SOME PHARMACOLOGICAL AND PHYTOCHEMICAL STUDIES OF *CLITORIA TERNATEA* EXTRACTS

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SOME PHARMACOLOGICAL AND PHYTOCHEMICAL STUDIES OF *CLITORIA TERNATEA* EXTRACTS

by

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To my Lord Sri Krishna

and

dearest family members

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LIST OF SYMBOLS AND ABBREVIATIONS

%	: Percent sign
°C	: Degree celcius
β	: Beta
δ	: Delta
γ	: Gamma
g	: Gravity
κ	: Kappa
σ	: Sigma
μ	: Micro
μ	: Mu
μ_1	: Mu type 1
μ_2	: Mu type 2
4x	: Microscope magnification at 4x
10x	: Microscope magnification at 10x
20x	: Microscope magnification at 20x
Aδ	: A-delta
ABPA	: Allergic bronchopulmonary aspergillosis
A. flavus	: *Aspergillus flavus*
A. fumigatus	: *Aseprgillus fumigatus*
A. nidulans	: *Aspergillus nidulans*
A. niger	: *Aspergillus niger*
$AlCl_3$	: Aluminium chloride
AmpB	: Amphotericin B
ANOVA	: One way analysis of variance

ANS	: Autonomic nervous system
A_0	: Absorbance of the control
ArOH	: Aromatic ring
A_S	: Absorbance of test extract solution
A. terreus	: *Aspergillus terreus*
BEA	: Benzene/ethanol/ammonium hydroxide
BHT	: Butylated hydroxytoluene
$(C_2H_5)_2O$	: Diethyl ether
CEF	: Chloroform/ ethyl acetate/ formic acid
CFU	: Colony forming unit
CFU/mL	: Colony forming unit per milliliter
CFU/organ	: Colony forming unit per organ sample
CH_3COOH	: Glacial acetic acid
CH3COONa•3H2O	: Sodium acetate trihydrate
CLSI	: Clinical and Laboratory Standards Institute
cm	: Centimeter
cm^{-1}	: Recripocal centimeter (units of wavenumber)
cm/s	: Centimeter per second
CNS	: Central nervous system
Conidio/mice	: *Aspergillus niger* conidio per mice sample
COX-1	: Cyclooxygenase 1
COX-2	: Cyclooxygenase 2
C. ternatea	: *Clitoria ternatea*
Cu(II)	: Copper(II)
DNA	: Deoxyribonucleic acid

DPPH	: 2,2-diphenyl-2-picryl-hydrazyl
EDTA	: Ethylenediaminetetra acetic acid
EFGW	: Ethyl acetate/formic acid/glacial acetic acid/water
e.g.	: For example
ELISA	: Enzyme-linked immunosorbent assay
EMEA	: European Medicines Agency
EMW	: Ethyl acetate/methanol/water
FCA	: Freund's complete adjuvant
FCR	: Folin-Ciocalteu reagent
Fe(II)	: Iron (II)
Fe^{2+}-TPTZ	: Ferrous tripyridyltriazine
Fe^{3+}-TPTZ	: Ferric tripyridyltriazine
$FeCl_3$	: Ferric chloride
FeSO4•7H2O	: Eisen II sulfat heptahydrate
FRAP	: Ferric reducing antioxidant power
FTIR	: Fourier Transform Infrared Red
g	: Gram
GC	: Gas Chromatography
GC-MS	: Gas Chromatography-Mass Spectroscopy
GHS	: Globally Harmonised System
g/kg	: Dose (weight of test substance in grams per unit weight of test animal)
GM	: Galactomannan
GMI	: Galactomannan index
GM/mL	: Galactomannan per milliliter
GMS	: Grocott's methenamine silver stain

GRAS	: Generally recognized as safe
h	: Hour
H	: Hydrogen
$H^{\bullet}$	: Hydrogen free radical
H_2O_2	: Hydrogen peroxide
H_2SO_4	: Sulphuric acid
HAT	: Hydrogen atom transfer
HCl	: Hydrochloric acid
HOCl	: Hypochlorous acid
HPLC	: High Performance Liquid Chromatography
HPTLC	: High Performance Thin Layer Chromatography
IA	: Invasive pulmonary aspergillosis
IASP	: International Association for the Study of Pain
IC_{50}	: Half maximal inhibitory concentration
i.e.	: That is
INT	: *p*-Iodonitrotetrazolium chloride
i.p.	: Intraperitoneal
IR	: Infrared
i.v.	: Intravenous
K_2HPO_4	: Dipotassium hydrogen phosphate
KBr	: Potassium bromide
KH_2PO_4	: potassium dihydrogen orthophosphate
kV	: Kilovolt
LC-MS	: Liquid Chromatography Mass Chromatography
LD_{50}	: Median lethal oral dose

LDL	: Low-density lipoprotein
m	: Meter
MBC	: Minimum bactericidal concentration
MFC	: Minimum fungicidal concentration
MIC	: Minimum inhibitory concentration
Mice/group	: Number of mice per group
min	: Minutes
mg CE/g	: Milligram catechin equivalents in 1 gram of sample
mg GAE/g	: Milligram gallic acid equivalents in 1 gram of sample
mg/kg	: Dose (weight of test substance in milligrams per unit weight of test animal)
mg/mL	: Concentration (weight of test substance in milligrams per volume of test concentration)
MHA	: Mueller–Hinton agar
mL	: Milliliter
mL/kg	: Milliliter per kilogram
mL/min	: Gas flow rate millimeter per minute
mm	: Millimeter
mm^2	: Millimeter x millimeter
mM	: Millimolar
mM/L	: Millimolar / Liter
mN/cm	: MilliNewtons / centimeter
mPa/s	: milliPascal / second
MRSA	: Methicillin or multi-resistant *Staphylcoccus aureus*
n	: Sample size
NaCl	: Sodium chloride

