

**CHEMICAL CONSTITUENTS AND FREE RADICAL  
SCAVENGING ACTIVITY OF EXTRACTS FROM *Ocimum  
basilicum* AND *Ocimum sanctum* GROWN IN MALAYSIA**

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**FACULTY OF SCIENCE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

**2017**

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MALAYSIA**

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**THESIS SUBMITTED IN FULFILMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF MASTER OF  
SCIENCE**

**FACULTY OF SCIENCE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

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**UNIVERSITY OF MALAYA**  
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## ABSTRACT

*Ocimum basilicum* (Thai basil) and *Ocimum sanctum* (Holy basil) both possess strong aromatic annual herb that is used in Asian cuisines and traditional medicine. The main constituents of *Ocimum basilicum* were identified to consist of estragole (> 35.71%), (*E*)- $\beta$ -ocimene (> 1.47%), *trans*- $\alpha$ -bergamotene (> 0.83%),  $\tau$ -cadinol (> 0.41%) eucalyptol (> 0.25%) and  $\alpha$ -caryophyllene (> 0.07%) while *Ocimum sanctum* were mainly of eugenol methyl ether (> 34.34%), (*E*)-caryophyllene (> 7.91%), germacrene D (> 5.58%),  $\beta$ -elemene (> 4.22%) and copaene (> 1.49%). The chemical compounds were extracted by hydrodistillation, headspace-solid phase microextraction (HS-SPME) and solvent extraction. The free radical scavenging activity of *Ocimum basilicum* leaves showed IC<sub>50</sub> values of 88  $\mu$ g/mL and 1178  $\mu$ g/mL for the methanol and dichloromethane extracts, respectively, while the methanol and dichloromethane extract of *Ocimum sanctum* showed a higher DPPH free radical scavenging activity with an IC<sub>50</sub> value of 12  $\mu$ g/mL and 369  $\mu$ g/mL, respectively. Interspecies differences between the two species were determined by calculating Nei's genetic distances with the value of 2.86. The calculated genetic distance between the two species showed that *Ocimum basilicum* and *Ocimum sanctum* are closely related species which share some of the same traits. Our results showed that *Ocimum basilicum* and *Ocimum sanctum* are promising to be used as natural antioxidants for food industry.

## ABSTRAK

*Ocimum basilicum* (Thai basil) dan *Ocimum sanctum* (Holy basil) merupakan herba yang mempunyai aroma yang kuat serta digunakan di dalam masakan Asia dan perubatan tradisional. Jujuk utama *Ocimum basilicum* yang dikenalpasti terdiri daripada estragole (> 35.71%), (*E*)- $\beta$ -ocimene (> 1.47%), *trans*- $\alpha$ -bergamotene (> 0.83%),  $\tau$ -cadinol (> 0.41%) eucalyptol (> 0.25%) dan  $\alpha$ -caryophyllene (> 0.07%) manakala *Ocimum sanctum* terdiri daripada eugenol metil eter (> 34.34%), (*E*)-caryophyllene (> 7.91%), germacrene D (> 5.58%),  $\beta$ -elemene (> 4.22%) dan copaene (> 1.49%). Komponen-komponen kimia tersebut telah diekstrak menggunakan beberapa teknik iaitu hidrodistilasi, headspace-fasa pepejal mikroekstraksi (HS-SPME) dan pengekstrakan pelarut. Aktiviti memerangkap radikal bebas oleh *Ocimum basilicum* masing-masing menunjukkan nilai IC<sub>50</sub> iaitu 88  $\mu$ g/mL dan 1178  $\mu$ g/mL untuk ekstrak metanol dan ekstrak diklorometana manakala ekstrak methanol dan ekstrak diklorometana *Ocimum sanctum* masing-masing menunjukkan aktiviti memerangkap radikal bebas DPPH yang lebih tinggi dengan nilai IC<sub>50</sub> iaitu 12  $\mu$ g/mL dan 369  $\mu$ g/mL. Perbezaan interspesis diantara kedua-dua spesis ditentukan dengan mengira jarak genetik Nei dengan nilai 2.86. Jarak genetik yang telah dikira menunjukkan kedua-dua spesis berkait-rapat dan berkongsi beberapa sifat yang sama. Hasil kajian kami menemui bahawa *Ocimum basilicum* and *Ocimum sanctum* berpotensi untuk dijadikan antioksidan semulajadi di dalam industri makanan.

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## LIST OF SYMBOLS AND ABBREVIATIONS

$\alpha$	:	alpha
$\beta$	:	beta
$\gamma$	:	gamma
$\delta$	:	delta
$\varepsilon$	:	epsilon
$\tau$	:	tau
3D	:	three dimensional
%	:	percentage
(v/w)	:	(volume/weight)
(w/w)	:	(weight/weight)
°C	:	degree Celsius
$\mu\text{g/mL}$	:	microgram/milliliter
$\mu\text{L}$	:	microliter
$\mu\text{m}$	:	micrometer
$^1\text{H-NMR}$	:	hydrogen-nuclear magnetic resonance
ASE	:	accelerated solvent extraction
BH	:	butylated hydroxyanisole
BHT	:	butylated hydroxytoluene
cm	:	centimeter
cm/s	:	centimeter/second
CUPRAC	:	cupric reducing antioxidant power
DCIS	:	ductal carcinoma <i>in situ</i>
DNA	:	deoxyribonucleic acid

DPPH	:	2,2-diphenyl-1-picrylhydrazyl
EP	:	European Pharmacopeia
eV	:	electron volt
FDA	:	Food and Drug Administration
FRAP	:	ferric reducing antioxidant power
g	:	gram
GC	:	gas chromatography
GC-MS	:	gas chromatography-mass spectroscopy
HFS	:	human fibrosarcoma
h	:	hour
HPLC	:	high performance liquid chromatography
HS-SPME	:	headspace- solid phase microextraction
IC <sub>50</sub>	:	50% inhibitory concentration
kPa	:	kilopascal
L	:	liter
LC-MS	:	liquid chromatography-mass spectroscopy
LC-NMR	:	liquid chromatography-nuclear magnetic resonance
LDLs	:	low-density lipoproteins
m	:	meter
min	:	minute
mL	:	milliliter
mL/min	:	milliliter/minute
mm	:	millimeter
mM	:	millimolar
MT-4	:	metallothionein 4
NIST	:	National Institute of Standards and Technology

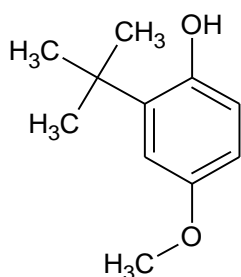
nm	:	nanometer
NMR	:	nuclear magnetic resonance
ORAC	:	oxygen radical absorbance capacity
RI	:	retention index
rpm	:	revolutions per minute
s	:	second
SFC	:	supercritical fluid chromatography
SPE	:	solid phase extraction
SPME	:	solid-phase microextraction
TBHQ	:	<i>tert</i> -butylhydroquinone
TEAC	:	trolox equivalent antioxidant capacity
TLC	:	thin layer chromatography
TRAP	:	total radical trapping antioxidant potential
USA	:	United States of America
USDA	:	U. S. Department of Agriculture
WR-2721	:	S-2-(3-aminopropylamino) ethylphosphorothioic acid



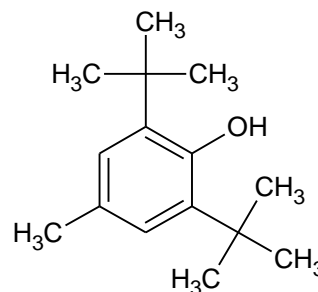
## CHAPTER 1: INTRODUCTION

### 1.1 Food Preservation

Food preservation is a process in keeping the food desired qualities for a longer period of time. It is important to preserve food in order to overcome inappropriate planning in agriculture, produce value-added products, and provide different choices in diet (Rahman, 1999). The desired level of quality, the preservation length and the targeted group the products are preserved for are the important keys in preserving food. The main reasons of food deterioration are mechanical, physical, chemical, and microbial effect (Rahman, 1999). The main food preservation techniques include inhibiting chemical deterioration and microbial growth, directly inactivating bacteria, yeasts, molds, or enzymes, avoiding re-contamination before and after processing (Gould, 1989; Gould, 1995). Additives are added in food as antimicrobial, antioxidants and pH controller. Synthetic antioxidants namely, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butylhydroquinone (TBHQ) and propyl gallate are added to decelerate oxidation reaction in food (Shahidi, 2000). Chemical structures of the synthetic antioxidants are illustrated in Figure 1.1 and Figure 1.2.

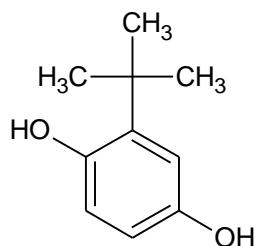


Butylated hydroxyanisole (BHA)

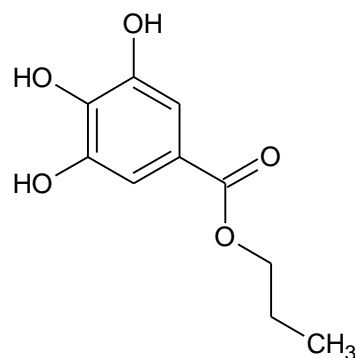


Butylated hydroxytoluene (BHT)

**Figure 1.1:** The chemical structure of BHA and BHT



*tert*-butylhydroquinone (TBHQ)



Propyl gallate

**Figure 1.2:** The chemical structure of TBHQ and propyl gallate

In reference to Andre' *et al.* (2010), BHA was found in food group such as meat, meat products, milk and milk products, cheese and cheese products, doughnuts, biscuits, cakes, fat and fat products, oil and oil products. BHT was added in meat, meat products, fish, fish products, cheese, cheese products, bakery products, fat, oil, fat and oil products, chewing gums and plastic products. TBHQ is contained in food such as meat, meat products, fish, fish products, cheese, milk, cheese and milk products, fat, oil, fat and oil products, shortening, fishery products, chewing gum, mayonnaise and breakfast cereal while propyl gallate was found in meat, meat products, fish, fish products, fat, oil, fat and oil products, cheese, milk, milk and cheese products, bakery goods and chewing gums.

Despite their effectiveness in delaying antioxidant activity, their use might be a contributing factor to cancer (Namiki, 1990). A study by Imaida *et al.* (1983) reported that BHT is promoters of the urinary bladder carcinogenesis initiated by N-butyl-N-(4-hydroxybutyl)nitrosamine in male Fisher 344 rats. In addition, it was reported that BHA is carcinogenic in the fore stomach of Fisher 344 rats (Ito *et al.*, 1983).

Consumption of antioxidant-rich food capable in preventing oxidative stress related diseases (Ames *et al.*, 1995; Kaur & Kapoor, 2001). Natural antioxidants are believed to be one of the dietary factors that may play an important role in preventing cancer. Green tea is well-known antioxidant and it has been speculated that it is due to consumption of green tea in Japan, their lung cancer rates are low despite having high smoking rates (Glensler, 1996). In addition, it was found that *Pleurotus pulmonarius* has high antioxidant activity, showed significant effects in the prophylaxis of liver cancer in mice and able to sensitize tumours to chemotherapeutic drugs (Xu *et al.*, 2014). Other than that, it was found that fresh apples have high antioxidant activity and the extract suppressed the growth of colon and liver cancer cells *in vitro* in a dose-dependent manner (Eberhardt, 2000).

## **1.2 The family Lamiaceae**

Lamiaceae or Labiatae consist of the mint family of flowering plants. The Lamiaceae contains about 236 genera and 6,900 to 7,200 species. This family has common characteristics of square stem, opposite and decussate leaves with many gland dots while the flowers are zygomorphic with two lips (Paton *et al.*, 1999). The aromatic plants are widely used as culinary herbs, such as basil, mint, rosemary, sage, savory, marjoram, oregano, thyme, lavender and perilla. These families of Lamiaceae are shrubs, trees, but rarely vines and most of the members are easy to be cultivated. By stem cuttings, these plants can be propagated (Raja, 2012).

### 1.2.1 The genus *Ocimum*

*Ocimum* L. (Lamiaceae) are native to the tropical and subtropical regions of Asia, Africa and Central South America (Labra *et al.*, 2004). It is estimated 30 (Paton, 1992) to 160 (Pushpangadan & Bradu, 1995) number of species in this genus, but the exact number is unknown due to taxonomic reason. In reference to Paton *et al.* (1999), *Ocimum* can be annual herbs, possessing only a tap root or suffrutices with a large or small perennating rhizome. They are sometimes shrub with woody stems or softer subshrubs. The members in genus *Ocimum* are strongly aromatic or very weakly scented. Among the common species grown are *Ocimum basilicum*, *Ocimum americanum*, *Ocimum gratissimum*, *Ocimum sanctum* and *Ocimum kilimandscharicum*. These species are being grown as they are useful as medicinal plants, culinary herbs and insect-controlling agents (Grayer *et al.*, 1996).

In the modern days, the cancer remained the main concerned and extensive research is being carried out in order to find prevention or cure for this disease. It is scientifically proven that the members of the *Ocimum* have anticancer property. It was reported that the essential oil from the leaves of *Ocimum kilimandscharicum* was investigated for anticancer, anti-inflammatory and antioxidant activity. The essential oil was tested in an anticancer assay against ten human cancer cell lines. The results showed that *in vitro* cytotoxicity screening *Ocimum kilimandscharicum* against the human ovarian cancer cell line displayed high selectivity and potent anticancer activity (Lima *et al.*, 2014).

Other than that, a study of the effects of *Ocimum gratissimum* leaves extract on human breast cancer utilizing *in vitro* and *in vivo* methodologies had been carried out by Nangia-Makker (2007). Tumour progression and angiogenesis related processes like chemotaxis, proliferation, apoptosis, three dimensional (3D) growth and morphogenesis, angiogenesis and tumour growth were studied in the presence or absence of the leaves *Ocimum gratissimum* extract and in some experiments, a comparison was made with eugenol, apigenin and ursolic acid standards. *Ocimum gratissimum* leaves extract inhibits proliferation, migration, anchorage independent growth, 3D growth and morphogenesis and induction of cyclooxygenase 2 protein in breast cancer cells. Furthermore, *Ocimum gratissimum* leaves extract reduced tumour size and neoangiogenesis in a MCF10 DCIS.com xenograft model of human ductal carcinoma *in situ* (DCIS).

### 1.2.1.1 *Ocimum basilicum*



**Figure 1.3:** *Ocimum basilicum*

Kingdom	: Plantae
Subkingdom	: Viridiplantae
Infrakingdom	: Streptophyta
Superdivision	: Embryophyta
Division	: Tracheophyta
Subdivision	: Spermatophytina
Class	: Magnoliopsida
Superorder	: Asteranae
Order	: Lamiales
Family	: Lamiaceae
Genus	: <i>Ocimum</i> L.
Species	: <i>Ocimum basilicum</i> L.

Source: Integrated Taxonomic Information System on-line database

*Ocimum basilicum* L. or commonly known as Thai basil has intense aroma that resembles cloves (Makri & Kintzios, 2008). In reference to Jaganath & Ng (2000), this species can grow up to 75 cm to 100 cm in height with numerous branch. The leaves are oval shaped, dotted with tiny oil cells, which gives its aromatic characteristic and arranged in opposite decussate pairs. The stems are erect and have four angles. The flowers are roughly 10 cm in length and produced at the end of vertices. In Malaysia, *Ocimum basilicum* are cultivated and could not find in the wild.

*Ocimum basilicum* is often being used in confectionery, baked goods, seasonings, spiced meats and sausages, oral care products and fragrance (Guenther & Althausen 1952). In Asian cuisines, the leaves are used in flavouring curries, noodles, salads and chickens. In addition, this herb is also a renowned traditional medicine. It is a remedy for treating a bowel complication in children, ringworm infection, weak indigestion and cough, irregular menstrual cycle, as nasal douche, high fever, headache, treating kidney troubles and diarrhoea. Other than that, it is being used as insecticide and African uses it for hair care (Jaganath & Ng, 2000).

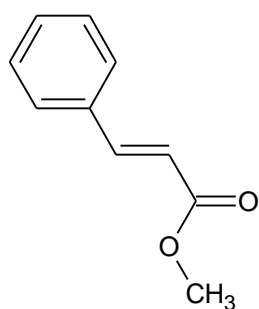
*Ocimum basilicum* provides benefits to health as the study showed that it has the ability to protect the myocardium against isoproterenol-induced infarction in rats (Fathiazad *et al.*, 2012) and it was reported that *Ocimum basilicum* had reduced systolic and diastolic blood pressure and cardiac hypertrophy in renovascular hypertensive rats (Umar *et al.*, 2010).

Besides that, *Ocimum basilicum* possesses anti-inflammatory and anti-ulcer activity. *Ocimum basilicum* was reported to possess significant anti-ulcer activity against aspirin, indomethacin, alcohol, histamine, reserpine, serotonin and stress-induced ulceration in experimental animal models (Singh, 1999). It was also reported that *Ocimum basilicum* was one of the Labiatae (Lamiaceae) members that showed significant inhibitory effects against human immunodeficiency virus-1 (HIV-1) induced cytopathogenicity in metallothionein 4 (MT-4) cells (Yamasaki *et al.*, 1998). *Ocimum basilicum* also was reported to have potent cytotoxicity against the human cervical cancer cell line, human laryngeal epithelial carcinoma cell line and mouse embryonic fibroblasts (Kathirvel & Ravi, 2012).

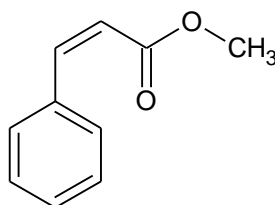
The chemical composition of *Ocimum basilicum* varies from each origin. Much research has shown that this species is rich in terpenes. It was reported that hydrodistillation of three samples of French Polynesia *Ocimum basilicum* yielded an essential oil that consisted mainly of (*E*)-methyl cinnamate (43.4-62.3%) and (*Z*)-methyl cinnamate (8.1-8.6%) (Adam *et al.*, 2009). It was also found that the composition of the essential oil of the aerial parts of *Ocimum basilicum* native to northeast India that was analysed by Gas Chromatography (GC) and Gas Chromatography-Mass Spectroscopy (GC-MS) consisted of camphor (42.1%), followed by limonene (7.6%) and  $\beta$ -selinene (5.6%) as the major components of the oil (Purkayastha & Nath, 2006). The chemical composition of the essential oil of aerial parts of Iranian *Ocimum basilicum* L. (green type) that was analysed by GC and GC-MS comprised of methyl chavicol (62.5%), geranial (12.5%) and neral (9.9%) as the main components while the main constituents of *Ocimum basilicum* essential oil from Serbia that was analysed by GC-MS were methyl



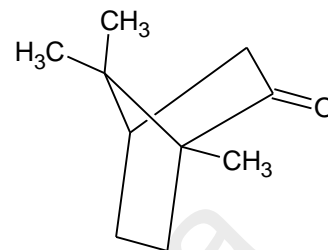
chavicol (45.8%) and linalool (24.2%) (Bozin *et al.*, 2006). Figure 1.4 shows the chemical structures of some of the main compounds from *Ocimum basilicum*.



