STUDY ON A CHINESE HERB FLOS MAGNOLIAE (XIN YI) – IDENTIFICATION AND PHARMACOLOGICAL ACTIONS

Ye Shen

(**Doctor of Philosophy**)

Division of Chinese Medicine

School of Health Sciences

RMIT University

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to quality for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Ye Shen _____

Date _____

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Abbreviations

5-HT	5-hydroxytryptamine
AA	Atomic Absorption
AC	Affinity chromatography
ADC	Automated developing chamber
AFLP	Amplified fragment length polymorphism
AMD	Automated multiple development system
ANOVA	Analysis of variance
AN	Arylnaphthalene
APC	Adsorption chromatography
AP-PCR	Arbitrarily-primed PCR
AR	Allergic rhinitis
As	Arsenic
AT	Aryltetralin
BK	Bradykinin
bp	Base pair
BSA	Bovine serum albumin
$CaCl_2$	Calcium chloride
CAM	Complementary and alternative medicine
CCD	Change couple device
Cd	Cadmium
cDNA	Chromosomal DNA
CE	Capillary electrophoresis
CGRP	Calcitonin gene-related peptide
CHM	Chinese Herbal Medicine

CN	Cyanoprophyl
CO_2	Carbon dioxide
COX	Cyclooxygenase
cpDNA	Chloroplast DNA
DAD	Diode array detector
DALP	Direct amplification of length polymorphism
DB	Dibenzylbutane
DBL	Dibenzylbutyrolactone
DBLE	Dibenzylbutyrolactol
DCO	Dibenzocyclooctadiene
DID	Discharge ionization detector
DNA	Deoxyribose nucleic acid
dNTP	2'-deoxyribonucleotide-5' triphosphates
ECD	Electron capture detector
ECL	Enterochromaffin-like
EDTA	Ethylenediaminetetraacetic acid
EICD	Hall Electrolytic conductivity detector
ELSD	Low temperature evaporative light scattering detector
EMEA	European Agency for the Evaluation of Medicinal Products
EPA	United States Environmental Protection Agency
EtBr	Ethidium bromide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FF	Furfurans
FID	Flame ionization detector
FPD	Flame photometric detector

FR	Furan
FTIR	Fourier transform IR spectrometer
GAP	Good agricultural practice
GC	Gas chromatography
GC-MS	Gas chromatography - mass spectrometry
GLC	Gas liquid chromatography
GMP	Good manufacturing practice
GPC	Gel permeation chromatography
GSC	Gas solid chromatography
HEPES	N-2-hydroxyehtylpiperazine-N'-ethanesulfonic acid
Hg	Mercury
HID	Helium ionization detector
HPLC	High performance liquid chromatography
HPTLC	High performance TLC
IEC	Ion-exchange chromatography
IFN	Interferon
IgE	Imunoglobulin E
IL	Interleukin
IL-1α	Interleukin - 1-alpha
iNOS	Inducible nitric oxide synthase
IR	Infrared
ITS	Internal transcribed spacers
KCl	Potassium chloride
KFDA	Korean Food and Drug Administration
LC	Liquid chromatography
LPS	Lipopolysaccharide

LT	Leukotrienes
MEGA 2	Molecular Evolutionary Genetics Analysis 2
$MgCl_2$	Magnesium chloride
MS	Mass spectrometry
MSD	Mass selective detector
MTB	Magnesium tanshinoate B
NaCl	Sodium chloride
NaH_2PO_4	Di-sodium hydrogen orthophosphate
NaOH	Sodium hydroxide
NCCAM	National Center for Complementary and Alternative Medicine
NGF	Nerve growth factor
NH_2	Aminoprophyl
NICPBP	National Institution for the Control of Pharmaceutical and Biological Products of China
NIR	Near IR
NIST	National Institute of Standard and Technology
NIH	National Institute of Health
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NOS	Nitric oxide synthase
NPC	Normal-phase chromatography
NPD	Nitrogen phosphorus detector
nrDNA	Nuclear ribosomal DNA
oligo	Oligonucleotide
OPA	o-phthalaldehide
ORP	Overlapping ratio of peak

PAF	Platelet-activating factor
Pb	Lead
PC	Partitioning chromatography
PCR	Polymerase chain reaction
PCR-RFLP	PCR-restriction fragment length polymorphism
PDD	Pulsed discharge detector
PG	Prostaglandin
PGE2	Prostaglandin E2
PID	Photoionization detector
R.S.D.	Relative standard deviation
RAPD	Randomly amplified polymorphic DNA
rbcL	Ribulose-1,5-bisphosphate carboxylase L
RBG	Royal Botanic Garden
rDNA	Ribosomal DNA or Sequences encoding rRNA
RID	Refractive index detector
RPC	Reversed-phase chromatography
RPMC	Rat peritoneal mast cell
rRNA	Ribosomal ribonucleic acid
SD	Sprague Dawley
S.D.	Standard deviation
S.E.M.	Standard error of measurement
SCCO2	Supercritical carbon dioxide
SCF	Supercritical fluids
SEC	Size-exclusion chromatography
SFC	Supercritical fluid chromatography
SPME	Solid phase microextraction

SSR	Simple sequence repeat
TCD	Thermal conductivity detector
TGA	Therapeutic Goods Administration
THM	Traditional herbal medicine
Tl	Thallium
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor-α
*trn*K	Transfer RNA for lysine
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultraviolet
WHM	Western herbal medicine
WHO	World Health Organization

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Summary

Flos Magnoliae, one of the commonly used Chinese herbal medicines (CHM), has a long history of clinical application for rhinitis, sinusitis and headache. More than 20 different Flos Magnoliae species can be found in the market. Thus, a systematic evaluation of the authentic species and quality assessment of Flos Magnoliae from different botanic sources could be a complicated process. The major aim of this thesis was to study the molecular, chemical and pharmacological profiles of different Flos Magnoliae species and varieties, as well as different Flos Magnoliae products. The main approaches included establishment of DNA and chromatographic fingerprinting profiles, determination of the contents of magnolin and fargesin, and comparison of the anti-histamine release effects of different Flos Magnoliae samples.

Firstly, DNA-based techniques were used to identify the genetic relationships between six *Magnolia spp.* The distinct genetic distances of their molecular profiles from Flos Magnoliae materials were demonstrated after amplification by random amplified polymorphic DNA (RAPD). The present study using ten random primers and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis by 5s-rRNA gene after digestion by restriction enzymes (*Hha*I, *Pst*I, *Sma*I, and *EcoR*V) represents the first report of DNA fingerprinting profiles of these Flos Magnoliae species.

Secondly, the optimised thin layer chromatography (TLC) approach is rapid, specific and simultaneous for qualitatively analysis of magnolin and fargesin in ethanol extracts of different Flos Magnoliae species and varieties. All *M. biondii* batches and *M. kobus* were found to contain magnolin and fargesin. Magnolin, but not fargesin was detected from *M. liliflora*. No magnolin and fargesin were found in other Flos Magnoliae species.

Thirdly, bioactive components, magnolin and fargesin were quantitatively determined by the newly developed high performance liquid chromatography (HPLC) method. By choosing an appropriate gradient elution system of acetonitrile and water with the UV detection wavelength at 278nm, magnolin and fargesin were baseline separated without interference peaks in the HPLC chromatograms. By using this method, magnolin was detected from the ethanol extracts of *M. biondii, M. kobus* and *M. liliflora*, while fargesin was only found from the ethanol extracts of *M. biondii* and *M. kobus*. Furthermore, the significant differences of the content of magnolin and fargesin were observed in samples from different Flos Magnoliae sources.

Fourthly, the HPLC fingerprinting profiles of different Flos Magnoliae sources were studied for the first time. Various parameters, including the retention time, the relative retention time, the peak area, the relative peak area, the total peak area of 13 common peaks and the overlapping ratio were evaluated. The consistency of the fingerprints was observed among *M. biondii* batches, whilst the chromatographic profiles among the other Flos Magnoliae species were distinguished. In addition, the Flos Magnoliae products, which were collected from different herbal suppliers, and the *M. biondii* batches, which were grown in different cultivation sites, were clearly identified and authenticated by their HPLC fingerprinting features.

Finally, the effects of different samples on mast cell derived histamine release induced by compound 48/80 in rat peritoneal mast cells (RPMC) were studied, using HPLC with post-derivatization. The significant differences in inhibition of histamine release were observed among various species or even among the same species but different varieties. Moreover, the inhibitory effects of the Flos Magnoliae sources from different herbal suppliers and cultivation sites on compound 48/80 induced histamine release were evaluated. To the best

knowledge, it is the first report of the anti-histamine release effects by the bioactive lignan, magnolin, and volatile oil from *M. biondii*.

In conclusion, the present study verified that the variations of botanic species and varieties, cultivation environments, and manufacturer process might cause the differences of the chemical compositions and pharmacological actions of Flos Magnoliae products. Thus, in order to insuring the quality, efficacy and consistency of Flos Magnoliae, proper quality control of Flos Magnoliae species is important. Furthermore, local grown Flos Magnoliae species may be used for anti-allergic and anti-inflammatory applications. For the future direction, it would have been interesting to characterise the chemical structures of these components, which may contribute to the anti-allergic effects of Flos Magnoliae, if it had not been for time constraints. Even though the inhibitory effects of mast cells derived histamine release were demonstrated in the present study, assays on mediators involved in the anti-allergic actions may provide additional evidences on the clinical application of Flos Magnoliae for rhinitis and sinusitis. Further to that, assays on anti-allergic effects of the major chemical compounds from Flos Magnoliae on these mediators may apply chances to discover new therapeutic agents.

Chapter One

General Introduction of Flos Magnoliae and Quality Control of Chinese Herbal Medicine

1.1 Introduction of Flos Magnoliae

Flos Magnoliae (Chinese name: Xin-yi) is one of the most commonly used Chinese medicinal herb, and has a long history of clinical application for rhinitis, sinusitis and headache (The Pharmacopoeia Commission of People's Republic of China, 2005, Bensky and Gamble, 1993). Few species from genus *Magnolia* (Family Magnoliaceae) have been considered as the original sourcE for medicinal Flos Magnoliae (Fu, 2000). The medicinal use of Flos Magnoliae was originally recorded in <Shennong's Classic of Materia Medica> about two thousand years ago (Yu et al., 1999). Owing to its versatile therapeutic properties, Flos Magnoliae has been widely used in modern practice of herbal medicines.

Recently, with the revival interested in Chinese herbal medicines (CHM), Flos Magnoliae and it is related therapeutic products have captured a considerable proportion of the global market. Owing to the significant therapeutic effects, the market for medicinal plants, including Flos Magnolia is ever-growing

1.1.1 The Genus *Magnolia*

Magnoliaceae, as one of the primitive flowering plants family, includes more than 200 species of well-defined trees and shrubs, which plays the key role for the researches of angiosperm phylogeny (Kim et al., 2001). Magnoliaceae family was natively appeared in Asia until the 18th century when it spread from the Himalayas to Japan and from North-eastern America to Central America and finally reached in Venezuela (Hutchinson, 1973, Pei and Chen, 1991) (Figure 1.1). Most of the plants from Magnoliaceae distribute in tropic and subtropic regions of the Northern Hemisphere, for instance in South-east Asia and America. The rest of the family reaches to the Southern Hemisphere (Wang et al., 2004b). Subsequently, it has been planted throughout the world, including Australia.

1.1.1.1 Taxonomy of Magnoliaceae

As the most primitive living family of flowering plants, the Magnoliaceae is an exclusive family diverse from any other plant families (Pei and Chen, 1991). There are 14 genera, forming three tribes in the Magnoliaceae (Pei and Chen, 1991). However, the taxonomy classification of the Magnoliaceae is still arguable (Table 1.1).

The *Magnolia spp*., considered as the medicinal sources for Flos Magnoliae, includes the largest genera, *Magnolia* (over 30 species), which is common to Asia and America. Other genera include the genera with terminal flowers, such as *Manglietia*, *Manglietiastrum, Talauma, Parakmeria, Kmeria, Alcimandra* and *Liridendron,* and as well as the genera with axillary flowers, such as *Michelia, Paramichelia* and *Tsoongiodendron.*

Among the species from genus *Magnolia*, more than 20 *Magnolia spp.* have been used in the commercial products or CHM practice as the sourcing of Flos Magnoliae. These species are located in the section *Yulania*, section *Buergeria*, section *Tulipastrum* and section *Theorhodon*

(Pei and Chen, 1991, Magnolia Society, 2003). Based on the Chinese Pharmacopoeia (Pharmacopoeia Commission of People's Republic of China, 2000), three species, namely, *M. biondii, M. denudata* and *M. sprengeri* are listed under the pharmaceutical name Flos Magnoliae. However, there are more than 20 different *Magnolia* species have been used as substitutes or adulterants (Fu, 2000, Zhao and Cao, 1990, Li et al., 1994a). It has been suggested that *M. sargentiana* (Sichuan Province, China) (State Administration of Traditional Chinese Medicine "Chinese Materia Medica" Editorial Committee, 1998), *M. liliflora* (Shanxi Province, China) (Zhao and Cao, 1990), *M. cylindrica* (Zhejiang Province, China) (Li et al., 1994a), *M. campbellii* (Yunnan Province, China) (Fu, 2000), *M. pilocarpa* (Hubei and Anhui Province, China) (Zhao et al., 1987), *M. wilsonii* (Sichuan and Yunnan Province, China) (Fu, 2000) and *M. kobus* (Japan) (PFAF, 2000) can be used as Flos Magnoliae in CHM practice. Furthermore, in Australia, *M. liliflora,* and *M. salicifolia* have been included as the 'listed' medicines by Therapeutic Goods Administration (TGA) (TGA, 2004).

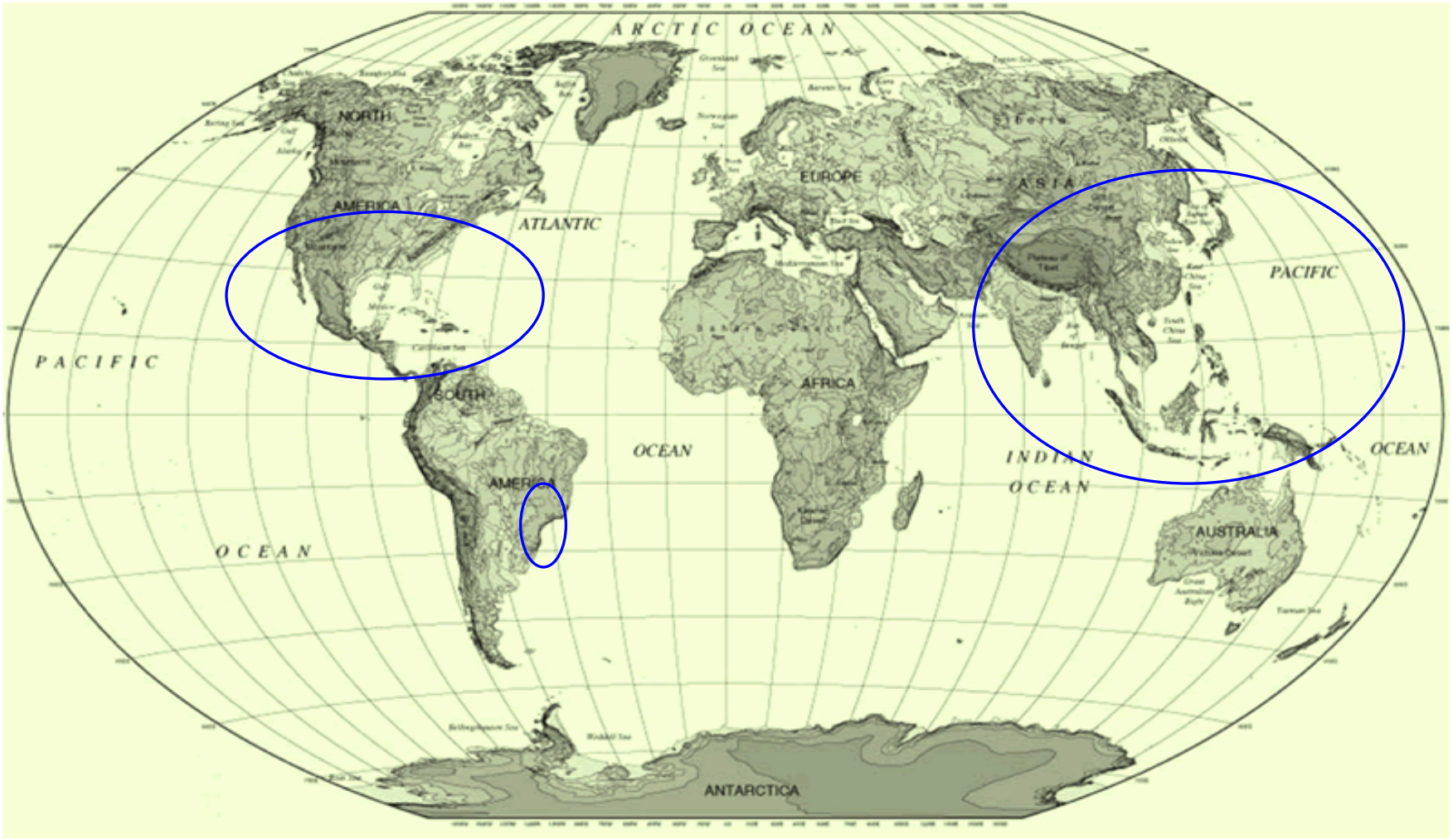


Figure 1.1 The distribution patterns of Family Magnoliaceae (Hutchinson, 1973, Pei and Chen, 1991). The blue circles indicate the approximate distribution ranges of *Magnolia spp.*

Table 1.1 Classification of the Magnoliaceae. Specified taxa, which have been considered as the botanic sources of Flos Magnoliae, are shown in the red characters.

Pei & Chen (Pei and Chen, 1991)	**Magnolia Society** (Magnolia Society, 2003)	**NCBI Taxonomy Database** (Bischoff et al., 2004)
Family: Magnoliaceae	**Family:** Magnoliaceae	**Family:** Magnoliaceae
Tribe: *Magnolieae*	**Subfamily:** Magnolioideae	
Genus: *Manglietia*		**Genus:** *Dugandiodendron*
Genus: *Manglietiastrum*		**Genus:** *Kmeris*
Genus: *Talauma,*		**Genus:** *Liriodendron*
Genus: *Parakmeria,*		**Genus:** *Manglietia*
Genus: *Kmeria*	**Genus:** *Pachylarnax*	**Genus:** *Michelia*
Genus: *Alcimandra*		**Genus:** *Pachylarnax*
Genus: *Michelia*		**Genus**: *Talauma*
Genus: *Paramichelia*		
Genus: *Tsoongiodendron*		
Genus: *Liriodendron*		
Genus: *Magnolia*	**Genus:** *Magnolia*	
Subgenus: *Magnolia*	**Subgenus:** *Magnolia*	
Subgenus: *Yulania*	**Subgenus:** *Yulania*	
Section: *Yulania*	**Section:** *Yulania*	**Genus:** *Magnolia*
M. campbellii	M. denudata	*M. biondii*
M. sprengeri	*M. campbellii*	*M. campbellii*
M. sargentiana	*M. sprengeri*	*M. denudata*
M. denudata	*M. sargentiana*	*M. kobus*
M. salicifolia	*M. salicifolia*	*M. liliflora*
Section: *Buergeria*	**Section:** *Buergeria*	*M. sargentiana*
M. biondii	*M. biondii*	*M. sprengeri*
Section: ***Tulipastrum***	*M. kobus*	*M. salicifolia*
M. liliflora	Section: ***Tulipastrum***	
Section: ***Theorhodon***	M. liliflora	
M. grandilora		

1.1.1.2 Distribution of Flos Magnoliae species used in this thesis

1.1.1.2.1 Flos Magnoliae species in China

M. biondii, which is the most commonly used Flos Magnoliae species in native of central China, but cultivated in many regions of the country, such as Gansu Province, Shanxi Province, western Henan Province, western Hubei Province and eastern Sichuan Province (State Administration of Traditional Chinese Medicine "Chinese Materia Medica" Editorial Committee, 1998, Fu, 2000). *M. denudata* has been cultivated for both medicinal and ornamental uses in China. It is believed to have originated in eastern China, and the wild species are only seen in the mountain regions, such as Anhui Province, Fujian Province, Guangdong Province, Guizhou Province, Hunan Province, Jiangsu Province and Zhejiang Province (Fu, 2000, State Administration of Traditional Chinese Medicine "Chinese Materia Medica" Editorial Committee, 1998, Pei and Chen, 1991). The home of *M. sprengeri* is in western China, with a range extending from western Hubei Province, western Henan Province, eastern Sichuan Province, Guizhou Province and Yunnan Province in China (Pei and Chen, 1991, Fu, 2000). As one of the similar taxa of *M. sprengeri*, *M. sargentiana* is also widely cultivated in central-southern Sichuan Province and northern Yunnan Province (Fu, 2000). *M. liliflora* is commonly seen in the region of south of Yangtze River in China (Pei and Chen, 1991). It is now widely cultivated in central and eastern China, such as Shanxi Province, Fujian Province and Sichuan Province (Fu, 2000). *M. campbellii*, as one of the rarest species, is cultivated ranged from the eastern Himalayas to Tibet and Yunnan Province, China (Pei and Chen, 1991).

1.1.1.2.2 Flos Magnoliae species in other countries

M. kobus replaces *M. biondii* as the authentic Flos Magnoliae species in Japan and Korea and widely distributes in southern Japan and on the coast of southern Korea (Table 5.1) (PFAF,

2000). Meanwhile, commonly used Flos Magnoliae species, including *M. liliflora* and *M. denudata*, have been cultivated in Japan and Korea (Pei and Chen, 1991).

As the native plants of United States, the species that are recognised as the botanic origins of Flos Magnoliae are widely cultivated around the country (Bailey and Bailey, 1976). *M. campbellii, M. denudata, M. kobus, M. liliflora, M. sargentiana* and *M. sprengeri* have been found in United States (Bailey and Bailey, 1976).

Although *Magnolia spp*. are not the native plants in Australia, the family has been imported and widely cultivated by local nurseries (Australian National Botanic Gardens, 2004, Australian Nurseries Online, 2006). Furthermore, *M. liliflora* has been included as the 'listed' medicine in TGA, Australia (TGA, 2004).

1.1.1.3 Genetic features of *Magnolia spp*.

Over the last two decades, with the development of molecular cloning and polymerase chain reaction (PCR) techniques, deoxyribose nucleic acid (DNA) based markers have been well established for the evaluation of taxonomic relationship and genetic mapping of plants. These techniques have been commonly used to identify and authenticate the species of medicinal materials (Joshi et al., 2004). It has been applied to validate reference strains of *Magnolia spp*. The sequence of chloroplast *mat*K gene has been introduced to screen different *Magnolia* species from East Asia, China and America (Wang et al., 2004d, Azuma et al., 2001). The findings support the phylogenetic relationship between different species from various genera based on their morphological and fossil evidence, and also generate the evolutionary history of Family Magnoliaceae by disjunction of more than 200 species (Azuma et al., 2001). Kim *et al* (2001) reported that the Chloroplast *ndhF* gene was employed to reveal the taxonomic relationship between different sections and genera. Meanwhile, the genomic outcomes have identified some diverse lineages according to certain taxa position, such as redefining the

polyphyletic genus *Magnolia*, section *Rytidospermum* and subgenus *Talauma*, and further recognising the relationships between *Michelia* and subgenus *Magnolia*.

As a simple and efficient molecular detection method, random amplified polymorphic DNA (RAPD) has been used to generate the genetic distances of medicinal plant of Flos Magnoliae. Wang *et al* (2004c) used the RAPD technique to revise the phenotypic relationship between 20 taxa from subgenus *Yulania*, by screening with fifteen 10-mer random primers. By combining the morphological observation and RAPD identification, the close relationship between those taxa were established. Further study also observed the significant genetic distance between the species derived from Asia and the species from America among 17 taxa of subgenus *Yulania* using RAPD analysis (Wang et al., 2004e).

1.1.2 Flos Magnoliae and traditional Chinese medicine

Clinical usage of Flos Magnoliae has a long history in traditional CHM and has been traced back to the Han Dynasty. The therapeutic functions of Flos Magnoliae were recorded in the oldest and comprehensive medical literatures, <Shen Nong Ben Cao Jing>, the earliest Chinese Materia Medica of the first century BC, which was preceded by a long verbal history of the application of Flos Magnoliae in ancient China (Yu et al., 1999). From the CHM perspective, it has a wind-dispelling property, pungent flavour and warm nature, and it enters the lung and stomach meridians (Bensky and Gamble, 1993). Based on the traditional Chinese medicine theory, pungent and warm properties of herbs have the effects to release the exterior disorders, which are defined as mild fever, severe chills, accompanied by headache, body and neck pains, and absence of thirst (Bensky and Gamble, 1993). Flos Magnoliae has the property of expelling wind cold from the *Five Zangs* (5 viscera) of the body. It can also relieve headache. Prolonged intake would descend the *Qi*, reduce body weight, brighten the eyes, as well as increase longevity. Since then, Flos Magnoliae has been appeared in most of

the classic CHM literatures. A summary of the appearances of Flos Magnoliae in the classic CHM literatures is presented in Table 1.2.

Modern scientific research has found that Flos Magnoliae is a key herb for symptomatic management of allergic rhinitis, sinusitis and headache (The Pharmacopoeia Commission of People's Republic of China, 2005). It has various pharmacological actions (see Section 1.1.5). In Japan, Flos Magnoliae is regarded as an important folk herb for headache treatment (PFAF, 2000). In Korean, Flos Magnoliae has been successfully demonstrated for the management of rheumatic arthritis (Kim et al., 2003).

Most of the *Magnolia spp.* have economic values. In addition to the Flos Magnoliae species, *M. officinalis* and *M. officinalis* var. *biloba* have also been introduced as another commonly used CHM, Cortex Magnoliae Officinalis (Ho-pu) (The Pharmacopoeia Commission of People's Republic of China, 2005). Furthermore, almost all the *Magnolia spp*. are valuable ornamental plants (Hutchinson, 1973). The species, which have the relatively high quality, are recognised as furniture timbers (Fu, 2000). Besides the above-mentioned economic importance, the essential oil of *Magnolia spp.* is also used as natural resource of stacte, spice, flavouring essence, food and cosmetics for a long history (Fu, 2000).

Table 1.2 Documentation of Flos Magnoliae in classic CHM literatures

Book Title (In Chinese)	Book Title (In English)	Author	Era
神农本草经	Shennong's Classic of Materia Medica	Anonymous	220 – 225 B.C
本草经集注	Collection of Commentaries on the Classic of Materia Medica	Tao, Hong Jing	502 – 536 A.D.
雷公炮炙论	Leigong's Discussion of Processing of Materia Medica	Lei, Xiao	420 – 479 A. D.
千金翼方	A Supplement to Recipes Worth A Thousand Gold	Sun, Si Miao	682 A.D.
本草图经	Illustrated classic of Materia Medica	Su, Song	1061 A.D.
证类本草	Classified Materia Medica	Tang, Shen Wei	1108 A.D.
本草衍义	Extension of Materia Medica	Kou, Zong Shuang	1116 A.D.
本草纲目	Compendium of Materia Medica	Li, Shi Zhen	1596 A.D.
本草备要	Essential of Materia Medica	Wang, Ang	1694 A.D.
本草从新	Thoroughly Revised Materia Medica	Wu, Yi Luo	1757 A.D.

1.1.3 Botanic features of Magnolia spp.

1.1.3.1 *Magnolia spp.*

Magnolia, trees or shrubs, is the most well known genus of Magnoliaceae family. Its leaves are evergreen and are simple, stipules large, deciduous and leaving an annular scar on the shoot, enclosing the young buds. Early in the spring, large flowers cover the tree before new leaves appear. The plants are solitary terminal or axillary. Flowers are usually unisexual; sepals and petals are frequently similar, in several series and imbricate; stamens numerous and opening lengthwise; carpels usually numerous; in fruit circumscissile and woody (Hutchinson, 1973). The images of different *Magnolia spp.*, which have been used as the sources of medicinal Flos Magnoliae illustrated in Figure 1.2.

1.1.3.2 Medicinal Flos Magnoliae

1.1.3.2.1 Morphological features

Based on the classification of genus *Magnolia* by Magnolia Society (Magnolia Society, 2003), the botanical origins of Flos Magnoliae belong to the genus *Magnolia*, subgenus *Yulania*, section *Yulania, Buergeria,* and *Tulipastrum*. Differences of their distributions and the appearances of the flowers from these sections show in Table 1.3. Table 1.4 further describes the distributions and habits of the Flos Magnoliae species, which were selected for the present study. The buds are collected in late winter and early spring before blooming, and dried in the shade (Fu, 2000). The buds of Flos Magnoliae are long and ovoid, having the shape of a writing brush, and texture light and delicate; odour, aromatic; taste bitter (State Administration of Traditional Chinese Medicine "Chinese Materia Medica" Editorial Committee, 1998) (Figure 1.3). Morphological identification shows considerable difference between different species of *Magnolia* (Table 1.5). Some morphological differences, including the shapes and sizes of the buds, and numbers of the segments, were distinguished between different Flos Magnoliae species, including *M. biondii, M. denudata, M. sprengeri,*

M. soulangeana, M. liliflora, M. cylindrica, M. steboidii, M. sinostellata M. amoena, and *M. elliptillimba* (Li et al., 1994b, Huang, 1990, State Administration of Traditional Chinese Medicine "Chinese Materia Medica" Editorial Committee, 1998).

M. denudata

M. sprengeri

M. liliflora

M. kobus

Figure 1.2 Images illustrating four medicinal Flos Magnoliae species

Table 1.3 Key to three sections of genus *Yulania*, which has been recognised as the sourcing of medicinal Flos Magnoliae (Pei and Chen, 1991, Hutchinson, 1973)

Section	**Distribution**	**Tree**	**Flower appurtenance**	**Colour of flowers**	Sepals and petals
Yulania	East and West China, Himalayas	Deciduous tree	Before new leaves appear	White, pink or purple	Similar
Buergeria	East China Japan and Korea	Deciduous shrub or tree	Before new leaves appear	Pink	Dissimilar
Tulipastrum	China and America	Deciduous shrub	Before new leaves appear or when leafing	Red or purple	Similar

Table 1.4 Taxonomy, distributions and habits of the Flos Magnoliae species, selected in this thesis

Taxon			Distribution	Habit	
Botanic name	Chinese name	Synonym		Type	Mature height (m)
M. biondii	望春玉兰	*M. fargesii*	Temperate Asia and China	Deciduous tree	6-12
M. campbellii	滇藏木兰	*M. mollicomata*	Temperate and tropical Asia, and China	Deciduous tree	24-30
		M. conspicua			
M. denudata	玉兰	*M. yulan*	Temperate Asia and China	Deciduous tree	15-22
		M. heptapeta			
M. kobus	日本辛夷	*M. praecocissima*	Temperate Asia	Deciduous tree	4-6
M. liliflora	紫玉兰	*M. quinquepeta*	Temperate Asia and China	Deciduous shrub	1-3
M. sargentiana	凹叶木兰		Temperate Asia and China	Deciduous tree	8-20
M. sprengeri	武当木兰	*M. diva*	Temperate Asia and China	Deciduous tree	10-20

M. biondii

M. sprengeri

M. sargentiana

Figure 1.3 Images illustrating the dried buds of three Flos Magnoliae species

Table 1.5 Morphological differences between the medicinal buds of different Flos Magnoliae species (Han, 2005, Li et al., 1994b, Li et al., 1994a, The Pharmacopoeia Commission of People's Republic of China, 2005).

Name of species	Shape	Pedicle	Lenticels	Perianth-segments	Outer and inner whorls	Small scaly buds between 2 layers of bracts
M. biondii	Long ovoid	Stouter	Whitish	9	Polygeneous	Yes
M. denudata	Long ovoid	Stouter	Brownish	9	Monogeneous	No
M. sprengeri	Long ovoid	Stouter	Red brownish	10-12-15	Monogeneous	No
M. soulangeana	Ovoid	Short, with grey-whitish short tomenta	Light brownish	6-9	Polygeneous	No
M. liliflora	Long ovoid	Short, with whitish or yellowish short tomenta	Grey-whitish	9	Polygeneous	Yes
M. cylindrica	Long ovoid or ovoid	Short, with light yellowish short tomenta	Grey-yellowish	9	Polygeneous	No
M. steboidii	Ovoid	Short, with yellowish short tomenta	Grey-whitish	9	Monogeneous	No
M. sinostellata	Long ovoid or ovoid	Short, with yellowish short tomenta	Grey-yellowish	12-18	Monogeneous	Yes
M. amoena	Long ovoid	Short, with grey-whitish short tomenta	Grey-yellowish	9	Monogeneous	No

1.1.3.2.2 Histological features

The macroscopic form of the transverse sections of Flos Magnoliae shows one layer of epidermal cells. Cortex is relatively broad and vascular bundles are scattered. Phloem bundles are wide and xylem bundles are relatively narrow. Numerous stone cells and oil cells can be found in cortex and pith (Huang, 1990). A recent study identified the microscopic structures of the cuticle at the corolla surface of four Flos Magnoliae species, including *M. biondii, M. denudata, M. sprengeri* and *M. liliflora* (Tang, 2006).

The macroscopic form of the powder of Flos Magnoliae has been listed in the Chinese Pharmacopoeia, as one of the major identification approaches in the monograph. Greyish-green or yellowish-green powder has abundant non-glandular hairs, which are scattered and regularly broken. The inflated image of the basal cells looks like stone cells. The grouped stone cells are elliptical, with obliterated pit canals. The brownish-yellow secretion appears in the stone cells. There are some oil droplets in the numerous oil cells. The anticlinal wall of the epidermal cells is bead (The Pharmacopoeia Commission of People's Republic of China, 2005).

1.1.4 Phytochemistry of Flos Magnoliae

Efficient detection and characterisation of the components from Flos Magnoliae are important in understanding of chemical and pharmacological basis of Flos Magnoliae. Various analytical techniques, such as thin liquid chromatography (TLC), high performance liquid chromatography (HPLC), gas chromatography (GC), mass spectrometry (MS), and nuclear magnetic resonance (NMR) have been used in studying the chemical components of Flos Magnoliae (Su et al., 2001, Xu et al., 2003, Ma and Han, 1995, Yang et al., 1998).

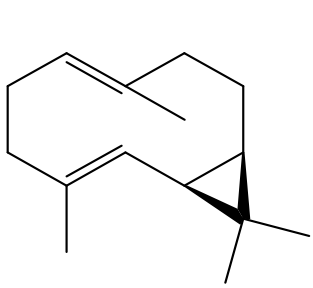
The major components of Flos Magnoliae can be grouped into two classes: lipid soluble and water soluble (Yang and Zhang, 1998). The lipid soluble, normally obtained by extraction with alcohol solvents, is rich in volatile oil and lignans (Yang and Zhang, 1998). In recent years, the water-soluble components of Flos Magnoliae, alkaloids and flavonoids, have also attracted some attentions (Chen et al., 1994b, Gao et al., 1994).

1.1.4.1 Lipid-soluble components

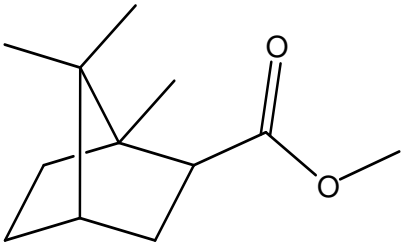
1.1.4.1.1 Volatile oil

Volatile oils are typically very complex mixtures and contain many classes of compounds, e.g. hydrocarbons, alcohols, aldehydes, ketones, or esters. The authentic Flos Magnoliae has been suggested containing more than 1% of volatile oil (The Pharmacopoeia Commission of People's Republic of China, 2005). The principle constituents of the volatile oil are bicyclogermacrene, bornyl acetate, calarene, camphene, camphor, caryophyllene, cineol, citral, citronellol, cymene, diethyl phthalate, elemol, eudesmol, farnesol, geraniol, limonene, linalool, methyl eugenol, methyl heptenone, muurolene, myrcene, nerolidol, pinene, sabinene, terpinene, tert-butylbenzene, and torreyol, which consist of more than 93% of the total volatile compositions (Chen et al., 1994a) (Figure 1.4).

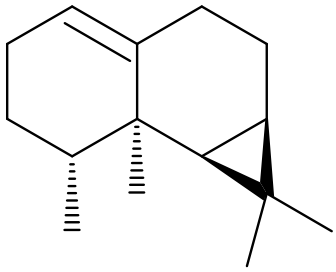
The yield of volatile oil varies with the species, growing locations and collecting seasons (Hu and Wu, 1995). Mass spectrometry, coupled with gas chromatography (GC-MS), has been demonstrated to be a valuable analytical technique for characterisation of volatile oil from Flos Magnoliae (Table 1.6). Quantitative evaluation of volatile ingredients in different species from *Magnolia spp.* have been intensively studied (Nagasawa et al., 1969). The routine methods of steam distillation and solvent extraction have been applied to its volatile oil analysis. In addition, a supercritical fluids (SCF) method has been employed to extract the volatile oil compounds from two Flos Magnoliae species, *M. biondii* and *M. liliflora*, by supercritical carbon dioxide ($SCCO_2$) (Zhang, 1999, Zhang et al., 2005b). The result showed that extraction rate of $SCCO_2$ flow was 3.8 - 4.2%, which was more than extraction by steam distillation (2.4%). At the same time, 54 components from the $SCCO_2$ extract of *M. biondii* were determined in comparison with the volatile oil by steam distillation extract, which contained 34 volatile compounds (Zhang, 1999). Differences of the main compounds from volatile oil from *M. liliflora* extracted by different methods have also been demonstrated (Table 1.7) (Zhang et al., 2005b). Previous research found that five Flos Magnoliae species, namely, *M. biondii, M. denudata, M. cylindrical, M. amoena* and *M. liliflora* have significant difference in volatile compositions, *M. denudate* contained highest content of volatile oil of 4.5%, whereas *M. liliflora* was least (1.2%) (Hu and Wu, 1995, Yang et al., 1998). Meanwhile, the analysis of three species, listed in the Chinese Pharmacopoeia, found that *M. biondii, M. denudata* and *M. sprengeri* contained 91, 71 and 88 volatile components, respectively, and the contents of the major components were varying (Table 1.8) (Yang et al., 1998).



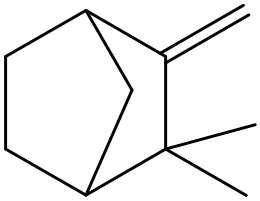
Bicyclogermacrene



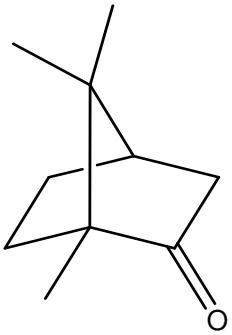
Bornyl acetate



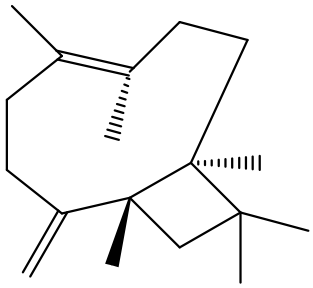
Calarene



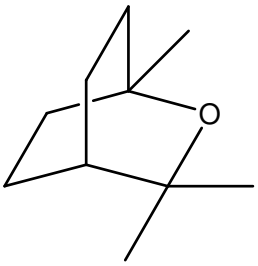
Camphene



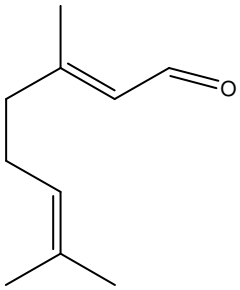
Camphor



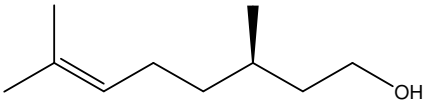
Caryophyllene



1,8-cineole

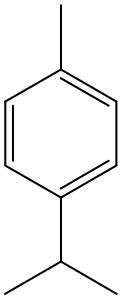


Citral

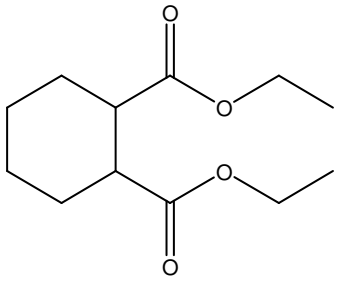


Citronelleol

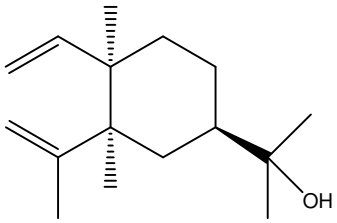
Figure 1.4 Chemical structures of some identified volatile components from Flos Magnoliae



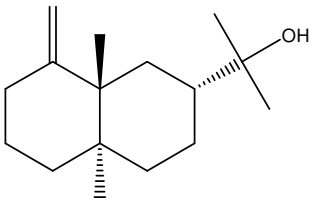
Cymene



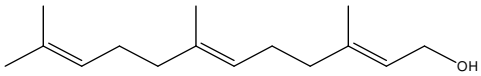
Diethyl phthalate



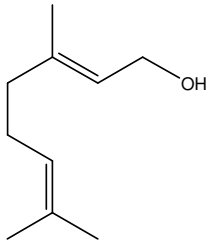
Elemol



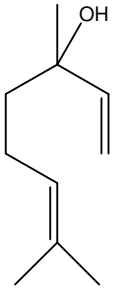
Eudesmol



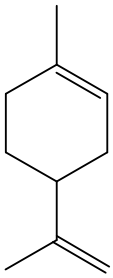
Farnesol



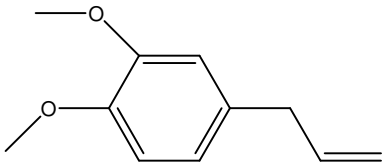
Geraniol



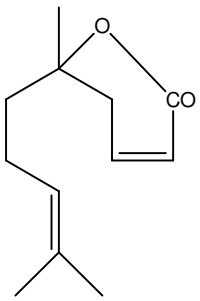
Linalool



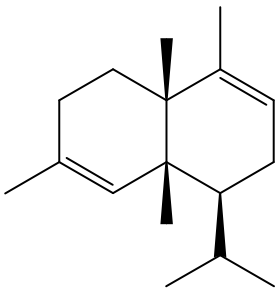
Limonene



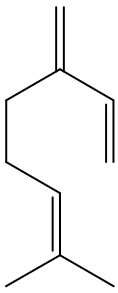
Methyl eugenol



Methyl heptenone

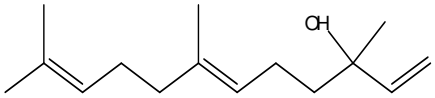


Muurolene

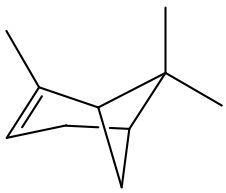


Myrcene

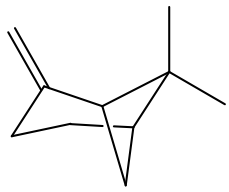
Continued Figure 1.4 Chemical structures of some identified volatile components from Flos Magnoliae



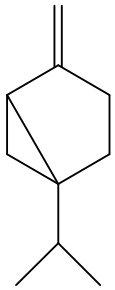
Nerolidol



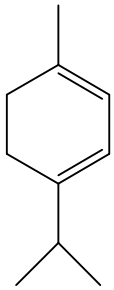
α-pinene



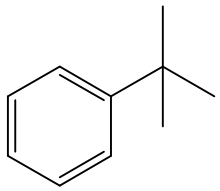
β-pinene



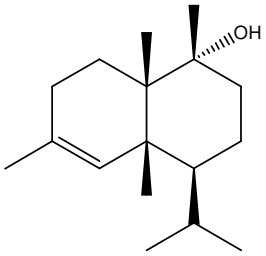
Sabinene



Terpinene



Tert-butylbenzene



Torreyol

Continued Figure 1.4 Chemical structures of some identified volatile components from Flos Magnoliae

Table 1.6 Different GC-MS systems for analysis of the volatile components from Flos Magnoliae

		Zhang *et al.* (1999)	Wei *et al.* (2000)	Yang *et al.* (1998)	Ma *et al.* (2005)
GC	Model	PH-5890	QP-5000	Pye-204	HP-6890
	Column	HP1 (SE-30) 60m×0.2mm	DB-1 30m×0.25mm	SE-54 30m×0.32mm	HP-1NNoωax 25m×0.2mm×0.25μm
	Temperature program	Rise from initial 60 °C (held for 1min) to 240 °C at 8°C/min (held for15min). Auxiliary oven：230 °C	Rise from initial 60 °C to 120 °C at 3°C/min, rise to 160 °C at 1.5°C/min, rise to 250 °C at 8°C/min, Auxiliary oven：230 °C	Rise from initial 50°C to 210 °C at 4°C/min	Rise from initial 60 °C to 80°C at 5°C/min (held for 5min), decrease to 60 °C at 5°C/min (held for 10min), rise to 230 °C at 10°C/min. Auxiliary oven：230 °C
	Carrier gas	He	He	He	He
	Pressure (kPa)	120	80	78.4	-
	Inject (μl)	0.2	1.0	0.2	0.2 - 0.4
	Distribution	10:1	10:1	40:1	80:1

Continued Table 1.6 Different GC-MS systems for analysis of volatile components from Flos Magnoliae

		Zhang *et al.* (1999)	Wei *et al.* (2000)	Yang *et al.* (1998)	Ma *et al.* (2005)
MS	Model	PH-5972	QP-5000	MM-7070H	PH-5973
	Interface temperature	280 °C	-	-	240 °C
	Electron Energy (eV)	70	70	70	70
	Scope (u)	12-550	40-400	20-350	20-4000
	Scanning speed	550 u/s	-	1 s/dec	-
Major findings		64 volatile compounds were identified.	33 volatile compounds were identified.	91 volatile compounds were identified.	80 volatile compounds were identified.

Table 1.7 Comparison of the content of the major volatile components from *M. liliflora* using GC-MS

Retention time (min)	Compound	Relative content (%)	
		$SCCO_2$	Steam distillation
5.14	α-pinene	4.03	3.26
6.14	Sabinene	7.61	7.62
6.23	β-pinene	6.37	6.65
7.49	1,8-cineole	17.88	17.13
10.15	Camphor	4.75	4.71
11.25	α-terpineol	3.55	3.74
16.31	β-caryophyllene	2.03	1.50
22.22	Farnesol	15.99	21.11

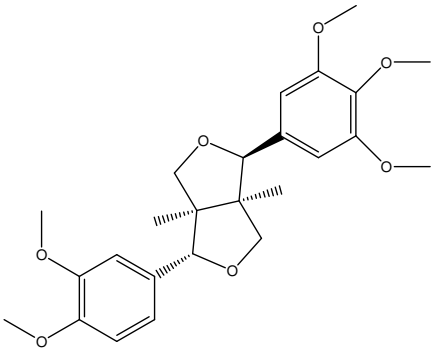
Table 1.8 Quantitative analysis of the total volatile oil and four volatile components from different Flos Magnoliae species using GS-MS

Name of species	Total volatile oil content (%)	Relative content (%)				References
		α-pinene	β-pinene	Camphene	1,8-cineole	
M. biondii	2.15	4.02	7.71	2.25	18.39	
M. sprengeri	1.70	9.65	13.62	0.99	0.24	(Yang et al., 1998)
M. denudata	2.60	4.50	11.77	0.78	15.00	
M. liliflora	1.2	-	2.02	-	13.39	
M. cylindrica	1.6	8.16	33.45	1.60	7.96	(Hu and Wu, 1995)
M. amoena	2.3	6.37	10.97	2.13	25.35	

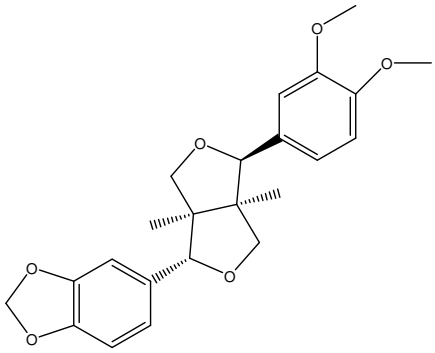
1.1.4.1.2 Lignans and neolignans

Lignans and neolignans are optically active dimeric natural products formed essentially by the union of two phenylpropane (C_6-C_3) units, and widely distributed in the plant kingdom (Moss, 2000). Lignans and neolignans have been considered as one of the major secondary metabolites found in genus *Magnolia* (Kakisawa, 1972). Over the last two decades, the methods of separation and isolation of lignans and neolignans from different Flos Magnoliae species have been established using different techniques, including TLC (Su et al., 2001), ultraviolet (UV) spectrometry (Ma and Han, 1995), HPLC (Fang et al., 2002) and NMR (Mitsuo et al., 1992a). Various lignans and neolignans, including fargesin, demethoxyaschantin, aschantin, pinoresinol dimethyl ether, magnolin, lirioresinol-β-dimerthyl ether, fargesone A, fargesone B, denudatin B, magnosalin and magnoshinin have been identified (Jung et al., 1998, Kobayashi et al., 1996b, Kobayashi et al., 1998, Mitsuo et al., 1992a, Mitsuo et al., 1996, Pan et al., 1987). The chemical structures of some lignans and neolignans are shown in Figure 1.5.

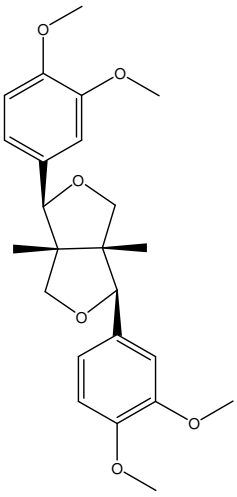
Recently, the bioactive compound, magnolin has been employed as a marker compounds for quality control of Flos Magnoliae using TLC and HPLC (The Pharmacopoeia Commission of People's Republic of China, 2005). The quantitative levels of magnolin and fargesin from three Flos Magnoliae sources have been analysed using TLC and HPLC as well (Xu et al., 2003, Su et al., 2001, Fang et al., 2002). Magnolin and fargesin from *M. biondii, M. denudata* and *M. sprengeri* were determined by a HPLC method using C_{18} column and with the mobile phase consisted with acetonitrile and water (50:50, %-w:w) (Table 1.9) (Xu et al., 2003). Moreover, there are significant differences of the compositions of lignans and neolignans in different Flos Magnoliae species (Table 1.10).



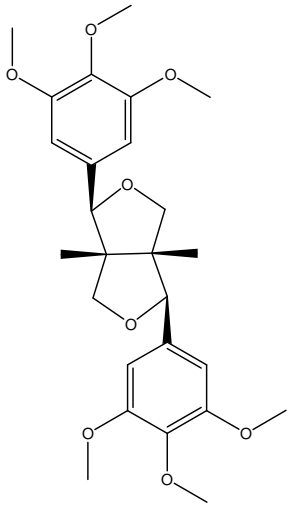
Magnolin



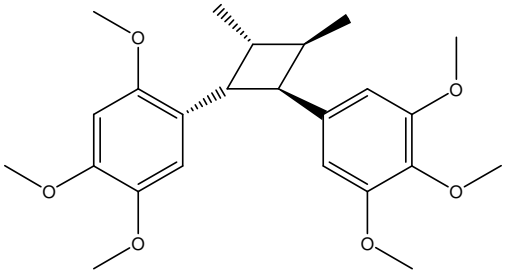
Fargesin



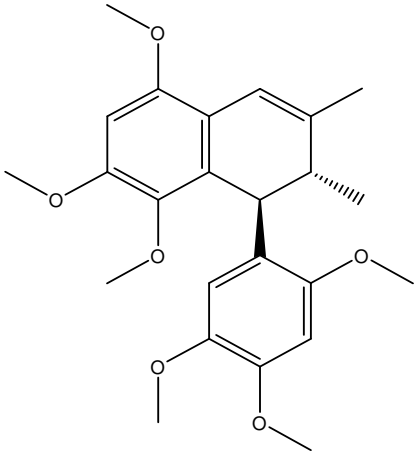
Pinoresinol dimethyl ether



Lirioresinol-β-dimerthyl ether

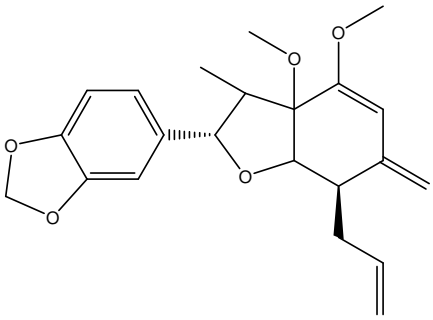


Magnosalin

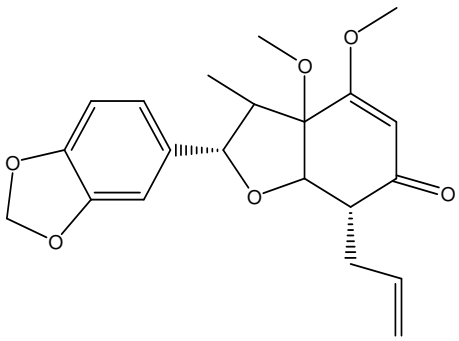


Magnoshinin

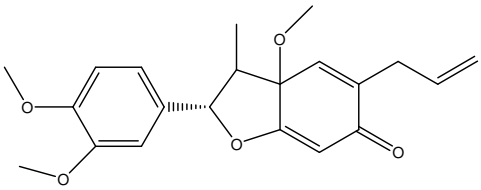
Figure 1.5 Chemical structures of some identified lignans and neolignans from Flos Magnoliae



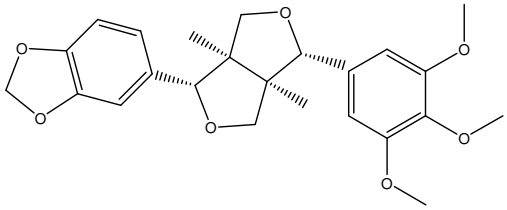
Fargesone A



Fargesone B



Denudation B



Aschantin

Continued Figure 1.5 Chemical structures of some identified lignans and neolignans from Flos Magnoliae

Table 1.9 Quantitative analysis of magnolin and fargesin from three Flos Magnoliae species using HPLC (Xu et al., 2003).

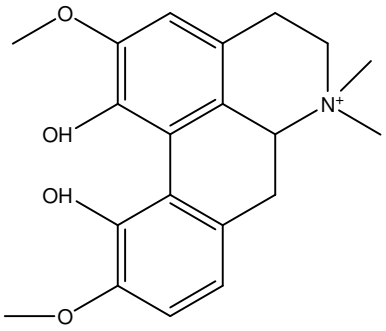
Name of species	Replicates	Magnolin (%)	Fargesin (%)
M. biondii	5 batches	1.26 ~ 1.77	0.078~0.129
M. denudata	2 batches	0.007 ~ 0.024	—
M. sprengeri	1 batch	0.016	—

Table 1.10 Identified lignans and neolignans from different Flos Magnoliae species

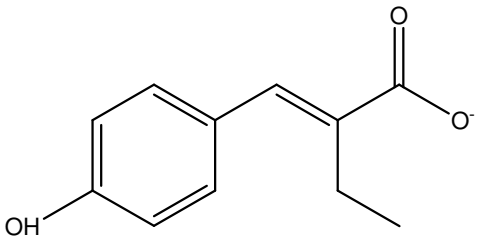
Name of species	Compounds	References
M. biondii	Biondinin B Biondinin E Pinoresinol dimethyl ether Fargesin Lirioresinol-β-dimerthyl ether Magnolin Demethoxyaschantin Fargesone A Fargesone B	(Ma et al., 1996)
M. denudata	Licarin A Denudatin B	(Iida et al., 1982, Kwon et al., 1999)
M. liliflora	Denudatin A Denudatin B	(Iida and Ito, 1983)
M. salicifolia	Magnosalin Magnoshinin	(Kimura et al., 1992)
M. soulangeana	Demethoxyaschantin Cyclohexadienone Denudatin A Denudatin B Saulangianin I Pinoresinol dimethyl ether	(Abdallah, 1993)

1.1.4.2 Water-soluble components

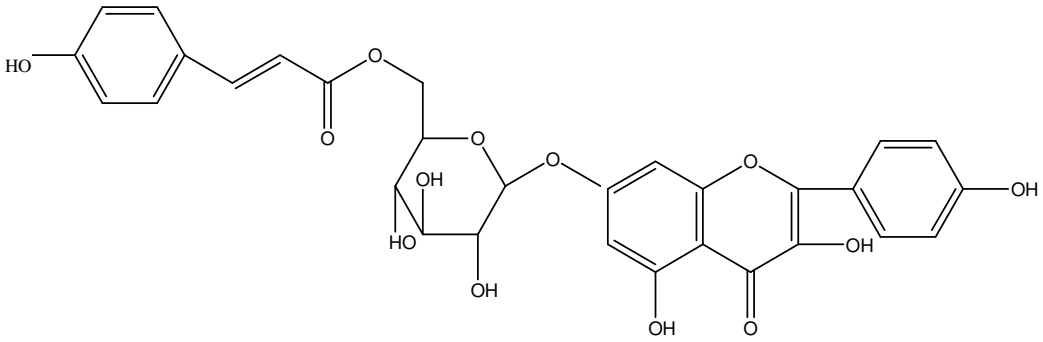
Three water-soluble compounds from aqueous extraction of Flos Magnoliae, including magnoflorine, biondnoid and ethyl-E-p-hydroxyl-cinnamate, have been isolated and their structures have been identified (Gao et al., 1994, Chen et al., 1994b) (Figure 1.6). However, the bioactivities of these compounds are still unclear.



Magnoflorine



ethyl-E-*P*-hydroxylcinnamate



Biondnoid I

Figure 1.6 Chemical structures of some identified water-soluble components from Flos Magnoliae

1.1.5 Pharmacological aspects of Flos Magnoliae

The pharmacological actions of Flos Magnoliae have been extensively studied (Yang and Zhang, 1998). The main pharmacological actions of Flos Magnoliae include anti-inflammation, anti-allergy, anti-angiogenesis, anti-proliferation, anti-hypertension, Ca^{2+} antagonist and anti-microbe activities (Bensky and Gamble, 1993).

1.1.5.1 Anti-inflammation and anti-allergy

The anti-inflammatory effects of Flos Magnoliae have been demonstrated by various *in vivo* and *in vitro* studies to be related to cellular events and inflammatory mediators during the acute or chronic inflammatory responses (Kim et al., 2003, Kim et al., 1999, Kimura et al., 1992, Kobayashi et al., 1998).

In vivo studies

Several acute inflammatory animal models have been used for determination of the anti-inflammatory or anti-allergic effects of Flos Magnoliae, including ear-swelling response induced by 2,4-dimethylphenol or compound 48/80 in $WBB6F_1$ +/+ mice (Wang et al., 2000b, Kim et al., 1999), and anti-dinitrophenyl IgE antibody-induced passive cutaneous anaphylaxis (Kim et al., 1999). Functional study showed the remarkable inhibitions by Flos Magnoliae of the capillary permeability of blood vessels and degranulation of mast cells (Kim et al., 1999). Liang and Yang (2005) reported that Flos Magnoliae has the astringent action and capillary dilation, which protected the mucosal surface and improved local blood circulation resulting in the suppression of inflammation, when injected to chronic maxillary sinusitis rabbits. The anti-inflammatory effects of *M. biondii* on Freund's adjuvant induced primary foot tumefaction on Wistar rats was studied (Wang et al., 2004a).

The active ingredients, magnoshinin and magnosalin have been shown to significantly inhibit croton oil-induced pouch granuloma formation and pouch exudation (Kimura et al., 1990, Kimura et al., 1991). Kimura *et al* (1990) suggesting a mechanism of selective inhibition of angiogenesis by magnosalin may be involved in the anti-inflammatory effects of Flos Magnoliae. Magnosalin and magnoshinin have also been demonstrated to induce fetal bovine serum (FBS) - stimulated and interleukin (IL)-1-alaph (IL-1α) - stimulated tube formations, which resulted in inhibition of endothelial cell proliferation (Kobayashi et al., 1996a, Kimura et al., 1992). These findings suggested that magnosalin and magnoshinin might have potential inhibitory effects on chronic inflammation.

In vitro studies

The anti-inflammatory effects of Flos Magnoliae are likely to be due to its effects on the secretion of the inflammatory and allergic mediators. Histamine is one of the most potent mediators that contributes to many processes of inflammation and hypersensitivity (Rang et al., 2003). Recent studies found that the volatile oil from Flos Magnoliae inhibited the histamine release from mice's swelling feet, which may cause decrease of capillary permeability (Wang et al., 2000c, Wang et al., 2000b). Similarly, the volatile oil from Flos Magnoliae has been found to inhibit Prostaglandin E_2 (PGE_2) formation from rat peritoneal macrophages (Lim et al., 2002), and other inflammatory mediators, such as platelet-activating factor (PAF), precursor of PAF phospholipase A_2, IL-1α, nitric oxide (NO) and tumour necrosis factor-α (TNF-α) (Lim et al., 2002, Wang et al., 2002).

Lignans and neolignans, including magnolin, lirioresinol-β-dimerthyl ether, pinoresinol dimerthyl ether, fargesin, demethoxyaschantin and aschantin, isolated from *M. biondii*, have been demonstrated to have potent PAF antagonistic activities (Pan et al., 1987). In addition, pinoresinol dimerthyl ether, magnolin, lirioresinol-β-dimerthyl ether were demonstrated

having inhibitory effects on TNF-α production in lipopolysaccharide (LPS) – stimulated murine macrophage cell line (Chae et al., 1998)

A recent report from Korea suggested that the ethanol extraction of Flos Magnoliae provoked apoptosis of RBL-2H3 cells through mitochondria and caspase, which may also be associated with the anti-inflammatory effect of Flos Magnoliae (Kim et al., 2003).

1.1.5.2 Anti-microbial and anti-fungal actions

The decoctions of Flos Magnoliae inhibited many pathogenic fungi *in vitro*, which commonly cause dermatophytosis (Bensky and Gamble, 1993). In addition to the anti-fungal effects, one of the major Flos Magnoliae species, *M. biondii,* has been demonstrated to have potential anti-bacterial effects on *Staphylococcus aureus, Beta Streptococcus* and *Diplococcus pneumonia et al* (Wang and Li, 1999).

1.1.5.3 Other effects

Anti-hypertension

The intravenous, intraperitoneal or intramuscular injection of Flos Magnoliae aqueous extraction reduced blood pressure in anaesthetised animals (Bensky and Gamble, 1993). However, it had no significant hypotensive effect on secondary hypertension in anaesthetised animals (Bensky and Gamble, 1993).

Effects on the Uterus

The decoction of Flos Magnoliae could stimulate the uterus of rabbits and dogs (Bensky and Gamble, 1993).

1.1.5.4 Toxicology

There is little published toxicology data on Flos Magnoliae. Flos Magnoliae decoction administered intravenously in dog or rabbit in the doses of 1g/kg or 4.75g/kg, respectively, resulted in no mortality. The LC_{50} of ethanol extracts from two Flos Magnoliae varieties, which were from different locations of China (Henan or Sichuan Province), was 38.21g/kg or 93.55g/kg in mice by oral administration, respectively (State Administration of Traditional Chinese Medicine "Chinese Materia Medica" Editorial Committee, 1998).

For the subacute toxicological investigation, oral administration of 18g/kg (ethanol extraction) or 30g/kg (decoction) of Flos Magnoliae in rats for one month did not cause any haematological, clinical, chemical or histological alteration (State Administration of Traditional Chinese Medicine "Chinese Materia Medica" Editorial Committee, 1998).

1.1.5.5 Adverse effects

Reports of harmful effects of Flos Magnoliae are rare, although two cases in China, reported oedema and red wheals on the patients' skin after oral administration of Flos Magnoliae decoctions (Zhang and Zhu, 1996). Further research found that alkaloids, tubocurarine and associated substances in Flos Magnoliae, may cause respiratory paralysis on experimenting animals (Dharmananda, 2004).

1.1.6 Clinical applications

1.1.6.1 Single herb or phytochemical compounds

The volatile oil of Flos Magnoliae has been demonstrated to produce symptomatic relief of seasonal allergic rhinitis and chronic allergic rhinitis (Gu et al., 1999). However, there is no report on randomised clinical trials for Flos Magnoliae as a single herb or herbal preparation.

1.1.6.2 CHM formula

On the other hand, the therapeutic efficacy of the CHM formulas and the commercial therapeutic products, which contained Flos Magnoliae, have been elucidated in several clinical studies over last decade (Table 1.11 and Table 1.12) (Chen and Li, 1998, Gu et al., 1999, Zheng, 1996, Han, 1998, Luo, 2000, Xue et al., 2003b). Recently, a CHM formula, which contained Flos Magnoliae, has been demonstrated to produce symptomatic relief and improvement of quality of life for patients with hay fever in a randomised placebo controlled trial (Xue et al., 2003a).

Table 1.11 The compositions of the formulas and therapeutic products, which contained Flos Magnoliae, used for clinical application for rhinitis and sinusitis

Name of formula	Composition		Additives (% - w/v)	Indications	Reference
Xin-yi Nose Drop	Flos Magnoliae	60g	Tweenum-80 0.5%	Seasonal and persistent allergic rhinitis and sinusitis	(Luo, 2000)
	Herba Centipedae	180g			
	Herba Menthae	180g			
	Radix Scutellariae	60g			
	Glycerol	200ml			
Compound Xinyi Nasal Drops	Herba Houttuyniae	15g	Glycerol 1%	Seasonal and persistent allergic rhinitis	(Li et al., 2000c)
	Herba Centipedae	9g			
	Flos Magnoliae	9g			
	Herba Schizonepetae	3g			
	Radix Angelicae Sinensis	9g			
	Herba Menthae	6g			
	Fructus Xanthii	6g			
Xin-yi Volatile oil Injection	Volatile oil of Flos Magnoliae		Tweenum-80 0.5%	Seasonal allergic rhinitis	(Hu et al., 1998)

Continued Table 1.11 The compositions of the formulas and therapeutic products, which contained Flos Magnoliae, used for clinical application for rhinitis and sinusitis

Name of formula	Composition		Additives (% - w/v)	Indications	Reference
Xin-yi Cang-er Tang I	Flos Magnoliae	12g	-	Sinusitis	(Chen and Li, 1998)
	Fructus Xanthii	12g			
	Radix Angelicae Dahuricae	12g			
	Rhizoma Et Radix Ligustici	12g			
	Rhizoma Chuanxiong	12g			
	Radix Platycodonis	10g			
	Caulis Akebiae	10g			
	Radix Et Rhizoma Glycyrrhizae	6g			
Xin-yi Ping Feng San	Radix Astragali	240g	-	Persistent allergic rhinitis	(Gu et al., 1999)
	Rhizoma Atractylodis Macrocephalae	180g			
	Flos Magnoliae	120g			
	Fructus Xanthii	60g			
	Rhizome Chuanxiong	60g			

Table 1.12 A review of clinical studies of Flos Magnoliae for rhinitis or sinusitis

Study design	Patients conditions	Concentration of Flos Magnoliae	Main outcomes	References
Randomised placebo controlled trial	Seasonal allergic rhinitis (placebo 27 and treatment, 28)	2.25%	After 8 weeks treatment, 89% of the patients completed the trial. Significant improvements of nasal or non-nasal symptomatic relief and quality of life in the treatment group compare with the placebo group. Eleven patients reported mild adverse event.	(Xue et al., 2003b)
Case study (placebo and treatment comparison)	Rhinitis (placebo 20, treatment 128)	Nasal drop (12.5%)	After three months treatment, the significant improvement of nasal symptoms of 92.5% of 128 patients, compare to the placebo group.	(Luo, 2000)
Case study	Allergic rhinitis or chronic rhinitis	Volatile oil (single herb)	The nasal symptoms were relieved for 82% of 82 patient with allergic rhinitis and 66% of 18 patient with chronic rhinitis	(Gu et al., 1999)
Case Study	Rhinitis and sinusitis	Compound nasal drop (15.8%)	After two sessions of treatment, compound Xinyi nasal drop were improve 86.3% of the patients with rhinitis or sinusitis.	(Li et al., 2000c)
Case study	Acute and chronic sinusitis	13.5%	After three sessions of treatment, significantly inhibitory effects on nasal symptoms from more than 90% patients with sinusitis.	(Chen, 1998)

1.2 Quality assessment of Chinese herbal medicine (CHM)

1.2.1 Prevalence / popularity

1.2.1.1 Complementary and alternative medicine (CAM)

The term CAM, is often " used interchangeably with traditional medicine in some countries, it refers to a broad set of health care practices that are not part of that country's own tradition and are not integrated into the dominant health care system" by World Health Organization (WHO) (WHO, 2001, WHO, 2004a). The definition of CAM from National Centre for Complementary and Alternative Medicine (NCCAM), United States, is " a group of diverse medical and health care systems, practices and products that are not presently considered to be part of conventional medicine" (NCCAM, 2002). The major therapeutical approaches involved in CAM therapies are shown in Table 1.13.

