CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The genus *Phyllagathis* of the family Melastomataceae comprises of about 60 species of which 35 species can be found in northern Laos, Vietnam and southern China, 12 species in Thailand, Peninsular Malaysia and Sumatra, and 13 species in Borneo (Lemmens and Bunyapraphatsara, 1999). *Phyllagathis rotundifolia* (Jack) is a creeping herb that can be found in shady or partly shady places in the lowland and montane forests of Peninsular Malaysia and Sumatra. Traditionally, a decoction of the leaves is used for the treatment of malaria, fever and stomachache, in parturition and as tonic. Another closely related species, *Phyllagathis praetermissa* (Weber) is also found in the same habitat as that of *P. rotundifolia*. The two species are known by the same local name as "Tapak Gajah" or "Tapak Sulaiman" because they are morphologically similar and it is difficult to differentiate them by their appearance. Nevertheless, one distinct characteristic difference between the two species is that *P. praetermissa* has silvery white spots on the lamina of juvenile leaves but this feature disappeared when matured (Weber, 1990). Thus, for the raw material suppliers in herbal industry, the two species are considered to be of the same species and they are often mixed together in the traditional medicines as well as in women's healthcare herbal products. This practice will affect the consistency and quality of the raw materials as well as their derived products. The routine botanical species identification represents an unfeasible approach here because the herbal manufacturers

often buy the pre-processed raw materials. As a result of that, phytochemical analysis and chemical fingerprint techniques become a necessity in the quality monitoring of the raw material.

The phytochemical analysis for the identification compounds is essential for the scientific validation of their uses and the preparation of standardized herbal products. At the same time, the level of active or main components as well as chemical fingerprinting has potential to determine the identity, authenticity and batch to batch consistency of processed plant materials or finished products. The World Health Organisation (WHO), the European Medicine Agency (EMA), the American Food and Drug Administration (FDA), and the Chinese Sate Food and Drug Administration (SFDA) have accepted fingerprint technology as a suitable methodology for the quality assessment of herbal products (Tistaert *et al.*, 2001b). The fingerprint is a characteristic profile of the analysed sample which emphasizes on the integral characterisation of a complex system with a quantitative degree of reliability and thus can be used as indicators of raw material and herbal product quality and consistency. Thus far, very few of the above studies have been reported for the *Phyllagathis* species.

Current development in improving sensitivity and analytical capacity of instruments such as Fourier transform infrared (FTIR) spectroscopy, high performance liquid chromatography (HPLC) and hyphenated technology such as liquid chromatography-mass spectrometry (LC-MS) make it feasible to establish analytical technologies for chemical fingerprinting of complex plant material. The IR spectroscopy combined with two-dimensional correlation analysis is able to effectively identify and discriminate complex plant material without going through tedious separation and extraction (Li *et al.*, 2004). This approach has been widely used especially in the identification of traditional Chinese medicinal materials (Wu *et al.*, 2008b; Liu *et al.*, 2006b), identification of herbal medicine

from different regions (Li *et al.*, 2004), discrimination of genuine and fake products (Yap *et al.*, 2008) as well as in quality control analysis (Lin *et al.*, 2003). Similarly, the chromatographic fingerprinting technique has been regarded as one of the most rational and powerful tools for the quality evaluation of herbal preparations (Yang *et al.*, 2011). Recently, chromatographic fingerprinting, especially HPLC fingerprint has been widely accepted and is attracting increasing attention owing to both its high separation efficiency and high sensitivity. It can give an overall view of all the components and it can show both the similarity and dissimilarity among various samples. By coupling with hyphenated techniques such as the HPLC-MS/MS, it becomes one of the most important techniques for both quantitative and qualitative analysis of complex samples due to its enhanced selectivity. The ability of MS to perform the compositional and structural analysis provided more information than is not possible with other techniques (Han *et al.*, 2009).

Thus far, several studies have been reported for the two *Phyllagathis* species. The leaves of *P. rotundifolia* were found to contain a series of prunasin based cyanogenic glucosides with different extent of galloyl esterification in addition to a number of known hydrolysable tannins (Ling *et al.*, 2002). However, there is yet no chemical study been reported for *P. praetermissa* and the comparative analysis of the chemical profiles for the two species has not been reported either. For that reasons, this study was initiated to identify the chemical constituents present in the leaves of *P. rotundifolia* and *P. praetermissa* which could be used for assessing their differences as well as for the comparative analysis of samples collected from different locations. Subsequent to that, multi-steps infrared macro-fingerprints together with HPLC and LC-MS fingerprinting were applied with the aim of developing a rapid identification and discrimination of *P. rotundifolia* and *P. praetermissa* from various locations. Besides, the IR and HPLC data were further analysed by means of principal component analysis (PCA) in order to

complete the identification of these two species more effectively.

In addition to the above, some of the isolated chemical constituents from *P. rotundifolia* and *P. praetermissa* that included galloylated cyanogenic glucosides, gallotannins, ellagitannins, ellagic acid derivatives and aromatic acid were evaluated for their potential on H_2O_2-induced neuroprotective activity against NG108-15 hybridoma cell line, *in vitro* cytotoxicity against colon carcinoma cell line (HCT 116), cervical epidermoid carcinoma cell line (Ca Ski) and breast carcinoma cell line (MCF 7), and the inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA).

1.2 PROSPECT OF MEDICINAL PLANTS

The use of herbs and medicinal plants as the first medicines is a universal phenomenon. Every society throughout the world, through written or oral tradition, has relied on the vast variety of natural chemistry found in healing plants due to their therapeutic properties. Even until now, the indigenous people are still greatly dependent on traditional medicines from natural resources such as leaves, berries, roots, animal parts or minerals for the treatment of life threatening diseases. Many of these traditional medicines indeed have beneficial effect. The World Health Organization (WHO) has defined traditional medicines as the sum total of the knowledge, skills, and practices based on the theories, beliefs, and experiences indigenous to different cultures, whether explicable or not, used in the maintenance of health as well as in the prevention, diagnosis, improvement or treatment for the physical and mental illness.

Today, the list of plants with known medicinal properties is rather long, about 5,800 in the Chinese Material Medica, 2,500 in India, at least 800 regularly collected from the

tropical forest of Africa, almost 300 currently detailed for the medical profession in Germany, and many thousands more known only to traditional healers in other parts of the world. At least 80 % of the world's populations in developing countries used the plant material as their source of primary health care (Farnsworth *et al.*, 1985; Cragg and Newman, 2002; Cordell and Colvard, 2005). Usually these medicinal plants are used as complex mixture such as extracts and essential oils or pure and chemically defined active compounds (Hamburger and Hostettmann; 1991). A survey conducted in USA found that about 40 % of the responded patients believed taking prescription medications together with herbal medicines was more effective than taking either alone (Kuo *et al.*, 2004). Whilst in Malaysia, 88.9 % of the population is using biological based therapies which included herbal therapy for health problem and 87.3 % are using it for health maintenance (Siti *et al.*, 2009). The remaining categories are consisted of manipulative and body based medicine (massage, midwifery and sinusitis treatment), mind body medicine (prayer for health reasons) and whole medical system (acupuncture, homeopathy and traditional Chinese medicine) (Siti *et al.*, 2009). The traditional Chinese medicines have been practiced in Tung Shin Hospital in Kuala Lumpur since 1967 (Chen, 1981). These indicated that people are still relying on and trusted the value of traditional medicine. On top of that, the inheritance of traditional healing practices by diverse ethnicity and rich natural resources were believed to be the dominant reasons for the herbal usage in Malaysia (Siti *et al.*, 2009).

Nevertheless, it was estimated that only about 5-15 % of the 250,000 species of higher terrestrial plants have been investigated chemically and pharmacologically for the presence of bioactive compounds (Balandrin *et al.*, 1993; Cragg and Newman, 2002). In the developed country such as USA, about 25 % of all prescriptions from community pharmacies from 1959 to 1980 were consisted of plant derived drugs (Farnsworth *et al.*,

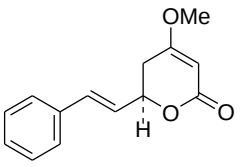
1985; Hamburger and Hostettmann, 1991; Cragg and Newman, 2002). Among the plant derived drugs 74 % were discovered from the isolation of active substances in traditional medicine (Cragg and Newman, 2002). Tremendous changes of the natural products chemistry strategies that included plant selection and collection, isolation technique, structure elucidation, biological evaluation, semi-synthesis, dereplication and biosynthesis have been made feasible for the discovery of new biological and medicinal agents from plant secondary metabolites (Cordell, 1995; Cordell and Colvard, 2005; Colvard *et al.*, 2006).

Malaysia is among the world's 12 mega biodiversity rich countries in terms of the number of plant species (Ang, 2004; Ang and Lee, 2006). However, the knowledge and information of plant constituents with related biological and medicinal properties are still scarcity. Therefore, more comprehensive chemical and biological documentations need to be generated for these traditional medicinal plants. The biological evaluation includes chemical biological assays, animal models and clinical studies that are required to provide scientific proof and clinical validation of these herbal medicines (Yuan and Lin, 2000). The biological properties of these medicinal plants are also influenced by variation in the chemical constituents due to the age of plants, time or season collected and their geographical origin (Farnsworth *et al.*, 1985). There is also a substantial need to collate the incidence of drug-herbs interaction, effects of traditional medicinal plants on human genome and difference of effectiveness between the pure active compounds and the whole or selected plant extracts (Cordell, 2003).

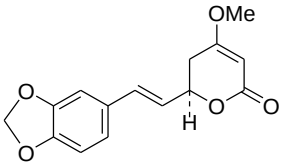
1.2.1 Plant Derived Medicinal Natural Products

Natural product has chemical compound or substance that usually has a pharmacological or biological activity for the usage in pharmaceutical drug discovery and drug design (Balunas and Kinghom, 2005). Plant derived natural products generally provide safe primary health care and lead to discovery of new biologically active agents. They have been considered as candidates for diverse biological activities such as anti-inflammatory, anti-cancer, anti-HIV, analgesic, immune-modulatory, anti-microbial and anti-depressant. Biologically active plant derived secondary metabolites are mainly contributed from the class of terpenoids, steroids, flavonoid, tannins, alkaloids and saponins.