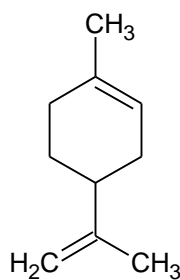
i. (*E*)-methyl cinnamate



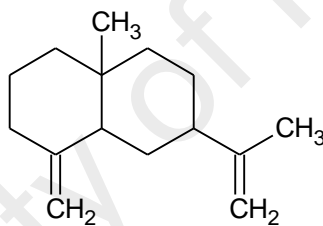
ii. (*Z*)-methyl cinnamate



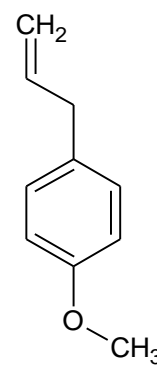
iii. Camphor



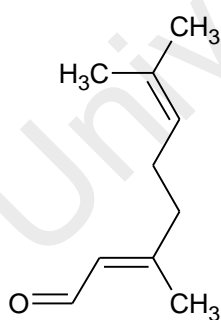
iv. Limonene



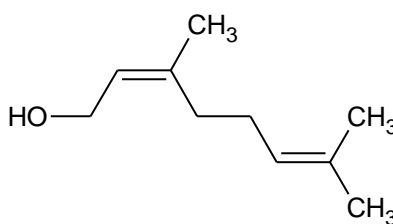
v.  $\beta$ -selinene



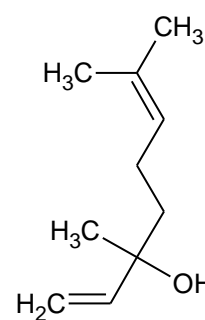
vi. Estragole



vii. Geranial



viii. Nerol



ix. Linalool

**Figure 1.4:** Some of the chemical structure of the main compounds from *Ocimum basilicum* from different origin.

### 1.2.1.2 *Ocimum sanctum*



**Figure 1.5:** *Ocimum sanctum*

Kingdom	: Plantae
Subkingdom	: Viridiplantae
Infrakingdom	: Streptophyta
Superdivision	: Embryophyta
Division	: Tracheophyta
Subdivision	: Spermatophytina
Class	: Magnoliopsida
Superorder	: Asteranae
Order	: Lamiales
Family	: Lamiaceae
Genus	: <i>Ocimum</i> L.
Species	: <i>Ocimum tenuiflorum</i> L. ( <i>Ocimum sanctum</i> L.)

Source: Integrated Taxonomic Information System on-line database.

*Ocimum sanctum* L. (Holy basil) has intensive and somewhat pungent smell (Makri & Kintzios, 2008). *Ocimum sanctum* is being cultivated in the garden of Hindu homes and temples as a sacred plant and known as “The Incomparable One”, “Mother Medicine of Nature” and “The Queen of Herbs” in Ayurveda (Cohen, 2014). In reference to Jaganath & Ng (2000), it is branched shrub with quadrangle hairy erect branches. The two typical types of *Ocimum sanctum* are a purple type of *Ocimum sanctum* and a green type of *Ocimum sanctum*. The leaves of purple type of *Ocimum sanctum* are dark green while the leaves of green type of *Ocimum sanctum* are green. For both types, the flowers are produced at the end of vertices and grow up to about 7 cm in length. Unlike *Ocimum basilicum*, this species grow wildly along roadsides and in wastelands. Being a sacred plant to the Hindus, *Ocimum sanctum* is commonly being cultivated in garden of Indian homes.

*Ocimum sanctum* is a very important plant, especially in traditional medicine. *Ocimum sanctum* is a remedy for gonorrhoea, malaria, cough, anorexia, chronic dyspepsia, flatulence, colic, bronchitis, gastric, fever, rheumatism, disorder of urinal-genital system, stings of bees, bite of mosquitoes and leeches, stimulate appetite, improve digestion, improve the milk secretion, hepatic affections and kill intestinal worms (Jaganath & Ng, 2000).

This species provides a vast array of health benefits such as prevention of mental stress, anti-malarial against *Plasmodium vivax* and *Plasmodium falciparum* and antibacterial against *E. coli*, *B. anthracis* and *P. aeruginosa in-vitro* (Mohan *et al.*, 2011). In addition, *Ocimum sanctum* showed a significant anti-inflammatory activity against carrageenan

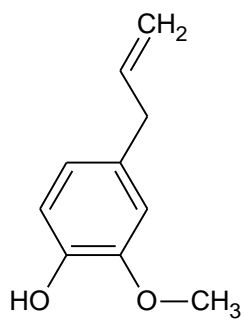
and different other mediator-induced paw edema in rats (Singh *et al.*, 1996). It was reported that *Ocimum sanctum* has enhanced radioprotection of bone marrow and reduced the toxicity of S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR-2721). The radioprotective effect of the leaf extract in combination with WR-2721 was investigated on mouse bone marrow. The pre-treatment with the leaf extract or WR-2721 individually resulted in a significant decrease in aberrant cells as well as different types of aberrations while the combination of the two further decreases the aberrant cells by resulting in a twofold increase in the protection factor (Ganasoundari *et al.*, 1998).

A study was conducted using the seed oil of *Ocimum sanctum* to evaluate the chemopreventive activity of the plant against subcutaneously injected 20-methylcholanthrene induced-fibrosarcoma tumours in the thigh region of Swiss albino mice. The oil has significantly reduced 20-methylcholanthrene induced tumour incidence and tumour volume, hence, increased the survival rate and delay in tumour incidence was observed in the seed oil supplemented mice (Prakash & Gupta, 2000).

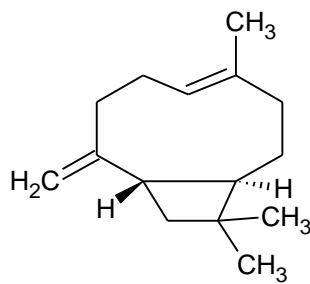
It was also reported that the ethanolic leaf extract of *Ocimum sanctum* has chemopreventive property against 7,12-dimethylbenz[a]anthracene induced skin papillomagenesis in male Swiss albino mice. There was a significant reduction in the values of tumour incidence, average number of tumours per tumour bearing mice and the cumulative number of papillomas observed in mice treated topically with the leaf extract of *Ocimum sanctum* at either the pre-initiation, post-initiation stages or continuously at pre- and post-initiation stages of papillomagenesis as compared to their control group (Prashar *et al.*, 1994).

A study has evaluated *Ocimum sanctum* against human fibrosarcoma (HFS) cells in culture. The ethanolic extract of *Ocimum sanctum* has induced cytotoxicity of the HFS cells. The cells showed shrunken cytoplasm and condensed nuclei. The deoxyribonucleic acid (DNA) was found to be fragmented on observation in agarose gel electrophoresis. The extract-treated HFS cells showed depleted intracellular glutathione, and increased levels of lipid peroxidation products. Administration of extracts of *Ocimum sanctum* to mice bearing Sarcoma-180 solid tumours showed a significant reduction in tumour volume and an increase in the mice lifespan (Kartikheyen *et al.*, 2008).

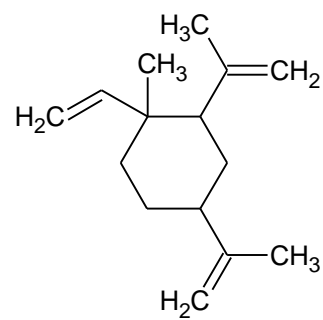
*Ocimum sanctum* consisted of terpenes such as monoterpene and sesquiterpene hydrocarbons. It was reported that the essential oil of the leaves from *Ocimum sanctum* L. from India comprised of eugenol (53.4%), *p*-caryophyllene (31.7%) and *p*-elemene (6.2%) as the major components after being analysed using GC and GC-MS (Raju *et al.*, 1999). In other research, the composition of the essential oils of the air-dried aerial parts of *Ocimum sanctum* L. from Poland that was harvested in four stages of vegetation, were isolated by hydrodistillation and analysed by capillary GC, GC-MS, and <sup>1</sup>H-NMR. Among the 57 identified compounds, the main constituents are found to be  $\beta$ -bisabolene (13–20%) 1,8-cineole (9–33%) and methyl chavicol (2–12%) (Kicel *et al.*, 2005). Other than that, it was reported that the volatile oil of the air-dried aerial parts of *Ocimum sanctum* from Cuba that was analysed by GC and GC-MS yielded forty compounds with their major constituents being represented by eugenol (34.3%),  $\beta$ -elemene (18.0%) and  $\beta$ -caryophyllene (23.1%) (Pino *et al.*, 1998). Figure 1.6 illustrates some of the chemical structures of the major constituents identified from *Ocimum sanctum* from different origin.



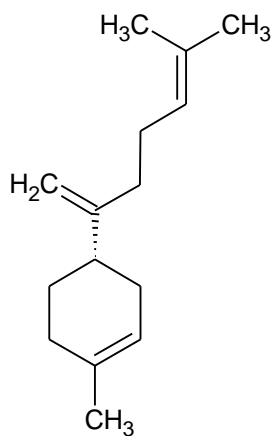
i. Eugenol



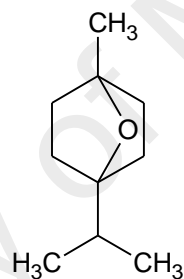
ii.  $\beta$ -caryophyllene



iii.  $\beta$ -elemene



iv.  $\beta$ -bisabolene



v. Eucalyptol

Figure 1.4 (vi)

Estragole

**Figure 1.6:** Some of the chemical structures of the major constituents identified from *Ocimum sanctum* from different origin.

### 1.3 Extraction Techniques of Aromatic Plants

Before the chemical compounds can be identified, the sample has to be prepared and the extraction techniques which are commonly applied in fragrances and aroma analysis are listed as below:

1. Plant tissue homogenization
2. Serial exhaustive extraction
3. Soxhlet extraction
4. Maceration
5. Decoction
6. Infusion
7. Digestion
8. Percolation
9. Sonication
10. Steam distillation
11. Hydrodistillation
12. Solid-phase microextraction (SPME)
13. Supercritical fluid extraction
14. Aqueous alcoholic extraction by fermentation
15. Solid phase extraction
16. Counter-current extraction

The advantages and disadvantages of some of the extraction techniques were shown in Table 1.1.

**Table 1.1:** The advantages and disadvantages of some of the extraction techniques. (Xie *et al.*, 2013; Tiwari *et al.*, 2011; Handa, 2008; Azwanida, 2015; Tandon & Rane 2008)

Extraction technique	Advantages	Disadvantages
Serial exhaustive extraction	<ul style="list-style-type: none"> <li>✓ Successive extraction with solvents of increasing polarity from a non-polar to a more polar solvent to ensure that a wide polarity range of compound could be extracted.</li> </ul>	<ul style="list-style-type: none"> <li>✓ Cannot be used for thermolabile compounds as prolonged heating may lead to degradation of compounds.</li> </ul>
Soxhlet extraction	<ul style="list-style-type: none"> <li>✓ Applicable on compounds that have limited solubility in solvent.</li> <li>✓ One batch of solvent is recycled instead of many portions of warm solvent being passed through the sample.</li> <li>✓ Requires a smaller quantity of solvent compared to maceration.</li> </ul>	<ul style="list-style-type: none"> <li>✓ Unsuitable for thermolabile compounds as prolonged heating may lead to degradation of compounds.</li> <li>✓ Exposure to hazardous and flammable liquid organic solvents, with potential toxic emissions during extraction.</li> <li>✓ Solvents need to be of high-purity.</li> <li>✓ May contribute to pollution problem.</li> <li>✓ Limited to a dry and finely divided solid sample.</li> <li>✓ Temperature, solvent-sample ratio and agitation speed need to be considered.</li> </ul>



Table 1.1, continued

<b>Extraction technique</b>	<b>Advantages</b>	<b>Disadvantages</b>
Maceration	<ul style="list-style-type: none"> <li>✓ Suitable for use in case of the thermolabile compounds.</li>   <li>✓ Applicable in small batches and in large scale.</li>   <li>✓ Easy and simple method.</li>   <li>✓ Alteration in temperature and choice of solvents can enhance the extraction process, reduce the volume needed for extraction and can be introduced in the maceration technique.</li> </ul>	<ul style="list-style-type: none"> <li>✓ Destroyed certain chemical compounds due to high temperature heating.</li>   <li>✓ Organic waste produced thus proper waste management needed.</li> </ul>
Sonication	<ul style="list-style-type: none"> <li>✓ Increases the permeability of cell walls and produces cavitation of the sample.</li>   <li>✓ Reduction in extraction time and solvent consumption.</li> </ul>	<ul style="list-style-type: none"> <li>✓ Larger scale application is limited due to the higher costs needed.</li>   <li>✓ Effect of ultrasound energy on the active constituents.</li>   <li>✓ Consequently undesirable changes in the chemical molecules.</li>   <li>✓ Formation of free radicals.</li> </ul>

Table 1.1, continued

<b>Extraction technique</b>	<b>Advantages</b>	<b>Disadvantages</b>
HS-SPME	<ul style="list-style-type: none"> <li>✓ Environmental friendly.</li> <li>✓ No solvent required.</li> <li>✓ Fast and efficient.</li> <li>✓ Amount of the plant material used for the HS-SPME analysis was much smaller.</li> <li>✓ Can be used in the laboratory or under field conditions.</li> <li>✓ Minimum disturbance to specimen.</li> </ul>	<ul style="list-style-type: none"> <li>✓ Relatively expensive to purchase the fiber.</li> <li>✓ Unattainable for the yield of the crude extract.</li> <li>✓ Lack of quantitative analysis ability.</li> </ul>
Steam distillation	<ul style="list-style-type: none"> <li>✓ More flexible and competent for preparative extraction both in small batches and in large scale.</li> </ul>	
Microwave assisted extraction	<ul style="list-style-type: none"> <li>✓ Reduced extraction time and solvent volume.</li> <li>✓ Improved recoveries of analytes and reproducibility.</li> </ul>	<ul style="list-style-type: none"> <li>✓ Thermal degradation of compounds.</li> <li>✓ Limited to phenolic compounds with small molecule.</li> <li>✓ Unsuitable for tannins and anthocyanins.</li> </ul>

Table 1.1, continued

<b>Extraction technique</b>	<b>Advantages</b>	<b>Disadvantages</b>
Accelerated solvent extraction	<ul style="list-style-type: none"> <li>✓ Able to control temperature and pressure for each individual samples.</li> <li>✓ Requires less than an hour for extraction.</li> </ul>	<ul style="list-style-type: none"> <li>✓ Critically depend on the solvent types.</li> </ul>
Supercritical fluid extraction	<ul style="list-style-type: none"> <li>✓ Excellent solvent for nonpolar analytes.</li> <li>✓ CO<sub>2</sub> is easily available at low cost with low toxicity.</li> </ul>	<ul style="list-style-type: none"> <li>✓ The initial cost of the equipment is very high.</li> </ul>
Hydrodistillation	<ul style="list-style-type: none"> <li>✓ Inexpensive.</li> <li>✓ Easy to construct.</li> <li>✓ Suitable for laboratory and field operation.</li> <li>✓ Suitable for plant material that has a tendency to agglomerate or to agglutinate into an impenetrable mass when steam is passed through.</li> </ul>	<ul style="list-style-type: none"> <li>✓ Complete extraction is not possible.</li> <li>✓ Certain esters are partly hydrolyzed and sensitive substances tend to polymerize.</li> <li>✓ Require experience and familiarity with the method.</li> <li>✓ The high-boiling and water-soluble constituents incompletely vaporized.</li> <li>✓ The chances of charring are greater for material that tends to form mucilage.</li> </ul>

### 1.3.1 Solid-Phase Microextraction (SPME)

In many conventional extraction methods, organic solvents are being used and it is known that most organic solvents are toxic. Therefore, an extraction method which is solvent-free is desperately in need. SPME is an extraction technique which involves no solvent and has two approaches which are direct SPME and headspace SPME. SPME extracts the analyte and releases the absorbed analyte in the fiber coating and further analyse by analytical instrument such as GC or GC–MS (Zhang *et al.*, 1994). In headspace SPME (HS-SPME), a fused silica fiber coated with polymer is injected into the headspace above the sample (Zhang & Pawliszyn 1993). By using SPME, a sample can be prepared without using solvent, automated and overcome the plugging problem encounter in solid phase extraction (SPE) (Zhang *et al.*, 1994).

Among the advantages of HS-SPME sampling are able to analyse low molecular weight volatiles with absence of solvent peaks, automatically easy to handle, controlled parameters, inexpensive per analysis, simple sample preparation and no solvent removal process needed (Wampler, 1997). It is important to take account the affinity of the compounds towards the fiber that was used (Harmon, 2002). The downsides of HS-SPME are only a fraction of the compounds of interest can be injected, lack of sensitivity for very low levels of analyte detection, capable of heating the sample to 150°C, low temperature may decrease the separation efficiency and sample equilibrium which eventually effects the reproducibility of the separation technique (Wampler, 1997).

HS-SPME had been used by researchers in extracting natural products, volatile compounds and essential oils. For example, in extracting of the essential oil from the

*Osmanthus fragrans* Lour, 35 compounds were found at different flowering stage (Wang *et al.*, 2009) and in the essential oil of Sachalinmint from Norway, 58 compounds were identified (Rohloff, 2002) with the aid of this technique.

### 1.3.2 Solvent Extraction

Maceration or solvent extraction is a simple and widely used procedure. In this technique, the whole or coarsely powdered plant is soaked in solvent in a stoppered container for a known period of time with frequent agitation until the soluble matter is dissolved (Tiwari *et al.*, 2011). The mixture then is strained, the solid sample is pressed and the combined liquids are filtered. The purity, safety, extraction efficiency, ease of impurity removal and odour alteration are important parameters to be considered in choosing the right solvent (Theimer, 1982). This method is more suitable for plant with thermolabile compounds (Ncube *et al.*, 2008). The main downside of this technique is that this procedure requires a long time, taking from a few hours up to several weeks. This procedure also requires large volumes of solvent and this technique can cause potential loss of metabolites and plant material. In addition to that, selection of suitable solvent is very important as some compounds may be extracted inefficiently if they have poor solubility if unsuitable solvent is used at room temperature (25 °C) (Seidel, 2012).

