IN VITRO ANTICANCER ACTIVITY GUIDED INVESTIGATION OF THE PHYTOCHEMICALS OF *TYPHONIUM FLAGELLIFORME* (LODD.) BLUME (ARACEAE)

by

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LIST OF SYMBOLS AND ABBREVIATIONS

δ	Chemical shift
J	Coupling constant
I	Spin quantum number
1D	One dimensional
2D	Two dimensional
ACN	Acetonitrile
AR	Analytical grade reagent
ATCC	American Type Culture Collection
BPI	Base peak intensity
BuOH	Butanol
$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
COSY	Homonuclear Shift Correlation Spectroscopy
DAB	Diaminobenzidine
DCM	Dichloromethane
dd	Doublet of doublet
DEPT	Distortionless Enhancement by Population Transfer
DMEM	Dulbeco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dq	Doublet of quadruplet
DQF	Double quantum filtered
dUTP	Biotin-16-deoxyuridine triphosphate

EDTA	Ethylenediaminetetracetic acid
EI	Electron impact ionisation
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fetal bovine serum
FDA	Food and Drug Administration, United States
FT	Fourier Transform
GC	Gas chromatography
GR	General purpose reagent, for analysis
HEPES	N-2 hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HMBC	Heteronuclear Multiple Bond Coherence
HPLC	High performance liquid chromatography
HSQC	Heteronuclear Single Quantum Correlation
IC_{50}	Concentration of a test substance that is required for 50 % inhibition *in vitro*
ICCVAM	Interagency Coordinating Committee on the Validation of Alternative Methods
i.d.	Internal diameter
IR	Infrared
IUPAC	International Union of Pure and Applied Chemistry
m	Multiplet
MEM	Eagle's Minimal Essential Medium
MeOH	Methanol
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National Cancer Institute, United States
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser effect
NOESY	NOE difference spectroscopy
OD	Optical density
PBS	Phosphate buffer saline
PDA	Photodiode array
PDT	Photodynamic therapy
ppm	Parts per million
prep-TLC	Preparative thin-layer chromatography
prep-HPLC	Preparative high performance liquid chromatography
q	Quadruplet
Q-TOF	Quadrupole-Time of flight
R_f	Retention factor
rpm	Revolution per minute
RPMI	Rosewell Park Memorial Institute
rTdT	Recombinant terminal deoxynucleotidyl transferase
s	Singlet
S.E.M.	Standard error mean
Streptavidin-HRP	Horseradish-peroxidase-labeled streptavidin
t	Triplet
TIC	Total ion chromatogram
TLC	Thin layer chromatography

TMS	Tetramethylsilane
TUNEL	rTdT mediated dUTP Nick-End Labelling
UV	Ultraviolet
v/v	Volume by volume
Vis	Visible
WHO	World Health Organisation

KAJIAN FITOKIMIA *TYPHONIUM FLAGELLIFORME* (LODD.) BLUME (ARACEAE) BERPANDUKAN AKTIVITI ANTIKANSER *IN VITRO*

ABSTRAK

Typhonium flagelliforme (Lodd.) Blume merupakan sejenis tumbuhan tempatan yang digunakan sebagai ubat herba tradisional terhadap berbagai jenis penyakit kanser. Untuk mengetahui tindakan farmakologi, keselamatan dan keberkesanan tumbuhan herba ini dengan lebih terperinci, kandungan kimia dan kesan biologinya perlu dikenalpasti dan dicirikan terlebih dahulu.

Suatu kaedah pengekstrakan yang sesuai telah dipilih dengan teliti pada peringkat awal kajian ini. Tiga jenis kaedah pengekstrakan yang mempunyai cara tindakan yang berlainan, iaitu soxhlet, pengekstrakan yang dibantui ultrasonik dan rendaman telahpun dikaji. Kaedah rendaman merupakan kaedah pengekstrakan yang paling sesuai kerana ekstrak yang diperolehi adalah lebih aktif dan mempunyai kepelbagaian kandungan kimia yang tinggi, walaupun ia mempunyai peratusan hasil yang lebih rendah berbanding dengan kaedah-kaedah lain. Kajian juga menunjukkan bahawa pelarut-pelarut tidak polar seperti heksana dan diklorometana paling sesuai digunakan untuk mengekstrak sebatian-sebatian aktif dalam tumbuhan ini.

Tiga fraksi daripada ekstrak diklorometana, iaitu D/F19, D/F21 dan D/F15 telah dikaji. D/F19 merupakan fraksi yang paling aktif terhadap turunan sel NCI-H23 (kanser paru-paru manusia) tetapi ia juga didapati aktif terhadap turunan sel bukan kanser BALB/c 3T3. D/F21 merupakan fraksi yang kedua paling aktif dan mempunyai spesifikasi yang bagus terhadap NCI-H23 berbanding dengan BALB/c 3T3. D/F15 merupakan fraksi utama *Typhonium flagelliforme* yang mempunyai

kesan antiproliferat yang sederhana. Feoforbid-*a* dan pirofeoforbid-*a* merupakan dua sebatian utama yang dipencilkan daripada fraksi D/F19. Metil heksadekanoat, 1-heksadekena, fitol dan suatu terbitan fitol merupakan kandungan kimia yang mungkin terdapat dalam D/F21. Kampesterol, stigmasterol, β-sitosterol, asid palmitik, asid oleik, asid linoleik dan asid linolenik merupakan kandungan kimia D/F15.

Kesan antiproliferat D/F19 didapati bukan dihasilkan oleh komponen tunggal tetapi adalah hasil tindakan sinergistik daripada campuran komponen-komponen. D/F19 megaruhkan kematian sel mengikuti laluan apoptosis. Di samping itu, D/F19 juga didapati mempunyai kesan pengfotopekaan terhadap NCI-H23 dan suatu turunan sel payu dara manusia, HS578T, yang dihasilkan daripada aktiviti feoforbid-*a*, pirofeoforbid-*a* dan suatu lagi subfraksinya, iaitu D/F19/04. Feoforbid-*a* dan pirofeoforbid-*a* merupakan agen pengfotopekaan cahaya yang telah dibuktikan berkesan secara *in vitro* dan *in vivo* untuk digunakan dalam terapi fotodinamik. Fraksi D/F21 didapati mempunyai aktiviti antiproliferat dan dapat mengaruhkan apoptosis. Kesan ini mungkin adalah disebabkan oleh aktiviti fitol. Walaupun kandungan kimia D/F15 merupakan sebatian-sebatian yang biasa ditemui dalam tumbuh-tumbuhan, sebatian-sebatian ini dipercayai dapat menyumbang kepada kesan rawatan *Typhonium flagelliforme* terhadap kanser berdasarkan kepada hasil-hasil penyelidikan lain yang telah dilaporkan.

