1 Introduction

The research programme described in this thesis involved the study of medicinal plant extracts and included work on their activity towards skin diseases. It is therefore pertinent to first introduce the topics of medicinal plants and skin diseases.

1.1 General Introduction to Medicinal Plants

Research into the chemical and biological properties of natural products over the past two centuries has not only yielded drugs for the treatment of human ailments but has provided the stimulus for the development of modern synthetic organic chemistry and the emergence of medicinal chemistry as a major route for the discovery of novel and more effective therapeutic agents [1]. Natural products especially those derived from higher plants, which are seed plants, including gymnosperms (in which the seed is 'naked') and angiosperms, or flowering plants (in which the seed is contained in an ovary) historically play a vital role in the history of medicine [2]. Plants represent a rich source of new molecules with pharmacological properties, which may be used as lead compounds for the development of new drugs such as taxol and camptothecin [3]. A lead is a prototype compound for a given biological activity. A natural product lead structure is subjected to chemical modification to arrive at the therapeutically important molecular fragment, the pharmacophore. Only a few natural products are directly used

as drugs but in many cases, the chemical modification of the lead structure gave more potent synthetic or semi-synthetic analogues.

Over three quarters of the world's population relies mainly on plants and plant extracts for healthcare [4]. Natural products once served as the only source of medicine for humankind. The structure determination and biological activity screening of natural products, especially those with a history of medicinal use taking clues from folklore medicines, Ayurveda, tribal medicine etc., has been an important activity in medicinal chemistry. Discovery and development of new therapeutic agents is a continuing process. In spite of the fact that at present, there is a formidable array of modern drugs [5], the need to discover and invent new agents is genuine and important. It has been estimated that satisfactory therapy is available only for about one third of all presently known human ailments, and several diseases such as cancer, AIDS, senile dementia, autoimmune diseases [6]. There has been an increasing risk due to the growing resistance of microbes to the anti-microbial agents, which could be contributed to many mechanisms like mutation, prolong exposure to the therapeutic agents etc. As a precautionary measure, it is very important to develop new strategies that could discover new therapeutic agents [7] [8]. Approximately one third of the prescription drugs in the United States contain plant components and more than 120 important prescription drugs are derived from plants [10]. Most of these drugs were developed because of their use in traditional medicine [9]. Recent World Health Organisation (WHO) studies indicate that over 30% of the world's plant species have at one time or another been used for medicinal purposes. Of the 2,50,000 higher plant species on Earth, more than 80,000 species have medicinal properties. Although, traditional medicine is widespread

throughout the world, it is an integral part of each individual culture. Its practice is based mainly on traditional belief handed down from generation to generation for hundreds or even thousands of years [11] [12].

India's use of plants for health care also dates back to 5000 years. About 8000 herbal remedies have been codified in Ayurveda, which is still in use in many dispensaries today [13]. For a long time, the only way to use plant medicine was either direct application or the use of crude plant extracts [14]. Now, it is possible to rapidly build up extensive libraries of certain classes of organic compounds by the method of combinatorial chemistry [15].

Table 1.1: Natural product drugs, leads and their synthetic or semi- synthetic analogues.

Natural product drugs or lead	Source	Drug activity	Synthetic or semi-synthetic analogues
atropine	*Atropa belladonna*	anti-cholinergic	tropicamide
benzyl penicillin	*Penicillin chrysogenum*	antibiotic	ampicillin amoxycillin
codeine	*Papaver somniferum*	analgesic	nalarphine meperidine
cephalosporin	*Cephalosporium acremonium*	antibiotic	cephalexin, cephadroxil, cefachlor.
camptothecin	*Camptotheca accuminata*	Anticancer	10-hydroxy camptothecin, α-aminocamptothecin topotecan, irinotecan
digoxin	*Digitalis lantana*	cardiovascular	-
ephedrine	*Ephedra sinica*	anti-asthama	salbutamol, salmeterol
morphine	*Papaver somniferum*	analgesic	heroin, naloxane
podophyllotoxin	*Podophyllum peltatum*	anticancer	etopside teniposide
quinine	*Cinchona succirubra*	Antimalarial	chloroquine meploquine pamaquine primaquine
reserpine	*Rauwolfia serpentina*	hypotensive anticholinergic	deserpidine
tubocurarine	*Chondrodendron species*	neuro-muscular blocking agent	decamethoxium soxamethonium laudexium
taxol	*Taxus baccata*	anticancer	Palcitaxel, docetaxol
vinblastin	*Catharanthus roseus*	anticancer	vindesine
vincristine	*Catharanthus roseus*	anticancer	-

artemisinin	*Artemisia annna*	antimalarial	artemether
papaverine	*Papaver somiferum*	antispasmodic	Nifedepine,verapamil(Ca.channel blocker)
khellin	*Ammi visnaga*	bronchodilator	cromoglycate

Natural products when directly used as drugs can be highly effective. The main disadvantage in the use of drugs obtained from natural products is that of short or limited supply and the complex chemical structure, which makes manufacturing impossible. Therefore, semi-synthetic analogues or synthetic analogues [15], which are closely related to the natural product drugs or leads, are synthesised and screened. It is expected that a close synthetic structural analogue of a natural product drug has a better chance of being similarly active than the more remote analogue. The synthesis and screening of structurally similar analogues to the natural drug or lead (or a synthetic lead) is referred to as analogue design, which is a major technique in modern drug discovery programmes. Analogue design includes synthesis of analogues, structures of lead with variation of branches, chain length increases or decreases, variation of the kind and position of the substituents, the replacement of rings by similar cyclic structures and empirical modification within the framework of reasonably close analogy. Functional groups in the lead are replaced by similar size, shape and reactive groups, the changes are known as biosteric replacements [16]. Quantitative structure-activity relationship studies, (QSAR) the process by which chemical structure is quantitatively correlated

with a well-defined process, such as biological activity or chemical reactivity, are made to find the relationship between the substituent introduced and the drug potency of the analogue. Many of the structure alternations are dictated by synthetic accessibility in a given lead series. The same techniques of analogue design are followed even in the case of a synthetic lead structure modification. In these studies, the success rate to discover a new drug is 1:10,000. However, the modification of the natural or synthetic lead by design of analogues is still the principal approach by the pharmaceutical companies to discover a new drug structure.

In India, about 20% of the drugs sold are plant derived and are particularly useful to treat chronic diseases of liver, kidney and skin. In India people prefer Ayurvedic or herbal medicines as they have practically fewer side effects, cheaper and equivalent medicines in allopathy are not available[17] [18]. There is a renewed interest world wide, in India, in particular after the General Agreement on Tariffs and Trade (GATT) agreement to discover new plant based drugs in view of diverse vegetation. It was designed to provide an international forum that encouraged free trade between member states by regulating and reducing tariffs on traded goods and by providing a common mechanism for resolving trade disputes. Several major Indian pharmaceutical companies have embarked on ambitious programmes to screen indigenous plants with a view to discover new drugs or lead structures and develop their synthetic analogues as drugs

1.2 Importance of Medicinal Plants

From thousands of years, humans have relied almost entirely on plants to treat all types of illness; from minor problems to life threatening diseases [11]. About 80% of people still depend largely on traditional plant derived drugs for their primary health care. In the developing countries, herbal medicine is deep rooted even today. This is because of traditional medicine is still practiced to some degree in all cultures. Traditional medicine is the only source of health for many people and for them "Primary health care" is synonymous with traditional medicine and traditional practitioners play an important role in their communities particularly in regard to common ailments and chronic disorders [14]. The discovery of penicillin from microbes and the isolation of anti-malarial alkaloids, cardiotonic glycosides, anti-cancer agents etc., from plants is significant evidence for the importance of natural products for the benefit of human kind [1-3].

Despite the belief that the majority of clinical drugs are synthetic in origin, it is interesting to note that six out of the top 20 pharmaceutical prescription drugs dispensed in 1996 were natural products and that over 50% of the top 20 drugs could be linked to natural product research [19]. It is estimated that in 1997 the world market for over the counter phytomedicinal products was US $10 billion, with annual growth of 6.5%. Presently, about 25% of drugs prescribed worldwide come from plants and 121 active compounds from natural products are being in current use. Of the 252 drugs considered

as basic and essential by WHO, 11% are exclusively of plant origin and there are a significant number of synthetic drugs obtained from natural precursors [20]. There are 119 drugs of known structure that are still extracted from higher plants and used globally in allopathic medicine. These 119 plant derived drugs are produced commercially from less than 90 species of higher plants [21]. It is estimated that 60% of anti-tumour and anti-infectious drugs already in the market or under clinical trial are of natural origin and the vast majority of these molecules cannot yet be synthesised economically and are still obtained from wild or cultivated plants [20]. The isolated compounds artemisinin, taxol and camptothecin are examples of natural products that under went development and clinical trials and are now on the market. Several natural products isolated from plants used in traditional medicine have potent anti-spasmodial action *in-vitro* and represent potential sources of new anti-malarial drugs [22]. In addition to the above, a good number of novel plant derived substances have entered into western drug markets and clinical plant based research has made rewarding progress in the important fields, such as in anti-cancer and anti-malarial therapies [23].

In addition to the above-mentioned facts, the extensive practice of traditional medicine in developing countries and the rapidly growing demand for alternative and basic therapeutic means in industrialised countries give rise to the international relevancy of research and development in the field of traditional herbal drugs. An additional motivation for such activities is found in the practical necessity to integrate the potential of traditional herbal medicine into current practice of modern health care [24].

1.3 Scope for Research on Medicinal Plants

Emphasis shifted away from plant-derived drugs with the tremendous development of synthetic pharmaceutical chemistry and microbial fermentation soon after 1945. Plant metabolites were mainly investigated from a phytochemical and chemotaxonomic viewpoint during this period [25]. However, during the later part of the 20th century herbalism was widely used worldwide. This reformation observed was due to several reasons. The most significant ones are as follows –

(a) the value of traditional and indigenous pharmacopoeias, which make healthcare affordable for all, (b) conventional medicine can be inefficient (e.g., side effects and ineffective therapy), (c) abusive and/or incorrect use of synthetic drugs results in side effects and other problems, (d) a large percentage of the world's population does not have access to conventional pharmacological treatment, (e) folk medicine and ecological awareness evidence suggests that the natural products are less harmful, (f) herbal medicine often complements conventional treatments and (g) provides safe, well-tolerated remedies for chronic illness [20] [26].

Over the last decade, interest in drugs of plant origin has been growing very fast and currently, there is a Worldwide upsurge in the use of herbal preparations and active ingredients of medicinal plants in health care. The consumption of medicinal plants has almost doubled in Western Europe in recent years. The ecological awareness and an increased demand for non-classical therapies may be involved as the main reasons for

this renewal. Equally, the efficacy of a number of phyto-pharmaceutical preparations, such as ginkgo, garlic or valerian, has been demonstrated by studies that applied the same scientific standards as for synthetic drugs. As a result of all these reasons, there is an enormous market for crude herbal medicines in addition to purified plant derived drugs [25].

The clinical applications of taxol, etoposide and artemisinin have helped to renew an interest in higher plants as sources of new drugs. Though there is a considerable development in the field of medicine, there still remains an urgent need to develop new clinical drugs for numerous diseases, which results from the malfunction of the central nervous system (CNS), e.g., Alzheimer's and Parkinson's disease, epilepsy, migraine, pain, schizophrenia, sleeping disorder, etc. Natural products have already proven a track record for CNS activities, e.g., caffeine, codeine, morphine, nicotine, reserpine and it is possible that there are further such drugs still to be found from nature [19] .

Besides, this industrial research, many institutes and universities are also concentrating much on herbal product research e.g., the NCI (National Cancer Institute, USA) has tested more than 50,000 plant samples for anti-HIV activity and 33,000 samples for anti-tumour activity [20].

The WHO considers phytotherapy in its health programmes and suggests basic procedures for the validation of drugs from plant origin in developing countries. Eastern countries, such as China and India have well established herbal medicines industries and

Latin American countries have been investing in research programmes in medicinal plants and the standardisation and regulation of phytomedicinal products following European countries such as France and Germany [20]. WHO has hosted inter regional workshops to address methodologies for the selection and use of traditional medicines in national “Primary health care” programmes [11].

However, the potential use of higher plants as a source of new drugs is still poorly explored. Despite all the activities described up to this point, it is still the case that of the estimated 2.5 to 5 million plant species, only a small percentage has been investigated phytochemically and even a smaller percentage has been properly studied in terms of their pharmacological properties. In most cases, only pharmacological screening or preliminary studies have been carried out and it is also estimated that only 5000 species have been studied for medicinal use [20] [25]. It is logical to presume that many more drugs that are useful will be found in the plant kingdom if the search for these entities is carried out in a logical and systematic manner [21].

1.4 Dermatology

1.4.1 Introduction to Dermatology

Dermatology is the science that is concerned with the diagnosis and treatment of diseases of the skin, hair and nails. Skin is the largest organ [29] in the body, both by weight and surface area. It is about 3 millimetres thick and protects us from all the harmful external factors. The skin separates the internal environment from the external [30]. In adults, the weight of the skin accounts for about 16% of the total body weight.

1.4.2 Skin's Architecture (Fig 1.1)

Skin comprises of three layers (Figure 1.1): the epidermis (outer), dermis (middle) and subcutaneous tissue (innermost) [29]. The skin contains a variety of appendages, mainly hair follicles, sweat glands, sebaceous glands, which are all embryological epidermal in origin [31]. The normal skin flora includes microorganisms of virulence such as *Staphylococcus*, *Micrococcus* and *Peptococcus* [32].

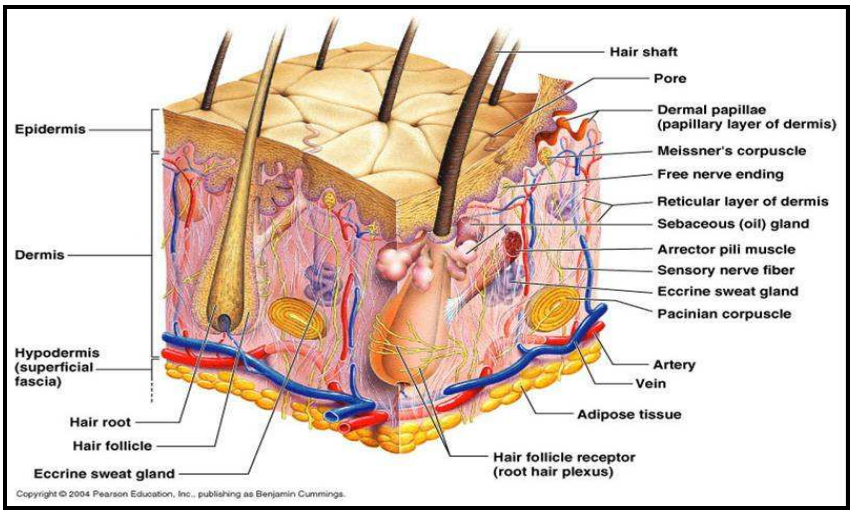


Figure 1.1: Skin Diagram (source ulty.ircc.edu/faculty/tfischer/AP1/skin.jpg)

The epidermis is the outermost layer, which produces keratin that protects the body against harmful environment and controls water loss. The dermis or middle layer of skin, contains a number of important substances such as collagen and reticulin, which prevent the skin from tearing; elastin, which makes the skin resilient; and jelly like substances that make the skin soft and compressible. The third layer, subcutaneous tissue, is primarily made up of fat which provides insulation, shock absorption and calorie reserves. Both the dermis and the subcutaneous tissue contain sensory and motor nerves. The vast network of blood vessels in skin provides oxygen and nutrients to sensory, motor nerves and to skin appendages including glands, nails and hair [31]. The skin's dry state is due to its low pH, and its pilosebaceous secretions that inhibit microbial growth [32]. The skin serves many purposes [31], as a barrier to the environment and some glands (sebaceous) may have weak anti-infective properties. Skin protects from

water loss, friction wounds and impact wounds. It also protects from UV rays of sun and produces vitamin D in epidermal layer. In addition to the above, skin also helps to regulate body temperature through sweat glands as well as in the regulation of metabolism.

1.4.3 Epidemiology

Specific body sites of skin diseases are hair, scalp, nails, sweat glands, genitals, face, hands, feet, skin folds. Skin diseases affect 20-30 % of the population at any one time, seriously interfering with activities in 10% [30-32]. Teenagers and young adults account for a significant number of dermatology patients. Acne is the leading skin disease, occurring in 20% of all patients seeking treatment for skin diseases. Contact skin disorders such as poisoning and burns or rashes caused by industrial or household chemicals are the second most common problem. Other common skin disorders include skin cancer, warts, fungal infections and psoriasis.

Causes of Skin Diseases – Any component of the skin can be affected by any category of disease. The complexity and variety of skin diseases is the fact that the skin comprises the distinct organs welded together i.e., dermis and epidermis. The causes for skin diseases [32] are due to the effects of mechanical, chemical, thermal radiant, parasitic, cosmetic and therapeutic environments. The other causes are due to accumulations in

skin e.g., jaundice (bile pigments), cutaneous side effects (drugs or metabolites), calcium accumulation (scrotal calcinosis).

1.4.4 Skin Disorders and Treatment

Skin diseases range in severity from mild disorders to severe diseases, which can be extremely debilitating and may cause physical and psychological scarring of the patient. Dermatological conditions commonly seen in primary care include acne vulgaris, atopic eczema, psoriasis, bacterial infections of the skin such as impetigo, fungal infections, such as tinea pedis (athletes' foot) and viral infections such as warts caused by the human papilloma virus (HPV)[34] [36].

There are many disorders of the skin like bed sores, dry skin, calluses and corns, keratosis pilaris, pityriasis rosea, rosacea, sebaceous cysts, albinism, birthmarks, dercum disease, erythema multiforme, hairloss, head lice, hidradenitis, suppurativa, hives, hyperhidrosis, contact with poison ivy, scabies, seborrheic dermatitis, vitiligo, warts, hyper pigmentation – a darkening of the skin that requires clinical care by a physician or other health care professional [35-40].

1.4.4.1 Acne vulgaris

Figure 1.2: Acne vulgaris of an older teenager (source eucerin)

Acne vulgaris (Fig. 1.2) is a common disease affecting around 40% of people aged between 16-18 years [33]. It is a chronic inflammatory disorder [41], of the pilosebaceous glands in which seborrhoea (greasy skin) and a rash of lesions are present. The rash may consist of closed and open comadones (white heads and black heads respectively) and inflammatory lesions such as papules, pustules, nodules and cysts. These lesions may lead to permanent scarring of the skin. Lesions commonly occur on the face, neck, shoulders and back. The pathogenesis of *acne vulgaris* is poorly understood but is thought to result from an abnormal response to physiological levels of the androgen, testosterone, which causes a high level of sebum production, in addition abnormal cornification of the keratinocytes within the follicular duct causes micro-comedone formation. The micro-comedone restricts the flow of sebum, which collects in the duct, becomes thickened and forms an open or closed comedone. Bacteria present in the duct, such as *Propionobacterium acne,* break down this sebum to release fatty

acids, which are irritant and this contributes to inflammation that occurs during lesion formation. The inflammatory reaction leads to neutrophil and polymorph infiltration and pus formation and so a pustule develops. Current therapy for *acne vulgaris* includes topical preparations such as benzoyl peroxide (panoxyl®) and azelaic acid which are effective in the treatment of mild to moderate acne. These preparations have anti-comedonal, antimicrobial and anti-inflammatory activity. Topical anti-bacterials such as erythromycin (Stiemycin®) and clindamycin (Duac®) once daily are used in the treatment of mild to moderate acne. However, *Propionibacterium acne* has developed some resistance to these agents. Other topical preparations used in the treatment of acne include the retinoids and related substances such as tretinoin (Retin A®). Tretinoin was one of the first topical retinoids available for acne and is still in use today [42] isotretinoin, (Isotrex®) and the retinoid – like compound adapalene (Differin®) Oral preparations for acne include the antibiotics oxytetracycline, tetracycline, minocycline, doxycycline and trimethoprim (where acne is resistant to other anti-bacterials), hormone treatment such as co-cyproterone (cyproterone acetate with ethinylestradiol) and the retinoid, isotretinoin. Isotretinoin reduces sebum secretion and is used in the systemic treatment of nodulo-cystic and conglobate acne, severe acne and scarring acne, which has not responded to systemic antibiotic. The retinoid isotretinoin (Roaccutane®) is teratogenic and must be used with caution in women of childbearing age and with the use of effective contraception [35].

1.4.4.2 Atopic eczema

Atopic eczema (Fig. 1.3) is an inflammatory condition which commonly occurs in childhood and continuous into adult life. It is characterised by red, hot, swollen and itchy skin that may have papules and blisters. The skin may suffer exudation and crusting or may be dry and scaly. This skin condition can cause lichenification of the skin, painful fissures and be particularly susceptible to infection by various pathogens e.g., bacteria, fungi, viruses. Up to 90% of atopic eczema lesions are colonised by *Staphylococcus aureus* [36] and can result in secondary bacterial infections that can be difficult to treat and may require systemic antibacterial therapy. The mainstay of treatment for eczema is the use of emollients such as aqueous cream, emulsifying ointment and bath oils e.g., Oilatum® to moisturise the skin and maximise its hydration to prevent the scaling and fissure that can lead to discomfort and infection for the patient. Other treatments include topical corticosteroids such as hydrocortisone, betamethasone and their esters, which modulate the inflammatory process involved in the disorder.

Figure 1.3: Atopic eczema and recurrent eczema accessed from http://www.superlanolin.com/eczema/images/recurrent-eczema.jpg

The use of topical steroids has some benefit in the treatment of atopic and other forms of eczema but can produce adverse effects such as skin atrophy and rebound eczema on cessation of the therapy. Examples of some of the products currently in use for the treatment of eczema include the topical corticosterioids such as hydrocortisone, betamethasone esters, clobetasone, desoximetasone, and triamcinolone acetonide [43]. The itching of eczema can cause the chronic scratching and rubbing of the patient to produce lichenification of the skin; this can be managed by using antihistamines and topical ichthammol to reduce the pruritis (itching) [45].

1.4.4.3 Bacterial infections

Bacterial skin infections (Fig. 1.4) such as impetigo are caused by the bacteria *Staphylococcus aureus* and/or *Streptococcus pyogenes*. Impetigo is characterised by yellow crusting blisters on the mouth or face. It commonly occurs in children and is highly contagious [34]. Topical treatment for this condition may be considered for small-localized areas. Treatment includes use of the antibacterial preparations like, fusidic acid 2% cream or ointment for short term use in mild cases, neomycin sulphate 0.5% cream for use where only small areas are affected (risk of ototoxicity in the treatment of large areas) and muprocin 2% cream and ointment (to be used for not more than 10 days to prevent development of bacterial resistance) [46]. For widespread infection with multiple lesions, systemic anti-bacterials may be used. These include

flucloxacillin and erythromycin (most strains of *Staphylococcus aureus* are resistant to penicillins).

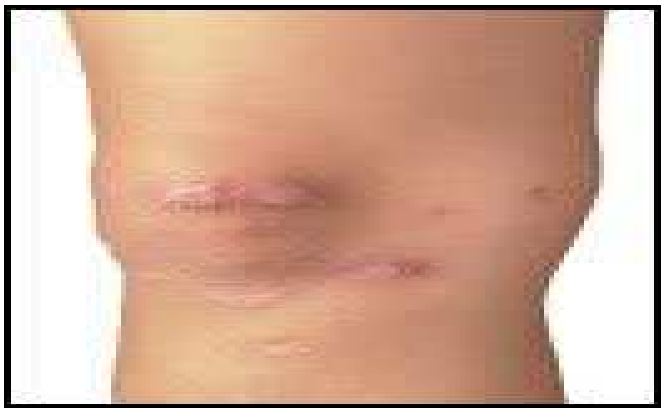


Figure 1.4: Diagram of Impetigo (source Net doctor/geir)

Other bacterial infections of the skin caused by *S.aureus* include cellulitis, folliculitis and *Staphylococcal* scalded skin syndrome. Cellulitis causes the skin to become reddened, hot and swollen, erthyma, a deeper more prolonged infection than impetigo, characterized by hard black crusts overlying localized areas of necrotic skin. Folliculitis is an infection of the hair follicles, which produces small pustules within the hair follicles and furuncles, which are abscesses within a hair follicle that can produce extensive lesions called carbuncles and may become ulcerated. *Staphylococcal* scalded skin syndrome is a skin infection in which a strain of *S. aureus* produces an epidermolytic toxin that causes a blistering eruption and skin peeling [33]. Systemic anti-bacterials are used in more severe infections of the skin or where topical therapy has not been successful [48] . Examples include flucloxacillin, fusidic acid, erythromycin

and metronidazole. Many strains of *Staphylococcus aureus* have become resistant to penicillin and other anti-bacterials [35].

1.4.4.4 **Fungal infections**

Fungal infections (Fig. 1.5) of the skin such as ringworm (Tinea) can be effectively treated with topical anti-fungals. Ringworm can affect the scalp, feet, hands and body or nails [34]. Useful anti-fungals include the imidazole preparations clotrimazole, ketoconazole, miconazole and other antifungals include terbinafine, amorolfine [49]. Candidiasis is a fungal infection of the skin caused by the yeast *Candida albicans*, such infections can be effectively treated with imidazole anti-fungals such as clotrimazole, miconazole , econozole and nystatin is useful in the treatment of candidiasis [35].

Figure1.5: Ringworm (Tinea on the arm) source
(http://www.research.usf.edu/cm/pics/ringworm_2.jpg)

1.4.4.5 **Rosacea**

Rosacea is a relatively common chronic dermatosis disorder affecting more than 40 million people worldwide [51]. It is common in middle age. The cause may be result of solar damage or a vascular problem. The signs are chalazia, styes, punctuate epithelial erosions, corneal infiltrates, inflamed eyelid margins, scales and crusts of the eyelid. Doxycycline, 100 mg daily will improve ocular disease and increase the tear break up time [39] [40].

Table 1.2: Drugs for skin diseases and some of side effects (BNF 55, SECTION 13, March 2008).

Sr.	Drug	Disease	Adverse effect
1.	clotrimazole	candidiasis	hypersensitivity
2.	clotrimazole, econazole,ketokonazole	dermatophytosis	erythema, itching
3.	permethrin,phenothrin, and malathion	*crab lice* (*Pthirus pubis*).	irritation of excoriated skin
4.	Permethrin	scabies	irritation
5.	Topical corticosteroids	Eczema (dermatitis)	itching, iritation
6	salicylic acid, formaldehyde, gluteraldehyde or silver nitrate	Warts (verrucas)	skin irritation
7	benzoyl peroxide, Azelaic acid	acne	local skin irritation
8	griseofulvin	tinea pedis; resistant fungal infections	nausea, vomiting, diarrhoea; headache hepatotoxicity, dizziness

2 Selection of Rutaceae Plants

2.1 Introduction

With the fast growing demand for herbal drugs during the last two decades in every branch of medical care, it was considered appropriate if not necessary to explore the therapeutic claims of reputed herbal drugs. A collection of Rutaceae plants was available within the University and there was an interest in the use of medicinal plants to treat skin diseases, given the large number of plants claimed to be useful in this therapeutic area [51-55]. Accordingly, in considering rutaceous plants their claimed dermatological uses [56-61] were kept in mind.

2.2 Literature Reports of Selected Rutaceous Plants

2.2.1 Tetractomia roxburghiana

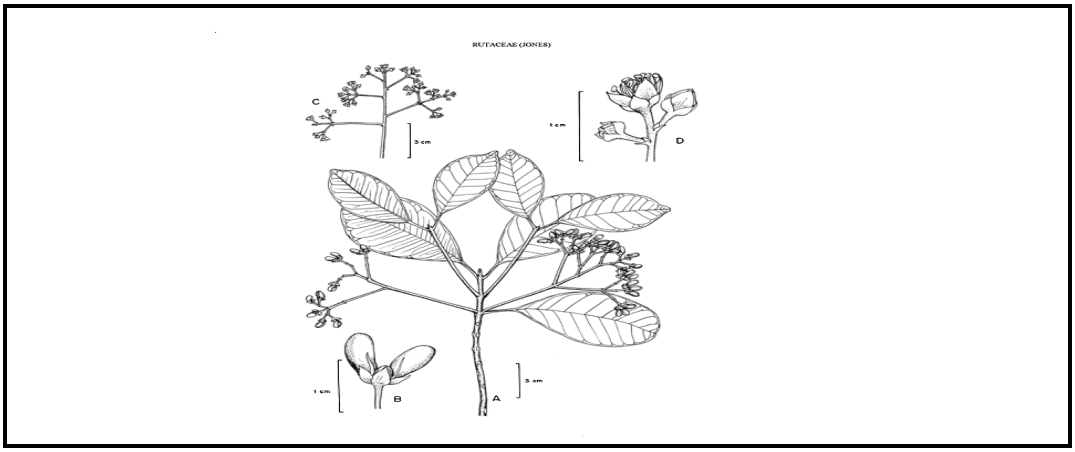


Figure 2.1: *Tetractomia roxburghiana, Hook*. (Tree Flora of Malaya 1972, Stone, B.C)

2.2.1.1 *Tetractomia roxburghiana, Hook* Classification

The genus Tetractomia belongs to the kingdom: plantae, division: magnoliophyta, class: magnoliopsida, order: sapindales, family: Rutaceae, species: roxburghiana and the plants of the genus *Tetractomia* are trees or shrubs.

2.2.1.2 The botanical aspects / morphological identification of Genus Tetractomia

The three species of the genus Tetractomia namely *Tetractomia majus*, *Tetractomia roxbughii*, *Tetractomia tetrandra* are natives of Malaysia,Peninsula and Borneo [62]

The morphological characters of Genus Tetractomia were described in the Tree Flora of Malaya 1972, S tone, B.C as follows –

"The leaves are opposite, petioled, one-foliolate, coriaceous, quite entire and punctate. The flowers are small in auxillary branched cymes. Calyx is small and four-partite. Petals are four, triangular-ovate, acute, persistent, volvate. Disk is broad, glandular, flatish or pulvinate, obtusely four-angled. Stamens are eight in number, four perfect alternate with the petals, four smaller with imperfect anthers (or zero) opposite the petals and partially adnate to them at the base; filaments are subulate and flattened at the base; the anthers are two-lobed and dorsifixed. Ovary immersed in the centre of the disk, four-celled with four free projecting lobes; styles are four in number, free at the base, connate above, stigma capitate obscurely four-lobed; ovules two, collateral in each cell. Fruit of four coriaceous laterally compressed oblong two-seeded carpels, splitting ventrally, the horny endocarp partially separates partially. Seeds inserted towards the base of the carpels (nucleus small with a large oblong obtuse membranous wing, embryo unknown

The leaflets of *Tetractomia roxburghiana (roxburghii),* Hook. (Family: Rutaceae) are 7.5 to 12.5 cm elliptic or cuneate-obovate obtusely acuminate, cymes pubescent. It differs from *Tetractomia majus* conspicuously in size, and the flowers are 0.3 cm in diameter and ripe carpels are 0.6 cm long. In texture and nervation, the leaflets are identical; the cymes of this are laxely clothed with a furfuraceous pubescence".

2.2.2 *Glycosmis calcicola,* B.C. Stone

Figure 2.2: *Glycosmis calcicola, B.C, Stone, (*source plant images database search)

2.2.2.1 *Glycosmis calcicola, B.C, Stone, Classification*

The genus Glycosmis belongs to the kingdom: plantae, division: magnoliophyta, class: magnoliopsida, order: sapindales, family: Rutaceae, species: calcicola. It is distributed in south-and-southeast Asia [63].

2.2.2.2 The botanical aspects/ morphological identification of Genus Glycosmis

The botanical aspects/ morphological identification of some genera of plants were described in (Benjamin C. Stone, (1985) as “A conspectus of the Genus Glycosmis

Correa Studies in Malaysian Rutaceae" *III Proceedings of the Academy of Natural Sciences of Philadelphia*) as follows. "The shrubs are small trees possess pinnate or simple leaves with translucent glands. The buds are covered by prominent short rusty-coloured hairs. Inconspicuous white flowers in recemose inflorences are followed by red berries, and the leaflets of this plant are mostly 5 to 15 inches long, pointed to rounded. These are shrubs or small trees. It can be easily distinguished from other species by its emargenate leaflets (terminated with depressed apex), tetramerous flowers and globose fruits and is found mostly in open and dryer limestone range" [63]. *Glycosmis arborea* (Roxb.) an Indian medicinal plant, which is locally used for the treatment of fever, liver and certain skin diseases [64].

2.2.3 *Clausena excavata* Burm. f. (Rutaceae)

2.2.3.1 *Clausena excavata* Classification

It belongs to the family Rutaceae, sub-family:aurantoideae, tribe:clauseneae, Genus: Clausena and species: excavata

2.2.3.2 The botanical aspects / morphological identification of Genus Clausena

The botanical aspects/ morphological identification of some genera of plants were described in tree flora of Malaysia, as “It is a slender tree up to 10 metre tall. Twigs are finely hairy. Leaves are pinnate up to 60 centimetres long, with 10 to 15 pairs of dark green narrowly oval oblique leaflets 3.5-7 cm long with pointed tips. Leaflets have a characteristic curry-like smell when crushed. Small white flowers occur in terminal clusters, followed by translucent pink berries 7-10 mm across, each containing one to two seeds”.

Fig 2.3: *Clausena excavata* Burm. f.

It is a wild shrub of the Rutaceae family distributed in South Asia. It is 1.5 metres high bush growing both wild and cultivated; it has been used as folic medicine for the treatment of cancers and several disorders in the east of Thailand [65]. Its leaves and stem are also used for the ailment of colic, cough, headache, rhinitis, sore, wounds, and detoxification purposes in some countries [65]. The leaves are particularly used as a traditional medicine to cure cold, abdominal pain, malaria and dysentery. The constituents of *Clausena excavata* have been frequently studied. The plant was reported to contain carbazole alkaloids, coumarins, limonoids [65-70]. The sap of the leaves is rubbed on all kinds of muscular pain; a decoction of the root is given in malarial fever, an infusion of the stem in colic, the leaves and stem bark are also used locally as a diuretic, tonic and astringent [68]. Coumarins reported from the leaves have been found to be possess inhibitory effects on tumour promotions [66].

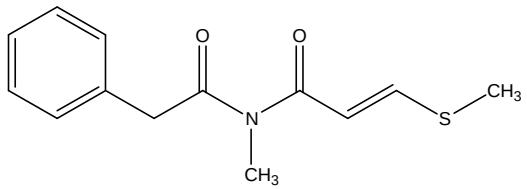
2.3 Literature review

This study was based on the ethnobotanical knowledge of three rutaceous plants and the chemical constituents of several genera of the family Rutaceae that had yet to be fully elucidated namely, *Tetractomia roxburghiana, Glycosmis calcicola and Clausena excavate.* Accordingly, these were to form the basis of phytochemical study. Ethnobotanical screening of the Rutaceae family has demonstrated important medicinal uses throughout several continents. The pharmacological activity of these plants is diverse and includes the reported anti-tumour activity of *Glycosmis cirifolia* [71], the

anti-coagulant components of *Murraya paniculata* [72] and the anti-trypanosomiasis activity of several of the Rutaceae family [73]. Research has identified several pharmacologically active constituents of this family, which include phenylpropanoids [74], methoxyflavones [75], furoquinoline alkaloids [76], coumarins [77] and acridone alkaloids [78].

