TRADITIONALLY USED THAI MEDICINAL PLANTS:

IN VITRO ANTI-INFLAMMATORY, ANTICANCER AND ANTIOXIDANT ACTIVITIES

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THIS THESIS DESCRIBES RESEARCH CONDUCTED IN THE SCHOOL OF PHARMACY, UNIVERSITY OF LONDON BETWEEN OCTOBER 2006 AND JUNE 2010 UNDER THE SUPERVISION OF PROF. MICHAEL HEINRICH. I CERTIFY THAT THE RESEARCH DESCRIBED IS ORIGINAL AND THAT ANY PARTS OF THE WORK THAT HAVE BEEN CONDUCTED BY COLLABORATION ARE CLEARLY INDICATED BY SUITABLE CITATION.

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SIGNATURE DATE

ABSTRACT

INTRODUCTION:

This thesis presents a panel of the anti-inflammatory, cytotoxic and antioxidant activities of nine plant species, which have been selected from Thai textbooks, in order to assess the traditional claims about the therapeutic potential and to select plants for further phytochemical research, of active compounds through bioassay-guided fractionation procedures.

METHODS:

Anti-inflammatory, Nuclear factor-kappa B (NF-κB) inhibitory effects on PMA-induced NF-κB activation in stably tranfected HeLa cells were determined by luciferase assay, and the effects on LPS-induced pro-inflammatory mediators prostaglandin E_2 (PGE_2), interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)α in primary monocytes were assessed by ELISA. Cytotoxic activities were examined against cervix cancer HeLa cells, human leukaemia CCRF-CEM cells and the multidrug-resistant CEM/ADR5000 cells using the MTT and XTT tests. However, as redox status has been linked with both inflammation and cancer, antioxidant effects were also assessed using the DPPH, lipid-peroxidation, and Folin- Ciocalteau methods. Phytochemical investigation of active compounds from the methanol extract of *G. pseudochina var. hispida* was carried out using Sephadex LH-20 column chromatography, TLC and HPLC as well as NMR and MS spectroscopic techniques.

RESULTS:

Among the nine species, the methanol extract of *Gynura pseudochina var. hispida* and the ethylacetate extract of *Oroxylum indicum* showed the most promising NF-κB-inhibitory effects with the lowest IC_{50} values (41.96 and 47.45 μg/ml, respectively). The ethyl acetate and methanol extracts of *Muehlenbeckia platyclada* did not inhibit the NF-κB activation but effectively inhibited the release of IL-6, IL-1β and TNF-α with IC_{50} values ranging between 0.28 and 8.67 μg/ml. The petroleum

ether extract of *Pouzolzia indica* was the most cytotoxic against CCRF-CEM cells and the multidrug resistant CEM/ADR5000 cells (9.75% and 10.48% viability, at 10 μg/ml, respectively). The ethylacetate extract of *Rhinacanthus nasutus* showed the most potent cytotoxicity against HeLa cells (IC_{50} 3.63 μg/ml) and the methanol extract of *R. nasutus* also showed specific cytotoxicity against the multidrug resistant CEM/ADR5000 cells (18.72% viability at 10 μg/ml, $p < 0.0001$ compared to its cytotoxicity against CCRF-CEM cells). Moreover, the ethylacetate extract of *O. indicum* showed a high level of antioxidant activity by inhibiting lipid-peroxidation ($IC_{50} = 0.08$ μg/ml).

As the most active anti-inflammatory species via the NF-κB signaling pathway, *G. pseudochina var. hispida* was selected for further investigation of active compounds. Through bioassay guided fractionation and isolation procedures, flavonoid glycosides; quercetin-rutinoside (rutin), dicaffeoylquinic acid derivatives and caffeoylquinic acid were isolated as the active NF-κB inhibitors.

CONCLUSIONS:

This thesis provides in vitro evidence for the use of the Thai plants, most importantly *Gynura pseudochina var. hispida, Oroxylum indicum* and *Muehlenbeckia platyclada* as Thai anti-inflammatory remedies. The active compounds isolated from the methanol extract of *G. pseudochina var. hispida*: the most potent NF-κB inhibitory extract, were identified as the known compounds quercetin-rutinoside and dicaffeoylquinic acid. Some of the results obtained might support the uses of the plants and are in agreement with previously reported literature, but some are in need of further investigation of either active compounds or their pharmacology.

This finding provides a new insight for understanding the anti-inflammatory activities of a panel of traditionally used anti-inflammatory plants. The active compounds isolated from the methanol extract of *G. pseudochina var. hispida*: the most potent NF-κB inhibitory extract, were identified as the known compounds quercetin-rutinoside, dicaffeoylquinic acid and caffeoylquinic acid derivatives. The active compounds are reported from this genus for the first time.

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LIST OF ABBREVIATIONS

Acetone-d_6	Deuterated Acetone
13C NMR	Carbon Nuclear Magnetic Resonance
COSY	Correlation spectroscopy
CD_3OD	Deuterated methanol
d	Duplet
dd	Duplet of duplet
D_2O	Deuterated water
DMSO-d_6	Deuterated Dimethylsulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
EA	Ethyl acetate
g	Gram
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Correlation
HNE	4- hydroxynonenal
1H NMR	Proton Nuclear Magnetic Resonance
hrs	Hours
IC_{50}	50 % inhibition concentration
IL	Interleukin
LC-MS	Liquid chromatography-Mass spectroscopy
LPS	Lipopolysaccharide
m/z	Mass to charge ratio
m	Multiplet
MDA	Malondialdehyde
ME/ MeOH	Methanol
MeOD4	Deuterated methanol
Methanol-D4	Deuterated methanol
mg	Milligrams
min	Minutes
mL	Millilitres
MPO	Myeloperoxidase

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
N/A	Data not available
ND	Not determined
NF-κB	Nuclear Factor Kappa B
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
PBS	Phosphate Buffered Saline
PE	Petroleum ether
PGE_2	Prostaglandin E_2
PMA	Phorbol myristate acetate
Pyridine-d_5	Deuterated pyridine
q	Quartet
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
s	Singlet
SAR	Structure activity relationships
t	Triplet
TLC	Thin layer chromatography
TNF	Tumor necrosis factor
XTT	2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilid
δ	Chemical shift
%	Percent
μM	Micro molar
μL	Micro litre

LIST OF POSTER PRESENTATIONS/ ABSTRACT PUBLICATIONS/ FULL PAPERS

Siriwatanametanon, N., Fiebich, B., Efferth, T., Prieto, J.M., Heinrich, M., 2010. Traditionally used Thai medicinal plants: *in vitro* anti-inflammatory, anticancer and antioxidant activities. *Journal of Ethnopharmacology*, 130 (2): 196-207.

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CHAPTER 1

INTRODUCTION

1.1 NATURAL PRODUCTS IN DRUG DISCOVERY

Natural products and their derivatives have been known to be an important source of new structures and starting materials for the discovery of new therapeutic agents. Drugs derived from natural products are used for the treatment of about 87% of all human disease categories (Newman *et al.* 2003). Approximately 25% of drugs in the modern pharmacopoeia are derived from medicinal plants, including currently use anticancer chemotherapy drugs such as vincristine, vinblastine, paclitaxel, podophyllotoxin, camptothecin and combretastatin (Ramawat & Goyal 2009). Moreover, about 12.5% of the 422,000 plant species documented worldwide are reported to have medicinal values (Rao *et al.* 2004).

Despite the rise in synthetic drugs designed as part of the process in today's drug lead discovery, natural products have advantages over synthetic drugs, in that natural products provide chemical diversity with structural complexity and biological potency (Clardy & Walsh 2004). A review published in 2007, covering years 1981-2006 showed that 63% of the 974 small molecule new chemical entities were natural products or semi-synthetic derivatives of natural products. With anticancer drugs, 77.8% were either natural products or mimicked natural products and only 22.2% were synthetic (Newman & Cragg 2007). Examples of drugs derived from natural products between 1980 and 2006 are shown in Table 1.

Table 1 Selected examples of drugs derived from natural products launched between 1985 and 2006 (Lam 2007; Newman & Cragg 2007).

Year	Trade name	Natural product	Indications
1985	Bactroban	Mupirocin	Antibacterial
1987	Artemisin	Artemisinin	Antimalarial
1989	Curaderm	Solamargines	Anticancer
1992	Actinex	Masoprocol	Anticancer
1993	Taxol	Paclitaxel	Anticancer
1994	Delivert	Angiotensin II	Anticancer
1999	Tamiflu	Oseltamivir	Antiviral
2001	Ketek	Erythromycin	Antibacterial
2002	Calsed	Doxorubicin	Anticancer
2002	Faslodex	Estradiol	Anticancer
2002	Reminyl	Galantamine	Alzheimer's disease
2003	Cubicin	Daptomycin	Antibacterial
2004	Laserphyrin	Talaporfin sodium	Anticancer
2005	Finibax	Carbapenem	Antibacterial

Furthermore, as of September 2007, a total of 91 plant-derived compounds were in clinical trials (Saklani & Kutty 2008). Most of the plant-derived compounds in the above table might have been known for long period before their commercial availability which can take many years from the experimental stage until approval. The search for new molecules, nowadays, has taken a slightly different route where the sciences of ethnobotany and ethnopharmacognosy are being used as guides to lead the chemist towards different sources and classes of compounds (Fakim 2006).

1.2 ETHNOBOTANICAL STUDY: RESEARCH INTO TRADITIONAL USES OF PLANTS

Research dealing with medicinal plants and their bioactive compounds can be distinguished as 2 different approaches which are bioprospecting and ethnopharmacology (Heinrich *et al.* 2004). A Bioprospecting approach is normally seen in more developed countries and mainly focuses on the screening of natural products using high-throughput system and enormous financial input to develop new drugs for commercial purposes (Soejarto *et al.* 2005; Heinrich *et al.* 2004). On the other hand, ethnopharmacological study deals with the documentation of a rather limited set of well-documented useful plants, mostly medicinal, with the aim of development or improvement of the preparations as used by local people, normally in developing countries (Heinrich *et al.* 2004).

Ethnopharmacology involves field studies of indigenous groups, contacting traditional healers, botanists, anthropologists, exploring traditional medical knowledge together with the biodiversity component to which such knowledge is attached, the documentation and conversion of the knowledge into a product, which could be an academic paper, a book, photos or a tangible product of commercial value (Soejarto *et al.* 2005). Analysis has shown that the uses of 80% of 122 plant-derived drugs are related to their original ethnopharmacological purposes (Fabricant & Farnsworth 2001) and thus it is challenging to research such traditional medicine for further development.

1.3 THAI TRADITIONAL MEDICINE AND ETHNOBOTANICAL KNOWLEDGE OF THAILAND

Thailand is a tropical country located in Southeast Asia. It is a developing country with a population of 67 million, with a forested area 167,591 km^3 (32.7% of the country), and unemployment rate of 1.2 % (data for November 2009 from UNDP 2009). Thailand is home to many traditional medicinal systems. According to the Department for Development of Thai Traditional and Alternative Medicine, Ministry of Public Health Thailand, Thai traditional medicine includes three main systems: 1) Thai Traditional Medicine, 2) Thai Indigenous Medicine, and 3) Thai-Chinese Medicine (Chokevivat & Chuthaputti 2005). In general when referring to the Thai Traditional Medicine system, it usually includes Indian Ayurveda and Southeast Asian medicines as well, in practice.

1.3.1 OVERVIEW OF THAI TRADITIONAL MEDICINE

The first historical evidence of Thai traditional medicine comes from the official textbook "Tamra Phra Osod Phra Narai", which compiled recipes of drugs used in the royal court of King Narai the Great (1656-1688). It noted that Thai people started to use herbal medicine for the treatment of various diseases, symptoms, and health promotion, before the Sukhothai period (1238) or during the Ayutthaya period (1350-1767). Over 1,000 drug formulations and the information regarding the cause of diseases, their treatments, and the principles of Thai traditional medicine were gathered and emblazoned on limestone and placed on the walls of Thai temples, namely Wat Po and Wat Raja Oros (Subcharoen 2003; Suchawan 1989)

Figure 1 The first official textbooks of Thai traditional medicine (photographed by the author).

Under the influence of Western medicine, especially Presbyterian physician-missionaries from the United States, biomedicine was introduced to Thailand in 1828 and Siriraj Hospital was officially opened in 1889 as the first Western-style hospital and medical school (Techatraisak & Gesler 1989). At the beginning, both Thai traditional medicine and modern medicine services were provided, and the medical school "Mahidol University" taught both disciplines of medicine. However, in 1916 the training of Thai traditional medicine and the provision of Thai traditional medicine services at Siriraj Hospital were discontinued as the two principles were considered incompatible and confusing to medical students (Chuthaputti 2007).

Thai traditional medicine and its practice, which is based on traditional philosophies and knowledge, is considered to be more in harmony with the Thai culture and way of life. The principles of Thai traditional medicines are summarized here in the two following paragraphs which were gathered from Subcharoen (2001); Sittitanyakit and Termwiset (2004) which were collected in Chuthaputti (2007). The main concept was reported to be that the human body is composed of four elements; earth, water, wind and fire. The body will be healthy when the four elements are in balance, but a person will be ill, if there is a lack, excess, or disability in any of them.

The imbalance of the four internal elements can be due to an imbalance of the four external elements and also can be influenced by one's age and inappropriate behaviors, location where one lives, time, or season. In addition, the following factors can also cause illness: 1) supernatural power such as evil spirits, ancestor's soul, punishment from a heavenly spirit of those who misbehave; 2) power of nature such as imbalances of the body, the heat and cold; 3) power of the universe and the influence from the sun, the moon and the stars; 4) the equivalent of microorganisms or parasites. Examination and diagnostic procedures of Thai traditional medicine are as follows.

1. Patient's history and chief complaint. In addition to asking about a patient's history, symptoms, chief complaints and usual behaviors or habits, Thai traditional medicine practitioners need to know a patient's date, time, month and year of birth in order to figure out the patient's dominant element and determine which element is causing the imbalance and illness.
2. Physical examinations, e.g., heart rate, pulse, fever, visual and manual examination of affected organs or areas of the body, structure of the body, degree of movement in joints and the extremities.
3. Diagnosis. Since each element of the body controls different organs or systems of the body, Thai traditional medicine practitioners can diagnose what is wrong with a patient's elements from their symptoms, chief complaints and physical examinations.
4. Astrological examination. Some Thai traditional medicine practitioners may also perform astrological examination of patients to determine if their illnesses are a result of the stars, a supernatural power or bad karma. If so, a form of rites may also be performed to psychologically boost the patient's morale.

The practice of Thai traditional medicine uses five main approaches: 1) medical practice, dealing with the diagnosis and treatment of diseases or symptoms; 2) pharmacy practice, dealing with the use of natural product derived drugs in various combinations and dosage forms; 3) traditional midwifery; 4) traditional Thai massage; 5) the application of Buddhism or rites and rituals for mental health care. Treatment of diseases and symptoms is considered to be holistic. The treatment focus on adjusting the equilibrium of the body elements and various factors including the use of prescribed herbal medicines, traditional Thai massage, post-partum care, mother and child care, hot herbal compresses, or herbal steam baths.

1.3.2 THAI INDIGENOUS MEDICINE AND ETHNOBOTANICAL KNOWLEDGE OF THAILAND

Thai indigenous medicine originated at about the same time as the country. It has evolved naturally by learning from experience and mistakes. The knowledge has been passed on from generation to generation. In different areas traditional knowledge may be different depending upon the environment, ecology, geography, economy, society, culture and beliefs. An interesting example is in the north and the northeast of Thailand where traditional medicine and practices are rather different. Several ethnobotanical studies have been conducted in Thailand especially regarding the indigenous knowledge originating from hill tribes in the North and Northeast of Thailand.

The 'I-SAN' traditional medicine (I-SAN is a common name meaning the northeast direction) is an example of Thai indigenous medicine which is only found in the Northeast of Thailand. This system is recognized as one of the oldest Thai indigenous medicine systems with many sub-categories divided by traditional practices, diagnostic patterns, and beliefs of etiology of disease. Some groups of healers treat diseases using natural products e.g. plants, animals, among others (with or without employing supra-natural powers) while other groups might only use magic formulas or ritual, involving Buddhist tradition mixed with other religions. A few examples are given below (some data in this topic were derived from PSU 2009; MSU 2009 and the interviews of traditional healers/ experienced people by the author).

1. ‘MOR-HAK-MAI’

[MOR = doctors, HAK = roots, MAI = plants]

Healers who are experts in using the roots of medicinal plants in the treatment of diseases and symptoms, especially hematological diseases, vertigo, headache, and post-partum symptoms. Some indigenous formulae are recorded in the adapted version of the first official textbook "Tamra Phra Osod Phra Narai" namely "Tamra Phad Sart Song Koa" which has been used until the present. This book comprised 3 volumes and consists of many Thai traditional/ indigenous medicinal recipes. The medicinal recipes contain either a single ingredient or combinations from two to more than ten ingredients. Selected recipes are presented here.

Example 1: **‘YA-SAM-RAK’**

[YA = drugs, SAM = three, RAK = roots] or 'TRI-SAN' [TRI = three, SAN= types]

A combination of three roots which includes the root of 'Cha-Phru' (*Piper sarmentosum* Roxb.), the root of 'Sa-Khan' (*Piper interruptum* Opiz. or *Piper ribesoides* Wall.), and the root of 'Jed-Ta-Mun-Preng' (*Plumbago indica* L.). This combination is used against menstrual cycle disorders and pre-mentrual symptom, as well as hematological diseases. The drug was prepared by rubbing the three ingredients with sand-rocks and soaking with clean-water, then drinking the resulting liquid as needed.

Piper sarmentosum (Piperaceae)

Piper ribesoides (Piperaceae)

Plumbago indica (Plumbaginaceae)

Figure 2 Identifications of the ingredients of Ya-Sam-Rak (www.samunprix.com; www.biogang.net and www.pharmacy.cmu.ac.th)

Note: There is a toxicological study showing that oral administration of the combination of these three plants, for 10 days at the usual dose, could induce renal and liver toxicities in Wister rats. Therefore, it was recommended not to use this recipe continuously for more than 10 days (Chavalittumrong *et al.* n.d.).

Example 2: **'YA-RAK-DEAW'**

[YA = drugs, RAK = roots, DEAW = one/single]

Use of the root of 'Lod-Tha-Nong' (*Trigonostemon reidioides* (Kurz) Craib.) for detoxification of snake bites, to induce vomiting for the elimination of toxic substances consumed, as well as to combat drug addiction. The root is macerated with clean-water. About 250-500 ml of this solution is drunk once to induce vomiting and this is followed by drinking clean water a little bit at a time, as often as possible, to maintain an equilibrium of water in the body.

Figure 3 *Trigonostemon reidioides* (Euphorbiaceae) - aerial parts and fruit

(www.forumkhonbaakpae.com)

Note: There is a study focusing on the interaction between Thai cobra venom and the active compounds found in the methanol extract of *T. reidioides*. In the in silico technique, principal compounds of the plant root extract could interact/combine with the neurotoxin-3 from the snake-toxin. The compounds could prevent the neurotoxin-3 interacting with the acetylcholine receptors in the human body. The use of the in-silico technique could explain the detoxification mechanism against Thai cobra snake bites (Temtrirath *et al.* 2005).

Example 3: **'YA-HA-RAK'**

[YA = drugs, HA = five, RAK = roots]

A combination of five plants' roots including the roots of 'Ching-Chi' (*Capparis horrida* L.), 'Tao-Yai-Mom' (*Tacca pinnatifida* Forst.), 'Ma-Due-Chum-Porn' (*Ficus glomerata* Roxb.), 'Kon-Tha' (*Harrisonia perforata* Merr.), and 'Yah-Nang' (*Tiliacora triandra* Diels.), is used against fevers, to purify blood, headaches, and muscle aches (PSU 2009).

Capparis horrida (Capperaceae)

Clerodendrum petasites (Verbenaceae)

Ficus glomerata (Moraceae)

Harrisonia perforata (Simaroubaceae)

Tiliacora triandra (Menispermaceae) Cutting and drying processes

Figure 4 Ingredients of 'YA-HA-RAK' and preparation (www.siamensis.org; www.songsaijang.com; www.southalltour.com; www.dnp.go.th; www.pharm.su.ac.th; www.gotoknow.org).

2. **'MOR-PAO'**

[MOR = doctors, PAO = blowing]

Healers who employ a procedure by means of blowing after chewing some remedies such as 'Mak', a combination of ripe areca catechu (normally yellow or orange yellow seeds are used), fresh piper betel leaves or steam betel leaves (in some seasons), and shell-lime paste. This formula is used against skin diseases e.g. herpes infections, abscesses, pustules whilst chewing with garlic and blowing onto the head of patients is usually used against colds.

Preparation of 'Mak'

Areca catechu nut

Piper betel leaves

Figure 5 Major ingredients and a preparation of 'MAK' (www.goldhips.com; www.trekkingthai.com)

3. **'MOR-NAM-MON'**

[MOR = doctors, NAM = water, MON = sacred]

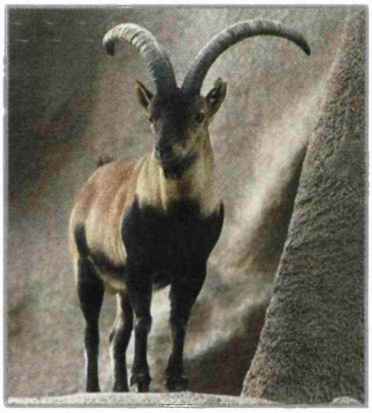
Healers that treat illness by using consecrated rain water with drops of melted yellow bee wax candle. This consecrated water is applied by sprinkle onto the heads of the patients. Sometimes the consecrated water can be drunk in the case of fevers. This could also apply onto the broken or dislocated joints resulting from an accidents e.g. falling from the trees, vehicle accidents. This is used when the healers agreed that the fractures are not very complicated.

Figure 6 Consecrated water in specific containers which are placed in Thai temples (www.amulet.in.th; www.luangpukahlong.com).

4) **'MOR-EN'**

[MOR = doctors, EN = the tendons]

Healers who apply aromatic oils, animal oils and use the thumb or index finger to touch or press onto the tendons which sore or stiff. The medicinal oil used during this procedure includes the oil from the bones or horns of the animal called 'mainland-serow', or using methyl salicylate oil, to make the tendon warm and relaxed (PSU 2009; MSU 2009).



Mainland-Serow

Figure 7 The animal which was used to make an oil for the treatment of sore tenders, the shops selling this product along the way in rural areas, and traditional healers called 'MOR-EN' (picture from www.thaivi.com; www.thaimtb.com).

5) **'MOR-PRA'**

[MOR = doctors, PRA = monks]

Healers in this group are usually monks or people who used to be monks. This kind of treatment is usually used when all the above treatments have failed and modern medical doctors cannot treat the patient. There are some beliefs regarding some diseases have unclear origins and cannot be diagnosed or treated by medical doctors. Therefore, the monks or healers use both medicinal plants and magical powers to treat patients.

'MOR-PRA' (the Monk in yellow costume on the left side)

Herbal drugs

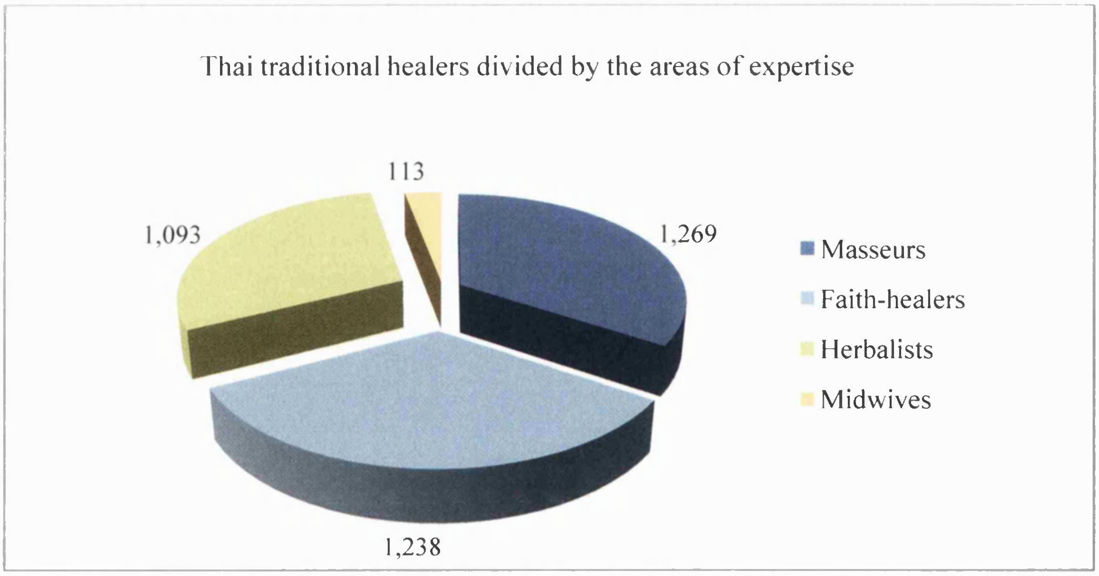
Herbal drugs storage

Figure 8 MOR-PRA, the healer and his helper, is treating a patient and pictures of herbal drugs stored in a temple (photos from kanchanapisek.or.th).

These are only examples of Thai traditional healers which might be of interest. A statistical figure from the provincial public health offices of 75 provinces of Thailand (HISO-Thailand 2009) estimated that in 2006 there were 24,538 traditional healers in Thailand. Approximately half of these resided in the northeast followed by the north and the west of Thailand. Moreover, about 20,011 traditional healers were registered by the Thai Ministry of Public Health and 14,854 out of this number received their licenses in traditional medicine. Interestingly, 78 of those receiving the license have their own traditional medicine clinics.

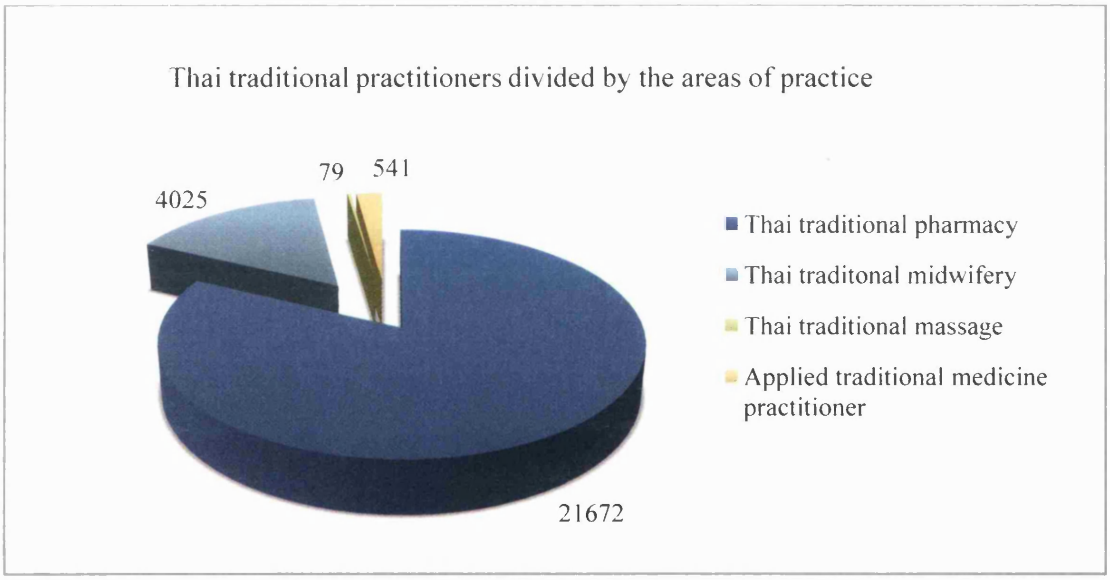
Another survey which focused on the distribution of traditional healers, conducted in 39 provinces of Thailand reported that from a total of 3,075 traditional healers, the majority were males (1,938 males and 1,124 females), and there were 13 monks in total. The average age of traditional healers was 53 years. Healers mainly worked as agriculturists, laborers, traders, and some of them earn some money from massaging. Regarding the expertise of traditional healers, there were 1,269 masseurs/masseuses, 1,238 faith-healers, 1,093 herbalists and 113 midwives (data from Office of Registration, Ministry of Public Health: PTMK 2009).

Figure 9 Thai traditional healers divided by their expertise (data from PTMK 2009)



Furthermore, there is a system to train Thai traditional medicine practitioners which can be either by apprenticeship with an authorized licensed practitioner or by institutional education. As of June 2007, there were 15,806 practitioners in Thai traditional medicine; 21,672 practitioners in Thai traditional pharmacy; 4,025 practitioners in Thai traditional midwifery and 79 in Thai traditional massage, as shown in the graph below (data from the Medical Registration Division, Ministry of Public Health, reported by Chuthaputti 2007).

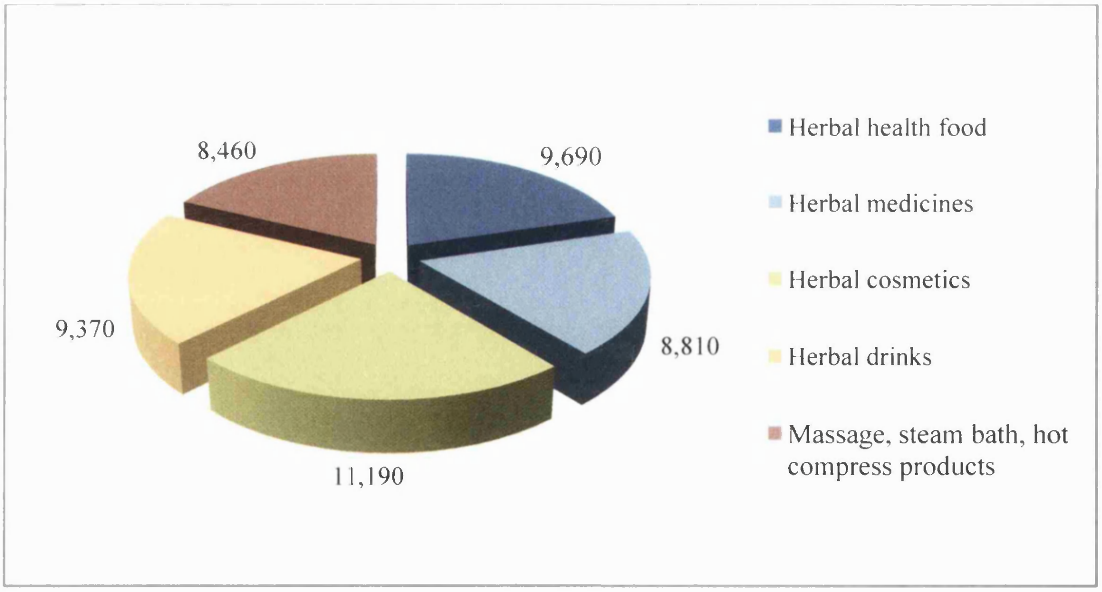
Figure 10 Thai traditional practitioners as of June 2007 divided by their expertise (data from PTMK 2009)



1.3.3 THE USE OF PLANTS IN THAI CONVENTIONAL MEDICINE AND HOSPITALS

The use of herbal medicine and herbal products by Thai people was estimated in 2005. The data on the consumption of herbal products was surveyed in all regions of Thailand. It was found that the total consumption of herbal products in 2005 was about 1,200 million US dollars (48,000 million Thai baht), and 220.2 million dollars US (8,810 million Thai baht) of that was on herbal medicines as shown in figure 11.

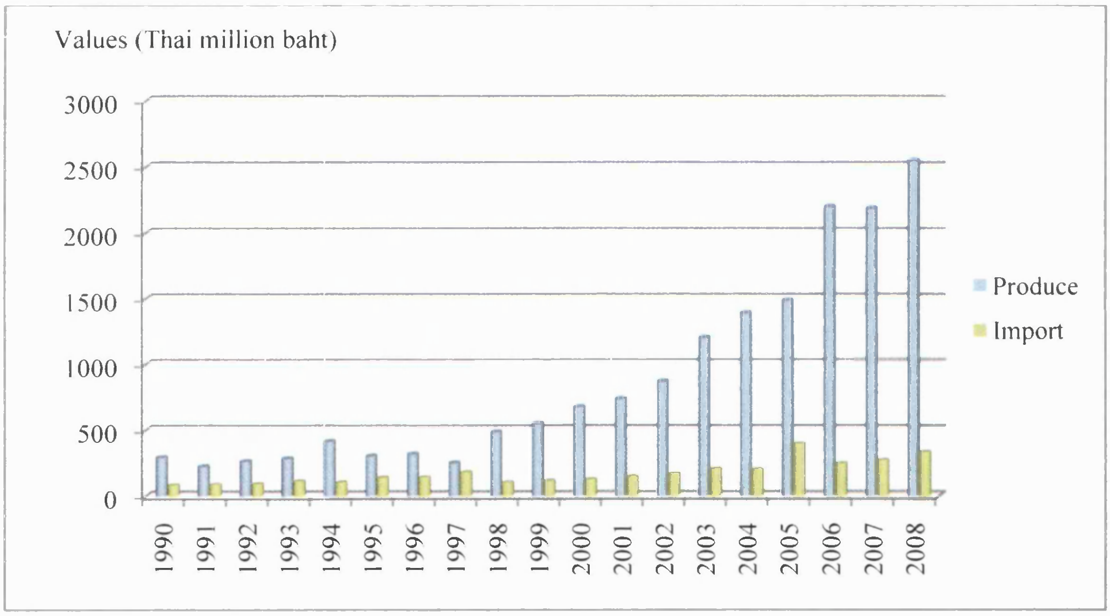
Figure 11 The value of herbal products in Thailand (in million Thai-baht) in 2005, grouped by type of product (Kasikorn Research Center in Chuthaputti, 2007).



Plant-derived pharmaceuticals and phytomedicines have been used by Thai people. Also some of the knowledge has been documented and studied scientifically. Many medicinal products have been used since the 19th century when there were no western or modern drugs. These medicines still play a crucial role especially in the remote areas of the country. The uses of herbal drugs are also accepted in hospitals.

As the use of medicinal plants has been supported by the Thai government during the past 20 years, there has been a significant increase in the production of Thai traditional medicine within the country while imported traditional medicines from other countries has also slightly increased. This can be clearly seen from the data reported by the Thai Food Drug Administration Office (Thai-FDA 2009), from 1990- 2008, as shown in figure 12.

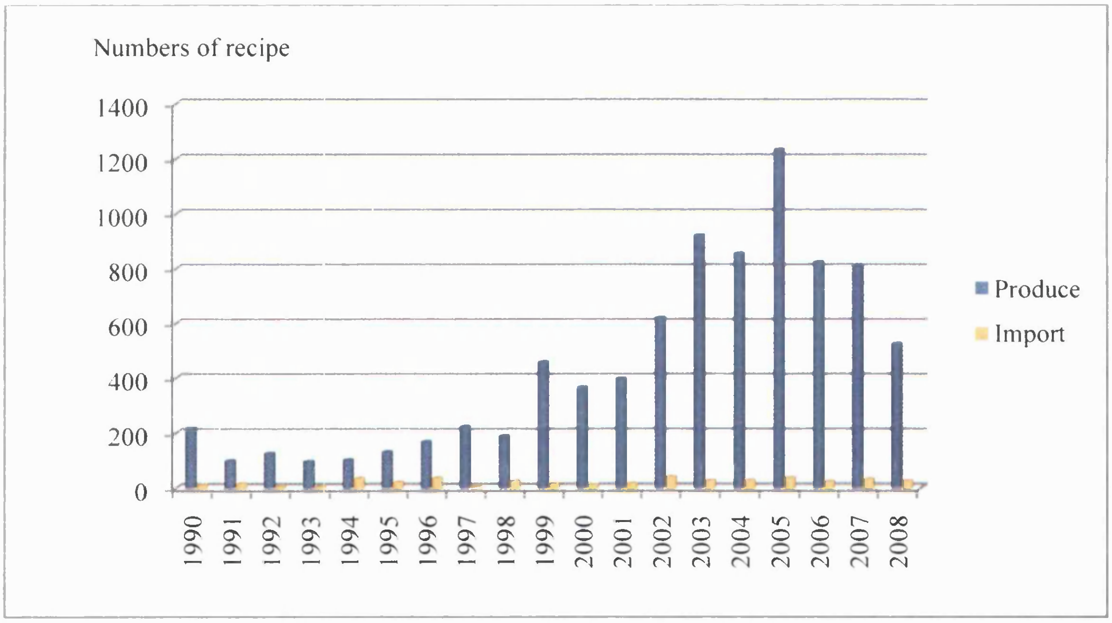
Figure 12 Thai traditional medicine production vs. imported from 1990 to 2008 (raw data from Thai-FDA 2009). Note that Y-axis presents values in Thai million baht.



Not only have the total values of traditional medicines produced increased, but also new traditional medicine formulae have been developed. Traditional medicine manufacturers, Government Pharmaceutical Organization, and some hospitals have increased the production of herbal medicines in easy forms, mainly capsule (1,593 formulae), tablet (1,593 formulae), capsules (339 formulae) and liquid (820 formulae) to meet the demand of consumers (Thai-FDA 2009).

The total number of Thai traditional medicine manufacturers increased by 43% from 616 in 1997, to 881 in 2006, of which 286 (32.46%) were in Bangkok and 595 (67.54%) were located in other provinces (Chuthaputti 2007). Data from 1990 to 2008, reported by Thai Drug Control Division, showed that Thai traditional medicine formulae have been increasing whereas the numbers of formulae imported have been stable. The amount of locally produced and imported traditional medicine recipes (reported from 1990 to 2008) are shown in figure 13.

Figure 13 Number of traditional medicine recipes registered in Thailand during 1999-2008 (raw data from Thai-FDA 2009).



From figure 13, although the numbers of recipes of domestically produced traditional medicines have decreased since 2005, the total amounts of products have continued to increase. This may be due to drug registration controls becoming more strict. Under the current law of Thailand, to register a traditional medicine product (including herbal drugs), the manufacturer has to submit results of microbial limit tests, presence of contaminants such as pesticides, heavy metals, etc. according to permitted levels (FDA-framework paper presented by Chuthaputti, in WHO 2004).

As of April 2007, Thailand has not produced its traditional medicine pharmacopoeia or formulary yet. However, many plants are included in the recent 'List of Herbal Medicinal Products A.D. 2006' as well as the National List of Essential Drugs of Thailand, prescribed by healthcare practitioners. There were two categories in the list as follows (data from Thaifda.com and Chuthaputti 2007).

1) Herbal medicines which are based on traditional/ethnobotanical knowledge. These herbal medicines are usually made of medicinal plants in combinations.

    1.1) Antitussive and expectorant formula named "Ya-Prasa-Mawaeng"
    1.2) Antipyretics (three formulae): "Ya-Ha-Rak", "Ya-Keaw-Hom", and "Ya-Chata-Leela".
    1.3) Drugs for obstetrics and gynecology problems called "Ya Prasa Plai"
    1.4) Drugs for gastrointestinal disorders (four formulae); "Ya-Tai-Dekleur-Farang", "Ya-Tard-Bunjob", "Ya-Prasa Karnplu", and "Ya-Leung-Pid-Samut".
    1.5) Drugs for cardiovascular diseases or refered to'wind' problems (two formulae): "Ya-Hom-Taeppajit" and "Ya-Hom-Nawakod".

2) Scientifically developed herbal medicines (note these are used as singles).

    2.1) Turmeric for dyspepsia
    2.2) *Senna alata* for constipation
    2.3) *Andrographis paniculata* for pharyngotonsillitis and diarrhea
    2.4) *Zingiber cassumunar* for bruising and muscle sprain
    2.5) *Clincanthus nutans* for herpes infection
    2.6) Ginger for gastrointestinal disorders
    2.7) *Centella asiatica* for wound healing
    2.8) Capsicum for joint and muscle pain

Although, according to the National List of Essential Drugs of Thailand, many inflammatory related diseases/conditions have been widely treated with Thai traditional medicines (Saralamp *et al.* 2000; Chuakul *et al.* 2000; van Valkenburg & Bunyapraphatsara 2001; Lemmen & Bunyapraphatsara 2003; Laupattarakasem *et al.* 2003) no anti-inflammatory plant species are included in it. However, anti-inflammatory plant species have been documented elsewhere in other Thai traditional textbooks and/or modern textbooks by academic professionals.

1.4 SELECTED THAI PLANTS & THEIR TRADITIONAL USES

At the beginning of this study the selection of anti-inflammatory species included 52 plant species which were found in Thai textbooks. However, some plant species have been used worldwide and have been studied scientifically previously in Thailand or other countries. Therefore, after excluding plants which had already had their bioactivities researched, and after excluding those species where the availability might be limited, there were 9 plant species included in the present study. The details of each species are as follows.

BASELLA ALBA L. (BASELLACEAE)

Common names: Ceylon spinach (English), Gendola (Indonesian), Tsuru murasa kai (Japanese), Phakkang (Laotian), Remayoung (Malay), Pak plang kwaw (Thai).

Botanical description of *Basella alba*

Figure 14 Identification of *Basella alba* (photographed by the author)

Ceylon spinach is not true spinach. It is a very common fast growing vegetable. It is a tender tropical vine; grown as a perennial up to 30 feet tall. Only the leaves and young stems are eaten. *Basella alba* has white flowers, while *Basella rubra* has red flowers. *B. alba* is green-leaved and *B. rubra* is a red-leaved variety (Prasuna *et al.* 2009). It usually has white-veined, red or green leaves depending on the region. This plant re-grows very well after cutting as long as it is planted in soil. The most common method of cooking is as a pot herb, mixed with stew or other vegetables. On cooking, the green stem/leaf species retains its fresh green colour. In some countries this species is sometimes called *Basella rubra* (www.tropicos.org).

Systematic classification of *Basella alba* (USDA Plant Database 2010a)**:**

Kingdom:	Plantae	(Plants)
Subkingdom:	Tracheobionta	(Vascular plants)
Superdivision:	Spermatophyta	(Seed plants)
Division:	Magnoliophyta	(Flowering plants)
Class:	Magnoliopsida	(Dicotyledons)
Subclass:	Caryophyllidae	
Order:	Caryophyllales	
Family:	Basellaceae	
Genus:	*Basella*	
Species:	*Basella alba* L.	(Ceylon spinach)

Chemistry of *Basella alba*

B. alba is a rich source of nutrients such as vitamin A or β-carotene and has been researched for the treatment of vitamin A deficiency in Bangladeshi men (Haskell *et al.* 2004). It has been reported to contain high amounts of calcium, magnesium, manganese, and zinc, and has been widely consumed by Hmong refugees from Southeast Asia living in Sacramento, California, the United States, as a diet for pregnancy and post-partum (Corlett *et al.* 2002).

<u>Medicinal use of Basella alba</u>

***Basella alba* in traditional medicine**

The fruits contain a red dye which has been used for official seals. This dye has also been widely used as rouge in many parts of Asia. In Thailand, the aerial parts were eaten to alleviate symptoms of appendicitis, smallpox fevers, and as laxatives while crushed leaves were used against topical skin problems e.g. wounds, itching, or abscesses. In addition, juice of the fruit was used for treatment of smallpox fevers, and skin inflammation (Theangburanatham 2005).

In India, the Paliyar tribes in the Theni district of Tamil Nadu, use the juice of the leaves externally against eye infections (Ignacimuthu *et al.* 2008). *B. alba* has also been used in the Dharward district in Karnataka India, to treat different types of oral ailment such as toothache, plaque and caries, pyorrhoea and aphthae by masticating the leaves and keeping them in the mouth for some time to obtain relief from such conditions (Hebbar *et al.* 2004).

In Cameroon, traditional healers use a mixture of *B. alba* and *Hibiscus macranthus* to prepare a crude extract which improves male virility and to cure male sexual asthenia and infertility (Moundipa *et al.* 2005; 2006). In the Sangmelima region of Southern Cameroon, two spoonfuls of the juice of crushed fresh leaves has been used as an abortifacient by drinking repeatedly, as needed. This administration often led to lacerations (multiple tears) of the vulva (Noumi & Tchakonang 2001).

Current pharmacology of *Basella alba*

B.alba extracts have been studied in relation to testosterone production *in vitro* on adult rat testes (sliced) and bull Leydig cells. The testosterone production in testes slices increased after incubation with an aqueous extract of a mixture of *B. alba* and *H. macranthus*. The *B. alba* aqueous extracts (10 g/mL and 100 g/mL) significantly enhanced testosterone production in bull and rat Leydig cells in a concentration-dependent manner (Moundipa *et al.* 2005; 2006). In Taiwan, the aqueous extracts of *B. alba* leaves showed weak to moderate mutagenicity on *Salmonella typhimurium* at the dose 5 mg/plate (Yen *et al.* 2001).

BASELLA RUBRA L. (BASELLACEAE)

Common names: Malabar spinach (English), Indian spinach (English), Ceylon spinach (English), Vine spinach (English), Malabar nightshade (English), Hong-Chan-Cai (Chinese), Tsuru-Murasaki (Japanese), Pak-Prang-Daeng (Thai).

Botanical description of *Basella rubra*

Figure 15 Identification of *Basella rubra* (photographed by the author)

Malabar spinach is a soft vegetable. Its leaves are almost circular to ovate, alternate, and short petiole. It has thick tender stems. Color of the leaves can be green, red or purple. The flowers borne on axillaries and are bisexual but inconspicuous. The plant re-grows quickly after cutting as long as it is planted in soil and has enough water.

The young succulent leaves and stems are eaten. The leaves are tasteless but the stems are slightly bitter. All parts become gelatinous or mucilaginous when cooked or crushed. It leaches red color pigment into water when cooked. Purple colour from crushed fruit was used as a dye for stamps, as rouge to brighten cheeks and as a food colouring. This species is sometimes called *Basella alba var. rubra* (The Herb Society of America 2010).

Systematic classification of *Basella rubra* (USDA Plant Database 2010b)**:**

Kingdom:	Plantae	(Plants)
Subkingdom:	Tracheobionta	(Vascular plants)
Superdivision:	Spermatophyta	(Seed plants)
Division:	Magnoliophyta	(Flowering plants)
Class:	Magnoliopsida	(Dicotyledons)
Subclass:	Caryophyllidae	
Order:	Caryophyllales	
Family:	Basellaceae	
Genus:	*Basella*	
Species:	*Basella rubra* L.	(Malabar spinach)

Chemistry of *Basella rubra*

Fresh aerial parts of *B. rubra* contain basellasaponins A, B, C, and D, oleanane-type triterpene, oligoglycosides having the dioxolane-type substituent, β-vulgaroside I, spinacoside C, and momordins IIb and IIc (Murakami *et al.* 2001). Saline extract of the seeds contains α-basrubrin and β-basrubrin and these compounds have shown antifungal activity with IC_{50} values ranged from 5.8 to 14.7 μM (Wang & Ng 2004; 2001).

Medicinal use of Basella rubra

Basella rubra in traditional medicine

The fruits, which are fleshy and purplish black, and their juice, have been used widely as a dye. In Thailand, *B. rubra* has traditional uses similar to those of *B. alba* in that the aerial parts were eaten to alleviate symptoms of appendicitis, smallpox fevers, and as laxatives, and the crushed leaves were used against topical skin problems, e.g. wounds, itching, or abscesses. Also the juice of the fruit was used for the treatment of smallpox fevers, and skin inflammation (Theangburanatham 2005). In Chinese traditional medicine, the leaves or the aerial parts have been used as diuretic, anti-inflammatory and the treatment of constipation (Murakami *et al.* 2001).

In Ayurveda, the leaves of *B. rubra* were prepared as a poultice by grinding them with sour buttermilk and salt. Habitual intake of the *B. rubra* preparation could cure 'arbuda' which is one type of tumor specified in Ayurveda (Balachandran & Govindarajan 2005). In Assam, a state in the north-east of India, crushed leaves, mixed with cheese are used in the treatment of skin infections, urticaria, and skin burns and sometimes the plant has been applied as a cosmetic (Murakami *et al.* 2001). In Uttara Kannada district of Karnataka India, the crushed leaves are used externally for the treatment of skin diseases and boils (Harsha *et al.* 2003).

Current pharmacology of *Basella rubra*

Two novel peptides, α-basrubrin and β-basrubrin, isolated from the saline extract of the seeds, demonstrated *in vitro* antifungal activity with IC_{50} values ranged from 5.8 to 14.7 μM (Wang & Ng 2004) whereas the aqueous extract of the leaves (10 and 20 mg/kg p.o.) showed significant and dose-dependent antiulcer activity against ethanol and pylorus legated induced ulcer in albino rats (Deshpande *et al.* 2003).

CAYRATIA TRIFOLIA (L.) DOMIN. (VITACEAE)

Basionym: *Vitis trifolia* L., *Cissus trifolia* (L.) K. Schum.

Common names: Three-leaf cayratia (English), Sorrel vine (English), Bushkiller (English), Taw kan kwaw (Thai)

Botanical description of *Cayratia trifolia*

Figure 16 Identification of *Cayratia trifolia* (photographed by the author)

Cayratia comprises about 50 species and is distributed in the tropics (Grubben 2004). This species is a loosely climbing vine with tendrils that are found opposite the leaves. The tendrils have a few branches each and have no adhesive disks. The leaves are tri-foliolate with petioles. The leaflets are ovate to oblong-ovate, pointed at the tip, and coarsely toothed at the margins. The flowers are small inflorescences, greenish white, and found opposite the leaves. The fruit is fleshy, juicy, dark purple or black (Wen 2007).

Systematic classification of *Cayratia trifolia* (USDA Plant database 2010):

Kingdom:	Plantae	(Plants)
Subkingdom:	Tracheobionta	(Vascular plants)
Superdivision:	Spermatophyta	(Seed plants)
Division:	Magnoliophyta	(Flowering plants)
Class:	Magnoliopsida	(Dicotyledons)
Subclass:	Rosidae	(Polypetalous flowers)
Order:	Rhamnales	(Thorny bearing fruits)
Family:	Vitaceae	(Grape family)
Genus:	*Cayratia*	(or Vitis as its basionym)
Species:	*Cayratia trifolia* (L.) Domin.	(Threeleaf cayratia)

Chemistry of *Cayratia trifolia*

The stems, leaves and roots contain cyanic acid and traces of this were also found in the flowers. The leaves contain several flavonoids including cyanidine, delphinidin, kaempferol, myricetin and quercetin. The aerial parts yielded triterpene epifriedelanol (Kundu *et al.* 2000; van Valkenburg & Bunyapraphatsara 2001).

Medicinal use of Cayratia trifolia

***Cayratia trifolia* in traditional medicine**

In Thailand, the leaves and roots are used against fever, and as an astringent. The stems are used as an antitussis (expectorant), carminative and they are also applied to relieve vertigo, dizziness, nose ulcers, internal bruises, and blood purifier. Heated leaves are used to treat inflammatory conditions (van Valkenburg & Bunyapraphatsara 2001). It was also reported in an ethnobotanical survey of 14 provinces of Thailand that the leaves have been applied externally for nose ulcers, muscle pains and abscesses (Chuakul *et al.* 2000). In the Philippines and Thailand, a decoction or juice from the fresh leaves is consumed for prevention or cure of scurvy (van Valkenburg & Bunyapraphatsara 2001).

Some other uses such as for asthma, catarrhal affection, and headache have also been recorded in the Sitamata wildlife sanctuary, Rajasthan, India (Jain *et al.* 2005). In Peninsular, Malasia and East-New Britain, the leaves of *C. trifolia* are typically applied as a nose ulcer poultice. The roots are also used as a counterirritant that produces erythematic when applied to the skin surface (rubefacient). The juice or decoction is used to foster fever, resulting in perspiration. In Java, the juice of leaves, added with the juice of young pineapple is employed against dandruff (van Valkenburg & Bunyapraphatsara 2001).

Current pharmacology of *Cayratia trifolia*

The triterpene epifriedelanol isolated from "*Vitis trifolia*" (species reported as its basionym in the original text) demonstrated 100% inhibition against crown gall tumours formation caused by *Agrobacterium tumefaciens* on potato disc at 40 and 60 μg/disc, as compared to 6.25 μg/disc of vincristine (Kundu *et al.* 2000).

GYNURA PSEUDOCHINA (L.) DC. VAR. HISPIDA THV. (ASTERACEAE)

Common names: Wan-maha-kan (Thai)

Botanical description of *Gynura pseudochina var. hispida*

Figure 17 Identification of *G. pseudochina var. hispida* (photographed by the author)

G. pseudochina var. hispida is an erect herb, perennial, and semi-succulent, growing up to about 1 meter high. It has tuberous rounded roots about 2 cm in diameter. The leaves are green or purple with shallow lobes arranged in a simple rosette. The leaf stalks are 0.3-3 cm long connecting with the leaf blades which are inverted-egg spatula shaped, elliptical or ovate. The upper leaves are smaller and paler. The flowers are in an inflorescence with a bell-shaped head, and are yellow or orange. Small florets are yellow, and about 1-1.3 cm long. The flowers produce an unpleasant musky smell. The fruits are very small, 3-4 mm long, and dry when mature (Beentje *et al.* 2005).

Systematic classification of *Gynura pseudochina var. hispida*

(Missouri Botanical Garden 2010):

Kingdom:	Plantae	(Plants)
Subkingdom:	Tracheobionta	(Vascular plants)
Superdivision:	Spermatophyta	(Seed plants)
Division:	Magnoliophyta	(Flowering plants)
Class:	Magnoliopsida	(Dicotyledons)
Subclass:	Magnoliidae	
Order:	Asterales	
Family:	Asteraceae	
Genus:	*Gynura*	
Species:	*Gynura pseudochina* (L.) DC. *var. hispida* Thwaites	

Chemistry of *Gynura species* (as no report for *G. pseudochina var. hispida*)

No chemical research on *G. pseudochina var. hispida* has so far been reported. However, some other Gynura species were phytochemically studied and their chemical structures were reported in publications; from 1969 until today, only seven species of Gynura including *Gynura divaricata* DC ; *Gynura divaricata sub-sp. formosana* ; *Gynura japonica*; *Gynura elliptica* ; *Gynura formosana*; *Gynura bicolor* DC ; *Gynura aurantiaca* ; *Gynura formosana* and *Gynura procumbens* have been investigated chemically.

G. divaricata contains cerebroside, 1-O-*β*-D-glucopyranosyl-2-[(2′R)-2′-hydroxyltri cosanoyl-amino]-10-octadecene-1,3,4-triol, quercetin, 3-O-β-D-glucopyranosyl quercetin, 3-O-β-D-glucopyranosyl-α-L-rhamnosyl quercetin, 3-O-β-D-glucopyranosyl-α-L-rhamnosyl kaempferol, epi-friedelinol, epi-friedelinol acetate, β-sitosterol, stigmasterol, adenosine, and uridine all isolated from the aerial parts (Chen *et al.* 2009; Hu *et al.* 2006).

G. divaricata sub-sp. formosana contains docosane, I-dotriacontanol, 7,11,15-trimethyl-3-methylene-1,2-hexadecanediol, methyl hexadecanoate, methyl oleate, methyl linoleate, 1,2-dihydroxypropyl hexadecanoate, friedelin, epi-friedelanyl acetate, 3-epi-friedelinol, glutinol, methyl-hydroxy-pheophorbide- α and -β, a mixture of β-sitosterol and stigmasterol, as well as a mixture of β-sitosterol-3-O-β-D-glucoside and stigmasterol-3-O-β-D-glucoside, all isolated from the chloroform extract of the whole plant (Chen *et al.* 2003).

G. japonica contains a quinonoid terpenoid, (-)-α-tocospirone, a chromanone (-)-gynuraone, three steroids; (22E,24S) -7 hydroperoxystigmasta– 5 , 22– dien -3-β- ol, (22E,24S)- stigmasta- 1, 4, 22- trien- 3- one, (24R)- stigmasta- 1,4- dien- 3- one, and caryophyllene oxide, 6- acetyl-2, 2- dimethylchroman- 4- one, vanillin,2,6-dimethoxy-1, 4- benzoquinone, and benzoic acid isolated from the rhizomes (Lin *et al.* 2003). *G. elliptica* contains p-hydroxyacetophenone-like derivative, (+)-gynunone, and a chromane together with six mentioned compounds isolated from the roots (Lin *et al.* 2000).

G. formosana was reported to contain caffeic acid, quercetin 3-O-rutinoside, kaempferol 3-O-rutinoside, and kaempferol 3-O-robinobioside (Hou *et al.* 2005) and a chromanone; 6-acetyl-2-hydroxymethyl-2'-methylchroman-4-one, isolated from the fresh aerial parts (Jong & Hwang 1997).

G. bicolor contains anthocyans; pelargonidin, delphinidin, malvidin, and oenin (malvidin 3-glucoside) (Hayashi *et al.* 2002).

G. aurantiaca yielded cyanidin tetra-glucoside acylated by three molecules of caffeic acid and one molecule of malonic acid (Yoshitama *et al.* 1994). Lastly, *G. procumbens* which is famous for its anti-diabeting biguanide-like activity (X. F. Zhang & B. K. Tan 2000a) has been reported to contain kaempferol-3-O-rutinoside and astragalin (Yam *et al.* 2008; Rosidah *et al.* 2008).

Medicinal use of Gynura pseudochina var. hispida

Gynura pseudochina var. hispida in traditional medicine

In Thailand, the leaves have been used for the treatment of inflammatory herpes infection, burning pains and used as a poultice for abscesses (TISTR 2010). Fresh leaves and rhizome are used externally against inflammation and viral infections (herpes). The root can also be used internally for pain and fever (Saralamp *et al.* 2000; Lemmen & Bunyapraphatsara 2003). In addition, the water extract of the leaves has been prescribed for treating AIDS (Woradulayapinij *et al.* 2005).

Current pharmacology of *Gynura pseudochina var. hispida*

The water and the methanol extracts of the leaves of *G. pseudochina var. hispida* demonstrated 58% and 35 % *in vitro* HIV-1 reverse transcriptase inhibitory activity at a concentration of 200 mg/ml (Woradulayapinij *et al.* 2005).

GYNURA PSEUDOCHINA VAR. PSEUDOCHINA (L.) DC. (ASTERACEAE)

Common names: Wan-hua-nuom (Thai), Beluntas cina (Indonesia)

Botanical description *Gynura pseudochina var. pseudochina*

Figure 18 Identification of *Gynura pseudochina* (photographed by the author)

G. pseudochina var. pseudochina is an erect herb with tuberous root. The stems are very short. It is perennial and semi-succulent. The leaves are simple, green or purple arranged as a rosette. The leaves are inverted egg and spatula shaped. Flower-heads are inflorescence, bell-shaped, yellow-orange loosely grouped in racemes or panicles. Fruits are very small, dry when mature (Davies 1980). The differences between *G. pseudochina var. pseudochina* and *var. hispida* is the leaf colour; in the *var. pseudochina* the front of the leaves are more greenish with purple veins, whereas the *var. hispida* has more purple pigments with the green vein.

Systematic classification of *Gynura pseudochina*

(Missouri Botanical Garden 2010):

Kingdom:	Plantae	(Plants)
Subkingdom:	Tracheobionta	(Vascular plants)
Superdivision:	Spermatophyta	(Seed plants)
Division:	Magnoliophyta	(Flowering plants)
Class:	Magnoliopsida	(Dicotyledons)
Subclass:	Magnoliidae	
Order:	Asterales	
Family:	Asteraceae	
Genus:	*Gynura*	
Species:	*Gynura pseudochina* (L.) DC.	

Chemistry of *Gynura pseudochina*

Not known. The chemistry of genus Gynura is discussed as above.

<u>Medicinal use of Gynura pseudochina var. pseudochina</u>

***Gynura pseudochina var. pseudochina* in traditional medicine**

In Thailand, the root is considered to be an antipyretic. Fresh roots and leaves, ground with water are applied externally to herpes simplex and herpes zoster infections. The water extract reduces both inflammation and recurrence of herpes infections (Missouri Botanical Garden 2010). Also the roots have been used as an anti-inflammatory, relieving hot pain symptoms, fevers, and treating herpes infections (TISTR 2010).

In Java the roots are used externally as a remedy for bruises while leaf poultice is applied against pimples. The leaves and roots are also used as a haemostatic and used against breast tumours. In Vietnam, the roots are used as a tonic, while the leaves have been used as an emollient and the sap of the leaves used against sore throat (Lemmen & Bunyapraphatsara 2003).

Current pharmacology of *Gynura pseudochina var. pseudochina*

No study has been reported for this sub species.

MUEHLENBECKIA PLATYCLADA (F. MUELL) MEISN. (POLYGONACEAE)

Basionyms: *Polygonum platycladum*, *Homalocladium platycladum*

Common names: Centipede Plant (English), Tapeworm Plant (English), Ribbon bush (English), Ta-kab-hin (Thai)

Botanical description of *Muehlenbeckia platyclada*

Figure 19 Identification of *Muehlenbeckia platyclada* (photographed by the author)

Muehlenbeckia platyclada can grow up to 2-3 meters as a garden shrub. The stems and branches are very flat, joining together at the nodes, making it looks like ribbons or tapeworms. Its glossy joined stems and branches are easily confused for leaves. The branches in the stems are tough but flexible. The simple leaves are arranged along the entire stem. The edges are thin and small, rolling back in the bud and are smooth. Flowers are tiny and generally hermaphrodite, borne compound inflorescences, whorled or acyclic. This species does not produce fruit or berries but is easily propagated by cuttings or by seed.

Systematic classification of *Muehlenbeckia platyclada* (Tropicos 2010a)**:**

Kingdom:	Plantae	(Plants)
Subkingdom:	Tracheobionta	(Vascular plants)
Superdivision:	Spermatophyta	(Seed plants)
Division:	Magnoliophyta	(Flowering plants)
Class:	Magnoliopsida	(Dicotyledons)
Subclass:	Caryophyllidae	
Order:	Caryophyllales	
Family:	Polygonaceae	
Genus:	*Muehlenbeckia*	
Species:	*Muehlenbeckia platyclada* (F. Mull.) Meisn.	

Chemistry of *Muehlenbeckia platyclada*

It contains several flavonoids, morin-3-O-α-rhamnopyranoside, kaempferol-3-O-α-rhamnopyranoside, kaempferol-3-O-β-glucopyranoside, quercetin-3-O-α-rhamno pyranoside and catechin, which were isolated from the methanolic extract of *Muehlenbeckia platyclada* (Yen *et al.* 2009).

