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Phytochemical Investigation of *Rhododendron lepidotum*

DISSERTATION

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS

PROVIDED FOR THE AWARD OF DEGREE OF

MASTER OF PHILOSOPHY

IN

CHEMISTRY

BY

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SRINAGAR (J&K) INDIA 190 005

2011

DEDICATED

TO

MY BELOVED PARENTS

FOR

THEIR LOVE AND PRAYERS

Indian Institute of Integrative Medicine
(CSIR) Sanatnagar, Srinagar-190 005
&
Department of Chemistry,
University of Kashmir, Srinagar-190 006

Certificate

This is to certify that the work presented in this dissertation entitled **"Phytochemical Investigation of *Rhododendron lepidotum*"** is original and has been carried out by **Mr Shakeel-u-Rehman** under our joint supervision. This work is suitable for submission for the award of M.Phil Degree in Chemistry. It is further certified that the work has not been submitted in part or full for award of any degree in this or any other University.

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Declaration

I hereby declare that the dissertation entitled **"Phytochemical Investigation of *Rhododendron lepidotum*"** submitted for M.Phil degree to the University of Kashmir has been carried out at Indian Institute of Integrative Medicine (CSIR), Srinagar, under the joint supervision of Dr K A Bhat (Scientist, IIIM, Srinagar) and Dr M A Khuroo (Department of Chemistry, University of Kashmir). The work embodied in this dissertation is original and has not been submitted in part or full for any degree or diploma to this or any other University.

Dated: **SHAKEEL-U-REHMAN**

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Chapter 1
Introduction

Introduction

1.1 General overview of Medicinal plants

Throughout the ages, humans have relied on Nature for their basic needs for the production of food-stuffs, shelters, clothing, means of transportation, fertilizers, flavours and fragrances, and, not the least, medicines. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. Although some of the therapeutic properties attributed to plants have proven to be erroneous, medicinal plant therapy is based on the empirical findings of hundreds and thousands of years. The first records, written on clay tablets in cuneiform, are from Mesopotamia and date from about 2600 BC; among the substances that were used were oils of *Cedrus* species (Cedar) and *Cupressus sempervirens* (Cypress), *Glycyrrhiza glabra* (Licorice), *Commiphora* species (Myrrh) and *Papaver somniferum* (Poppy juice), all of which are still in use today for the treatment of ailments ranging from coughs and colds to parasitic infections and inflammation (Fakim, 2006).

Natural products have, until recently, been the primary source of commercial medicines and drug leads. A recent survey revealed that 61% of the 877 drugs introduced worldwide can be traced to or were inspired by natural products (Newman *et al*., 2003). However, beginning in the 1990s, natural product drug discovery was virtually eliminated in most big pharmaceutical companies. This was primarily due to the promise of the then-emerging field of combinatorial chemistry (Cseke *et al*., 2004), whereby huge libraries of man-made small molecules could be rapidly synthesized and evaluated as drug candidates.

Thus far, this approach has led to lukewarm results at best. From 1981 to 2002, no combinatorial compounds became approved drugs, although several are currently in

late-stage clinical trials. At the same time, the number of new drugs entering the market has dropped by half, a figure of which the large pharmaceutical corporations are painfully aware. The haystack is larger, but the needle within it is more elusive. This has led only recently to a newfound respect for the privileged structures inherent within natural products (DeSimone *et al*., 2004).

Of the roughly 250,000 species of plants believed to exist, one-third of those have yet to be discovered. Of the quarter million that have been reported, only a fraction of them have been chemically investigated. Many countries have become aware of the value of the biodiversity within their borders and have developed systems for exploration as well as preservation. At the same time, habitat loss is the greatest immediate threat to biodiversity (Frankel *et al*., 1995).

Products of natural origins can be called ‘‘natural products.’’ Natural products include: (1) an entire organism (e.g., a plant, an animal, or a microorganism) that has not been subjected to any kind of processing or treatment other than a simple process of preservation (e.g., drying), (2) part of an organism (e.g., leaves or flowers of a plant, an isolated animal organ), (3) an extract of an organism or part of an organism, and exudates, and (4) pure compounds (e.g., alkaloids, coumarins, flavonoids, glycosides, lignans, steroids, sugars, terpenoids, etc.) isolated from plants, animals, or microorganisms (Samuelsson, 1999). However, in most cases the term natural products refers to secondary metabolites, small molecules (mol wt <2000 amu) produced by an organism that are not strictly necessary for the survival of the organism. Concepts of secondary metabolism include products of overflow metabolism as a result of nutrient limitation, shunt metabolism produced during idiophase, defense mechanism regulator molecules, etc. (Cannell, 1998). Natural products can be from any terrestrial or marine source: plants (e.g., paclitaxel [Taxol]

from *Taxus brevifolia*), animals (e.g., vitamins A and D from cod liver oil), or microorganisms (e.g., doxorubicin from *Streptomyces peucetius*).

Strategies for research in the area of natural products have evolved quite significantly over the last few decades. These can be broadly divided into two categories:

1. Older strategies:

a. Focus on chemistry of compounds from natural sources, but not on activity.

b. Straightforward isolation and identification of compounds from natural sources followed by biological activity testing (mainly in vivo).

c. Chemotaxonomic investigation.

d. Selection of organisms primarily based on ethnopharmacological information, folkloric reputations, or traditional uses.

2. Modern strategies:

a. Bioassay-guided (mainly in vitro) isolation and identification of active ‘‘lead’’ compounds from natural sources.

b. Production of natural products libraries.

c. Production of active compounds in cell or tissue culture, genetic manipulation, natural combinatorial chemistry, and so on.

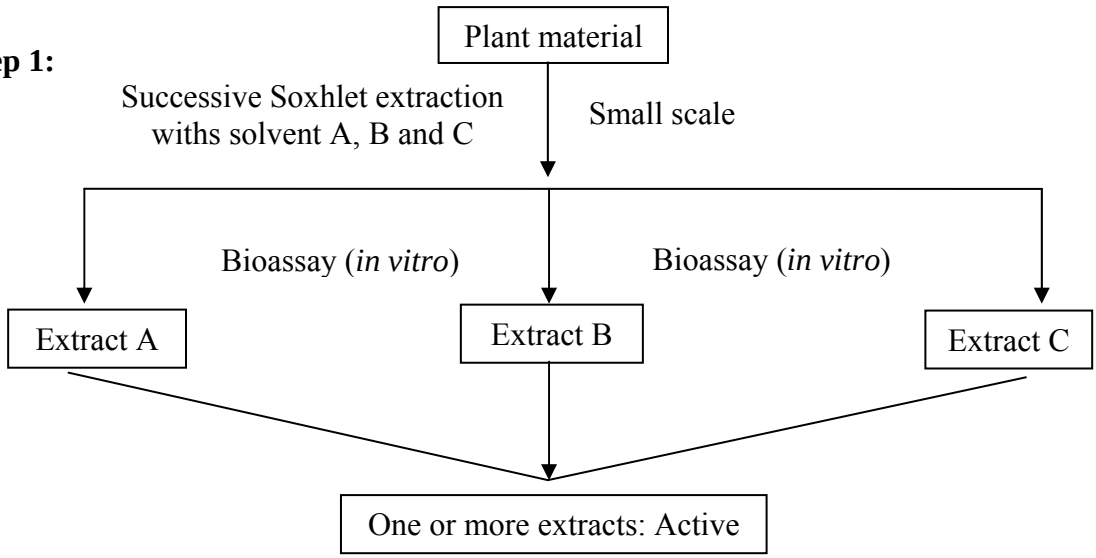
d. More focused on bioactivity.

e. Introduction of the concepts of dereplication, chemical fingerprinting, and metabolomics.

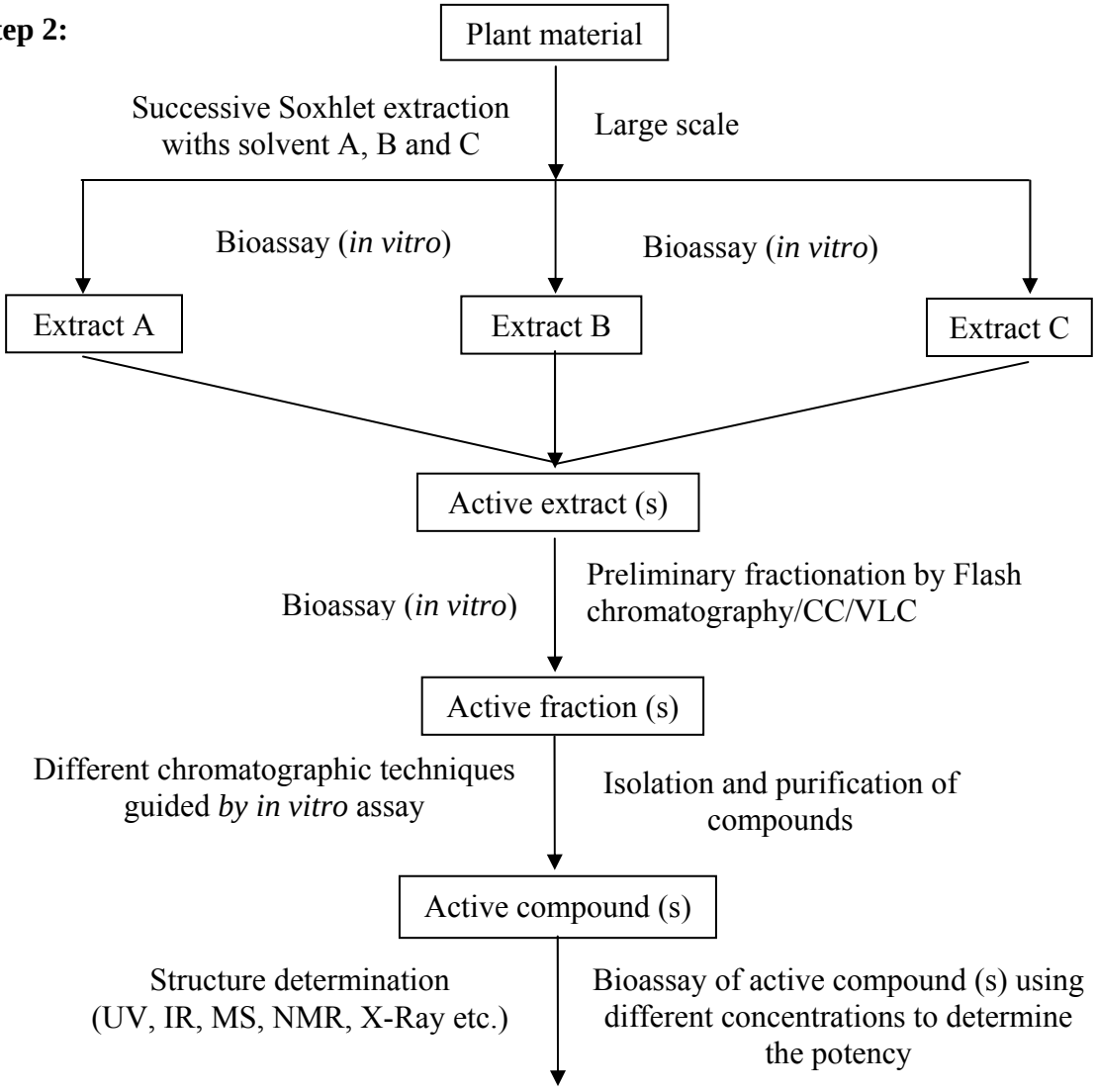
f. Selection of organisms based on ethnopharmacological information, folkloric reputations, or traditional uses, and also those randomly selected.

A generic protocol for the drug discovery from natural products using a bioassay-guided approach is presented in Fig: 1.1.

Step 1:


Plant material
Successive Soxhlet extraction withs solvent A, B and C
Small scale
Bioassay (*in vitro*)
Bioassay (*in vitro*)
Extract A
Extract B
Extract C
One or more extracts: Active

Step 2:


Plant material
Successive Soxhlet extraction withs solvent A, B and C
Large scale
Bioassay (*in vitro*)
Bioassay (*in vitro*)
Extract A
Extract B
Extract C
Active extract (s)
Bioassay (*in vitro*)
Preliminary fractionation by Flash chromatography/CC/VLC
Active fraction (s)
Different chromatographic techniques guided *by in vitro* assay
Isolation and purification of compounds
Active compound (s)
Structure determination (UV, IR, MS, NMR, X-Ray etc.)
Bioassay of active compound (s) using different concentrations to determine the potency

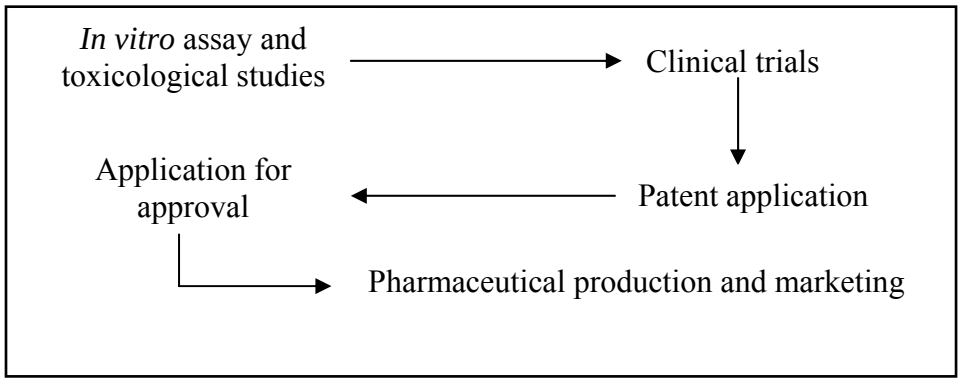


Fig. 1.1 An example of natural product drug discovery process (bioassay guided approach)

1.2 Natural Products: Historical Perspective

The use of natural products, especially plants, for healing is as ancient and universal as medicine itself. The therapeutic use of plants certainly goes back to the Sumerian civilization, and 400 years before the Common Era, it has been recorded that Hippocrates used approximately 400 different plant species for medicinal purposes. Natural products played a prominent role in ancient traditional medicine systems, such as Chinese, Ayurveda, and Egyptian, which are still in common use today. According to the World Health Organization (WHO), 75% of people still rely on plant-based traditional medicines for primary health care globally. A brief summary of the history of natural product medicine is presented in Table 1.1

1.3 Current status of Natural Products

Nature has been a source of therapeutic agents for thousands of years, and an impressive number of modern drugs have been derived from natural sources, many based on their use in traditional medicine. Over the last century, a number of top selling drugs have been developed from natural products (vincristine from *Vinca*

rosea, morphine from *Papaver somniferum*, Taxol from *T. brevifolia*, etc.). In recent years, a significant revival of interest in natural products as a potential source for new medicines has been observed among academia as well as pharmaceutical companies. Several modern drugs (~40% of the modern drugs in use) have been developed from natural products.

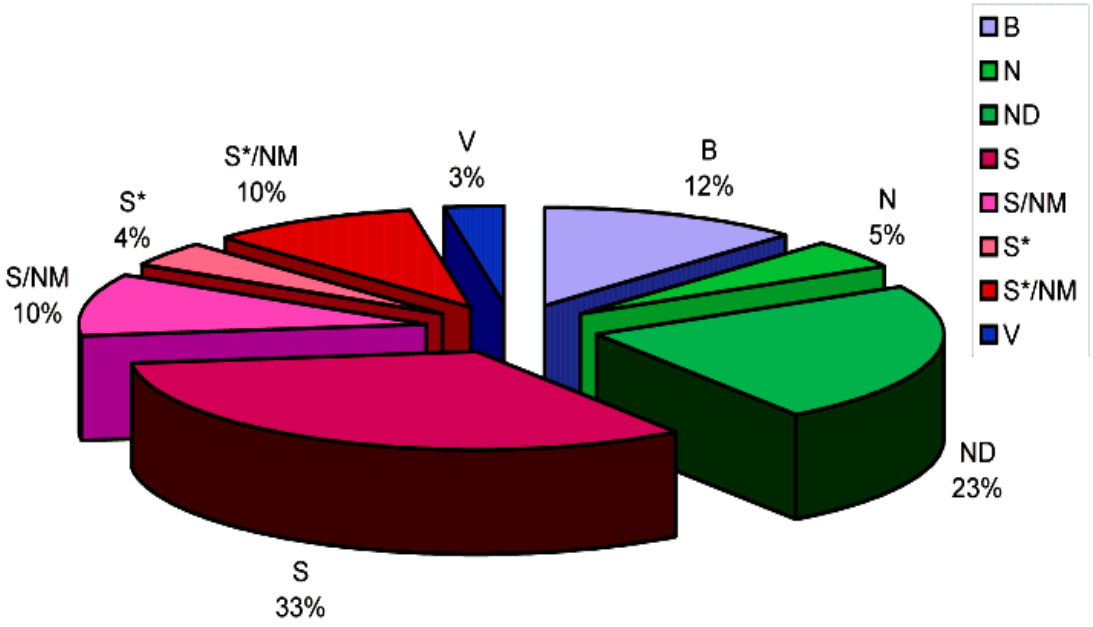
Table 1.1 History of Natural Product Medicine

Period	Type	Description
Before 3000 BC	Ayurveda (knowledge of life)	Chinese traditional medicine Introduced medicinal properties of plants and other natural products
1550 BC	*Ebers Papyrus*	Presented a large number of crude drugs from natural sources (e.g., castor seeds and gum arabic)
460–377 BC	Hippocrates, ‘‘The Father of Medicine’’	Described several plants and animals that could be sources of medicine
370–287 BC	Theophrastus	Described several plants and animals that could be sources of medicine
23–79 AD	Pliny the Elder	Described several plants and animals that could be sources of medicine
60–80 AD	Dioscorides	Wrote De Materia Medica, which described more than 600 medicinal plants
131–200 AD	Galen	Practiced botanical medicines (Galenicals) and made them popular in the West
15th century	Krauterbuch (herbals)	Presented information and pictures of medicinal plants

More precisely, according to Cragg *et al*., 39% of the 520 new approved drugs between 1983 and 1994 were natural products or their derivatives, and 60–80% of antibacterial and anticancer drugs were from natural origins. In 2000, approximately 60% of all drugs in clinical trials for the multiplicity of cancers had natural origins (Cragg *et al*., 1997). In 2001, eight (simvastatin, pravastatin, cvamoxycillin, clavulanic acid, azithromycin, ceftriaxone, cyclosporin, and paclitaxel) of the 30 top-selling medicines were natural products or their derivatives, and these eight drugs together totaled US $16 billion in sales. Apart from natural product-derived modern medicine, natural products are also used directly in the ‘‘natural’’ pharmaceutical industry, which is growing rapidly in Europe and North America, as well as in traditional medicine programs being incorporated into the primary health care systems of Mexico, the People’s Republic of China, Nigeria, and other developing countries. The use of herbal drugs is once again becoming more popular in the form of food supplements, nutraceuticals, and complementary and alternative medicine.

