VALIDATION OF GC-MS METHOD FOR STANDARDIZATION OF *CURCUMA XANTHORRHIZA* EXTRACTS USING BIOCHEMICAL MARKERS, AR-CURCUMENE AND XANTHORRHIZOL

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BY

MOHD ROHAIMI BIN AB HALIM

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Master of Science

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LIST OF SYMBOLS AND ABBREVIATIONS

^{1}H	:	Proton
^{13}C	:	Carbon
ATR	:	Attenuated Total Reflectance
cm	:	Centimeter
CE	:	Catechin Equivalent
d	:	Doublet
DAD	:	Diode array detector
EI	:	Electron ionization
eV	:	Electron volt
FTIR	:	Fourier Transform Infrared Spectroscopy
FDA	:	Food and Drug Administration
g	:	gram
GCC	:	Gravity column chromatography
GAE	:	Gallic Acid Equivalent
GC-MS	:	Gas Chromatography Mass Spectrometry
h	:	Hour
Hz	:	Hertz
HPLC	:	High Performance Liquid Chromatography
ICH	:	International Conference on Harmonization
i.d	:	Internal diameter
J	:	Coupling constant in Hz
L	:	Liter
LOD	:	Limit of detection
LOQ	:	Limit of quantification
LLOQ	:	Lower limit of quantification
mL	:	Milliliter
m	:	Meter
mm	:	Millimeter
m	:	Multiplet
m/z	:	Ratio of mass per charge
min	:	Minutes

M^+	:	Molecular ion
M	:	Molarity
MCC	:	Mini column chromatography
n	:	Number of Replicate
N	:	Normality
NMR	:	Nuclear Magnetic Resonance
NIST	:	National Institute of Standard Technology
ppm	:	Parts per million
q	:	Quartet
RSD	:	Relative Standard Deviation
Rf	:	Retardation Factor
s	:	Singlet
SD	:	Standard deviation
SIM	:	Single ion monitoring
t	:	Triplet
TPC	:	Total Phenol Content
TFC	:	Total Flavonoid Content
TSC	:	Total Saponin Content
TAC	:	Total Alkaloid Content
TLC	:	Thin Layer Chromatography
TMS	:	Tetramethylsilane
UV-Vis	:	Ultraviolet-visible
USP	:	US Pharmacopeia
UPLC	:	Ultra Performance Liquid Chromatography
v/v	:	Volume over Volume
WHO	:	World Health Organization
ºC	:	Degree in Celsius
µg	:	Microgram
µL	:	Microliter
δ	:	Chemical shift

LIST OF PUBLICATIONS

Journals

1. Ab Halim, M. R., Zabri Tan, M. S. M., Ismail, S., & Mahmud, R. (2012). Standardization and Phytochemical Studies of *Curcuma Xanthorrhiza* Roxb., *International Journal of Pharmacy and Pharmaceutical Sciences*, **4**, 606-610

2. Zabri, Tan M. S. M., Ab Halim, M. R., Ismail, S., Mustaffa, F., Mohd Ali, N. I. & Mahmud. R., (2011). Inhibitory Effect of Selected Malaysian Herbal Plants on Gluthathione S-Transferase Activity, *International Journal of Pharmacology*, 7 (3), 349-355.

Conference

1. Ab Halim, M.R., Ismail, S., & Mahmud, R. (2012). Standardization and Phytochemical Studies of *Curcuma Xanthorrhiza* Roxb. (Temulawak), HS05-P, Universiti Malaysia Terengganu 11th International Annual Symposium on Sustainability Science and Management (UMTAS). 9-11th July 2012 at Kuala Terengganu, Malaysia.

In Progress

1. Ab Halim, M.R., Ismail, S., & Mahmud, R. (2013). Development and validation of a gas chromatography-mass spectrometry (GC-MS) for simultaneous determination and quantification of marker compounds in *Curcuma xanthorrhiza* extracts and pharmaceutical preparations. (Manuscript completed in progress for submission at Journal of Association of Official Analytical Chemists (JAOAC)).

PENGESAHAN KAEDAH GC-MS UNTUK STANDARDISASI EKSTRAK *CURCUMA XANTHORRHIZA* MENGGUNAKAN PENANDA BIOKIMIA, AR-KURKUMENE DAN XANTHORIZOL

ABSTRAK

Dalam tesis ini, juzuk kimia daripada rizom *C. xanthorrhiza* Roxb. telah dikaji. Pengekstrakan rizom *C. xanthorrhiza* telah dijalankan dengan menggunakan kaedah pengekstarakan rendaman dan sohlet yang memberikan tiga ekstrak berbeza dikenali sebagai ekstrak etanol, ekstrak akues dan ekstrak heksana. Pengasingan dan penulenan juzuk kimia telah dijalankan keatas ekstrak heksana *C. xanthorrhiza*. Dua sebatian tulen telah berjaya dipencilkan dan dicirikan sebagai ar-kurkumene dan xantorhizol. Sebatian yang dipencilkan ini telah dipilih sebagai sebatian penanda dalam standardisasi ekstrak *C. xanthorrhiza*. Satu kaedah analitikal kromatografi gas spektrometri jisim baru telah dibangunkan dan disahkan untuk menganalisa sebatian penanda dalam ekstrak *C. xanthorrhiza* dan produk farmaseutikalnya. GC-MS dalam mod pemantauan ion tunggal (SIM) memberikan masa analisis yang pendek, had pengesanan (LOD) dan had kuantifikasi (LOQ) yang mencukupi serta ketepatan dan kebolehpulihan pengujian yang baik. Kandungan ar-kurkumene di dalam ekstrak etanol dan ekstrak akues masing-masing adalah 136.02 ± 5.11 μg mL^{-1}dan 21.08 ± 0.10 μg mL^{-1}. Kandungan xantorhizol bagi ekstrak etanol didapati sebanyak 228.86 ± 16.10 μg mL^{-1} dan 34.09 ± 0.93 μg mL^{-1} bagi ekstrak akues. Penenalpastian sebatian menggunakan kaedah yang telah disahkan tersebut memberikan ar-kurkumene, α-cedrena, β-elemenona, xantorhizol, kamfor, zingiberena, γ-elemena, trans β-farnesena dan benzofuran. Ar-kurkumene dan xantorhizol telah dikenal pasti sebagai sebatian utama di dalam ekstrak-ekstrak tersebut. Pemeriksaan awal kualitatif fitokimia menunjukkan bahawa ekstrak *C. xanthorrhiza* mengandungi terpenoid,