Na_2CO_3 : Sodium carbonate

ng : Nanogram

NIR : Near infrared

NIST : National Institute of Standard and Technology

nm : Nanometer

No : Number

NSAIDs : Non-steroidal anti-inflammatory drugs

$^1O_2$: Singlet oxygen

O_2 : Oxygen

$O_2^{\bullet}$: Superoxide anion

O_3 : Ozone

OD : Optical density

OECD : Organization for Economic Co-operation and Development

$OH^{\bullet}$: Hydroxyl

$OOH^{\bullet}$: Hydroperoxyl

ORAC : Oxygen radical absorbance capacity

OsO_4 : Osmium tetroxide

p : Pearson

PAG : Peri-aqueductal gray

PAS : Periodic acid-Schiff

PCR : Polymerase chain reaction

PDA : Potato dextrose agar

PDB : Potato dextrose broth

PLC : Preparative layer chromatography

p.o. : Oral administration

Ramp/min : Rate of temperature increases per minute

R_f : Retention factor

$RO^{\bullet}$: Alkoxyl

$ROO^{\bullet}$: Peroxyl

ROOH : Lipid hydroperoxide

ROS : Reactive oxygen species

RSA : Radical scavenging activity

s : Seconds

s.c. : Subcutaneous

SD : Standard deviation

SDA : Sabouraud dextrose agar

SEM : Scanning Electron Microscopy

SET : Single electron transfer

SMs : Secondary metabolites

Sp1 : Active spot 1

SPE : Solid phase extraction

spp. : Species

SPSS : Statistical Package for the Social Sciences

TEAC : Trolox equivalence antioxidant capacity

TLC : Thin Layer Chromatography

TPTZ : 2,4,6-tris(2-pyridyl)-s-triazine

t_R : Retention time

TRAP : Total radical trapping antioxidant

µg : Microgram

µg/mL : Microgram of plant extract per milliliter of assay mixture

µL	: Microliter
U/mL	: Unit per milliliter
µm	: Micrometer
µM Fe[II]/g	: Micromolar ferric reducing ability in 1 g of dry weight plant material
µM/L	: Micromolar per liter
USFDA	: United States Food and Drug Administration
UV	: Ultraviolet
UV-254nm	: Ultraviolet at 254 nm wavelength
UV-365nm	: Ultraviolet at 365 nm wavelength
vis.	: Visible
VRE	: Vancomycin resistant *enterococci*
vs.	: Versus
v/v/v	: Volume per volume per volume
v/v/v/v	: Volume per volume per volume per volume
W	: Watt
WHO	: World Health Organization

LIST OF APPENDICES

LIST OF PUBLICATIONS AND CONFERENCES

PUBLICATIONS

1. Kamilla, L., Mansor, S. M., Ramanathan, S., & Sasidharan, S. (2009). Antimicrobial activity of *Clitoria ternatea* (L.) extracts. *Pharmacologyonline, 1*, 731-738. (Peer review journal-Scopus).

2. Kamilla, L., Mansor, S. M., Ramanathan, S., & Sasidharan, S. (2009). Effects of *Clitoria ternatea* leaf extract on growth and morphogenesis of *Aspergillus niger*. *Microscopy and Microanalysis*, *15*, 366-372. ISI journal (Impact factor: 2.99).

PUBLICATIONS IN PROGRESS

1. Kamilla, L., Ramanathan, S., Sasidharan, S., Murugaiyah, V., & Mansor, S. M. (2011). *In vivo* antifungal efficacy of *Clitoria ternatea* leaf extract against experimental aspergillosis. *Phytotherapy Research* (Manuscript submitted) Impact factor: 1.746.

2. Kamilla, L., Ramanathan, S., Sasidharan, S., & Mansor, S. M. (2011).Antinociceptive studies of *C. ternatea* leaf and root extracts , *Journal of Natural Medicine*. (Manuscript completed in progress for submission) Impact factor: 1.027.

CONFERENCE PRESENTATION

1. Kamilla, L., Ramanathan, S., Sasidharan, S., Murugaiyah, V., & Mansor, S. M. (2009). *In vitro* antioxidant activities of *Clitoria ternatea*'s extracts, 4th Global Summit on Medicinal and Aromatic Plants, Kuching, Sarawak (Oral presentation).

KAJIAN FARMAKOLOGI DAN FITOKIMIA *CLITORIA TERNATEA* EKSTRAK

ABSTRAK

Dalam tesis ini, penyaringan fitokimia, antimikrob, antioksida dan aktiviti analgesik telah dijalankan ke atas pelbagai bahagian pokok *C. ternatea* dengan menggunakan pelbagai teknik bioassai dan model eksperimental yang sesuai. Penyaringan fitokimia menunjukkan kehadiran tanin, flobatanin, flavonoid, antrakuinon, alkaloid, saponin, glikosida kardiak, minyak meruap, steroid and terpenoid. Kandungan flavonoid dan fenol dalam pelbagai bahagian tumbuhan juga telah ditentukan. Analisis menunjukkan ekstrak bunga *C. ternatea* mengandungi kandungan fenol (104.77 ± 2.64 mg asid gallik/g jisim tumbuhan) dan flavonoid (28.10 ± 4.13 mg katecin/g jisim tumbuhan) yang tinggi apabila dibandingkan dengan bahagian-bahagian lain. Hal ini telah terbukti apabila ekstrak bunga *C. ternatea* menunjukkan aktiviti antioksida yang paling tinggi (FRAP: 6.80 ± 0.32 μM Fe(II)/g jisim tumbuhan; DPPH IC_{50} : 9.17 ± 0.003 μg/mL) berbanding dengan bahagian-bahagian lain tumbuhan ini. Kaempferol-3-glukosida (K3G) yang merupakan sebatian flavonoid telah dikenalpasti sebagai salah satu bioaktif dalam ekstrak daun, batang dan bunga *C. ternatea*. Biaoktif ini mungkin merupakan penyumbang kepada aktiviti antioksida. Bagi penyaringan antimikrob *in vitro*, kedua-dua ekstrak daun dan akar *C. ternatea* menunjukkan aktiviti antimikrob yang tinggi. Lebih-lebih lagi, ekstrak daun *C. ternatea* ini menunjukkan aktiviti antikulat yang bagus terhadap *Aspergillus niger* dengan nilai perencatan minimum (MIC) 0.4 mg/mL dan nilai kepekatan maut minimum (MFC) 0.8 mg/mL. Hal ini telah terbukti apabila analisis mikroskop pengimbasan elektron (SEM) telah menunjukkan perubahan morfologi yang ketara ke atas dinding hifa dan konidiofor selepas pendedahan kepada ekstrak daun *C.*

