BIOCHEMICAL STUDIES ON LICHENS AS POTENTIAL SOURCE OF AGROCHEMICALS

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CERTIFICATE

This is to certify that the thesis titled **"Biochemical Studies on Lichens as Potential Source of Agrochemicals"** submitted to the Faculty of Technology, University of Delhi, Delhi, in fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Applied Chemistry embodies the original research work carried out by Ms. Mayurika Goel under my supervision and Co supervision of Dr. Prem Dureja. This work has not been submitted in part or full for any degree or diploma of this or any other University. It is further certified that the scholar has devoted more than two years, the minimum stipulated period, for the completion of this work.

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Affectionately Dedicated to My Beloved

Papa, Mummy and Bhai

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(Mayurika Goel)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Introduction

Introduction

Agrochemical is a generic term that refers to the substances used in agriculture to improve crop productivity quantitatively and qualitatively. It includes fertilizers; plant growth regulators/promoters like hormones; herbicides (substances that suppress the growth of undesirable competitive plants); plant protection agents, broadly termed as pesticides (a substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest). Depending upon the pest they control, these are classified as fungicides, insecticides, algaecides etc.

Due to the intensive use of these chemicals worldwide, the productivity of the agriculture has increased dramatically during the last three and half decades of the twentieth century. Yield of the main food crops, rice and wheat, almost doubled, and the yield of other food as well as non-food crops too increased considerably. The yield-augmenting technological changes also led to the self-sufficiency in the food production in most parts of the world. Yet, the concerns of food security have not vanished altogether. By 2030, in India, the demand for food grains is expected to increase to 260-265 million tonnes (Paroda and Kumar, 2000) to meet the food security needs of the growing population. The incremental production to meet this demand would have to come from the substantial increase in the agricultural productivity, as the scope for bringing additional land under cultivation is limited. Concurrently, a number of biotic and abiotic factors also continue to constrain the growth in the agricultural productivity.

According to Oerke *et al.*, 1995, among various biotic factors, insect pests, diseases and weeds are the main yield-limiting factors in most parts of the world. So the chemical protection of plants (use of pesticides) has become an integral part of the agro-technology in ensuring sufficient food supply to a growing world population.

In India, chemical pest control has become the preferred strategy in practice from the beginning of the green revolution era in 1965-66. During this period, introduction of the new varieties of crops necessitated intensive use of purchased inputs including chemical pesticides for better yield response. Thus, the pesticide consumption increased to 90 gm ha^{-1} in 1965-66 as compared to 15 gm ha^{-1} in 1955-56. In a span of 10 years, from 1965-66 and 1974-75, pesticide use has increased four-fold (Chand and

Birthal, 1997). Concurrently, the pest problem also kept on multiplying and, the effectiveness of the pesticides was so unambiguous that soon these overshadowed the traditional methods of pest control. Pesticide use kept on increasing till 1990-91, albeit at a slower rate (Chand and Birthal, 1997). In 1990-91, the pesticide use reached a peak of 405 gm ha^{-1}. It is worth mentioning here, that, Indian pesticide industry has now become the second largest manufacturer of the pesticides in Asia after China and third largest in the world (Vladimir Radyuhin, 2011).

The contribution of these pesticides in controlling the insect pests, diseases, weeds, is outstanding. A study by the National Council of Applied Economic Research (NCAER, 1967) showed that every rupee invested in chemical pest control returned Rs 3 in crops saved. Nonetheless, indiscriminate and continuous use of these chemicals has posed a great threat to agriculture, environment and human health globally. In most of the areas, the fungi and weeds have developed chemical resistance and as a consequence higher dose of chemicals are needed which increases the level of toxic residues in soil, water and the ecosystem. In addition, injudicious application of synthetic chemicals have resulted in emergence of many new problems like pest resurgence (pest outbreak), destruction of non-target organisms, phytotoxicity, (Mercier and Manker, 2005) environmental pollution and poisoning cases including accidental poisoning (Fernando *et al.*, 2005). Empirical evidences suggest that the cost of the measurable negative externalities of the synthetic pesticides outweigh their perceived economic benefits (Pimental *et al*., 1993; Rola and Pingali, 1993).

With rising public concerns about the economic and ecological externalities of the chemical pesticides, the emphasis of plant protection research and development strategies has been gradually shifting from chemical to nonchemical alternative approaches. Consequently, researches have yielded new technologies, such as,

- Development of high yielding seeds;
- Development of disease-resistant varieties of plants (Schestibratov and Dolgov, 2005; Dong *et al*., 2007); and
- Use of biocontrol agents (naturally occurring enemies of pests as parasitoids, predators and pathogens). Some important commercially available products are *Trichogramma, Bracons, Crysoperla carnea, Crytaemus montrouzieri, Bacillus*

thuringiensis, Bacillus sphaericus, Nuclear polyhedrosis viruses (NPV) and *Trichoderma*

These techniques though have proved functional; there are various likely constraints on their adoption world-wide such as skill requirement, system dependence, income and employment implications. Therefore, the scientists reoriented their research towards allelochemicals (chemicals produced by algae, fungi, bacteria and certain plants that influence the growth of other organisms) (Molisch, 1937). The application of these chemicals to influence the growth, survival and reproduction of other organisms is termed as Allelopathy. These products in crude form as plant extracts (Bowers and Locke, 2004) or as isolated pure substances like azadirachtin (neem), pyrethrum, nicotine and fungicides from antagonistic strains of fungi and bacteria (Lemessa and Zeller, 2007; Paulitz and Belanger, 2001; Minuto and Spadaro, 2006, Andersson *et al*., 2011, Molla´*et al*., 2011) can be relatively less damaging for environment and more efficient in disease control (Surajit *et al.*, 2006). According to a survey by National Institute of Science Technology and Development Studies (NISTADS, 2008), the consumption of biopesticides is still meager in comparison with the chemical pesticides.

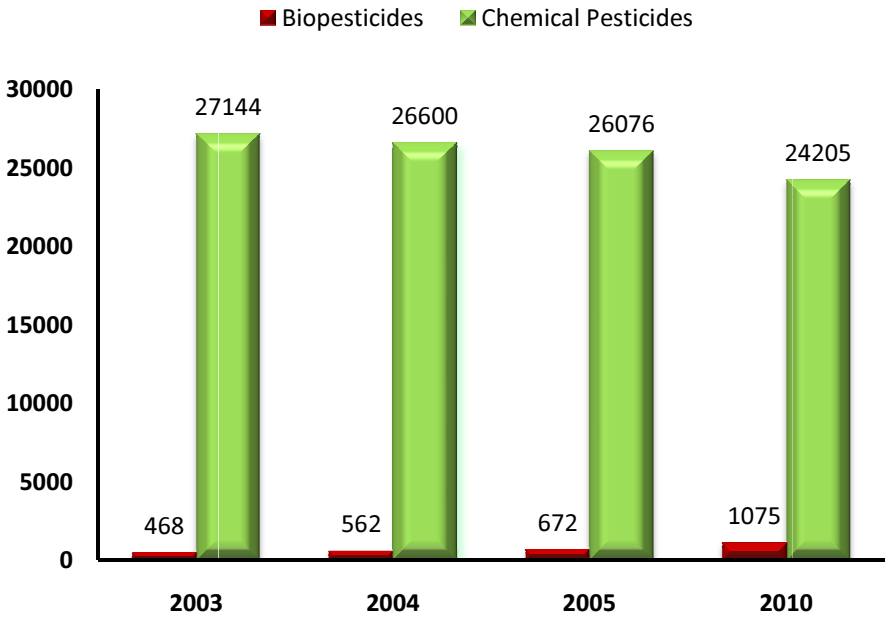


Figure 1.1: Trend of global pesticide market
(Source: http://www.nistads.res.in/indiasnt2008)

With the development of novel and sensitive techniques to detect biological activity of natural products and improved techniques to isolate, purify, and structurally characterize

active constituents collectively, the efforts focused on exploring newer sources of allelochemicals gained momentum.

Lichens, in this context, are catching the attention of scientists worldwide as a source of vast, virtually untapped reservoir of chemical compounds. Lichens are unusual intriguing symbiotic organisms composed of a fungal partner (mycobiont) in association with one or more photosynthetic partners (photobiont). The photobiont can be green algae, cyanobacteria, or both. They are deposited on the surface as thread like hyphae, the tiny filaments that connect fungi to a food source or host rather than being compartmentalized in the cells. The symbiotic relationship among such diversified organisms is undoubtedly very successful, the result, lichens are found worldwide (covering about 8% of the earth's surface), they colonize a wide range of substrata (i.e. rocks, bare ground, leaves, bark, metal, glass). Lichens occur in some of the most extreme environments on Earth and they are able to survive in these environmental conditions because of slow metabolism, capacity for survival in a metabolic resting state for months and production of many particular bioactive molecules which offer chemical protection to them. These compounds typically arise from the secondary metabolism of the fungal part of the lichen (Wörgötter, 2008; Jason and Townsend, 2010). These secondary metabolites are unique with respect to those of higher plants (Lawrey, 1986 and Huneck, 1999) and possess diverse biological activities (Molnár and Farkas, 2010; Muller, 2001). Of the more than 20,000 known lichen species, only a few have been analyzed and identified containing biologically active compounds.

Biodiversity of lichen species as potential source of agrochemical, particularly from Himalayan geographical zones is still unexplored. It provides a compelling justification for the proposed plan of work aimed to explore the constituents from Himalayan lichen species in order to increase the natural product pool and uses thereof as future agrochemicals.

Thus, *Parmelia reticulata* Taylor, *Ramalina roesleri* Nyl, *Usnea longissima* Ach. Articus and *Stereocaulon himalyense* Lamb were collected from the Kilberi forest ranges, Himalayas in the Uttarakand province of India. The samples were deposited in the Herbarium of the Department of Botany, University of Delhi, Delhi, India. The voucher numbers for the lichen species are *Parmelia reticulata* Tayl (DUH 13531), *Ramalina roesleri* Nyl (DUH 13532), *Usnea longissima* Ach. Articus (DUH 13533), *Stereocaulon himalayense* Lamb (DUH 13534).

The dried and powdered lichen thalli were subjected to the soxhlet extraction with hexane, ethyl acetate and methanol for 6 hrs each. The extracts were concentrated in vacuum and the following allelopathic activities of the crude solvent extracts were evaluated at different concentrations.

1) *In vitro* antifungal activity against devastating, polyphagous plant pathogenic fungi namely *Sclerotium rolfsii* ITCC 5226, *Rhizoctonia solani* ITCC 5395, *R. bataticola* ITCC 0482, *Fusarium udum* ITCC 2042, *Pythium debaryanum* ITCC 4744, *Pythium aphanidermatum* ITCC 4746 procured from the Indian Type Culture Collection Centre (ITCC), Division of Plant Pathology, Indian Agricultural Research Institute (IARI), New Delhi.
2) Herbicidal efficacy of the solvent extracts and isolated constituents was studied against one of the devastating weeds, *Phalaris minor* Retz., collected from Division of Agronomy, Indian Agricultural Research Institute, New Delhi.
3) Effect on seed germination and early growth of seedlings of major crop plants, wheat (*Triticum aestivum*), lentil (*Lens culinaris* Medikus) and chick pea (*Cicer arietinum* L.) collected from Division of Genetics, Indian Agricultural Research Institute, New Delhi.
4) Algaeicidal activity against microalgae *Chlorella vulgaris*, *C. sorokiniana* and *Scenedesmus subspicatus*, collected from SERA Heinsberg, Germany.

In vitro antifungal activity of solvent extracts of lichen species were evaluated using poisoned food technique at different concentrations. The results have been reported in terms of effective dose for 50 % inhibition (ED_{50}) value, calculated by Probit analysis The antifungal activities of different solvent extracts of lichen species against different pathogenic fungi were found to be selective. Thus, in general, the hexane extract of all the lichen species were most active, followed by the ethyl acetate and methanol extracts. The finding revealed that antifungal activity of these extracts may be due to the presence of antifungal compounds. Therefore, these extracts were subjected to column chromatography for isolation and identification of antifungal compounds.

Herbicidal potential of solvent extracts of *P. reticulata* and *R. roesleri* were studied in a biological assay at different concentrations (50, 25 and 12.5 µg ml^{-1}) on the germination and early seedling growth of weed *Phalaris minor* Retz. Results are reported in terms of percentage of seed germination (and inhibition %), shoot and root length in cm

(and inhibition %). Maximum shoot and root length inhibition was recorded in seedlings treated with hexane extract followed by the ethyl acetate and methanol extracts.

To assess the influence of crude extracts of *P. reticulata* and *R. roesleri* on the growth of major food crops, wheat (*Triticum aestivum* L.), chick pea (*Cicer arietinum* L.) and lentil (*Lens culinaris* Medikus), a bioassay was carried out at different concentrations (2.5, 1.25 and 0.625 μg ml^{-1}) under laboratory conditions. Mean percentage seed germination (%), shoot length (cm) and root length (cm) was measured to observe the growth of the plant.

To find the components responsible for the diverse activities of crude extracts, a bioassay guided approach was followed. Chemical constituents were isolated using various chromatographic techniques and were characterized on the basis of spectral analysis and comparison with the literature reports. In total, fourteen compounds (Pr Ia-Pr IIIa, Pr IVb- Pr XIVb) were isolated from *P. reticulata*, six compounds (Rr Ia, Rr IIb-Rr VIb) were isolated from *R. roesleri*, three compounds (Uℓ Ia, Uℓ IIb- Uℓ IIIb) were isolated from *U. longissima*, four compounds (Sh Ia-Sh IIa, Sh IIIb-Sh IVb) were isolated from *S. himalayense*.

The herbicidal activity of isolated compounds was evaluated against *P. minor* at three different concentrations. Among the compounds tested against the growth of *P. minor*, maximum inhibition was observed with usnic acid followed by homosekikaic acid and divaricatinic acid. In current study, usnic acid was found most effective in suppressing the germination and early seedling growth of *P. minor*.

Isolated compounds were tested against microalgae namely *Chlorella vulgaris*, *C. sorokiniana* and *Scenedesmus subspicatus* purchased from S.E.R.A, Heinsberg, Germany. The activity was determined at 40 μg/disc using disc diffusion assay. The diameter of the inhibition zone was taken as the measure of extent of activity. Isousnic acid and protolichesterinic acid was found to be very active against *Chlorella vulgaris* and *C. Sorokiniana*. This is first report of algaeicidal activity of metabolites isolated from lichen species.

Encouraged by these results, the investigation has been extended to evaluate the effect of lichen solvent extracts and isolated compounds on pathogenic bacteria, *Bacillus subtilis* (ATCC 6051), *Staphylococcus aureus*, *Streptomyces viridochromogenes* (Tu 57),

Escherichia coli procured from the Culture Collection Centre, Institute of Organic and Bimolecular Chemistry, University of Göttingen, Göttingen, Germany. A preliminary biological screening was carried out using the method recognized by the National Committee for Clinical Laboratory Standards (NCCLS). Usnic acid and protolichesterinic acid showed potent inhibition against *E. coli* and *S. aureus*. Among metabolites isolated from *R. roesleri*, homosekikaic acid was found to be the most active against *E. coli* and *S. aureus*. Crude extracts and isolated compounds showed varying results against test organisms.

The results indicate that the investigated lichen extracts manifested strong, but varying degree of agrochemical activity. In the present scenario, when the scientist community is compelled to reduce the load of hazardous synthetic chemicals and to develop environmentally benign strategies for protection of crops as well as human from diseases, current investigation has established the lichens as potential source of bioactive agents.

Review of Literature

Agriculture was developed at least 10,000 years ago and it has undergone significant developments since the time of the earliest cultivation. Agriculture in India has a significant history. Indian agriculture contributes to 8% global agricultural gross domestic product (GDP) to support 18% of world population on only 9% of world's arable land and 2.3% of geographical area. Agriculture is the key sector for generating employment opportunities for the vast majority of the population; most of them depend for their livelihoods. It is the pivotal sector for ensuring food and nutritional security, sustainable development and for alleviation of poverty. Pioneering work by agriculture scientists and the efforts of farmers had helped achieve a breakthrough in the agriculture sector in the 1960s, popularly known as the '*Green Revolution'*. High agricultural production and productivity achieved in subsequent years has been the main reason for attaining food security to a large extent. The country has not witnessed any big technological breakthrough in agriculture since then.

In global perspective, the production environment and natural resources are continuously deteriorating. Across the larger part of the world, inadequate attention to agriculture has led to steep rise in food prices and increased food riots. Food crisis has aggravated further because of climate change and diversion of arable lands to urbanization and industrialization. Therefore, conservation of natural resources, maintenance of biological wealth and acceleration of agricultural growth are considered of paramount importance in the present context as well as of the future.

With limited land available for cultivation and concerns about environmental sustainability, growth of agricultural output must come through productivity improvements on the existing land. This can only be achieved in parts by suitable control of losses due to various abiotic and biotic agents summarized in Fig 2.1.

2.1 Deteriorating production environment and growing food demand

The food safety net for each of the over a billion citizens a number that is growing requires enhanced agricultural production and productivity in the form of a Second Green Revolution. The demand for food and processed commodities is increasing due to growing population and rising per capita income. There are projections that demand for

food grains would increase from 192 million tonnes in 2000 to 345 million tonnes in 2030. Hence in the next 20 years, production of food grains needs to be increased at the rate of 5.5 million tonnes annually. Urbanisation has led to a fall in natural resources. Thus, the key challenges would be to develop promising technologies and sustainable resource management options to raise productivity to meet growing food demand in a situation of deteriorating production environment (NAAS, 2009).

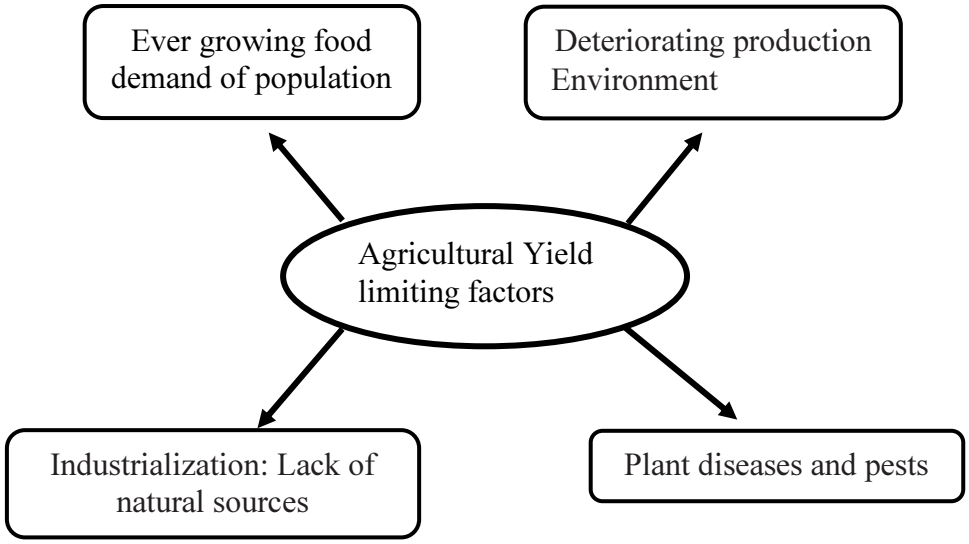


Figure 2.1: Factors that governs the agriculture yield worldwide

2.2 Climate change and agriculture

Inter-Governmental Panel on Climate Change has projected that by the end of this century, global earth temperature is likely to increase by 1.8 to 4.0°C. This would lead to more frequent hot extremes, floods, droughts, cyclones and recession of glaciers. Dynamics of pests and diseases would be significantly altered. The projected increase in these events will result in greater instability in food production and will threaten farmers' livelihood security. Producing enough food for increased demand against the changing climate scenario is a challenging task for agricultural research.

2.3 Pests in Agriculture

With the advent of agriculture, plant diseases, thousands of insects, fungi, viruses, bacteria, nematodes and other living forms pose potential hazards to crops. The agricultural pests and pathogens known so far include 2,000 species of insects and 800 species of fungi. It has been estimated that around 38-42 % loss in the global output is due to insect pests, diseases and weeds, despite use of various plant protection tactics

(Oerke, 1995; Agrios, 1997; Montesinos, 2003). The loss could have increased to 70 % if the pests were left uncontrolled. Similar observations have been made by Pimental (1993) who estimated 40 % pre-harvest production loss; the post harvest loss adds another 10-20 %.

Owing to the tropical climate, the loss is even higher in the Asian countries (Oerke *et al*., 1995). In India, the pre-harvest production loss due to insect pests is estimated to be 25 % for rice, 5 to 10 % for wheat and 30 % for pulses (Dhaliwal and Arora, 1993). For cotton, rapeseed-mustard and sugarcane, the loss is estimated at 50, 35 and 20 %, respectively. The overall comparative losses of crop yield in India and world are summarized in Table 2.1.

