vitamin B2	190 µg
	niacin	100 µg
	vitamin C	29 mg
Colubrina asiatica	nd	nd
P. indica	nd	nd
P. cordifolia	protein	3.6 mg
	fat	0.4 mg
	carbohydrate	14 g
	phosphorus	55 mg
	potassium	393 mg
	calcium	116 mg

nd = not determined

However, other factors such as physical exercise and healthy lifestyle i.e. not smoking and lower fatty food intake, also contribute to human health (Panagiotakos *et al*. 2006, Ignarro *et al*. 2007). These factors as well as dietary intake of fruits and vegetables could be a solution to the quote “Prevention is better than cure”. It is certainly a more effective strategy than treatment of chronic diseases. Fibre, vitamins and non-vitamins contained in fruits and vegetables also exhibit other actions on human health other than as antioxidants. For example, fiber has shown to be able to bind bile salts which may reduce blood cholesterol levels. There is possibility of eating a lot of fruits and vegetables that will

decrease the intake of fat diet which could probably reduce the risk of chronic diseases (Lampe 1999).

1.4 Phytochemicals in plants

Plants have their own capacity to synthesise a diverse array of chemical compounds, for instance they can synthesise vitamin C which is different from humans and a number of animals (Valpuesta and Botella 2004). Understanding how phytochemicals function in plants may further our understanding of the mechanisms by which they impact on human health (Lampe 2003). Phytochemicals, the bioactive non-nutrient compounds in fruit, vegetables and other plant-based foods have been linked to a reduced risk of major chronic diseases (Kris-Etherson *et al.* 2002, Hu 2003, Liu 2003). It is estimated that more than 25,000 terpenoids, 12,000 alkaloids and 8,000 phenolics have been identified in plants (Lampe 2003), but a very large number still remain unknown and need to be identified and quantified before their health benefits can be evaluated (Hollman and Arts 2000). However, recent evidence suggests that the benefits of phytochemicals in fruit and vegetables may be even greater than anticipated because oxidative stress induced by free radicals which is involved in the aetiology of a wide range of chronic diseases can be stabilised by antioxidants (Liu 2003). To understand the contribution of the phytochemicals towards health effects, some of the important phytochemicals will be discussed i.e. flavonoids, chlorogenic acids, alkaloids, carotenoids, vitamins and volatile compounds.

1.4.1 Phenolic compounds - flavonoids

Phenolic compounds are widely distributed in plants and abundant in fruit and vegetables (Hollman and Arts 2000, Manach *et al.* 2005, Crozier *et al.* 2006a). One of the major groups of polyphenolic compounds, the flavonoids, are important in contributing to the flavours and colour of many fruit and vegetables (Hertog *et al.* 1993, Block and Langseth 1994). Flavonoids are widely distributed in leaves, fruits and barks of the plants (Heim *et al.* 2002). Over 5000 types of flavonoids out of approximately 8000 types of phenolics have been identified (Kris-Etherton *et al*. 2002; Marchand 2002), mainly as flavones, flavanones, flavan-3-ols, flavonols, anthocyanins, flavonones and isoflavones (*Figure 1.7 and 1.8*) (Rice-Evans *et al.* 1996, Bravo 1998). Flavonoids are C15, benzo-α-pyrone derivatives consisting of phenolic and pyrane rings (*Figure 1.7*) and they are classified according to substitutions. Flavonoids differ in the arrangements of hydroxyl, methoxy, and glycosidic

side groups, and in the conjugation between the B- and C- rings. During metabolism, hydroxyl groups are added, methylated, and glycosylated typically forming as 3-*O*-glycosides in foods (Heim *et al.* 2002, Crozier *et al*. 2006a).

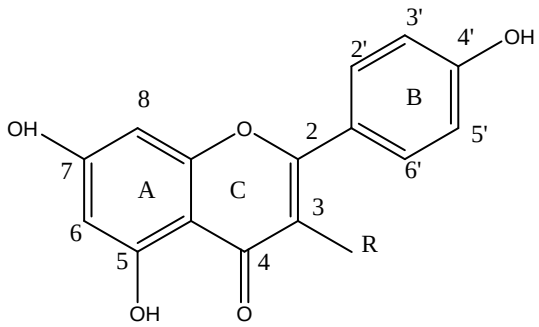


Figure 1.7 Basic structure of flavonoids (Cook and Samman 1996)

Flavonoids have been reported to exhibit wide range of biological effects including antibacterial, antiviral, anti-inflammatory, antiallergic and vasodilatory actions (Cook and Samman 1996, Di Carlo *et al.* 1999, Cushnie and Lamb 2005). In addition, they also inhibit lipid peroxidation, platelet aggregation as well as the activity of enzyme systems including cyclo-oxygenase and lipoxygenase (Korkina and Alfanas'ev 1997, Awad *et al.* 2001). Flavonoids exert these effects as antioxidants, free radical scavengers and chelators of divalent cations (Afanas'ev *et al*. 1989, Hollman and Katan 1999) and are reported to have unique cardioprotective effects (Rajadurai and Prince 2007). For example, rats fed a flavonoid-rich diet are reported to exhibit reduced myocardial post-ischemic damage (Heim *et al*. 2002). As the flavonoids are believed to be beneficial and occur in food, fruit and vegetables, investigation of flavonoids in Malaysian traditional vegetables was one of the objectives of the present study.

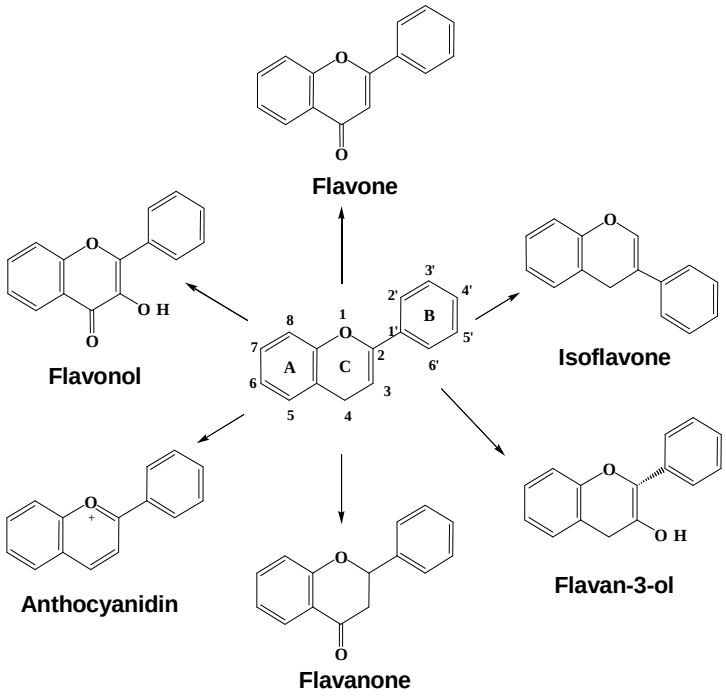


Figure 1.8 Structures of the six main classes of flavonoids (Crozier *et al.* 2006a)

1.4.1.1 Flavonols

Flavonols are the most ubiquitous flavonoids in foods, fruit and vegetables (Hollman and Arts 2000, Manach *et al*. 2004). The main representatives of flavonols are kaempferol, quercetin, isorhamnetin and myricetin and these components are shown in *Figure 1.9, 1.10 and 1.11.* Flavonols usually present in fruit and vegetables as glycosylated conjugates usually with the sugar moiety being either glucose or rhamnose. However, conjugation with other sugars such as galactose, arabinose, xylose and glucuronic acid does occur (Hollman *et al.* 1996, Crozier *et al*. 2006a). The levels of flavonols in plants vary greatly depending on the type of fruits and vegetables (Hertog *et al*. 1992). Fruit often contains between 5 and 10 different flavonol glycosides (Manach *et al*. 2004). Onions are one of the richest sources of flavonols (Hertog *et al*. 1992, Crozier *et al.* 1997a, 1997b) and other fruit and vegetables that contain high flavonols are the curly kale, leeks, broccoli, and blueberries (Craig, 1997). Over 200 flavonol aglycones have been identified in plants and the most common in fruits and vegetables are quercetin, kaempferol, myricetin and isorhamnetin (Robards and Antolovich 1997). In plants, *O*-glycosylation occurs preferentially at C-3 and the

predominant types in fruits are monoglycosides in the following order: 3-glucosides > 3-galactosides > 3-rhamnosides > 3-glucuronides (Llorach *et al*. 2003). The only diglycosides observed with any frequency in fruit and vegetables are the 3-rutinosides of quercetin and kaempferol.

1.4.1.2 Flavones

Flavones have a similar C-ring structure as flavonols with a 2-3 double bond but they lack a 3-hydroxyl group. They have a relatively limited distribution in fruits and vegetables compared to flavonols (Hollman and Arts 2000). Flavones are also found in plants as *O*-glycosides, but they also occur as *C*-glycosides (Hollman and Arts 2000, Manach *et al.* 2004). Examples of flavone glycosides are luteolin and apigenin of which parsley and celery are the main edible sources identified to date. Cereals such as millet and wheat contain of *C*-glycosides of flavones (Rice-Evans and Miller 1995, Crozier *et al.* 2006a). The skin of citrus fruit contains large quantities of polymethoxylated flavones such as tangeretin, nobiletin, and sinensetin. These polymethoxylated flavones are the most hydrophobic flavonoids (Manach *et al.* 2004). The structure of luteolin and apigenin are shown in *Figure 1.11*.

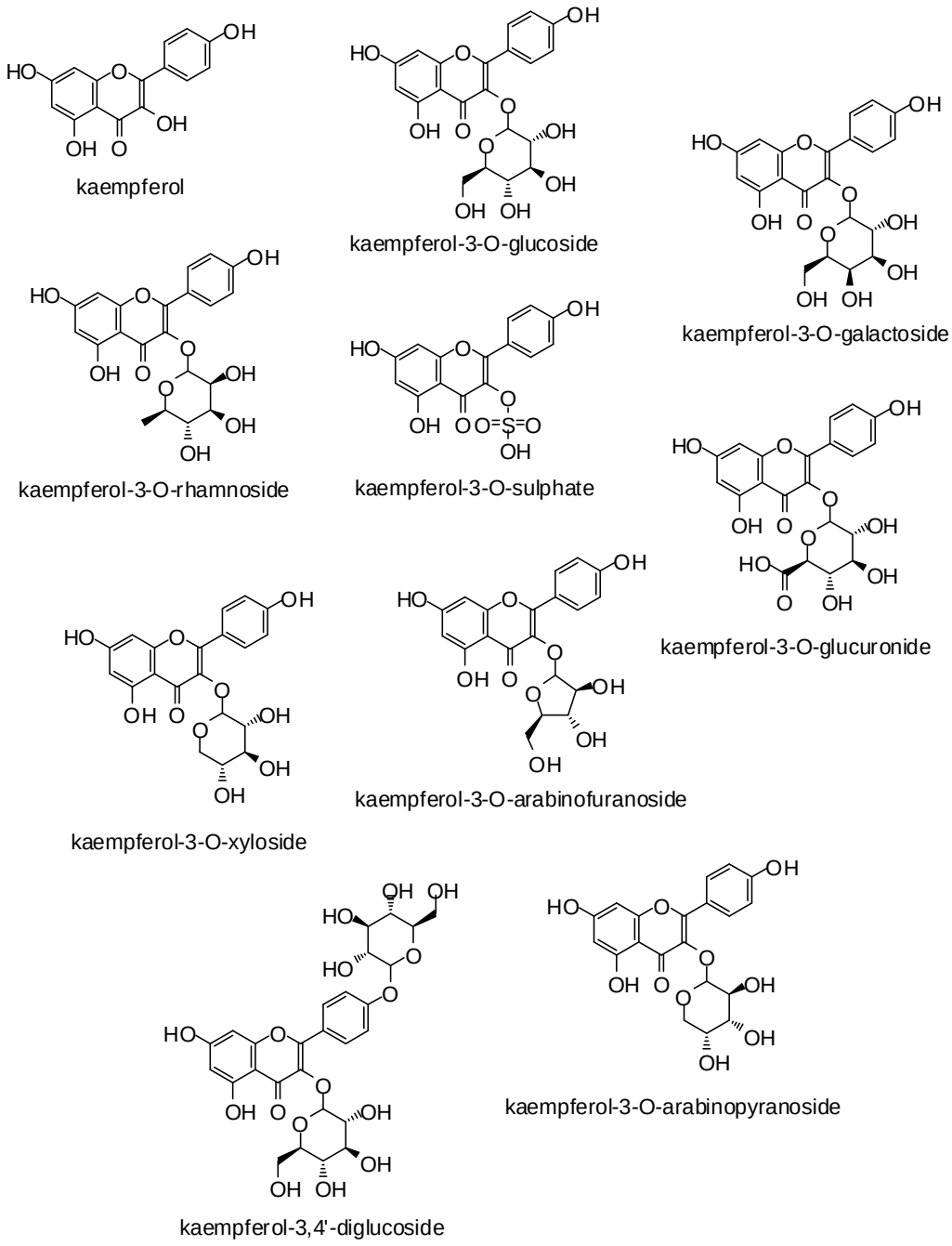


Figure 1.9 Structures of kaempferol and naturally occurred kaempferol glycosides

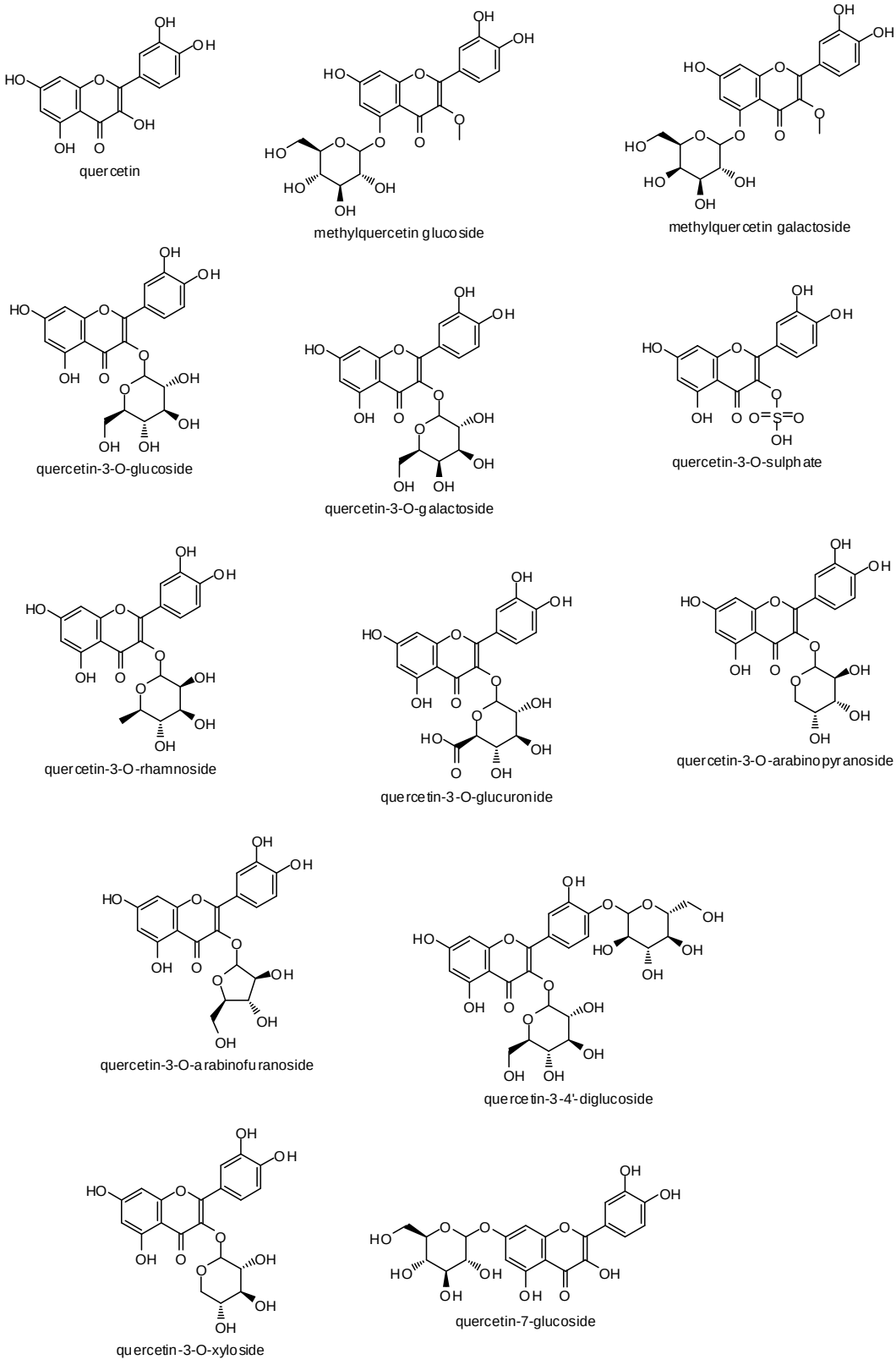


Figure 1.10 Structures of quercetin and naturally occurred quercetin glycosides

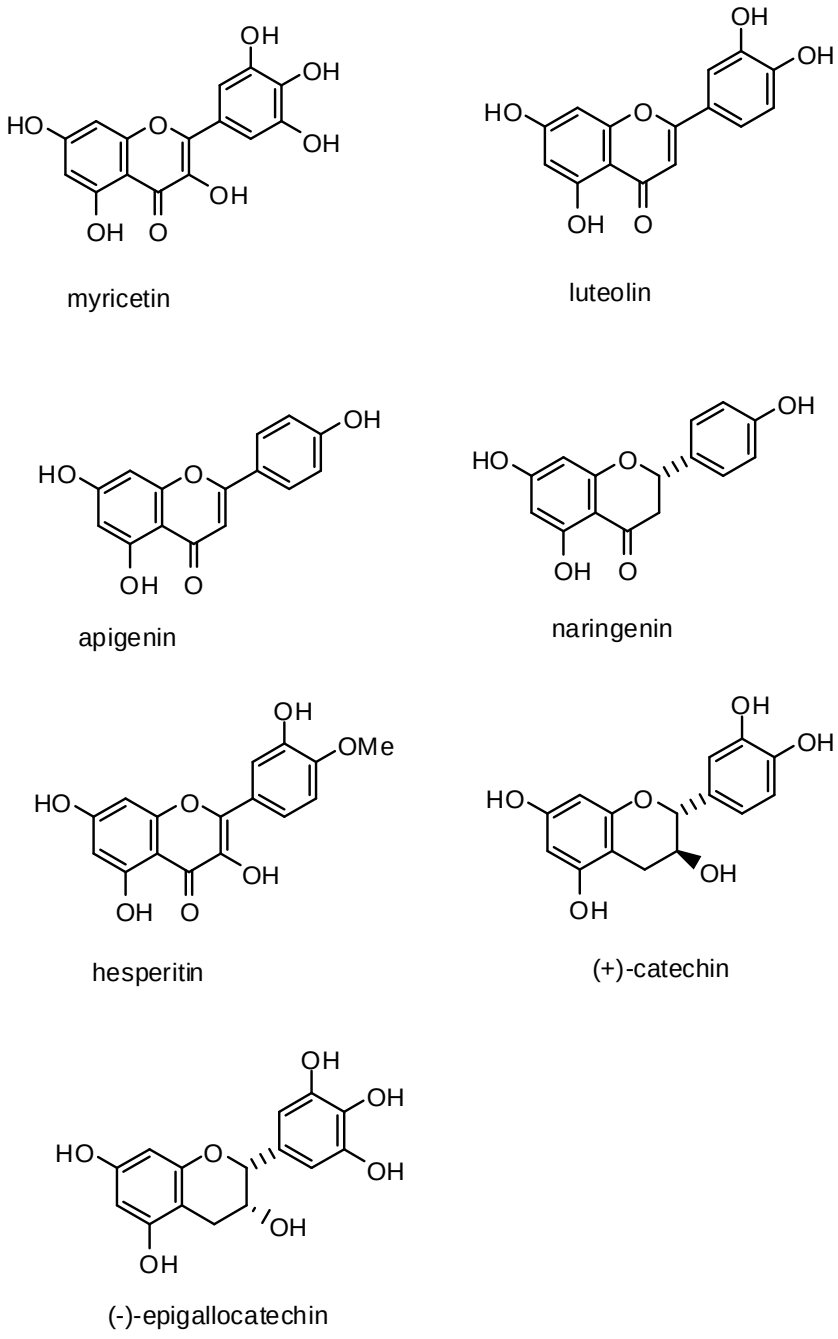


Figure 1.11 Other types of flavonoids

1.4.1.3 Isoflavones

Isoflavones are flavonoids with similar structure to oestrogens having hydroxyl groups in C-4 and C-7 in a configuration analogous to that of the hydroxyls in the estradiol molecule (Manach *et al*. 2004). This confers pseudo-hormonal properties including the ability to bind to oestrogen receptors and consequently, they are classified as phytoestrogens (Yasuda *et al.* 2004). Isoflavones are mainly found in leguminous plants such as soybeans and its processed products such as tofu and soy milk. Soybeans contain principally genistein, daidzein, and glycitein derivatives (Kudou *et al.* 1991). These isoflavones are found in four forms i.e. the aglycones, 7-O-glucosides, 6'-O-acetyl-7-O-glucosides and 6'-O-malonyl-7-O-glucosides of which the 6'-O-malonylglucoside derivatives are responsible for the unpleasant, bitter and astringent taste (Hollman and Arts 2000).

1.4.1.4 Flavanones

Flavanones are found in tomatoes and some aromatic plants such as mint. However, they are present in very high concentrations only in citrus fruits (Kanaze *et al.* 2003). The main aglycone is naringenin (*Figure 1.11*), which is present in grapefruit, hesperetin is found in oranges, and eriodictyol in lemons (Manthey *et al.* 2001). Glycosylation of flavanones typically to a disaccharide at C-7. Naringin-7-O-neohesperidose (naringin) in grapefruit has a strong better taste while hesperitin-7-rutinoside (hesperidin) and naringin-7-O-rutinoside (narirutin) in oranges, are tasteless (Manthey *et al.* 2001, Manach *et al.* 2004). It is reported that orange juice contains 200-600 mg hesperidin/L and 15–85 mg narirutin/L and a single glass of orange juice may contain between 40 and 140 mg flavanone glycosides, however the whole fruit may contain up to five times more flavanones than a glass of orange juice (Manach *et al.* 2004).

1.4.1.5 Flavan-3-ols

Flavan-3-ols are the most complex subclass of flavonoids existing both as monomers, such as catechins, and polymers such as proanthocyanidins (Manach *et al*. 2004, Crozier *et al*. 2006a). Catechins are predominantly found in green tea but also in many types of fruit as well as red wine and chocolate. An infusion of green tea contains up to 200 mg catechins, however black tea contains fewer monomers of flavan-3-ols than green tea which due to their oxidization during the fermentation leading to the appearances of more complex

condensation products known as theaflavins (dimers) and thearubigins (polymers) (Manach *et al.* 2004). The flavan-3-ols (+)-catechin and (−)-epicatechin are the main components in fruit whereas (+)-gallocatechin, (−)-epigallocatechin, and (−)-epigallocatechin gallate are found in high concentration in green tea (Shahidi and Naczk 2004). In contrast to other types of flavonoids, flavan-3-ols are not glycosylated in foods, fruit or vegetables (Manach *et al*. 2004, Crozier *et al.* 2006b).

Proanthocyanidins, which are also known as condensed tannins, are dimers, oligomers, and polymers of flavan-3-ol monomers that are linked between C-4 and C-8 (or C-6) (Guo He 2000). Their mean degree of polymerization in foods was until recently, rarely determined, however, it has been reported that in cider apples, the mean degree of polymerization ranges from 4 to 11 (Manach *et al.* 2004). As the condensed tannins can form a complex with salivary proteins, they are responsible for the astringent character of fruit, such as in grapes, peaches, kakis, apples, pears and berries and beverages including wine, cider and tea. They are also responsible for the bitter taste of dark chocolate (Crozier *et al.* 2006a).

1.4.1.6 Anthocyanidins

Anthocyanins are a conjugated form of anthocyanidins and are the pigments dissolved in the vacuolar sap of the epidermal tissues of flowers and fruit which exhibit a pink, red, blue, or purple colour (Milbury *et al.* 2002). Anthocyanidins are more unstable than anthocyanins. The most commonly found anthocyaninidins are cyanidin, pelargonidin, delphinidin, peonidin, petunidin and malvidin, which are invariably found as sugars conjugates in plants (Crozier *et al*. 2006a). In the human diet, anthocyanins are found in red wine and certain leafy and root vegetables but they are most abundant in fruits (Shahidi and Naczk 2004). Anthocyanins accumulate mainly in the skin of fruits except for certain types of red fruit, such as cherries and strawberries, where they also occur in the flesh. Red wine contains up to 200–350 mg anthocyanins per litre and they are transformed into various complex structures as the wine ages (Manach *et al.* 2004)

1.4.2 Chlorogenic acids

Chlorogenic acids are a family of esters formed between certain trans-cinnamic acids and quinic acid, which has axial hydroxyls at C-1 and C-3 and equatorial hydroxyls at C-4 and C-5. The best-known conjugate is 5-caffeoylquinic acid, commonly referred to as chlorogenic acid and another conjugate called neochlorogenic acid which is 3-caffeoylguinic acid, a term which should now be used to refer only to the family of related quinic acid conjugates (Clifford 2000). Chlorogenic acids are characteristic components of coffee beans and commercial coffee products of which caffeoylquinic, p-coumaroylquinic, feruloylquinic, dicaffeoylquinic, and caffeoylferuloylquinic acids have been reported to occur (Clifford *et al.* 2003). The structures of chlorogenic acids are shown in *Figure 1.12*. Chlorogenic acids are also widely distributed in the plant kingdom and are common constituents of beverages, fruits and vegetables common in the human diet (Newmark 1984, Kitts and Wijewickreme 1994). Chlorogenic acids have been reported to possess protective effects on methylazoxymethanol acetate-induced intestinal and hepatic tumorigenesis in hamsters (Mori *et al*. 1986). It was also reported that chlorogenic acids have little effect in modulating hepatic xenobiotic activating-detoxification exzymes, P-450 and glutathione transferase (GSH) in benzo(α)pyrane-induced mice suggesting the integral role in inhibiting and modulating the carcinogenic potential of reactive xenobiotics (Kitts and Wijewickreme 1994).

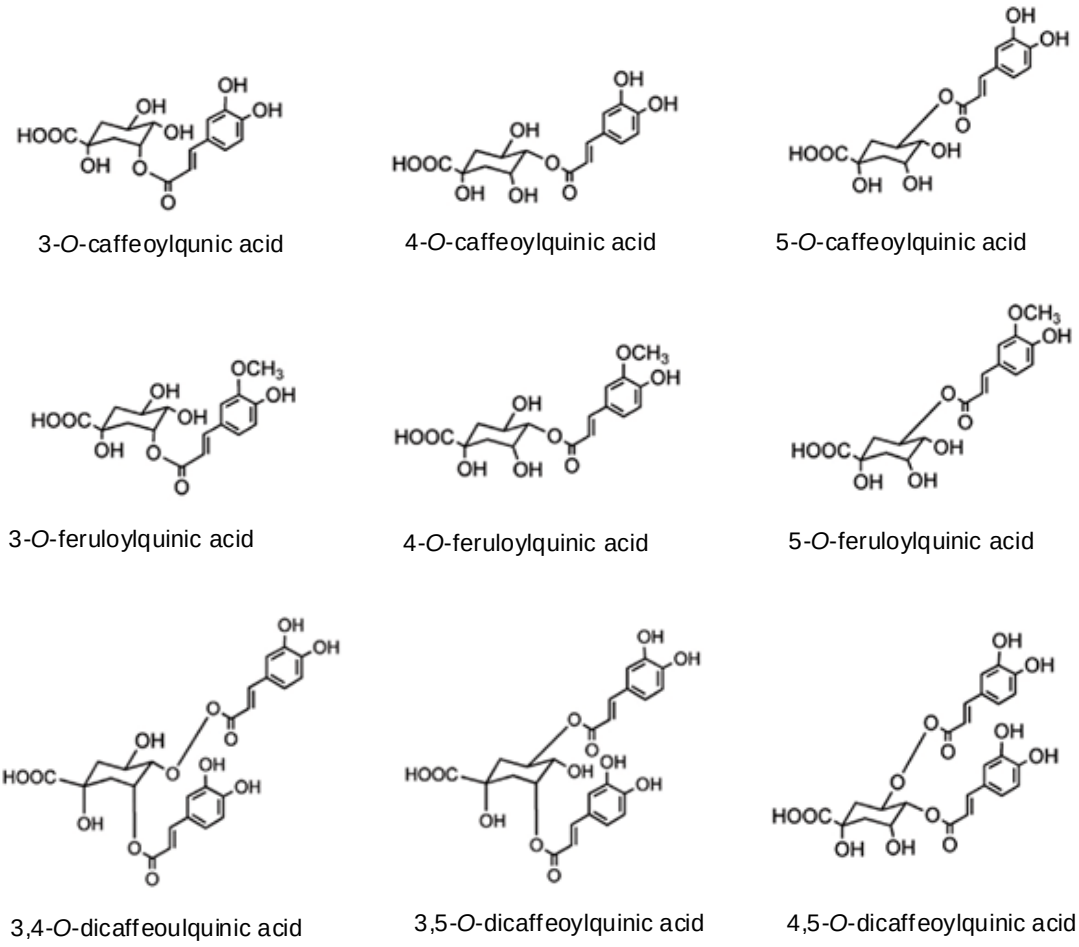


Figure 1.12 Family of chlorogenic acids

1.4.3 Vitamins

1.4.3.1 Carotenoids as pro-vitamin A

Phytochemicals may be important in preventing health problems but vitamins are essential in the diet to prevent deficiency disease and some of the vitamins are important antioxidants. Carotenoids are a family of pigmented compounds that are responsible for yellow, orange and red colour in plants. They are synthesized by plants and microorganisms, but not animals and humans (Rao and Rao 2007). Fruits and vegetables constitute the major sources of carotenoids in the human diet, which are thought to be responsible for the beneficial effects in preventing human diseases including CVD, cancer and other chronic diseases (Agarwal and Rao 2000, Cooper 2004). More than 600

carotenoids have so far been identified in nature, although only about 40 are present in a typical human diet. Of these, approximately 20 carotenoids have been identified in human blood and tissues. Ninety percent of dietary carotenoids intake is represented by α-carotene, β-carotene, lycopene, lutein and cryptoxanthin (Rao and Rao 2007).

Epidemiological studies have associated the increased consumption of carotenoid-containing fruit and vegetables with a lower risk of certain cancers, age-related macular degeneration and CVD (Erdman 1999, Cooper 2004). However, whether it is the carotenoids themselves or the fact that carotenoid intake or blood carotenoid concentrations are markers for other protective, bioactive components of those fruit and vegetables, is yet to be determined. Recently, the antioxidant properties of carotenoids has been the major focus of research as their antioxidant activity has been suggested as being the main mechanism by which they afford their beneficial effects (Lee *et al.* 2004, Rao and Rao 2007). Several *in vitro* animal and human experiments have demonstrated the antioxidant properties of carotenoids such as β-carotene and lycopene (Cooper 2004). Intake of these compounds also have been shown to be inversely related to the risk of cardiovascular diseases and cancers, whereas, lutein and zeaxanthin reduce the risk of disorders related to the eyes (Agarwal and Rao 2000).

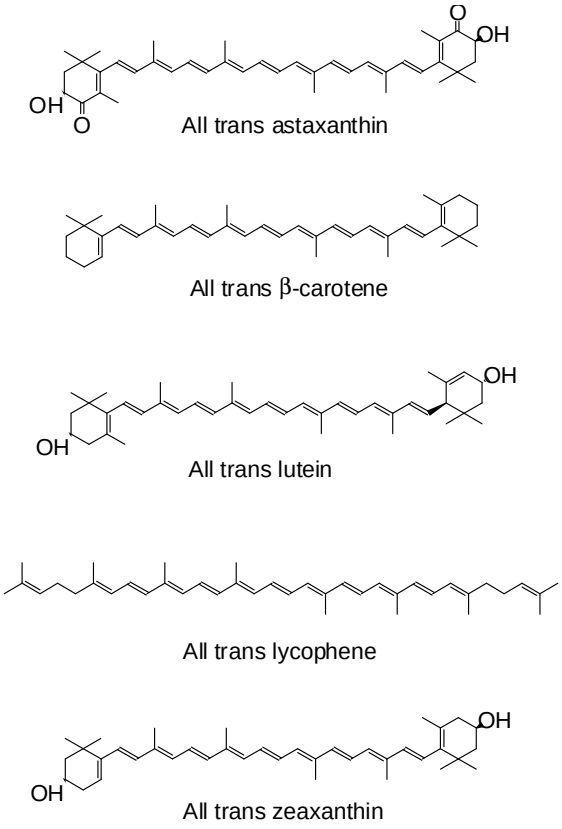


Figure 1.13 Structures of carotenoids

1.4.3.2 Vitamin C

Vitamin C also known as ascorbic acid is a 6-carbon lactone ring structure with 2,3-enediol moiety (*Figure 1.14*) (Lee *et al*. 2004) and L-ascorbic and dehydroascorbic acid are the major dietary forms. Ascorbic acid is widely distributed in fruits such as orange, lemons, grapefruit, watermelon, papaya, strawberries, mango, pineapple, raspberries, cherries and in green leafy vegetables such as tomatoes, broccoli, green and red peppers, cauliflower and cabbage (Naidu 2003). Most plants and animals synthesize ascorbic acid from D-glucose or D-galactose. Humans do not, due to the absence of the enzyme L-gulono-1,4-lactone oxidase. A majority of animals produce relatively high levels of ascorbic acid from glucose in liver but the guinea pigs, fruit eating bats and apes, like man do not synthesize ascorbic acid. Therefore, these animals, and human specifically need to be supplemented with vitamin C through food and/ or tablets (Kim and Lee 2004). Ascorbic acid in foods is mainly in the reduced form approximately 80% to 90% and is absorbed in the human intestine by a sodium-dependent active transport system. It is believed to be absorbed

better than dehydroascorbic acid (Lee *et al.* 2004). As a labile molecule which is sensitive to heat, ascorbic acid can be lost from foods during cooking or processing, even though it has the ability to preserve foods by virtue of its reducing property (Naidu 2003). Therefore, it is advised to eat fresh fruit and vegetables and avoid cooking as far as possible to preserve the vitamin C.

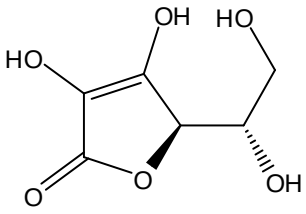


Figure 1.14 Structure of vitamin C

1.4.3.3 Vitamin E

Like vitamin C, vitamin E (i.e. α-tocopherol) functions as an antioxidant in various chemical reactions due to its characteristic structure (*Figure 1.15*) (Kim and Lee 2004) which consist of a chroman ring and a long, saturated phytyl chain (Lee *et al*. 2004). Vitamin E is made up of eight related compounds, classified as tocopherols and tocotrienols of which each class consists of four forms i.e. α, β, γ and δ. α-Tocopherol is among the most widely available form in food and is also found in many supplements. It has been shown to have anticancer activity and cholesterol-lowering ability, whereas, tocotrienols have been reported to inhibit low density lipoprotein (LDL) oxidation better than tocopherols (Watkins *et al.* 1999).

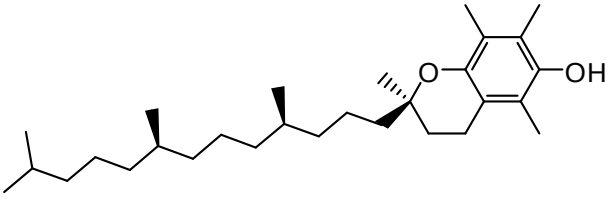


Figure 1.15 Structure of α-tocopherol

1.4.4 Alkaloids

The majority of drugs currently in common use are derived from alkaloids, for example paclitaxel or Taxol® (*Figure 1.16*) from the plant *Taxus brevifolia*. Alkaloids, which are nitrogen containing compounds, created via diverse biosynthetic pathways, continue to provide an extensive range of therapeutic compounds (Bruneton 1995, Rathbone *et al.* 2002).

There is much interest in the identification of new alkaloids either as drugs or as pivotal intermediates in the synthesis of new drugs. The use of alkaloids as drugs started in the early 1950s, when camptothecin isolated from the stem of the Chinese ornamental *tree Camptotheca acuminate* was shown to be an effective anticancer agent (Srivastava *et al.* 2005). Many alkaloids derived from plants have anticancer properties. The anticancer drug Taxol® has been reported to exhibit side effects including numbness, nausea and migraine (Singla *et al*. 2002). This is not uncommon, and as a consequence, there is a continuing search for new anticancer compounds that are active and selective with minimal side effects.

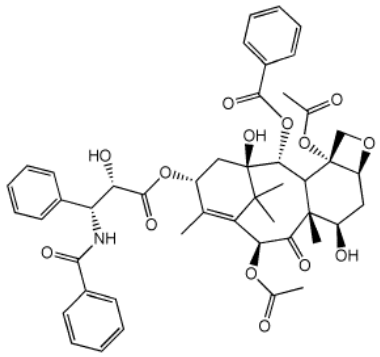


Figure 1.16 Structure of Taxol®

1.4.5 Plant essential oils and volatile compounds

Plant volatile compounds are usually isolated through mechanical pressing and steam distillation (Sacchetti *et al*. 2004). They are chemicals responsible for characteristic odours and have important ecological functions for the plants in attracting pollinators, assisting seed dissemination and in resisting attack by predators or pathogens (Vaughn 2001). Plant essential oils, as well as their purified or synthesized constituents, are used nowadays on a large scale in the food and cosmetics industries due to their attractive flavours and scents. In addition to a characteristic flavour, many essential oils and their ingredients have been shown to exhibit a range of biological activities including antibacterial and antifungal properties (Stammati *et al.* 1999).

There are several groups of compounds primarily responsible for the odours associated with essential oils. Terpenes or terpenoids, are compounds formed by the linking of two or more five-carbon isoprene units which are generally present in the highest amounts in

plant essential oils (Vaughn 2001). Terpenes are grouped according to the number of isoprene units, however, only the monoterpenes (two isoprene units), sesquiterpenes (three units) and, to a much lesser extent, the diterpenes (four units) are found in nature as volatile compounds (Grabmann 2005). Citrus fruits contain among the highest quantities of volatile compounds (Choi *et al.* 2000) and tea tree oil, the popular topical antiseptic and fragrance, contains a lot of volatile compounds. More descriptive studies on tea tree oil are discussed in *Chapter 5*. Examples of some of the predominant volatile compounds present in fruit and vegetables are carvacrol, limonene, terpinen-4-ol, terpinene and citral. The structures of some of the volatile compounds are shown in *Figure 1.17*.

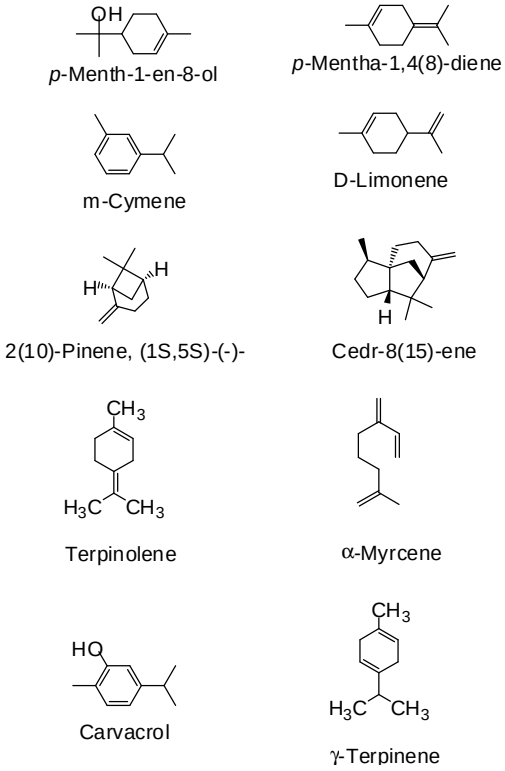


Figure 1.17 Structures of volatile compounds

1.5 Phytochemicals and health effects

CVD and cancer continues to be a leading cause of morbidity and mortality among adults in Europe and in the US (McGovern *et al.* 1996) and it was recently reported that Scotland has the highest mortality rate from CVD for men and the second highest for women amongst other industrial countries (Anon 2001). Fruit and vegetables that contain significant amounts of

bioactive phytochemicals may provide desirable health benefits beyond basic nutrition, and play important roles in the prevention of chronic diseases (Hollman *et al*. 1996, Galati *et al.* 2000, 2002). The key question is whether a purified phytochemical derived from plants has the same health benefit as a whole fruit or vegetable or a mixture of them in which the phytochemical is present. Different parts of plants contain different levels of phytochemicals and bioactivities. For example, it was reported that vitamin C in apple skin accounted for only 0.4% of total antioxidant activity of the fruit even though total antioxidant activity of apples is higher compared to other fruits (Liu 2003). Many researchers have suggested that most of the antioxidant activity of apples may come from phytochemicals such as phenolics and flavonoids rather than vitamin C (Pearson *et al*. 1999, Chinnici *et al*. 2004, Crozier *et al.* 2006a). It is also proposed that the additive and synergistic effects of phytochemicals in fruits and vegetables are responsible for their substantial antioxidant and anticancer properties as well as other relevant biological activities in humans (Liu 2003, Kris-Etherton *et al.* 2004). However, to answer this, more research is needed to investigate the relationship of the specific phytochemicals and their contribution to human health.

1.5.1 Flavonoids, dietary intake, bioavailability, absorption and health effects

Flavonoids are the most abundant antioxidants in the diet and their total dietary intake could be as high as 1 g per day, which is much higher than that of all other classes of phytochemicals and known dietary vitamin antioxidants (Scalbert *et al*. 2005). The *in vitro* antioxidant activities have been extensively studied, but it is still not clear whether there are significant *in vivo* beneficial effects of flavonoids (Harborne and Williams 2000). Many other biological activities for flavonoids have been described, but it is not clear to what extent flavonoids actually contribute to human health. However, some recent studies have indicated that flavonoids have value as anticancer agents, and as antimicrobial and anti-inflammatory compounds (Marchand 2002, Cragg and Newman 2005, Cushnie and Lamb 2005). For example, the antimicrobial properties of propolis against *Streptococcus* and *Bacillus* species have been attributed to its high flavonoid content and in particular the presence of the flavonoids galangin and pinocembrin (Pepeljnjak *et al*. 1982, Bosio *et al.* 2000). Another example, a novel flavonoid structure, rohitukine, isolated as the constituent responsible for anti-inflammatory and immunomodulatory activity from *Dysoxylum binectariferum* Hook (Meliaceae), was found to possess tyrosine kinase activity and potent growth inhibitory activity against a series of breast and lung carcinoma cell lines (Cragg and Newman 2005).

Epidemiology studies conducted by Duthie *et al.* (2003) have shown that dietary intakes of flavonoids in Scotland are as presented in *Table 1.4*, which indicate that vitamin C and catechins are the major compounds in the Scottish daily diet from drinking tea and eating fruit and vegetables, which contain high amounts of vitamin C. However, such studies are lacking in Malaysia and information is not available in international journals. Therefore, research needs to be increased in this field to estimate the dietary intake of fruit and vegetables in Malaysia, in particular traditional vegetables. Hollman and Katan (1999) reported that estimation of the total flavonoid intake is difficult due to individual variations, limited data on contents of fruits and vegetables and different species or source of the plant tested. Based on the studies conducted in The Netherlands (Hertog *et al*. 1992, 1993), estimation of flavonoids intake can be used as a guidance and a starting point of further research. Tea has been reported to be the major source of phenolics in The Netherlands accounting for approximately 48% of total intake followed by onions (29%) and apples (7%).

Table 1.4 Estimated daily intake of flavonoids, vitamin E and vitamin C in North East Scotland (Duthie *et al.* 2003)

Flavonoid	Median (mg)	Minimum (mg)	Maximum (mg)
Flavonols	19	2	51
Flavones	0.1	0	7
Procyanidins	23	0	145
Catechins	59	2	263
Flavanones	1	0	239
Vitamin E	8	5	11
Vitamin C	65	37	108

Data obtained from 4 days weighted intakes (n=81)

There is evidence from epidemiological studies that intake of flavonoid containing fresh fruit and vegetables is associated with low rates of cancer (Hollman *et al.* 1996, Hollman and Katan 1999, Williamson and Manach 2005). It is estimated that the daily intake of all flavonoids is a few hundreds of milligram per day expressed as the aglycones (Hollman *et al.* 1997). This is in contrast with data of Kuhnau (1976) who reported that the total intake of flavonoids in the USA was 1 g per day per person expressed as glycosides or 650 mg per day expressed as aglycones. However, these large differences may reflect the use of inaccurate-analytical methodology by Kuhnau (1976), as well as other factors such

as population, race and individual variation among participants (Hertog *et al.* 1993, Manach *et al.* 2004). A lot of questions need to be answered as the content of flavonoids in fruit and vegetables also vary due to the factors previously mentioned. For instance, it was shown the quercetin levels in edible vegetables were below 10 mg/kg, except for onions, kale, broccoli and beans, which contain up to 486 mg/kg. In most fruits, quercetin averaged 15 mg/kg, except for apples which had between 21 to 72 mg/kg. However, these values may be low since there may be loss of flavonols during acid hydrolysis used prior to analysis (Manach *et al*. 2004). Even though the estimations of flavonoids content vary with each study, flavonoids are nonetheless receiving interest from consumers, food processors and researchers because of their perceived importance and association with the prevention of chronic diseases (Amiot *et al.* 1997).

The absorption and subsequent distribution, metabolism and excretion of flavonoids in humans are now the subject of much research (Manach *et al.* 2004). The flavonoids are present in foods as β-glycosides conjugates and originally it was thought that only the aglycones were able to be transported across the intestinal wall (Kuhnau 1976). However, it is now known that some flavonoids glycosides can be absorbed intact in the small intestine via the sodium-dependent glucose transporter SGLT1 (Hollman *et al.* 1995a, Walle *et al.* 2000). In the wall of the small intestine, they are hydrolysed and appear in the bloodstream as methylated, sulphated and glucuronidated metabolites (Donovan *et al.* 2006). The studies described by Hollman *et al.* (1995a, 1995b) were focused only on quercetin and quercetin glycosides. In the study with ileostomy volunteers, absorption of orally administered quercetin aglycone was 24% and the absorption of quercetin glycosides from onions was 52%, and 17% for pure quercetin rutinose (Hollman *et al*. 1995a, 1995b). It can be suggested that humans absorb appreciable amounts of quercetin and the absorption of glycosides is possible. Analysis of quercetin metabolites in plasma dan urin after the ingestion of onions has been described by Mullen *et al*. (2006). Five flavonol glycosides, quercetin-3'sulphate, quercetin-3-glucuronide, isorhamnetin-3-glucuronide, a quercetin diglucuronide and a quercetin glucuronide sulphate have been detected in quantifiable amount in plasma after 0.6 to 0.8 h indicating the absortion and metabolism of flavonols from onions in humans. Intake of other flavonoids should be investigated, to get a more complete view and understanding of their impact on disease prevention as well as their fate in human following ingestion.

1.5.2 Vitamins and health effects

There is evidence of correlation of vitamin intake with a reduced risk of chronic diseases (Rimm *et al.* 1993, Stampfer *et al.* 1993, Naidu 2003). Vitamin A performs important biological functions being essential for vision, deficiency can result in eye diseases such as macular degeneration (Grabmann 2005). Vitamin E supplement for a longer period has been associated with a reduced incidence of coronary disease but in contrast, the benefits of vitamin C supplements have proved inconsistent in reducing the risk of myocardial infarction in epidemiological studies (Kaikkonen *et al*. 2001). The low risk of having these diseases is in part, due to the antioxidant activity of compounds that lowers lipid peroxidation in man. Vitamin A, C and E have been reported to show antioxidant activities, however they can also act as pro-oxidants (Schneider 2005). The inconsistent results of the epidemiology studies with these vitamins may be a reflection of the lack of long-term clinical trials into the effects of vitamin C or E supplementation on lipid peroxidation *in vivo*. Kaikkonen *et al*. (2001) conducted a study on the long-term effects of vitamin C (500 mg) and E (200 mg α-tocopheryl acetate) on plasma F2-isoprostane, a biomarker of lipid peroxidation *in vivo*. It was shown in this study with 100 healthy men that vitamin E but not vitamin C lowered lipid peroxidation by 17%. This finding may provide a mechanism for the observed ability of vitamin E supplements to prevent atherosclerosis.