In some Asian countries, CAM is the primary source of healthcare for up to 80% of the populations, such as China, North and South Korea and Viet Nam, and has been incorporated into the health system (Bagozzi, 1998). Meanwhile, CAM is also getting popular in the western countries in recent decades (Eisenberg et al., 1998, Eisenberg et al., 1993, Barnes et al., 2004, Thomas et al., 2003, Thomas et al., 2001, Yamashita et al., 2002). Recent surveys from United States in 1990 and 1997 found increased use of CAM therapies during the previous year from 33.8% to 42.1% (Eisenberg et al., 1998, Eisenberg et al., 1993). The national population health surveys in United States in 2002 found 62% of the interviewers had been used some forms of CAM (Barnes et al., 2004). Similar trends of the popularity of the use of CAM have been revealed in surveys from other countries, including United Kingdom (Thomas et al., 2003, Thomas et al., 2001), Canada (Ramsay et al., 1999) and Japan (Yamashita et al., 2002). In Australia, the population used the CAM in last 12 months, increased from 48.5% in 1993 (MacLennan et al., 1996) to approximately 52% in 2000 (MacLennan et al., 2002) and 2004 (MacLennan et al., 2006), based on the Omnibus Health

Surveys conducted in the state of South Australia. Recent study also found more than 75% of general practitioners had recommended their patients to a CAM therapist in the state of Western Australia in 1998 (Hall and Giles-Corti, 2000). Due to the considerable increase of CAM usage, the global market of CAM has gained dramatically. For instance, in United States, US$21.2 billion has been spent on the CAM related market in 1997 (Eisenberg et al., 1998). In Australia, the annual cost for CAM in 1993 was AU$621 millions (MacLennan et al., 1996), which has been increased to AU$2.3 billions in 2000 (MacLennan et al., 2002).

Table 1.13 The major therapeutic approaches of CAM (NCCAM, 2002).

Catalogues	Therapies and techniques
Alternative medical systems	❖ Homeopathic medicine ❖ Naturopathic medicine ❖ Traditional Chinese Medicine ➢ *Chinese herbal medicine (CHM)* ➢ *Acupuncture* ➢ *Remedial massage* ➢ *Qi gong* ❖ Ayurveda (Traditional Indian Medicine) ➢ *Diet and herbal remedies* ➢ *Yoga*
Mind-body interventions	❖ Patient support groups ❖ Cognitive-behavioural therapy
Dietary supplements	❖ Vitamins ❖ Minerals ❖ Herbs or other botanicals ❖ Amino acids ❖ Other natural products
Manipulative and body-based methods	❖ Chiropractic ❖ Osteopathic ❖ Massage

1.2.1.2 Herbal medicine (HM)

HM is one of the important therapeutical approaches of CAM. It includes western herbal medicine (WHM), Chinese herbal medicine (CHM), Ayurvedic and medicinal herbal materials and products from different countries (Wohlmuth et al., 2002). The application of HM has a long history in many eastern countries, such as China, India, Vietnam, Korea, and Japan, and it also gains popularity in many western countries, such as Australia, New Zealand and United States (MacLennan et al., 2006, Eisenberg et al., 1998, Barnes et al., 2004, Thomas et al., 2003, Thomas et al., 2001, Ramsay et al., 1999, Yamashita et al., 2002, Bagozzi, 1998). In Australia, a public survey found 26% of CAM users in South Australia in 1993 had been prescribed HM (MacLennan et al., 1996).

Western herbal medicine (WHM)

WHM, including botanical sources of popular western herbs, which contain active ingredients or herbal drug preparations, has been used in Europe, U.S.A., Australia and other counties (EMEA, 2001).

Chinese herbal medicine (CHM)

CHM, including Chinese herbal materials and products of Chinese patent herbal medicines, has been used in China for more than 2000 years (Bensky and Gamble, 1993). More than 5000 medicinal herbs have been documented (SATCM, 2000) and 992 herbs and herbal patent products are listed in the current Chinese Pharmacopoeia (The Pharmacopoeia Commission of People's Republic of China, 2005).

Global growing of HM

The global market of herbal medicines has increased dramatically, which is in the range of US$ 16-20 billion per year (Mahady, 2001). China, has a US$1.8 billion HM manufacturing

industry (Bagozzi, 1998). In UK, HM market represents an important share of the pharmaceutical market, with 57% of annual retails, which was in the range of £115 million in 2000 (Barnes, 2003a). In 2000, Korea exported HM approximately about US$7.4 million and imported US$80.6 million (Choi et al., 2002). Meanwhile, the HM market of Japan in 2000 is worth about US$2.4 billions (Bagozzi, 1998). With the significant increase in the use of HM, more countries have committed to develop better access to HM with improved quality and efficacy.

1.2.2 Complexity of CHM

Herbs, as used in CHM, include barks, duramen, flowers, flower-buds, fruits, leaves, petals, roots, root-barks, seed and stems (The Pharmacopoeia Commission of People's Republic of China, 2005). They can contain a single herb, but more commonly several herbs in order to achieve complementary effects (Liang et al., 2004). Current herbal products are existed as the forms of raw herbs or herbal extracts that are often maintained after extracted with water, alcohol or other different solvents to achieve better therapeutic effects (Lu, 2003). The complexity of raw herbs and herbal products often cause the difficulties to identify or determine the bioactive components for clinical application (Rousseaux and Schachter, 2003). Alkaloids, coumarins, flavonoids, lignans, neolignans, terpenoids, fatty acids, sterols, glycosides, saponins, tannins and terpenens have been found as the bioactive components of herbal products (State Administration of Traditional Chinese Medicine "Chinese Materia Medica" Editorial Committee, 1998). A number of intrinsic as well as extrinsic factors have significant effects on the botanical quality, in terms of the yields of bioactive components and potencies of bioactivities (Figure 1.7).

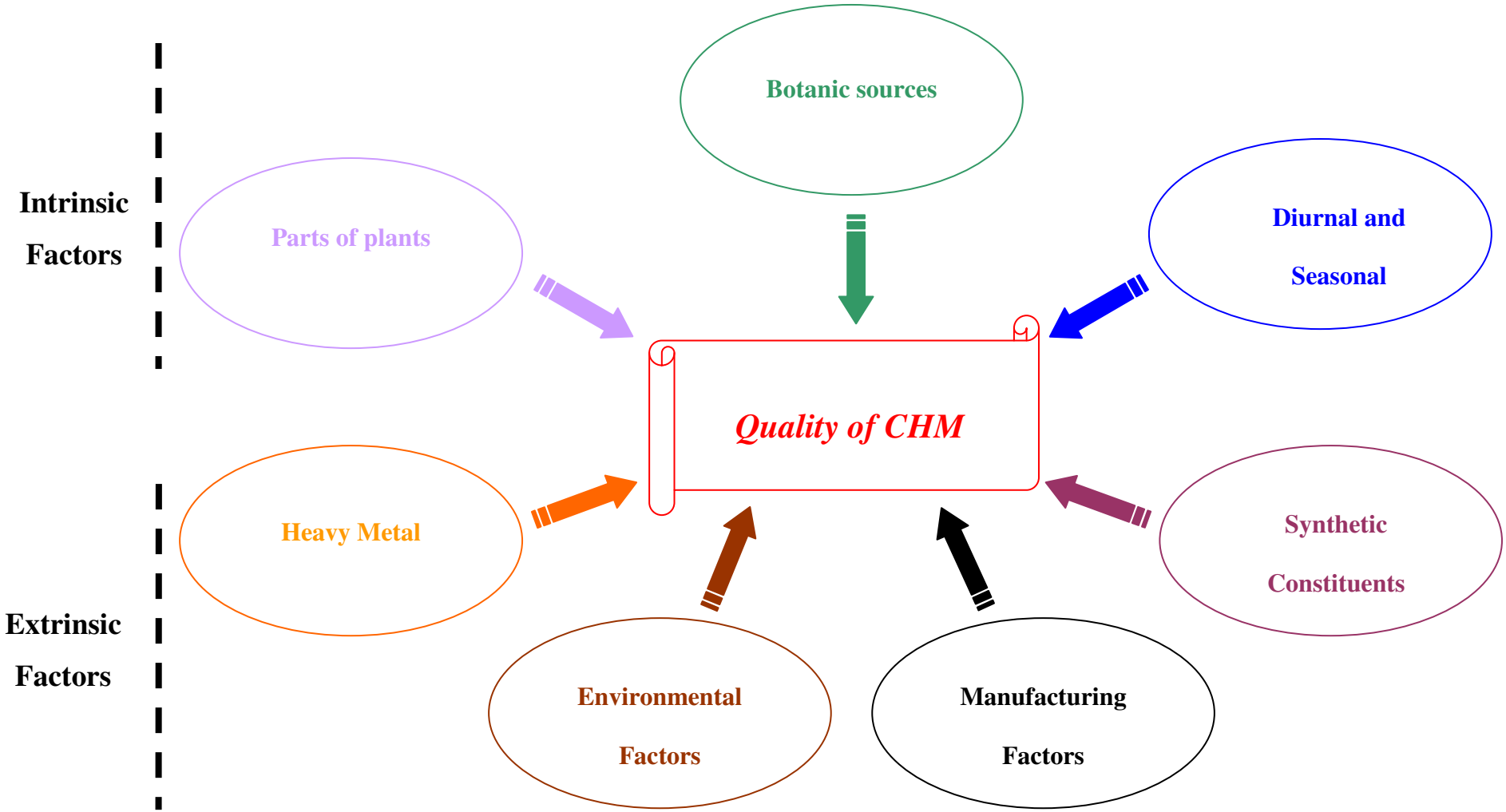


Figure 1.7 The factors may affect the quality of CHM

1.2.2.1 Botanic sources

1.2.2.1.1 Species and varieties differences

More than 800 raw herbs, which are derived from thousand of botanic sources, can be found in the global CHM market (Li, 1996). Based on the existing evidences from academic and authoritative references, 522 herbal products have been included the authentic species and listed in the Chinese Pharmacopoeia (The Pharmacopoeia Commission of People's Republic of China, 2005). Moreover, British Herbal Pharmacopoeia (BHMAPublications, 2000) and American Herbal Pharmacopoeia (AHP, 2004) have been advocated the authentic botanic origins of 169 and 300 monographs, respectively. Frequently, more than one botanic species or varieties are named in a CHM scientific name (Latin name), which are commonly labelled for the herbal products in the global market. Such clinical practices of using different but related botanic species or varieties that have the same pharmaceutical names have created difficulties for CHM evaluation and identification.

Firstly, the molecular profiles of different species and varieties are varying. Recent studies demonstrated that different Rhizoma Atractyloids (Cang-zhu) sources, which were found in the market, came from either the genuine source of plants *Atractylodes lancea* and *A. chinenesis*, or the three substitutes or adulterants (*A. koreana, A. ovata* and *A. japonica*), based on their DNA fingerprinting profiles (Ren et al., 2000, Mizukami et al., 2000, Kohjyouma et al., 1997). The significant genetic diversities also have been found with eight species in plants of *Isodon* (Schrad. Ex Benth.) Kudo (Xiang-cha-cai), a commonly used CHM (Fang et al., 2003). Radix Astragali (Huang-qi), regarded as one of the most important CHM for *Qi* deficiency syndromes, has been used as CHM for more than 2000 year in China. The similar genetic relationships of four commonly used *Astragalus* species under the same CHM pharmaceutic name, Radix Astragali (Huang-qi), were recently sequenced and evaluated (Ma et al., 2000b) Fu *et al* (1999) reported the significant genetic differences between three

Codonopsis spp., including *C. tangshen, C. modesta,* and *C. nervosa var. macrantha,* which are known as the botanic sources of CHM Radix Codonopsis Pilosulae (Dang-shen).

Furthermore, the bioactive chemical compositions of medicinal plants vary from species to species, varieties to varieties, within or without a genus as demonstrated by the presence of structurally different ephedrine alkaloids from two different species of Herba Ephedrae (Ma-huang), *Ephedrae sinica* and *E. aspera* (Schaneberg et al., 2003). This significant variation in bioactive chemicals was also revealed in recent study on Magnesium tanshinoate B (MTB), Danshensu, Isotanshinon II_A, and Cryptotanshin I, after analysis of 57 batches of Radix Salviae Miltiorrhizae (Dan-shen) species or varieties, using a HPLC method (Zhang et al., 2002a). Significant differences of the content of two anti-convulsive components, α-asarone and β-asarone, from three Rhizoma Acori (Shi-chang-pu) species, including *Acori gramineus, A. calami* and *Anemone altaica,* were investigated using RP-HPLC (Ke and Fang, 2004). The findings indicated that the contents of α-asarone and β-asarone of *A. gramineus* were 1.967% and 0.317%, respectively, while as 0.070% of α-asarone and 0.018% of β-asarone from *A. calami*, were detected. However, no α-asarone and β-asarone were detected from *Anemone altaica*, which was suggested as the adulterant of Rhizoma Acori (Shi-chang-pu). *Phellodendron amurense* and *P. chinense* have been recognised as the botanic sources of Cortex Phellodendri (Huang-bai), which was clinically applied for jaundice and morbid leucorrhoea caused damp-heat, urinary infection, oedema, consumptive fever, night sweating and skin infection (Bensky and Gamble, 1993). Ma *et al* (2006) reported that the content of major bioactive compound, obaculactone, was 0.269% from *P. amurense* and 0.154% from *P. chinense* using RP-HPLC method. Wang *et al* (2005) analysed the content of α-ligustilide from three Radix Angelicae Sinensis (Dang-gui) varieties from Gansu province, China. The range of the content of α-ligustilide from three *Angelica sinensis* was from 1.69% to 3.77%. Radix Ligustici Chuanxiong (Chuan-xiong), which has been used to invigorate the blood, has

also been found to contain α-ligustilide (Wang et al., 2006a). Recent study found that the content of α-ligustilide were varying between the Chinese species, *Ligusticum chuanxiong* (0.532 – 1.482 %), and the Japanese species *Cnidium officiniale* contained lower content of α-ligustilide, ranged from 0.059 – 0.761% (Wang et al., 2006a). Furthermore, recent study demonstrated differences of the content of ferulic acid, another bioactive compound from Radix Ligustici Chuanxiong (Chuan-xiong) between the Chinese species (0.6530 – 1.3271%) and the Japanese species (0.7360 – 0.9507%) (Wang and Kuang, 2002). Flos Chrysanthemi Indici (Ye-ju-hua), one of the commonly used CHM for respiratory diseases, contained anti-biotic compound cyclohexanecarboxylic acid. It has been elucidated differences of the content of cyclohexanecarboxylic acid in 14 *Chrysanthemum indicum* varieties in China (Tan et al., 2004). Similar variation also appeared in protocatechuic acid in five *Schisandra chinensis* varieties, as the botanic sources of Fructus Schisandrae Chinensis (Wu-wei-zi) . The highest content of protocatechuic acid was 0.276% from the Korean variety, while as the lowest content was 0.099% from one of the Chinese varieties, grown in Hebei Province (Xu and Zhao, 2004). The previous study on selected 23 Radix Sophorae Flavescentis (Ku-shen) varieties from *Sophora flavescens* showed that oxymartine contents ranged from 1.25% to 3.63% (Zhang et al., 2004). Oxymartine has been known as one of the bioactive compounds for anti-inflammatory, anti-arrhythmic and anti-fungal effects. Based on the Chinese pharmacopeia (The Pharmacopoeia Commission of People's Republic of China, 2005), one of the well-known CHM Radix Astragali (Huang-qi) are the dried roots from two species, *Astragalus membranaceus (Fisch.)* Bge. var. *mongholicus (Bge.) and A. membranaceus (Fisch.)Bge.* The varying quantities of one of the key bioactive compounds, astragaloside IV, were demonstrated between two Radix Astragali (Huang-qi) species (Zhang et al., 2005c, Xu et al., 2005). Previous study on the quality of Radix et Rhizoma Rhei (Da-huang) species, including *Rheum palmatum* L.*, R. tanguticum* and *R. ficinale* showed that content of aloe-emodin ranged from 0.28% to 0.56%, content of rhein ranged from 0.32% to 0.84%, content

of emodin ranged from 0.16% to 0.61%, content of chrysophanol ranged from 0.44% to 1.33%, and content of physcion ranged from 1.74% to 3.69% (Lin et al., 2005). Thus, to insure the chemical consistency of a CHM, it is necessary for establishment of the chemical profiles of the medicinal sources of different species and varieties.

Although the evidences of differences of the pharmacological functions among the botanical sources of CHM are still lacking, the changes of chemical compositions, which are the foundation of pharmacological actions of CHM, suggest varying efficacies of their bioactivities. Due to the rapid development of isolation and separation techniques of bioactive constituents from CHM, there are increasing interests in the researches of the therapeutic effects of active components from various CHM species (Choi et al., 2002, Li, 2003). As mentioned before, the considerable diversity of the quality and the quantity of chemical compositions from various Flos Magnoliae species may cause differences of the pharmacological effects. However, such systematic research on evaluation of the pharmacological actions by Flos Magnoliae sources has not been established.

1.2.2.1.2 Wild and domesticated populations

Due to the elevated pressure on the protection of natural ecosystems, increased harvesting of domesticated population of CHM has been highly recommended and developed (Zou, 2000). Genetic variation occurred in the process of domesticated populations may alter the amount and structure of chemical compositions of CHM populations. This chemical variation of quantities and qualities was shown in previous studies, such as on artemisinin, the anti-malarial agent, in *Artemisia annua* populations of CHM Herbal Artemisiae Annuae (Qing-hao) (Cao and Chen, 1996). The highest content of artemisinin in the domesticated *Artemisia annua* was 0.6%, which was higher than the content of artemsinin from the wide populations. In contrast, the quantitative chemical variations are superior in wild than in domesticated

populations in some CHM species, for instance, on ergosteral in Cordyceps (Dong-chong-xia-cao) (Li et al., 2001b). Xie *et al* (2004) also revealed that the content of bioactive compound, gastrodin, was higher in the wild Rhizoma Gastrodiae (Tian-ma) species than the domesticated populations.

1.2.2.1.3 Substitutes and adulterants

When adverse effects from CHM products are reported, often they are the results of the occurrence of substitutions or adulterations (Ernst, 2004). The failure to sufficiently guarantee the correct botanical identity has been revealed as one of the most frequent limitations of botanical quality assurance (Leon et al., 2004). Even since Chinese started employing CHM to cure diseases two thousand years ago, choosing effective and safe botanic sources as herbal remedies had already been a big issue that appeared in some reputable CHM references (Yu et al., 1999).

In some cases, the authorised CHM species may be substituted by an inferior species. A number of cases presented serious consequences of Aristolochia substitutions. Investigation of the 33 cases of nephropathy in Belgian revealed that *Aristolochia westlandii*, which contained a known nephrotoxin aritolochia acid, had been substituted for Radix Stephaniae Tetrandrae (Han-fang-ji), dried roots of *Stephania tetrandra* populations (Depierreux et al., 1994). Based on the Chinese Pharmacopoeia, *Clematis armandii* and *C. montana* are the authorised species for the CHM Caulis Clematidis Armandii (Chuan-mu-tong). *A. manshuriensis*, as the substitutes of Caulis Clematidis Armandii (Chuan-mu-tong), have been reported to cause terminal kidney failure in Canada (Health Canada, 2001b, Health Canada, 2001c, Health Canada, 2001d, Health Canada, 2001a). Recently, significant differences of the qualities and quantities of four bioactive compounds, including cinnamaldehyde, cinnamic acid, cinnamyl alcohol and coumarin, were elucidated in CHM Cortex Cinnamomi Cassiae (Rou-gui), from

13 authorised *Cinnamomum cassia* and 16 substitutes or adulterants including *C. cassia*, *C. wilsonii*, C. *japonicum*, *C. burmannii*, *C. mairei* and *C. loureirii* (He et al., 2005).

1.2.2.2 Parts of plants

Use different parts of plants may alter the quality of CHM, in which the site of biosynthesis, accumulation and storage are usually varying. An example of organ-specific accumulation as well as species specificity is that of the compounds considered responsible for the anti-inflammatory effect of Radicis Angelicae Sinensis (Dang-gui). Different plant parts of *Angelica sinensis* are used for varying medical conditions. The entire root, Radicis Angelicae Sinensis, is usually prescribed for tonifying the blood and regulating the menses. The body of the herb, Corpus Radicis Agnelicae Sinensis (Dang-gui-shen), has effects on tonifying and invigorating the blood. The head of the herb, Caput Radicis Agnelicae Sinensis (Dang-gui-tou), is suggested to be the most tonifying part, and worst in blood movements. On the other hand, the tail, Extremitas Radicis Agnelicae Sinenesis (Dang-gui-wei), is considered as the least tonifying part, but is most powerful in moving the blood. Previous studies indicated the different content of the bioactive compounds derived from four sites of the herb (Table 1.14) (Qiao and Xiang, 2005, Wu et al., 2004b). On the basis of the quantitative analysis, significant differences of the main flavonoids were demonstrated from five Herba Epimedii (Yin-yang-huo) species, included *Epimedium brevicornum*, *E. sagittatum*, *E. pubescens*, *E. wushanense* and *E. koreanum* (Guo and Xiao, 1996, Li et al., 1996, Guo et al., 1996). The components of flavonoids between five sources of Herba Epimedii (Yin-yang-huo) had significant differences between rhizome and roots (Guo and Xiao, 1996).

Table 1.14 Quantitative analysis of the contents of the bioactive components from different *Angelica sinensis* sites (Qiao and Xiang, 2005, Wu et al., 2004b).

The sites of the herb	Ferulic acid (%, w/w)	The level of the compounds	
		α-pinene	β-ocimene
Radicis Angelicae Sinensis	0.1190	**	*
Corpus Radicis Angelicae Sinensis	0.0813	***	**
Coput Radicis Angelicae Sinensis	0.1085	****	****
Extremitas Radicis Angelicae Sinensis	0.1262	*	***

The levels of the compound are ****>***>**>*.

Similar findings are also showed in volatile compositions of CHM, for instance, Fructus Alpiniae Oxyphyllae (Yi-zhi-ren), the fruits of *Alpinia axyphylla*. The content of volatile oil from the fruit was five times higher than it from the shell of the fruit, 1.77% and 0.38%, respectively (Yang and Xing, 2003). *Asafum sieboldii*, regarded as the botanic origin of Herba cum Radice Asari (Xi-xin), were used for clinical application of headache. The volatile composition accumulate and storage in the sites of roots and stems, where chemical biosynthesis usually takes place, the content of that was more than seven times higher than the content of the volatile oil in the leaves and fruits (Xia, 2005). Palmatic acid and linoleic acid are present in Caulis Lonicerae (Ren-dong-teng), the stems of *Lonicera japonica*, and Flos Lonicerae (Jin-yin-hua), the flowers of the same plant. Thirty-six volatile compounds were determined using GC-MS, whereas all 18 of them were both found in Caulis Lonicerae (Ren-dong-teng) and Flos Lonicerae (Jin-yin-hua) (Li et al., 2003).

1.2.2.3 Diurnal and seasonal variations

Due to the influences on accumulation and storage of the chemical synthesis, diurnal and seasonal variations are the other factors affecting the quality of CHM. Based on the different accumulation of bioactive compounds on the medicinal plants, the best harvesting and collecting time can occur at any time during the plants' growth. The harvesting and collecting period usually takes place before or at the time of flower blossoming (Bensky and Gamble, 1993). Previous studies elucidated that the diurnal and seasonal variations of harvesting and collecting can vary the quality of CHM. For example, the middle of April and the end of May, when stems have a purple hue, were considered as the best season for harvesting for Radix Ligustici Chuanxiong (Chuan-xiong), based on the content of bioactive compounds, senkyunolide A, coniferyl ferulate and Z-ligustilide (Li et al., 2006). The anti-oxidant compound luteolin-7-O-glycoside, derived from *Dracocephalum rupestra* (Yan-qing-lan), was reached highest level before blossoming (Ren et al., 2004). In other cases, medicinal plants are harvested immaturely, such as Pericarpium Citri Reticulatae Viride (Qing-pi), dried barks of *Citrus spp.,* and Fructur Immaturus Citri Aurantii (Zhi-shi), dried fruits from *Citrus aurantium* (Bensky and Gamble, 1993).

Thus, it is essential to establish the time for harvesting and collecting, when the highest amount of the bioactive compositions of CHM is achieved. However, the correlation of the content of the bioactive compounds and the seasons of harvesting and collecting are hardly available for most CHM. In such cases, the medicinal parts harvest traditionally, when the specific site reach its finest level of development (Bensky and Gamble, 1993). In the majority cases, roots and rhizomes are collected at the beginning of the spring or the end of the summer, barks are collected in spring, the leaves and whole plants are collected before or at the flower blossoming stage, and fruits and seeds are collected when they are fully ripe (Nanjing Botanical Garden, 2006).

1.2.2.4 Environmental factors

Besides the intrinsic factors, the quality of CHM may affect by a number of extrinsic factors as well, such as the environmental factors during the cultivation process. Certain amount of sunlight and moisture, optimal soil conditions, appropriate climate and adequate nutrients are necessary for the medicinal plants to achieve the best quality. Existing classic and authorised CHM literatures have suggested the suitable area for the cultivation of the particular CHM, based on the differences in climate and geography. For instance, the high attitude mountain areas are the most suitable sites for the cultivation of Rhizoma Coptidis (Huang-lian) and Bulbus Fritullariae Cirrhosae (Chuan-bei-mu), where as the best quality of Herba Cistanches (Rou-cong-rong) and Herba Cynomorii Songarici (Suo-yang) from the desert. Furthermore, some of the CHM are suitable grown from damp areas, for example, Radix Rehmanniae Glutinosae (Sheng-di-huang) and Flos Carthami Tinctorii (Hong-hua) (Bensky and Gamble, 1993, The Pharmacopoeia Commission of People's Republic of China, 2005, Yu et al., 1999).

Resent researches studied on the relationship between the environmental conditions of growth and the quality of the chemical properties of soil for the cultivation of *Panax notoginseng*, medicinal sources of Radix Notoginseng (San-qi) in Yunan and Guangxi, China. The neutral weakly acidic soil with loam clay, and with carbonate was suggested as the most suitable soil conditions for Radix Notoginseng (San-qi) growing (Cui et al., 2005). The bioactive compound, berberine, was analysed for establishment of the most optimal environmental conditions for cultivation of Rhizoma Coptidis (Huang-lian), dried plant of *Coptis chinensis*. It was recommended that the low shading ratio was more suitable for *Coptis chinensis*, to obtain the better quality of Rhizoma Coptidis in the harvest (Guo et al., 2004b). It also has been reported that the proper shading method and the optimal level of fertilizer nitrogen was needed for achieving the highest content of the bioactive compounds, citronellol and geraniol, of Geranium (Tian-zhu-kui) (Ram et al., 2003).

1.2.2.5 Manufacturing factors

Some manufacturing factors also affect the quality of CHM, such as post-harvest processing, shipping and storage. Those extrinsic factors can affect the morphological features and the chemical compositions of the medicinal plants.

One of the reasons for processing the medicinal materials is to increase the potency. Proper processing is also aimed to minimize side effects or alter a substance's properties to meet the particular clinical requirements. Dried roots and rhizomes of *Polygonum cuspidatum* is one of the commonly used CHM, Rhizoma Picrorhizae (Hu-zhang). Different manufacturing processes of Rhizoma Picrorhizae (Hu-zhang), including products fried with salt, vinegar or wine, have been found in the market and used for different clinical applications (Bensky and Gamble, 1993). Resent study found that among three processed products, the products processed by salt contained the highest level of piceid (1.114%), which has effects on reducing blood fat and thrombosis (Jiang et al., 2004a). The findings are in line with the suggestions appeared in the classic and authorised literatures. On the other hand, overheat process lead to the low quality of Fructus Arctii (Niu-bang-zi). Zhang (2004) indicated that crude Fructus Arctii (Niu-bang-zi) contained higher content of arctiin, as the anti-cancer and anti-biotic agent, than the products which have been boiled or burnt.

Meanwhile, differences of the chemical contents of same CHM products from different marketing sources have been analysed by a number of studies. For instance, the contents of glycyrrhizin from five Radix Glycyrrhizae (Gan-cao) samples from different pharmaceutical suppliers were varying, ranged from 5.58% - 9.81% (Liu et al., 2003a). The poisonous compounds, aconitine and hypaconitine have been found in both Radix Aconiti Preparata (Chuan-wu) and Radix Aconiti Kusnezoffii (Cao-wu). The content of aconitine from three commercial suppliers ranged from 0 - 0.000233%, while the content of hypaconitine from

Radix Aconiti Preparata (Chuan-wu) ranged from 0 – 0.007462% (Zhang and Feng, 2005). In the other hand, HPLC method was employed for determined the aconitine and hypaconitine, derived from Radix Aconiti Kusnezoffii (Cao-wu), from three suppliers in the market, ranged from 0 – 0.002100% and 0.010231% – 0 .028950%, respectively (Zhang and Feng, 2005).

1.2.2.6 Contaminations

It's likely that unintentional or accidental substances may contaminate the CHM products. Potential contaminations include microbial and chemical agents (pesticides and herbicides) during any stage of CHM production. In addition, the heavy metal is also regarded as other major concerns, which may lead to poor quality and unsafe CHM application.

1.2.2.6.1 Microbial

Contamination of bacterial and fungal of CHM may cause health risk and financial loss. The improper sanitary conditions during post-harvesting process, storage, packaging and shipping usually result in the certain level of microbial contamination, which cannot meet the authorise standards. Some of the microbial contaminations, even at very low level, can induce server infections. A fluorescent staining method was employed for rapid assessment of total bacterial and esterase-active bacteria level of CHM (Nakajima et al., 2005). The outcomes indicated that the total bacterial from Radix Ginseng (Ren-shen) reached more than 10^7 cells/g and almost 10^6 cells/g of esterase-active bacteria.

Furthermore, bacterial and fungal endotoxins can also cause harmful effects. For example, Aflatoxin, which is produced by a fungus, Aspergillus, is one of the most harmful substances for human being. Acute necrosis, cirrhosis and carcinoma of the liver exhibited by hemorrhage, acute liver damage, edema, alteration in digestion, absorption and metabolism of nutrition will be produced by exposure of high level of Aflatoxin, including Aflatoxin B_1 and

B_2, and Aflatoxin G_1 and G_2 (Smith et al., 1975). Recently, the contamination level of Aflatoxin in nine CHM patent product and 14 crude herbs were investigated using HPLC method (Zheng et al., 2005). Four patent products of nine were found contained Aflatoxin B_1 and B_2, whose levels ranged from 0.48-1.21μg/kg and 0.49-2.43μg/kg, respectively. Only one of the 14 crude CHM herbs, was contained Aflatoxin B_1 (1.54 μg/kg).

1.2.2.6.2 Pesticide and herbicides

Different pesticides and herbicides contamination of medicinal plants during the cultivation process is another safety concern. A pesticide can be used against pest, including insects, plant pathogens and microbes, and the herbicides are employed against weeds. Not only pesticides and herbicides are dangers to the environment, the pesticides and herbicides residue on CHM are also harmful to the health of general public. There are evidences that higher chances of brain cancer, leukaemia and birth defects after exposure to pesticides and herbicides (Hepworth et al., 2006, Lee et al., 2005, Miligi et al., 2006, Mahajan et al., 2006, Garry et al., 2002).

In China, a study employed multiresidue analytical method was applied for analysis of the organochlorine pesticides from CHM products (Hao and Xue, 2005). Thirteen organchloride pesticides from CHM products were examined by the SCF extraction followed by GC-MS confirmation method (Ling et al., 1999). CHM products, including *Panax. notoginseng*, botanical origins of Radix Notoginseng (San-qi); *Panax. quinguefolium*, botanical origins of Radix Panacis Quinquefolii (Xi-yang-shen); *Paeonia lactiflora,* botanical origins of Radix Paeoniae Alba (Bai-shao) and *Angelica. sinensis*, botanical origins of Radix Angelicae Sinensis (Dang-gui) were contained ranged from 0 - 3.6×10^{-10}g of metalaxyl residues (Dong et al., 2004).

1.2.2.6.3 Heavy metals

Heavy metal contamination can happen at any stages of the cultivation and manufacturing stages. Clinical consequences of heavy metal poisoning have been documented by a number of studies.

Arsenic (As)

Short-term exposure of *As* may cause neuropathy and megaloblastic anemia, and long-term exposure can lead to the development of cancer (Lerman et al., 1980, Tapio and Grosche, 2006). In Singapore, 74 cases and 3 cases were reported for *As* poisoning, in 1975 and 1998, resulting to two patients died, after taking various CHM preparation, mainly for asthma treatment (Tay and Seah, 1975, Wong et al., 1998b, Wong et al., 1998a).

Cadmium (Cd)

Nephropathy and emphysema are usually seen after short-term exposure of *Cd*. Long-term exposure also can induce the development of cancer (Lee and White, 1980). In 1996, a 34-year-old female patient was reported have permanent nephrogenic diabetes insipidus, caused by *Cd* poisoning, after three months CHM mixture treatment (Wu et al., 1996a).

Mercury (Hg)

The major symptoms and signs of *Hg* poisoning are depression, irritability, forgetfulness, confusion, tremor, sensory disturbances, visual deficits, hearing loss, movement disorders and cognitive disturbances (Magos and Clarkson, 2006). In Hong Kong, one case reported that a CHM mouth spray was responsible for the *Hg* poisoning for a five-year-old male patient, who recovered in two weeks (Li et al., 2000a). Unfortunately, it was reported that three *Hg* poisoning cases led to two death in United States (Kang-Yum and Oransky, 1992).

Lead (Pb)

Pb poisoning leads to the gastrointestinal and neurologic symptoms for the adults, and lead encephalopathy, including lethargy, seizures, coma and death, for the children (Lidsky and Schneider, 2006, Toscano and Guilarte, 2005, Papanikolaou et al., 2005). A number of *Pb* poisoning cases, which caused by CHM, have been reported since 1977 (Lightfoote et al., 1977, Chan et al., 1977, Levitt et al., 1984, Yu and Yeung, 1987, Markowitz et al., 1994, Wu et al., 1996b). Most of the patients were fully recovered, however, a two-months-old male infant was suffered by mildly delayed development (Yu and Yeung, 1987).

Thallium (Tl)

Gastrointestinal complaints can be initially caused by exposure of *Tl*, followed by sensory paresthesia (Jha et al., 2006, Meggs et al., 1994). Two middle-aged women, who had symptoms, including paresthesia, hair loss and vomiting, were diagnosed as *Tl* poisoning after being treated by CHM in 1992 (Schaumburg and Berger, 1992).

1.2.2.7 Synthetic adulterants

In addition to the adulteration by other medicinal plants, undeclared pharmaceuticals to CHM products are another quality concern. Commonly seen compounds includes a wide range of pharmaceuticals, such as analgesics, antibiotics, antidiabetics, antiepileptics, aphrodisiacs, hormones and anabolic drugs, psychotropic drugs, and weight reducer that have been found in CHM products.

The chemical analysis of some CHM that led to the finding of nine adulteration, including sildenafil, famotidine, ibuprofen, promethazine, diazepam, nifedipine, captopril, amoxicillin and dextromethorphan, was performed using a robust LC-MS-MS method from over 200 CHM products (Liang et al., 2006b). Recent study also showed that nine herbal products were

adulterated by pharmaceuticals, contained tadalafil, sildenafil, testosterone, decanoate, glibenclamide, fenfluramine, phentermine, caffeine, phyenylbutazone and dipyrone using HPLC-MS-MS (Bogusz et al., 2006). In Taiwan, 618 CHM samples from eight hospitals were found adulterated by pharmaceuticals, including caffeine, acetaminophen, indomethacin, hydrochlorothiazide, prednisolone, ethoxybenzamide, phenylbutazone, betamethasone, theophylline, dexamethasone, diazepam, bucetin, chlorpheniramine maleate, prednisone, oxyphenbutazone, diclofenac sodium, ibuprofen, cortisone, ketoprofen, phenobarbital, hydrocortisone acetate, niflumic acid, triamcinolone, diethylpropion, mefenamic acid, piroxicam and salicylamide (Huang et al., 1997). Moreover, 52.8% of the adulterated CHM products contained more than two synthetic therapeutic agents (Huang et al., 1997).

1.2.3 Regulation and quality control of CHM

Although the majority of CHM has a safe record, some still carry potential risk. Standardisation by most of manufacturers may help to maintain consistently high level of active ingredients of CHM products (Corns, 2003).

This situation leads WHO and other authorities in many countries to address the issues of regulation and evaluation of quality, safety and efficacy of CHM. In China, CHM is fully covered in the health system. The Chinese Pharmacopoeia (The Pharmacopoeia Commission of People's Republic of China, 2005) described 920 monographs of CHM as therapeutic substances, which include details of Chinese materia medica and traditional Chinese patent medicines. Surveillance and legislation have been required to control the use of these herbal products (Akerele, 1984). In Korea, the authorities have regulated and evaluated herbal material medica by the Korean Pharmaceutical Affairs Law; directed manufacturers to conform to the Korean Good Manufacturing Practice (GMP) standard; and developed researches of new herbal therapeutic products by the Korean Food and Drug administration

(KFDA) (Choi et al., 2002, Corns, 2003). In Japan, Pharmaceutical Affairs Law ensures the quality standards of herbal therapeutical goods, regulated by Pharmaceutical administration. More than 90% of the products have been announced in the Japanese Pharmacopoeia (Saito, 2000). In Australia, phytomedicines, including CHM, are regulated under the Therapeutic Goods Act 1989, which is administered by TGA (Drew and Myers, 1997, Briggs, 2002). To ensure better access to safe and proven CHM to the public, TGA has jurisdiction over licensing of manufacturers, pre-market assessment and post-market regulatory activity (Briggs, 2002).

Nevertheless, harmonisation of regulation and evidence-based data for quality control are the biggest challenges in most countries. The good agricultural practices (GAP) can be maintained the quality production of medicinal plant during the commercial cultivation (Leung et al., 2006). Based on the GMP guideline, the medicinal materials have to be processed to ensure the quality, efficacy and safety (Leung et al., 2006). The WHO has published general guidelines for the GMP production of botanic therapeutic products (WHO, 2004a). Moreover, regulatory authorities must employ the surveillance of post-marketing quality guarantees. Further steps are needed to institute and develop rapid, sensitive and reliable assessment methods to screen the quality of CHM products, including botanical taxonomic identification, morphological and histological analysis, analytical procedures of molecular profiles and chemical compositions and pharmacological and clinical evaluation.

1.3 Techniques for identification and authentication of CHM

The quality of CHM products has been constantly acknowledged as a priority issue in the development of CHM (WHO, 2004b). Thus, establishing selective and efficient analytical methods are important not only for authentication of the origins of medicinal plant materials, but also for the quality control and safety assurance of CHM (Xue et al., 2004, Barnes, 2003a, Barnes, 2003b).

There is an extraordinary history for CHM clinical application in China. Since early 220-225 B.C., organoleptic identification was introduced in the first classic CHM literature, "Shennong's Classic of Materia Medica" (Yu et al., 1999). In 420-479 A.D., 'Leigong's Discussion of Processing of Materia Medica' was published, which firstly recorded a number of identification methods for assurance the quality of CHM (Yu et al., 1999). In 1500 years ago, the famous CHM practitioner, Tao, Hongjing firstly addressed in his publication 'Collection of Commentaries on the Classic of Materia Medica' that the misidentification, adulteration and substitution were found in the market, which might lead to the unsafe clinical application of CHM (Yu et al., 1999). The first Pharmacopoeia was issued in order to regulating the CHM, in 659 A.D. Since then, numerous classic literatures have emphasised on quality and safety of CHM, and attempted to develop the sensitive and reliable methods for the identification and authentication of CHM.

With the development of biology, chemistry and physics in the early 19th century, the Austrian physician, Schmidt firstly introduced pharmacognosy, which was used for dealing with crude or unprepared phytomedicines. Researchers started drawing attention to the bioactive components from plants. In 1803, French Derosne discovered the alkaloid from plants, and German Sertüner isolated morphine from opium in 1806 (Huxtable and Schwarz, 2001). Therefore, modern techniques were used to assure and assess the quality, efficacy and

safety of herbal therapeutic agents. Microscopes have been extensively applied for the identification of herbal materials since 18th century (Techen et al., 2004). Until middle of last century, chromatographic and spectrophotometric techniques have been employed widely (Akerele, 1984). In 1985, Kary Mullis developed the PCR technique (Mullis, 1993). In recent decades, PCR-based molecular markers have been become one of the most accepted techniques for CHM identification and authentication.

1.3.1 Traditional techniques

Macroscopic and microscopic examinations of morphological and histological characteristics of CHM are regarded as the first step to authenticate CHM (Li, 1996). The quality parameters for botanicals continued are mainly based upon macroscopic and microscopic visual evaluations of herbal materials (Techen et al., 2004). For raw botanicals, above examinations of the characteristics of medicinal plants are usually very quick and inexpensive. However, a requirement of experienced and competent personnel to produce accurate assessment is the disadvantages of these approaches.

1.3.1.1 Morphological identification

The macroscopic description of CHM appeared in 'Shennong's Classic of Materia Medica' two thousand years ago (Yu et al., 1999). Until now, description of organoleptic markers such as shape, colour, texture and odour of CHM is still an important part in the botanic monographs from most of the authorised literatures and guidelines (Akerele, 1984, Bensky and Gamble, 1993, The Pharmacopoeia Commission of People's Republic of China, 2005, WHO, 1998, Yu et al., 1999).

1.3.1.2 Histological identification

The introduction of microscopic technique to CHM identification resulted in the distinct improvement in CHM quality. Microscopic identification involves examination of internal structures of different botanic sources, for instances, skin, phloem, cambium, vessels, fibres, crystals, and calcium oxalate (Li, 1996). Optical microscopes and electron microscopes are two most commonly used instruments for CHM identification.

1.3.2 DNA-based molecular markers

Based on molecular cloning and PCR techniques, DNA-based molecular markers have been widely used for identification and authentication of plant and animal species (Joshi et al., 2004). Moreover, since the mid-1990s, DNA fingerprinting techniques have been considered as an independent approach to identify and authenticate the medicinal plants (Cheung et al., 1994). Various types of DNA-based techniques, includes PCR-based markers, hybridisation-based markers and sequencing-based markers, have been employed to reveal the molecular information from CHM (Fowler et al., 1994). The various methods involved in DNA-based markers are shown in Table 1.15.

Compared with other identification methods, DNA-based molecular marker techniques are much less affected by some factors, such as age, physiological circumstance, environments, climates and other issues. As the most basic signature of an organism, DNA sequences, even a few molecular DNA, could be the powerful evidence contributing to the quality control of CHM (Joshi et al., 2004). Furthermore, in most organisms, the DNA is an extended double-stranded polymer, which institutes and keeps the cellular and biochemical functions of organisms (Glick and Pasternak, 2003). DNA-based markers are DNA-specific, not tissue-specific, that can be identified at any stage of the organism progress. These markers have non-restrictive physical forms and requiring only small quantities of testing samples (Fan et al.,

2004). Therefore, they are particularly applicable for identification and authentication of the CHM.

Table 1.15 Classification of diverse arrays of DNA-based markers (Fowler et al., 1994)

Types	Representative
PCR-Based Markers	Random Amplified Polymorphic DNA (RAPD)
	Arbitrarily-primed PCR (AP-PCR)
	Simple Sequence Repeat (SSR)
	Amplified Fragment Length Polymorphism (AFLP)
	Direct Amplification of Length Polymorphism (DALP)
	PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)
Hybridisation-Based Markers	RFLP
	Minisatellite / microstatellite
Sequencing-Based Markers	Assessed the differentiation directly and obtained information by a defined locus
	Internal transcribed spacers (ITS) of ribosomal DNA (rDNA) or chloroplast gene (*trn*K)

1.3.2.1 PCR-based markers

PCR

In 1944, Avery, Macleod and McCarty announced the revolution of genetics by DNA, which started and founded the molecular genetic science (Lederberg, 1994). In 1953, the structure of DNA was published by Watson and Crick (Watson and Crick, 2003). Since the successful development of PCR in 1988, (White et al., 1989), it has been considered as a valuable approach for producing large quantities of a specific DNA sequence that is useful for the molecular genetic analysis of plant, animals and other organisms, as three-step cycling procedure accomplishes the amplification of a specific DNA sequence that can be more than a million fold (Fowler et al., 1994).

Components for PCR amplification

Several crucial components are involved in PCR amplification (Table 1.16): (1) primers that are harmonising to regions on opposite strands that flank the target DNA sequence; (2) a DNA template, which contains a target sequence from 100 to 35,000 base pairs (bp) in length; (3) a heat-stable DNA polymerase, which would synthesize DNA at elevated temperatures from single-stranded templates in the presence of a primer; and (4) four 2'-deoxyribonucleotide-5' triphosphates (dNTP) (including dAPT, dCTP, dGTP and dTTP).

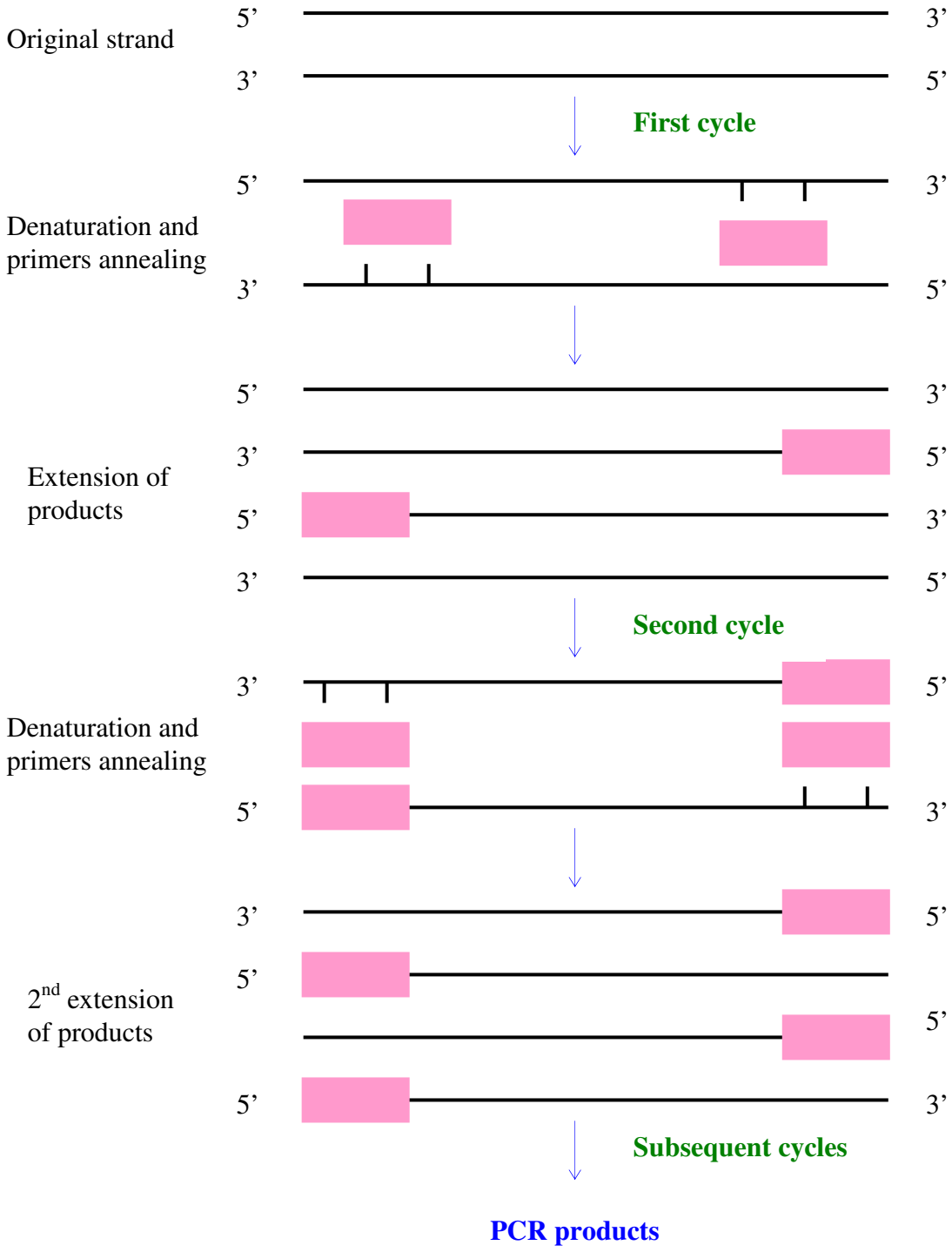
As a critical issue in any PCR to obtain high and specific amplification products, the optimisation of the combination of the constituents is essential, for instances, the balance of dNTPs and magnesium ion, the concentration of the PCR buffer, quantity of template DNA and Taq DNA polymerase, amount of primers and the quality and quantity of DNA templates (Markoulatos et al., 2002).

Table 1.16 Typical reaction set-up of routine PCR (Glick and Pasternak, 2003)

PCR Master Mix	PCR buffer	Containing the cations in the buffer, which neutralise the negatively charge phosphate groups on the DNA backbone and decline the repulsive, and obliging the annealing process between the primer and the template.
	Magnesium chloride ($MgCl_2$)	The magnesium ion, which influences on primer annealing, the melting temperature of the PCR products and products specificity, not only bound by the template DNA, the nucleotides and the primer, but *Taq* DNA polymerase requires free magnesium ions as well.
	dNTPs	The concentration of deoxyribonucleotides depends on the concentrations of primers and magnesium ions, thus decreasing the concentration of dNTPs results in a significant improvement in PCR amplification, but reduction of the PCR products.
	Taq DNA polymerase	Heat-stable DNA polymerase from *Thermus aquaticus,* synthesizing DNA at specific temperatures from single-stranded templates in the presence of a primer.
Primers		The proportion of the primer is critical for the generation of PCR products.
DNA template		The amount, purity and integrity of the DNA template can be critical.

Steps of PCR amplification

On the basis of denaturation, annealing and extension, a PCR is a relatively simple technique by which a DNA template is amplified many thousand or million fold quickly and reliably, generating sufficient material for subsequent analysis. The basic PCR reactions take place in a thermal cycler that has been preheated to 95°C (initial denaturation) for approximately 2min to ensure that the target DNA is completely denatured. This is followed by a number of cycles for amplifying a specific DNA sequence, with each cycle having three steps: denaturation (maintaining at 95°C), annealing (at a lower temperature which should be optimised for each primer set based on the primer melting temperature), and extension (maintaining at 72°C, a step catalysed by the *Taq* DNA polymerase), followed by the final extension at 72°C. Finally the reaction is maintained at 4°C. Repeating the denaturation-annealing-extension cycles multiplies an exponential accumulation of the DNA fragment of the defined sequence (McPherson and Moller, 2000) (Figure 1.8). The presence of primers, single-stranded polynucleotides that recognise and bind to the complementary DNA sequence on template DNA attributed to the specific duplication. The annealing primer, initiating from the 3' hydroxyl end of each primer sequence, is extended by a thermostable DNA polymerase and persists though the region of the template DNA strand that is complementary for the next PCR cycle. The amplified PCR products are fractionated by electrophoresis on agarose or polyacrylamide gel and illuminated under UV after ethidium bromide (EtBr) staining. Due to the capacity to amplify DNA from a small quantity of materials *in vitro*, a range of PCR-derived techniques have been established.



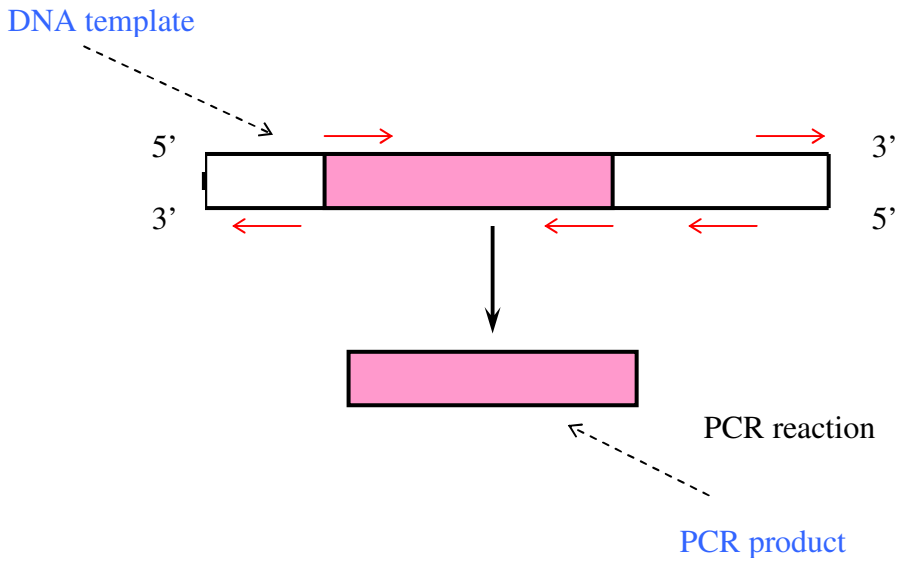
‘ ’, represents primers. The primer used may be a specific target sequence (e.g. in PCR-RFLP) or an arbitrarily sequence (e.g. in RAPD).

Figure 1.8 Schematic diagram of PCR

1.3.2.1.1 Random amplified polymorphic DNA (RAPD)

General principles of RAPD

In 1990, Williams reported the development of PCR-based genetic screening technique, RAPD (Williams et al., 1990). The method processes PCR amplification by only a single arbitrary oligonucleotide (oligo) primer, as both the forward and reverse primer in a PCR reaction. At a comparable low annealing temperature, a single random oligo, generally 10 nucleotides long, can be bound to the chromosomal DNA (cDNA) templates, when two multiple copies of the oligo on opposite strands are oriented facing one another (Williams et al., 1990). Sequences between these positions will be amplified by PCR (Figure 1.9).



' → ', represent multiple copies of a primer (all primers have the same sequence).

Figure 1.9 The phenomenon of RAPD

RAPD analysis, which is simple and rapid, can be employed to identify entire genomic information, individual chromosomes, and unusually specific genes (Williams et al., 1990). The advantages of RAPD include: (1) smaller quantity of DNA is required compared to other PCR-based techniques; (2) a universal set of primers, even the same primer can be conducted and displayed in a short period of time, and samples from various plants, animals or microbes can be tested; (3) there is no requirements for isolation of cloned DNA probes, genomic libraries, radioactivities, southern transfers or preparation of hybridisation filers; (4) it is amenable to automation; and (5) low cost involved (Hadrys et al., 1992).

However, RAPD technique is sensitive to contaminants in DNA and pre-PCR preparation and slight alteration during experiment, due to its low stringency of the annealing step (Sun and Yu, 2000). Apart from precautions to avoid contamination, there are few disadvantages: (1) purified, high molecular weight DNA is compulsory; (2) band profiles can not be translated in terms of loci and alleles; (3) low reproducibility; and (4) similar sized fragments may not be homologous (Sun and Yu, 2000).

The arbitrary primers

PCR is used to amplify a known sequence of DNA, while RAPD reactions amplify segments of DNA, which are essentially unknown. In other words, a ten-oligo sequence is simply made by experimenters or is generated by a computer randomly, then used to synthesise the primer. The replacement of a single oligo in a primer can cause a whole change in the RAPD products. Furthermore, the same sets of primers can be used to perform the RAPD fingerprints of different plants. However, it is still necessary to carry out the RAPD procedure with a number of different arbitrary primers until differences are exposed. The random primers that were used in this study are from RAPD 10-mer Kits specifically developed for

RAPD analysis from Operon (German). Operon manufactures 26 kits, each containing 20 different arbitrary primers, which are currently available in the market.

Application of RAPD approach

RAPD technique can be used in molecular ecology to establish taxonomic characteristics, review association relationships, analyse varied genome samples and generate specific probes (Hadrys et al., 1992). The RAPD technique has been commonly applied for the analysis and identification of the fingerprinting genomes from plants, animals or microbes (Williams et al., 1990). A number of studies assessing the phylogenetic relationships of organisms have been reported using RAPD. For instances, the genetic maps of tea, grapes, bush mango and strawberries have been screened by RAPD method (Wachira et al., 1995, Siles et al., 2000, Lowe et al., 2000, Congiu et al., 2000). RAPD has also been employed to generate the genetic maps from animals, such as fish, cattle and sheep (Kantanen et al., 1995, Asensio et al., 2002, Bardakci and Skibinski, 1994). In addition, this technique has been popularly employed in bacterial and fungal pathology (Satokari et al., 2003, Cohen et al., 2001, Rychlik and Pavlik, 1997, Sullivan et al., 1996).

RAPD for identification and authentication of CHM

Genomic fingerprinting assays using RPAD have already been shown to be useful for the differentiation of Radix Ginseng (Ren-shen) and Radix Panacis Quinquefolii (Xi-yang-shen). Different random decamer primers were selected to reveal the genetic diversity of *Panax spp.* among different species and cultivation regions (Ma et al., 2000a, Jiang et al., 1998). Various primers were used as to their applicability in establishing RAPD assays as a rapid identification system for CHM, for instance, Rhizoma Atractyloids (Cang-zhu), from either the genuine source of plants *Atractylodes lancea* and *A. chinenesis*, or other three substitutes or adulterants (*A. koreana, A. ovata* and *A. japonica*) (Ren et al., 2000, Mizukami et al., 2000,

Kohjyouma et al., 1997). RAPD analysis also has been used for the identification and authentication of Radix Angelicae Sinensis (Dang-gui), Flos Lonicerae (Jin-yin-hua) and Fructus Lycii (Gou-qi-zi) (Gao et al., 2001, Yu and Shi, 2000, Zhang et al., 2001). In addition to single herb identifications, RPAD was also employed to identify the CHM components in herbal formula, Yu-Ping-Feng San, which contained Rhizoma Atractylodis Macrocephalae (Bai-zhu), Radix Saposhnikoviae (Fang-feng) and Radix Astragali (Huang-qi) (Cheng et al., 1998).

1.3.2.1.2 Arbitrarily-primed PCR (AP-PCR)

Almost the same year as RAPD technique invented, another group reported the establishment of another method, AP-PCR (Welsh and McClelland, 1990). The general principles and the procedures of AP-PCR are similar to RAPD that is the technique processes PCR amplification by only a single arbitrary oligo primer. Compared to a 10-oligo primer used in RAPD, AP-PCR amplification used a 20-30 necleotides long primer (Welsh and McClelland, 1990). The identity of CHM samples can be determined, and the substitutions of CHM can be detected by this method. For example, the authentic Radix Ginseng (Ren-shen) and Radix Panacis Quinquefolii (Xi-yang-shen) species and their adulterants were identified and authenticated using AP-PCR (Shaw and But, 1995).

1.3.2.1.3 Simple sequence repeat (SSR)

SSR, also known as microsatellites, is repeated in tandem, and spread throughout eucaryotic genomes (Grist et al., 1993). The PCR amplified fragments were obtained using a unique pair of primers, followed by detection on high-resolution gels. These days, SSR has been commonly used for genetic mapping with populations of plants species (Garnica et al., 2006). SSR also has been successfully revealed the genetic characteristics of CHM, including

Rhizoma Gastrodiae (Tian-ma) and Radix Ginseng (Ren-shen) (Xu et al., 2006, Kim and Lee, 2004).

1.3.2.1.4 Amplified fragment length polymorphism (AFLP)

AFLP is a highly sensitive technique used in the study of genomic fingerprints (Thomas et al., 1995). Three stages are involved in AFLP procedures, including digestion of the genomic DNA with one or more restriction enzymes, amplification of some restriction fragments using two PCR primers that represent adaptor and restriction site sequences, and followed by electrophoresis on high-resolution polyacrylamide gel. Since its discovery, AFLP has been used for the identification of species, molecular evolution and biological diversity, the comparison of the differential expression of genes, and gene mapping for bacteria, fungi, plans, and human (Latorra and Schanfield, 1996, Lin et al., 1996, Meksem et al., 1995, Mueller et al., 1996).

AFLP has been employed for the identification and authentication of CHM. For instances, Radix Ginseng (Ren-shen) and Radix Panacis Quinquefolii (Xi-yang-shen), from different growing locations in China or Korea (Ha et al., 2002). AFLP was also used for studying the complexity of application of CHM in Singapore market that included Randix Quinquefolii (Xi-yang-sheng), Radix Astragli (Huang-qi), Radix Notoginseng (San-qi), Cortex Cinnamomum (Gui-pi), Radix Isatidis (Ban-lan-gen), Radix Codonopsis (Dang-shen) and Radix Rehmannia (Sheng-di) (Yuan and Hong, 2003).

Compared to other PCR-based technologies, ALFP is more reproducible due to the inflexible condition by used specific primers. However, the requirements of the genetic knowledge of the samples and high quality of DNA become the barriers for application of SSR for CHM identification and authentication (Glick and Pasternak, 2003).

1.3.2.1.5 Direct amplification of length polymorphism (DALP)

This method resembles AP-PCR to produce DNA fingerprinting and sequence of the polymorphic loci (Desmarais et al., 1998). It has been employed for detection of the polymorphism of virus, plants and animals (Desmarais et al., 1998, Langar et al., 2003, Hu et al., 1999).

The method has been adapted for analysis of the genomic diversity between Radix Ginseng (Ren-shen) and Radix Panacis Quinquefolii (Xi-yang-shen). A 636-bp DALP band was generated from *Panax. ginseng*, the botanic sources of Radix Ginseng (Ren-shen) sample, which may contribute to authenticate the Radix Ginseng (Ren-shen) products (Ha et al., 2001).

High resolution of genomic profiles and high efficiency in isolation of large number of polymorphic fragments are regarded as the advantages of DALP. Moreover, no prior knowledge of the amplified site is required. However, the considerable requirements of time for sequencing the polymorphic DALP fragments is assuming as the disadvantage of this technique (Glick and Pasternak, 2003).

1.3.2.1.6 PCR – restriction fragment length polymorphism (PCR-RFLP)

General principles of PCR-RFLP

PCR-RFLP technique is based on a distinct DNA fragment, which is amplified by PCR, using a pair of universal primers, including forward and reversed primers. The selected restriction endonuclease is applied for digestion, followed by gel electrophoresis and illumination under UV (Maeda et al., 1989). A specific recognition region for a particular restriction endonuclease is present or absent in one DNA template so that differences of the DNA fragments of different sizes are produced (Glick and Pasternak, 2003).

PCR-RFLP is one of the most commonly used approaches for genetic testing, due to its: (1) small amount DNA templates needed for analysis; (2) more loci and exclusive alleles to perform valuable linkage studies; (3) better repetition; and (4) sensitive and efficient amplification of DNA from organisms (Glick and Pasternak, 2003, Arens, 1999). However, there are still some shortcomings involved in PCR-RFLP. For instances, the RFLP loci are not allocated consistently from every chromosome, and the maintenance of the protocols can be inconvenient and cumbersome (Glick and Pasternak, 2003).

Primers selection for PCR-RFLP

As the basic requirements of PCR amplification, the region for PCR analysis flanking the target sequence is known (McPherson et al., 1991). Due to the preservation of specific region, this region can be willingly amplified from many species by a 'universal primer'(Glick and Pasternak, 2003). Universal primers, which are designed to provide a 'universal specificity', can anneal to the preserved region of the target sequence and amplify DNA section in a wide range of taxa (Baker et al., 2003). Some candidates for 'universal primers' have been applied for PCR-RFLP analysis. The PCR-RFLP of rDNA (Carvalho et al., 2004, Abliz et al., 2004, Rachman et al., 2004) and the ribulose-1, 5-bisphosphate carboxylase L (rbcL) region (Van Droogenbroeck et al., 2004) have been successfully used to differentiate and identify the genetic profiles of organisms.

Application of PCR-RFLP

PCR-RFLP technique has been well established for genetic mapping study and evaluation of taxonomic relationship. It has been applied for a number of studies in various areas, such as ecology, virology, epidemiology, zoology and phytobiology (Glick and Pasternak, 2003). Botstein *et al* (1980) established a structure for reorganisation of polymorphic single-base-pair probes, and using them as a 'DNA marker loci', they have contributed to map human

chromosomes. Over more than two decades, PCR-RFLP has been used as one of the powerful methods to validate reference strains of different phytogenic organisms, such as beech (Vettori et al., 2004), papaya (Van Droogenbroeck et al., 2004), ananas (Duval et al., 2003), etc. Furthermore, PCR-RFLP used in human genetic mapping contributes to the clinical microbiology, for instances, the identification of *Burkhoderia spp.* (van Pelt et al., 1999), dermatomycoses (Machouart-Dubach et al., 2001), TNF (Keso et al., 2001), etc.

PCR-RFLP for identification and authentication of CHM

The PCR-RFLP of the 18s ribosomal ribonucleic acid (rRNA) gene has been proposed to distinguish *Panax spp*., whose dried roots are recognised as Radix Ginseng (Ren-shen) (Um et al., 2001). The approach has also been suggested conveniently to identify Rhizoma Menispermi (Ban-xia), the dried roots from *Pinellia ternate (Chung et al., 2002)*. Furthermore, it can be applied to the authentication of morphologically similar medicinal *Codonopsis* species for a CHM Radix Codonopsis (Dang-shen) (Fu et al., 1999).

1.3.2.2 Hybridization-based markers

A DNA fragment or synthetic oligo is used as a probe, which is labelled with radioisotopes or with conjugated enzymes that catalyse a colored reaction, to hybridise DNA (Huang and Kochert, 1994, Meyer et al., 1993). RFLP and minisatellite/microsatellite markers are representative of this technology and are employed in gene mapping and systematic and evolution studies in a wide range of organisms, plants, animals, and humans (Cheng et al., 2003, Haberfeld et al., 1991, Iwasaki et al., 1992).

Difficult to find the DNA probe may cause the failure to differentiate the genetic diversity between different *Glehnia littoralis* varieties of CHM Radix Glehniae (Bei-sha-shen) that

cultivated in different geographic conditions (Mizukami et al., 1993). In addition, the high quantity and quality of DNA from CHM are necessary for using RFLP techniques.

1.3.2.3 Sequencing-based markers

Sequencing-based markers focus on a defined locus, and the variation can be compared, based on the PCR techniques (Gillespie et al., 2006). The nuclear (5S, 18S and 28S) and mitochondrial (12S and 16S) sequences encoding rRNA (rDNA), related internally transcribed spacer (ITS) from nuclear ribosomal DNA (nrDNA), transfer RNA for lysine (*trn*K), rubisco large subunit (*rbc*L) from chloroplast DNA (cp DNA) are the locus frequently used as molecular markers (Gillespie et al., 2006, Bena et al., 1998, Cao and Komatsu, 2003, Soltis et al., 1992). Although the requirements of the genetic knowledge of the target genome may affect the application of sequencing-based markers for CHM identification, the reliability and reproducibility are the significant advantages of this approach over other molecular markers.

Recently, the spacer region of 5S rRNA was sequenced to verify that the crude CHM Radix Adenophorae (Sha-shen) was derived from roots of *Adenophora stricta* rather than *A. tetraphylla, A. hunanensis* and *Glihnia littorlis* (Zhao et al., 2003a). A sequence of 250bp amplified fragment from the conserved sequences of the 5S rRNA gene was used to identify the *Adenophora spp*. The sequence of 5S rRNA has also been used to authenticate Radix Notoginseng (San-qi), Radix Astragali (Huang-qi) and Radix Angelica (Dang-gui) (Cui et al., 2003, Ma et al., 2000b, Zhao et al., 2003b). The ITS of rDNA was selected for authentication of Herba Dendrobii (Shi-hu) (Lau et al., 2001). 18S rRNA has been used for verifying several *Panax* species, medicinal plants for Radix Ginseng (Ren-shen) and Radix Panacis Quinquefolii (Xi-yang-shen) (Fushimi et al., 1996). For *Curcuma* species, the 18S rRNA and *trn*K have been used to authenticate several related herbal medicines (Sasaki et al., 2002, Cao

et al., 2001). The molecular markers of 11 *Curcuma spp.,* including *C. longa* and *C. chuanhuangjiang* for Rhizoma Curcumae Longae (Jiang-huang)*, C. phaeocaulis, C. wenyujin, C. aromatica, C. sichuanensis* and *C. chuanujin* for Radix Curumae (Yu-jin)*,* and *C. zedoaria, C. kwangsiensis, C. chuanezhu* and *C. yunnanensis* for Rhizoma Curcumae (E-zhu) were revealed. Furthermore, based on the sequence analysis of 18S rRNA and *trn*K genes, 25 CHM formulations contained *Curcuma* species were identified as well (Sasaki et al., 2002).