ternatea berbanding dengan kumpulan kawalan. Sebelum kajian analgesik dan antikulat dikaji, ketoksikan akut ekstrak daun *C. ternatea* telah dijalankan dengan menggunakan mencit. Nilai LD_{50} ekstrak daun *C. ternatea* adalah melebihi daripada dos berat badan 2000 mg/kg yang menunjukkan esktrak daun *C. ternatea* ini berada pada zon selamat yang tinggi. Selanjutnya, model haiwan telah digunakan untuk mengkaji aktiviti analgesik dan antikulat ekstrak daun *C. ternatea*. Kedua-dua ekstrak daun dan akar *C. ternatea* menunjukkan aktiviti analgesik yang lebih baik apabila berbanding dengan kumpulan kawalan. Namun begitu, aktiviti analgesik adalah tidak setanding dengan tikus yang di rawat dengan morfina. Dalam kajian yang lain, aktiviti *in vivo* antikulat ekstrak daun *C. ternatea* telah dikaji dengan menggunakan mencit yang telah dijangkiti dengan *A. niger*. Aktiviti antikulat ekstrak daun *C. ternatea* adalah setanding dengan amfoterisin B. Kajian fraksinasi bioaktiviti berpandu menyarankan yang aktiviti antikulat ekstrak daun *C. ternatea* mungkin disebabkan oleh aktiviti sinergisme pelbagai bioaktif yang terdapat di dalam ekstrak tersebut. Fraksi bioaktif telah dianalisis selanjutnya dengan menggunakan kromatografi gas-spektroskopi jisim (GC-MS) dan spektroskopi inframerah (FTIR). Analisis ini menunjukkan yang fraksi bioaktif ini mungkin tergolong dalam kumpulan lipid dan/ atau terpenoid (seskuiterpena).

SOME PHARMACOLOGICAL AND PHYTOCHEMICAL STUDIES OF *CLITORIA TERNATEA* EXTRACTS

ABSTRACT

In this thesis, detailed work was carried out to explore and determine the phytochemical screening, potential anti-infective, antioxidant and analgesic properties of *C. ternatea* plant parts using various bioassays techniques and appropriate experimental models. The initial qualitative phytochemical screening shows that *C. ternatea* plant parts contain tannin, phlobatannin, flavonoid, anthraquinone, alkaloid, saponin, cardiac glycosides, volatile oils, steroids and terpenoids. The flavonoids and phenolic content of the various plant parts were also determined in the plant parts. Analysis shows that *C. ternatea* flower extract has both high phenolic (104.77 ± 2.64 mg GAE/g) and flavonoid (28.10 ± 4.13 mg CE/g) content than other plant parts. This is evident by the fact that *C. ternatea* flower extract exhibited the highest antioxidant activity (FRAP: 6.80 ± 0.32 μM Fe(II)/g ; DPPH IC_{50} : 9.17 ± 0.003 μg/mL) when compared to other parts of the plant. One of the bioactives identified in the flower extract was kaempferol-3-glucoside (K3G) which is a flavonoid compound. Kaempferol-3-glucoside was also found in *C. ternatea* stem and leaf. This could be one of the identified bioactives responsible for the antioxidant activity. As for the *in vitro* anti-infective screening, both leaf and root crude extracts demonstrated marked antimicrobial activities. Interestingly, the leaf extract demonstrated a remarkable antifungal activity against *Aspergillus niger* with a MIC and MFC of 0.4 and 0.8 mg/mL, respectively. This was evident by the scanning electron microscope (SEM) analysis which showed a marked morphological alteration of the *A. niger* hyphal wall and conidiophores after exposure to *C. ternatea* leaf extract when compared to control. The acute toxicity of

C. ternatea leaf extract was assessed in mice prior to antinociceptive and *in vivo* antifungal study. After oral administration in mice, the LD_{50} was found to be higher than 2000 mg/kg body weight which indicated some high level of safety for *C. ternatea* leaf extract. Subsequently C. *ternatea* leaf extract was further tested for its antinociceptive and *in vivo* antifungal activities in animal models. Studies in rats showed that both *C. ternatea* leaf and root extracts demonstrated antinociceptive activity at the studied dosage regimens when compared with control but the analgesic effect was not comparable to morphine treated rats. In another study, the *in vivo* antifungal activity of *C. ternatea* leaf extract was investigated in *A. niger* infected mice. *C. ternatea* leaf extract antifungal activity was comparable to clinically used amphotericin B. Bioactivity guided fractionation studies of the *C. ternatea* leaf extract suggest that the antifungal activity could be a synergistic activity of various bioactives presence in the extract. Further gas chromatography-mass spectroscopy (GC-MS) and fourier transform infrared (FTIR) analysis of the fractions indicated these bioactives could be lipids and/or terpenoids (sesquiterpenes) group of compounds.

CHAPTER 1

1.0 INTRODUCTION

Traditional medicinal practice has been known for centuries in many parts of the world for the treatment of various human ailments (Arora & Kaur, 2007). In developing countries, the World Health Organization (WHO) has estimated that 80% of the population rely almost on traditional medicine for their primary health care needs (World Health Organization, 2010). It is assumed that a major part of traditional therapy involves the use of plant extracts or their active principles (Ignacimuthu, Ayyanar, & Sivaraman, 2006). Hence, medicinal plants play a crucial role and constitute the backbone of traditional medicine (Mukherjee, 2002a).

Medicinal plants have formed an essential element of the materia medica of various formal and informal systems of medicine (Suneetha & Chandrakanth, 2006) as they have fitted the immediate personal need and are easily accessible and inexpensive (Mukherjee, 2002a). The demand for medicinal plants for medicinal purposes or as health supplements has been increasing worldwide and not to exclude, Malaysia has great potential to develop her abundant natural resources. However, the medicinal plants available have not been extensively exploited or studied (Jamal, 2006). Among the promising medicinal plant that should receive more attention is *Clitoria ternatea* Linn. which is one of the medicinal plants used as an ingredient in 'Medha Rasayana' a rejuvenating recipe for the treatment of neurological disorders in Ayurvedic traditional medicinal system (Sivaranjan & Balachandran, 1994).