1.6 Environmental effects on the level of phytochemicals

Phytochemicals are ubiquitous in plants which have multiple functions in plant protection, attraction of insects for seed dispersion and pollination or in self-defence (Dey and Harborne 1997). Environmental conditions can influence the level and types of phytochemicals present in plants. Sunlight can greatly influence the level of phytochemicals inducing the accumulation of flavonoids (Hertog *et al*. 1992). Flavonols have been reported to accumulate in the outer and aerial tissues of plants i.e. skin and leaves and their biosynthesis is stimulated by light (Herrmann 1976). Marked differences in concentration exist between individual fruit on the same tree and even between different sides of a single piece of fruit, depending on exposure to sunlight (Robards and Antolovich 1997). Similarly, in leafy vegetables, such as lettuce and cabbage, the glycoside concentration can be up to ten-fold higher in the green outer leaves than in the inner light-colour leaves (Manach *et al.* 2004). Quantitative studies on fruit flavonols and anthocyanins particularly in relation to genetic and environmental variability have also been reported (Milbury *et al.* 2002). Hertog

et al. (1992) found that the level of flavonols varied in leafy vegetables, such as lettuce, endive and leek, due to seasonal influences with levels in summer being 3- to 5-times higher than in other seasons. Flavonoids are synthesised in plants via the shikimic pathway (Dixon and Paiva 1995) and the key enzyme, phenylalanine ammonialyase (PAL) that catalyzes the biosynthesis of phenolic compounds, is responsive to environmental stresses such as high UV light, low temperature, pathogen infection or nutrient deficiency (Dey and Harborne 1997, Winkel-Shirley 2002). It was hypothesised that an organic agricultural production system would increase the level of phytochemicals especially phenolic compounds, but it was shown that there was no significant different of the level of total phenolic content between lettuce and collard samples grown under organic and non-organic environments (Young *et al.* 2005). Understanding of the effects of the environmental factors on the level of phytochemicals is essential for the production of the fruit and vegetables to get the maximum and optimum levels of phytochemical which in the end, will benefit the consumers.

1.7 Biological activities

In recent years, investigation of biological activities of phytochemicals has increased as searches for novel compounds have raised attention to understand the bioactivity and health effects. To this end, a number of methodological approaches have been used (Horan *et al.* 2003, Kris-Etherton *et al*. 2004). Early stages of experimental design are of priority as shown in *Figure 1.18*. Different types of experimental approaches are used with *in vitro* studies and investigations with animals and humans. At early stages, *in vitro* and *in vivo* studies are used to attempt to understand the mechanisms of action before the long-term effect studies. However, this process can take several years before the final stage - a clinical trial is reached (Gurib-Fakim 2006). Advance techniques such as the availability of cDNA micro and macro arrays, modern proteomic methods and comprehensive cell signalling analysis allows for thorough and simultaneous examination of many potentially affected metabolic pathways in one experiment (Kris-Etherton *et al.* 2004). Identification of bioactive compounds from plants and establishing their health effects are a priority as there are exciting prospects that selected beneficial components will reduce the risk of many diseases such as cardiovascular disease and cancer (Ong 2004). Recent evidence has established that cardiovascular disease can be due to inflammation and consequently can be treated through compounds that exhibit anti-inflammatory effects. The protective effects

of antioxidant compounds are also related to reduced inflammation *in vitro* and *in vivo* (Heim *et al.* 2002).

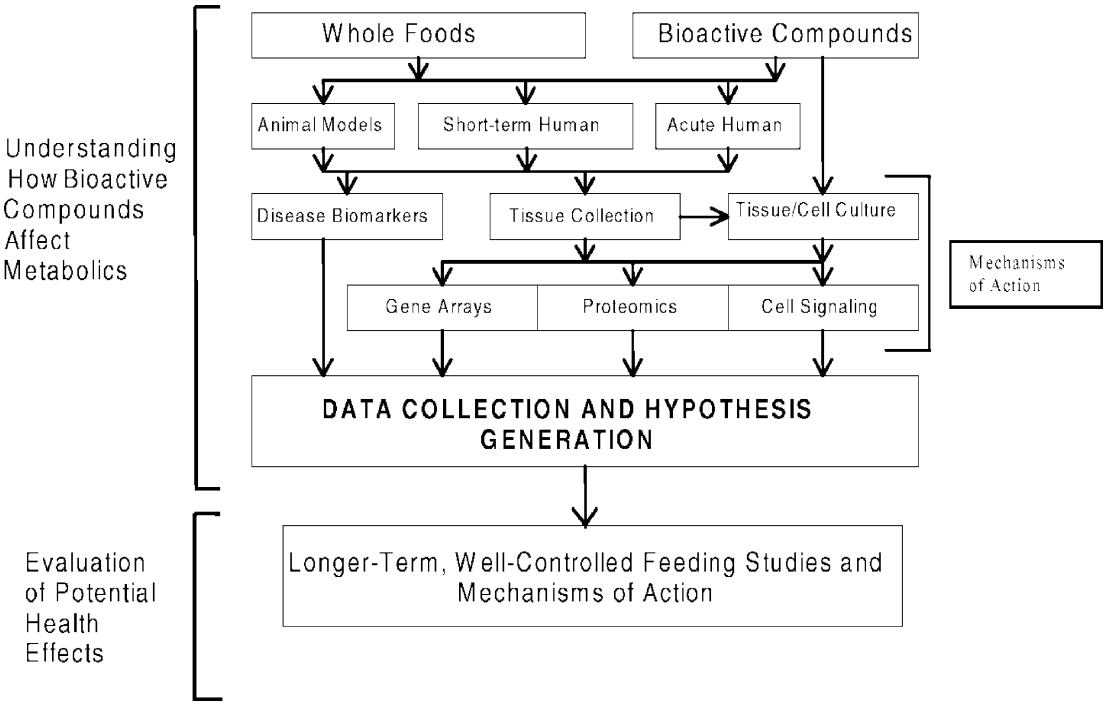


Figure 1.18 Experimental approach for assessing biological effects of bioactive compounds (Kris-Etherton *et al.* 2004)

1.7.1 Antioxidants

Oxidative stress is caused by the imbalance between oxidants and antioxidants, potentially leading to damage in plants and in humans (Grabmann 2005). Approximately 1-3% of the oxygen consumed in human's body is converted to superoxide and other reactive oxygen species (ROS) called free radicals under physiological conditions (Fridovich 1986, Halliwell 1996). Even though these free radicals perform many important physiological processes such as cell signalling, microbial killing and gene transcription (Dröge 2002, Grabmann 2005), they may also damage DNA, protein or lipids in the human body (Halliwell 1996). These deleterious effects are reported to be responsible for chronic diseases mentioned before (Halliwell 1994, Heim *et al*. 2002). To counteract the threat of free radical-induced damage, the human body has developed an antioxidant defence system, which consists mainly of antioxidant enzymes such as superoxide dismutase (SOD) or catalase and radical

scavengers like ascorbic acid or tocopherols (Grabmann 2005). However, during physical stress, such as exercise or certain disorders i.e. fever, this antioxidant system is affected and enhanced, which request the body to keep the balance between antioxidants and prooxidants (Clarkson 1995, Clarkson and Thompson 2000). Therefore, to improve the antioxidant defense, one easy way is to increase the dietary intake of antioxidants from food mainly fruits and vegetables, which contain bioactive compounds such as vitamin C, carotenoids and importantly polyphenolics (Harborne and Williams 2000, Kim and Lee 2004).

Recent studies have emphasised the importance of antioxidants and the mode of action of specific flavonoids as bioactive components of the diet *in vivo* and *in vitro*. Thus, it is important to have a clear idea of the major phenolic antioxidant compounds and their levels in fruit and vegetables (Hollman *et al*. 1996). Proteggente *et al*. (2002) reported the antioxidant capacities of extracts from selected fruit and vegetables assessed using the Trolox Equivalent Antioxidant Capacity (TEAC), the Ferric Reducing Antioxidant Potential (FRAP) and Oxygen Radical Absorbance Capacity (ORAC) assays. Fruit such as strawberry, raspberry and red plum, which are rich in anthocyanins had the highest antioxidant activities, followed by those rich in flavanones, such as orange and grapefruit, and flavonols (e.g. onion, leek, spinach and green cabbage), while the hydroxycinnamate-containing fruit (e.g. apple, tomato, pear and peach) consistently elicited lower antioxidant activities (Proteggente *et al*. 2002). *In vitro* studies have shown the relationships between flavonoid structure and antioxidant activity (Rice-Evans *et al.* 1996, Awad *et al.* 2001). Quercetin, the most potent antioxidant among the flavonoids, has three structural properties which contribute to its activity. First, the number and configuration of hydroxyl groups on A and B rings especially the dihydroxycathecol structure of the B ring. Secondly, the planarity of the molecule and third, the double bond in relation to the 4-oxo group of the C ring (Rice-Evans *et al*. 1996). Other antioxidant properties of flavonoids were reported to stabilise unpaired electrons (Duthie *et al.* 2003), scavenge free radicals from lipid peroxidation (Nijveldt *et al.* 2001), reduce the incidence of DNA damage (Anderson *et al.* 2000) and the ability to chelate with transition metal ions, which results in the inhibition of the reactive oxygen species production (Duthie and Crozier 2000). There is limited information on the antioxidant activities of Malaysian traditional vegetable although recently, the screening of biological activities such as anticancer, antioxidant and anti-inflammatory *in vitro* has been reported (Chang *et al.* 2000). For example, extracts from *Alpinia galanga* and *Cayratia*

japonica, used traditionally to treat cancer, have been shown to possess cytotoxicity against human lung and breast cancer cell lines (COR L23 and MCF 7) with the IC_{50} 7.8 and 23.9 µM respectively (Lee and Houghton 2005). Extracts from *Centella asiatica* have also been shown to exhibit high antioxidant activities using linoleic acid and TBARS assays (Hussin *et al*. 2007). Therefore, investigation of the biological activities of these plants should be a priority to understand the potential health effects of Malaysian traditional vegetables.

1.7.2 Anticancer

Phytochemicals derived from plants have been an important source of several clinically useful anti-cancer agents such as vinblastine, vincristine, camptothecin derivatives, topotecan and irinotecan, etoposide and paclitaxel (Taxol®) (Cragg and Newman 2005, Gurib-Fakim 2006). The search for anti-cancer agents from plants started in 1950s with the discovery of the vinca alkaloids, vinblastine and vincristine. As a result, the United States National Cancer Institute (NCI) initiated an extensive plant collection programme in 1960 that focused mainly in temperate regions, to expand the search of plants with anticancer properties (Cragg and Newman 2005). This has led to the discovery of many novel compounds showing a range of cytotoxic activities including the taxanes and camptothecins which spanned a period of 30 years from the early 1960s to the 1990s to be developed clinically (Mukherjee *et al.* 2001). The plant collection program was terminated by NCI in 1982. However, the development of new screening technologies led to the revival of plant collections with a focus on the tropical and sub-tropical regions of the world (Gurib-Fakim 2006). To date, new plant derived clinical anticancer agents have not yet reached the stage of general use but a number of anticancer agents are in a preclinical development but may take several years before they can be fully applied in medical treatment (Mukherjee *et al.* 2001, Cragg and Newman 2005). Phenolic compounds have also being reported to possess anticancer properties (Galati *et al.* 2000, Ren *et al.* 2003). The role of phenolic compounds particularly flavonoids in the prevention of cancer is associated with their ability to influence cancer-inducing processes *in vivo*. Quercetin and myricetin have been reported to suppress hydrogen peroxide-induced DNA damage in isolated human lymphocytes (Kim *et al.* 2003), inhibited protein kinase which is responsible for regulating tumour promotion and regulation of cell growth (Srivastava 1985), exhibit antiproliferative effects and induce apoptosis, a programmed cell death in cancer cell lines (Kuo 1996, Iwashita *et al.* 2000).

1.8 Future drugs from plants

Plants have played a significant role in human life as food, shelter and stability of the ecosystem. Most importantly to humans, it is currently estimated that 50% of all drugs in clinical use has been derived from natural products, and at least 25% of all prescription drugs contain ingredients extracted from higher plants (Carr *et al.* 1993, Mohammed 1999). A well known example is Taxol®, a potent anticancer drug derived from *Taxus brevifolia*. Traditionally, according to WHO, 80% of the world population, primarily in developing countries, rely on plant-derived medicines for their healthcare. In terms of medicinal usage, it does not mean that synthetic drugs are not used in western medicine rather than some of them derived are from plants (Gurib-Fakim 2006). Anticancer drugs like Taxol® can be very profitable as it is estimated to be worth an average of $94 million to a private drug company (Mohammed 1999). In the US, pharmaceutical chemicals constitute a $11.4 billion market and in Europe, the industry is worth about $9.3 billion (Mohammed 1999). With the increasing demand of herbal medicine products, nutraceuticals and natural products for health care all over the world, plant extract and essential oil manufacturers and producers have started to use the latest extraction technology to produce high quality products to meet demands (ICS-UNIDO 2006). However, these products are labelled as nutraceuticals and supplements as the development of pure drugs will take a longer time frame for research and development especially in clinical trial stages (Cragg and Newman 2005) from 10 to 20 years (Cragg 1998). *In vitro* bioassays have resulted in the discovery of some novel therapeutic agents and are continually revealing compounds from plants used in traditional medicines, which help explain their traditional usage. They may also be valuable in providing evidence on the modes of action of plant compounds, which have shown activity in clinical or *in vivo* studies (Houghton *et al.* 2005). In the context of the scenario in Malaysia, research searching for novel compounds from Malaysian plants is in its infancy and should be a priority as Malaysia has extensive plant diversity but probably lacks management, support and facilities. However recently, the government has started to highlight the importance of biotechnology and bio-prospecting of medicinal plants as the demands on this industry have increased.

CHAPTER 2

MATERIALS AND METHODS

Materials and methods described in this Chapter are used generally for all the Chapters but specifically for Chapter 3 and Chapter 4. Materials and Methods used in this study in *Chapter 5* are described independently in that particular chapter.

2.1 Planting material

Two batches of seven varieties from five species of selected Malaysian traditional vegetables were chosen in these studies. There are *Centella asiatica* (L.) (pegaga), *Anacardium occidentale* L. (gajus), *Colubrina asiatica* (peria pantai), *Pluchea indica* (beluntas) and *Premna cordifolia* (bebuas). Fresh samples of *Centella asiatica* (L.) Urban (pegaga) were collected from Durian Tanah, Melaka, Malaysia from a local farmer. The first batch was harvested in September 2004 (wet season) and second batch was in February 2006 (dry season). Fresh samples of *Anacardium occidentale* L. (gajus), *Colubrina asiatica* (peria pantai), *Pluchea indica* (beluntas) and *Premna cordifolia* (bebuas) were collected from the Traditional Vegetables Garden, MARDI Seberang Perai, Penang, Malaysia and characterization of the accessions of the specimens were verified with MARDI Seberang Perai and MARDI Herbarium, Serdang, Selangor, Malaysia. The Passport data for all the specimens are shown in *Table 2.1*. All the fresh samples were weighted and oven-dried 40°C overnight. The moisture content was determined as shown in *Table 2.2*. The dried vegetables were weighed and ground using a Waring blender (Buchi, Japan). The powdered samples were weighed, sealed in an aluminum bag and stored at -80°C.

2.2 Chemicals and reagents

Quercetin-3-*O*-glucoside, quercetin-3-*O*-rutinoside, myricetin-3-*O*-rhamnoside and gallic acid were obtained from Sigma (Poole, Dorset, UK). Kaempferol, kaempferol-3-*O*-rutinoside and 5-*O*-caffeoylquinic acid (chlorogenic acid) were supplied by AASC Chemicals (Southampton, UK). Quercetin-3-*O*-glucuronide was extracted and purified by partitioning against ethyl acetate and fractioned using preparative reversed-phase HPLC from French beans (*Phaselous vulgaris*) by Alison Sutcliffe. Methanol (AR and HPLC grade), ethanol,

hexane and acetonitrile (HPLC grade) were purchased from Rathburn Chemicals (Walkerburn, Peebleshire, UK). Acetic acid was from BDH (Poole, UK). Formic acid, phosphoric acid, metaphosphoric acid and Folin-Ciocalteu's phenol reagent were supplied by Sigma (Poole, Dorset, UK).

Table 2.1 Information on the Malaysian traditional vegetables and their origin.

Specimens	Accession Number	Catalogue Number	Origin
Centella asiatica (L.) Urban (pegaga)	-	9102/ 9728/ 9729	Bumbung Lima, Melaka, Malaysia
Anacardium occidentale L. (gajus)	153/184/185	45/ 5189	Pajak Song, Kepala Batas, Malaysia
Colubrina asiatica (peria pantai)	6/128	9976	Bumbung Lima & Permatang Pauh, Perai, Malaysia
Pluchea indica (beluntas)	13	8039	Bumbung Lima, Perai, Malaysia
Premna cordifolia (bebuas)	15/ 147	8615/ 9215	i) Kg MakNan, Penaga, Perai, Malaysia ii) Kg. Keda, Sg. Tengas, Kedah, Malaysia

Table 2.2 Moisture content of selected Malaysian traditional vegetables

Batch 1

Species	Fresh Weight (g)	Dry Weight (g)	Weight reduction (%)	Moisture Content (%)
Centella asiatica	243	42	17	83
Pluchea indica A	238	44	19	81
Pluchea indica B	210	41	20	80
Anacardium occidentale (Red)	44	15	35	65
Anacardium occidentale (Yellow)	166	38	23	77
Premna cordifolia	320	76	24	76
Colubrina asiatica	263	62	24	77

Batch 2

Species	Fresh Weight (g)	Dry Weight (g)	Weight reduction (%)	Moisture Content (%)
Centella asiatica	352	37	11	89
Pluchea indica A	206	37	18	82
Pluchea indica B	174	31	18	82
Anacardium occidentale (Red)	274	61	22	78
Anacardium occidentale (Yellow)	202	40	20	80
Premna cordifolia	130	27	20	79
Colubrina asiatica	156	34	22	78

Disodium carbonate (Na_2CO_3), di-sodium hydrogen ortho-phosphate and sodium dihydrogen orthophosphate dihydrate were obtained from BDH Chemicals Ltd (Poole, UK). Concentrated hydrochloric acid, disodium hydrogen phosphate (Na_2HPO_4) and sodium hydroxide (NaOH) were obtained from Fisher Scientific (Loughborough, Leicestershire, UK). Ammonium dihydrogen phosphate buffer was purchased from Fluka Biochemika, UK. Pentane was purchased from Fisher Scientific (UK). All other chemicals and reagents were obtained from Sigma-Aldrich (Poole, Dorset, UK) unless otherwise stated.

2.3 Extraction of plant materials

2.3.1 General extraction for phytochemical analysis

Five grams of powdered sample was extracted with 40 mL of methanol containing 0.1% HCl for 1 h using a shaker (IKA KS 130 basic, UK). After which the samples were centrifuged (SORVALL® LEGEND RT, UK) at 3000g for 20 min. The supernatant was filtered using 0.22 µm membrane (MILLIPORE Millex GP, Ireland) before being reduced to dryness in vacuo using a rotavapor (Buchi Rotavapor R200, Japan). The extracts were weighted and dissolved in 10 mL methanol to get the concentration and kept in -80 ºC prior to analysis.

2.3.2 Extraction for phytochemical analysis and antimicrobial screening

The extraction procedure used was as described in *Section 2.3.1* except that the tissue remaining after the original methanol extraction was dried and further extracted on shaker with 40 mL of distilled water for 1 h. After centrifugation at 3000g for 20 min, the aqueous supernatant (aqueous fraction) was collected and stored at -80°C prior to analysis. A similar procedure was followed for methanol and distilled-water to get a methanol and aqueous extracts.

2.3.3 Extraction for GC-MS analysis

Essential oils from fresh leaves of selected Malaysian traditional vegetables, harvested in February 2006 were extracted using a modified simultaneous distillation extraction technique based on Licken Nickerson apparatus and steam distillation (Togari *et al.* 1995) at the Food Technology Centre, MARDI. Two hundred g of fresh samples were added with 200 mL of distilled water and extracted with 40 mL of pentane. The extraction process was

carried out for 2 h. Then, 2 g of anhydrous sodium sulphate was added to pentane to remove residual water. A Whatman filter paper no. 141 was then used to separate the sodium sulphate from the oils. The oil was concentrated under a stream of nitrogen gas and kept at -80ºC until use. All of the essential oils were transported in a special container from Malaysia to Glasgow for GC-MS analysis. As a control, *Melaleuca alternifolia* oil was purchased from Boots Plc, Byres Road, Glasgow, UK.

2.4 Determination of total phenolic content

This method determines phenols and oxidized substances by producing a blue colour from reducing yellow heteropolyphosphomolibdate-tungstate anions (Singleton and Rossi 1965). In brief, 200 µL of a different dilution of sample was added to 10 mL of a 1:10 diluted Folin and Ciocalteu reagent and 1.8 mL of distilled water. After 5 min, 7.0 mL of a Na_2CO_3 solution (115 g/L) was added and the reaction mixture was left at room temperature for 2 h. The absorbance of the solution was read at 765 nm against water blank on a UNICAM UV500 (ThermoSpectronic, UK) spectrophotometer. The optical density (OD) was compared to a standard curve prepared with 50 to 500 mg/ml gallic acid and results are expressed as gallic acid equivalents (GAE).

2.5 Extraction and analysis of carotenoids and α-tocopherol

The levels of carotenoids (lutein, zeaxanthin, *t*-canthaxanthin, β-cryptoxanthin, *trans*-lycopene, an α- and β-carotene mixture) and α-tocopherol were measured in vegetables extracts by reversed phase HPLC with absorbance and fluorescence detectors (Hess *et. al.* 1991). A 200 µL sample was added to 200 µL water and 400 µL ethanol and mixed for 10 s. To this 700 µL hexane was added, mixed and centrifuged for 5 min. The upper hexane layer was removed, reduced to dryness and dissolved in 200 µL of 1,4-dioxan:ethanol:acetonitrile (1:1:3, v/v). After shaking for 5-10 min, a 150 µL of sample was injected onto a 4 µm Phenomenex Synergy Hydro-RP 250 x 20 mm (i.d.) column with a Phenomenex C18 guard cartridge and eluted at a flow rate of 0.3 mL/min. Echinenone was used as an internal standard, which was provided by Mr. Stephen Combe, Division of Environmental and Evolutionary Biology. The coefficient of variation of the recovery of compound from the extraction was less than 5%. Analysis of total carotenoids was carried out without a column attached but connected with a guard cartridge. Analysis of total and

individual carotenoids was carried out using Finnigan Spectra System SP8880 ternary HPLC pump, UV6000LP UV detector, SN4000 interface, AS3000 auto-injector, SCM1000 degasser and Waters 474 fluorescence detector at a flow rate of 0.6 ml/min for 55 min with the gradient system shown in *Table 2.3*. Spectrum data was analysed by Xcalibur software.

Identification was carried out by comparing the retention time of standards and absorbance spectra (λ_{max}). The carotenoids (lutein, zeaxanthin, *t*-canthaxanthin, mix-carotene, lycopene and β-cryptoxanthin) were monitored at 450 nm. α-Tocopherol was analysed with a fluorescence detector (290/310 nm) and quantified at λ_{max} = 290 nm. Quantification of each compounds was based on the standard curve ranged from 0 – 500 ng.

Table 2.3 Gradient solvent system for the analysis of carotenoids and α-tocopherol using HPLC

Time (min)	Composition of mobile phase (%)	
	A (acetonitrile 2.5% H_20)	**B** (ethyl acetate 2.5% H_20)
0	100	0
30	60	40
40	10	90
45	10	90
46	100	0
55	100	0

2.6 Extraction and analysis of ascorbic acid

Analysis of ascorbic acid was based on the method described for fruit and vegetables with a slight modification (Robert and Gordon 2003). Five g of plant materials were mixed with 50 mL of 5% metaphosporic acid (MPA) (Sigma, UK): methanol (70:30 v/v) and mixed using a mechanical shaker (IKA KS 130 basic, UK). The extracts were filtered through Whatman No. 1 filter paper (Whatman, UK). Both filtrates were combined and reduced to dryness in vacuo. The dried extracts were stored at -80 ºC prior to analysis.

For HPLC-analysis, the extracts were dissolved in MPA:methanol (1:10 w/v). The samples were analysed using Surveyor gradient HPLC system comprising an HPLC pump, photodiode array absorbance (PDA) detector scanning from 250 to 700 nm, and an autosampler cooled to 6°C (Thermo Finnegan, USA). Separations were carried out using a Phenomenex Nucleosil ODS 5 μm 250 x 4.6 mm i.d. C18 reverse-phase column with Phenomenex C18 guard cartridge (Torrance, USA) maintained at 15°C, eluted isocratically at 1 ml/ min for 15 min a mobile phase of 20 mM ammonium dihydrogen phosphate buffer pH 2.4 containing 0.15 % MPA. The injection volume was 20 µl, the detection was at 245 nm and data were analysed by Xcalibur software. Quantification of vitamin C was based on the ascorbic acid standards ranged from 0 – 500 ng.

2.7 HPLC-PDA-MS^2 analysis of selected Malaysian traditional vegetables

The extracts were analysed using a Surveyor gradient HPLC system comprising an HPLC pump, photodiode array absorbance (PDA) detector scanning from 250 to 700 nm, and an autosampler cooled to 6°C (Thermo Finnegan, USA). Separations were carried out using a Phenomenex RP-MAX 4 μm 250 x 4.6 mm i.d. C12 reverse-phase column with a Phenomenex C18 guard cartridge (Torrance, USA) maintained at 40°C, eluted at 1 ml/ min with a 90 min gradient of a 10–20% gradient of acetonitrile in water containing 0.1% formic acid for *A. occidentale* and 60 min gradient of a 5-40% gradient of acetonitrile in water containing 1.0% formic acid for other species. Separation of phenolics was detected by PDA analysis at 365, 330 and 280 nm. After the mixture passed through the flow cell of the PDA detector, the column eluate was split and 20% was directed to a Finnegan LCQ Advantage mass spectrometer with an electrospray interface (ESI), operating in full scan MS mode from 150 to 1000 amu. Samples were analysed using both positive and negative ionisation modes. ESI-MS parameters were as follows: potential of the ESI source, 4 kV; capillary temperature, 400°C. Spectrum data was analysed by Xcalibur software.

2.8 HPLC and HPLC-PDA-MS^2 analysis of triterpenes in *Centella asiatica*

The analysis was carried out as described in *Section 2.7* but with a 60 min gradient system of a 20-60% gradient of acetonitrile in water containing 0.05% phosphoric acid. Separation of triterpenes was detected by PDA at 205 nm. Spectrum data was analysed by Xcalibur software. Tunning and calibration were based on four types of triterpenes standards - asiatic acid, madecassic acid, asiaticoside and madecasosside.

2.9 GC-MS analysis of essential oils of selected Malaysian traditional vegetables

The samples were analysed by GC-MS (Thermo Finnigan and Trace DSQ) using ZB-5MS (30 m x 0.25 id. X 0.25 µm) capillary column (Phenomenex, USA). The GC-MS conditions were as follows; injection volume (1 µL), temperature programme 80˚C to 160˚C for 5 min at 10˚C/min; 160˚C to 235˚C for 5 min at 5˚C/min and 235˚C to 290˚C for 5 min at 50˚C/min.; injector temperature (280˚C), MS transfer line (290˚C), ion source (200˚C) spit ratio (1:10) and mass range at 50-450. Data was analysed by Xcalibur software and compared to a SI (standard index) from the NIST library available and for certain compounds were confirm by co-chromatogram of standards.

2.10 2, 2'-Azinobis - 3 - ethylbenzothiazoline- 6 - sulphonic acid ($ABTS^+$) decolourisation assay

The antioxidant activities of phenolics in Malaysian traditional vegetables were measured using the radical cation ($ABTS^{\bullet+}$) on-line decolourisation assay (*Figure 2.1*) (Dapkevicius *et al*. 2001, Koleva *et al*. 2001). A 2 mM $ABTS^+$ stock solution containing 3.5 mM potassium persulphate was prepared and incubated at room temperature in darkness overnight to allow the stabilisation of the radical. $ABTS^+$ reagent was prepared by diluting the stock solution 8-fold in phosphate buffer at pH 8. Triplicate of five µL samples were injected into a HPLC system comprising a LC pump, a PDA detector and a UV-VIS detector (Surveyor HPLC, Thermo Finnigan). The eluent from the autosampler was mixed with the $ABTS^+$ reagent at a flow rate of 0.5 mL/min supplied by a Shimadzu LC-10 AP VP liquid chromatography pump. A Shimadzu GT-1543 vaccuum degasser was used to remove any

oxygen in the reagent prior to mixing. After mixing through a 3 m x 0.25 mm i.d. loop, the absorbance was measured by a UV detector at 720 nm (Nemphlar Bioscience, Lanark, UK). Data were analysed using Thermo Finnigan Chromquest™ software version 4.0. The chromatography column was removed to obtain a large peak representing the total area of the phenolics indicating the total antioxidant activity of the compounds in the extracts. The total antioxidant activity was quantified against a Trolox standard calibration curve and expressed as Trolox equivalent.

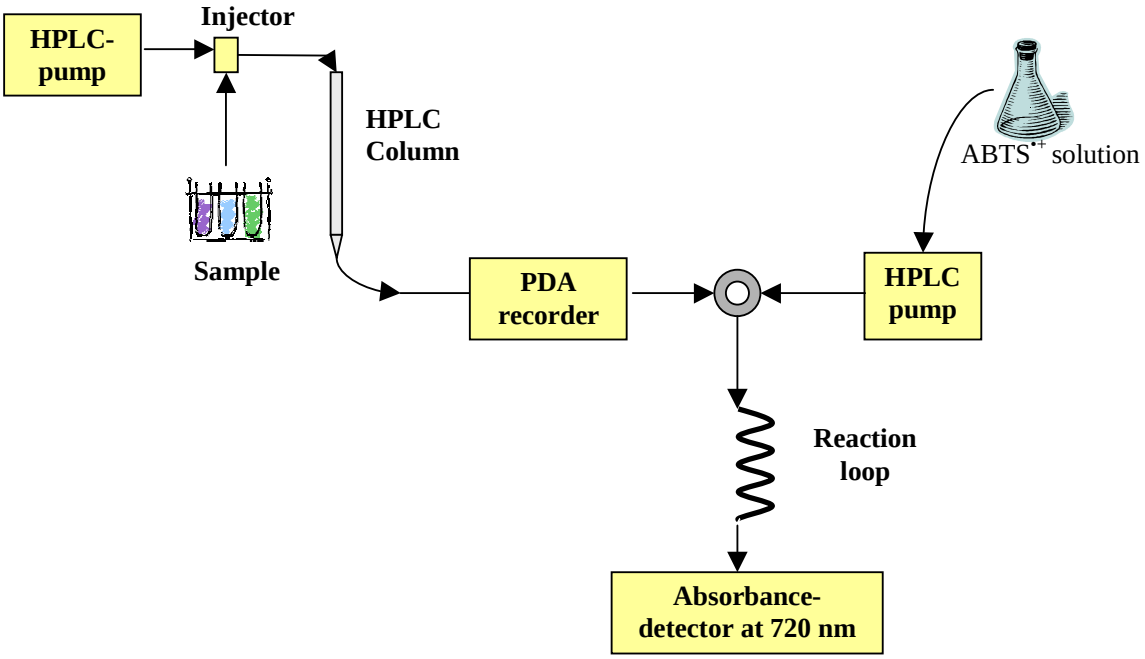


Figure 2.1 ABTS-online HPLC system for the measurement of antioxidant activities of plant extracts.

2.11 Ferric reducing antioxidant potential (FRAP) assay

Three different antioxidant assays were used in this study as they measure different aspects of antioxidant actitvity (Pellegrini *et al.* 2003, MacDonald-Wicks *et al*. 2006) and sometimes lipid soluble compunds were used and needed DPPH for measurement . The FRAP assay was used in this study to estimate the antioxidant power of vegetables extracts (Benzie and Strain 1996). This method measures the ability of the extracts to reduce a ferric-2,4,6-tri-2-pyridyl-s-triazine (TPTZ) complex (Fe^{3+} TPTZ) to the ferrous form Fe^{2+}, producing an intense blue colour with absorption at 593 nm. The reaction is non-specific

and any half life reaction, which has a less positive redox potential, under reaction conditions, than the Fe^{3+}/ Fe^{2+}-TPTZ half-life reaction will drive the Fe^{3+}-TPTZ reduction. In the FRAP assay excess Fe^{3+} is used and the rate limiting factor of the Fe^{2+}-TPTZ, and hence the colour formation, is the reducing ability of the sample. 1.5 mL in volume, was added to 50 µL of sample and 150 µL water. The absorbance at 593 nm was measured 4 min after addition of the reactant (FRAP reagent - acetate buffer, pH 3.6; $FeCl_3$, TPTZ in 40 mM HCl). This absorbance was compared to a standard curve prepared with 0 to1 mM ferrous sulphate ($FeSO_4$) and results are expressed as the mean concentration of Fe^{2+} produced / mM.

On a separate occasion, to measure the contribution of vitamin C in the samples toward antioxidant potential, the above methods were followed. However ascorbate oxidase (EC 1.10.3.3) solution 4U/ ml (Sigma, UK), the enzyme that can oxidize the vitamin C to dehydroascorbate and H_20 was added (20 µl). In this way, the contribution of vitamin C antioxidant could be measured.

2.12 Measurement of radical-scavenging activity by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) colourimetry assay for essential oils

This method was based on reports previously described (Yamaguchi *et al*. 1998, Choi *et al*. 2000, Sacchetti *et al.* 2004). 200 µl oil was mixed with 800 µl 100 mM Tris-HCl buffer (Sigma, UK) (pH 7.4) and 50 µL of 0.5% (v/v) Tween 20 (Sigma, UK) solution as oil-in-water emulsifier. Then, 1 mL of 500 µM DPPH (Sigma, UK) in methanol was added to the solution (final concentration of 250 µM). The mixture was shaken vigorously and left immediately in the dark for 20 min at room temperature. The absorbance at 517 nm was measured by a Thermospectronic UV500. A distilled water (10 µL) was used as a blank and 50 µL Trolox (1 mM) was used as a positive control. Inhibition percentages (*Ip*) were calculated according to the formula of DPPH:

$$I_p^{DPPH}\ \% = (A_B\text{-}A_A)/A_B \times 100 \times D$$

where,

A_B and A_A absorbance value for blank and test sample and D for dilution.

2.13 Statistical analysis

All experimental results were expressed as mean values ± standard error (SD) of n experiments, where n = the number of samples. Analysis was carried out using either t-test or one-way and two-way analysis of variance (ANOVA). Values at $p < 0.05$ were considered statistically significant. ns = not significant, as compared to relevant control. Calibration curves of standards were determined using a simple regression test. Correlation coefficients of samples and variable parameters were analysed by Pearson-correlation test. Analysis was carried out using Minitab software version 12 (Minitab Inc., Addison-Wesley Publishing Co., Reading MA, USA).

CHAPTER 3

ANALYSIS OF PHENOLICS IN SELECTED MALAYSIAN TRADITIONAL VEGETABLES

3.1 Introduction

Evidence suggests that a diet high in fruits and vegetables can decrease the risk of chronic diseases, including cardiovascular disease and cancer (Hollman *et al*. 1996, Hollman and Katan 1999, Halliwell *et al*. 2005). Phytochemicals such as phenolics, flavonoids and carotenoids may play a key role in these protective effects (Heim *et al.* 2002, Ross and Kasum 2002, Chun *et al.* 2005). It is estimated that more than 8000 phenolics, 25000 terpenoids and 12,000 alkaloids have been identified in plants (Lampe 2003), but a large number still remain unknown and need to be identified before the potential health benefits of fruits and vegetables can be better evaluated. Recent evidence suggests that the benefits of phytochemicals in fruit and vegetables, which are believed to act as antioxidants may be even greater than is currently appreciated because oxidative stress induced by free radicals is involved in the aetiology of a wide range of chronic diseases (Liu 2003). However, more studies at all levels are needed to characterize the potential health benefits of individual plant phenolics especially flavonoids in traditional vegetables and medicinal plants which have been claimed to exhibit therapeutic effects. Given the mounting data in support of the role of phenolics in the prevention of cancer and other chronic diseases, methods to improve the phenolic content of plant foods could be of benefit to human health (Young *et al*. 2005). Plant geneticists have attempted to improve phytochemical content through traditional breeding programs and bioengineering leading to secondary metabolite accumulation (Chun *et al*. 2005, Liu 2003). However, accumulating evidence suggests that phytochemical content can also be affected by the environmental conditions in which plants are grown (Lampe 2003, Liu 2003).

Flavonoids, a major group of phenolics are predominantly found *in planta* not as aglycones but as glycosylated forms conjugated to a variety of sugars. As a consequence, there are many hundreds present in the diet and their study in foods is analytically complex (Herrmann 1976, Wollenweber and Dietz 1981, Chun *et al*. 2005). However, it is important

to determine the amounts of polyphenols in vegetables, fruits, and beverages in order to investigate the beneficial health effects of these components. The bioactivity of flavonoids is attributed to aglycone structures, not to sugar moieties and the antioxidative potency is due mainly to the orthodiol (catechol) structure of the aglycones (Bors *et al*. 1990, Sakakibara *et al*. 2002). The specificity of interaction with proteins depends on the steric structures of the aglycones, with the sugar moieties disrupting the interaction (Ferte *et al.* 1999, Casagrande and Darbon 2001). Therefore, a better understanding of the structures involved and levels of aglycone moieties is needed.

Malaysian traditional vegetables constitute a significant part of the food intake of local people particularly among the Malay and indigenous communities, thereby justifying their importance for scientific study (Mansor 1988). Even though these vegetables are popular in Malaysia, only a few have been studied scientifically and the information has not been disseminated widely. Nutritional studies have indicated that many of the traditional vegetables are a rich source of protein, amino acids, dietary fibre, vitamins and minerals (Tee 1985, Zanariah *et al*. 1986, Candlish *et al*. 1987, Bautista *et al*. 1988). Carotenoids, which have been attributed with anticancer properties, are present at high levels in several of the Malaysian traditional vegetables (Tee 1985, Mansor 1988). On the other hand, reports on ethnobotanical studies have ascribed the vegetables with a diverse range of medicinal features such as astringent, antiulcer, antiviral, antiseptic and anti-inflammatory properties. They represent an untapped source of potentially bioactive compounds (Burkill 1966, Perry 1980). Unfortunately, many of the claims of medicinal value have yet to be scientifically substantiated. No chemical, pharmacological and biological studies have been undertaken to investigate the traditional vegetables as a collective group.

The need for profiling and identifying individual phenolics and other compounds has seen traditional methods replaced by HPLC while the limited volatility of many phenolic has restricted the application of GC (Robards 2003). However, HPLC-MS and GC-MS currently represent the most popular and reliable techniques for analysis of phenolic compounds (Hvattum 2002, Martinez-Valverde *et al*. 2002, Olthof *et al*. 2003). This chapter reports on the investigation and analysis of phenolics in seven varieties of Malaysian traditional vegetables using HPLC-MS^2 and measured their antioxidant activities using FRAP and ABTS assays to see their correlation with the total phenolic content measured by Folin-Ciocalteu assay.

3.2 HPLC-tandem mass spectrometry analysis of phenolic compounds in selected Malaysian traditional vegetables

The levels of individual phenolics were determined by HPLC-MS^2 and diode array detection as described in *Chapter 2*. Generally, extracts of Malaysian traditional vegetables were analysed using HPLC-PDA-MS^2 which revealed the presence of a number of flavonoids and phenolic compounds. The compounds were identified by their retention time, absorbance spectra (λ_{max}), parent mass and fragmentation patterns as described by Mullen *et al*. (2002a, 2002b, 2003), and where possible, by comparison with an authentic standard. General rules concerning the MS fragmentation and the chromatographic behaviour of the highly glycosylated and acylated flavonols have been reported by Llorach *et al.* (2003).

3.2.1 HPLC-PDA-MS^2 analysis of phenolics in *Anacardium occidentale*

Analysis of the red and yellow varieties of *A. occidentale* showed that they have similar HPLC-PDA profiles (*Figure 3.2.1*). Therefore, only fragmentation and mass spectra data obtained with the red variety are presented. Fifteen compounds were identified based on their MS^2 data and absorbance spectra (λ_{max})(*Table 3.2.1*).

Peak 1 and *Peak 2* (t_R = 32.4 and 34.0 min, λ_{max} = 355 nm) had a $[M-H]^-$ at *m/z* 479, with MS^2 producing three charged fragment ions at *m/z* 317 (162 *amu*, loss of a glucosyl unit), *m/z* 271 and *m/z* 179. According to the fragmentation pattern and λ_{max}, these compounds are myricetin-*O*-glycosides. As the authentic standard was not available, the identifications are based on the HPLC elution order which indicates that peak 1 is myricetin-3-*O*-galactoside and peak 2, myricetin-3-*O*-glucoside (Schieber *et al.* 2001).

Peak 3 (t_R = 37.6 min, λ_{max} = 355 nm) had a $[M-H]^-$ at *m/z* 447 which on MS^2 yielded a fragment ion at *m/z* 315, a 132 *amu* loss suggesting cleavage of a pentose unit. According to the MS fragmentation pattern and λ_{max}, this compound is a methylquercetin pentose conjugate. This compound has similar molecular weight to that of isorhamnetin-3-*O*-rhamnoside. However, co-chromatography with the authentic standard showed that the retention time of isorhamnethin-3-*O*-rhamnoside was t_R = 60.3 min very different from that of peak 3. Thus, peak 3 is an unknown methylquercetin pentose-like conjugate.

Peak 4 (t_R = 41.3 min, λ_{max} = 365 nm) had a $[M-H]^-$ at *m/z* 615 with MS^2 yielding a charged fragment ion at *m/z* 463 with the loss of 152 *amu* unit, an unknown substituent which in turn fragmented with a loss of 162 *amu* (glucose) to *m/z* 301 quercetin ion. Therefore, this peak is quercetin glucoside with an additional unknown conjugating moiety.

Peak 5 (t_R = 45.3 min, λ_{max} = 345 nm) had a $[M-H]^-$ at *m/z* 463, with MS^2 producing a charged fragment ion at *m/z* 317 due to the loss of a 146 *amu* rhamnose unit. The fragmentation pattern, λ_{max} and co-chromatography with authentic standard demonstrate this peak is myricetin-3-*O*-rhamnoside.

Peak 6 (t_R = 47.9 min, λ_{max} = 345 nm) had a $[M-H]^-$ at *m/z* 463 which on MS^2 exhibited a 162 *amu* loss, corresponding to cleavage of a glucosyl unit, producing a quercetin fragment ion at *m/z* 301. *Peak 7* (t_R = 50.6 min, λ_{max} = 350 nm) also had a $[M-H]^-$ at *m/z* 463 with yielded a charged MS^2 fragment ion at *m/z* 301. Co-chromatography with a standard of quercetin-3-*O*-glucoside showed that peak 7 is quercetin-3-*O*-glucoside which elutes after quercetin-3-*O*-galactoside (Schieber *et al*. 2001). Peak 6 is, therefore, quercetin-3-*O*-galactoside.

Peak 8 (t_R = 56.6 min, λ_{max} = 355 nm) had a $[M-H]^-$ at *m/z* 433 which on MS^2 yielded a charged fragment ion at *m/z* 301, suggesting that this peak is a quercetin pentose. *Peak 9* (t_R = 59.4 min, λ_{max} = 350 nm) also had a $[M-H]^-$ at *m/z* 433 and MS^2 fragment ion at *m/z* 301. The elution order indicates that peak 8 is quercetin-3-*O*-xyloside and peak 9, quercetin-3-*O*-arabinopyranoside (Schieber *et al*. 2001, Alonso-Salces *et al.* 2004).

Peak 10 (t_R = 61.5 min, λ_{max} = 350 nm) had a $[M-H]^-$ at *m/z* 447 with MS^2 yielding a kaempferol fragment ion at *m/z* 285 (a 162 *amu* loss which is a glucosyl unit). Co-chromatography with a standard confirmed the presence of kaempferol-3-*O*-glucoside.

Peak 11 (t_R = 65.8 min, λ_{max} = 350 nm) had a $[M-H]^-$ at *m/z* 433 with MS^2 yielding a fragment ion at *m/z* 301 exhibited a 132 *amu* loss which is a pentose unit. Identification is similar to peak 8 and peak 9. The mass spectra, elution order and co-chromatography with available standard, indicate this peak is quercetin-3-*O*-arabinofuranoside.

Peak 12 (t_R = 67.1 min, λ_{max} = 345 nm) had a $[M-H]^-$ at *m/z* 447 with MS^2 yielding a fragment ion at *m/z* 301 exhibited a 146 *amu* loss which is a rhamnosyl unit. The mass spectra and co-chromatography with a standard, revealed this peak as quercetin-3-*O*-rhamnoside.

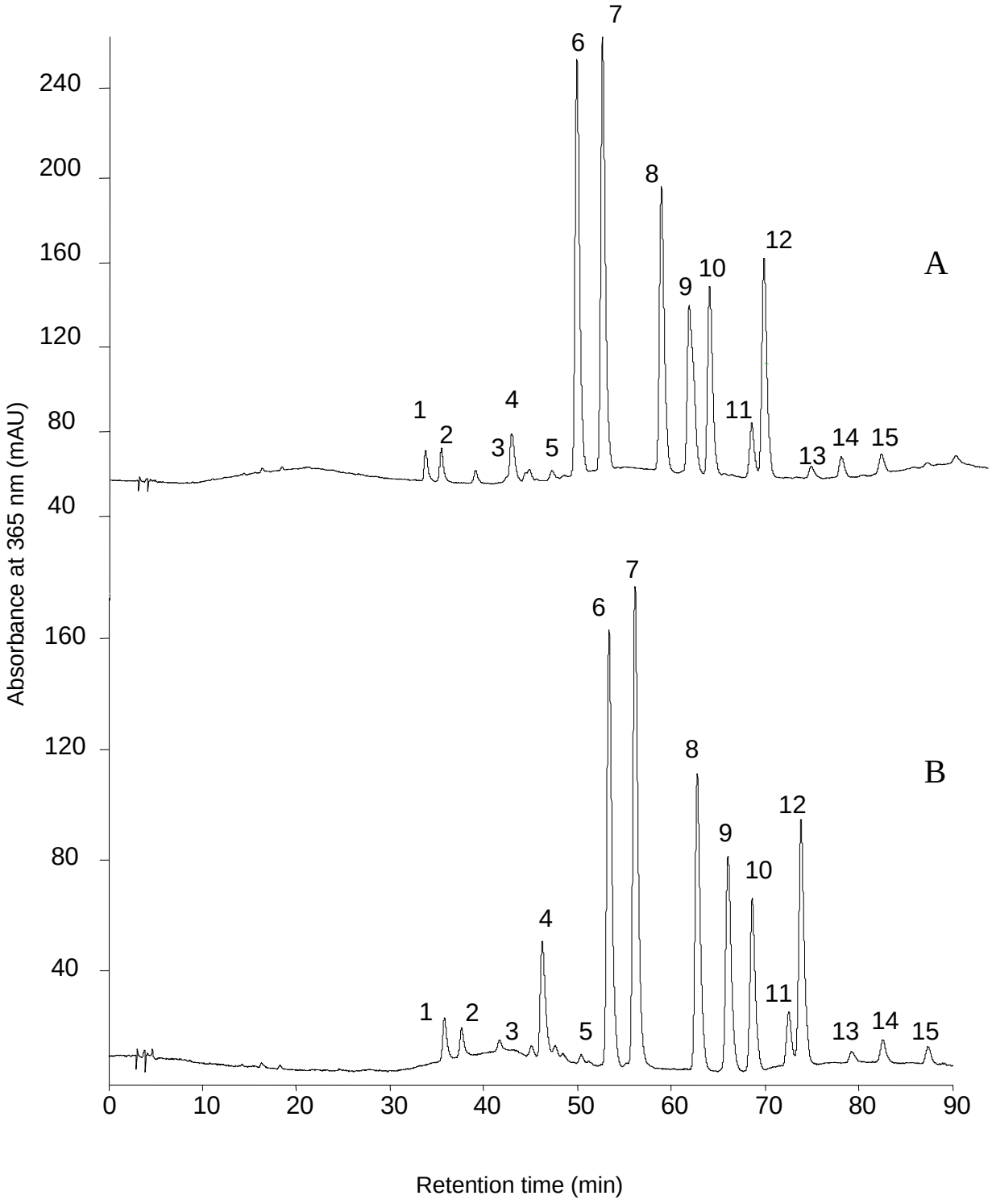


Figure 3.2.1 Chromatogram of phenolics in batch 1 of *Anacardium occidentale* [**A** - red , **B** - yellow] obtained using HPLC-MS^2 at 10-20% acetonitrile over 90 minutes with 0.1% formic acid at absorbance 365 nm. Identification of numbered peaks is in *Table 3.2.1*. Note: Minor variation of retention times.