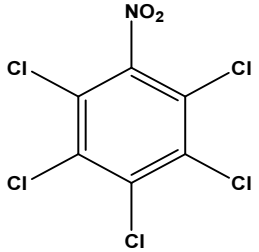
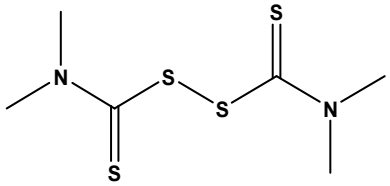
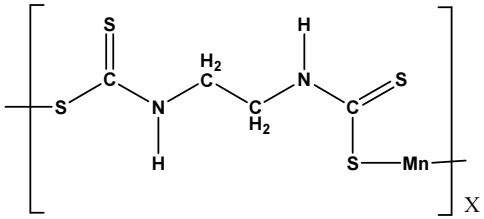
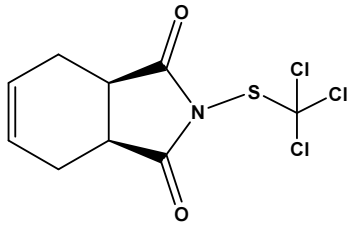
Table 2.1: Loss in crop yield caused by insect pests and fungal pathogens

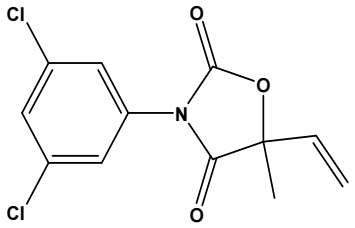
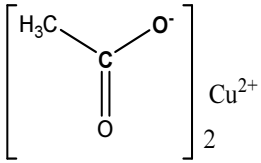
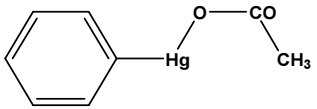
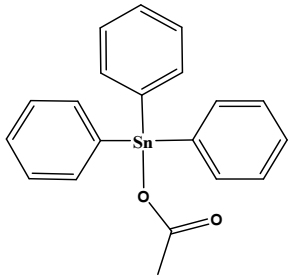
Crop	Loss in Yield	
	In India (%)	**Worldwide (%)**
Wheat	30.0	5.0
Rice	50.0	26.7
Millet/Sorghum	30.0	9.6
Cotton	>40.0	11.0
Pulses	>35.0	30.0
Sugarcane	>40.0	30.0
Vegetables	30.0	30.0

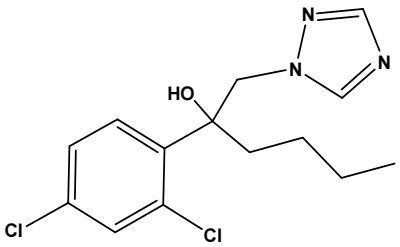
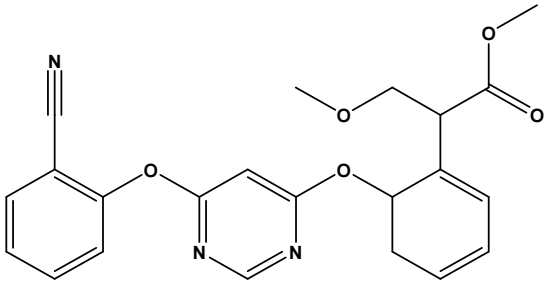
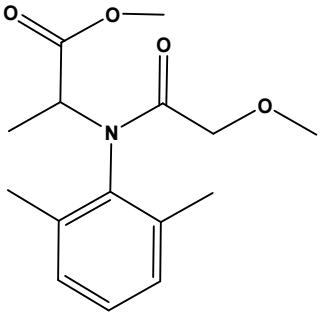
Pest management: current scenario

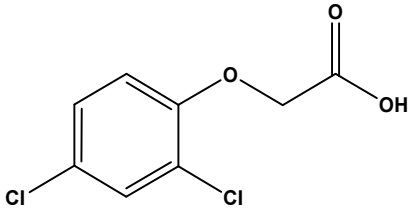
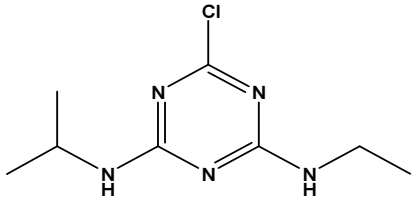
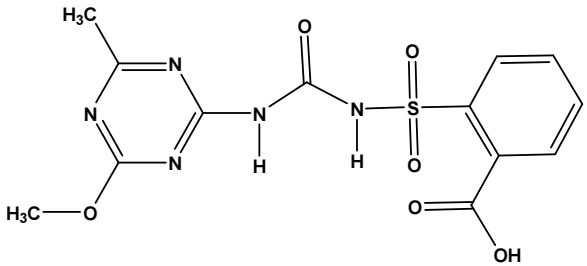
Among various pest control measures like traditional crop rotation, use of natural enemies of pests and development of resistant varieties, application of the chemical pesticides is the preferred method for pest management. Table 2.2 summarizes some common chemical pesticides with their major chemical groups currently used for protection against pests.

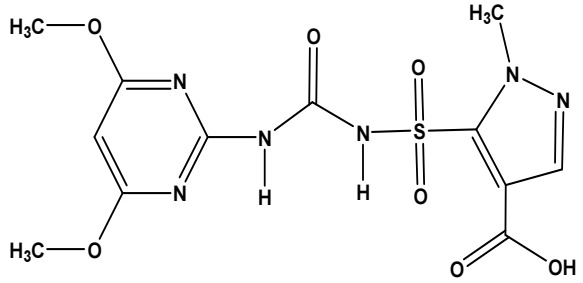
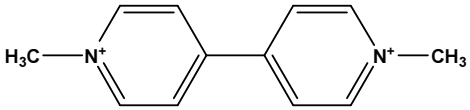
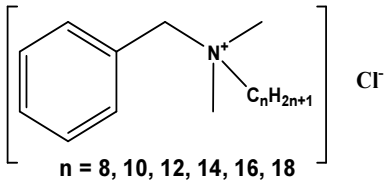
Table 2.2: List of common pesticides.

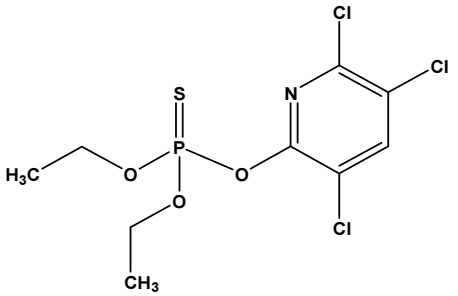
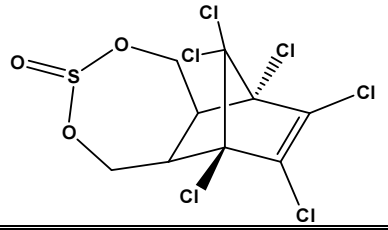
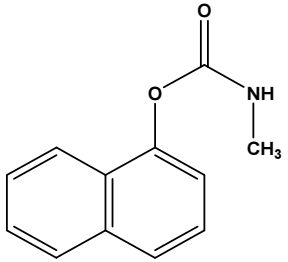
Class of Fungicides	Examples	Structure
Aromatic Hydrocarbons (Substituted benzene) chlorophenyls	Quintozene PCNB, Blocker	
Dithiocarbamates	Thiram	
Ethylenebis Dithiocarbamate	Mancozeb, Maneb	
Thiophtalimides	Captan	

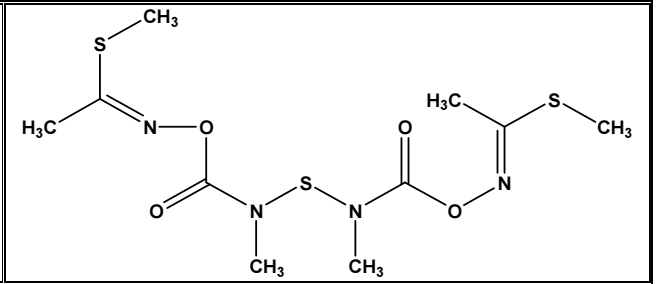
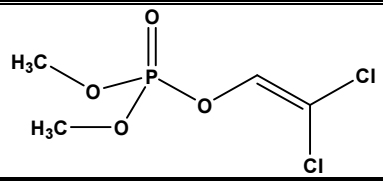
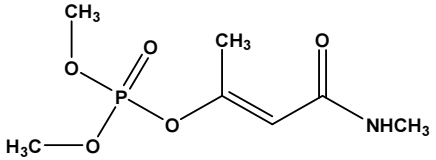
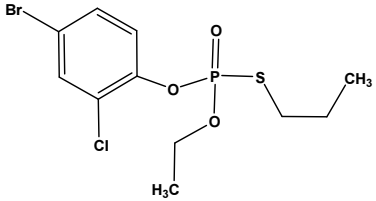
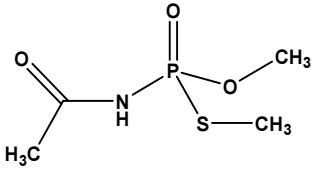
Dicarboximide (dicarboxamide)	Vinclozoline	
Copper compounds	Kocide	
	Bordeaux mixture	$CuSO_4.3Cu(OH)_2.3CaSO_4$
Organomercury compounds	Phenyl mercury acetate	
Organotin compounds	Fentin acetate (Triphenyltin acetate)	

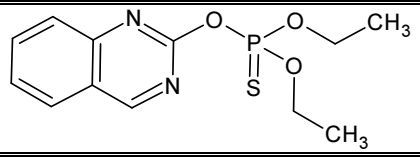
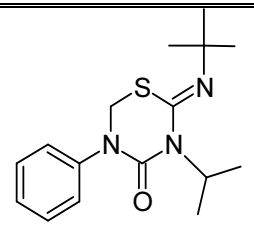
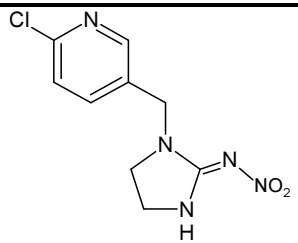
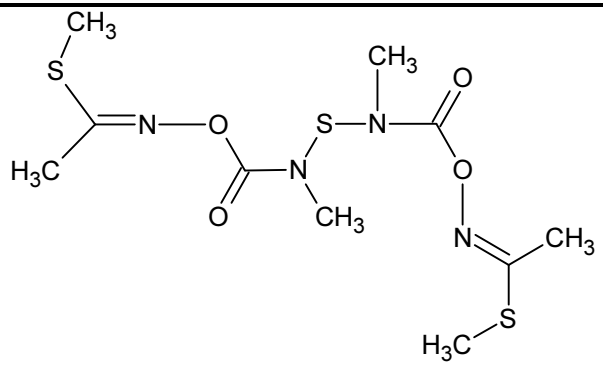
Triazoles	Hexaconazole	
Strobilurin	Azoxystrobin	
Miscallaneous organic compounds	Metaxyl	

Herbicides	Examples	Structure
Phenoxy acetic	2,4- Dichlorophenoxyacetic acid	
Chlorotriazines	Atrazine	
Triazinyl sulfonylurea herbicides	Metasulfuron	

Pyrazole derivatives	Pyrazosulfuron	
Algaecide	**Examples**	**Structure**
Quaternary ammonium herbicides	Paraquat dichloride	
n-alkyl-dimethyl benzyl ammonium chloride (benzalkonium chloride)	Polyquat	
Copper(II) sulfate	Copper Sulphate	$CuSO_4$
Colloidal Silver		Ag

Insecticides	Examples	Structure
Chlorinated hydrocarbons	Chloropyiphos	
	Endosulfan	
Carbamates	Carbaryl	

	Thiodicarb	
Organophosphates	Dichlorvos	
	Monocrotophos	
	Profenophos	
	Acephate	

	Quinalphos	
Others	Buprofezin	
	Imidaclorprid	
	Thiodicarb	

2.4 Limitations of chemical control measures

Chemical pesticides and herbicides are the preferred methods for pest management, due to their efficacy and cost effectiveness. Data from Pesticide Manufacturers and Formulators Association of India revealed that the Indian Pesticide market exceeded 3.5 billion dollar by usage in 2009. The total industry reached more than $3.35 billion in global sales in 2009. The following figure shows the different class of pesticides and their consumption in Indian agriculture.

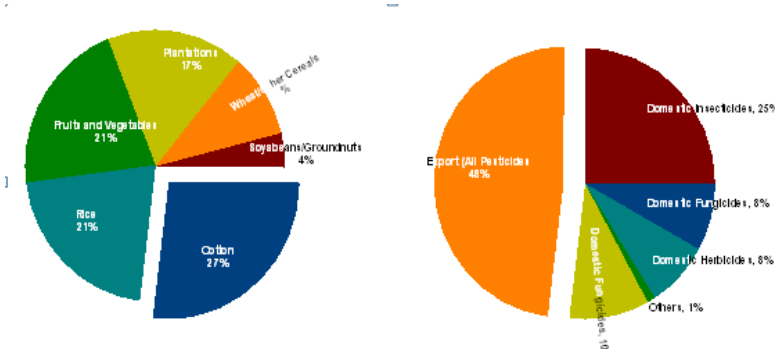


Figure 2.2: Consumption of pesticides in India
(Source:http://www.farmchemicalsinternational.com/magazine/)

However, the increased use of pesticides poses serious environmental hazards and public health concerns (Mancini *et al*., 2008). Numerous reports have indicated direct exposure to the pesticides can induce allergies and affect various body organs such as liver, kidneys, nervous, respiratory and gastro-intenstinal systems. Chronic exposures to pesticides are also causative for diseases such as cancer, genetic mutations, and male sterility. According to a report by WHO/UNEP 1989, though the pesticide-use-intensity is low in the developing countries, most of the pesticide related accidents, such as poisoning and deaths occur there. This is mainly because of the misuse of the pesticides and lack of awareness about precautionary measures during its application (Mencher, 1981). Mohan, (1987) observed a high positive correlation between per hectare pesticide use and physical disabilities in India.

Apart with the above health hazards, their indiscriminate and injudicious application has resulted in the development of problems of the pest resistance, resurgence (pest outbreak), and residue.

A number of insect pests, pathogenic microbes and weeds have become resistant to pesticides intended to control them. Worldwide, about 504 insect pests are reported to have acquired resistance to pesticides. This figure was 7 in 1938. Resistance in the weeds was non-existent before 1970, but now 273 weed species have developed resistance to the herbicides (Pimental *et al*., 1993). Fungicide resistance is reported in nearly 150 plant pathogens. These are serious obstacles to raising the agricultural productivity. Furthermore, the development of resistance to pesticides by pests particularly in developing nations is a serious matter of concern (De Weger *et al*., 1995; Gerhardson, 2002).

Repeated applications of higher doses of pesticides to overcome the problem of resurgence have increased the cost of pest control. Their exposure inevitably leads to bioaccumulation, biomagnification and adverse effect on non target organisms. Pesticides residues not only affect the ecosystem but also pollute the atmosphere with risky chemicals, contaminate the water we drink, air we breathe and the food we eat. Residues of the pesticides in food grains, fruits and vegetables, fish, milk, water and soil have often been reported to exceed their acceptable limits which have endangered the health of the consumers (ICMR, 1993). These, besides limiting the pest populations, also adversely affect the populations of the beneficial non target organisms such as insects and the microorganisms, predatory birds and the natural enemies of the insect pests.

These health and environmental hazards associated with the use of chemical pesticides are of primary concern to international organizations and governments. Therefore, many countries have banned or severely restricted the use of several hazardous chemicals, including some pesticides used to suppress plant diseases. For example, methylbromide, an odorless, colorless gas used as agricultural fumigant to control a wide variety of pests throughout the world, has been reported by the U.S. Environmental Protection Agency (EPA) to deplete the stratospheric ozone layer and is classified as a class I ozone-depleting substance and banned worldwide (EPA, 2006).

Natural products are now being considered as an alternative to the arsenal of synthetic compounds currently available. These products contribute not only to overcome new diseases but also provide challenges to the synthetic chemists (Cutler

and Hill, 1994; Dayan *et al.*, 1999). Increasing public concern on environmental issues requires alternative plant protection and pest management systems, which are less dependent on synthetic pesticides and are based on naturally-occurring compounds (Cuthbertson and Murchie, 2005).

2.5 Alternative pest control strategies

After severe setback arising from the detrimental effects associated with the use of conventional chemical pesticides, a safer approach to protect the crops from these highly devastating polyphagous pests is urgently needed. Consequently, efforts to obtain pesticides from natural sources have gained momentum (Cutler 1994, 1999). These products can be relatively broad spectrum, bioefficacious, biodegradable and environmentally safe and can serve as ideal candidates for crop protectants to be used in agriculture (Fawcett and Spencer 1970; Gilbert, 1977).

Among various natural sources, researchers have turned their interest towards the isolation of bioactive molecules from microorganisms. Their metabolites are structurally unique and highly active in comparison to plant metabolites. After substantial research over the past 60 years, about 28,000 natural products have been isolated from microorganisms. More than 10,000 of these compounds have been found to be biologically active and more than 8000 are antibiotic and antitumor agents (Fenical, 1993; Berdy, 2005; Betina, 1983; Gustafson *et al*., 1983). Lichens, in this context, are also catching the attention of scientists worldwide as a source of bioactive molecules of natural origin.

2.6 Brief account of lichens

Lichens are symbiotic organisms composed of a fungal partner (mycobiont) in association with one or more photosynthetic partners (photobiont). Lichens and their products have been used in traditional medicines for centuries and still hold considerable interest as alternative treatments in various parts of the world (Richardson 1991). The morphology, physiology and biochemistry of lichens are very different from those of the isolated fungus and alga in culture. Lichens are probably the earliest colonizers of terrestrial habitats on the earth, with fossil records tracing back to 400-600 million years ago (Taylor *et al.* 1995; Yuan *et al*. 2005). There are about 300 genera and 18000 species of presently recognised lichens, and they account

for about 20% of all fungi (Galun 1988). DeBary (1831-1888) hypothesized that lichens are symbiotic association (mutualism) between fungi and algae: the symbionts benefit from each other. The photobiont can be green algae, cyanobacteria, or both.

Figure 2.3: Mycobiont – Photobiont association in lichen

This unique symbiotic relationship among such diversified organisms makes them flourish in some of the extreme environmental conditions on earth, they can adapt to extreme temperatures, drought, inundation, salinity, high concentrations of air pollutants, nutrient poor and highly nitrified environments (Müller, 2001; Nash, 2008). Thus, these are found worldwide (covering about 8% of the earth's surface) and their habitat ranges from arctic to tropical regions, from the plains to the highest mountains and from aquatic to xeric conditions. In addition, both fungal and algal cells in the lichen thallus are known for their ability to survive in space (Sancho *et al.*, 2007). They are able to survive in a particular metabolic state for months, due to the slow rate of metabolism and production of many distinctive bioactive molecules which offer chemical protection to them.

According to Conner (2003), lichens grow slowly because the algal and fungal components of the lichens need to grow at similar rates for their survival. If one among them grows faster than the other, the lichens break down. The fungal component of the lichen is very specific about the alga and usually a species of lichen has the same species of alga. Cyanobacteria are particularly useful for lichens living in nitrogen poor environments because the cyanobacteria can fix nitrogen into a form useable by the lichen. In this mutual association, the fungus provides the alga with a more steady supply of moisture, structure and sun protection (presence of the sunscreen pigments). In return, the alga provides the fungus with food. This

association makes the cell wall of the fungus permeable, so that the carbohydrates produced by alga can be absorbed by the fungal tissue.

2.6.1 Morphology of lichens

Lichens are morphologically completely different from their constituent parts, the alga and the fungi. Lichen morphology is extremely diverse; these organisms come in a fantastic array of colors and can vary in size from very small individuals to large structures The morphology of lichens, which is determined by the mycobiont, can be subdivided into two main categories: (Dayan *et al.*, 2001)

1) **Macrolichens** are relatively large in form and easily visible by naked eyes. They include:

   Foliose lichens which are two-dimensional leafy structures, typically with an upper and lower side, and are commonly divided into lobes.

   Fruticose lichens which grow preferentially in wet and humid climates are three-dimensional organisms that stand out from their substrate and are characterized as hair-like, shrubby, moss-like, or pendulous.

   Sqamulose lichens have scales like tiny leaves and sometimes resemble match stick.

2) **Microlichens** include crustose lichens, which have a thallus (flat structure) tightly adhered to the substrate at the lower surface and cannot easily be removed.

2.6.2 Versatile applications of lichens

Lichens have been used in folk medicines for centuries, and their biological activities were recognized by Native Americans, Haitian, Indians, Chinese and Europeans to treat a variety of ailments in their traditional medicines (Dayan and Romagni, 2002). Since Egyptians, lichens were used as dyes, perfumes and remedies in folk medicine (Vartia, 1973). Lichens dyed textile reached considerable economic importance in 18th century in some parts of the world as in the Canary Islands (Muggia *et al*., 2009). Litmus, a blue coloring matter from lichen fermentation, was used as dye for textile and beverages (Beecken *et al.*, 1961). Also, paper strips

impregnated with litmus, a water extracted dyes from *Roccella* sp., are used as pH indicators in laboratories. Extracts of some species of lichens, like *Evernia prunastri*, are used in cosmetics industry as contents of perfumes (Trease and Evans, 1978).

In a study of two lichen species by Munoz *et al.*, 1981, *Parmelia caperata* and *Umbilicaria sp.* are reported as Chilean traditional medicine. *Ramalina thrausta* is used in Finland for treatment of wounds, athlete's foot or other skin diseases and taken to relieve sore throat and toothache (Vartia, 1973). Various species of lichen are known for treating dyspepsia, bleeding piles, diabetes, bronchitis, pulmonary tuberculosis, spermatorrhoea and other diseases of the blood and heart (Richardson, 1988). For instance, New Zealand Maori traditionally use long, pendulous species of *Usnea* for nappies and sanitary pads (Perry *et al.*, 1999). Also, *Usnea* species have been used in Asia, Africa and Europe for pain relief and fever control (Okuyama *et al*., 1995). *Usnea densirostra*, known as "barba de la piedra" served as a cure for various disorders in Argentina's folk medicine (Correche *et al*., 1998). *Cetraria islandica* is ancient cough remedy known as "tonicum amarum" accepted as a mucilage drug (Muller, 2001). Thus, the most important and studied application of lichens is the one in traditional medicine for treatment of animals and humans.