2.3.1 Structures of the Previously Isolated Compounds from the Rutaceae Family

Although there is much phytochemical work still to be done on the Rutaceae family, a wide range of interesting compounds have already been found in the related genera of *Glycosmis, and Clausena etc.,*



penimide A

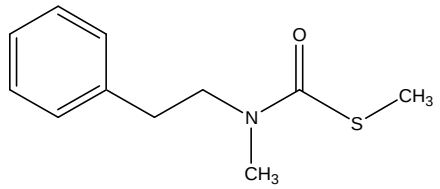
Source of the plant: *Glycosmis cyanocarpa, Glycosmis crassifolia*

Part of the plant: leaves

Class of compound: amide

Pharmacological activity: anti-fungal

Reference [57]



niranin

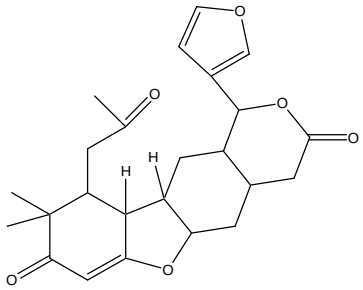
Source of the plant: *Glycosmis cyanocarpa, Glycosmis crassifolia*

Part of the plant: leaves

Class of compound: amide

Pharmacological activity: anti-fungal

Reference [57]



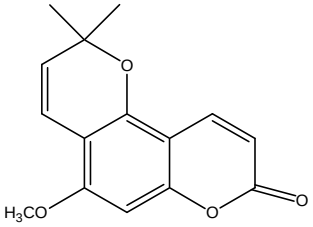
hortiolide A

Source of the plant: *Hortia columbiana*

Part of the plant: wood

Class of compound: triterpenoids

Reference [79]



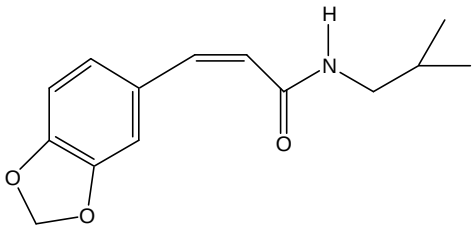
alloxanthoxyletin

Source of the plant: *Hortia columbiana*

Part of the plant: wood

Class of compound: coumarin

Reference [79]



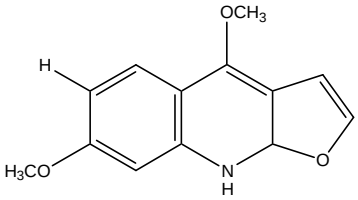
cis-fagaramide

Source of the plant: *Zanthoxylum schnifolium*

Part of the plant: leaves

Class of compound: amide

Reference [80]



skimmaianine

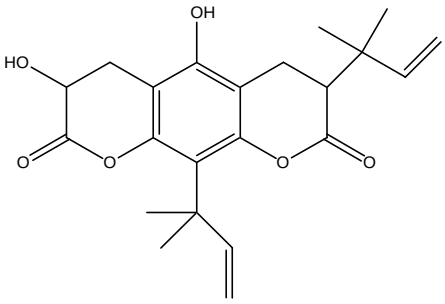
Source of the plant: *Raulia echinata Cowan*

Part of the plant: leaves

Class of compound: quinoline alkaloid

Pharmacological activity: anti-fungal

Reference [81]



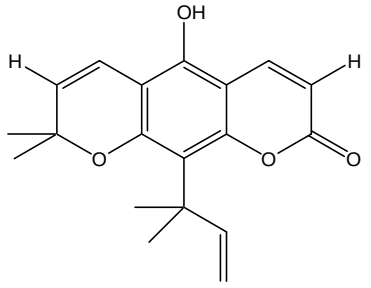
claucavatin A

Source of the plant: *Clausena excavata*

Part of the plant: root bark

Class of compound: pyrano-coumarin

Reference [82]



nordentatin

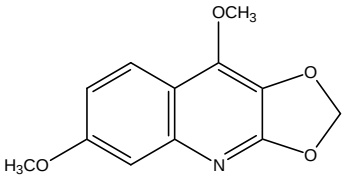
Source of the plant: *Clausena excavata*

Part of the plant: root bark

Class of compound: pyrano-coumarin

Pharmacological activity: anti-bacterial

Reference [84]



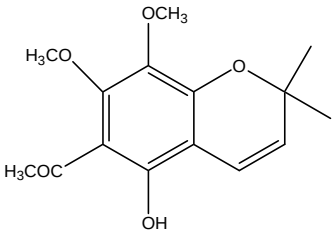
2, 3-methylenedioxy-4, 7-dimethoxy quinoline

Source of the plant: *Acronychia laurifolia*

Part of the plant: root

Class of compound: quinoline alkaloid

Reference [85]



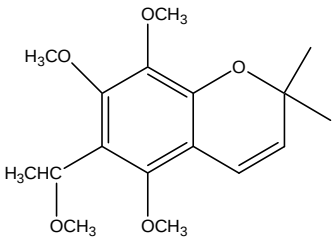
leptol

Source of the plant: *Evodia lepta*

Part of the plant: herb

Class of compound: chromene

Reference [86]



methyl leptol A

Source of the plant: *Evodia lepta*

Part of the plant: herb

Class of compound: chromene

Reference [86]

2.3.2 Reported Biological Activities of Some Related Genera of Rutaceous Plants

The lipophilic leaf extracts of *Glycosmis cyanocarpa*, *Glycosmis crassifolia* and *Glycosmis mauritiana* contain anti-fungal phenethyl- and styrylamine derived amides and imides, most of them with sulphur [57]. Greger *et al.*, reported that *Glycosmis* leaf compounds are the possible compounds for naturally derived fungicides against the rice blast disease (*Pyricularia oryzae*). Each compound was assayed in broth serial micro dilutions (2% malt extract) in 96-well micro plates (200–0.8 mg/ml). Growth inhibition was determined after hours by microscopical observation of ten conidiospores /concentration [57].

Ming-Jencheng. *et al*. reported anti-platelet aggregation activity [80] of the methanolic leaf extract of *Zanthoxylum schinifolium.*

Biavatti and Vieria reported anti-fungal activity [81] of furoquinoline alkaloids namely skimmaianine, kukarafinine, maculine, flindersamine against *Leucogaricus gongylophorous*, the symbiotic fungus of leaf cutting ants (*Atta sexdens*) from the stem and leaf extract of south Brazilian endemic plant, *Raulina echinata*. They were inactive or displayed weak inhibitory activity when assayed *in-vitro* against trypomastigate forms of *Trypanasoma cruzi.*

Mlijoana Ranbrianarivebjosia and Harison Rabarison reported strongest anti-plasmodial activity [87] of γ-fagarine and weak anti-plasmodial activity of n-benzoyltyramine, and weak anti-plasmodial activity of n-benzoyltyramine, skimmaianine, dictamine when tested *in vitro* against malarial parasite, *Plasmodium falciparasum* (strain FCM29) from the stem of bark of *Zanthoxylum tsihanimposa.*

Rahman *et al.* reported anti-nociceptive [68] activity of the ethanolic leaf extract of *Clausena excavata.* Given orally at doses 125.25 and 500 mg/kg it showed significant anti-nociceptive activity on acetic acid induced writhing in mice. The ethanolic extract of *Clausena excavata* leaves significantly and dose dependently suppressed the frequency of acetic acid induced writhing in mice. Rahman suggested that further studies were required to confirm this preliminary finding, which might also provide a pharmacological rationale for some ethnobotanical uses of the plant.

A. Manosroi *et al.*, reported immuno modulatory [65-67] activities of aqueous acetone extract of *Clausena excavata*.

Sohrab *et al.* reported anti-bacterial activity of clausimarin and lunamarins (puran-coumarins) from the cold methanolic extract of *Clausena hetaphylla* [88]. Clausimarin and lunamarins showed significant zones of inhibition (9-14 mm) against *Staphylococus aureus*, *Aeromonas hydrophila* and *Salmonella paratyphi*.

Douglas *et al.* reported a weak inhibitory cytotoxic activity [85] of a new quinoline alkaloid, 2,3-methylenedioxy-4, 7-dimethoxy quinoline against a panel of human b cancer cells BC 1(Human breast cancer) and kb (Human epidermoid carcinoma) from the methanolic leaf extract of *Acronychia laurifolia*.

2.3.3 Ethnobotanical uses of Rutaceae family

The uses of the *Rutaceae* in ethnopharmacology are to relieve symptoms of malaria, topical infections, skin inflammation, diuretic, toxic and astringent, stomachie, febrifuge, anti-periodic [65-70] and the bark is used to cure bites of poisonous animals and to check eruptions. Sap of leaves of *Clausena excavata* is used for all kinds of muscular pain. Decoction of the root is given in malarial fever, infusion of the stem is given in colic, leaves and stem bark are also used locally as diuretic, toxic and stem bark are also used as diuretic, toxic and astringent [68-70]. Several members of the *Rutaceae* family are aromatic and used as foods as well as in the pharmacy, perfumes, and essential oils are common in citrus and ruta [89].

Glycosmis citrifolia is used in folk medicine for the treatment of skin itch, scabies, boils and ulcers [71]. In Indonesia, the leaves of *M. paniculata* are considered astringent and useful for the treatment of venereal diseases. Leaves of *Murraya koenigii* [90] are used as stomachic, febrifuge, anti-vomiting, anti-dysenteric, stimulant, carminative and also used to cure bites of poisonous animals and to check eruptions.

The literature supported the importance of several genera of *Rutaceae* family with respect to pharmacological activity. However, little evidence had been reported for those that were selected for the current phytochemical and biological investigations in order to identify the active constituents present in them.

2.4 Aims and Objectives

Given the background as described, the aim of the investigation was to separate, characterise and identify the major chemical constituents of three rutaceous plants by combining traditional and state-of-the-art techniques in separation science and spectroscopic characterisation and interpretation. As the programme evolved a secondary aim emerged of exploring scientific evaluation of traditional use of these plants as drugs as well as to support the phytochemical investigation. Also it became necessary to study and improve upon the analytical methodology used in the isolation and characterisation.

3 Analytical Methodology-Liquid Chromatography

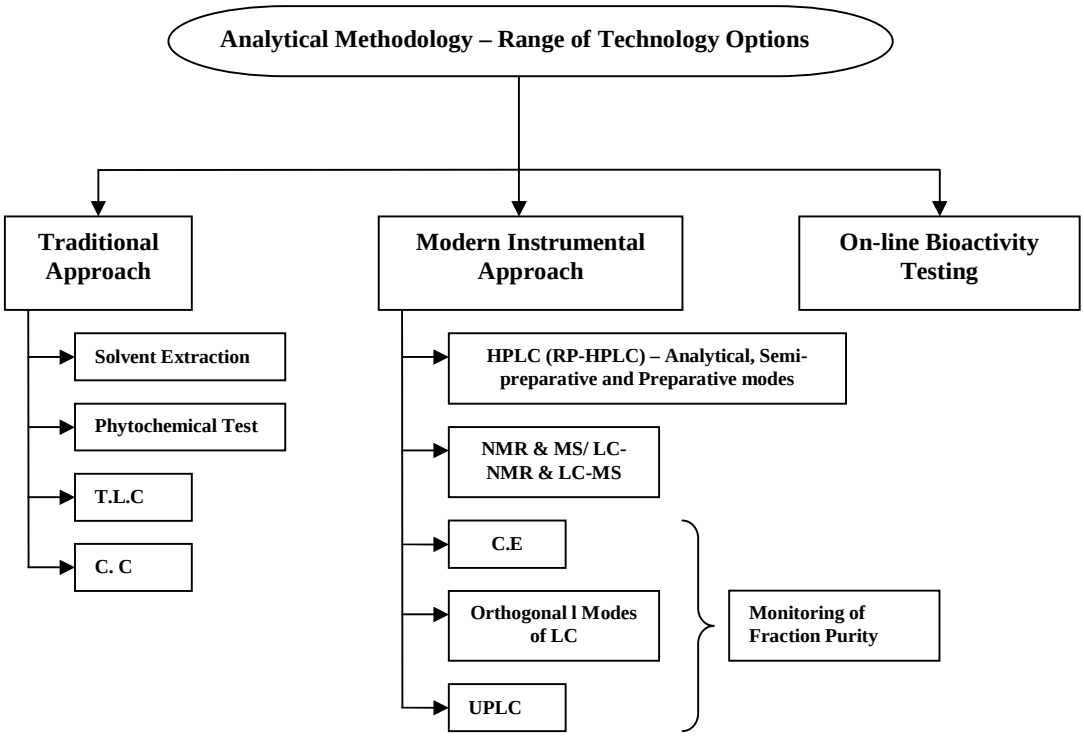


Fig. 3.1 - Flow Chart-Analytical methodology Used for Isolation and Identification of Active constituents of Rutaceous Plant Extracts

3.1 Introduction

3.1.1 Background

While in an ideal world it would be best to work throughout with state-of-the-art technology for the isolation and characterisation of constituents of medicinal plants, such technology was not always readily at hand. Accordingly, throughout the project there were ongoing efforts being made to develop and exploit the best of what was available. Ultimately, such studies accounted for a significant proportion of the overall research programme.

3.1.2 Range of Technology Options

The isolation and characterisation of active components from plants requires various techniques and methods, ranging from traditional solvent extraction methods to state-of-the-art techniques such as NMR, MS and LC-NMR-MS. Thus, the plan was to subject each plant to solvent extraction with several different solvents of increasing polarity. The crude extracts would then require fractionation by column chromatography and then evaluation by HPLC for the number of components in each fraction, followed by HPLC purification. The purified extracts were subjected to NMR and MS methods to

characterise and identify the components. The programme of work involves an understanding of the basis for the extractions and separative science, which fuses organic and analytical chemistry. The characterisation and identification of the components requires an ability to recognise the most appropriate technique and analyse the spectroscopic data to suggest a plausible structure, which are desirable pharmaceutical skills. It would also require knowledge of the literature and databases against which the suggested structures can be evaluated. The best possible option for any research laboratory studying medicinal plant extracts would be the one that makes the most of the resources available

3.1.3 Traditional Approach

The traditional approach [91] to isolating constituents from medicinal plants involves solvent extraction (Fig. 3.2) and open column chromatography; highly selective procedures are established but they are somewhat laborious. Typically, botanically identified, part air-dried, leafy plant material may be powdered and homogenised for five minutes in methanol-water (4:1) and then filtered. The residue is extracted with ethyl acetate five times in order to remove the fibre (mainly polysaccharide, fats and waxes). On the other hand, the filtrate may be evaporated to one-tenth of the original volume below 40°C and acidified with 2 M sulphuric acid. The acidified filtrate can then be extracted three times with chloroform. The chloroform extract is evaporated to dryness to get moderately polar extracts (terpenoids and phenolics).

The aqueous acid layer is basified to pH 10 with ammonium hydroxide and again extracted with chloroform-methanol (3:1, twice) and chloroform. The chloroform - methanol extract may be evaporated to dryness to get the basic extract (most alkaloid).

The aqueous basic layers formed may be evaporated and extracted with methanol in order to isolate the polar compounds found in the plants (quaternary alkaloids and N-oxides)

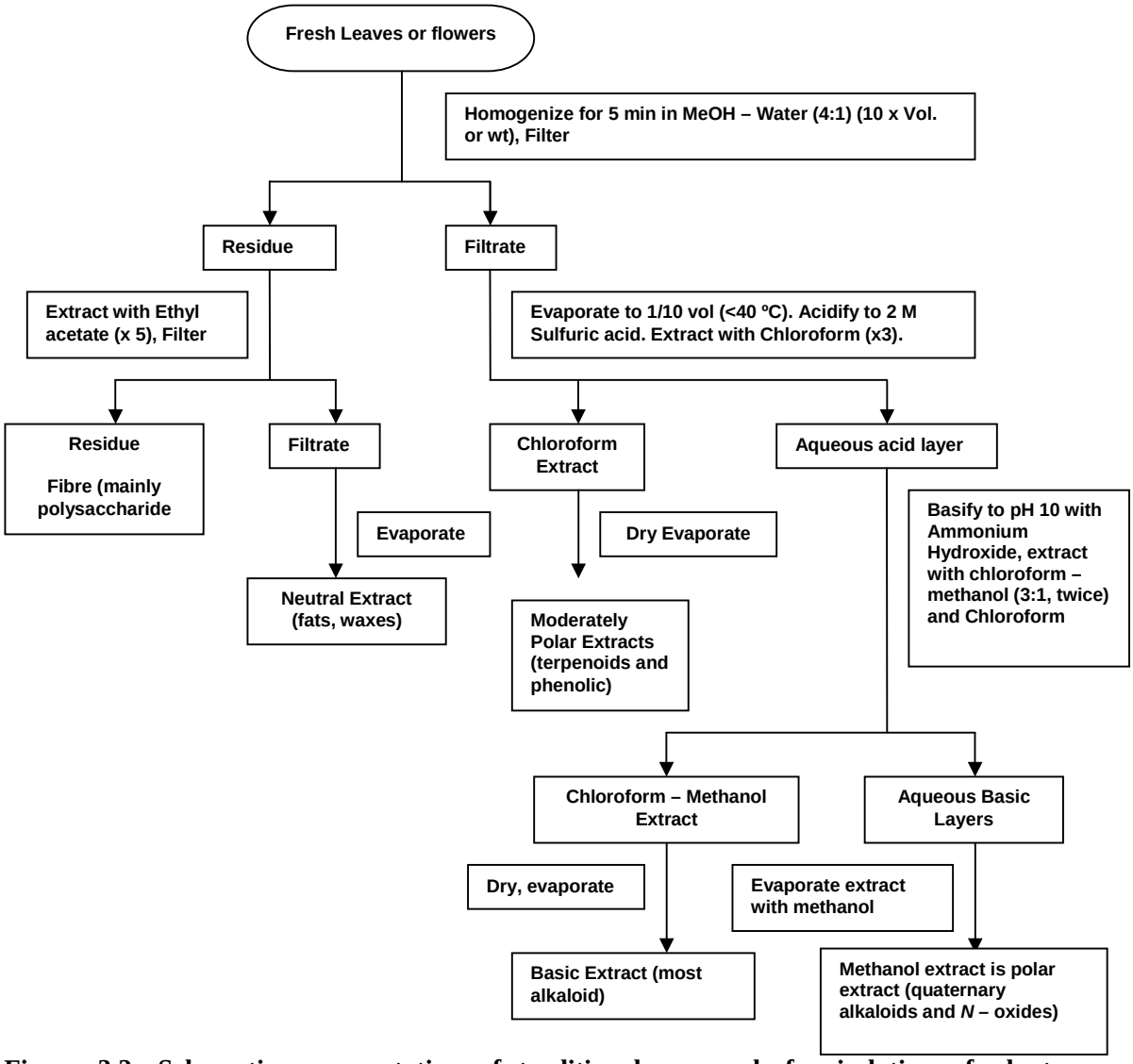


Figure 3.2: Schematic representation of traditional approach for isolation of plant constituents. (from Harborne, J.B. Phytochemical methods: A guide to modern techniques of plant analysis. July 1998, 3 rd edition, page no.214-216(Chapman and Hall Limited).

3.1.4 Modern Instrumental Approach

Over the years, things have moved on from the traditional approach, particularly with respect to identification. In the past few years, LC-NMR studies[92-107] have become popular in the identification of chemical constituents from both the crude and semi-purified mixtures. Due to high sensitivity now available from LC-NMR methodology, it is possible to identify individual components from crude extracts and mixtures wherein they are present in minute amounts (micrograms to nanograms).

LC-NMR is one of the best available analytical methods for the separation and identification of unknown compounds in mixtures. For example, it was used for the characterisation of xanthone and flavone constituents from *Gentiana ottonis,* a Gentianaceae species [92].

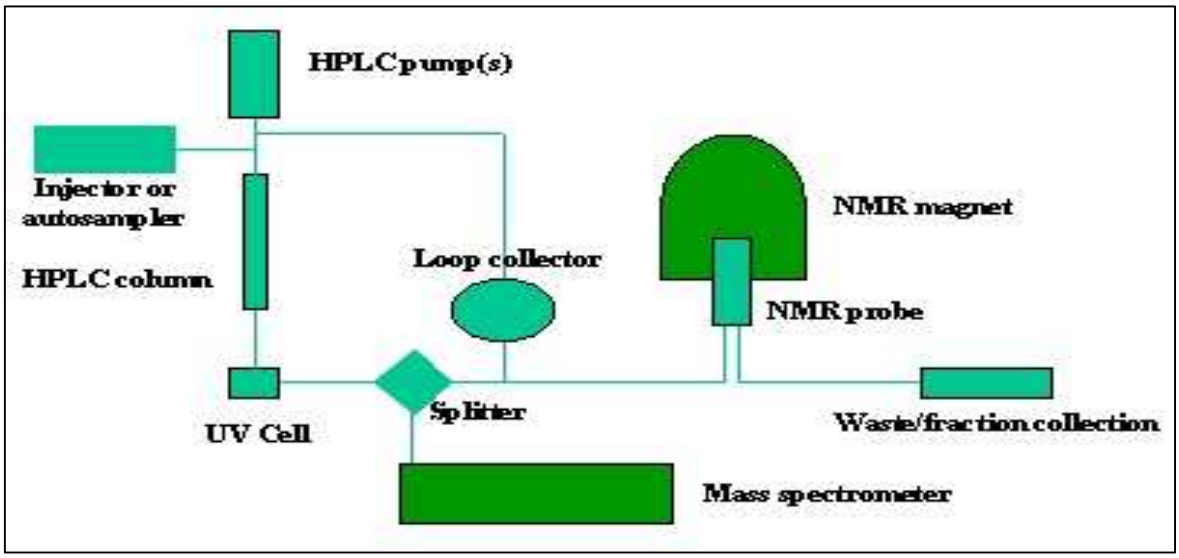


Figure 3.3: LC-NMR-MS schematic accessed from www.spectroscopy.com

LC-hyphenated techniques as mentioned above have become the most important tools in phytochemical investigation. These techniques yield enormous amount of information about the content and nature of constituents of crude plant extracts. Other examples include anti-fungal flavanones and iso-flavones being isolated and identified from two African plants *Erythrina vogelii* (leguminosae) [93] and M*onotes engleri* (dipterocarpaceae) [94]. Similarly, four betacyanin pigments were analysed by LC/NMR [95] and determination of the structure of the photo isomerisation product of the bioinsecticide, azadirachtin [96]. However, the major drawback of these techniques is unavailability of LC/MS and LC/MS/MS libraries of spectra [97].

LC/NMR is one of the most recent 'hyphenated' techniques wherein HPLC has been practically interfaced directly to NMR. The technique uses higher field magnets (500 MHZ and greater) and digital signal processing which have helped address the lack of sensitivity of the traditional technique [98]. Another reported advance of the development of LC/NMR is of on-flow and stopped–flow modes, which was used to characterise sesquiterpene lactones [99] in *Zaluzania grayana.*

LC/NMR now finds wide application in many areas [100-104], which include the determination of marine alkaloids, drug metabolites, degradation products and food analysis.

3.1.5 On-line Bioactivity Testing

At the very other end of the scale from traditional methods, with the right facilities, it can be possible to carry out LC-NMR-MS routinely and even carry out the accompanying subsequent biological testing online[105]. LC/UV/MS and LC/NMR can be used in a complementary manner for metabolite profiling [106]. Based on these methods, LC-bioactivity assays being performed in parallel, the targeted isolation of interesting compounds can be efficiently performed. These hyphenated techniques playing an impotant role in support of the phytochemical investigation. However it will be difficult to implement this approach generally since on-line biological testing for a range of different activities would be very difficult. Also not all biological tests are amenable to the rapid on-line approach.

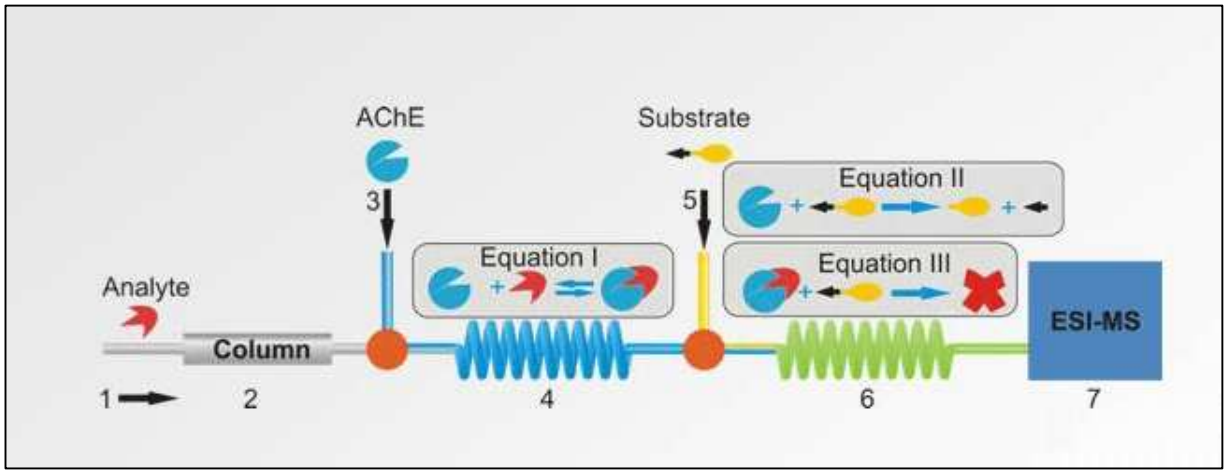


Figure 3.4: LC- Online Bioactivity Testing

("Screening of enzyme inhibitors by coupled liquid chromatographic continuous-flow enzyme assay and mass spec" Arjen de Boer, Camiel de Jong, Ben Bruyneel Collaboration with Kiadis BV (Groningen, The Netherlands); also prominently, Irth, Lingerman.)

3.1.6 Aims and Objectives

At the outset of the programme, it was only possible to use methodology that was slightly advanced from traditional methods. Therefore, in parallel with ongoing work on the chosen plants, it was aimed to evaluate other more advanced analytical options that might prove useful for routine use in the plant programme.

3.1.7 Experimental

3.1.7.1 Instrumentation

The HPLC system used consisted of a Shimadzu LC-6A pump, Shimadzu SPD-6AV variable wavelength UV/ Vis detector, and using low dead volume connections, and a Shimadzu CR5-A integrator (all supplied by Dyson Instruments, Hetton-le-Hole, Tyne & Wear, UK). Injections were made manually using a Rheodyne 7125 injection valve (Anachem, Luton, Beds., UK) fitted with a 20 µl, 50 µl, 1 ml and 2 ml loops for analytical and semi-preparative scale up, respectively.

A1-450 Dionex chromatographic system integrated with Shimadzu LC-10AD with UV/Vis detector. Agilent 1200 series chromatographic system integrated with HP Compaq L1706 monitor. It is inbuilt with preparative pump A (G1361A), pump B (G1361A), Auto sampler (G2260A), Fraction collector (G 1364B) and DAD (diode

array detector) (G1315B). Acquity Ultra pressure Liquid chromatography (UPLC)™ system (Waters)

3.1.7.2 Materials

HPLC grade methanol and ammonium formate (Sigma-Aldrich, Poole, and Dorset, UK). Water was distilled and doubly de-ionised using and Elgostat option 3 (Vivendi Water Systems, High Wycombe, Bucks., UK). Short column, C18 (100 X 3.0) µm for LC-NMR analysis was used. Luna NH_2 column (250 × 4.6 mm)), LUNA SCX column(Phenomenex) (250 × 4.6 mm) for orthogonal modes . Short C18 (5 × 2.1 mm i.d) columns, containing sub-2 µm particles for UPLC analysis. ACE-5-C18 column (250 mm × 4.6 mm), ACE-5-C18 column (250 mm × 7 mm), ACE-5-C18 column (250 mm × 22 mm).

3.2 Results and Discussion

3.2.1 Initial Approaches and Subsequent directions

Initially crude extracts were subjected to open-column ‘flash’ chromatography followed by analytical-scale high performance liquid chromatography and overloading of analytical columns. However, later it was possible to go to direct HPLC of crude extracts with scale-up to semi-prep and prep columns. Subsequent monitoring fractions

could be done by reversed phase high performance liquid chromatography but the use of orthogonal modes of LC, UPLC and capillary electrophoresis separations were also evaluated. These offered high efficiency, orthogonal selectivity respectively. Much of the actual identification was done by accurate mass MS with searches of spectral libraries but eventually it was possible to carry out some LC-NMR work.

3.2.2 Preparative Liquid chromatography

High performance liquid chromatography is the most useful tool for the separation of complex mixtures of small molecules. Reversed phase HPLC on octadecylsilane (ODS or c_{18}) has come to be recognised as the most broadly applicable of bonded phases for this purpose [108]. Reversed phase HPLC of the crude extracts was carried out using an ACE-5-C18 column (250 mm × 22 mm), chosen for high retentivity and low silanol activity at room temperature with the scaled up parameters developed from the analytical method. This approach was used for methanolic leaf extract of *Clausena excavata* and is described in Chapter 6. For the methanolic extract of *Glycosmis calcicola,* it was possible to carry out the method development and the scale-up work on the same semi-preparative column (described in the Chapter 5). Given the increasing experience that had been developed, *Tetractomia roxburghiana* was revisited and the extract analysed using a generic RP-HPLC method (Fig 3.11). In principle, this could have been scaled up directly but by this time in the programme the semi-preparative and preparative ACE-5-C 18 were in poor condition.

3.2.3 Monitoring fraction purity

Even when using the strategy of loading up preparative columns while still maintaining resolution it is still possible to get impure fractions and therefore there is a need to check the purity of fractions. Several approaches to this monitoring of fraction purity were evaluated.

3.2.3.1 RP-HPLC and RP-HPLC Method development for LC-NMR

Analytical reversed phase high performance liquid chromatography of the methanolic semi-purified fractions of extracts was carried out in order to determine the number of constituents present in each fraction for some of the work on *Clausena excavata*. The reversed phase HPLC of the semi-purified fractions were carried out using a short column, Luna C18 (100×2.0 nm) suitable for direct transfer to LC-NMR, at room temperature, using an isocratic mobile phase [methanol-water (49:51, v/v,)] at a flow rate of flow rate of 0.3 ml/min, concentration 0.5 mg/ml, with the injection volume of 20 µl. The detection was carried out at 254 nm and 215 nm using a UV detector. The Sample was dissolved in mobile phase: methanol-water (49:51, v/v), the sample concentration used was 0.5 mg/ml, (high concentration for a short narrow column was needed for good sensitivity in subsequent LC-NMR) and the analysis time was 60 minutes as the run time was not an issue.

The experimental parameters developed on the semi-purified fraction CE 3 using short C18 column, the conditions were simulated on the LC-NMR and LC-MS experiments and attempts were made to identify the major components from the semi-purified CE 3 fraction of *Clausena excavata.* From the LC-NMR and LC-MS data, the mass of the twelve peaks was noted on the ESI positive mode. On the basis of spectral analysis and on comparison with the reported data four lager peaks were named as peak five, peak seven, peak eight, and peak ten. Out of the four larger peaks , two peaks (peak eight and seven) were identified and characterised as 2-(3,4-dimethoxy-phenyl)-5,7-dimethoxy-chromen-4-one, 2-(3,4-dimthoxy-phenyl) 3,7-dihydroxy-5-methoxy-chromen-4-one (chrysin). It was the first time these flavanol compounds were identified from the leaves of *Clausena excavata.*

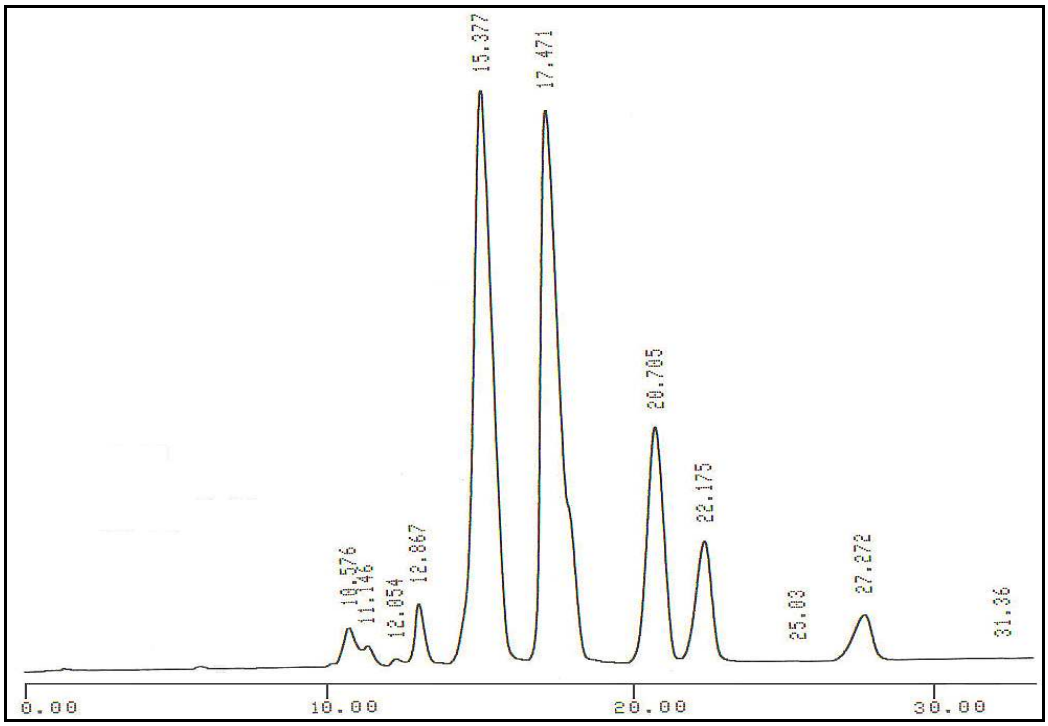


Figure 3.5 : Analytical reversed phase HPLC profile of methanolic leaf extract of semi purified CE3 fraction of *Clausena excavata* with a mobile phase of methanol-water (49:51, v/v) short column, C18 (x 3) µm; sample concentration 0.5 mg/ml, flow rate: 0.3ml/min; UV detection λ_{max}:254 nm.

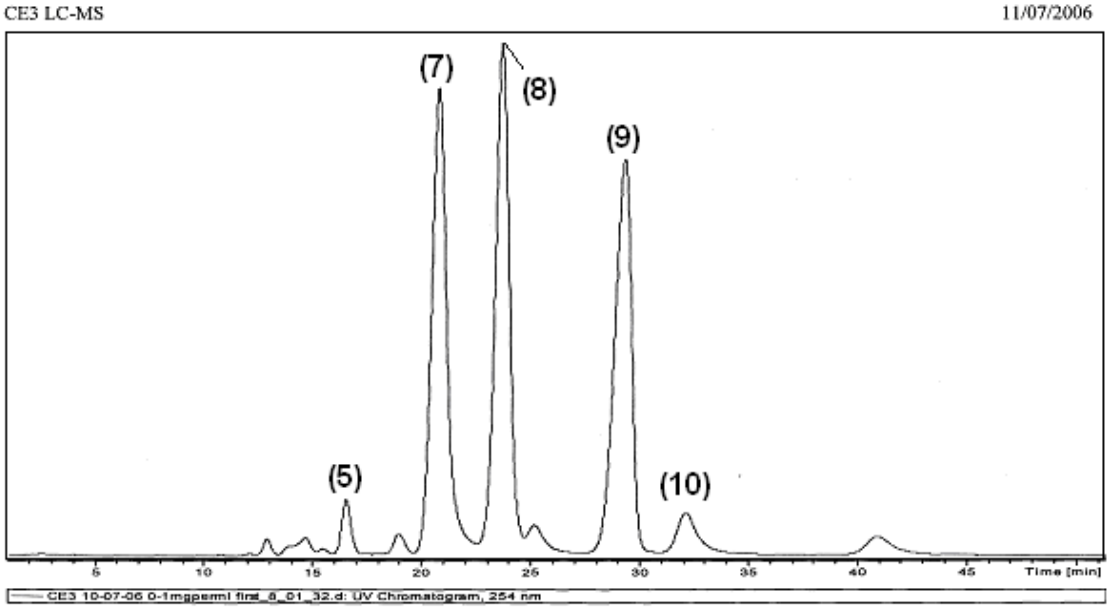


Figure 3.6 : Analytical RP-HPLC-NMR profile of methanolic leaf extract of semi purified CE3 fraction of *Clausena excavata* with a mobile phase of methanol-water (49:51, v/v) short column, C18 (100 X 2.0) µm; sample concentration 0.5 mg/ml, flow rate: 0.3ml/min; max: λ_{max} 254 nm.

Having developed RP-HPLC conditions on a short narrow column was suitable for LC-NMR, the semi-purified fractions were analysed. In some cases it had been known that more than one peak in the preparative run was being collected but also some impurities were revealed in several of the collected from a single peak. This was probably because of the degree to which the column had loaded up for the isolation work. The (100 X 2 mm) 3µparticle system subsequently proved suitable for LC-NMR. These results are discussed later (Chapter6)

3.2.3.2 Orthogonal Modes of LC

Given that the short RP-HPLC column with 3 µm particles would not have that much greater efficiency than the RP-HPLC columns being used for preparative isolation, it was thought that a better way of revealing any 'hidden' impurities in collection fractions would be to analyse then on a stationary phase showing different selectivity.

Orthogonal LC methods with a different separation mechanism RP-HPLC are not only potentially very useful in terms of resolution. Since the most readily accessible orthogonal LC phases are ion-exchangers [109] useful, they give some indication of the nature of the constituents and the chromatograms obtained would yield information on the nature of the constituents retained [110]. For example, for an anion-exchange phase, bases and neutral compounds will be unretained. However, it was also found that phenols were retained accordingly to the nature of phenolic groups and carboxylic acids were much more strongly retained than phenols.