Clitoria ternatea Linn. (*C. ternatea*) belongs to family Fabaceae and is a very well known Ayurvedic medicine used for various different ailments. It has been used for centuries as antidepressant, antistress, anxiolytic, memory enhancer, nootropic, anticonvulsant, tranquilizer, sedative agent and also to treat fever, arthritis, eye ailments, indigestion, constipation, ear-diseases and swollen joints. *C. ternatea* extracts is known to possess a wide range of pharmacological activities which includes anti-inflammatory, analgesic, antipyretic, antimicrobial, insecticidal, diuretic, antidiabetic, local anesthetic, blood platelet aggregration-inhibiting and vascular smooth muscle relaxing properties (Mukherjee, V. Kumar, N. S. Kumar, & Heinrich, 2008). Various secondary metabolites such as polyphenolic flavonoids, anthocyanin glycosides, pentacyclic triterpenoids, phytosterols and fatty acids have been reported in this plant (Mukherjee, Kumar, & Heinrich, 2007). Flavonols i.e. kaempferols, quercetin and myricetin and their glycosides have been also isolated from this plant (Kazuma, Noda, & Suzuki, 2003).

A review paper on *C. ternatea* by Mukherjee et al. (2008) has well documented various traditional and potential pharmacological uses of this plant. An evaluation of *C. ternatea* for its potential anti-inflammatory, antimicrobial, central nervous system (CNS) related effects are imperative for development of an effective natural remedy. Previously, finotin (a protein) isolated from *C. ternatea* was found to be potent inhibitor against various important plant fungal pathogens (Kelemu, Cardona, & Segura, 2004). Though the antifungal activity is not of immediate relevance to humans, nevertheless the finding indicates its possible antifungal effects. With this in view, it has prompted us to systematically evaluate and determine antimicrobial spectrum of *C. ternatea* against human pathogenic

microorganisms. Further to this, numerous studies have reported the presence of flavonoids and phenolic compounds in *C. ternatea* plant parts thus these suggest their potential applications as antimicrobial, antioxidant, anti-inflammatory, anticarcinogen, antiallergic, antiviral, immune-stimulating agents (Rice-Evans, Miller, & Paganga, 1996). Despite these activities, there were also studies that demonstrated the analgesic activity of methanolic *C. ternatea* roots extract using acetic-acid induced writhing test and ethanolic *C. ternatea* aerial extracts (Parimaladevi, Boominathan, & Mandal, 2003; Kulkarni, Pattanshetty, & Amruthraj, 1988) in rats.

As of to date not much work was carried out on the antimicrobial activity of various *C. ternatea* plant extracts on human pathogenic microorganism, while studies on antioxidant activity was limited only to water extract of *C. ternatea* plant parts. In addition, analgesic activity of various *C. ternatea* plant parts crude extracts have been also documented but using limited experimental models. Therefore in this thesis, detailed work was undertaken to explore and determine the potential toxicology, anti-infective, antioxidant and analgesic properties of *C. ternatea* plant parts using various bioassays techniques and appropriate experimental models. In this thesis, the chemical constituents responsible for its antifungal activity was isolated and identified.

1.1 Objectives

The objectives of the work carried out in this thesis are as follows:

- To obtain various crude extracts from *C. ternatea* leaves, stems, flowers, seeds and roots by means of maceration extraction method.
- To conduct qualitative phytochemical, antimicrobial and antioxidant assays on various crude extracts of *C. ternatea* leaf, stem, flower, seed and root.
- To evaluate the antinociceptive activities of *C. ternatea* leaf and root extracts using hot plate, tail-flick and formalin test models.
- To conduct oral acute toxicity in mice using *C. ternatea* leaf extract.
- To perform Scanning Electron Microscopy (SEM) and *in vivo* studies of *C. ternatea* leaf extract against *Aspergillus niger*.
- To identify the antifungal active fraction(s) from *C. ternatea* leaf extract.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 Herbal medicine

Plants, parts of plants and isolated phytochemicals have been used since ancient times for prevention and treatment of various health ailments (Sahoo, Machikanti, & Dey, 2010). Plants still contribute significantly to health care sector, in spite of the great advances observed in modern medicines in recent decades (Calixto, 2000). Plants may be defined as food, functional food, dietary supplement or as herbal medicine (Sahoo et al., 2010). Herbal medicines include herbs, herbal materials, herbal preparations and finished herbal products that contain active ingredients of plant parts or other plant materials or combinations (World Health Organization, 2010). Worldwide, it is estimated that 25% of the drugs prescribed are derived from plants. Besides this, 11% of the total 252 drugs in WHO's essential medicine list is exclusively of plant origin (Rates, 2001).

Herbal drugs can be categorized into three types based on the nature of their active metabolites. The first category of plant drugs are usually simple botanicals employed in their crude form. Examples are opium, belladonna, aloe and cinchona. The second category of herbal drugs is represented by the active constituents (pure molecules) isolated after the processing of plant extracts. Some of the prominent examples are reserpine from *Rauvolfia*, quinine from *Cinchona* and taxol from *Taxus* species. The third category of herbal drugs is drugs with acute and chronic toxicity data available. Examples are mineralo-herbal drug Amlena and green tea extract

(Veregen) (Iwu, Duncan, & Okunji, 1999; Chandra, Sachan, Ghosh, & Kishore, 2010; Wu, Ghantous, & Birnkrant, 2008).

WHO (2003) classified the herbal drugs into four categories such as indigenous herbal medicines, herbal medicine in systems, modified herbal medicines and imported products with an herbal medicine base. Indigenous herbal medicines are used in local community and are well known in terms of their composition, treatment and dosage. Herbal medicines in systems (Ayurveda, Unani and Siddha) have been in use for a long time. Modification of indigenous herbal medicine or herbal medicines in systems in terms of shape or form, dosage form, mode of administration, herbal medicinal ingredients, methods of preparation and medical indications represents modified herbal medicines.

In developing countries, WHO has estimated that the 80% of the population rely almost on traditional medicine for their primary health care needs. Herbal plants play a crucial role and constitute the backbone of traditional medicine (Mukherjee, 2002a). The demand for medicinal plants for medicinal purposes or as health supplements has been increasing worldwide and not to exclude, Malaysia has great potential to develop her abundant natural resources. However, the medicinal plants available herein have not been extensively exploited or studied (Jamal, 2006).

2.2 Plant introduction

2.2.1 *Clitoria ternatea*

Clitoria ternatea Linn, is a very renowned Ayurvedic medicine used to treat various ailment. *C. ternatea* belongs to family Fabaceae. It is commonly known as butterfly pea, conch flower, shankapushpi and bunga telang. In Indian traditional medicine, it is known as Aparajit (Hindi), Aparajita (Bengali) and Kankattan (Tamil). Initially, *C. ternatea* is a native of the Caribbean, Central America and Mexico and currently it is well distributed in India subcontinent, Phillipines, Malaysia and other tropical Asian countries, South and Central America, Madagascar and Caribbean (Mukherjee et al., 2008).