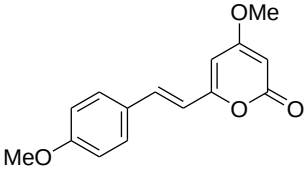
1.2.1.1 Polyphenols



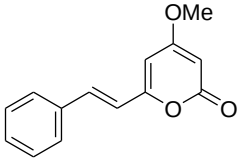
Kawain



Methysticin



Yangonin

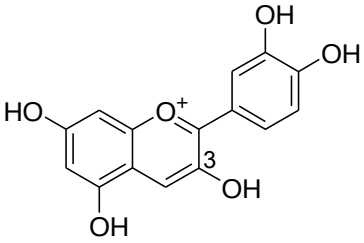


Demethoxyyangonin

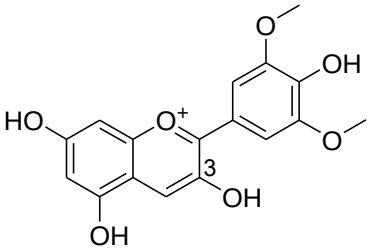
Polyphenols occur naturally as diverse compounds which contain various phenolic functionalities (Tuckmantel *et al.*, 1999). These naturally occurring polyphenolic compounds are classified into proanthocyanidin derivatives, galloyl and hexahydroxydiphenyl ester derivatives, hydroxy cinnamic acid derivatives and phloroglucinol derivatives (Handique and Baruah, 2002). Examples are styrylpyrone derivatives or kavapyrones which were found in *Piper methysticum* and the kava extracts indicated the analgesic effect and central muscle relaxing. These styrylpyrone derivatives included enolides kawain, methysticin, dienolides yangonin and demethoxyyangonin.

Numerous of these compounds also showed the effect on neurotransmitter system (Paul, 2002).

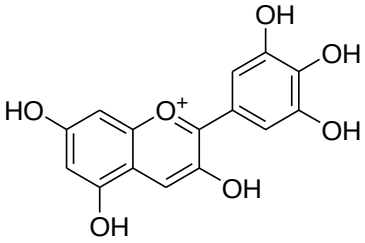
1.2.1.2 Flavonoids



Cyanidin



Malvidin

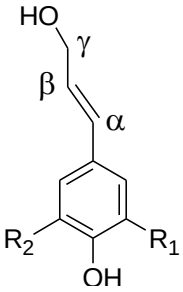


Delphinidin

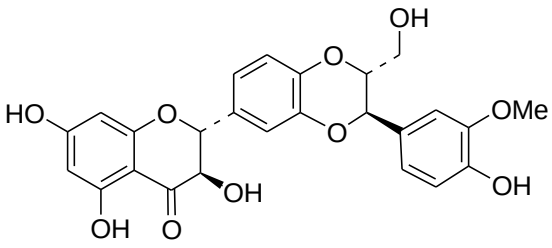
Flavonoids are benzo-γ-pyrone derivatives which encompass dihydroflavonol, flavone, flavonol, flavanone, isoflavone and anthocyanidin. They exhibited unique cardioprotective effects by their ability to inhibit the LDL oxidation (Heim *et al.*, 2002), anti-inflammatory, anti-hepatotoxic and anti-ulcer actions (Narayana *et al.*, 2001). Anthocyan is another class of flavonoids, comprising of anthocyanins with glycosides and anthocyanidins for the aglycon form (Cooke *et al.*, 2005; Wang and Stoner, 2008). The commom anthocyanidins are delphinidin, petunidin, cyanidin, pelargonidin, peonidin and malvidin, while sugar components of anthocyanins commonly are glucose, galactose and arabinose. In most of the cases, the sugar conjugates *via* C3 hydroxyl group in the C skeleton. Anthocyan rich plants has many beneficial effects such as protecting brain cognitive function (Kang *et al.*, 2006; Shin *et al.*, 2006), obesity prevention (Tsuda *et al.*, 2003), gastroprotective effect (Matsumoto *et al.*, 2004), cardiovascular risk (Xia *et al.*, 2006) and cancer prevention such as skin, lung, breast, uterus and colon (Cooke *et al.*, 2005; Wu *et al.*, 2007). The other class of flavanol is proanthocyanidins which contribute as second most abundant natural phenolic after lignin. The polymeric forms of proanthocyanidins are categorized as condensed tannins (Landete, 2011). They have been

investigated for activities such as anti-oxidant activity, decrease of LDL-cholesterol fraction and oxidative stress-derived substances, improvement of endothelium vasodilatation and decrease of blood pressure (Espín *et al.*, 2007).

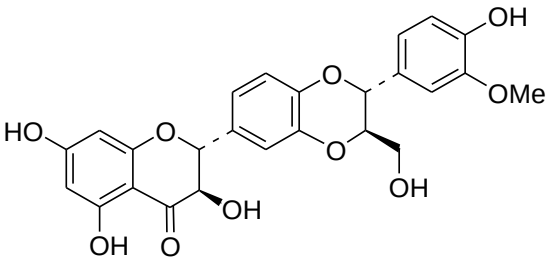
1.2.1.3 Lignins and Lignans



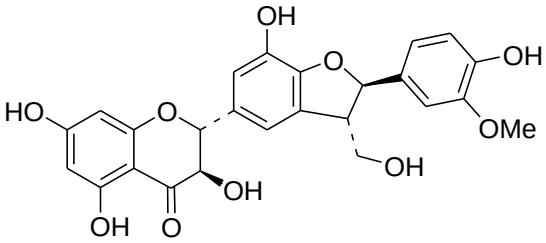
R_1=OME, R_2=H (Coniferyl)
R_1=R_2=OMe (Syringyl)
R_1=R_2=H (*p*-Coumaryl alcohol)



Silybin



Isosilybin

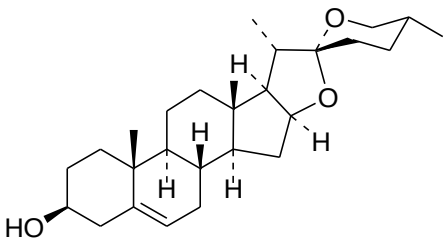


Silychristin

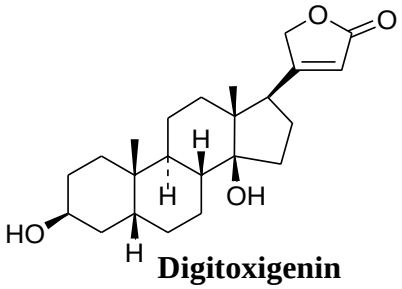
Lignins are the polymerization of three types of phenylpropane units or monolignols. These units are coniferyl, sinapyl and *p*-coumaryl alcohol (Lewis, 1999). The lignins in plants are essential to regulate the transportation of liquid, provide mechanical support and defend against pathogens. Certain polysaccharides in the cell wall are linked to lignin to form lignin-carbohydrate complexes (LCCs). These lignin derived substances have shown diverse pharmacological activities such as anti-tumor, anti-microbial and anti-HIV (Sakagami *et al.*, 2010). Lignans are polyphenolics derived from phenylalanine *via* dimerization of monolignols and are found highly in the flax seed and sesame seed. The lignan such as niranthin from *Phyllanthus amarus* has been claimed for the anti-inflammatory and anti-allodynic action (Kassuya *et al.*, 2006). Other flavonolignans or

silymarin such as silybin, silychristin, silydianin and isosilybin in *Silyburn marianum* (milk thistle) have also been used to treat various hepatic diseases, protect liver against damaging effect including drug and free radicals and inhibit absorption of toxins (Paul, 2002).

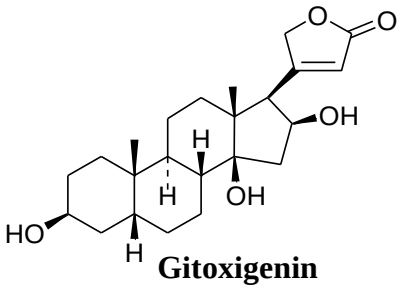
1.2.1.4 Saponins



Diosgenin



Digitoxigenin



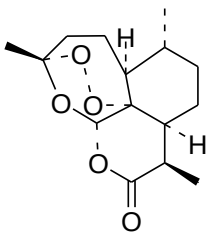
Gitoxigenin

Ginsenoside

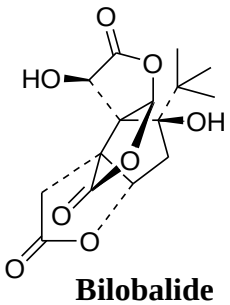
Saponins are natural occurring glycosides which are characterised by a skeleton derived from a 30 carbon precursor, oxidosqualene and attached to glycosyl residues (Vincken *et al.*, 2007). They are classified into triterpenoid saponins and steroidal saponins which consist of the spirostanol saponins and furostanol saponins (Sparg *et al.*, 2004). In biosynthetic pathway, saponins are further distinguished into eleven classes which consist of dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes and steroids (Vincken *et al.* 2007). Studies showed that saponins not only protected the human body against cancers and lower cholesterol levels by affecting the immune systems, but also decrease blood lipids, lower cancer risks and lower blood glucose response (Shi *et al.*, 2004; Wong *et al.*, 2011). Other properties of saponins include anti-inflammatory, anti-microbial, anti-viral, insecticidal, haemolytic,

anti-fungal, anti-parasitic, anti-tumour, and molluscicidal activity (Sparg *et al.*, 2004; Vincken *et al.*, 2007). Diosgenin, a furostanol saponin was discovered for the potential in apoptosis of HCT-116 human colon carcinoma cells (Raju and Bird, 2007). Saponins with cholesterol-modulating properties might show the anti-cancer activities by targeting the 3-hydroxy-3-methylglutaryl Co-enzyme A (HMG-CoA) reductase (Raju and Bird, 2007). The cardioactive glycosides such as the aglycones of digitoxigenin, gitoxigenin and gitaloxigenin were contributing as major components in *Digitalis purpurea*. The digitoxin derived from the hydrolysis of digitoxigenin glycosides has been regularly utilised as agent for congestive heart failure and treatment of atrial fibrillation (Paul, 2002). Other saponins such as ginsenosides or panaxosides also appeared to be the main components in *Panax ginseng* and *P.* notoginseng. These triterpenoid saponins have been widely used for treatment of anaemia, diabetes and improving stamina.

