CHEMICAL AND BIOLOGICAL STUDIES OF SIDDHA MEDICINAL PLANTS

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by

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DECLARATION

The work presented in this thesis has not been submitted, either in whole or in part, for a higher degree to any other university or institution, and to the best of my knowledge is my own and original work, except as acknowledged in the text.

Unnikrishnan K P
October 2010

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ABSTRACT

Siddha is a traditional system of medicine being practiced in the southern part of India. Siddha medicines are predominantly plant based, usually containing polyherbal combinations. Following the establishment of a collaborative research partnership with a Siddha practitioner, Dr R. Velmurugan, information on some medicinal plants that he identified to be effective in treating cancer, pain and swelling were provided. The overall aim of this study was to isolate and identify biologically active molecules from some of these plants. This study consisted of reviewing the literature on the Siddha medicinal plants identified by Dr Velmurugan, biological studies and isolation and characterisation of bioactive constituents from a selection of these plants.

A broad literature search was undertaken on these plants to identify those plants that have not been well studied phytochemically and/or biologically, thus having the potential for further investigations. This led to the selection of nine plants for biological screening studies.

Antiproliferative and antiinflammatory studies were employed for the evaluation of the biological activities. The antiproliferative screenings were performed against SKNMC (neuroepithelioma), MCF 7 (Breast Cancer) and MRC 5 (normal) cell lines using the MTT assay. Antiinflammatory studies were carried out by using cyclooxygenase inhibitory assays. Significant antiproliferative activity of *Cardiospermum halicacabum* and *Caralluma fimbriata* was identified in the MTT assay. Both these plants also exhibited potent COX 1 and COX 2 inhibitory activity.

Inhibition studies with the enzyme indoleamine 2,3-dioxygenase (IDO), which is involved in cancers and inflammatory diseases, were also conducted on all plants. IDO inhibitory studies revealed the IDO inhibitory activity of *Marsdenia tinctoria*, *Evolvulus alsinoides* and *C. halicacabum.*

Based on the initial antiproliferative screening results, *C. halicacabum* and *C. fimbriata* were selected for further biological and chemical investigations. The bioassay guided isolation of the water partition of the ethanolic extract of the leaves of C. halicacabum led to the isolation of five compounds: quercetin 3-*O*-*β*-D-rutinoside, apigenin 7-*O*-*β*-D-

glucoside, chrysoeriol, luteolin and scutellarein. The antiproliferative activities of the isolated compounds were evaluated against the SKNMC, MCF 7 and MRC 5 cell lines. Chrysoeriol and scutellarein exhibited the highest antiproliferative activity. The other three molecules also demonstrated antiproliferative activity. A comparison of the activity exhibited by the compounds against the MCF 7 and SKNMC cell lines indicated some selectivity of these molecules for the breast cancer cell line. This is the first report of apigenin 7-*O*-*β*-D-glucoside and scutellarein from *C. halicacabum*.

Bioassay guided studies on the ethyl acetate fraction of *C. fimbriata* led to the isolation of cleomiscosin A, *N*-(*p*-*trans*-coumaroyl)tyramine, aristolochic acid 1 and aristolactam1a-*N*-*β*-D-glucoside . This is the first report of all these molecules from this plant and the first report of aristolochic acid 1 and aristolactam1a-*N*-*β*-D-glucoside from the Ascelpedeaceae family. The compounds were tested for antiproliferative activities. Aristolochic acid 1 demonstrated the most potent activity. Cleomiscosin A and *N*-(*p*-*trans*-coumaroyl)tyramine also demonstrated antiproliferative activity and aristolactam1a-*N*-*β*-D-glucoside was found to be less active. No remarkable selectivity in activity was observed with these compounds.

This study also identified the IDO inhibitory activity of *N*-(*p*-*trans*-coumaroyl)tyramine for the first time.

The biological activities of the plant extracts and the isolated molecules were consistent with the use of these plants by Dr Velmurugan, thus providing strong support for their use in the Siddha system of medicine for treatment of cancers.

ABBREVIATIONS

$(CD_3)_2CO$	Deuterated acetone
δ	Chemical shift (NMR)
^{13}C NMR	Carbon Nuclear Magnetic Resonance Spectroscopy
^{1}H NMR	Proton Nuclear Magnetic Resonance Spectroscopy
2D NMR	Two-Dimensional Nuclear Magnetic Resonance Spectroscopy
br	Broad (NMR)
BSI	Botanical Survey of India
$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
COSY	(Proton-Proton) Correlation Spectroscopy
d	Doublet (NMR)
DCM	Dichloromethane
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl Sulfoxide
EI	Electronic impact
EtOAc	Ethyl acetate
EtOH	Ethanol
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Correlation
IDO	Indoleamine 2,3-dioxygenase
IBRG	Indigenous Bioresources Research Group
IC_{50}	Half maxima Inhibitory Concentration
J	Coupling constant
m	Multiplet (NMR)
m/z	Mass to charge ratio
Me	Methyl
MeOH	Methanol
MeOD	Deuterated methanol

MEM	Minimal Essential Media
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MS	Mass Spectrometry
n-BuOH	*n*-Butanol
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance
ºC	Degrees Celsius
R_f	Retention factor
s	Singlet (NMR)
SGC	Silica Gel Chromatography
SPE	Solid Phase Extraction
SEC	Size Exclusion Chromatography
t	Triplet (NMR)
TLC	Thin Layer Chromatography
UNESCO	United Nations Educational, Scientific and Cultural Organisation
UV	Ultraviolet
µg	Microgram
µM	Micromolar

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Chapter 1

Introduction

The major objective of this PhD project was to isolate biologically active compounds from a selection of plants used in the Siddha traditional system of medicine for the treatment of cancers. This chapter describes the importance of medicinal plants in human healthcare and drug discovery and the significance of ethnopharmacological leads in the process of drug discovery. The specific aims of this project are also detailed.

1.1 Background

Plants have formed the basis of most of the traditional medicine systems that have been used for thousands of years[1]. Ayurveda, Traditional Chinese Medicine, Siddha and Unani are some of the important traditional systems of medicines. Traditional medicines play an essential role in healthcare and it is estimated that approximately 80% of the world's total population still rely mainly on traditional medicines for their primary healthcare[2-4].

All recorded civilisations have traditionally used plants as a source of medicine and their application in the form of crude drugs such as tinctures, teas, poultices, powders and other formulation dates back centuries[5]. For many of these civilisations, the medicinal plant knowledge has been passed down the generations in a written format[6, 7]. The Ebers Papyrus, the Egyptian pharmaceutical documentation of 1550 BC, has information on over 800 herbal remedies involving the use of plant extracts, animal organs and minerals[6].

Atharvaveda (1200 BC), *Charak Samhita* and *Sushrut Samhita* (1000-500 BC) are the classic texts of India that formed the basis for the Science of Ayruveda (Traditional Indian Medicine) and provide detailed descriptions of about 700 herbs[8]. Ayurveda, meaning "knowledge of life" encompasses more than just medicinal aspects, also including psychological, cultural, religious and philosophical concepts[8].

The Chinese Materia Medica, which forms the basis of Traditional Chinese Medicine (TCM) dates back to 1100 BC, and incorporates information on 6000 herbs, which are

used to make polyherbal formulations[9]. TCM and Ayurveda still remain popular as the most ancient herbal systems of medicine[10].

The treatise, Tirumantiram, written in 5th century AD, forms the basis of another Indian traditional healthcare system known as Siddha medicine and describes numerous potent herbs [11]. In 100 AD, *De Materia Medica*, compiled by the Greek physician, Dioscorides, recorded the use of 600 plants and plant products[9]. In Europe, the compilation of knowledge was initially carried out by Constantinople and then by lbn al-Baytar (1197-1248) in his collection named *Corpus of Simples*, which describes around 800 herbal remedies[6].

On the American continent, the earliest text of medicinal plants is the 'Badianus Manuscript' collated by Aztec scholars in 1500 AD, which has information on 184 important plants used in Aztec medicine[12].

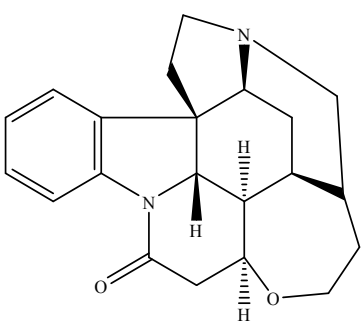
All these ancient texts have invaluably contributed towards the enrichment of medicinal plant knowledge and traditional medicine systems of different cultures all over the world.

1.1.2 Natural Products from Plants

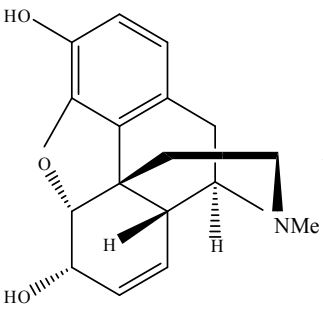
Plants contain a variety of secondary metabolites serving as hormones, attractants, poisons and other functional agents of the plants, and a large number of them display pharmacological properties that can be and have been used by humans. The cardiac glycoside digitoxin, the anticancer drugs paclitaxel and vinblastine, the antimalarial drug artemisinin, and the acetylcholinesterase inhibitor galantamine are just a small selection of the many examples of bioactive natural products originating from plants that are used as medicines.

It was in the early 1800s that the concept of pure active principles from plants emerged. Some of the remarkable achievements in this period were the isolation of the analgesic morphine (1816), the psychostimulant strychnine (1817), the anticholinergic atropine (1819), the antipyretic quinine (1820) and the antiinflammatory colchicine (1820)[6]. Such discoveries attracted the attention of the world to plants as a source of potential bioactive molecules. The development of aspirin (1899), the first synthetic drug based on a natural

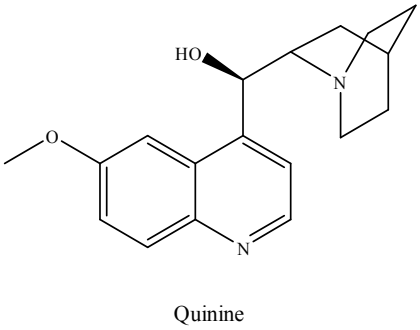
product lead (salicin, from the bark of the willow tree) and one of the highest selling drugs of all time, was a major breakthrough in natural product chemistry[13]. This was followed by a series of bioactive natural compounds including the anticholinergic compounds atropine and hyoscine from *Atropa belladonna*, the antihypertensive agent reserpine from *Rauwolfia serpentine*, and the anticancer molecules vinblastine and vinscristine from *Catharanthus roseus*[7].



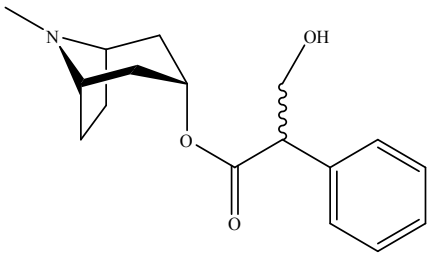
Strychnine



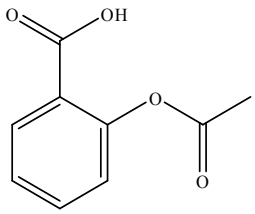
Morphine



Quinine



Atropine



Aspirin

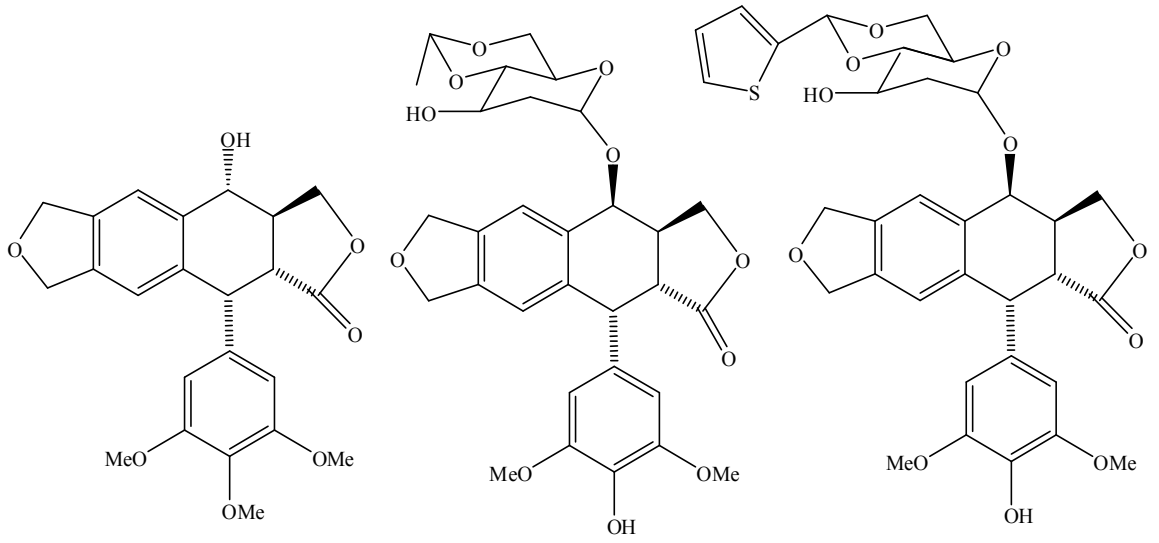
In the time span of 1981-2006 (25 years) a total of 1010 chemical entities were introduced to the pharmaceutical market. Out of this, 275 molecules (28%) were either natural products or derivatives of natural products. The significance of natural product chemistry in drug discovery becomes more evident with the statistics of all available anticancer drugs up until 2006. Out of the 175 drugs available about 42% are natural products or natural product derivatives[14].

Despite the great successes already achieved in natural products chemistry and drug discovery, the potential of molecular diversity has not been fully tapped[15]. It is estimated that there are approximately 350,000 different species of plants growing on earth and out of these, only two-thirds have been discovered[15]. Out of this two-thirds, only 15% of

plant species has been studied for bioactive molecules[15]. Multidrug resistance of targets, weak or narrow spectrum of bioactivities, and side effects of drugs are a few factors that impose limitations on the usage of some existing drugs[16]. The need for structurally novel therapeutic categories/efficacious drugs/leads, especially those with new mechanisms of action is growing. Nature, with its immense potential of unexplored plant species, continues to be an important source for bioactive molecules[17].

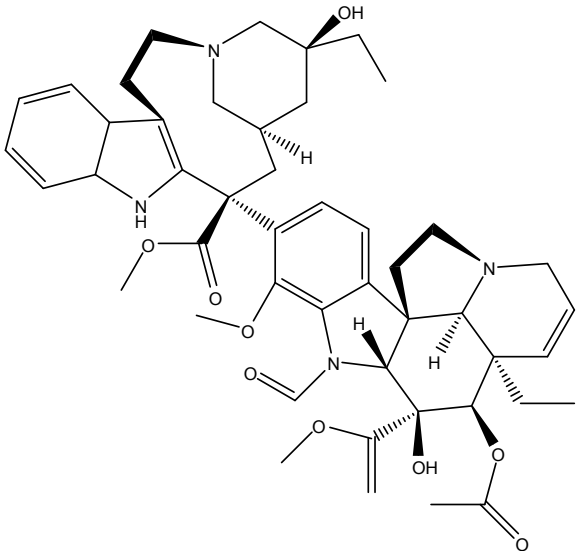
1.1.3 Role of natural products in the treatment of cancer

Single drug molecules isolated from plants have played a vital role in the treatment of cancer. Podophyllotoxin, isolated from *Podophyllum peltatum* in 1944, is one of the earliest examples of a plant derived anticancer agent[18, 19]. Synthetic analogues, including, etoposide and teniposide, are now commonly used for a wide range of cancer treatments[20].

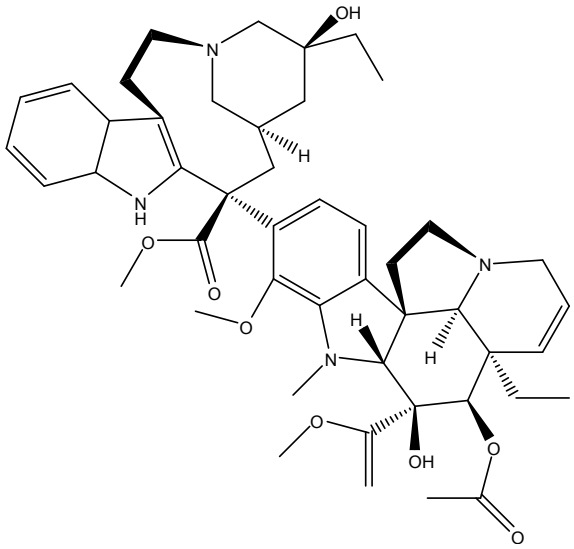


Podophyllotoxin Etoposide Teniposide

Catharanthus roseus, belonging to the family Apocynaceae, is rich in indole alkaloids including vincristine and vinblastine, which block cell multiplication by inhibiting mitosis due to irreversibly binding to tubulin[21]. These alkaloids are important therapeutics for the treatment of leukemia and lymphomas[21].

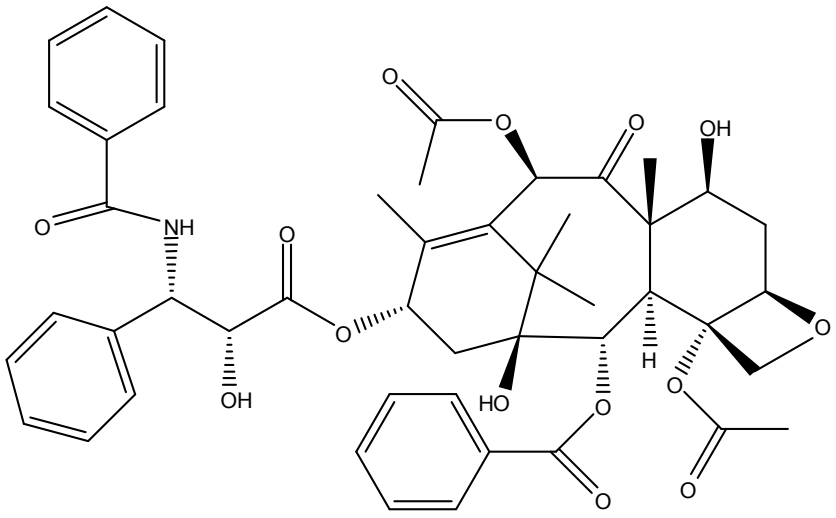


Vincristine



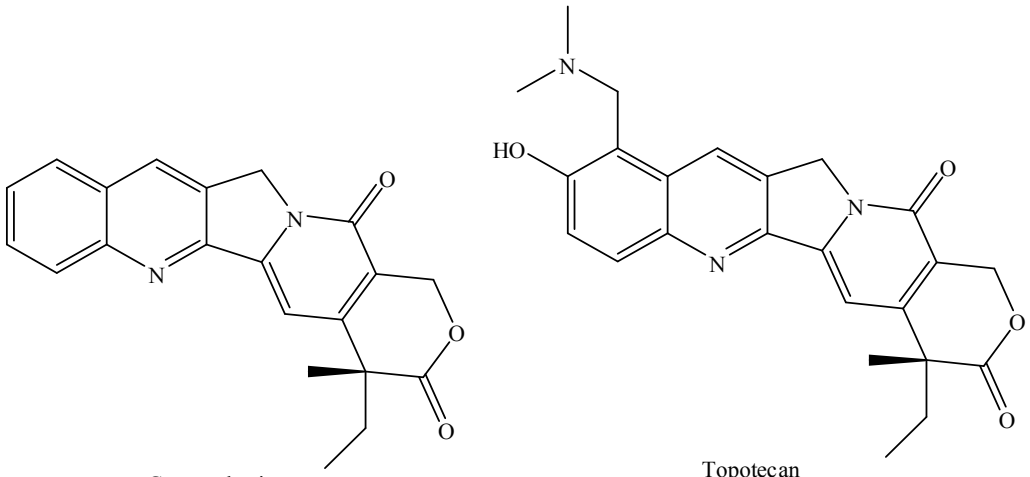
Vinblastine

The discovery of paclitaxel from the Pacific yew tree, *Taxus brevifolia*, is another outstanding example[22]. Paclitaxel was the first microtubule stabilising natural product discovered and has become an invaluable drug, especially against the treatment of ovarian and breast cancer[22].



Paclitaxel

Camptothecin, isolated from *Camptotheca acuminata*, is an anticancer molecule that inhibits topoisomerase[23]. Its more water soluble synthetic analogues, topotecan and irinotecan, are now being used clinically as anticancer drugs[23].



1.1.4 Ethnopharmacological approach - An effective method of selecting plants for drug discovery

Ethnopharmacology is an interdisciplinary area of research that focuses on the validation of traditional medicine either by isolating bioactive molecules or by pharmacologically proving the efficacy of Indigenous drug preparations[24]. In the past few decades, ethnopharmacology has emerged as an interdisciplinary approach to drug discovery involving the integrated efforts of western medicine practitioners, phytochemists, pharmacologists and Indigenous healers practicing traditional systems of medicine[25].

A good example of the relationship between traditional medicine and drug discovery is the plant *Chandra* (*Rauwolfia serpentina*). The term *Chandra* (Sanskrit term for 'moon') was used because of the use of this plant in the treatment of moon disease or lunacy in Ayurveda. It is also used for hypertension, insomnia and epilepsy[26]. For centuries, the Indian sadhus used the root of this plant for its calming effects and claimed that it helped in reaching 'spiritual enlightment'[26]. Later, Emil Sclittler in 1949 isolated reserpine, an indole alkaloid from the roots of *R. serpentina*[27]. Reserpine was the first major drug to be reported to treat hypertension, one of the most serious illnesses of the western world[27].

Another example is *Adhatoda vasica,* the leaves of which were traditionally used as an antispasmodic agent[26]. Later vasicine, a quinazoline alkaloid, was isolated as the lead molecule exhibiting bronchodilatory and respiratory stimulant properties[5]. Numerous examples illustrate the significance of the ethnopharmacological approach for drug discovery. These include the isolation of the anticancer molecule podophyllotoxin (described earlier), the antiasthmatic ginkgolides from *Gingko biloba,* the memory enhancing baccosides from *Bacopa monieri*, and the anticancer and antiinflammatory withanolides from *Withania somnifera*[10]. Statistical analysis shows that over 70% of the plant based drugs currently in use were isolated from plants used in traditional medicine[1, 5].

1.2 Objectives of this research

This PhD project is based on a collaborative partnership between Dr Velmurugan, a Siddha practitioner, and the Indigenous Bioresources Research Group (IBRG) of Macquarie University. The major objective of this project was to isolate and structurally identify biologically active compounds from a selection of plants used by Dr Velmurugan in the Siddha traditional system of medicine for the treatment of cancers.

The specific aims of the project were to:

- Undertake a literature survey on plants used by Dr Velmurugan for treating cancer, pain and swelling and to shortlist promising plants for biological studies.
- Evaluate the antiproliferative, indoleamine 2,3-dioxygenase inhibitory and antiinflammatory properties of selected plants through the use of bioassays.
- Isolate and structurally elucidate bioactive compounds from these plants, using bioassay guided fractionation.

The initiation of this project, including establishing the collaboration with Dr Velmurugan, a literature review carried out on Siddha medicinal plants used by Dr Velmurugan, and the consolidation of a shortlist of plants based on the literature, is detailed in Chapter 2. Chapter 3 describes the biological screening studies conducted on the plants shortlisted from Chapter 2, which were used to select plants for further

bioassay guided studies. Chapters 4 and 5 describe the isolation of bioactive compounds from those selected plants. Conclusions and future directions of this research are presented in Chapter 6.

Chapter 2

Literature Studies on Siddha Medicinal Plants

This chapter outlines the establishment of the collaboration with Dr Velmurugan, a Siddha practitioner from South India, and the literature review carried out on the Siddha medicinal plants provided by Dr Velmurugan. The shortlisting of plants for biological studies, based on the literature, is also detailed.

2.1 An introduction to Siddha Medicine

Siddha is one of the most ancient medical systems of India. In Sanskrit language 'Siddha' means 'achievement'[28]. It was Siddhars (the accomplished ones) who established the Siddha school of thought. The Siddha medicinal system is mainly practiced in Tamilnadu, a southern state in India (Fig 2.1). The formulations used in this system are predominantly combinations of herbs, minerals and metals[29]. The Siddha medicinal system considers that all psychological and physiological functions of the body are a combination of seven elements, plasma, blood, muscle, fatty tissue, bone, nerve and semen[30]. Pulse reading and urine analysis are the diagnostic methods used in this system. Similar to Ayurveda, the physiological components of the human body, as defined by the Siddha system, are air (Vatha), fire (Pitha), earth and water (Kapha) and a normal equilibrium is believed to exist between these factors in a healthy individual and disease conditions emerge when the equilibrium is altered[31].

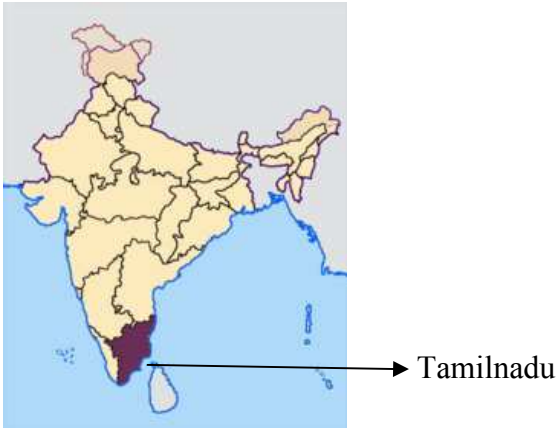


Fig 2.1 Map of India (Tamilnadu highlighted)[32]

While the translated versions of Ayurveda literature have been available to western scholars for centuries and have contributed to its worldwide popularity[33], there have been no such efforts with the Siddha literature and hence this system is less known to the west. Most of the Siddha literature is still in the Tamil language and is documented in palm leaf manuscripts[33]. Siddha is however slowly gaining recognition in the world due to the growing popularity of traditional medicine[34]. The vast knowledge of this system of medicine, similar to other traditional medicinal systems, is considered as an important avenue for seeking bioactive molecules. For example, there are now many scientific studies that have shown the efficacy of Siddha medicines. Semecarpus *'lehyam'* is a Siddha herbal medicine that contains six herbs formulated in the form of a paste with palm sugar and butter. Studies carried out on the MCF 7 and MDA 231 breast cancer cell lines showed that Semecarpus *'lehyam'* exhibits potent antiproliferative activity[35]. An anticancer molecule, (8'*Z*)-3-pentadec-10-enyl-benzene-1,2-diol was isolated as the major compound from this herbal medicine[36]. It is also interesting to note that five (*Semecarpus anacardium*, *Strychnos nux vomica*, *Nigella sativa*, *Smilax chinensis* and *Plumbago zeylanica*) of the six plants in semacarpus '*lehyam'* are reported to possess anticancer activity[37].

2.2 Dr Velmurugan - Siddha Practitioner

Dr Velmurugan (Fig 2.2) belongs to a family of Siddha practitioners who have practiced the Siddha system of medicine for the past five generations. He is based in Chennai, India, and treats patients with cancers and inflammation related conditions. He has a large collection of ancient text books and palm leaves on which the knowledge of this system of medicine is stored. His treatments are based on polyherbal formulations prepared by following the methods prescribed and refined by the clinical experience of his forefathers and himself. The formulations used by Dr Velmurugan and his forefathers are combinations of plant powders, mixed in proportions dependent on the patients condition and age, and administered orally in the form of capsules or pills. The formulations have shown good results in his clinic, particularly in cases of cancers of the breast, liver, larnyx, nasopharynx, tongue, colon, skin and ducts. In his experience these formulations have helped to reduce secondary metastases and are also used as palliatives.

Fig 2.2: Dr Velmurugan (R) with his teacher (L) and former Indian President (C)

The value of Dr Velmurugan and his forefather's knowledge is highlighted by the following example. *Phyllanthus urinaria* is one plant that Dr Velmurugan uses in his formulations for cancer treatment. Based on this information, scientists from Anna University, Chennai and the National Institute of Immunology, New Delhi undertook bioassay guided studies on this plant to identify bioactive molecules. This resulted in isolation of the novel compound, 7'-hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylenedioxylignan (Fig 2.3), with potent antiproliferative activity[38]. This compound exhibited marked inhibition of the alveolar epithelial carcinoma cell line Hep 2, cervical cancer cell line HeLa, breast cancer cell line MCF 7 and monocyte cell line ELI[38].

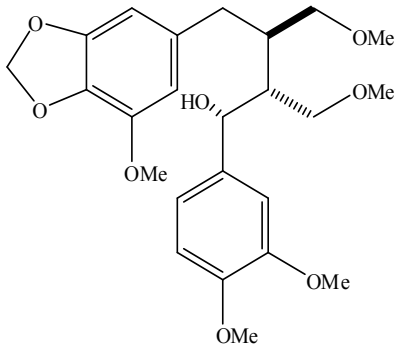


Fig 2.3: 7'-Hydroxy-3',4',5,9,9'-pentamethoxy-3,4-methylenedioxylignan

2.2.1 Collaboration between the IBRG and Dr Velmurugan

The Indigenous Bioresources Research Group (IBRG) is a group of researchers at Macquarie University interested in the documentation, preservation and validation of customary (traditional and contemporary) medicinal plant knowledge of Indigenous people. The primary focus of the IBRG is on Australian Indigenous medicine, but it has also established a collaborative partnership with tribal elders from Nagaland, India. Having learnt about the activities of the IBRG, Dr Velmurugan expressed his interest in sharing his information to promote research on medicinal plants used in Siddha medicine. After several personal discussions, a formal collaborative agreement was established between the IBRG and Dr Velmurugan.

2.2.2 The Collaborative Agreement

With their knowledge about the therapeutic properties of plants and other natural resources, Indigenous peoples have made invaluable contributions to biodiversity conservation and healthcare systems of the world[39]. A review on commercially available plant derived drugs identified that approximately 80% of these were developed following their traditional usage[40]. The anticancer drugs vincristine and vinblastine, the bronchodilatory agent vasicine, the antihypertensive drug reserpine and the antimalarial drug artemisinin, all highlight the value of the study of traditional medicines for drug discovery[5, 40].

The enormous commercial interest in medicines derived from traditional leads has, however, also led to the exploitation of Indigenous people. Merchandising the traditional

knowledge or the end product of this knowledge without giving any benefit to the owners of this information is an issue that has been debated worldwide[41]. The international community has developed guidelines on best practices for collaborative research with Indigenous people in an attempt to stop this exploitation[39]. The United Nations have also intervened as a watchdog putting forward "The Participatory Action Research Methods" and "General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine" to ensure that the Intellectual Property Rights of the Indigenous people are protected and the traditional knowledge is used properly[41, 42].

A collaborative research agreement between the IBRG and Dr Velmurugan was established on the basis of benefit sharing and upholding proper ethics. The collaboration was framed in such a way that the duties of both the parties were carried out in accordance with generally accepted professional scientific and ethical principles. The agreement regarding the particular herbs and their application for specific ailments was developed to recognise ownership of the traditional knowledge by Dr Velmurugan and clauses ensured that any intellectual property arising from the project shall be jointly owned by both the IBRG and Dr Velmurugan. Special emphasis was given to confidentiality with both parties agreeing to treat all information as confidential and to use all this information only for the purposes of the project. A sample agreement between Dr Velmurugan and the IBRG is given in Appendix 1.

2.3 Literature Survey on Siddha Medicinal Plants

Based on the agreement with the IBRG, Dr Velmurugan provided a list of plants which, from his experience, were the best candidates for treating cancers, pain and swelling. Dr Velmurugan uses dried powders of these plants in the form of capsules, usually containing a combination of two or three plants mixed in a particular proportion. A literature search was performed on each plant in the list to identify the chemical and biological studies reported for these plants. Table 2.1 summarises the relevant literature known for these plants.

Table 2.1 List of Siddha medicinal plants recommended by Dr Velmurugan and the reported biological activities and phytochemicals

Name of plant/ family/ part used by Dr Velmurugan	Use	Major biological activities*	Major reported chemical constituents
***Abutilon indicum*/ Malvaceae/ roots, leaves**	cancer treatment	Antinociceptive, antioxidant[43]; immunomodulatory[44], **cytotoxic^,[45]**	*β*-sitosterol, geraniol, caryophylline, abutilin A, (*R*)-*N*-(10-methoxycarbonyl-20-phenylethyl)-4-hydroxybenzamide[45]; methylcoumarate[43]; 4-hydroxyacetophenone[46]; aurantiamide acetate[47]; methylindole-3-carboxylate[48]; 3,7-dihydroxychromen-2-one[49]; scopoletin, syringaldehyde,1-methoxycarbonyl-*β*–carboline[45].
***Aerva lanata*/ Amaranthaceae/ whole plant**	cancer treatment	analgesic[20]; anthelmintic, antimalarial[50]; antimicrobial[51]; antiinflammatory[52]; antivenin, antioxidant[51]; antidiabetic[53]; **antitumour**[54]; **cytotoxic**; diuretic[55]	aervine, aervoside, aervolanine, methylaervine[56]; aervitrin, aervolanine, α-amyrin, betulin, campesterol[57]; canthin-6-one[a], *β*-carboline[a, 54]; 10-hydroxy-canthin-6-one, carboline-1-propionic acid, chrysin[58]; *β*-ecdysone, daucosterol, hentriacontane, narcissin[59]; *β*-sitosterol[60]; syringic acid, feruloyl tyramine, vanillic acid, canthine-6-one[61]
***Azima tetracantha*/ Salvadoraceae/ leaves**	pain and swelling	anticancer[62]; **antiinflammatory**[63]; antifungal[64]; analgesic[65]; antiulcer; antispasmodic, antioxidant[66]	friedelin[64,62]; azimine, azcarpine, carpine, isorhamnitine-3-*O*-rutinoside, lupeol, glutinol, *β*-sitosterol[66]
***Borreria hispida*/ Rubiaceae/ whole plant**	cancer treatment	antihyperlipidemic[67]	no chemical studies reported so far

Bryonopsis laciniosa/ **Cucurbitaceae/ whole plant**	cancer treatment	antimicrobial, **cytotoxic**[68]	goniothalamin[a,68]; bryonin, punicic acid[69]
Caralluma fimbriata/ **Ascelpedeaceae/ leaves**	cancer treatment	appetite suppressant[70]	no chemical studies reported so far
Cardiospermum halicacabum/ **Sapindaceae/ roots, leaves**	cancer treatment	analgesic, anti-HIV[71]; antiinflammatory, antispasmodic vasodepressant [72]	rutin, luteolin[a], apigenin[a,b], pinitol[73]
Cassia tora/ **Fabaceae/ seeds**	cancer treatment	**anticancer**[74, 75, 76]; antifungal[77]; antihepatotoxic[78]; antiinflammatory[79]; antimutagenic[80]; antioxidant[81]; antimutagenic[76]	1,3,8-trihydroxy-6-methyl-9,10-anthracenedione[81]; 9-[(*β*-D-glucopyranosyl-(1→6)-*O*-*β*-D-glucopyranosyl)oxy]-10-hydroxy-7-methoxy-3-methyl-1H-naphtho[2,3-c]pyran-1–one, 6-[(*α*-apiofuranosyl-(1→6)-*O*-*β*- glucopyranosyl)oxy]-rubrofusarin, cassiaside, rubrofusarin-6-*β*-gentiobioside[78]; cassitoroside[82]; chrysophanic acid-9-anthrone[77]; emodin[a], chrysophanol[a], rhein[a,83]
Cleome viscose/ **Capparidaceae/ roots, leaves**	cancer treatment	analgesic, antipyretic, hepatoprotective[84]; hypoglycaemic[85]	viscocic, viscosin[86]; cleosandarin[87]; cleomiscosin A, B, C and D[88, 89, 90, 91]; dihydrokaempferide-3-glucuronide, dihydrokaempferol-4'-xyloside, docosanoic acid[92]; naringenin-4-galactoside[93]; naringenin-4-(xylosyl-*β*-(1,4)-glucoside)[94]; 5'-trihydroxyflavanone-7-*O*-*α*-l-

			rhamnopyranoside, *β*-amyrin, lupeol[95, 96]; eriodictyol-5-rhamnoside[96]; 3',4'-dihydroxy-5-methoxyflavanone-7-*O*-α-l-rhamnopyranoside[94]; stigmasta-5,24(28)-diene-3 *β*-*O*-α-l-rhamnoside[97]; ergast-5-ene-3-*O*-*α*-l-rhamnopyranoside, 5,4'-di-*O*-methyleriodictyol-7-*O*-*β*-d-glucopyranoside[98]; glucocapparin, glucocleomin[99]; cleomaldeic acid[100]
***Crataeva religiosa*/ Capparaceae/ roots, leaves**	cancer treatment	antibacterial[101]; antiarthritic[102, 103, 104]	lupeol[102]; oleanolic acid, 4-*epi*-hederagenin[101]
***Evolvulus alsinoides*/ Convolvulaceae/ leaves**	cancer treatment	adaptogenic[105]; antiulcer, anticatatonic activity[106]; antioxidant[24, 107]; antiamnesic[105]; immunomodulatory[108]	scopoletin, scopolin, umbelliferone, 2-methyl-1,2,3,4-butanetetrol[109]; ferulic acid, 2-*C*-methyl erythritol, kaempferol-3-*O*-*β*-glucopyranoside, kaempferol-7-*O*-*β*-glucopyranoside,6-methoxy-7-*O*-*β*-glucopyranoside, quercetin-3-*O*-*β*-glucopyranoside, pentatriacontane, triacontane and *β*- sitosterol. 2,3,4-trihydroxy-3-methylbutyl 3-[3-hydroxy-4-(2,3,4-trihydroxy-2-methylbutoxy)-phenyl]-2-propenoate, 1,3-di-*O*-caffeoyl quinic acid methyl ester, 6-methoxy-7-*O*-*β*-glucopyranoside coumarin[109]; betaine, shankpushpin[110];. evolvulin, evolvoids A and B[105]
***Heliotropium indicum*/ Boraginaceae/ leaves**	cancer treatment	**antitumor**[111]; antiinflammatory[112]; antihepatotoxic[113]; antiproliferative[114];	indicine-*N*-oxide[a,111]; heliotrine[116]; indicine[117]; helindicine, lycopsamine[118]; acetyl indicine, indicinine[119]; phytol, 1-dodecanol, linalool[120]; phenylacetaldehyde, (*E*)-2-nonenal, (*E*,*Z*)-2-

		antimicrobial[115]	nonadienal, hexahydrofarnesylacetone[121]
***Hemidesmus indicus*/ Apocynaceae/ roots**	cancer treatment	antifungal, antiinflammatory, antioxidant[122]; antipyretic, antiulcer, **anticancer**[123]; antibiotic[123]; antinociceptive[124]; hepatoprotective[125]	2-hydroxy-4-methoxybenzaldehyde[126]; ledol[127]; nerolidol, emidine, hemidescine[128]; hemidesminin, indicine, hemidine[129]; indicusin, medidesmine, hemisine, desmisine[130]; denicunine, heminine[131]
***Holarrhena antidysenterica*/ Apocynaceae/ bark**	pain and swelling	antibacterial, expectorant[132]	pubadysone, puboestrene, pubamide, morphaladiene, pubescine, pubescimine[133, 134]; kurchinine, kurchinidine[135]; kurchinicin[136]
***Indigofera aspalathoides*/ Fabaceae/ whole plant**	cancer treatment	**anticancer**[137, 138]; antiinflammatory, **antitumour**[139]; antihepatotoxic[140]; antimycobacterial, antiviral, **cytotoxic**[141]	5,4-dihydroxy-6,8-dimethoxy-7-*O*-rhamnosyl flavone[a,142]; indigocarpan, mucronulatol[143]
***Pogostemon parviflorus*/ Fabaceae/ aerial parts**	cancer treatment	antimutagenic[144]; antibacterial[145]; **cytotoxic**[146]	epoxyparvinolide[147]; parvinolide, *trans*-phytol, friedelin, friedelan-3-*β*-ol, sitosterol, 7,4‘-di-*O*-methyleriodictyol, 7,3‘,4‘-tri-*O*-methyleriodictyol, 3,7,4‘-tri-*O*-methylkaempferol, ombuine, pachypodol, kumatakenin[144]; licochalcone A, 5,7-dihydroxy-3′,4′-dimethoxyflavanone[146]; 15*α*-hydroperoxy-guaia-1(10),11-diene, 1*α*-hydroperoxy-guaia-10(15),11-diene, 10*α*-hydroperoxyguaia-1,11-diene[148]; 5,6,7,8,2’,4’,5’-octamethoxyflavone[145]