The scientific name of the genus was derived from Greek kentron, a spur, prickle, sharp point, the center, and sema, a signal, referring to the spurred standard petal (Austin, 2004). Meanwhile, its species name was named after the island of Ternata in Moluccaa archipelago (Mukherjee et al., 2008).

2.2.2 Botany and pharmacognosy of *C. ternatea*

C. ternatea (Figure 2.1) is an ornamental perennial climber which climbs up to 2-3 m in height, grows wild and also in gardens. It bears conspicuous blue or white flowers resembling a conch-shell. Though it is presumably of American origin, today it is cultivated and naturalized throughout the humid tropics of the old and new world below 1600 m elevation (Morton, 1981).

The plant possesses imparipinnate leaves consisting of five to seven leaflets, 6-13 cm long. The leaflets are oblong or ovate, 2-5 cm long and subcoriaceous, rubiaceous stomata with wavy cell walls are present on both upper and lower epidermis of the leaflets (Mukherjee et al., 2008).

The plant bears papilionaceous, axillary, solitary flowers. The flowers are available in white or bright blue in colour with a yellow or orange center (Figure 2.2). *C. ternatea* pods are flat, nearly straight, sharply beaked and are 5-10 cm long. Each pod can bear 6-11 seeds. The seeds are yellowish-brown or blackish in color and subglobose or oval in shape (Mukherjee et al., 2008).

The *C. ternatea* root system consists of a fairly stout taproot with few branches and many slender lateral roots. The thick horizontal root can grow up to more than 2 m long, bears one to several purplish, glaucous, wiry stems. The root is woody, cream white with a few lenticels united to form transverse cracks. The fresh root is slightly bitter and acrid in taste (Mukherjee et al., 2008).

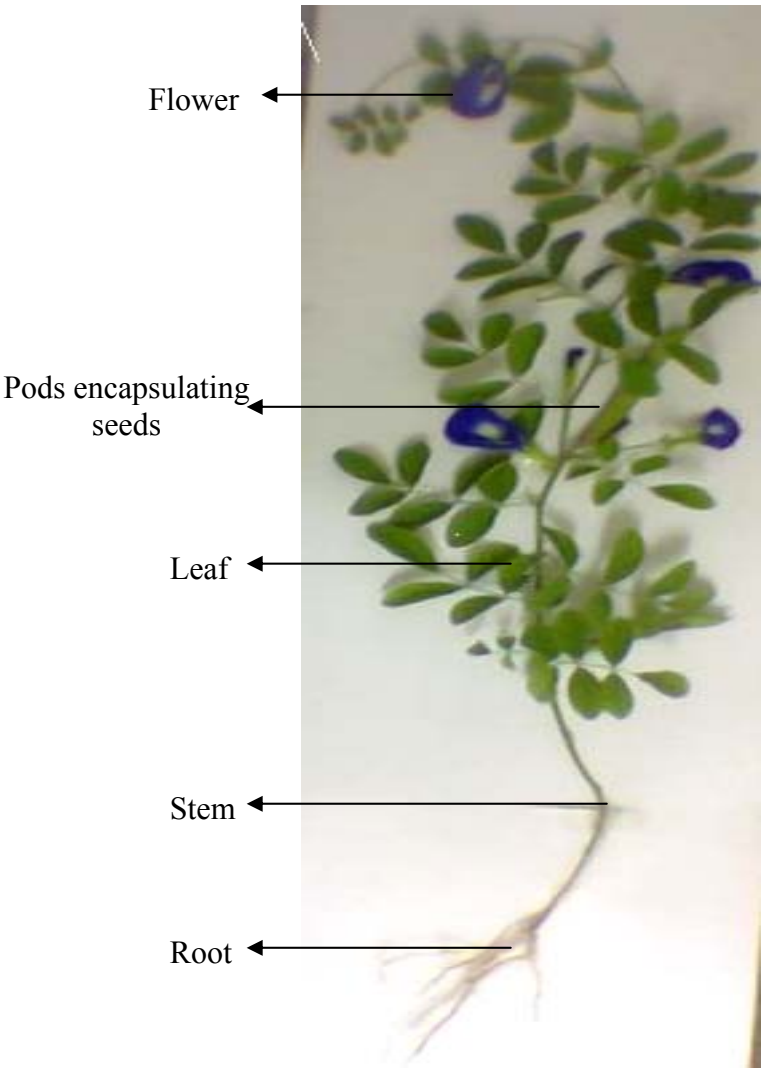

Figure 2.1: *C. ternatea* Linn plant.

Figure 2.2: *C. ternatea* Linn flower in blue colour.

2.2.3 Traditional uses of *C. ternatea*

C. ternatea has been used for centuries in traditional Ayurvedic system of medicine to treat several diseases. *C. ternatea* has been used as an ingredient in 'Medhya Rasayana' a rejuvenating recipe used for treatment of neurological disorders and strengthens a person's intellect. In Ayurveda, the drug 'Sankhapushpi' consists of the roots and seeds of *C. ternatea* and is used as a 'tonic of the nerves', alterative and laxative (Mukherjee et al., 2008).

The roots and leaves are used in the treatment of a number of ailments including body aches, infections, urinogenital disorders, and as an anthelmentic and antidote to animal stings. In addition, the roots have an acrid and bitter taste and are credited with diuretic, purgative and laxative properties. The root is used in the treatment of various diseases, like indigestion, constipation, fever, arthritis and eye ailments. It is also employed in cases of ascetics, enlargement of the abdominal viscera, sore throat, skin diseases and chronic bronchitis. The decoction or powder of the root is given in rheumatism, and ear-diseases (Mukherjee et al., 2008).

Powdered seeds are mixed with ginger and given as laxative, the action, however, is accompanied by gripping in lower abdomen. The seeds are considered for colic, dropsy and enlargement of abdominal viscera; they are also used in swollen joints. The root, stem and flower are recommended for the treatment of snakebite and scorpion sting in India (Mukherjee et al., 2008).