Analytical HPLC of semi-purified *Clausena excavata* fractions was carried out using a Luna NH_2 column (250mm × 4.6 mm) at room temperature. [Methanol-water (90:10, v/v,)] with 0.002 M ammonium formate was used as mobile phase at a flow rate of 1.0 ml/min, with injection volume of 20 µl. The detection was done using UV detector at 254 nm. Sample preparation was done in the mobile phase. Concentration of the sample used was 0.5mg/ml and the analysis time was 30 minutes. It was carried out in order to find out number of phenolic or carboxylic acid groups present in the components. The chromatograms developed by this method are shown in Fig 3.7.

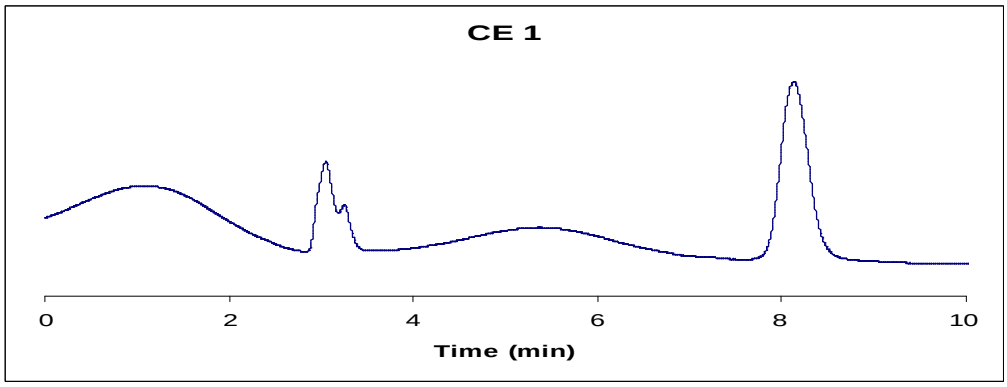


Figure 3.7 (a): -HPLC profile of semi purified fraction (CE1)of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.002 M ammonium formate LUNA NH2 C18 (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume 20 µl , flow rate: 1ml/min; λ_{max} 254 nm.

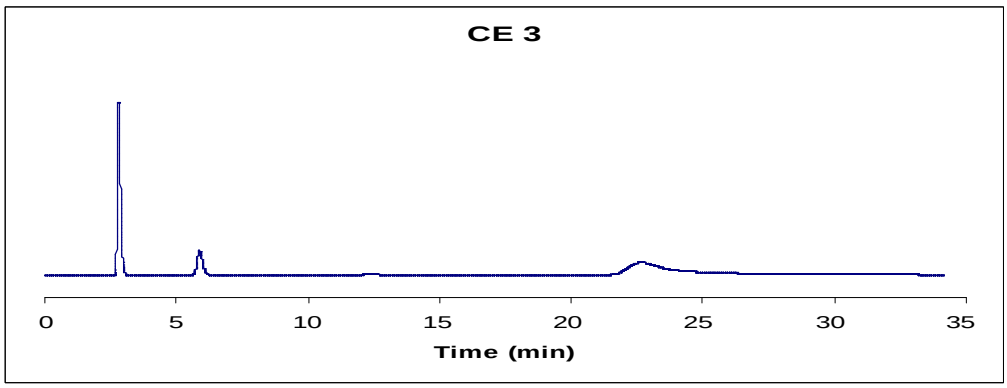


Figure 3.7 (b): -HPLC profile of semi purified fraction (CE 3) of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.002 M ammonium formate LUNA NH2 C18 (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume 20 µl , flow rate: 1ml/min; λ_{max} 254 nm.

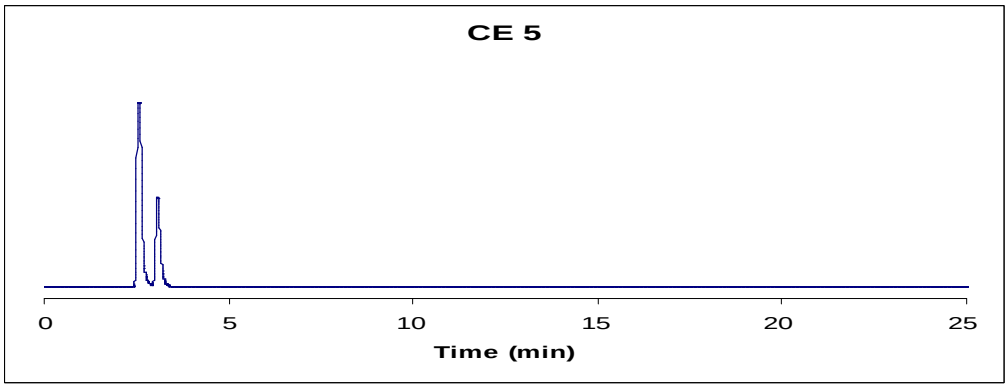


Figure 3.7 (c): -HPLC profile of semi purified fraction (CE 5) of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.002 M ammonium formate LUNA NH2 C18 (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume 20 µl , flow rate: 1ml/min; λ_{max} 254 nm.

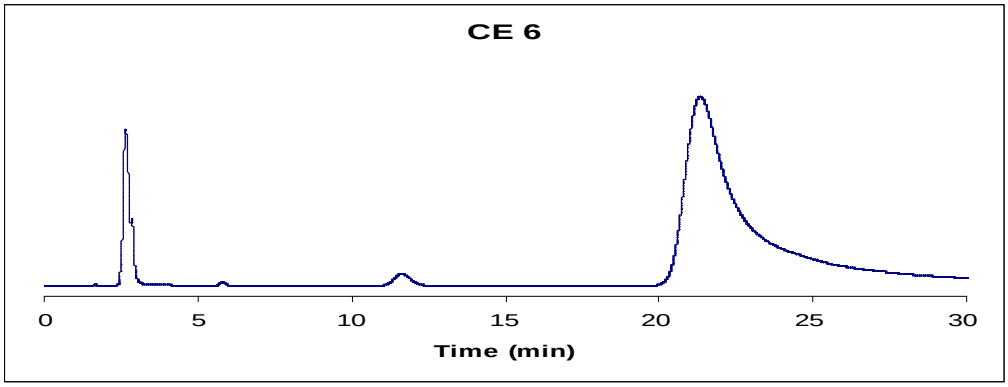


Figure 3.7 (d): -HPLC profile of semi purified fraction (CE6) of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.002 M ammonium formate LUNA NH2 C18 (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume 20 µl ,flow rate: 1ml/min; λ_{max} 254 nm.

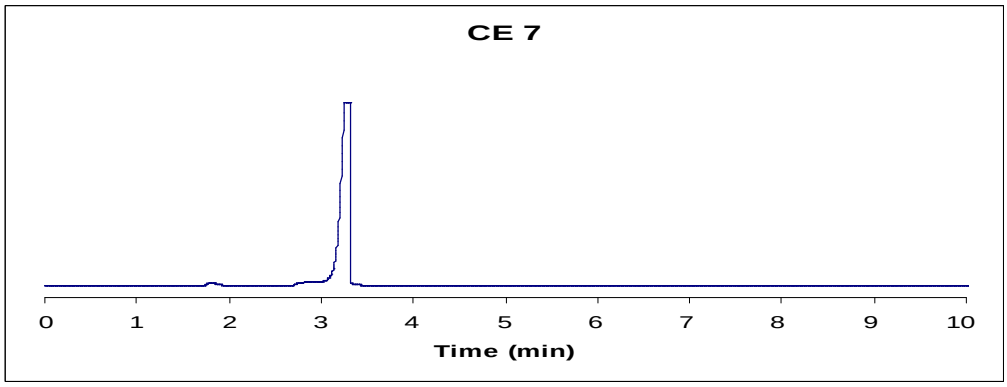


Figure 3.7 (e): -HPLC profile of semi purified fraction (CE7) of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.002 M ammonium formate LUNA NH2 C18 (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume 20 µl , flow rate: 1ml/min; λ_{max} 254 nm.

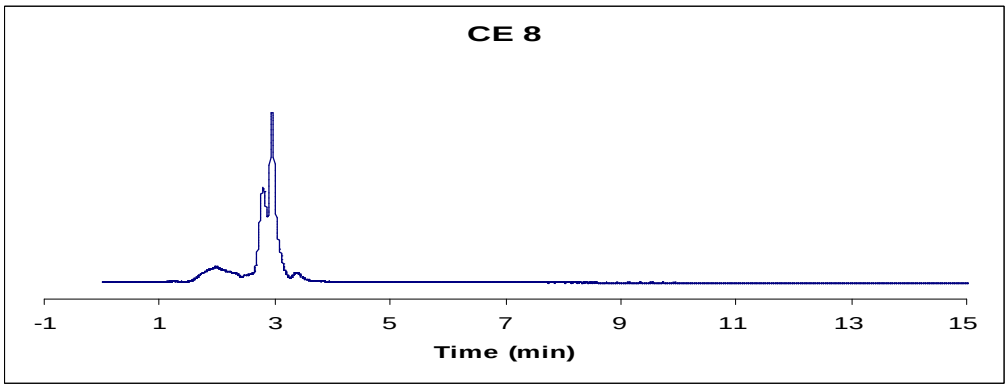


Figure 3.7 (f): -HPLC profile of semi purified fraction (CE88) of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.002 M ammonium formate LUNA NH2 C18 (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume of 20 µl , flow rate: 1ml/min; λ_{max} 254 nm.

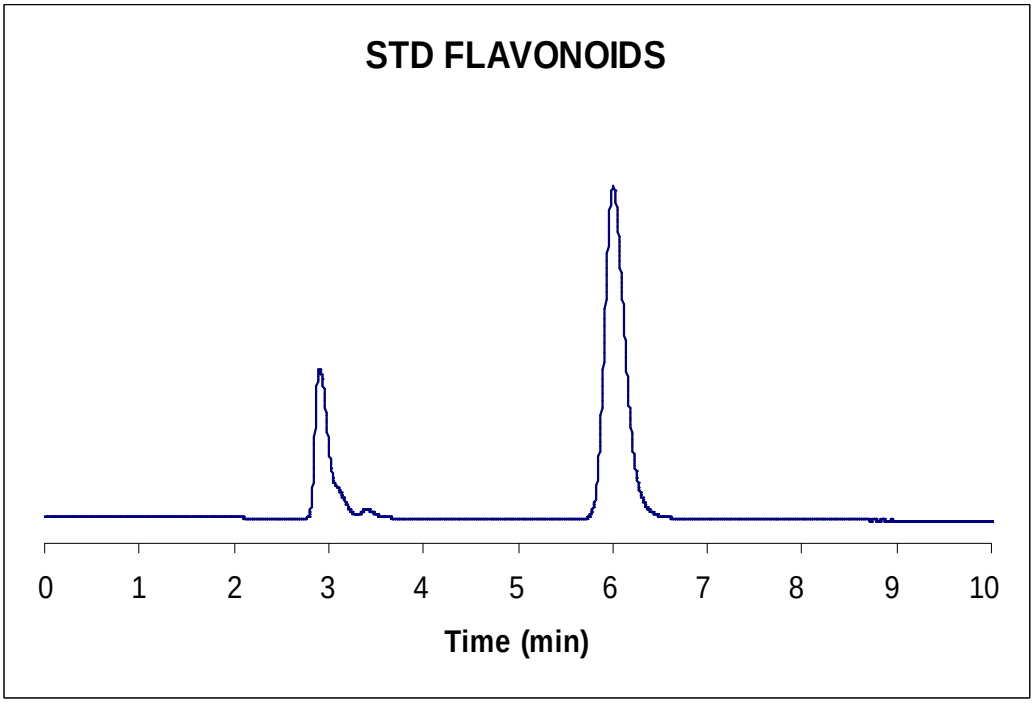


Figure 3.7 (g): HPLC profile separation of mixtures of typical flavonoids, rutin and chrysin; of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.002 M ammonium formate LUNA NH2 C18 (250 X 4.6 mm) sample concentration 0.5, mg/ml, injection volume of 20 µl ,flow rate: 1ml/min; max: λ_{max} 254 nm.

The chromatograms show the purity of the fractions and by comparison with standard flavonoids (rutin and chrysin) it appears that all fractions contain neutral compounds or those with a single phenolic group (t_r ~ 3 min) and the compounds with phenolic groups (CE 1). The longer retained peaks in CE 3 and CE 6 almost certainly contain compounds with carboxylic acid groups. CE 1 probably contain one more phenlic group than other fractions.

Analytical HPLC of the semi-purified fractions was carried out using a SCX column (250mm × 4.6 mm) at room temperature. [Methanol-water (90:10 v/v,)] with 0.005 M ammonium formate was used as mobile phase at a flow rate of 1.0 ml/min, with the injection volume of 5 µl. The detection was carried out using UV detector at 254 nm. Sample preparation was done in the mobile phase. Concentration of the sample used was 0.5mg/ml. This was carried out in order to find out any alkaloidal separations groups present in the components and it was found that all sample constituents eluted at t_0 showing that alkaloids were not present.

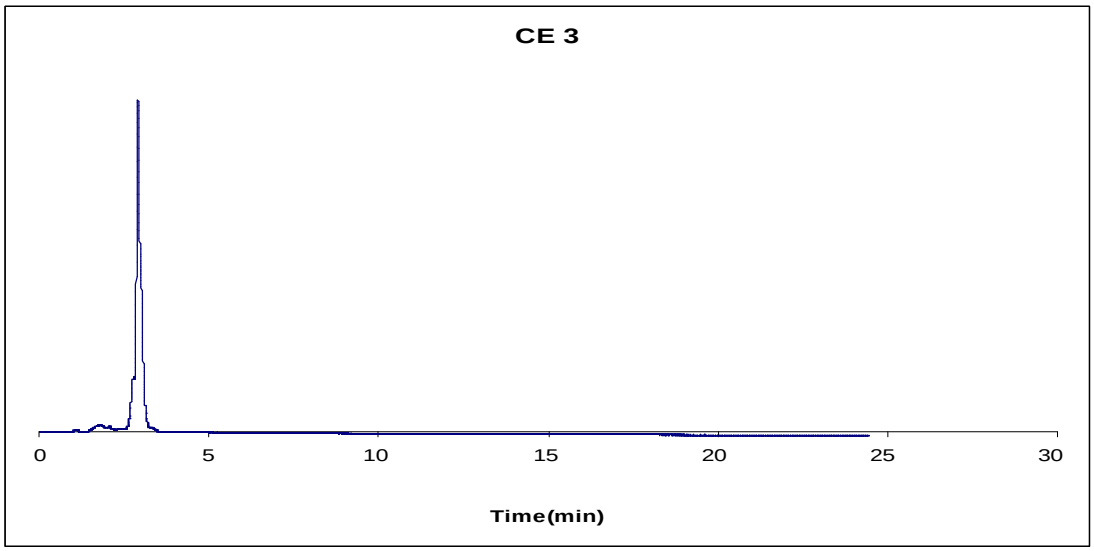


Figure 3.8 (a): HPLC profile of semi purified (CE 3) fraction of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.005 M ammonium formate LUNA SCX (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume 5 µl , flow rate: 1ml/min; λ_{max} 254 nm.

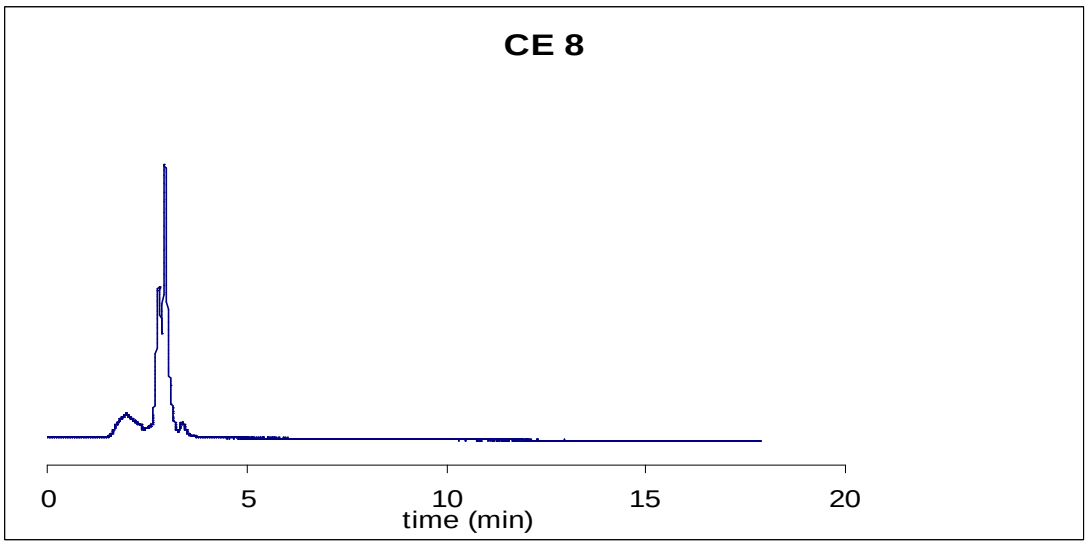


Figure 3.8 (b): HPLC profile of semi purified (CE 8) fraction of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.005 M ammonium formate LUNA SCX (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume 5 µl , flow rate: 1ml/min; λ_{max} 254 nm.

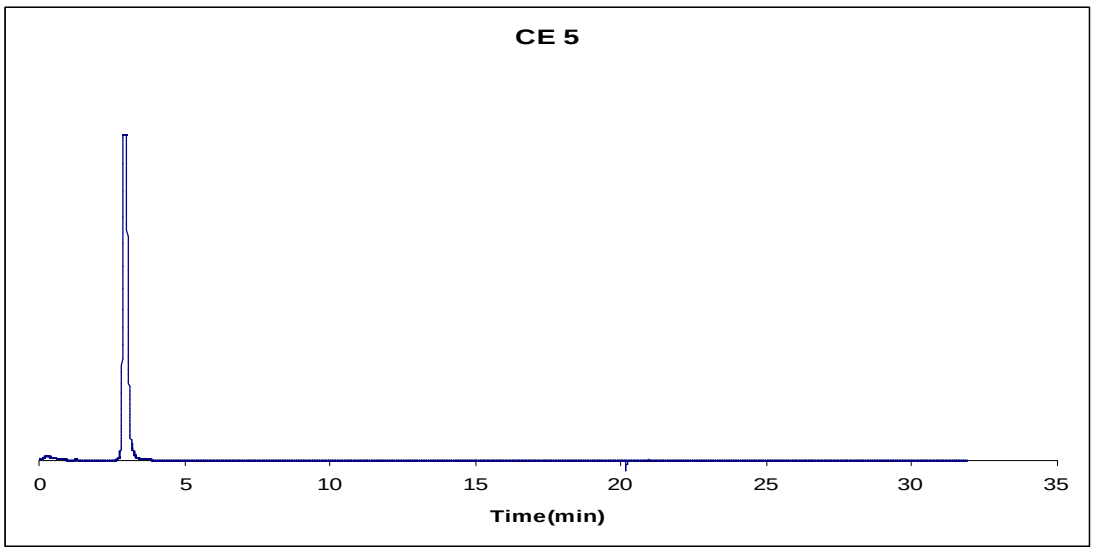


Figure 3.8 (c): HPLC profile of semi purified (CE 5) fraction of *Clausena excavata* with a mobile phase of methanol-water (90:10, v/v) with 0.005 M ammonium formate LUNA SCX (250 X 4.6 mm) sample concentration 0.5 mg/ml, injection volume 5 µl , flow rate: 1ml/min; max: λ_{max} 254 nm.

3.2.3.3 Ultra Performance Liquid Chromatography

Towards the final stages of the research programme, it was possible to use a Water Acquity Ultra pressure Liquid chromatography (UPLC)^{TM} system. Using UPLC it is possible to use short (5 cm × 2.1 mm i.d) columns, containing 2 µm particles at very high pressures (up to 16000 psi) to obtain high efficiency separations in very short run times [111]. This very much proved to be the case not only for rapid monitoring of fractions but also the very rapid profiling of the crude extracts themselves with similar resolution to that could be obtained on a 25 cm column. The chromatograms shown in Fig 3.8, demonstrate the high-resolution power and reduced analysis time of UPLC. The analysis was carried out using gradient UPLC system. The following experimental parameters were used.

Mobile Phase	:Gradient, methanol-water (90:10, v/v)
Injection volume	: 2 µl
Column	: ODS
Flow rate (ml/min)	: 1.2 ml/min
Wavelengths	: 254 nm
Analysis time	: 4 min

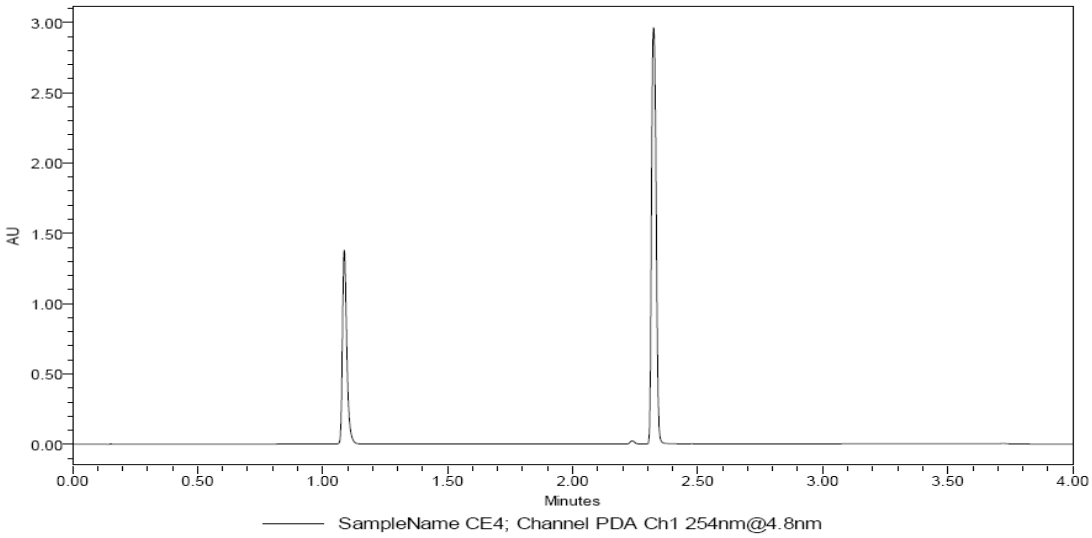


Figure 3.9 (a): UPLC profile of CE 4 fraction collected from preparative HPLC of methanolic leaf extract of *Clausena excavata* with a short column, C18 5 X 2.0 mm; sample conc. 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

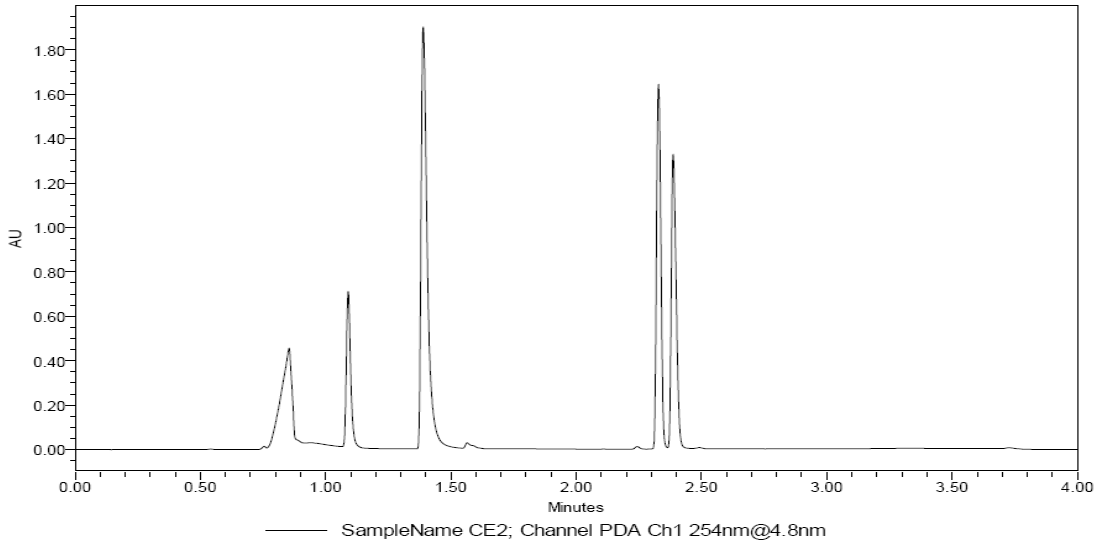


Figure 3.9 (b): UPLC profile of CE 2 fraction collected from preparative HPLC of methanolic leaf extract of *Clausena excavata* with a short column, C18 5 X 2.0 mm; conc. 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection λ_{max}:254 nm.

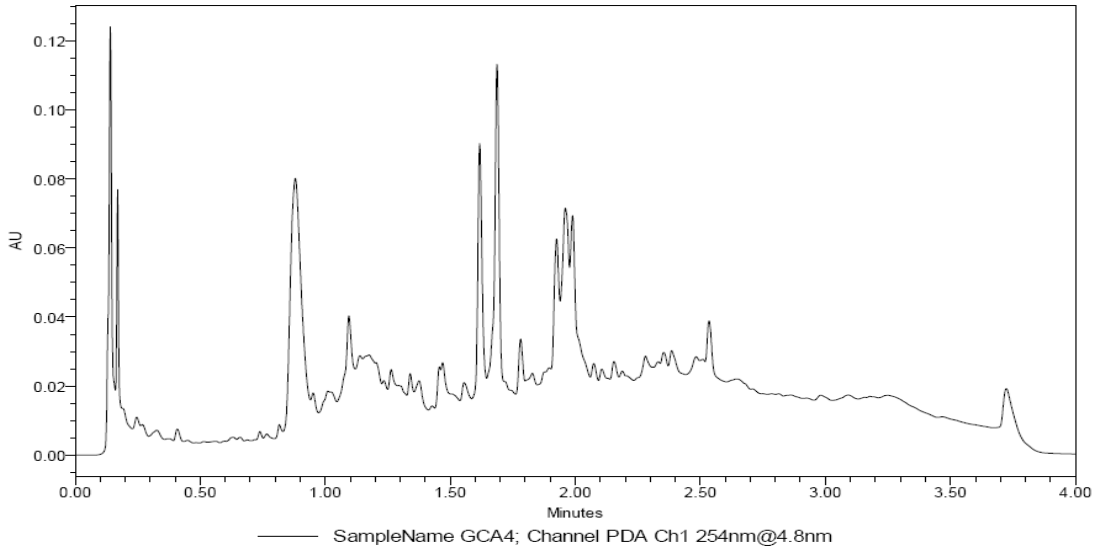


Figure 3.10 (a): UPLC profile of GCA 4 fraction collected from semi-preparative HPLC, of methanolic leaf extract of *Glycosmis calcicola* with a short column, C18 5 X 2.0 mm; conc. 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

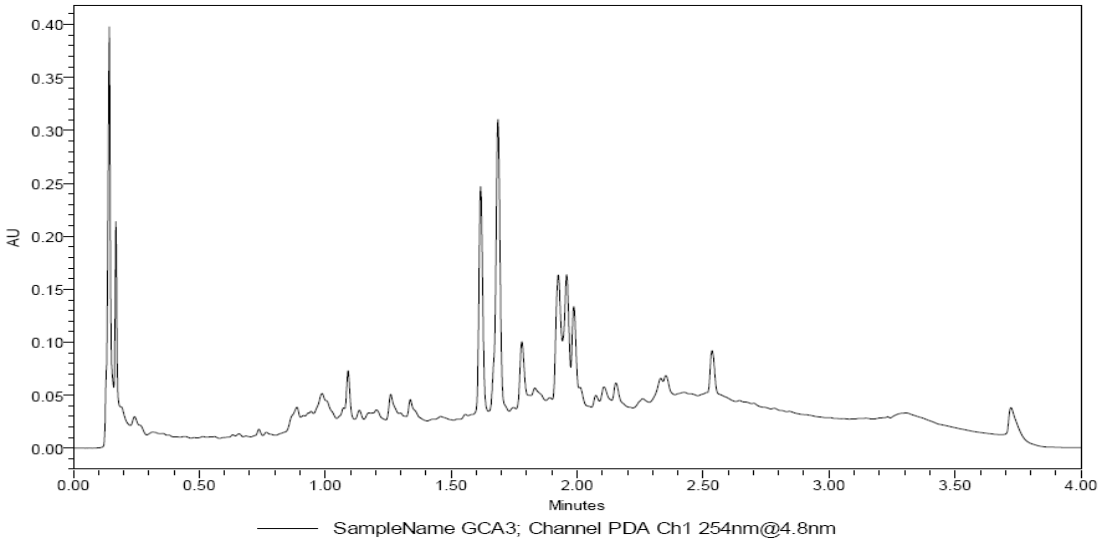


Figure 3.10 (b): UPLC profile of GCA 3 fraction collected from semi-preparative HPLC, of methanolic leaf extract of *Glycosmis calcicola* with a short column, C18 5 X 2.0 mm; conc. 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

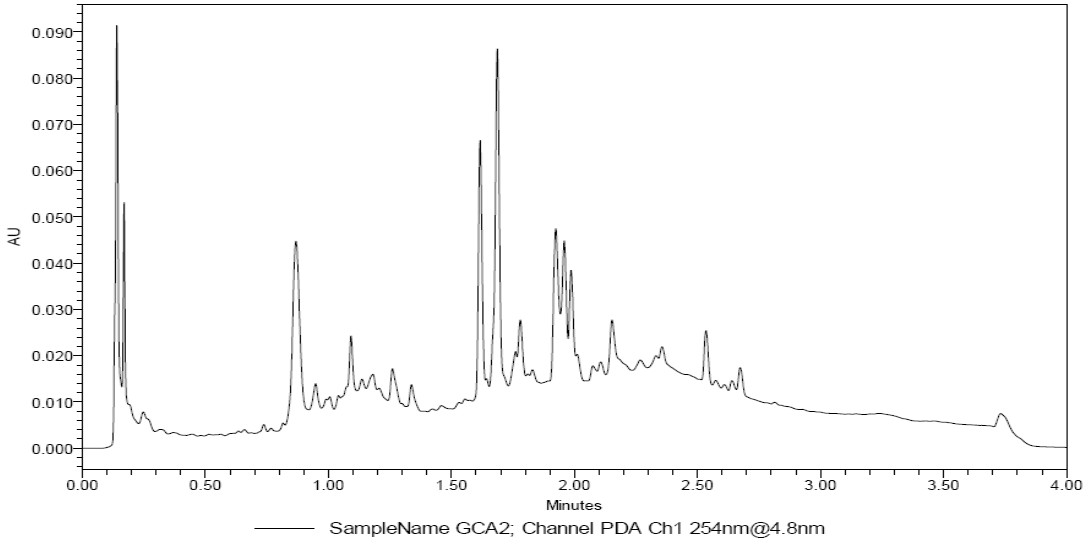


Figure 3.10 (c): UPLC profile of GCA 2 fraction collected from semi-preparative HPLC, of methanolic leaf extract of *Glycosmis calcicola* with a short column, C18 5 X 2.0 mm; conc. 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

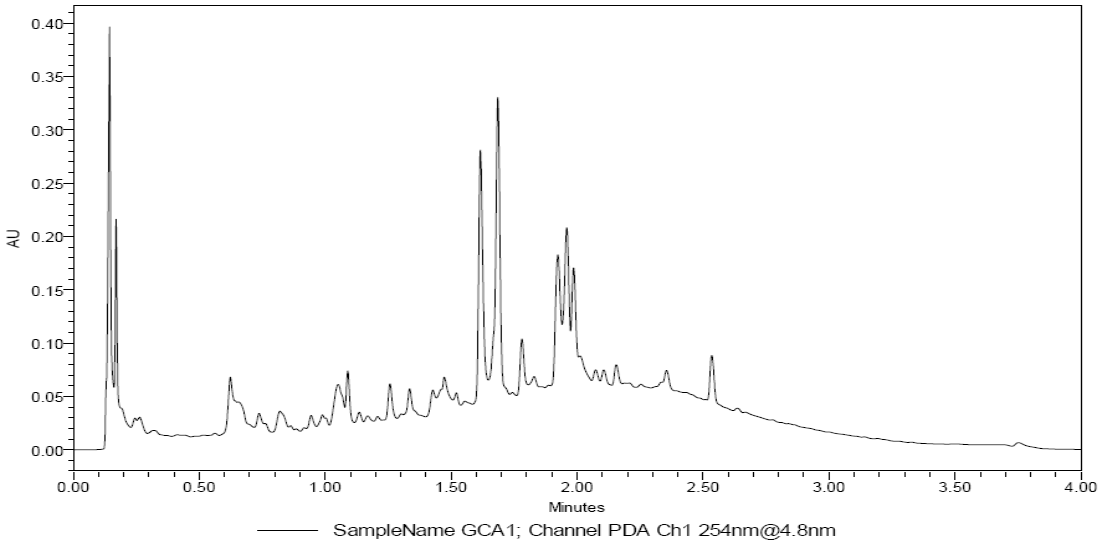


Figure 3.10 (d): UPLC profile of GCA 1 fraction collected from semi-preparative HPLC, of methanolic leaf extract of *Glycosmis calcicola* with a short column, C18 5 X 2.0 mm; conc. 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

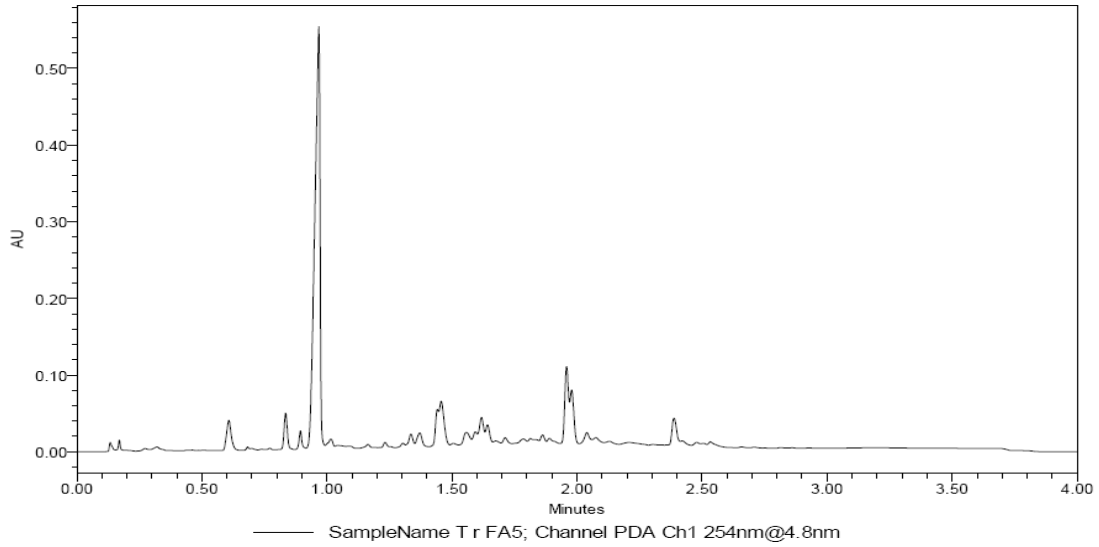


Figure 3.11 (a): UPLC profile of TRFA 5 fraction collected from flash column (methanolic leaf extract of *Tetractomia roxburghiana)* with a short column, C18 5 X 2.0 mm; conc. 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

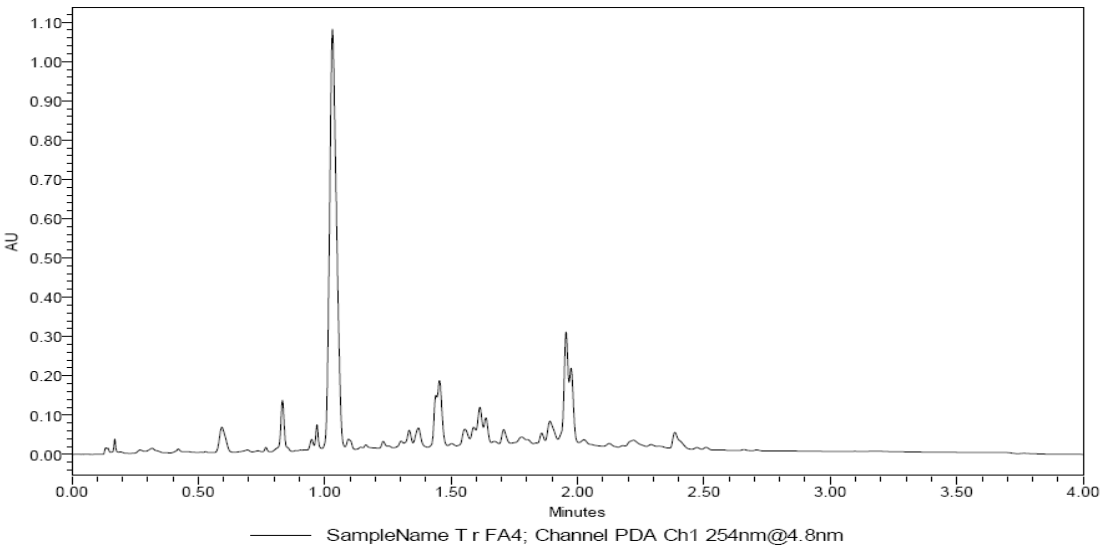


Figure 3.11 (b): UPLC profile of Tr FA4 fraction collected from flash column (methanolic leaf extract of *Tetractomia roxburghiana)* with a short column, C18 5 X 2.0 mm; con 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