Since solvent extraction is such a simple technique, many researchers had chosen this technique in extracting the phytochemicals in their studied plant. In investigating the 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging capacity of phenolic extracts from African yam bean (*Sphenostylis stenocarpa*), raw and processed ground seed of the African yam bean were extracted by stirring with 100 mL of acidic 70% acetone, 80%

acetone and 70% ethanol at 25°C for 24 h and filtered through filter paper. The residues were extracted again with 50 mL of 70% acetone for 3 h. The frozen and dried extract was used directly for their investigation (Victor *et al.*, 2012). In another study, finely ground leaf powder of *Ocimum basilicum* was homogenized at 1500 rpm for 5 min with hexane, methanol, acetone, ethyl acetate, ethanol and water separately at room temperature in preparing the crude extract prior to phenolics composition and antioxidant activity investigation of sweet basil (Jayasinghe *et al.*, 2003).

### 1.3.3 Hydrodistillation

Steam distillation and hydrodistillation is part of vapor distillation and these techniques are traditional procedure for isolating the volatile compounds. Hydrodistillation has the ability to separate volatile organics from non-volatile materials in the sample (Walradt, 1982). Hydrodistillation is suitable for compounds that are slightly volatile and water insoluble with boiling point of less than 100°C (Parliament, 2002). The downsides of using this method are time consuming, may introduce contaminant to the sample, requires high temperature and unsuitable for thermally labile compounds (Harmon, 2002).

Although hydrodistillation was discovered decades ago, this technique still remained the selected technique by the researchers in investigating the composition of the aromatic plants. In investigating chemical composition, toxicity and mosquito repellency of *Ocimum selloi*, the essential oil was obtained by hydrodistillation of powdered leaves for 6 h by using a Clevenger apparatus (de Paula *et al.*, 2003). In another study, the essential oils were extracted by hydrodistillation of dried *Haplophyllum Robustum* for 8 h using a

Clevenger-type apparatus, dried over anhydrous sodium sulphate and stored in sealed glass vials prior to analysis of the chemical composition of the leaves and flowering aerial parts of *Haplophyllum Robustum* (Rahimi-Nasrabadi *et al.*, 2009).

As there are many extraction techniques available in investigating the chemical composition of the aromatic plants, researchers had made comparisons between the extraction techniques in determining the most suitable extraction method. In reference to Xie *et al.* (2013), volatile compounds from *Exocarpium Citri Grandis* were extracted by steam distillation, HS-SPME and solvent extraction, respectively. A total of 81 compounds were identified by GC-MS (77 compounds from steam distillation, 56 compounds from HS-SPME and 48 compounds from solvent extraction). Based on the study, steam distillation gave an entire profile of volatile with longer extraction time while solvent extraction enabled the analysis of low volatility and high molecular weight compounds but lose some volatile components. HS-SPME gave satisfactory extraction efficiency, requiring a less amount of sample amount and shorter extraction time.

It was also reported that different extraction methods affect the yield and chemical compounds of gaharu oil (Sulaiman *et al.*, 2015). The research had investigated the extraction yields and percentages of five specific chemical compounds, namely, 3-phenyl-2-butanone,  $\alpha$ -guaiene,  $\alpha$ -agarofuran, 10-*epi*- $\gamma$ -eudesmol and agarospirol using hydrodistillation, Soxhlet and accelerated solvent extraction, respectively. Results showed that accelerated solvent extraction method exhibited the highest oil recovery percentage (2.12%) compared to the other two methods. The total percentage of the five chemical compounds was found highest in gaharu oil extracted using accelerated solvent

extraction (21.60%), followed by Soxhlet (9.51%) and lastly, hydrodistillation (5.08%). Hydrodistillation exhibited the least chemical compounds compared with Soxhlet and accelerated solvent extraction methods.

#### **1.4 Chromatographic Techniques**

Investigations of the leaf pigments by M.S. Tswett have led to the development of a special adsorption technique that permitted the separation of the leaf pigments. M.S. Tswett had further refined this technique, which eventually became known as chromatography (Ettre, 2003).

Chromatography is a separation technique in separating the mixture into its component and relative amount distributed between a mobile phase and the stationary phase. Some of the common chromatographic techniques are listed as follows:

1. High Performance-Liquid Chromatography (HP-LC)
2. Liquid Chromatography- Mass Spectroscopy (LC-MS)
3. Liquid Chromatography- Nuclear Magnetic Resonance (LC-NMR)
4. Gas Chromatography (GC)
5. Gas Chromatography Fourier Transform Infrared Spectrometry
6. Gas Chromatography-Mass Spectroscopy (GC-MS)
7. Supercritical Fluid Chromatography (SFC)
8. Nuclear Magnetic Resonance (NMR)
9. Thin Layer Chromatography (TLC)



### 1.4.1 Gas Chromatography-Mass Spectroscopy (GC-MS)

In GC-MS, ions are created from the eluted sample by electron impact or chemical ionization and stored in a radio frequency field. The trapped ions are then ejected from the storage area to an electron multiplier detector. The ejection is controlled so that scanning on the basis of mass-to-charge ratio is achievable. The ion trap detector is very compact and inexpensive than quadrupole instruments. GC-MS instruments have been used for identification of components that are present in natural and biological system, (Oleszek & Marston, 2000; Philipson, 2007; Daffre *et al.*, 2008).

Being reliable chromatography techniques, GC and GC-MS is much preferred by the researchers in analysing and separating the studied extract into their components. It was reported that 104 compounds were successfully detected by GC and GC-MS in chemical analysis of French beans (*Phaseolus vulgaris* L.) by headspace solid phase microextraction and simultaneous distillation/extraction (Barra *et al.*, 2007).

It was also reported that a total of 56 compounds was successfully identified by GC and GC-MS in investigating the chemical composition of the essential oils of inflorescences from *Origanum vulgare* ssp. *hirtum* (Link) Ietswaart growing wild in Calabria, southern Italy. The main compounds of the essential oil were thymol and carvacrol, while *p*-cymene and  $\gamma$ -terpinene, were the most abundant monoterpenes (Russo *et al.*, 1998).

## 1.5 Different Parts of Plant

Flower is very important as it is the plant organs of sexual reproduction and plays an important role in pollination. The flower protects the pollen and stigma, delivering and capturing pollen, helping in pollen hydration and germination, giving way to the entry of suitable pollen tubes into the stigma and guiding the tubes to the ovary (Edlund *et al.*, 2004). Leaves function as photosynthetic organs of the plant and must be able to control water potential in order to allow carbon dioxide intake by opening the stomata (Farquhar & Sharkey, 1982). Inflorescence helps in pollen transferring, provide maximum success in reproduction, and provide nutrients in fruits and flower development, supporting the fruits before dispersal when the fruits are matured and allowing success fruit and seed dispersal (Kirchoff & Classen-Bockhoff 2013).

Every part of the plant serves different functions, thus different chemical composition was expected. In order to report a chemical composition of a plant, part of the plant used as material is very important as different parts of the plant would provide a different profile of chemical constituents. It was reported that there was a difference in composition of germacrene D (27.2% and 41.0%, respectively) in the essential oils of fresh leaf and inflorescences of *Conium maculatum* from Serbia (Radulovic *et al.*, 2008).

In addition, a study on the composition of the essential oils of inflorescence and leaf from *Eupatorium cannabinum* L. reported that Germacrene D has been found as a first principal component in five out of six investigated oils (9.1–12.3%) and in one inflorescence oil as third main compound (7.7%). Neryl acetate was a second major constituent in all leaf oils (average mean: 9.4%) and in two samples of inflorescence oils

(7.9% and 8.2%), while another ester neryl isobutyrate was the third dominant component in one flower oil. Methyl thymol was the first main compound in one flower oil and the fourth in all investigated leaf oils (average mean: 5.7%).  $\beta$ -bisabolene comprising 7.1% and 5.6% was second or fourth constituent in two inflorescences, and as third compound (6.7% and 8.6%) in two leaf oils (Judzentiene, 2007).

## 1.6 Evaluation of Antioxidant Activity

Antioxidants in food help to delay the oxidation process. Oxidation reaction in food resulted in an unpalatable taste and products that may be harmful to the human consumption (Brand-Williams *et al.*, 1995). The primary sources of natural antioxidants are whole grains, fruits and vegetables while plant sources food antioxidants are vitamin C, vitamin E, carotenes, phenolic acids, phytate and phytoestrogens.

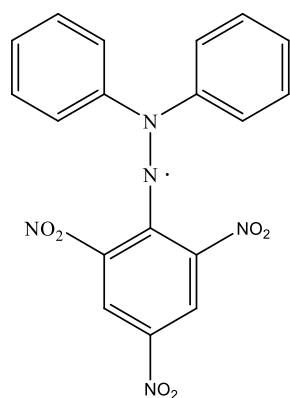
Since synthetic antioxidants might be a contributing factor for cancer, antioxidants from natural resources are widely sought after to replace the synthetic antioxidants. Furthermore, research had shown that the increased consumption of antioxidant-rich foods were found to correlate with prevention of oxidative stress diseases (Ames *et al.*, 1995; Kaur & Kapoor, 2001).

Many *in vitro* methods have been developed to evaluate the effectiveness of natural antioxidants. *In vitro* methods can be divided into hydrogen atom transfer reactions and electron transfer reactions. Some of the antioxidant assay that were developed were listed as the following:

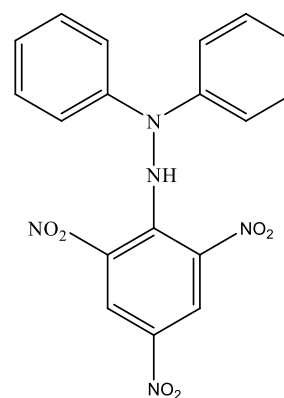
1. 2,2- diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay
2. Oxygen radical absorbance capacity (ORAC)
3. Total radical trapping antioxidant potential (TRAP)
4.  $\beta$  carotene bleaching
5. Trolox equivalent antioxidant capacity (TEAC)
6. Ferric reducing antioxidant power (FRAP)
7. Superoxide anion radical scavenging assay
8. Hydroxyl radical scavenging assay
9. Nitric oxide radical scavenging assay
10. Total phenol assay
11. Cupric reducing antioxidant power (CUPRAC)
12. Low-density lipoproteins (LDLs) oxidation

### **1.6.1 2,2 diphenyl-1-picryl-hydrazyl (DPPH) Radical Scavenging Assay**

DPPH is a stable free radical by the delocalisation of the spare electron over the molecule preventing the molecule from dimerise. The delocalisation also gives the deep violet colour of the free radical (Molyneux, 2004). In this method, DPPH in methanol solution is reduced in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H (Blois, 1958). The free radical DPPH• is reduced to the corresponding hydrazine when it reacts with hydrogen donors (Contreras-Guzma'n & Strong, 1982). Figure 1.7 illustrates the chemical structure of the DPPH molecule and its reduced chemical structure.

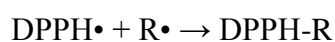
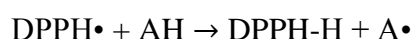


DPPH molecule



Reduced DPPH molecule

**Figure 1.7:** The chemical structure of the DPPH molecule and its reduced chemical structure.



Where, antioxidant = (AH)

radical species = (R•) (Brand-Williams *et al.*, 1995)

The reduction process results in a colour change from purple to yellow (picryl group still present). The colour change is due to the molar absorptivity of the DPPH radical at 517 nm reduces from 9660 to 1640 when the odd electron of DPPH radical becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced DPPH-H (Prakash, 2001). The sample was incubated in the dark and the absorbance is measured spectrophotometrically at 517 nm. Among of the positive controls that can be used are ascorbic acid, gallic acid (Blois, 1958), BHA, tocopherol (Shimada *et al.*, 1992), quercetin (Shon *et al.*, 2003), BHT (Liyana-Pathirana & Shahidi, 2005), rutin (Yamasaki *et al.*, 1994), catechin (Astudillo *et al.*, 2000) or glutathione (Kato *et al.*, 1988).

The percentage of the DPPH remaining is calculated as %DPPH<sub>rem</sub>. %DPPH<sub>rem</sub> is proportional to the antioxidant concentrations, and the concentration that causes a decrease in the initial DPPH concentration by 50% is defined as IC<sub>50</sub> or EC<sub>50</sub> (Abuja *et al.*, 1997). The equation for the calculation of %DPPH<sub>rem</sub> is as follows:

$$\%DPPH_{rem} = 100 \times [DPPH]_{rem}/[DPPH]_{T=0}$$

Where [DPPH]<sub>rem</sub> = Remaining concentration of DPPH

[DPPH]<sub>T=0</sub> = Initial concentration of DPPH

DPPH method is recommended as it is easy, accurate with regard to measuring the antioxidant activity of fruit and vegetable juices or extracts and the results are highly reproducible and comparable to other free radical scavenging methods (Gil *et al.*, 2000). This method is unsuitable in measuring the antioxidant activity of plasma as the protein precipitates in the alcoholic reaction medium (Sa ´nchez-Moreno, 2002).

In an investigation of the antioxidant activities of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae), the DPPH method had been applied and the results showed that the oil strongly reduced the diphenylpicrylhydrazyl radical with the IC<sub>50</sub> value of 1.56 µg/mL (Candan *et al.*, 2003). It was reported in the investigation of the antioxidant activities of *Melissa officinalis* L. (Lamiaceae) essential oil using DPPH• assay and OH• assay, the essential oil exhibited very strong radical scavenging capacity by reducing the DPPH radical with the IC<sub>50</sub> of 7.58 µg/mL and OH radical generation with the IC<sub>50</sub> value of 1.74 µg/mL in a dose-dependent manner (Mimica-Dukic *et al.*, 2004).

## 1.7 Significant of the Present Study

The present study is significant in determining the chemical constituents of *Ocimum basilicum* and *Ocimum sanctum* grown in Malaysia as different origin of the plant would have different chemical composition (Dambolena *et al.*, 2010). Since most of the research would only focus on the leaves of the species (Lee *et al.*, 2005; Gupta *et al.*, 2007; Devedran and Balasubramanian, 2011), the study for comparison of the other parts of this plant would be significant in determining the composition difference. Besides that, the present study showed the comparison between different extraction procedures in extracting chemical constituents of *Ocimum basilicum* and *Ocimum sanctum*. Other than that, the study on genetic distance is important in determining how closely *Ocimum basilicum* and *Ocimum sanctum* are related and whether the species has the same ancestor. Lastly, the study would be important in determining the strength of the studied species in being free radical scavenger in determining their promising nutrient values.

## 1.8 Research Objectives

The objectives of the present study are as follows:

1. To extract and characterize the chemical constituents from the leaves, inflorescence and flowers of *Ocimum basilicum* and *Ocimum sanctum* grown in Malaysia by using different extraction techniques.
2. To investigate the interspecies relationship between *Ocimum basilicum* and *Ocimum sanctum* by calculating Nei's genetic distance.
3. To determine the antioxidant activities from the leaves extracts of *Ocimum basilicum* and *Ocimum sanctum* by using DPPH radical scavenging assay.

## CHAPTER 2: MATERIALS AND METHODS

### 2.1 Plant Materials

Healthy and flowering *Ocimum basilicum* and *Ocimum sanctum* of approximately four months of age were purchased from a nursery located in Sungai Buloh, Selangor. The detached leaves, flowers and inflorescence were cleaned using distilled water to remove dirt and soil, then were air-dried at room temperature. Figure 2.1 shows the different parts of *Ocimum basilicum*.



**Figure 2.1:** Leaf, flower and inflorescence of *Ocimum basilicum*

Source: Picture was taken from [www.mygardeninsider.com](http://www.mygardeninsider.com)

### 2.2 Chemicals and Materials

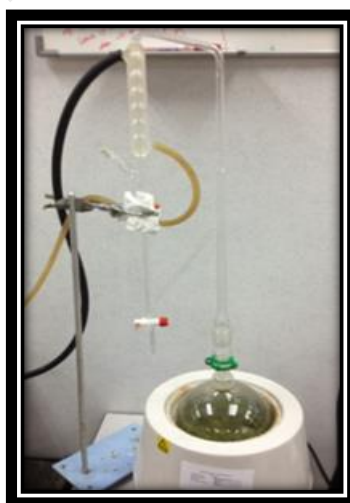
Estragole (methyl chavicol) 98%, 2, 2-diphenyl-1-picrylhydrazil (DPPH) and European Pharmacopeia (EP) grade butylated hydroxytoluene (BHT) were purchased



from Aldrich, (Steinheim, Germany). C<sub>7</sub>-C<sub>30</sub> saturated alkane analytical standard was purchased from Supelco (Pennsylvania, USA). Methanol, hexane and anhydrous sodium sulfate were purchased from System (Selangor, Malaysia). Analytical grade dichloromethane was purchased from Merck (Darmstadt, Germany). A SPME fibre holder with a 1 cm fibre assembly coated with a 100 µm polydimethylsiloxane (PDMS) was purchased from Supelco (Bellefonte, PA, USA).

### 2.3 Hydrodistillation

A 100.00 g of blended fresh leaves in 500 mL of distilled water was subjected to hydrodistillation in a Clevenger-type apparatus for 2 h. The obtained essential oils were dried over anhydrous sodium sulfate to eliminate humidity. The aliquot was collected in triplicates and analysed using GC-MS. Figure 2.2 shows the Clevenger-type apparatus setup.



**Figure 2.2:** Hydrodistillation using Clevenger-type apparatus.

## 2.4 Solvent Extraction

A 18.00 g of fresh leaves, 0.20 g of flowers and 5.20 g of inflorescence were extracted separately using two different solvents, namely, dichloromethane and methanol at a ratio of (1 g plant materials: 3 mL of solvent) and supernatant were collected after 24 h, then they were passed through a column of anhydrous sodium sulfate to eliminate humidity. The solvent was removed using rotatory evaporator. The crude extracts were analysed individually in triplicate by GC-MS.

## 2.5 Headspace-Solid Phase Microextraction (HS-SPME)

A 1.00 g of leaves, 0.02 g of flowers and 0.02 g of inflorescence were sealed separately in a 20 mL capped vial. The fibre was exposed to the headspace and the system was left for 2 min at 80°C to allow equilibrium. Volatile compounds were extracted from the headspace with 15 min extraction time and the extraction temperature was set at 50°C. The trapped volatile compounds were desorbed at 250°C in the GC injection port for 7 min and flushed into the GC column. The contents were analysed in triplicates by GC-MS. Figure 2.3 shows the leaves in vials prior to HS-SPME.