Table 3.2.1 Mass spectral characteristics and identity of phenolics present in batch 1 of red and yellow varieties of *Anacardium occidentale*

Peak	t_R (min)		λ max	Compound	[M-H]⁻ (m/z)	MS² fragments ions (*m/z*)
	Red	Yellow				
1	32.4	32.8	355	Myricetin-3-*O*-galactoside	479	317(M;[M-H]⁻-Glc), 271, 179
2	34.0	34.6	355	Myricetin-3-*O*-glucoside	479	317(M;[M-H]⁻-Glc), 271, 179
3	37.6	37.5	355	Unknown methylquercetin pentose-like conjugate	447	315 (Mq;[M-H]⁻-Pen), 301
4	41.3	41.7	365	Unknown quercetin conjugate	615	463 (Q;[M-H]⁻-152), 301
5	45.3	45.5	345	Myricetin-3-*O*-rhamnoside	463	317 (M;[M-H]⁻-Rham), 179
6	47.9	48.4	345	Quercetin-3-*O*-galactoside	463	301 (Q;[M-H]⁻-Gal)
7	50.6	51.1	350	Quercetin-3-*O*-glucoside	463	301 (Q;[M-H]⁻-Glc)
8	56.6	56.8	355	Quercetin-3-*O*-xyloside	433	301 (Q;[M-H]⁻-Pen)
9	59.4	59.7	350	Quercetin-3-*O*-arabinopyranoside	433	301 (Q;[M-H]⁻-Pen)
10	61.5	61.6	350	Kaempferol-3-*O*-glucoside	447	285 (K;[M-H]⁻-Glc)
11	65.8	65.3	350	Quercetin-3-*O*-arabinofuranoside	433	301 (Q;[M-H]⁻-Pen)
12	67.1	67.8	345	Quercetin-3-*O*-rhamnoside	447	301 (Q;[M-H]⁻-Rham)
13	71.9	79.2	345	Kaempferol-3-*O*-xyloside	417	285 (K;[M-H]⁻- Pen)
14	75.0	82.6	345	Kaempferol-3-*O*-arabinopyranoside	417	285 (K;[M-H]⁻-Pen)
15	79.1	87.4	345	Kaempferol-3-*O*-arabinofuranoside	417	285 (K;[M-H]⁻-Pen)

Peak numbers and HPLC retention times refer to HPLC trace in *Figure 3.2.1.* t_R – retention time; [M-H]⁻- negatively charged molecular ion; Q - quercetin; K – kaempferol; M – myricetin; Mq – methylquercetin; Gal – galactosyl unit; Glc – glucosyl unit; Pen – pentosyl unit; Rham – rhamnosyl unit; Cou – coumaroyl unit

Peak 13, 14 and 15 (t_R = 71.9, 75.0 and 13.1 min, λ_{max} = 345 nm) all had a $[M-H]^-$ at *m/z* 417 with MS^2 yielding a kaempferol fragment ion at *m/z* 285 with the 132 *amu* loss indicating cleavage of a pentose unit. Identification of this compound based on the mass spectral fragmentation pattern suggests these peaks are kaempferol pentose conjugates. The elution order implies that peak 13 as kaempferol-3-*O*-xyloside, peak 14 as kaempferol-3-*O*-arabinopyranoside and peak 15 as kaempferol-3-*O*-arabinofuranoside (Schieber *et al*. 2001, Alonso-Salces *et al*. 2004).

3.2.2 HPLC-PDA-MS^2 analysis of phenolics in *Centella asiatica*

Eleven phenolic compounds were identified in *C. asiatica* based on MS^2 spectra, co-chromatography with authentic standards and λ_{max}. The results are summarised below and presented in *Table 3.2.2* and the chromatogram is illustrated in *Figure 3.2.2*. Chlorogenic acids were shown to be predominant in *C. asiatica*. Due to the lack of chlorogenic acids standards, the mass spectra fragmentation pattern and elution order of these compounds in *C. asiatica* were compared with that of coffee analysed with similar HPLC conditions (Stalmach *et al*. 2006). However, distinguishing MS^2 characteristics of chlorogenic acids have also been described by Clifford *et al.* (2003) and these assisted the identifications.

Peak 1 (t_R = 13.6 min, λ_{max} = 325 nm). MS analysis of this peak revealed a negatively charged $[M-H]^-$ ion at *m*/*z* 353 that fragmented to yield a MS^2 spectrum with ions at *m*/*z* 191(162 *amu* loss of a quinic acid) and a caffeoyl moiety at *m*/*z* 179. Peak 1 is therefore a caffeoylquinic acid. Comparative study has been conducted using coffee extract to see the elution order of caffeoylquinic acid. HPLC chromatogram of coffee showed that 3-*O*-caffeoylquinic acid elutes before 5-*O*-caffeoylquinic acid (Clifford *et al*. 2003). Peak 1 is, therefore 3-*O*-caffeoylquinic acid (Stalmach *et al.* 2006). When commercial standards were not available, peak identification were assigned primarily by means of their parent ion and supported by their absorbance spectrum and sequence of elution order relative to 5-*O*-caffeoylquinic acid.

Peak 2 (t_R = 19.0 min, λ_{max} = 325 nm). MS analysis of this peak revealed a negatively charged $[M - H]^-$ ion at *m*/*z* 353 that fragmented to yield a MS^2 spectrum with ions at *m*/*z* 191 and *m*/*z* 179. Co-chromatography of authentic standard verified the identification of this peak as 5-*O*-caffeoylquinic acid.

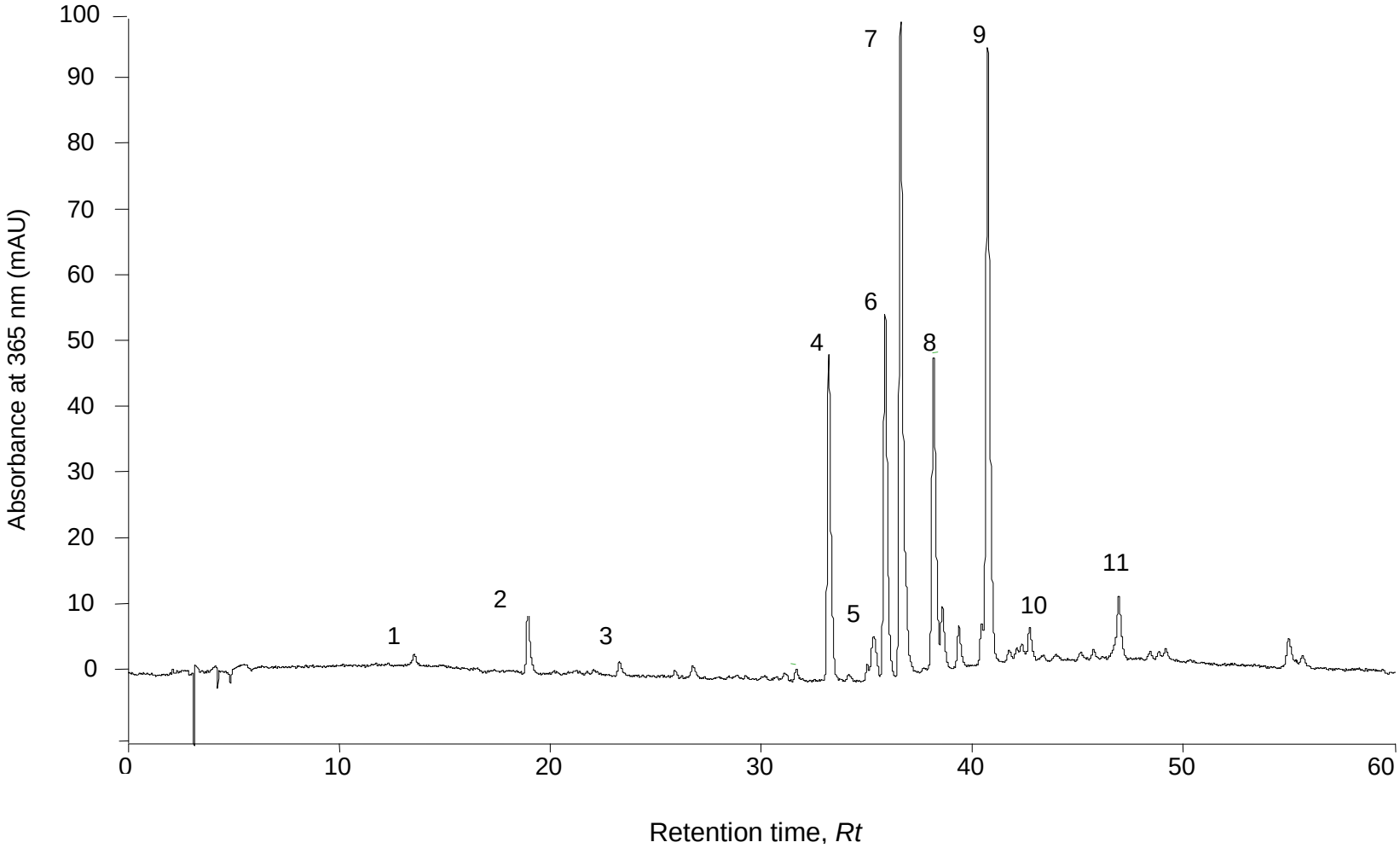


Figure 3.2.2 Chromatogram of detectable compounds in batch 1 of *Centella asiatica* obtained using HPLC-MS^2 at 5-40% acetonitrile over 60 minutes with 1.0% formic acid at absorbance 365 nm. Identification of numbered peaks is in *Table 3.2.2.*

Table 3.2.2 Mass spectral characteristics and identity of phenolics present in batch 1 of *Centella asiatica*

Peak	λ_{max}	t_R (min)	Compound	$[M-H]^-$ (*m/z*)	MS^2 fragments ions (*m/z*)
1	325	13.6	3-*O*-Caffeoylquinic acid	353	191 (quinic acid; $[M-H]^-$-caffeoyl), 179, 135
2	325	19.0	5-*O*-Caffeoylquinic acid	353	191 (quinic acid; $[M-H]^-$-caffeoyl), 179
3	325	26.8	5-*O*-Feruloylquinic acid	367	191 (quinic acid; $[M-H]^-$-caffeoyl), 173
4	360	33.2	Quercetin-3-*O*-glucuronide	477	301 (Q;$[M-H]^-$-GlcUA), 179
5	350	35.3	Kaempferol-3-*O*-glucoside	447	285 (K;$[M-H]^-$-Glc)
6	325	35.9	3,4-*O*-Dicaffeoylquinic acid	515	353 ($[M-H]^-$-162), 335, 173
7	345	36.6	Kaempferol-3-*O*-glucuronide	461	285 (K;$[M-H]^-$-GlcUA)
8	330	38.2	3,5-*O*-Dicaffeoylquinic acid	515	353 ($[M-H]^-$-162), 191, 173
9	330	40.7	4,5-*O*-Dicaffeoylquinic acid	515	353 ($[M-H]^-$-162),
10	325	42.7	3-*O*-Feruloyl-5-*O*-caffeoylquinic acid	529	367 ($[M-H]^-$-162), 335
11	325	46.9	3-*O*-Caffeoyl-5-*O*-feruloylquinic acid	529	353 ($[M-H]^-$-176)

Peak numbers and HPLC retention times refer to HPLC trace in *Figure 3.2.2*. t_R – retention time; $[M-H]^-$ - negatively charged molecular ion; Q - quercetin; K - kaempferol; Glc - glucoside; GlcUA – glucuronide unit

Peak 3 (t_R = 26.8 min, λ_{max} = 325 nm). MS analysis of this peak revealed a $[M - H]^-$ at *m/z* 367 that fragmented to yield a MS^2 exhibiting a 176 *amu* loss, corresponding to the cleavage of a feruloyl unit, producing a fragment ion at *m/z* 191 which is an indicative of feruloylquinic acid. Based on the analysis of coffee chlorogenic acids (Stalmach *et al*. 2006) this compound is 5-*O*-feruloyl quinic acid.

Peak 4 (t_R = 33.2 min, λ_{max} = 350 nm) had a $[M-H]^-$ at *m/z* 477 with MS^2 exhibiting a 176 unit loss, corresponding to the cleavage of a glucuronide unit, producing a quercetin fragment ion at *m/z* 301. This indicates the presence of a quercetin glucuronide. Co-chromatography with a standard purified from French bean revealed this peak as quercetin-3-*O*-glucuronide.

Peak 5 (t_R = 35.3 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 515, with MS^2 producing a major charged fragment ions at *m/z* 353 and *m/z* 173. Based on the mass spectral data reported previously, this compound is a 3,4-*O*-dicaffeoylquinic acid.

Peak 6 (t_R = 35.9 min, λ_{max} = 325 nm) fragmented differently to *Peak 5* with the *m/z* 515 $[M-H]^-$ yielding MS^2 ions at *m/z* 353 and *m/z* 191. This is in keeping with the presence of dicaffeoylquinic acid as described by Clifford *et al.* (2003). The elution order suggests this peak is 3,5-*O*-dicaffeoylquinic acid.

Peak 7 (t_R = 36.6 min, λ_{max} = 345 nm) had a $[M-H]^-$ at *m/z* 461, which on MS^2 produced a charged ion at *m/z* 285 indicative of the 176 *amu* loss of a glucuronide unit. The peak is, therefore, a kaempferol-3-*O*-glucuronide.

Peak 8 (t_R = 38.2 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 515 which yielding a MS^2 charged fragment ion at *m/z* 353 with the loss of a 162 *amu* quinic acid unit. This indicates that this compound is a dicaffeoylquinic acid similar to peaks 5 and 6. The elution order and the retention times imply this peak as 4,5-*O*-dicaffeoylquinic acid.

Peak 9 (t_R = 40.7 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 515 which on MS^2 yielded a charged fragment ion at *m/z* 353 with the loss of 162 *amu*. This indicates that the compound is a dicaffeoylquinic acid but the fragmentation pattern and HPLC elution properties do not allow further elucidation of the structure.

Peak 10 (t_R = 42.7 min, λ_{max} = 330 nm had a $[M-H]^-$ at *m/z* 529 which on MS^2 exhibited a 162 amu loss, corresponding to the cleavage of a caffeoyl unit, producing a fragment ion at *m/z* 367 and as well as relatively intense ions at *m/z* 335 and *m/z* 193 which corresponds to 3-*O*-feruloyl-5-*O*-caffeoyl quinic acid (Clifford *et al*. 2003).

Peak 11 (t_R = 46.9 min, λ_{max} = 330 nm had a $[M-H]^-$ at *m/z* 529 which on MS^2 exhibited a 176 amu loss, corresponding to the cleavage of a feruloyl unit, producing fragment ions at *m/z* 353 and *m/z* 179 which corresponds to caffeoylferuloyl quinic acid. However in contrast to *Peak 10*, the cleavage of the fragmentation was on feruloyl moiety, thus, this compound is 3-*O*-caffeoyl-5-*O*-feruloyl quinic acid (Clifford *et al*. 2003).

When standards were not available, peak identities were assigned primarily by means of their parent ion and supported by their absorbance spectrum and HPLC elution order relative to 5-CQA using methods validated in our laboratory. The use of MS^3 and MS^4 for the fragmentation of chlorogenic acids (*Table 3.2.3*) will help to identify and quantify the compounds by means of the main ions and base ions. However in this study, the use of MS^2 and comparison of absorbance spectra and elution order between the samples and those obtained with coffee (Stalmach *et al.* 2006) have confirmed the identity of the chlorogenic acids especially in *C. asiatica*.

Table 3.2.3. Identification of individual chlorogenic acids in coffee, according to retention time and negative ionization mass spectra (Clifford *et al.* 2003, Stalmach *et al.* 2006)

Chlorogenic acids	$[M-H]^-$ (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)	MS^4 ions (*m/z*)
3-*O*-Caffeoylquinic acid	353	191, 179, 135	191→ 85, 127, 173	
4-*O*-Caffeoylquinic acid	353	173, 179, 135	173 → 93, 111	
5-*O*-Caffeoylquinic acid	353	191, 179	191→127, 93, 85, 173	
3-*O*-Feruloylquinic acid	367	193	193→ 134, 149	
4-*O*-Feruloylquinic acid	367	173, 193	173→ 93, 111	
5-*O*-Feruloylquinic acid	367	191	191→ 85, 127, 173	
3,4-*O*-Dicaffeoylquinic acid	515	353, 335	353→ 173	173→ 93, 111
3,5-*O*-Dicaffeoylquinic acid	515	353	353→ 191	191→ 85, 127, 171
4,5-*O*-Dicaffeoylquinic acid	515	353	353→ 173	173→ 93, 111

3.2.3 HPLC-PDA-MS^2 analysis of phenolics in *Colubrina asiatica*

Acidified methanol extracts of *Colubrina asiatica* were analysed on a Surveyor HPLC system using a 60 min 5-40 % gradient of acetonitrile in 1 % aqueous formic acid. Three

compounds were identified based on MS/MS data and λ_{max}. The results are summarised below and presented in *Table 3.2.4* and *Figure 3.2.3*.

Peak 1 (t_R = 29.8 min, λ_{max} = 355 nm) had a $[M-H]^-$ at *m/z* 609 with MS^2 yielding a charged fragment ion at *m/z* 301. With the lost of 308 *amu* which consisted of rutinose unit. This compound was confirmed by co-chromatography as quercetin-3-*O*-rutinoside.

Peak 2 (t_R = 33.5 min, λ_{max} = 345 nm) had a $[M-H]^-$ at *m/z* 593 with MS^2 exhibited a 308 *amu* loss, corresponding to cleavage of a rutinose unit, producing a kaempferol fragment ion at *m/z* 285. The fragmentation pattern and λ_{max} are in keeping with the presence of kaempferol-3-*O*-rutinoside.

Peak 3 (t_R = 35.7 min, λ_{max} = 345 nm) had a $[M-H]^-$ at *m/z* 447, with MS^2 producing a charged fragment ion at *m/z* 285 which is in keeping with the presence of kaempferol-3-*O*-glucoside.

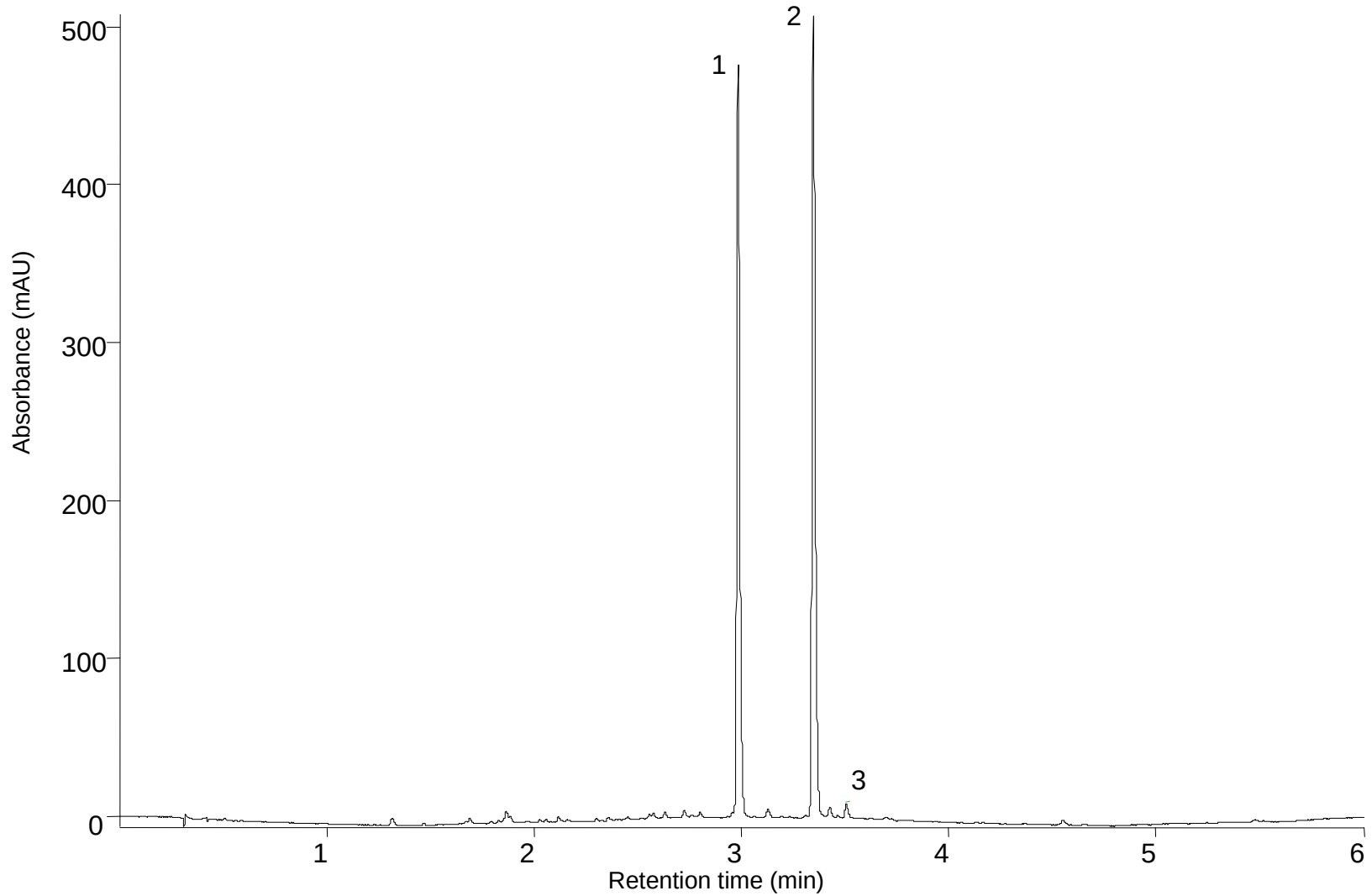


Figure 3.2.3. Chromatogram of phenolics in batch 1 of *Colubrina asiatica* obtained using HPLC-MS^2 at 5-40% acetonitrile over 60 minutes with 1.0% formic acid at absorbance 365 nm. Identification of numbered peaks is in *Table 3.2.4*.

Table 3.2.4. Mass spectral characteristics and identity of phenolics present in batch 1 of *Colubrina asiatica*

Peak	t_R (min)	λ max	Compound	$[M-H]^-$ (*m/z*)	MS^2 fragments ions (*m/z*)
1	29.8	355	Quercetin-3-*O*-rutinoside	609	301 (Q;$[M-H]^-$-Hex-Rham)
2	33.5	345	Kaempferol-3-*O*-rutinoside	593	285 (K;$[M-H]^-$-Hex-Rham)
3	35.7	345	Kempferol-3-*O*-glucoside	447	285 (K;$[M-H]^-$-Glu)

Peak numbers and HPLC retention times refer to HPLC trace in *Figure 3.2.3.* t_R – retention time; $[M-H]^-$, negatively charged molecular ion. Q - quercetin; K - kaempferol; Glc – glucosyl unit; Hex – hexosyl unit; Rham – rhamnosyl unit

3.2.4 HPLC-PDA-MS^2 analysis of phenolics in *Pluchea indica*

The individual phenolics in *P. indica* were analysed by HPLC-PDA-MS^2. Acidified methanol extracts of *Pluchea indica* were analysed on a Surveyor HPLC system using an 80 min 5-40 % gradient of acetonitrile in 1 % aqueous formic acid. In total, nine compounds were identified which was determined based on MS^2 data, λ_{max} and co-chromatography with standards. The results are summarised below and presented in *Table 3.2.5* and *Figure 3.2.4*.

Peak 1 (t_R = 13.9 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 353 with MS^2 yielding two charged fragment ions at *m/z* 191 (loss of 162 *amu*), *m/z* 179 and *m/z* 135. On the basis of the fragmentation data of Clifford *et al*. (2003), this compound is 3-*O*-caffeoylquinic acid, a chlorogenic acid.

Peak 2 (t_R = 20.7 min, λ_{max} = 325 nm) co-chromatographed with 5-*O*-caffeoylquinic acid and had a $[M-H]^-$ at *m/z* 353 which yielded a *m/z* 191 MS^2 fragment ion.

Peak 3 (t_R = 37.0 min, λ_{max} = 355 nm) had a $[M-H]^-$ at *m/z* 463, with MS^2 producing a fragment ion at *m/z* 301. With the lost of 162 *amu*, the glycosyl unit and based on the mass spectral data and λ_{max}, this compound is quercetin-3-*O*-galactoside.

Peak 4 (t_R = 37.6 min, λ_{max} = 355 nm) had a $[M-H]^-$ at *m/z* 463, with MS^2 producing charged fragment ion at *m/z* 301. With the lost of 162 *amu*, the glycosyl unit and based on the mass spectral, λ_{max} and co-chromatography with authentic standard, this compound is quercetin-3-*O*-glucoside.

Peak 5 (t_R = 42.1 min, λ_{max} = 325 nm), *Peak 6* (t_R = 42.9 min, λ_{max} = 325 nm) and *Peak 7* (t_R = 46.6 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 515 with MS^2 yielding a charged fragment ion at *m/z* 353 with a 162 *amu* loss. Based on the findings of Clifford *et al.* (2003), these peaks are dicaffeoylquinic acids. By comparison with the chlorogenic acids in coffee run under similar HPLC condition, Peak 5, 6 and 7 are identified 3,4-*O*-dicaffeoylquinic acid, 3,5-*O*-dicaffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid respectively (see *Table 3.2.3*).

Peak 8 (t_R = 51.8 min, λ_{max} = 325 nm) is a tricaffeoylquinic acid, had a $[M-H]^-$ at *m/z* 677 with MS^2 yielding a charged fragment ion at *m/z* 515, which exhibited the loss of 162 *amu*. This compound was revealed as tricaffeoylquinic acid on the basis of the fragmentation pattern and λ_{max} as reported by Clifford *et al.* (2003).

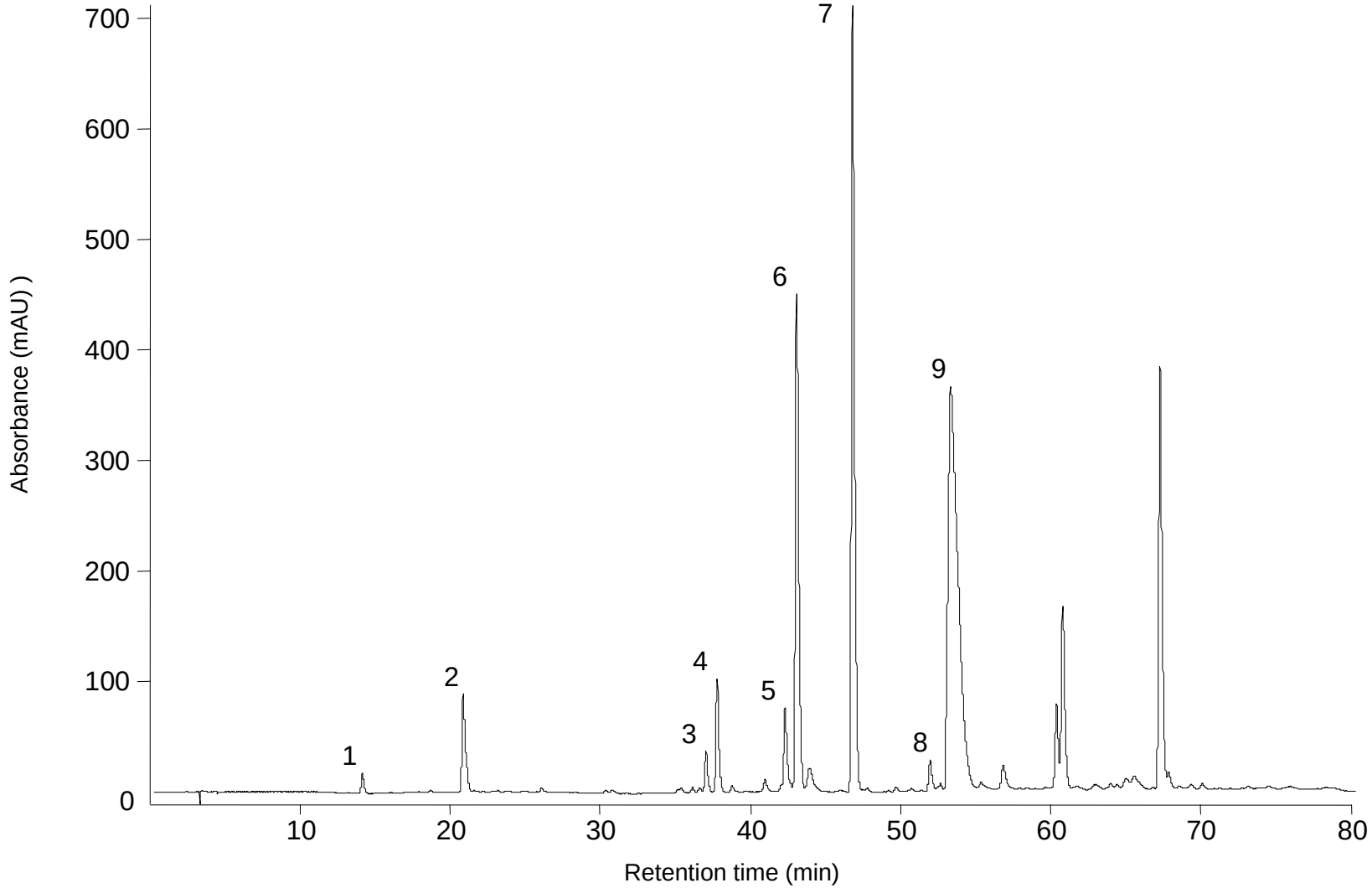


Figure 3.2.4 Chromatogram of phenolics in batch 1 of *Pluchea indica* var. A obtained using HPLC-MS^2 at 5-40% acetonitrile over 80 minutes with 1.0% formic acid at absorbance 365 nm. Identification of numbered peaks is in *Table 3.2.5*.

Table 3.2.5 Mass spectral characteristics and identity of phenolics present in batch 1 of var. A and B of *Pluchea indica*

Peak	t_R *(min)*		λ max	Compound	$[M-H]^-$ (*m/z*)	MS^2 fragments ions (*m/z*)
	var. A	var.B				
1	13.9	13.9	325	3-*O*-Caffeoylquinic acid	353	191 (quinic acid; $[M-H]^-$ -caffeoyl), 179, 135
2	20.7	20.6	325	5-*O*-Caffeoylquinic acid	353	191 (quinic acid; $[M-H]^-$ -caffeoyl)
3	37.0	36.8	355	Quercetin-3-*O*-galactoside	463	301 (Q;$[M-H]^-$-Gal)
4	37.6	37.5	355	Quercetin-3-*O*-glucoside	463	301 (Q;$[M-H]^-$-Glc)
5	42.1	42.1	325	3,4-*O*-Dicaffeoylquinic acid	515	353 ($[M-H]^-$-162), 179, 335
6	42.9	42.8	325	3,5-*O*-Dicaffeoylquinic acid	515	353 ($[M-H]^-$-162), 191
7	46.6	46.6	325	4-5-*O*-Dicaffeoylquinic acid	515	353 ($[M-H]^-$ -162), 173
8	51.8	51.7	325	Tri-caffeoylquinic acid	677	515 ($[M-H]^-$-162)
9	54.1	53.1	355	Quercetin-3'-*O*-sulphate	381	301(Q;$[M-H]^-$-Sul)

Peak numbers and HPLC retention times refer to HPLC trace in *Figure 3.2.4*. t_R – retention time; $[M-H]^-$- negatively charged molecular ion; Q - quercetin; Glc – glucosyl unit; Gal – galactosyl unit; Sul – sulphate unit

Peak 9 (t_R = 54.1 min, λ_{max} = 355 nm) had a $[M-H]^-$ at *m/z* 381 with MS^2 yielding a charged fragment ion at *m/z* 301 with the lost of 80 *amu*, a sulphate unit and co-chromatography with a standard confirmed this peak is quercetin-3-*O*-sulphate.

3.2.5 HPLC-PDA-MS^2 analysis of phenolics in *Premna Cordifolia*

Acidified methanol extracts of *P. cordifolia* were analysed on a Surveyor HPLC system using a 60 min 5-40 % gradient of acetonitrile in 1 % aqueous formic acid (*Figure 3.2.5*). Three compounds were identified based on MS/MS data and λ_{max}(*Table 3.2.6*). The results are summarised below:

Peak 1 (t_R = 31.2 min, λ_{max} = 325 nm) had a $[M-H]^-$ at *m/z* 623 with MS^2 yielding two charged fragment ions at *m/z* 461 and *m/z* 315. With the lost of 162 *amu*, a glucosyl unit and also a 146 *amu* of a rhamnosyl unit, this compound is a methylquercetin glucosylrhamnose conjugate with the sugars attached at different positions.

Peak 2 (t_R = 33.7 min, λ_{max} = 330 nm) had a similar fragmentation pattern to peak 1, $[M-H]^-$ at *m/z* 465 with MS^2 exhibited a 162 amu loss, corresponding to cleavage of a glycosyl unit producing a fragment ion at *m/z* 463 and *m/z* 315 which also unknown methylquercetin glycoside conjugate.

Peak 3 (t_R = 34.7 min, λ_{max} = 330 nm) had a $[M-H]^-$ at *m/z* 577, with MS^2 producing a fragment ion at *m/z* 269 (loss of a 308 *amu* rutinose unit). Based on this fragmentation pattern and co-chromatography with a reference compound, peak 3 is the flavone conjugate apigenin-7-rutinoside.

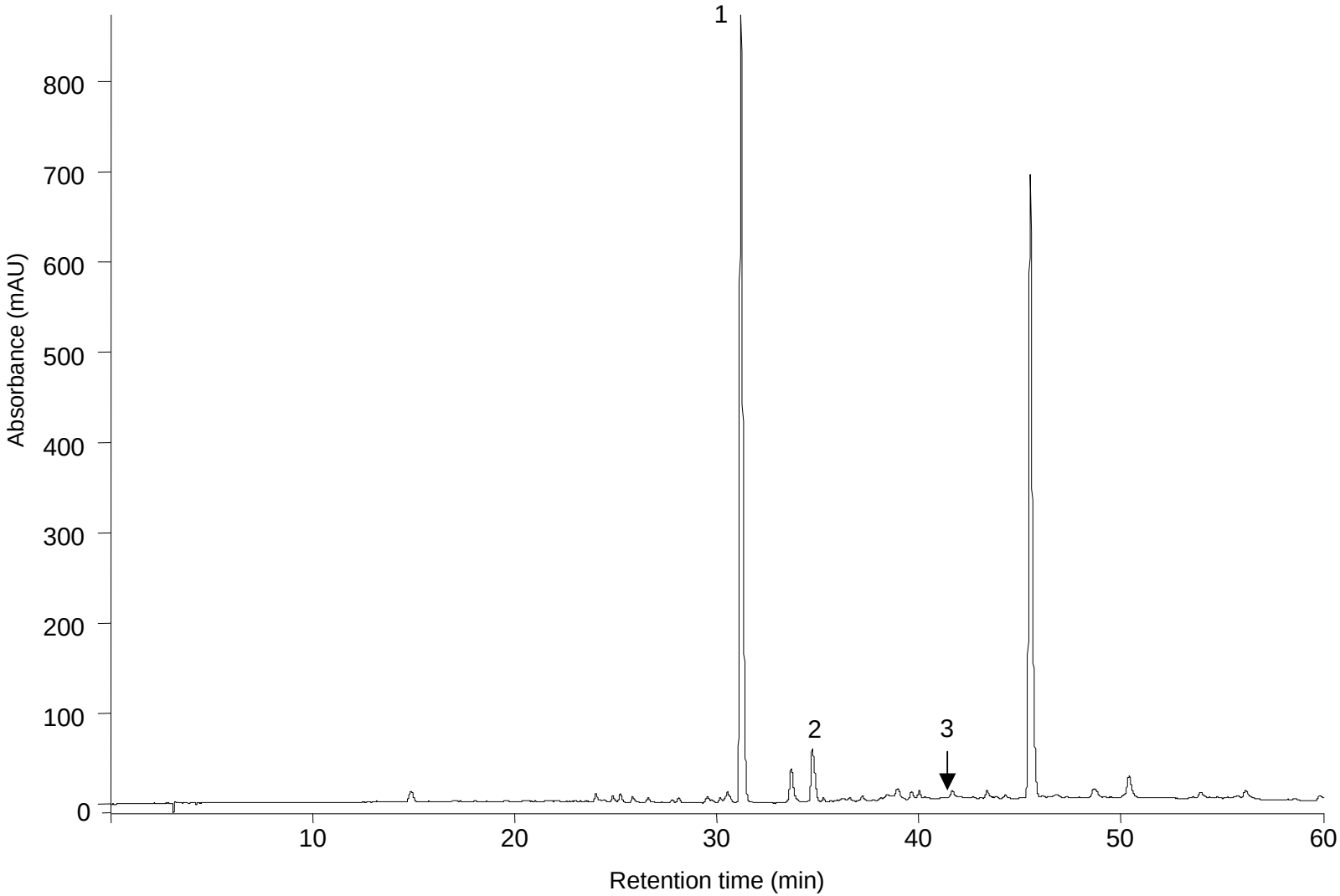


Figure 3.2.5 Chromatogram of phenolics in batch 1 of *Premna cordifolia* obtained using HPLC-MS^2 at 5-40% acetonitrile over 60 minutes with 1.0% formic acid at absorbance 365 nm. Identification of numbered peaks is in *Table 3.2.6*.

Table 3.2.6 Mass spectral characteristics and identity of phenolics present in batch 1 of *Premna cordifolia*

Peak	t_R (min)	λ max	Compound	$[M-H]^-$ (*m/z*)	MS^2 fragments ions (*m/z*)
1	31.2	325	Methylquercetin glycoside conjugate	623	461 (Mq;$[M-H]^-$-Glc), 315 ($[M-H]^-$-Rham)
2	33.7	330	Methylquercetin glycoside conjugate	625	463 (Mq;$[M-H]^-$-Glc), 315
3	34.7	330	Apigenin-7-*O*-rutinoside	577	269 (Ap;$[M-H]^-$-Rham)

Peak numbers and HPLC retention times refer to HPLC trace in *Figure 3.2.5*. t_R – retention time; $[M-H]^-$, negatively charged molecular ion. Mq – methylquercetin; Ap – apigenin; Glc – glucosyl unit; Rham – rhamnosyl unit

3.3 Levels of phenolic compounds in Malaysian traditional vegetables: comparison of two batches

The levels of total phenolics in Malaysian vegetables measured with the Folin-Ciocalteu assay varied between species and between batches as shown in *Table 3.3.1*. *A. occidentale* contained the highest total phenolic content compared than the other vegetables in batch 1 and 2 which are depicted in *Figure 3.3.1*. *C. asiatica* has shown to be contained the lowest total phenolic content, 100 ± 7.8 mg/g GAE fw in batch 1 and 72 ± 0.9 mg/g GAE fw in batch 2. In the red variety of *A. occidentale*, there are no significant differences between the levels of total phenolics of the two batches. However, in the yellow variety, levels of phenolics in batch 2 (353 ± 26 mg/g GAE fw) were significantly lower ($p>0.05$) to that of batch 1 (415 ± 20 mg/g GAE fw). All other species showed that the level of total phenolics in batch 2 material was lower to that in batch 1. However, HPLC-derived phenolics did not show any correlation to the total penolic content in Folin-Ciocalteu assay. This could be due to some of the unknown peaks which could not be quantified and to the compounds which were retained in the reverse-phase HPLC column.

As shown in *Table 3.3.2*, the predominant compounds identified in *A. occidentale* were flavonols. The profile of compounds identified in the red and yellow varieties and also in the two batches was similar. The level of flavonols in the red variety of batch 1 ranged from 20 ± 0.1 to 4308 ± 111 µg/g fw, with kaempferol-3-*O*-glucoside showing the highest concentration. In batch 2, phenolic compounds ranged from 41 ± 1.5 to 1564 ± 143 µg/g fw with quercetin-3-*O*-glucoside rather than kaempferol-3-*O*-glucoside being the predominant component. In batch 1 of the yellow variety, kaempferol-3-*O*-glucoside also showed the highest concentration, and the concentration of phenolics ranged from 12 ± 0.8 to 2592 ± 160 µg/g fw. Similarly to batch 2 of the red variety, level of quercetin-3-*O*-glucoside was the highest and the phenolics concentration ranged from 35 ± 0.8 to 1672 ± 25 µg/g fw respectively. The total phenolics derived from the HPLC-PDA-MS^2 analysis varied and were significantly different to each other, which showed that total phenolics was the highest in batch 1 of the red variety. In contrast, the level of phenolics in batch 2 was the highest in the yellow variety. This was also in contrast to the total phenolic content analysed by Folin-Ciocalteu method. In batch 1 of *C. asiatica*, chlorogenic acids were present in substantial amounts but flavonols were the major components present (63% of the total phenolics, ranged from 55 ± 1.1 to 1633 µg/g fw) to that of the chlorogenic acids (*Table 3.3.3*).

However, in contrast, in batch 2, chlorogenic acids were predominant ranging from 27 ± 5.2 to 1229 ± 24 µg/g fw (84% of the total phenolics). In *Colubrina asiatica* as shown in *Table 3.3.4*, only three phenolic compounds were identified and quantified. In batch 1, the level of individual phenolics ranged from 103 ± 1.5 to 398 ± 2.3 µg/g fw where quercetin-3-*O*-rhamnoside was the major compound. In batch 2 material, kaempferol-3-*O*-rutinoside was the main constituent. The HPLC-derived total phenolics of this plant was the lowest among the species investigated and was different between two batches where batch 1 contained more phenolics than batch 2 plants. This is in agreement with the total phenolic content analysed by Folin-Ciocalteu method. Two varieties of *P. indica*, var. A (grown under shade) and var. B (grown exposed to sunlight) showed different profiles of compounds and the level of individual phenolics varied (*Table 3.3.5*). Chlorogenic acids were the major constituents (91% of the HPLC-derived total phenolics) with 3,5-*O*-dicaffeoylquinic acid predominantly in var. A of batch 1. The level of individual phenolics in batch 2 was lower to that of batch 1 of var. A, which was similar to the yellow variety. However, the level of total phenolics analysed by HPLC-PDA-MS^2 was significantly higher in batch 1 of the yellow variety (9723 µg/g fw^{-1}) compared to other variety of both batches. Quercetin glycosides were identified only in batch 2 of both varieties indicating the different effect of season to the metabolism of plant metabolites. 3-*O*-caffeoylquinic acid and 4,5-*O*-dicaffeoylquinic acid also were also not present in batch 2 of both varieties. Four phenolic compounds were identified in *P. cordifolia* (*Table 3.3.6*) in concentrations ranging from 341 ± 1.4 to 997 ± 10 µg/g fw in batch 1 and from 93 ± 5.4 to 5959 ± 37 µg/g fw in batch 2. Flavonols were the predominant compounds (77% in batch 1 and 73% in batch 2 from the total individual phenolics). One flavone, apigenin-7-*O*-rutinoside was identified and quantified. The level of HPLC-derived total phenolics in batch 2 was significantly higher to that of batch 1. Isorhamnethin was present only in batch 2. In batch 1, two types of methylquercetin glycoside conjugates were detected and quantified based on quercetin standard.

Table 3.3.1 Total phenolic content in selected Malaysian traditional vegetables

Traditional Vegetables	Folin-Ciocalteau Method (mg/kg GAE fw)[a]		HPLC-derived phenolics (mg/g fw)[b]	
	Batch 1	**Batch 2**	**Batch 1**	**Batch 2**
Anacardium occidentale (Red variety)	361 ± 18	386 ± 41	12.4	7.6*
Anacardium occidentale (Yellow variety)	415 ± 20	353 ± 26*	6.4	8.5*
Centella asiatica	100 ± 7.8	72 ± 0.9*	3.5	3.2*
Colubrina asiatica	105 ± 5.6	62 ± 2.5*	0.9	0.7*
Pluchea indica A	294 ± 39	97 ± 2.7*	2.5	1.6*
Pluchea indica B	316 ± 18	135 ± 8.9*	9.7	2.3*
Premna cordifolia	187 ± 1.0	170 ± 9.1*	4.4	8.3*

[a]Total phenolic content analysed by Folin-Ciocalteu method. [b]Accumulation of individual phenolics analysed by HPLC-PDA-MS^2. *indicates significantly different ($p<0.05$) between batches. Result expressed as value ± standard deviation, SD (n=6).

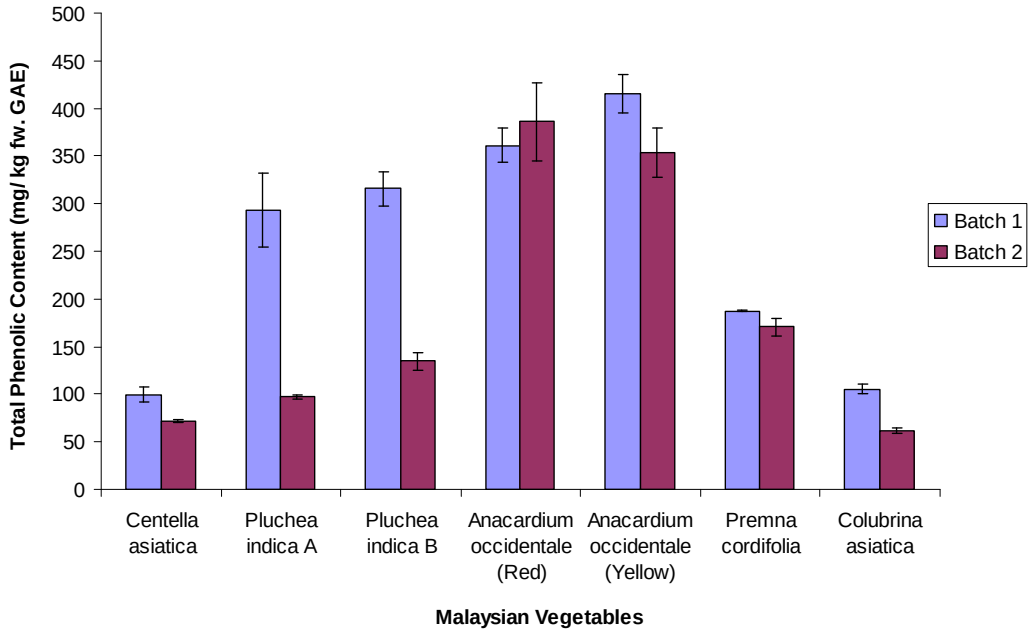


Figure 3.3.1 Total phenolic content of two batches in selected Malaysian traditional vegetables. Result expressed as value ±standard deviation, SD (n=6).