***Marsdenia tinctoria*/ Asclepiadaceae/ roots, leaves**	cancer treatment	anti-fertility agent[149]	tinctoroside A, B and C[150, 151]; lupenyl palmitate, lupenyl acetate, lupenone, lupeol, *β*-sitosterol, 5,7-dihydroxy-2,6,8-trimethylchromone, 2,6-dimethoxybenzoquinone, tinctoramine, tinctoralactone[152]
***Pergularia pillida*/ Asclepiadaceae/ roots**	cancer treatment	**anticancer**[153]; antiviral[154]	pergularinine[a], tylophorinidine[a,153]; tylophorinicine[155]; sarcogenin[156]; tylophorine, 3,6,7-trimethoxyphenanthrindolizidlne, *O*-acetylpergularinine, desoxypergularinine, perguiarinine methiodide[157]; pallidine, pallidinine[158]
***Phyllanthus urinaria*/ Euphorbiaceae/ whole plant**	pain and swelling, cancer treatment	antitumour, antiangiogenic[159]; **antiinflammatory**, antidiarrheal, cytotoxic, hepatoprotective[160]	corilagin, rutin[a], brevifolincarboxylic acid, isostrictiniin, geraniin, gallic acid, ellagic acid[161]; 5-demethoxyniranthin, urinatetralin, dextrobursehernin, urinaligran[162]
***Poinciana elata*/ Fabaceae/ aerial parts**	pain and swelling	no biological activities reported	no chemical studies reported so far
***Sagittaria obtusifolia*/ Alismataceae/ leaves**	cancer treatment	antibacterial[163]; antimicrobial[164]; immunosuppressant[165]	sagittines A−G, 13-*epi*-manoyloxide-19-*O*-α-l-2‘,5‘-diacetoxyarabinofuranoside[163]; sandaracopimaric acid[a,165]; sagittariol[166]; hexahydrofarnesyl acetone, tetramethylhexadecenone, myristaldehyde, *n*-pentadecane, 2-hexyldecanol[164]
***Toddalia asiatica*/ Rutaceae/**	pain and swelling	antipyretic[167]; **antiinflammatory**,	7-geranyloxy-5-methoxycoumarin, 8-geranyloxy-5,7-dimethoxycoumarin[169];

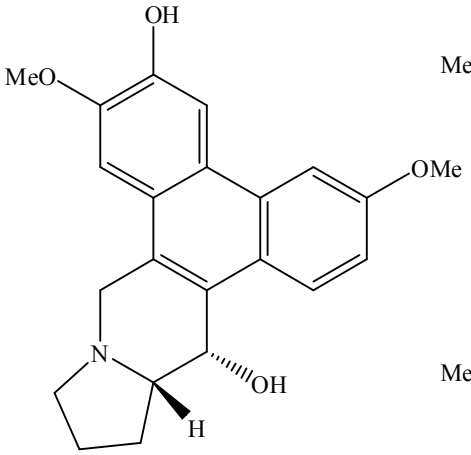
leaves		larvicidal, spasmolytic[168]	artanin, norbraylin, 5,7,8-trimethoxycoumarin, toddalosin[169]; toddalactone, toddanone[167]
Trichodesma indicum/ Boraginaceae / **leaves**	cancer treatment	antidiarrheal[170]; antiinflammatory[170]; antispasmodic[171]; cough suppressant[172]	hexacosane, ethyl hexacosanoate, 21,24-hexacosadienoic acid[173]; oleic, linoleic, palmatic, stearic and linolenic acid[174]
Vernonia cinerea/ **Asteraceae/** **leaves**	cancer treatment	antiinflammatory[175, 176]; antibacterial[177]; antiarthritic[178]; analgesic, anxiolytic, antidepressant[176]; antimalarial[179, 180]; antipyretic[181]; **cytotoxic**[182, 183]	vernolide A[a], vernolide B[182]; stilpnotomentolide-8-*O*-tiglate, 8α-[hydroxymethacryloyloxy]-hirsutinolide-13-*O*-acetate[184]; 3*β*-acetoxyurs-19-ene, lupeol acetate[185]; stigmast-5,17(20)-dien-3*β*-ol, stigmasterol, 26-methylheptacosanoic acid, isomontanic acid[186]; 6-amyrin acetate, α-amyrin acetate, 3*β*-acetoxyurs- 13(18)-ene , *β*-amyrin acetate, *β*-amyrin, *α*-amyrin[187]; 24-hydroxytaraxer-14-ene[188]

*Activity in **bold** indicates congruence between usage by Dr Velmurugan and biological activity reported in literature.
[a]anticancer, [b]antiinflammatory, ^ activity reported in 2008

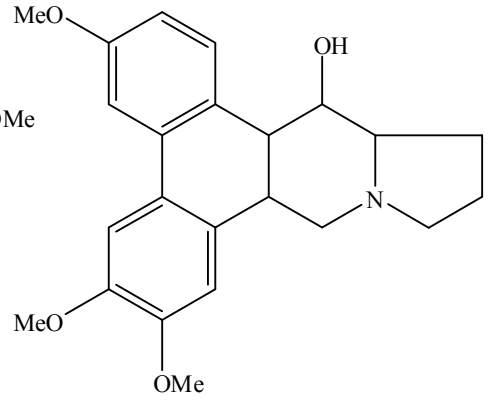
The list of plants provided by Dr Velmurugan comprised nineteen plants used for treating cancer conditions, four plants for treating pain and swelling (antiinflammatory related conditions) and one for treating both cancer and inflammatory conditions. Some of the plants in the list were identified to be widely used for similar conditions in other traditional medicinal systems. For example, roots of *H. indicus* are used by Ayurvedic medical practitioners in Sri Lanka to treat cancer[189, 190]. Plants belonging to the genus *Vernonia* are used in South African and Tanzanian traditional medicines for cancer related conditions[191, 192] and *I. suffruticosa,* a species closely related to *I. aspalathoides*, is used as an alternative cancer therapy in Brazilian traditional medicines[193]. *C. halicacabum* is being used in many folk and traditional medicine systems including Ayurveda for the treatment of inflammatory conditions[194]. *Pergullaria pallida* has been reported to be used for cancer therapy in folk medicines[195]. In Thailand, *Phyllanthus urinaria* is traditionally used as an adjuvant or alternative medicine for cancer patients, including for liver cancer[160].

Table 2.1 shows that some of the plants used by Dr Velmurugan have been extensively studied, both biologically and phytochemically from a perspective similiar to that of Dr Velmurugan's usage. Eleven plants in the list that are used by Dr Velmurugan for treating cancer have been well studied for anticancer activity and have had bioactive constituents isolated. Three plants used for treating pain and swelling have been reported for their antiinflammatory activity. This information strongly supports the value of the knowledge of Dr Velmurugan and the Siddha medicinal system. The details of the chemical and biological studies supporting the anticancer and antiinflammatory activities of these plants are described below.

Bioassay guided fractionation studies to isolate anticancer molecules have been reported for *Pergullaria pallida*, *Aerva lanata*, *Casssia tora*, *Phyllanthus urinaria*, *Vernonia cinerea*, *Pogostemon parviflorus* and *Heliotropium indicum*. Anticancer studies carried out on roots of *P. pallida* resulted in isolation of two phenanthroindolizidine alkaloids, pergularinine and tylophorinidine[153]. Both of these compounds were found to inhibit thymidylate synthase, a key target enzyme in cancer chemotherapy[153].

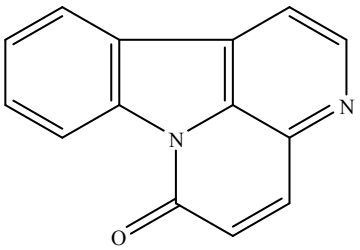


Tylophorinidine

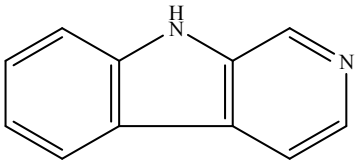


Pergularinine

The petroleum extract of the whole plant of *A. lanata* exhibited cytotoxicity on Dalton's lymphoma ascites, Ehrlich ascites and B16F10 cell lines and these were attributed to the presence of alkaloids in the active fraction that included canthin-6-one and *β*-carboline[54].

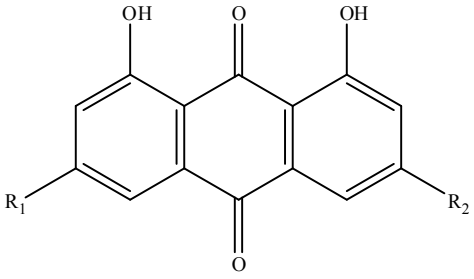


Canthin-6-one



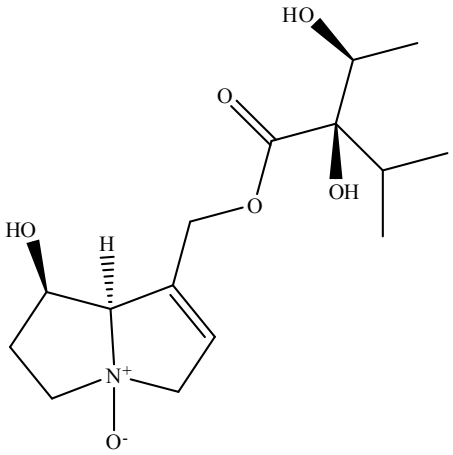
β-carboline

The water extract of seeds of *C. tora* was found to be inhibitory to the HepG2 cell line and its potent activity was due to the presence of the anthraquinones chrysophanol, emodin and rhein[83]. Emodin and rhein have been previously reported as the major chemical constituents of *C. tora*[83].



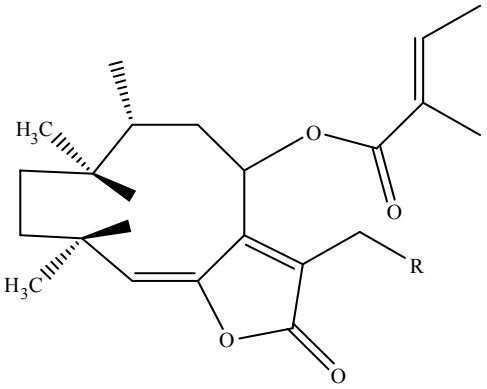
Rhein, $R_1 = H$, $R_2 = CO_2H$
Emodin, $R_1 = CH_3$, $R_2 = OH$
Chrysophanol, $R_1 = H$, $R_2 = CH_3$

The methanol extract of whole plant of *H. indicum*, showed significant activity in several tumour systems such as Walker-256 carcinosarcoma, melanoma B-16, leukemia L-1210, leukemia P-388, and leukemia P-1534[111]. Indicine-*N*-oxide, a pyrrolizidine alkaloid was isolated as the antitumour principle of this plant[111]. Indicine-*N*-oxide has undergone clinical trials in humans for cancer treatment[196]. Even though the drug showed complete remission for patients suffering from acute lymphocytic leukemia and acute myelocytic leukemia, drug induced toxicities such as bone marrow suppression, mild anorexia, nausea and liver failure were limiting factors.



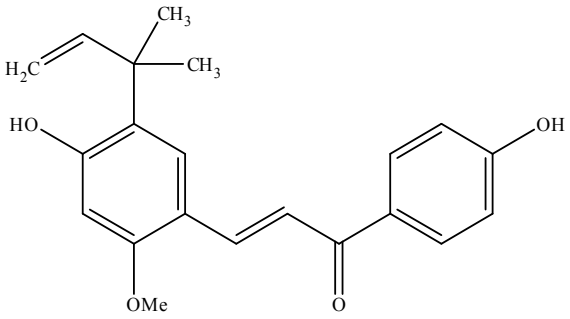
Indicine-*N*-oxide

Bioassay guided studies on the ethanolic extract of leaves of *V. cinerea* have resulted in the isolation of two sesquiterpene lactones, vernolide A and B. Vernolide A was identified as the bioactive compound, exhibiting significant cytotoxicity against human KB, DLD-1, NCI-661 and HeLa tumour cell lines[182].

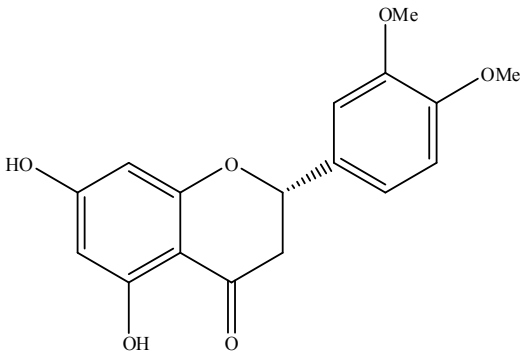


Vernolide A, R = OH, Vernolide B, R = OAc

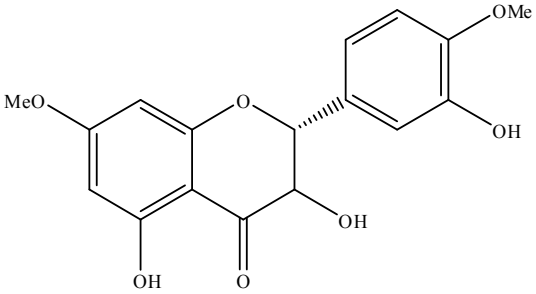
Bioassay guided fractionation studies have resulted in the isolation of licochalcone A, ombuin and 5,7-dihydroxy-3',4'-dimethoxyflavanone from *P. parviflorus*[146]. Licochalcone A showed *in vitro* cytotoxicity and PI-PLCγ1 inhibition activity[146]. The molecule is also reported to inhibit promyelocytic leukemia cells (HL-60)[146].



Licochalcone A



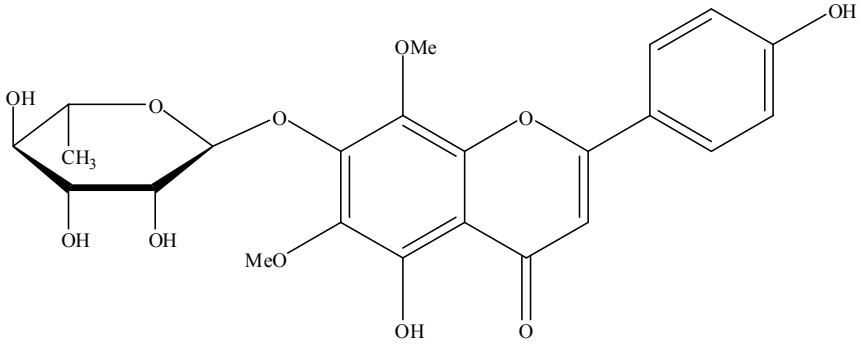
5,7-Dihydroxy-3',4'-dimethoxyflavanone



Ombuin

Another four plants in the list, *Indigofera aspalathoides, Hemidesmus indicus, Bryonopsis laciniosa* and *Sagittaria obtusifolia* have been well studied for anticancer activity and anticancer substances have been isolated from these plants.

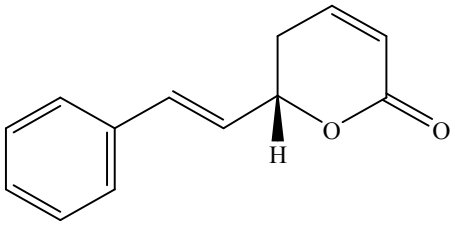
Alcoholic extracts of whole plant of *I. aspalathoides* have been reported to significantly increase the life span and decrease the cancer cell count and body weight in Dalton's ascitic lymphoma-induced mice. 5,4'-Dihydroxy-6,8-dimethoxy7-*O*-rhamnosylflavone was isolated from *I. aspalathoides* and was found to be cytotoxic against 57 human tumour cell lines representing leukemia, lung, colon, central nervous system, melanoma, ovarian, renal, prostate and breast cancers[197].



5,4'-Dihydroxy-6,8-dimethoxy7-*O*-rhamnosylflavone

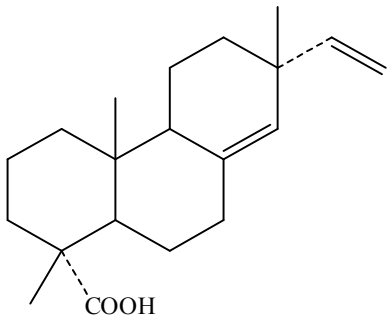
The modulating effect of *H. indicus* on cumene hydroperoxide-mediated cutaneous oxidative stress and tumour promotion response in murine skin has been reported[198]. The *H. indicus* extract reduced the cumene hydroperoxide-mediated increase in xanthine oxidase activity suggesting its role as an antitumor promoting agent[199]. The water decoction of *H. indicus* is reported to possess powerful cytotoxic properties towards HepG2 human liver cancer cell lines[200]. The methanolic extract is also reported to exhibit significant cytotoxicity against MCF 7 breast cancer cell lines[200].

Goniothalamin, isolated from *B. laciniosa*, has displayed antiproliferative activity against a variety of cancer cell lines, including cervical (Hela), gastric (HGC-27), kidney (786-0), breast carcinomas (MCF 7, T47D and MDA-MB-231), and leukemia (HL-60, Jurkat and CEM-SS)[201].



Goniothalamin

S. obtusifolia is phytochemically well characterised with many bioactive molecules isolated[163, 165]. The diterpene sandaracopimaric acid has been reported to be a potential antitumor promoting compound. It has also been reported to exert a significant inhibitory effect on lymphoblastoid Raji cells[202].



Sandaracopimaric acid

Another three plants in the list, *Toddalia asiatica, Azima tetracantha* and *Phyllanthus urinaria*, used by Dr Velmurugan for treating inflammation related conditions such as pain and swelling, have also been reported for antiinflammatory activity. The volatile oil of *T. asiatica* leaves obtained by steam distillation has been reported to exhibit good antiinflammatory activity comparable to ketorolac tromethamine, a non-steroidal antiinflammatory drug[203]. An antiinflammatory study carried out on leaves of *A. tetracantha* has reported the suppression of the transudative, exudative and proliferative components of chronic inflammation by the drug. It is also reported to protect the lysosomal membrane system during chronic inflammation[63]. The preventive effect of *P. urinaria* on paw edema in rats with adjuvant-induced arthritis (AA) has been reported[160]. The aqueous extract of *P. urinaria* was found to inhibit the neutrophil migration into inflamed tissues[160].

The literature available on *P. pallida*, *A. lanata*, *C. tora*, *V. cinerea*, *P. parviflorus*, *I. aspalathoides, H. indicus, B. laciniosa, P. urinaria, S. obtusifolia* and *H. indicum* provide scientific evidence for their anticancer activity, thus supporting the use of these plants by Dr Velmurugan for cancer treatment. Similarly, antinflammatory studies reported for *T. asiatica, A. tetracantha* and *P. urinaria* support the use of these plants by Dr Velmurugan for treating inflammatory conditions such as pain and swelling. However, it is noteworthy that so far chemical studies for identifying the bioactive molecules responsible for the antiinflammatory activities have not yet been carried out on these three plants.

The literature also identified that some of the plants in Dr Velmurugan's list have not been studied biologically or chemically in a manner relating to Dr Velmurugan's usage. *C. halicacabum, C. fimbriata, T. indicum, A. indicum, B. hispida, E. alsinoides, C. religiosa, M. tinctoria* and *C. viscosa* had no reports relating to their anticancer properties. *T. asiatica, A. tetracantha, P. urinaria, P. elata* and *H. antidysentrica* require further investigation on their antiinflammatory properties. These plants were considered as promising plants, worthy of further research to identify anticancer and antiinflammatory molecules. The five plants with potential antiinflammatory activity have not been further investigated in this study as it was decided to focus on the plants used by Dr Velmurugan for cancer treatment, *i.e. C. halicacabum, C. fimbriata, T. indicum, A. indicum, B. hispida, E. alsinoides, C. religiosa, M. tinctoria* and *C. viscose*. The morphological and taxonomical features of these nine plants, along with the available information on their ethnomedicinal use, phytochemistry and pharmacology are discussed in detail in the next section.

2.3.1 *Cardiospermum halicacabum*[1, 2]

Family: *Sapindaceae*
Common name: Balloon vine
Growth form: Vine/herb

Cardiospermum halicacabum is a flowering vine from a small genus of 12 species. It is a perennial climber that can grow up to 10 m and is characterised by its large seed capsules that are balloon shaped[204]. The species is Indigenous to North and Central America, but also found across South East Asia and Australia[204].

C. halicacabum has been cited as a medicinal and food resource in the Indian and Chinese systems of medicine[205]. The species is commonly reported as an antiinflammatory remedy, especially for rheumatoid arthritis[206]. Juice from the leaves of this plant taken internally with castor oil is found to be highly effective against inflammatory conditions[206]. There are also reports indicating that the juice is used in traditional medicine to reduce hardened tumours[207]. In addition, the aqueous extract of the species is used to treat chronic immune disorders[72]. This property is reportedly due to its ability to inhibit the release of histamine and nitric oxide production[72].

Earlier pharmacological studies have reported this plant as an analgesic, antiinflammatory, vasodepressant and antispasmodic[72]. The shoots and fruit of this plant are found to inhibit the virus induced cytopathogenicity in MT-4 cells infected with HIV[71]. The antioxidant activity of the methanolic extract of leaves of *C. halicacabum* has also been reported[207]. This plant has additionally been reported for its vermicidal and antidiarrhoeal activity[208,209]. Rutin, chrysoeriol and luteolin have been isolated from the leaves of *C. halicacabum*[210]. To date no anticancer related studies has been reported for this plant.

[1] Photos reproduced with permission from Dr Velmurugan; Tushar K V, Taxonomist, CMPR, Kottakkal; Wikipedia Commons
[2] A glossary of taxonomical terms are given in Appendix 2

2.3.2 *Evolvulus alsinoides*

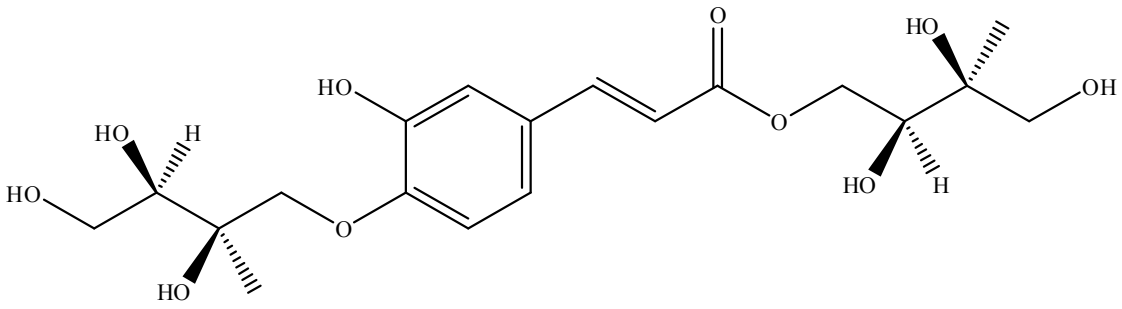
Family: Convolvulaceae
Common name: Dwarf morning-glory
Growth form: Perennial herb

Evolvulus alsinoides is a diffuse perennial herb belonging to the family of Convolvulaceae, which grows up to 35-40 cm. Its branches are annual, numerous, more than 30 cm long, often prostrate, slender and wiry with long hairs[211]. Native to South India, its also reported from Sri Lanka, tropical Africa and South Eastern Asia[211, 105]. *E. alsinoides* is well known for its memory enhancement, antiepileptic and immunomodulatory properties in Ayurveda system of medicine[105]. In Sri Lanka, roots and stem extracts of the plant are used to treat dysentery and depression[211]. The Valaiyan community of Piranmalai hills, Tamilnadu uses the leaf juice of *E. alsinoides* for fever[212]. The whole plant is used by the Kani group in South India for venereal diseases[213].

The alcoholic extract of *E. alsinoides* is reported for its marked antiulcer and anticatatonic activity [214]. The immunomodulatory properties of crude aqueous extract of roots and aerial parts of *E. alsinoides* is also reported[108]. The antioxidant activity of ethanolic extracts and water infusion of roots and leaves of *E. alsinoides* was tested in the 2,2′-azinobis-3-ethyl-benzothiazoline-6-sulfonic acid radical cation decolourisation assay[24, 107]. The ethanolic extract was found to possess significant antioxidant activity[24, 107, 211]. Adaptogenic and antiamnesic properties have also been reported from the whole plant[105].

Various phytochemical studies have been reported for *E. alsinoides*. The chemical constituents reported include scopoletin, scopolin, umbelliferone, 2-methyl-1,2,3,4-butanetetrol[109]; ferulic acid, 2-*C*-methyl erythritol, kaempferol-3-*O*-*β*-glucopyranoside, kaempferol-7-*O*-*β*-glucopyranoside, 6-methoxy-7-*O*-*β*-glucopyranoside, quercetin-3-*O*-*β*-

glucopyranoside, pentatriacontane, triacontane and *β*-sitosterol. 2,3,4-Trihydroxy-3-methylbutyl 3-[3-hydroxy-4-(2,3,4-trihydroxy-2-methylbutoxy)-phenyl]-2-propenoate, 1,3-di-*O*-caffeoyl quinic acid methyl ester, 6-methoxy-7-*O*-*β*-glucopyranoside coumarin[109]; betaine, shankpushpin[110]; evolvulin, evolvoids A and B[105]; are also reported from this plant. 2,3,4-Trihydroxy-3-methylbutyl 3-[3-hydroxy-4-(2,3,4-trihydroxy-2-methylbutoxy)-phenyl]-2-propionate was found to exhibit significant antistress activity in acute stress induced biochemical changes in rats[109]. No anticancer studies have been reported on this plant.



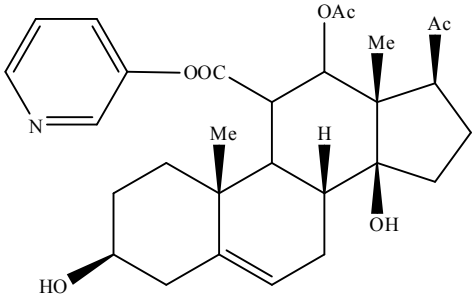
2,3,4-Trihydroxy-3-methylbutyl 3-[3-hydroxy-4-(2,3,4-trihydroxy-2-methylbutoxy)-phenyl]-2-propionate

2.3.3 *Marsdenia tinctoria*

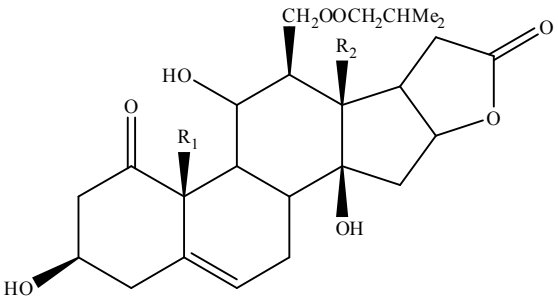
Family: *Asclepiadaceae*
Common name: Milkweed
Growth form: Climber

Marsdenia tinctoria is a perennial climber belonging to the family Asclepiadaceae. The genus *Marsdenia* consists of four reported species. *M. tinctoria* is distributed in India, Bangladesh, South China, Taiwan and Tibet[149]. *M. tinctoria* demonstrates significant medicinal properties and is cited widely as a medicinal resource in India and Bangladesh, usually as an abortative or anti-fertility agent[149] and the traditional healers from Sikkim Himalayas use the leaf juice of this plant for treating stomach ache[215].

Bioassay guided studies on oxytocic, antiimplantation and abortifacient activities have resulted in the isolation of tinctoramine and tinctoralactone from this plant[149]. Tinctoroside A, B and C[150]; lupenyl palmitate, lupenyl acetate, lupenone, lupeol, β-sitosterol, 5,7-dihydroxy-2,6,8-trimethylchromone and 2,6-dimethoxybenzoquinone[216] have also been reported from this plant. No anticancer studies have been reported for this plant.



Tinctoramine



Tinctoralactone ($R_1 = CH_2OH$, $R_2 = COOCH_3$)

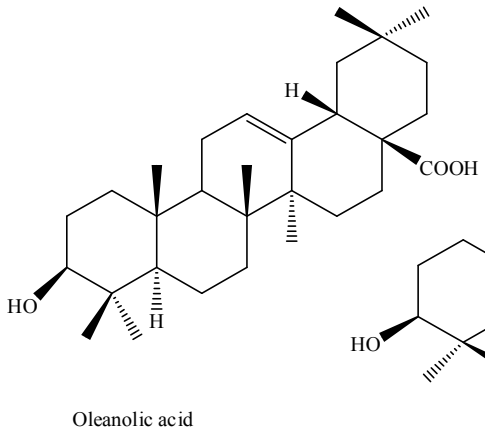
2.3.4 *Crateava religiosa*

Family: *Capparaceae*
Common name: Temple plant
Growth form: Small tree (shrub)

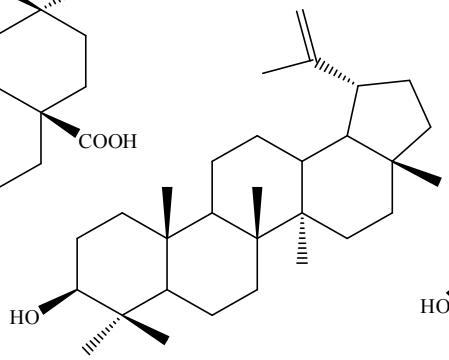
Crateava religiosa is a moderate-sized deciduous tree named after Cratevas, a Greek botanist[217]. The genus *Crateava* consists of eight reported species[217]. *C. religiosa* is distributed through Bhutan, Cambodia, India, Indonesia, Myanmar, Nepal, Philippines, Sri Lanka, Thailand, Vietnam and the Pacific islands[218]. The leaves are petiolate and covered with spiny stipules. They have distinctive flowers having hypogynous disks, from which long stamens emerge[218, 219]. *C. religiosa* is traditionally used extensively for various ailments. Crushed leaves of this plant are applied to relieve swelling and burning sensations[220]. Fresh leaves along with the bark of fresh root applied with vinegar act as a rubefacient and vesicant[26]. The

aqueous decoction of the bark is used in the disorders of urinary organs and urinary calculi[26, 221]. The bark is also reported to be used for calculi, flatulence, anaemia and heart complaints[103].

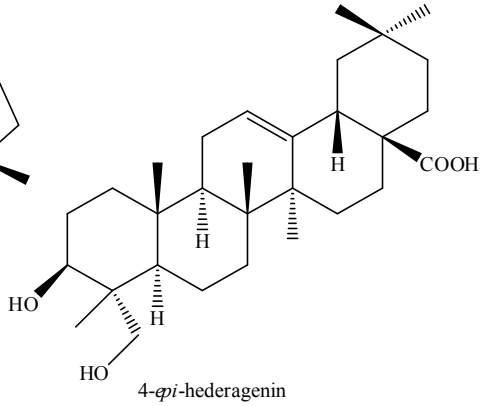
So far, lupeol[102] oleanolic acid and 4-*epi*-hederagenin[101] have been reported from this plant. Lupeol isolated from the stem bark of *C. religiosa* exhibits antiarthritic activity through possible suppression of T–lymphocytes[102]. The dichloromethane-methanol extract of the seeds of this plant was evaluated for its toxicity to brine shrimp. Bioassay guided studies resulted in the isolation of oleanolic acid and 4-*epi*-hederagenin[101]. The aqueous extract of *C. religiosa* is reported to be totally inactive against *Bacillus cereus, Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas pseudoalcaligenes* [103, 104]. No anticancer studies have been reported for this plant.



Oleanolic acid



Lupeol



4-*epi*-hederagenin

2.3.5 *Borreira hispida* (Syn: *Spermacoce neohispida*)

Family: Rubiaceae
Common Name: Shaggy buttonweed
Growth form: Herb

Borreira hispida is a small plant distributed in China, Indo-China and the Indo-Malaysian region. It is found throughout India as a weed in cultivated fields, fallow lands and in pastures. It is a procumbent herb with subterete stems. The leaves are ovate-elliptic, with marginal spinous-ciliates[221, 222]. The flowers are pinkish white with a purple tinge in the axillary fascicles[221, 222].

Seeds of this plant are cooling and demulcent and are given for treating diarrhoea and dysentery in Ayruveda[221]. Seeds are used in the treatment of haemorrhage and haemorrides in Siddha medicine[223]. The whole plant is used in Siddha preparations for treating fever[223]. It is also reported as an effective natural drug for the treatment of hypertension[67]. Tribal people living in the Western ghats of Kerala, India use this plant for purifying blood and improving vitality[224]. No phytochemical studies have been reported on this plant so far. A recent *in vitro* study has reported the potent antihyperlipidemic effect of *B. hispida* seeds[67].

2.3.6 *Cleome viscosa*

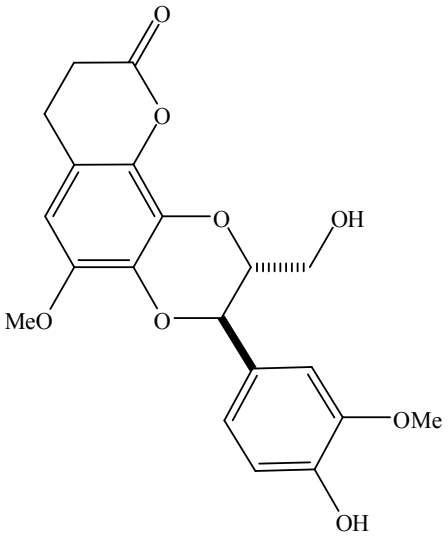
Family: Capparidaceae
Common Name: Wild mustard
Growth form: Herb

Cleome viscosa, belonging to the Capparidaceae family, is a weed widely distributed in tropical regions and plains of India. The genus *Cleome* consists of 275 species[225]. *C. viscosa* is an annual herb with a strong penetrating odour[222]. It grows up to 30-90 cm. The leaves are 3-5 foliate and obtuse and gradually get shorter upward[226]. The plant is extensively used in the Ayurvedic system of medicine, and has been reported to possess cooling, diuretic and anthelmintic properties[227]. The juice of the leaves is used in Unani medicine (a traditional medicinal system originating from the middle east) for earache, malaria, piles, fever, diarrhoea and lumbago[228,229]. The leaves are used by Australian aboriginals as a remedy for headache[229].

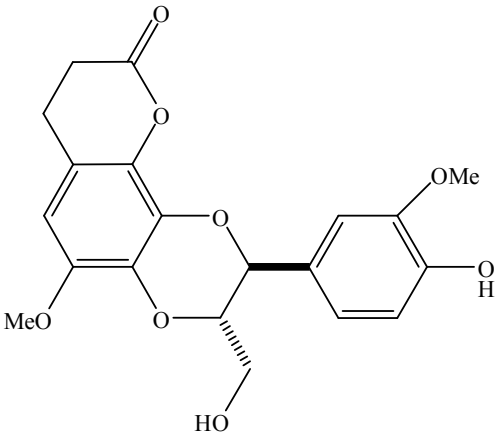
No phytochemical and pharmacological studies have been reported on this plant from an anticancer perspective. However, a number of phytochemicals have been isolated from various parts of this plant. These include viscocic, viscosin[86]; cleosandarin[87]; cleomiscosin A, B, C and D (seeds)[88,89,90,91]; dihydrokaempferide-3-glucuronide, dihydrokaempferol-4'-xyloside, docosanoic acid[92]; naringenin-4-galactoside[93]; naringenin-4-(xylosyl-*β*-(1,4)-glucoside)[94]; 5'-trihydroxyflavanone-7-*O*-*α*-l-rhamnopyranoside, *β*-amyrin, lupeol[95, 96] (roots); eriodictyol-5-rhamnoside[96]; 3',4'-dihydroxy-5-methoxyflavanone-7-*O*-*α*-l-rhamnopyranoside[94]; stigmasta-5,24(28)-diene-3*β*-*O*-α-l-rhamnoside[97]; ergast-5-ene-3-*O*-α-l-rhamnopyranoside,5,4'-di-*O*-methyleriodictyol-7-*O*-β-d-glucopyranoside[98]; glucocapparin, glucocleomin[99]; and cleomaldeic acid[100] (whole plant). Cleomiscosin A and B were identified as the antihepatotoxic principles from this plant[230].