**Figure 2.3:** Leaves sealed in 20 mL capped vials ready for HS-SPME.

## 2.6 GC-MS Analysis

The extracts were analysed by a Hewlett Packard HP 6890 series mass selective detector linked to a GCMS-QP 2010 Plus Shimadzu gas chromatograph that operates in a splitless injection mode and was fitted with a DB-5 ms column coated with 5% phenyl 95% dimethyl arylene siloxane with column length of 30.0 m, a diameter of 0.25 mm and film thickness of 0.25  $\mu\text{m}$ . The oven temperature was set at 50°C and the injection temperature was set at 250°C. The flow control mode was at linear velocity and the helium pressure was set at 68.1 kPa. The helium total flow was 58.2 mL/min and the column flow was 1.2 mL/min. The linear velocity was 39.7 cm/s and the purge flow was 3 mL/min with the split ratio of 45. The initial temperature was set at 50°C and the hold time at 2 min, the temperature was programmed to 180°C at a rate of 3°C/min and held for 3 min, and then heated at a rate of 8°C/min to a final temperature of 280°C and held for 10 min. Ionization of the sample components was performed in the EI mode at 70 eV. The ion source temperature and interface temperature were kept at 200°C and 300°C, respectively. Mass spectra were scanned from  $m/z$  50 to 600 with a scan speed of 1250. Injected volume was 2  $\mu\text{L}$ . All the samples were analysed in triplicates. Figure 2.4 shows the GC-MS instrument used throughout the study.



**Figure 2.4:** Hewlett Packard HP 6890 series mass selective detector linked to a GCMS-QP 2010 Plus Shimadzu gas chromatograph.

## 2.7 Kovats Indices

The compounds from both species were identified by comparing its mass spectra with the National Institute of Standards and Technology (NIST) library and further confirmed with Kovats retention indices. The retention indices for the compounds were determined by co-injection of the samples with a solution containing the homologous series of C<sub>7</sub>-C<sub>30</sub> *n*-alkanes (Van Den Dool & Kratz, 1963). Qualitative analysis was based on comparison of retention times and mass spectra with corresponding data provided in the literature (Adams, 2001). Retention index (*I*) of all the volatile analytes were calculated relative to the series of homologous hydrocarbons using the following equation:

$$I = [100 (t - t_n) + 100 (n)] / [(t_{n+1} - t_n)]$$

where  $t$  = retention time of analyte

$t_n$  = retention time of alkane before analyte

$t_{n+1}$  = retention time of alkane after analyte

$n$  = number of carbon atoms of analyte

## 2.8 Genetic Distance

Genetic distance is a measure of the evolutionary divergence between copies of homologous genes which share a common ancestor (Beaumont *et al.*, 1998). An ideal measure of genetic distance assumes that the difference between two genes is proportional to the time since they shared a common ancestor. Genetic distance ( $D$ ) between two populations can be measured using a statistical method developed by Nei's study (Nei, 1972). In the study, Nei had defined the normalized identity of genes between populations, then related it to the accumulated number of gene differences per locus. The genetic distance between two populations was being calculated using the following equation:

$$D = -\log_e J_{XY} / \sqrt{J_X J_Y}$$

Where  $D$  = genetic distance between X and Y population

$J_{XY} / \sqrt{J_X J_Y}$  = normalized identity of genes between X and Y population

According to the equation developed by Nei, X and Y are two populations. Let  $x_i$  and  $y_i$  be the frequencies of the  $i$ th alleles in X and Y, respectively. The probability of identity of two genes that has been chosen randomly is  $j_x = \sum x_i^2$  in population X, while for population Y it is  $j_y = \sum y_i^2$ . The probability of identity of a gene from X and a gene from Y is  $j_{xy} = \sum x_i y_i$ . The normalized identity of genes between X and Y with respect to loci is defined as  $I_j = j_{xy} / \sqrt{j_x j_y}$ . Loci, plural of locus, is the place or position on a chromosome. The normalized identity of genes between X and Y with respect to all loci is defined as  $I = J_{XY} / \sqrt{J_X J_Y}$ , where  $J_X$ ,  $J_Y$  and  $J_{XY}$  are the arithmetic means of  $j_x$ ,  $j_y$  and  $j_{xy}$ , respectively, over all loci.

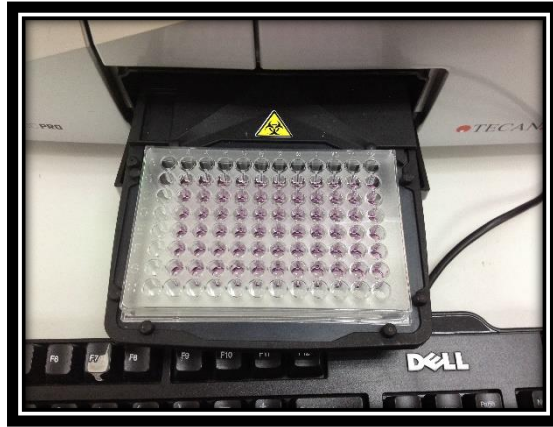
## 2.9 Antioxidant Assay on Leaves Extracts

DPPH assay was used to determine the free radical scavenging capacity of the leaves extracts as described as (Brand-Williams *et al.*, 1995). A series of concentration (6.75 µg/mL to 5000.00 µg/mL) of leaves extracts were prepared respectively with methanol as solvent. The prepared samples were then mixed with 90 mM DPPH in methanol. A blank solution was prepared by using 90 mM DPPH in methanol. The absorbance at 517 nm was recorded after an incubation period of 30 min by using a microplate reader (TECAN infinite M200 PRO). The condition for microplate reader was agitated for 5 s in orbital mode with 3 mm amplitude at the speed of 44.3 rpm and a measurement bandwidth of 9 nm. A 96-well flat bottom, polypropylene plate was used. Different concentrations of BHT (range from 6.75 µg/mL to 500.00 µg/mL) were used as positive control. Each series of concentration was analysed in triplicate. The free radical scavenging of DPPH percentage was calculated using the following equation:

$$\text{Free radical scavenging of DPPH (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / (A_{\text{blank}})] \times 100$$

Where  $A_{\text{blank}}$  = Absorbance of blank  
 $A_{\text{sample}}$  = Absorbance of sample

A graph was plotted to determine the IC<sub>50</sub> values of the extracts after the free radical scavenging of DPPH percentage was calculated. The IC<sub>50</sub> value represented the concentrations of the extracts that caused 50% inhibition on DPPH assay. Figure 2.5 shows the sample prior to absorbance measuring using a microplate reader.



**Figure 2.5:** Measuring absorbance of samples using microplate reader.

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## CHAPTER 3: RESULTS AND DISCUSSION

### 3.1 Analysis of Chemical Constituents of *Ocimum basilicum* and *Ocimum sanctum*

In this study, the chemical constituents of two species of basil, *Ocimum basilicum* and *Ocimum sanctum* grown in Malaysia were investigated to determine their chemical composition. Different extraction techniques were applied in extracting the chemical constituents, namely, solvent extraction using methanol and dichloromethane, hydrodistillation and HS-SPME. Other than that, the chemical constituents of different parts of the plant, namely, leaves, flowers and inflorescence were investigated to determine the major compounds of each part. The appearance and colour that can be observed from the different extracts were summarized in Table 3.1.

**Table 3.1:** Appearance and colour of different extracts from *Ocimum basilicum* and *Ocimum sanctum*.

Species	Part of plant	Type of extract	Colour and appearance
<i>Ocimum basilicum</i>	Leaves	Hydrodistillation	Viscous yellow liquid
<i>Ocimum basilicum</i>	Leaves	Methanol extract	Brown sticky solid



Table 3.1, continued

<b>Species</b>	<b>Part of plant</b>	<b>Type of extract</b>	<b>Colour and appearance</b>
<i>Ocimum basilicum</i>	Leaves	Dichloromethane	Brown sticky solid
<i>Ocimum basilicum</i>	Flowers	Methanol extract	Brown sticky solid
<i>Ocimum basilicum</i>	Flowers	Dichloromethane	Brown sticky solid
<i>Ocimum basilicum</i>	Inflorescence	Methanol extract	Brown sticky solid
<i>Ocimum basilicum</i>	Inflorescence	Dichloromethane	Brown sticky solid
<i>Ocimum sanctum</i>	Leaves	Hydrodistillation	Viscous yellow liquid
<i>Ocimum sanctum</i>	Leaves	Methanol extract	Brown sticky solid
<i>Ocimum sanctum</i>	Leaves	Dichloromethane	Brown sticky solid
<i>Ocimum sanctum</i>	Flowers	Methanol extract	Brown sticky solid
<i>Ocimum sanctum</i>	Flowers	Dichloromethane	Brown sticky solid
<i>Ocimum sanctum</i>	Inflorescence	Methanol extract	Brown sticky solid
<i>Ocimum sanctum</i>	Inflorescence	Dichloromethane	Brown sticky solid

*Ocimum basilicum* and *Ocimum sanctum* were subjected to detailed GC-MS analysis in order to determine the chemical constituents and were further confirmed with Kovats indices (Adam, 2001). Kovats index is a system of retention indices that the data is accepted for identification of chemical compounds by gas chromatography (Babusok *et al.*, 2007).

### 3.1.1 Chemical Composition of *Ocimum basilicum*

The chemical constituents identified from Malaysian grown *Ocimum basilicum* are listed in Table 3.2. A total of 47 chemical compounds was identified in *Ocimum basilicum*. The peak number and molecular weight of compounds according to GC-MS chromatogram profile of *Ocimum basilicum* are shown in Appendix A. The GC-MS chromatogram profiles of leaves, flowers and inflorescence from *Ocimum basilicum* using hydrodistillation, solvent extraction and HS-SPME are shown in Appendix B. The percentage mean area peaks of different extraction techniques and different parts from *Ocimum basilicum* are illustrated in Figure 3.1 (A-C).

#### 3.1.1.1 Chemical Composition of *Ocimum basilicum* leaves

The percentage yields of leaves extract obtained by hydrodistillation, dichloromethane extraction and methanol extraction were 0.10% (v/w), 0.13% (w/w) and 0.83% (w/w), respectively. Hydrodistillation of *Ocimum basilicum* leaves yielded estragole (35.71%), eucalyptol (13.26%), (*E*)- $\beta$ -ocimene (7.99%), *trans*- $\alpha$ -bergamotene (5.82%), and  $\tau$ -cadinol (5.71%) as the main components and the finding is consistent with Thai *Ocimum basilicum* (Bunrathep *et al.*, 2007). The leaves methanol extract of *Ocimum basilicum* yielded estragole (82.69%), (*Z*)- $\beta$ -ocimene (1.26%), *trans*- $\alpha$ -bergamotene (0.83%) and eugenol methyl ether (0.40%) as the major compounds. The leaves dichloromethane extract of *Ocimum basilicum* yielded estragole (73.16%), eucalyptol (6.17%), *trans*- $\alpha$ -bergamotene (5.26%), (*E*)- $\beta$ -ocimene (4.52%) and  $\tau$ -cadinol (2.56%) as main components. HS-SPME analysis of the volatiles from *Ocimum basilicum* leaves showed estragole (59.67%), eucalyptol (9.02%), *trans*- $\alpha$ -bergamotene (8.60%),  $\alpha$ -caryophyllene (2.65%) and camphor (1.62%) as the major compounds. HS-SPME analysis of basil

leaves from Czech Republic also showed estragole as the major compound (Klimánková *et al.*, 2008). Only one plant was used throughout the entire research to avoid inconsistent parameters that might affect the chemical composition of the plant and this includes difference in geographical origin or nutrient availability (Lim *et al.*, 2012; Nguyen & Niemeyer, 2008), thus, hydrodistillation was conducted only on leaves due to insufficient material of flowers and inflorescence.

### **3.1.1.2 Chemical Composition of *Ocimum basilicum* flowers**

Extractions of *Ocimum basilicum* flowers were conducted using dichloromethane extraction, methanol extraction and HS-SPME. The percentage yield of flower extracts obtained by dichloromethane extraction and methanol extraction was 0.05% (w/w) and 0.34% (w/w), respectively. Estragole (98.88%) and (*Z*)- $\beta$ -farnesene (1.11%) were the two main compounds identified from dichloromethane extraction while estragole (99.22%) and (*Z*)- $\beta$ -farnesene (0.77%) were identified from methanol extract. The major compounds that were identified in HS-SPME analysis were estragole (88.18%), *trans*- $\alpha$ -bergamotene (2.82%), (*E*)- $\beta$ -ocimene (1.47%), eugenol methyl ether (0.72%) and  $\alpha$ -bulnesene (0.51%). Estragole was also reported as the main constituent in Turkey *Ocimum basilicum* L. flowers (Chalchat & Özcan, 2008).

### **3.1.1.3 Chemical Composition of *Ocimum basilicum* Inflorescence**

Extraction of *Ocimum basilicum* inflorescence was conducted using HS-SPME, dichloromethane and methanol extraction. The percentage yields of inflorescence extract obtained by dichloromethane extraction and methanol extraction were 0.45% (w/w) and

2.75% (w/w), respectively. The major compounds that were identified in the dichloromethane extract of *Ocimum basilicum* inflorescence were estragole (57.85%), *trans*- $\alpha$ -bergamotene (7.24%), (*E*)- $\beta$ -ocimene (6.59%), linalool (1.84%) and eucalyptol (1.71%). Five compounds that were identified from the methanol extract of *Ocimum basilicum* inflorescence were estragole (77.65%), (*Z*)- $\beta$ -ocimene (3.89%), *trans*- $\alpha$ -bergamotene (2.35%), eucalyptol (0.42%) and camphor (0.30%) while estragole (86.39%),  $\alpha$ -cubebene (1.86%) and (*E*)- $\beta$ -ocimene (1.70%), *trans*- $\alpha$ -bergamotene (1.44%) and linalool (0.81%) were the main components identified in HS-SPME analysis. A report by Hassanpouraghdam *et al.* (2010) on Iranian *Ocimum basilicum* L inflorescence also revealed that estragole was the main constituent.

**Table 3.2:** Chemical constituents of leaves (L), inflorescence (I) and flowers (F) from *Ocimum basilicum*.

Peak #	*Compounds	Retention Index (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
1	$\alpha$ -thujene	932	n.d	n.d	n.d	0.05 $\pm$ 0.04	n.d	n.d	n.d	n.d	n.d	n.d
2	$\alpha$ -pinene	936	0.94 $\pm$ 0.03	n.d	n.d	n.d	n.d	n.d	n.d	0.32 $\pm$ 0.07	n.d	n.d
3	$\alpha$ -fenchene	944	0.01 $\pm$ 0.00	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
4	camphene	947	0.23 $\pm$ 0.40	n.d	n.d	n.d	n.d	n.d	n.d	0.09 $\pm$ 0.01	n.d	n.d
5	sabinene	972	0.30 $\pm$ 0.40	n.d	n.d	0.03 $\pm$ 0.01	n.d	n.d	n.d	0.35 $\pm$ 0.08	n.d	n.d
6	$\beta$ -pinene	976	1.26 $\pm$ 0.42	0.80 $\pm$ 0.19	n.d	0.12 $\pm$ 0.03	n.d	n.d	n.d	0.66 $\pm$ 0.20	n.d	n.d
7	$\beta$ -myrcene	988	1.59 $\pm$ 0.01	0.57 $\pm$ 0.14	n.d	0.14 $\pm$ 0.40	n.d	n.d	n.d	0.55 $\pm$ 0.09	n.d	0.02 $\pm$ 0.01

Table 3.2, continued

Peak #	*Compounds	Retention Index (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
8	(Z)-3-Hexen-1-ol acetate	1004	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.03 ± 0.01	n.d
9	Limonene	1026	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.04 ± 0.02	0.06 ± 0.01
10	eucalyptol	1031	13.26 ± 0.84	6.17 ± 0.81	n.d	1.71 ± 0.38	n.d	n.d	0.42 ± 0.01	9.02 ± 1.38	0.25 ± 0.12	0.44 ± 0.13
11	(Z)-β-ocimene	1034	n.d	n.d	n.d	n.d	1.26 ± 0.08	n.d	3.89 ± 0.06	0.27 ± 0.06	0.03 ± 0.01	0.03 ± 0.01
12	(E)-β-ocimene	1046	7.99 ± 0.43	4.52 ± 0.94	n.d	6.59 ± 0.60	n.d	n.d	n.d	2.44 ± 0.62	1.47 ± 0.47	1.70 ± 0.45
14	γ-terpinene	1056	0.13 ± 0.00	n.d	n.d	n.d	n.d	n.d	n.d	0.05 ± 0.01	n.d	n.d
15	trans-β-terpineol	1069	0.20 ± 0.01	n.d	n.d	n.d	n.d	n.d	n.d	0.11 ± 0.01	n.d	n.d

Table 3.2, continued

Peak #	*Compounds	Retention Index (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
16	terpinolene	1082	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.16 ± 0.03	0.06 ± 0.02	0.08 ± 0.02
17	2-carene	1083	0.50 ± 0.01	n.d	n.d	n.d	n.d	n.d	n.d	0.03 ± 0.01	n.d	n.d
18	linalool	1100	1.06 ± 0.01	0.44 ± 0.12	n.d	1.84 ± 0.23	n.d	n.d	n.d	0.41 ± 0.18	0.50 ± 0.21	0.81 ± 0.16
19	camphor	1145	3.15 ± 0.06	1.24 ± 0.25	n.d	1.13 ± 0.14	n.d	n.d	0.30 ± 0.01	1.62 ± 0.35	0.43 ± 0.25	0.55 ± 0.27
20	borneol	1171	n.d	n.d	n.d	0.43 ± 0.08	n.d	n.d	n.d	n.d	0.10 ± 0.07	0.37 ± 0.15
21	estragole	1205	35.71 ± 1.81	73.16 ± 2.24	98.88 ± 0.13	57.85 ± 3.53	82.69 ± 1.14	99.22 ± 0.12	77.65 ± 0.17	59.67 ± 0.31	88.18 ± 4.51	86.39 ± 2.38
22	chavicol	1251	n.d	n.d	n.d	0.50 ± 0.08	n.d	n.d	n.d	n.d	n.d	n.d