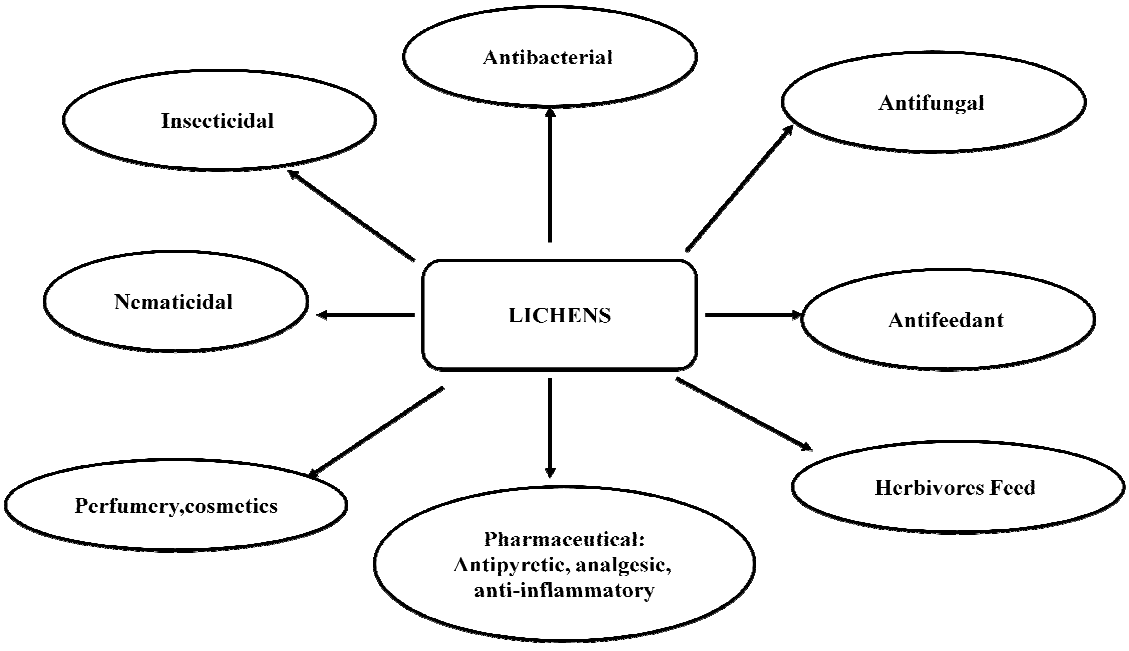


Figure 2.4: Manifold biological activity of lichens

During the last part of the 20th century, numerous studies have established manifold biological activities of lichens (figure 2.5). In a work by Esimone and

Adikwn, 1999a, b; Perry *et al*., 1999 monitored *Ramalina farinacea* and 69 species of lichens from New Zealand and showed their inhibitory effect against various Bacillus species such as *Pseudomonas*, *Escherichia coli*, *Streptococcus*, *Staphylococcus*, *Enterococcus*, *Mycobacterium*. Behera *et al*., (2005) reported that acetone, methanol and petroleum ether extracts of lichen *Usnea ghattensis* were effective against *Bacillus licheniformis*, *B. megaterium*, *B. subtilis* and *Staphylococcus aureus*. Also, Karagoz *et al*., (2009) evaluated antibacterial activity of the aqueous and ethanol extracts of some lichens from Turkey against six pathogenic bacteria namely *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. Ethanol extracts showed better antibacterial activity than the aqueus extracts.

Halama and Van Haluwin (2004) reported that acetone extracts of *Evernia prunastri* and *Hypogymnia physodes* showed a strong inhibitory effect on the growth of plant pathogenic fungi viz., *Phytophthora infestans*, *Pythium ultimum*, and *Ustilago maydis*. Aqueous, ethanol and ethyl acetate extracts of *Alectoria sarmentosa* and *Cladonia rangiferina* were found to have moderate antifungal action against different species of fungi namely *Aspergillus flavus*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Candida albicans*, *Fusarium udum*, *Mucor mucedo*, *Paecilomyces variotii*, *Penicillium purpurescens*, *Penicillium verruc*osum and *Trichoderma harzianum* (Rankovic´ and Mišic´, 2007). Activity was determined against three gram positive bacteria, *Bacillus mycoides*, *Bacillus subtilis*, and *Staphylococcus aureus* and against three gram negative bacteria *Enterobacter cloacae*, *Escherichia coli* and *Klebsiella pneumonia*. Also, in a subsequent report by Ranković *et al*., (2007b) the minimal inhibitory concentrations (MICs) for acetone and methanol extracts of the lichens *Cladonia furcata*, *Parmelia caperata*, *P. pertusa*, *Hypogymnia physodes*, *Umbilicaria polyphylla* and *Umbilicaria cylindrical* were determined. The study revealed that the bacterial inhibitions varied within the lichen extract, solvent used for extraction and bacteria tested. Extracts of *P. pertusa* exhibited braod spectrum antimicrobial activities. *Bacillus mycoides* and *Candida albicans* were the most sensitive microbe species tested.

Paudel *et al.*, (2008) reported a target-specific strong antibacterial activity of methanol extracts from Antarctica lichens namely *Caloplaca regalis*, *Caloplaca* sp., *Lecanora* sp., *Ramalina terebrata*, *Stereocaulon alpinum* against five clinical microorganisms, two gram positive bacteria, *Bacillus subtilis* and *Staphylococcus*

aureus, two gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* and a fungus, *Candida albicans*.

According to Schmeda Hirschmann *et al.*, (2008) dichloromethane and methanol extracts of *Protousnea poeppigii* exhibited strong antifungal effects against the fungal pathogens, *Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. rubrum*. The extracts were also active against the yeasts *Candida albicans*, *C. tropicalis*, *Saccharomyces cerevisiae* and the filamentous fungi *Aspergillus niger*, *A. flavus* and *A. fumigatus* but with much higher strength. In the same assay, extracts of *Usnea florida* also showed strong antifungal properties.

2.7 Chemical screening of lichens

The first studies on "lichen substances" dates back to the origins of organic chemistry. Wilhelm Zopf, a German chemist (Zopf, 1895; and subsequent publications) provided the initial contributions to lichen chemistry. The first review was published as early as 1858 (Gmelin, 1858). Zopf (1907) published a research work on the chemical, pharmacological, and technical aspects of lichen substances. He described about 150 lichen substances based on their chemical properties. In those days little was known about the chemistry of lichen substances, and it was only in the era between 1920 and 1945 that Asahina and Shibata (1954) succeeded in elucidating the structural features of these compounds (Asahina, 1936; Shibata, 1958, 2000; and citations therein). In the second half of the 20th century, thin layer chromatography became a routine technology, and was broadly applied to study the presence of compounds in diverse lichens. The advent of modern analytical techniques, viz., TLC and HPLC, in the 1960s also contributed significantly towards the identification of a large number of lichen substances. This technical improvement helped taxonomists in classifying lichens, many of which differ only in chemical characters. Among the score of literature, particularly important was the publication of Culberson (1969) on the chemistry of lichen substances, followed by two supplementary volumes (Culberson, 1970; Culberson *et al.*, 1977). The number of known substances from lichens increased to more than 800 (Huneck and Yoshimura, 1996; Huneck, 2001). Approximately 1050 secondary compounds have been identified to date (Stocker-Wörgötter, 2008).

2.8 Types of lichen metabolites and their bio activities

According to Asahina's "Chemistry of Lichen Substances", 1973, the lichen substances may be classified into the following groups: Aliphatic lichen substances (includes acids and polyhydric alcohols) and aromatic lichen substances (includes pulvinic acid derivatives, depsides, depsidones, Anthraquinones, xanthone derivatives). Lichens secondary metabolites are usually insoluble in water and can be extracted into organic solvents. They amount to between 0.1% and 10% of the dry weight of the thallus. The following types of metabolites are found:

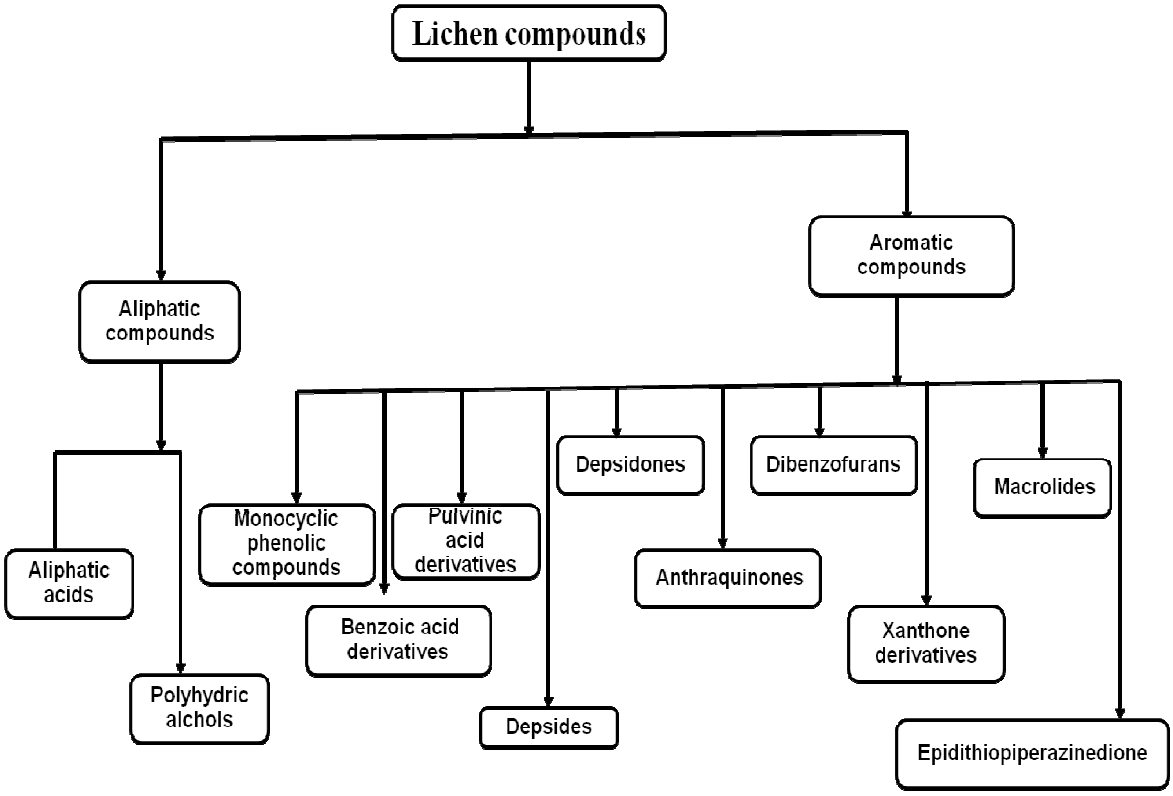
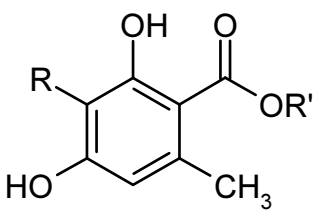


Figure 2.5: Types of lichen compounds

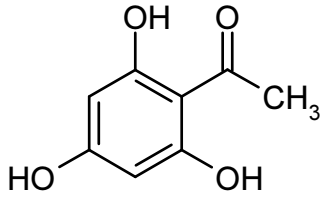
2.8.1 Mononuclear phenolic compounds from lichen

Mononuclear phenolic compounds comprise methyl orsellinate (1) (orsellinic acid), phloroacetophenone (2) and phthalide derivatives (3). The lichen derived mononuclear phenolic compounds such as methyl orsellinate (1a) and ethyl orsellinate (1b) display antibiotic activity against *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Candida albicans* (Ingolfsdottir *et al*., 1985; Fujikawa *et al*., 1970). Among other monocyclic phenolic compounds, Methyl hematommate (1c) and Ethyl hematommate (1d) showed activity against *Epidermophyton floccosum, Microsporum canis, M. gypseum, Trichophyton rubrum,*

T. mentagrophytes, Verticillium achliae; (Hickey *et al*., 1990). They are rarely found in lichens and are produced by the partial hydrolysis of depsides.



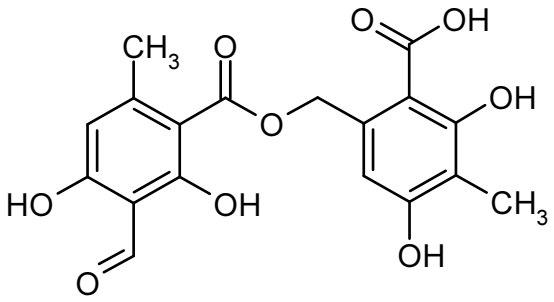
(1a) R=H, R'=CH_3
(1b) R=H, R'=C_2H_5
(1c) R=CHO, R'=CH_3
(1d) R=CHO, R'=C_2H_5



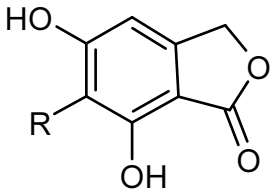
(2)

2.8.2 Phthalide derivatives

Several phthalide derivatives, like 5, 7-dihydroxy-6- methylphthalide (4a) and 5, 7-dihydroxy-6-formylphthalide (4b), were isolated together with alectorialic acid (3) from the lichens *Alectoria nigricans* and *A. capillaries* (Solberg, 1970).



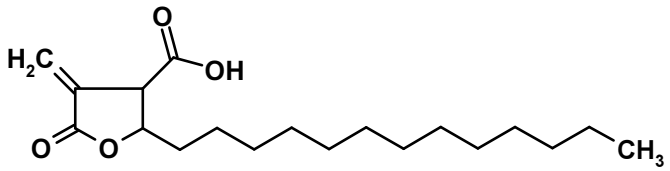
(3)



(4a), R=Me
(4b), R=CHO

2.8.3a Aliphatic acids

Besides the generally wide-spread fatty acids, many lichens accumulate fatty acids with branched chains and 17, 19, or 20 carbon atoms. Unsaturated acids such as arachidonic acid have also been detected in lichen extracts (Yamamoto and Watanabe, 1974). The prototype of monobasic acids is protolichesterinic acid (5), an aliphatic α-methylene butyrolactone. Protolichesterinic acid is chemically unstable and easily rearranges into its tautomeric form, lichesterinic acid, which has the double bond at the α-carbon within the lactone ring.



(5)

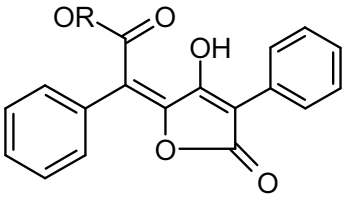
2.8.3b Polyhydric alcohol

The high tolerance of lichens at sub zero temperature is because of the osmotic activity of sugar alcohols. Polyols help lichens to maintain their physiological activity by protecting lichen thallus from desiccation at subzero temperatures. Natural concentration of polyols in lichen thalli are species specific and depend on the site of collection (Roser *et al*., 1992b). Actual amount of these sugar alcohols varies with orientation of the thallus and leaching due to rainfall (Dudley, 1987) Mannitol, ribitol and arabitol are abundant in large quantities in lichen thallus (da silva et al., 1993, chapman *et al*., 1994).

2.8.4 Pulvinic acid derivatives

Numerous lichens contain yellow- or orange-colored pigments that are composed of two phenylpropane units, in which the C_3 unit of these lichen-derived acids is isopropyl rather than propyl. A study conducted by Foden *et al*., 1975, derivative of vulpinic acid (7), a methyl ester of the prototype pulvinic acid (6), were evaluated as antiinflammatory agents in the adjuvant arthritis test in rats. Abo-Khatwa *et al*., (1996) showed that vulpinic acid itself possess antimicrobial activity against aerobic and anaerobic microorganisms (*Drechslera rostrata, Alternaria alternata)*. It induces uncoupling of oxidative phosphorylation (Lauterwein, 1995; Raju and Rao, 1986a, b; Raju *et al.*, 1985; Rao *et al.*, 1989).

Emmerich *et al*., (1993) and Ahad *et al*., (1991) have studied the antifeedant activity and acute toxicity of vulpinic acid against the highly resistant polyphagous, herbivorous insect *Spodoptera littoralis.* The metabolite retarded the growth as well as increased the larval period (delayed the pupation) in a dose-dependent manner. The slug-repelling activity of vulpinic acid is being investigated as a seed-treatment for protecting higher plants. According to Hesbacher *et al.*, (1995) application of lichen metabolites in insects may be utilized for chemical defense.

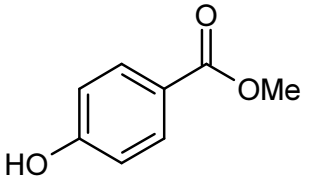


(6) R=Me (7) R=H

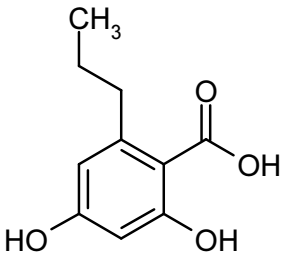
2.8.5 Hydroxybenzoic acid derivatives

Esters of 4-hydroxybenzoic acid such as methyl paraben (8) are used as preservatives in pharmaceutical preparations. As these agents are rapidly hydrolyzed *in vivo* to the corresponding acid, which is then conjugated and excreted, their toxicity is generally low (Wilson *et al.*, 1998). Some of the lichen derived benzoic acid derivatives, Divaric acid and divaricatinic acid (9, 10), 2-methoxy-4-hydroxy-3, 6-dimethyl benzoic acid (11) and 2-Hydroxy-4-methoxy-3, 6-dimethyl benzoic acid (12) are shown below.

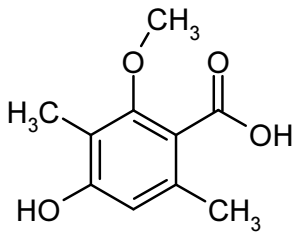
Schmeda Hirschmann *et al.*, (2007) studied antifungal activity of extracts of Andean lichens *Protousnea poeppigii* and *Usnea rigida* against *Microsporum gypseum*, *Trichophyton mentagrophytes* and *T. rubrum*. The main constituents, divaricatinic acid, isodivaricatic acid, and 5-propyl resorcinol also displayed activity against these fungi.



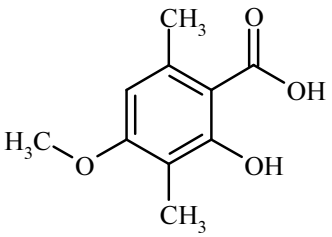
(8)



(9) R=H (10) R=CH_3



(11)



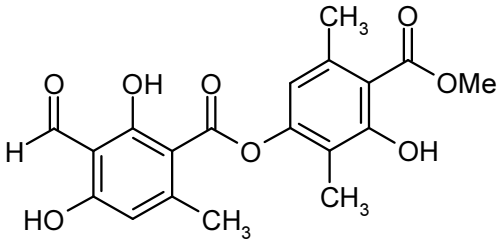
(12)

2.8.6 Depsides

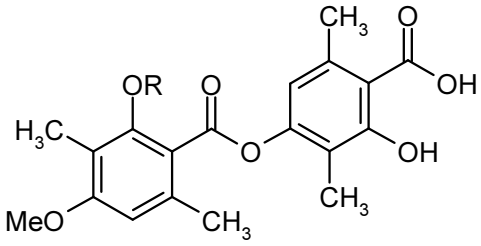
A distinct class of lichen metabolites is the depsides. These types of compounds are formed by condensation of two or more hydroxybenzoic acids whereby the carboxyl group of one molecule is esterified with a phenolic hydroxyl group of a second molecule. These are two different types, with either an orcinol moiety and with a β-orcinol moiety (Asashina and Shibata, 1954). Of the β-orcinol depsides, atranorin (13), diffractaic acid (14) and barbatic acid (15) are moderate inhibitors of arachidonate 5-lipoxygenase (Kumar and Müller, 1999a, b). Accordingly, novel analogues of lichen depsides were synthesized to improve their activity. Kumar and Müller, (1999a, 2000) found that among the depsides, ethyl 4-*O*-demethyl barbatate (16) and propyl 4-*O*-demethyl obtusate (17) turned out to be the most potent inhibitors, with IC_{50} values in the submicromolar range. Kumar and Müller, (1999c) studied several lichen metabolites; the tridepside gyrophoric acid (18) was a potent inhibitor of the growth of human keratinocytes. The activity may be related to its ability to interfere with electron transport systems (Rojas *et al.*, 2000).

Use of lichen metabolites as potential source of herbicides was studied by Dayan (2001). The depsides barbatic acid (15), lecanorin and the tridepside gyrophoric acid (18) act by interrupting photosynthetic electron transport in isolated chloroplasts (Nash, 1996). Lawrey, (1989) studied antibacterial activity of evernic acid (16a), leprapinic acid derivatives like leprapinic acid glycinamide against gram-positive and gram-negative bacteria (Raju *et al*., 1985).

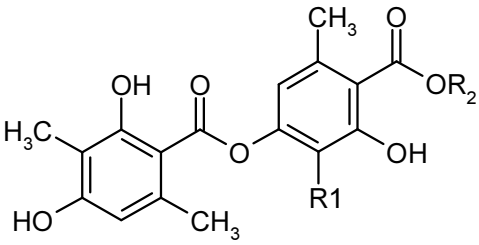
In a study by Rankovic´ *et al.*, (2008) isolated Atranorin (from *Physcia aipolia*), fumarprotocetraric acid (from *Cladonia furcata*), gyrophoric acid (18) isolated from *Umbilicaria polyphylla*, lecanoric acid (17a) from *Ochrolechia androgyna* showed strong antimicrobial effects against six plant and human pathogenic bacteria and ten food spoiling fungi.