The *n*-hexane extract of the leaves and stems of *C. viscosa* have been studied for antibacterial activity by a bioautographic assay[231]. Bioassay guided studies resulted in the identification of a cembranoid diterpene, cleomaldeic acid, as the bioactive compound[231].

Analgesic activity of the methanolic extract of this plant has also been studied *in vivo* by acetic acid-induced writhing and the tail flick, tail clip and tail immersion methods in mice[232]. Even though the plant has been phytochemically well characterised, no anticancer studies have been reported for this plant.



Cleomiscosin A



Cleomiscosin B

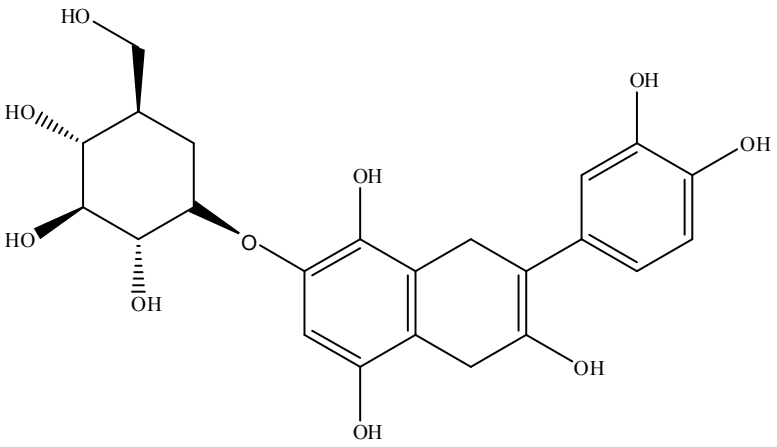
2.3.7 *Abutilon indicum*

Family: Asclepiadaceae
Common name: Rosy milkweed vine
Growth form: Twining herb

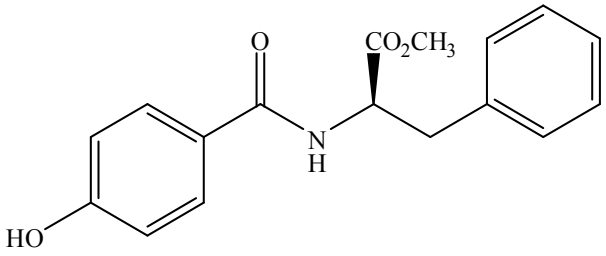
Abutilon indicum (Linn) is a small shrub belonging to the family of Malvaceae commonly known as 'Thuthi or Atibala'[233]. The leaves are ovate, acuminate, toothed, rarely subtrilobate and 1.9-2.5 cm long. This plant is distributed throughout the hotter parts of India, Sri Lanka, topical regions of America and Malaysia[43, 234].

The roots of the plant are considered to have demulcent, diuretic activity and have been used to treat urethritis[26]. It is used as a remedy for jaundice, piles, ulcer and leprosy in the Siddha system of medicine[43]. The juice of the leaf is used in toothache, tender gums and internally for inflammation of the bladder[235, 236]. The antinociceptive and antioxidant activity of *A. indicum* has been reported[43]. The alcohol and water extracts of *Abutilon indicum* leaves have been reported to possess significant hypoglycemic effects in normal rats[237]. The whole plant fine powder has been reported to exhibit significant immunomodulatory activity in albino mice[44]. In a study subsequent to the shortlisting of plants for anticancer screening the chloroform fraction of *A. indicum* was found to be cytotoxic against MCF 7, NCI-H460, and SF-268 tumor cell lines[45].

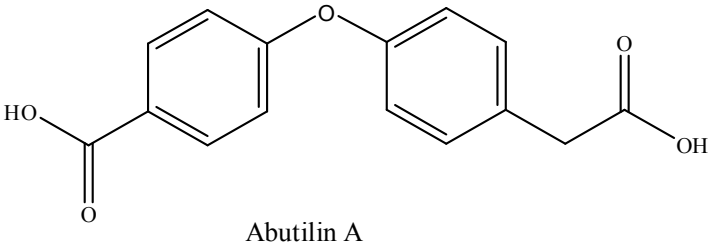
Chemical constituents reported from *A. indicum* include *β*-sitosterol, geraniol, caryophylline[45]; abutilin A and B[238]; (*R*)-*N*-(1'-methoxycarbonyl-20-phenylethyl)-4-hydroxybenzamide[45]; methylcoumarate[43]; 4-hydroxyacetophenone[46]; aurantiamide acetate[47]; methyl indole-3-carboxylate, gossyseptin-7-*O*-glucoside[48]; 3,7-dihydroxychromen-2-one[49]; scopoletin, syringaldehyde, and 1-methoxycarbonyl-*β*-carboline[45].



Gossypetin-7-*O*-glucoside



(*R*)-*N*-1'-methoxycarbonyl-2'phenylethyl-4-hydroxybenzamide



Abutilin A

2.3.8 *Caralluma fimbriata*

Family: Asclepiadaceae
Common name: Famine food
Growth form: Perennial herb

The genus Caralluma consists of 57 species and all are leafless[239]. *Caralluma fimbriata* is an erect, fleshy small plant with grooved, round shaped stems[240]. It forms small dark coloured flowers[70, 241]. It is traditionally used by tribal Indians to suppress hunger and enhance endurance[70]. The plant is also used for treating diabetes, pain, fever and inflammation[242].

The effect of *C. fimbriata* on appetite and anthropometry has been studied. The study showed that regular intake of the leaves resulted in a significant reduction in waste circumference and hunger level[70]. No other pharmacological and phytochemical work has been reported on this plant. Other plants belonging to this genus are well known for the presence of cytotoxic and tumouricidal pregnane esters and glycosides[241, 243].

2.3.9 *Trichodesma indicum*

Family: Acanthaceae
Common Name: Acanthus, King of bitters
Growth form: Annual herb

Trichodesma indicum is a hispid, erect or diffuse annual herb belonging to the family Boraginaceae and is found throughout the greater part of India[170]. Its also distributed in Afghanistan, Pakistan, Philippines and Mauritius[26]. The plant grows up to 2 m tall, much-branched, with lower leaves opposite, and upper leaves alternate[26]. The whole plant and roots are reportedly used to treat arthritis, anorexia, dysentery, skin diseases, snakebite poisoning and fever[244]. The root is

pounded into a paste and is applied to reduce swelling and the extract is given to children suffering from dysentery and fever[245, 246]. Tribal people of Madhya Pradesh, India use the plant for treatment of breast cancer[247]. The methanol extract of the whole plant of *T. indicum* has shown significant cough suppressant activity in Swiss Albino mice[172]. The chloroform extract of *T. indicum* root has been reported for antiinflammatory activity against oedema produced by carrageenan[170]. The methanol extract of the whole plant of *T. Indicum* has been reported for significant inhibition in frequency of cough on sulphur dioxide (SO_2) induced cough reflex in Swiss albino mice[172]. Other pharmacological activities reported include antidiarrheal[171] and antispasmodic activities[248]. Hexacosane, ethyl hexacosanoate and 21,24-hexacosadienoic acid (leaves)[173], and oleic, linoleic, palmatic, stearic and linolenic acid (seed oil)[174] are reported from this plant. No anticancer studies have been reported for this plant.

2.4 Conclusion

An agreement was established between the IBRG and Dr Velmurugan, a Siddha practitioner. Based on the agreement, Dr Velmurugan provided information of some plants used by him in the treatment of cancers and inflammation related conditions. An exhaustive literature search for any reports on anticancer/antiinflammatory activity was performed on these plants. This search identified fourteen plants in the list that have been investigated and reported for anticancer and/or antiinflammatory activity. This was in accordance with the plants' use by Dr Velmurugan and therefore supports the traditional use of these plants in Siddha medicine.

Nine plants used by Dr Velmurugan for treating cancer and five plants used for treating conditions related to inflammation were unexplored from a chemical and/or biological perspective, thus making them potential candidates for further investigations, including bioassay guided isolation and identification of bioactive substances.

As the primary focus of this project was on anticancer studies, the nine plants, *C. halicacabum, C. fimbriata, T. indicum, A. indicum, B. hispida, E. alsinoides, C. religiosa, M. tinctoria* and *C. viscose,* which had no reports on anticancer studies or relevant bioactive molecules at the time of this study, were selected for further investigation. A

more detailed review of the literature encompassing ethnomedicinal use, phytochemical and bioactivities related to these plants was undertaken. The collection, processing and extraction of plant materials and the preliminary biological screening studies carried out on these plants are discussed in Chapter 3. Even though further studies on the plants with potential antiinflammatory activity, *T. asiatica, A. tetracantha, P. urinaria, P. elata* and *H. antidysentrica* were not considered, these plants warrant further investigation for their potential bioactive molecules.

Chapter 3

Biological Studies on Siddha Medicinal Plants

This chapter describes the biological screening studies conducted on ethanolic extracts of the nine plants shortlisted from Chapter 2. The selection of the bioassays and the results that guided the identification of potential plants for further bioassay guided studies is also detailed.

3.1 Background

The major objective of this component of the study was to investigate the selected plants from Chapter 2 for biological activities consistent with their use by Dr Velmurugan for treatment of patients with cancer. Antiproliferative and indoleamine 2,3-dioxygense (IDO) inhibitory assay studies were chosen for the biological screening of all the plants. Abnormal cell proliferation is a necessary feature of the formation of tumours. Assessment of the antiproliferative activity of cells grown in culture is therefore an important parameter in the discovery of anticancer therapeutics[249]. IDO is an enzyme that has been found to be overexpressed in many tumour cells and IDO inhibition has been recently recognised as an effective strategy for the treatment of cancer[250, 251].

Cellular proliferation and neoangiogenesis are closely associated with inflammation and the beneficial effect of antiinflammatory drugs as potential anticancer agents is being investigated[252]. The crude plant extracts that showed potent antiproliferative activity were therefore also tested for their antiinflammatory activity.

The selection of bioassays, the assay techniques and results are discussed in detail in the next sections.

3.2 Antiproliferative Studies

Cancer prevention is generally associated with inhibition, reversion or retardation of cellular hyperproliferation[253]. Many anticancer molecules have been demonstrated to inhibit proliferation in different cultured human cancer cell lines[253].

3.2.1 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

A variety of strategies including direct cell counting, measuring DNA content and measuring metabolic viability are being used for assessing the cell proliferative activity of anticancer molecules[249]. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay method, which measures metabolic viability, is one of the most frequently used methods for determining cellular proliferation[249, 254]. It is regarded as a reliable, relatively cheap and user friendly assay[249, 255] and has been used to evaluate the antitumour activity of a large number of natural products, permitting the isolation of biologically active principles[256].

The MTT assay is based on the reduction of the tetrazolium salt, MTT, to a coloured formazan by mitochondrial dehydrogenases in metabolically active cells[257] (Fig 3.1). It was first described by Mosman in 1983[258]. As it allows determination of viable cells, it has been used to determine cell proliferation in response to growth factors, cytokines, mitogens and nutrients[259], to analyse anticancer drugs[260, 261] and to assess growth inhibitory antibodies and physiological mediators[262, 263].

For examining anticancer agents, cells are incubated in the presence of test substances and the cell viability is determined by measuring the amount of the formazan (λ_{max} 580 nm) formed at the end of the incubation period[263].

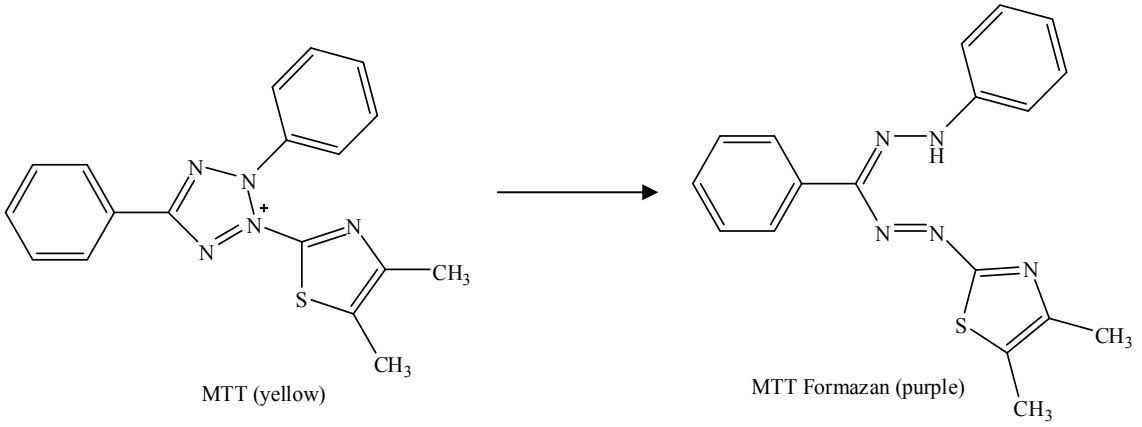


Fig 3.1 Enzymatic conversion of MTT to MTT formazan by living cells

3.3 Indoleamine 2,3-dioxygenase inhibitory studies

The mammalian enzyme indoleamine 2,3-dioxygenase (IDO) catalyses the oxidative cleavage of the essential amino acid L-tryptophan in the kynurenine pathway, which is the major tryptophan catabolic pathway in humans[264](Fig 3.2). IDO is an important immunoregulatory enzyme that is significantly induced under various pathological conditions, including viral, bacterial and protozoan infections[265]. This is believed to suppress the growth of pathogens *via* in part depletion of the essential amino acid L-tryptophan. This depletion of L-tryptophan has also been shown to inhibit T-cell proliferation[266, 267].

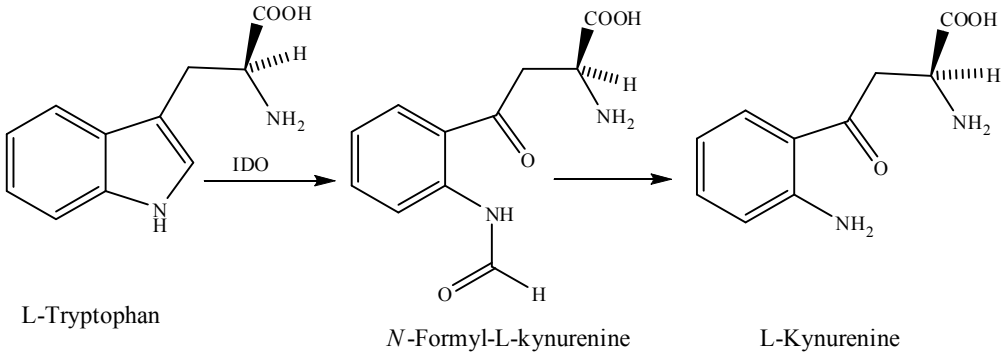


Fig 3.2 First step of the kynurenine pathway

IDO is expressed in various human tumours[251, 268] and IDO expression by ovarian cancer cells[269] and colorectal cancer cells[270, 271] are both associated with decreased patient survival. It is believed that the ability of IDO to control T-cell function protects the

tumours from immune eradication[266, 272]. A number of studies have shown that IDO inhibitors in combination with cancer therapeutic agents can significantly reduce the size of tumours, presumably *via* decreasing the cancer cells ability to evade T-cells[274]. Thus, IDO inhibition has emerged as an attractive strategy for the treatment of cancers[250, 273, 274].

3.3.1 IDO inhibitory Assay

The most common method for assessing IDO inhibitory activity is the absorbance based assay[275]. In this assay IDO is incubated with the substrate, L-tryptophan and test substance in an artificial reducing system consisting of ascorbic acid, catalase in potassium phosphate buffer (pH 6.5) and methylene blue[276-278]. This reducing system keeps the enzyme in its active state. After the incubation period, trichloroacetic acid (TCA) is added to the reaction mixture to halt the reaction and hydrolyse the *N*-formyl-L-kunurenine formed to L-kynurenine[275, 277](Fig 3.3). *N*-Formylkynurenine is highly unstable and cannot be quantified directly. *p*-Dimethylaminobenzaldehyde (Ehrlich's reagent) is then added, which rapidly reacts with L-kynurenine to form a yellow coloured imine that is quantified by measuring the absorbance at 480 nm.

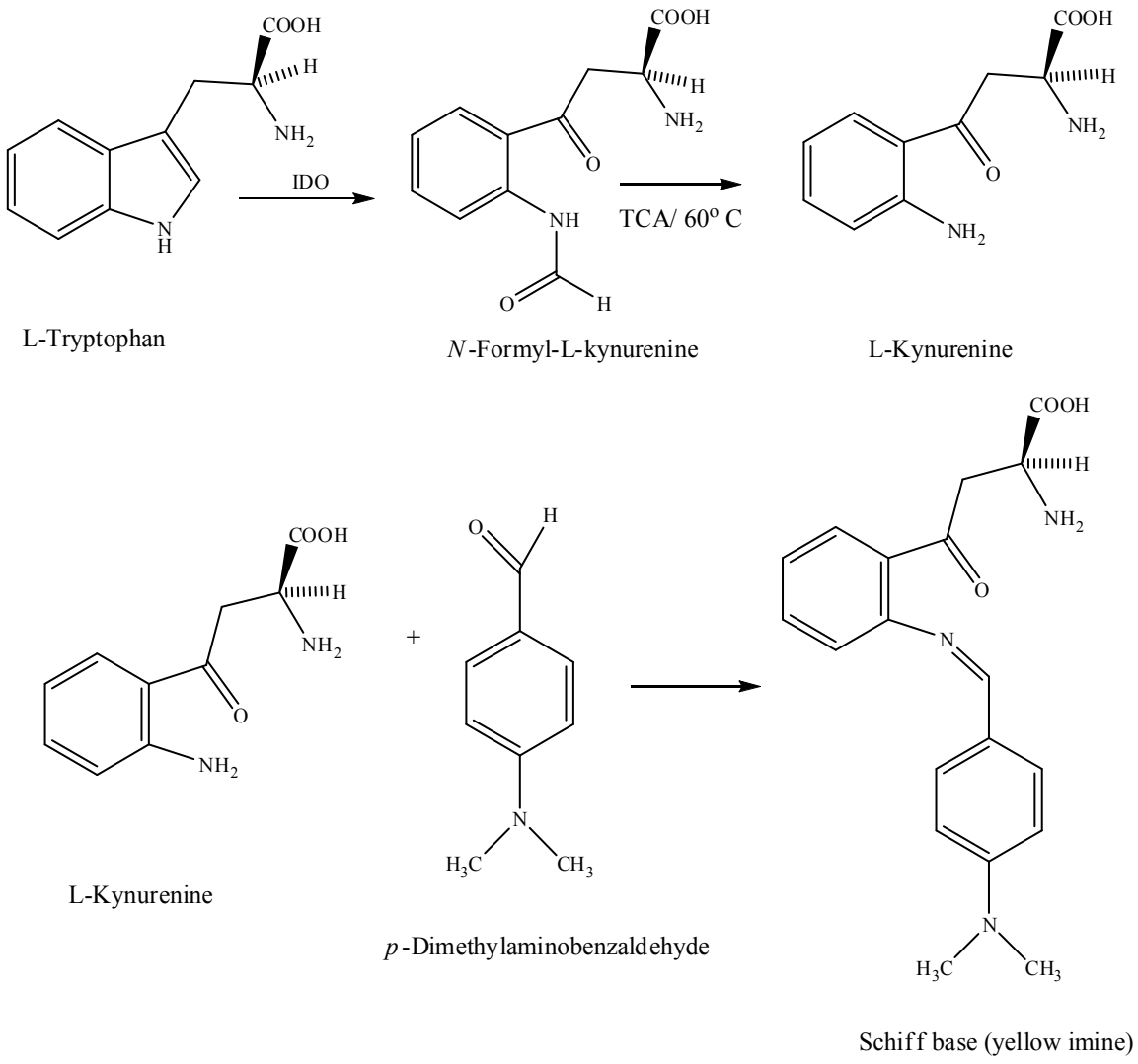


Fig 3.3 Molecular representation of IDO inhibitory assay

3.4 Antiinflammatory studies

Inflammation is a complex set of interactions that can arise in any tissue as part of the body's normal response to trauma, infection or injury[279]. Numerous cellular and plasma mediators like prostaglandins, leukotrienes, amines, purines, cytokines, chemokines, kinins and adhesion molecules are associated with this process[280]. Even though inflammation is an essential defense mechanism of the body, if not properly targeted it can lead to serious tissue damage due to the chronic activation of leukocytes, lymphocytes or collagen[279]. Prostaglandins (PG) and leukotrienes are the critical inflammatory mediators that lead to increased vascular permeability and increased

vasodilation in the inflammatory site and cause swelling and increased sensitivity to pain[280, 281]. Arachidonic acid (Fig 3.4), a 20:4 omega fatty acid, is the precursor for prostaglandins and leukotrienes[282]. The first step in the synthesis of prostaglandins is catalysed by cyclooxygenase (COX) by converting arachidonic acid, an essential fatty acid, into prostaglandin H_2, which is a common substrate for specific prostaglandin synthases. Three isoforms of COX exist and two of them COX 1 and COX 2 shares 80% homology[283].

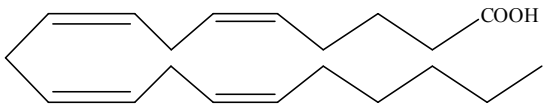


Fig.3.4 Arachidonic acid

Various pharmacological studies in animals and humans have shown that there is a significant increase in mRNA, protein and activity levels of COX 2 during inflammation, leading to the concept that COX 2 is the isoform that plays the pivotal role in inflammation[284, 285].

3.4.1 Relationship between inflammation and cancer

There is increasing scientific evidence to support the role of COX 2 in cell proliferation and neoangiogenesis. It has been reported that there is a 2 to 50 fold increase in the COX 2 expression in 85% of adenocarcinomas[286, 287]. The increased COX 2 expression associated with colon cancer has also been reported in carcinogen induced and genetic animal models. *In vitro* studies have shown that cells overexpressing COX 2 exhibit greater affinity to extracellular matrix proteins, resistance to apoptosis and stimulate endothelial cell migration, proliferation of cancer cells and *in vitro* angiogenesis by producing higher amounts of vascular endothelial growth factor[288, 289]. Since tumour promotion is closely linked to inflammation, a compound that exhibits antiinflammatory properties is expected to act as an antitumour promoter[290].

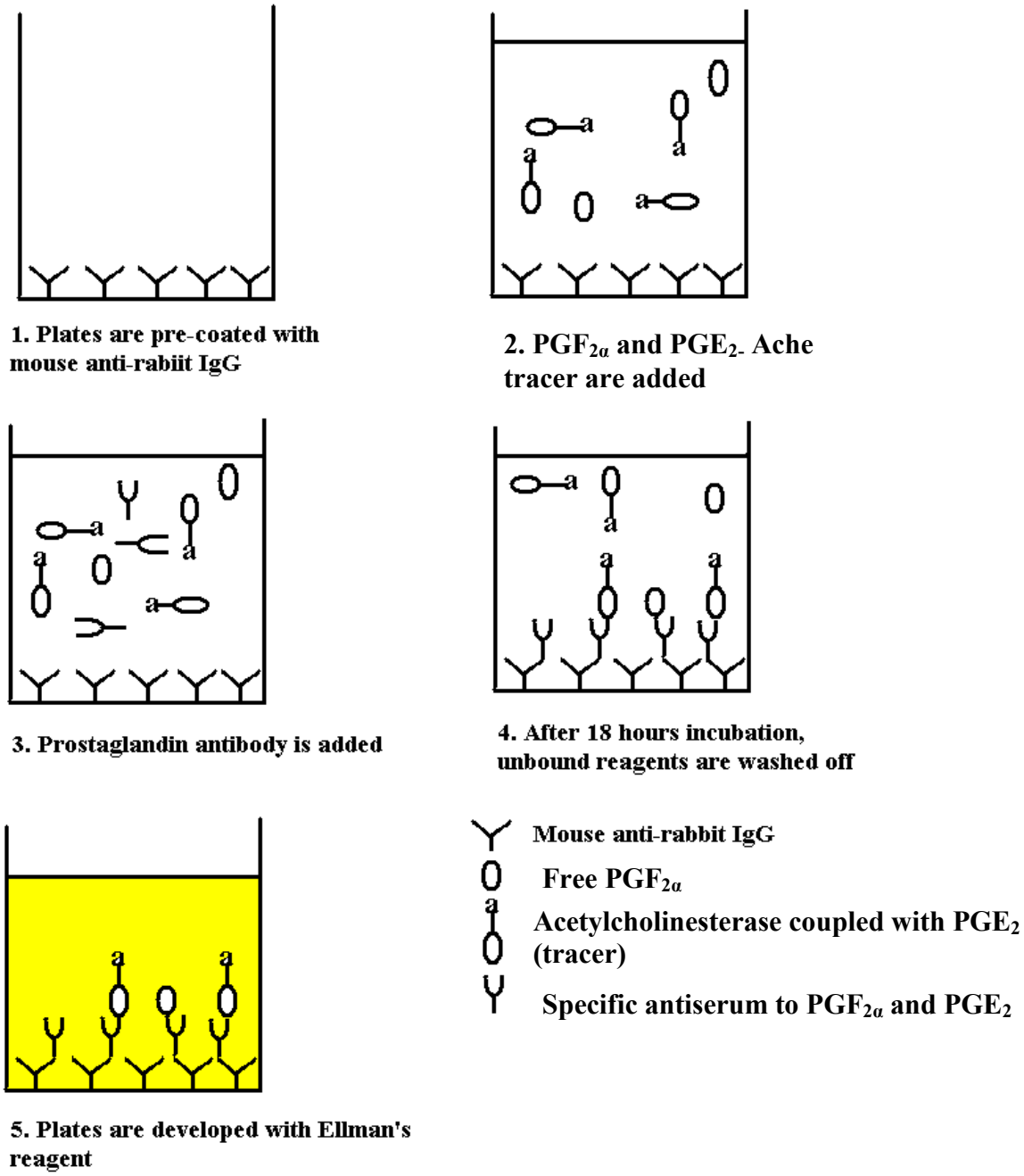
Because of the above associations, the beneficial effect of COX 2 inhibitors as potential anticancer agents is now being investigated[291]. Regular long term consumption of aspirin, a non steroidal antiinflammatory drug (NSAID), is found to be associated with a 40-50%

reduced risk of colon cancer and also reduces the risk of lung, oesophagus and stomach cancer[292]. The efficacy of COX inhibitors such as piroxicam, indomethacin, ibuprofen and ketoprofen in reducing colon cancer in rats and mice has also been reported[285]. Celecoxib, a selective COX 2 inhibitor, has been shown to enhance the effect of conventional chemotherapy and radiation[293, 294]. Even though many of the molecular and cellular mechanisms mediating the fundamental relationship between inflammation and cancers are not yet resolved, the search for new therapeutic approaches to treat cancers directed at antiinflammatory activity is being actively pursued[295].

3.4.2 COX inhibitor screening assay

Commercially available COX inhibitor screening assay kits are commonly used to assess COX inhibition[296]. For these assays, COX 1 and COX 2 are treated with the test samples and then allowed to react with arachidonic acid. The amount of prostaglandin H_2 (PGH_2) formed in this reaction depends on the enzyme inhibitory activity of the test sample. The PGH_2 formed is reduced by $SnCl_2$ to the more stable compound prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The amount of $PGF_{2\alpha}$ produced by the treated COX 1 or COX 2 is quantified by an enzyme immunoassay (Scheme 3.1 and Fig 3.5) and is compared to that of the untreated COX enzymes. This enzyme immunoassay is based on the competitive affinities of $PGF_{2\alpha}$ and a PG acetyl cholinesterase conjugate (PG tracer) for a limited amount of PG antiserum[297]. The concentration of the PG tracer is maintained constant whereas the concentration of $PGF_{2\alpha}$ will vary depending upon the COX inhibitory activity. Hence, the amount of PG tracer that can bind to PG antiserum is inversely proportional to the amount of $PGF_{2\alpha}$ formed. The antibody-PG complex formed in the wells binds to a mouse anti-rabbit monoclonal antibody that is coated inside the wells [297]. The plate is washed to remove all unbound $PGF_{2\alpha}$ and PG tracer reagents and then Ellman's reagent is added to the well. Ellman's reagent is composed of acetylthiocholine and 5,5'-dithio-bis(2-nitrobenzoic acid). The bound acetylcholine esterase reacts with acetylthiocholine to form thiocholine, which further reacts with DTNB to form thionitrobenzoic acid which is yellow in color and can be quantified by measuring the absorbance spectrophotmetrically at 405 nm. The intensity of yellow colour is directly proportional to the amount of PG tracer bound to the well, which is inversely

proportional to the amount of PG formed[297]. A higher inhibitory activity is indicated by an intense yellow color and vice versa. The above chemical reactions are shown in Scheme 3.1 and Fig 3.5.



Scheme 3.1 Enzyme immunoassay in the COX inhibitor screening assay

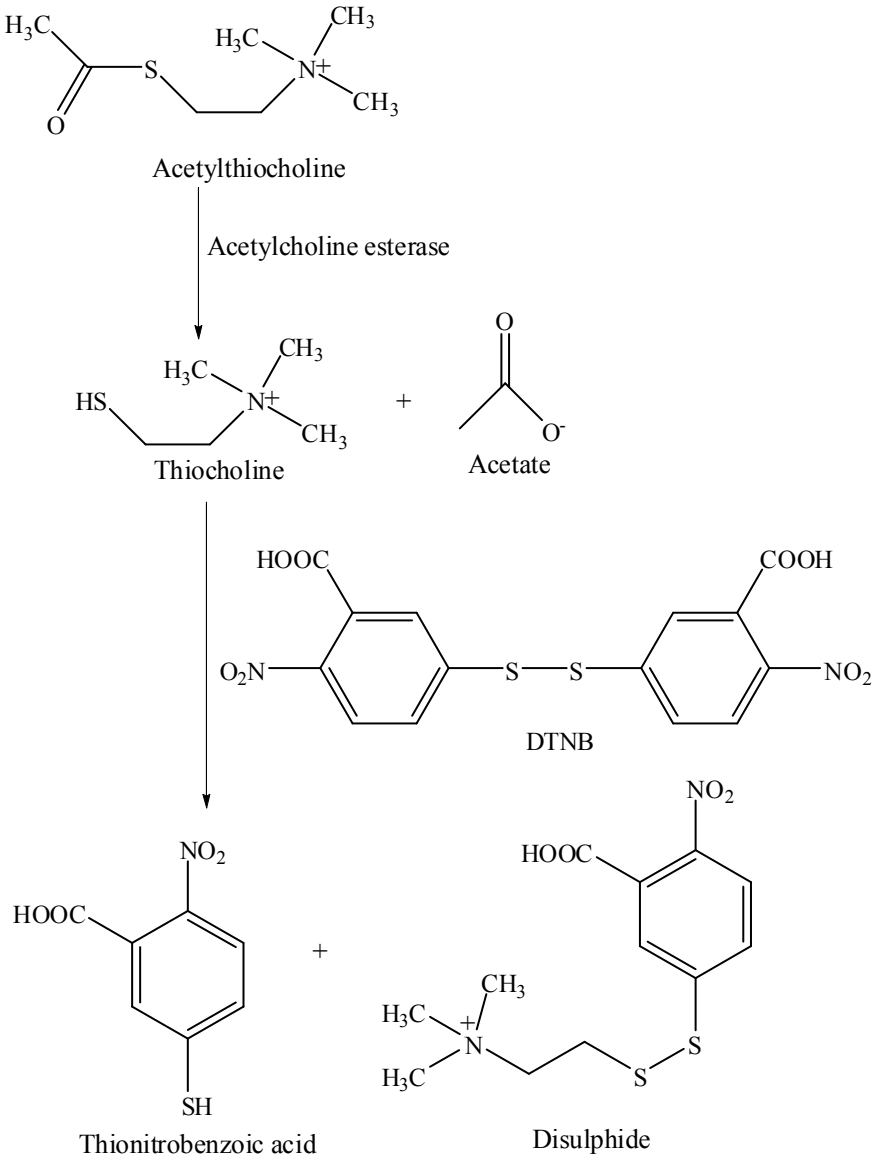


Fig.3.5 Reaction scheme for COX inhibitor screening assay

3.5 Results and Discussion

3.5.1 Collection, authentication and processing of plant materials

The collection of plant materials was carried out by Dr Velmurugan from different parts of South India. A voucher specimen was obtained for each plant and the authentication

was carried out at the Botanical Survey of India (BSI), Coimbatore, India. The voucher specimens were deposited at the BSI herbarium, Coimbatore for future reference.

The nine plants shortlisted, as described in Chapter 2, were collected, *i.e. Abutilon indicum* (roots and leaves), *Borreria hispida* (roots and leaves), *Caralluma fimbriata* (leaves), *Cardiospermum halicacabum* (roots and leaves), *Cleome viscose* (leaves), *Crataeva religiosa* (roots and leaves), *Evolvulus alsinoides* (leaves), *Marsdenia tinctoria* (roots and leaves), *Trichodesma indicum* (leaves). Additionally, *Vernonia cinerea* (leaves) was also collected. Previous bioassay guided studies carried out on *V. cinerea* (positive control) have identified the presence of two sesquiterpene lactones, vernolide A and vernolide B, which demonstrated potent cytotoxicity against KB, DLD-1, NCI-661 and Hela tumour cell lines[298]. *V. cinerea* was included in this study as a positive control. The collected plant materials were dried, powdered and sent to Australia under moisture free conditions. The protocol used by Dr Velmurugan for processing of the plants is described in the materials and method section.

3.5.2 Extraction of plant material

As described in Chapter 2, Dr Velmurugan provides his medicines as mixtures. However bioassay guided studies to isolate active molecule from a mixture of plants was regarded as unfeasible. Instead, all of the ten shortlisted plants were individually extracted to allow subsequent testing.

Extraction of dried plant material is commonly done with 70% aqueous ethanol[299-301]. This solvent system generally allows extraction of a wide range of secondary metabolites present in plant material, and was therefore used in this study. For screening, small scale extractions were conducted and the extracts were evaporated under reduced pressure and low temperature (40^{o}C) to decrease degradation of compounds. The yields (%, *w/w*) of the crude extracts are shown in Table 3.1.

Sample	Voucher Number	Crude extract % yield (*w/w*)
Abutilon indicum, roots, leaves	SRC/5/23/10-11/1240-4	14.9
Borriera hispida, roots, leaves	BSI3	19.5
Caralluma fimbriata, leaves	SRC/5/23/10-11/1240-3	8.2
Cardiospermum halicacabum, roots and leaves	BSI9	8.6
Cleome viscose, leaves	BSI4	7.7
Crateava religiosa, roots, leaves	BSI1	20.1
Evolvulus alsinoides, leaves	SRC/5/23/10-11/1240-1	11.7
Marsdenia tinctoria, roots, leaves	BSI6	12.6
Trichodesma indicum, leaves	SRC/5/23/10-11/1240-2	18.8

Table 3.1 70% aqueous ethanol crude extracts yields (%, *w/w*)

3.5.3 Antiproliferative studies

All antiproliferative assays in this study were carried out in the laboratory of Professor Des Richardson, Department of Pathology, University of Sydney, under the guidance of Dr Danuta Kalinowski. The MTT assay was carried out with the SKNMC cell line to test the antiproliferative activity of the crude plant extracts. SKNMC is a human neuroblastoma cell line and is one of the most commonly used cell lines for determining the anticancer activity of plant extracts and natural products[302, 303]. The anticancer drug doxorubicin was chosen as a positive control[301] along with the 70% crude extract of *V. cinerea*. A concentration range of 10 to 100 µg/ml of all the extracts was used for testing [304, 305].

C. halicacabum and *C. fimbriata* were found to be the most potent plants, exhibiting the highest antiproliferative activities against the SKNMC cell line at IC_{50} values of 22.0 ± 3.0 and 17.1 ± 1.0, µg/ml, respectively (Fig 3.6). Doxorubicin, the positive control, exhibited an IC_{50} value of 0.12 µg/ml. As expected, the crude extract of *V. cinerea* also showed inhibitory activity (63 ± 4.6 µg/ml). *M. tinctoria* exhibited moderate activity (60% reduction of cell proliferation at 100 µg/ml). The other plants did not exhibit any significant antiproliferative activity, even at the highest concentration of 100 µg/ml. The National Cancer Institute (NCI) has set criterias to identify plants from preliminary

screening results that should be further evaluated for potential bioactive molecules. According to them, an IC_{50} value of less than 30 µg/ml is recommended for crude extracts to warrant further investigation[306]. Both *C. halicacabum* and *C. fimbriata* were therefore identified as plants worth further investigation for their bioactive molecules.