Table 3.2, continued

Peak #	*Compounds	Retention Index (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
23	bornyl acetate	1282	0.27 ± 0.00	n.d	n.d	0.53 ± 0.05	n.d	n.d	n.d	0.03 ± 0.01	0.16 ± 0.14	0.05 ± 0.03
24	$\alpha$ -terpineol acetate	1340	0.36 ± 0.01	n.d	n.d	n.d	n.d	n.d	n.d	0.23 ± 0.12	n.d	n.d
26	copaene	1369	0.14 ± 0.00	n.d	n.d	n.d	n.d	n.d	n.d	0.14 ± 0.06	n.d	n.d
27	$\beta$ -cubebene	1390	0.28 ± 0.01	0.73 ± 0.47	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
31	eugenol methyl ether	1398	2.85 ± 0.04	0.76 ± 0.14	n.d	0.34 ± 0.06	0.40 ± 0.07	n.d	n.d	0.77 ± 0.10	0.72 ± 0.79	0.53 ± 0.44
32	<i>cis</i> - $\alpha$ -bergamotene	1409	0.12 ± 0.00	n.d	n.d	7.24 ± 0.12	n.d	n.d	n.d	0.15 ± 0.06	n.d	0.03 ± 0.01
33	( <i>E</i> )-caryophyllene	1414	1.37 ± 0.03	0.75 ± 0.15	n.d	0.65 ± 0.05	n.d	n.d	n.d	0.86 ± 0.40	0.19 ± 0.13	n.d



Table 3.2, continued

Peak #	*Compounds	Retention Index (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
34	<i>β</i> -cedrene	1418	0.10 ± 0.01	n.d	n.d	n.d	n.d	n.d	n.d	0.10 ± 0.04	n.d	n.d
35	<i>trans-α</i> -bergamotene	1431	5.82 ± 0.06	5.26 ± 0.40	n.d	7.24 ± 0.12	0.83 ± 0.03	n.d	2.35 ± 0.03	8.60 ± 2.99	2.82 ± 2.12	1.44 ± 2.22
36	( <i>Z</i> )- <i>β</i> -farnesene	1437	0.16 ± 0.00	0.39 ± 0.08	1.11 ± 0.13	n.d	n.d	0.77 ± 0.12	n.d	0.17 ± 0.06	0.18 ± 0.13	0.22 ± 0.04
37	<i>α</i> -cubebene	1441	0.53 ± 0.01	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	1.86 ± 1.57
38	<i>ε</i> -muurolene	1446	0.16 ± 0.00	1.98 ± 0.36	n.d	0.78 ± 0.04	n.d	n.d	n.d	n.d	0.30 ± 0.18	n.d
39	<i>α</i> -caryophyllene	1451	n.d	n.d	n.d	0.27 ± 0.02	n.d	n.d	n.d	2.65 ± 1.50	0.09 ± 0.06	0.07 ± 0.05

Table 3.2, continued

Peak #	*Compounds	Retention Index (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
40	<i>β</i> -farnesene	1462	n.d	n.d	n.d	0.52 ± 0.02	n.d	n.d	n.d	0.65 ± 0.22	n.d	n.d
43	<i>α</i> -amorphene	1483	0.11 ± 0.01	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d
47	<i>α</i> -bulnesene	1496	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.48 ± 0.15	0.51 ± 0.47	0.59 ± 0.13
44	geranyl propionate	1500	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.24 ± 0.08	0.33 ± 0.14
45	<i>β</i> -bisabolene	1502	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.18 ± 0.06	n.d	n.d
46	linalyl isovalerate	1504	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.43 ± 0.14	0.40 ± 0.10

Table 3.2, continued

Peak #	*Compounds	Retention Index (RI)	Relative Area (%)										
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME			
				L	L	F	I	L	F	I	L	F	I
48	$\delta$ -cadinene	1512	0.38 $\pm$ 0.01	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.09 $\pm$ 0.06
49	(-)-calamenene	1515	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.29 $\pm$ 0.11	n.d	n.d
50	$\beta$ -sesquiphellandrene	1519	0.45 $\pm$ 0.01	n.d	n.d	0.40 $\pm$ 0.03	n.d	n.d	n.d	n.d	0.43 $\pm$ 0.15	0.15 $\pm$ 0.10	0.19 $\pm$ 0.04
52	$\alpha$ -cadinene	1535	n.d	n.d	n.d	n.d	n.d	n.d	n.d	n.d	0.08 $\pm$ 0.02	n.d	n.d
53	$\tau$ -cadinol	1637	5.71 $\pm$ 0.05	2.56 $\pm$ 0.50	n.d	1.06 $\pm$ 0.05	n.d	n.d	n.d	n.d	1.75 $\pm$ 0.34	0.41 $\pm$ 0.21	0.65 $\pm$ 0.26

Table 3.2, continued

Peak #	*Compounds	Retention Index (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
54	$\alpha$ -cadinol	1648	0.82 ± 0.02	n.d	n.d	n.d	n.d	n.d	n.d	0.06 ± 0.03	n.d	n.d
57	$\alpha$ -bisabolol	1682	0.29 ± 0.01	n.d	n.d	n.d	n.d	n.d	n.d	0.03 ± 0.01	n.d	0.02 ± 0.00
Total			86.25	99.33	99.99	89.42	85.18	99.99	84.61	93.40	97.29	96.92

\*Compounds identified by using Mass Spectra (MS) data and confirmed by Kovats Index

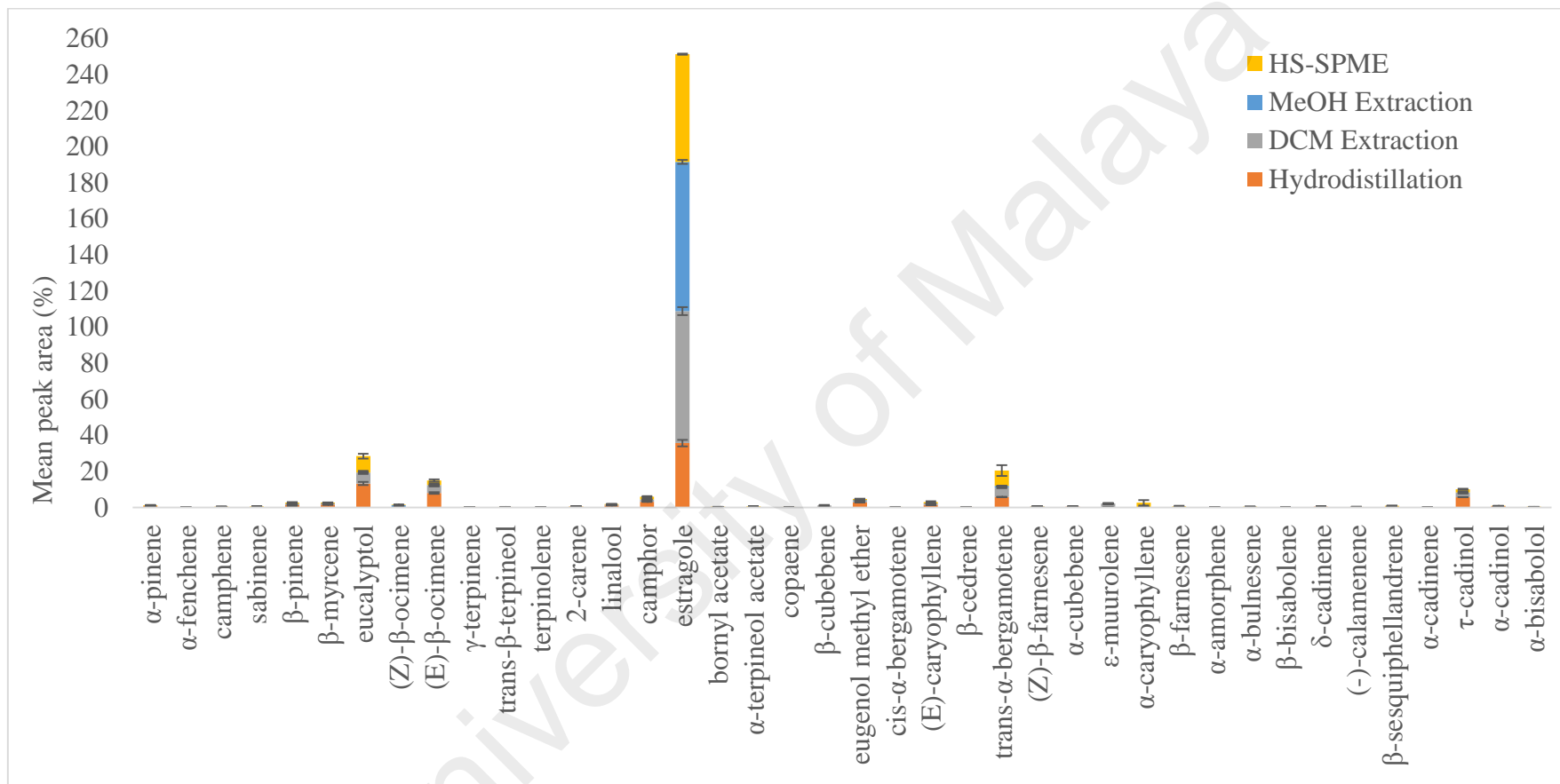
n.d = not detected

HS-SPME = Headspace-Solid Microextraction

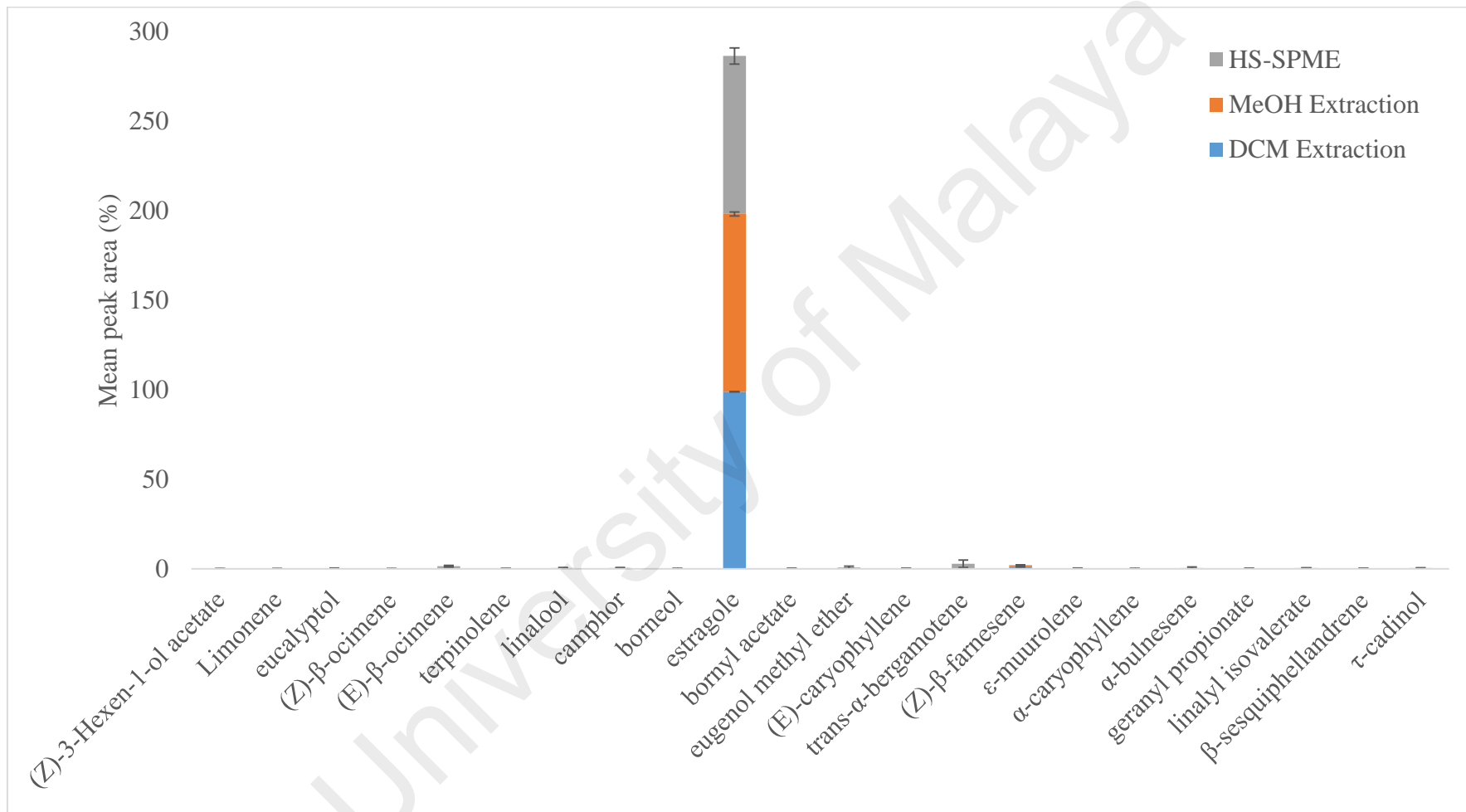
L = Leaves

F = Flowers

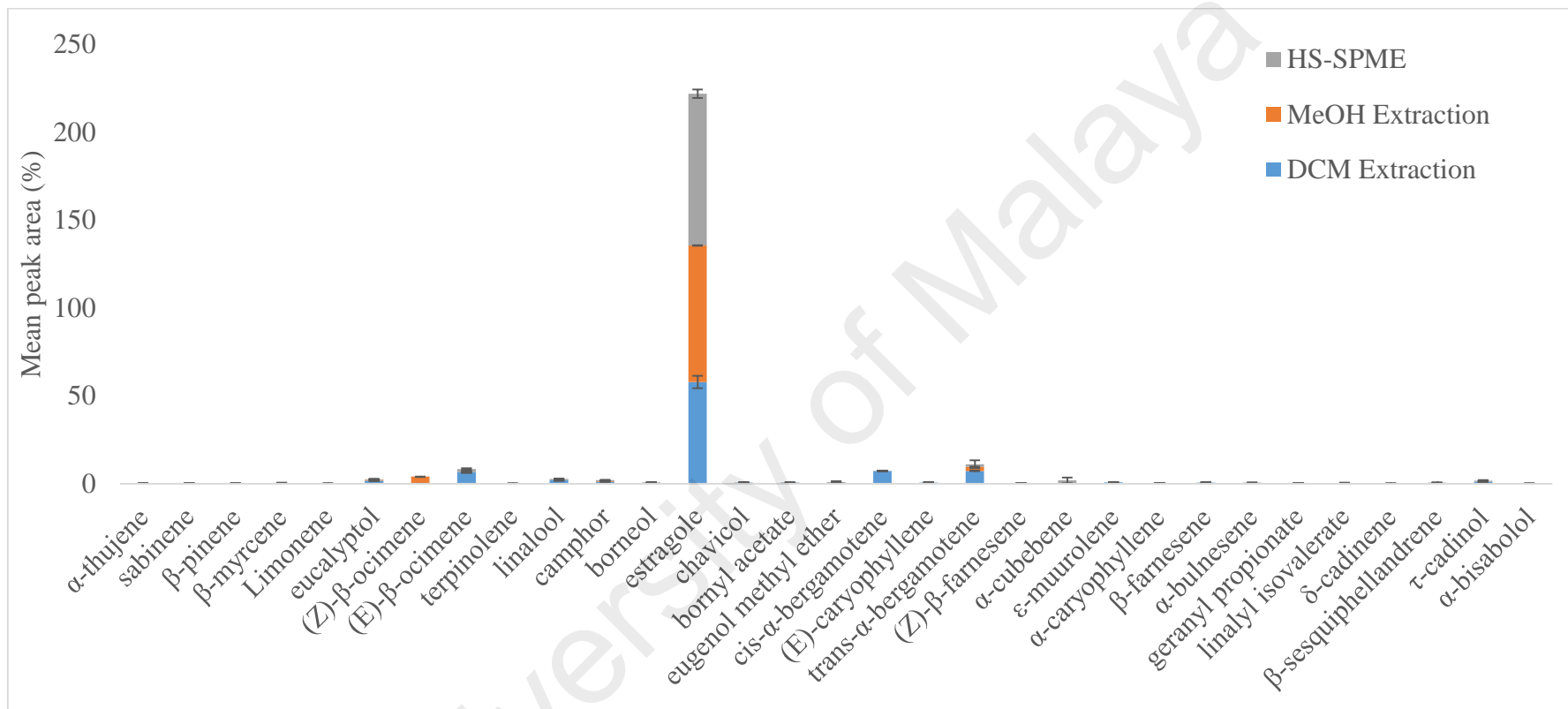
I = Inflorescence



**Figure 3.1A:** Percentage mean peak area (%) of chemical constituents of *Ocimum basilicum* leaves using different extraction techniques.



**Figure 3.1B:** Percentage mean peak area (%) of chemical constituents of *Ocimum basilicum* flowers using different extraction techniques.



**Figure 3.1C:** Percentage mean peak area (%) of chemical constituents of *Ocimum basilicum* inflorescence using different extraction technique.

### 3.1.2 Chemical Composition of *Ocimum sanctum*

The chemical constituents identified from the Malaysian grown *Ocimum sanctum* are listed in Table 3.3. A total of 34 chemical compounds were identified in *Ocimum sanctum*. The peak number and molecular weight of compounds according to GC-MS chromatogram profile of *Ocimum sanctum* are shown in Appendix A. The GC-MS chromatogram profiles of leaves, flowers and inflorescence from *Ocimum sanctum* using hydrodistillation, solvent extraction and HS-SPME is shown in Appendix C. The percentage mean area peaks of different extraction techniques and different parts from *Ocimum sanctum* are illustrated in Figure 3.2 (A-C).

#### 3.1.2.1 Chemical Composition of *Ocimum sanctum* Leaves

The percentage yield of *Ocimum sanctum* leaves extracts obtained by hydrodistillation, dichloromethane extraction and methanol extraction were 0.20% (v/w), 0.21% (w/w) and 0.36% (w/w), respectively. The profile of hydrodistillation of *Ocimum sanctum* leaves is characterized by the presence of eugenol methyl ether (39.90%), caryophyllene (27.51%), germacrene D (9.62%),  $\beta$ -elemene (4.59%) and copaene (4.22%) as the major compounds. It was reported that Thai *Ocimum sanctum* leaves contain the highest percentage of eugenol methyl ether (Bunrathep *et al.*, 2007). The methanolic extract of *Ocimum sanctum* leaves yielded eugenol methyl ether (57.46%), caryophyllene (18.02%), germacrene D (5.58%),  $\beta$ -elemene (5.26%) and copaene (1.80%) while the dichloromethane extract on leaves of *Ocimum sanctum* yielded eugenol methyl ether (50.12%), caryophyllene (29.95%), germacrene D (6.58%),  $\gamma$ -muurolene (5.18%) and copaene (3.51%) as the main constituents. HS-SPME analysis of the volatiles from



*Ocimum sanctum* leaves showed eugenol methyl ether (34.34%), caryophyllene (22.15%), germacrene D (11.54%),  $\beta$ -elemene (9.16%) and copaene (4.62%) as the major compounds.