fenol, saponin, flavonoid, glikosida, kardioaktif, alkaloid dan koumarin manakala antrakuinon, antron dan tanin tidak ditemui. Analisis kuantitatif keatas jumlah kandungan fenol (TPC) menunjukkan bahawa ekstrak etanol (199.00 ± 1.31 mg GAE g^{-1}) mengandungi sebatian polifenolik yang lebih tinggi berbanding dengan ekstrak akues (19.99 ± 0.16 mg GAE g^{-1}). Satu corak yang sama seperti TPC diperhatikan pada jumlah kandungan flavonoid (TFC) dengan ekstrak etanol menunjukkan TFC yang lebih tinggi berbanding dengan ekstrak akues dengan nilai masing-masing adalah 101.66 ± 0.83 mg CE g^{-1} dan 10.58 ± 0.83 mg CE g^{-1}. Jumlah kandungan saponin adalah 80.90 mg g^{-1} dan jumlah kandungan alkaloid adalah 14.06 mg g^{-1}. Hasil daripada kajian kestabilan menunjukkan bahawa sebatian penanda (ar-kurkumene dan xantorhizol) adalah sensitif terhadap pH dan berkurang dengan kadar yang cepat pada suhu yang lebih tinggi tetapi sedikit stabil dalam pendedahan fotokimia. Profil sebatian penanda berdasarkan kaedah kromatografi cecair prestasi tinggi menunjukkan ar-kurkumene dan xantorhizol di dalam etanol mempamerkan penyerapan puncak yang boleh diterima pada 270 nm dengan masa pengekalan masing-masing adalah 9.03 min dan 3.21 min.

VALIDATION OF GC-MS METHOD FOR STANDARDIZATION OF *CURCUMA XANTHORRHIZA* EXTRACTS USING BIOCHEMICAL MARKERS, AR-CURCUMENE AND XANTHORRHIZOL

ABSTRACT

In this thesis, the chemical constituents from rhizomes of *C. xanthorrhiza* Roxb. were investigated. Extraction of rhizomes of *C. xanthorrhiza*was conducted using maceration and Soxhlet extraction method that gives three different extracts namely ethanol, aqueous and hexane extracts. Isolation and purification of chemical constituents were done on the *C. xanthorrhiza* hexane extract. Two pure compounds was successfully isolated and characterized as ar-curcumene and xanthorrhizol. These isolated compounds were chosen as the chemical markers in the standardization of *C. xanthorrhiza* extracts. A new Gas Chromatography Mass Spectrometry analytical method was developed and validated for the assay of chemical markers in *C. xanthorrhiza* extracts and its pharmaceutical products. The GC-MS in the single ion monitoring (SIM) mode gave short analysis time, sufficient limit of detection (LOD) and limit of quantification (LOQ) and good assay precision and accuracy. Ar-curcumene content in the ethanol and aqueous extract was 136.02 ± 5.11 μg mL^{-1} and 21.08 ± 0.10 μg mL^{-1}, respectively. The xanthorrhizol content for ethanol extract was found to be 228.86 ± 16.10 μg $mL^{-1}$and 34.09 ± 0.93 μg mL^{-1} for the aqueous extract. Identification of compounds using the validated method revealed the present of ar-curcumene, α-cedrene, β-elemenone, xanthorrhizol, camphor, zingiberene, γ-elemene, transβ-farnesene and benzofuran. Ar-curcumene and xanthorrhizol were found to be the major compounds in the extracts. Initial qualitative phytochemical screening showed that *C. xanthorrhiza* extracts contain terpenoids, phenols, saponins, flavonoids, cardiac glycosides, alkaloids and

coumarins while anthraquinones, anthrones and tannins were absent. Quantitative analysis of the total phenol content (TPC) showed that ethanol extract (199.00 ± 1.31 mg GAE g^{-1}) contained higherpolyphenolic compounds compared to aqueous extract (19.99 ± 0.16 mg GAE g^{-1}). A similar pattern as TPC was observed for total flavonoid content (TFC) with ethanol extract showing a higher amount of TFC compared to the aqueous extract with values of 101.66 ± 0.83 mg CE g^{-1} and 10.58 ± 0.83 mg CE g^{-1}, respectively. Total saponin content was 80.90 mg g^{-1} and total alkaloid content was14.06 mg g^{-1}. The result from stability studies revealed that the marker compounds (ar-curcumene and xanthorrhizol) were pH sensitive and degraded rapidly at higher temperature but were slight stable under photochemical exposure. Profiling of chemical markers based on developed High Performance Liquid Chromatography method showed ar-curcumene and xanthorrhizol in ethanol exhibited acceptable absorption peaks at 270 nm with retention times of 9.03 min and 3.21 min, respectively.

CHAPTER 1

1.0 INTRODUCTION

1.1 Natural products as Herbal Medicine

Natural products chemistry is a science, which studies different products from living material, animals or plants. Natural products may be defined simply as any chemicals, which are produced by living matter, and human have utilized such compounds since the beginning of time (Killop, 1970). A natural product also can be considered as such even if it was prepared by total synthesis (Bensky et al., 2004). According to their nature, natural products can be used for different purposes. This include for preparing foodstuff, coloring matters, flavors and fragrances, insecticides, extraction of enzymes, pheromones and so on (Cox, 1990, Brunetton, 1999). It can also be considered and use as traditional herbal medicine. The World Health Organization (WHO) define herbal medicine as herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredients of plant parts or other plant materials or combination (WHO, 2008).

The use of plants as food, as a source of beverages, dietary supplement and medicines depends on the knowledge of phytochemicals that are present (Sahoo et al., 2010). Plants have an extensive record as a source of drugs for treating human illness (Chin et al., 2006). A large number of plants have been used in traditional medicinal practices for more than 3000 years, such as in traditional chinese medicine, ayurvedic medicine, unani medicine, etc. Some of the plants could probably exert therapeutics effect and would be proven as such if they were properly

evaluated by Western standards (Farnworth and Soejarto, 1985). Since plant materials are composed of thousand of chemical constituents, chemist have a compelling curiosity to discover the actual compounds in a plant extract used as herbal medicines which responsible for the therapeutic effects. To do this, it is necessary to isolate pure compounds from their natural sources and determine their structures. This, however, is not an easy task, especially when the compounds of interest are present in low concentration. In these cases, enormous quantities of raw materials are required to extract even a few milligrams of the desired compounds. Therefore a high degree of skills and technology is required in both the isolation processes and in subsequent investigations to establish their structures.