Interest in herbal drugs and natural medicine is undergoing a renaissance in the present age. Higher plant derived products represent around 25% of the total number of clinically used drugs (Bames *et al*., 2007). It has been reported that plants and other sources of natural products are superior sources of molecular diversity and novel molecular chemotypes, particularly in the areas where good synthetic leads do not exist (Raskin *et al*., 2002). Despite competition from other drug discovery methods, NPs are still providing their fair share of new clinical candidates and drugs. For example, between 1981 and 2002, 5% of the 1,031 new chemical entities approved as drugs by the US Food and Drug Administration (FDA) were natural products, and another 23% were natural-product-derived molecules (Newman *et al*., 2003) Fig: 1.2.

About 250000 living plant species contain a much greater diversity of bioactive compounds than any chemical library made by humans.



"B": Biological; usually a large (>45 residues) peptide or protein either isolated from an organism/cell line or produced by biotechnological means in a surrogate host. **"N":** Natural product. **"ND":** Derived from a natural product and is usually a semisynthetic modification. **"S":** Totally synthetic drug, often found by random screening/modification of an existing agent. **"S*":** Made by total synthesis, but the pharmacophore is/was from a natural product.**"V":** Vaccine. **"NM":** Natural product mimic.

Fig: 1.2 All new chemical entities, 1981-2002, by source (Total =1031)

Natural products can contribute to the search for new drugs in three different ways:

1. By acting as new drugs that can be used in an unmodified state (e.g., vincristine from *Catharanthus roseus*).

2. By providing chemical ''building blocks'' used to synthesize more complex molecules (e.g., diosgenin from *Dioscorea floribunda* for the synthesis of oral contraceptives).

3. By indicating new modes of pharmacological action that allow complete synthesis of novel analogs (e.g., synthetic analogs of penicillin from *Penicillium notatum*).

Natural products will certainly continue to be considered as one of the major sources of new drugs in the years to come because:

1. They offer incomparable structural diversity.

2. Many of them are relatively small (<2000 Da).

3. They have ‘‘drug-like’’ properties (i.e., they can be absorbed and metabolized).

Only a small fraction of the world’s biodiversity has been explored for bioactivity to date. For example, there are at least 250,000 species of higher plants that exist on this planet, but merely 5–10% of these have been investigated so far. In addition, reinvestigation of previously studied plants has continued to produce new bioactive compounds that have drug potential.

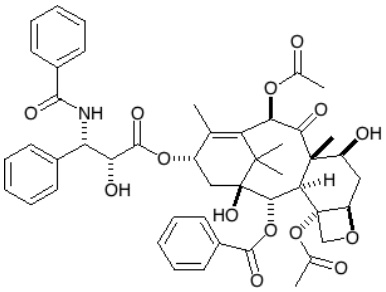
Much less is known about marine organisms than other sources of natural products. However, research up to now has shown that they represent a valuable source for novel bioactive compounds. With the development of new molecular targets, there is an increasing demand for novel molecular diversity for screening. Natural products certainly play a crucial role in meeting this demand through the continued investigation of the world’s biodiversity, much of which remains unexplored (Cragg and Newman, 2001a). With less than 1% of the microbial world currently known, advances in technologies for microbial cultivation and the extraction of nucleic acids from environmental samples from soil and marine habitats will offer access to an untapped reservoir of genetic and metabolic diversity (Cragg and Newman, 2001b). This is also true for nucleic acids isolated from symbiotic and endophytic microbes associated with terrestrial and marine macroorganisms.

Discovery of vincristine and vinblastine (Fig 1.3) in 1963 by R. L. Noble and his Canadian co-workers (Neuss and Neuss, 1990) and its successful patent by Eli Lilly launched the pharmaceutical industry into the search for natural product leads for the

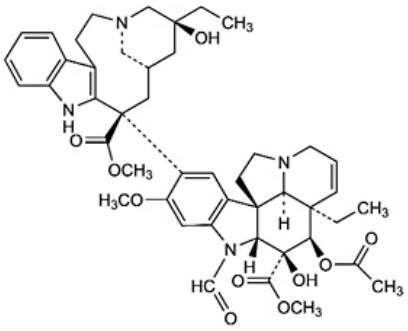
treatment of various cancers. Recent natural product discovery and development of avermectins (anthelmintic), cyclosporine and FK-506 (immunosuppressive), mevinolin and compactin (cholesterol-lowering), and Taxol and camptothecin (anticancer) (Fig. 1.3) have revolutionized therapeutic areas in medicine (Kirst *et al*., 1992). Similar successful development of azoxystrobin (β-methoxyacrylate) fungicides and spinosad (tetracyclic macrolides) pesticides have created a renewed interest in natural product agrochemical discovery. Because biologically derived chemicals are perceived by consumers as having less environmental toxicity and lower mammalian toxicity, chemical and pharmaceutical companies currently have a greater desire to discover and develop natural product-based plant protectants.

The versatility of plants in chemistry and biosynthesis is arguably unmatched by any other group of living organisms and potentially represents the most wide ranging source of novel pharmacologically active chemical entities in the living world. While the majority of natural products used therapeutically are derived from plants, it is argued that we have as yet only scratched the surface of what may be an Aladdin's cave. This of course is true but we must strongly advocate *expanding*, not decreasing, the exploration of nature as a source of novel active agents that may serve as the leads and scaffolds for elaboration into desperately needed efficacious drugs for a multitude of disease indications.

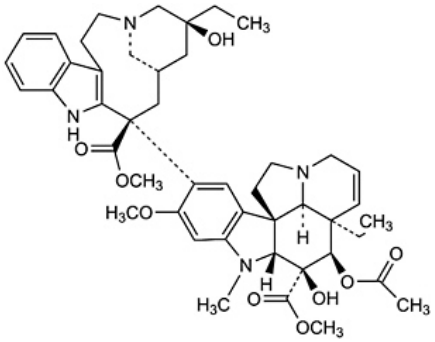
Advent, introduction, and development of several new and highly specific in vitro bioassay techniques, chromatographic methods, and spectroscopic techniques, especially nuclear magnetic resonance (NMR), have made it much easier to screen, isolate, and identify potential drug lead compounds quickly and precisely. Automation of these methods now makes natural products viable for high-throughput screening (HTS).



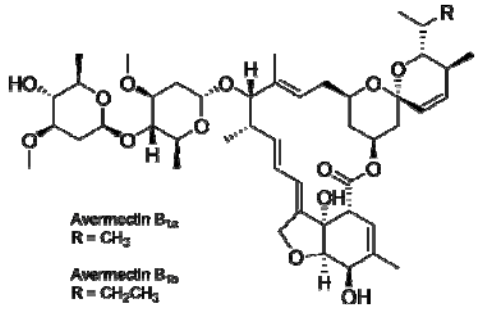
Paclitaxel (Taxol)



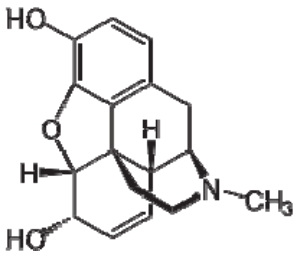
Vincristine



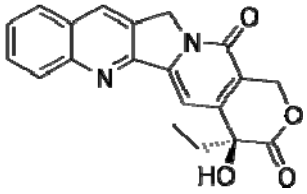
Vinblastine



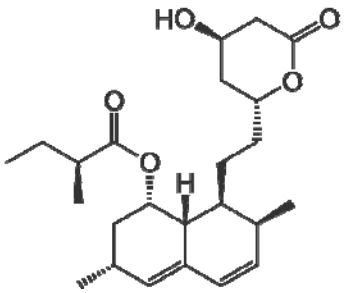
Avermectins



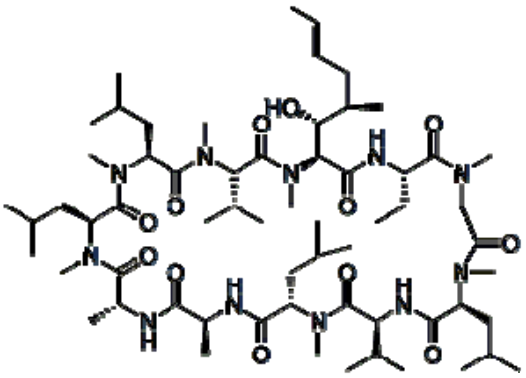
Morphine



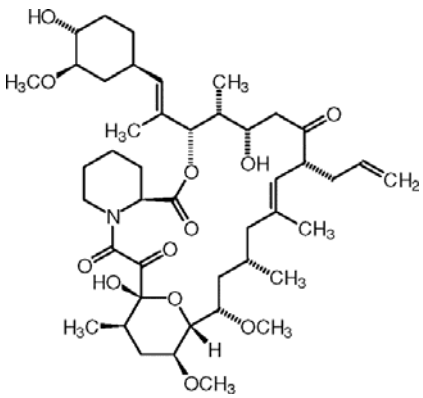
Camptothecin



Mevinolin



Cyclosporine



FK-506

Fig: 1.3 Some drugs from natural sources

1.4 Overview of the Genus *Rhododendron*

The genus *Rhododendron* (Ericaceae) consists of over 850 species, occurring throughout the world (Alan *et al*., 2010). An exhaustive survey of literature revealed that the different species of *Rhododendron* have a vast range of biological activities including anti-HIV, anti-inflammatory, anti-arthritic, anti-rheumatic, diaphoretic, anti-hypertensive, hypotensive, diuretic, anti-asthmatic, cytotoxic, antibacterial, antifungal and antioxidant activity. Some very important bioactive molecules have been discovered from this genus, notably the anti-HIV rhododaurichromanic acid A, daurichromenic acid, hypotensive andromedotoxin and cytotoxic rhodomolleins and rhodojaponins. Terpenoids, flavonoids, coumarins, steroids, amines, phenolic acids and glycosides constitute major classes of phytoconstituents of the genus.

Various species of *Rhododendron* offer an opportunity for bioprospection and need to be explored for the isolation of bioactive constituents for various biological activities, especially their effects on immune and circulatory and respiratory systems.

Chapter 2
Review of Literature

2.1 The Genus *Rhododendron*

The generic name *Rhododendron* is derived from two Greek words "rhodon", a rose, and "dendron", a tree. It is a large genus of herbs, shrubs and trees, including some epiphytes and is distributed in the Northern hemisphere especially in temperate regions of North America, Europe and Asia with over 900 species, 600 of which grow in south to north of China- the *Rhododendron* emperor country (Alan *et al*., 2010; Chinese Forestry Science and Technology Message, 1998). Maximum concentration of species occurs in the eastern Himalayas, China and Japan, extending into the mountains of South-East Asia. The genus has about 50 species in India (Singh *et al*., 2003) mainly distributed in the Himalayan region, of which only four occur in the Western Himalayas; a few exotics have also been introduced. Several Rhododendron species are widely cultivated for their diverse flower colours or evergreen foliage. Many hybrids have been raised and are extensively cultivated in Europe.

2.1.1 Ethnopharmacology

R. dauricum L. and *R. aureum* Georgi. are used in Tibetan medicine to treat several pathological conditions(Gammerman and Semichov, 1963) and as an expectorant to treat acute and chronic bronchitis (Iwata *et al*., 2004; Kashiwada *et al*., 2001). The roots of *R. dauricum* are used for therapy of acute bacteroidal dysentery (Cao *et al*., 2004). A decoction of *R. luteum* and *R. ponticum* leaves is externally used to treat fungal foot infection in Giresun province (Alan *et al*., 2010). The leaf of *R. ellipticum* is used in Taiwan as a folk medicine to treat high blood pressure (Chiu, 1987). The leaves and flowers of *R. lepidotum* are used as a snuff in headache (Dar *et al*., 2002). The leaves and flowers of *R. ferrugineum* are used for rheumatism in Germany

(Beauquesne *et al*., 1990). The leaves of *R. campanulatum* are mixed with tobacco and used as a snuff in the treatment of colds and headaches that affect only one side of the head (Chopra *et al*., 1986). The poulticed leaves of *R. maximum* are used to relieve arthritic pain, headaches etc (Usher, 1974; Foster and Duke, 1990). The flowers and leaves of *R. latoucheae* have been traditionally used to treat skin festers and the roots as a paregoric and antidote (Fan *et al*., 2001). *R. barbatum* has been used as fish poison (Chopra *et al*., 1957). The flowers and fruits of *Rhododendron molle* G. Don, a well-known poisonous plant, have been recorded in ancient and modern monographs as analgesics and insecticides (Li *et al*., 2000; Chen and Zheng, 1987). The roots, leaves and flowers of *R. arboreum* are important crude source of drugs in traditional system of medicine and are used in dysentery, fever and headaches (Swaroop *et al*., 2005; Chauhan, 1999). *Rhododendron przewalskii* Maxim. has been used as a folk medicine in China for the treatment of hypertension and coronary heart diseases (Li and Jia, 2003). *R. ponticum*, is a common folk medicine of the Black Sea region, widely used as analgesic for the treatment of rheumatic or dental pain, common colds and edema, both internally and externally (Baytop, 1999). The leaves and flowers of *R. primulaeflorum* are used to clear away heat, for detumescence, and tonification of kidneys. In Tibet and the western part of China, it is widely used to cure pulmonary disease, dropsy, indigestion, gastroptosis, and gastrectasis. The essential oils from it are used in curing chronic tracheitis (Li *et al*., 2010). The dried stems, leaves, and flowers of *R. spinuliferum* have been used as Chinese folk medicine for eliminating phlegm, relieving cough, and as an anti-asthmatic (Chen *et al*., 2009). The leaves and flowers of *Rhododendron simsii* have been used in Chinese medicine to treat irregular menstruation, traumatic injuries, rheumatism and subcutaneous swelling (Fenglin *et al*., 2004).

Rhododendrons are not only of high value in view but are also of good medicinal importance because of the essential oils present in their twigs, leaves and flowers and can be used as high grade spices and cosmetics, especially due to efficacies such as detoxification, relieving cough and calming asthma, dispelling phlegm, diminishing inflammation and depressing blood pressure (Jiangsu New Medicinal Academy, 2003; Kurokawa, 1995; Klingeman *et al*., 2000). In Tibet and China, the essential oils from various species of the genus *Rhododendron* are used to cure pulmonary diseases, laryngitis, urethritis, indigestion, gastroptosis, gastrectasis gastric cancer, hepatoma, dropsy and chronic tracheitis (Zhao *et al*., 2006; Klingeman *et al*., 2000).

2.1.2 Phytochemistry

Previous phytochemical work on genus *Rhododendron* has resulted in the isolation of various classes of compounds which is presented in the form of table 2.1.

Table 2.1 Phytoconstituents of various species of *Rhododendron*.

Species	Phytoconstituents
R. adamsii	**Essential oil** (Fig. 1): α-pinene, β-pinene, β-myrcene, *cis*-β-ocimene, isoledene, aromadendrene, humulene, β-farnesene, δ-cadinene, *trans*-nerolidol, spathulenol, β-elemenone, and germacrone. (Rogachev *et al*., 2006)
R. anthopogon	quercetin-3-*O*-α-L-rhamnopyranoside, kaempferol, 24-methylenecycloartenyl acetate, betulinic acid, ursolic acid, epifriedelinol, β-sitisterol, rutin, friedelin, quercetin (Joshi *et al*., 1981); 2, 6, 10, 14, 18, 22-tetracosahexaene, umbelliferone, 3-hydroxy-3-phenylproponamide, orcin, quercetin, myricetin, myricetin-3-O-β-D-xylopyranoside, hyperin (Zhou *et al*.,

	2010a); **Essential oil**: N-acetyl-1, 2, 3, 4-tetrahydroisoquinoline, 2-Ethoxypropane, 3-methyl-6-tert-butylphenol, 3-Methyl-5-phenylisothiazole, diphenylamine, N-ethyl-1, 2, 3, 4-tetrahydronaphthalenamine, pentacosane and tricosane (Zhou *et al*., 2010b)
R. anthopogonoides	fatty acids and esters, terpenic alcohols and steroids (Liu, 2007)
R. arboreum	rutin, coumaric acid, ursolic acid, epifriedelinol, friedelin, hyperin, quercetin, quercetin rhamnoside, taraxerol, betulinic acid, campanulin, α-amyrin, β-sitosterol, 3, 10- epoxyglutinane, flavone 5, 2′- dihydroxy-7-methoxy-4′-*O*- glucoside, dimethyl ester of terephthalic acid (Swaroop *et al*., 2005)
R. aureum	**Essential oil** (Fig. 1): calarene, aristolene, burbonanes, cadinanes, *trans*-caryophyllene, aromadendrene, α-Selinene, Caryophyllene oxide, hexanoic acid, carvacrol, and α-pinene (Olennikov *et al*., 2009; Rogachev *et al*., 2006)
R. bakeri	taxifolin-7-galactoside (King, 1977)
R. barbatum	epifriedelinol, friedelin, β-amyrin, ursolic acid, n-triacontane, hentriacontanol, oleanolic acid, betulonic acid, 5, 6, 7, 4′-tetra methoxyflavone, β-sitosterol (Bahuguna and Jangwan, 1987; Thapliyal and Bahuguna, 1993)
R. brachycarpum	campanulin, α-amyrin, β-amyrin, uvaol, simiarenol, ursolic acid and oleanolic acid (Youn and Cho, 1991)
R. campanulatum	campanulin, epifriedelinol, ursolic acid, quercitin, friedelin, α-amyrin (King, 1977), oleanane triterpene (Tantry *et al*, 2011)
R. capitatum	Haplogenin (Batirov, 1980)