1.3.3 Spectrophotometric and chromatographic techniques

With the development of chemical and physical sciences, and analytical instruments, analytical chemistry is now widely used in the quality and quantity analysis of chemical compounds (Chun, 2003). Compared to the classic electroanalytical methods, a number of instrumental approaches have been employed as powerful analytical tools including spectrophotometry, atomic spectroscopy, MS, NMR, X-ray spectroscopy and chromatography (Banasal et al., 2004). The brief principles and classifications of various analytical methods are presented in Table 1.17.

Analytical chemistry deals with the identification of quantity and quality of various chemical compositions that contributes to different areas, which involve pharmaceuticals, food industry, and environmental improvement (ACS, 2003). Recently, chemical analytical techniques have become a valid approaches to identify marker compounds of CHM, and have been contributed to qualitative and quantitative analysis of the bioactive constituents and quality evaluation of CHM (Mahady, 2001). In current CHM regulatory requirements in pharmacopoeias and authorised guidelines, UV spectrophotometry, atomic absorption (AA) spectrophotometry, TLC, HPLC, gas liquid chromatography (GLC), MS, GC-MS and LC-MS are commonly quoted techniques (AHP, 2004, BHMAPublications, 2000, Akerele, 1984, The Pharmacopoeia Commission of People's Republic of China, 2005).

Nevertheless, there are limitations in chemical analysis. Physiological circumstances, cultivation period, drying conditions and preparation circumstances could affect the variability and quantity of the active compositions of CHM. In addition, the similarities of chemical components among related medicinal species and varieties as well as the high cost of the advanced instruments, for instances HPLC, GC-MS and NMR, are also considered (Joshi et al., 2004).

Table 1.17 The classifications and brief principles of the commonly used analytical approaches (Kwak, 1997, Skoog et al., 2004)

Method	**Brief principles**	Common types of instruments
Electroanalytical chemistry	Based on the Redox reaction and inter-relationship between electrical and chemical energy	Potentiometer Coulometer Electrogravimeter Voltammeter
Spectroscopy	Electromagnetic radiation, treated as photons, is absorbed by analytical samples that associated with wavelength, frequency and wavenumber.	X-ray spectroscopy Spectrophotometry NMR spectroscopy
Spectrophotometry	More specific than general spectroscopy that is the study of spectra.	UV spectrophotometers Visible spectrophotometers IR spectrophotometers

Continued Table 1.17 The classifications and brief principles of the commonly used analytical approaches (Kwak, 1997, Skoog et al., 2004)

Method	Brief principles	Common types of instruments
Atomic spectroscopy	The amount of radiation absorbed by ground-state atoms created in a flame or a minifurnace is measured.	Atomic emission spectroscopy AA spectroscopy Atomic fluoresecne spectorcopy
Mass spectrometry (MS)	Small molecular is converted to ions so that they can be operated by external electric and magnetic fields	Tandem MS Chromatography – MS
Chromatography	Based on the differences in separating characteristics between a mobile phase and a stationary phase, separate the compounds.	TLC, LC, HPLC, GC Ion-exchange chromatography (IEC) Gel permeation chromatography (GPC) Affinity chromatography (AC)
Electrophoresis	Based on the movement of ions, which have various migration rate, separate the compound	Paper / gel electrophoresis Capillary electrophoresis (CE)

1.3.3.1 Spectroscopy

Spectroscopy is production, measurement and analysis of matter and its properties by investigating light, sound or particles as a result of the emission, absorption or scattering. Common types of spectroscopy, including flame spectroscopy, visible spectroscopy, UV spectroscopy, IR spectroscopy, thermal IR spectroscopy, NMR, photoemission spectroscopy are used for different measurement processes (Skoog et al., 2004). Recently, with the employment of the electromagnetic and non-electromagnetic radiations, such as microwaves, radiowaves, x-rays and other spectroscopy is broadly used in physical and analytical analysis (Skoog et al., 2004). X-ray spectroscopy, NMR and spectrophotometry are the most commonly used categories of spectroscopy for study the chemical profiles of CHM.

1.3.3.1.1 X-ray spectroscopy

X-ray spectroscopy technology has been employed in the Chinese Pharmacopoeia to identify the CHM products (The Pharmacopoeia Commission of People's Republic of China, 2005). X-ray spectroscopic fingerprinting has been applied for the identification of CHM, including Radix Gentianae Macrophyllae (Qin-jiao), Herba Oldenlandia (Bai-hua-she-she-cao), Rhizoma Ligusticum Chanxiong (Chuan-xiong), Radix Rehmanniae Preparata (Shu-di), Radix Paeoniae Alba (Bai-shao) and Radix Glycyrrhizae (Gan-cao) (Zhang and Gong, 2004, Liu et al., 2003b, Jia et al., 2003, Wang and Li, 2002, Wu and Wang, 2002b, Wu and Wang, 2002a).

1.3.3.1.2 NMR spectroscopy

NMR is a powerful technique to maintain physical, chemical and electronic information on the three-dimensional structural of molecules, based on the magnetic properties of an atom's nucleus (Skoog et al., 2004). NMR spectroscopy is widely used for medical diagnosis and chemical analysis of biological samples, including nucleic acid (RNA and DNA) or protein.

Proton NMR and carbon-13 NMR are the most commonly used techniques for revealing the chemical compositions of CHM. The methods have been employed to establish the fingerprinting profiles of Radix Ginseng (Ren-shen), Randix Quinquefolii (Xi-yang-sheng) and Radix Notoginseng (San-qi), which may contribute to standardise the herbal products contained *Panax spp.* (Qin and Zhao, 1999). NMR also has been applied for revealing the chemical compositions of Radix Rhapontici (Lou-lu), Semen Cuscutae (Tu-si-zi) and *Aloe vera* (Wang and Fang, 2001, Cheng and Zhang, 2002, Zhang and Sun, 2000). Furthermore, NMR-MS has been used for isolation of new chemical components from CHM. Seven saponins from *Astraglus memkranaceus*, as one of the botanic sources of Radix Astragali (Huang-qi), were isolated using NMR-MS (Cao, 2002).

1.3.3.2 Spectrophotometry

Spectrophotometry is more specific than the general spectroscopy. Different types of spectrophotometers are available for measuring the wavelength of light (Banasal et al., 2004, Skoog et al., 2004). Three major classes of spectrophotometry are visible-region spectrophotometers, UV spectrophotometers and IR spectrophotometers, which are commonly used for CHM identification and authentication.

1.3.3.2.1 Ultraviolet (UV) spectrophotometers

UV spectrophotmeter uses light in the visible and adjacent near UV and near infrared (NIR) ranges (Skoog et al., 2004). UV spectrophotometry is routinely used in the quantitative determination of organic compounds, with a high degree of conjugation. Due to the organic solvents may have signifiant UV absorption, water usually is used for water soluble compounds, while ethanol is used for organic soluble compounds. UV spectrophotometer is also recognised as one of the most commonly seen detectors for HPLC system (Toussaint et al., 2000).

As the basic part of a UV spectrophotometer, an incandescent bulb for visible wavelengths, or a deuterium arc lamp for the UV wavelenth is applied with a holder for the sample. Testing samples are placed in cuvette, which made from high quality quartz, sometimes galss and plastics are also employed. A photodiode and a change couple devide (CCD) are two commonly used UV spectrometer detectors (Skoog et al., 2004).

UV spectrophotometry has been applied for the identification of CHM, including Radix Puerariae (Ge-gen), Pericarpium Trichosanthis (Gua-lou-pi), Flos Buddlejae (Mi-meng-hua) and Rhizoma Cyperi (Xiang-fu) (Yu et al., 2005a, Li and Cao, 2005, Tu, 2005 , Xun and Chen, 2000). With the rapid development of UV spectrophotometry, this approach is becoming one of the main techniques to establish CHM fingerprinting profiles.

1.3.3.2.2. Infrared (IR) spectrophotometers

IR spectrophotmeter uses light in the near-, mid- and far- IR ranges to measure the wavelenth and intensity of the absorption of IR light (Skoog et al., 2004). The IR region is devided into three sections, incluidng, far-IR (400 – 10 cm^{-1}), mid-IR (4000 - 400 cm^{-1}) and near-IR (14000 - 4000 cm^{-1}). In the 1970s, Fourier transform IR spectrometer (FTIR) was developed as a single-beam instrument (Lopez-Avila et al., 1993). It becomes widely used in both research and industry for quality control and dynamic measurement. In order to make the assessment quicker and more accurate, advanced technologies, such as specular reflection, diffuse reflection, attenuated total reflectance and photoacoustic spectroscopy have also been developed (Drozd et al., 2006, Smith et al., 2003, Hutson et al., 1988, Graves and Luo, 1994).

IR spectrometer has been used for the identification of a number of CHM, including Radix et Rhizoma Rhei (Da-huang), Rhizoma Coptidis (Huang-lian) and Cornu Cervi (Lu-rong) (Tian and He, 1992, Zheng and Zhou, 1996, Fan et al., 2005). The approach has also been applied to

differentiate *Cyathula officinalis*, authentic species for CHM Radix Cyathulae seu Achyranthis (Niu-xi), from its adulterant (*Arctium tappa*) (Li et al., 2000b).

1.3.3.3 Mass spectroscopy (MS)

MS is an analytical technique used to measure the molecular mass of the analytes (Kwak, 1997). In early 19th century, English scientist J. J. Thomson created the first mass spectrograph. A. J. Dempster and F. W. Aston devised the modern version of MS in 1918-1919 (Skoog et al., 2004). Three basic parts involve in a typical MS, including an ion source, a mass analysor and a detector.

MS has been applied for the determination of isotopic composition of elements (Danesi et al., 2003), trace gas analysis (Fontanals et al., 2006), pharmacokinetics (Liang et al., 2006a) and the characterisation, identification and quantitation of protein (Areces et al., 2004). In resent decade, hyphenated MS has been used in a wide range, for instances, GC-MS, LC-MS and CE-MS. These technologies have been intensively employed for CHM identification and authentication, which will be reviewed in the following sections.

1.3.3.4 Chromatography

In 1906, the term "chromatographic experiments" firstly appeared in Mikhail Semenovich Tswett's paper that established chromatography as a technique for the analysis of chlorophyll (Zolotov, 2003, UMICH, 2005). Since then, chromatography has been applied in various research areas, such as organic and inorganic chemistry, biochemistry and analytical chemistry, in the fields of medicine, food, cosmetics, and environmental industries (Skoog et al., 2004).

Chromatography is classified in two types: column chromatography and planar chromatography (IUPAC, 1993a). Planar chromatography includes paper chromatography and TLC (IUPAC, 1993b). Based on the various types of mobile phase, the sub-classification of column chromatography involves LC, GC and supercritical fluid chromatography (SFC) (Skoog et al., 2004). The differences of mobile phase, stationary phase, and column between different types of chromatography are shown in Table 1.18.

The past century has been a vast undertaking in the development of chromatographic techniques. By comparison, TLC and HPLC provide rapid and reliable chemical approaches for ensuring botanical identity for most of CHM. However, GC procedures are more suitable for evaluating the volatile components (Li et al., 1998). In the current Chinese pharmacopoeia, TLC method has been adopted for the identification in 602 monographs, and HPLC method appeared in 105 monographs (The Pharmacopoeia Commission of People's Republic of China, 2005).

Table 1.18 Classification of column chromatographies

Classification	Application	Mobile phase	Stationary phase	Specific methods
Liquid chromatography (LC)	Organic or inorganic molecular dissolved in the solvent	Liquid	Solids	Adsorption chromatography (APC)
			Ionic groups on a resin	Ion-exchange chromatography (IEC)
			Liquids on an insert solid support	Partitioning chromatography (PC)
			Porous insert particles	Size-exclusion chromatography (SEC)
			Immobilised solids	Affinity chromatography (AC)
Gas chromatography (GC)	Organic volatile components	Gaseous	Immobilised liquid	Gas liquid chromatography (GLC)
			Solid	Gas solid chromatography (GSC)
Supercritical fluid chromatography (SFC)	Thermally unstable components	Supercritical fluid chromatography	A highly viscous liquid on a solid surface	

1.3.3.4.1 Thin layer chromatography (TLC)

Principles and application of TLC

In 1951, American scientist J. G. Kirchner firstly reported a new chromatographic technique, and he called it 'chromatostrip' (Kirchner et al., 1951). In 1954, R. H. Reitsema applied similar approach to separate the essential oil that was named as 'chromatoplate' (Reitsema, 1954). In the 1960s, E. Stahl contributed to the optimisation and standardisation of TLC technology (Ettre, 2005). High performance TLC (HPTLC) was born in late 1970s, which significantly increase the performance capacity, compared to the classic TLC technique (Kaiser and Rieder, 1977).

The TLC technique involves using a solid fixed or stationary phase (the thin-layer), which is usually made from adsorbent material (silica gel, aluminium oxide, or cellulose) applied to a glass or a plastic plate, and a liquid mobile phase (elute solvent) that is applied to separate the analytes, via capillary action. The experimental solution is placed above the bottom of the TLC plate. After placed the plate in a developing chamber, in which the test solvent will either remain with the solid phase or dissolve in the elute solvent (Skoog et al., 2004).

The TLC technique has been widely used for the determination and identification of biological and toxic samples (Raharjo and Verpoorte, 2004, Matsumura and Mori, 1998), analysis environmental hazardous pollutant (LeBlanc et al., 2003), and chemical compounds of food, cosmetic and pharmaceutical (Komsta and Misztal, 2005, Simko, 2002, Biju et al., 2005).

Stationary phase and mobile phase

The stationary phase for TLC divides into two categories: self-coating preparation and pre-coated TLC layers. A wide range of absorbents can be obtained that are used for preparation

of TLC plates, mainly including silica gel, aluminium oxide and cellulose. Self-coating plates need to be activated before sample application. Therefore, the pre-coated TLC plates are utilised due to its high quality and convenience to that of self-coating layers. TLC/HPTLC silica gel plates, TLC/HPTLC aluminium oxide plates and TLC/HPTLC cellulose plates are generally available in the market. The commonly sizes of plates are 10cm x 10cm, 10cm x 20cm, 20cm x 10cm and 20cm x 20cm. Based on the polarity of the each solvent, the mobile phase has its own solvent strength. To maintain the best separation results, the optimal mobile phase is crucial.

TLC system

Basic TLC kit includes capillary pipettes for sampling, a developing chamber, a UV cabinet for detection and sprayer for colour developing reagents. In order to achieve the best test conditions, more and more new components have been invented (CAMAG, 2006a). Auto-sampler has been employed to minimize the size of sample application on the plate, while maximum the volume of sample application. Automated developing chamber (ADC) was invented to keep the development fully automatic and reproducible. The automated multiple development system (AMD) has lead gradient elution and multiple development firstly appeared in TLC technology. High-resolution camera with the documentation system and the advanced scanner for densitometric evaluation were developed for measurements of refection and documentation of images. In addition, the computer technology has been hyphenated with TLC system. For instance, the planar chromatography manager, such as winCATs from CAMAG, now is popularly used for automatical information analysis.

Application of TLC in identification and authentication of CHM

TLC has been widely used for the identification and authentication of CHM. Rhaponticin, the toxic compounds, which were found in the adulterants of Radix et Rhizoma Rhei (Da-huang),

was determined on silica gel plates with mobile phase composed of ethyl acetate : methanol (8:2) (Yue et al., 1986). Bioactive compounds, matrine and oxymatrine, from Radix Sophorae Tonkinensis (Shan-dou-gen) were successfully separated on silica gel using chloroform : methanol : aminoprophyl (NH_2) (4:1:0.1) (Zhang and Gu, 2002). Moreover, TLC has been applied for quantitative and qualitative analysis of chemical compositions of CHM. Four saponins from Radix Notoginseng (San-qi) were determined on methanol extraction using silica gel with the mobile phase composition of 1,2-dichloroethane : 1-butanol : methanol : water (30:40:15:25) (Zhou et al., 1981). Berberine, from Rhizoma Coptidis (Huang-lian), was quantitatively determined on silica gel using ethyl acetate : 2-butanol : formic acid : water (10:7:1:1) (Zang, 1986). TLC method has also been employed to assess the quality of particular species in order to indicate the clinical preference of CHM. Three Radix Bupleuri (Chai-hu) species, including *Bupleurum polyclonum. B. kunmingense* and *B. luxiense*, in Yunan Province, China, were found to contain high content of bioactive saponins using TLC (Pan et al., 1983). Although those species are not listed in the authorised literatures, the findings suggested that those species might be considered for clinical application in certain regions. With the development of TLC system, such as CAMAG TLC system coupled with the winCATs software, the method for determination of trigonelline hydrochloride from Semen Juglandis (Hu-lu-ba) was validated with the good linearity, precision and recovery (Chopra et al., 2006).

1.3.3.4.2 High performance liquid chromatography (HPLC)

Principles and application of HPLC

In the 1960s, one of the modern analysis methods, HPLC started to evolve in the development of modern chemical analysis (Horvath, 1980). During the early stage of development of LC, low plate heights and low flow rates were major obstacles to increase the efficiency of separation (Skoog et al., 2004). Since then, the diameter of packing materials that

dramatically decreased to several micrometer, and the development of high pressure pump system, have been recognised as the evolution of the column chromatography (Michaelis et al., 1973). Therefore, C. Horváth built the first HPLC instrument to separate the biological substances in Professor S. R. Lipsky's laboratory at Yale University (Guttmán, 2004). Now, a HPLC system involves a reservoir of mobile phase, a sample injection system, a high-pressure pumping system, a separation column and detectors.

Stationary phase and mobile phase

The selection of stationary phase and mobile phase is critical for development of a HPLC method. The commonly used stationary phase is considered as porous particles that are made from high purity silica and permeable to solvent (ASRG, 2004). Particles consideration dictates a wide selection, for instances, hydrophobic groups bonded to a silica support commonly used for reversed-phase chromatography (RPC), unbonded silica or bonded amino or cyano group regularly applied for normal phase chromatography (NPC) and bonded ionic groups utilized for IEC (Agilent, 2004). In HPLC system, mobile phase carries the analytes through the stationary phase, and then on account of the their polarity, produces separation (Skoog et al., 2004). Different HPLC separation modes are shown in Table 1.19.

Table 1.19 Various HPLC separation modes (Agilent, 2004, Skoog et al., 2004, Phenomenex, 2003).

Stationary phase	Columns	Mobile Phase	Separation mode
Silica	Luna Silica (Phenomenex, U.S.A); ZORBAX Rx-SIL (Agilent, U.S.A).	Hexane, alcohols	AC, NPC
Octadecylsilyl and Octylsilane	Gemini C_8 and C_{18}; Luna C_8 and C_{18} (Phenomenex, U.S.A); ZORBAX Eclipse XDB C_8 and C_{18} (Agilent, U.S.A)	Water, methanol, acetonitrile	RPC
Cyanoprophyl (CN)	Luna CN, Capcell CN (Phenomenex, U.S.A); ZORBAX Eclipse XDB-CN (Agilent, U.S.A)	Water, alcohol for RPC Hexane, ether for NPC	NPC
Aminopropyl (NH_2)	Capcell NH_2 (Phenomenex, U.S.A); ZORBAX NH_2 (Agilent, U.S.A)	Water, alcohol	RPC
Other polymers	SHODEX (Phenomenex, U.S.A); Ultron chiral column, ZORBAX PSM, ZORBAX SAX and ZORBAX SCX (Agilent, U.S.A)	Modifiers	IEC, NPC, AC, RPC, SEC

Detectors

HPLC requires high sensitive detectors to detect the eluted analytes and consequently indicate a peak on the chromatogram (Ho and Stuart, 2003). Examples of detectors include UV detectors, refractive index detectors (RID), fluorescence, low temperature evaporative light scattering detectors (ELSD), chemiluminescence, electrochemical, MS, NMR, etc (Wu et al., 2004a, Sato et al., 1990, Li et al., 2004b, Henderson et al., 1999, Ikarashi et al., 2001b).

The UV detector has been considered as one of the most commonly used HPLC detectors (Toussaint et al., 2000). Based on the UV absorption by analytes passing through a flow cell, the voltage changes are illustrated as a peak and recorded at the retention time for the peak as area in a chromatogram (Ho and Stuart, 2003). Compared to the measurements of only one single wavelength by fixed wavelength detectors or one wavelength a time over specific range of wavelengths by variable wavelength detectors, diode array detectors (DAD) measure a wide range of wavelengths simultaneously (Valentao et al., 1999).

Development of HPLC — evolution of reversed phase chromatography and gradient elution

In the early stage of LC, a polar stationary phase and a relatively non-polar mobile phase such as hexane were focused, thus named NPC (Skoog et al., 2004). However, RPC, a relatively polar mobile phase and non-polar stationary phase, becomes the most popular separation mode (Hagan, 1994). The RPC is prevalently used for analysis of organic components, most of which can be dissolve in the modified mobile phase, containing aqueous soluble substances mixed with various concentrations of organic solvent, such as methanol, ethanol, acetonitrile, etc. (Ho and Stuart, 2003). RPC has been popularly applied for the chemical fingerprints of the botanics (Arnoldi et al., 2004, Schaneberg et al., 2003).

Obtaining the balance of sufficient separation and satisfactory retention time for each analytes is not easy for the optimisation of HPLC. The isocratic elution mode, which means the composition of the mobile phase kept constant during the whole analysis, often causes poor isolation of early peaks, expansion the later peaks, or prolonging the whole analytical procedure (Lee and Row, 2004). The new gradient elution chromatography opens a new era. Progressively increasing the proportion of organic solvent and the ionic strength of the mobile phase with time during the analysis is called gradient elution (Snyder, 1980). Gradient elution has been suggested as a solution to problems, which can be found in the usage of isocratic elution system, by improving the early eluting peaks, limiting the broadening of the later peaks and decreasing the time of analysis (Kress et al., 2002, Purcell et al., 1999).

Application of HPLC in identification and authentication for CHM

During past decades, HPLC has been applied for the isolation and purification of organic compounds, and the identification and quantification of chemical components (Horvath, 1980). As one of the main approaches of chemical analysis, HPLC provides a reliable, sensitive and reproducible technique (Li et al., 1998).

The application of HPLC to the quantitative and qualitative analysis of herbal medicines has been listed in the WHO guideline for regulation of the herbal medicine, the Chinese Pharmacopeia, the American herbal pharmacopeia and the British herbal Pharmacopeia (WHO, 2003, WHO, 2004a, AHP, 2004, BHMAPublications, 2000, The Pharmacopoeia Commission of People's Republic of China, 2005). HPLC method has been employed for the determination, identification, and evaluation of CHM. Moreover, it is recognised that modern HPLC method can be one of the main approaches to generate fingerprints of numerous compounds that are found in analysis of high complex CHM (Gong et al., 2003). Numbers of studies reported that the identification and authentication of CHM using HPLC (Table 1.20).

HPLC has also been used to isolated the bioactive components from CHM, including MTB, Danshensu, isotanshione II_A and cryptotanshinone in Salvia Miltiorrhizae (Dan-shen); rutin, quercitrin, quercetin, kaempferol and isorhamnetin in *Ginkgo biloba*, saponins in Radix Ginseng (Ren-shen); and andrographolide and neoandrographolide in Herba Andrographis (Chuan-xin-lian) (Zhang et al., 2002a, Zhang et al., 2005a, Dubber and Kanfer, 2004, Samukawa et al., 1995, Srivastava et al., 2004). Furthermore, detection of the declared drugs in CHM has been regarded as another potential advantage of HPLC (Liu et al., 2001a, Liu et al., 2000).

Table 1.20 Recent studies on the identification and authentication of CHM using HPLC

Name of CHM	**Standard compounds**	**Column**	**Mobile phase**	**UV wavelength**	**Reference**
Radix Paeoniae Alba (Bai-shao)	Paeoniflorin	Supelco Discovery (150mm x 4.6mm, 5µm)	Methanol : potassium phosphate: isopropanol : acetic acid (67:173:4:4)	230nm	(Liu et al., 2001b)
Cordyceps (Dong-chong-xia-cao)	Ergosterol	Allsphere ODS-II, 5µm	Methanol : water (95:5)	275nm	(Li et al., 2001b)
Fructus Chinensis (Wu-wei-zi)	Schizandrin Gomisin, Deoxyschizandrin γ-schizandrin Gomisin Wuweizisu C	Nucleosil 100 $C_{18,}$ 5µm	Acetonitrile : water (50:50 – 70:30) Methanol : water (70:30 – 95:5)	254nm	(Bartlova et al., 2002)
Rhizoma Ligusticum Chuanxiong (Chuan-xiong)	Ferulic acid	ODS C_{18} (250mm x 4.6mm)	Methanol : water : acetic acid (30:67:3)	322nm	(Wang and Kuang, 2002)

Continued Table 1.20 Recent studies on the identification and authentication of CHM using HPLC

Name of CHM	Standard compounds	Column	Mobile phase	UV wavelength	Reference
Radix Salviae Miltiorrhizae (Dan-shen)	MTB Danshensu Isotanshinone II_A Eryptotanshinone	Discovery RP – Amide C_{18} (250mm x 4.6mm, 5µm)	Methanol : water (85:15)	270nm	(Zhang et al., 2002b)
Radix Astragali (Huang-qi)	Astragaloside IV	Phenomenex ODS (250mm x 4.6mm)	Acetonitrile : water (32:68)	203nm	(Zhang, 2003)
Fructus Arctii (Niu-bang-zi)	Arctiin	Bondapak C_{18} (250mm x 4.6mm, 5µm)	Methanol : water (1:1.1)	280nm	(Zhang, 2004)
Flos Chrysanthemi Indici (Ye-ju-hua)	Cyclohexanecarboxylic acid	Diamonsil™ C_{18} (250mm x 4.6mm, 5µm)	Acetonitrile : water : phosphoric acid (12:88:0.04)	327nm	(Tan et al., 2004)
Radix Sophorae Flavescentis (Ku-shen)	Martine Oxymartine	Hypersil ODSII (200mm x 4.6mm, 5µm)	Methanol: acetonitrile : water : phosphoric acid (10:30:65:0.05)	210nm	(Zhang et al., 2004)

Continued Table 1.20 Recent studies on the identification and authentication of CHM using HPLC

Name of CHM	Standard compounds	Column	Mobile phase	UV wavelength	Reference
Herba Andrographis (Chuan-xin-lian)	Andrographolide Neoandrographolide	Merck RP_{18c} (100mm x 4.6mm)	Acetonitrile : water (50:50)	220nm	(Srivastava et al., 2004)
Radix et Rhizoma Rhei (Da-huang)	Aloe-emodin Rhien Emodin Chrysophanol	Kromasil ODSII-1 (200mm x 4.6mm, 5μm)	Methanol : water : acetic acid (92:8:0.4)	254nm	(Li, 2005)
Semen Astragali Complanati (Sha-yuan-zi)	Complanatuside	VP-ODS (150mm x 4.6mm, 5μm)	Acetonitrile : 1% HAC solvent (18:82)	266nm	(Liu et al., 2005)
Radix Angelicae Sinensis (Dang-gui)	Ligustilide	Diamonsil C_{18} (250mm x 4.6mm, 5μm)	Methanol : water (70:30)	324nm	(Wang et al., 2005)
Cortex Phellodendri (Huang-bai)	Obaculactone	Diamonsil C_{18} (250mm x 4.6mm, 5μm)	Acetonitrile : water : phosphoric acid (50:50:0.2)	210nm	(Ma et al., 2006)

Identification and authentication of CHM using LC-MS

LC-MS is the technology that combines the separation capabilities of LC with the mass analysis capabilities of MS. Compared to other identification techniques, LC-MS not only isolate and detects the chemicals, but also potentially identifies the chemical structures of these chemicals. LC/MS is very commonly used in pharmacokinetic and proteomic studies (Liu et al., 2007, Shen et al., 2006). LC-MS has been used for the identification and authentication of CHM dealing with a complex mixture of chemicals. Moreover, the technology contributes to the discovery of the new bioactive compounds of CHM and revealing the chemical structures of these compounds. LC-MS has been used to identify the glycyrrhizin in Radix Glycyrrhizae (Gan-cao), andrographllide and 14-deoxy-11,12-didehydroandrographolide in Herba Andrographis (Chuan-xin-lian), lipophilic and hydrophilic components in Radix Salivae Miltirorrhizae (Dan-shen), and flavonol glycosides and aglycones in *Ginkgo biloba* (Yin-xing) (Dubber et al., 2005, Liu et al., Li and Wang, 2002).

1.3.3.4.3 Gas chromatography (GC)

Principles and GC systems

In 1945, Germen scientist F. Prior devloped the world first solid state GC (Bobleter, 1996). Based on the thermal stability and volatility of the chemical compounds, the anayltes are seperated and analysed (Skoog et al., 2004). In GC, the mobile phase is a carrier gas, which usually is helium or nitrogen, and the stationary phase is high bioling liquid, which is packed in a long narrow glass or metal column (Kwak, 1997). Based on the different staitionary phases, solid state GC and liquid state GC are the two types of GC. GC is commonly used for separation of volatile components and even some involatile compounds, such as amino acids, steroids, and high molecular fatty acides (Haiyan et al., 2007, Hope et al., 2005, Crook, 1972).

A basic GC system involves a gas source, a flower controller, a sampe injector, a column and its oven and a detector (Kwak, 1997). As one of the commonly used gas source, hydrogen is suggested given the best efficiency and best serperation (Ray, 1958). However, helium as a non-flammable gas, has wide flowrates and is suitable for most of detectors (Ray, 1958). Split, splitless, or cool on column intel system is generally applied (Garrett, 1998). Recently, purge and trap system and solid phase microextraction (SPME) have been introduced to GC technique that offered a conventient and low-cost pre-concentration and purification steps (Rosell et al., 2006, Motlagh and Pawliszyn, 1993). Generally, a 1.5–10m long stainless steel or glass column, packed with solide support material coated with a liquid or solid stationary phase (Kwak, 1997). For instance, the capillary columns are usually made of fused-silica with a polyimide outer coating in order to allowing the components remaining longer and better seperation (Skoog et al., 2004). The flame ionization detector (FID) and the thermal conductivity detector (TCD) are the most commonly used detectors (Tvrzicka et al., 2002). TCD is less sensitive than FID towards organic molecules, but TCD is non-destructive for preparative applications. Other detectors include discharge ionization detector (DID) (May et al., 2003), electron capture detector (ECD) (Bigdeli and Collins, 1975), flame photometric detector (FPD) (Hoshika, 1982), hall electrolytic conductivity detector (ElCD) (Dynes and Thorburn Burns, 1987), helium ionization detector (HID) (Roberge et al., 2004), nitrogen phosphorus detector (NPD) (Beljean-Leymarie and Bruna, 1988), mass selective detector (MSD) (Doyle et al., 1990), photoionization detector (PID) (Hill and Baim, 1982) and pulsed discharge detector (PDD) (Forsyth, 2004).

GC-MS

In 1959, Holmes and Morrel invented the first GC-MS system (Gohlke and McLafferty, 1993), based on the chemical properties of the components, GC separates the molecular, and followed by MS identify them based on their specific fragment spectrum, with the connection

of the database of standard references (Skoog et al., 2004). The most universal database NIST/EPA/NIH mass spectral library is maintained by the National Institute of Standards and Technology (NIST), the United States Environmental Protection Agency (EPA) and the National Institutes of Health (NIT) (NIST, 2005). GC-MS has been commonly used in medicines, evironmental analysis, forensics and astrochemistry (Panteghini and Forest, 2005, Betty and Karasek, 1978, Lachenmeier et al., 2006, Nuevo et al.).

Application in identification and authentication of CHM

A number of CHMs, mainly containing high content of volatile components, have been identified using GC-MS, such as 27 chemical compounds from Folium Perillae (Zi-su-ye) (Cui et al., 2002), and 20 volatile components from Herba Menthae (Bo-he) (Li et al., 2001a). Fifty volatile components from Rhizoma Curcumae Longae (Jiang-huang) were separated and 13 of them were identified using GC-MS system (Chen, 2001). Qin *et al* (2001) reported that 65 volatile compounds from Pericarpium Zanthoxyli (Hua-jiao) were identified from 80 separated components. Moreover, the approach was also used to separate and identify other CHMs, including Rhizoma Ligusticum Chuanxiong (Chuan-xiong) (Yang et al., 2002), Cacumen Platycladi (Ce-bai-ye) (Wei and Wang, 2001), Cortex Cinnamomi (Rou-gui) (Li and Yuan, 2000) and Herbal Artemisiae Anuae (Qing-hao) (Guo et al., 2004a).

1.3.3.5 Capillary electrophoresis (CE)

CE is the method based on the conflicting electrophoretic mobility, in a conductive buffer and narrow capillary. The CE instrument consists of a sample vial, source and destination vials, a capillary, electrodes, a high-voltage power supply, a detector and a data analysor (e.g. a computer). CE technology has been applied for the basic researches of pharmaceuticals (Macia et al.), proteins (Laing et al., 2006), forensics (Cengiz et al., 2004), toxicology (Thormann et al., 1998), etc.

Recently, CE or CE-MS have been used for the identification and authentication of CHM, for example, three active compounds, cryptotanshinone, tanshinone I and tanshinone IIA in Radix Salivae Miltiorrhizae (Dan-shen) (Gu et al., 2004). The procedure was also used for the chemical electrophoregrams of *Ginkgo biloba* (Yin-xing) using CE with DAD (Ji et al., 2006). Three bioactive components, baicalin, baicalein and wogonin in Radix Scutellariae (Huang-qin), were successfully separated by CE (Yu et al., 2007). The active components, magnolol and honokiol from Cortex Magnoliae Officinalis (Ho-pu), were separated under the optimised CE conditions (Tian and Chen, 2006).

1.3.4 Fingerprinting techniques

Fingerprints are chromatograms, electrophotograms and/or other illustrative grams that represent the unique characteristics of CHM by various analysing techniques. The application of fingerprinting techniques offers consistent and reproducible approach in studying pharmaceuticals (Welsh et al., 1996, Collantes et al., 1997, Aksenova et al., 1999), environmental substances identification (Wang et al., 2004f), classification of food products (Berente et al., 2000), forensic drug samples (Klemenc, 2001), etc.

In 1996, fingerprints were introduced to quality control and standardise the CHM products by WHO (WHO, 1996). Food and Drug Administration (FDA) issued that the chromatographic fingerprints of herbal products were sufficed if the identities of the active constituents or characteristic markers in drug substance was unknown (CDER, 2004). The European Anency for the Evaluation of Medicinal Products (EMEA) also addressed that the chemical fingerprints of herbal products as a whole would be reflected the quality of the drug substances (The European Anency for the Evaluation of Medicinal Products, 2002).

Fingerprints of CHM need to be exclusive and with unique features. Furthermore, stability is also required from CHM fingerprints, and especially in the main common peaks and the characterised peaks is relatively constant. Moreover, the well-established CHM fingerprints are also reproducible within a limit error. Although the fingerprinting technique is considered as an efficient and reliable quality control approach, there are some limitations. The intrinsic and extrinsic factors, including confusion of usage of medicinal plants, various environmental factors, different harvesting seasons, post-manufacture factors, which are detailed in Section 1.2.2, are the main encountered obstacles for establishment of CHM fingerprints. Therefore, high quality and reliable herbal sources are essential for this technique. In some cases, the lack of correlation between the fingerprints and pharmacological actions is another major concern. Generally, the fingerprints of CHM products can represent the consistence and content of their molecular and chemical characteristics, regardless if the bioactivities of these chemical compositions are unknown.

1.3.4.1 Main techniques

There are two categories for CHM fingerprints: chemical fingerprints (eg. TLC, HPLC) and molecular fingerprints (eg. RAPD, PCR-RFLP). Chromatographic approaches are commonly reported for a chemical fingerprint of CHM (Li et al., 2004a). TLC fingerprints has been recognised as a fast, economic and widely used technique (Cai et al., 2000b). However, the lack of sensitivity and difficulty to detect the microconstituents are the limitations for TLC fingerprints (Cai et al., 2000b). HPLC and GC, which may coupled with MS, have been applied to identify and authenticate the CHM products sensitively and reliably, to make them becoming the most commonly used methods (Fan et al., 2006). Spectrophotometric technologies, including UV and IR, have also been used to reveal the chemical fingerprints for a wide range of CHM. However, poor resolution of these techniques is discouraged application for similar botanic origins (Jiang et al., 2004b). Other techniques, for instances,

CE and NMR, have been commonly employed for CHM fingerprints (Han et al., 2004). With the rapid development of molecular sciences, more and more DNA-based markers, which were described in Section 1.3.2, become widely used for CHM fingerprints (Shaw et al., 2002).

1.3.4.2 Application in CHM

There are a number of chromatographic fingerprinting studies on CHM, such as, Radix Scutellariae (Huang-qin) (Yu et al., 2007), Radicis Angelicae Sinensis (Dang-gui) (Wang et al., Wang et al., 2006b), Fructus Xanthii (Cang-er-zi) (Ruan and Li), Rhizoma et Radix Notopterygii (Qiang-huo) (Jiang et al.), *Ginkgo biloba* (Yin-xing) (Ji et al., 2005), Ganoderma (Ling-zhi) (Di et al., 2003), Radix Slaviae Miltiorrhizae (Dan-shen) (Liang et al., 2005), Radix Astragli (Huang-qi) (Hu et al., 2004), Herba Ephedrae (Ma-huang) (Schaneberg et al., 2003), etc.

1.4 Aims of This Thesis

Flos Magnoliae is an important CHM used in the clinical application for rhinitis, sinusitis and headache (The Pharmacopoeia Commission of People's Republic of China, 2005). As mentioned through this chapter, molecular and chemical profiles, and pharmacological actions of different Flos Magnoliae species may vary, which may cause the confusion of its clinical application. In addition, there is little information about the locally grown Flos Magnoliae species, in terms of their genetic and chemical data, and pharmacological actions. Therefore, a systematic evaluation for different species and varieties is essential for developing quality control procedures for Flos Magnoliae. Furthermore, some intrinsic and extrinsic factors may contribute to the influences of the quality of Flos Magnoliae. Firstly, six Flos Magnoliae species and six *M. biondii* varieties were employed in the present study. Secondly, the Flos Magnoliae products from different herbal suppliers were included as well. At last, the qualities of the different *M. biondii* and *M. sprengeri* samples, collected from different cultivation sites in China or Australia, were used in this thesis.

The major aim of this thesis was to develop systematic quality profiles of different Flos Magnoliae sources. The specific aims include: (1) identification of six Flos Magnoliae species from Australia and China using DNA-based markers; (2) qualitative identification of different Flos Magnoliae samples using TLC/HPTLC, while magnolin and fargesin were regarded as the marker compounds; (3) simultaneous determination of content of the bioactive lignans, magnolin and fargesin, in different Flos Magnoliae samples; (4) identification of Flos Magnoliae samples using HPLC fingerprinting techniques; (5) evaluation of the pharmacological actions of Flos Magnoliae and its active components, including the volatile oil and the lignans (magnolin and fargesin), on compound 48/80 induced histamine release from rat peritoneal mast cells (RPMC).

Chapter Two

Methodology

Five major methods were used in this study, including: DNA fingerprinting using RAPD and PCR-RFLP techniques; qualitative identification of the herbal extracts using TLC/HPTLC; determination of contents of the bioactive compounds using HPLC; identification of the herbal extracts using HPLC and pharmacological studies on compound 48/80 induced histamine release in RPMC.

2.1 Sample collection

2.1.1 Sample collection from China

2.1.1.1 Flos Magnoliae species from Henan Province

Eleven *M. biondii* samples from six varieties were collected in Nanzhao, Henan Province, China in February 2006. Four *M. biondii* samples were collected from four different locations of the cultivation sites in Nanzhao, Henan Province, China. A packed Flos Magnoliae product from Hualong Magnolia Development Co. Ltd. was employed in this study. Furthermore, the essential oil from Flos Magnoliae was used for testing. The samples were donated by Hualong Magnolia Development Co. Ltd. The altitude of the cultivation site is 300 – 1000m. The mean annual temperatures are 13˚C-15˚C and the mean annual precipitations are around 600 – 1000mm. The cultivation site and collection procedures of *M. biondii* are shown in Figure 2.1 and the morphological figures of the samples are illustrated in Figure 2.2.

2.1.1.2 Flos Magnoliae species from Sichuan Province

Two *M. sprengeri* samples and *M. sargentiana* were collected from two different locations (Jiangyou and Mabian) of Sichuan Province, China (Figure 2.3). The samples were supplied and identified by Prof. Zhongzhen Zhao from School of Chinese Medicine, Hong Kong Baptist University (HKBU), Kowloon Tong, Hong Kong Special Administrative Region (SAR), China in June 2005.

2.1.1.3 Flos Magnoliae species from Hong Kong SAR

The Flos Magnolia products were from three herbal suppliers, including Tung Fong Hung Medicine Co. Ltd., Tsang Fook Kee Medicine Co., and Eu Yan Sang International Ltd., from Hong Kong SAR, China (Figure 2.4). The samples were supplied and identified by Prof. Zhongzhen Zhao from School of Chinese Medicine, HKBU in June 2004.

2.1.2 Sample collection from Australia

2.1.2.1 *Magnolia spp*. from Royal Botanic Garden (RBG), Melbourne

The fresh buds of *M. kobus* were kindly supplied by RBG, Melbourne, Australia (Figure 2.5). With the assistance from Izabella Meraviglia-Crivelli, the curator of the Magnolia garden, RBG, Melbourne, the samples were collected in June 2003.

2.1.2.2 *Magnolia spp.* from Ferny Creek Nursery, Mt. Dandenong, Victoria

The fresh samples of *M. campbellii, M. denudata, M. liliflora* and *M. sprengeri* were purchased from the Ferny Creek Nursery, Mt. Dandenong, Victoria in June 2003 (Figure 2.6). All the tax invoices were kept in the RMIT Chinese Medicine Research Group. All the purchased specimens were planted in the Herbarium, RMIT.

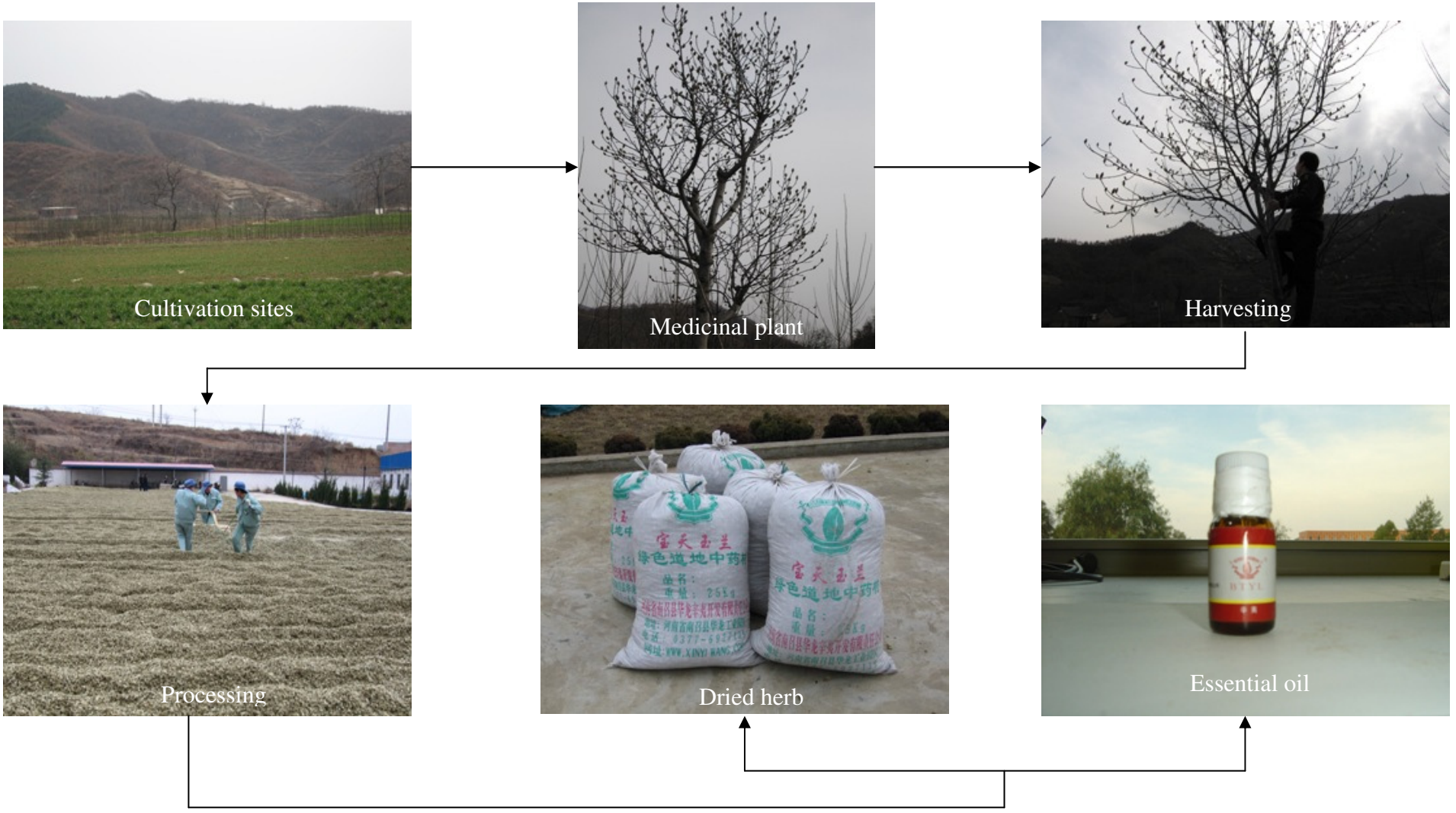


Figure 2.1 The process of the Flos Magnoliae samples, collected from Nanzhao, Henan Province, China

Chuan Yu

Huang Geng Chuan Yu

Er Mao Tao

Xiao Mao Tao

Hua Long Wu Hao

Biao Zhun Chuan Yu

Figure 2.2 The images of different varieties of *M. biondii,* collected from Nanzhao, Henan Province, China

M. sprengeri

M. sargentiana

Figure 2.3 The images of *M. sprengeri* and *M. sargentiana* colleted from Sichuan Province, China

Tung Fong Hung
Medicine Co. Ltd.

Tsang Fook Kee
Medicine Co.

Eu Yan Sang
International Ltd.

Figure 2.4 The images of three different Flos Magnoliae products from Hong Kong SAR, China

RBG, Melbourne

M. kobus

Figure 2.5 The images of *M. kobus*, collected from RBG, Melbourne, Australia

M. denudata

M. liliflora

Figure 2.6 The images of *M. denudata* and *M. liliflora*, from Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia

2.2 Plant materials

2.2.1 Flos Magnoliae samples for DNA fingerprinting

The plant materials used in this study were five fresh *Magnolia spp.* and six dried Flos Magnoliae species (Table 2.1). The five fresh samples sourced locally from Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia and Royal Botanic Garden (RBG), Melbourne, Victoria, Australia. *Schisandra chinensis*, as the positive control, was obtained from White Cottage Nursery, Victoria, Australia.

For the dried samples, five above samples, except *M. kobus*, were dried in RMIT plant biotechnology laboratory under room temperature and in darkness condition for one month after collection. The other two dried samples, *M. biondii* and *M. sargentiana* were supplied and identified by Prof. Zhongzhen Zhao from School of Chinese Medicine, HKBU.

Studies on PCR of 5S rRNA region were only conducted for five Flos Magnoliae species, except *M. sargentiana*.

Table 2.1 Fresh and dried Flos Magnoliae samples used in RAPD and PCR-RFLP analysis

Section	Species	Nature	Source of material
Yulania	*M. campbellii*	Fresh	Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia
Yulania	*M. denudata*	Fresh/dried	Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia
Yulania	*M. liliflora*	Fresh/dried	Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia
Tulipastrum	*M. sprengeri*	Fresh/dried	Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia
Buergeria	*M. kobus*	Fresh/dried	Royal Botanic Garden (RBG) Melbourne, Victoria, Australia
Schisandra	*Schisandra chinensis*	Fresh	White Cottage Nursery, Victoria, Australia
Buergeria	*M. biondii*	Dried	Nanzhao, Henan Province, China
Yulania	*M. sargentiana*	Dried	Mabian, Sichuan Province, China

2.2.2 Flos Magnoliae samples for chemical determination and identification

Different Flos Magnoliae samples from six different *Magnolia spp.* (Table 2.2) were employed, including *M. sargentiana* and *M. biondii* from China. *M. sprengeri, M. denudata* and *M. liliflora* were purchased from Ferny Creek Garden, Victoria, Australia. *M. kobus* was kindly donated by RBG, Melbourne, Victoria, Australia.

Moreover, 11 batches of *M. biondii* from six varieties were used in this study (Table 2.3). Five different Flos Magnoliae products were obtained from different herbal suppliers, namely, Hualong Magnolia Development Co. Ltd. in Henan Province, China, Tung Fong Hung Medicine Co. Ltd., Tsang Fook Kee Medicine Co. and Eu Yan Sang International Ltd. in Hong Kong SAR, China, and KODA International in Australia (Table 2.4). Four *M. biondii* samples were collected from different growing locations, in Nanzhao, Henan Province, China (Table 2.5).

Two of three *M. sprengeri* samples were collected from Jiangyou and Mabian, Sichuan Province, China. Another *M. sprengeri* sample was purchased from Ferny Creek Garden, Victoria, Australia (Table 4.6).

The identities of all samples from China were confirmed by Prof. Zhongzhen Zhao, School of Chinese Medicine, HKBU, Hong Kong SAR, China. All Flos Magnoliae samples collected from Australia were identified by Dr. Edwin C. K. Pang, School of Applied Sciences, RMIT.

Table 2.2 Different species of Flos Magnoliae, collected from Australia and China

Species Name	Sources
M. biondii	Nanzhao, Henan Province, China
M. denudata	Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia
M. kobus	RBG, Melbourne, Victoria, Australia
M. liliflora	Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia
M. sargentiana	Mabian, Sichuan Province, China
M. sprengeri	Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia

Table 2.3 Different varieties of *M. biondii*, collected from Nanzhao, Henan Province, China.

No	Variety Name		
	Sample Name	English Name	Chinese Name
1	HLWH	Hua Long Wu Hao	华龙五号
2	EMT	Er Mao Tao	二毛桃
3	EMT	Er Mao Tao	二毛桃
4	XMT	Xiao Mao Tao	小毛桃
5	HGCY	Huang Geng Chuan Yu	黄梗串鱼
6	CY	Chuan Yu	串鱼
7	CY	Chuan Yu	串鱼
8	CY	Chuan Yu	串鱼
9	EMT	Er Mao Tao	二毛桃
10	HLWH	Huang Long Wu Hao	华龙五号
11	BZCY	Biao Zhun Chuan Yu	标准串鱼

Table 2.4 Different Flos Magnoliae products from five herbal suppliers

Sample Name	Herbal Suppliers
HL	Hualong Magnolia Development Co. Ltd., Henan Province, China
TFH	Tung Fong Hung Medicine Co. Ltd., Hong Kong SAR, China
TFK	Tsang Fook Kee Medicine Co., Hong Kong SAR, China
EYS	Eu Yan Sang International Ltd., Hong Kong SAR, China
KODA	KODA International, Australia

Table 2.5 Different *M. biondii* samples, collected from different cultivation sites in China

Sample Name	Cultivation sites
DHY	Eastern Garden, Nanzhao, Henan Province, China
XHY	Western Garden, Nanzhao, Henan Province, China
YYS	Mt. Yanyi, Nanzhao, Henan Province, China
TQ	Tian Bridge, Nanzhao, Henan Province, China

Table 2.6 Different *M. sprengeri* samples, collected from different cultivations sites in China and Australia

Sample Name	Cultivation sites
JY	Jiangyou, Sichuan Province, China
MB	Mabian, Sichuan Province, China
MD	Ferny Creek Nursery, Mt. Dandenong, Victoria, Australia

2.2.3 Flos Magnoliae samples for pharmacological study

The Flos Magnoliae samples used for anti-histamine release studies were previously identified using TLC and HPLC in this thesis. Furthermore, the volatile oil of Flos Magnoliae, which was supplied by Hualong Magnolia Development Co. Ltd., was employed for anti-histamine release test. The anti-histamine release effects of two isolated compounds, magnolin and fargesin were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) of China.

2.3 Reagents and chemicals

2.3.1 Reagents and chemicals for DNA fingerprinting

DNeasy Plant Mini Kit and DNeasy Plant Maxi Kit were purchased from Qiagen (Victoria, Australia). Tris-base, boric acid and ethylenediaminetetraacetic acid (EDTA) were obtained from Sigma-Aldrich (New South Wales, Australia). GeneRuler 1kb DNA Ladders and GeneRuler 100bp DNA Ladders Plus were acquired from Fermentas (Queensland, Australia). dNTPs kits (dATP, dCTP, dTTP and dGTP), 10×PCR buffer, 50mM $MgCl_2$ and Taq polymerase were purchased from Invitrogen (Victoria, Australia). EtBr was acquired from BioRad (New South Wales, Australia). Ten-mer oligo primers (GeneWorks, South Australia, Australia) were used for RAPD analysis. A pair of 5S rRNA primers was purchased from Invitrogen, Australia.

2.3.2 Reagents and chemicals for TLC analysis

Diethyl ether for TLC / HPTLC analysis was HPLC grade (Merck, New South Wales, Australia); and chloroform for TLC / HPTLC analysis was HPLC grade (Sigma-Aldrich, New South Wales, Australia) also. HPLC grade methanol was purchased from Merck New South Wales, (New South Wales, Australia). The marker compounds magnolin and fargesin were purchased from the NICPBP of China.

2.3.3 Reagents and chemicals for HPLC analysis

Acetonitrile for HPLC analysis was HPLC grade (Sigma-Aldrich, New South Wales, Australia); and water for HPLC analysis was purified by a Milli-Q water purification system (Millipore, New South Wales, Australia). ACS grade ethanol was purchased from Ajax Finechem (New South Wales, Australia). The marker compounds magnolin and fargesin were purchased from the NICPBP of China.

2.3.4 Reagents and chemicals for histamine release determination

Compound 48/80, spermidine trihydrochloride, o-phthalaldehide (OPA), histamine, toluidine blue, N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), sodium chloride (NaCl), potassium chloride (KCl), $MgCl_2$, L-(+)-tartaric acid, sodium tartrate dihydrate, 1-octanesulfonic acid sodium salt, boric acid and sodium hydroxide (NaOH) were obtained from Sigma-Aldrich (New South Wales, Australia). Calcium chloride ($CaCl_2$), di-sodium hydrogen orthophosphate (NaH_2PO_4) and D-glucose anhydrous were acquired from Ajax Chemicals (New South Wales, Australia). Heparin sodium injection was obtained from BDL (Australia). HPLC grade methanol was purchased from Merck (New South Wales, Australia). The marker compounds magnolin and fargesin were purchased from the NICPBP of China.

2.4 Identification of different Flos Magnoliae species using DNA fingerprinting

2.4.1 DNA extraction

DNAs from fresh samples were extracted using the DNeasy Plant Mini Kit (Qiagen, Victoria, Australia), and DNA templates from dried medicinal materials were extracted using the DNeasy Plant Maxi Kit (Qiagen, Victoria, Australia). The extraction procedures were based on the protocols provided by the supplier. Briefly, 100mg of fresh plant tissues or 500mg of dried buds were ground to a fine powder in liquid nitrogen. After adding specific buffers at

various stages to lyse the cells and precipitate the detergent, proteins and polysaccharides, the debris and precipitates were retained in the filter of either QIAshredder Mini or Maxi Spin Column. The DNA that was bound on the membrane of either DNeasy Mini or Maxi Spin Column was then washed by the washing buffer, followed by an elution step.

Aliquots of 5µl or 10µl of DNA templates were analysed using 1.5% DNA grade agarose gel electrophoresis in the 1× TBE (10.8g of Tris-base, 5.5g of boric acid and 0.93g of EDTA dissolved in 1L distilled water) buffer system. The 1kb GeneRuler™ DNA Ladders was used as a molecular weight marker. The electrophoretic gel plate was stained in a 10µg/ml solution of EtBr and visualised under UV illumination. The Discovery Series Quantity One 1-D Analysis Software (BioRad, New South Wales, Australia) was used for imaging the electrophoresis gels. The remaining DNA was kept at -20°C for further analysis. The DNA stocks were adjusted to a concentration of about 10ng/µl for PCR amplification.

2.4.2 RAPD

Fifteen 10-mer oligo primers (GeneWorks, South AustraliaAustralia) were used for RAPD analysis of fresh samples, and ten 10-mer oligo primers (Operon, Cologne, Germany) were employed for analysis of dried herbal materials (Table 2.7). The RAPD method was modified from previous studies (Shaw and But, 1995, Cui et al., 2003). Briefly, 40ng of plant DNA was amplified in a 25µl PCR mixture consisting of 2.5µl of 10×PCR buffer (100mM Tris-HCl, 500mM KCl, 0.01% gelatin), 0.75µl of 50mM $MgCl_2$, 6µl of 1mM dNTPs, 1µl of 10µM primer and 1U *Taq* polymerase. The amplified reaction took place in a P×2 Thermal Cycler (Thermal Electron Corporation, Waltham, MA, United States) and PCR was performed using the following cycles: initial 1 cycle of 3min at 94°C, followed by 15s denaturing at 94°C, 1min annealing at 36°C and 1min elongation at 72°C for 38 cycles and 1 cycle of 5min extension at 72°C. The RAPD fragments were separated on 1.5% DNA grade agarose gel

electrophoresis. The Discovery Series Quantity One 1-D Analysis Software (BioRad, New South Wales, Australia) was used for imaging the electrophoresis gels.

Table 2.7 Decamer oligo primers used for RAPD analysis

Oligo Name	Sequence (5' → 3')	Suggested annealing temperature	Usage
OPA-07	GAA ACG GGT G	35°C	Dried samples
OPA-11	CAA TCG CCG T	32°C	Fresh / Dried samples
OPA-14	TCT GTG CTG G	35°C	Dried samples
OPB-03	CAT CCC CCT G	32°C	Fresh / Dried samples
OPB-04	GGA CTG GAG T	35°C	Fresh / Dried samples
OPB-06	TGC TCT GCC C	32°C	Fresh / Dried samples
OPB-07	GGT GAC GCA G	35°C	Fresh / Dried samples
OPB-08	GTC CAC ACG G	32°C	Fresh samples
OPB-12	CCT TGA CGC A	35°C	Dried samples
OPB-16	TTT GCC CGG A	35°C	Dried samples
OPB-17	AGG GAA CGA G	35°C	Dried samples
OPC-19	GTT GCC AGC C	32°C	Fresh samples
OPG-13	CTC TCC GCC A	32°C	Fresh samples
OPM-04	GGC GGT TGT C	32°C	Fresh samples
OPN-04	GAC CGA CCC A	32°C	Fresh samples
OPP-10	TCC CGC CTA C	32°C	Fresh samples
OPU-03	CTA TGC CGA C	32°C	Fresh samples
OPV-06	ACG CCC AGG T	32°C	Fresh samples
OPW-04	CAG AAG CGG A	32°C	Fresh samples
OPW-09	GTG ACC GAG T	32°C	Fresh samples

2.4.3 PCR-RFLP

A pair of PCR-primers (GeneWorks, South Australia, Australia), 5SP1=5' GTG CTT GGG CGA GAG TAG TA 3' (forward) and 5SP2=5' TTA GTG CTG GTA TGA TCG CA 3' (reverse), was used for PCR-RFLP. They were designed to amplify the 5S ribosomal RNA (5s rRNA) spacer (Wolters and Erdmann, 1988). A 25µl PCR mixture contained the following 40ng plant DNA, 2.5µl of 10×PCR buffer, 0.75µl of 50mM $MgCl_2$, 6µl of 1mM dNTPs, 1µl of 10µM forward primer, 1µl of 10µM reverse primer and 1 units *Taq* polymerase. PCR was performed using the following cycles: initial 3min at 94°C, followed by 1min denaturing at 94°C, 1min annealing at 62°C and 1min elongation at 72°C repeated for 38 cycles and with 10min extension at 72°C. The RFLP fragments were separated on 1.5% agarose gel by electrophoresis in 1×TBE buffer system and stained with EtBr (Lin et al., 2001).

The PCR products were digested by restriction enzymes *Afl*III, *Apo*I, *Dde*I, *EcoR*I, *EcoR*V, *Hae*III, *Hha*I, *Hinf*I, *Mnl*I, *Nco*I, *Nla*IV, *Pml*I, *Pst*I, *Rsa*I and *Sma*I (Table 2.8). Briefly, 4µl of PCR products was digested for 2hr at 37°C with 5 units of restriction enzymes (10 units/µl), 1µl RE 10×buffer and 0.1µl of acetylated bovine serum albumin (BSA) (10µg/µl) in a 10µl total volume (Lin et al., 2001). The digested fragments were separated on 1.5% agarose gel, stained with EtBr and visualised under UV illumination. The Discovery Series Quantity One 1-D Analysis Software (BioRad, New South Wales, Australia) was used for imaging the electrophoresis gels.

Table 2.8 The details of 15 restrictions enzymes for PCR-RFLP analysis

Restriction Enzymes	Sequence (5' → 3')	Suppliers
*Afl*III	A▼CRYGT	New England Biolabs, Ipswich, MA, USA
*Apo*I	R▼AATTY	New England Biolabs, Ipswich, MA, USA
*Dde*I	C▼TNAG	Promega, New South Wales, Australia
*EcoR*I	G▼AATT C	Promega, New South Wales, Australia
*EcoR*V	GAT▼TAC	Promega, New South Wales, Australia
*Hae*III	GG▼CC	Promega, New South Wales, Australia
*Hha*I	GCG▼C	Promega, New South Wales, Australia
*Hinf*I	G▼ANTC	Promega, New South Wales, Australia
*Mnl*I	CCTC$(N)_7$▼	New England Biolabs, Ipswich, MA, USA
*Nco*I	C▼CATG G	Promega, New South Wales, Australia
*Nla*IV	GGN▼NCC	New England Biolabs, Ipswich, MA, USA
*Pml*I	CAC▼GTG	New England Biolabs, Ipswich, MA, USA
*Pst*I	CTGCA▼G	Promega, New South Wales, Australia
*Rsa*I	GT▼AC	Promega, New South Wales, Australia
*Sma*I	CCC▼GGG	Promega, New South Wales, Australia

2.5 Identification of different Flos Magnoliae species and varieties using TLC/HPTLC

2.5.1 Herbal extraction and preparation

Samples were crushed into powder with a mortar and pestle. Precisely, 1g of fine powder of each sample was extracted with 60ml of absolute ethanol using an Accelerated Solvent Extraction machine (ASE-100, Dionex, Sunnyvale, CA, U.S.A.). The herb was extracted three times at a temperature of 100°C and under the pressure of 1500psi. The static time was 5 min and the flush volume was 60% of the 33ml cell. After filtered by Whatman No1. paper filter (150mm Dia) (Crownscientific, Victoria, Australia), the combined extract was evaporated at 60°C under vacuum to 20ml approximately. The concentrated extract was then diluted by HPLC grade ethanol to the final volume as 25ml using a volumetric flask. The ethanol extract was filtered with PTFE syringe filters (25mm, 0.45μm) (Alltech Associates, New South Wales, Australia) and stored at -20°C for further analysis. Before the TLC application, the test samples were sonicated for 15min.

2.5.2 Preparation of standard references, magnolin and fargesin

A stock solution of the standard was prepared by weighing 1.5mg each of magnolin and fargesin into a 1.5ml Eppendorf tube. Precisely 1.5ml of ethanol was added to each tube and the solution was sonicated for 15min. After the solution had cooled to the room temperature, it was diluted from the stock solution to the concentrations of 2.5mg/ml for magnolin and 1mg/ml for fargesin.

2.5.3 Chromatographic conditions

2.5.3.1 TLC/HPTLC plates preparation

TLC silica gel 60 F_{254} plates (20cm x 10cm, E. Merck, Darmstadt, Germany) and HPTLC silica gel 60 F_{254} plates (10cm x 10cm, E. Merck, Darmstadt, Germany) were employed. The

plates were pre-washed with HPLC grade methanol, and then dried for 30min at 105°C before sample application.

2.5.3.2 Sample application

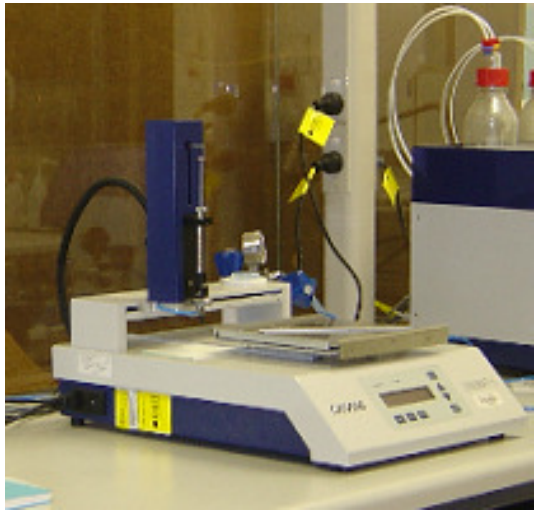
Test solution of the reagent blank (methanol, 5µl), reference standard and samples were applied on the plates with a TLC system (CAMAG, Muttenz, Switzerland) (Figure 2.7 and Table 2.9). With Linomat 5, maximum of 18 tracks were applied. Band width was required at the minimum of 8mm. Varying amount of analytes were applied of 8mm space from the lower edge of the plates. The distances from the side of the plates were at least 15mm, while the space between two bands were at least 10mm. The spraying rates for the samples were 20µl.

2.5.3.3 Chromatographic conditions

The TLC plates and HPTLC plates were then developed in a 20cm x 10cm and a 10cm x 10cm horizontal developing chambers with glass lids, respectively. The twin-trough chamber was pre-equilibrated for 30min with the filter paper soaked with 10ml developing solvent. For TLC/HPTLC separation, chloroform – ether (5:1) was used as the developing solvent. The migration distance was 7cm from lower edge. Subsequent to the development, TLC/HPTLC plates were dried in a current of air.

2.5.3.4 Documentation

After developing, densitometric scanning was performed by CAMAG TLC scanner III in the absorbance mode at 254nm. The source of radiation utilized was a deuterium lamp.



Linomat 5
(semi-automatic sample application)

20cm x 10cm plates

10cm x 10cm plates
Horizontal developing chamber

DigiStore 2 documentation system with high-resolution 12-bit CCD camera (HV-C20A, Hitachi, Japan)

Figure 2.7 CAMAG TLC system (CAMAG, Switzerland)

Table 2.9 The compositions of CAMAG system

Components	Model
Sample application	Linomat 5 (semi-automatic sample application)
Chromatogram development	Horizontal developing chamber 20cm x 10cm plates (for TLC) Horizontal developing chamber 10cm x 10cm plates (for HPTLC)
Evaluation: detection	TLC scanner III
Evaluation: documentation	DigiStore 2 documentation system with high resolution 12 bit CCD camera (HV-C20A, Hitachi, Japan) UV cabinet dural wavelength, 254/366nm
Chromatographic layer	Merck, TLC plates silica gel 60F 254 20 x 10cm Merck, HPTLC plates silica gel 60F 254 10 x 10cm
Software	winCATS (Version: 1.2.5)

2.6 Quantitative determination of Magnolin and Fargesin from different Flos Magnoliae species and varieties using HPLC

2.6.1 Herbal extraction and preparation

The procedure for extraction and preparation are described in Section 2.5.1. Briefly, the fine powder of each sample was extracted with absolute ethanol using an Accelerated Solvent Extraction machine (ASE-100, Dionex, Sunnyvale, CA, U.S.A.). The concentrated ethanol extracts were filtered with PTFE syringe filters (25mm x 0.45μm, Alltech Associates, New South Wales, Australia) and stored at -20°C. After being sonicated for 15min, the 20 times ethanol (HPLC grade) dilution of the stock solution was used for HPLC injection.

2.6.2 Preparation of standard references, magnolin and fargesin

A stock solution of the standard was prepared by accurately weighing about 1.5mg each of magnolin and fargesin into a 1.5ml Eppendorf tube. Precisely 1.5ml of ethanol was added to each tube and the solution was sonicated for 15min. After the solution cooled to the room temperature, it was diluted from the stock solution to five concentrations, ranging from 10-100 ng/ml for magnolin and 1-10 ng/ml for fargesin.