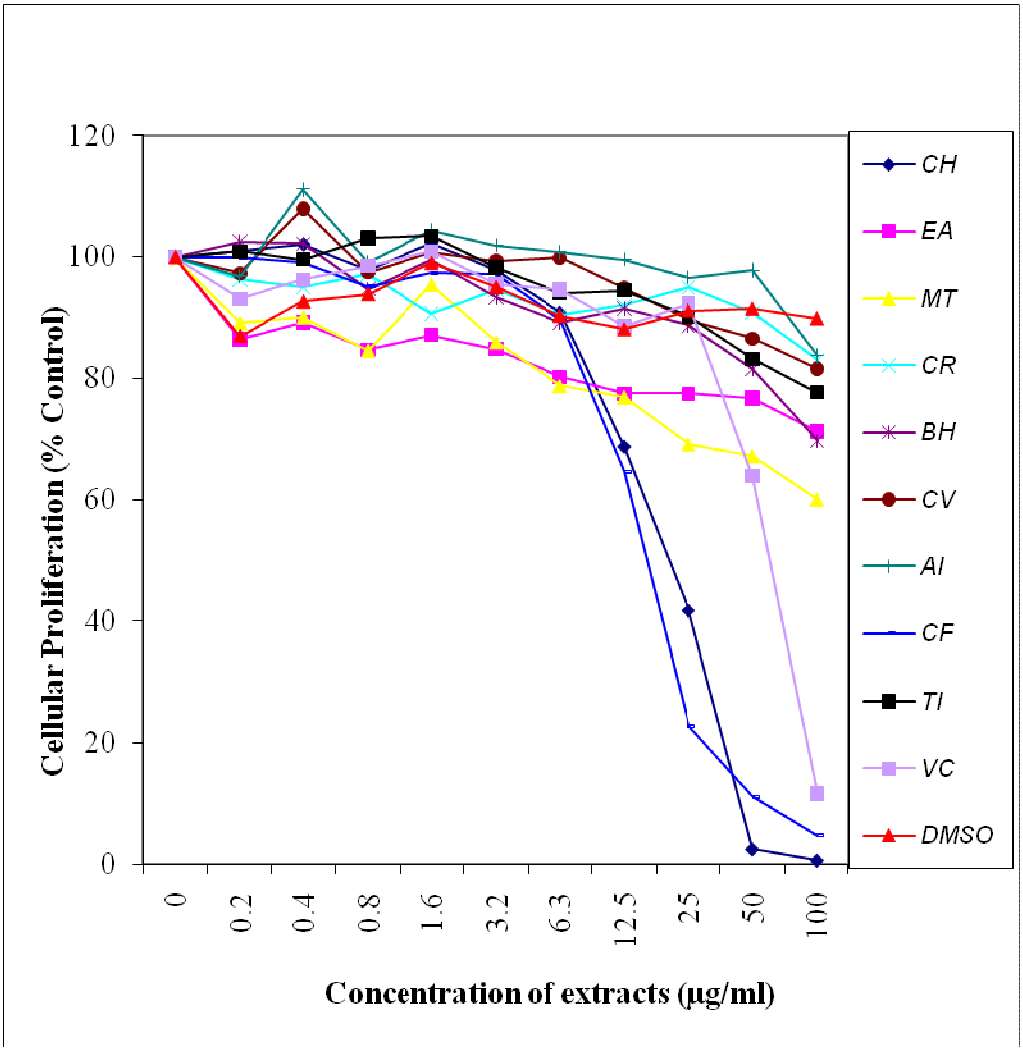


Fig 3.6 Antiproliferative activity of plant extracts against SKNMC cell line

The moderate level of activity exhibited by *M. tinctoria* could be a consequence of the presence of active molecules at comparatively low final concentration[301]. The fact that the other plants did not exhibit any significant antiproliferative activity against the SKNMC cell line does not necessarily mean that those plants are ineffective for cancer treatment. Possibly, if tested against a panel of other cell lines they may exhibit inhibitory activity. For example, the methanolic extract of *Aegiceras corniculatum* exhibited

significant cytotoxic activity against the colorectal adenocarcinoma cell line HT-29, whereas under identical conditions the same extract was found to be inactive against the gastric adenocarcinoma cell line AGS[307]. Also, herbal mixtures can exhibit therapeutic efficacy mediated through a synergistic mode of action[308, 309]. As mentioned earlier, Dr Velmurugan uses polyherbal combinations (usually a mixture of 2-3 plants) in capsule or pill form to treat his patients.

The antiproliferative activity of *C. halicacabum* and *C. fimbriata* were demonstrated for the first time in this study and these findings supports the use of these plants in Siddha medicine for treating cancers.

3.5.4 IDO inhibition studies

Recombinant human IDO (6His tagged, 6His-rhIDO) was used for the IDO inhibitory assays. This has been shown to be an excellent model for native human IDO[310, 311]. As this is not commercially available, it was expressed and purified using the method developed by Dr C. Austin, a former researcher of our group[310, 312, 313].

The expression was done in *Escherichia coli* EC538 (pREP4, pQE9-IDO), a strain selective for kanamycin and ampicillin resistant bacterial colonies. Luria Bertani (LB) medium was used for culturing *E. coli* at 37^0C. The medium contained isopropyl *β*-D-thiogalactopyranoside to elicit the induction of rhIDO, phenylmethylsulfonylflouride as a protease inhibitor to prevent the formation of truncated rhIDO and aminolevulinic acid as the heme source[311]. The initial purification was done using Ni-NTA (nickel nitrilotriacetic acid) affinity chromatography, which relies on having a high affinity for the nitrogen of imidazole groups such as the histidine imidazole groups of the 6-His tagged protein[311]. Initial elution with low concentrations of imidazole (10, 30, 40 50 and 70 mM) removed the non-specific proteins bound to the column resin, while a higher concentration of imidazole (300 mM) allowed elution of the 6His-rhIDO. 6His-rhIDO, which is unstable in the prolonged presence of imidazole, was immediately desalted by a NAP-10 (size exclusion) column and concentrated[313-315]. The specific activity of the enzyme was found to be 149 μmoles of kynurenine/h/mg of protein, and along with the gel electrophoresis (Fig 3.8) showed that this was sufficiently active and pure to use for

the IDO inhibitory studies (a specific activity of 150-160 µmoles of kynurenine/h/mg of protein is considered as pure for IDO [310, 312]).

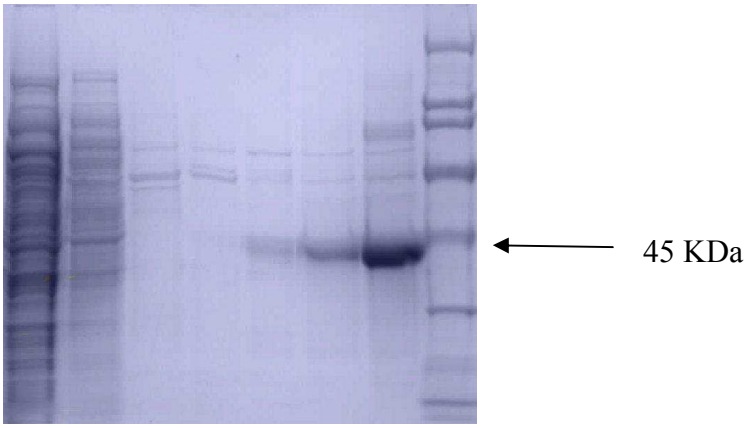


Fig 3.8 Purification of rhIDO using Ni-NTA column chromatography illustrated by SDS-PAGE analysis, L1: supernatant from cell lysate; L2: 10 mM imidazole, L3: 30 mM imidazole; L4: 40 mM imidazole; L5: 50 mM imidazole; L6: 70 mM imidazole; L7: 300 mM imidazole; L8: molecular weight markers

The crude plant extracts were tested at two different concentrations, 50 and 100 µg/ml. Unlike antiproliferative assays, no definite concentration ranges for testing crude extracts or threshold activity limits for identifying potent IDO inhibitory activity from plants are reported. The highest concentration of crude extracts tested in the antiproliferative assay was 100 µg/ml and was therefore chosen for IDO inhibitory studies as well.

The known IDO inhibitor 1-methyl-L-tryptophan was used as the positive control. The concentration of L-tryptophan used was 0.2 mM. The IDO inhibitory activity exhibited by these nine plants is shown in Table 3.2.

Plant	% inhibition of IDO activity	
	at 100 µg/ml	at 50 µg/ml
A. indicum	85 ± 4	46 ± 4
B. hispida	78 ± 6	32 ± 6
C. fimbriata	76 ± 3	46 ± 3
C. halicacabum	92 ± 5	62 ± 7
C. religiosa	86 ± 1	48 ± 4
C. viscose	64 ± 4	34 ± 3
E. alsinoides	85 ± 3	72 ± 2
M. tinctoria	79 ± 3	64 ± 4
T. indicum	84 ± 3	40 ± 3
1-methyl-L-tryptophan (0.2 mM)	100 ± 5	

* Assay performed in duplicate with samples tested in triplicates

Table 3.2 IDO inhibitory activity of plant extracts

The level of activities observed for virtually all the plants was high at 100 µg/ml, with none of them showing markedly different activities. No definite dose dependent activity was observed with the two tested concentrations, 100 and 50 µg/ml *C. halicacabum* showed the highest activity with 92 ± 5 inhibition. *C. viscose* exhibited the least inhibition of all the plants (64 ± 4.2%). At 50 µg/ml, *C. halicacabum, E. alsinoides* and *M. tinctoria* were found to inhibit IDO by 62 ± 7, 72 ± 2 and 64 ± 5%, respectively.

E. alsinoides, *C. halicacabum* and *M. tinctoria* also showed significant activities, indicating the presence of IDO inhibitory molecules. The remaining six plants exhibited relatively low inhibition of less than 50% at a concentration of 50 µg/ml. *E. alsinoides*, *M. tinctoria* and *C. halicacabum* therefore can be considered as potential candidates for seeking IDO inhibitory molecules.

The IDO inhibitory studies performed in this project are preliminary. No definite dose dependent activity was observed with the two tested concentrations, 100 and 50 µg/ml. To ascertain our observations from the IDO studies, analysis using a broader range of concentrations is required.

3.5.5 Antiinflammatory studies

C. halicacabum and *C. fimbriata* were identified as the most active plants in the MTT assay screening assays, exhibiting significant antiproliferative activity. These two plants have also been reported for their use in traditional medicine for treating conditions related to inflammation[242, 316]. This, along with the increasingly recognised link of inflammation and cancer, prompted investigation of the antiinflammatory activity of these two plants. Even though the inducible isoform, COX 2, is important in inflammation and pain, the constitutively expressed isoform, COX 1, has also been suggested to play a role in inflammatory processes[317]. The 70% ethanolic extract of these plants were therefore tested for their activity against COX 1 and COX 2 as the target enzymes. Crude extracts of plants are generally screened for antiinflammatory activity at concentrations between 0.1 and 10 mg/ml and those extracts that exhibit activities at concentrations < 2 mg/ml are generally considered to be potent and worth investigating for bioactive molecules[280]. Celecoxib, a selective COX 2 inhibitor and ibuprofen, a selective COX 1 inhibitor, were used as the positive controls[318, 319].The results of the assays are given in Table 3.3.

Plant	% Inhibition of	
	COX 1	COX 2
Cardiospermum halicacabum		
50 µg/ml	65	55
100 µg/ml	100	84
Caralluma fimbriata		
50 µg/ml	82	100
100 µg/ml	100	100

*Assay was performed once with sample tested in duplicate

Table: 3.3 Antiinflammatory activities of *C. halicacabum* and *C. fimbriata*

The crude extract of *C. halicacabum* inhibited COX 1 and COX 2 by 65 and 55% respectively at 50 µg/ml, whereas at 100 µg/ml the inhibition observed was 100 and 84% respectively (Table 3.3). *C. fimbriata* exhibited 100% inhibition of both COX 1 and COX 2 at 100 µg/ml. At 50 µg/ml, the inhibition of COX 1 was lowered to 82%, but COX 2 was completely inhibited. Celecoxib, the positive control, inhibited COX 2 by 100% at a concentration of 1.8 µg/ml. Ibuprofen inhibited COX 1 by 80% at a concentration of 6

µg/ml. *C. halicacabum* and *C. fimbriata* appeared to be active against both inflammation pathway enzymes. *C fimbriata* exhibited particularly high inhibition of COX 2.

The level of activity exhibited by *C. halicacabum* and *C. fimbriata* indicated the possible presence of antiinflammatory molecules in the crude extract of these plants. Our study is supported by the recent finding that the ethanolic extract of the whole plant of *C. halicacabum* showed 70% inhibition of COX 2 at a concentration of 200 µg/ml in the mouse macrophage cell line RAW264.7 [194]. The study indicated that *C. halicacabum*. is a potent inhibitor of induced COX 2 protein, iNOS and TNF-α via gene expression by blocking NF-κB activation[194]. These results support the use *C. halicacabum* for treating inflammation related conditions in Siddha medicine. *C. halicacabum* is also being used in Ayurvedic medicines and by aboriginals in Sri Lanka to treat rheumatism[194, 320].

The antiinflammatory activity of *C. fimbriata* has been demonstrated for the first time in this study. The COX inhibitory activity exhibited by *C. fimbriata* also supports the use of this plant in various traditional medicines for treating inflammatory conditions.

The cyclooxygenase inhibitory activity exhibited by these plants is consistent with their ethnomedicinal use for inflammatory related conditions. Even though a further detailed study was not performed due to time constraints, the present findings serve as a lead for investigating antiinflammatory molecules from these plants.

3.6 Conclusion

The antiproliferative studies performed with the SKNMC cell lines on the crude extracts of nine plants identified *C. halicacabum* and *C. fimbriata* as promising plants for further bioassay guided studies. The crude extracts of these two plants were also found to exhibit potent COX inhibitory activity. The crude extracts of *C. halicacabum*, *E. alsinoides* and *M. tinctoria* exhibited strong IDO inhibitory activity. Bioassay guided fractionation studies of *C. halicacabum* and *C. fimbriata* to isolate and characterise antiproliferative molecules are described in Chapters 4 and 5 respectively.

3.7 Materials and Methods

3.7.1 Reagents and equipment

Ethanol used for extraction was of analytical grade and distilled prior to use. MilliQ water was used. Organic solvents were evaporated using a Büchi rotary evaporator (Germany) under reduced pressure and 40ºC and traces of solvent were removed using a Pascal 2005 SD high vacuum pump from Alcatel (UK). A Labconco (USA) freeze dryer was used for removal of water. Cyclooxygenase 1 (ovine) and cyclooxygenase 2 (human recombinant) were used as the target enzymes in the antiinflammatory assays (Cayman Chemical, Catalogue No. 60100 and 60122, respectively). Imidazole was from BDH; ampicillin, DNase and kanamycin were from Bohringer Ingelheim; EDTA-free protease cocktail inhibitor was from Roche; Coomassie Blue, ethylenediaminetetraacetic acid (EDTA), ethylene glycol, isopropanol β-D-thiogalactopyranoside (IPTG), phenylmethylsulfonylfluoride (PMSF), sodium dodecyl sulfate (SDS), tris(hydroxymethyl)methylamine (Tris), doxorubicin, 1-methyl-L-tryptophan and ibuprofen were purchased from Sigma-Aldrich. Eagle's minimum essential medium, foetal calf serum, non-essential amino acids, sodium pyruvate, L-glutamine, streptomycin and penicillin were from Gibco, Australia and fungizone from Squibb Pharmaceuticals, Canada. Bio-Rad dye reagent was from Bio Rad, Australia. The cyclooxygenase inhibitor screening assay kit (Cayman Chemical, Catalogue No. 560131) was used for the assays. Celecoxib was obtained as a gift sample from Ranbaxy Lab, Mumbai, India. A Spectramax Spectrophotometer and Fluostar Microplate Reader were used for absorbance measurements.

3.7.2 Processing of plant samples and importation to Australia

All plant materials were collected by Dr Velmurugan from different regions of south India. The voucher specimens of plants collected were deposited at the BSI herbarium, Coimbatore, India for future reference. The collected plant materials were separated from foreign particles and other impurities. Samples were washed and shade dried for 24 hours. The plants were then dried in a vacuum drier at 75-85^{o}C and then passed through a micropulveriser to obtain powders of 130-200 mesh size. The plant powders were again

dried under vacuum to remove any moisture. The processed samples were stored in air-tight and water-proof containers protected from direct sunlight and heat and dispatched to Australia. Appropriate clearances were obtained from Indian Customs and the Australian Quarantine and Inspection Service (AQIS) (Permit no - IPO7010190).

3.7.3 Extraction and sample preparation

Dried powdered plant material 3 g was extracted with 50 ml 70% aqueous ethanol at 40-50^{o}C for 8 hours. The extraction was repeated three times. The filtrates were combined and were concentrated by rotary evaporation at 40^{o}C under reduced pressure and freeze dried. Crude extracts were dissolved in 5% dimethylsulfoxide (DMSO) at a concentration of 1 mg/ml to give the desired stock solutions to perform the assays. A stock solution of doxorubicin was prepared in Eagle's minimal essential media. Ibuprofen, celocoxib and 1-methyl-L-tryptophan were prepared in DMSO.

3.7.4 SKNMC Cell lines

The human SKNMC neuroepithelioma cell line was purchased from the American Type Culture Collection. All cell lines were grown in Eagle's minimum essential medium supplemented with 10% foetal calf serum, 1% non-essential amino acids, 1% sodium pyruvate (all (*v/v*)), 2 mM L-glutamine, 100 µg/ml of streptomycin, 100 µg/ml penicillin, and 0.28 µg/ml of fungizone. Cells were grown in an incubator at 37°C in a humidified atmosphere of 5% CO_2 and 95% air and sub-cultured as described previously. Phase contrast microscopy was used for assessing the cell growth and viability[321].

3.7.5 MTT Assay

The methodology for performing the MTT assay is explained below[321].

Day 1

I. SKNMC neuroepithelioma cell suspension was prepared by detaching cells from the monolayer using 5 mM EDTA. The cells were spun to make a pellet and the pellet resuspended in supplemented minimal essential media (MEM) media (20 ml). The cells present per 10 µl were counted using a hemocytometer. Using this

value a suspension of 15000 cells per 100 µl was prepared in a total volume of 40 ml.

II. 100 µl of the above cell suspension was added to each lane except lane 12 of a 96 well cell culture plate.

III. The plate was incubated at 37°C overnight to allow the cells to adhere.

Day 2

I. 75 µl of MEM media was added to all wells of a new plate except lane 11.

II. Working solutions 10-100 µg/ml of extracts were prepared by adding 2 ml of extract into 3 ml media. 150 µl of extract working solution was added to lane 11. The extract/controls were serially diluted from lane 11 to lane 2 by mixing and transferring 75 µl. Lane 1 acted as a control for cells with no treatment.

III. 75 µl of a transferrin (Tf) solution (0.24 mg/ml) was added to all wells.

IV. 100 µl of the above test solution (extracts/control) in the dilution plate was added to the corresponding wells in the cell plate. Lane one (no treatment - 100% cell growth) and lane 12 (media only) acted as controls.

V. The cell plate was incubated at 37°C for 48 hours.

Day 5

I. 100 µl of media was removed from the top of all wells except lane 12 (this had a total of 100 µl to begin with).

II. 10 µl of a 5 mg/ml solution of MTT in PBS was added to all wells. After 2 hours incubation, all medium from all wells was removed by pipetting without disturbing cells at the bottom.

III. 100 µl of lysis buffer was added to all wells to disrupt the cells. Once the cells were disrupted the absorbance measurement was taken at 580 nm.
The samples were tested in duplicates.

3.7.6 Luria Bertani (LB) agar plates

A mixture of Tryptone 2% (*w/v*), yeast extract 1% (*w/v*), 1% NaCl (*w/v*) and bacteriological agar 1.5% (*w/v*) was made in MilliQ water. The resulting mixture was autoclaved at 121°C for 20 minutes. The autoclaved medium was cooled to 55°C

followed by the addition of a filter-sterilised antibiotic mixture of ampicillin and kanamycin at a final concentration of 100 μg/ml and 50 μg/ml, respectively. The cooled molten agar was poured into sterile petri dishes to set[312].

3.7.7 LB medium

A mixture of tryptone 2% (*w/v*), yeast extract 1% (*w/v*), NaCl 1% (*w/v*) and bacteriological agar 1.5% (*w/v*) was made in MilliQ water. The resulting mixture was autoclaved at 121°C for twenty minutes. The autoclaved medium was cooled to 55°C followed by the addition of filter-sterilised antibiotic mixture, ampicillin (100 μg/ml) and kanamycin (50 μg/ml).

3.7.8 Phosphate buffered saline

NaCl 8% (*w/v*), KCl 0.2% (*w/v*), Na_2HPO_4 1.1% (*w/v*) and KH_2PO_4 0.2% (*w/v*) in MilliQ water were thoroughly mixed and the pH adjusted to pH 7.4 with concentrated HCl. The 10 times concentrated stock solution was diluted 1:10 with MilliQ water before use.

3.7.9 Re-suspension buffer

25 mM Tris(hydroxymethyl)methylamine (Tris-HCl pH 7.4), containing 150 mM NaCl, 10 mM imidazole and 10 mM $MgCl_2$ in MilliQ water. Phenylmethylsulfonylfluoride (PMSF) was added to a final concentration of 1 mM before use.

3.7.10 Basal buffer

25 mM Tris-HCl pH 7.4 containing 150 mM NaCl in MilliQ water with PMSF added to a final concentration of 1 mM before use.

3.7.11 Cracking buffer

SDS 1% (*w/v*), *β*-mercaptoethanol 1% (*v/v*), glycerol 10% (*v/v*), bromophenol blue 0.01% (*w/v*) in 60 mM Tris-HCl adjusted to pH 6.8 with 6.0 M HCl.

3.7.12 SDS running buffer

Tris base 1.5% (*w/v*), glycine 7.2% (*w/v*) and SDS 0.5% (*w/v*) in MilliQ water. For final dilution, 5 times stock solution was diluted 1:5 with MilliQ water.

3.7.13 Gel fixing solution

Ethanol 50% (*v/v*) and glacial acetic acid 10% (*v/v*) in MilliQ water.

3.7.14 Gel staining solution

Methanol 40% (*v/v*), glacial acetic acid 10% (*v/v*) and Coomassie blue R-250, 0.00002% (*w/v*) in MilliQ water.

3.7.15 Gel de-stain solution

Glacial acetic acid 10% (*v/v*) and glycerol 4% (*v/v*) in MilliQ water.

3.7.16 Gel drying solution

Ethylene glycol 4% (*v/v*) and ethanol 35% (*v/v*) in MilliQ water.

3.7.17 Expression of 6His-rhIDO

Stock culture of *E. coli* EC538 (pQE-9-IDO, pREP4) stored at -80°C was thawed on ice, inoculated on Luria-Bertani (LB) agar plates and grown overnight at $37^{o}C$[312]. A single colony of *E. coli* cells isolated from an LB agar plate was re-inoculated onto an LB plate and grown overnight. A single colony from the second growth was inoculated into 100 ml LB medium and cultured overnight at 37°C (200 *g*). 40 ml of the overnight culture was added to 1 l of LB medium and grown to a density of 0.6 OD at 600 nm (OD_{600nm}). *δ*-Aminolevulinic acid (ALA, 500 mM), isopropanol β-D-thiogalactopyranoside (IPTG, 100 mM) and phenylmethylsulfonylfluoride (PMSF, 1M) were then added at a final concentration of 0.5 mM, 0.1 mM and 1 mM, respectively. The culture was shaken for another 3 hours at 37°C. After this incubation, cells from the culture were collected as a pellet by centrifuging at 5000 *g* for twenty minutes at $4^{o}C$. The pellet obtained was suspended in 20 ml of ice cold Dulbecco's phosphate-buffered saline (PBS, pH 7) containing 1 mM PMSF and 1 mM ethylenediaminetetraacetic acid (EDTA). The

resultant mixture was centrifuged at 5000 *g* for 15 minutes at 4°C to obtain a pellet. The pellet was stored at -20°C[312].

3.7.18 Purification of 6His-rhIDO

The pellet was resuspended in ice cold resuspension buffer[312]. The suspension was then centrifuged at 5000 *g* for 20 minutes at 4°C and the supernatant discarded. This washing process was to remove EDTA. The washed pellets were resuspended in 20 ml of ice cold buffer, along with EDTA free cocktail inhibitor tablets (2 tablets per pellet) and DNase (1 mg). The suspension was lysed by passing through a French press three times at a pressure of 16000 psi and then centrifuged at 5000 *g* for 20 minutes to produce a reddish brown supernatant and pellet.

The reddish brown supernatant was then applied to a 5 ml Ni-NTA column (chelating column charged with Ni^{2+} ions). Prior to this, the column was equilibrated with buffer (25 mM Tris pH 7.4, 500 mM NaCl) solution containing imidazole at a final concentration of 10 mM. Further washing was carried out with buffer containing increasing imidazole concentrations of 10 mM, 30 mM, 40 mM 50 mM and 70 mM respectively. IDO was eluted with Tris buffer containing 300 mM imidazole. The IDO was collected as intense red fractions, combined and then buffer exchanged with 50 mM Tris-HCl pH 7.4 using a Sephadex G25 desalting column (NAP-10). The column was prewashed with 5 column volumes of 50 mM Tris buffer at pH 7.4, and then the IDO was loaded on to the column. The column was washed with 50 mM Tris buffer at pH 7.4.

The desalted, red fractions were pooled and concentrated to a volume of 200 µl using a 15 ml Amicon Ultra-15 centrifugal device with a 30 kDa molecular weight cut off. The freshly obtained IDO was mixed with 80% glycerol in a 1:1 ratio and stored at -20°C[310].

3.7.19 Bradford assay

Bio-Rad dye reagent was prepared by diluting 1 part the reagent with 4 parts MilliQ water. The standard dilutions (100, 200, 300, 400, 500 and 600 µg/ml) of bovine serum albumin (BSA) were prepared. Each standard or IDO solution (10 µl) was pipetted into a 96 well plate (Greiner) and Coomassie Plus reagent (200 µl) was added. The samples

were incubated for 10 minutes at room temperature and the absorbance was measured at 595 nm with a Spectramax microplate reader. Using the standard curve the concentration of IDO sample was estimated using the standard curve.

3.7.20 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS PAGE was performed according to the method described by Laemmli[322]. To the sample solution, an equal volume of 2 times cracking buffer was added. Sample solutions were incubated at above 90^{o}C for 5-10 minutes and centrifuged to remove any solids. Samples were separated through a polyacrylamide gel (Tris-Glycine-SDS Gel, 4-20%), immersed in SDS running buffer. Samples were electrophoresed for a period of 60-90 minutes at a voltage of 120 V. Gels were removed from their casing, immersed in a gel fixing solution for 10 minutes, and then in rapid Coomassie blue stain. Gels were destained by immersing in gel destain solution for 5 hours and soaked in gel drying solution and dried.

3.7.21 IDO inhibition assay

The standard medium (200 µl) contained 0.5 M potassium phosphate buffer (20 µl, pH 6.5, final concentration of 50 mM), 0.2 M ascorbic acid (20 µl, neutralised with 1 M NaOH solution, final concentration of 20 mM), 0.5 mM methylene blue (4 µl, final concentration of 10 µM), 5 mg/ml catalase (4 µl, final concentration of 100 µg/ml), 2 mM L-tryptophan (20 µl, final concentration of 0.2 mM), MilliQ water (112 µl), 20 µl rhIDO, and 20 µl inhibitor solutions (crude plant extracts and positive control 1-methyl-L-tryptophan) in DMSO. The solutions were incubated at 37°C for 1 hour, and the reaction was stopped by adding trichloroacetic acid (30% *w/v*, 40 µl). The solutions were then incubated at 60°C for 15 minutes to hydrolyse *N*-formylkynurenine to kynurenine. After the incubation period the samples were centrifuged for 5 minutes at 12000 *g* to remove denatured protein. The supernatant (125 µl) of each sample was transferred to a 96 well microtitre plate. 125 µl of Ehrlich's reagent (2% (*w/v*) in glacial acetic acid), was added into each well. The absorbance at 480 nm was measured with a Spectramax microplate reader[323]. The samples were tested in triplicates.

3.7.22 Cyclooxygenase inhibitor screening assay

The COX assay was performed according to the manufacturer's (Cayman Chemicals, USA) instructions. COX 1 and COX 2 were incubated separately with the plant extracts and controls in a reaction buffer (100 mM Tris-HCl buffer, pH 8.0) containing 1 μM heme, for 10 minutes at 37°C. Arachidonic acid was added (final concentration of 100 μM) to initiate the reaction. HCl solution (1 M) was added after 2 minutes to terminate the reaction, followed by saturated $SnCl_2$ solution to reduce the reaction products to $PGF_{2\alpha}$. The amounts of $PGF_{2\alpha}$ produced in the COX reactions were quantified using an enzyme immunoassay. $PGF_{2\alpha}$ and PGE_2-acetylcholine esterase tracer were added to a 96-well plate coated with mouse anti-rabbit IgG and the specific antibody (rabbit anti-$PGF_{2\alpha}$ and PGE_2) was added. The plate was incubated for 18 hours at room temperature. After the incubation period the plate was washed 5 times with 10 mM potassium phosphate buffer containing 0.05% Tween 20 to remove any unbound reagents. Ellman's reagent was added to each well and the plate was developed in the dark for about 60 minutes until the maximum binding control yielded an optimum absorbance of 0.3-0.8 A.U. at 405 nm. Ibuprofen and celecoxib were used as controls[280]. The samples were tested in duplicates.

Chapter 4

Bioassay guided studies on Cardiospermum halicacabum

This chapter describes the bioassay guided fractionation studies carried out with the crude ethanolic extract of Cardiospermum halicacabum to isolate bioactive molecules responsible for its antiproliferative activity and their structure elucidation.

4.1 Introduction

Cardiospermum halicacabum belongs to the Sapindaceae family. This family includes many economically important fruits such as the lychee, rambutan, longan and gaurana. The genus Cardiospermum comprises of 14 species, mostly herbaceous vines, whose seeds are often used for treating skin related diseases[209]. *C. halicacabum* is traditionally used to treat stiffness of limbs (rheumatism), snake bite, fever and earache (whole plant); nervous diseases (roots); diarrhoea, dysentery, headache and swellings (leaves and stalks)[204, 324, 325, 194]. Antiinflammatory, analgesic and antipyretic activities of *C. halicacabum* have been reported[194]. Dr Velmurugan uses a mixture of the dried root and leaf powder of *C. halicacabum* in combination with the dried whole plant of *Marsdenia tinctoria* and dried leaves of *Vernonia cinerea*, usually in the proportion of 4:2:5 (*w/w*) as an anticancer formulation. These proportions, as well as the relative proportions of the root and leaf powders of *C. halicacabum* are varied however, to suit individual cases (depending on the severity of the disease and age and weight of the individual). So far rutin, luteolin, pinitol and chrysoeriol have been reported from the leaves of this plant[325-327].

The crude extract of a mixture of the roots and leaves of *C. halicacabum* was shown to possess significant antiproliferative activity against the SKNMC (human neuroblastoma) cell line (IC_{50} of 22.0 ± 3.0 µg/ml) in the preliminary MTT assay (Section 3.3). The activity level exhibited by this plant was within the range suggested by the National Cancer Institute for selecting potential candidate plants for further biological and chemical investigation[306]. The aim of this chapter was to describe the bioassay guided

studies carried out on *C. halicacabum* to isolate bioactive molecules responsible for the antiproliferative activity and to determine their structures.

4.2 Results and Discussion

4.2.1 Bioassay guided investigation of partitions

As described in Chapter 3, a small scale extraction (3.0 g) of dried powdered *C. halicacabum* roots and leaves with 70% aqueous ethanol was carried out for initial screening studies. For the bioassay guided studies, a similar extraction was carried out with 500 g of the dried powdered roots and leaves. The larger scale crude extract was tested to confirm the activity and was found to be consistent with the earlier crude extract (IC_{50} of 19 ± 3 µg/ml). The crude aqueous ethanolic extract was then successively partitioned with solvents of increasing polarities to afford four partitions, *i.e. n*-hexane, ethyl acetate, *n*-butanol and water partitions (Scheme 4.1).

The partitions were tested by the MTT assay at concentrations of 10-100 µg/ml against the SKNMC cell line[304, 305]. The water partition (Ch-water) showed the most potent antiproliferative activity with an IC_{50} value of 21 ± 4 µg/ml. The *n*-hexane, ethyl acetate, and *n*-butanol partitions were found to be inactive (IC_{50} > 100 µg/ml). Further separation of the active water partition was therefore carried out to identify the antiproliferative compounds.

4.2.2 Fractionation of water partition

A reversed phase (C_{18}) HPLC profile of the water partition Ch-water (Fig 4.1) showed one major peak as well as several medium intensity and minor peaks. Analysis of the HPLC photodiode array (PDA) UV spectra of the peaks between retention time 30 to 45 minutes identified (using a gradient elution of water:acetonitrile (100-40%) that these peaks absorbed at the wavelengths characteristic of flavonoids with a bimodal absorption pattern, with the first peak in the range 230-300 nm and the second peak between 300-350 nm[328]. Flavonoids such as rutin, chyrsoeriol and luteolin have been previously reported from this plant[325-327].

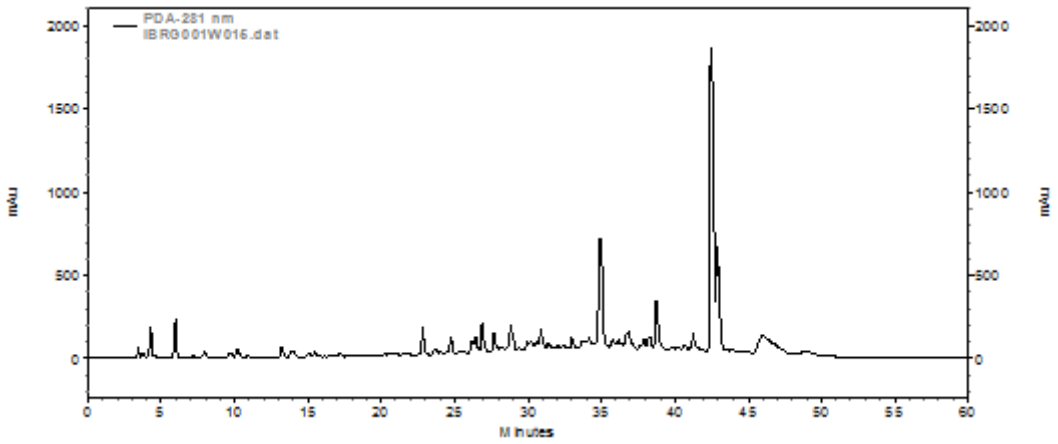


Fig 4.1 HPLC chromatogram of water fraction (Ch-water) of *C. halicacabum*

The water partition being highly polar was expected to contain large quantities of sugars[329]. As sugars are known to interfere with the fractionation process[330], reversed phase (C_{18}) solid phase extraction (SPE) was chosen for the initial clean up. Reversed phase SPE is widely used for the purification of flavanoids, alkaloids, saponins and sesquiterpenoids[331]. The water partition, Ch-water, was loaded on to a C_{18} SPE column and the column eluted with varying proportions of water and acetonitrile (100::0 to 40:60). Fractions were collected and monitored by reversed phase thin layer chromatography (TLC), visualising under UV light at 365 nm and derivatising using anisaldehyde-sulfuric acid spray[332]. Anisaldehyde-sulfuric acid spray reagent is the normally used TLC stain for the detection of phenols, sugars, steroids and terpenes[332]. Fractions having similar R_f values were pooled to yield six fractions (Ch-water-1 to -6). The MTT assay was performed with these fractions against the SKNMC, MCF 7 (human breast cancer) and MRC 5 (normal) cell lines. Our collaborator and Siddha practitioner, Dr Velmurugan, has seen good clinical results with his formulations against breast cancer (Dr Velmurugan, personal communication), hence the MCF 7 breast cancer cell line was included in the study. To determine the selectivity, the normal fibroblastic MRC 5 cell line was also included in the cell line panel.

The antiproliferative activities exhibited by fractions Ch-water-1 to -6 were less than that of Ch-water and crude ethanol extract. The higher level of activity can possibly be attributed to a mixture of compounds working in synergi[308]. This additive effect might decrease or even be lost during the fractionation process[308].

Ch-water-5 exhibited the highest level of activity with IC_{50} values of 38 ± 3, 28 ± 5 and 34 ± 6 µg/ml and Ch-water-6 showed moderate activity with IC_{50} values of 75 ± 8, 78 ± 5 and 78 ± 6 µg/ml against the three cell lines SKNMC, MCF 7 and MRC 5, respectively. The active fractions did not exhibit any remarkable selectivity to the cancer cell lines. However, it should be noted that most of the currently used anticancer cytostatic drugs also have a strong effect on all proliferating cells including normal cells[333].

The remaining fractions did not exhibit any significant antiproliferative activity even at the highest tested concentration of 100 µg/ml. Ch-water-5 was therefore selected for further chromatographic separation to isolate antiproliferative molecules.

4.2.3 Bioassay guided isolation of compounds from Ch-water-5

The UV spectral data of Ch-water obtained from the HPLC PDA analysis indicated the presence of flavonoids[328] with a bimodal absorption pattern between ~ 230-300 nm and ~300-350 nm. The presence of flavonoids in Ch-water-5 was confirmed by the formation of a yellowish white precipitate following reaction with a saturated aqueous lead acetate solution[122]. Reversed phase HPLC is the most popular and reliable method used for the isolation of phenolic (flavonoid) compounds[334], hence, preparative scale reversed phase HPLC was carried out on the Ch-water-5 fraction (Fig 4.2). This afforded compounds 4.1, 4.2, 4.3, 4.4 and 4.5. Compounds 4.1 and 4.2 were isolated with minor impurities and were further purified using Sephadex LH 20 size exclusion column chromatography. Size exclusion chromatography (SEC) using the lipophilic organic resin Sephadex LH 20 is another commonly used method for the purification of flavonoids and has the advantage of high sample recovery[335, 336]. Isolation of the pure compounds 4.1 and 4.2, with almost complete sample recovery was obtained with this method. The peak at retention time of 28.5 minutes was collected with minor impurities, but due to limited sample further purification could not be achieved. A flow chart for the process of the bioassay guided isolation of the compounds is shown in Scheme 4.1.