R. catawbiense	quercetin 3-galactoside, sitosterol (Doss *et al*., 1982)
R. chamaecistus	Gossytrin (Harborne, 1969; De Loose, 1970)
R. championae	ursolic acid (Arthur and Hui, 1954b)
R. cinnabarinum	ursolic acid, quercetin (King, 1977)
R. collettianum	8′-epi-cleomiscosin A, 8-*O*-β-*D*-glucopyranosyl-6-hydroxy-2-methyl-4H-1- benzopyrane-4-one, cleomiscosin A, aquillochin, 5, 6, 7- trimethoxycoumarin (Ahmad *et al.*, 2004)
R. concinnum	(2R)-farrerol-7-O-glucopyranoside, (2R, 3R)-(-)-dihydroquercetin-3-O-β-D-xylopyranoside, (2S, 3S)-(-)-dihydroquercetin-3-O-β-D-glucopyranoside, eriodictyol-7-O-β-D-glucopyranoside, (2R, 3R)-(+)-dihydroquercetin (Zhao *et al*., 2010); (2S)-4',5,7-trihydroxy-flavanone, (2R, 3R) (+)taxifolin, avicularin, quercetin-3-O-α-L-rhamnoside, hyperoside, quercetin, 19-kaurane acid, isoscopoletin, ledol, stigmast-4-en-6β-ol-3-one, β-amyrin, α-amyrin, 28-hydroxy-β-amyrin, uvaol, oleanolic acid, β-sitosterol and β–daucosterol (Yang and Tian, 2007)
R. dauricum	rhododaurichromanic acid A, rhododaurichromanic acid B, daurichromenic acid, p-bromophenacyl rhododaurichromanic acid A, ursolic acid, oleanolic acid, daurichromenes A, B, C and D, confluentin, grifolin, orcinol, farrerol, quercetin, syringic acid, vanillic acid, 4-hydroxy benzoic acid and protocatechuic acid (Committee of Jiangsu New Medical College, 1995; Kashiwada *et al*., 2001; Iwata *et al*., 2004); 4, 5-Dihydro-5-oxo-3-(p-tolyl) isoxazole, 1,3-Benzenediol, 5-

	methyl-2-(3, 7, 11-trimethyl-2, 6, 10-dodecatrienyl)-(E, E)-3, 6-Diphenyl-1, 2, 3, 4, 5, 6, 7, 8-octahydro-1, 8-acridinedione, 6H-[1, 2, 4] Triazolo [1, 5-a] indole, 4a, 5, 7, 8, 8a, 9-hexahydro-9-methylene, 7-Amino-4-methyl-1, 8-naphthyridin-2-ol, 4-Methyl-2, 6-dihydroxyquinoline, 2, 4, 6-triaminoquinazoline, 2(1H) quinolinone, 4-hydroxy-1-methyl- (Mo-long and Tian-miao, 2011); 1-menthol, β-sitosterol, tricosa tricosoate, docosanol, 1-heptadecanol, 3β,14-dihydroxy-oleanane-12-ene, octadecanoic acid, hyperin, quercetin-3-O-β-D-xylofuranoside (Fu et al., 2010); aavicularin, hyperoside (Mino et al., 2002); **Essential oil** (Fig. 1): *trans*-caryophyllene, γ-cadinene, β-gurjunene, humulene, α-amorphene, limonene, α-pinene, *p*-cymene, tetradecane, 1,2-benzenedicarboxylic acid, caryophyllene, caryophyllene oxide (Olennikov *et al*., 2009; Rogachev *et al*., 2006; Li *et al*., 2000)
R. decipiens	campanulin, ursolic acid, friedelin, quercetin (Rangaswami and Sambamurthy, 1961)
R. delavayi	3′, 4′, 7-trihydroxy-3, 5-dimethoxyflavone , 3′, 4′, 5, 7-tetrahydroxy-3-methoxyflavone , quercetin-3-O-β-D-arabinopyranoside, catechin, epicatechin, epicatechin-(2 β→O→7, 4 β→8)-ent-epicatechin, (2S)-4-(3,4-dihydroxyphenyl)-2-butanol, (3,4-dihydroxyphenol)-2-ethanol (Song *et al*., 2009)
R. edgeworthii	Sesquiterpene-germacrone (Dass *et al*., 1980)

R. ellipticum	β-carotene, sitosterol, uvaol, quercetin, myricetin, quercitrin, myricitrin, hyperin, quercetin 3- glucoside, quercetin 5, 4′-dimethyl ether (Ho and Lin, 1995)
R. falconeri	friedelin, α-amyrin, campanulin, betulinic acid, taraxerol, taraxeryl acetate, β-sitisterol, uvaol-3-acetate (Gupta *et al*., 1969)
R. farrerae	ursolic acid, cerin, farrerol (Arthur and Hui 1954b; Arthur *et al.,* 1956)
R. ferrugineum	phloracetophenone, phloracetophenone-4′-glucoside, hyperoside, myricetin 3-*O*- β- galactopyranoside, kaempferol 3-*O*-(6″-*O*-acetyl)-glucoside, quercetin 3-*O*-(6″-O-acetyl)-glucoside, quercetin 3-*O*-(6″-*O*-acetyl)- galactoside, quercetin 3-*O*-(3″,6″- *O*-diacetyl)-galactoside, trans-taxifolin 3-*O*-α-arabinopyranoside, cis-taxifolin 3-*O*-α-arabinopyranoside (Chosson *et al*., 1998a & b)
R. flavum	**Essential oil**: β-pinene, α-pinene, bornyl acetate, limonene, β-elemene, sabinene, citronellol, myrcene, γ-terpinene, acoradiene, p-cymene and terpineol (Pu and Liang, 1999)
R. formosanum	5, 6α -epoxy-5α -stigmastan-3β-ol, 5, 6β -epoxy-5β -stigmastan-3β-ol (Krishna *et al*., 2006)
R. formosum	leucocyanidin, epicatechin, dihydrotaraxerone, ursolic acid, taxifolin (King, 1977)
R. grande	campanulin, friedelin, ursolic acid, dihydroflavonol, taxifolin, taraxeryl acetate, friedelin, taraxerol, β- sitosterol, betulinic acid, 2-acetyl-1- naphthol (King, 1977)

R. hodgsonsii	campanulin, friedelin, α and β-amyrin, uvaol, ursolic acid (King, 1977)
R. irroratum	kaempferol, quercetin, myricetin, quercetin-3-*O*-β-*D*-galactopyranoside, quercetin -4′-*O*- β-*D*-galactopyranoside (Yang and Kong, 2008)
R. latoucheae	rhodolatouside A, rhodolatouside B, scandoside, galioside, 4-(4′-*O*-β-*D*- glucopyranosyl-3′-methoxyphenyl)-2-butanone, dihydrodehydrodiconiferyl alcohol, 2-hydroxy-5-(2′-hydroxy ethyl)- phenyl-*O*-β-*D*-gluco-pyranoside, tachioside, stratioside II, ursolic acid, daucosterol, β-sitosterol (Higuchi and Dervilla, 1977; Jensen and Nielsen, 1982; Bianco *et al*., 1983; Shogo *et al*., 1987; Masataka and Masao, 1992; Fukuyama *et al*., 1996; Marina *et al*., 1996; Fan *et al*., 2001); (+)-rhodolatouchol, and isoepirhododendrin 4-[4′-O-β-D-glucopyranosylphenyl]-2(S)-butanol, were identified. Six other known compds., (+)-rhododendrol, epirhododendrin, 4-[3′-O-β-D-glucopyranosyl-4′-hydroxyphenyl]-2-butanone, 4-(4′-O-β-D-glucopyranosylphenyl)-2-butanone, benzyl-O-β-D-glucopyranoside, arbutin (Fan *et al*., 1999)
R. lepidotum	rhodonin, rhodonetin (Khan *et al*., 2008); 7-O-β-D-glucopyranosyl-8-methoxy benzopyranone , 7-hydroxy-8-O-β-glycosyl benzopyranone , daphnetin (Ahmad *et al*., 2010); shamimarin, acetoxy-8-methyl-chromen-2H-one, daphnin,

	daphnetin 8-O-b-D-glucopyranoside, umbelliferone (Tantry *et al*., 2010)
R. luteum	**Essential oil:** β-caryophyllene, methyl benzoate, (*E*)-β-ocimene, *α*-pinene, β-pinene, trans-methyl isoeugenol, benzyl alcohol, octadecanol, α-humulene, β-elemene; **andromedotoxin** (Alan *et al*., 2010)
R. maximum	d-betuligenol, andromedotoxin (Wood *et al.,* 1954; Tallent, 1964)
R. micranthum	hyperin, astragalin, kaempferol, and quercetin. (Xia *et al*., 1999)
R. molle	everninic acid methyl ester-2-*O*-β-*D*-xylopyranosyl-(1→6)-β-*D*-glucopyranoside, 7-hydroxy-5-methoxyphthalide-7-β-*D*-xylopyranosyl(1→6)-β-*D*- glucopyranoside, taraxerol, β-sitosterol, rhodomolleins I, IX , X , XI , XII, XIII, XIV, XV, XVI, XVII, XVIII and XIX, grayanotoxin II, rhodojaponins II, rhodojaponins III, rhodojaponins VI, kalmanol, rhodomolins A and B (Kaiya and Sakakibara, 1985; Burke *et al.*,1989; Nishida *et al*.,1990; Klocke *et al.*,1991; Chen *et al*.,1992; Li *et al.*, 2000; Bao *et al*., 2002 ; Chen *et al.*, 2004; Xiang *et al*., 2004; Zhong *et al*., 2005)
R. mucronatum	Azalein, azaleatin (Wada, 1956)
R. mucronulatum	scopoletin, (+)-taxifolin, quercetin, (-)-catechin, (+)-epicatechin, scopolin, lyoniside, fraxin, (+)-lyoniresinol-3*α*-O-β-D-glucopyranoside, (+)-taxifolin-3-O- *α*-L-arabinopyranoside and astragalin (Lee *et al*., 2005)

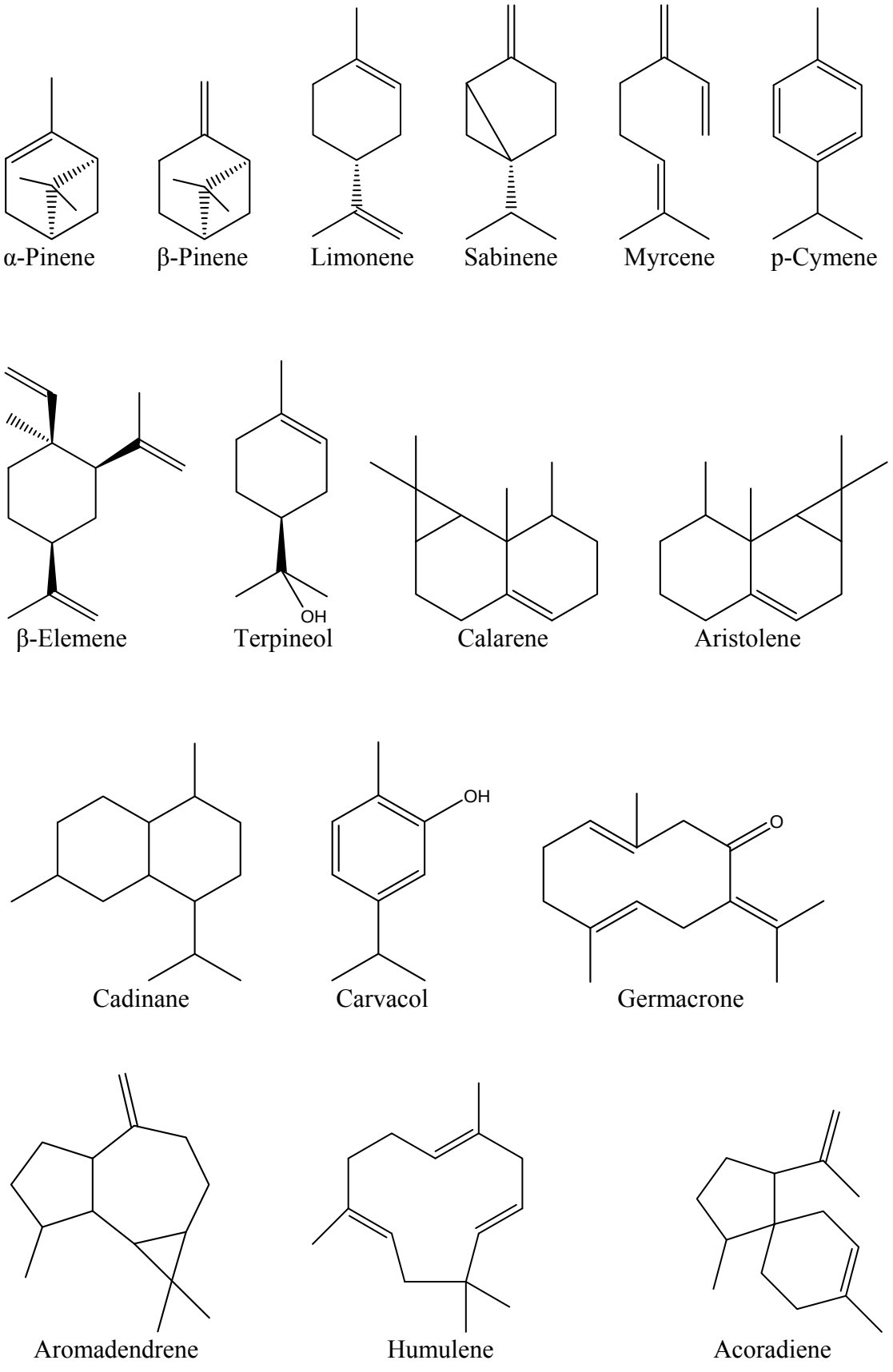


Fig. 2.1: Molecular structures of the major components of essential oils

R. nilagiricum	friedelin, campanulin, ursolic acid, leucocyanidin (King, 1977)
R. nivale	**Essential oil:** δ-cadinene, α-cadinol, cis, trans-farnesol, methyl carvacrol, α -cadinol isomer (Liu and Chen, 2008)
R. niveum	β-sitisterol, vitexin, friedelin, hentriacontane, hentriacontanol, α- amyrin, epifriedelinol, quercetin-3- galactoside, quercetin-3-glucoside (King, 1977)
R. obtusum	azaleatin 3-galactoside (Horhammer *et al*., 1969)
R. ovatum	3,5,7-trihydroxylchromone 3-*O*- β -*D*-xylopyranoside, taraxerol, β-sitosterol, betulinic acid, quercetin, quercetin-3-*O*-α -L- rhamnopyranoside, *D*-glucose (Feng *et al*., 2005)
R. oveodoxa	grayanotoxin-I, grayanotoxin-N, 5,6-acetonyl grayanotoxin-I, 5,6-acetonyl grayanotoxin-N and betuloside (Liu and Chen, 1994)
R. ponticum	catechin-(4α→6)-epicatechin (Fletcher, 1977); isoquercitrin, quercitrin, 6-C-glycosylnaringenin (Erdemoglu *et al*., 2008)
R. primulaeflorum	farrerol, 4′,5,7-trihydroxy-8-methylflavanone, dihydrokaempferol, isorhamnetin, quercetin, hirsutine, reynoutrin, taxifolin-3-O-*α*-L-arabinopyranoside, (2R,3S)-taxifolin-3-O-*α*-L-arabinopyranoside, (2S,3R)-taxifolin-3-O-*α*-L-arabinopyranoside (Li *et al*., 2009); lyoniside, nudiposide, ssioriside, umbelliferone, scopoletin, isovanillin, noregenin, 3,5,7-trihydroxychromone-3-*O*- *α*-L-arabinopyranoside, *cis*-taxifolin, *trans*-taxifolin, (+)-catechin, (–)-epicatechin, proanthocyanidin A-1, proanthocyanidin A-2, avicularin, usinc acid, uvaol, ursolic acid, oleanolic acid, lupeol, β-sitosterol, and

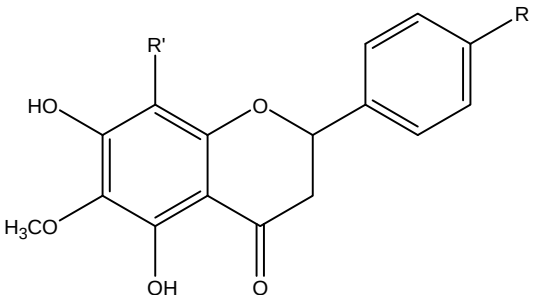
	daucosterol (Li *et al*., 2010); **Essential oil:** β-pinene, *α*-pipene, limonene, β-caryophyllene, myrcene, naphthalene, (+)-ledol, (+)-aromadendrene and *α*-terpineol (Wu *et al*., 2010)
R. prunifolium	taxifolin 7-galactoside (King, 1977)
R. przewalskii	rhododendrone, rhododendronside (Li and Jia, 2003)
R. purdomii	quercetin 3-O-*α*-L-rhamnoside, hyperin, quercetin, scopoletin, *α*-amyrin, β-amyrin, oleanolic acid, β-sitosterol and β-daucosterol (Liu *et al*., 2009)
R. racemosum	kaempferol 5-methyl ether, kaempferol 5-methylether 3-galactoside (Harborne, 1969; De Loose, 1970)
R. simiarum	ursolic acid, α-Amyrin, lupeol, simiarol, aromadendrin, quercetin (Arthur and Hui, 1954b; Arthur and Tam, 1960a)
R. simsii	19, 24-dihydroxyurs-12-en-3-one-28-oic acid, 7-*O*-β-*D*-apiofuranosyl- (1→6)-β-*D*-glucopyranosyl matteucinol, 3, 4-dihydroxybenzoic acid, 3, 4-dihydroxybenzoate, matteucinol, azaleatin 3-galactoside, azaleatin 3-glucoside, azaleatin 3-rutinoside, myricetin 5-methyl ether 3-galactoside, myricetin 5-methyl ether 3- rhamnoside, cyanidin 3-galactoside-5-glucoside, cyanidin 3-arabinoside-5-glucoside, matteucinol, ursolic acid, matteucinin (Arthur and Hui,1954a; Arthur and Tam,1960a; Asen and Budin, 1966; De Loose, 1969; Horhammer *et al*., 1969; De Loose, 1970; Takahashi *et al*., 2001)
R. spiciferum	quercetin-3-O-α-L-arabinfuranside, quercetin-3-O-α-L-rhamnopyranoside, trans-taxifolin-3-O-α-arabinopyranoside,