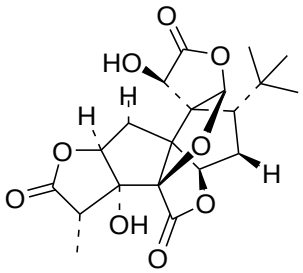
1.2.1.5 Terpenoids



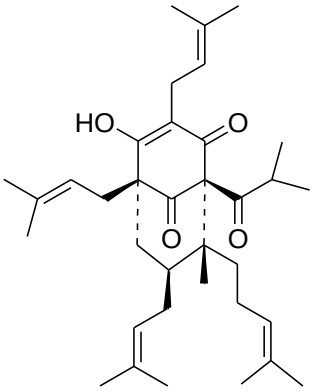
Artemisinin



Bilobalide



Ginkgolide A

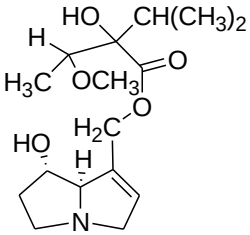


Hyperforin

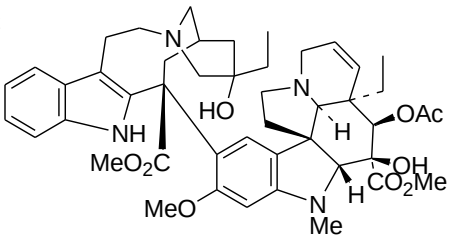
Terpenoids are classified into hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), and tetraterpenes (C_{40}) according to the number of isoprenoid $(C_5H_8)_n$ units. They have been used for the treatment of various diseases such as cancer, malaria and virus or bacterial

infection (Wong *et al.*, 2011). The modified triterpenoids are steroids that contained the tetracyclic ring system of lanosterol but lack of three methyl groups at C-4 and C-14. The diterpenoid taxol have been known for the anti-cancer property. The pharmacologically active diterpene ester paclitaxel in the bark from *Taxus brevifolia* was also exploited as anti-tumour agents for the treatment of ovarian and breast cancers, non-small cell lung cancer, small-cell lung cancer and cancers of the head and neck (Hamburger *et al.*, 1991, Da Rocha *et al.*, 2001; Paul, 2002). The artemisinin (qinghaosu), a sesquiterpene lactone found in *Artemisia annua* has been discovered for anti-malarial activity against drug resistant strains without virtual toxicity (Hamburger *et al.*, 1991; Paul, 2002). In tradition, *Ginkgo biloba* has been used to enhance the memory processes by improving blood circulation (Mazza and Oomah, 2000; Paul, 2002). The active constituents in ginkgo were the mixture of diterpenoid, sesquiterpenoid and flavanoids such as ginkgolides, bilobalide, glycosides of kaempferol and quercetin (Paul, 2002). St John's Wort (*Hypericum perforatum*) has been claimed for treating mild anti-depressant with fewer side effects by increasing the levels of serotonin, noradrenaline and dopamine (Mazza and Oomah, 2000; Paul, 2002). These anti-depressant constituents were derived from napthodiantrone structure such as prenylated phloroglucinol derivative hyperforin (Paul, 2002).

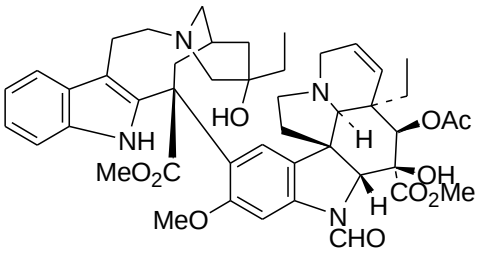
1.2.1.6 Alkaloids



Heliotrine



Vinblastine



Vincristine

Alkaloids were nitrogen-containing organic substances of natural origin. Many bioactive alkaloids derived from medicinal plants such as morphine, strychnine and quinine are used as medicine. The pyrrolizidine alkaloids, heliotrine was found mainly in *Heliotropium ramosissimum* and it has been well documented as the remedy for snake and scorpion bites (Schoental, 1982). The bis-indole alkaloid vinblastine from *Catharanthus roseus* has been used for Hodgkin's disease, non-Hodgkin's lymphomas and treatment for various type of cancer affecting lymph glands, spleen and liver. Vincristine, another superior anti-tumour alkaloids from *C. roseus* has been used in combination with other anti-cancer agents for cancer treatment particularly childhood leukemia. It presented more potent anti-tumour activity than vinblastin (Hamburger *et al.*, 1991; Paul, 2002).

1.3 MELASTOMATACEOUS PLANTS

More than one third of dicotyledons plants are belonging to Rosidae. This subclass consists of 18 orders, 114 families and about 58,000 species which represents the largest subclass in angiosperms. Three-fourths of the species in the Rosidae belongs to five large orders that include Fabeles (about 14,000 species), Myrtales (9,000 species), Euphorbiales (7,600 species), Rosales (6,600 species) and Sapindales (5,400 species), while the

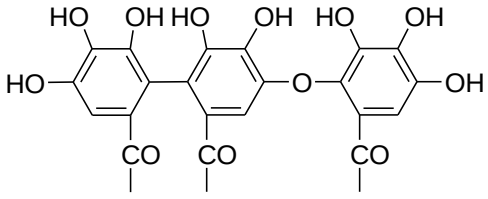
remaining of about 15,000 species come from other order (Cronquist, 1981). Myrtales order consists of 14 families with more than 9000 species. Three-fourths of the species belong to two families, the Melastomataceae (4000 species) and Myrtacea (3000 species), while the Onagraceae, Combretaceae, Lythraceae and Thymelaeaceae have 400-650 species each and the remaining six families (Sonneratiaceae, Penaeaceae, Crypteroniaceae, Trapaceae, Punicaceae, Oliniaceae) have only about 60 species (Cronquist, 1988). Plants in Myrtales order are woody or herbaceous dicotyledons with assorted repellency for discouraging predators (Cronquist, 1981). Henderson in 1954 has classified Melastomataceae family into 14 genera that include *Sonerila*, *Sarcopyramis*, *Phyllagathis*, *Clidemia*, *Marumia*, *Dissochaeta*, *Anplectrum*, *Medinilla*, *Pogonanthera*, *Pachycentria*, *Melastoma*, *Allomorphia*, *Blastus* and *Ochthocharis*. The genus *Phyllagathis* comprises of about 60 species of which 35 species occur in northern Laos, Vietnam and southern China, 12 species in Thailand, Peninsular Malaysia and Sumatra, and 13 species in Borneo (Lemmens and Bunyapraphatsara, 1999). Besides *P. rotundifolia* and *P. praetermissa*, the other *Phyllagathis* species were *P. griffithii*, *P. tuberculata*, *P. magnifica*, *P. stonei*, *P. nanakorniana*, *P. hispida*, *P. cordata*, *P. scortechinii*, (Henderson, 1954; Weber, 1987, 1990; Wangwasit *et al.*, 2010).

1.3.1 Chemotaxonomy of Melastomataceous Plants

Both *P. rotundifolia* and *P. praetermissa* are classified in the Myrtales order and Melastomataceae family. The order of Myrtales, Myrtaceae, Lythraceae, Onagraceae, Melastomataceae and Combretaceae are rich in ellagitannins and ellagic acid (Cronquist, 1981; Okuda *et al.*, 1993; Yoshida *et al.*, 2010). Among these families, only the species from Myrtaceae and Combretaceae show the presence of proanthocyanins (Cronquist, 1981). Other families rich in ellagitannins are Fagaceae, Betulaceae and Cornaceae (Bate-

Smith, 1973). Some plants in the Myrtales have been studied and results suggested hexahydroxydiphenoyl and dehydrohexahydroxydiphenoyl ester of D-glucopyranose have their chemotaxonomic values (Haddock *et al.*, 1982).

The chemistry of Melastomataceae is still not very well known according to Cronquist's classification. Generally, it shows the presence of hydrolysable tannins, proanthocyanins, ellagic acid, acylated anthocyanins and rarely cyanogenics and alkaloids but lack of saponins and iridoids (Cronquist, 1968; 1981; 1988; Okuda *et al.*, 1993). The hydrolysable tannins have been claimed to be observed only in dicotyledons (Hernes and Hedges, 2003). They also produce oligomers that are distinct from those of any other plant family and it has been suggested that ellagic acid and its derivatives appear to facilitate the chemotaxonomic character or definitive systematic marker in the family (Lowry, 1968; Hillis and Yazaki, 1973; Okuda *et al.*, 1993; Yoshida *et al.*, 2000; Calderon *et al.*, 2003). The alkylated ellagic acids had been suggested as potential taxonomic indicator for the family of Melastomataceae and the appearance of highly alkylated 3,3',4-tri-*O*-methylellagic acid and 3'-*O*-methyl-3,4-methylenedioxyellagic acid are more often as compared to 3,3'-di-*O*-methylellagic acid (Lowry, 1968).