Secara keseluruhan, kandungan kimia *Typhonium flagelliforme* yang mempunyai aktiviti antikanser secara *in vitro* telah berjaya dikenalpasti melalui pendekatan bioaktiviti berpandu. Kajian ini menyokong penggunaan *Typhonium flagelliforme* secara etnoperubatan terhadap kanser.

IN VITRO ANTICANCER ACTIVITY GUIDED INVESTIGATION OF THE PHYTOCHEMICALS OF *TYPHONIUM FLAGELLIFORME* (LODD.) BLUME (ARACEAE)

ABSTRACT

Typhonium flagelliforme (Lodd.) Blume is an indigenous plant of Malaysia used as a traditional herbal medicine to treat various types of cancer. In order to effectively understand its pharmacological action, safety and efficacy, it is essential to firstly identify and characterise its chemical constituents and their biological relevance to the healing effect of the plant.

A suitable extraction methodology was carefully selected at the preliminary stage of this study. Three types of extraction methods with different modes of action, namely soxhlet, ultrasonic-assisted extraction and maceration methods were evaluated. The maceration method proved to be the most suitable extraction methodology for the purpose of this study with regard to the better activity and greater diversity of the chemical constituents obtained in the extract although the extraction yields were lower. The study also found that extraction carried out using non-polar solvents like hexane and dichloromethane is most suitable in obtaining the active chemical constituents.

Three fractions of the dichloromethane extracts, namely D/F19, D/F21 and D/F15 were evaluated. D/F19 was the most active fraction on NCI-H23 (human lung cancer) cell line but the fraction was also active on BALB/c 3T3 non-tumourous cell line. D/F21 was the second most active fraction with good specificity for NCI-H23 as compared to BALB/c 3T3. D/F15 was the major fraction of *Typhonium*

flagelliforme with moderate antiproliferative effect on NCI-H23. Pheophorbide-*a* and pyropheophorbide-*a* were the two major constituents isolated from D/F19. Methyl hexadecanoate, 1-hexadecene, phytol and a derivative of phytol were tentatively suggested to be the constituents in D/F21. Campesterol, stigmasterol, β-sitosterol, palmitic acid, oleic acid, linoleic acid and linolenic acid were the chemical constituents of D/F15.

The antiproliferative effect of D/F19 did not originate from a single component but was due to synergistic action from a mixture of components. Cell death was induced by D/F19 via the apoptotic pathway. In addition, D/F19 was also found to possess photosensitizing effect on NCI-H23 and a breast cancer cell line, HS578T, mainly due to the activity of pheophorbide-*a*, pyropheophorbide-*a* and another of its subfraction, D/F19/04. Pheophorbide-*a* and pyropheophorbide-*a* are photosensitizers proven *in vitro* and *in vivo* to be effective for use in photodynamic therapy. Fraction D/F21 was found to be antiproliferative and apoptosis inducing. This effect may be due to the result of the activity of phytol. Although the chemical constituents of D/F15 were commonly found in plants, these compounds are believed to also contribute to the healing effect of *Typhonium flagelliforme* against cancer as supported by many reported studies.

In short, the chemical constituents of *Typhonium flagelliforme* which possess *in vitro* anticancer activity have been identified based on a bioactivity guided approach. The study supports the ethnomedical use of *Typhonium flagelliforme* as a herbal remedy towards cancer.

CHAPTER 1

INTRODUCTION

1.1 Medicines from Plants

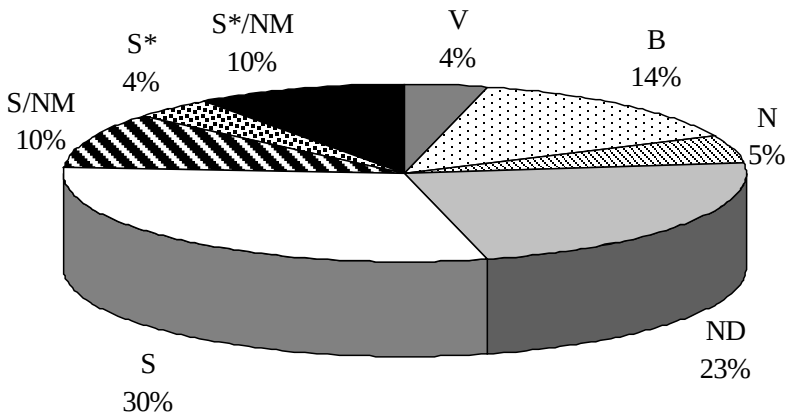
Plants have formed the basis for treatment of diseases in traditional medicine for thousands of years. It is the oldest and most natural form of medicine even at the present time. The World Health Organisation (WHO) estimated that about 80% of the world's inhabitants today still rely on plants as their primary source of medicine and health care agents (Farnsworth et al., 1985). Early drugs or so called 'folk medicine' took the form of crude drugs prepared as tinctures, teas, poultices, powders, infusions and inhalants (Geddes & Grosset, 2000). The drug discovery trend then evolved into the isolation of their active constituents such as codeine, aspirin, quinine and morphine of which some are still in use today (Ng, 2004). Out of 119 chemical substances that were isolated as drugs, above 74 % were discovered as a direct result of chemical studies which focused on the isolation of active substances from plants used in traditional medicine (Farnsworth et al., 1985). However, despite the many successes of plants as a source of medicine, the process of drug discovery from natural products has been a historically slow process, and the traditional natural product extract libraries were not compatible with high-throughput screening (Koehn & Carter, 2005).

In the beginning of 1990s, with the advent of combinatorial chemistry, many big pharmaceutical companies switched their drug discovery strategies to include a large proportion of combinatorial chemistry (Balkenhohl et al., 1996). The ability of combinatorial chemistry to generate huge amount of compound libraries which would then undergo high-throughput screening to identify the lead compounds has been thought to be an efficient approach and thus carries great promise (Lee &

Breitenbucher, 2003). In addition, most of the synthetic compound libraries have no issues with intellectual property right and biodiversity conservation as those experienced with natural products (Baker et al., 1995; Fischli et al., 2002). However, by the mid to end of 1990s, results from the early combinatorial libraries had been a great disappointment with almost no lead compounds ever generated despite a few hits (Lahana, 1999). The inability of the synthetic libraries to generate lead compounds of biological relevance is mainly due to the different chemical space occupied by the synthetic compounds compared to natural products (Butler, 2004). Synthetic compounds have lower molecular weights, higher number of nitrogen, halogen and sulphur atoms but lower content of oxygen than natural products (Henkel et al., 1999). In addition, these compounds have less chiral centres, less complex structures and are greater in flexibility compared to natural products. Structural flexibility results in entropic losses that weaken the binding with protein molecules where the therapeutic effect is exerted (Feher & Schmidt, 2003).