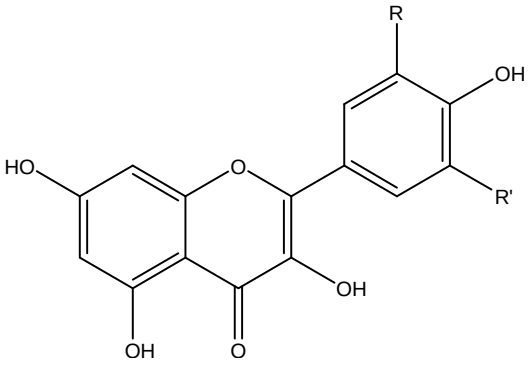
	astilbin, 3-O-rhamnoside engeletin, naringenin, (2S)-5,7,4′-trihydroxy-8-methylflavanone, epicatechin-(2 β→O→7,4 β→8)-ent-epicatechin, proanthocyanidin A-1, bis-8, 8′-catechinylmethane, catechin and epicatechin (Wang *et al*., 2010)
R. spinuliferum	quercetin-3-0-[3″, 4″-O-(isopropylidene)-β-D-xylopyranoside], taxifolin, taxifolin 3-O-β-D-xylopyranoside, isoengeletin, astilbin and proanthocyarlidin A-2 (Luo *et al*., 2009); ursolic acid, oleanolic acid, quercetin, catechin, epicatechin, Umbelliferone, Scopoletin, Farrerol, (2S)-4′, 5, 7-Trihydroxy-8-methylflavanone, Myricetin, Dihydromyricetin, Kaempferol, Dihydrokaempferol, Isorhamnetin, Hirsutrin, Hyperin, Avicularin, Quercetin-3-*O*-α-D-arabinofuranoside, Kaempferol-3-*O*-α-D-arabinopyranoside, (2*R*, 3*R*)-Taxifolin-3-*O*-α-D-arabinoside, (2*S*, 3*R*)-Taxifolin-3-*O*-α-D-arabinoside, (2*R*, 3*S*)-Taxifolin-3-*O*-α-D-arabinoside (Chen *et al*., 2009)
R. speciosum	taxifolin 3-galactoside (King, 1977)
R. thomsonii	andromedotoxin, cyanidin 3-galactoside, cyanidin 3-arabinoside (Harborne, 1962)
R. veitchianum	ursolic acid, quercetin (King, 1977)
R. westlandii	ursolic acid, epifriedelano1, friedelin, epoxyglutinane, α- and β-amyrin, lupeol, myricetin, quercetin (Arthur and Hui,1954b; Arthur *et al*.,1956; Arthur and Tam,1960a; Arthur and Hui, 1960b)
R. wiltonii	ursolic acid, (+)-catechin, quercetin, kaempferol,

	polystachoside and kaempferol-3-O-arabinoside (Hu *et al*., 1990)
R. yedoense	myricitin-5-methyl ether, quercetin-5-*O*-β-*D*-glucopyranoside, quercetin, quercitrin (Jung *et al*., 2007)

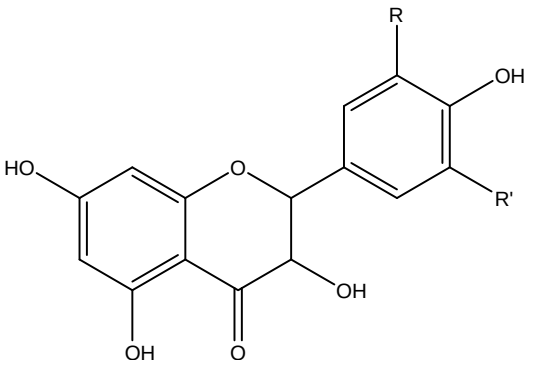
2.1.3 Pharmacology

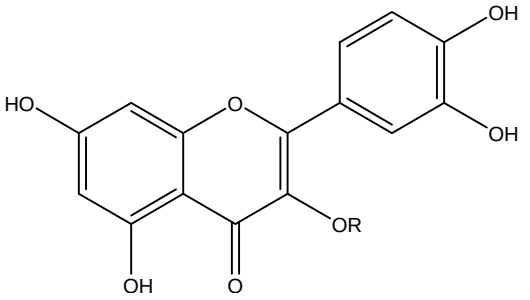
Apart from their worldwide aesthetic and ethnic uses, several species have commercial and medicinal values (Leach, 1986). The dried leaves of *R. dauricum* are used medicinally as expectorant and in treatment of acute-chronic bronchitis (Jiangsa, 1977; Yasuda *et al*., 1984). Methanol extract of the leaves and twigs of *R. dauricum* L. was found to display significant anti-HIV activity ($EC_{50} \leq 20\mu g/mL$, TI >5) (Xiao, 2002). Pathological experiments have demonstrated that farrerol and 8-desmethlyfarrerol (Fig 2.2) from *R. dauricum* have significant ability to move the phlegm (Cao *et al*, 2004), hyperoside and isohyperoside can alleviate cough (Yin, 1997), quercetin (Fig. 2.2), as a major active component of this plant, can prevent LPS-induced tumor necrosis factor-α and NO overproduction, which result in many immunomodulatory, infectious and inflammatory diseases such as sepsis syndrome, bacterial meningitis, cerebral malaria, adult respiratory distress syndrome, AIDS and rheumatoid arthritis (Manjeet and Ghosh, 1999; Morikawa *et al*., 2003). Aavicularin and hyperoside (Fig 2.2) from *R. dauricum* have antinociceptive action (Mino et al., 2002).





Farrerol	:R = OH, R′ = Me	Kaempferol	:	R = R′ = H
Matteucinol	:R = OMe, R′ = Me	Quercetin	:	R = OH, R′ = H
8-Desmethylfarrerol	:R = OH, R′ = H	Myricetin	:	R = R′ = OH





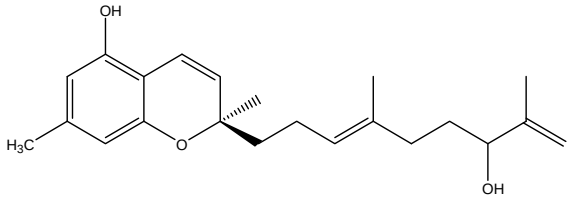
Dihydroaempferol	:R = R′ = H	Isohyperoside	:	R = gala (α)
Dihydroquercetin	:R = OH, R′ = H	Hyperoside	:	R = gala (β)
Dihydromyricetin	:R = R′ = OH	Avicularin	:	R = ara

Fig. 2.2: Molecular structures of the bioactive flavonoids

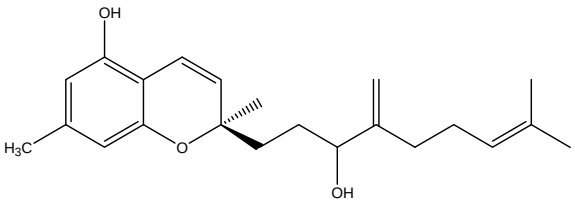
Prenylated orcinol derivatives i.e., daurichromenes A-D, along with confluentin and grifolin (Fig 2.3) from *R. dauricum* have been found to show histamine release inhibitory effects, which can make them potential candidates in the treatment of inflammatory diseases (Iwata *et al*., 2004). Daurichromenic acid (Fig. 2.4) from *R. dauricum* has been found to possess potent anti-HIV activity with an EC_{50} value of 0.00567 mg/mL and therapeutic index (TI) of 3.710. Rhododaurichromanic acid A

also shows relatively potent anti-HIV activity with an EC_{50} value of 0.37 mg/mL, and a TI of 91.9, whereas rhododaurichromanic acid B displays no anti-HIV activity (Kashiwada et al., 2001). The methanolic extract of its leaves and twigs was found to display significant anti-HIV activity (Kashiwada *et al*., 2001). 4-*O*-Methylphloroacetophenone from *R. dauricum* has shown strong activity against all fungal species while as arbutin has an inhibitory effect on tyrosinase activity, and has been extensively used as a kind of skin-lightening agent in cosmetics (Cao *et al*, 2004).

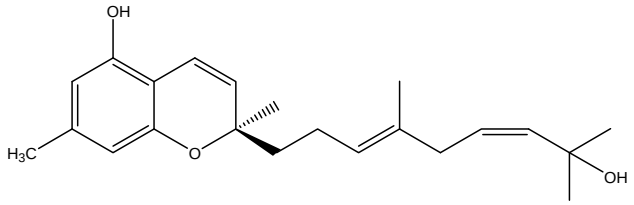
Coumarins i.e., daphnin, daphnetin, daphnetin glucoside, rhodonetin, rhodonin and umbelliferone (Fig. 2.5) and their acetyl derivatives isolated from the methanolic extract of *Rhododendron lepidotum* have shown good antibacterial activity against *Staphylococcus aureus*, methicillin resistant *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Shakeel-u-Rehman *et al*., 2010).



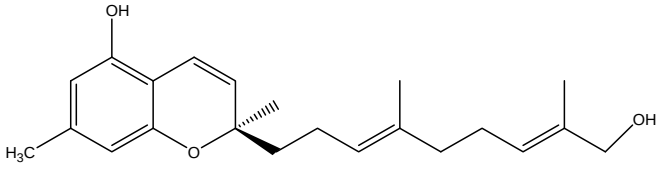
Daurichromene A



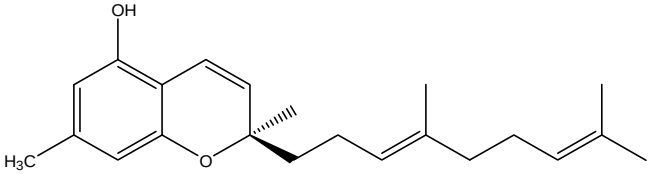
Daurichromene B



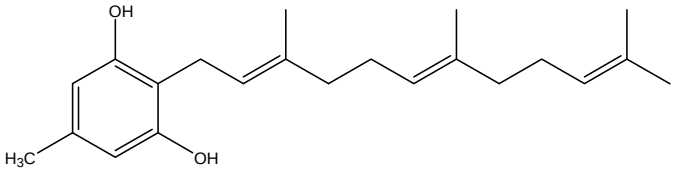
Daurichromene C



Daurichromene D

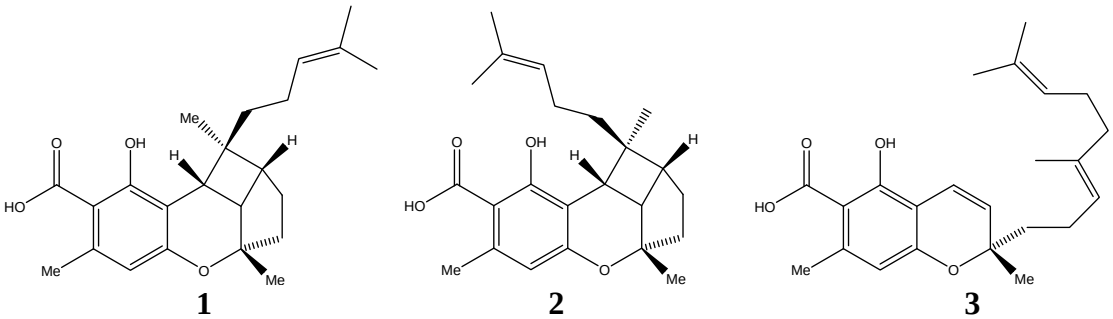


Confluetin



Grifolin

Fig. 2.3: Molecular structures of histamine release inhibitory chemical constituents



Fiq. 2.4: Molecular structures of rhododaurichromanic acid A (1), rhododaurichromanic acid B (2) and daurichromenic acid (3)

R_1	R_2	R_3	
H	OGlc	OH	: Daphnin
H	OH	OH	: Daphnetin
H	OH	OGlc	: Daphnetin glucoside
OGal	H	OH	: Rhodonetin
H	Opentose	OMe	: Rhodonin
H	OH	H	: Umbelliferone

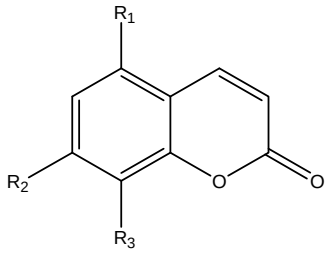
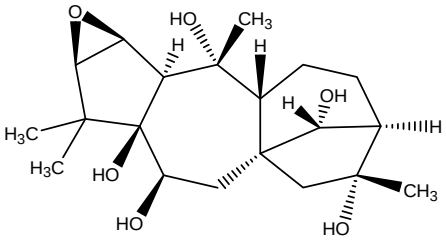
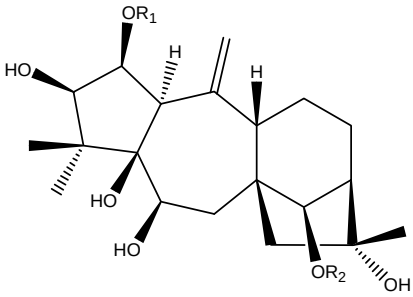


Fig. 2.5: Molecular structures of the antibacterial coumarins

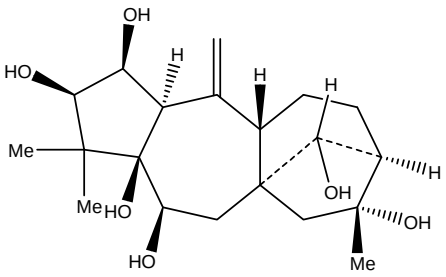
A physiologically active substance andromedotoxin (acetylandromedol) (Fig. 2.6) present in leaves of *Rhododendron maximum* has shown potent hypotensive activity at very low concentrations. The same compound is believed to be responsible for the reported high toxicity of *Rhododendron* honey (mad honey) which indicates that the flowers may be a particularly rich source of andromedotoxin (Wood *et al*., 1954). Andromedotoxin exerts toxic effects by binding to sodium channels in cell membranes and increases the permeability of sodium ions in excitable membranes (Sutlupinar *et al*., 1993). The chemical compound rhodojaponin-III (Fig 2.6) isolated from *R. molle* has been shown to have significant blood pressure lowering and heart rate slowing effects (Li *et al*., 2000). Grayanane diterpenoids, rhodomolins A and B, together with diterpenoids, rhodomollein I and rhodojaponin III (Fig 2.6), isolated from the flowers of *Rhododendron molle* have shown significant cytotoxic activity against the *Spodoptera frugiperda* cell line Sf-9 (Zhong et al., 2005).



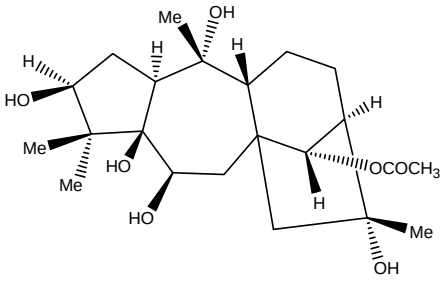
Rhodojaponin III



Rhodomolins A: R1 = Me; R2 = H
Rhodomolins B: R1 = H; R2 = Ac



Rhodomollein I



Andromedotoxin

Fig. 2.6: Molecular structures of the bioactive diterpenes

The ethanolic extract of *R. arboretum* possesses hypotensive effect. Its flowers are also used in dysentery, fever, headache and are known to possess anti-inflammatory activity (Agarwal and Kalpana, 1988). The flowers and leaves of *R. ferrugineum* are antirheumatic, diaphoretic and diuretic (Usher, 1974; Launert, 1981; Chiej, 1984). Leaves and flowers of *R. latoucheae* have been used to treat skin festers and the roots as antidote. The water extract of its leaves, used to treat chronic tracheitis, have shown antitussive and expectorant effects in clinical practice (Han, 1984). The methanol extract along with the chemical constituents of *R. simsii* showed potent antioxidative activity (Takahashi *et al*., 2001). An unusual oleanane triterpene (Fig 2.7) isolated from the aerial parts of *R. campanulatum* has shown prominent antibacterial and immunomodulatory activities (Tantry *et al*., 2011). Courmarins isolated from *R. collettianum* are effective inhibitors of tyrosinase enzyme and have a

potential to be used for the treatment of hyperpigmentation associated with the high production of melanocytes (Ahmad *et al.*, 2004). Sitosterol and quercetin 3-galactoside from *R. catawbiense* have been found to be phagostimulants in nature (Doss *et al*., 1982). Tibetan medicine Dali, consisting of two Tibetan plants, *R. anthopogonoides* and *R. primulaeflorum* containing a mixture of essential oils, flavonoids and triterpenoids have been successfully used in eliminating phlegm, relieving cough, relieving inflammation, relaxing smooth muscle and curing asthma. As a clinical drug, it has also been used for treatment of chronic bronchitis and coronary artery disease (Li *et al*., 2008). *Rhododendrons* are a good source of pharmacologically active pentacyclic triterpenic acids like oleanolic acid, ursolic acid and betulinic acid (Fig 2.7) (King, 1977; Youn and Cho, 1991; Bahuguna and Jangwan, 1987; Thapliyal and Bahuguna,1993). Oleanolic acid and ursolic acid have been shown to act at various stages of tumor development to inhibit tumor initiation and promotion, as well as to induce tumor cell differentiation and apoptosis (Laszczyk, 2009). Oleanolic acid and ursolic acid are well known for their anti-HIV and hepatoprotective effects (Liu, 2005). Betulinic acid Shows a broad spectrum of activity against various cancer cell types (Schmidt *et al*., 1997) and is also known for its anti-HIV (Fujioka *et al*., 1994), anti-inflammatory (recio *et al*., 1995), antimicrobial (Schuhly *et al*., 1999), antimalarial (Bringmann *et al*., 1997), spasmogenic (Bejar *et al*., 1995), antinoceceptive (Kinoshita *et al*., 1998), antihelmintic (Enwerem *et al*., 2001), and anti-HSV-1 (Ryu *et al*., 1992) activities.

