CHAPTER 1

INTRODUCTION

Cancer is a major public health problem in many parts of the world (Siegel *et al.,* 2013) and about 12.7 million cancer cases worldwide were reported with 7.6 million cancer deaths in 2008 (Jemal *et al.,* 2011). Cancer, as defined by World Health Organization (2013) and American Cancer Society (2012), is a group of disease characterized by uncontrolled growth of the cells which leads to accumulation of cells and producing tumour and spread of the abnormal cells.

Chemotherapy is one of the approaches widely used in treating cancer. However, the treatment is reported to have negative side effects (Naidu *et al.,* 2013). According to Plenderleith (1990), the negative side effect including toxicity might be caused by the frequent use of combinations of anticancer agents. The toxic effects that are commonly observed include nausea, vomiting, hair loss, irritation, ulcer, and bone marrow toxicity. Cardiac toxicity is associated with the use of the anthracycline groups of anticancer agents that include doxorubicin, daunorubicin, epirubicin, and mitoxantrone. Due to these side effects, studies in developing new anticancer agents that have less or no negative side effects and toxicity will always be important.

Usage of medicinal plants is one of the alternative treatments which play a significant role and it can be exploited into discovering novel anticancer drugs since plant-based or natural anticancer drugs have no negative side effects or toxicity (Kaur *et al.,* 2011). The potential and the value of medicinal plants had been identified for many years by traditional healers and old-folklore practices. Scientist too believe that plants have great biomedical potential and possessed broadrange of bioactive chemical compounds that can be used to treat various human diseases and offer protection against

a variety of chronic ailments including cardiovascular diseases, obesity, diabetes, and cancer (Cragg & Newman, 2005; Bernhoft, 2010; Kaur *et al.,* 2011). Plants' chemical compounds are secondary metabolites that can be classified into three main families, phenolics, terpenes and steroids, and alkalaoids (Harborne, 1999; Bourgaud *et al.,* 2001).

Approximately 60% of available cancer chemotherapeutic drugs were discovered from natural products produced by medicinal plants such as vinblastine, vincristine, paclitaxel, and combretastin (Newman *et al.,* 2003; Cragg *et al.,* 2005). According to Cragg & Newman (2005), the search for anticancer agents from plant sources started in the 1950s with the discovery and development of the vinca alkaloids, vinblastine, vincristine, and the isolation of the cytotoxic podophyllotoxins. Vinblastine and vincristine were isolated from the Madagascar periwinkle, *Catharanthus roseus* G. Don. (Apocynaceae), which was used by various cultures for the treatment of diabetes (Gueritte & Fahy, 2005). While under investigation, the plant extracts were found to be active against lymphocytic leukaemia in mice. This led to the isolation of vinblastine and vincristine as the active agents and development of synthetic analogues of these agents, vinorelbine and vindesine. These agents are primarily used in combination with other cancer chemotherapeutic drugs for the treatment of a variety of cancers, including leukaemia, lymphomas, advanced testicular cancer, breast and lung cancers, and Kaposi's sarcoma (Cragg & Newman, 2005).

Malaysia is rich with various types of flora and fauna and is listed as one of the 17 mega-biodiversity country. According to the Forestry Department of Peninsular Malaysia (FDPM), this "mega-store" is the habitat to approximately 17,631 species of flora including 377 algae, 1,387 bryophytes, 1,600 ferns and its families, 61 gymnosperms, 4,180 monocotyledons and 10,026 dicotyledons. Many plants in Malaysia remains relatively under studied and their phytochemical and cytotoxicity

potential virtually unknown. Even documented information of a large number of plants on their pharmacognostic parameters including the macroscopic characteristics and their phytochemical profiles are severly lacking. In view of this, the current study is been undertaken to inch through and narrow the huge gap of basic studies on native plants of Malaysia. Due to funding and time factors only four species, all belonging to the same family (Melastomataceae) were choosen for this work. These plants are *Melastoma muticum* Ridl., *Melastoma sanguineum* Sims, *Memecylon caeruleum* Jack, and *Phyllagathis rotundifolia* Bl. Thus, the specific objectives of this study are:

i. to characterize their macroscopic characteristic of the vegetative structures;

ii. to screen the phytochemicals of the crude methanol extract;

iii. to investigate the cytotoxicity potential of the crude methanol, hexane and ethyl acetate extracts by *in vitro* growth assay on human breast MCF-7 cancer cells and human ovarian SKOV-3 cancer cells;

iv. to determine the major chemical constituents of the cytotoxically active crude extract(s) in relation to their cytotoxic activity by LC-MS/MS system, and

v. to evaluate the antioxidant activities of crude methanol, hexane and ethyl acetate extracts.

CHAPTER 2

LITERATURE REVIEW

2.1 Plants and their Medicinal Properties

Natural sources are gaining attention as a source for new compounds with potential therapeutic values. This is due to large chemical diversity found in different species of plants, animals, marine organisms and microorganisms (Nor Hazalin *et al.*, 2012). Plants produce a broad range of bioactive chemical compounds via secondary metabolism and these compounds may have medicinal properties beneficial to humans and animals (Bernhoft, 2010). The use of plant-derived compounds has a long tradition in the history of mankind both as medicines and as supplements to support normal physiological functions of the body (Flatlandsmo, 2010). However many wild plants from the forest and actively used by traditional healers have not been thoroughly investigated for their bioactivities in order to identify the bioactive compounds that possess their medicinal properties (Paulsen, 2010).

Since thousands of years ago, plants have been manipulated as medicines (Samuelsson, 2004) in countries like Egypt, China, India, and Greece (Kaur *et al.*, 2011) and the application of the plant materials to treat specific illnesses were a secret passed down to close associates only by oral communication. However, as time passed by much of the information regarding plants and their therapeutic values have been recorded (Balunas and Kinghorn, 2005). The best known Egyptian pharmaceutical record is the "Ebers Papyrus", which documented over 700 drugs, marked the Egyptian medicine dated from 1500 B.C. The Chinese "Materia Medica" has described more than 600 medicinal plants with the first record dating to about 1100 B.C (Kaur *et al.*, 2011). The Ayurvedic system was documented in *Susruta* and *Charaka* dates from about 1000

B.C and Dioscorides, the Greek physician (100 A.D) described more than 600 medicinal plants in his work "*De Materia Medica*" (Kappor, 1990).

Till today, plant-derived natural products still remain as an essential component in the search for medical treatments (WHO, 2008) to fight against various pharmacological targets including cancer, HIV/AIDS, Alzheimer's, malaria, and pain (Balunas and Kinghorn, 2005). Aspirin, a drug that is derived from the willow tree (the family Salicaceae), is a well known plant-derived natural product. In the book "Aspirin: The Remarkable Story of a Wonder Drug" authored by Jeffereys D. (2004), it is indicated that the ancient Egyptians used willow bark as a remedy for aches and pains. He further elaborated that they did not know that what was reducing body temperature and inflammation was the salicylic acid. In the late 1890s, Felix Hoffmann, a chemist at Bayer developed aspirin in the form of acetylsalicylic acid (Landau, 2010). Jeffereys also added that nowadays, aspirin is universally recognized as a heart-attack prevention and potential anticancer drug (Schnedorf *et al.,* 1936).

World Health Organization (WHO) reported that medicinal plants are supplied through wild collections and cultivation (Bagozzi, 2004). According to Kumar *et al*., (2006), natural resources remain as a main choice for drug development and approximately 60–80 % of the world's population still believe in traditional medicine and much of these are plant-based. The tropical rain forest has been the source for the discovery of new compounds and drugs. Although these studies have been going on for a long time, it is only still done for a relatively small number of plants (Sakong *et al.,* 2011). Malaysia is listed as one of the mega-biodiversity countries of the world and plant life is enormous. This makes it even harder to choose a candidate species for studies and normally the "lead" is through ethnobotanical records of the plants used by *orang asli* (native people) for therapeutic purposes. The current study was based on these records as well as choosing sister species or closely related species that are found

only in the forest. Since these sister-species are not so frequently encountered, it is worthwhile to investigate whether it has potent therapeutic values compared to its well-recorded relatives.

There are large numbers of species or families to choose candidate species for this study and four species from the flowering plant family Melastomataceae were chosen for a number of reasons. Firstly, Melastomataceae is a large family with among the most abundant and diversified groups of plants throughout the tropics. They comprise of shrubs, woody climbers, herbs, or trees and occur in a variety of habitats. This family comprises about 4570 species in 150–166 genera (Clausing & Renner, 2001; Renner *et al.,* 2001). Secondly, the four species that were chosen belong to three genera; *Melastoma*, *Memecylon*, and *Phyllagathis*. All three genera have species that has records of therapeutic values therefore making the related species to potentially possess these values as well. Thirdly, three of the species investigated, *M. muticum*, *M. sanguineum*, and *M. caeruleum*, are relatively rare species occurring in restricted geographical range. These species have no records of therapeutic values or other ehtnobotanical uses perhaps due to its very limited availability.

2.2 Therapeutic Values of the Species in the Genus *Melastoma*

The genus *Melastoma* is widely distributed in tropical and subtropical Asia and northern Australia and with about 50 species (Meyer, 2001). In the Southeast Asia region alone, the genus *Melastoma* comprises of 22 species, 2 subspecies, and 3 varieties (Rajenderan, 2010). One of the most common and widely occurring species is *M. malabathricum*. This species has also been widely used in folklore remedies for the treatment of numerous sicknesses (Omar *et al.* 2012). This species locally known as '*senduduk*' have gained herbal status in the Malay folklore and have been widely studied for its phytochemical properties and pharmacological potentials in Malaysia

(Joffry *et al.,* 2012). Different communities/tribes using *M. malabathricum* as medicine based on their traditional beliefs is listed in Table 2.1. The whole plant including its leaves, shoots, and roots could be used as herbal medicine to heal different diseases.

Even though *M. malabathricum* demonstrated various types of bioactive compounds and various pharmacological activities, other species of *Melastoma* remains relatively under studied and their phytochemical and cytotoxicity potential remains unknown. Table 2.2 shows bioactivity records of several species in this genus.

Table 2.1: Reported medicinal uses of *M. malabathricum* by various communities/tribes around the world.

Communities/ Tribes	Country	Plant parts	Medicinal uses
Gayo and Alas	Aceh, Sumatera, Indonesia	Flowers	The cold infusion of *M. malabathricum* flowers is an optional ingredient to oral remedy for anemia associated with gastrointestinal bleeding and epigastric pain (Elliott & Brimacombe, 1987).
Talang Mamak	Riau Sumatera, Indonesia	Leaves	The ground leaves are applied to cuts and wounds (Grosvenor *et al.,* 1995).
Sundanese	Bogor, West Java, Indonesia	Leaves	The leaves are used as topical application or oral ingestion to treat toothache and for postpartum remedy (Roosita *et al.,* 2008).
Malay	Machang, Kelantan, Malaysia	Fruits	The fruit juice is applied on dry lips (Ong & Nordiana, 1999).
	Gemencheh, Negeri Sembilan, Malaysia	Leaves	The pounded leaves are applied onto wounds to accelerate healing (Ong & Norzalina, 1999).
Jah Hut	Jerantut, Pahang, Malaysia	Roots	The roots are applied as decoction to treat diarrhea (Lin, 2005).
Lakher and Pawi	Mizoram, India	Leaves	The decoction of the leaves or its juice is taken orally to treat diarrhea and dysentery (Sharma *et al.,* 2001).
Naga	Manipur, India	Leaves	The fresh and dried leaves are used to treat cuts and wounds, stomach disorder, and fever (Ringmichon *et al.,* 2010)
Didayi	Malkangiri, Orissa, India	Leaves	The leaves are applied externally as paste to treat cuts and wounds (Pattanaik *et al.,* 2008)
Marmas	Bandarban, Bangladesh	Roots	The root juice is used to treat jaundice ([a]Rahmatullah *et al.,* 2009)

Table 2.1, continued

Garo	Netrakona, Bangladesh	Leaves	The juice of the leaves is used as a diuretic and to treat various urinary problems ([b]Rahmatullah *et al.,* 2009)
Murong	Rangamati, Bangladesh	Roots	The juice or water extract of the roots are used orally to treat leucorrhea (Rahmatullah *et al.,* 2010)

(Adapted from Joffry *et al.,* 2012)

Table 2.2: Scientific findings related to pharmacological properties of selected *Melastoma* species.

Plant	Distribution	Bioactivity
Melastoma malabathricum	Southeast Asia region (Malaysia and Indonesia)	70 % methanol extracts of combined *M. malabathricum* leaf, stem, and flower is reported to have antibacterial activity against *Staphylococcus aureus*, *Saccharomyces cerevisiae*, and *Fusarium oxysporum* using the agar diffusion assay (Grosvenor *et al.,* 1995). Methanolic extract of *M. malabathricum* plant had nematocidal activity (antiparasitic) against *Bursaphelenchus xylophilus* with minimum effective dose of approximately 5 mg/bl (Alen *et al.,* 2000). Antioxidant potential of hexane, ethyl acetate, and methanol extracts of leaves of *M. malabathricum* with white flowers (Susanti *et al.,* 2008). Methanol extracts of leaves of *M. malabathricum* exhibited cytotoxic activity with IC_{50} value <25 µg/ml against murine cell lines (Lewis lung carcinoma cells (3LL) and leukaemic cells (L1210)) (Lohèzic-Le Dèvèhat *et al.,* 2002). Hot and cold water extract, and methanol extract of leaves of *M. malabathricum* were assayed for anticoagulant property using blood samples drawn from healthy volunteer donor and they were reported to have anticoagulant activity (Manicam *et al.,* 2010). Aqueous extract of the leaves (concentration range from 10 – 100 %) were assayed using carrageenan-induced paw edema in rats and demonstrated anti-inflammatory activity in a concentration-independent manner (Zakaria *et al.,* 2006). Aqueous extract of the leaves exhibited antipyretic activity as temperature of pyretic-induced rats was reduced for the first 6 hours after Brewer's Yeast administration (Zakaria *et al.,* 2006).

Table 2.2, continued

Melastoma decemfidum	Malaysia, Madagascar, India and Australia	Flavonoids (naringenin and kaemferol-3-*O*-(2',6'-di-*O*-*p*-trans-coumaroyl) glucoside have been shown to inhibit the proliferation of human breast MCF-7 cancer cells (Susanti *et al.*, 2007) Flavonoids (naringenin and kaemferol) detected in leaves of *M.decemfidum* is reported to serve as promising anticancer agent against cervical cancer (HeLa) cell lines (Sarju *et al.*, 2010)
Melastoma candidum	Southern China, Taiwan, Japan and Philippines	Three active compounds (castalagin, procyanidin B-2, and helichrysoside) isolated from leaf is reported to lower blood pressure through decreasing the sympathetic tome and causing direct vasodilation in adult hypertensive rats (Cheng *et al.*, 1993). Four flavonoids (quercitrin, isoquercitrin, rutin, and quercetin) isolated from leaf exhibited an inhibitory effect on monoamine oxidase B and free radical scavenging activities (Lee *et al.*, 2001). Water, acetone, ethanol, and ethyl acetate extracts of dried mixed stems and roots of *M. candidum* exhibited antibacterial properties against several species of Gram-positive and Gram-negative bacteria (Wang *et al.*, 2008).

2.2.1 Brief Description of the Species in the Genus *Melastoma* used in this Study (*Melastoma muticum* and *Melastoma sanguineum*)

Melastoma muticum is a shrub growing up to 3 m tall with smooth brownish bark (Figure 2.1(a)). The leaves are elliptic to lanceolate and the petals are violet (Figure 2.1(b)). The fruits have fleshy capsule and rupture irregularly at maturity (Rajenderan, 2010).

(a)

(b)

Figure 2.1: (a) Shruby habit of *M. muticum*, (b) Flowers of *M. muticum* with violet petals.

M. sanguineum is a shrub reaching up to 10 m tall with grey or brownish bark. The leaves are lanceolate or elliptic (seldom ovate (Figure 2.2 (b)), petals violet and fruit capsules are campanulate. The fruit pulps are yellow with orange seeds (Rajenderan, 2010).

(a)

(b)

Figure 2.2: (a) Shruby habit of *M. sanguineum*, (b) Leaves of *M. sanguineum*

2.3 Brief Description and Traditional Folklore uses of *Phyllagathis rotundifolia*

The whole genus of *Phyllagathis* comprises about 60 species of which 12 species occur here in Peninsular Malaysia and the neighboring countries and another 13 species in Borneo (Lemmens & Bunyapraphatsara, 1999; Tan *et al.*, 2010). *Phyllagathis rotundifolia* is locally known as *Tapak Sulaiman, Tapak Gajah*, *Bawal Hutan* or *Akar Serau Malam*, and is commonly found in lowland and hill forest of Peninsular Malaysia and Sumatera ([a]Tan *et al.*, 2011; [b]Tan *et al.*, 2011; Nor Hazalin *et al.*, 2012). It has

large, blue green metallic sheen, leathery, and heart-shaped-leaves; short stem, and pink flowers (Figure 2.3) (Henderson, 1978; Ridley, 1992). In traditional folklore, a decoction of the whole plant is given to women immediately after birth to hasten the process of delivery of the placenta (Nor Hazlin *et al.,* 2012). Other than that, decoction of leaves is used to treat malaria, stomach ache, fever, and also taken as a tonic ([a]Tan *et al.,* 2011; [b]Tan *et al.,* 2011; Tan *et al.,* 2012).

Figure 2.3: *P. rotundifolia* is a small plant usually found growing as forest floor herbs.

2.4 The Genus *Memecylon* and Associated Species with Therapeutic Records

The genus *Memecylon* consists of about 150 species, mainly occurring in the old world tropics (Kumar *et al.,* 2003). *Memecylon edule* Roxb., commonly known as Iron wood tree, *kaayam*, *delek bangas*, *delek air*, *miat*, and *nemaaru*, is a small shrub commonly found in the Northeast forest of Thailand (Nualkaew *et al.,* 2009) and India, especially the Deccan Plateau, including most of Karnataka, Andhra Pradesh, and parts of Tamil Nadu (Naidu *et al.,* 2013). The leaves of this plant have been used in the dyeing industry as wool and silk dye (Nadkarni, 1996).

M. edule has been used in folklore or traditional medicine practices to treat burns and accelerate wound healing (Nualkaew *et al.,* 2009). Secondary metabolites or biologically active compounds that possess the medicinal properties include triterpenes, tannins, and flavonoids (Kongkachuichay *et al.,* 2002). Nualkaew *et al*., (2009) have pointed out that leaf extracts of this plant showed to have anti-inflammatory, analgesic, and antioxidant activity.

Recently Naidu *et al* (2013) investigated the apoptogenic activity of ethyl acetate extract of leaves of *M. edule* on human gastric carcinoma cells via mitochondrial dependent pathway. They concluded that the extract induces apoptosis selectively in gastric cancer cells and their findings could be developed as novel bioactive anticancer molecules for the treatment of gastric cancer. Works on *M. edule* and other selected sister-species are summarized in Table 2.3.

Table 2.3: Medicinal properties of selected *Memecylon* species.

Plant	Location of distribution	Medicinal Properties
Memecylon edule	Thailand and India	The leaf of this plant is used to treat burns as well as for its anti-inflammatory effect; roots and heartwood have been used to treat fever symptoms of several diseases such as common cold, measles, and chicken-pox. (Wuthidhammvej, 1997; Suriyajuntrathong *et al.,* 1999). Leaves of *M. edule* are used to treat leucorrhea, gonorrhea, wound, and gastrointestinal ailments (Elavazhagan & Arunachalam, 2011). Lotion prepared from leaves is used to treat bruises and cure eye ailments (Said *et al.,* 1998). Apoptogenic activity detected from ethyl acetate extract of leaves of *M. edule* on human gastric carcinoma cells via mitochondrial dependent pathway (Naidu *et al.,* 2013).
Memecylon umbellatum	India	Methanolic extract of the leaves showed high antioxidant property, good antibacterial activity, and moderate antifungal activity (Puttaswamy & Achur, 2013).

Table 2.3, continued

		The leaves have been reported to exhibit astringent properties and are given to treat leucorrhea and gonorrhea; a lotion prepared from the leaves is used to treat eyes problems (Sastri, 1962; Amalraj & Ignacimuthu, 1998). Oral administration of an alcoholic extract of the leaves of *M. umbellatum* led to a significant lowering of serum glucose levels (hypoglycaemic) in normal and alloxan-induced diabetic mice (Amalraj & Ignacimuthu, 1998).
Memecylon malabaricum	India	Traditionally used for the treatment of various skin diseases including Herpes (Hullatti & Rai, 2004). Methanol extract of the leaves has shown activity against both gram –ve and gram +ve bacteria, and fungi (Hullatti & Rai, 2004).

2.4.1 A Short Description of *Memecylon caeruleum*

M. caeruleum is a shrub that can grow up to about 3–6 m tall. The leaves are oblong to elliptic and the petals are white or yellowish with pale-blue base (Figure 2.4 (a)). The fruits are pink to dark red and maturing fruits are purple to black in color (Figure 2.4 (b)). So far there has been no reported study on biological and phytochemical content of this particular species.

(a) **(b)**

Figure 2.4: (a) Part of the flower of *M. caeruleum* with an attractive bluish base, (b) Pink to dark red fruits of *M. caeruleum*.

2.5 Plant Natural Products

In plants, bioactive compounds are naturally produced as secondary metabolites (Bernhoft, 2010). Plants are in stationary state and yet they have to survive from numerous challenges such as pathogen infections, coexistence of herbivores, and fluctuation in supply of simple nutrient that they need for synthesizing of their food (Reymond *et al.,* 2000; Kennedy & Wightman, 2011). The secondary metabolites can be unique to specific species or genera and do not play any role in the plants' primary metabolic requirement. In fact, they are important for their survival and overcoming local challenges (Harborne, 1993). Plant secondary metabolites can be classified into three main families, according to their biosynthetic pathway which are phenolics, terpenes and steroids, and alkaloids (Harborne, 1999; Bourgaud *et al.,* 2001).

Most common bioactive compounds are used as pharmaceuticals (antibiotics), agrochemical, flavours, fragrances, biopesticides, food grade pigments or food colourant, food additives, and plant growth factors (Rao & Ravishankar, 2002; Joffry *et al.,* 2012). Plant secondary metabolites have proved to be an excellent reservoir of new medical compounds (Nirmala *et al.,* 2011) and they have been used for centuries in traditional medicine because of their large biological activities. Since than, different strategies have been extensively studied with the objectives of improving the production of secondary plant compounds and in the hope that the isolated novel and valuable compounds or molecules can cure various diseases (Bourgaud *et al.,* 2001).

All the natural compounds found in plants have strongly been associated with its ethnomedicinal values (Joffry *et al.,* 2012). In a study, Sakong *et al* (2011) concluded that therapeutic activities or medicinal properties of plant are in relation with the plants' phytochemical and bioactive compounds. The study was done on selected traditional medicinal plants in Northeast Thailand and plants that contain high alkaloid content are documented in their folklore practices as anti-tussive (to suppress coughing) and

indigestion. Plants with high tannin contents were reportedly used to treat diarrhea (Sakong *et al.,* 2011). Such studies and many more scientific investigations have shown that secondary metabolites are important for medicinal purposes and researchers nowadays are showing considerable interest in screening various plants from different genus and species to find novel compounds including new anticancer agents (Kinghorn, 2000; Nirmala *et al.,* 2011).

2.5.1 The Discovery and Development of Natural Products as Anticancer Agents

As reported by Hartwell (1982) in his review paper plants have been used in cancer treatment for a long period of time and he listed more than 3000 plant species that have reportedly been used against cancer. Massive research was done by different research groups in finding novel natural cancer fighting agents or anticancer agents derived from plants (Harborne, 2000) and it was discovered largely by testing for cytotoxic activity against cancer cell lines grown either *in vitro* or using *in vivo* models (Cragg & Newman, 2005).

Approximately 60 % of available cancer chemotherapeutic drugs were discovered from natural products produced by medicinal plants (Newman *et al.,* 2003; Cragg *et al.,* 2005). Chemotherapy is the latest cancer treatment, but the clinical applications are limited because of severe toxic effects. Because of that, it is important to find novel anticancer agents with fewer negative-side effects and plant based medicine is gaining attention because of low side effects and much lower production cost (Naidu *et al.,* 2013). Interestingly no new plant-derived clinical anticancer agents have, as yet, reached the stage of general use, but several numbers of agents are in preclinical or clinical development stage as shown in Table 2.4. But, development of

effective drugs required dedicated research and patience, and considerable resources to ultimately prove their efficacy as clinical agents (Cragg & Newman, 2005).

Table 2.4: Plant-derived anticancer agents used in preclinical and clinical stages.

Compound	Plant species	Cancer treatment	Development Stage
Vinblastine and Vincristine	*Catharanthus roseus*	Leukemia, lymphoma, testicular cancer, breast and lung cancer, and Kaposi's sarcoma (cancerous tumour of connective tissue) (Gueritte & Fahy, 2005).	Clinical use
Paclitaxel	*Taxus brevifolia*	Ovarian, breast, and lung cancer (Hartwell, 1982).	Clinical use
Homohariingtonine	*Cephaloyaxus harringtonia*	Leukemia (Itokawa *et al.,* 2005).	Clinical use
Elliptinium	*Bleekeria vitensis*	Breast cancer (Itokawa *et al.,* 2005).	Clinical use
Etoposide and teniposide	*Podophyllum peltatum* and *Podophyllum emodi*	Lymphoma, bronchial, and testicular cancer (Lee & Xiao, 2005).	Clinical use
Flavopiridol	*Dysoxylum binectariferum*	Leukemia, lymphoma, breast, and lung (Sausville *et al.,* 1999).	Phase I and Phase II clinical trials
Combretastin	*Combretum caffrum*	Anti-angiogenic agents – causing vascular shutdown in tumour and lead to tumour necrosis (Li & Sham, 2002).	Clinical trials and Preclinical development
Roscovitine	*Raphamus sativus*	Inhibit cyclin-dependent kinases (Chang *et al.,* 1999).	Phase II clinical trials

(Modified from Cragg & Newman, 2005; Kaur *et al.*, 2011)

As pointed out earlier, the chemical compounds in plants can be divided into three main families according to their biosynthetic pathway; alkaloids, terpenoids and steroids, and phenolic compounds. All the compounds will be explained in detail in the next topic.