Table 3.3.2 Individual phenolics in two batches of *Anacardium occidentale* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (µg/g fw^{-1})			
	var. red		var. yellow	
	Batch 1	***Batch 2***	***Batch 1***	***Batch 2***
Myricetin glycoside	65 ± 0.5	212 ± 18	61 ± 4.4	160 ± 2.3
Myricetin glycoside	75 ± 2.4	148 ± 14	46 ± 4.1	173 ± 3.9
Unknown quercetin conjugate*	20 ± 0.1	28 ± 2.0	12 ± 0.8	35 ± 0.8
Unknown quercetin conjugate*	77 ± 3.4	664 ± 84	125 ± 2.5	637 ± 34
Myricetin-3-*O*-rhamnoside	25 ± 0.5	59 ± 3.4	nd	79 ± 0.6
Quercetin-3-*O*-galactoside	771 ± 9.3	1321 ± 122	600 ± 32	1503 ± 23
Quercetin-3-*O*-glucoside	829 ± 5.8	1564 ± 143	663 ± 39	1672 ± 25
Quercetin-3-*O*-xyloside	566 ± 4.5	1034 ± 90	481 ± 20	1149 ± 18
Quercetin-3-*O*-arabinofuranoside	459 ± 7.7	718 ± 48	338 ± 11	897 ± 11
Quercetin-3-*O*-arabinopyranoside	109 ± 2.5	597 ± 43	60 ± 0.2	822 ± 23
Quercetin-3-*O*-rhamnoside	481 ± 13	892 ± 77	374 ± 21	967 ± 16
Kaempferol-3-*O*-glucoside	4308 ± 111	145 ± 7.3	2592 ± 160	171 ± 3.1
Kaempferol-3-*O*-xyloside	388 ± 27	41 ± 1.5	257 ± 22	48 ± 0.4
Kaempferol-3-*O*-arabinofuranoside	633 ± 46	71 ± 2.6	477 ± 43	96 ± 0.4
Kaempferol-3-*O*-arabinopyranoside	3307 ± 39	86 ± 3.7	350 ± 53	101 ± 1.5
Kaempferol coumaroyl glucoside	310 ± 30	nd	nd	nd
HPLC-derived total phenolics	**12420**	**7578**	**6434**	**8509**

Note: nd = not detected. *Unknown quercetin conjugates were quantified based on quercetin standard's calibration curve. Result expressed as value ± standard deviation, SD (n=6).

Table 3.3.3 Individual phenolic in two batches of *Centella asiatica* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (µg/g fw^{-1})	
	Batch 1	***Batch 2***
Quercetin-3-*O*-glucoronide	55 ± 1.1	16 ± 0.7
Kaempferol-3-*O*-glucoside	526 ± 34	493 ± 8.3
Kaempferol-3-*O*-glucuronide	1633 ± 145	nd
Total flavonol	**2214**	**509**
3-*O*-Caffeoylquinic acid	14 ± 0.5	27 ± 5.2
5-*O*-Caffeoylquinic acid	86 ± 1.6	113 ± 5.5
5-*O*-Feruloylquinic acid	18 ± 0.5	47 ± 1.2
3,4-*O*-Dicaffeoylquinic acid	408 ± 4.2	126 ± 1.7
3,5-*O*-Dicaffeoylquinic acid	671 ± 16	718 ± 30
4,5-*O*-Dicaffeoylquinic acid	nd	163 ± 4.7
3-*O*-Feruloyl-5-*O*-caffeoylquinic acid	41 ± 1.2	1229 ± 24
3-*O*-Caffeoyl-5-*O*-feruloylquinic acid	72 ± 1.0	106 ± 10
Total chlorogenic acids	**1310**	**2664**
HPLC-derived total phenolics	**3524**	**3173**

Note: nd = not detected. Result expressed as value ± standard deviation, SD (n=6).

Table 3.3.4 Individual phenolic in two batches of *Colubrina asiatica* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (µg/g fw^{-1})	
	Batch 1	***Batch 2***
Quercetin-3-*O*-rhamnoside	398 ± 2.3	242 ± 17
Kaempferol-3-*O*-glucoside	103 ± 1.5	29 ± 2.0
Kaempferol-3-*O*-rutinoside	371 ± 1.7	392 ± 18
HPLC-derived total phenolics	**872**	**663**

Result expressed as value ± standard deviation, SD (n=6).

Table 3.3.5 Individual phenolic in two batches of *Pluchea indica* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (µg/g fw^{-1})			
	var. A		*var. B*	
	Batch 1	***Batch 2***	***Batch 1***	***Batch 2***
Quercetin-3-*O*-galactoside	5.8 ± 0.8	nd	47 ± 0.3	nd
Quercetin-3-*O*-glucoside	29 ± 0.3	nd	134 ± 2.8	nd
Quercetin-3-*O*-sulphate	187 ± 6.6	303 ± 5.5	1496 ± 17	35 ± 1.1
Total flavonol	**222**	**303**	**1677**	**35**
3-*O*-Caffeoylquinic acid	13 ± 0.4	nd	181 ± 0.5	nd
5-*O*-Caffeoylquinic acid	132 ± 0.1	9.8 ± 0.2	881 ± 4.2	48 ± 2.3
3,4-*O*-Dicaffeoylquinic acid	131 ± 3.7	662 ± 21	573 ± 4.8	676 ± 25
3,5-*O*-Dicaffeoylquinic acid	1011 ± 27	586 ± 7.8	3128 ± 52	1361 ± 31
4,5-*O*-Dicaffeoylquinic acid	958 ± 7.0	nd	3214 ± 67	nd
Tricaffeoylquinic acid	68 ± 0.8	68 ± 4.6	69 ± 2.1	164 ± 16
Total chlorogenic acids	**2313**	**1326**	**8046**	**2249**
HPLC-derived total phenolics	**2535**	**1629**	**9723**	**2284**

Note: nd = not detected. Result expressed as value ± standard deviation, SD (n=6).

Table 3.3.6 Individual phenolic in two batches of *Premna cordifolia* analysed by HPLC-PDA-MS^2

Phenolic compounds	Level of individual phenolics (µg/g fw^{-1})	
	Batch 1	***Batch 2***
Isorhamnetin	nd	93 ± 5.4
Methylquercetin glycoside conjugate*	3046 ± 30	nd
Methylquercetin glycoside conjugate*	341 ± 1.5	5959 ± 37
Total flavonol	**3387**	**6052**
Apigenin-7-*O*-rutinoside	997 ± 10	2287 ± 434
HPLC-derived total phenolics	**4384**	**8338**

*Quantified based on quercetin standard, nd = not detected. Result expressed as value ± standard deviation, SD (n=6).

3.4 Total antioxidant activities of Malaysian traditional vegetables

Total antioxidant activities of the traditional vegetable extracts were analysed by ABTS assay in comparison to the synthetic vitamin E analogue, Trolox using on-line HPLC antioxidant detection system without the HPLC column. Total antioxidant activity was also determined by the FRAP assay (refer to *Chapter 2*).

3.4.1 Correlations between total phenolic content and total antioxidant activities

The total phenolic content of Malaysian traditional vegetables which was measured by Folin-Ciocalteu assay is shown in *Figure 3.4.1* and the total antioxidant activities measured by FRAP assay are shown in *Figure 3.4.2*. Among all the species*, A. occidentale* showed the highest total antioxidant activity in batch 1 and 2 with the yellow variety showed the highest activity. This was correlated to the total phenolic content which showed the highest concentration among the vegetables tested. *Colubrina asiatica* exhibited the lowest total antioxidant activity for both batches (0.1 ± 0.1 mM in batch 1 and 0.2 ± 0 mM in batch 2). The rank of these vegetables based on FRAP-derived antioxidant activity was *A. occidentale* (yellow) > *A. occidentale* (red) > *P.* indica (var B) > *P. indica* (var A) > *P. cordifolia* > *C. asiatica* > *Colubrina asiatica.* In the ABTS on-line antioxidant assay (*Figure 3.4.3*), a similar profile was observed where *A. occidentale* showed the highest activities in batch 1 and 2, *P indica* was ranked second and *Colubrina asiatica* and *C. asiatica* exhibited the lowest antioxidant activities.

There were significant correlations between the total phenolic content and the total antioxidant activities in FRAP and ABTS assays. For example, in the red variety of *A. occidentale,* FRAP-derived antioxidant activity was highly and significantly correlated with total phenolic content, $r = 0.987$, $p < 0.01$ in batch 1 and $r = 0.974$, $p < 0.01$ in batch 2. Tsao and Deng (2004) have reported that high antioxidant activity was correlated with total phenolic content. This was supported by the fact that phenolic compounds are more effective antioxidants than other compounds such as vitamin C and E in fruit and vegetables which contribute to the protective effects mechanism in human (Rice-Evans *et al.* 1997, Miller and Ruiz-Larrea 2002). In contrast, it was reported that there was no correlation between total phenol content and the total antioxidant activities of 92 species of fruits and vegetables analysed by Folin-Ciocalteu for total phenolic content and oxidised Melo method for *in vitro* antioxidant activity (Kahkonen *et al.* 1999). Therefore, quantification of the

contribution of phenolic compounds to the total antioxidant activity would be required to understand the correlation between the phenolic compounds and the antioxidant activities. However, comparison or judgement could not be made as the samples measured in the studies reported on the correlation between total phenolic content and antioxidant activity were different to that of traditional vegetables used in the present study. On the other hand, the method used to analyse the samples also influenced the outcome (Puupponen-Pimia *et al*. 2001)

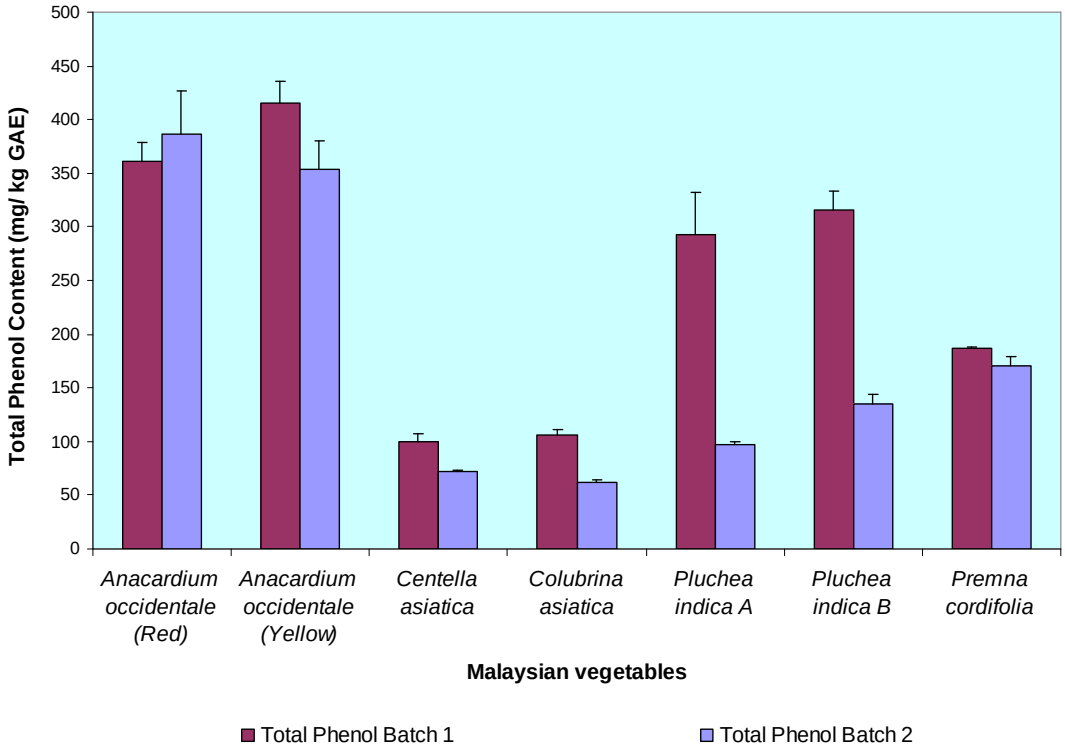


Figure 3.4.1 Total phenolic content of Malaysian traditional vegetables. Result expressed as value ± standard deviation, SD (n=6).

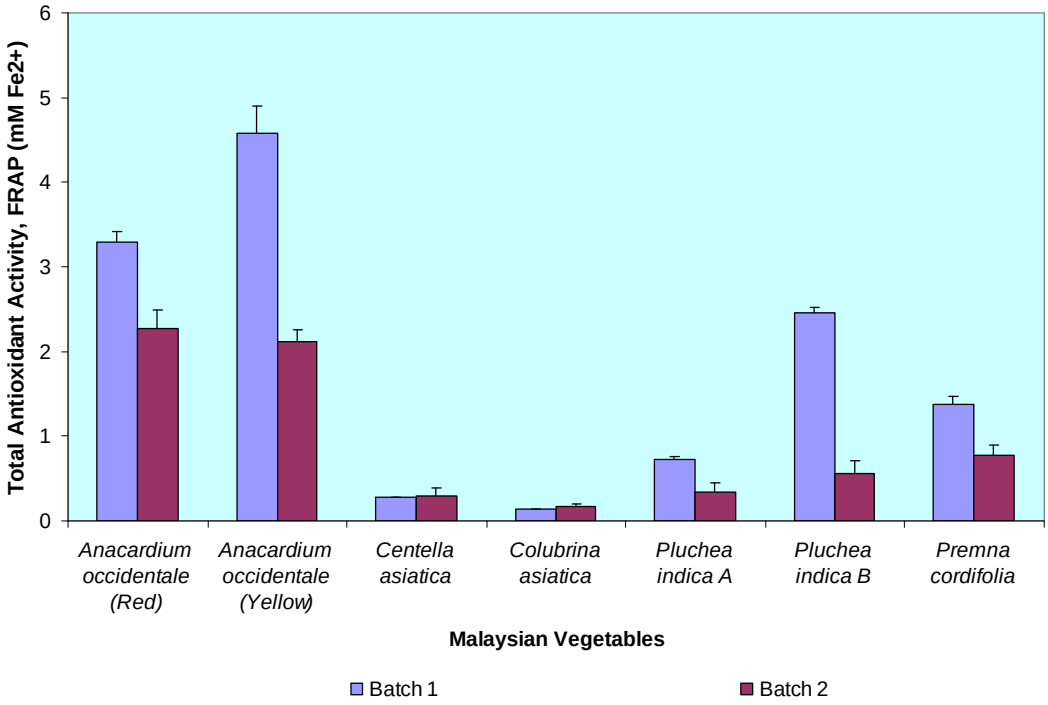


Figure 3.4.2 Total antioxidant activity (FRAP) of Malaysian traditional vegetables. Result expressed as value ± standard deviation, SD (n=6).

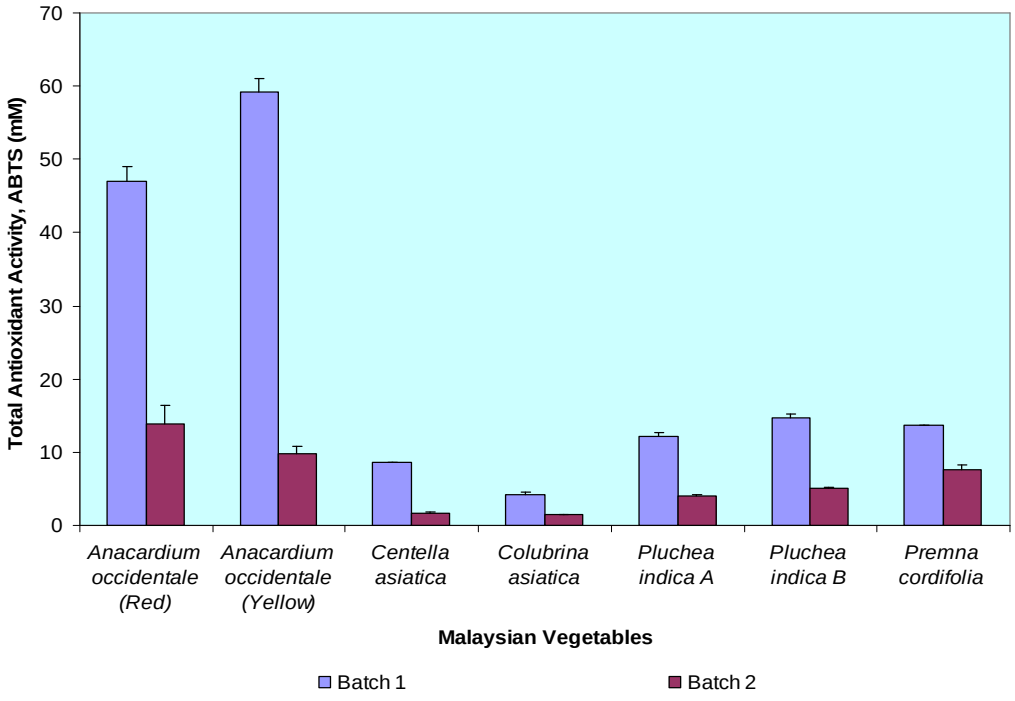


Figure 3.4.3 Total antioxidant activity (ABTS) of Malaysian traditional vegetables. Result expressed as value ± standard deviation, SD (n=6).

3.5 Discussion

There is convincing epidemiological evidence in support of the association between diet and chronic diseases (Rao and Rao 2007). Based on such evidence, dietary guidelines have been formulated around the world to reduce the incidence of chronic diseases such as cancer, CVD, diabetes and osteoporosis. One of the main recommendations of these dietary guidelines is to increase the consumption of fruit and vegetables that are good sources of phenolics, carotenoids and other biologically active phytochemicals. However, determination of the active phytochemicals in plants should be a priority, in order to help understand the effects of these components on human health, following the ingestion of five portions a day of fruits and vegetables. Information of phytochemicals in Malaysian vegetables is still in an early stage, even though a database of medicinal plants of Malaysia was documented in 1966 (Burkhill 1966). Lack of a database of chemical components in Malaysian plants, especially beneficial compounds responsible for improving human health, has led this present study to investigate the level of phytochemicals in selected Malaysian traditional vegetables.

All the seven species in the present study have shown high concentrations of total phenolics which varied greatly between two batches of harvesting. The total phenolic content was statistically lower ($p<0.05$) in batch 2 which was harvested in the dry season compared to batch 1 that was obtained in the rainy season (*Figure 3.3.1*). This could be due to the metabolism of secondary metabolites in the plants affected by various factors such as light intensity, humidity and different harvesting periods, that are complex and beyond unclear. For instance, the increased of total phenolic content in the rainy season (batch 1) could be due to phenolic esterification as reported by Solecta *et al*. (1999). Acclimatisation especially low temperature and high humidity are reported to influence the modification of metabolic pathways in plants (Kacperska 1989) especially phenylpropanoid metabolism (Dixon and Paiva 1995). The key enzyme of phenylpropanoid biosynthesis, phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) is reported to increase in tomatoes (Rhodes and Wooltorton 1977) and potatoes (Tanaka and Uritani 1977) after the temperature is reduced by chilling. Chalker-Scott and Fuchigami (1989) also reported accumulation of water-soluble phenolics in frost-stressed rhododendron leaves and there was increased lignification of grapevine, apple trees and sugar cane subjected to low temperature. In the present study, even though there was variability of total phenolic content between species, the

identification of individual phenolics has revealed a number of flavonoids in each species which contribute to the high amount of total phenolic content. However, HPLC-derived total phenolic content were lower compared to total phenolics content analysed by Folin-Ciocalteu assay but in line with the previous observation, that batch 1 had a higher phenolic content than batch 2. The lower values of the HPLC-derived total phenolic content and some variations between the results of batch 1 and batch 2 could be due to the unknown phenolic compounds that could not be quantified and high molecular weight compounds, such as polymeric procyanidins, that do not elute from the HPLC column. Furthermore, some of the flavonoid conjugates were quantified on the basis of their parent aglycones as no standards available, for example, methylquercetin glucoside conjugate (*Table 3.3.6*) was quantified by reference to a quercetin standard curve. The level of total phenolics in the traditional vegetables can be ranked in the following order: *Anacardium occidentale* > *Pluchea indica* > *Premna cordifolia* > *Centella asiatica* > *Colubrina asiatica*.

Among the flavonoid groups, flavonol glycosides have been shown to be predominant in most of the species particularly *A. occidentale, Colubrina asiatica* and *P. cordifolia*. The flavonols identified in the present study were similar to the previous studies on other vegetables (Justesen *et al.* 1998, Crozier *et al*. 2000). Other flavonoids glycosides are also reported to be predominant forms of naturally occurring flavonoids in plants (Cuyckens *et al*. 2000), which represent a large group of secondary plant metabolites (Harborne *et al*. 1988, Harborne 1994). Analysing these compounds by HPLC-MS^2 has facilitated identification based on mass spectra fragmentation pattern; however, there are limitations when it comes to identify compounds with a similar molecular weight. Therefore, the need to compare absorbance spectra and retention times (elution order) with available standards helps to verify the identity of compounds. Bloor (2001) has discussed general rules of reverse-phase HPLC elution order to confirm identities of flavonol glycosides. The order of elution from most polar through to least polar means that flavonol triglycosides (and higher glycosides) elute early, followed by di- and monoglycosides and then acylated or alkylated glycosides and aglycones. Hydrolysis with HCl can also be proposed for the analysis of flavonoids as the sugar moieties are cleaved releasing the aglycone (Hertog *et al*. 1992). However, as most of the flavonoids detected in these traditional vegetables were flavonols in particular quercetin- and kaempferol-based compounds, which are reported to be predominant in plants, the need of acid hydrolysis was not necessary and acid hydrolysis removes the possibility of identifying the conjugation of the compound. This is in agreement

with previous reports (Harborne *et al*., 1988, Heim *et al*. 2002). There is increasing interest not only in the phytochemical identification, but also in the bioactivities of these compounds *in vitro* and *in vivo* especially with regard to antioxidant and anticancer properties. The bioactivities have been reviewed elsewhere (Fleuriet and Macheix 2003, Proteggente *et al.* 2003). The present study has indicated that quercetin and kaempferol are among the most important flavonoids that could contribute to such bioactivities. These two flavonols have been reported to possess the highest antioxidant activity ranking after myricetin (Heim *et al*. 2002, Kim and Lee 2004). Myricetin glycosides were found in moderately high amounts in *A. occidentale* and could contribute to the high antioxidant activity of this species.

Chlorogenic acids which are characteristic components of coffee beans and commercial coffee products, in which caffeoylquinic, *p*-coumaroylquinic, feruloylquinic, dicaffeoylquinic and caffeoylferuloylquinic acids have been reported (Clifford 2000, Clifford *et al.* 2003). In the present study, chlorogenic acids were the major components found in *C. asiatica* and *P. indica*. Identification of these compounds was based on the MS^2 fragmentation patterns of Clifford *et al*. (2003) and comparison of the elution order of chlorogenic acids found in coffee analysed under similar HPLC conditions. The level of total chlorogenic acids in *C. asiatica* was found highest in batch 2 compared to batch 1 (*Table 3.3.3*). This is in contrast with *P. indica*, where the level of total chlorogenic acids was higher in batch 1. However, the level of total chlorogenic acids in var. B samples, which were grown under direct sunlight, was approximately 4-fold higher than var. A samples, grown under shade. The effect of sunlight on the production of chlorogenic acids in plant has been reported by Zucker (1963). When longer light exposures at higher intensities are given, chlorogenic acid synthesis is stimulated, and light appears to enhance chlorogenic acid formation by virtue of its effects on protein synthesis (Zucker 1963). The level of different types of chlorogenic acids also varied greatly in these two species which arguably indicates an internal factor such as the metabolism of chlorogenic acid (5-caffeoylquinic acid) to dicaffeoyl- and tricaffeoylquinic acids (Clifford 2000). Genetic engineering has also increased chlorogenic acid levels in plants such as in tomato and tobacco, in order to increase antioxidant potential for health benefits (Niggeweg *et al*. 2004). Like other dietary polyphenols, chlorogenic acid is an antioxidant, which has been reported to scavenge radicals generated in the aqueous phase *in vitro* (Clifford 1999, Rice-Evans *et al.* 1996), increase the resistance of LDL to lipid peroxidation (Castellucio *et al.* 1995, Nardini *et al.* 1995, Abu-Amsha Caccetta *et al.* 1996) and inhibit DNA damage (Shibata *et al.* 1999, Kasai

et al. 2000). Apart from showing an antioxidant activity *in vitro*, chlorggenic acids when added to the diet treatment *in vivo*, inhibited chemically induced carcinogenesis of the large intestine, liver and tongue in rats and hamsters (Mori *et al.* 1986, Tanaka *et al.* 1990, 1993) which could be potential as anticancer agent.

There were correlations between total phenolic content and FRAP-derived total antioxidant activities in these plants (*Table 3.5.1*). Significant correlations between total phenolic content and total antioxidant activities in batch 2 of all the species were observed in the present study, but no significant correlations in *C. asiatica, Colubrina asiatica* and *P. cordifolia* in batch 1. The discrepancy could be due to the lower number of samples in the analysis (*n = 3*). Many reports have indicated a correlation between total phenolic contents and total antioxidant activities *in vitro* in vegetables, and phenolics have been attributed to be the main contributor for these biological activities (Proteggente *et al*. 2002, Ismail *et al.* 2004, Mansouri *et al*. 2005). However, Puupponen-Pimia *et al.* (2001) did not observe any correlation between total phenolic and total antioxidant activities in 92 fruit and vegetables tested. The reason being was, different phenolic compounds have different responses in the Folin-Ciocalteu method (Satue-Gracia *et al.* 1997). This is also supported by Kahkonen *et al.* (1999) and may be a consequence of fruits and vegetables containing other phytochemicals such as vitamins and alkaloids that has been reported to exhibit potent antioxidant activity (Rice-Evans and Miller 1995, Heim *et al*. 2002, Grabmann 2005).

Table 3.5.1 Pearson correlation of total phenolic content and total antioxidant activities (FRAP) of traditional vegetables (a = p<0.001, b = p<0.005)

	Anacardium occidentale		***Centella asiatica***	***Colubrina asiatica***	***Pluchea indica***		***Premna cordifolia***
	Red	**Yellow**			**A**	**B**	
Batch 1	0.987[a]	0.602	-0.614	-0.950[a]	0.851[b]	0.101	-1.000[a]
Batch 2	0.974[a]	0.946[a]	0.267	0.945[a]	0.921[a]	0.149	0.968[a]

A. occidentale has been shown to have the highest antioxidant activity in all the assays tested and in line with the Folin-Ciocalteu assay. This plant is popular in Malaysia, and the highest antioxidant activity exhibited by *A. occidentale* in the present study is in agreement with the previous reports (Vimala *et al.* 2003, Abas *et al.* 2006). However, these earlier studies used thiobarbituric acid (TBA), ferric thiocyanate (FTC) and DPPH assays, which

were different methods to that of the present study. Maia *et al.* (2000) reported that *A. occidentale* also contained other compounds that could contribute to the antioxidant activity, for example, anacardic acids which were found to be abundant in fruits of *A. occidentale* (Kubo *et al*. 2006, Schultz *et al*. 2006). Surprisingly, *C. asiatica,* which is also a very popular traditional vegetable in Malaysia and reported to have high antioxidant activity (Vimala *et al.* 2003, Hussin *et al*. 2007) and to increase the levels of antioxidant enzymes i.e. SOD, glutathione peroxidase (GSHPx) and GSH significantly in rats *in vivo* after 200 mg/ kg extract for 14 days (Veerendra Kumar and Gupta 2002, Jayashree *et al.* 2003), showed the lowest antioxidant activities among the vegetables tested in the present study. This, however, could be influenced by several factors such as different samples used, different extracts and methodologies and also the effects of environmental factors such as different harvesting season, sunlight and storage (Harborne and Williams 2000, Robards 2003).

The total antioxidant activities exhibited by phenolic compounds in fruit and vegetables, as stated, have been correlated to the protective effects, which reduced the risk of chronic diseases in man (Cook and Samman 1996, Arts and Hollman 2005). The antioxidant activities of phenolic compounds are closely related to their structures. The common flavonoids such as myricetin, quercetin and rutin have been shown to exert greater antioxidant activities than the conventional antioxidant vitamin, α-tocopherol (vitamin E) but the bioavailability may be much less (Afanas'ev *et al*. 1989, Rice-Evans *et al.* 1996, 1997). This is due to the number of hydroxyl groups which enable the compound to donate H^+ and delocalise the resulting free electron (Awad *et al*. 2001, Heim *et al.* 2002). The more hydroxyl groups attached in a molecule, the greater the antioxidant activitiy (Salah *et al.* 1995). Thus, similar amounts of quercetin and myricetin have very high TEAC values, 4.7 and 3.1 mM (Rice-Evans *et al*. 1996, 1997). In the present study, *A. occidentale* exhibited very high antioxidant activities, which is probably due to the presence of myricetin- and quercetin glycosides. However, it was reported that the aglycones are more potent antioxidants than their corresponding glycosides as the attached glucose to lower the radical scavenging activity (Heim *et al.* 2002). Nevertheless, synergistic effects from other compounds presence in plant can also influent the total antioxidant activity (Liu 2003). In the present study, the total aglycones were not quantified, and flavonoid glycosides were the predominant components in the plants under study.

Flavonoid glycosides will be highly metabolised in the human stomach, intestine and colon, which will affect the protective effects *in vivo* after consumption of fruit and vegetables. The absorption and metabolism of phenolic compounds are still unclear, however it was reported that quercetin glycosides are not affected by low pH and are probably able to resist acid hydrolysis in the stomach, and so, pass intact into the small intestine (Gee *et al*. 1998). The glycosides are then hydrolysed and appear in the bloodstream as methylated, sulphated and glucuronidated metabolites (Donovan *et al.* 2006). However, the glycosides of anthocyanins have been showed to be rapidly and efficiently absorbed through the gastric wall of rats perfused *in situ* with berry anthocyanin extracts (Talavera *et al.* 2003). More extensive research is required in order to understand and determine the mechanism of flavonoids uptake, absorption and metabolism, whether in the form of glycosides or aglycones from the small intestine, or whether they enter the colon to be metabolised through the degradation by the colonic micro-flora. The present study had been limited to the analysis of phytochemicals in the plants and the bioactivities *in vitro,* as the human intervention studies were not approved, due to the lack of information and availability or surveillance studies of the Malaysian traditional vegetables. However, information obtained on the levels of phenolic compounds in the fruit and vegetables and their contribution to the total antioxidants can assist us in calculating the suggested portion of daily dietary intake and thus, could help promote the importance of a healthy plant-rich diet that can be easily found in Malaysia, but at the moment is largely neglected by the local people. However, *in vitro* assay of antioxidant may not reflect antioxidant activity *in vivo*. Therefore, there is a need of animal model studies as a representative of *in vivo* before human intervention studies are conducted.

3.6 Conclusion

The use of advanced analytical instruments such as HPLC, HPLC-MS^2 and different antioxidant assays has led to identification of phenolic compounds and their contribution to the total antioxidant activities in Malaysian traditional vegetables, which have been reported to have medicinal properties. The phenolic contents of these vegetables were low except for *A. occidentale*. However, this study has given much information for a preliminary database to increase interest among researchers. From this study, it is suggested that consumption of traditional vegetables could possibly offer some dietary benefits since they contain constituents which are able to protect against lipid peroxidation and to scavenge free

radicals. Consumption of the traditional vegetables can be as good as the exotic vegetables which are normally expensive especially in the developing countries. The traditional vegetables are ranked as shown in *Table 3.6.1* according to popularity and, from this study, their phytochemical content.

Table 3.6.1 Rank of Malaysian traditional vegetables based on popularity and also based on phenolic content after the study.

Species	Rank	
	Popularity*	**Phenolic content and antioxidant activity**
Anacardium occidentale	2	1
Centella asiatica	1	3
Colubrina asiatica	5	5
Pluchea indica	3	2
Premna cordifolia	4	4

* *Ref*: Jaganath and Teik (2000), Saidin (2000).

CHAPTER 4

ANALYSIS OF OTHER PHYTOCHEMICALS IN SELECTED MALAYSIAN TRADITIONAL VEGETABLES

4.1 Introduction

It is known that the roles of fruit and vegetables diet in lowering the risk of serious diseases have been attributed in part, to the antioxidant properties of their constituent polyphenols (Rice-Evans *et al.* 1997, Prior 2003), which have been discussed in the previous chapter. Even though recent studies have shown that many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than vitamins E or C, and thus may contribute significantly to the protective effects *in vivo* (Rice-Evans *et al.* 1997, Miller and Ruiz-Larrea 2002), the contribution of the protective effects of other phytochemicals in humans cannot be ruled out (Tsao and Deng 2004). Several factors that influence the relationship between fruit and vegetables and their protective effects include the bioavailability and absorption and the impact of their constituent phytochemicals in humans (Lin and Tang 2007). Although *in vivo* studies are important, *in vitro* experiments have been a key to understanding the mechanism of protective effects and their relationship to human health, especially their correlation to reduce the risk of chronic diseases. Many researchers have carried out bioassay-guided fractionation to investigate the bioactivities of phytochemicals in the search for novel compounds that exhibit for instance, anticancer, anti-aging and antimicrobial properties (Gebhardt 2000, Houghton 2000, Houghton *et al.* 2005). Understanding the mechanism of bioactivities *in vitro* is essential in developing *in vivo* studies, in particular, the relationships of antioxidant activities of phenolic compounds and other phytochemicals, which in turn are responsible for the protective effects in humans.

Polyphenols have been reported to be the major phytochemicals contributing to health effects. For example, quercetin, which is ubiquitous in fruit and vegetables, has been reported to contribute to antioxidant capacity (Hertog *et al*. 1992, Wang *et al.* 1996). However, there may be more than 100 compounds in the fruit and vegetables, that can be analysed and identified by analytical instruments such as HPLC, that have potential antioxidant activities (Guo *et al*. 1997, Prior 2003). The contribution of other phytochemicals

towards antioxidant potential of fruit and vegetables should not be neglected. Terpenoids, such as carotenoids have been shown to have potent antioxidant activities (Tsao and Deng 2004). Consumption of a carotenoid-rich diet has reported to reduce the risk of eye diseases, such as cataract and macular degeneration (Rao and Rao 2007). This, however, is also influenced by vitamin C and E (Guo *et al.* 1997).

As the information of phytochemicals and bioactivities of Malaysian traditional vegetables is scarce, this chapter reports on the analysis of phytochemicals and their contribution to the antioxidant capacity of Malaysian traditional vegetables. Seven species of traditional vegetables were analysed by the HPLC, HPLC-MS and GC-MS and their antioxidant activities *in vitro* were determined using the FRAP and DPPH assays.

4.2 HPLC and HPLC-tandem mass spectrometry analysis of other phytochemicals in selected Malaysian traditional vegetables

4.2.1 Qualitative and quantitative analysis of triterpenes in *Centella asiatica*

Triterpenes have been reported as biomarkers of *C. asiatica* which is a very popular medicinal plants or traditional vegetables in India and Southeast Asia (Singh and Rastogi 1968, Kartnig 1988, Goh *et al*. 1995). In order to investigate the level of triterpenes in *C. asiatica* grown in Malaysia, analyses were conducted using both HPLC and HPLC-PDA-MS^2 to investigate the differences between the two different batches. By using the RP-HPLC with PDA detector, all triterpenes were detected, separated and quantified at 200 nm (*Figure 4.2.1*). The MS data obtained in this study has facilitated the fragmentation pattern of madecassoside and asiaticoside to their aglycones as shown in *Figure 4.2.2*.

Madecasoside (t_R = 44.2 min, λ_{max} = 205 nm). This peak had a $[M-H]^-$ at *m/z* 974 with MS^2 yielding a charged fragment ion at *m/z* 469 which is its aglycon, madecassic acid, lost of 505 *amu* units [3 x 162 *amu* (glucose) + 18 *amu* (H_2O) + 1 *amu* (H)]. The fragmentation pattern was compared to (Mauri and Pietta 2000) and the structure is given in *Figure 4.2.2*, where *R = OH* and three molecules of glucose are attached to the basic skeleton. During the fragmentation, the *C-O* bond which attached to the glucose molecules breaks down and produces two molecules of water which will form a molecule of madecassic acid.

Asiaticoside (t_R = 48.7 min, λ_{max} = 205 nm). This peak had a $[M-H]^-$ at *m/z* 957 with MS^2 yielding a charged asiatic acid fragment ion at *m/z* 469 with loss of 488 *amu* unit [3 x 162 *amu* (glucose) and 2 *amu* (2H)]. The structure of asiaticoside is given in *Figure 4.2.2*, where R = OH and three glusoce molecules are attached to the basic skeleton.

For the quantification of the triterpenes, as mentioned, HPLC-PDA was used and the retention times of the compounds eluted were different from the HPLC-MS^2 above. The levels of triterpenes between two batches as shown in *Table 4.2.1* varied with madecasoside having the highest level in both batches with 91 ± 4.8 µg/g fw in batch 1 and 77 ± 3.4 µg/g fw in batch 2 respectively. The level of madecassoside in batch 2 was significantly lower ($p<0.05$) to that of batch 1. The level of asiaticoside was the lowest among all the triterpenes quantified both batches, however, the total triterpenes were not significantly different between batches in *C. asiatica*.

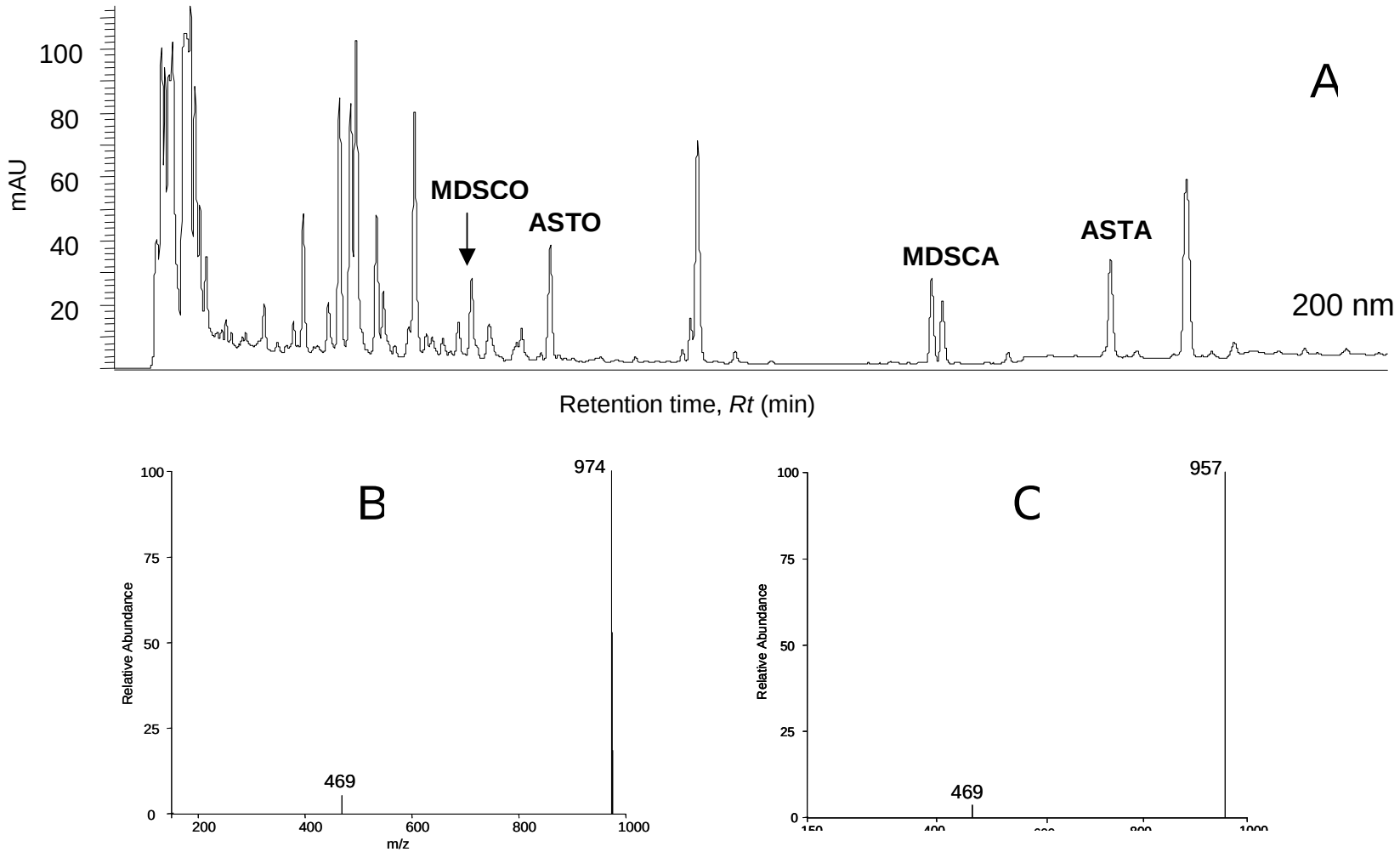
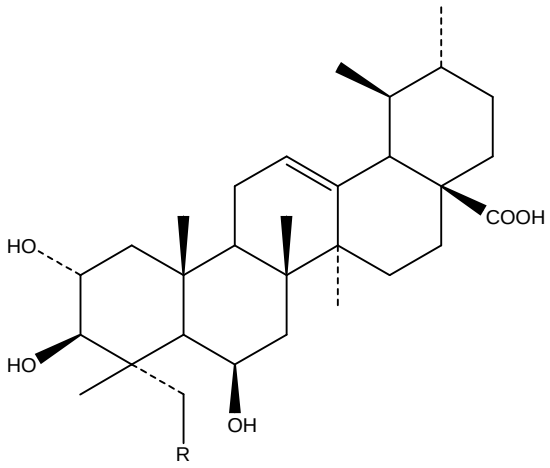
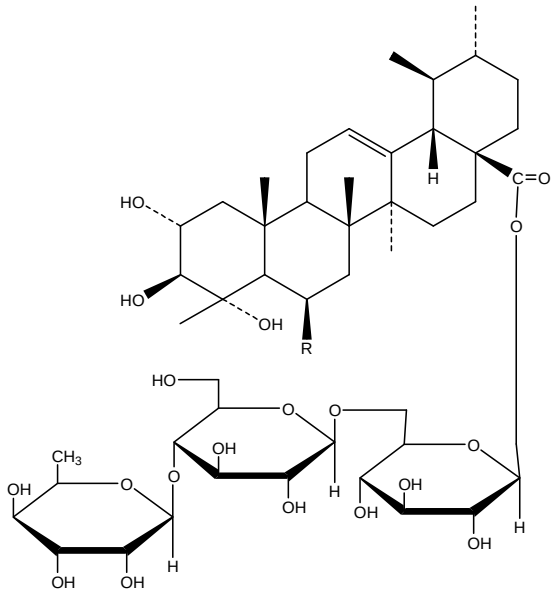


Figure 4.2.1 (A) Chromatogram of triterpenes in batch 1 of *Centella asiatica* obtained using HPLC at 5-60% acetonitrile over 60 minutes with 0.05% phosphoric acid at absorbance 200 and 365 nm, (B) Fragmentation of madecassoside, (C) Fragmentation of asiaticoside



Triterpene	R
Asiatic acid	R = H
Madecassic acid	R = OH



Triterpene glycoside	R
Asiaticoside	R = H
Madecassoside	R = OH

Figure 4.2.2 Structure of triterpene and triterpene glycosides in *Centella asiatica* (Inamdar *et al.* 1996)

Table 4.2.1 Level of triterpenes (µg/ g fw) in batch 1 and 2 of *Centella asiatica*

Triterpenes	R_t (min)	Level of triterpenes	
		Batch 1	Batch 2
Madecasosside	16.8	91 ± 4.8	77 ± 3.4*
Asiaticoside	20.5	11 ± 0.5	13 ± 0.6*
Madecassic acid	39.1	54 ± 4.1	71 ± 5.0 *
Asiatic acid	47.5	46 ± 2.9	42 ± 2.4
Total triterpenes		202 ± 12	209 ± 11

*(p<0.05) shows statistically significant analysed by two-way ANOVA. Result expressed as value ± standard deviation, SD (n=6).

4.2.2 Level of carotenoids and α-tocopherol

The levels of total and individual carotenoids and α-tocopherol in traditional vegetables were measured using HPLC with a fluorescence detector (refer to *Chapter 2*). The results are presented in *Table 4.2.2. P. indica* contained the highest amount of total carotenoids (ranged from 21 ± 0.2 to 28 ± 0 µg/kg fw) in two batches. *P. cordifolia* had the lower total carotenoids level. The level of total carotenoids in the vegetables can be ranked in the following order: In batch 1, *P. indica* > *A. occidentale* > *C. asiatica* > *Colubrina asiatica* > *P. cordifolia*. In batch 2, *P. indica* > *C. asiatica* > *A. occidentale* > *Colubrina asiatica* > *P. cordifolia.* There was a slight difference in the rank of vegetables between batches. *A. occidentale* was ranked second in batch 1, but ranked third in batch 2 after *C. asiatica*. This discrepancy could be due to the environmental effects on the level of carotenoids in the vegetables (Dixon and Paiva 1995).

The chromatograms of individual carotenoids are shown in *Figures 4.2.3 – 4.2.7*. The level of individual compounds of carotenoids varied in batch 1 and 2 as shown in *Table 4.2.3*. Lutein was the predominant carotenoids detected in the vegetables ranged from 0.4 ± 0 to 3.9 ± 0.2 µg/ kg fw in batch 1 and 0.3 ± 0 to 5.8 ± 0 µg/ kg fw in batch 2. Zeaxanthin, *t*-canthaxanthin and *t*-lycopene were not detected in batch 1 of all species compared to batch 2, which was probably presence in trace amounts that were below the detection level of the

HPLC. The levels of α-tocopherol which were detected by fluorescence detector (*Peak 7*), varied greatly between batches with *A. occidentale* having the highest concentration and *Colubrina asiatica* ranked last with the lowest amount in both batches. In batch 2, no trace of α-tocopherol was detected in *Colubrina asiatica*. The level of total carotenoids in the vegetables can be ranked in the following order: In batch 1, *P. indica* > *A. occidentale* > *C. asiatica* > *Colubrina asiatica* > *P. cordifolia*. In batch 2, *P. indica* > *C. asiatica* > *A. occidentale* > *Colubrina asiatica* > *P. cordifolia.*

Table 4.2.2 Total carotenoids in selected Malaysian traditional vegetables

Plant Species	**Level of total carotenoids (µg/ kg fw)**	
	Batch 1	**Batch 2**
Anacardium occidentale (Yellow)	16 ± 0.6	3.9 ± 0.4
Anacardium occidentale (Red)	7.4 ± 0.6	9.0 ± 0.7
Centella asiatica	6.8 ± 0.3	9.3 ± 0.2
Colubrina asiatica	6.8 ± 0	2.8 ± 0.1
Pluchea indica A	28 ± 0	24 ± 2.5
Pluchea indica B	25 ± 0	21 ± 0.2
Premna cordifolia	1.6 ± 0	1.2 ± 0

Level of carotenoids expressed as µg per kg fresh weight (fw) *Expressed as value ± standard deviation, SD (n=6) in µg/ kg lutein equivalent.

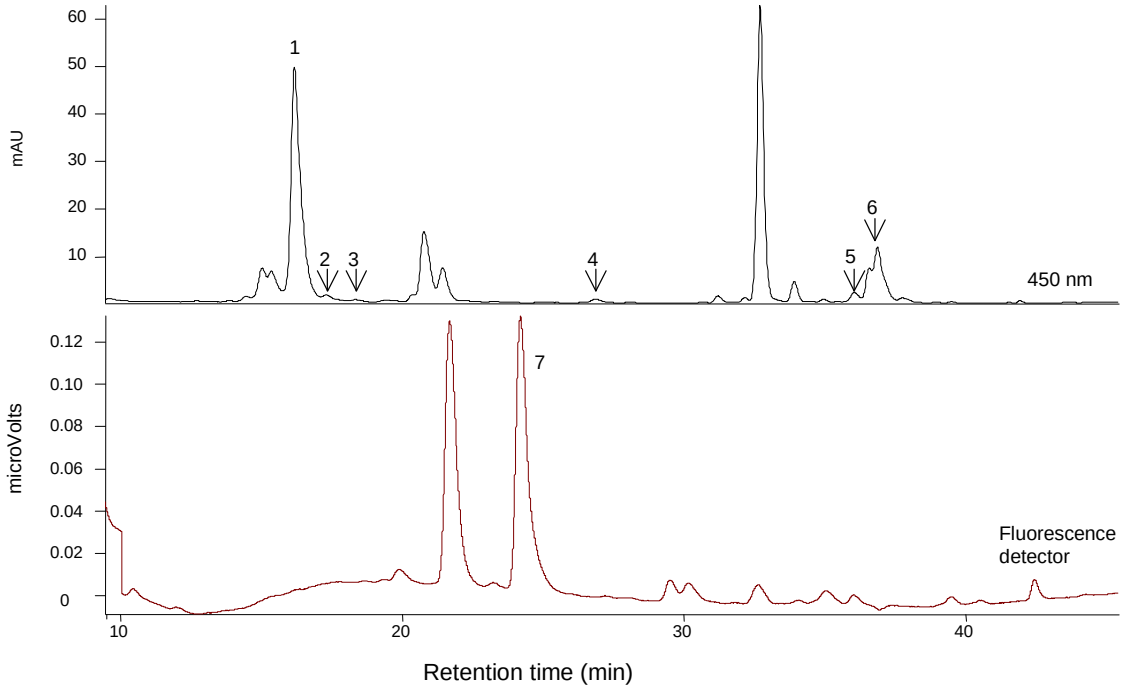


Figure 4.2.3 Chromatogram of carotenoids and α-tocopherol of *Anacardium occidentale* (red var.) analysed by HPLC observed at 450 nm and fluorescence detector (290/310 nm). For peak numbers see *Table 4.2.3*.