***m*-DOG**

Okuda *et al.* in 2000 has not only classified the hydrolysable tannins in Melastomataceae into gallotannins and ellagitannins but also various additional transformations such as *C*-glucosidic ellagitannins, complex tannins, ether linkage of ellagitannins with polyphenol and gluconic acid of ellagitannins. The *C*-glycosidic

ellagitannins were also found in the families of Lythraceae, Myrtaceae, Combretaceae, Punicaceae, Fagaceae, Betulaceae, Casuarinaceae, Rosaceae, Theaceae and Elaeagnaceae (Yoshida *et al.*, 2010). Complex tannins are polyphenols consisting of *C*-glycosidic tannins with condensed tannins and present in the family of Combretaceae, Myrtaceae, Fagaceae and Theaceae (Okuda *et al.*, 1993; Yoshida *et al.*, 2010). The oligomeric ellagitannins are commonly found in plant from Fagaceae, Rosaceae, Coriariaceae, Onagraceae, Myrtaceae and Lythraceae families (Okuda *et al.*, 1993; Yoshida *et al.*, 2010). More than 20 characteristic ellagitannin oligomers in Melastomataceae were characterised from *Medinilla*, *Heterocentron*, *Tibouchina*, *Melastoma*, *Bredia* and *Monochaetum* genera (Yoshida *et al.*, 2010). The Melastomataceous plants were discriminated from other Myrtales species by the position of the galloyl in the *m*-DOG (oxidative coupling between hexahydroxydiphenoyl oxygen of a monomer and the galloyl carbon of another monomer) group at O-4 of the monomeric unit (Okuda *et al.*, 1993). However, the correlation of oligomerization of hydrolysable tannins with plant evolution remained unclear (Okuda *et al.*, 1993).

1.3.2 *Phyllagathis rotundifolia* and *Phyllagatis praetermissa*

Taxonomy	**Taxonomy**
Division: Magnoliophyta	Division: Magnoliophyta
Class: Magnoliopsida	Class: Magnoliopsida
Subclass: Rosidae	Subclass: Rosidae
Order: Myrtales	Order: Myrtales
Family: Melastomataceae	Family: Melastomataceae
Genus: *Phyllagathis*	Genus: *Phyllagathis*
Species: *rotundifolia*	Species: *praetermissa*

P. rotundifolia (Jack) Bl. is a creeping herb found in Peninsular Malaysia and Sumatra. It has heart-shaped leaves with dark green upper surface and reddish lower surface, short stem and pink or magenta flower (Henderson, 1954; Ridley, 1922). Locally, it is known as "Tapak Gajah" and "Tapak Sulaiman". Other vernacular names are "akar serau malam", "bawal hutan", "seri bulan" and "kacip Fatimah" (Henderson, 1954; Lemmens and Bunyapraphatsara, 1999). *P. rotundifolia* is closely related to *P. praetermissa* (Weber). The differences between these two species have been described by Weber in 1990 (Table 1.3.2.1). Ecologically, both species occupy similar habitats in the

lowland, montane forest, shady or partly sun localities and sloping ground. However, *P. praetermissa* is dominant in montane area of the central part of Peninsular Malaysia and the northern Sumatra. In contrast, *P. rotundifolia* is distributed throughout Peninsular Malaysia and also common in Sumatra.

Traditionally, a decoction of the leaves of *P. rotundifolia* is used in the treatment of malaria, fever and stomachache, in parturition and as tonic. The leaves are often added to bath water for a refreshing and relaxing dip (Burkill, 1966; Grosvenor *et al.*, 1995; Lemmens and Bunyapraphatsara, 1999). It also contributes as one of the ingredients in an indigenous contraceptive herbal formulation which contains the mixture of *Lepidagathis longifolia* and *Palaquium* sp. (Mohd *et al.*, 2007). Although plants of the Melastomataceae family are rarely documented for medicinal purposes, several *Melastoma*, *Medinilla* and *Osbeskia* species are known to be astringent for stomach ailments, wounds, hemorrhoids, diarrhea, and dysentery in the tropical and subtropical Asian countries (Isaza *et al.*, 2003).

Table 1.3.2.1: Differences between *Phyllagathis rotundifolia* and *P. praetermissa*

P. rotundifolia	***P. praetermissa***
Habit	
Stem ± or prostrate, apex usually lying ground	Leafy part of stem usually erect, base decumbent
Petiole	
Loosely to very densely setose, emerging from the whole upper part of petiole Colour usually deep purplish or red-brown, surface densely scurfy Upper surface a narrow canal	Glabrous or a few fine, pale, straight hairs emerging strictly from the petiole margins Petioles colour usually green, reddish green, sometimes brown, surface scarcely scurfy Upper surface flat or v-shaped
Lamina	
Orbicular to broadly elliptical Margin with fine teeth Nerves mostly 7+2 Margin meeting at one point on the upper side of the petiole Juvenile leaves without white spots, later usually with strong bluish metallic sheen	Round to ovate Margin almost smooth Usually with 5 main and 2 thinner sub-marginal nerves Lamina margins merging into the petiole margins Juvenile leaves with silvery white spots, adults leaves green, rarely with a bluish metallic sheen
Inflorescence	
Cyme axes often very contracted	Cyme axes mostly well discernible
Flower receptacle	
As wide as long, usually 2-4 bristles in each calyx sinus	Elongate, usually one or no bristle in each calyx sinus

(Source: Weber, 1990)

1.3.3 Cyanogenic Glucosides and Hydrolysable Tannins

Very few studies have been reported for the *Phyllagathis* species. The leaves of *P. rotundifolia* were found to contain a series of prunasin based cyanogenic glucosides with different extent of galloyl esterification in addition to a number of known hydrolysable tannins (Ling *et al.*, 2002). About 11 % of the plant species contain cyanogenic compounds (Jones, 1998) and these are found in more than 2500 plant species from the ferns, palms, woody, herbaceous plants, angiosperms and gymnosperms (Conn, 1980; Møller, 2010; Zagrobelny, 2010). Cyanogenic plants are capable of liberating toxic cyanide which acts as effective herbivore deterrent when the leaves are invaded by herbivores and pathogens (Goodger *et al.*, 2006; Møller, 2010). In most of the cases, cyanide is released by hydrolysis of cyanogenic glycosides (Goodger *et al.*, 2002). These cyanogenic glycosides are particularly found in the families of Rosaceae, Leguminosae, Graminae, Araceae, Compositae, Euphorbiaceae and Passifloraceae (Paul, 2002). The prunasin based cyanogenic glycosides also existed in *Eucalyptus* species (Goodger *et al.*, 2002; Gleadow *et al.* 2008) while taxiphyllin was found occurring in *Henriettella fascicularis*. Other species such as *Clerodendrum grayi* (Lamiaceae) has also shown the presence of cyanogenic diglycoside lucumin and monoglucoside prunasin (Rebecca *et al.*, 2006).

Tannins are divided into condensed and hydrolysable tannins which are widely distributed in the plant kingdom (Piao *et al.*, 2009). The hydrolysable tannins are the polyesters of glucose with gallic acid or hexahydroxydiphenic acid (HHDP) which form gallotannins and ellagitannins, respectively (Chung *et al.*, 1998; Piao *et al.*, 2009). The name hydrolysable means that they are readily hydrolyzed by acids, bases or certain enzymes (Kumar and Vaithiyanathan, 1990; Landete, 2011). The different possible binding sites of galloyl or HHDP group with glucose core leads to enormous structural diversity (Pietta *et al.*, 2003; Yoshida *et al.*, 2005). The classification of the oligomeric hydrolysable

tannins are based on the structures of the unit linking monomers, structures of constituent monomers, conformation of constituent glucose core and degree of oligomerization (Yoshida *et al.*, 2000, Okuda *et al.*, 1993). Up to 2005, more than 500 hydrolysable tannins have been characterised and about 40 % are consisting of oligomeric ellagitannins (Yoshida *et al.*, 2005). Several Melastomataceous plants such as *Tibouchina semidecandra*, *Bredia tuberculata*, *Heterocentron roseum*, *Melastoma normale* and *M. malabathricum* have been studied for their hydrolysable tannins and nobotanins (Yoshida *et al.*, 1991a; 1991b; 1991c; 1992; 1994; 1995; 1999).

1.3.4 Ellagic Acid Derivatives

Ellagic acid is a free ellagitannin which is resulted from the spontaneous lactonization of hexahydroxydiphenic acid, in more or less complex combination with gallate and sugar moieties, resulting in poor water solubility (Lowry, 1968; Landete, 2011). These compounds are distributed in dicotyledonous plants, mainly in Fagales. Methylellagic acids and their derivatives are seldom reported possibly due to the complexity in isolation, purification and identification procedures. However, several studies have shown that methylellagic acids were observed in *Eucalyptus punctata*, *E. megacornuta*, *E. occidentalis* and *E. morrissii*, *Diplopanax stachyanthus* and *Miconia myriantha* (Hillis *et al.*, 1973; Khac, *et al.*, 1990; Li *et al.*, 1999). These compounds have drawn the attention for its systematic significance due to its distribution and association to flavonoids biosynthesis and potential taxonomic value (Lowry, 1968). The ellagic acid or ellagic acid derivatives together with their derived metabolites have been claimed for various biological properties such as anti-oxidant, anti-inflammatory and anti-microbial. The efficacy of the anti-oxidant of these compounds was strongly correlated with their degree of hydroxylation and reduction of sugar moiety (Landete, 2011).

1.4 BIOLOGICAL ACTIVITIES OF HYDROLYSABLE TANNINS

In plants, tannins play a role as repellents to predators such as animals or microbes (Bate-Smith, 1973; Kouki and Manetas, 2002). The presence of tannins as anti-nutritional substances has drawn attention due to nutrient digestibility (Kumar and Vaithiyanathan, 1990; Kouki and Manetas, 2002). This is because tannins have protein-precipitating capabilities which inactivate several digestive enzymes in ruminant animals. However, this perspective has not been investigated thoroughly. The tannins also demonstrate a variety of biological activities including marked anti-viral, anti-HIV, inhibition of lipid peroxidation, anti-tumour (Yokozawa *et al.,* 1998) and anti-bacterial against *Helicobacter pylori* and diminish the resistance of methicillin-resistant *Staphylococcus aureus* (MRSA) to β-lactam antibiotic and anti-leishmanial activity (Chung *et al.*, 1998; Yoshida *et al.*, 2000; 2005). Anti-leishmanial activity of hydrolysable tannins has marked potency evidence as compared to proanthocyanidins and flavan-3-ol derivatives (Kolodziej and Kiderlen, 2005). In addition, the study of monomeric type hydrolysable tannins has shown potential suppression on *H. pylori* without affecting gastric epithelial cells and nonpathogenic intestinal bacteria (Yoshida *et al.,* 2005). Other study also showed that ellagitannins exhibited *in vitro* and *in vivo* anti-carcinogenic properties through induction of cell-cycle arrest, apoptosis and inhibition of tumour formation and growth in animals (Heber, 2008).