2.6.3 Chromatographic conditions

HPLC was performed on a Shimadzu Model SCL-10A*vp* HPLC system (Shimadzu, Japan) equipped with a Shimadzu Model SPD-M10A*vp* photodiode array detector (Figure 2.8 and Table 2.10). The separations were obtained with a RP-C_{18} column (ODSII, L 250 × 4.6 I.D. mm, 5μM, Phenomenex, Torrance, CA, U.S.A.) and a pre-column of the same packing material (Security Guide cartridge; 3.0mm; Phenomenex, Torrance, CA, U.S.A.) were used for analysis. A linear gradient elution of acetonitrile and water was used. The gradient program is presented in Table 2.11. Among of these, 20min for determination, and 10min for pre-equilibration. Detection wavelength was set at 278nm. The solvent flow rate was 1ml/min

and the column temperature was set at 30°C (Figueiredo et al., 1999, Xu et al., 2003). The injection volume was 10µl of the prepared solution. Total running time was 30.50min.

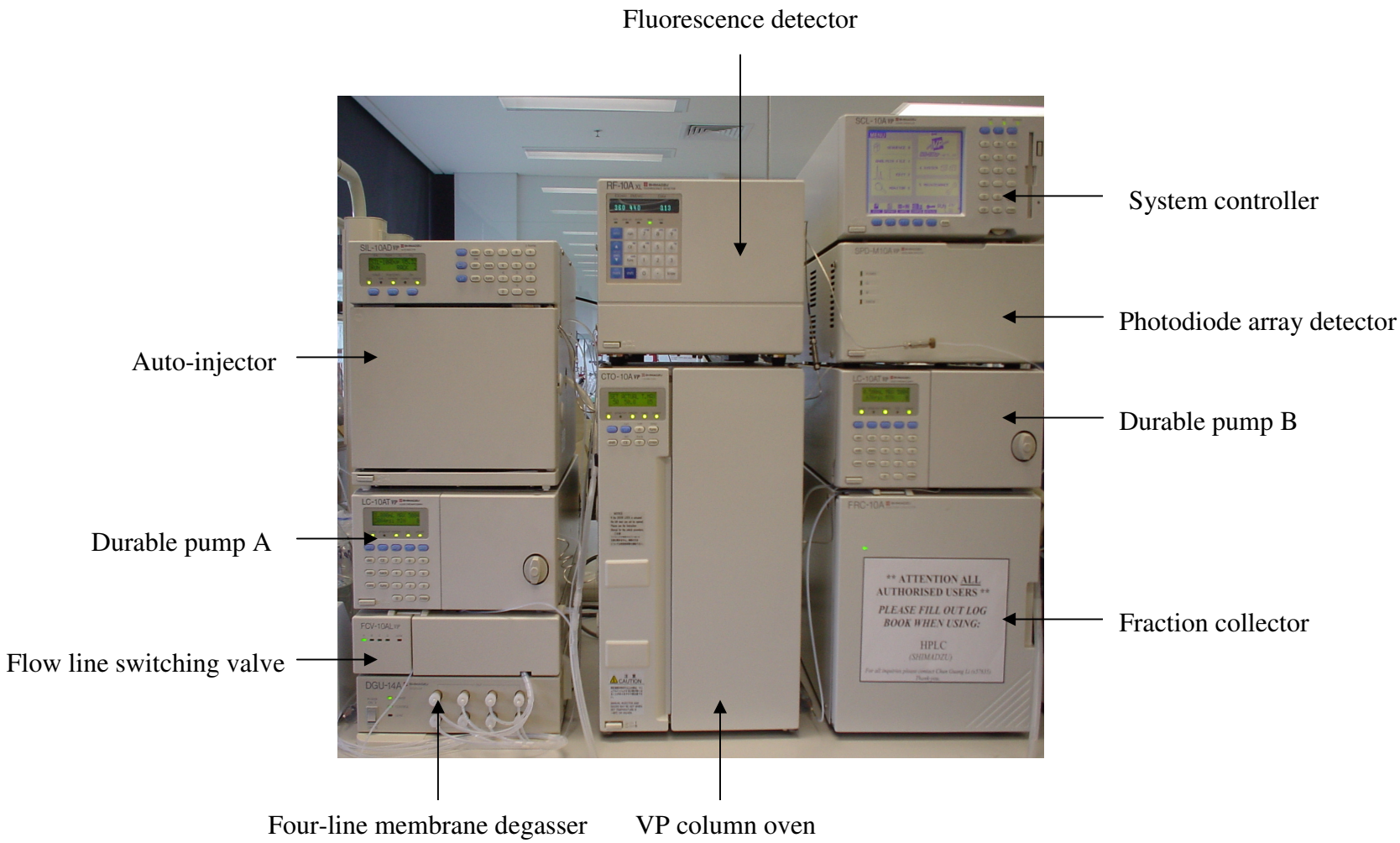


Figure 2.8 Shimadzu Model SCL-10A*vp* HPLC system (Shimadzu, Japan)

Table 2.10 The compositions of Shimadzu system

Components	Model
System controller	SCL-10A*vp*
Auto-injector	SIL-10AD*vp*
Durable pump	LC-10AT*vp*
Flow line switching value	FCV-10AL*vp*
Four-line membrane degasser	DGU-14A
VP column oven	CTO-10A*vp*
Photodiode array detector	SPD-M10A*vp*
Fluorescence detector	RF-10Axl
Software	Class-*vp*5.0

Table 2.11 A linear gradient solution for HPLC determination

Time (min)	Acetonitrile (%)	Water (%)
0.01	45	55
10.00	65	35
18.00	70	30
20.00	45	55
30.00	45	55
30.50	Stop	

2.6.4 Validation parameters

2.6.4.1 Linearity

Linear calibration curves were constructed by analysis of a mixture containing magnolin and fargesin at five levels and plotting peak area against the concentration of each reference standard. The standard solution (10µl) was used for HPLC injections. Calibration curves were constructed at the relevant wavelength of maximum absorption of each reference compound.

2.6.4.2 Precision

The Flos Magnoliae sample was prepared as described in Section 2.5.1. The intermediate precisions of injections were evaluated with the test sample five times on the same day. All of the measurements of precisions were expressed as relative standard deviations (R.S.D.s).

2.6.4.3 Stability

Same analyte, which was conducted the precision test, was employed for the stability test. The stability of injection was calculated with the test sample at the room temperature for five times. The HPLC injections were taken during 48hr. All of the measurements of stabilities were expressed as R.S.D.s.

2.6.4.4 Repeatability

Five replicates analyses of the same Flos Magnoliae sample were carried out to sequentially evaluate the repeatability of the quantitative procedure. All of the measurements of repeatabilities were expressed as R.S.D.

2.6.4.5 Recovery

Standards of 200µg/ml of magnolin and 15µg/ml of fargesin in solutions were spiked to the Flos Magnoliae sample solution of which the content of magnolin and fargesin had been

determined before the addition of the two chemical standards. Then, two marker compounds in Flos Magnoliae sample solutions were extracted, processed and quantified in accordance with the established procedure, and finally the recovery rates were calculated.

2.7 Identification of different Flos Magnoliae species and varieties using HPLC

2.7.1 Herbal extraction and preparation

The procedure for extraction and preparation of Flos Magnoliae samples were described in Section 2.5.1. After being sonicated for 15min, the stock solution was directly used for HPLC injection.

2.7.2 Chromatographic conditions

A Shimadzu Model SCL-10A*vp* HPLC system (Shimadzu, Japan) equipped with a Shimadzu Model SPD-M10Avp photodiode array detector was used to perform HPLC analysis (details see Figure 4.1 and Table 4.10). The HPLC fingerprint was carried out on a RP-C18 column (ODSII, L 250 × 4.6 I.D. mm, 5μM, Phenomenex, Torrance, CA, U.S.A.) and a pre-column of the same packing material (Security Guide cartridge; 3.0mm; Phenomenex, Torrance, CA, U.S.A.). A linear gradient mobile phase of acetonitrile and water was used. The gradient program is presented in Table 2.12. Among of these, 60min was used for determination, and 15min was used for pre-equilibration. Detection wavelength was set at 278nm. The solvent flow rate was 1.0ml/min and the column temperature was set at 30°C. The injection volume was 10μl of the prepared solution. Total running time was 75.50min.

Table 2.12 A linear gradient solution for HPLC identification

Time (min)	Acetonitrile (%)	Water (%)
0.01	30	70
15.00	30	70
25.00	60	40
35.00	70	30
45.00	75	25
60.00	75	25
61.00	30	70
75.00	30	70
75.50	Stop	

2.7.3 Validation parameters

2.7.3.1 Precision

The intra-day precisions of the HPLC-DAD method were validated with the analyte under the selected optimal conditions five times consistently. All measurements of precisions were expressed as R.S.D.s.

2.7.3.2 Stability

For stability, measurements on three consecutive days were conducted. All of the measurements of stabilities were expressed as R.S.D.s.

2.7.3.3 Repeatability

Five independently prepared sample solution of Flos Magnoliae with the same amount were analysed and the variations within five measurements were calculated for evaluation of the repeatability. All of the measurements of repeatabilities were expressed as R.S.D.s.

2.8 Determination of compound 48/80 induced histamine release in RPMC

2.8.1 Herbal extraction and preparation

Samples were crushed into powder with a mortar and pestle. Precisely, 1g of fine powder of each sample was extracted with 60ml of absolute ethanol using an Accelerated Solvent Extraction machine (ASE-100, Dionex, Sunnyvale, CA, U.S.A.). The herb was extracted three times (i.e. three static cycles), at a temperature of 100°C and under a pressure of 1500psi. After filtered by Whatman No1. paper filter (Crownscientific, New South Wales, Australia), the combined extract was evaporated at 65°C under vacuum to 5ml approximately. Ather transferred to a tube, the concentrated extracts then were evaporated at 80°C to dryness using a multi-block heater. The dried concentrated powder were precisely weighed and followed by

diluted in absolute ethanol. The ethanol extract was filtered with PTFE syringe filters (25mm x 0.45µm, Alltech Associates, New South Wales, Australia) and stored at -20°C for further analysis.

2.8.2 Animals

Male Sprague Dawley (SD) rats at 10 – 12 weeks of age (250 – 350g) were used for experiments. All animals were purchased from Monash Animal Services, Victoria, Australia. All procedures involved in experimental animals had the prior approval from the Animal Experimentation Ethics Committee of RMIT University and conformed to the Australian National Health and Medical Research Council guidelines. Animals were housed in the RMIT Bundoora animal facility located in building 201, level 1, under controlled conditions. The temperature (19-22°C) and lighting (12 hour on and 12 hour off) conditions of the animal accommodation were controlled. Groups of 2-4 animals were placed in a standard rat box and were fed a diet consisting of adlib water and standard rat chow.

2.8.3 Isolation and preparation of peritoneal mast cells from rats

On the day of the experiment, the male SD rats (250 – 350g, Monash Animal Services, Victoria, Australia) were transferred to the treatment laboratory of Building 223, level 2, Module E, Room 46 where they were sacrificed by asphyxiation in a saturated carbon dioxide (CO_2) atmosphere followed by decapitation.

The peritoneal mast cells were prepared as described previously (Ikarashi et al., 2001b). Briefly, 20ml of Tyrode's buffer (NaCl, 137.0mM; KCl, 1.0mM; $MgCl_2$, 1.0mM; $CaCl_2$, 1.6mM; $NaH_2PO_4 \cdot 2H_2O$, 0.41mM; HEPES, 10mM and L-(-)-Glucose, 1%; pH 7.4) containing 0.3% BSA and 5.0 units/ml heparin sodium was injected intraperitoneally. The

abdomen was gently massaged for 2min, followed by collection of peritoneal solutions, which contained mast cells. The solution was then centrifuged at 1200rpm for 5min at 4°C.

The pellet containing RPMC was washed three times with 15ml of Tyrode's solution and resuspended in 10ml Tyrode's buffer, PH 7.4, containing 0.1% BSA. Fifty microliter of 0.05% toluidine blue in saline was added to an equal volume of the cell suspension. This solution was employed to determine the number of mast cells in the suspension to stain mast cells by counting microscopically using a Burker-Turk counting chamber. Finally, the number of RPMC in the suspension was adjusted to 5×10^5 cells/ml for further experiments.

2.8.4 Effects of herbal extraction on compound 48/80 induced histamine release in RPMC

To study the effects of herbal extracts on mast cell-derived histamine release, 10µl of various concentrations of herbal extracts (final concentrations, 0.01, 0.05, 0.1, and 0.5 µg/ml) or an equal volume of absolute ethanol (vehicle controls) was added to the cell suspension (5×10^5 cells/ml, pre-incubated in the water bath at 37°C for 10 min) to a final volume of 500µl and incubated in the water bath at 37°C for 10min (Ikarashi et al., 2001a, Ikarashi et al., 2001b).

Histamine release in RPMC was then elicited by the addition of 25µl of compound 48/80 (final concentration of 0.5 µg/ml) into the cell suspension. After further incubation at 37°C for 10min, the suspension was chilled in ice for 10min to stop the reaction of histamine release in RPMC. Residual histamine in the cells was released by disrupting the cells with 30% $HClO_4$ and centrifugation at 12000rpm for further 2min. Then, 50µl of aqueous spermidine trihydrochloride (1.0 mg/ml) was then added as an internal standard into the supernatant. The basal and total levels of histamine release were determined using Tyrode's

buffer (10 mM) or 30% $HClO_4$, respectively, as previously described (Ikarashi et al., 2001b). Ca^{2+} chelating agent EDTA (100 μM) was used as a positive control.

2.8.5 HPLC system for determination of compound 48/80 induced histamine release in RPMC

The level of histamine released in RPMC was determined by HPLC using a Shimadzu HPLC instrument SCL-10A*vp* (Shimadzu, Japan) with the following conditions (details see Figure 2.1 and Table 2.10): a fluorescent detector (RF-10Axl, Shimadzu, Japan), a STR reverse-phase column (ODS-II; L 150 × 4.6 I.D. mm; 5μM; Shimadzu, Japan.) and an attached post-column for derivatization with the OPA, which is a reaction coil (5.0×0.5mm stainless steel tubing). Mobile phase to the main column was made up of a mixture of 100mM sodium tartaric acid buffer (pH 4.4), containing 25mM L-(+)-tartaric acid, 75mM sodium tartrate dihydrate and 10mM 1-octanesulfonic acid sodium salt, and HPLC grade methanol (2:1), and the flow-rate was maintained at 1.0ml/min (Table 2.13). The post column solution for derivatization contained a mixture of 400mM sodium borate buffer (pH 9.2), containing 400mM boric acid and 200mM NaOH, and 10mM OPA in HPLC grade methanol (2:1) (Table 2.14). The post column flow-rate was 0.5ml/min. The column temperature was set at 50°C and the injection volume was 10μl. The wavelengths for excitation and emission were 360nm and 440nm, respectively. Under these conditions, the retention time of histamine was 3.5 min, while that of the internal standard (spermidine) was 6.0 min.

Table 2.13 The constituents of the mobile phase for determination of histamine release. Consisted a mixture A:B = 2:1.

	Buffer	Gradients
A	100mM Sodium tartaric acid buffer with 10mM 1-octanesulfonic acid sodium salt, dissolved in distilled water, (pH 4.4)	25mM L-(+)-Tartaric acid 75mM Sodium tartrate dihydrate 10mM 1-octanesulfonic acid sodium salt
B	Methanol (HPLC Grade)	

Table 2.14 The constituents of the post column solution for determination of histamine release. Consisted a mixture C:D = 2:1.

	Buffer	Gradients
C	400mM Sodium borate buffer dissolved in distilled water, (pH 9.2)	400mM Boric acid 200mM NaOH
D	OPA dissolved in Methanol (HPLC Grade)	10mM OPA

2.9 Data analysis and statistics

2.9.1 Analysis of genetic distances

Only clear, coherent and consistent banding patterns were scored. RAPD and PCR-RFLP bands were scored as present (1) or absent (0) for each genotype. A matrix of genetic distances estimation between different *Magnolia spp.*, based on dissimilarity ($D=1-S_{XY}$) indices was obtained by POPGENE *v* 1.31, a Microsoft Window-based free software for population genetic analysis (Yeh et al., 1999). On the basis of Nei's coefficient, the genetic similarity indices were calculated as $S_{XY}=2n_{XY}/(n_X+n_Y)$, where n_X and n_Y represent the numbers of fragments in individuals X and Y, respectively, and n_{XY} represents the number of the fragments shared between individuals X and Y.

The free software package Molecular Evolutionary Genetics Analysis 2 (MEGA 2) was used for reconstruction which for comparison the genetic distances between all individuals by an Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (Kumar et al., 2001, Kumar and Tamura, 2000). The linearised tree was expressed as percentage of the dissimilarity indices.

2.9.2 Data analysis of identification using TLC/HPTLC

The chromatograms were imaged and analysed by winCATs (CAMAG, Muttenz, Switzerland). The R_f values of the resolved bands were recorded. Furthermore, testing samples were in quadruplicates and all of the measurements were expressed as R.S.D.

2.9.3 Data analysis of quantitative determination of magnolin and fargesin using HPLC

Based on the liner regression models from the calibrations of standards, magnolin and fargesin, the linear relationships were established between the measured peak area and the

known concentration. According to the equation $Y=a \times X + b$, a represents the intercept value of the axis of the measured peak area (Y), and b represents the slope of the line. The concentration of the test sample (X) was calculated as the area under the peak as compared to the reference area. Testing samples were in triplicates and were represented as mean ± standard deviation (S.D.). Furthermore, all of the measurements were expressed as R.S.D.

2.9.4 Data analysis of identification using HPLC

The HPLC chromatograms were documented by Class-*vp*5.0 (Shimadzu, Japan). The retention time, relative retention time, peak area and relative peak area of each characteristic peak were also calculated in the chromatograms. Testing samples were in triplicates and all measurements were expressed as R.S.D.

2.9.5 Data analysis and statistics for determination of histamine release

The effect of Flos Magnoliae species on compound 48/80-induced histamine release in RPMC was calculated using the following formula: Percentage (%) inhibition = (level of compound 48/80 induced histamine release in the present of equal volume of ethanol – level of compound 48/80 induced histamine release in the presence herbal ethanol extract) / (level of total histamine release – basal level of histamine release). The effect of EDTA was calculated use the same formula except using Tyrode's buffer as the vehicle.

Data were presented as mean ± standard error of measurement (S.E.M.). Statistics of comparisons between groups were carried out by one-way analysis of variance (one-way ANOVA) followed by Tukey's test using the Prism 4.03 (GraphicPad Software, Inc., San Diego, CA, U.S.A.). Values of $p < 0.05$ indicated significant difference.

Chapter Three

Identification of Different Flos Magnoliae Species Using DNA-Based Fingerprinting Markers

3.1 Background

As described in Section 1.3.2, DNA based markers have been considered as one of the commonly used methods to identify and authenticate medicinal species (Joshi et al., 2004). DNA-based markers are more distinct, standard, and definite means of CHM authentication and authentication, including PCR-based markers, hybridisation-based markers and sequencing-based markers (Fowler et al., 1994, Fan et al., 2004).

As one of the commonly used DNA-based molecular markers, RAPD has been extensively applied to study the genetic diversities and authenticate different species and varieties of CHM. The main principles and the application of CHM identification and authentication using RAPD are described in Section 1.3.2.1.1. RAPD fingerprints of a number of CHMs, including Radix Ginseng (Ren-shen) and Radix Panacis Quinquefolii (Xi-yang-shen) (Ma et al., 2000a, Jiang et al., 1998), Rhizoma Atractyloids (Cang-zhu) (Ren et al., 2000, Mizukami et al., 2000, Kohjyouma et al., 1997), Radix Angelicae Sinensis (Dang-gui) (Gao et al., 2001), Flos Lonicerae (Jin-yin-hua) (Yu and Shi, 2000), and Fructus Lycii (Gou-qi-zi) (Zhang et al., 2001), have been revealed.

Compared to RAPD, PCR-RFLP provides better reproducibility and sensitivity of DNA amplification from organisms (Glick and Pasternak, 2003, Arens, 1999). PCR-RFLP analysis of the 18S rRNA region has been used for the identification of various species of Radix Ginseng (Ren-shen) (Fushimi et al., 1997, Um et al., 2001). The rDNA ITS regions have been employed to differentiate the various sources of Radix Codonopsis (Dang-shen) (Fu et al., 1999). Nakai *et al* (1996) reported the genetic fingerprints of different Herba Epimedii (Ying-yang-huo) in Japan using PCR-RFLP of the *rbcL* region.

5S rRNA is a cellular protein synthesis apparatus that can be found in prokaryotes and eukaryotes (Barciszewska et al., 2001). The gene encoding 5S rRNA occurs in eukaryotes in tandem repeating units, possessing a coding region of 120bp (Figure 3.1) (Barciszewska et al., 2001, Ma et al., 2000b). The structure and the function of 5S rRNA have been detailed based on the electronic information from URL: http://biobases.ibch.Poznan.pl/5Sdata/ and http://rose.man.poznan.pl/5Sdata/ (Ma et al., 2000b). Recent studies indicated that the nucleotide sequence of 5S rRNA could be useful in resolving the phylogenetic relationships between related CHM species, including Radix Notoginseng (San-qi) (Cui et al., 2003), Radix Astragli (Huang-qi) (Ma et al., 2000b) and Radix Angelicae Pubescentis (Du-huo) (Mizukami, 1995).

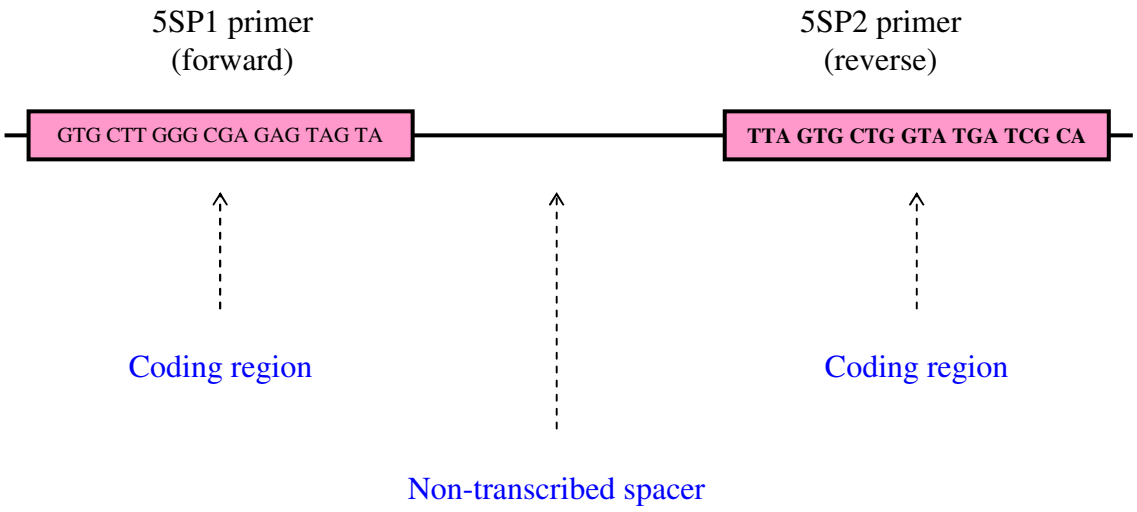


Figure 3.1 Basic structures of 5S rRNA gene clusters

Aim of this study

Although DNA fingerprinting technique has been used to determine the taxonomic relationship of *Magnolia spp.*, there is no report of the genetic identification of various medicinal Flos Magnoliae species. Therefore, the aim of this study was to develop DNA fingerprinting profiles of different Flos Magnoliae species, namely *M. biondii, M. denudata, M. sprengeri, M. kobus, M. liliflora* and *M. sargentiana* that were collected in Australia or China, which may contribute to the identification and authentication of Flos Magnoliae species.

Thus, the present DNA fingerprinting study was divided into three components. Firstly, the DNA fingerprinting profiles of fresh materials from various *Magnolia spp.* in Australia were established using RAPD analysis, in order to confirm the phylogenetic relationships. Secondly, the DNA fingerprints of six dried medicinal *Magnolia spp.* in Australia or China were generated for the identification of different Flos Magnoliae species using RAPD. Finally, in addition of RAPD analysis, another fingerprinting technique, PCR-RFLP of the 5S rRNA region was employed to identify the six dried medicinal *Magnolia spp.* for establishment of DNA fingerprints of six Flos Magnoliae species. In additional, *Schisandra chinensis*, which was in genus *Schisandra,* tribe *Shisandreae*, family Magnoliaceae, was introduced as a standard to identify the genetic similarity among analytical species.

3.2 Method

3.2.1 Collection of plant materials

3.2.1.1 Fresh samples from *Magnolia spp*.

The fresh materials from five *Magnolia spp*., *M. campbellii, M. denudata, M. kobus, M. liliflora* and *M. sprengeri,* and *Schisandra chinensis* were collected in Australia (as described in Section 2.2.1). In addition, due to the difficulties of obtaining fresh materials from China, two species, *M. biondii* and *M. sargentiana* were not included in the present study.

3.2.1.2 Dried medicinal Flos Magnoliae species

The dried medicinal parts of Flos Magnoliae were from six Flos Magnoliae species, *M. biondii, M. denudata, M. kobus, M. liliflora, M. sprengeri* and *M. sargentiana* (as described in section 2.2.1).

3.2.2 Reagents and chemicals

The details of the reagents and chemicals are described in Section 2.3.1.

3.2.3 DNA extraction

DNA extraction from fresh samples was conducted using DNeasy Plant Mini Kit (Qiagen, Australia). DNA templates from dried medicinal materials were extracted using DNeasy Plant Maxi Kit (Qiagen, Australia). The protocol of the extraction are described in section 2.4.1.

3.2.4 RAPD analysis of fresh samples from *Magnolia spp*.

Fifteen 10-mer oligo primers (GeneWorks, Australia) (details see Table 2.7) were used for RAPD analysis of fresh samples. The PCR amplified reaction took place in a P×2 Thermal Cycler (Thermal Electron Corporation, UK). The details of method are described in Section 2.4.2. RAPD marker has been previously demonstrated to establishment the DNA fingerprints

of *Cicer arietinum* (chickpea) (Sudupak et al., 2002). Thus, DNA extraction from *Cicer arietinum* was used for positive control and conducted in all PCRs.

3.2.5 RAPD analysis of crude medicinal materials of Flos Magnoliae

Ten 10-mer oligo primers (Operon, Germany) (details see Table 2.7) were used to identify the Flos Magnoliae sources. The protocol is described in Section 2.4.2.

3.2.6 PCR-RFLP analysis of crude medicinal materials of Flos Magnoliae

A set of 5S rRNA primers was applied for PCR-RFLP. For the details of the method, see Section 2.4.3.

3.2.7 Data collection and analysis of genetic distances

POPGENE *v* 1.31, and MEGA 2 software packages were used for analysis of the genetic diversities between different species (see Section 2.9.1). All PCRs were in triplicates.

3.3 Results

3.3.1 DNA extracted from fresh and dried samples of different Flos Magnoliae species

The average size of total DNAs from individual fresh materials of *Magnolia spp*. was ≥10 kb, as shown in Figure 3.2. The electrophoresis profiles of DNAs from dried buds of different Flos Magnoliae species showed the similar size of DNA fragments (≥10kb) for all species, except *M. biondii*, which DNA profiles was ranged from 0.25kb to 10kb, as shown in Figure 3.3.

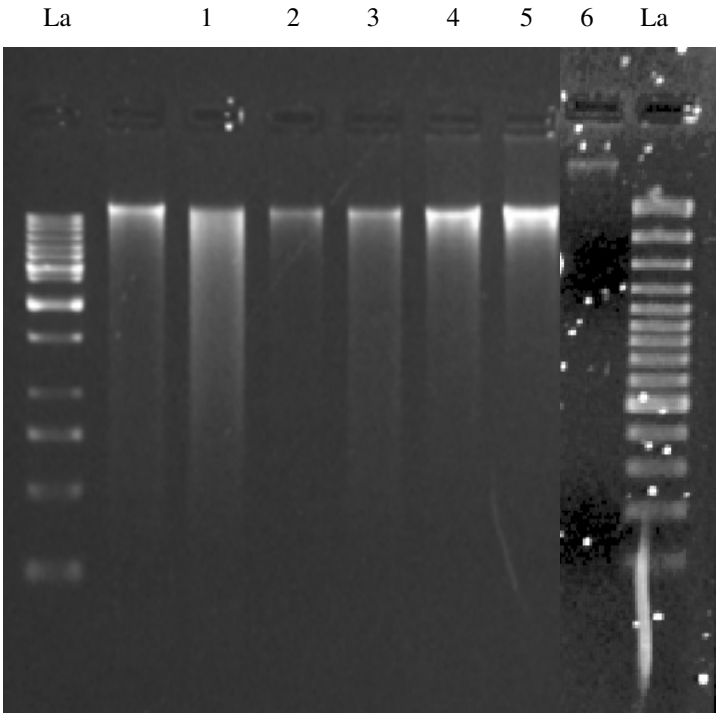


Figure 3.2 Total DNA extractions from five fresh *Magnolia spp.* and *Schisandra chinensis* (5µl of DNA extraction)

La: GeneRuler 1kb DNA Ladder; Track 1: *M. campbellii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; Track 5: *M. sprengeri*; and Track 6: *Schisandra chinensis*.

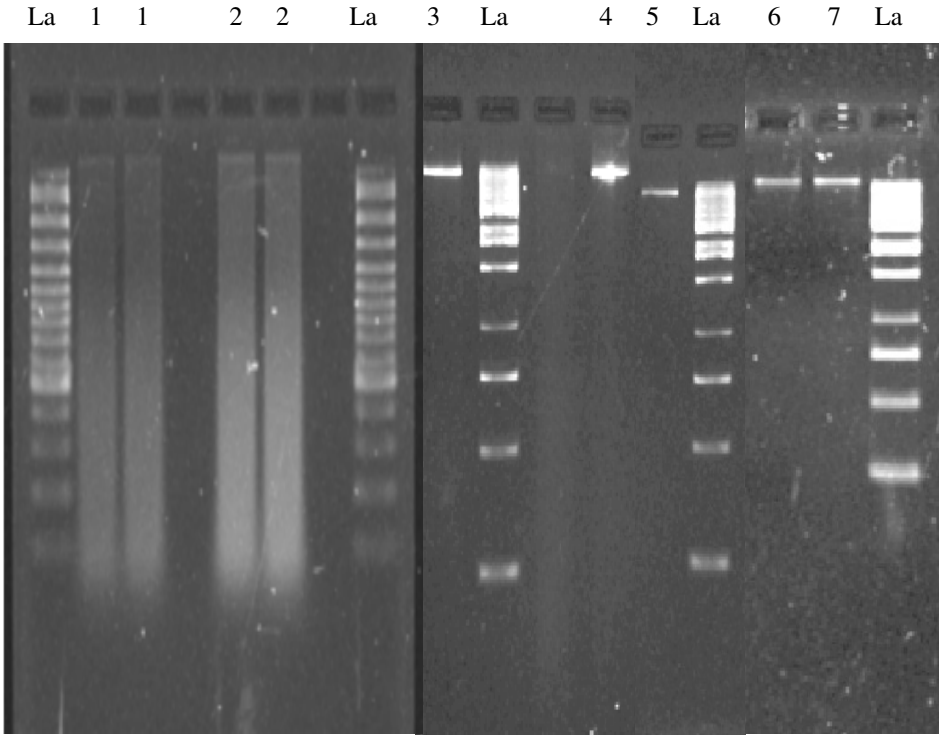


Figure 3.3 Total DNA extractions from six dried Flos Magnoliae species

La: GeneRuler 1kb DNA Ladder (Fermentas, U.S.A.); Track 1: *M. biondii* (5µl of DNA extraction); Track 2: *M. biondii* (10µl of DNA extraction); Track 3: *M. denudata* (5µl of DNA extraction); Track 4: *M. liliflora* (5µl of DNA extraction); Track 5: *M. sargentiana* (5µl of DNA extraction); Track 6: *M. sprengeri* (5µl of DNA extraction); and Track 7: *M. kobus* (5µl of DNA extraction).

3.3.2 Genetic divergence among five *Magnolia spp.* using fresh materials in Australia by RAPD

Fifteen RAPD primers (details see Table 2.7) were chosen to measure the genetic distances among the five *Magnolia spp.* and *Schisandra chinensis*. There was a very high level of polymorphism amongst five species of Magnolia and *Schisandra chinensis*. Of the total of 139 loci were scored, 98.6% were polymorphic. RAPD electrophoresis profiles of individual species demonstrated distinguishing DNA fingerprints of five *Magnolia spp.* and *Schisandra chinensis*, as shown in Figure 3.4 (a)-(o).

Based on the Nei's coefficient, the genetic identities and genetic distances among five *Magnolia spp.* and *Schisandra chinensis*, the specific plants that were grown in Australia, to RAPD data are presented in Table 3.1. The genetic distances among five *Magnolia spp.* and *Schisandra chinensis* ranged from 0.2160 to 0.7601. The maximum genetic distance was 0.7601 between *M. campbellii* and *Schisandra chinensis*. The maximum genetic distance among five *Magnolia spp.* was 0.4918 between *M. campbellii* and *M. sprengeri*, and the minimum genetic distance was 0.2160 between *M. campbellii* and *M. denudata*. *Schisandra chinensis* had bigger genetic distance with other species.

A dendrogram was constructed using MEGA 2 software using the UPGMA method (Figure 3.5). Six species were grouped into two main clusters. Cluster I contains *Schisandra chinensis,* which was found to be the most distinct (34% of genetic dissimilarity) species among six species. Within another main cluster that contained five *Magnolia spp.,* 11% of genetic dissimilarity indicated *M. campbellii* was closely related to *M. denudata,* and *M. kobus* was closely related to *M. liliflora* (13% of genetic dissimilarity). However, *M. sprengeri* was clustered more distantly than other four species within this cluster.

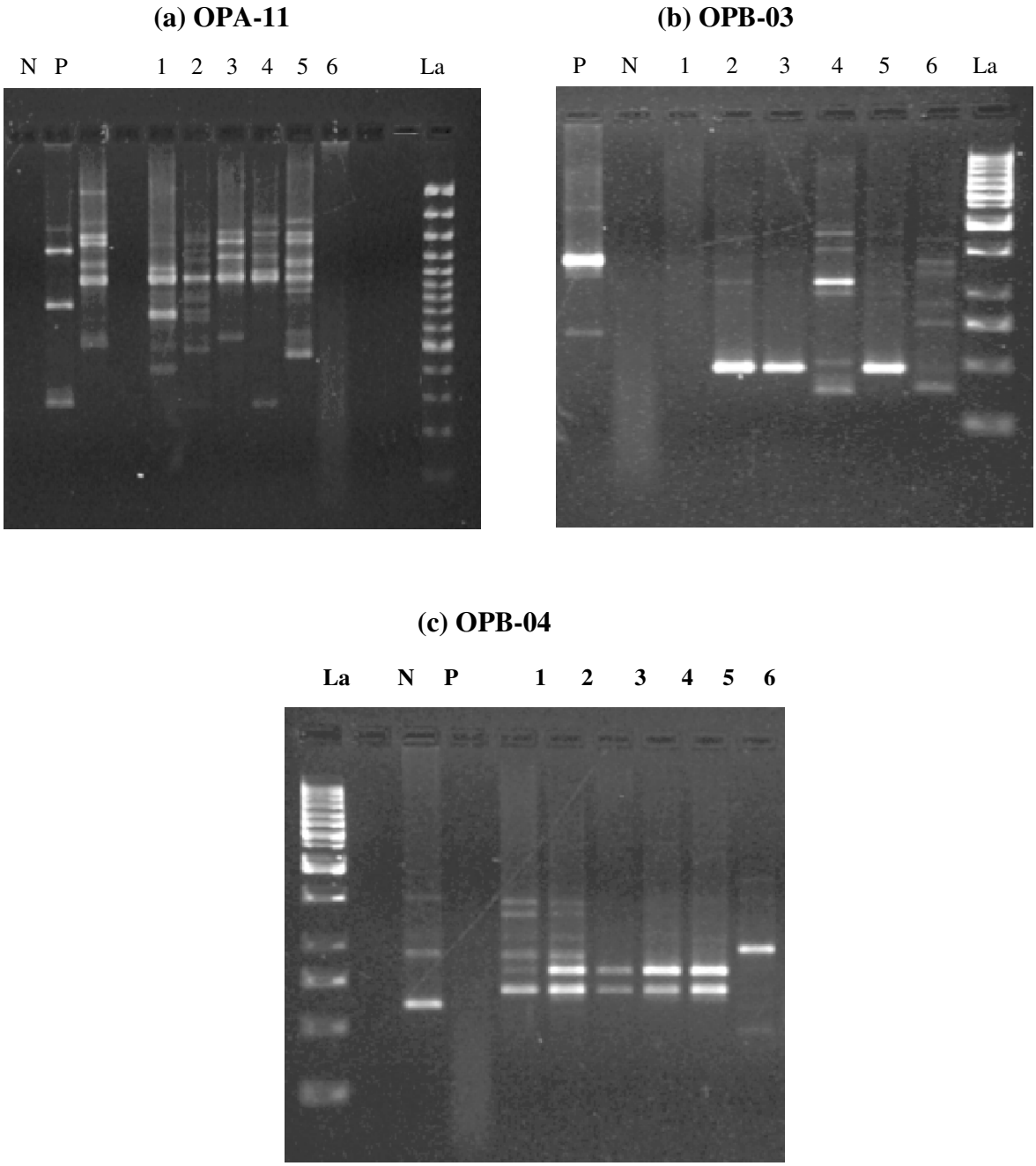
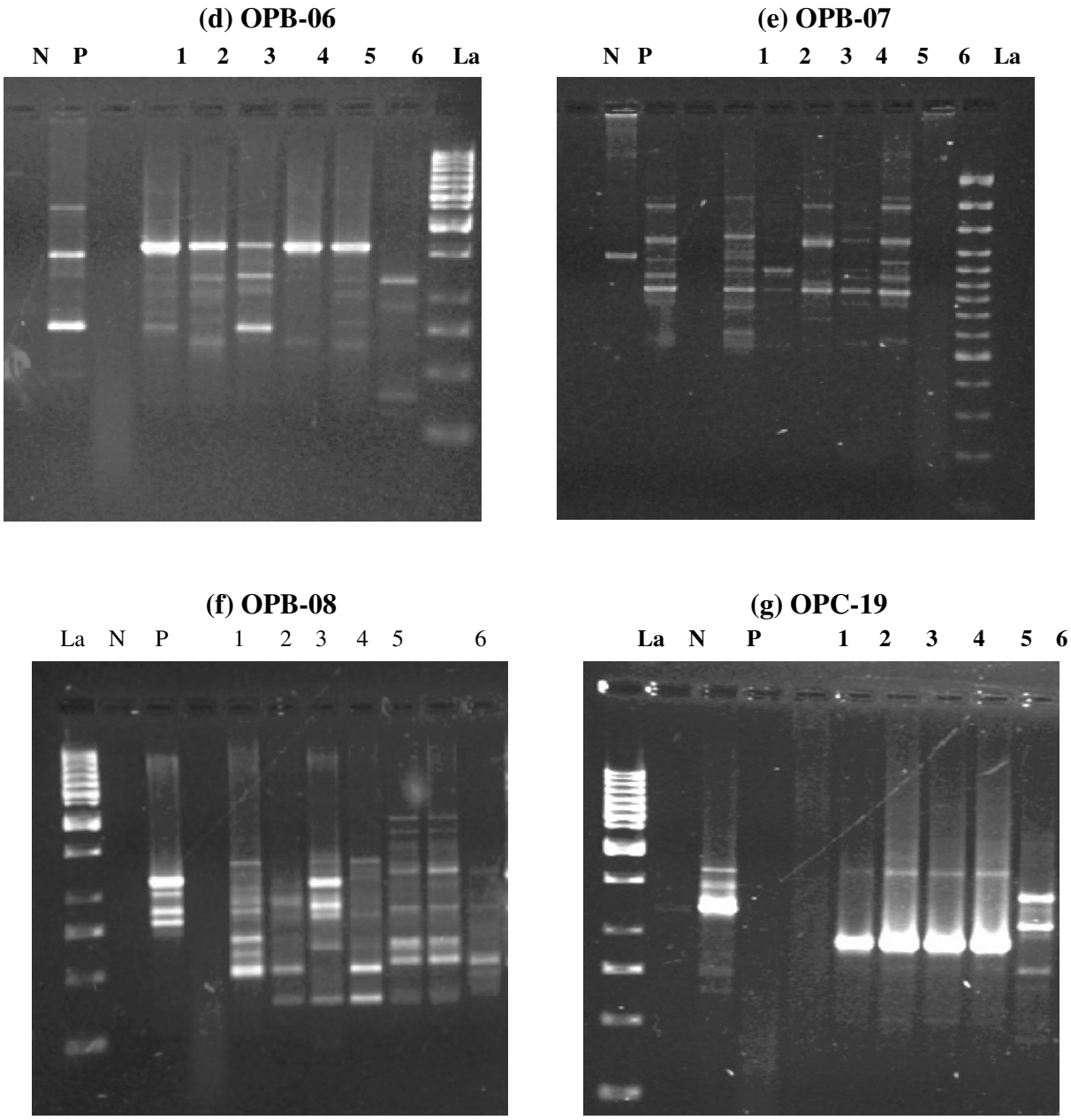


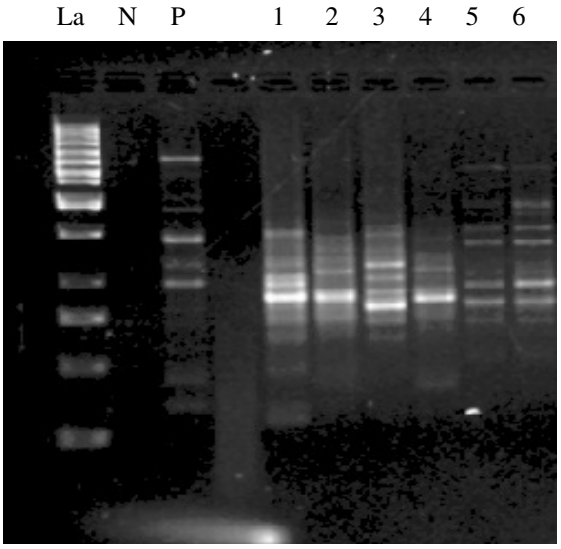
Figure 3.4 Gels of five *Magnolia spp.* and *Schisandra chinensis* (fresh materials) were tested by RAPD analysis using 15 random primers La: GeneRuler 1kb DNA Ladder or GeneRuler 100bp DNA Ladder Plus (Fermentas, U.S.A.); N; negative control (distilled water); P: positive control (chickpea); Track 1: *M. campbellii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; Track 5: *M. sprengeri* and Track 6: *Schisandra chinensis*.



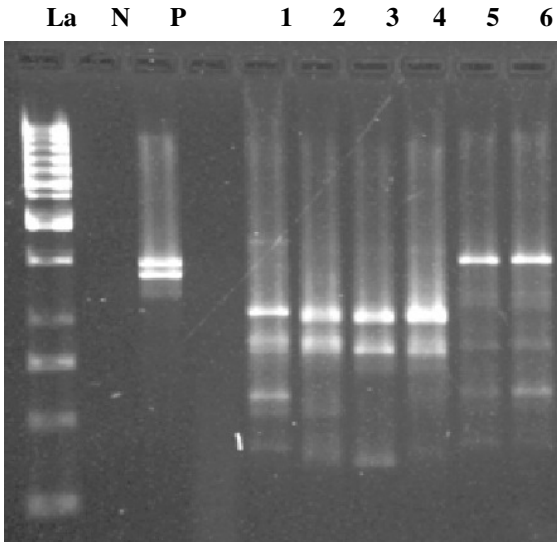
Continued Figure 3.4 Gels of five *Magnolia spp.* and *Schisandra chinensis* (fresh materials) were tested by RAPD analysis using 15 random primers

La: GeneRuler 1kb DNA Ladder or GeneRuler 100bp DNA Ladder Plus (Fermentas, U.S.A.); N; negative control (distilled water); P: positive control (chickpea); Track 1: *M. campbellii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; Track 5: *M. sprengeri* and Track 6: *Schisandra chinensis*.

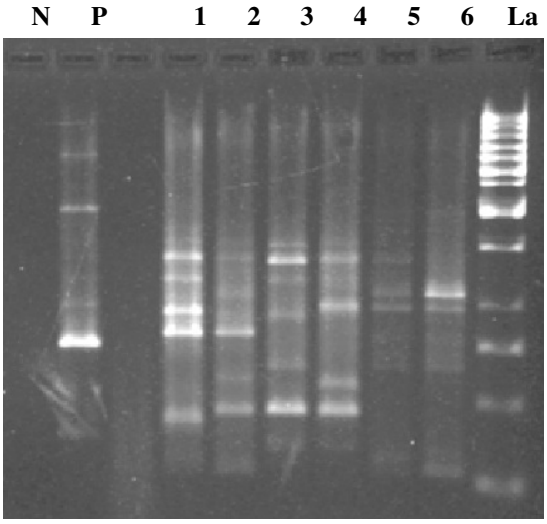
(h) OPG-13



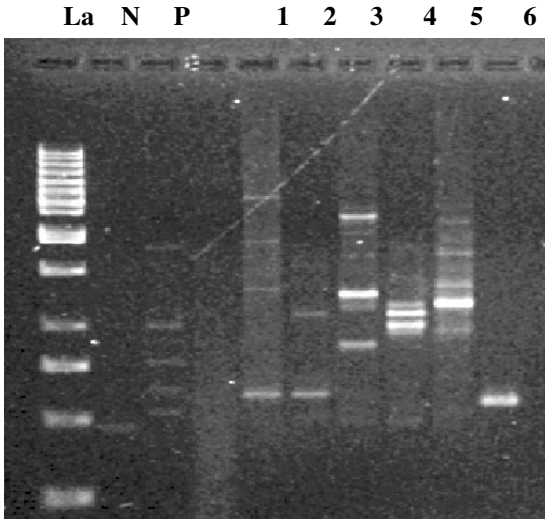
(i) OPM-04



(j) OPN-04

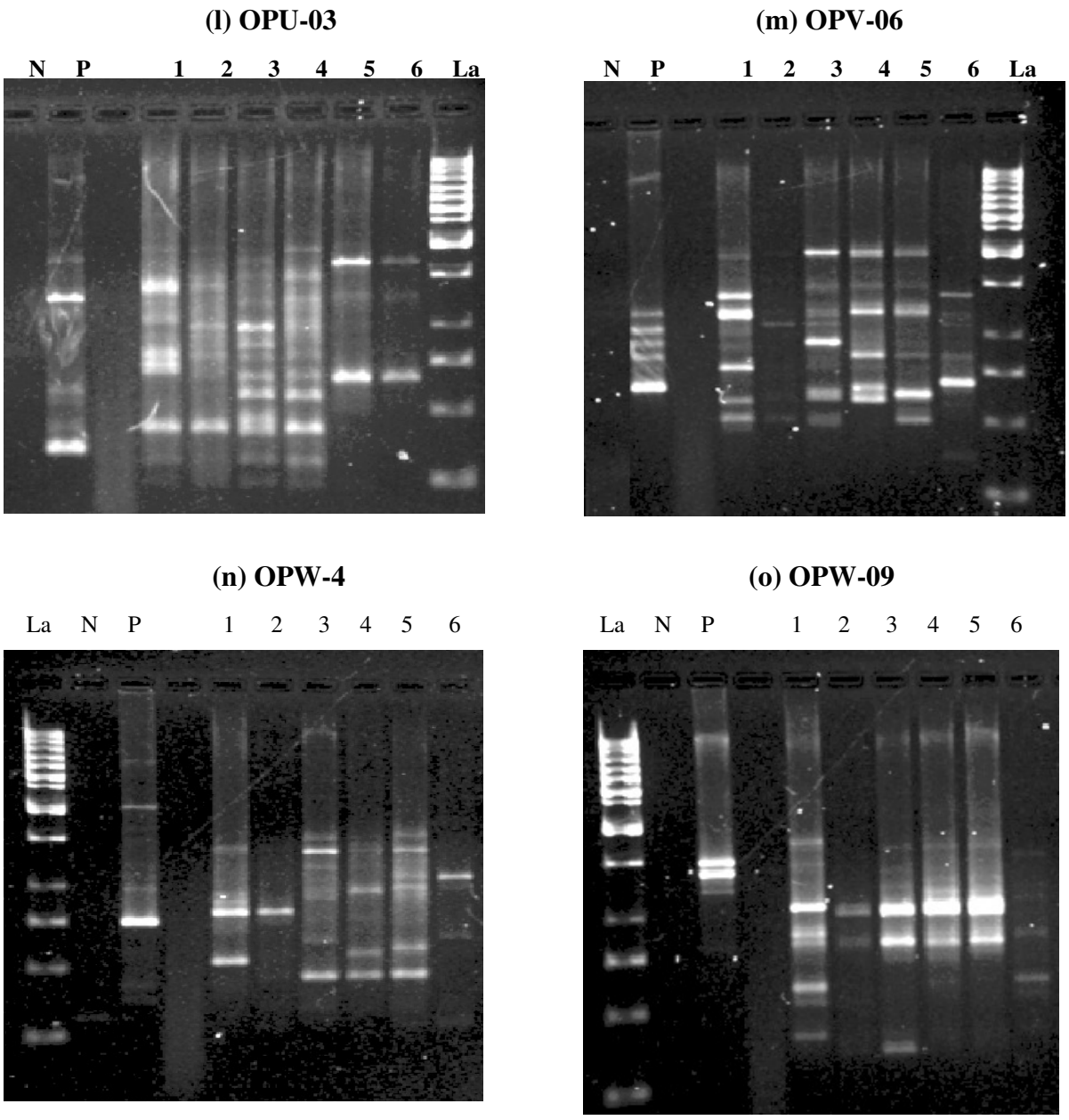


(k) OPP-10



Continued Figure 3.4 Gels of five *Magnolia spp.* and *Schisandra chinensis* (fresh materials) were tested by RAPD analysis using 15 random primers

La: GeneRuler 1kb DNA Ladder or GeneRuler 100bp DNA Ladder Plus (Fermentas, U.S.A.); N; negative control (distilled water); P: positive control (chickpea); Track 1: *M. campbellii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; Track 5: *M. sprengeri* and Track 6: *Schisandra chinensis*.

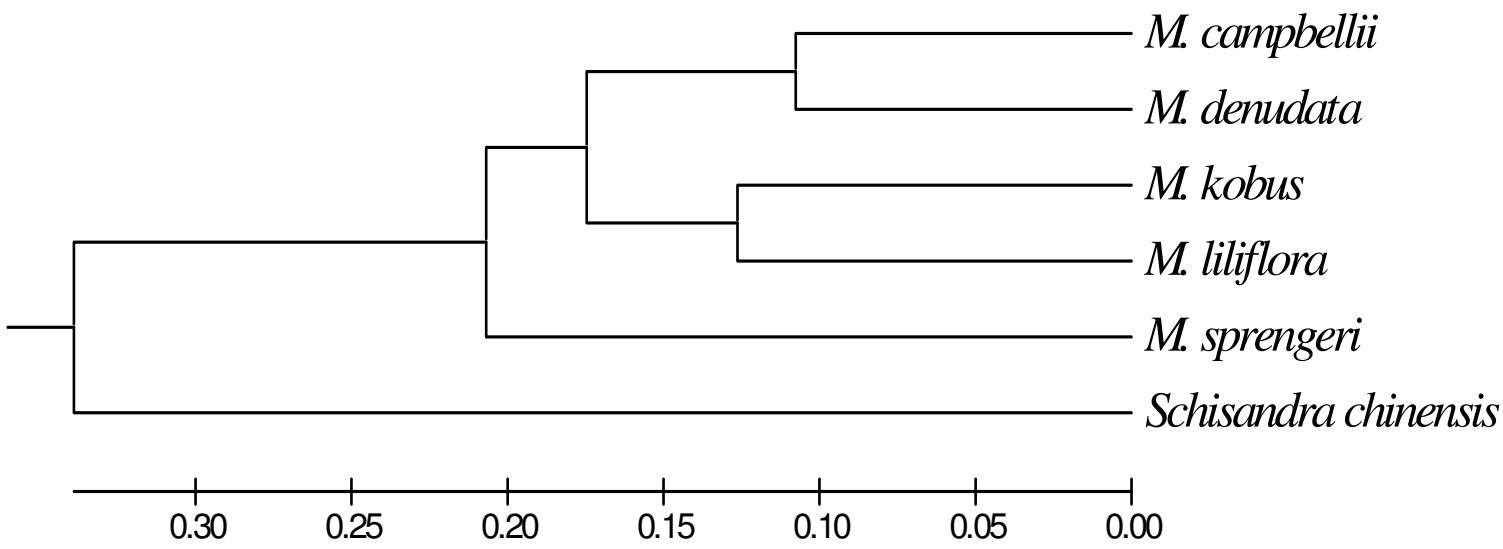


Continued Figure 3.4 Gels of five *Magnolia spp.* and *Schisandra chinensis* (fresh materials) were tested by RAPD analysis using 15 random primers

La: GeneRuler 1kb DNA Ladder or GeneRuler 100bp DNA Ladder Plus (Fermentas, U.S.A.); N; negative control (distilled water); P: positive control (chickpea); Track 1: *M. campbellii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; Track 5: *M. sprengeri* and Track 6: *Schisandra chinensis*.

Table 3.1 Matrix of genetic identity and genetic distance among five *Magnolia spp.* and *Schisandra chinensis* (fresh materials), based on Nei's coefficient (Nei, 1972). The genetic identities (upper diagonal) and genetic distances (under diagonal) among six species, according to RAPD data.

Species	*M. campbellii*	*M. denudata*	*M. kobus*	*M. liliflora*	*M. sprengeri*	*Schisandra chinensis*
M. campbellii	****	0.8058	0.6978	0.6906	0.6115	0.4676
M. denudata	0.2160	****	0.6906	0.7410	0.6763	0.4748
M. kobus	0.3598	0.3701	****	0.7770	0.6978	0.5108
M. liliflora	0.3701	0.2997	0.2523	****	0.6619	0.4748
M. sprengeri	0.4918	0.3912	0.3598	0.4127	****	0.6259
Schisandra chinensis	0.7601	0.7448	0.6718	0.7448	0.4689	****



The scale bar represented genetic dissimilarity.

Figure 3.5 The UPGMA dendrogram showed the estimated genetic distance among five *Magnolia spp.* and *Schisandra chinensis* (fresh materials) in Australia using RPAD.

3.3.3 Genetic divergence among six species of Flos Magnoliae using dried materials by RAPD analysis

Dried buds of six commonly used medicinal Flos Magnoliae species were used in RAPD analysis. *M. campbellii* was not included, due to the absence of the buds of *M. campbellii* from grown plants at the time of the study. Ten 10-mer oligo primers were tested for the identification of the DNA fingerprints of six Flos Magnoliae species. Of a total 86 loci, 98.8% were polymorphic (85 polymorphic loci), among the six Flos Magnoliae species. Figure 3.6 (a) – (i) show those RAPD electrophoresis profiles of six Flos Magnoliae species demonstrated unique DNA fingerprints. However, there is no amplification product for OPB-03 from six Flos Magnoliae species (the electrophoresis gel did not show).

The matrix (Table 3.2) based on Nei's coefficient revealed the polymorphism among six Flos Magnoliae species. Genetic identities between six species varied from 0.5698 to 0.7907. Genetic distances among six species ranged from 0.2348 to 0.5625. The maximum genetic distance was 0.5625 between *M. biondii* and *M. denudata*, and the minimum genetic distance was 0.2348 between *M. denudata* and *M. kobus*. *M. biondii* and *M. sargentiana*, which were collected from China, had more genetically divergent from the other species.

A dendrogram indicating the genetic relationships among six Flos Magnoliae species was constructed using MEGA 2 software (Figure 3.7). *M. denudata* was closely related to *M. kobus* with 12% of genetic dissimilarity. However, two preparations of Flos Magnoliae species, *M. biondii* and *M. sargentiana* were clustered more distantly than other four species.

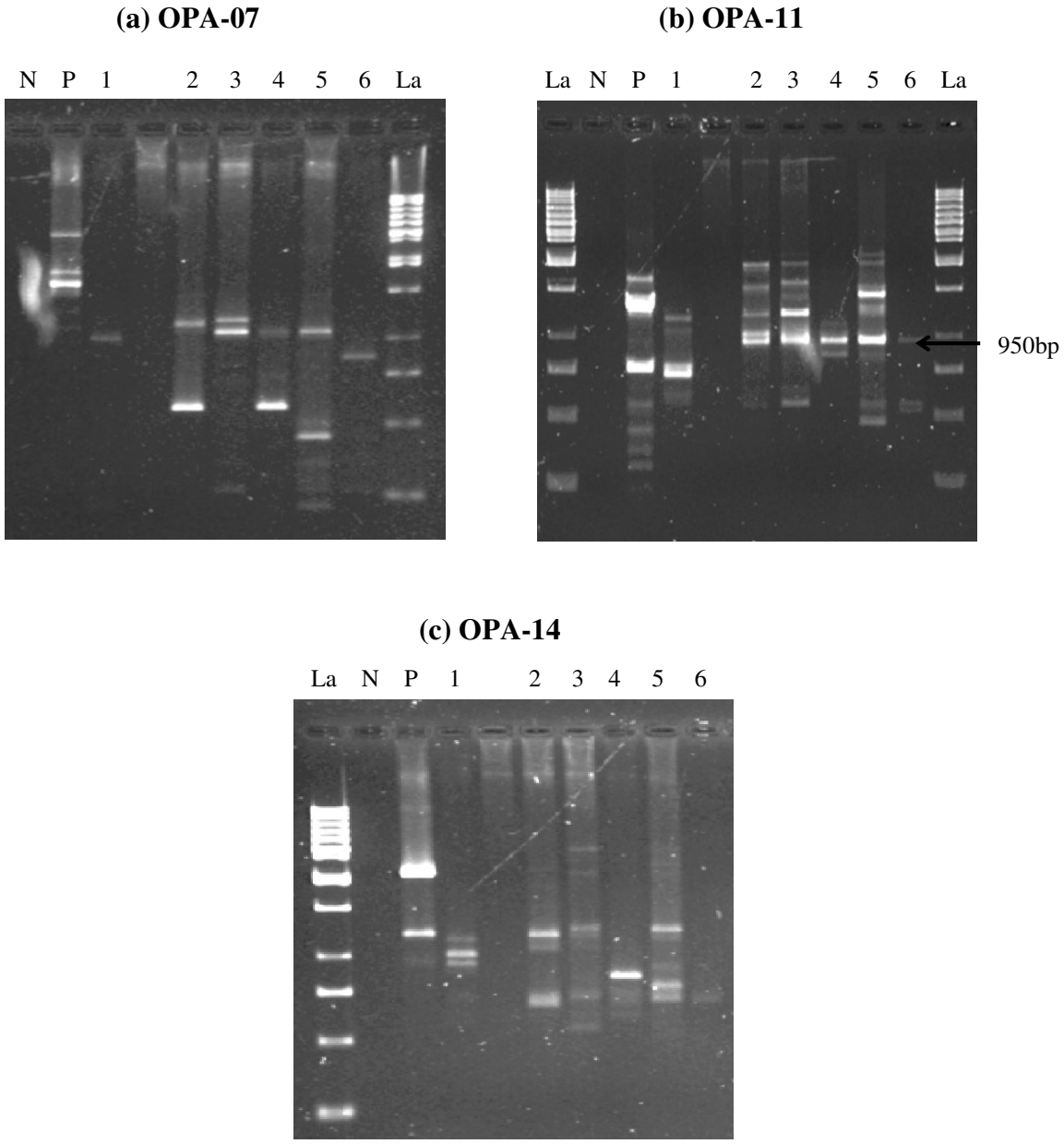
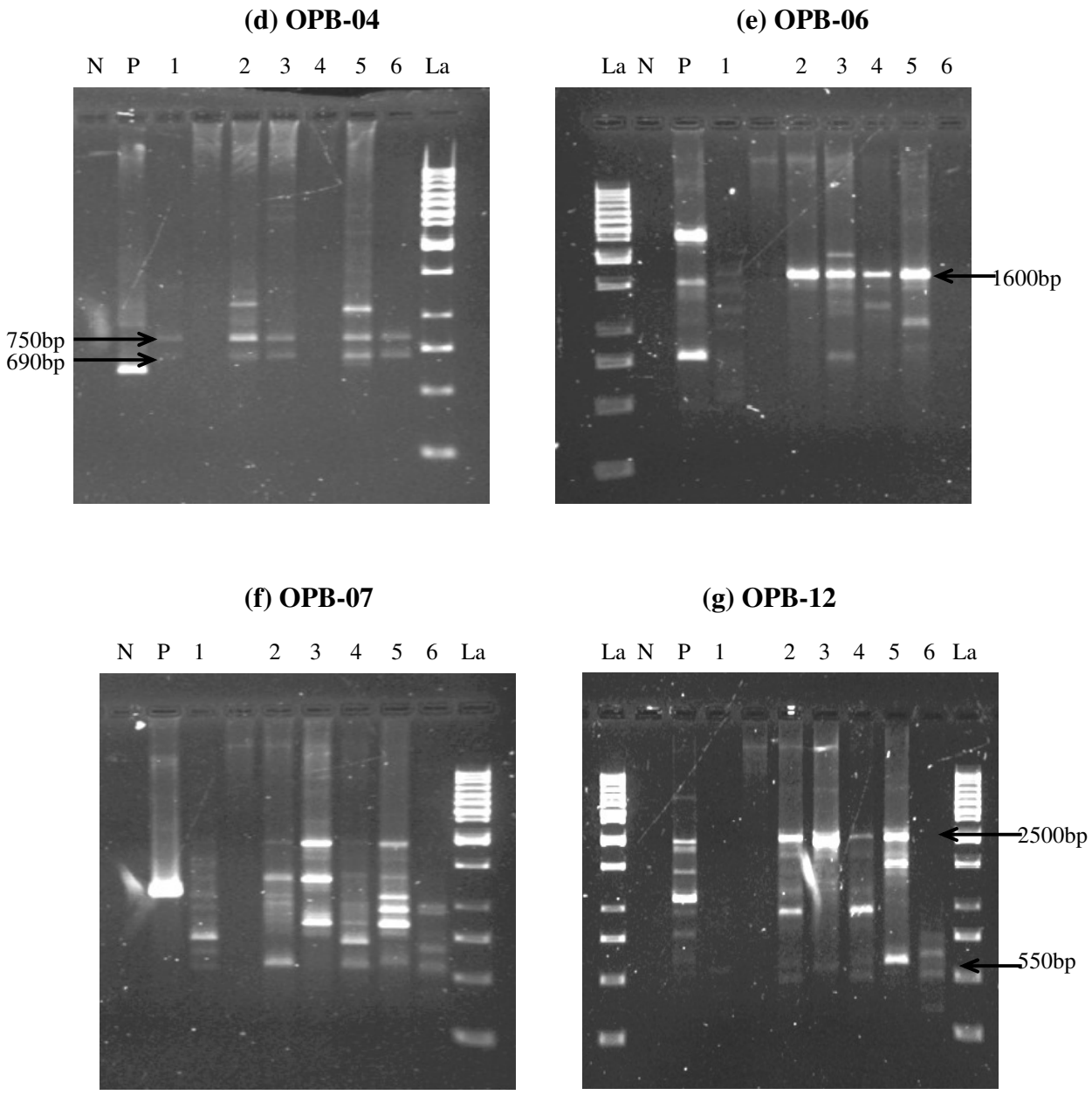


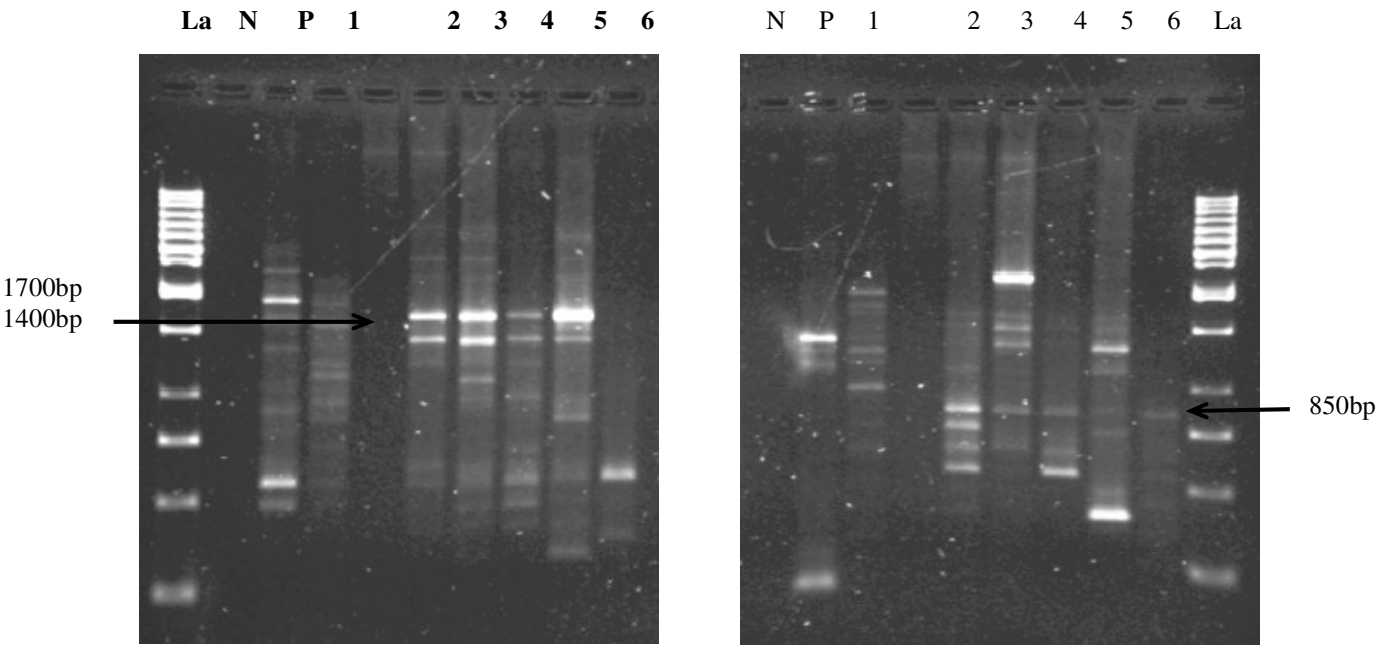
Figure 3.6 Gels of six dried Flos Magnoliae species (dried materials) were tested by RAPD analysis using nine random primers

La: GeneRuler 1kb DNA Ladder or GeneRuler 100bp DNA Ladder Plus; N; negative control (distilled water); P: positive control (chickpea); Track 1: *M. biondii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; Track 5: *M. sprengeri* and Track 6: *M. sargentiana*.



Continued Figure 3.6 Gels of six dried Flos Magnoliae species (dried materials) were tested by RAPD analysis using nine random primers

La: GeneRuler 1kb DNA Ladder or GeneRuler 100bp DNA Ladder Plus; N; negative control (distilled water); P: positive control (chickpea); Track 1: *M. biondii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; Track 5: *M. sprengeri* and Track 6: *M. sargentiana*.