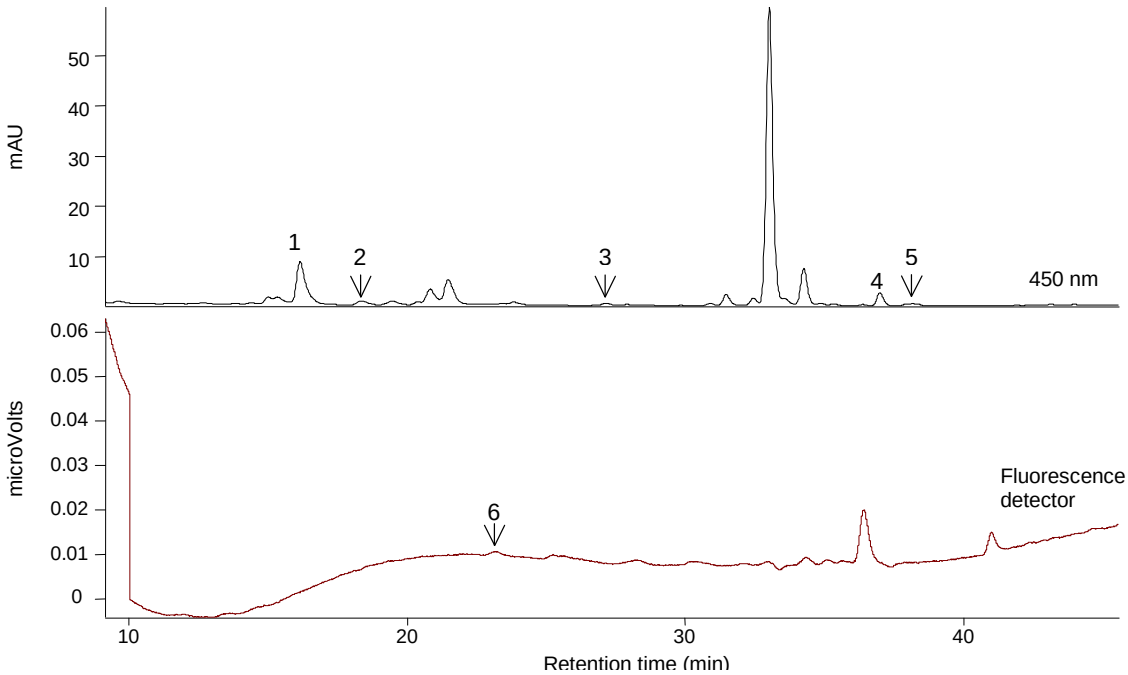


Figure 4.2.4 Chromatogram of carotenoids of *Centella asiatica* and α-tocopherol analysed by HPLC observed at 450 nm and fluorescence detector (290/310 nm). For peak numbers see *Table 4.2.3.*

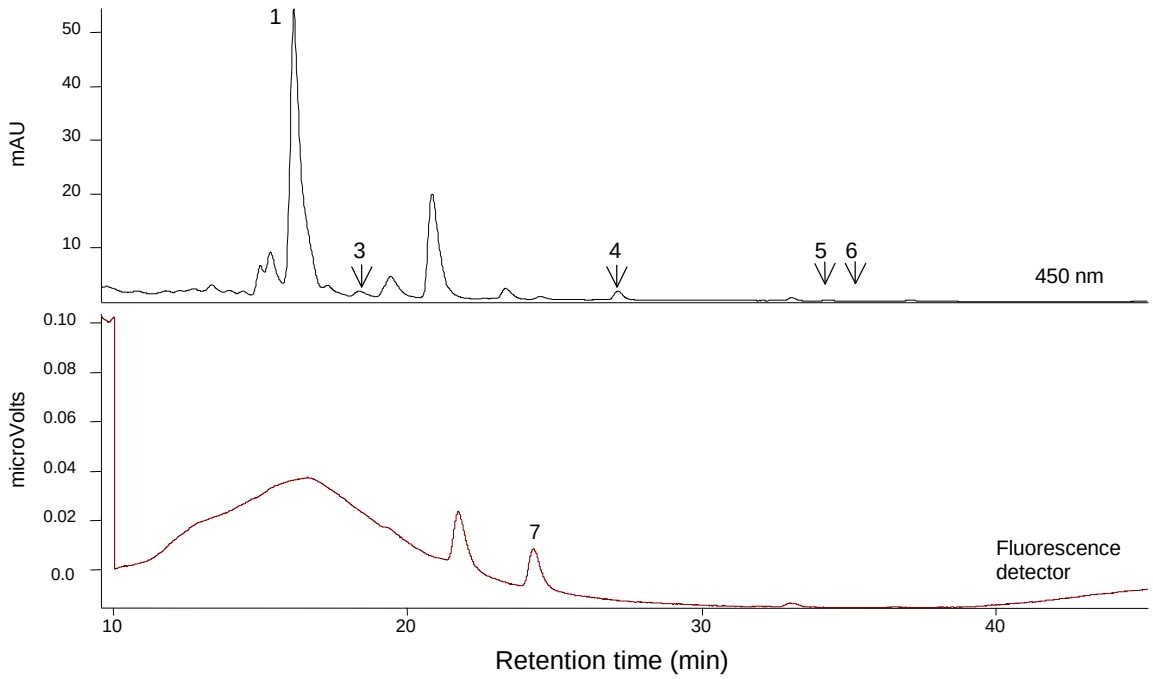


Figure 4.2.5 Chromatogram of carotenoids and α-tocopherol of *Colubrina asiatica* analysed by HPLC observed at 450 nm and fluorescence detector (290/310 nm). For peak numbers see *Table 4.2.3.*

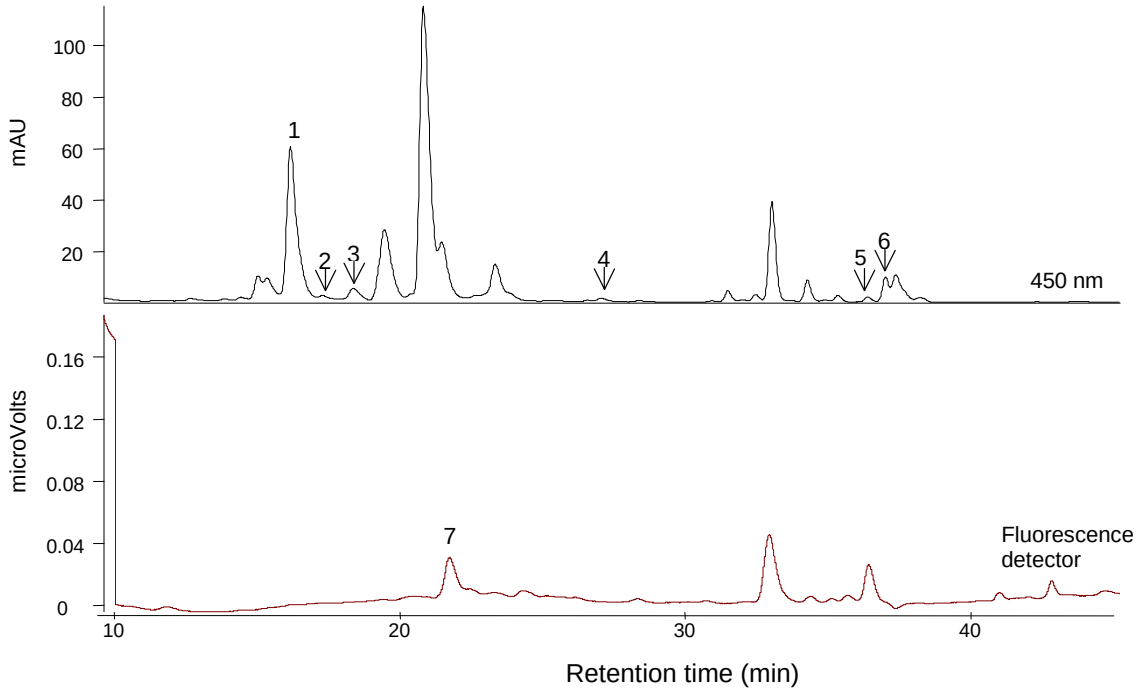


Figure 4.2.6 Chromatogram of carotenoids and α-tocopherol of *Pluchea indica* (A – var. A and B – var. B) analysed by HPLC observed at 450 nm and fluorescence detector (290/310 nm). For peak numbers see *Table 4.2.3.*

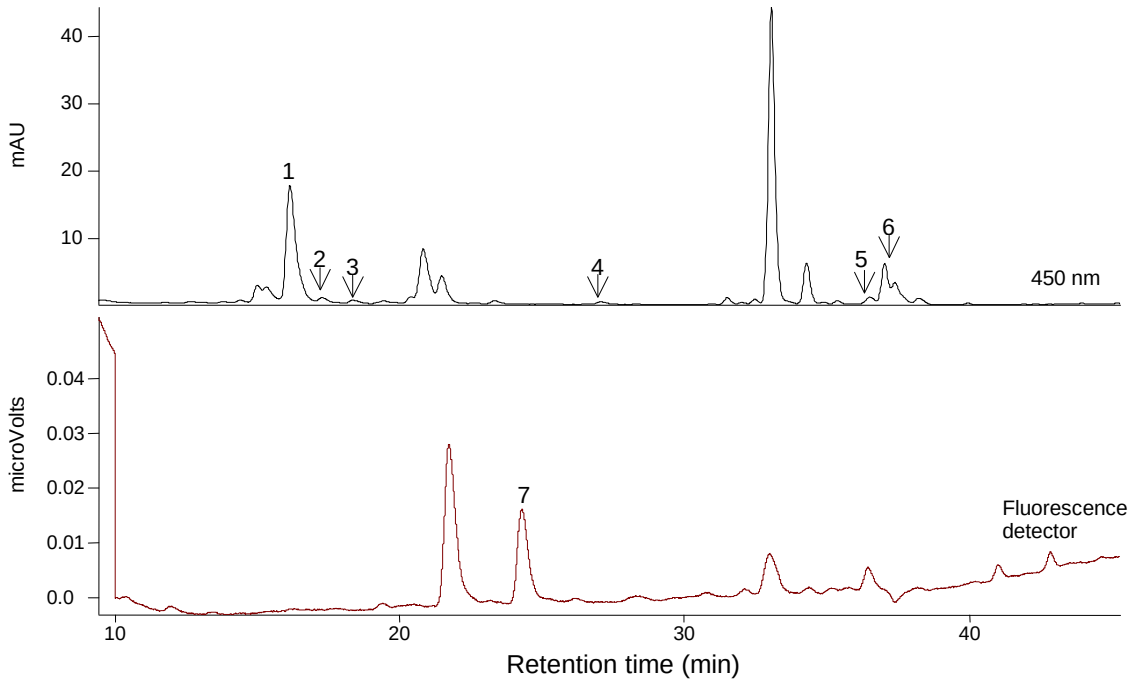


Figure 4.2.7 Chromatogram of carotenoids and α-tocopherol of *Premna cordifolia* analysed by HPLC observed at 450 nm and fluorescence detector (290/310 nm). For peak numbers see *Table 4.2.3.*

Table 4.2.3 Level of individual carotenoids in Malaysian traditional vegetables in Batch 1 (rainy season) and Batch 2 (dry season)

Batch 1

Peak	Carotenoids	*Anacardium occidentale*		*Centella asiatica*	*Colubrina asiatica*	*Pluchea indica*		*Premna cordifolia*
		Red	Yellow			A	B	
1	Lutein	2.1 ± 0.1	3.4 ± 0.1	2.8 ± 0.2	1.8 ± 0.1	3 .9 ± 0.2	4.2 ± 0.1	0.4 ± 0
2	Zeaxanthin	nd	nd	nd	nd	nd	nd	nd
3	*t*-Canthaxanthin	nd	nd	nd	nd	nd	nd	nd
4	β-Cryptoxanthin	0.1 ± 0	0.4 ± 0	0.4 ± 0	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.1 ± 0
5	*t*-Lycopene	nd	nd	nd	nd	nd	nd	nd
6	Mix-carotene	0.8 ± 0.1	1.2 ± 0	1.4 ± 0.1	0.5 ± 0	0.8 ± 0	1.2 ± 0	0.2 ± 0
7	α-Tocopherol	0.1 ± 0	0.2 ± 0	0.1 ± 0	0.03 ± 0	0.2 ± 0	0.3 ± 0	0.1 ± 0

Batch 2

Peak	Carotenoids	*Anacardium occidentale*		*Centella asiatica*	*Colubrina asiatica*	*Pluchea indica*		*Premna cordifolia*
		Red	Yellow			A	B	
1	Lutein	2.5 ± 0.2	5.8 ± 0	0.7 ± 0.2	4.0 ± 0.1	4.6 ± 0	4.7 ± 0	0.3 ± 0
2	Zeaxanthin	0.2 ± 0	1.2 ± 0	nd	nd	0.6 ± 0	0.5 ± 0	0.1 ± 0
3	*t*-Canthaxanthin	0.1 ± 0	0.1 ± 0	0.3 ± 0	0.5 ± 0	1.6 ± 0	1.5 ± 0	0.04 ± 0
4	β-Cryptoxanthin	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.3 ± 0	0 .01 ± 0	0.01 ± 0	0
5	*t*-Lycopene	0.3 ± 0	0.7 ± 0	0.9 ± 0	0.01 ± 0	0.5 ± 0	0.5 ± 0	0.06 ± 0
6	Mix-carotene	0.6 ± 0	1.0 ± 0	0.1 ± 0	0.1 ± 0	1.1 ± 0	1.1 ± 0	0.1 ± 0
7	α-Tocopherol	0.3 ± 0	0.7 ± 0	0.02 ± 0	nd	0.5 ± 0	0.4 ± 0	0.01 ± 0

Level of carotenoids expressed as µg per kg fresh weight (fw). Chromatograms of numbered peaks are shown in *Figure 4.2.4 – 4.2.8*. nd = not detected. Result expressed as value ± standard deviation, SD (n=6).

4.2.3 Level of vitamin C

Nutritional studies have indicated that fruit and vegetables are a rich source of protein, amino acids, dietary fibre, vitamins and minerals (Tee 1985; Zanariah *et al*. 1986; Candlish *et al*. 1987; Bautista *et al*. 1988). The levels of vitamin C detected in traditional vegetables in the present study are shown in *Table 4.2.4*. Within batch 1, the yellow variety of *A. occidentale* contained the highest vitamin C concentration (537 ± 27 µg/g fw). However, the level of vitamin C in this variety was 13-fold higher than in batch 2. The red variety of *A. occidentale* had the highest amount of vitamin C in batch 2 (65 ± 1.5 µg/g fw) compared to other species. The vitamin C content in batch 1 was higher than that of batch 2 in most of the species with the exception to *P. cordifolia*. The level of vitamin C in the vegetables can be ranked in the following order: In batch 1, *A. occidentale* (yellow) > *A. occidentale* (red) > *Colubrina asiatica* > *P. indica (A)* > *C. asiatica* > *P. cordifolia* > *P. indica (B)*. In batch 2, *A. occidentale* (red) > *A. occidentale* (yellow) > *Colubrina asiatica* > *P. indica (A)* > *P. cordifolia* > *C. asiatica* > *P. indica (B).* As the samples used in this study were dry samples, it is advised to use fresh samples to obtain optimum level of vitamin C.

Table 4.2.4 Level of vitamin C in selected Malaysian traditional vegetables

Plant Species	Level of Vitamin C (µg/ g fw)	
	Batch 1	**Batch 2**
Anacardium occidentale (Yellow)	537 ± 27	44 ± 0.5
Anacardium occidentale (Red)	291 ± 8.4	65 ± 1.5
Centella asiatica	11 ± 0.4	4.8 ± 0.1
Colubrina asiatica	98 ± 6.9	8.5 ± 0.3
Pluchea indica A	12 ± 2.2	11 ± 0.2
Pluchea indica B	4.9 ± 0.4	4.1 ± 0.1
Premna cordifolia	6.1 ± 1.5	11 ± 0.3

Level of vitamin C expressed as value ± standard deviation, SD (n=6) in µg per g fresh weight (fw).

4.3 GC-tandem mass spectrometry analysis of the volatile compounds in selected Malaysian traditional vegetables

Plant volatile oils in Malaysian vegetables are mixtures of a large number of terpenoids. These oils were extracted in February 2006 using steam distillation and transported from Malaysia for GC-MS analysis. Oils from different plant species may share similar components which can be identified based on the retention time and spectral index from the NIST library and where possible co-chromatograph with available standards.

Results of the volatile oil compounds in *A. occidentale* are shown in *Table 4.3.1* and *Figure 4.3.1*. Twenty three types of compounds were identified and quantified. The main component was γ-terpinene (28%) and the lowest β-phellandrene (0.3%) which represents the class of terpenes. In *C. asiatica*, 23 oil components also identified (*Table 4.3.2* and *Figure 4.3.2*) with α-humulene and γ-murolene were the major components (22%) and β-trans-ocimene and terpene-4-ol showed the lowest at 0.1%. Identified components in *Colubrina asiatica* are shown in *Table 4.3.3* and *Figure 4.3.3*. From the eleven components quantified, dodecamethylcyclohexasiloxane (D_6) has showed the highest (19%) and decamethylcyclopentasiloxane (D_5) showed the lowest percentage (1.9%). These compounds were reported to be in many personal care products such as toiletries, but not toxic compared to D_4, which was tested *in vivo* on rats when exposed at higher dose of more than 160 ppm. The effects were reduced numbers the pups their general health (Siddiqui *et al.* 2007). As the recovery of this oil was very low, the compounds in *Colubrina asiatica* appeared to be in the lower limit of detection, with a high noise to ratio backround, as shown in *Figure 4.3.3*. Another volatile compound identified in this plant was α-cubebene, which comprised of 12% of the total oil. *In P. indica*, 24 compounds were identified and the results are shown *Table 4.3.4* and *Figure 4.3.4.* Caryophyllene was present in largest amount with 49% and globulol in the lowest quantity (0.01%) in *P. indica*. In *P. cordifolia*, 24 components of the essential oil were identified (*Table 4.3.5* and *Figure 4.3.5*), which 6,6-dimethyl-2-methylenebicycloheptane as the major component (26%). Murolan-3,9 (11)-diene-10-peroxy, 2-methoxy-4-vinylphenol and 3-octanol were found to be the lowest percentage components in *P. cordifolia* with 0.4% respectively.

Most of the identification of the volatile compounds was based on the NIST library as it was very costly to co-chromatograph with all the standard compounds. Identification of

volatile compounds is essential to determine the predominant components and their composition in order to investigate their bioactivity including antioxidant and antibacterial activities. A number of reports have shown that plant volatile compounds exhibited potent antioxidant and antibacterial activities (Deans and Ritchi 1987, Choi *et al.* 2000, de Lourdes *et al.* 2005).

Table 4.3.1 Volatile compounds in *Anacardium occidentale* essential oil

Peak	*Rt*	Compound	Spectral Index (SI)*	Level (%)
1	5.4	γ-terpinene	935	28
2	6.0	Camphene	943	0.4
3	7.0	β-Pinene	938	2.2
4	7.4	β-Myrcene	883	1.1
5	7.9	β-Phellandrene	833	0.3
6	8.5	*D*-Limonene	917	1.4
7	8.9	β-trans-ocimene	712	0.1
8	9.6	1-Bromoallene	927	1.1
9	11.1	Butanoic acid	710	0.5
10	11.3	Terpinen-4-ol	902	4.2
11	11.6	*n*-Valeric acid *cis*-3-hexenylester	853	0.9
12	12.8	*p*-Menth-3-ene,2-isopropenyl-1-vinyl	858	0.6
13	13.3	α-Cubebene	907	3.6
14	13.9	Isocaryophyllene	914	4.5
15	14.8	Isoledene	892	8.0
16	15.3	Cadiena-1(10), 4-diene	871	2.6
17	15.5	Cadiena,3-9-diene	847	0.5
18	16.7	Globulol	856	0.5
19	16.9	Guaiol	822	1.4
20	17.6	Cubenol	845	1.5
21	18.4	α-Cadinol	910	2.2
22	18.5	Selna-6-en-4-ol	802	1.1
23	19.8	Eudesm-7(11)-en-4-ol	843	0.7

*Peak chromatogram is shown in **Figure 4.3.1**. *SI – mass spectra and retention index with a NIST library.

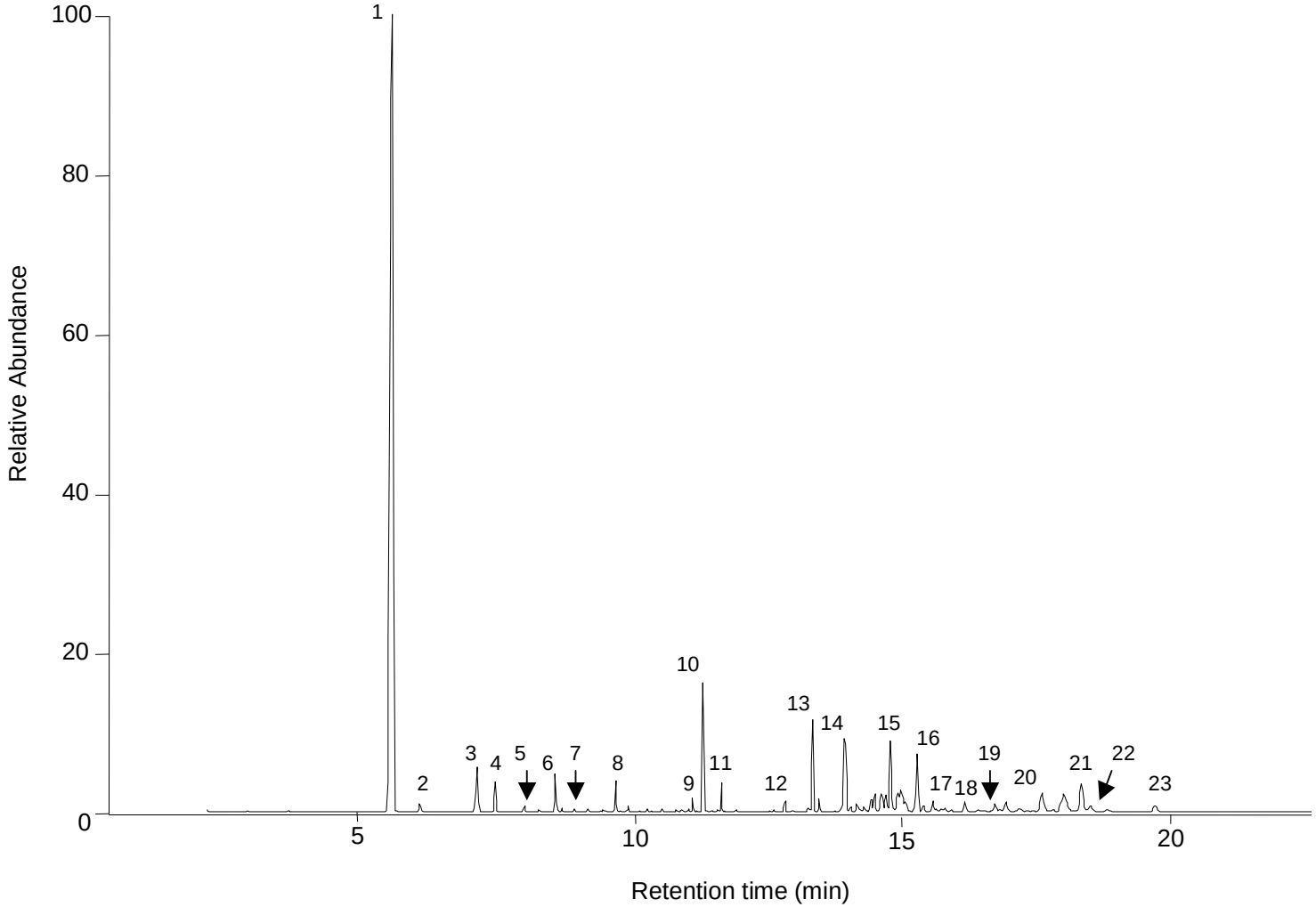


Figure 4.3.1 Chromatogram of volatile compounds in *Anacardium occidentale* essential oil analysed using GC-MS. For peak numbers see *Table 4.3.1.*

Table 4.3.2 Volatile compounds in *Centella asiatica* essential oil

Peak	*Rt*	Compound	Spectral Index (SI)*	Level (%)
1	5.4	α-Pinene	925	1.2
2	7.4	β-Pinene	902	0.4
3	8.4	*m*-Cymene	905	0.8
4	8.5	*D*-Limonene	894	0.3
5	8.9	β-trans-Ocimene	604	0.1
6	9.1	γ-Terpinene	848	0.3
7	9.6	1-Bromoallene	852	0.2
8	9.9	β-Linalool	903	0.4
9	10.5	*trans*-3-Nonen-2-one	869	0.2
10	11.3	Terpinen-4-ol	824	0.1
11	15.0	*o*-Menth-8-ene,4-isopropylidene-1-vinyl-	914	0.2
12	15.5	α-Cubebene	915	0.3
13	16.6	β-Cubebene	912	17
14	17.8	n-Decyl Acetate	811	0.2
15	18.5	α-Caryophyllene	957	17
16	18.8	β-Cedrene	911	0.1
17	20.1	β-Farnesene	948	4.8
18	20.3	α-Humulene	928	22
19	21.2	γ-Murolene	887	22
20	21.6	Germacrene D	903	1.6
21	22.0	(+)-Cyclosativene	836	2.3
22	22.2	δ-Cadinene	844	0.2
23	23.7	Caryophyllene oxide	896	0.9

*Peak chromatogram is shown in **Figure 4.3.2**. *SI – mass spectra and retention index with a NIST library.

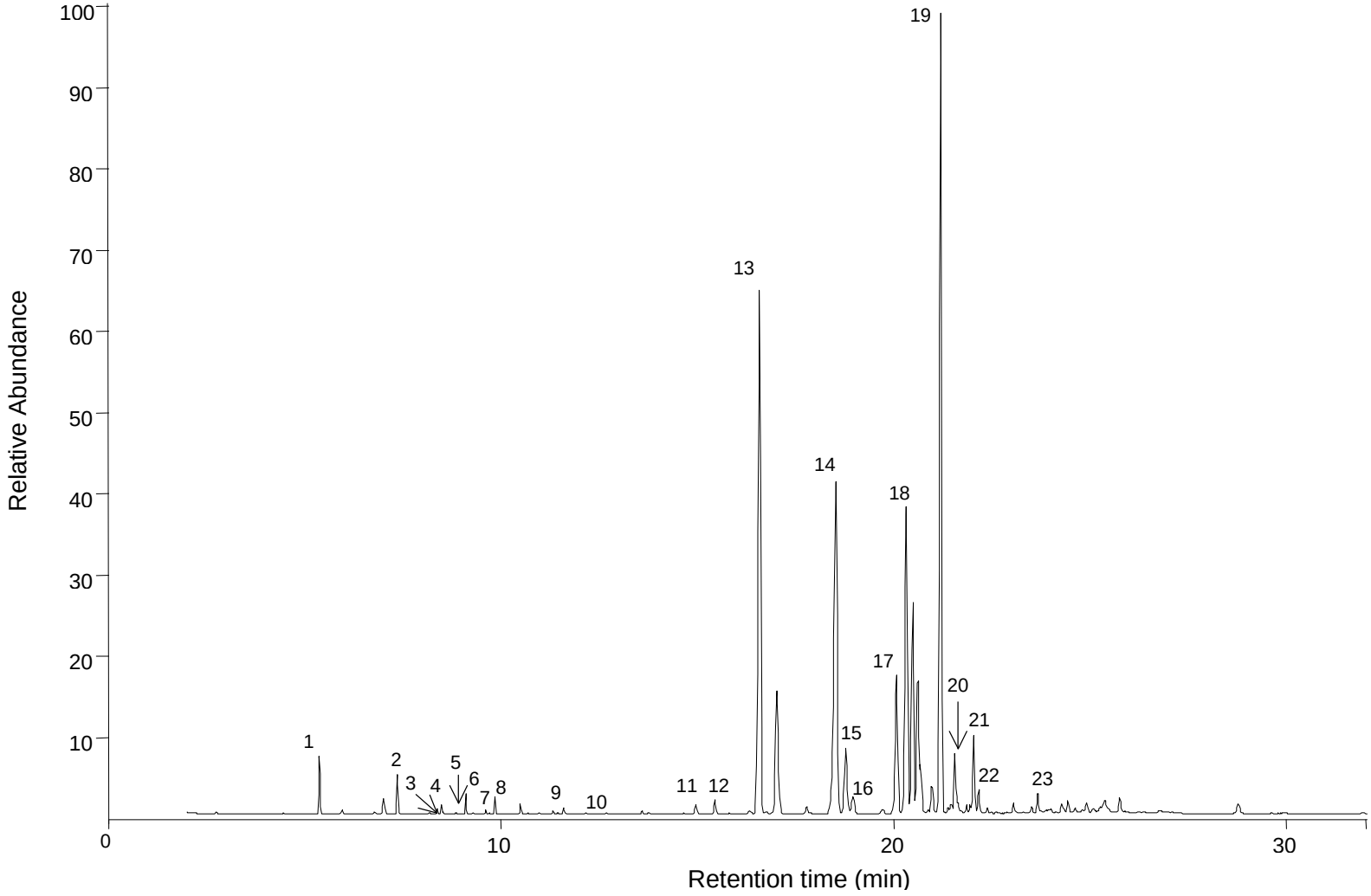


Figure 4.3.2 Chromatogram of volatile compounds in *Centella asiatica* essential oil analysed by GC-MS. For peak numbers see *Table 4.3.2.*

Table 4.3.3 Volatile compounds in *Colubrina asiatica* essential oil

Peak	*Rt*	Compound	Spectral Index (SI)*	Level (%)
1	2.7	2,4-Dimethylhexane	768	15
2	5.4	6,6-Methylenebicyclo[3.1.1]heptane	862	16
3	6.9	Octamethylcyclo-tetrasiloxane	796	4.6
4	7.0	Dehydro-N-[4,5-methylenedioxy-2-nitrobenzylidene]-tyramine	414	2.3
5	10.0	Decamethylcyclopentasiloxane	810	1.9
6	13.7	Dodecamethylcyclohexasiloxane	761	19
7	16.5	α-Cubebene	453	12
8	18.4	Isocaryophillene	585	3.4
9	20.4	Tetradecamethyl-cycloheptasiloxane	740	15
10	22.4	Cadina-1(10),4-diene	652	6.8
11	27.7	Hexadecamethyl-cyclooctasiloxane	639	5.4

*Peak chromatogram is shown in **Figure 4.3.3**. *SI – mass spectra and retention index with a NIST library.

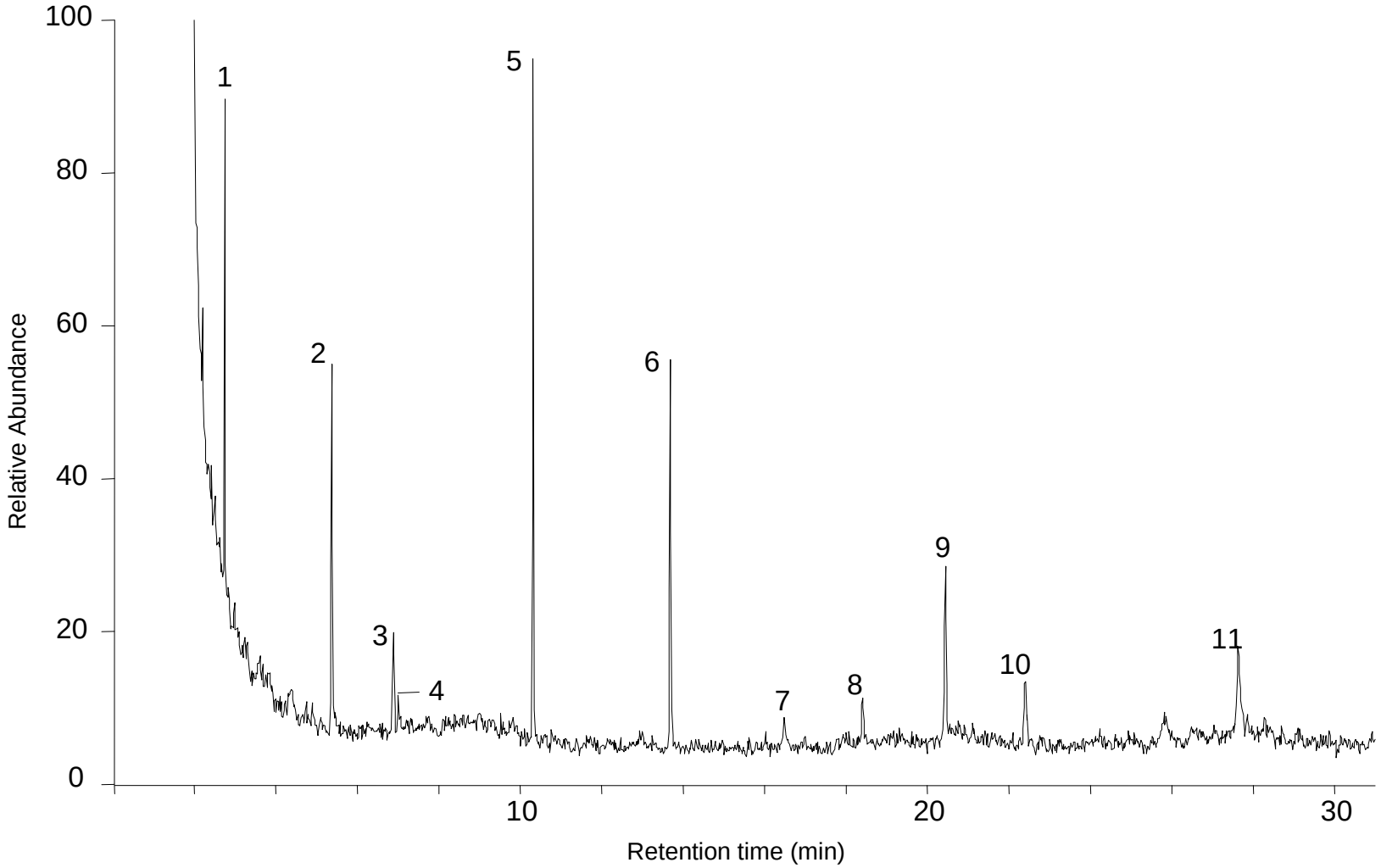


Figure 4.3.3 Chromatogram of volatile compounds in *Colubrina asiatica* essential oil analysed by GC-MS. For peak numbers see *Table 4.3.3.*

Table 4.3.4 Volatile compounds in *Pluchea indica* essential oil

Peak	*Rt*	Compound	Spectral Index (SI)*	Level (%)
1	4.4	Artemesiatriene	874	0.2
2	5.1	3-Thujene	861	0.02
3	5.4	3-Carene	916	0.1
4	6.8	β-Phellandrene	929	0.8
5	8.2	*p*-Mentha-1,4(8)-diene	844	0.1
6	8.4	*m*-Cymene	892	0.1
7	9.1	γ-Terpinene	885	0.2
8	9.4	*p*-Menth-1-en-3-ol, cis	735	0.1
9	9.9	Terpineol, cis-β	787	0.2
10	10.5	2,5-Furandione,3-methyl-4-propyl	797	2.0
11	11.1	*p*-Menth-1-en-4-ol	904	0.5
12	12.9	Cedrene	745	0.1
13	13.3	Copaene	914	3.9
14	13.9	α-Caryophyllene	945	49
15	14.1	β-Farnesen	893	0.1
16	14.4	1,4,7-Cycloundecatriene,1,5,9,9-tetramethyl-Z,Z,Z-	919	2.1
17	15.3	γ-Murolene	862	1.6
18	16.0	Caryophyllene oxide	858	0.6
19	17.2	Globulol	847	0.01
20	17.6	Isoaromadendrene epoxide	804	1.0
21	17.9	Tetracyclo[6.3.2.0(2.50.0(1.8)]tridecan-9-ol,4,4-dimethyl-	909	27
22	18.4	Isoaromadendrene epoxide	830	1.9
23	18.5	*t*-Himachalane	805	5.1
24	25.0	Eudesma-5,11(13)-diene-8,12-olide	730	2.1

*Peak chromatogram is shown in **Figure 4.3.4**. *SI – mass spectra and retention index with a NIST library.

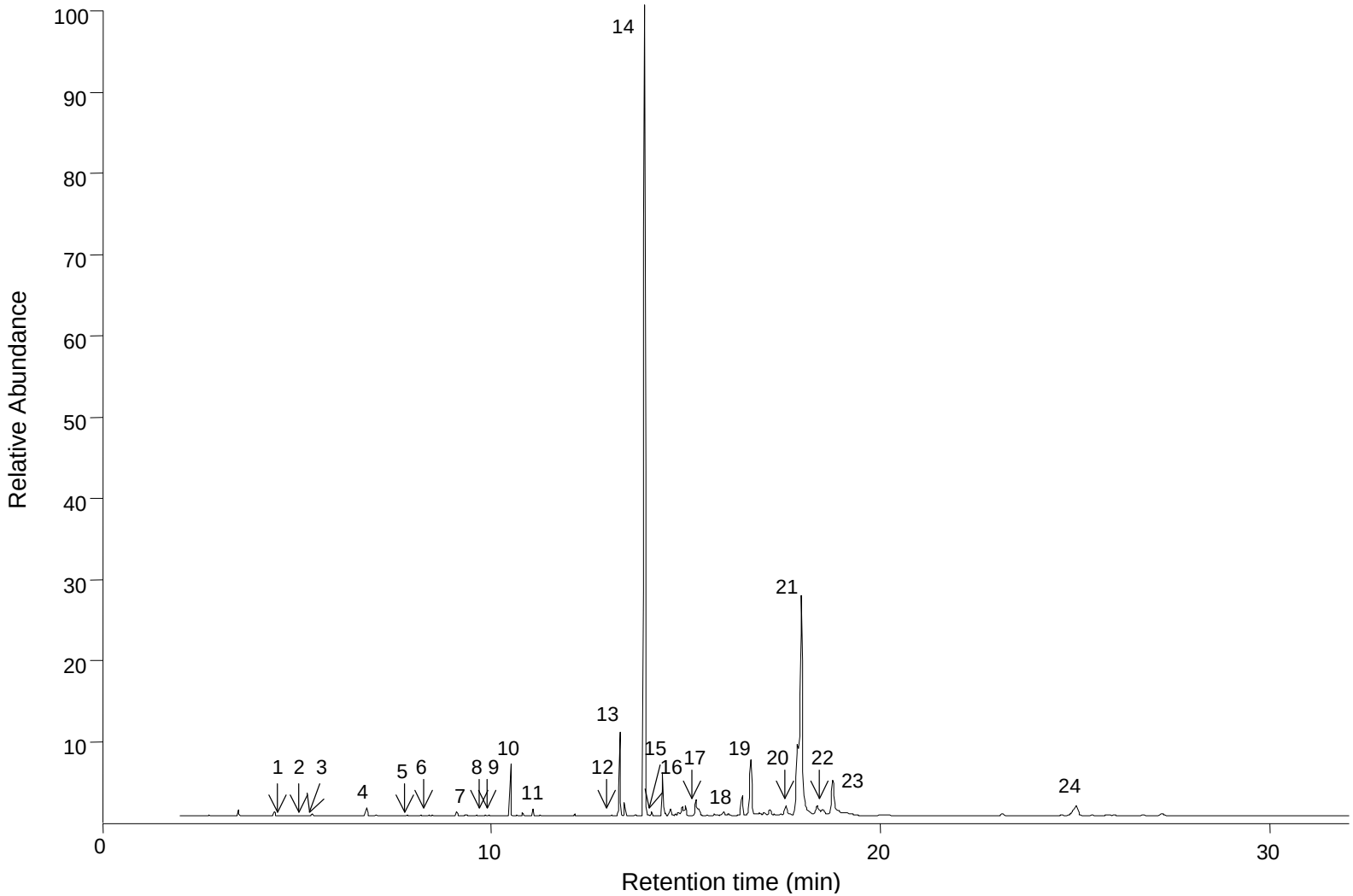


Figure 4.3.4 Chromatogram of volatile compounds in *Pluchea indica* essential oils analysed by GC-MS. For peak numbers see *Table 4.3.4.*

Table 4.3.5 Volatile compounds in *Premna cordifolia* essential oil

Peak	*Rt*	Compound	Spectral Index (SI)*	Level (%)
1	3.5	3-Hexen-1-ol	889	0.9
2	3.7	2-Hexen-1-ol	856	1.0
3	5.4	6,6-Dimethyl-2-methylenebicycloheptane	931	26
4	7.1	1,6-Octadien-3-ol,3,7-dimethyl-propionate	744	8.3
5	7.4	β-Myrcene	882	0.8
6	7.6	3-Octanol	910	0.4
7	8.5	*D*-Limonene	915	0.7
8	8.6	6,6-Dimethyl-2-methylenebicycloheptane	895	0.4
9	8.9	6,6-Dimethyl-2-methylenebicycloheptane	866	0.5
10	9.8	Epoxy-α-terpenylacetate	677	1.2
11	10.7	Anisole,p-vinyl	912	1.8
12	11.3	*p*-menth-1-en-8-ol	680	1.2
13	12.5	2-methoxy-4-vinylphenol	840	0.4
14	13.0	Durenol	688	0.8
15	13.3	Copaene	913	9.4
16	13.9	α-Caryophyllene	959	14
17	14.4	β-Caryophyllene	928	2.8
18	14.6	*o*-Meth-2-ene,4-isopropylidene-1-vinyl-	857	0.8
19	14.9	Eudesma-49140,11-diene	911	6.7
20	15.3	Cadina-1(10)-4-diene	885	19
21	15.7	α-Calacorene	793	0.5
22	16.7	Soaromadendrene epoxide	763	0.5
23	17.5	Murolan-3,9(11)-diene-10-peroxy	753	0.4
24	18.3	α-Cadinol	746	0.6
25	18.8	Murolan-3,9(11)-diene-10-peroxy	747	1.1

*Peak chromatogram is shown in **Figure 4.3.5**. *SI – mass spectra and retention index with a NIST library.

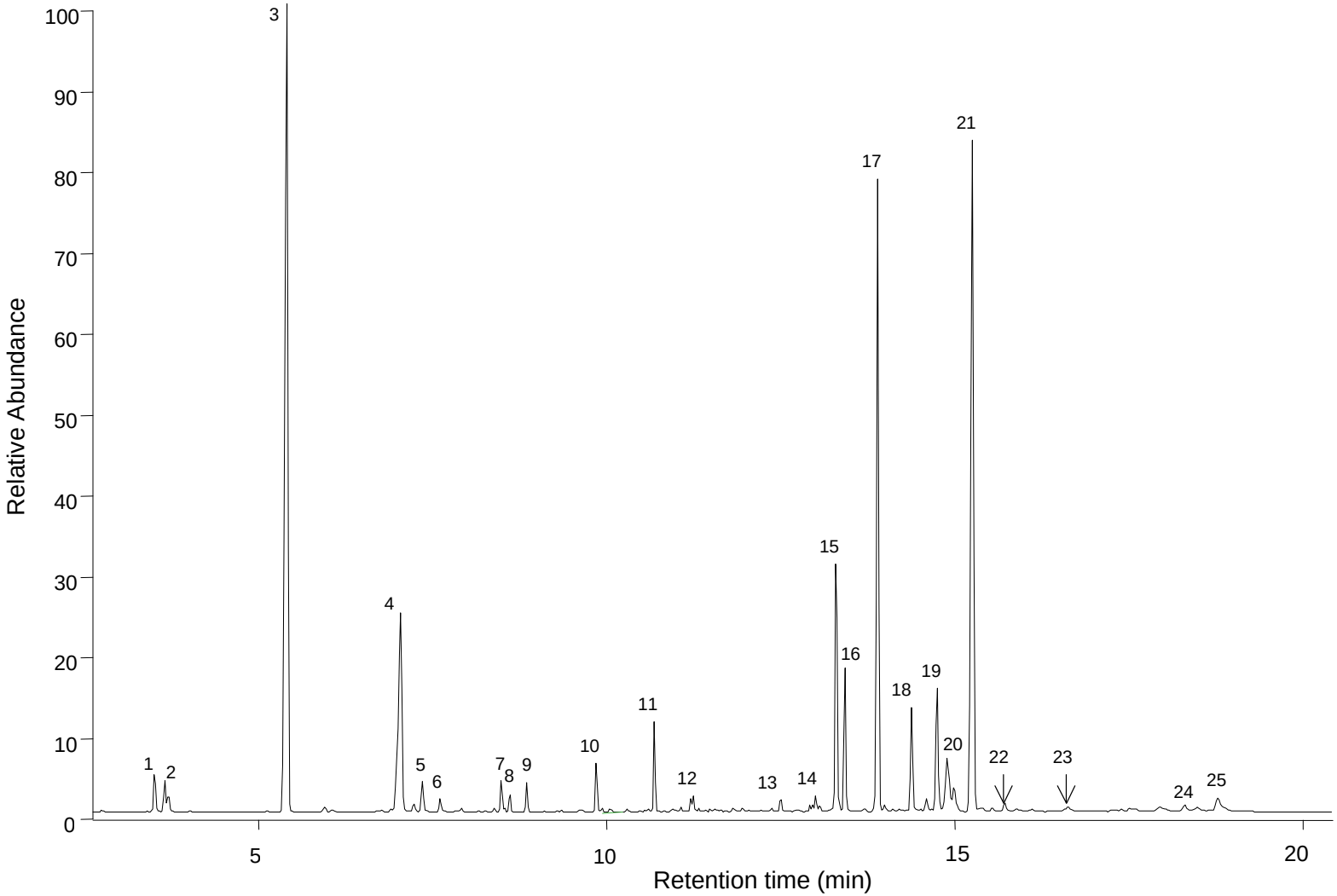


Figure 4.3.5 Chromatogram volatile compounds in *Premna cordifolia* essential oil analysed by GC-MS. For peak numbers see *Table 4.3.5.*

4.4 Measurement of antioxidant activities of vitamin C and volatile compounds

4.4.1 Measurement of vitamin C antioxidant using FRAP-ascorbic acid assay

The antioxidant activity and the contribution of vitamin C to the total antioxidant activity were evaluated with the FRAP assay but with and without addition of ascorbate oxidase to samples in order to oxidise ascorbic acid in the plant extracts as described in *Chapter 2*. The results are shown in *Table 4.4.1*.

Table 4.4.1 Contribution of vitamin C to the total FRAP-antioxidant activity (TAA)

Vegetables	FRAP total antioxidant with vitamin C*		Antioxidant activity of vitamin C*		Contribution of vitamin C to TAA (%)	
	Batch 1	Batch 2	Batch 1	Batch 2	Batch 1	Batch 2
A. occidentale (red)	3296 ± 117	2276 ± 219	181 ± 28	98 ± 7.8	5.5	4.3
A. occidentale (yellow)	4583 ± 318	2121 ± 133	220 ± 19	74 ± 23	4.8	3.5
C. asiatica	272 ± 4.9	290 ± 101	5.2 ± 0.6	3.2 ± 0.9	1.9	1.1
Colubrina asiatica	137 ± 4.8	170 ± 38	1.2 ± 0.3	3.1 ± 0.6	0.9	1.8
P. indica (A)	730 ± 34	336 ± 109	20 ± 1.4	8.4 ± 0.5	2.8	2.5
P. indica (B)	2455 ± 65	562 ± 150	74 ± 6.5	18 ± 9.6	3.0	3.3
P. cordifolia	1384 ± 91	767 ± 123	19 ± 3.7	6.9 ± 0.5	1.4	0.9

*Result expressed as value ± standard deviation, SD (n=6) in µM Fe^{2+}

The total antioxidant activity measured by FRAP assay ranged from 137 ± 4.8 to 4583 ± 318 µM Fe^{2+} in batch 1 and from 170 ± 38 to 2276 ± 219 µM Fe^{2+} in batch 2 with *A. occidentale* showing the highest level and *Colubrina asiatica* contained the lowest antioxidant activity. This was also observed in the absence of vitamin C, where the antioxidant activities were lower and varied between species. Overall, the contribution of vitamin C to the overall antioxidant capacity of the vegetable extracts was small, ranging

from 0.9 to 5.5% (*Table 4.4.1*). The antioxidant activities of vitamin C in the vegetables can be ranked in the following order: *A. occidentale* > *P. indica* > *P. cordifolia* > *C. asiatica* > *Colubrina asiatica.*

4.4.2 Measurement of antioxidant activities of volatile compounds using DPPH assay

The method used to measure the antioxidant activity of essential oils of Malaysian traditional vegetables are described by Yamaguchi *et al.* (1998) and Sacchetti *et al*. (2004). DPPH is one of a few stable and commercially available organic nitrogen radicals and has a UV-VIS absorption maximum at 515 to 517 nm. This assay is based on the measurement of the scavenging ability of antioxidants towards the stable radical DPPH. The free radical DPPH is reduced to hydrazine of which the solution colour fades when it reacts with hydrogen donors and the reaction progress is conveniently monitored by a spectrophotometer.