1.4.1 Neuroprotection

Oxidative stress-induced cell damage mediated by reactive oxygen species (ROS), mainly superoxide anion and hydrogen peroxide, has been shown to be involved in severe neuronal diseases (Weecharangsan *et al.*, 2006) and other degenerative diseases such as

cancer, atherosclerosis and gastric ulcer (Kumaran and Karunakaran, 2007; Das *et al.*, 1997). Neurodegenerative diseases such as Alzheimer's disease, Parkison's disease, stroke, and dementia is causing 8 % of total death rate in all over the world (Dajas *et al.*, 2003). Almost all organisms have natural anti-oxidant defenses such as glutathione peroxidase, catalase and superoxide dismutase (Aruoma, 1994; Lim *et al.*, 2007) to protect them against oxidative stress. However these systems are insufficient to prevent the damage entirely (Simic, 1988; Chyau *et al.*, 2002). Anti-oxidants present in plants have important role in scavenging free radicals in the body by prevention of cancer, cardiovascular disease, cataract formation, the aging process, inflammatory diseases, and neurological disorder diseases (Alothman *et al.*, 2009). These anti-oxidants revealed multi-functional properties such as anti-bacterial, anti-virus, anti-allergic, estrogenic and immune stimulating effects (Larson, 1988; Abas *et al.*, 2006). Consequently, plant anti-oxidants have received increasing attention and great interest since they are beneficial to human health. Plant polyphenols play an important role as anti-oxidants (Yoshida *et al.*, 2005). Various biological activities have been demonstrated in hydrolysable tannins (Binkley *et al.*, 2009; Yoshida *et al.*, 2000). They also act as anti-oxidant in cardiovascular activity which is known to enhance the synthesis of nitric oxide and relax vascular segments pre-contracted with norepinephrine (Dwivedi, 2007; Ali *et al.*, 2008). The scavenging effects of tannins are generally dependent on the molecular size and number of phenolic hydroxyl groups with and ortho-trihydroxyl structure in the molecule (Yokozawa *et al.*, 1998; Yoshida *et al.*, 2000). The potential of anti-oxidants in hydrolysable tannins was also depended on their oxidation level (Okuda *et al.*, 2000). The anti-oxidative properties of polyphenols can also be modified by proteins (Richard *et al.*, 2006; Arts *et al.*, 2001). Consequently, tannins may also function as potent anti-oxidants to scavenge free radicals to prevent the damage reaction on deoxyribonucleic acid, proteins and lipids. However, it is not clear how these

complex polyphenolic substrates contribute to these biological actions (Chung *et al.*, 1998). Nevertheless, they have been appreciated for their beneficial effect without being troubled by any obvious toxicity (Okuda *et al.*, 1993).

1.4.2 *In Vitro* Cytotoxicity against Cancer Cells

Chronic disease such as cancer has affected approximate 11 million people in the world and has become the second major cause of mortality after the cardiovascular disease (Saunders and Wallace, 2010). Reactive oxygen species (ROS) are considered as one of the cancer promoting factor which plays an important role in mediating apoptosis and cellular damage (Chen *et al.*, 2009). Plant derived polyphenolic compound such as tannins act as natural anti-oxidant and induce apoptosis in various cancer cell lines (Azmi *et al.*, 2006). Most plant polyphenols act as antioxidants and could be important in anti-cancer induction, but these compounds might not act as anti-cancer agents (Gali *et al.*, 1992). Several studies on anthocyanins and ellagic acid have shown their inhibition on cancer proliferation *in vitro* (Losso *et al.*, 2004; Cooke *et al.*, 2005; Ross *et al.*, 2007). Apart from that, Yang et al in 2000 has also reported that 1-*O*-galloylcastalagin and casuarinin (hydrolysable tannins) significantly inhibited human promyelocytic leukemia cell line HL-60 and showed less cytotoxicity to human adenocarcinoma cell line SK-HEP-1 and normal cell lines of human lymphocytes and Chang liver cells. Other hydrolysable tannins, punicafolin was able to inhibit the invasion of HT1080 fibrosarcoma cells (Tanimura *et al.*, 2005). A wide range of potential natural chemotherapeutic agents have been proposed and the discovery from plant source is highly demanded to enhance the effectiveness of cancer treatment. Thus, phytochemicals which were able to induce apoptotic cell death in various cancer cell lines were greatly sought after and extensively investigated for their potential.

1.4.3 Methicillin-Resistant *Staphylococcus aureus* (MRSA)

MRSA is a global problem (Sipahi *et al.*, 2005) and has been recognized as a nosocomial-associated pathogen in the world (Mohtar *et al.*, 2009). *Staphylococcus aureus* is one of the gram positive pathogens that threaten human health and caused a variety of pyogenic infection. This was due to its exceptional virulence, stress tolerance and capacity to accumulate anti-microbial resistances. Other microorganisms which caused the infections were penicillin-resistant *Streptococcus pneumonia*, vancomycin-resistant *Enterococcus* and *Mycobacterium tuberculosis* (Shridhar *et al.*, 2009). The extensive use of antibiotics has led to the resistance of microorganism against antibiotics. Methicillin-resistant *Staphylococcus aureus* (MRSA) is not only resistant to methicillin and other penicillin-related antibiotic (β-lactams) but also other anti-microbial agents such as tetracycline, rifampicin and chloramphenicol (Saiful et al., 2006). Nowadays, vancomycin and its analog teicoplanin are the most effective antibiotics for the treatment of MRSA infections (Hiramatsu, 2001; Jeong *et al.*, 2010). However, several treatments of infectious diseases have shown notable increases in the incidence of bacterial resistance against antibiotics (Finch, 1998). Therefore, the search for anti-microbial agents is still actively pursued for many phytochemicals. Tannins have noticeable and synergistic anti-microbial activity of β-lactams against MRSA (Okuda *et al.*, 2011). Complementary activity of the existing anti-microbial drugs with photochemical may remains to be practiced. The action mode of tannins against microorganisms, especially the structure-activity relationship of tannins components also needs to be studied. Efflux-mediated resistance has been introduced as the main contributor of antibiotic resistance in MRSA isolates (Poole, 2005).

1.5 QUALITY CONTROL OF MEDICINAL PLANTS

Over the past decade, there was an increasing popularity and number of products derived from various traditional medicines that enter the commercial market in the developed and developing countries. However, the quality control of these products is still very poor. In the past, many traditional medicine consumers neglected the importance of quality assurance in the products and assumed that all herbal products are safe to be consumed. This notion is no longer valid now as consumers are becoming more literate and aware which demands a greater effort in research and development by scientist towards the credibility of these products.

All plant derived products including the raw materials, extracts (aqueous, ethanol, distilled, condensed and dried extracts) or end products (pills, capsules, solutions, gels, liquors, powders and granulates) require careful quality monitoring in order to ensure the medicinal value and safety of the traditional medicine. The chemical constituents in the raw materials may vary depending on the factors such as harvesting season, plant origins, drying process and others (Liang *et al.*, 2004). For instance, some studies have shown that the contents of hydrolysable tannins may be affected by nutrients and water stress (Kouki and Manetas, 2002; Horner, 1990; Haukioja *et al.*, 1998). Thus, appropriate evaluation methods followed by substantial scientific study will increase the value of plants that are frequently used in health care. Accordingly, the scientific literatures relating to analytical procedures, chemical and biological information, clinical trials on phytotherapeutic and traditional agents thus becomes a very important aspect of safety and efficacy (Cordell *et al.*, 2005). For comprehensive standardization of the plant-based products, authentication, adulteration, contamination, qualification and quantification, consistency on a batch to batch basis of the plant species have to be established to ensure the safety, quality,

reliability and efficacy of the products to the consumers. This will determine the efficiency of the herbal products in therapeutic effect. Currently, the identification of herbal extracts is based on marker or bioactive compounds and the ideal chemical markers in herbs should be attributed with the biological or therapeutic effect (Jiang *et al.*, 2010). Therefore, the combination of qualification and quantification approach in medicinal plant is important to authenticate, and define the content of active constituents that able to exert the biological properties for disease prevention or body health, respectively.

The Ministry of Health in Malaysia implemented registration for traditional medicines under the Control of Drugs and Cosmetics Regulations 1984 and the quality and safety requirements include the limits for heavy metals (Poison Act 1952, Revised 1989), limits for microbial contamination, absence of steroids and other adulterants (Poison Act 1952, Revised 1989), limits of disintegration time (Pharmacopoeial Standards), claimed indications (Medicine Act-Advertisement and Sale, 1956, Revised 1983), prohibition of herbs with known adverse effects and prohibition of endangered animal species (Wildlife Protection Act, 1972), compliance to Good Manufacturing Practice (GMP) and approved marketing authorization from the importing countries (Ang, 2004; Ang and Lee, 2006; Saw *et al.*, 2006). The Drug Control Authority (DCA) has limited maximum five years validation for pharmaceutical products registration, after which the reassessment of quality, safety and efficacy are required (Ang, 2004; Ang and Lee, 2006). In China, the State Food and Drug Administration (SFDA) has proposed to use fingerprint concept for quality assurance of herbal extracts preparation and product consistency (Mok and Chau, 2006). Besides, other authorities such as Food and Drug Administration (FDA) of the China, United States, European Agency for the Evaluation of Medicinal Products (EMEA), herbal pharmacopoeia of Britain and India and World Health Organization (WHO) also suggested

that the fingerprints should be used as guidelines in manufacturing of herbal products (Mok and Chau, 2006).