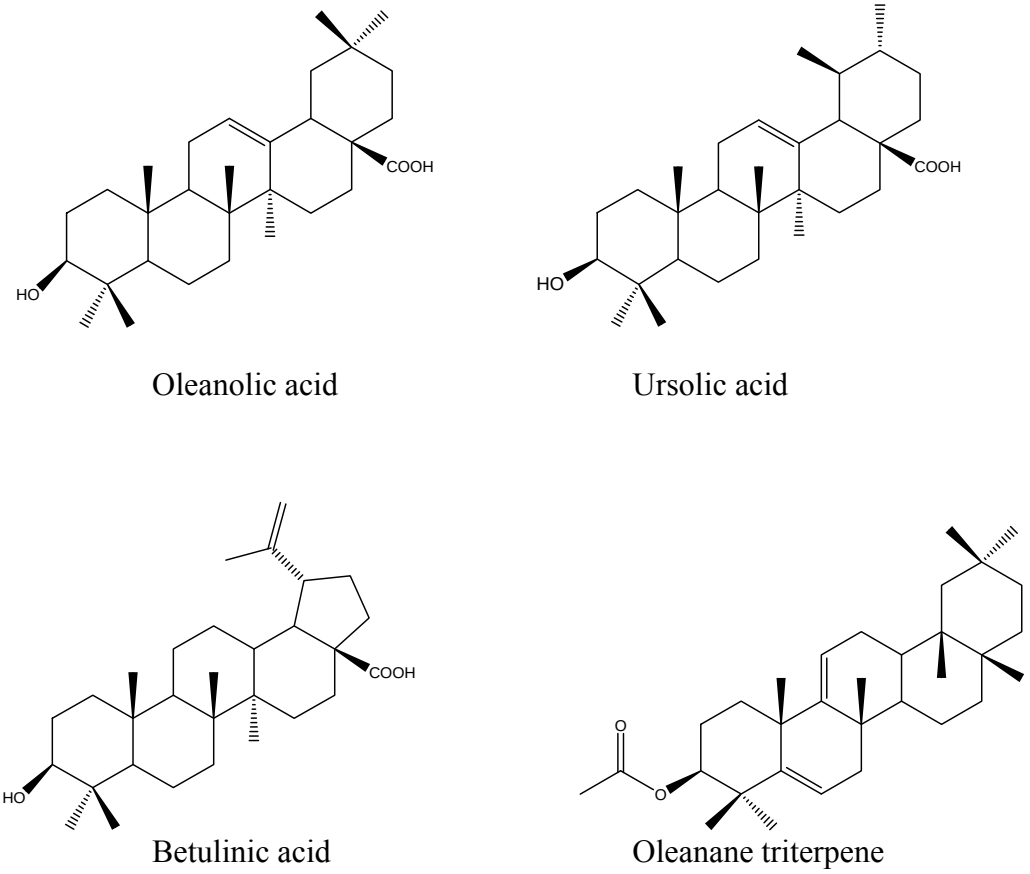
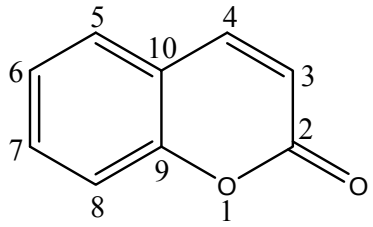


Fig. 2.7: Molecular structures of the bioactive triterpenoids

2.2 Coumarins

Since the compounds isolated as a result of this research work were coumarins, a brief review of coumarins is given below.

Coumarin, the simplest member of the group of oxygen heterocyclics called benzo-2-pyrones, is represented by structure (1). They are lactones possessing a 2H-1-benzopyran-2-one nucleus which opens on treatment with base to cis-*O*-hydroxycinnamic acid and spontaneously re-cyclize on acidification. The skeleton (1) for coumarins was deduced by several researchers (Fittig, 1868; Strecker, 1868; Tiemann and Herzfeld, 1877).

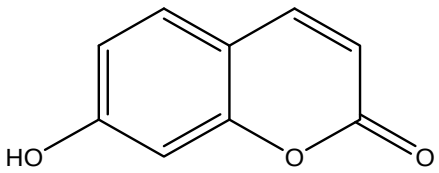


(1)

Coumarins owe their class name to 'coumarou', the common name of the *Coumarona odorata* (syn. *Dipteryx odorata*) from which coumarin itself was isolated for the first time by Vogel in 1820. It is a large group of naturally occurring compounds widely distributed in many families of plants such as *Asteraceae, Fabiaceae, Rosaceae, Rubiaceae, Solanaceae,* particularly in the *Umbelliferae* and *Rutaceae*. More than 1800 different natural coumarins have been discovered and described to date (Maes *et al*., 2008).

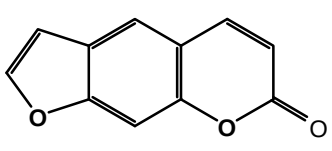
2.2.1 Classification

A feature common to most coumarins is oxygenation at C-7 position. The 7-hydroxycoumarin, commonly known as umbelliferone (2), is often regarded as the parent coumarin both structurally and biogenetically, of the more complex coumarins. Another common feature among coumarins is the presence of isoprenoid chains, frequently of one, but often of two or three units, attached to a carbon or oxygen or both. The prenyl group may be found as the simple 3-methylbut-2-enyl unit, but it is often encountered as the corresponding epoxide or vicinal glycol or in a variety of oxidized and skeletally rearranged forms (Murray *et al*., 1982). Biogenetically an additional heterocyclic ring can be formed when the prenyl group interacts with an *O*-phenolic group. The structural variations of this type encountered in the natural coumarins mostly include dihydrofuran, hydroxydihydropyran and their derivatives, furan and dihydropyran.

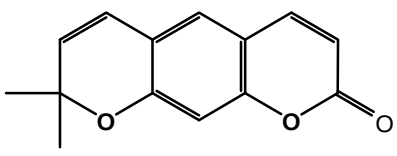


(2)

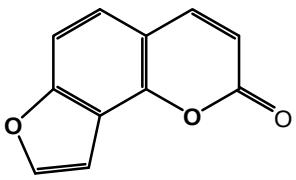
Prenylation at C-6 and C-8 can lead to linear coumarins such as psoralen (3) and xanthyletin (4) or angular forms like angelicin (5) and seselin (6) respectively.



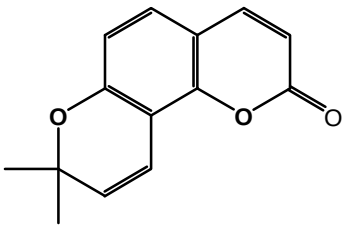
(3)



(4)



(5)



(6)

In this regard coumarins are classified as following:

a) Simple, which includes coumarin, its hydroxylated, alkoxylated and alkylated derivatives and their glycosides

b) Furanocoumarins, including the typical linear form psoralen and angular type angelicin

c) Pyranocoumarins, which have a six membered ring attached to the benzoid part like seselin and xanthyletin

d) Pyrone-ring substituted coumarins like 4-hydroxycoumarins and 3-phenylcoumarins.

2.2.2 Coumarins in Pharmaceutical and Chemical Industry

Coumarins have attracted intense interest in recent years because of their diverse pharmacological properties. The presence of coumarins in plant extracts that have been used to treat human ailments is well known (Kostova, 2005). These plant extracts which were employed as herbal remedies in early days have now been extensively studied for their biological activities. These investigations have revealed their potentials as versatile biodynamic agents. For example, coumarins with phenolic hydroxyl groups have the ability to scavenge reactive oxygen species and thus prevent

the formation of 5-HETE (hydroxyeicosatetraenoic acid) and HHT (hydroxy heptadecatrienoic acid) in the arachidonic pathway of inflammation suppression (Kulkarni, *et al*., 2006; Kontogiorgis *et al.*, 2006; Torres *et al*., 2006). Coumarin enhances the glycolytic enzymes and controls the glucose metabolism in the liver tissues of diabetic rats. Therefore, coumarin possesses antidiabetic activity by stimulating the insulin production from the existing β-cells of pancreas (Pari and Rajarajeswari, 2009). Naturally occurring coumarins possess a range of anticarcinogenic activities in rodent models. A recent study has shown that 7-hydroxycoumarin inhibits the release of Cyclin D1, which is over expressed in many types of cancer. Several natural 5, 6, 7-trioxygenated coumarins have the capacity to induce cell differentiation in human leukemia U-937 cells which make them potential lead compounds in cancer therapy (Baez *et al*., 2005; Prince *et al*., 2009). Imperatorin and isoimperatorin, isolated from the umbelliferous Chinese crude drugs *Tang-Bai-Zai* and *Ashita-ba*, possess strong antitumour promoter activity in cultured cells (Okuyama, *et al*., 1990). Coumarins have also inhibitory effect on DNA gyrase which may be linked to their anti-HIV activity (Kostova *et al.,* 2006; Yu *et al.,* 2003; Wu *et al*., 2001). Pyranocoumarins isolated from *Clausena excavata* and *Zanthoxylum schinifolium* exhibit anti-HBV activity (Tsai *et al*., 2000; Su *et al.,* 2009). Some of the coumarins isolated from *Angelica gigas* exhibited significant protection against glutamate-induced toxicity in primary cultured rat cortical cells (Kang and Kim, 2007). Coumarins are the classical anti-thrombotic agents. Oral anticoagulants of the 4-hydroxycoumarin class of warfarin type are used worldwide to treat thromboembolic disease. They represent the platform upon which newer drugs holding the promise of greater efficacy and less toxicity are being developed. Even as new drugs arrive into clinical practice, coumarins remain remarkable for their

decades-long pre-eminence. They have saved countless lives and also served as essential probes into basic mechanisms of thrombosis. Testament to their clinical importance is that these agents are the only drugs profiled on a regular basis in special scientific statements by the American Heart Association/American College of Cardiology and by the American College of Chest Physicians (Mueller, 2004; Au and Rettie, 2008).

Immunomodulatory activities of some coumarins have also been reported. For example, isopimpinellin contributes to the immuno-enhancing activity of lymphocyte activation whereas bergapten, scoparone and xanthotoxin serve as candidates for the immuno-stimulating activity of IFN-γ secretion (Huang *et al*., 1992; Cherng *et al*., 2008). Coumarins isolated from the aerial part of *Artemisia capillaries* showed antiplatelet aggregation activity (Wu *et al*., 2001). Coumarins also have good antimicrobial activity (Cespedes *et al*., 2006; Stein *et al*., 2006; Ostrov *et al*., 2007). A synthetic 8-Iodo-5, 6-dihydroxycoumarin has a MIC value close to that of the antibiotic vancomycin against *MRSA* (Smyth *et al*., 2009). Antimalarial activity has been addressed to daphnetin extracted from the plant *Daphne* as well as dentatin and clausarin isolated from *Clausem harmandiana* (Yang *et al*., 1992; Yenjai *et al*., 2000). Coumarin derivatives have also been shown to be novel lipid lowering agents that possess moderate triglyceride lowering activity (Madhavan *et al*., 2003). The bioactivities of phototoxic psoralens and of dicoumaroul derivatives are well known and several of these compounds are used in antipsoriatic therapy and anticoagulant therapy respectively. Besides psoriasis, skin diseases like cutaneous T-cell lymphoma, atopic dermatitis, alopecia areata, urticaria pigmentosa and lichen planus are treated with the photochemotherapy with linear furanocoumarins and UVA (Goodman and Gilman, 1996; Matern *et al*., 1999).

Coumarins like coumachlor and bromadiolone are potent and long-lasting commercially available rodenticides which last for a long time only with their single administration (Sato, 2005). According to Pillar, coumarin or its metabolic products have the potential to become the future treatement of scalds and other forms of thermal wounding because it facilitates the removal of extravasated protein through proteolytic by stimulated macrophages (Piller, 1997). Coumarins also reduce all high protein edemas, including lymphoedema and elephantiasis (Casley-Smith *et al.,* 1993; Kontogiorgis *et al.*, 2006). Some 3-alkyl and 3-phenyl isocoumarins have been reported to have diuretic and antihypertensive activities (Sassa *et al*., 1968).

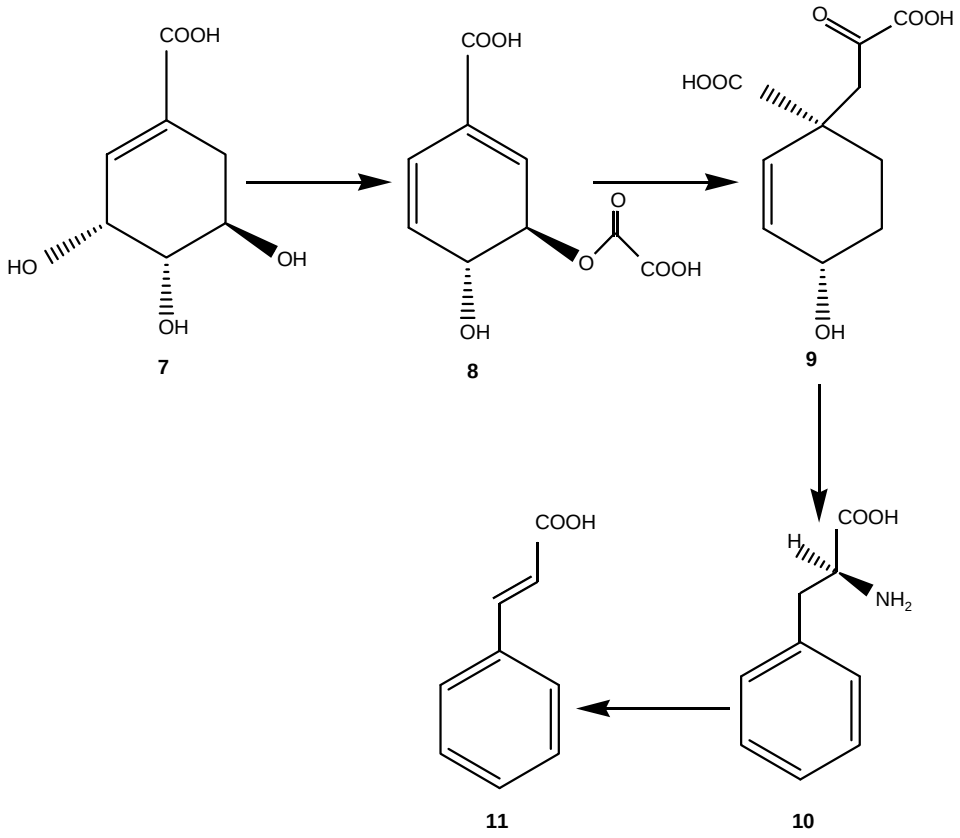
Coumarins have a wide variety of uses in industry, mainly due to its strong fragrant odour. Its uses include that of a sweetener and fixative of perfumes, an enhancer of natural oils, such as lavender, a food additive in combination with vanillin, a flavour/odour stabilizer in tobaccos, an odour masker in paints and rubbers and finally it is used in electroplating to reduce the porosity and increase the brightness of various deposits such as nickel. 6-Methylcoumarin is mainly used as a flavour enhancer and 7-hydroxycoumarin in sunscreens (Egan *et al*., 1990).

2.2.3 Biosynthesis

The early studies on the biosynthesis of simple coumarins trace its origin in shikimic acid pathway via cinnamic acid. Shikimic acid was first obtained in 1885 from the plant *Illicium religiosum* (shikimi-no-ki) and has since been identified in many plants. The shikimic acid pathway is the major metabolic route leading to the formation of aromatic compounds in living systems. It operates both in microorganisms and in higher plants but not in animals, which is why the latter are dependent on dietary supply of the aromatic amino acids phenylalanine, tyrosine and trytophan. In addition

to providing these essential protein constituents, the shikimate pathway is necessary for the synthesis of essential factors like folic acid.

Shikimic acid (7) is phosphorylated at the C-3 position, attachment of a 3-carbon side chain supplied by another molecule of phosphoenol pyruvate, followed by a 1, 4-elimination of the elements of phosphoric acid leads to the formation of chorismic acid (8). Chorismic acid is converted into prephenic acid (9) via claisen rearrangement which after reductive amination is transformed into L-phenylalanine (10) (Scheme-1). The conversion of L-phenylalanine into trans-cinnamic acid (11) is catalysed by enzyme phenylalanine ammonia-lyase (PAL) in all vascular plants. The enzyme plays an important role in the metabolism of higher plants.



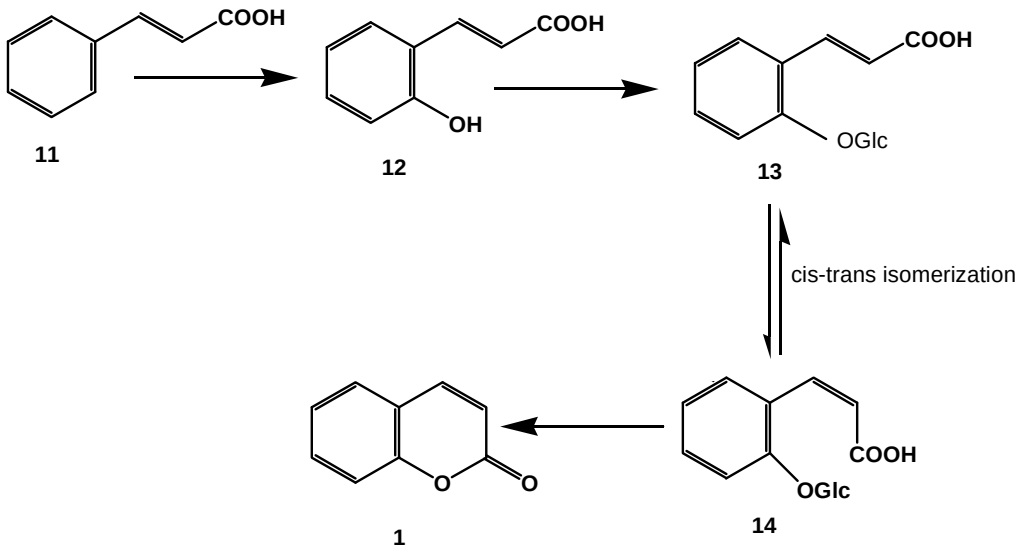
Scheme 1

The shikimate pathway is involved in the formation of a vast array of so called secondary metabolites in plants and microorganisms like phenyl propanoids (coumarin), alkaloids, flavones, xanthones or many antibiotics. Cinnamic acid however represents a branch point in the biosynthesis of coumarin. There are many classes of coumarins, the biosynthesis of only the simple coumarins is described below.

2.2.3.1 Simple coumarin (1)

The simple coumarins are those in which the entire carbon skeleton has no ring additional to the benzopyrone nucleus. These can be further divided into two main groups: those with an oxygenated substituent at the C-7, e.g. umbelliferone (2) and herniarin (20) and those in which this substituent is absent, e.g. coumarin (1).

Biosynthetic pathways leading to the formation of coumarins had been elaborated by Brown (1966) by using tracer experiments. Although the biosynthetic routes leading to 7-oxygenated and non-oxygenated coumarins are different in details, however the overall pathways have much in common. Scheme-2 illustrates the biosynthetic pathways for coumarin (1), while scheme-3 describes the biosynthesis of umbelliferone (2) and herniarin (20). The stage common in the biosynthesis of all coumarins from plant sources is the isomerization of trans-glucoside (13) to cis-glucoside (14) as illustrated in schemes 2 and 3.