Systematic investigations, particularly during the current century have resulted in identification of a growing number of active constituents (from natural products) many of which are now routinely employed in modern medicines. Benzoic acid was the first chemical substance to be isolated from a plant source in 1560. Morphine was isolated in 1806 and its structure was established by Sertuner whereas cocaine was isolated by Neiman in 1859 (Manitto and Sammes, 1981). According to Russel (1963), extraction of *Cinchoa liedgeriana* (Rubiacea) gave quinine and quinidine, which have been used in antimalarial and antiarrythmic therapy, respectively. *Curcuma longa* (Zingiberacea) produced mostly active curcumin, which has been used clinically as choleretic agent (Masaki et al., 1990). A number of screening programs for bioactive compounds exist and have led to a new drug, paclitaxel (taxol), which is used for the treatment of various cancers (Slichenmyer and Von Hoff, 1991). In addition, *Ginkgo Biloba* L., a breakthrough herb in the late 1990's, was found to posses the effective neuroprotective properties under conditions

such as hypoxia/ischemia, seizure activity and peripheral nerve damage (Smith et al., 1996). Based on this fact, people are becoming increasingly interested in medicinal plants because of their good therapeutic performance and low toxicity (Bensky et al., 2004). The study on medicinal plants continues to this day.

The world market of herbal medicines based on traditional knowledge is estimated at US$ 60 thousand million. In fact, the use of herbal medicine contributes significantly to primary health care, especially in developing countries (WHO, 2003). Malaysia has about 12000 species of flowering plants of which about 1300 are claimed to be medicinal, however, the medicinal plants available have not been investigated fully for their potential (Jamal, 2006). Research and development of herbal medicine are focusing on plants that have specific biological potential. In fulfilling the demand of the raw material requirement of local herbal industries, domestication and cultivation of medicinal plants of economic value on a plantation scale is needed. Therefore, in June 1999, Malaysia's First Herbal Monograph was prepared by a committee comprising representatives from the Health Ministry, universities, research institution and the industries (Malay. Tim. Bull., 1999).

1.2 Plant Introduction

1.2.1 Genus Curcuma

Curcuma species are found wild and cultivated in South East Asia. The rhizomes of these species are locally used in traditional medicine for the treatment of several kinds of diseases. In tropical countries Curcuma species are cultivated and not only used for traditional medicine but also for spices, dyes, foods, perfume and ornamental plants. Basically, the base of each arial stem of

Curcuma consists of an erect avoid primary tuber ringed with base of old scale leaves, bearing when matured. The leaves are usually oblong and flowers in dense compound spikes. Curcuma needs filtered light shade and moist soil. Most will thrives in a well watered soil with plentiful rain or in part sun and some will take full sun, but a few prefer shadier conditions (Kirtikar and Basu, 1975). Curcuma species are mostly grown for their foliage, as most of its inflorescence is close to the ground underneath the big fan shaped leaves. In fact many are called "Hidden Lillies" for that reason. The leaves of many species have a distinct dark red or burgundy stripe along the centre and the foliage of many is ruffled. The flowers usually come in summer. Most are pink to burgundy red, but some are the purest white (Kirtikar and Basu, 1975).

The Curcuma species are typically having large rhizomes. The rhizomes are aromatic, stomachic and carminative, where various secondary metabolites are accumulated and show biological activities. The orange color of the Curcuma rhizomes is always associated with curcumin and its derivatives in some cases, which are cytotoxic. Some of the curcuminoids from ginger were found to be natural antioxidants (Pandji et al., 1993; Jitoe et al., 1992). Chemical studies have revealed that genus Curcuma are known to be rich in sesquiterpenoids and bioactive curcuminoids (Ichiro et al., 1995; Kouno et al., 1985; Itokawa et al., 1985). It should also be noted that some of the Curcuma rhizomes have a large amount of essential oils. These oils have been reported to possess antibacterial and antifungal properties, while the antitumor activity of some of their constituents has also been studied (Zwaring and Bos, 1990).

1.2.2 *Curcuma xanthorrhiza* Roxb.

C. xanthorrhiza is a traditional herbs originated from the ginger family (Zingiberaceae) and popularly known as 'temulawak' in Malaysia. It is one of the important gingers cultivated in tropical areas (Masuda et al., 1992). It is a low growing plant with a root (rhizome) similar to ginger, with an aromatic, bitter taste and pungent odor. *C. xanthorrhiza* can grow up to 2 meter height on hill slope and in teak forest. It has a large leaves with a dark strip that runs up at the centre and outsized rhizomes that contribute to its various herbal qualities (Sears, 2005). Both rhizomes and tuberous roots of *C. xanthorrhiza* are deep yellow (Burkill, 1966).

Rhizomes of *C. xanthorrhiza* are usually aromatic and carminative, traditionally used to treat hepatitis, jaundice, atherosclerosis, diabetes and bacterial infections (Yasni et al., 1993; Lin et al., 1995). It was also used in traditional health supplement known as 'maajun' or 'jamu'. The juice of *C. xanthorrhiza* rhizomes was individually used to remedy certain health problem as such indigestion and rheumatism or applied to the body after childbirth. In South-East Asia, it is traditionally utilized for a range of illness including liver complaints, cancer, hepatitis, hypertension and heart disorders. Apart from that, *C. xanthorrhiza* are commonly used as medicines, flavoring agents, spices and source of dyes (Burkhill, 1966).

The methanol extract of this plant display anti-inflammatory activities on acetic acid-induced vascular permeability as well as writhing symptom in mice and carrageenin-induced edema in rats (Ozaki, 1990). Furthermore, *C. xanthorrhiza* shows a potent cancer chemopreventive potential (Park et al., 2008). The

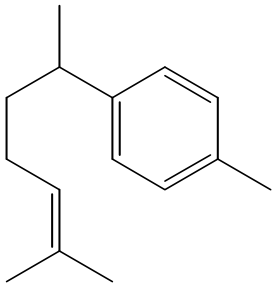
antibacterial activity of *C. xanthorrhiza* extract on *Streptococcus mutans* biofilm formation and in animal studies was also reported (Kim et al., 2008; Hentschel et al., 1996). Recent investigations indicate that the extract of *C. xanthorrrhiza* possessed effective hepatoprotective activity against ethanol-induced liver toxicity in rats (Devaraj et al., 2010). Though they are many uses of *C. xanthorrhiza* as a medicine but it is better known as a spice more than a drug (Ruslay et al., 1997).