Chromatographic fingerprint has been introduced as a rational strategy for assessing complex herbal sources (Ding *et al.*, 2011). There are several chromatographic techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC), capillary electrophoresis (CE) and thin layer chromatography (TLC) which can be applied in for the chromatographic fingerprinting (Liang *et al.*, 2004). In general, samples with similar chromatographic fingerprints have similar chemical characteristics. Such analytical profiles have the potential to determine the identity, authenticity and batch to batch consistency of substances such as herbal medicines. As a result, the development of chromatography fingerprint for the purpose of herbal quality control is definitely a progress in many countries. Recent hyphenated technology such as liquid chromatography mass spectrometry (LC-MS), high performance liquid chromatography coupled with diode array (HPLC-DAD), capillary electrophoresis coupled with diode array (CE-DAD) and liquid chromatography coupled with nuclear magnetic resonance (LC-NMR) have provided highly efficient and less tedious experimental techniques. The analytical approaches of quality control are tabulated in Table 1.5.1. The selected chromatographic and spectroscopic methods utilised for the quality control are discussed in the following section.

Table 1.5.1: Analysis of natural products in quality control.

	Analysis	References
a) Chromatographic		
Thin layer chromatography (TLC)	-Separation and identification of triterpenoids in plant	Martelanc *et al.*, 2009
	-Investigation of *Symphytum cordatum* alkaloids	Mroczek *et al.*, 2006
	-Separation of iridoid glycosides from *Veronica officinalis*	Dallenbach-Toelke *et al.*, 1987
	-Fast screening and classification of spirulina	Zarzyckia *et al.*, 2011
	-Separation of anti-oxidant components from *Psoralea Corylifolia*	Xiao *et al.*, 2010
	-The chemical composition of *Hippophae rhamnoides*	Guliyev *et al.*, 2004
	-Determination of *Gardenia* herbal active constituents	Wang *et al.*, 2004
High performance thin layer chromatography (HPTLC)	-Estimation of conessine in herbal extract	Kaur *et al.*, 2008
	-Determination of iridoid glycoside in *Gmelina arborea*	Akhilesh *et al.*, 2008
	-Quantification of tetrahydroamentoflavone in *Semecarpus anacardium*	Aravind *et al.*, 2008
	-Separation of iridoid glycosides from *Veronica officinalis*	Dallenbach-Toelke *et al.*, 1987
Microemulsion thin layer chromatography (ME-TLC)	-Fingerprinting of licorice	Cui *et al.*, 2005
Rotation planar chromatography (RPC)	-Analysis of oak (*Quercus robur* L.) bark	Vovka *et al.*, 2003

Ultra-micro-chamber centrifugal layer chromatography (UCLC)	-Fractionation tool for fast screening of raw extracts	Zarzycki *et al.*, 2011
Overpressured layer chromatography (OPLC)	-Separation of iridoid glycosides from *Veronica officinalis*	Dallenbach-Toelke *et al.*, 1987
High performance liquid chromatography (HPLC)	-Separation and identification of triterpenoids in plant	Martelenc *et al.*, 2009
	-Quantification of tetrahydroamentoflavone in *Semecarpus anacardium*	Aravind *et al.*, 2008
	-Separation of anti-oxidant phytochemicals	Tsao and Deng, 2004
	-Chemical compositional analysis of *Hippophae rhamnoides*	Guliyev *et al.*, 2004
	-Determination of adulterated steroids in liquid herbal medicine	Klinsunthorn *et al.*, 2011
Ultra performance liquid chromatography (UPLC)	-Rapid quantitation of curcuminoids in *Curcuma longa*	Jin *et al.*, 2010
High speed counter-current chromatography (HSCCC)	-Separation of anti-oxidant components from *Psoralea Corylifolia*	Xiao *et al.*, 2010
	-Fingerprinting of *Salvia miltiorrhiza*	Gua *et al.*, 2004
	-Separation of proanthocyanidins from tea leaves	Kumar *et al.*, 2009
	-Isolation of secondary metabolites from *Hortia oreadica*	Severino *et al.*, 2009
	-Isolation of coffee diterpenes	Scharnhop and Winterhalter, 2009
	-Isolation and purification of theaflavins and catechins	Wang *et al.*, 2008
	-Isolation of alkaloids from *Sceletium tortuosum*	Shikanga *et al.*, 2011

Gas chromatography (GC)	-Chemical compositional analysis of *Hippophae rhamnoides*	Guliyev *et al.*, 2004
	-Determination of *Gardenia* herbal active constituents	Wang *et al.*, 2004
	-Determination of matrine-type alkaloids in *S. flavescens* root	Chen *et al.*, 2004
Capillary electrophoresis (CE)	-Application of CE in phytochemical analysis	Gotti, 2011
	-Fingerprinting of *Salvia miltiorrhiza*	Gua *et al.*, 2004
	-Chemical compositional analysis of *Hippophae rhamnoides*	Guliyev *et al.*, 2004
	-Determination of aristolochic acids in herbal medicines using microemulsion electrokinetic chromatography (MEEK)	Zhai *et al.*, 2006
	-Separation of cardiac glycosides using micellar electrokinetic chromatography (MEKC)	Debusschere *et al.*, 1997
	-Determination of carnosic acid and rosmarinic acid in *Salvia Officinalis* using capillary zone electrophoresis (CZE)	Başkan *et al.*, 2007
	-Analysis of diarylheptanoids and α-tetralone in *Juglans regia* using CZE	Li *et al.*, 2008a
	-Determination of kava lactones and flavonoid glycoside in *Scorzonera austriaca* using CZE	Jiang *et al.*, 2007
	-Rapid determination of aristolochic acid I and II in medicinal plants	Wei and Feng, 2008
	-Determination of bioactive triterpenes in Chinese herbs	Qi *et al.*, 2006
	-Determination of matrine-type alkaloids in *S. flavescens* root using capillary zone electrophoresis (CZE).	Chen *et al.*, 2004

	-Determination of *Gardenia* herbal active constituents using micellar electrokinetic chromatography (MEKC)	Wang *et al.*, 2004
	-Determination of bioactive triterpenes in Chinese herbs using non-aqueous capillary electrophoresis (NACE)	Qi *et al.*, 2006
b) <u>Spectroscopic</u>		
Fourier transform infrared (FTIR)	-Discrimination of different *Chrysanthemums* species	Liu *et al.*, 2008
	-Rapid discrimination of Chinese propolis and poplar buds	Wu *et al.*, 2008a
	-Differentiation and quality estimation of cordyceps	Yang *et al.*, 2009
Fourier transform infrared-attenuated total reflectance (FTIR-ATR)	-Identification of brown and red seaweeds.	Gómez-Ordóñez and Rupérez, 2011
Near-infrared (NIR)	-Determination of free amino acid in *Radix pseudostellariae*	Lin *et al.*, 2009
	-Discrimination of *Camellia sinensis* in different geographical origin	Chen *et al.*, 2009
Nuclear magnetic resonance (NMR)	-Characterisation of triterpenoid and minor phenolic in *Anisophyllea dichostyla*	Khallouki *et al.*, 2009
c) <u>Hyphenated technique</u>		
Thin layer chromatography (TLC)-densitometry and colorimetry	-Determination of α-tocopherol in *Pistacia* species	Klvçak and Akay, 2005

High performance liquid chromatography-diode array detection (HPLC-DAD)	-Analysis of caffeic acid derivatives in *Echinacea* species	Pellati *et al.*, 2004
	-Determination of ginsenosides	Shi *et al.*, 2010
High performance liquid chromatography-ultraviolet (HPLC-UV)	-Determination of *Gardenia* herbal active constituents	Wang *et al.*, 2004
	-Determination of matrine-type alkaloids in *S. flavescens* root	Chen *et al.*, 2004
High performance liquid chromatography-mass spectrometry (HPLC-MS)	-Investigation of *Symphytum cordatum* alkaloids	Mroczek *et al.*, 2006
	-Alkaloids analysis in plant extracts	Aehle and Dräger, 2010
	-Chemical compositional analysis of *Hippophae rhamnoides*	Guliyev *et al.*, 2004
	-Determination of *Gardenia* herbal active constituents	Wang *et al.*, 2004
	-Determination of phenolic compounds in *Prunella* species	Şahin *et al.*, 2011
	-Characterisation of triterpenoid and minor phenolic in *Anisophyllea dichostyla*	Khallouki *et al.*, 2009
High performance liquid chromatography-diode array detection- mass spectrometry (HPLC-DAD-MS)	-Tentative chemical identification and quantitation of *Camellia sinensis*	Zhao *et al.*, 2011
Ultra performance liquid chromatography-photodiode array detector (UPLC-PAD)	-Chemical fingerprint of *Rhizoma coptidischinensis*	Kong *et al.*, 2009
Ultra performance liquid chromatography-ultraviolet (UPLC-UV)	-Rapid determination of active compounds in silymarin	Liu *et al.*, 2009
Ultra performance liquid chromatography-evaporative light scattering detector (UPLC-ELSD)	-Determination of bile acid derivatives in *Calculus bovis*	Kong *et al.*, 2010