In the present decade, there has been a renewed interest in natural product research due to the failure of other alternative drug discovery methods to produce lead compounds in key therapeutic areas particularly immunosuppresion, antiinfectives, anticancer and metabolic diseases (Butler, 2004). Although natural products isolated as the active compounds were sometimes not suitable for direct development into an effective drug, it can provide a suitable lead for conversion into clinically useful agents (Young, 1999; Hall et al., 2001). Some of the examples of new drugs derived from natural sources are apomorphine, which is a morphine derivative isolated from *Papaver comniferum* used to treat Parkinson's disease; arteether, an antimalarial drug developed from artemisinin isolated from *Artemisia annua*; vinflunine, an improved anticancer agent derived from vinblastine isolated

from *Catharanthus roseus*; tiotropium, a derivative of atropine isolated from *Atropa belladonna* for the treatment of bronchospasm associated with chronic obstructive pulmonary disease and many others (Balunas & Kinghorn, 2005; Chin et al., 2006). Statistics have shown that among the drugs approved by FDA from 1981 to 2006, those of natural products origin contributed a substantial amount of 52 % (Cragg et al., 1997; Newman et al., 2003; Newman & Cragg, 2007) as shown in Figure 1.1. Thus this indicates that natural products still play an important and irreplaceable role as a source of modern pharmaceuticals.



Legends:
V = vaccine
B = biological such as peptide or protein
N = natural product
ND = derived from a natural product (semisynthetic)
S = totally synthetic
S* = synthetic but pharmacophore was from a natural product
NM = natural product mimic

Figure 1.1: Drugs approved by FDA from Jan 1981 to June 2006 by source (total number of drugs, *N* = 1184). (Source: Newman & Cragg, 2007, p.472).

1.2 Plants as a Source of Anti-cancer Agents

According to the National Cancer Institute (NCI) of the United States, cancer is defined as 'a disease in which abnormal cells divide without control and are able to invade other tissues' (National Cancer Institute, 2009). This disease occurs mainly due to the mutation of certain genes that resulted in the disruption of cell growth regulation, repair of cell damage and growth inhibition (King & Robins, 2006). Human cancers can generally be classified into several categories. Carcinomas are malignant tumours that originated from epithelial tissues. Sarcoma refers to cancer that is mesenchymal in origin, such as the bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Neoplasms of the hematopoietic system are termed leukemia if it originates from the blood forming tissue, or lymphoma if it begins from the cells in the immune system (Ruddon, 1995).

Cancer is one of the major diseases affecting mankind with approximately 10.9 million people around the world diagnosed with cancer every year of which 45% are from Asia (Ferlay et al., 2004). Mortality as a result of cancer is estimated to be 6.7 million per year, which is 12% of total deaths that occur annually (Ferlay et al., 2004; Ma & Yu, 2006). In Malaysia, cancer is the fourth leading cause of death among the medically certified deaths with an annual incidence of 30,000 people (Lim, 2002). It is estimated that out of four Malaysians, one is expected to get cancer in his/her lifetime. The most common cancers found among men are lung, nasopharynx, colon, leukaemia, rectum and prostate while in women the common types of cancer are breast, cervix, colon, ovary, leukaemia and lung (Lim et al., 2003).

The approaches to the treatment of cancer are surgery, radiotherapy, immunotherapy and chemotherapy. Surgery is usually the first choice of treatment with the goal to remove totally or at least reduce the mass of tumour. However, in

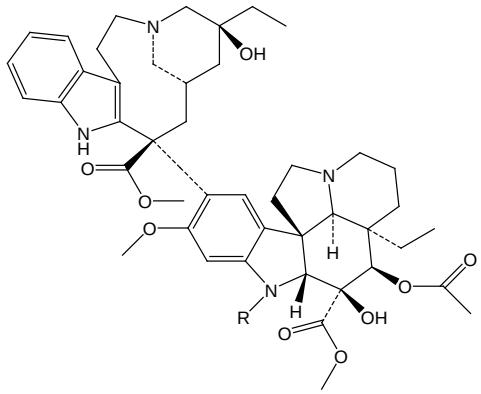
many cases, secondary tumours have established by the time the primary tumour is detected, thus chemotherapy and radiotherapy usually follow after surgery (King & Robins, 2006). Chemotherapy involves the use of cytotoxic or cytostatic drugs to kill or prevent malignant cells from proliferating (Cragg et al., 1996). The efficacy of the drug depends on the concentration of the drug that reaches the tumour, the duration of exposure and the proportion of the population that is proliferating. In many cases however, the action of chemotherapeutic drugs are not specific towards malignant cells alone but affects proliferating cells throughout the body and can be toxic to vital organs such as the heart and kidney, resulting in numerous untoward side effects. To minimise these unwanted effect, many chemotherapists use combinations of drugs which have additive effects against tumours but have less toxicities to normal cells (Boyer & Tannock, 1998).

Alkylating agents are drugs that act through covalent bonding of the alkyl groups to intracellular macromolecules. The formation of a positively charged intermediate compound combines with the electron-rich nucleophilic groups such as amino, phosphate, sulfhydryl or hydroxyl moiety that are present in the DNA, resulting in lethal toxicity to the cell. Examples of anticancer drugs in this category are such as the nitrogen mustards and nitrosoureas (Neidle & Thurston, 2005).

Antimetabolites are drugs that inhibit nucleic acid synthesis and prevent normal cell division from occurring. Examples of drugs in this category are methotrexate, 5-fluorouracil and cytosine arabinoside. These drugs are cell cycle-specific and exert toxicity on other proliferating cells such as the bone marrow cells and gastrointestinal mucosa (Highley et al., 2009).

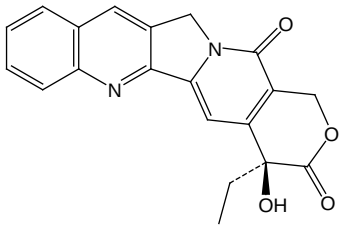
The third category of anticancer drugs is the natural products. It covers a substantial percentage of the total amount of anticancer drugs introduced in the time

frame of 1981–2006, whereby 77.8% were either natural products per se or derived from natural products (Newman & Cragg, 2007). There are four major classes of natural products based anticancer drugs which are derived from plants and these have quite diverse mechanism of actions. The first anticancer drugs of this category are the vinca alkaloids, vincristine and vinblastine, isolated from the Madagascar periwinkle, *Caranthus roseus*. These compounds block mitosis with metaphase arrest by binding specifically to tubulin resulting in its depolymerization (Okouneva et al., 2003) and are useful for the treatment of leukaemia, lymphoma, breast and lung cancers (Cragg & Newman, 2005). Another class of compounds which also affect the microtubules by causing tubular bundling are the taxanes, paclitaxel and docetaxel, extracted from pacific yew tree *Taxus brevifolia* (Hirose & Takiguchi, 1995). Paclitaxel is used primarily for the treatment of breast, ovarian and non-small cell lung cancer (Wall & Wani, 1996). The epipodophyllotoxins, etoposide and teniposide, derived from the mandrake plant *Podophyllum peltatum* are DNA topoisomerase II inhibitors. These drugs are used against lymphoma, bronchial and testicular cancers (Gordaliza et al., 2004). Camptothecin is a selective inhibitor of topoisomerase I which was isolated from the stem bark of the Chinese tree *Camptotheca acuminate*. It is used against breast, lung and colorectal cancers (Wall & Wani, 1996). Many other drugs which are derived from these four major classes of compounds are currently under clinical trials (Balunas & Kinghorn, 2005). The chemical structures of some of the anticancer agents derived from plants are given in Figure 1.2.