The total antioxidant activity of essential oils was determined by the DPPH assay and the results are shown in *Table 4.4.2*. *A. occidentale* possessed the highest activity of I_p = 40 ± 7.4 % followed by *P. cordifolia* (38 ± 9.0 %), *P. indica* (9.1 ± 7.5 %), *Colubrina asiatica* (6.6 ± 3.3 %) and *C. asiatica* (6.2 ± 1.7 %). The activities were considered low compared to 1 mM of Trolox (I_p = 93 ± 0.9%) (*Figure 4.4.1*). However, the differences could be due to the solubility as the plant essential oils were emulsified in Tween 20 for the determination of DPPH scavenging activity. *Colubrina asiatica* was shown to have poor radical-scavenging activity probably due to this plant not containing compounds, such as γ-terpinene and terpinolene that are abundant in other species, as shown in *Section 4.3*. These compounds have also shown high radical scavenging activity (*Table 4.4.3*). Some other components exhibited high activity such as terpinolene, terpinen-4-ol and *D*-limonene, which are the main constituents of *A. occidentale*. Overall, the antioxidant activities of volatile compounds in the vegetables can be ranked in the following order: *A. occidentale* > *P. cordifolia* > *P. indica* > *Colubrina asiatica* > *C. asiatica.*

Table 4.4.2 Radical scavenging activity of Malaysian traditional vegetables oils using DPPH assay

	Sample	Percentage inhibition of DPPH (I_p, %)
	Trolox 1 mM	93 ± 0.9
Oil	*Anacardium occidentale*	40 ± 7.4
	Centella asiatica	6.2 ± 1.7
	Colubrina asiatica	6.6 ± 3.3
	Pluchea indica	9.1 ± 7.5
	Premna cordifolia	38 ± 9.0

Result expressed as percentage ± standard deviation, SD (n=6).

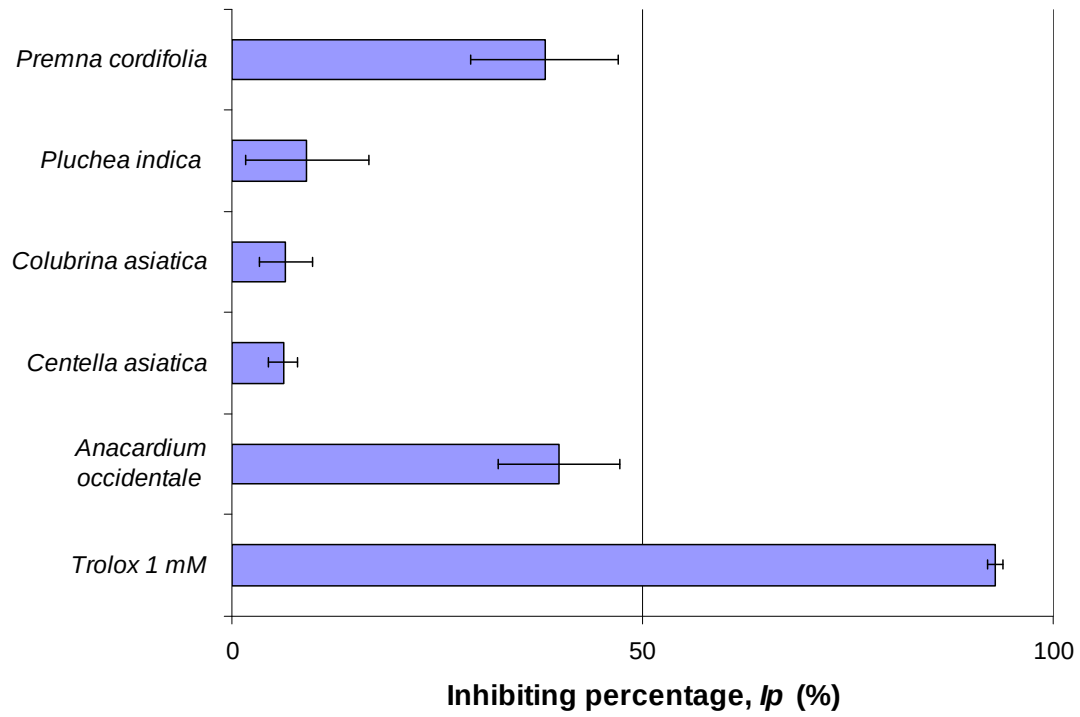


Figure 4.4.1 Radical scavenging activity of Malaysian traditional vegetables oils using DPPH assay. Results expressed as percentage ± standard deviation, SD (n=6).

Table 4.4.3 DPPH scavenging activity of standard compounds (1 mM)

Standard	*Ip* (%)
Citral	36 ± 0.4
Citronellal	34 ± 0.5
D-Limonene	68 ± 1.1
β-Pinene	25 ± 1.2
γ-Terpinene	91 ± 1.4
Terpinen-4-ol	73 ± 0.7
Terpinolene	76 ± 0.8
Trolox	93 ± 0.9

Result expressed as percentage ± standard deviation, SD (n=6).

4.5 Discussion

4.5.1 Analysis of other phytochemicals in Malaysian traditional vegetables

Asiaticoside, madecassoside, asiatic acid and madecassic acid are the main triterpene compounds presence and have been reported as biomarkers in *C. asiatica* (Singh and Rastogi 1968). Several methods have been used to isolate and analyse the triterpenes in *C. asiatica*, which were mainly by thin-layer chromatography (TLC) and HPLC (Singh and Rastogi 1968, Kartnig 1988, Goh *et al*. 1995). In the present study, identification of triterpenes was achieved by MS with quantification using PDA detection at 200 nm. The level of total triterpenes was not significantly different between two batches, even though there were variations between the levels of individual triterpenes. In the present study, the most predominant compound was madecassoside, which is in contrast to a previous report that asiaticoside showed the highest content (Inamdar *et al.* 1996). This could be due to variations in the samples, environmental effects, extraction and analytical methods used, influencing the levels of phytochemicals (Craig 1997, Grabmann 2005). Triterpenes have been reported to show antioxidant activity *in vitro* (Kartnig 1988, Goh *et al*. 1995), protective effects *in vivo* (Hussin *et al.* 2007), antiaging (Hong *et al.* 2005) and anticancer by inducing apoptosis in melanoma cell lines (Park *et al.* 2005). These findings may be the reason for

increasing research interest in the biological activities of *Centella asiatica*. In Malaysia, this plant is the most popular medicinal plant being extensively investigated as the triterpenes of this plant have shown to exhibit high antioxidant activities using several in vitro methods i.e MDA, TBARS and DPPH (Abas et al. 2006, Hussin et al. 2007). This plant also contained very high total phenolic content (Zainol *et al*. 2003, Abas *et al*. 2006) and showed protective effects as shown in *Figure 1.2* (*Chapter 1*).

Interest in carotenoids has increased over the past decade and some nutritionists have focused their efforts on the potential role of carotenoids in chronic disease prevention (Erdman 1999). In the present study, investigation on the level of carotenoids was carried out to differentiate the effect of harvesting season and varieties of species in the Malaysian traditional vegetables. It was revealed that the level of total carotenoids in this study was very low, ranging from 1.6 ± 0 to 28 ± 0 µg/ kg fw in batch 1 and 1.2 ± 0 to 24 ± 2.4 µg/ kg fw in batch 2 for all seven species (*Table 3.3.14*) compared to previously reported carotenoids level in fruits and vegetables. However, it could be the compounds were present in low amount in these vegetables as fresh samples were not analysed for comparison. Hart and Scott (1995) have reported that more than 10 mg/ kg fw of lutein and β-carotene in broccoli, lettuce, parsley and spinach, with the level of lutein in these vegetables being up to 240 mg/ kg fw (Heinonen *et al.* 1989). The low level of carotenoids in the present study is probably due to the use of dry samples for extraction and analysis as some of the carotenoids might have broken down while the tissues were being dried. The source of fresh samples from Malaysia was an issue, however the results obtained from the present study can be used to compare the level of carotenoids in traditional vegetables under different seasons. The content of different components of carotenoids in the samples may be affected by variety, maturity, growing conditions, season of the year and the part of the plant being analysed, as reported elsewhere (Khachik *et al.* 1986, Taylor *et al*. 1990, Chen and Chen 1992). Carotenoids are responsible for the colours of plants and are the biomarkers of some of the vegetables, such as the red colour due to lycopene in tomatoes, and the orange colour of β-carotene in carrots (Krinsky and Johnson 2005). Other factors that contribute to the variable level of carotenoids are the different plant parts analysed (Chen and Chen 1992). Outer leaves and skin contain higher levels of carotenoids than the inner parts of the plants (Hart and Scott 1995, van Breeman 1997). This was shown by Hart and Scott (1995) in an analysis of carrots, which revealed that the outer part contained twice as much β-carotene as the inner part, and the outer leaves of a savoy cabbage were

found to contain 150 times more lutein, up to 200 times more β-carotene than the inner portion. Lutein was found to be predominant in all the Malaysian vegetables, whereas, β-carotene was present in low amount in some species. The low level of β-carotene could be due to preferential conversion of the substrate to lutein (Lakshminarayana *et al*. 2005). Approximately 20-40% of the total carotenoids in this study consisted of unknown carotenoids, which could not be identified by HPLC. To resolve this issue, HPLC-MS analysis using atmospheric pressure chemical ionisation (APCI) mode, has been proposed (van Breeman 1997). However, it was shown that the identification of carotenoids was not fully achieved in some instances due to unexpected molecular ion heterogeneity (Lakshminarayana *et al.* 2005). For instance, polar solvents such as alcohols lead to an increased abundance of protonated carotenoids such as β-carotene, and less polar solvents such as methyl-tert-butyl ether facilitate the formation of more abundant molecular ions (van Beerman 1997). It was advised that analysis of carotenoids needs to be carefully evaluated in analytical procedures, in order to avoid factors which cause variation and inaccuracies in the quantitative determination of carotenoids (Hart and Scott 1995).

In vitro and *ex vivo* tests suggest that carotenoids are excellent antioxidants (Gerster 1991) and epidemiological studies suggest that higher intakes of certain green leafy vegetables containing lutein and zeaxanthin decrease the risk of macular degeneration. This is a medical condition predominantly found in elderly adults, in which the center of the inner lining of the eye, known as the macula area of the retina suffers thinning, atrophy and bleeding (Handelman *et al.* 1998). Isolation, identification and quantification of carotenoids is unusually challenging because of the instability of these compounds when exposed to heat, light and air (van Breemen 1997). Because carotenoids are thermally labile, their analysis is usually carried out by reversed phase HPLC rather than gas chromatography (Taylor *et al*. 1990).

L-Ascorbic acid is the main biologically active form of vitamin C and widely distributed in plants, (Hernandez *et al.* 2006). The level of vitamin C in seven species of traditional vegetables varied greatly, in which generally the level of vitamin C in batch 1 was higher than batch 2 plants particularly in *A. occidentale*, which showed 10-15 fold differences between batches. This could be due to the vitamin C being broken down during drying. Another possibility could be the external factors that have influenced the level of vitamin C in all the species. It was reported that in *A. occidentale*, the young leaves which

need a really humid environment, contain high vitamin C level in rainy seasons (Saidin 2000). This could be one reason why the level of vitamin C was found to be very high in batch 1 compared to batch 2, harvested in the dry season. The level of vitamin C in all the vegetables was relatively low, with the exception of *A. occidentale*, compared to other fruits and vegetables. Fruits are usually rich in vitamin C compared to vegetables (Henshall 1981), however Vanderslice and Higgs (1991) showed that fresh broccoli was higher in vitamin C (89 ± 2.0 to 148 ± 3.0 mg/ 100 g fw) compared to orange (52 ± 1.6 to 78 ± 1.4 mg/ 100g fw). The level of vitamin C in fresh cabbage and spinach were found to be comparable to that of orange (Vanderslice and Higgs 1991). It was also reported that the richest sources of vitamin C are West Indian cherry, rose hip and blackcurrant, which contain about 3-5 times more than citrus fruits (Henshall 1981). The level of vitamin C can vary widely, and the factors which influence this include, cultivar, cultivation practices, growing area and probably the most significant is the interval between harvest and analysis (Henshall 1981, Hernandez *et al.* 2006).

In the present study, identification and quantification of vitamin C using HPLC was based only on ascorbic acid and not total vitamin C, which includes dehydroascorbic acid. To ensure that the subsequent HPLC analysis is effective, it is very important to optimise sample extraction when analysing vitamin C in fruits and vegetables. It has been reported that vitamin C is unstabile and influenced by various factors such as pH and heat (Kall and Andersen 1999, Hernandez *et al.* 2006). It is also essential to inactivate degradative enzymes which can destroy ascorbic acid during the extraction and to fix the ascorbic acid/dehydroascorbic acid redox equilibrium (Hernandez *et al.* 2006). Therefore the use of pH plays a role in the degradation of these enzymes. Ascorbic acid is readily oxidised under alkaline conditions, so the use of a high ionic strength, acidic extraction solvent is required to suppress metabolic activity upon disruption of the cell and to precipitate proteins. Metaphosphoric acid is traditionally used for this purpose (Cano *et al.* 1997, Kall and Andersen 1999, Franke *et al*. 2004) and was utilised in the present study.

4.5.2 Analysis of volatile compounds

There has been a recent resurgence in interest into bioactive properties of the volatile oils extracted from aromatic and medicinal plants. Plant volatiles are usually complex mixtures of a wide variety of organic compounds, including saturated and unsaturated hydrocarbons,

organic acids, esters, aldehydes, ketones, amines, oxides and sulfur compounds, which are formed via several biochemical pathways such as the shikimic acid pathway (Vaughn 2001). In the present study, the components identified in all species of traditional vegetables are representative of different chemical classes as shown in *Table 4.5.1* and their constituents in the essential oil based on species are shown in *Figure 4.5.1*. The essential oils from these traditional vegetables such as γ-terpinene, *D*-limonene, terpen-4-ol, α-cubebene and β-myrcene were reported to show potent antibacterial properties (Burt 2004, Prabuseenivasan *et al*. 2006) and antioxidant properties (Ruberto and Baratta 2000). These compounds have demonstrated antibacterial activities against *Listeria monocytogenes, Salmonella typhimurium, Escherichia coli* O157:H7, *Shigella dysenteria, Bacillus cereus* and *Staphylococcus aureus* having MICs of 0.05–5 µl/ ml (Burt 2004). γ-Terpinene, *D*-limonene and terpen-4-ol have showed the highest antioxidant activities using two *in vitro* models, TBARS and 2,2'-azobis (2-amidinopropane) dihydrochloride assays in which at 1000 ppm their activities was between 64.6 to 79.4 % (Ruberto and Baratta 2000). γ-Terpinene was predominantly found in *A. occidentale* (*Figure 4.5.1*) has contributed substantial amount to the antioxidant and antimicrobial activities of this plant. High composition of potent volatile compounds in the plant oils contributed to high bioactivities *in vitro*. This was evidenced by the study reported by Brady *et al.* (2006) showing that terpinen-4-ol, which was the highest constituent of tea tree oil, exhibited strong antimicrobial activity against *S. aureus* and also showed high antioxidant activity tested using TBARS assay (Ruberto and Baratta 2000).

Extraction procedure plays important role to obtain quality and high recovery of essential oils, which steam distillation is the most commonly used method for producing essential oils (Burt 2004), and a Likens-Nickerson apparatus was used in this study. Advantages were that the desired substances were concentrated thousands-of-fold in a single 1 h operation and a relatively very small quantity of organic solvent was used, thus minimizing the possibility of artifacts from the solvent (Schultz *et al.* 1977). However, the recovery is low as the composition of oils in the plants is about less than 7% (Manzan *et al*. 2003). The yields from plants for commercial production needs to be very high (Schultz *et al.* 1977) for the production of perfumes and topical antiseptics (Williams *et al*. 1998).

Table 4.5.1 Chemical classes of volatile components in Malaysian traditional vegetables

	Classes	Sub-classes	Compound
1	Aliphatic	acyclic	β-myrcene
2	Terpenes	monocyclic	γ-terpinene, β-phellandrene
		bicyclic	β-pinene, carene, camphene
3	Sesquiterpenes		β-caryophyllenes, isocaryophyllene, γ-murolene, isoledene
4	Sesquiterpenes alcohol		globulol, β-humelene, α-cadinol, guaiol
5	Terpenoid	aldehydes	citronellal
		ketones	menthone
6	Alcohols	aliphatic	7-octen-4-ol
		acyclic	linalool and geraniol
		cyclic	terpineols and terpinen-4-ol
7	Phenols		eugenol
8	Esters	aliphatic	geranyl acetate
		cyclic	*p*-menth-1-en-8-ol acetate (terpinyl acetate), myristic acid methyl ester
		bicyclic	bornyl acetate

4.5.3 Analytical methodologies for quantitative analysis of Malaysian vegetables

In order to investigate the beneficial phytochemicals in Malaysian traditional vegetables as a source of potentially bioactive extracts, characterisation and identification of phytochemicals mainly flavonoids and chlorogenic acids, triterpenes, terpenes, vitamin C and carotenoids were determined by using HPLC-PDA-MS^2, HPLC and GC-MS. These techniques especially HPLC-PDA-MS^2 with electrospray ionisation (ESI) facilitated the routine identification of phenolics and triterpenes whereas, GC-MS was used to identify volatile compounds in Malaysian traditional vegetables. The extraction procedures also play

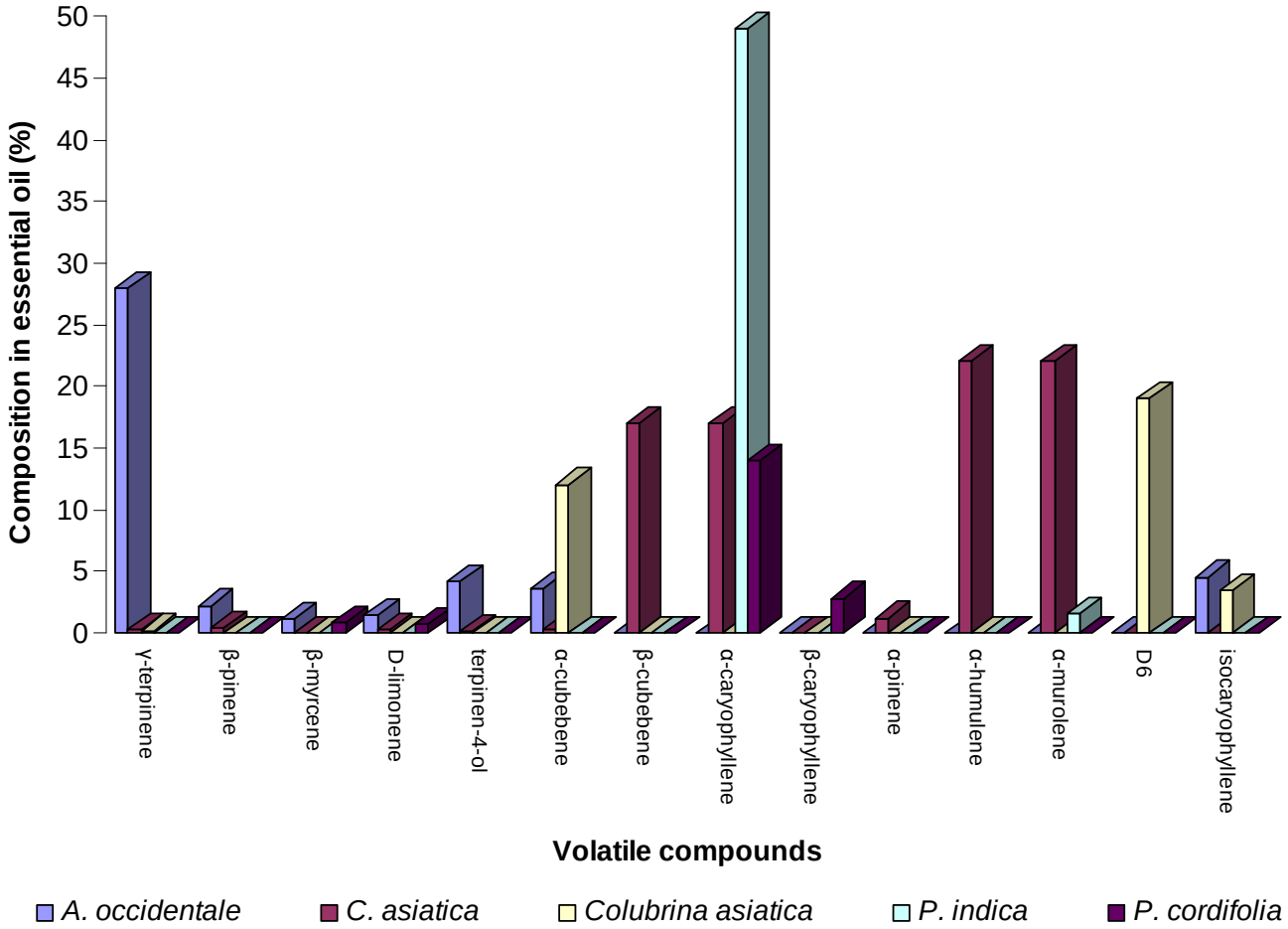


Figure 4.5.1 Composition of important volatile compounds in the essential oils of Malaysian traditional vegetables

an important role in obtaining better recoveries of the compounds of interest. This was applied to vitamin C as it is sensitive to internal (i.e. pH) (Henshall 1981) and external factors (i.e. light) (Hernandez *et al.* 2006). Metaphosphoric acid (MPA) was used to increase recovery and to stabilise the extract (Kall and Andersen 1999). In the present study, the challenge in the GC-MS analysis of volatile components was the ability for the oils to evaporate, which need a special container to be used during injection. The NIST library was able to help identify the unknown components in volatile oils instead of having to compare the compounds with thousands of volatile standards, which would increase the cost of analysis. However, in this study, important volatile components such as γ-terpinene, *D*-limonene and terpinolene were used as reference compounds. This study has led to identification of different phytochemicals in traditional vegetables which will help future research. Due to scarcity of information, especially on chemical compounds and bioactivities of Malaysian traditional vegetables, this study will generate interest among researchers to evaluate vegetables and to promote healthy diets containing fruits and vegetables from our local produce.

4.5.4 Influence of environmental factors and varieties on the level of phytochemicals in Malaysian vegetables

Environmental factors such as climate change, soil types and post-harvest handling process have been reported to influence the phytochemicals content in plants (Fleuriet and Macheix 2003), however not much information can be obtained on the effects of these factors on Malaysian plants particularly traditional vegetables. Malaysia has regular climate changes throughout the year with high and uniform temperature, high humidity and abundant rainfall (Tawang *et al*. 2001). In this study, analysis of phytochemicals from two batches of Malaysian vegetables which were collected from two different seasons, first batch in September 2004, which was in the rainy season (rainfall distribution of above 204.5 mm) and the second batch, which was harvested in February 2006 (dry season with the rainfall distribution of over 56.7 mm) (Malaysian Materiological Service 2001) were investigated and compared. Generally, the level of phytochemicals varied between species and between batches, however it was shown that the level of phytochemicals were much higher in batch 1. This could largely depend on external factors such as light, temperature and various stresses (Fleuriet and Macheix 2003, Dixon and Paiva 1995). Due to these factors, the accumulation of phenolics in these vegetables varies greatly and the changes involve the regulation of phenolic metabolism (biosynthesis and degradation) and its integration in the

metabolism of cells and tissues (Winkel-Shirley 2002, Fleuriet and Macheix 2003). In order to achieve valid and consistant results in the present investigation, plants from similar areas and from similar trees were collected and processed before being analysed for their phytochemical content and an evaluation of their biological activities. Levels of phytochemicals can also vary with storage conditions. Solecta *et al.* (1999) have shown that plants subjected to cold and then freezing treatments, the levels of soluble phenolic acids increased 70% after the frost-thaw treatment. Acclimation of plants in cold and the frost-thaw treatment resulted in the promotion of phenolic esterification.

4.5.5 Total antioxidant activities of vitamin C and volatile compounds

Vitamin C was reported to have similar antioxidant potential to that of vitamin E, which is being categorised as 'rapid kinetic behaviour' due to their structures (Brand-Williams *et al.* 1995). In the present study, vitamin C was shown to contribute a smaller percentage, less than 5.8% to the total antioxidant activities. The main contribution could be from phenolic compounds (Rice-Evans *et al.* 1997). This was also in agreement with Prior (2003) that phenolics have shown 4-fold higher antioxidant activities than vitamin C and vitamin E. Chu *et al.* (2002) have reported that contribution of vitamin C towards total antioxidant activity ranged from 1.2 to 24% in vegetables of which cucumber and red pepper exhibited the highest content of vitamin C. However, Szeto *et al*. (2002) showed that contribution of vitamin C antioxidants was 1 to 58% in vegetables of which cauliflower, turnip and tomato exhibited the highest vitamin C contributions (*Table 4.4.2*). However, contribution from other compounds should not be neglected and synergistic effects from all the compounds could contribute to the potent antioxidant activity, thus, might show beneficial effects to human health (Liu 2003). Variations that occur in the vitamin C content and the contribution to total antioxidant activities were reported to be due to environmental factors, different variety, harvest season, post-harvest handling and method used for analysis (Hollman and Arts 2000, Thoss *et al*. 2004).

Determination of antioxidant activities of volatile compounds in plants are quite challenging due to the volatility of the oils. Therefore, the DPPH assay was used, and Tween 20 was acted as an emulsifier to stabilise the oil-solvent mixture prior to addition of DPPH (Yamaguchi *et al.* 1998, Choi *et al*. 2000). The radical scavenging activities of the vegetable oils were comparatively low to that of Trolox. *A. occidentale* and *P. cordifolia*

showed high radical scavenging activity compared to other species. *A. occidentale* contained substantial amount of γ-terpinene, terpinen-4-ol and terpinolene and in *P. cordifolia*, caryophyllene was the major component (44%). These components have been reported to show strong antioxidant activities (Deans and Ritchie 1987, Dorman *et al*. 1995, Ruberto and Baratta 2000, Mevy *et al.* 2007). On the other hand, the synergistic combination of compounds could contribute to the antioxidant activities (Liu 2003).

Table 4.5.2 Contribution of vitamin C to total antioxidant activity (TAA)

Vegetable	Chu *et al*. (2002)		Szeto *et al.* (2002)	
	Vitamin C content (mg/kg)	Contribution to TAA (%)	Vitamin C content (mg/kg)	Contribution to TAA (%)
Broccoli	930	12	10	4
Cabbage	320	10	10	4
Carrot	90	1.2	10	5
Cauliflower	nd	nd	140	56
Celery	70	7.8	<10	1
Cucumber	50	24	nd	nd
Garlic	nd.	nd	60	27
Lettuce	40	8.1	<10	1
Onion	nd	nd	80	22
Pak choy	nd	nd	90	17
Potato	110	13	<10	1
Red pepper	1900	23	nd	nd
Spinach	280	3.8	nd	nd
Spring onion	nd	nd	150	30
Tomato	nd	nd	120	58
Turnip	nd	nd	140	44

nd = not determined

A possible explanation for the differences in antioxidant activities found in this study may be from the substantial variation in the composition of the traditional vegetable essential oils. It was shown that the important components, such as γ-terpinene, terpinolene and caryophyllene were found to be absence or less than 1% in *C. asiatica*, *Colubrina asiatica*

and *P. indica*. The antioxidant activities of these components are also related to the structures and the ability to scavenge free radical by donating oxygen or receiving hydrogen (Brand-Williams *et al*. 1995). The antioxidant activities of the essential oils also depend on the types of antioxidants, test system, emulsion system, concentration, oxidation time and method used (Choi *et al.* 2000). In the present study, several pre-cautionary steps during sample preparation were very important. For instance, samples needed to be properly stirred to form an emulsion and immediately added the DPPH. Delay in adding the DPPH solution will affect the antioxidant activities of the vegetables (Yamaguchi *et al.* 1998. The antioxidant capacities of various essential oils were previously reported (Dorman *et al.* 1995, Choi *et al.* 2000, Ruberto and Baratta 2000, Mevy *et al*. 2007). However, even though the antioxidant activities of the volatile oils were high *in vitro*, the antioxidant activities *in vivo* could be debatable as the volatile components are reported toxic if consumed at higher doses (Stammati *et al*. 1999). This has been reported in the case of tea tree oil (Hammer *et al*. 2006). Therefore, the application of oil is usually for topical use i.e. for antiseptic properties or for wound healing (Burt 2004). The potential of essential oil for antimicrobial activities are described in *Chapter 5*.

4.6 Conclusion

In this study, the use of HPLC, HPLC-MS^2 and GC-MS has led to the indentification and quantification of plant terpenoids, vitamins and volatile compounds. The total antioxidant activities of vitamin C and the contribution to the total antioxidant activity assessed by the FRAP-ascorbic acid assay indicated a small contribution in the Malaysian traditional vegetables. The total antioxidant activity of volatile compounds determined by DPPH assay was in agreement to the total antioxidant activity of plant extracts described in the previous chapter with *A. occidentale* contained the highest amount. It can be concluded that Malaysian traditional vegetables are a rich source of beneficial phytochemicals and antioxidants.

CHAPTER 5

ANTIMICROBIAL ACTIVITIES OF MALAYSIAN TRADITIONAL VEGETABLES AND THEIR MODE OF ACTION

5.1 INTRODUCTION

The healing power of plants is well established in traditional medicine but the mechanism of action remains to be elucidated (Iwu *et al*. 1999), not least for the antimicrobial properties of plant extracts and plant oils. With limited access to antibiotics in developing countries and with the upsurge in multi-resistant bacteria around the world (Gibbons 2004), alternative therapies derived from plant extracts merit investigation (Lee *et al.* 2003). Extracts of medicinal plant species, especially the volatile compounds, are already used for the production of soaps, perfumes and toiletries, in addition, their role in traditional medicine and as insecticidal, antibacterial and antifungal activities have all been demonstrated (Cimanga *et al*. 2002). The potential contribution of these materials to control hospital acquired infection has not yet been explored.

5.1.1 Bacteria

Bacteria are subdivided into two types based on the Gram stain reaction, the Gram-negatives and Gram-positives which reflects the composition of their outer cell wall (Jawetz 1991, Singleton 2004). Gram-negative bacteria are characterized by a thin, inner layer of peptidoglycan and an outer membrane of phospholipids, lipopolysaccharide with lipoprotein in the bacterial cell wall (Mann and James 1996). Due to certain components of this outer membrane, particularly the lipopolysaccharide (endotoxin) layer, Gram-negative bacteria can be extremely pathogenic especially if they invade the bloodstream (Jawetz 1991). Representatives of this group of bacteria used in this study were *Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa.*

Gram-positive bacteria are characterized by a thick cell wall 90% of which is comprised of peptidoglycan. No outer membrane is present (Jawetz 1991, Mann and James 1996). Unlike the Gram-negatives, this group of bacteria retain Gram's stain and appear

blue-violet on microscopy. The Gram-positive organisms *Enterococcus faecalis*, *Staphylococcus aureus* and coagulase negative staphylococcus (CoNS) were used in this study. Scientifically, it is important to understand the relationship between bacteria and their hosts and the processes that lead to disease. Not all bacteria are harmful, some have been used to produce antibiotics or enzymes for biological washing powder, while others form the basis of microbial insecticides (Sleigh and Morag 1998, Singleton 2004).

5.1.2 Bacterial Infections

5.1.2.1 Community-acquired infections

Antibiotic resistance is an emerging problem worldwide, and this applies to pathogenic bacteria such as *E*. *coli* and *S. aureus* (Zetola *et al*. 2005). Infections with resistant bacteria, especially in infants, small children and the elderly can be life-threatening. Nevertheless, epidemiological data on prevalence and determinants of antibiotic resistance in young children and the elderly, particularly studies in the community setting, are very sparse (Lietzau *et al.* 2007).

The antibiotic susceptibility of common community-acquired bacteria in Thailand and Malaysia has been studied (Danchaivijitr *et al.* 2005, Noor Azian *et al*. 2007). From a total of 9,091 isolates of target bacteria, community and hospital acquired bacteria accounted for 54.9% and 45.1%, respectively. Bacteria isolated from the bloodstream were more susceptible to antimicrobials compared to those from the lower respiratory tract, urinary tract and surgical sites. Community-acquired *Escherichia coli, Klebsiella pneumoniae, Acinetobacter spp., Enterobacter spp* and *Staphylococcus aureus* were more susceptible to antimicrobials compared to hospital-acquired strains (Danchaivijitr *et al*. 2005).

In developing countries such as Thailand and Malaysia, the high prevalence of community-acquired infections was attributed to poor environmental management, poor personal hygiene and lack of health education (Noor Azian *et al.* 2007). Other factors such as intra-familial transmission have also been reported (Zetola *et al.* 2005). Severely infected patients could die due to lack of antibiotics and the medical support in a rural area. Therefore, the control of community-acquired infections using plant products and extracts derived from medicinal herbal remedies, which have been practiced traditionally, may be helpful. Novel antimicrobial agents from plants, which are available in developing countries

could in future be used to tackle increasing number of community-acquired bacterial infections.

5.1.2.2 Nosocomial infections

Nosocomial infections are those acquired in or associated with hospitals, which are common and may be serious or fatal. They are also known as hospital-acquired infections, or more recently, healthcare-associated infections (Breathnach 2005). Any community infection can also occur in hospital, but there are many factors in the hospitals environment that lead to a particular spectrum of infective problems (*Table 5.1.1*). The most common bacterial infections in the hospital are caused by *Staphylococcus spp*. Staphylococcal infections are a problem throughout the healthcare environment and the scale of the public health problem posed, especially by meticillin-resistant *Staphylococcus aureus* (MRSA) in the UK is huge. MRSA infection in England and Wales increased significantly during the 1990s (Johnson *et al*. 2001). Most dramatically, the proportion of cases of *S. aureus* bacteraemia due to MRSA increased from 1-2% in 1990-1992 to 40% in 2000 (Speller *et al*. 1997, Reacher *et al*. 2000). The incidence of MRSA around the world is shown in *Table 5.1.2.*

Invasive, potentially life-threatening MRSA infections are derived from local colonisation and infection of areas of broken skin (Diekema *et al*. 2004, Brady *et al*. 2006). Attempts are made to interrupt this sequence of events in hospital patients with topical antimicrobials and antiseptics, for example, mupirocin and chlorhexidine are currently used in an attempt to eradicate superficial infection, but have been associated with increased resistance (Boyce 2001). Recent reports have shown that these two antimicrobial agents are ineffective against bacterial strains isolated from superficial skin wounds and leg ulcers (Colsky *et al.* 1998, Valencia *et al.* 2004). In addition to the morbidity and mortality related to complicated skin and suture structure infection, treating these infections is estimated to cost an additional £3246 per patient with an additional hospital stay of 6.5 days (Plowman *et al*. 2001). Current data for Scottish hospitals indicate that of 1691 *Staphylococcus* isolates recovered from patients with invasive disease during 2004, 41% were meticillin resistant *Staphylococcus aureus* (MRSA) (Coyne *et al*. 2004).

Table 5.1.1 Typical hospital organisms (Breathnach 2005)

	Usual location	Risk factors for acquisition	Typical infections	Comments
Methicillin-resistant *Staphylococcus aureus*	Nasal or cutaneous	Antibiotic use, skin lesions, overcrowding, poor infection control	Skin/wound, orthopaedic, intravenous devices, bacteraemia, endocarditis, respiratory tract, prosthetic devices	Occasional case reports of developing resistance to glycopeptides, which are the agents of choice for invasive infections
Enterococci and vancomycin-resistant enterococci	Gut, hospital environment	Antibiotics – particularly cephalosporins, glycopeptides	Low virulence; typically, vulnerable ICU patients or renal patients, in whom use of glycopeptide antibiotics is common	Vancomycin-resistant enterococci are more correctly, but rarely, termed 'glycopeptide-resistant enterococci';heat and disinfectant tolerant, so survive well in hospital environment
Clostridium difficile	Gut, hospital environment, elderly care wards	Antibiotics, chemotherapy, poor hygiene	Antibiotic-associated diarrhoea/ pseudomembranous colitis	Large outbreaks with fatal cases reported; predisposed by loss of natural bowel flora, hence several suggested 'alternative' remedies based on the principle of replacing this flora (e.g. live yoghurt, donor faeces)
Multi-resistant Gram-negatives	Gut, hospital environment, high-dependency units	Antibiotics, poor hygiene	Intra-abdominal, respiratory and bloodstream infections in vulnerable patients	Various species (e.g. *Acinetobacter*, *Klebsiella*, *Enterobacter*, *Stenotrophomonas*); many areindolent opportunists, but some (e.g. *Klebsiella*, *Pseudomonas aeruginosa*) can cause aggressive, virulent infections

Notes: Most nosocomial infections are caused by the patient's own flora or by cross-infection with more sensitive organisms not listed above.

Table 5.1.2 Incidence of methicillin-resistant *Staphylococcus aureus* (MRSA) in different parts of the world in 1990s (Panlilio *et al.*, 1992; SMAC Report, 1998). For some countries all MRSA isolates have been included; for others only bacteraemia isolates

Country	MRSA incidence (%)
Northern European countries, the Netherlands	<1
Germany	5–24
USA	15–38
United Kingdom	32
Spain	30–39
Belgium	25–40
Japan, Korea	70

5.1.3 *Staphylococcus* species

The staphylococci are a group of Gram-positive cocci that are often associated with nosocomial infections and antibiotic resistance (Nicolson *et al.* 1999, Sa-Leao *et al*. 2001). They are divided into two groups based on production of the enzyme coagulase which induces clotting of fibrin and contributes to virulence by isolating sites of infection from the immune factors present in the blood. The most pathogenic strain of staphylococcus is the coagulase positive *Staphylococcus aureus* which is commonly found on the skin and in the nasal passages of carriers (Kloos and Schleifer 1975, Speller *et al*. 1997). *S. aureus* infections are often nosocomial or opportunistic and can cause infection ranging from pimples, furuncles and impetigo in newborns to pneumonia, endocarditis, and septicemia (Le Loir *et al*. 2003). There are several virulence factors that contribute to the pathogenicity of *S. aureus* among which, are several toxins that can cause serious ill-effects including toxic shock syndrome and food poisoning (Mann and James 1996).

Resistance to antibiotics appeared in *S. aureus* shortly after their introduction, and by 1953, resistance to streptomycin, tetracycline, chloramphenicol, and novobiocin had been reported (Robinson *et al*. 2005). Penicillin resistant *S. aureus* strains initially emerged in hospitals but these strains moved rapidly into the community and it has been reported that more than 90% of *S. aureus* are currently resistant to penicillin (Gemmell *et al.* 2006). Through the 1950s, a pandemic was caused by a particularly virulent and penicillin resistant

strain of *S. aureus* belonging to phage type 80/81. After the discovery of penicillinase-resistant drugs such as meticillin, the pandemic was brought under control (Robinson *et al.* 2005, Gemmell *et al.* 2006).

5.1.3.1 Meticillin-resistant *Staphylococcus aureus* (MRSA)

S. aureus strains resistant to penicillinase-stable β-lactam drugs, such as meticillin, began to appear in hospitals by the 1950s (Srinivasan *et al*. 2002). MRSA quickly spread around the world and are now a major nosocomial and emerging community pathogen. In 2000, 47% of *S. aureus* and 75% of coagulase-negative staphylococcal isolates from intensive care units in the US, and 48% of hospital isolates of *S. aureus* in Portugal were meticillin resistant (Sa-Leao *et al.* 2001, Srinivasan *et al.* 2002). Until recently, MRSA was mainly a hospital problem; however, there have been reports of community-acquired infection in several countries.

Currently, fewer drugs are available for the treatment of MRSA infections than meticillin-sensitive *S. aureus* (MSSA) and vancomycin has been the mainstay for therapy (Williams *et al.* 1996, Srinivasan *et al.* 2002). In 1997, in Japan, vancomycin resistant *S. aureus* (VRSA) was first isolated (McCormick 1998) and this discovery was quickly followed by the appearance of VRSA isolates in the U.S. and other countries (Srinivasan *et al*. 2002). As many of the VRSA strains that have been isolated do not meet the NCCLS standards (Speller *et al*. 1997) for full resistance, the term, vancomycin intermediate *S. aureus* (VISA), has come into use (Speller *et al*. 1997, Srinivasan *et al.* 2002, Gibbons 2004, Gemmel *et al.* 2006). The discovery of VISA prompted fears that vancomycin resistance had come from vancomycin resistant enterococci (VRE), especially after it was reported that the vancomycin resistance gene *vanA* could be transferred from *E. faecalis* to *S. aureus in vitro* (Noble *et al*. 1992). For several years, no connection to VRE could be found, but since 2004, strains of VISA containing the *vanA* gene have been isolated from patients in the US (Ruef 2004). However, none of these VISA strains have yet been reported in the UK (Gemmell *et al*. 2006).

A major change in the epidemiology of MRSA infections in the UK was associated with the emergence in the early 1990s of two ‘epidemic’ EMRSA strains (designated EMRSA-15 and EMRSA-16), which were remarkably successful at spreading between

hospitals (Richardson and Reith 1993, Cox *et al.* 1995, Johnson *et al*. 2005,). EMRSA-15 was initially identified in Southeast England and in the Midlands in 1991, subsequently spreading to hospitals in the North of England, while EMRSA-16 emerged at about the same time in Northamptonshire. Both strains have spread widely to affect a large number of hospitals across the UK. They remain the dominant strains of MRSA, accounting for 93-95% of MRSA isolates seen in recent years (Johnson *et al.* 2001, 2005).

5.1.3.2 Coagulase-negative staphylococci

The coagulase-negative staphylococci particularly *Staphylococcus epidermidis*, are part of the skin microbiota, they were long regarded as harmless skin commensals and were generally believed to be simply opportunistic pathogens (Archer and Climo 1994). Based on their inability to produce the virulence factor coagulase, CoNS were often referred to as apathogenic staphylococci (Kloos and Schleifer 1975, Huebner and Goldmann 1999).

In 1975, Kloos and Schleifer extended the existing classification scheme by adding seven new species to the already known *S. epidermidis* and *S. saprophyticus*. However, today there are 32 coagulase-negative staphylococcal species; about 15 species are indigenous to humans, while the remainder are animal pathogens (Huebner and Goldmann 1999). During the past few decades, the importance of coagulase-negative staphylococci as nosocomial pathogens has been recognized (Boyce 1997). In 1990 to 1995, the National Nosocomial Infection Surveillance (NNIS) program reported that coagulase-negative staphylococci were the causative agent in 11% of all nosocomial infections, making this pathogen the third most common nosocomial isolate after *S. aureus* and *E. coli* (Boyce 1997, Huebner and Goldmann 1999).

The increasing incidence of infections caused by CoNS can be attributed to their particular affinity for the foreign materials that are integral to modern medicine (Boyce 1997). The increasing use of prosthetic devices, intravascular catheters, and other invasive technologies in severely ill patients who may be immuno-suppressed or at the extremes of life has increased survival rates but has resulted in CoNS becoming a prime opportunistic pathogen resulting in considerable morbidity and excess medical costs (Breathnach 2005). Coagulase-negative staphylococci have become increasingly resistant to antibiotics, the

most recent threat being the emergence of strains with moderate levels of resistance to vancomycin (Archer and Climo 1994, Boyce 1997, Huebner and Goldmann 1999).

5.1.4 *Enterococcus* species

The enterococci are a second group of Gram-positive cocci that are part of the normal resident flora of both humans and some animals (Melhus and Tjernberg 1996, van den Bogaard *et al.* 2002). They are generally low-grade pathogens; however, their intrinsic resistance to many antibiotics (including cephalosporins, penicillin, and aminoglycosides) has made them important opportunistic pathogens (Williams *et al*., 1996) and of interest in the investigation of novel antimicrobial agents. It has been reported that 12% of nosocomial infections in US are caused by enterococci, with *Enterococcus faecium* and *Enterococcus faecalis* the most important and common species (Wagener *et al*. 1997). Although the enterococci are opportunistic pathogens, they are a frequent cause of urinary tract infections, bacteremia, and endocarditis. All of which can be difficult to treat due to antibiotic resistance, with mortality rates for enterococcal bacteremia reaching up to 70% (Melhus and Tjernberg 1996).

Traditionally, treatment for serious infections such as endocarditis has been a combination of an aminoglycoside and ampicillin. By the 1970's, only vancomycin (a glycopeptide) was an effective treatment option in some cases (Frieden *et al*. 1993). Since 2000, high level resistance to ampicillin and aminoglycosides has been common and treatment options are now limited in a large number of strains (Jeljaszewicz *et al*. 2000). In this study, one of the species, *E. faecalis* was used to assess the activity of plant extracts and oils against this species.

5.1.5 Gram-negative bacilli

The effect that food and waterborne diseases have had on world populations cannot be overstated. Through history, millions of people have died, armies have faltered, and populations have been changed by diseases transmitted through food or water. Today, gastrointestinal diseases continue to cause malnutrition and death in developing countries. In industrial countries, while there are fewer deaths, many people are affected each year by these diseases. The majority of food and waterborne infections are caused by Gram-negative bacterial pathogens e.g. *Campylobacter* spp., *Salmonella* spp., *Shigella* spp.,

Vibrio spp., and *Escherichia coli*. These species cause disease around the world, and antibiotic resistance is an increasing problem, which impinges on effective therapy (Schaechter 2001).

While *Escherichia coli* is a very common Gram-negative rod normally found in the intestinal tract of mammals, some strains are pathogenic, causing sepsis, urinary-tract infections, meningitis, and gastroenteritis (Schaechter 2001, Kaper *et al.* 2004). While antibiotic treatment of gastro-intestinal disease is not always appropriate, the inhibitory effect of novel agents may exert on progression of infection is of interest since a great deal of morbidity is associated with GI disease. A strain of *E. coli* was therefore included in this investigation.

5.1.6 Normal flora

Not all bacteria are harmful, some for example the so called probiotic bacteria, may be beneficial, as are the normal flora. A probiotic generally means a live microbial food supplement, which beneficially affects the host animal by improving its intestinal microbial balance. Pathogenic and non-pathogenic bacteria can be and are affected by changes in the intestinal environment. Stress, travelling, antibiotic treatment, alcohol and poor diet may disturb the balance in our intestinal tract, often decreasing the number of beneficial bacteria while allowing an increase in pathogenic bacteria. Some common symptoms of this imbalance include flatulence, constipation and diarrhoea (MacFarlane and McBain 1999). Probiotic supplements contain beneficial bacteria, and may help to readjust the balance (Irene and Mark 2003).

It is reported that the intestinal microflora consists of approximately 10^{14} bacteria of over 400 species. It plays vital roles in metabolism and bioavailability of plant phenolics such as flavonoids, the importance of which is so far largely unknown (Puupponen-Pimia *et al.* 2001). It has been possible to ease the symptoms of lactose intolerance, to prevent diarrhoeal diseases and to stimulate the immune response by increasing the number of activity of probiotic bacteria i.e. *Lactobacillus* and *Bifidobacterium* in the colon through eating yogurt (Salminen and Saxelin 1996, Salminen *et al*. 1996).