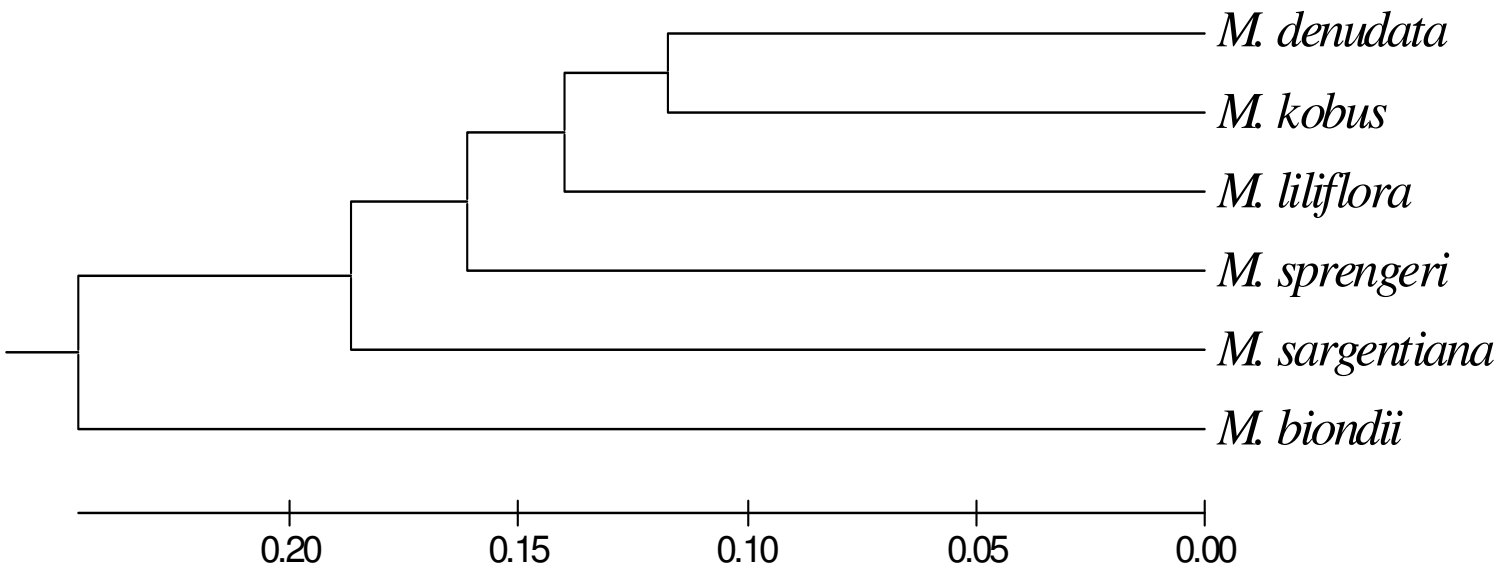


Continued Figure 3.6 Gels of six dried Flos Magnoliae species (dried materials) were tested by RAPD analysis using nine random primers

La: GeneRuler 1kb DNA Ladder or GeneRuler 100bp DNA Ladder Plus; N; negative control (distilled water); P: positive control (chickpea); Track 1: *M. biondii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; Track 5: *M. sprengeri* and Track 6: *M. sargentiana*.

Table 3.2 Matrix of genetic identity and genetic distance among six Flos Magnoliae species (dried materials), based on Nei's coefficient (Nei, 1972). The genetic identities (upper diagonal) and genetic distances (under diagonal) among six Flos Magnoliae species, according to RAPD data.

Species	*M. biondii*	*M. denudata*	*M. kobus*	*M. liliflora*	*M. sprengeri*	*M. sargentiana*
M. biondii	****	0.5698	0.6395	0.6047	0.5930	0.6512
M. denudata	0.5625	****	0.7907	0.7558	0.7442	0.6860
M. kobus	0.4470	0.2348	****	0.7558	0.7442	0.6628
M. liliflora	0.5031	0.2800	0.2800	****	0.6860	0.7442
M. sprengeri	0.5225	0.2955	0.2955	0.3768	****	0.6628
M. sargentiana	0.4290	0.3768	0.4113	0.2955	0.4113	****



The scale bar represented genetic dissimilarity.

Figure 3.7 The UPGMA dendrogram showed the estimated genetic distance among six Flos Magnoliae species (dried materials) using RAPD

3.3.4 Genetic divergence among five species of Flos Magnoliae using dried materials by PCR-RFLP analysis

The PCR of 5S rRNA region was with five Flos Magnoliae species, except *M. sargentiana*. PCR products having a similar size of about 650bp were obtained from five Flos Magnoliae species, as shown in Figure 3.8. A DNA fragment of 500bp was also presented in *M. liliflora* after amplification.

Fifteen different restriction enzymes were used. Only four of the 15 enzymes (*Hha*I, *Pml*I, *Sma*I and *EcoR*V) (New England Biolabs, USA and Promega, Australia) (details described in Table 2.8) were suitable for PCR-RFLP analysis. The DNA fingerprinting profiles from digestions generated clear, coherent banding patterns, which enabled to determine the differences between five Flos Magnoliae species [Figure 3.9 (a)-(d)].

According to the Nei's coefficient, the genetic identities and genetic distances among five Flos Magnoliae species to PCR-RFLP data are presented in Table 3.3. The genetic identities among five Flos Magnoliae species ranged from 0.6400 to 0.9200. The genetic distances between five Flos Magnoliae species ranged from 0.0834 to 0.4463. The maximum genetic distance was 0.4463 between *M. biondii* and *M. liliflora*, and the minimum genetic distance was 0.0834 between *M. denudata* and *M. sprengeri*.

A dendrogram indicating the genetic relationships among five Flos Magnoliae species was constructed using MEGA 2 software (Figure 3.10). The scale bar was inflated in order to clearly illustrate the branching patterns. There is little genetic distance among five Flos Magnoliae species, compared to the genetic distance, found in RPAD profiles. However, *M. liliflora* was clustered more distantly than other four species.

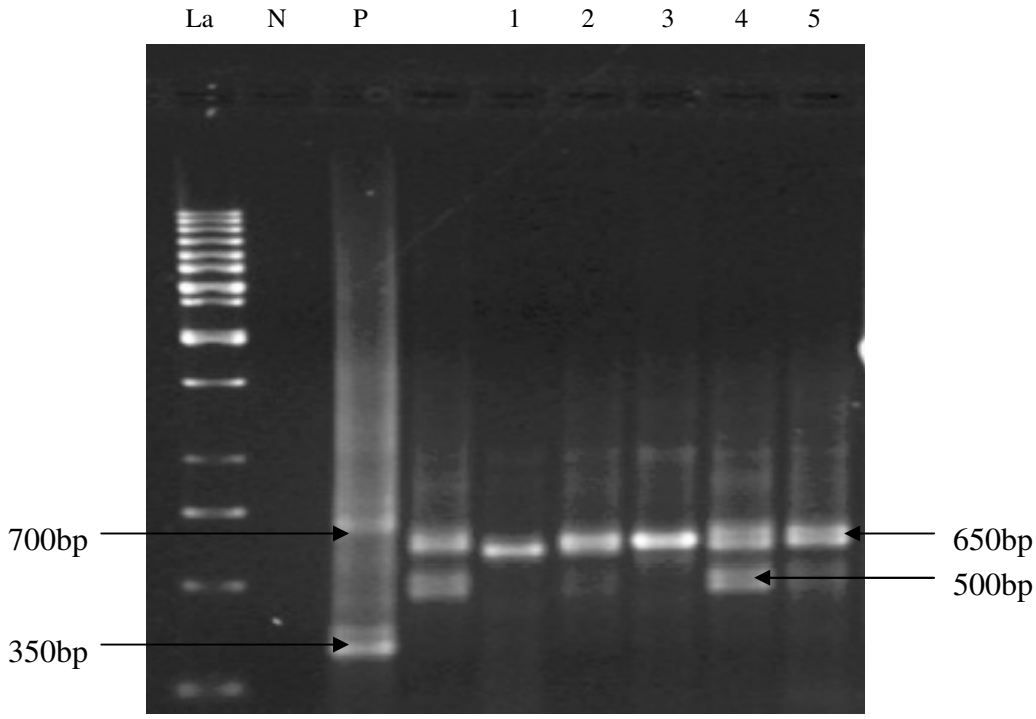


Figure 3.8 PCR products of five Flos Magnoliae species (dried materials) after amplified with 5S rRNA genes

La: GeneRuler 1kb DNA ladder; N; negative control (distilled water); P: Positive control (chickpea); Track 1: *M. biondii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; and Track 5: *M. sprengeri*.

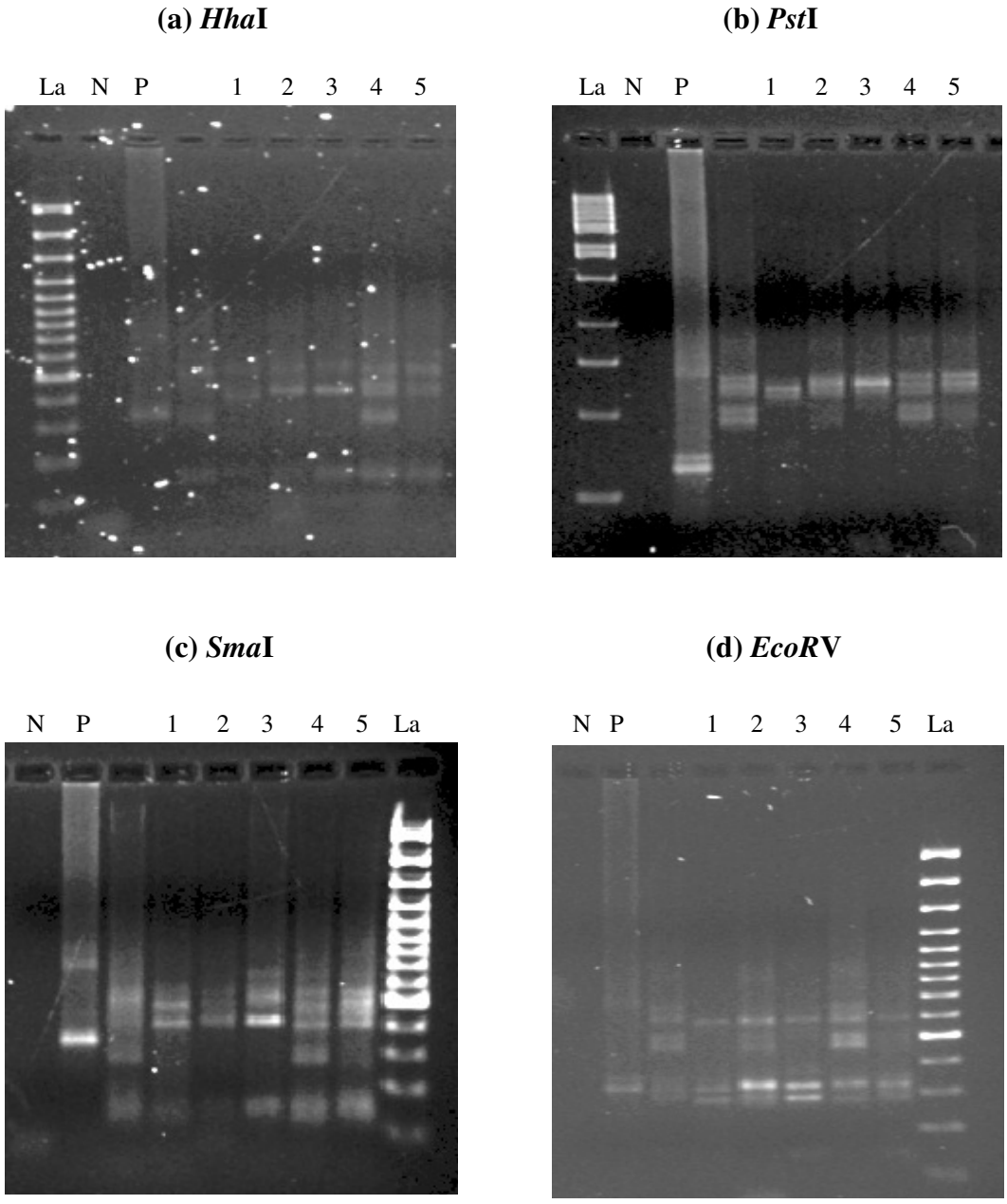
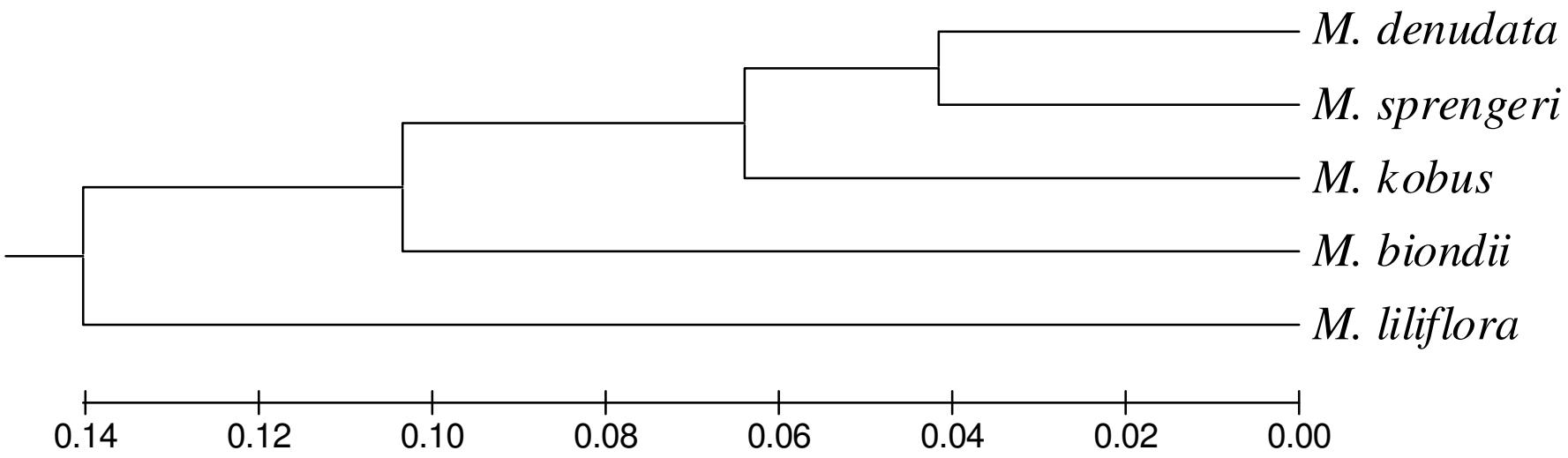


Figure 3.9 After amplified with 5S rRNA genes, DNA fingerprinting profiles of five Flos Magnoliae species (dried materials), digested by four restriction enzymes

La: GeneRuler 1kb DNA Ladder or GeneRuler 100bp DNA Ladder Plus; N; negative control (distilled water); P: Positive control (chickpea); Track 1: *M. biondii*; Track 2: *M. denudata*; Track 3: *M. kobus*; Track 4: *M. liliflora*; and Track 5: *M. sprengeri*.

Table 3.3 Matrix of genetic identity and genetic distance among five Flos Magnoliae species (dried materials), based on Nei's coefficient (Nei, 1972). The genetic identities (upper diagonal) and genetic distances (under diagonal) among five Flos Magnoliae species, according to PCR-RFLP data.

Species	*M. biondii*	*M. denudata*	*M. kobus*	*M. liliflora*	*M. sprengeri*
M. biondii	****	0.8000	0.8400	0.6400	0.8000
M. denudata	0.2231	****	0.8800	0.8400	0.9200
M. kobus	0.1744	0.1278	****	0.7200	0.8800
M. liliflora	0.4463	0.1744	0.3285	****	0.8400
M. sprengeri	0.2231	0.0834	0.1278	0.1744	****



The scale bar represented genetic dissimilarity.

Figure 3.10 The UPGMA dendrogram showed the estimated genetic distance among five Flos Magnoliae species (dried materials) using PCR-RFLP of 5S rRNA region

3.4 Discussion

Species of fresh Flos Magnoliae buds, collected in Australia, are from the family Magnoliaceae, tribe *Magnolieae*, subtribe *Magnolieae*, genus *Magnolia* subgenus *Yulania,* except *Schisandra chinensis* (Magnolia Society, 2003). *M. campbellii, M. denudata* and *M. sprengeri* are recognised under section *Yulania* (Magnolia Society, 2003, Pei and Chen, 1991). *M. kobus* and *M. liliflora* are classified in the section *Buergeria* and section *Tulipastrum*, respectively. *Schisandra chinensis* (Family Magnoliaceae, tribe *Schisandreae*, genus *Schisandra*) was selected as an out-group genus, thereby providing a yardstick for comparisons within *Magnolia spp*. High-level genetic dissimilarity (34%) among *Schisandra chinensis* to other five *Magnolia spp* was in agreement with the taxonomy system.

The finding of the close genetic distance between *M. denudata* and *M. campbellii* is in line with the classical taxonomy system (Magnolia Society, 2003). Although *M. liliflora* and *M. kobus* are under two different sections, *Buergeria* and *Tulipastrum*, respectively, dissimilarity as mentioned in this study was found very small. More species under these two sections need to be accurately analysed the genetic distances of the species from *Buergeria* and *Tulipastrum.* However, *M. sprengeri* has the highest genetic distance in this cluster compared to other species. This finding disagrees with the finding in previous studies that the genetic distances were 0.17 or 0.18 between *M. sprengeri* and *M. denudate,* (Wang et al., 2004c, Wang et al., 2004e) compared to the finding (genetic distance: 0.3912) of this study. In addition, Wang *et al* (2004c) also found larger genetic distance, 0.591, between *M. kobus* and *M. sprengeri,* compared to the findings (genetic distance: 0.3598) from present study. The samples used in the present study were locally collected, and the samples used in the previous studies were collected from China. Hybridization could be the factor causing the big genetic distance between the Australian *M. sprengeri* variety and the Chinese variety. Furthermore, these findings may be due to the misidentification of *M. sprengeri* by the local nursery. The

future study on investigation of the molecular profiles from different *M. sprengeri* varieties is necessary for a better understanding of the present existing findings.

The RAPD analysis demonstrated a slight genetic dissimilarity among six Flos Magnolia species. For instance, the DNA fingerprinting profiles of *M. biondii* and *M. sargentiana*, from the Chinese market, have larger genetic distances compared to other species. These results are in disagreement with the previous studies (Wang et al., 2004e, Wang et al., 2004c) and Magnoliaceae taxonomy (Magnolia Society, 2003). However, due to the low concentration and poor quality of dried materials, present study was not able to investigate further to explain the present genetic data. The distinct DNA fingerprinting profiles, which were obtained after amplification by primers OPA-07, OPA-14 and OPB-07, may be used for distinguishing different *Magnolia spp.* in the future. A number of DNA fragments identified by OPA-11 (950bp), OPB-04 (690bp and 750bp), OPB-06 (1600bp), OPB-12 (550bp and 2500bp), OPB-17 (1400bp and 1700bp) and OPB-18 (850bp) (black arrows as shown in Figure 3.6), could be regarded as species-specific markers. Therefore, the shared RAPD fragments between species can be cloned, sequenced and then designed as probes to be used for the identification and authentication of Flos Magnoliae species.

There were slight differences between the DNA fingerprinting profiles from fresh samples and crude buds in this study, although most of the DNA fragments are same between fresh and dried samples. For instance, no DNA fragments of dried Flos Magnoliae were visualised after amplification by primer OPB-03, compared to distinct DNA patterns in the fresh samples, that probably as a result of the physical conditions and individual characteristics of the DNA extraction from crude materials (Shaw et al., 2002).

PCR-RFLP analysis was also performed for the identification of the genetic profiles of Flos Magnoliae in this study. The extraction conditions of dried materials were optimised in this study, because the DNAs obtained from crude drugs were degraded. The low concentration and poor purity of DNA from *M. sargentiana* may cause the failure of the amplification by 5S rRNA, which may be due to over-heating of the preparation under high temperature by the herbal suppliers. This may indicate that the physical conditions of heat and dryness and the content of polysaccharides and polyphenolic could directly affect the accuracy and sensitivity of PCR amplification (Shaw et al., 2002).

The distinct DNA fingerprints from PCR-RFLP of 5S rRNA region may contribute to the identification of Flos Magnoliae species. The closer genetic dissimilarity was demonstrated in the PCR-RFLP profiles, compared to the RAPD profiles. PCR-RFLP method, detected a single genomic locus in which recombination has been shown to occur, in present study the 5S rRNA region (Glick and Pasternak, 2003). In the contrast, RAPD method explores genetic dissimilarity with the whole genomic DNA, resulting in the high degree of polymorphism among individuals (Lynch and Milligan, 1994).

The molecular bases of the RAPD methods and PCR-RFLP analysis are different. The RAPD method explores genetic dissimilarity with the whole genomic DNA, which can detect greater genetic diversity, compared to PCR-RFLP method (Betancor et al., 2004). Such multi loci can be exploited in ways largely analogous to the uses of PCR-RFLP (Glick and Pasternak, 2003). RAPD does allow the better identification of different species, compared to PCR-RFLP (Yakoob et al., 2001). These factors are especially valuable for the identification of different Flos Magnoliae species. However, the low reproducibility and sensitivity were considered for RAPD method (Penner et al., 1993). Compared to RAPD, the PCR-RFLP of multi copy

genes, such as 5S rRNA, may estimate different medicinal sources with the high reproducibility and sensitivity (Shaw and But, 1995).

In conclusion, the present study established the genetic profiles of different Flos Magnoliae species using RAPD and PCR-RFLP, which represents the first report on these particular species. The findings may be useful for the identification and authentication of different Flos Magnoliae species. Furthermore, the phylogenetic relationship of *Magnolia spp.* in Australia was confirmed, indicating locally grown Flos Magnoliae species may be used for medicinal application.

Chapter Four

Qualitative Identification of Flos Magnoliae Using TLC

4.1 Background

Since 1960s, optimal and standardised TLC technology has been widely applied for analysis of chemical compositions of food, cosmetics and pharmaceuticals (Ettre, 2005). The principle of the techniques has been described in Chapter 1 (see Section 1.3.4.4.1). Recently, with the rapid development of the HPTLC instrumentation and computer assistance, more advanced TLC systems, such as CAMAG, have been used for analysis of a numbers of CHM (CAMAG, 2006b). TLC has now been frequently used for the identification and authentication of CHM and other herbal medicines. Various authorised references, for instances the Chinese Pharmacopoeia and the American Herbal Pharmacopoeia (The Pharmacopoeia Commission of People's Republic of China, 2005, AHP, 2004). Compared to other chromatographic techniques, it has been recognised with the characterisations of rapidity and simultaneousness (Kotiyan and Vavia, 2000).

As one of the commonly used CHM, many substitutes and adulterants of Flos Magnoliae have been found in the market (Fu, 2000). According to the current Chinese Pharmacopoeia, TLC is used for the identification of Flos Magnoliae, in which magnolin as the marker compound (The Pharmacopoeia Commission of People's Republic of China, 2005). Recently, magnolin, pinoresinol dimethyl ether and lirioresinol-B dimethyl ether from the chloroform extracts of *M. biondii, M. denudata* and *M. sprengeri*, have been identified using TLC (Su et al., 2001).

Amis of the study

The aim of the present study is to establish the method to identify Flos Magnoliae species, in regard to qualitative analysis of two marker compounds, including magnolin and fargesin, in the ethanol extracts from different Flos Magnoliae species and varieties. Meanwhile, to use the technique as a quality control tool to identify the various batches of *M. biondii* from different suppliers and cultivation sites.

4.2 Method

4.2.1 Samples extraction and preparation

Dried Flos Magnoliae samples from six different *Magnolia spp.* (Table 2.2) were used, including *M. biondii, M. denudata, M. kobus, M. liliflora, M. sprengeri* and *M. sargentiana*. Twenty *M. biondii* samples, including 11 batches from six varieties (Table 2.3), five batches from different herbal suppliers (Table 2.4), and four batches from different cultivation sites (Table 2.5) were used in the present study.

The protocol of samples extraction and preparation is described in Chapter 2 (Section 2.5.1).

4.2.2. Reagents and chemicals

The details of the reagents and chemicals are described in Chapter 2 (Section 2.3.2).

4.2.3 Preparation of standard references, magnolin and fargesin

Briefly, 1.5mg of marker compounds, magnolin and fargesin, were dissolved in 1.5ml of ethanol, and then sonicated for 15min (details see Section 2.5.2). The final concentrations of 2.5mg/ml for magnolin and 1mg/ml for fargesin were used for standard solutions.

4.2.4 Chromatographic conditions

The TLC system (CAMAG, Switzerland) (Figure 2.7 and Table 2.9) consisted of the semi-automatic sample application (Linomat 5), the horizontal developing chambers, the TLC scanner III, the documentation system with the camera (HV-C20A, Hitachi, Japan) and the workstation (winCATS, version: 1.2.5). TLC silica gel 60 F_{254} plates (20cm x 10cm, E. Merck, Germany) and HPTLC silica gel 60 F_{254} plates (10cm x 10cm, E. Merck, Germany) were used. The mobile phase was a mixture of chloroform – ether (5:1, %-v:v). The protocols of the chromatographic conditions are described in Section 2.5.3.

4.2.5 Data analysis

Magnolin and fargesin from the testing samples were qualitatively determined based on the R_f values of the resolved bands. Testing samples were in quadruplicates and were represented as mean ± S.D. Furthermore, all measurements were expressed as R.S.D.

4.3 Results

4.3.1 Method optimisation

The mixture of chloroform – ether (5:1) resolved magnolin and fargesin spots with a better peak shape. The combination of chloroform and ether was found to produce an optimal migration of magnolin and fargesin (R_f values of 0.41±0.01 for magnolin and 0.62±0.02 for fargesin). The repeatability of the method was tested by five applications of the same sample (*M. biondii*, Hualong Magnolia Development Co. Ltd) on the same TLC plates (Figure 4.1). The R_f value of magnolin was 0.41±0.00 and the R_f value of fargesin was 0.60±0.00.

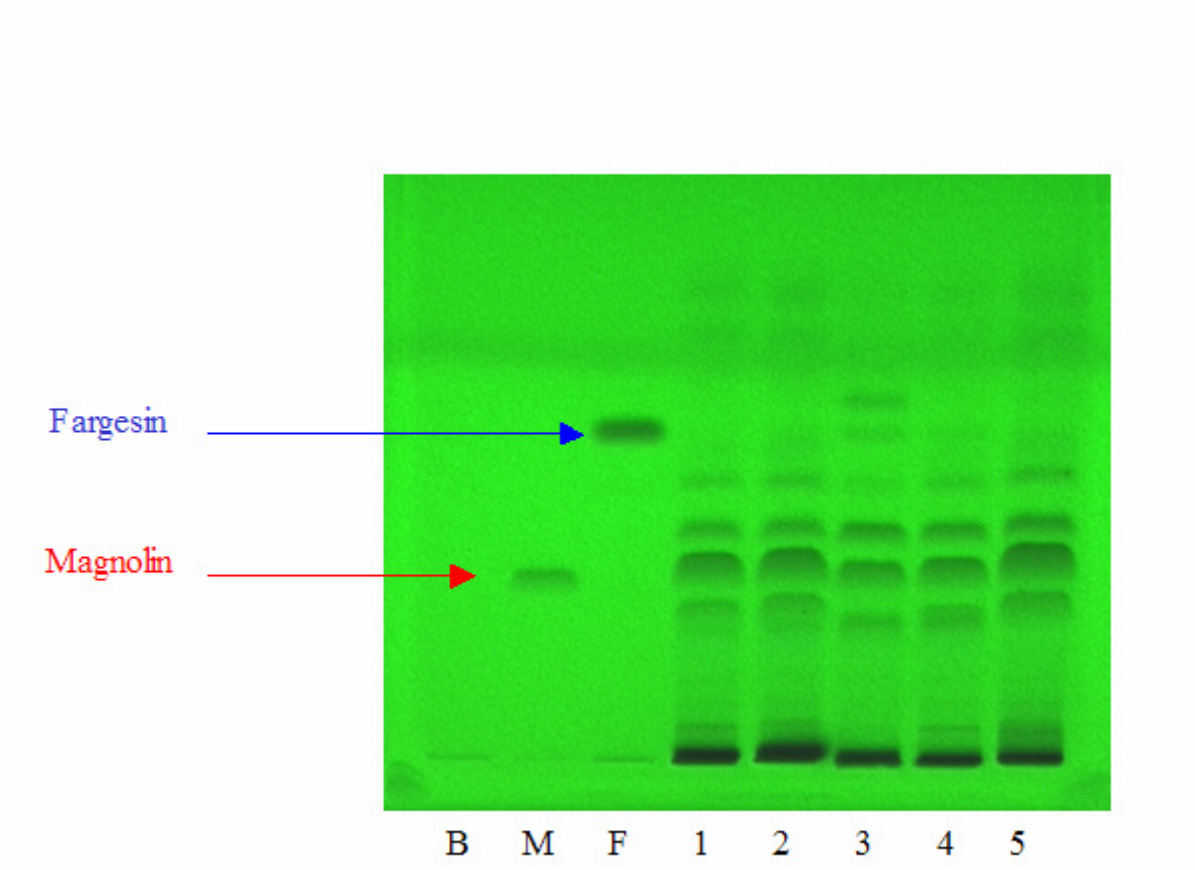


Figure 4.1 The repeatability of the method for TLC identification

B: Blank (10µl, methanol); M: Magnolin standard (2.5mg/ml, 10µl); F: Fargesin standard (1mg/ml, 10µl); Track 1-5: *M. biondii* (50mg raw herb/ml, 30µl).

4.3.2 Identification of different Flos Magnoliae species

Six Flos Magnoliae species were studied, namely, *M. biondii, M. denudata, M. kobus, M. liliflora, M. sprengeri* and *M. sargentiana* (Figure 4.2). The chromatograms of two species, including *M. biondii* and *M. kobus* showed the bands that indicated magnolin and fargesin. Magnolin was detected from the ethanol extract of *M. liliflora*, although fargesin was not found. Table 4.1 showed the values of R_f for magnolin and fargesin from the samples. For positive identification, the testing sample must exhibit bands with the R_f values (0.41 – 0.42 for magnolin and 0.60 – 0.61 for fargesin), similar to those (% R.S.D. < 3%) to those of reference marker compounds.

Table 4.1 The values of R_f for magnolin and fargesin in different Flos Magnoliae species. Data are presented as mean ± S.D. (n=4). 'n/a' indicated not detected.

		M. biondii	*M. denudata*	*M. kobus*	*M. liliflora*	*M. sargentiana*	*M. sprengeri*
Magnolin	R_f (S.D.)	0.41 (0.00)	n/a	0.41 (0.00)	0.42 (0.00)	n/a	n/a
	% R.S.D.	0.9	n/a	0.7	0.8	n/a	n/a
Fargesin	R_f (S.D.)	0.60 (0.00)	n/a	0.61 (0.00)	n/a	n/a	n/a
	% R.S.D.	0.7	n/a	0.9	n/a	n/a	n/a

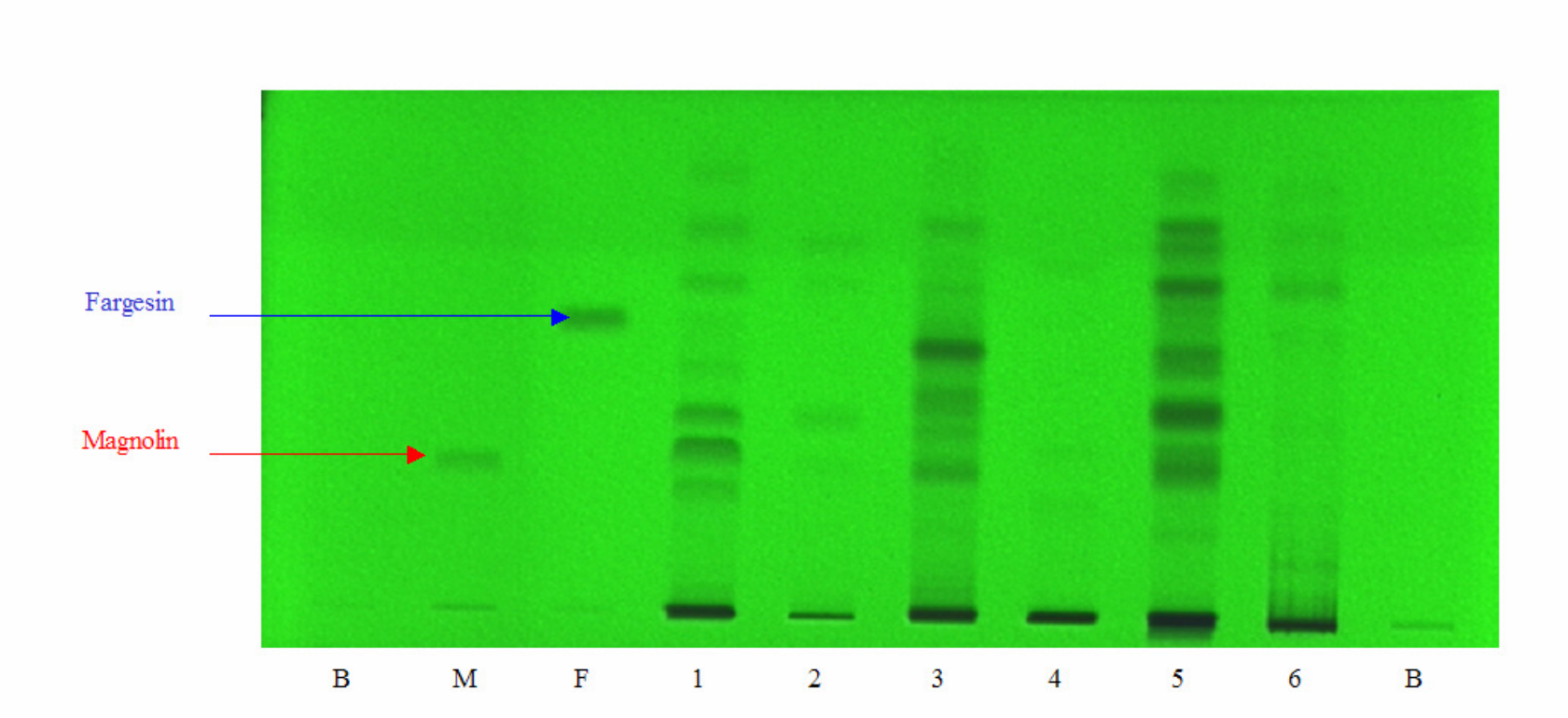


Figure 4.2 TLC chromatograms of six Flos Magnoliae species

B: Blank (10µl, methanol); M: Magnolin standard (2.5mg/ml, 10µl); F: Fargesin standard (1mg/ml, 10µl); Track 1: *M. biondii* (50mg raw herb/ml, 30µl); Track 2: *M. denudata* (50mg raw herb/ml, 30µl); Track 3: *M. kobus* (50mg raw herb/ml, 30µl); Track 4: *M. sprengeri* (50mg raw herb/ml, 30µl); Track 5*: M. liliflora* (50mg raw herb/ml, 30µl); Track 6: *M. sargentiana* (50mg raw herb/ml, 30µl).

4.3.3 Identification of different *M. biondii* varieties

Eleven *M. biondii* samples were studied (details see Table 2.3). The bands of magnolin and fargesin were observed from all 11 samples in the TLC chromatogram (Figure 4.3). The HPTLC chromatogram has also identified that all the samples contained magnolin and fargesin (Figure 4.4). The R_f values of magnolin and fargesin are shown in Table 4.2. For positive identification, the testing sample must exhibit bands with the R_f values (0.37 – 0.43 for magnolin and 0.60 – 0.64 for fargesin), similar to those (% R.S.D. < 3%) to those of reference marker compounds.

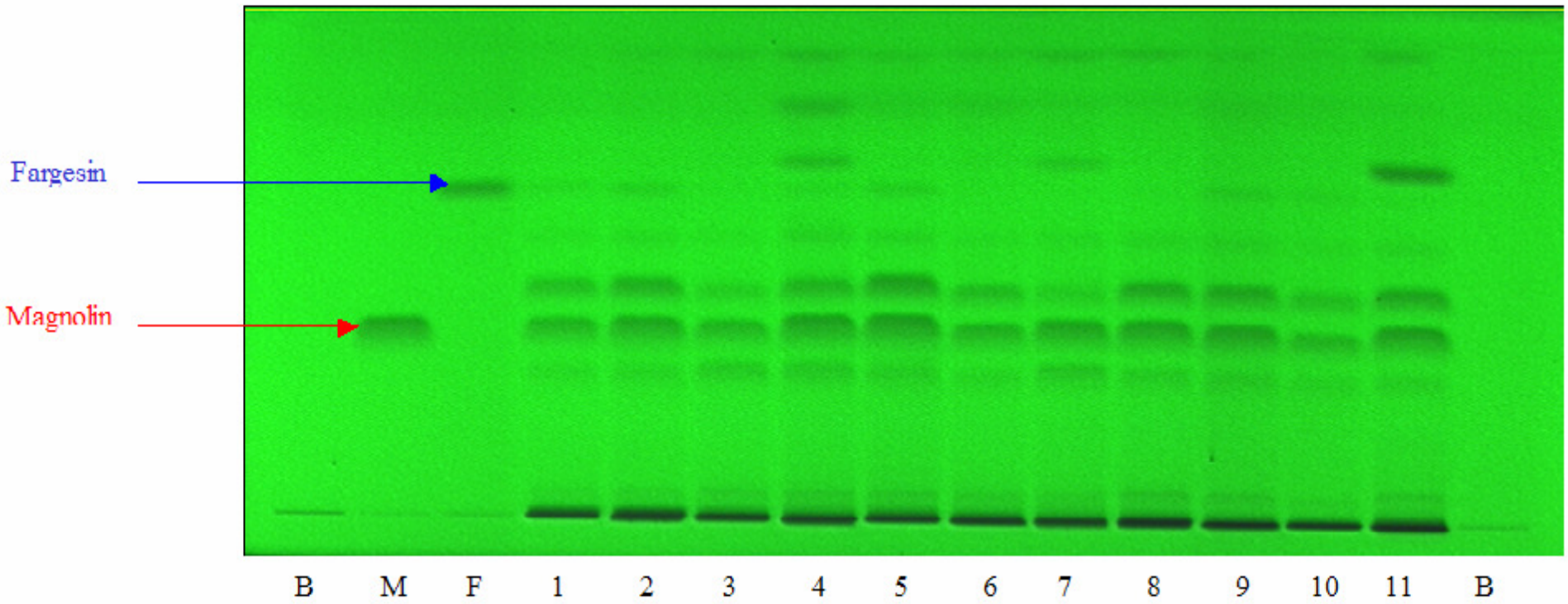


Figure 4.3 TLC chromatograms of 11 batches of *M. biondii* varieties

B: Blank (10µl, methanol); M: Magnolin standard (2.5mg/ml, 10µl); F: Fargesin standard (1mg/ml, 10µl); Track 1: No1 (50mg raw herb/ml, 30µl); Track 2: No2 (50mg raw herb/ml, 30µl); Track 3: No3 (50mg raw herb/ml, 30µl); Track 4: No4 (50mg raw herb/ml, 30µl); Track 5: No5 (50mg raw herb/ml, 30µl); Track 6: No6 (50mg raw herb/ml, 30µl); Track 7: No7 (50mg raw herb/ml, 30µl); Track 8: No8 (50mg raw herb/ml, 30µl); Track 9: No9 (50mg raw herb/ml, 30µl); Track 10: No10 (50mg raw herb/ml, 30µl); Track 11: No11 (50mg raw herb/ml, 30µl).

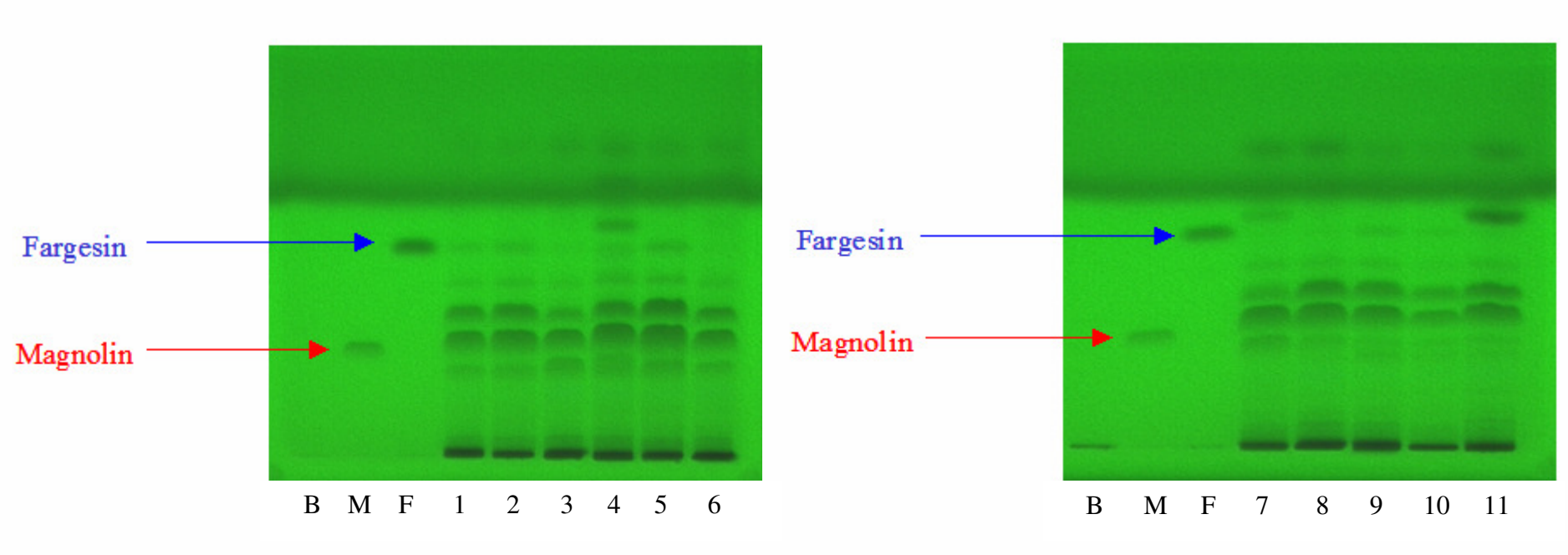


Figure 4.4 HPTLC chromatograms of 11 batches of *M. biondii* varieties

B: Blank (10µl, methanol); M: Magnolin standard (2.5mg/ml, 10µl); F: Fargesin standard (1mg/ml, 10µl); Track 1: No1 (50mg raw herb/ml, 30µl); Track 2: No2 (50mg raw herb/ml, 30µl); Track 3: No3 (50mg raw herb/ml, 30µl); Track 4: No4 (50mg raw herb/ml, 30µl); Track 5: No5 (50mg raw herb/ml, 30µl); Track 6: No6 (50mg raw herb/ml, 30µl); Track 7: No7 (50mg raw herb/ml, 30µl); Track 8: No8 (50mg raw herb/ml, 30µl); Track 9: No9 (50mg raw herb/ml, 30µl); Track 10: No10 (50mg raw herb/ml, 30µl); Track 11: No11 (50mg raw herb/ml, 30µl).

Table 4.2 The values of R_f for magnolin and fargesin in different *M. biondii* varieties. Data are presented as mean ± S.D. (n=4).

		1	2	3	4	5	6	7	8	9	10	11
		HLWH	EMT	EMT	XMT	HGCY	CY	CY	CY	EMT	HLWH	BZCY
Magnolin	R_f	0.40	0.41	0.41	0.42	0.42	0.39	0.40	0.40	0.39	0.37	0.43
	(S.D.)	(0.00)	(0.00)	(0.01)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.01)
	% R.S.D.	0.7	0.5	1.4	1.0	0.7	0.6	0.6	0.7	1.0	1.2	2.1
Fargesin	R_f	0.61	0.61	0.61	0.61	0.61	0.61	0.61	0.61	0.61	0.60	0.64
	(S.D.)	(0.00)	(0.00)	(0.00)	(0.00)	(0.00)	(0.02)	(0.01)	(0.01)	(0.01)	(0.00)	(0.00)
	% R.S.D.	0.5	0.3	0.5	0.3	0.3	2.5	1.3	1.0	1.5	0.7	0.6

4.3.4 Identification of Flos Magnoliae products from different herbal suppliers

The chromatograms from five Flos Magnoliae herbal products from five herbal suppliers (details see Table 2.4) show similar chromatographic patterns (Figure 4.5). Magnolin and fargesin were observed from all five Flos Magnoliae products, based on the R_f values (Table 4.3). For positive identification, the testing sample must exhibit bands with the R_f values (0.40 – 0.43 for magnolin and 0.61 – 0.65 for fargesin), similar to those (% R.S.D. < 3%) to those of reference marker compounds.

4.3.5 Identification of *M. biondii* samples from different cultivation sites

Four *M. biondii* samples, which were collected from different cultivation sites, were used in the present study (details see Table 2.5). The magnolin and fargesin were identified by the optimised TLC approach (Figure 4.5). The R_f values of magnolin and fargesin are shown in Table 4.4. For positive identification, the testing sample must exhibit bands with the R_f values (0.40 – 0.42 for magnolin and 0.62 – 0.63 for fargesin), similar to those (% R.S.D. < 3%) to those of reference marker compounds.

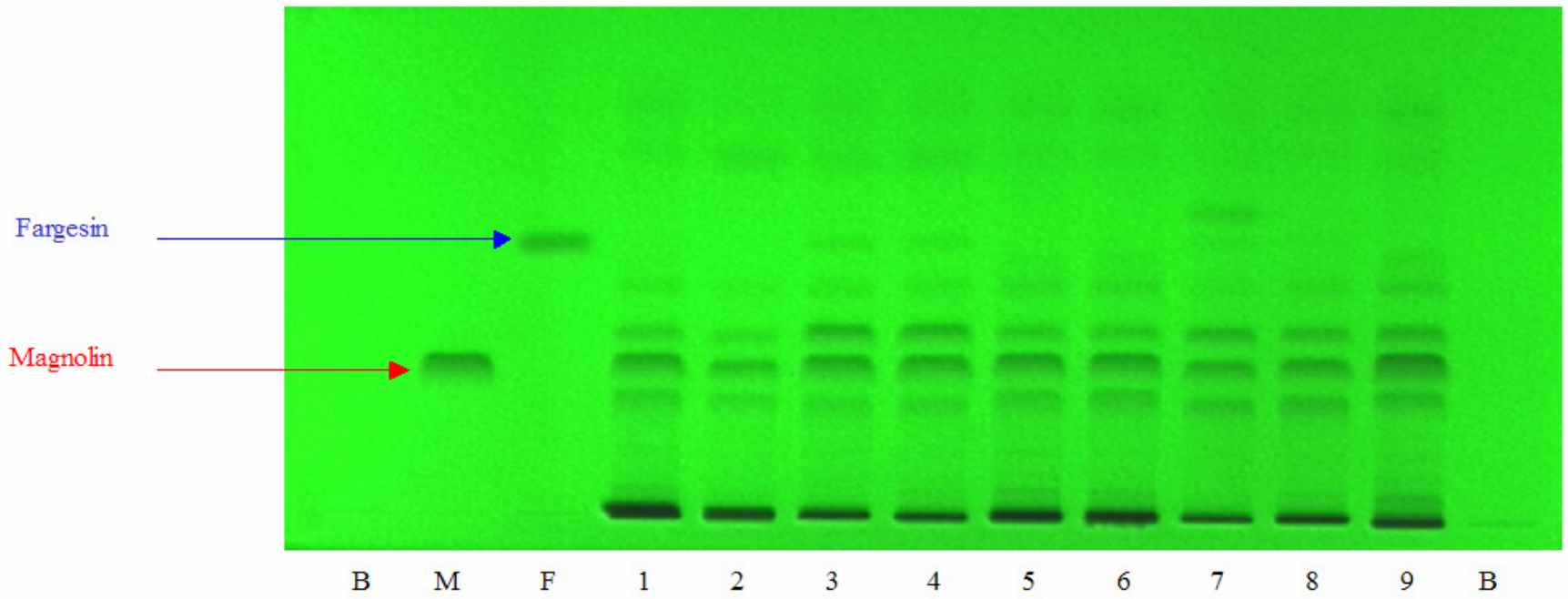


Figure 4.5 TLC chromatograms of five Flos Magnoliae products from different herbal suppliers and four *M. biondii* batches, collected from different cultivation sites

B: Blank (10µl, methanol); M: Magnolin standard (2.5mg/ml, 10µl); F: Fargesin standard (1mg/ml, 10µl); Track 1: EYS (50mg raw herb/ml, 30µl); Track 2: KODA (50mg raw herb/ml, 30µl); Track 3: TFH (50mg raw herb/ml, 30µl); Track 4: TFK (50mg raw herb/ml, 30µl); Track 5: HL (50mg raw herb/ml, 30µl); Track 6: DHY (50mg raw herb/ml, 30µl); Track 7: XHY (50mg raw herb/ml, 30µl); Track 8: YYS (50mg raw herb/ml, 30µl); Track 9: TQ (50mg raw herb/ml, 30µl).

Table 4.3 The values of R_f for magnolin and fargesin in different Flos Magnoliae products from five herbal suppliers. Data are presented as mean ± S.D. (n=4).

		HL	TFH	TFK	EYS	KODA
Magnolin	R_f	0.43	0.40	0.41	0.42	0.41
	(S.D.)	(0.00)	(0.01)	(0.02)	(0.02)	(0.01)
	% R.S.D.	1.0	1.7	3.6	3.7	1.5
Fargesin	R_f	0.65	0.61	0.62	0.63	0.62
	(S.D.)	(0.01)	(0.01)	(0.01)	(0.01)	(0.01)
	% R.S.D.	1.1	1.8	1.4	1.7	1.5

Table 4.4 The values of R_f for magnolin and fargesin in different *M. biondii* samples, collected from four cultivation sites. Data are presented as mean ± S.D. (n=4).

		DHY	XHY	YYS	TQ
Magnolin	R_f	0.42	0.42	0.40	0.40
	(S.D.)	(0.01)	(0.01)	(0.01)	(0.01)
	% R.S.D.	2.2	1.6	2.1	1.4
Fargesin	R_f	0.62	0.63	0.63	0.62
	(S.D.)	(0.01)	(0.01)	(0.00)	(0.00)
	% R.S.D.	1.3	1.1	0.4	0.7

4.4 Discussion

A simple, sensitive and robust TLC method for the identification of different Flos Magnoliae species has been developed in the present study using magnolin and fargesin as marker compounds. After initial experiments on selection of a suitable solvent system for the best separation of Flos Magnoliae samples, chloroform – ether in varying rations (1:1, 2:1, 4:1 and 5:1) were used, and chloroform – ether (5:1) was found as the optimised mobile phase for the development of the chromatogram, for achieving the dense and compact bands of magnolin and fargesin. The present findings are in agreement with that suggested by a previous study (Su et al., 2001). It was also observed in the present study that well-defined spots of Flos Magnoliae samples were obtained when the chamber was pre-equilibrated with the mobile phase for a period of 30min. The percentage R.S.D. for repeatability of magnolin and fargesin from five samples application on the same plates were found to be 0.9% and 0.7%, respectively, indicating an excellent repeatability of the method developed.

Six Flos Magnoliae species were studied using the optimised TLC method. Two species, *M. biondii* and *M. kobus* were found to contain magnolin and fargesin, based on the analysis by winCATS workstation (CAMAG, Switzerland). Locally grown *M. liliflora*, which was commonly used as the substitutes of Flos Magnoliae in China, was found to contain magnolin. All six species showed unique chromatographic fingerprints, which may be used to identify and authenticate different Flos Magnoliae species. The good separation of the chromatogram of *M. biondii* was achieved, compared to the chromatograms of other five Flos Magnoliae species. Moreover, 11 batches from six *M. biondii* varieties, five Flos Magnoliae herbal products and four *M. biondii* batches from different cultivation sites, were qualitatively analysed using the present TLC method. Magnolin and fargesin were detected in all samples. Five bands, whose R_f values ranged from 0.40 to 0.75, were well separated from all *M. biondii* varieties. So it seems the present TLC condition may be specific for the identification of *M.*

biondii. Furthermore, the existences of magnolin and fargesin from all five Flos Magnoliae products, purchased from different herbal suppliers in Australia and China, indicate that *M. biondii* may be the botanic sources of those marketed products.

High opportunities of contaminations of the neighbouring analytes, which might be occurred during the promotion by the solvents, has been considered as one of the major limitations of TLC method (Hilaire et al., 1998). Furthermore, although TLC is a fast, economic and widely used technique, the lack of sensitivity and difficulty to detect the microconstitutes is another advantage of this approach (Cai et al., 2000a). Recently, the use of CAMAG video store system improved the sensitivity and reproducibility of the present method. Further research will be focused on the quantitative analysis of the bioactive compounds from Flos Magnoliae samples. The well-established and validated TLC or HPTLC method will be required with the help of image analysis and digitalized techniques with the CAMAG TLC system (Chopra et al., 2006, Sek et al., 2001).

In summary, the well-established TLC method is rapid, specific and simultaneous for the qualitative analysis of magnolin and fargesin in ethanol extracts of different Flos Magnoliae species and varieties, and the authentication of the Flos Magnoliae products in the market.

Chapter Five

Simultaneous Determination of Magnolin and Fargesin in Flos Magnoliae Using HPLC

5.1 Background

HPLC has been distinguished as the most commonly used approach for the identification and quantification of chemical components (Horvath, 1980). Due to its high reliability, sensitivity and reproducibility, the technique has been frequently used in the quality control of CHM (Li et al., 1998). The applications of HPLC for determination of the active components from CHM and related chromatographic conditions are shown in Chapter 1 (Section 1.3.3.4.2).

In 1936, Haworth named systematic compounds, the lignans that contained two β,β'-linked cinnamic acid residues (Moss, 2000). With more new lignans and neolignans revealed by the investigation of bioactive compounds from plants, they are categorised into eight subgroups of lignans, including furofurans (FF), furan (FR), dibenzylbutane (DB), dibenzylbutyrolactone (DBL) dibenzylbutyrolactol (DBLL), aryltetralin (AT), arylnaphthalene (AN) and dibenzocyclooctadiene (DCO) (Umezawa, 2003).

Pharmacological and clinical investigations have demonstrated that FFs have anti-tumour and anti-allergic effects (Brown et al., 2001). One of the AT lignans, podophyllotoxin, has significant cytotoxic properties (Gordaliza et al., 2004, Chang et al., 1992). Although the toxic side effects of podophyllotoxin are recognised by clinical investigation, it has been regarded as one of the major parent component for the powerful anti-cancer agent, etoposide and related medicinal products (Arroo et al., 2002).

The investigation of CHM has led to the discovery of the potent bioactive components, lignans and neolignans (Homma et al., 2000). The active lignans and neolignans, including forsythin and forsythoside in Fructus Forsythiae (Lian-qiao), arctiin and arctigenin in Fructus Arctii (Niu-bang-zai), schisandrene in Fructus Schisandrae (Wu-wei-zi), asarinin in Herba Asari (Xi-xin) and honokiol and magnolol in Cortex Magnoliae Officinalis (Ho-pu) have been

demonstrated to have potent pharmacological function and potential clinical effects (Ho et al., 2001, Iwakami et al., 1992, Kim et al., 2004, Sun et al., 1992)

Extracts of dried buds from different *Magnolia spp.* have been reported to contain different yield of bioactive lignans and neolignans (Kimura et al., 1991, Ma and Han, 1995). The chemical structures of these lignans and neolignans are shown in Figure 1.5 of Section 1.1.4.1.2. The investigation of lignans and neolignans started with *M. biondii*. In the early stage, six different FF lignans, which were magnolin, fargesin, demethoxyaschantin, aschantin, pinoresinol dimethyl ether, and lirioresinol-B-dimethyl ether, were reported as the active components that were responsible for the anti-inflammatory and anti-allergic actions of Flos Magnoliae (Ma and Han, 1995, Brown et al., 2001, Mitsuo, 2001). With the development of isolation and separation techniques, more and more lignans from Flos Magnoliae have been identified, including biondinin A, fargesone A and B, magnone A and B, and denudatin B (Chen et al., 1988, Pan et al., 1987, Ma et al., 1996, Ma and Han, 1992, Jung et al., 1998). However, the pharmacological actions of these compounds are still unclear. In Japan, two neolignans, magnosalin and magnoshinin were reported as the major bioactive components of Flos Magnoliae (*Magnolia salicifolia* Maxim) (Kikuchi et al., 1983). The anti-inflammatory and anti-allergic effects of magnosalin and magnoshinin were demonstrated through inhibition of angiogenesis and proliferation of vascular endothelial cells by magnosalin and granuloma formation by magnoshinin *in vivo* and *in vitro* (Kobayashi et al., 1998, Kobayashi et al., 1996a, Kimura et al., 1992, Kimura et al., 1991, Kimura et al., 1990).

HPLC has been applied to quantitatively and qualitatively analyse of the lignans and neolignans of different Flos Magnoliae species. Magnolin, pinoresinol dimethyl ether, and lirioresinol-B-dimethyl ether were qualitatively identified by RP-HPLC using a C_8 column with the mobile phase containing acetonitril-tetrahydrogenfuran-water and at 278nm

wavelength (Fang et al., 2002). Different HPLC system was also employed to identify magnolin and fargesin using a C_{18} column, with the mobile phase of acetonitrile-water (50:50) mixture as mobile phase, at 278 nm wavelength (Xu et al., 2003). Xu *et al* (2003) also found that *M. biondii* contained significantly higher content of magnolin than *M. denudata* and *M. sprengeri*, while only *M. biondii* contained fargesin.

Aims of this study

The present study aimed to establish a HPLC system to simultaneously determine two bioactive compounds, magnolin and fargesin in different Flos Magnoliae sources. Magnolin has been listed as the marker compound in the current Chinese Pharmacopoeia (The Pharmacopoeia Commission of People's Republic of China, 2005). The qualities of different Flos Magnoliae species and varieties were used in the present study, including six Flos Magnoliae species, batches of *M. biondii* from different varieties, herbal suppliers and cultivation sites, and batches of *M. sprengeri* grown in different locations from Australia and China.

5.2 Method

5.2.1 Samples collection, extraction and preparation

Six Flos Magnoliae species were used, including *M. biondii, M. denudata, M. kobus, M. liliflora, M. sprengeri* and *M. sargentiana* (Table 2.2). Meanwhile, 20 *M. biondii* samples, including 11 batches from six varieties (Table 2.3), five batches from different herbal suppliers (Table 2.4) and four batches collected from different cultivation sites (Table 2.5) were used in the present study. Furthermore, three *M. sprengeri* batches collected from China and Australia were employed as well (Table 2.6).

The method of extraction and preparation are described in Chapter 2 (Section 2.6.1).

5.2.2 Reagents and chemicals

The details of the reagents and chemicals are described in Chapter 2 (Section 2.3.3).

5.2.3 Preparation of standard references, magnolin and fargesin

A stock solution of the standard compounds was prepared by accurately weighing each of magnolin and fargesin. The protocols of the preparation of the standard compounds are described in Section 2.6.2.

5.2.4 Chromatographic conditions

The HPLC system (Shimadzu Model SCL-10A*vp*, Shimadzu, Japan) consisted of a photodiode array detector (Shimadzu Model SPD-M10*Avp*, Shimadzu, Japan). A RP-C_{18} column (ODSII, L 250 × 4.6 I.D. mm, 5μM, Phenomenex, U.S.A.) and a pre-column of the same packing material (Security Guide cartridge; 3.0mm; Phenomenex, U.S.A.) were used for separation of magnolin and fargesin. The mobile phase was a mixture of acetonitrile and water. The protocols of the chromatographic conditions are described in Section 2.6.3.

5.2.5 Validation parameters

The calibration curves and the linearity relationships between peak areas and concentrations were established. Meanwhile, the intra-day precision, stability, reproducibility and recovery experiments are described in Section 2.6.4.

5.2.6 Data analysis of quantitative determination of magnolin and fargesin

The contents of magnolin and fargesin from the testing samples were analysed based on the linearity relationship between the peak area and the concentration. Details are described in Section 2.9.3. Testing samples were in triplicates and were represented as mean ± S.D.

5.3 Results

5.3.1 Method optimisation

The effectiveness of HPLC separation was tested using the ethanol extracts of *M. biondii* and the herbal products from Hualong Magnolia development Co. Ltd. in Henan Province, China. The gradient elution profile was optimised to obtain the highest resolution of magnolin and fargesin within a short analysis period. Different mobile phase compositions were tested. Acetonitrile and water (65:35) did not achieve a good separation of both compounds (Figure 5.1). Then, the effect of acetonitrile and water composition of the mobile phase was studied as a gradient elution (Figure 5.2). The gradient elution program was 10% to 100% of acetonitrile (0 – 90min). The good separation of magnolin and fargesin were achieved when the concentrations of acetonitrile and water were 64% : 36% and 71% : 29%, respectively. As a result, the best resolution of the main peaks was achieved to use a gradient elution of the mobile phase consisting of acetonitrile and water within 20min (the elution program see Table 2.11). The retention times of magnolin and fargesin are 11.038min and 14.297min, respectively (Figure 5.3). Furthermore, the detection of a mixture of magnolin and fargesin was tested at 250nm, 278nm and 290nm, under the above conditions. It was found that the best sensitivity was achieved when the detection wavelength was 278nm (Figure 5.4).

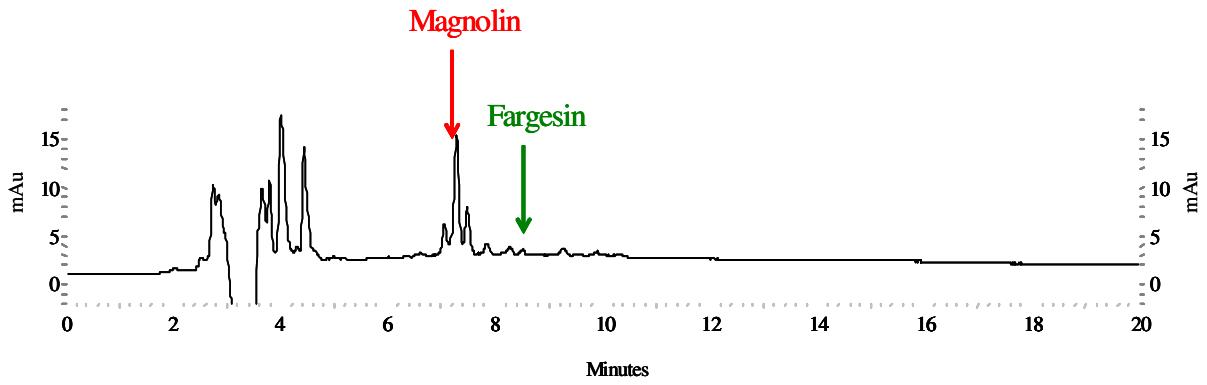


Figure 5.1 The chromatogram of *M. biondii* from the mobiles phase of a mixture of acetonitrile and water (65:35).

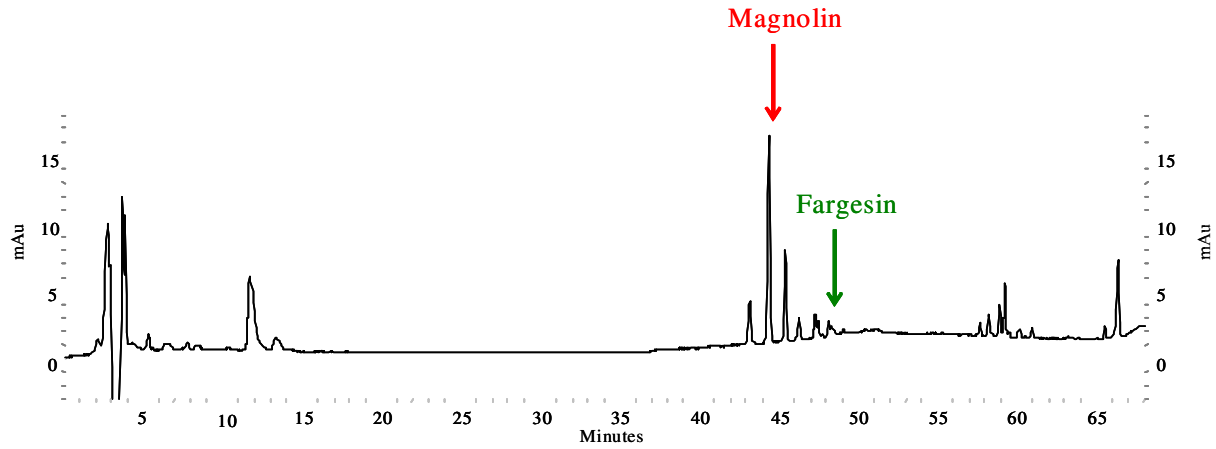


Figure 5.2 The chromatogram of *M. biondii* from the mobiles phase of a mixture of acetonitrile and water with a gradient elution

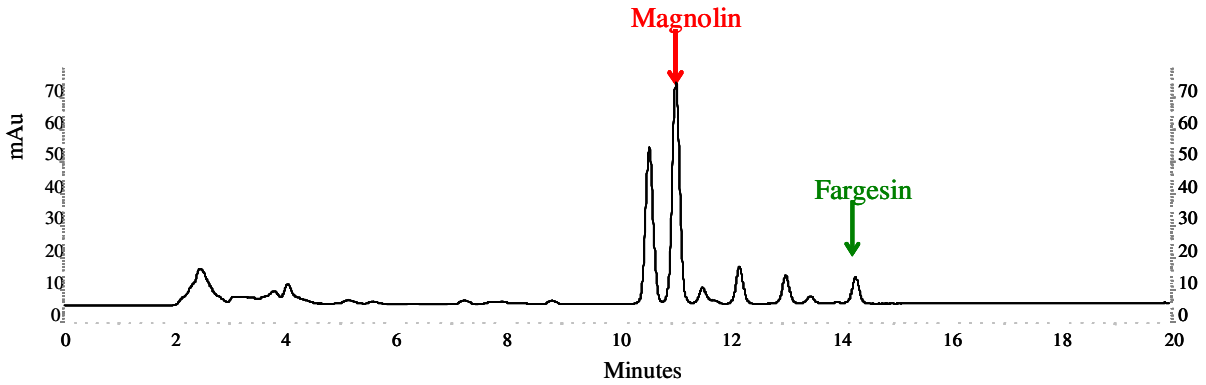


Figure 5.3 The chromatogram of *M. biondii* from the mobiles phase of the optimised gradient elution for 20min. The program details are described in Table 2.11.

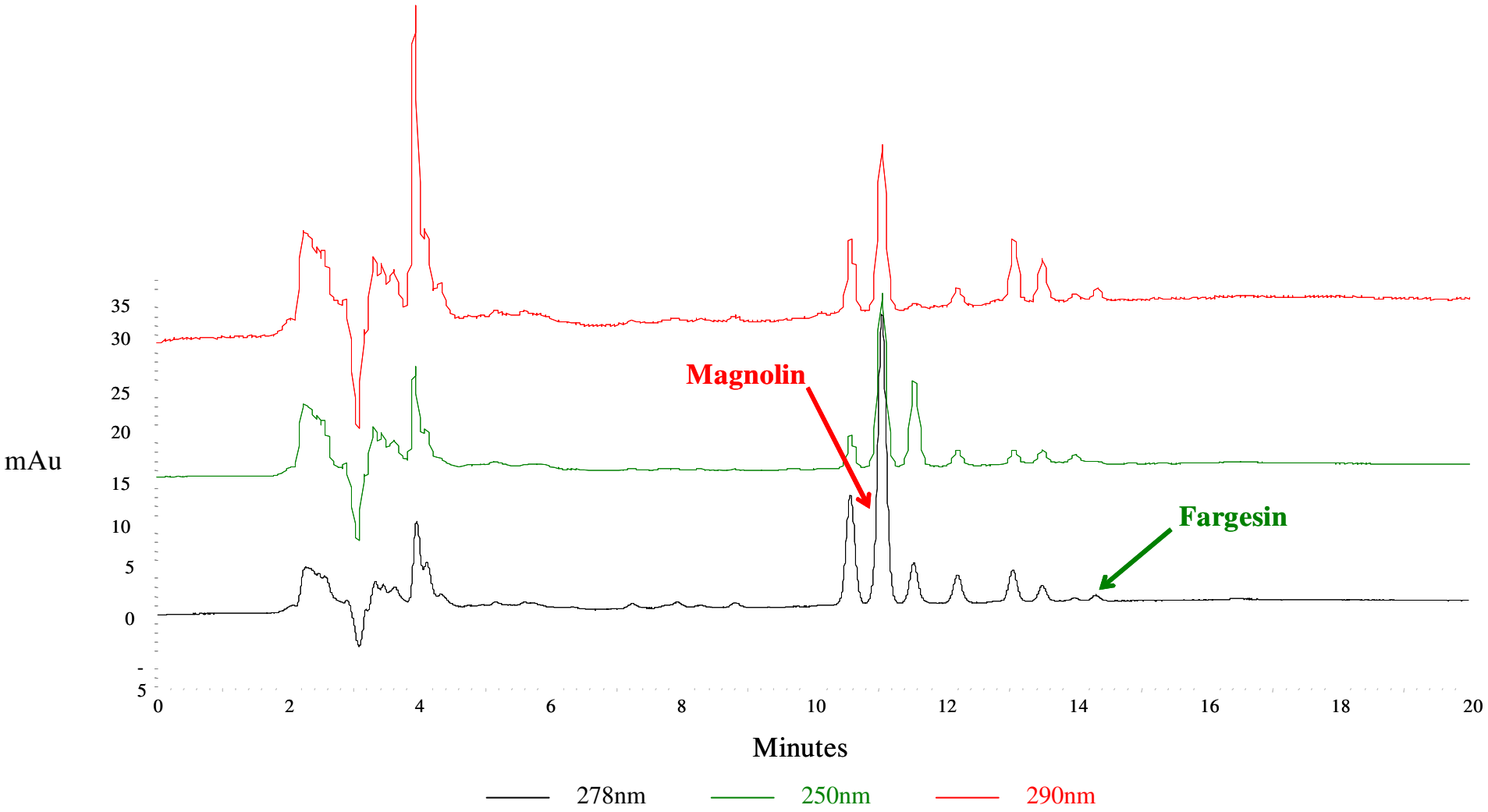


Figure 5.4 The chromatograms of *M. biondii*, using optimised HPLC determination conditions with different detection wavelengths at 250nm, 278nm and 290nm.