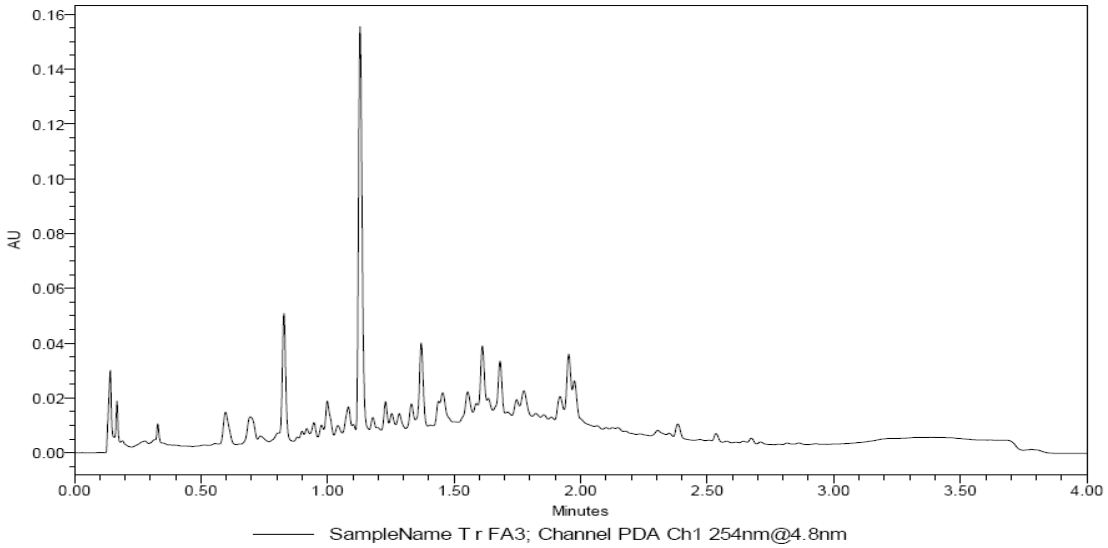


Figure 3.11 (c): UPLC profile of TrFA 3 fraction collected from flash column (methanolic leaf extract of *Tetractomia roxburghiana)* with a short column, C18 5 X 2.0 mm; con 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

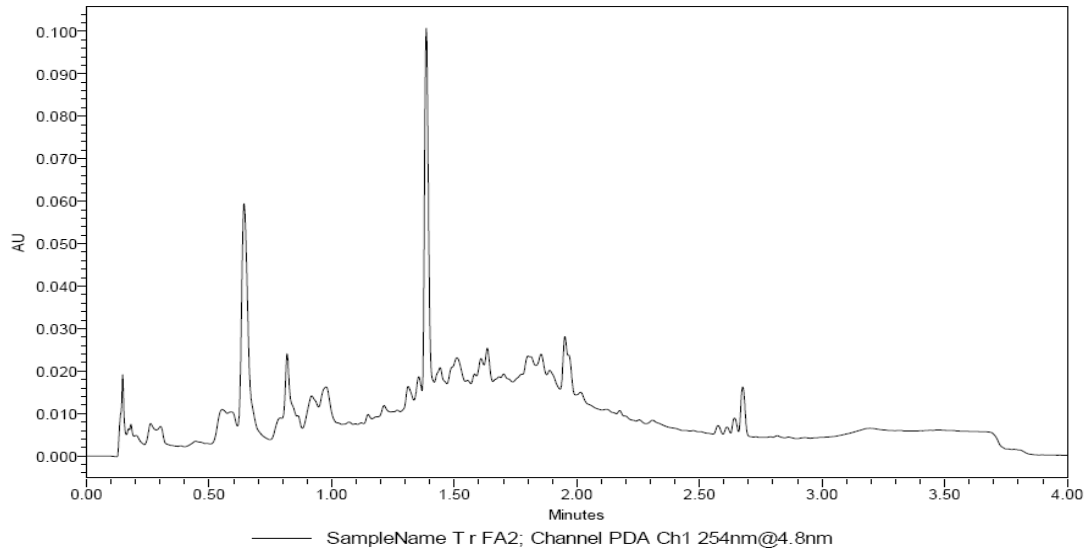


Figure 3.11(d): UPLC profile of Tr FA2 fraction collected from flash column (methanolic leaf extract of *Tetractomia roxburghiana)* with a short column, C18 5 X 2.0 mm; con 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

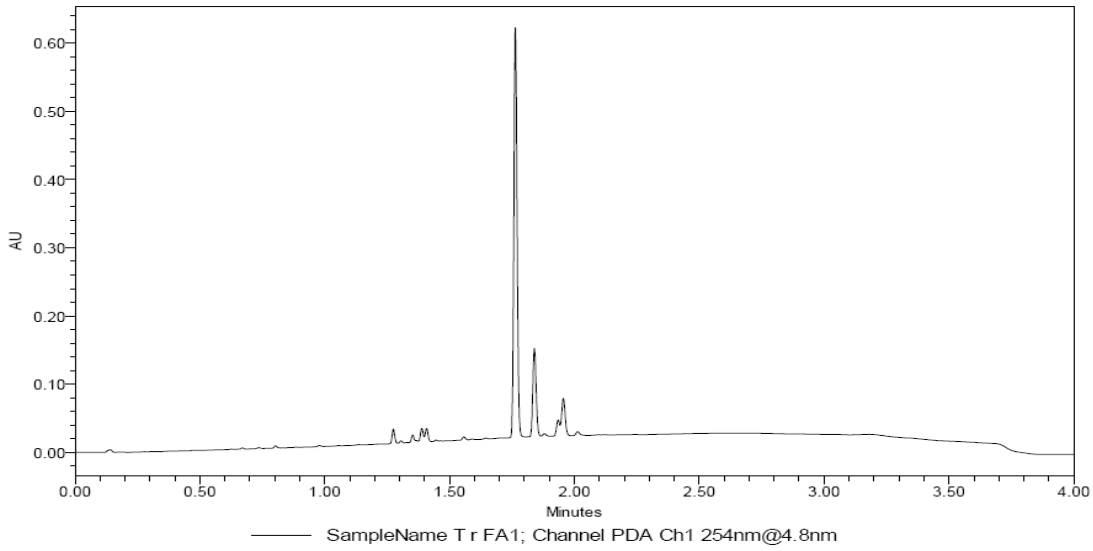


Figure 3.11 (e): UPLC profile of T RFA 1 fraction collected from flash column (methanolic leaf extract of *Tetractomia roxburghiana)* with a short column, C18 5 X 2.0 mm; con 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

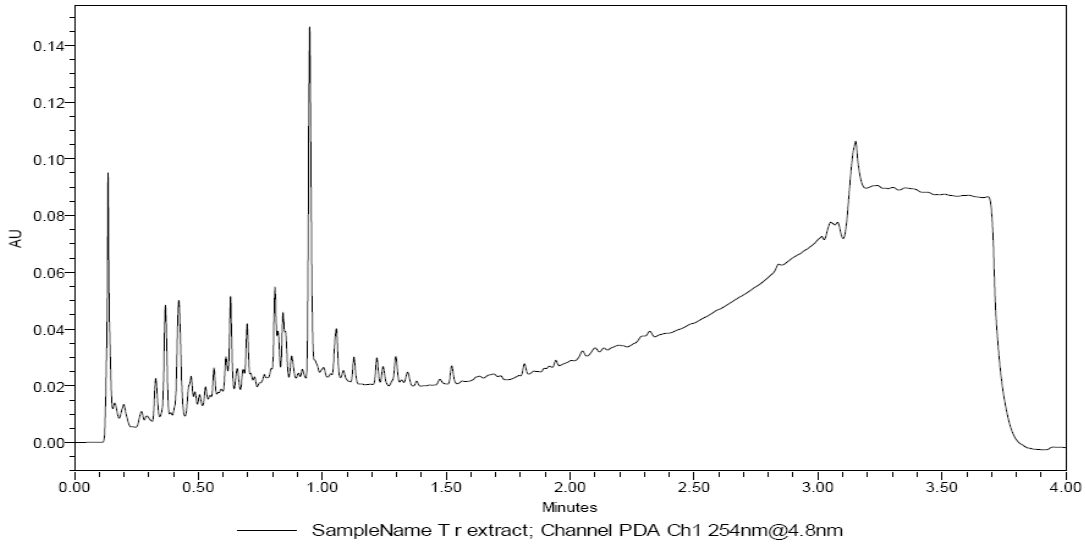


Figure 3.11 (f): UPLC profile of methanolic leaf extract of *Tetractomia roxburghiana,* with a short column, C18 5 X 2.0 mm; con 0.5 mg/ml, flow rate: 1.2ml/min; Injection volume 2µl, UV detection at λ_{max}:254 nm.

The samples, which were analysed by UPLC, were the ones, which had failed to give a positive match with library spectra when using accurate mass MS because they were mixtures. It was confirmed that this was very much the case for these fractions which had in general been from the peaks in between the major peaks.

All the LC approaches to monitoring fraction purity (RP-HPLC for RP-HPLC-NMR, ion exchange LC, UPLC) were successful in terms of being viable means of rapidly reviewing the composition of collected fractions. From the above chromatograms, (Figure 3.10(a), 3.10(b), 3.10(k)) it can be observed that better resolution (when compared to RP-HPLC) could be achieved within a short analysis time. Thus UPLC could serve as fast and economical analytical tool for isolation and identification of complex plant mixtures. Ultimately, the use of ion exchange LC-NMR (or perhaps even UPLC-NMR) might improve methods further with better resolution to accompany the NMR.

3.2.3.4 Reversed Phase Gradient High Performance Liquid Chromatography

Gradient reversed phase HPLC (grp-HPLC) was carried out on an integrated Agilent 1200 HPLC with a UV-diode array detector (DAD) and chemstation software (Agilent Technologies, Delaware, USA), using a C18 Ace column (250 ×4.6 mm i.d.,) . Mobile phase (A) was Methanol and Mobile phase (B) was water. Following parameters were used –

Mobile Phase	: Methanol-Water (gradient profile described in Table 3.1)
Injection volume	: 20µl
Column	: C18 Ace column (250 ×4.6 mm i.d.,)
Flow rate (ml/min)	: 1.0ml/min
Wavelengths	: 254 nm
Analysis time	: 110 min

Table 3.1 HPLC gradient profile

Time(min)	% solvent A (methanol)	% solvent B (Water)
0.00	10	90
10.00	15	85
70	85	15
80	90	10
110	0.00	100

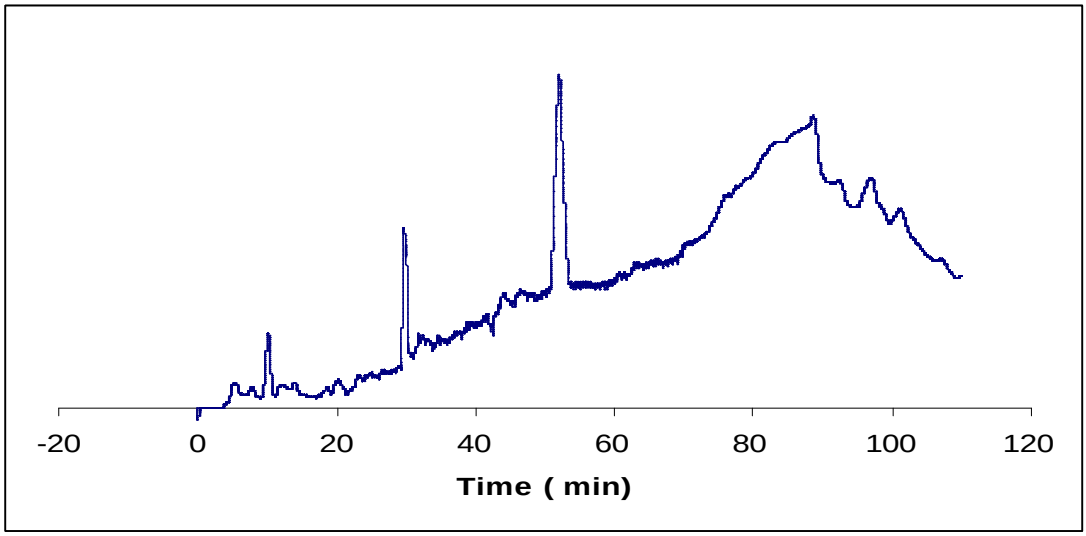


Figure 3.12: Reversed Phase Gradient High Performance Liquid Chromatography of methanolic leaf extract of *Tetractomia roxburghiana*, with a column, C18 250 X 4.6 mm; con 1 mg/ml, flow rate: 1.0 ml/min; Injection volume 2o µl, UV detection at λ_{max}:254 nm.

3.2.3.5 Micellar ElectroKinetic Chromatography (MEKC)

3.2.3.5.1 Introduction

Due to the very complex nature of plant extracts it was thought that it might be useful to go for a high-resolution separation technique like capillary electrophoresis as well as the LC approaches already described, in order to better assess the number of constituents in isolated fractions, the purity of components and also ultimately qualitative identification of the individual constituents based upon the relative retention times and more importantly with UV profile. This technique is very convenient and has more resolving power, which is quite essential for analysing multi-component mixtures usually found in plants [112].

Due to its high resolving power, it can overcome the problems of non-baseline resolution associated with high performance liquid chromatography. The main disadvantage of using CE analysis was that it could not be used for preparative work but doing capillary electrophoresis preparative work had never remotely been a consideration.

Capillary electrophoresis has been used for a long time as a highly efficient and alternative technique to HPLC in areas such as pharmaceutical analysis. It is widely used for separation to separate a complex array of large and small molecules like protein, peptides, urine metabolites etc. Compared to HPLC, CE provides fast and better

separation and requires less solvent. However, sensitivity of CE is less compared to HPLC.

Using Capillary electrophoresis in both its capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) modes allow the analyst to resolve both ionic (by CZE) and neutral (by MEKC) compounds on the same capillary using buffers with or without an organic modifier, a micelle or cyclodextrin. When compared to high performance liquid chromatography, capillary electrophoresis techniques simpler, faster, and more easily carried out.

According to literature reports [112-116] CE has been used for the analysis of widely different natural compounds from extracts of leaves and needles, bark, roots, marine organisms, moss, soil. CE and micellar electrokinetic chromatography (MEKC) had already been used for the separation of different chemical compounds like alkaloids, flavonoids, iso-flavonoids, coumarins, steroids, and saponins from different plant sources MEKC and CE with both UV and MS detection had been used for separation of different groups of alkaloids. Coumarins are biologically active compounds found in different plant families [114]. Seven coumarin derivatives were resolved with MEKC using borate / phosphate buffer at pH 7. Flavonoids are a large group of naturally occurring phenolic compounds consisting mainly of flavones, flavanols and their glycosides. These compounds are widely spread in most of the plant families. Dantuluri *et al.* [116] reported the separation of a mixture of sulphated flavanoids and flavonoids, (-) - epicatechin sulphate (ECS), (+) - catechin sulphate (CS), quercetin sulphate (QS),

apigenin sulphate (AS), and 6,2′,3′-flavonoid sulphate (FS). Olesjek reported the optimal conditions for separation of standard saponins [113] were found to be 150 mM sodium phosphate buffer at 25° under these conditions complete separation was achieved in seven min. Li *et al.*, reported rapid determination of aristolochic acid I and II [117] in *Aristolochia* plants from different regions by β-cyclodextrin modified capillary zone electrophoresis using 120 mM sodium borate buffer (pH 8.8) containing 10 m M β-CD under 10°C temperature and 30 KV applied voltage. Y. Cao *et al*., reported separation and quantification of swertiamarin, mangiferin and chlorogenic acid for the differentiation of *Swertia musoti franch* from *Artemisiae capillaris herba* by a capillary electrophoresis method with electrochemical detection [118].

3.2.3.5.2 Basic Principles

Electrophoresis is the migration of charged analytes in an electrically conducting medium under the influence of an electric field. In capillary electrophoresis, the medium consists of a solution of buffer salts adjusted to a desired pH. This buffer solution is contained within a fused silica capillary. When a current is applied, cations in the middle are attracted to the cathode. These hydrated cations will drag aqueous buffer with them towards cathode. Then the uncharged species move at the same speed as the electro osmotic force, positively charged ions move faster and negatively charged ions move slower. A basic CE system consists of two buffer reservoirs, a detector, a capillary with a transparent detection window, and a high voltage power supply. Commonly the sample is introduced into the capillary by hydrodynamic or electrokinetic means. This involves

either applying a small positive pressure in the inlet or a potential difference across the capillary.

3.2.3.5.3 Aims and Objectives

The general aim of the use of CE was to carry out high resolution profiling of extracts and in particular to assess the purity of "semi-purified" or preparative LC purified fractions of extracts. This would be achieved by first carrying out a more typical application of CE as a familiarisation exercise, using that application to study the effect of changing variables in several generic MEKC systems and then using the MEKC systems to profile a prepared mixture of "typical medicinal plant constituents".

3.2.3.5.4 Experimental

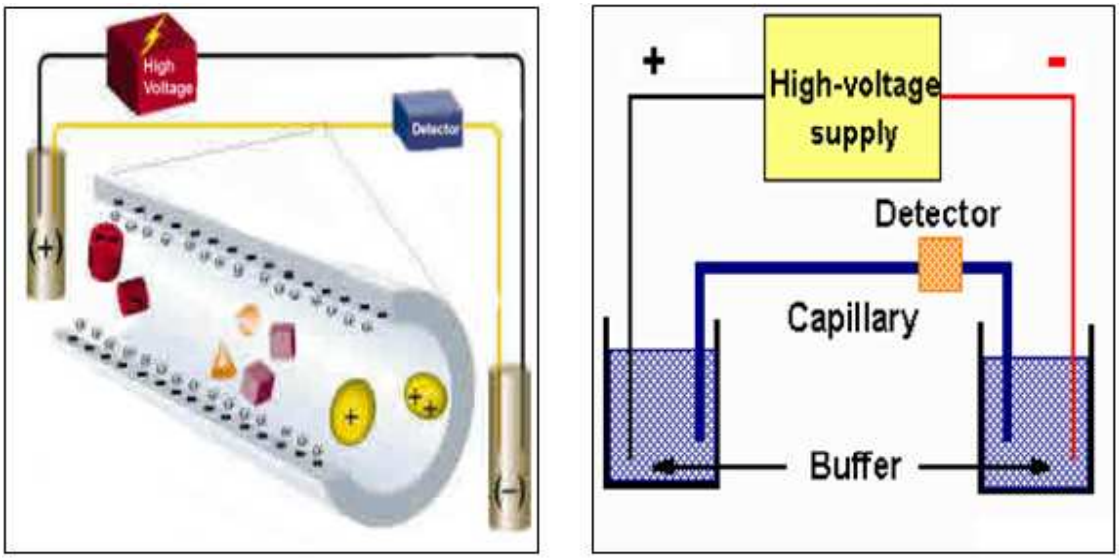


Figure 3.13: Schematic of a capillary electrophoresis System
Source: www.cstl.nist.gov/biotech/strbase/NTST pub.html

3.2.3.5.4.1 CE Instrumentation

Capillary electrophoresis was carried out on an Agilent CE system, which was equipped with a UV diode-array detector. Bare, fused silica capillaries (56 cm × 50 μm) micro extended light path were used for the separations. Sample and buffer solutions were filtered with 0.45 μm Acrodisc syringe filters (Pall Corporation, Ann Arbor, MI, USA) prior to use.

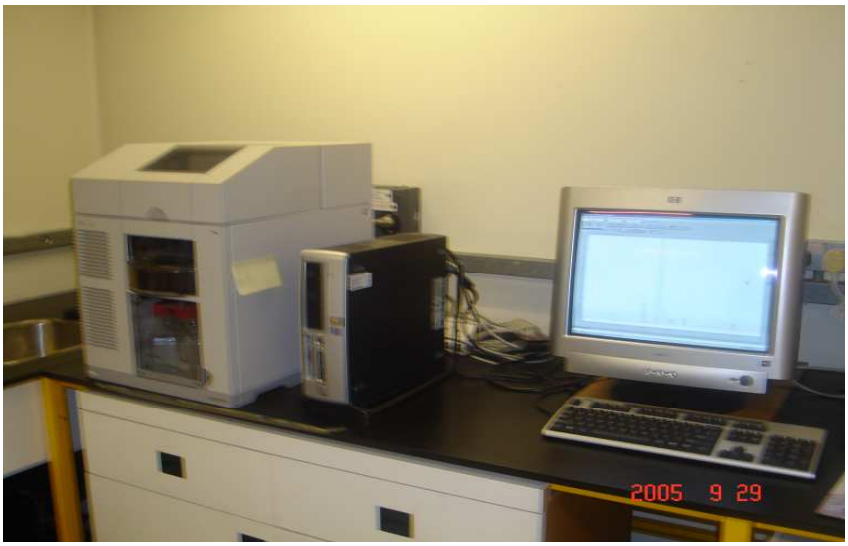


Figure 3.14: Agilent 1200 CE system

3.2.3.5.4.2 Materials and Reagents

In the CE experiments, buffers were prepared using sodium dihydrogen phosphate and sodium borate that was purchased from Sigma-Aldrich (poole, Dorset, UK) and water that had been double distilled and deionised with an Elga Option 3 system followed by an Elgastat UHQPS system (Elga Limited, High Wycombe, UK). Sodium tetraborate

(Sigma-Aldrich), sodium dodecyl sulphate (Sigma-Aldrich), sulphated β-cyclodextrin (Sigma-Aldrich), NaOH, sodium dihydrogen phosphate (Sigma-Aldrich), sodium cholate (Sigma-Aldrich), were used in the preparation of run buffers.

3.2.3.5.4.3 Run buffer systems evaluated

Cholate / SCD buffer [119]: 0.1 M sodium cholate and 6.25 mM sulphated β-cyclodextrin with each 20 mM sodium dihydrogen phosphate and sodium tetraborate (20 mM MEKC).

SDS / SCD [119]: Electrolyte comprised of 25 mM sodium borate, 75 mM SDS and 6.25 mM sulphated β-cyclodextrin at pH adjusted to 9.50 with 1M NaOH after the addition of SDS and sulphated β-cyclodextrin.

Cholate buffer [120]: Sodium cholate MEKC system consists of 20 mM Sodium tetra borate at pH 8.9 with 0.2 M sodium cholate.

SDS buffer [120]: 20 mM SDS in 200 mM sodium tetraborate at pH 9.1

Temp. 25°C, voltage 14 kV.

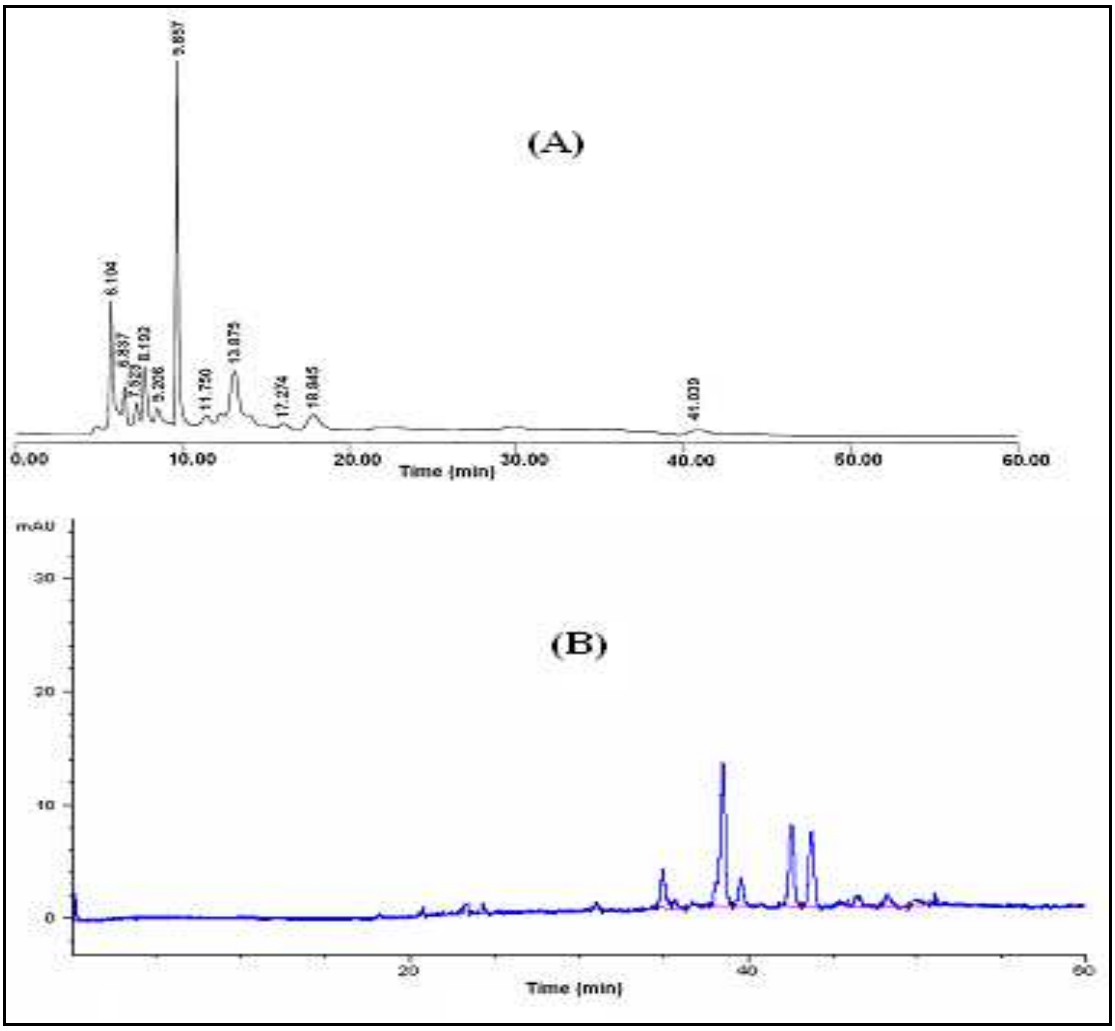


Figure 3.15: (A) Analytical reversed phase HPLC profile of methanolic leaf extract of *Tetractomia roxburghiana* with a mobile phase of methanol-water (20:80, v/v) C18 250 X 4.6 mm; con 1mg/ml, flow rate: 1.5ml/min; 254 nm. (B) Electropherogram of methanol leaf extract of *Tetractomia roxburghiana* Capillary: (56 cm × 50 µm), Voltage 14 kV, Temp 25°C. Run buffer: Sodium cholate MEKC system consists of 20 mM sodium tetraborate at pH 8.9 with 0.2 M sodium cholate.

Note: Increased resolution observed in the electropherogram(marked increase in separation and had longer elution time) than in the HPLC proile

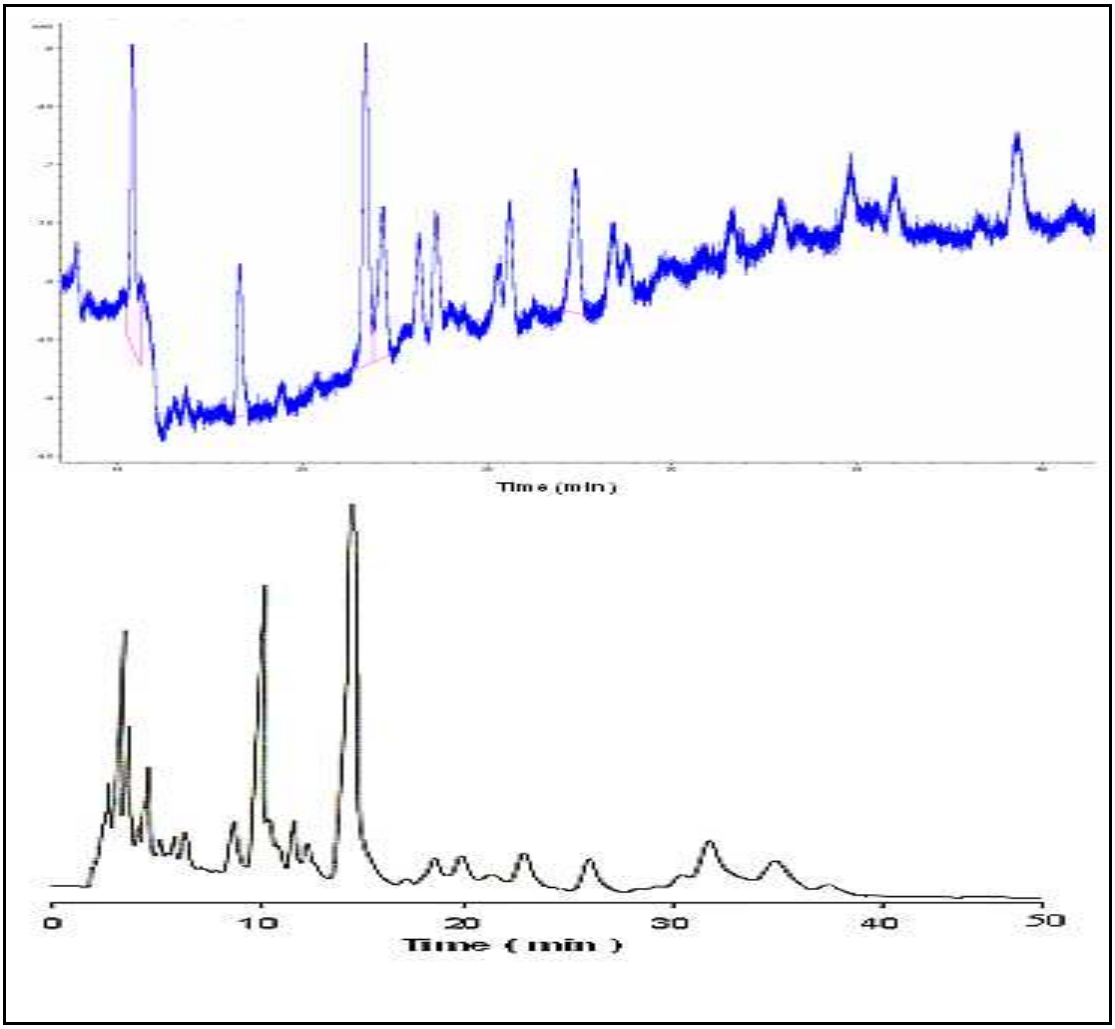


Figure 3.16: (A) Electropherogram of methanol leaf extract of *Glycosmis calcicola* Capillary: (56 cm × 50 µm), Voltage 14 kV, Temp 25°C. Run buffer: Sodium cholate MEKC system consists of 20 mM sodium tetraborate at pH 8.9 with 0.2 M sodium cholate. and (B) semi-preparative reversed phase HPLC profile of methanolic leaf extract of *Glycosmis calcicola* with a mobile phase of methanol-water (20:80, v/v) C_{18} 250×7 mm; conc. 1. 5 mg/ml, flow rate: 1.5ml/min; λ_{max}: 245 nm.

Note: Better resolution was observed in electropherogram but the base line noise was high.

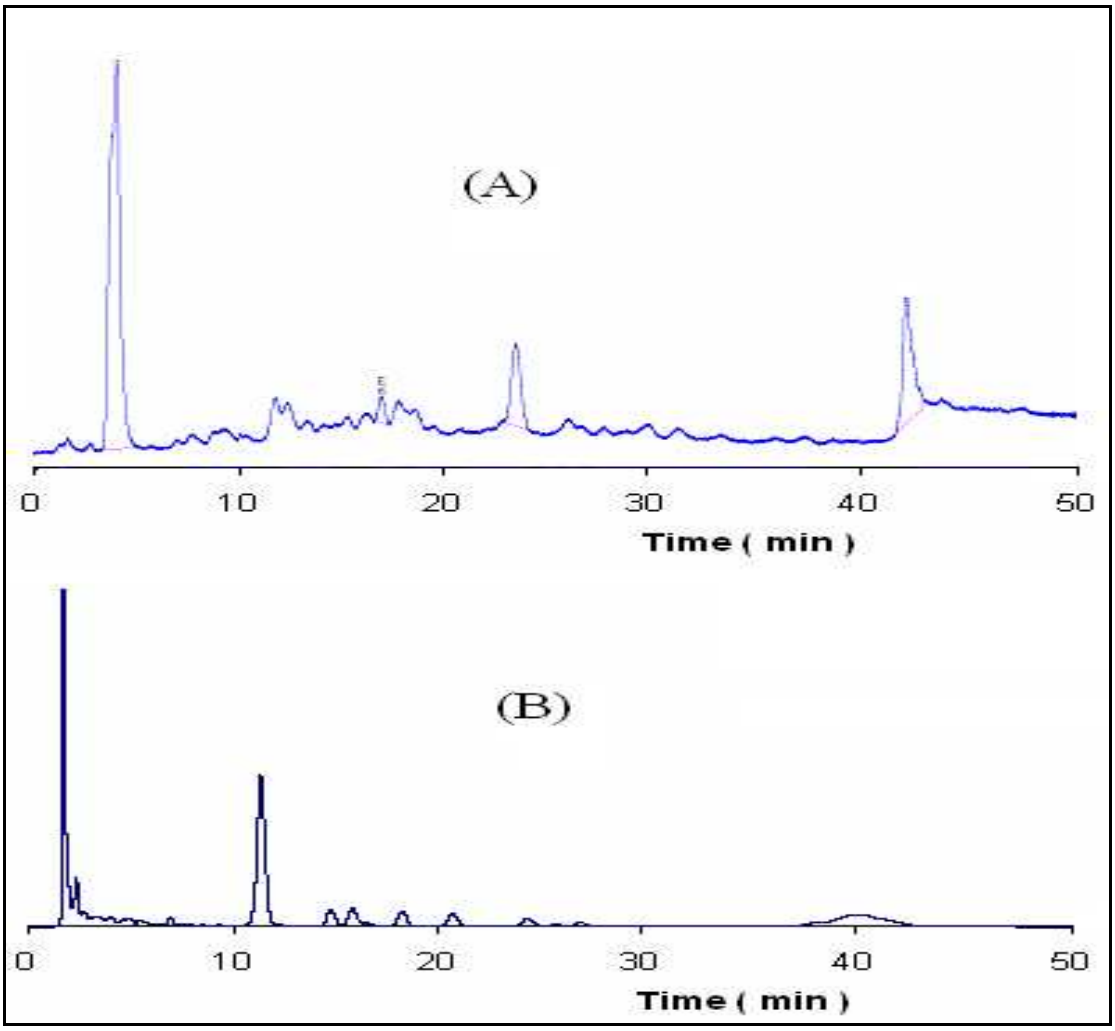


Figure 3.17: (A) Electropherogram of methanol leaf extract of *Clausena excavata* Capillary: (56 cm × 50 µm), Voltage 14 kV, Temp 25°C. Run buffer: Sodium cholate MEKC system consists of 20 mM sodium tetraborate at pH 8.9 with 0.2 M sodium cholate. and (B) Analytical reversed phase HPLC profile of methanolic leaf extract of *Clausena excavata* with a mobile phase of methanol-water (20:80, v/v) C_{18} 250×4.6 mm; conc. 1. 5 mg/ml, flow rate: 1.5ml/min; λ_{max}: 245 nm.

Note: As an exception here, HPLC profile was much better resolved than the electropherogram.