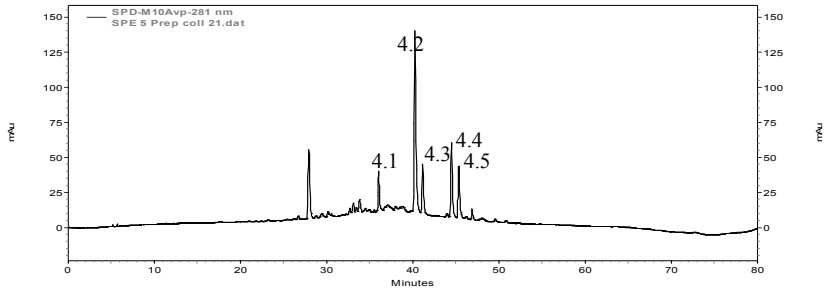
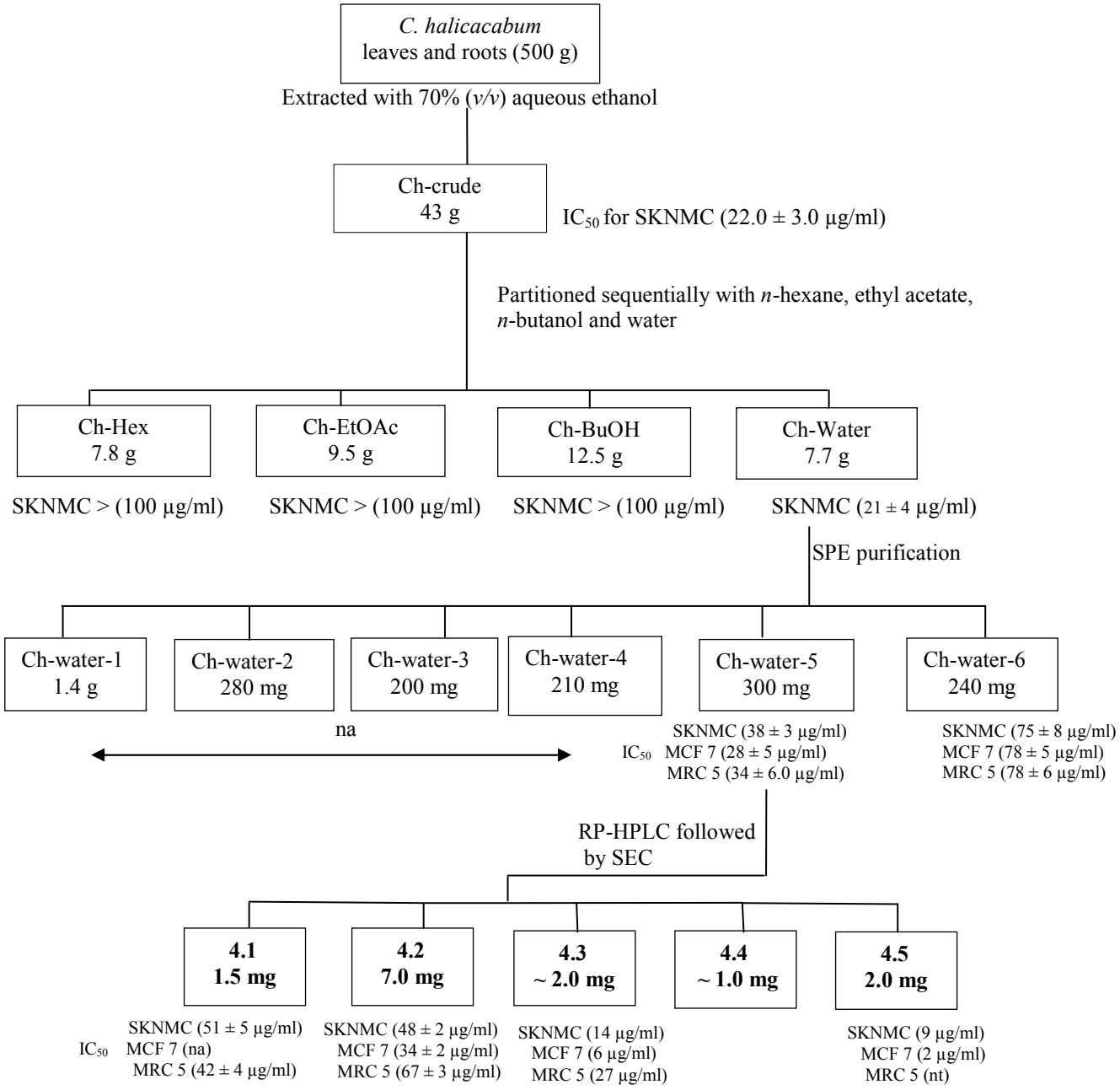


Fig 4.2 HPLC PDA-UV chromatogram of Ch-water-5 fraction of *C. halicacabum*

Scheme 4.1 Bioassay-guided fractionation of *C. halicacabum*

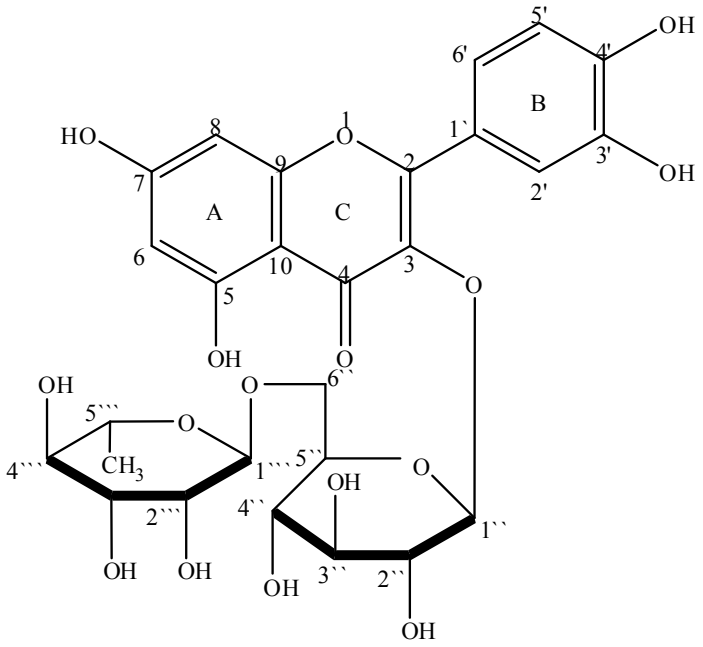


Scheme 4.1 Antiproliferative activity results shown in the scheme are from the MTT assay. Cell lines used: SKNMC (neuroblastoma), MCF 7 (breast cancer) and MRC 5 (normal). SPE: solid phase extraction, RP-HPLC: reversed phase high performance liquid chromatography, SEC: size exclusion chromatography, nt: not tested, na: not active. The values following the cell lines are the IC_{50} values.

4.2.4 Characterisation of isolated compounds

The structures of the compounds were elucidated by 1D and 2D NMR, mass spectrometry, UV spectroscopy and by comparison with reported data. The structural elucidation of these compounds is described below.

4.2.4.1 Quercetin 3-*O*-*β*-D-rutinoside (4.1)

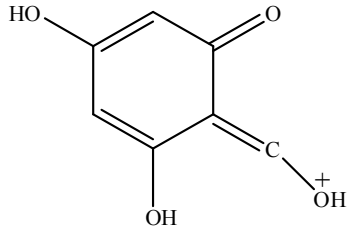


Quercetin 3-*O*-*β*-D-rutinoside

Compound 4.1 was obtained as a yellow solid (3.5 mg, 0.018% *w/w*, mp 192-194^{o}C). Electrospray Ionisation Mass Spectrometry (ESI MS) (negative ion mode) indicated a deprotonated molecular ion at *m/z* 609 $[M-H]^-$ consistent with the molecular formula $C_{27}H_{30}O_{16}$. The ^{13}C NMR data, along with HSQC and ^{1}H NMR data identified 27 carbon atoms (Table 4.1), in agreement with one carbonyl, two aromatic rings (5 methines and 7 quaternary carbons), one tetrasubstituted double bond and 12 aliphatic carbon atoms (1 methylene, 1 methyl and 10 methines). The ^{1}H NMR spectrum (recorded in methanol-d4) confirmed the presence of five aromatic protons, 12 non-exchangeable protons consistent with a sugar moiety, and a methyl group. The PDA-UV absorption spectrum of 4.1 exhibited λ_{max} of 258 and 362 nm, indicative of a flavonoid type molecule[337, 338].

One of the aromatic rings contained only two aromatic proton signals, at δ 6.39 (1H, *d*, *J* = 2.1 Hz, H-8) and 6.20 (1H, *d*, *J* = 2.1 Hz, H-6), with characteristic *meta* coupling (*J* = 2.1 Hz). Since there were no other proton signals on this ring, this suggested the aromatic ring was tetrasubstituted. Both protons showed HMBC correlations to the quaternary carbons at δc 104.5 (C-10) and 164.8 (C-7). Additionally, the proton at δ 6.20 (H-6) showed correlations to a quaternary carbon at δc 161.8 (C-5), while the proton at δ 6.39 (H-8) showed a correlation to the carbon signal at δc 161.9 (C-9). The chemical shifts and coupling of the protons at δ 6.39 and 6.20 and the chemical shifts of the three carbon signals in the range of 160-165 ppm, implied a 1,3,5-trioxygenation pattern around the aromatic ring.

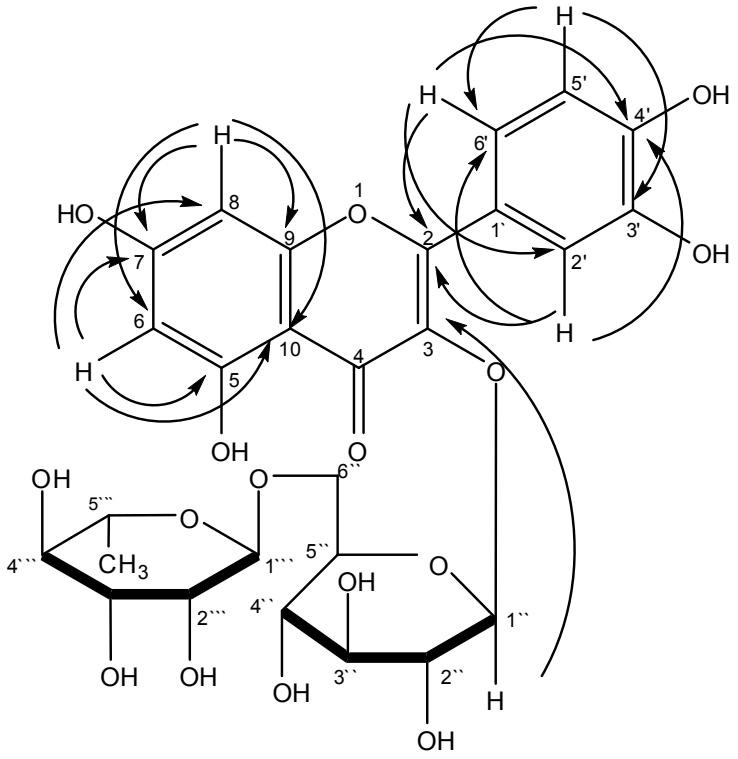
The second aromatic ring showed an *ortho* coupled doublet at δ 6.85 (1H, *d*, *J* = 8.5 Hz, H-5'), a doublet of doublets at δ 7.62 (1H, *dd*, *J* = 8.5, 2.1 Hz, H-6') and an *ortho* coupled doublet at δ 7.66 (1H, *d*, *J* = 2.1 Hz, H-2'), consistent with a 1,3,4-substitution pattern. The carbon resonances at δc 144.8 (C-3') and 148.6 (C-4') indicated a dioxygenated phenyl ring. The HMBC spectrum showed correlations of the protons at δ 7.62 (H-6') and 7.66 (H-2') to δc 158.0. This carbon chemical shift is characteristic of a C-2 double bond of ring C of a flavonoid skeleton and the HMBC correlations were consistent with a 3',4'-dioxygenated phenyl ring (ring B) being connected to C-2. The flavonoid skeleton was further supported by the presence of a second double bond signal at δc 134.3 (C3) and a characteristic carbonyl signal at δc 178.4 (C10). While there were no HMBC connectivities between the proposed A and C rings, the characteristic signal at δc 104.5 supported its connectivity to the carbonyl carbon of a flavonoid. A dihydroxyated A ring of a flavonoid was further supported by the fragment ion at *m/z* 153 $(A_1+1)^+$ in the ESI MS spectra[339].



(*m/z* 153) $(A_1+1)^+$

The above data were found to be consistent with the spectral data of quercetin[340]. The ESI MS spectrum also showed the presence of a fragment ion at *m/z* 301 which further supported this observation[337, 341]

The presence of 11 non-exchangeable protons in the region of 5.10 to 3.20 ppm was consistent with a sugar moiety. The two protons at δ 5.10 (1H, *d*, *J* = 7.5 Hz, H-1'') and 4.51 (1H, *d*, *J* = 1.5 Hz, H-1''') were indicative of anomeric protons and implied that the molecule contained two sugar units. The chemical shift of δ 5.10 was consistent with a glucose moiety and δ 4.51 for a rhamnose group[342]. The presence of a three-proton doublet at δ 1.11 (d, *J* = 6.2 Hz), representing a methyl group, along with the proton signal at δ 4.51 (H-1'''), confirmed the presence of a rhamnosyl unit[343, 344]. The small coupling constant (*J* = 1.5 Hz) for the anomeric proton of the rhamnosyl moiety supported an α configuration for the anomeric proton[343, 344]. The unusually low field (δc 134.4) signal for C-3 of the C ring, along with a HMBC correlation between the proton at δ 5.10 (1'') of the glucosyl portion and this carbon, supported attachment of the sugar at C-3. The coupling constant of *J* = 7.6 Hz implied a *β* configuration of the glucose attachment[343, 344]. The 1→6 linkage between the rhamnosyl group and the glucosyl unit was confirmed by the HMBC correlation between the anomeric proton of the rhamnosyl moiety and the deshielded methylene carbon at δc 68.5 (C-6'') of the glucosyl moiety[344]. These observations were consistent with a rutinoside structure[344]. The molecular mass suggested by the ESI MS supported the presence of hydroxy groups at C-5, 7, 3' and 4'. Compound 4.1 was confirmed as quercetin 3-*O*-*β*-D-rutinoside (rutin)[345] by comparison with the literature MS, NMR, UV and melting point data, which were found to be in agreement[344, 346, 347]. Unfortunately there was insufficient sample for optical rotation measurements so that absolute configuration was uncertain.



Key HMBC correlations of quercetin 3-*O*-*β*-D-rutinoside

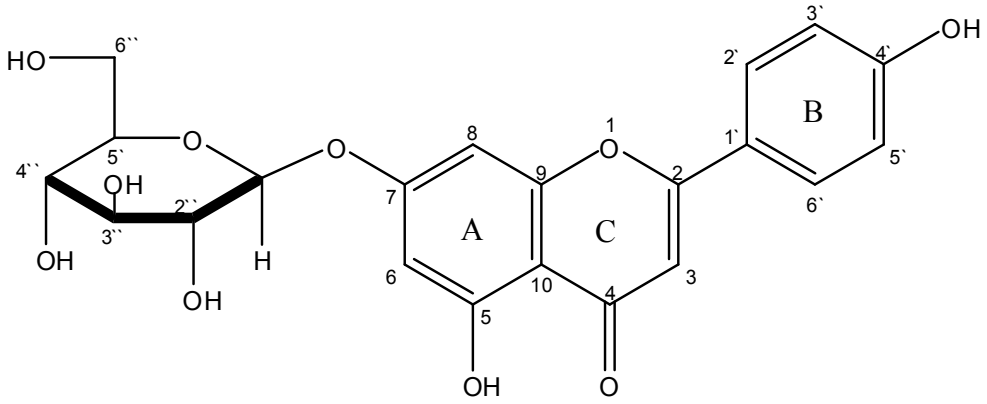
Table 4.1 NMR data of quercetin 3-*O*-*β*-D-rutinoside (4.1)

Compound 4.1

Position	δc	δ_H multiplicity	HMBC correlations
2	158.0		
3	134.3		
4	178.4		
5	161.8		
6	98.9	6.20 *d*, *J* = 2.1 Hz	C-8, C-10, C-5, C-7
7	164.8		
8	93.7	6.39 *d*, *J* = 2.1 Hz	C-6, C-10, C-9, C-7
9	161.9		
10	104.5		
1’	122.4		
2’	116.3	7.66 *d*, *J* = 2.1 Hz	C-6’, C-2, C-4’
3’	144.8		
4’	148.6		
5’	114.7	6.85 *d*, *J* = 8.5 Hz	C-3’, C-6’
6’	122.2	7.62 *dd*, *J* = 8.5, 2.1 Hz	C-2, C-2’, C-4’
1’’	103.5	5.10 *d*, *J* = 7.5 Hz	C-3
2’’	74.6	3.22-3.49 *m*	
3’’	77.1	3.22-3.49 *m*	
4’’	72.8	3.22-3.49 *m*	
5’’	76.1	3.22-3.49 *m*	
6’’	67.4	3.80 *m*	
1’’’	101.2	4.51 *d*, *J* = 1.5 Hz	C-6’’
2’’’	70.2	3.22-3.49 *m*	
3’’’	70.9	3.62 *m*	
4’’’	71.0	3.53 *m*	
5’’’	67.3	3.22-3.49 *m*	
6’’’	16.7	1.11 *d*, *J* = 6.2 Hz	C-1’’’

**recorded in methanol-d4*

4.2.4.2 Apigenin 7-O-*β*-D-glucoside (4.2)



Apigenin 7-*O*-*β*-D-glucoside

Compound 4.2 was obtained as a yellow amorphous solid (7.0 mg, 0.048% *w/w,* mp 196-201^{o}C). ESI MS (positive ion mode) indicated a protonated molecular ion at *m/z* 433 $[M+H]^{+}$, consistent with the molecular formula $C_{21}H_{20}O_{10.}$ The ^{13}C, along with HSQC and ^{1}H NMR data identified 21 carbon atoms (Table 4.2), consistent with one carbonyl, two aromatic rings (6 methines and 6 quaternary carbons), one trisubstituted double bond and 6 aliphatic carbon atoms (1 methylene and 5 methines). The ^{1}H NMR spectrum (recorded in methanol-d4) confirmed the presence of seven aromatic protons and seven non exchangeable protons consistent with a sugar moiety. The PDA UV absorption spectrum of 4.2 exhibited λ_{max} of 263 and 337 nm indicative of a flavonoid type molecule[348]. The NMR spectral data also showed many features similar to compound 4.1, further supporting a flavonoid skeleton.

The ^{1}H and the ^{13}C NMR signals and HMBC correlations for one of the aromatic rings were found to be highly similar to that determined for the A ring of compound 4.1, strongly supporting a 5,7-dioxygenated flavonoid A ring. Key features consistent with this are described below.

The presence of only two aromatic protons in the ring at δ 6.44 (1H, *d*, *J* = 2.1 Hz, H-6) and 6.83 (1H, *d*, *J* = 2.1 Hz, H-8), with characteristic *meta* coupling, indicated a tetrasubstituted aromatic ring. Their upfield shifts were indicative of oxygenation on the ring, which was further supported by HMBC correlations to the quaternary carbons at δc

163.2 (C-7), 161.8 (C-5) and δc 157.5 (C-9). Both of these aromatic protons also showed HMBC correlations with the carbon signal at δc 106.1 (C-10), which also showed a HMBC correlation with the proton at δ 6.85 (typical of the flavone skeleton proton at C-3[349]). These features, along with the carbonyl signal at δc 182.8 (C-4), were all consistent with a 5,7-dioxygenated flavonoid skeleton unsubstituted at C-3 on the C ring. The fragment ion at *m/z* 153 $(A_1+1)^+$ in the ESI MS spectrum supported dioxygenation of the A ring[339].

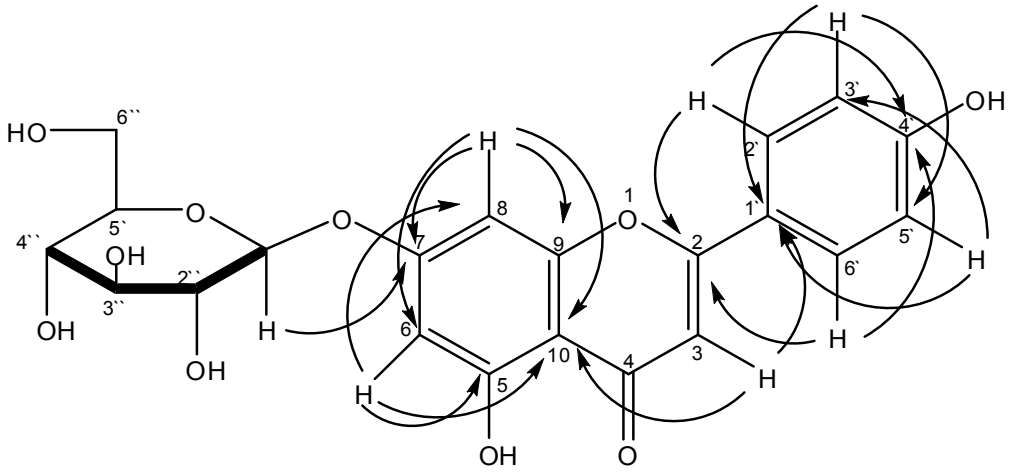
The second aromatic ring showed A_2B_2 proton signals at δ 6.95 (2H, *d*, *J* = 8.4 Hz, H-3', H-5') and 7.92 (2H, *d*, *J* = 8.4 Hz, H-2', H-6'). These protons showed HSQC correlations to the signals at δc 129.4 (C-2' and C-6') and 116.6 (C-3' and C-5') respectively, and along with their HMBC correlations to a biogenetically expected oxygenation at δc 161.9 (C-4'),[350, 351] were consistent with a 4'-oxygenated phenyl group of the B ring of a flavonoid.

The HMBC spectrum showed correlations of the protons at δ 7.92 (H-2' and H-6') to the carbon signal at δc 164.9 (C-2) confirming the phenyl group attachment to C-2 of ring C of the flavonoid skeleton. An observed HMBC correlation between the proton at δ 6.85 (H-3) and the quaternary carbon at δc 121.7 (C-1') further confirmed the connectivity.

This flavonoid portion of the molecule was found to be consistent with the spectral data of apigenin. This was further confirmed by EI MS analysis which had peaks at *m/z* 270, 242 $(270\text{-CO})^-$ and 153 $((A_1+1)^+)$ that are consistent with that of apigenin[352].

The proton signals for seven non-exchangeable protons between δ 5.10 and 3.10 in the ^{1}H NMR spectrum were consistent with a sugar moiety. The proton at δ 5.10 (1H, *d*, *J* = 7.5 Hz, H-1''), corresponding to a carbon signal at δc 99.9 in the HSQC spectrum, along with the rest of the sugar protons, were consistent with a glucose moiety[342]. The coupling constant of *J* = 7.2 Hz implied a *β* configuration of the glucose attachment.

A HMBC correlation between δc 163.2 (C-7) and the proton at δ 5.10 (1’’), supported attachment of the sugar on the A ring at C-7[353, 354]. This was further confirmed by the downfield shift of the protons at δ 6.44 (H-6) and 6.83 (H-8) relative to the corresponding protons in the aglycone (apigenin)[353]. The molecular mass suggested by the ESI MS supported the presence of hydroxy groups at C-4’ and C-5. The molecule 4.2 was therefore identified as apigenin 7-*O*-*β*-D-glucoside, a flavone glycoside. All spectral data were found to be consistent with the literature for apigenin 7-*O*-*β*-D-glucoside[355]. Unfortunately there was insufficient sample for optical rotation measurements so that absolute configuration was uncertain.



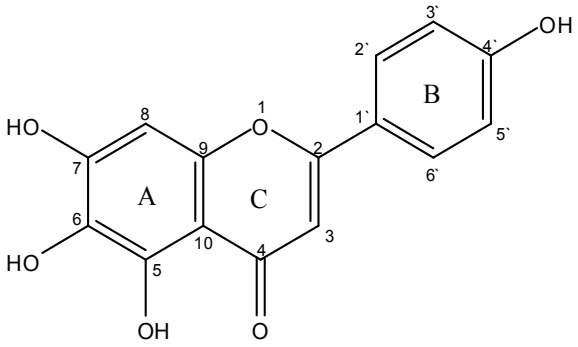
Key HMBC correlations of apigenin 7-O-*β*-D-glucoside

Table 4.2 NMR data of apigenin 7-*O*-*β*-D-glucoside (4.2)

Compound 4.2			
Position	δc	δ_H multiplicity	HMBC correlations
2	164.9		
3	103.9	6.85 *s*	C-10, C-1’
4	182.8		
5	161.8		
6	100.1	6.44 *d*, *J* = 2.1 Hz	C-8, C-7, C-10, C-5
7	163.2		
8	95.4	6.83 *d*, *J* = 2.1 Hz	C-6, C-10, C-9, C-7
9	157.5		
10	106.1		
1’	121.7		
2’	129.4	7.92 *d*, *J* = 8.4 Hz	C-6’, C-2, C-4’
3’	116.6	6.95 *d*, *J* = 8.4 Hz	C-5’, C-1’, C-4’
4’	161.9		
5’	116.6	6.95 *d*, *J* = 8.4 Hz	C-5’, C-1’, C-4’
6’	129.4	7.92 *d*, *J* = 8.4 Hz	C-2’, C-2, C-4’
1’’	99.9	5.10 *d*, *J* = 7.2 Hz	C-7
2’’’	73.4	3.40 *m*	
3’’’	76.4	3.40 *m*	
4’’’	70.2	3.16 *m*	
5’’’	75.8	3.69 *m*	
6a’’’	61.4	4.58 *dd J*=11.0 Hz, 6.5 Hz	
6b’’’		4.60 *dd J* = 11.0 Hz, 1.5 Hz	

recorded in methanol-d4

4.2.4.3 Scutellarein (6-hydroxyapigenin) (4.3)



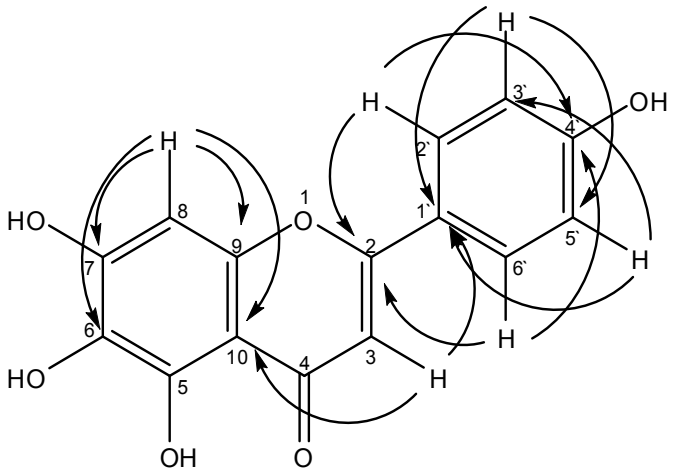
Scutellarein

Compound 4.3 was obtained as a pale yellow amorphous solid (2.0 mg, 0.012 % *w/w*, mp 332-337^{o}C). ESI MS (positive ion mode) indicated a protonated molecular ion at *m/z* 287 $[M+H]^+$, consistent with the molecular formula $C_{15}H_{10}O_6$. The ^{13}C, along with HSQC and ^{1}H NMR data identified 15 carbon atoms in agreement with one carbonyl, two aromatic rings (5 methines, 7 quaternary carbons) and one trisubstitued double bond. The ^{1}H NMR spectrum (recorded in methanol-d4) also confirmed the presence of six non exchangeable protons with five aromatic protons and one deshielded vinylic proton. The spectral data observed for 4.3 were similiar to that observed for the aglycone portion of compound 4.2, indicating that compound 4.3 was a derivative of apigenin[356]. Compared to apigenin, compound 4.3 lacked a non exchangeable proton and had one additional quaternary carbon signal, implying an additional hydroxy group. The PDA UV absorption spectrum exhibited λ_{max} of 284 and 337 nm, indicating a flavonoid type molecule[357].

One of the aromatic rings showed proton signals at δ 6.92, (2H, *d*, *J* = 8.9 Hz, H-3', H-5' and 7.85 (2H, *d*, *J* = 8.9 Hz, H-2', H-6'), along with ^{13}C signals and HSQC and HMBC correlations highly similar to the B ring of apigenin. This included HMBC correlations with the C2-C3 double bond of the flavonoid (δ 6.92 (H-5') to δc 165.4 (C-2) and δ 6.65 (H-3) to δc 121.9 (C-1')). The other aromatic ring contained only one aromatic proton signal at δ 6.61 (1H, *s*, H-8), indicating a pentasubstituted aromatic ring. The carbon signals were highly characteristic of a 5,6,7 trihydroxyflavone[358], supporting the presence

of the aromatic proton at C-8. The HMBC correlations of this proton, including its correlation to the signal at δc 103.9 (C-10), which further correlated to the vinylic proton at δ 6.65 (H-3), supported this ring as a tetraoxygenated A ring of a flavonoid.

The molecular mass suggested by the ESI MS supported the presence of hydroxy groups at C-4', C-5, C-6 and C-7. Based on these observations and in comparison with the NMR, UV and melting point data for compound 4.3 and published literature, compound 4.3 was identified as scutellarein[357, 359] (Table 4.6).



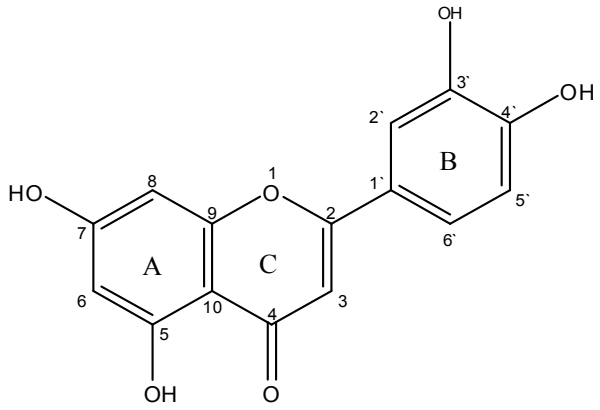
Key HMBC correlations of scutellarein

Table 4.3 NMR data of scutellarein (4.3)

Compound 4.3			
Position	δc	δ_H multiplicity	HMBC
2	165.4		
3	102.4	6.65 *s*	C-10, C-1', C-2
4	182.1		
5	147.0		
6	130.7		
7	153.1		
8	93.9	6.61 *s*	C-6, C-10, C-7
9	149.6		
10	103.9		
1'	121.9		
2'	128.0	7.85 *d, J* = 8.90 Hz	C-6', C-2, C-4'
3'	115.0	6.92 *d*, *J* = 8.90 Hz	C-5', C-1', C-4'
4'	161.5		
5'	115.0	6.92 *d*, *J* = 8.90 Hz	C-5', C-1', C-4'
6'	128.0	7.85 *d*, *J* = 8.90 Hz	C-2', C-2, C-4'

**recorded in methanol-d4*

4.2.4.4 Luteolin (4.4)



Luteolin

Compound 4.4 was obtained as a yellow powder (~1.0 mg, 0.006% *w/w*, mp 321-324^{o}C). ESI MS (positive ion mode) indicated a protonated molecular ion at *m/z* 287 $[M+H]^+$, consistent with the molecular formula $C_{15}H_{10}O_6$. The ^{13}C NMR data, along with HSQC and ^{1}H NMR data identified 15 carbon atoms (Table 4.4), consistent with one carbonyl and two aromatic rings (5 methines and 7 quaternary carbons) and one trisubstituted double bond. The ^{1}H NMR signals showed six non-exchangeable protons consistent with 5 aromatic protons and a deshielded vinylic proton. The PDA UV absorption spectrum of 4.1 with λ_{max} of 256 and 352 nm indicated a flavonoid type molecule[360].

The NMR spectral data were consistent with the presence of a 5,7-dioxygenated A ring and a 3',4'-dioxygenated B ring. Key features supporting this are described below.

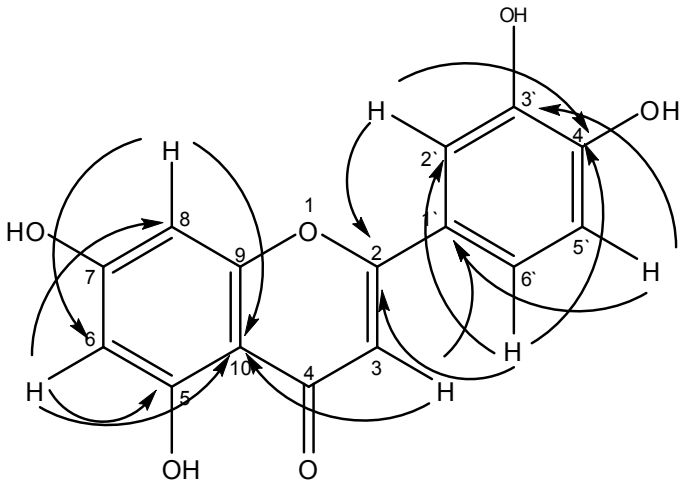
One of the aromatic rings contained only two aromatic proton signals (*meta* coupled) at δ 6.54 (1H, *d*, *J* = 2.1 Hz, H-8) and 6.25 (1H, *d*, *J* = 2.1 Hz, H-6), indicating a tetrasubstituted aromatic ring. Both protons showed HMBC correlations to the quaternary carbons at δc 105.8 (C-10) and 165.2 (C-7). Additionally, the proton at δ 6.25 showed correlations to a quaternary carbon at δc 163.6 (C-5), while the proton at δ 6.54 showed a correlation to the carbon signal at δc 159.1 (C-9). These were all consistent with the data observed for the A ring of compounds 4.1 and 4.2. Along with the carbonyl signal at δc 183.5 (C-4), the quaternary signal at δc 165.7 (C-2) and the methine carbon at 104.5 (C-3), these data supported a 5,7-dioxygenated flavonoid skeleton[361]. The presence of a

trisubstituted double bond for ring C was confirmed by the characteristic singlet at δ 6.56 (H-3)[362].

The second aromatic ring showed an *ortho* coupled doublet at δ 6.99 (1H, *d*, *J* = 8.4 Hz, H-5'), a doublet of doublets at δ 7.45 (1H, *dd*, *J* = 8.4, 2.1 Hz, H-6') and an *ortho* coupled doublet at 7.50 (1H, d, *J* = 2.1 Hz, H-2'), consistent with a 1,3,4-trisubstitution pattern. The carbon resonances at δc 146.9 and 150.5 (C-3' and C-4') indicated a dioxygenated phenyl ring[363]. The HMBC spectrum showed correlations of the protons at δ 7.45 (H-6') and 7.50 (H-2') to the carbon signal δc 165.7 (C-2), confirming that the phenyl ring was attached to C-2 of ring C of the flavonoid skeleton. An observed HMBC correlation between the proton at δ 6.56 (H-3) and the quaternary carbon at δc 124.1 (C-1') further confirmed the connectivity.

The molecular mass suggested by the ESI MS supported the presence of hydroxy groups at C-5, 7, 3' and 4'.

Literature examination revealed that the compound was the flavone luteolin, and the NMR, UV and mp data were in agreement with the published literature[364, 365]. The NMR data is presented in Table 4.4.



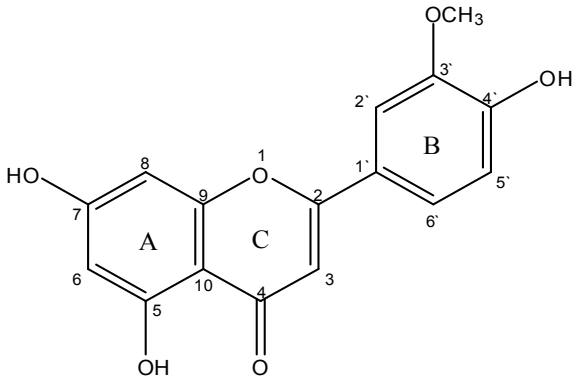
Key HMBC correlations of luteolin

Table 4.4 NMR data of luteolin (4.4)

Compound 4.4			
Position	δc	δ_H multiplicity	HMBC
2	165.7		
3	104.5	6.56 *s*	C-10, C-1’
4	183.5		
5	163.6		
6	100.1	6.25 *d, J* = 2.1 Hz	C-8, C-7, C-10, C-5
7	165.2		
8	95.2	6.54 *d*, *J* = 2.1 Hz	C-6, C-10, C-9, C-7
9	159.1		
10	105.8		
1’	124.1		
2’	114.6	7.50 *d, J* = 2.1 Hz	C-6’, C-2, C-4’
3’	146.9		
4’	150.5		
5’	117.2	6.99 *d*, *J* = 8.4 Hz	C-3’, C-1’, C-4’
6’	120.3	7.45 *dd, J* = 8.4, 2.1 Hz	C-2, C-2’, C-4’

**recorded in methanol-d4*

4.2.4.5 Chrysoeriol (4.5)

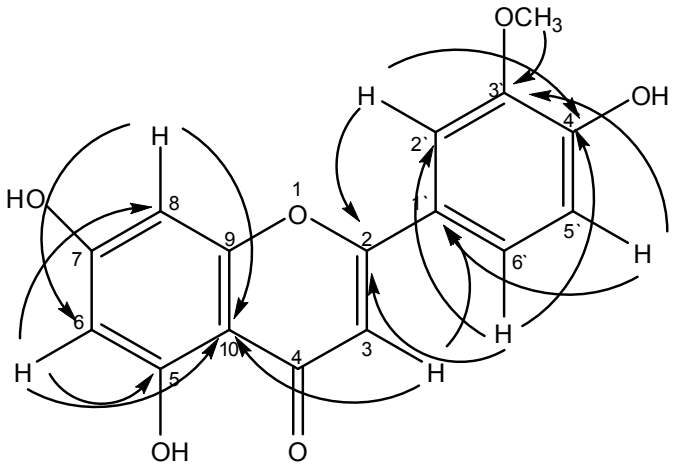


Chrysoeriol

Compound 4.5 was obtained as a yellow amorphous solid (2.0 mg, 0.012% *w/w*). ESI MS (positive ion mode) indicated a protonated molecular ion at *m/z* 301 $[M+H]^+$, consistent with the molecular formula $C_{16}H_{12}O_6$. The PDA UV absorption spectrum of 4.1, with λ_{max} of 251 and 344 nm, indicated a flavonoid type molecule[360].