2.2.4 Other uses

C. ternatea is a highly palatable forage legume generally preferred by livestock over other legumes. It exhibits excellent re-growth after cutting or grazing within short period of time and produce high yields. It is grown with tall grasses for rotational grazing, hay or silage. It is grown either alone or with other perennial grasses in Uttar Pradesh, Gujarat, Maharashtra, Madhya Pradesh, Andhra Pradesh, Punjab, Rajasthan, Tamilnadu and Karnataka in India. Besides suppressing many perennial weeds, it enriches the soil by fixing nitrogen. It is also used as drought-resistant pasture in arid and semi-arid regions (Mukherjee et al., 2008).

Butterfly pea is also used as a cover crop and green manure. It is also grown as ornamental plant due to its attractive flower colours (Kalamani & Michael, 2003). In Kerala (India) and Philippines, the young shoots, leaves, flowers and tender pods are eaten as vegetable. In Malaysia, the leaves are traditionally employed to impart a green colour to food and the flowers to impart a bright blue colour to rice cakes (Mukherjee et al., 2008). The medicinal potential of this use in Malaysian traditional practice warrants investigation.

2.2.5 Pharmacological activities of *C. ternatea*

C. ternatea has been widely screened for various pharmacological activities. It has been documented for its neuropharmacological actions such as nootropic, antistress, antidepressant, anticonvulsant, enhancing acetylcholine content, anxiolytic, tranquilizing and sedative activities which justify its use in CNS diseases in Ayurvedic system of medicine. It has antimicrobial, antipyretic, anti-inflammatory, analgesic, diuretic, local anesthetic, antidiabetic, insecticidal, blood platelet

aggregation inhibiting and vascular smooth muscle relaxant properties (Mukherjee et al., 2008). The various reported pharmacological activities of *C. ternatea* (Table 2.1) have been summarized below.

Table 2.1: Pharmacological activities of *C. ternatea* Linn. (Source: Mukherjee et al., 2008).

Plant part	Extract/compound	Activity
Roots and aerial parts	Aqueous extract, alcoholic extract	Learning and memory enhancing, enhancement of acetylcholine content.
Roots and aerial parts	Alcoholic extract, methanol extract	Nootropic activity, anxiolytic, anxiolytic activity, antidepressant activity, tranquilizing property, sedative activity, anticonvulsant activity, and antistress activity
Flowers	Anthocyanins (Ternatin A1, A2, B1, B2, D1, and D2)	Blood platelet aggregation-inhibiting and vascular smooth muscle relaxing activities.
Roots, aerial part	Methanol extract, alcoholic extract	Antipyretic activity
Roots	Methanol extract	Anti-inflammatory and analgesic activity
Seeds	Finotin Flavonol glycoside from ethyl acetate soluble fraction	Antimicrobial activity
Flowers	Ethanol extract	Antidiabetic activity
Seeds	Finotin	Insecticidal activity
Roots	Alcoholic extract	Diuretic activity in dogs
Aerial part	Alcoholic extract	Local anesthetic effect

Though the pharmacological activities are thoroughly documented but the bioactives responsible for its pharmacological activities are still lacking. Studies on the absorption and disposition of this bioactives in preclinical and clinical trials are imperative (Mukherjee et al., 2008). Therefore *C. ternatea* merits further phytochemical, pharmacological and clinical investigations for development of therapeutically effective lead compounds or standardized extracts. In this thesis, the phytochemical, toxicity, antioxidant, analgesic and antimicrobial investigation were carried out for *C. ternatea.*

2.3 Quality control of extracts

Herbal extracts are in great demand for primary healthcare in developed as well as developing countries. This is because of their extensive biological activities, lesser cost and high safety margins. The most important challenges faced by herbal extracts formulations arise because of their lack of standardization (Palav & D'mello, 2006). Various factors such as the age of the plant, part of the plant used, time of harvest, soil conditions, weather conditions, extraction and drying methods, type of solvents used, etc. greatly affects the quality, composition and bioactivity of the herbal extracts (Hendriks, Juarez, Bont, & Hall, 2005). Since these factors affect the end composition and consistency of the finished product, the standardization and quality control of the herbal extracts are required.

With the tremendous growth of herbal extracts, the health authorities and the public are concern on the quality control of herbal extracts used for treatment (Fan, Cheng, Ye, Lin, & Qian, 2006). The herbal extracts have distinctive characteristics from synthetic drugs in which the herbal extracts contain more than one active compound and the active principle is frequently unknown (Sahoo et al., 2010). Currently, the analysis of one or more selected marker compounds is used to predict the quality of herbal extracts. Ideally, the marker compounds could be responsible for the efficacy and activity of the herbal extract. However, the selected marker compounds that are often chemical characteristics of the herb may not be related to the bioactivity of the herb. Furthermore, the synergistic effect of several compounds may also contribute to the bioactivity of certain plant. The common practice of using one marker compound neglects the synergistic effect. As an outcome, the marker-based approach is found to be incomplete and unsatisfactory method for quality

control of herbal materials (Hendriks et al., 2005). Liang, Xie, & Chan (2004) have stated that this kind of determination does not give a complete picture of a herbal product because multiple constituents are usually responsible for its therapeutic effects.

Quality control of herbal preparations is the requirement of credible clinical trials. As stated in the draft guidelines by United States Food and Drug Administration (USFDA) and The European Agency for the Evaluation of Medicinal Products, various aspects of analysis must be performed for the purpose of certification of herbal preparations (Ong, 2004). The standardization methods should take into consideration all aspects that contribute to the quality of herbal drugs. The methods include correct identity of the sample, organoleptic evaluation, pharmacognostic evaluation, volatile matter, quantitative evaluation (ash values, extractive values), phytochemical and biological activity evaluation. The herbal preparations should also be tested for the presence of xenobiotics, microbial contaminations, toxicity testing, pesticides, arsenic and heavy metals contents (Rajani & Kanaki, 2008; Mukherjee, 2002b).