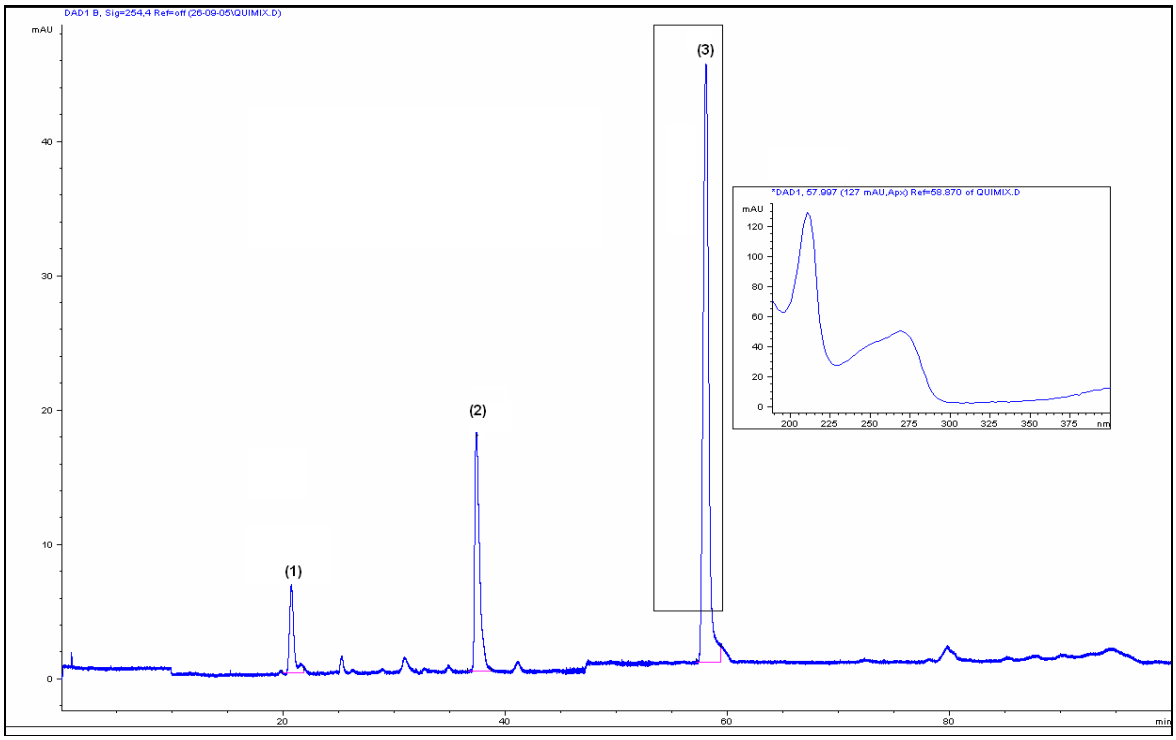


Figure 3.18: Typical electropherogram of mixture of three flavonoids, rutin, 2, 6-dihydroxy flavone and quercitin. concentration ranging from 100-200 µg/ml. CE conditions Capillary: (56 cm × 50 µm), Voltage 14 kV, Temp 25°C. Run buffer: SDS / SCD buffer. Electrolyte comprised of 25 mM sodium borate, 75 mM SDS and 6.25 mM sulphated β-cyclodextrin at pH adjusted to 9.50 with 1M NaOH after the addition of SDS and sulphated β-cyclodextrin

Table 3.2: Migration times of typical plant metabolites in cholate/SCD buffer. (0.1 M sodium cholate and 6.25 mM sulphated β-cyclodextrin with each 20 mM sodium dihydrogen phosphate and sodium tetraborate (20 mM MEKC)).

Migration time (mins) of Plant Metabolites in B1 Buffer		
Flavonoid	Rutin	18.3 mins
	Quercetin	30.4
	Chrysin	44.8
	Myricitin	47.1
	Apigenin	40.1
Flavonoid Mixture	Rutin	17.9
	Quercetin	28.0
Alkaloids	Morphine	42.8
	Quinine	72.0
	Physostigmine	78.0
	Reserpine	82.1
A.Mixture	Morphine	41.8
	Quinine	73.0
	Physostigmine	82.1
	Reserpine	92.0
Steroids	Lanosterol	78.5
	Stigmasterol	80.0
	Sitosterol	82.0
Quinolide Mixture	plumbagin	58.0
	Juglone	20.7
Others	Theobromine	13.1
	Piperine	78.6
	Digoxin	72.5

Note: Migration times of plant metabolites in B 1 buffer provide a useful dataset to compare the migration time of unknown sample and conclude which family it might belong.

Table 3.3: Migration times of typical plant metabolites in SDS / SCD buffer. Electrolyte comprised of 25 mM sodium borate, 75 mM SDS and 6.25 mM sulphated β-cyclodextrin at pH adjusted to 9.50 with 1M NaOH after the addition of SDS and sulphated β-cyclodextrin

Migration time (mins) of Plant Metabolites in B2 Buffer		
Flavonoid	Rutin	39.4
	Quercetin	73.6
	Chrysin	75.0
	Myricitin	70.0
	Apigenin	45.0
Flavonoid Mixture	Rutin	25.0
	Quercetin	28.0
Alkaloids	Morphine	42.8
	Quinine	72.0
	Physostigmine	78.0
	Reserpine	82.1
A.Mixture	Morphine	52.8
	Quinine	34.6
	Physostigmine	21.7
	Reserpine	No data
Steroids	Lanosterol	26.9
	Stigmasterol	No data
	Sitosterol	No data
Quinolide Mixture	plumbagin	39.9
	Juglone	42.1
Others	Theobromine	17.9
	Piperine	59.6
	Digoxin	60.1

Note: Migration times of plant metabolites in B 1 buffer provide a useful dataset to compare the migration time of unknown sample and conclude which family it might belong . It can be observed that migration time of flavonoids increased , steroids decreased compared to the B 1 buffer, whereas alkaloids and quinolides did not much effected.

Table 3.4: Migration times of typical plant metabolites in cholate buffer. Sodium cholate MEKC system consists of 20 mM sodium tetraborate at pH 8.9 with 0.2 M sodium cholate

Migration time (mins) of Plant Metabolites in B3 Buffer		
Flavonoid	Rutin	41.2
	Quercetin	26.4
	Chrysin	21.2
	Myricitin	No data
	Apigenin	32.5
Flavonoid Mixture	Rutin	35.0
	Quercetin	26.0
Alkaloids	Morphine	27.9
	Quinine	3 4.7
	Physostigmine	21.8
	Reserpine	43.0
A.Mixture	Morphine	52.8
	Quinine	34.6
	Physostigmine	21.7
	Reserpine	42.5
Steroids	Lanosterol	No data
	Stigmasterol	No data
	Sitosterol	No data
Quinolide Mixture	plumbagin	No Data
	Juglone	No Data
Others	Theobromine	21.9
	Piperine	57.2
	Digoxin	37.7

Note: Migration times of plant metabolites in B 3 buffer provide a useful dataset to compare the migration time of unknown sample and conclude which family it might belong plus when compared to B 1 and SDS buffers the migration time of flavonoids and alkaloids were decreased.

3.3 Results and Discussion

The main problem encountered using HPLC of plant extracts and fractions was that with the higher loads, the resolution achieved with the microinjections could not be seen in the higher loads due to the complex nature of plant extracts and multi-component mixtures.

It was clearly desirable to search for a high-resolution technique so that it could be used to resolve all the components of plant extracts and also to assess the purity of the components. With the selection of different micellar electro kinetic chromatography (MEKC) buffers, the first aim was to know how the buffers performed when changes in experimental variables were made. This was done with the urine components because capillary electrophoresis had long been used for analysis of urine. Once after applying all the generic MEKC methodologies to the urine components it could be seen how. e.g. the resolution and peak sensitivity parameters will be affected.

It was observed that in the case of cholate buffer good resolution was obtained but the main disadvantage was the long migration times. However, the initial aim was to establish resolution so that, after achieving this, higher voltages would then be applied to shorten the migration times of the components. A comparative study of the selected buffers using some of the peaks of urine (labelled as B, C, D, E, F, G, H, I, J) to investigated the peak parameters like migration time, peak area, peak height, and half height peak width was conducted (Appendix, Tables 3.5, 3.6 and 3.7). By using these buffers it was possible to assess how different peak parameters responded to the different load times of 10 sec, 20 sec, 30 sec, 40 sec and 50 seconds in order to establish

for each buffer the extent to which limits of detection could be improved by using peak stacking. Based on the investigation it was concluded that the cholate buffer gave better resolution and peak sensitivity whereas, at the other end at the scale, SDS buffer blocked the capillaries. CE work on urine was concluded by carrying out the MEKC analysis of standard urine metabolites in order to establish the migration times and build up libraries. In this way the capabilities, advantages and disadvantages of each buffer system had been well explored for the complex mixture urine samples, to which MEKC is more often applied, before moving on to the complex mixture extract samples, for which MEKC has less frequently been applied. The migration times of these have been shown in the (Appendix table 3.8). Monitoring of fraction purity was assessed by capillary electrophoresis and the electropherograms were shown in the (3.19 (a) and 3.19 (b) 3.19 (c))

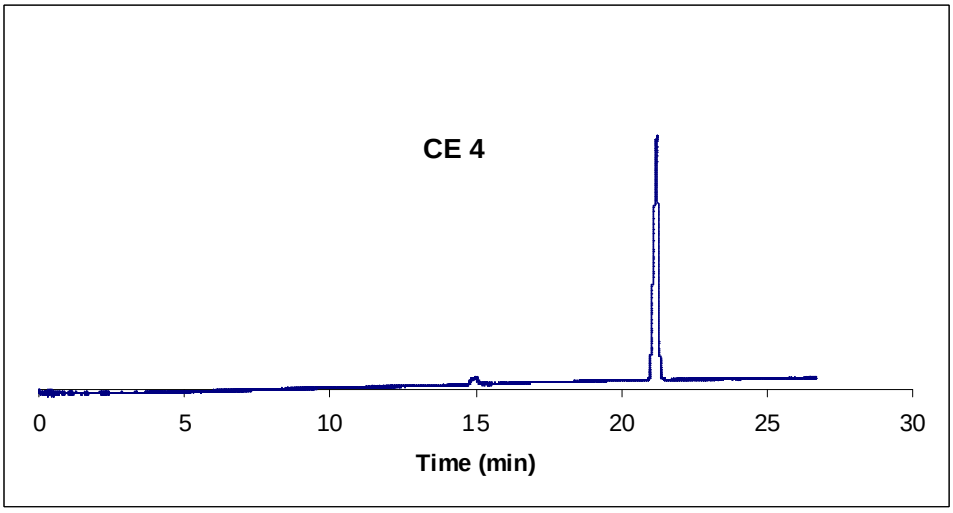


Figure : 3.19 (a) Electropherogram of CE 4 fraction obtained from preparative HPLC , concentration ranging from 100-200 µg/ml. CE conditions Capillary : (56 cm × 50 µm), Voltage 14 kV, Temp 25°C. Sodium cholate MEKC system consists of 20 mM sodium tetraborate at pH 8.9 with 0.2 M sodium cholate

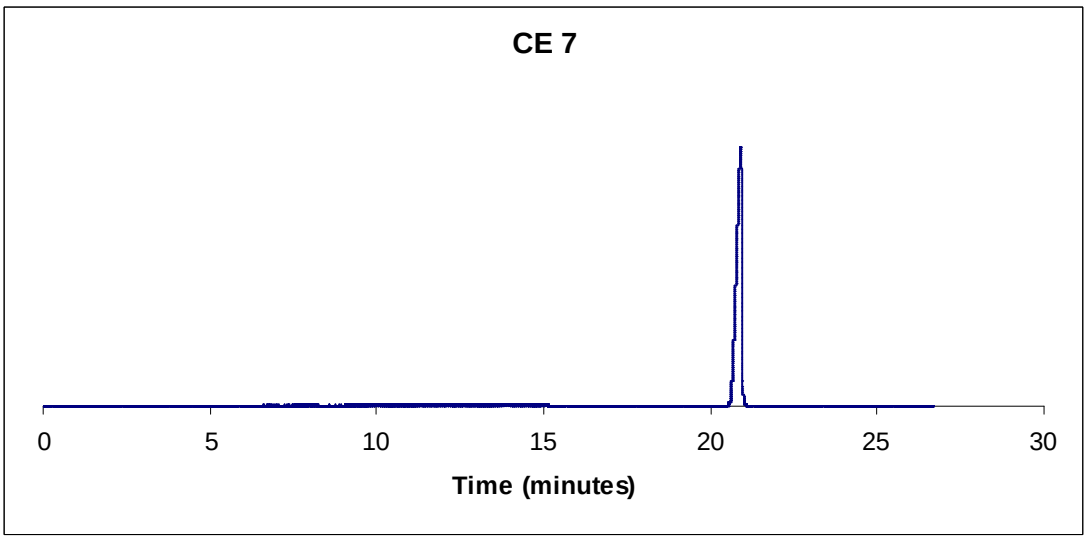


Figure: 3.19 (b) Electropherogram of CE 7 fraction obtained from preparative HPLC, concentration ranging from 100-200 µg/ml. CE conditions Capillary: (56 cm × 50 µm), Voltage 14 kV, Temp 25°C. Sodium cholate MEKC system consists of 20 mM sodium tetraborate at pH 8.9 with 0.2 M sodium cholate

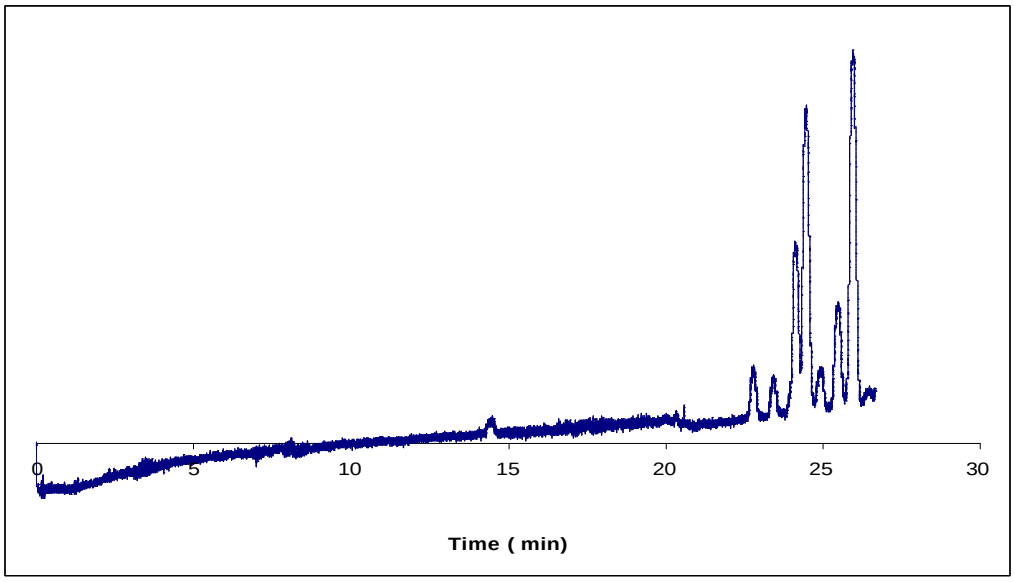


Figure: 3.19 (c) Electropherogram of CE 4 fraction obtained from preparative HPLC, concentration ranging from 100-200 µg/ml. CE conditions Capillary: (56 cm × 50 µm), Voltage 14 kV, Temp 25°C. Sodium cholate MEKC system consists of 20 mM sodium tetraborate at pH 8.9 with 0.2 M sodium cholate

Initial work on plant extracts was performed by running standard mixtures of typical plant metabolites for the comparison of these buffers with respect to the migration of different classes of plant metabolites. Separation of standard mixtures of flavonoids, quinolids, steroids and other mixtures were established and it was observed that, in general alkaloids came first followed by flavonoids (Tables 3.2, 3.3 and 3.4). Capillary electrophoresis separations, using the optimum cholate / SCD buffer, of methanolic leaf extracts of rutaceous plants were carried out and compared with separation with that of the HPLC profile. CE had given higher resolution as expected but still the non-polar components had not been resolved, particularly the standard steroidal compounds, with MEKC buffers. Nonetheless a suitable generic MEKC system, along with some possible alternatives, had been established and this was used to study the composition of other extracts, both semi-purified and purified.

Capillary electrophoresis and MEKC provided high-resolution separations for the methanolic leaf extracts of three rutaceous plants when compared with their HPLC profiles. Although CE with UV photodiode array detection is a good confirmation method of known compounds. CE-ESI –MS is a good characterisation method of unknown compounds. At this point capillary electrophoresis method development for identification of plant metabolites is confined to the achievements of high-resolution separations, and establishment of migration times of common known typical plant metabolites have been achieved. In the case of plant metabolites no single buffer is capable of eluting all the classes of compounds in three buffer used (This area needs further investigation) Alkaloids best resolved in Sodium cholate system (B3 buffer) In

B2 buffer alkaloids are not resolved properly. The migration times are quite high in three buffers run.

One more problem encountered for identification of plant metabolites was that UV profiles could not be established for all the eluted peaks; this area still needs to be investigated. However, the combined use of the profiles of HPLC and MEKC that gave an idea about the efficiency of preparative separation. With these CE experiments, it could be said that polar compounds had been easily eluted where as non-polar components elution was needed to be further investigated. Further work could be carried out by altering the composition of sulphated beta cyclodextrin in MEKC method, or by looking at non-aqueous CE.

3.4 Conclusions

In general, the usefulness of the high resolving power of MEKC had been confirmed. However, experimental / instrumental difficulties were encountered more often than in the LC approaches to checking fraction purity. More importantly (a) it was much slower than the equally highly resolving UPLC, and (b) the selectivity of MEKC was similar to that of RP-HPLC rather than orthogonal like ion-exchange HPLC. Nonetheless, given that the UPLC was not permanently available, MEKC was a useful complimentary approach to the LC approaches.

4 Isolation, Identification and Structural elucidation of components of *Tetractomia roxburghiana*

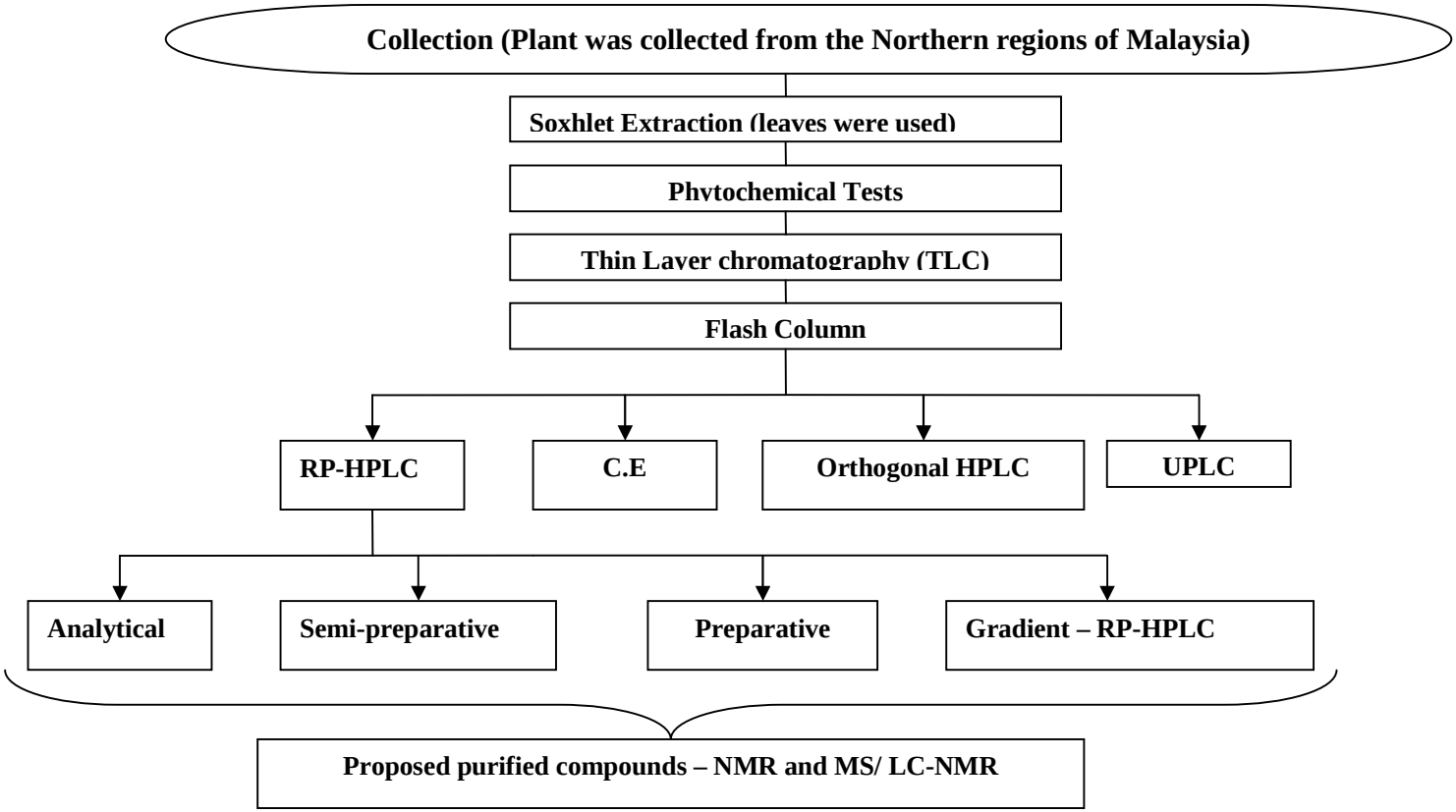


Fig 4.1 – Flow Chart of Separation techniques and Test Used in Chronological Order For The Methanolic Leaf Extract of Tetractomia roxburghiana

4.1 Introduction

The genus Tetractomia belongs to the kingdom: plantae, division: magnoliophyta, class: magnoliopsida, order: Sapindales, family: Rutaceae, species: roxburghiana. The plants of the genus *Tetractomia* are trees or shrubs. The current investigation aims to separate, characterise and identify the major chemical constituents of methanolic leaf extracts of *Tetractomia roxburghina* by combining traditional and state-of-the-art techniques in separation science and spectroscopic characterisation and interpretation.

The literature search suggests that phytochemical characterisation has not been reported for this plant. Not even a single chemical constituent has been identified or isolated. Therefore, the phytochemical investigations were carried out on the methanol leaf extract of this plant in order to identify and isolate the plant metabolites. Thus, the strategy was to subject leaves to solvent extraction with several different solvents of increasing polarity. The crude extracts were first separated by column chromatography and then evaluation by HPLC for the number of components in each fraction, followed by HPLC purification. The purified extracts were subjected to NMR and MS methods to characterise and identify the components.

4.2 Experimental

4.2.1 Materials and methods

The plants were collected from the Northern regions of Malaysia, leaves were separated and the plants were authenticated by the Department of Pharmacognosy, University of Sunderland, identified by Bowen *et al* [58] and a voucher specimen sample was deposited. The powdered materials were stored in airtight plastic containers for further studies. The samples were dried in a shade and powdered. The powdered leafy material was extracted in Soxhlet apparatus using methanol as a solvent. The crude extracts were evaporated to dryness in a rotary-evaporator (Buchii) under lower temperature and reduced pressure to obtain crude dried extract of individual plant.

4.2.2 Plant Material

Part air-dried, leafy plant material (100 g) was powdered and extracted with different solvents (from non-polar to polar). The solvents were partly removed *in vacuo* below 50° C and freeze-dried to give extracts. The solvents used were hexane, petroleum ether, chloroform, dichloromethane, methanol, ethanol and water. The extractive values of different solvents were tabulated below.

Table 4.1: Percent (%) Extractive values of leaves of *Tetractomia roxburghiana* using different solvents.

Plant	Part	Solvent for extraction	Extractive values (% w/w)
Tetractomia roxburghiana	Leaves	Hexane	0.05
		petroleum ether	0.09
		chloroform	1.11
		Dichloromethane	0.9
		methanol	5.4
		ethanol	1.25
		water	2.4

4.2.3 Soxhlet Extraction

The powdered leafy plant material (100 g) was successively extracted with petroleum ether, hexane, chloroform, dichloromethane, ethanol and water. The volume of the solvent used in each case was 500 ml. Each time before extraction with the next solvent, the powdered material was air-dried, and then weighed. In the above extracts the largest quantity of the extract was obtained with methanol whereas with the petroleum ether, hexane, chloroform, dichloromethane, ethanol solvents the extract obtained was in smaller amounts. Further study was therefore carried out on the methanolic leaf extract of *T. roxburghiana.*

Table 4.2: Three plants selected for phytochemical and microbiological study, their voucher no. part used, method, solvent for extraction, nature and colour of extract tabulated.

Sr. No.	Plant	Voucher No.	Plant Part	Method of extraction	Solvent for extraction	Extract colour and nature
1	*T.roxburghiana*	TRB-1	Leaf	Hot	Methanol	yellowish powdery
2	*Glycosmis calcicola*	GCM-2	Leaf	Hot	Methanol	Dark Green Gummy
3	*Clausena excavata*	CEM-3	Leaf	Hot	Methanol	Dark Brown Gummy

4.3 Preliminary Phytochemical screening

4.3.1 Introduction

Plants consist of two types of chemicals, primary metabolites such as sugars, amino acids, proteins and chlorophyll. The second category is called secondary metabolites such as alkaloids, glycosides, flavonoids, steroids terpenoids, saponins, tannins and phenolic compounds. These secondary plant metabolites often have a significant physiological role in the mammalian system.

4.3.2 Preparation of Reagents for Phytochemical study

The following reagents and solutions, which are required for the preliminary phytochemical study, were prepared. The methanolic leaf extracts obtained from *Tetractomia roxburghiana*, preliminary phytochemical screening for identification of range of chemical constituents / plant secondary metabolites were studied qualitatively by methods of Harborne [121]. The results of the phytochemical tests were shown in Table-4.3. Preparation procedure for the reagents and solutions are described in the appendix chapter.

Table 4.3: Phytochemical tests for secondary metabolites (Rutaceous extracts)

Sr. no	**Plant**	**Part of plant**	**Solvent for extraction**	**Secondary metabolites**					
				S	**A**	**F**	**P**	**G**	**Sa**
1	*Tetractomia roxburghiana*	leaves	methanol	-	-	+	+	-	+

S – Steroids; A – Alkaloids; F – Flavonoids; P – Phenolics; G – Glycosides; Sa – Saponins. + – Positive, - – Negative

4.3.3 Results and Discussion

The methanolic leaf extract of *Tetractomia roxburghiana* was used for the preliminary phytochemical screening of the identification of plant secondary metabolites/active constituents. The results indicate that preliminary phytochemical investigation of the

methanolic leaf extracts showed positive results for phenolics, sterols, flavonoids and tannins. In the consequent chapters, the analytical technology used for the identification and isolation of major constituents from the above methanolic leaf extracts were discussed.

4.4 Thin Layer Chromatography studies

Thin layer chromatographic studies were carried out on extracts prior to column chromatography not only to assess the number of constituents present but also to help select a suitable solvent system for the column chromatography that would follow [122].

4.4.1 Preparation of Extract solution

Powdered material (1g) was extracted with 10 ml methanol for 30 minutes under reflex on the water bath. The filtrate was evaporated to about 1 ml and 20 μl was used for the investigation.

4.4.2 Application of spots

The spots of extract were applied on the TLC plate at a distance of about 2 cm from the bottom and allowed to dry in air. The distance between two spots was kept to at least 10 mm so that the components did not mix.

4.4.3 Saturation of chamber

The solvent was poured into a chamber and a filter paper was placed into the chamber to get rapid saturation.

4.4.4 Detection reagents

The following reagents were prepared 5 %, 10 % ethanolic H_2SO_4, 50 % ethanolic H_2SO_4 , conc. H_2SO_4, iodine reagent was prepared by about two to three grams of solid iodine was spread on the bottom of a chromatographic tank; the developed plate was placed into the tank and exposed to iodine vapour. All compounds containing conjugated double bonds gave yellow-brown zones. vanillin-sulphuric acid reagent (vs.)1% ethanolic vanillin (solution 1), 10% ethanolic sulphuric acid (solution II)The plate was sprayed with 10ml solution I followed by solution II Immediately by 10 ml solution II. After heating at 110° C for 5-10 min under observation, the plate was evaluated [122].

The methanolic extract obtained from *T.roxburghiana* was subjected to thin layer chromatography. Broad bands with tailing were observed with different solvent proportions petroleum ether and ethyl acetate (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100.) and methanol and ethyl acetate (95:5, 90:10, 85:15, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 100:0, v/v) but satisfactory results were not obtained with TLC, as a tailing effect was observed. Typical solvent

systems described in the literature for the separation of various secondary metabolites were tried. The TLC plates were developed by the ascending technique. After development, the plates were sprayed with detection reagent. The various mobile phase mixtures tried, resolution patterns and the number of spots and their resolution factor values, are given in the (Table 4.4 and 4.5).

The methanolic leaf extract of *Tetractomia roxburghiana* was subjected to column chromatography on silica gel (E-Merck 60-120 mesh), hexane and ethyl acetate were used as an eluting solvent in different proportions, fractions were collected, TLC was performed and then further purification was carried out by high performance liquid chromatography (HPLC).

4.5 Isolation of Compound fractions by Column Chromatography

4.5.1 Preparation of Column

A glass column (150 ml) was cleansed and dried. A piece of glass wool was inserted with the help of glass rod to the base of column. The column was placed in a vertical position with the help of clamps and stand.

4.5.2 Preparation of Extract

The extract was dissolved in small quantity of methanol and applied above the silica gel layer into the column. Elution was done using chloroform (20 × 10 ml) and 10%

increments of ethyl acetate (EA) in chloroform were used. The column was eluted with various solvents (Table 4.4).

The fractions were collected and evaporated to small volumes for all the solvent systems. Thin layer chromatography (TLC) studies of the fractions were performed to ascertain the number of compounds present in the fraction.The homogenous fractions were mixed and the number of spots and R_f values for those fractions were noted and were subjected to further studies. The details of fractions obtained from the column chromatography are given in the (Table 4.4 and 4.5.)

Table 4.4: Elution pattern of column chromatography using chloroform and ethyl acetate (EA)

Eluting solvents	Ratios	Volume
$CHCl_3$	100 %	20×10
$CHCl_3$: EA	90:10 v/v	20×10
$CHCl_3$: EA	80:20 v/v	20×10
$CHCl_3$: EA	70:30 v/v	20×10
$CHCl_3$: EA	60:40 v/v	20×10
$CHCl_3$: EA	50:50 v/v	20×10
$CHCl_3$: EA	40:60 v/v	20×10
$CHCl_3$: EA	30:70 v/v	20×10
$CHCl_3$: EA	20:80 v/v	20×10
$CHCl_3$: EA	10:90 v/v	20×10
EA	100 v/v	20×10

Fraction	Solvent System	No. of Spots	R_f Values	Quantity	Description
fraction 1	Chloroform (100 %)	2	0.33, 0.4	5 mg	light yellow colored substance
fraction 2	Chloroform:ethyl acetate (90:10)	2	0.5,0.7	10 mg	yellow coloured substance
fraction 3	Chloroform:ethyl acetate (80:20)	4	0.21,0.28, 0.5, 0.8	800 mg	white coloured sticky substance
fraction 4	Chloroform:ethyl acetate (70:30)	2	0.34,0.71	30 mg	brownish white residue
fraction 5	Chloroform:ethyl acetate (60:40)	2	0.7,0.5	40 mg	yellowish white residue
fraction 6	Chloroform:ethyl acetate (50:50)	2	0.5, 0.4	120 mg	white yellowish residue
fraction 7	Chloroform:ethyl acetate (40:60)	2	0.35,0.5	negligible	brownish white residue
fraction 8	Chloroform:ethyl acetate (30:70)	3	0.4, 0.5, 0.6	120 mg	white coloured
fraction 9	Chloroform:ethyl acetate (20:80)	2	0.7, 0.8	20 mg	dark brown coloured substance
fraction 10	Chloroform:ethyl acetate (10:90)	2	0.65, 0.85	150 mg	yellow coloured substance

Table 4.5: Details of fractions obtained from column chromatography of methanolic leaf extract of *T. roxburghiana*

Table 4.6: TLC studies of crude methanolic leaf extract of T*etractomia roxburghiana*

Solvent	**Ratio**	**No. of spots**	**R_f values**	**Pattern**
hexane: ethyl acetate	70:30	4	0.3, 0.4, 0.7, 0.9	tailing was observed
hexane: ethyl acetate	50:50	4	0.21, 0.3, 0.4, 0.6	not satisfactory
hexane: ethyl acetate	30:70	3	0.3,0.4,0.7	not satisfactory
chloroform:methanol	90:10	3	0.24, 0.4, 0.5	moderate separation
chloroform:methanol	80:20	4	0.21, 0.28,0.3,0.8	moderate separation
chloroform:methanol	50:50	2	0.44,0.52	less resolution
chloroform:methanol	20:80	3	0.4,0.6,0.7	not satisfactory
chloroform:ethyl acetate	90:10	4	0.3,0.4,0.6,0.7	moderate separation
chloroform:ethyl acetate	80:20	6	0.2,0.4,0.5,0.7,0.8,0.9	best separation
chloroform:ethyl acetate	60:40	2	0.4, 0.7	not satisfactory
ethyl acetate:methanol: water	55:30: 15	3	0.2, 0.41,0.8	not satisfactory
methanol: ethyl acetate	80:20	2	0.23,0.78	less separation with tailing
methanol: ethyl acetate	60:40	3	0.24, 0.71, 0.8	tailing was observed

4.6 High Performance Liquid Chromatography

4.6.1 Introduction

Often in crude plant extracts, the compounds of interest are not present in very high concentrations; HPLC has been by far the most useful tool for the separation of complex mixtures of components present in crude plant extracts. Reversed phase HPLC on octadecylsilane (ODS or c18) has been recognized as the most common applicable of bonded phases for this purpose [108]. For the past twenty years High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance Spectroscopy (NMR) have been the primary tools used by natural product chemist for the isolation and identification of the compounds[92-105].

Reversed phase HPLC (RP-HPLC) consists of a non-polar stationary phase and an aqueous, moderately polar mobile phase. RP-HPLC allows polar components elute first where as retention time is quite longer for molecules which are non-polar in nature.

4.6.2 Experimental

4.6.2.1 Instrumentation

The HPLC system used consisted of a Shimadzu LC-6A pump, Shimadzu SPD-6AV variable wavelength UV/ Vis detector, and using low dead volume connections, and a Shimadzu CR5-A integrator (all supplied by Dyson Instruments, Hetton-le-Hole, Tyne & Wear, UK). Injections were manually using a Rheodyne 7125-injection valve (Anachem, Luton, Beds., UK) fitted with a 20 µl, 50 µl, 1 ml and 2 ml loops for analytical and semi-preparative scale up, respectively.

4.6.2.2 Materials and reagents

ACE-5-C 18(250×4.6mm I.D.), Hichrom Limited, (Reading, Berkshire, UK) and Waters Spherisorb S5 ODS 1 (250mm × 4.6 mm) columns were used during course of the study. Mobile phases were prepared by using HPLC-grade Methanol, HPLC far-UV grade (Sigma-Aldrich, Poole, Dorset, UK). Water was distilled and doubly de-ionised using and Elgostat option 3 (Vivendi Water Systems, High Wycombe, Bucks., UK).

4.6.2.3 Liquid Chromatographic analysis

The analytical and semi-preparative reversed phase high performance liquid chromatography of the methanolic crude extract and fractions from column

chromatography (flash columns) were carried out in order to determine constituents both qualitatively and quantitavely. Semi-preparative HPLC of the fraction (collected from flash column) was carried out using a ACE-5-C18 column (250mm × 7 mm) at room temparature, using mobile phase methanol-water (50:50 %, v/v, with 0.1 % TFA)] at a flow rate of 4.5 ml/min, with the injection volume of 1ml. The detection UV trace measured at 254 nm. The sample was prepared in methanol: water, 50:50, v/v, concentration of the sample used was 5 mg/ml, method: isocratic and the analysis stop time was 30 minutes. The fractions exhibiting peaks at similar retention times were combined to isolate the pure compounds by semi-preparative method and the fractions were collected, evaporated and the masses of the proposed compounds were noted.

4.6.2.3.1 Analytical reversed phase high performance liquid chromatography of the *Tetractomia roxburghiana*.

The analytical reversed phase high performance liquid chromatography of the *T. roxburghiana* (T.R) crude leaf extract and fractions of crude extracts was carried out in order to determine the number of constituents present in each fraction.

Initial chromatographic separations were achieved by using Waters Spherisorb S5 ODS 1 column (250mm × 4.6 mm) with flow rate of 1.5 ml/min, with the UV trace measured at 215 nm and 254 nm, which involved analytical method development as described below.