2.5.1.1 Alkaloids as Therapeutic Drugs and Pharmaceutical Agents

Alkaloids are a generic term of basic organic compounds containing nitrogen atom and a ring structure. In most cases, the nitrogen atom is located inside the heterocyclic ring structure (Miura *et al.*, 2010; Lu *et al.*, 2012). They are widely distributed in the plant kingdom and mainly exist in higher plants, such as those belonging to Ranunculaceae, Leguminosae, Papaveraceae, Menispermaceae, and Loganiaceae (Lu *et al.*, 2012). Alkaloids, a toxic substance found mostly in fungi and plants, possessed various pharmacological effects and are used as medication (Solihah *et al.*, 2012).

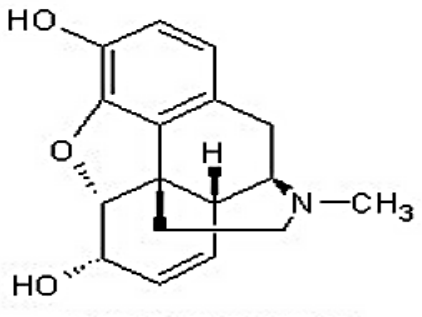

Figure 2.5: Structure of morphine, containing Nitrogen atom and ring structure (www.sciencebase.com).

Alkaloids were first isolated in the nineteenth century where in 1803, the French apothecary Derosne, isolated alkaloids known as narcotine. The Hanoverian apothecary Sertûrner further investigated opium in 1806 and isolated morphine in 1816 (Figure 2.5). Over time, alkaloids featured strongly in the search for plant drugs with anticancer activity. A notable success was the introduction of *Catharanthus* alkaloids (vinblastine and vincristine) and paclitaxel into medicine and there is much current interest in other alkaloids having anticancer properties as well as those exhibiting anti-aging and antiviral possibilities (Evans, 1996).

Isolated from the plant *Catharanthus roseus,* vinblastine and vincristine are alkaloids that exhibit significant biological anticancer effects, which interact with tubulin, while some of them have already been successfully developed into chemotherapeutic drugs such as camptothecin (CPT), topoisomerase I (TopI) inhibitor (Lu *et al.,* 2012). The primary mode of action for vinblastine and vincristine could be considered as one of the most strategic subcellular targets for chemotherapeutic action because it consists of an interaction with α- and β-tubulin heterodimeric subunits of microtubules (Himes, 1991). This interaction prevents the normal process of polymerization of the subunits into microtubules and produced mitotic arrest, which ultimately resulted in cell death (Lu *et al.,* 2012). Dong *et al*., (1995) reported that both alkaloids have achieved a prominent role in modern cancer chemotheraphy because they exhibit significant clinical antitumour activity against Hodgkin's and non-Hodgkin's lymphomas, acute lymphoblastic leukemia, breast carcinoma, Wilm's tumour, Ewing's sarcoma, neuroblastoma, heptoblastoma, and small cell lung cancer.

Other than vinblastine and vincristine, several naturally derived alkaloids, such as berberine, evodiamine, matrine, piperine, sanguinarine, and tetrandrine (Figure 2.6), have been noted to have anticancer properties. Each of the compounds has different mechanisms of action in which the compound responsible to restrain cancer by

modulating signaling pathways, resulting in the inhibition of carcinogenesis, induction of cell cycle arrest, apoptosis, autophagy, or differentiation, and inhibition of metastasis, angiogenesis, and so forth. Table 2.5 summarizes the various alkaloids and their specific mode of cancer inhibiting mechanism. Further study is needed to accelerate discovery of anticancer drugs derived from alkaloids (Lu *et al.*, 2012).

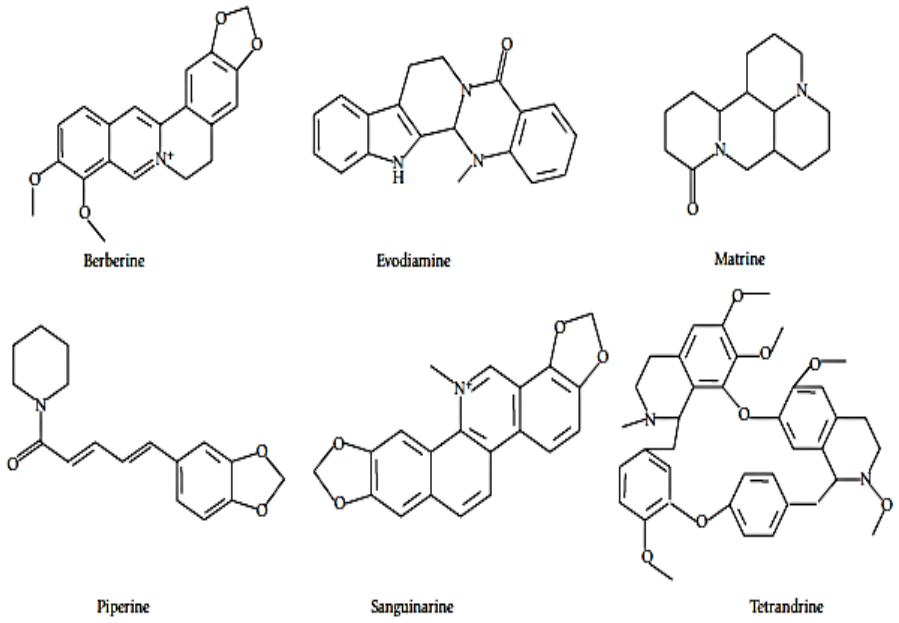

Figure 2.6: The chemical structure of berberine, evodiamine, matrine, piperine, sanguinarine, and tetrandrine (Lu *et al.*, 2012).

Table 2.5: Different mechanisms of anticancer action by naturally derived alkaloids.

Naturally derived alkaloids	Mechanism of anticancer
Autophagy	Berberine, Evodiamine, Matrine
Apoptosis	Berberine, Evodiamine, Matrine, Piperine, Sanguinarine, Tetrandrine
Cell cycle arrest	Berberine, Evodiamine, Matrine, Piperine, Sanguinarine, Tetrandrine
Differentiation	Berberine, Matrine, Piperine
Anti-multi drug resistant	Matrine, Piperine, Sanguinarine, Tetrandrine
Chemoprevention	Berberine, Piperine, Sanguinarine
Antimetastasis	Berberine, Evodiamine, Matrine, Piperine, Sanguinarine, Tetrandrine
Antiangiogenesis	Berberine, Evodiamine, Matrine, Sanguinarine, Tetrandrine

(Adopted from Lu *et al.*, 2012)

2.5.1.2 Terpenoids as Therapeutic Drugs and Pharmaceutical Agents

Terpenoids, one of the largest groups of natural products (Solihah *et al.,* 2012) has shown to possess various bioactivity including inhibiting the growth of *Candida albicans*, anti-inflammatory properties as well as antioxidant activities (Houghton *et al*., 2003; Zore *et al*., 2011). They are secondary metabolites occurring mostly in plant, formed from five-carbon isoprene units (C_5H_8) and are also called isoprenoids (Salminen *et al.,* 2008; Thoppil & Bishayee, 2011).

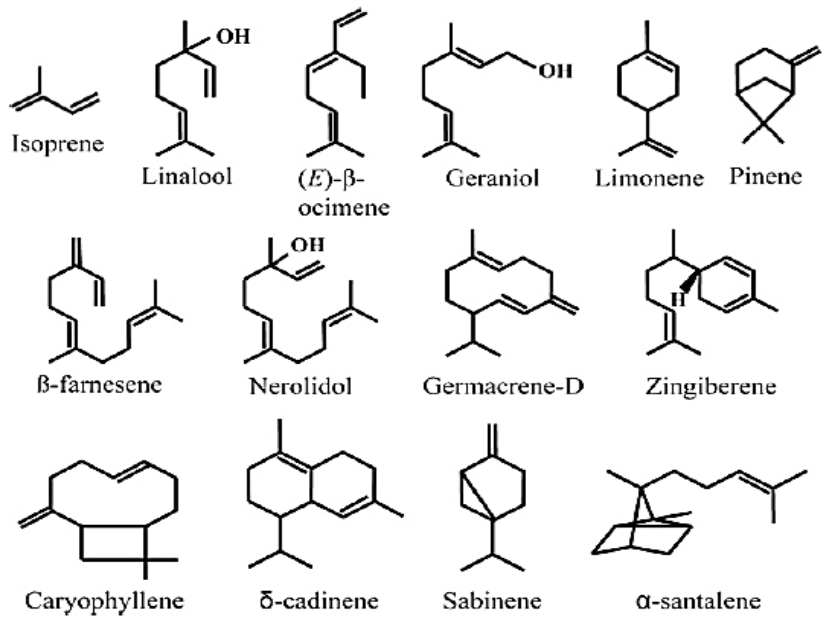

Figure 2.7: Structures of representative volatile terpenoid compounds (Nagegowda, 2010).

Volatile terpenoids (Figure 2.7) play important roles in direct and indirect plant defense against herbivores and pathogens, in reproduction by attraction of pollinators, and seed disseminatior, and in plant thermotolerance (Dudareva *et al.,* 2006; Nagegowda, 2010). Terpenoids are classified into several classes, such as monoterpenes (e.g., carvone, geraniol, *d*-limonene, and perillyl alcohol), diterpenes (e.g., retinol and *trans*-retinoic acid), triterpenes (e.g., betulinic acid, lupeol, oleanic acid, and ursolic acid) and tetraterpenes (e.g., *α*-carotene, *β*-carotene, lutein, and lycopene) (Rabi & Bishayee, 2009).

Terpenoids are major constituents usually present fruits, vegetables, and other different species having high antioxidative activities, anti-inflammatory and anticancer properties (Wagner *et al.,* 2003). According to Salminen *et al*., (2008), natural terpenoids are powerful inhibitors of nuclear factor- κB (NF-κB) signallng and subsequently could represent suppressive agents for inflammatory disease and cancer. NF-κB, the major regulator in the pathogenesis of inflammatory disease and cancer, has

major role in anti-apoptotic signaling and the development of cellular resistance against apoptosis (Dutta *et al.*, 2006).

Paclitaxel is an example of a terpenoid compound that is currently being used widely in cancer chemotheraphy (Kim & Park, 2002; Spiridon, 2006). The Paclitaxel (formerly called taxol), is a natural product derived from the bark of the Pacific Yew tree, *Taxus brevifolia* (Mastropaolo *et al.*, 1995; Salminen *et al.*, 2008). Mode of action for this compound is by promoting polymerization and stabilization of microtubules and interferes with the mitotic spindle. Excessive stabilization of the microtubules blocks mitosis, and this leads to the apoptotic cell death of proliferating cancer cells (Salminen *et al.*, 2008). This drug passed clinical trials and it (paclitaxel) is effective to treat ovarian cancer, breast cancer, malignant, melanoma, and probably other solid tumours (Slichenmyer & Von Hoff, 1991; Xiao *et al.*, 2006).

2.5.1.2.1 Saponins as Therapeutic Drugs and Pharmaceutical Agents

Saponins are glycosides containing one or more sugar chains on a triterpens or steroid aglycone backbone (Figure 2.8) and are believed to form the main constituents of many plant drugs and folk medicines (Thakur *et al.*, 2011; Solihah *et al.*, 2012). Saponins are glycosides with a distinctive foaming characteristic found in many plants. The name was obtained from the soapwort plant (*Saponaria*), the root of which was used historically as a soap (Sparg *et al.*, 2004).

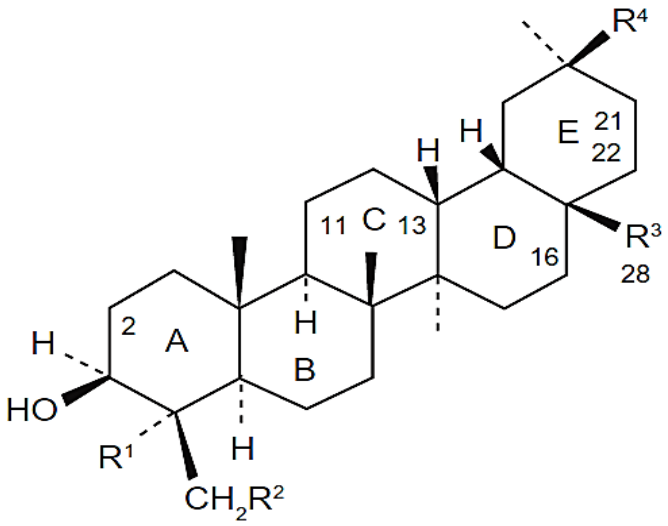

Figure 2.8: Core structure of triterpenoid sapogenins with arrangement of ring ABCDE (Thakur *et al.,* 2011).

Sparg *et al*., (2004) have reported that saponins have antiviral activity, antitumour, antifungal, antiparasitic and antibacterial capacities. First reported antitumour studies using saponins from the sea cucumber was done in 1960 by Friess *et al*. Most of the studies were tested in mice, with only 24% of studies being done in human cell lines – HeLa (cervical cancer cells), MCF-7 (breast cancer cells), MDA-MB43 (breast cancer cells), Caco-2 (colon cancer cells), and Hep-G2 (hepatic carcinomas) cells. General anticancer mechanisms of saponins are by inducing apoptosis and autophagy, act as antimicrotubule agents, induction of endoreduplication and mitotic arrest, suppressing MMP-2 and MMP- 9 (key factors in promotion of tumour-associated proteolysis) production, and activation of caspase 2 (apoptosis inducer) (Thakur *et al.,* 2011).

2.5.1.3 Phenolic Compounds as Therapeutic Drugs and Pharmaceutical Agents

Phenolic compounds (phenolics) are substances that contain an aromatic ring bearing one or more hydroxyl substituents, as shown in Figure 2.9, including their functional derivatives (Shahidi & Naczk, 1995; Strack, 1997). There are three main categories of phenolic compounds: phenolic acids, flavonoids, and tannins (Chung *et al.,* 1998).

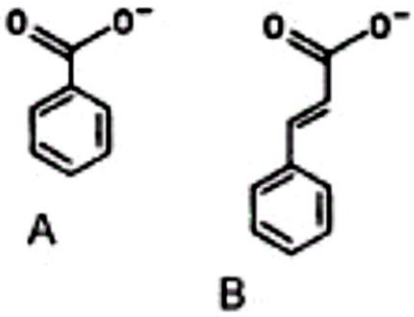

Figure 2.9: Basic structure of phenolic compounds
(A = cinnamic acid; B = benzoic acid) (Waniska, 2000).

A total of 51 purifies phenolic constituents, isolated from Canadian maple syrup (grade C and D) extracts were tested against human tumorigenic (HT-29, HCT-116, and Caco-2) and non-tumourigenic (CCD-18Co) colon cells. The results showed that the extracts are effective against the tumourigenic colon cells in comparison to non-tumourigenic cells. Among the isolates, gallic acid, carechaldehyde, syringaldehyde, and catechol were most active against cancer cells (Sarrias *et al.,* 2012). It was reported that phenolics can inhibit growth, induce cell-cycle arrest, and exert pro-apoptotic effects on colon cancer cells (Larrosa *et al.,* 2006; Kern *et al.,* 2007; Gonzalez *et al.,* 2009). Because of that, it is not surprising that the isolation and identification of plant phenolics, and the evaluation of their mechanisms of anticancer action, has received significant scientific attention (Sarrias *et al.,* 2012).

2.5.1.3.1 Flavonoids as Therapeutic Drugs and Pharmaceutical Agents

Flavonoids are the largest group of plant phenolics that account for over half of the eight thousand naturally occurring phenolic compounds (Harbone *et al.*, 1999). Phenols are naturally occurring compounds in plants and they are groups of antioxidant that inhibit various stages of cancer process (Wattenberg, 1992) by scavenging free radicals or quenching reactive oxygen species (Halliwell, 1997), give protection against cardiovascular disease and can protect against lipoprotein oxidation (Hollman, 2001). Flavonoids are known to have medicinal properties such as free radical scavenging activity, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action (Atanassova *et al.*, 2011).

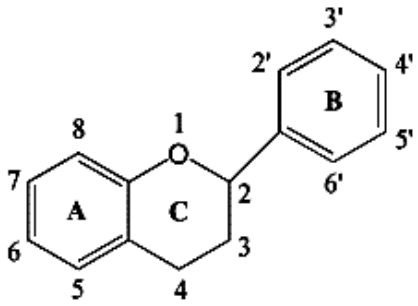

Figure 2.10: General structure of flavonoids (Wan *et al.*, 2009).

Flavonoids are among the most popular anticancer agent used worldwide and they are present in different plant parts including the leaves, stems, roots, flowers, and seeds (Amir & Mousa, 2007). Flavonoids may be divided into several different major classes such as flavonols, flavans, proanthocyanidins, anthocyanidins, flavanones, flavones, isoflavones, and, neoflavonoids based on differences in molecular backbone structure (Harborne *et al.*, 1974; Amić *et al.*, 2003; Kanadaswami *et al.*, 2005; Pinheiro & Justino, 2012). Figure 2.10 is the general structure of flavonoids having the common phenylbenzopyrone structure (C6-C3-C6), and they are categorized according to the saturation level and opening of the central pyran ring (Amić *et al.*, 2003).

Flavonoids are reported to inhibit specific enzymes, which include hydrolases, oxidoreductase, DNA synthases, RNA polymerase, lipoxygenase, and gluthation *S*-transferase. They also block several digestive enzymes, including α-amylase, trypsin, and lipase (Koshira *et al.*, 1984; Griffith, 1986; Reddy & Aggarwal, 1994; Sadik *et al.*, 2003). As a result, a rising number of authorized physicians are prescribing pure flavonoids to treat many important common diseases (Amir & Mousa, 2007).

2.6 Cancer – A Brief Overview

Cancer is a major public health problem in many parts of the world (Siegel *et al.*, 2013). Cancer is a group of disease characterized by uncontrolled growth leading to accumulation of cells producing tumour and spread of the cells (American Cancer Society, 2012; WHO, 2013). It can affect almost any part of the body since the growth often invade surrounding tissue and can metastasize to distant sites (Figure 2.11). According to Kroll *et al*., (2010), there are four main 'characteristics' of cancer cells: i) self-sufficiency in growth signals; ii) ignoring of anti-growth signals; iii) evading apoptosis; and iv) immortalization. When all the four characteristics have been acquired, the cancer cells can become malignant. In addition, angiogenesis (the process of generating new blood vessels which infiltrate the tumour) and metastasis (invasion and colonization of essential organs by the cancer cells or secondary tumours) of the tumour cells will lead to death of an organism (Figure 2.11).

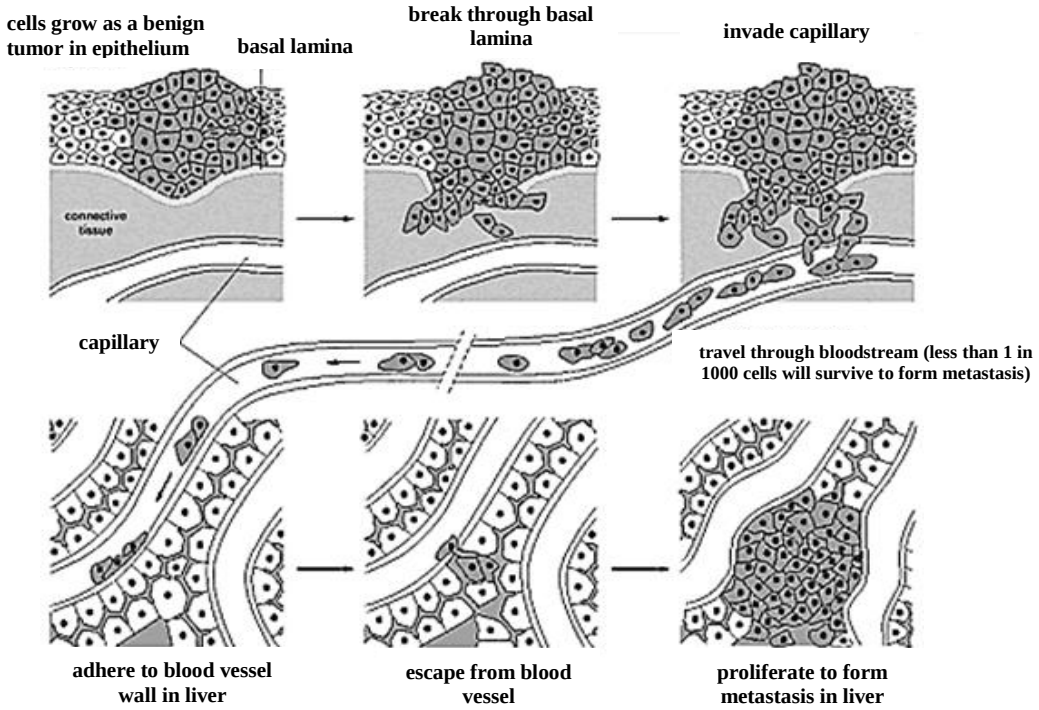

Figure 2.11: Process of metastasis. Cells grow as a benign tumour in epithelium in which they breakthrough the basal lamina and invade the capillary. The tumour cells travel through bloodstream (less than 1 in 1000 cells will survive to form metastasizes). The survived cells adhere to blood vessel wall in liver. Then, they escape from the blood vessel and proliferate to form metastasis in liver (Alberts *et al.*, 2002).

Cancer is caused by both external factors (tobacco, infectious organisms, chemicals, and radiation) and internal factors (inherited mutations, hormones, immune conditions, and mutations that occur from metabolism) (American Cancer Society, 2012). Cancer can be avoided by early detection and removal of precancerous lesions can be done through regular screening examinations (Center *et al.*, 2011). In addition, a significant proportion of cancers can be cured by surgery, radiotherapy, chemotherapy, hormone therapy, biological therapy, and targeted therapy especially if they are detected early (American Cancer Society, 2012; WHO, 2013).

About 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide (Jemal *et al.,* 2011). Cancer cause more death than AIDS, tuberculosis, and malaria combined. When countries are grouped according to economic development, cancer is the leading cause of death in developed countries and the second leading cause of death in developing countries (following heart diseases) (Center *et al.,* 2011). WHO (2013) reported that 5 most common types of cancer that kill men are (in order of frequency): lung, stomach, liver, colorectal, and esophagus. While, the 5 most common types of cancer that kill women are (in the order of frequency): breast, lung, stomach, colorectal, and cervical.

2.6.1 Brief Introduction to Breast Cancer

Breast cancer is the most commonly occurring cancer in women, comprising almost one third of all malignancies in females (Richie & Swanson, 2003). Breast cancer cases in the West have become stabilized or even decreased. However, breast cancer cases in most Asian countries are increasing over the past two decades (Ravdin *et al.,* 2007; Center *et al.,* 2011; Jemal *et al.,* 2011; Pathy *et al.,* 2011). This might be due to the fact that lifestyle in most Asian countries are changing over the time and delayed introduction of effective breast cancer screening programs (Center *et al.,* 2011).

There are several risk factors for development of breast cancer – gender and age, lifestyle, and genetics. Gender is by far the greatest risk factor in which it occurs 100 times more frequently in women than in men. In addition, the rates of breast cancer rise accordingly with age. This is because of hormonal change (menopause) that occurs at the age of 45–50 (Richie & Swanson, 2003; Center *et al.*, 2011, Jemal *et al.,* 2011). Unhealthy lifestyle that could lead to cancer development is overweight or obesity, use of MHT (combined estrogen and progestin hormone therapy), physical inactivity, and

alcohol consumption (Jemal *et al.*, 2011). Mutation of *BRCA1* and *BRCA2* genes (genes that help repair damaged DNA and destroy irrepairable cells) and personal or family history of breast cancer increase the risk of breast cancer incidents (Metcalfe *et al.*, 2009).

As mentioned earlier, internal and external factors can cause this disease to develop. Despite that, breast cancer is preventable if early detection could be done. Mammography and clinical breast examination can identify cancer at an early stage where treatment may be more effective. Besides this the risk of developing cancer could be reduced by having a healthy and active lifestyle such as eating balance and nutritious food and exercising regularly (American Cancer Society, 2012).

2.6.2 Ovarian Cancer – Women's Silent Killer

National Cancer Institute defined ovarian cancer as cancer that forms in tissues of the ovary. Most ovarian cancers are either ovarian epithelial carcinomas (cancer that begins in the cells on the surface of the ovary) or malignant germ cell tumours (cancer that begins in the eggs cells). Ovarian cancer is categorized under gynecological tumours and it is one of the causes of cancer death in women (Clinton & Hua, 1997; Zhang *et al.*, 2012). According to the American Cancer Society (2013), ovarian cancer accounts for about 3 % of cancer among women, but it causes more deaths than any other cancer of the female reproductive system.

Women that have personal or family history of ovarian cancer are at high risk of obtaining this disease and it is related to mutation of *BRCA1* and *BRCA2* genes (Negri *et al.*, 2003). Individuals at high risk are those with a first degree relative (mother, father, sister, brother, daughter, or son). Potential screening tests for ovarian cancer include the bimanual pelvic examination, serum CA 125, and ultrasound imaging

(Daniilidis & Karagiannis. 2007). Unlike breast cancer, there is currently no sufficiently accurate screening test proven to be effective in the early detection of ovarian cancer. Pelvic examination only occasionally detects ovarian cancer, generally when the disease is advanced (American Cancer Society, 2012). Treatment includes surgery (salpingo-oophorectomy: removal of one or both ovaries and fallopian tubes) and usually chemotherapy. However, current chemotherapies for ovarian cancer often lead to resistance or relapse, making it necessary for identification of novel treatments and therapeutic agents (Zhang *et al.,* 2012).

2.6.3 Cell Cycle – Phases of Cells Development and Proliferation

Proper regulation of cell cycle is important for all eukaryotes to modulate cell growth and development (Xiong *et al.,* 2012). Cells reproduce (production of two daughter cells) by duplication of their contents and subsequent division. This cycle of duplication and division is called the cell cycle, and the events of the cell cycle are generally understood to correspond to the mitotic cycle (Imoto *et al.,* 2011). Cells proliferate by going through the mitotic cells cycle that consist of four distinct phases (Figure 3.2): Gap 1 phase (G_1 phase), DNA synthesis phase (S phase), Gap 2 phase (G_2 phase), and mitotic phase (M phase) (Imoto *et al.,* 2011; Komaki & Sugimoto, 2012). Figure 2.12 and table 2.6 explain nature of events that occur in each phase of the cell cycle.