Figure 1.1 *Curcuma xanthorrhiza* Roxb. (Picture is adapted from http://herbalandhealthy.wordpress.com/2012/05/10/temulawak-curcuma-xanthorriza-roxb/)

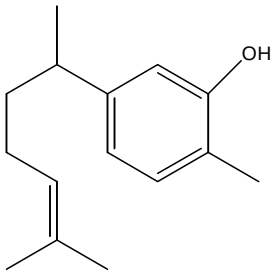
Figure 1.2 Rhizomes of *Curcuma xanthorrhiza* Roxb. (Picture is adapted from http://learnherbalife.com/herbal-medicine/steps-of-making-herbal-medicine-for-liver).

1.2.2.1 Previous Work on the Phytochemicals of *C. xanthorrhiza*

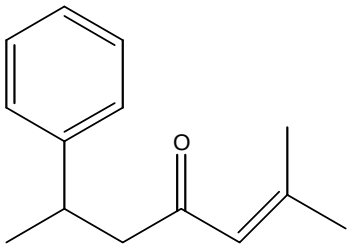
Phytochemical investigations on *C. xanthorrhiza* have revealed that this plant was rich in secondary metabolites. These investigations have led to the isolation of many known and new compounds. This include curcuminoid, bisabolene and terpenoid group of compounds. For instance, a research by Itokawa et al. (1985) has succesfully isolated four bisabolene sesquiterpenoids known as ar-curcumene (1), xanthorrhizol (2), ar-turmerone (3) and β-atlantone (4) which are a major antitumor constituent (against Sarcoma 180 ascites in mice) from the rhizomes of *C. xanthorrhiza*.



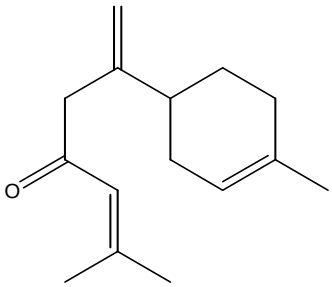
ar-curcumene (1)



xanthorrhizol (2)



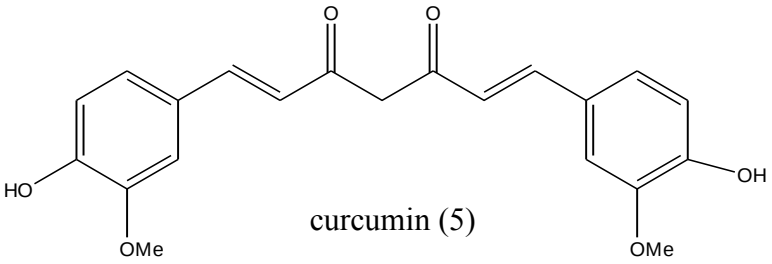
ar-turmerone (3)



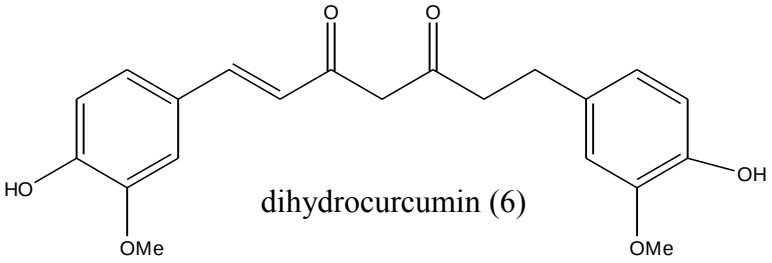
β-atlantone (4)

In 1987, Uehara et al. have isolated five compounds from *C. xanthorrhiza* which is curcumin (5), dihydrocurcumin (6), hexahydrocurcumin (7), octahydrocurcumin (8) and 1-hydroxy-1,7-bis(4-hydroxy-3-1-hydroxy-1-methoxyphenyl)-6-heptene-3,5-dione (9). Further studies on the chloroform extracts

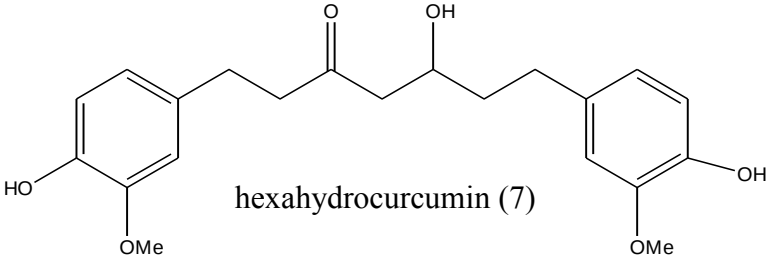
of *C. xanthorrhiza* produced curlone (10), bisacurol (11), bisacurone (12) and bisacumol (13) (Uehara et al., 1989).



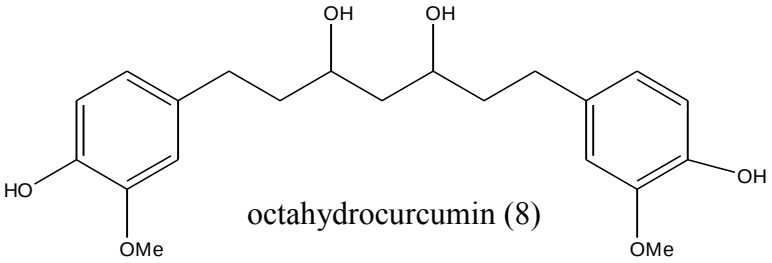
curcumin (5)



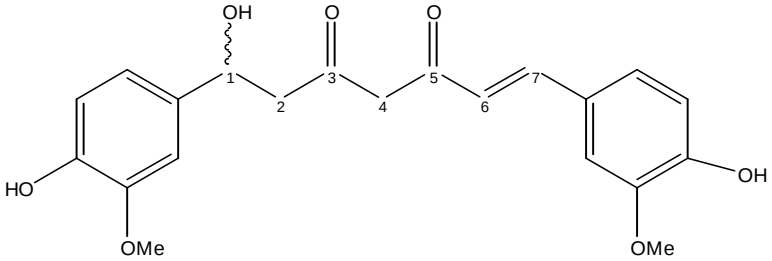
dihydrocurcumin (6)