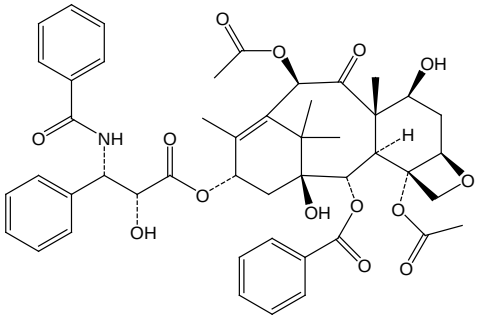


Vinblastine: R = CH_3

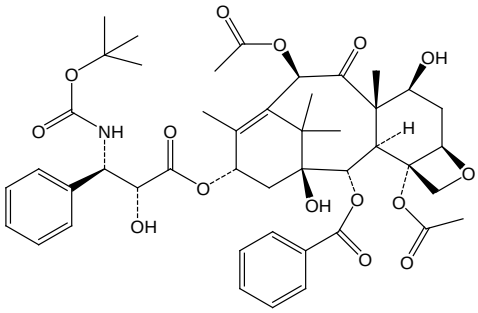
Vincristine: R = CHO



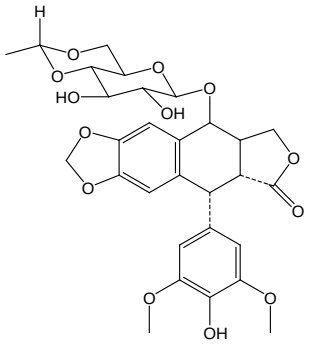
Campthothecin



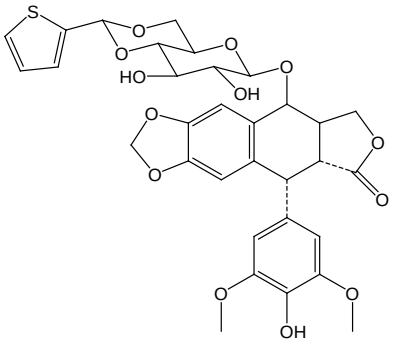
Paclitaxel



Docetaxel



Etoposide



Teniposide

Figure 1.2: Anticancer agents derived from plants.

There are approximately 250,000 plant species distributed throughout the world and more than half of these are found in the tropical rainforests (Wilson, 1988).

So far, only 1% of tropical species have been explored for their pharmaceutical potentials and there remain a substantial opportunity for finding more new compounds of pharmaceutical interests (Zuhud, 1991; Fakim, 2006). The vast variety of flora and fauna hosted by the tropical rainforests provide natural product chemists with numerous compounds as starting point for the development of new anticancer drugs.

Malaysia is home to about 40,000 species of higher plants of which about 36,000 are flowering plants, 3,600 are ferns and 87 are conifers (Soepadmo, 1998). In addition to the magnificent array of plant species, Malaysia has a unique confluence of traditional medicine system due to the diverse ethnic communities in the country. The rich ethnobotanical and ethnopharmacological knowledge in the use of plants has provided a good starting point for Malaysian scientists for locating useful tropical plants for phytochemical investigation. To make use of these advantages, the present study selected *Typhonium flagelliforme* as the subject of study since it has been used locally as an alternative treatment for cancer but has not been investigated in depth scientifically.

1.3 *Typhonium flagelliforme* (Lodd.) Blume

1.3.1 Taxonomy and Distribution

Typhonium flagelliforme (Lodd.) Blume is a herbal plant indigenous to Malaysia which belongs to the family of Araceae under the subfamily of Aroid. The tuber of the plant is nearly round and measures about 1 cm in diameter. The leaves are green and smooth, typically narrowly hastate with spreading basal lobes but may be elliptic sometimes. The size of the blade is about 6 × 4 cm. The petiole is yellowish and thin, it measures about 6–20 cm long, partially enclosed in the spathe.

The colour of the spathe is green with brownish margin, the limb is linear acute, dilated and round at the base. The flowers are unisexual (Hsuan et al., 1998; Nicolson & Sivadasan, 1981). A picture of *Typhonium flagelliforme* is given in Figure 1.3.