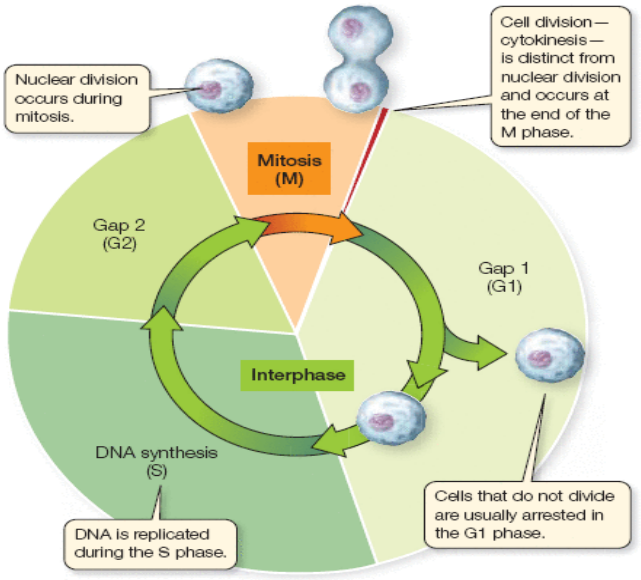

Figure 2.12: Phases of cell cycle (O'Connor, 2008).

Table 2.6: Overview of the cell cycle.

Phase	Events
Gap 1 (G_1)	• Represents a critical period in which the decision is made either to proliferate or enter G_0 (a period in the cell cycle in which cells exit the cycle in response to environmental changes such as depletion of nutrition or growth factors, changes in cell adhesion, and increased cell density during the early G_1 phase) (Oki *et al.*, 2014). • Growth occurs, and a cell's metabolic requirements are at their highest. • The duration of G_1 is quite variable (major determinant of the overall length of the cell cycle.
DNA Synthesis (S)	• DNA replication occurs in this phase. • Takes about 8 hours, although this time can be extended considerably in the presence of DNA damage or if replication is hindered in any way.
Gap 2 (G_2)	• This phase lasts around 4 hours. • Provides the cell with an opportunity to complete the DNA replication process by appropriately packaging chromosomes and sister chromatids. • Cell growth continues, and proteins required for mitosis are synthesized.
Mitosis (M)	• Duration of this phase is about 30 minutes to an 1 hour (less than 5 % of the total duration of the cell cycle). • Strictly, mitosis refers only to nuclear division, with the subsequent formation of two daughter cells being termed cytokinesis.

(Adapted from Bury & Cross, 2003)

Cell division is tightly regulated and because of that, the cells possess a defense system called checkpoints to resist from DNA damage and genotoxic insult, which are present at the G_1/S boundary, intra-S, and G_2/M transitions (Zhou & Elledge, 2000; Hyun *et al.*, 2012). DNA damage activates cell cycle checkpoints to prevent replication and segregation of damaged genome. DNA damage also inhibits cellular differentiation, activates apoptosis, and induces premature senescence. Defects in these checkpoints response to DNA damage that contributes to tumour progression, resistance to radiation/chemotherapy, and the development of degenerative disease. In the event of DNA damage, sensor proteins and signal transducer protein associated with each checkpoint detect DNA damage and transmit signals to their appropriate effectors, which initiate cell cycle arrest, DNA repair, or apoptosis. When repair of DNA damage is completed, the cells restart the cell cycle. If cells do not successfully complete DNA repair, the cells continue cell cycle arrest and then must be removed by apoptosis or cellular senescence or oncogenesis (Bury & Cross, 2003; Hyun *et al.*, 2012; Weitzman & Wang, 2013).

2.7 Overview of Antioxidants

Antioxidants as defined by the National Cancer Institute (2013) are chemicals that block the activity of other chemicals known as free radicals. Antioxidant refers to a wide spectrum of compounds, which are able to donate electron and neutralize free radicals, resulting in scavenging and preventing cell injuries (Saeidnia & Abdollahi, 2013). Naturally occurring antioxidants are mostly preferred due to their little or no toxicity in comparison with some synthetic antioxidants which have been documented to have toxic or mutagenic effects (Alnajar *et al.*, 2012). With respect to their mechanism of action, antioxidants are divided into two major groups: (i) enzymatic (eg.,

superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GSR), NADPH: quinone oxidoreductase (NQO), and thioredoxin reductase (TrxR)) and (ii) non-enzymatic (eg., CoQ10, glutathione, non-protein thiols, vitamin C and vitamin E) (Goodman *et al.*, 2011; Thapa & Ghosh, 2012).

The body makes some of the antioxidants (endogenous antioxidants) and uses them to neutralize free radicals. But, the body relies on exogenous antioxidants, commonly known as dietary antioxidants (Figure 2.13), to obtain the rest of the antioxidants needed by the body. Fruits, vegetables, and grains are rich sources of dietary antioxidants and some available as dietary supplements (Thaipong *et al.,* 2006; National Cancer Institute, 2013). According to Thaipong *et al*., (2006), fruits and vegetables are main sources or natural antioxidant as they contain vitamins, phenolics, and carotenoids. Ascorbic acid and phenolics are known as hydrophilic antioxidants, while carotenoids are known as lipophilic antioxidants (Halliwell, 1996; Thaipong *et al.*, 2006).

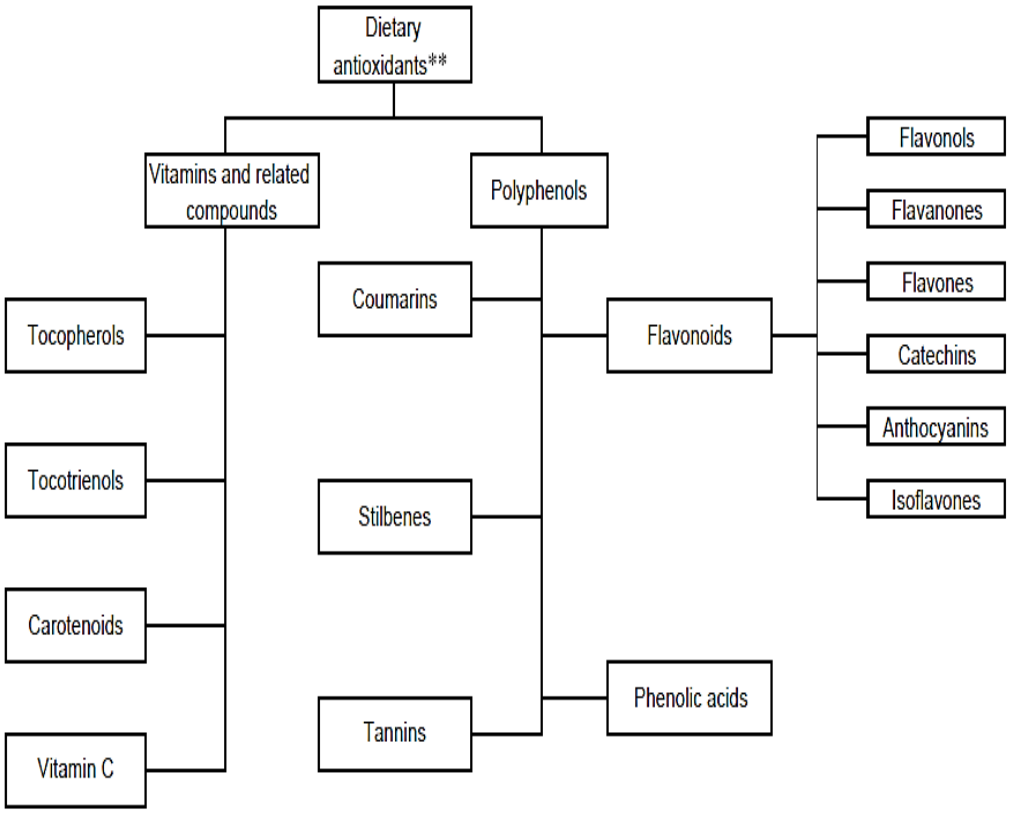

Figure 2.13: Classification of dietary antioxidant. ** This classification excludes components of enzymatic antioxidants (eg: selenium and molybdenum) and dietary factors (eg: fatty acids) that may decrease oxidative stress via anti-inflammatory effects (Adapted from Goodman *et al.*, 2011).

2.7.1 Free Radicals in Relation to Cancer

Free radicals are highly reactive chemicals that have the potential to harm cells (National Cancer Institute, 2013). Free radicals are produced when a molecule gives its electron to unattached radicals or "oxidant compounds" that are defined as atoms with one or more unpaired electrons. These oxidant compounds are unstable and need to 'steal' electron from other compounds to become more stable. Our body produced free radicals through mitochondrial oxidative metabolism, such as oxy-radical species, hydroxyl radical ($^{\bullet}OH$) and peroxide radical ($O_2^{\bullet}$). Other than that, external factors like pollution, radiation, cigarette smoke, and pesticide, are also producing free radicals that

are harmful to cells and tissues in the body (Kowluru & Chan, 2007; Saeidnia & Abdollahi, 2013). For example, auto-oxidation of polyphenols in saliva of tobacco users is crucial in the initiation and promotion of oral cancer (Patel *et al.*, 2008).

Free radicals that contain the element oxygen are the most common type of free radicals produced in living tissues. Another name for them is "reactive oxygen species" or ROS (Diplock *et al.*, 1998; Valko *et al.*, 2007; National Cancer Institute, 2013). In normal conditions, ROS have an important role in signal transduction and gene transcription, nevertheless, ROS may act as a trigger for carcinogenesis *via* persistent DNA injuries (Figure 2.14) as well as mutations in *p*53 (tumour suppressor gene) such as conditions observed in skin, hepatocellular, and colon cancers. However, interestingly, adequate levels of ROS may show opposite effects to inhibit carcinogenesis by enhancing *p*53 expression and inducing apoptosis in the tumour cells (Khosravi-far & White, 2008; Liu *et al.*, 2008; Alawadi *et al.*, 2011; Saeidnia & Abdollahi, 2013). Free radicals are thus able to increase or decrease the risk of cancer based on diverse situations as shown in Figure 2.15 below (Saeidnia & Abdollahi, 2013).

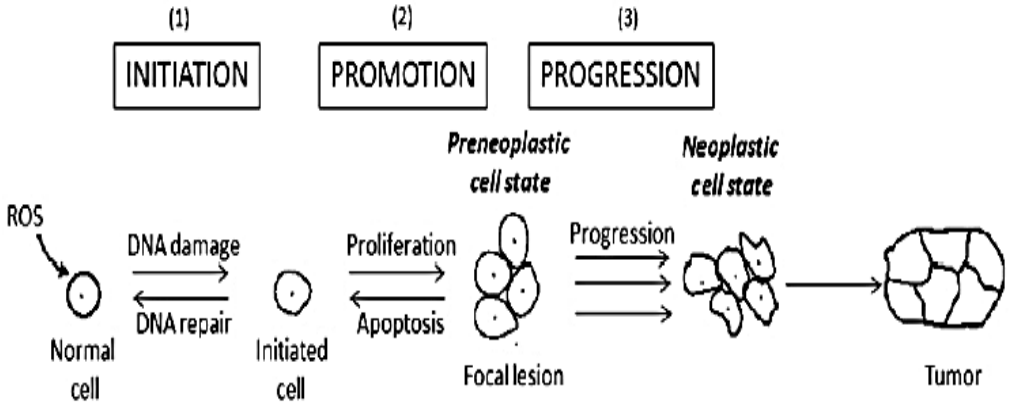

Figure 2.14: Three-stage model of carcinogenesis and the level of carcinogenic effects (Vineis & Husgafvel-Pursiainen, 2005; Vanessa, 2013).

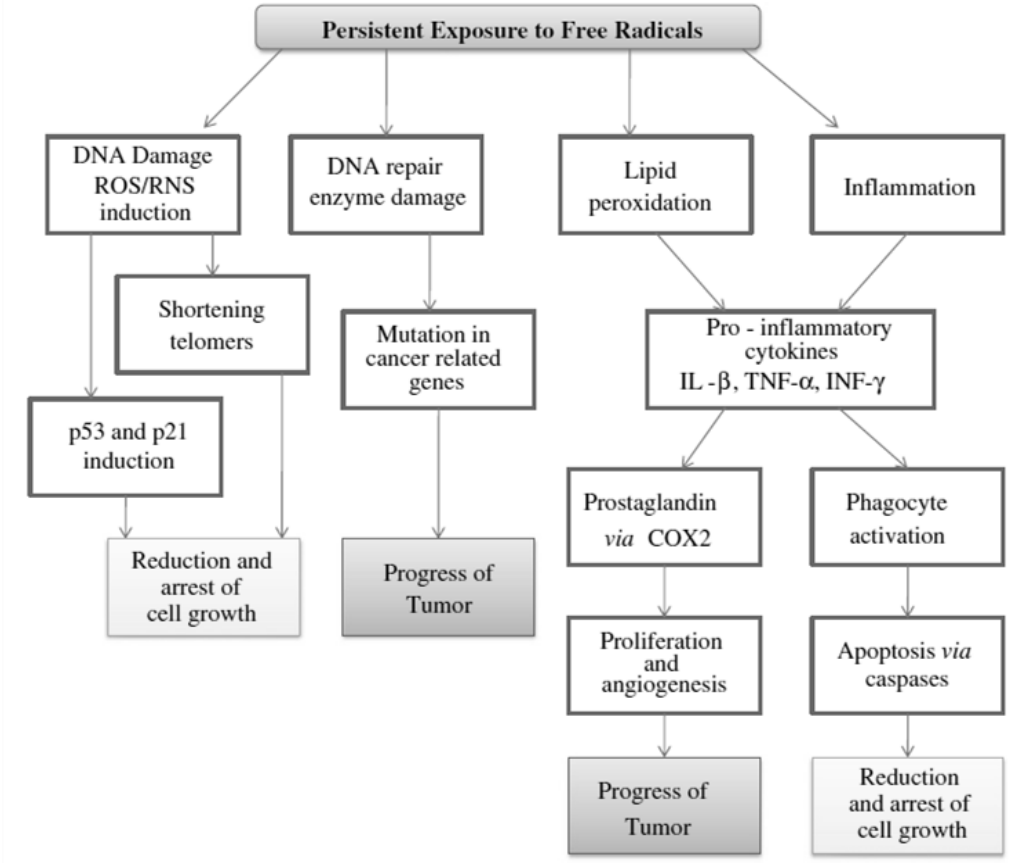

Figure 2.15: Free radicals can act dual roles as cancer inhibitors or accelerators under different condition. COX-2: cyclooxygenase 2; IL-*β*: interleukin beta; INF-*γ*: interferon-gamma; *p*21: tumour protein 21; *p*53: tumour protein 53; RNS: reactive nitrogen species; ROS: reactive oxygen species; TNF-*α*: tumour necrosis factor alpha (Saeidnia & Abdollahi, 2013).

2.7.2 Antioxidants as Anticancer Agents

Free radicals causing oxidative stress play important roles in the pathogenesis of many cancers. Flavonoids and resveratrol are examples of antioxidant sources, which can be obtained with a well-balanced diet rich in fruits and vegetables evidently showing to lower the incidence of prostate, breast, renal, and other cancers in *in vitro* and small trials. However, further research through larger clinical trials is needed to determine the optimal dosage and formulation that elicit antioxidant and anticarcinogenic effects (Bennett *et al.*, 2012).

In general, antioxidants have shown promising results in various preclinical models. Antioxidative compounds such as lycopene, selenium, vitamin E, genistein, green tea polyphenols, and other novel synthetic compounds have been tested and proven successful in various rodent models for treatment of prostate cancer. Other preclinical studies have demonstrated the efficacy of antioxidants (lycopene, β-carotene, curcumin, resveratrol, vitamin E, vitamin D) as potential chemopreventive agents as evidenced by decreased incidences as well as reduced tumour growth and metastasis in animal models (rats/mouse) (Thapa & Ghosh, 2012). Several anticancer agents are developed to have similar mechanism as antioxidants and are used in cancer treatment (refer to Table 2.7 for a summary).

Table 2.7: Mechanism of action for well-known natural and semi-synthetic anticancer medicines regarding to their antioxidant abilities.

Anticancer drugs	Mechanism of anticancer activity	Antioxidant ability	References
Berberine	Binding to oligonucleotides and stabilizing DNA triplexes or G-quadruplexes, inhibiting telomerase and topoisomerase; effective in osteosarcoma and in lung, liver, prostate, and breast cancer.	Scavenging ROS; inhibiting lipid peroxidation; protecting against LDL oxidation; binding to catalyzing metal ions and reducing the concentration of metal ions in lipid peroxidation.	(Xu *et al.*, 2006; Zhang *et al.*, 2008; Xie *et al.*, 2009; Patil *et al.*, 2010; [c]Tan *et al.*, 2011; Ji & Shen, 2011)
Betulinic acid	Acting as a trigger of mitochondrial apoptosis; showing anti-neoplastic activity in therapy and prevention of cancer in humans.	Weak radical scavenging activity has been observed with a minimum inhibition at 38.07 µg/ml.	(Fulda, 2008; Nguemfo *et al.*, 2009)
Cucurbitacin	Cucurbitacin I and B are able to inhibit both signal transducer/Januse Kinase 2 (JAK2) activity and activator of transcription 2 (STAT3) pathways.	Indicating antioxidant activity in a dose-dependent manner probably through the involvement of a direct scavenging effect on several free radicals.	(Tannin-Spitz *et al.*, 2007; Bernard & Olayinka, 2010)
Curcumin	Modulating the cell cycle pathway and inducing apoptosis of various cancer cells. Phase I/II trials are going on for colorectal and pancreatic cancers as well as multiple myeloma.	This is a classic phenolic chain- breaking antioxidant, donating H-atoms from the phenolic group.	(Litwinienko & Ingold, 2004; Goel *et al.*, 2008; Sa *et al.*, 2010)
Daphnoretin	Suppression of protein and DNA synthesis in Ehrlich ascites carcinomas.	Daphnoretin is antioxidative only at high concentrations.	(Deiana *et al.*, 2003; Diogo *et al.*, 2009)
Ellipticine	Intercalates with DNA; inhibits topoisomerase II and cell growth to result in apoptosis of human hepatocellular carcinoma HepG2 cells.	This compound is not an antioxidant, while its other analogs showed potent antioxidant activity (eg., 9-hydroxy ellipticine).	(Kuo *et al.*, 2006)

Table 2.7, continued

Sanguinarine	Inducing cyclin kinase inhibitors 21/WAF1 and p27/KIL1; down-regulating of cyclin E, D1, D2, and cyclin0dependent kinase 2, 4, and 6.	Inhibition of oxidative burst by impacting on NADPH oxidase activity directly; preventing the formation of NADPH oxidase protein complex.	(Adhami *et al.*, 2004; Vrba *et al.*, 2004)
Taxol®	Docetaxel and paclitaxel; effective in first- and second-lines of metastasis, breast, and ovarian cancers; active against lung, prostate, and lymphoid malignancies; binding to the polymerized microtubules which prevent normal mitosis (anti-mitotic).	Enhancing superoxide, hydrogen peroxide, nitric oxide (NO), oxidative DNA adducts, G2-M arrest, and fragmented nuclei cells.	(Ramanathan *et al.*, 2005; Nirmala *et al.*, 2011)
Topotecan	Inhibiting DNA topoisomerase I; clinically effective in second line therapy of epithelial ovarian and small cell lung cancers.	Augmenting the oxidative status; enhancing lipid peroxidation and protein oxidation; lowering GSH and sulfhydryl levels in MCF-7 cells.	(Timur *et al.*, 2005; Kisa *et al.*, 2005; Nirmala *et al.*, 2011)

GSH: glutathione peroxidase; JAK2: Janus kinase 2; LDL: Low-Density Lipoprotein; NADPH: Nicotinamide Adenine Dinucleotide Phosphate Hydrogen; ROS: reactive Oxygen Species; STAT3: signal transducer and activator of transcription 3; WAF1: wild-type activating fragment-1

(Adapted from Saeidnia & Abdollahi, 2013)

CHAPTER 3

METHODOLOGY

3.1 Macroscopic Studies

Leaves and stem were taken for morphological study. Observations of the macroscopical characteristics of the plants were done by naked eyes, aided by magnifying glass. The studies were carried out by simple determination technique, to study morphology of the leaves, flowers and fruits of each plant. The data obtained were compared with literature for identification and thus confirming the identity of the species (Henderson, 1978; Ridley, 1992; Meyer, 2001).

3.2 Preparation of Crude Extracts

3.2.1 Solvents

Organic solvents with analytical grade were used for extraction and fractionation process of the plant samples. The organic solvents such as hexane, ethyl acetate (EtOAc), and methanol (MeOH) were purchased from Fisher Scientific, UK.

3.2.2 Sample Collection

The leaves of *Melastoma muticum* Ridl. and *Melastoma sanguineum* Sims (leaves) were collected from Ulu Kali, Selangor in the month of March 2013. The leaves of *Memecylon cearuleum* Jack were collected from Lumut, Perak in the month of November 2013, and leaves of *Phyllagthis rotundifolia* (Jack) Bl. were collected from Kuala Langat, Selangor in the month of October 2013. Authentication of *M. muticum* (KLU47788), *M. sanguineum* (KLU47792), *M. caeruleum* (KLU48120), and *P. rotundifolia* (KLU48130) was carried out in the herbarium of the Rimba Ilmu Botanical Garden, Institute of Biological Sciences, University of Malaya by Dr. Sugumaran Manickam.

3.2.3 Extraction of Plant Materials

Figure 3.1 shows the schematic extraction of the plant material. The leaves of the selected species were cleaned immediately to remove any extraneous material and dried at room temperature (27 °C). The dried leaves were then ground into powder and soaked in methanol (MeOH) (Fisher Scientific, UK) with ratio 1:10 (powder of dried plants:methanol) for 72 hours at room temperature with shaking. After 72 hours, the solution was filtered through Whatman No.1 filter paper (Whatman, England). The MeOH filtrate was collected and excess solvent was evaporated under reduced pressure using a rotary evaporator (Buchi, Switzerland) at 40 °C to dryness producing dark-greenish MeOH extract.

A small portion of the MeOH extract was reserved for the assay. While hexane (Fisher Scientific, UK) was added to the remaining MeOH extract and further shaken. The hexane soluble solution was filtered and this step was repeated few times until the resultant hexane become almost colourless. The hexane soluble solution was pooled, followed by concentration under reduced pressure by a rotary evaporator to yield the hexane extract. The remaining hexane insoluble was subjected to solvent-solvent extraction with a mixture of ethyl acetate (EtOAc) (Fisher Scientific, UK) and distilled water (1:1, v/v) followed by fairly vigorous mixing. This mixture was then successively fractionated using a separating funnel in which two distinct layers formed.

The bottom layer (water layer) was discarded while the EtOAc phase (top layer) was released into a clean beaker. This filtrate was concentrated under reduced pressure using a rotary evaporator to yield the EtOAc extract. In all experiment, extracts was dissolved in dimethylsulfoxide (DMSO) (Sigma) as stock solution and stored at -20 °C. Prior to analysis, the final concentration of each sample was prepared by diluting the stock solution in 10% DMSO.

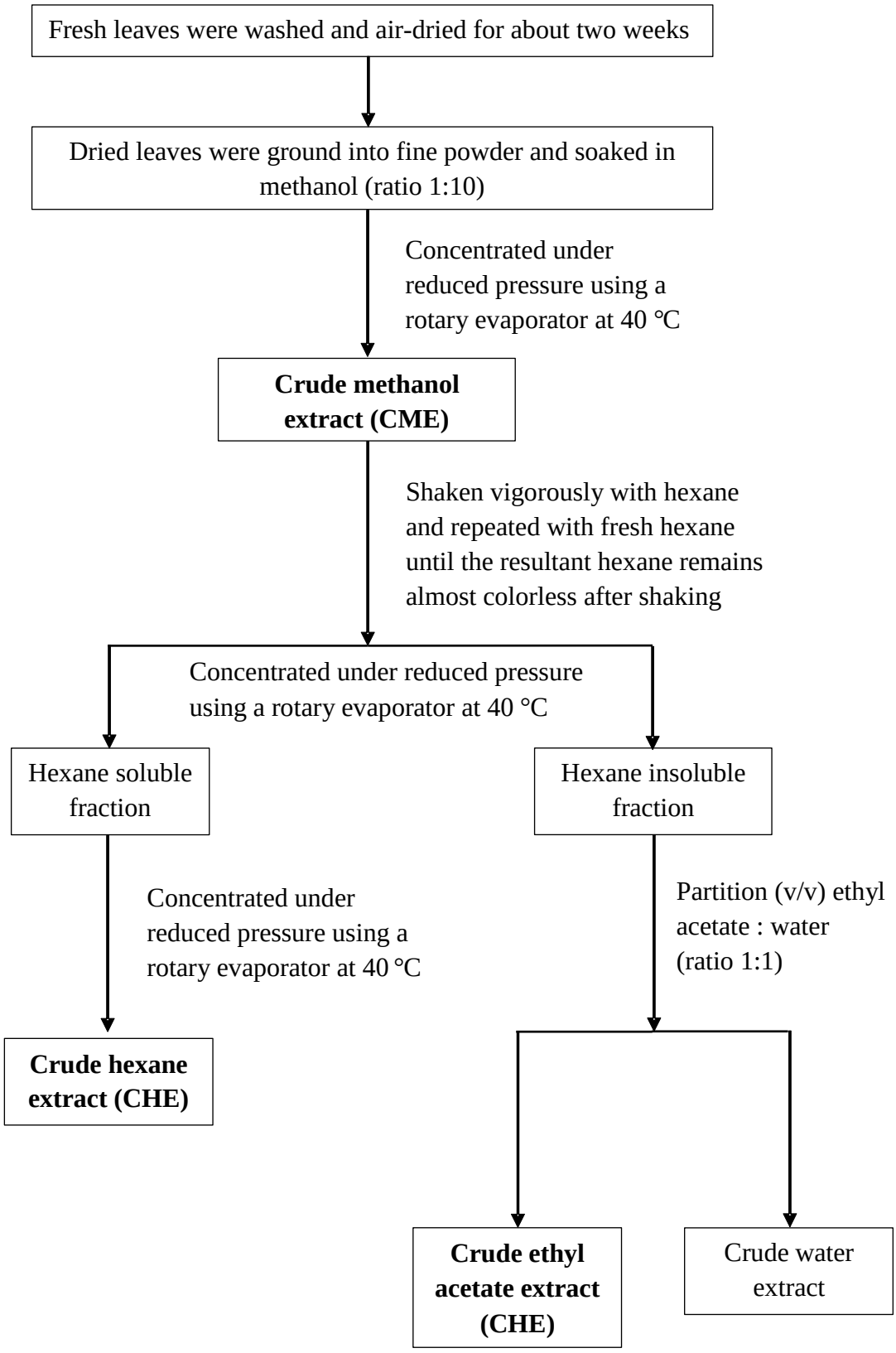

Figure 3.1: Schematic extraction of the selected species from the family Melastomataceae.