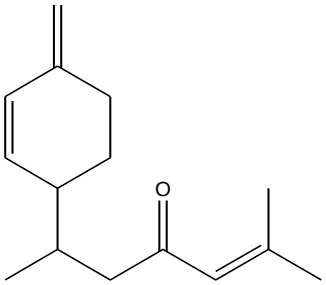
hexahydrocurcumin (7)



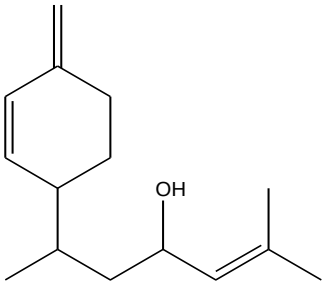
octahydrocurcumin (8)



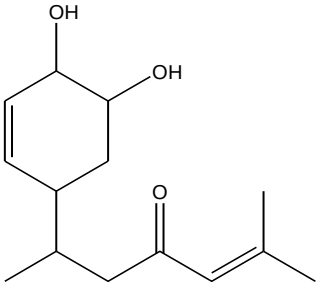
1-hydroxy-1,7-bis(4-hydroxy-3-1-hydroxy-1-methoxyphenyl)-6-heptene-3,5-dione (9)



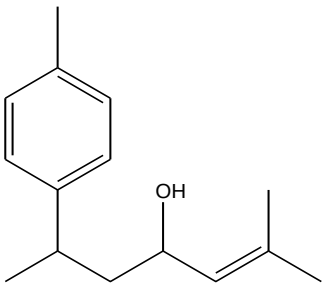
curlone (10)



bisacurol (11)

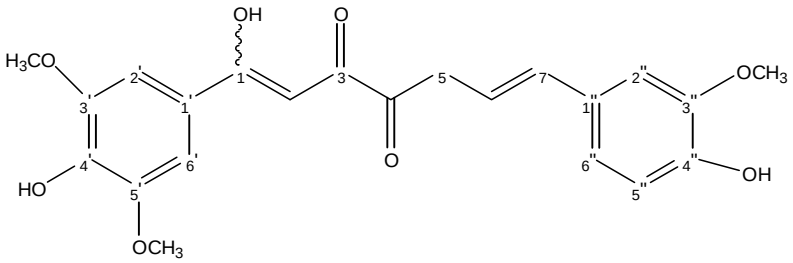


bisacurone (12)

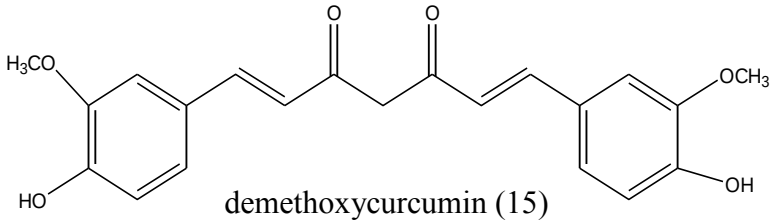


bisacumol (13)

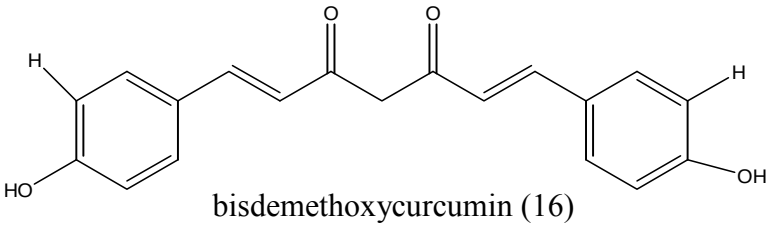
An oxidative study was carried out using rhizomes of *C. xanthorrhiza* and a new curcumin analogue has been isolated from the acetone extract along with three other known curcuminoids. Characterization of the new analogue by spectroscopic data give 1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(1E,6E)-1,6-heptadiene-3,4-dione (14). The other three compounds were identified as curcumin (5), demethoxycurcumin (15) and bisdemethoxycurcumin (16). All curcuminoids showed potent antioxidant activity against autoxidation of linoleic acid in a water-alcohol system (Masuda et al., 1992).



1-(4-hydroxy-3,5-dimethoxyphenyl)-7-(4-hydroxy-3-methoxyphenyl)-(1E,6E)-1,6-heptadiene-3,4-dione (14)

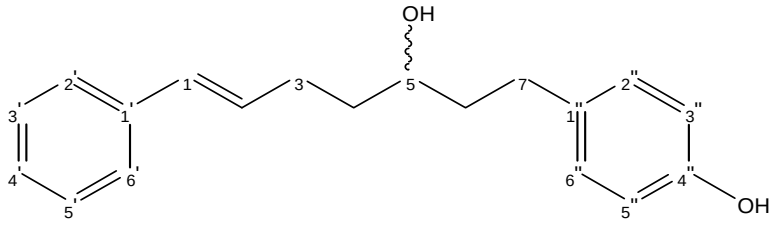


demethoxycurcumin (15)

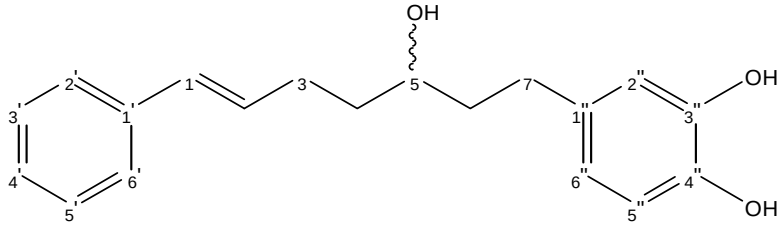


bisdemethoxycurcumin (16)

In addition, two diarylheptanoids, 5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-1(1E)-1-heptene (17) and 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-1(1E)-heptene (18) was isolated from ethyl acetate extract of *C. xanthorrhiza* by chromatographic separation using chloroform and mixtures of chloroform-methanol as eluents (Suksamrarn et al., 1994).