The ^1H and ^{13}C NMR spectra were almost identical to that of luteolin except for the presence of signals for a methoxy group at δ 3.96 (3H, *s*) and δc 56.2. The HMBC

experiment showed a correlation between the methoxy protons and the carbon at δc 148.6. This carbon signal was known to be in ring B based on HMBC correlation with the proton at 6.92 (H-5'). This supported the methoxy group being placed at the C-3' position (4',5,7-trihydroxy-3'-methoxyflavone, chrysoeriol) as opposed to the C-4' position (3',5,7-trihydroxy-4'-methoxyflavone, diosmetin)[238]. Reported literature showed that there is a marked difference in the melting point of these compounds (330-331^{o} C for chrysoeriol and 256-259^{o} C for diosmetin)[238, 366]. The melting point of 4.5 was determined to be 326-331^{o} C, which was consistent with the methoxy group at the 3′ position, thus establishing the molecule as chrysoeriol. The assignment as chrysoeriol was further supported by EI MS which exhibited characteristic *m/z* signals at 300 (M^{+}), 257 (M-$COCH_3$) and 153 ($(A_1+1)^{+}$), identical to reported literature for chrysoeriol[367]. The NMR, MS, UV data and melting point were in agreement with the published literature[368-370] (Table 4.6). The NMR data are presented in Table 4.5.



Key HMBC correlations of chrysoeriol

Table 4.5 NMR data of chrysoeriol (4.5)

Compound 4.4			
Position	δc	δ_H multiplicity	HMBC
2	165.3		
3	103.4	6.63 *s*	C-10, C-1’
4	183.0		
5	162.4		
6	99.7	6.21 *d, J* = 2.0 Hz	C-8, C-7, C-10, C-5
7	165.1		
8	94.2	6.47 *d*, *J* = 2.0 Hz	C-6, C-10, C-9, C-7
9	158.5		
10	104.5		
1’	122.8		
2’	110.0	7.48 *d, J* = 2.0 Hz	C-6’, C-2, C-4’
3’	148.7		
4’	151.0		
5’	116.4	6.92 *d*, *J* = 8.5 Hz	C-3’, C-1’, C-4’
6’	120.5	7.50 *dd, J* = 8.5, 2.1 Hz	C-2, C-2’, C-4’
-OCH_3	56.2	3.96 s	C-3’

**recorded in methanol-d4*

A comparison of the carbon shift values of the isolated compounds to those reported in the literature is given in Table 4.6. The literature values were measured in DMSO-d6, while in this study the spectra were recorded in methanol-d4. The values were in agreement with the reported literature data.

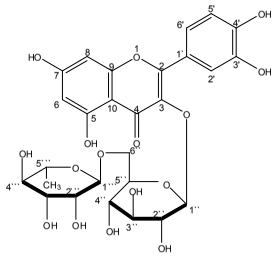
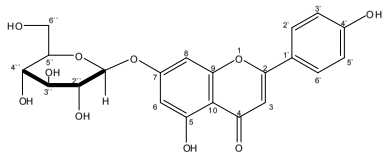
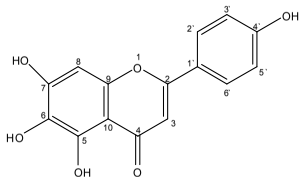
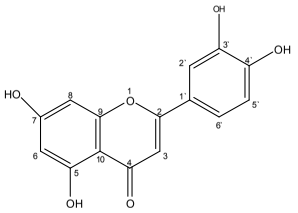
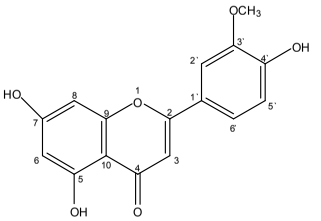
Position	 **Quercetin 3-*O*-*β*-D-rutinoside**	 **Apigenin 7-*O*-*β*-D-glucoside**	 **Scutellarein**	 **Luteolin**	 **Chrysoeriol**
	MeOD (DMSO)	MeOD (DMSO-d6)	MeOD (DMSO-d6)	MeOD (DMSO-d6)	MeOD (DMSO-d6)
2	158.0 (157.3)[338, 371, 372]	164.9 (164.3)[373, 374]	165.4 (164.2)[359]	165.7 (166.5)[375]	165.3 (165.5)[369, 376]
3	134.3 (134.1)	103.9 (102.8)	102.4 (102.6)	104.5 (104.2)	103.4 (103.8)
4	178.4 (178.2)	182.8 (181.7)	182.1 (182.4)	183.5 (184.1)	183.0 (182.1)
5	161.8 (161.4)	161.8 (161.5)	147.0 (146.9)	163.6 (163.0)	162.4 (161.8)
6	98.9 (99.5)	100.1 (99.7)	130.7 (130.1)	100.1 (100.1)	99.7 (99.5)
7	164.8 (164.9)	163.2 (162.9)	153.1 (153.2)	165.2 (165.2)	165.1 (164.1)
8	93.7 (94.5)	95.4 (94.9)	93.9 (93.8)	95.2 (96.2)	94.2 (94.6)
9	161.9 (162.1)	157.5 (156.8)	149.6 (149.6)	159.1 (159.1)	158.5 (157.8)
10	104.5 (104.8)	106.1 (105.4)	103.9 (104.0)	105.8 (107.1)	104.5 (103.5)
1'	122.4 (122.5)	121.7 (121.3)	121.9 (121.4)	124.1 (123.5)	122.8 (121.9)
2'	116.3 (116.1)	129.4 (128.4)	128.0 (128.2)	114.6 (114.2)	110.0 (110.6)
3'	144.8 (145.6)	116.6 (116.0)	115.0 (115.9)	146.9 (147.1)	148.7 (148.5)
4'	148.6 (149.3)	161.9 (161.1)	161.5 (160.9)	150.5 (151.3)	151.0 (151.3)
5'	114.7 (116.3)	116.6 (116.0)	115.0 (115.9)	117.2 (116.8)	116.4 (116.2)
6'	122.2 (122.0)	129.4 (128.4)	128.0 (128.2)	120.3 (120.3)	120.5 (120.8)
1''	103.5 (102.1)	99.9 (100.2)			
2''	74.6 (74.9)	73.4 (73.1)			
3''	77.1 (77.3)	76.4 (76.5)			
4''	72.8 (72.7)	70.2 (69.8)			
5''	76.1 (76.7)	75.8 (76.3)			
6''	67.4 (67.9)	61.4 (61.0)			
1'''	101.2 (102.2)				
2'''	70.2 (70.8)				
3'''	70.9 (71.2)				
4'''	71.0 (71.4)				
5'''	67.3 (69.1)				
6'''	16.7(17.7)				
-OCH_3					56.2 (56.4)

Table 4.6 Carbon NMR data for isolated compounds compared with literature values (literature values in brackets)

While the flavonoids quercetin 3-*O*-*β*-D-rutinoside (4.1), luteolin (4.4) and chrysoeriol (4.5) have been reported earlier from the leaves of *C. halicacabum*[325-327], this is the first report of isolation of apigenin 7-*O*-*β*-D-glucoside (4.2) and scutellarein (4.3) from this plant.

4.4 Antiproliferative activity of flavonoids isolated from Ch-water-5

Flavonoids are one of the major classes of natural products and are well documented for their important pharmacological activities including antiinflammatory, antioxidant, antimutagenic, anticarcinogenic and antitumour activities[377, 378]. The biological activities of flavonoids occur mainly through their interaction with regulatory enzymes[379-381]. All the five compounds isolated from the active water fraction have been reported for their anticancer activity. Quercetin 3-*O*-*β*-D-rutinoside has been shown to exhibit significant activity in reducing azoxymethanol (AOM) induced hyperproliferation of colonic epithelial cells[382]. Apigenin 7-*O*-*β*-D-glucoside has been reported to possess strong antiproliferative activity against the human prostate cancer cells LNCaP, DU145 and PC 3[383]. Scutellarein has been reported to selectively inhibit proliferation of malignant glioma and breast adenocarcinoma cell lines[384, 385]. *In vivo* experiments have shown that luteolin suppresses growth of tumours formed from human skin carcinoma, hepatoma and human ovarian cancer cells[386]. Chrysoeriol has been reported to exhibit strong antiproliferative activity against lung, breast and colon cancer cell lines[387].

Quercetin 3-*O*-*β*-D-rutinoside, apigenin 7-*O*-*β*-D-glucoside, scutellarein and chrysoeriol were tested for their antiproliferative activities against the SKNMC and MCF 7 cancer cell lines. To determine whether their antiproliferative activities were selective towards cancer cell lines, they were also tested against the normal cell line MRC 5. Due to a low yield, luteolin was not included in the study. The antiproliferative activities of the tested molecules are summarised in Table 4.7.

The aglycones, scutellarein and chrysoeriol exhibited higher activity compared to the glycosides quercetin 3-*O*-*β*-D-rutinoside and apigenin 7-*O*-*β*-D-glucoside. Chrysoeriol

exhibited the strongest activity especially against the MCF 7 cell line (IC_{50} of 2 µg/ml). The IC_{50} value against the SKNMC cell line was determined to be 9 µg/ml. Scutellarein also exhibited significant antiproliferative activity with IC_{50} values of 14 µg/ml and 6 µg/ml against the SKNMC and MCF 7 cell lines, respectively. The IC_{50} value for apigenin 7-*O*-*β*-D-glucoside for the same cell lines was determined to be 48 ± 2 and 34 ± 2 µg/ml, respectively. Quercetin 3-*O*-*β*-D-rutinoside inhibited proliferation of the SKNMC cell line, with an IC_{50} value of 51 ± 5 µg/ml, but was not active against the MCF 7 cell line, even at 100 µM, the highest concentration tested.

Table 4.7 Antiproliferative activity of isolated molecules

Compound tested*	SKNMC	MCF 7	MRC 5
		IC_{50} values in µg/ml	
quercetin 3-*O*-*β*-D-rutinoside (4.1)	51 ± 5	na	42 ± 4
apigenin 7-*O*-*β*-D-glucoside (4.2)	48 ± 2	34 ± 2	67 ± 3
scutellarein (4.3)	14	6	27
chrysoeriol (4.5)	9	2	nt

* The results for 5.1 and 5.2 are from two independent assays (done in duplicate) and for 4.4 and 4.3 are from a single assay (done in duplicate), nt: not tested, na: not active

A comparison of the activity exhibited by the compounds against the MCF 7 and SKNMC cell lines indicated some selectivity of these molecules for the breast cancer cell line.

The higher level of activity exhibited by the aglycones, compared to the glycosides is in accordance with the literature[387]. The presence of a sugar moiety is believed to be more likely to hinder the entry of flavonoids into cells and can also sterically inhibit their binding to receptors involved in gene expression[387]. However studies have shown that *β*-glucosidase in the human small intestine and liver can efficiently hydrolyse naturally occurring flavonoid glycosides to their aglycones[388]. This deglycosylation is an important first step in the uptake, metabolism, excretion and biological activity of these compounds[388]. Hence, even though flavonoid glycosides often show little effect on the proliferation of cancer cells *in vitro*, it is highly likely that they can exert a greater activity under *in vivo* conditions.

Studies using rat intestine have reported 49.9% conversion of apigenin 7-*O*-*β*-D-glucoside to apigenin by *β*-glucosidase[383]. Apigenin has been demonstrated to possess significant anticancer properties and to cause selective growth inhibition and apoptosis in cancer cells[389]. This includes inhibition of proliferation of various human cancer cell lines, such as Caco 2, SW480, and HT 29 (colon), OCM 1 (skin), MCF 7 and MDA MB 468 (breast), prostate, thyroid and HL 60 cancer cell lines[390-394].

Quercetin, the aglycone moiety of quercetin 3-*O*-*β*-D-rutinoside, has been shown to exert antiproliferative activity in Caco 2 and Hep G2 cells by covalently binding to the cellular DNA and protein[395]. Quercetin (3′(*N*-carboxymethyl)carbomyl-3,4′,5,7-tetrahydroxyflavone), a water soluble prodrug of quercetin, is currently under clinical trials[253].

Structure activity relationship studies have demonstrated the importance of the C2-C3 double bond and the C-4 carbonyl group of flavonoids for exhibiting antiproliferative activity[377, 396]. Flavonoids function as antioxidant molecules as well. *In vitro* studies have demonstrated the antioxidant potential of flavonoids by scavenging the superoxide anion, singlet oxygen and lipid peroxy radicals[253]. Limiting the damaging oxidative reactions in the cells can be one of the mechanism by which flavonoids exert anticancer activity[253]. They are also reported to protect the microvascular endothelium during oxidative stress, particularly in cases involving cancer and inflammation[387].

4.5 Conclusions

The use of roots and leaves of *C. halicacabum* in the Siddha system of medicine for anticancer treatment was the rationale for this study. The antiproliferative activity of *C. halicacabum* has been demonstrated for the first time *in vitro* using two different cancer cell lines. The presence of five flavonoids, quercetin 3-*O*-*β*-D-rutinoside, apigenin 7-*O*-*β*-D-glucoside, chrysoeriol, luteolin and scutellarein were detected following a bioassay guided fractionation study. All these molecules have earlier been reported to possess significant antiproliferative activity. Apigenin 7-*O*-*β*-D-glucoside was identified as the major constituent in *C. halicacabum*. This is the first report of apigenin 7-*O*-*β*-D-glucoside and scutellarein from this plant.

Flavonoids are reported to regulate proliferation and cell death pathways leading to cancer and are emerging as prospective anticancer drug candidates[397]. The *in vitro* findings are consistent with the traditional medicinal use of *C. halicacabum* in the Siddha system of medicine for treatment of cancers.

4.6 Materials and Methods

4.6.1 Reagents and equipment

All the solvents used for extraction and chromatographic separations were of analytical grade and distilled prior to use. Milli Q water was used for preparing 70% ethanol and other reagents. HPLC grade solvents were used for HPLC studies. Analytical normal phase thin layer chromatography (TLC) was performed on fluorescent Merck silica gel F254 plates (Germany) and reversed phase TLC on Merck Silica gel 60 RP-18 F_{254s} plates. Size exclusion chromatography was carried out using LH20100 Sephadex LH 20 (Sigma Aldrich). The TLC plates were visualised using UV light (254 nm and 365 nm). Solid Phase Extraction was carried out using Waters Sep Pak Vac 35cc (10 g) cartridges. The ^{1}H (600 MHz), ^{13}C (100 MHz), HSQC, COSY and HMBC NMR spectra were recorded on a Bruker DRX600K 600 MHz NMR Spectrometer (Germany) using standard pulse sequences. Chemical shifts were calculated relative to the methanol-d4 (^{1}H δ 3.31 and ^{13}C δ 49.0) solvent peaks. A Shimadzu LC 10 AVP HPLC system was used for chromatographic separations and a Shimadzu 2010 LC MS system was used for ESI MS analysis. A Shimadzu GC-17 system was used for EI MS analysis. A Stuart Scientific melting point detector (UK) was used for determining melting points.

4.6.2 Preparation of plant material

500 g of powdered *C. halicacabum* was extracted successively with 70% aqueous EtOH (3 x 1.5 l, 8 hr each) at 40-50^{o}C and the EtOH was evaporated under vacuum leaving the aqueous extract. The resultant extract was freeze dried to obtain a brown solid mass (43 g, crude extract, Ch-crude, sticky on exposure to air). A portion (40 g) of the crude extract was suspended in water and successively partitioned with *n*-hexane (2 x 0.5 l), ethyl acetate (3 x 0.5 l) and *n*-butanol (3 x 0.5 l) to afford four partitions. Each partition was concentrated to dryness, under reduced pressure, and dried under high vacuum to

afford their corresponding partitions as Ch-Hex (19.5% *w/w*, green gum, 7.8 g), Ch-EtOAc (23.7% *w/w,* green partial solid, 9.5 g), Ch-BuOH (31% *w/w*, brown solid mass, 12.5g) and Ch-Water (19.2% *w/w*, dark brown solid, 7.7 g).

4.6.3 Fehlings Solution

Solution A: 7 g of hydrated copper(II) sulfate was dissolved in 100 ml of distilled water.

Solution B: 35 g of potassium sodium tartrate and 10 g of sodium hydroxide were dissolved in 100 ml of distilled water.

Equal volumes from each stock solution were mixed freshly before use.

A small amount (2-3 ml) of crude extract dissolved in water (0.5 mg/ml) was treated with Fehling's solution. The formation of a brown color indicated the presence of carbohydrates[398].

4.6.5 Anisaldehyde-Sulfuric acid reagent

Anisaldehyde-sulfuric acid reagent was prepared fresh by slowly adding 9 ml of 98% H_2SO_4 to an ice cooled mixture of 85 ml of methanol and 10 ml of glacial acetic acid. To this solution 0.5 ml of anisaldehyde was added and thoroughly mixed[398].

TLC plates were dipped in anisaldehyde reagent and heated at 100^{o}C for 1-2 minutes.

4.6.4 Fractionation of Ch-water partition

A C_{18} Sep Pak (35cc, 10 g) cartridge was preconditioned with 3 column volumes of methanol and 2 column volumes of water. The Ch-water-5 sample (350 mg in 5 ml of water) was loaded on to the Sep Pak column and was fractionated by eluting with varying proportions of water:acetonitrile (100-40% water) under a small vacuum. 20 ml fractions were collected and monitored by reversed phase TLC (water:acetonitrile, 8:2; visualisation at UV 365 nm and by derivatising using anisaldehyde-sulfuric acid reagent). The fractions that showed poor resolution on reversed phase TLC plates were monitored by normal phase TLC (ethyl acetate:acetic acid:formic acid:water, 100:11:11:26; visualisation at UV 365 nm after exposing plates to ammonia[399]). Similar fractions were

pooled together and evaporated under vacuum then dried on a freeze dryer to obtain Ch-water-1 (38% *w/w*, off white solid crystalline mass, 1.4 g), Ch-water-2 (8% *w/w*, 280 mg), Ch-water-3 (6% *w/w*, 200 mg), Ch-water-4 (6.6% *w/w*, 210 mg), Ch-water-5 (9.5% *w/w*, 300 mg), Ch-water-6 (7.6% *w/w*, 240 mg) (Ch-water-2 to -6 were all yellow fluffy powders).

4.6.6 Fractionation of Ch-water-5

Ch-water-5 was further fractionated by preparative high performance liquid chromatography. HPLC analysis was carried out using a Shimadzu HPLC system consisting of a LC-10 AVP pump with a SPD M10AVP photodiode array detector and an autosampler. Chromatographic separation was performed at ambient temperature (23 – 25°C) on a Synergi 10u Hydro RP 80A column (250x10 mm i.d., 10 micron, Phenomenex) connected with a guard column (Phenomenex Fusion 4x3 mm). The mobile phase consisted of water (A) and acetonitrile (B) and was eluted in a gradient mode (0.01 minutes - 85% A, 10 minutes - 85% A, 50 minutes - 30% A, 60 minutes - 15% A, 70 minutes - 15%, 75 minutes - 85% A continued until 80 minutes). The injection volume was set as 400 µl and the total run time was 80 minutes at a flow rate of 4.0 ml/minute.

4.6.6.1 Isolation of compounds

Five peaks corresponding to retention times 36.0, 40.3, 41.1, 44.5 and 45.4 minutes were repeatedly collected using an automatic fraction collector. The solvent was evaporated under vacuum and the remaining water was removed by freeze drying. The fractions collected at retention times 36.0 and 40.3 minutes were obtained with minor impurities and were further purified by size exclusion chromatography on a Sephadex LH 20 column by using methanol as the eluting solvent to obtain compounds 4.1 (3.5 mg, yellow amorphous powder) and 4.2 (7.5 mg, yellow amorphous powder). The other compounds 4.3 (2 mg), 4.4 (1 mg) and 4.5 (2 mg) were also obtained as yellow amorphous powders.

4.1 Quercetin 3-*O*- *β*-D-rutinoside (3.5 mg, ESI MS *m/z* 609 $[M-H]^-$, mp 192-194^oC (lit. 188-192^oC[347]), EI MS *m/z* 301, 272, 256, 1D and 2D NMR data in Table 4.1)

4.2 Apigenin 7-O-*β*-D-glucoside (7 mg, ESI MS *m/z* 433 $[M+H]^+$, mp 196-201^oC (lit. 204^oC[348]), EI MS *m/z* 270, 242, 213, 153, 1D and 2D NMR data in Table 4.2)

4.3 Scutellarein (2 mg,ESI MS *m/z* 287 $[M+H]^+$, mp 335-337^oC (lit. 340^oC[400]), 1D and 2D NMR data in Table 4.3)

4.4 Luteolin (1 mg, ESI MS *m/z* 287 $[M+H]^+$, mp 321-324^oC (320-324^oC[363]), 1D and 2D NMR data in Table 4.4)

4.5 Chrysoeriol (2 mg,ESI MS *m/z* 301 $[M+H]^+$, mp 326-331^oC (330-331^oC[238]), EI MS *m/z* 300, 257, 229, 153 1D and 2D NMR data in Table 4.5)

4.6.7 MTT assay

The MTT assay was carried out by following the method as explained in Chapter 3 (Section 3.7.5). Sample solutions were prepared in DMSO and tested at concentrations of 1-100 µg/ml. The crude extract, partitions, quercetin 3-*O*-*β*-D-rutinoside and apigenin 7-*O*-*β*-D-glucoside were assayed in duplicate and repeated twice. Due to a limited amount of sample, chrysoeriol and scutellarein were assayed in duplicate only once. The assays were performed against the SKNMC, MCF 7 and MRC 5 cell lines.

Chapter 5

Bioassay guided isolation of bioactive compounds from Caralluma fimbriata

This chapter describes the bioassay guided studies carried out with the crude ethanolic extract of Caralluma fimbriata to isolate bioactive molecules responsible for its antiproliferative activity and their structure elucidation.

5.1 Introduction

The genus *Caralluma* belongs to the family Asclepiadaceae and consists of 87 species distributed throughout Asia and Africa, the majority being indigenous to India and Arabian sub-regions[70, 401]. Plants from the genus *Caralluma* are rich in bioactive constituents with various biological activities and have been used as a food and a source of medicine by tribal people in India, Arabia and East Africa for centuries[402]. Bedouins (an Arab ethnic group) use *Caralluma spp.* for the treatment of chronic lung diseases such as lung cancer and tuberculosis[403]. *Caralluma spp.* are also used in Nigerian folklore medicine as a tonic, aphrodisiac, analgesic and antiemetic[404].

C. fimbriata is found in the hilly regions of India, especially in the western and southern states. Tribal people in South India use *C. fimbriata* as a vegetable. The aerial parts of *C. fimbriata* are eaten raw, cooked with spices as a curry, or as a pickle. During drought or famine, *C. fimbriata* is consumed as a substitute for food[402]. This is interesting because *C. fimbriata* is also claimed to possess appetite suppressant activities[70]. A number of products have been recently introduced to the market as dietary supplements for weight loss with *C. fimbriata* as the major ingredient. In Ayurveda this plant is used for the treatment of migraine and diabetes[405].

The crude extract of the leaves of *C. fimbriata* was shown to possess significant antiproliferative activity against the SKNMC (human neuroblastoma) cell line (IC_{50} of 17.1 ± 1.0 μg/ml) in the preliminary MTT assay (Section 3.3). The activity level exhibited by this plant was within the range recommended by the National Cancer

Institute for selecting potential candidate plants for further biological and chemical investigation[306]. The aim of this chapter was to describe the bioassay guided studies carried out on *C. fimbriata* to isolate bioactive molecules responsible for the antiproliferative activity and to determine their structures.

5.2 Results and Discussion

5.2.1 Bioassay guided investigation of partitions

The extraction was repeated, as described in Chapter 3, except on a larger scale. One kilogram of dried powdered *C. fimbriata* leaves was extracted with 70% aqueous ethanol (40-50^{o} C) and the aqueous residue, after evaporation of ethanol, was dried under vacuum on a freeze dryer. The dried crude extract was suspended in water and partitioned with increasing polarities of solvents to afford four partitions, *i.e*. *n*-hexane (Cf-Hex), ethyl acetate (Cf-EtOAc), *n*-butanol (Cf-BuOH) and water (Cf-water) partitions (Scheme 5.1).

The partitions were tested by the MTT assay at concentrations of 10-100 µg/ml against the SKNMC cell line[304, 305] (Scheme 5.1). The larger scale crude extract was also tested to confirm the activity and was found to be consistent with the earlier crude extract (IC_{50} of 22 ± 3 µg/ml). Of the partitions, the ethyl acetate partition was found to exhibit the greatest antiproliferative activity with an IC_{50} value of 12.5 ± 3.0 µg/ml. The water partition also showed activity with an IC_{50} value of 33 ± 5 µg/ml. The *n*-hexane and *n*-butanol partitions were found to be inactive (IC_{50} > 100 µg/ml). The ethyl acetate partition, being the most active, was therefore selected for further bioassay guided isolation studies.

5.2.2 Isolation and identification of bioactive molecules from ethyl acetate partition

The ethyl acetate partition (15 g) was applied to a normal phase silica gel column. The column was eluted with a solvent gradient of chloroform:methanol, in order of increasing polarity (100:0 to 35:65). Similar fractions were pooled according to their TLC profiles, to yield 7 fractions (Cf-EtOAc-1 to Cf-EtOAc-7), which were then tested for antiproliferative activity by the MTT assay.

Fractions Cf-EtOAc-1 to Cf-EtOAc-7 were analysed for antiproliferative activity against the SKNMC (human neuroblastoma) and MCF 7 (human breast adenocarcinoma) cell lines. As described earlier, Dr Velmurugan, has seen good clinical results with his formulations against breast cancer, hence the MCF 7 breast cancer cell line was included in the study.

Cf-EtOAc-7 showed significant activity against both the cell lines tested. The IC_{50} was determined as 5 ± 1 and 27 ± 2 µg/ml for the SKNMC and MCF 7 cell lines respectively. Fraction Cf-EtOAc-6 also showed good activity with IC_{50} values of 22 ± 2 and 48 ± 3 µg/ml for the SKNMC and MCF 7 cell lines, respectively. The level of activity exhibited by Cf-EtOAc-5 was moderate with IC_{50} values of 82 ± 6 and 70 ± 4 µg/ml. Cf-EtOAc-6 and Cf-EtOAc-7 exhibited greater activity against the SKNMC cell line in comparison to MCF 7 (Table 5.1).

To determine the selectivity, Cf-EtOAc-5, Cf-EtOAc-6 and Cf-EtOAc-7 were further tested against the normal fibroblastic MRC 5 cell line. Fraction Cf-EtOAc-6 exhibited selectivity against the SKNMC cell line (IC_{50} 22 ± 2 µg/ml) in comparison with the normal cell line (IC_{50} 47 ± 5 µg/ml). Fraction Cf-EtOAc-7 was also found to be more active against the SKNMC (IC_{50} 5 ± 1 µg/ml) and MRC 5 (IC_{50} 7 ± 4 µg/ml) cell lines compared to MCF 7 (27 ± 2 µg/ml). No remarkable selectivity was observed for Cf-EtOAc-5. However, it should be noted that most of the currently used anticancer cytostatic drugs also have a strong effect on all proliferating cells including normal cells[333].

Cf-EtOAc-6 and Cf-EtOAc-7 were selected for further chromatographic separation due to their strong antiproliferative activities.

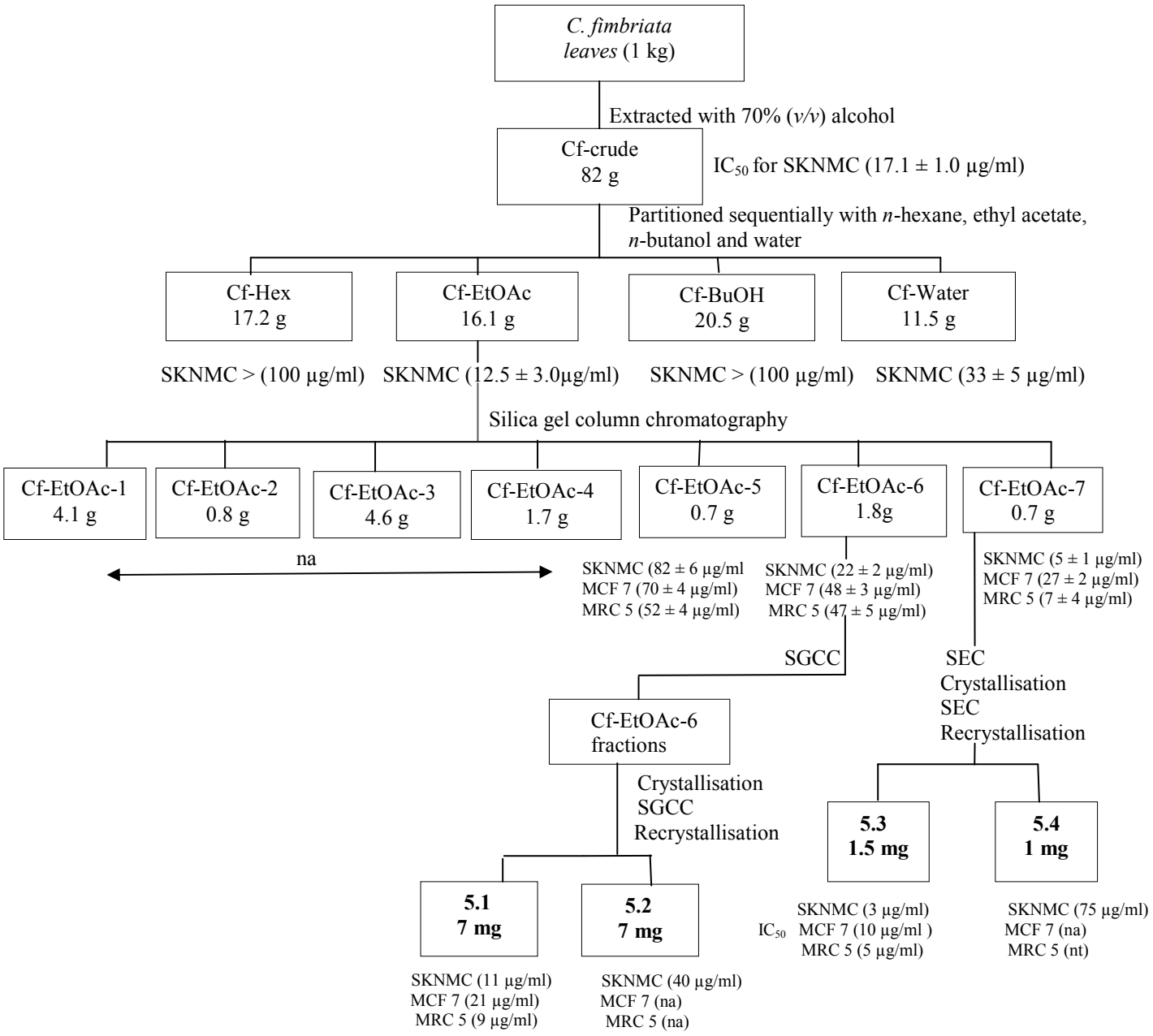
Table 5.1 Antiproliferative activities of fractions of ethyl acetate partitions

Fraction	**IC_{50} (µg/ml)** SKNMC	MCF 7	MRC 5
Cf-EtOAc-1	> 100	> 100	nt
Cf-EtOAc-2	> 100	> 100	nt
Cf-EtOAc-3	> 100	> 100	nt
Cf-EtOAc-4	> 100	> 100	nt
Cf-EtOAc-5	82 ± 6	70 ± 4	52 ± 4
Cf-EtOAc-6	22 ± 1	48 ± 3	47 ± 5
Cf-EtOAc-7	5 ± 1	27 ± 2	7 ± 4

The results are from two independent assays (samples tested in duplicate), nt: not tested
Cf-EtOAc-1-7 were not active against cancer cell lines and were not tested against MRC 5

Cf-EtOAc-6 was further separated by normal phase column chromatography. The column was developed with a gradient solvent system of chloroform:methanol, in order of increasing polarity (10:0 to 9:1). This led to the isolation of cleomiscosin A (5.1) and *N*-(*p-trans*-coumaroyl)tyramine (5.2). Normal phase column chromatography was attempted to isolate the active molecules from fraction Cf-EtOAc-7, but as the sample was found to be too polar it was unable to be easily eluted. Sephadex LH 20 size exclusion chromatography (SEC) was attempted. Size exclusion chromatography (SEC) using the lipophilic organic resin Sephadex LH 20 has the advantage of high sample recovery[335, 336]. Cf-EtOAc-7 was eluted with methanol to provide two major constituents, identified as aristolochic acid 1 (5.3) and aristolactam1a-*N*-*β*-D-glucoside (5.4). Sephadex LH-20 SEC was repeatedly carried out for the further purification of the isolated molecules 5.3 and 5.4.

The bioassay guided studies towards the isolation of cleomiscosin A, *N*-(*p-trans*-coumaroyl)tyramine, aristolochic acid 1 and aristolactam1a-*N*-*β*-D-glucoside are summarised in Scheme 5.1.

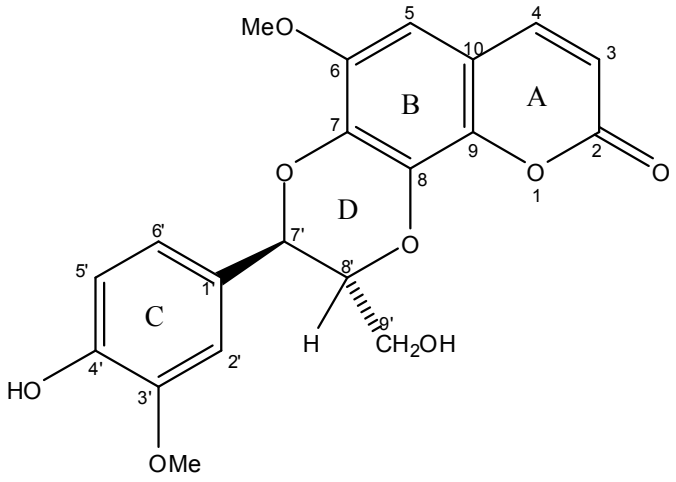


Scheme 5.1 Antiproliferative activity results shown in the scheme are from the MTT assay. Cell lines used: SKNMC (neuroblastoma), MCF 7 (breast cancer) and MRC 5(normal). Recrystallisation solvents: Methanol/acetone. nt: not tested. na: not active. The values following the cell lines are the IC_{50} values

5.2.3 Characterisation of bioactive compounds

The structures of the bioactive compounds were elucidated by 1D and 2D NMR and mass spectrometry and by comparison with reported data. The structural elucidation of these compounds is described below

5.2.3.1 Cleomiscosin A (5.1)



Cleomiscosin A

Compound 5.1 was obtained as a colourless amorphous solid (7 mg, mp 246-250^{o}C). Electrospray Ionisation Mass Spectrometry (ESI MS) (positive ion mode) revealed a protonated molecular ion at *m/z* 387 $[M+H]^+$, consistent with the molecular formula $C_{20}H_{18}O_{8.}$ The ^{13}C NMR data, along with HSQC and ^{1}H NMR data, identified 20 carbon atoms (Table 5.2), consistent with one carbonyl, two aromatic rings (4 methines and 8 quaternary carbons), two methine groups of a double bond, two aromatic methoxy groups, and two methines and a methylene group attached to oxygens. The ^{1}H NMR spectrum (recorded in acetone-d6) confirmed the presence of four aromatic protons, two methine protons of a *cis* double bond, two methoxy groups and four deshielded aliphatic protons. This suggested that the two remaining protons expected based on the proposed molecular formula were exchangeable heteroatom protons.

The aromatic ring C showed three proton signals at δ 6.90 (*d*, *J* = 8.0 Hz, H-5'), 7.01 (*dd*, *J* = 8.0, 2.0 Hz, H-6'), δ 7.15 (*d*, *J* = 2.0 Hz, H-2') in agreement with a 1,3,4-substituted aromatic ring. The proton at δ 7.15 (H-2') exhibited HMBC correlations with the

aromatic carbons at δc 127.6 (quaternary, C-1'), 121.1 (methine, C-6') and 147.4 (quaternary, C-4') and the deshielded methine at 76.8 (C-7'). Its *meta* coupled partner at δ 7.01 (H-6') also showed a HMBC correlation with the carbon at δc 76.8 (C-7'), along with the aromatic signals at δc 111.3 (methine, C-2'), 147.4 (C-4') and 115.0 (methine, C-5'). The proton at δ 6.90 (H-5') showed HMBC correlations with the carbons at δc 148.0 (methine, C-3'), 127.6 (C-1') and 121.1 (C-6'). The chemical shift of the quaternary carbons at δc 147.4 (C-4') and 148.0 (C-3') were consistent with two oxygen substituents on the aromatic ring. The HMBC correlations of both of the protons at δ 7.15 (H-2') and 7.01 (H-6') with the signal at δc 76.8 (C-7') implied that these two protons were adjacent to the non-aromatic link and, along with the diagnostic coupling patterns, confirmed a 3,4-dioxygenated substitution pattern on the phenyl ring. A HMBC correlation was also observed for a methoxy group (δ 3.83, s) and the carbon signal at δc 148.0 (C-3'). The methoxy group at δ 3.87 did not correlate to any carbons in aromatic ring C, leading to the conclusion that the oxygenation next to C-4' was likely an OH group with the proton being exchanged with the trace moisture in the sample.