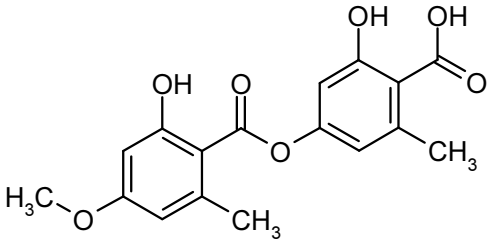
(13)



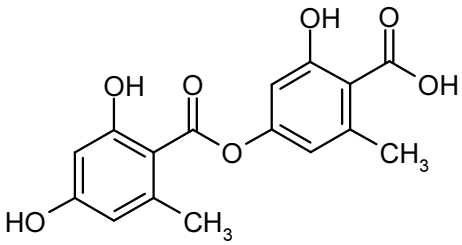
(14) R=Me
(15) R=H



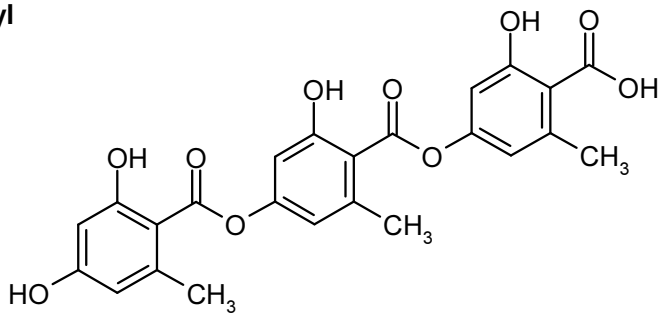
(16) R1=Me, R2=Et
(17) R1=H, R2=Propyl



(16a)

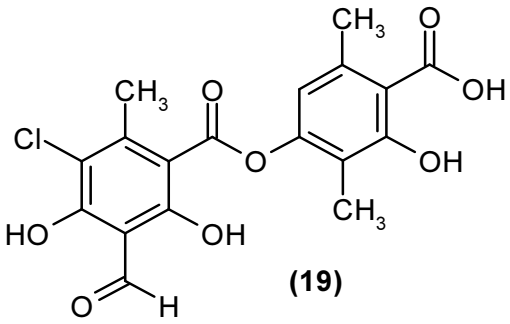


(17a)

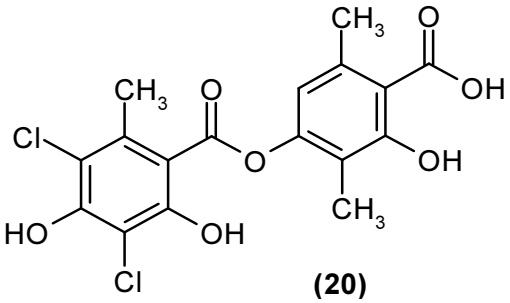


(18)

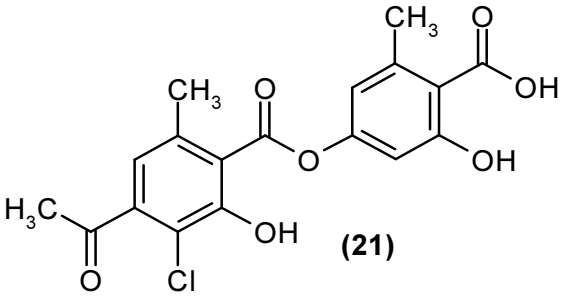
Among several halogenated depsides in lichens, chloroatranorin (19), tumidulin (20), wrightin (21), other depsides (23-28) were isolated from *Pseudocyphellarie pickeringii*, (22) isolated from the lichen *Lecanara sulpurella,* 3-chlorodivaricatinic acid (23), 3-chlorostenosporic acid (24), 3-chloroperlatolic acid (25) were isolated from *Thelomma mammosum* and *Dimelaena* spp, are reported. Among them, Diploicin (26) was isolated from an unidentified species by Zopf (1904).



(19)



(20)



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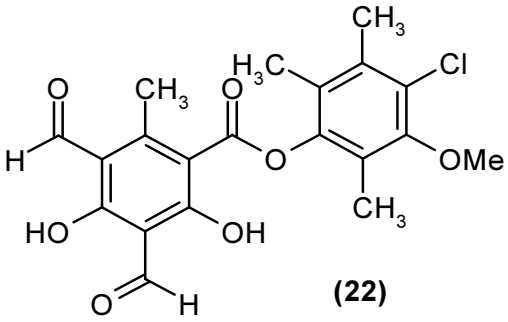
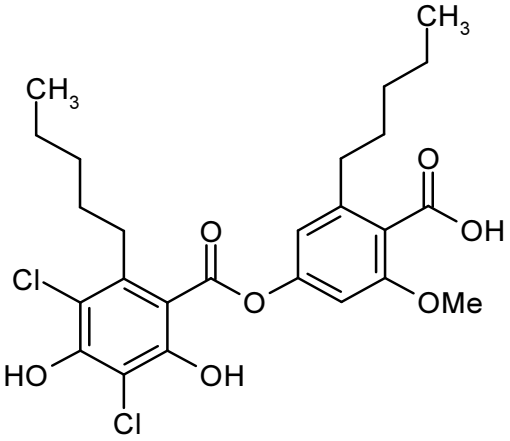
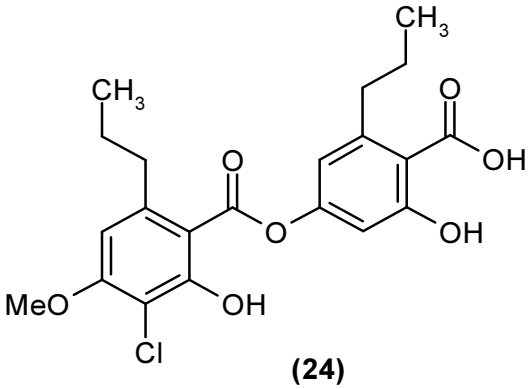


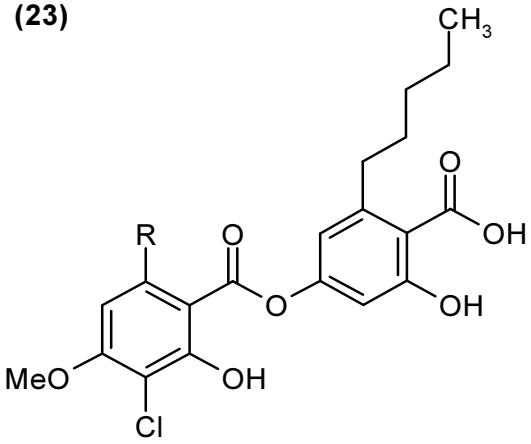
(22)



(23)



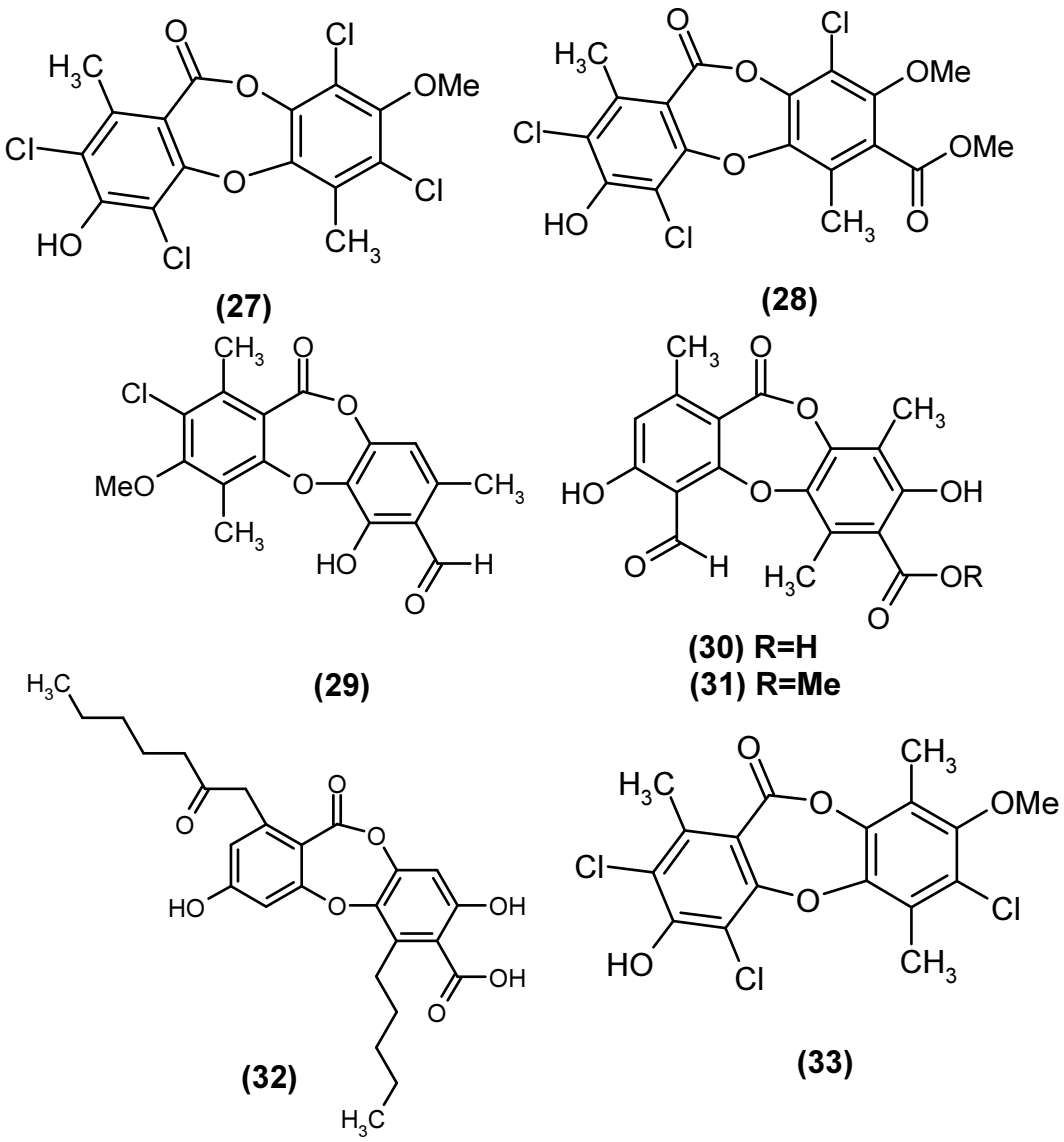
(24)



(25) R=n-C_3H_7
(26) R=n-C_5H_{11}

2.8.7 Depsidones

Depsidones have an ether linkage in addition to the ester linkage of the depsides, resulting in a rigid polycyclic system. Therefore, they are based on an 11*H*-dibenzo [*b*,*e*][1,4]dioxepin-11-one ring system. This chemical feature was shown to be important for the inhibitory activity of this class of lichen metabolites against HIV-1 integrase (Neamati *et al*., 1997).



The first representative of depsidones, diploicin (27), was isolated in 1904 by Zopf from an unidentified species of lichen (Huneck and Santensson, 1969). Subsequent research showed that it is present in *Buellia canescens* and *Lecidea cargaleoides* (Culberton, 1963, Bendz *et al*., 1965a, b). Gangaleoidin (28) was

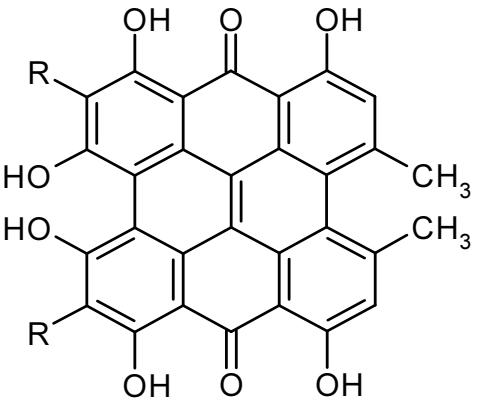
discovered in *Lecanora gangaleoides* lichen. Pannarin (29) was isolated from the lichens of Pannaria genus: *P. lanuginosa*, *P. fulvescens*, and *P. lurida* (Elix *et al.*, 1987a, b), *P. pityrea*, *P. rubiginosa*. Pannarin (29), together with the depside atranorin (13), were shown to possess photoprotector capacity and thus may be useful agents to protect against damaging effects of Ultra Violet light (Fernández *et al.*, 1998a, b).

While the open-chain depsides were generally inactive, the β-orcinol depsidones virensic acid (30) isolated from *Alectoria tortuosa* Merr, its methyl ester granulatine (31) and the orcinol-type depsidones physodic acid (32) was found active in the low micromolar range (Neamati *et al.*, 1997). The lichen of Caloplaca sp. is found to include caloploicin (33), the structure of which was confirmed by synthesis (Elix *et al.*, 1986 and Jackman *et al*., 1979).

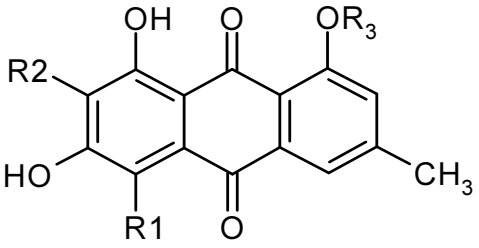
2.8.8 Anthraquinones, naphthoquinones and related compounds

Anthraquinones are widely distributed in nature, and they are particularly prominent in higher plants and fungi (Thomson, 1987). Also, they are important constituents of lichens (Cohen and Towers, 1995, 1996). Several active anthraquinones have been isolated and characterized from some species of the lichen genus *Xanthoria*. Anthraquinones are of interest as antiviral agents against HIV (Schinazi *et al.* 1990), in particular, hypericin (38) is of pharmaceutical relevance because of its remarkable antiretroviral activity (Lavie *et al*., 1989). The derivative 7,7′-dichlorohypericin (39) and the anthraquinone 5,7-dichloroemodin (42) isolated from the lichen *Heterodermia obscurata* (Cohen and Towers, 1995), were shown to exhibit strong inhibitory activity against herpes simplex virus type 1 (Cohen *et al*., 1996). Partial inactivation of the virus was shown by emodin (39), 7-chloroemodin (40) and 7-chloro-1-O-methylemodin (43)**.** Furthermore, derivatives of emodin and chrysophanol (44), which were found in the lichen *Asashinea chrysantha* (Mishchenko *et al.*, 1980), exhibited anticancer activity against leukemia cells in a dose-dependent manner (Koyama *et al.*, 1989). Emodin (39) and its analogues also exhibit strong herbicidal activity on grasses, causing malformation

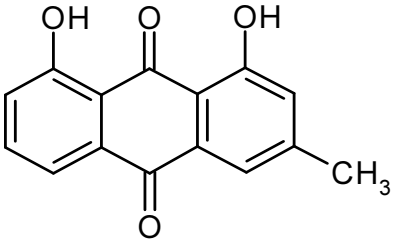
and bleaching in early seedlings. Emodin and physcion was found active against *Bacillus brevis* (Anke *et al*., 1980a, b)



(38) R=H
(39) R=Cl



(40) R1=H,R2=H, R^3=H
(41) R1=H, R2=Cl, R$_3$=H
(42) R1=Cl, R2=Cl, R$_3$=H
(43) R1=H, R2=Cl, R$_3$=Me



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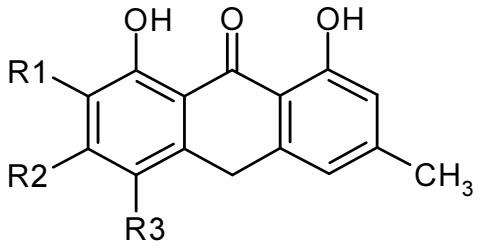
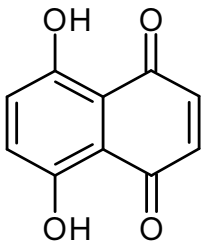
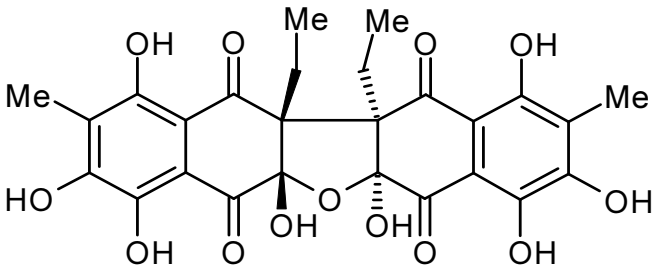


(45) R1=H, R2=OH, R3=H
(46) R1=Cl, R2=OH, R3=H
(47) R1=Cl, R2=OH, R3=Cl
(48) R1=H, R2=H, R3=H



(49)



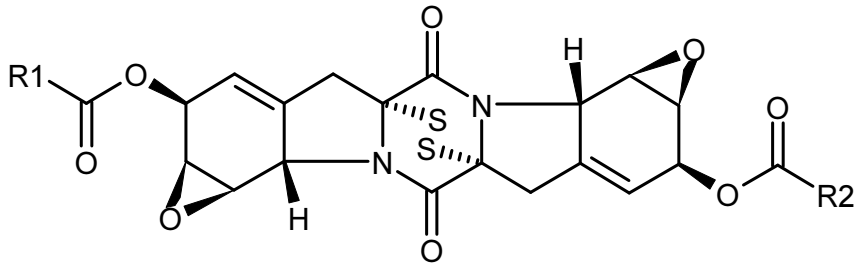
(50)

The reduced form of anthraquinones, the anthrone class of compounds, have been detected in several lichen species (Huneck, 1971) like the 2-chloro- and 2, 4-dichloro-substituted derivatives of emodin anthrone (46, 47). The antipsoriatic anthrone chrysarobin (48) is the reduced form of chrysophanol (Müller *et al*., 1994).

The naphthoquinone naphthazarin (49), derivatives of which have been isolated from *Cetraria islandica* (Stepanenko *et al*, 1997), was found to have cytotoxic activity against human epidermal carcinoma cells (Paull *et al.*, 1976). A dimer of naphthazarin, the highly substituted pentacyclic hybocarpone (46) was isolated from mycobiont cultures derived from the lichen *Lecanora hybocarpa*, was shown to be a potent cytotoxic agent against a murine mastocytoma cell line. Also, the naphthopyrone euplectin (50), a lichen metabolite from *Flavoparmelia euplecta*, exhibited moderate activity in this assay (Ernst-Russell *et al.,* 2000).

2.8.9 Epidithiopiperazinediones

Recently, compounds that contain an epidithiopiperazinedione moiety have been identified in lichenized fungi. Several scabrosin esters (51-55) from the lichen *Xanthoparmelia scabrosa* were found to exhibit potent cytotoxic activity against murine P815 mastocytomia cells and human breast MCF7 carcinoma cells (Ernst-Russell *et al*., 1999a).



(51) R1= Me, R2=Me
(52) R1=Me, R2=n-prop
(53) R1=n-prop, R2=n-prop
(54) R1=Me, R2= n-pent
(55) R1=n-prop, R2= n-pent

2.8.10 Dibenzofuran derivatives

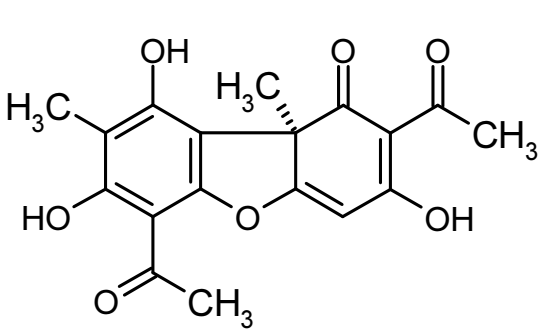
These compounds are quite unusual in nature and are produced almost exclusively by certain lichen species such as *Cladonia* spp., *Sterercaulon* spp., *Lepraria* spp., *Haematomma* spp, *Lecidea* spp. and *Roccellina* spp. Usnic acid (56) is the major and most studied metabolite of this group. Usnic acid is known to have broad spectrum activity against viruses, microbes, protozoans, insects and fungi (Ingólfsdóttir, 2002). It also absorbs efficiently in the ultraviolet (UV) range of the

spectrum (Rancan *et al*., 2002), and therefore probably renders some protection against UV-B radiation (Bjerke *et al.*, 2002; Buffoni Hall *et al*., 2002).

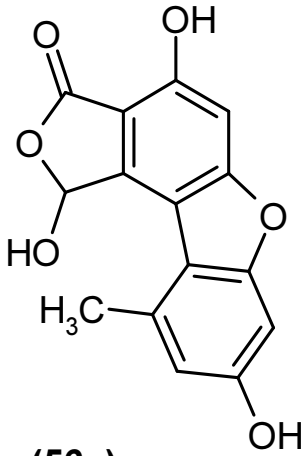
In a study by Rankovic´ *et al.*, (2008) usnic acid isolated from *Flavoparmelia caperata* showed strong antimicrobial effects against six plant and human pathogenic bacteria and ten food spoiling fungi. The activity of usnic acid was found to be better than standard streptomycin.

Emmerich *et al*., (1993) and Ahad *et al*., (1991) have explored usnic acid against the polyphagous and herbivorous insect *Spodoptera littoralis* and observed that usnic acid demonstrated acute toxicity and feeding deterrence to insect larvae. It retarded the growth as well as increased the larval period (delayed the pupation) in a dose-dependent manner. Both (±) usnic acid, exhibited strong larvicidal activity against the larvae of the house mosquito *Culex pipiens* in a dose-dependent manner (Cetin *et al.*, 2008). According to Hesbacher *et al.*, (1995) application of lichen metabolites in insects may be utilized for chemical defense.