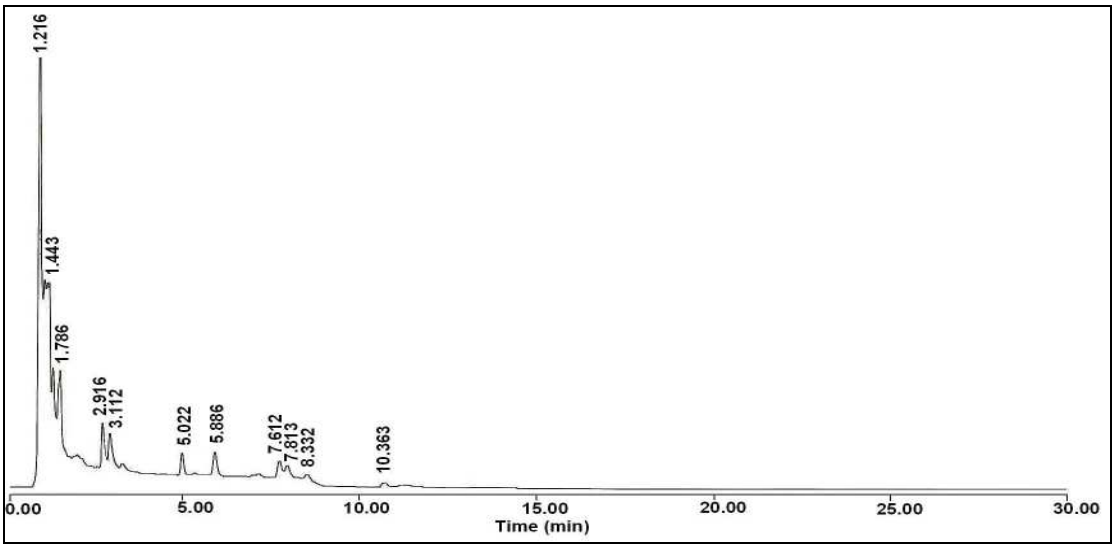


Figure 4.2: Analytical HPLC profile of MTR (methanolic *Tetractomia roxburghina* fraction (collected from flash column) of *Tetractomia roxburghiana* with a mobile phase of methanol-water (90:10, v/v) C18 250 X 4.6 mm; conc. 1 mg/ml, flow rate: 1.5ml/min; 254 nm.

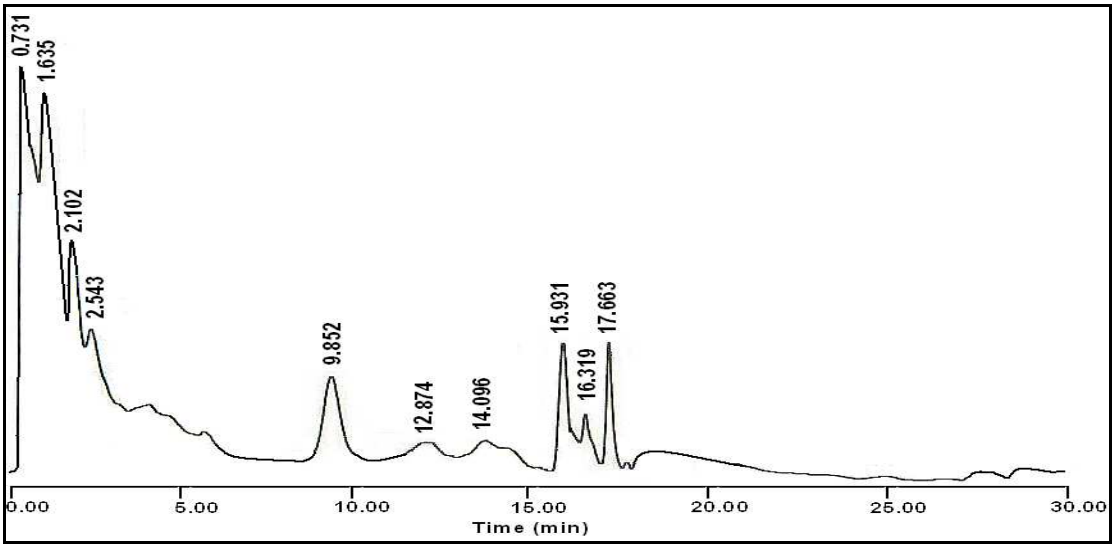


Figure 4.3: Analytical HPLC profile of MTR fraction (collected from flash column) of *Tetractomia roxburghiana* with a mobile phase of methanol-water (80:20, v/v) C18 250 X 4.6 mm; conc. 1 mg/ml, flow rate: 1.5ml/min; 254 nm.

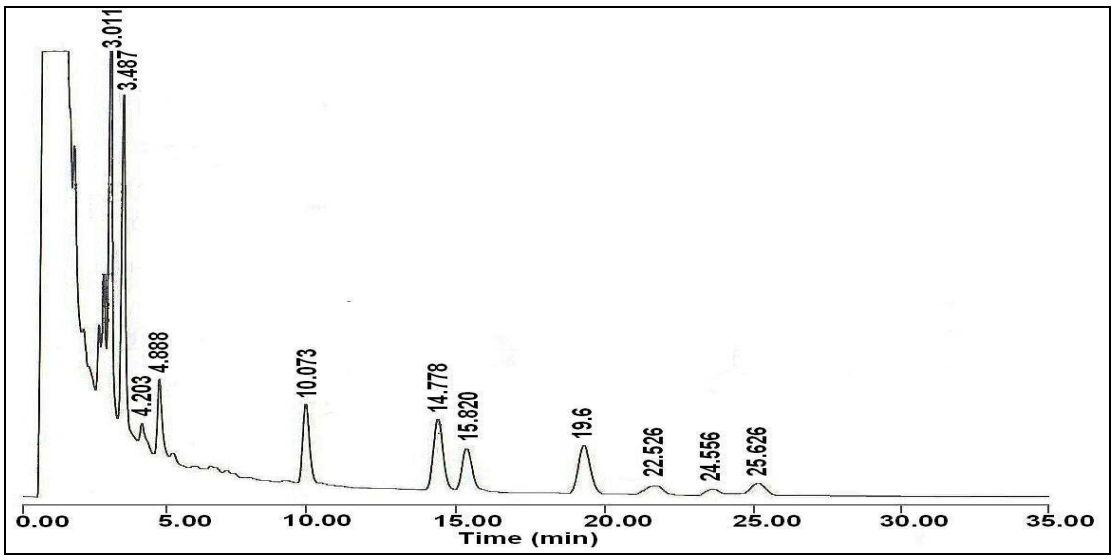


Figure 4.4: Analytical HPLC profile of MTR fraction (collected from flash column) of *Tetractomia roxburghiana* with a mobile phase of methanol-water (70:30, v/v) C18 250 X 4.6 mm; conc. 1 mg/ml, flow rate: 1.5ml/min; 254 nm.

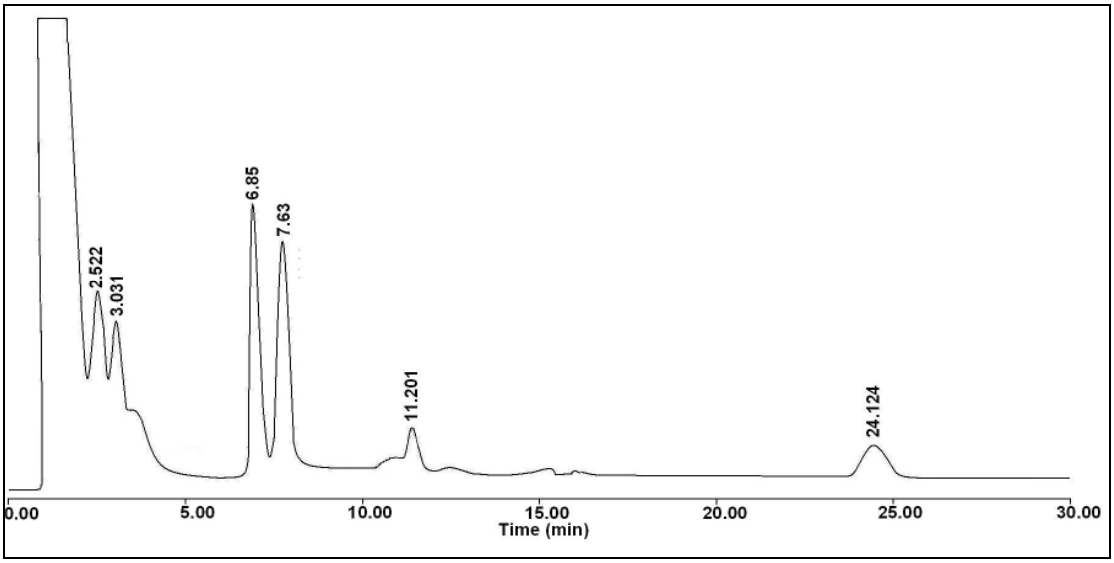


Figure 4.5: Analytical HPLC profile of MTR fraction (collected from flash column) of *Tetractomia roxburghiana* with a mobile phase of methanol-water (60:40, v/v) C18 250 X 4.6 mm; conc. 1 mg/ml, flow rate: 1.5ml/min; 254 nm.

When comparing the above chromatograms, it can be observed that from Fig.(4.2,4.3,4.4,4.5) best resolution was obtained with the mobile phase (60:40, v/v).

4.6.2.3.2 Semi preparative HPLC of crude extract of *Tetractomia roxburghiana*

From the semi-preparative scale work, it was observed that in one of the column chromatography fractions there were unretained peaks and two other major peaks, with retention times 19.057min and 28.507 min (Figure 4.6). These two peaks were collected at λ_{max} 254 nm and flow rate of 4.5 ml/min (reversed phase HPLC). In the process of semi-preparative method development, the experimental parameters recorded are shown below.

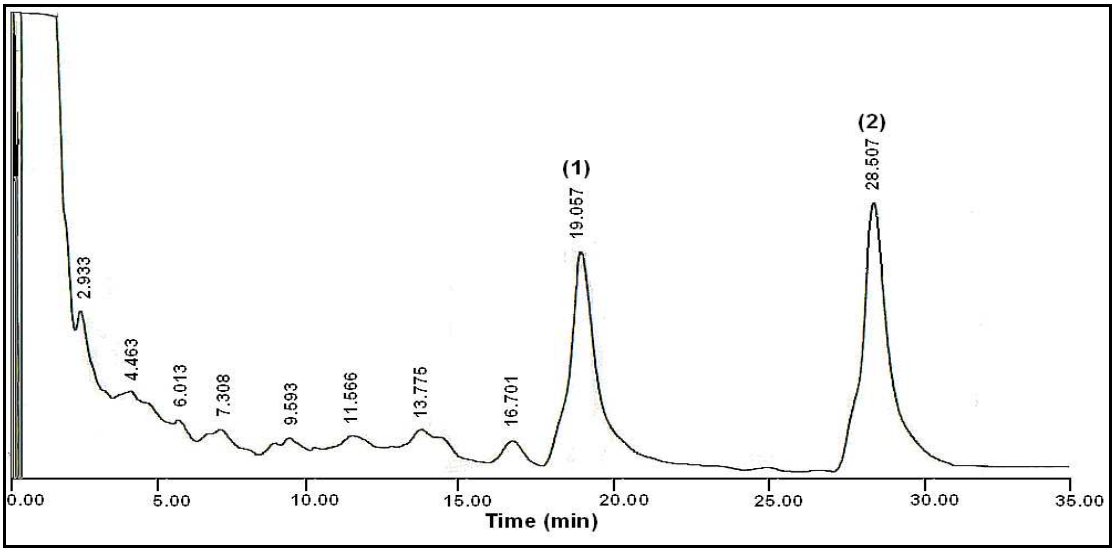


Figure 4.6: Semi preparative HPLC profile of MTR fraction (collected from flash column) of *Tetractomia roxburghiana* with a mobile phase of methanol-water (50:50, v/v) C18 250 X 7.0 mm; conc. 5 mg/ml, flow rate: 4.5 ml/min; 254 nm.

Experimental Parameters

Sample Code	: MTR
Plant name and Family	: *Tetractomia roxburghiana* (Rutaceae)
Sample preparation	: Methanol: water (50:50, v/v)
Concentration of the sample	: 5 mg/ml
Method (Isocratic/Gradient)	: Isocratic
Analytical/Preparative work	: Semi preparative
Pump	: Shimadzu LC-6A
Detector	: Shimadzu SPD-6AV
Integrator	: Shimadzu C-R5A
Mobile Phase	: Methanol: water (50:50 v/v, 0.1% TFA)
Loop size	: 1.5 ml
Injection volume	: 1.0 ml
Column	: ACE 5 C18 A11334 (250 X 7.0 mm id)
Flow rate (ml/min)	: 4.5 ml/min
Wavelengths	: 254 nm
Analysis time	: 35 min

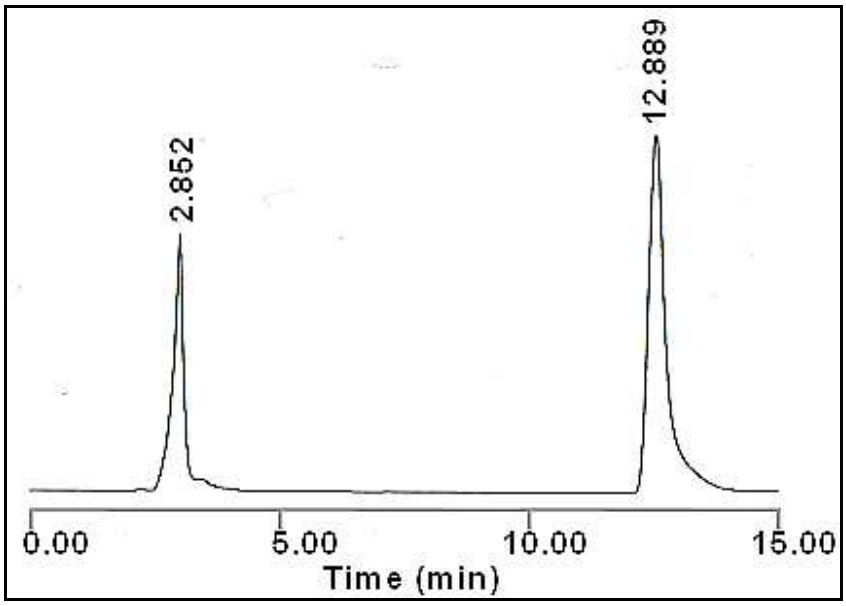


Figure 4.7: Semi-preparative phase HPLC profile of crude fraction mixtures of peak two and peak three (which were collected from Fig 4.5 retention times 6.85 and 7.63 minutes) with a mobile phase of methanol-water (30:70, v/v) 250×7mm; con 5mg/ml, flow rate: 4.5ml/min ; λ_{max} : 254nm, Analysis time 15 min.

Experimental Parameters

Sample Code	: TRUPM(Unretained Peak Mixtures)
Plant name and Family	: *Tetractomia roxburghiana* (Rutaceae)
Sample preparation	: Methanol: water (30:70, v/v)
Concentration of the sample	: 5 mg/ml
Method (Isocratic/Gradient)	: isocratic
Analytical/Preparative work	: Semi preparative
Pump	: Shimadzu LC-6A
Detector	: Shimadzu SPD-6AV
Integrator	: Shimadzu C-R5A

Mobile Phase	: Methanol: water (30:70 v/v, 0.1% TFA)
Loop size	: 1.5 ml
Injection volume	: 1 ml
Column	: ACE 5 C18 A11334 (250 X 7.0 mm id)
Flow rate (ml/min)	: 4.5 ml/min
Wavelengths	: 254 nm
Analysis time	: 15 min

The semi-preparative method was carried out using scaled up experimental parameters. The concentration of the sample was 5mg/ml, the injection volume: 1 ml, and the flow rate: 4.5 ml/min. The two retained peaks collected were evaporated using a rotary evaporator to 1/3rd of the initial volume and the remaining solvent was evaporated by freezedrier. The first collected compound (1) one was light brown in colour and amorphous in nature .The weight of the compound was noted (1.1 mg) and the compound MTR was subjected to NMR and mass methods. On the basis of the spectral analysis and on comparison with the authentic sample, one compound was characterised as swertisin (confirmed with LC-NMR).The retention time of this compound was established as shown in the above figure 4.6(Rt 28.507 min). The second collected compound (2) was yellow crystalline substance and was identified as gallic acid (2.1 mg)(Confirmed with NMR and MASS spectra)

At this point, it was clear that carrying out HPLC of the column chromatography fractions would be a very time consuming process. I therefore decided to make use of

the availability of analytical (250 x 4.6 mm i.d.), semi-preparative (250 x 7 mm i.d.) and preparative (250 x 22 mm i.d.) columns in the same chromatographic material (ACE-C18, 5 μm) to carry out high-resolution separations direct on crude extracts.

Semi-preparative HPLC was then carried out on the ACE 5 C18 column for the isolation of peaks using the scaled up parameters (Fig 4.7). The purified compounds were subjected to accurate mass MS and NMR methods. α-asarone (1.8 mg) and angelicin (2.2 mg,). On the basis of spectral analysis (accurate mass, library match of fragmentation pattern and then confirmed by NMR) the compounds were identified as gallic acid, α-asarone and angelicin (a furanocoumarin) which was the first time that these compounds had been found in the methanolic leaf extract of *T. roxburghiana.* The retention times of α -asarone (Rt 2.852 min), angelicin (12.889 min) were established are as shown in the (fig 4.7)

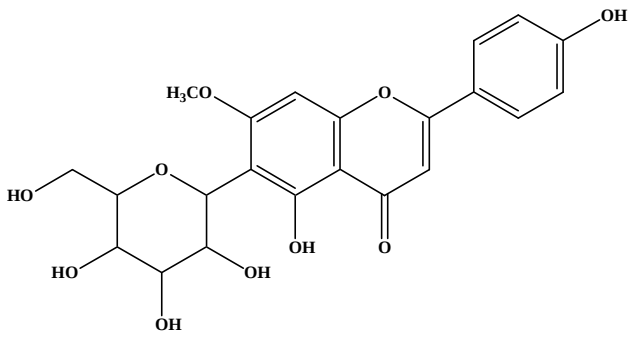
4.7 Results and Discussion

4.7.1 Spectral Studies on Isolated Compounds

4.7.1.1 compound one (MTR)

The fraction collected from the methanolic leaf extract of *Tetractomia roxburghiana* in the hexane - ethyl acetate (85:15,v/v) elution by column chromatography and it was further purified by HPLC using mobile phase: methanol – water (50:50, v/v), Stationary

phase Waters Spherisorb S5 ODS column, $\lambda_{max;}$ 254nm. It is a white amorphous powder, melting point: 153-155°C, soluble in methanol, ethanol and chloroform and partially soluble in ether. Molecular formula $C_{22}H_{22}O_{10}$.

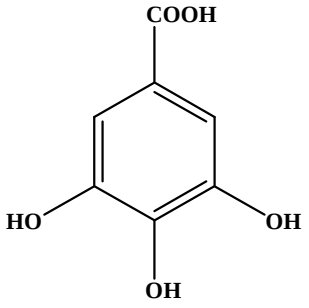


Swertisin [124], [125]

^{1}HNMR(500MHz,D2O-CH_3CN) 6.73(1H,S,8-H),6.65(1H,S,3-H), 9.0(2H,d,J8.7Hz,1-H), 7.85(2H,d,J 8.7Hz,2-H) .

4.7.1.2 compound two (TRF 1)

It was isolated from the methanolic leaf extract of TR by HPLC using mobile phase: methanol – water (50:50, v/v) Stationary phase Ace 5 C18 A11334 (250 x 7 mm i.d.) $\lambda_{max;}$ 254nm. It was yellow crystalline substance, soluble in water, CH_3OH, C_2H_5OH, acetone, insoluble in benzene, chloroform and petroleum ether. Its melting point was found to be 250-251°C. Molecular formula $C_7H_6O_5$.

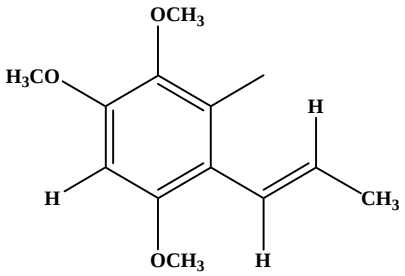


Gallic acid

^{1}HNMR (300MHz, $CDCl_3$) 4.7 (3H, s, 3×OH) 6.9 (2H, s, 2×CH) IR (KBr) γ max cm^{-1} 3386 (C-H), 3288 (O-H) 1616 (C=O), 1263 (C-O), 865 (C_6H_5); UV λ_{max} nm: 216, 272; Mp (250-251) °C.

4.7.1.3 compound three (TRF 2)

It was isolated from the methanolic leaf extract of TR by HPLC using mobile phase: methanol – water (30:70, v/v), stationary phase Ace 5 C18 A11334 (250 x 7 mm i.d.) column. λ_{max} ; 254 nm. It was yellow powder, soluble in alcohol, chloroform, and petroleum ether, practically insoluble in water. Its melting point was found to be 60-62°C.

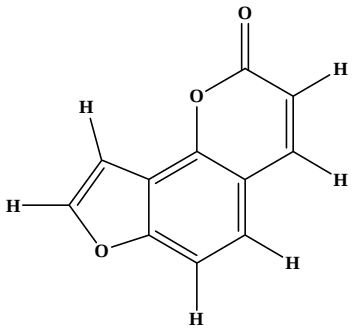


1, 2, 4-trimethoxy-5 (E)-(1-propenyl) benzene

^{1}HNMR (300 MHz, $CDCl_3$) 1.85 (1H, dd, J =1.6, 6.6 Hz, $CHCH_3$) 6.10 (1H, dq, J= 15.9, 6.6Hz, $CHCH_3$) 6.62 (1H, dq, J = 15.9, 1.6 Hz, CH_3CHCH) 6.62 (1H, s, 3-H), 7.00 (1H,s,6-H) IR (KBr) γ max cm^{-1} 3434 C-H (Ph), 2350 C-H (OCH_3), 1268(C-O), 1029 (C-O-C), 825 (C_6H_5); UV λ_{max} nm: 220,256,312; Mp (60-61)°C.

4.7.1.4 compound four (TRF 3)

It was isolated from the methanolic leaf extract of TR by HPLC using mobile phase: methanol – water (30:70, v/v), Stationary phase ACE 5 C18 A11334 (250 x 7 mm i.d) C18 column. λ_{max} ; 254 nm. It was yellow amorphous powder. Its melting point was found to be: 137-138°C. It was soluble in alcohol, chloroform, and acetone and sparingly soluble in petroleum ether, ether etc.



8 –oxo-8H-furo (2, 3-f) (1) benzopyran

^{1}HNMR (300MHz, $CDCl_3$) 6.42 (1H, d, J 9.6 Hz, 4′-H) 7.18 (1H, dd, J = 2.25,0.8Hz, 3′-H) 7.55 (1H, dd, J= 8.6, 0.8 Hz, 6′-H) 7.58 (1H, d, J = 8.6 Hz, 7-H), 7.93 (1H,d, J = 2.25; 2-H) 8.08 (1H,d, J = 9.6 Hz,5′-H) IR (KBr) γ max cm^{-1} 3430 (C-H), 1615 (C=O), 1271(C-O-C), 831 (C_6H_5); UV λ_{max} nm: 212, 244, 296; Mp(137-138)°C.

4.8 Conclusions

Following the phytochemical investigations already described, four compounds had been isolated and chemically characterized from the methanolic leaf extract of *Tetractomia roxburghiana.* These were swertisin, gallic acid, α-asarone and angelicin (furanocoumarin), this being the first time that these compounds had been found in the methanolic leaf extract of *Tetractomia roxburghiana.*

Although, by this time it had been decided to carry out biological testing as well as characterization, it was still worthwhile to check the literature for and reported activity of the individual isolated constituents as obviously that would have a bearing on the overall activity of the extract itself. It was found that, swertisin was reported to possess free radical scavenging activity as evidenced by the DPPH (1,1-diphenyl-2-picrylhydrazyl) assay [124]. Activities reported for α-asarone include effects on the central nervous system, cardiovascular system and its smooth muscle depressant action. Hence it shows sedative [126], anti-acetylcholine, hypocholesterolaemic [127] [128], and anti-microbial [129] activity. Angelicin was reported to exhibit catalytic topoisomerase II inhibition [131] in the SV40 bioassay and to be both inducer and an inhibitor of cytochrome P450 1A1 in rat hepatocytes [132]. Gallic acid (phenolic acid) was also reported to possess anti- flowering inhibitor [133] and anti-carcinogenic [134] activity. It also exhibits anti-oxidant activity [135] anti-inflammatory [136] activity due to its inhibitory effect on release of myeloperoxidase enzyme by polymorphonuclear lymphocytes [137]. Most importantly, the nature of the compounds that had been

isolated suggested that there might be some interesting activity with respect to skin diseases; for example,furanocoumarins (angelicin) are recognised anti-psoriatic / anti-proliferative compounds, when used in conjunction with ultraviolet – A light [139-142]. This was a potential link with the strong interest at Sunderland in the use of medicinal plants in the treatment of skin diseases and something that would be looked at, given the number of microbiological assays in this therapeutic area that had been established.

5 Isolation, Identification and structural elucidation of constituents of Glycosmis *calcicola*, B.C Stone.

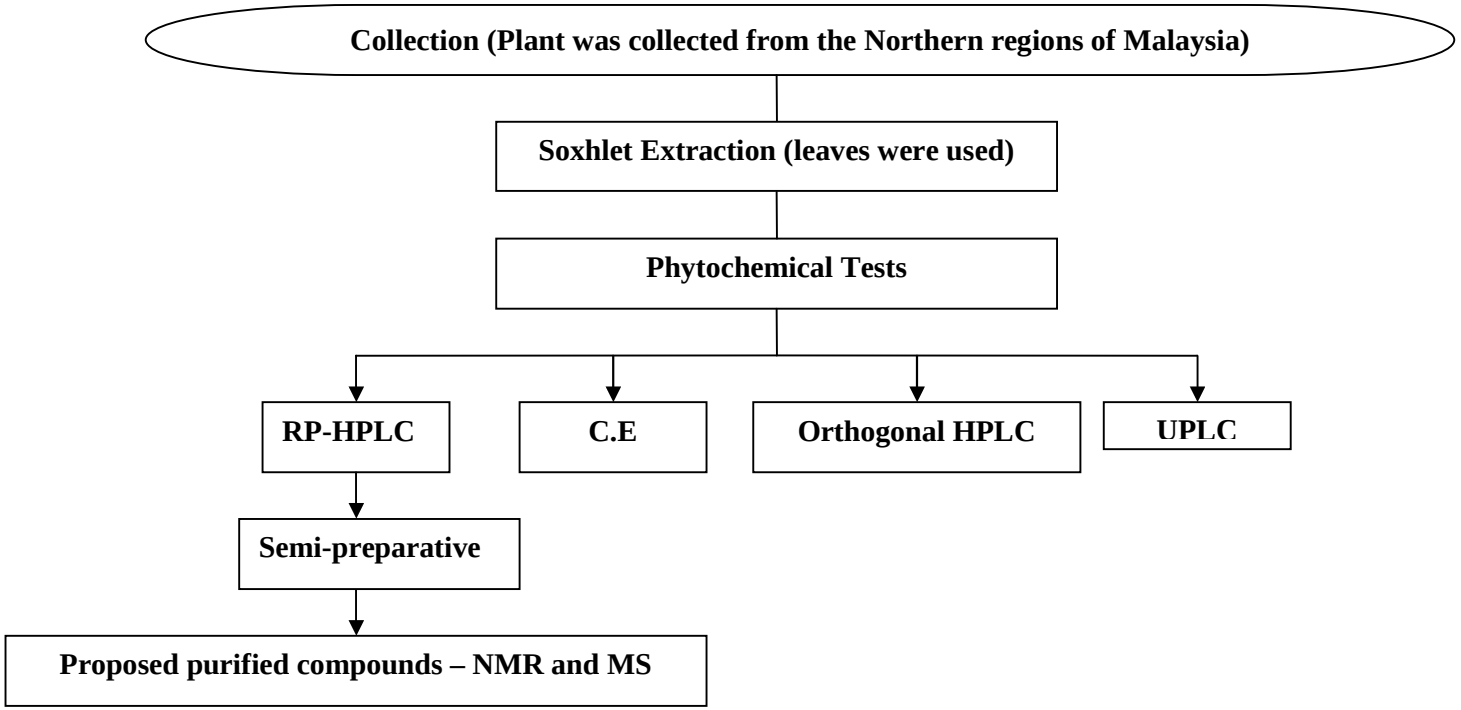


Fig 5.1 – Flow Chart of Separation techniques and Test Used in Chronological Order For The Methanolic Leaf Extract of Glycosmis calcicola

5.1 Introduction

Glycosmis is a genus of 40 species of trees and shrubs of the family Rutaceae. It is distributed in South-and-Southeast Asia [63]. The shrubs are small trees possess pinnate or simple leaves with translucent glands. The members of the genus are aromatic (contain volatile oils) and traditionally used for the treatment of fever, swollen spleen, and stimulant to digestion and skin diseases [57]. The chloroform rot extracts of *Glycosmis calcicola* and *Glycosmis rupestris* reported to have anti-fungal activity and the anti-fungal compounds identified as pyranoquinoline, flindersine and desmethoxyanthophylline [56].

5.2 Experimental

5.2.1 Plant material

The leaf plant material was collected from Penang district of Malaysia. Botanically identified, part air-dried, leafy plant material (50 g) was powdered and extracted with different solvents (from non-polar to polar). The solvents were partly removed *in vacuo* below 50° C and freeze-dried to give extracts. The solvents used were hexane, petroleum ether, chloroform, dichloromethane, methanol, ethanol and water. The extractive values of different solvents were tabulated below.

Table 5.1: Percentage extractive values of leaves of *Glycosmis calcicola* using different solvents.

Plant	Part	Solvent for extraction	Extractive values (% w/w)
Glycosmis calcicola	leaves	Hexane	0.05
		petroleum ether	0.03
		chloroform	0.06
		Dichloromethane	0.01
		methanol	3.23
		ethanol	1.5
		water	1.9

5.2.2 Soxhlet extraction

The powdered leafy plant material (25 g) was successively extracted with petroleum ether, hexane, chloroform, dichloromethane, ethanol and water. The volume of the solvent used in each case was 125 ml. Each time before extraction with the next solvent, the powdered material was air-dried, and then weighed. In the above extracts the largest quantity of the extract was obtained with methanol whereas with the petroleum ether, hexane, chloroform, dichloromethane, ethanol solvents the extract obtained was in smaller amounts. Further study was therefore carried out on the methanolic leaf extract of *G. calcicola.*

5.3 Preliminary Phytochemical screening

The methanolic leaf extract used for the preliminary phytochemical screening of the identification of plant secondary metabolites/active constituents (refer to the section 4.3). The results indicate that preliminary phytochemical investigation of the methanolic leaf extracts showed positive results for phenolics, sterols, flavonoids and tannins. The results were tabulated in the table-5.2.

Table 5.2: Phytochemical tests for secondary metabolites (Rutaceous extracts)

S r. No.	Plant	Part of plant	Solvent for extraction	Secondary metabolites					
				S	A	F	P	G	Sa
1	*Glycosmis calcicola*	leaves	methanol	+	-	+	+	-	-

S – Steroids; A – Alkaloids; F – Flavonoids; P – Phenolics; G – Glycosides; Sa – Saponins. + – Positive, - – Negative

5.3.1 Results and Discussion

The methanolic leaf extract of *G. calcicola* was used for the preliminary phytochemical screening of the identification of plant secondary metabolites/active constituents. Preliminary phytochemical investigation of the methanolic leaf extracts showed positive results for phenolics, sterols, flavonoids and tannins. In the following chapters, the analytical technology used for the identification and isolation of major constituents from the above methanolic leaf extracts is discussed.

5.4 High Performance Liquid Chromatography of *Glycosmis calcicola*

In the case of *G. calcicola*, it was convenient to carry the chromatographic separations on a semi-preparative scale because of the problems encountered with the analysis of *T. roxburghiana* that it was a time consuming and high solvent consumption, first developing an analytical and then scaled up semi/ preparative analysis. The sample was dissolved in methanol-water (50:50 %, v/v).

5.4.1 Experimental

5.4.1.1 Instrumentation

The HPLC system used consisted of a Shimadzu LC-6A pump, Shimadzu SPD-6AV variable wavelength UV/ Vis detector, and using low dead volume connections, and a Shimadzu CR5-A integrator (all supplied by Dyson Instruments, Hetton-le-Hole, Tyne & Wear, UK). Injections were manually using a Rheodyne 7125-injection valve (Anachem, Luton, Beds., UK) fitted with a 20 µl, 50 µl, 1 ml and 2 ml loops for analytical and semi-preparative scale up, respectively.

5.4.1.2 Materials

An ACE-5-C 18(250×4.6mm I.D.), Hichrom Limited, Reading, Berkshire, UK) column was used during course of the study. Mobile phases were prepared by using HPLC-grade methanol, HPLC far-UV grade (Sigma-Aldrich, Poole, and Dorset, UK). Water was distilled and doubly de-ionised using and Elgostat option 3 (Vivendi Water Systems, High Wycombe, Bucks., UK).

5.4.1.3 Liquid Chromatographic analysis

Semi-preparative reversed phase high performance liquid chromatography of the methanolic crude extract was carried out. The reversed phase HPLC of the crude extracts was carried out using a ACE-5-C18 column (250mm × 7 mm) at room temparature, using mobile phase [methanol-water (20:80 %, v/v, with 0.1 % TFA)] at a flow rate of flow rate of 4.5 ml/min, with the injection volume of 1ml. The detection UV trace measured at 254 nm.The sample was prepared in methanol-water (20:80, v/v), the concentration of the sample used was: 5mg/ml and the method was isocratic with the analysis stop time of 60 minutes. The fractions exhibiting peaks at similar retention times were combined to isolate the pure compounds by semi-preparative method and the fractions were collected evaporated and the mass of the proposed compounds were noted.

In this case it was convenient to carry out the initial analytical method development on the “semi-preparative” column (Figure 5.1). Using the same gradient and scaled up

conditions the retained four main peak fractions were collected, evaporated and subjected to the accurate Mass and NMR methods.

The rest of the peak fractions, which were found to be mixtures, will be subjected to further study. On the basis of spectral analysis (accurate mass, library match of fragmentation pattern) one compound was identified as angelicin. Other fractions that were found to be mixtures and needed to be further investigated.

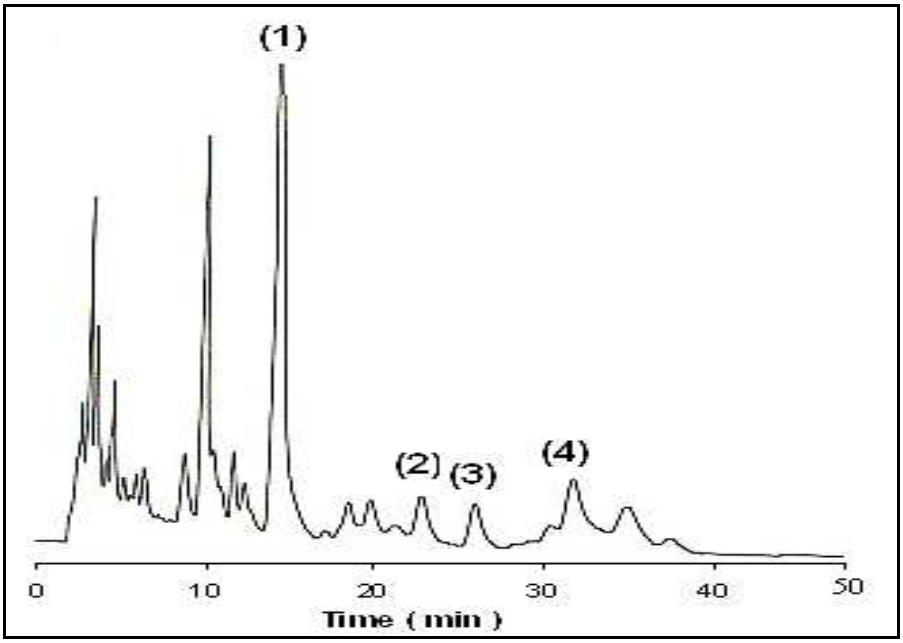


Figure 5.2: Semi-preparative HPLC profile of methanolic leaf extract of *Glycosmis calcicola* with a mobile phase of methanol-water (20:80, v/v) C18 250 X 7 mm; con 5 mg/ml, flow rate: 4.5ml/min; 254 nm.