<u>Medicinal use of Muehlenbeckia platyclada</u>

***Muehlenbeckia platyclada* in traditional medicine**

In Thailand, the aerial parts mixed with whisky or alcohol has been applied externally for skin swelling, sores, and insect bites (Chuakul *et al.* 2000). In Taiwan and China, it has been used in the treatment of poisonous snake bites and fracture injuries, alleviating fever and detoxification (Je-Chian *et al.* 1961 in Yen *et al.* 2009).

Current pharmacology of *Muehlenbeckia platyclada*

There is only one report relating to the pharmacology of this species. The methanol extract of *M. platyclada* contained flavonoids which demonstrated anti-inflammatory activities. The isolated morin-3-O-α-rhamnopyranoside, kaempferol-3-O-β-glucopyranoside, and catechin, could inhibit the release of neutrophil elastase with IC_{50} values of 3.82, 8.61 and 4.37 μg/ml, respectively, and were 15-fold more potent than phenylmethylsulfonyl fluoride (PMSF), the positive control used in this anti-inflammatory assay. In addition, Kaempferol-3-O-α-rhamnopyranoside showed moderate inhibition of superoxide anion generation with an IC_{50} value of 6.11 μg/ml (Yen *et al.* 2009).

OROXYLUM INDICUM (L.) KURZ. (BIGNONIACEAE)

Basionym: *Bignonia indica* L.

Common names: Midnight Horror (English), Broken bones (English), Indian trumpet flower (English), Tree of Damocles (English), Mu hu die (Chinese), Phe kaa (Thai).

Botanical description of *Oroxylum indicum*

Figure 20 Identification of *Oroxylum indicum* (photographed by the author)

O. indicum is a tree 6-10 m tall with the trunk 15-20 cm in diameter. The bark is dark-brown and the young branches are fresh green. Compound leaves are borne pinately at the apex of the stem. Leaflets are triangular-ovate, glabrous, green, but changing to dark blue after drying. Leaves base is subrounded, margin entire, apex short acuminate; lateral veins 5 or 6 on each side of midrib. Flowers are 2-lobed upper and 3-lobed lower, usually bloom at night with foul smell. Fruit pods hang down from the branches. Within the pod are the seeds which become sub-woody when dry and flutter to the ground when the pod bursts (Caldecott 2006).

Systematic classification of *Oroxylum indicum* (Tropicos 2010b)**:**

Kingdom:	Plantae	(Plants)
Subkingdom:	Tracheobionta	(Vascular plants)
Superdivision:	Spermatophyta	(Seed plants)
Division:	Magnoliophyta	(Flowering plants)
Class:	Magnoliopsida	(Dicotyledons)
Subclass:	Magnoliidae	
Order:	Lamiales	
Family:	Bignoniaces	
Genus:	*Oroxylum*	
Species:	*Oroxylum indicum* (L.) Kurz.	

Chemistry of *Oroxylum indicum*

The various parts of *O.indicum* are rich in flavonoids. The leaves contain flavonoids baicalein, scutelarein, and their glycosides bicalin and scutellarin. The stem and root bark contain baicalein, scutelarein, oroxylin A, chrysin and p-coumaric acid. Baicalein and oroxindin have been isolated from the seed. Other compounds mentioned in the literature included the prenylated naphthoquinone lapachol, the anthraquinone derivative aloe-emodin and baicalin-7-O-glucoside (Miao *et al.* 2006).

Medicinal use of Oroxylum indicum

Oroxylum indicum in traditional medicine

In India, a root preparation called "Dasamoola" is used as an astringent, anti-inflammatory, anti-helminthic, antibronchitic, antileucodermatic, anti-rheumatic, anti-anorexic and for the treatment of leprosy and tuberculosis (Gupta *et al.* 2008). In the Sikkim and Darjeeling group (Himalaya), the stem bark decoction or juice is taken 2-3 times a day as an antidiabetic (Chhetri *et al.* 2005). In Indukantha Ghritha, a polyherbal Ayurvedic formula of 17 plants including this species is used against respiratory disorders, fevers, gastric disorders, cough, dyspnoea (George *et al.* 2008). Also in the Similipal Biosphere Reserve-Orissa of India, the bark is used against diarrhea, rheumatism and stomach-ache (Thatoi *et al.* 2008).

In Ayurvedic medicine, it has been used for the treatment of 'granthi' which in Ayurveda is one type of tumour (Balachandran & Govindarajan 2005). The methanol extracts of the flowers and fruits have been used against stomach disorders, diarrhea, dysentery and rheumatic swelling (Nakahara *et al.* 2002). In Far-West Nepal, a root decoction is used against diarrhea and dysentery. The seeds are used as a digestive. In addition, the bark is used as a diuretic and stomachic (Baral & Kurmi 2006; Kunwar *et al.* 2009).

In Bangladesh, *O. indicum* is used as an anti-cancer agent. The ethanol extract of the stem bark showed the highest toxicity against all tumour cell lines tested, exhibiting lower IC_{50} values as compared to 11 plant species (IC_{50} = 14.2 μg/ml for HL-60, 19.6 μg/ml for CEM leukaemia cells, 17.2 μg/ml for B-16 murine melanoma cells, and 32.5 μg/ml for HCT-8 human colon carcinoma cells) (Costa-Lotufo *et al.* 2005).

In Central Laos, the plant is used as an anti-allergic. Also the roots are mixed with the roots of *Bi kheuy ton*, and the roots of *Kok bi hon* to be used against diabetes (Libman *et al.* 2006). In China, it has been widely used as anti-inflammatory, anti-pyretic and anti-hypersensitivity (Roy *et al.* 2007).

In Thailand, the stem bark boiled in water has been used to treat arthritis (Laupattarakasem *et al.* 2003). It is also used against abscesses, skin inflammation, and for purifying blood, and as an expectorant. When the stem bark is mixed with alcohol it can be used in children for treating fevers, tongue inflammation, bruises and swellings. Fresh stem bark mixed with citric acid is also used against vomiting, and used in combination with other herbal medicines for the treatment of diabetes (Wuthithamvech 1997). The seeds are used against coughs, tumors, diarrhoea, and also used as a tonic (Palasuwan *et al.* 2005).

Current pharmacology of *Oroxylum indicum*

According to Palasuwan *et al.* (2005), the seeds boiled in water showed antioxidant activity. It was found that *O. indicum* extract could completely inhibit the Heinz body formation at the dilution of 1:20 and its total antioxidant was 7.5 mM Trolox equivalent in 1 g of herb. The leaves and the shoots were also reported to have *in vitro* antioxidant activities (at 1mg/25ml) in the β-carotene bleaching assay.

The root bark alcoholic extract was found to have gastroprotective effects against ethanol and water-immersion restraint stress (WIRS)-induced gastric ulcer in rats (Zaveri & Jain 2007) as well as anti-ulcer effects against experimental gastric ulcers. It was suggested that the antiulcer activity may be due to the presence of baicalein which was found to be the major component of the active fraction (Khandhar *et al.* 2006) and also due to its flavonoid glycosides which were tested for their ulcer protective effects against various gastric ulceritis models in rats (Babu *et al.* 2010).

Anti-inflammatory and anti-analgesic activities were also found in the alcoholic and aqueous extract of the stem bark which showed anti-inflammatory activity by inhibiting the release of myeloperoxidase (Laupattarakasem *et al.* 2003). The alcoholic extract of the stem bark also showed significant anti-inflammatory activity against carrageenan induced rat paw edema comparable to the effect of phenylbutazone (Prasad *et al.* 1989) and the leaves demonstrated analgesic activity in the writhing and hot plate tests (Upaganlawar *et al.* 2007).

The dichloromethane extracts of the root and stem bark, showed potent antifungal activity against dermatophytes- *Microsporum gypsum* and slight activity against *Trichophyton mentagophytes* by inhibiting the development of mycelium and conidia in filamentous fungi, this was probably due to lapachol which was identified by TLC (Ali *et al.* 1998). Its compounds, chrysin (5, 7-dihydroxy flavone), and three chrysin analogues also exhibited moderate antibacterial activity against a panel of susceptible and resistant gram-positive and gram-negative organisms (Babu *et al.* 2006) including *Shigella flexneri* (Thatoi *et al.* 2008).

Anticancer and antiproliferative activities of *O. indicum* have been widely studied. A nitrosated *O. indicum* fraction exhibited *in vivo* genotoxic and cell proliferative activities in the pyrolic mucosa of rat stomach (Tepsuwan *et al.* 1992). Similar effects were found in the fraction of methanolic extract from the fruits, baicalein as an active compound, demonstrated antiproliferative effects on human cancer cells HL-60 (Roy *et al.* 2007). Baicalein, isolated from the flowers and fruits also showed anti-mutagenicity activity against Trp-P-1 in an Ames test (Nakahara *et al.* 2002).

Stem bark ethanolic extract has an antiproliferative effect against MCF7 and MDA-MB-231 breast cancer cell lines (Lambertini *et al.* 2004) as well as cytotoxicity against B-16 (murine melanoma), HCT-8 (human colon carcinoma), CEM and HL-60 (leukaemia) tumour cell lines (Costa-Lotufo *et al.* 2005). *O. indicum* was shown to increase the life span of WBC, RBC and TLC count in Dalton's lymphoma ascites tumour cell lines transplanted Swiss albino mice (Sam & Ganesh 2005).

The n-butanol extract of the root bark (100 mg/kg body weight, per oral) showed *in vivo* immunostimulant/ immunomodulatory activity by enhancing specific immune response (humoral immunity) and non-specific immune response (phagocytosis) of the body (Gohil *et al.* 2009). A polyherbal preparation called "Indukantha Ghritha", which consists of 17 plants including *O.indicum* was found to have an activity which reversed cyclophosphamide-induced myelosuppression in control tumor bearing animals (George *et al.* 2008).

POUZOLZIA INDICA (L.) GAUDICH. (URTICACEAE)

Basionym: *Parietaria indica* L., synonym *Pouzolzia zeylanica* (L.) Benn.

Common name: Khob-cha-nang-dang (Thai), Dudhmor-goch (Assam)

Botanical description of *Pouzolzia indica*

Figure 21 Identification of *Pouzolzia indica* (photographed by the author)

P. indica is a shrub without stinging hairs. It is a monocotyledon with little hairs on its stem and leaves. Its leaves are alternated or rarely opposite. The leaf is blade-liked or stipules-lanceolated shape or sometimes rhombic-ovale with a papery look, often persistent, lateral, leaf blade 3-veined, margin serrate, dentate, or entire. Its secondary veins comprise 2 apical pairs. The leaf base is round with a group of tiny flowers in between the corners of the leaves and the branches. The fruit is small and longitudinally ribbed (van Valkenburg & Bunyapraphatsara 2001).

Systematic classification of *Pouzolzia indica* (Tropicos 2010c)**:**

Kingdom:	Plantae	(Plants)
Subkingdom:	Tracheobionta	(Vascular plants)
Superdivision:	Spermatophyta	(Seed plants)
Division:	Magnoliophyta	(Flowering plants)
Class:	Magnoliopsida	(Dicotyledons)
Subclass:	Magnoliidae	
Order:	Rosales	
Family:	Urticaceae	
Genus:	*Pouzolzia*	
Species:	*Pouzolzia indica* (L.) Gaudich.	

Chemistry of *Pouzolzia indica*

Not known

Medicinal use of *Pouzolzia indica*

***Pouzolzia indica* in traditional medicine**

In Malaysia, a poultice of the leaves is used against stomach-ache and sores. In Indonesia, a poultice of the leaves is used against ulcers. In Java, juice or a decoction of the leaves is used as a galactogogue. In Vietnam, the whole plant is used against coughs and sore throat, or used as a diuretic and galactogogue. In the Philippines, the leaves are used against gangrene. In India, the whole plant is used against gonorrhoea, syphilis and wounds. Moreover, in China, the roots are used against sores, abscesses, and swellings (van Valkenburg & Bunyapraphatsara 2001).

In Assam, Nate people use the whole plant against snake bites, use in convalescence after child birth, syphilis, and gonorrhoea (Sikdar & Dutta 2008). In Thailand, it has been used for the treatment of parasite infections in children, expelling menstrual blood, against discharge in urine and a treatment for pus. It has also been used in dermatological and urological diseases (Roongruangchai *et al.* 2009). The leaves have been applied externally as an anti-inflammatory, while the aerial parts can be used internally as emmenagogue, diuretic and insecticide (Saralamp *et al.* 2000).

Current pharmacology of *Pouzolzia indica*

Several fractions of methanolic extract of *P. indica* have cysticide effects (kill the cysts) of Acanthamoeba which is a group of single-celled free-living amoeba that are opportunistic pathogens of humans, causing infections of the eyes, contact lenses and contact lens cases. It is suggested that *Pouzolzia indica* might be beneficial as a disinfectant solution for contact lens cases if the active ingredients are sufficiently purified (Roongruangchai *et al.* 2009).

RHINACANTHUS NASUTUS (L.) KUNTZE. (ACANTHACEAE)

Common name: Daun burung (Indonesia), Tong-pan-chang (Thai), Tereba (Malay), Tagak-tagak (Tagalog), Anitia (Burma), Thong kan sang (Laos).

Botanical description of *Rhinacanthus nasutus*

Figure 22 Identification of *Rhinacanthus nasutus* (photographed by the author)

R. nasutus is a small shrub which grows up to about 1 m tall having many branches and green leaves. The leaves are simple with an oblong-lanceolated shape, narrowed and pointed at both ends. Flowers are usually in clusters, subsessile calyx with 5 narrow lobes, short connate at base, the upper 2-lipped corolla is white, erect and oblong. Lower lip has 3 large lobes, white with red-brownish dots near the base. The fruit is club-shaped with the basal part sterile and 4 seeds. Seeds held up on well developed hooks, orbicular, flat, and pubescent (de Padua *et al.* 1999)

Systematic classification of *Rhinacanthus nasutus* (Tropicos 2010d):

Kingdom:	Plantae	(Plants)
Subkingdom:	Tracheobionta	(Vascular plants)
Superdivision:	Spermatophyta	(Seed plants)
Division:	Magnoliophyta	(Flowering plants)
Class:	Magnoliopsida	(Dicotyledons)
Subclass:	Magnoliidae	
Order:	Lamiales	
Family:	Acanthaceae	
Genus:	*Rhinacanthus*	
Species:	*Rhinacanthus nasutus* (L.) Kuntze	

Chemistry of *Rhinacanthus nasutus*

R. nasutus is reported to contain several naphthoquinones: rhinacanthin-A, and –B, isolated from roots, and naphthoquinones rhinacanthin-C and –D, isolated from the aerial parts. Most of these showed significant cytotoxicity, particularly rhinacanthin -D, -H, -K, -M, and –Q, and also showed inhibition of rabbit platelet aggregation (de Padua *et al.* 1999). Other compounds such as Sesquiterpenoids, Naphthoquinone esters have also been found (Cheeptham & Towers 2002).

<u>Medicinal use of *Rhinacanthus nasutus*</u>

***Rhinacanthus nasutus* in the traditional medicine**

The stem and leaves are used for the treatment of ringworms and other skin diseases caused by fungi (Kodama *et al.* 1993; Awai *et al.* 1995). The leaves and roots, soaked in vinegar or alcohol, pounded with lemon or tamarind, or made into decoction are applied externally as a remedy for certain skin disorders such as ringworm, eczema, skin infections, antipyretic, and anti-inflammation. In Vietnam, its infusion has a reputation in folk medicine for the treatment of hypertension (de Padua *et al.* 1999; Farnsworth & Bunyapraphatsara 1992).

In China, the stem and leaves are applied against ringworm infections and early stages of tuberculosis. The leaves are also taken internally as an antipyretic, antihypertensive, anti-inflammatory, detoxicant and used against snake venom (Sendl *et al.* 1996). In South China and India, ethanol extracts of the roots and water extracts of the leaves were reported to be used for the treatment of hepatitis, diabetes, and hypertension, and in Taiwan, it is used against skin diseases (Gotoh *et al.* 2004).

In Thailand, its fresh leaves are soaked with alcohol, prepared as a solution, and have been reported as an excellent herbal drug for various skin conditions such as ringworm, severe eczema and *Tinea* infections (Saralamp *et al.* 2000; Suchawan 1989). *R. nasutus* has been reported as a remedy against cancers (Farnsworth & Bunyapraphatsara 1992) and the part use for against cancer is the root (Siripong *et al.* 2006).

This plant has also been used for the treatment of mental disorders, inflammation, rheumatism, circulatory problems, asthma and bronchitis, epilepsy and immune system deficiency (Punturee *et al.* 2004). The tea made from this plant has been used for treating colds, fevers, sore throat, to refresh the lungs, against the early stages of TB, relieve from headache and hypertension, to reduce blood pressure, and constipation (Cheeptham & Towers 2002).

Current pharmacology of *Rhinacanthus nasutus*

The methanol extracts of the leaves and stems have been found to have antifungal activity against *Pyricularia oryzae* which is the pathogen of rice blast disease. The active compound isolated was identified as a naphthopyran derivative; 3,4-dihydro-3,3-dimethyl-2H-naphtho [2,3-b] pyran-5,10-dione (Kodama *et al.* 1993). An extract from the leaves (extract solvent not reported) showed antifungal effects against various dermatophytes; *Trichophyton mentagrophytes*, *T.mentagrophytes var. interdigitale*, *T. rubrum*, *Microsporum canis* and *M. gypseum* (Darah & Jain 2001).

The ethanol extract of the leaves showed moderate antimicrobial activities against *Bacillus subtilis*, *Staphylococcus aureus* K147 methicillin-sensitive, *Escherichia coli* (wild type), and *Pseudomonas aeruginosa* 187 (wild type) (Cheeptham & Towers 2002). The ethylacetate extract of the leaves was also tested for antimicrobial activities and was found to have a potent bactericidal activity against *Streptococcus mutans*, and a potent bacteriostatic activity against *Streptococcus epidermidis, Propionibacterium acnes* and *Staphylococcus aureus*. However, it was not active against *Candida albicans*. The active antibacterial compound was identified as rhinacanthin-C (Puttarak *et al.* 2010).

The aerial parts, extracted with 1:1 dichloromethane: isopropanol, were evaluated for antiviral activity. It was found that Rhinacanthin-C and Rhinacanthin-D are antiviral active compounds against cytomegalovirus (Sendl *et al.* 1996). Other studies also reported that the dichloromethane-2-propanol (1:1) extract of the aerial parts yielded active compounds Rhinacanthin E, and rhinacanthin-F which showed *in vitro* antiviral activity against influenza type A in the anti-Flu-A cytopathic effect (CPE) assay, with the EC_{50} of 7.4 and 3.1 μg/ml, respectively (Kernan *et al.* 1997).

The ethanol extract of the root and the aqueous extract of the leaves yielded Rhinacanthin C which was found to have an anti-proliferative activity (Gotoh *et al.* 2004). Also another rhinacanthone identified as 3,4-dihydro-3,3-dimethyl-2H-naphtho-[1,2-B] pyran-5,6-dione, isolated from the petroleum ether hot percolation of the aerial parts, was found to be an active compound against tumor growth in Swiss albino mice undergoing intra-peritoneal inoculation with Dalton's ascetic lymphoma (Thirumurugan *et al.* 2000).

Moreover, rhinacanthin-M, -N, and -Q, isolated from the methanol extract of the roots, showed significant anticancer activity against human carcinoma cell lines (epidermoid carcinoma, HeLa, and HepG2) and vero cell line (African green monkey kidney cell) (Kongkathip *et al.* 2004). There was also a report that rhinacanthones could also induce apoptosis in human leukemic cell lines (Senthilkumar, et al, 2004). Furthermore, rhinacanthins-C, -N and -Q have been found to induce apoptosis in tumour cells by being involved in the activation of caspase-3 (Siripong *et al.* 2006).

Rhinacanthin Q, A, B, C, D, G, H, I, K, M, N as well as wogonin showed significant cytotoxicity against P-388, A-549, HT-29 and HL-60 tumour cells. On the other hand, Rhinacanthin Q, A, B, C, G, H, I, K, M and wogonin showed 36–100% inhibition of the rabbit platelet aggregation induced by arachidonic acid. Rhinacanthin A, B, C and wogonin also showed 72–100% inhibition against the rabbit platelet aggregation induced by collagen. Only rhinacanthin-B exhibited antiplatelet aggregation induced by platelet activation factor (Wu *et al.* 1998)

The ethanol extract of the aerial parts demonstrated an analgesic activity in the acetic acid induced-writhing test (Karunambigai & Sugumaran 2005). Also it was reported that the water and ethanol extracts of the stem and leaves showed immunomodulatory activity in both non specific cell-mediated and humoral immune responses (Punturee *et al.* 2005). The extracts were reported to have no effects on nitric oxide production in J774.2 mouse macrophages at concentrations between 62.5 and 1000 μg/ml. The extracts (100 ng/ml) were found to lead to a small increase in TNF-α expression but did not change iNOS (Punturee *et al.* 2004).

On the contrary, rhinacanthin C, D and N isolated from the ethanol extract of the leaves showed potent anti-allergic activity by inhibiting TNF-α and IL-4 gene expression in antigen-induced TNF-α and IL-4 releases RBL-2H3 cells (Tewtrakul *et al.* 2009). These three compounds also showed anti-inflammatory activity against LPS-inducing the release of nitric oxide, PGE_2 and TNF-α in RAW264.7 cells by inhibiting the iNOS and COX-2 gene expressions (Tewtrakul *et al.* 2009). Other activity reported was a significant hepatoprotective effect against paracetamol induced-liver damage in rats on a basis of a decrease in serum enzymes levels (Suja *et al.* 2004).

1.5 PRINCIPLES OF INFLAMMATION

Inflammation is a protective response of human tissues from injuries, irritation or infections. It is characterized by vasodilation; the capillaries become more permeable in order to facilitate fluid, large molecules and white blood cells (especially neutrophiles and monocytes) to the affected region. This can cause heat or increased temperature, redness, and swelling. Also the release of inflammatory mediators and the compression of nerves can cause pain and sometimes the affected tissues may be altered or changed in their functions (Braun & Anderson 2006; Aggarwal *et al.* 2006)

The inflammatory response can be acute or chronic as well as local or systemic, depending on the cause, the organs involved and individual conditions. After the white blood cells and contributors migrating into damaged tissues and finish eliminating the pathogens that caused the tissue injury, they will return to normal and resume their usual functions. Thus, it is likely to be beneficial if the inflammatory reaction is acute, inducing immune response in a local area within a short period of time through a complex sequence of actions including local leukocyte recruitment, death and migration (Buckley *et al.* 2001).

In acute inflammation, the initial white blood cell (mainly neutrophil) will last about one or two days, after that monocytes are more dominant. IL-6 is an important factor to switch between acute and chronic inflammation (Kaplanski *et al.* 2003). If there is a prolonged infection or severe inflammation both neutrophils and monocytes can be toxic to normal surrounding tissues. Neutrophils have been found to be a major source of free radical at inflammatory sites (Johar *et al.* 2004). The increased neutrophil count or increased monocyte counts as well as toxic granulation can be found in a patient's blood film (Bain 2004). An example is shown in the photo below.

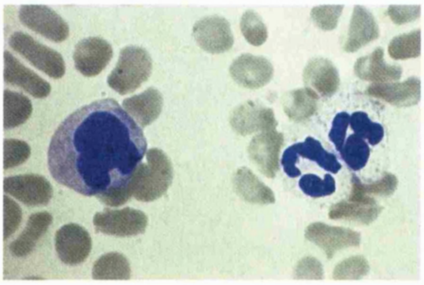


Figure 23 A toxic monocyte (a dark-purple cell on the left), and toxic granulation of neutrophils (dark-purple granules in cytoplasm of two neutrophils) are found in the blood film of a patient who is suffering from chronic inflammation (photo from pathmicro.med.sc.edu).

1.5.1 PRO-INFLAMMATORY MEDIATORS AND THEIR ROLES IN INFLAMMATION

The inflammatory response requires communication between different types of immune cells to maintain their functions. Therefore, the immune system produces inflammatory mediators by a variety of cell types, but mainly from monocytes and macrophages, in order to interact with the immune system cells and generate the response against disease and infection (Gabay 2006). Several studies reported cytokines that were produced during inflammatory process, includes TNF-α, IL-1β, IL-6, and IL-8. Elevated levels of cytokines have been found in many diseases and conditions, such as subarachnoid hemorrhage (Kimura *et al.* 2003), rheumatoid arthritis (Zangerle *et al.* 1992), and even in menopause (Yasui *et al.* 2008).

However, to balance the effects of pro-inflammatory cytokines, the body produces anti-inflammatory cytokines such as IL-4, IL-10, and IL-13, to suppress the production of IL-1, TNF, and IL-8 (Dinarello 2000). Therefore, it has been suggested that the determination of disease and the severity depends on the balance between the effects of pro-inflammatory and anti-inflammatory cytokines (Malaviya 2006). This thesis focuses on the screening of the selected Thai plants which have activity to inhibit pro-inflammatory cytokines. The pro-inflammatory cytokines used to examine such activities and their detailed functions and effects, are as follows.

Interleukin-6 (IL-6)

Interleukin-6 is produced at the site of inflammation and plays a key role in controlling the extent of local and systemic acute inflammatory responses (Xing *et al.* 1998; Gabay 2006). It has been found that IL-6 influences the control of individual leukocytes such as neutrophils, monocytes and lymphocytes by dictating the recruitment, activation and apoptotic clearance (Jones et al 2005). IL-6 and its soluble receptor may dictate the transition from acute to chronic inflammation by changing polymorphonuclear neutrophils to monocytes or macrophages. IL-6 has dual effects as during the acute phase response it acts as a defense mechanism but in chronic inflammation it is pro-inflammatory (Gabay 2006). In type 2 diabetes, plasma levels of IL-6 has been shown to be elevated in obese people with insulin resistance (Kern *et al.* 2001; Vgontzas *et al.* 2000).

Interleukin-1β (IL-1β)

Interleukin-1β is a one of the potent pyrogens and pro-inflammatory cytokines of the IL-1 family primarily released by activated monocytes but also by macrophages, dendritic cells and a variety of other cells in the body (Dickinson 2002; Sasaki *et al.* 2002; Dinarello 1996). IL-1β has both paracrine (peripheral) and endocrine (hormonal) effects when binding to its receptors. Its paracrine effects are the stimulation of blood clotting factors and the synthesis of interleukins, whereas its endocrine effects are the stimulation of the release of prostaglandins inducing hypotension and fever. Besides this, it induces the release of adrenocorticoids and other cytokines involving in inflammatory and immune responses (Li *et al.* 2008).

Evidence of elevated IL-1β has been found in the acute phase of rheumatoid arthritis, heat-burns, septic shock, and Alzheimer's disease (Li *et al.* 2008; Casey *et al.* 1993; Lanzrein *et al.* 1998). IL-1β production also leads to stimulation of insulin resistance in the obese (Maedler *et al.* 2009). In addition, IL-1β plays a role in the creation and maintenance of the state of pain (White & Jones 2008; Takeda *et al.* 2009). IL-1α and IL1-β, but not other pro-inflammatory cytokines, strongly induced primary and secondary Cd4 responses in mice (Ben-Sasson *et al.* 2009). However, IL-1α and IL1-β appear not to influence killing of virus-infected cells but enhance migrations of IgM antibody and $CD4^+$ T cells to the site of infection (Schmitz *et al.* 2005).

Tumor necrosis factor-α (TNF-α)

TNF- α is a multifunctional pro-inflammatory cytokine that belongs to the tumor necrosis factor (TNF) super-family. It is produced by several cell types including macrophages, monocytes, T-cells, smooth muscle cells, adipocytes, and fibroblasts. TNF-α plays a crucial role in the innate and adaptive immunity, cell proliferation, and apoptotic processes. Generation of TNF-α at high levels leads to the development of inflammatory responses that are the hallmarks of a variety of diseases as well as cancer (Popa *et al.* 2007). TNF-α causes insulin resistance and has been found to be elevated in obese people with type 2 diabetes (Hotamisligil *et al.* 1995; Kern *et al.* 2001).

There is growing evidence that the effect of one cytokine is often regulated by another as a network in many physiological and pathological conditions (Haddad 2002). It has been reported that TNF-α and IL-6 have synergistic actions and sometimes overlap although some of the effects are regulated by distinct mechanisms (Fox 2000; Vassalli 1992). Interestingly, an *in vivo* study found that TNF-α can increase the production of IL-6 while, in contrast, IL-6 does not increase the production of TNF-α (Matsuno *et al.* 2002). TNF also has synergistic effects with IL-1 and has been reported commonly since both cytokines are created at the same local site of inflammation (Dinarello 2000).

Prostaglandin E_2 (PGE_2)

PGE_2 is one of the most abundant eicosanoid lipid metabolites of arachidonic acid. It is synthesized by most of the cells in the body and is control led by cyclooxgenase enzymes in response to cell-specific trauma, stimuli, or signaling molecules (Park *et al.* 2006). PGE_2 has a role in numerous homeostatic biological functions including the increase of vascular permeability, fever generation, and hyperalgesia (Chizzolini & Brembilla 2009; Funk 2001) and is also a potent inducer of pro-inflammatory cytokines such as IL-10 (Harizi & Gualde 2006) and IL-23 (Sheibanie *et al.* 2004). During the pain states, PGE_2 is stimulated by IL-1 or by the combination of IL-1 and TNF (Dinarello 1996). IL-1 also lowers the threshold of pain primarily by increasing PGE_2 synthesis (Schweizer *et al.* 1988).

1.5.2 NUCLEAR FACTOR KAPPA B (NF-κB) AND ITS ROLE IN INFLAMMATORY DISEASES AND CANCERS

Chronic inflammation leads to many diseases including cancers. Thus, controlling inflammation is becoming a key for curing and preventing such diseases. In the past fifteen years, a protein nuclear factor kappa B (NF-κB) has been studied largely as the key factor which causes inflammation. The presence of activated NF-κB has been found in the tissues of most cancers including leukemia, lymphoma, and cancers of the oral cavity, liver, pancreas, colon, prostate, breast, and ovary (Aggarwal & Gehlot 2009).

NF-κB has been known as a redox sensitive transcriptional factor that controls expression of genes involved in the regulation of inflammatory related diseases and survival of the cells. As already identified in many studies, NF-κB controls the expression of genes encoding the pro-inflammatory cytokines (e.g. IL-1, IL-2, IL-6, TNF-α), chemokines (e.g. IL-8, MIP-1α, eotaxin), adhesion molecules (e.g. ICAM, VCAM, E-selectin), inducible enzymes (COX-2 and iNOS), growth factors, and immune receptors, all of which play major roles in controlling most inflammatory diseases (Nam 2006).

Therefore, NF-κB is responsible for promoting the expression of genes that are required for the resolution of inflammation (Gilroy *et al.* 2004), maintaining cell proliferation, preventing apoptosis and increasing blood flow to ensure cell survival. It also controls transformation of cells from one type to another type, controls cell migration and invasion from one site to a specific site before entering the tissue in order to promote inflammation (Van Waes 2007). As such, NF-κB protects the body from infection and inflammatory triggering agents, maintains good health and longevity as aging is also protected (Salvioli *et al.* 2006).

However, these effects seem to be similar to the principles of cancer development. In addition to its role in promoting inflammation, NF-κB also plays a role in the development of cancer. In cancer cells, the cells replicate without proper control, cell death is restrained, blood flow is increased to promote cancer survival, inflammation occurs and the cells travel to other sites and invade different types of normal cells such as bone and brain (Karin & Greten 2005; Sethi *et al.* 2008). As a result, prolonged activation of NF-κB has been found to be associated with many types of cancer (Van Waes 2007; Sethi *et al.* 2008).

Not only cancers, but autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus, Crohn's disease, multiple sclerosis, autoimmune thyroid disease, and psoriasis, have also been associated with over activation of NF-κB (Orozco *et al.* 2005; Di Sabatino *et al.* 2005; Kuryłowicz & Nauman 2008). Also in viral infections such as hepatitis C (not hepatitis B), it has been found that NF-κB increases hepatitis C virus loads which can promote chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Sato *et al.* 2006).

In autoimmune type 1 diabetes, NF-κB activation has a pro-apoptotic role following exposure to cytokines such as IL-1β and TNF-α (Ortis *et al.* 2008) leading to pancreatic β cell death and thus diabetes sufferers lack of insulin. It has been suggested that inhibition of this process could be beneficial as an effective strategy to protect β-cells (Eldor *et al.* 2006; Melloul 2008). On the other hand, in type 2 diabetes which is associated with insulin resistance, there is evidence which suggests that NF-κB activation leads to muscle insulin resistance (Sriwijitkamol *et al.* 2006).

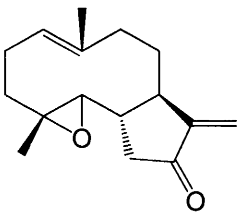
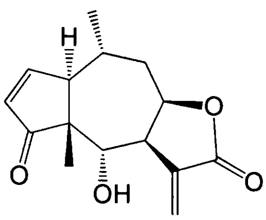
The NF-κB family consists of five protein subunits; RelA (p65), RelB, c-Rel, NF-κB1 (p50 and its precursor, p105), and NF-κB2 (p52 and its precursor, p100) (Jacque *et al.* 2005). NF-κB subunits normally remain in the cytoplasm binding to inhibitory proteins called IκBs (or known as inhibitors of NF-κB) in its inactive form. When stimuli such as stress conditions, free radicals, UV radiation and pathogens, binding to Toll-like receptors (TLRs), the release of chemical signals e.g. cytokines are stimulated inducing NF-κB to be activated (Salminen *et al.* 2008).

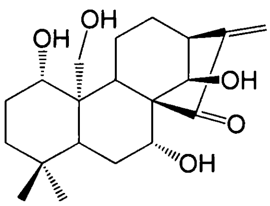
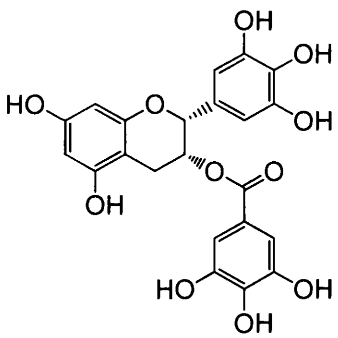
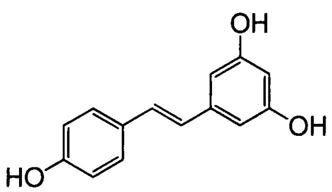
There are three pathways that can activate NF-κB. First, the typical pathway which can be triggered by infections or pro-inflammatory cytokines leads to IkB degradation upon phosphorylation by IκB Kinase (IKK). Second, the alternative pathway activated by the TNF family through selective activation of IKK-α homodimers by the upstream kinase NIK. The third pathway is called CK2 and is IKK independent (Escárcega *et al.* 2007). However, NF-κB is activated mostly through IκB kinase–dependent (IKK-dependent) phosphorylation. All these pathways control cell proliferation and cell death. It has been found that NF-κB inhibits caspase enzymes which seem to directly induce apoptosis (Uzzo *et al.* 2001).

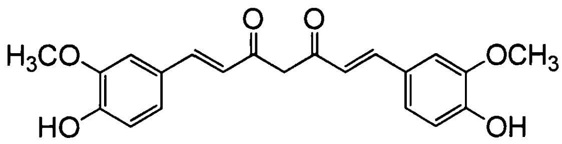
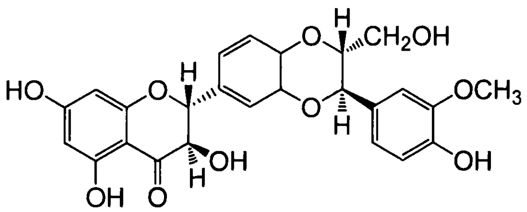
1.5.3 NATURAL PRODUCTS AS NF-KB INHIBITORS

A large number of natural products from various chemical classes have been determined to have NF-κB inhibitory properties. This includes sesquiterpene lactones, kaurene diterpenes, triterpenes, phenolics, and γ-lactams (Folmer *et al.* 2008). A selection of natural products reported as potent NF-κB inhibitors are listed in table 2.

Table 2 A selection of natural products that act as NF-κB inhibitors, their sources and their minimum inhibitory concencentrations.

Names/structures	**Sources/species name**	**MIC**	**References**
Sesquiterpene lactones:			
Parthenolide 	Feverfew (*Tanacetum parthenium*)	30 μM	Bremner & Heinrich (2002)
Helenalin 	*Arnicae spp.*	10 μM	Bremner & Heinrich (2002); Siedle *et al.* (2004)

Names/structures	Sources/species name	MIC	References
di- and tri-terpenoids:			
Kamebakaurin 	*Isodon japonicas*	27 µM	Bremner & Heinrich (2002)
Phenolics :			
Epigallocatechin-3-gallate 	Green tea (*Thea sinensis*)	20 µM	Bremner & Heinrich (2002)
Resveratrol 	Red wines (*Vitis spp.)*	5 µM	Bremner & Heinrich 2002

Names/structures	Sources/species name	MIC	References
Phenolics (continue):			
Curcumin	*Curcuma longa*	10 µM	Bremner & Heinrich (2002)
			
Silymarin (family of silybin A)	Milk thistle (*Silybum marianum*)	25 µM	Agarwal *et al.* (2006)
			

1.5.3 REACTIVE OXYGEN SPECIES, OXIDATIVE STRESS AND LIPID PEROXIDATION

Reactive oxygen species (ROS) is a term used to explain a variety of 'free radical' molecules with one unpaired electron. Normally an oxygen molecule is stable as a bi-radical, containing two unpaired electrons. As a result of the two single electrons having the same spin, it can only react with one electron at a time. If one of the two unpaired electrons is excited and changes its spin, singlet oxygen will become more reactive, reacting with other molecules, especially ones containing double bonds (Turrens 2003). Once the process has begun, it can result in a chain reaction causing trouble in a living cell.

Normally the body is able to produce antioxidants which can neutralize free radicals that might damage cells. However, sometimes excess exposure to oxidants (which are increased in the environment) can overwhelm the body's ability to detoxify, thus oxidative stress conditions may occur as a consequence. Chronic inflammation can also introduce oxidative stress, nitrosative stress and lipid peroxidation, causing excess reactive oxygen species (ROS), reactive nitrogen species (RNS), accumulations and massive DNA damage, along with deregulation of cell homeostasis, leading to malignant diseases (Bartsch & Nair 2006).

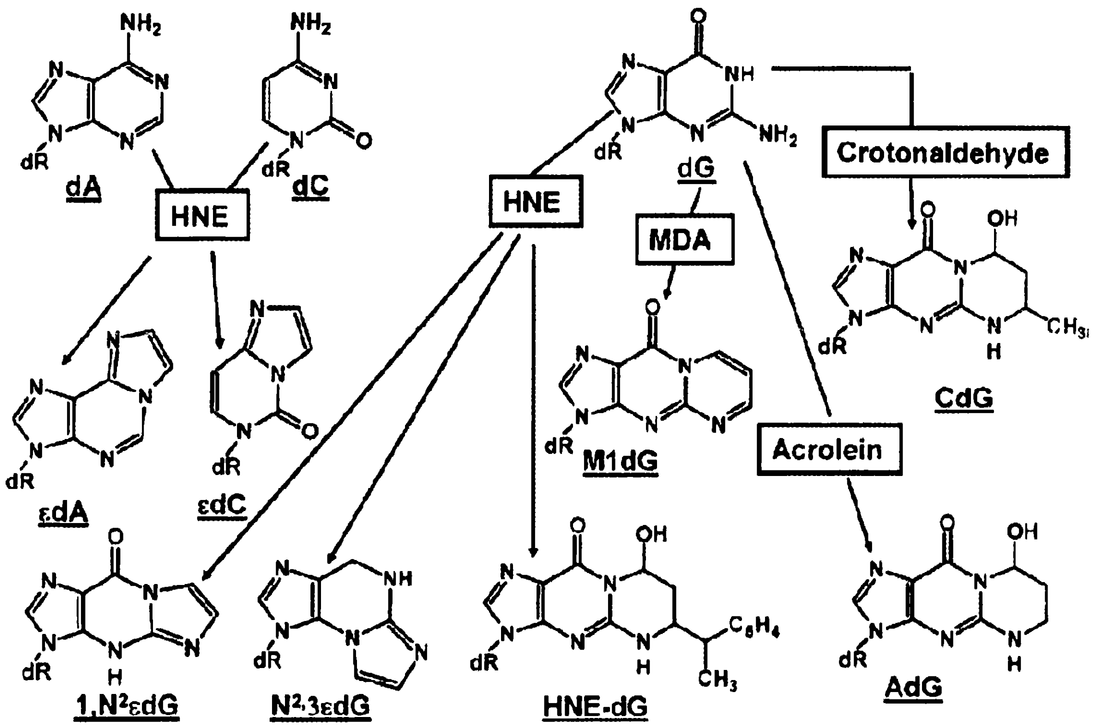
Table 3 Reactive oxygen species of interest in oxidative stress

Oxidant	**Product-origins**	**References**
O_2^-, superoxide anion	• Mitochondrial respiration through reduction of molecular oxygen. • Various enzymatic oxidation reactions e.g., reactions by cytochromes P450. • During the innate immune response.	Finkel & Holbrook (2000); Pryor (1986)

Oxidant	Product-origins	References
$NO^{\cdot}$, nitric oxide radicals	a product of nitric oxide synthases (NOS)	Yao *et al.* (2004)
H_2O_2, hydrogen peroxide	Produced by neutrophils during reactions engendered by phagocytosis	Sibille *et al.* (1987)
•OH, hydroxyl radical	Fenten reaction, wherein Fe^{2+} or Cu^{2+} functions as a reducing agent. Note that this radical is very powerful, and damages most cellular macromolecules, even though it has a very short life.	Toyokuni (1996)
RO•, alkoxy and ROO•, peroxy radicals	Lipid forms produced by lipid peroxidation reactions.	Kelly *et al.* (1998)

ROS involve many signalling pathways of inflammation, most importantly the four pathways known as NF-κB, activating protein-1 (AP-1), mitogen-activating protein kinase (MAPK), and phosphotidyl inositol-3 kinase (PI3K) pathways, acting as either primary or secondary stimuli leading to different cell functions including cell growth, cell proliferation, cell survival, and inflammation (Gwinn & Vallyathan 2006). Toxic products of lipid peroxidation are peroxyl radicals which propagate through cellular membranes during oxidative stress (Poli *et al.* 2008) can react with DNA bases and produce mutagenic adducts including malondialdehyde-deoxyguanine (M1dG), and etheno- and propano-DNA adducts causing DNA damage and mutations in human cells, as shown in figure 25 (Bartsch & Nair 2004; Nair *et al.* 2007).

Figure 24 Chemical structure of lipid peroxidation products found in human tissue and their adducts which causes changes in the DNA bases resulting in the development of cancers (Nair *et al.* 2007).



Notes of abbreviations used in the diagram:

εdA = 1,N6-etheno-2′-deoxyadenosine;

εdC = 3, N4-etheno-2′-deoxycytidine;

N2,3εdG = N2,3-etheno-2′-deoxyguanosine;

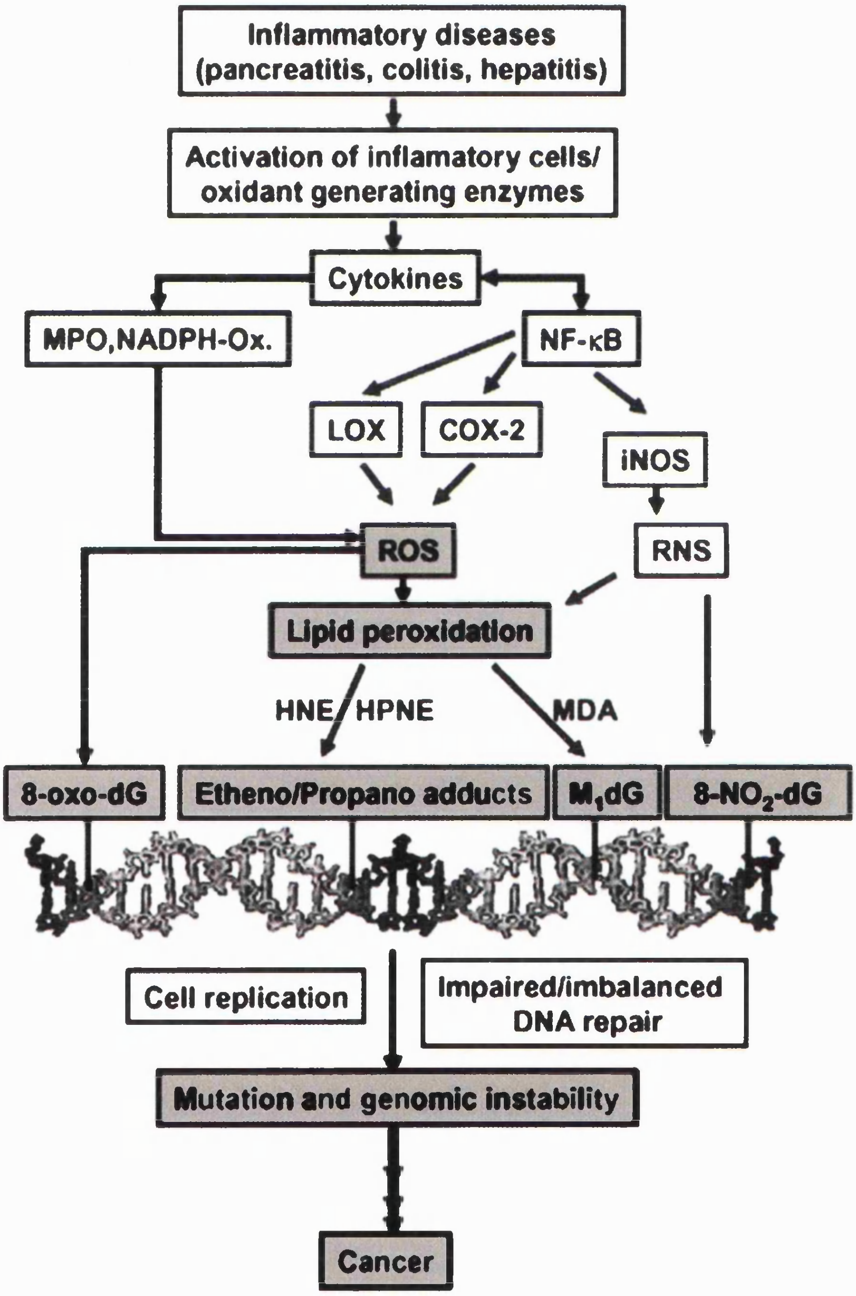
1N2εdG = 1N2-etheno-2′-deoxyguanosine;

M1dG = pyrimido[1,2-α]purine-10(3H)-one-2′-deoxyribose;

AdG = acrolein-dG;

CdG = crotonaldehyde-dG

Figure 25 The diagram illustrates the relationships of reactive oxygen species and lipid peroxidation resulting from chronic inflammatory conditions and their sequence products causing changes in DNA bases, mutations of genes, and cancers (Bartsch & Nair 2004; Nair *et al.* 2007).



It has been confirmed by many studies that oxidative stress is the major cause of several human diseases (Thomas *et al.* 2007; Mimura *et al.* 2007). Antioxidants have been explained as any substance which delays or inhibits oxidative damage to a target molecule. In general, an antioxidant may work in one of five ways: 1) replacing damaged target molecules, 2) keeping formation of reactive species to a minimum, 3) repairing damaged target molecules, 4) binding metal ions required for formation of highly reactive species, 5) scavenging reactive species either by using enzymes or directly by reaction whereby the antioxidant itself would be used up (Gutteridge *et al.* 1994).

Individual antioxidants may, in some cases, act by multiple mechanisms in the same system (Ishige *et al.* 2006) or by a different single mechanism depending on the reaction system. Furthermore, antioxidants may respond in a different manner to different radical or oxidant sources. For example, carotenoids are not particularly good quenchers of peroxyl radicals as compared to phenolics and other antioxidants but are exceptional in quenching singlet oxygen, at which most other phenolics and antioxidants are relatively ineffective (Prior *et al.* 2005)

Antioxidant properties of plants, thus, can be investigated in many different ways. For the present study, three methods were applied. DPPH assay is one of the most popular among all the tests and it is considerably easy and straightforward. Lipid-peroxidation is also valid and possibly suitable for extracts in lipophilic systems. Phenol content determination by the Folin-Ciocalteau method is likely to be the best method to determine phenol contents relating to anti-oxidant effects.

The detailed bioassays employed for the investigation of *in vitro* pharmacological activities including anti-inflammatory, anticancer and antioxidant activities of the selected plants species can be seen in the following method section.

1.6 AIMS OF THE STUDY

This thesis investigates pharmacological activities of the selected medicinal plants based on their reported use recorded in Thai textbooks in order to gather pharmacological evidence related to their popular use and provide selection criteria for further investigations of their active compounds. The selection of the plant for further phytochemical study has been prioritized on the NF-κB anti-inflammatory activities. The species which show potential medicinal utility are subjected to bio-assay guided fractionation using NF-κB as a lead. The structures of the active components were then elucidated using phytochemical and spectroscopic techniques.

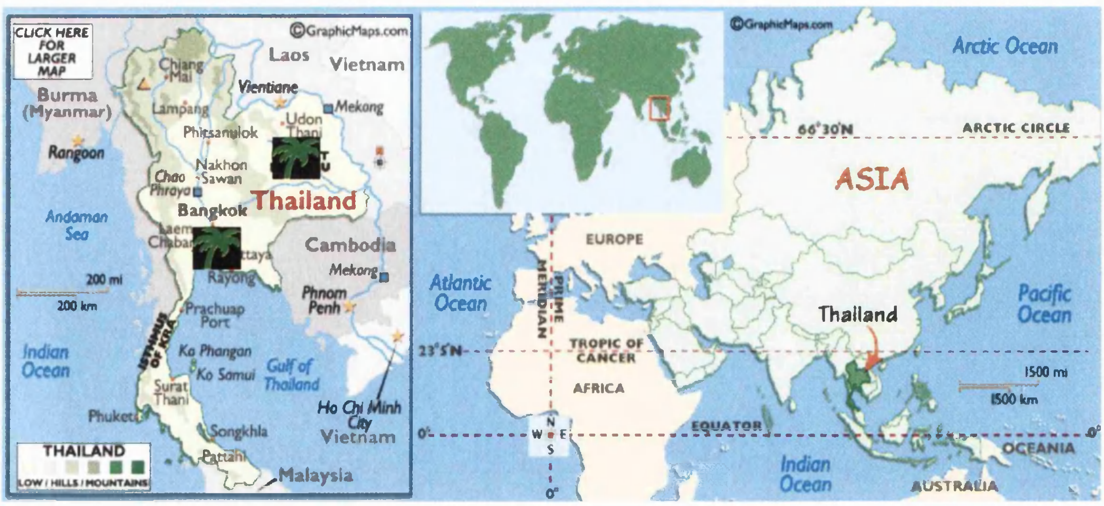
CHAPTER 2

MATERIALS AND METHODS

2.1 PLANT COLLECTION

Fresh leaves of *P. indica* and aerial parts of *M. platyclada* were collected from the Sirirukhachart Botanical Garden, Mahidol University, Thailand. Stem bark of *O. indicum* and leaves of *C. trifolia* were collected in suburban areas of Buriram Province, while leaves of *B. alba, B. rubra, G. pseudochina, G. pseudochina var. hispida* and *R. nasutus* were collected from farmland in the northeastern part of Thailand, mainly in Buriram Province. The plants were gathered during September to October 2006.

Figure 26 Map of Thailand and the areas where the plants were gathered (map from www.worldatlas.com).



 This symbol represents the places where the plants were gathered in Sep - Oct 2006.

2.2 IDENTIFICATION OF PLANT MATERIALS

The fresh and dried plants were identified and compared with specimens at the Forest Herbarium of the Thai Royal Forest Department, Bangkok, Thailand. Voucher specimens were deposited at the Centre for Pharmacognosy and Phytotherapy, School of Pharmacy, University of London. (Accession no. NS06/00001 to NS06/00009).

Figure 27 Identifications of the fresh Thai medicinal plants involved in this study

Basella alba
(Basellaceae)

Basella rubra
(Basellaceae)

Cayratia trifolia
(Vitaceae)

Gynura pseudochina
(Asteraceae)

Gynura pseudochina var. hispida
(Asteraceae)

Muehlenbeckia platyclada
(Polygonacae)

Oroxylum indicum
(Bignoniaceae)

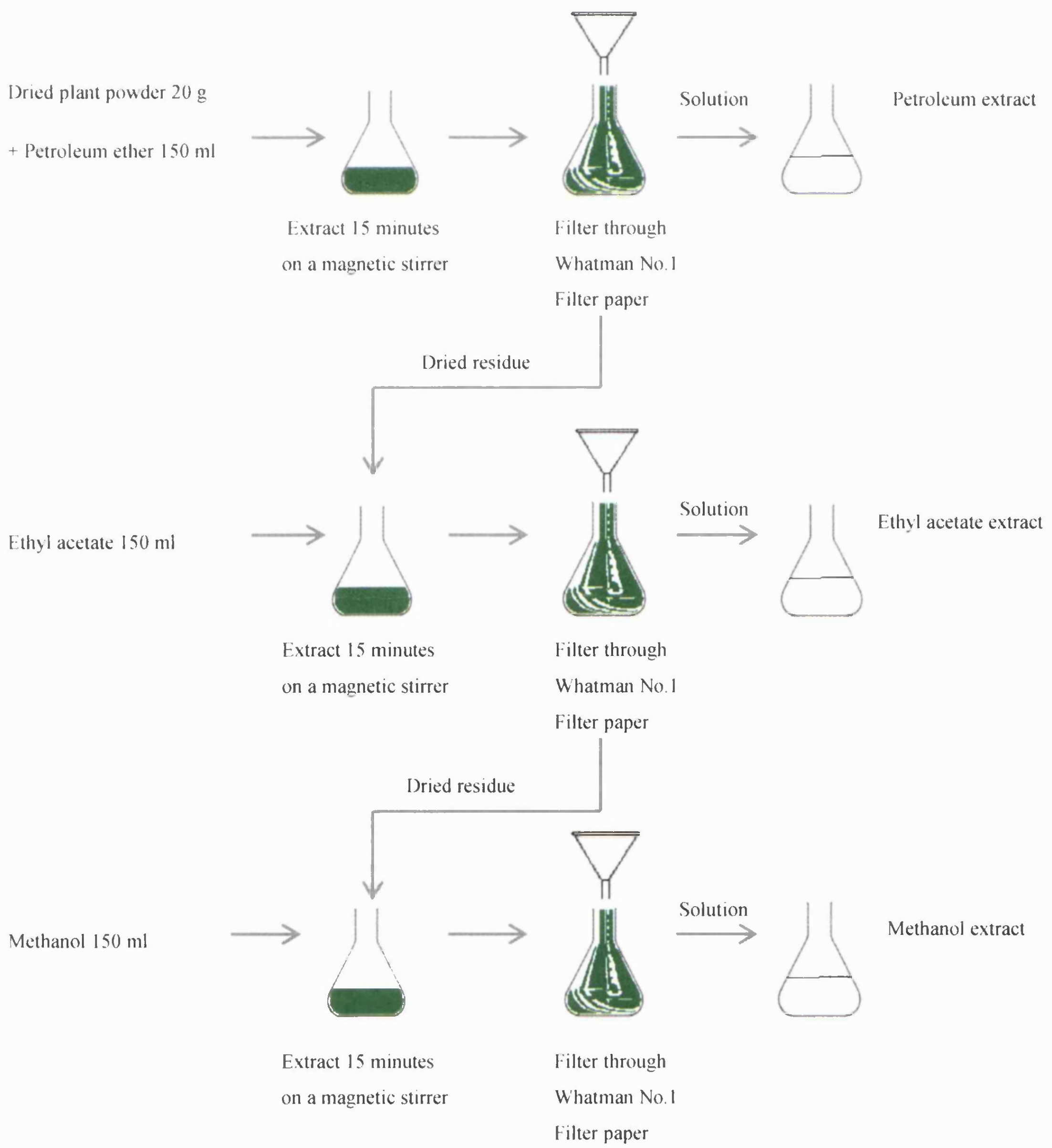
Pouzolzia indica
(Urticaceae)

Rhinacanthus nasutus
(Acanthaceae)

2.3 EXTRACT PREPARATION

After all plant material were collected, washed with water, and dried in the shade at about 35-40°C for several days then processed to a fine powder using a laboratory scale mill. The dried powder, 20 g of each plant, was sequential extracted with petroleum ether, ethyl acetate and methanol following a standardised protocol of the AINR (Anti-Inflammatory Natural Products from Plants) project (Bremner et al 2009).

Figure 28 Extraction process using sequential solvents of increasing polarity



Each solvent extraction was repeated 3 times and each of the extract solutions were combined and dried under pressure using a rotary evaporator. All the dried extracts were kept in tightly stopperd bottles in a fridge (-20°C) until used for the pharmacological testing. Thereafter, the extracts were re-dissolved in 96% ethanol to make a concentration of 20mg/ml then filtered by a mini disk filter (0.45 μm), and stored in amber glass bottles for bioassays.

2.4 CELL CULTURE

This research is based mainly on micro-bioassays and cell culture. Therefore, it must be ensured that the procedures were not contaminated with mycoplasma, bacteria, and fungi, or cross contamination with other cell lines. Processes, material and equipment used in this research are described as follows.

Chemicals used in cell culture and their references;

1. Foetal Bovine Serum -inactivated (Gibco®, Fisher Scientific UK)
2. Dulbecco's modified Eagle medium (D-MEM)- liquid, high glucose with L-glutamine, D-glucose, sodium pyruvate (Gibco®, Fisher Scientific UK)
3. Phosphate Buffer Saline (PBS), pH 7.4 with potassium phosphate, sodium chloride and dibasic sodium phosphate (Gibco®, Fisher Scientific UK)
4. 0.25%Trypsin-EDTA liquid (Gibco®, Fisher Scientific UK)
5. Penicillin-Streptomycin (10000:10000 units)(Gibco®, Fisher Scientific UK)
6. Dimethyl sulphoxide: DMSO (AnalaR BDH, UK)

2.4.1 THAWING CELL LINES FROM LIQUID NITROGEN

Many cell lines, bought commercially normally arrive frozen. In order to use them the cells must be thawed and put into culture. Cells should be thawed rapidly and then diluted slowly into warm growth medium in order to eliminate DMSO which is toxic to the cells. Note that DMSO is used as a cryoprotectant. It prevents crystallization of water that would cause cells to lyse during cryopreservation. Cell-thawing procedures are as follows.

Cell thawing procedures;

1. A sterile cabinet and water-bath were warmed up in advance and media was prepared by adding 50 ml of FBS, and 5ml of Penicillin-Streptomycin into 500 ml of D-MEM.
2. A vial of cells was taken from the liquid nitrogen (or -70° C freezers) and placed in a water bath until it becomes molten.
3. The cells were transferred into pre-warmed media and then centrifuged at 1000 rpm for 2 min.
4. The supernatant was aspirated off and the cell were re-suspended in 5 ml medium and transferred to a culture flask containing 10 ml media for growth.
5. The flask was then placed in the incubator at appropriate temperature and CO_2 level.
6. Cells were observed under the microscope and sub-culture das necessary.

2.4.2 SUBCULTURE OF ADHERENT CELL LINES (CELL PASSAGING)

Adherent cell lines will grow *in vitro* until they have covered the surface area available or the medium is depleted of nutrients. At this point the cell lines should be sub-cultured in order to prevent the culture dying and to increase the number of cells sufficiently for assays. Cell passaging procedures are as follows.

Cell passaging procedures;

1. Old media in the flask was aspirated off and the cells were washed with sufficient amount of PBS solution.
2. The PBS was then aspirated off and 4 ml trypsin-EDTA was added in to the flask for detaching cells from the bottom of the container.
3. The flask was swirled slowly in order to let the trypsin-EDTA cover completely and the flask was incubated for about 5 minutes.
4. The cells were transferred into a falcon tube and 10 ml of media was added.
5. The falcon tube was centrifuged at 1,000 rpm for about 3 minutes. Then the supernatant was aspirated off and the cells were re-suspended with media.
6. The cells were transferred into and a new flask containing 10 ml media and the flask was placed into an incubator to let the cells grow

2.4.3 FREEZING CELLS IN LIQUID NITROGEN FOR STORAGE

The main concept of cell-freezing is not to freeze the cells too quickly. The reason for this is to avoid osmotic shock caused by extracellular and intracellular ice formation, as well as to minimize the heat that is generated when DMSO is added to the water. Cells are frozen in liquid nitrogen, therefore, it is necessary to ensure that a freezer container is always filled with a sufficient amount of nitrogen.

Cell freezing procedures;

1. Viability of the cells should be ensured (not less than 90% before freezing).
2. The cells were trypsinized by adding 4 ml trypsin-EDTA. The flask was swirled and then placed in the incubator for 3-5 minutes.
3. Cells in trypsin-EDTA solution were transfered into a falcon tube and then centrifuged at 1,000 rpm for 3 min.
4. The supernatant was aspirated off out without disturbing the cell pellet, and then 2 ml of 90% fetal bovine serum with 10% DMSO were added. The cells were re-suspended and transferred into cryogenic vials.
5. The cryogenic vial with cells were placed into a Nalgene Cryo-1 container (a Styrofoam container) and placed in a -70°C freezer overnight.
6. The cryogenic vial was then put into a liquid nitrogen container until needed. Note, the cells can be kept for more than six months in liquid nitrogen.

2.5 MEASUREMENT OF ANTI-INFLAMMATORY EFFECTS

In this thesis, in order to determine an anti-inflammatory activity of the plant extracts two assays were used. In brief, the NF-κB inhibitory effects on PMA-induced NF-κB activation in stably tranfected HeLa cells was determined by luciferase assay, and effects on LPS-induced pro-inflammatory mediators PGE_2, IL-6, IL-1β, and TNF-α in primary monocytes was assessed by ELISA. The procedures and chemicals used are described as follows.

2.5.1 DETERMINATION OF ANTI-INFLAMMATORY ACTIVITY ON THE NF-κB PATHWAY IN HELA CELLS

HeLa cells were stably transfected with a luciferase reporter gene controlled by the IL-6 promoter which is one of the target genes for activated NF-κB. The luciferase product can be investigated as an IL-6 dependent determination of the activation or inhibition of NF-κB (Bremner et al, 2004). Before starting the experimental processes all extracts are prepared by dissolving in ethanol at a starting concentration which is less toxic to cells (< 20% cytotoxic, observing by the MTT assay).