One interesting aspect of the allelopathic potential of lichens is related to the ability of (–)-usnic acid, to inhibit carotenoid biosynthesis through the enzyme 4-hydroxyphenylpyruvate dioxygenase (HPPD). The *in vitro* activity of usnic acid is superior to that of other synthetic inhibitors of this herbicide target site (Dayan, 2001). Gollapudi *et al.*, (1994) isolated a novel antimicrobial dibenzofuranoid lactol (alectosarmentin) from *Alectoria sarmentosa*, which was found active against *Staphylococcus aureus* and *Mycobacterium smegmatiti*



(56)

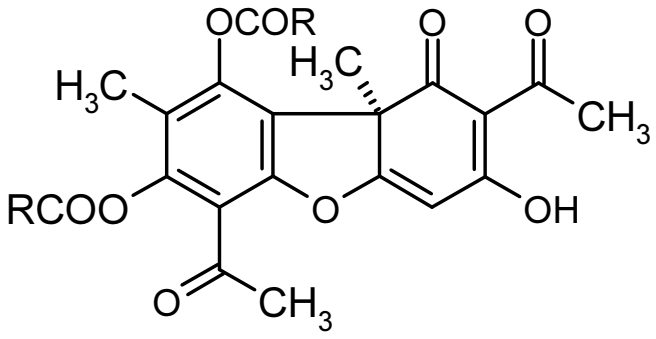


(56a)

Synthesis of usnic acid derivatives

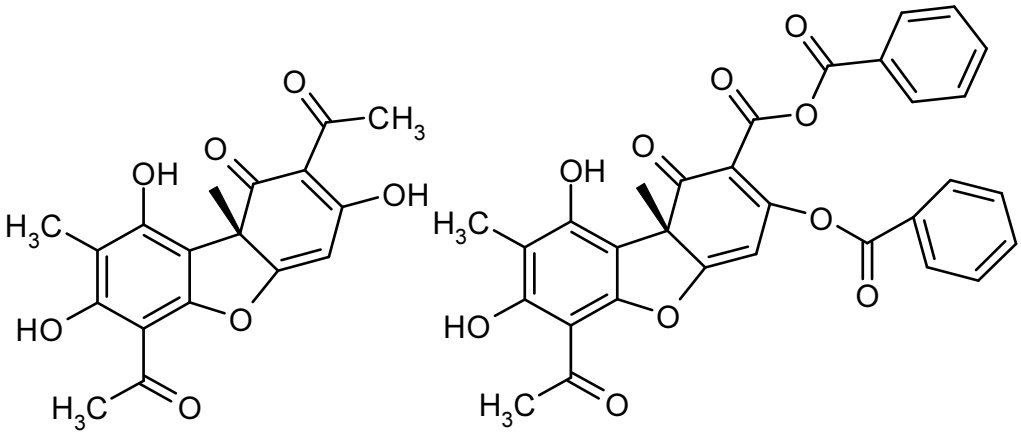
The many functional groups of usnic acid make the molecule a good target for structural modification. The compound reacts with amines, hydrazines and acyl hydrazides to form condensation products, undergoes esterification, gives numerous degradation derivatives and forms dihydrousnic acid on reduction. Usnic acid salts are usually unstable. A large number of studies from 1962 to 1985 by the work of Japanese researchers on the chemistry of usnic acid and reaction mechanisms were conducted (Takani and Takahashi, 1985).

Erba *et al.*, (1998) synthesized dipropionate derivative of usnic acid to develop new derivatives for pharmacological evaluation. Preparation of new esters was investigated starting from *R* (-) usnic acid. Only the monoacetate and the diacetate esters (57a) of usnic acid have been described and prepared by reaction of usnic acid (56) with acetic anhydride, in the presence of an acidic catalyst. By a similar procedure, dipropionate was prepared (57b) in a 60% yield. A better yield of 2b (80%) was obtained by using propionyl chloride and pyridine as acylating reagents. Similarly, starting from chloroacetyl chloride, compound 57c was produced in 82% yield.



57a: R=CH_3
57b: R=C_2H_5
57c: R=CH_2Cl

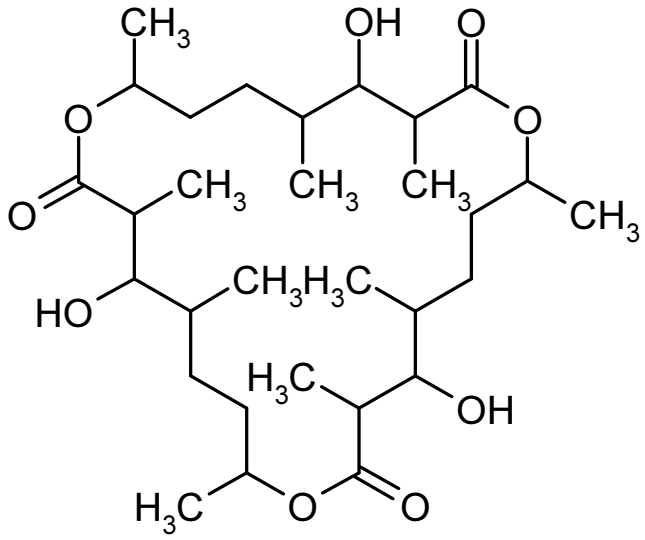
Derivatization aimed at obtaining enhanced biological activity has been attempted by many workers. Shibata *et al.*, (1948) found that antitubercular activity decreased with acetylation of the two hydroxyls in ring A as well as with dihydro-usnic acid.



R (+) Usnic acid **Dibenzoylusnic acid**

2.8.11 Macrolides

Macrolides (large ring compounds), representing aliphatic polyketides, are often formed from 9 units (acetyl-CoA and 8 units of malonyl-CoA). So far, many different macrolides of various complexities have been found in prokaryotic and eukaryotic organisms, and recently a new macrolide was found in lichens. Dasypoga-lactone (59) is unusual in being a 24 membered trilactide, named after the lichen *Usnea dasypoga* (now *Usnea filipendula*) and can be recognised as white spots after spraying with sulfuric acid in TLC analyses, and crystallises as white needles. (Suwarso *et al.*, 1999)



(59) Dasypoga-lactone

2.9 Advanced studies on lichens

Lichen studies are primarily limited due to the difficulties observed in their occurrence in extreme habitats and because of their slow growth. In recent decades, improvement in tissue culture conditions for lichen forming fungi has achieved a remarkable progress which has evaded the difficulties encountered with collecting substantial amounts of lichen materials from nature. Ahmadjian, (1974) for the first time suggested the way for isolation of mycobiont using ascospores discharged from the fruit bodies. Since then, spore isolations were conducted by many researchers with different purposes (Ahmadjian and Jacobs, 1987; Stocker-Wörgötter and Türk, 1991, 1993; Culberson *et al.*, 1992; Ahmadjian, 1993; Honegger *et al.*, 1993, Crittenden *et al,.* 1995, Stocker-Wörgötter, 1995, 1997, 1998; Molina and Crespo, 2000; Stocker-Wörgötter and Elix, 2002). In contrast to the fast growth of non- lichen fungi in culture, slow growth of lichen fungi renders difficult to study them as axenic cultures. However, later, this was circumvented by Yamamoto *et al.*, (1985) who introduced a faster 'tissue culture-method' for the regeneration of lichen fragments that enables larger amounts of lichen material to be formed within a shorter period of time. However, it should be noted that lichen metabolites produced in mycobiontic cultures are not always identical to those produced from lichens themselves (Brunauer *et al.*, 2007; Yoshimura *et al.*, 1994, Muller, 2001). Mycobionts grown without their photobionts synthesize specific secondary lichen compounds under certain conditions (Culberson and Armaleo, 1992; Fazio *et al.*, 2007; Hager *et al.*, 2008; Mattsson 1994; Stocker-Wörgötter and Elix, 200. Consequently, the number of lichen compounds is much higher than that found in previous literature sources (Culberson and Elix, 1989; Elix, 1996; Elix and Stocker-Wörgötter, 2008; Galun and ShomerIlan, 1988; Huneck, 1999; Huneck and Yoshimura 1996; Lumbsch, 1998). The large increase is due to the fact that, previously, only "natural" substances occurring in intact lichen thalli were counted, but now, substances identified from cultures are also being included.

2.10 Distribution pattern of lichen metabolites

It has been shown that the distribution pattern of secondary metabolites is greatly affected by varying conditions of climatic, availability of nutrients, substrates, pH, temperature, light, humidity, stress, topography and varied altitude of the area. The first detailed field survey of the spatial variability of levels of lichen secondary

metabolites (usnic acid levels) with varying climatic conditions was reported by Bjerke *et al.*, (2004). The study showed that the usnic acid concentrations in the arctic lichen *Flavocetraria nivalis* vary considerably between sites along local longitudinal and altitudinal transects, and the variability is correlated with diverge climatic factors, with the coldest sites having the highest concentrations of metabolites. Thus, the trend towards higher concentrations of usnic acid in colder environments may be a protective response against freezing stress (Kappen *et al*., 1996; Avalos and Vicente, 1987; Longton, 1988; Rikkinen, 1995; Huneck, 1999). The study confirmed that the usnic acid levels are altered by numerous stimuli, among which low temperatures and humidity are important factors for usnic acid synthesis. As for usnic acid, there may be some abiotic or biotic differences on microscale causing the high variability. Previous studies along spatial gradients in the field have included comparatively fewer sites with lower control of environmental variables, and have therefore not been able to detect such relationships. The positive correlations with altitude found in some lichen studies was considered as an effect of higher Ultra violet radiation fluxes at high altitudes (Fernández *et al.*, 1998; Quilhot *et al.*, 1998; Bjerke *et al.*, 2002; Rubio *et al.*, 2002). The above study however contradicts this idea and suggests the production of usnic acid may be instead related to the lower temperatures at higher altitudes.

2.11 Descriptive details of the test lichens and test organisms

A detailed survey of literature revealed, that despite, manifold pharmaceutical activities of lichens, studies on potential of lichen metabolites as agrochemicals are scarce. It is also revealed that metabolites production is dependent on certain parameters like the extreme environmental conditions, high altitudes, extreme temperature and varied climate. In addition, the Indian Himalayan region is well known for the richness and diversity of valuable but highly sensitive species of lichens (Kala, 2009). So the present work was undertaken aiming at the survey and collection of lichens from the under collected habitats of higher ridges of Himalayas. These lichens are found upto an altitude of 1000-2000 m in the vicinity of various forest ranges. For the present work, four lichen species namely *Parmelia reticulata* Tayl., *Ramalina roesleri* Nyl., *Usnea longissima* Ach. Articus

and *Stereocaulon himalyense* Lamb were collected from Kilberi forest ranges (Northern Himalayas) in the Uttaranchal State (Uttarakhand). These species are typically found in extreme stressful environment where any other forms of life can't exist.

Parmelia reticulata **(caperata)** is a medium to large, loosely adnate foliose lichen that has a very distinctive pale yellow green upper cortex when dry. The rounded lobes, measuring 3–8 mm (0.1–0.3 in) wide, usually has patches of granular soredia arising from pustules. The lobes of the thallus may be smooth, but quite often have a wrinkled appearance especially in older specimens. The lower surface is black except for a brown margin; rhizines attached to the lower surface are black and unbranched.

Ramalina roesleri is characterised by fruticose thalli, the lichen genus Ramalina Ach (Ascomycetes; Lecanorales, Ramalinaceae) (Hale, 1983). There are about 200 species of Ramalina worldwide (kirk *et al*., 2008) In Ramalina, the thalli, attached to the substrate by a basal holdfast that are erect, tufted (Kashiwadani and Kalb, 1993), and irregularly branched. Ramalina grow preferentially in humid climates and its stand out from the substrate and appear as hair-like, shrubby moss. Fresh thalli of Ramalina are usually gray, greenish gray to yellowish-gray to dark brown when dried. Their branches are much closer to the true branches of plants, however unlike most plants; the lichen branch has no specialized vascular system for transporting fluids (Dayan *et al.*, 2001).

Usnea longissima **(now *Dolichousnea longissima*)** is fruticose lichen with very long stems and short side branches. It is considered as the longest lichen in the world. It is generally found hanging from the tree branches, resembling grey, greenish hair. It's also called as old man's beared. The most common host of this lichen is spruce trees. It is currently comes under threatened species (Source: www.wikipedia.org/wiki/*dolichousnea longissima*).

Stereocaulon himalayense is characterized by an upright growth of primary thallus, which is called as 'pseudopodetium' and the genus was segregated into its own family Stereocaulaceae. The pseudopodetia attached to the substratum (rocks)

usually are erect and simple or branched. Abundant apothecia (reproductive bodies of lichen thallus) can be seen lateral or terminal to the lichen that is more or less rounded and brown coloured.

The solvent extracts and isolated metabolites of these lichens were evaluated for their agrochemical potential against following test organisms:

Plant Pathogenic fungi

Fungal phytopathogens pose serious problems worldwide in the cultivation of economically important plants, especially in the subtropical and tropical regions and thereof pose threat for global food security (Brimner and Boland, 2003; Strange and Scott, 2005). Phytopathogenic fungi are difficult to control because their populations are variable in time, space and genotype. Among various fungal phytopathogens soil borne fungi such as *Rhizoctonia bataticola*, *R. solani*, *Fusarium udum*, *Pythium aphanidermatum*, *P. debaryanum* and *Sclerotium rolfsii* are the main pathogens responsible for the diseases and causes high yield losses (Table 2.3) (Park *et al*., 2005; Pereira *et al*., 2007; Shenoy *et al*., 2007; Soares and Barreto, 2008; Than *et al*., 2008a, b).

In addition, most of them produce mycotoxins, which are harmful to humans and livestock. To a certain extent, pathogens can be controlled by use of suppressive soil, crop rotation and removal of potential inocula, but all these have a limited effect. Alteration in agricultural practices, such as monoculture cropping and the use of synthetic fertilizers and pesticides, have led to a decline in soil structure and an increase in soil borne plant diseases (Bailey and Lazarovits, 2003). The soil-borne pathogens can rapidly colonize even in pasteurized soil and can invade crops through seeds, roots, stalks and cause substantial losses (Wheeler and Rush, 2001; Stephens *et al*., 1983). Table 2.2 summarizes diseases caused by some important fungal pathogens on various crops. Therefore, these fungi were chosen for evaluating the antifungal potential of lichens species under the study.

Table 2.3: Major phytopathogens and the diseases caused

Phytopathogen	Major crop host	Disease caused
Fusarium udum	Pigeonpea	*Fusarium* wilt
Pythium debaryanum	Tomato, Onion, Peas, Cabbage, Chickpea	Foot rot/damping-off, Root rot, Damping off, Powdery mildew, Seedling blight
Rhizoctonia bataticola	Groundnut/oil seed crops, Cotton, Pigeonpea	Dry root rot/dry wilt Root rot
Rhizoctonia solanii	Potato	*Rhizoctonia* stem lesion
Sclerotium rolfsii	Lentil, Groundnut, Onion, Gram, Rice (nursery bed)	Collar rot, Stem rot *Sclerotium* wilt, White rot, Collar rot of gram, Seedling blight

Weeds

Weeds are the next main agricultural yield limiting factors in most parts of the world. Weeds are any undesirable plants grown in field along with the main crop plant. In particular, *Phalaris minor* Retz has been identified as a serious weed of wheat and barley fields in India, USA, Canada, Africa, Australia, France, Pakistan, Iran, Iraq and Mexico (Holm *et al.*, 1979; Shad and Siddiqui, 1996). Furthermore, a rice wheat crop rotation also stimulated its emergence, growth and development (Chhokar and Malik, 1999). Weed seeds of many weed species may be killed during the summer or by rainy season flooding under rice cultivation, *P. minor* remains unaffected due to an impermeable seed coat. The weed is highly competitive and can cause significant yield losses under favourable conditions. Depending upon density of *P. minor*, 10-65% yield losses (in India) in wheat have been reported by various workers (Mehra and Gill, 1988; Dhaliwal *et al.*, 1997; Dhima and Eleftherohorinos, 2003; Chhokar *et al.*, 2008). Due to morphological similarity of *P. minor* with wheat, manual weeding at early stages is difficult.

Microalgae

Microalgae are the first algae emerged over 2 billion years ago and first to carry out photosynthesis and converted earth's oxidizing atmosphere into reducing one. Algae are of particular interest for studies because they inhabit wide range of

ecological habitats. They are well endowed with effective protective mechanisms against different biotic stresses and have remarkable adaptability to varying environmental conditions (Jabir, 2009). Another major global aspect is determining the effects of these algae in natural fresh water ecosystem, because of the continuing widespread growth of them. Although algae don't influence the agriculture directly but may hinder the agricultural yield in many indirect ways. By covering the water surface, they alter the life cycle of aquatic flora and fauna, also, disturbs the irrigation requirements of any field.

Microalgae used in the study namely *Chlorella vulgaris*, *Chlorella sorokiniana* and *Scenedesmus subspicatus* belongs to the chlorophyceae family of algae. It's a large and important group of freshwater green algae. The Chlorophyceae are one of the classes of green algae, distinguished mainly on the basis of ultrastructural morphology. Chlorophyceae algae include some of the ecologically important species of algae like *Chlorella vulgaris* and *Chlorella sorokiniana*. *Chlorella* is a genus of single cell green algae, belonging to the phylum 'Chlorophyta'. It is spherical in shape, about 2 to 10 μm in diameter, and is without flagella. *Chlorella* contains the green photosynthetic pigments chlorophyll a and b in its chloroplast. It requires only carbon dioxide, water, sunlight and a small amount of minerals to reproduce. *Scenedesmus* is another genus of the chlorophyceae group. These are usually colonial and non motile. These green algae usually have a rigid cell wall of an inner layer of cellulose and outer layer of pectose.

Materials & Methods

3.1. Materials

3.1.1 Lichen samples

Samples were collected from bark of trees, shrubs and on rocks of Kilberi forest ranges (Northern Himalayas) in the Uttaranchal State (Uttarakhand), (Figure 3.1) India and air-dried. A specimen of *Parmelia reticulata* Tayl., *Ramalina roesleri* Nyl, *Usnea longissima* Ach.Articus and *Stereocaulon himalyense* Lamb (Figure 2) was deposited in the herbarium of the Department of Botany, University of Delhi, Delhi, India. The voucher numbers for the lichen species are *Parmelia reticulata* Tayl, (DUH 13531) *Ramalina roesleri* Nyl, (DUH13532) *Usnea longissima* Ach. Articus, (DUH 13533) *Stereocaulon himalayense* Lamb (DUH 13534).