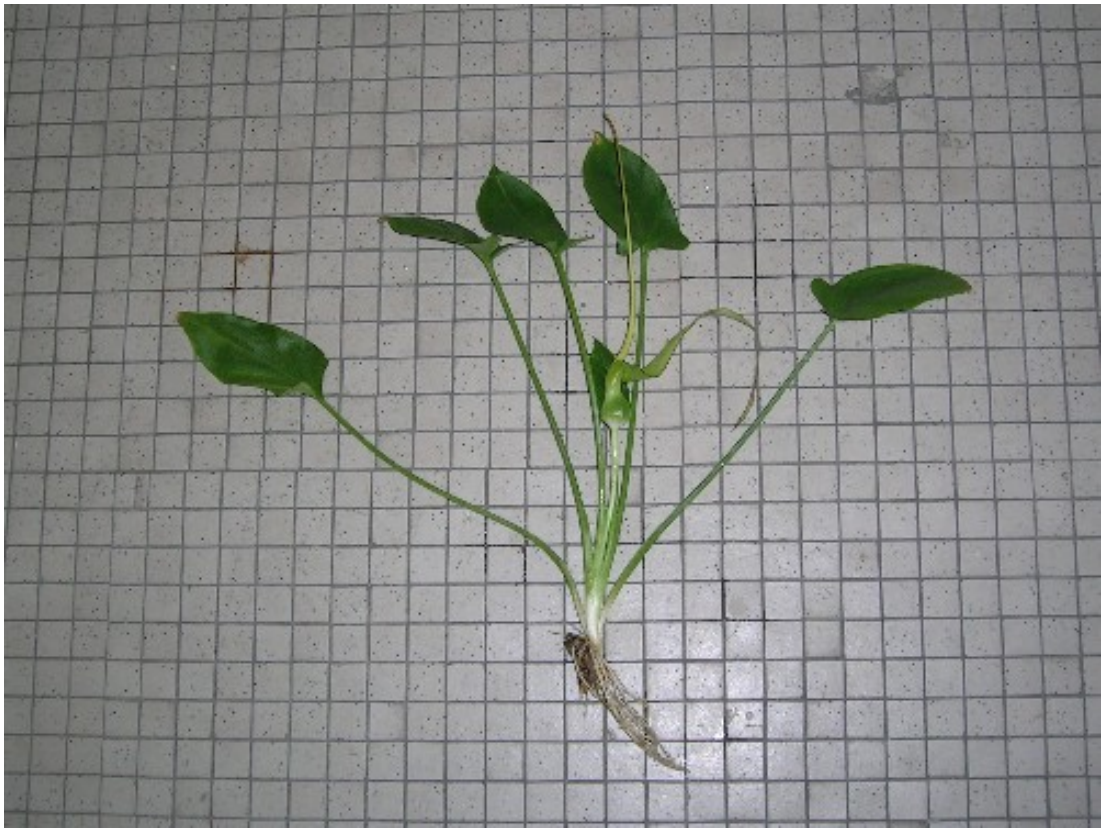


Figure 1.3: Picture of *Typhonium flagelliforme*.

Typhonium flagelliforme, which was previously mistakenly regarded as *Typhonium divaricatum*, is distributed across IndoChina, Peninsular Malaysia, Northern Queensland, South India, Sri Lanka and Luzon as shown in Figure 1.4 (Nicolson & Sivadasan, 1981). It grows wild in waste grounds and is native to several parts of Peninsular Malaysia such as the ditches in Melaka town and the Sepoy lines in Penang (Ridley, 1967). However, due to developments and

deforestations, these plants are no longer found in the mentioned areas but are mostly cultivated in pots and garden yards for personal consumption.

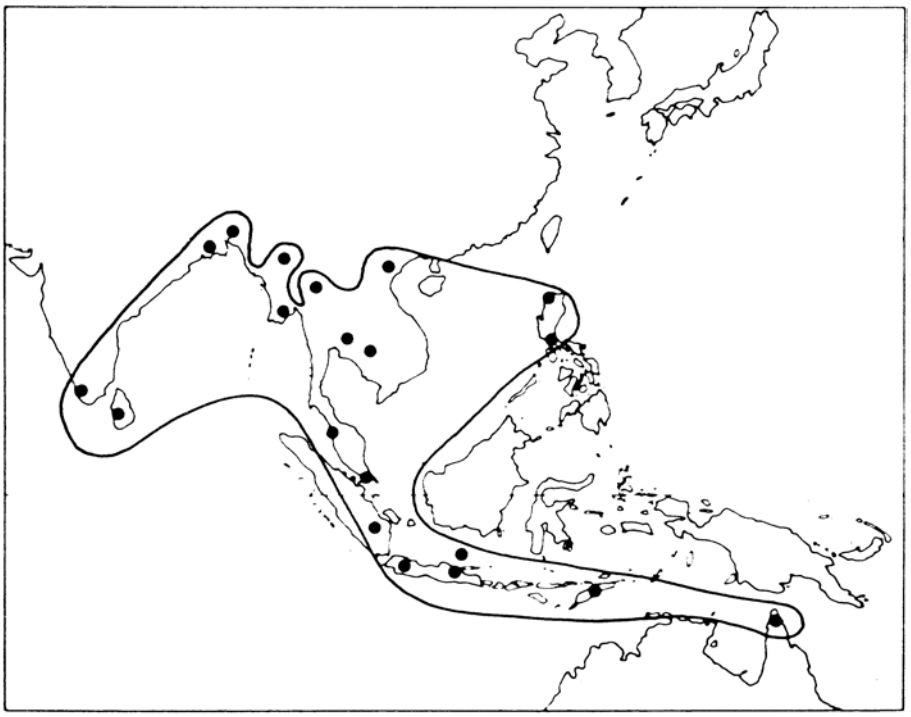


Figure 1.4: Geographical distribution of *Typhonium flagelliforme* (Source: Nicolson & Sivadasan, 1981, p.491).

1.3.2 Ethnomedical Background

Typhonium flagelliforme, known by its vernacular name of Keladi Tikus (Malay) or Rodent Tuber (English), is described as toxic, warming and phlegm-resolving in the Malaysian Traditional Chinese Medicine. The leaves or whole plant is often applied over swellings and used against various kinds of malignancy. The roots are used to eliminate expectoration (Lee & Wong, 2004). There have been many accounts on the uses of *Typhonium flagelliforme* for treating cancer, including colon, breast, prostrate, liver, lung and bone cancer. Many cancer patients who have tried such alternative therapy have claimed successes while others who took this

during the course of chemotherapy have experienced significant improvement in appetite, reduction in hair loss with no cases of nausea and vomiting (Teo & Ch'ng, 1999).

The use of three other species of *Typhonium* has also been documented in the Traditional Chinese Medicine and Ayurvedic system for the treatment of cancer. In China, the tuber of *Typhonium giganteum*, the major component of Herbal Medicine Rhizoma Typhonii is applied externally in the form of paste to the lesion for the treatment of reticulocytic sarcoma and lymphoma (Chang, 1992). In India, the poultice of *Typhonium trilobatum* is used to treate Scirrhous tumours and cancerous tumours, while *Typhonium orixense* is used by the Indians and Pakistanis to treat Scirrhous tumours (Hartwell, 1982).

Each preparation of *Typhonium flagelliforme* requires the use of approximately 50 g of the fresh whole plant. The plant is pounded briefly using a mortar and pestle to break the cell walls and the juice is then forced out through a thin cloth. The juice extract is then mixed with honey in order to prevent irritation to the throat and is recommended to be consumed immediately. Initially, the juice is to be drunk 3 times daily and can be reduced to a maintenance dose of twice a week if improvement is observed. This can either be used as a single therapy or taken while undergoing the course of chemotherapy or radiotherapy (Neoh, 1992; Teo & Ch'ng, 1999). There are also other less commonly used preparative methods of *Typhonium flagelliforme*, such as wrapping 2–6 pieces of the plant leaves in Longan flesh for direct consumption (Lee & Wong, 2004) and pulverising the dried plant material to prepare as a drink which is not documented. Of late, encapsulated dried plant powder of the plant is also available commercially (Global Bioscience, 2007).