Chemicals and equipments used with their references

1. Phorbol 12-myristate 13-acetate: PMA (Sigma Aldrich UK, P-8139-1MG)
2. Luciferase Cell Culture Lysis Reagent (Promega, E1531)
3. Luciferase Assay System (Promega, E1501)
4. PBS pH 7.4 (Gibco®, Fisher Scientific UK)
5. Parthenolide (Sigma Aldrich UK, P0667-5MG)
6. 96- well microplates- white color (Nunc®, Thermo Fisher Scientific UK)
7. 24- well microplates- clear color (Nunc®, Thermo Fisher Scientific UK)
8. Anthos Lucy-1 luminometer/photometer

Luciferase assay procedure;

1. Extracts were prepared at 10 mg/ml in ethanol in order to give a working concentration 100 μg/ml when dilute in cell medium.
2. PMA was prepared to 50 ng/ml in purified water and pre-warmed.
3. Luciferase lysis solution is prepared by diluting the stock solution in distilled water (5-fold) (or 2 ml of lysis reagent in 10 ml purified water).
4. Stably transfected HeLa cells were cultured in a flask with medium until obtaining a confluence of about 60-80% before harvesting.
5. The cells were transferred to a 24-well plate containing 0.5 ml of media in each well and incubated at 37°C for 24 hrs until cell viability = 80-90% .
6. Extracts in several concentrations were added to each well. Positive controls were made without a sample. Negative controls were made without PMA-stimulation. Parthenolide was used as a reference (a positive control).
7. After 1 hr of incubation, 14.3 μl PMA (50 ng/ml, final concentration) was added and the plate was incubated again for 7 hrs.
8. Cells were washed with PBS and 50 μl of prepared luciferase lysis solution were added to each well. 15 min was allowed to complete cell lysis.
9. 15 μl from each well was transferred to a 96-wells plate. The enzymatic reaction was made with luciferase reagent, measured and recorded using an Anthos Lucy-1 luminometer/photometer.

Interpretation of results:

$$\% \text{ Luciferase inhibition} = 100 \times [1-\{(A_S - C_B)/(C - C_B)\}]$$

Where C = intensity of control (positive control)

C_B = intensity of blank (negative control)

A_S = intensity of sample

2.5.2 DETERMINATION OF ACTIVITY OF THE EXTRACT ON PRO-INFLAMMATORY CYTOKINE RELEASE IN PRIMARY HUMAN MONOCYTES

These experiments were done in the Department of Psychiatry, University of Freiburg Medical School, Germany, by Dr. Bernd L. Fiebich. Experiments were repeated with at least two buffy coats from different blood donors in triplicate. Detailed experiments can be found in Bremner *et al.* (2004).

Chemicals and equipment used with their references;

1. ELISA kit (IL-6, IL-1β, TNF-α: Pelikine, HISS, Freiburg, Germany).
2. EIA kit (Assay Design, distributed by Biotrend, Koln, Germany).
3. DNA Wizard (Promega, Mannheim, Germany)
4. LPS-free extract (PSH 69, purified SteiHap 69)
5. Hydrocortisone (Sigma), used as a standard reference

Experimental procedures;

1. Isolation of human peripheral monocytes was prepared following a standardised protocol (Ficoll gradient preparation, Amersham Biosciences) using a completely endotoxin-free cultivation.
2. 25 ml Ficoll gradient preparation was loaded with 25 ml blood of buffy coats from healthy blood donors in a 50 ml tube.
3. Cells were seeded in a 24-well plate for EIA/ELISA measurements and the extracts were prepared in DMSO.
4. The cells were incubated with the purified, LPS-free extract, and the prepared extracts, for 30 min before stimulation with LPS (10 ng/ml) and then incubated for another 24 hrs.
5. After incubation for 24 hrs, the supernatant was removed, centrifuged and prepared for IL-6, IL-1β, TNF-α, and PGE_2 measurement according to the manufacturer's instructions.

6. The levels of cytokines IL-6, IL-1β, TNF-α, and PGE_2 in the supernatant were measured using ELISA and EIA kits.

2.6 MEASUREMENT OF CYTOTOXICITY

Cytotoxicity of the extracts was examined against cervix cancer HeLa cells, human leukaemia CCRF-CEM cells and the multidrug-resistant CEM/ADR5000 cells using the quantitative colorimetric method to determine cell proliferation. MTT and XTT reagents were used and the procedures were described as follows.

2.6.1 MTT-REDUCTION ASSAY ON HELA CELLS

Cytotoxic activity was assessed by the following method described by Mosmann (1983) using MTT 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), a yellow tetrazolium salt, which turns into a purple formazan complex after reduction by enzymes in the mitochondria of living cells.

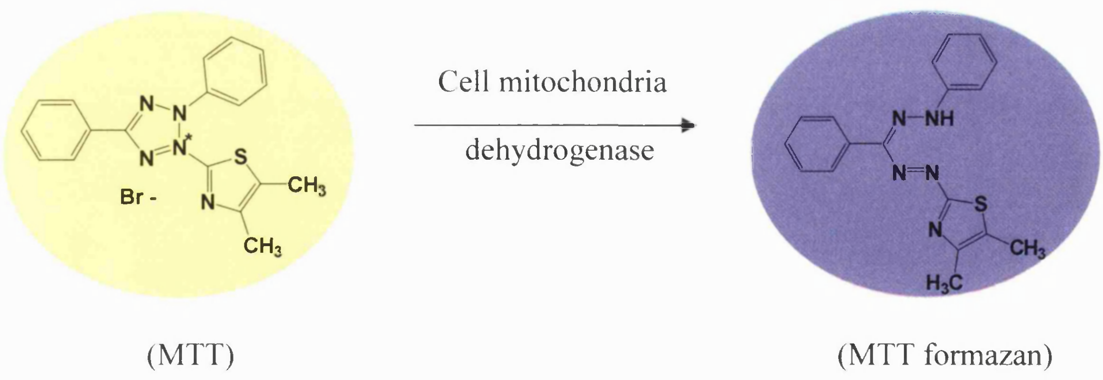


Figure 29 Yellow tetrazolium salt is metabolized by mitochondrial (succinate-) dehydrogenase of proliferating cells giving a purple formazan reaction product. The formazan products can be re-dissolved again before measurements.

Chemicals and equipment used in the MTT assay with their references;

1. MTT Formazan 98% (Sigma Aldrich UK, M2003-1G)
2. PBS pH 7.4 (Gibco®, Fisher Scientific UK)
3. Isopropanol- HPLC grade (Fisher Scientific UK)
4. Dimethyl sulphoxide: DMSO (AnalaR BDH, UK)
5. Doxorubicin hydrochloride (Sigma Aldrich UK, D-1515)
6. 96- well microplates- clear color (Nunc®, Thermo Fisher Scientific UK)
7. Anthos Lucy-1 luminometer/photometer

Experimental procedures in the MTT assay;

1. The cells were seeded into 96-well plates at a density of 10,000 cells in 100 μL medium per well, and were allowed 24 hrs to attach to the bottom.
2. Extracts were prepared in ethanol at appropriate concentrations, usually 10-20 mg/ml in ethanol and then diluted in medium to make 100-200 μg/ml.
3. 200 μl of the test solution was added into column 4, to make a starting concentration. 100 μl of media was added into column 2 as a control, and column 3 was a blank.
4. 10 μl extracts were added into column 4, obtaining 100 μg/ml (if the extracts were 10 mg/ml), or 200 μg/ml (if the extracts were 20 mg/ml).
5. Double dilutions were made by transferring 100 μl from column 4 to columns 5-11.
6. The plates were incubated at 37°C (5% CO_2, 95% humidity) for 24 hrs. Then the media was removed and the cells were washed with 200 μl PBS.
7. 200 μl MTT working solution (0.5 mg/ml in PBS) was added and the plates were incubated again for 2 hrs.
8. The MTT solution was removed and the formazan product was solubilized by adding 200 μl of 10% DMSO plus 90% isopropanol. The plates were wrapped with aluminium foil and allowed to sit for 10 minutes.
9. The solution in each well was re-mixed again before measuring absorbance at 570 nm using the Lucy-1 spectrophotometer (Anthos).

Interpretation of results:

$$\% \text{ Viability} = 100 \times [(A_S - C_B)/(C - C_B)]$$

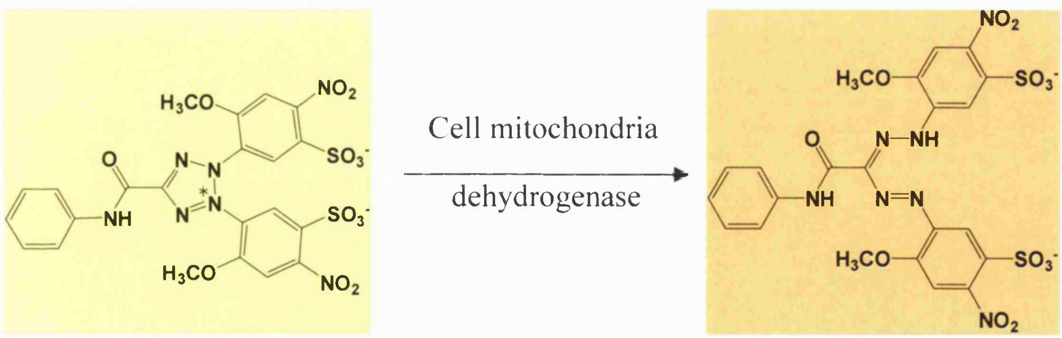
Where C = absorbance of control

C_B = absorbance of blank

A_S = absorbance of sample

2.6.2 XTT-REDUCTION ASSAY ON HUMAN LEUKEMIC CELLS

The XTT assay was first described by Scudiero *et al* (1988). The assay is based on the ability of metabolic active cells to reduce the tetrazolium salt XTT (2, 3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide) by ubiquitous dehydrogenases leading to the formation of an orange formazan dye to orange colored compounds of formazan (Konkimalla & Efferth 2010). The chemical reaction could be explained as follows.



(XTT) (XTT formazan)

Figure 30 XTT is metabolized by mitochondrial dehydrogenase (succinate tetrazolium reductase) in the cell mitrochondria of active cells giving an XTT soluble orange formazan dye.

The dye formed is water soluble and the dye intensity can be read at a given wavelength with a spectrophotometer. The intensity of the dye is proportional to the number of metabolic active cells. The greater the number of active cells in the well, the greater the activity of mitochondria enzymes, and the higher the concentration of the dye formed, which can then be measured. In this experiment the amount of dye is commensurate to the number of metabolically active cells (Konkimalla *et al.* , 2008).

This assay was conducted in a German cancer research center, Heidelberg Germany, by supervisions of Prof. Thomas Efferth. The assay procedures are very similar to that of the MTT assay, which can be briefly described as follows.

Chemicals and equipments used in the XTT assay with their references;

1. XTT assay kits (Roche, Indianapolis, IN)
2. Cell Proliferation Kit II (Roche Diagnostics, Mannheim, Germany)
3. 96-well culture plates (Costar, Corning, USA)
4. ELISA plate reader (Bio-Rad, München, Germany)

Experimental procedures of the XTT assay;

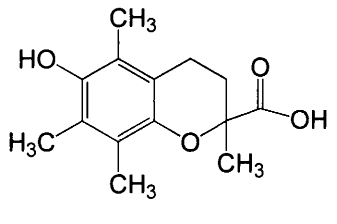
1. Fresh stock solutions of each compound were prepared in DMSO at a concentration of 100 mM. A dilution series ranging from 10-3 M to 10-9 M was prepared using DMEM medium to perform the XTT test.
2. Cells were diluted to a final concentration of 1×10^5 cells/ml. 100 μl of the cell suspension was added into the wells of a 96-well culture plate.
3. Marginal wells were filled with 100μL of pure medium in order to minimize effects of evaporation. The wells filled with medium were required to determine the background absorbance caused by non-metabolized XTT.
4. A row of wells containing cells was left untreated and another row of wells containing cells was treated with 1μL DMSO and this served as solvent control.

5. The other rows of wells containing cells were supplemented with different concentrations of compound. Each concentration was tested in at least two independent plates containing different batches of cells.
6. After incubation with compounds at 37°C, 5% CO_2 in humidified atmosphere, freshly prepared XTT reagent was added to each well as specified by the manufacturer:
7. XTT-labeling reagent and electron-coupling reagent were mixed in a ratio of 50:1 and 50μL of this mixture was added to each well of the 96-well.
8. The plates were incubated for about 3h at 37°C, 5% CO2 in humidified atmosphere and read after incubation.
9. Quantification of cell cytotoxicity was performed in an ELISA plate reader at 490nm with a reference wavelength of 655nm.
10. Absorbance values at both wavelengths were subtracted. The cytotoxic effect of the treatment was determined as percentage of viability compared to untreated cells. Simple ligand binding module of Sigma plot software (version 10.0) was used for analysis.

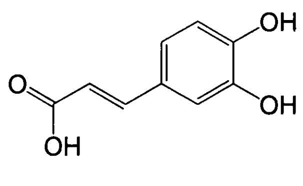
2.7 MEASUREMENT OF ANTIOXIDANT ACTIVITY

Antioxidant properties of the plant extracts were investigated using three methods; DPPH, lipid-peroxidation, and Folin-Ciocalteau. DPPH assay is one of the most popular among all the tests and it is easy and straightforward. Lipid-peroxidation is also valid and possibly suitable for extracts in lipophilic systems. Phenol content determination by the Folin-Ciocalteau method is a method to determine phenol contents relating to anti-oxidant effects.

Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as a positive control for testing suitability and proper performance of the DPPH and lipid peroxidation assays, while caffeic acid was used as a standard reference for the Folin-Ciocalteau method. Trolox is a registered trademark of Hoffman La Roche for a cell-permeable, water-soluble derivate of vitamin E with potent antioxidant properties, used only for research purposes.



(A)



(B)

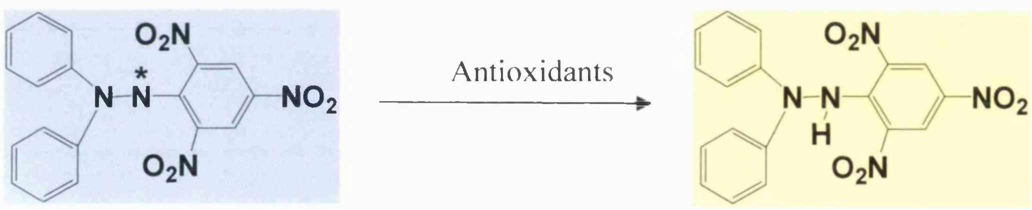
Figure 31 (A) Chemical structure of Trolox ($C_{14}H_{18}O_4$, molecular weight 250.30)

(B) Chemical structure of Caffeic acid ($C_9H_8O_4$, molecular weight 180.16)

2.7.1 DPPH ASSAY

Free radical scavenging activities of the extracts were determined by a method based on the reduction of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH). DPPH is a stable free radical that has a purple appearance with a free electron and this has been used in measurement of antioxidant activity. As the antioxidant compound binds onto the unpaired electron of DPPH, it results in the purple color changing to yellow.

In order to determine the efficacy of the antioxidant this color change may be measured as it is proportional to the number of unpaired electrons that have been bound by the antioxidant as shown in a diagram below;



(DPPH) (DPPH-H)

Figure 32 Structure of DPPH in reaction with the antioxidant agent giving DPPH-H

The DPPH free radical gives a strong absorption maximum at 517 nm. The resulting decolourization depends on the amounts of reactants and products in a chemical reaction with respect to the number of electrons captured, antioxidant concentration and the reaction time. The experimental procedures of DPPH assay and chemicals used can be explained as follows.

Chemicals and equipment used in the DPPH assay with their references

1. DPPH (1,1-Diphenyl-2-picryl-hydrazyl)(Sigma Aldrich UK, D-9132)
2. 96- well microplates- clear color (Nunc®, Thermo Fisher Scientific UK)
3. Trolox® (Fluka, 56510)
4. Quercetin (Sigma)

Experimental procedures in the DPPH assay;

1. The DPPH reagent was prepared by dissolving 4.2 mg of DPPH in 3.15 ml of methanol (stock solution). 150 µl of stock solution was diluted in 3 ml of methanol (test solution).
2. The extract solution was made by dissolving the extract in ethanol 97% to make a concentration of 20 mg/ml.
3. For the first column, each row contained test solution= 5 µl of extract solution + 95 µl DPPH test solution (x 3 rows served as 3 replicates). Blank = 5 µl extract solution + 95 µl ethanol 96%. Control = 100 µl DPPH test solution. Blank control = 5 µl ethanol 96% + 95 µl extract solution.
4. Dilutions were made by adding solutions twice into the first columns before pipetting half of the solution into the next column, continuing the same manner until the last column. The color changes from the reaction can be seen immediately as shown in the picture below.

High concentrations-----------→ Low concentration

(e.g. 100 µg/ml, 50 µg/ml, 25 µg/ml and so on)

1
2
3
4
5
6
7
8

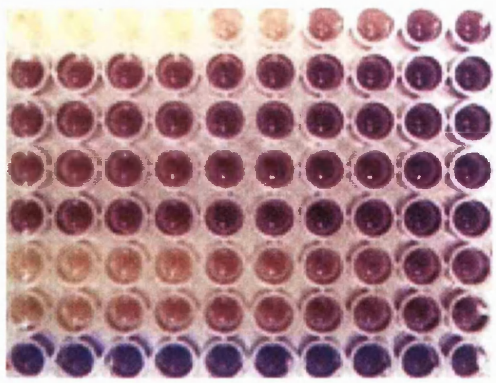


Figure 33 An example of a microplate containing different samples in each row and different concentrations in each column

5. After the dilutions the absorbance was measured at 492 nm using a UV spectrophotometer (Anthos Lucy-1 machine) at time zero, 20 minutes, 40 minutes and 60 minutes, respectively.

Interpretation of results:

$$\% \text{ Oxidation inhibition} = 100 \times [1-\{(A_S - A_{SB})/(C - C_B)\}]$$

Where C = absorbance of control (ethanol + DPPH)

C_B = absorbance of blank control (pure ethanol)

A_S = absorbance of sample (extract + DPPH)

A_{SB} = absorbance of blank (extract + ethanol)

2.7.2 LIPID PEROXIDATION ASSAY

Lipid peroxidation is a well-established mechanism of cellular damage in biological systems, and is used as an indicator of oxidative stress in cells and tissues (Barsch & Nair 2004). It is a radical initiated chain reaction with self-propagation in cellular membranes. The primary lipid oxidation products are unstable and decompose to form secondary products.The direct detection of lipid-peroxidation is complicated, therefore, it is normally analysed by measuring the level of secondary oxidation products, particularly malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE).

Malondialdehyde (MDA) has been used as a biomarker of lipid peroxidation (Esterbauer *et al.* 1990). The most used assay for lipid peroxidation is the thiobarbituric acid reactive substances (TBARS) test. The TBARS test relies on the production of a colored adduct from the reaction of lipid peroxidation product MDA and thiobarbituric acid which can be explained as in the diagram below.

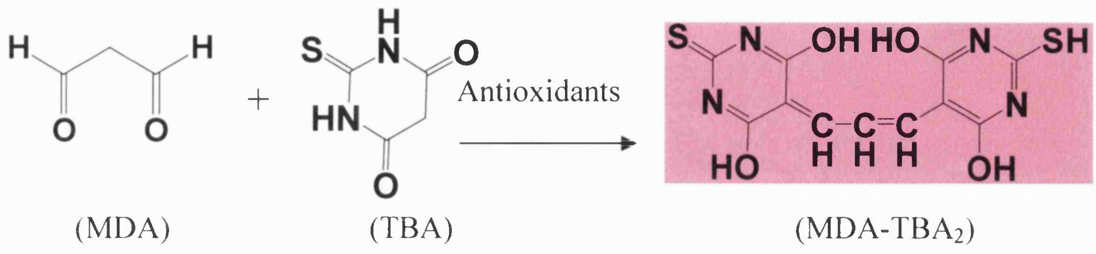


Figure 34 The reaction between MDA and the TBA reactive substance giving MDA-TBA_2 complex (pink). Antioxidants inhibit the formation of MDA-TBA_2, thus, reducing pink color to yellow or orange, depends on the strength of the antioxidants.

Lipid peroxidation measurement was carried out following the procedures of Houghton *et al.* (1995); Burits and Bucar (2000), using the thiobarbituric acid reactive substances (TBARS), with the principle that $FeCl_3$ solution is a source of Fe^{3+} ions. Ascorbic acid reduces Fe^{3+} to Fe^{2+}. Thiobarbituric acid forms coloured complexes with aldehydes that are formed as degrading products of fatty acids. Trichloracetic acid and trichloroacetic acid are used to precipitate interfering proteins. The experimental procedures are as follows.

Chemicals and equipments used in the lipid-peroxidation assay with their references

1. Bovine Brain Extract Type VII (Sigma Aldrich UK, B-3635)
2. PBS: Buffer solution pH 7.5 for HPCE (Fluka Bio Chemika, 82592)
3. Trichloracetic acid (Fluka Bio Chemika, 91228)
4. 2,6-Di-tert-butyl-p-cresol (Fluka Chemika, 34750)
5. Thiobarbituric acid (Fluka Chemika, 2- 88481)
6. n-butanol 99% (Sigma Aldrich UK, 1- BT-105)
7. Sodium Hydroxide pellets (AnalaR BDH UK,102524X)
8. Ascorbic acid Sodium salt (Fluka Bio Chemika,11140)
9. Iron(III) Chloride 97% (Sigma Aldrich UK, 15774-0)
10. Trolox® (Fluka, 56510)

Experimental procedures in the lipid-peroxidation assay;

1. Liposomal suspension was prepared from Type VII Folch bovine brain extracts (Sigma), suspended in phosphate buffer in concentration 5mg/ml, and sonicated until a milky solution was obtained.
2. Then 30 µl sample solution (5 µl plant extract + 25 µl PBS) was added and mixed before adding 10 µl FeCl3 (1mM in water) and 10 µl ascorbic acid (1mM in water) and incubate at 37°C for 1 hour.
3. 100 µl thiobarbituric acid (1% in 50 mM NaOH), 100 µl trichloroacetic acid (2.8% in water) and 10 µl 2, 6-di-t-butyl-p-kresal were added and mixed thoroughly in a serial manner in order to precipitate interfering substances.
4. The mixture was then heated at 80°C for 20 min, allowed to cool down, and then 250 µl n-butanol was added and centrifuged.
5. After centrifugation, 100 µl of organic layer was transferred into a microplate and absorbance was measured at 540 nm by colorimeter.
6. The absorbance was recorded against blanks and the results were expressed as a ratio of the formation of TBARS in the presence of plant extract compared to control.
7. The color changes from the reaction can be seen after heating at 80°C.

Interpretation of results:

$$\%\ \text{Lipid-peroxidation Inhibition} = \frac{(\text{control-sample})}{(\text{control-blank})} \times 100$$

Where control = absorbance of control

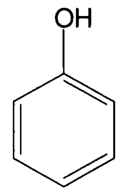
Blank = absorbance of blank

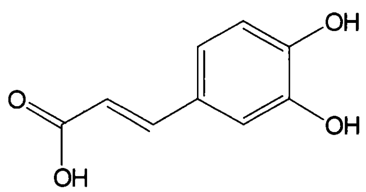
Sample = absorbance of sample

2.7.3 TOTAL PHENOLIC CONTENT BY FOLIN-CIOCALTEAU METHOD

Phenol compounds are a basic structure of natural products and diets. Significant amounts of phenolics have been reported in vegetables, fruits and traditional plants in many studies. Antioxidant phenolics may scavenge reactive oxygen and nitrogen species and, therefore, potentially modify pathogenic mechanisms relevant to oxidative stress related diseases. (Bahorun et al, 2006).

In order to determine a total phenolic content, the Folin-Ciocalteau method was first established by Lowry et al (1951). This method is an electron transfer based assay with an oxidation/ reduction reaction, and gives reducing capacity, which has normally been expressed as phenol contents, in this study, equivalent to caffeic acid.





Structure of phenol   Structure of a caffeic acid (MW = 180.16)

Before processing the assay, all the extracts were prepared in appropriate concentrations and were then filtered. Caffeic acid solution was also prepared by dissolving in ethanol to make an appropriate concentration which to be recorded for calculations. In this study, 20.6 mg caffeic acid was dissolved in 25 ml ethanol (97%), obtaining the concentration of 0.824 mg/ml. Na_2CO_3 solution (20% in water) was prepared, 2 gm of Na_2CO_3 dissolved in 10 ml water. The experimental processes and chemical used are explained below.

Chemicals and equipments used in the Folin-Ciocalteau assay with their references

1. Folin-Ciocalteau reagent (Sigma Aldrich UK, F9252)
2. Sodium Carbonate 99.95% (Sigma Aldrich UK, 71627)
3. 96- well microplates- clear color (Nunc®, Thermo Fisher Scientific UK)

Experimental procedures in the Folin-Ciocalteau assay;

1. Sterile water 150 µl was added to each well of a 96-Wells plate.
2. 2.5 µl of each extract solution was added to the well and mixed pipetting. Caffeic acid solution was also processed in the same manner.
3. 12.5 µl of Folin-Ciocalteau reagent was added to each well and mixed.
4. 1-8 minutes was allowed for the Folin reagent in oxidation with phenolates in the extracts to change to a blue complex.
5. Blank experiments contained all the solutions in the same amounts except the Folin-Ciocalteau reagent
6. After 8 minutes, 37.5 µl of Na_2CO_3 solution was added, and this was recorded as time zero.
7. The solution in each well was mixed before adding 47.5 µl of sterile water to fill up the volume to exactly 250 µl. For the blanks, 60 µl of water was used to fill up the volume.
8. After 2 hours, the solution was mixed again, and then UV absorption was measured at 550-850 nm using the Lucy-1 machine.

Table 4: Summary of the amount of reagents used, in order to process 24-wells, 96-wells plates, or volumetric flasks in experimental order.

Experiments	For volumetric	For 24-Wells	For 96-Wells
First volume of	3 ml (3,000 μL)	300 μL	150 μL
Sample solution	50 μL	5 μL	2.5 μL
Mix by vortex		Mix by pipettes	
Folin's solution	250 μL	25 μL	12.5 μL
Mix by vortex		Mix by pipettes	
Wait 1-8 minutes		Wait 1-8 minutes	
Na_2CO_3 (20% in	750 μL	75 μL	37.5 μL
Mix by vortex and record time zero		Mix by pipettes and record time zero	
Sterile water to fill	950 μL	95 μL	47.5 μL
Final volume	5 ml (5,000 μL)	500 μL	250 μL
Measure UV absorption after 2 hours from time zero.			

Interpretation of results

The absorbance of blank sample was subtracted from the absorbance of the samples. The content of total phenols in percentage was based on the caffeic acid standard, using the following equation:

$$\% \text{ Total Phenols} = (A_{sample} \times C_{standard})/(A_{standard} \times C_{sample}) \times 100$$

Where A = absorption of sample/ standard

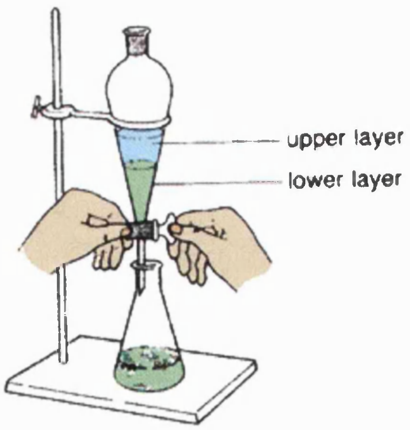
C = concentration of sample/ standard solution

2.8 TECHNIQUES USED IN SEPARATION AND IDENTIFICATION OF COMPOUNDS

In this study, bioassay guided isolation and identification of active compounds was carried out using NF-κB as a lead assay. The techniques used in separation and identification of compounds can be described as follows.

2.8.1 LIQUID-LIQUID PARTITIONING

Liquid-liquid partitioning was used in the separation of water-soluble and fat-soluble compounds. This method was used initially to eliminate fat, protein and chlorophylls or other interfering substances that usually have no pharmacological activities. By this process substances that dissolve better in one solvent will migrate to the solvent in which they are more soluble. After the compounds were properly migrated into the two phases already, the bottom phase will be drained out first, and the top phase will be poured out from the top in order to avoid a contamination.



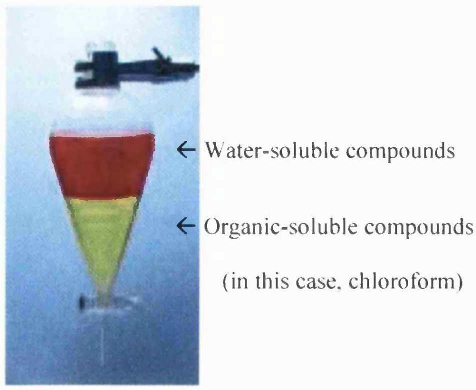


Figure 35: Liquid-liquid extraction using two immiscible solvents (picture adapted from http://firstyear.chem.usyd.edu.au).

Chemicals and equipment used in the liquid-liquid partitioning technique;

1. Chloroform HPLC grade (Fisher Scientific UK)
2. Methanol HPLC grade (Fisher Scientific UK)
3. Purified water obtained from Millipore® machine with a 0.22 μM filter
4. Separating funnel (250 ml)

Experimental procedures in the liquid-liquid partitioning technique;

1. The methanol extract was dissolved in the smallest amount of methanol as possible (about 1-2 ml) and then diluted more with water about 5-10 ml. Then this solution was poured into a separating funnel with closed stopper.
2. 50 ml chloroform was added and mixed by shaking or swirling. Pressure produced in the funnel was released by opening the stopper occasionally.
3. The bottom layer was collected in a round bottom flask and the process was repeated 3 times as the same manner.
4. All the bottom layers were then combined and dried under pressure using a rotary evaporator.

2.8.2 SEPHADEX GEL FILTRATION CHROMATOGRAPHY WITH GRADIEN ELUTION

In phytochemistry, various methods based on chromatography for purification of plant extracts have been widely discussed. Silica gel with gradient elution is one of the most commonly used chromatography, separating compounds by their polarities. However, concerning large quantity of solvent used, time consuming, and the potential for decomposition of some natural compound during chromatography, a combination of two methods; size-exclusion chromatography with gradient elution, has been used in this study.

Sephadex is a monopolysaccharide dextran composed of little beads with many spherical, pores that can trap small molecules inside the beads. This technique is also called size-exclusion chromatography. The beads of a single-pore-size can exclude only a specific molecular size, so a variety of pore sizes is commercially available. Large molecules will be eluted first and small molecules elute last. The concept of this technique is demonstrated as in the diagram below.

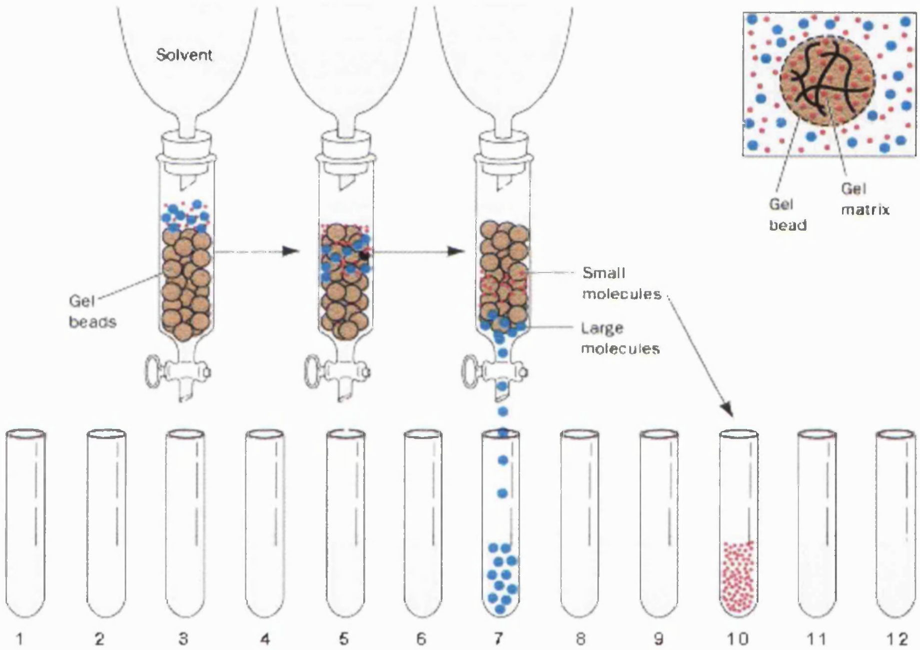


Figure 35 Gel filtration chromatography (picture modified from www.siumed.edu)

Sephadex™ LH-20 is designed for molecular sizing of natural products such as steroids, terpenoids, lipids, and low molecular weight peptides (GE Healthcare, 2009). Therefore, it was used in this experiment. Sephadex™ LH-20 can be swelled in water and a number of organic solvents can be used but chloroform and methanol are the ones most commonly used (GE Healthcare, 2009). In this experiment, gradient solvents elution between dichloromethane, methanol and water were used as explained in the following sections.

Chemicals and equipment used in the Sephadex Gel filtration technique;

1. Sephadex™ LH-20 (GE Healthcare Life Sciences UK)
2. Methanol HPLC grade (Fisher Scientific UK)
3. Dichloromethane HPLC grade (Fisher Scientific UK)
4. Purified water obtained from Millipore® machine with a 0.22 μM filter
5. A glass column with a stopper (2 inches in diameter x 100 cm in length)

Experimental procedures in the Sephadex Gel filtration technique;

1. Sephadex LH-20 was allowed to swell in methanol and was sonicated to eliminate air bubbles for 5-10 min until no more air bubbles appeared.
2. The slurry Sephadex was poured into the column and allowed to sit for at least 24-48 hrs in order to obtain a tightly packed column.
3. The packed column was eluted without the extract, using only a mixture of dichloromethane: methanol (70:30) in order to eliminate any impurities that might be in the column and materials.
4. The extract was dissolved in 1-2 ml of methanol and load on to the top of the bed using a glass pipette.
5. The mixture of dichloromethane: methanol (70:30) was dripped slowly at the beginning and loaded for 400 ml and the diluents (fractions) were collected in round-bottom flasks for every 10 ml.
6. The column was then eluted with dichloromethane: methanol (50:50) for about 400 ml and then eluted with methanol: water (50:50) for 400 ml. Then finally the column was washed again with pure methanol until no bands in the column were observed.
7. Thin layer chromatography (TLC) of each fraction was monitored and similar fractions were then combined as appropriate.
8. All the fractions were dried separately using a rotary evaporator and transferred to a sample bottle, dried with nitrogen gas and kept at -20°C.

2.8.3 THIN-LAYER CHROMATOGRAPHY (TLC)

TLC is a chromatographic method used to identify or test for the purity of compounds with small quantities of materials used. TLC can be prepared as preparative or analytical. The aim of preparative TLC used in this thesis is to separate/purify compounds out of a mixture. The separation depends on the distribution of compounds onto a stationary phase and a mobile phase and the results are usually expressed as Rf value or "ratio of front" which is used for comparing unknown compounds with known compounds for their identification.

Rf can be estimated as the distance that the mobile phase traveled from starting point to the solvent front divided by the distance that of the compound traveled.

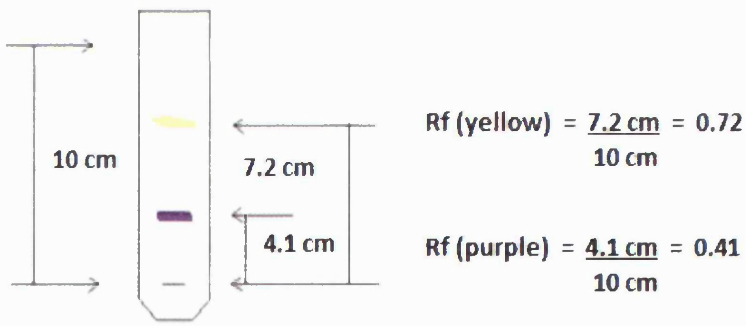


Figure 36 An example of Rf calculation of the spots on the TLC plate.

In this study, TLC technique was used in the detection of compounds presence in all the 27 extracts at the beginning of the project, after the small-scale extraction process has been done. TLC experiments with different solvent systems were used again in the investigation of compounds present in the methanol of *G. pseudochina var. hispida*, the active NF-κB inhibitor. As the methanol extract yielded high polarity compound, the reverse phase TLC and the reverse phase solvent were used. The TLC technique was also used to isolate the most active compounds. For the detection of compounds, sprayed reagents were used as explained as follows.

Chemicals and equipment used in the TLC technique;

1. TLC Silica gel 60 on aluminium sheets 20 x 20 cm (Merck Chemicals UK)
2. TLC Silica gel 60 RP-18 F254 Aluminium sheets 20 x 20 cm (Merck Chemicals UK)
3. Toluene HPLC grade (Fisher Scientific UK)
4. Ethyl acetate HPLC grade (Fisher Scientific UK)
5. Dichloromethane HPLC grade (Fisher Scientific UK)
6. Methanol HPLC grade (Fisher Scientific UK)
7. Acetic acid 100% (AnalaR, BDH UK)

Detection reagents (Wagner et al, 1965; Mattocks & Jukes1987)

1. 4% Vanillin in sulphuric acid (w/v)
2. Natural product reagent: 1% diphenylboryloxyethylamine in methanol (v/v), and subsequent spraying with 5% polyethylene glycol-4000 (PEG-4000) in ethanol (w/v) to lower the detection limit from 10μg (the average TLC detection imit for flavonoids) to about 2.5 μg.
3. Ehrlich reagent (ready to use solution, from Sigma Aldrich, UK) or otherwise can be prepared by dissolving 4-dimethylaminobeddehyde (5 g) in a mixture of acetic acid (60 ml), water (30 ml), and 60% perchloric acid (10 ml) (life, one week if kept dark).

Experimental procedures in the TLC technique

1. The solvent system was prepared, mixed and loaded into the TLC chamber.
2. Filter paper or white clean A4 sized paper was put insides the chamber.
3. Small spots of the extract solution were applied onto a plate, about one centimeter from the base. The plate was then dipped in to a suitable solvent.
4. After the solvent travelled for 10-15 cm, the plate was taken out and observed under UV lights. Spots seen under the UV light were marked.
5. A suitable detection reagent may be applied and the plate may be heated.

2.8.4 VACUUM SOLID-PHASE EXTRACTION

Solid phase extraction (SPE) is a method for the isolation and purification of compounds. This method has been popular and effective for the extraction, clean-up and concentration of analytes from a variety of herbal materials (Huie 2002). It is often used before employing other chromatographic techniques such as HPLC. The common process is to load a sample solution onto the SPE cartridge surface, wash away undesired components, and then wash off the desired analytes with another solvent into a collection tube (Boyce 2006). The SPE system is shown below.

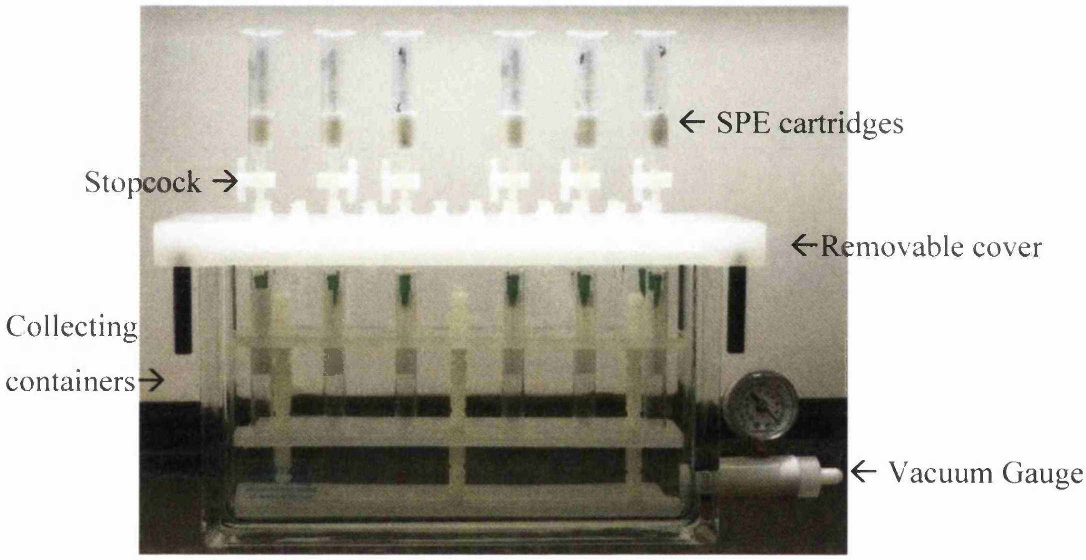


Figure 37 Vacuum solid-phase extraction and an illustration of how the system was set up (photo adapted from commons.wikimedia.org)

Chemicals and equipment used in the SPE technique;

1. Strata C18-E (20g/60ml) Giga-tube cartridges (Phenomenex, USA)
2. SPE vacuum chamber
3. Methanol HPLC grade (Fisher Scientific UK)
4. Dichloromethane HPLC grade (Fisher Scientific UK)
5. Purified water obtained from Millipore® machine with a 0.22 μM filter

Experimental procedures for the SPE technique;

1. SPE chamber was set up and a sample was prepared in a small amount of an appropriate solvent.
2. The cartridge was rinsed with the solvent that will be used, and the sample solution was dripped onto the cartridge base and the solvent was loaded slowly as well as the pressure was observed and adjusted.
3. Each fraction obtained was collected and dried separately using a rotary evaporator or nitrogen gas and was kept in a sample bottle, stored in a fridge -20°C for further analysis.

2.8.5 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC is a powerful tool in analytical chemistry which has been widely used for the isolation and identification of bioactive natural products (Heinrich *et al.* 2004). In this thesis, it was used for separation of aromatic/phenolic compounds found in two of the active fractions of the methanol extract of the *G. pseudochina var. hispida*. Chemicals and equipment used are listed below.

Chemicals and equipment used in the HPLC technique;

1. Agilent 1200 Series Quaternary HPLC system (Agilent Technology UK)
2. Agilent ZORBAX Eclipse XDB-C18, 125 x4 mm (Agilent Technology UK)
3. Acetonitrile HPLC grade (Fisher Scientific UK)
8. Acetic acid 100% (AnalaR, BDH UK)
9. Purified water obtained from Millipore® machine with a 0.22 μM filter

Details of experiment and detection method used;

1. All samples were prepared in methanol at concentrations of 1-5 mg/ml.
2. Mobile phase: Solvent A: 0.05% acetic acid in water,
   Solvent B: Acetonitrile
3. Gradient method from 1% Solvent A to 99% Solvent A, for 30 min.
4. Flow rate: 0.5-1 ml/min

2.9 SPECTROSCOPIC TECHNIQUES FOR STRUCTURE ELUCIDATION

There are many spectroscopic techniques which have been used in phytochemical research including ultraviolet spectroscopy (UV), infrared spectroscopy (IR), nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS). In brief, UV spectroscopy uses electromagnetic radiation in the UV range and IR spectroscopy employs the electromagnetic radiation in the infrared range to make electrons move from the steady state to an excite state. These techniques are usually used for detection of certain functional groups and conjugation in the molecules (Heinrich, 2004).

In this study, two spectroscopic techniques were used in order to gain information about proton/carbon arrangements, chemical structures and chemical properties of known and unknown compounds. The NMR and electrospray ionization measurements were performed by the group of Dr. Mire Zoh, the School of Pharmacy, University of London. The two techniques can be explained as follows.

2.9.1 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR)

Unlike IR and UV spectroscopies where absorption peaks are unique and located by a frequency or wavelength, the location of the NMR peaks depend the strength of external magnetic field and the resonance frequencies. Examples of Proton (1H)-NMR ranges are in the following diagram.

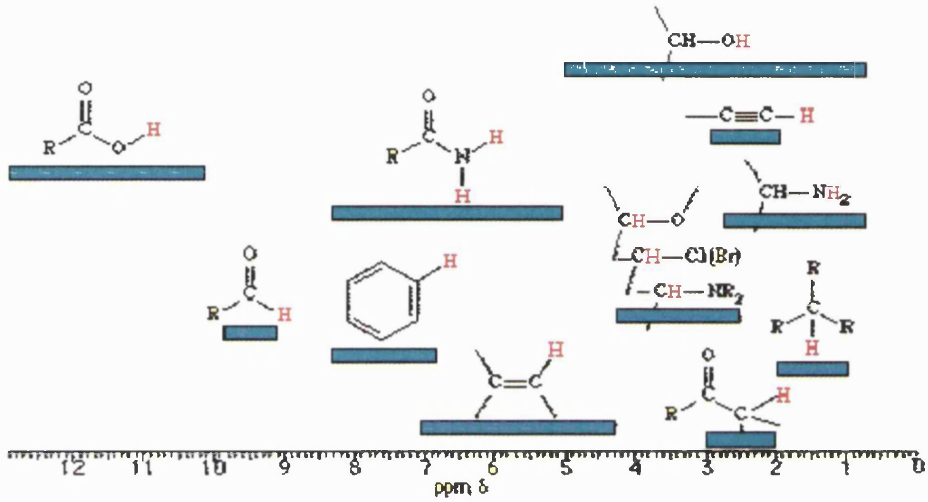


Figure 39 Typical chemical shifts ranges for 1H atoms (www.chemistry.nmsu.edu).

In this thesis, 1H NMR spectroscopy was used in the determination of the structure of unknown natural compounds. Normally the 1H NMR spectrum provides information about the number of protons present in the molecule, the number of proton neighbours and the environment of the different types of protons. The chemical shift, the spin-spin coupling and the peak intensities enable the identification of the links between atoms through the bonds and how the atoms combine to form a molecular structure (Williams & Fleming 2008).

13C NMR was also used in the determination of the number of different types of carbon present in a molecule. In addition to the 13C, the DEPT experiments were used in the identification types of carbon present in the molecules, particularly DEPT-90 and DEPT-135 which showed very useful information of how many CH, CH_2 and CH_3 present in the molecule. In the DEPT-135, the peaks above zero (peak up) represent CH and CH_3 groups whereas peaks down represent CH_2 groups.

However, DEPT-90 normally shows peaks up representing only CH groups (as shown in table 5).

Table 5 Types of DEPT experiments and the characteristics of peaks shown

Types	CH peaks	CH_2 peaks	CH_3 peaks
DEPT-45	⇧(peak up)	⇧(peak up)	⇧(peak up)
DEPT-90	⇧(peak up)	-	-
DEPT-135	⇧(peak up)	⇩ (peak down)	⇧(peak up)

2D experiments were used to identify the correlation between protons and protons, as well as protons and carbons. 2D experiments used in this thesis includes COSY (Correlation Spectroscopy) experiments which present cross peaks in the spectrum showing the direct correlation between a proton and its coupled partners. HMQC (Heteronuclear Multiple-Quantum Correlation) experiments show correlations between boned carbons and protons. HMBC (Heteronuclear Multiple-Bond Correlation) experiments present long-range couplings between protons and carbons in the distance of two or three bonds. NOESY (Nuclear Overhauser Effect Spectroscopy) experiments show correlations between protons, via bonds and via spaces. The information of solvents and equipment used are listed here.

Chemicals and equipment used in the NMR experiment;

1. Bruker Avance 500 MHz NMR spectrometer equipped with broadband and triple resonance (1H, 13C and 15N) inverse probes
2. Bruker Avance 400 MHz NMR spectrometer equipped with broadband and selective (1H and 13C) inverse probes
3. Methanol-d4, 99.8% (Cambridge Isotopes Limited, Goss Scientific)
4. NMR tubes (Wilmad LabGlass)

Detailed experiments;

1. Dried samples were dissolved in about 0.5 ml of a deuterated solvent
2. The NMR experiments were run and the numbers of spins applied were;
   - 128 for 1H experiments, and 16 for NOESY experiments.
   - 5k for 13C experiments, and 3k for DEPT experiments,
   - 24-32 for HMQC and HMBC, and 12 for COSY experiments,

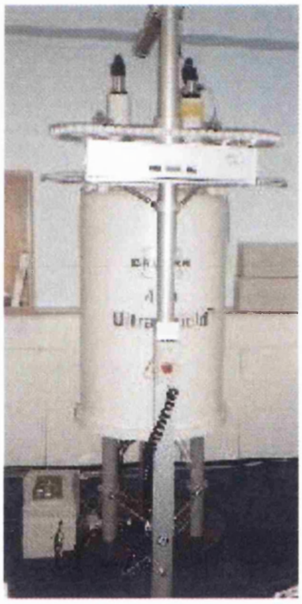


Figure 40 Bruker Avance 500 MHz NMR spectrometer (www.pharmacy.ac.uk)

Solvent peaks in the NMR spectra which were considered as an impurity

Methanol-D4 gives absorption signals in the 1H spectra at chemical shifts around 3.35 ppm as a quintet (split into 5 peaks) from proton of its CH_3 group, and at 4.8 ppm as a single peak from proton of its OH group , as well as giving a signal around 49 ppm as a septet (split into 7 peaks) in the C13 spectra. However, these values may vary depending upon the solvent and its concentration but the above data was regarding to the NMR book (Williams & Fleming 2008). The above signals were considered as impurity peaks in the spectra.

2.9.2 ELECTROSPRAY IONIZATION - MASS SPECTROSCOPY

Mass spectroscopy (MS) is used for the measurement of the molecular mass of a sample as well as fragmentation of a molecule. MS spectra obtained were used to confirm identities of a compound because each molecule or compound is likely to produce different ion fragments. The spectra usually present in the fragments give a clue as to the molecular structure, assuming the compound tested is pure. MS experiments in this thesis were performed by the Mass spectroscopy service, London School of Pharmacy.

Detailed experiments in brief, acidic hydrolysis of sample was performed with 1% formic acid in water and was fed into an electro-spray needle to force charged droplets towards the counter electrode. As the droplets travel from the needle tip to the cone on the counter electrode, solvent was evaporated producing smaller droplets and causing fragmentations of the molecules and molecular ion which can be detected by the detector. The uncharged particles were removed by vacuum letting only the charged particles to be accelerated and detected. Charged particles are presented as a line on the diagram.

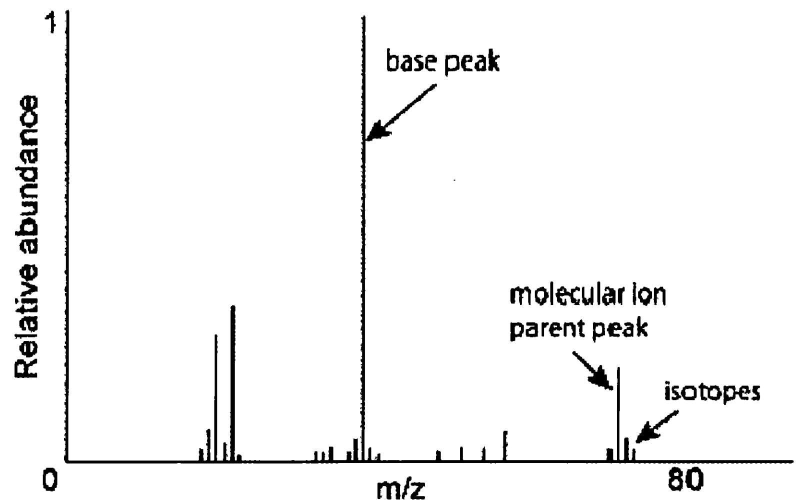


Figure 41 Mass spectroscopy and characteristics/ types of peaks shown in the spectrum (diagram adapted from MCAT-Review.org).

In the MS spectrum, each peak or each line represents an ion with a specific mass-to-charge ratio (m/z). The height of the peak indicates the relative abundance of the ion. The tallest line in the diagram is usually a base peak which may be expressed as the height of 100, and other peaks are measured relative to this base peak. The base peak is the most abundant ion to be formed during fragmentation of the molecules and usually is a certain stable ion. In this thesis, the patterns of the expected compounds were identified by comparing with established spectra in publication.

Solvents used to prepare samples:

1. Formic acid (AnalaR, BDH UK)
2. Methanol HPLC grade (Fisher Scientific UK)
3. Water HPLC grade (Fisher Scientific UK)

Equipment used in the MS experiment;

1. Instrument: Micromass Q-TOF Global Tandem Mass Spectrometer
    - Electrospray and MALDI ion sources
    - MS and MS/MS modes
    - Accurate mass capability in MS and MS/MS mode

Figure 42 Micromass Q-TOP Global Tandem Mass Spectrometer (Photo and instrument specifications from www.pharmacy.ac.uk)

2. Instrument: Thermo Navigator Mass Spectrometer
   - Operated under Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces for liquid samples
   - Quadrupled mass filter; m/z 10 - 2000
   - Operates in positive and negative-ion mode

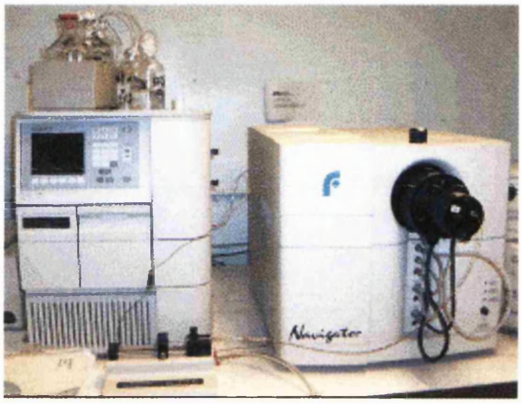


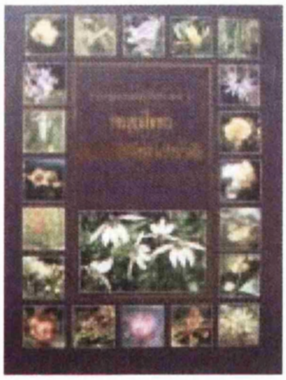
Figure 43Thermo Navigator Mass Spectrometer (www.pharmacy.ac.uk)

CHAPTER 3

RESULTS

3.1 PLANT SELECTION

Nine plant species were selected on the basis of their use, as anti-inflammatory medicines, reported in Thai textbooks. The majority of them were reported in the book "Medicinal Plants in Thailand Volume I" (Saralamp *et al.* 2000): a collection of common species that have been commonly used as medicines in Thailand. The plants in the book are also cultivated in the "Sirirukhachart Botanical Garden" Bangkok Thailand, for study and research purposes. The species reported in this book include:



- *Basella alba* L. (Basellaceae)
- *Gynura pseudochina* (L.) DC. var. hispida Thv. (Asteraceae)
- *Oroxylum indicum* (L.) Kurz. (Bignoniaceae)
- *Pouzolzia indica* (L.) Gaudice.(Urticaceae)
- *Rhinacanthus nasutus* (L.) Kuntze. (Acanthaceae)

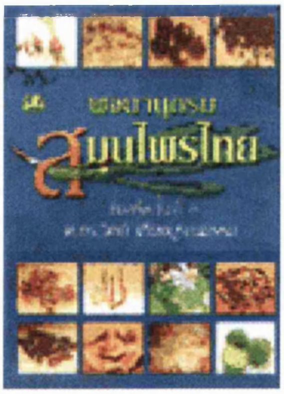
(The book was photographed by the author)

Similarly, the book "Medicinal Plants in Thailand Volume II" (Chuakul et al, 2000): the ethnobotanical survey of 14 provinces of Thailand was written by the same team as above but the collection was from the interview of healers. The plants documented in this book include:

- *Cayratia trifolia* (L.) Domin. (Vitaceae)
- *Muehlenbeckia platyclada* (F.) Muelll,Meisn. (Polygonaceae)

(The book was photographed by the author)

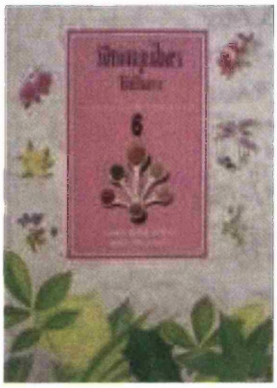
Another interesting book is the "Dictionary of Thai Herbal Medicine" (Theangburanatham 2005): a collection of medicinal Thai plants which have been used for more than 100 years. The plants in this book have been used singularly or in combination with other plant species. The plants presented in this book include;



- *Basella rubra* L. (Basellaceae)
- Most species included here were also recorded in this book.

(The book was photographed by the author)

A fourth book, "Medicinal Plants Used as Drugs Volume 6" (Suchawan 1989) was also consulted. This book contains a short summery of each plant with large photos of the plants. The plants reported in this book include:



- *Pouzolzia indica* (L.) Gaudice. (Urticaceae)
- *Rhinacanthus nasutus* (L.) Kuntze. (Acanthaceae)

(The book was photographed by the author)

A further two sources were the "Encyclopedia of Thai Herbal Medicine and Fundamentals of Thai Pharmaceutics" (Wuthithamvech 1997), in which *Oroxylum indicum* (Bignoniaceae) was reported, as well as "Plant Genetic Conservation Project of Her Royal Highness Princess Maha Chakri Sirindhorn" (Plant Genetic Conservation Project 2009), in which *Gynura pseudochina* (L.) DC. (Asteraceae) was included. These two sources exist as electronic databases.

3.2 SMALL SCALE EXTRACTION

The bark of the stem of *O. indicum*, aerial parts of *M. platyclada*, and the leaves of all the other plant species and were dried and ground. Then 20 g of each was exhaustively extracted at room temperature three times using petroleum ether, ethyl acetate and methanol sequently. After the extracts were completely dried, in a rotary evaporator, the yields obtained range between 0.040 and 1.690 g, giving yield percentages of approximately 0.2- 8.45% as shown in table 6.

Table 6 The 27 crude extracts of the nine plants and their percentage of yield obtained from petroleum ether (PE), ethyl acetate (EA) and methanol (ME) extracs.

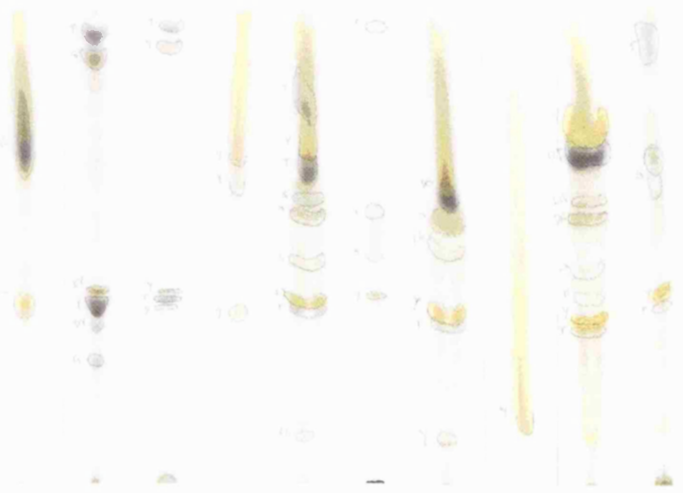
Plant species	Plant parts	Extracts	Yield (g)	% Yield
B. alba	Leaves	PE	0.455	2.28
		EA	0.483	2.42
		ME	1.015	5.08
B. rubra	Leaves	PE	0.552	2.76
		EA	0.124	0.62
		ME	1.088	5.44
C. trifolia	Leaves	PE	0.240	1.20
		EA	0.116	0.58
		ME	1.523	7.62
G. pseudochina var. hispida	Leaves	PE	0.180	0.90
		EA	0.225	1.12
		ME	0.182	0.91
G. pseudochina	Leaves	PE	0.196	0.98
		EA	0.162	0.81
		ME	0.599	2.99
M. platyclada	Aerial parts	PE	0.040	0.20
		EA	0.319	1.59
		ME	0.301	1.50
O. indicum	Stem bark	PE	0.046	0.23
		EA	0.067	0.34
		ME	1.690	8.45
P. indica	Leaves	PE	0.105	0.52
		EA	0.089	0.44
		ME	0.802	4.01
R. nasutus	Leaves	PE	0.124	0.62
		EA	0.072	0.36
		ME	0.704	3.52

After the extraction, thin layer chromatographies (TLC) of all the 27 crude extracts were obtained and the images of their fingerprinting are presented below. Note that the solvent system used in this observation is suitable for the detection of phenolics and flavonoids (Wagner *et al.* 2009) and might not be the most suitable for individual extracts, especially for high-polarity compounds. However, by performing this, we could get some information of how similar or different the constituents found in each extract were.

Figure 44 TLC of the 27 crude extract on normal phase TLC plates after spraying with natural product reagent.

Solvent system toluene: ethyl acetate: acetic acid= 60: 38: 2

BA1 BA2 BA3 BR1 BR2 BR3 GP1 GP2 GP3 GH1 GH2 GH3 CT1 CT2 CT3 OI1 OI2 OI3



MP1 MP2 MP3 PI1 PI2 PI3 PI2 RN1 RN2 RN3

Note from the TLC photo:

BA1= *B. alba* (PE),

BA2= *B. alba* (EA),

BA3= *B. alba* (MeOH),

BR1= *B. rubra* (PE),

BR2= *B. rubra* (EA),

BR3= *B. rubra* (MeOH),

GP1= *G. pseudochina* (PE),

GP2= *G. pseudochina* (EA),

GP3= *G. pseudochina* (MeOH),

GH1= *G. pseudochina var. hispida* (PE),

GH2= *G. pseudochina var. hispida* (EA),

GH3= *G. pseudochina var. hispida* (MeOH),

CT1= *C. trifolia* (PE),

CT2= *C. trifolia* (EA),

CT3= *C. trifolia* (MeOH),

OI1 = *O. indicum* (PE),

OI2 = *O. indicum* (EA),

OI3 = *O. indicum* (MeOH),

MP1= *M. platyclada* (PE),

MP2 = *M. platyclada* (EA),

MP3= *M. platyclada* (MeOH),

PI1 = *P. indica* (PE),

PI2 = *P. indica* (EA),

PI3 = *P. indica* (MeOH),

RN1= *R. nasutus* (PE),

RN2= *R. nasutus* (EA),

RN3= *R. nasutus* (MeOH),

3.3 ANTI-INFLAMMATORY ACTIVITIES OF THE 27 PLANT EXTRACTS

Among all the tested extracts, *G. pseudochina var. hispida* (MeOH) and *O. indicum* (EA) showed the strongest NF-κB inhibitory effects. In addition, they also inhibited the release of IL-1β and PGE_2 (as summarised in Table 2). Interestingly, *M. platyclada* (EA and ME) showed the highest level of inhibition on the release of several pro-inflammatory cytokines such as IL-6, IL-1β and TNF-α, but did not present any inhibitory effects on the activation of NF-κB. In addition, a number of extracts activated NF-κB or increased the synthesis of the pro-inflammatory mediators.