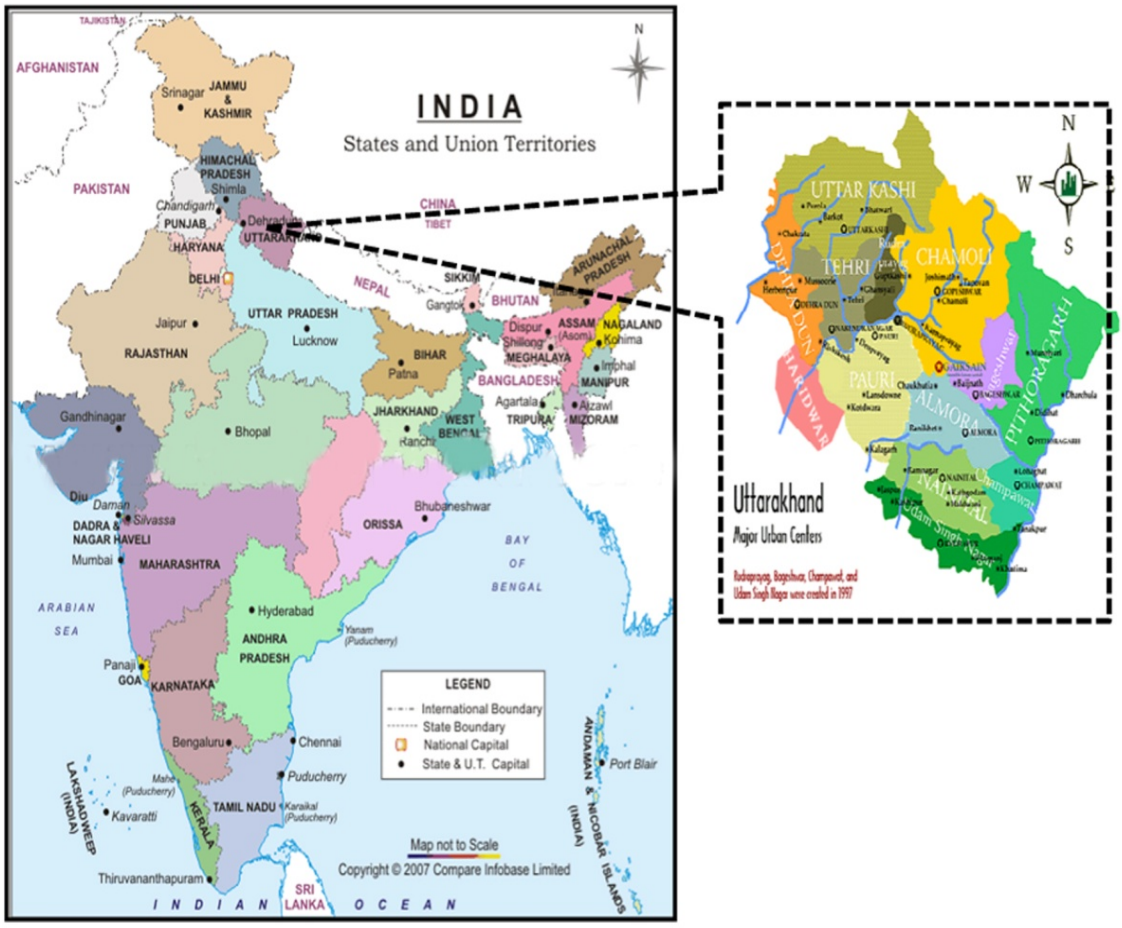


Figure 3.1: Sampling sites for collection of lichen

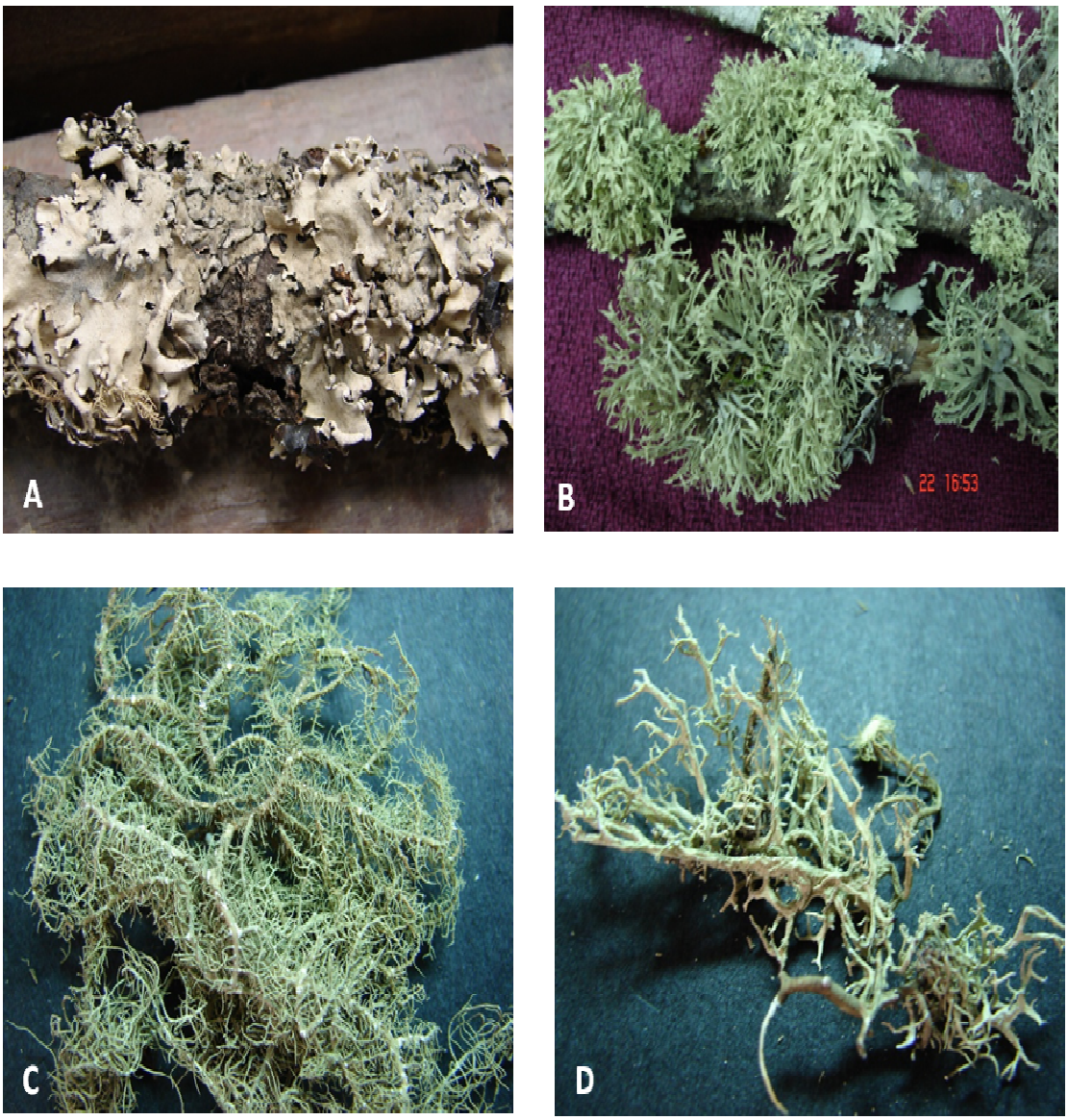


Figure 3.2: Lichen Samples; (A): *Parmelia reticulata*; (B): *Ramalina roesleri*; (C): *Usnea longissima*; (D): *Stereocaulon himalyense*.

3.1.2 Solvents and chemicals

Laboratory grade reagents were procured locally. The solvents used were of analytical grade.

3.1.3 Biological agents

3.1.3.1 Plant pathogenic fungi

Plant pathogenic fungi namely, *Sclerotium rolfsii* ITCC 5226, *Rhizoctonia solani* ITCC 5395, *Rhizoctonia bataticola* ITCC 0482, *Fusarium udum* ITCC 2042,

Pythium debaryanum ITCC 4744, *Pythium aphanidermatum* ITCC 4746 were collected from the Indian Type Culture Centre, Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi, India. Pathogenic fungi were maintained on PDA (potato dextrose agar) at 27°C and were sub-cultured on PDA Petri dishes for 5-6 days at 27°C prior to use as inoculums.

3.1.3.2 Weed seed

Seeds of *Phalaris minor* Retz. were collected from Division of Agronomy, Indian Agricultural Research Institute, New Delhi.

3.1.3.3 Seeds of major food crop plants

Wheat (*Triticum aestivum* L., Var. HW 2004), Chick pea (*Lens culinaris* Medikus, Var. Pusa 391) and lentil (*Cicer arietinum* L.) seeds were collected from Division of Genetics, Indian Agricultural Research Institute, New Delhi.

3.1.3.4 Micro algae

Algae namely *Chlorella vulgaris*, *C. sorokiniana* and *Scenedesmus subspicatus* were collected from SERA Heinsberg, Germany.

3.1.3.5 Pathogenic bacteria

Pathogenic bacteria, *Bacillus subtilis* (ATCC6051), *Staphylococcus aureus*, *Streptomyces viridochromogenes* (Tü 57), *Escherichia coli* were procured from the Culture Collection Centre, Institute of Organic and Bimolecular Chemistry, Georg August University of Göttingen, Göttingen, Germany.

3.2 Identification of lichens

3.2.1 Morphological characteristics

Fruiting bodies of the lichens were examined with a microscope. Preparations were made in distilled water and then mounted in lacto phenol-cotton blue and sealed with polyvinyl alcohol. Morphological characters were identified with lichen identification key. The micro and macrolichen keys of Awasthi (1991) are the important literature referred for identification of lichens. The colour of the cortex or medulla changes due to presence of particular lichen substances in lichen thallus.

3.2.2 Spot test

Lichen Spot tests procedure (Huneck and Yoshimura, 1996)

1. Apply the reagent solutions to the lichen's cortex or medulla (or both) using a small glass needle. Keep a separate needle for each reagent.

2. Observe the colour.

3. Wait at least half a minute before concluding that the test is negative. The following table describes the reagents used for test:

Table 3.1: Reagents used for the spot test

Test	Reagent*
K test	10% water solution of potassium hydroxide
C Test	a solution of commercial bleach
P Test	a saturated alcohol solution (95% ethanol) of *p*-phenylenediamine (1,4 diamino benzene) Steiner's solution: 1 gm of *p*-phenylenediamine, 10g of sodium sulfite in 100ml distilled water with 2ml liquid detergent.
KC	K and C are applied together (called the KC test when the K is applied first)
CK	C is applied first

Where, *K: Turns yellow then red with most o-hydroxy aromatic aldehydes red to purple with anthraquinone pigments

*C: Turns red with m-dihydroxy phenols and green with dihydroxy dibenzofurans

*KC (K followed by C): Turns red with depsides and depsidones

*P: Turns yellow, orange, or red with aromatic aldehydes

3.3 Solvent extraction of lichens

The lichen thalli were washed thoroughly with water to remove dust particles and dried at room temperature. The air-dried mass was powdered with a mortar and pestle. The powdered material (50 g) was extracted in a soxhlet extractor with hexane followed by ethyl acetate and finally with methanol (500 ml) 6 h for each. The solvent was evaporated *in vacuo*, and the dried residues obtained were stored at 4°C for further biological screening and isolation of compounds (Scheme 3.1).

3.4 Isolation of chemical constituents

Chromatography: Separation techniques

3.4.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was performed on TLC cards (Macherey-Nagel and Co., Düren, Germany) and pre-activated glass plates (20 cm × 20 cm, coated with 0.5 mm silica gel G containing 10% of gypsum as binder).The plates were developed in the solvent systems given below usually employed in the TLC of lichen substances

Solvent A: Toluene: dioxane: acetic acid (180:45:5, v/v);

Solvent B: Toluene: acetic acid (170:30, v/v)

The compounds were detected from their UV absorbance at 254 and 366 nm and by spraying with following reagents:

a) **10% sulfuric acid**: TLC plates were visualized by spraying with 10% sulfuric acid followed by heating at 110 °C in an oven for 10 minutes to develop the spots.

b) **Anisaldehyde/sulfuric acid:** TLC plates were visualized by spraying anisaldehyde/sulfuric acid reagent (prepared by dissolving anisaldehyde (1ml) to a solution (100ml) containing methanol (85 ml), acetic acid (14ml) and sulfuric acid (1ml) followed by heating at 110 °C in an oven for 10 minutes to develop the spots.

c) **Iodine vapour:** TLC plates were also visualized by placing plates in iodine chamber.

TLC was used to optimize the solvent system that would be applied for column chromatography and to monitor the fractions and their qualitative purity.

3.4.2 Preparative Thin Layer Chromatography

Preparative thin layer chromatography (PTLC) was performed by making a homogenous suspension of silica gel (55 g) (Macherey-Nagel and Co.) and demineralized water (120 ml) with continuous stirring for 15 min. Then 60 ml of the above suspension was evenly distributed on a horizontally held 20 cm × 20 cm glass

plate. The plates were dried at ambient temperature for 24 h and were activated by heating at 130 °C for 2 h.

3.4.3 Column Chromatography

Chemical constituents from crude extracts were separated using a glass column (75cm x 2 cm id.) packed with pre-activated silica gel (50 g, 60-120 mesh) in hexane and was eluted with different ratios of hexane, hexane/ dichloromethane, dichloromethane/ ethyl acetate, ethyl acetate/ methanol and lastly with methanol. Different fractions (25 ml each) were collected and evaporated to dryness on a rotary evaporator. The fractions containing similar compounds (as monitored by TLC) were combined and purified further for characterization.

3.4.4 High Performance Liquid Chromatography (HPLC)

Analytical HPLC was used to identify interesting peaks from extracts and fractions as well as to evaluate the purity of isolated compounds. Samples were analyzed by HPLC (Varian Prostar) equipped with a variable wavelength UV-150 UV VIS detector and a Rheodyne injector (20 µl loop) and connected to a datajet reporting integrator. Stationary phase consisted of a Lichrosorb C-18 column (250 mm x 4.6 mm id) and the mobile phase was acetonitrile: water (0.1 % phosphoric acid) maintained at a flow rate of 1 ml/ min with detector wavelength set at λ 280 and 320 nm.

3.4.5 Liquid chromatography – Mass Spectrometry (LC-MS)

Crude extracts were analyzed using Knauer HPLC equipment (RP-C12 column with stationary phase Hypersil ODS 120 and UV Vis-Diode-Array-Detector at the wavelength range of 200-800 nm coupled with mass detector (ESI) in the range of 100-2000 amu. The mobile phase used was buffer A: MeOH+0.05% HCOOH and buffer B: H_2O+0.05% HCOOH at a flow rate of 300 µl/min. HPLC raw data were analyzed by Borwin HPLC-software.

3.4.6 Instrumentation: Characterization techniques

3.4.6.1 Nuclear Magnetic Resonance Spectroscopy (^{1}H NMR and ^{13}C NMR)

The proton nuclear magnetic resonance spectra were recorded on a Bruker 400 AC (400 MHz) instrument. All 1D and 2D spectra were obtained using the standard Bruker software. The chemical shift values were recorded on the δ scale. The solvents used were deuterated chloroform ($CDCl_3$), deuterated methanol (CD_3OD) and dimethyl sulfoxide (DMSO-d_6) containing tetramethylsilane [$(CH3)_4$ Si, TMS] as internal standard. All ^{13}C multiplicities were deduced from 90° and 135° DEPT (Distortionless Enhancement by Polarization transfer) experiments which distinguishes and classifies the carbons as doublets (CH), triplets (CH_2), or quartets (CH_3). The two dimensional COSY, (^{1}H-^{1}H correlation spectroscopy) HSQC (^{1}H-^{13}C heteronuclear single quantum coherence) and HMBC (^{1}H-^{13}C heteronuclear multiple bond correlation) experiments were performed. HSQC was used to directly correlate proton and carbon nuclei through one bond and the Hetero Multinuclear Bond Correlation (HMBC) was utilized to obtained long range correlations of proton and carbon nuclei through two, three or four bonds.

3.4.6.2 Mass Spectrometry

EI MS was recorded on Varian MAT 731 mass spectrometer at 70 eV and a HRGC-MEGA series instrument with FISONS-TRIO 1000 Ion Trap and HP 5790 detetctor. Elecrospray ionization mass spectrometry (ESI MS) and high resolution mass spectra (HR ESI MS) were recorded on a time of flight mass spectrometer (Bruker Daltonics, Bremen, Germany), as well as on an Apex IV 7 Tesla Fourier transform ion cyclotron resonance mass spectrometer (Bruker Daltonics, Billerica, MA).

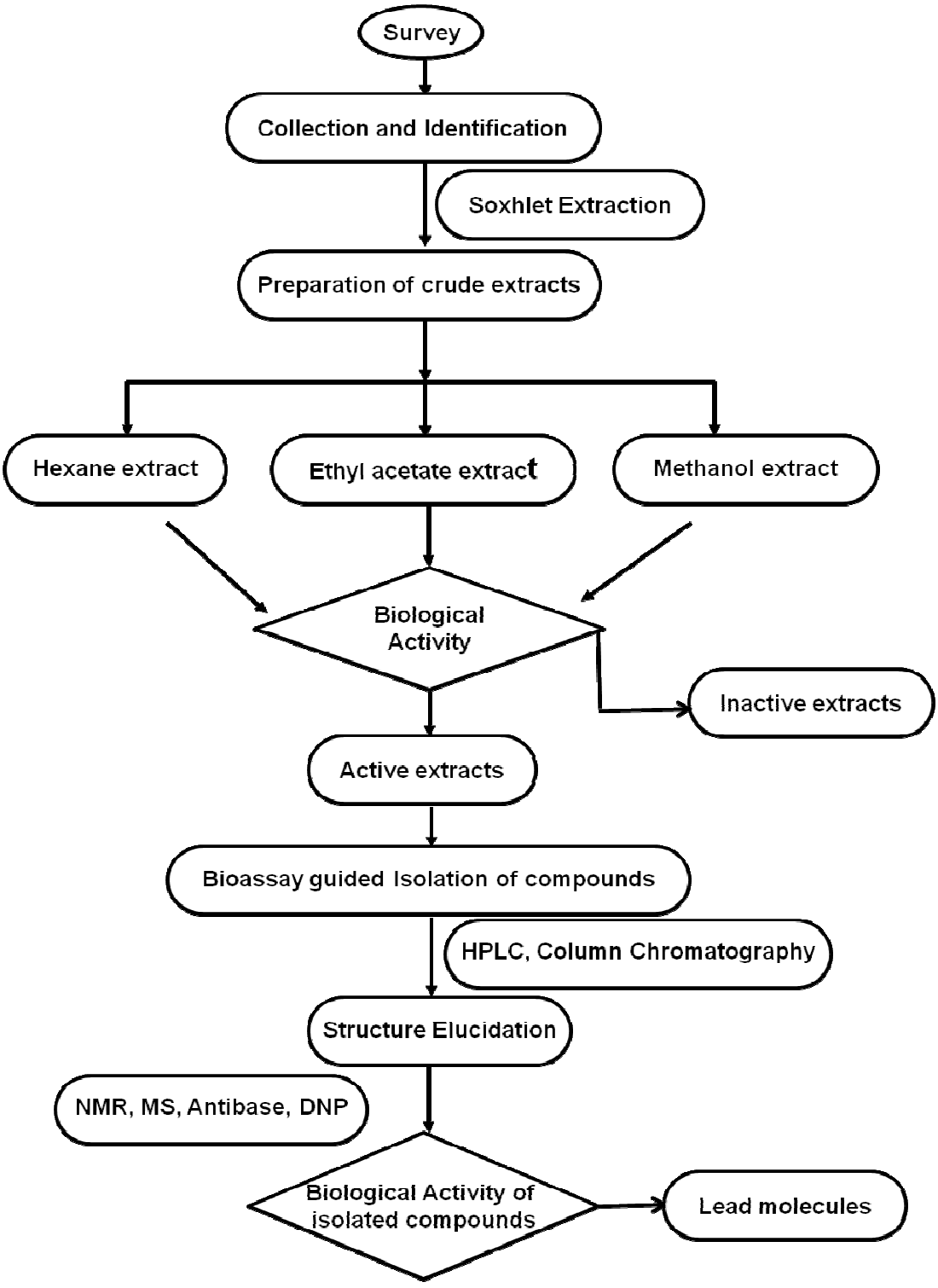
3.4.6.3 Melting Point

Melting points of the isolated compounds were determined on a JSW melting point apparatus with an open capillary and are uncorrected.

3.5 Confirmation of Structures

Identification of the compounds was finally confirmed using the AntiBase, a database of molecular structures of natural compounds produced by microorganisms, developed by Prof. Hartmut Laatsch, Institute of Organic and Biomolecular

Chemistry, George August University of Göttingen, Germany, was used as a tool to find possible chemical structures to aid the identification of the metabolites.



Scheme 3.1:.Layout of the work plan

Structures were drawn using ISIS ^{TM}/ Draw version 2.4, MDL information systems lnc and ChemDraw Ultra, Version 8.0, CambridgeSoft Corporation.

The IUPAC nomenclature was ascertained following ACD chem sketch, version 10.2 (2006).

3.6 Biological activity

3.6.1 Antifungal activity

Antifungal activity of crude extracts and pure compounds was evaluated by poisoned food technique using potato dextrose agar media (Nene and Thaplyal, 1979) against phytopathogenic fungi namely; (i) *Rhizoctonia bataticola* (ii) *Rhizoctonia solani* (iii) *Fusarium udum* (iv) *Pythium debaryanum* (v) *Pythium aphanidermatum* (vi) *Sclerotium rolfsii*

3.6.1.1 Preparation of potato dextrose agar (PDA) media

The PDA media was prepared using following materials:

(1) Potato (peeled) 200 g

(2) Dextrose (anhydrous) 20 g

(3) Agar 20 g

The potatoes (200 g) were peeled, cut into small pieces and boiled with water (600 ml) for 20 min, till the potato pieces became soft. The extract was strained through a muslin cloth and volume was made upto 1000 ml. The weighed quantities of agar (20 g) and dextrose (20 g) were added to the filtrate and the mixture was heated with stirring till uniform media was obtained. The media (65 ml) was transferred to conical flasks (150 ml). The flasks were plugged with non-absorbent surgical cotton. The media was sterilized in an autoclave at 15 Psi and 121.5^{o}C for half an hour.

3.6.1.2 Preparation of test samples

A stock solution of 1000 μg ml^{-1} of the test compound was prepared, which was further diluted with acetone to give the required concentrations of 250, 125, and 62.5 μg ml^{-1}. Acetone (1 ml) was used as the control. These solutions were added to the media (65 ml) contained in conical flasks to obtain the desired concentrations of

the test samples. The media (65 ml) was thoroughly mixed and poured into Petri dishes (4 replications) under aseptic conditions in a Laminar Flow Chamber. The plates were kept under UV light in the Laminar Flow Chamber for solidification of media. After the solidification of the media, a 5 mm disc of the desired pathogenic fungus containing spores and mycelia were inoculated aseptically to the centre of the Petri dishes.

3.6.1.3 Incubation

The inoculated Petri dishes were kept in a BOD incubator, maintained at 27^{o}C till the fungal growth was almost complete in the control plates. The incubation periods for different pathogenic fungi are given below

Incubation periods for pathogenic fungi used

Fungus	Incubation period (days)
Rhizoctonia bataticola	4-5
Rhizoctonia solani	5-6
Fusarium udum	4-6
Pythium aphanidermatum	6-7
Pythium debaryanum	6-7
Sclerotium rolfsii	3-4

3.6.1.4 Recording of observations

The mycelial growth of fungus (cm) in both treated (T) and control (C) Petri dishes were measured diametrically and the percentage inhibition of growth (I%) was calculated by using the following formula:

$$\textit{Percentage Inhibition } I(\%) \frac{C - T}{C} X100 \qquad \text{(I)}$$

3.6.1.5 Calculation of ED_{50} values

For calculation of ED_{50} values, the percent inhibition was converted to corrected percent inhibition by using the Abbott's formula:

$$\textit{Corrected Inhibition } (\%) \frac{\%I - CF}{100 - CF} X100 \qquad \text{(II)}$$

Where CF is the correction factor obtained by the equation:

$$Corrected\ factor\ (\%) \frac{9 - C}{C} X100 \quad \text{(III)}$$

Where 9 is the diameter of the Petri dish in cm, ED_{50} was calculated from the concentration (µg ml^{-1}) and corresponding corrected percentage inhibition data of each sample. ED_{50} values were calculated statistically by Probit analysis (MSTATC software; Basic LD_{50} program version 1.1).