3.3 Phytochemical Screening

Iron (III) chloride ($FeCl_3$); lead (II) acetate ($(CH_3COO_2)_2Pb$); hydrochloric acid (HCl); concentrated sulfuric acid (H_2SO_4) and nitric acid (HNO_3) were all purchased from R & M Chemical, UK. Chloroform ($CHCl_3$) and glacial acetic acid (CH_3COOH) were purchased from Fisher Scientific, UK. Dilution of crude methanol extracts (CME) was done at 1:100 ratio, (w/v) with distilled water. The phytochemical screening of the CME was carried out according to the method describes by Solihah *et al.,* (2012) with slight modification.

a) Test for phenols

2 ml of CME was taken into water and warmed at 45–50 °C. 2 ml of 3 % $FeCl_3$ was added to the extract solution. Formation of green or blue color indicated the presence of phenols.

b) Test for flavonoids (I)

1 ml of CME was added to 1 ml of 10 % $(CH_3COO_2)_2Pb$ and gently shaken. Formation of muddy brownish precipitate indicated the presence of flavonoids.

c) Test for flavonoids (II)

1 ml of CME was added to 10 % of $FeCl_3$. The mixture was shaken. Formation of woolly brownish precipitate indicated the presence of flavonoids.

d) Test for tannins

1 ml of CME was added to 1 ml of 3 % of $FeCl_3$. A greenish black precipitate signified the presence of tannins.

e) Test for phlobatannins

1 ml of CME was boiled with 2 ml of 1 % HCl. The formation of red precipitate signified the presence of phlobatannins.

f) Test for alkaloids

1 ml of CME was stirred with 5 ml of 1 % HCl on a steam bath (60 °C) for 15 minutes and filtered.

Test for alkaloids I: 1 ml of Dragendorff reagent was added to 1 ml filtrate. The formation of cloudy orange indicated the presence of alkaloids.

Test for alkaloids II: 1 ml of Mayer reagent was added to 1 ml of filtrate. Appearance of slight yellow color indicated the presence of alkaloids.

Test for alkaloids III: 1 ml of Wagner reagent was added to 1 ml of filtrate. The formation of turbid brown color indicated the presence of alkaloids.

g) Test for terpenoids

5 ml of CME was mixed in 2 ml of $CHCl_3$. Then, 3 ml of concentrated H_2SO_4 was carefully added. Formation of the reddish brown coloration between upper and lower layer indicated the presence of terpenoids.

h) Test for saponins

Approximately 0.2 ml of CME was mixed with 5 ml distilled water. It was shaken vigorously for 5 minutes. Persistence of foams appearance was the indicator for saponins.

i) Test for sterols (Salkowski's test)

2 ml of concentrated H_2SO_4 was added to 2 ml of CME. A red precipitate formation indicated the presence of steroidal ring compound(s) in sample.

j) Test protein-xanthoprotein

A few drops of HNO_3 were added by the side of the test tube containing 1 ml of CME. A yellow color formed indicated the presence of protein-xanthoprotein.

k) Test cardiac-glycosides

100 mg of CME was dissolved in 1 ml CH_3COOH containing 1 drop of 3 % $FeCl_3$. Then, 1 ml of concentrated H_2SO_4 was slowly added to the mixture until formation of brown ring at the interface that indicated the presence of de-oxy sugar characteristic of cardenolides compound(s).

3.4 Cytotoxicity Screening of Crude Extracts

3.4.1 Chemicals

Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI 1640), and Minimum Essential Medium (MEM) powder were purchased from Sigma-Aldrich, USA. Foetal Bovine Serum (FBS), penicillin-streptomycin (100×) and amphotericin B (250 µg/ml) were obtained from PAA Lab, Austria. Accutase in DPBS, 0.5 mM EDTA was purchased from iCT, CA. HEPES was purchased from Molekula, UK. Neutral Red was purchased from ICN, Ohio. All other chemicals and solvents used were of the highest purity and grade available purchased from BDH AnalaR, UK and Sigma-Aldrich, USA. Cell culture plastic ware was from Nunc (Denmark).

3.4.2 Cell Lines

The human ovarian SKOV-3 cancer cell line, human breast MCF-7 cancer cell line, and human fetal lung fibroblast MRC-5 cell line were purchased from American Culture Collection (ATCC, USA). SKOV-3, MCF-7, and MRC-5 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM), Roswell Park Memorial Institute 1640 (RPMI 1640), and Minimum Essential Medium (MEM) (Sigma, UK), respectively. All the media were supplemented with 10 % (v/v) Foetal Bovine Serum (FBS) (PAA Lab, Austria), 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 1 ml of fungizone (PAA Lab, Austria). Cells were maintained in 25 cm^3 flasks with 10 ml of media and were incubated at 37 °C in an incubator with 5 % CO_2 in a humidified atmosphere

(Jouan, France). The culture was sub-cultured every 2 or 3 days and routinely checked under an inverted microscope (Labscope, Jerman) for any contamination.

3.4.3 Cytotoxic Assay - Neutral Red Uptakes (NRU) Cytotoxic Assay

The cytotoxicity of the CHE, CEE, and CME were measured by Neutral Red Uptake (NRU) assay which was based on the uptake and subsequent lysosomal accumulation of the supravital dye, neutral red in the viable and uninjured cells. The quantification of the dye extracted from the cells has shown to be linear with the cell numbers, both by direct cell count and by protein determinations of the cell population (Borenfreund & Puerner, 1984). Cells were detached from the flask with 1 ml solution of accutase in phosphate buffered saline solution (PBS) pH 7.4. The cell pellet was obtained by centrifugation at 1000 rpm for 5 minutes (Kubota 2010, Tokyo) and the density of the viable cells was counted by 0.4 % of tryphan blue exclusion method using a haemocytometer (Hirschmann Techcolor, Germany) as describe by Freshney, (1994). Cells were then seeded in 96-well micro titer plate (Orange Scientific, Belgium), at a concentration of 30 000 cell/ml and then incubated in a CO_2 incubator at 37 °C for 3 hours to allow the cells to adhere before addition of the test agents. After 3 hours, the crude extracts were then added to the wells at six different concentrations of 1, 10, 25, 50, 75, and 100 µg/ml. Incubation with the extracts were carried out for 72 hours. Wells containing untreated cells (without addition of any extract) were regarded as a negative control, whereas cells treated with doxuribicin (0.5–10.0 µg/ml) served as a positive control.

At the end of the incubation period, the medium was replaced with medium containing 50 µg/ml Neutral Red (NR) solution (50 µg/ml NR in culture media, 24 hours pre-incubated in the dark at room temperature and then centrifuge at 1500 rpm for 10 minutes before use) and incubated for further 3 hours to allow for uptake of the vital dye into the lysosomes of viable and uninjured cells. The medium was then removed and cells were rapidly washed with calcium chloride-formaldehyde mixture. The dye within viable cells was eluted with from the cells with a mixture of acetic acid, ethanol, and water (1:50:49) (0.2 ml). The optical density (OD) was measured at 540 nm using microplate reader (Sunrise Tecan). Three replicate plates were used to determine the cytotoxicity of each extracts. The plates were agitated on a micro titer plate shaker (Wisd Laboratory Instrument) for 30 minutes and then absorbance against a blank reference was measured at 540 nm using a micro plate reader (Sunrise Tecan, Switzerland).

NR uptake, proportional to the number of viable cells within the well, was expressed as a percentage of uptake by control cells [$(OD_{control} - OD_{sample}) / (OD_{control}) \times 100$ %]. IC_{50} values (concentration required to reduce cells viability by 50 % as compared to the control cells) for each extract was extrapolated from the graphs plotted using the OD values obtained. Based on the US National Cancer Institute guidelines, a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value in carcinoma cells, following incubation between 48 and 72 hours, is ≤ 20 µg/ml, while for a pure compound the IC_{50} value is ≤ 4 µg/ml (Geran *et al.*, 1972; Boik, 2001).

In order to investigate whether the cytotoxic activity will be specific to the cancer cells, the cytotoxic activity of the extract was determined. The selectivity index (SI = IC_{50} on normal cells/IC_{50} on cancer cells) of the extracts is defined as the ratio of cytotoxicity (IC_{50} values) on normal cells to cancer cells (SKOV-3 and MCF-7).

Samples with an SI greater than 3 was considered to have high selectivity towards cancer cells (Mahavorasirikul *et al.*, 2010).

3.5 LC-MS/MS Analysis

CHE of *M. caeruleum* and *P. rotundifolia* was analyzed on a LC-MS/MS system equipped with the 3200 QTrap mass spectrophotometer and uHPLC system. The chromatographic separation was performed on a 150 mm × 4.6 mm × 5 µM Aqua C18 column, eluted with a mobile phase consisting of a water (A) and acetonitrile (B) containing 0.1 % formic acid and 5mM ammonium formate. A gradient elution starting from 10 % B to 90 % B from 0.01 min to 8.0 min, hold for 3 min and back to 10 % B in 0.1 min and re-equilibrated for 5 minutes, was used to separate the compounds of interest prior to mass spectral analysis. The mass spectrophotometer analysis was performed in a negative ion mode for detection of secondary compounds. Identities of the compounds were obtained by matching their molecular ions (*m/z*) obtained by LC-MS/MS with reference standards where available and by correlation with previous literature reports.

3.6 Antioxidant Assay

3.6.1 Chemicals and Reagents

Folin-Ciocalteau reagent, sodium carbonate (Na_2CO_3), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), ferric chloride hexahydrate ($FeCl_3.6H_2O$), sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) and gallic acid ($C_7H_6O_5$) were purchased from R & M Chemicals, UK. Ascorbic acid was purchased from Sigma-Aldrich, USA; 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma, Germany; 2, 4, 6-tripyridyl-*s*-triazine (TPTZ) was purchased from Sigma, Switzerland and glacial acetic acid was purchased from Fisher Scientific, UK. The other chemicals and solvents used were analytical grade.

3.6.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The DPPH free radical scavenging activity assay was carried out according to the method described by Blois (1958) with slight modifications. 25 µl of CHE, CEE, and CME were diluted appropriately in methanol (0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) mixed with 175 µl of 0.175 mM DPPH solution in methanol in wells of a 96-well plate. The plate was kept in the dark for 30 minutes, absorbance of the solution was measured at 517 nm with Multiskan Go plate reader (Thermo Scientific). Antioxidant activity was tested over a range of concentrations to establish IC_{50} (the concentration reducing DPPH absorbance by 50 %). Ascorbic acid was used as positive control.

3.6.3 Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was modified from Benzie and Strain (1996). FRAP reagent was freshly prepared by mixing 300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ in the volume ratio 10:1:1 (v/v), respectively. FRAP reagent was placed in 37 °C water bath before using it. For determination of FRAP activity, 20 µl of CHE, CEE, and CME were diluted appropriately in methanol (0.02, 0.04, 0.06, 0.08, and 0.10 mg/ml) was mixed with 180 µl of FRAP reagent in wells of a 96-well plate and left for 4 minutes at room temperature. The absorbance was measured at 595 nm with Multiskan Go plate-reader (Thermo Scientific, USA). Ferrous sulfate ($FeSO_4$) solution (0.1, 0.2, 0.4, and 0.6 mM) was used as standard ($y = 1.436x – 0.0024$, $R^2 = 0.9996$) and FRAP activity was calculated as Ferrous Equivalent (FE). Butylated hydroxyanisole (BHA) was used as positive control.

3.6.4 Determination of Total Phenolic Content (TPC)

The total phenolics content of the extracts was determined by using Folin-Ciocalteou (F-C) reagent method with slight modifications (Singleton & Rossi, 1965). 10 µl of CHE, CEE, and CME werw diluted in methanol (0.1, 0.2, 0.3, 0.4, and 0.5 mg/ml) and mixed with 150 µl of F-C reagent freshly. After five minutes, the solution was mixed with 100 µl of 7.5 % Na_2CO_3 solution, and left for 60 minutes at room temperature. Absorbance was read at 650 nm with Multiskan Go plate-reader. The TPC of each extract was calculated by comparing the absorbance with the gallic acid calibration curve according to the following formula:

TPC (mg/g) = C × V / g

where,

C = concentration of the gallic acid equivalent from standard curve (mg/ml)

V = volume of the extract (ml)

g = weight of extract (g)

The contents were expressed as gallic acid equivalent (mg GAE/g)

3.7 Data Analysis

Each measurement was carried out in triplicate, and the results were shown as mean ± S.D. Correlation coefficients between the total phenolic contents and reducing activity or free radical scavenging activity were calculated by using SPSS, version 16.0. The probability of $P < 0.05$ was considered to be statistically significant.

CHAPTER 4

RESULTS

4.1 Collection of the Selected Species from the Family Melastomataceae

Four plants namely, *Memecylon caeruleum* Jack, *Melastoma sanguineum* Sims, *Melastoma muticum* Ridl. and *Phyllagathis rotundifolia* Bl. from the Melastomataceae were selected in this study. *M. caeruleum* was collected from Lumut, Perak, *M. sanguineum* and *M. muticum* were collected from Ulu Kali, Selangor and collection of *P. rotundifolia* was done at Kuala Langat, Selangor.

Authentication of the plants was carried out in the herbarium of the Rimba Ilmu Botanical Garden, Institute of Biological Sciences, University of Malaya and voucher materials for this study were deposited at the same herbarium. Selection of the plants were made based on literature survey and ethno-medicinal data which reported the usage of those particular plants including plants under the same family or genus in traditional medicine. Table 4.1 summarizes the list of plant species and part of the plants used in this study, voucher numbers and also the collection sites.

Table 4.1: Selected species of Melastomataceae used in this study.

Plant species	Plant's part used	Voucher Number	Collection site
Memecylon caeruleum	Leaves	KLU 48120	Lumut, Perak
Melastoma muticum	Leaves	KLU 47788	Ulu Kali, Selangor
Melastoma sanguineum	Leaves	KLU 47792	Ulu Kali, Selangor
Phyllagathis rotundifolia	Leaves	KLU 48130	Kuala Langat, Selangor

4.2 Macroscopic Characteristics of the Plants

4.2.1 *Memecylon caeruleum* Jack

Figure 4.1: *Memecylon caeruleum* Jack

Memecylon caeruleum (Figure 4.1) is widely grown throughout Malaya, found near the sea in Indo-China, South and South East Thailand, the Philippines and throughout the Malay Archipelaga to New Guinea. *M. caeruleum* is a shrub c. 4 m tall with smooth bark and the twigs are flat close to the nodes. The leaves are simple, opposite, ovate to elliptic, cordate base, blunt or pointed apex, plain margin, thin-coriaceous, and smooth upper and lower surface. The midrib is prominent below, flat above; secondary veins are inconspicuous on both surfaces and the leaf stalks are 1–3 mm long. This plant has axillary inflorescence with a many-flowered, multi-branched cyme. The flowers are bisexual, very short pedicel 1–1.5 mm long, cup-shaped calyx 2–4 mm long that is glabrous and pink to purple color. Corolla is dark purple or deep blue, 2.5–3.5 mm in diameter. The stamens are 3–4 mm long; u-shaped anthers deep blue with white locules, style 5–6 mm long. The fruit is ellipsoid to obovoid, smooth and glaborous, 10–15 mm long, 6–9 mm wide, pink to dark red and turning deep purple to blackish upon maturity.

4.2.2 *Melastoma muticum* Ridl.

Figure 4.2: *Melastoma muticum* Ridl.

M. muticum (Figure 4.2) is widely distributed in Peninsular Malaysia and Borneo (Sabah). The plant is commonly found in lower or upper montane forest at 800–1700 m altitude. *M. muticum* is a shrub with c. 3 m tall. The plant has smooth bark and subquandrangular twigs. The leaves are simple, opposite, elliptic to lanceolate, 8.5–13.0 cm long, 3.0–5.0 cm wide, cuneate base, acute apex, plain margin, thin-coriaceous, and velvety hairy on the upper and lower surfaces. The midrib is flat to slightly raised below; 5-nerved, parallel to each other, upper side faint and lower side visible; tertiary veins inconspicuous and the leaf stalks are 7–21 mm long. This plant has axillary inflorescence with a few flowered, cyme. The flowers are bisexual; campanulate hypanthium, 12–15 mm long, hairy, and pink in color. Corolla is bright violet, obovate to cuneate, 19–20 mm long, 11–12 mm wide, dimorphic stamens – outer filaments c. 5.5 mm, connectives c. 5.0 mm long with a ventral bilobed appendage c. 2 mm long, inner filaments c. 5 mm long, connectives not prolonged, with 2 ventral appendages; 5–6 mm long anthers. The fruit is a fleshy capsule 10–14 mm long, 10–11 mm wide, rupturing irregularly at maturity.

4.2.3 *Melastoma sanguineum* Sims

Figure 4.3: *Melastoma sanguineum* Sims

M. sanguineum (Figure 4.3) is widely distributed in Burma, Thailand, Cambodia, Vietnam, South China, Malaysia, Sumatra, Java, Borneo, Sulawesi, Lesser Sunda Islands and Philippines (Leyte). The plant is commonly found in forest, along stream and roads, in open places and savannas at elevations of up to 2300 m. *M. sanguineum* is a shrub c. 2–3 m tall with smooth brown or grey barks and quandrangular twigs covered with appressed or spreading, red to brown scales or bristles. The leaves are simple, opposite, elliptic to lanceolate, cuneate base, acute apex, plain margin, thin-coriaceous, and bristly hairy upper surface and long velvety hairy with a tinge of red on the lower surface. The midrib is prominent below, flat above; 5-nerved, parallel to each other, upper side faint and lower side prominent; tertiary veins inconspicuous and the leaf stalks are 3–4.1 mm long, covered with erect flexuose red bristles, 5–9 mm long. This plant has terminal inflorescence with a few flowered, cyme. The flowers are bisexual; campanulate hypanthium, 7–15 mm long, 5–14 mm wide, covered with spreading, reddish bristles, 2–9 mm long. Corolla is violet, cuneate to obovate, 33–46 mm long, 22–32 mm wide, dimorphic stamen – outer filaments 8–12 mm long, yellow connectives 10–16 mm long ventrally curved with 2 yellow, ventral lobes 1–3 mm long and inner filaments 5–8 mm long, yellow connective not prolonged, basal with 2 ventral appendages 1–2 mm long; anthers 8–14 mm long, ± straight,

yellow. The fruit is a fleshy capsule 8–19 mm long, 8–18 mm wide, rupturing irregularly at maturity, exposing the yellow pulp with orange seeds.

4.2.4 *Phyllagathis rotundifolia* Bl.

Figure 4.4: *Phyllagathis rotundifolia* Bl.

P. rotundifolia (Figure 4.4) is commonly found in lowlands throughout Malaya. *P. rotundifolia* is a creeping herb occasionally becoming shrubby and growing upright. The leaves are simple, opposite, heart-shaped 5–23 cm long and 3.8–20 cm wide, cordate or wedge-shaped base, blunt or shortly pointed tip, wavy margin and thin-coriaceous. The midrib is prominent below, flat above; 9–11 main nerves, secondary nerves many and parallel to each other, upper side faint and lower side prominent; tertiary veins are prominent and the leaf stalks are 2.5–10 cm long. The inflorescence crowded in a head surrounded by magenta or pink, leafy bracts, on a stalk 2.5–7.6 cm long. The flowers are bisexual, corolla pinkish, c. 1.5 cm long stamens, anthers versatile, 5–6 mm long and the style is 1.6–1.8 cm long.

4.3 Yield of Crude Extracts

Yields of crude extracts obtained from hexane (CHE), ethyl acetate (CEE), methanol (CME) extraction are shown in Table 4.2. Yields of CME were the highest (5.27 % – 14.57 %) in all the species compared to CHE and CEE. Yields of CHE was the lowest (0.20 % – 0.92 %) in all species except for *P. rotundifolia*. Methanol extraction yielded the highest yield since it is high in polarity in comparison to ethyl acetate (middle polarity) and hexane (low polarity). Therefore, greater amount of compounds from the plants can be extracted by using methanol.

Table 4.2: Yield (%, w/w) of crude hexane (CHE), ethyl acetate (CEE), and methanol (CME) extracts prepared from selected species from the family Melastomataceae.

Species	Yield (%, w/w)		
	CHE	CEE	CME
M. caeruleum	0.31	1.53	5.27
M. muticum	0.34	0.87	10.80
M. sanguineum	0.20	1.36	14.57
P. rotundifolia	0.92	0.71	6.36

4.4 Phytochemical Screening

In the present study, the qualitative screening with crude methanol extracts revealed the presence of a wide range of phytochemical compounds in *M. caeruleum, M. muticum, M. sanguineum,* and *P. rotundifolia*. As shown in Table 4.3, it is clear that all of the four plant species contain phenolic groups including phenols, flavonoids and tannins while alkaloids and protein-xanthoprotein were absent. Phlobatannins were only present in *M. muticum* and *P. rotundifolia*. Whereas, terpenoids were detected in all plant species except *P. rotundifolia*. Saponins were present in CME of *M. sanguineum*

and *P. rotundifolia* while sterols were noted in *M. sanguineum* and *M. caeruleum*. Cardiac-glycoside was detected only in *M. sanguineum*. All of these phytochemical compounds had been that highly reported to possess some pharmacological benefits (Mensah *et al.*, 2009; Masih & Singh, 2012).

Table 4.3: Qualitative analysis of the phytochemical properties of crude methanol extracts (CME) of selected species from the family Melastomataceae.

Phytochemical group	Crude methanol extract (CME)			
	M. caeruleum	*M. muticum*	*M. sanguineum*	*P. rotundifolia*
Phenols	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Phlobatannins	-	+	-	+
Alkaloids	-	-	-	-
Terpenoids	+	+	+	-
Saponins	-	-	+	+
Sterols	+	-	+	-
Protein-Xanthoprotein	-	-	-	-
Cardiac-glycosides	-	-	+	-

+ = present

- = absent

4.5 Cytotoxic Activity of Crude Extracts on Human Cancer Cell Lines

In the present study, the cytotoxic activities of all the crude extracts were screened by Neutral Red Uptake (NRU) assay against human breast MCF-7 cancer cells and human ovarian SKOV-3 cancer cell lines; and human fetal lung fibroblast MRC-5 cell lines. The crude extracts were tested at concentrations range from 1–100 µg/ml and complete dose-response curve was plotted to determine the IC_{50} value. Figure 4.5 shows the dose-response curve of CME of *M. muticum* tested against human ovarian SKOV-3 cancer cell line. All the crude extracts demonstrated a significant concentration-dependent increase in growth on inhibition at concentration after 72 hours incubation period.

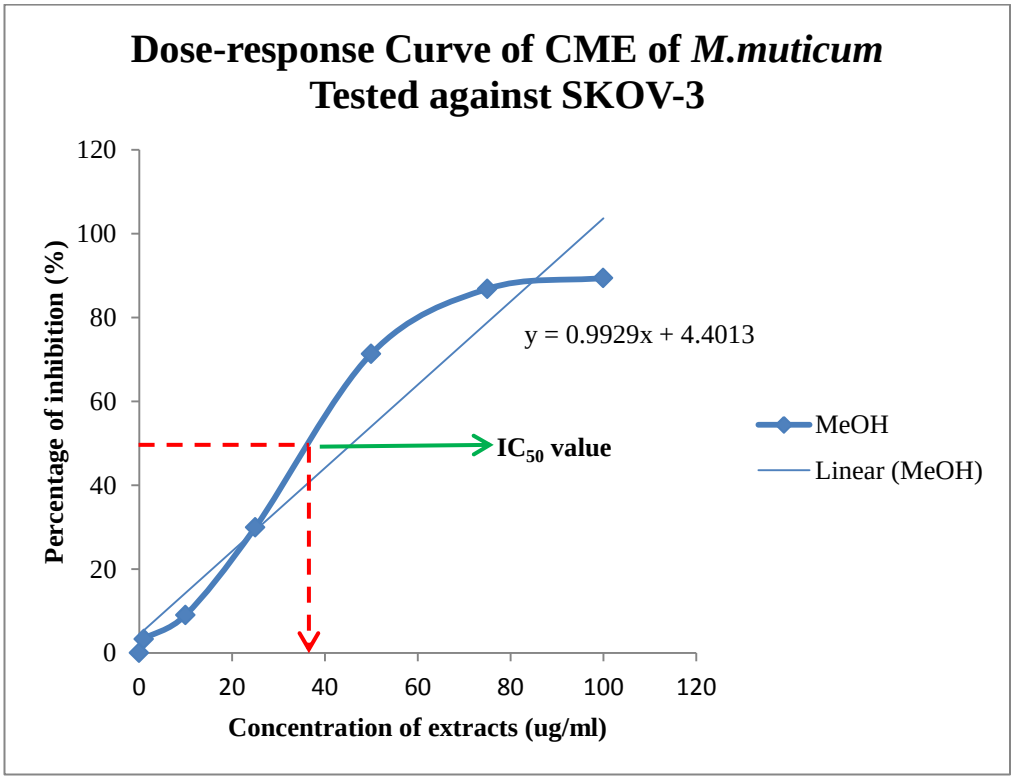

Figure 4.5: Dose-response curve of CME of *M. muticum* tested against human ovarian SKOV-3 cancer cell line.

Cytotoxicity (IC_{50} value in μg/ml) of all the crude extracts towards human breast MCF-7 and human ovarian SKOV-3 cancer cell lines; and human fetal lung fibroblast MRC-5 cell lines are summarized in Table 4.4.

Table 4.4: IC_{50} values of crude extracts tested against human cell lines.

Plant species	Extracts	IC_{50} values[@] (μg/ml) on different cell lines*		
		SKOV-3	MCF-7	MRC-5
M. caeruleum	CME	46.3 ± 0.84	31.16 ± 1.40	NT[x]
	CHE	>100	>100	NT[x]
	CEE	**16.04 ± 1.68**	**14.87 ± 0.31**	51.89 ± 0.54
M. muticum	CME	47.43 ± 1.35	46.03 ± 0.19	NT[x]
	CHE	74.48 ± 18.49	70.68 ± 4.01	NT[x]
	CEE	82.80 ± 8.22	47.11 ± 1.39	NT[x]
M. sanguineum	CME	46.08 ± 1.71	46.19 ± 0.34	NT[x]
	CHE	>100	>100	NT[x]
	CEE	55.58 ± 8.86	51.02 ± 0.46	NT[x]
P. rotundifolia	CME	21.53 ± 4.22	**17.83 ± 0.33**	95.61 ± 10.33
	CHE	24.76 ± 4.78	34.30 ± 3.09	NT[x]
	CEE	**5.23 ± 0.07**	**0.92 ± 0.01**	**7.54 ± 0.22**
Doxorubicin	-	0.42 ± 0.24	0.72 ± 0.03	1.72 ± 0.08

[x]NT = Not Tested

*SKOV-3 = Human ovarian cancer cell

*MCF-7 = Human breast cancer cell

*MRC-5 = Human fetal lung fibroblast cell lines (normal cell)

[@]Values in bold characters are considered to have cytotoxic activity. Crude extracts with IC_{50} value < 20 μg/ml are considered active (Geran *et al*., 1972; Boik, 2001).