The graphs below show activities of the traditional anti-inflammatory plant extracts on the NF-κB activation and pro-inflammatory cytokines/mediators release. Each value represents the averaged IC_{50} of 3 independent experiments and the missing/disappearing bars mean activating effects or increased biosynthesis at all the tested concentrations.

Figure 45 Effects on PMA induced NF-κB activation on HeLa cells. Note: the shorter bars indicate the better inhibitory activities.

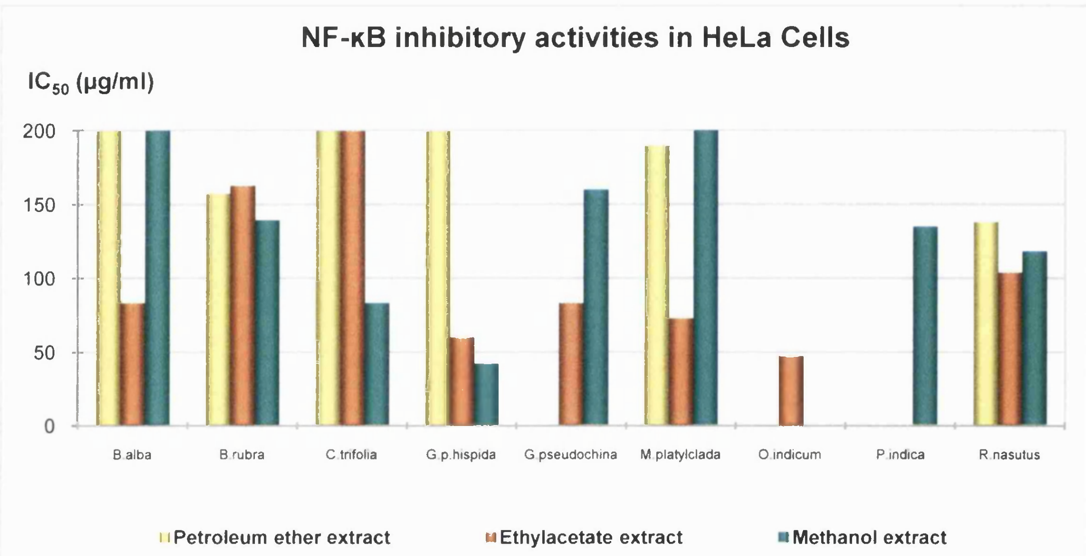


Figure 46 Effects on pro-inflammatory cytokines IL-6 release in human monocytes. Note: the shorter bars indicate the better inhibitory activities.

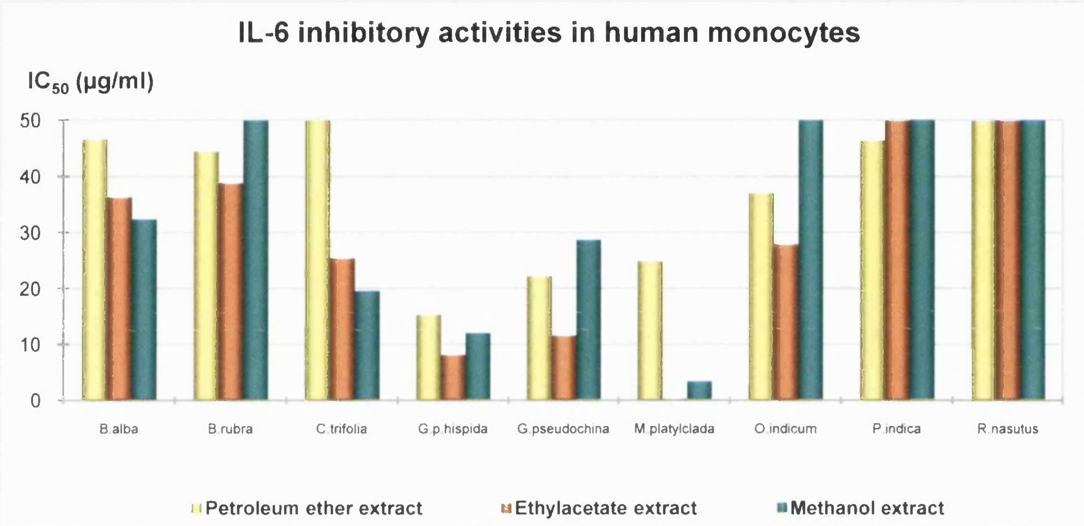


Figure 47 Effects on pro-inflammatory cytokines IL-1β release in human monocytes. Note: the shorter bars indicate the better inhibitory activities.

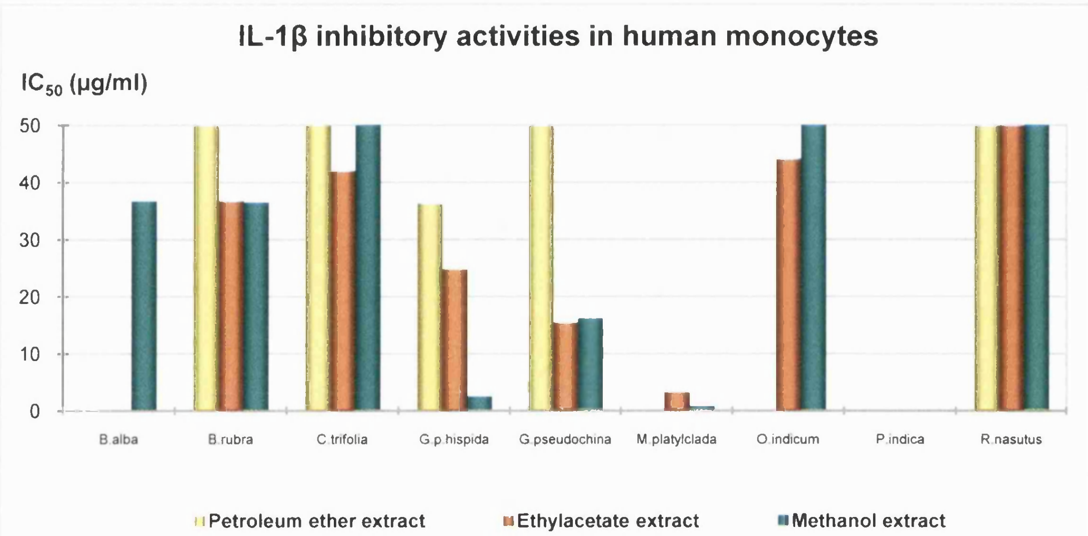


Figure 48 Effects on LPS-induced PGE_2 release in human monocytes. Note: the shorter bars indicate the better inhibitory activities.

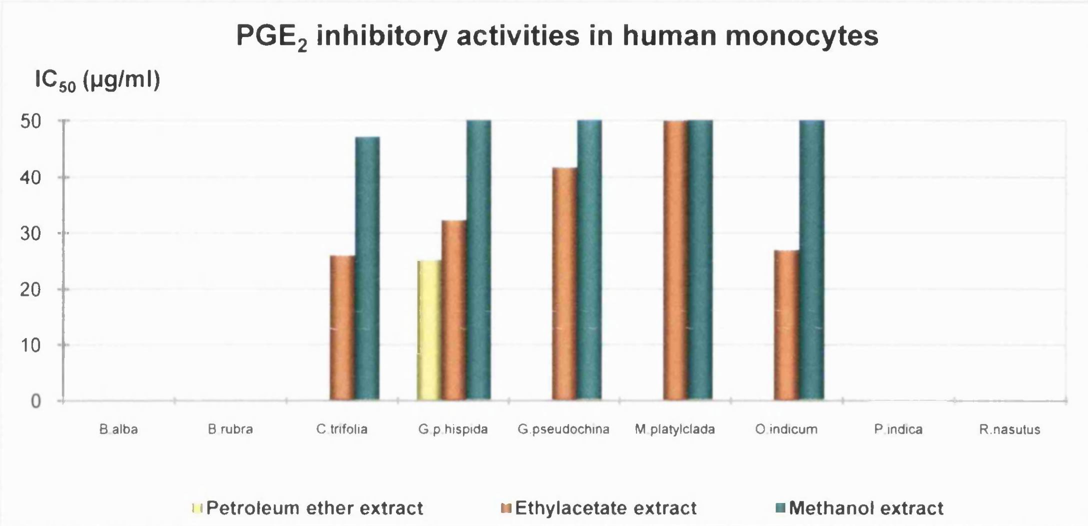


Figure 49 Effects on LPS-induced TNF-α release in human monocytes. Note: the shorter bars indicate the better inhibitory activities.

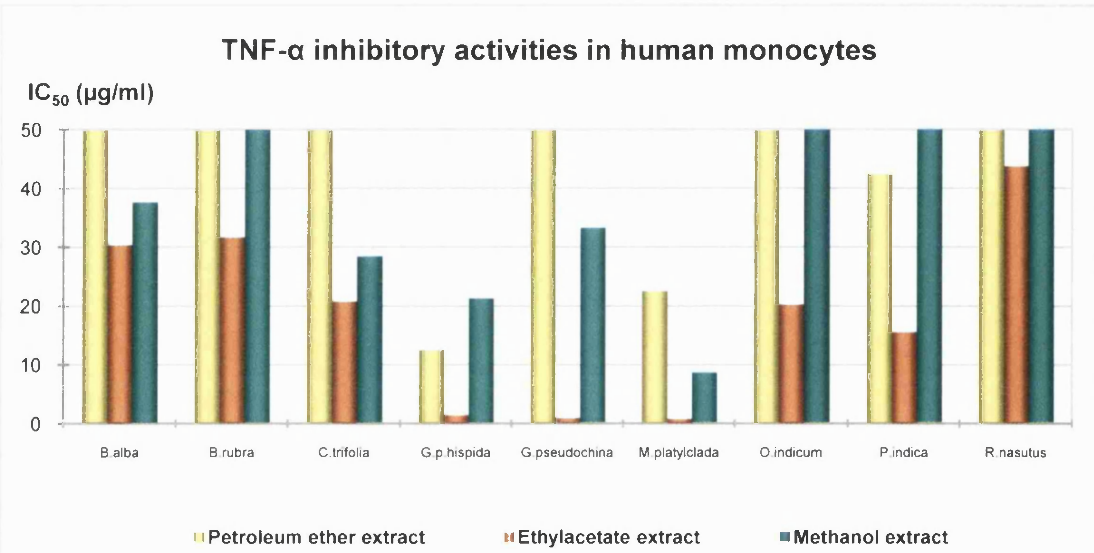


Table 7 Summary of NF-κB inhibitory activities on HeLa cells, and inhibitory effects on LPS-induced IL-1β, IL-6, TNF-α and PGE_2 release in human monocytes (values represent means, n =3).

Species	**Extracts**	**IC$_{50}$ (μg/ml)**				
		NF-κB	**PGE_2**	**IL-6**	**IL-1β**	**TNF-α**
B. alba	PE	> 200.00	↑	46.74	↑	> 50.00
	EA	83.28	↑	36.40	↑	30.42
	ME	> 200.00	↑	32.38	36.73	37.68
B. rubra	PE	157.31	↑	44.49	> 50.00	> 50.00
	EA	162.83	↑	38.87	36.76	31.72
	ME	139.21	↑	> 50.00	36.49	> 50.00
C. trifolia	PE	> 200.00	↑	> 50.00	> 50.00	> 50.00
	EA	> 200.00	26.04	25.47	42.04	20.83
	ME	83.16	47.14	19.53	> 50.00	28.45
G. pseudochina var. hispida	PE	> 200.00	25.23	15.30	36.32	12.63
	EA	60.18	32.35	8.14	24.87	1.49
	ME	41.96	> 50.00	12.01	2.46	21.24
G. pseudochina	PE	↑	↑	22.23	> 50.00	> 50.00
	EA	83.20	41.77	11.63	15.44	1.04
	ME	159.76	> 50.00	28.62	16.11	33.28
M. platyclada	PE	190.25	↑	24.95	↑	22.59
	EA	72.94	> 50.00	0.28	3.27	0.86
	ME	> 200.00	> 50.00	3.38	0.73	8.67
O. indicum	PE	↑	↑	37.13	↑	> 50.00
	EA	47.45	26.98	27.98	44.12	20.33
	ME	↑	> 50.00	> 50.00	> 50.00	> 50.00
P. indica	PE	↑	↑	46.51	↑	42.52
	EA	↑	↑	> 50.00	↑	15.68
	ME	134.69	↑	> 50.00	↑	> 50.00
R. nasutus	PE	138.16	↑	> 50.00	> 50.00	> 50.00
	EA	104.04	↑	> 50.00	> 50.00	43.83
	ME	118.03	↑	> 50.00	> 50.00	> 50.00
Parthenolide		1.97	ND	ND	ND	ND
Hydrocortisone		ND	0.77	0.32	1.44	0.89

Note: ↑ - activating effects or increased biosynthesis at all the tested concentrations.

3.4 CYTOTOXIC ACTIVITIES OF THE 27 EXTRACTS

The cytotoxicity of the extracts was examined against cervix cancer HeLa cells, human leukaemia CCRF-CEM cells and multidrug-resistant CEM/ADR5000 cells using the MTT and XTT assays to determine cell proliferation.

Overall the results suggest that *P. indica* (PE) strongly inhibits cell mitochondria activity of both CCRF-CEM and CEM/ADR5000 cells at a concentration of 10 μg/ml followed by *R. nasutus* (ME) and *G. pseudochina var. hispida* (EA) which more specifically inhibited the multidrug resistant CEM/ADR5000 subline. *R. nasutus* (EA and PE) showed the highest cytotoxicity against HeLa cells, followed by *O. indicum* (EA) (as summarized in Table 8).

Some of the extracts showed cytotoxic effects on both leukaemia cells and cervix cancer cells, but some extracts only acted on one of the two cell lines. For example, *R. nasutus* (EA) expressed high cytotoxicity against HeLa cells, CCRF-CEM cells and CEM/ADR5000 cells, while *P. indica* (PE) only showed high level of cytotoxicity on leukaemia cells (both) but not on HeLa cells.

The following graphs show cytotoxic effects of the extracts at 10 μg/ml against human leukaemia CCRF-CEM cells (Fig.1) and multidrug resistant CEM/ADR5000 cells (Fig. 2). Note that the Y-axes are the % viability of the cells. X-axes are the different plant extracts at a concentration of 10 μg/ml.

Figure 50 Cytotoxicity of the extracts against CCRF-CEM leukemia cells.

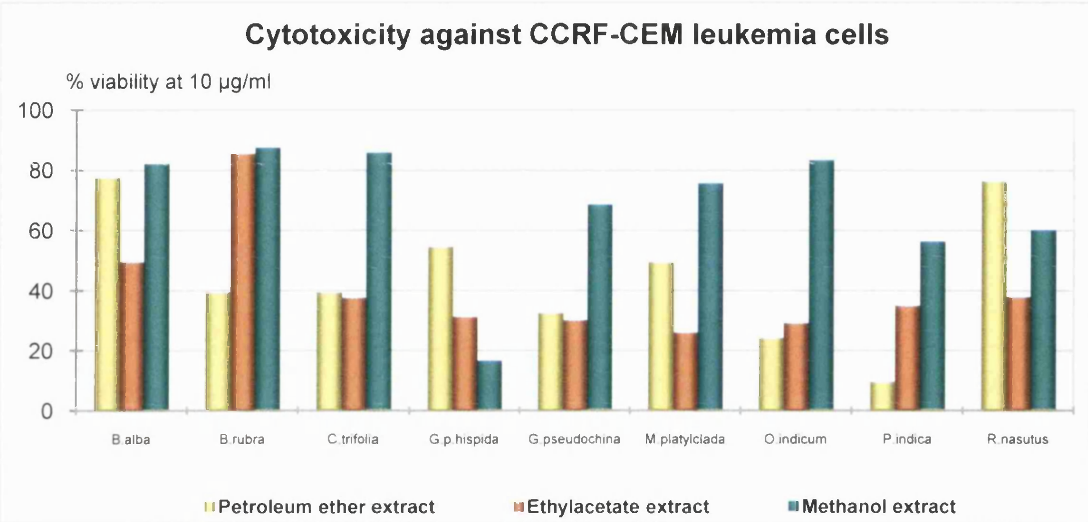
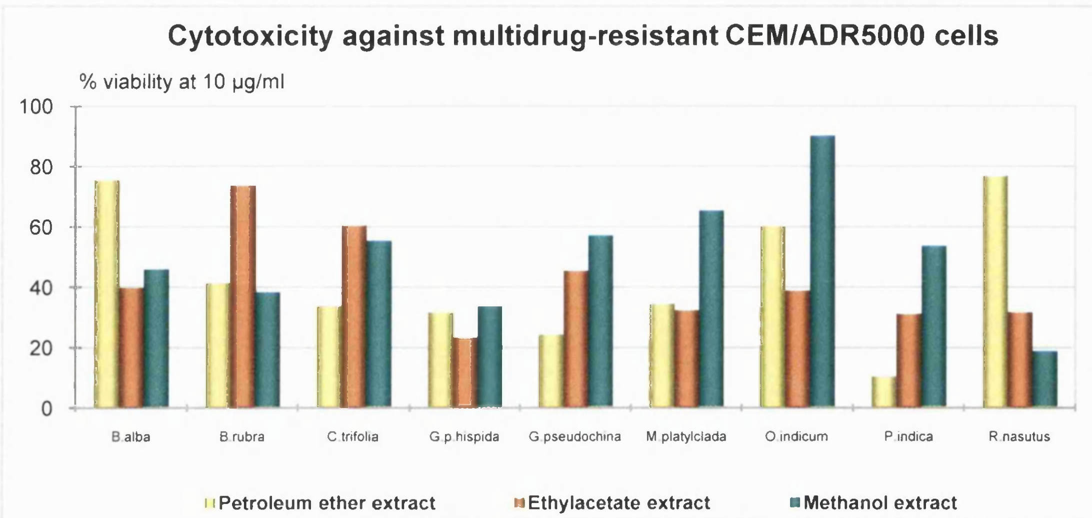


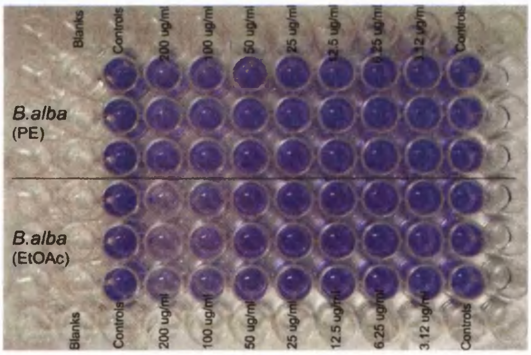
Figure 51 Cytotoxicity of the extracts against CEM/ADR5000 leukemia cells.



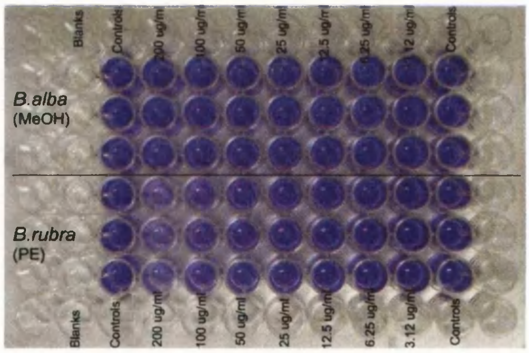
Cytotoxic activities of the plant extracts on HeLa cells were estimated by the change in color after the cells were exposed to the extracts for 48 hrs. Results are shown in the figure below. On each plate, the 2nd column is blank (medium only), the 3rd column is control (non-toxicity), the 4th column has samples at a starting concentration of 200 μg/ml followed by their serial dilution from the 5th-10th column, with the final column as another control (non-toxicity). Each extract was tested in 3 rows which served as 3 duplicates. Cytotoxicity of doxorubicin (positive control) is shown in the last figure. The photos were taken from only one experiment out of the three independent experiments. Less purple means higher toxicity.

Figure 52 Cytotoxicity of the plant extracts against Hela cells

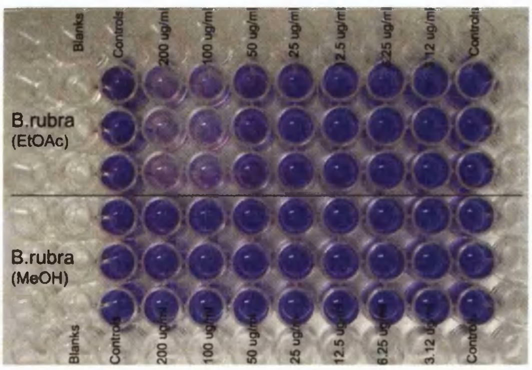
B. alba (PE, EA)



B. alba (ME), **B. rubra** (PE)



B. rubra (EA, ME)



C. trifolia (PE, EA)

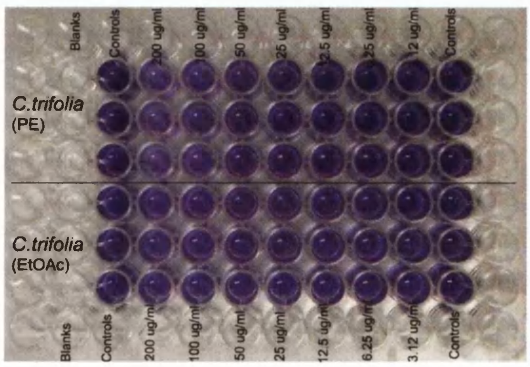
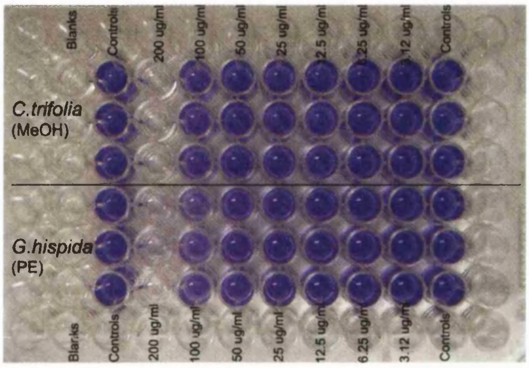
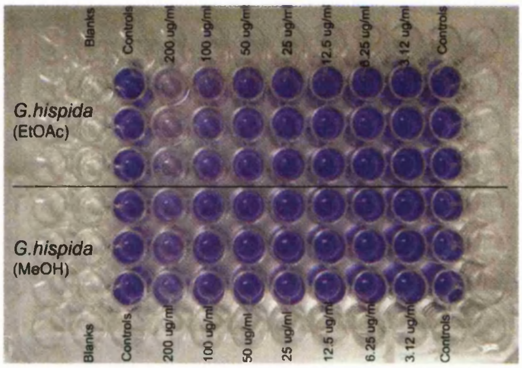


Figure 52 Cytotoxicity of the plant extracts against Hela cells (continued)

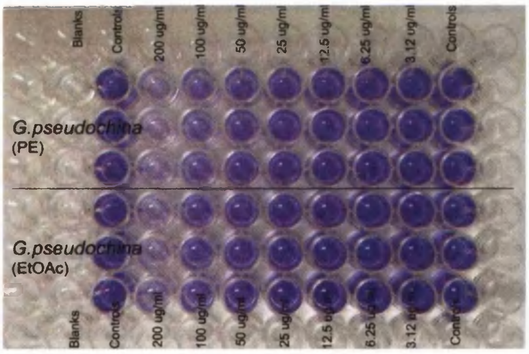
C. trifolia (ME), **G.P hispida** (PE)



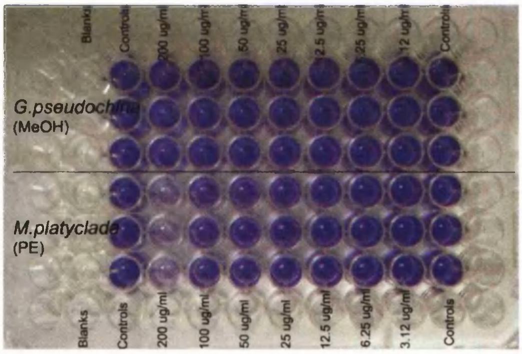
G.P. hispida (EA, ME)



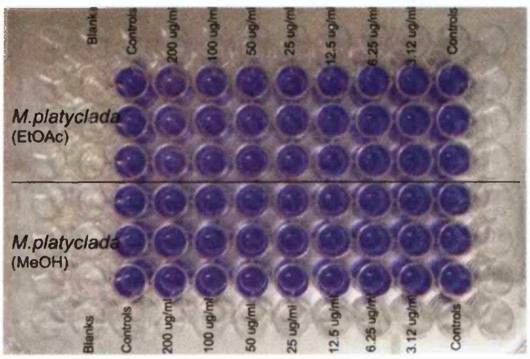
G.pseudochina (PE, EA)



G.ps (ME), **M. platyclada** (PE)



M. platyclada (EA, ME)



O. indicum (PE, EA)

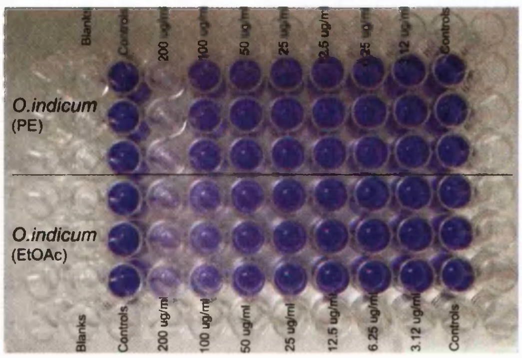
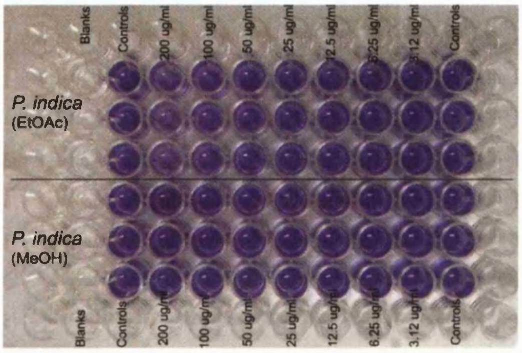
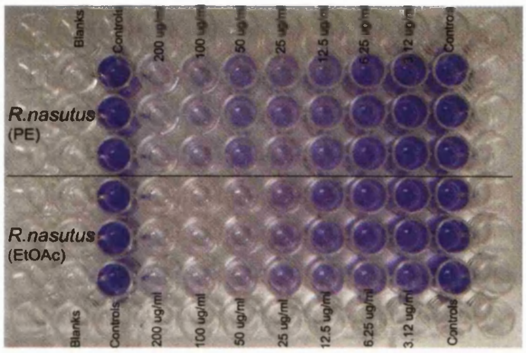


Figure 52 Cytotoxicity of the plant extracts against Hela cells (continued)

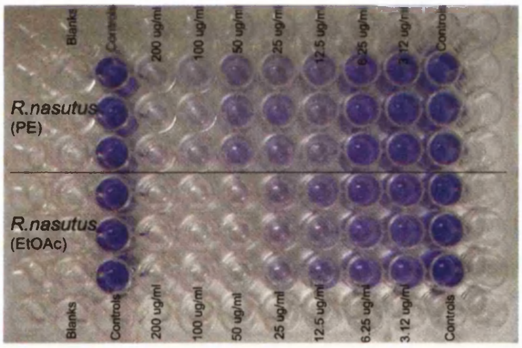
P. indica (EA, ME)



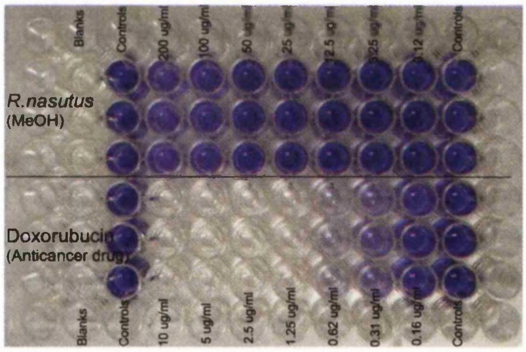
R. nasutus (PE, EA)



R. nasutus (PE, EA)



R. nasutus (ME), **Doxorubicin***



* Doxorubicin starting concentration= 10 μg/ml, final concentration= 0.15 μg/ml

Table 8 Summary of cytotoxic activities on HeLa cells, CCRF-CEM and multidrug resistant CEM/ADR5000 cells (values represent means ± S.D., *n* = 3)

Species		**Hela cells**		**Leukemia cells (%viability at 10 µg/ml)**	
		IC_{50} (µg/ml)	**%Viability at 10µg/ml**	**CCRF-CEM**	**CEM/ADR5000**
B. alba	PE	197.23 ± 1.23	91.22 ± 1.09	77.66 ± 0.01	75.56 ± 0.02**
	EA	130.89 ± 1.09	89.15 ± 0.78	49.52 ± 0.37	39.97 ± 0.13**
	ME	1024.24 ± 0.87	98.35 ± 0.67	82.13 ± 0.18	45.91 ± 0.18**
B. rubra	PE	145.39 ± 0.81	96.40 ± 1.06	39.56 ± 0.13	41.51 ± 0.19
	EA	114.89 ± 1.37	89.58 ± 1.77	85.63 ± 0.32	73.82 ± 0.21**
	ME	711.56 ± 2.34	97.18 ± 2.09	87.48 ± 0.46	38.37 ± 0.24**
C. trifolia	PE	128.37 ± 4.09	84.47 ± 1.23	39.41 ± 0.26	33.88 ± 0.12**
	EA	194.70 ± 0.19	83.33 ± 1.76	37.74 ± 0.02	60.49 ± 0.15
	ME	127.35 ± 1.34	93.89 ± 2.01	85.87 ± 0.53	55.37 ± 0.05**
G. pseudochina var. hispida	PE	93.38 ± 1.39	87.82 ± 0.54	54.67 ± 0.60	31.66 ± 0.04**
	EA	114.05 ± 1.84	100.00 ± 1.23	31.42 ± 0.42	23.50 ± 0.12**
	ME	181.85 ± 2.71	93.31± 0.89	16.72 ± 0.13	33.69 ± 0.22
G. pseudochina	PE	96.81± 0.80	88.63 ± 0.76	32.74 ± 0.27	24.47 ± 0.03**
	EA	119.56 ± 1.41	96.67 ± 0.34	30.29 ± 0.01	45.53 ± 0.12
	ME	397.15 ± 2.55	97.41 ± 0.66	68.56 ± 0.37	57.03 ± 0.04**
M. platyclada	PE	123.59 ± 1.15	91.27 ± 0.32	49.52 ± 0.24	34.51 ± 0.17**
	EA	194.34 ± 1.65	93.63 ± 1.66	26.13 ± 0.09	32.45 ± 0.18
	ME	605.66 ± 5.33	92.88 ± 2.65	75.61 ± 0.08	65.31 ± 0.23**
O. indicum	PE	96.18 ± 1.32	85.61 ± 0.99	24.35 ± 0.26	60.37 ± 0.16
	EA	55.22 ± 0.58	87.81 ± 0.47	29.35 ± 0.02	38.99 ± 0.04
	ME	417.95 ± 1.77	100.00 ± 0.55	83.32 ± 0.39	89.94 ± 0.27
P. indica	PE	214.27 ± 1.39	88.14 ± 0.79	9.75 ± 0.29	10.48 ± 0.12
	EA	199.72 ± 2.07	95.51 ± 0.46	35.12 ± 0.52	31.30 ± 0.19*
	ME	1108.54 ± 2.82	99.71 ± 0.59	56.35 ± 0.18	53.59 ± 0.22**
R. nasutus	PE	24.88 ± 0.69	45.05 ± 0.74	76.49 ± 0.57	77.03 ± 0.48
	EA	3.63 ± 1.99	36.77 ± 0.81	38.13 ± 0.25	31.81 ± 0.24**
	ME	171.21 ± 2.41	92.38 ± 0.49	60.10 ± 0.10	18.72 ± 0.10**
Doxorubicin		0.11 ± 0.33	0.00 ± 0.25	(IC_{50}=11.8 nmol/L)[a]	(IC_{50}=12.2 mmol/L)[a]
Vincristine				(IC_{50}=1.7 nmol/L)[a]	(IC_{50}= 1,043 nmol/L)[a]

Note: *indicates significantly greater cytotoxicity against multidrug resistant CEM/ADR5000 cells when compared to CCRF-CEM cells at *p=0.0003 or **p<0.0001, respectively. [a] Efferth *et al.* (2008a).

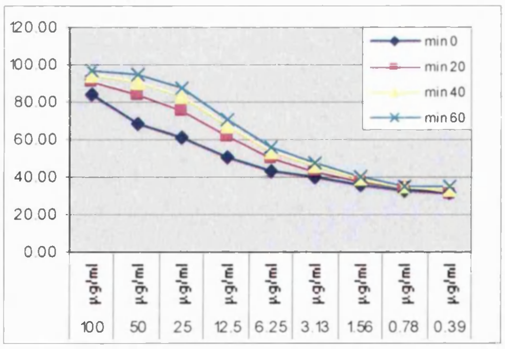
3.5 ANTIOXIDANT ACTIVITIES & PHENOLIC CONTENTS

Overall results show that the highest activity in the DPPH assay were found in *C. trifolia* (ME), followed by *P. indica* (ME) and *O. indicum* (EA). On the other hand, *O. indicum* (EA and ME) showed the most potent inhibition of lipid-peroxidation, followed by *C. trifolia* (ME). Moreover, the phenolic contents were found at high levels in *C. trifolia* (ME), *G. pseudochina var. hispida* (EA), and *O. indicum* (EA). Individual extracts presented unique patterns of DPPH scavenging activity at different concentrations and different time periods of 0, 20, 40 and 60 minutes, as shown in the graphs below.

Figure 53 DPPH scavenging activities of the plant extracts. Note that on the X axes are the concentration of the extracts. Y axes are the % oxidation inhibition. Values represent means (n = 3).

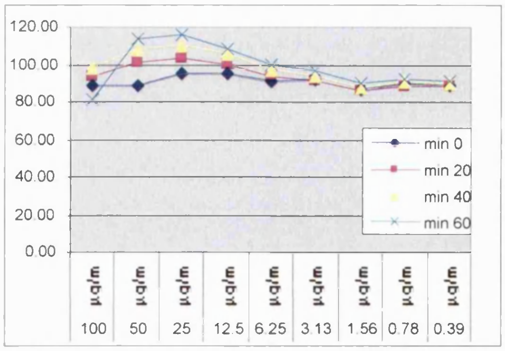
B. alba (EA)

% inhibition



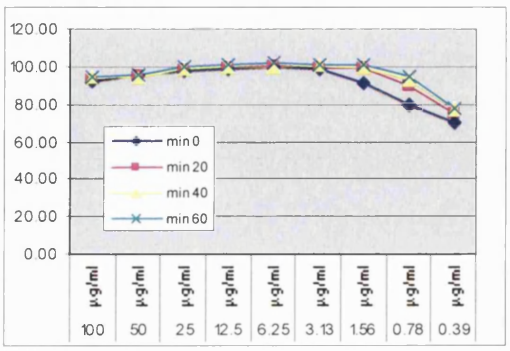
B. rubra (EA)

% inhibition



C. trifolia (EA)

% inhibition



C. trifolia (ME)

% inhibition

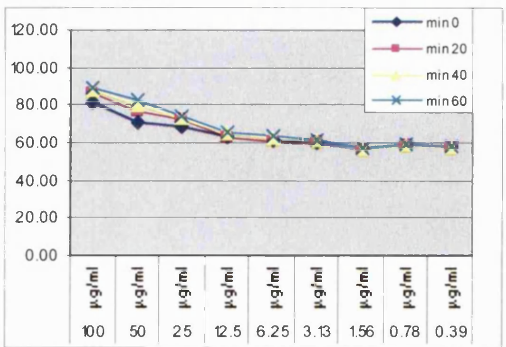
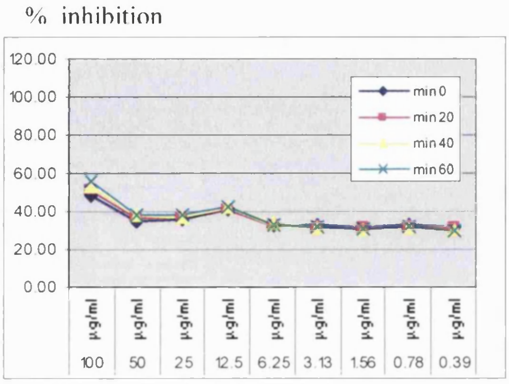
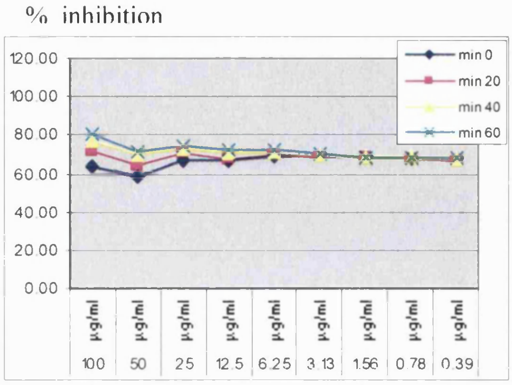


Figure 53 DPPH scavenging activities of the plant extracts (continued)

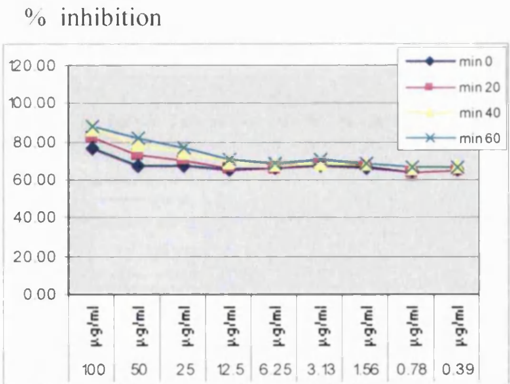
G. P. hispida (PE)



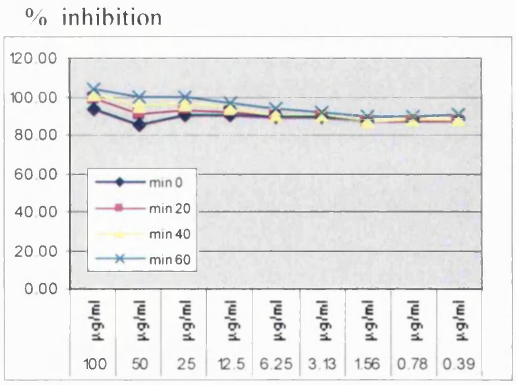
G. P. hispida (EA)



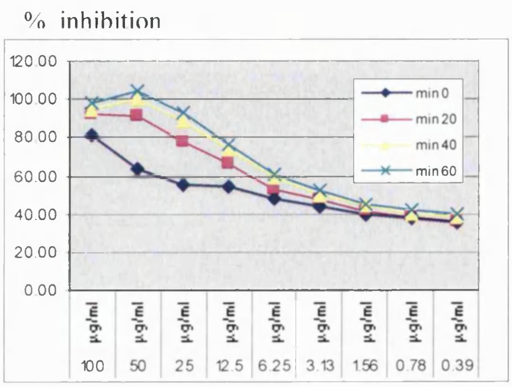
G. P. hispida (ME)



M. platyclada (PE)



M. platyclada (EA)



M. platyclada (ME)

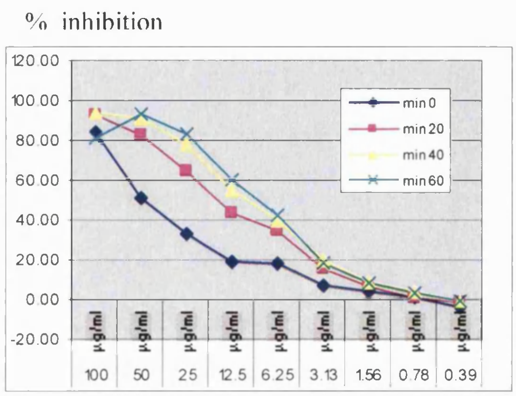
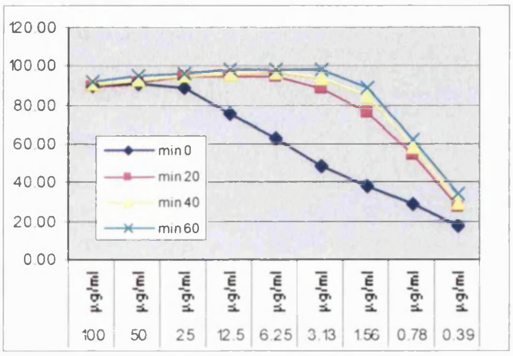


Figure 53 DPPH scavenging activities of the plant extracts (continued)

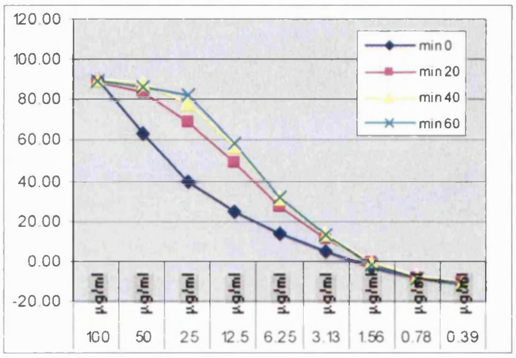
O. indicum (PE)

% inhibition



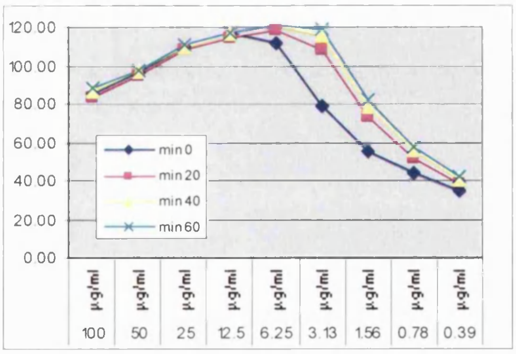
O. indicum (EA)

% inhibition



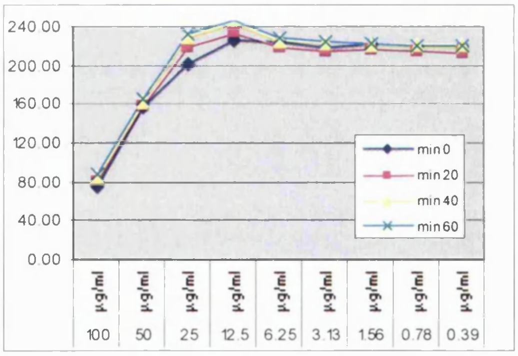
P. indica (ME)

% inhibition



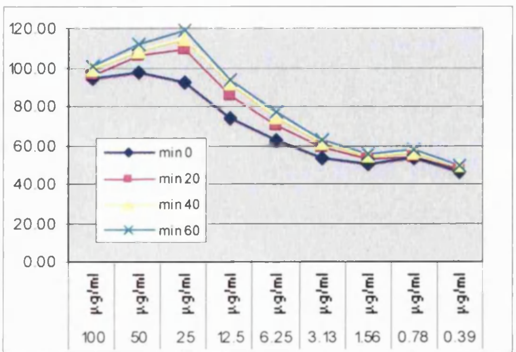
R. nasutus (EA)

% inhibition



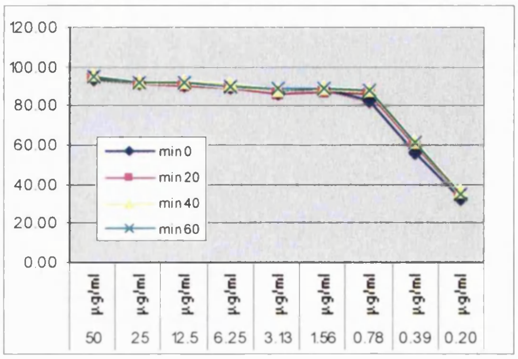
R. nasutus (ME)

% inhibition



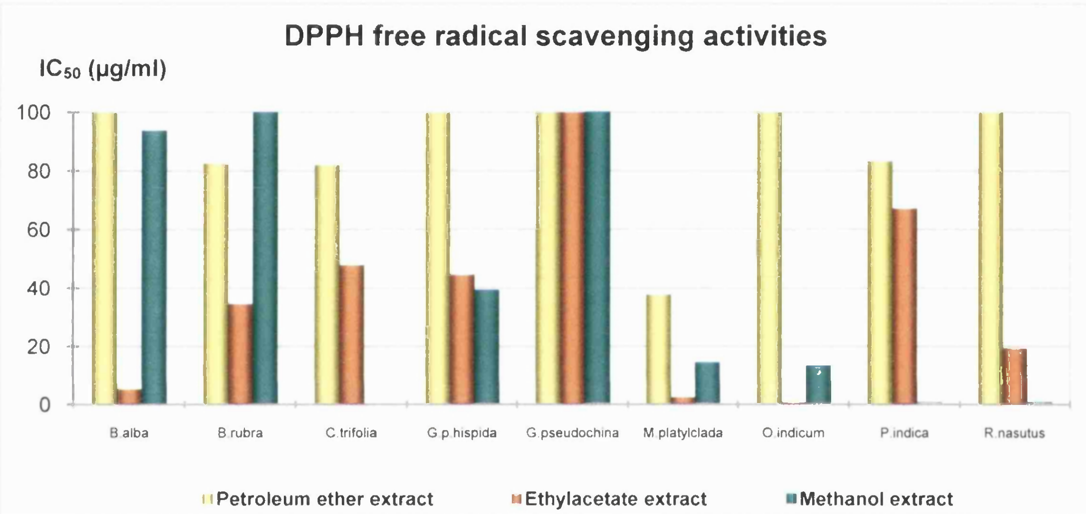
Trolox (standard Vitamin E)

% inhibition



The concentrations that showed 50% inhibition (IC_{50}) were calculated with linear regression from results at the time 40 minutes after the reactions, as shown in the graph below.

Figure 54 DPPH free radical scavenging activities of the 27 plant extracts (all values averaged from 3 independent experiments). Note: the shorter bars indicate the better scavenging activities.

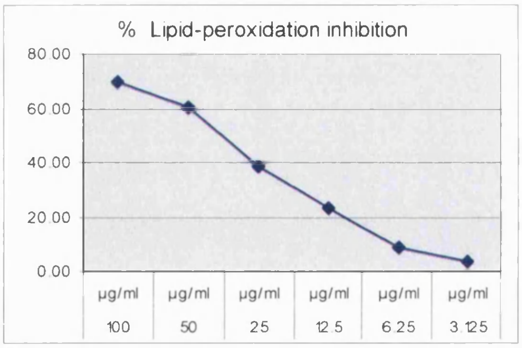


3.5.2 LIPID-PEROXIDATION INHIBITORY EFFECTS OF THE PLANT EXTRACTS

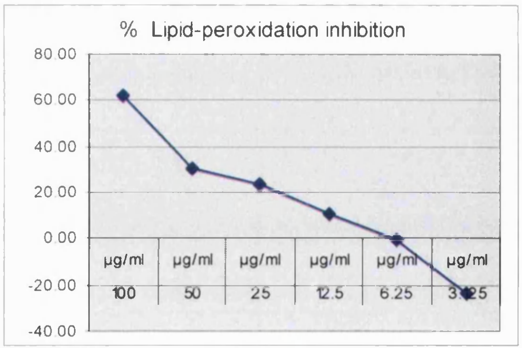
The capability of the extracts to protect membrane lipids against peroxidation were tested at a starting concentration of 100 μg/ml. At this concentration the inhibitory effects of the extract on lipid-peroxidation were found to vary in a range of 19.94-99.80%. The graphs below show inhibitory behaviors of some of the plant extracts.

Figure 55 Lipid-peroxidation inhibitory effects of the extracts. Note that on the X axes are the concentration of the extracts. Y axes are the % inhibition. Each value is an average of 3 independent experiments.

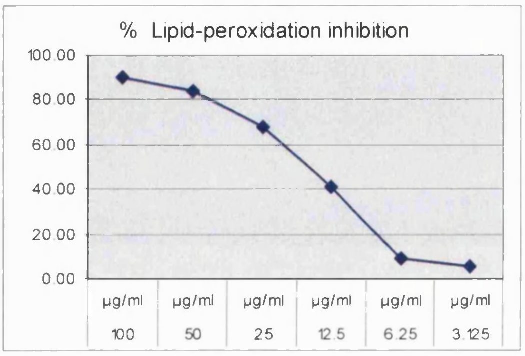
B. alba (EA)



C. trifolia (EA)



C. trifolia (ME)



G. P. hispida (PE)

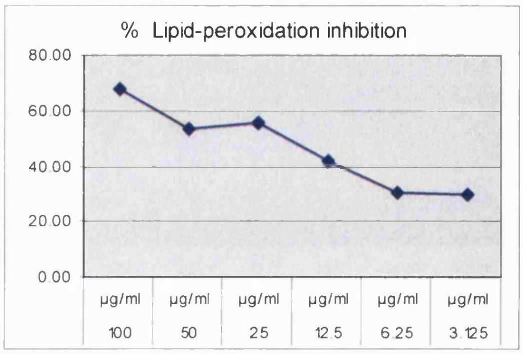
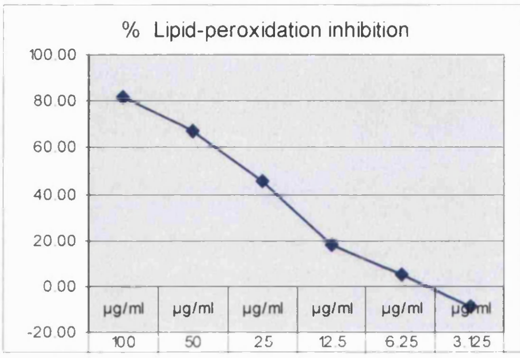
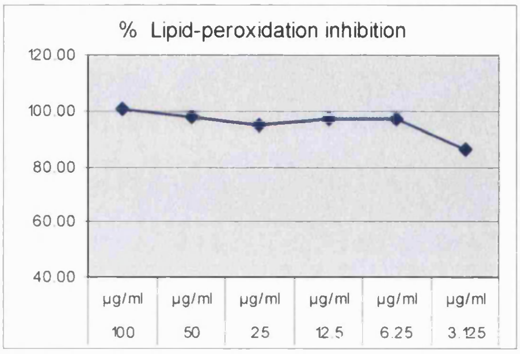


Figure 55 Lipid-peroxidation inhibitory effects of the extracts (continued)

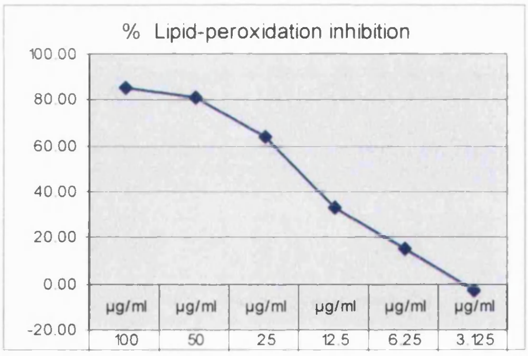
M. platyclada (PE)



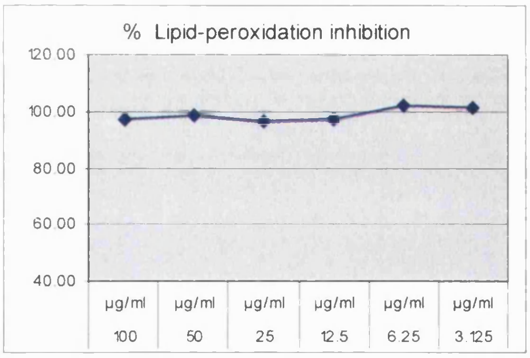
M. platyclada (EA)



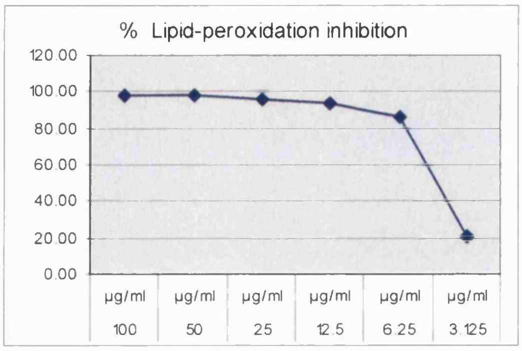
M. platyclada (ME)



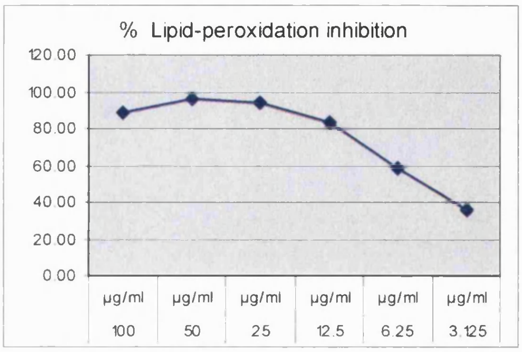
O. indicum (PE)



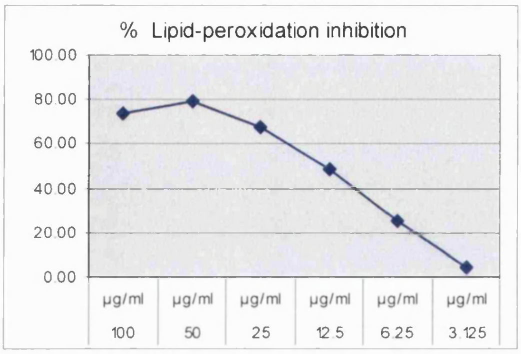
O. indicum (EA)



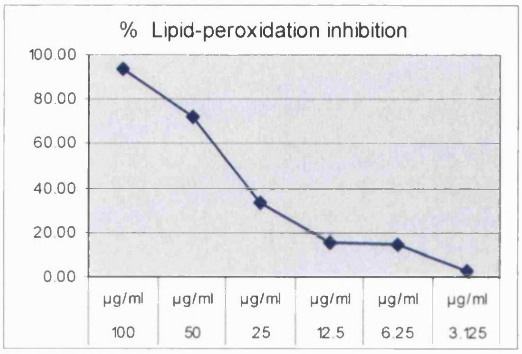
P. indica (ME)



R. nasutus (EA)

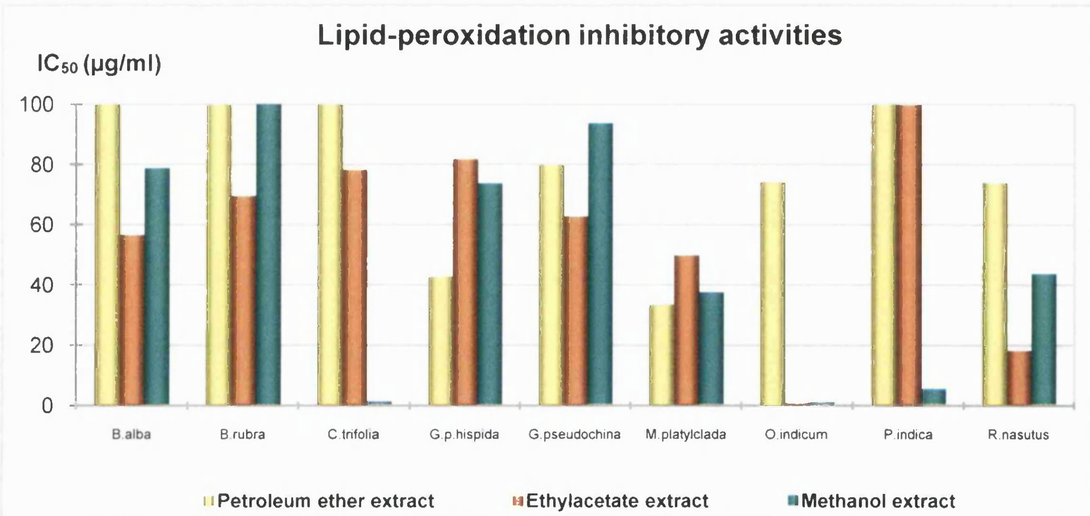


R. nasutus (ME)



Serial dilutions were made to obtain the concentration that inhibited lipid peroxidation at 50%, the IC_{50} were calculated using the appropriate calibration curves. The IC_{50} values of all the extracts are shown in the graphs below.

Figure 38 Lipid-peroxidation inhibitory effects of the extracts. Note: the shorter bars indicate the better inhibitory activities.

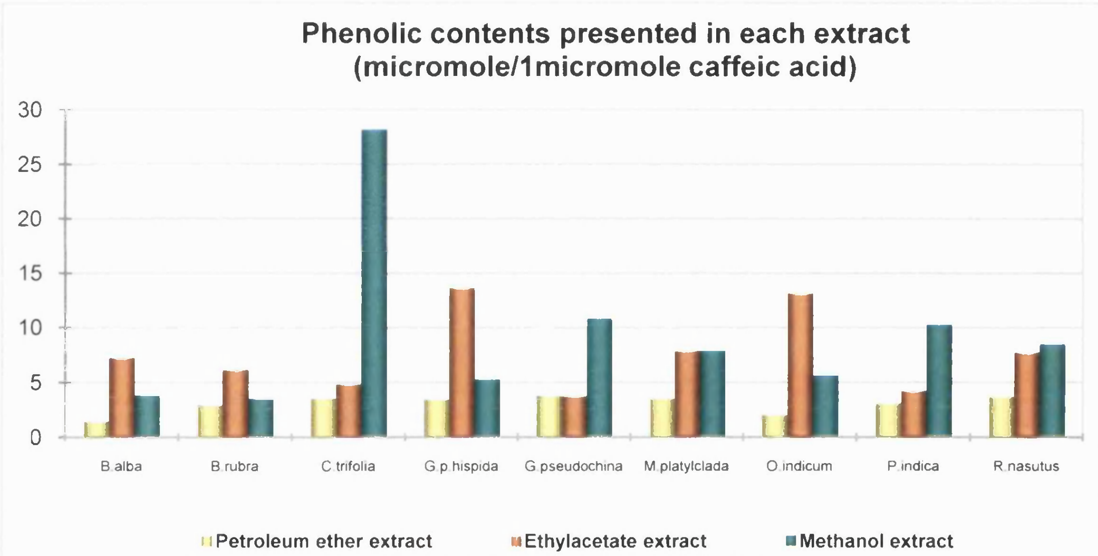


From the graph, the highest lipid-peroxidation inhibition activity was found in the ethyl acetate and methanol extracts of *O. indicum* (IC_{50} = 0.08 and 1.05 µg/ml, respectively) followed by the methanol extract of *C. trifolia* (IC_{50} = 1.36 µg/ml) and the methanol extract of *P. indica* (IC_{50} = 5.44 µg/ml).

3.5.3 PHENOLIC CONTENTS DETERMINED BY FOLIN-CIOCALTEAU METHOD

The amount of total phenolics in the 27 extracts was determined using the Folin-Ciocalteau assay with some modifications. The amount of phenolics present in the extracts were found to range from 1.44 to 28.14 μMol/ caffeic acid 1μMol dry material, as shown in the graph below. Each value represent mean (*n = 3*).

Figure 57 Total phenolic contents presented in each extract.



It can be seen that the methanol extract of *C. trifolia* contains the highest amount of phenolics when compared to other extracts and other plant species. The second highest phenolic content is in the ethylacetate extracts of both *G. pseudochina var. hispida* and *O. indicum*, followed by the methanol extracts of *G. pseudochina* and *P. indica*. However, these figures do not imply that those extracts containing higher amounts of phenolics will always present higher pharmacological activities or antioxidant activities. This is because the assay detected all the phenolics compounds including sugars, carbohydrates, etc, which may or may not express pharmacological activities.

Table 9 Overall antioxidant capacities and total phenolic contents of the extracts (all values averaged from 3 independent experiments).

Species	Extracts	IC_{50} in DPPH assay (µg/ml)	IC_{50} in Lipid-peroxidation assay (µg/ml)	Total phenolic content[b]
B. alba	PE	>100	>100	1.44 ± 0.97
	EA	5.32	56.65	7.25 ± 0.76
	ME	93.72	78.70	3.81 ± 1.51
B. rubra	PE	82.64	>100	2.93 ± 0.32
	EA	34.58	69.59	6.17 ± 0.58
	ME	>100	>100	3.50 ± 0.07
C. trifolia	PE	82.06	>100	3.51 ± 0.62
	EA	47.89	78.27	4.81 ± 0.55
	ME	0.48	1.36	28.14 ± 0.71
G. pseudochina var. hispida	PE	>100	42.76	3.43 ± 0.09
	EA	44.56	81.90	13.66 ± 0.32
	ME	39.27	73.63	5.27 ± 1.08
G. pseudochina	PE	>100	79.94	3.77 ± 0.63
	EA	>100	62.88	3.76 ± 0.76
	ME	>100	93.56	10.82 ± 0.25
M. platyclada	PE	37.74	33.61	3.56 ± 0.71
	EA	2.45	49.87	7.89 ± 0.45
	ME	14.42	37.36	7.85 ± 0.12
O. indicum	PE	>100	74.28	2.01 ± 0.91
	EA	0.73	0.08	13.17 ± 1.01
	ME	13.39	1.05	5.59 ± 0.78
P. indica	PE	83.37	>100	3.09 ± 1.90
	EA	67.23	>100	4.24 ± 0.23
	ME	0.60	5.44	10.25 ± 0.82
R. nasutus	PE	>100	74.01	3.72 ± 1.34
	EA	19.34	18.26	7.73 ± 1.09
	ME	0.78	43.56	8.45 ± 0.76
Quercetin		0.17	0.13	ND
Trolox®		0.31	0.28	ND

[a] Equivalent to caffeic acid 1 µMol and express in µg of extract. ND = not determined.

3.6 BIOASSAY-GUIDED ISOLATION & IDENTIFICATION OF NF-KB INHIBITORS FROM THE METHANOL EXTRACT OF GYNURA PSEUDOCHINA VAR. HISPIDA

The methanol extract of *G. pseudochina var. hispida* showed the highest NF-κB inhibitory effect compared to the other extracts or other plant species. This extract showed low antioxidant activity, which makes this extract interesting. The ethyl acetate extract (not the methanol extract) of this species also showed the second most potent and specific cytotoxic effects on the multidrug resistant CEM/ADR5000 subline. To date, no research has reported such activities or the chemistry of this species. Therefore, this plant was chosen for further investigation of active compounds using NF-κB as a lead bioassay-guided isolation.