Among the various quality control methods, the chromatographic fingerprinting has gained a lot of attention recently. Various bodies like WHO, USFDA, European Medicines Agency (EMEA), German Commission E, British Herbal Medicine Association, Indian Drug Manufacturers and others have accepted the usage of chromatographic fingerprinting to assess and standardize the herbal medicines (Fan et al., 2006). Chromatographic fingerprinting is a chromatogram that represents the chemical characteristics or pattern of a herbal extract (Cheng, Chen, &

Tong, 2003). The chemical profiling is a versatile technique and is of good use in standardization method (Mukherjee, 2002e). The common analytical techniques used are Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC), Gas Chromatography-Mass Spectroscopy (GC-MS), Liquid Chromatography Mass Chromatography (LC-MS), near infrared (NIR) and spectrophotometer. A chemical fingerprint by hyphenated chromatography is used as the primary tool for quality control of herbal medicines (Liang et al., 2004) and serve as guideline to the phytochemical profile of the drug in ensuring the quality (Rajani & Kanaki, 2008). Also, the full herbal product can be regarded as the active compound (Liang et al., 2004). Chromatographic fingerprinting has potential to determine the identity, authencity and batch-to-batch consistency of herbal medicines. Generally, samples with similar chromatographic fingerprint have similar properties (Fan et al., 2006). The chromatographic techniques enable rapid, reproducible and efficient semiquantitative and quantitative analysis of the chemical constituents in complex mixtures (Rajani & Kanaki, 2008).

2.4 Phytochemistry

Phytochemistry is termed as plant chemistry. The phytochemistry studies are essential for various research disciplines such as plant physiology, plant biochemistry, chemosystematics (often referred to as chemotaxonomy), plant biotechnology and pharmacognosy (Hajnos, Sherma, & Kowalska, 2008). Phytochemistry studies are concerned with a variety of organic substances (primary and secondary metabolites) present in plant and deal with the chemical structures of these substances, their biosynthesis, turnover, metabolism, natural distribution and biological function (Harborne, 1973a).

Plants are biosynthetic producer of chemical substances such as primary and secondary metabolites (Nyiredy, 2004). Primary metabolites include amino acids, simple sugars, lipids and nucleic acids which are necessary for cellular processes and are found in all plant species (Wink, 2006; Zwenger & Basu, 2008). Secondary metabolites (SMs) differ between species and occur in mixtures. Unlike primary metabolites, SMs are not essential for energy metabolism and life but they play an important role for the ecological fitness and survival of the plants (Wink, 2006). Plant SMs are usually categorized according to their biosynthetic pathways. Phenolics, terpenes and steroids, and alkaloids are the three large molecule families that are generally considered (Bourgaud, Gravot, Milesi, & Gontier, 2001). Other SMs include lignins, flavonoids, amino acid derivatives, organic acids, polyketids and sugar derivatives (Nyiredy, 2004).

Plants SMs serve important biological and ecological roles mainly as chemical messengers and defensive compounds (Jones & Kinghorn, 2005). These molecules are able to protect plants from pathogens (phytoalexins), therefore expressed as antibiotic, antifungal and antiviral. They also act as toxic or anti-germinative for other plants (allelopathy) (Bourgaud et al., 2001). Notably, SMs constitute important ultraviolet (UV) absorbing compounds, thus preventing serious leaf damage from the light (Li, Ou-Lee, Raba, Amundson, & Last, 1993).

Plant SMs have been used in traditional medicines for centuries. Currently, SMs are used in pharmaceutics, cosmetics, fine chemicals and also in nutraceutics (Bourgaud et al., 2001). In western countries, studies have shown that 25% of the

molecules used in pharmaceutical industry are of natural plant origin (Payne, Bringi, Prince, & Shuler, 1991).

2.4.1 Sample selection, collection and identification

In drug discovery from plants, four standard approaches are available for selecting plants: (1) follow-up of ethnomedicine or traditional uses of plants used as food, medicine, or poison based on literature review or interviews, (2) random or systematic collection of a biodiverse set of plant samples, typically from an ecological region that is comparatively uncharted as regard to secondary metabolite production, (3) the selection of species based on phylogenetic relationship to a species known to produce a compound or compound class of interest, (4) follow up of species based on reports of biological activity in the literature (Jones & Kinghorn, 2005). A fifth, non-systematic approach is serendipity, where plant is selected based on ethnomedical use, but the bioactivity of the plant is new and unexpected (Cos, Vlientinck, Berge, & Maes, 2006).

In phytochemical analysis, the botanical identity of the plants studied should be authenticated to identify and confirm the plant identity (Harborne, 1973a). The voucher specimens (which include reproductive organs, when feasible) should be deposited in the herbarium of major institutions. The identity of the material should include observations such as its habitat, local uses of the species, microenvironment (e.g. shaded vs. sunny location), overall state of the plant health, reproductive stage and also other information that may be useful for future investigations (Jones & Kinghorn, 2005).

2.4.2 Extraction of plant constituents

The most crucial step in drug discovery is processing of the plant material. During the preparation of the extract, careful measurements must be adopted to ensure the potentially active constituents are not lost, destroyed or altered. Extraction procedures such as maceration, percolation and infusion are the traditional techniques used for the extraction of medicinal plants (Cos et al., 2006). The sole purpose of such basic extraction procedures is to obtain the therapeutically desired portion and eliminate the inert material by treatment with a selective solvent known as menstruum (Singh, 2008). In pharmaceutical industry, extraction techniques such as maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication) and supercritical fluid extraction are generally used (Handa, 2008).

2.4.2 (a) Maceration extraction

Specific extraction procedures are usually employed to produce enriched extracts when a particular phytochemical constituent or compound class is targeted to be extracted for an investigation (Jones & Kinghorn, 2005). In this thesis, the maceration extraction method was used. Maceration is the extraction process of drug/ plant extracts with a solvent with several daily shakings or stirrings at room temperature. Maceration is one of the most widely used extraction method and can be carried out with small samples (Mukherjee, 2002c).

The maceration system is static except during occasional shaking. The occasional shaking removes the concentrated solution accumulating around the

surface of the particles and mixes it with the rest of the menstruum whereby it brings fresh menstruum to the particle surface for further extraction. The shaking also assists in diffusion. A closed system is used as it prevents the evaporation of menstruum over a prolonged period and this avoids batch-to-batch variation (Sambamurthy, 2005).

2.4.2 (b) Types of solvent

Interactions between the solvent and solute can be assessed by several indicators for example the dipole moment, polarity index, viscosity and surface tension (Lalman & Badley, 2004). The chemical properties of frequently used solvent for extraction purpose are tabulated in Table 2.2.

Table 2.2: Chemical properties of solvents.