According to the US National Cancer Institute Plant Screening Program, a crude extract is generally considered to have active cytotoxic activity if the IC_{50} value in carcinoma cells, following incubation between 48–72 hours, is equal or less than 20 μg/ml, while it is equal or less than 4 μg/ml for pure compounds (Geran *et al.*, 1972; Boik, 2001). All extracts of *M. muticum* and *M. sanguineum* did not show any cytotoxic

activity when tested against MCF-7 and SKOV-3 cancer cell lines since the IC_{50} values for each of the crude extract was greater than 20 μg/ml. On the other hand, CME and CEE of *P. rotundifolia* exhibited potent cytotoxic activity when tested against MCF-7 cell lines with IC_{50} values of 17.83 ± 0.33 μg/ml and 0.92 ± 0.01 μg/ml, respectively. CEE of *M. caeruleum* was also found to be cytotoxically active on MCF-7 cell lines with an IC_{50} of 14.87 ± 0.31 μg/ml.

CEE of *M. caeruleum* and *P. rotundifolia* were considered to have strong cytotoxic activity on SKOV-3 cells. Both CEE from *M. caeruleum* and *P. rotundifolia* exhibited IC_{50} values of 16.04 ± 1.68 and 5.23 ± 0.07 μg/ml respectively. In comparison between the cytotoxically active extracts, cytotoxic activity of *P. rotundifolia* was greater than *M. caeruleum*. CEE of *P. rotundifolia* exhibited IC_{50} values < 10.0 μg/ml in both SKOV-3 and MCF-7 cells.

The cytotoxic potency of the four tested species in descending order is as follows: *P. rotundifolia* > *M. caeruleum* > *M. muticum* and *M. sanguineum*. The cytotoxic effects of the crude extracts were more pronounced towards MCF-7 cells than SKOV-3 cells.

CME of *P. rotundifolia* and CEE of *M. caeruleum* and *P. rotundifolia* were further selected to be tested against MRC-5 human fetal lung fibroblast cells in order to determine the selectivity index. CEE of *M. caeruleum* and CME of *P. rotundifolia* can be considered less toxic on the MRC-5 cells with the IC_{50} values of 51.89 ± 0.54 and 95.61 ± 10.33 μg/ml, respectively. However, the IC_{50} value of CEE of *P. rotundifolia* on MRC-5 cells was 7.54 ± 0.22 μg/ml which can be considered as toxic.

Table 4.5: Selectivity of the cytotoxically active crude extracts of *M. caeruleum* and *P. rotundifolia* in comparison with MRC-5 cells.

Plant species	Crude extracts	Selectivity index (SI[y])	
		SKOV-3	MCF-7
M. caeruleum	CEE[z]	3.26	3.49
P. rotundifolia	CME[z]	NT[x]	5.37
	CEE[z]	1.44	8.22
Doxorubicin	-	4.1	2.4

[x]NT = Not tested

[y]SI = Ratio of the IC_{50} values of extract on MRC-5 cells to those in the cancer cell lines. Sample with SI greater than 3 were considered to have high selectivity (Mahavorasirikul *et al.*, 2010).

[z]CME = Crude Methanol Extract

[z]CEE = Crude Ethyl acetate Extract

Table 4.5 displays the Selective Index (SI) of the cytotoxically active crude extracts of *M. caeruleum* and *P. rotundifolia*. The SI was determined by comparing the cytotoxic activity of the crude extracts tested against normal human lung fibroblast MRC-5 cells to those in the cancer cell lines. Extracts with SI value greater than 3 was considered to be highly selective towards cancer cells.

CEE of *P. rotundifolia* was considered to be the most potent and most selective against MCF-7 (IC_{50} = 0.92 ± 0.01 µg/ml, SI = 8.22). However the extract was not selective against MCF-7 (IC_{50} = 5.23 ± 0.07 µg/ml, SI = 1.44) as the SI value was less than 3. CME of *P. rotundifolia* also showed selectivity towards MCF-7 cells (IC_{50} = 17.83 ± 0.33 µg/ml, SI = 5.37). The SI values for CEE of *M. caeruleum* can be considered slightly selective towards SKOV-3 and MCF-7 cells with the SI values of 3.26 and 3.49, respectively. Thus, the extract showed selectivity towards both SKOV-3 and MCF-7 cells. However, the SI was slightly higher when tested against MCF-7 cells (SI = 3.49) and it showed selectivity towards MCF-7.

Overall, the cytotoxic property of CEE of *M. caeruleum* and CEE of *P. rotundifolia* was shown to be selective towards MCF-7 cells. The selectivity towards MCF-7 cells in descending order is as follows: *P. rotundifolia* > *M. caeruleum*. Only CME of *M. caeruleum* was identified to be selective towards SKOV-3 cells.

4.6 Identification of Major Compound by using LC-MS/MS System in Crude Ethyl Acetate Extracts (CEE) of *M. caeruleum* and *P. rotundifolia*

The identification of the major compounds by using LC-MS/MS system was carried out on the CEE of *M. caeruleum* and *P. rotundifolia* since both extracts showed the highest cytotoxic activity. The data obtained were compared with the previous published data of chemical constituents of both plants and the LC-MS/MS profiles of compounds in the extract obtained using Agilent Zorbax C18 column (150 mm × 4.6 mm × 5 µM) are shown in Figure 4.3 and Figure 4.4. The spectrometric analysis and tentatively identified major compounds are listed in Table 4.6. Peaks at R_t 4.59, 6.43, 7.08, and 8.40 minutes of CEE of *M. caeruleum* and peaks at R_t 6.16, 6.42, and 7.08 minutes of CEE of *P. rotundifolia* were characterized because they were present in more than 50% concentration in the mixture. Peaks which are not able to be identified are not listed in this table and they might be new or novel compound(s).

Referring to the full chromatogram of *M. caeruleum* (Figure 4.3), the major peak with a retention time of 4.59 min was identified as isohamnetin-3-*O*-hexoside (MS *m/z* = 477) and kaempferol glucoside (MS *m/z* = 447). Meanwhile, the major peak for *P. rotundifolia* with a retention time of 7.08 min was identified as tetramethoxychalcone derivative (MS *m/z* = 343) (Figure 4.4). Initial qualitative phytochemical investigation

revealed the present of polyphenols and flavonoids as major compounds in both CEE of *M. caeruleum* and *P. rotundifolia*.

Table 4.6: Identification of compounds in CEE of *M. caeruleum* and *P. rotundifolia* by using LC-MS/MS data.

Plant species	Retention time (min)	MW (*m/z*)	Tentative ID
M. caeruleum	4.59	477.1	Isorhamnetin-3-*O*-hexoside
		447.1	Kaempferol glucoside
	6.43	329.3	3,3'-di-*O*-methyl ellagic acid
	7.08	343.1	Tetramethoxychalcone derivatives
	8.40	311.2	15,16-dihydroxy-9Z, 12Z-octadecadienoic acid
P. rotundifolia	6.16	327.3	Methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate
	6.42	329.3	3,3'-di-*O*-methyl ellagic acid
	7.08	343.1	Tetramethoxychalcone derivatives

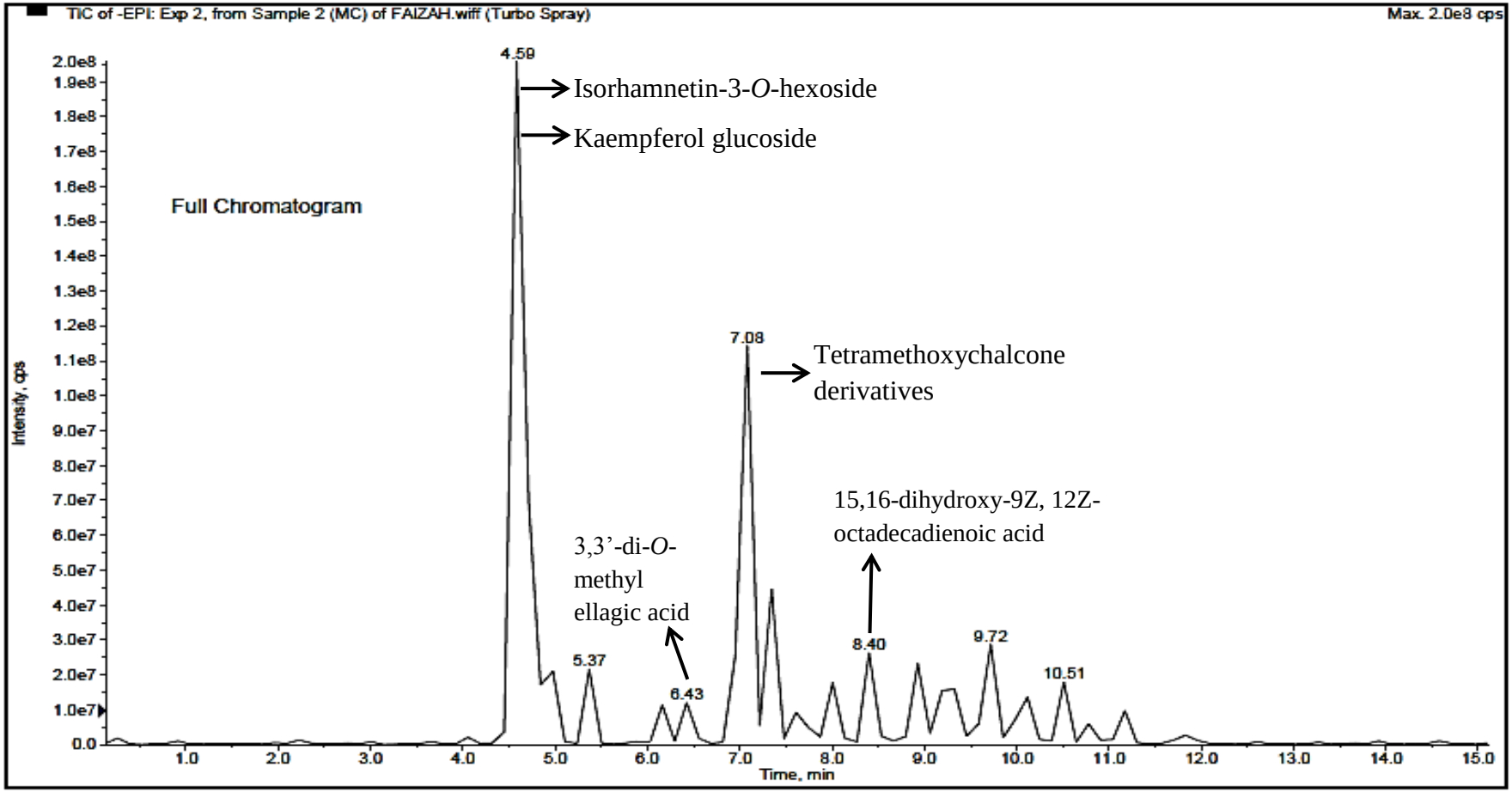

Figure 4.3: LC-MS/MS profiles of chemical compounds in CEE of *M. caeruleum.*

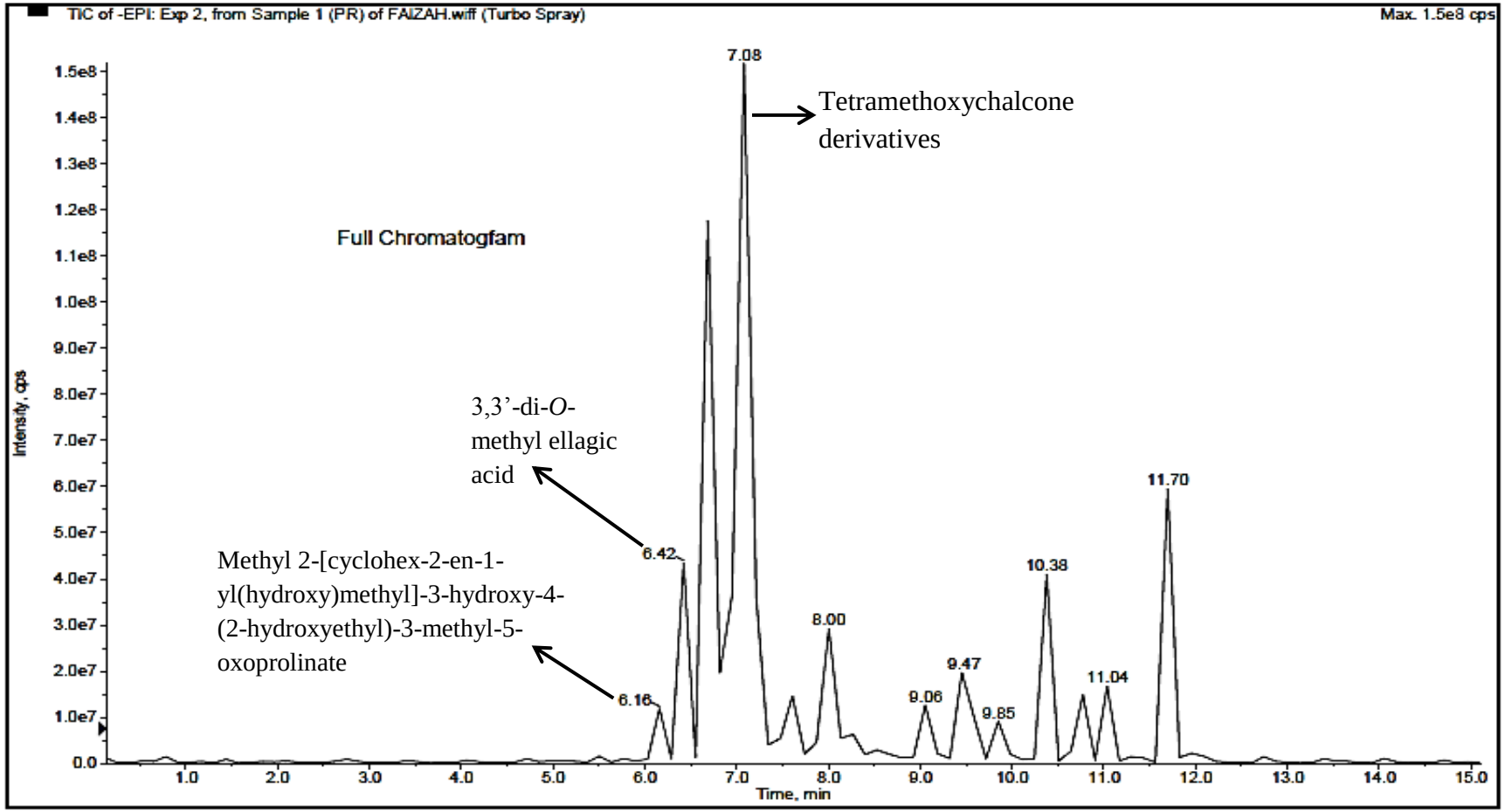

Figure 4.4: LC-MS/MS profiles of chemical compounds in CEE of *P. rotundifolia.*

4.7 Antioxidant Potential of Crude Extracts

4.7.1 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The free radical scavenging activity of the selected species were expressed as IC_{50} µg per ml of each extract. The antiradical activity was defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50 %. Table 4.7 shows the free radical scavenging ability of CME, CHE, and CEE on the basis of percentage inhibition. It is evident from the table that, CME of *M. sanguineum* exhibited the highest free radical scavenging activity (77.1 ± 2.8 µg/ml), followed by *M. muticum, P. rotundifolia,* and *M. caeruleum*. CHE of *M. sanguineum* was observed to have the lowest free radical scavenging potential (2224.3 ± 321.2 µg/ml).

` **Table 4.7:** Free radical scavenging activity of crude extracts.

Plant species	IC_{50} (µg/ml)		
	CME	CHE	CEE
M. caeruleum	309.6 ± 4.7	1131.7 ± 37.6	446.5 ± 10
M. muticum	121.5 ± 2.1	457.0 ± 49.0	156.1 ± 1.8
M. sanguineum	77.1 ± 2.8	2224.3 ± 321.2	175.5 ± 5.2
P. rotundifolia	251.6 ± 5.6	2123.4 ± 193.6	2172.0 ± 272.8

4.7.2 Ferric Reducing Antioxidant Power (FRAP) Assay

Reducing potential was expressed as mM Fe (II) per 100 g of each extract. In general, the reducing potential increased as the concentration of the extract increased and the highest reducing potential was observed in CME of all the selected species followed by CEE and CHE (Table 4.8). CME of *M. sanguineum* had the highest reducing potential (68.89 ± 0.50 mM Fe (II)/100 g) followed by *M. muticum*, *M. caeruleum* and *P. rotundifolia*.

Table 4.8: Reducing potential of crude at different concentrations.

Plant species	Concentration of extract (mg/ml)	FRAP value (mM Fe (II)/ 100 g)		
		CME	CHE	CEE
M. caeruleum	0.02	4.37 ± 0.34	1.67 ± 0.14	3.34 ± 0.40
	0.04	6.52 ± 0.44	2.52 ± 0.33	5.38 ± 0.24
	0.06	12.86 ± 0.65	3.34 ± 0.19	7.35 ± 0.30
	0.08	13.01 ± 0. 54	4.43 ± 0.37	11.00 ± 0.26
	0.10	16.78 ± 0. 67	4.15 ± 0.49	10.25 ± 0.25
M. muticum	0.02	7.64 ± 0.60	1.49 ± 0.70	8.31 ± 0.27
	0.04	11.81 ± 0.14	1.93 ± 0.60	20.51 ± 0.48
	0.06	30.84 ± 0.93	3.70 ± 0.14	23.48 ± 0.99
	0.08	42.13 ± 1.66	4.47 ± 0.12	27.59 ± 1.27
	0.10	45.64 ± 1.80	5.41 ± 0.25	37.86 ± 0.35
M. sanguineum	0.02	13.34 ± 0.79	1.69 ± 0.21	6.79 ± 0.41
	0.04	25.79 ± 1.02	1.90 ± 0.10	15.42 ± 1.10
	0.06	38.11 ± 1.47	2.77 ± 0.77	22.71 ± 1.12
	0.08	53.15 ± 3.17	2.85 ± 0.10	29.54 ± 0.95
	0.10	68.89 ± 0.50	3.02 ± 0.80	30.85 ± 1.19
P. rotundifolia	0.02	5.14 ± 0.30	1.01 ± 0.26	1.10 ± 0.17
	0.04	8.46 ± 0.30	1.60 ± 0.11	1.68 ± 0.13
	0.06	12.00 ± 0.28	1.87 ± 0.14	2.17 ± 0.19
	0.08	13.83 ± 0.83	2.85 ± 0.15	2.32 ± 0.16
	0.10	17.08 ± 1.47	3.08 ± 0.14	2.63 ± 0.70

4.7.3 Total Phenolic Content (TPC) Assay

The total phenolic content was expressed as mg GAE per 100 g of each extract. In general, the total phenolic content increased as the concentration of the extract increased and the highest phenolic content was observed in CME of all the selected species followed by CEE and CHE, as shown in Table 4.9. At concentration range from 0.1–0.5 mg/ml, CME of *M. sanguineum* had the highest total phenolic content ranging from 718.0 ± 9.0 – 801.0 ± 10.0 mg GAE / 100 g, followed by *M. muticum, M. caeruleum* and *P. rotundifolia*.

Table 4.9: Total phenolic content of crude extracts at different concentrations.

Plant Species	Concentration of extract (mg/ml)	Gallic Acid Equivalent (mg GAE/ 100 g)		
		CME	CHE	CEE
M. caeruleum	0.1	234.0 ± 5.0	79.0 ± 1.0	234.0 ± 5.0
	0.2	244.0 ± 1.0	82.0 ± 2.0	240.0 ± 6.0
	0.3	251.0 ± 1.0	87.0 ± 3.0	251.0 ± 2.0
	0.4	257.0 ± 4.0	96.0 ± 2.0	285.0 ± 8.0
	0.5	264.0 ± 5.0	99.0 ± 2.0	299.0 ± 4.0
M. muticum	0.1	676.0 ± 33.0	76.0 ± 4.0	571.0 ± 12.0
	0.2	693.0 ± 11.0	88.0 ± 1.0	606.0 ± 4.0
	0.3	708.0 ± 5.0	91.0 ± 1.0	610.0 ± 5.0
	0.4	714.0 ± 2.0	95.0 ± 6.0	618.0 ± 4.0
	0.5	725.0 ± 3.0	103.0 ± 1.0	625.0 ± 2.0
M. sanguineum	0.1	718.0 ± 9.0	55.0 ± 9.0	476.0 ± 6.0
	0.2	735.0 ± 2.0	56.0 ± 1.0	572.0 ± 9.0
	0.3	747.0 ± 10.0	58.0 ± 2.0	593.0 ± 6.0
	0.4	761.0 ± 12.0	61.0 ± 3.0	602.0 ± 11.0
	0.5	801.0 ± 10.0	69.0 ± 4.0	613.0 ± 11.0
P. rotundifolia	0.1	203.0 ± 5.0	29.0 ± 2.0	35.0 ± 2.0
	0.2	207.0 ± 2.0	36.0 ± 1.0	39.0 ± 1.0
	0.3	217.0 ± 2.0	38.0 ± 0.0	43.0 ± 2.0
	0.4	224.0 ± 6.0	40.0 ± 2.0	45.0 ± 1.0
	0.5	227.0 ± 4.0	45.0 ± 2.0	50.0 ± 1.0

4.7.4 Comparison of Antioxidant Activities

Total phenolic content, reducing activities, and free radical activities of the studied plants are shown in Table 4.10. Analysis of distribution of phenolic content in the four selected species were variable and revealed that the CME of *M. sanguineum* contained the highest phenolic content (718.18 ± 8.91 mg GAE/100 g).

The reducing potentials of the different crude extracts were determined by the FRAP assay. The results indicated that the CME of *M. sanguineum* had the highest reducing power (68.89 ± 0.50 mM/100 g) when compared to the other extracts and the positive control (Butylated hydroxyanisole). In the DPPH assay, all the crude extracts were tested for their ability to reduce the stable radical DPPH into formation of non-radical form of DPPH. As seen in Table 4.7, the CME of *M. muticum* showed the highest activity in this assay (IC_{50} = 77.1 ± 2.8 µg/ml). However, the IC_{50} was slightly higher than the positive control (ascorbic acid).

Table 4.10: Antioxidative properties of crude extracts at 1.0 mg/ml.

Plant species	Extract	Gallic Acid (mg GAE/100 g)	FRAP value (mM Fe (II)/100 g)	IC_{50} (µg/ml)
		TPC	FRAP	DPPH
M. caeruleum	CME	234.19 ± 4.74^{c}	16.78 ± 0.66^{d}	121.5 ± 2.1^{a}
	CHE	79.19 ± 1.22^{b}	4.15 ± 0.49^{ab}	457.0 ± 49.0^{b}
	CEE	234.48 ± 4.87^{c}	10.25 ± 0.25^{c}	156.1 ± 1.8^{a}
M. muticum	CME	676.03 ± 32.85^{f}	45.64 ± 1.8^{g}	77.1 ± 2.8^{a}
	CHE	76.20 ± 3.62^{b}	5.41 ± 0.24^{b}	2224.3 ± 321.2^{a}
	CEE	571.45 ± 11.97^{e}	37.86 ± 0.35^{f}	175.5 ± 5.2^{a}
M. sanguineum	CME	718.18 ± 8.91^{g}	68.89 ± 0.50^{h}	309.6 ± 4.7^{a}
	CHE	54.58 ±8.92^{ab}	3.02 ± 0.08^{ab}	1131.7 ± 37.6^{c}
	CEE	475.97 ± 5.55^{d}	30.85 ± 1.19^{e}	446.5 ± 10^{a}
P. rotundifolia	CME	202.91 ± 5.26^{c}	17.08 ± 1.47^{d}	251.6 ± 5.6^{a}
	CHE	28.51 ± 2.12^{a}	3.08 ± 0.14^{ab}	2123.4 ± 193.6^{c}
	CEE	34.63 ± 2.40^{a}	2.63 ± 0.07^{a}	2172.0 ± 272.8^{c}
Ascorbic Acid	-	-	-	73.9 ± 1.6
BHA	-	-	18.76 ± 0.46	-

Each value is the mean ± standard deviation (S.D) consisting of three replicates.

$^{a, b, c, d, e, f, g, h}$ Mean values in a column indicates significance differences at $P<0.05$.

BHA = Butylated hydroxyanisole

4.7.5 Correlation between Total Phenolic Content and Antioxidant Properties

Correlational analyses (Table 4.11) were done to study the relationship between the total phenolic content (TPC) of CME, CHE, and CEE of the four species and their antioxidant activities were based on FRAP (reducing potential) and DPPH assay (free radical scavenging activity). There was a significant positive correlation between TPC and reducing activity (r = 0.96, n = 36, P < 0.01). This suggests that phenolics might play major roles in contributing the reducing activity.

In contrast, TPC and DPPH were negatively correlated (r = -0.719, n = 36, $P < 0.01$). Results indicated a strong inverse relationship between TPC and free radical scavenging activity. In other words the DPPH method yields inversely proportional results with IC_{50} values, in which the lower the IC_{50} value, the higher the free radical scavenging activity.

Table 4.11: Pearson's correlation coefficients (r) between total phenolic content and antioxidant capacity.

		FRAP	DPPH
TPC	Pearson's correlation (r)	0.963*	-0.719*
	Sig. (2-tailed)	0.000	0.000
	N	36	36

*****significant at $P < 0.01$

CHAPTER 5

DISCUSSION

5.1 Botanical Identity and Pharmacognostical Study

The most fundamental step in the scientific study of medicinal plants is establishing their botanical identity and assigning correct taxonomy name to the plant specimen (Bennet & Balick, 2008). Botanical specimens, known as vouchers or voucher specimens, are deposited in a properly curated location known as herbaria. Herbaria are collections of dried plants that are labeled, mounted on archival quality paper, and arranged systematically for scientific study and documentation (Ballick, 1999; Bennet & Balick, 2008).