As shown in the following diagram, the methanol extract was fractionated by Sephadex column with gradient elution between dichloromethane, methanol and water, resulting 60 fractions (F1-F60). The fractions were monitered using TLC with the solvent system of dichloromethane: methanol: acetic acid = 80: 20: 1 and sprayed with 4% vanillin in sulfuric acid. Thereafter the fractions which showed similar compounds on the TLC were combined together, giving only 12 fractions in total. All 12 fractions were tested for the NF-κB and it was found that fractions F24-34 and F38 showed the highest NF-κB inhibitory effects with IC_{50} values less than 50 μg/ml, as shown in the diagram. Note, the figures in brackets are the weights of the fractions, figures with * (asterisks) are IC_{50} of the NF-κB inhibitory effects.

TLC fingerprints of the active fractions were observed along with their neighboring fractions (as shown in the TLC photos). The detailed TLC experiments are explained underneath the photos. After obtaining the active compounds from TLC scraping, purifications of the active compounds were done using TLC and HPLC techniques depending on the amount of compounds obtained. Detailed purifications and identification of those compounds are presented in the following sections which show each individual compound separately.

Figure 58 Bioassay guided fractionation scheme of the methanol extract of *G. pseudochina var. hispida*. Note that figures in the brackets are weight of the fractions and figures with * (asterisks) are the IC_{50} values (µg/ml)

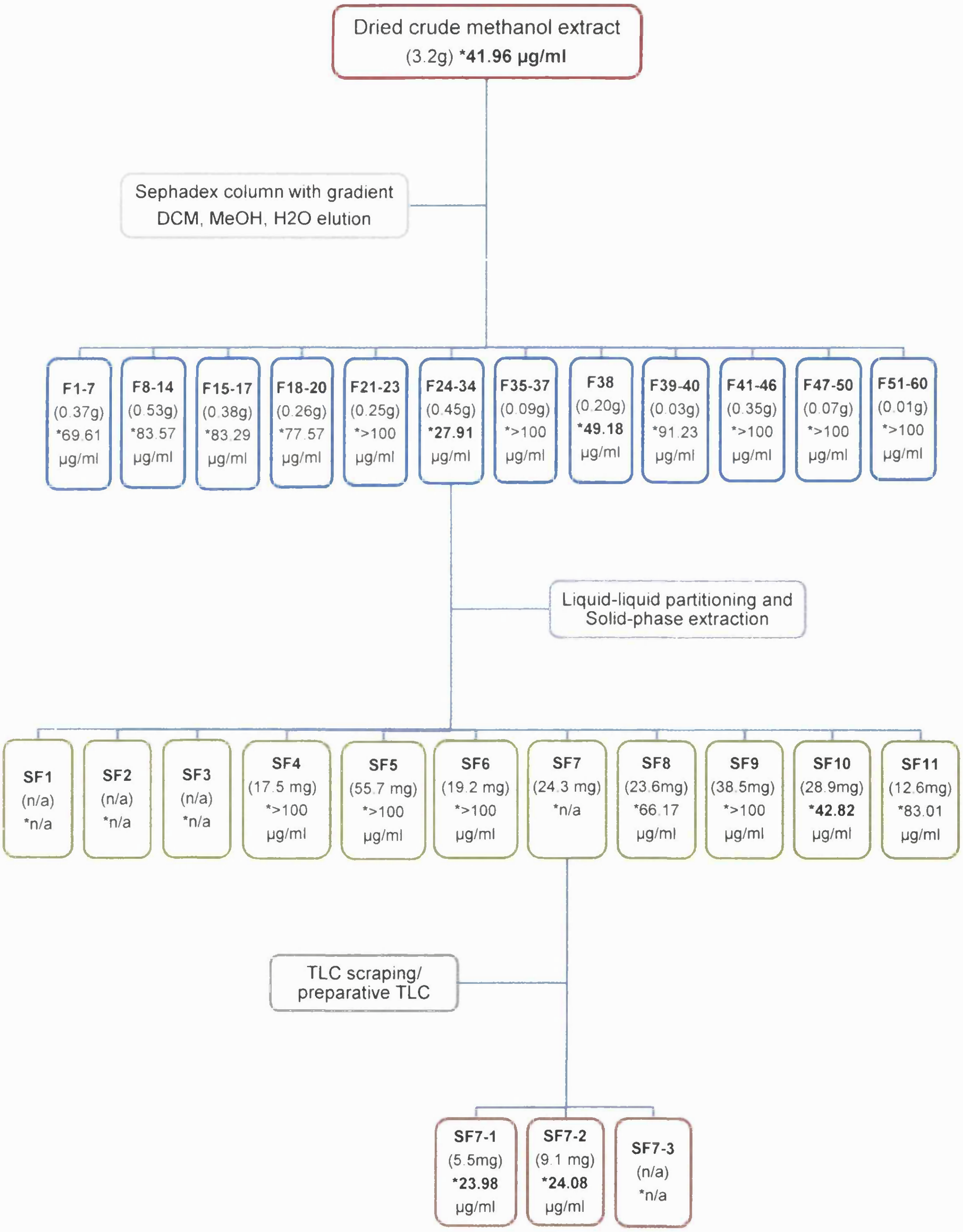
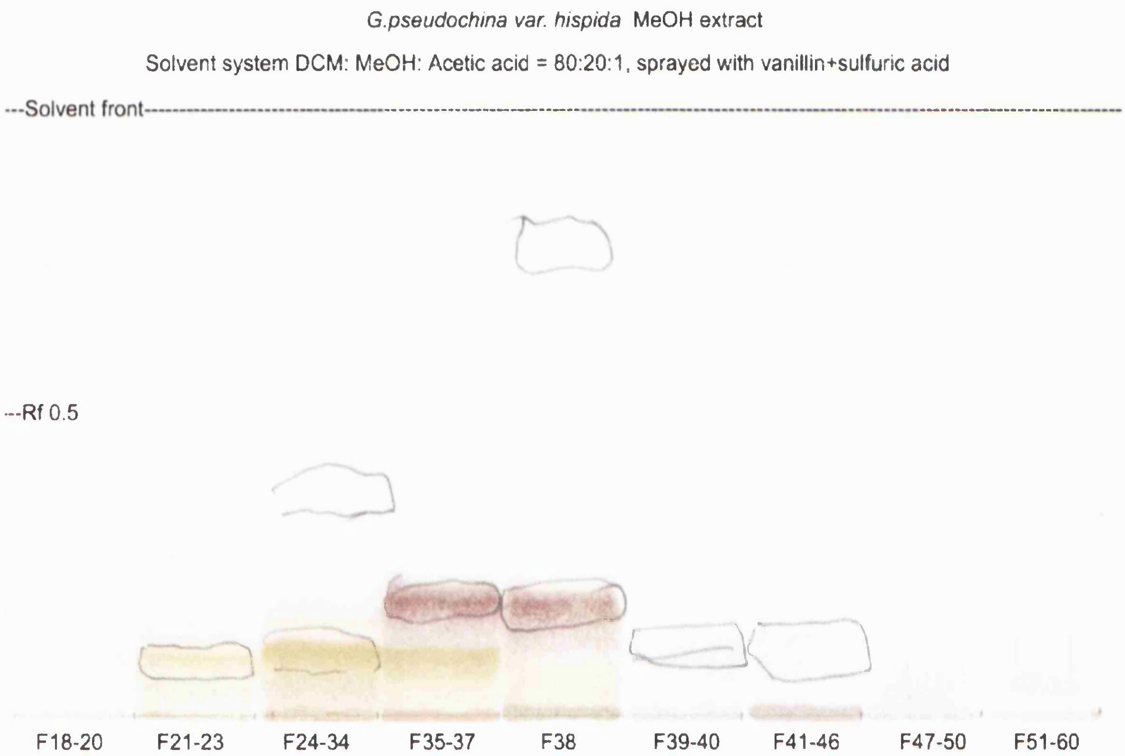


Figure 59 TLC of *G. pseudochina var. hispida* fractions.

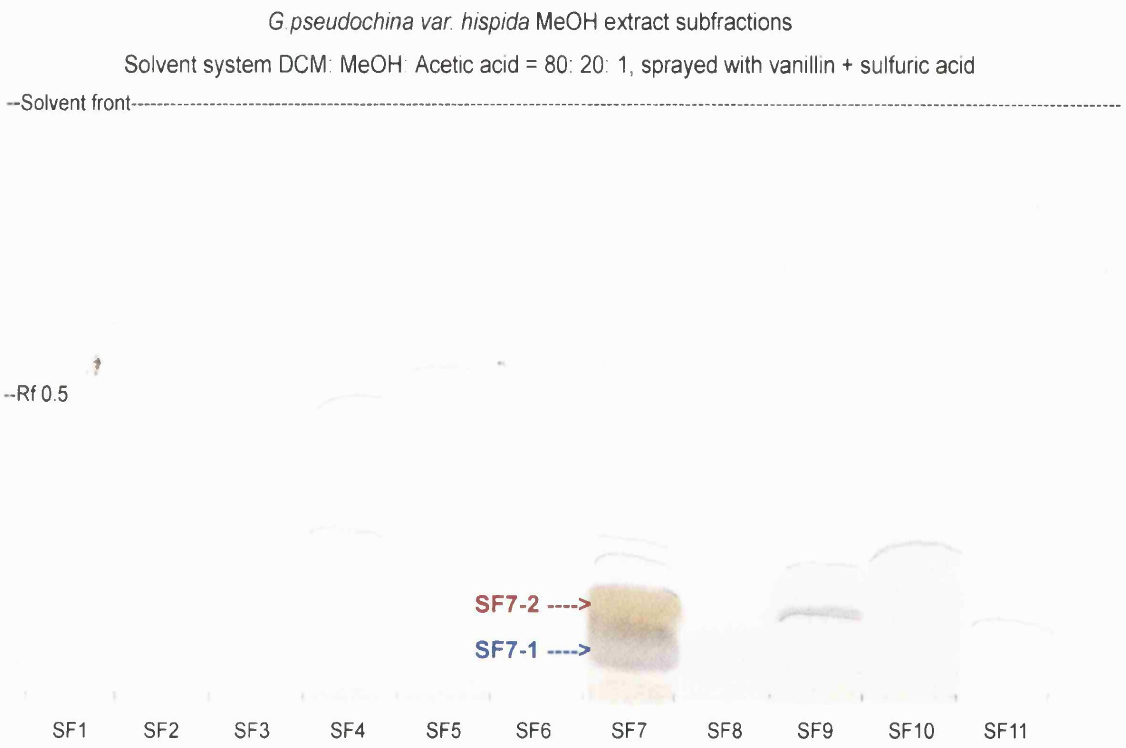
Note that the pencil-circled compounds were the compounds that could be seen only under UV light at 254 nm (as dark spots on green background). The plate was also sprayed with 4% vanillin in sulfuric acid in order to detect some groups of compounds, such as steroids and saponins (Fried & Sherma 1996).



As mentioned above, F24-34 and F38 were endowed with the most potent NF-κB inhibitory effects. Therefore, they were observed for the presence of compounds. In this TLC plate, the active F24-34 and F38 fractions contain 2-3 main compounds which can be easily detected. The neighboring fractions: F21-23 and F35-37 also contain similar compounds. F24-34 was then sub-fractionated again using solid-phase extraction with gradient solvents elution between methanol and water. Then they were defatted by liquid-liquid partitioning.

Fingerprints of sub-fractions of F24-34 are shown in the photo below. Note that the solvent system and TLC conditions were the same as used with the previous TLC experiment shown earlier.

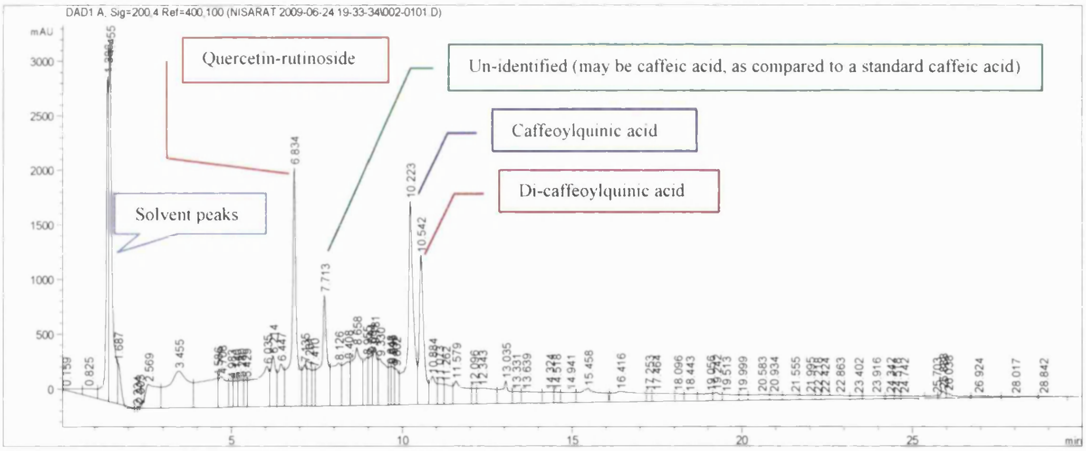
Figure 60 TLC of *G. pseudochina var. hispida* sub-fractions of F24-34



As seen in this photo, sub-fraction SF7 contains 2-3 main compounds which could be detected by 4% vanillin-sulfuric spray detecting reagent. Neighboring sub-fractions contain different compounds with different colors. The compounds SF7-1 and SF7-2 were then separated again on another TLC application under the same conditions. The compounds SF7-1 and SF7-2 were then scraped off, and without further purification, they were submitted for NMR experiments and then tested in the NF-κB assay. It was found that SF7-2 showed the most potent NF-κB inhibitory effects, followed by SF7-1, as shown previously in the fractionation scheme.

HPLC chromatogram of F24-34 showed 4 main compounds including quercetin-rutinoside (a peak at 6.8 min), caffeoylquinic acid (a peak at 10.2 min) and di-caffeoylquinic acid (a peak at 10.5 min). The peak of quercetin-rutinoside was confirmed by comparing with that of a standard rutin (Sigma) in the same conditions. The peaks of caffeoylquinic acid and dicaffeoylquinic acid derivatives were identified by the NMR and MS interpretation which are presented individually on the following pages.

Figure 61 HPLC chromatogram of the fraction F24-34 (5mg/ml in MeOH), injected volume 10 μl. Solvent A: water plus 0.05% acetic acid, B: acetonitrile. Gradient: 99% A -1% A in 30 min.

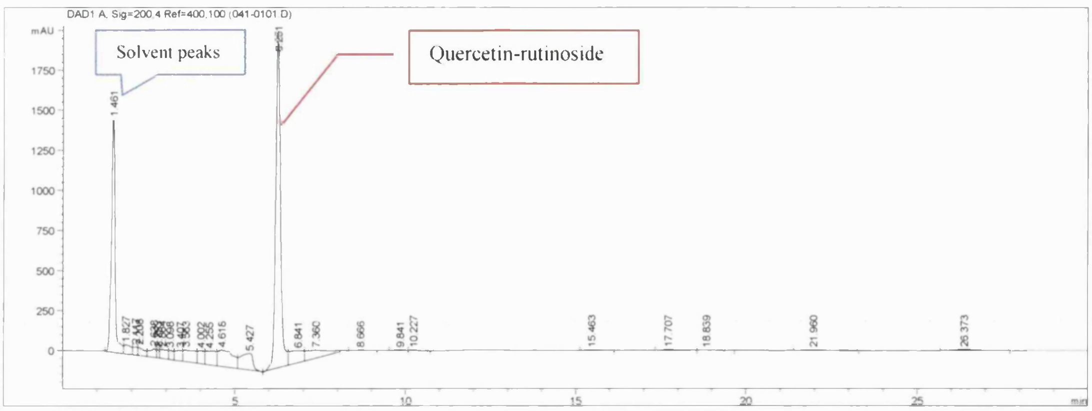


3.6.1 IDENTIFICATION OF COMPOUND SF7-1 AND SF7-2 (QUERCETIN- RUTINOSIDE DERIVATIVES)

Results from the NF-κB assay suggested that compound SF7-2 and SF7-1 were the most active NF-κB inhibitors (IC_{50}= 24.08 and 23.98 μg/ml, respectively). The two compounds were isolated from the sub-fraction SF7 of F24-34 using TLC scraping technique after finishing Sephadex column chromatography with gradient elution between dichloromethane, methanol, and water. The compounds were obtained as a yellow solid, dissolved well in methanol plus water, and was visible on the TLC plate with an Rf value about 0.20- 0.25 in the solvent system of dichloromethane: methanol: acetic acid = 80:20:1.

Compound SF7-2 was checked for its purity using HPLC. The HPLC chromatogram below shows the presence of quercetin-rutinoside (retention time 6.3 min) as compared to a standard rutin (Sigma). The chemical structure of this compound was identified by 1D and 2D NMR as well as ESI-MS experiments.

Figure 62 HPLC chromatogram of isolated quercetin-rutinoside (5mg/ml in MeOH), injected volume =10 μl. Solvent A: water plus 0.05% acetic acid, B: acetonitrile. Gradient: 99%A -1%A in 30 min.



1H NMR spectrum of the compound SF7-2 after integrations of existing peaks suggested that this compound has five aromatic signals between 6 to 8 ppm including signals at δ 6.88 (J = 8.4 Hz), δ 7.63 (J = 2.1, 8.4 Hz) and δ 7.67 (J = 2.1 Hz) each integrating for one proton, having an identical environment, showing the same coupling constants, thus indicating an ABX system. Also an adjacent pair of meta-coupled doublets at δ 6.41 (J = 2.0 Hz) and 6.22 (J = 2.0 Hz) each integrating for one proton, indicating a 2H AX system, in accordance with a flavone backbone with 3, 5, 7, 3'and 4'- substituted group (see H1 NMR spectra page 159).

The 13C NMR spectrum of this compound in comparison with DEPT spectra showed 27 carbon signals in total, including one signal at δ 17.85 (peak up in DEPT-135) representing one CH_3 group, one signal at δ 68.56 (peak down in DEPT-135) representing one CH_2 group, 14 tertiary carbons (-CH groups) as well as 13 quaternary carbons which were absent in both DEPT experiments. A signal at δ 179.44 indicates the presence of either a ketone or aldehyde group. Also 13 signals ranging between δ 100- 170 indicate the presence of carbons in an aromatic ring.

The 13C NMR spectrum also showed signals at δ 166.02, 162.96, 149.79, 145.84, and 135.63 which were assignable to be in an aromatic ring connected with hydroxyl groups (-OH). This was confirmed by long-length C-H correlations in HMBC spectra (see HMBC spectra in Appendix). A correlation of C-4' (δ 149.79) and H-5'(δ 6.88 d) and H-6' (δ 7.63 dd); a correlation of C-3' (δ 145.84) and H-2' (δ 7.67 d) and H-5' (δ 6.88 d); a correlation of C-5 (δ 162.96) and H-6 (δ 6.22 doublet), as well as a correlation of C-7 (166.02) and H-8 (δ 6.41 d) in the HMBC spectra are particularly useful for assignment of the quaternary carbons in the aglycone.

All the sugar proton resonances were assigned by the COSY experiment, using the anomeric protons and the sugar H-6 protons as entry points. 1H NMR spectra also shows a doublet signal at δ 1.12 (J = 6.2 Hz) integrating for three protons was in accord with rhamnose H-6" protons. HMQC spectra confirm that the signal at δ 1.12 correlates with the 13C signal at δ 17.85. 1H NMR signals between δ 3.00- 4.00 indicate the presence of single-bond hydrocarbons of sugar moieties. Those signals showed as mixed signals which are difficult to assign for each position in the sugar moieties. The 13C NMR spectra also indicated the presence of two sugar moieties.

However, based on 13C and 1H NMR shift values and coupling constants, the sugar unit was identified as rhamnosyl. The HMBC spectrum indicates a connection between C-3 position of aglycone and H-1' proton of rhamnosyl. The HMBC correlation of the rhamnosyl H-1''' to the glucosyl C-6'' also supported an existence and a connection of the rutinose moiety. The complete chemical structure of this compound and their correlations are shown in the structure below (all the NMR spectra can be seen in the appendix).

1H and 13C NMR spectra of SF7-1 were identical with those of the SF7-2 but had extra proton signals at δ 1.20 (d, J = 8.9) which integrated for 3 protons, as well as three extra carbons at δ 38.28, δ 38.07 and δ 29.24. A crosspeak in HMQC showed a connection between δ 1.20 and δ 29.24 indicates a fat impurity in the spectra (this is well known among phytochemists but not in today's literature).

However, from the TLC photos of F24-34 (first TLC photo on page 153), SF7-1 cannot be seen on the TLC plate, while it showed up as a gray spot in the second TLC photo (of sub-fractions SF7, on page 154). The possible reasons to explain this is that (1) the compound SF7-2 might decompose or transform during chromatography, or (2) oxidation reaction might occur if preparative silica TLC is used for fractionation. The possibility that active compounds might loss or decompose or transform to less active during fractionation process has recently been published (Houghton et al 2007).

The molecular formula $C_{27}H_{30}O_{16}$ and molecular weight 610 of the compound SF7-2 as well as SF7-1 were confirmed by their ESI-MS data (see Appendix for the ESI-MS spectra).

Figure 63 Chemical structure of quercetin-rutinoside (SF7-2) with their chemical shifts, correlations and 1H spectra. Note that full-lined arrows mean COSY correlations and dashed arrows mean HMBC correlations.

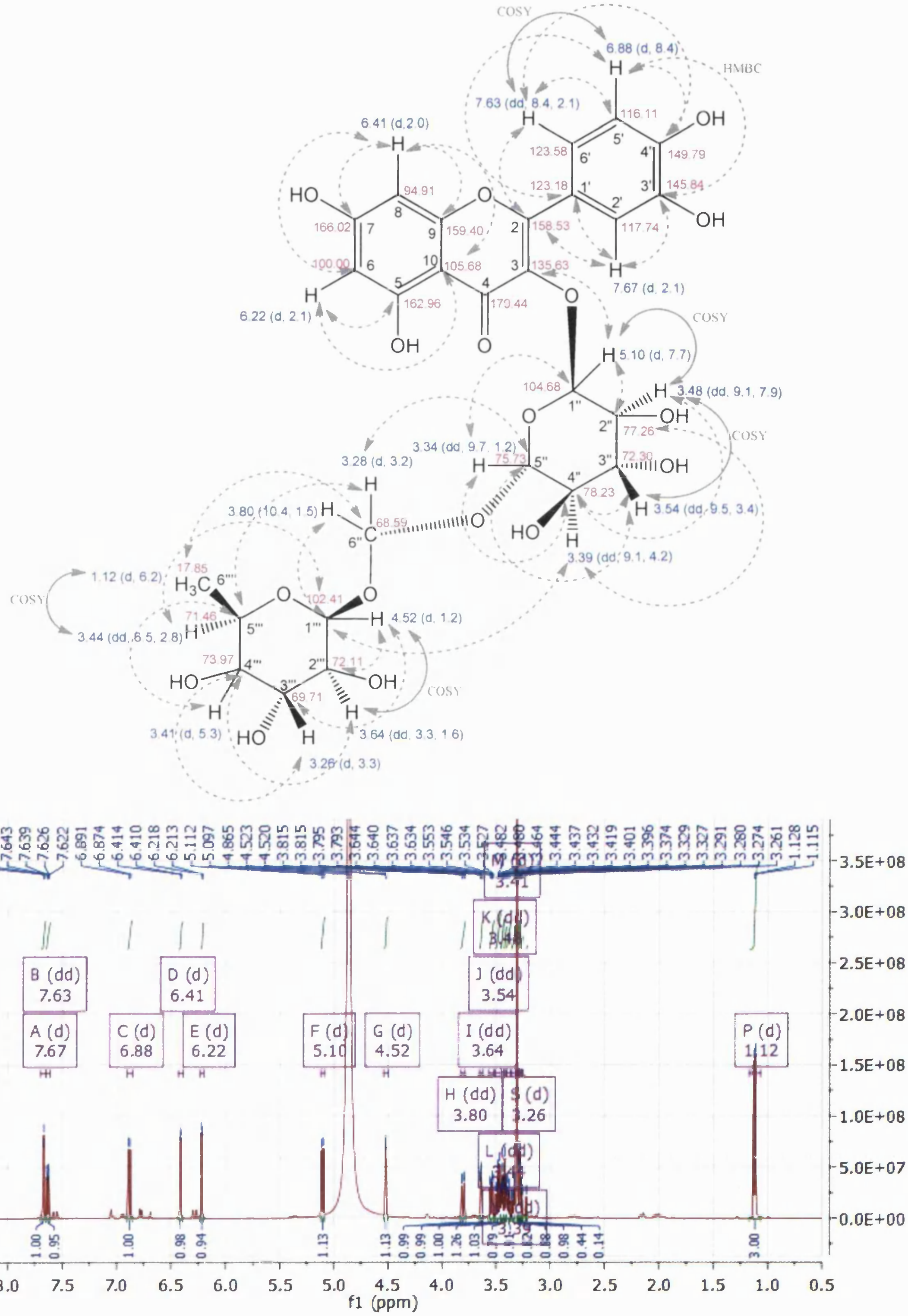


Table 10 Summary of NMR spectral data of compound SF7-2

Position	13C	DEPT	1H	HMBC	COSY	NOESY
2	158.53	C				
3	135.63	C				
4	179.44	C				
5	162.96	C				
6	100.00	CH	6.22 (d, 2.1, 1H)	105.68, 162.96	6.10	6.10
7	166.02	C				
8	94.91	CH	6.41 (d, 2.0, 1H)	100.00, 105.68, 159.40, 166.02	6.28	6.28
9	159.40	C				
10	105.68	C				
1'	123.18	C				
2'	117.74	CH	7.67 (d, 2.1, 1H)	123.18, 145.84, 158.53		
3'	145.84	C				
4'	149.79	C				
5'	116.11	CH	6.88 (d, 8.4, 1H)	123.18, 145.84, 149.79	7.63	7.63
6'	123.58	CH	7.63 (dd, 8.4, 2.1, 1H)	116.11, 149.79, 158.53	6.88	6.88
1''	104.68	CH	5.10 (d, 7.7, 1H)	77.26, 135.63	3.48	3.48
2''	77.26	CH	3.48 (dd, 9.1, 7.9, 1H)	78.23	3.54	3.54
3''	72.30	CH	3.54 (dd, 9.5, 3.4, 1H)	75.73, 78.23	3.48	3.47
4''	78.23	CH	3.39 (dd, 9.1, 4.2, 1H)	77.26, 102.41		
5''	75.73	CH	3.34 (dd, 9.7, 1.2, 1H)	78.23, 104.68		
6''	68.59	CH2	3.80 (dd, 10.4, 1.5, 1H) 3.28 (d, 3.2, 1H)	71.46, 75.73, 102.41		
1'''	102.41	CH	4.52 (d, 1.2, 1H)	69.71, 72.11	3.64	3.64
2'''	72.11	CH	3.64 (dd, 3.3, 1.6, 1H)	73.97	4.52	4.52
3'''	69.71	CH	3.26 (d, 3.3, 1H)	73.97		
4'''	73.97	CH	3.41 (d, 5.3, 1H)	71.46		
5'''	71.46	CH	3.44 (dd, 6.5, 2.8, 1H)	102.41	1.12	1.12
6'''	17.85	CH3	1.12 (d, 6.2, 3H)	68.59	3.44	3.44

Figure 64 Chemical structure of quercetin-rutinoside (SF7-1) with their chemical shifts and the 2D NMR correlations. Note that full-lined arrows mean COSY correlations and dashed arrows mean HMBC correlations. NOESY is in orange.

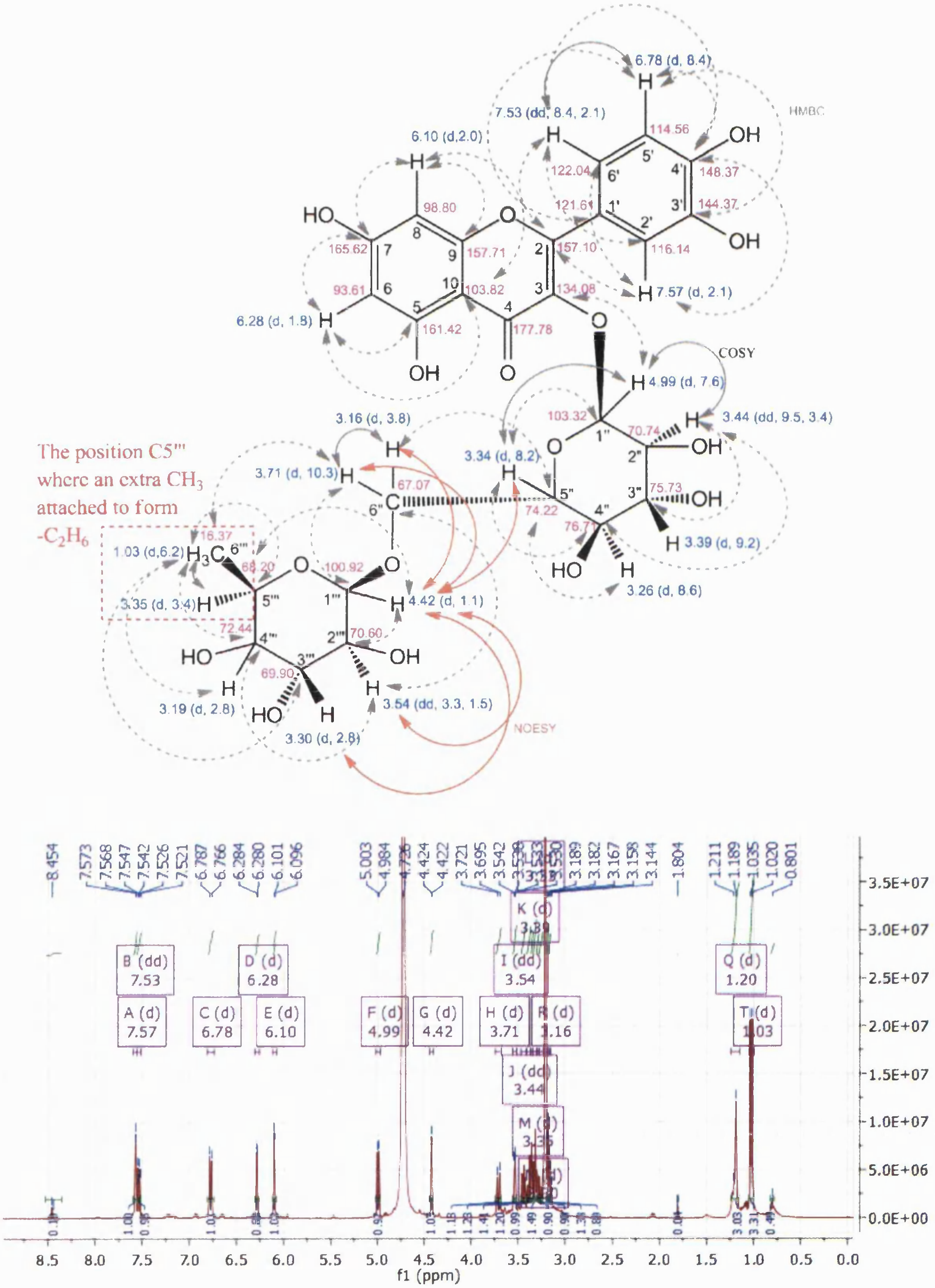
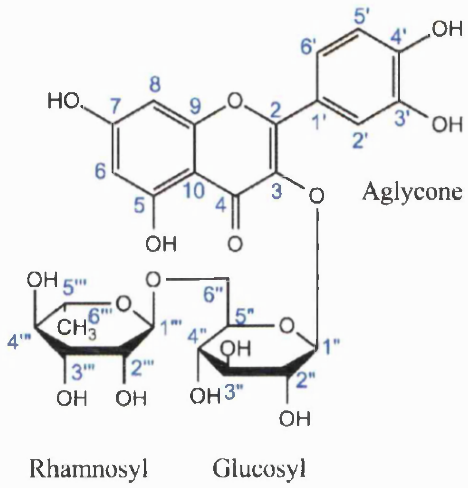


Table 11 Summary of NMR spectral data of compound SF7-1

Position	13C	DEPT	1H	HMBC	COSY	NOESY
2	157.71	C				
3	134.08	C				
4	177.78	C				
5	161.42	C				
6	93.61	CH	6.28 (d, 1.8, 1H)	103.82, 161.42, 165.62	6.10	6.10
7	165.62	C				
8	98.80	CH	6.10 (d, 2.0, 1H)	103.82, 157.10, 165.62	6.28	6.28
9	157.10	C				
10	103.82	C				
1'	121.61	C				
2'	116.14	CH	7.57 (d, 2.1, 1H)	122.04, 148.37, 157.71		
3'	144.37	C				
4'	148.37	C				
5'	114.56	CH	6.78 (d, 8.4, 1H)	121.61, 144.37, 148.37	7.53	3.21
6'	122.04	CH	7.53 (dd, 8.4, 2.1, 1H)	116.14, 148.37, 157.71	6.78	6.78
1''	103.32	CH	4.99 (d, 7.6, 1H)	134.05	3.44	3.44, 3.34, 3.26 or 3.35, 3.39
2''	70.74	CH	3.44 (dd, 9.5, 3.4, 1H)	76.71, 75.73	4.42, 3.17, 3.54	3.17
3''	75.73	CH	3.39 (d, 9.2, 1H)	72.44	1.03?	3.17, 4.99? , 1.03
4''	76.71	CH	3.26 (d, 8.6, 1H)	74.22	3.71?	4.42
5''	74.22	CH	3.34 (d, 8.2, 1H)	76.71, 103.32, 16.37	4.99	4.99, 3.17?
6''	67.07	CH2	3.71 (d, 10.3, 1H) 3.16 (d, 3.8,1H)	70.60 (or, 69.90), 100.92, 74.22, 16.37	3.29? 3.71? 3.17	3.26, 3.30 or 3.33 or 3.35
1'''	100.92	CH	4.42 (d, 1.1, 1H)	68.20, 70.60	3.54, 3.44	3.26 or 3.30 or 3.33 or 3.35, 3.54, 3.71
2'''	70.60	CH	3.54 (dd, 3.3, 1.5, 1H)	74.22, 72.44, 67.07, (76.71)	3.44, 1.13, 3.35, 4.42	4.42
3'''	69.90	CH	3.30 (d, 2.8, 1H)	75.73, 100.92	3.71, 4.99	3.71
4'''	72.44	CH	3.19 (d, 2.8, 1H)	69.90, 70.74 , 74.22 or 75.73, 76.71	3.33, 3.44	3.34, 1.03, 3.44
5'''	68.20	CH	3.35 (d, 3.4, 1H)	70.60,(72.44),	1.03, 3.54	1.03
	38.28	C				
	38.07	C				
6'''a?	29.24	CH2	1.20 (d, 8.9, 3H)	68.20	3.54	3.35, 3.17, 1.03
6'''b	16.37	CH3	1.03 (d, 6.2, 3H)	68.20, 72.44, 69.90	3.35	1.20, 3.34

Table 12 1H NMR of compound SF7-2 and SF7-1 in comparison with the literature



Positions	Kazuma *et al.* (2003) in MeOD$_4$, NMR 400 MHz (ppm)	SF7-2 in MeOD$_4$, NMR 500 MHz (ppm)	SF7-1 in MeOD$_4$, NMR 400 MHz (ppm)
Aglycone			
H-6	6.21 (d, 2.0)	6.22 (d, 2.1)	6.28 (d, 1.8)
H-8	6.40 (d, 2.0)	6.41 (d, 2.0)	6.10 (d, 2.0)
H-2’	7.66 (d, 2.1)	7.67 (d, 2.1)	7.57 (d, 2.1)
H-3’	-	-	-
H-5’	6.87 (d, 8.5)	6.88 (d, 8.4)	6.78 (d, 8.4)
H-6’	7.62 (dd, 2.1, 8.5)	7.63 (dd, 2.1, 8.4)	7.53 (dd, 2.1, 8.4)
3-Glucosyl			
H-1’’	5.10 (d, 7.7)	5.10 (d, 7.7)	4.99 (d, 7.6)
H-2’’	3.46 (dd, 7.7, 8.9)	3.48 (dd, 7.9, 9.1)	3.44 (dd, 3.4, 9.5)
H-3’’	3.40 (t, 8.9)	3.54 (dd, 3.4, 9.5)	3.39 (d, 9.2)
H-4’’	3.26 (t, 8.9)	3.39 (dd, 4.2, 9.1)	3.26 (d, 8.6)
H-5’’	3.32 (ddd, 1.2, 6.1, 8.9)	3.34 (dd, 1.2, 9.7)	3.34 (d, 8.2)
H-6a’’	3.80 (dd, 1.2, 11.0)	3.80 (dd, 1.5, 10.4)	3.71 (d,10.3)
H-6b’’	3.38 (dd, 6.1, 11.0)	3.28 (d, 3.2)	3.16 (d, 3.8)
6’’-Rhamnosyl			
H-1’’’	4.51 (d, 1.5)	4.52 (d, 1.2)	4.42 (d, 1.1)
H-2’’’	3.62 (dd, 1.5, 3.4)	3.64 (dd, 1.6, 3.3)	3.54 (dd, 1.5, 3.3)
H-3’’’	3.53 (dd, 3.4, 9.6)	3.26 (d, 3.3)	3.30 (d, 2.8)
H-4’’’	3.27 (t, 9.6)	3.41 (d, 5.3)	3.19 (d, 2.9)
H-5’’’	3.44 (dq, 6.2, 9.6)	3.44 (dd, 2.8, 6.5)	3.35 (d, 3.4)
H-6’’’ (CH_3)	1.11 (d, 6.2)	1.12 (d, 6.2)	1.03 (d, 6.2)
Un-identified	-	-	1.20 (d, 8.9)

Table 13 13C NMR of compound SF7-2 and SF7-1 compared with the literature

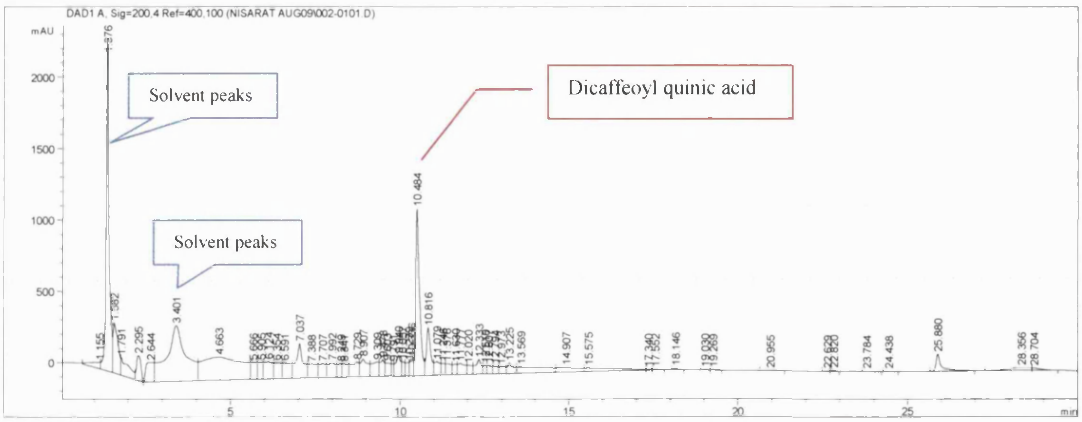
Positions	Kazuma *et al*, (2003), in $MeOD_4$, NMR 100 MHz (ppm)	SF7-2 in $MeOD_4$, NMR 126 MHz (ppm)	SF7-1 in $MeOD_4$, NMR 100 MHz (ppm)
Aglycone			
C-2	158.52	158.53	157.71
C-3	135.62	135.63	134.08
C-4	179.44	179.44	177.78
C-5	162.98	162.96	161.42
C-6	99.95	100.00	93.61
C-7	166.01	166.02	165.62
C-8	94.87	94.91	98.80
C-9	159.35	159.40	157.10
C-10	105.66	105.68	103.82
C-1'	123.15	123.18	121.61
C-2'	117.69	117.74	116.14
C-3'	145.84	145.84	144.37
C-4'	149.81	149.79	148.37
C-5'	116.06	116.11	114.56
C-6'	123.55	123.58	122.04
3-Glucosyl			
C-1"	104.69	104.68	103.32
C-2"	75.74	77.26	70.74
C-3"	78.20	72.30	75.73
C-4"	71.42	78.23	76.71
C-5"	77.25	77.53	74.22
C-6"	68.56	68.59	67.07
6"-Rhamnosyl			
C-1'''	102.42	102.41	100.92
C-2'''	72.12	72.11	70.60
C-3'''	72.26	69.71	69.90
C-4'''	73.94	73.97	72.44
C-5'''	69.71	71.46	68.20
C-6'''	17.87	17.85	16.37
Un-identified	-	-	29.24
Un-identified	-	-	38.07
Un-identified	-	-	38.20

3.6.2 STRUCTURE DETERMINATION OF COMPOUND SF10 (3, 5- DICAFFEOYL QUINIC ACID)

Compound SF10 showed NF-κB activity with the IC_{50} = 42.82 μg/ml. It was sub-fractionated from the fraction F24-34 which was the most active NF-κB inhibitor compared to other fractions of the methanol extract of *G. pseudochina var. hispida.* After Sephadex column with gradient solvents elution; dichloromethane: methanol: water, this compound was defatted using chloroform and water, liquid-liquid partitioning. It was visible on the TLC as a pink band after spraying with spray detecting reagent (4% vanillin in sulfuric acid), with an Rf value 0.30, in the solvent system of dichloromethane: methanol: acetic acid, 80:20:1.

This isolated compound dissolved well in water and it was checked for purity using HPLC. The HPLC chromatogram below showed the presence of a pure compound 3, 5- dicaffeoyl quinic acid (a peak at retention time 10.4 min). This peak was further identified using NMR and ESI-MS interpretations which will be presented on the following pages.

Figure 65 HPLC of an isolated dicaffeoyl quinic acid derivative (5mg/ml in MeOH), injected volume =10 μl. Solvent A: water plus 0.05% acetic acid, B: acetonitrile. Gradient: 99% A - 1% A in 30 min.



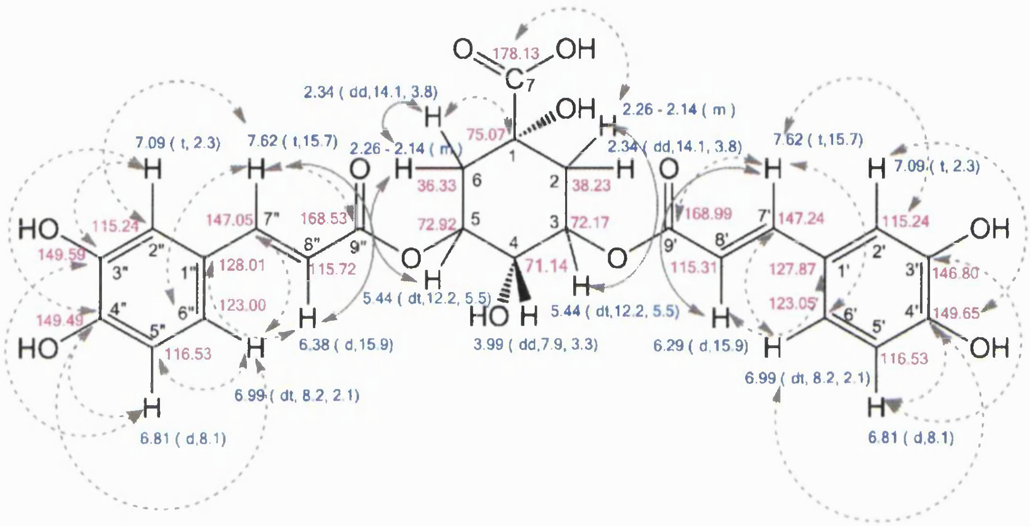
The 1H NMR spectrum of this compound showed a pair tri-substituted and an aromatic moiety, which was indicated by two pairs of three ABX protons at δ 7.09 (t, *J* = 2.3, 2H), δ 6.99 (dt, *J* = 8.2, 2.1, 2H) and δ 6.81 (d, *J* = 8.1, 2H) each one integrating for 2 protons, thus indicating two ABX systems. The large coupling constant between the caffeoyl group double bond at δ 7.62 (t, *J* = 15.7, 2H), δ 6.38 (d, *J* = 15.9, 1H) and δ 6.29 (d, *J* =15.9, 1H) indicates the trans-configuration of the pairs of double bond which are characteristics of caffeic acid moieties (see 1H NMR spectra on page 167). The relative number of caffeoyl ester groups is evident from the number of characteristic ester carbonyl carbon resonances observed in the 13C.

The 13C NMR spectrum of this compound contained one carbon resonance for the free carboxylic acid (at 178.13 ppm for C-7) as well as two carbon signal for the ester carbonyl (168.99 ppm for C-9' and 168.53 ppm for C-9") where di-substituted nature of the two dicaffeoylquinic acid were evident (see 13C NMR spectra in Appendix). The substitution patterns of the caffeoyl ester moieties were identified based on the characteristic downfield chemical shifts (1 ppm or greater) (Arbiser *et al.* 2005) of the proton signals at the C-3, C-4, C-5 positions of the quinic acid, in the 1H NMR spectrum, compared to that of quinic acid (Pauli *et al.* 1998).

The protons at C-3 and C-5 position of this compound showed identical signal at 5.44 ppm (dt, J = 12.2, 5.5 Hz) while the C-4 position contained proton signal at 3.99 (dd, J = 7.9, 3.9 Hz). This indicates that the two caffeoyl groups were linked to the C-3 and C-5 position of quinic acid moiety. This compound was identified as 3, 5-dicaffeoylquinic acid, confirmed by comparison with the NMR spectral data reported in the literature (Pauli *et al.* 1998).

Figure 66 Chemical structure of 3, 5- dicaffeoylquinic acid and the chemical shifts.

Note that full-lined arrows mean COSY correlations and dashed arrows mean HMBC correlations.



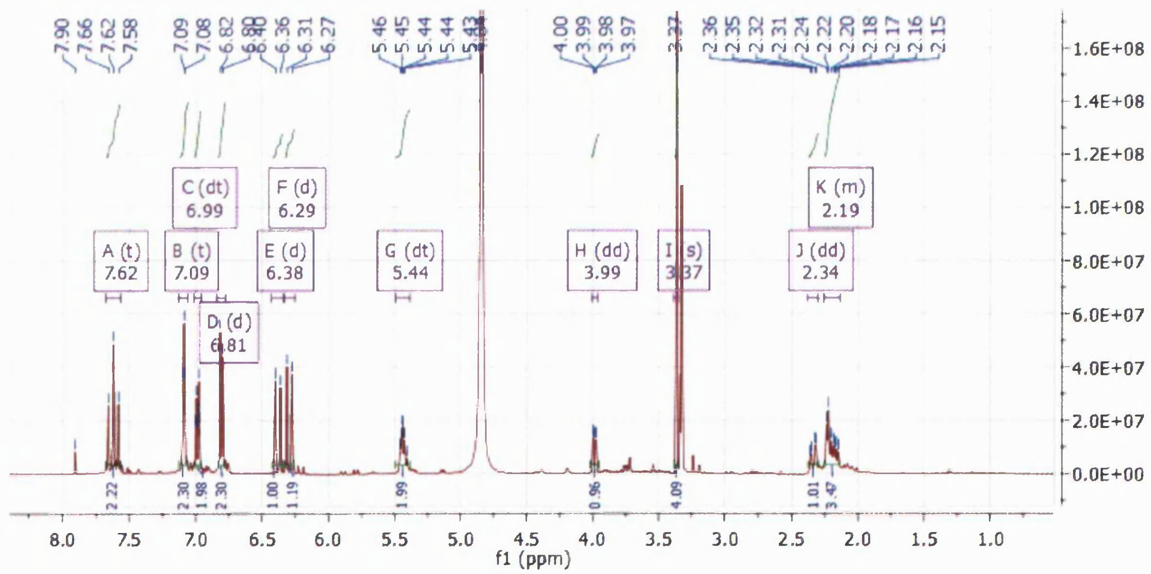
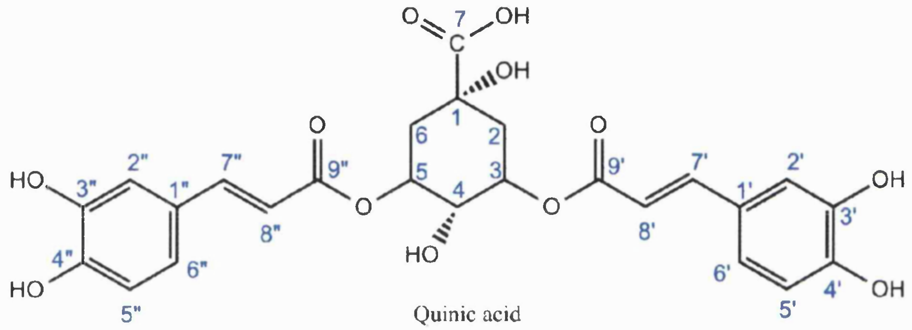


Table 14 Summary of NMR spectral data of compound SF10

Position	13C	DEPT	1H	HMBC	COSY	NOESY
1	75.07	C				
2	38.23	CH2	2.26 – 2.14 (m, 2H) 2.34 (dd, 14.1, 3.8, 2H)	178.13	5.44	
3	72.17	CH	5.44 (dt, 12.2, 5.5, 1H)	38.23 or 36.33,	2.22, 3.99	
4	71.14	CH	3.99 (dd, 7.9, 3.3, 1H)			
5	72.92	CH	5.44 (dt, 12.2, 5.5, 1H)	38.23 or 36.33	2.22, 3.99	
6	36.33	CH2	2.26 – 2.14 (m, 2H) 2.34 (dd, 14.1, 3.8, 2H)	75.07	2.26	
7	178.13	C				
1'	127.87	C				
2'	115.24	CH	7.09 (t, 2.3, 1H)	149.65, 147.24, 6.99 123.00 or 123.05		
3'	146.80	C				
4'	149.65	C				
5'	116.53	CH	6.81 (d, 8.1, 2H)	123.00 or 123.05	6.99	
6'	123.05	CH	6.99 (dt, 8.2, 2.1, 2H)	149.65, 147.24 or 147.05	6.81	
7'	147.24	CH	7.62 (t, 15.7, 2H)	123.05 or 123.00	6.29	
8'	115.31	CH, CH3	6.29 (d, 15.9, 1H)	147.24 or 147.05	7.62	
9'	168.99	C				
1''	128.01	C				
2''	115.24	CH	7.09 (t, 2.3, 2H)	149.59, 147.05 or 147.24, 123.00 or 123.05		
3''	149.59	C				
4''	149.49	C				
5''	116.53	CH	6.81 (d, 8.1, 2H)	123.00 or 123.05		
6''	123.00	CH	6.99 (dt, 8.2, 2.1, 2H)	149.49, 147.24 or 147.05, 116.53	6.81	
7''	147.05	CH	7.62 (t, 15.7, 2H)	123.05 or 123.00, 168.53		
8''	115.72	CH	6.38 (d, 15.9, 1H)			
9''	168.53	C				

Table 15 1H NMR of compound SF10 in comparison with the reported data



	Arbiser *et al.* (2005) in $MeOD_4$, NMR 400 MHz	Compound SF10 $MeOD_4$,NMR 400 MHz
Quinic acid		
H-2a	2.15-2.34 (m)	2.14-2.26 (m)
H-2b	2.15-2.34 (m)	2.34 (dd, 3.8, 14.1)
H-3	5.44 (m)	5.44 (dt, 5.5, 12.2)
H-4	3.99 (dd, 3.1, 7.4)	3.99 (dd, 3.3, 7.9)
H-5	5.40 (br.d)	5.44 (dt, 5.0, 12.2)
H-6a	2.15-2.34 (m)	2.14-2.26 (m)
H-6b	2.15-2.34 (m)	2.34 (dd, 3.8, 14.1)
3-Caffeyol		
H-2'	7.07 (br.s)	7.09 (t, 2.3)
H-5'	6.79 (d, 8.0)	6.81 (d, 8.1)
H-6'	6.97 (m)	6.99 (dt, 2.1, 8.2)
H-7'	7.62 (d, 16.0) or 7.58 (d, 16.0)	7.62 (t, 15.7)
H-8'	6.35 (d, 16.0)	6.29 (d, 15.9)
5-Caffeyol		
H-2"	7.07 (br.s)	7.09 (t, 2.3)
H-5"	6.79 (d, 8.0)	6.81 (d, 8.1)
H-6"	6.97 (m)	6.99 (dt, 2.1, 8.2)
H-7"	7.62 (d, 16.0) or 7.58 (d, 16.0)	7.62 (t, 15.7)
H-8"	6.35 (d, 16.0)	6.38 (d, 15.9)

Table 16 1H NMR of compound SF10 in comparison with the literature

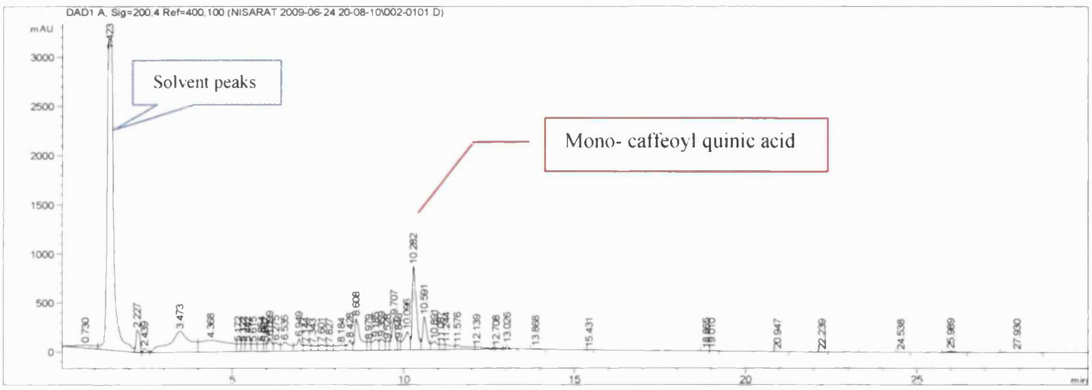
	Arbiser et al (2005) in $MeOD_4$, NMR 400 MHz	Compound SF10 in $MeOD_4$, NMR 400 MHz
Quinic acid		
C-1	74.8	75.07
C-2	37.7	38.23
C-3	72.2	72.17
C-4	70.7	71.14
C-5	72.6	72.92
C-6	36.1	36.33
C-7	177.5	178.13
3-Caffeyol		
C-1'	128.0	127.87
C-2'	115.5 or 115.7	115.24
C-3'	146.9	146.80
C-4'	149.7	149.65
C-5'	116.6	116.53
C-6'	123.1 or 123.2	123.05
C-7'	147.2 or 147.4	147.24
C-8'	115.2 or 115.4	115.31
C-9'	168.5 or 168.3	168.99
5-Caffeyol		
C-1''	128.0	128.01
C-2''	115.5 or 115.7	115.24
C-3''	146.9	149.59
C-4''	149.7	149.49
C-5''	116.6	116.53
C-6''	123.1 or 123.2	123.00
C-7''	147.2 or 147.4	147.05
C-8''	115.2 or 115.4	115.72
C-9''	168.5 or 168.3	168.53

3.6.4 STRUCTURE DETERMINATION OF COMPOUND SF11 (5- CAFFEOYLQUINIC ACID OR CHLOROGENIC ACID)

Compound SF11 was endowed with an intermediate NF-κB activity with the IC_{50} 83.01 μg/ml. It was isolated using Sephadex column chromatography with gradient solvents elution; dichloromethane: methanol: water. Then this compound was observed on the TLC plate using the solvent system of dichloromethane: methanol: acetic acid, 80:20:1. The compound was visible on the plate as a pale pink band after spraying with 4% vanillin in sulfuric acic, and with an Rf value about 0.3. This compound was obtained as a pink solid that dissolved well in water.

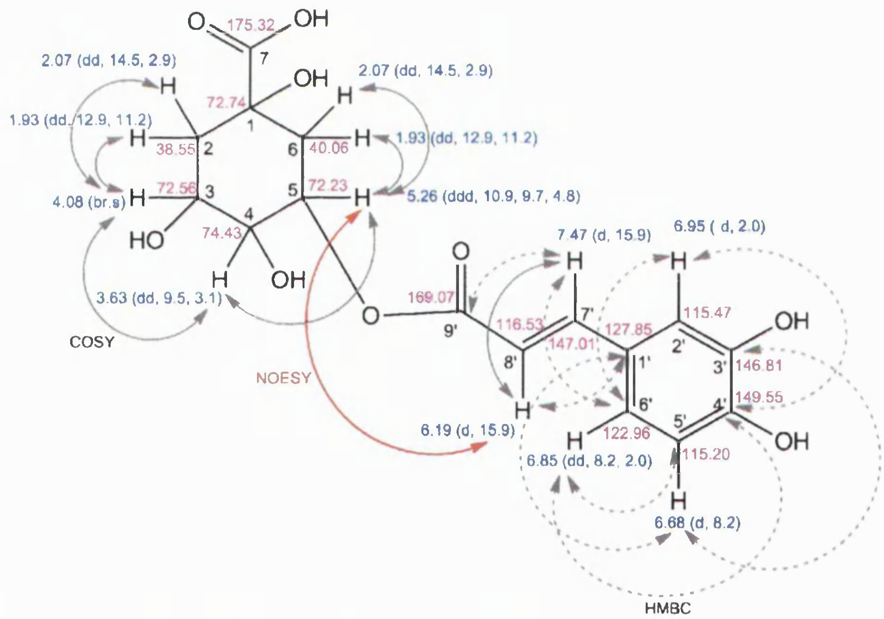
The compound was further investigated using analytical HPLC and further purified using preparative HPLC in the same conditions used to analyze the compounds as shown below. The presence of a peak at a retention time 10.282 min was identified as a caffeoylquinic acid derivative by the 1H NMR 13C and two dimension NMR spectral interpretations. ESI-MS spectrum of this compound confirmed the structure. Details of the structure elucidation by NMR and ESI-MS will be presented on the following pages.

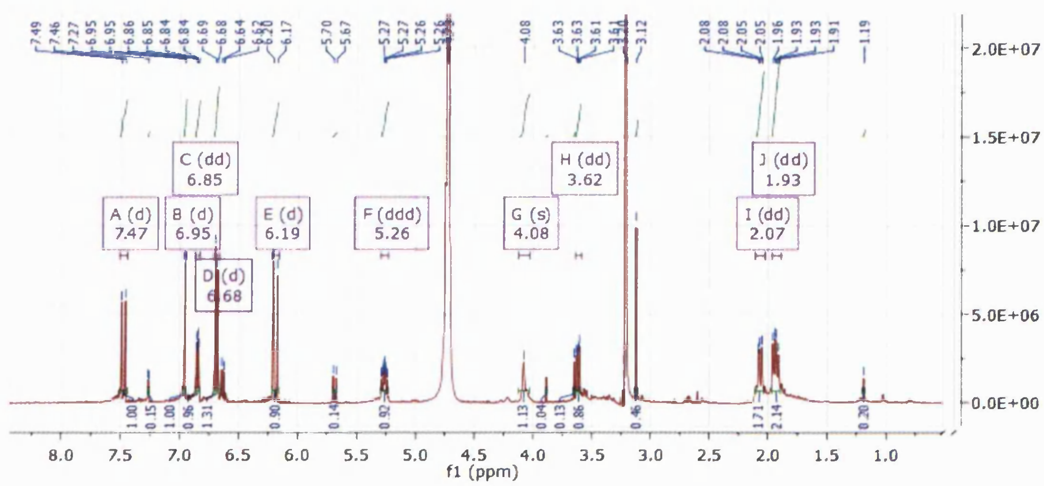
Figure 67 HPLC of an isolated caffeoyl quinic acid derivative (1mg/ml in MeOH), injected volume =10 μl. Solvent A: water plus 0.05% acetic acid, B: acetonitrile. Gradient: 99% A - 1% A in 30 min.



The 1H NMR spectrum of this compound showed three aromatic proton signals at chemical shifts 6.68 (d, *J*=8.2, 1H), 6.85 (dd, *J*=8.2, 2.0, 1H) and 6.95 (d, *J*=2.0, 1H) which indicates an ABX system of an aromatic ring. The large coupling constant at the chemical shifts 7.47 (d, *J*=15.9, 1H) and 6.19 (d, *J*=15.9, 1H) indicates the pair of protons trans to each other and this is a characteristic of a caffeoyl group.

The 13C NMR spectrum this compound contained one carbon resonance for the free carboxylic acid at 175.32 ppm for C-7 and one carbon signal for the single ester carbonyl 169.07 ppm for C-9'. The substitution pattern of the caffeoyl ester moieties was identified based on the characteristic downfield chemical shift (1 ppm or greater) in the 1H NMR spectra compared to quinic acid without any substitutions (Arbiser et al, 2005). The proton signals and their coupling patterns at C-3, C-4, C-5 of this compound indicated that the caffeoyl group was linked at position 5.