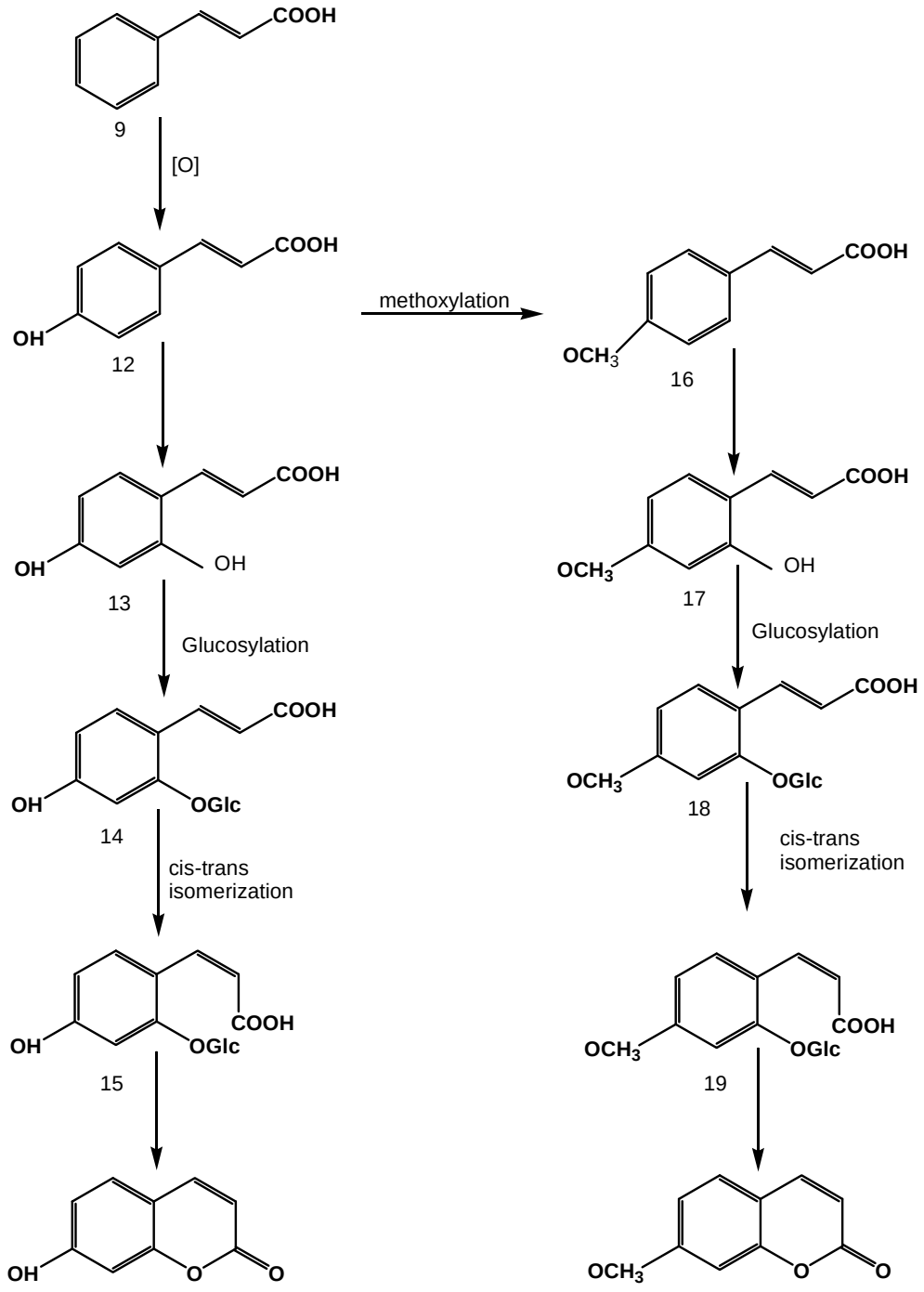


Scheme 2

2.2.3.2 7-oxygenated coumarins

O-hydroxycinnamic acid (12) and *O*-coumaryl glucoside (13) are selectively converted into coumarin (1) (Scheme-2), whereas p-coumaric acid (15) and 2-glucosyloxy-4-methoxy cinnamic acid (21) are selectively converted into umbelliferone (2) and herniarin (20) (Scheme-3). That places cinnamic acid at a branching point of the biosynthetic sequence. The first step to herniarin (20) is a *p*-hydroxylation of cinnamic acid while the first step to coumarin is an *O*-hydroxylation. It was also found that in the intact cell herniarin is predominantly present as a glycoside, presumably the cis-2-β-glycoside. The stage at which methylation occurs has not been determined with certainty. Studies of the biosynthesis of umbelliferone (2) gave analogous results (Torsell, 1997) (Scheme 3).

The formation of di- and tri-hydroxycoumarins and of their ethers involves the hydroxylation of umbelliferone (2) rather than the lactonization of the corresponding cinnamic acids.

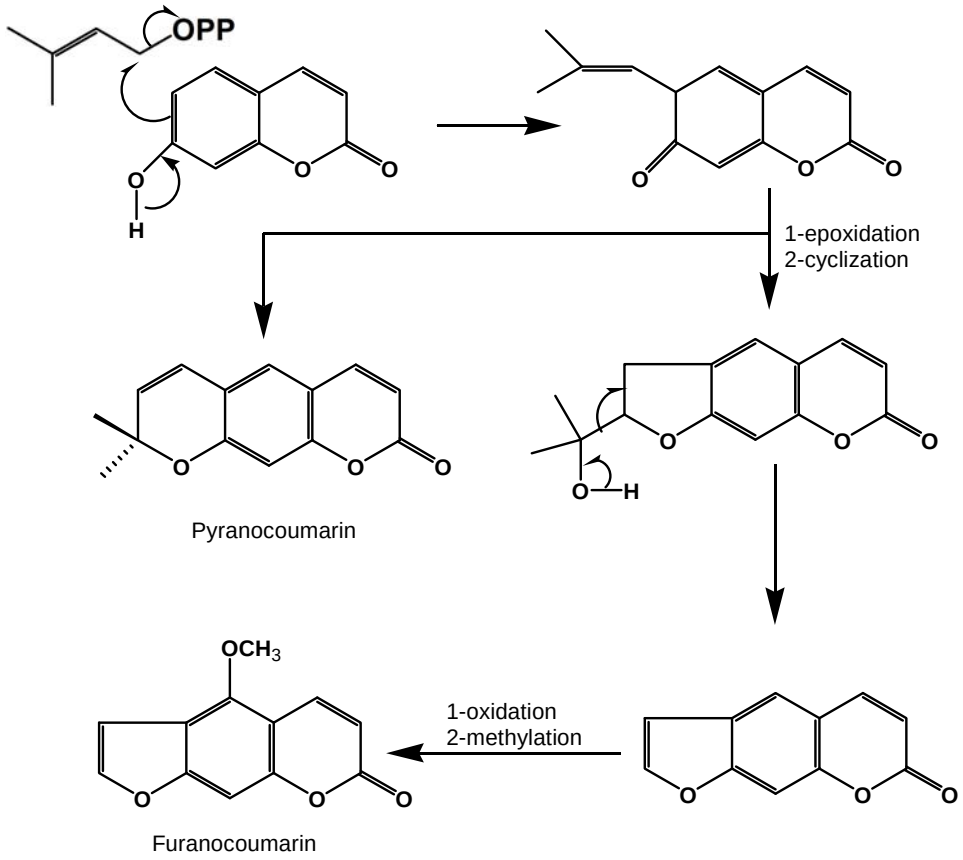


Scheme 3

2.2.3.3 Furano and pyranocoumarins

Prenylation of the benzene ring by dimethylallyl pyrophosphate in the 6- position of a 7-hydroxycoumarin yields the so called linear furano and pyrancoumarins and in the

8- position it affords the angular homologues. The formation of furanocoumarins includes two successive steps: stereospecific oxidation and elimination of the hydroxyisopropyl residue by retroaldol condensation. Substitution in the 5- or 8- position or in both positions of furanocoumarins occurs later and is catalysed by oxidases and *O*- methyltransferases (Bruneton, 1999; Matern *et al*., 1999) (Scheme 4).

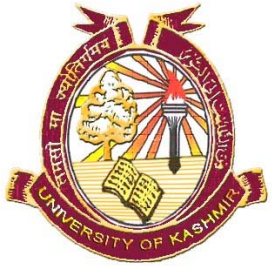


Scheme 4

Chapter 3
Results and Discussion

Chapter 4
Experimental

Summary



References

Appendices

Rhododendron lepidotum

3. Results and Discussion

3.1 RL-1

RL-1 was obtained as sharp crystalline needles, melting point 225 °C. Mass spectrum showed the molecular ion peak at m/z 162 which corresponds to molecular formula $C_9H_6O_3$. It gave characteristic greenish-blue spot under UV light and the absorption bands at 216, 245 and 322 nm in the UV spectrum, suggesting the presence of a coumarin skeleton (Murray *et al*., 1982). The IR spectrum exhibited the characteristic bands for -OH absorption at 3411 cm^{-1} and carbonyl absorption at 1680 cm^{-1}. Its purity was checked both by TLC and HPLC (Fig. 3.1).

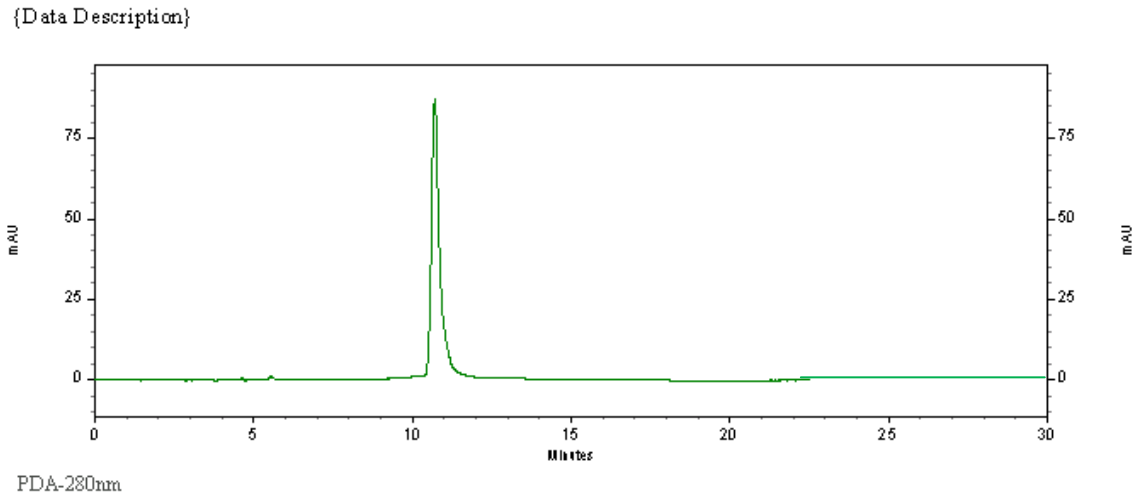


Fig. 3.1: HPLC spectrum of RL-1

The ^{1}HNMR spectrum confirmed the presence of a coumarin skeleton, showing two doublets at δ 6.18 and 7.87 (each integrating for one proton, J = 9.5 Hz) attributed to H-3 and H-4 respectively and the signals at δ 7.51 and 6.84 (each integrating for one proton, J = 8.4 Hz) and a singlet at δ 6.72 attributed to protons of benzene aromatic

system. Further ^{13}C–DEPT showed the presence of five methines and four quaternary carbons. RL-1 was positive to ferric chloride test and was thought to be phenolic which was supported by the chemical shifts of C-6, C-7 and C-8 in the ^{13}C NMR spectrum (Table 3.1). It was further confirmed by the acetylation of RL-1 which yielded a monoacetate derivative, confirming the presence of a phenolic group.

Finally RL-1 was characterized as **Umbelliferone** by comparison of spectral data (UV, IR, ^{1}H and ^{13}C NMR) with literature values and finally confirmed by Co-TLC and mixed m.p. with authentic sample (Reisch and Achenbach, 1992) (Fig. 3.2).

Table 3.1: ^{1}H and ^{13}C NMR of RL-1

Position	δH	δC	DEPT
2	-	162.0	C
3	6.18 (1H, d, *J* = 9.5 Hz)	113.1	CH
4	7.87 (1H, d, *J* = 9.5 Hz)	145.3	CH
5	7.51(1H, d, *J* = 8.4 Hz)	130.7	CH
6	6.84 (1H, dd, *J* = 8.4, 2.3 Hz)	114.1	CH
7	-	157.2	C
8	6.72 (1H, d, *J* = 2.3 Hz)	103.4	CH
9	-	155.0	C
10	-	112.9	C

Coupling constants in Hertz are given in parenthesis

Solvent used: MeOD

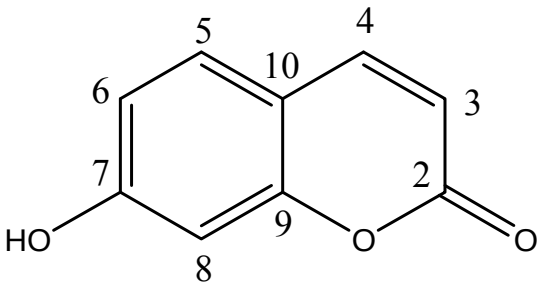


Fig. 3.2: Structure of RL-1

3.1.1 Antimicrobial activity of RL-1

RL-1 showed mild to poor antibacterial activity against the following bacterial strains: Gram positive *Staphylococcus aureus* ATCC 29213, *MRSA* ATCC 15187; Gram negative *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027 obtained from American Type Cultures Collection.

Table 3.2: **Antibacterial activity of RL-1 against bacterial pathogens**

Organism	**Inhibitory zone MIC (µg/ml)**	
	RL-1	**Standard**
S. aureus ATCC-29213	500	0.12
MRSA ATCC-15187	1000	8.0
E. coli ATCC-8739	1000	<0.03
P. aeruginosa ATCC-9027	500	0.06

3.2 RL-2

RL-2 crystallized as colourless needles from methanol, melting point 255°C. The molecular ion peak at m/z 178 corresponded to molecular formula $C_9H_6O_4$. Its purity

besides checking by TLC was also checked by HPLC (Fig. 3.3). The UV spectrum suggested it to be a coumarin as it was similar to those compounds already discussed in literature (Murray *et al.*, 1982) with absorption bands at 218, 267 and 302 nm. It gave characteristic greenish-blue spot on silica gel coated TLC plates under UV light. Absorption bands for -OH at 3419 cm^{-1} and for carbonyl at 1725 cm^{-1} were exhibited in the IR spectrum of **RL-2**.

The ^{1}H NMR spectrum of **RL-2** was quite similar to that of **RL-4** except that the peaks for mannose moiety were absent. It showed four doublets of benzopyran nucleus (Table 3.3). The two doublets at δ 6.19 and δ 7.84 (each integrating for one proton, J = 9.4 Hz) were attributed to H-3 and H-4 respectively and the signals at δ 7.00 (1H, J = 8.4 Hz) and δ 6.82 (1H, J = 8.4 Hz) were attributed to H-5 and H-6. In DEPT, $^{13}CNMR$ showed the presence of four methines and five quaternary carbons. Acetylation of **RL-2** yielded a diacetate derivative confirming the presence of two phenolic groups.

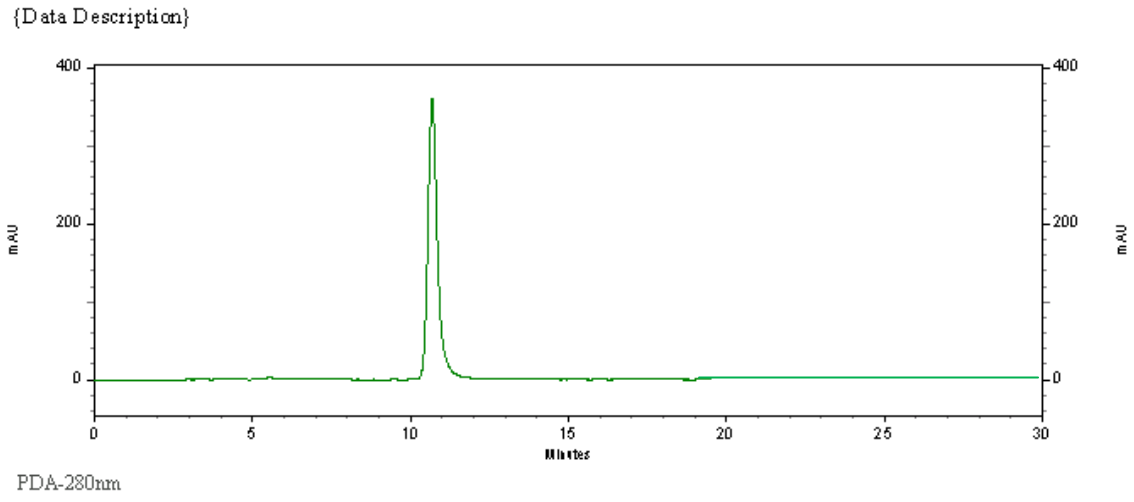


Fig. 3.3: HPLC spectrum of RL-2

Finally **RL-2** was identified as **daphnetin** on comparing its spectral data with that reported in literature (Thusoo *et al.*, 1981) (Fig. 3.4). Daphnetin is reported to have antimalarial activity (Yang *et al*., 1992; Huang *et al*., 2006).

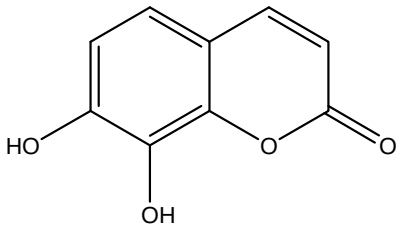


Fig. 3.4: Structure of RL-2

Table 3.3: ^{1}H and ^{13}C NMR of RL-2

Position	δH	δC	DEPT
2	-	160.1	C
3	6.19 (1H, d, *J* = 9.4 Hz)	110.3	CH
4	7.84 (1H, d, *J* = 9.4 Hz)	144.2	CH
5	7.00 (1H, d, *J* = 8.4 Hz)	117.7	CH
6	6.82 (1H, d, *J* = 8.4, Hz)	112.1	CH
7	-	145.7	C
8	-	130.1	C
9	-	143.3	C
10	-	111.2	C

Coupling constants in Hertz are given in parenthesis

Solvent used: MeOD

3.2.1 Antimicrobial activity of RL-2

RL-2 showed good antibacterial activity against various bacterial strains. Gram positive *Staphylococcus aureus* ATCC 29213, MRSA ATCC 15187; Gram negative *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027 were used to test the activity. The cultures were maintained as per standard procedures. The antibacterial activity of the compound was expressed as minimum inhibitory concentration.