Ultra performance liquid chromatography-mass spectrometry (UPLC-MS)	-Metabolite profiling of *Panax notoginseng*	Dan *et al.*, 2008
Ultra performance liquid chromatography-ultraviolet- mass spectrometry (UPLC-UV-MS)	-Fingerprinting of *Hoodia* species	Avula *et al.*, 2008
Rapid resolution liquid chromatography-mass spectrometry (RRLC-MS)	-Identification of major constituents in Deng's herbal tea	Deng *et al.*, 2011
	-Simultaneous quantification of constituents in traditional Chinese medicine	Zhang *et al.*, 2011
Gas chromatography-mass spectrometry (GC-MS)	-Alkaloids analysis in plant extracts	Aehle and Dräger, 2010
	-Characterisation of volatile constituents of *Scaligeria tripartita*	Tabanca *et al.*, 2007
	-Chemical compositional analysis of *Hippophae rhamnoides*	Guliyev *et al.*, 2004
	-Determination of *Gardenia* herbal active constituents	Wang *et al.*, 2004
	-Characterisation of triterpenoid and minor phenolic in *Anisophyllea dichostyla*	Khallouki *et al.*, 2009
Capillary electrophoresis-diode array detection (CE-DAD)	-Isolation of xanthones from *Securidaca inappendiculata*	Wu *et al.*, 2004
Capillary electrophoresis-mass spectrometry (CE-MS)	-Identification and quantification of isoquinoline alkaloids in *Fumaria officinalis*	Sturm *et al.*, 2006
	-Determination of alkaloids in *Strychnos pierrian* and *Radix aconiti praeparata*	Feng *et al.*, 2003

Liquid chromatography-nuclear magnetic resonance (LC-NMR)	-Rapid identification of metabolites in *Dioncophyllum thollonii* -Structure elucidation of isoflavones in *Radix astragali*	Bringmann *et al.*, 1999 Xiao *et al.*, 2005
d) <u>Chemometric methods</u>		
Principal component analysis (PCA)	-Metabolite profiling of *Panax notoginseng* -Chemical fingerprint of *Rhizoma coptidischinensis* -Tentative identification and quantitation of *Camellia sinensis* -Discrimination of *Camellia sinensis* in different geographical origin -Detection and quantification of secondary metabolite in *Hypericum*	Dan *et al.*, 2008 Kong *et al.*, 2009 Zhao *et al.*, 2011 Chen *et al.*, 2009 Kusari *et al.*, 2009
Similarity analysis (SA)	-Chemical fingerprint of *Rhizoma coptidischinensis*	Kong *et al.*, 2009
Hierarchical clustering analysis (HCA)	-Chemical fingerprint of *Rhizoma coptidischinensis*	Kong *et al.*, 2009
Hierarchical agglomerative cluster analysis (HACA)	-Detection and quantification of secondary metabolite in *Hypericum*	Kusari *et al.*, 2009
Partial least squares (PLS)	-Determination of free amino acid in *Radix pseudostellariae* -Qualitative and quantitative analysis in tea	Lin *et al.*, 2009 Chen *et al.*, 2006
Partial least squares–discriminant analysis (PLS-DA)	-Quality evaluation of *Angelica acutiloba* Kitagawa roots -Classification and discrimination of *Rhizoma Corydalis*	Tarachiwin *et al.*, 2008 Lai *et al.*, 2011
Linear discriminant analysis (LDA)	-Classification of *Phyllanthus niruri* in different localities -Classification of active compounds in medicinal plants	Dharmaraj *et al.*, 2006 Xue *et al.*, 2005

Soft independent modeling of class analogy (SIMCA)	-Qualitative and quantitative analysis in tea	Chen *et al.*, 2006
	-Classification of *Phyllanthus niruri* in different localities	Dharmaraj *et al.*, 2006
Artificial neural networks (ANN)	-Discrimination of tea varieties	Li and He, 2008
	-Classification and discrimination of *Rhizoma Corydalis*	Lai *et al.*, 2011
	-Species identification of higher plants	Clark, 2003
K nearest neighbors (KNN)	-Classification and discrimination of *Rhizoma Corydalis*	Lai *et al.*, 2011
Probabilistic neural networks (PNN)	-Classification of active compounds in medicinal plants	Xue *et al.*, 2005
Orthogonal projections to latent structures–discriminant analysis (OPLS-DA)	-Discrimination of *Pelargonium sidoides* and *P. reniforme*	Maree and Viljoen, 2011

1.5.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a simple and low cost separation technique which requires minimal amount of sample. Apart from that, it also significantly reduces the separation time. Generally, the stationary phase of TLC consists of a thin layer of adsorbent such as silica gel, aluminium oxide or cellulose which is coated on a sheet of glass, plastic or aluminum foil. The mobile phase in TLC separates the sample through the capillary action. The separation ends when the mobile phase is evaporated and the analytes become immobilized. The presence of targeted analyte is observed *via* UV detection or various reagents such as Emerson reagent for detection of phenols and aryl amines, aniline phthalate spray reagent for detection of reducing sugars, Dragendorff reagent for detection of nitrogen compounds and alkaloids, iodine vapour as universal reagent, rhodamin B reagent for detection of lipids and vanillin reagent for detection of amines and amino acids. The retention factor, or R_f, is obtained by dividing the distance traveled by the compound over distance traveled by the solvent. The two-dimensional TLC increases the resolution and efficiency of separation by resolving the sample over entire TLC plate. Another technique high performance thin layer chromatography (HPTLC) is an improved method of TLC which is able to increase the resolution and permit more information and parameters for comprehensive identification and assessment of medicinal plants (Jiang *et al.*, 2010). TLC approach has been applied for quantitative determination of medicinal plants (Vanhaelen and Vanhaelan-Fastre; 1983), detection of pesticides or insecticides in food (Rezić *et al.*, 2005) and dye composition in forensic (Djozan *et al.*, 2008). Other applications of TLC and HPTLC have been described in Table 1.5.1a.

1.5.2 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a chromatographic analytical technique used for the separation, identification, quantification and purification of components in the mixtures. A HPLC pump is utilised to pump the mobile phases and analyte at higher pressure through the small particle size and dense column. Combination of water or buffer together with various organic solvents such acetonitrile, methanol are generally used in HPLC. The separation types in HPLC can be in normal-phase chromatography, reversed-phased chromatography, adsorption chromatography, partition chromatography, ion-exchange chromatography, ion-pair chromatography and size-exclusion chromatography (Bélanger *et al.*, 1997). Various type of materials such as C_{18}, C_8, NH_2 and CN columns have been improved to enhance the resolution, reduce high backpressure and increase the usage life. The elution chromatography is graphically represented in chromatogram by various detection systems such as diode array detection (DAD), refractive index (RI) and ultraviolet (UV). The samples retain and interact with stationary phase in column at different periods. Then they are eluted by the mobile phase at different rates and hence characterised by specific retention time for each component. The variation of retention time is not only affected by sample capacity and resolution, but also influenced by the composition of solvents, flow rate of mobile phase and type of column used. Besides HPLC (Martelenc *et al.*, 2009; Aravind *et al.*, 2008; Tsao and Deng, 2004; Guliyev *et al.*, 2004; Klinsunthorn *et al.*, 2011), other chromatographic techniques such as ultra-micro-chamber centrifugal layer chromatography (UCLC), ultra performance liquid chromatography (UPLC), overpressured layer chromatography (OPLC) and high speed counter-current chromatography (HSCCC) were also been used for plant analysis (Table 1.5.1a).

1.5.3 Liquid Chromatography-Mass Spectrometry (LC-MS)

Liquid chromatography-mass spectrometry (LC-MS) is a powerful and high sensitivity technique that combined the liquid chromatography with mass analysis. Commonly, this technique has been applied in pharmacokinetics, metabolomics, natural products and drug identification. The mass spectrometry (MS) ionizes the samples and produces ion in mass-to-charge (*m*/*z*) ratio either in single or multiple charged with positive or negative species. Various analysers such as magnetic sector, quadrupole, time-of-flight, ions trap and Fourier transform-ion cyclotron resonance are used in mass spectrometry. High resolution mass spectrometry data permits more accuracy for species in the same unit mass. The tandem mass spectrometry (MS^n) is able to give more useful data such as the expected and directed fragmentation pathways. Certain isomers could also be distinguished by comparing the fragmentation pattern and their elemental isotopic compositions. The chemical ionisation (CI) complements with electron impact (EI) have been used in analysing organic compounds. However both ionisation techniques have shown the limitation in complex organic compounds because they are only suitable for stable compounds. Thus, electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and fast atom bombardment (FAB) have been introduced in chemical analysis. ESI represents a soft and rapid desorption ionisation technique. The liquid sample effluent from liquid chromatography is nebulised by a flow of nitrogen gas and then sprayed at atmospheric pressure using spray voltage before formation of fine droplets. The resulting droplets are then electrostatically charged in high vacuum with the presence of strong electrostatic field (Crews *et al.*, 1998). In positive mode, an intense $[M + H]^+$ is derived whereas $[M - H]^-$ is derived in negative mode. Generally, this ionisation technique is applied for biological macromolecules and effective for samples which are not responsive to EI or CI. The LC-MS analysis has been utilised extensively for the identification of

natural products in plants (Mroczek *et al.*, 2006; Aehle and Dräger, 2010; Guliyev *et al.*, 2004; Wang *et al.*, 2004; Şahin *et al.*, 2011; Khallouki *et al.*, 2009). The other hyphenated techniques are also tabulated in Table 1.5.1c.