The ESI-MS spectra fingerprint of this compound presented the ions of m/z 355.11 [caffeoylquinic acid + H], m/z 339.14 [caffeoylquinic acid - OH] and m/z 181 [caffeic acid + H] which are the unique fragments of 5-caffeoylquinic acid as previously reported (Moco *et al.* 2006)

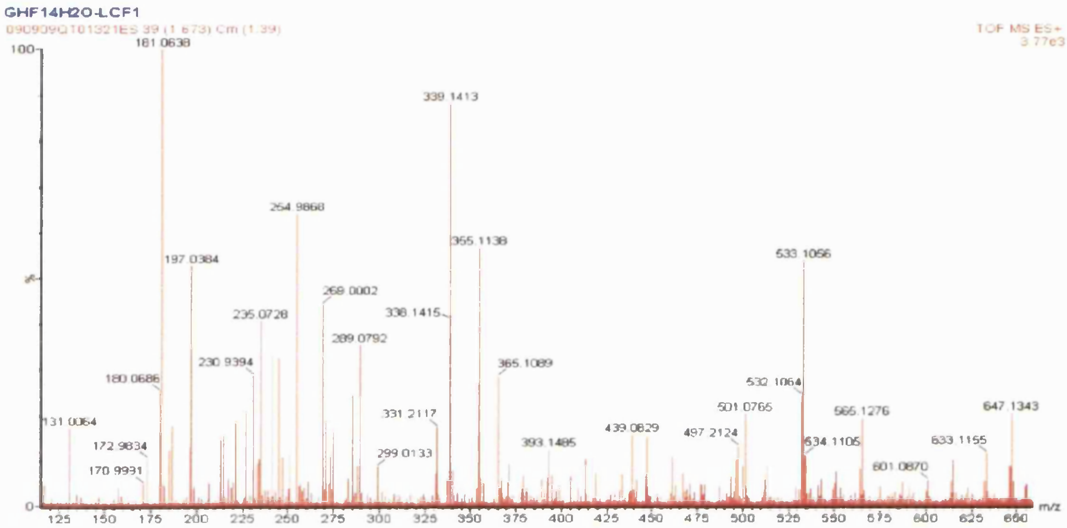


Table 17 Summary of NMR spectral data of compound SF11

Position	13C	DEPT	1H	HMBC	COSY	NOESY
1	72.74	C				
2	38.55	CH2	2.07 (dd, 14.4, 3.0, 2H) 1.93 (dd, 12.9, 11.2, 2H)		1.93, 2.07, 4.08	
3	72.56	CH	4.08 (br.s)		1.93, 2.07, 3.63	
4	74.43	CH	3.62 (dd, 9.5, 3.1, 1H)		4.08, 5.26	1.93
5	72.23	CH	5.26 (ddd, 11.0, 9.6, 4.8, 1H)		1.93, 2.07, 3.63	1.93, 2.07, 6.19
6	40.06	CH2	2.07 (dd, 14.4, 3.0, 2H) 1.93 (dd, 12.9, 11.2, 2H)		1.93, 2.07, 5.26	
7	175.32	C				
1'	127.85	C				
2'	115.47	CH	6.95 (d, 2.0, 1H)	122.96, 149.55		
3'	146.81	C				
4'	149.55	C				
5'	115.20	CH	6.68 (d, 8.2, 1H)	127.85, 146.81		
6'	122.96	CH	6.85 (dd, 8.2, 2.0, 1H)	115.20, 149.55, 147.01	6.68	
7'	147.01	CH	7.47 (d, 15.9, 1H)	116.53, 122.96, 169.07	6.19	6.19
8'	116.53	CH	6.19 (d, 15.9, 1H)		7.47	5.26
9'	169.07	C				

Table 18 1H of compound SF11 in comparison with the literature

	Arbiser *et al.* (2005) in CD_3OD, NMR 400 MHz	Compound SF11 in CD_3OD,NMR 500 MHz
Quinic acid		
H-2a	1.97 (dd, 10.4, 13.2)	1.93 (dd, 11.2, 12.9)
H-2b	2.17 (m)	2.07 (dd, 3.0, 14.4)
H-3	4.18 (m)	4.08 (br.s)
H-4	3.66 (dd, 3.2, 8.6)	3.62 (dd, 3.1. 9.5)
H-5	5.37 (br.d, 4.8)	5.26 (ddd, 4.8, 9.6, 11.0)
H-6a	2.17 (m)	1.93 (dd, 11.2, 12.9)
H-6b	2.17 (m)	2.07 (dd, 3.0, 14.4)
3-Caffeyol		
H-2'	7.05 (d, 1.2)	6.95 (d, 2.0)
H-5'	6.78 (d, 8.2)	6.68 (d, 8.2)
H-6'	6.94 (dd, 1.5, 8.2)	6.85 (dd, 2.0, 8.2)
H-7'	7.58 (d, 15.9)	7.47 (d, 15.9)
H-8'	6.31 (d, 15.9)	6.19 (d, 15.9)

Table 19 13C NMR of compound SF11 in comparison with the literature

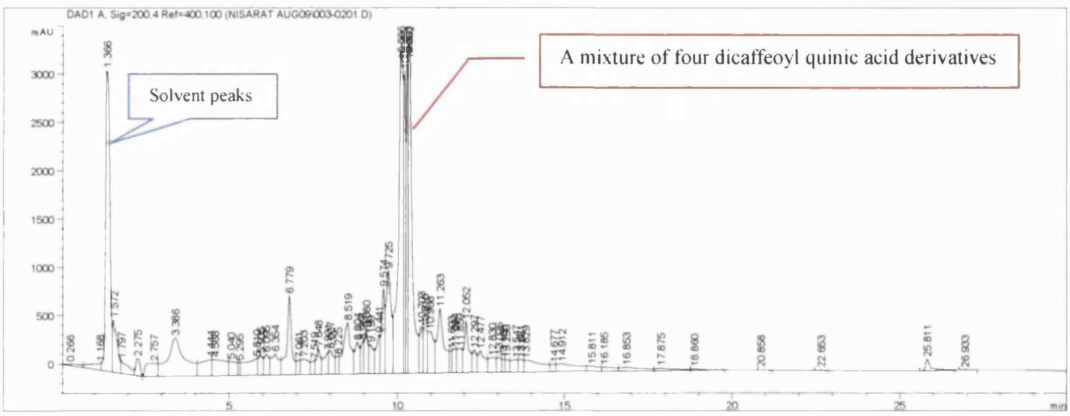
	Arbiser et al (2005) in CD_3OD, NMR 100 MHz	Compound SF10 in CD_3OD, NMR 125 MHz
Quinic acid		
C-1	75.5	72.74
C-2	41.7	38.55
C-3	68.3	72.56
C-4	75.0	74.43
C-5	73.2	72.23
C-6	36.8	40.06
C-7	178.4	175.32
5-Caffeyol		
C-1'	128.1	127.85
C-2'	115.9	115.47
C-3'	146.8	146.81
C-4'	149.5	149.55
C-5'	116.6	115.20
C-6'	123.0	122.96
C-7'	147.0	147.01
C-8'	115.2	116.53
C-9'	169.2	169.07

3.6.5 STRUCTURE DETERMINATION OF COMPOUNDS F38 (4,5- DICAFFEOYLQUINIC ACID DERIVATIVE)

Fraction F38 was fractionated from the methanol extract of *G. pseudochina var. hispida* using Sephadex and gradient solvent elution as shown in the previous diagram. This fraction was a mixture of dicaffeoyl quinic acid derivatives and other minor compounds. The fraction F38 showed the NF-κB inhibitory effect with an IC_{50} of 49.18 μg/ml. As shown in the TLC plate (page 153), this fraction was likely to contain rutin (a yellow band), dicaffeoylquinic acid derivatives (a dark pink band) and caffeoylquinic acid derivatives (a pale pink band) which can be seen easily, in the solvent system of dichloromethane: methanol: acetic acid, 80:20:1.

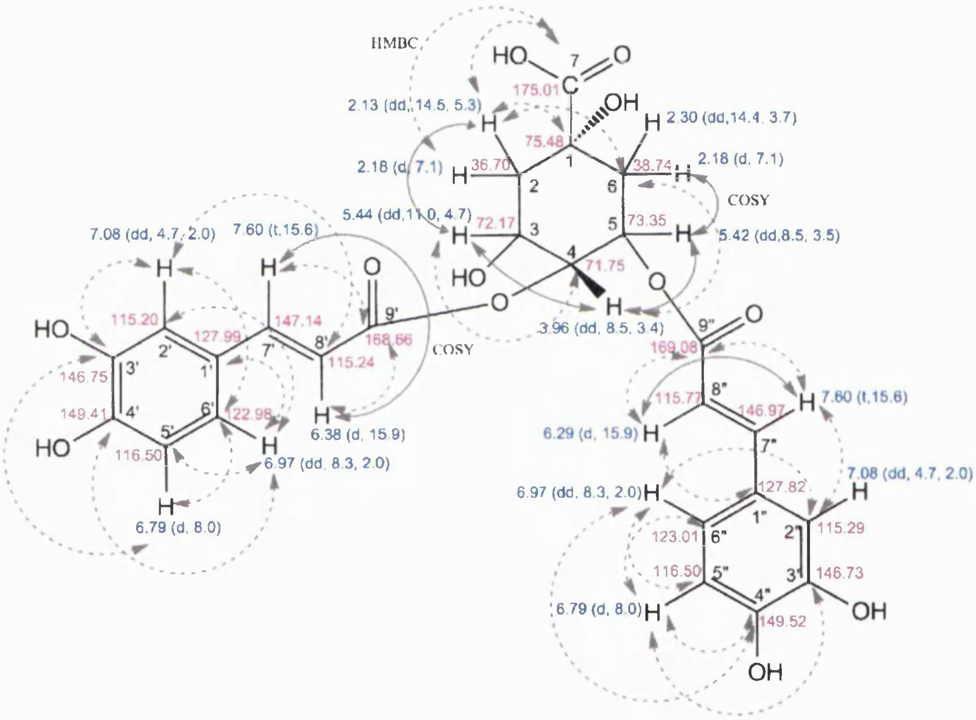
This fractionated mixture was observed for a number of compounds, using HPLC with the same manner as other compounds reported above. The peaks at retention time 10.096, 10.206, 10.303, and 10.352 min were found to be a mixture of dicaffeoylquinic acid derivatives which were identified later by the NMR and ESI-MS spectra which can be seen in the following pages. The HPLC chromatogram below showed a group of large peaks which are major components of fraction F38.

Figure 68 HPLC of F38, a mixture of dicaffeoylquinic acid derivative and other minor compounds (5mg/ml in MeOH). Injected volume =10 μl. Solvent A: water plus 0.05% acetic acid, B: acetonitrile. Gradient: 99% A -1%A in 30 min.



1H NMR analysis of this compound showed signals for two caffeic acid moieties and one quinic acid moiety. H-3, H-4 and H-5 protons in the quinic acid moiety were observed between 4 and 5.5 ppm. Signals for protons in caffeoyl moieties appeared between 6 and 8 ppm. Four doublets with the largest coupling constant (15.6 and 15.9 Hz) showed trans double bond protons to their couple. Proton signals at position H-3, H-4, H-5 and their coupling constants indicated that two caffeoyl groups were linked to the 4, 5 position of quinic acid moiety.

Figure 69 Chemical structure of 4,5 dicaffeoylquinic acid isolated from fraction F38.



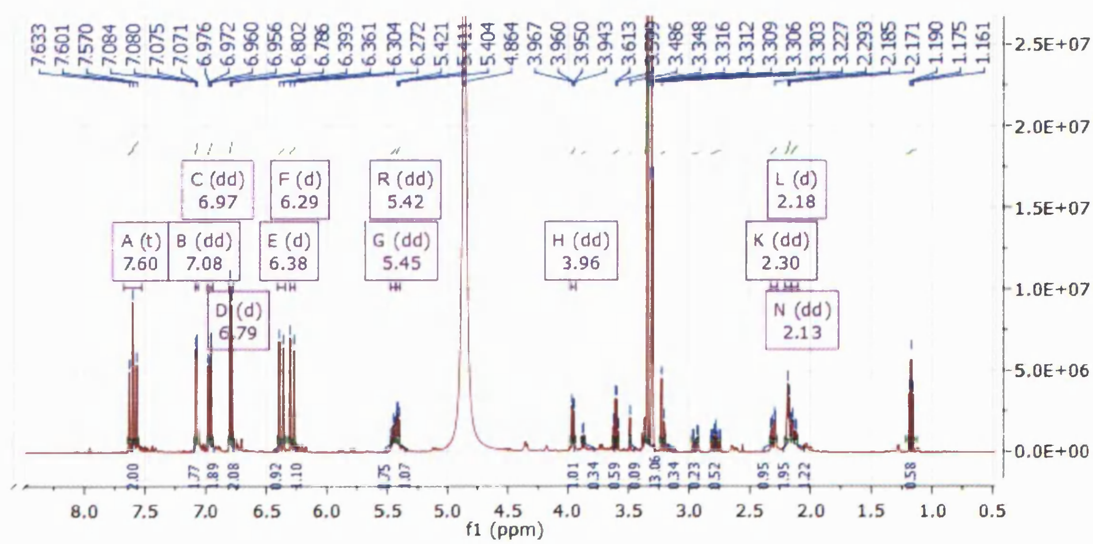
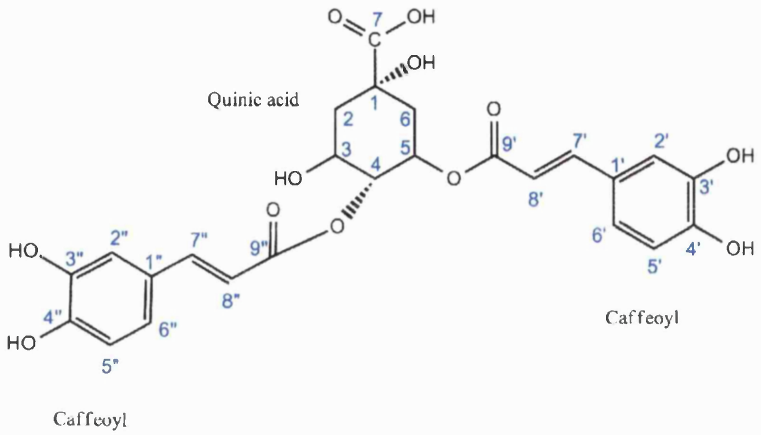


Table 20 Summary of NMR spectral data of compound F38

Position	13C	DEPT	1H	HMBC	COSY	NOESY
1	75.48	C				
2	36.70	CH2	2.13 (dd, 14.5, 5.3, 1H) 2.18 (dd, 7.1, 1H)	38.74, 75.48, 175.01	5.44	
3	72.17	CH	5.44 (dd, 11.0, 4.7, 1H)	71.75	2.13 or 2.18	
4	71.75	CH	3.96 (dd, 8.5, 3.4, 1H)	38.74	5.42, 5.44	
5	73.35	CH	5.42 (dd, 8.5, 3.5, 1H)		2.18	
6	38.74	CH2	2.18 (d, 7.1, 1H) 2.30 (dd, 14.4, 3.7, 1H)		5.42	
7	175.01	C				
1'	127.99	C				
2'	115.20	CH	7.08 (dd, 4.7, 2.0, 2H)	115.24, 122.98, 146.75		
3'	146.75	C				
4'	149.41	C				
5'	116.50	CH	6.79 (d, 8.0, 2H)	122.98, 146.75		
6'	122.98	CH	6.97 (dd, 8.3, 2.0, 1H)	115.20, 116.50, 127.99		
7'	147.14	CH	7.60 (t, 15.6, 1H)	168.66	6.38 or 6.29	
8'	115.24	CH	6.38 (d, 15.9, 1H)	168.66		
9'	168.66	C				
1''	127.82	C				
2''	115.29	CH	7.08 (dd, 4.7, 2.0, 2H)	146.73		
3''	146.73	C				
4''	149.52	C				
5''	116.50	CH	6.79 (d, 8.0, 2H)	123.01, 149.52		
6''	123.01	CH	6.97 (dd, 8.3, 2.0, 1H)	116.50, 149.52		
7''	146.97	CH	7.60 (t, 15.6, 1H)	169.08	6.38 or 6.29	
8''	115.77	CH	6.29 (d, 15.9, 1H)	127.82, 169.08		
9''	169.08	C				
-	18.35	CH2	1.18 (t, *J*=7.1, 1H)	58.32	An impurity peak from ethanol	

Table 21 1H NMR of compound F38 in comparison with the literature



	Wang & Liu (2007) DMSO-d6, NMR 500 MHz	Compound F38 $MeOD_4$,NMR 500 MHz
Ring with carboxylic acid		
H-2a	2.15–2.17 (m)	2.13 (dd, 5.3, 14.5)
H-2b	2.15–2.17 (m)	2.18 (d, 7.1)
H-3	4.17 (dd, 3.8, 9.5)	5.44 (dd, 4.7, 11.0)
H-4	5.11(ddd, 3.8, 9.5, 10.5)	3.96 (dd, 3.5, 8.5)
H-5	5.36 (ddd, 3.8, 9.5, 10.5)	5.42 (dd, 3.5, 8.5)
H-6a	2.13 (m)	2.30 (dd, 3.7, 14.4)
H-6b	2.13 (m)	2.18 (d, 7.1)
3-Caffeyol		
H-2'	7.02 (d, 1.5)	7.08 (dd, 2.0, 4.7)
H-5'	6.75 (d, 8.1)	6.79 (d, 8.0)
H-6'	6.98 (dd, 1.5, 8.1)	6.97 (dd, 2.0, 8.3)
H-7'	7.48 (d, 16.0)	7.60 (t, 15.6)
H-8'	6.24 (d, 16.0)	6.38 (d, 15.9)
5-Caffeyol		
H-2"	7.00 (d, 1.5)	7.08 (dd, 2.0, 4.7)
H-5"	6.73 (d, 8.1)	6.79 (d, 8.0)
H-6"	6.99 (dd, 1.5, 8.1)	6.97 (dd, 2.0, 8.3)
H-7"	7.42 (d, 16.0)	7.60 (t, 15.6)
H-8"	6.14 (d, 16)	6.29 (d, 15.9)

Table 22 1H NMR of compound F38 in comparison with the literature

	Wang & Liu (2007) DMSO-d6, NMR 500 MHz	Compound F38 in $MeOD_4$, NMR 100 MHz
Quinic acid		
C-1	73.6	75.48
C-2	38.6	36.70
C-3	67.4	72.17
C-4	73.4	71.14
C-5	71.2	73.35
C-6	38.5	38.74
C-7	174.8	175.01
4-Caffeyol		
C-1'	121.5	127.99
C-2'	115.8	115.20
C-3'	148.5	146.75
C-4'	145.6	149.41
C-5'	114.8	116.50
C-6'	125.4	122.98
C-7'	131.6	147.14
C-8'	113.8	115.24
C-9'	166.0	168.66
5-Caffeyol		
C-1''	121.4	127.82
C-2''	115.7	115.29
C-3''	148.5	146.75
C-4''	145.6	149.52
C-5''	114.8	116.50
C-6''	125.4	123.01
C-7''	131.6	146.97
C-8''	113.6	115.77
C-9''	165.6	169.08

3.7 DETECTION OF PYRROLIZIDINE ALKALOIDS IN G. PSEUDOCHINA VAR. HISPIDA

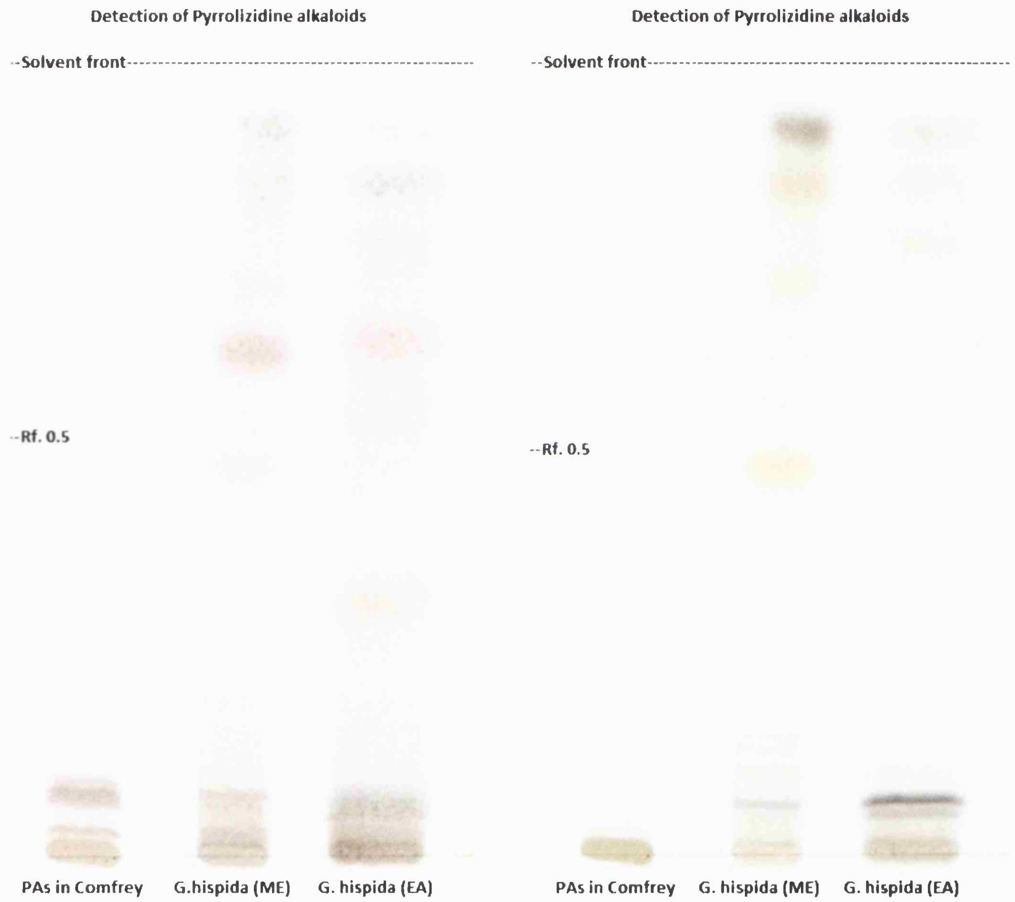
Pyrrolizidine alkaloids (PAs) have been reported to cause hepatotoxicity and have been found in some Asteraceae species especially of the tribe Senecioneae. Therefore, the methanol extract of *G. pseudochina var. hispida* was investigated for pyrolizidine alkaloids using TLC with spray detection; in comparison with the pyrolizidine alkaloid fraction from *Symphytum officinalis,* following the method of Mroczek *et al.* (2006). Further observation of overall pyrolizidine compounds present in both samples were examined using HPLC. The HPLC chromatograms of both samples were compared.

Five grams of *Symphytum officinalis* (Comfrey) roots were extracted in 250 ml of 1% tartaric acid in methanol for 2 hr. The extracts were dried using a rotary evaporator. The dry residue was dissolved in 10 ml of 0.05 M hydrochloric acid and transferred into the separation funnel for liquid–liquid partitioning clean up. At first, chloroform extraction was performed (2 × 50 ml). The water fraction was alkalised with 25% ammonia (pH = about 10.0) followed by extraction with a mixture of chloroform-*n*-butanol (2:1, v/v) (5 × 10 ml). Organic fractions were collected and evaporated to dryness. An oily alkaloid mixture was obtained as a PAs fraction.

The PAs fraction of Comfrey, the methanol and the ethylacetate extracts of *G. pseudochina var. hispida* were applied onto the TLC plate under the same conditions using chloroform- methanol- 25% Ammonia (100: 10: 2) as a mobile phase. Then natural product reagent was sprayed onto the TLC plate and the plate was heated at 70°C for 1 min using a hairdryer. After that the plate was sprayed again with Ehrlich reagent and heated for a further 1 min. The development of a magenta color in the sample compared with the blank indicates the presence of an unsaturated PA N-oxide (Mattock & Jukes, 1987), as shown in figure 71.

Figure 70 TLC of the pyrrolizidine alkaloids (PAs) fraction of *Symphytum officinalis* (Comfrey) – a positive control containing PAs compounds, in comparison with the methanol (ME) and ethylacetate (EA) extracts of *G. pseudochina var. hispida.*

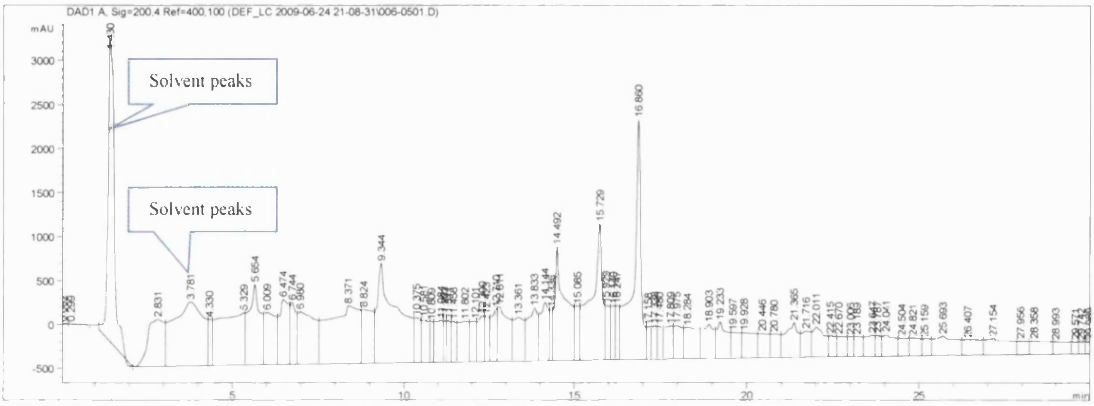
After applying the detecting reagent | Before applying the detecting reagent



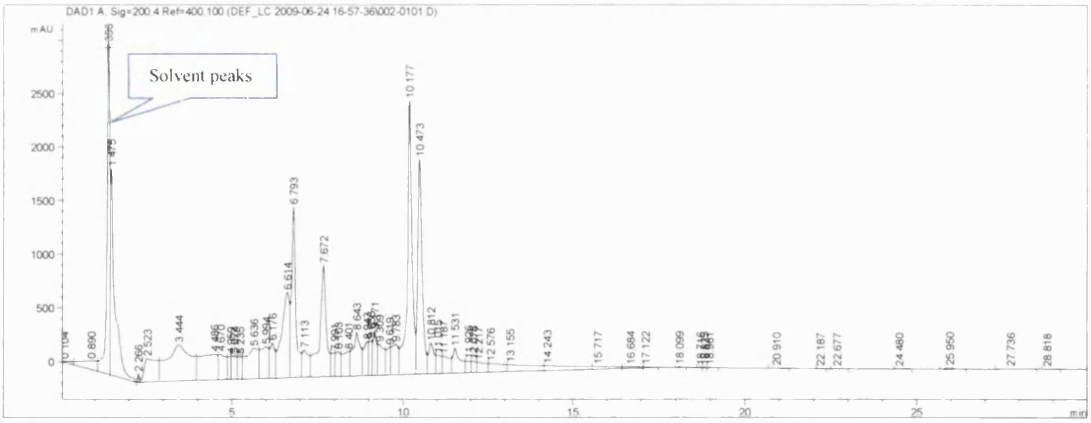
As seen in the TLC photos, the extracts of *G. pseudochina var. hispida* showed one or two bands that gave positive reactions with the spray reagent similar to compounds in the PAs fraction of Comfrey. Therefore, the extracts of *G. pseudochina var. hispida* were analysed further using HPLC in order to confirm the similarity of the compound presented in it, compared with the compound in the PAs fraction, as shown in the following HPLC chromatograms.

Figure 71 HPLC chromatogram of the PAs fraction of *Symphytum officinalis* (Comfrey) and the extracts of *G. pseudochina var. hispida*. Solvent A: water plus 0.05% acetic acid, B: acetonitrile. Gradient: 99% A -1% A in 30 min.

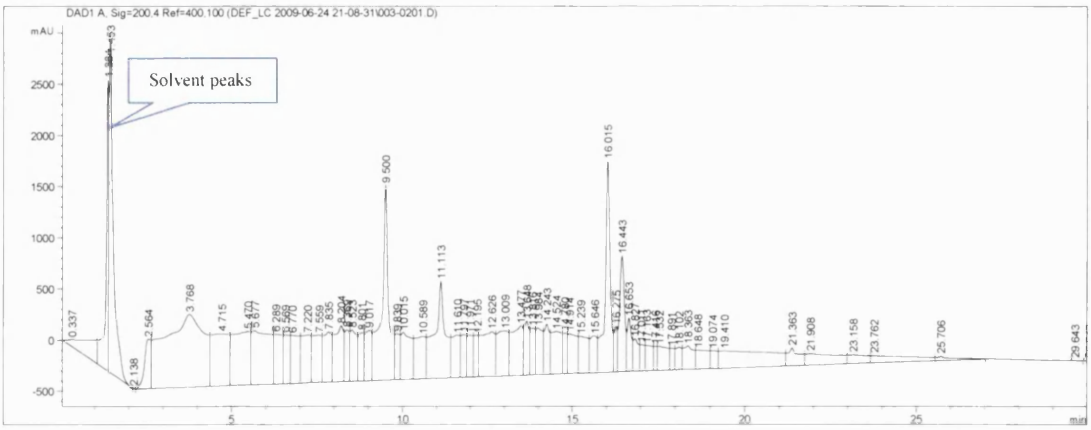
Symphytum officinalis (Comfrey) – a positive control containing PAs compounds



G. pseudochina var. hispida (methanol extract)



G. pseudochina var. hispida (ethylacetate extract)



From the HPLC chromatogram and the TLC photos, it may be concluded that the pyrrolizidine alkaloids presented in *S. officinalis* (Comfrey) did not exist in the methanol extract of *G. pseudochina var. hispida* while those compounds might or might not present in the ethylacetate extract of *G. pseudochina var. hispida* since the retention time of some peaks (e.g. the peaks shown at about 9 min and 16 min) are very close to that of the pyrrolizidine alkaloids. However, further phytochemical investigation is required for the identification of these compounds.

CHAPTER 4

DISCUSSION

4.1 PHARMACOLOGICAL ACTIVITIES OF THE NINE PLANT SPECIES

4.1.1 BASELLA ALBA (BASELLACEAE)

The petroleum ether and methanol extracts of *B. alba* did not show anti-inflammatory activities via the NF-κB pathway in the transfected HeLa cells (IC_{50} > 200 μg/ml). Moreover, all three extracts of this plant were found to increase synthesis of PGE_2 and IL-1β at the tested concentrations (except the methanol extract which did not increase the production of IL-1β). Only the ethyl acetate extract of this species showed moderate NF-κB inhibitory activity (IC_{50} = 83.23 μg/ml). This may be due to its free radical scavenging activity detected in the DPPH assay; the ethyl acetate extract demonstrated moderate DPPH free radical scavenging effects but did not express antioxidant effects in the lipid-peroxidation test.

Although this species did not have significant *in vitro* anti-inflammatory activities, this plant is well known in Thailand as a medicinal vegetable. The aerial parts are consumed for alleviating the symptoms of appendicitis and fevers as well as a laxative. The crushed leaves and the juices of the flowers have been used externally against skin inflammations and other topical skin problems such as wounds/ulcers, itching and abscesses (Theangburanatham 2005). In addition, according to an ethnobotanical survey among the Paliyar, Tamil Nadu, India, this species was reported as a remedy for external treatment of eye infections (Ignacimuthu *et al.* 2008) but so far its mechanism of action is unknown.

Among the three extracts of this plant, the ethyl acetate extract yielded the highest amount of phenolic contents compared to the other extracts but it was not high when compared to the other species in this study. However, as reported in several studies, the water extract of this species is more commonly used. Local forms of aqueous extraction reported include juice of the flower (Theangburanatham 2005), leaves masticated in the mouth (Hebbar *et al.* 2004), and juice from crushed fresh leaves (Ignacimuthu *et al.* 2008; Noumi & Tchakonang 2001; Theangburanatham 2005).

In Cameroon, this species is used in females for abortions and used in males for improving virility and to cure anasthernia and infertility. Therefore, it has continuously been investigated by (Moundipa *et al.* 2005; 2006) for such activities particularly its effects on the production of testosterone. So far, the results have shown that the aqueous extract of this plant tested in combination with another plant species- *Hibiscus macranthus* demonstrated significant enhancement of testosterone production in bull and rat Leydig cells in a concentration-dependent manner.

None of the extracts of *B. alba* showed cytotoxic effects against Hela cells. At the concentration of 10 µg/ml, the viability of the cells was still higher than 90%. The methanol extract of this species seemed to be less cytotoxic and was the second least toxic among all the tested species. The extracts of *B. alba* showed significant and specific cytotoxicity against the multidrug resistant CEM/ADR500 cells compared to CCRF-CEM leukaemia cells ($p < 0.0001$), although the petroleum ether only showed low level of cytotoxicity.

Interestingly, the ethyl acetate extract showed high toxicity against both leukaemia and multidrug resistant leukaemia cells whereas the methanol extract showed mild toxicity on CCRF-CEM cells but was specifically toxic to the multidrug-resistant CEM/ADR5000 cells (about two times greater when compare to its toxicity on CCRF-CEM cells). However, as far as we know, no study has reported such activity or the use for treatment of leukaemia or other types of cancers. The only report on this species was the antimutagenicity activity towards two direct mutagens; 2-Amino-3-methyl-imidazo [4, 5-f] quinoline (IQ) and Benzo[a] pyrene in the Ames test in which the extract markedly reduced the mutagenicity of IQ towards *S. typhimurium* TA98 and TA100 (Yen *et al.* 2001).

To conclude, this species demonstrated mild *in vitro* anti-inflammatory effects via the NF-κB pathway. The anti-inflammatory effect of this species might be due to its DPPH antioxidant property which can be linked to the redox sensitive NF-κB signalling regulation as already identified in some cases (Schreck *et al.* 1992). Although, the DPPH test model used in this thesis is a non-specified antioxidant system, this species is a good example of food plants that could effectively alleviate inflammatory symptoms due to its antioxidant potential.

4.1.2 BASELLA RUBRA (BASELLACEAE)

All the *B. rubra* extracts showed very weak anti-inflammatory effects via the NF-κB pathway with the IC_{50} values ranging from about 140 to160 μg/ml. The ethyl acetate extract slightly inhibited pro-inflammatory mediators which include IL-6, IL-1β and particularly TNF-α (IC_{50} values range from 32 to 39 μg/ml). Other extracts apart from the ethyl acetate did not show any inhibitory effects at the tested concentrations. The most active anti-inflammatory extract of this species was the ethyl acetate extract, which more specifically inhibited the TNF-α.

B. alba and *B. rubra* showed specific cytotoxic effects against multidrug resistant leukemic cells as well as an increased biosynthesis of PGE_2. Although, the activity to enhance the PGE_2 production seems to be one of negative effects inducing inflammation, there was some explanation which might link the effect of increasing PGE_2 production and the resulting anticancer potentials.

It has been reported that PGE_2 can be a potent enhancer of IL-12 production on human dendritic cells which have a role in developing immune responses and have anti-angiogenic activity (Rieser *et al.* 1997). In the pre-clinical models of IL-12 anti-tumor immunotherapy, good results have raised much hope that IL-12 could be a powerful therapeutic agent against cancer (Colombo & Trinchieri 2002). Although PGE_2 alone stimulated low amounts of IL-12 production, with TNF-α it could induce high levels of IL-12, while TNF-α alone had no effect on IL-12 production (Rieser *et al.* 1997). However, further studies are necessary to explore such potential effects.

There were reports of traditional uses of this species which lend some support to the activity of this plant as an anticancer agent. For instance, the leaves of *B. rubra* ground with sour buttermilk and salt prepared as a poultice has been reported to cure 'Arbuda' one type of tumour which is specified in Ayurvedic medicine (Balachandran & Govindarajan 2005). The aqueous extract of the leaves of *B. rubra* demonstrated antiulcer activity (Deshpande *et al.* 2003).

B. rubra and *B. alba* have medicinal properties for reducing dental diseases and they are also used as a remedy for piles. The two species were reported to help in the development of mental maturity (Vasishta 1978 in Prasuna *et al.* 2009). *B. rubra* decoction, cooked fresh leaf, and bulblet have also been used for stomachache (Lee *et al.* 2008). The α- and β- basrubrins isolated from these species showed antifungal activity (Wang & Ng 2004; 2001).

4.1.3 CAYRATIA TRIFOLIA (VITACEAE)

The methanol extract of *C. trifolia* exhibited the most potent DPPH free radical scavenging activity (IC_{50} = 0.48 μg/ml) compared to all the species investigated. The extract also strongly inhibited lipid peroxidation (IC_{50} = 1.36 μg/ml) and contained the highest amount of phenolics (28.14 μMol equivalent to 1μMol of caffeic acid). This plant was reported previously to yield cyanic acid from its stems, leaves, roots and flowers. Besides this, cyanidin, delphinidin, kaempferol, myricetin, and quercetin were found, mainly in the leaves. A triterpene-epifriedelanol was also reported from the aerial parts (Kundu *et al.* 2000; van Valkenburg & Bunyapraphatsara 2001).

Flavonoids and phenolic compounds are known to possess antioxidant effects and in the past decades the capacity of flavonoids to act as *in vitro* antioxidants has been a subject of many studies. Structure-activity relationships of flavonoids and their antioxidant activity have been established (Pietta 2000). This could explain the activity of this plant which may be due to the ability of its flavonoids to reduce free radical formation and scavenge DPPH reactive substance by virtue of the structural arrangements and hydrogen-donating potential of their phenolic groups (Miller & Ruiz-Larrea 2009). Therefore, it is not surprising that this species was not only found to contain the highest amount of phenolics but also exhibited the most potent free radical scavenging activity.

The methanol extract of *C. trifolia* exhibited a moderate inhibitory effect via the NF-κB pathway in Hela cells (IC_{50} =83.16 μg/ml) but did not show inhibitory effects on IL-1β and PGE_2 in human monocytes. However, in activated human monocytes, the ethylacetate extract showed the strongest inhibitory effect on the synthesis of PGE_2 when compared to other species in this thesis. Both, methanol and ethyl acetate extracts also showed moderate inhibitory effects on the activations of IL-6 (IC_{50} = 19.53 and 25.47 μg/ml, respectively) and the TNF-α (IC_{50} = 28.45 and 20.83 μg/ml, respectively) in human monocytes.

The petroleum ether extract of this plant did not show anti-inflammatory effects in any models tested. However, it exhibited cytotoxicity on both leukemic CCRF-CEM and CEM/ADR5000 cells (viability = 39% and 34% respectively, at 10μg/ml). On the other hand, the methanol extract showed mild to moderate cytotoxic effects on both cell lines, but more cytotoxicity against the multidrug-resistant leukemia cells. The ethyl acetate extract induced apoptosis of CCRF-CEM cells but not the multidrug-resistant cells. The cytotoxic effects of this plant were very different between the solvent extractions. Therefore, further studies are required to search for active compounds responsible for the activities of each extract.

The anti-inflammatory effect of this species might be due to flavonoids which have been reported to possess antioxidant activity, NF-κB inhibitory activity as well as inhibitory effects on the release of pro-inflammatory mediators. For example, cyanidin, isolated from tart cherries, possess antioxidant activity in a liposomal model system lipid peroxidation, comparable to commercial antioxidants (BHA and BHT) and also superior than that of vitamin E at the same concentrations (2-mM). Cyanidin also showed better anti-inflammatory activity than that of aspirin on the cyclooxygenase model, measured by inhibitions of the enzymes prostaglandin H endoperoxide synthase-1 and synthase-2 (Wang *et al.* 1999).

Cyanidin isolated from cherries, protected against the rat paws swelling model from adjuvant induced arthritis and could alleviate inflammations of the joint. After 14 days of adjuvant arthritis induction, the swellings were significantly reduced, as compared with a control group. Its mechanism of action might be via the increased activity of glutathione (GSH), superoxide dismutase (SOD) and total antioxidative capacity (T-AOC) activity, that improved the total antioxidative capacity and scavenge the free radicals and perhaps as a result of the decrease of the levels of the PGE_2 in paw tissues and TNF-α contents in serum (He *et al.* 2005).

Delphinidin has recently been investigated for NF-κB modulatory activity using immunoblot, ELISA and EMSA analysis in human colon cancer HCT116 cells. It was found that treatment of cells with delphinidin resulted in the inhibition of IKK, phosphorylation and degradation of IB, phosphorylation of NF-κB/p65, nuclear translocation of NF-κB/p65, NF-κB/p65 DNA binding activity, and transcriptional activation of NF-κB. These results suggested that delphinidin could suppress the NF-κB pathway in the human colon cancer HCT116 cells, resulting in G2/M phase arrest and apoptosis (Yun *et al.* 2009).

Kaempferol has been found in many berries and Allium species. Kaempferol was found to suppress NF-κB activation and expression of cyclooxygenase-2, inducible nitric oxide synthase, monocyte chemoattractant protein-1, and regulated upon activation, and normal T-cell expressed and secreted in aged rat kidney and in tert-butylhydroperoxide-induced YPEN-1 cells. Furthermore, it was found that kaempferol suppressed the activation of NF-κB through NIK/IKK and MAPKs in aged rat kidney (Park *et al.* 2009).

Myricetin induces apoptosis in human leukemia HL-60 cells, characterized by the occurrence of DNA ladders and hypodiploid cells. Western blotting and caspase activity assays showed that it activated caspases 3 and 9 but not caspases 1, 6 or 8. However, no significant induction of intracellular reactive oxygen species levels by myricetin was observed by the DCHF-DA assay, DPPH assay or plasmid digestion assay. Moreover, treatment with antioxidants including N-acetyl-cysteine, catalase, and superoxide dismutase showed no protective effects on myricetin-induced apoptosis (Ko *et al.* 2005).

A study on structure-activity relationship (SAR) demonstrated that the presence of OH at C3′, C4′, and C5′ is important for the apoptosis-inducing activities of myricetin. Myricetin also induced apoptosis in the HL-60 leukemia cell line, Jurkat cells, but not in primary human polymorphonuclear (PMN) cells or in murine peritoneal macrophages (PMs). The results of this study suggested myricetin induced apoptosis through a mitochondrion-dependent, ROS-independent pathway. The compound called 12-O-tetradecaoylphorbol-13-acetate (TPA) could protect the cells from myricetin-induced apoptosis via PKC activation which prevents mitochondrial destruction during apoptosis (Ko *et al.* 2005).

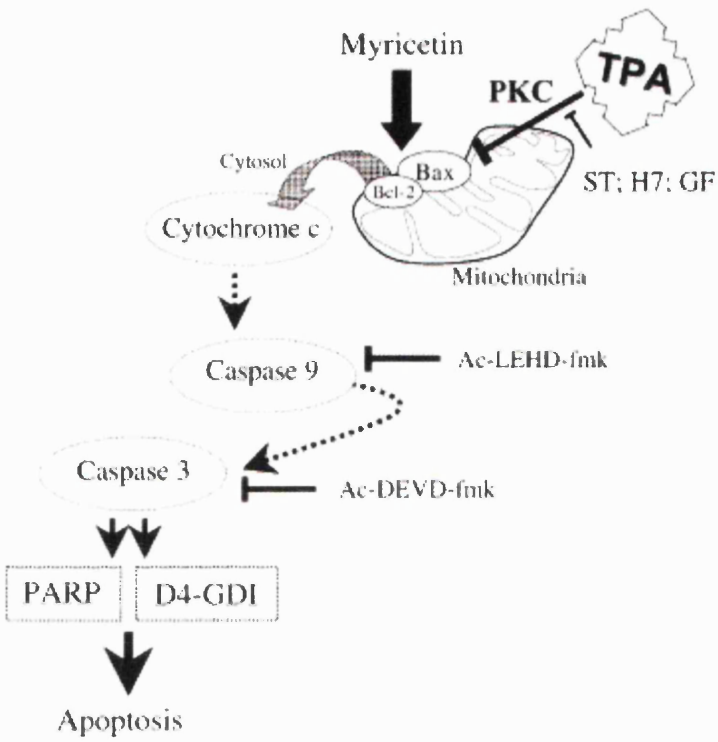


Figure 72 The model of myricetin-induced apoptosis in HL-60 leukemia cells as proposed in Ko et al (2005).

Abbreviation used are TPA = 12-O-tetradecanoylphorbol 13-acetate; PKC= protein kinase C; ST = staurosporine; H-7= isoquinoline-5-sulfonic 2-methyl-1-piperazide; GF= GF-109203X, and PARP = poly (ADP-ribose) polymerase.

Quercetin was reported as one of the main constituents in the leaves of *C. trifolia*. Quercetin is a strong antioxidant and a major dietary flavonoid (Hollman *et al.* 1997). It inhibits the oxidation of low-density lipoproteins (LDL) in patients with hyperlipidemia by scavenging free radicals and chelating transition metal ions (Chopra *et al.* 2000). However, in healthy volunteers, daily supplementation with graded concentrations of quercetin for 2 wk dose-dependently increased plasma quercetin concentrations but did not affect antioxidant status (serum uric acid or plasma- and g-tocopherols), oxidized LDL, inflammation (TNF-α), or metabolism (Egert *et al.* 2008).

A triterpene epifriedelanol isolated from this species showed significant *in vitro* inhibitory effects on the crown gall tumors formed by *Agrobacterium tumefaciens*, which is a plant disease and a great concern to the agriculture industry (Moore *et al.* 1997). However, epifriedelanol isolated from *Artemisia annua* has been tested against five human tumor cells (P-388, A-549, HT-29, MCF-7, and KB tumor cells) but no cytotoxicity was observed at 10 μg/ml (Zheng 1994). In addition, epifriedelanol did not show cytotoxic effects on Hela, A375 (human melanoma), and MCF-7 (human breast carcinoma) cells (Wang *et al.* 2006).

The above reports suggest that the pharmacological activities of *C. trifolia* including the high level of antioxidant activities especially on the lipid peroxidation and the highest PGE_2 inhibitory effect, might be due to its reported flavonoid content as discussed above. More studies also confirmed that reactive oxygen species have a regulatory role in the expression of COX-2 and a subsequent synthesis of PGE_2 (Wang *et al.* 2004; Martinez *et al.* 2000). Therefore, this might be the reason of why this plant showed antioxidant activity as well as PGE_2 inhibitory effect.

Our findings might support traditional uses of this plant species against conditions reported in two sources. For example, the plant leaves have been used externally against nose ulcers, muscle pains, and abscesses (Chuakul et al, 2000). Also the heated leaves have been applied externally for inflammatory conditions (Valkenburg & Bunyapraphatsara, 2001). It seems that all the findings here are in agreement with the results obtained from these studies although its active components have not been purified using, for example, bio-assay guided fractionation, and mechanism of actions have not been studied.

4.1.4 GYNURA PSEUDOCHINA (ASTERACEAE)

The ethyl acetate extract of *G. pseudochina* (*var. pseudochina*) showed low level of NF-κB and PGE_2 inhibition (IC_{50} = 83.20 and 41.77 μg/ml, respectively), but strongly inhibited the release of TNF-α (IC_{50} = 1.04 μg/ml). The similarity of this species and *G. pseudochina var. hispida* is that, both species possess inhibitory effects on the release of pro-inflammatory cytokines in human monocytes including IL-6, PGE_2 and TNF-α (but not IL-1β). However, *G. pseudochina var hispida* showed superior effects compared to the subspecies *pseudochina*.

The physical appearance of the two species, *G. pseudochina var. pseudochina* and *G. pseudochina var. hispida* are similar, apart from the purple colour of the leaves of the sub-species *hispida*. It has been reported that anthocyanins, the brightly-coloured compounds, are responsible for much of the red, blue, and purple colouring of fruits and plants (Alkema & Seager 1982). Therefore, it might be an anthocyanin that is responsible for the greater activity of the subspecies *hispida* and correspond to its purple color. However, this hypothesis needs to be investigated further.

The petroleum ether, ethyl acetate and methanol extracts of this species did not show free radical scavenging activity in the DPPH test. They only demonstrated lipid peroxidation inhibition at high concentrations (IC_{50} > 60 μg/ml) which are considerbly lower than those of the other species. The total phenolic contents of the three extracts of this species were at a low level and lower than those of the subspecies *hispida*. Therefore, it is not surprising that the antioxidant activities of the subspecies *pseudochina* were lower than those of the subspecies *hispida*.

However, the petroleum extract of *G. pseudochina var. pseudochina* showed intermediate, but specific, cytotoxic effects against multidrug resistant CEM/ADR5000 cells (IC_{50} = 32.74 and 24.47 μg/ml, respectively, $p < 0.0001$), which were similar to the cytotoxicity effects of the ethyl acetate extract of another subspecies *G. pseudochina var. hispida* (IC_{50} = 31.42 and 23.50 μg/ml, respectively, $p < 0.0001$). In Java, the leaves and roots of this species are used against breast tumours. The roots were also used to treat bruises whereas poultice made from the leaves was used externally against pimples (Lemmen & Bunyapraphatsara 2003).

In Thailand, the root of this species has been used against inflammation, including the use for relieving hot pain symptoms, fevers and viral infections (Plant Genetic Conservation Project 2009; Lemmen & Bunyapraphatsara 2003). However, due to the limited amount of root material available for collection, we had to use leaves. Therefore, these results are not directly relevant to the validation of the popular belief and the low pharmacological activities should not be surprising.

4.1.5 GYNURA PSEUDOCHINA VAR. HISPIDA (ASTERACEAE)

Our investigation identified *G. pseudochina var. hispida* as the most potent inhibitor of NF-κB activation in the stably transfed Hela cells. Its methanol extract showed NF-κB inhibitory effect with the lowest IC_{50} value of 41.96 μg/ml without any toxicity to this cell type at this concentration (viability > 90%). The methanol extract also effectively inhibited the release of IL-1β (IC_{50} = 2.46 μg/ml). On the other hand, the ethylacetate extract effectively inhibited the IL-6 and TNF-α production (IC_{50} = 8.16 and 1.49 μg/ml, respectively). These results support the use of fresh leaves for treating inflammation (Saralamp et al, 2000; Lemmens & Bunyapraphatsara, 2003).

At the concentrations tested, extracts of *G. pseudochina var. hispida* did not show high levels of cytotoxicity against either HeLa cells or leukaemia cells. Besides, they did not show relevant antioxidant effects although the ethyl acetate extract possesses high level of total phenolic contents. However, the ethyl acetate extract showed intermediate, but specific cytotoxicity against multidrug resistant CEM/ADR5000 cells (IC_{50} = 31.42 and 23.50 μg/ml, respectively, $p < 0.0001$), which is similar to the cytotoxic effect of the petroleum ether extract of another subspecies *G. pseudochina var. pseudochina* (IC_{50} = 32.74 and 24.47 μg/ml, respectively, $p < 0.0001$).

As far as we know, no pharmacological study has reported such anti-inflammatory activity of this species. The only available report related to the moderate HIV-1 reverse transcriptase inhibitory effect of the water extract of the leaves and the use of this plant in Thai folkloric medicine for treating AIDS (Woradulayapinij *et al.* 2005). The methanol extract of this plant, therefore, was selected for phytochemical investigation for its active NF-κB inhibitory compound.

Since a species of Gynura was selected for phytochemical research, the literature on this genus is reviewed accordingly. Gynura species grow well in tropical humid areas. Some species have been studied for bioactivities and phytochemistry. It was found that the most popular *Gynura spp.* is the species named *Gynura procumbens* which has been used traditionally as a remedy for kidney diseases, eruptive fevers, rashs, hypertension and diabetes mellitus and hyperlipidemia in many parts of South-East Asia (Perry 1980 in Lee *et al.* 2007).

The aerial parts of *G. procumbens* have been used in Thailand as a remedy against inflammation, rheumatism and viral infections, and have been found to possess anti-inflammatory activity in a croton oil-induced mouse ear inflammation model, from the compounds in the ethanolic fractions. It is expected that steroids might be one class of anti-inflammatory compounds in this plant (Iskander *et al.* 2002).

The leaves of this species demonstrated the effects in lowering blood sugar and lipid levels. The ethanol extract significantly suppressed the elevated serum glucose levels in streptozotocin-induced diabetic rats, however did not significantly suppress the elevated serum glucose levels, unlike Glibenclamide (a first-line antidiabetes drug). However, the extract significantly reduced serum cholesterol and triglyceride levels and these results indicate that the leaves of *G. procumbens* may have biguanide (Metformin) like activity (Zhang & Tan 2000).

The water extract of *G. procumbens* was found to possess the activity of lowering blood pressure in experimental rats and may be useful for prevention and treatment of hypertension through the increase of nitric oxide production in blood vessels (Mi-Ja Kim *et al.* 2006). The water extract also inhibited proliferation, DNA synthesis, and expressions of PDGF-BB (platelet-derived growth factor-BB), CDK1 (cyclin-dependent kinase-1), and CDK2 mRNA, as well as expressions of TGF-β1 (transforming growth factor beta-1) protein in human mesangial cells (MCs).

It is concluded that the inhibitory effect of *G. procumbens* on MCs proliferation, may be mediated through suppression of PDGF-BB and TGF-β1 expressions and the modulation of CDK1 and CDK2 expression. Therefore, this study claimed that *G. procumbens* might be promising as an adjunct therapy in preventing progressive renal diseases (Lee *et al.* 2007).

G. bicolor was reported to contain several anthocyanins. The anthocyans pelargonidin, delphinidin, malvidin and oenin (malvidin 3-glucoside) inhibited the HL60 human leukemia cell growth and induced apoptotic cell bodies and oligonucleosomal DNA fragmentation of the cells (Hayashi *et al.* 2002). *G. bicolor* also showed strong antioxidant effects superior to those of α-tocopherol at the same concentration, for the 72 hr duration of the linoleic acid oxidation experiment. However, it showed lower antimutagenicity as compared to that of the same concentration of BHA and α-tocopherol. Moreover, *G. bicolar* could inhibit PGE_2 induced release by LPS in primary splenocyte culture (Lin *et al.* 2006).

G. formosana was tested for antioxidant effects using DPPH and superoxide radical scavenging assays. The active compounds were isolated and identified as caffeic acid and quercetin 3-O-rutinoside which exhibited good hydroxyl radical activity (Hou *et al.* 2005).

G. elliptica chloroform fraction of the root contained p-hydroxyacetophenone-like derivative, (+)-gynunone, and a chromane, together with six known compounds. Among the isolated compounds, 6-acetyl-2,2-dimethylchroman-4-one and vanillin showed *in vitro* anti-platelet aggregation activity (induced by arachidonic acid) (Lin *et al.* 2000).

G. divaricata sub-species formosana was found to have antiproliferative activity. Among the 17 isolates, methyl 132-hydroxy-(132-S)-pheophorbide A and A exhibited potent antiproliferative effects against HL-60 cell lines (IC_{50} = 0.9 and IC_{50} < 0.5 μg/ml, respectively) (Chen *et al.* 2003).

G. japonica sup-species formazana rhizome was extracted and its isolates caryophyllene oxide, 6-acetyl-2,2-dimethylchroman-4-one, vanillin, 2,6-dimethoxy-1,4-benzoquinone, and benzoic acid exhibited significant *in vitro* anti-platelet aggregation activity (Lin *et al.* 2003).

Pyrrolizidine alkaloids (PAs) have been found in more than 6,000 plant species, including species in the Compositae (Asteraceae), Boraginaceae and Leguminosae (Fabaceae) families, from which, more than 660 PAs have been identified (Roeder, 1995 & 2000; Fu *et al.* 2002 in Qi *et al.* 2009). *G. segetum* showed the presence of senecionine, seneciphylline, sineciphyllinine and (EZ)-seneciphylline, classified as retronicine-type, which are known to possess hepatic toxicity (Yuan *et al.* 1990).

Ingestion of *G. segetum*, a Chinese herb with pyrrolizidine alkaloids can cause sinusoidal obstruction syndrome which is an infrequent liver disease. The symptom of toxicity includes portal hypertension and light injury of liver function. It was suspected that the PAs in *G. segetum* might be responsible for the main causes of hepatic veno-occlusive disease (Chen *et al.* 2007).

4.1.6 MUEHLENBECKIA PLATYCLADA (POLYGONACEAE)

The ethyl acetate and methanol extracts of *M. platyclada* did not possess high inhibitory activities in the case of PMA-induced NF-κB activation in Hela cells. However, these extracts expressed the most active inhibitory effects on pro-inflammatory cytokines IL-1β, IL-6 and TNF-α release with the lowest IC_{50} values ranging from 0.28 to 8.67 μg/ml.

This species showed greater DPPH scavenging activity compared to the other plants species in this study. However, the petroleum ether, ethylacetate and methanol extracts of this species possessed lower antioxidant activity than that of the methanol extract of *C. trifolia* and the methanol extract of *P. indica.* The activities at inhibiting lipid-peroxidation of the three extract of this species were low and their phenolic contents were at an intermediate level when compared to the other species.

In Taiwan and China, *M. platyclada* has been used for alleviating fever and for detoxification (Je-Chain et al, 1961 in Yen *et al.* 2009) whilst in Thailand the alcoholic extract of the aerial parts are usually applied to skin swellings, sores, and insect bites (Chuakul *et al.* 2000). However, from our results the overall anti-inflammatory effect of this plant species was unlikely to be mediated by the NF-κB pathway or by unspecific antioxidant mechanisms. This plant species illustrated the inhibitory effect on the release of pro-inflammatory cytokines and a consequent overall anti-inflammatory effect independent of direct NF-κB signaling control.

Yen *et al.* (2009) found that the methanol extract of *M. platyclada* demonstrated inhibitory effects on the generation of superoxide anions, and inhibitory effects on the release of neutrophil elastase. Active compounds isolated were flavonols, morin-3-O-α-rhamnopyranoside (1), kaempferol 3-O-α-rhamnopyranoside (2), kaempferol 3-O-β -glucopyranoside (3), quercetin 3-O-α-rhamnopyranoside (4) and catechin (5). Compound 2 showed moderate inhibition of superoxide anion generation while compound 1, 3 and 5 showed inhibition of the neutrophil elastase released which was 15-fold more potent than the positive control, phenylmethylsulfonyl fluoride used in the anti-inflammatory assay.

The results gained from this thesis, particularly the high activities of this species on inhibiting pro-inflammatory cytokines release, could support the traditional uses of this plant for the treatment of inflammatory conditions. It is not surprising that this species showed such a high level of anti-inflammatory effects since some of the active compounds isolated from this species belong to the flavonoid group that has been reported previously to possess anti-inflammatory effects (Nijveldt *et al.* 2001).

4.1.7 OROXYLUM INDICUM (BIGNONIACEAE)

O. indicum was found to be the second most potent NF-κB inhibitor in this study. Its ethyl acetate extract not only showed potent NF-κB inhibitory effect, but also showed PGE_2 inhibitory activity. In addition, it was endowed with the most potent *in vitro* antioxidant activity in the lipid-peroxidation assay. It may be that they both act via different mechanisms or it might demonstrate the link between redox status and NF-κB inhibitory activity which has been found previously (Rahman *et al.* 2004).

NF-κB mediated cyclooxygenase-2 (COX-2) expression has been observed in many studies (Lim *et al.* 2001). COX-2 expression controls the synthesis of PGE_2 through the metabolic conversion of arachidonic acid in response to inflammatory stimuli (Surh *et al.* 2005). Therefore, this could be a reason why this plant species showed both NF-κB and PGE_2 inhibitions.

Our results on the biological activities of *O. indicum* are in agreement with previous investigations by Palasuwant et al (2005) and Yang et al (2006) that *O. indicum* possessed very high levels of antioxidant activity although different approaches were used for investigation. Its anti-inflammatory activities have also been reported incuding the anti-inflammatory activity against carrageenan induced rats' paw edema (Prasad *et al.* 1989) and the inhibitory effect on the release of myeloperoxidase (Laupattarakasem *et al.* 2003).

Many studies have investigated this plant and reported its potential activities such as antimicrobial activity (Houghton *et al.* 1997; Suresh *et al.* 2006; Thatoi *et al.* 2008), antifungal activity (Ali *et al.* 1998), immunostimulant/ immunomodulatory activity (Gohil *et al.* 2008; Zaveri *et al.* 2006), analgesic activity (Upaganlawar *et al.* 2007), hepatoprotective activity (Tenpe *et al.* 2009; Sohn *et al.* 2008), ulcero-protective activity (Zaveri & Jain, 2007) and antiulcer activity (Khandhar *et al.* 2006). *O. indicum* seed or bark decoction have been used internally for Hepatitis and lumbago (Lee *et al.* 2008).

Its anti-proliferative activity against breast cancer cells (Lambertini *et al.* 2004) and cytotoxicity activity against B-16 (murine melanoma), HCT-8 (human colon carcinoma), CEM and HL-60 (leukemia) tumor cell lines (Costa-Lotufo et al, 2005) has also been observed. However, at our test concentrations this plant did not show promising cytotoxic activity against cervix cancer Hela cells, and CCRF-CEM, CEM/ADR5000 leukaemia cells.

O. indicum has been reported as having a flavonoid- baicalein which inhibited proliferation of HL-60 cells *in vitro* (Roy *et al.* 2007) as well as the mutagenicity of Trp-P-1 in an Ames test (Nakahara *et al.* 2001-2). Baicalein demonstrated growth suppression of primary myeloma cells through the downregulation of NF-κB (Otasuyama *et al.* 2005). It suppressed IL-6 and IL-8 production, and inhibited IL-1β -induced IL-6 and IL-8 mRNA and protein production in human retinal pigment epithelial cells (Nakamura 2003). Baicalein also effectively protected RAW264.7 cells from hydrogen peroxide-induced cytotoxicity (Lin 2007) and showed significant DPPH, superoxide, and hydroxyl radical scavenging capacity protection against ischemia/reperfusion injury of cardiomyocytes (Chang 2007).

O. indicum also contains a naphthoquinone- lapachol which is well known for its therapeutic potential, such as antineoplastic/antitumor capability (Balassiano *et al.* 2005; Houghton *et al.* 1994), antibacterial and antifungal activities (Binutu 1996; Houghton *et al.* 1997), antileishmanial activity (Lima *et al.* 2004). Lapachol is a vitamin K antagonist (Dinnen & Ebisuzaki 1997) and is bioactivated by P450 reductase to reactive species, which promote DNA scission through the redox cycling based generation of superoxide anion radical (Kumagai 1997).

Many derivatives synthesized from lapachol have also shown anti-malarial activity (de Andrade-Neto *et al.* 2004), and are active against Epstein Barr virus (Sacau *et al.* 2003). *O. indicum* also contains other compounds such as oroxylin A (Chen et al, 2000) and chrysin derivatives (Dao *et al.* 2004; Woo *et al.* 2005) which have been reported of having anti-inflammatory activity.

4.1.8 POUZOLZIA INDICA (URTICACEAE)

P. indica did not show *in vitro* anti-inflammatory activity that involved the NF-κB pathway , and did not show effects on the release of pro-inflammatory mediators except TNF-α which the ethyl acetate extract inhibited at an intermediate to high level (IC_{50} = 15.68 μg/ml). The petroleum ether and the methanol extracts did not show inhibitory effects on the release of pro-inflammatory mediators in human monocytes and NF-κB inhibition (except the methanol extract that showed very low NF-κB effect with IC_{50} = 135 μg/ml).