Types of solvent	**Polarity index** [a]	**Surface tension (mN/cm)** [b]	**Viscosity (mPa/s)** [c]	**Dipole moment (Debye)** [d]
n-Hexane	0	17.9	0.30	0
n-Butanol	4.0	24.2	0.41	1.521
Chloroform	4.1	26.5	0.53	1.155
Ethyl acetate	4.4	23.1	0.43	1.831
Methanol	5.1	22.3	0.54	1.621
Ethanol	5.2	21.9	1.08	1.550
Water	9.0	72.8	0.89	1.870

[a] Retrieved from Gupta et al., 1997 and Synder 1974
[b] Retrieved from Markom et al., 2007
[c] Retrieved from Synder 1974
[d] Retrieved from Sheng et al., 2011

The dipole moment measures the polarity of a molecule as a net sum of the dipole moments of its polar bonds (Sheng, Vannela, & Rittman, 2011). The interaction between the solvent and solute also resulted in the overall increase of dipole moment for the solution (Markom, Hasan, Daud, Singh, & Jahim, 2007).

The polarity index is defined as the measure of the ability of the solvent to interact with various polar test solutes, and it increases with solvent polarity. The higher polarity index value demonstrates their stronger capability do dissolve more polar solutes. Vice versely, solvents with lower polarity index value exhibits weaker ability in interacting with polar solutes. Solvents with low viscosity have low density and high diffusivity, and can easily diffuse into the pores of the plant materials (Sheng et al., 2011). Temperature is another important factor that contributes to the interaction between the solvent and solute. High temperature breaks the solute-matrix, reduces the viscosity and surface tension and therefore improves the extraction yield (Markom et al., 2007). However, higher temperatures are avoided as it can cause degradation of thermolabile compounds (Sheng et al., 2011).

2.4.3 Purification of plant constituents

Purification of the extract is the next step and a vital one in the sample preparation after the extraction process. The existing purification method includes analytical and preparative process. In analytical purification, the most widely used method is the solid-phase extraction (SPE) meanwhile in preparative purification, the liquid-liquid extraction method is used (Nyiredy, 2004). As for liquid-liquid extraction method extraction is based on the partition between the immiscible solvents (Nyiredy, 2004). The extract usually dissolved in water is mixed with another solvent which does not mix with the aqueous phase. The plant constituents solubilise in each of the phases and after a time, equilibrium between the phases is reached. The solvent added is replaced with another immiscible solvent to the aqueous phase (Houghton & Raman, 1998b). The frequently used immiscible phases include diethyl ether/water, chloroform/water, ethyl acetate/water and dichloromethane/water (Nyiredy, 2004).

Vigorous mixings of the two phases in a stoppered separating funnel can shorten the time taken for the equilibrium to be achieved (Houghton and Raman, 1998b). In this thesis, the liquid-liquid extraction method was used to purify the *C. ternatea* constituents.

2.4.4 Separation and identification of plant constituents

In search of plant active constituents, various combinations of separation methods such as extraction, purification and chromatography are essential. The first two methods belong to sample preparation meanwhile the chromatographic methods are used to evaluate the qualitative and quantitative analysis, as well as for isolation purposes (Nyiredy, 2004). In this thesis, bioassay guided TLC was used to separate and purify the bioactive fraction. Subsequently the GC-MS and Fourier Transform Infrared Red (FTIR) were used to identify the purified bioactive fraction.

2.4.4 (a) Thin Layer Chromatography (TLC)

TLC is one of the most extensively used chromatography methods in an organic laboratory (David, 2001). The TLC is a quick method that would separate small quantity of compounds (Raaman, 2008a). TLC is simple, low in cost, flexible and allows multiple detections. It is also used as an ideal screening method in chemical and biological analysis, provides identification and qualitative results and determines adulteration together with quantitative and semi-quantitative determination. Furthermore, TLC is the only technique in which all the components of the sample can be displayed in the chromatogram. In contrast, Gas Chromatography (GC) is selective and not all of the compounds in the sample are included in the display (Marston, 2007).

TLC is classified as solid-liquid chromatography since a solid is used as the stationary phase and a liquid as the mobile phase. The separation of compounds in a sample involves the adsorption principle, thus TLC is regarded as adsorption chromatography and the principle is exactly the same as column chromatography. The compounds are separated due to their different adsorption strengths to the adsorbent (David, 2001).

In simple terms, the mobile phase solvents desorb and dissolve the compounds from their binding to the adsorbent and carry the compounds upwards, in the direction of the movement of the solvent. Thus the compounds move due to the dynamic adsorption and desorption (David, 2001). Compounds which have great affinity for the mobile phase is desorbed and moved from the stationary phase and deposited near the solvent front whereas compounds with a lower affinity is deposited nearer to the origin (R. J. Hamilton & S. Hamilton, 1987). The distance travelled by the compounds are measured and reported as R_f value where

$$R_f = \frac{\text{Distance moved by the compound}}{\text{Distance moved by the solvent front}}$$

2.4.4 (b) Gas Chromatography-Mass Sectroscopy (GC-MS)

GC-MS is an essential analytical technique used for qualitative and quantitative analysis of solid, liquid and gaseous samples (Raaman, 2008b) and also to characterize and identify the volatile organic compounds in complex mixtures (Zhu et al., 2009). This technique also provides information about the structure of a wide variety of complex molecular structure, the isotopic ratios of atoms in samples and the structure and composition of solid surfaces. In general, the samples injected are

converted into gaseous ions and then separated on the basis of their mass-to-charge ratio (Raaman, 2008b).

In gas chromatography, the sample is vapourised and injected onto the head of the chromatographic column (Raaman, 2008b). The column contains a liquid stationary phase which is either coated on to the walls of a narrow column (as in capillary column GC) or on to small particles of an inert, solid material which is packed in wider column (packed-column GC) (Houghton & Raman, 1998c). The samples are transported through the column by the flow of an inert gas (often nitrogen, helium and argon) mobile phase. The components of the mixtures are distributed between a gas and a liquid phase (Raaman, 2008b) and separated due to differences in their relative affinities for the liquid and gaseous phases (Houghton & Raman, 1998c). The rate of migration of the components is determined by how much of it is distributed into the gas phase. The higher the percentage of material in the gas phase, the faster it will migrate. For example, a component that distributes itself 100% into the gas phase will migrate at the same rate as the flowing gas. On the other hand, a species that distributes 100% into the stationary phase, will not migrate at all. Components that distribute themselves partly in both phases will migrate at an intermediate rate (Raaman, 2008b).

The primary criterion for the peaks identification is the retention time (t_R) which is termed as the time between sample injection and an analyte peak reaching a detector at the end of the column (Hu et al., 2006; Raaman, 2008b). The chromatographic detector (mass spectrometer) provides additional data for the