Experimental parameters:

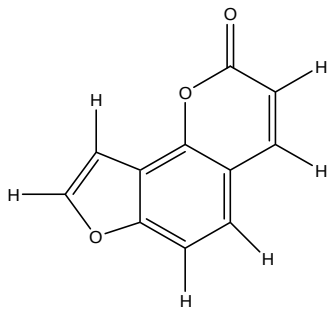
Sample Code	: GCM
Plant name and Family	: *Glycosmis calcicola*
Sample preparation	: methanol-water (20:80, v/v)
Concentration of the sample	: 5 mg/ml
Method (Isocratic/Gradient)	: Isocratic
Analytical/Preparative work	: Semi preparative
Pump	: Shimadzu LC-6A
Detector	: Shimadzu SPD-6AV
Integrator	: Shimadzu C-R5A
Mobile Phase	: methanol-water (20:80, v/v) 0.1% TFA
Loop size	: 1.5 ml
Injection volume	: 1 ml
Column	: ACE 5 C18 A11334 (250 X 7.0 mm id)
Flow rate (ml/min)	: 4.5 ml/min
Wavelengths	: 254 nm
Analysis time	: 50 min

5.5 Results and Discussion

5.5.1 Spectral studies on isolated compounds

5.5.1.1 Compound five (GCZE4)

It was isolated from the methanolic leaf extract of *G. calcicola* by HPLC using mobile phase: methanol – water (20:80, v/v) stationary phase ACE 5 C18 A11334 (250 x 7 mm i.d.) $\lambda_{max;}$ 254 nm. Molecular formula $C_{11}H_6O_3$.



angelicin

^{1}HNMR: δ_H/ppm (300 MHz, D_2O): 6.37 (1H, d, J = 9.6, Ar-H), 7.10 (1H, d, J = 2.1, Ar-H), 7.47-7.53 (2H, m, Ar-H), 7.76 (1H, d, J = 2.1, Ar-H), 8.04 (1H, d, J = 9.6, Ar-H). *m/z* (EI): 186 ($[M]^+$, 100%), 158 (80%), 129 (10%), 102 (20%), 51 (10%).

Semi-preparative HPLC of the methanolic leaf extract of *G.calcicola* afforded one compound in sufficient quantity and purity to be identified, namely, angelicin. The structure was determined by spectroscopic methods and on comparison with the

authentic sample. This was the first time that this compound had been found in the methanolic leaf extract of *G. calcicola.*

As already been discovered in the literature search carried out on compounds found in *T. roxburghiana*, furanocoumarins are recognised anti-psoriatic / anti-proliferative compounds, when used in conjunction with ultraviolet – A light [139-142]. As already intimated, it had already been decided by this point to screen the extracts against a range of microbiological assays appropriate for the treatment of skin diseases and this literature report gave some hope for success.

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6 Isolation, Identification and Structural elucidation of constituents of *Clausena excavata* Burm.f. (Rutaceae)

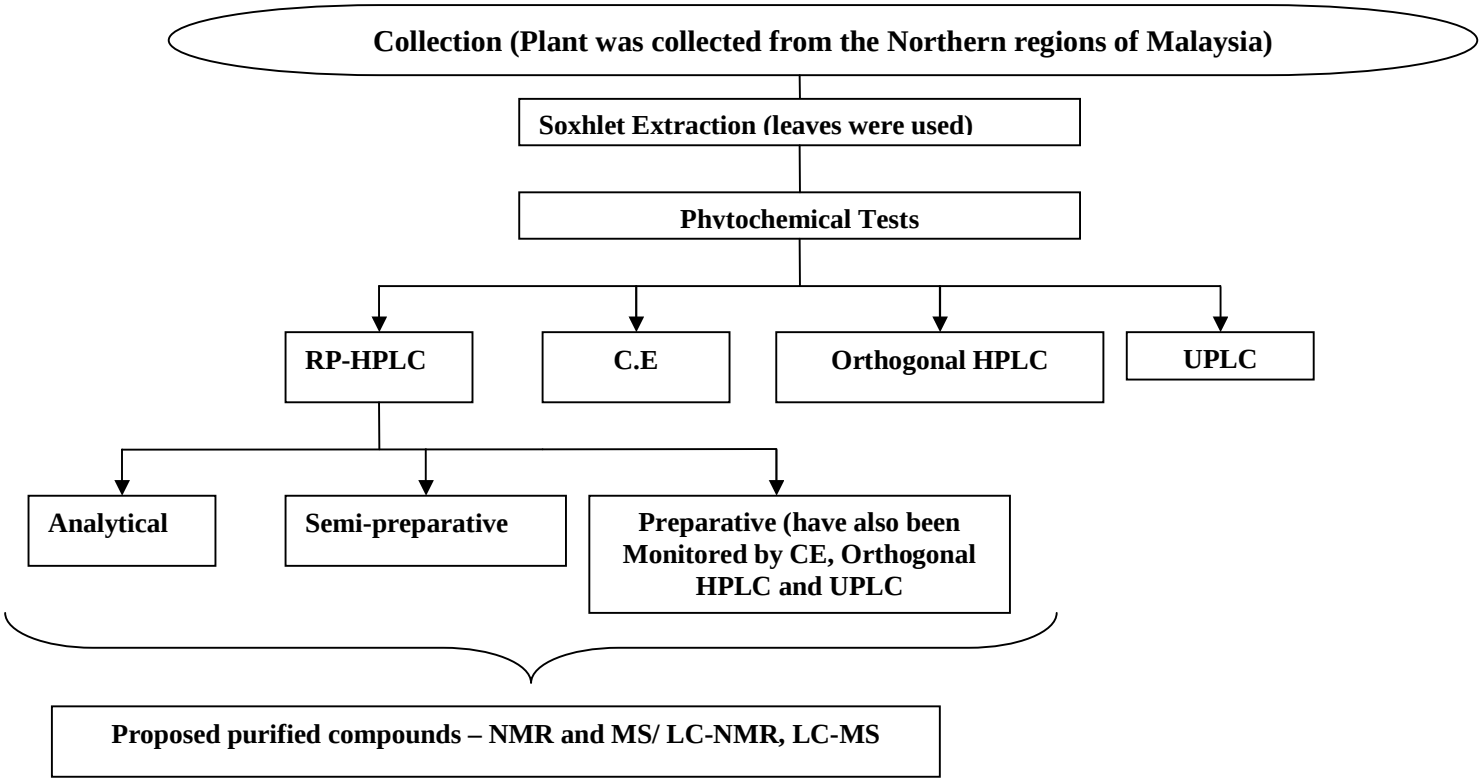


Fig 6.1 – Flow Chart of Separation techniques and Test Used in Chronological Order For The Methanolic Leaf Extract of C.E

6.1 Introduction (Plant introduction described earlier in the chapter 2, section 2.4)

6.2 Experimental

6.2.1 Plant material

The leaf plant material was collected from Penang district of Malaysia. Botanically identified, part air-dried, leafy plant material (100 g) was powdered and extracted with different solvents (from non-polar to polar). The solvents were partly removed *in vacuo* below 50° C and freeze-dried to give extracts. The solvents used were hexane, petroleum ether, chloroform, dichloromethane, methanol, ethanol and water. The extractive values of different solvents were tabulated below.

Table 6.1: Percentage extractive values of leaves of *Clausena excavata* using different solvents.

Plant	Part	Solvent for extraction	Extractive values (% w/w)
Clausena excavata	leaves	hexane	0.04
		petroleum ether	0.02
		chloroform	0.79
		dichloromethane	0.13
		methanol	2.3
		ethanol	1.2
		water	1.22

6.2.2 Soxhlet extraction

The powdered leafy plant material (100 g) was successively extracted with petroleum ether, hexane, chloroform, dichloromethane, ethanol and water. The volume of the solvent used in each case was 500 ml. Each time before extraction with the next solvent, the powdered material was air-dried, and then weighed. In the above extracts the largest quantity of the extract was obtained with methanol whereas with the petroleum ether, hexane, chloroform, dichloromethane, ethanol solvents the extract obtained was in smaller amounts. Further fractionation of the extracts was therefore carried out on the methanolic leaf extract of *Clausena excavata* by high performance liquid chromatography.

6.3 Preliminary Phytochemical screening

The methanolic leaf extract was used for the preliminary phytochemical screening and for the identification of plant secondary metabolites / active constituents (refer to the appropriate sections 4.3). The results indicate that preliminary phytochemical investigation of the methanolic leaf extracts showed positive results for phenolics, sterols, flavonoids and tannins. The results were tabulated in the table-6.3.

Table 6.3: Phytochemical tests for secondary metabolites (Rutaceous extracts)

S r. No.	Plant	Part of plant	Solvent for extraction	Secondary metabolites					
				S	A	F	P	G	Sa
1	*Clausena excavata*	leaves	methanol	-	-	++	+	+	-

S – Steroids; A – Alkaloids; F – Flavonoids; P – Phenolics; G – Glycosides; Sa – Saponins. + – Positive, - – Negative

6.3.1 Results and Discussion

The methanolic leaf extract of *G. calcicola* was used for the preliminary phytochemical screening of the identification of plant secondary metabolites/active constituents. The results indicate that preliminary phytochemical investigation of the methanolic leaf extracts showed positive results for phenolics, flavonoids and tannins. In the appropriate sections, the analytical technology used for the identification and isolation of major constituents from the above methanolic leaf extracts were discussed.

6.4 High Performance Liquid Chromatography of *Clausena excavata*

6.4.1 Introduction

In case of *C.excavata* chromatographic separations were carried out on an analytical scale. From the analytical method developed (Figure 6.1), the retained peaks were collected using the preparative column (250 x 22 mm i.d.) and also using the same conditions with the scaled up parameters (sample concentration 10 mg/ml, injection volume 10 ml and flow rate 9.5 ml/min).

6.4.2 Experimental

6.4.2.1 Instrumentation

The HPLC system used consisted of a Shimadzu LC-10 AD pump, Shimadzu SPD-6AV variable wavelength UV/ Vis detector, and using low dead volume connections, and a A1-450 Dionex integrator (all supplied by Dyson Instruments, Hetton-le-Hole, Tyne & Wear, UK). Injections were manaually using a Rheodyne 7125-injection valve (Anachem, Luton, Beds., UK) fitted with a 20 µl, 50 µl, 1 ml and 2 ml loops for analytical, semi-preparative and preparative scale up, respectively.

6.4.2.2 Materials

ACE-5-C18 (250×4.6mm I.D.), ACE-5-C 18(250×7mm I.D.), ACE-5-C 18(250×22 mm I.D.) columns supplied by Hichrom Limited,(Reading, Berkshire, UK) were used during course of the study. Mobile phases were prepared by using HPLC-grade methanol, HPLC far-UV grade (Sigma-Aldrich, Poole, and Dorset UK). Water was distilled and doubly de-ionsied using and Elgostat option 3 (Vivendi Water Systems, High Wycombe, Bucks., UK).

6.4.2.3 Liquid chromatographic analysis

The analytical reversed phase high performance liquid chromatography of the methanolic crude extract as carried out in order to determine number of constituents present in the extract. The reversed phase HPLC of the crude extracts was carried out using a ACE-5-C18 column (250mm × 7 mm) at room temparature, using the mobile phase [methanol-water (70:30 %, v/v, with 0.1 % TFA)] at a flow rate of 1.5 ml/min, with the injecton volume of 20 µl. The detection UV trace measured at 245 nm.

Sample was prepared in methanol-water (20:80, v/v), concentration of the sampleused: 1.5mg/ml, method: isocratic and the analyis stop time were 60 minutes. The fractions exhibiting peaks at similar retention times were combined to isolate the pure compounds by preparative methods and the fractions were collected evaporated and the mass of the proposed compounds were noted.

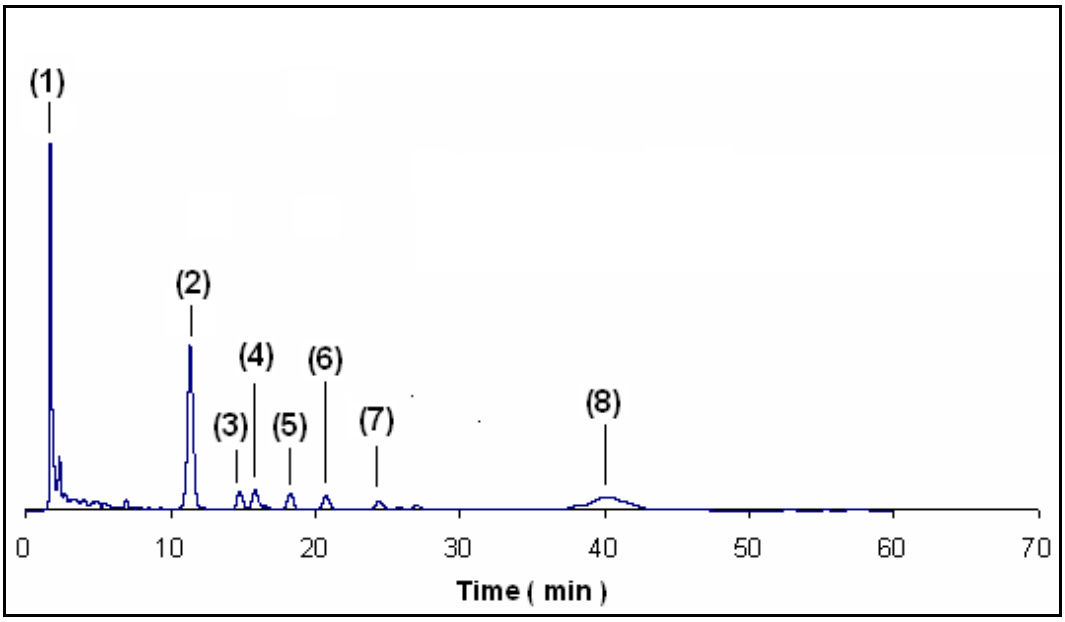


Figure 6.2: Analytical reversed phase HPLC profile of methanolic leaf extract of *Clausena excavata* with a mobile phase of methanol-water (20:80, v/v) C_{18} 250×4.6 mm; conc 1. 5 mg/ml, flow rate: 1.5ml/min; λ_{max}: 245 nm.

Experimental Parameters

Sample Code	: CE
Plant name and Family	: *Clausena excavata*
Sample preparation	: methanol-water (20:80, v/v)
Concentrationof the sample	: 1.5 mg/ml
Method (Isocratic/Gradient)	: isocratic
Analytical/Preparative work	: Analytical
Pump	: Shimadzu LC-10AD
Detector	: UV/VIS

Integrator	: A1-450 Dionex
Mobile Phase	: methanol-water (20:80, v/v) 0.1% TFA
Loop size	: 20 µl
Injection volume	: 20 µl
Column	: ACE 5 C18 A11334 (250 X 4.6 mm id)
Flow rate (ml/min)	: 1.5 ml/min
Wavelengths	: 245 nm
Analysis time	: 70 min

6.4.2.4 Preparative HPLC

From the analytical method developed (Figure 6.1), the retained peaks were collected using the preparative column (250 x 22 mm i.d.) using the same conditions with the scaled up parameters (sample concentration 10 mg/ml, injection volume 10 ml and flow rate 9.5 ml/min). Eight peak fractions were collected, evaporated and the purified compounds were subjected to accurate mass MS and NMR methods. Masses of the peak fractions were noted as as CE1 (0.5 mg), CE2 (1.7 mg), CE 3 (5 mg) CE4 (3.3 mg), CE5 (4.1 mg), CE6 (1.4 mg), CE7 (2.2 mg), CE8 (1.5 mg). On the basis of spectral analysis compound (CE7) was identified as 5, 7- dihydroxy flavone and the remaining seven peak fractions were found to be mixtures. Therefore, further study was carried out on the fractions using short column in order to identify the components present in the mixtures. LC-NMR and LC-MS analysis (Chapter-3, section-3.2.3.1) of the peak fraction collected from preparative HPLC (CE 3) afforded identification of two

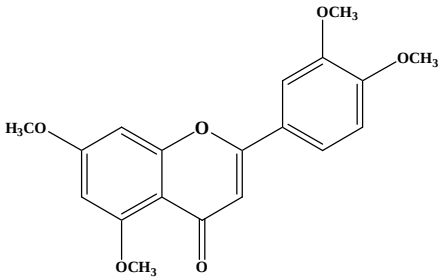
compounds namely 2-(3, 4-dimethoxy-phenyl)-5, 7-dimethoxy-chromen-4-one, 2-(3, 4-dimthoxy-phenyl) 3, 7-dihydroxy-5-methoxy-chromen-4-one and 5,7-dihydroxy-2-phenyl-chromen-4-one (chrysin)

6.5 Results and Discussion

6.5.1 Spectral studies on isolated compounds

6.5.1.1 Compound six

It was identified from the CE 3 peak fraction which is collected from the methanolic leaf extract of *C.excavata* using preparative HPLC, mobile phase: methanol – water (20:80, v/v) stationary phase ACE 5 C18 (250 x 22 mm i.d.) λ_{max} ; 254 nm. The mass of the ion was found to be 343. Molecular formula – $C_{19}H_{18}O_6$.

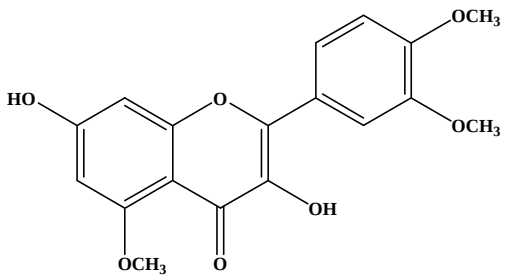


2-(3, 4-dimethoxy-phenyl)-5, 7-dimethoxy-chromen-4-one

^{1}HNMR: δ_H/ppm (500 MHz, MeOHD$_2$O): 3.88-3.93 [12H, m, (CH$_3$)$_4$], 6.56 (1H, s, Ar-H), 6.67 (1H, s, Ar-H), 6.82 (1H, s, Ar-H), 7.15 (1H, d, J = 8.5, Ar-H), 7.40 (1H, s, Ar-H), 7.65 (1H, d, J = 8.5, Ar-H).

6.5.1.2 Compound seven

It was identified from the CE 3 fraction of methanolic leaf extract of *C.excavata* by preparative HPLC using mobile phase: methanol – water (20:80, v/v) Stationary phase ACE 5 C18 (250 x 22 mm i.d.) $\lambda_{max;}$ 254 nm. Molecular formula $C_{18}H_{16}O_7$

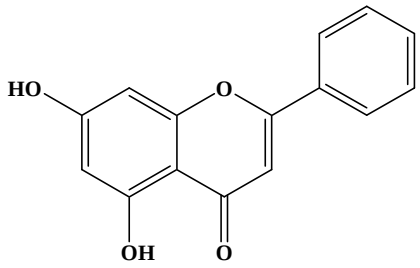


2-(3, 4-dimthoxy-phenyl) 3, 7-dihydroxy-5-methoxy-chromen-4-one

^{1}HNMR: δ_H/ppm (500 MHz, MeOHD$_2$O): 3.82(3H, s, CH$_3$), 3.92 (3H, s, CH$_3$), 3.97 (3H, s, CH$_3$), 6.71 (1H, s, Ar-H), 6.89 (1H, s, Ar-H), 7.13 (1H, d, J = 9.0, Ar-H), 7.45 (1H, d, J = 2, Ar-H), 7.59 (1H, dd, J = 9.0 + 2.0, Ar-H), 8.29 (1H, s, Ar-0H). The peak for the second OH-was not found in the spectra.

6.5.1.3 Compound eight (CE 7)

It was isolated from the methanolic leaf extract of *Clausena excavata* by HPLC using mobile phase: methanol – water (20:80, v/v) stationary phase ACE 5 C18 (250 x 22 mm i.d.) $\lambda_{max;}$ 254 nm. Molecular formula $C_{15}H_{10}O_4$.



5,7-dihydroxy-2-phenyl-chromen-4-one

^{1}HNMR: δ_H/ppm (500 MHz, CD_3OD): 6.235 (1H, d, J =2, Ar-H), 6.50 (1H, d, J =2, Ar-H), 6.75 (1H, s, Ar-H), 7.54-7.61 (3H, m, Ar-H), 7.99 (2H, d, J = 10Hz, Ar-H).
^{13}CNMR: δ_C/ppm (125 MHz, CD_3OD): 95.2(CH), 100.3(CH), 106(CH C-3), 127.5(CH), 130.3(CH), 132.6(quat.), 133.1(quat.), 159.6(quat.), 163.3(quat.), 165.7(quat.), and 166.4(quat.)

Following the extensive chromatographic separation techniques three compounds had been identified from the methanolic leaf extract of *C.excavata* and chemically characterised as 2-(3,4-dimethoxy-phenyl)-5,7-dimethoxy-chromen-4-one, 2-(3,4-dimthoxy-phenyl) 3,7-dihydroxy-5-methoxy-chromen-4-one and 5,7-dihydroxy-2-phenyl-chromen-4-one, which were confirmed by spectroscopic methods. This is the first time these compounds had been identified from this plant.

Then an extensive literature search was carried out on the isolated compounds. Flavanoids have been reported to possess a variety of pharmacological activities including analgesic, anti-inflammatory, antimicrobial, anti-histaminic, anti-cancer and anti-diabetic [143-145]. These reports gave further encouragement to proceed to biological testing of the extracts, with emphasis to be given on anti-oxidant activity and anti-microbial evaluations.

6.6 Conclusions

In summary, by the end of the characterisation element of the research programme, reversed phase HPLC had been carried out on three Rutaceous extracts. It had been thought that, given the range of components in the extracts, that reversed phase gradient conditions would be needed. However, in the case of both analytical and semi-up-scale separations it had been possible to arrive at suitable isocratic conditions without having too long a run time. Importantly, in each case, the sample concentration and injection volume were sequentially increased up to the point where baseline resolution of the major peaks was just maintained. From these points, calculations were carried out to ascertain the optimum conditions for direct scale-up for the semi-preparative or preparative ACE-5-C18 column to be used. By following the LC approach the major components of the extract had been separated, purified, identified by accurate MS, and in the majority of cases confirmed by NMR. However, fractions found to be mixtures needed to be separated to eliminate the minor components of the mixtures and within the time constraints of the research programme, there had not been time, and in some instances, not enough sample, to scale up the analytical separations achieved.

7 Biological Testing of the Rutaceous crude extracts and its Isolated components

This study was based on the ethnobotanical knowledge of rutaceous plants [65-78] in order to support the phytochemical investigations and for the scientific evaluation of traditional use of plants as drugs. The biological testing of the three rutaceous leaf extracts were investigated with the following tests.

7.1 Anti-oxidant activity using lipid peroxidation assay

7.1.1 Introduction

A reactive oxygen species (ROS) and oxygen free radicals plays important roles, both beneficial and detrimental, in aerobic life. Excess ROS have been implicated in a variety of patho-physiological phenomena, such as inflammation, ageing, cardiovascular diseases, cancer and atherosclerosis [146]. Considering the importance of this area, methanolic leaf extracts of *Tetractomia roxburghiana, Glycosmis calcicola* and *Clausena excavata* were tested for antioxidant activity. This was done by lipid peroxidation assay[148].

7.1.2 Experimental

7.1.2.1 Materials and reagents

1 % Thiobarbituric Acid (TBA): 0.5 g of TBA was dissolved in 50 ml of 50 mM NaOH with warming and stirring.

Propylgallate (0.1m M): 0.0106 g of PG in 500 ml distilled water.

Phosphate buffered saline (PBS): Prepared from purchased tablets and the solution was stored in the refrigerator at approx. 4°C.

1 mM $FeCl_{3:}$ 0. 0270 g of $FeCl_3$ was dissolved in 100 ml water with continuous stirring.

1 mM ascorbic acid: 0.0176 g of ascorbic acid was dissolved in 100ml water with continuous stirring and the solution was stored in the refrigerator at approx. 4°C.

2 % butylated hydroxy toluene (BHT) in ethanol, n – butanol (Fisher Scientific),

Bovine brain extract (Sigma product: B 3635), HCL (25% Hydrochloric acid),

Glass balls (about 2mm diameter) were also used.

7.1.2.2 Preparation of liposomes

50 mg brain extract was weighed into a clean universal container. About 7 glass balls and 10ml of PBS were added to it, sonicated in an ice-water bath until the entire lipid has been suspended and the suspension appeared homogeneous and milky in appearance.

7.1.2.3 Preparation of extracts

50 mg of leafy plant methanol extract was dissolved in 10 ml of methanol and filtered; the filtrate was used for the experiment.

7.1.2.4 Setting up the assays

Using disposable plastic test tubes, the assay was carried out as follows: 0.2ml of liposomes (5mg/ml, prepared as above), 0.5 ml PBS, water for control, 0.1ml $FeCl_3$ (1mM), 0.1ml ascorbic acid (1mM). For a control, the iron source was omitted and replaced by H_2O and incubated at 37 ˚C for 20 min.

7.1.2.5 Procedures

Chemical were purchased from BDH and Sigma-Aldrich. The liposomes were prepared as a 5mg/mL suspension of bovine brain extract (Sigma) in PBS and were stored at –80 °C. Ascorbic acid and ferric chloride (1 mM aqueous solutions) was freshly prepared before each test. A stock solution of each compound was prepared in 10 mL methanol and six serial 1 in 10 dilutions were made. Four replicates were carried out for each reaction mixture, resulting in 68 tubes (Sterilin) per assay (blank, full reaction mixture (FRM), positive control and seven compound concentrations with and without the FRM).

Stock solution (0.1 mL) and each of the six dilutions were added into eight consecutive tubes each, arranged into two rows of four replicates. Liposomes (0.2mL), 0.5mL PBS, 0.1mL $FeCl_3$ solution and 0.1 mL of ascorbic acid solution were added in the first row of each concentration (test compound). Distilled water (0.4 mL) and 0.5 mL of PBS were added in the second row of each concentration (compound alone). The positive control tubes contained 0.1 mL propyl gallate, 0.2 mL of liposomes, 0.5 mL of PBS, 0.1 mL $FeCl_3$ solution and 0.1 mL ascorbic acid solution. The blank reaction mixture tubes contained 0.3 mL distilled water, 0.2 mL of liposomes and 0.5 mL of PBS. The tubes were incubated at 37 °C, covered with foil paper, for 30 min and then 0.1 mL of BHT, 0.5mL of TBA and 0.5 mL of HCL solutions were added. The tubes were heated in a water-bath (Gallenkamp) at 85-90 °C for 30 min and, after they had cooled, 2.5mL of n-butanol was added. They were shaken individually with a vortex mixer (Gallenkamp) and centrifuged at 3500 rpm (MISTRAL-3000i) at room temperature, for 10 min. The top, n-butanol, layer (2.5 mL) was removed from each tube into a cuvette, and its absorbance was measured with a UNICAM UV/VIS spectrophotometer operating at 532 nm, using n-butanol as internal standard. The data were processed with the aid of Microsoft Excel 2000 and the percentage inhibition of each compound, at a given concentration was calculated [14]. The IC_{50} values were obtained from LOWESS analysis of percentage inhibition of peroxidation versus logarithmic concentration of each inhibitor, using PRISM GraphPad. The results were tabulated in (tables 7.1, 7.2 and 7.3)

Table 7.1: Antioxidant Activity Using Lipid Peroxidation Assay (*Tetractomia roxburghiana*).

Percentage inhibition of methanolic extract of *Tetractomia roxburghiana*										
	Conc mg/ml	Conc mM/L	Absorbances at 532nm,(n=4)				Mean	Std Deviation	% Relative Std Deviation	% Inhibition
Blank			0.1110	0.0860	0.0610	0.0460	0.0760	0.0247	32.5641	
FRM			0.1830	0.2010	0.1770	0.1830	0.1860	0.0090	4.8387	
Propylgallate		0.1000	0.1400	0.1150	0.2090	0.1110	0.1438	0.0393	27.3232	84.3100
Substance test	5.0000	18.7041	0.1340	0.1390	0.1380	0.1290	0.1350	0.0039	2.9163	75.4545
Substance alone	5.0000	18.7041	0.0410	0.0400	0.0230	0.0240	0.0320	0.0085	26.6084	
Substance test	2.5000	9.3521	0.1580	0.1490	0.1567	0.1399	0.1509	0.0072	4.7863	61.6363
Substance alone	2.5000	9.3521	0.0473	0.0392	0.0223	0.0218	0.0327	0.0110	33.6339	
Substance test	1.2500	4.6760	0.1400	0.2190	0.1700	0.1720	0.1753	0.0283	16.1261	56.3636
Substance alone	1.2500	4.6760	0.1180	0.0110	0.0190	0.0570	0.0513	0.0423	82.4881	
Substance test	0.6250	2.3380	0.2080	0.2010	0.2060	0.1120	0.1818	0.0404	22.2013	48.1818
Substance alone	0.6250	2.3380	0.0810	0.0470	0.0280	0.0390	0.0488	0.0198	40.6230	
Substance test	0.3125	1.1690	0.1710	0.2110	0.1470	0.2200	0.1873	0.0297	15.8440	42.7273
Substance alone	0.3125	1.1690	0.0600	0.0100	0.0723	0.0510	0.0483	0.0234	48.3871	
Substance test	0.1563	0.5845	0.2140	0.2300	0.2150	0.2319	0.2227	0.0083	3.7086	29.4545
Substance alone	0.1563	0.5845	0.0664	0.0637	0.0790	0.0673	0.0691	0.0059	8.4910	
Substance test	0.0781	0.2923	0.2650	0.2300	0.2350	0.2570	0.2468	0.0146	5.9312	10.3636
Substance alone	0.0781	0.2923	0.0782	0.0703	0.0794	0.0610	0.0722	0.0074	10.1953	

Table 7.2: Antioxidant Activity Using Lipid Peroxidation Assay (*Glycosmis calcicola)***.**

Percentage inhibition of methanolic extract of *Glycosmis calcicola*										
	Conc mg/ml	Conc mM/L	Absorbances at 532nm,(n=4)				Mean	Std Deviation	% Relative Std Deviation	% Inhibition
Blank			0.0657	0.0586	0.0537	0.0725	0.0626	0.0082	13.1297	
FRM			0.1685	0.1857	0.1895	0.2156	0.1898	0.0195	10.2522	
Propylgallate		0.1000	0.1152	0.1245	0.1159	0.1351	0.1227	0.0093	7.5813	84.3100
Substance test	5.0000	18.7041	0.1256	0.1452	0.1395	0.1352	0.1364	0.0083	6.0634	77.1226
Substance alone	5.0000	18.7041	0.0521	0.0429	0.0386	0.0452	0.0447	0.0056	12.6195	
Substance test	2.5000	9.3521	0.1675	0.1467	0.1506	0.1731	0.1595	0.0128	8.0303	68.8679
Substance alone	2.5000	9.3521	0.0585	0.0567	0.0527	0.0613	0.0573	0.0036	6.2892	
Substance test	1.2500	4.6760	0.1592	0.1558	0.1429	0.1424	0.1501	0.0087	5.7889	55.7390
Substance alone	1.2500	4.6760	0.0315	0.0328	0.0339	0.0265	0.0312	0.0033	10.4808	
Substance test	0.6250	2.3380	0.1357	0.1927	0.2257	0.1867	0.1852	0.0372	20.0803	50.5503
Substance alone	0.6250	2.3380	0.0689	0.0539	0.0552	0.0609	0.0597	0.0068	11.4366	
Substance test	0.3125	1.1690	0.2247	0.2173	0.1951	0.1935	0.2077	0.0157	7.5714	32.1540
Substance alone	0.3125	1.1690	0.0568	0.0674	0.0495	0.0616	0.0588	0.0076	12.8826	
Substance test	0.1563	0.5845	0.2219	0.2467	0.1978	0.2328	0.2248	0.0207	9.1922	26.5723
Substance alone	0.1563	0.5845	0.0627	0.0687	0.0687	0.0752	0.0688	0.0051	7.4176	
Substance test	0.0781	0.2923	0.2617	0.2586	0.2637	0.2547	0.2597	0.0039	1.5114	9.9057
Substance alone	0.0781	0.2923	0.0819	0.0873	0.0849	0.0758	0.0825	0.0050	6.0238	

Table 7.3: Anti-oxidant Activity Using Lipid Peroxidation Assay (*Clausena excavata)***.**

Percentage inhibition of methanolic extract of *Clausena excavata*										
	Conc mg/ml	Conc mM/L	Absorbances at 532nm,(n=4)				Mean	Std Deviation	% Relative Std Deviation	% Inhibition
Blank			0.0880	0.0480	0.0910	0.1040	0.0828	0.0242	29.2266	
FRM			0.2810	0.2930	0.2660	0.2630	0.2758	0.0139	5.0543	
Propylgallate		0.1000	0.1690	0.1210	0.1810	0.1170	0.1470	0.0327	22.2731	84.31
Substance test	5.0000	18.7041	0.1270	0.1740	0.1500	0.1740	0.1563	0.0225	14.4284	62.9534
Substance alone	5.0000	18.7041	0.0020	0.0040	0.0010	0.0010	0.0020	0.0014	70.7107	
Substance test	2.5000	9.3521	0.1980	0.1750	0.1860	0.1920	0.1878	0.0098	5.2254	56.9948
Substance alone	2.5000	9.3521	0.0030	0.0760	0.0070	0.0020	0.0220	0.0361	163.9307	
Substance test	1.2500	4.6760	0.2180	0.2480	0.2580	0.2150	0.2348	0.0215	9.1587	33.1606
Substance alone	1.2500	4.6760	0.0810	0.0060	0.0030	0.0020	0.0230	0.0387	168.2783	
Substance test	0.6250	2.3380	0.3140	0.1560	0.2600	0.2350	0.2413	0.0657	27.2345	32.3834
Substance alone	0.6250	2.3380	0.0910	0.0110	0.0070	0.0030	0.0280	0.0421	150.4528	
Substance test	0.3125	1.1190	0.2974	0.3035	0.2721	0.2291	0.2755	0.0338	12.2689	20.1554
Substance alone	0.3125	1.1690	0.0295	0.0349	0.0421	0.0478	0.0386	0.0080	20.8136	
Substance test	0.1563	0.5845	0.3139	0.3160	0.2600	0.2350	0.2812	0.0403	14.3183	10.0027
Substance alone	0.1563	0.5845	0.0191	0.0266	0.0234	0.0298	0.0247	0.0046	18.4852	
Substance test	0.0781	0.2923	0.2673	0.3126	0.2842	0.2420	0.2765	0.0297	10.7224	3.8342
Substance alone	0.0781	0.2923	0.0093	0.0070	0.0011	0.0150	0.0081	0.0058	71.3744	

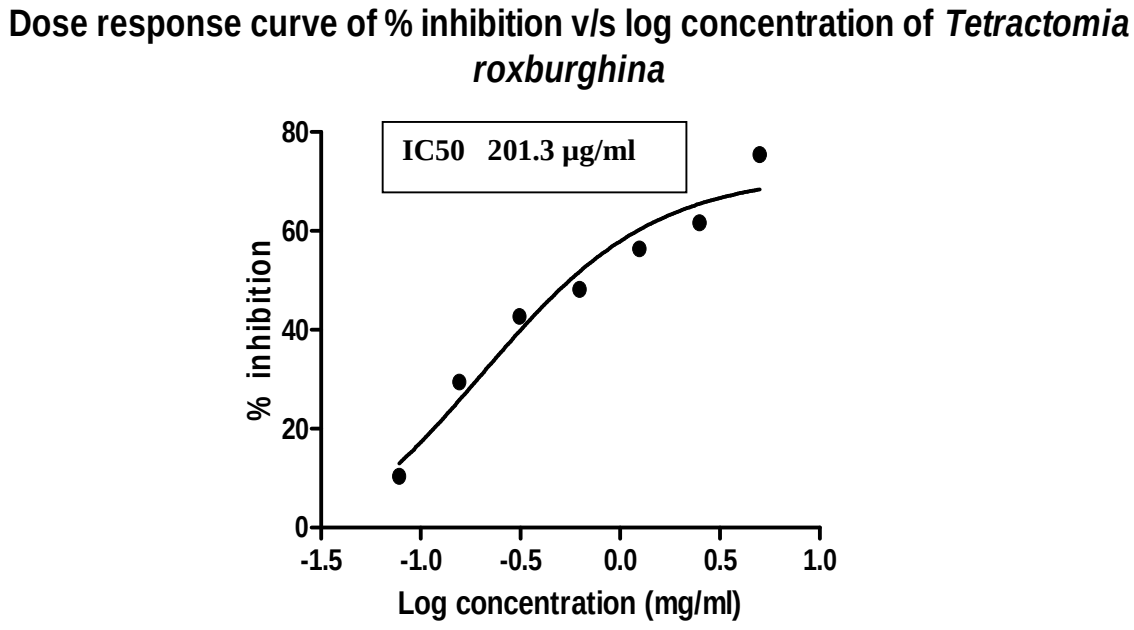


Figure 7.1: Data from *Tetractomia roxbhurghiana with* the Graph-pad prism

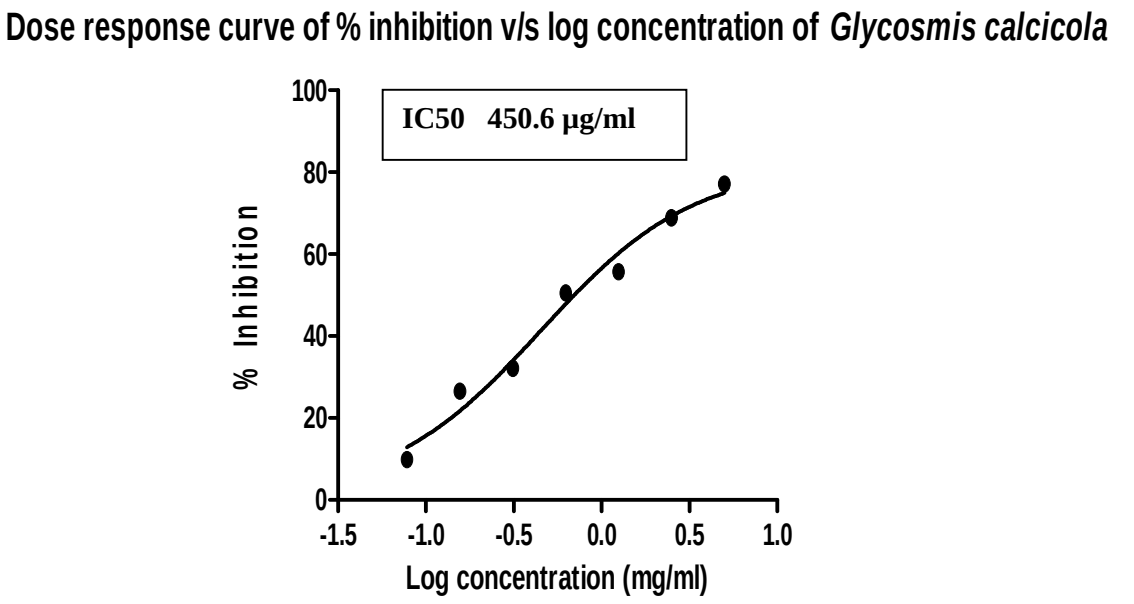


Figure 7.2: Data from *Glycosmis calcicola* with the Graph-pad prism

Dose response curve of % inhibition v/s log concentration of *clausena excavata*

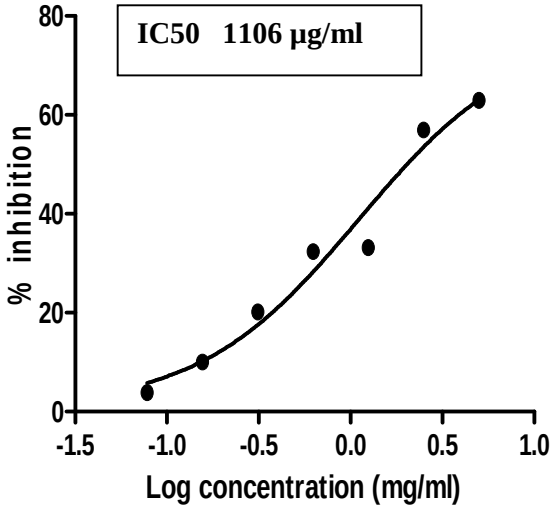


Figure 7.3: Data from *Clausena excavata with* the Graph-pad prism

7.1.2.6 Results and discussion

The results indicated that the methanolic leaf extract of *T.roxburghiana* showed marked anti-oxidant activity (Table 7.1), whereas methanolic leaf extract of *G. calcicola* and *C. excavata* showed moderate anti-oxidant activity (Table 7.2 and 7.3). The $IC_{50\ value}$ (fig. 7.1) of methanolic leaf extract of *T. roxburghiana* was found to be 201.3 µg/ml; whereas for *G. calcicola* and *C. excavata* (fig. 7.2 and 7.3) were found to be 450.6 µg/ml and 1106 µg/ml. The extended degree of anti-oxidant activity displayed by methanolic extract of *T. roxburghiana* could be contributed to the presence of swertisin[124], gallic acid[135] and angelicin[139], which are proven anti-oxidants.