Table 3.4: **Antibacterial activity of RL-2 against bacterial pathogens**

Organism	**Inhibitory zone MIC (µg/ml)**	
	RL-2	**Standard**
S. aureus ATCC-29213	125	0.12
MRSA ATCC-15187	125	8.0
E. coli ATCC-8739	500	<0.03
P. aeruginosa ATCC-9027	500	0.06

3.3 RL-3

RL-3 was obtained as a whitish amorphous powder, melting point 185°C; $[\alpha]_D^{25}$ –62° (c 1.0, MeOH). It was assigned the molecular formula $C_{16}H_{18}O_9$ (Fig. 3.5) as determined from ESI-MS, m/z at 354 which is supported by its elemental analysis and NMR data. Its purity was checked both by TLC and HPLC (Fig. 3.6).

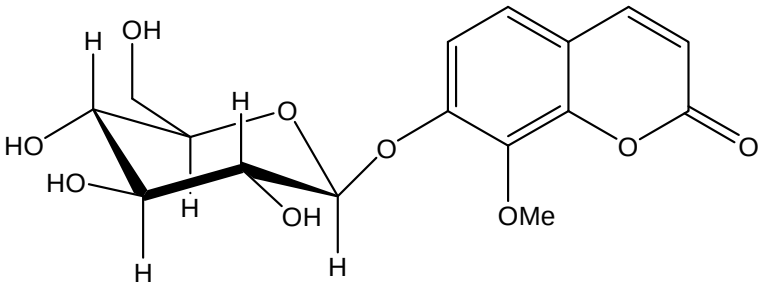


Fig. 3.5: Structure of RL-3

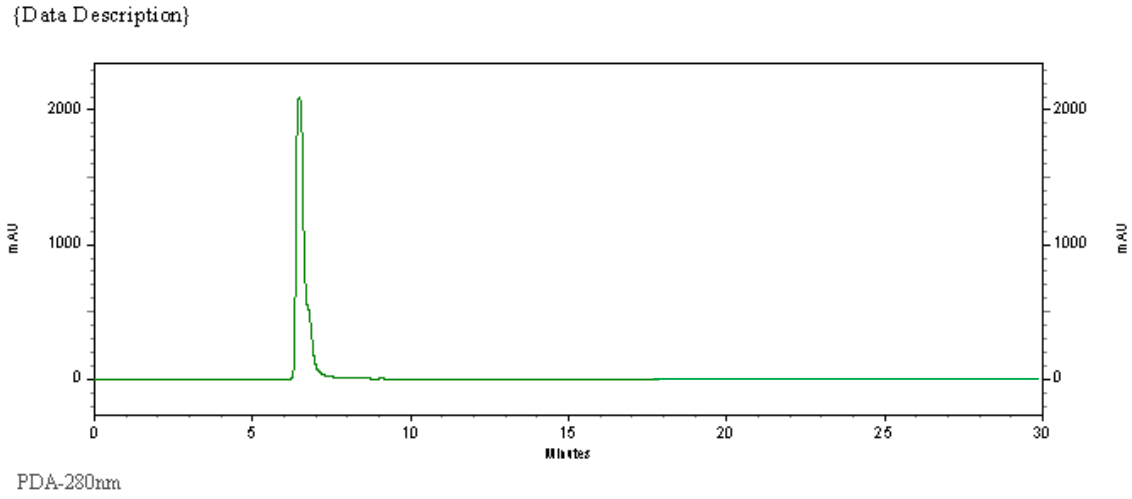


Fig. 3.6: HPLC spectrum of RL-3

The IR spectrum showed the absorption bands of OH group (3463cm^{-1}), a CO group (1724cm^{-1}) and an aromatic moiety (1610 and 1578). The ^{1}H NMR (Table 3.5) spectrum showed two doublets at δ 7.90 (1H, *d*, *J* = 9.5 Hz) and δ 6.28 (1H, *d*, *J* = 9.5 Hz) and a quartet at δ 7.15 (2H, q, *J* = 8.6 Hz) besides other signals in nonaromatic range. In the aromatic region the ^{13}CNMR and DEPT spectra (Table 3.5) showed 9 signals in the range of δC 113 to δC 160 (aromatic region) and six resonances at δ 103.3 (C-1'), 78.3 (C-3'), 76.7 (C-5'), 74.2 (C-2'), 70.9 (C-4'), 61.8 (C-6') (glycosidic

carbons) and a methoxyl carbon at δ55.3. From the above data the compound **RL-3** can be taken as a coumarin glycoside, in which the two doublets at δ 6.28 and 7.90 account for H-3 and H-4 respectively. The proton resonances at δ 4.96 (1H, *d*, *J* 7.15 Hz), 3.68-3.93 (3H, m), 3.52 (5H, m) are attributed to hexose and a methoxyl group. The above spectral data is more or less similar to that of Rhodonin except the signals in glycosidic region (Khan *et al*., 2008). The quartet at δ 7.15(2H, q, J = 8.6 Hz) is due to two protons at C-5 and C-6. Unlike that of Rhodonin which bears a pentose moiety at C-7, the glycan part in **RL-3** can be clearly characterised as glucose on the basis of above ^{1}H and ^{13}C NMR spectrum. The coupling constant 7.1 Hz between H-1′ and H-2′ and the ^{13}C NMR value of anomeric carbon at δ103.3 indicates that the sugar moiety is attached to coumarin skeleton through β-linkage. Furthermore acidic hydrolysis of **RL-3** afforded 7-hydroxy-8-methoxy coumarin which was identified by comparison of its ^{13}C NMR and melting point with that reported in literature (Zhang *et al*., 2007). Thus the structure of **RL-3** was elucidated as ***7-O-β-D-glucopyranosyl-8-methoxy benzopyranone***.

Table 3.5: ^{1}H and ^{13}C NMR of RL-3

Position	δH	δC	DEPT
2	-	160.2	C
3	6.28 (1H, d, *J* = 9.5 Hz)	113.2	CH
4	7.90 (1H, d, *J* = 9.5 Hz)	145.2	CH
5	7.15 (1H, d, *J* = 8.6 Hz)	118.9	CH

6	7.14 (1H, d, *J* = 8.6 Hz)	114.3	CH
7	-	149.1	C
8	-	135.2	C
9	-	143.6	C
10	-	115.5	C
1'	4.96 (1H, d, *J* = 7.15 Hz)	103.3	CH
2'	3.68 (1H, m)	74.2	CH
3'	3.71 (1H, m)	78.3	CH
4'	3.93 (1H, m)	70.9	CH
5'	4.21 (1H, m)	76.7	CH
6'	3.52 (2H, m)	61.8	CH_2
-OMe	3.52 (3H, m)	55.3	CH_3

Coupling constants in Hertz are given in parenthesis

Solvent used: DMSO (d_6)

3.3.1 Antimicrobial activity of compound RL-3

RL-3 showed mild to poor antibacterial activity against the following bacterial strains: Gram positive *Staphylococcus aureus* ATCC 29213, *MRSA* ATCC 15187; Gram negative *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027 obtained from American Type Culture Collection.

Table 3.6: **Antibacterial activity of RL-3 against bacterial pathogens**

Organism	**Inhibitory zone MIC (µg/ml)**	
	RL-2	**Standard**
S. aureus ATCC-29213	250	0.12
MRSA ATCC-15187	250	8.0
E. coli ATCC-8739	500	<0.03
P. aeruginosa ATCC-9027	500	0.06

3.4 RL-4

RL-4 was obtained as a yellow powder, melting point 195 °C; $[\alpha]_D^{25}$ –25° (c 1.0, MeOH). Its molecular formula was determined as $C_{15}H_{16}O_9$ on the basis of ESI-MS peak at 363 $[M+Na]^+$ which was confirmed by elemental analysis. The purity of the compound was checked both by TLC and HPLC (Fig. 3.7)

The IR (KBr) spectrum showed the absorption bands of OH group (3535 cm^{-1}), a CO group (1723 cm^{-1}) and an aromatic moiety (1613, 1579 cm^{-1}). The UV spectrum with λ_{max} at 220nm and 368nm correspond to a coumarin nucleus. The ^1H NMR spectrum showed four equivalent doublets in the aromatic region at δ 7.78 and δ 6.14 (J = 9.5 Hz) and δ 7.20 and δ 6.79 (J = 8.6 Hz). This ^1H NMR spectrum is quite similar to that of daphnetin except a singlet at δ 4.68 (1H) and two multiplets at δ 3.63-3.73 and δ 3.34-3.38 (equivalent to 3H each). These signals can be attributed to a hexose moiety. The nature of the sugar moiety was determined by hydrolyzing RL-4 with 1.0N HCl and comparing its ^1H NMR values with that of mannose. Thus the structure of RL-4

was elucidated as ***7-hydoxy-8-O-β-D-mannosyl benzopyranone*** and is shown in Fig. 3.8.

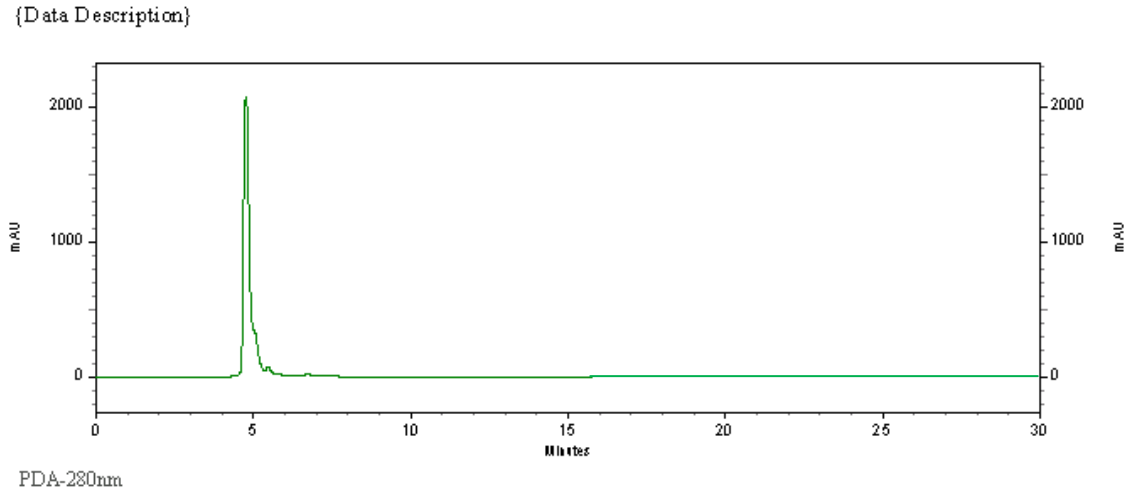


Fig. 3.7: HPLC spectrum of RL-4

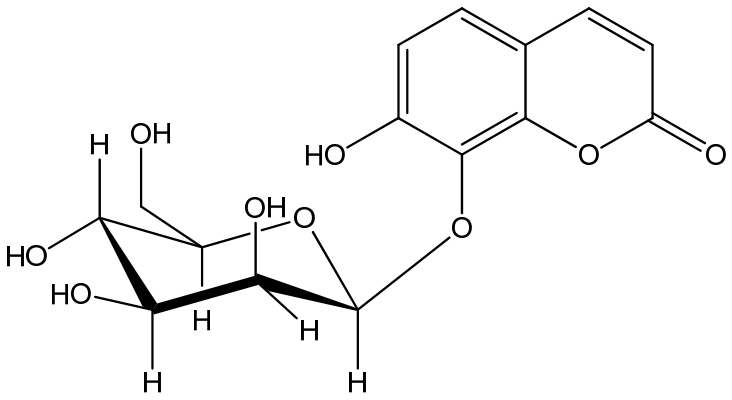


Fig. 3.8: Structure of RL-4

Table 3.7: ^{1}H and ^{13}C NMR of RL-4

Position	δH	δC	DEPT
2	-	161.8	C
3	6.14 (1H, d, *J* = 9.5 Hz)	113.5	CH
4	7.78 (1H, d, *J* = 9.5 Hz)	145.0	CH
5	7.20 (1H, d, *J* = 8.6 Hz)	124.5	CH
6	6.79 (1H, d, *J* = 8.6 Hz)	111.3	CH
7	-	154.1	C
8	-	131.6	C
9	-	147.9	C
10	-	112.7	C
1'	4.68 (1H, s)	105.3	CH
2'	3.73 (1H, m)	74.1	CH
3'	3.63 (1H, m)	77.1	CH
4'	3.34 (1H, m)	69.5	CH
5'	3.67 (1H, m)	76.4	CH
6'	3.38 (2H, m)	60.8	CH_2

Coupling constants in Hertz are given in parenthesis

Solvent used: DMSO (d_6)

3.4.1 Antimicrobial activity of compound RL-4

RL-4 showed mild to poor antibacterial activity against the following bacterial strains: Gram positive *Staphylococcus aureus* ATCC 29213, *MRSA* ATCC 15187; Gram negative *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027 obtained from American Type Cultures Collection.

Table 3.8: **Antibacterial activity of RL-4 against bacterial pathogens**

Organism	**Inhibitory zone MIC (µg/ml)**	
	RL-2	**Standard**
S. aureus ATCC-29213	250	0.12
MRSA ATCC-15187	250	8.0
E. coli ATCC-8739	250	<0.03
P. aeruginosa ATCC-9027	500	0.06

3.5 Antibacterial activity of the extracts of *Rhododendron lepidotum*

The three extracts namely A001 (ethanol), A002 (1:1 ethanol/water) and A003 (water) of *Rhododendron lepidotum* were tested against following bacteria: Gram positive *Staphylococcus aureus* ATCC 29213, *MRSA* ATCC 15187; Gram negative *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027 obtained from American Type Cultures Collection. Ciprofloxacin was used as a standard antibacterial agent for this study. The antibacterial activity of the compound was expressed as minimum inhibitory concentration (MIC). Both A001 and A002 extracts showed good inhibitory effect against *Staphylococcus aureus* ATCC 29213, *MRSA*

ATCC 15187 and moderate inhibitory effect against *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027. However A003 failed to show any significant activity against any of the tested pathogens.

Table 3.9: **Antibacterial activity of the extracts of *R lepidotum* against bacterial pathogens**

Organism	**Inhibitory zone MIC (µg/ml)**			
	A001	A002	A003	Standard
S. aureus ATCC-29213	250	1000	>2000	0.12
MRSA ATCC-15187	500	1000	>2000	8.0
E. coli ATCC-8739	1000	2000	>2000	<0.03
P. aeruginosa ATCC-9027	1000	2000	>2000	0.06

Chapter 4
Experimental

4. Experimental

4.1 Materials and Methods

Melting points are uncorrected and were determined in glass capillary tubes using Buchi B- 545 melting point apparatus. Optical rotations were measured using Rudolph Autopol IV digital polarimeter. UV spectra were scanned in methanol on specord S100. Infrared spectra were recorded as KBr pellets in cm^{-1} on a Hitachi 270-30 spectrophotometer. ^{1}H NMR and ^{13}C NMR, HMBC were recorded on 200 and 500 MHz Bruker Daltonics spectrometers respectively. The chemical shifts (δ) are reported in ppm relative to tetramethylsilane (TMS) as internal standard and coupling constants were measured in Hz using deuterated methanol and DMSO as solvents and TMS as internal standard. Mass spectra were recorded by using a Bruker Daltonics electrospray ionization. Thermo Finnegan HPLC system consisting of high pressure liquid chromatography pump (P-4000), an auto sampler (AS-3000), a column oven, a diode array detector (UV-6000 LP), vacuum membrane degasser (SCM-1000), system integrator (SN-4000) and RP-18 (250mm x 4.6mm; particles 5µm; Merck Germany) was used for checking the purity of compounds. Column chromatography was carried out on silica gel (Merck, 60-120 and 120-240 mesh). The purity of samples was checked on precoated silica gel plates (Merck). Acetic anhydride, pyridine and dimethylamino pyridine (DMAP) were used for acetylation. Solvents of LR grade (Merck) were used for chromatographic isolation.

Thin layer chromatographic (TLC) plates were visualised under ultraviolet light at 254 nm for fluorescence quenching spots and at 366 nm for fluorescent spots. Cerric sulphate, sulphuric acid and $FeCl_3$ were used as spraying reagents to visualize the spots. Iodine was also used to detect the spots.

4.2 Plant Material

The aerial parts of *Rhododendron lepidotum* were collected from Sonamarg, Kashmir in September 2007. After proper identification a voucher specimen (No. 1455/93) was deposited in the herbarium of the institute.

4.3 Extraction and Isolation

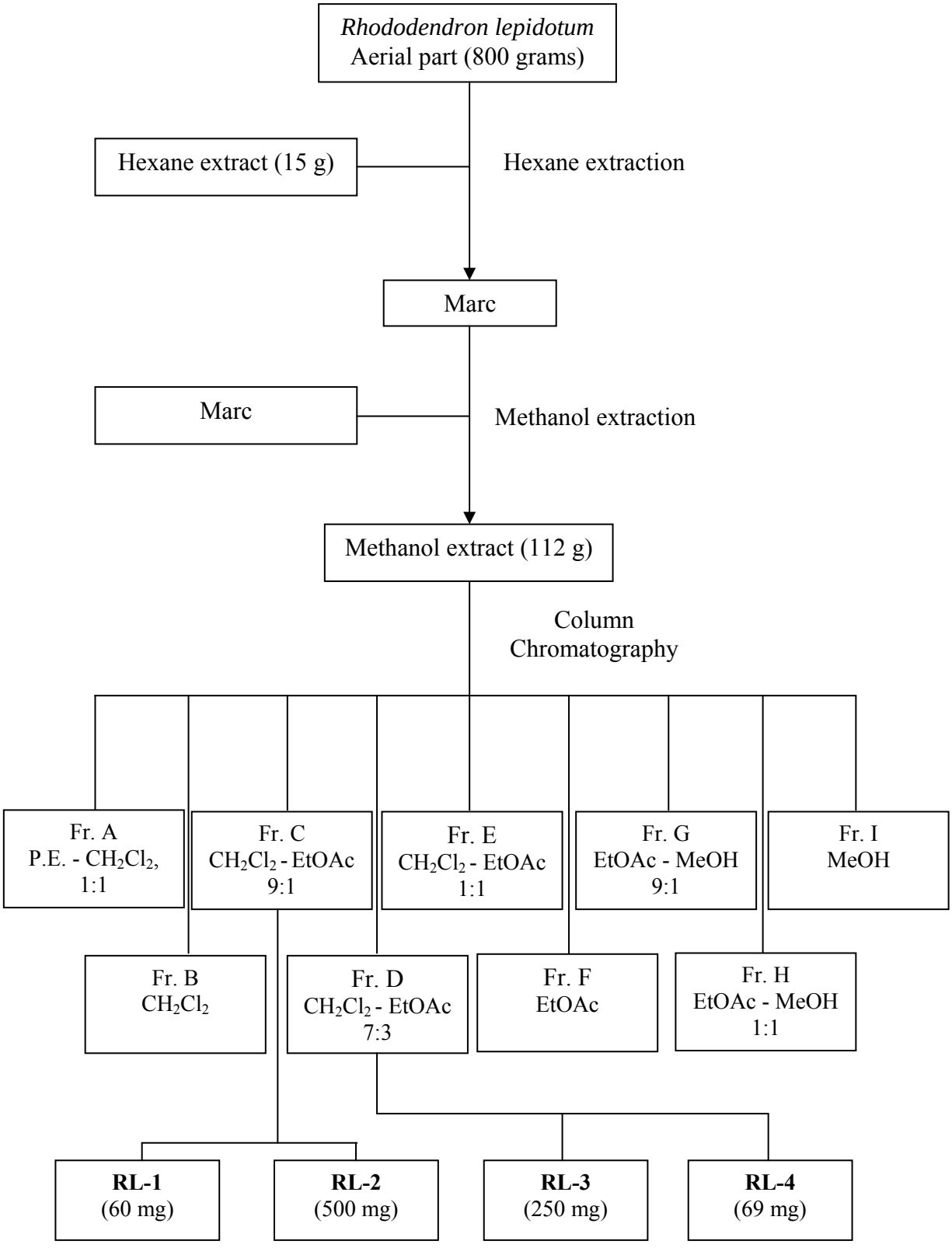
Shade-dried and coarsely powdered plant material (aerial part, 800 g) was defatted with hexane at room temperature for 48 hours to give 15g of hexane extract (1.87%). The defatted plant material was dried and extracted with methanol for 48 hours. The methanolic extract thus obtained was concentrated under reduced pressure to give crude extract, 112 g (14%).