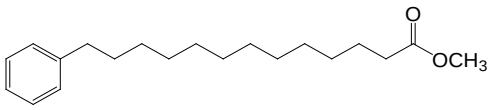
1.3.3 Pharmacological Studies

In vitro studies carried out on the various extracts of *Typhonium flagelliforme* (then wrongly referred as *Typhonium divaricatum* (Nicolson & Sivadasan, 1981) revealed that the hexane, chloroform ($CHCl_3$) and ethyl acetate (EtOAc) extracts exhibited significant cytotoxic effect on a murine lymphoid cell line with the rate of inhibition above 90%. However, the concentration of the extract tested and the cell line designation was not specified (Neoh, 1992). Choo et al. (2001a) had also observed cytotoxic activity for the hexane and $CHCl_3$ extracts on P388 leukemia cells and the IC_{50}s were found to be 15 μg/ml and 6 μg/ml respectively for the root extracts, 65 μg/ml and 8 μg/ml respectively for the stems/leaves extracts. Chan et al. (2005) also found the hexane, $CHCl_3$ and *n*-butanol (*n*-BuOH) extracts to be active towards NCI-H23 human lung cancer cell line at IC_{50} 1.8, 5.2 and 1.2 μg/ml, respectively, and T-47D human breast cancer cell lines at IC_{50} 12.2, 23.0 and 54.6 μg/ml, respectively.

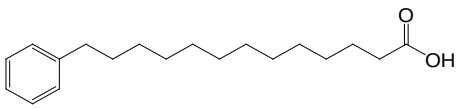
A study on the effect of *T. flagelliforme* on the immune system of cat fish found that the plant, given as intraperitoneal injections, was able to increase phagocytic activity and other non-specific immune response. Resistance of the cat fish against live *Vibrio parahaemolyticus* was also found to have increased (Lam, 1998). Another *in vivo* study conducted in rats showed that *T. flagelliforme* juice extract had a similar effect as glycyrrhizin in preventing hepatocarcinogenesis (Karuppiah et al., 1999). Other pharmacological studies also showed that the water, alcohol and ester extracts of *Typhonium flagelliforme* is antiasthmatic, antiinflamatory, analgesic, sedative, antiexpectoration and cough relieving (Zhong et al., 2001).

1.3.4 Phytochemicals

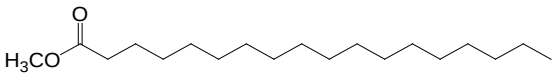
Chen et al. (1997) reported the isolation of benzenetridecanoic acid (synonym: 13-phenyltridecanoic acid) and methyl benzenetridecanoate (synonym: methyl 13-phenyltridecanoate) from the ethyl acetate extract of *Typhonium flagelliforme*. Four other esters, namely methyl hexadecanoate, methyl octadecanoate, methyl 9-octadecenoate and methyl 9, 12-octadecadienoate were also identified by Choo et al. (2001b) from the hexane extract of *Typhonium flagelliforme* in addition to methyl 13-phenyltridecanoate. However, despite the earlier report by Choo et al. (2001a) which showed that the $CHCl_3$ extracts were more active than the hexane extracts, no study was carried out on the $CHCl_3$ extracts. Huang et al. (2004a,b) had isolated several glycosides, namely coniferin, methylconiferin, 12-hydroxy-*N*-(3-hydroxy-1-O-β-glucopyranosyloctadeca-4*E*,8*E*-dienyl)octadecanamide and two sterols, β-sitosterol and β-daucosterol from the *n*-BuOH fraction of *Typhonium flagelliforme*. The chemical structures of the constituents are illustrated in Figure 1.5. Except for methyl 13-phenyltridecanoate that was found to be inactive *in vitro*, none of the chemical constituents had been tested for anticancer activity in the studies mentioned and none was used to support its ethnopharmacological application against cancer.



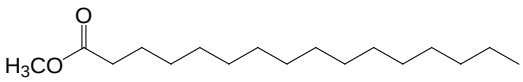
Methyl 13-phenyltridecanoate



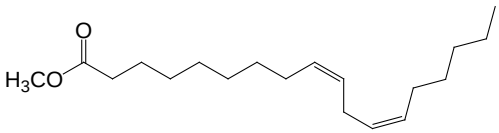
13-Phenyltridecanoic acid



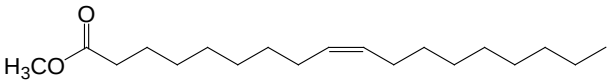
Methyl octadecanoate



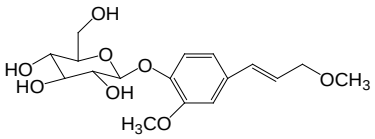
Methyl hexadecanoate



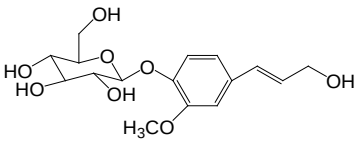
Methyl 9,12-octadecadienoate



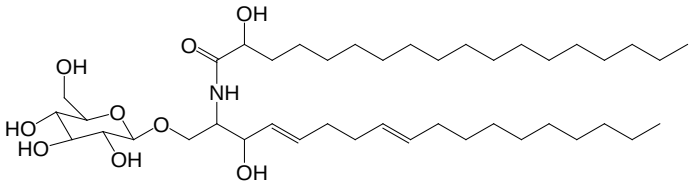
Methyl 9-octadecenoate



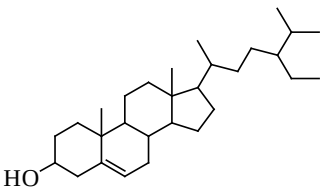
Methyl coniferin



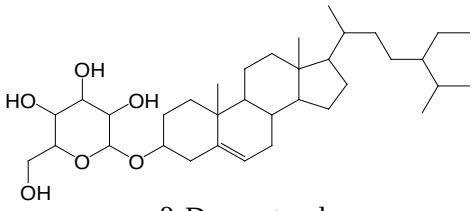
Coniferin



2-Hydroxy-*N*-(3-hydroxy-1-O-β-glucopyranosyloctadeca-4*E*, 8*E*-dienyl)octadecanamide



β-Sitosterol



β-Daucosterol

Figure 1.5: Chemical structures of the compounds isolated from *Typhonium flagelliforme*.

1.4 Methods of Plant Extraction

Extraction can be carried out on either fresh or dried plants. Ideally, fresh plant tissues should be used for phytochemical investigation right after collection (Harborne, 1998). However, if extraction is to be carried out a few days after collection, it should be stored dry in a plastic bag (Harborne, 1998). Alternatively, dried plants can also be used and has several advantages. Unlike in the case of fresh plant, solubility of the chemical constituents in the dried plants is not affected by the presence of water. It is also easier to handle when large scale extraction is carried out due to the lower mass to plant ratio (Eloff, 1998). However, when dried plant is intended for use, the material needs to be dried rapidly to prevent degradation of the components by air or microorganisms. If a vacuum system is used for drying, care should be taken in order to avoid excessive loss of the volatile constituents which may possess interesting biological activity (Fakim, 2006).