5.3.2 Method validation

5.3.2.1 Linearity

Appropriate dilutions of magnolin and fargesin dissolved in ethanol, were made to prepare the working solutions containing 10, 25, 50, 75, and 100 ng/ml, and 1, 2.5, 5, 7.5, and 10 ng/ml, respectively. The chromatogram of the standard solution is shown in Figure 5.5. Calibration curves were plotted by linear regression of peak area vs. concentration. The regression equations of magnolin and fargesin are shown in Figure 5.6. The linearity relationship between peak areas and concentrations were good, and the regression coefficients (γ) were 0.9995 for the curves (Table 5.1).

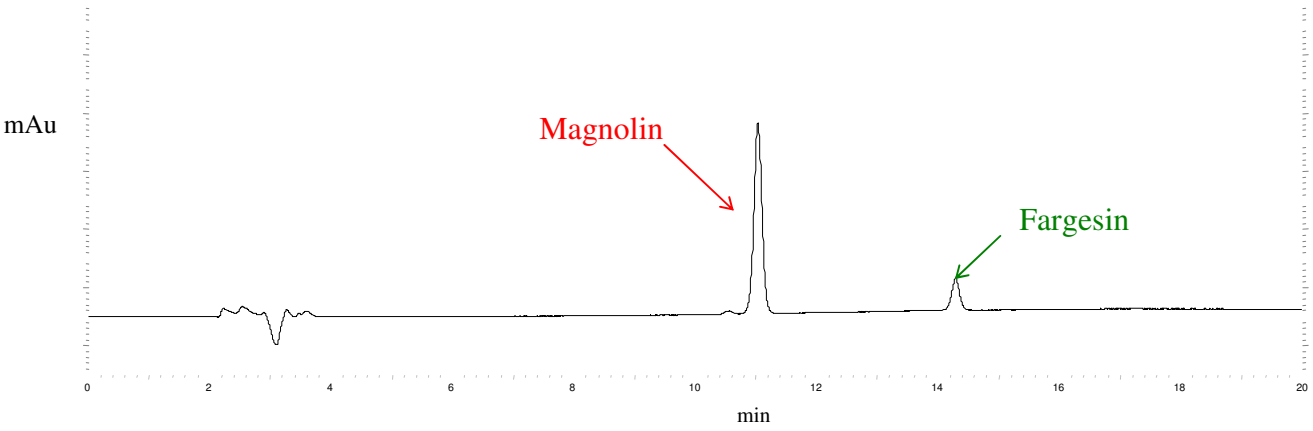
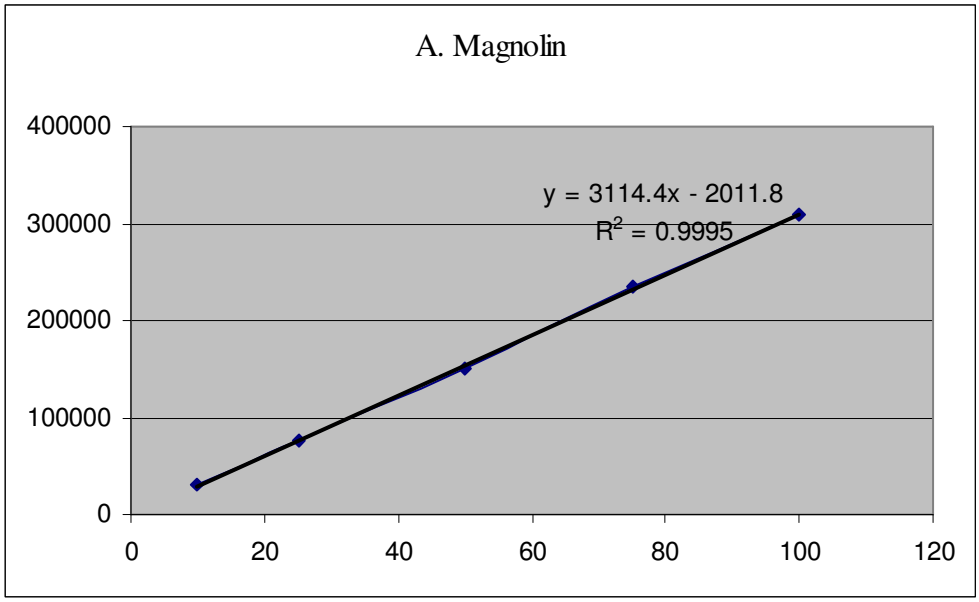


Figure 5.5 The chromatogram of the standard solution containing 100ng/ml of magnolin and 10ng/ml of fargesin (20min)

Table 5.1 The linear ranges and correlation coefficients of calibration curves of magnolin and fargesin

Standard compound	Range	Retention time	Standard curve	γ
Magnolin	10-100 ng/ml	11.038 min	y=3114.4x-2011.8	0.9995
Fargesin	1-10ng/ml	14.297 min	y=5168.2x-141.8	0.9995



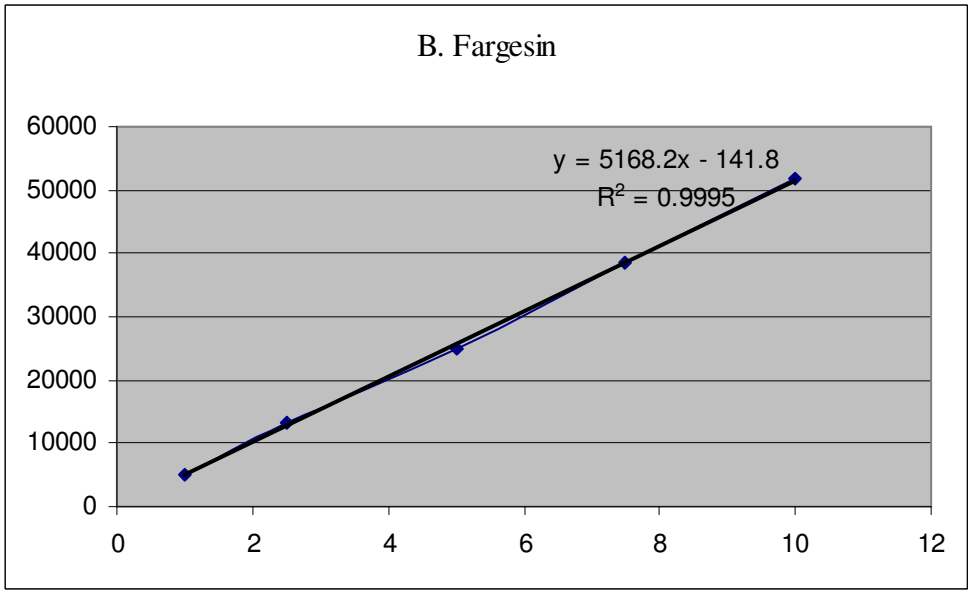


Figure 5.6 The standard curves of magnolin (A) and fargesin (B)

5.3.2.2 Precision

Injection of five replicates of a *M. biondii* sample produced a consistent result (Table 5.2). The R.S.D.s for the determination of the magnolin and fargesin were 0.22% and 0.36%, respectively.

Table 5.2 The precision test for HPLC determination method

No	Weight (g)	Magnolin		Fargesin	
		Peak area	% (w/w)	Peak area	% (w/w)
1		652571	6.50	73468	0.40
2		655172	6.53	73188	0.40
3	1.5559	654099	6.52	72788	0.39
4		653565	6.51	73259	0.40
5		651350	6.49	72966	0.39
Average			6.513		0.396
SD			0.015		0.001
% RSD			0.22		0.36

5.3.2.3 Stability

Injection of a *M. biondii* sample was performed at room temperature for five times within 48hrs (Table 5.3). The R.S.D.s for magnolin and fargesin were 1.09% and 1.75%, respectively.

Table 5.3 The stability test for HPLC determination method

Time	Weight (g)	Magnolin		Fargesin	
		Peak area	% (w/w)	Peak area	% (w/w)
0hr	1.5559	652571	6.50	73468	0.40
2hr		653565	6.51	73259	0.40
18hr		651308	6.50	74488	0.40
24hr		649147	6.48	72483	0.39
42hr		638792	6.34	72365	0.38
48hr		642414	6.38	72593	0.39
Average			6.446		0.393
SD			0.070		0.007
% RSD			1.09		1.75

5.3.2.4 Repeatability

Five replicates of one batch of *M. biondii* sample were extracted and determined to evaluate the repeatability of the quantitative procedure. All of the measurements of repeatabilities were expressed as R.S.D.s (Table 5.4).

Table 5.4 The reproducibility test for HPLC determination method

No	Weight (g)	Magnolin		Fargesin	
		Peak area	% (w/w)	Peak area	% (w/w)
1	1.5946	653988	6.80	74163	0.42
		656151	6.82	75778	0.43
2	1.5466	648172	6.94	72941	0.43
		649104	6.95	73897	0.43
3	1.5389	649742	6.99	75064	0.44
		654432	7.04	75308	0.44
4	1.5558	635510	6.76	73832	0.43
		637324	6.78	74914	0.43
5	1.5559	664401	6.78	74610	0.41
		661880	6.75	74228	0.41
Average			6.859		0.427
SD			0.107		0.010
% RSD			1.55		2.46

5.3.2.5 Recovery

The recovery rates for magnolin and fargesin were evaluated by investigating maker compounds, 200μg/ml of magnolin and 15μg/ml of fargesin, which were added to a sample. Spiked samples were prepared in five duplicates. The results are shown in Table 5.5 and 5.6. The average recoveries for magnolin and fargesin were 98.373% and 94.226, respectively.

Table 5.5 The recovery rate of magnolin and related experiment conditions

No	Weight (g)	Concentration (μg/ml)	Added standard (μg/ml)	Peak area	Concentration (μg/ml)	Recovery (%)
1	1.5946	216.75	200.00	627652	204.59	98.18
		217.47	200.00	636998	207.64	99.47
2	1.5466	214.82	200.00	620397	202.22	97.50
		215.13	200.00	617247	201.20	96.93
3	1.5389	215.34	200.00	628237	204.78	98.61
		216.90	200.00	636880	207.60	99.59
4	1.5558	210.63	200.00	637324	207.74	101.18
		211.23	200.00	618881	201.73	98.11
5	1.5559	210.88	200.00	620656	196.99	95.89
		210.08	200.00	634749	201.47	98.26
Average						98.373
SD						1.482
% RSD						1.51

Table 5.6 The recovery rate of fargesin and related experiment conditions

No	Weight (g)	Concentration (μg/ml)	Added standard (μg/ml)	Peak area	Concentration (μg/ml)	Recovery (%)
1	1.5946	13.41	15.00	75648	13.50	95.01
		13.70	15.00	75859	13.54	94.31
2	1.5466	13.19	15.00	74792	13.34	94.68
		13.36	15.00	73597	13.13	92.59
3	1.5389	13.57	15.00	75480	13.47	94.26
		13.62	15.00	76793	13.70	95.75
4	1.5558	13.35	15.00	74914	13.37	94.29
		13.55	15.00	75022	13.39	93.78
5	1.5559	12.84	15.00	75112	12.93	92.86
		12.77	15.00	76432	13.15	94.72
Average						94.226
SD						0.951
% RSD						1.01

5.3.3 Determination of contents of magnolin and fargesin in different Flos Magnoliae species

The content of magnolin and fargesin in six Flos Magnoliae species, *M. biondii, M. denudata, M. kobus, M. liliflora, M. sargentiana* and *M. sprengeri* were determined (Table 5.7). Among the six different species, only *M. biondii* and *M. kobus* contained magnolin and fargesin. Magnolin was also detected in the ethanol extract of *M. liliflora*. However, this sample did not contain fargesin. No magnolin and fargesin were detected from ethanol extract of other three species. There were great variations in the concentrations of magnolin and fargesin among different sources of Flos Magnoliae. *M. biondii* was found to possess the highest amount of magnolin and fargesin, as 6.513±0.015% and 0.396±0.001%, respectively. In addition, there were remarkable differences between the chromatographic profiles of six Flos Magnoliae species (Figure 5.7).

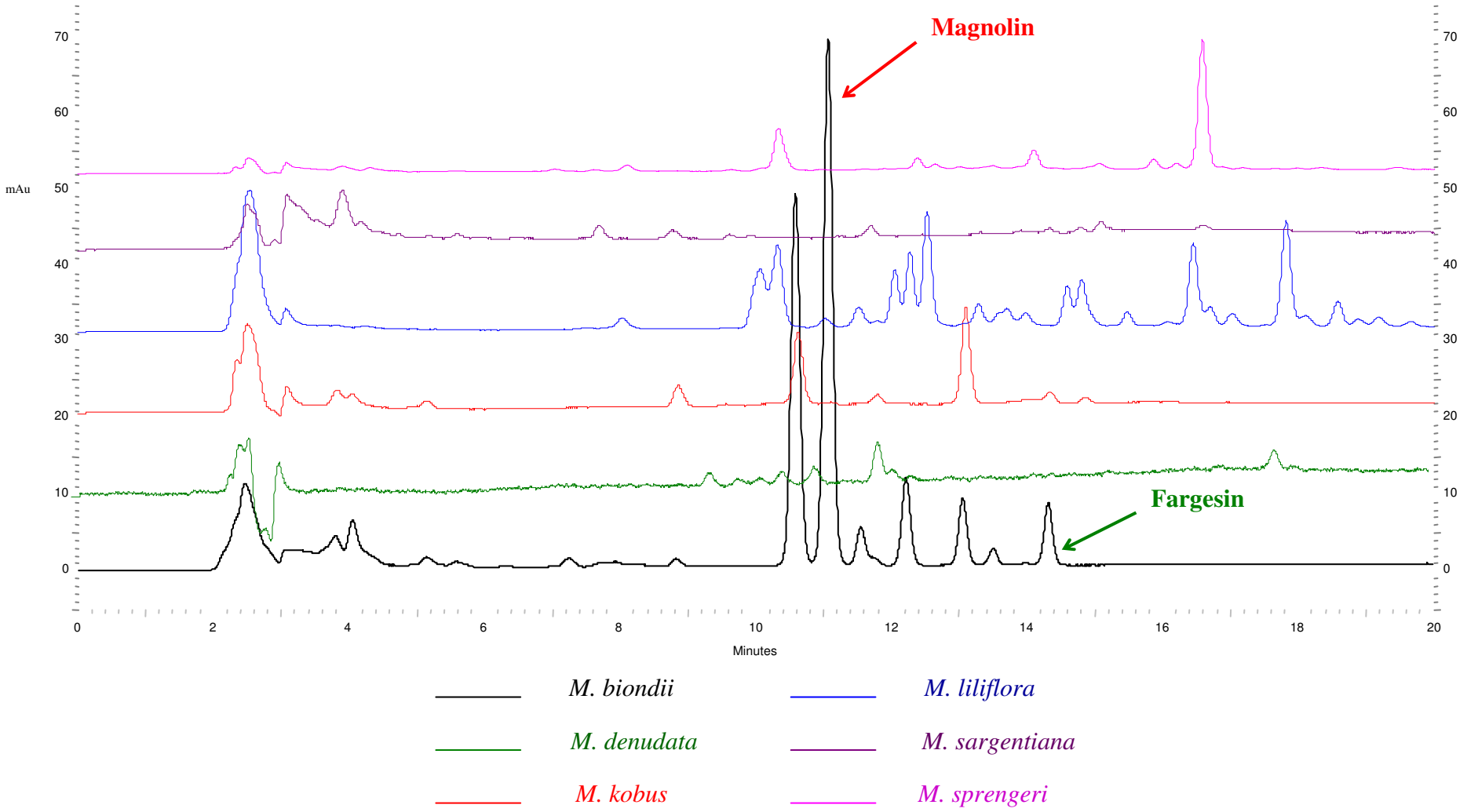


Figure 5.7 HPLC chromatograms of six Flos Magnoliae species.

Table 5.7 The contents of magnolin and fargesin in six Flos Magnoliae species. (n=3)

		M. biondii	*M. denudata*	*M. kobus*	*M. liliflora*	*M. sargentiana*	*M. sprengeri*
Magnolin	Mean (%, w/w)	6.513	-	0.108	0.244	-	-
	(S.D.)	(0.015)		(0.003)	(0.014)		
	% R.S.D.	0.2	-	3.8	3.5	-	-
Fargesin	Mean (%, w/w)	0.396	-	0.210	-	-	-
	(S.D.)	(0.001)		(0.002)			
	% R.S.D.	0.4	-	1.3	-	-	-

5.3.4 Determination of contents of magnolin and fargesin in different *M. biondii* varieties

Eleven batches of six *M. biondii* varieties were studied (details see Table 2.3) and each samples was determined in triplicates. The chromatographic profiles of the ethanol extracts of all batches showed significantly different in the content of magnolin and fargesin (Table 5.8), ranging from 4.800±0.022% (Sample 5: HGCY, 黄梗串鱼) to 2.304±0.044% (Sample 3: EMT, 二毛桃) for magnolin and 0.427±0.002% (Sample 5: HGCY, 黄梗串鱼) to 0.051±0.000% (Sample 7: CY, 串鱼) for fargesin.

Table 5.8 The contents of magnolin and fargesin in 11 batches of *M. biondii* varieties. (n=3)

		1	2	3	4	5	6	7	8	9	10	11
		HLWH	EMT	EMT	XMT	HGCY	CY	CY	CY	EMT	HLWH	BZCY
Magnolin	Mean (%, w/w)	3.949	3.662	2.304	2.914	4.800	3.709	4.156	3.555	3.700	3.067	3.757
	(S.D.)	(0.107)	(0.104)	(0.044)	(0.023)	(0.022)	(0.066)	(0.024)	(0.074)	(0.020)	(0.078)	(0.061)
	% R.S.D.	2.7	2.8	1.9	0.8	0.4	1.8	0.6	2.1	0.6	2.6	1.6
Fargesin	Mean (%, w/w)	0.351	0.355	0.056	0.141	0.427	0.063	0.051	0.090	0.321	0.302	0.098
	(S.D.)	(0.006)	(0.013)	(0.002)	(0.009)	(0.002)	(0.002)	(0.000)	(0.005)	(0.004)	(0.010)	(0.002)
	% R.S.D.	1.6	3.6	3.2	6.6	0.5	3.9	0.8	5.5	1.3	3.2	2.4

5.3.5 Determination of contents of magnolin and fargesin in Flos Magnoliae products from different herbal suppliers

Five batches of Flos Magnoliae products from five herbal suppliers were studied (Details of the herbal suppliers see Table 2.4). All samples were identified from the botanic origin, *M. biondii*. The contents of magnolin and fargesin are shown in Table 5.9.

Table 5.9 The contents of magnolin and fargesin in five Flos Magnoliae products from different herbal suppliers. (n=3)

		HL	TFH	TFK	EYS	KODA
Magnolin	Mean (%, w/w) (S.D.)	6.513 (0.015)	4.483 (0.007)	5.368 (0.023)	3.669 (0.023)	2.205 (0.024)
	% R.S.D.	0.2	0.2	0.4	1.2	1.1
Fargesin	Mean (%, w/w) (S.D.)	0.396 (0.001)	0.038 (0.001)	0.493 (0.002)	0.345 (0.010)	0.121 (0.002)
	% R.S.D.	0.4	2.5	0.5	0.3	1.6

5.3.6 Determination of contents of magnolin and fargesin in samples of *M. biondii* and *M. sprengeri* from different cultivation sites

Four batches of *M. biondii* samples from different cultivation sites in Nanzhao, Henan Province, China, were studied (details see Table 2.5). The contents of magnolin and fargesin, determined by the optimised method, are shown in Table 5.10.

Two batches of *M. sprengeri* samples from two cultivation sites in China and one sample from Australia were determined (details see Table 2.6). However, no magnolin and fargesin were detected from all three batches of *M. sprengeri*.

Table 5.10 The contents of magnolin and fargesin in four *M. biondii* batches, collected from different cultivation sites. (n=3)

		DHY	XHY	YYS	TQ
Magnolin	Mean (%, w/w) (S.D.)	1.988 (0.012)	2.686 (0.025)	2.860 (0.066)	2.369 (0.003)
	% R.S.D.	0.6	0.9	2.3	0.1
Fargesin	Mean (%, w/w) (S.D.)	0.017 (0.000)	0.023 (0.002)	0.286 (0.013)	0.117 (0.003)
	% R.S.D.	1.3	7.0	4.6	2.5

5.4 Discussion

Initially, the isocratic elution chromatographic conditions recommended by the current Chinese Pharmacopoeia employing acetonitrile – water (35:65) was used to separate components in Flos Magnoliae ethanol extract (The Pharmacopoeia Commission of People's Republic of China, 2005). However, separation of peaks was unsatisfactory, because there were poor resolutions of peaks, broadening of late peaks and long running time. These difficulties were explained by previous studies (Lee and Row, 2004), and might result in misidentification and/or inaccurate quantification.

Thus, in order to achieve a better separation of these compounds, a gradient elution method was used in the present study. Gradient elution has been shown to improve the early eluting peaks, limit the broadening of the later peaks and decrease the time of analysis (Kress et al., 2002, Purcell et al., 1999, Snyder, 1980). With acetonitrile and water as the mobile phase (ratio 64% and 71%, respectively), a good separation was achieved for magnolin and fargesin. Moreover, a detection wavelength of 278nm was found to be the best wavelength to measure absorbance for all the peaks. Thus, an optimised acetonitrile-water gradient system has been developed for simultaneous determination of magnolin and fargesin. Under these conditions linear calibration curves, excellent intra-day precision and stability, high repeatability and good recovery were achieved (R.S.D. less than 3%).

Among the six *Magnolia spp.*, *M. biondii, M. denudata* and *M. sprengeri,* have been listed in the Chinese Pharmacopoeia (The Pharmacopoeia Commission of People's Republic of China, 2005). The Chinese species *M. biondii* was found to contain magnolin and fargesin, and matched the chemical specification of Flos Magnoliae required by the Chinese Pharmacopoeia (The Pharmacopoeia Commission of People's Republic of China, 2005). The dried buds from *M. kobus* that has been used as authentic Flos Magnoliae species in Japan

were also shown to contain magnolin and fargesin. It produced similar chromatographic profile to the *M. biondii* (the chromatogram see Figures 5.7), although *M. kobus* has a lower content of magnolin and fargesin than *M. biondii*. However, compared to *M. biondii*, *M. kobus* has a relative higher content of fargesin than magnolin. Another locally grown species, *M. liliflora*, which is one of the common substitutes of Flos Magnoliae in China, only contained one of the bioactive compounds, magnolin. In addition, the chromatogram of *M. liliflora* was significantly different from *M. biondii* and *M. kobus*.

It is surprising that some of the Flos Magnoliae species, *M. denudata, M. sprengeri* and *M. sargentiana,* did no contain magnolin and fargesin. This negative finding of magnolin has not been reported previously (Chae et al., 1998, Page, 1990, Pan et al., 1987). The species variation may lead to the different quantities of active components of CHM. Consequently, such differences in the quantity of the lignans and neolignans may affect the clinical efficacy.

In the present study, 11 batches of six *M. biondii* varieties were contained both magnolin and fargesin, including HLWH (华龙五号), EMT (二毛桃), XMT (小毛桃), HGCY (黄梗串鱼), CY (串鱼) and BZCY (标准串鱼). All *M. biondii* batches were collected from Nanzhao, Henan Province, China on the same day. The chromatograms from the 11 batches all revealed the same patterns. However, the content of magnolin and fargesin varied. The content of magnolin and fargesin ranged from 2.304 – 4.800% and 0.051 – 0.427%, respectively. Among six varieties, HGCY (黄梗串鱼) has the highest concentration of magnolin (4.8%) and fargesin (0.427%). Especially, the content of magnolin in HGCY (黄梗串鱼) were 12 times above the standard mentioned in the current Chinese Pharmacopoeia that suggested the content of magnolin no less than 0.4% (The Pharmacopoeia Commission of People's Republic of China, 2005). High contents of magnolin (3.757%) and fargesin (0.098%) were also found in BZCY (标准串鱼), which is recognised as a high quality Flos Magnoliae

source by local farmer. Three batches of CY (串鱼) varieties, which are the majority variety for the cultivation site, have a high content of magnolin, but low content of fargesin. Furthermore, one of the three batches of EMT (二毛桃) contained much lower magnolin and fargesin that the other two batches from the same variety. This support a number of previous studies indicating that the chemical compositions of CHM differ from various botanic species and varieties (Xu and Zhao, 2004, Zhang et al., 2002a). Thus, where multiple species and varieties of Flos Magnoliae are used as CHM, it is necessary for establishment of the content of the bioactive compounds in these plants.

On the other hand, differences of the contents of magnolin and fargesin among five Flos Magnoliae products from five different herbal suppliers were observed. The batch from Hualong Magnolia Development Co. Ltd. contained higher level of magnolin, compared to other three batches from the herbal suppliers in Hong Kong. As one of the major manufactures, Hualong Magnolia Development Co. Ltd., cultivated more than 50% of the Flos Magnoliae products for the Chinese market (Hualong Magnolia Development Co. Ltd., 2003). The Flos Magnoliae sample from KODA International was claimed to be the high quality herbal extract in powdered form. However, it contained the least content of magnolin and second least content of fargesin compared to other four batches. It is not known if these changes were caused by manufacturing factors, such as processing, shipping and storage (Liu et al., 2003a, Zhang and Feng, 2005). Nevertheless, those extrinsic factors can influence the chemical compositions of herbal products, and may result in changes of content of bioactive ingredients, and lead to varying quality and efficacy of Flos Magnoliae.

The influences of environmental factors during the cultivation process on the contents of lignans were also tested in the present study. Four batches of *M. biondii*, collected from four cultivation sites in Nanzhao, Henan Province, China, were studied using the optimised HPLC

method. Three batches, collected from Western Garden, Mt. Yanyi and Tian Bridge, contained higher levels of magnolin compared to another batch from Eastern Garden. Moreover, the other two batches, collected from Mt. Yanyi and Tian Bridge contained significantly higher levels of fargesin than that in two batches collected from Eastern Garden and Western Garden. These findings indicate that the differences of the amount of sunlight, the soil conditions and climates may alter the accumulation and the chemical compositions of Flos Magnoliae. Furthermore, three *M. sprengeri* batches, two of which were collected from China (from Mabian and Jiangyou, respectively), and one from Mt. Dandenong, Australia, were found no containing of magnolin and fargesin, indicating these two compounds may not be a suitable marker for this species.

In conclusion, an optimised and validated method has been developed and demonstrated to be a useful procedure to quantitatively analyse the bioactive lignans, magnolin and fargesin, in different samples of Flos Magnoliae. The RP-HPLC coupled with a gradient elution system was found to be a rapid, sensitive and reproducible for simultaneous determination of bioactive compounds. Present findings also verified that the variations of botanic species and varieties, the cultivation environments, and post-cultivation manufacturing process might cause significant change of the content of bioactive compounds, which might affect the quality and efficacy of related herbal products.

Chapter Six

Identification of Flos Magnoliae Using HPLC Fingerprinting

6.1 Background

The chromatographic fingerprint of CHM has been recognised as one of the most representing approaches, to identify and characterise chemical compositions of CHMs (Drasar and Moravcova, 2004). As mentioned in Chapter 1 (Section 1.3.4), the CHM fingerprints, including chromatograms, electrophotograms and other illustrative grams, have been widely used in pharmaceuticals, environmental substances, foods and cosmetics, and forensic subjects (Aksenova et al., 1999, Wang et al., 2004f, Berente et al., 2000, Klemenc, 2001). Since 1996, the chromatographic fingerprints have been widely used as the quality control tool for the authentication and identification of CHMs (CDER, 2004, The European Anency for the Evaluation of Medicinal Products, 2002, WHO, 1996). Compared to other commonly used approaches that only consider one or several chemical components, the fingerprints of CHM products can study the content and consistence of complex chemical compositions.

Previous studies have used various HPLC systems to study the chemical compositions of Flos Magnoliae species and determined the levels of bioactive components, magnolin and fargesin, *M. biondii, M. denudata* and *M. sprengeri* (Table 6.1). However, there is still no systematic study on HPLC fingerprints of Flos Magnoliae.

Table 6.1 Various HPLC systems for qualitative and quantitative analysis of Flos Magnoliae

HPLC system	Detector	Column	Temperature of the oven	Elution mode	Mobile phase	Flow rate	Wavelength	Reference
Waters, United States	Photodiode array detector	μBondapak C_{18} (3.9mm x 300mm, 10μM, Waters, United States)	25°C	Isocratic	Methanol : water (60:40)	1.0ml/min	283nm	(Yu et al., 2005b)
Waters, United States	Photodiode array detector	Hypersil ODS C_{18} (4.6mm x 200mm, 5μM, Agilent, United States)	30°C	Isocratic	Acetonitrile : water (50:50)	1.0ml/min	278nm	(Xu et al., 2003)
Shimadzu, Japan	SPD-6A (V) detector	Zorbax SB-C_8 (4.6mm x 150mm, 5μM, Agilent, United States)	Room temperature	Isocratic	Acetonitrile – tetrahydrogenfuran – water (35:1:64)	1.0ml/min	278nm	(Fang et al., 2002)
Spectra-physics, United States	Spectra –200 UV detector	Supelcosil^{TM} LC_{18} (4.6mm x 250mm, 5μM, Supelco, United States)	Room temperature	Isocratic	Acetonitrile – tetrahydrogenfuran – water (35:1:64)	1.0ml/min	290nm	(Zhu et al., 2002)

Aims of this study

The aim of the present study is to establish and evaluate the HPLC fingerprinting profiles of different species or varieties of Flos Magnoliae, including *M. biondii, M. denudata, M. kobus, M. liliflora, M. sargentiana* and *M. sprengeri* and different varieties of *M. biondii*. In addition, HPLC fingerprinting profiles of different Flos Magnoliae herbal products, and different batches of *M. biondii* grown in different cultivation sites were also studied. The following parameters were selected, including the total peak areas of the common peaks, the retention time, the relative retention time, the peak area and the relative peak area. The percentage of the single peak in the common peaks and the overlapping ratio of peak (ORP) (Lijuan et al., 2007) were also employed for elucidation of the HPLC fingerprints of Flos Magnoliae.

6.2 Method

6.2.1 Samples extraction and preparation

The details of sample collection and preparation are described in Chapter 2, Section 2.2.2 and Section 2.7.1, respectively.

6.2.2 Reagents and chemicals

The details of the reagents and chemicals are described in Section 2.3.3.

6.2.3 Preparation of standard references, magnolin and fargesin

The details of preparation of standard references are described in Section 2.6.2.

6.2.4 Chromatographic conditions

The HPLC system (Shimadzu Model SCL-10A*vp*, Shimadzu, Japan) consisted of a photodiode array detector (Shimadzu Model SPD-M10A*vp*, Shimadzu, Japan). A RP-C_{18} column (ODSII, L 250 × 4.6 I.D. mm, 5μM, Phenomenex, U.S.A.) and a pre-column of the

same packing material (Security Guide cartridge; 3.0mm; Phenomenex, U.S.A.) were used for qualitative and quantitative analysis of the Flos Magnoliae sources. The mobile phase was a mixture of acetonitrile and water (programs see Table 2.12). The protocols of the chromatographic conditions are described in Section 2.7.2.

6.2.5 Validation parameters

The intra-day precision, stability and reproducibility of the method were tested using the protocols as described in Section 2.7.3.

6.2.6 Data analysis

The details of the data analysis are described in Section 2.9.4. Testing samples were in triplicates and were represented as mean ± S.D. Furthermore, all measurements were expressed as R.S.D.s. The retention time and the relative retention time (the ratio between retention time of sample constituents to that of internal standard), and the peak area and the relative peak area (the ratio between peak of sample constituents to that of internal standard) of the common peaks were used to evaluate the quality and quantity of the chemical compositions of Flos Magnoliae samples. In addition, the ORPs were used to study the correlation of two comparative samples according to their respective total number of peaks and common peaks, based on the reference sample. It is defined as follows: ORP (%) = $2\times c/(a+b) \times 100$, where ORP (%) represents the overlapping ratio; c represents the number of common peaks in two samples; a and b represent the number of total peaks in reference sample and compared sample, respectively.

6.3 Results

6.3.1 Optimisation of chromatographic conditions

The ethanol extract of *M. biondii*, from Hualong Magnolia development Co. Ltd. in Henan Province, China, was used for optimisation of the chromatographic fingerprinting method. The resolution of 13 common peaks was obtained using a gradient mobile phase consisting of acetonitrile and water within 60min, based on their relative retention time (Figure 6.1). Using reference standards authenticated two peaks of magnolin (Peak 6) and fargesin (Peak 11) (Figure 6.2). The retention times of magnolin and fargesin were 29.64±0.01min and 32.82±0.01min, respectively. Furthermore, the chromatographic fingerprints of the Flos Magnoliae extracts were detected using photodiode array detector at 250nm, 278nm and 290nm (Figure 6.3). The best resolution was achieved when the detection wavelength was 278nm.

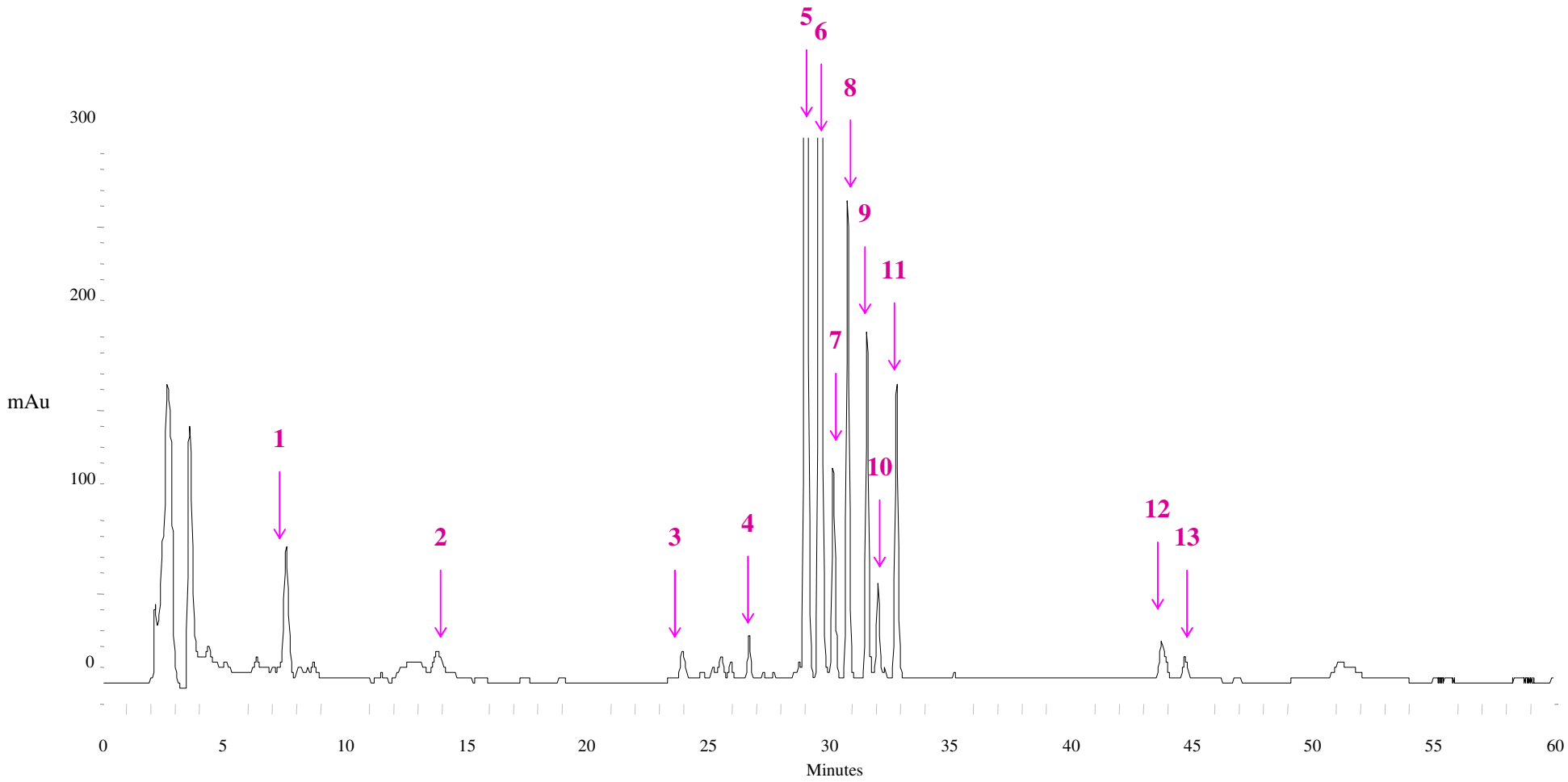


Figure 6.1 The chromatogram of *M. biondii* from the optimised mobile phase with the gradient elution for 60min (details see Table 2.12). Thirteen common peaks were indicated in the chromatogram, among that peak No 6 was indicated as magnolin and peak No 11 was indicated as fargesin.

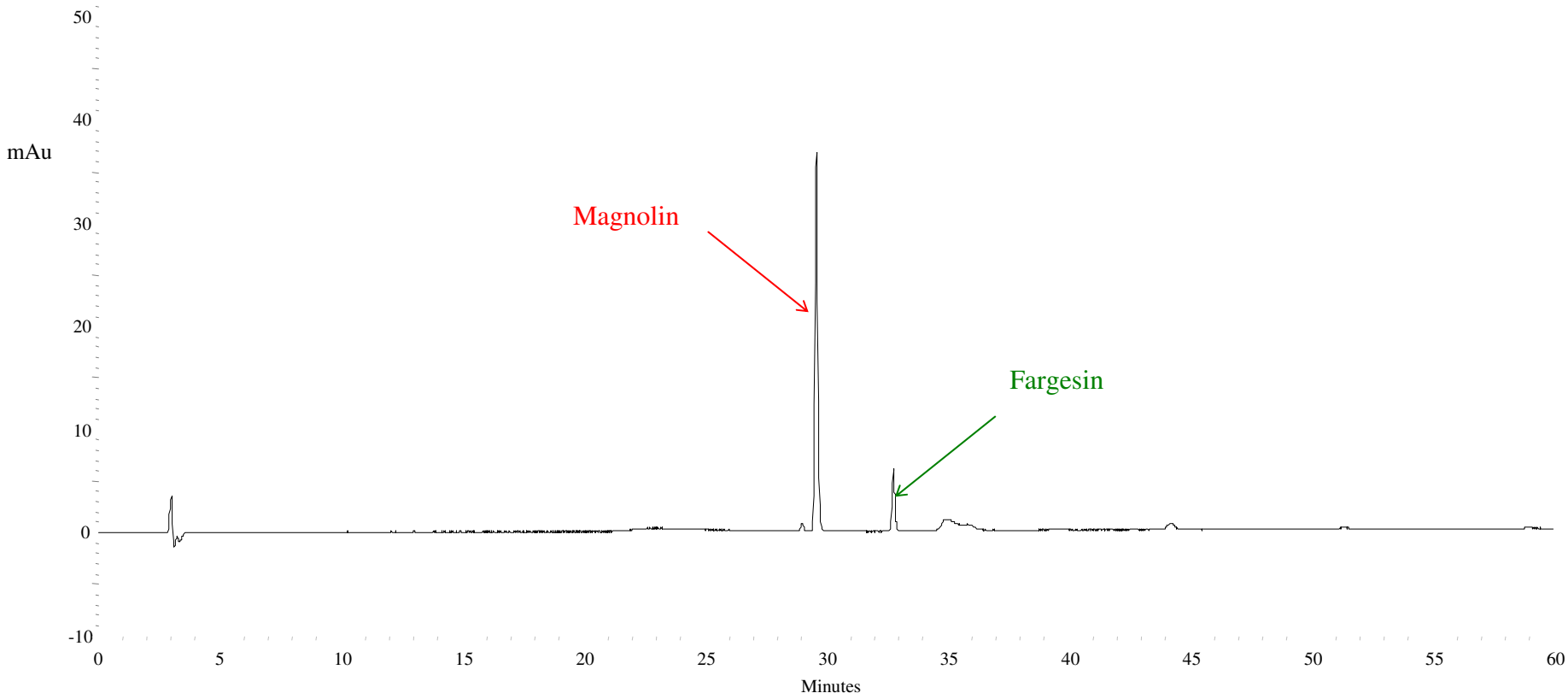


Figure 6.2 The chromatogram of the standard solution containing 100ng/ml of magnolin and 10ng/ml of fargesin (60min)

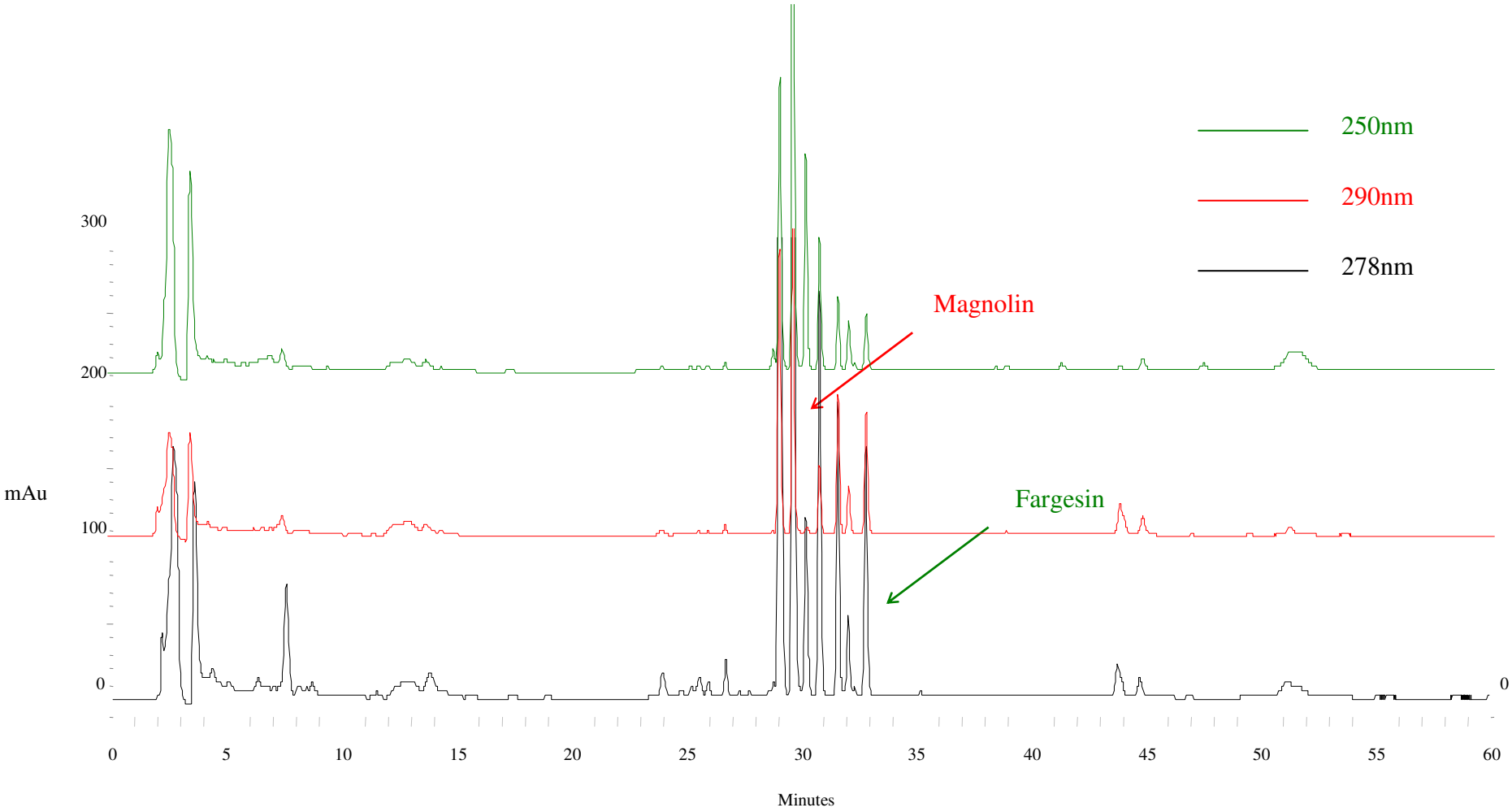


Figure 6.3 The chromatograms of *M. biondii*, using optimised HPLC conditions with different detection wavelengths at 250nm, 278nm and 290nm.

6.3.2 Validation parameters

6.3.2.1 Precision

Injections of five replicates of the same *M. biondii* sample produced a consistently result (Table 6.2). The validation practices were focused on the relative retention time and relative peak area of nine major common peaks. In the present study, magnolin (peak 6) was regarded as the internal standard. The R.S.D.s for the determination were less than 3%.

6.3.2.2 Stability

Five replicates of one *M. biondii* sample were performed at room temperature every 12hrs for five times within three days and the results are shown in Table 6.3. The validation practices were focused on the relative retention time and relative peak area of nine major common peaks. In the present study, magnolin (peak 6) was regarded as the internal standard. The R.S.D.s for the determination were less than 4%.

6.3.2.3 Repeatability

Five replicates of the same *M. biondii* sample were extracted and determined to evaluate the repeatability of the identification method. The results are shown in Table 6.4. In the present study, magnolin (Peak 6) was regarded as the internal standard. The R.S.D.s for the determination were less than 4%.

Table 6.2 The precision test for HPLC identification method (n=5). RRT represents relative retention time, and RPA represents relative peak area.

		Peak No.								
		4	5	6	7	8	9	10	11	12
RRT	Average	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48
	S.D.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% R.S.D.	0.14	0.13	0.00	0.13	0.13	0.13	0.13	0.13	0.16
RPA	Average	0.02	0.74	1.00	0.10	0.19	0.13	0.04	0.12	0.03
	S.D.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% R.S.D.	2.49	0.04	0.00	0.29	0.23	0.20	1.09	0.79	1.45

Table 6.3 The stability test for HPLC identification method (n=5). RRT represents relative retention time, and RPA represents relative peak area.

		Peak No.								
		4	5	6	7	8	9	10	11	12
RRT	Average	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.49
	S.D.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.02
	% R.S.D.	0.04	0.01	0.00	0.01	0.02	0.02	0.02	0.06	1.08
RPA	Average	0.02	0.74	1.00	0.10	0.19	0.13	0.04	0.13	0.03
	S.D.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% R.S.D.	3.16	0.21	0.00	0.52	0.26	0.33	1.51	1.98	2.38

Table 6.4 The reproducibility test for HPLC identification method (n=5). RRT represents relative retention time, and RPA represents relative peak area.

		Peak No.								
		4	5	6	7	8	9	10	11	12
RRT	Average	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48
	S.D.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% R.S.D.	0.04	0.01	0.00	0.01	0.02	0.02	0.02	0.02	0.03
RPA	Average	0.02	0.74	1.00	0.10	0.18	0.13	0.04	0.12	0.03
	S.D.	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	% R.S.D.	3.76	0.25	0.00	1.10	0.85	0.50	1.06	1.01	1.87

6.3.3 Identification of different Flos Magnoliae species

The HPLC chromatograms of six Flos Magnoliae species showed significantly different pattens (Figure 6.4). As shown in Table 6.5, Peak 1-4, 6-7 and 9-13 were not found in the chromatogram of *M. denudata*, Peak 3, 8 and 10 were absent in the chromatogram of *M. kobus*, Peak 1-3 and 11 were not found in the chromatogram of *M. liliflora*, Peak 1, 3, 4, 6, 10 and 11 were absent in the chromatogram of *M. sargentiana*, and Peak 1-6 and 9-11 were absent in the chromatogram of *M. sprengeri*. Compared to the quantity of chemical components in the chromatograms of six Flos Magnoliae species (Table 6.6), Peak 6 (magnolin) was the highest peak (7551815±11255.9) that was found in the chromatogram of *M. biondii*. Peak 8 was the highest peak in the chromatograms of *M. denudata* and *M. sprengeri*. Peak 9 was the highest peak (7232110.0±26758.8) in the chromatogram of *M. kobus*. Peak 5 was the highest peak (605316.6±7701.2) in the chromatogram of *M. liliflora*, while Peak 2 was the highest peak (263557.2±32598.8) in the chromatogram of *M. sargentiana*.

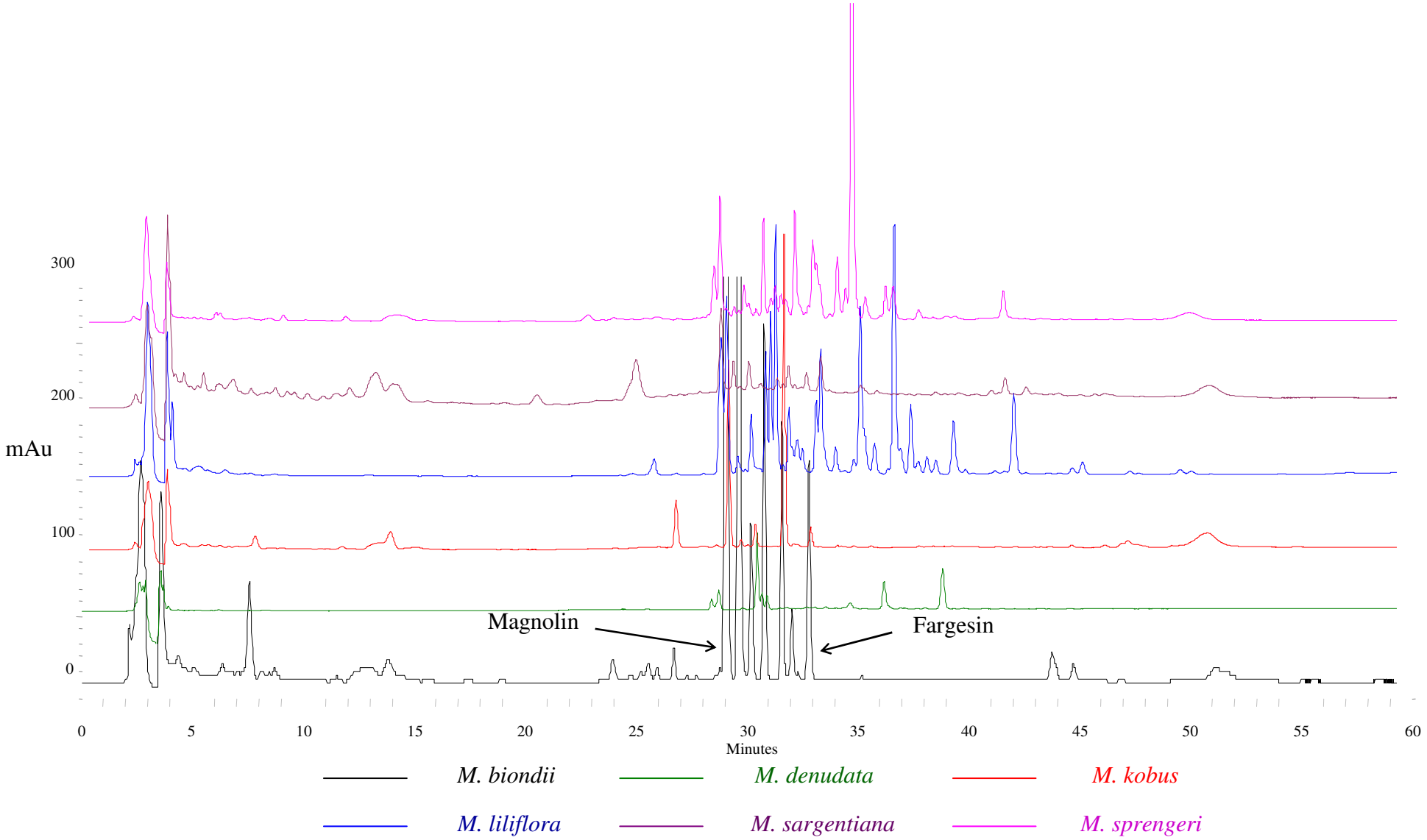


Figure 6.4 HPLC chromatograms of six Flos Magnoliae species (with 10μl injection volume of stock solution; peak 6, magnolin; peak 11, fargesin)

Table 6.5 The retention time of 13 common peaks of six Flos Magnoliae species. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks												
	1	2	3	4	5	6	7	8	9	10	11	12	13
M. biondii (HL)	7.57	13.37	23.99	26.72	29.07	29.63	30.20	30.80	31.60	32.06	32.82	43.80	44.78
M. denudata	-	-	-	-	29.04	-	-	30.81	-	-	-	-	-
M. kobus	7.55	13.52	-	26.65	29.03	29.62	30.25	-	31.56	-	32.77	43.68	44.62
M. liliflora	-	-	-	26.67	28.96	29.47	30.08	30.75	31.53	32.19	-	43.70	44.67
M. sargentiana	-	13.42	-	-	29.08	-	30.35	30.77	31.55	-	-	43.74	44.65
M. sprengeri	-	-	-	-	-	-	30.24	30.92	-	-	-	43.65	44.97
Mean	7.563	13.448	23.992	26.682	29.036	29.575	30.223	30.809	31.558	32.122	32.795	43.715	44.739
%R.S.D.	0.18	0.58	0.09	0.14	0.15	0.29	0.32	0.22	0.09	0.28	0.11	0.13	0.32

Table 6.6 The peak areas of 13 common peaks of six Flos Magnoliae species. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks Mean (%, w/w) (% R.S.D.)						
	1	2	3	4	5	6	7
M. biondii (HL*)*	833987.4 (4.04)	942559.9 (6.90)	177831.0 (4.21)	176575.0 (2.55)	5734722.8 (5.00)	7551815.8 (0.15)	738912.3 (0.43)
M. denudata	-	-	-	-	35693 (1.03)	-	-
M. kobus	1109179.9 (2.57)	1028542.3 (1.86)	-	2106445.5 (0.62)	5672324.8 (0.41)	125524.2 (0.61)	751598.8 (0.40)
M. liliflora	-	-	-	73386.0 (1.94)	605316.6 (1.27)	247061.7 (2.31)	409213.3 (1.50)
M. sargentiana	-	263557.2 (12.37)	-	-	14133.2 (4.02)	-	18526.9 (0.84)
M. sprengeri (JY)	-	-	-	-	-	-	68070.9 (1.10)

Continued Table 6.6 The peak areas of 13 common peaks of six Flos Magnoliae species. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks Mean (%, w/w) (% R.S.D.)						
	8	9	10	11	12	13	Total
M. biondii (HL)	1400263.5 (0.26)	1012449.6 (0.37)	300903.1 (1.09)	931563.3 (0.76)	251961.6 (7.02)	133588.4 (8.50)	20187133.7
M. denudata	181251.8 (0.39)	-	-	-	-	-	216944.8
M. kobus	-	7232110.0 (0.37)	-	513318.2 (2.05)	58502.5 (5.42)	40384.5 (4.47)	18637930.7
M. liliflora	577489.8 (0.83)	66190.5 (2.85)	552216.5 (1.02)	-	109290.8 (1.96)	128156.2 (1.98)	2768321.4
M. sargentiana	9792.6 (7.39)	9249.4 (5.44)	-	-	14254.2 (8.92)	3200.5 (38.92)	332714.0
M. sprengeri (JY)	218513.6 (0.77)	-	-	-	65736.5 (3.35)	37293.2 (8.79)	389614.2

6.3.4 Identification of different varieties of *M. biondii*

Replicate extracts of 11 batches of *M. biondii* from six different varieties were employed in the present study (details see Table 2.3). Triplicate HPLC runs were performed for each ethanol extract. Thirteen common peaks (defined in Figure 6.1) of ethanol extractions were all found in the chromatograms of 11 batches of *M. biondii* samples (Figure 6.5 – 6.10). Peak 6 in the chromatogram indicates the bioactive component, magnolin, which was used as internal standard for fingerprinting analysis. Peak 11 represents another bioactive lignan, fargesin. Based on the R.S.D. (less than 1%) of the relative retention time of each common peak in 11 *M. biondii* samples, these peaks were authenticated as the same peaks (Table 6.7).

Six non-common peaks (A-F) were also identified based on the retention time of the peaks in the chromatograms of 11 samples of *M. biondii* (Table 6.8). In the present study, ORP in 11 *M. biondii* samples were calculated, when sample 5 (HGCY) was as the reference standard (Table 6.9). ORP of each sample was no less than 70.27%.

Furthermore, it was also demonstrated that the peak area and the peak area percentage of single peak owned in 13 common peaks in the chromatographic fingerprints of 11 *M. biondii* samples (Table 6.10). The total peak area of the common peaks from Sample 5 (HGCY) was the highest among 11 *M. biondii* samples, while the total peak area of Sample 3 (EMT) was the lowest. Peaks 5-11 were also identified as the main common peaks that the total peak areas of those dominant peaks were more than 85%. Moreover, in the chromatographic fingerprints, peak 6 (magnolin) was use as the internal standard. The relative peak area were elucidated in Table 6.11

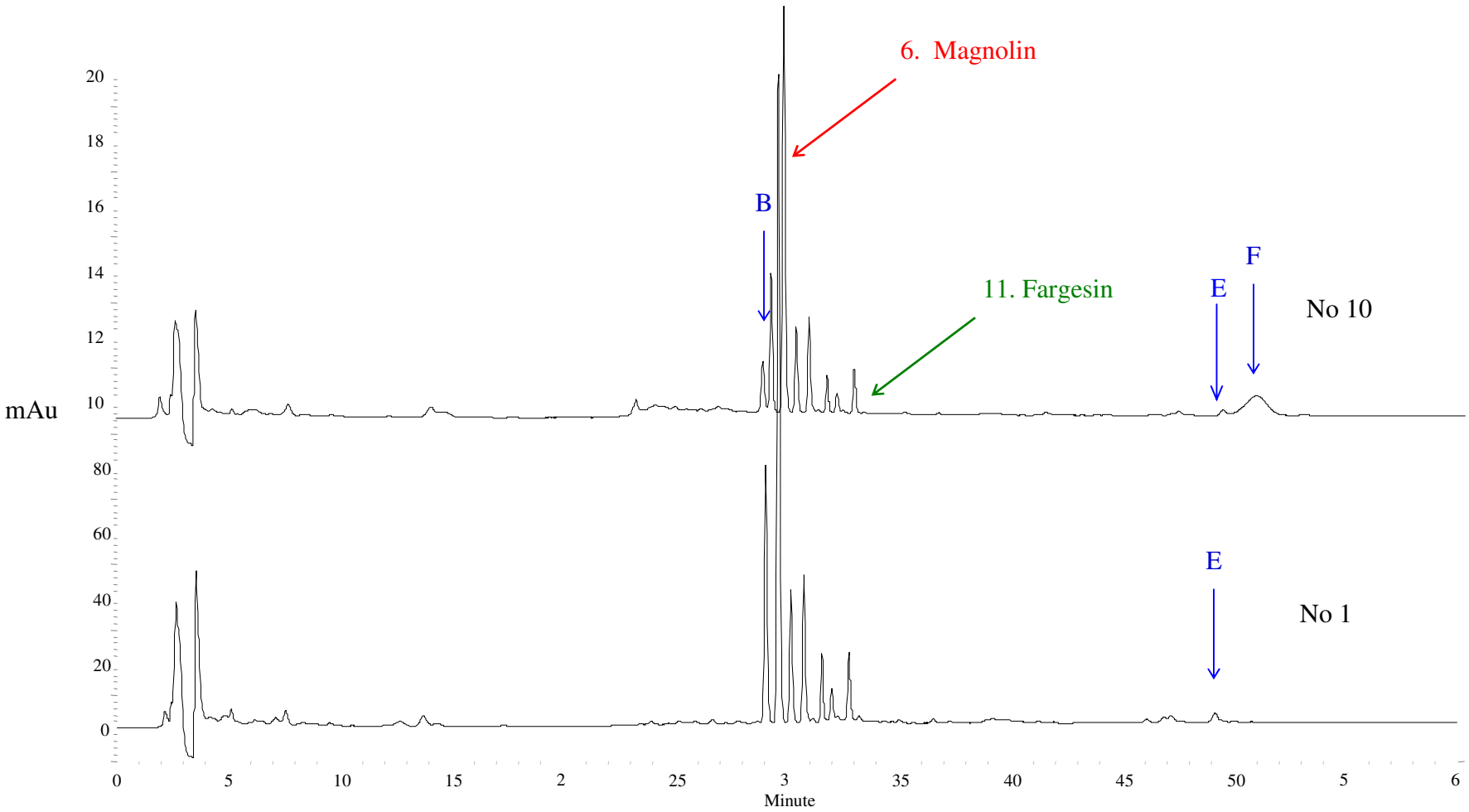


Figure 6.5 HPLC chromatograms of Sample 1 and Sample 10 (HLWH) (with 10µl injection volume; peak 6, magnolin; peak 11, fargesin)

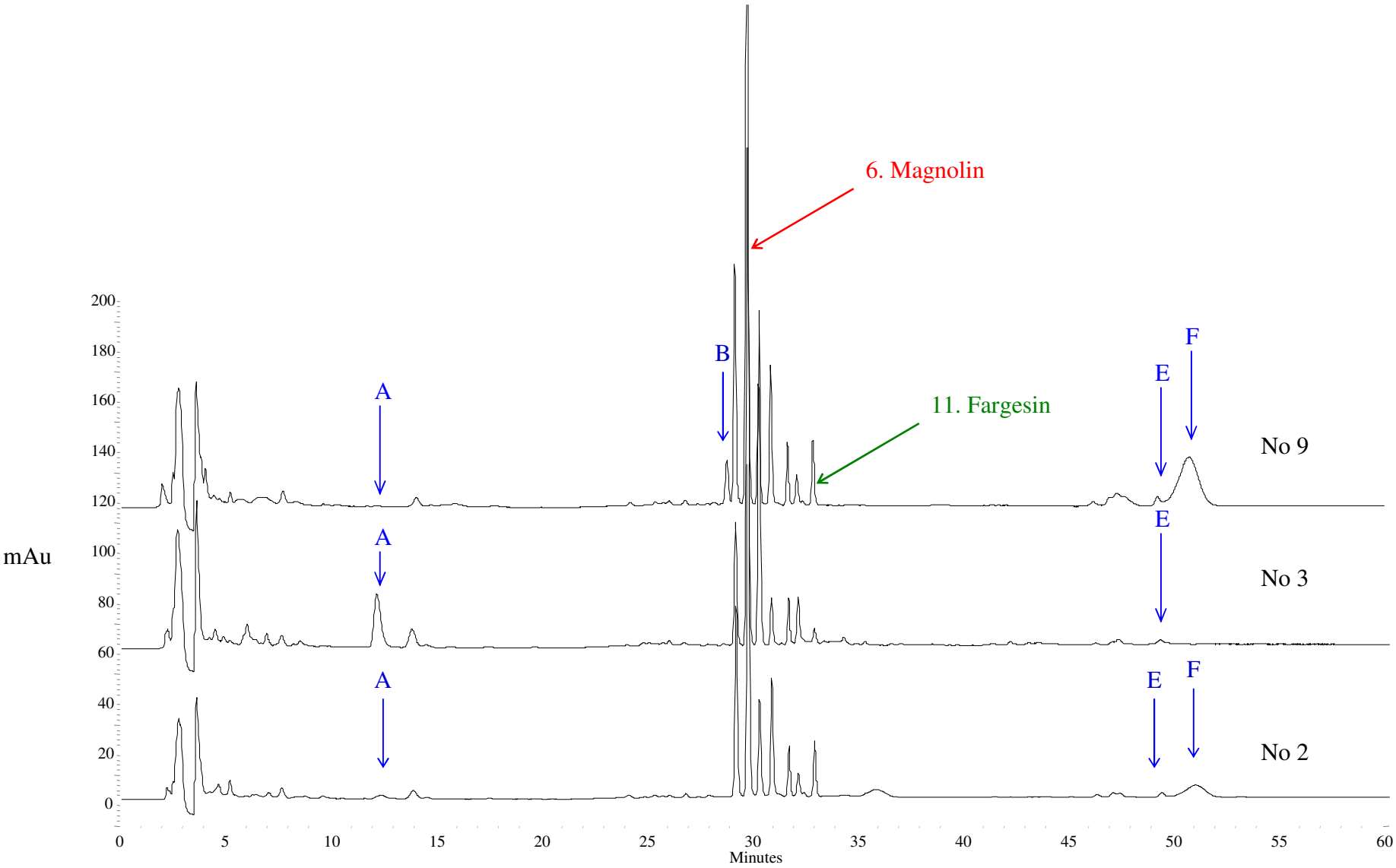


Figure 6.6 HPLC chromatograms of Sample 2, Sample 3 and Sample 9 (EMT) (with 10μl injection volume; peak 6, magnolin; peak 11, fargesin)

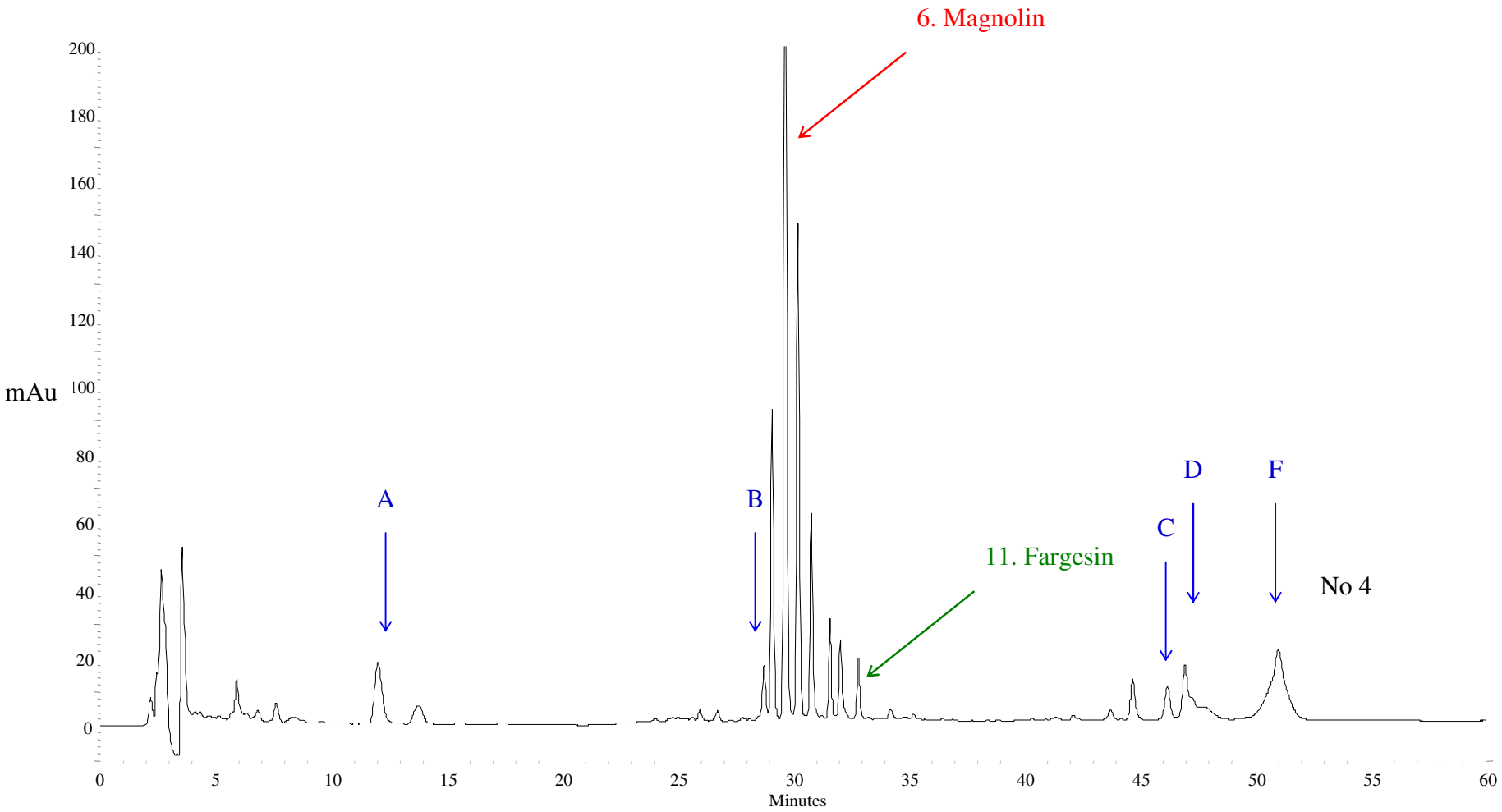


Figure 6.7 HPLC chromatogram of Sample 4 (XMT) (with 10µl injection volume; peak 6, magnolin; peak 11, fargesin)