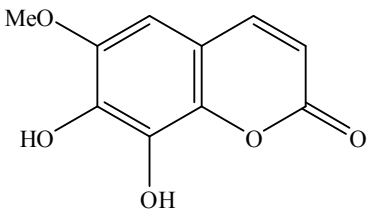
The deshielded protons in ring D at δ 5.01 (H-7'), 4.25 (H-8'), 3.89 (H-9') and 3.86 (H-9') were found to be consistent with the following bonding sequence –CH(O)-–CH(O)-–CH_2OH, namely C(H-7'), C(H -8') and $C(H\text{-}9')_2$ [406]. The presence of methines C(H-7'), C(H -8') and one methylene $C(H\text{-}9')_2$ and their connection sequence was confirmed by HSQC and HMBC correlations. The proton at δ 4.25 showed coupling to all three protons, confirming its central placement. The doublet at δ 5.01 (H-7') was typical of a benzylic CH substituted with an O-atom[407]. Its benzylic placement was confirmed by the proton at δ 5.01 (H-7') showing HMBC correlation to aromatic carbons at δc 121.1 (C-6') and 111.3 (C-2') and by the carbon connected to this proton (at δc 76.8 as seen by HSQC spectrum) having HMBC spectrum correlation to the aromatic protons at δ 7.01 (H-6') and 7.15 (H-2').

The proton at δ 5.01 (H-7') also showed a HMBC correlation to C-7 at δc 137.8 confirming the connection of ring C and B via C-7' and an oxygen. A coupling constant of 8.2 Hz for the doublet at δ 5.01 (H-7') indicated a *trans* relative configuration between it and the proton at δ 4.25 (H-8'). The confirmation of the 1,4-dioxane ring D fused with ring B was achieved by careful comparison of the published NMR data [408, 409].

The methoxy group at δ 3.87 was found to have no HMBC correlation to aromatic ring C but observed to correlate to C-6 in the aromatic ring B. Ring B contained only one aromatic proton at δ 6.84 (1H, *s*, H-5), indicating pentasubstitution in the ring. This proton, shown by a HSQC correlation to be connected to the carbon at δc 100.8, exhibited HMBC correlations to quaternary carbon atoms at δc 146.0 (C-6), 137.8 (C-7), 138.7 (C-9) and 111.5 (C-10).

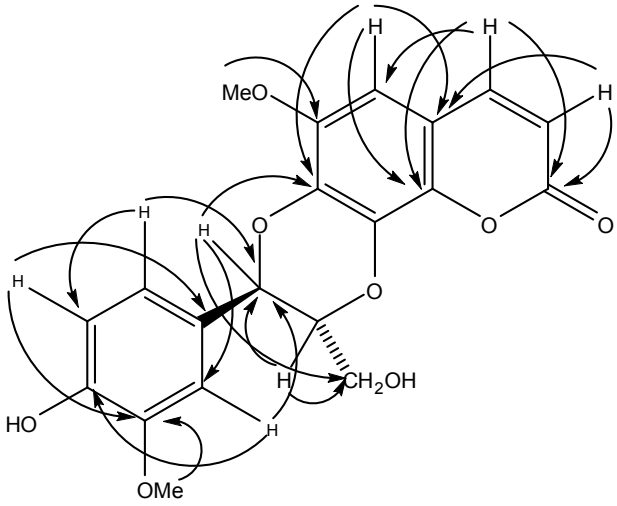
The presence of a pair of doublets at δ 6.26 (H-3) and 7.88 (H-4) with the *cis* coupling (*J* = 9.5 Hz), their correlations to the carbonyl carbon at δc 160 (C-2) in HMBC, and aromatic ring B were characteristic of a coumarin moiety[410]. The assignment of ring A in a coumarin moiety was further supported by HMBC correlations of the proton at δ 6.26 (H-3) to the quaternary aromatic carbon at δc 111.5 (C-10) and the proton at δ 7.88 (H-4) to the aromatic carbon at δc 100.8 (C-5). The remaining carbon at δc 132.2 (C-8) was assigned to the final quaternary aromatic carbon of the second aromatic ring. The chemical shifts of the quaternary carbon signals at δc 146.0 (C-6), 137.8 (C-7), 138.7 (C-9), 132.2 (C-8), along with the correlation observed for the aromatic methine carbon signal with the coumarin vinylic proton, were consistent with a 6,7,8-trioxygenated coumarin moiety[411]. This was also consistent with this aromatic ring being fused to ring D.

The presence of a 6,7,8-trioxygenated coumarin moiety was further supported by the UV maxima at 324 and 287 nm, which are characteristic absorptions for 6,7,8-trioxygenated coumarins[412, 413]. The attachment of the methoxy group at δ 3.87 to the carbon adjacent to the aromatic singlet at δ 6.84 was confirmed from the HMBC correlation of the methoxy protons with the carbon signal at δc 146.0 (C-6). The fragment ion at *m/z* 208 in the EI MS was also indicative of a coumarin moiety with a methoxy group[407].

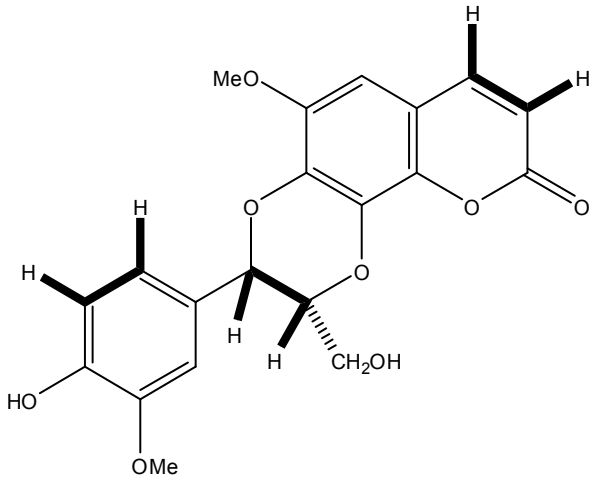


m/z 208^{+}

This compound was identified as cleomiscosin A, a coumarinolignan, and the spectroscopic data were in agreement with published literature[91, 414]. Further confirmation was obtained from melting point determination (246-250^{o} C)[410]. Unfortunately there was insufficient sample for optical rotation measurements so that absolute configuration was uncertain.



Key HMBC correlations of cleomiscosin A



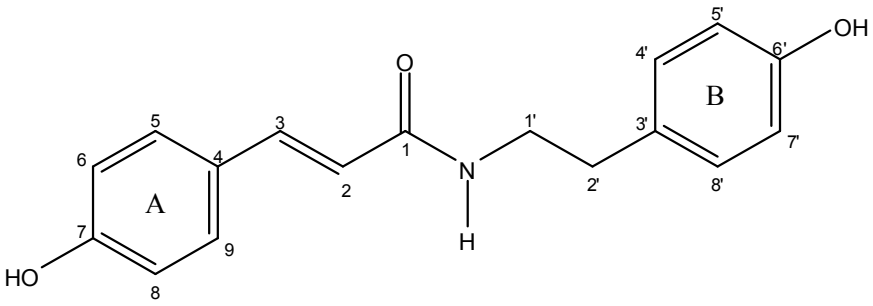
Key COSY correlations of cleomiscosin A

Table 5.2 NMR data of cleomiscosin A (5.1)*

Compound 5.1			
Position	δc	δ_H multiplicity	HMBC correlations
2	160		
3	113.5	6.26 *d J* = 9.5 Hz	C-2, 10
4	144.4	7.88 *d J* = 9.5 Hz	C-2, 5, 9, 10
5	100.8	6.84 *s*	C-6, 7, 9, 10
6	146.0		
7	137.8		
8	132.2		
9	138.7		
10	111.5		
1’	127.6		
2’	111.3	7.15 *d J* = 2.0 Hz	C-4’, 7’, 1’, 6’
3’	148.0		
4’	147.4		
5’	115.0	6.90 *d J* = 8.0 Hz	C-3’, 1’, 6’
6’	121.1	7.01 *dd J* = 8.0, 2.0 Hz	C-7’ 2’, 4’, 5’
7’	76.8	5.01 *d J* = 8.2 Hz	C- 7, 6’, 9’, 2’, 8’
8’	78.6	4.25 *ddd J* = 8.1, 3.0, 2.0 Hz	C-9’, 7’
9’	60.6	3.56 *dd*, 3.89 *dd* Hz, *J* = 12.0, 3.0 Hz	C-8’, C-7’
-OCH_3	56.4	3.83 *s*	C-3’
-OCH_3	56.5	3.87 *s*	C-6

recorded in *acetone-d6**

5.2.3.2 *N*-(*p-trans*-coumaroyl)tyramine (5.2)

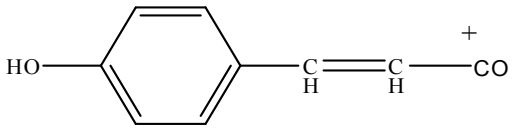


***N*-(*p-trans*-coumaroyl)tyramine**

Compound 5.2 was obtained as off-white crystals (7 mg, mp 238-241^{o}C). ESI MS revealed a protonated molecular ion (positive ion mode) at *m/z* 284 $[M+H]^+$, consistent with the molecular formula $C_{17}H_{17}NO_3$. The ^{13}C NMR data, along with HSQC and ^{1}H NMR data identified 17 carbon atoms (Table 5.2), in agreement with one carbonyl, two aromatic rings (8 methines and 4 quaternary carbons), two methine groups of a double bond, and two deshielded aliphatic carbons. The ^{1}H NMR spectrum (recorded in methanol-d4) confirmed the presence of eight aromatic protons, two methine protons of a *trans* double bond, and two deshielded aliphatic protons, and based on the proposed molecular mass implied the presence of two exchangeable protons.

The ^{1}H NMR spectrum identified two A_2B_2 spin systems accounting for the eight aromatic protons. Aromatic ring A showed signals at δ 7.41 (2H, *d*, *J* = 8.5 Hz, H-5, H-9) and 6.84 (2H, *d*, *J* = 8.5 Hz, H-6, H-8) and ring B at δ 7.06 (2H, *d*, *J* = 8.5 Hz, H-4', H-8') and 6.75 (2H, *d*, *J* = 8.5 Hz, H-5', H-7'), respectively. Both ring A and B were assigned as *para* disubstituted by COSY correlations. In the HMBC experiment, the protons at δ 7.41 (H-5, H-9) and 6.84 (H-6, H-8) showed correlations to C-4 at δc 127.0 and C-7 at 158.8, while the protons at δ 7.06 (H-4', H-8') and 6.75 (H-5', H-7') correlated with the quaternary carbons at δc 130.3 (C-3') and 156.0 (C-6'). The chemical shifts of the quaternary carbons at 158.8 and 156.0 ppm were consistent with oxygen substituents, and along with the A_2B_2 spin systems observed and biogenetically expected oxygenation at the *para* positions[350, 351], were consistent with the presence of two 4-hydroxyated aromatic rings.

The presence of a *trans* double bond was confirmed by the proton signals at δ 6.46 (*d*, *J* = 15.5 Hz, H-2) and 7.45 (*d*, *J* = 15.5 Hz, H-3), with the diagnostic large *trans* coupling, and their corresponding methine carbons at δc 118.8 and 139.2, respectively, in the HSQC spectrum. The chemical shifts of these proton and carbon signals were consistent with an α,β-unsaturated carbonyl and more specifically with a cinnamoyl moiety[415]. The linkage of this double bond to one of the aromatic rings was confirmed by HMBC correlations of the proton signal at δ 7.45 (H-3) with the aromatic methine carbon at δc 129.2 (C-5, C-9) and of δ 6.46 (H-2) with the quaternary carbon atom of the aromatic ring at δc 127.0 (C-4). It was further supported by the protons on this aromatic ring at δ 7.41 (H-5, H-9) exhibiting HMBC correlations with the double bond carbon at δc 118.8 (C-3). The connection of the double bond to a carbonyl was confirmed by HMBC correlations of both double bond protons to a carbonyl carbon at δc 165.5 (C-1). The chemical shift of this carbonyl was characteristic of an amide carbonyl group adjacent to double bond[416]. The connectivity of the double bond to the aromatic ring was indicative of the presence of a *p*-coumaroyl (*p*-hydroxycinnamoyl) moiety in the molecule. The *m/z* signal at 147 in the EI MS spectra was consistent with this observation[415].



m/z 147

A broad singlet at δ 7.20, without any correlation in the HSQC spectrum, suggested its connection to a heteroatom. This singlet however was correlated to the carbonyl carbon (δc 165.5) in the HMBC experiment. The uneven molecular weight and the chemical shift of the carbonyl supported the presence of an amide group.

The chemical shift of the aliphatic proton signals at δ 3.47 (*dd*, 2H, H-1') and 2.73 (*t*, 2H, H-2'), which corresponded to the carbon signals at δc 41.1 and 34.9 in the HSQC spectrum, respectively, were consistent with these methylene protons being adjacent to each other and next to a nitrogen and aromatic ring, respectively. The 'quartet' for the signal at δ 3.47 also implied coupling to the amide proton. Its connection to the amide nitrogen was confirmed by its HMBC correlation to the carbonyl (C-1), which was absent

for the other methylene protons. HMBC correlations were observed for both sets of methylene protons to the quaternary aromatic carbon at δc 130.3 (C-3'). The protons at δ 2.73 (H-2') also showed correlations with the aromatic carbons at δc 129.6 (C-4' and C-8'), indicating the attachment of this methylene group to the aromatic ring. The connectivity of the aromatic ring with the hydroxy group and aliphatic groups in the *para* positions, along with the amide proton, was consistent with a tyramine moiety[417]. The EI MS fragment ion at *m/z* 120 confirmed the presence of tyramine moiety in the molecule[416, 418].

Based on these observations and by comparison with reported spectral data, compound 5.2 was assigned as *N*-(*p*-*trans*-coumaroyl)tyramine[419, 420].

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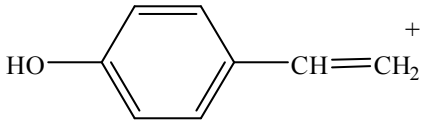
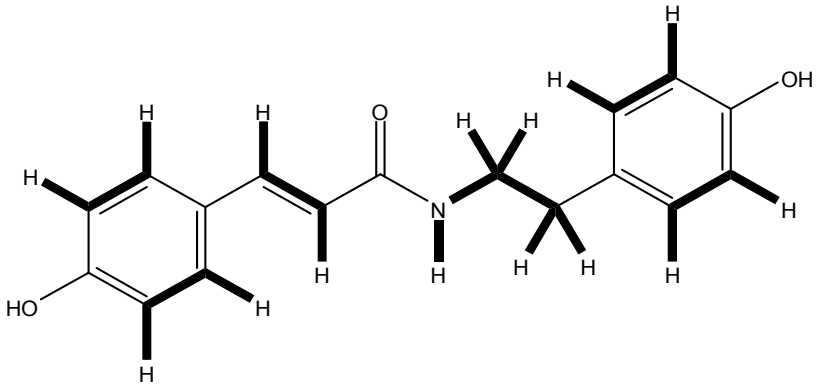
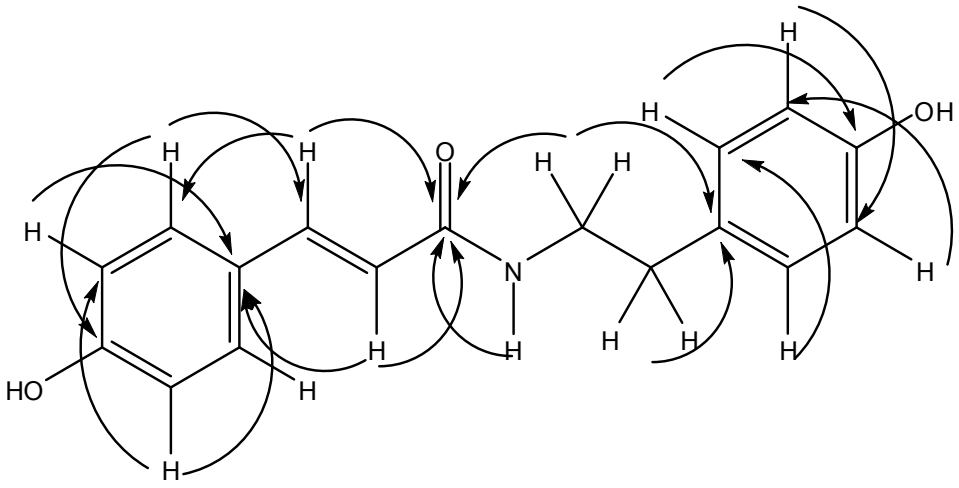


m/z 120



Key COSY correlations of *N*-(*p*-*trans*-coumaroyl)tyramine



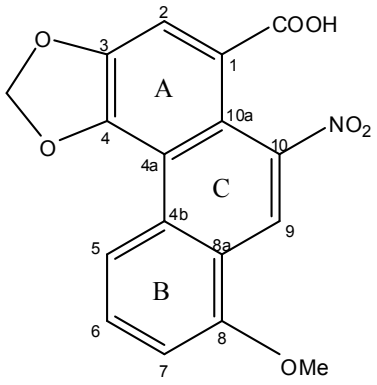
Key HMBC correlations of *N*-(*p-trans*-coumaroyl)tyramine

Table 5.3. NMR data of *N*-(*p-trans*-coumaroyl)tyramine*

Compound 5.2			
Position	δc	δ_H multiplicity	HMBC correlations
1	165.5		
2	118.8	6.46 *d J* = 15.5 Hz	C-4, 1, 3
3	139.2	7.45 *d J* = 15.5 Hz	C-1, 2, 5, 9
4	127.0		
5	129.2	7.41 *d J* = 8.5 Hz	C-3, 6, 7, 9
6	115.7	6.84 *d J* = 8.5 Hz	C-8, 4, 7
7	158.8		
8	115.7	6.84 *d J* = 8.5 Hz	C-6, 7, 4
9	129.2	7.41 *d J* = 8.5 Hz	C-5, 3, 7
1’	41.1	3.47 *dd J* = 7.2, 6.8	C-1, 2’, 3’
2’	34.9	2.73 *t J* = 7.3 Hz	C-1’, 3’, 4’ 8’
3’	130.3		
4’	129.6	7.06 *d J* = 8.5 Hz	C-8’, 6’
5’	115.2	6.75 *d J* = 8.5 Hz	C-7’, 3’, 6’
6’	156.0		
7’	115.2	6.75 *d J* = 8.5 Hz	C-5’, 6’, 3’
8’	129.6	7.06 *d J* = 8.5 Hz	C- 4’, 6’
-NH		7.20 *bs*	

*recorded in *methanol-d4*

5.2.3.3 Aristolochic acid 1 (5.3)



Aristolochic acid 1

Compound 5.3 was isolated as a yellow amorphous powder (1.5 mg, mp 264-268^{o}C). The ESI MS indicated a deprotonated molecular ion (negative ion mode) at *m/z* 340 $[M-H]^-$, consistent with the molecular formula $C_{17}H_{11}NO_7$. The ^{13}C NMR data, along with HSQC and ^{1}H NMR data, identified 17 carbon atoms (Table 5.2), consistent with one carbonyl carbon atom, three aromatic rings (5 methines and 9 quaternary carbons), one aromatic methoxy group, and one methylenedioxy carbon. The ^{1}H NMR spectrum (recorded in methanol-d4) confirmed the presence of five aromatic protons, one methoxy group and a methylenedioxy singlet proton. This suggested that the remaining proton expected based on the proposed molecular formula was an exchangeable heteroatom proton.

Ring B showed three proton signals at δ 8.73, (*d*, *J* = 8.3 Hz, H-5); 7.69 *(dd*, *J* = 8.3 Hz, H-6); 7.20, (*d*, *J* = 8.3 Hz, H-7) in agreement with a 1,2,3-trisubstituted aromatic ring. The proton at δ 8.73 (H-5) exhibited HMBC correlations with the aromatic carbons at δc 117.7 (quaternary, C-4a), 107.4 (methine, C-7) and 119.9 (quaternary, C-8a). The proton at δ 7.69 showed HMBC correlations with a quaternary carbon atom at δc 130.7 (C-4b) and an oxygenated carbon at δc 156.0 (C-8) and the proton at δ 7.20 (H-7) showed HMBC correlation to aromatic carbon atom at δc 119.9 (quaternary, C-8a) and 118.8 (methine carbon, C-5). These correlations indicated a trisubstituted fused aromatic ring system. The chemical shift of the quaternary carbon at δc 156.0 (C-8) was consistent with an oxygen substituent on the aromatic ring. The presence of a HMBC correlation for the methoxy proton signals at δ 4.09 with the carbon signal at δc 156.0 (C-8) confirmed the

methoxy group as the oxygen substituent on this ring. The large difference in the chemical shifts of the protons at δ 8.73 (H-5) and 7.20 (H-7) was attributed to the influence of the strong deshielding effect of a fused aromatic ring and the strong shielding effect of a methoxy group and confirmed the placement of the proton at δ 8.73 next to the fused ring and the proton at δ 7.20 next to the methoxy group[421].

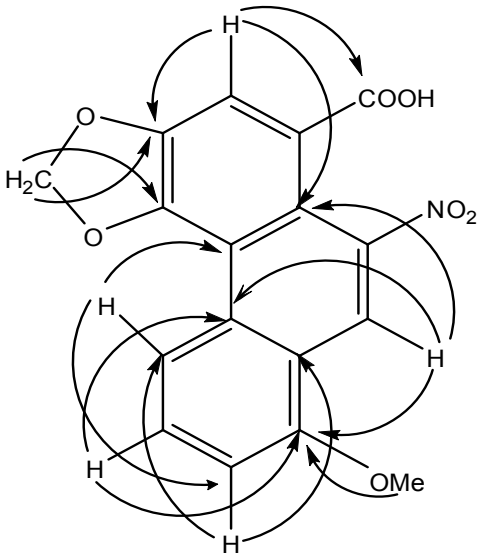
Ring C contained only one aromatic proton signal, as a singlet at δ 8.55. This corresponded to a carbon at δc 117.8 (C-9) in the HSQC spectrum. This proton exhibited HMBC correlations to the oxygenated carbon at δc 156.0 (C-8) and quaternary carbons at δc 130.7 (C-4b) and 116.8 (C-10a), indicating that H-8 was part of the fused aromatic ring. The low field signal of this proton (δ 8.55) indicated the presence of a strong deshielding group next to it[421]. This observation, along with the low field carbon value of δc 147.2 (C-10), indicated the presence of a nitro group at C-10. The presence of a fragment ion peak at *m/z* 295 in the EI MS spectrum (formed by the elimination of a nitro group) was in agreement with this observation.

Ring A contained only one proton singlet at δ 7.64, which showed a HSQC correlation to the carbon at δc 111.2. This proton exhibited HMBC correlations to the oxygenated carbon atoms at δc 146.2 (C-3) and 144.1(C-4). H-2 also showed a HMBC correlation to the quaternary carbon at δc 116.8 (C-10a), confirming that compound 5.3 is a phenanthrene derivative consisting of three fused aromatic rings. The proton signal at δ 7.64 (H-2) showed a correlation to the carbonyl signal at δc 171.0 characteristic of a carboxylic acid group. The formation of an ammonium adduct, as indicated by the ESI MS spectrum (*m/z* 359, $[M+NH_4]^+$), and the ease of formation of a deprotonated molecular ion in the ESI MS (negative mode), were consistent with a carboxylic acid.

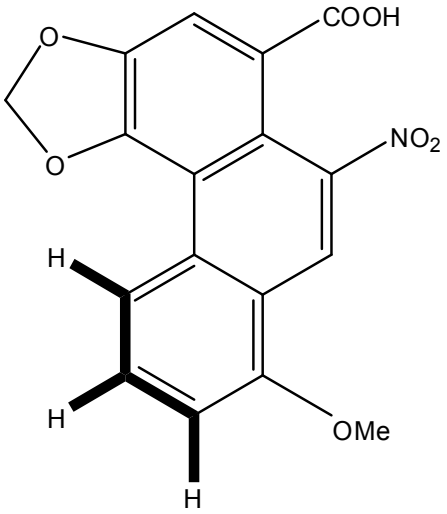
The two proton singlet at δ 6.31 and the corresponding carbon signal at δc 102.1 indicated a methylenedioxy group[421, 422]. HMBC correlations of the protons at δ 6.31 to two low field carbons at δ 146.2 (C-3) and 143.2 (C-4) and the absence of other long bond correlations confirmed the presence of the methylenedioxy group[421, 422]. These observations, along with the HMBC correlation of the proton at δ 7.64 (H-2) to the

carbon signals at δc 146.2 (C-3) and 143.2 (C-4), indicated that the methylenedioxy group was attached to the aromatic ring containing this proton (H-2).

EI MS also showed the presence of fragment ions at *m/z* 278 $(341 - NO_2 - CH_3)^+$ and 250 $(341 - NO_2 - CH_3 - CO)^+$. This compound was identified as aristolochic acid 1 and the spectral data were identical to the literature[422-424]. The 1D and 2D NMR data are presented in the Table 5.4.



Key HMBC correlations of aristolochic acid 1



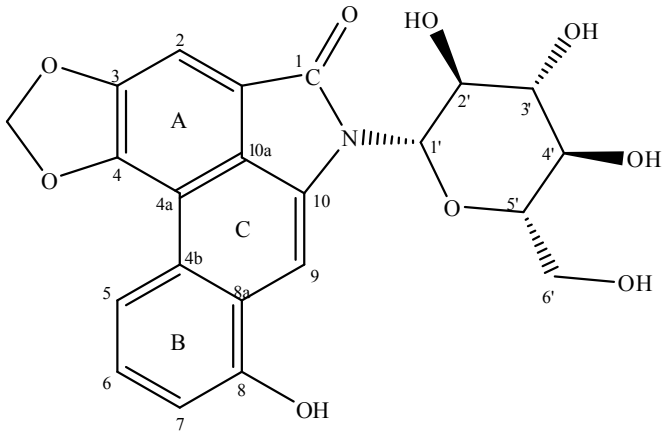
Key COSY correlations of aristolochic acid 1

Table 5.4 NMR data of Aristolochic acid 1*

Compound 5.3			
Position	δc	δ_H multiplicity	HMBC correlations
1	122.0		
2	111.2	7.64 *s*	C-3, 10a
3	146.2		
4	144.1		
4a	117.7		
4b	130.7		
5	118.8	8.73 *d J* = 8.3 Hz	C-4a, 7, 8a
6	129.8	7.69 *dd J* = 8.3, 8.3 Hz	C-4b, 8
7	107.4	7.20 *d J* = 8.3 Hz	C-5, 8a
8	156.0		
8a	119.9		
9	117.8	8.55 *s*	
10	147.2		
10a	116.8		
COOH	171.0		
3,4-CH_2O_2	102.1	6.31 *s*	C-3, 4
-OMe	55.1	4.09 *s*	C-80

recorded in *methanol-d4**

5.2.3.4 Aristolactam1a-*N*-*β*-D-glucoside (5.4)



Aristolactam1a-*N*-*β*-D-glucoside

Compound 5.4 was obtained as a yellow amorphous solid (1.0 mg, mp 275-278^{o}C) with an intense greenish yellow fluorescence visualised at 365 nm. ESI MS indicated a deprotonated molecular ion (negative ion mode) at *m/z* 440 ([M-H]$^{-}$), consistent with the molecular formula $C_{22}H_{19}NO_9$. The ^{13}C and HSQC NMR data identified 22 carbon atoms (Table 5.2), consistent with one amide carbonyl carbon, three aromatic rings (5 methines and 9 quaternary carbons), one methylenedioxy carbon and six sugar carbon atoms. The ^{1}H NMR spectrum (recorded in methanol-d4) confirmed the presence of five aromatic protons and two methylenedioxy protons and also contained seven protons in the region 5.57 and 3.56 ppm consistent with a sugar moeity. The ^{1}H NMR and ^{13}C NMR data for compound 5.4 possessed some similarities to the spectral data of aristolochic acid (5.3), along with signals consistent with a sugar moiety.

Ring B showed three aromatic proton signals at δ 8.17 (*d*, *J* = 8.0 Hz, H-5), 7.40 (dd *J* = 8.0, 8.0 Hz, H-6) and 7.04 (d, *J* = 8.0 Hz, H-7). The coupling patterns were consistent with the trisubstituted aromatic ring of aristolochic acid, although there were significant differences in the proton chemical shifts with all protons more upfield for compound 5.4. The aromatic protons showed HMBC correlations consistent with a fused aromatic ring similar to aristolochic acid. This included correlations for the proton at δ 8.17 (*d*, *J* = 8.0 Hz, H-5) to quaternary carbon atoms at δc 126.2 (4a) and 123.5 (8a) and a methine carbon at δc 112.0 (C-7); correlations to a quaternary carbon atom at δc 126.1 (4b) and

the oxygenated carbon at δc 154.8 (C-8) for the proton at δ 7.40 (H-6); and correlations to the quaternary carbon atoms at δc 117.6 (C-5) and 123.5 (8a) for the proton at δ 7.04 (H-6). The carbon signal at δc 154.8 indicated an oxygenated carbon atom. The absence of a methoxy signal suggested that this was a hydroxy substituent.

Ring C contained only one aromatic proton signal at δ 7.87 (H-9), which corresponded to the carbon at δc 102.5 (C-9) in the HSQC spectrum. This proton exhibited HMBC correlations to the oxygenated carbon at δc 154.8 (C-8) and quaternary carbons at 126.1 (C-4b) and 125.0 (C-10a), indicating that the proton was part of a fused aromatic ring. Its placement at H-9 was confirmed by the correlation with C-8. Its significantly upfield shift compared to aristolochic acid again supported the lack of a highly electron withdrawing group next to this proton. The chemical shift was however downfield to that expected for a simple aromatic proton on a fused aromatic ring and its shift was consistent with the presence of a phenolic group at C-8 leading to *peri* interactions[425].

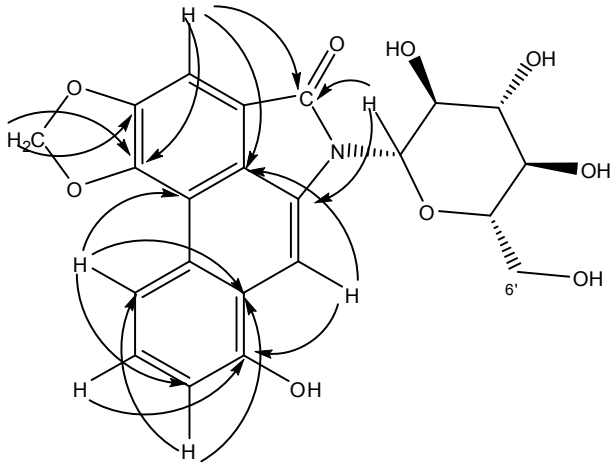
Ring B contained only one proton singlet at δ 7.62 (H-2), which showed correlation to the aromatic carbon signal at δc 105.0 in the HSQC spectrum. This proton showed HMBC correlations to the methylenedioxy carbons at δc 149.1 (C-3) and 148.3 (C-4), to the carbonyl carbon at δc 168.0 (C-1) and to a quaternary carbon at 125.0 (C-10a). The carbon shift at δc 168.0 is characteristic of a carbonyl carbon of an amide. The molecule was therefore identified as a phenanthrene derivative with a methylenedioxy and an amide group attached.

Similar to aristolochic acid, a two proton singlet at δ 6.42, with the corresponding ^{13}C signal at δc 103.3 was present, indicative of a methylenedioxy group[425]. The HMBC correlation of these protons to two low field quaternary carbons at δc 149.1 (C-3) and 148.3 (C-4) confirmed this observation.

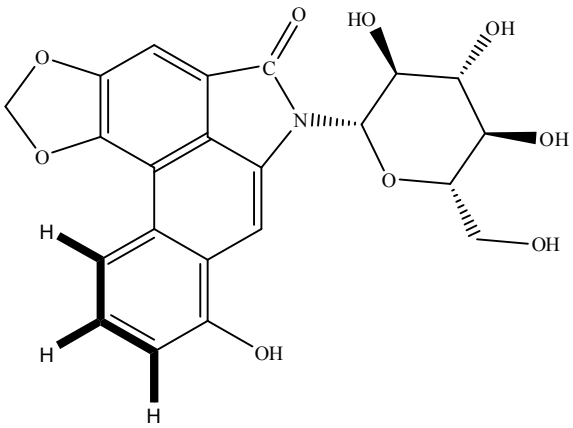
The carbonyl carbon (δc 168.0) exhibited a difference in chemical shift in comparison to the carbonyl carbon of aristolochic acid (5.3) (δc 171.0). This shift difference is an elegant method to distinguish between aristolochic acids and aristolactams[426]. The carbonyl carbon of aristolactams usually resonates with an upfield shift compared to the

carbonyl carbon of aristolochic acid[426]. These observations were consistent with the presence of an aristolactam moiety in the molecule.

The presence of six non-exchangeable protons was consistent with a sugar moiety. The anomeric proton with a doublet signal at δ 5.57 (*d*, 1H, *J* = 9.3 Hz, H-1') corresponded to the carbon signal at δc 82.3 in the HSQC spectrum. Both the anomeric proton chemical shift at δ 5.57 and vicinal coupling constant supported the *N*-*β*-glycosidic linkage of the sugar moiety[427,428]. This was further substantiated by the ^{13}C chemical shift of δc 82.3, which is indicative of its attachment to a nitrogen atom[427, 429]. The proton signals at δ 4.27 (H-2'), 3.58 (H-3'), 3.56 (H-4'), 3.56 (H-5'), 3.75 (H-6a) and 3.94 (H-6b) correlated to the carbon signals at δ 70.4 (C-2'), 77.8 (C-3'), 70.1 (C-4'), 79.9 (C-5') and 61.7 (C-6') respectively, in the HSQC spectrum, confirming the presence of a glucose moiety[429]. The anomeric proton also showed HMBC correlations to the amide carbonyl carbon at δc 168.0 (C-1) and to 132 (C-10). These correlations confirmed that the molecule was an *N*-*β*-glucoside. Based on these observations and by comparison of the spectral data with the literature, compound 5.2 was identified as aristolactam1a-*N*-*β*-D-glucoside[428-431]. Unfortunately there was insufficient sample for optical rotation measurements so that absolute configuration was uncertain.



Key HMBC correlations of aristolactam1a-*N*-*β*-D-glucoside

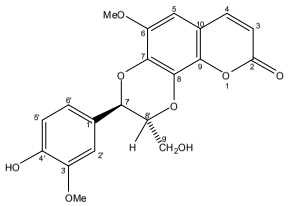
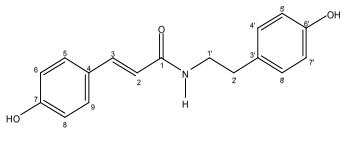
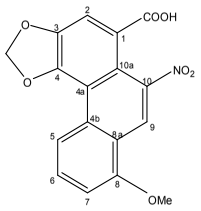
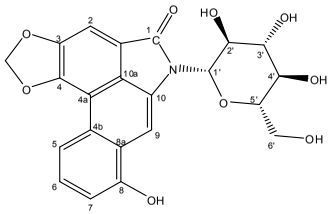


Key COSY correlations of aristolactam1a-*N*-*β*-D-glucoside

Table 5.5 NMR data of Aristolactam1a-*N*-*β*-D-glucoside (5.4)*

Compound 5.4			
Position	δc	δ_H multiplicity	HMBC correlations
1	168.0		
2	105.0	7.62 *s*	C-3, 4, 1, 10a
3	149.1		
4	148.3		
4a	126.2		
4b	126.1		
5	117.6	8.17 *d J* = 8.0 Hz	C- 4a, 7, 8a
6	126.1	7.40 *dd J* = 8.0, 8.0 Hz	C-4b, 8
7	112.0	7.04 *d J* = 8.0 Hz	C-5, 8a
8	154.8		
8a	123.5		
9	102.5	7.87*s*	
10	132.0		
10a	125.0		
10b	132.0		
3,4-CH_2O_2	103.3	6.42 *s*	C-3, 4
1’	82.3	5.57 *d J* = 9.3 Hz	
2’	70.4	4.27 *d J* = 8.9 Hz	
3’	77.8	3.58 *m*	
4’	70.1	3.56 *m*	
5’	79.9	3.56 *m*	
6’	61.7	3.75 *dd J* = 12.0 Hz, 5.6 Hz 3.94 *dd J* = 12.0 Hz, 1.5 Hz	

*recorded in *methanol-d4*

Position	 Cleomiscosin A	 *N*- (*p-trans*-coumaroyl)tyramine	 Aristolochic acid 1	 Aristolactam1a-*N*-*β*-D-glucoside
	Acetone-d6 (C_5D_5N)[410]	MeOD (MeOD)[420]	MeOD (DMSO-d6)[432, 433]	MeOD (DMSO-d6)[429]
		165.5 (165.6)	122.0 (121.9)	168.0
2	160.0 (160.8)	118.8 (119.2)	111.2 (112.2)	105.0 (105.6)
3	113.5 (113.9)	139.2 (139.3)	146.2 (146.0)	149.1 (148.2)
4	144.4 (144.5)	127.0 (127.2)	144.1 (145.9)	148.3
4a			117.7 (116.7)	126.2
4b			130.7 (129.9)	126.1(125.2)
5	100.8 (101.2)	129.2 (129.4)	118.8 (118.4)	117.6 (117.0)
6	146.0 (146.5)	115.7 (115.4)	129.8 (131.4)	126.1 (126.1)
7	137.8 (138.5)	158.8 (159.0)	107.4 (108.7)	112.0 (112.2)
8	132.2 (133.1)	115.7 (115.4)	156.0 (156.2)	154.8 (154.2)
8a			119.9 (119.3)	123.5 (122.6)
9	138.7 (139.4)	129.2 (129.4)	117.8 (118.9)	102.5 (100.8)
10	111.5 (111.9)		147.2 (145.9)	132.0 (133.1)
10a			116.8 (117.2)	125.0 (124.1)
10b				132.0
1'	127.6 (127.6)	41.1 (41.3)		82.3 (81.6)
2'	111.3 (112.3)	34.9 (34.7)		70.4 (69.5)
3'	148.0 (148.9)	130.3 (129.6)		77.8 (77.0)
4'	148.6 (149.0)	129.6 (129.4)		70.1 (69.5)
5'	114.7 (116.6)	115.2 (115.5)		79.9 (80.0)
6'	121.1 (121.7)	156.0 (156.0)		61.7 (60.8)
7'	76.8 (77.6)	115.2 (115.5)		
8'	78.6 (79.9)	129.6 (129.4)		
9'	60.6 (60.7)			
3'-OCH_3	56.5 (56.2)		55.1 (57.2)	
6-OCH_3	56.4 (55.8)			
COOH			171.0 (169.1)	
3,4-CH_2O_2			102.1 (103.8)	103.0

5.2.4 Antiproliferative activity of isolated compounds

The pure compounds isolated from *C. fimbriata*, cleomiscosin A, *N*-(*p-trans*-coumaroyl)tyramine, aristolochic acid 1 and aristolactam1a-*N*-*β*-D-glucoside were examined for their antiproliferative activities against the SKNMC (neuroblastoma), MCF 7 (breast adenocarcinoma) and MRC 5 (normal) cell lines. Of the four compounds, aristolochic acid 1 was found to be the most active compound exhibiting IC_{50} values of 3, 10 and 5 µg/ml against the SKNMC, MCF 7 and MRC 5 cell lines, respectively (Table 5.6). Comparison of the IC_{50} values of aristolochic acid 1 against the SKNMC and MRC 5 cell lines indicated a selective mode of activity against the neuroblastoma cell line. Cleomiscosin A also exhibited significant antiproliferative activity against the cell lines tested, with IC_{50} values of 11 and 21 µg/ml against the SKNMC and MCF 7 cell lines, respectively. *N*-(*p-trans*-coumaroyl)tyramine showed moderate activity against the SKNMC cell line (IC_{50} of 40 µg/ml) and was inactive against the MCF 7 cell line. Aristolactam1a-*N*-*β*-D-glucoside showed weak activity against the SKNMC cell line (IC_{50} of 75 µg/ml) and was inactive against the MCF 7 cell line. Due to low yield, aristolactam1a-*N*-*β*-D-glucoside was not tested against the MRC 5 cell line. The IC_{50} of doxorubicin, the positive control, was determined as 0.06, 1.2 and 2.1 µg/ml against the SKNMC, MCF 7 and MRC 5 cell lines, respectively. It is noteworthy that doxorubicin, a recognised anticancer drug is also considerably active against the normal cell line, showing poor selectivity.