5-hydroxy-7-(4-hydroxyphenyl)-1-phenyl-1(1E)-1-heptene (17)



7-(3,4-dihydroxyphenyl)-5-hydroxy-1-phenyl-1(1E)-heptene (18)

Two most major compounds in the literature for *C. xanthorrhiza* were xanthorrhizol and curcumin. The rhizomes are rich in essential oil and 46.3% from it corresponds to xanthorrhizol (Cheah et al., 2009) along with > 30.0% of ar-curcumene. Xanthorrhizol displays biological properties such as antifungal (Rukayadi et al., 2006), neuroprotective (Lim et al., 2005) and antimetastasis (Choi et al., 2005). In the article by Hwang et al. (2000), the isolated xanthorrhizol from the methanol extract of *C. xanthorrhiza* was subjected for an evaluation of antibacterial activity against oral microorganisms in comparison with chlorhexidine. The result suggested that it could be used as food supplements and dental products for prevention of oral diseases.

On the other hand, curcumin, a major yellow pigment isolated from the rhizome of Curcuma species exhibits antimicrobial, antioxidant, anti-inflammatory and hepatoprotective activity. The oxygen free radicals such as hydroxyl radicals and superoxide anions are responsible for the formation or initiator to lipid peroxidation which contributes to heart diseases, inflammation and cancer. Curcumin is capable to scavenging such free radicals as reported by Pulla Reddy and Lokesh (1992, 1994) where it shows antioxidant activity in the inhibition of lipid peroxidation of rat liver microsomes. Furthermore, a combined mixture of xanthorrhizol-curcumin exhibited a synergistic growth inhibitory activity via apoptosis induction in human breast cancer cells (Cheah et al., 2009).

1.3 Phytochemistry

Phytochemistry is studies concerned with a variety of organic substances present in plant including their chemical structures, biosynthesis, metabolism, natural distribution and biological function (Harborne, 1973a). In common usage today, many phytochemicals are associated with health benefits which continue today, as medicines (Rouhi, 2003b). In western countries, studies shows that 25% of the molecules used in pharmaceutical industry are of natural plant origin (Payne et al., 1991). Phytochemical studies mainly involve extractions, isolations, purifications and characterization of the active compounds in plants. Before starting the extraction process, identity of the plants studied should be authenticated to identify and confirm the plant identity (Harborne, 1973a). The information obtained must be kept for future references.

1.3.1 Extraction of Plant Materials

Nowadays, the study of natural products as part of drug discovery programs is growing rapidly. There are various methods available for the extraction of secondary metabolites from plants. For example, Jones and Kinghorn (2005) discussed widely on the specific extraction protocols for certain classes of compounds. However, the efficiency and effectiveness of the extraction depends mostly on the method selections and suitable solvents used. During the preparation of the extract, careful measurements must be adopted to ensure the potential active constituents are not destroyed, lost or altered. According to Cos et al. (2006), extraction procedures such as percolation, maceration and infusion are the traditional techniques used for the extraction of medicinal plants. The main objective of such basic extraction

procedures is to obtain the desired portion (therapeutically active) by eliminating the inert material using a selective solvent known as menstruum (Singh, 2008).

In this thesis, the maceration extraction method was employed. The procedure involves leaving the powdered plant material to soak in suitable solvent in a closed container at room temperature. A closed system is essential to avoid the evaporation of menstruum over a prolonged period. To increase the efficiency of the extraction, several occasional or constant stirring is required (Siedel, 2005). Another extraction method employed was Soxhlet extraction method. This method is widely used in the extraction of plant material based on the advantages of its continuous process, less time and solvent-consumption. The powdered plant is placed inside a thimble which is loaded into the main chamber (on top of a collecting flask beneath a reflux condenser). The collecting flask is filled with a suitable solvent and heated under reflux. The vapor travels up a distillation arms and floods into the chamber, housing the thimble. Some of the desired compound will dissolved in the warm solvent and brings back to flask. Regardless of the extraction technique used, the resulting solution should be filtered to remove any remaining particulate matter.

1.3.2 Isolation of Plant Material

Chromatography is the method of choice in separating the problem related to the isolation of compound of interest from natural mixture. Various methods are available, from basic to advance techniques which support for isolation and separation effectively. The basic concept all the chromatographic techniques involve the distribution or separation of components in the extracts between two phases which is a moving mobile phase that is passed through an immobile stationary phase.

Separation depends on the difference affinity of the components towards mobile phase and stationary phase. In this thesis, two most common methods for isolation of bioactive compounds were adapted. This includes thin layer chromatography (TLC) and column chromatography (CC).

CC is the most common form of chromatography. The stationary phase is solid (usually silica or alumina) and packed in a column made of glass. Sample were prepared either by dissolving it in a solvent and applied to the front of the column (wet packing) or adsorbed on a coarse silica gel (dry packing). The sample mixture is applied to the top of the column and the mobile phase passes through the column either by vacuum, pressure or gravity. For an easy separation, ratio of 100g of silica gel / g of crude sample is an option. The mobile phase consist of selected solvent elutes the samples through the column and separate the samples into components (separation band). Fractions are collected according to the separation by the solvent or mixture of solvent that is usually changed in the order of increasing polarity. The fractions collected are commonly monitored by TLC.

TLC is one of the most extensively used chromatography methods in an organic laboratory (David, 2001). It advantages in simple sample preparation, low in cost and allow multiple detections using reactive spray reagents, make it ideal for screening method in chemical and biological analysis. It is also used to provide identification and qualitative results. Furthermore, purified samples can be scraped off the plate and analyzed by other techniques because of it is a non-destructive detection (fluorescent indicators in the plates, examination under UV lamp).

1.3.3 Characterization of Isolated Compounds

The main objectives of the extraction and isolation of natural products is to identify, characterize and elucidate the conclusive structure of the isolated compounds. There are many useful spectroscopic methods of getting information about chemical structures that can be utilized such as ultraviolet-visible (UV-Vis), infrared (IR), mass spectrometry (MS) and nuclear magnetic resonance (NMR). It is easy to compare preliminary spectroscopic data with literature data or direct comparison with the standard sample if the isolated compound is a known constituent. However, comprehensive and systematic approaches involving a variety of chemical, physical, and spectroscopic techniques is required if the compounds is an unknown constituent.

Briefly, the data collected from each spectroscopy techniques provide useful explanation about the characteristic of particular compound. In the UV-Vis spectroscopy analysis, information on chromophores present in the molecules can be obtained. For example, some natural products like coumarins, flavonoids and isoquinoline alkaloids can be primarily characterized (chemical class) from characteristic absorption peaks. However, UV-Vis spectra are not very specific; they are at best indicative. Different functional groups present in a molecule such as –OH, C=C, aromaticity and so on can be determined using analysis data from IR spectra. Like a fingerprint, no two unique molecular structures produce the same infrared spectrum (Coates, 2000).