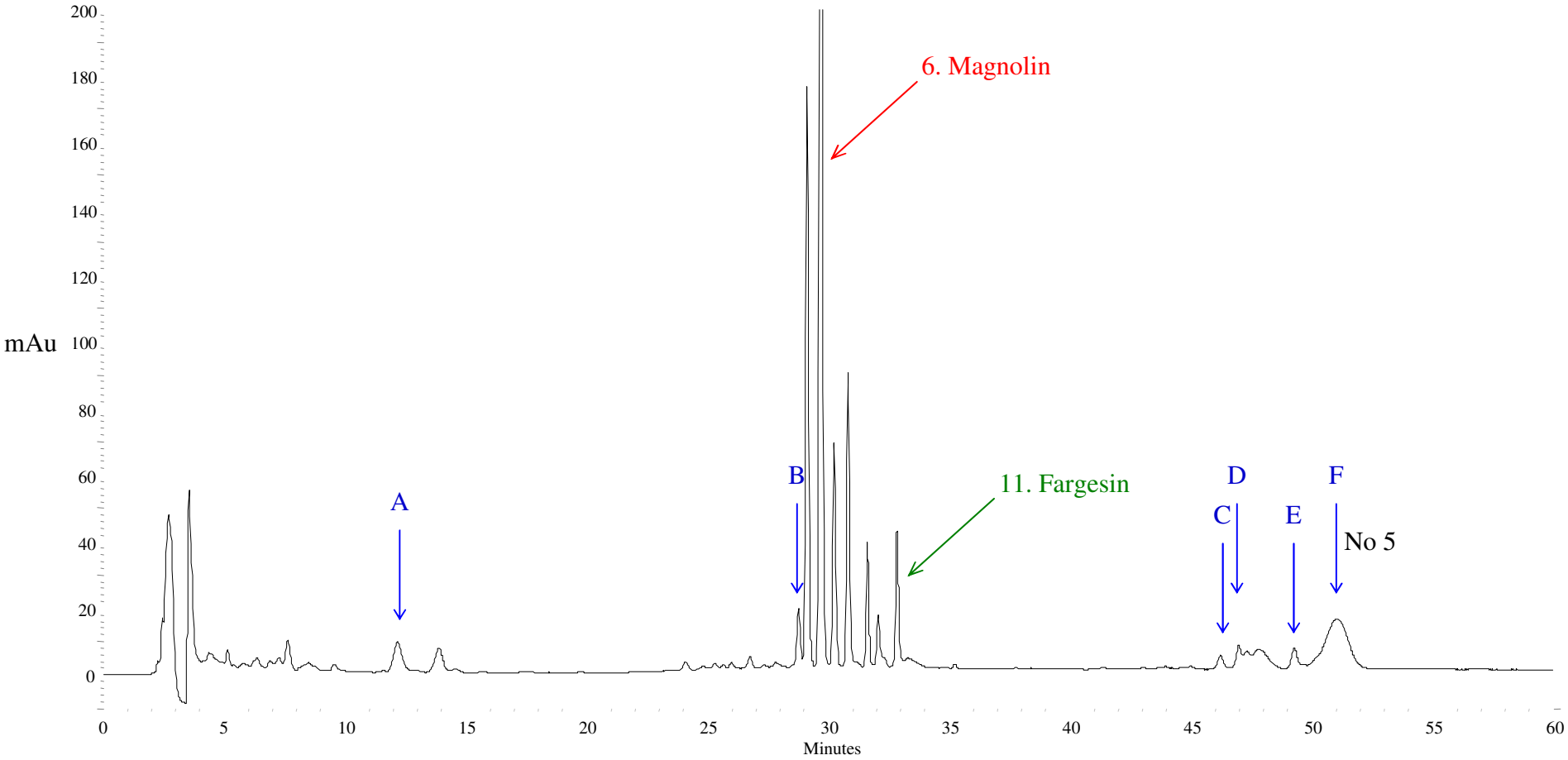


Figure 6.8 HPLC chromatogram of Sample 5 (HGCY) (with 10µl injection volume of stock solution; peak 6, magnolin; peak 11, fargesin)

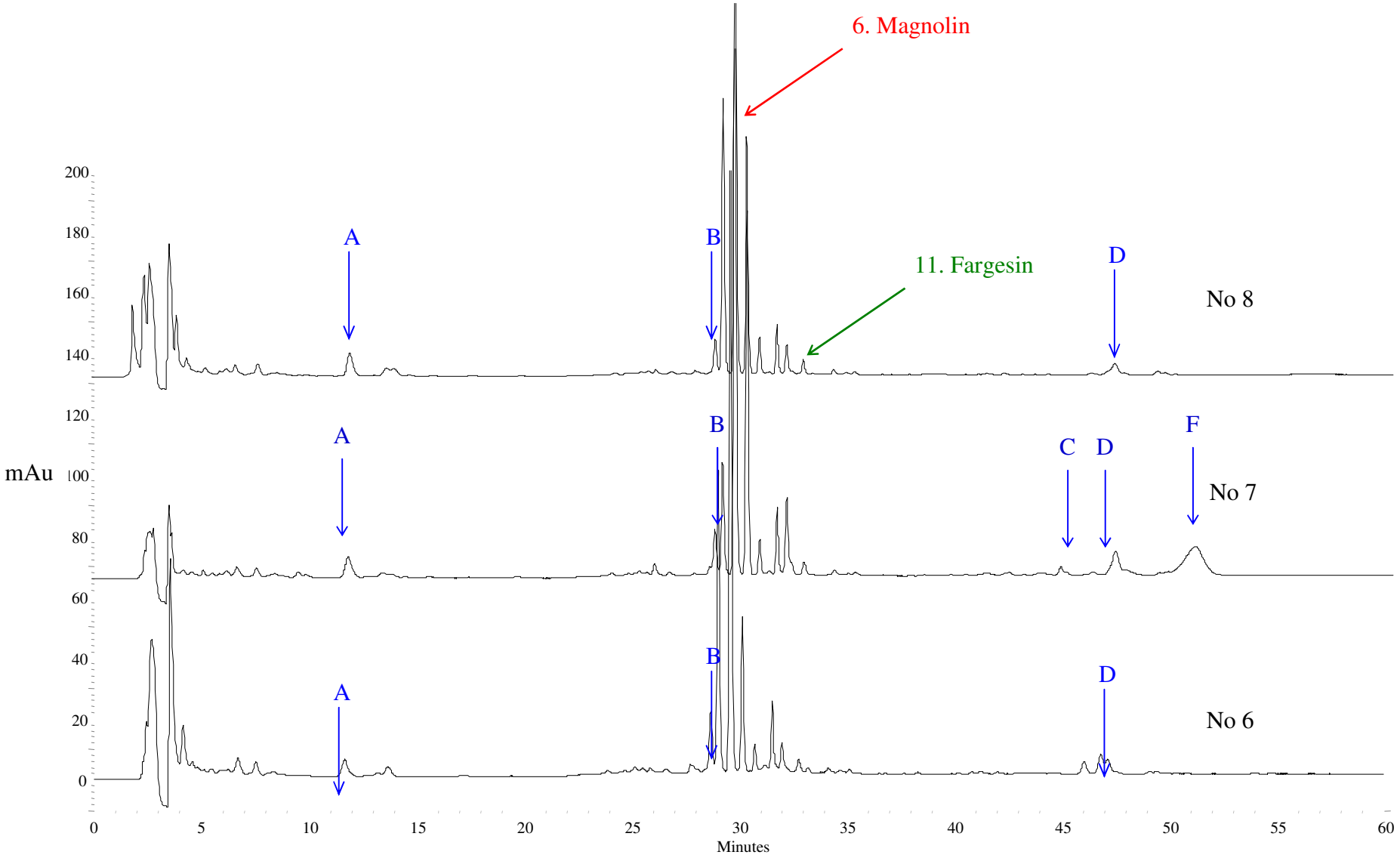


Figure 6.9 HPLC chromatograms of Sample 6, Sample 7 and Sample 8 (CY) (with 10μl injection volume; peak 6, magnolin; peak 11, fargesin)

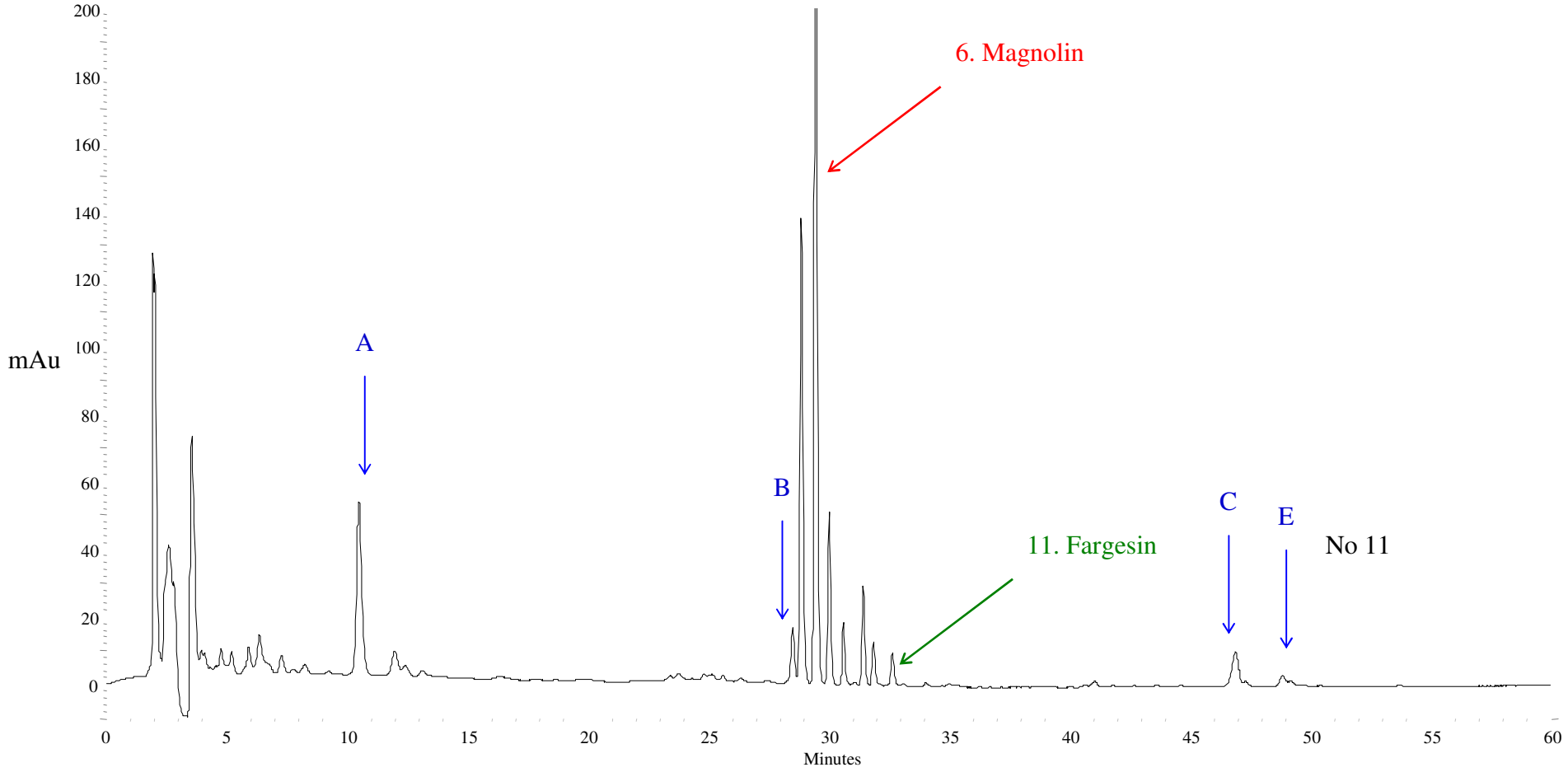


Figure 6.10 HPLC chromatogram of Sample 11 (BZCY) (with 10µl injection volume; peak 6, magnolin; peak 11, fargesin)

Table 6.7 The relative retention times of 13 common peaks of 11 batches of *M. biondii*. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1 (HLWH)	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.52
2 (EMT)	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.52
3 (EMT)	0.26	0.46	0.81	0.90	0.98	1.00	1.02	1.04	1.06	1.08	1.11	1.48	1.51
4 (XMT)	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.08	1.47	1.51
5 (HGCY)	0.26	0.47	0.82	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.51
6 (CY)	0.26	0.46	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.50
7 (CY)	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.03	1.07	1.08	1.11	1.48	1.51
8 (CY)	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.04	1.06	1.08	1.11	1.48	1.50
9 (EMT)	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.04	1.06	1.08	1.11	1.48	1.51
10 (HLWH)	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.04	1.06	1.08	1.11	1.48	1.51
11 (BZCY)	0.25	0.46	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.51
Mean	0.257	0.467	0.811	0.901	0.980	1.000	1.018	1.038	1.065	1.080	1.104	1.479	1.509
% R.S.D.	0.46	0.65	0.39	0.09	0.02	0.00	0.02	0.25	0.03	0.03	0.60	0.11	0.33

Table 6.8 The non-common peaks appeared in the chromatograms of 11 batches of *M. biondii* samples

	Peak No.					
	Retention time (min)					
	A	B	C	D	E	F
	12.18	28.76	46.22	46.89	49.27	51.01
1 (HLWH)	-	-	-	-	+	-
2 (EMT)	+	-	-	-	+	+
3 (EMT)	+	-	-	-	+	-
4 (XMT)	+	+	+	+	-	+
5 (HGCY)	+	+	+	+	+	+
6 (CY)	+	+	-	+	-	-
7 (CY)	+	+	+	+	-	+
8 (CY)	+	+	-	+	-	-
9 (EMT)	+	+	-	-	+	+
10 (HLWH)	-	+	-	-	+	+
11 (BZCY)	+	+	+	-	+	-

Table 6.9 The overlapping ratio of peaks (ORPs) of 11 batches of *M. biondii*

Sample ID	Number of common peaks	Total number of peaks	ORP (%)
1 (HLWH)		14	78.79
2 (EMT)		16	74.28
3 (EMT)		15	76.47
4 (XMT)		18	70.27
5 (HGCY) (references standard)	13	19	-
6 (CY)		16	74.28
7 (CY)		17	72.22
8 (CY)		16	74.28
9 (EMT)		17	72.22
10 (HLWH)		16	74.28
11 (BZCY)		17	72.22

Table 6.10 The peak area of 13 common peaks of 11 batches of *M. biondii*. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks Peak area (% peak area of single peak owned in 13 common peaks)						
	1	2	3	4	5	6	7
1 (HLWH)	775599.6 (5.18)	194547.7 (1.30)	110766.0 (0.74)	104695.1 (0.70)	4257723.7 (28.46)	5735934.5 (38.34)	440699.5 (2.95)
2 (EMT)	655833.3 (4.54)	260214.5 (1.80)	137585.0 (0.95)	121267.8 (0.84)	4105156.6 (28.39)	5392253.2 (37.29)	425414.1 (2.94)
3 (EMT)	524837.7 (6.62)	323379.2 (4.08)	35435.8 (0.45)	44275.6 (0.56)	1657387.2 (20.89)	3508641.4 (44.23)	637043.6 8.03)
4 (XMT)	444537.1 (4.09)	365699.8 (3.37)	58788.8 (0.54)	122701.5 (1.13)	2470228.7 (22.74)	4484932.6 (41.28)	586129.9 (5.40)
5 (HGCY)	715848.1 (4.57)	338031.9 (2.16)	181427.2 (1.16)	164151.5 (1.05)	4940026.6 (31.52)	5630288.3 (35.93)	411538.9 (2.63)
6 (CY)	750958.8 (5.80)	403681.7 (3.12)	125714.9 (0.97)	102172.8 (0.79)	4680505.9 (36.15)	5128337.6 (39.60)	379363.7 (2.93)
7 (CY)	463599.4 (4.83)	200307.2 (2.09)	59983.4 (0.63)	61208.1 (0.64)	1484744.1 (15.47)	4945111.8 (51.53)	802954.5 (8.37)
8 (CY)	665625.7 (5.13)	255934.5 (1.97)	78983.5 (0.61)	69136.7 (0.53)	4985640.0 (38.39)	5031863.8 (38.75)	450630.7 (3.47)
9 (EMT)	588843.1 (5.04)	170787.5 (1.46)	108463.8 (0.93)	102104.9 (0.87)	3363808.9 (28.78)	4468813.6 (38.24)	331356.1 (2.84)
10 (HLWH)	745212.4 (7.20)	236582.8 (2.28)	105917.5 (1.02)	84351.2 (0.81)	2677608.7 (25.86)	3813064.8 (36.82)	328350.5 (3.17)
11 (BZCY)	489723.1 (4.01)	230201.2 (1.89)	162551.4 (1.33)	61181.3 (0.50)	4658796.2 (38.16)	4890809.1 (40.06)	275597.8 (2.26)

Continued Table 6.10 The Peak area of 13 common peaks of 11 batches of *M. biondii*. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks Peak area (% peak area of single peak owned in 13 common peaks)						
	8	9	10	11	12	13	Total
1 (HLWH)	1300037.1 (8.69)	813256.4 (5.44)	180547.6 (1.21)	973867.3 (6.51)	61055.3 (0.41)	10605.3 (0.07)	14959335.1
2 (EMT)	1352464.2 (9.35)	782052.8 (5.41)	171540.7 (2.94)	994686.4 (2.13)	52134.8 (0.26)	9177.6 (0.04)	14459781.0
3 (EMT)	342359.5 (4.32)	433242.4 (5.46)	232859.1 (2.94)	169180.4 (2.13)	20354.9 (0.26)	3273.1 (0.04)	7932269.7
4 (XMT)	837020.3 (7.70)	530902.3 (4.89)	221549.4 (2.04)	383838.8 (3.53)	221476.9 (2.04)	135734.2 (1.25)	10863540.4
5 (HGCY)	1336726.7 (8.53)	753290.5 (4.81)	144138.2 (0.92)	995866.7 (6.35)	54322.1 (0.35)	9425.8 (0.06)	15675082.7
6 (CY)	252271.9 (1.95)	735269.2 (5.68)	159963.1 (1.24)	178081.7 (1.38)	40184.7 (0.31)	12523.5 (0.06)	12949029.4
7 (CY)	260925.5 (2.72)	606904.5 (6.32)	387891.9 (4.04)	120793.6 (1.26)	118146.5 (1.23)	84369.8 (0.88)	9596940.1
8 (CY)	371471.2 (2.86)	623008.6 (4.80)	176709.4 (1.36)	225039.8 (1.73)	42760.2 (0.33)	9744.2 (0.08)	12986548.3
9 (EMT)	1021971.8 (8.74)	606503.8 (5.19)	127389.8 (1.09)	744811.5 (6.37)	45957.3 (0.39)	6595.8 (0.06)	11687407.9
10 (HLWH)	971923.5 (9.39)	506817.9 (4.89)	127079.5 (1.23)	708575.9 (6.84)	30663.9 (0.30)	19703.1 (0.19)	10355851.8
11 (BZCY)	338888.6 (2.78)	670243.5 (5.49)	128646.4 (1.05)	247622.7 (2.03)	43574.9 (0.36)	9763.7 (0.08)	12207599.8

Table 6.11 The relative peak areas of seven main common peaks of 11 batches of *M. biondii*. Peak 6 indicated magnolin and Peak 11 indicated fargesin. RPA represents relative peak area. (n=3)

	Number of the common peaks RPA (% R.S.D.)						
	5	6	7	8	9	10	11
1 (HLWH)	0.74 (0.05)	1.00	0.08 (1.34)	0.23 (0.51)	0.14 (1.22)	0.03 (2.27)	0.17 (0.94)
2 (EMT)	0.76 (0.10)	1.00	0.08 (0.86)	0.25 (0.35)	0.15 (0.59)	0.03 (1.34)	0.18 (0.21)
3 (EMT)	0.47 (0.34)	1.00	0.18 (0.26)	0.10 (0.76)	0.12 (1.20)	0.07 (3.51)	0.05 (4.63)
4 (XMT)	0.55 (4.48)	1.00	0.13 (0.76)	0.19 (0.36)	0.12 (0.37)	0.05 (0.55)	0.09 (1.41)
5 (HGCY)	0.88 (0.14)	1.00	0.07 (0.07)	0.24 (0.18)	0.13 (0.37)	0.03 (0.39)	0.18 (0.47)
6 (CY)	0.91 (0.13)	1.00	0.07 (1.08)	0.05 (1.71)	0.14 (0.31)	0.03 (3.36)	0.03 (2.26)
7 (CY)	0.30 (0.02)	1.00	0.16 (0.08)	0.05 (0.43)	0.12 (0.49)	0.08 (0.93)	0.02 (1.01)
8 (CY)	0.99 (0.03)	1.00	0.09 (0.58)	0.07 (1.54)	0.12 (0.45)	0.04 (1.39)	0.04 (1.81)
9 (EMT)	0.75 (0.03)	1.00	0.07 (0.27)	0.23 (0.05)	0.14 (0.18)	0.03 (0.28)	0.17 (0.23)
10 (HLWH)	0.70 (0.19)	1.00	0.09 (2.79)	0.25 (0.38)	0.13 (0.63)	0.03 (2.04)	0.19 (0.05)
11 (BZCY)	0.95 (0.14)	1.00	0.06 (0.72)	0.07 (0.73)	0.14 (0.21)	0.03 (1.53)	0.05 (1.01)

6.3.5 Identification of different Flos Magnoliae products from five herbal suppliers

The chromatograms of five Flos Magnoliae products samples (details see Table 2.4) are shown in Figure 6.11. The results demonstrated that the five samples contained all 13 common peaks, based on the percentage of R.S.D (less than 1%) of the relative retention time of these peaks (Table 6.12).

The peak area and the peak area percentage of single peak owned in 13 common peaks show quality differences of the chemical compositions of five Flos Magnoliae products (Table 6.13). Among five herbal products, the total peak area of the common peaks of the product from Hualong Magnolia Development Co. Ltd. was the highest, while the total peak area of the concentrated herbal powder from KODA International was the lowest.

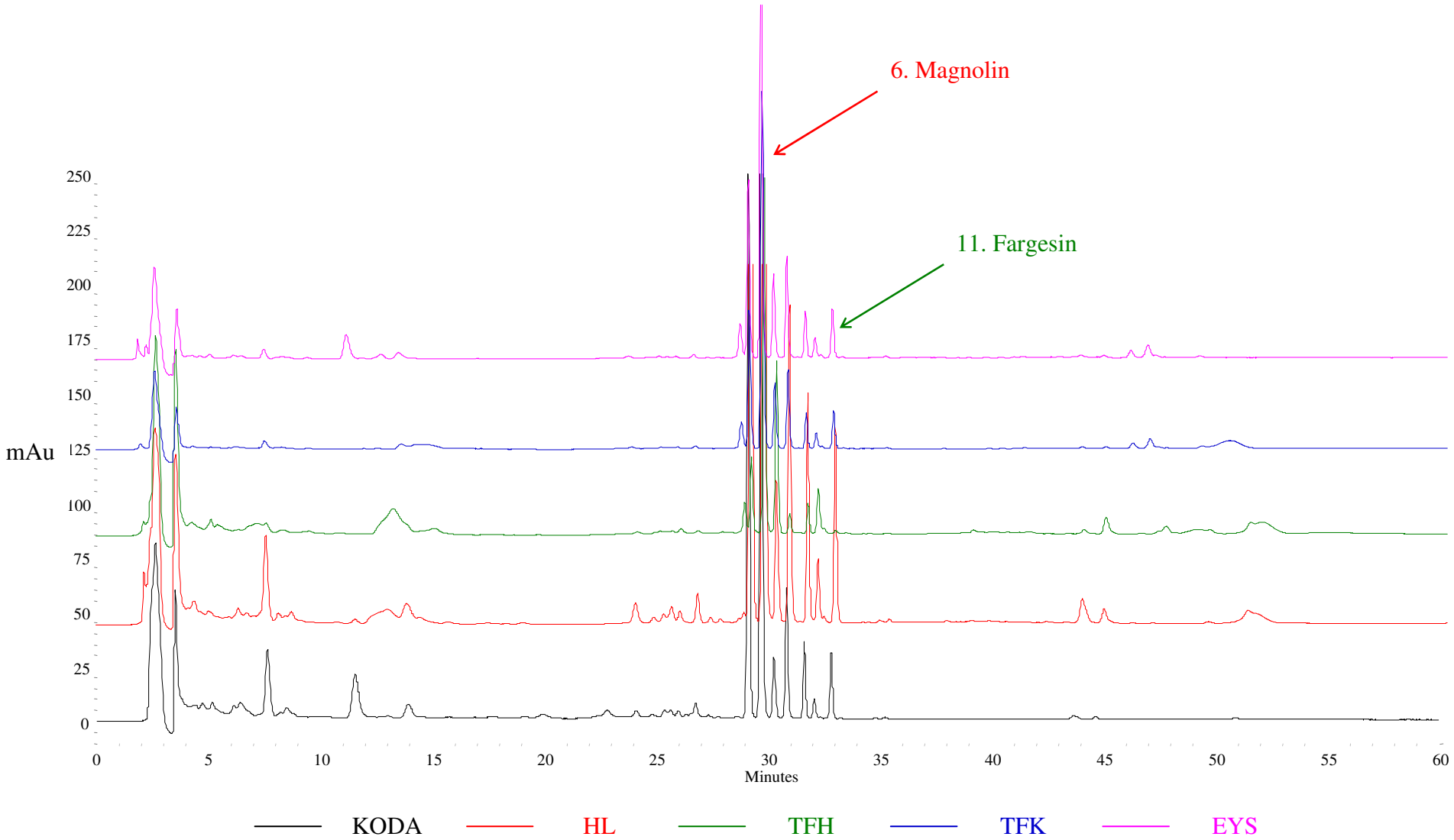


Figure 6.11 HPLC chromatograms of five Flos Magnoliae products from different herbal suppliers (with 10μl injection volume; peak 6, magnolin; peak 11, fargesin)

Table 6.12 The relative retention times of 13 common peaks of five Flos Magnoliae products from different herbal suppliers. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks												
	1	2	3	4	5	6	7	8	9	10	11	12	13
HL	0.26	0.44	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.51
TFH	0.26	0.45	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.51
EYS	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.52
TFK	0.25	0.46	0.80	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.52
KODA	0.26	0.45	0.80	0.90	0.98	1.00	1.02	1.04	1.06	1.09	1.11	1.47	1.50
Mean	0.256	0.454	0.807	0.901	0.981	1.000	1.018	1.038	1.065	1.083	1.106	1.477	1.511
% R.S.D.	0.0013	0.0105	0.0063	0.0009	0.0018	0.0000	0.0002	0.0003	0.0005	0.0044	0.0007	0.0042	0.0055

Table 6.13 The peak area of 13 common peaks of five Flos Magnoliae products from different herbal suppliers. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks Peak area (% peak area of single peak owned in 13 common peaks)						
	1	2	3	4	5	6	7
HL	833987.4 (4.13)	942559.9 (4.67)	177831.0 (0.88)	176575.0 (0.87)	5734722.8 (28.41)	7551815.8 (37.41)	738912.3 (3.66)
TFH	891973.6 (6.96)	1482802.4 (11.56)	131394.6 (1.02)	79678.0 (0.62)	2202398.2 (17.17)	5307902.4 (41.39)	725149.6 (5.65)
EYS	415711.9 (5.59)	200368.5 (2.69)	67079.6 (0.90)	68112.8 (0.92)	2181897.5 (29.34)	2672591.0 (35.93)	202148.5 (2.72)
TFK	703555.3 (4.40)	287457.6 (1.80)	131091.5 (0.82)	124861.9 (0.78)	4644081.3 (29.06)	6040824.0 (37.80)	437553.1 (2.74)
KODA	754508.5 (10.67)	125333.8 (1.77)	16050.7 (0.23)	90375.1 (1.28)	2056309.5 (29.07)	2615908.1 (36.99)	256609.0 (3.63)

Continued Table 6.13 The peak area of 13 common peaks of five Flos Magnoliae products from different herbal suppliers. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks Peak area (% peak area of single peak owned in 13 common peaks)						
	8	9	10	11	12	13	Total
HL	1400263.5 (6.94)	1012449.6 (5.02)	300903.1 (1.49)	931563.3 (4.61)	251961.6 (1.25)	133588.4 (0.66)	20187133.7
TFH	323756.2 (2.52)	617985.3 (4.82)	398412.0 (3.11)	86955.6 (0.68)	352080.9 (2.75)	222809.5 (1.74)	12823298.3
EYS	692136.0 (9.31)	340040.5 (4.57)	68026.8 (0.91)	483385.3 (6.50)	40864.7 (0.55)	5212.7 (0.07)	7437575.8
TFK	1394983.7 (8.73)	823886.0 (5.15)	163940.1 (1.03)	1037603.6 (6.49)	164727.1 (1.03)	28551.8 (0.18)	15983117.1
KODA	472705.0 (6.68)	283600.0 (4.01)	79065.2 (1.12)	265520.4 (3.75)	39153.9 (0.55)	17467.0 (0.25)	7072606.1

6.3.6 Identification of *M. biondii* samples from four different cultivation sites

Four *M. biondii* samples, which were collected from four different cultivation sites from Nanzhao, Henan Province, China, were employed in the present study (details see Table 2.5). Figure 6.12 showed the chromatograms of four *M. biondii* batches. The results demonstrated that the four samples contained all 13 common peaks, based on the percentage of R.S.D (less than 1%) of the relative retention time of these peaks (Table 6.14).

Under the optimised chromatographic conditions, the peak area and the peak area percentage of single peak owned in 13 common peaks were shown quality differences of the chemical compositions in four *M. biondii* batches collected from different cultivation sites (Table 6.15). The peak area percentages of Peak 5 and Peak 6 (magnolin) were more than 59% of the total peak area of 13 common peaks. The total peak area of the common peaks of the samples, from Mt. Yanyi (YYS), was the highest among four *M. biondii* samples, while the total peak area of the batch that was collected from Eastern Garden (DHY), was the lowest.

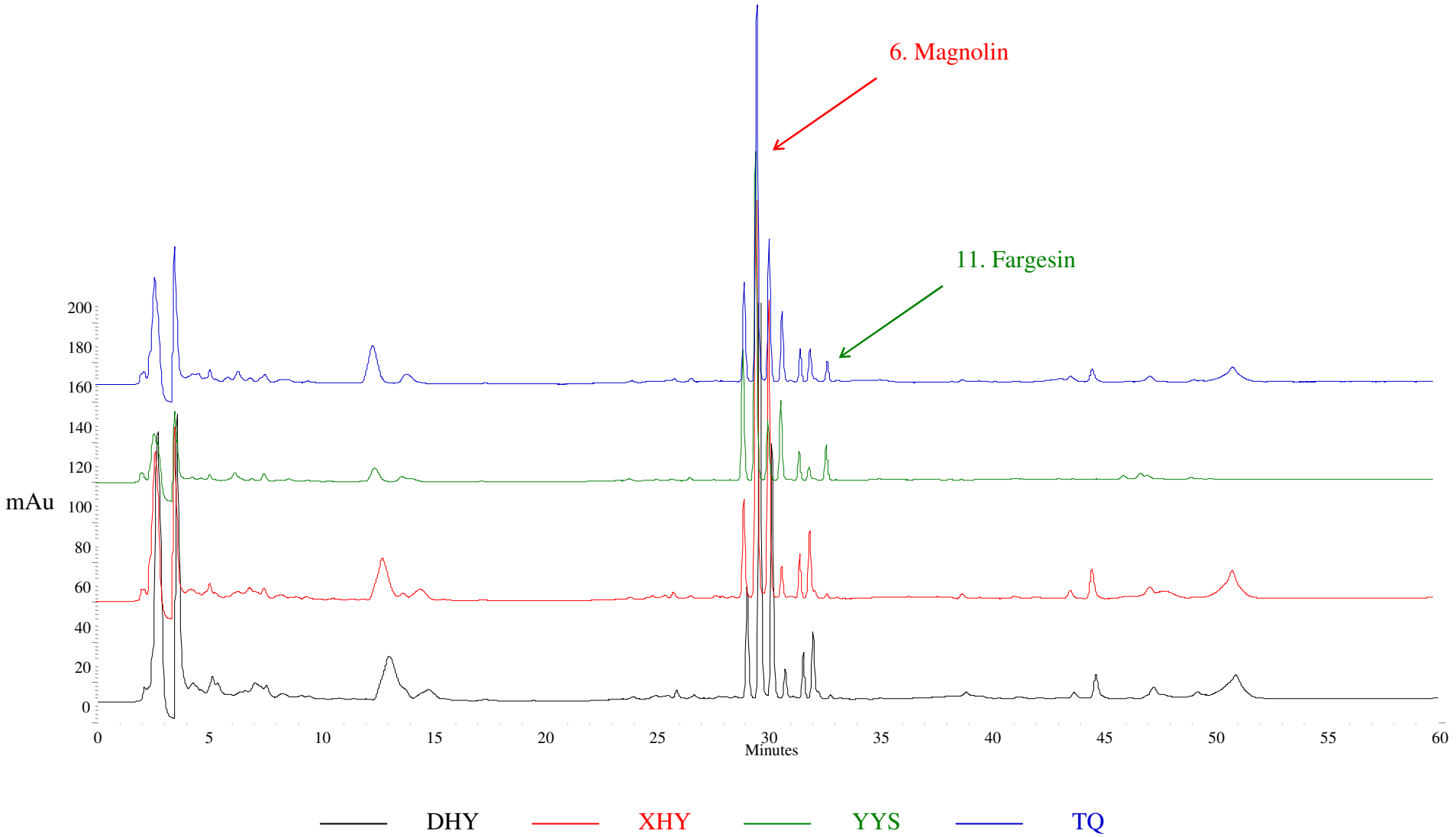


Figure 6.12 HPLC chromatograms of four *M. biondii* sample, collected from four cultivation sites (with 10μl injection volume; peak 6, magnolin; peak 11, fargesin)

Table 6.14 The relative retention time of 13 common peaks of four *M. biondii* samples, collected from different cultivation sites. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks												
	1	2	3	4	5	6	7	8	9	10	11	12	13
DHY	0.26	0.45	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.51
XHY	0.26	0.45	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.47	1.51
YYS	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.04	1.07	1.08	1.11	1.48	1.51
TQ	0.26	0.47	0.81	0.90	0.98	1.00	1.02	1.04	1.06	1.08	1.11	1.47	1.51
Mean	0.256	0.460	0.810	0.901	0.980	1.000	1.018	1.038	1.065	1.081	1.106	1.476	1.507
% R.S.D.	0.202	2.236	0.128	0.028	0.004	0.000	0.016	0.014	0.019	0.021	0.018	0.182	0.050

Table 6.15 The peak area of 13 common peaks of four *M. biondii* samples collected from different cultivation sites. Peak 6 indicated magnolin and Peak No11 indicated fargesin. (n=3)

	Number of the common peaks						
	Peak area						
	(% peak area of single peak owned in 13 common peaks)						
	1	2	3	4	5	6	7
DHY	391973.6	395715.1	43915.7	39939.7	1062447.6	2574979.0	346171.8
	6.76	6.82	0.76	0.69	18.32	44.40	5.97
XHY	481167.7	434144.0	63264.5	47609.1	1217948.6	3284572.6	517179.5
	6.51	5.87	0.86	0.64	16.48	44.45	7.00
YYS	546050.6	263190.3	88124.3	89468.3	2865990.8	3510532.1	265516.3
	5.59	2.69	0.90	0.92	29.34	35.94	2.72
TQ	520897.1	285579.0	77090.2	81429.7	1574373.7	2897464.3	349956.7
	6.97	3.82	1.03	1.09	21.07	38.77	4.68

Continued Table 6.15 The peak area of 13 common peaks of four *M. biondii* samples collected from different cultivation sites. Peak 6 indicated magnolin and Peak 11 indicated fargesin. (n=3)

	Number of the common peaks Peak area (% peak area of single peak owned in 13 common peaks)						
	8	9	10	11	12	13	Total
DHY	152717.4 2.63	298607.5 5.15	186175.9 3.21	35276.6 0.61	166324.9 2.87	104950.9 1.81	5799195.8
XHY	209623.8 2.84	372950.7 5.05	254576.1 3.44	47294.0 0.64	296856.0 4.02	163008.8 2.21	7390195.4
YYS	909142.3 9.31	446653.8 4.57	89355.3 0.91	634941.7 6.50	53677.1 0.55	5887.2 0.06	9768530.3
TQ	582325.7 7.79	356923.4 4.78	156396.7 2.09	263313.3 3.52	228229.9 3.05	98618.7 1.32	7472598.5

6.4 Discussion

In order to develope an accurate, valid and optimised chromatographic fingerprint, proper selections of HPLC parameters, including mobile phase, column, column temperature, flow rate of mobile phase and detection wavelength are necessary. Two steps, which are modelling of retention and optimisation, are required for establishment of a HPLC gradient method (Dolan et al., 1999a, Dolan et al., 1999b, Dolan et al., 1999c). Based on the results from Chapter 5 and previous literatures (Fang et al., 2002, Xu et al., 2003, Yu et al., 2005b, Zhu et al., 2002), a gradient acetonitrile – water system was used as the mobile phase to achieve a better HPLC separation. Hence, with a stringent demand on resolution and peak capacity, the well separation of the 13 common peaks from *M. biondii* samples was achieved. Furthermore, the detection wavelengths, as one of the key factors to contribute to a reliable and reproducible HPLC fingerprint, were tested at 250nm, 278nm and 290nm. It was found that the chromatographic fingerprints at 278nm produced the best resolution of a sufficiently large number of detectable peaks, under this condition.

The R.S.D.s of relative retention times and relative peak areas of nine of the 13 common peaks for the injections of five independently prepared samples were found less than 4%. The R.S.D.s of relative retention times and relative peak areas of the common peaks for five times injections consistently were detected less than 3%, while the R.S.D.s of relative retention times and relative peak areas of the common peaks in stability test were less than 4%. All results indicated that the present method of the HPLC fingerprinting analysis was valid and satisfactory.

It is well known that *M. biondii* is the authentic species of Flos Magnoliae (Fu, 2000). For each separated peak of the 13 common peaks, the R.S.D. of retention time should be less than 1 %, if not, these peaks could not be identified as the same peak (Cai and Liu, 2005). Among

the common peaks, two peaks were authenticated using reference standards. Peak 6 and Peak 11 represented magnolin and fargesin, respectively. The HPLC profiles of *M. kobus* and *M. liliflora*, commonly used as the substitutes of Flos Magnoliae in Japan and China, were found to contain the ten and nine common peaks, respectively. Meanwhile, magnolin were identified from the ethanol extracts of *M. kobus* and *M. liliflora*, while fargesin were only found in *M. kobus*, which are in agreement with the findings in Chapter 5. Another three species, including *M. denudata, M. sargentiana* and *M. sprengeri* were discovered having significant differences in the quality and quantity of the chemical compositions. Therefore, the HPLC profiles can be used for the identification and differentiation of different Flos Magnoliae species.

In the present study, the HPLC fingerprints of 11 batches of *M. biondii* samples were also conducted. Thirteen common peaks were all found in the chromatograms of 11 *M. biondii* batches. The similar relative retention time of each peak in all samples indicated the peak locality in the chromatographic fingerprints, which may provide qualitative and quantitative information on the chemical compositions of ethanol extracts of *M. biondii*, and thus, contribute to quality control of different botanic varieties of *M. biondii* (Cai and Liu, 2005). Further findings from another parameter of chromatographic fingerprints, the ORPs, indicate slight differences of the chemical compositions among 11 *M. biondii* batches. These findings can be used further in compare of the diversities between various *M. biondii* samples. The total peak area of 13 common peaks of Sample 5 (HGCY, 黄梗串鱼) was almost two-folded as the total peak area of Samples 3 (EMT, 二毛桃). This suggests that differences exist in the quality of these *M. biondii* samples. As mentioned before, Peak 6 (magnolin) and Peak 11 (fargesin) were detected from all 11 batches of *M. biondii* samples, while Peak 6 (magnolin) owned the highest percentage (35.93% - 51.53%) in 13 common peaks. The *M. biondii* batches also had high percentage of Peak 5 (15.47% - 38.39%). These two chemical

compounds may play key roles in the therapeutic effects of Flos Magnoliae. Moreover, the dominant common peaks have been recognised as one of the fingerprinting parameters to ensure the consistency of the CHM quality (Cai and Liu, 2005). It has been suggested that the percentage of the toil peak area of the dominant common peaks is more than 80% of the total peak area of all the common peaks (Cai and Liu, 2005). Therefore, the HPLC fingerprints of relative peak areas of seven main common peaks (Peak 5-11) in 11 *M. biondii* batches were demonstrated in the present study, when Peak 6 (magnolin) was used as the internal standard. The quality consistency was not found in different varieties *of M. biondii*, collected from the same cultivation sites. Thus, botanic sources may influence the quality of the chemical compositions of *M. biondii*.

All 13 common peaks were detected from five Flos Magnoliae products, based on the relative retention times. The findings are in agreement with the results from the previous Chapter that identified *M. biondii* as the source of these five products. In addition, differences of the content of the chemical compositions of five Flos Magnoliae products from different herbal suppliers were demonstrated. For instance, the total peak area of 13 common peaks of the Flos Magnoliae products from Hualong Magnolia Development Co. Ltd., China was more than 2.8-fold higher than the total peak areas of the local sample from KODA International. It is not clear about the reason of this change, but the manufacturing process may influence the quality of Flos Magnoliae products (Liu et al., 2003a, Zhang and Feng, 2005).

The environmental factors during the cultivation process, including the amount of sunlight and moisture, soil conditions, nutrients and climate are important in affecting the chemical compositions of CHM (Bensky and Gamble, 1993, Yu et al., 1999). This is supported by the present findings that among four *M. biondii* batches, differences of the peak area and the percentage peak area of each single peak of 13 common peaks were demonstrated.

In conclusion, a validated HPLC fingerprinting method has been developed for qualitative and quantitative analysis of the Flos Magnoliae samples for the first time. Under the optimised chromatographic conditions, qualitative and quantitative differences of the chemical compositions among six Flos Magnoliae species were observed. Moreover, 13 common peaks were obtained for the identification of the authentic species, *M. biondii*. These chromatographic fingerprinting data may help to ensure the consistency of the quality of Flos Magnoliae products. The findings also support the suggestions that botanic species and varieties, cultivation sites and manufacturing process may influence the quality of Flos Magnoliae.

Chapter Seven

Effects of Flos Magnoliae and Its Active Components on Mast Cell-Derived Histamine Release in Rat Peritoneal Mast Cells

7.1 Background

7.1.1 Mast cell-dependent allergy

7.1.1.1 Allergy

The immune system is very important to defence the body against invaded pathogens (such as bacteria, viruses, fungi and parasites) (Roitt and Delves, 2001). It is also involved in various inflammatory conditions, including allergy. The inflammatory process of immunopathological reactions, can be clarified as the acute and the chronic inflammatory responses (Willoughby et al., 2000). The acute responses are often initiated by hypersensitivity reactions, microbial infections, irritant and corrosive substances (Brook, 1996, Umetsu and DeKruyff, 1997, Kligman and Kligman, 1998), involving changes in blood flow, permeability of blood vessels, and leukocytes emigration (Rang et al., 2003). The chronic inflammation may be caused by infectious microbes (e.g. mycobacterium tuberculosis) that can avoid phagocytosis and continue to exist in the tissue for a long-lasting period (Drennan et al., 2004), irritant inhalation of toxic materials (e.g. mineral particles) that cannot be eliminated by enzymic breakdown, or phagocytosis (Muhle and Mangelsdorf, 2003) or auto-immune disease (autoimmune hepatitis) (Frank, 2002). The chronic inflammation can last for weeks, months even an indefinite period (Roitt and Delves, 2001).

Hypersensitive response is also a kind of inflammatory responses. It has been classified into four categories, immediate or anaphylactic hypersensitivity (allergy) (Type I), antibody-dependent cytotoxic hypersensitivity (Type II), immune complex-mediated hypersensitivity (type III) and cell-mediated (delayed-type) hypersensitivity (Type IV) (Roitt and Delves, 2001).

In 1902, Richet and Portier firstly used the term anaphylaxis to describe sensitivity to relatively foreign antigens (such as grass pollen, dead house dust mites and certain food),

contrary to prophylaxis (Cohen and Zelaya-Quesada, 2002). Anaphylaxis occurs by gathering of Immunoglobulin E (IgE) receptors through cross-linking after re-exposure to a same antigen (Noone and Osguthorpe, 2003). Binding IgE to the high-affinity IgE receptor (FcεRI) immediately triggers release of inflammatory mediators from mast cells, basophils, eosinophils, etc. (Venarske and deShazo, 2003).

A number of cells have been shown involved in the allergic process (Table 7.1), including vascular endothelial cells, mast cells, macrophages, platelets and leucocytes that are believed to contribute to inflammatory reactions in tissues (Roitt and Delves, 2001). Studies have indicated that mast cells play a central role in mediating Type I hypersensitivity (Marone et al., 1998). A number of mediators, such as histamine, leukotrienes and cytokines, are involved in allergic reactions (Table 7.2).

Table 7.1 Cells involved in allergy

Cell types			Roles in allergic inflammation
Mast cells			Degranulation triggers secretion of mediators, e.g. histamine, leukotrienes, interleukins, prostaglandins, PAF and nerve growth factor (NGF) (Hart, 2001) (Details see Section 7.1.1.2).
Leucocytes	Polymorphonuclear leucocytes	Neutrophils	Gulfs and kills the micro-organisms, however, it also contributes to the allergic inflammation through the potential inflammatory function of variable enzymes released from neutrophils (Monteseirin et al., 2001).
		Eosinophils	Similar as neutrophils. It is a primary pathogenesis of inflammation by producing a number of potent granule constituents (Venarske and deShazo, 2003).
		Basophils	It concerns basophils involved in the late inflammatory reaction, while it has the similar characteristics to the mast cells (Chugh, 1998).
	Mononuclear cells	Monocytes/ macrophages	They ingest and kill pathogens through their highly adherent, motile and phagocytic functions, nevertheless, they are implicated in the chronic inflammation (O'Connor et al., 2004).
Vascular endothelial cells			It releases NO that contributes to vasodilation, chemokines and cytokines during allergic inflammation (Yamamoto and Nagata, 1999, Laberge and El Bassam, 2004).
Platelets			Low-affinity receptors for IgE are found in it, and a number of inflammatory substances (e.g. PAF) are released from platelets, that are believed to contribute to both early and late phases of allergic inflammation (Klinger, 1997).
Neurons			The neuropeptides develops during airway hypersensitivity released from activated neurons, which is termed to “neurogenic inflammation” (Richardson and Vasko, 2002).

Table 7.2 Mediators involved in allergy

Mediators type	Roles in allergic inflammation
Histamine	Mediating allergic reaction, such as urticaria and hay fever (Repka-Ramirez and Baraniuk, 2002). (Details see section 7.1.1.3)
Prostaglandin (PG) PGD_2, PGE_2, PGF_2, PGI_2	PGE_2 plays a predominant role in the acute and chronic inflammation through its vasodilation functions, and is a major mediator of fever (Ushikubi et al., 2000). Both PGI_2 and PGD_2 have effects on vasodilation and inhibition of platelet aggregation (Takahashi et al., 2002, Matsuoka et al., 2000).
Leukotrienes (LT) LTA_4, LTB_4, LTC_4, LTD_4, LTE_4	LTB_4 is an important mediator during allergic inflammatory process, particularly in asthma through its adherence by leukocyte, thereby stimulating the proliferation of cytokines (Hart, 2001). Also LTC_4 and LTD_4 have been found to play roles in inflammation due to their potent bronchoconstrictory actions (Wenzel, 2003).
Platelet-activating factor (PAF)	PAF is an important mediator during the acute and persisting allergic inflammation that include bronchial constriction, vasodilation, erythema etc. (Page, 1990).
Bradykinin (BK)	The pathological functions, including vasodilation, increased vascular permeability, etc., contribute to many allergic and inflammatory phenomenon (Kaplan et al., 2002).
Nitric oxide (NO)	iNOS – induced NO, is considered to be a potent inflammatory mediator through its vasodilation and vascular permeability (Guzik et al., 2003).
Neuropeptides	Substance P and Neurokinin A, two of the main neuropeptides, perform on mast cells to release histamine and other inflammatory mediators (Joos et al., 2003). Moreover, another constituent calcitonin gene-related peptide (CGRP), which is a potent vasodilator, also contribute to the inflammation (Holzer, 1998).
Cytokines Interleukins (IL) chemokines, interferons (IFN), colony-stimulating factors, growth factors and tumour necrosis factors (TNF)	TNF-α and IL-1α are regarded as primary pro-inflammatory cytokines (Bachert et al., 1995). Thus a cascade of numerous cytokines (chemokines) are initiated and defined performing in the inflammatory site (Sindern, 2004). Other cytokines that inhibit the inflammation are the anti-inflammatory cytokines, e.g. IL-10, IL-12 and IFN-γ (Chung, 2001).

7.1.1.2 Mast cells

Mast cells, whose precursor cells originate from bone marrow, are distributed abundantly in the tissues (Galli and Hammel, 1994, Marone et al., 1997). Mast cells have been identified playing a central role in allergic diseases (Marone et al., 1998), such as allergic rhinitis and conjunctivitis, (Parikh et al., 2003), asthma (Robinson, 2004), dermatitis (Leung, 1995), food allergy (Metcalfe, 1984), and anaphylactic shock caused by drug allergy (Orta et al., 2003).

Mast cells are classified into two sub-types, mucosal mast cells and connective tissue mast cells. The mucosal mast cells are mainly located in intestine and lung, while the connective mast cells are distributed in most tissues (Roitt and Delves, 2001). There are some differences between these two types of mast cells. Notably, mucosal mast cells are T-cell dependent, while connective tissue mast cells are T-cell independent (Welle, 1997). Additionally, certain serine proteinases, eg. chymase, were found only in the connective tissue mast cells, but not in mucosal mast cells, although both types of cells contain tryptase (Roitt and Delves, 2001). Nevertheless, the high affinity receptor of IgE are expressed on the surfaces of both types of mast cells (Marone et al., 1997).

Mast cells can be stimulated or degranulated by various pathways, one of the best clarified mechanisms of activation is through the high affinity IgE receptor, FcεRI, cross-linked by IgE and antigen (Galli and Hammel, 1994). Primary exposure to the allergens, such as pollen, drugs, foods, insect products, mould and animal hair, leads to the formation of a pool of specific IgE antibodies, which then bound to the surface of mast cells and its circulating counterpart, basophils (Parikh et al., 2003). After secondary exposure to same allergen, mast cells become activated to trigger degranulation (Reed and Kita, 2004, Parikh et al., 2003), leading to the rapid release of inflammatory mediators (Bradding, 2003, Brightling et al., 2003, Galli and Hammel, 1994), including preformed mediators [e.g. histamine, 5-

hydroxytryptamine (5-HT), protease (tryptase and chymase), and proteoglycans] (Forsythe and Ennis, 1998, Alfaro, 2004), and newly synthesized mediators, such as arachidonic acid metabolites, including PGD_2 and LTs (LTB_4, LTC_4 and LTD_4), and lipid-derived mediator, PAF (Hart, 2001, Henderson, 1991). Moreover, activated mast cells synthesized and secreted various cytokines, including TNF-α, IL-3, IL-4, IL-5, IL-6, IL-8, IL-13, IL-16, IL-18 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Lorentz and Bischoff, 2001, Hart, 2001).

7.1.1.3 Histamine

Histamine is an essential vasoactive amino acid. Histamine [2-(4-imidazolyl ethylamine)], formed by decarboxylation of L-histidine (Weltman, 2000), primarily presents in the mast cells granules and basophils, and binds to the carboxyl sulphate groups of the cytoplasmic secretory granules (Graff et al., 2002) (Figure 7.1). The content of histamine in human mast cells is 3pg/cell, and that in basophil is 1pg/cell (Marone et al., 2003).

Histamine involves in a number of allergic diseases, including seasonal allergic rhinitis (hay fever), asthma, food or drug allergy and dermatitis (Storms, 2004, Turner et al., 2005, Mittag et al., 2004, Thong and Yeow, 2004, Ikoma et al., 2005). Histamine also exists ingastric enterochromaffin-like (ECL) cells, as a regulator of stomach acid production (Qvigstad and Waldum, 2004). Histamine is also considered as a neurotransmitter in histaminergic neurons in brain (Watanabe and Yanai, 2001). Its pharmacological actions include vasodilatation, smooth muscle contraction, mucus hypersecretion and increased vascular permeability (MacGlashan, 2003), which are likely to mediate some inflammatory responses, eg. nasal effects (sneezing, swelling, itching and congestion), skin sensations (itching, burning and flushing), contraction of bronchial, stomach pain (acid secretion increases, small intestine contraction and pain) and headache (Roitt and Delves, 2001). Histamine acts via histamine

receptors, which have four subtypes (Table 7.3). The subtype involved in allergy is mainly H1 receptor (Akdis and Blaser, 2003, MacGlashan, 2003).

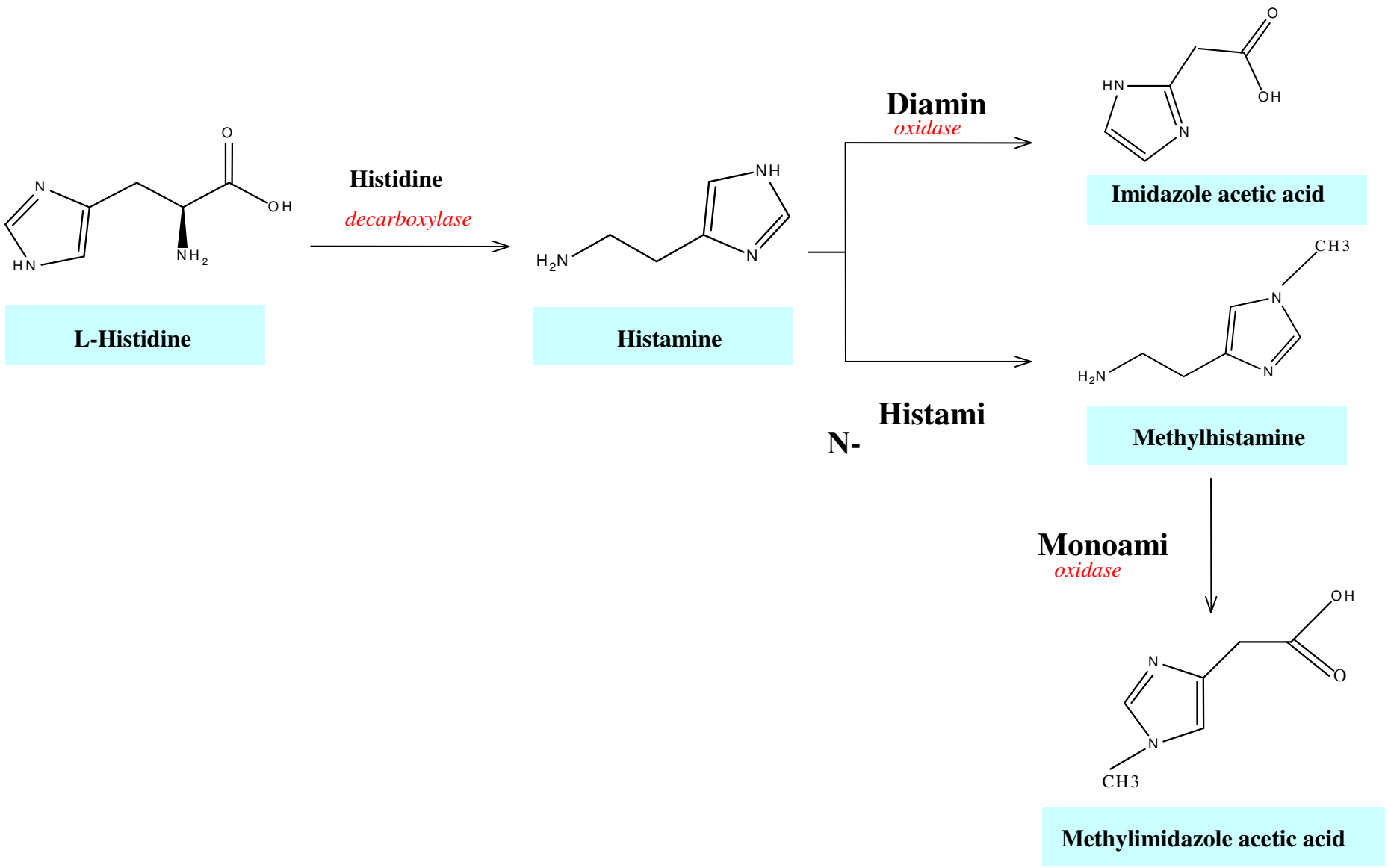


Figure 7.1 The biosynthesis and metabolism of histamine

Table 7.3 Classification of histamine receptors and their functions

Classification	Functions	Reference
H1 receptor	• Vasodilation • Bronchial smooth muscle contraction • Mucous secretion • Increased vascular permeability • Pruritus (bronchial obstruction) • Wheal and flares in urticaria	(MacGlashan, 2003, Akdis and Blaser, 2003)
H2 receptor	• Vasodilation • Elevation cAMP • Gastric acid secretion • Inhibition of chemotaxis in eosinophils • Inhibition of chemotaxis and activation of neutrophils • Inhibition of superoxide development • Inhibition degranulation	(Akdis and Blaser, 2003)
H3 receptor	• Modulation of neurotransmitters in the central / peripheral nervous system • Regulation of histamine synthesis and release • Increased gastric mucous production	(Bertaccini et al., 1991)
H4 receptor	• Meditation of histamine signalling and chemotaxis of mast cells • New member of the family, and the pharmacological functions have not been identified clearly.	(Nguyen et al., 2001)

7.1.2 Actions of Flos Magnoliae on mast cell-dependent allergy

The anti-allergic and anti-inflammatory actions of Flos Magnoliae have been demonstrated in various *in vivo* and *in vitro* studies (Kim et al., 2003, Kim et al., 1999, Kimura et al., 1992, Kobayashi et al., 1998). For example, *M. biondii* inhibited the capillary permeability of blood vessels (Kim et al., 1999, Kimura et al., 1990, Kimura et al., 1991), and secretions of the inflammatory and allergic mediators, including histamine, PGE_2, PAF, NO, and pro-inflammatory cytokines IL-1α and TNF-α (Lim et al., 2002, Wang et al., 2000b). Among these, the inhibition of mast cells derived histamine release by Flos Magnoliae was considered as the main mechanism of its anti-allergic reactions (Li and Zhang, 2002). Furthermore, some active components of Flos Magnoliae, such as magnolin and fargesin have been suggested that they may have effects on the anti-allergic actions (Ma and Han, 1995, Brown et al., 2001, Mitsuo, 2001). However, still little studies have been done in terms of differences of the anti-histamine release effects by different Flos Magnoliae species.

7.1.3 Aims of this study

The aim of this study was to investigate and compare the effect of six commonly used Flos Magnoliae species, namely, *M. biondii, M. denudata, M. sprengeri, M. kobus*, *M. liliflora* and *M. sargentiana,* and 11 *M. biondii* batches from six varieties on compound 48/80 induced histamine release in RPMC. In addition, the effects of five Flos Magnoliae products, four *M. biondii* batches and three *M. sprengeri* batches, collected from different cultivation sites, as well as Flos Magnoliae volatile oil and the active lignans, magnolin and fargesin, on mast cell derived histamine release in RPMC were determined.

7.2 Method

7.2.1 Plant materials and herbal products

See Chapter 2 (Section 2.2.3). Same plant materials as in qualitative and quantitative studies (in Chapter 5-7) were used in this study. Furthermore, the essential oil of Flos Magnoliae and two isolated chemical components, magnolin and fargesin, were used.

7.2.2 Reagents and chemicals

The details of the reagents and chemicals are described in Section 2.3.4.

7.2.3 Samples extraction and preparation

Samples were extracted using an Accelerated Solvent Extraction machine (ASE-100, Dionex, U.S.A.). The details of the extraction and preparation procedure are described in Section 2.8.1. The ethanol extract was filtered with PTFE syringe filters (25mm, 0.45μm) (Alltech Associates, Australia) and stored at -20°C for further analysis.

7.2.4 Animals

Male SD rats at 10 – 12 weeks of age (250 – 350 g) were used for experiments (see Section 2.8.2).

7.2.5 Isolation and preparation of RPMC

Briefly, after injection of the Tyrode's Buffer containing 0.3% BSA and 5.0 units/ml heparin sodium, the peritoneal solution was collected. After being washed by the Tyrode's Buffer for three times, the RPMC were resuspended in the Tyrode's Buffer, which contained 0.1% BSA (see Section 2.8.3).

7.2.6 Effects of herbal extracts on compound 48/80 induced histamine release in RPMC

After being pre-incubated at 37°C for 10min, different concentration of herbal extracts was added, followed by 10min incubation, then 0.5μg/ml compound 48/80 was added. After stopping the histamine release in RPMC, spermidine was added as an internal standard for further analysis (see Section 2.8.4).

7.2.7 HPLC system for determination of compound 48/80 induced histamine release in RPMC

A Shimadzu HPLC instrument with a post-derivatization system using a fluorescent detector was employed for the present study. For details, see Section 2.8.5.

7.2.8 Data analysis and statistics

The details of the data analysis and statistics are described in Section 2.9.5. Data were represented as mean ± S.E.M.

7.3 Results

7.3.1 Effects of compound 48/80 on histamine release in RPMC

Unstimulated RPMCs in Tyrode's buffer had a basal level of histamine at 32.4±2.4ng/ml (n=6). Compound 48/80 (0.0001–0.5μg/ml) caused a concentration-dependent increase of histamine release in RPMC, with the maximum response at 0.5μg/ml being 529.1±67.6ng/ml (n=7). Consequently, 0.5μg/ml of compound 48/80 was used for the subsequent experiments (Figure 7.2). The vehicle control (equal volume of absolute ethanol) slightly inhibited the compound 48/80 induced histamine release (9.5±1.2%, n=6, $P>0.05$, compared with compound 48/80 alone group, T-test). The increase in histamine release by compound 48/80 was significantly inhibited by 100μM EDTA (35.3±1.1%, n=7, $P<0.01$, compared with compound 48/80 alone group, T-test) (Figure 7.3). The 30% $HClO_4$ caused the total release of histamine in RPMC, being 566.5±85.8ng/ml.

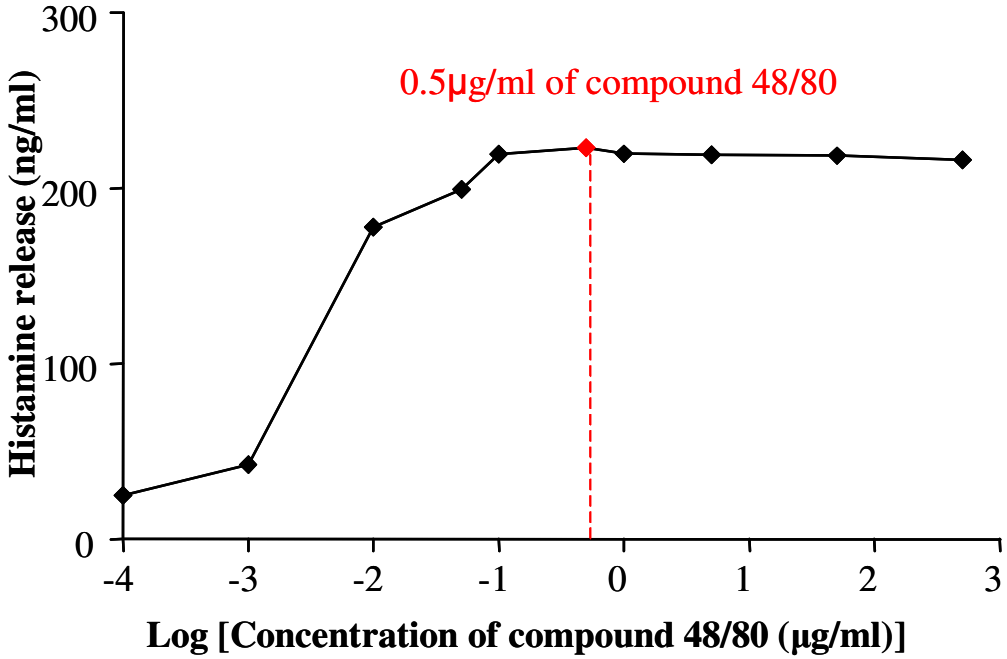


Figure 7.2 Dose-response curve of compound 48/80 induced histamine release in RPMC

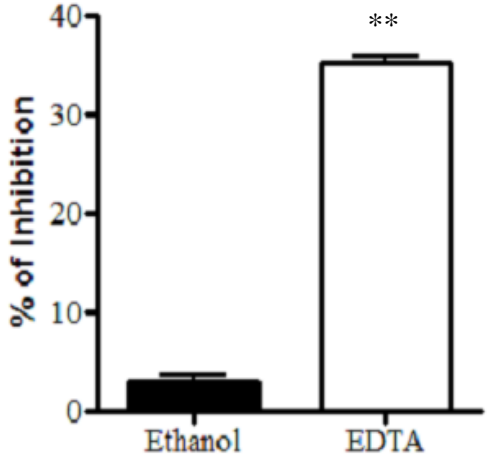


Figure 7.3 The effects of 100µM EDTA on compound 48/80 induced histamine release in RPMC. Each value represents the mean ± S.E.M. of 8 preparations. **P<0.01, t-test.

7.3.2 Effects of different species of Flos Magnoliae on compound 48/80 induced histamine release in RPMC

The typical HPLC chromatograms, which present the baseline, control vehicle (ethanol), total release (30% $HClO_4$), positive control (100μM EDTA), showing the effects of Flos Magnoliae extracts on compound 48/80 (0.5μg/ml) induced histamine release in RPMC, are illustrated in Figure 7.4. The retention time of histamine is 3.5min of the whole running time (8min), while the internal standard (spermidine) was detected at 6.0min.

Four Flos Magnoliae species, *M. biondii* (HL), *M. denudata, M. kobus* and *M. sprengeri* (MD) showed a concentration-dependent (0.1–0.01mg/ml) inhibition of histamine release. However, at a higher concentration (0.5mg/ml) the effect was partly reduced. Other two Flos Magnoliae species, *M. liliflora* and *M. sargentiana*, showed a concentration-dependent (0.5–0.01mg/ml) inhibitory effect. All six Flos Magnoliae species significantly ($P<0.05$, one-way ANOVA followed by the Tukey's test) inhibited the histamine release at the concentration of 0.05μg/ml or above. At the lowest concentration tested, no significant ($P>0.05$, one-way ANOVA followed by the Tukey's test) effects were observed for *M. sargentiana* and *M. sprengeri* (MD).

The comparison of the inhibitory effects of six Flos Magnoliae species was shown in Figure 7.5 (A-D). At the highest concentration (0.5mg/ml), *M. liliflora* has the most potent inhibitory effects on histamine release (61.1±1.0%), which significantly ($P<0.05$, one-way ANOVA followed by the Tukey's test) higher than the anti-histamine release effects of *M. biondii, M. denudata, M. kobus* and *M. sargentiana*. At the concentrations from 0.1–0.01mg/ml, *M. biondii* and *M. kobus* showed significantly ($P<0.05$, one-way ANOVA followed by the Tukey's test) higher inhibition, than *M. sargentiana* and *M. sprengeri*. On the other hand, *M. sargentiana* was the least potent among six Flos Magnoliae species tested at a given

concentration (0.1 – 0.01mg/ml), although at high concentration (0.5mg/ml), it significantly inhibited the histamine release up to 47.7±1.0%.