Table 5.6 Antiproliferative activity of isolated molecules

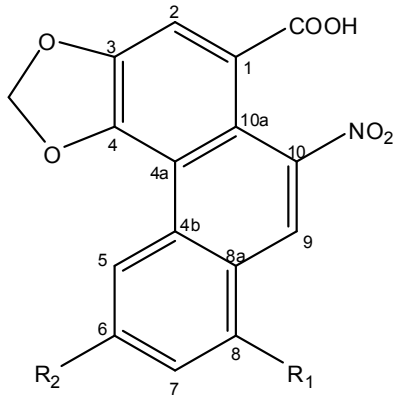
Compound tested	**IC_{50} values in µg/ml**		
	SKNMC	MCF 7	MRC 5
cleomiscosin A (5.1)	11	21	9
N-(*p-trans*-coumaroyl)tyramine (5.2)	40	na	na
aristolochic acid (5.3)	3	10	5
aristolactam1a-*N*-*β*-D-glucoside (5.4)	75	na	nt
doxorubicin (positive control)	0.06	1.2	2.1

Assay was performed once, with the samples tested in duplicates, na: not active, nt: not tested

The anticancer activity of aristolochic acid 1 has been previously reported. Aristolochic acid was isolated as the major antitumour principle from the 95% ethanolic extract of

roots of *Aristolochia indica*, which demonstrated reproducible tumour inhibitory activity against adenocarcinoma in mice[434]. Aristolochic acid 1 and its derivatives are the major characteristic compounds of plants belonging to the genus *Aristolochia* and it is interesting to note that plants belonging to this genus have been used in the treatment of cancers since the Graeco-Roman period[434]. Aristolochic acid 1 has been found to be active *in vitro* with the P-388 lymphocytic leukemia system and with the NSCLNC6 (bronchial epidermoid carcinoma) cell line[435]. Of all the compounds isolated from *A. fangchi* following a bioassay guided study, aristolochic acid 1 was found to be the most cytotoxic, with an IC_{50} of 3.7 μg/ml against the LLC-PK_1 (porcine kidney epithelial) cell line[436].

Arsitolochic acids are structurally related nitrophenanthrene carboxylic acids (Fig 5.3). The most common aristolochic acids are aristolochic acid 1 and aristolochic acid II. One of the unique and unusual characteristic features of aristolochic acids is the presence of a nitro group. There are very few naturally occurring nitro compounds reported. These include chloramophenicol, *β*-nitropropionic acid and 1-amino-2-nitrocyclopentane carboxylic acid. Structure activity relationship studies have shown that the potent cytotoxicity of aristolochic acid 1 is due to the presence of the nitro group at C-10 and the methoxy group at the C-8 position[436, 437]. Any modification on the structure of aristolochic acid 1, such as addition, deletion, substitution or replacement of the position of these functional groups drastically reduces the cytotoxicity[438].



	R_1	R_2
Aristolochic acid 1	OMe	H
Aristolochic acid 1a	H	OH
Aristolochic acid 11	H	H
Aristolochic acid III	OMe	H
Aristolochic acid IIIa	OH	H
Aristolochic acid IV	OMe	OMe
Aristolochic acid IVa	OH	OMe

Fig 5.3 Structure of aristolochic acids

Other reported activities of aristolochic acid 1 include antiinflammatory, antimicrobial, antimalarial and herbicidal activities[439, 440]. Aristolochic acid 1 was widely used in various antiinflammatory drug formulations until the 1980's[438].

Cleomiscosin A was first isolated from the seeds of *Cleome viscosa*[412]. It has been shown to be active against P-388 lymphocytic leukemia cells with an effective dose (ED_{50}) of 3.8 μg/ml[412, 414]. Cleomiscosin A has also been reported to possess antihepatotoxic, antioxidant and immunomodulatory activities[230, 441].

N-(*p*-*trans*-coumaroyl)tyramine was found to be moderately active against the SKNMC cell line. It has previously been reported for its inhibitory effect on platelet aggregation, which is a contributing factor to thrombosis and arteriosclerosis[419].

Aristolactam1a-*N*-*β*-D-glucoside showed weak activity against the SKNMC cell line but was inactive against the MCF 7 cell line. It is noteworthy that aristolactams (phenanthrene lactam alkaloids) have recently received attention due to an interesting

array of biological properties including cytotoxic, antiinflammatory, antiplatelet, antimycobacterial and neuroprotective activities[442, 443]. Aristolactams have also been reported for treating neurological disorders, including Parkinson's disease and, Alzheimer disease and impotence[444].

The antiproliferative activity of extracts of *C. fimbriata* has been demonstrated for the first time in this study. This is the first time that cleomiscosin A, *N*-(*p-trans*-coumaroyl)tyramine, aristolactam1a-*N*-*β*-D glucoside and aristolochic acid 1 have been reported from the Asclepiadaceae family. Isolation of the bioactive molecules from *C. fimbriata* supports the use of this plant in Siddha medicine for treating cancer related conditions.

Aristolochic acid 1 and its derivatives are characteristic of *Aristolochia* species and are seldom reported from plants outside the Aristolochiaceae family. Previous reports of aristolochic acid 1 and its derivatives from plants beyond the Aristolochiaceae family are from *Antidesma pentandrum* (Euphorbiaceae family), *Stephania cepharantha* (Menispermaceae) and *Shefferomitra subaequalis* (Annonaceae)[445, 446].

5.2.5 Nephrotoxicity of aristolochic acid 1 and concerns

Aristolochic acid 1 has been associated with aristolochic acid nephropathy (also known as Chinese herb nephropathy (CHN)), a progressive form of renal interstitial fibrosis that may subsequently lead to urothelial cancer[447, 448]. Acute tubular necrosis and renal failure have also been reported in rats and mice administered with aristolochic acid 1[448]. The first report of CHN came from Belgium where around 100 people who had attended a weight-loss program, which included the use of a regimen containing Chinese herbs, were diagnosed with interstitial fibrosis and renal failure[437]. About 3-5% of the population who got exposed to this weight loss treatment were diagnosed with CHN. The observed nephrotoxicity has been traced to the ingestion of *Aristolochia fangchi* containing aristolochic acids unintentionally included in weight loss pills[449]. Later, aristolochic acid 1 and aristolochic acid II were reported to be genotoxic mutagens, forming DNA adducts after metabolic activation through simple reduction of the nitro group[449]. The metabolic activation has been reported to be mediated through a cyclic

nitrenium ion which forms purine adducts bound to the exocyclic amino groups of deoxyadenosine and deoxygaunosine (Fig 5.3)[447]. The aristolochic acid 1 adduct is highly persistent and is a mutagenic lesion leading to AT→TA transversions[447, 449]. The International Agency for Research on Cancer has classified herbal remedies that contain a high level of aristolochic acids as carcinogenic in humans[450]. Pharmaceutical preparations containing aristolochic acids have been withdrawn from the market in many countries and countries like USA, Australia, Singapore and many European countries have banned herbal products known to contain aristolochic acids[449]. A recent study carried out on human subjects has reported that a cumulative consumption of more than 150 mg of aristolochic acid 1 is associated with an increased risk of urinary tract cancer[450].

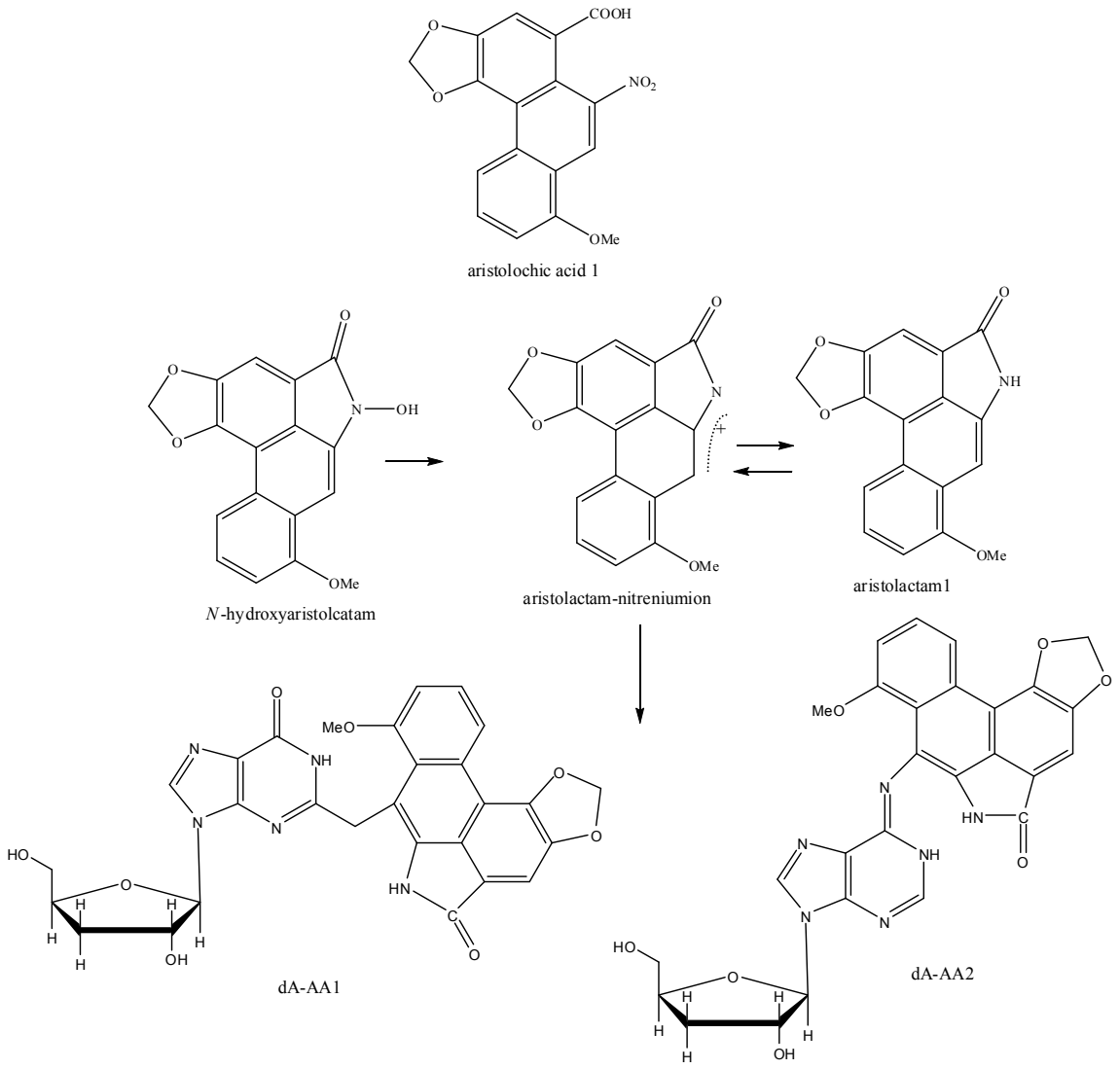


Fig 5.4 Metabolic activation and DNA adduct formation of aristolochic acid I,7-(deoxyadenosin-N^6-yl)aristolactam I (dA-AAI), 7-(deoxyguanosin-N^2-yl)aristolactam I (dG-AAI).

C. fimbriata is now widely being used as a dietary supplement agent, especially in western countries. The leaves also have application as a vegetable in some parts of south India. The finding of the presence of aristolochic acid 1 in *C. fimbriata* is a health concern in this regard. Many health supplements are formulations consisting only of *C. fimbriata* extracts and generally very large doses of ~500 mg/ day (product information on Caralluma actives, www.carallumaactives.org, accessed on 30/09/10) are consumed.

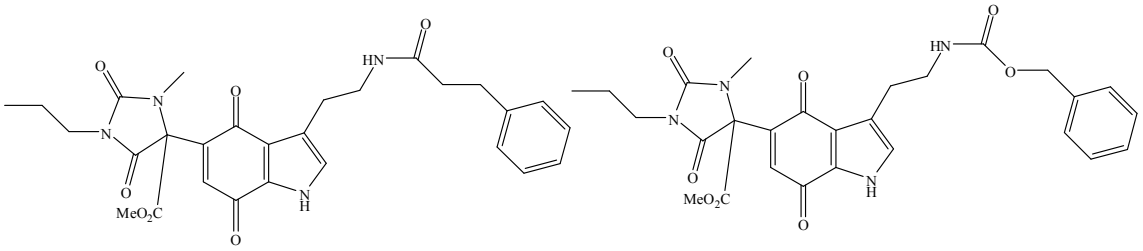
HPLC quantification was therefore carried out to determine the amount of aristolochic acid 1 in the methanolic extract of *C. fimbriata* (methanol was used as the extraction solvent to be consistent with a reported quantification method[456]). A previously reported method involving reversed phase C_{18} HPLC chromatography with a gradient elution of sodium acetate and acetonitrile was followed for the analysis[451]. This method has been shown to be a rapid and reliable method for the routine analysis of aristolochic acids and derivatives[451]. The methanolic extract of C. *fimbriata* was found to contain 1.3 x 10^{-2}% (*w/w*) of aristolochic acid 1. This is approximately 1/20th of the amount of aristolochic acid 1 found in *Aristolochia fangchi,* the plant responsible for CHN. This is a very low quantity and an average consumption of 500 mg/day for three years would be required to get exposed to 150 mg of aristolochic acid. However, proper toxicity studies need to be carried out on the formulations containing *C. fimbriata.*

A HPLC study was carried out to identify the presence of aristolochic acid 1 in a commercial dietary supplement prepared using *C. fimbriata*. Caralluma Actives, a commercially available product marketed through the internet was purchased from the USA. The formulation was in the form of capsules and contained 500 mg of 'Slimaluma' (a registered *C. fimbriata* extract, as per the manufacturers claim). A methanolic extract of the capsules was analysed for aristolochic acid 1 by HPLC. The extraction process and HPLC method were identical to that carried out for *C. fimbriata*. However the presence of aristolochic acid was not detected in the methanolic extract. It is possible that a different method of extraction or sample processing is followed in preparing the capsules or the *C. fimbriata* used for these capsules does not contain aristolochic acid. Due to time constraints we were unable to test more commercial products. This study, however,

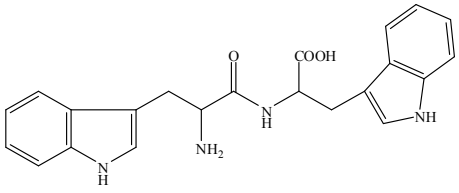
highlights the importance of monitoring herbal drugs for their safety/toxicity by drug safety authorities.

5.2.6 Indoleamine 2,3-dioxygenase (IDO) inhibitory activity of *N*-(*p-trans*-coumaroyl)tyramine

IDO has emerged as an attractive drug target for the treatment of cancer[452]. Recently molecules with some structural similarities to *N*-(*p-trans*-coumaroyl)tyramine and possessing potent IDO inhibitory activity have been isolated from natural sources[453]. These include analogues of exiguamine A, a hexacyclic alkaloid isolated from a marine sponge *Neopetrosia exigua* [454] and L-tryptophyl-L-tryptophan[455] (Fig 5.3). These possessed some structural smilarities to *N*-(*p-trans*-coumaroyl)tyramine, with each compound containing aromatic rings on either side of an amide linkage. The preliminary IDO screening studies described in Chapter 3 identified the inhibitory activity (76% inhibition at 100 µg/ml) of the 70% ethanolic extract of *C. fimbriata*. Given the structural similarities with IDO inhibitors, *N*-(*p-trans*-coumaroyl)tyramine was therefore screened for IDO inhibitory activity using the absorbance assay[310].



IDO inhibitory analogues of exiguamine A



L-tryptophyl-L-tryptophan

N-(*p-trans*-coumaroyl)tyramine exhibited micromolar levels of inhibitory activity, with an IC_{50} value of 0.14 mM (39 µg/ml). This was comparable to the known IDO inhibitor 1-methyl-L-tryptophan (IC_{50} value of 0.11 mM (24 µg/ml) that was used as the positive control. The preliminary IDO screening studies described in Chapter 3 identified the

inhibitory activity (76% inhibition at 100 µg/ml) of the 70% ethanolic extract of *C. fimbriata* (Section 3.5.4). *N*-(*p-trans*-coumaroyl)tyramine is abundantly available from natural sources and is also relatively easy to synthesise as are it analogues. This finding of inhibitory activity therefore opens the possibility for future structure activity relationship studies.

5.3 Conclusion

The traditional Siddha medicinal knowledge on *C. fimbriata* was used as a guide for a targeted chemical and biological investigation of this plant. The antiproliferative activities of the extracts of *C. fimbriata* have been demonstrated for the first time in this study. The bioactive compounds, cleomiscosin A, *N*-(*p-trans*-coumaroyl)tyramine, aristolochic acid 1 and aristolactam1a-*N*-*β*-D-glucoside were isolated from the fractions that showed significant antiproliferative activity. This is the first time that these compounds have been isolated from *C. fimbriata*. *N*-(*p-trans*-coumaroyl)tyramine additionally exhibited good IDO inhibitory activity. These research findings provide strong support for the use of *C. fimbriata* in Siddha medicine for anticancer treatments. For the first time aristolochic acid and its derivatives are reported from the family of Ascelpedeaceae. However, the presence of aristolochic acid and its derivatives in this plant is a matter of concern and further toxicity studies to ensure the safety of this plant is essential.

5.4 Experimental

5.4.1 Reagents and equipment

Normal phase column chromatography was performed using silica gel 60 (0.040-0.063 mm) from Merck (Germany). Analytical normal phase thin layer chromatography (TLC) was performed on fluorescent Merck silica gel F_{254} plates (Germany). Size exclusion chromatography was carried out using LH20100 Sephadex LH 20. TLC plates were visualised using UV light (254 nm and 365 nm) and with the vanillin-sulfuric acid stain[456]. Aristolochic acid and vanillin were purchased from Sigma Aldrich and Fluka respectively.

5.4.2 Vanillin-Sulfuric acid reagent

1% solution of vanillin was prepared in concentrated sulfuric acid. The plates were dipped in the reagent and heated at 100^{o}C[456].

5.4.3 Aristolochic acid standard

1 mg of standard aristolochic acid 1 was dissolved in 1 ml of methanol to prepare the stock solution.

5.4.4 Preparation of *Caralluma fimbriata* plant material

One kilogram of powdered leaves (1 kg) was extracted successively with 70% aqueous ethanol (3 x 1.5 l, 8 hr each) at 40-50^{o}C and the ethanol was evaporated, leaving the aqueous extract. The aqueous extract was freeze dried to obtain the crude extract (82 g, brownish green solid). A portion (70 g) was suspended in water, which was then partitioned with *n*-hexane (3 x 0.5 l), ethyl acetate (4 x 0.5 l) and *n*-butanol (3 x 0.5 l) to afford four partitions. Each partition was concentrated to dryness under reduced pressure and dried under high vacuum to afford their corresponding partitions as Cf-Hex (17.2 g, 24.5% *w/w*, green gum), Cf-EtOAc (16.1 g, 23% *w/w*, green partial solid), Cf-BuOH (20.5 g, 29% *w/w*, greenish brown sticky solid) and Cf-Water (11.5 g, 16.4% *w/w*, brown solid).

5.4.5 Fractionation of ethyl acetate partition (Cf-EtOAc)

The ethyl acetate partition (15 g) was applied to a flash silica gel column. The column was developed with a solvent gradient of chloroform:methanol in order of increasing polarity (10:0 (1 l), 8:2 (500 ml), 7:3 (500 ml), 6:4 (800 ml), 5:5 (1 L) and 4:6 (500 ml)) and 41 fractions (120 ml each) were collected. Similar fractions were pooled together according to the TLC profile (TLC plate was developed in chloroform:methanol, 9:1 and 7:3, visualising under UV 254 and 365 nm and derivatising with vanillin-sulfuric acid reagent). This afforded Cf-EtOAc-1 (4.1 g, 27% *w/w*, green sticky gum), Cf-EtOAc-2 (0.8 g, 5.3% *w/w*, green sticky gum), Cf-EtOAc-3 (4.6 g, 30% *w/w*, light yellow semi-solid), Cf-EtOAc-4 (1.7g, 11% *w/w*, yellow solid), Cf-EtOAc-5 (0.7 g, 4.6% *w/w*, yellow

solid), Cf-EtOAc-6 (1.8g, 12% *w/w*, light yellow solid) and Cf-EtOAc-7 (0.7 g, 4.6% *w/w*, yellow brown solid).

TLC analysis of Cf-EtOAc-6, eluting with 7:3 chloroform and methanol showed two major constituents with R_f values 0.62 (intense blue fluorescence at 365 nm) and 0.28 (quenching at UV 254), respectively. These two compounds were isolated using silica gel column chromatography of Cf-EtOAc-6 (1.2 g, dissolved in chloroform with a few drops of methanol), eluting with a solvent gradient of chloroform:methanol in order of increasing polarity (10:0 to 9:1) to give cleomiscosin A (5.1) and *N*-(*p-trans*-coumaroyl)tyramine (5.2).

Cleomiscosin A was obtained as a white paste (amorphous) and was further purified by repeated silica gel column chromatography eluting with a solvent gradient of chloroform:methanol in order of increasing polarity (10:0 to 9:1). Cleomiscosin A was recrystallised in acetone. *N*-(*p-trans*-coumaroyl)tyramine was obtained as off-white crystals and was further purified by recrystallisation in methanol.

5.1 Cleomiscosin A (7.0 mg, ESI MS *m/z* 387 $[M+H]^+$, mp 246-250^oC (lit. 251-253^oC[88]), EI MS *m/z* 208, 137, 1D and 2D NMR data in Table 5.2)

5.2 *N*-(*p-trans*-coumaroyl)tyramine (7.0 mg, ESI MS *m/z* 284 $[M+H]^+$, mp 238-241^oC, (lit. 240-245^o C[418]), EI MS *m/z* 283, 164, 120, 147, 1D and 2D NMR data in Table 5.3)

Cf-EtOAc-7 (500 mg) was applied to a size exclusion gel column (Sephadex LH 20, packed in methanol) and was eluted with methanol. 20 ml fractions were collected and the fractions were monitored by normal phase silica gel TLC developed with 4:6 chloroform and methanol, followed by visualisation under UV 365 nm. The eluted fractions consisted of two major constituents, identified as aristolochic acid 1 and aristolactam1a *N-β*-D glucoside. Both compounds were obtained as amorphous yellow solids exhibiting intense yellowish green fluorescence under UV 365 nm. Further size exclusion chromatography on Sephadex by elution with methanol was repeatedly carried out to purify the isolated compounds.

5.3 Aristolochic acid 1 (1.5 mg, ESI MS *m/z* 359 $[M+NH_4]^{+1}$, mp 269-272^oC (lit. 274^o C[457]), EI MS *m/z* 295, 278, 251, 1D and 2D NMR data in Table 5.4)

5.4 Aristolactam1a-*N*-*β*-D-glucoside (1 mg, ESI MS, *m/z* 440 $[M-H]^-$, 1D and 2D NMR data in Table 5.5)

5.4.6 MTT assay

The MTT assay was carried out by following the method as explained in Chapter 3 (Section 3.7.5). Sample solutions were prepared in DMSO and tested at concentrations of 1-100 µg/ml. Doxorubicin was used as the positive control. The assays were performed against the SKNMC, MCF 7 and MRC 5 cell lines.

5.4.7 HPLC quantification of aristolochic acid 1

5.4.7.1 Preparation of methanolic extract of *C. fimbriata*

The powder of *C. fimbriata* (10 g) was refluxed with 30 ml of 75% methanol (40-50^o C) for one hour. The extraction process was repeated three times, each time with fresh solvent. The combined extract, after filtration, was evaporated to dryness. The residue was dissolved in HPLC grade methanol to prepare a stock solution of 1 mg/ml[451].

5.4.7.2 Preparation of methanolic extract of Caralluma actives

15 g of Caralluma actives powder was extracted in 50 ml methanol following the process explained in Section 5.4.7.1[451].

5.4.7.3 Plotting standard curve for aristolochic acid 1 by HPLC Method

1 mg of standard aristolochic acid 1 was dissolved in 1 ml methanol to prepare the stock solution. Concentrations of 10, 25, 50, 80 and 100 µg/ml were injected and chromatographed under conditions described in Section 5.4.7.4. A calibration curve of aristolochic acid 1 was prepared by plotting peak areas *vs* concentration.

5.4.7.4 HPLC analysis

HPLC analysis was carried out using a Shimadzu HPLC system consisting of a LC-10 AVP pump with a SPD M10AVP photodiode array detector and an autosampler.

Chromatographic separations were performed at ambient temperature (23–25^{o}C) on a Waters 4.6×150 mm (particle size - 3.5 μm) SunFire C_{18} column. The mobile phase consisted of 0.01 M sodium acetate buffer at pH 5.0 (mobile phase A) and acetonitrile (mobile phase B) eluted in a gradient mode. The gradient elution started with 20% B for 5 minutes. The percentage of mobile phase B was increased to 44% at 33 minutes, 50% at 43 minutes, then to 68% at 53 minutes and finally to 80% at 59 minutes. The total run time was 60 minutes at a flow rate of 1.0 ml/min. A retention time of 19.7 minutes was obtained for aristolochic acid 1. The mass (% *w/w*) of aristolochic acid 1 was calculated by analysing the area under the peak of aristolochic acid 1 in the sample and using the linear regression equation derived from the calibration curve.

Chapter 6

Conclusions and Future Directions

This PhD study was based on a collaborative research partnership between Macquarie University (IBRG) and Dr Velmurugan, a practitioner of Siddha traditional medicine. Dr Velmurugan has identified, through his clinical experience, some plants for the treatment of cancer, pain and swelling. Based on a collaborative agreement, the names of 24 such plants were provided.

A broad literature study was carried out on these plants. For some of these plants, the available literature provided scientific evidence for their anticancer activity and/or antiinflammatory activity, thus supporting the use of these plants by Dr Velmurugan for treatment of cancers and/or pain and swelling. The literature search also identified that some of the plants in the list have not been studied biologically or chemically in line with Dr Velmurugan's usage of the plants. These plants were identified as potentially promising plants that are worth considering for further research to identify anticancer and antiinflammatory molecules.

Plants identified as promising candidates	**Potential bioactivity**
Abutilon indicum	Cancer treatment
Azima tetracantha	Pain and swelling
Borreria hispida	Cancer treatment
Caralluma fimbriata	Cancer treatment
Cardiospermum halicacabum	Cancer treatment
Cleome viscose	Cancer treatment
Crateava religiosa	Cancer treatment
Evolvulus alsinoides	Cancer treatment
Holarrhena antidysenterica	Pain and swelling
Marsdenia tinctoria	Cancer treatment
Phyllanthus urinaria	Pain and swelling
Poinciana elata	Pain and swelling
Toddalia asiatica	Pain and swelling
Trichodesma indicum	Cancer treatment

Antiproliferative screening with SKNMC cell lines was performed on 70% ethanolic extracts of the nine plants shortlisted as potential anticancer candidates. The highest antiproliferative activities were exhibited by *C. halicacabum* and *C. fimbriata*, with IC_{50} values of 22.0 ± 3.2 and 17.1 ± 1.1 µg/ml respectively. The activities exhibited by the rest of the plants were not significant even at a concentration of 100 µg/ml. Based on the antiproliferative screening results, *C. halicacabum* and *C. fimbriata* were selected for further biological and chemical investigations of their antiproliferative constituents.

The bioassay guided isolation of the water partition of the ethanolic extract of *C. halicacabum* led to the isolation of five compounds: quercetin 3-*O*-*β*-D-rutinoside, apigenin 7-*O*-*β*-D-glucoside, scutallarein, luteolin and chrysoeriol. Their structures were elucidated by 1D and 2D NMR and mass spectrometric data. This is the first reported isolation of apigenin 7-*O*-*β*-D-glucoside and scutallarein from *C. halicacabum*. The antiproliferative activities of the isolated compounds were evaluated against two different cancer cell lines, SKNMC (neuroepithelioma) and MCF 7 (breast cancer).

The most potent activity was shown by chrysoeriol (IC_{50} of 9 and 2 µg/ml against SKNMC and MCF 7 respectively). Scutellarein (IC_{50} of 14 and 6 µg/ml against SKNMC and MCF 7 respectively), also exhibited antiproliferative activity. The activities exhibited by apigenin 7-*O*-*β*-D-glucoside and quercetin were moderate.

The bioassay guided isolation of the ethyl acetate partition of the ethanolic extract of the leaves of *C. fimbriata* led to the isolation of cleomiscosin A, *N*-(*p*-*trans*-coumaroyl) tyramine, aristolochic acid 1 and aristolactam1a-*N*-*β*-D-glucoside. This is the first report of all these compounds from *C. fimbriata*. These compounds were tested for their antiproliferative activity against the SKNMC and MCF 7 cell lines. Aristolochic acid 1 was found to exhibit the most potent activity (IC_{50} of 3 and 10 µg/ml against SKNMC and MCF 7 respectively). Cleomiscosin A (IC_{50} of 11 and 21 µg/ml) also exhibited antiproliferative activity. The presence of aristolochic acid 1, a potent carcinogen in *C. fimbriata*, is a matter of concern. Extracts of *C. fimbriata* have recently gained popularity as a dietary supplement. Further toxicity studies to ensure the safety of this plant needs to be considered. It is also noteworthy that aristolochic acid and its derivatives are reported very rarely from families other than Aristolochiaceae.

The antiproliferative activity of *C. halicacabum* and *C. fimbriata* is reported for the first time in this study. The biological activities exhibited by *C. halicacabum* and *C. fimbriata* and the molecules isolated through bioassay guided studies were consistent with their traditional medicinal applications. Traditional knowledge therefore provided a successful lead towards the isolation of bioactive compounds from Siddha medicinal plants.

Indoleamine 2,3-dioxygenase (IDO) has emerged as an important anticancer drug target. For this reason, the IDO inhibitory activity of the shortlisted plants was also determined. Crude extracts of *M. tinctoria*, *E. alsinoides* and *C. halicacabum* were identified to possess significant IDO inhibitory activity and are worth considering for further studies to identify IDO inhibitory molecules

Tumour promotion is closely linked to inflammation and the search for new antiinflammatory therapeutic approaches for cancer treatment is in progress in several research laboratories. The crude extracts of *C. halicacabum* and *C. fimbriata*, which showed significant antiproliferative activity, were tested for their antiinflammatory activity by the cyclooxygenase inhibitory assay. Both plants exhibited good cyclooxygenase inhibitory activity. At 50 µg/ml, *C. halicacabum* inhibited the activity of cyclooxygenase 1 and 2 by 65% and 55%, respectively. *C. fimbriata* inhibited cyclooxygenase 1 and 2 by 82 and 100%, respectively.

The MTT studies have also identified other partitions and fractions showing antiproliferative activities. The water partition of *C. fimbriata* was found to exhibit antiproliferative activity against the SKNMC cell line with an IC_{50} value of 33 ± 5 µg/ml. Fraction Cf-EtOAc-5 from the ethyl acetate partition of *C. fimbriata* also exhibited potent antiproliferative activity, with IC_{50} values of 82 ± 6, 70 ± 4 and 52 ± 4 µg/ml against SKNMC, MCF 7 and MRC 5 cell lines, respectively. However no more pure compounds were isolated from these fractions due to time constraints. There is potential to isolate more compounds from these bioactive fractions in the future. Also, future studies can further explore the antiprolierative activity of these plants on various other cell lines to ensure that the biological effects are more widely applicable.

Even though most of the major constituents in the active fraction Ch-water-5 have been isolated, further pursuing of the purification of the peak at retention time 28.0 needs to be considered. Ch-water-6 should also be investigated for bioactive molecules. In a subsequent report after the selection of plants for our initial antiproliferative screening studies, the chloroform extract of *A. Indicum* was reported to exhibit significant cytotoxicity against MCF 7 cell lines. However no chemical studies to isolate the bioactive molecules responsible for the activity has been carried out till now. This study needs to be considered.

The crude extract of *C. fimbriata* and *C. halicacabum* demonstrated antiinflammatory activity and should also be investigated in future. The finding that *N*-(*p*-*trans*-coumaroyl)tyramine inhibited IDO opens the possibility for structure activity relationship studies with this molecule.

In conclusion, this PhD study has successfully achieved its primary aim of supporting the use of medicinal plants by Dr Velmurugan for the treatment of cancer through bioassay guided isolation of bioactive compounds from these plants. The positive results obtained from *in vitro* bioassays performed in this project are encouraging. However, as in vitro studies are carried out under controlled conditions, further validation through *in vivo* studies is warranted to account for various factors such as bioavailability, enzyme interactions and molecular mechanisms.

Appendix 1

The intellectual property and confidentiality clauses in the collaborative research agreement between Dr Velmurugan and Macquarie University

5. Intellectual Property and Copyright

The Parties agree that:

a) any Background Intellectual Property shall remain the property of the relevant Party;

b) each party warrants that the use of any Background Intellectual Property or Project Intellectual Property will not infringe the intellectual property rights of any third party;

c) where use of Background IP is necessary for the conduct of the Project, each Party grants to the other an exclusive, royalty-free licence to use its Background IP solely for the conduct of the Project;

d) any Project Intellectual Property shall be jointly owned by the Parties in equal shares as tenants in common;

e) each Party agrees to grant the other Party a non-exclusive royalty-free license to use the Project Intellectual Property for non-commercial, internal business and research purposes;

f) should the results of the Project have commercial value to either Party, then both Parties agree to enter into a further agreement (**Further Agreement**) upon terms to be negotiated between the Parties in good faith, including, among other matters, how the Project Intellectual Property will be protected, and how it will be commercially exploited within 12 months of the expiration or termination of this Agreement. In the event that the Parties fail to enter into the Further Agreement within 12 months of the expiration or termination of this Agreement, the University shall be free to commercialise the Project Intellectual Property itself, as it sees fit and, in such circumstances, Dr Velmurugan agrees to execute such further documents as is reasonably necessary to enable the University to commercialise the Project Intellectual Property in accordance with this sub-clause 5 f).

6. Confidentiality

6.1 The Parties undertake:

a) to treat as confidential and keep secret all Confidential Information which has been or may be disclosed or which is created or discovered in the course of the Project and to use the Confidential Information only for the purposes of the Project;

b) not to copy or reduce to writing any part of the Confidential Information except as may be reasonably necessary for the purposes of the Project and that any such copies or reduction are the property of the Party which made available that part of the Confidential Information;

c) where there is involvement in the Project by a Participating Student, the University will ensure that this Participating Student is, where necessary, aware of and subject to a separate Confidentiality agreement with regards to the Project.

6.2 The obligation of Confidentiality does not extend to information which:

a) was already well known in the public domain or becomes so at a future date without the fault of the recipient or parties to whom the information was disclosed, directly or indirectly, by the recipient;

b) was a matter of written record in the files of the recipient prior to disclosure to the recipient;

c) was received by the recipient from a third person under circumstances permitting its disclosure by the recipient;

d) was independently developed by the recipient; or

e) becomes known to the recipient from a source other than the disclosing party without breach of a similar agreement by the outside source.

Appendix 2

Glossary of taxonomical terms

Ciliates - describing a structure, such as a leaf margin, fringed with fine hairs

Corolla - the petals of a flower considered as a group or unit

Fascicles - bundle or a cluster

Florets - a small or reduced flower

Foliate - having or resembling a leaf or having a specified kind or number of leaves

Hypogynous disks - designating petals, sepals, and stamens that are attached to the receptacle

Obtuse - having a blunt or rounded tip

Ovate-elliptic - intermediate between ovate and elliptic

Petiolate - small stalk attaching the leaf blade to the stem

Raceme - an inflorescence having stalked flowers arranged singly along an elongated-unbranched axis

Serrated leaves - *leaves* with little bent teeth like those of a saw

Stamens - pollen-producing reproductive organ of a flower

Stipules - paired appendages at the base of a leaf stalk

Subterete - slightly tapered at both ends, circular in cross section, and smooth-surfaced

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