By analyzing the MS spectrum, information about the molecular formula, molecular mass and mass fragmentation pattern can be obtained. Electron impact mass spectrometry (EIMS) is the technique that commonly use in MS. Generally, the samples injected are converted into gaseous ions and then separated on the basis of their mass-to-charge ratio (Raaman, 2008b). The MS provides additional data for the identification of the separated compounds. The data obtained will be compared with the MS library and/or standard compound if available (Hu et al., 2006).

NMR spectroscopy is one of the important and advanced spectroscopic methods available to chemists for determination of the detailed chemical structure of the compounds they were isolating from natural sources. It reveals information on the number and types of protons and carbons present in the molecule and the relationship among these atoms (van de Ven, 1995). Two major categories in NMR spectroscopy are one-dimensional techniques (1D-NMR) for simple molecules and two-dimensional techniques (2D-NMR) for more complicated molecules. 1D-NMR includes proton NMR (^{1}H) and carbon NMR (^{13}C). Homonuclear correlated spectroscopy (COSY) as well as Nuclear Overhauser Enhancement SpectroscopY (NOESY) and Heteronuclear Multiple Quantum Correlation (HMQC) as well as Heteronuclear Multiple Bond Correlation (HMBC) are the examples of 2D-NMR.

1.4 Method Development and Validation

The objective of an analytical measurement can be qualitative or quantitative (De Smet et al., 1997). Qualitative analysis is utilized to provide basic information about the composition of sample. Simple chemical reactions can be conducted to identify the analytes in the samples. In contrast, quantitative analysis provides

information not only about composition but also about the concentration of the specific analytes present in the sample and often require more complex analytical techniques to obtain accurate and reliable information of the sample. The main analytical phases are method development, validation and method application. The vast development of analytical methods among the global community has marked acceptance internationally where various analytical methods such as GC-MS, HPLC, LCMS and so on are suitable for the analysis of compound(s) of interest (Liang et al., 2004). Assessment and optimization of the different steps in sample preparation, chromatographic separation and quantification is necessary in method development.

Before a new analytical method or sample preparation technique is to be implemented, it must be validated. The objective of validation of an analytical method is to demonstrate that the method is suitable for the intended use. According to the guidelines proposed by International Conference on Harmonization (ICH), the validation characteristic that should be considered includes linearity, range, limit of detection and limit of quantification, precision, accuracy and robustness (ICH, 2005). In general, method validation provides a comprehensive data or information about the parameters involve in a new method and a basis for comparison with existing methods.

1.4.1 Validation Characteristic

Method validation has received considerable attention in the literature and from regulatory agencies and industrial committees. For example the United States Pharmacopeia (USP) develops methodology for specific applications and general chapters on different analytical aspects of Food and Drug Administration (FDA)

regulated industry. Two general chapters regarding method validation is provided by USP and another one with information on allowed method changes without the need for revalidation (USP, 2009). The extent of guidelines for validation requirements provided by different organizations may vary, but the objective of validation is always to achieve valid analytical test results to ensure the quality and safety of products that are measured. In this thesis, the validation was performed according to the ICH Guidelines (ICH, 2005). Typical validation characteristics that should be taken into consideration are listed below:

1.4.1.1 Linearity and Calibration Curve

ICH defines linearity of an analytical procedure is its ability to obtain test results (within a given range) which are directly proportional to the amount (concentration) of analyte in the sample. This can be evaluated by visual inspection of a plot of signals as a function of analyte concentration or content. Often, a calibration curve will be constructed that shows the relation between analyte concentration in the sample and the detected response. To define adequately the relationship between response and concentration, a sufficient number of calibration points (n=6) is crucial (Shah et al., 1992). It is a standard practice in an analysis that a standard curve should be able to cover the entire range of the concentration of the unknown samples. A graph is plotted using the relative responses (y-axis) and the corresponding concentrations (x-axis). The calibration equation is as follows:

$$Y = mX + C \quad \text{……. (Equation 1)}$$

The slope of the regression line (m), y-intercept (C) and correlation coefficient value is established. The regression line data may be helpful to provide mathematical estimates of the degree of linearity. A correlation coefficient of > 0.999 is considered as evidence of an acceptable fit of the data to the regression line (Green, 1996).

1.4.1.2 Limit of Detection and Limit of Quantification

Based on the definition in ICH guideline, limit of detection (LOD) is the lowest amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. In contrast, limit of quantification (LOQ) is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. To determine the LOD and LOQ, three approaches are given in the guideline which is based on the visual evaluation, signal to noise ratio and standard deviation of the response and the slope. In this thesis, visual approach together with signal to noise ratio is used. Signal to noise ratio of approximately 2:1 or 3:1 and 10:1 were used in estimating LOD and LOQ, respectively.

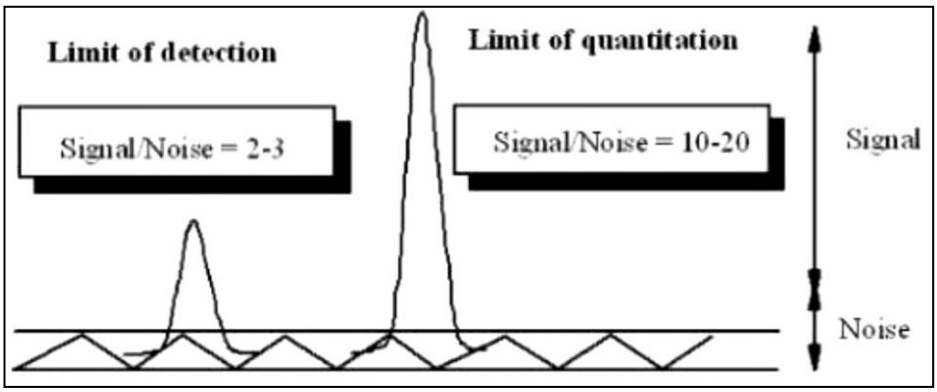


Figure 1.3 Limit of detection and limit of quantification via signal to noise ratio (ICH, 1996b).