3.6.2 *In vitro* herbicidal activity on *Phalaris minor*

The effect of different concentrations (50, 25 and 12.50 µg ml^{-1}) of solvent extracts and/or compounds was studied on germination and early seedling growth of *P. minor*. The experiments were carried out in portray maintained in natural environment in the National Phytotron Facility, Indian Agricultural Research Institute (IARI). The required concentrations of each solvent extract/compounds were prepared containing 1% Tween 20 in minimum amount of acetone to dissolve the extract and the volume was made up to 5 ml in water. Thirty seeds of equal size were soaked overnight in above solutions of different extracts/compounds. These soaked seeds were removed from the aqueous solutions and dried in the air, to give a uniform layer of the extract/compound on the seed coat. The control treatments for all sets contained the same quantities of 1% Tween 20 and distilled water. These coated seeds were placed in the protrays maintained in natural environment of growth room at 18 ^{o}C and 12 hours daily light periods. Seeds were considered germinated, if a radical protruded at least 1 mm. Root and shoot lengths of the seedlings were measured after 15 days. Each bioassay was replicated 3 times with a set of 10 seeds each in a complete randomized design. Control of growth of the weed is calculated in terms of inhibition percentage as depicted in Formula IV.

$$Inhibition\ (\%) \frac{Ci - Ti}{Ci} X100 \quad \text{(IV)}$$

Where,

Ci = Number of seeds germinated in control and/or root/shoot length in control

Ti = Number of seeds germinated in treatment and/or root/shoot length in control

3.6.3 Allelopathic potential of solvent extracts

In order to find the effect of solvent extracts on the growth of major food crops, seeds of wheat (*Triticum aestivum* L.) (Var. HW 2004), Chick pea (*Lens culinaris* Medikus) (Var. Pusa 391) and lentil (*Cicer arietinum* L.), a bioassay is set up under laboratory conditions.

Three concentrations of each solvent extract viz. 2.5, 1.25 and 0.625 (μg ml^{-1}) were prepared containing 1% Tween 20 in minimum amount of acetone to dissolve the extract and the volume was made up to 5 ml in water. Thirty seeds of equal size were soaked overnight in above solutions of different extracts. These soaked seeds were removed from the aqueous solutions and dried in the air, to give a uniform layer of the extract on the seed coat. These coated seeds were placed in the Petri dishes (9 cm) lined with moist sheet of Whatman No. 1 filter paper and sealed with paraffin. The plates containing seeds were kept in a BOD incubator at 25±1 ^{o}C. Seeds were considered germinated if a radical protruded at least 1 mm. The observations were recorded on 15th day. Each bioassay was replicated 3 times with a set of 10 seeds each in a complete randomized design. Root and shoot lengths were measured by scale. The percentage phytotoxicity and percentage growth were calculated as shown in Formula IV and V respectively:

$$\textit{Growth (\%)} = \frac{Cg - Tg}{Cg} X100 \qquad \text{(V)}$$

Where,

Cg = Number of seeds germinated in control and/or root/shoot length in control

Tg = Number of seeds germinated in treatment and/or root/shoot length in control

3.6.4 Algaeicidal activity

The isolated compounds were tested on the lawn of unicellular algae namely *Chlorella vulgaris*, *C. sorokiniana* and *Scenedesmus subspicatus*. The disc diffusion assay was performed and the diameter of the inhibition zone was taken as the measure of extent of toxicity.

3.6.4.1 Disc diffusion assay

Aliquots of the test solution were applied to sterile filter-paper discs (5 mm diameter, Hi Media) to give a final disc loading concentration of 40 µg of samplefor crude extracts and for pure compounds. The impregnated discs were placed on agar plates previously seeded with the selected test organisms, along with discs containing solvent blanks. The plates were incubated at 37 ^{o}C for 24 hours; the activity was recorded as the zone of inhibition surrounding the disc and was measured in mm.

3.6.5 Antibacterial activity

3.6.5.1 Culture preparation

Prior to testing, a few colonies (3 to 10) of the organism to be tested were sub-cultured in 4 ml of Nutrient agar broth (Hi Media)) and incubated for 24 h to produce a bacterial suspension of moderate cloudiness. The suspension was diluted with sterile saline solution to a density visually equivalent to that of a barium sulfate standards, prepared by adding 0.5 ml of 1% barium chloride to 99.5 ml of 1% sulfuric acid (0.36 N). The prepared bacterial broth is inoculated onto Müller-Hinton-Agar plates (Difco, USA) and dispersed by means of sterile beads.

3.6.5.2 Disc diffusion assay

The diffusion assay was performed in a same manner as described above for algaecidal activity.

3.7 Statistical analysis of the data

The experimental data were statistically analyzed by analysis of variance (ANOVA) using Statistical Package for Social Services (SPSS version 10.0), and treatment means were compared by using Duncan's Multiple Range Test (DMRT) at 5% levels.

Results & Discussion

PART I

Investigation of agrochemical potential of lichens

This part is based on the studies carried out on four lichen species, *Parmelia reticulata* Taylor, *Ramalina roesleri* Nyl., *Usnea longissima* Ach. Articus and *Stereocaulon himalayense* Lamb collected from the Kilberi forest ranges (Northern Himalayas) in the Uttaranchal state (Uttarakhand) India. The samples were identified based on thallus characters (growth form, shape of the thallus, color of cortex), reproductive characters (shape and color of the apothecia (if present) and ecological characters (distribution, elevation, substrate).

4.1 Extraction of lichen samples

The dried samples of *P. reticulata* Taylor, *R. roesleri* Nyl*, U. longissima* Ach. Articus and *S. himalayense* Lamb were powdered and subjected to soxhlet extraction using hexane followed by ethyl acetate and methanol for about 6 hrs successively. The extracts were filtered and concentrated in vacuum under reduced pressure and the extract thus obtained was subjected to various biological activities and isolation of chemical constituents.

4.2 Biological activities

The crude solvent extracts and isolated chemical constituents were evaluated for the following allelopathic activities:

1. *In vitro* antifungal activity against devastating, polyphagous plant pathogenic fungi *Rhizoctonia bataticola*, *R. solani, Fusarium udum, Pythium debaryanum, P. aphanidermatum* and *Sclerotium rolfsii.*
2. Herbicidal activity against a devastating weed *Phalaris minor* Retz.
3. Effect on seed germination and early seedlings growth of major crop plants, wheat (*Triticum aestivum* L), lentil (*Lens culinaris* Medikus) and chick pea (*Cicer arietinum* L.)
4. Algaeicidal activity against microalgae *Chlorella vulgaris*, *Chlorella sorokiniana*, *Scenedesmus subspicatus*

4.2.1 *In vitro* antifungal activity

The antifungal activity of hexane, ethyl acetate and methanol extracts of *P. reticulata, R. roesleri, U. longissima* and *S. himalayense* against *S. rolfsii, R. bataticola*, *R. solani, F. udum, P. debaryanum* and *P. aphanidermatum* was evaluated using poisoned food technique at different concentrations (250, 125, 62.5, 31.2, 12.5) (Goel *et al*., 2011) and the results have been reported in terms of effective dose for 50 % inhibition (ED_{50}) value, calculated by Probit analysis with the help of MSTATC software (Basic LD_{50} program version 1.1). In the current study, the following scale has been followed to present the potentiality of the crude extracts against pathogenic fungi (Table 4.1).

Table 4.1: Scale to present antifungal potentiality of extracts

ED_{50} ranges (µg ml^{-1})	**Potentiality of the crude extract**
0-25	Good
25-50	Significant
50-75	Moderate
75-100	Mild
above 100	Insignificant

In case of ***P. reticulata***, hexane extract showed maximum activity against *R. bataticola* (ED_{50} = 25.1 µg ml^{-1}), followed by *R. solani* (ED_{50} = 29.4 µg ml^{-1}), and a considerable activity in the range of 48.4-76.8 µg ml^{-1} observed for other tested fungi. However, the ethyl acetate extract showed maximum inhibition against *F. udum* (ED_{50} = 43.7 µg ml^{-1}) and *S. rolfsii* (ED_{50} = 43.7 µg ml^{-1}), a notable activity against *P. debaryanum* (ED_{50} = 48.4 µg ml^{-1}) and a mild activity against *P. aphanidermatum* (ED_{50} = 78.4 µg ml^{-1}). Methanol extract of *P. reticulata* also exhibited a moderate inhibition against *S. rolfsii* (ED_{50} = 51.2 µg ml^{-1}) followed by *F. udum* (ED_{50} = 59.2 µg ml^{-1}) and rest of the fungi in the range of 79.5-96.4 µg ml^{-1} (Figure 4.1). All the three extracts were found to be equally active against *S. rolfsii* as shown in Table 4.2, Figure 4.1.

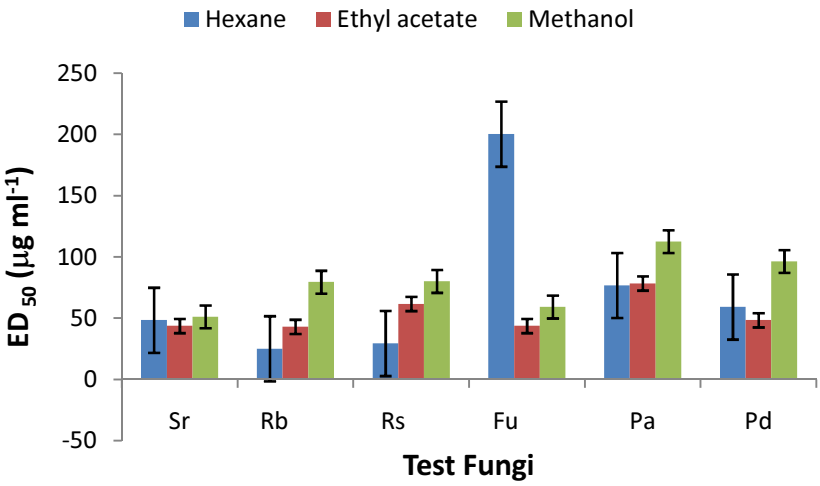


Figure 4.1: Comparison of antifungal activity of hexane, ethyl acetate and methanol extracts of *P. reticulata*

Table 4.2: Antifungal activity of solvent extracts of *P. reticulata* against pathogenic fungi

Solvent Extracts	[a]ED_{50} (µg ml^{-1}) against pathogenic fungi					
	Rb	***Rs***	***Fu***	***Pa***	***Pd***	***Sr***
Hexane	25.1 ± 2.03	29.4 ±0.66	200.3±0.67	76.86 ±0.66	59.2 ±0.53	48.4 ±0.69
Ethyl acetate	43.0 ±0.64	61.7 ±0.64	43.7 ±0.63	78.4 ±0.63	43.7 ±0.63	43.7 ±0.63
Methanol	79.5 ±0.54	80.1 ±0.63	59.2 ±0.70	112.6 ±0.64	96.4 ±0.64	51.2± 1.20

[a]Mean percentage inhibition, used for ED_{50} calculation, is an average of four replicates, where *Rb = Rhizoctonia bataticola; Rs=Rhizoctonia solani*; *Fu=Fusarium udum,; Pa =Pythium aphanidermatum*; *Pd=Pythium debarynum; Sr=Sclerotium rolfsii.*

The antifungal activity of solvent extracts of ***R. roesleri*** is tabulated in Table 4.3 and shown in Figure 4.2. Among all the extracts of *R. roesleri*, the hexane extract showed maximum activity against *R. bataticola* (ED_{50} = 24.5 µg ml^{-1}). Hexane extract was also effective against *R. solani* (ED_{50} = 27.7 µg ml^{-1}) and *F. udum* (ED_{50} = 28.4µg ml^{-1}), found significantly active against *S. rolfsii* (ED_{50} = 39.9 µg ml^{-1}) (Figure 4.2). The ethyl acetate extract showed maximum antifungal activity against *F. udum* (ED_{50} = 57.0 µg ml^{-1}) followed by *S. rolfsii* (ED_{50} = 67.5 µg ml^{-1}), and moderate to negligible activity against rest of the test fungi in the range of 72.8-239.1 µg ml^{-1}. The methanol extract showed only mild activity against *F. udum* followed by *S. rolfsii* with ED_{50} values of 76.1 and 83.4 µg ml^{-1} respectively and inactive against rest of the test fungi in the range of 108.7-230.8 µg ml^{-1}.

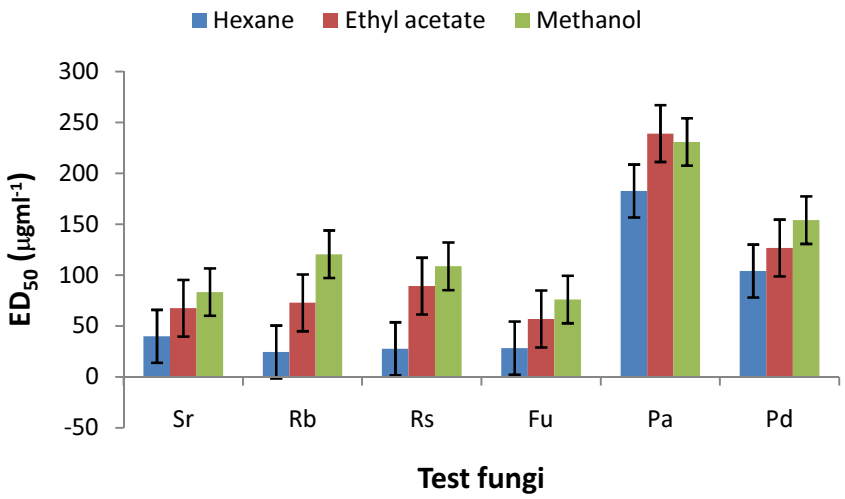


Figure 4.2: Comparison of antifungal activity of hexane, ethyl acetate and methanol extracts of *R. roesleri*

Table 4.3: Antifungal activity of solvent extracts of *R. roesleri* against pathogenic fungi

Solvent Extracts	[a]ED_{50} (µg ml^{-1}) against pathogenic fungi					
	Rb	***Rs***	***Fu***	***Pa***	***Pd***	***Sr***
Hexane	24.5±0.60	27.7 ±0.67	28.4 ±1.30	182.6 ±0.65	104.0 ±1.34	39.9 ±0.61
Ethyl acetate	72.8±0.51	89.3 ±0.56	57.0 ±0.66	239.1 ±0.54	126.7 ±0.64	67.5 ±0.66
Methanol	120.5±0.65	108.7 ±0.52	76.1 ±0.64	230.8 ±0.63	154.0 ±0.66	83.4 ±0.65

[a]Mean percentage inhibition, used for ED_{50} calculation, is an average of four replicates, where *Rb = Rhizoctonia bataticola*; *Rs=Rhizoctonia solani; Fu=Fusarium udum,; Pa = Pythium aphanidermatum*; *Pd = Pythium debarynum; Sr= Sclerotium rolfsii*

For ***U. longissima*** (Table 4.4 and Figure 4.3) among all the extracts, hexane extract was found to be most active against *R. bataticola* (ED_{50} = 18.9 µg ml^{-1}), *S. rolfsii* (ED_{50} = 24.8 µg ml^{-1}) and *P. debaryanum* (ED_{50} = 28.3 µg ml^{-1}) and *P. aphanidermatum* (ED_{50} = 37.0 µg ml^{-1}). However, it exhibited a moderate to mild inhibitory potency against rest of the two fungi in the range of ED_{50}= 52.8-96.5 µg ml^{-1}. Ethyl acetate extract showed moderate activity only against *F. udum* (ED_{50} =72.0 µg ml^{-1}) and it was only slightly active against rest of the fungi with ED_{50} in the range of 74.3-99.0 µg ml^{-1}. The methanol extract may be considered inactive against all the fungi with ED_{50} in the range of 125.6-168.8 µg ml^{-1} (Table 4.4).

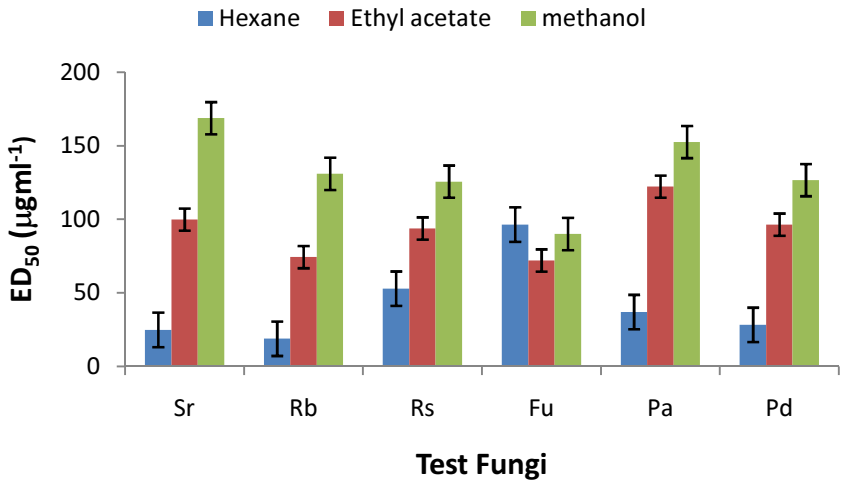


Figure 4.3: Comparison of antifungal activity of hexane, ethyl acetate and methanol extracts of *U. longissima*

Table 4.4: Antifungal activity of solvent extracts of *U. longissima* against pathogenic fungi

Solvent extracts	[a]ED_{50} (µg ml^{-1}) against pathogenic fungi					
	Rb	***Rs***	***Fu***	***Pa***	***Pd***	***Sr***
Hexane	18.9 ±0.63	52.8 ±0.64	96.5 ±0.69	37.0 ±0.55	28.3 ±0.61	24.8 ±0.63
Ethyl acetate	74.3 ±0.64	93.8 ±0.65	72.0 ±0.66	122.3 ±0.54	96.5 ±0.66	99.9 ±0.63
Methanol	131.0 ±1.12	125.6 ±0.61	90.1 ±0.65	152.6 ±0.76	126.7 ±0.64	168.8 ±0.55

[a]Mean percentage inhibition, used for ED_{50} calculation, is an average of four replicates, where *Rb = Rhizoctonia bataticola*; *Rs = Rhizoctonia solani; Fu= Fusarium udum,; Pa = Pythium aphanidermatum*; *Pd = Pythium debarynum; Sr= Sclerotium rolfsii*

As is the case with other lichens discussed above, the hexane extract of ***S. himalayense*** showed maximum inhibition against *S. rolfsii* with ED_{50} = 29.2 µg ml^{-1} (Figure 4.4). Ethyl acetate and methanol extract may be considered to be inactive against all the fungi with ED_{50} in the range of 126.7-225.2 µg ml^{-1} and 140.6-287.6 µg ml^{-1} respectively (Table 4.5).

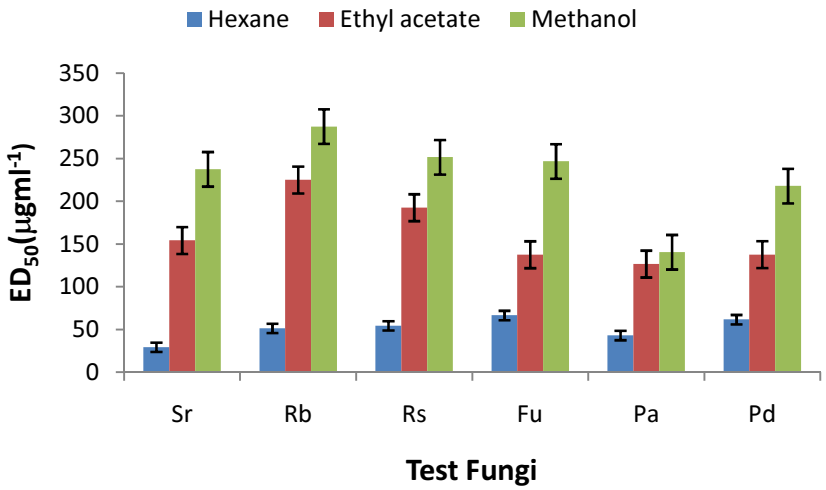


Figure 4.4: Comparison of antifungal activity of hexane, ethyl acetate and methanol extracts of *S. himalayense*

Table 4.5: Antifungal activity of solvent extracts of *S. himalayense* against pathogenic fungi

Solvent Extracts	[a]ED_{50} (µg ml^{-1}) against pathogenic fungi					
	Rb	***Rs***	***Fu***	***Pa***	***Pd***	***Sr***
Hexane	51.3 ±2.31	54.3 ±0.64	66.5 ±0.67	43.0 ±0.63	61.7 ±0.66	29.2 ±0.61
Ethyl acetate	225.2 ±0.57	192.7 ±0.55	137.6 ±0.62	126.7 ±0.63	137.7 ±0.64	154.3 ±0.51
Methanol	287.6 ±0.63	251.8 ±0.63	246.9 ±0.57	140.6 ±0.65	218.0 ±0.53	237.6 ±0.61

[a]Mean percentage inhibition, used for ED_{50} calculation, is an average of four replicates, where *Rb = Rhizoctonia bataticola*; *Rs =Rhizoctonia solani; Fu= Fusarium udum,; Pa = Pythium aphanidermatum*; *Pd = Pythium debarynum; Sr= Sclerotium rolfsii*

Antifungal potential of solvent extracts: Discussion

Plant pathogenic fungi are one of the major reasons for losses of crop worldwide because of their wide host range. These fungi infect seeds, seedlings and mature plants in the field causing collar rot, wilt, damping off, dry root rot and spoilage (Okonkwo, 1989; Brenneman *et al.,* 1991; Thammaiah *et al.,* 1997; Raneshwaran and Prasad, 1999; Bhowmik *et al.,* 2002; Tsahouridou and Thanassoulopoulos, 2002; Pande *et al.,* 2004). The current investigation is an effort to identify newer, safer and eco-friendly agents against these pathogenic fungi.

Detailed analysis of antifungal data showed that lichen extracts exhibited strong effects against certain fungi, even in relatively low concentrations. Among the three extracts of all the lichens species, hexane extract exerted the strongest inhibitory action. The antifungal activity of different solvent extracts of the lichen species against different pathogenic fungi was found to be selective. *R. bataticola, R. solani* and *S. rolfsii* were found sensitive towards the hexane extract of all the lichens, hence, can be used for checking the growth of these fungi (Figure 4.1- 4.4). Differences in the activity of these extracts against various fungi indicated that the activity is species-specific and also depend on the solvent used for extraction. Current study suggests hexane and ethyl acetate as the solvents of choice for extraction and the possibility of presence of similar class of compounds as shown by their promising antifungal activity.