Preparation of the vouchers is one of the crucial steps in achieving "good botanical practices" in plant medicinal studies (Balick, 1999). Plant vouchers play an important role in scientific documentation and identification, as well as for studies of variability within plant taxa. In the present study, parts of *Memecylon caeruleum, Melastoma muticum, Melastoma sanguineum*, and *Phyllagathis rotundifolia* were collected from wild habitat in Peninsular Malaysia, dried, preserved, mounted onto herbarium card, and finally labeled before been assigned with correct taxonomy name and voucher number. Authentication of the specimens including measurements of the botanical features (habit – size or physical appearance of the plant and structure of leaves, inflorescence, flower, corolla, stamens, style, stigma, and fruits of the plant) were carried out in the herbarium of Rimba Ilmu Botanical Garden, Institute of Biological Science, University of Malaya and the voucher materials were deposited at the same herbarium.

Proper documentation and identification of the plant materials used in a study is crucial in order to avoid any mistakes in research or clinical studies, thus reducing the value of the study. Frequent misuse or misspell of binomials is causing researchers to cannot adequately find the literature or electronic database. Another problem is lack of author citations for binomials. Their inclusion is mandatory for publications in systematics and ethnobotany. Failure in including the author citation is causing confusion or unclear as to which taxon the plant belongs to (Bennet & Balick, 2008; Khasim *et al.,* 2013). In addition, more accurate features or parameters of the medicinal plants must be developed to provide pharmacognostic standards that will help in the identification and quality control of raw material of the medicinal plants in future (Saralla *et al.,* 2012; Khasim *et al.,* 2013). Pharmacognostic standard included the evaluation of morphological and anatomical features, physicochemical constants and qualitative phytochemical analysis which is required for quality control of the crude drug (Kaneria & Chandra, 2011).

5.2 Preparation of Crude Extracts

In the present study, crude extracts were prepared from air-dried leaves of *M. caeruleum, M. muticum, M. sanguineum,* and *P. rotundifolia*. Leaves are perishable and may deteriorate within few days after harvest and one way to preserve them is by air or oven drying at room temperature. These methods will help to conserve the desirable qualities, reduce storage volume, and to extend the shelf-life of the harvested leaves (Lim & Murtijaya, 2007). Temperature plays an important role in drying process due to the present of polyphenol oxidases, a degradative enzyme in leaves that can be deactivated by sun drying or oven heating at 50 °C. Moreover, sun drying can lead to an uneven loss of phenolic compounds and high temperature causes some polyphenols and

other phytochemicals to be degraded (Okuda *et al.,* 1989). According to Nicoli *et al*., (1999) intense and/or prolonged thermal treatment may be responsible for a significant loss of natural antioxidants, as most of these compounds are relatively unstable. Investigation by Capecka *et al*., (2005) reported that air drying resulted in a considerable increase of total phenolic content (TPC) in oregano and peppermint leaves.

Preparation of the crude extracts was achieved by the cold extraction method in three organic solvents with increasing polarity – hexane, ethyl acetate, and methanol. Cold extraction method allows for maximum extraction of compounds from plant. This observation was based on the ability of the organic solvents to increase cell wall permeability, facilitating the efficient extraction of large amounts of polar and medium-to-lower-polarity constituents (Sarker *et al.,* 2005; Fakim, 2006). It is known that the main groups of chemical compounds that can be extracted by using methanol were anthocyanins, terpenoids, saponins, tannins, xanthoxyllines, totarol, lactones, flavones, phenones, and polyphenols (Tiwari *et al.*, 2011). Waxes, fats, and volatile oils are among the chemical compounds that can be extracted by hexane, while ethyl acetate can be used to extract alkaloids, aglycones, and glycoside (Houghton & Raman, 1998). It can be concluded that the different yields of crude extracts obtained in these three different solvents used in the study is due to the differences in their solvent polarity.

5.3 Phytochemical Screening

The phytochemical screening was carried out with the crude methanol extract (CME) of the selected species of *Memecylon caeruleum, Melastoma muticum, Melastoma sanguineum,* and *Phyllagathis rotundifolia* according to the standard phytochemical screening methods (Solihah *et al.*, 2012). The presence of various phytochemical compounds belonging to the groups of phenols, flavonoids, and tannins were observed after screening. These groups have been previously reported to possess various pharmacological benefits in many other studies. The presence of these groups can be differentiated from one plant to another but their therapeutic properties to cure certain illnesses could vary (Mensah *et al.*, 2009; Solihah *et al.*, 2012; Masih & Singh, 2012).

A literature survey indicated that no phytochemical work as well as biological activity has been reported on *Melastoma muticum*, and *Melastoma sanguineum*. Therefore, the phytochemical data obtained in the current study were compared with their sister species from the genus *Melastoma*.

Melastoma malabathricum is the only plant from Melastomataceae that has gained herbal status in Malay folklore and has been widely used in the treatment of various diseases. Joffry *et al.,* (2012) presented a comprehensive review of ethnomedicinal uses, phytochemistry, and pharmacological aspect of this plant. The leaves part of *M. malabathricum* consists of flavonoids, triterpenes, tannins, saponins, steroids, glycosides, and phenolics (Zakaria *et al.,* 2006; Faravani, 2009). The current results on the preliminary phytochemical screening of *M. muticum* and *M. sanguineum* were parallel to the sister species in which group of similar compounds such as phenolics, tannins, terpenoids, saponins, and flavonoids were detected.

Melastoma candidum is commonly found throughout southern China, Taiwan, Japan, and Philippines and is widely used in folklore medicine in Taiwan to eliminate stasis, clean serum of toxins treat traumatic injury, and cure bacterial dysentery (Wang *et al.,* 2008). Several studies had been done to study active compounds in this plant. According to Cheng *et al*., (1993), three active compounds were isolated from the leaves (castalagin, procyanidin B-2, and helichrysosode) as well as four flavonoids (quercitrin, isoquercitrin, rutin, and quercetin) were also isolated from the leaves (Lee *et al.,* 2001). *Melastoma decemfidum* is locally known as *'senduduk putih'* and is widely distributed throughout Madagascar, India, and Australia. Phytochemical analysis of leaves of *M. decemfidum* showed the presence of three compounds (naringenin, kaempferol and kaempferol-3-*O*-D-glucoside) in the ethyl acetate extract and two compounds (kaempferol-3-*O*-2″,6″-di-*O*-*p*-*trans*-coumaroyl glucoside and kaempferol-3-*O*-d-glucoside) from the methanol extracts (Sarju *et al.,* 2010).

No phytochemical work and biological activity of *Memecylon caeruleum* have been reported. Because of that, the current phytochemical data were compared with sister species from genus *Memecylon*. Phytochemical screening of ethyl acetate of *Memecylon edule*, showed the presence of alkaloids, flavonoids, tannins, saponins, and terpenoids (Naidu *et al.,* 2013). *Memecylon malabaricum* is used in traditional practices to treat various skin diseases including herpes. Phytochemical screening of the leaves showed the presence of steroids, triterpenes, flavonoids, saponins, tannins, and resins (Hullatti & Rai, 2004). *Memecylon umbellatum* is reported to have good hypoglycemic effect (Amalraj & Ignacimuthu, 1998). The phytochemical analysis revealed the presence of significant levels of terpenoids, flavonoids, tannins, and moderate amount of phenols, and glycosides (Puttaswamy & Achur, 2013).

Phytochemical analysis of leaves of *P. rotundifolia* by using LC-ESI-MS/MS system (electrospray ionization), identified the presence of prunasin based cyanogenic glucosides with galloyl esterification together with prunasin and polyphenols such as gallotannins, ellagitannins, ellagic acid derivatives, and gallic acid (Ling *et al.,* 2002; [b]Tan *et al.,* 2011). In comparison, phenolics groups and saponins were detected in the phytochemical screening and our results were in tandem with the reported phytochemical data of *P. rotundifolia* (Ling *et al.,* 2002; [b]Tan *et al.,* 2011). A literature survey also indicated that no phytochemical work as well as biological activity has been reported on other species in this genus.

5.4 Cytotoxic Screening of Crude Extracts on Various Human Cancer Cell Lines

Natural sources are the reservoir of novel anticancer finding since huge number of studies had been conducted in search for active materials to fight against human tumours. Paclitaxel was isolated from pacific *yew* plant and since then, it has been used for lung and breast cancer treatment (Alkhamaiseh *et al.,* 2012). The present study was undertaken to evaluate the cytotoxic activity of crude methanol (CME), crude hexane (CHE), and crude ethyl acetate (CEE) of selected species of *M. caeruleum, M. muticum, M. sanguineum,* and *P. rotundifolia* from the family Melastomataceae. To date, this is the first study on the cytotoxicity investigation reported on human breast MCF-7 cancer cells and human ovarian SKOV-3 cancer cells for the species mentioned before.

The cytotoxicity test was carried out by Neutral Red Uptake (NRU) assay which is based on the ability of viable cells to incorporate and bind the supravital dye, neutral red. Besides NRU assay, several *in vitro* cytotoxic assays that are commonly used in these kinds of studies are colony formation (CF) assay, crystal-violet staining (CV)

assay, lactate dehydrogenase (LDH) leakage assay, protein assay, and methyl tetrazolium (MTT) assay. NRU assay was selected because it is more sensitive and much cheaper than other cytotoxic assays. Once the cells have been treated, the assay can be completed in less than 3 hours (Repetto *et al.,* 2008).

The current study indicated that the crude extracts of *M. muticum* and *M. sanguineum* were not cytotoxically active when tested against SKOV-3 and MCF-7 cells. In contrast, CME of *P. rotundifolia* and CEE of *M. caeruleum* and *P. rotundifolia* demonstrated high cytotoxic activity towards SKOV-3 and MCF-7 cell lines. However, the extracts of *M. caeruleum* and *P. rotundifolia* exhibited different degree of cytotoxicity and sensitiveness towards the different cell lines.

The observed differentiation in the cytotoxic activity between all the crude extracts of *M. caeruleum, M. muticum, M. sanguineum*, and *P. rotundifolia* might be primarily due to their differences in the phytochemical profiles. Kontogianni *et al*., (2013) reported that the ethyl acetate extract of rosemary (*Rosmarinus officinalis*) and ethyl acetate extract of sage (*Salvia officinalis*) have different cytotoxic activity when tested against rat insulinoma RINm5F cells. Rosemary extract was more potent in reducing RINm5F cell viability with an IC_{50} value of 35.6 µg/ml compared to the sage extract (IC_{50} value = 101.0 µg/ml). Both *R. officinalis* and *S. officinalis* belong to the Lamiaceae family. The major differences in their phytochemical profiles of these two extracts were fourfold higher concentration of carnosic acid and also the presence of a significantly higher amount of betulinic acid in the rosemary extracts. The study showed that both extracts have different concentration of carnosic acid and betulinic acid was identified only in rosemary extracts. The differences in the phytochemical profiles of both extracts might be responsible for the differential cytotoxic activity observed.

Different cancerous cells showed different degree of response or sensitivity toward anticancer drugs and these are due to different genetic profiles or molecular characteristics of that particular cancer or drugs (Szakacs *et al.*, 2004). *Curcuma* is a genus of the ginger family, Zingiberaceae. One of the active constituents reported in *Curcuma* is curcumin (diferuloylmethane) and it has been reported to possess chemopreventive effect against various human cancer cells (Aggarwal *et al.*, 2003). Sertel *et al*., (2012) did a study to evaluate factors that determine the response of tumours (60 human tumour cell lines in the Developmental Therapeutics Program of the National Cancer Institute (NCI) consisted of leukemia, lung, colon, renal, ovarian, central nervous system, prostate carconima and breast cancer) towards curcumin. The results showed that different genes from diverse functional groups such as *m*RNA metabolism, folate metabolism, signal transduction, DNA repair invasion, angiogenesis, apoptosis, proliferation and transporter genes, determine the sensitivity or resistance toward curcumin.

In this study, the CEE and CME of *P. rotundifolia* were considered to be the most potent and selective against human breast MCF-7 cancer cells. The CEE of *M. caeruleum* exhibited selectivity towards both human ovarian SKOV-3 cancer cells and human breast MCF-7 cancer cells. Selective index (SI) indicated selectivity of the sample or extract to the cell lines tested. Samples with an SI greater than 3 were considered to have a high selectivity towards cancerous cells. SI is important to detect the most promising and most selective cytotoxic activity against cancerous cells (Mahavorasirikul *et al.*, 2010).

5.5 LC-MS/MS Analysis of Cytotoxically Active Crude Extracts

The present study was preceded with further phytochemical analyses to identify the major compound(s) in cytotoxically active extracts that is crude ethyl acetate (CEE) for *M. caeruleum* and *P. rotundifolia*. Investigation and identification of major compounds present in crude ethyl acetate extracts (CEE) of these two species were done by LC-MS/MS analyses which indicated the present of phenolics (isorhamnetin-3-*O*-hexoside, kaempferol glucoside, 3,3'-di-*O*-methyl ellagic acid, tetramethoxychalcone derivatives). The detected compounds may be responsible for the observed cytotoxic activity of *M. caeruleum* and *P. rotundifolia.* LC-MS/MS approach is one of the most reliable methods in separating and detecting a broadrange of chemical constituents and secondary metabolites that are present in plant kingdom (Allwood & Goodacre, 2009).

Phenolic compounds are one of the major chemical compounds in the cytotoxically active crude ethyl acetate extract (CEE). The phenolic compounds in the crude ethyl acetate extracts (CEE) of *M. caeruleum* were identified as isorhamnetin-3-O'-hexoside, kaempferol glucoside, 3,3'-di-*O*-methyl ellagic acid, and tetramethoxychalcone derivatives. In crude ethyl acetate extract (CEE) of *P. rotundifolia*, 3,3'-di-*O*-methyl ellagic acid, and tetramethoxychalcone derivatives were identified. Many studies showed that phenolic compounds possessed antitumour properties and by inhibiting growth of the cells, inducing cell-cycle arrest, and exerting pro-apoptotic effects on colon cancer cells (Larrosa *et al.,* 2006; Kern *et al.,* 2007; Gonzalez *et al.,* 2009).

The phytochemical screening of CEE of *Memecylon edule* showed the presence of alkaloids, saponins, and phenolic compounds that include flavonoids, tannins, and terpenoids. Cytotoxicity of the extract was assessed by using calorimetric MTT assay method and it was reported that the extracts had the potential to inhibit the growth of the

human gastric NUGC cancer cells and human gastric MKN-74 cancer cells, with IC_{50} 51.0 ± 3.6 µg/ml and 49.2 ± 6.0 µg/ml, respectively (Naidu *et al.*, 2013). Zakaria *et al* (2011) reported that the aqueous and methanol extracts of leaves of *M. malabathricum* contain the highest phenolic content, which are approximately 3344 and 3055 mg/100 g gallic acid, respectively. Results showed that methanol extracts of *M. malabathricum* exhibited antiproliferative activity against human breast MCF-7 cancer cells, human cervical HeLa cancer cells, human ovarian CAOV-3 cancer cells, human promyelocytic leukemia HL-60 cells, human T-lymphoblastic leukemia CEM-SS cells, and human breast MDA-MB-231 cancer cells. The aqueous extract inhibited the proliferation of human ovarian Caov-3 cancer cells and human promyelocytic leukemia HL-60 cells.

In CEE of *M. caeruleum*, it was revealed that the highest peak was at retention time 4.59 min and the detected compound is isorhamnetin-3-*O*-hexoside and kaempferol glucoside and both compounds fall in the family of flavonoids (N'Dri *et al.*, 2010; Kontogianni *et al.*, 2013). While, in CEE of *P. rotundifolia*, the highest peak was at 7.08 min and identified as tetramethoxychalcone derivatives. In general, the major compounds detected from the CEE of both the species were flavonoids. Flavonoids belong to polyphenolic compounds which have been reported to be abundantly found in many fruits and vegetables and possessed anticancer properties (Liu *et al.*, 2010; Mansuri *et al.*, 2014).

Bennett *et al*., (2012) reported that flavonoids have the ability to prevent initiation, promotion, and progression of cancer development through selective interaction with protein kinase signaling cascades involved in cell defense (Hou & Kumamoto, 2010; Katiyar, 2011; Yao *et al.*, 2011). Susanti *et al*., (2007) reported that two flavonoids, which is naringenin and kaempferol-3-*O*-(2",6"-di-*O*-*p*-*trans*-coumaroyl) glucosides, from the ethyl acetate extract of flowers of *Melastoma malabathricum* exhibited significant cytotoxic effects when tested against human breast

MCF-7 cancer cells line by using MTT assay. The IC_{50} value for naringenin and kaempferol glucoside was 1.3 µM and 0.28 µM, respectively. Flavonoids have the potential to prevent cancer by suppression of nuclear transcription factor-κB (NF-κB) pathway. This pathway is of the attractive therapeutic targets since NF-κB is the main regulator for the expression of more than 200 diverse genes (Natarajan *et al.,* 1996; Bennett *et al.,* 2012; Momtaz *et al.,* 2013).

Ellagic acid was one of the detected compounds in the CEE of *M. caeruleum* and *P. rotundifolia*. According to the American Cancer Society (2008), ellagic acid seems to have some anticancer properties and has been found to cause cell death in cancer cells in the laboratory. In addition, ellagic acid seems to reduce the effect of estrogen in promoting growth of tissue culture breast cancer cells. Ellagic acid was reported to be present in the barks (Lowry, 1976) and flowers of *M. malabathricum* (Wong *et al.,* 2004; Ali *et al.,* 2010; Joffry *et al.,* 2012). Ellagic acid exhibited strong growth-inhibiting and apoptosis promoting activities *in vitro* when tested against human cervical CaSki carcinoma cells, human breast MCF-7 cancer cells, human breast Hs 578T cancer cells, human colon Caco-2 cancer cells, and human prostatic DU 145 cancer cells (Narayanan *et al.,* 1999; Losso *et al.,* 2004). It was observed that the mechanism of apoptosis induction in ellagic acid-treated cancer cells was associated with decreased of ATP production, which is crucial for the viability of cancer cells (Losso *et al.,* 2004).

5.6 Antioxidant Evaluation of Crude Extracts

Today, naturally occurring antioxidants are mostly preferred due to their little or no toxicity in comparison with some synthetic antioxidants which have been documented to have toxic or mutagenic effects (Alnajar *et al.,* 2012). In this study, the antioxidant activity and total phenolic content (TPC) of the crude methanol (CME), crude hexane (CHE), and crude ethyl acetate (CEE) of *M. caeruleum, M. muticum, M. sanguineum,* and *P. rotundifolia* were determined. CME of all the plant extracts have the highest TPC and was found to exhibit high antioxidant capacity when assessed by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay and Ferric Reducing Antioxidant Power Assay (FRAP). Rufino *et al*., (2010) mentioned that FRAP, 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), DPPH, and Oxygen Radical Absorbance Capacity (ORAC) are the most widely used methods for determining antioxidant capacity *in vitro*. It is recommended that at least two (or even all) of these assays be combined, as a single method may have limitations in terms of sensitivity in detecting the activities (Perez-Jimenez *et al.,* 2008). DPPH and FRAP assays were selected in this study because both assays showed no differences in determination as well as both methods were highly reproducible, simple and can be rapidly performed. They also showed high correlation with both ascorbic acid and TPC assays.

2,2-diphenyl-1-picrylhydrazyl (DPPH) method is a convenient method to measure common natural antioxidants (Blois, 1958). DPPH is a stable free radical that possesses an unpaired electron, shows a strong absorption band at 517 nm in methanol solution and exhibits a stable deep violet color. As this electron becomes paired, the absorption vanishes and the resulting decolorization (yellow color) is stoichiometric with respect to the number of electrons taken up (Blois, 1958, Oyaizu, 1986, Li *et al.*,

2012). As reported by Suhaj (2006) scavenging of the stable radical (DPPH) is considered a valid and easy assay to evaluate scavenging activity of antioxidants (Maizura *et al.,* 2011).

The present study showed that CME of *M. sanguineum* and *M. muticum* exhibited high free radical scavenging activity when assessed by DPPH assay. It was reported that chloroform, methanol, and aqueous extract of leaves of *M. malabathricum* exhibited different degree of antioxidant capacity when assessed by the DPPH radical scavenging assay. The 20, 100, and 500 µg/ml methanol extract produced approximately 13–98 % of DPPH free radical scavenging activities, while the aqueous extracts and chloroform extracts produced 7–70 % and 0–18 % DPPH free radical scavenging activities (Zakaria *et al*., 2011). Puttaswamy & Achur (2013) reported that methanol extracts of *Memecylon umbellatum* showed good free radical scavenging activity (estimated IC_{50} = 5.0 µg/ml) as compared to the standard, butylated hydroxyanisole (BHA). Another study by Alnajar *et al*., (2012) also showed that the aqueous extract of *M. malabathricum* exhibited good antiradical activity with IC_{50} value of 10.573 ± 0.58 µmol/L and the findings confirmed the previous report by Susanti *et al*., (2007).

FRAP assay measures the ferric reducing ability. At low pH, when a ferric-tripyridyltriazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous (Fe^{2+}) form, an intense blue color with an absorption maximum at 593 nm develops. The FRAP assay is inexpensive, reagents are simple to prepare, results are highly reproducible, and the procedure is straightforward and speedy (Benzie & Strain, 1996). In this study, CME of *M. sanguineum* had the highest reducing power when compared with other crude extracts. The reducing power is determined on the basis of the ability of antioxidant in this plant extract to reduce ferric (Fe^{3+}) iron to ferrous (Fe^{2+}) iron in FRAP reagent (Wong *et al.,* 2006). Fu *et al*., (2010) did a study on antioxidative capacity and TPC of

56 wild fruits from South China including *Melastoma candidum* and *M. sanguineum*. Their results showed that both species of *Melastoma* had high reducing activities when assessed by FRAP assay i.e., 141.0 ± 8.77 µmol Fe (II)/g and 288.0 ± 10.4 µmol Fe (II)/g respectively. This is also observed in the current study with CME leaf extracts of *M. sanguineum*. In another study involving another species of Melastomataceae, that is *Osbeckia parvifolia*, it was reported that successive soxhlet methanol extract (7075.4 µmol Fe (II)/g) of *O. parvifolia* showed higher ferric reducing ability compared to ascorbic acid (6341.84 µmol Fe (II)/g) and quercetin (7461.14 µmol Fe (II)/g) (Murugan & Parimelazhagan, 2013). *O. parvifolia* is widely distributed in South India and it is regarded as an important medicinal plant there.

In this study, TPC is assessed by the Folin-Ciocalteau method and CME of *M. sanguineum* had the highest total phenolic content (718.18 ± 8.91 mg GAE/100 g at 1.0mg/ml) among all the extracts tested. Polyphenols in plant extracts react with specific redox reagents (Folin-Ciocalteau reagent) to form a blue complex that can be quantified by visible light spectrophotometry (Blainski *et al.,* 2013). It was reported that methanol-acetic acid-water extract of *M. candidum* and *M. sanguineum* have high total phenolic content, 16.2 ± 0.98 mg GAE/g and 23.3 ± 0.49 mg GAE/g, respectively (Fu *et al.,* 2010). Zakaria *et al*., (2011) reported the total phenolic content for the aqueous, methanol, chloroform extracts of *M. malabathricum*, were 3344.2 ± 19.1, 3055.1 ± 8.7, and 92.5 ± 7.3 mg/100 g gallic acid, respectively. In another study, Alnajar *et al*., (2012) reported that aqueous and ethanol extracts of *M. malabathricum* revealed to have high phenolic content, 379.33 ± 0.007 mg/g and 384.33 ± 0.005 mg/g respectively. Fractionation of the ethanol extract and maceration of the methanol extract of *O. parvifolia* also reported to have high phenolic content, 36.72 g GAE/100 g and 24.93 g GAE/100 g respectively.

Polyphenols are one of the major chemical groups reported to attribute to the antioxidant activity (Thomas & Chimie, 2000). As mentioned earlier, methanol extracts of *M. umbellatum* reportedly exhibited good free radical scavenging activity and the phytochemical analysis revealed the presence of significant levels of terpenoids, flavonoids, tannins, and moderate amount of phenols and glycosides (Puttaswamy & Achur, 2013). Phenolic compounds are the secondary metabolites produced by plant that have aromatic ring structure containing one or more phenolic hydroxyl groups. They have diverse phenolic structures, from simple phenols to complex polymers, with flavonoids, anthocyanins, hydroxycinnamic acids, and tannins being the main groups of phenolics (Dewick, 2009; Dai & Mumper, 2010; Saednia & Adbollahi, 2013). Studies by Rice-Evan *et al*., (1997) stated that all the herbal phenolic compounds (30 of known flavonoids and phenolic acids) possessed higher scavenging activity compared to Trolox (an analog of Vitamin E) which can be considered as a standard antioxidant.

Phenolics are considered as efficient antioxidants mainly because of the structure that has the ability to donate the electron from the hydroxyl (-OH) group (Saeidnia & Abdollahi, 2013). Fukumoto & Mazza (2002) reported that flavonoids and hydroxycinnamic acid are commonly more effective than vitamin C in scavenging aqueous phase radicals. Preliminary phytochemical screening of selected berry extracts showed that they contain phenolic compounds that include phenols, flavonoids and tanins, terpenoids, saponins, sterols, and cardiac-glycosides. In comparison, *M. malabathricum*, also showed the presence of these chemical groups (Zakaria *et al.*, 2006; Faravani, 2009; Joffry *et al*., 2012).

In this study, all the extracts from the four selected species exhibited different antioxidant potential. Several reports indicated that the antioxidant potential of medicinal plants may be related to the concentration of their phenolic compounds depending on their growing environments and varying proportions of various

antioxidant compounds synthesized by the plants for self-protection (Diaz *et al.,* 2001; Wong *et al.,* 2006). In a related study, methanol and chloroform extracts of *Memecylon umbellatum* showed different antioxidant activities even though both extracts have high phenolic contents. This might be due to the different concentration of flavonoids and terpenoids in the extracts (Puttaswamy & Achur, 2013).

A positive correlation was found in this study between TPC and FRAP (r = 0.963). However, TPC and DPPH were negatively correlated (r = - 0.719) due to the fact that the DPPH method yields inversely proportional results. Zakaria *et al*., (2006) did a study on the correlation of total phenolic contents and antioxidant activities of the *M. malabathricum* extracts. From the study, it was revealed that extracts with high phenolic contents, that is aqueous and methanol extracts, were found to exhibit high antioxidant capacities when assessed by DPPH radical scavenging and super oxide scavenging assay. The study by Fu *et al*., (2010), involving 56 wild fruits from south China also the results showed positive correlation between antioxidant capacities (measured by FRAP assay) and total phenolic contents. In general, phenolic compounds might contribute to the reducing and free radical scavenging capabilities. The linear co-relation between the high total phenolic contents and the antioxidant activities observed were in line with previous reports (Wu *et al.,* 2006; Zakaria *et al.,* 2006; Faravani, 2009; Rufino *et al.,* 2010; Joffry *et al.,* 2012). This may be interpreted that medicinal properties against oxidative stress might be related to the free radical scavenging properties and reducing power activities.