1.5.4 Fourier Transform Infrared (FTIR) Spectroscopy

Infrared (IR) region of the electromagnetic spectrum is usually divided into the near-IR (14000-4000 cm^{-1}), mid-IR (4000-400 cm^{-1}) and far-IR (400-10 cm^{-1}). In this study, the chemical constituents of the plant materials were studied using mid-IR spectroscopic technique. Fourier transform infrared (FTIR) spectroscopy is one of the common multi-purpose tool that is used in various studies such as ecotoxicology (Mecozzi *et al.*, 2007), study of nano-material at the molecular scale (Baudot *et al.*, 2010) and thermal degradation of polymer (Hemvichian *et al.*, 2002; Cervantes-Uc *et al.*, 2006). As shown in Table 1.5.1b, it is also used for identification, discrimination and quality surveillance of medicinal herbs (Liu *et al.*, 2008; Wu *et al.*, 2008a; Yang *et al.*, 2009). The development of IR spectroscopy also opened up new perspective in food industry by evaluating the quality, authenticity, adulteration and deterioration of food products (Fügel *et al.*, 2005). Currently, the multi-steps infrared macro-fingerprinting which included IR spectroscopy, the second derivative infrared spectroscopy and two-dimensional (2D) correlation infrared spectroscopy is being utilised for quality control, authentication and examining the adulteration in traditional medicine (Jiang *et al.*, 2010). The 2D-IR correlation spectroscopy (Noda, 1986; 2004; 2010) has been extensively utilised in the quality control of traditional Chinese medicines (Zuo *et al.*, 2003; Li *et al.*, 2004; Jiang *et al.*, 2010). The 2D-correlation analysis is able to enhance spectral resolution and generate new features by characterizing the dynamic variables of the data related to external perturbation (Harrington *et al.*, 2000; Mecozzi *et al.*, 2007; Oh *et al.*, 2009). Synchronous

spectrum provides information about the change in-phase data whereas the asynchronous spectrum displays information regarding out-of-phase data (Harrington *et al.*, 2000). The peaks in the synchronous spectrum indicate the relative similarity of variation behavior of spectral intensity for two separate wavenumbers whereas that of asynchronous spectrum represents the dissimilarity of the intensity variation behavior (Noda, 2004). The diagonal line in 2D-IR synchronous correlation spectrum reveals the auto peaks which show the variation of the absorption band or a functional group with the external perturbation. In the case of thermal perturbation, the auto peaks represent the alleviation of chemical constituent transformation and consequently contribute to molecular vibration. The closer the correlativity between the absorption bands with thermal susceptibility, the stronger intensity variation will be (Liu *et al.*, 2006a; 2008). However, no auto peaks are found when no self-correlativity and susceptibility of the functional group with thermal perturbation. The cross peaks located at off diagonal synchronous plot and reveals intensity variation occurring at two different wavenumbers (Wu *et al.*, 2008b). It shows the correlativity of vibration of different group or different normal vibration for even same kind of group. The positive cross peaks indicate the intensity variation from the two wavenumbers with thermal perturbation which proceed in the same direction. In contrast, the negative cross peak means that the intensity variation are in opposite directions; (i.e, the intensity of one wavenumber is increasing and the other one is decreasing). The more coincidence the reorientation direction is, the stronger the intensity of cross peaks will be. The absence of any cross peak indicates the lack of chemical coupling or interaction among various functional groups (Li *et al.*, 2004).

1.5.5 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) spectrometer has been introduced for acquisition of ^{1}H spectra of organic molecules in the early 1950 (Crews *et al.*, 1998). The NMR spectroscopic technique relies on the magnetic properties of the atomic nucleus and provides variation in resonant frequency which allows the determination of molecular structure. Some of the important nuclei that are commonly used are ^{1}H, ^{13}C, ^{15}N, ^{19}F and ^{31}P. The resonant frequencies vary depending on the position of that atom within a molecule in an external magnetic field. These variation is called chemical shift (δ) and measured in parts per million (ppm). The NMR spectrum represents the resonant frequencies over a very narrow range of frequencies centered on the fundamental resonant frequency of the nucleus of interest (Jacobsen, 2007). Electronegative groups shift resonance to the left (downfield or higher resonance frequency) whereas hydrocarbons shift the resonance to the right (upfield or lower resonance frequency). Valuable coupling constant, *J* Hz is obtained from spin-spin splitting phenomenon where the number of the splitting peaks is equal to one more than the number of neighboring protons and the intensity ratio is determined by Pascal's triangle (Jacobsen, 2007). The different atoms within a molecule can be identified and determined using chemical shifts and coupling constant. The INEPT (insensitive nuclei enhanced by polarization transfer) is used in advanced 1D experiment such as DEPT (distortionless enhancement by polarization transfer), as well as in a number of 2D experiments such as COSY (correlation spectroscopy), DQF-COSY (double-quantum filtered correlation spectroscopy), HETCOR (heteronuclear correlation experiment), HMQC (heteronuclear multiple quantum coherence) and HMBC (heteronuclear multiple bond correlation). DEPT-45 experiment gives all positive ^{13}C peaks, a DEPT-90 gives CH peaks, and a DEPT-135 gives positive peaks for CH and CH_3 and negative peaks for CH_2. These experiments are able to assign

each carbon in the molecule to quaternary, methine, methylene or methyl based on its exact number of attached protons. Most 2D experiments can be divided into auto-correlated or cross-correlated types. A homonuclear spin system represents auto-correlated experiment and produces off-diagonal correlation peaks. The common auto-correlated experiments are ^{1}H-^{1}H COSY (correlated spectroscopy) serve to correlate protons along one axis to protons along a second and the NOESY (nuclear overhauser effect spectroscopy) where correlations between axes occur *via* homonuclear dipole-dipole interaction. ^{1}H-^{13}C COSY or HETCOR experiments such as HMQC and HMBC correlate the H-C and H-C-C or H-C-C-C, respectively. TOCSY (total correlation spectroscopy) identifies all mutually coupled protons, whereas the HMQC-TOCSY method reveals connectivity between a CH and all other proton in the same spin system. The ^{1}H–^{1}H COSY experiments gives correlations between germinal and vicinal protons, a ^{1}H–^{1}H TOCSY can give correlations between protons that are up to seven bonds away. ROESY (rotating-frame overhauser effect spectroscopy) is different with TOCSY which transfers the magnetization through space whereas TOCSY transfer the magnetization through bond. The NOESY and ROESY correlate protons with other protons *via* their homonuclear NOE interaction. For small molecules, ROESY tends to replace NOESY experiment for NOE measurements. The cross peaks present in NOESY but not in COSY or TOCSY spectra show that the pairs of protons are close in space but not close enough in the bonding network to be *J* coupled. Thus, the NMR experiments are important in plant analysis for the structural elucidation of the chemical constituents (Table 1.5.1b).

1.5.6 Principal Component Analysis (PCA)

Chemometrics is the application of mathematical and statistical techniques to retrieve more information from large number of data (Mok and Chau, 2006) in order to understand the multivariate data. It is useful in quality control as well as classification and discrimination among herbal fingerprints (Tistaert *et al.*, 2011a). Principal component analysis (PCA) is a multivariate chemometric method which is able to reduce the large data size without removing essential information in mathematical calculation (Brereton, 2003). Previously, the selection of peak detection parameters in the chromatograms might cause difficulties in data interpretation (Nielsen *et al.*, 1998; Mok and Chau, 2006). However by introducing the chemometrics analysis, the entire chromatographic profiles could be utilised directly (Nielsen *et al.*, 1998). The combination of fingerprints such as the IR spectra and chromatographic data with chemometrics approach has been extensively used in quality control (Jiang *et al.*, 2010) which greatly improved the quality of the resulting fingerprint. PCA is generally used to determine relationship between data, while other similarity analysis such as correlation coefficient, Euclidean distance, Manhattan distance and Mahalanobis distance have also been utilised. As presented in Table 1.5.1d, several statistical analyses for plant analysis have also been introduced. Hierarchical clustering analysis (HCA) and Hierarchical agglomerative cluster analysis (HACA) are presented in dendogram or tree diagram using the nearest neighbor linkage and correlation coefficient for their similarities (Brereton, 2003; 2007). Partial least squares (PLS) represent a major regression technique for multivariate data and become an important tool when there is partial knowledge of the data. Both Linear discriminant analysis (LDA) and Soft independent modeling of class analogy (SIMCA) belong to the supervised pattern recognition and are presented in a distance plot. SIMCA is regarded as a form of soft modeling used in chemical pattern recognition where two classes can be overlapped.

Therefore, an object can belong to both or neither class simultaneously. The *K* nearest neighbors (KNN) is a conceptually much simple method and do not require elaborate statistical computation which is different with SIMCA and discriminant analysis. An Artificial neural network (ANN) is a mathematical model or computational model that is inspired by the structure and or functional aspects of biological neural network. The other neural network, Probabilistic neural networks (PNN) has an advantage where the training samples can be added or removed without extensive retraining. Hence, the integration of chemometrics in chemical analysis becomes important and is able to foster the development of scientific information in natural products more rapidly.

1.6 OBJECTIVES OF THE STUDY

1. To isolate and characterise the natural products in the leaves of *Phyllagathis rotundifolia* (Jack) and *Phyllagathis praetermissa* (Weber).

2. To generate the chemical fingerprints for *Phyllagathis rotundifolia* (Jack) and *Phyllagathis praetermissa* (Weber) from different locations by multi-steps infrared spectroscopy and high performance liquid chromatography combined with principal component analysis.

3. To evaluate the H_2O_2-induced neuroprotective activity, *in vitro* cytotoxicity of colon carcinoma cell, cervical epidermoid carcinoma cell and breast carcinoma cell and inhibition on methicillin-resistant *Staphylococcus aureus* (MRSA) of the isolated compounds from the leaves of *Phyllagathis rotundifolia* (Jack) and *Phyllagathis praetermissa* (Weber).

1.7 PUBLICATION LIST

The following represents the list of research publications in international journals during the course of this study. The published papers are attached in the reprints section.

I. Tan, H.P., Ling, S.K. and Chuah, C.H. (2010). Multi-step infrared macro-fingerprinting on leaves of *Phyllagathis praetermissa* from different localities in Peninsular Malaysia, *Vibrational Spectroscopy* 52: 48-53.

II. Tan H.P., Ling, S.K. and Chuah, C.H. (2011). Characterisation of galloylated cyanogenic glucosides and hydrolysable tannins from leaves of *Phyllagathis rotundifolia* by LC-ESI-MS/MS. *Phytochemical Analysis* 22: 516-525.

III. Tan H.P., Ling, S.K. and Chuah, C.H. (2011). One- and two-dimensional Fourier transform infrared correlation spectroscopy of *Phyllagathis rotundifolia*. *Journal of Molecular Structure* 1006: 297-302.

IV. Tan H.P., Wong, D.Z.H., Ling, S.K., Chuah, C.H. and Abdul Kadir H. (2012). Neuroprotective activity of galloylated cyanogenic glucosides and hydrolysable tannins isolated from leaves of *Phyllagathis rotundifolia*. *Fitoterapia* 83: 223-229.