Our bodies are composed of more bacterial cells than human cells, while the human body is made up of about 10^{13} human cells and we harbour near 10^{14} bacteria. This group of organisms, traditionally referred to as normal flora is composed of a fairly stable set of genera, mostly anaerobes. While each person has a relatively unique set of normal flora, members of the *Streptococcus* and *Bacteroides* make up a large percentage of the inhabitants. These organisms contribute in several ways in which they may help us by competing with pathogens such as *Salmonella*. They also provide vitamins or eliminating toxins, for example *Bacteroides*. However, the normal flora also can harm us by promoting disease such as dental caries and they may cause neither help nor harm (e.g. commensals such as coagulase-negative Staphylococcus) (Sleigh and Morag 1998).

Investigating the effect of plant extracts on the normal flora merits consideration. Plant compounds could be toxic to normal flora, which could be used as a biomarker to test the toxicity of plant products. Ideally, novel agents would be more effective against potential pathogens but act selectively without affecting the normal flora. To ascertain the effect of the plant extracts obtained in this study, two strains of a probiotic *Lactobacillus acidophilus* were included in the investigation, even though this strain is not a representative of overall normal flora, but information obtain of the effects (if any) would be valuable. In the present study, skin normal flora, coagulase-negative Staphylococcus was used to see the effects of the plant extracts as this strain also known to be opportunistic pathogen.

5.1.7 Biofilms

From an infection point of view, an area of antimicrobial research which is becoming increasingly investigated, particularly in the context of surgical devices is biofilms. A biofilm is a layer of microorganisms embedded in a matrix of secreted polysaccharides and glycoprotein (Watnick and Kolter 2000) also described as surface associated bacterial communities comprising exopolysaccharide-surrounded microcolonies (Pratt and Kolter 1999). They represent a further weapon in the armamentarium of bacterial and fungal pathogens to enhance their resistance. Many antimicrobials are highly effective against planktonic microbes i.e. organisms not growing in a biofilm. Once a biofilm is formed, those microbes so incorporated are exposed to far lower levels of the drug, and their heterogeneity reduces the effectiveness of monotherapy or narrow-spectrum antibiotic drugs (Donlan 2001, Jefferson 2004). This is compounded by the additional problem of

bacterial promiscuity and ‘quorum sensing’, which allows interspecies sharing of genetic resistance vectors and communication between the organisms promoting phenotypic alteration (Suntharalingam and Cvitkovitch 2005). It is now known that many medically significant infections are caused by mixed-organism biofilms, the most devastating of which from a surgical perspective, are the infection of endoprostheses (heart valves, joints and vascular grafts) or other indwelling medical devices, such as vascular cannulae or bladder catheters (Donlan 2001). The morbidity and mortality associated with endoprosthetic infection is significant, often involving longer stays in hospital, long-term antibiotic use, and occasionally repeat surgery to explant and eventually replace affected components where possible (Donlan 2001, 2002).

As the initial stage in biofilm formation relies on adhesion through cell-surface protein-substrate interaction, any anti-adhesive properties of plant compounds such as flavonoids, terpenes and alkaloids may prove crucial in reducing rates of biofilm formation. Tea tree oil which contains mainly terpenes has been reported to have anti-adhesive effects on *S. aureus* biofilms (Brady *et al.* 2006). Investigation of other plant products which have similar effect to that of tea tree oil could merit consideration and in this study, extracts and oil from *Anacardium* species, were tested.

5.1.8 Biofilms, medical devices, MRSA infection and antibiotic challenges

MRSA is recognised as a major nosocomial pathogen and causes infections in hospitals worldwide. The UK is one of the highest rates of MRSA in Europe (Johnson *et al.* 2005, Brady *et al.* 2006). Mortality rates associated with these types of infections remain unacceptably high. Vancomycin remains the antibiotic of choice in the treatment of serious staphylococcal infections but with the emergence of resistance to this glycopeptide future use remains uncertain. Linezolid and daptomycin offer alternative options, but once again there are reports of resistance and treatment failure (Wagenlehner 2005). This may be in part due to the mode of growth that *S. aureus* is able to adopt in the presence of various biomaterials, i.e. biofilms (Tunney *et al.* 1998a, 1998b). There is no question that the use of various medical devices has greatly facilitated the management of serious medical and surgical conditions, but their use has also resulted in a concomitant rise in their infection (Costerton 1999).

Recently, there has been an increasing recognition of the role that microbial biofilms play in human medicine and it has been estimated that about 65% of all human microbial infections involve biofilms (Khardori and Yassien 1995, Costerton 1999, Donlan 2001, 2002, Douglas 2002). The introduction of artificial materials into several body locations has been accompanied by the ability of micro-organisms, including *S. aureus*, to colonise them and form biofilms that protect them from antibiotics and host defences leading to persistent infections (Darouiche *et al.* 1992, 1997, Raad 1995, Darouiche 2004). Devices and foreign materials such as shunts, prostheses (voice, heart valve and knee), stents, implants (lens, breast and denture) endotracheal tubes, pacemakers, various types of catheters and suture structures have all been shown to support colonisation and biofilm formation by *S. aureus*, which is comprised of a multi-layer of cells embedded within a glycocalyx matrix of complex polysaccharides (Stickler 1999, Donlan 2001, Stewart and Costerton 2001). These infections continue to be a major cause of morbidity and mortality in patients with implanted medical devices (Donlan 2001). Once biofilms are formed on these surfaces, therapeutic interventions only rarely achieve clinical cure because the sessile bacteria which make up the biofilm are intrinsically resistant to the actions of antimicrobial challenge and elevated concentrations of antimicrobial agents are required to kill them compared with their equivalent free-floating planktonic counterparts (Gilbert *et al.* 1997, Amorena *et al.* 1999. Habash and Reid 1999). Therefore, removal of the infected device is required, often involving surgery. Therefore, development of novel antimicrobial agents with alternative modes of action may provide a means of managing hard-to-treat biofilm related infections, for this reason, the oils and extracts of *A. occidentale* were tested against MRSA strains grown as biofilms.

5.1.9 Novel compounds from plant as antimicrobial agents

Natural resources are now being sought as a source of novel antimicrobial agents as conventional agents are increasingly ineffective due to the acquisition of resistance mechanisms (Cowan 1999, Gibbons 2004, 2005, Lewis and Ausubel 2006). These complex plant based products contain various secondary metabolites with varying modes of action and complex interactions, to which bacteria and fungi are less likely to develop resistance. Traditionally the search for novel antimicrobial agent to tackle bacterial resistance has focused around products which arise from microbial sources. For example, the development of antibiotics such as streptomycin, aureomycin and chloromycetin followed on from the

discovery of penicillin (Trease and Evans 1972). Though most of the antibiotics used clinically are produced by soil microorganisms, natural plant resources are now under investigation as a source of novel agents. The presence of various secondary metabolites with different modes of action, reduces the likelihood that resistance to the products will develop in bacteria and fungi.

Plant based antimicrobials have enormous therapeutic potential as they are effective in the treatment of infectious diseases while simultaneously reducing many of the side effects that are often associated with synthetic antimicrobials. They are effective, yet gentle (Cowan 1999, Iwu *et al*. 1999). The healing power of medicinal plants is in most instances not understood and the mechanisms of healing are in need of investigation (Iwu *et al.* 1999).

5.1.9.1 Antimicrobial activity of phytochemicals

Plants have been a source of beneficial phytochemicals which have different biological activities (Cowan 1999, Sara 2004, Rios and Recio 2005) (*Table 5.1.3*). Extensive research has been conducted to assess the ability of green tea and its constituents to inhibit MRSA (Yam *et al*. 1998, Hamilton-Miller and Shah 1999, Gibbons 2004). Epicatechin gallate was reported to have antibacterial activity which reverses meticillin-resistance by inhibiting the synthesis of penicillin binding protein 2' (PBP2') and is selective, affecting only staphylococci that synthesise PBP2' (Hamilton-Miller and Shah 1999). Another plant metabolite, totarol, a diterpene has also been shown to potentiate meticillin activity against MRSA via interference of PBP2' expression (Nicolson *et al.* 1999). Flavonoids, a major group of C_6-C_3-C_6 phenolic compounds have also shown some activity against *S. aureus* and MRSA, especially the flavones apigenin and luteolin (Rahman and Gray 2002).

Table 5.1.3 Major classes of antimicrobial compounds from plants (Adapted from Cowan 1999)

Common name	Scientific name	Compound	Class	Activity	References
Thyme	*Thymus vulgaris*	Caffeic acid	Hydroxycinnamate	Viruses, bacteria, fungi	
		Thymol	Terpenoid		
		Tannins	Flavones		
Tree bard	*Podocarpus nagi*	Totarol	Terpenoid	*P. acnes*, other gram-positive bacteria	Kubo *et al*. (1994)
		Nagilactone	Lactone	Fungi	Kubo *et al.* (1992)
Tua-Tua	*Jatropha gossyphiifolia*	?		General	Kubo *et al.* (1993)
Turmeric	*Curcuma longa*	Curcumin	Cinnamoyl-methane	Bacteria, protozoa	Apisariyakul *et al.* (1995)
		Turmeric oil			
Valerian	*Valeriana officinalis*	Essential oil	Terpenoid	General	
Willow	*Salix alba*	Salicin	Phenolic glucoside	General	
		Tannins	Polyphenols		
		Essential oil	Terpenoids		
Wintergreen	*Gaultheria procumbens*	Tannin	Polyphenols	General	Bose (1958), Berkada (1978), Hamburger and Hostettman (1991), Scalbert (1991)
Woodruff	*Gallium odoratum*	-	Polyphenols	General, viruses	
Yarrow	*Achille millefolium*	?		Viruses, helminths	
Yellow dock	*Rumex crispus*	?		*E. coli, Salmonella, Staphyllococcus*	

* 'General' denotes activity against multiple types of microorganisms (e.g bacteria, fungi and protozoa). 'Bacteria' denotes activity against gram-positive and negative bacteria.

One of the species used in this study, *A. occidentale*, has been reported to have medicinal healing properties and have been used in the treatment of diabetes, and as a remedy for fever (Kamtchouing *et al*. 1998, Maia *et al.* 2000) and, most interestingly from the point of view of MRSA treatment, has been shown to have wound healing and antimicrobial activity. Information on the bioactivity of *A. occidentale* extracts is scarce and reports on antimicrobial activity of this plant relate to the bark (Akinpelu 2001), and the cashew-nut apple or shell (Muroi *et al*. 1993, Muroi and Kubo 1993, Laurens *et al.* 1997). Akinpelu (2001) reported that methanolic extract of *A. occidentale* bark exhibited antimicrobial activity, using an agar diffusion method, at a concentration of 20 mg/ml on selected bacteria isolates (*Bacillus spp., Kl. pneumoniae, Pseudomonas spp., S. aureus* and *E. faecalis*) and Schmourlo *et al.* (2005) reported antifungal activity of extracts from the leaves and fruits (*Table 1.2* in *Chapter 1)*.

The antimicrobial properties of the plant appear to be species specific, with no activity demonstrated against *E. coli* and *C. albicans* with bark extracts. Muroi *et al.* (1993) has reported some antimicrobial activity from non-isoprenoid alkyl side chain phenolic compounds such as anacardic acids, methylcardols and cardols from the cashew-nut shell oil. Anacardic acids were found to exhibit potent antibacterial activity against Gram-positive bacteria and contrary to Schmourlo *et al.* (2005), weak antifungal activity was also detected.

Tea tree oil, the essential oil of *Melaleuca alternifolia*, a native Australian plant has been suggested as a potential agent for MRSA decolonization (Halcon and Milkus 2004, Brady *et al*. 2006). It has been reported to show an effective broad-spectrum antimicrobial response with good activity *in vitro* against a variety of bacteria including MRSA (Gustafson *et al.* 1998, Hammer *et al.* 2003). Tea tree essential oil was tested in this study as a comparitor for cashew-nut essential oil. Leaf extracts and essential oil were screened for antimicrobial activity.

One group of important phytochemicals is the flavonoids. The antibacterial activity of flavonoids is being increasingly documented (Cowan 1999). Crude extracts from plants with a history of use in traditional medicine have been screened *in vitro* for antibacterial activity by many research groups (Cowan 1999, Cushnie and Lamb 2005). Flavonoid-rich plant extracts from species of *Hypericum*, *Capsella* and *Chromolaena* have been reported to

possess antibacterial activity. Many other phytochemical preparations with high flavonoid content have also been reported to exhibit antibacterial activity (Cushnie and Lamb 2005).

Many research groups have gone one step further and either isolated and identified the structure of flavonoids that possess antibacterial activity, or quantified the activity of commercially available flavonoids. Examples of such flavonoids are apigenin, naringin, naringenin, (-)-epigallocatechin gallate (EGCG), quercetin, 3-*O*-methylquercetin and various quercetin and kaempferol glycosides, and their derivatives. Other flavones, flavone glycosides, isoflavones, flavanones, isoflavanones, isoflavans, flavonols, flavonol glycosides and chalcones with antibacterial activity have also been identified (Basile *et al.* 2000, Cushnie and Lamb 2005).

Propolis from honey has been analysed on many occasions and samples containing high concentrations of flavonoids are frequently reported to show antibacterial activity. The antimicrobial properties of propolis have been attributed to its high flavonoid content and in particular the presence of the flavonoids galangin and pinocembrin (Grange 1990, Grange and Davey 1990). Huangchin (*Scutellaria baicalensis*) is yet another example. This herbal medicine has been used systemically and topically for thousands of years in China for the treatment of periodontal abscesses and infected oral wounds. The flavone baicalein is reported to be largely responsible for this plant's antimicrobial effects (Tsao *et al*. 1982).

5.1.9.2 Antimicrobial activity of plant volatiles

The volatile compounds contain variable mixtures of principally terpenoids, specifically monoterpenes [C10] and sesquiterpenes [C15] although diterpenes [C20] may also be present, as some variety of low molecular weight aliphatic hydrocarbons, acids, alcohols, aldehydes, acyclic esters or lactones and exceptionally nitrogen and sulphur-containing compounds, coumarins and homologues of phenylpropanoids. Terpenes are amongst the chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants. Most terpenes are derived from the condensation of branched five-carbon isoprene units and are categorized according to the number of these units present in the carbon skeleton (Dorman 1999, Dorman and Deans 2000).

It is clear from the reports that these plant secondary metabolites have potential in medical procedures and applications in the cosmetic, food and pharmaceutical industries. Investigations into the antimicrobial activities, mode of action and potential uses of plant volatile extracts have regained momentum. There appears to be a revival in the use of traditional approaches to protecting livestock and food from disease, pests and spoilage in industrial countries. This is especially true with regard to plant volatile oils and their antimicrobial evaluation, as can be seen from the comprehensive range of organisms against which volatile oils have been tested. These have included food spoilage organisms and food poisoning organisms, spoilage and mycotoxigenic filamentous fungi, pathogenic and dimorphic yeasts and animal and plant viruses (Lee *et al*. 2003).

5.1.10 Evaluation of antimicrobial activity

5.1.10.1 Colourimetric assay to measure biomass and biofilms

Antimicrobial activities of planktonic cells can be easily determined using an universal method such as agar diffusion or disc diffusion methods, minimum inhibitory concentration (MIC) and electron microscopy observations (CLSI 2006a, 2006b), however given the tremendous clinical importance of biofilms, it is somewhat surprising that is no standard method for investigating the drug susceptibility of bacterial biofilms (Pettit *et al*. 2005). Several methods are available but are limited by long processing times, incompatibility with high throughput, expensive reagents and equipment, or the fact that the method measures mass instead of viability (Amorena *et al.* 1999, Baillie and Douglas 1999).

A common method of assessing susceptibility in bacteria is to quantitate the mass of biofilms by crystal violet or safranin staining, followed by extraction of bound dye with a solvent and measurement of absorption (Balaban *et al.* 2003). However, this method does not provide information on the viability of the cells. Another common method of assessing bacterial biofilm susceptibility is to disrupt the biofilm by sonication, vortexing or scraping, followed by dilution plating for determination of CFU/ml (Amorena *et al.* 1999, Polonio *et al*. 2001). This method has serious limitations; biofilm clumps can be difficult to dissociate into single-cell suspensions for plate counting, it is extremely laborious, and antibiotic carryover is a concern.

5.1.10.2 XTT assay to measure biofilm viability

The most common method used to measure biofilm viability is an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)- 2*H*-tetrazolium-5-carboxanilide] reduction assay (Ramage *et al.* 2001a, 2001b, Tunney *et al.* 2004). This method has also been used for such bacterial and fungal biofilms (Brady *et al.* 2006). While XTT reduction does measure metabolic activity, it requires the addition of an electron coupling reagent. Tetrazolium salt i.e XTT is heterocyclic organic compound that substitutes the natural final acceptor (oxygen) in the biological redox process and is reduced to formazan derivatives by receiving electrons enzymically from substances in the hydrogen transport system or nonenzymically from artificial electron transporters (menadione) which enhance the reaction. XTT can penetrate rapidly into intact cells and directly into subcellular membranes with dehydrogenase activity, where it is converted to coloured formazan derivative. Therefore, XTT was used as indicator of reducing systems (Tunney *et al.* 2004). Clearly, there is a need for an easy, cost-effective, reproducible assay of biofilm susceptibility that measures viability, and XTT has been proven to be a suitable assay for biofilm susceptibility testing (Ramage *et al.* 2001c, 2002). In this study, this assay was used to investigate the effects of plant extracts and oils against MRSA biofilms. The effects (if any) could be shown by the orange-coloured solution formed in the 96-well plate due to the reduced formazan which can be measured spectrometrically at 490 nm and this showed the effects of the plant products on the viability of the cells.

5.1.11 Modes of action and mechanism of inhibition of antimicrobial agents

A common way to classify antimicrobial agents is according to their mode of action. Antibiotics act on bacteria by interfering with processes that are essential for normal bacterial structure and function. One example is inhibition of bacterial cell wall synthesis. This structure and composed of peptodoglycan which is structurally unique to bacteria and therefore has become an important target for antibiotics such as beta-lactams and glycopeptides (Williams *et al.* 1996). Antimicrobial agents work in one of two ways. Bacteriostatic agents inhibit bacterial multiplication but multiplication resumes upon removal of the agent. Bactericidal agents kill the bacteria irreversibly. Typically, bactericidal agents cause lysis of the cells or disruption of DNA as the genetic element of reproducibility (Jawetz 1991, Mann and James 1996).

Antibacterial agents may affect cells in a variety of ways, many of which are poorly understood (Williams *et al*. 1996, Cowan 1999). At high concentrations many agents are so destructive that, among other things, the cell proteins precipitate from the colloidal state (coagulate). Under certain conditions, some agents may specifically disrupt the cell membrane. Many of the cell's essential enzymes possess sulphydryl (-SH) groups and can only function if these remain free and reduced: hence agents which oxidise or combine with sulfhydryl groups are strongly inhibitory (Jawetz 1991, Hawkey 2000). Finally, many agents may act by interfering with one or a few specific enzymatic reactions which is called chemical antagonism (Mann and James 1996, Normark and Normark 2002). There are three principle mode of actions of antimicrobial agents, inhibition of the cell wall synthesis, inhibition of DNA replication and disruption of the cell wall membrane function in intracellular metabolism.

Some of the compounds e.g. epicathechin (Hamilton-Miller and Shah 1999) and flavonoids (Cowan 1999) appear to make the external structure of the cell wall permeable thus changing the physiology of bacteria. This was observed structurally by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) and these techniques were employed in this study. Most of the compounds acted preferentially on Gram-positive bacteria. This may be due to the high lipid and low peptidoglycan content in Gram negative bacteria (Mann and James 1996, Hawkey 2000).

5.1.12 Aims of this chapter

With a dwindling arsenal of effective antibiotics and increasing rates of bacterial resistance, alternative agents are required. The first research section of this chapter aimed to evaluate the antibacterial activities of five species of Malaysian traditional vegetables, which are widely known to contain various beneficial phytochemicals such as phenolics, vitamins, sterols, terpenes and carotenoids as discussed in the previous chapter. This was based on the hypothesis that these phytochemicals exhibit antibacterial effects. The methods employed in this study were based on the Clinical Laboratories Standards Institute (CLSI) and the investigation of the mode of actions of the antimicrobial activity is discussed based on the qualitative and microscopic techniques. Different types of bacteria, Gram-positive and Gram-negative as well as normal flora tested in this study will provide information on

the potential effects of plant compounds as future antimicrobials. This will allow more thorough research to be conducted in the future.

In the second section of this chapter, the study aimed to address the increasing recognition of the role of biofilm infections particularly of implanted medical devices used in surgery by investigating the efficacy of novel antimicrobial components from the extracts of *A. occidentale* (cashew-nut leaves) against four EMRSA strains isolated from the Scottish hospital environment when growing as adherent biofilm populations. These extracts were compared to tea tree oil extracts, which have been used more extensively in research to date. It was shown that antibiotics are able to treat bacterial infection planktonically, but need 1000x higher doses to treat biofilm infections. Therefore, novel antimicrobials which can treat infection of both planktonic organisms and biofilms merits consideration. Qualitative and quantitative measurements of the effect of the extracts on the biofilms viability and structures were conducted using colourimetric assay and microscopic evaluation.

5.2 MATERIALS AND METHODS

5.2.1 PART 1: THE EFFECTS OF MALAYSIAN TRADITIONAL VEGETABLES EXTRACTS AND OILS AGAINST PLANKTONIC (FREE-FLOATING) BACTERIA

5.2.1.1 Samples

The extracts and oils used were extracted as described in *Chapter 2* and diluted to working concentrations in DMSO, distilled water, methanol, acidified methanol or hexane, as appropriate.

5.2.1.2 Reagents and bacteria

Where available, ATCC strains were tested. However, to extend the spectrum of organisms investigated, laboratory strains collected in the Microbiology Department at Yorkhill Hospital were also included in the study. The strains of bacteria used in this study are listed in *Table 5.2.1*.

The following culture media were used and prepared according to the manufacturers' instructions. Mueller Hinton (MH), Columbia Agar and Horse Blood (CBA) [E & O Laboratories Ltd (UK)], Nutrient Agar (NA [Oxoid, UK]) and Brain Heart Infusion (BHI [Bioconnections, Leeds, UK]) were used for MIC (minimum inhibitory concentration) testing, prepared as described in *Appendix 5.1* and stored at 4˚C prior to use.

5.2.1.3 Antimicrobial susceptibility test by disc diffusion method

The disc diffusion method has been carefully standardised by the Clinical Laboratory Standard Institute (CLSI) methodology (CLSI 2006a, 2006b). Suspensions of the test organisms were prepared in sterile saline to a density equivalent to a McFarland 0.5 using a densitometer (BioMerieux, UK). A sterile cotton swab was dipped into the suspension and inoculated evenly over the entire surface of the medium by rotating the plate. The discs containing different concentration of extracts and oils were applied (5 and 10 µl) to the plates individually using sterile forceps and then gently pressed down onto the agar. Generally, no more than 4 disks were placed on a 90-mm plate. This prevents overlapping

of the zones of inhibition and possible error in measurement. After the disks were placed on the plate, the plate was inverted and incubated at 35℃ (Air incubator, LTE Scientific, UK) for 16 to 24 h dependent on strains being tested. After incubation, the diameters of the zones of complete inhibition were measured and recorded in millimetres (mm).

Table 5.2.1 Different strains of bacteria and fungi used in the study

Gram Type / Type	Strain of organism	Source
Gram-positive	*Enterococcus faecalis* (EF)	ATCC 29212
	Staphylococcus aureus (SA)	ATCC 29213
	Meticillin resistance *Staphylococcus aureus* (MRSA)	
	• Laboratory control	Yorkhill Hospital
	• EMRSA 15	Clinical isolate No. 11450A
	• EMRSA 16	Clinical isolate No. 8733A
	Staphylococcus spp. (coagulase negative, CoNS)	Yorkhill Hospital
	Lactobacillus acidophilus (LA1)	Clinical isolate PO22017
	Lactobacillus acidophilus (LA2)	Lambert Food Capsule
Gram-negative	*Escherichia coli* (EC)	ATCC 25922
	Klebsiella pneumoniae (KP)	ATCC 13883
	Pseudomonas aeruginosa (PA)	ATCC 27853
Fungi	*Candida albicans*	Yorkhill Hospital

5.2.1.4 MIC

One millilitre of extract of each different concentration, 125, 250, 500 and 1000 mg/ml for aqueous and organic extracts and 25, 50 and 100% (v/v) for essential oil was mixed with nutrient agar in serial decimal dilutions diluting 1 ml in 9 ml of agar to get the concentration range of 0.125 to 100 mg/ml and 0.25 to 10% (v/v), respectively. The agar was then poured into sterile Petri-dishes and allowed to solidify on the bench prior to drying in the incubator to prevent bacterial spread on moist plates. Bacterial suspensions were prepared equivalent to 0.5 MacFarland standards, giving approximately 10^6 bacteria/ ml. One ml of each suspension was placed into a well on a sterile block on the multipoint inoculator (Denley - Multipoint Inoculator A400, Sussex UK). The inoculation pins were sterilised in the Bunsen burner. The robot automatically transferred 0.1 ml of the bacteria suspension from the well to the MIC plates. The plates were then incubated overnight at 35 ºC and the MIC of the extract was determined as the concentration which bacterial growth was ≤ 10 colonies per inoculum.

5.2.1.5 Bacterial growth measurement

Bacterial growth was determined using the method of Miles & Misra or drop-plate method (Miles *et al.* 1938, Herigstad *et al.* 2001). This experiment was performed using *S. aureus* to determine the effects of *A. occidentale* extracts and oils on the inhibition of bacterial growth. Ten millilitres of fresh BHI medium were inoculated with *S. aureus*. The suspension used was standardized to McFarland standard 0.5. Methanol extracts and oils of the vegetables, γ-terpinene and DMSO were added to each bottle containing 10 ml of suspension bacteria in BHI broth to give a final concentration of 50 mg/ml for extracts and 10% (v/v) of oils, γ-terpinene and DMSO. One bottle of *S. aureus* culture alone was used as control.

The bottles were incubated at 34 ± 1ºC for 24 h. Twenty microlitres of each suspension was taken at 1, 2, 6, 18 and 24 h to be used for inoculating into 96-well microplates containing 180 µl BHI for serial dilution prior to performing Miles & Misra. The suspension was serially decimal diluted from 10^{-1} to 10^{-7}. Then, six aliquots of 10 µl of suspension were spotted onto a dried-CBA agar plate and incubated overnight to allow bacterial growth. The colonies present on the agar were counted and based on the serial dilution allowed calculation of the colony forming unit per ml bacterial count.

5.2.1.6 Scanning electron microscope (SEM) and transmission electron microscope (TEM) analysis for the observation of bacteria structures and bacteria cell walls

SEM and TEM analysis were carried out in the Electron Microscopy Unit (Division of Infection and Immunity, IBLS) to determine the effect of *A. occidentale* extracts and oil on the bacterial cell walls. Bacterial suspensions containing cell density equivalent to 0.5 McFarland standard were treated with 25 mg/ml of extract, 2.5% v/v for essential oil and untreated (10% v/v DMSO). Following centrifugation, the samples were fixed for 10 min in a 0.1 M phosphate buffer containing buffer of 2.5% glutaraldehyde (Sigma, UK, EM Grade), and then rinsed (3 x 10 min) in 0.1 M phosphate buffer (2% sucrose v/v).

For SEM, the samples were placed onto poly-L-lysine coated cover slips for 30 min. The samples were soaked with 1% osmium tetraoxide (Oxkem Ltd, UK) and rinsed with distilled water (3 x 10 min). Each sample was dehydrated for three times of 10 min each in 30, 50, 70 and 90% ethanol (Fisher Scientific). The samples were then soaked with absolute ethanol and dried for 10 min. Hexamethyldisilazane (HMDS [Taab Lab, UK]) was then added to the cover slips (2 x 2 min), which then left to dry in a desicator. These were mounted on a stub (Agar Scientific, Cambridge UK) with double sided copper tape and coated with Au/Pd (20 nm thick) (Polaron SC515 SEM coating system) for 15 min. At this stage these were ready to be observed under SEM (J 6400, Japan).

For TEM, propylene oxide (PO) was added (three times of 5 min each) to the samples, which were embedded in PO: epon resin (1:1) for 24 h. The samples were embedded in epon resin with no accelerator with several changes (8 h) and reembedded in pure epon resin (6 h). The samples were reembedded in epon with accelerator for 6 h and fresh embedded samples were put in moulds (60ºC/ 48 h) before sectioning and stained with 2% methanolic uranyl acetate (5 min). The samples were rinsed with distilled water and restained with Reynolds citrate (5 min). The samples then ready for TEM analysis (Leo 912 TEM, Germany).

5.2.2 PART 2: THE EFFECTS *ANACARDIUM OCCIDENTALE* EXTRACTS AND OIL AGAINST EMRSA BIOFILMS

In this study, all the experiments were performed under Biohazard Safety Cabinet Class II (MICROFLOW, Hampshire, UK). The tools used were sterilised by autoclaving or by the Bunsen burner or exposed to UV for 15 min.

5.2.2.1 Extracts and oil of *A. occidentale* and *Melaleuca alternifolia*

Methanol extracts of the yellow and red varieties and essential oil of *A. occidentale* were extracted as described in *Chapter 2.* The extracts were diluted in dimethyl sulfoxide (DMSO) and brain heart infusion (BHI) broth. The oil (100%) was diluted in BHI broth. Commercially available tea tree oil (*Melaleuca alternifolia*) mouthwash was purchased for these studies (Herbal Authority, Holland and Barrett, UK).

5.2.2.2 Isolates used and growth condition

Four isolates of epidemic meticillin resistant *Staphylococcus aureus* (EMRSA) strains were used in this study. These were obtained from the Scottish MRSA Reference Laboratory (SMRSARL) at Stobhill Hospital, Glasgow. These isolates were selected because they had been shown to produce reproducibly coherent biofilm structures (Perez *et al.* 2007). All strains were maintained on BHI agar plates (Bioconnections, Leeds, UK) and subcultured weekly. For all experiments, each strain was grown overnight at 37^{o}C on BHI agar. All isolates were stored on microbead cryostorage systems (Fisher Scientific, UK) and stored at -80˚C. When required, the isolates were sub-cultured onto BHI agar and incubated overnight at 37˚C. The strains used in this study are listed in *Table 5.2.2.*

5.2.2.3 Preparation of XTT (metabolic substrate) and menadione (electron coupler)

A metabolic dye, XTT (3-bis[2-methyloxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide]) was utilised in this study to assess the effects of cashew-nut extracts and oil. The XTT assay utilises the reduction of a tetrazolium salt, XTT by metabolically active cells to a coloured water- soluble formazan derivative that can be easily quantified colorimetrically. XTT (Sigma, UK) was prepared in phosphate buffer saline (PBS [Sigma,

UK]) in a saturated solution at 0.5 mg/ml. The solution was filter sterilised through a 0.22 µm-pore size filter, aliquoted and stored at -80˚C. Menadione (Sigma, UK), the electron coupling agent, was prepared in acetone (Fisher Scientific, UK) at a stock concentration of 10 mM and stored at -80˚C. When required, the desired concentrations of menadione were prepared in XTT immediately prior to use.

Table 5.2.2 EMRSA strains used in the study

Strain	No. of Isolate	Resistance Profile	Notes
EMRSA 15	66	Pn Mt Cx Cp Rf (ErMp)	Mp resistant
	84	Pn Mt Cx Cp Tr	Very sticky
EMRSA 16	100	Pn Mt Cx Er Cl Gn Cp Tr Mp Km NoTb	Mp resistant
	547	Pn Mt Cx Er Cl Gn Cp Tr Km No Tb	

Pn = penicillin Mt = methicillin Cx = cefotaxime Cp = ciprofloxacin Rf = rifampicin Er = erythromycin Mp = mupiricin Tr = trovafloxacin Cl = chloramphenicol Gn = gentamicin Km = kanamicin No = novobiocin Tb = tobramicin

5.2.2.3.1 XTT optimisation

Colourimetric assays are increasingly used to investigate cellular viability of metabolising cells and the susceptibility of cells to antimicrobial agents (Roslev and King 1993, Ramage *et al.* 2001a, 2001c, Kuhn *et al.* 2003). The tetrazolium salts (MTT or XTT), have both proven to be useful as the water solubility of the formazan product results in a simple assay (Roehm *et al.* 1991). Principally, XTT is converted to a coloured formazan in the presence of metabolic activity (the primary mechanisms of XTT-to-formazan conversion are the mitochondrial succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidases). Since the formazan product is water soluble, it is easily measured in cellular supernatants. This point is important in biofilm research because it allows the study of intact biofilms, as well as examination of biofilm drug susceptibility without disruption of biofilm structure (Kuhn *et al.* 2003).

A series of initial experiments were performed with the four clinical strains (66, 84, 100 and 547) to optimise the concentrations of menadione required to accurately quantify MRSA metabolic activity. Aliquots of XTT were thawed and menadione was added to give the desired final concentration. A suspension of each strain was prepared in PBS to a density of approximately 1×10^8 cfu/ml. The suspension was serially diluted ten-fold to obtain 5 sequential dilutions. Each dilution was subsequently evaluated using the XTT metabolic assay in an attempt to obtain a correlation between the total number of cells and the relative metabolism of the strain tested. Initially, one millilitre samples were pelleted by mild centrifugation (6,500 rpm). 200 µL aliquots of XTT (0.5 mg/ml) containing different concentrations of menadione (0, 50, 100, and 200 mM) were then used to resuspend the various pellets. The samples were incubated in the dark for 3h at 37℃, after which a colorimetric change in the XTT reduction assay was measured using a microtitre plate reader (Dynex MRXII Microplate Reader) at 490 nm. Quadruplicate samples were evaluated for each strain and menadione concentration.

5.2.2.3.2 Determination of optimal XTT incubation time

Experiments were performed as described above, but on this occasion the incubation time for XTT was varied using the optimised menadione concentration. The cells were pelleted and resuspended in XTT containing 50 mM menadione, which were then incubated in the dark at 37℃ for 0.5, 1, 3, 6 and 24 h. XTT colometric changes were quantified as described above.

5.2.2.4 Effects of plant extracts on MRSA biofilms

Fresh EMRSA colonies were inoculated into sterile BHI broth. 200µl of the suspension was aliquoted into each well of 96-well plate. A Nunc Immuno Maxisorp 96 peg plate lid was placed into the plate and sealed. The plate was sealed with Parafilm™ and incubated overnight at 37℃ on a rocking platform. The following day the lid was removed, containing bacteria on each peg, and used to inoculate a new plate containing 150 µl of fresh BHI broth. Subsequently, a new Nunc Immuno Maxisorp 96 peg plate lid was placed into the newly inoculated plate. This plate was then sealed with Parafilm™ and incubated for 48 h at 37℃ on a rocking platform.

For biofilm treatments, 25 mg/ml concentration of plant extract and 2.5% (v/v) of oil were prepared in sterile BHI broth. 150 µl of the suspension was aliquoted into each well. The lids with biofilms on each peg were removed from their growth media and placed into a fresh Nunc microtitre plate containing the extract, sealed and incubated overnight at 37℃. Control biofilms were placed into wells containing BHI broth only. For the assay, 200 µl of optimised XTT-menadione solution was added to each wells of a new microplate. The lid was then placed into the plate and incubated for 3 h in the dark. The colorimetric reading was performed by using a microtitre plate reader at 490 nm, as described above.

5.2.2.5 Quantification of the biofilm biomass

Plant extract treated biofilms on pegs and appropriate controls upon lids were washed in PBS and dried for 1 h in an incubator at 37℃. The biofilms were subsequently stained in 0.5 % crystal violet (w/v in dH_2O [Sigma, UK]) for 5 min and washed thoroughly under tap water until no residual crystal violet stain was noted. The biofilm laden pegs (n = 8) were snapped from the lid and placed into 100% ethanol (100 µl per peg) within a 20 ml glass Universal bottle. The bottle was vortexed for 15 seconds to decolourise the biofilm. 100 µl of this solution was then transferred to a new microtitre plate and the absorbance measured at 570 nm.

5.2.2.6 Total viable cell counts

5.2.2.6.1 Total viable counts of planktonic cell

Total viable counts were estimated using the method described by Miles and Misra (Miles *et al*. 1938). 100 µl of each suspension of planktonic cells used in the experiments was diluted in 900 µl sterile PBS and serial ten fold dilutions were performed. Measured volumes of each dilution were dispensed onto BHI agar plates and then incubated for 18-24 h at 37˚C. Colonies were counted the following day to estimate the total viable cell counts from each suspension (n = 6).

5.2.2.6.2 Total viable counts of biofilms

The pegs of biofilms were snapped from the lid of the microtitre plate (*Figure 5.2.1*) using a set of plyers and added to sterile PBS in glass bottles. These were sonicated for 5 min to disaggregate clumps and then vortexed for 30 sec. Total viable counts were then estimated

using the method described by Miles and Misra. Briefly, serial ten fold dilutions in sterile PBS were made for each sample. Measured volumes of each dilution were dispensed onto BHI agar plates and then incubated for 18-24 h at 37˚C. Colonies were counted the following day to estimate the total viable cell counts from each peg.

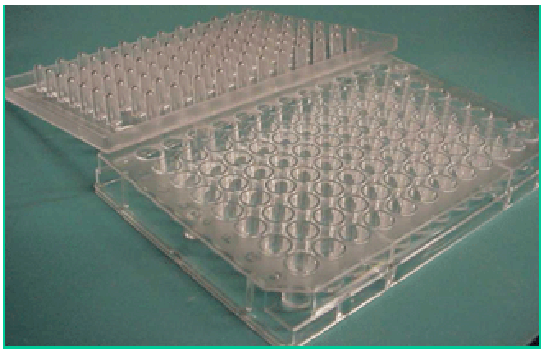


Figure 5.2.1 96-well plate with pegs lid for biofilm studies

5.2.2.7 Scanning electron microscopy analysis of biofilms with and without treatment

The solvents and procedures for fixation, staining and processing for SEM are similar to section *5.2.1.6*, however the samples were different. The pegs containing the control and treated biofilms from the 96-well plate lid were fixed in 96-well plate containing the solvents before further processing as described in *5.2.1.6.*

5.3 RESULTS

5.3.1 PART 1: THE EFFECTS OF MALAYSIAN TRADITIONAL VEGETABLES EXTRACTS AND OILS AGAINST PLANKTONIC (FREE-FLOATING) BACTERIA

5.3.1.1 Disc diffusion method to assess antimicrobial property of crude extracts of Malaysian traditional vegetables on selected bacterial strains

Seven types of vegetables, which were analysed for their phytochemical content, were screened for antimicrobial activity. Due to scarcity of material, the antimicrobial properties of these vegetables were tested against only a small number of bacterial strains (*E. coli* ATCC 25922, *E. faecalis* ATCC 29212, *Kl. pneumoniae* ATCC 13883, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and clinical isolates of MRSA. The disc diffusion method used in the experiment was standardised in accordance to CLSI. Methanol, aqueous and acidified methanol extracts were assessed in this study.

The methanol, acidified methanol, aqueous and hexane extracts of all the vegetables had no effect on all the bacterial strains tested with the exception of *A. occidentale*. The results for the disc diffusion method of methanol extracts at 25 mg/ml of *A. occidentale* on eight strains of bacteria are shown in *Table 5.3.1*. Red and yellow varieties of *A. occidentale* showed inhibition, mostly of Gram-positive bacteria. Acidified methanol extracts of both varieties showed only minor inhibition against the Gram-negative bacteria, *Kl. pneumonia* ATCC 13883 (7.5 ± 0.6 and 7.8 ± 0.2 mm for red and yellow varieties, respectively). Methanol and DMSO, which were used to dilute the extracts did not induce any inhibition of bacterial growth. Cefoxitin, a widely used antibiotic was used as a positive control at 30 µg, which showed > 20 mm inhibition zones with the bacteria tested. This was more effective than the crude extracts of *A. occidentale*.

5.3.1.2 Disc diffusion method to assess antimicrobial activity of crude extracts of *A. occidentale* on MRSA and coagulase-negative staphylococci

Due to the promising effects of *A. occidentale,* especially on Gram-positive bacteria compared to other vegetable extracts, an extension of screening was carried out for MRSA strains (including EMRSA) and coagulase-negative *Staphylococci*. Three MRSA strains

Table 5.3.1 Antibacterial activity of crude extracts (*M - methanol, AM - acidified methanol*) of *Anacardium occidentale* (AO) against Gram-positive and Gram-negative bacteria

Strain/ Extracts	Extract	Gram-negative			Gram-positive					
		EC	***KP***	***PA***	***EF***	**SA**	**MRSA (control)**	**EMRSA 15**	**EMRSA 16**	**CoNS**
AO (red)	*M*	-ve	-ve	-ve	-ve	8.6 ± 0.1^{a}	8.3 ± 0.3^{a}	8.1 ± 0.1^{a}	8.1 ± 0.1^{a}	10.8 ± 0.5^{a}
	AM	-ve	7.5 ± 0.6^{a}	-ve	-ve	7.1 ± 0.1^{b}	7.3 ± 0.3^{b}	7.1 ± 0.1^{b}	6.6 ± 0.1^{b}	11.9 ± 0.3^{b}
AO (yellow)	*M*	-ve	-ve	-ve	-ve	8.6 ± 0.1^{a}	9.3 ± 0.5^{c}	9.3 ± 0.3^{c}	9.1 ± 0.1^{c}	12.3 ± 0.3^{b}
	AM	-ve	7.8 ± 0.2^{a}	-ve	-ve	8.6 ± 0.1^{a}	8.1 ± 0.1^{a}	7.8 ± 0.3^{a}	7.6 ± 0.1^{d}	13.1 ± 0.6^{c}
DMSO *	*M*	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Methanol *	*AM*	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Cefoxitin (30 µg)						≥ 20				

-ve = no inhibition zone was observed, results expressed as diameter of inhibition zone in mm (mean ± standard deviation, SD) (n=9). Concentration tested of the extracts at 25 mg/ml except for CoNS at 100 mg/ml. [Similar superscript letter in the same column shows not significant ($p > 0.05$) statistically]

were tested; the laboratory control strain and isolates of EMRSA 15 and EMRSA 16. Coagulase negative *Staphylococci* which are the predominant commensal microorganisms found on the skin were also investigated, as it was of interest to investigate the effects that *A. occidentale* extracts might have on the normal skin flora.

As shown in *Table 5.3.1*, in both varieties, the methanol extracts had significantly more inhibitory effect ($p<0.05$) against MRSA, EMRSA 15 and 16 at 25 mg/ml when compared to acidified methanol extracts. However, there was no significant difference in the effectiveness of both extracts on *S. aureus* ATCC 29213 strain. Against coagulase-negative *Staphylococci, A. occidentale* extracts exhibited mild inhibitory effects at 100 mg/ml, with the acidified methanol extract of the yellow variety showing the largest inhibition zone (13.1 ± 0.6 mm). The difference between two varieties was not significant. DMSO and methanol used as controls did not show any effects on the MSSA, MRSA and coagulase-negative *Staphylococci* tested.

5.3.1.3 Determination of the MIC to assess antimicrobial activity of *A. occidentale* extracts against Gram-positive bacteria

Different concentrations ranging from 1 to 100 mg/ml of extracts were tested on the Gram-positive bacteria *S. aureus* ATCC 29213, MRSA, EMRSA strains and coagulase-negative *Staphylococci*. Colonies are observed if the extracts permit bacterial growth but not if the concentration of the extracts completely inhibits the growth of the bacteria, i.e. the lowest concentration to completely inhibit microbial growth (MIC). The results are shown in *Table 5.3.2.* MIC testing revealed that the MIC of methanol extracts for *S. aureus* and EMRSA strains was 5 mg/ml which was lower than the acidified methanol extracts at 25 mg/ml. The MIC value for coagulase-negative *Staphylococci* was 100 mg/ml, which was higher than the MIC values for *S. aureus* and MRSA strains.

5.3.1.4 Disc diffusion method to assess antimicrobial property of essential oils of Malaysian traditional vegetables on selected bacterial strains

The essential oils of the vegetables were tested on the same strains as the crude extracts; these results are shown in *Table 5.3.3***.** The concentration of *Ulam* oils tested was 100% (v/v), but the application on the disc was 5 µl due to scarcity of the materials. At this volume, inhibitory effects were observed on almost all strains tested. *C. asiatica* oil showed very

minor activity on *S. aureus* ATCC 29213 only with an inhibition zone of 6.6 ± 0.6 mm. *P. indica* oil had showed inhibitory effects against the Gram-positive bacteria (*S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212) with inhibition zones of 8.5 ± 0.0 mm and 6.8 ± 0.4 mm, respectively. A similar spectrum of activity was observed for *P. cordifolia* and *Colubrina asiatica* oils. *A. occidentale* essential oil showed inhibition of all bacterial strains tested. Overall, the activity of A. occidentale was greater than the activity of tea tree oil for all strains tested apart from CoNS. Representative plates are shown in *Figure 5.3.1* and *5.3.2*.

Table 5.3.2 Minimum Inhibitory Concentration (MIC) of *Anacardium occidentale* extracts (*M = methanol, AM = acidified methanol*) against *Staphylococcus spp.*

Strain	Extracts	SA	MRSA (control)	EMRSA 15	EMRSA 16	CoNS
Red variety	*AM*	25.0	25.0	25.0	25.0	100
	M	5.0	5.0	5.0	5.0	100
Yellow variety	*AM*	25.0	25.0	25.0	25.0	100
	M	5.0	5.0	5.0	5.0	100

Results expressed as minimum inhibitory concentration (mg/ml) (n=9)

Table 5.3.3 Antibacterial activity of essential oils of Malaysian vegetables against Gram-positive and Gram-negative bacterial strains

Strain/ Extracts	Gram-negative			Gram-positive					
	EC	***KP***	***PA***	***EF***	***SA***	**MRSA (control)**	**EMRSA 15**	**EMRSA 16**	**CoNS**
Centella asiatica	-ve	-ve	-ve	-ve	6.6 ± 0.6	-ve	-ve	-ve	nd
Pluchea indica	-ve	-ve	-ve	6.8 ± 0.4	8.5 ± 0.0	-ve	-ve	-ve	nd
Anacardium occidentale	20.5 ± 0.7	16.0 ± 0.0	7.5 ± 1.4	15.0 ± 1.4	21.3 ± 0 .4	21.8 ± 0.4	24.5 ± 0.7	20.3 ± 0.4	7.3 ± 0.3
Premna cordifolia	-ve	-ve	-ve	7.3 ± 1.1	6.6 ± 0.6	-ve	-ve	-ve	nd
Colubrina asiatica	-ve	-ve	-ve	7.5 ± 0.7	10.0 ± 0.0	-ve	-ve	-ve	nd
Tea tree oil	10.3 ± 0.4	9.3 ± 0.4	-ve	-ve	7.0 ± 0.0	8.5 ± 0.0	6.5 ± 0.0	7.8 ± 0.4	8.3 ± 0.3
Cefoxitin (30 µg)					≥ 20				

-ve = not detected; nd = not determined, results expressed as diameter of inhibition zone in mm (mean ± standard deviation, SD) (n=9).

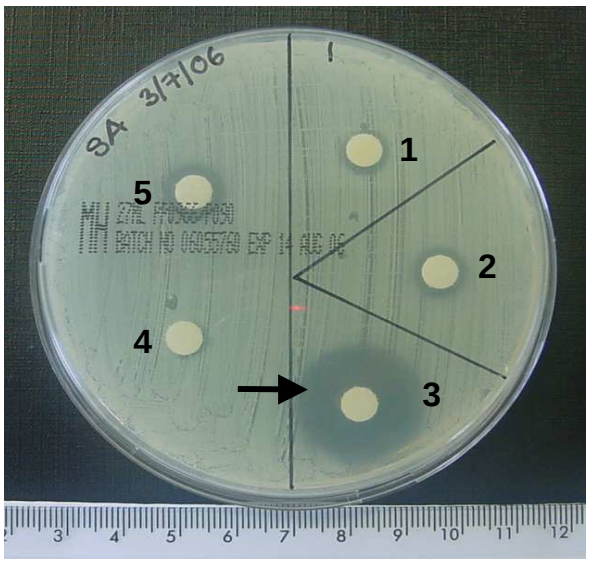


Figure 5.3.1 The disc diffusion method to assess the effect of plant essential oils (100% v/v; application on the disc = 5 µl) against Gram-positive bacteria, *S. aureus* ATCC 29213 on MH agar plate.