Nowadays, many extraction methods are available. The more commonly used methods are such as cold extraction, hot percolation, soxhlet, supercritical fluid extraction, ultrasonic-assisted solvent extraction and pressurized liquid extraction (Péres et al., 2006; Barnes et al., 2009). However, careful selection of the right extraction methodology is needed so that it is adaptable to the intended study (Fakim, 2006). The improper extraction methods may result in degradation and loss of bioactive natural products which seriously hampers the outcome of the study (Davey et al., 2009). Three common and inexpensive extraction methodologies that were evaluated in this study are briefly introduced in the following sections.

1.4.1 Maceration

The maceration method is the simplest but yet still widely used procedure. Pulverised plant material is allowed to soak in solvents of differing polarity in a closed container at room temperature (Fakim, 2006). The metabolites are allowed to diffuse into the solvent and the period of extraction may differ from few hours to several weeks. Occasional or constant stirring is used sometimes to speed up the extraction. The disadvantage of this method is that it consumes large volumes of solvents and some of the poorly soluble metabolites may not be extracted efficiently since extraction is carried out at room temperature (Seidel, 2006).

1.4.2 Soxhlet Extraction

Soxhlet extraction is a classical and most widely used extraction method. Pulverised plant material is placed in a cellulose thimble in an extraction chamber which is located above a collecting flask but beneath a reflux condenser. A suitable solvent is then added into the soxhlet apparatus and the solvent is heated to a boil. The solvent vapour rises and condenses into the thimble. When the thimble is filled with the solvent, it is siphoned back into the distillation flask and the whole cycle is then repeated (Pavia et al., 1995). The main advantage of Soxhlet extraction is that the solvent, when saturated with solubilized metabolites, is emptied into the distillation flask and fresh solvent is recondensed into the extraction thimble continuously, thus making the extraction procedure more efficient and solvent saving. However, the major disadvantage of this method is that the extraction carried out at high temperature may degrade thermolabile compounds and initiate the formation of artefacts (Seidel, 2006).

1.4.3 Ultrasonic-assisted Solvent Extraction

The ultrasonic-assisted solvent extraction is a technique modified from the maceration method. This solvent extraction method is facilitated by the use of ultrasound (frequency pulses approximately 20 kHz) and is one of the well established and commonly used methods today for extracting plant materials for bioactive principles (Sališová et al., 1997; Trusheva et al., 2007). The pulverised plant material is soaked in a suitable solvent in a container and ultrasound is used to induce mechanical stress on the plant which results in the formation of cavitations in the sample. The cellular breakdown allows easier access of the solvent into the cells thereby increases the solubility of the metabolites in the solvent. In addition the ultrasound also facilitates mass transfer and thus is a more efficient method of extraction (Vinatoru et al., 1997). However, the huge amount of energy generated increases extraction temperature and thus a cooling system is required in order to control the temperature.

1.5 Techniques of Separation

1.5.1 Thin-layer Chromatography

Thin-layer chromatography (TLC) is a simple, cheap and quick method for analysis and screening of natural products. Thin layers of adsorbents, usually silica gel or alumina, is coated on glass, plastic and aluminium sheets. The TLC sheets can be cut into strips of various sizes according to the desire of the user. A mixture of natural products is then spotted near the bottom of the thin strip and developed in a jar that contains saturated atmosphere of the eluting solvent. The eluting strength of the solvent is varied by mixing two or more solvents with different polarity. As the mobile phase moves up the plate through capillary action, the components of the

mixture are carried along at different rates and hence separate from one another. The development of the plate is complete when the solvent front has advanced nearly to the top of the plate. The separated compounds on the TLC plate can be detected by ultraviolet (UV) illumination or spray with suitable detection reagent. TLC is frequently used to determine the optimal combinations of solvent for preparative column chromatography separations and to monitor the progress of separation by column chromatography (Gilbert & Martin, 1998).

Preparative thin-layer chromatography (prep-TLC) is a scaled up procedure of TLC and is used to separate compound mixtures at a larger scale. Prep-TLC is normally used as a final purification step in compound isolation, most frequently after column chromatography. The chromatographic conditions for prep-TLC can be predetermined using a TLC plate by trial and error experimentation and then scale up in proportion using a similar or slightly less polar solvent combination. The separated compounds are either detected by the non-destructive UV method or by using a spray reagent. When using a reagent, only a small section at the edge of the plate is sprayed, and the unreacted section of a particular separation band is scraped from the plate and desorbed from the silica sorbent using an appropriate solvent that is able to dissolve the compound (Gibbons, 2006).

1.5.2 Column Chromatography

Column chromatography plays a very important role in the separation of compounds from a complex mixture of natural products. The principle of separation takes place through selective distribution of the components between the mobile phase, which is the eluting solvent, and the stationary phase, which is usually the silica gel. Different compounds have different adsorption affinities for the surface of

the stationary phase and the extent of adsorption is governed by the types of interaction between the compound molecule and the stationary phase such as hydrogen bonding, Van der Waals forces, dipole-dipole interactions, acid-base properties and others (Gilbert & Martin, 1998). The column can be developed using various eluting techniques such as gravity, application of nitrogen pressure at the inlet, vacuum suction or controlled by a pump. A solvent gradient is applied for separation of complex mixtures of compounds where the composition of the solvents is varied in steps so as to gradually increase the eluting strength of the mobile phase. The different rate of movement of the chemical constituents along the column then results in the separation of the compound mixture (Reid & Sarker, 2006). However, for a complex mixture of natural products such as crude extracts, compounds with similar polarity often co-elute in the same fraction and thus, chromatography may be carried out several times using different chromatographic conditions in order to obtain reasonably pure compounds.

Nowadays, the use of flash column chromatography has largely replaced classic column chromatography techniques. Flash column chromatography is an air pressure driven column chromatography which has been optimized for rapid separations. The smaller particle size of the silica gel and rapid flow rate was able to overcome poor separation recovery which is a problem faced in classic column chromatography due to band tailing (Landgrebe, 1993). In addition, the technique is inexpensive, easy to operate and allows separation of samples weighing between 0.01–10.0 g in a short period of time (Still et al., 1978). This method is currently a preferred technique for routine large scale purification of natural products.

1.5.3 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a modern day analytical technique derived from the same working principle as for column chromatography. Compared to classical column chromatography, HPLC offers major advantages in convenience, speed, accuracy, selectivity and the ability to carry out difficult separations owing to the improvements in equipment sophistication, material, technique and the application of theory (Rouessac & Rouessac, 2007). The general setup of a HPLC system consists of a high-pressure pump, an injection port, a column which is the stationary phase, and a detector. The sample is introduced into the injection port and is carried by the stream of mobile phase delivered by the pump which then passes through the column where the separation takes place. Separation is then monitored with a detector.