7.2 Anti-microbial assays

7.2.1 Introduction

For growth and multiplication, bacteria require a suitable host. They become pathogenic (i.e., ability to cause disease) when established upon or within an individual, by pass the defensive barrier (antibiotic, phagocytes) of the host, apart from the normal state of health as when the host is visibly or sensibly injured. Thus, the recognizable interaction between the infectious agent (microorganism) and host (individual) is called disease. The broken skin, burn or other injury may allow the pathogenic microorganism to penetrate the body defenses to cause infection [149].

"The microorganisms chosen" to study for the evaluation of anti-microbial properties of three selected leafy plants extracts are *Staphylococcus aureus, Streptococcus pyogenes, Propionobacterium acne,* yeast, *Candida albicans,* dermatophytes namely *Trichophyton rubrum* and *Trichophyton mentagrophytes.*

i) *Staphylococcus aureus*

Staphylococcus aureus is a Gram + ve coccus, facultative anaerobe organism that occur in grape like clusters. The infections range from mild to life threatening [150]. Populations prone to infection include newborns, infected drug users, breast feeding

women and people with skin disorders, surgical incisions, weak immune systems or chronic diseases (depending upon the immunological status of the patients).

S. aureus frequently causes sepsis in wounds and burns. The majority of hospital cross infections are of staphylococci origin. Stripping the superficial layers of the skin from the underlying tissues by the action of the exfoliative (epidermolytic) toxins is a pathogenic mechanism for the various exfoliative syndromes caused by *Staphylococcus*. Diseases caused by *Staphylococcus* are bullous impetigo, pemphigus neonatrum, Ritten's disease, toxin epidermal necrolysis, erysipelas, cellulitis, folliculitis, and furunculosis.

S. aureus also causes other serious and potentially life-threatening toxin mediated conditions [151].

Treatment (BNF , 55,MARCH 2008)

The following drug therapies are used to treat *S.aureus* infections: broad-spectrum antipseudomonal penicillin; vancomycin (if methicillin resistant *S. aureus* suspected); flucloxacillin *or* clindamycin if penicillin-allergic (*or* vancomycin if resistant *Staphylococcus epidermidis* or meticillin-resistant *Staph. aureus*). For purulent conjunctivitis, chloramphenicol *or* gentamicin eye-drops are used. Amoxicillin, doxycycline, Phenoxymethylpenicillin are used but if allergic to pencillin, erythromycin is suitable. For Impetigo, topical fusidic acid is used or if methicillin–resistant

Staphylococcus aureus, mupirocin is the alternative therapy. For oral therapy flucloxacillin or erythromycin are used if there is widespread infection.

ii) *Streptococcus pyogenes*

Streptococcus pyogenes are Gram- + aerobic cocci arranged in chains or pairs. These cause pyogenic infections with a characteristic tendency to spread, as opposed to *Staphylococcus* lesions that are typically localised [150].

It is prone to causing skin infections like cellulitis, erysipelas, impetigo and necrotizing fascilities. These strains have earned notoriety under the name 'Flesh Eating Bacteria' in subcutaneous infections.

Treatment (BNF, 55, MARCH 2008)

The following drugs are used for *Streptococcus pyogenes* infection: tonsillitis or pharyngitis can be treated by telithromycin. Erythromycin, azithromycin, clarithromycin, vancomycin, pencillin (oral or i.v), and quinolones (nalidixic acid ,norfloxacin, ofloxacin, moxifloxacin) ampicillin, amoxicillin and co-amoxiclav.

Table 7.4: Anti-microbial drugs, classification and mechanism of action(BNF, 55 March 2008) [152, 153].

Class of drug	Mechanism of action	Drug
1. allylamines and other non-azole ergosterol biosynthesis inhibitors	reduce ergosterol biosynthesis in fungi	terbinafine
2. anti-mycotic drug	small molecule is a DNA substrate analogue that leads to incorrect DNA synthesis in fungi	Flucytosine(5-fluorocytosine)
3. azoles	inhibit the synthesis of ergosterol by blocking the action of 14-alpha demethylase in fungi	fluconazole, miconazole, ketaconazole,
4. glucan synthesis inhibitors	Inhibit glucan synthesis in fungi	caspofungin,echinocandins (anti-fungal)
5. polyenes	Act on fungal cell membrane and causes the of leak electrolytes	amphotericin-b
6. miscellaneous other systemic fungal drugs	disputing the mitotic spindle of fungal cell	griseofulvin
7. antibiotics	cell wall synthesis	bacitracin, cephalosporins, pencillins
	disrupt membrane function	nystatin, bacitracin
	RNA metabolism	griseofulvin, streptomycin
	DNA metabolism	quinolones, actinomycin
	protein synthesis	chloramphenicol, erythromycin

Table 7.5: Some important anti-microbial drugs and their adverse effects

Drug	Common uses	Adverse effects
amphotericin b	wide variety of fungal infections	chills, fever, headache, vomiting, lowered blood potassium levels, kidney damage and anaemia
fluconazole	candida and other fungal infections	liver toxicity
mucytosine	candida infections	bonemarrow and kidney damage
ketaconazole	fungal infections	nausea and vomiting, blocked production of testosterone and cortisol, liver toxicity
itraconazole	Fungal infections	nausea, diarrhoea, liver toxicity

7.2.2 Evaluation of methanolic leafy rutaceous plant extracts for antimicrobial screening

Anti-microbial activity of plant extracts can be evaluated by observing the growth response of various microorganisms that are placed in contact with them. Various anti-microbial susceptibility tests [include:

i) Diffusion [154] – Stokes method, Kirby-Baurer (disc diffusion)

ii) Dilution– broth dilution, agar dilution [155]

iii) Bioautographic [156]

Dermatophytes will grow on sabouraud dextrose agar (SDA, Oxoid CM 41) producing filamentous growth of varying colours.

Three rutaceous plant extracts were studied in the present investigation for their antimicrobial potential against bacteria (*Staphylococcus aureus, Streptococci pyogenes, Propionibacterium acne*), fungi (*Candida albicans, Trichophyton rubrum, Trichophyton mentagrophytes, Epidermatophyton floccussum*). During our present investigation, the dilution susceptibility testing method was undertaken for detailed study of rutaceous methanol leafy plant extracts.

7.2.2.1 Anti-microbial screening of rutaceous plant extracts and isolated components against *Staphylococcus aureus and Streptococcus pyogenes*

The anti-bacterial activities of the extracts for *Staphylococcus aureus and Streptococcus pyogenes* were assessed by agar incorporation technique [154]. All the three rutaceous extracts were subjected to anti-bacterial screening. This method was based on diffusion of anti-bacterial components from the reservoir well to the surrounding inoculated nutrient agar medium, so that the growth of microorganisms is inhibited as circular zone around the well.

7.2.2.1.1 Aims and Objectives

The aim was to determine the minimum inhibition concentration (MIC) of crude plant extracts against, *Staphylococcus aureus* and *Streptococcus pyogenes* by using an agar incorporation technique.

7.2.2.1.2 Materials and reagents

The following reagents, equipment and organisms were used for anti-microbial assay. Disposable 30 ml plastic universal containers were used, sterile petri dishes, purified water (pH 7), dimethylsulphoxide (GC) 99+% (Sigma-Aldrich EEC Number 200-664-3). Batch no 0041018 was used as a solvent control, a vortex mixer was used for the homogenization of extract solution, automatic multichannel pipettes Eppendorf, sterile tips 200 µl, sterile plastic 5 ml and 10 ml pipettes, Nutrient Agar (plates) and nutrient broth (liquid culture) were used for the growth of the inoculum, Gentamicin 40 mg/ml DBL Mayne Pharma, Warwickshire U.K. Exp. June 07 Code 2732B, batch no. P012732 was used as a standard drug, *S. aureus and S. pyogenes* was used for this investigation.

7.2.2.1.3 Experimental procedures

The dilution susceptibility testing method was used to determine the minimal concentration of anti-microbial to inhibit or kill the microorganisms *(S. aureus and S. pyogenes)*. A series of dilutions of the original sample in culture medium (agar/broth) was prepared and then inoculated with the test organism. The highest dilution (lowest concentration of antimicrobial agent) which inhibited perceptible growth of test organism was recorded. This dilution is considered as the minimum inhibitory concentration (MIC).

The agar dilution minimum inhibitory concentration test method uses doubling dilutions of extract suspended in an agar growth medium. The agar dilution method has advantages over a broth dilution method. Due to colour and potential precipitation of various components in the extract, incorporation into agar medium allows better homogenization of the extract and the colour or precipitate does not impair the visual assessment of surface growth. The MIC value for the extract in the screening will be quantified in micrograms per millilitre (µg/ml).

Extract stock solution: 200 mg crude plant extract was dissolved in 10 ml dimethyl sulfoxide (DMSO) with glass beads. Vortex was used to homogenise the extract solution (20,000 µg/ml).

Dilution series: 5 ml of stock solution was pipetted into 5 ml of purified water. It was vortexed for 30 seconds. Doubling dilutions were repeated with successive 5ml aliquots of purified water.

Table 7.6: Plant extract dilution in agar medium

Solution	volume of stock solution (ml)	diluent (ml)	concentration in dilution (µg/ml)	concentration of extract in agar (µg/ml)
a	5 ml of stock	5 ml pw	10000	1000
b	5 ml of a	5 ml pw	5000	500
c	5 ml of b	5 ml pw	2500	250
d	5 ml of c	5 ml pw	1250	125
e	5 ml of d	5 ml pw	625	62.5
f	5 ml of e	5 ml pw	312	31.2
g	5 ml of f	5 ml pw	156	15.6
h	5 ml of g	5 ml pw	78	7.8
i	5 ml of h	5 ml pw	39	3.9
j	5 ml of i	5 ml pw	19.5	1.9

Pw – purified water, a-j - plant extract / dilution in serial order

Table 7.7: MIC values of standard drugs, by agar dilution against selected microorganisms

Standards	microorganisms MIC in μg/ml						
	t.r	*T.m*	*E.f*	*C.a*	*S.a*	*S.p*	*P.a*
Ketaconazole	0.04	5	10	<3.91	-	-	-
Miconazole	0.08	0.63	1.25	-	-	-	-
Griseofulvin	1.25	20	20	-	-	-	-
amphotericin b	2.5	5	5	-	-	-	-
Ciclopirox	5	5	5	-	-	-	-
Ciclopirox olamine	5	10	5	-	-	-	-
Ciprofloxacin	-	-	-	-	<3.91	<3.91	-
Gentamycin	-	-	-	-	<3.91	<3.91	-
Erythromycin	-	-	-	-	-	-	0.0625

T. r - Trichophyton rubrum; *T.m-Trichophyton mentagrophytes*;

E. f - Epidermatophyton floccusum; *S. a - Staphylococcus aureus*;

S. p -Streptococcus pyogens; *P. a - Propionibacterium acne;*

C. a - Candida albicans

Table 7.8: MIC values for three rutaceous crude extracts by agar dilution method

Plant	Part	Solvent for extraction	MIC values in µg/ml of microorganisms						
			C.a	P.a	S.a	S.p	T.r	T.m	E.f
Tetractomia roxburghiana	Leaves	Hexane	500	250	250	250	500	500	500
		petroleum ether	250	1000	1000	500	125	250	250
		chloroform	1000	1000	125	250	1000	>1000	1000
		methanol	250	1000	1000	500	62.5	62.5	250
		ethanol	500	1000	1000	500	<500	<500	500
		water	500	1000	1000	500	<500	<500	500
		hexane	1000	>1000	500	500	500	1000	1000
Glycosmis calcicola	Leaves	petroleum ether	1000	1000	>1000	1000	>1000	>1000	1000
		chloroform	1000	>1000	1000	1000	1000	1000	1000
		methanol	1000	1000	500	1000	31.2	31.2	1000
		ethanol	1000	1000	500	1000	1000	1000	1000
		water	1000	250	500	250	1000	1000	1000
		Hexane	250	250	500	500	>1000	>1000	>1000
Clausena excavata	Leaves	petroleum ether	1000	1000	1000	1000	1000	1000	1000
		chloroform	1000	1000	500	500	2000	1000	1000
		methanol	>1000	250	250	250	1000	1000	500
		ethanol	500	1000	500	500	1000	>1000	1000
		water	1000	1000	500	1000	>1000	>1000	1000

C. a - Candida albicans; P. a - Propionibacterium acne; S. a - Staphylococcus aureus S. p -Streptococcus pyogenes; T. r - Trichophyton rubrum; *T. m - Trichophyton mentagrophytes*; *E. f - Epidermatophyton floccusum.*

Table 7.9: MIC values for isolated compounds and semi-purified fractions

Plant	Isolated Compounds/ Fractions	MIC values in µg/ml of microorganisms			
		C.a	T.r	T.m	E.f
Tetractomia roxburghiana	Angelicin	1000	125	62.5	>1000
	Swertisin	>1000	>1000	>1000	>2000
	α-asarone	>1000	>1000	>1000	>1000
	Gallic acid	>1000	125	125	1000
Glycosmis calcicola	GCA1 (Fraction)	>1000	500	500	1000
	GCA2 (Fraction)	1000	125	62.5	>1000
	GCA3 (Fraction)	1000	>1000	500	1000
	GCA4 (Fraction)	>1000	500	250	1000
	GCA5 (Fraction)	1000	>1000	1000	500
Clausena excavata	CE1 (Fraction)	>1000	1000	1000	500
	CE2 (Fraction)	1000	2000	1000	>1000
	CE3 (Fraction)	>1000	1000	>1000	500
	CE4 (Fraction)	500	>1000	500	>1000
	CE5 (Fraction)	>1000	>1000	>1000	500
	CE6 (Fraction)	500	>1000	500	500

7.2.2.1.4 Results and discussion

All the crude extracts showed no significant activity against *S. aureus* and *S. pyogens* when compared with that of the stadandard drugs (ciprofloxacin and gentamycin, <3.91 µg/ml). However, minimum inhibition concentration value of the extracts were established which were shown in the (table 7.8). The values seem to be very high when compared to the MIC values obtained from ciprofloxacin and gentamycin.

7.3 Antimicrobial screening of rutaceous plant extracts and isolated components against dermatophytes.

7.3.1 Introduction

Dermatophytes[157] are a group of phylogenetically related fungi, which have the ability to colonise the fully keratinised stratum corneum, hairs and nails resulting in the disease dermatophytosis (ringworm or tinea). They comprise three asexual genera, *Trichophyton, Microsporum* and *Epidermophyton* and belong to a family of largely soil–borne fungi, which break down keratinous material. The genera are defined according to the shape of the large, multicellular macroconidia they form in culture. *Microsporum* is a rough walled fusiform to obovate, *Trichophyton is a* smoothwalled cylindrical, clavate or fusiform and *Epidermophyton* is a rough walled clavate.

The morphology of *Trichophyton rubrum* species is variable. The form most frequently isolated has white, cottony, domed colonies. The reverse is initially brown with a paler border, but after two to three weeks develops the red pigment which gives the species its name. Microscopically the oval to elongate microconidia along the sides of the hyphae may be scanty.

Colonies of the anthropophilic form of *T. mentagrophytes* have a white powdery surface, cream in colour in the centre. The colony is usually flatter and more spreading than *T.rubrum.* Abundant spherical microconidia are arranged in clusters. Spiral hyphae and thin walled, smooth macroconidia may also be present. A less powdery, downy form may only show elongate microconidia along the sides of the hyphae.

Candida albicans

Single celled fungi are called yeast. *Candida albicans* is an ovoid or spherical celled, which produces pseudomycelia both in culture and in tissues. Colonies are creamy white, smooth and yeasty odour. Mature in 3 days. Candidosis is an opportunist endogenous infection, the commonest predisposing factor being diabetes [32]. Other diseases are paromychia, onchychia, (occupation hands immersed in water), vaginitis. Current treatment includes nystatin, amphotericin-b, 5-fluorocytosine, clotrimazole, miconazole, ketaconazole, fluconazole, voriconazole.

7.3.2 Aims and objectives

The aim was to determine the minimum inhibition concentration (MIC) of crude plant extracts and isolated components against dermatophytes; *Trichophyton rubrum* and *Trichophyton mentagrophytes*, by using an agar incorporation technique.

7.3.3 Experimental

7.3.3.1 Materials and reagents

The following reagents, equipment and organisms were used for anti-fungal assay.

Disposable 30 ml plastic universal containers were used. Sterile petri dishes (compartmentalised) were used for preparation of test plates. Dimethylsulphoxide (Fisher Chemicals, D/4121/PB17) Batch no 0197233 (Expire date 13-11-08) was used as solvent control in this experiment. Purified water pH 7.3 and Maximum Recovery Diluent (Oxoid CM733 Lot No.290569) Batch No.X04/056 (Expire date 20-05-04) were also used. Vortex mixer was used for the homogenization of extract solution. Automatic multichannel pipettes Eppendorf, sterile tips 200 µl, sterile plastic 5ml and 10 ml pipettes for transfer of inoculum into the Petri dishes were used. Sabouraud Dextrose Agar (SDA) (Oxoid CM41 Batch No 304644 Expire date 2008/05) pH 5.55 was used as a medium for the dermatophyte growth. Griseofulvin (97 %) Acros Organics, 16084/3, C.A. S. 126-07-8 was used as standard drug. *T.rubrum, T.mentagrophytes Epidermatophyton floccusum* and *Candida albicans* were used as test organism.

7.3.3.2 Experimental procedures

7.3.3.3 Preparation of extract dilution series

Extract stock solution: 200 mg crude plant extract was dissolved in 10 ml dimethyl sulfoxide (DMSO) with glass beads. Vortex was used to homogenise the extract solution.

Dilution series: 5 ml of stock solution was pipetted into 5 ml of purified water. It was vortexed for 30 seconds. Doubling dilutions were repeated with successive 5ml aliquots of purified water.

7.3.3.4 Preparation of dermatophyte inoculum

Using a 7-10 day culture of dermatophyte, grown on SDA at 30º C, enough 0.45% saline solution was added (BioMerieux cat. No.V1204) onto the surface of the growth and then gently agitated with the tip of a sterile cotton swab to create a suspension and the spore concentration was determined with the aid of a Neubar haeamocytometer. From the stock suspension, dilutions in maximum recovery diluent were performed. (MRD, Oxoid Cat No. CM 733), in order to achieve a final concentration of $1x10^6$ cfu/ml. 20 μl (equivalent to $2x10^4$ cfu/ml) of inoculum was used.

7.3.3.5 Preparation of test plates

A suitable number of sterile 9 ml aliquots of molten Sabouraud Dextrose Agar (SDA, Oxoid Cat. No.CM41), were prepared in 30 ml glass universals (temperature held at 50-55°C). To each SDA aliquot, 1ml of extract dilution was added and then gently inverted to mix (without introducing air bubbles). For each series of extract/agar suspensions a number of double-compartment plastic petri dishes were suitably labelled. Into one-half of the petri dish (compartment) the extract/agar suspension was added. The plates were left to set for 30 minutes at room temperature. Each plate was dried under laminar flow for 5-10 minutes with lid open and stored in the fridge for future use.

7.3.3.6 Inoculation of test plates

From each dermatophyte working suspension a 20 μl aliquot was pipetted onto the agar surface of each petri-dish compartment. Each compartment had two aliquots representing the two different dermatophytes. Plates were left to absorb the dermatophyte spore. Once dry, plates were inverted and incubated at 30^{0}C for minimum of 4 days or until growth was seen on control plates. Plates were incubated in loosely bound plastic bags to prevent excessive moisture loss.

7.3.3.7 Controls

TVAC control: A serial pour plate method, with SDA, was used to determine total viable aerobic Count (TVAC) of dermatophyte stock suspension. The plates were incubated along with test samples. (to demonstrate sufficient organism concentration).

Media controls (for each batch of media used): SDA plates (without extract dilution or dermatophyte) were incubated along with the test sample (to demonstrate media sterility). SDA plates (without extract dilution) were inoculated with dermatophytes, as in the test procedure, and incubated along with the test samples. (to demonstrate media suitability and organism viability within the test system).

Solvent control: Series of plates using dilutions of DMSO (without extract) were prepared according to test procedure. Plates were incubated along with test samples. (to demonstrate lack of solvent interference).

Antibiotic/ process control: MIC of griseofulvin was determined during every experiment. A stock solution of griseofulvin in DMSO was prepared (1000 μg/ml). This was diluted in purified water to produce a doubling dilution series and plates prepared according to the test procedure (concentrations: 0.2 to 100 μg/ml) and incubated along with test samples. (to demonstrate process repeatability between day-to-day testing and comparability to published MIC values).

The test plates were removed from the incubator and laid out in such a manner that the dilution series was apparent. The MIC value is the concentration of first plate in the dilution series to exhibit no growth (negative). No growth is interpreted as no visible growth or a very faint haze, when compared to control plates. The MIC value is the concentration of the first plate in the dilution series to exhibit no growth (negative). The presence of colonial growth of dermatophytes on the area where dilution was applied is recorded as growth).

7.3.3.8 Results and Discussion

A total of three crude methanolic plant extracts, four isolated compounds and eleven semi-purified fractions were tested for *in-vitro* efficacy, using an agar incorporation method to determine the minimum inhibition concentration (MIC), against the dermatophyte species; *T.rubrum, T.mentagrophytes, E. floccusum* and *C. albicans*. The results are tabulated in table 7.8 and 7.9. The MIC value for crude methanolic extracts of *T.roxbhurghiana* and *G. calcicola* was found to be 62.5 µg/ml and 31.2 µg/ml against *T.rubrum* and *T.mentagrophytes*, whereas methanol extract of *C. excavata* did not show any activity against dermatophytes. The isolated compounds (from methanol extract of *Tetractomia roxbhurghiana)* angelicin and gallic acid also displayed significant activity against *T.rubrum* and *T.mentagrophytes.* Based on the results and literature reports, it may be possible that the ant-fungal activity of *T. roxbhurghiana and G.calcicola* methanol extracts arose at least in part from angelicin and gallic acid. Out of the eleven semi-purified fractions, GCA2 showed anti-fungal activity.

By this time, the *G. calcicola* extract was in very short supply. However, the anti-fungal activity of the relatively abundant *T. roxburghiana* extract would be worth investigating further. Time did not permit the confirmation of anti-oxidant activity but this extract is certainly of interest given the anti-fungal activity and the anticipated (from its constituents) anti-oxidant activity.

7.4 Propionibacterium acne testing

7.4.1 Introduction

Acne or acne vulgaris is a most common dermatological disorder. It is a chronic medical condition [41] that may last for years and lead to severe emotional distress and permanent scarring. It is commonly seen in both sexes at the age of puberty and in some cases continues for a long period.

Despite the fact that there are numerous existing treatments for *Acne Vulgaris*, not everyone seeks medical care. Reasons for this include poor adherence, side effects and resistance to *P. acne,* which increases with prolonged use of antibiotics. There is no perfect algorithm in acne treatment. A drug that is efficacious and completely free of side effects does not exist. It has been reported that *P. acne*-induced mediators of inflammation is inhibited by the Indian herbs [161].

P. acne is an anaerobic, Gram-positive rod, non-spore forming cornyebacterium. *P. acne* causes acne, commonly seen in both sexes at the age of puberty and in some cases continues for long period. The development of acne is clinically proven to be related to excess sebem production, abnormal keratinization of sebaceous glands, bacteria colonisation and inflammation. It can also cause ulcers of the cornea and infections in the heart valves and osteoarticular infections. Current treatment includes tretinoin, benzoylperoxide, tetracyclines, doxycycline, minocycline, azelic acid and salicylic acid.

7.4.2 Aims and objectives

The aim was to determine the minimum inhibition concentration (MIC) of crude plant extracts of three related Rutaceae plants namely *Tetractomia roxbhughiana, Glycosmis calcicola and Clausena excavata* against *P.acne.*

7.4.3 Experimental

7.4.3.1 Materials and reagents

The following reagents, equipment and organisms were used for *P. acne* testing. Disposable plastic universals were used. Sterile petri dishes (compartmentalised).Purified water at pH 7 was used. DMSO Fisher Chemicals, code: D/4121/PB17, batch no: 0196153.VITEK Colorimeter, product no 52-1210, Jan 87.Colorimeter cuvets were used. Oxoid TSA 03/020/15 expire 21/07/04 JAT received 02/09/03.Maximum Recovery Diluent (MRD) x03/207. Vortex mixer was used for

mixing purpose. Manual pipettes, sterile tips, sterile 5ml and 10 ml pipettes. Anaerobic generator kits were used, BioMerieux Genbox anaerobe (Product code 96124), for invitro diagnostic use. Oxoid Anaerogen™ (Product code AN 0035a), an in-vitro diagnosticum. Anaer indicator (Product code 961184) Oxoid Anaerobic indicator (Product code BR55).12) Methanol, Fisher Chemicals (Code: M/4056/17, Batch No: 0253417) was used.

7.4.3.2 Procedures

7.4.3.3 Preparation of the sample

200 mg crude plant extract was dissolved in 10 ml DMSO, glass beads were added to it, and vortex mixer to help dissolution / uniform suspension. First dilution was prepared by 5 ml stock was pipetted into 5 ml purified water taken in disposable plastic universals, shaken well for uniform dilution/suspension. Whereas second dilution was prepared by diluting 5 ml first dilution to 10 ml with purified water. Dilutions were repeated with the resulting new dilution until X dilutions were achieved.

7.4.3.4 Preparation of the inoculum

Cultured *P. acne* was swabbed into 2 ml of 4.5% saline and observed its transmittance using a colorimeter with filter 450 nm. Saline was added to the culture until 74%

transmission was obtained. This serves as *P. acne* stock suspension (74% transmission = $1x10^8$ cfu/ml). From the stock 1ml was pipetted into 9 ml of MRD and shaken thoroughly to get a concentration of $1x10^7$ cfu/ml. From the new dilution the process was repeated until a concentration of $1x10^1$ cfu/ml was obtained. The required inoculum concentration was 20 µl. 20 µl. = $2x10^4$ cfu/ml.

7.4.3.5 Preparation of the plates

Petri dishes were prepared (carefully marked with suitable appropriate dilution that was used) with 1ml of the test dilution and 9 ml of molten TSA previously prepared and autoclaved in glass universals and maintained at 50-55°C. (Care was taken to mix the test solution and TSA thoroughly without introducing much air bubbles). The plates were then left to set for 30 min. The surface of the petri dishes was then dried in the laminar flow chamber.

7.4.3.6 Inoculation of the plates

From the *P. acne* stock suspension standard volume (20 µl) was added on to the surface of petri dishes prepared, the plates were dried and incubated anaerobically (using anaerobic kits) at 36°C for a minimum of 48 hrs or until growth was seen on the control plates.

7.4.3.7 Controls

Total viable aerobic count (TVAC) of *P. acne* stock suspension from concentrations $1x10^4$ cfu to $1x10^1$ cfu using the pour plate method (TSA media). The plates were incubated anaerobically along with the test samples. Media sterility was done without the plant extract using pour plate method (TSA media). The plates were incubated anaerobically along with the test samples.

DMSO control: 11 dilutions of DMSO were carried out in a similar way as that of the test solutions. The standard volume of inoculum was added onto plates prepared with dilution of DMSO and TSA media. After drying standard volume of the inoculum was added and then incubated anaerobically along with the test samples.

MIC of erythromycin standard was determined during every experiment. Stock of erythromycin was prepared and dilutions were carried out such that the actual inhibition concentration of erythromycin was in the middle of the dilution series. Each of the dilution was then mixed with separate TSA media and poured to the petri dishes. Standard volume of inoculum was then added and left aside for drying later incubated anaerobically along with the test samples.

7.4.3.8 Results and Discussion

The MIC value is concentration of first plate in dilution series to exhibit no growth (negative). The presence of colonial growth of *P. acne* on the area where dilution was applied is recorded as growth (positive). All the crude extracts showed no marked or significant activity against *P. acne* when compared with that of the standard drug. However minimum inhibition concentration values of the extracts were established which was >250 (µg/ml) and <1000(µg/ml) (Table 7.8.)

8 General Conclusions and Recommendations for Further Work

The main general conclusion that can be made is that the separation, characterisation and identification of major chemical components from three rutaceous plants namely *T.roxburghiana, G. calcicola and C. excavata* that had been embarked upon at the outset was achieved using instrumental analytical technologies such as HPLC, NMR and MS. Importantly, while there had been limitations in the initial analytical and preparative approaches, these were gradually improved upon as a result of parallel studies on analytical methodology. Microbiological studies were also carried out and for *T. roxburghiana* it was possible to attribute the anti-oxidant activity to the major constituents that had been found.

As already discussed, eight major constituents were identified. Four compounds were isolated from methanolic extracts of *T. roxburghiana* and identified as swertisin, angelicin, gallic acid and α-asarone. One compound namely *angelicin* was isolated and identified from the methanolic leaf extract of *G. calcicola*. Three compounds“2-(3, 4-dimethoxy-phenyl)-5, 7-dimethoxy-chromen-4-one, 2-(3, 4-dimethoxy-phenyl) 3, 7-dihydroxy-5-methoxy-chromen-4-one and 5, 7-dihydroxy-2-phenyl-chromen-4-one” were identified from methanolic extracts of *C. excavata*.

As mentioned above, the interesting biological activity found could, in part, be attributed to the constituents found. The anti-oxidant activity of methanolic leaf extract of *T. roxbhurghiana* was found to be significant, whereas the methanolic extract of *G. calcicola* and *C. excavata* showed moderate activities. The anti-oxidant activity could be attributed to the presence of isolated and identified compounds of these extracts. swertisin, angelicin and gallic acid have been already reported as potent anti-oxidants. The MIC results of anti-dermatophyte activity indicated that methanolic leaf extract of *G. calcicola* has significant inhibitory effect on *T.rubrum and T.mentagrophytes* in comparison to the effect of methanolic leaf extract of *T. roxburghiana and C. excavata* . As these studies were sample limited, it was not possible to test individual components but it is possible that the anti-dermatophytic activity of *C. excavata* might arise from the flavonoid nature of some of the constituents. Coumarins are considered as phytoalexins since plants produce them as defence substances when wounded or attacked by other organisms. Furthermore, coumarin compounds (angelicin isolated both from *T. roxburghiana* and *G. calcicola*) have been suggested as possible medicinal agents of importance in the case of hyper proliferative skin diseases like psoriasis [121].

The other main conclusions that can be drawn from the research programme lie within the studies on supporting analytical methodology. No direct comparisons could be made but it was clear that the approach of taking solvent extracts direct to RP-HPLC was much faster and more effective in that in the early and open column chromatography work, that had been very little difference between many of the fractions collected.

Having found by accurate mass MS and NMR that not all of the fractions collected were single components, advances were also made in methods for rapidly monitoring the 'purity' of the collected fractions. Due to the very complex nature of plant extracts and inability of reverse phase HPLC to resolve impure fractions, CE was tried as an alternative analytical tool for separation and identification of the plant constituents [106]. HPLC profiles of the extracts were compared with the capillary electrophoresis separations and found that higher resolutions were achieved with CE as anticipated [with reference to the Chapter3, (Figures 3.9(a), 3.9(b), 3.9(c)]. However the analysis time was quite high and elution of some of the constituents could not be observed. Since it is very difficult to identify plant constituents based on their UV spectra and relative migration time, CE cannot be used as an identification tool. However CE was used to monitor the purity of impure fractions collected from reverse phase preparative HPLC (with reference to the chapter- 3, Fig 3.10 (a), 3.10 (b)). Hyphenated systems like CE-MS (aqueous and non-aqueous CE) might play an important role in separation and identification of complex plant mixtures.

UPLC, ion-exchange chromatography (orthogonal to RP-HPLC) provided better resolution compared to routine RP-HPLC. The analysis time was drastically reduced (4 min) in case of UPLC analysis which proved to be a faster and more economical analytical tool than RP-HPLC. Hyphenation of these techniques to detection systems like NMR would ease and enhance the identification of plant constituents. Use of class separations by LC, particularly using ion-exchange phases can speed up preparative

work. Fractions from preparative HPLC may be rapidly monitored. Alternatively, crude extracts can be fractionated by preparative ion-exchange prior to preparative RP-HPLC.

It is suggested that suitable future work should be focussed on the anti-dermatophytic activity of *T. roxburghiana*. While *Glycosmis calcicola and C. excavata* showed better activity, it would be necessary now to collect fresh samples. There are still plentiful supplies of *T. roxburghiana.*

This future work would include scale up of the developed analytical gradient method for *T. roxburghiana* to a preparative method (now have the capability for direct scale-up from gradient analytical RPLC to prep-scale using an Agilent 1200 HPLC) for isolation and identification of plant constituents from rutaceous extracts and also the use of appropriate straight phase system for further separation of unresolved mixtures (impure fractions). Straight phase could help in separation of compounds, which were not resolved using reverse phase systems. Selection of solvents is to be considered mainly based on solubility of the extracts and volatility of the solvent. Monitoring fraction purity and structural elucidation could be done with LC-NMR-MS using orthogonal LC modes and scale up orthogonal LC modes for isolation of constituents from impure fractions for microbiological and anti-fungal activity testing. It is also logical to extend the testing of the extracts and isolated compound for other biological activities like ant-inflammatory, analgesic and anti-psoriatic because flavonoids have also been reported to possess a variety of pharmacological activities [143] including analgesic, ant-inflammatory, anti-microbial, anti-histaminic, anti-cancer, anti-diabetic.

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