CHAPTER 6

CONCLUSION

6.1 Conclusions

CME, CHE, and CEE of selected four species from the family Melastomataceae were studied mainly to investigate their cytotoxic activity against two human cancer cell lines, MCF-7 and SKOV-3, to study their TPC and antioxidant potentials by using DPPH and FRAP assay.

Results indicated that potent anticancer properties were found in *M. caeruleum* and *P. rotundifolia*. CEE of *M. caeruleum* and *P. rotundifolia* were shown to have high selectivity on human breast MCF-7 cancer cell lines. However, CME of *M. caeruleum* was only shown to be selective for human ovarian SKOV-3 cancer cell lines. As with the antioxidant potential, CME of *M. sanguineum* have the highest reducing activity and CME of *M. muticum* have the highest free radical scavenging activity.

6.2 Future Study

Each plant may have its own and unique phytochemical compounds and these unique compounds can be recommended to be used as therapeutic agent to certain illness. Two of the four species that is *M. caeruleum* and *P. rotundifolia* have demonstrated to have good medicinal values and should be subjected to deeper scientific studies such as understanding the compound structures, cancer cell inhibition modes as well as molecular level understanding.

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APPENDIX 1

Preparation of Solutions and Reagents

Phytochemical screening

Preparation of Solutions and Reagents

3 % Iron (III) chloride, $FeCl_3$

3.0 g of Iron (III) chloride (R & M Chemicals, UK) was dissolved in 100 ml of distilled water. The solution was kept in the dark at room temperature.

10 % of Iron (III) chloride, $FeCl_3$

10.0 g of Iron (III) chloride (R & M Chemicals, UK) was dissolved in 100 ml of distilled water. The solution was kept in the dark at room temperature.

10 % Lead (II) acetate, (CH_3COO_2) $Pb.Pb(OH_2)$

10.0 g of lead (II) acetate (R & M Chemicals, UK) was dissolved in 100 ml of distilled water. The solution was kept in the dark at room temperature.

1 % Hydrochloric acid, HCl

1.0 ml of concentrated hydrochloric acid (R & M Chemicals, UK) was mixed with 100 ml of distilled water. The solution was kept at room temperature.

30 % Nitric acid, HNO_3

30 ml of nitric acid (R & M Chemicals, UK) was diluted with 70 ml of distilled water.

Dragendorff Reagent

8.0 g of bismuth nitrate (R & M Chemicals, UK) was dissolved in in 12 ml of 30 % nitric acid (R & M Chemicals, UK). 27.2 g of potassium iodide (R & M Chemicals, UK) was dissolved in 50 ml of distilled water and the solution was added into the bismuth nitrate solution. The volume was adjusted to 100 ml with distilled water.

Mayer Reagent

1.36 g of mercury (II) chloride (R & M Chemicals, UK) was dissolved in 60 ml of distilled water (Solution A) and 5.0 g of potassium iodide (R & M Chemicals, UK) was dissolved in 10 ml of distilled water (Solution B). Solution A and solution B were mixed and the volume was adjusted to 100 ml with distilled water.

Wagner Reagent

2.0 g of potassium iodide (R & M Chemicals, UK) was dissolved in distilled water and 1.27 g of iodine (R & M Chemicals, UK) was added into the solution. The solution was mixed thoroughly and the volume was adjusted to 100 ml with distilled water.

Cell Culture

Preparation of Media and Solutions

Basic DMEM Media

One sachet of DMEM powder (Sigma-Aldrich, USA) that containing Earle's salt with L-glutamine and HEPES (N-2-Hydroxylethyl-Piperazine-N-2-Sulfonoc Acid, without sodium bicarbonate was dissolved in 1 liter with distilled water. 2 g of sodium bicarbonate ($NaHCO_3$, R & M Chemicals, UK) was added to the media. The media was filter sterilized using a 0.22 µm filter membrane (Orange Scientific) and stored at 4 °C for up to 4 months.

Basic RPMI 1640 Media

10.39g of RPMI 1640 powder (Sigma-Aldrich, USA) was dissolved in 1 liter of distilled water. 2 g of sodium bicarbonate ($NaHCO_3$, R & M Chemicals, UK) was added to the media and pH of the media was calibrated to pH 7.4 (Thermo Scientific). The media was filter sterilized using a 0.22 µm filter membrane (Orange Scientific) and stored at 4 °C for up to 4 months.

Basic MEM Media

One sachet of MEM powder (Sigma-Aldrich, USA) that containing Earle's salt with L-glutamine, without HEPES (N-2-Hydroxylethyl-Piperazine-N-2-Sulfonoc Acid, without sodium bicarbonate (Sigma-Aldrich, USA) was dissolved in 1 liter of distilled water. 0.2603g of HEPES (Molekula, UK), 2 g of sodium bicarbonate (R & M Chemicals, UK) and 1 ml of sodium pyruvate (PAA Lab, Austria) were added into the media. The media was filter sterilized using a 0.22 µm filter membrane (Orange Scientific) and stored at 4 °C for up to 4 months.

10 % Supplemented DMEM Media

100 ml of 10 % supplemented DMEM media were prepared using 90 ml of basic media, supplemented with 10 ml of inactivated Foetal Bovine Serum (FBS, PAA Lab, Austria), 1 ml (100 µg/ml) and 1 ml (100 IU/ml) of streptomycin and penicillin (PAA Lab, Austria) respectively and 1 ml of fungizone (PAA Lab, Austria). The media was filter sterilized using a 0.22 µm filter membrane and stored at 4 °C for up to 2 weeks.

10 % Supplemented RPMI 1640 Media

100 ml of 10 % supplemented RPMI 1640 media were prepared using 90 ml of basic media, supplemented with 10 ml of inactivated Foetal Bovine Serum (FBS, PAA Lab, Austria), 1 ml (100 µg/ml) and 1 ml (100 IU/ml) of streptomycin and penicillin (PAA Lab, Austria) respectively and 1 ml of fungizone (PAA Lab, Austria). The media was filter sterilized using a 0.22 µm filter membrane and stored at 4 °C for up to 2 weeks.

10 % Supplemented MEM Media

100 ml of 10 % supplemented MEM media were prepared using 90 ml of basic media, supplemented with 10 ml of inactivated Foetal Bovine Serum (FBS, PAA Lab, Austria), 1 ml (100 µg/ml) and 1 ml (100 IU/ml) of streptomycin and penicillin (PAA Lab, Austria) respectively and 1 ml of fungizone (PAA Lab, Austria). The media was filter sterilized using a 0.22 µm filter membrane and stored at 4 °C for up to 2 weeks.

20 % Supplemented DMEM

50 ml of 20 % supplemented DMEM media was prepared using 45 ml of 10 % supplemented media added with 5 ml of inactivated FBS. The media was filter sterilized using a 0.22 µm filter membrane and stored at 4 °C for up to 2 weeks. This 20 % supplemented media was used to revive cells.

20 % Supplemented RPMI 1640

50 ml of 20 % supplemented RPMI 1640 media was prepared using 45 ml of 10 % supplemented media added with 5 ml of inactivated FBS. The media was filter sterilized using a 0.22 µm filter membrane and stored at 4 °C for up to 2 weeks. This 20 % supplemented media was used to revive cells.

20 % Supplemented MEM

50 ml of 20 % supplemented MEM media was prepared using 45 ml of 10 % supplemented media added with 5 ml of inactivated FBS. The media was filter sterilized using a 0.22 µm filter membrane and stored at 4 °C for up to 2 weeks. This 20 % supplemented media was used to revive cells.

Phosphate Buffered Saline (PBS) pH 7.2

The phosphate buffered saline (PBS) was prepared using 1.52 g of sodium phosphate anhydrous ($NaHPO_4$, Merck), 0.58 g of potassium dihydrogen orthophosphate (KH_2PO_4, Merck) and 8.5 g of sodium chloride (BDH AnalaR, UK) that were dissolved in distilled water and the volume was made up to 1 liter. The pH of the buffer was adjusted to 7.2 using a pH meter (Thermo Scientific). The buffer was then filter sterilized using a 0.22 µm filter membrane, autoclaved, and stored at room temperature.

Tryphan Blue Solution 0.4%

0.2 g of tryphan blue powder was dissolved in 50 ml of distilled water.

Neutral Red Cytotoxicity Activity Assay

Preparation of Solutions

Neutral Red Stock Solution

0.4 g of Neutral Red (ICN, USA) was dissolved in 100 ml sterile distilled water. The solution was kept the in dark at 4 °C.

Neutral Red Medium

The Neutral Red stock solution was diluted (80:1) in treatment culture medium to give a final concentration of 50 µg/ml. Prepared Neutral Red medium were incubated overnight at room temperature in the dark. This solution was centrifuged (Thermo Scientific) twice at 1500 g for 10 minutes before use to remove any fine, needle-like precipitate of dye crystals.

Neutral Red Washing Solution

1.0 g calcium chloride (Systerm) was dissolved in 500 µl of formaldehyde (R & M Chemicals, UK) and 99.5 ml of distilled water. The solution was kept in the dark at 4 °C.

Neutral Red Resorb Solution

1 ml of glacial acetic acid (Fisher Scientific, UK) was dissolved in 500 ml of absolute ethanol (Fisher Scientific, UK) and 49 ml of distilled water.

Antioxidant

Preparation of solutions and reagents

300 mM acetate buffer, pH 3.6

1.55 g of sodium acetate trihydrate (R & M Chemicals, UK) was dissolved in 8 ml of glacial acetic acid (Fisher Scientific, UK) and top up to 500 ml with distilled water.

40 mM Hydrochloric acid, HCl

1.46 ml of concentrated hydrochloric acid (R & M Chemicals, UK) and it was top up with 1 L of distilled water.

10 mM 2, 4, 6-tripyridyl-*s*-triazine (TPTZ)

0.031 g of TPTZ (Sigma, Switzerland) was dissolved in 10 ml of 40 mM hydrochloric acid (R & M Chemicals, UK) and must be prepared fresh on the day of assay.

20 mM Iron (III) chloride anhydrous, $FeCl_3.6H_2O$

0.054 g of $FeCl_3.6H_2O$ (R & M Chemicals, UK) was dissolved in 10 ml of distilled water and must be prepared fresh on the day of assay.

1 mM stock of Iron (II) sulphate heptahydrate, $FeSO_4.7H_2O$

0.0278 g of $FeSO_4.7H_2O$ (R & M Chemicals, UK) was dissolved in 100 ml of methanol (Fisher Scientific, UK).

0.175 mM 2, 2-diphenyl-1-picrylhydazyl (DPPH)

0.0069 g of DPPH powder (Sigma, Germany) was dissolved in 100 ml of methanol (Fisher Scientific, UK). The solution was kept in the dark at 4 °C overnight.

10 % Folin-Ciocalteaou reagent

10 ml of Folin-Ciocalteaou reagent (R & M Chemicals, UK) was added into 90 ml of distilled water. The solution was kept in the dark at room temperature.

7.5 % sodium carbonate, Na_2CO_3

7.5 g of sodium carbonate (R & M Chemicals, UK) was dissolved in 100 ml of distilled water.

APPENDIX 2

Cytotoxic Activity Raw Data

Cytotoxic activity of crude extracts of Melastomataceae species on various human cancer cell lines

IC_{50} values (µg/ml) of Melastomataceae crude extracts on MCF-7 cell lines

Plant species	Crude extracts	IC50 value (µg/ml)			
		Test 1	Test 2	Test 3	Average
M. caeruleum	CME	32.46	31.34	29.67	31.16 ± 1.40
	CHE	>100	>100	>100	>100
	CEE	15.06	15.04	14.52	14.87 ± 0.31
M. muticum	CME	45.89	45.96	46.24	46.03 ± 0.19
	CHE	69.73	75.08	67.22	70.68 ± 4.01
	CEE	47.90	47.92	45.51	47.11 ± 1.39
M. sanguineum	CME	46.44	45.80	46.34	46.16 ± 0.34
	CHE	>100	>100	>100	>100
	CEE	50.50	51.38	51.19	51.02 ± 0.46
P. rotundifolia	CME	17.46	18.08	17.96	17.83 ± 0.33
	CHE	36.75	35.32	30.83	34.30 ± 3.09
	CEE	0.92	0.92	0.91	0.92 ± 0.01
Doxorubicin	-	0.7	0.75	0.7	0.72 ± 0.03

IC_{50} values (µg/ml) of Melastomataceae crude extracts on SKOV-3 cell lines

Plant species	Crude extracts	IC50 value (µg/ml)			
		Test 1	Test 2	Test 3	Average
M. caeruleum	CME	45.35	46.57	46.97	46.3 ± 0.84
	CHE	>100	>100	>100	>100
	CEE	14.36	17.71	16.04	16.04 ± 1.68
M. muticum	CME	48.77	46.07	47.44	47.43 ± 1.35
	CHE	90.14	79.23	54.08	74.48 ± 18.49
	CEE	75.03	81.97	91.41	82.80 ± 8.22
M. sanguineum	CME	44.57	47.93	45.74	46.08 ± 1.71
	CHE	>100	>100	>100	>100
	CEE	49.10	51.96	65.67	55.58 ± 8.86
P. rotundifolia	CME	17.01	25.37	22.22	21.53 ± 4.22
	CHE	24.85	29.49	19.94	24.76 ± 4.78
	CEE	5.31	5.22	5.17	5.23 ± 0.07
Doxorubicin		0.15	0.6	0.5	0.42 ± 0.24

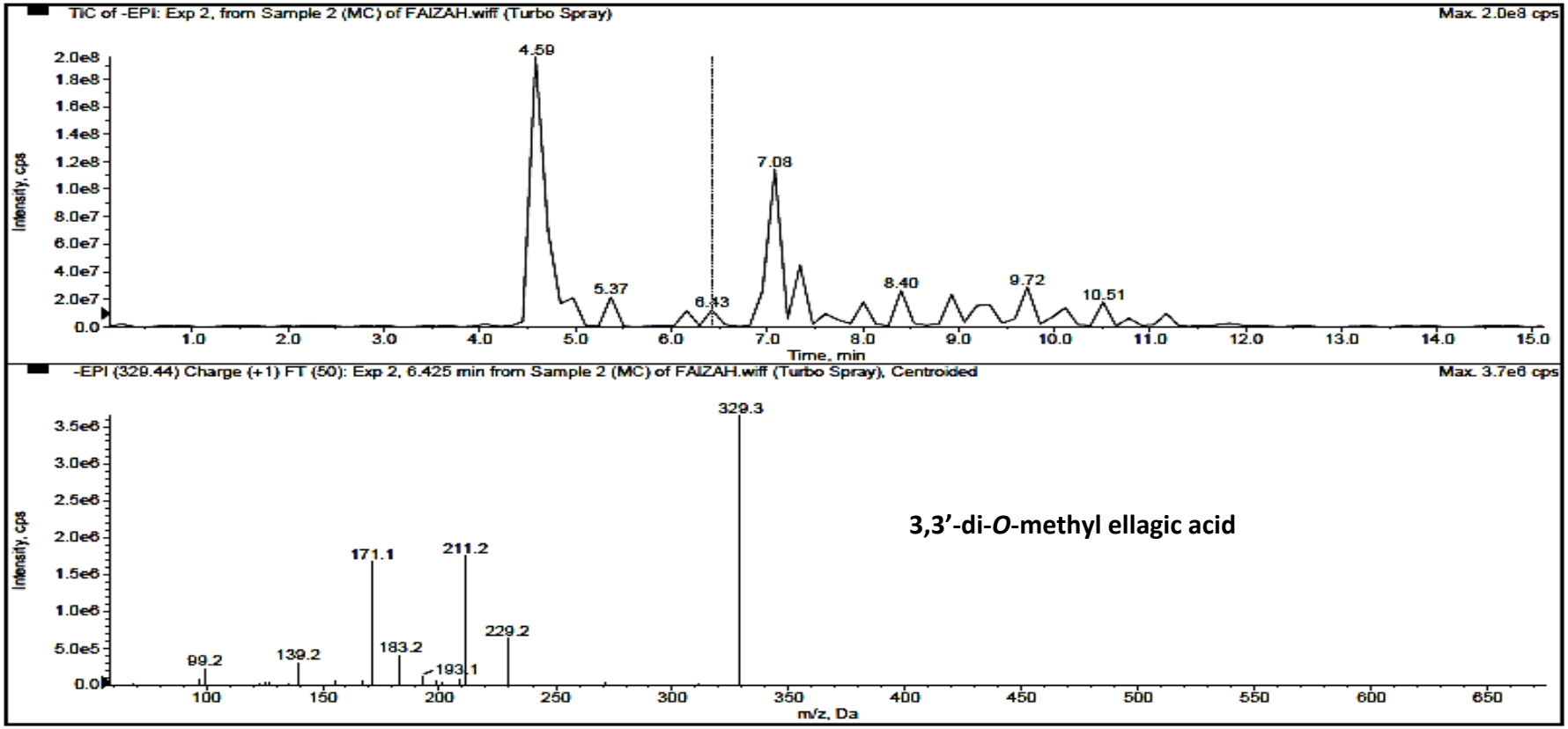
IC_{50} values (µg/ml) of Melastomataceae crude extracts on MRC-5 cell line

Plant species	Crude extracts	IC50 value (µg/ml)			
		Test 1	Test 2	Test 3	Average
M. caeruleum	CME	NT	NT	NT	NT
	CHE	NT	NT	NT	NT
	CEE	51.34	51.94	52.40	51.89 ± 0.54
M. muticum	CME	NT	NT	NT	NT
	CHE	NT	NT	NT	NT
	CEE	NT	NT	NT	NT
M. sanguineum	CME	NT	NT	NT	NT
	CHE	NT	NT	NT	NT
	CEE	NT	NT	NT	NT
P. rotundifolia	CME	99.31	83.94	103.59	95.61 ± 10.33
	CHE	NT	NT	NT	NT
	CEE	7.31	7.55	7.74	7.54 ± 0.22
Doxorubicin	-	1.80	1.65	1.70	1.72 ± 0.08

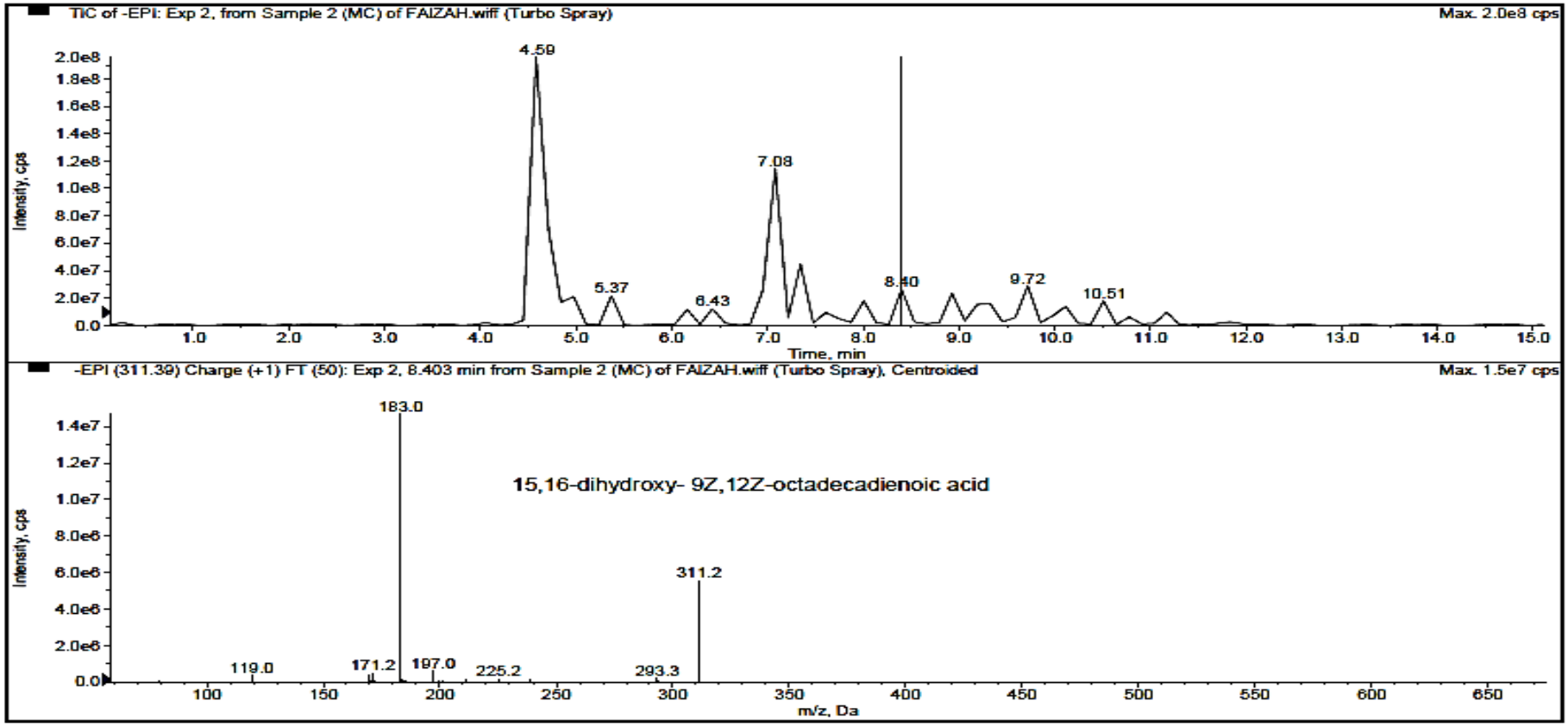
NT = Not Tested

APPENDIX 3

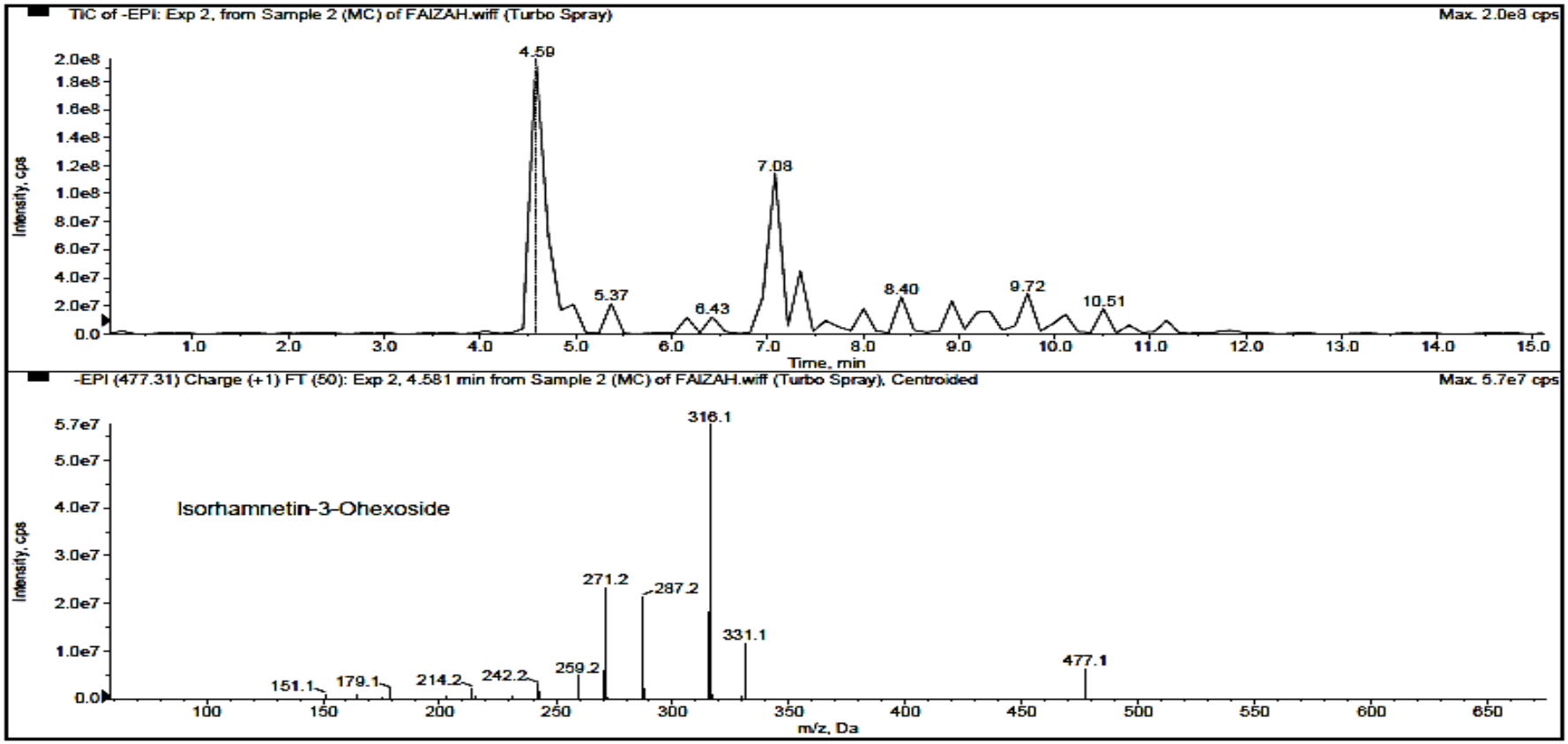
The Mass Spectrum of Detected Compounds in CEE of *M. caeruleum* and *P. rotundifolia* by LC-MS/MS