On the other hand, the petroleum ether extract was endowed with the most potent cytotoxicity against both CCRF-CEM cells and the multidrug resistant CEM/ADR5000 cells (viability = 9.75% and 10.48% at 10 μg/ml, respectively). The ethyl acetate was also toxic to the leukemia cells but more specifically cytotoxic to the multidrug resistant subline (viabilities = 35.12% and 31.30%, respectively, with $p = 0.003$). The methanol extract which possesses a low level of NF-κB inhibitory effect, showed less toxicity against the leukemia cells (viabilities = 56.35% and 53.59%, respectively).

The most active anti-inflammatory extract of this species was the ethyl acetate extract. This extract which showed anti-inflammatory effects via an inhibition of TNF-α, did not possess antioxidant activities in both DPPH and lipid peroxidation assays. However, the most active antioxidant extract seemed to be the methanol extract which showed high levels of scavenging activity in the DPPH and lipid-peroxidation tests (IC_{50} = 0.60 and 5.44 μg/ml, respectively).

The averaged total phenolic contents of the ethyl acetate and petroleum ether extracts were within a range, similar to those of other plant species, but the methanol extract had a higher level of phenolic content, which might imply good antioxidant activities as well as NF-κB inhibitory activity as discussed above.

According to Valkenburg and Bunyapraphatsara (2001), this plant has been reported to be used in many Asian countries in the treatment of many inflammatory-related symptoms, including sores (Malaysia), ulcers (Indonesia), sore throat (Vietnam), gangrene (the Philippines), gonorrhea, syphilis, and wounds (India), abscesses and swelling (China). In Thailand, its leaves have been applied externally against skin inflammation.

These reports indicated that this plant might have anti-inflammatory properties via some pathway or might process through a compound or a mixture of compounds that are responsible for TNF-α inhibition. Also antioxidant compounds might be involved. All of the extracts of *P. indica* showed activatory effects on the release of pro-inflammatory mediators including PGE_2 and IL-1β, which is interesting, and might be worth further investigation.

There is no report on the phytochemistry of this species. The only report is on a pharmacological activity of the methanol extract that exhibited a cysticidal effect on amoeba cysts (Acanthamoeba), an opportunistic pathogen of humans causing eye infections (Roongruangchai *et al.* 2009), which is still ongoing. Therefore, it is interesting to explore the phytochemical constituents of this species that were active as antileukemic or anti-inflammatory agents, most importantly the petroleum extract of this species which showed the most potent cytotoxicity against both CCRF-CEM cells and the multidrug resistant CEM/ADR5000 cells, but did not have anti-inflammatory or antioxidant effects.

4.1.9 RHINACANTHUS NASUTUS (ACANTHACEAE)

The ethyl acetate extract of *R. nasutus* demonstrated the most potent cytotoxicity against Hela cells (IC_{50} = 3.63 μg/ml) and intermediate to high levels of cytotoxicity to leukemia cells and the multi-drug resistant leukemia cells (viability = 38.13 and 31.81 μg/ml, $p < 0.0001$). On the other hand, the methanol extract showed highly specific cytotoxicity against the multidrug resistant CEM/ADR5000 cells (viability = 18.72%) when compared to CCRF-CEM cells (viability = 60.10%) ($p < 0.0001$). However, the petroleum ether extract only show mild cytotoxic effects to both cell types.

Previous studies reported that *R. nasutus* contained several naphthoquinone-rhinacanthin derivatives which possess many pharmacological properties such as the activity of inducing apoptosis in tumor cells via activation of caspase-3 (Siripong *et al.* 2004), anti-proliferative activity (Gotoh *et al.* 2004), antitumour activity against Dalton's ascitic lymphoma in mice (Thirumurugan *et al.* 2000), cytotoxicity against epidermoid carcinoma, Hela, and HepG2 cell lines (Kongkathip *et al.* 2004), cytotoxicity against P-388, A-549, HT-29 and HL-60 tumor cells, antiplatelet activities (Wu *et al.* 1988), and antiviral activity against influenza type A (Kernan *et al.* 1997).

However, in our stably transfected HeLa cells, *R. nasutus* showed poor inhibitory effects on the NF-κB pathway, as well as poor inhibitory effects on the release of IL-1β , IL-6, TNF-α or PGE_2 in primary human monocytes. As previously reported by Tewtrakul et al, (2009), some rhinacanthins demonstrated anti-inflammatory activity by inhibiting iNOS and COX-2 gene expressions against lipopolysaccharide induced release of nitric oxide, PGE_2 and TNF-α in RAW264.7 cells.

In J774.2 mouse macrophages, either water or ethanol extracts of *R. nasutus* alone had no effect on nitric oxide production, although when the ethanol extract of *R. nasutus* was used in combination with LPS, production of nitric oxide was increased. TNF-α secretion was correlated with nitric oxide production and increases were associated with an elevation in TNF-α mRNA (Punturee *et al.* 2004).

Therefore, it is not surprising that we have found that the extracts of *R. nasutus* showed poor anti-inflammatory effects on NF-κB activation in our stably transfected Hela cells, as well as poor inhibitory effects on the release of IL-1β , IL-6, TNF-α or PGE_2 in primary human monocytes.

The methanol extract of *R. nasutus* demonstrated a high level of antioxidant activity in the DPPH scavenging assay (IC_{50} = 0.78 μg/ml) but only showed a low level of antioxidant effects in the lipid peroxidation assay (IC_{50} = 43.56 μg/ml). Total phenolic contents of the three extracts of *R. nasutus* were in average levels compared to other plants species. Surprisingly, no such antioxidant activity of this plant species has been reported, although the plant has been very famous in Thailand as a remedy for various skin diseases and many other activities have extensively been studied.

4.2 DISCUSSION ON THE ISOLATION OF ACTIVE NF-KB INHIBITORS FROM THE METHANOL EXTRACT OF *G. PSEUDOCHINA VAR. HISPIDA*

In this study the active NF-κB inhibitory compounds isolated from *G. pseudochina var. hispida* were identified as quercetin rutinoside, dicaffeoyl quinic acid derivatives, and a monocaffeoyl quinic acid derivative. Their isolation was guided by the NF-κB assay as well as the fingerprints on the TLC aiding by their strong coloration with vanillin and sulphuric acid and their UV light activity. All of the isolated compounds have been reported previously in related species but this is the first time they have been reported from this species.

4.2.1 CHEMICAL AND BIOLOGICAL STUDIES OF QUERCETIN-RUTINOSIDE (RUTIN)

Previously, rutin has been isolated from *G. formosana* by extracting the plant with 70% aqueous acetone (Hou *et al.* 2005). This isolation procedure was guided by the DPPH assay and the active antioxidant compounds were purified using Sephadex LH-20 column and a preparative HPLC to obtained rutin (Hou *et al.* 2005). The procedures were similar to that of our study, in the way, that they used gel filtration methods with gradient solvents elution but with an exception that in our study, a preparative HPLC was not used.

Rutin is a quercetin with glucose and rhamnose, and is frequently found in many plant species. Significant amounts of rutin have been reported from tea, spinach, chokeberries and buckwheat (Materska 2008). Rutin has been considered a micronutrient and is proven to be a potent antioxidant and has many important biological, pharmacological and medicinal properties (Lacopini *et al.* 2008). In many countries, rutin has been added to products as an active ingredient of a variety of multivitamin and herbal preparations (Erlund *et al.* 2000).

Rutin is widely present in plants but is relatively rare in their edible parts (Kreft *et al.* 2006). Rutin was first detected in *Ruta graveolens* which gave the common name to this pharmaceutically important substance (Chen *et al.* 2001). The rutin content of buckwheat was reported to accumulate mostly in the inflorescence (up to 12%), in stalks (0.4–1.0%) and in upper leaves (8–10%). Also ecological factors, such as UV irradiation, may also have a great influence on rutin content in many plant species (Kreft *et al.* 2006).

Although the amount of rutin obtained in our study was very small, there is a report suggesting a solution that might help to increase the amount of rutin, in further experiments. Paniwnyk *et al.* (2001) reported that an application of ultrasound to the methanolic extraction of rutin from flower buds of *Sophora japonica* gave a significant increase in maximum extraction yield, compared to the conventional refluxed extraction. It is believed that further increase in yield when ultrasound is applied is due to the disruption of cell walls by cavitational effects.

There were reports relating to the pharmacological activities of rutin which are similar to the results from our study. A study on NF-κB inhibitory activity of rutin isolated from *Hancornia speciosa* has been performed (Endringer *et al.* 2009). Determination of NF-κB inhibitory activity in that study was carried out in 293 cells transfected with NF-κB luciferase plasmid, and induced NF-κB activation by TNF-α. Rutin was found to possess NF-κB inhibitory activity with IC_{50} = 16.4 ± 3.8 μg/ml which is greater than that of the NF-κB activity in our stably transfected Hela cells.

Endringer *et al.* (2009) also conducted an assay for testing rutin on antiproliferative assays. It was found that at a concentration of 20 μg/ml, rutin was non-toxic to the tested cells (viability > 90%). The tested cells included human lung carcinoma (LU1), hormone-dependent human prostate carcinoma (LNCaP), human hepatoma cells (HepG2), and human breast carcinoma (MCF-7) cells. This report is in agreement with our study in that, rutin was also non-toxic to Hela cells (viability > 90%) at all tested concentrations (100- 10 μg/ml).

4.2.2 CHEMICAL AND BIOLOGICAL STUDIES OF DI-CAFFEOYL QUINIC ACID DERIVATIVES

Di-caffeoyl quinic acid derivatives were isolated from the methanol extract of *G. pseudochina var. hispida* using Sephadex LH-20 column chromatography with gradient elution, and guided by the NF-κB assay, in the same manner as the isolation for rutin. Di-caffeoyl quinic acid was obtained from the very last fraction which is much more polar than other fractions. The two derivatives of di-cafferoyl quinic acid were obtained and were tested in the NF-κB assay. Both compounds were found to be active NF-κB inhibitors but less than that of rutin.

Di-caffeolyquinic acid was less soluble in deuterated-methanol but dissolved quite well in deuterated-water. However, when preparing these compounds in deuterated-water, and submitting for the NMR experiments, the NMR spectra did not show. It seemed that the compounds were less stable and may have decomposed. Therefore, the compounds were resubmitted again in deuterated methanol and the NMR spectra were much better although the compound was not completely dissolved.

Peluso *et al.* (1995) reported three caffeoylquinic acids, isolated from the Peruvian plants *Tessaria integrifolia* and *Mikania cordifolia*, which are used medicinally as anti-inflammatory agents. They were tested for their activities on monocyte migration and superoxide anion production. It was found that 3,5-di-O-caffeoylquinic and 4,5-di-O-caffeoylquinic acids exhibited an appreciable *in vitro* anti-inflammatory activity, while the tricaffeoyl derivative was inactive.

It has been found that 3,4-di-O-caffeoylquinic acid and 3,5-di-O-caffeoylquinic acid, isolated from the methanol extract from *Dipsacus asper* (Dipsacaceae) are potent scavengers of 1,1-diphenyl-2-picrylhydrazyl (DPPH), and are more potent than butylated hydroxyl toluene (BHT). The compounds also inhibited Cu(2+)-mediated low-density lipoprotein (LDL) oxidation in the TBARS assay in a dose-dependent manner (Hung *et al.* 2006).

3,5- dicaffeoylquinic acid and 4,5- di-caffeoylquinic acids, isolated from *Lychnophora ericoides* (Asteraceae), showed analgesic activity in the acetic acid-induced mouse writhing test at low dose (but not at high doses). On the other hand, 3,4,5-tri-caffeoylquinic acid did not contribute to analgesic activitiy (Santos *et al.* 2005). Also 4,5-dicaffeoylquinic acid, isolated from *Phagnalon rupestre*, strongly inhibited the release of elastase from polymorphonuclear leukocytes with an IC_{50} value of 4.8 μM (Góngora *et al.* 2002).

The method used to isolate dicaffeoylquinic acid derivatives in this study, was similar to that used in a previous study by Li *et al.* (2005). In Li's study, *Schefflera heptaphylla* was first extracted with a different method from our study but the methods used to isolate mono-caffeoyl quinic acid and dicaffeoyl quinic acid derivatives were similar to our study, using Sephadex LH-20 and obtained the same sequence of compounds as we found in our study. The procedures are shown below.

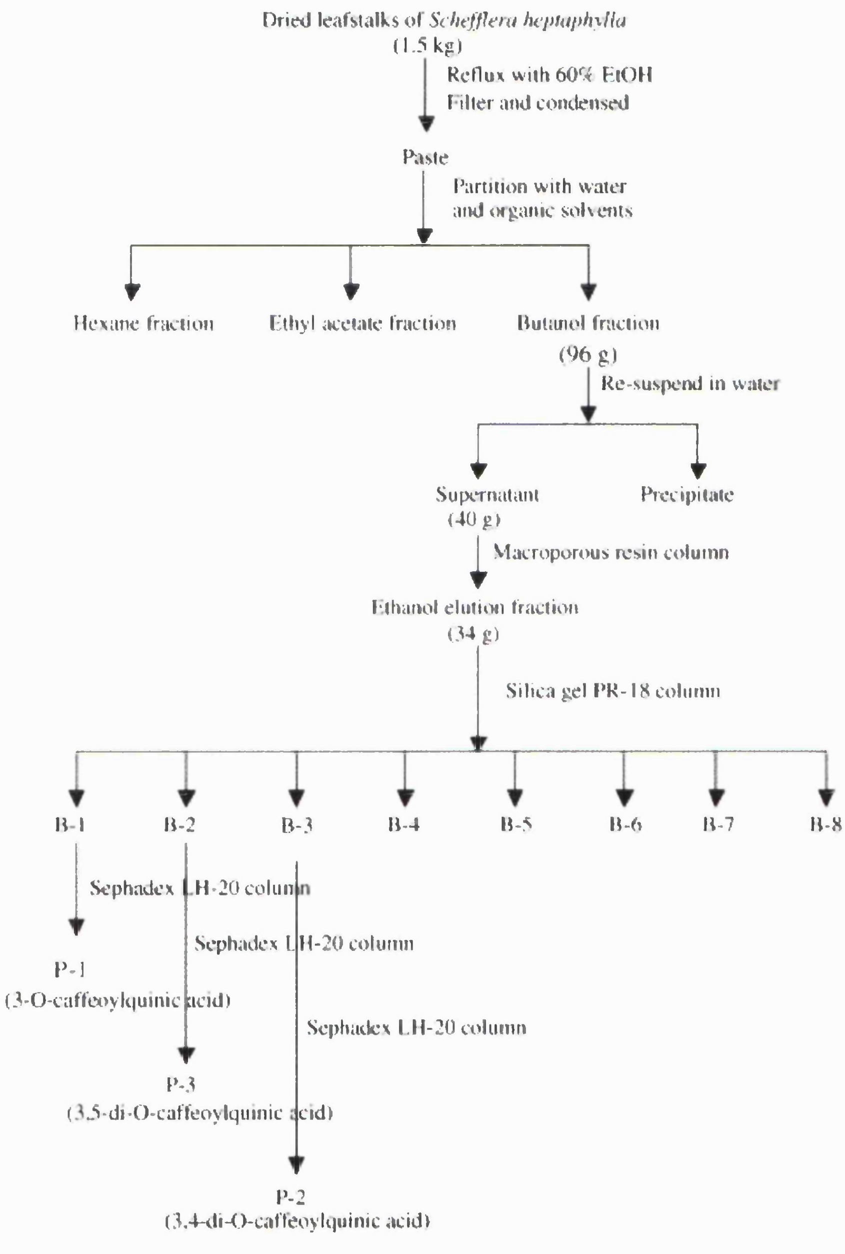


Figure 73 Procedures to isolate three caffeoylquinic derivatives by Li et al (2005)

3,4-di-O-caffeoylquinic acid and 3,5-di-O-caffeoylquinic acid from *Schefflera heptaphylla* were found to possess potent antiviral activity against respiratory syncytial virus (RSV), a major cause of lower respiratory tract infections in infants, young children, and even adults, with the IC_{50} = 1.2 and 0.6 μg/ml, respectively. 3-O-caffeoylquinic acid was not as active as 3, 4-di-O-caffeoylquinic acid and 3, 5-di-O-caffeoylquinic acid (the IC_{50} values were not indicated) (Li *et al.* 2005).

However, the above isolates 3, 4- and 3, 5- dicaffeoylquinic acid showed low cytotoxicity against HEp-2 cells (IC_{50} higher than 1000 μM). Therefore, it is suggested the anti-RSV effect was not due to their cytotoxicity. Also these two compounds did not inhibit RSV that was attached to host cells and could not protect HEp-2 cells from RSV infection at low concentrations. Moreover, they did not have antiviral activity against influenza A (Flu-A), coxsackie B3 (Cox-B3), and herpes simplex type one (HSV-1) viruses (Li *et al.* 2005).

4.2.3 CHEMICAL AND BIOLOGICAL STUDIES OF CAFFEOYL QUINIC ACID (CHLOROGENIC ACID)

The procedures used in isolating mono-caffeoyl quinic acid in this study were similar to the ones used for isolating di-caffeoyl quinic acid derivatives. Mono-caffeoyl quinic acid was eluted from a Sephadex LH-20 column before a mixture of di-caffeoyl quinic acid. To purify this compound, preparative HPLC was used. It is not too difficult to gain pure caffeoyl quinic acid from the HPLC, but unfortunately when running this experiment, the monitor and detector of the HPLC failed. Therefore, the separation was done, instead, in an analytical HPLC equipped with a fraction collector (Agilent) and the amount obtained was very little (2-3 mg only).

This compound was found to be very difficult to manage since it was very unstable and decomposed easily in deuterated solvents (either water or methanol), or when exposed to light. The compound was first obtained in a sufficient amount (approximately 7-10 mg) to obtain a good NMR spectrum (as seen from NMR spectra of other compounds), but the spectrum did not appear, showing only solvent peaks. This may be due to the long waiting-time for the NMR experiments. Although it was on the feeding-chain of the NMR instrument already, the compound was in a crystal clear NMR tube, unavoidably exposing it to light.

However, after this problem was taken into account and extra care was taken, a quick submitting and short running NMR experiment helped to obtain the 1H NMR spectra. However, the 13C NMR and HMBC experiments, which need to be run overnight, were still difficult to obtain a result from. Therefore, the interpretation of monocaffeoyl quinic acid structure in this study mainly focused on its 1H NMR spectra and ESI-MS spectra instead of relying solely on the 13C and 2D NMR experiments.

A study about how solvents affect structure dereplication of caffeoylquinic acids by Pauli *et al.* (1999) found that pyridine-d_5 and acetone-d_6 showed the strongest signal overlap in the region of the aromatic and methylene resonances (as shown in the figure below), therefore, theses are not solvents of primary choice.

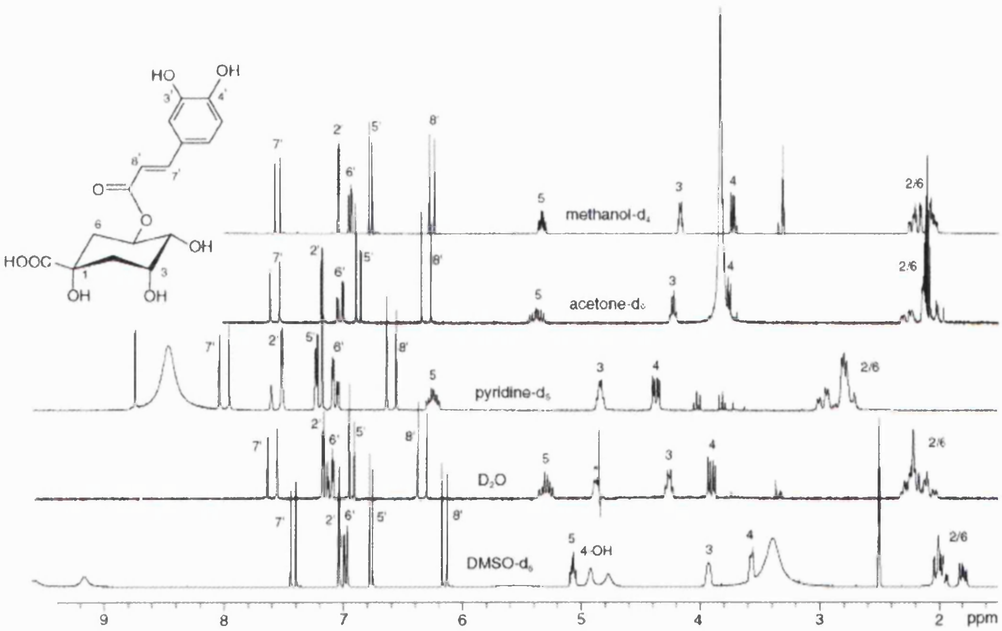


Figure 74 1H NMR spectra of chlorogenic acid in different solvents (Pauli *et al.* 1999).

Generally, the most important information required for the identification of isomers of monocaffeoylquinic or dicaffeoylquincic acid, is the coupling constant patterns of the H-3, H-4, and H-5 position of compounds where the caffeoyl moiety may attach to the quinic acid moiety. As seen in the figure below, CD_3OD (or methanol-d_4) showed a distinct detection of coupling partners.

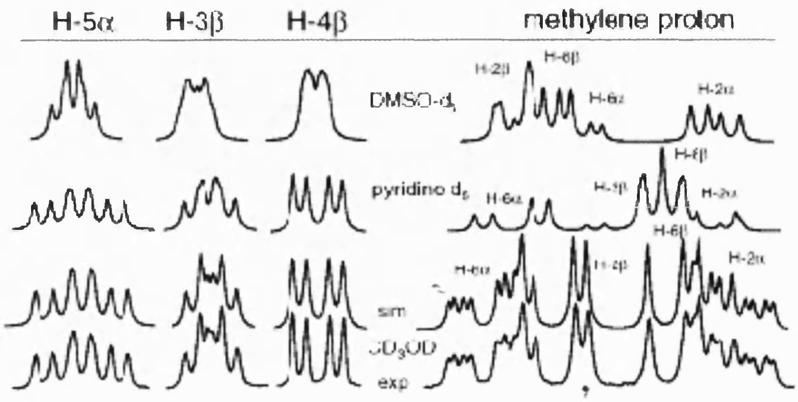


Figure 75 1H NMR spectra of chlorogenic acid in different solvents (Pauli *et al.* 1999).

This study draw a conclusion that methanol-D4 (or CD3OD) should be used as the solvent of first choice, which in the case of solubility problems can be modified with small amounts (<10%) of D_2O and DMSO-d_6. Nevertheless, DMSO-d_6 has to be considered in the case of poorly soluble samples (Pauli *et al.* 1999).

There were also a few studies reported on LC-MS experiments of mono-caffeoyl quinic acid (Clifford *et al.* 2003; 2005) which are more suitable for analyzing this type of compound in term of discriminating between the isomers of chlorogenic acid as well as the isomers of dicaffeoylquinic acid, but as the School of Pharmacy's LC-MS service did not work effectively, this study had to rely on the ESI-MS experiments and the comparison between our 1H NMR data and previous reported NMR as well as MS spectroscopic data.

Pharmacological activities of mono-caffeoyl quinic acid or chlorogenic acid have been reported widely since this compound was found abundantly in many plant species (Gonthier *et al.* 2003). Chlorogenic acid isolated from an antioxidant plant *Lonicera japonica* was found to contribute to the antioxidant activities in the DPPH assay and ferric reducing antioxidant power (FRAP) assay (Wu 2007).

In addition, there is a study on various plant extracts, such as carrot, burdock (gobou), apricot and prune focusing on their inhibitory effects in an *in vitro* assay of lipid peroxide-induced 8-hydroxydeoxyguanosine (8-OH-dG) formation. The major inhibitor purified from various plants extracts was identified as chlorogenic acid. The 8-OH-dG level in the DNA of the rat tongue, the target organ, was significantly reduced in the chlorogenic acid treated group (Kasai *et al.* 2000).

Bandyopadhyay *et al.* (2004) reported that chlorogenic acid induces apoptosis of several Bcr-Abl–positive chronic myelogenous leukemia cell lines and primary cells from myelogenous leukemia patients. In contrast, this compound has no effect on the growth and viability of Bcr-Abl–negative lymphocytic and myeloid cell lines and primary myelogenous leukemia cells. It was concluded that chlorogenic acid inhibits Bcr-Abl tyrosine kinase and triggers p38 mitogen-activated protein kinase–dependent apoptosis in chronic myelogenous leukemic cells.

It was reported that chlorogenic acid could protect against environmental carcinogenesis and suggest that the chemopreventive effects of chlorogenic acid may be through its up-regulation of cellular antioxidant enzymes and suppression of ROS-mediated NF-κB, AP-1, and MAPK activation (Feng *et al.* 2005).

Furthermore, there is an interesting report by Shan et al (2009) who found that chlorogenic acid suppresses LPS-induced COX-2 expression but not iNOS in RAW264.7 cells via attenuating the activation of NF-κB and JNK/AP-1 signaling pathways, suggesting that chlorogenic acid could exert anti-inflammatory effects through inhibiting PGE_2 production. The result from MTT assay showed that chlorogenic acid, even at 50 μg/ml, did not affect cell viability in RAW264.7 cells and no cytotoxicity of chlorogenic acid was detected from dose of 12.5 to 50 μg/ml.

4.3 DISCUSSION ON METHODS USED THIS STUDY

4.3.1 PLANT SELECTION

In the field of ethnobotanical studies, information on the use of the plant in the treatment of diseases can be gathered from intensive field work or from many valuable historical sources. The historical sources might be diaries, travel accounts, and treatises on medicinal plants written by explorers, botanists or physicians etc (De Natale *et al.* 2009) which act as guides to the discovery of potential pharmaceutical agents.

The plant selection in this thesis was based on textbooks which reviewed traditional uses of the plants and people in specific areas. There are interesting studies using similar approaches to intensively describe traditional knowledge and verified such knowledge for the development of medical and pharmaceutical approaches for the treatment of diseases. For example, a study by (Touwaide *et al.* 1997; Leonti *et al.* 2009) using the Greek "Dioscorides; De Materia Medica" which was distributed among healthcare professionals in the past since pre-Linnean times.

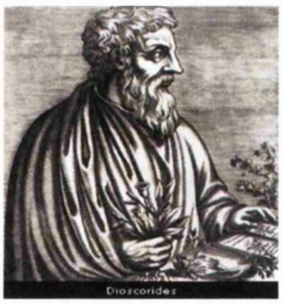


Figure 76 the book De Materia Medica by Dioscorides (ca. AD 40–80); standard guide to drugs until the 17th century, containing around 1000 natural product drugs from the plant kingdom, as well as animals and minerals (Leonti *et al.* 2009)(photos from www.greekmedicine.net and www.artlex.com).

Although textbooks, which are considered as a secondary source, might provide incomplete data or limited descriptions or identifications of species, which sometimes has been a matter of controversy (De Natale *et al.* 2009), these kinds of databases are still a great source for the development of medical and pharmaceutical sciences and also many other fields such as agricultural and environment studies.

This limitation did not exist in this thesis since a majority of the selected plants were reported in the books which were written by a team consisting of at least one academic ethnobotanist/taxonomist (Saralamp *et al.* 2000; Chuakul *et al.* 2000).

4.3.2 METHODS USED IN THE ASSESSMENT OF ANTI-INFLAMMATORY ACTIVITIES; THE NF-KB AND PRO-INFLAMMATORY CYTOKINE INHIBITORY ASSAYS

NF-κB is one of many transcriptional factors that have been a target of drug discovery as already described in the first chapter. Many well-known conventional anti-inflammatory drugs have been assessed for their effect on NF-κB activation and TNF-α formation (Yamamoto & Gaynor 2001). For example, glucocorticoid drugs such as dexamethasone were found to significantly inhibit two sepsis-induced inflammatory mediators, NF-κB and TNF-α, in the early phase of peritoneal sepsis in experimental rats.

Anti-inflammatory natural compounds that have been assessed using NF-κB as a target; were terpenoids including monoterpenoid, sesquiterpenoid, diterpenoid, triterpenoid and carotenoid groups. They were found to suppress the NF-κB pathway (Salminen *et al.* 2008). An NF-κB inhibitory pharmacophore is often associated with adverse side effects. However, NF-κB inhibitors from natural products such as salinosporamide, verracurin and curacin rely on more reactive functional groups such as g-lactams and epoxide rings that have no, or at least only mild side effects (Folmer *et al.* 2008).

4.3.3 METHOD USED IN ASSESSMENT OF CYTOTOXICITY OF THE EXTRACTS: MTT AND XTT

Many extracts showed positive correlations between cytotoxic effects in the XTT assay on leukemia cells and the MTT assay on cervix cancer cells, but for some extracts, the results are contradictory. A possible explanation is that leukemia cells and cervix cancer cells are rather different in their histology and their sensitivity to a variety of chemical compounds present in individual plant extracts. For example, in the case of *Cayratia trifolia* (methanol extract), it seemed to have potent free radical scavenging and high anti-cervical cancer activities but a weak anti-leukemic effect.

These two assays involve the use of tetrazolium salts. There are seemingly only three differences between XTT and MTT methods. Firstly, in both assays, the reagents used to form formazan product in order to determine cell densities are clearly different. Another point is that MTT is metabolized by all cells; therefore, the assay can be used with all cell types, in contrast, this is a major limitation of XTT assay.

XTT is not metabolized by all cell types particularly *Candida spp.* and *yeasts* in which the relationship between cell number and XTT signal is not constant (Kuhn *et al.* 2003). Moreover, XTT formazan derived from XTT in cell culture significantly inhibits the fusion of HIV-1-infected cells with uninfected cells. In other words, XTT formazan itself has anti-HIV-1 activity, thus it is not suitable to use with this cell type (Zhao *et al.* 2002).

However, XTT assay has greater benefits in terms of its formazan product, which is water soluble. As a result, it can be measured easily in supernatants and results can be obtained at multiple time points in the same assay. Advantages of both essays are; the entire assay is performed in a single multiwell plate, therefore, there is no need to transfer the cells, and no radioisotope or organic solvent are required. MTT is best suited for use with adherent cell lines since it is less expensive. XTT is soluble in culture medium, and is therefore suitable for non-adherent and adherent cell lines.

4.3.4 METHODS USED IN THE ASSESSMENT OF ANTIOXIDANT ACTIVITIES OF THE EXTRACTS

Free radical scavenging properties of plants were subjected to intensive examination since it is generally possible to predict the potential NF-κB inhibition and anticancer effects of compounds or extracts. In this study, two antioxidant assays and total phenolics determinations were used.

The advantage of the DPPH assay is that the test is easy, quick and requires only a DPPH reagent and a UV spectrophotometer to perform. Moreover, assays can be based on other time periods during the reaction. However, interpretation of the results is quite complicated when the test compounds have spectra that overlap with DPPH at 515 nm. Carotenoids, in particular, interfere (Noruma *et al.* 1997).

Moreover, the assay is not a competitive reaction because DPPH is both a radical probe and an oxidant. DPPH color can be lost via radical reaction or reduction as well as unrelated reactions, and steric accessibility is a major determinant of the reaction. Thus, small molecules that have better access to the radical site have higher apparent antioxidant capacity with this test (Prior *et al.* 2005).

Lipid peroxidation is a well-established mechanism of cellular damage in biological systems, and is used as an indicator of oxidative stress in cells and tissues. It is a radical-initiated chain reaction with self-propagation in cellular membranes. As a result, isolated oxidative events may have profound effects on membrane function. The reactions of this process involve three distinct steps: initiation, propagation and termination (Kelly *et al.* 1998). Antioxidants, therefore, will act as a scavenger to inhibit the peroxidation processes.

Although much controversy has appeared in the literature regarding the specificity of TBARS toward compounds other than MDA, it remains the most widely employed assay used to determine lipid peroxidation. The TBARS test can work reasonably well when the systems are controlled and defined such as liposomes and microsomes (Armstrong & Browne 1994). In this thesis, the factors that may interfere with the results of the TBARs test, such as proteins and lipids, were controlled throughout the experiment.

Although a wide variety of antioxidant assays have been used in the examinations of antioxidant properties of plants, methods which directly identify specific molecular target and pathways are still limited. There are no magic antioxidant assays which specifically determine antioxidant effects and their mechanism of action at this moment. However, the limitations of each method have been considered throughout the study.

4.4. DISCUSSION ON METHODS USED IN PHYTO-CHEMICAL INVESTIGATIONS

4.4.1 PLANT EXTRACTION

In this study, the leaves of *G. pseudochina var. hispida* were ground using a laboratory scale mill and then extracted with three solvents of increasing polarity: petroleum ether, ethay acetate and methanol, using a cold extraction method. The cold extraction was achieved by adding the ground plant material and the selected solvent into a conical flask which were mixed by a magnetic stirrer apparatus. After 15 min of extraction, the resulting solution was removed and filtered. Then the remaining materials on the filter paper were scraped back to into the flask to minimize the lost of material and this was extracted again in the same manner for three times in total.

Although percentages yields obtained from the extraction were relatively low, this extraction method has an advantage over the Soxhlet extraction in that, compounds from cold extraction do not degrade or change their structures. Soxhlet extraction has been a popular method in phytochemical research since the recovered amount after extracted is usually greater than using cold extraction. However, prolonged heating of plant materials possibly leds to degradation and decomposition or even results in synthetic compounds, and has been questionable. As a result, in this study, we avoided using heat to maximizing the percentages of yield.

4.4.2 LIQUID – LIQUID PARTITIONING IN SEPARATING COMPOUNDS

Liquid- liquid partitioning is very useful in separating polar and non-polar components and is straightforward requiring only suitable solvents and separating funnels. However, the two solvents used for this method should have different partition coefficients in order to maximize the separation. This method was found to be a very useful in separating the contents of the methanol extract of *G. pseudochina var. hispida*, especially the red and purple compounds (soluble in water), which could be separated out from the contents soluble in organic solvents.

In this study, dichloromethane-water or chloroform-water was used. Methanol was used minimally, only for dissolving the extract before adding the two solvents, otherwise the separation might be difficult since methanol can dissolve both non polar and polar compounds and result in the compounds not separating much, as well as the compounds tend to remain as a layer between the two phases.

Also it has been found experimentally that using a small amount of solvent to extract each time, but to do the separation repeatedly, resulted in a better separation than using a large amount of solvent but extracting only once or twice. To use large amount of solvent, not only did not increase separation, but this can also create a problem in drying process at later stages. For large samples with high water contents, there is no need to add further water in a washing step, as this procedure uses smaller volumes of organic solvents; it has proved to be both economical and convenient (Christie 1997).

4.4.3 SEPHADEX GEL FILTRATION CHROMATOGRAPHY

Sephadex gel filtration separates compounds by a size-exclusion method. This means that the large sized compounds were eluted out first. The smaller sized compounds which can be trapped by Sephadex's beads remained in the column and can be eluted out later using an appropriate solvent. The disadvantage of this method is that Sephadex is very expensive when compared to silica gels. Also the column-packing process needs extra care and it is time consuming since the gel needs to be well settled and stable before use.

In this study, Sephadex gel was packed into an appropriate sized column and was allowed to sit, for at least two days in the column with a suitable solvent prior to use. It has been experimentally found in this study that if Sephadex gels were used repeatedly for more than 2-3 times or if they were left to dry or kept in inappropriate solvents, the pore-size of the beads may change, resulting in poorer separation in the latter experiments compared to new Sephadex.

4.4.4 THIN LAYER CHROMATOGRAPHY

Thin layer chromatography was used to identify the number of compounds present in the extract as well as separate the compounds in the later stage of phytochemical investigation of the active NF-κB inhibitors. It is crucial to choose an appropriate type of TLC plate. Once the mixture of polar compounds were applied onto the normal-phase TLC, all the polar compounds were bound tightly to the stationary phase and could not be recovered, although excessive solvent was used to wash the compounds out of the scraped out materials.

It has been a concern that after scraping off the compound and submitted them for NMR or MS experiments, the impurities from the TLC such as fluorescence detective agents, might interfere as unidentified peaks in the spectra. However, this problem has been controlled in this thesis. Every time the compounds were scraped off the TLC plates, the TLC plate (silica gel) was submitted in parallel, in order to identify if there may be peaks of impurities appearing in the spectra. The solvents used were also submitted for the same reason.

5. GENERAL CONCLUSION

Ethnopharmacological knowledge is still beneficial in guiding which plants may have the potentials to yield anti-inflammatory and/or anticancer products. In this thesis, *Gynura pseudochina var. hispida* (Asteraceae), *Oroxylum indicum* (Bignoniaceae), and *Muehlenbeckia platyclada* (Polygonaceae) were found to possess anti-inflammatory activity and could serve as leads for the development of future anti-inflammatory drugs. Also *Rhinacanthus nasutus* (Acanthaceae) and *Pouzolzia indica* (Urticaceae) might yield novel natural compounds as anticancer products.

Interestingly, multidrug-resistant, P-glycoprotein expressing CEM/ADR5000 cells reveal high levels of resistance to doxorubicin, vinblastine, paclitaxel and many other established anti-cancer drugs (Efferth et al. 2008b), but no or only weak cross-resistance was found to the present panel of Thai medicinal plants. This suggests that the plant extracts might yield valuable adjuncts for use in standard chemotherapy in the cases of drug-resistance and refractory tumours (Siriwatanametanon et al. 2010).

This thesis provides evidence for the use of Thai plants and the findings provide a new insight into understanding the anti-inflammatory activities of a panel of traditionally used Thai plants, most importantly *Gynura pseudochina var. hispida.* The active compounds isolated from the methanol extract of *G. pseudochina var. hispida* were identified as quercetin-rutinoside, dicaffeoylquinic acid and monocaffeoylquinic acid derivatives. The results obtained support the uses of the plants as anti-inflamatory remedies and are in agreement with previous reports.

It should be noted that the results from in vitro tests might not guarantee in vivo activity because many factors may be responsible for the activity. Adsorption and metabolism are the most important factors that make the in vitro and in vivo systems so much different (Houghton et al, 2007). Therefore, what might be done in the future for this project is an in vivo study or clinical study if the plants of interest have been used in human for long time and has no evidence of their toxicity.

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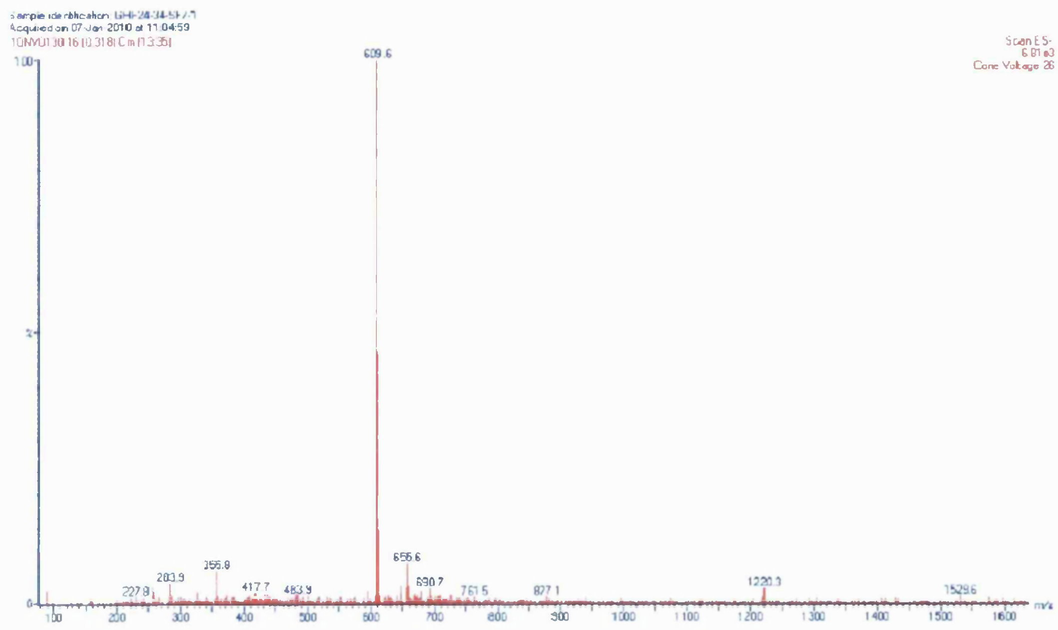
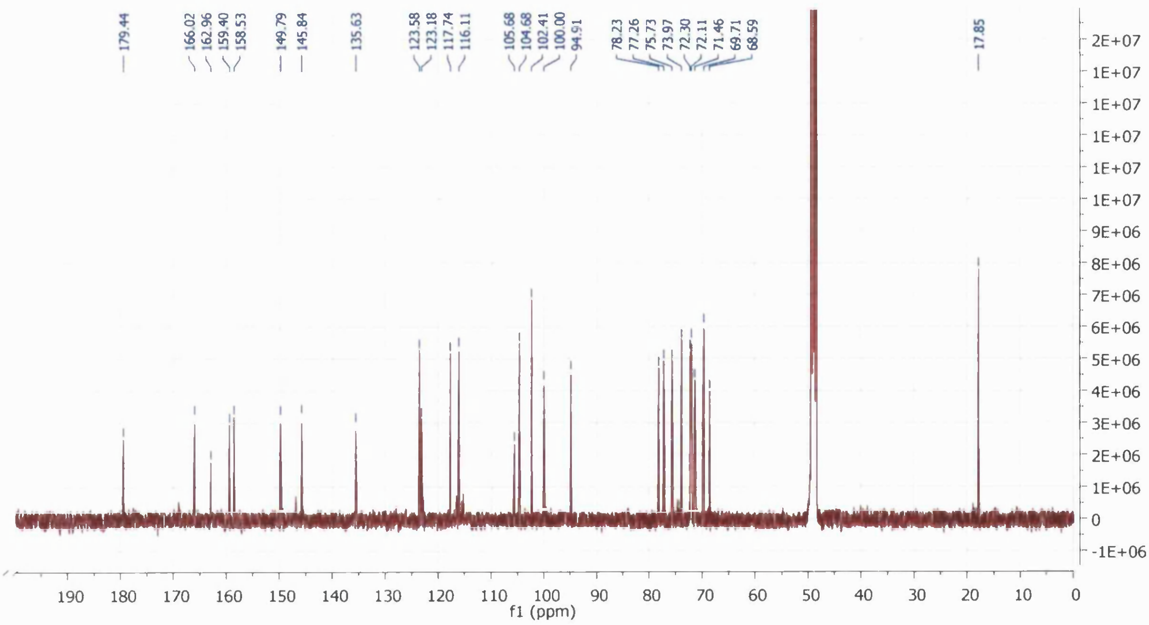
APPENDIX

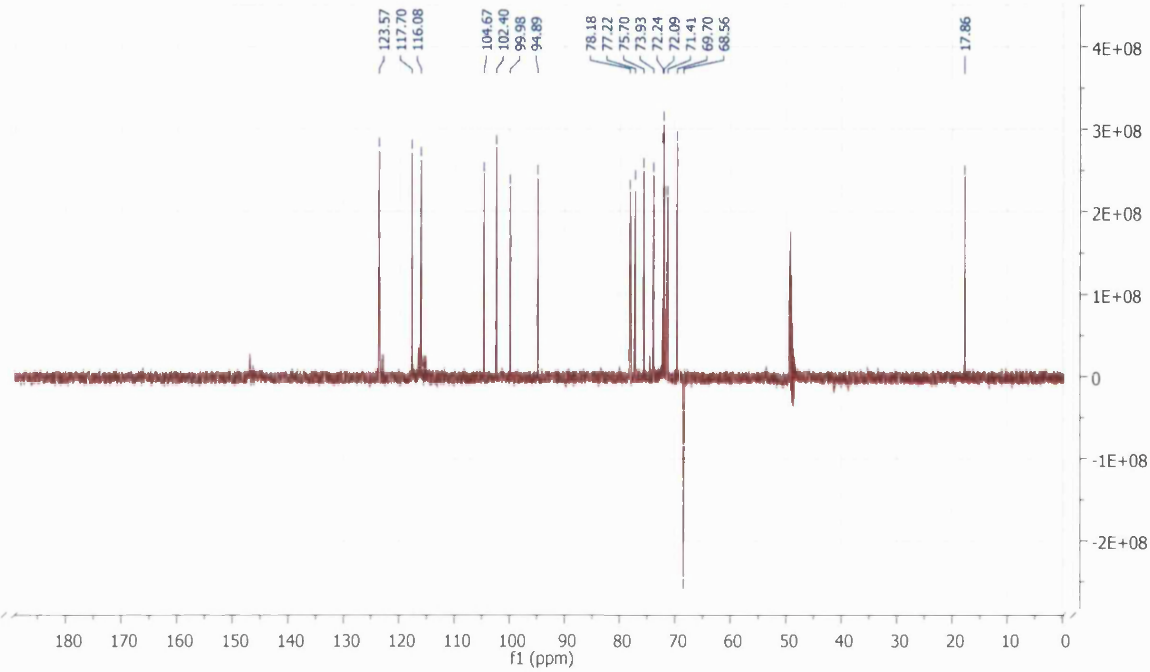
ESI-MS OF COMPOUND SF7-2 (NEGATIVE MODE)



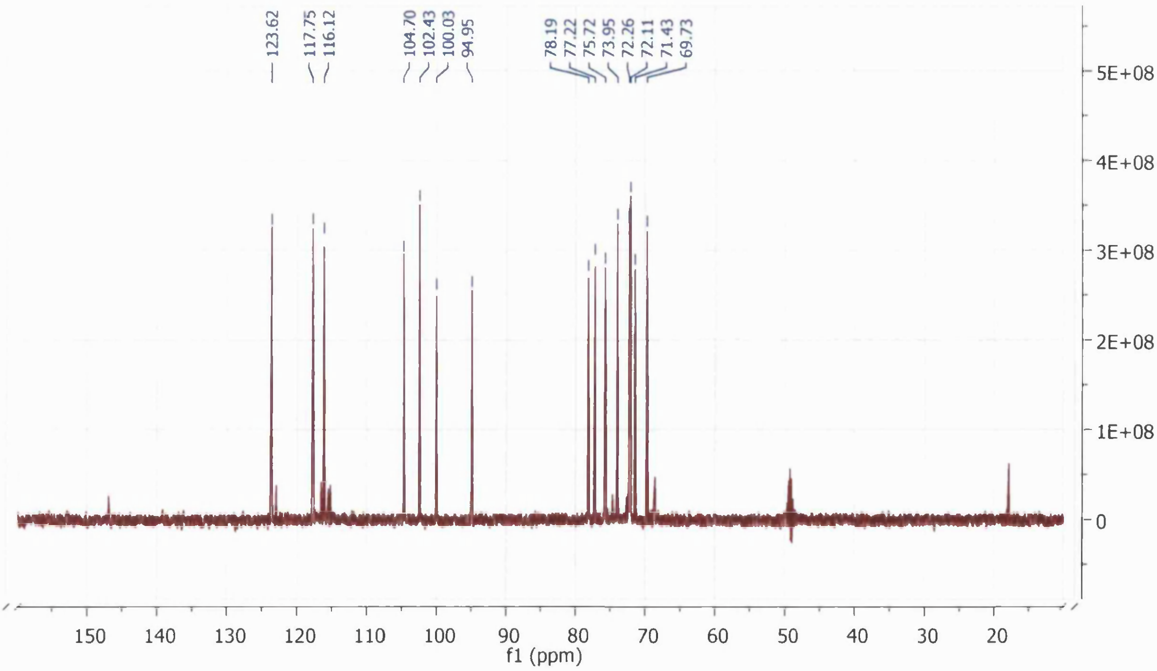
C13 NMR OF COMPOUND SF7-2 (IN METHANOL-D4) 126 MHZ



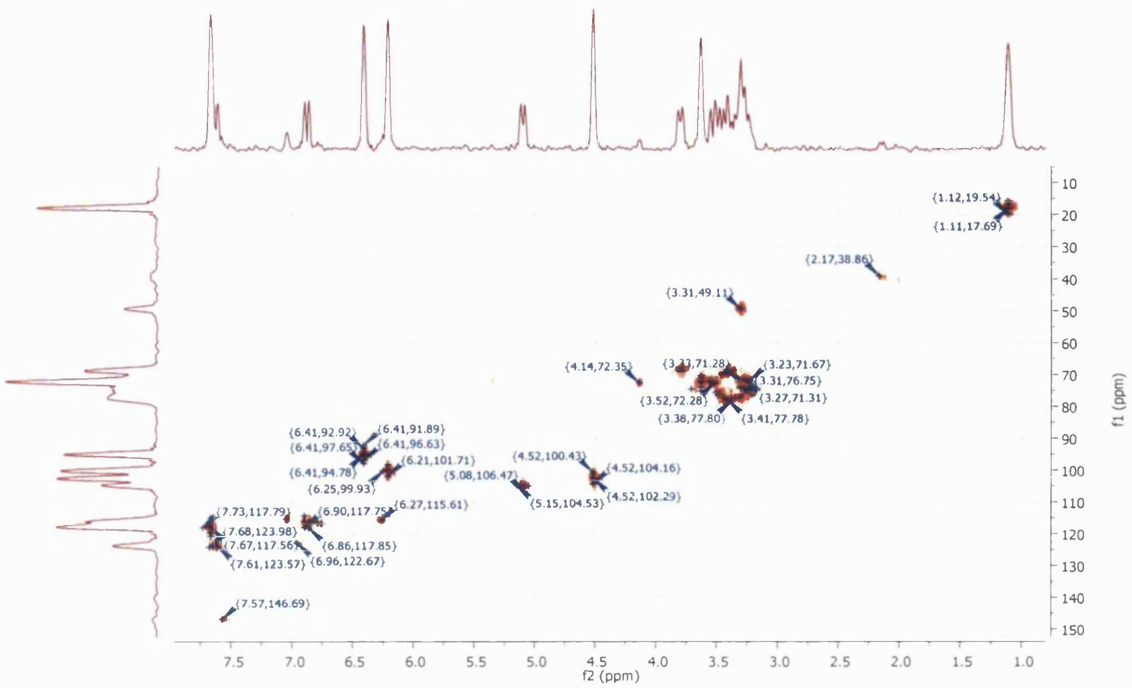
DEPT-135 OF COMPOUND SF7-2 (IN METHANOL-D4) 126 MHZ



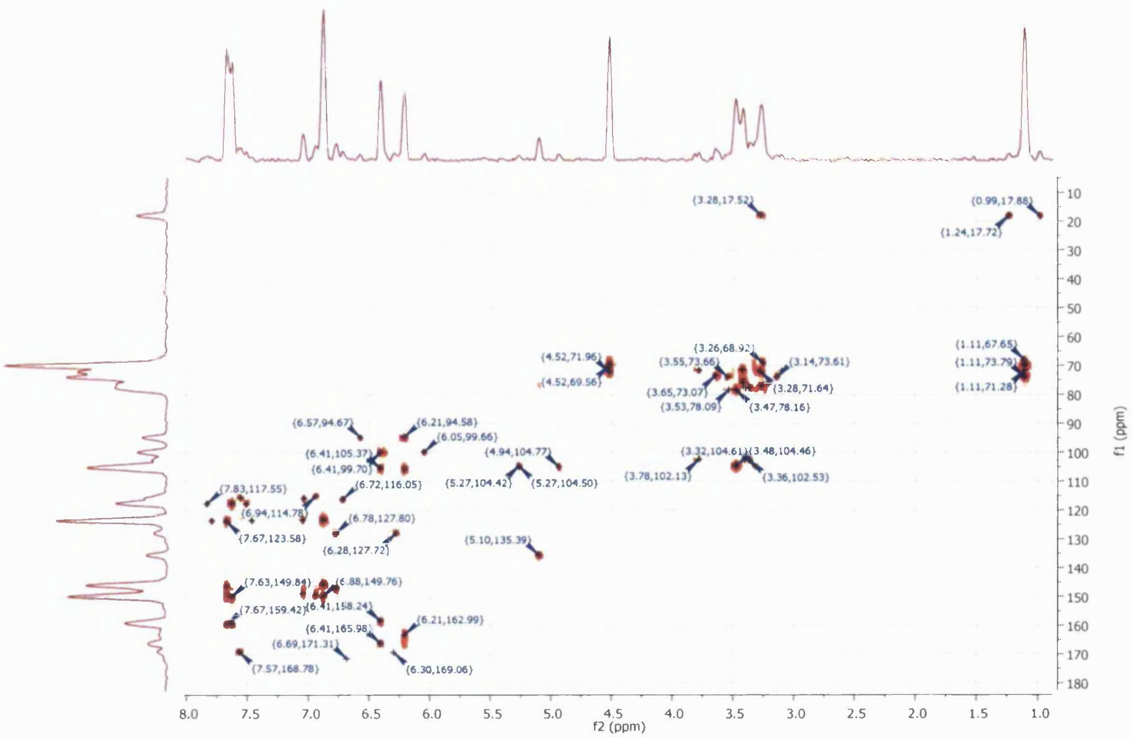
DEPT-90 OF COMPOUND SF7-2 (IN METHANOL-D4) 126 MHZ



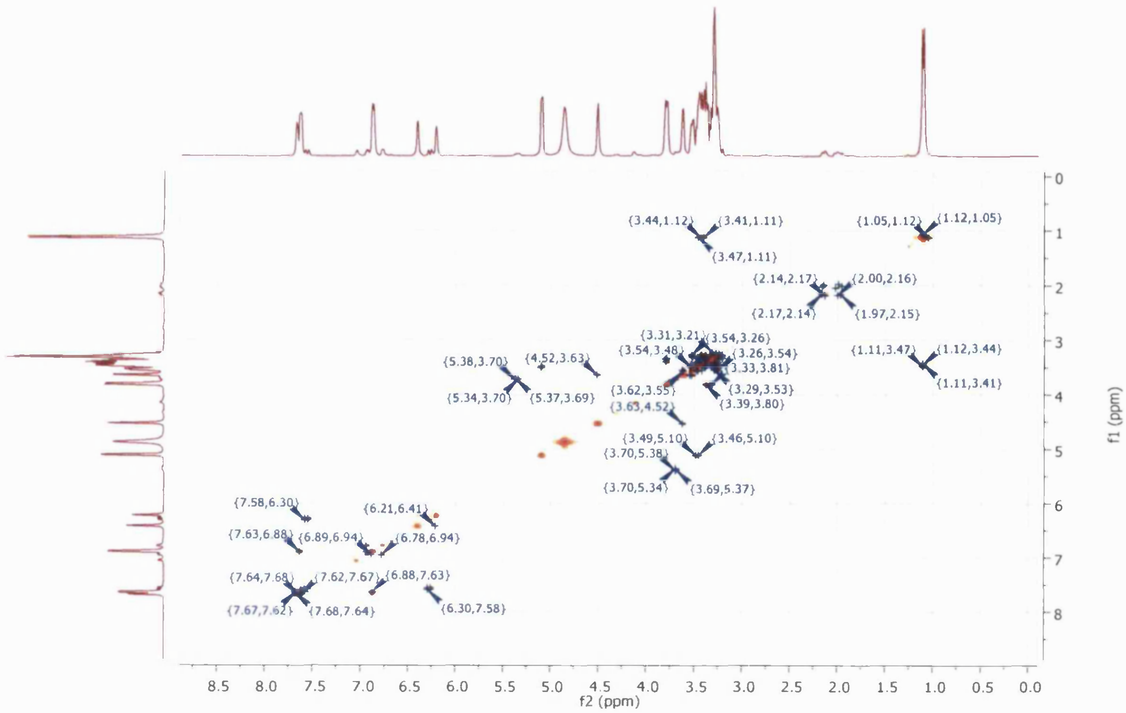
HMQC OF COMPOUND SF7-2 (IN METHANOL-D4) 126 & 500 MHZ



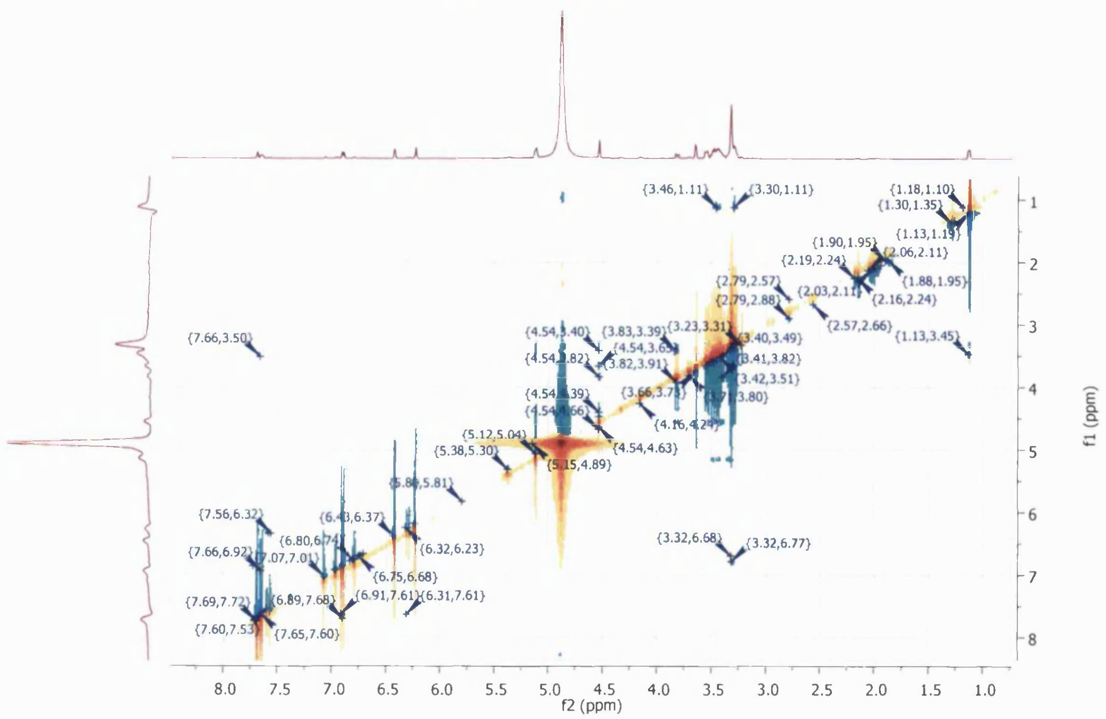
HMBC OF COMPOUND SF7-2 (IN METHANOL-D4) 126 & 500 MHZ



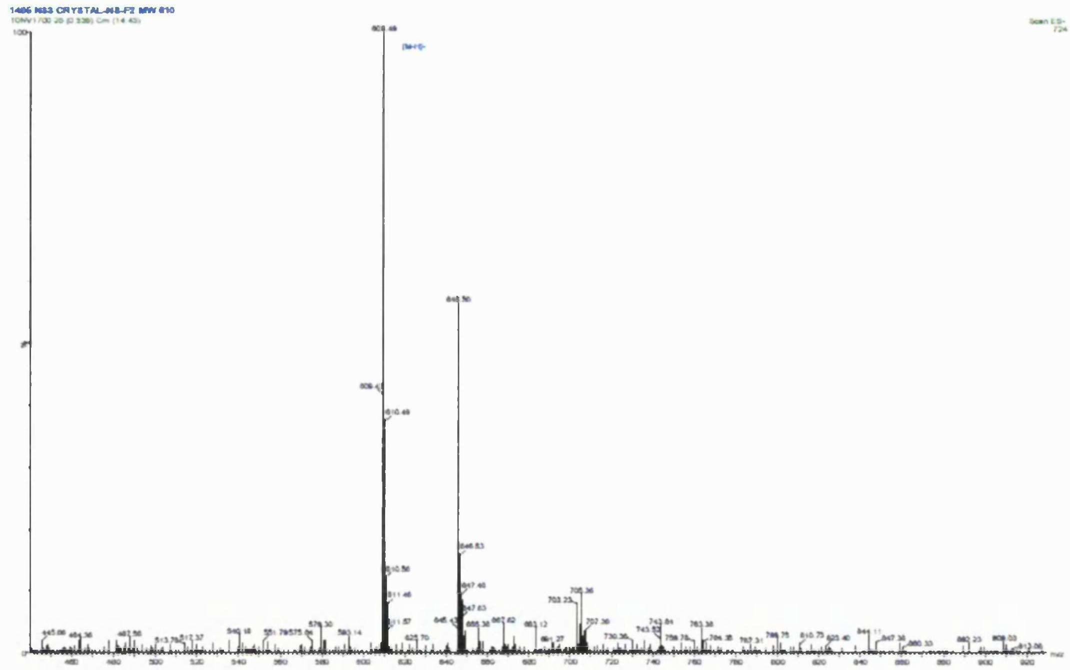
COSY OF COMPOUND SF7-2 (IN METHANOL-D4) 500 MHZ



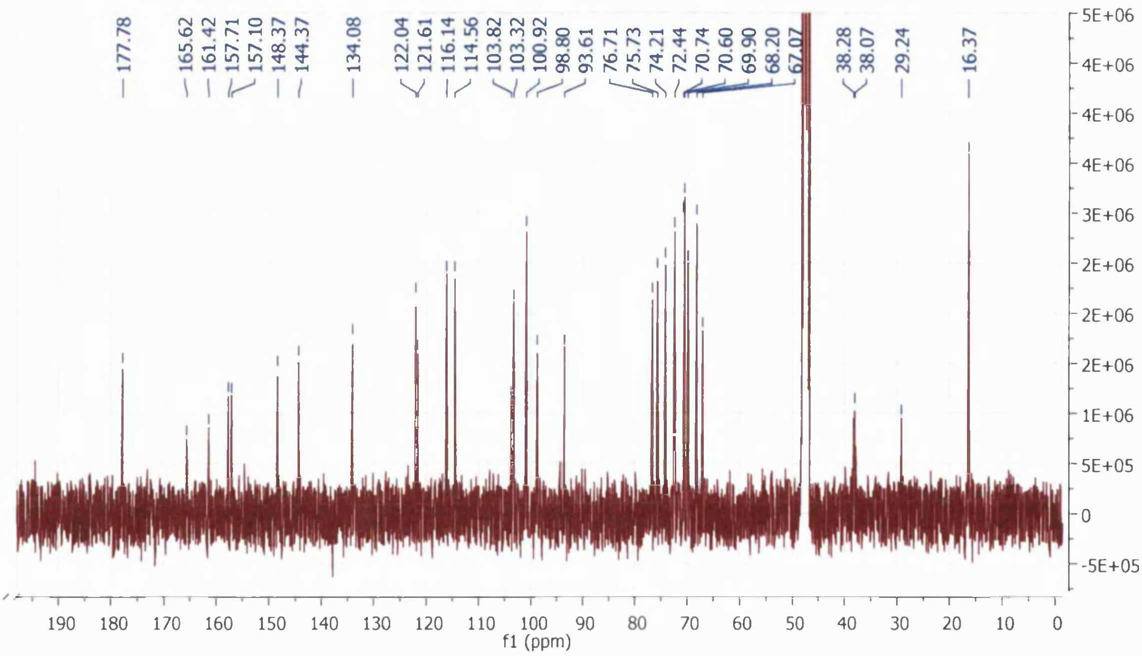
NOESY OF COMPOUND SF7-2 (IN METHANOL-D4) 500 MHZ