The methanolic extract was dissolved in the minimum amount of methanol and adsorbed on silica gel to form slurry. The dried slurry was subjected to column chromatography over silica gel. The column was eluted with different percentages of petroleum ether, dichloromethane, ethyl acetate and methanol in different proportions and the following fractions (100 ml each) were collected: 1-250 (petroleum ether - CH_2Cl_2, 1:1) (Fraction A), 251-375 (CH_2Cl_2) (Fraction B), 376-455 (CH_2Cl_2 - EtOAc, 9:1) (Fraction C), 456-557 (CH_2Cl_2 - EtOAc, 7:3) (Fraction D), 558-591 (CH_2Cl_2 - EtOAc, 1:1) (Fraction E), 592-670 (EtOAc) (Fraction F), 671-720 (EtOAc - MeOH, 9:1) (Fraction G), 721-773 (EtOAc - MeOH, 1:1) (Fraction H) and 774-797 (MeOH) (Fraction I).

Fraction C (1.5 g) on keeping overnight deposited impure crystals, which were separated through filtration and purified by recrystallization to afford compound **RL-2** (500 mg). Sub fraction C-1 (850 mg) of fraction C was rechromatographed over silica

gel and eluted with solvents like petroleum ether, chloroform and ethyl acetate in increasing order of polarity and afforded compound **RL-1** (60 mg).

Recolumn chromatography of fraction D (2 g) and elution with different solvents (hexane, chloroform and methanol) in increasing order of polarity afforded compound **RL-3** (250 mg) and **RL-4** (69 mg). Flow chart depicting isolation of compounds from the aerial part of *R. lepidotum* is shown in scheme 1.



Scheme 1: Flow chart depicting preparation of extracts and isolation of compounds from the aerial part of *Rhododendron lepidotum*.

4.3.1 RL-1

Physical state	:	Sharp needles
Yield	:	0.0075%
M.p.	:	225°C
R_f	:	0.54 ($CHCl_3$: MeOH; 8:2, v/v)
UV (MeOH, λmax, nm)	:	216, 245 and 322
IR (KBr, ν , cm^{-1})	:	3411, 1680
ESI-MS (m/z)	:	162 [M^+], (Calcd. for $C_9H_6O_3$)
1H NMR	:	Table 3.1
13C-NMR	:	Table 3.1

4.3.2 RL-2

Physical state	:	Colourless needles
Yield	:	0.062%
M.p.	:	255°C
R_f	:	0.66 (CH_2Cl_2: EtOAc; 1:1, v/v)
UV (MeOH, λmax, nm)	:	218, 267 and 302
IR (KBr, ν , cm^{-1})	:	3419, 2870, 1725, 1463, 1023, 827
ESI-MS (m/z)	:	178 [M^+], (Calcd. for $C_9H_6O_4$)
1H NMR	:	Table 3.3
13C-NMR	:	Table 3.3

4.3.3 RL-3

Physical state	:	Whitish amorphous powder
Yield	:	0.031%
M.p.	:	185°C
R_f	:	0.72 ($CHCl_3$: MeOH; 9:1, v/v)
UV (MeOH, λmax, nm)	:	219, 261 and 309
IR (KBr, ν , cm^{-1})	:	3463, 1724, 1610, 1578)
ESI-MS (m/z)	:	354 [M^+], (Calcd. for $C_{16}H_{18}O_9$)
$[\alpha]_D^{25}$	:	–62° (c 1.0, MeOH)
1H NMR	:	Table 3.5
13C-NMR	:	Table 3.5

4.3.4 RL-4

Physical state	:	Yellow powder
Yield	:	0.0086%
M.p.	:	195°C
R_f	:	0.70 ($CHCl_3$: MeOH; 9:1, v/v)
UV (MeOH, λmax, nm)	:	221, 264 and 307
IR (KBr, ν , cm^{-1})	:	3535, 3320, 1723, 1613, 1579, 1084
ESI-MS (m/z)	:	340 [M^+], (Calcd. for $C_{15}H_{16}O_9$)
$[\alpha]_D^{25}$	:	–25° (c 1.0, MeOH)
1H NMR	:	Table 3.7
13C-NMR	:	Table 3.7

4.4 Preparation of extracts for biological screening

The shade dried aerial part of *Rhododendron lepidotum* (150 g) was chopped, grinded and divided into three equal parts of 50 g each. One part was soaked in ethanol and labelled as A001, second part was soaked in 1:1 ethanol and water and labelled as A002 and the remaining part was soaked in water and labelled as A003. Each part was extracted four times at room temperature and concentrated under reduced pressure to give 5.5 g of A001, 3.5 g of A002 and 2.2 g of A003.

4.5 Minimum inhibitory concentration assay

All the four compounds isolated and the extracts prepared were tested against the following bacterial strains: Gram positive *Staphylococcus aureus* ATCC 29213, *MRSA* ATCC 15187; Gram negative *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 9027. The bacterial cultures were maintained on Tryptone soya agar and stored at -70°C containing 50% glycerol. Ciprofloxacin obtained from Sigma-Aldrich was used as standard antibacterial agent for this study. Stock solution was prepared at 1mg/ml.

MIC was determined as per the guidelines of Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards) (Clinical and Laboratory Standards Institute, 2006)**.** Bacterial suspensions were prepared by suspending 18-24 hrs grown bacterial cultures in sterile normal saline. The turbidity of the bacterial suspension was adjusted to 0.5 McFarland standards (equivalent to 1.5 × 108 CFU/ml) at wavelength 625nm. The 2-fold serial of compounds and extracts (stock solution prepared in DMSO) were prepared in MHB (Mueller Hinton Broth; DIFCO laboratories) in 100µl volume in 96-well U bottom microtitre plates (Tarson, Mumbai, India). The above mentioned bacterial suspension

was further diluted in the MHB and 100µl volume of this diluted inoculum was added to each well of the plate resulting in the final inoculum of 5×10^5 CFU/ml in the well and the final concentrations of the samples ranged from 2000 to 3.90µg/ml till the 10^{th} column. Column 11 and column 12 containing 100µl and 200µl of medium without drug served as growth and media control respectively. The plates were incubated at 37°C for 18 hrs and were read visually and the minimum concentration of the compound showing no turbidity was recorded as MIC.

4.6 HPLC conditions

The purity of all the isolated four compounds was checked by Thermo Finnigan HPLC system consisting of high pressure liquid chromatography pump (P 4000), an auto sampler (AS 3000), a column oven, a diode array detector (UV 6000 LP), Vacuum membrane degasser (SCM 1000) and System Integrator (SN 4000). ChromQuest 4.0 software was used for data analysis and processing. The measurements were carried out on RP 18 column (250mm x 4.6mm; particle size 5mm; Merck, Germany) at 30°C. The injection volume was 5.0 µL, the mobile phase was Methanol: Acetonitrile: Water (45:15:40, v/v/v) and the flow rate was 0.5 ml/min. A typical chromatogram with smooth base line was obtained using the isocratic elution of the compounds.

Summary

Summary

The present work describes the isolation, identification and antibacterial activity studies of the chemical constituents of *Rhododendron lepidotum*.

This dissertation is divided into four chapters: The Introduction, Review of Literature, Results and Discussion and Experimental. The first chapter describes an introduction to medicinal plants and their use in traditional system of medicine, current status of medicinal plants and natural products isolated thereof and isolation of phytochemicals/bioactive molecules from these plants which act as therapeutic agents. The second chapter includes the review of literature of ethno-pharmacology, chemistry and pharmacology of genus *Rhododendron.* The third chapter highlights the phytochemical investigation of *Rhododendron lepidotum.* Overall four natural compounds were isolated which were characterized and identified on the basis of physical properties, spectroscopic data analysis and chemical derivatisation. Out of four isolates two compounds were found as new molecules.

The experimental part (fourth chapter) describes the collection of plant material, identification of the plant, preparation of extracts and isolation of natural products using column chromatography. The natural compounds thus isolated were subjected to evaluation of antibacterial activities. The four compounds isolated through column chromatography were identified as coumarins and were labelled as **RL-1**, **RL-2**, **RL-3** and **RL-4**.

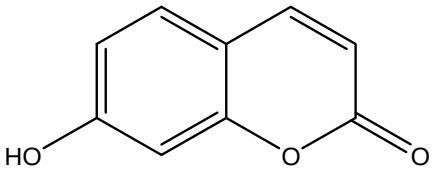
RL-1 was obtained as colorless crystalline needles from $CHCl_3$-EtOAc fraction of methanol extract of the aerial part of *Rhododendron lepidotum*. The molecular formula was established as $C_9H_6O_3$. It was identified as **umbelliferone** based on its physico-chemical properties and by comparing its spectral data with that reported in the literature. This compound is known to literature but is being reported for the first

time from this genus. Its antibacterial activity was not significant against the available Gram positive and Gram negative bacterial strains.

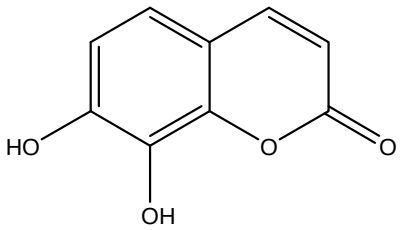
RL-2 was obtained as colourless crystalline compound from $CHCl_3$-EtOAc fraction of methanol extract of the aerial part of *Rhododendron lepidotum*. Its molecular formula was established as $C_9H_6O_4$ and its structure was identified as **daphnetin** based on physicochemical properties and spectral data analysis. Although it is known to literature but is being reported for the first time from the genus *Rhododendron*. Its antibacterial activity was found to be good with MIC value of 125µg/ml against Gram positive *Staphylococcus aureus*, *MRSA* and Gram negative *Escherichia coli* and *Pseudomonas aeruginosa*.

RL-3 was isolated as whitish amorphous powder from $CHCl_3$-EtOAc fraction of methanol extract of the aerial parts of *Rhododendron lepidotum*. Its molecular formula was established as $C_{16}H_{18}O_9$ and the structure was characterized as ***7-O-β-D-glucopyranosyl-8-methoxy benzopyranone*** based on chemical and spectral data. The compound is new to literature and is being reported for the first time. It showed a broad range of antibacterial activity against Gram positive *Staphylococcus aureus*, *MRSA* and Gram negative *Escherichia coli* and *Pseudomonas aeruginosa* with MIC 250 µg/ml.

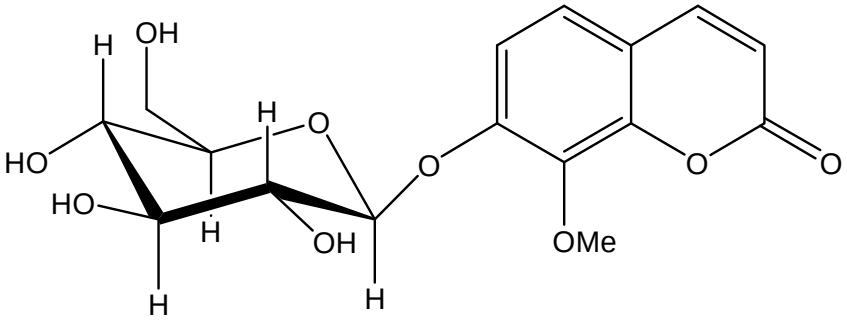
RL-4, corresponding to molecular formula $C_{15}H_{16}O_9$, was isolated as yellow amorphous powder from $CHCl_3$-EtOAc fraction of methanol extract of the aerial part of *Rhododendron lepidotum*. Its structure was characterized as ***7-hydoxy-8-O-β-glycosyl-benzopyranone*** based on chemical and spectral data. It is also new to literature, reported for the first time. It showed moderate antibacterial activity against the above mentioned organisms.



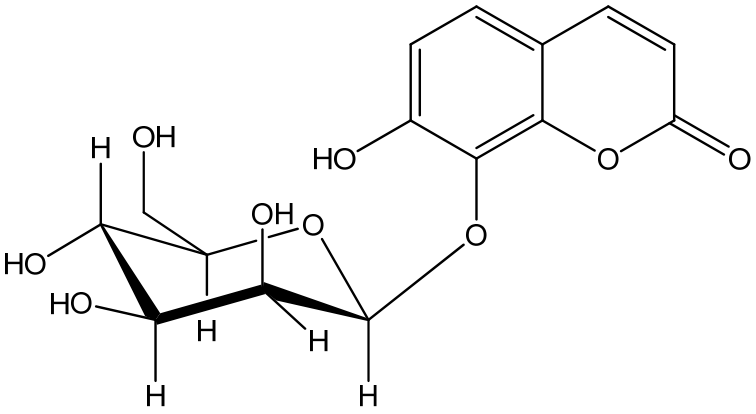
RL-1



RL-2



RL-3



RL-4

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Appendices

Appendix I : List of Symbols/Abbreviations

ATCC	American Type Culture Collection
CC	Column chromatography
$CDCl_3$	Deuterated Chloroform
CFU	Colony forming units
cm^{-1}	Centimeter inverse
°C	Degree Celsius
d	Doublet
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO (d_6)	Deuterated dimethyl sulphoxide
ESI-MS	Electrospray Ionization Mass Spectrometry
Fig.	Figure
g/gm	Gram
Hz	Hertz
IR	Infrared
J	Coupling constant
m	Multiplet
M	Molecular mass
MeOD	Deuterated Methanol
mg	Milligram
µg	Microgram
MHz	Mega Hertz
MIC	Minimum Inhibitory Concentration
mL/ml	Milliliter

mM	Millimolar
M.p.	Melting point
m/z	Mass to Charge ratio
Na	Sodium
NAM	Nutrient agar medium
NBGP	Nutrient broth containing 0.05% phenol red and supplemented with 10% glucose
NMR	Nuclear Magnetic Resonance
ppm	Parts Per Million
RA	Reference antibiotic
Rf	Retention factor
s	Singlet
TLC	Thin Layer Chromatography
UV	Ultra Violet
v/v	Volume by Volume
α	Alpha
β	Beta
δ	Delta
$[\alpha]_D$	Specific rotation
λ_{max}	Lamda maximum

Appendix II : List of Figures

No. **Title**

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1.2 All new chemical entities, 1981-2002, by source (Total =1031).

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2.2 Molecular structures of the bioactive flavonoids

2.3 Molecular structures of histamine release inhibitory chemical constituents

2.4 Molecular structures of rhododaurichromanic acid A (1), rhododaurichromanic acid B (2) and daurichromenic acid (3)

2.5 Molecular structures of the antibacterial coumarins

2.6 Molecular structures of the bioactive diterpenes

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3.1 HPLC spectrum of RL-1

3.2 Structure of RL-1

3.3 HPLC spectrum of RL-2

3.4 Structure of RL-2

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Appendix III : List of Tables

No. **Title**

1.1 History of Natural Product Medicine

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3.1 ^{1}H and ^{13}C NMR of RL-1

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3.3 ^{1}H and ^{13}C NMR of RL-2

3.4 Antibacterial activity of RL-2 against bacterial pathogens

3.5 ^{1}H and ^{13}C NMR of RL-3

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3.7 ^{1}H and ^{13}C NMR of RL-3

3.8 Antibacterial activity of RL-4 against bacterial pathogens

3.9 Antibacterial activity of RL-4 against bacterial pathogens

Appendix IV : Phytochemical Tests

1. **Test for Carbohydrates:** 100 mg of the substance is dissolved in 1 ml water and 1drop of 1 % alcoholic solution of α-naphthol is added. 1 ml of conc. H_2SO_4 is added through the sides of test tube so that it forms a heavy layer at the bottom. A deep violet colour at the liquid junction confirms the presence of carbohydrates

2. **Test for Coumarins:** A few mg of the compound is dissolved in methanol/ethanol and alcoholic KOH is added. Yellow colour which disappears on adding conc. HCl indicates the presence of coumarins.

3. **Test for Flavonoids:** Dissolve a few mg of the compound in methanol or ethanol, add Mg powder/turnings and 5 M HCl. Flavonoids give deep red or magneta colour

4. **Test for Terpenoids:** A few mg of substance is dissolved in chloroform and acetic anhydride, followed by sulphuric acid. Pink colour indicates the presence of terpenoids.

Appendix V : Spray Reagents

1. Anisaldehyde-sulphuric acid reagent: 0.5 ml of anisaldeyde is mixed with 10 ml glacial acetic acid, followed by 85 ml methanol and 5 ml conc. H_2SO_4

2. Cerric suplphate reagent: 1 g of $Ce(SO_4)_2$ is dissolved in 10 ml of conc. H_2SO_4 and made upto 100 ml with distilled water

3. Ferric chloride reagent: To 5 % solution of $FeCl_3$, NH_4OH solution is added with shaking drop by drop till the permanent precipitate is obtained (or) 1% Ferric (III) Chloride in Methanol/water (1:1).