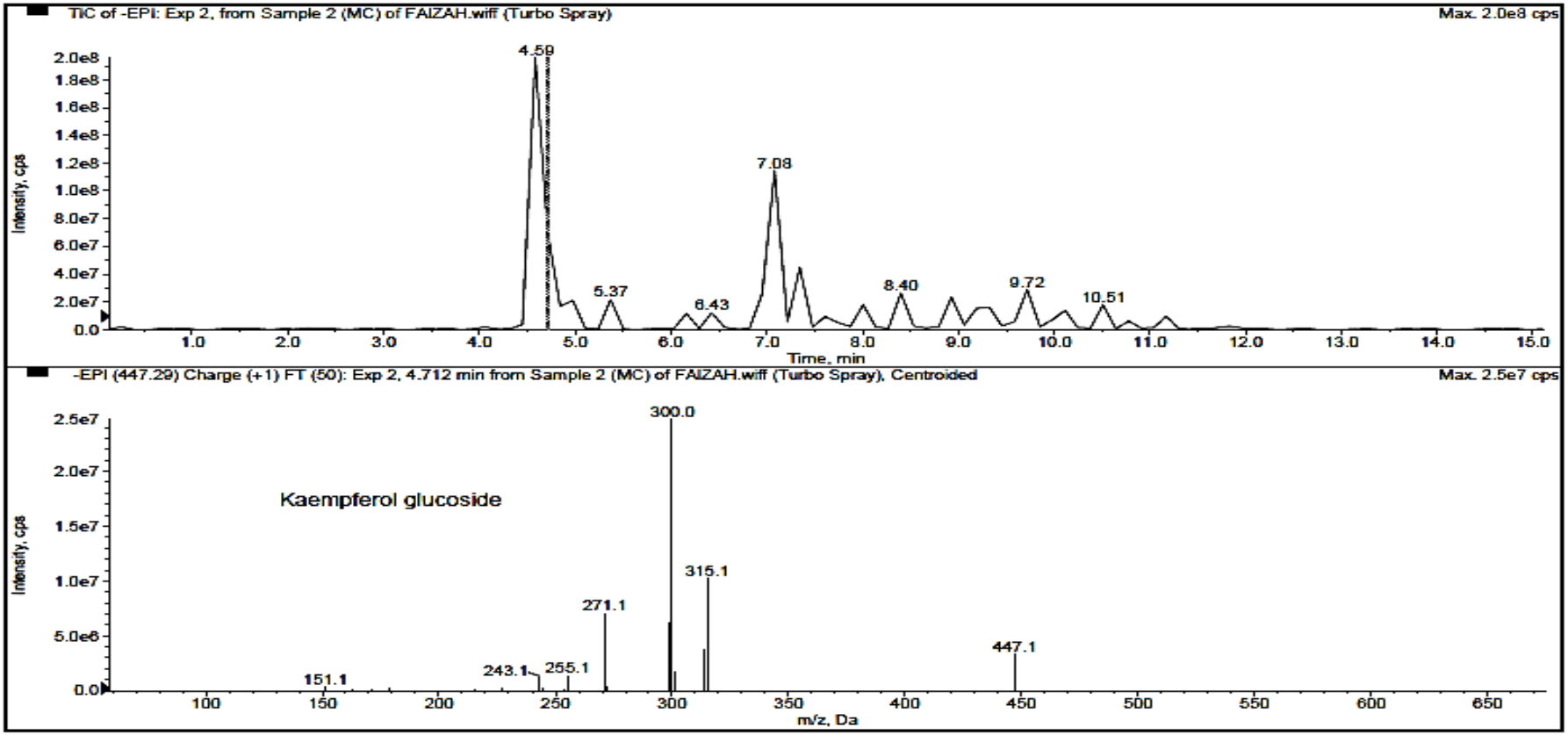
The Mass Spectrum of 3,3'-di-*O*-methyl ellagic acid (*M. caeruleum*)



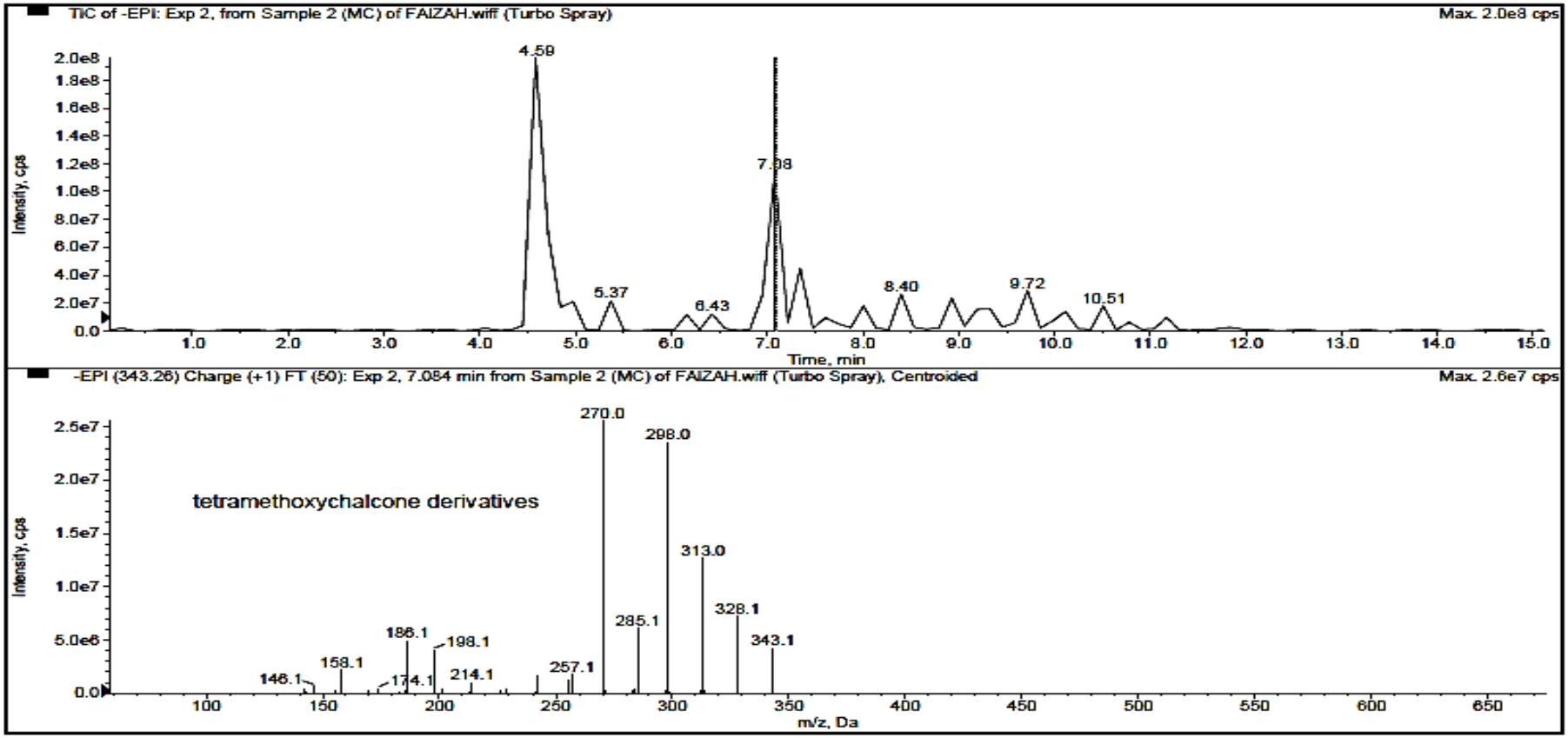
The Mass Spectrum of 15,16-dihydroxy- 9Z,12Z-octadecadienoic acid (*M. caeruleum*)



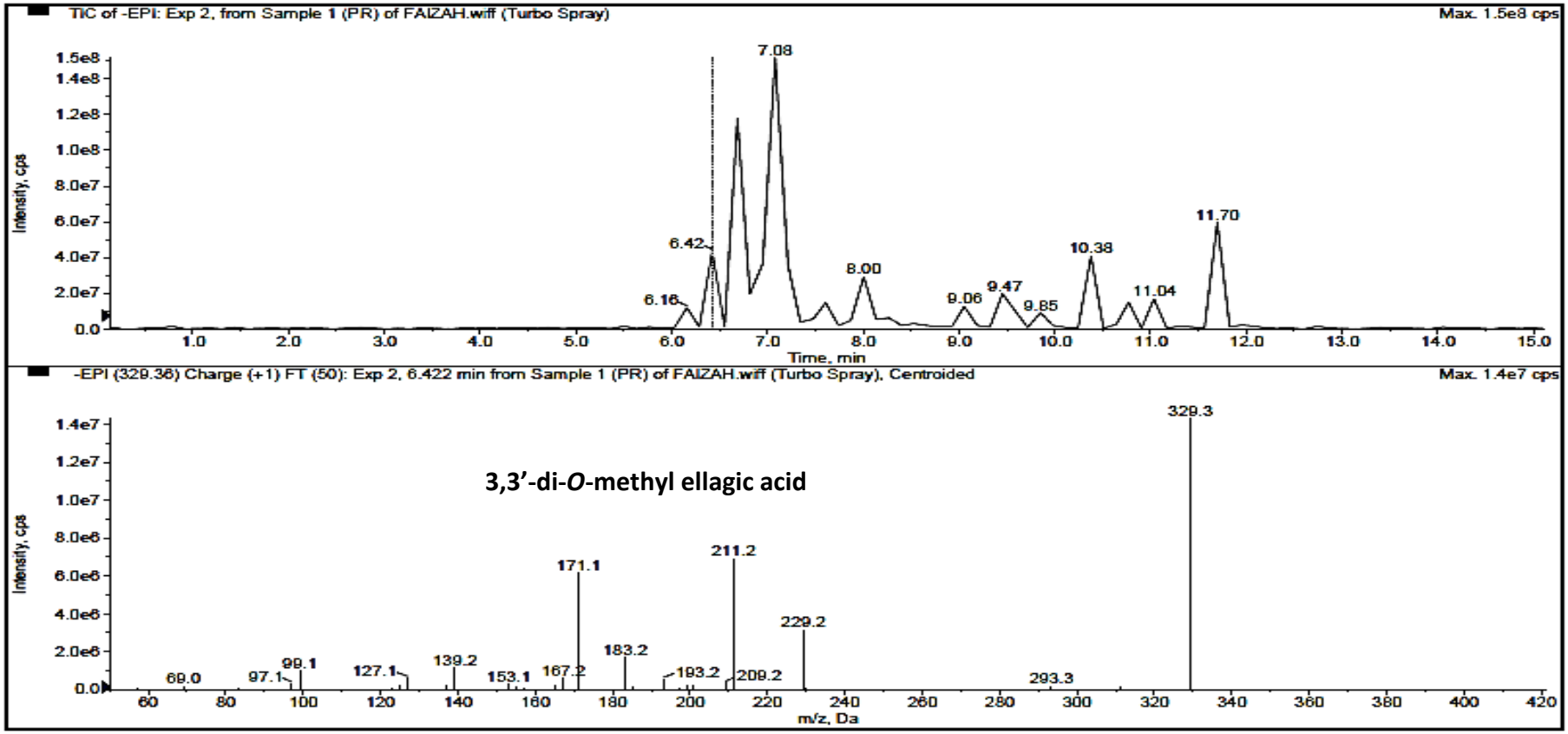
The Mass Spectrum of Isorhamnetin-3-*O*-hexoside (*M. caeruleum*)



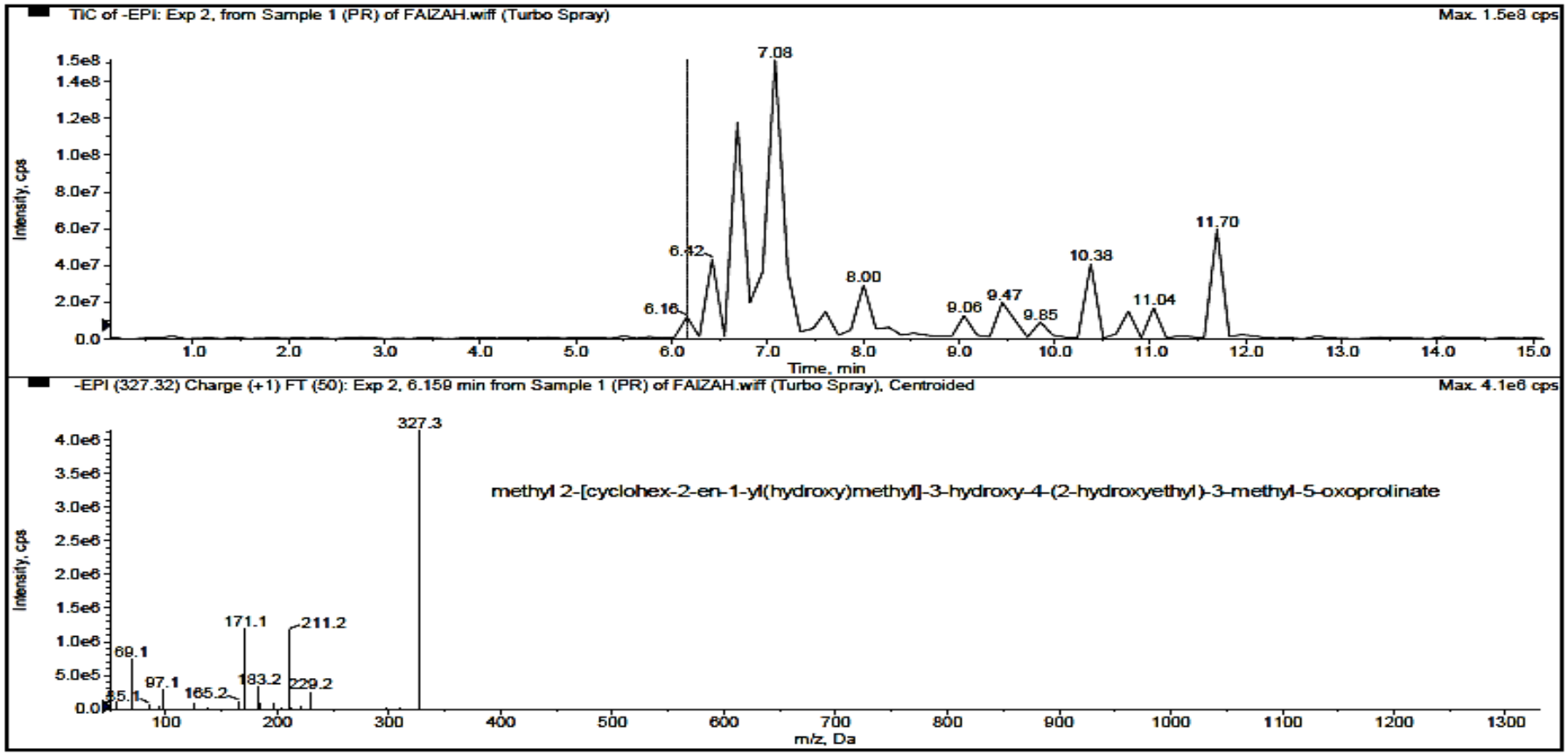
The Mass Spectrum of Keampferol glucoside (*M. caeruleum*)



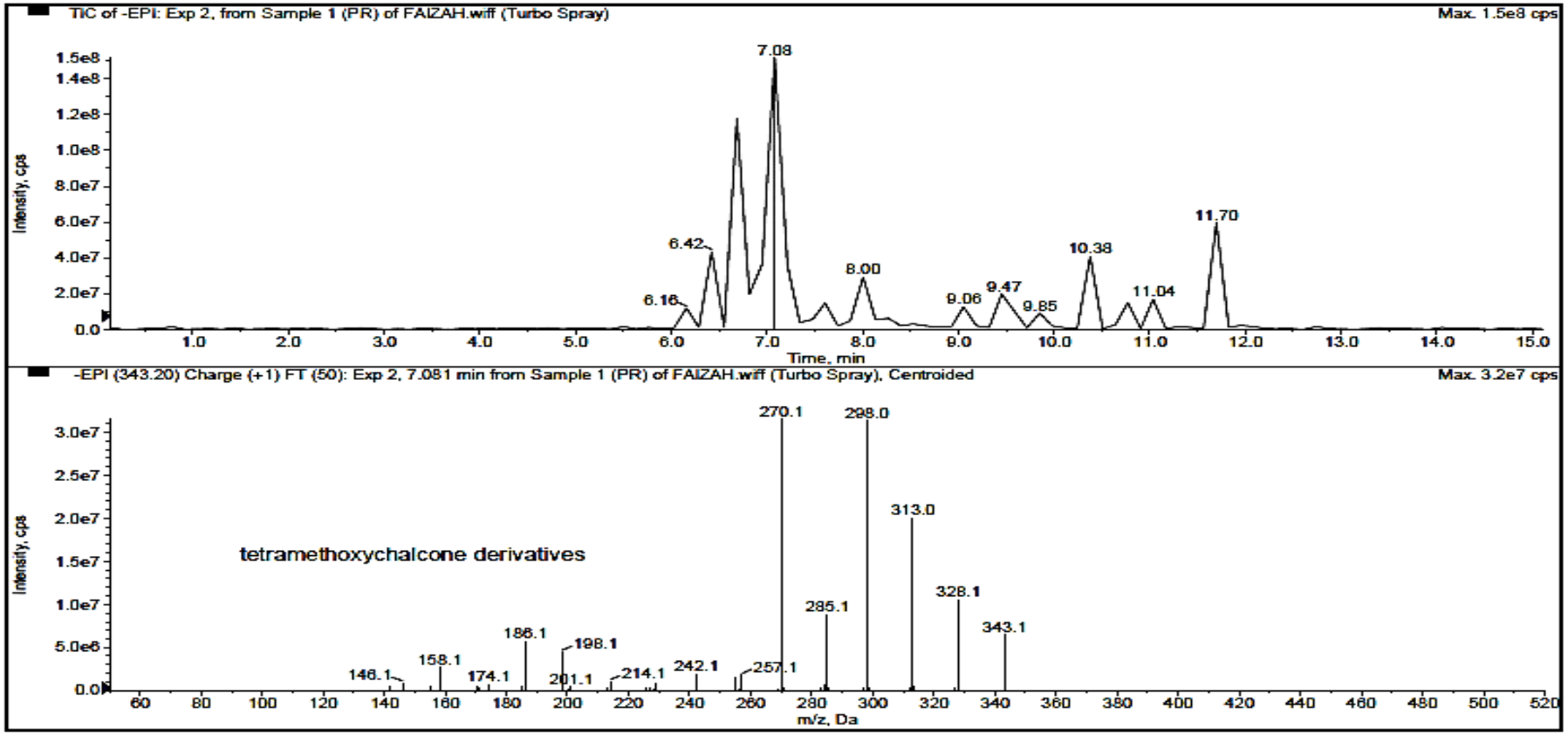
The Mass Spectrum of Tetramethoxychalcone derivatives (*M. caeruleum*)



The Mass Spectrum of 3,3'-di-*O*-methyl ellagic acid (*P. rotundifolia*)



The Mass Spectrum of
methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate (*P. rotundifolia*)



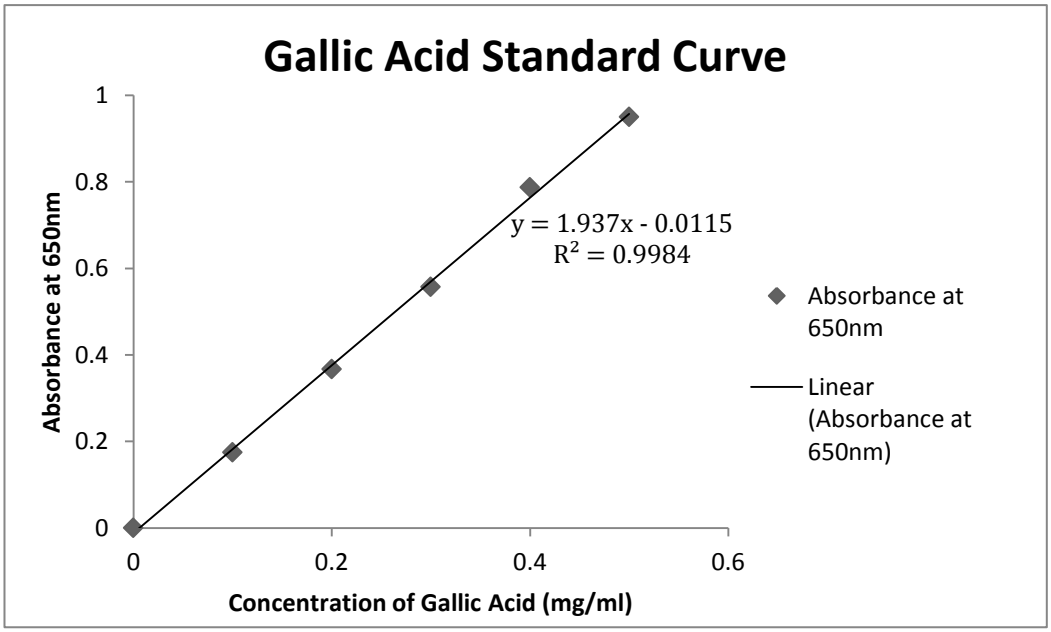
The Mass Spectrum of Tetramethoxychalcone derivatives (*P. rotundifolia*)

APPENDIX 4

Antioxidant Potential Raw Data

Total Phenolic Content (TPC) Assay

Standard curve of gallic acid for Total Phenolic Content assay. Each value is the mean ± standard deviation (S.D) consisting of three replicates.

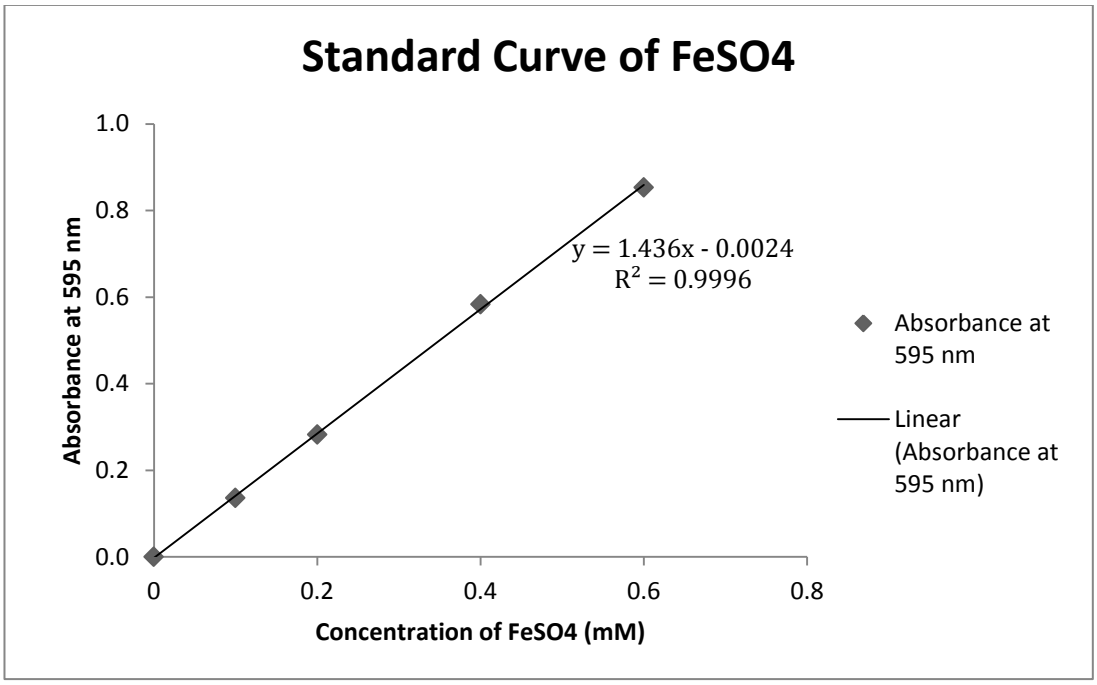


Total Phenolic Content of each crude extract was expressed as gallic acid equivalent (mg GAE/ 100 g).

Plant Species	Extract	Gallic Acid Equivalent (mg GAE/ 100 g)			
		Test 1	Test 2	Test 3	Average
M. caeruleum	CME	231.69	231.23	239.66	234.19 ± 4.74
	CHE	80.59	78.55	78.41	79.19 ± 1.22
	CEE	230.83	232.60	240.00	234.48 ± 4.87
M. muticum	CME	694.87	695.12	638.10	676.03 ± 32.85
	CHE	80.37	73.88	74.35	76.20 ± 3.62
	CEE	563.04	585.15	566.17	571.45 ± 11.97
M. sanguineum	CME	711.94	714.21	728.38	718.18 ± 8.91
	CHE	54.58	63.50	45.65	54.58 ± 8.92
	CEE	473.87	482.26	471.77	475.97 ± 5.55
P. rotundifolia	CME	208.86	198.89	200.99	202.91 ± 5.26
	CHE	28.86	30.44	26.24	28.51 ± 2.12
	CEE	34.11	37.26	32.54	34.63 ± 2.40

Ferric Reducing Antioxidant Power (FRAP) Assay

Ferrous sulfate standard curve as reference for FRAP assay. Each value is the mean ± standard deviation (S.D) consisting of three replicates.



Reducing activity of crude extracts of the selected species.

Plant Species	Extract	FRAP value (mM Fe (II)/ 100 g)			
		Test 1	Test 2	Test 3	Average
M. caeruleum	CME	17.00	17.31	16.04	16.78 ± 0.66
	CHE	4.70	3.81	3.92	4.15 ± 0.49
	CEE	10.52	10.20	10.03	10.25 ± 0.25
M. muticum	CME	46.91	43.58	46.45	45.64 ± 1.8
	CHE	5.32	5.23	5.69	5.41 ± 0.24
	CEE	38.25	37.75	37.57	37.86 ± 0.35
M. sanguineum	CME	68.88	68.39	69.39	68.89 ± 0.50
	CHE	2.94	3.02	3.11	3.02 ± 0.08
	CEE	31.98	30.97	29.61	30.85 ± 1.19
P. rotundifolia	CME	18.30	15.45	17.50	17.08 ± 1.47
	CHE	3.03	3.24	2.96	3.08 ± 0.14
	CEE	2.68	2.66	2.56	2.63 ± 0.07
BHA	-	17.92	18.69	18.76	18.76 ± 0.46

2,2-diphenyl-1-picrylhydazyl (DPPH) Radical Scavenging Activity Assay

Free radical scavenger activity of crude extracts of the selected species.

Plant Species	Extract	IC_{50} value (µg/ml)			
		Test 1	Test 2	Test 3	Average
M. caeruleum	CME	305.7	314.9	308.3	309.6 ± 4.7
	CHE	1103.9	1174.5	1116.9	1131.7 ± 37.6
	CEE	454.1	435.2	450.3	446.5 ± 10
M. muticum	CME	119.4	123.5	121.6	121.5 ± 2.1
	CHE	493.7	475.9	401.4	457.0 ± 49.0
	CEE	154.5	155.8	158.1	156.1 ± 1.8
M. sanguineum	CME	76.7	80.2	74.6	77.1 ± 2.8
	CHE	2420.7	2398.7	1853.6	2224.3 ± 321.2
	CEE	181.6	172.5	172.5	175.5 ± 5.2
P. rotundifolia	CME	251.2	246.3	257.4	251.6 ± 5.6
	CHE	2346.6	2022.7	2001.0	2123.4 ± 193.6
	CEE	2309.5	1857.8	2348.7	2172.0 ± 272.8
Ascorbic Acid	-	74.80	74.90	72.10	73.93 ± 1.6