Two modes of adsorption chromatography are available in HPLC, i.e. normal-phase and reversed-phase chromatography. Reversed-phase HPLC is generally preferred by many researchers as it is applicable to a wide range of chemical compounds. This mode of separation utilises the combination of a non-polar stationary phase and a polar mobile phase which is usually a combination of acetonitrile and/or methanol with an aqueous solution. Acids, bases or buffers are frequently used as the aqueous solution with the purpose of suppressing compound ionisation and to control the degree of ionisation of free unreacted silanol groups in the column that can result in peak tailing which in turn affect the separation (Snyder & Kirkland, 1979).

For a long time the size of the HPLC column have been standardised around the internal diameter of 3–4.6 mm, which requires a mobile phase flow rate of 0.5–2 ml/min. However, a wide choice of column now exists such as narrow-bore (2–4 mm

i.d.), microbore (1–2 mm i.d.) and even packed capillaries (<1 mm), for which the flow rate descends to a few μl/min and thus requires special pumps and detectors. Such developments were necessary as there has been increased demand for the determination of analyte at trace level concentrations over the recent years (Rouessac & Rouessac, 2007). Ultra high-performance liquid chromatography with the trade name of UPLC (Waters) or UHPLC (Agilent) is an improved version of HPLC. With the UPLC or UHPLC pump capable of operating at extremely high pressure limit, the system is designed to be used with sub-2 μm particle columns leading to exceptionally better resolution, sensitivity and separation speed due to the much improved column efficiency. Furthermore, when the system is integrated with a mass spectrometer, often termed as 'hyphenation', it allows provisional identification of an unknown natural product sample, thus further expand the potential and versatility of the analytical instrument (Niessen, 1999).

While an analytical HPLC is used for qualitative and quantitative determination of a compound, a preparative HPLC (prep-HPLC) is meant for isolation and purification of a compound. Purification of natural products using prep-HPLC has been widely adopted by many synthetic and natural product chemists to date as the demand for higher throughput could be better met in an automated prep-HPLC system. Moreover, the reproducibility, efficiency and consistency in compound separation are better in prep-HPLC compared to conventional column chromatography. Depending on the amount of sample intended to be purified, a wide range of prep-HPLC columns are available to cater for sample from μg scale up to kg level (Huber & Majors, 2004).

1.6 Compound Identification and Structure Elucidation

1.6.1 Ultraviolet-visible Spectroscopy

Natural products absorb light in the ultraviolet or visible (UV-Vis) region of the electromagnetic spectrum. Absorption of the UV-Vis light occurs when the energy of the incident radiation is the same as the electronic excitation of the molecule (Lambert et al., 1976). An unknown natural product can be identified according to the complexity of its spectrum and the general position of the wavelength maxima. Different classes of compounds have their characteristic light absorption spectra and the identification of the class of compounds can be enhanced by performing measurements on the sample solution at different pH or in the presence of a particular inorganic salt and then observing the shift of the absorption band (Harborne, 1998).

1.6.2 Infrared Spectroscopy

Infrared (IR) radiation is the part of electromagnetic spectrum in between the visible and microwave regions. The portion which is of greatest practical use for a natural product chemist is the mid-IR region, which ranges from 4000 – 400 cm^{-1}. A molecule that possesses an oscillating dipole absorbs energy from the oscillating electric field of a beam of infrared radiation when the IR frequency of the applied electric field is similar to the frequency of the oscillating dipole. Except for homonuclear diatomics, practically all molecules have some vibrations that produce a change of dipole moment (Lambert et al., 1976). The wavelength of absorption depends on the relative masses of the atoms, the force constants of the bonds between the atoms and the geometry of the atoms (Silverstein, et al., 2005).

A mid-IR spectrum can generally be evaluated in two parts. The region between 4000 – 1300 cm^{-1} is called the functional group region where characteristic stretching frequencies for important functional groups, such as O-H, N-H, C-H, C=O and C=C, are observed. The region between 1300–900 cm^{-1} is referred to as the "fingerprint" region where the complex absorption bands originated from interacting vibrational modes. Absorption at this region is unique for every type of molecule and can be used to cross examine the types of vibration in reference to the other regions. Due to its simplicity and reliability in identifying the functional groups, IR spectroscopy is an extremely useful method of assigning a compound to its class (Silverstein et al., 2005; Harborne, 1998).

1.6.3 Nuclear Magnetic Resonance Spectroscopy

Nuclear Magnetic Resonance (NMR) Spectroscopy is practically an indispensable tool used for the identification and structure elucidation of natural products. Akin to IR and UV spectroscopy, it is another form of absorption spectroscopy that takes place in the radio frequency region. All nuclei are charged particles and some in addition have spin angular momentum. The nuclei of interest are those that have appropriate spin properties (spin quantum number, $I \neq 0$) and generate a magnetic field. ^{1}H and ^{13}C nuclei (both having $I = ½$) have the widest application for identification of organic molecules.

In the presence of an external magnetic field, a spinning nucleus absorbs radiofrequency radiation, which has a magnitude that is identical to the Larmor frequency, resulting in nuclear transition to a higher energy spin state (Silverstein et al., 2005). However, a nucleus within a molecule experiences a lesser magnetic field than the applied external field. This phenomenon is due to the presence of electron

cloud around the nucleus which generates an induced magnetic field opposed to the external field termed as the “shielding effect”. The density of the shielding varies with the chemical environment of the nucleus and the variation gives rise to differences in chemical shift positions and the ability to distinguish among individual absorptions, hence the usefulness of NMR spectroscopy (Macomber, 1998). Chemical shift is measured relative to a reference compound, which is usually tetramethylsilane (TMS).

Based on a ^{1}H NMR spectrum, the relative positions of the various types of protons in a molecule can be determined by evaluation of its chemical shifts, spin-spin couplings and peak integral. Similarly, a ^{13}C NMR spectrum provides information on the relative positions of various types of carbons in a molecule. Due to the low abundance of ^{13}C nucleus and high abundance of ^{1}H nucleus in nature as well as the large C-H coupling constants, a proton-coupled carbon spectrum usually shows complex overlapping multiplets which are difficult to interpret. Thus, for routine analysis of sample, a broadband proton-decoupled ^{13}C spectrum is frequently used, where each type of carbon nucleus is represented by a single peak, making the interpretation much simpler (Silverstein et al., 2005). Distortionless enhancement by population transfer (DEPT) ^{13}C NMR displays separate subspectra for CH, CH_2 and CH_3 carbon signals. Three types of DEPT experiments using three different pulse angles, θ, at 45°, 90° and 135° are available. A combination of DEPT experiments helps distinguish the types of protonated carbons. Any remaining carbon signal in the normal ^{13}C spectrum which is not present in the DEPT subspectra would belong to the quaternary carbons (Macomber, 1998).

Two-dimensional (2D) NMR spectroscopy is also referred to as correlation spectroscopy where the correlation between two or more types of the same or