### 3.1.2.2 Chemical Composition of *Ocimum sanctum* Flowers

The percentage yield of *Ocimum sanctum* flowers extract obtained from dichloromethane extraction and methanol extraction were 4.28% (w/w) and 9.04% (w/w), respectively. The main constituent of *Ocimum sanctum* flowers from methanol extract were eugenol methyl ether (74.51%), caryophyllene (13.76%), germacrene D (9.34%), and copaene (2.39%) while eugenol methyl ether (62.94%), caryophyllene (13.35%), germacrene D (8.29%) and  $\beta$ -elemene (4.22%) were the main constituents in *Ocimum sanctum* flowers dichloromethane extract. HS-SPME analysis of *Ocimum sanctum* flowers yielded eugenol methyl ether (62.44%), germacrene D (12.73%), caryophyllene (11.09%),  $\beta$ -elemene (6.61%) and copaene (1.49%) as the main components.

### 3.1.2.3 Chemical Composition of *Ocimum sanctum* Inflorescence

The percentage yields of inflorescence extract obtained by dichloromethane extraction and methanol extraction were 11.76% (w/w) and 2.23% (w/w), respectively. The methanolic extract of *Ocimum sanctum* inflorescence yielded eugenol methyl ether (59.84%), germacrene D (11.01%), caryophyllene (9.77%),  $\beta$ -elemene (5.28%) and  $\beta$ -cubebene (3.67%) as the main components while the dichloromethane extract of *Ocimum sanctum* inflorescence yielded eugenol methyl ether (51.68%), caryophyllene (16.58%),

germacrene D (13.71%),  $\beta$ -elemene (9.60%) and copaene (5.08%) as the main components. HS-SPME analysis of *Ocimum sanctum* inflorescence yielded eugenol methyl ether (63.96%), germacrene D (13.71%), caryophyllene (7.91%),  $\beta$ -elemene (7.70%), and copaene (2.35%) as major compounds. The finding of eugenol methyl ether as the major constituent in inflorescence of *Ocimum sanctum* was concurrent with a report by Kothari *et al.* (2005) on the Indian *Ocimum sanctum* inflorescence.

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**Table 3.3:** Chemical constituents of leaves (L), inflorescences (I) and flowers (F) from *Ocimum sanctum*.

Peak #	*Compounds	Retention Indices (RI)	Relative Area (%)										
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME			
				L	L	F	I	L	F	I	L	F	I
1	$\alpha$ -thujene	931	0.26 $\pm$ 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4	camphene	948	0.24 $\pm$ 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	$\beta$ -pinene	976	0.65 $\pm$ 0.86	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	(Z)-3-Hexen-1-ol acetate	1005	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.26 $\pm$ 0.07	n.d.	n.d.	n.d.
9	limonene	1036	0.08 $\pm$ 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	0.13 $\pm$ 0.01	0.35 $\pm$ 0.03	n.d.	n.d.	n.d.
10	eucalyptol	1040	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.11 $\pm$ 0.01	n.d.	n.d.	n.d.
12	(E)- $\beta$ -ocimene	1046	n.d.	n.d.	n.d.	0.54 $\pm$ 0.02	n.d.	n.d.	n.d.	0.02 $\pm$ 0.00	n.d.	n.d.	n.d.

Table 3.3, continued

Peak #	*Compounds	Retention Indices (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
13	benzeneacetaldehyde	1051	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.22 ± 0.07	0.21 ± 0.13
14	γ-terpinene	1062	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.03 ± 0.00	n.d.	n.d.
18	linalool	1099	0.09 ± 0.02	n.d.	n.d.	0.88 ± 0.17	n.d.	n.d.	2.19 ± 0.60	0.51 ± 0.08	1.14 ± 0.14	0.75 ± 0.18
19	camphor	1154	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.03 ± 0.01	n.d.	n.d.
20	borneol	1179	0.31 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	1.73 ± 0.02	0.57 ± 0.23	0.87 ± 0.08	0.86 ± 0.60
21	estragole	1203	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04 ± 0.01	n.d.	n.d.
25	α-cubebene	1349	0.19 ± 0.03	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.43 ± 0.03	n.d.	0.13 ± 0.01

Table 3.3, continued

Peak #	*Compounds	Retention Indices (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
26	copaene	1379	4.22 ± 0.09	3.51 ± 0.13	n.d.	5.08 ± 0.07	1.80 ± 0.19	2.39 ± 0.83	3.31 ± 0.14	4.62 ± 0.19	1.49 ± 0.00	2.35 ± 0.27
27	$\beta$ -cubebene	1383	2.13 ± 0.09	n.d.	n.d.	n.d.	1.76 ± 0.47	n.d.	3.67 ± 0.66	n.d.	n.d.	n.d.
28	$\beta$ -elemene	1384	4.59 ± 0.11	n.d.	4.22 ± 0.85	9.60 ± 0.46	5.26 ± 0.76	n.d.	5.28 ± 4.90	9.16 ± 0.45	6.61 ± 0.33	7.70 ± 1.27
29	$\beta$ -bourbonene	1387	2.16 ± 0.09	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.28 ± 0.11
30	$\gamma$ -muurolene	1392	n.d.	5.18 ± 0.58	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 3.3, continued

Peak #	*Compounds	Retention Indices (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
31	eugenol methy ether	1403	39.90 ± 0.56	50.12 ± 0.80	62.94 ± 4.48	51.68 ± 1.19	57.46 ± 8.69	74.51 ± 2.27	59.84 ± 4.92	34.34 ± 2.60	62.44 ± 0.22	63.96 ± 3.83
33	( <i>E</i> )-caryophyllene	1417	27.51 ± 0.74	29.95 ± 0.11	13.35 ± 0.09	16.58 ± 0.17	18.02 ± 3.06	13.76 ± 0.04	9.77 ± 0.88	22.15 ± 1.61	11.09 ± 0.74	7.91 ± 1.43
35	<i>trans</i> - $\alpha$ -bergamotene	1429	0.21 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
38	$\epsilon$ -muurolene	1446	0.45 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	0.60 ± 0.15	n.d.	n.d.	n.d.
39	$\alpha$ -caryophyllene	1459	2.82 ± 0.02	1.43 ± 0.04	n.d.	n.d.	0.93 ± 0.99	n.d.	0.60 ± 0.09	2.66 ± 0.08	0.76 ± 0.01	0.49 ± 0.11
41	$\gamma$ -gurjunene	1479	0.24 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 3.3, continued

Peak #	*Compounds	Retention Indices (RI)	Relative Area (%)									
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME		
				L	L	F	I	L	F	I	L	F
43	$\alpha$ -amorphene	1480	0.43 $\pm$ 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.34 $\pm$ 0.04	n.d.	n.d.
42	germacrene D	1484	9.62 $\pm$ 0.16	6.58 $\pm$ 0.18	8.29 $\pm$ 0.27	13.71 $\pm$ 0.40	5.58 $\pm$ 0.69	9.34 $\pm$ 1.48	11.01 $\pm$ 0.40	11.54 $\pm$ 0.58	12.73 $\pm$ 0.76	13.71 $\pm$ 1.35
47	$\alpha$ -bulnesene	1511	0.09 $\pm$ 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
51	eugenol acetate	1525	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.10 $\pm$ 0.14	n.d.	n.d.	n.d.
48	$\delta$ -cadinene	1535	1.08 $\pm$ 0.03	n.d.	n.d.	0.58 $\pm$ 0.03	n.d.	n.d.	0.77 $\pm$ 0.16	1.05 $\pm$ 0.05	1.32 $\pm$ 0.01	1.07 $\pm$ 0.08
55	caryophyllene oxide	1574	0.75 $\pm$ 0.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.18 $\pm$ 0.02	n.d.	n.d.

Table 3.3, continued

Peak #	*Compounds	Retention Indices (RI)	Relative Area (%)										
			Hydrodistillation	Solvent Extraction (Dichloromethane)			Solvent Extraction (Methanol)			HS-SPME			
				L	L	F	I	L	F	I	L	F	I
56	isocaryophyllene	1580	n.d.	0.62 ± 0.16	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
53	$\tau$ -cadinol	1643	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.00 ± 0.07	n.d.
54	$\alpha$ -cadinol	1649	0.12 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Total			98.14	97.39	88.80	98.65	90.81	100.00	100.00	88.39	99.67	99.42	

\*Compounds identified by using Mass Spectra (MS) data and confirmed by Kovats Index

n.d = not detected

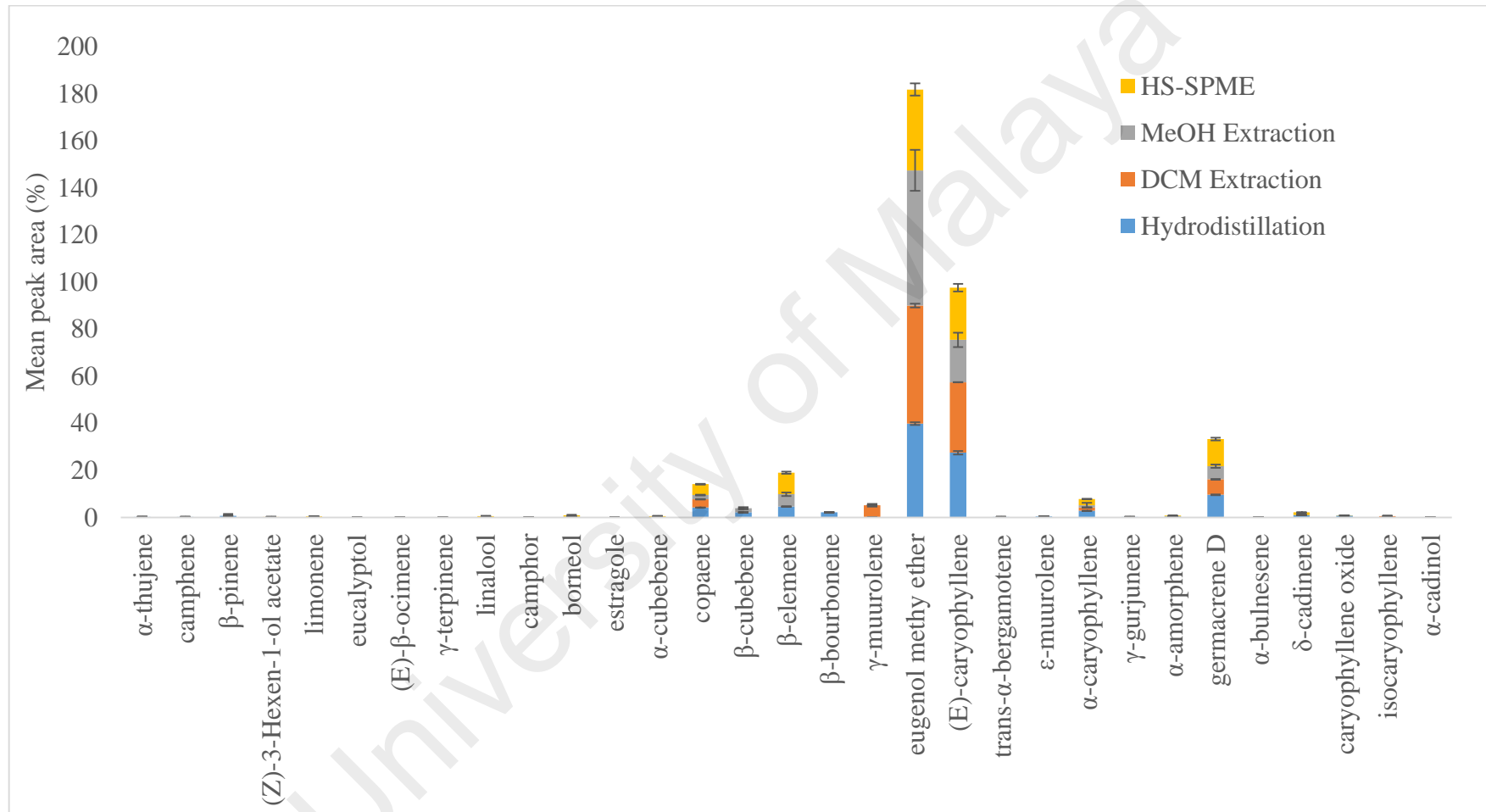
HS-SPME = Headspace-Solid Microextraction

L = Leaves

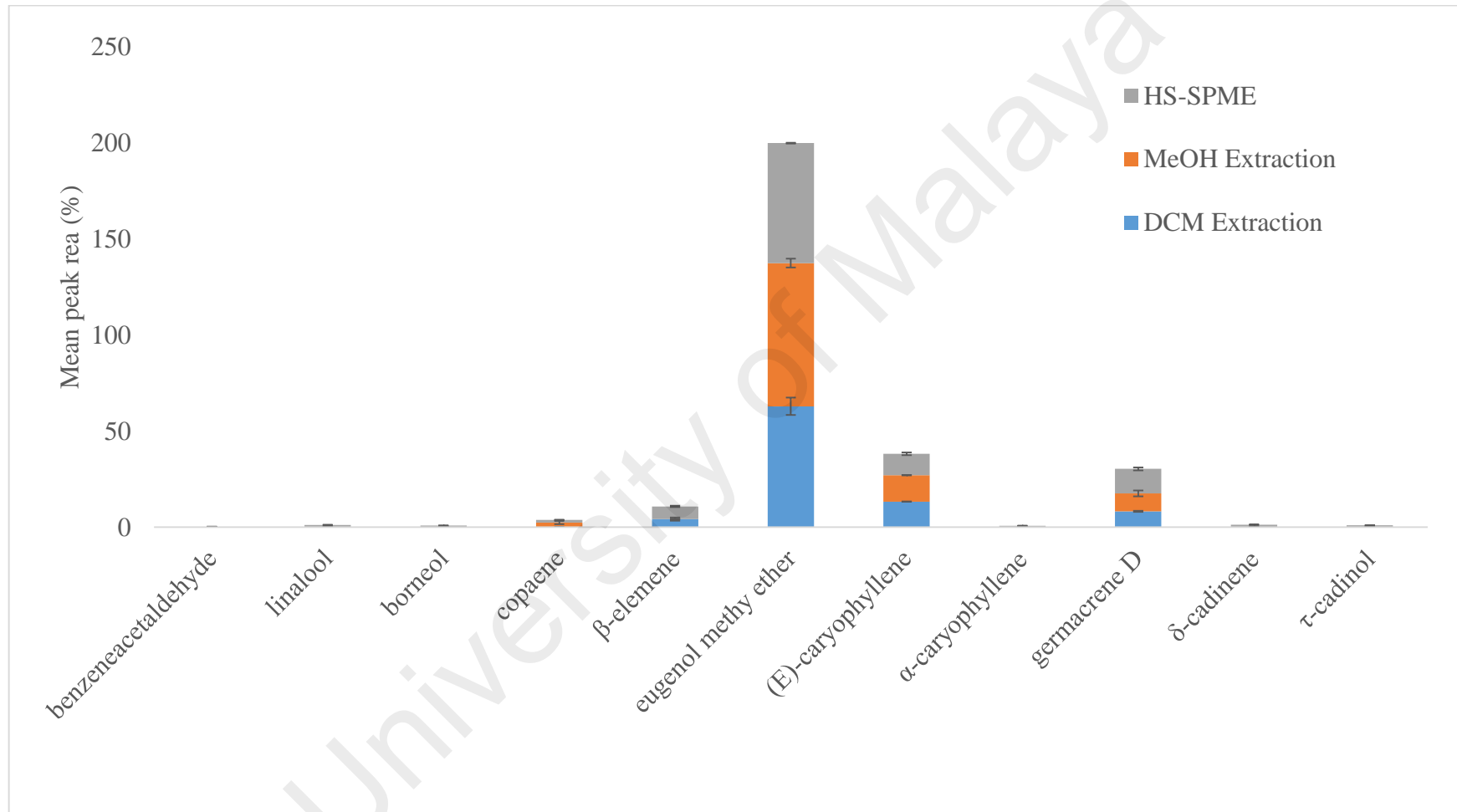
F = Flowers

I = Inflorescences

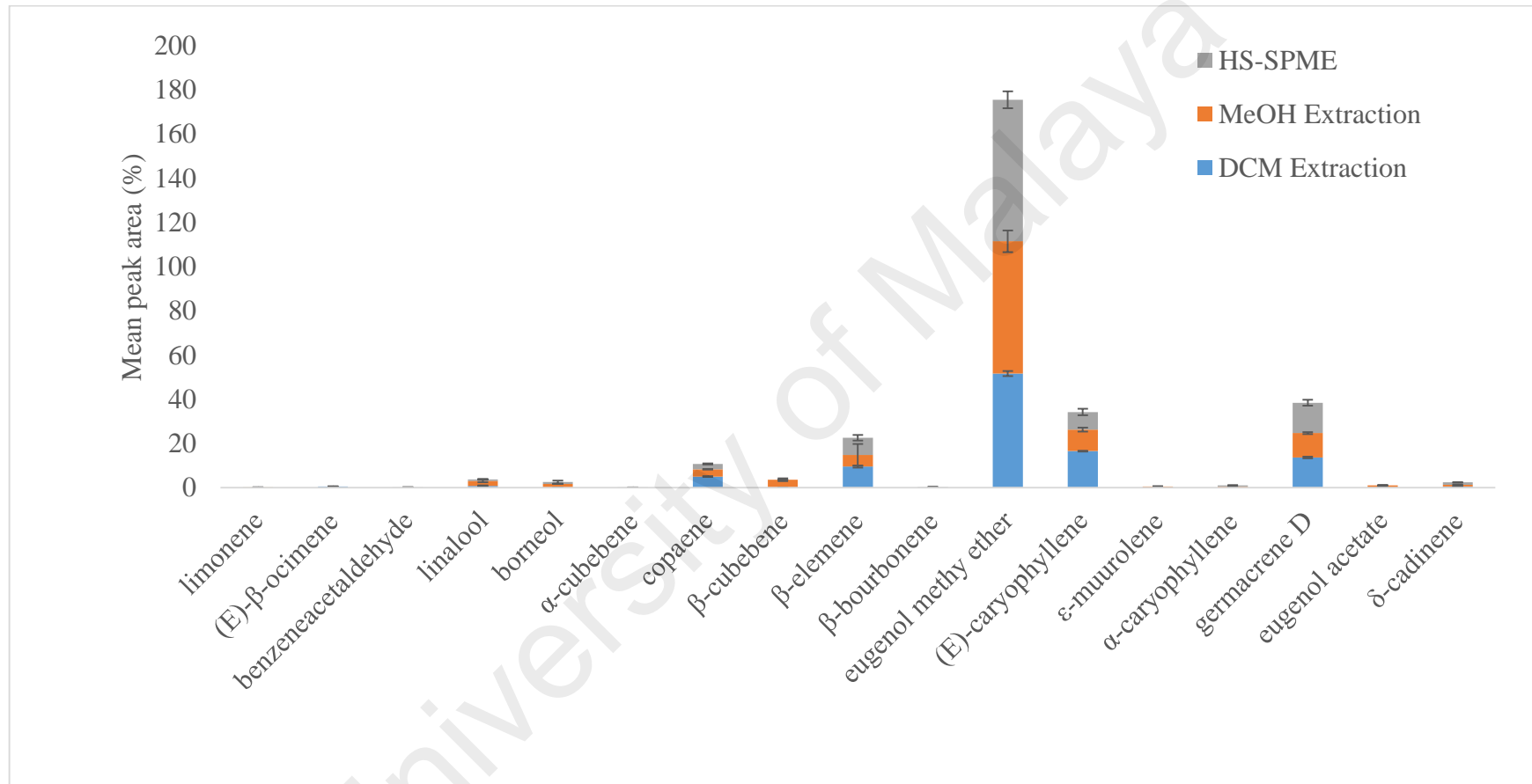




**Figure 3.2A:** Percentage mean peak area (%) of chemical constituents of *Ocimum sanctum* leaves using different extraction techniques.