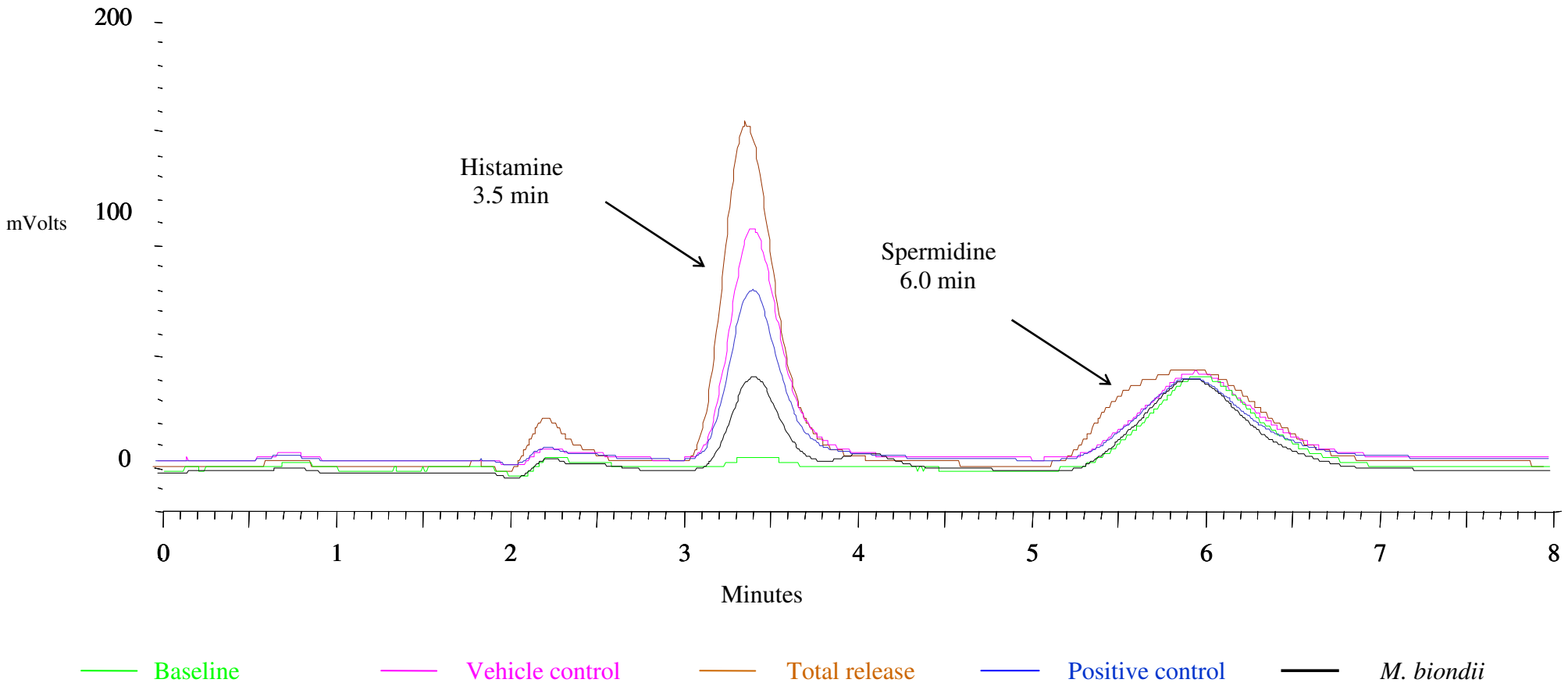


Figure 7.4 The representative HPLC chromatograms of compound 48/80 (0.5µg/ml) induced histamine release in RPMC. The chromatograms characterised baseline, vehicle control (ethanol), total release (30% $HClO_4$) positive control (100µM EDTA) and the ethanol extracts of *M. biondii* (0.1mg/ml) sample on histamine release.

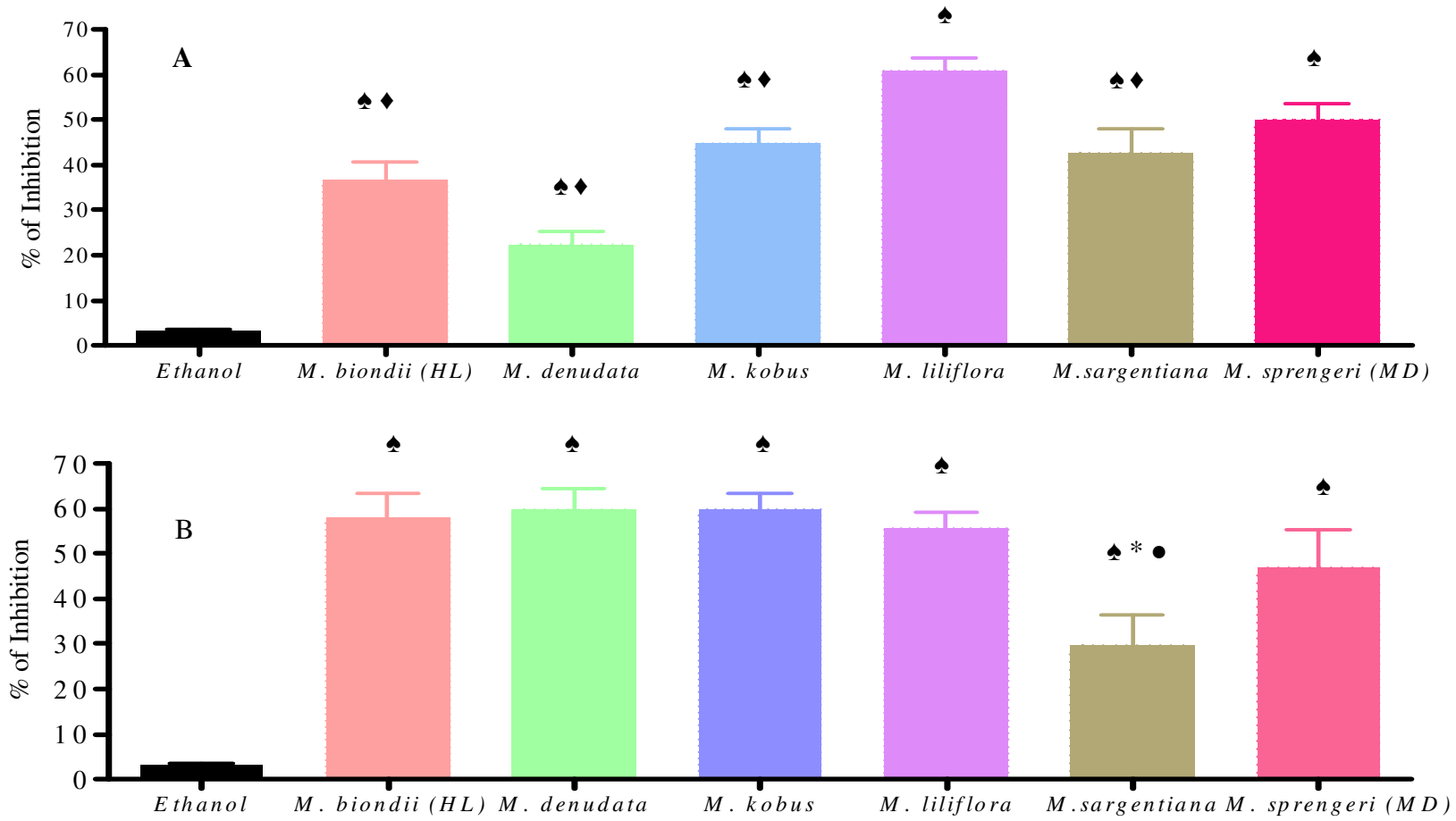
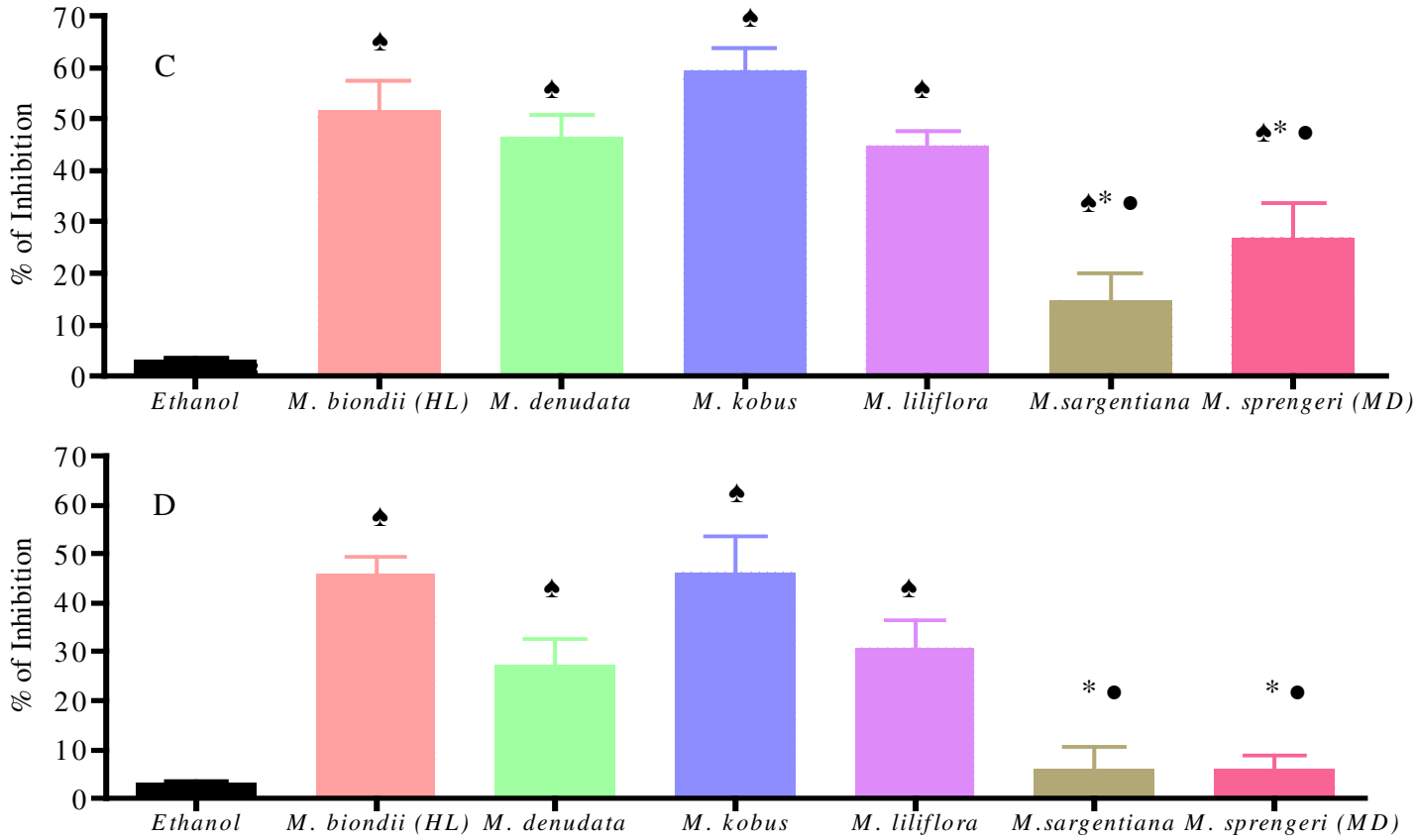


Figure 7.5 The effects of ethanol extracts of six Flos Magnoliae species on compound 48/80 induced histamine release in RPMC. The concentrations of the herbal extract included 0.5mg/ml (A), 0.1mg/ml (B), 0.05mg/ml (C), and 0.01mg/ml (D). ♠ significantly different from vehicle control (ethanol), * significantly different from *M. biondii*, ● significantly different from *M. kobus*, ♦ significantly different from *M. liliflora.* ♠ *, ●, ♦ $P<0.05$, one-way ANOVA, followed by a Tukey's test. Each value represents the means ± S.E.M. of 5-6 preparations.



Continued Figure 7.5 The effects of ethanol extracts of six Flos Magnoliae species on compound 48/80 induced histamine release in RPMC. The concentrations of the herbal extract included 0.5mg/ml (A), 0.1mg/ml (B), 0.05mg/ml (C), and 0.01mg/ml (D). ♠ significantly different from vehicle control (ethanol), * significantly different from *M. biondii*, ● significantly different from *M. kobus*, ♦ significantly different from *M. liliflora.* ♠ *, ●, ♦ P<0.05, one-way ANOVA, followed by a Tukey's test. Each value represents the means ± S.E.M. of 5-6 preparations.

7.3.3 Effects of different varieties of *M. biondii* on compound 48/80 induced histamine release in RPMC

Eleven *M. biondii* batches from six varieties (details see Table 2.3) showed a concentration-dependent (0.1–0.01mg/ml) inhibition on histamine release. All 11 *M. biondii* batches significantly ($P<0.05$, one-way ANOVA followed by the Tukey's test) inhibited the histamine release at the concentration of 0.1mg/ml and 0.05mg/ml. At the lowest concentration 0.01mg/ml, Sample 7, 8 (CY, 串鱼) and Sample 10 (HLWH, 华龙五号) have no significant effects.

The comparisons of the inhibitory effects of these *M. biondii* samples are illustrated in Figure 7.6 (A – C). At the highest concentration (0.1mg/ml) tested, Sample 11 (BZCY, 标准串鱼) had the most potent inhibitory effect on histamine release (39.3±1.9%). Sample 7 (CY, 串鱼) was the least potent (19.3±0.9%), being significantly ($P<0.05$, one-way ANOVA followed by the Tukey's test) weaker than the anti-histamine release effects of Sample 1 (HLWH, 华龙五号), Sample 2 (EMT, 二毛桃), Sample 5 (HGCY, 黄梗串鱼) and Sample 11 (BZCY, 标准串鱼). At the concentrations of 0.05mg/ml, Sample 11 (BZCY, 标准串鱼) had the most potent inhibitory effect (27.7±1.2%), while Sample 7 (CY, 串鱼) was the least potent (15.4±0.5%). At the lowest concentration (0.01mg/ml) tested, Sample 5 (HGCY, 黄梗串鱼) was the most potent inhibitor (21.9±2.7%), while Sample 8 (CY, 串鱼) was the least potent (7.3±1.7%). However, at the concentrations of 0.05mg/ml and 0.01mg/ml, no significant differences of the anti-histamine release effects were found among 11 *M. biondii* batches.

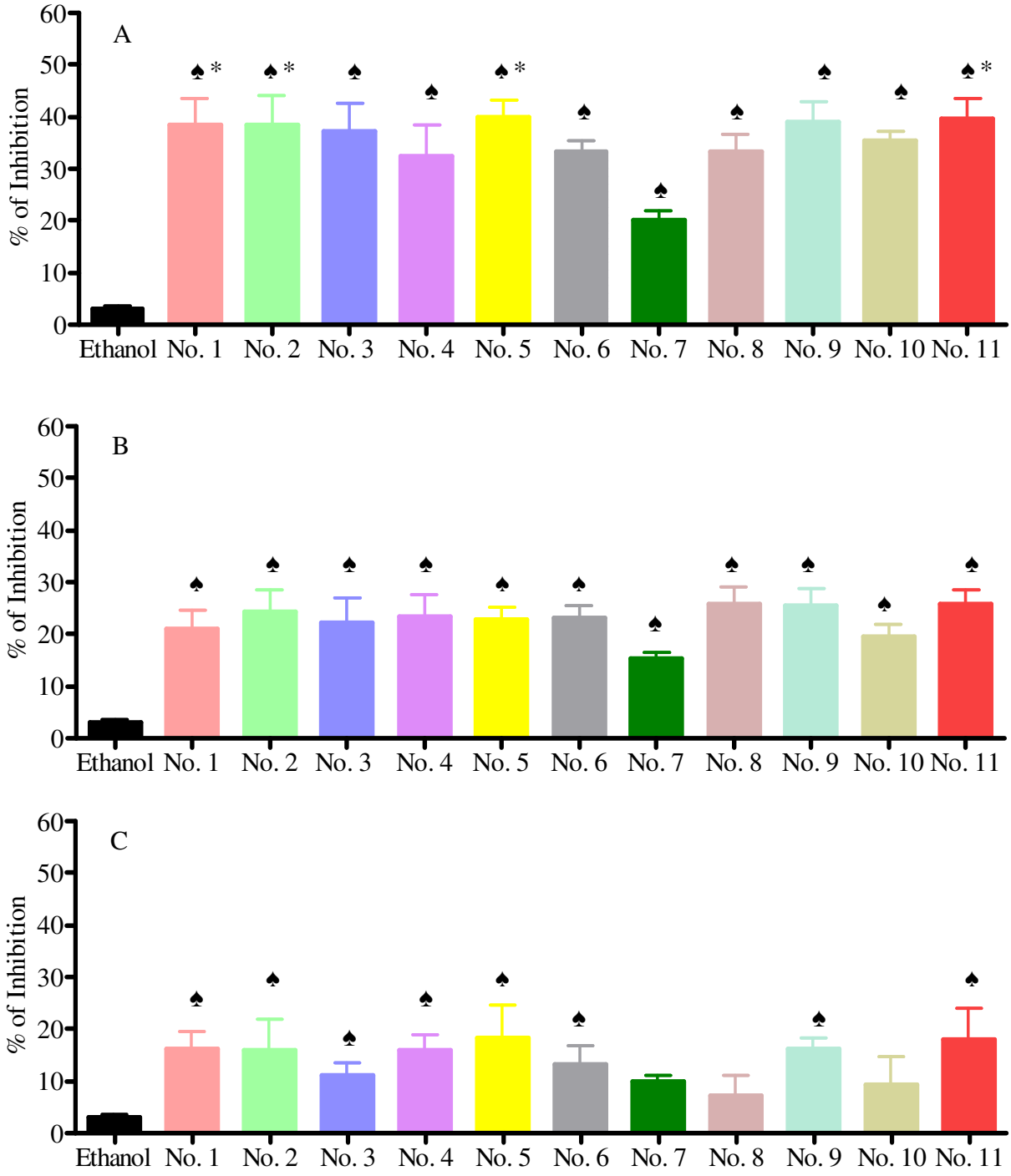


Figure 7.6 The effects of ethanol extracts of 11 *M. biondii* batches on compound 48/80 induced histamine release in RPMC. The concentrations of the herbal extract included 0.1mg/ml (A), 0.05mg/ml (B), and 0.01mg/ml (C). ♠ significantly different from vehicle control (ethanol), * significantly different from Sample 7 (CY, 串鱼). ♠, * P<0.05, one-way ANOVA, followed by a Tukey's test. Each value represents the means ± S.E.M. of 5-6 preparations

7.3.4 Effects of different Flos Magnoliae products from five herbal suppliers on compound 48/80 induced histamine release in RPMC

Five Flos Magnoliae products containing *M. biondii*, were provided by five different herbal suppliers in China or Australia (details see Table 2.4). Five Flos Magnoliae products showed concentration-dependent inhibitory effects on histamine release with the concentration range from 0.1 – 0.01mg/ml. All batches significantly ($P<0.05$, one-way ANOVA followed by the Tukey's test) inhibited the compound 48/80 induced histamine release in RPMC at the concentrations of 0.5mg/ml and 0.1mg/ml. No significant ($P>0.05$, one-way ANOVA followed by the Tukey's test) anti-histamine release effects were found from the products from Tung Fong Hung Medicine Co. Ltd and KODA International at the lowest concentration (0.01mg/ml).

The comparison of the inhibitory effects of five Flos Magnoliae products on histamine release induced by compound 48/80 in RPMC are illustrated in Figure 7.7 (A-D). At the highest tested concentration (0.5mg/ml), sample from Tsang Fook Kee Medicine Co. had the most potent inhibitory effects (42.7±2.2%) on histamine release, which was significantly ($P<0.05$, one-way ANOVA followed by the Tukey's test) higher than the anti-histamine release effects of Tung Fong Hung Medicine Co. Ltd. The sample has the lowest anti-histamine release effect (14.7±5.3%). At concentrations 0.1mg/ml to 0.01mg/ml, the sample, provided by Hualong Magnolia Development Co. Ltd., had the highest anti-histamine release effects. In contrast, at the lowest concentrations of 0.1mg/ml and 0.05mg/ml, the sample from KODA International has the lowest anti-histamine effects (12.2±1.1% and 6.3±0.9%, respectively) among all five batches. This product had significantly weaker anti-histamine release effects than the effects of the samples from Hualong Magnolia Development Co. Ltd., Tung Fong Hung Medicine Co. Ltd. and Tsang Fook Kee Medicine Co. At the lowest concentration (0.01mg/ml), the sample from Hualong Magnolia Development Co. Ltd. has the most potent

inhibitory effects on histamine release (46.1±1.3%), which significantly ($P<0.05$, one-way ANOVA followed by the Tukey's test) higher than other four Flos Magnoliae products.

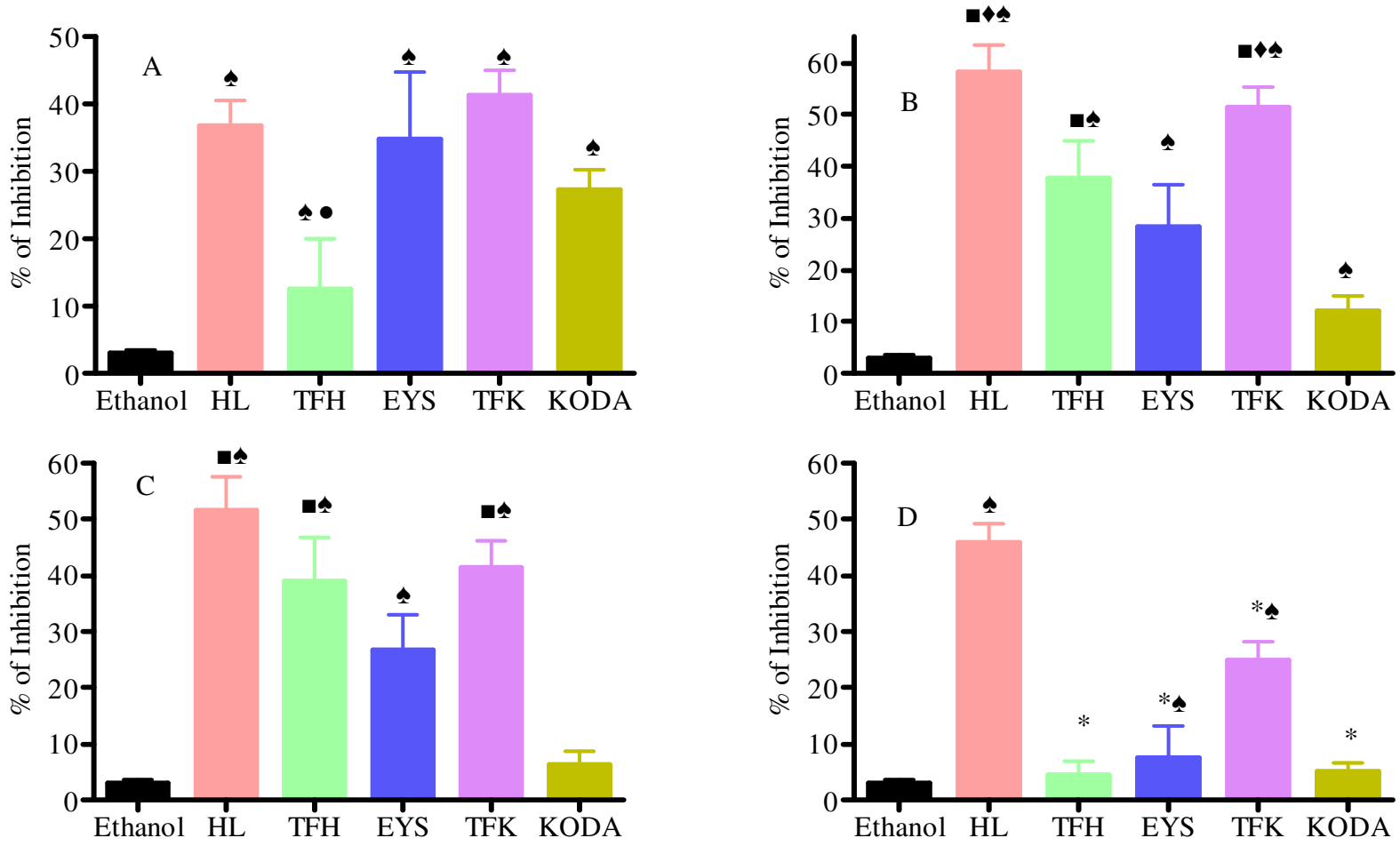


Figure 7.7 The effects of ethanol extracts of five Flos Magnoliae products on compound 48/80 induced histamine release in RPMC. The concentrations of the herbal extract included 0.5mg/ml (A), 0.1mg/ml (B), 0.05mg/ml (C), and 0.01mg/ml (D). ♠ significantly different from vehicle control (ethanol), * significantly different from HL, ● significantly different from TFK, significantly different from EYS, ■ significantly different from KODA. ♠, *, ●, ♦, ■ P<0.05, one-way ANOVA, followed by a Tukey's test. Each value represents the means ± S.E.M. of 5-6 preparations

7.3.5 Effects of different *M. biondii* and *M. sprengeri* samples, from different cultivation sites, on compound 48/80 induced histamine release in RPMC

Four *M. biondii* batches, grown in different cultivation sites in Nanzhao, Henan Province, China, were included in the present study (details see Table 2.5). At the highest concentration (0.1µg/ml) tested, the batch from Tian Bridge (TQ) had the highest anti-histamine release effect (40.2±2.2%), while the sample Eastern Garden (DHY) has the lowest anti-histamine release effects (32.6±1.0%). At the concentration 0.05mg/ml and 0.01mg/ml, the sample Eastern Garden (DHY) and Tian Bridge (TQ) had the lowest anti-histamine release effects among four samples, respectively. In contrast, the sample Mt. Yanyi (YYS) had the highest anti-histamine release effect. However, no significant difference of the anti-histamine release effects were determined among these four *M. biondii* samples (Figure 7.8).

Three *M. sprengeri* batches, grown in different cultivation sites in China and Australia, were included in the present study (details see Table 2.6). At the tested concentrations of 0.1mg/ml and 0.05mg/ml, the batch JY cultivated from Jiangyou, Sichuan Province, China, had the highest anti-histamine release effects. At the highest tested concentration of 0.1mg/ml, another Chinese sample, MB, which was cultivated in Mabian, Sichuan Province, China, had the lowest anti-histamine release effects (44.3±3.4%). At the tested concentrations 0.05mg/ml and 0.01mg/ml, the sample, MD, cultivated in Mt. Dandenong, Victoria, Australia, had the lowest anti-histamine release effects. However, no significant difference of the anti-histamine release effects was found among three *M. sprengeri* batches (Figure 7.9).

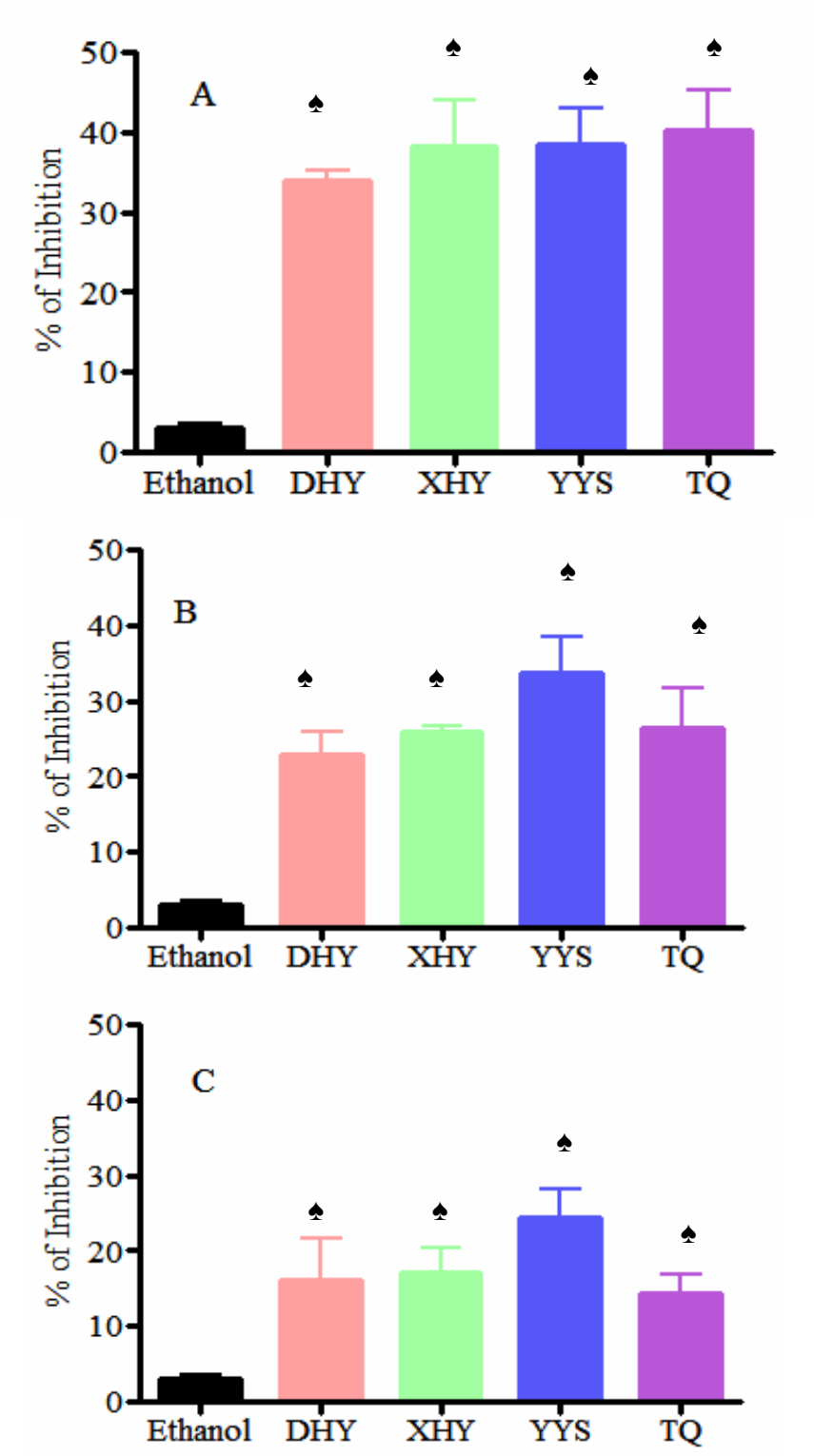


Figure 7.8 The effects of ethanol extracts of four *M. biondii* batches, collected from different cultivation sites, on compound 48/80 induced histamine release in RPMC. The concentrations of the herbal extract included 0.1mg/ml (A), 0.05mg/ml (B), and 0.01mg/ml (C). ♠ significantly different from vehicle control (ethanol). ♠, $P<0.05$, one-way ANOVA, followed by a Tukey's test. Each value represents the means ± S.E.M. of 5-6 preparations

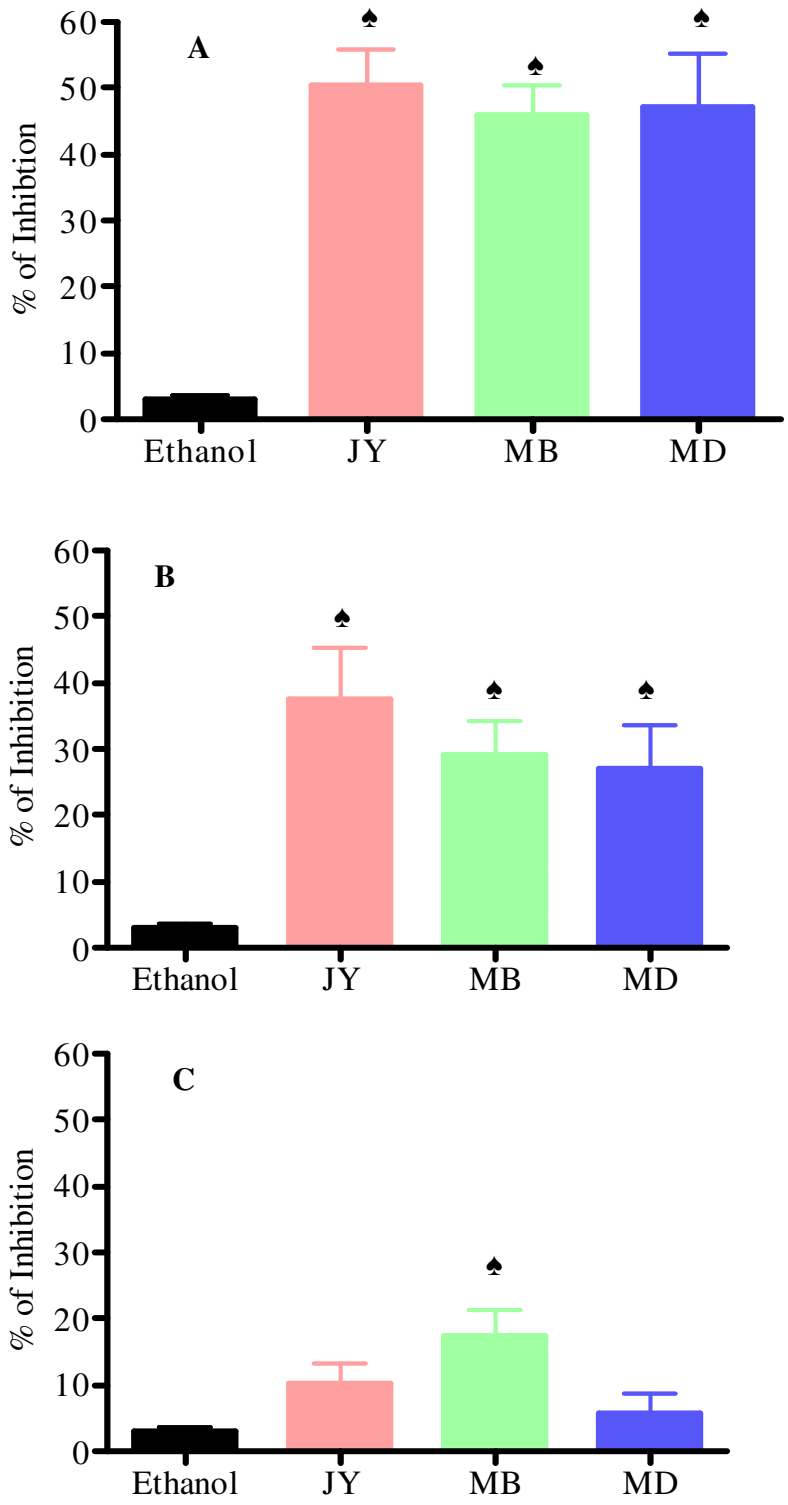


Figure 7.9 The effects of ethanol extracts of three *M. sprengeri* batches, collected from different cultivation sites, on compound 48/80 induced histamine release in RPMC. The concentrations of the herbal extract included 0.1mg/ml (A), 0.05mg/ml (B), and 0.01mg/ml (C). ♠ significantly different from vehicle control (ethanol). ♠, $P<0.05$, one-way ANOVA, followed by a Tukey's test. Each value represents the means ± S.E.M. of 5-6 preparations.

7.3.6 Effects of volatile oil from *M. biondii* on compound 48/80 induced histamine release in RPMC

The effect of a volatile oil from *M. biondii,* provided by Hualong Magnolia Development Co. Ltd. was studied of five different concentrations, ranged from 10% to 0.001%. At higher tested concentrations (10% and 1%), it had no significant effect on histamine release, while at the lower tested concentrations (0.1 – 0.001%), it showed a significant ($P<0.05$, one-way ANOVA followed by the Tukey's test) concentration-dependent inhibitory effect (Figure 7.10).

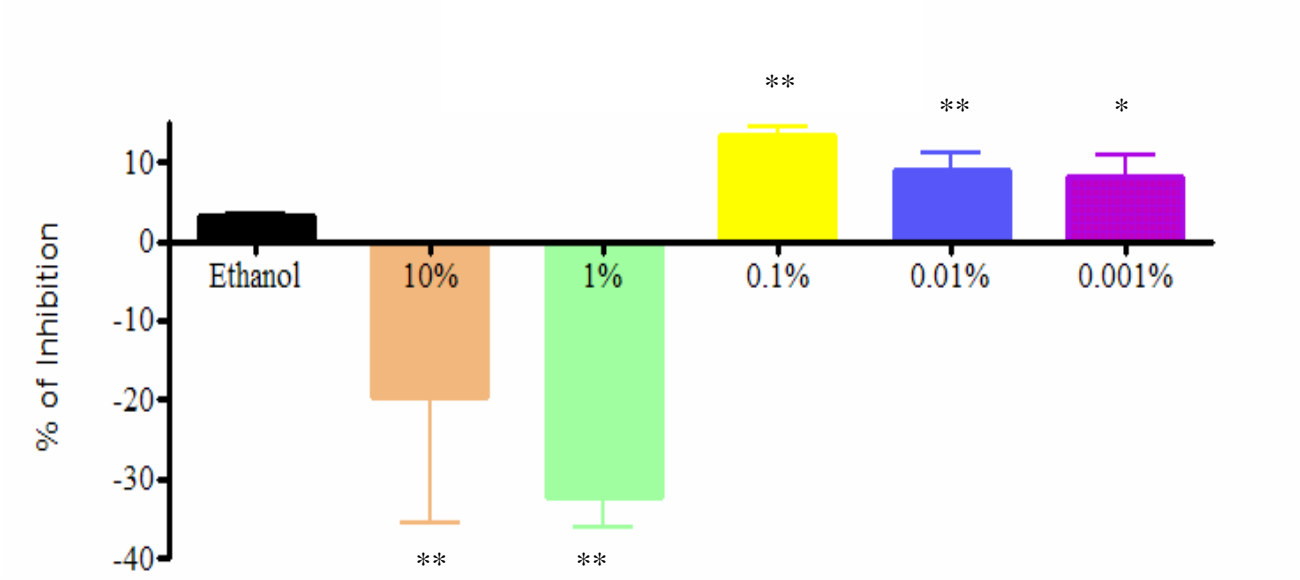


Figure 7.10 The effects of volatile oil from *M. biondii* on compound 48/80 induced histamine release in RPMC. *, $P<0.05$, and **, $P<0.01$, one-way ANOVA, followed by a Tukey's test. Each value represents the means ± S.E.M. of 7-8 preparations.

7.3.7 Effects of magnolin and fargesin on compound 48/80 induced histamine release in RPMC

Magnolin showed a concentration-dependent (1 – 0.1μg/ml) inhibition on histamine release. However, at the highest concentration (5μg/ml) the effect was partly reduced (Figure 7.11). In contrast, fargesin (5 – 0.1μg/ml) had no significant effect on histamine release (Figure 7.11).

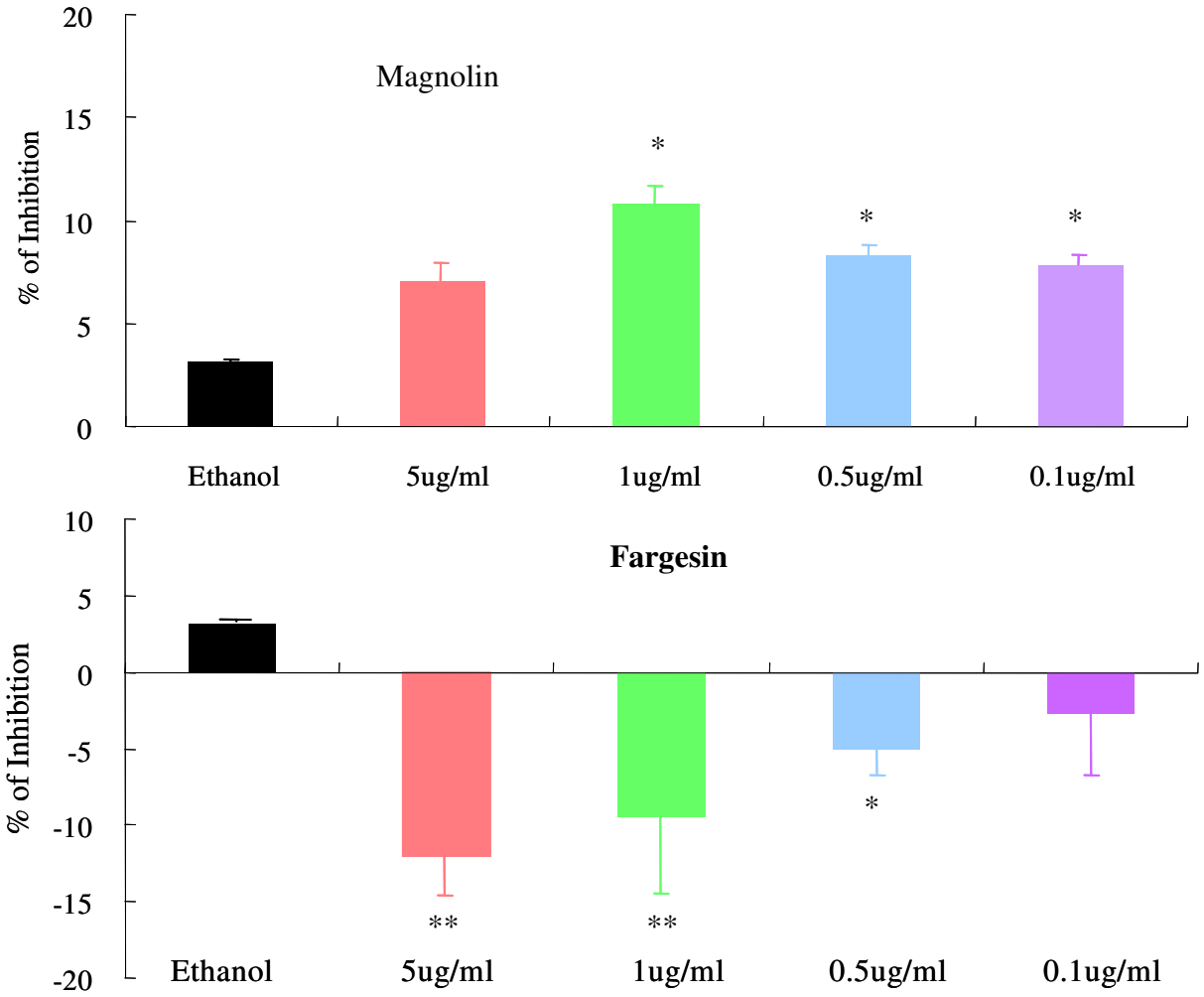


Figure 7.11 The effects of magnolin and fargesin on compound 48/80 induced histamine release in RPMC. *, P<0.01, one-way ANOVA, followed by a Tukey's test. Each value represents the means ± S.E.M. of 7-8 preparations.

7.4 Discussion

To our best knowledge, this is the first report to evaluate the anti-allergic actions of different Flos Magnoliae species and varieties on mast cell-derived histamine release in RPMC. The present study compared anti-histamine release effects of six Flos Magnoliae species, 11 *M. biondii* batches from six varieties, five Flos Magnoliae products, as well as four *M. biondii* samples and three *M. sprengeri* samples, collected from different cultivation sites, using a HPLC post-derivatization method. Furthermore, the anti-histamine release effects of the volatile oil and lignans, including magnolin and fargesin, were studied. The major finding is that different Flos Magnoliae species had the anti-histamine release effects in RPMC, which is in agreement with previous studies on the inhibition of mast cells derived histamine release (Li and Zhang, 2002)

Compound 48/80 was used in this study as a histamine releaser. Compound 48/80 selectively targeted connective tissue mast cells, such as peritoneal mast cells, by increasing intracellular Ca^{2+} concentrations, hence inducing the release of histamine (Chakravarty and Yu, 1986, Marshall et al., 1994). It has been reported that the stimulation of immunity-independent histamine release from mast cells by compound 48/80 involved phospholipase C activation, and changes of GTPase activity and Ca^{2+} recruitment through guanine nucleotide-binding regulatory protein (Wu et al., 1993, Barrocas et al., 1999, Koibuchi et al., 1985). It is a calmodulin antagonists (Gietzen et al., 1983). It was also reported that compound 48/80 increased permeability of the lipid bilayer membrane of mast cells by causing the perturbation, as an crucial trigger for the release of mediators from activated mast cells (Tasaka et al., 1986). Compound 48/80 has been employed widely as a histamine releaser and degranulators for a number of *in vivo* or *in vitro* studies in rats or mice (Yuzurihara et al., 2000a, Yuzurihara et al., 2000b, Ikarashi et al., 2001b, Kim et al., 1999).

In the present study, Flos Magnoliae inhibited the compound 48/80 induced histamine release in mast cells, possibly by acting on the intracellular calcium channel or the lipid bilayer membrane, which may affect the prevention of the perturbation being induced by compound 48/80 and regulate the degranulation of the mast cells.

Two Flos Magnoliae species, *M. liliflora* and *M. sargentiana,* showed a concentration-dependent inhibitory effect between 0.01 – 0.5mg/ml, while other four samples, *M. biondii* (HL), *M. denudata, M. kobus* and *M. sprengeri* (MD) had a concentration-dependent effect between 0.01-0.1mg/ml, but at a higher concentration (0.5mg/ml), the effects of these herbal extracts were partly reduced. It is not clear if additional ingredient(s), which may exist in higher concentration Flos Magnoliae extract, is involved in these effects, or some active ingredients of Flos Magnoliae extract may have multiple actions on RPMC function. Nevertheless, the present findings indicated for certain Flos Magnoliae species the best anti-mast cell-derived histamine release effect may be achieved at lower concentrations. Further study is necessary to reveal the relationships between the potency of anti-histamine release and composition of chemical components of different Flos Magnoliae species. Furthermore, this study demonstrated different potencies among six Flos Magnoliae species. For example, *M. kobus* and *M. biondii* seem to be more potent than *M. sargentiana, M. denudata, M. sprengeri* and *M. liliflora* against the mast cell-derived histamine release at lower concentrations. Although the present findings may not directly apply to a clinical condition, but if such differences also exist in action of these herbas in human, the potential changes of anti-allergic actions by different substitutes or adulterants of Flos Magnoliae species may occur. Thus, there may be implications for interchangeable use of different Flos Magnoliae species for the same conditions.

In addition, *M. biondii* from Nanzhao area has been recognised as the authenticated species, which is the most commonly used Flos Magnoliae in China (HuaLong, 2003). All 11 batches tested, had a similar dose-dependent anti-histamine release effect. Only at the highest tested concentration (0.1mg/ml), the anti-histamine release effects of Sample 7 (CY, 串鱼) showed a significantly lower effect than other *M. biondii* batches. Therefore, the present findings indicate *M. biondii* varieties tested may have similar pharmacological actions and chemical compositions, as shown in previous chapters.

As described in Chapter 1, a number of factors, including manufacturing process, may affect the CHM quality. Thus, five Flos Magnoliae products from different herbal suppliers were tested in the present study. The significant differences of the anti-histamine release effects were found among five batches, indicating the importance of quality control of manufacturer products. Although the reasons of this difference is not known, it is likely to involve the different use of herbal sources and manufacturing processes as described previously (Chapter 1, Section 1.2.2).

An interesting finding from the present study is the observation that *M. biondii* and *M. sprengeri,* cultivated in different locations in China or Australia, showed similar inhibitory effects on histamine release. These finding may provide a useful example of comparable pharmacological actions of same herbs grown at different locations. However, cautions should be taken when apply this to other Flos Magnoliae species, since the environmental conditions for CHM cultivation may affect the quality of CHM (Bensky and Gamble, 1993, Yu et al., 1999).

The anti-histamine release effect of Flos Magnoliae is likely to be caused by component(s) existed in Flos Magnoliae. Volatile oil and lignans have been recognised as the active

components for anti-allergic reactions in Flos Magnoliae (Yang and Zhang, 1998). The present findings indicated that the volatile oil from Flos Magnoliae has the significant anti-histamine release effects at the lower concentration ranges (0.1 – 0.001%). However, increase of histamine release in RPMC was found from the higher tested concentrations (10 – 1%) of volatile oil. The reason for this is not clear, but it may be due to the complex nature of volatile oil, containing ingredients which either antagonist or having an opposite effects of other active compositions. Previous studies showed that Flos Magnoliae contains about 1.0 % - 4.5 % of volatile oil (Chen et al., 1994a, Wang et al., 2000a). The yield of volatile oil may vary with the species, growing locations and cultivation seasons (Hu and Wu, 1995). Analysis of the quantity of volatile oil in *M. biondii, M. sprengeri*, *M. denudate* and *M. liliflora,* by GC-MS have found that *M. denudate* contained 4.5% of volatile oil, whereas that of M. *liliflora* was least (1.2%). However, it is not clear about the exact nature of the active component(s) involved in volatile oil in these species, for example *M. biondii, M. denudata* and *M. sprengeri,* have been shown to contain 91, 71 and 88 volatile components, respectively (Hu and Wu, 1995, Yang et al., 1998). It is possible multiple ingredients may be involved in the anti-histamine release effects of Flos Magnoliae. Other major components in Flos Magnoliae species include lignans, which are regarded having Ca^{2+} antagonist activities (Mitsuo, 2001, Mitsuo et al., 1992b). Given compound 48/80 may target RPMC by increasing intracellular Ca^{2+} concentrations hence inducing the release of histamine (Chakravarty and Yu, 1986, Marshall et al., 1994). In the present study, magnolin showed a concentration-dependent effect between 1 – 0.1μg/ml, but at a higher concentration (5μg/ml), the effect were partly reduced. However, no significant anti-histamine release effect of fargesin in RPMC was not found. This finding support to use magnolin, but not fargesin as bioactive marker for anti-histamine release effects of Flos Magnoliae. Present findings are in line with the previous findings that the lignans derived from Flos Magnoliae may contribute to the regulation of mast cells degranulation (Ma and Han, 1995, Brown et al., 2001, Mitsuo, 2001). It would be

interesting to investigate the like of magnolin with other known pharmacological actions of Flos Magnoliae. For instances, recent studies reported the inhibitory effects of Flos Magnoliae on PGE_2 and pro-inflammatory cytokines IL-1α and TNF-α (Wang et al., 2000c). Therefore, the anti-histamine release effects demonstrated in the present study, together with the inhibitory effects of Flos Magnoliae on other allergic mediators, may help to elucidate the mechanism of anti-allergic actions of Flos Magnoliae.

Quality of herbal materials is critical for ensure the efficacy and safety of herbal products. It is known that the herb quality is affected by many factors, including grown conditions, processing and preparation procedures, even the storage conditions. Thus, different Flos Magnoliae, even the same species from different cultivations and post-harvesting backgrounds may possess different biological actions. There is evidence in the literature regarding the changes of active ingredients in the herbal species(Choi et al., 2002). It would be interesting to further investigate the correlations between the biological actions and the chemical compositions of Flos Magnoliae. Although differences of the pharmacological actions between the commonly used Flos Magnoliae species and varieties are yet to be well studied, the present study certainly provides some foundation work contributing to the quality control of Flos Magnoliae products.

In conclusion, all six species of Flos Magnoliae and 11 *M. biondii* batches have a similar but different potency in inhibition of compound 48/80 induced histamine release in RPMC. These findings may impact on the clinical application of these species if they are used interchangeable. *M. kobus* and *M. biondii* seem act better than other Flos Magnoliae species against mast cell-derived histamine release in PRMC. In addition, the findings of differences in actions of some Flos Magnoliae commercial products suggest a proper quality control procedure is needed to regulate the CHM productions in order to ensure the efficacy and

safety of these products. Finally, the demonstration of pharmacological effect of magnolin and volatile oil may be important for future development of a proper method for quality control of Flos Magnoliae products.

Chapter Eight

General Discussion and Conclusion

8.1 Background

With increasing prevalence of CHM, the risks associated with CHM emerge as an urgent problem for the expanding CHM industry (WHO, 2002). For instance, between 1997 and 1998, 140 cases of adverse effects were reported that the health risks might associate with the botanic dietary products containing one of the CHMs Herba Ephedrae (Ma-huang) in the United States [2]. Considering the gaining complaints of the usage of herbal products containing Herba Ephedrae, the public sale of this herb was stopped by FDA [3]. Recent scandals that were involved CHM included an eight-herb product, PC-SPES, which was discovered contaminated with feminizing hormone and anticoagulant [4]. In Singapore, six death and more others seriously ill were directly led to the withdraw of the herbal remedy, Slim-10, in 2002 [5]. Those infamous cases are associated with the poor manufacturing practice and lack of quality control system. Therefore, scientists attempt to launch reliable and precise data that manufacturers and regulators can use for ensure the quality, safety and efficacy of CHM and its related products.

In other cases, the amount of the CHM or the active ingredients in some commercial CHM products is considerably variable [1]. As one of the commonly used CHM, more than 20 Flos Magnoliae species have been found in the market for clinical application (Bensky & Gamble, 1993; Fu, 2000). Therefore, the identification and authentication of varying botanic sources of Magnolia spp. may contribute to the quality control of Flos Magnoliae (Ernst, 2004; Leon et al., 2004). The major aims of this thesis include establish DNA (RAPD and PCR-RFLP) and chromatographic (TLC and HPLC) fingerprinting profiles of different Flos Magnoliae species and varieties. Then, the anti-histamine release effects induced by compound 48/80 in RPMC of different Flos Magnoliae species and varieties were demonstrated using HPLC with post-derivatization. Furthermore, in order to understand the influence of other factors on the quality of Flos Magnoliae, such as manufacturing process and cultivation conditions, different

Flos Magnoliae samples from various herbal suppliers and cultivation sites were used for the present study.

8.2 Main achievements

8.2.1 Application of advanced techniques for Flos Magnoliae identification and authentication

DNA fingerprinting techniques have been introduced for authentication and evaluation of CHM for last two decades (Shaw, Wang, & But, 2002). It is particularly important, since DNA-based techniques are less affected by geographic differences, climatic variations, cultivation conditions, manufacturing processes and other factors (Hon, Chow, Zeng, & Leung, 2003). In the present study, the DNA fingerprinting profiles of fresh and dried medicinal Flos Magnoliae species were firstly reported using RAPD and PCR-RFLP of 5S rRNA region.

With the express development of instrumentation, TLC has been frequently employed for the identification and authentication of CHM, due to its features of rapidity and simultaneousness (CAMAG, 2006). In the present study, the optimised and simple TLC approach was specifically used for the identification and authentication of *M. biondii*, in which magnolin and fargesin was used as marker compounds.

Fast and sensitive HPLC determination of the active components or marker compounds has been recognised as one of the fundamental approaches for CHM quality control (Drasar & Moravcova, 2004). The contents of magnolin and fargesin from different Flos Magnoliae samples were demonstrated using HPLC with a gradient acetonitrile – water system. In addition to the determination of the single or several chemical components from CHM,

chromatographic fingerprinting techniques have been commonly used for revealing the consistence and the content of multiple chemical compositions (Drasar & Moravcova, 2004). The optimised and validated RP-HPLC method with a gradient acetonitrile – water system for the identification of different Flos Magnoliae samples was introduced as the first time.

The regulation of the safety and quality of CHM is also suggested to be supported by reliable data of their pharmacological and toxicological activities (Briggs, 2002). Anti-histamine release effect of Flos Magnoliae is regarded as one of the important anti-allergic actions (W.K. Wang, Shen, & Qi, 2000; W.K. Wang, Shen, Qi, & Nie, 2000). It is firstly reported that the inhibitory effects of different Flos Magnoliae samples on compound 48/80 induced histamine release in RPMC were revealed using HPLC with post-derivatization.

8.2.2 Quality differences of Flos Magnoliae species and varieties

The present results suggested that the variations of botanic species and varieties might cause quality differences of Flos Magnoliae species. Chinese species, *M. biondii*, is the most commonly used Flos Magnoliae species (Fu, 2000). With the largest genetic dissimilarity among all Flos Magnoliae species, *M. biondii* was determined containing the highest contents of magnolin, fargesin and total peak area of 13 common peaks. Later, the most potent anti-histamine release effects were demonstrated from the ethanol extract of *M. biondii*. Another Chinese species, *M. sargentiana*, which was found to have the second largest genetic distances among all Flos Magnoliae species, no magnolin and fargesin were found to use TLC and HPLC. Based on the present findings, bioactive lignans, magnolin and fargesin, were detected from *M. kobus*, while only magnolin was found in the ethanol extract of *M. liliflora*. Further, high content of chemical compositions and potent anti-histamine release effects of *M. kobus* and *M. liliflora* were found. The findings suggested that locally grown *Magnolia spp.* could be applied for clinical usage for anti-allergy.

However, significant differences of the total peak area indicated that the qualities of the lipid-soluble from different *M. biondii* varieties are varying. The highest total peak area was found in the ethanol extract of Sample 5 (HGCY, 黄梗串鱼), which had the highest potent anti-histamine release effects induced by compound 48/80 in RPMC. In contrast, Sample 7 (CY, 串鱼), which was found has lowest total peak area of the 13 main peaks, had the weakest anti-histamine release effects in RPMC. Interestedly, the high amount of magnolin was found in the ethanol extract of Sample 7 (CY, 串鱼), which suggested that the anti-histamine release effects of different *M. biondii* varieties may relate to the content of the main lipid compositions of the ethanol extracts, rather than the magnolin or fargesin only. Thus, quality differences of the different species and varieties of Flos Magnoliae, in terms of the quality of the chemical compositions and the potency of the anti-histamine release effects, need to be concerned in order to insuring the quality, efficacy and consistency of CHM products.

8.2.3 Other factors may influence the quality of Flos Magnoliae

Although five Flos Magnoliae products were authenticated from *M. biondii*, qualitative and quantitative differences of the chemical compositions were identified using HPLC fingerprinting. It may lead to different potencies of the anti-histamine release effects of five Flos Magnoliae products. For instances, the products, which were provided by Hualong Magnolia Development Co. Ltd., contained the highest amount of 13 common peaks, as well as had the highest potency of anti-histamine release effects in RPMC. On the other hand, the local product, which was found to contain the lowest content of the main components, had the weakest effects on anti-histamine release from RPMC. Moreover, different content of magnolin and fargesin were detected from *M. biondii* samples, collected from different cultivation sites. Once again, the correlation between the qualities and quantities of the chemical compositions and the potencies of anti-histamine release effects were revealed. For

instances, the sample from Mt. Yanyi, contained the highest amount of main components, was found the strongest inhibitory effect on histamine release in RPMC. In contrast, the sample from Eastern Garden, contained the lowest content of the main components, had the weakest anti-histamine release effect. Thus, the present findings suggested that the other factors, including manufacturing factors and cultivation conditions, might influence on the quality of Flos Magnoliae products, while as the cautions should be taken when apply this to other CHMs.

8.3 Limitation of this thesis and future direction

There are some unexplained facts in the present study. For example, the larger genetic divergence between Chinese Flos Magnoliae species and local species, as well as the local *M. sprengeri* sample and another local *M. denudata* sample, were shown in the present study. These findings are not in agreement with previous studies (Wang et al., 2004a, Wang et al., 2004b) and Magnoliaceae taxonomy (Magnolia Society, 2003). Meanwhile, except magnolin and fargesin, the chemical structures of other 11 common peaks are remained unknown. Due to the time constraints, the present study has focused on molecular (RAPD and PCR-RFLP) and chromatographic (TLC and HPLC) fingerprinting profiles of Flos Magnoliae. Coupled with other potential identification approaches (Figure 8.1), the systematic quality profiles may produce a more comprehensive understanding of the quality and therapeutic actions of Flos Magnoliae. For instance, with the extensive investigation of molecular profiles using sequencing-based markers and DNA microarrays have been previously reported for the identification and authentication of CHM (Cui, Lo, Yip, Dong, & Tsim, 2003; Jayasinghe et al., 2007; Ma, Duan, Zhu, Dong, & Tsim, 2000; Zhao et al., 2003). Therefore, combined with the chromatographic fingerprinting profiles of different Flos Magnoliae sources, by using TLC, HPLC and GC techniques, comprehensive, it will be ideally established a integrated fingerprints approaches for the continuation of this thesis.

Flos Magnoliae has a long history for clinical application for Allergic rhinitis (AR) treatment (The Pharmacopoeia Commission of People's Republic of China, 2005). AR is one of the major chromic respiratory disorders, which has been estimated to affect between 10% and 40% of the global population (Bousquet et al., 2001). In Australia, 15.6% of the national population are affected by AR (Australian Institute of Health and Welfare, 2005). AR has been reported that it considerably reduced the quality of life in several important domains, included work productivity and limits social activities (Bousquet et al., 1994). Furthermore, AR also can severely impact on concentration and learning performance and is a main cause of school absenteeism in adolescents for children, who are also recognized as major sufferers, with the prevalence of current AR at 12% in 6-7 years old and 19.6% in 13-14 years old, in Australia (Robertson et al., 1998). Substantial direct and indirect medical cost has been reported to be associated with the high prevalence of AR. In Australia, the significant economic burden of respiratory diseases, included AR, has been reported, with an estimated 8.0% of total healthcare costs in 1993-1994 (Australian Institute of Health and Welfare, 1998). Furthermore, AR is commonly associated with other disorders, for instances, otitis media, Eustachian tube dysfunction, sinusitis, nasal polyps, allergic conjunctivitis, sleep disorders and fatigue (Berger, 2003). Orthodox management of AR includes the administration of H1-antihiatamines, decongestants, antibiotics and nasal steroids. However, unwanted side effects such as local dryness, minor epistaxis, skin thinning, cataract, glaucoma, metabolic changes and impairment on performance have been revealed in previous studies (Wiseman and Benfield, 1997). In recent years, the treatment of allergic rhinitis has been revolutionized by the nasal steroids spray, which is quickly metabolized to less-active metabolites, has minimal systemic absorption, and has minimal systemic adverse effects (LaForce, 1999). However, side effects, including suppressive effects on adrenal function and serum osteocalcin, and considerable cost of these therapeutic products have been reported (Mehle, 2003). These limitations associated with existing western medial approaches are yet

to be addressed fully. In other hand, the use of CAM in AR management has increased recently (Schafer et al., 2002). Previous studies have demonstrated the efficacy and safety of CHM in the treatment of AR (Brinkhaus et al., 2004, Xue et al., 2003b, Xue et al., 2003a). Based on the literature review (details see Chapter 1), due to the outstanding anti-histamine release effects and high concentration of volatile oil contained, clinical studies indicate that Flos Magnoliae has excellent applicability in the treatment of AR by a single herb or combined with other CHM (Xue et al., 2003b, Wang et al., 2000c, Wang et al., 2000a, Wang et al., 2000b, Yang and Zhang, 1998). Meanwhile, even though the inhibitory effects of mast cells derived histamine release were demonstrated in the present study, other factors, such as effects on the production of PGE_2, NO and cytokines (such as, TNF-α and IL-1α) may also be responsible for the anti-allergic effects of Flos Magnoliae (Bachert, Wagenmann, & Hauser, 1995; Guzik, Korbut, & Adamek-Guzik, 2003; Ushikubi, Sugimoto, Ichikawa, & Narumiya, 2000).

Towards the end of this thesis, the findings suggest that with the largest genetic dissimilarity among all Flos Magnoliae species, *M. biondii* was determined containing the highest contents of magnolin, fargesin and total peak area of 13 common peaks. Later, potent anti-histamine release effects were demonstrated from the ethanol extract of *M. biondii*. The findings suggested that *M. biondii* could be the best candidate for further research on anti-allergic and anti-inflammatory actions of Flos Magnoliae. Interestedly, the high amount of magnolin was found in the ethanol extract of *M. biondii*, which may contribute to the anti-histamine release effects. Unlike western medications whose therapeutic effects generally derive from synthetic chemicals, the bioactivities of CHMs arise rather from a series of chemical compositions than a single compound. For instance, this thesis demonstrated the potencies of the anti-histamine release effects from six *M. biondii* varieties may relate to the content of the main lipid compositions of the ethanol extracts, rather than the magnolin or fargesin only. Six varieties

of *M. biondii* were collected from Nanzhao, Henan Province, China. It would have been interesting to characterise the chemical structures of these components, which may contribute to the anti-allergic effects of Flos Magnoliae, if it had not been for time constraints. Thus, development an herbal therapeutic product that offer greater topical activity with lower systemic exposure and improved safety by administering target organ directly has highly marketing potential in Australia. In order to achieve better understanding of the therapeutic potential of *M. biondii* or its bioactive components, the future direction is designed to separate and identify the major chemical components and further pharmacological evaluation of their anti-allergic and anti-inflammatory actions (Figure 8.1). It may also involve the development a new nasal formulation of bioactive components from *M. biondii,* which could appear to be a relatively safe treatment and rapidly improve nasal allergenic tolerance, reduce symptom, reflecting a general improvement in patients' well-being in Australia.

Typically, an analytical liquid chromatography – electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) will be used to identify the containing seven chemical components (Peak 5 – Peak 11). Among selected seven peaks, Peak 6 and Peak 11 indicate magnolin and fargesin, respectively. And then the analytical LC conditions will be linearly scaled up to afford target lignans on the preparative-HPLC. The optimized the compositions of the chemical fractions that are collected from the lab-scale preparative chromatography experiments, a preparative – HPLC system, will be used for further anti-allergic testing, including effects on PGE_2 production and COX-2 protein expression, effects on NO production and NOS expression, effects on cytokines production from RAW 264.7 cell lines, etc. The pharmacokinetics parameters and absolute bioavailability in rat plasma samples with sufficient sensitivity to facilitate analysis of sample collected after an intravenous or intranasal administration of the potential therapeutic product containing the bioactive

components from *M. biondii* will be evaluated by a LC-MS/MS method. oral and intravenous administration (Hsieh et al., 2006).

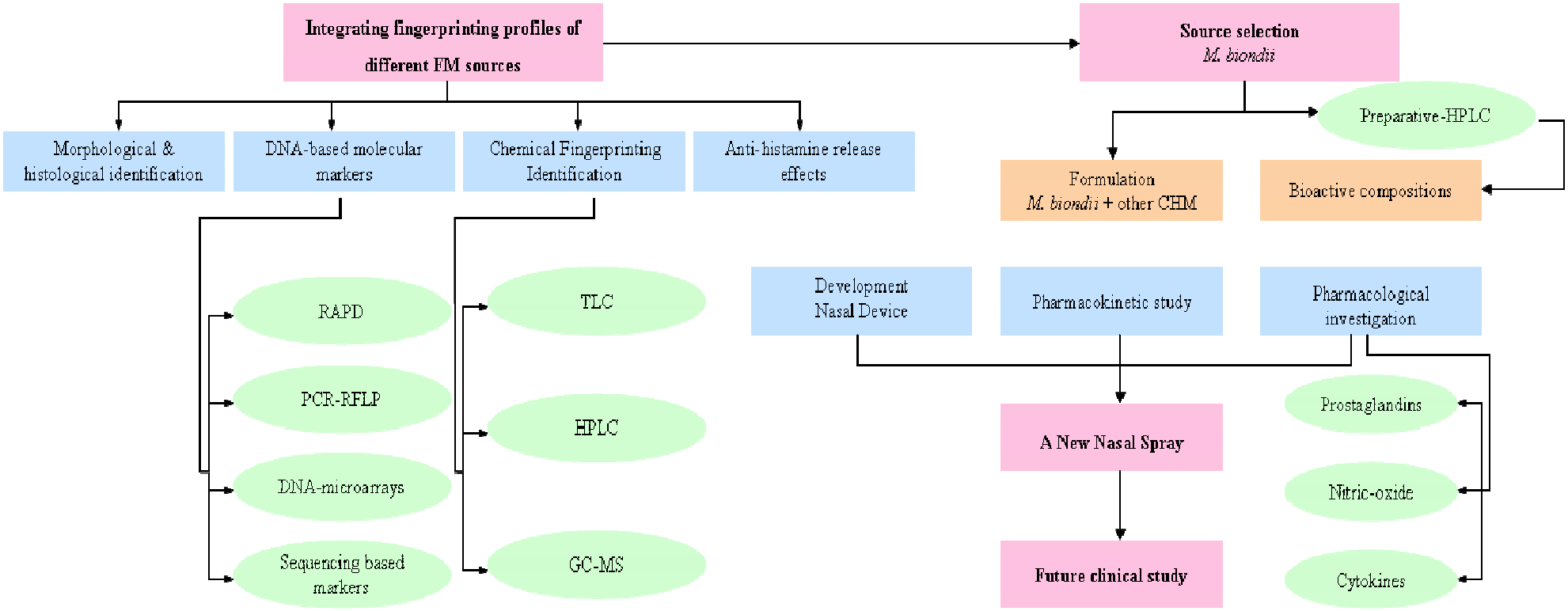


Figure 8.1 Schematic diagram of quality control of Flos Magnoliae

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