1 - *Centella asiatica*
2 - *Pluchea indica*
3 - *Anacardium occidentale*
4 - *Premna cordifolia*
5 - *Colubrina asiatica*

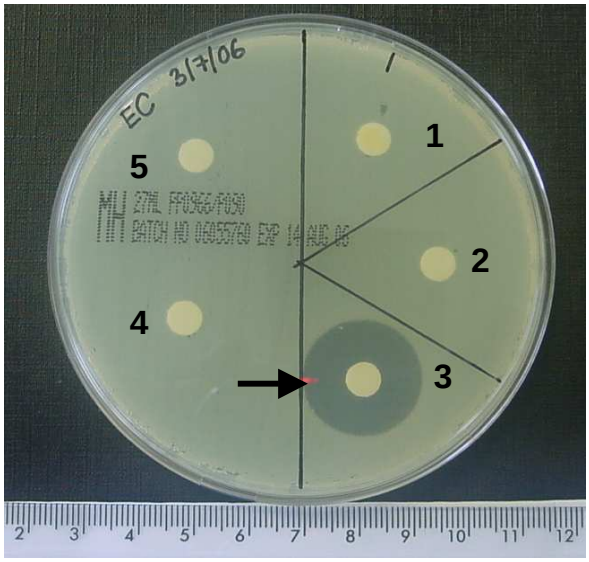


Figure 5.3.2 The disc diffusion method to assess the effect of plant essential oils (100% v/v; application on the disc = 5 µl) against Gram-negative bacteria, *E. coli* ATCC 25922 on MH agar plate.

1 - *Centella asiatica*
2 - *Pluchea indica*
3 - *Anacardium occidentale*
4 - *Premna cordifolia*
5 - *Colubrina asiatica*

5.3.1.5 Determination of MIC's to assess antimicrobial activity of *A. occidentale* essential oil

The essential oil of *A. occidentale* was analysed further and screened to determine the MIC. The MIC of *A. occidentale* oil and tea tree oil was shown in *Table 5.3.4.* The MIC of the oil was 2.5 % v/v for all the bacterial strains tested. However, the MIC for tea tree oil varied slightly. The MIC of tea tree oil was similar to that of *A. occidentale* oil against *E. coli* ATCC 25922*, S. aureus* ATCC 29213 and MRSA strains. However, the tea tree oil MIC values were higher for *Kl. pneumoniae* ATCC 13883 at 12.5 % v/v, 5.0 % v/v for *E. faecalis* ATCC 29212, and *P. aeruginosa* ATCC 27853.

5.3.1.6 Assessment of the antimicrobial properties of the essential oil of *Anacardium occidentale* and its components on selected bacterial strains

The results of antibacterial activity evaluated by disc diffusion method are shown in *Table 5.3.5***.** *D*-Limonene, γ-terpinene and terpinolene had substantial inhibitory effects on almost all strains of bacteria tested. γ-Terpinene was more active against *S. aureus* ATCC 29213 and MRSA strains. Terpinolene inhibited all strains except for *E. faecalis* ATCC 29212 but overall was not as effective as γ-terpinene. Myrcene and β-pinene were the least active against all bacteria tested. The effect of γ-terpinene against coagulase-negative *Staphylococci* was minor at 7.1 ± 0.3 mm compared to terpinolene and *D*-limonene which inhibited more bacterial growth at 15.3 ± 1.0 and 10.2 ± 0.8 mm, respectively. β-Myrcene and β-pinene showed the poorest inhibitory effects, with β-pinene demonstrating no antibacterial activity, and myrcene only showing minor activity against MRSA control strain. The activity of all components of the oil was less than the complete oil.

Table 5.3.4 Minimum Inhibitory Concentration (MIC) of *Anacardium occidentale* and *Melaleuca alternifolia* essential oils against Gram-positive and Gram-negative bacterial strains

Strain/ Extracts	Gram-negative			Gram-positive				
	EC	***KP***	***PA***	***EF***	***SA***	**MRSA (control)**	**EMRSA 15**	**EMRSA 16**
Anacardium occidentale oil	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Tea tree oil	2.5	12.5	5.0	5.0	2.5	2.5	2.5	2.5

Results expressed as minimum inhibition concentration (% v/v) (n=9)

Table 5.3.5 Antibacterial activity of compounds identified in *Anacardium occidentale* essential oil against Gram-positive and Gram-negative bacterial strains

Strain/ Extracts	***EC***	***EF***	***KP***	***PA***	***SA***	**MRSA (control)**	**EMRSA 15**	**EMRSA 16**	**CoNS**
γ-Terpinene	-ve	-ve	7.0 ± 0.7	6.5 ± 0.0	10.0 ± 1.4	10.0 ± 4.2	12.3 ± 2.5	15.5 ± 0.7	7.1 ± 0.3
α-Cubebene	-ve	7.5 ± 0.0	-ve	-ve	6.8 ± 0.4	6.5 ± 0.0	-ve	7.5 ± 0.0	-ve
Terpinolene	8.3 ± 0.4	-ve	11.5 ± 0.7	6.5 ± 0.0	8.5 ± 1.4	9.5 ± 0.7	8.3 ± 1.1	8.0 ± 0.7	15.3 ± 1.0
D-Limonene	21.0 ± 0.0	-ve	7.0 ± 0.0	-ve	8.0 ± 0.0	10.5 ± 0.7	10.3 ± 0.4	10.5 ± 1.4	10.2 ± 0.8
Myrcene	-ve	-ve	-ve	-ve	-ve	6.5 ± 0.7	-ve	-ve	-ve
β-Pinene	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Cefoxitin (30 µg)					≥ 20				

-ve = not detected, results expressed as diameter of inhibition zone in mm (mean ± standard deviation, SD) (n=9).

5.3.1.7 The effects of selected *Anacardium occidentale* crude extracts and essential oil on *Lactobacillus spp.*

The antibacterial effects of the extracts and oil of *A. occidentale* against two strains of *Lactobacillus acidophilus*, LA1 which were clinically isolated from patient, and LA2, isolated from a food capsule, are shown in *Table 5.3.6.* All crude extracts were inactive against these bacteria, as were the essential oils. The component compounds terpinolene and *D*-limonene had inhibitory effects of 12.1 ± 0.2 mm and 10.8 ± 0.3 for LA1 and 9.3 ± 0.5 mm and 8.3 ± 0.3 mm for LA2, respectively. LA1 was more sensitive to these compounds ($p<0.05$). Even though the components expressed some activity against this strain, the whole oils from both plants did not show any inhibitory effects probably because the concentrations of terpinolene and *D*-limonene in *A. occidentale* are very low (trace amount of terpinolene and 1.4% *D*-limonene analysed by GC-MS in *Chapter 4.*

5.3.1.8 The effects of *Anacardium occidentale* crude extracts and essential oil on the fungi (*Candida albicans*)

Fungi are resistant to antibiotics, and infections such as thrush may be encouraged by prolonged use of antibiotics as they can be treated topically. It may be beneficial to treat these infections with essential oils if those have anti-fungal properties. *A. occidentale* extracts and oil showed greater antibacterial effects against all strains tested, therefore this plant was chosen to be tested for antifungal activity. Crude extracts of *A. occidentale* showed promising antifungal activity against *C. albicans* (*Table 5.3.7*). The oil also exhibited mild antifungal activity. Terpinolene and *D*-limonene, which are present in the oil, showed the highest antifungal activity and were equivalent to that of tea tree oil.

Table 5.3.6 Antibacterial activity of *Anacardium occidentale* extracts, essential oils and oil components against *Lactobacillus acidophilus*

Strain/ Extracts	***Lactobacillus acidophilus* (LA1)**	***Lactobacillus acidophilus* (LA2)**
Methanol extract (red)	-ve	-ve
Methanol extract (yellow)	-ve	-ve
Acidified methanol (red)	-ve	-ve
Acidified methanol (yellow)	-ve	-ve
Anacardium occidentale oil	-ve	-ve
Tea tree oil	-ve	-ve
γ-Terpinene	-ve	-ve
α-Cubebene	-ve	-ve
Terpinolene	12.1 ± 0.2	9.3 ± 0.5
D-Limonene	10.8 ± 0.3	8.3 ± 0.3
Myrcene	-ve	-ve
β-Pinene	-ve	-ve

-ve = not detected, results expressed as diameter of inhibition zone in mm (mean ± standard deviation, SD) (n=9). Concentration tested of the extracts at 25 mg/ml and the oils / oil components at 100% v/v and the application of the extracts and oils on the blank disc was 10 µl.

Table 5.3.7 Antifungal activity of *Anacardium occidentale* extracts, essential oils and oil components against *Candida albicans*

Strain/ Extracts	***Candida albicans***
Methanol extract (red)	11.3 ± 0.4
Methanol extract (yellow)	13.8 ± 0.4
Acidified methanol (red)	13.0 ± 0.0
Acidified methanol (yellow)	12.0 ± 0.0
Anacardium occidentale oil	8.5 ± 0.4
Tea tree oil	24.5 ± 0.7
γ-Terpinene	13.5 ± 0.7
α-Cubebene	-ve
Terpinolene	27.0 ± 0.8
D-Limonene	38.0 ± 1.8
Myrcene	7.1 ± 0.8
β-Pinene	11.0 ± 0.8

-ve = not detected; results expressed as diameter of inhibition zone in mm (mean ± standard deviation, SD) (n=9). Concentration tested of the extracts at 100 mg/ml and the oils / oil components at 100% v/v and application of the extracts and oils on the blank disc was 10 µl.

5.3.1.9 Time-kill experiments: The effect of *A. occidentale* crude extracts and oil on *S. aureus* ATCC 29213 growth against exposure time

Methanol extracts of red and yellow varieties of *A. occidentale* were tested against *S. aureus* ATCC 29213 over 24 h incubation to investigate the effect on the growth of this strain with time. This was based on the results of the disc diffusion method and MIC testing which showed that methanol extracts of this plant exhibited strong inhibition at 25 mg/ml. The drop-plate method allows quantification of viable bacteria over 24 h incubation, thus the killing curve can be drawn. The bactericidal or bacteriostatic effects can be evaluated and thus mode of action may be revealed. *S. aureus* ATCC 29213 suspension with and without DMSO were examined at the same time during incubation to compare the growth curve profile. The growth curve of *S. aureus* ATCC 29213 is shown in *Figure 5.3.3*. As expected, the control underwent different phases of growth - lag, exponential, stationary and decline phases. However, adding the DMSO, which was used as a diluent, did effect substantially on the initial growth, but bacterial growth resumed after 18 h. The methanol extract of the yellow variety had a marked and more intense effect on the growth of the bacteria. Within two hours, the CFU/ ml was reduced from 1.0×10^6 to 3.7×10^5 cfu/ ml. A further reduction was observed at 6 h and bacterial counts reduced nearly 99% by 24 h. Whether a bacteriostatic or bactericidal mechanism is involved remains to be determined. The effect of the red variety was also very strong over the time frame but significantly low compared to the yellow variety. A one-log reduction from 1.0×10^7 to 1.0×10^6 cfu/ ml can be seen by 3 h, which levelled by 24 h.

A similar experiment was conducted to investigate the effects of *A. occidentale* oil and its main component, γ-terpinene. The effect of the oil against *S. aureus* ATCC 29213 growth was greater than the crude extracts as the inhibition was shown after 2 h of incubation (*Figure 5.3.4).* γ-Terpinene which is the predominant compound present in the oil exhibited gradual inhibition after 1 h to 24 h which was not significant compared to the oil indicating that the effect of this compound as good as the complete *A. occidentale* oil. The mode of action of these effects was investigated by electron microscopy to examine the effect of this extracts and oil on bacterial cell walls.

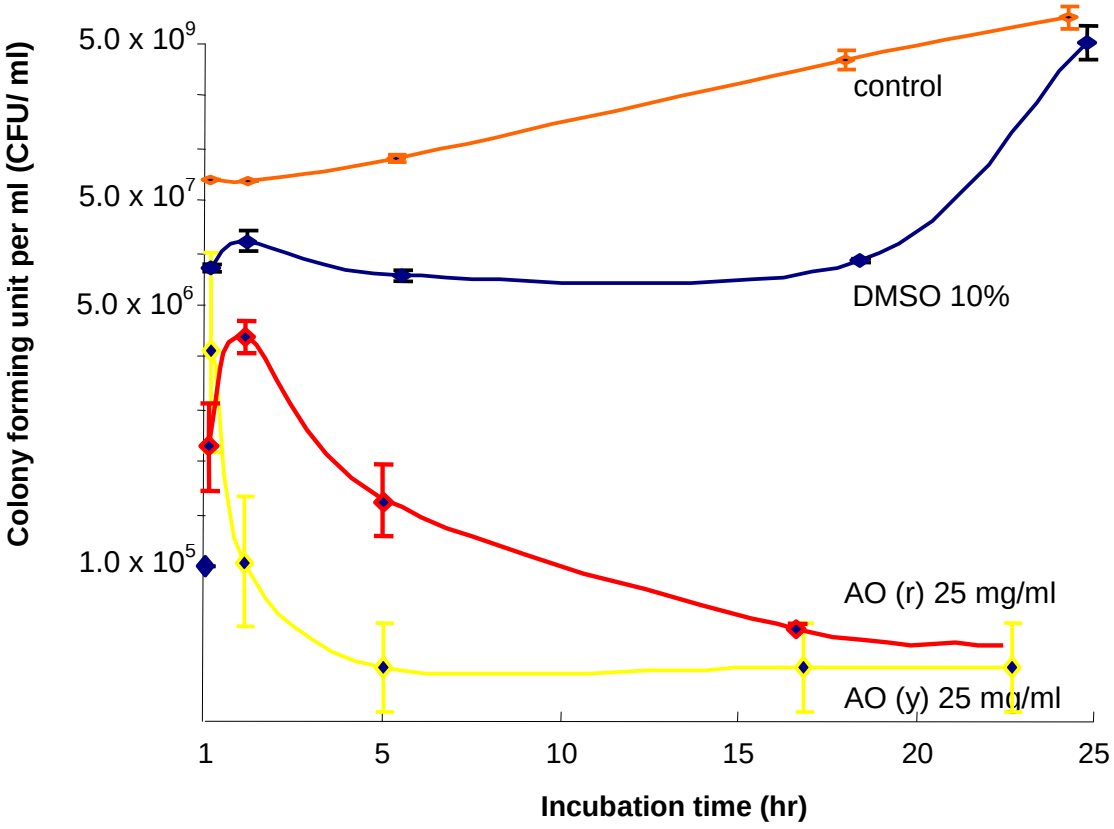


Figure 5.3.3 Growth-inhibition curve of *S. aureus* ATCC 29213 with and without treatment [DMSO 10%, AO (*A. occidentale*) methanol extracts – red (r) and yellow (y) varieties at 25 mg/ml] incubated over 24 h in BHI broth. Result expressed as value ± standard deviation, SD (n=3).

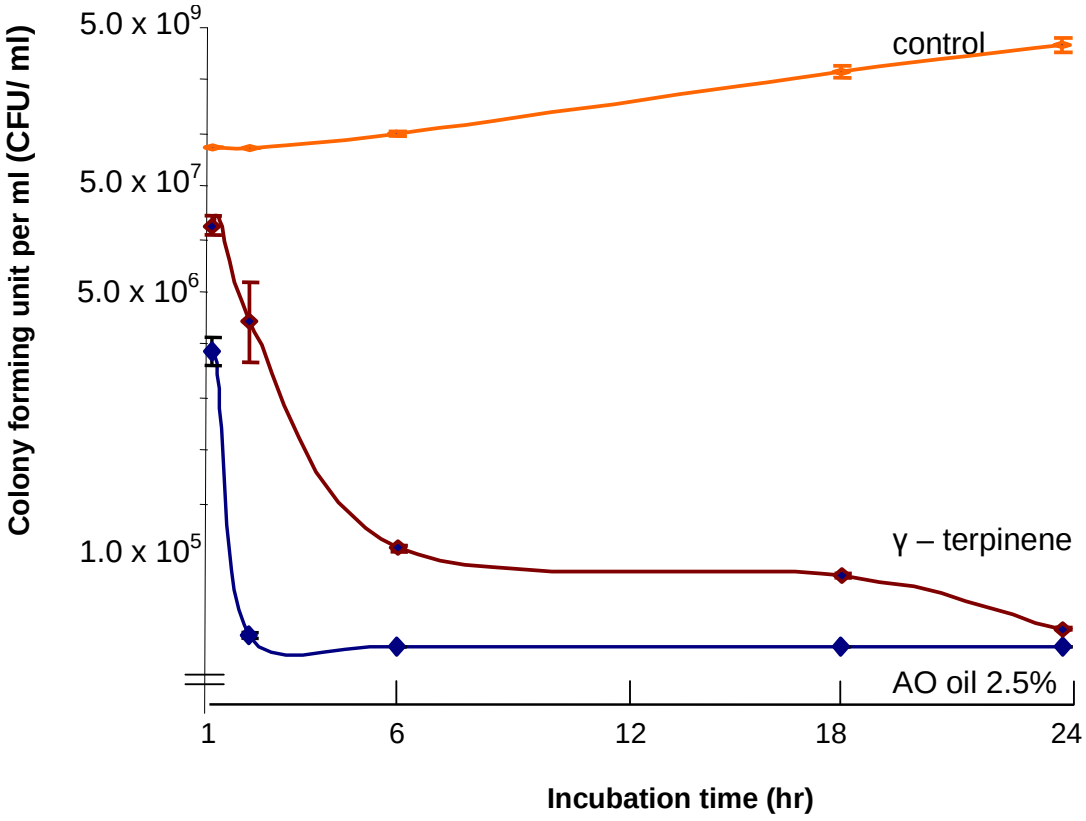


Figure 5.3.4 Growth-inhibition curve of *S. aureus* ATCC 29213 with and without treatment [AO (*A. occidentale*) essential oil and γ-terpinene at 2.5% v/v] incubated over 24 h in BHI broth. Result expressed as value ± standard deviation, SD (n=3).

5.3.1.10 Physical valuation of the effect of plant extracts and oils on *S. aureus* ATCC 29213 by SEM and TEM

The effects of *A. occidentale* extracts and essential oil on the cell wall of *S. aureus* ATCC 29213 were observed by SEM and TEM. The bacteria incubated with both the extracts and the oil for periods of 1, 2, 6, 18 and 24 h. Differences between the control and treated *S. aureus* ATCC 29213 are shown in *Figure 5.3.5***.** The control *(a, b* and *c*) demonstrated a normal spherical structure over the full duration of incubation and in the presence of DMSO. Incubation of the bacteria with crude extracts at 25 mg/ml and essential oil at 2.5% (v/v) for 24 h showed dramatic effects on the morphology and structure of the bacteria, which appear to be lysed at 24 h (*Figure 5.3.5 - d, e* and *f*)

Transmission electron microscopy of similar samples clearly shows the differences in the bacterial cell walls before (*Figure 5.3.6 - a, b* and c) and after the incubation with crude extracts and essential oil of cashew-nut (*Figure 5.3.6 - d, e* and *f*). Substantial effects were observed after 1 h incubation with both crude extracts and the essential oil of cashew-nut. In fact, no normal cell wall was visible in any of the experiments.

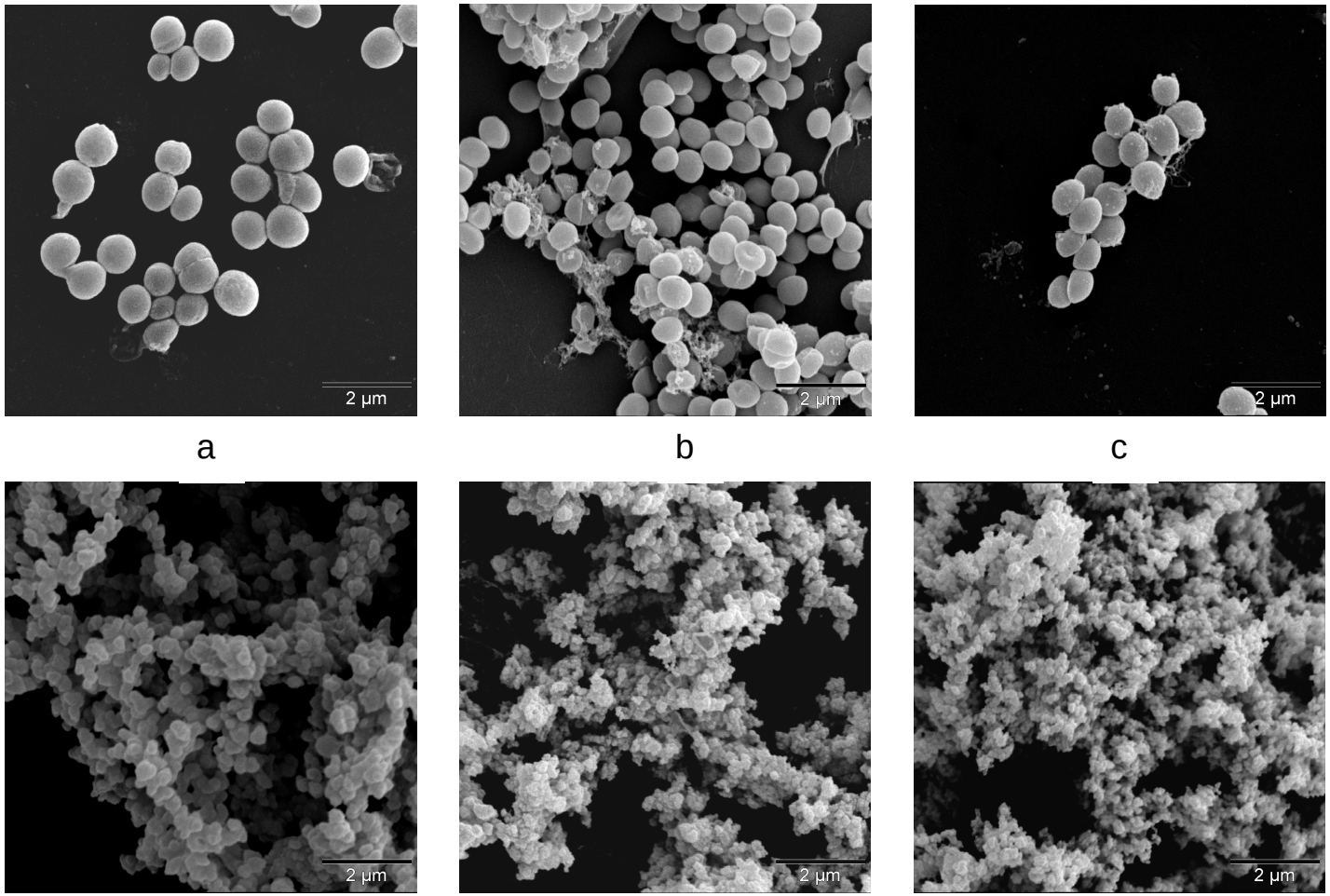


Figure 5.3.5 Scanning electron microscopy images of *S. aureus* ATCC 29213 control (**a** = 1 h, **b** = 24 h incubation) and **c** = treatment with DMSO 10% at 1 h incubation; and treated *S. aureus* ATCC 29213 (with **d** = *A. occidentale* oil at 2.5% v/v, **e** = methanol extract of red variety and **f** = methanol extract of yellow variety at 25 mg/ml incubated at 24 h) (Magnification = x 10,000 µm).

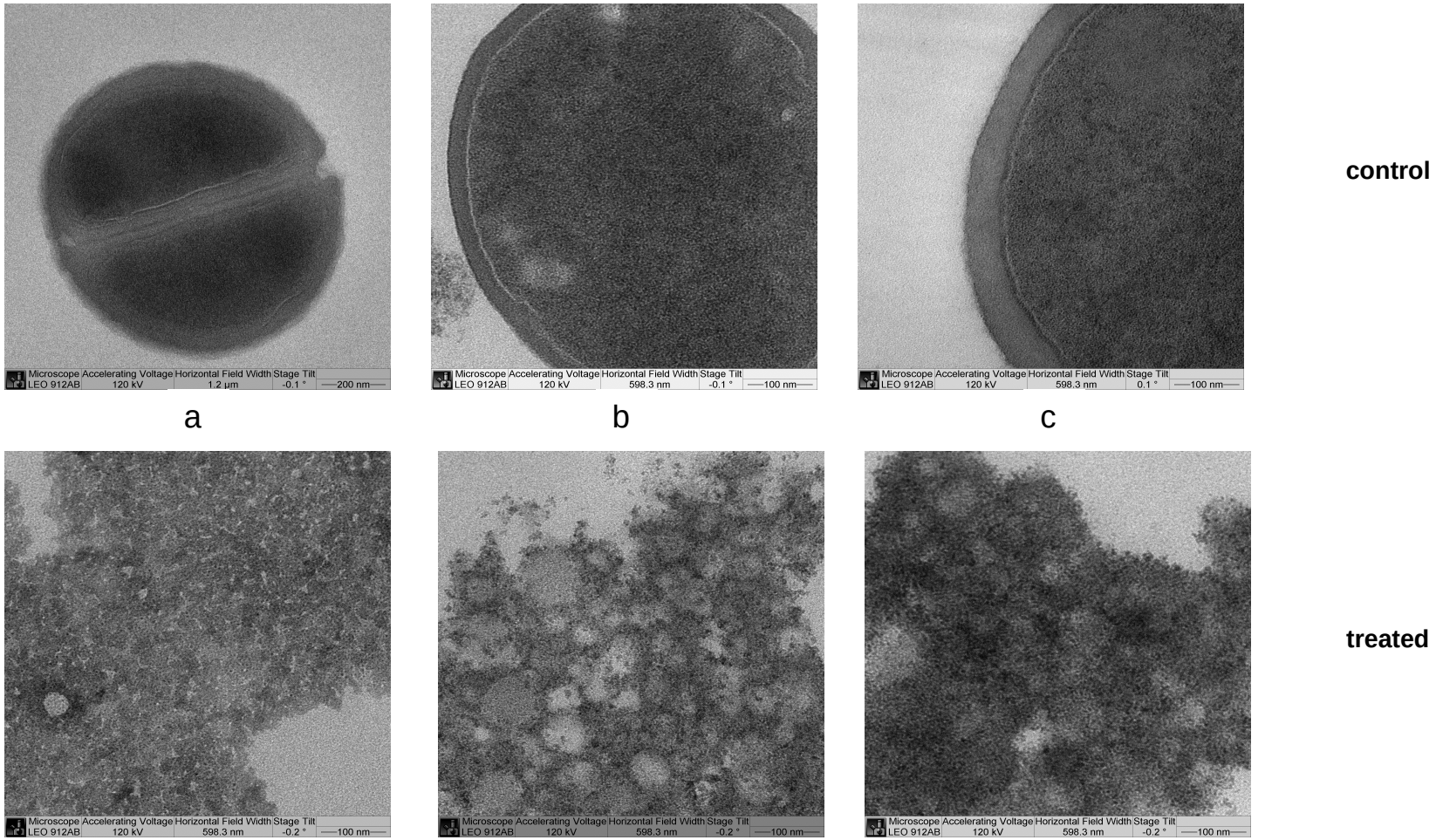


Figure 5.3.6 Transmission electron microscopy images of *S. aureus* ATCC 29213 control (**a** = 1 h (x 20,000), **b** = 24 h incubation (x 40,000) and **c** = treatment with DMSO 10% at 1 h incubation and treated *S. aureus* ATCC 29213 with (**d** = *A. occidentale* oil, **e** = red variety extract and **f** = yellow variety extract) at 1 h incubation (x 40,000).

5.3.2 PART 2: THE EFFECTS *ANACARDIUM OCCIDENTALE* ESSENTIAL OIL AGAINST EMRSA BIOFILMS

In this section, biofilm formation and development in EMRSA was observed using a 96 well microtitre plate model. The effect of plant extracts and essential oils were measured by microscopic observations and a colorimetric method based on the use of a modified tetrazolium salt (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide, XTT) to monitor the metabolic activity of cells within the biofilm.

5.3.2.1 Optimisation of XTT assay

5.3.2.1.1 Determination of optimal menadione concentration in planktonic cells

The effect of different concentrations of menadione on the XTT-absorbance at 490 nm in all 4 EMRSA strains – 66, 84, 100 and 547 are shown in *Figure 5.3.7*. There is a marked increase in XTT-absorbance after the addition of 50 µM of menadione compared to the control in all strains tested. Higher concentrations (250 µM and 500 µM) also resulted in higher XTT-absorbance but less than 50 µM. The XTT-absorbance decreased significantly ($p<0.05$) with the addition of a higher concentration of menadione. Acetone which was used to dissolve the menadione was used as positive control and showed no effect on the XTT-absorbance of the assay.

5.3.2.1.2 Determination of optimal XTT-menadione incubation time in planktonic cells

Experiments were performed similarly to those described in section *5.3.2.1.1* using the optimised menadione concentration, but on this occasion the incubation time for XTT was varied. Incubation for 3 h showed the highest XTT-absorbance. Incubation times longer than 3 h (6, 12, 18 and 24 h) resulted in the spectrophotometer giving a reading error as it was beyond the absorbance spectra of the instrument. Therefore, throughout the experiments, 3 h incubation time was chosen to be the optimal incubation time to assess the effect of plant extracts and essential oils against EMRSA biofilms. The results are summarised in *Figure 5.3.8.*

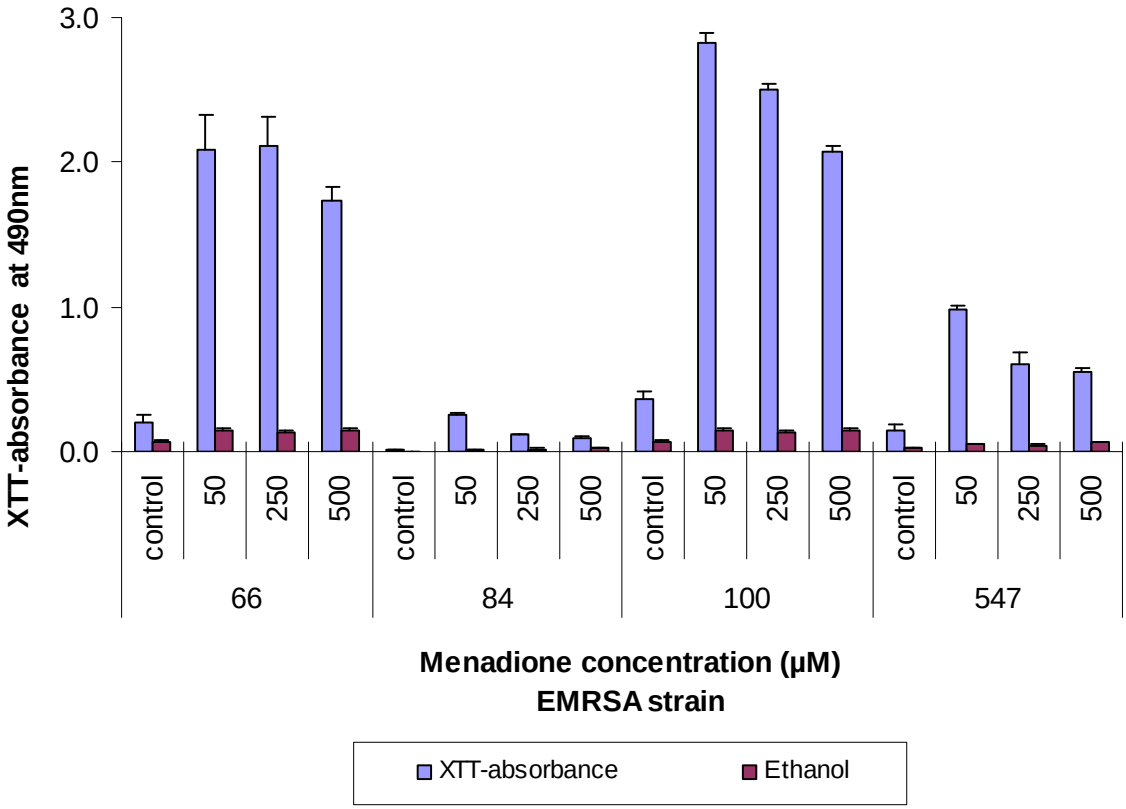


Figure 5.3.7 Colourimetric readings of XTT-absorbance at 490 nm of different menadione concentrations incubated for 3 h with 4 types of EMRSA strains. Control = without menadione. Values represent the mean ± standard deviation, SD of absorbance (n = 8).

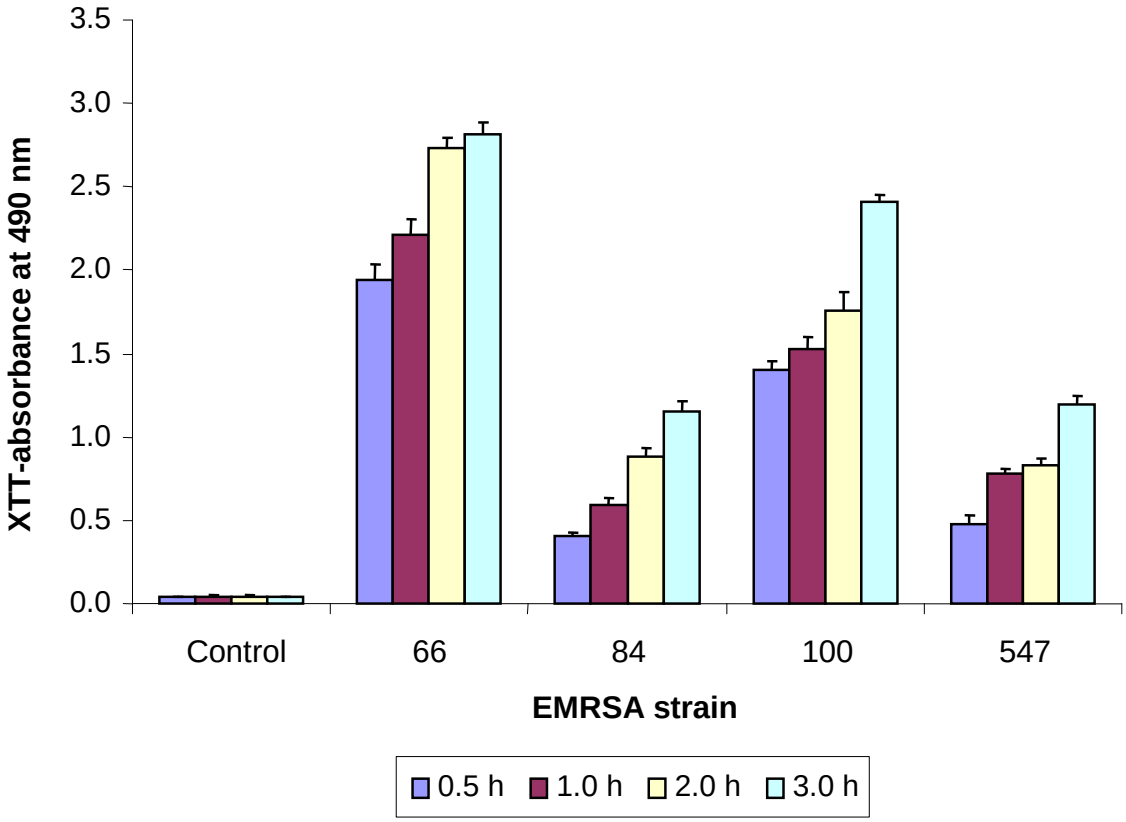


Figure 5.3.8 Colourimetric readings of XTT-absorbance at 490 nm of different planktonic EMRSA strains at different incubation time at 37℃ with 50 µM menadione. Control = without menadione. Values represent the mean ± standard deviation, SD of absorbance (n = 8)

5.3.2.1.3 The effect of planktonic cell density on the XTT-absorbance

Different cell densities of planktonic cells of all strains were prepared and incubated with different concentration of menadione (50, 250 and 500 µM) for 3 h. The results of the effects of different cell densities of strain 100 on the log percentage of XTT-absorbance compared to the control are shown in *Figure 5.3.9*. It was shown that, higher cell densities at 6.4 x 10^9 per ml resulted in significantly ($p<0.05$) higher percentage of XTT-absorbance at 490 nm compared to the control.

5.3.2.2 The effects of essential oils of *A. occidentale* and *M. alternifolia* on the viability of EMRSA biofilms

The effects of *A. occidentale* and *M. alternifolia* oils on the percentage of XTT-absorbance are shown in *Figure 5.3.10.* The essential oil of *A. occidentale* produced significant effects on strain 66, 100 and 547 biofilms. The XTT-absorbance percentages were reduced significantly ($p<0.05$) compared to the control. γ-Terpinene, which is present in both oils, significantly ($p<0.05$) reduced the XTT-absorbance in strains 66, 84 and 100. However, only γ-terpinene affected the metabolic activity of strain 84 compared to cashew-nut and tea tree oils. In strain 547, only *A. occidentale* oil was shown to have an effect.

5.3.2.3 The effects of essential oils of *A. occidentale* and *M. alternifolia* on the biomass of EMRSA biofilms

The effects of essential oils on the biomass of four EMRSA strains of biofilms are shown in *Figure 5.3.11*. *A. occidentale* oil showed a significant increase ($p<0.05$) of the biomass in EMRSA 66, 84 and 547 but decreased significantly ($p<0.05$) the biomass in strain EMRSA 100. Tea tree oil also reduced significantly the biomass in EMRSA 100 but not in other strains. γ-Terpinene was shown to reduce the biomass significantly ($p<0.05$) in EMRSA 66 and 100 but increased the biomass in strains 84 and 547.

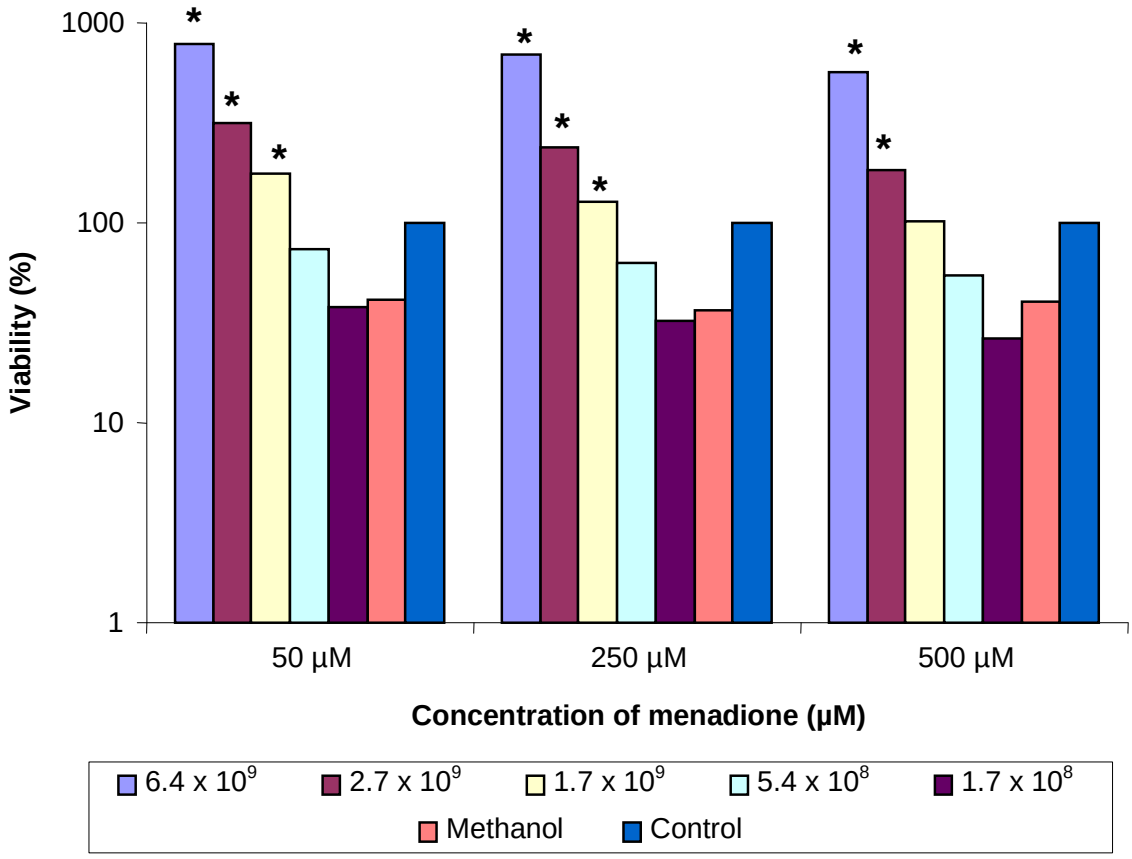


Figure 5.3.9 Percentage of viability of planktonic EMRSA 100 cells at different cell density (cfu/ml) incubated with different concentration of menadione for 3 h. Control = EMRSA cells without any treatments. (n=8) *($p<0.05$) – statistically significant analysed by two-way ANOVA.

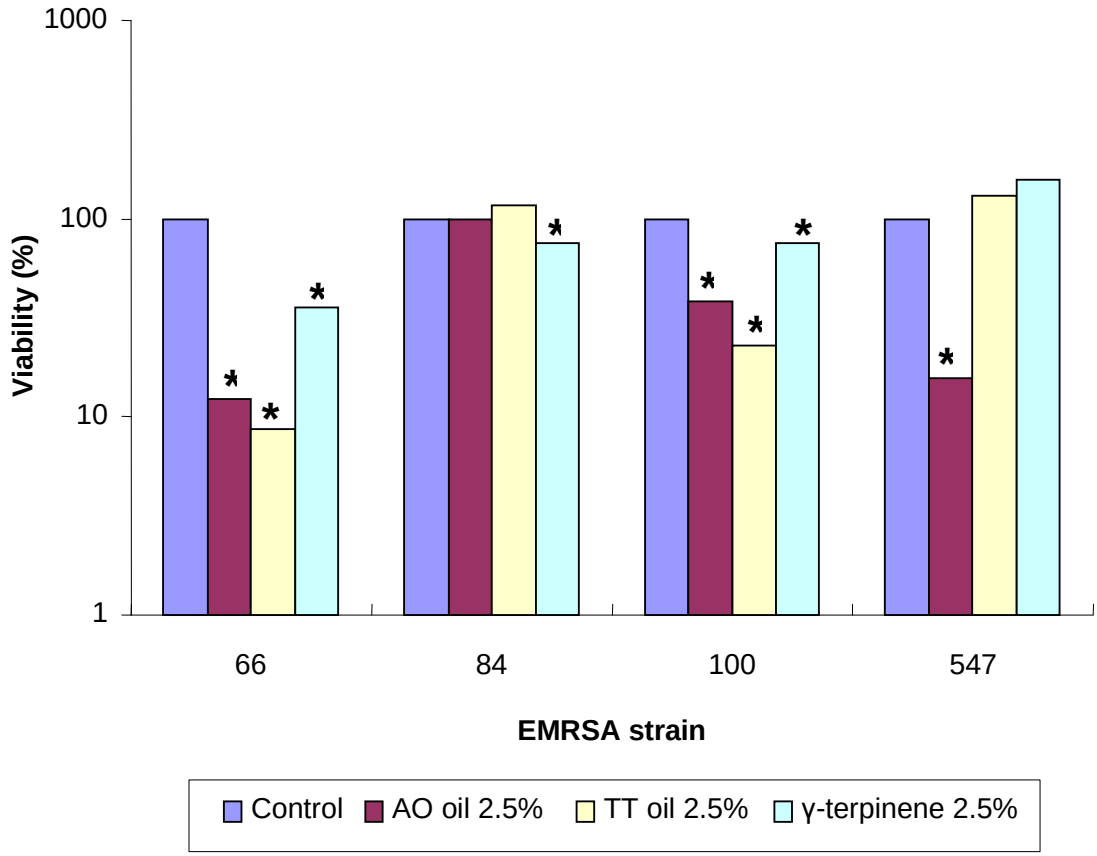


Figure 5.3.10 Percentage of viability of biofilms treated with *A. occidentale* (AO) oil, tea tree (TT) oil and γ-terpinene to that of control (100%). Values represent the mean percentage of absorbance of multiple independent biofilms formed on the pegs of each strain. (* = significant different, $p<0.05$ analysed by two=way ANOVA). Control = EMRSA biofilms without any treatments.

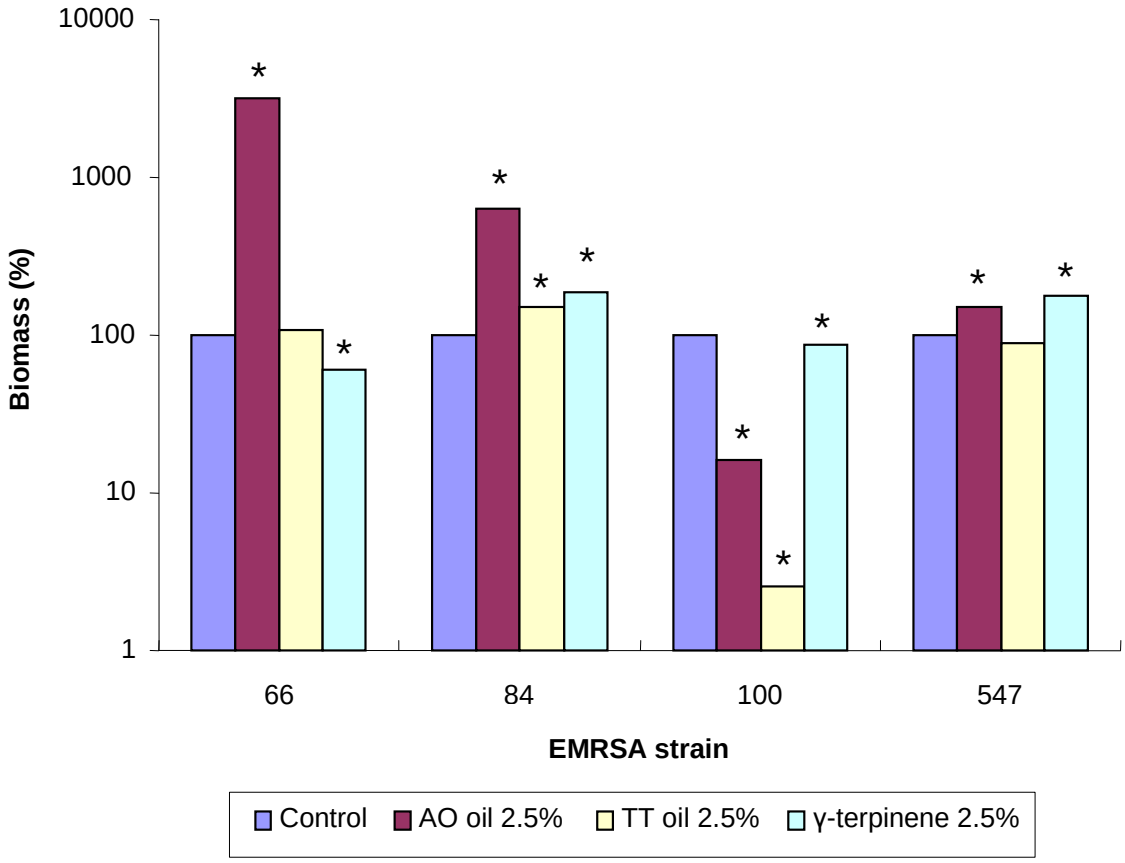


Figure 5.3.11 Percentage of biomass of the EMRSA biofilms treated with *A. occidentale* (AO) oil, tea tree (TT) oil and γ-terpinene to that of control (100%) Values represent the mean percentage of absorbance of multiple independent biofilms formed on the pegs of each strain. (* = significant different, $p<0.05$ analysed by two-way ANOVA). Control = EMRSA biofilms without any treatments.

5.3.2.4 Physical valuation of the effect of the essential oils on EMRSA biofilms by SEM

The SEM images of the effects of overnight incubation of EMRSA biofilm with *A. occidentale* and tea tree oils are shown in *Figure 5.3.12* and *Figure 5.3.13*. The structures of the biofilms were varied based on the different strains tested. The control EMRSA was noted to be spherically shaped, condensed and attached to each other. After treatment with *A. occidentale* and tea tree oils, in EMRSA 66, more extrapolymeric substances were produced. The cells were not attached to each other when compared to the control, where there were more spaces between the cells due to extrapolymeric substances produced. This can also be seen in EMRSA 84 and 100. However, the effects of the oils were more apparent on EMRSA 100 biofilms. The structures of the cells were changed and the colonies were eradicated from the surface of the pegs.