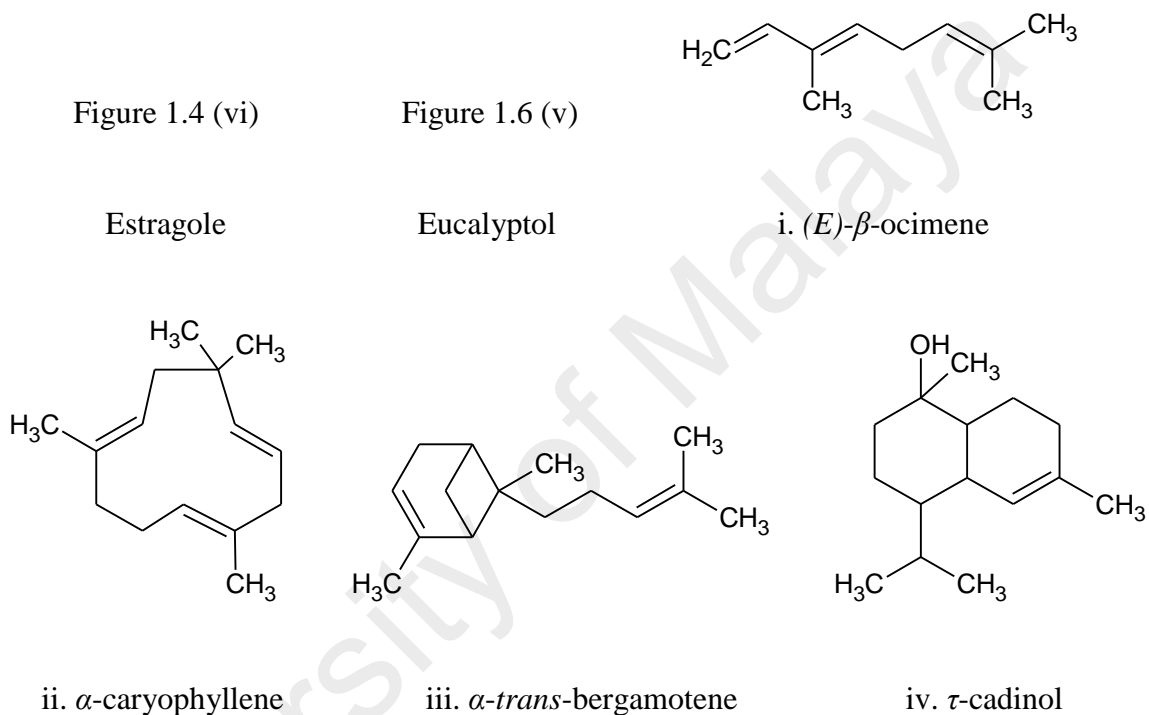


**Figure 3.2B:** Percentage mean peak area (%) of chemical constituents of *Ocimum sanctum* flowers using different extraction techniques.

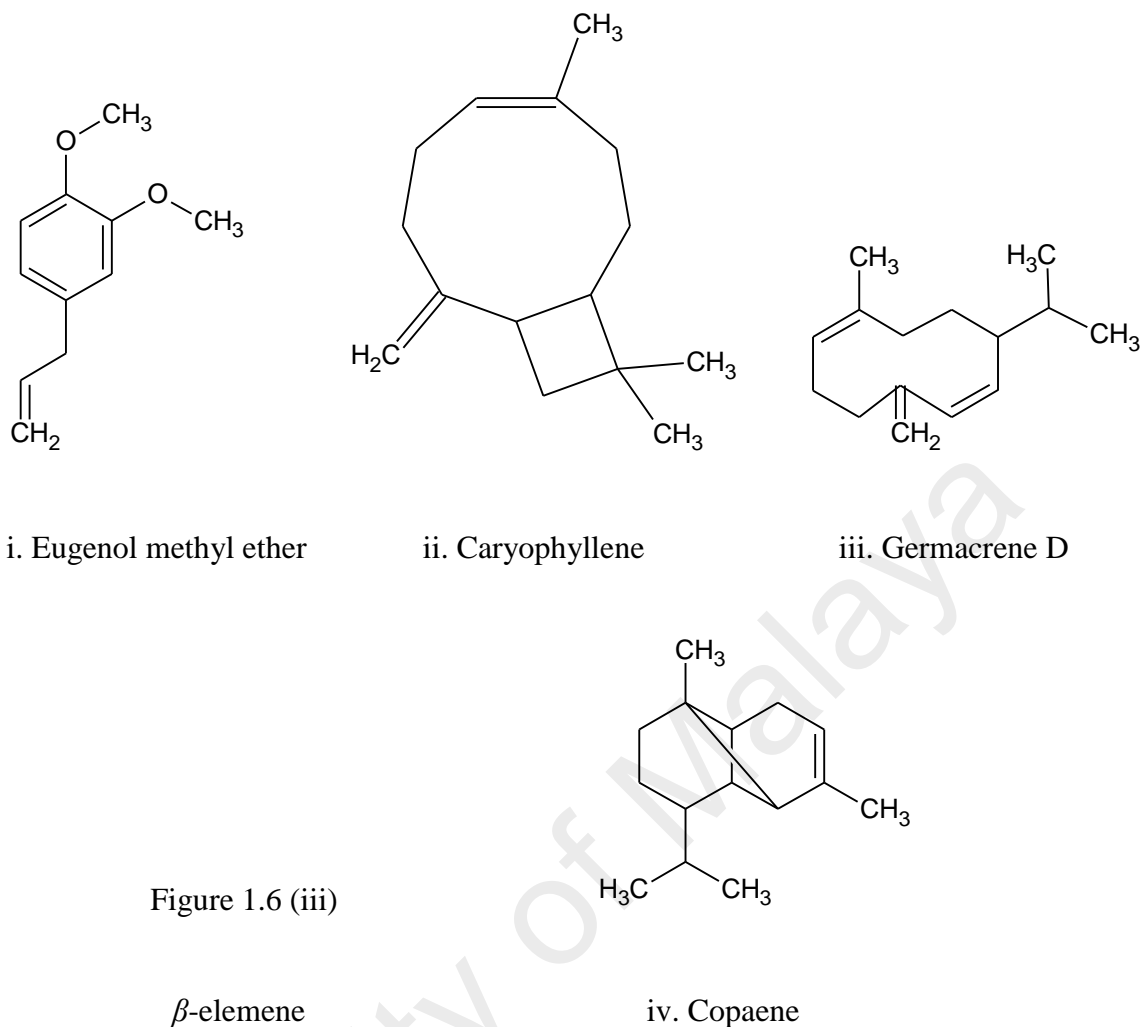


**Figure 3.2C:** Percentage mean peak area (%) of chemical constituents of *Ocimum sanctum* inflorescence using different extraction techniques.

From the identified chemical constituents in *Ocimum basilicum* and *Ocimum sanctum*, it was found that these two species were rich in phenylpropene and terpenes. The chemical structures of the major constituents of the two herbs are illustrated in Figure 3.3 and Figure 3.4.



**Figure 3.3:** The chemical structures of the major chemical constituents from *Ocimum basilicum* grown in Malaysia.



**Figure 3.4:** The chemical structures of the major chemical constituents from *Ocimum sanctum* grown in Malaysia.

Based on the present study, it was found that estragole was the major compound in *Ocimum basilicum*. *Ocimum basilicum* leaves contain the most chemical constituents, followed by inflorescence and flowers. Estragole was found abundantly in all three parts of *Ocimum basilicum*. In *Ocimum sanctum*, eugenol methyl ether was found to be the major compound. The similar trend observed in *Ocimum basilicum* was observed as well in *Ocimum sanctum* as the leaves contain more chemical constituents compared to inflorescence and flowers and eugenol methyl ether was found abundantly in all three parts. Table 3.4 shows the summary of the major compounds from the leaves, flowers and

inflorescence of *Ocimum basilicum* and *Ocimum sanctum* using different extraction techniques.

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**Table 3.4:** The major compounds from the leaves, flowers and inflorescence of *Ocimum basilicum* and *Ocimum sanctum* using different extraction techniques.

Species	Part of plant	Extraction technique	Major compound	Area percentage (%)
<i>Ocimum basilicum</i>	Leaves	Hydrodistillation	estragole	35.71
			eucalyptol	13.26
			( <i>E</i> )- $\beta$ -ocimene	7.99
			<i>trans</i> - $\alpha$ -bergamotene	5.82
			$\tau$ -cadinol	5.71
<i>Ocimum basilicum</i>	Leaves	Solvent extraction (Methanol)	estragole	82.69
			( <i>Z</i> )- $\beta$ -ocimene	1.26
			<i>trans</i> - $\alpha$ -bergamotene	0.83
			eugenol methyl ether	0.40
<i>Ocimum basilicum</i>	Leaves	Solvent extraction (Dichloromethane)	estragole	73.16
			eucalyptol	6.17
			<i>trans</i> - $\alpha$ -bergamotene	5.26
			( <i>E</i> )- $\beta$ -ocimene	4.52
			$\tau$ -cadinol	2.56

Table 3.4, continued

Species	Part of plant	Extraction technique	Major compound	Area percentage (%)
<i>Ocimum basilicum</i>	Leaves	HS-SPME	estragole eucalyptol <i>trans</i> - $\alpha$ -bergamotene $\alpha$ -caryophyllene camphor	59.67 9.02 8.60 2.65 1.62
<i>Ocimum basilicum</i>	Flowers	Solvent extraction (Dichloromethane)	estragole ( <i>Z</i> )- $\beta$ -farnesene	98.88 1.11
<i>Ocimum basilicum</i>	Flowers	Solvent extraction (Methanol)	estragole ( <i>Z</i> )- $\beta$ -farnesene	99.22 0.77
<i>Ocimum basilicum</i>	Flowers	HS-SPME	estragole <i>trans</i> - $\alpha$ -bergamotene ( <i>E</i> )- $\beta$ -ocimene eugenol methyl ether $\alpha$ -bulnesene	88.18 2.82 1.47 0.72 0.51
<i>Ocimum basilicum</i>	Inflorescence	Solvent extraction (Dichloromethane)	estragole <i>trans</i> - $\alpha$ -bergamotene ( <i>E</i> )- $\beta$ -ocimine linalool eucalyptol	57.85 7.24 6.59 1.84 1.71



Table 3.4, continued

<b>Species</b>	<b>Part of plant</b>	<b>Extraction technique</b>	<b>Major compound</b>	<b>Area percentage (%)</b>
<i>Ocimum basilicum</i>	Inflorescence	Solvent extraction (Methanol)	estragole ( <i>Z</i> )- $\beta$ -ocimene <i>trans</i> - $\alpha$ -bergamotene eucalyptol camphor	77.65 3.89 2.35 0.42 0.30
<i>Ocimum basilicum</i>	Inflorescence	HS-SPME	estragole $\alpha$ -cubebene ( <i>E</i> )- $\beta$ -ocimene <i>trans</i> - $\alpha$ -bergamotene linalool	86.39 1.86 1.70 1.44 0.81
<i>Ocimum sanctum</i>	Leaves	Hydrodistillation	eugenol methyl ether caryophyllene germacrene D $\beta$ -elemene copaene	39.90 27.51 9.62 4.59 4.22

Table 3.4, continued

<b>Species</b>	<b>Part of plant</b>	<b>Extraction technique</b>	<b>Major compound</b>	<b>Area percentage (%)</b>
<i>Ocimum sanctum</i>	Leaves	Solvent extraction (Methanol)	eugenol methyl ether caryophyllene germacrene D $\beta$ -elemene copaene	57.46 18.02 5.58 5.26 1.80
<i>Ocimum sanctum</i>	Leaves	Solvent extraction (Dichloromethane)	eugenol methyl ether caryophyllene germacrene D $\gamma$ -muurolene copaene	50.12 29.95 6.58 5.18 3.51
<i>Ocimum sanctum</i>	Leaves	HS-SPME	eugenol methyl ether caryophyllene germacrene D $\beta$ -elemene copaene	34.34 22.15 11.54 9.16 4.62
<i>Ocimum sanctum</i>	Flowers	Solvent extraction (Methanol)	eugenol methyl ether caryophyllene germacrene D copaene	74.51 13.76 9.34 2.39

Table 3.4, continued

<b>Species</b>	<b>Part of plant</b>	<b>Extraction technique</b>	<b>Major compound</b>	<b>Area percentage (%)</b>
<i>Ocimum sanctum</i>	Flowers	Solvent extraction (Dichloromethane)	eugenol methyl ether caryophyllene germacrene D $\beta$ -elemene	62.94 13.35 8.29 4.22
<i>Ocimum sanctum</i>	Flowers	HS-SPME	eugenol methyl ether germacrene D caryophyllene $\beta$ -elemene copaene	62.44 12.73 11.09 6.61 1.49
<i>Ocimum sanctum</i>	Inflorescence	Solvent extraction (Methanol)	eugenol methyl ether germacrene D caryophyllene $\beta$ -elemene $\beta$ -cubebene	59.84 11.01 9.77 5.28 3.67

Table 3.4, continued

<b>Species</b>	<b>Part of plant</b>	<b>Extraction technique</b>	<b>Major compound</b>	<b>Area percentage (%)</b>
<i>Ocimum sanctum</i>	Inflorescence	Solvent extraction (Dichloromethane)	eugenol methyl ether caryophyllene germacrene D $\beta$ -elemene copaene	51.68 16.58 13.71 9.60 5.08
<i>Ocimum sanctum</i>	Inflorescence	HS-SPME	eugenol methyl ether germacrene D caryophyllene $\beta$ -elemene copaene	63.96 13.71 7.91 7.70 2.35

In the present study, hydrodistillation and HS-SPME were found to extract more chemical constituents compared to solvent extraction. The solubility of the chemical constituents in different extraction procedures are affected by the polarity of solvent(s) used (Naczka & Shahidi, 2006). Dichloromethane and methanol used in solvent extraction and water used in hydrodistillation are categorized under polar solvents while PDMS used in HS-SPME is non-polar fibre. The least polar solvents are frequently considered to be suitable for the extraction of lipophilic phenols unless very high pressure is applied (Allothman *et al.*, 2009) and while polar solvents are commonly suitable for the extraction of polyphenols (Do *et al.*, 2014).

It is worth mentioning that the chemical compounds in methanol and dichloromethane extract might be lost during solvent removal process, due to highly volatile properties and having a small molecular weight (Walradt, 1982). Hydrodistillation required large amount of materials compared to HS-SPME which required the least amount of material between the other methods. Therefore, hydrodistillation is a more suitable method providing abundant access to material plant while HS-SPME is much preferable when the plant material is limited. In addition, no solvent peak present in HS-SPME chromatogram as HS-SPME requires no solvent in extracting process (Zhang *et al.*, 1994). Besides that, HS-SPME is used mainly for analysis or to test the quality of fruits and food and it was reported successfully applied to the quality assessments of apples (Lavilla *et al.*, 1999) and vegetable oils (Jeleń *et al.*, 2000). The quality of fruits and food can be tested by analysing the volatile compounds released by the fruits or food after a period of time.

### 3.1.3 Genetic Distances between *Ocimum basilicum* and *Ocimum sanctum*

Genetic distance is a statistical method measuring the genetic varieties between species or population (Nei, 1987). Species or populations with many similar alleles will have small genetic differences, and thus closely related to each other and have a recent same ancestor. Zero genetic distance indicates that there are no difference alleles or alleles with exact match. Genetic distance is important in understanding the origin of biodiversity. Different proposed methods that commonly used in measuring the genetic distance are Nei's standard genetic distance, Cavalli-Sforza chord distance and Reynolds, Weir, and Cockerham's genetic distance.

In Nei (1972), the effects of polymorphism within populations had been considered and the normalized identity of genes between populations had been defined. Nei (1972) then related it to the accumulated number of gene differences per locus. This method has several advantages compared to some other methods and these include related the method to Malecot's coefficient of kinship in a simplified manner, measures the accumulated number of gene substitutions per locus, the rate of gene substitution is linear to evolutionary time if it is constant and in some migration models it is linearly related to geographical distance or area. Nei (1972) had reported that when the two populations have the same alleles in identical frequencies, the normalized identity of genes between the two populations with respect to the locus is unity. On the contrary, when the two populations have the different alleles, it is zero.

The genetic distance was calculated by using Nei's statistical method to show interspecies relationship between *Ocimum basilicum* and *Ocimum sanctum*. The genetic distance was calculated based on compounds identified by GC-MS when hydrodistillation was carried out (Katrina *et al.*, 2015). X in the equation developed by Nei represents *Ocimum basilicum* while Y represents *Ocimum sanctum*. The probability of identity of two genes that has been chosen randomly is  $j_x = 1618.11$  in *Ocimum basilicum*, while for *Ocimum sanctum* it is  $j_y = 2500.06$ . The probability of identity of a gene from *Ocimum basilicum* and a gene from *Ocimum sanctum* is  $j_{xy} = 115.06$ . The normalized identity of genes between *Ocimum basilicum* and *Ocimum sanctum* with respect to all loci is defined as  $I = J_{XY}/\sqrt{J_X J_Y} = 0.0572$ , where  $J_X$ ,  $J_Y$  and  $J_{XY}$  are the arithmetic means of  $j_x$ ,  $j_y$  and  $j_{xy}$ , respectively, over all loci. Genetic distance,  $D$  was calculated by substituting the value of  $I$  in the equation below:

$$D = -\log_e J_{XY}/\sqrt{J_X J_Y}$$

$$= 2.86$$

Based on this study, the calculated genetic distance between the two species was 2.86. Therefore, the finding showed that *Ocimum basilicum* and *Ocimum sanctum* are related to each other, shared some similar alleles and these two species have a recent same ancestor.