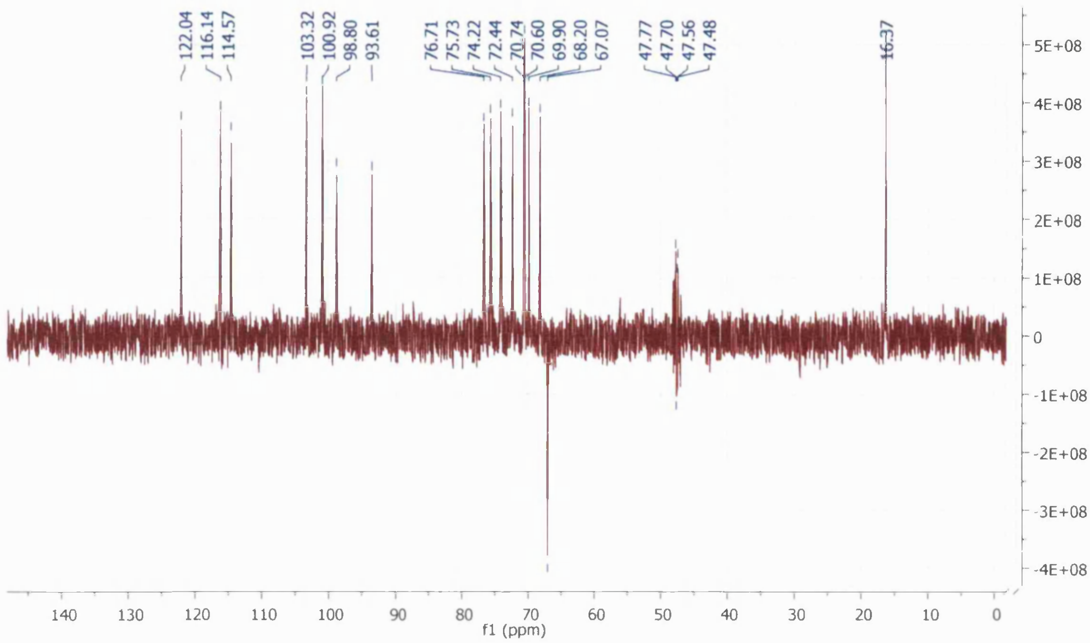
ESI-MS OF COMPOUND SF7-1 (NEGATIVE MODE)



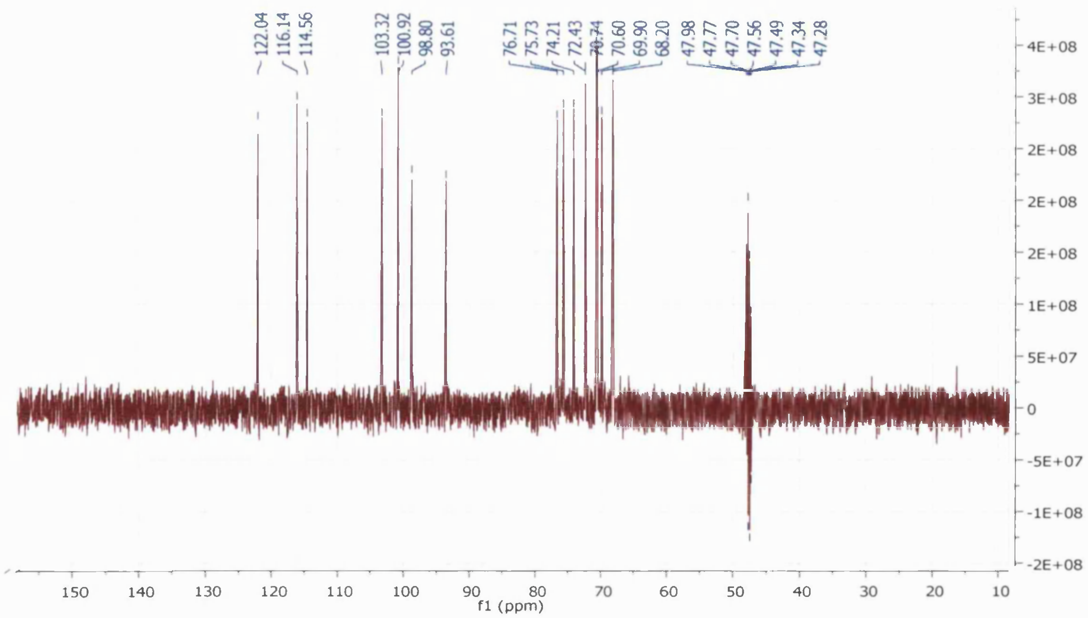
C13 OF COMPOUND SF7-1 (IN METHANOL-D4) 100 MHZ



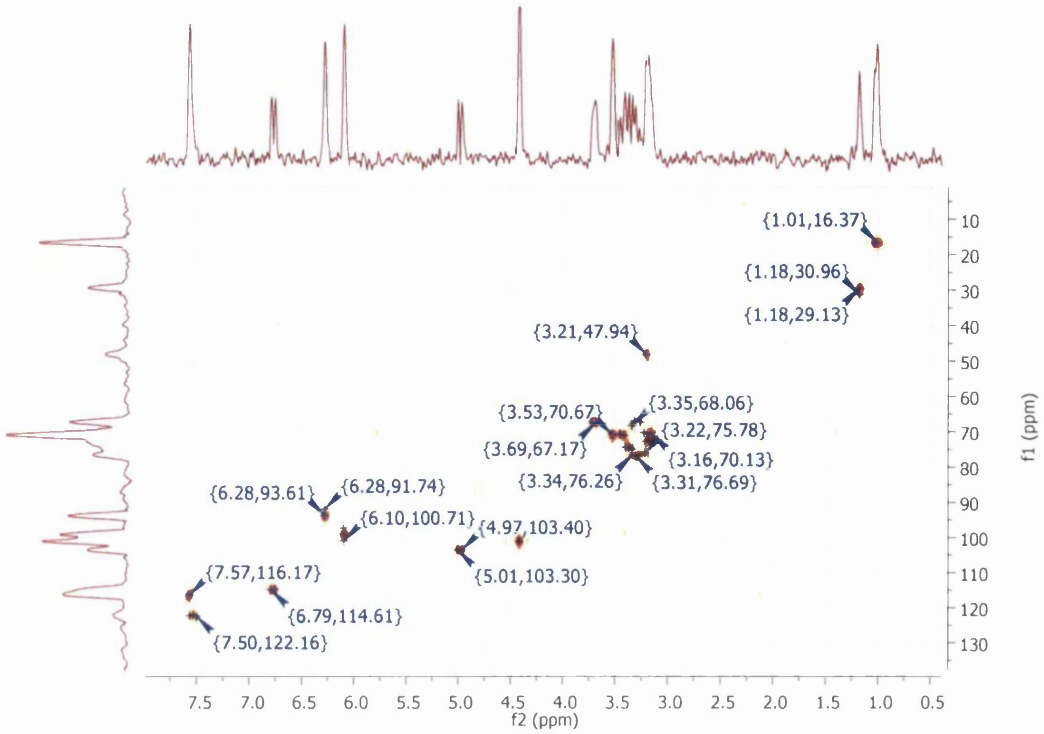
DEPT-135 OF COMPOUND SF7-1 (IN METHANOL-D4) 100 MHZ



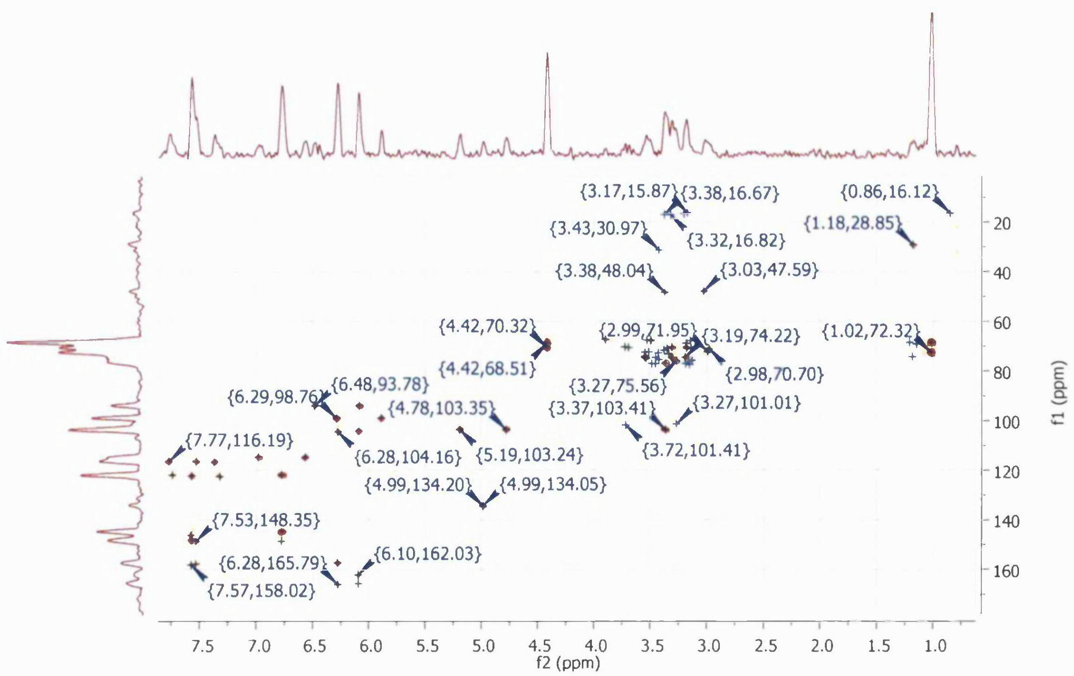
DEPT-90 OF COMPOUND SF7-1 (IN METHANOL-D4) 100 MHZ



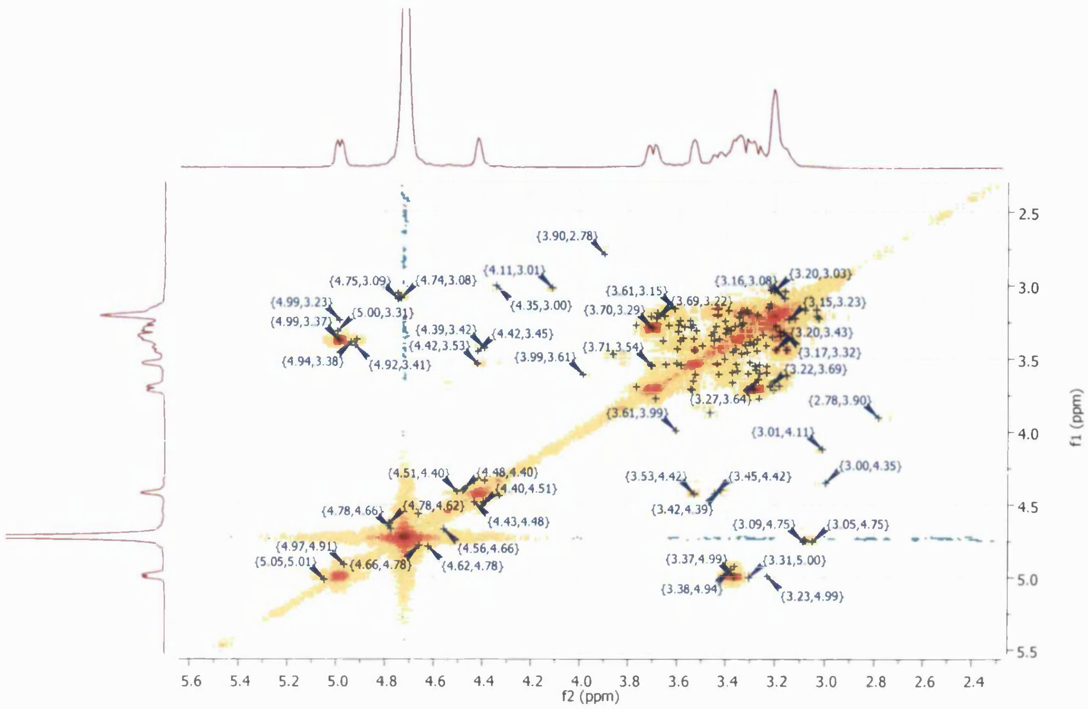
HMQC OF COMPOUND SF7-1 (IN METHANOL-D4) 100 & 400 MHZ



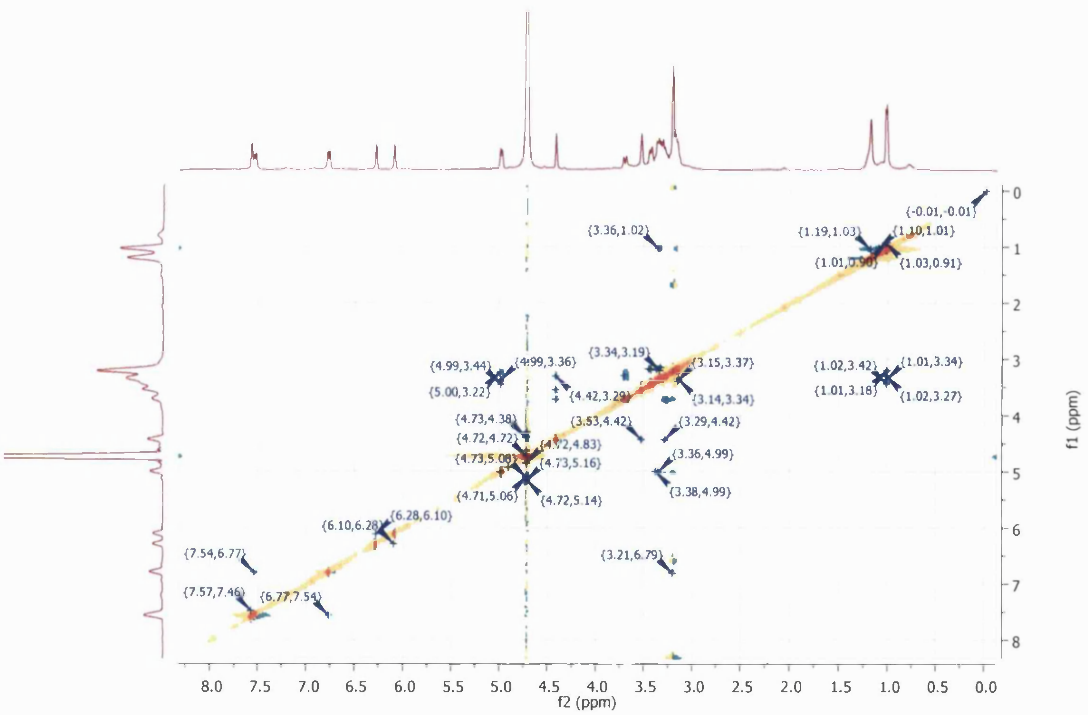
HMBC OF COMPOUND SF7-1 (IN METHANOL-D4) 100 & 400 MHZ



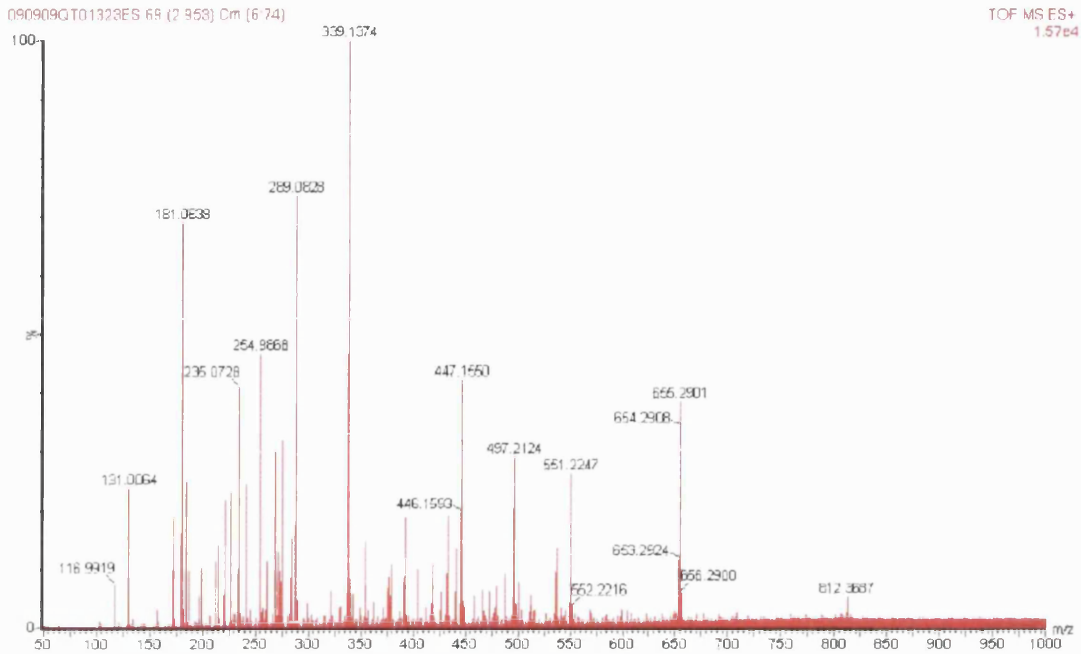
COSY OF COMPOUND SF7-1 (IN METHANOL-D4) 400 MHZ



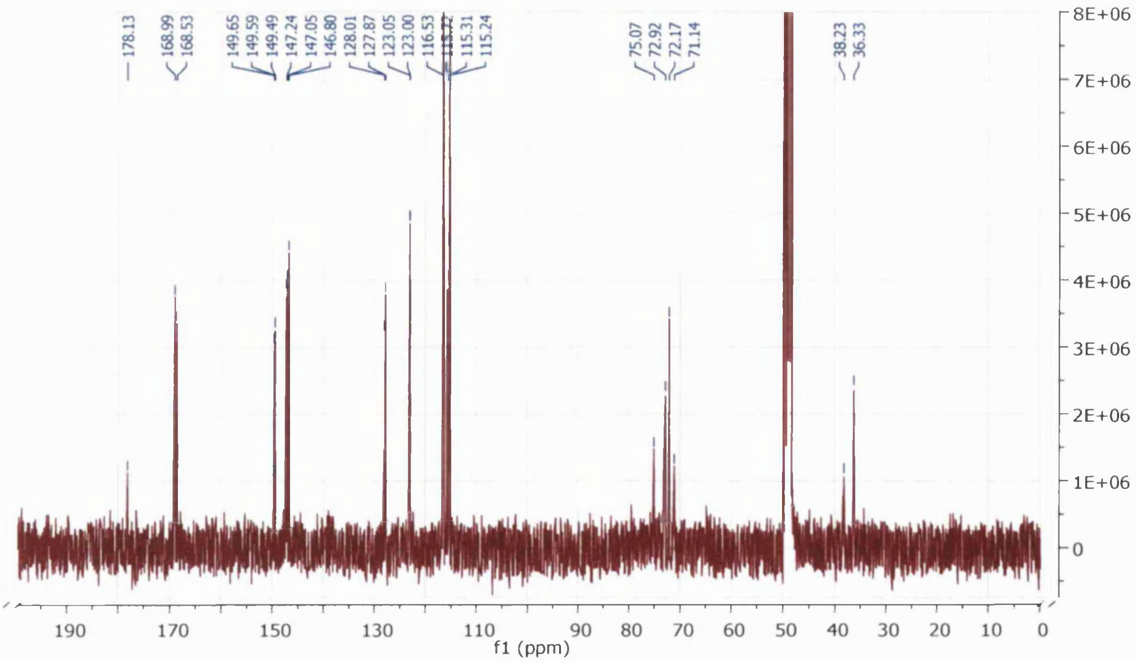
NOESY OF COMPOUND SF7-1 (IN METHANOL-D4) 400 MHZ



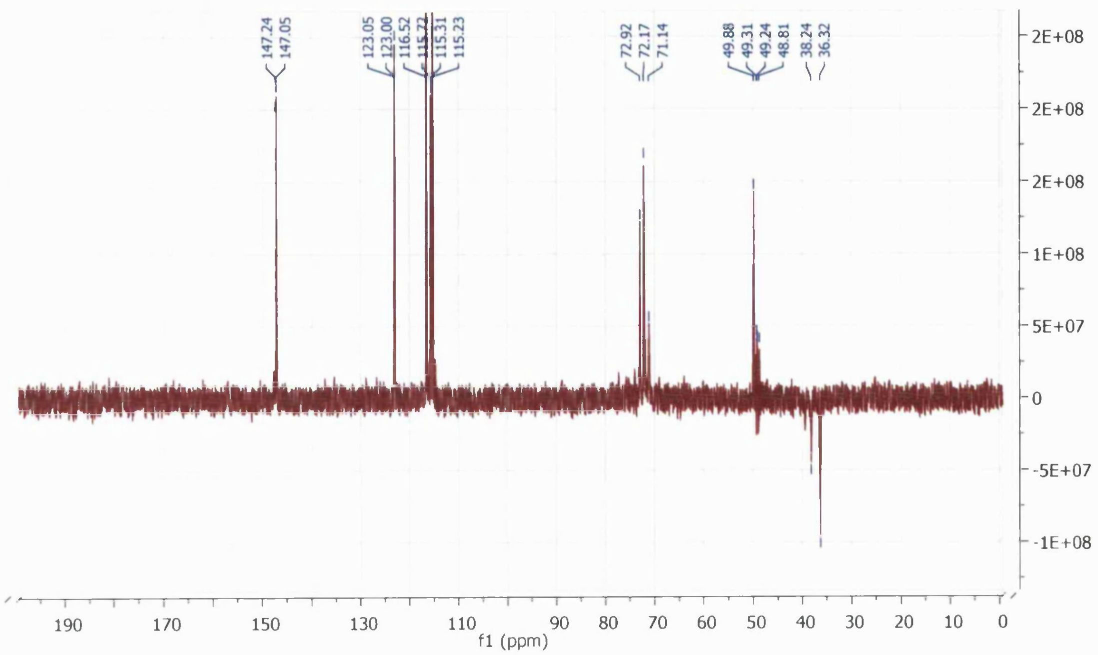
ESI-MS OF COMPOUND SF10 (POSITIVE MODE)



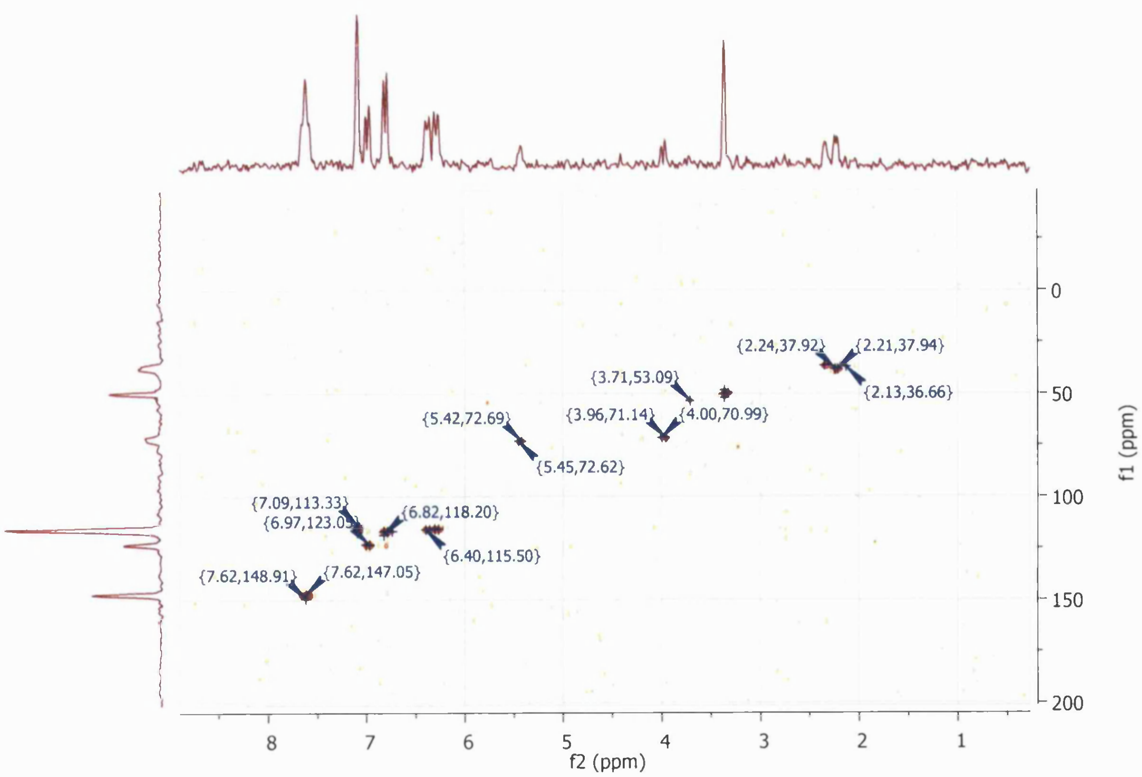
1C3 NMR OF COMPOUND SF10 (IN METHANOL-D4) 100 MHZ



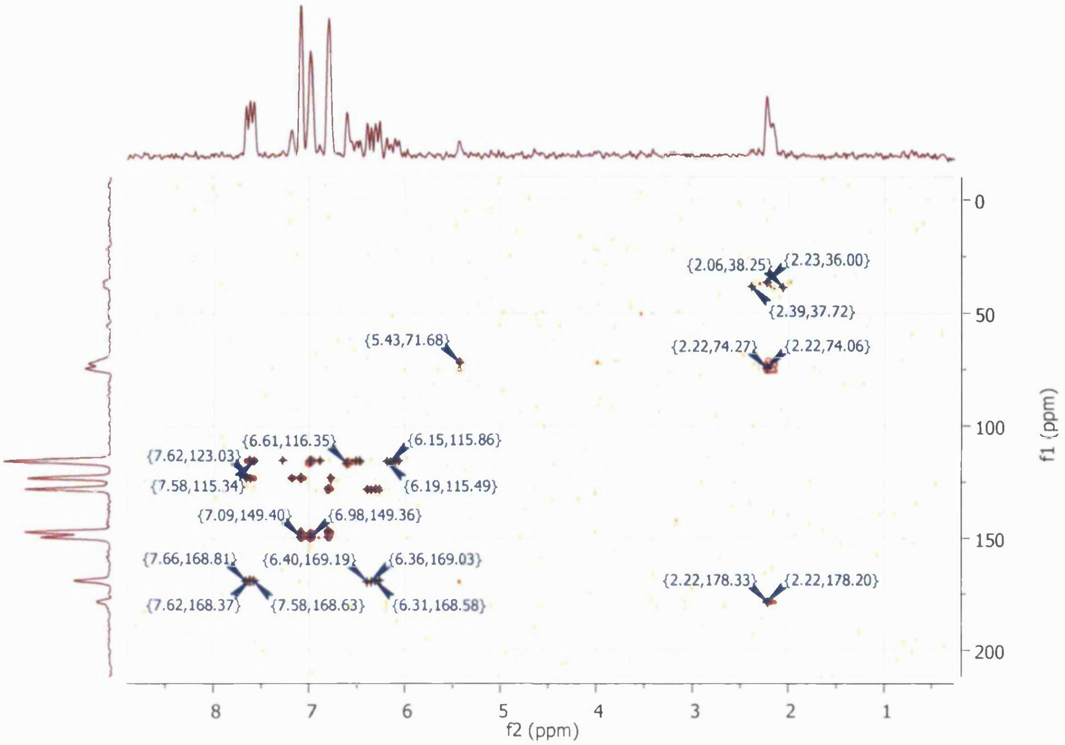
DEPT-135 OF COMPOUND SF10 (IN METHANOL-D4) 100 MHZ



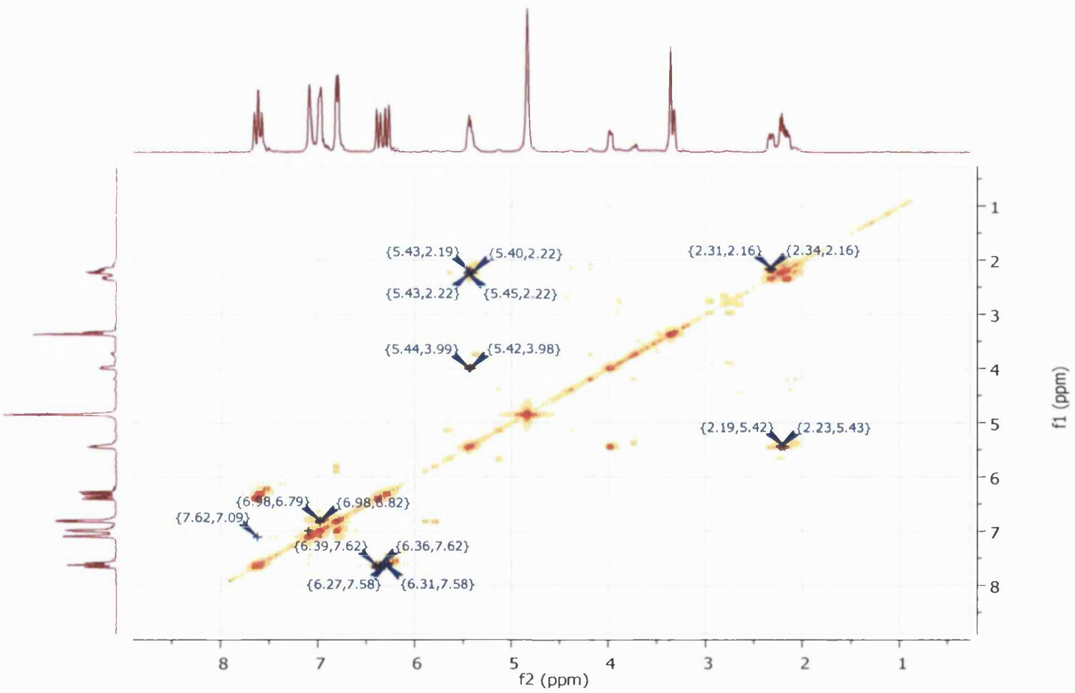
HMQC OF COMPOUND SF10 (IN METHANOL-D4) 100 & 400 MHZ



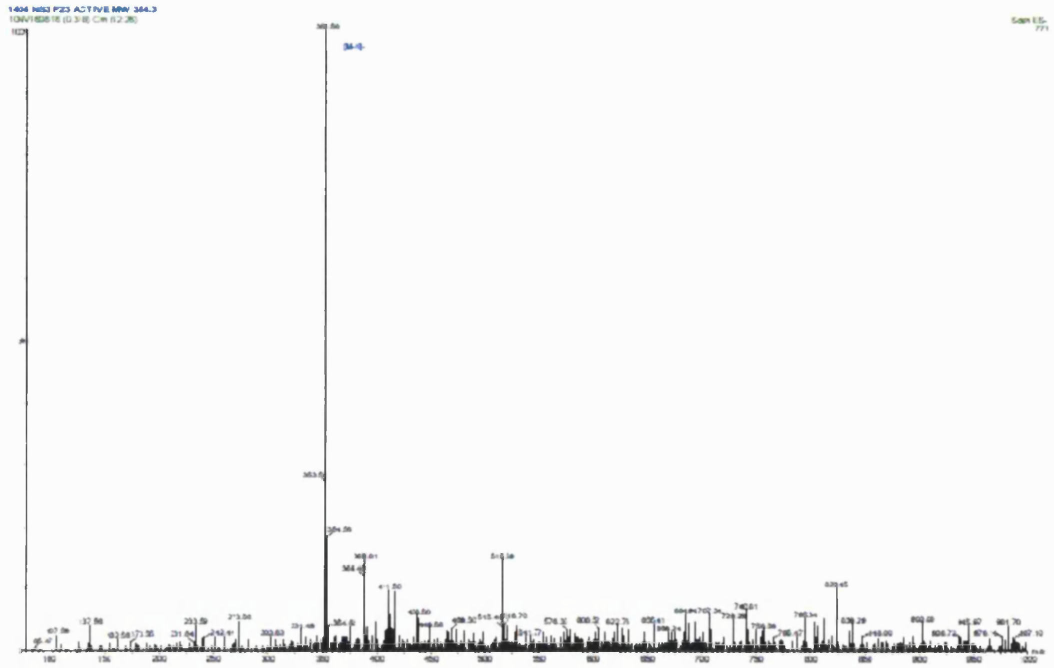
HMBC OF COMPOUND SF10 (IN METHANOL-D4) 100 & 400 MHZ



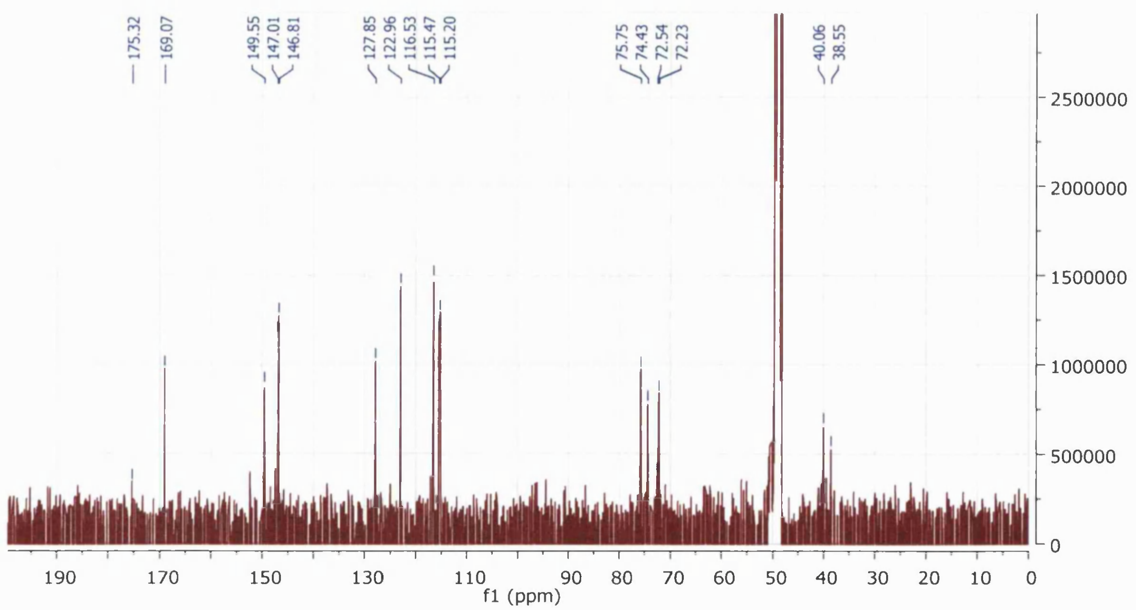
COSY OF COMPOUND SF10 (IN METHANOL-D4) 400 MHZ



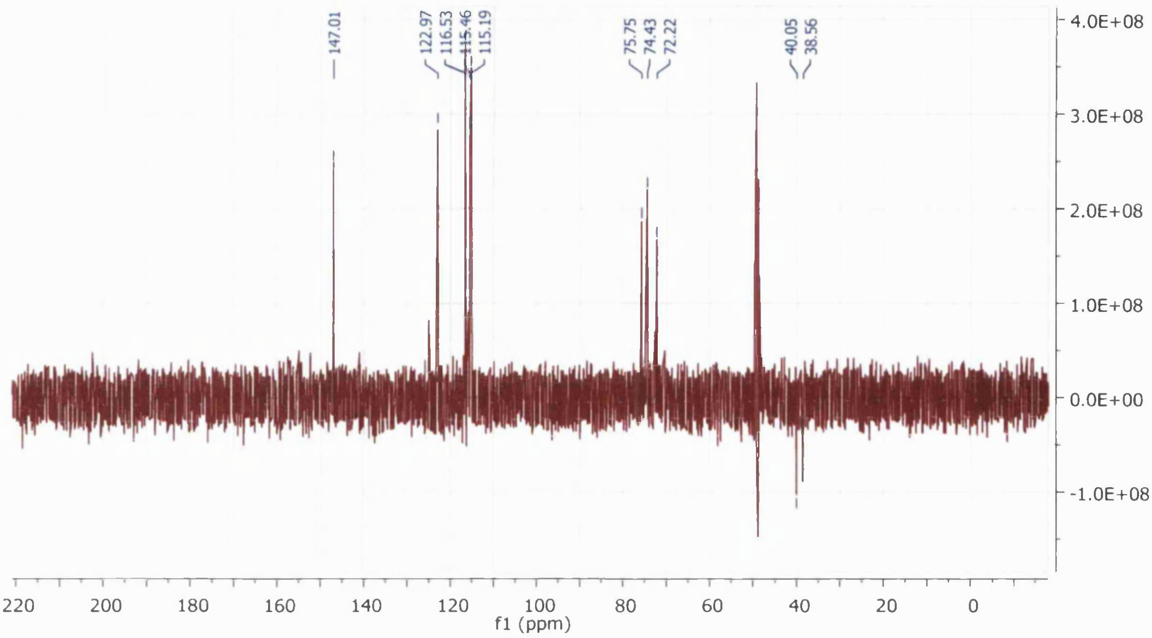
ESI-MS OF COMPOUND SF11 (NEGATIVE MODE)



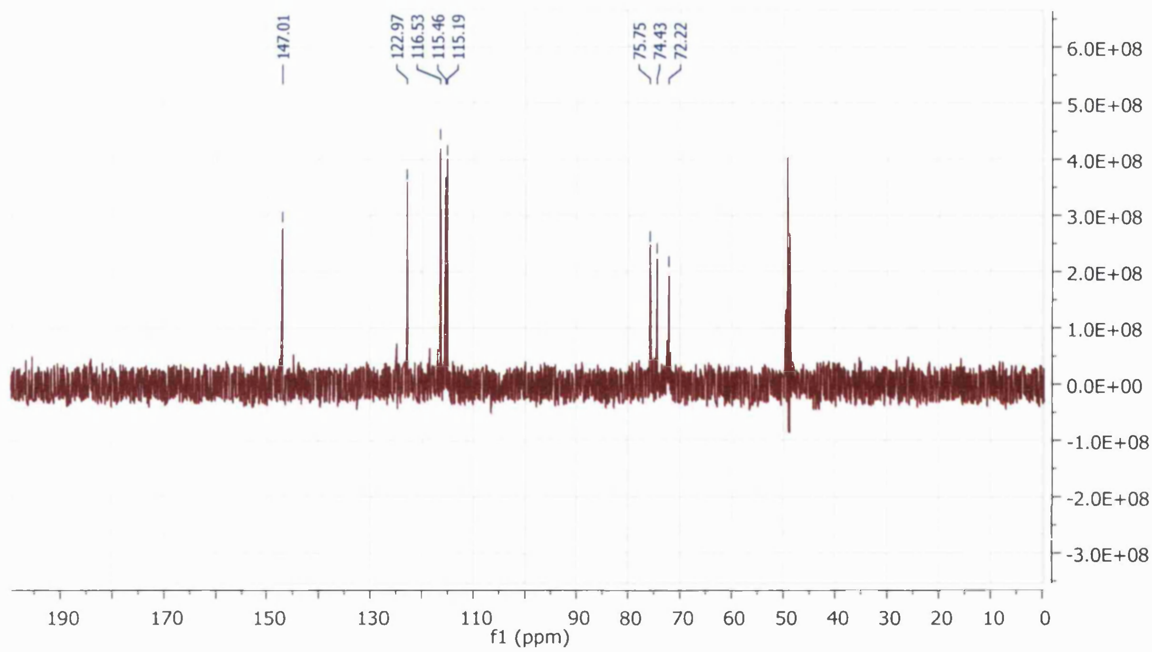
1C3 NMR OF COMPOUND SF11 (IN METHANOL-D4) 100 MHZ



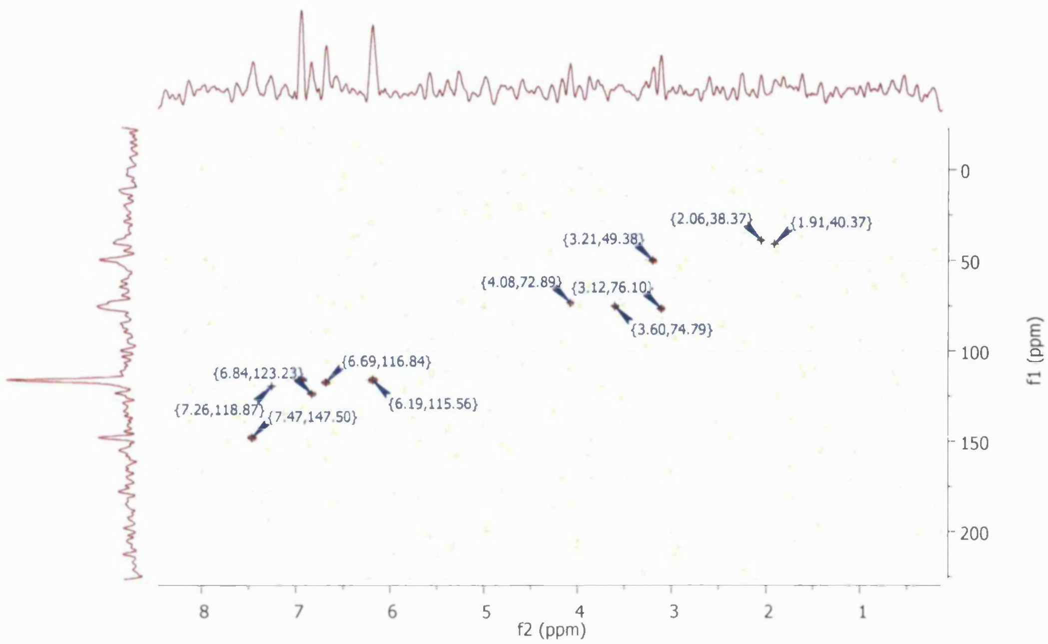
DEPT-135 OF COMPOUND SF11 (IN METHANOL-D4) 100 MHZ



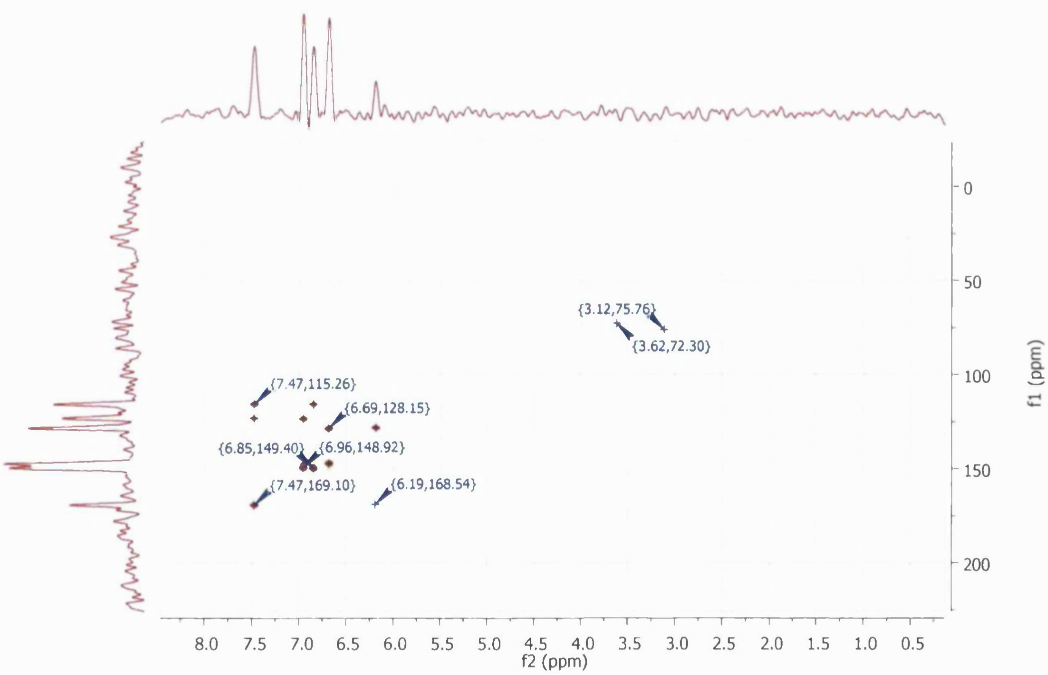
DEPT-90 OF COMPOUND SF11 (IN METHANOL-D4) 100 MHZ



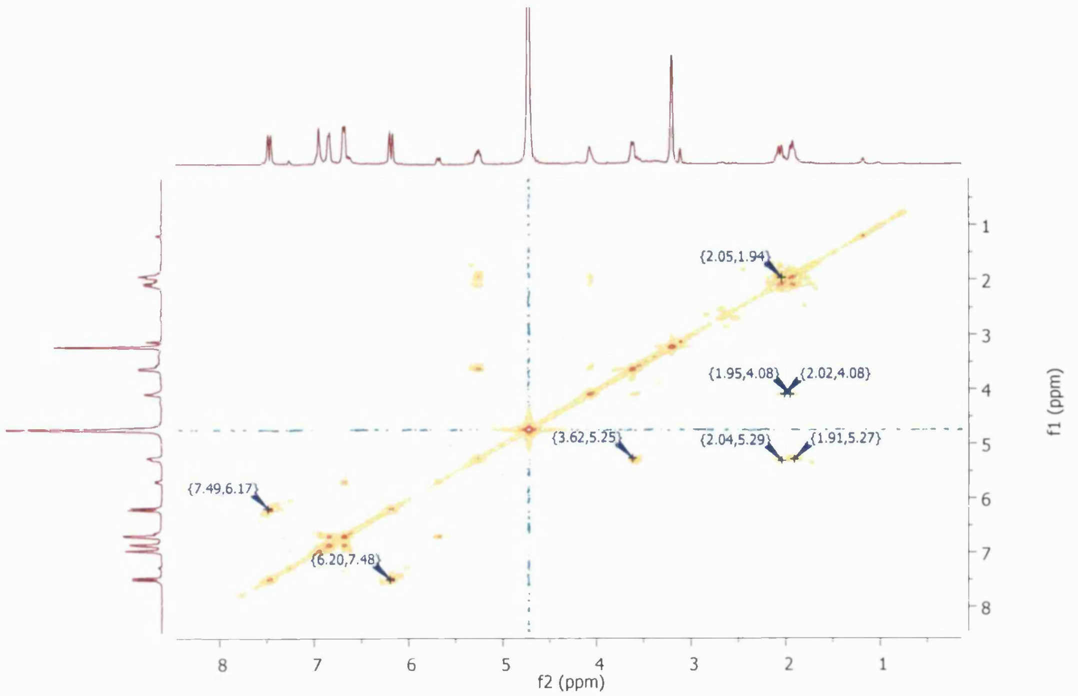
HMQC OF COMPOUND SF11 (IN METHANOL-D4) 100 & 400 MHZ



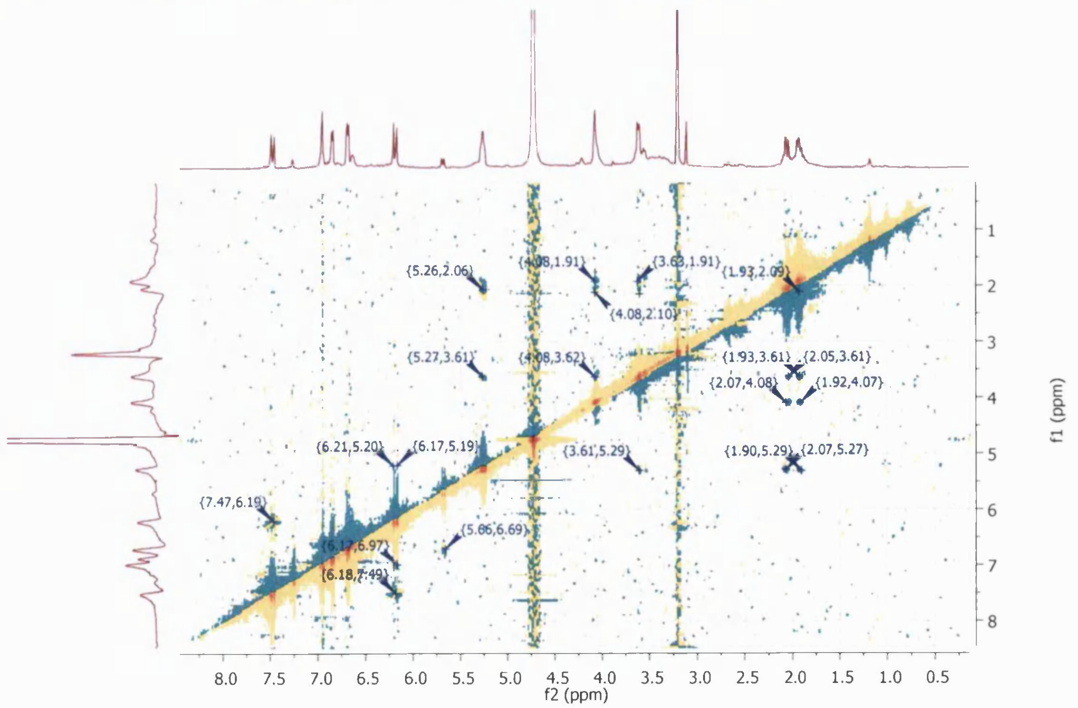
HMBC OF COMPOUND SF11 (IN METHANOL-D4) 100 & 400 MHZ



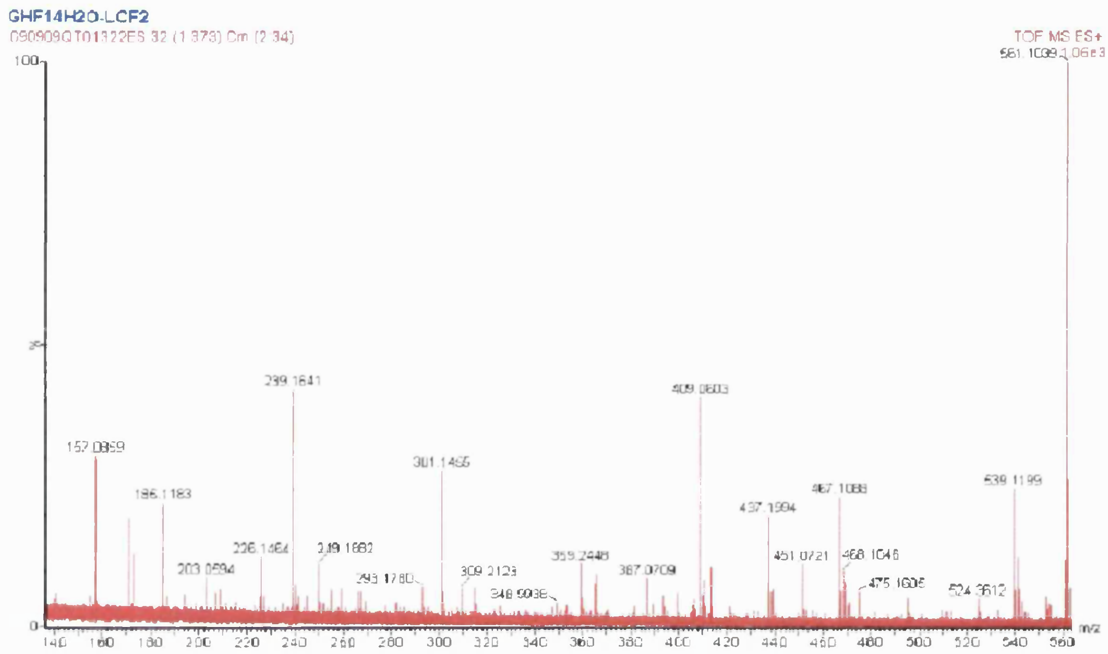
COSY OF COMPOUND SF11 (IN METHANOL-D4) 400 MHZ



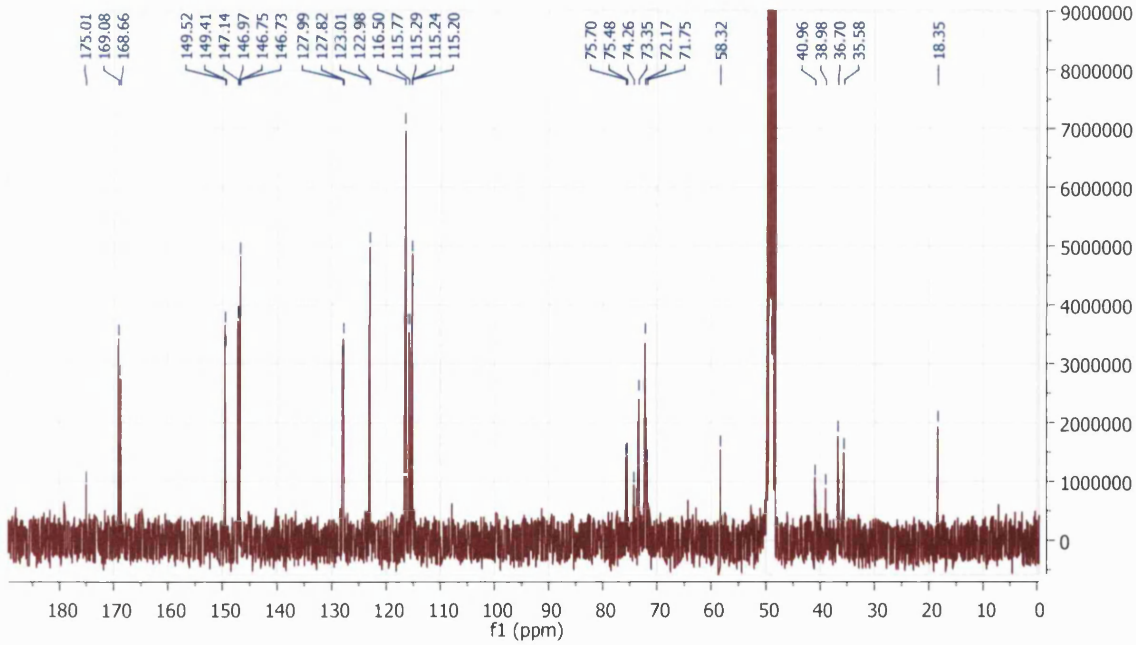
NOESY OF COMPOUND SF11 (IN METHANOL-D4) 400 MHZ



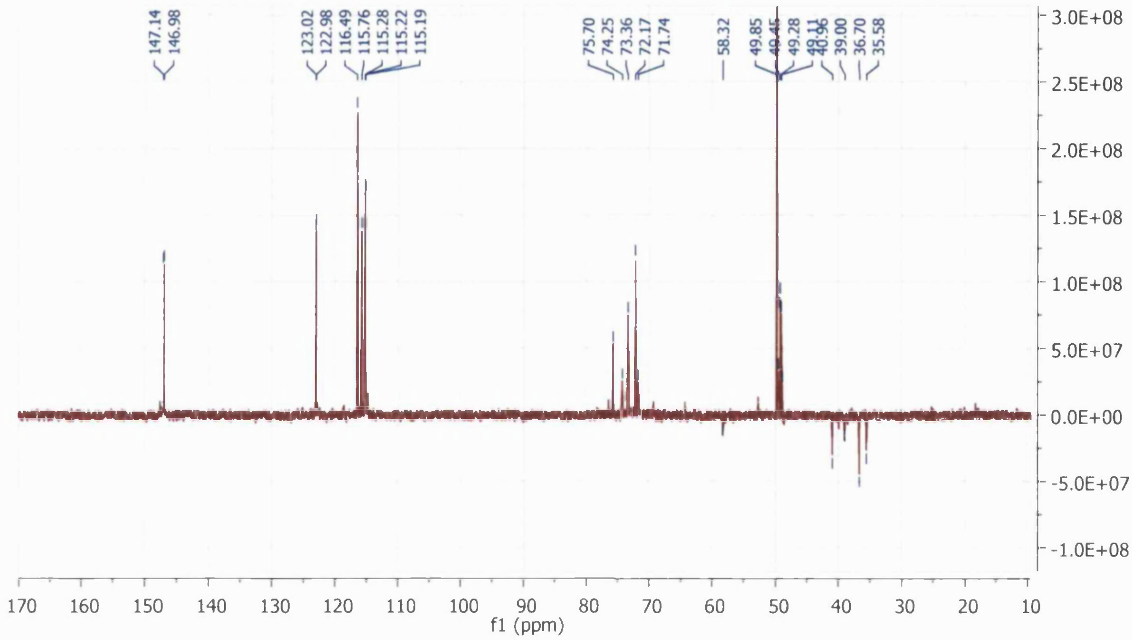
ESI-MS OF COMPOUND F38 (POSITIVE MODE)



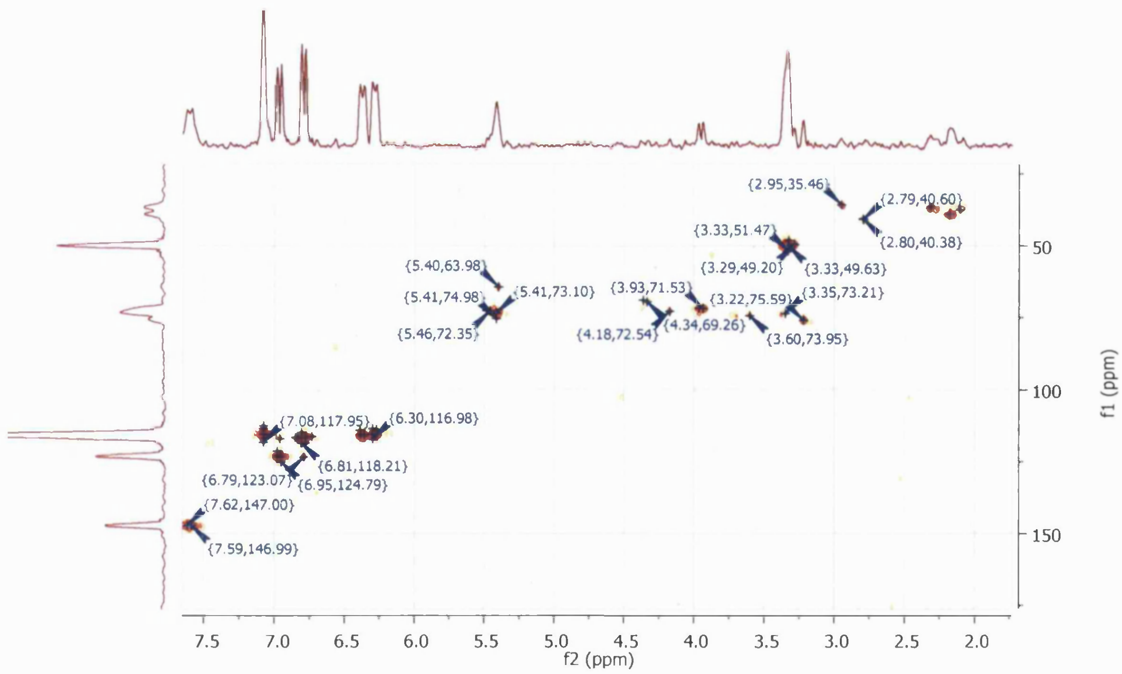
1C3 NMR OF COMPOUND F38 (IN METHANOL-D4) 126 MHZ



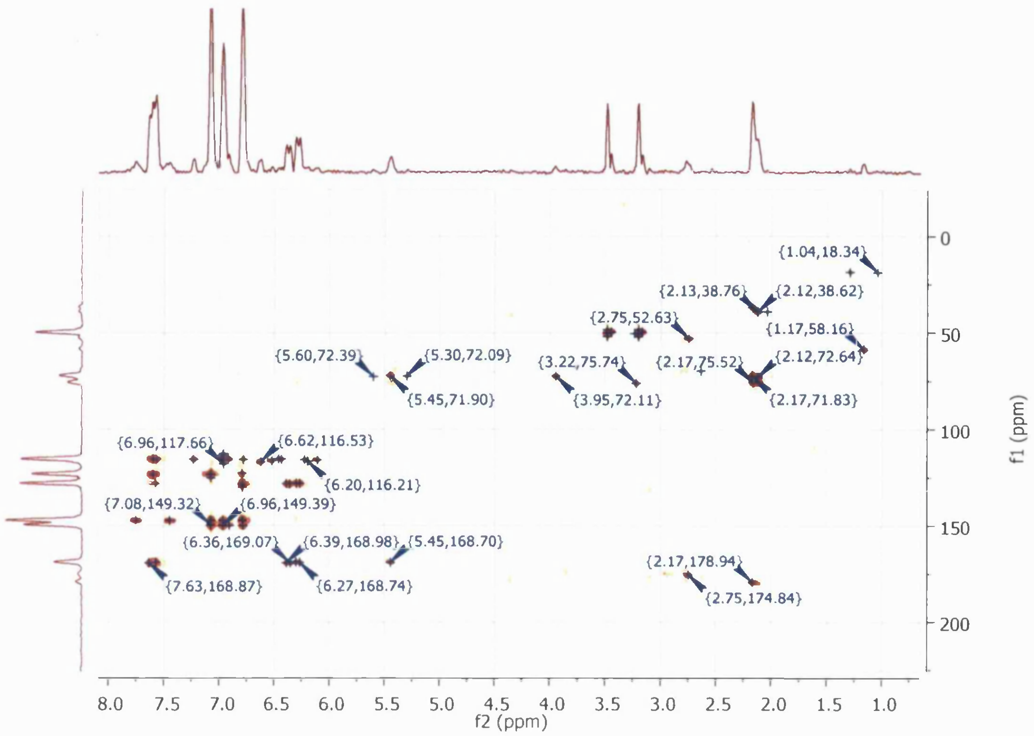
DEPT-135 OF COMPOUND F38 (IN METHANOL-D4) 126 MHZ



HMQC OF COMPOUND F38 (IN METHANOL-D4) 126 & 500 MHZ



HMBC OF COMPOUND F38 (IN METHANOL-D4) 126 & 500 MHZ



COSY OF COMPOUND F38 (IN METHANOL-D4) 500 MHZ