1.4.1.3 Precision

Precision of an analytical procedure is defined as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Three levels of precision may be considered which are repeatability, intermediate precision and reproducibility. Intra-assay precision (repeatability) expresses the precision under the same operating conditions over a short interval of time. Intermediate precision on the other hand deals with variations within laboratories, such as different analysts, different days, different equipment, and so on while reproducibility expresses the precision between laboratories.

The result is expressed as the relative standard deviation (RSD) or percentage coefficient of variance (% CV) of the replicate measurement (Causon, 1997). A minimum of three concentrations with three replicates per concentration is recommended in the range of expected concentrations. The intra-day (repeatability) and inter-day (intermediate) precision should fulfill the requirement where % RSD should not exceed 15% (20% for LLOQ) (FDA guidelines, 2001).

1.4.1.4 Accuracy

The accuracy of an analytical method is the extent to which test results generated by the method and the true value agree. Results for accuracy are best reported as percentage bias (% Bias).

% Bias = (measured value – true value / true value) x 100 ..... (Equation 2)

A minimum of three concentrations with three replicates per concentration is recommended in the range of expected concentrations. Furthermore, accuracy also will be examined by using standard addition method for recovery studies where known but varying amount of analytes is introduced in the sample. This study is usually performed at three concentrations which are low, medium and high (Bressolle et al., 1996). Results are expressed as percentage of recovery (% recovery).

1.4.1.5 Robustness

ICH defines the robustness as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters. It provides an indication of the procedure's reliability during normal usage. Robustness may be evaluated during the development phase and depends on the type of procedure under study. In the case of gas-chromatography, typical variations are temperature, different columns (different lots and/or suppliers) and flow rate.

1.5 Standardization of Extract

World Health Organization (WHO) estimate about 80% of the world population are still using herbs and other traditional medicines for their primary health care needs. Herbal formulations as therapeutic agents have reached widespread acceptability for cough remedies, liver diseases, diabetics, adoptogens and memory enhancers (Patel et al., 2006). Herbal medicine products are dietary supplements to improve the health of the people who are taking it. These products are available in the form of fresh or dried plants, extracts, powders, capsules, tablets and teas. With the tremendous growth of herbal extracts, the health authorities and

the public are concern on the quality control of herbal extracts used for treatment (Fan et al., 2006). There is a need to control and assure the quality of such preparations through systematic scientific studies including chemical standardization, biological assays and validated clinical trials (Firenzuoli and Gori, 2007).

Nowadays, herbal extracts formulations faces high challenges due to the lack of standardization (Palav et al., 2006). Given the nature of plant origin based products that are not usually constant and are dependent on and influenced by many factors, the survival of the industry are much depends on ensuring consistent quality of products (Bauer, 1998). The European Agency for the Evaluation of Medicinal Products (EMEA) and The United States Food and Drug Administration (USFDA) have drafted guidelines on various aspects of quality control of medicinal plants such as identification, assay of active ingredients, water content, heavy metal and inorganic impurities, microbial limits, pesticides, and so on for validation and standardization of herbal preparations (EMEA, 2001; USFDA 2000). Amongst these, the chemical standardization with respect to its major constituent (active compounds) has emerged as the most sought after parameter.

In herbal formulation, the therapeutic activity of a product is depending on the phytochemical constituents present. The development of authentic analytical methods which can reliably profile the phytochemical composition, including qualitative and quantitative analysis of bioactive compounds (marker) as well as other major constituents is crucial. Thus, standardization process is needed for the establishment of a consistent chemical profile, a consistent biological activity or simply a quality assurance program for production and manufacturing of an herbal

drug (Patra et al., 2010). Standardization can be simplified as a system that ensures a predefined amount of quantity, quality and therapeutic effect of ingredients in each dose (Zafar et al., 2005). In this thesis, an effort is made to achieve the standardization purposes by conducting the phytochemical standardization. It encompasses all possible information generated with regard to the chemical constituents present in an herbal drug / extract. This includes evaluation on:

- Preliminary testing for the presence of different chemical groups.
- Quantification of chemical groups of interest (e.g., total phenol, total alkaloid, total tannin, total flavonoid).
- Establishment of fingerprint profiles based on single or multiple marker fingerprints.
- Quantification of important chemical constituents.

1.6 Chromatogram Fingerprinting

Significant expansion of the use of herbal medicine as an alternative to modern medicine require a high standard of quality control in assessing the active components in raw plant materials to guarantee their identity, consistency and authenticity (WHO, 2000). In the quality control of traditional medicine, a few markers or pharmacologically active compounds are used as standards. However, there are tens of unknown components exist within the same herbal material and are often present in low amounts (Bauer, 1998; Tyler, 1999). The variety of the chemical components may vary depending on several factors such as plant origin, drying processes, harvest seasons and other factors (Mahady et al., 2001). Thus, it is impossible to isolate and purify the whole of phytochemical constituents from herbal medicines (Bauer, 1998; Tyler, 1999; Liang et al., 2004). Furthermore, identifying

only one or several compounds hardly describes the complex extracts. The result might not be reliable enough for the quality control of these extracts. Moreover, extracts of different species from a given genus, e.g. *Curcuma*, can have a very different composition.

Recently, the chromatographic fingerprint technique was introduced as a more meaningful quality control method of herbal samples or their derived products to evaluate its consistency and quality (Gu et al., 2004; Zhao et al., 2005; Alaerts et al., 2007). Chromatographic fingerprint provide the chemical information of medicines with chromatograms and other graphs by analytical techniques (Gan and Ye, 2006). It can be used to characterize both the marker compounds and the unknown components in a complex mixture (complete sample composition). WHO has acknowledged the usage of chromatographic fingerprint as a strategy for the quality assessment of herbal medicines (WHO, 2000). Up to now, varieties of chromatographic techniques involving fingerprint include TLC, HPLC (Pietta et al., 1991; Hasler et al., 1992), GC (Aichholz, and Lorbeer, 2000), and CE (Ji et al., 2006). The chromatographic techniques enable rapid, reproducible and efficient semi quantitative and quantitative analysis of the chemical constituents in complex mixtures (Rajani and Kanaki, 2008).

1.7 Qualitative and Quantitative Analysis of Secondary Metabolites

Plants are biosynthetic producer of chemical substances such as primary and secondary metabolites (Nyiredy et al., 2004). Primary metabolites include lipids, simple sugars, amino acids and nucleic acids which are necessary for cellular process are found in all plant species (Wink, 2006). On the other hand, secondary metabolites