### 3.2 Free Radical Scavenging Activity of *Ocimum basilicum* and *Ocimum sanctum* Leaves

The antioxidant activities of the various extracts of *Ocimum basilicum* and *Ocimum sanctum* leaves were determined by the DPPH radical scavenging ability of the extracts. Microplate reader was used to measure the absorbance of the assay. The results are expressed as IC<sub>50</sub> value, the concentration that causes a decrease in the initial DPPH concentration by 50%. A lower IC<sub>50</sub> value signifies a greater free radical scavenger by having the ability to scavenge the free radical at lower concentration. In this study, the free radical scavenging study was only executed on the leaves owing to a limited yield of flowers and inflorescence extracts.

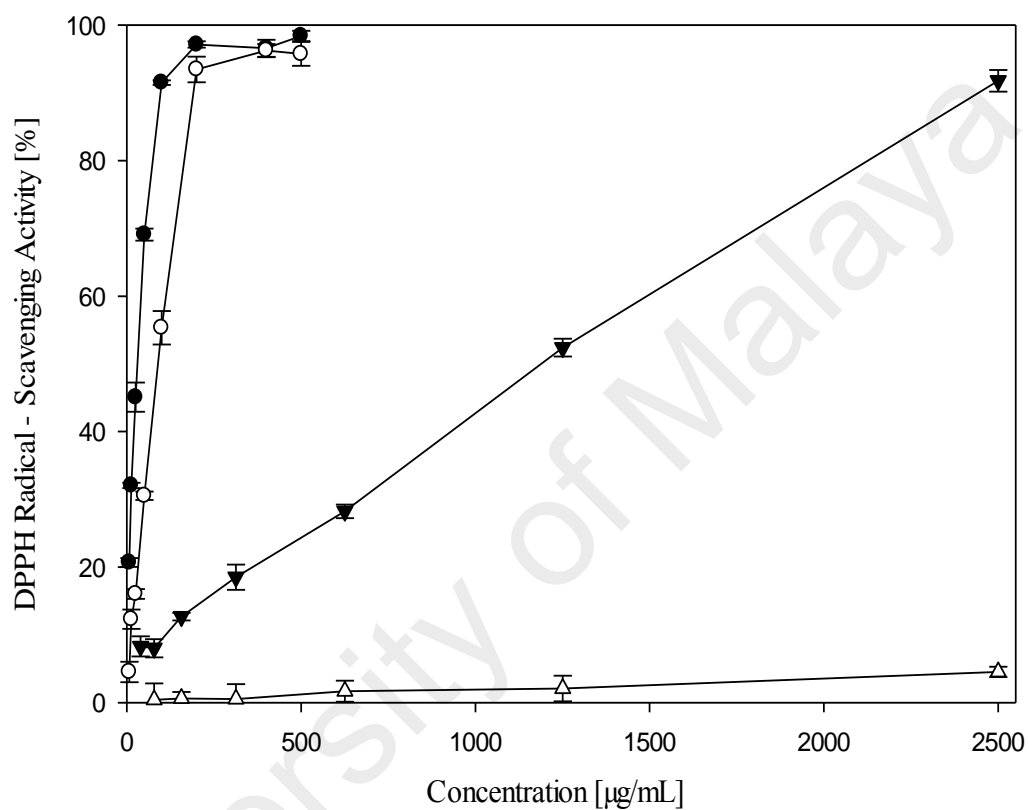
The violet colour of DPPH disappeared and turned to a pale yellow when the solution was tested with the methanol and dichloromethane extracts of *Ocimum basilicum* and *Ocimum sanctum* leaves. However, different observation was obtained when the solution was tested with the leaves hydrodistillation extract of the two species. The violet colour of DPPH changed to a lighter shade of violet. There is a significant positive relationship between concentration of *Ocimum basilicum* and *Ocimum sanctum* leaves extracts with the DPPH radical scavenging activity ( $p < 0.05$ ). *Ocimum basilicum* leaves methanol extract showed a significantly lower IC<sub>50</sub> value of 88 µg/mL as compared to dichloromethane extract (IC<sub>50</sub> value of 1178 µg/mL). BHT that was used as positive control in this investigation showed an IC<sub>50</sub> value of 29 µg/mL. Figure 3.5 shows graph of the antioxidant activity of *Ocimum basilicum* leaves extracts. BHT was expected to show a lower IC<sub>50</sub> value being a competent synthetic antioxidant. The hydrodistillation extract could not provide IC<sub>50</sub> values even though the concentration had been increased



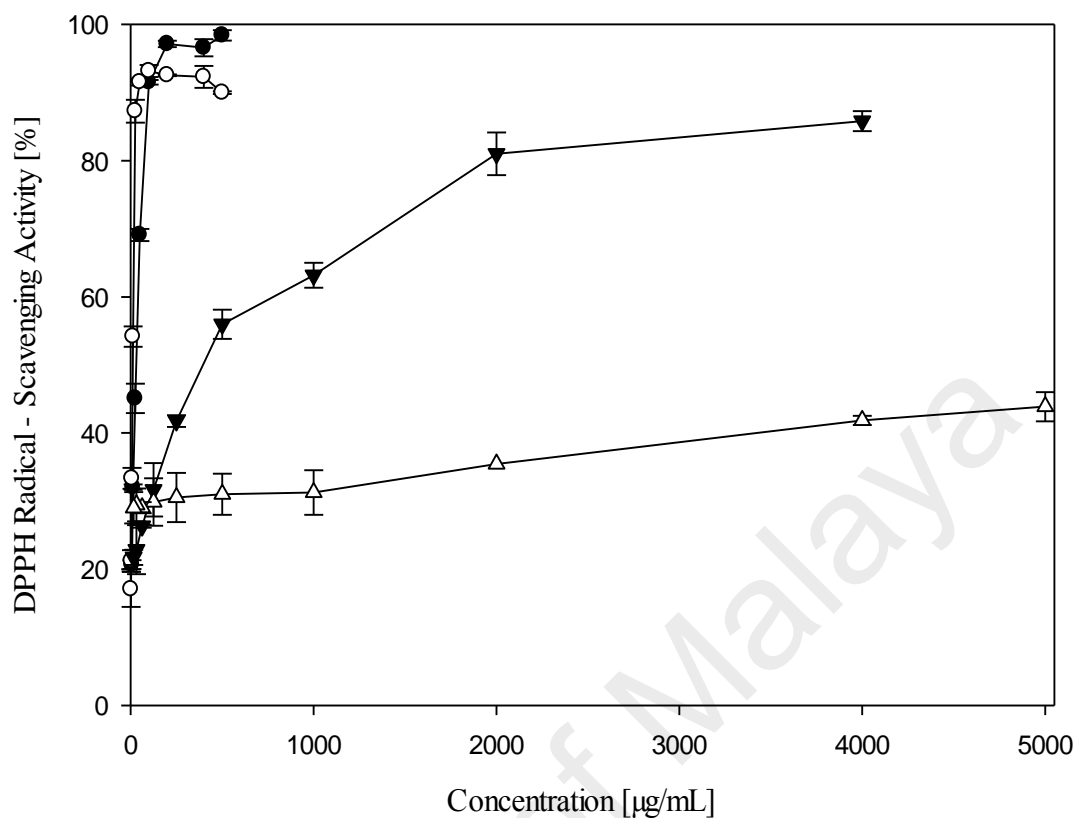
to 2500  $\mu\text{g/mL}$ . The hydrodistillation extract was expected to have lower radical scavenging activity due to lower concentration of oxygenated compounds and the presence of hydrocarbons (Kulisic *et al.*, 2004) as compared to the methanol and dichloromethane extracts of *Ocimum basilicum* leaves. It was reported that compounds with hydroxyl groups sterically hindered by a *t*-butyl group does affect the antioxidant activity (McGowan *et al.*, 1959). *Ocimum sanctum* leaves methanol extract showed a lower  $\text{IC}_{50}$  value of 11  $\mu\text{g/mL}$  compared to its dichloromethane extract which was 369  $\mu\text{g/mL}$ . Similar to hydrodistillation leaves extract of *Ocimum basilicum*, hydrodistillation extract of *Ocimum sanctum* showed a very weak free radical scavenging activity, even though the concentration had been increased to 5000  $\mu\text{g/mL}$ . Figure 3.6 shows the antioxidant activity of *Ocimum sanctum* leaves extracts. The results showed that *Ocimum sanctum* had a stronger free radical scavenging capacity compared to *Ocimum basilicum*. The major compounds that contributed to the free radical scavenging activities of *Ocimum basilicum* and *Ocimum sanctum* were possibly eugenol methyl ether, estragole,  $\beta$ -ocimene and  $\alpha$ -caryophyllene (Ruberto & Baratta, 1999).

Figure 3.7 shows the bar chart of the  $\text{IC}_{50}$  values of the different extracts from *Ocimum basilicum* and *Ocimum sanctum* leaves. The  $\text{IC}_{50}$  value of *Ocimum sanctum* leaves methanol extract was found lower than BHT and this finding showed that the extract has stronger DPPH scavenging activity compared to BHT. The finding is maybe due to the presence of eugenol methyl ether as this compound has strong DPPH scavenging activity against BHT. It was reported that the essential oil composition of three *Melaleuca* species with eugenol methyl ether identified as the principal component, showed lower  $\text{IC}_{50}$

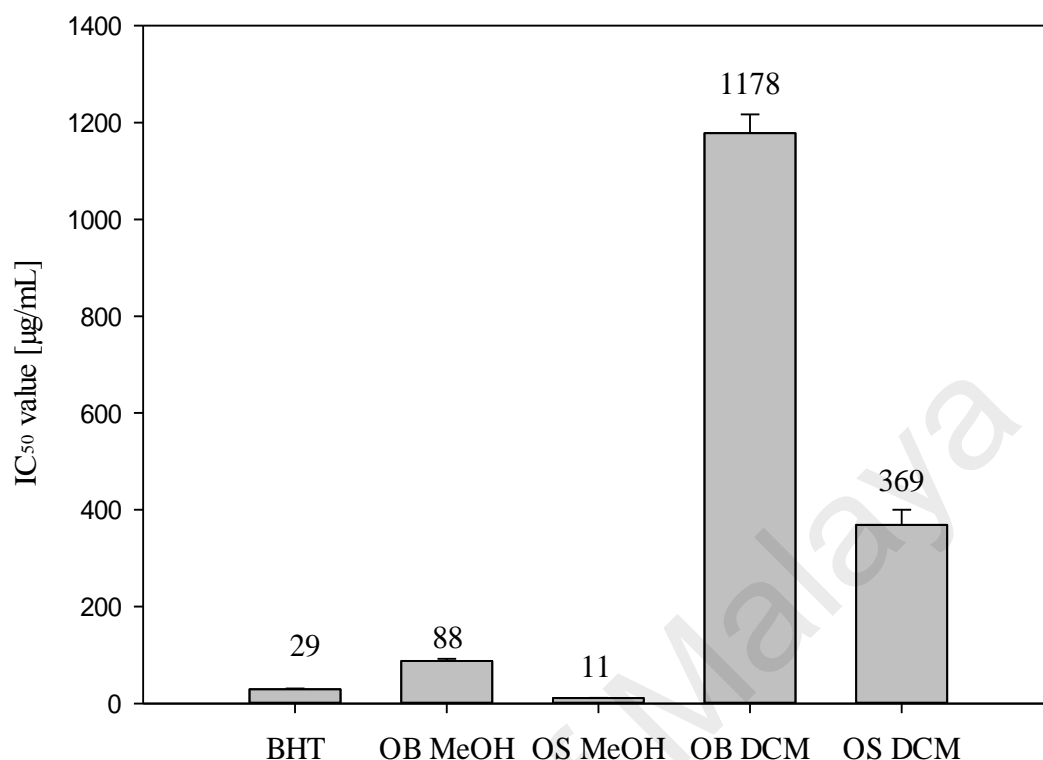
values ( $37.30 \pm 0.90 \mu\text{g/mL}$ ,  $37.80 \pm 1.60 \mu\text{g/mL}$  and  $39.10 \pm 0.30 \mu\text{g/mL}$ ) compared to BHT ( $41.50 \pm 0.50 \mu\text{g/mL}$ ) (Siddique *et al.*, 2017).



**Figure 3.5:** Antioxidant capacity of *Ocimum basilicum* leaves of (○) methanol extract, (▼) dichloromethane extract and (△) hydrodistillation extract as compared to (●) butylated hydroxytoluene standard.



**Figure 3.6:** Antioxidant capacity of *Ocimum sanctum* leaves of (○) methanol extract, (▼) dichloromethane extract and (Δ) hydrodistillation extract as compared to (●) butylated hydroxytoluene standard.



**Figure 3.7:** IC<sub>50</sub> values of butylated hydroxytoluene (BHT), methanol extract of *Ocimum basilicum* leaves (OB MeOH), methanol extract of *Ocimum sanctum* (OS MeOH), dichloromethane extract of *Ocimum basilicum* leaves (OB DCM) and dichloromethane extract of *Ocimum sanctum* (OS DCM).

It is worth mentioning that the adverse health effects of synthetic antioxidants remain the main concern in their usage even though they are extremely effective as an antioxidant. BHA and BHT are competent antioxidants at the lower range of concentrations. However, at high concentrations, they are pro-oxidant (Reische *et al.*, 2008; Halliwell *et al.*, 1995). BHT has detrimental effects on the liver (Simàn & Eriksson, 1996) and enhanced the cell death of lung tumour cells (Sarafian *et al.*, 2002). Besides that, TBHQ was proven to be cytotoxic in human monocytic leukaemia U937 cells (Okubu *et al.*, 2003). It was also reported that BHT and propyl gallate restrain humoral immunity by suppressing regulation of T cells or action of macrophages on B cells (Kim *et al.*, 1996). Considering the health effects of the addition of the antioxidants in food,

various regulations on the usage level are implemented in different countries. Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA) allowed maximum usage level of 0.02% and 0.01% of weight fat, respectively, for general use individually of BHT, BHA, TBHQ and propyl gallate (Shahidi, 2003). On the other hand, Europe, United Kingdom, Norway, Denmark, Sweden, Switzerland and Japan have banned the usage of TBHQ as a food additive in their countries.

Antioxidant is one of the dietary factors that play an important role in preventing cancer. *Ocimum basilicum* and *Ocimum sanctum* leaves were reported to have reduced the number of azoxymethane induced Aberrant Crypt Foci in Fisher 344 male rats. Therefore, the *Ocimum basilicum* and *Ocimum sanctum* leaves have potential in being chemopreventive agents (Gajula *et al.*, 2009).

## CHAPTER 4: CONCLUSION

### 4.1 The Chemical Constituents from the Leaves, Inflorescence and Flowers of *Ocimum basilicum* and *Ocimum sanctum* Grown in Malaysia by Using Different Extraction Techniques.

A total of 47 chemical compounds were identified in *Ocimum basilicum* while a total of 34 chemical compounds were identified in *Ocimum sanctum*. From the identified chemical constituents in *Ocimum basilicum* and *Ocimum sanctum*, it was found that these two species were dominated by the presence of phenylpropenes, monoterpenes, oxygenated monoterpenes, sesquiterpenes and oxygenated sesquiterpenes.

Based on this study, it was found that estragole was the major compound in *Ocimum basilicum*. *Ocimum basilicum* leaves contain the most chemical constituents, followed by inflorescence and flowers. Estragole was found abundantly in all three parts of *Ocimum basilicum*. The similar trend was observed in *Ocimum sanctum* as the leaves contain more chemical constituents compared to inflorescence and flowers and eugenol methyl ether was found abundantly in all three parts as the major compound.

The chemical constituents extracted depend highly on the extraction methods as the solubility of the chemical constituents in different extraction procedures affected by the polarity of solvent used. The least polar solvents are commonly suitable for the extraction

of lipophilic phenols unless very high pressure is applied while polar solvents are commonly suitable for the extraction of polyphenols.

Solvent extraction is an easy and simple method. Hydrodistillation is a more suitable method providing abundant access to material plant while HS-SPME is much preferable when the plant material is limited and requires no solvent in extracting process.

#### **4.2 The Interspecies Relationship between *Ocimum basilicum* and *Ocimum sanctum***

The calculated Nei's genetic distance between *Ocimum basilicum* and *Ocimum sanctum* grown in Malaysia was 2.86. Based on the calculated genetic distance, it can be concluded that *Ocimum basilicum* and *Ocimum sanctum* are related to each other, shared some similar alleles and these two species have a recent same ancestor.

#### **4.3 Free Radicals Scavenging Activity of *Ocimum basilicum* and *Ocimum sanctum* Leaves Extracts by Using 2,2-diphenyl-1-picryl-hydrazyl (DPPH) Radical Scavenging Assay.**

For the antioxidant study, *Ocimum basilicum* leaves methanol extract showed a significantly lower IC<sub>50</sub> value of 88 µg/mL as compared to its dichloromethane extract with the value of 1178 µg/mL. BHT was used as positive control in this investigation showed an IC<sub>50</sub> value of 29 µg/mL. The hydrodistillation extract could not provide IC<sub>50</sub>

values even though the concentration had been increased to 2500 µg/mL. *Ocimum sanctum* leaves methanol extract showed a lower IC<sub>50</sub> value of 11 µg/mL compared to its dichloromethane extract which was 369 µg/mL. Similar to hydrodistillation leaves extract of *Ocimum basilicum*, hydrodistillation extract of *Ocimum sanctum* showed a very weak free radical scavenging activity. Based on these findings, it can be concluded that *Ocimum sanctum* has a much stronger radical scavenging activity compared to *Ocimum basilicum* and BHT. Even though *Ocimum basilicum* showed a lower free radical scavenging activity, it still contains a considerable amount of activity. In the nutshell, *Ocimum basilicum* and *Ocimum sanctum* are considerable good antioxidant and free radical scavengers.

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

### Proceedings

Khairun Fadila Saaban, Sook Mei Khor and Cheng Hock Chuah. (2014).  
Chromatographic Analysis and Antioxidative Activity of Fragrances from *Ocimum  
basilicum*. Poster presented at The 9<sup>th</sup> Mathematics Physical Science Graduate Congress  
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