4.2.2 Herbicidal activity of solvent extracts against *Phalaris minor* Retz.

The effect of solvent extracts of ***P. reticulata*** and ***R. roesleri*** at 50, 25 and 12.5µg ml^{-1} concentrations on seed germination, shoot length and root length of *Phalaris minor* was studied. *Phalaris minor* is a common devastating weed of wheat-rice cropping system in north western India. The experiments were carried out in portrays maintained in natural environment in the National Phytotron Facility, Indian Agricultural Research Institute.

Effect on seed germination

The effect of extracts on seed germination at three concentrations is described in the Table 4.6, Figure 4.5. A significant decrease in seed germination of *P. minor* was recorded as compared to control, when hexane, ethyl acetate and methanol extracts were applied at various concentrations. There was only 56, 57 and 79 % seed germination, when hexane extract was applied at a dose of 50, 25 and 12.5 µg ml^{-1} respectively, as compared to control (90 %). Maximum inhibition of seed germination was observed, when hexane extract was applied at 50 µg ml^{-1} concentration. Similar observations were recorded with ethyl acetate and methanol extracts. There were 56, 56 and 66 % seed germination when hexane, ethyl acetate and methanol extracts were applied at 50 µg ml^{-1} concentration respectively. The above results revealed that

maximum inhibition of seed germination was observed at higher concentration as compared to lower concentrations and maximum seed germination was inhibited with hexane and ethyl acetate extracts than methanol extract. This indicates the semi polar nature of the chemical constituents responsible for the inhibition of the seed germination.

Table 4.6: Effect of hexane, ethyl acetate and methanol extract of *P. reticulata* on germination, shoot length and root length of *P. minor*

Extract	Concentration (µg ml^{-1})	% Germination (% Inhibition)	Shoot length in cm (Inhibition %)	Root length in cm (Inhibition %)
Hexane	**Control**	90 ± 1.86 (0.00)	10.7 ± 0.08 (0.00)	3.4 ± 0.16 (0.00)
	50	56 ± 2.64 (37.77)	2.5 ± 0.08 (76.63)	0.9 ± 0.14 (73.52)
	25	57 ± 1.73 (36.66)	4.7 ± 0.11 (56.07)	1.4 ± 0.17 (58.82)
	12.5	79 ± 1.00 (12.22)	6.0 ± 0.15 (43.92)	2.6 ± 0.16 (23.52)
Ethyl acetate	**Control**	90 ± 1.86 (0.00)	10.7 ± 0.08 (0.00)	3.4 ± 0.16 (0.00)
	50	56 ± 0.81 (26.66)	4.6 ± 0.09 (57.00)	1.5 ± 0.12 (55.88)
	25	63 ± 1.24 (25.55)	5.4 ± 0.09 (49.53)	1.8 ± 0.12 (47.05)
	12.5	77 ± 1.73 (15.55)	7.4 ± 0.12 (30.84)	1.9 ± 0.10 (44.11)
Methanol	**Control**	90 ± 1.86 (0.00)	10.7 ± 0.08 (0.00)	3.4 ± 0.16 (0.00)
	50	66 ± 1.73 (26.66)	6.3 ± 0.11 (41.12)	1.5 ± 0.11 (55.88)
	25	67 ± 2.64 (30.00)	6.8 ± 0.09 (36.44)	2.7 ± 0.17 (20.58)
	12.5	76 ± 2.64 (14.44)	7.3 ± 0.11 (31.77)	2.9 ± 0.13 (14.70)

Mean percentage seed germination, shoot length and root length used for calculation, is an average of three replicates and standard deviation ranged from 0.81 to 2.64, 0.08 to 0.15 and 0.10 to 0.17 respectively. Values given in parentheses indicate % inhibition in terms of seed germination, shoot length and root length as compared to the control

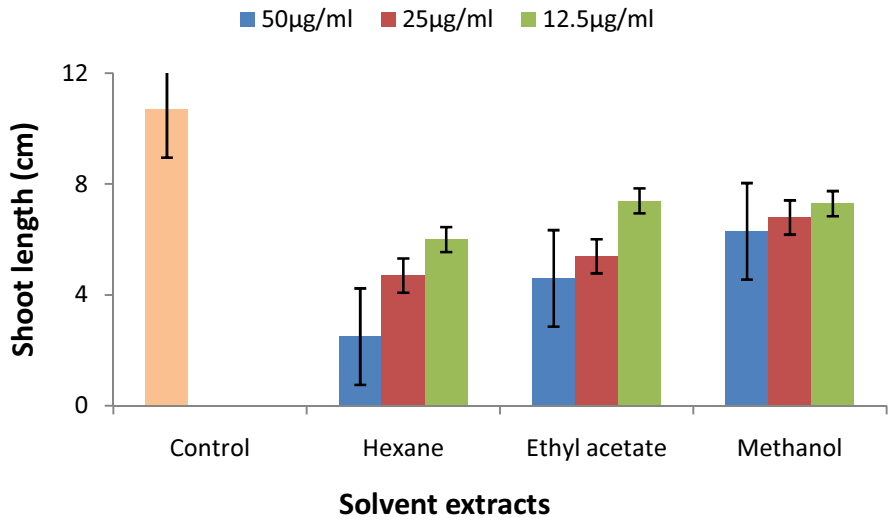


Figure 4.5: Effect of hexane, ethyl acetate and methanol extract of *P. reticulata* on seed germination of *P. minor*

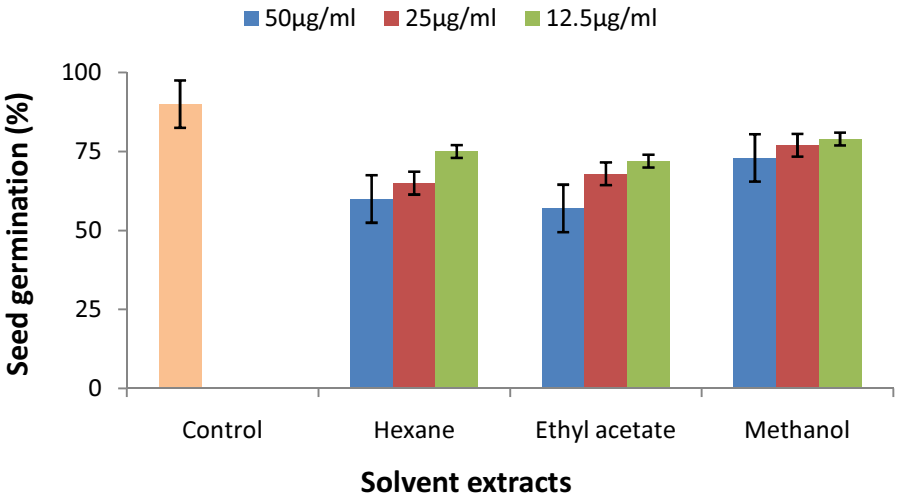


Figure 4.6: Effect of hexane, ethyl acetate and methanol extract of *R. roesleri* on seed germination of *P. minor*

In case of ***R. roesleri***, a significant decrease in germination of *P. minor* from 90 % (control) to 60, 65 and 75 % was observed, when hexane extract was applied at 50, 25 and 12.5 µg ml^{-1} concentrations respectively (Table 4.7 and Figure 4.6). Similar data were recorded with the ethyl acetate extract. The seed germination was found to be 57, 68 and 72 % at 50, 25 and 12.5 µg ml^{-1} concentrations. The methanol extract was found to be less effective in comparison to hexane and ethyl acetate extracts.

Effect on shoot length

Effect of solvent extracts of ***P. reticulata*** on shoot length was studied and a significant decrease in shoot length to 2.5, 4.7 and 6.0 cm was observed as compared to the control (10.7 cm), when hexane extract was applied at 50, 25 and 12.5 µg ml^{-1} concentrations respectively (Table 4.6 and Figure 4.7). Similarly, in case of ethyl acetate extract, the shoot length reduced to 4.6, 5.4 and 7.4 cm at 50, 25 and 12.5 µg ml^{-1} concentrations respectively. In general, maximum decrease was observed with hexane extract followed by ethyl acetate and methanol extracts at 50 µg ml^{-1} concentrations.

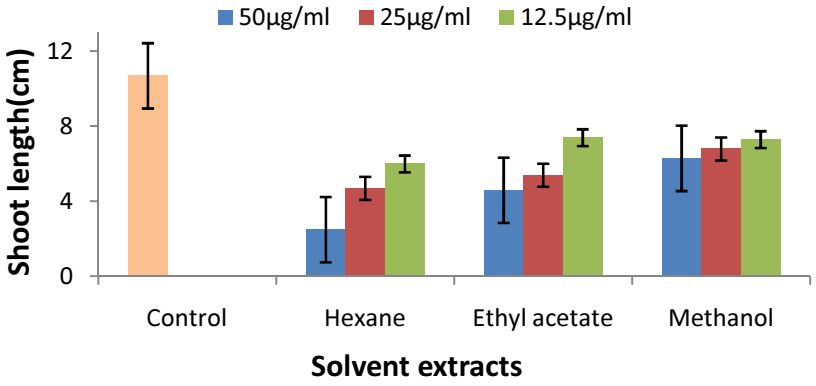


Figure 4.7: Effect of solvent extracts of *P. reticulata* on shoot length of *P. minor*

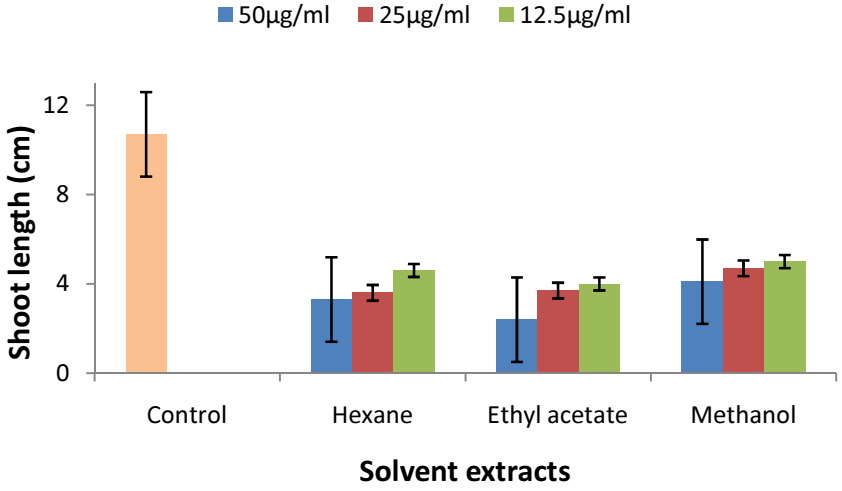


Figure 4.8: Effect of solvent extracts of *R. roesleri* on shoot length of *P. minor*

In case of ***R. roesleri***, the hexane extract suppressed the shoot length to 3.3, 3.6 and 4.6 cm, as compared to control (10.7 cm) (Table 4.7, Figure 4.8). Similarly, the growth was only 2.4, 3.7 and 4.0 cm when ethyl acetate extract was applied at 50,

25 and 12.5 μg ml^{-1} concentrations respectively. A comparable pattern was observed, with methanol extract, where 4.1, 4.7 and 5.0 cm shoot length was recorded for 50, 25 and 12.5 μg ml^{-1} concentrations, respectively. Among all the extracts, maximum decline in shoot length was recorded with ethyl acetate extract at 50 μg ml^{-1} concentration.

Table 4.7: Effect of solvent extracts of *R. roesleri* on germination, shoot length and root length of *Phalaris minor* seeds

Extract	Concentration (μg ml^{-1})	% Germination (Inhibition %)	Shoot length (cm) (Inhibition %)	Root length (cm) (Inhibition %)
Hexane	**Control**	90 ± 1.86 (0.00)	10.7 ± 0.08 (0.00)	3.4 ± 0.16 (0.00)
	50	60 ± 1.00 (33.33)	3.3 ± 0.09 (69.15)	0.5 ± 0.14 (85.29)
	25	65 ± 1.00 (27.77)	3.6 ± 0.17 (66.35)	0.8 ± 0.20 (76.47)
	12.5	75 ± 2.08 (16.67)	4.6 ± 0.11 (57.00)	2.9 ± 0.11 (14.70)
Ethyl acetate	**Control**	90 ± 1.86 (0.00)	10.7 ± 0.08 (0.00)	3.4 ± 0.16 (0.00)
	50	57 ± 1.00 (36.66)	2.4 ± 0.20 (77.57)	1.2 ± 0.25 (64.70)
	25	68 ± 2.64 (24.44)	3.7 ± 0.12 (65.42)	1.8 ± 0.11 (47.05)
	12.5	72 ± 1.00 (20.00)	4.0 ± 0.18 (62.61)	1.9 ± 0.08 (44.11)
Methanol	**Control**	90 ± 1.86 (0.00)	10.7 ± 0.08 (0.00)	3.4 ± 0.16 (0.00)
	50	73 ± 3.60 (18.88)	4.1 ± 0.18 (61.68)	0.9 ± 0.15 (73.52)
	25	77 ± 1.73 (14..44)	4.7 ± 0.13 (56.07)	1.1 ± 0.12 (67.64)
	12.5	79 ± 1.00 (12.22)	5.0 ± 0.12 (53.27)	1.9 ± 0.08 (44.11)

Mean percentage seed germination, shoot length and root length used for calculation, is an average of three replicates and standard deviation ranged from 1.0 to 3.60, 0.08 to 0.20 and 0.08 to 0.25 respectively. Values given in parentheses indicate % inhibition in terms of seed germination, shoot length and root length as compared to the control.

Effect on root length

A detrimental effect was observed in root length with hexane extract of ***P. reticulata*** at 50 and 25 μg ml-1 concentrations (Table 4.6, Figure 4.9). Root length

was reduced to 0.9 cm as compared to control (3.4 cm), when hexane extract was applied at 50 µg ml-1 concentrations. Considerable differences in the root length, 1.5, 1.8 and 1.9 cm was recorded, when ethyl acetate extract was applied at 50, 25 and 12.5 µg ml-1 concentrations respectively. Methanol extract had an insignificant on root growth at higher concentration when compared with lower concentrations.

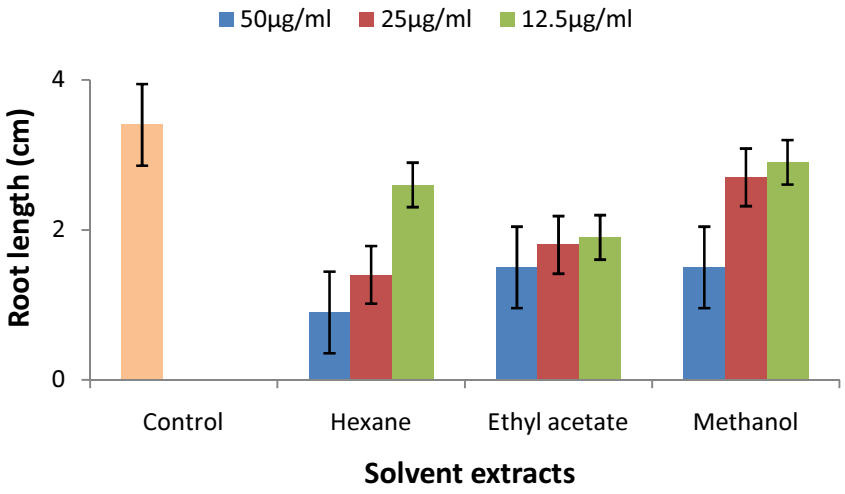


Figure 4.9: Effect of hexane, ethyl acetate and methanol extract of *P. reticulata* on root length of *P. minor*

In case of ***R. roesleri***, most significant decrease in root length was recorded with hexane extract. At 50 and 25 µg ml^{-1} concentrations, hexane extract hampered the root growth to 0.5 and 0.8 cm from 3.4cm in control. Alike the hexane extract, ethyl acetate and methanol extract also decreased the root length to 1.2, 1.8 cm and 0.9, 1.1 cm growth for 50 and 25 µg ml^{-1} concentrations, respectively (Table 4.7 and Figure 4.10).

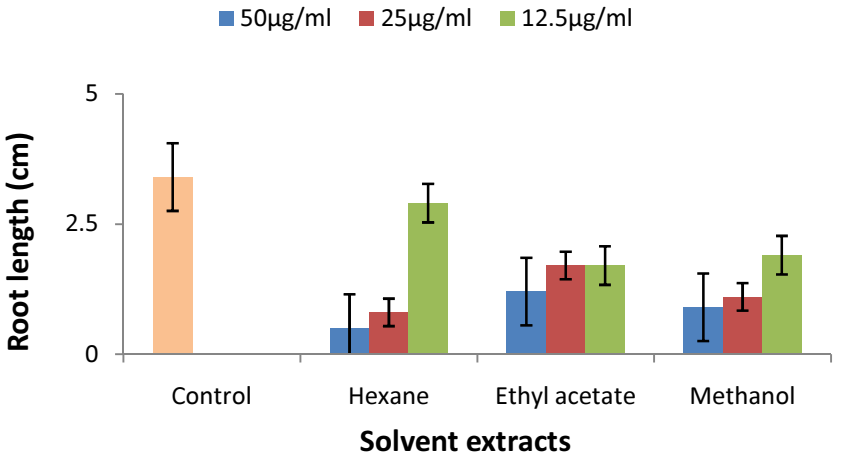


Figure 4.10: Effect of hexane, ethyl acetate and methanol extract of *R. roesleri* on root length of *P. minor*

Herbicidal potential of solvent extracts: Discussion

Solvents extracts of *P. reticulata* and *R. roesleri* exhibited markedly variable herbicidal activity against seedling growth of the weed species. More inhibition of seed germination and seedling growth occurred at higher concentration. In general, the methanol extract of both the lichens exhibited insignificant effect on the weed growth. These findings coincided with the report of Macías *et al*., 2000 and Daniel 1999, who reported that allelopathy includes both promoting and inhibitory activities and is a concentration-dependent phenomenon.

Extracts of *R. roesleri* inhibited the germination of *P. minor* seeds to a certain extent. Overall growth rate of seedlings was also reduced in almost all the treatments as compared to the control. Mortality of the seedlings and percentage growth under laboratory conditions indicated the presence of allelochemicals (chemicals having allelopathic potential) of the donor plant can be harmful to the growth of seedlings of receptor plants. Marked reduction in root length was noticed in most of the seedlings compared to shoot length and germination. However, few works have been done for evaluating allelopathic effect of lichens on crop plants and weeds. So, it was inferred that, *P. reticulata* and *R. roesleri* contains phytotoxin or allelochemicals impairing the growth of weed under the study, which may also act as a potential inhibitory agent on field weed.

4.2.3 Allelopathic potential of solvent extracts on major food crops

Allelopathy is an important interference mechanism wherein one organism releases chemicals into the surrounding environment that affect the growth of other plants (Rice 1984). It plays a significant role in agro-ecosystems, and affects the growth, quality and quantity of the produce (Batish *et al.*, 2007; Kohli *et al.*, 1998 and Singh *et al.*, 2001).

As the extracts of *P. reticulata* and *R. roesleri* exhibited a detrimental effect on growth of highly resistant weed (*P. minor*) of major food crop wheat. In order to assess their effect on the major food crops (wheat, lentil and chickpea), a bioassay, was therefore, carried out under laboratory conditions.

(I) Test on wheat seeds (*Triticum aestivum)*

Seed germination

A significant increase in seed germination from 88% (in control) to 91 and 93 % was observed at low concentrations (0.625 and 1.25 µg ml^{-1}) of hexane extract of ***P. reticulata***. The seed germination at higher concentration (2.5 µg ml^{-1}) was comparable to that of the control (88%) (Table 4.10, Figure 4.11). On the other hand, ethyl acetate and methanol extracts suppressed the germination at the applied concentrations.

The ethyl acetate extract showed 69, 71 and 79 % while methanol extract exhibited 52, 58 and 72 % seed germination at 2.5, 1.25 and 0.625 µg ml^{-1} concentrations, respectively (Figure 4.11). Thus, 0.625 µg ml^{-1} of hexane extract is found to be the optimum concentration for promoting germination of the wheat seeds.

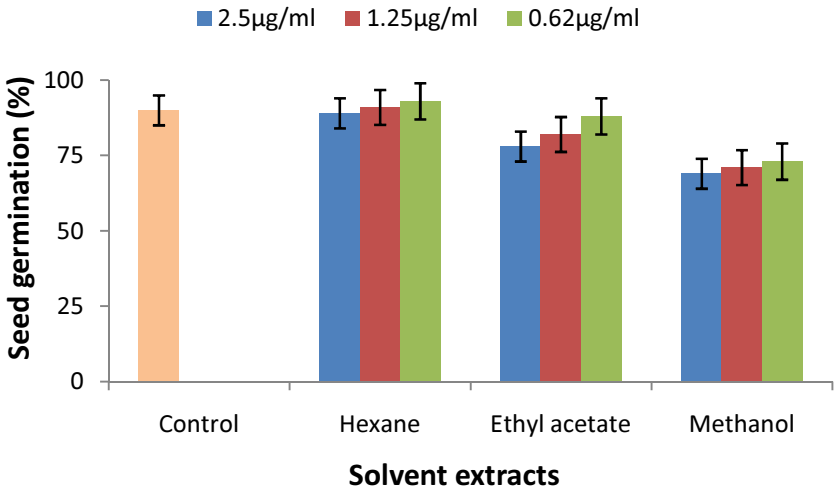


Figure 4.11: Effect of solvent extracts of *P. reticulata* on germination of wheat seeds

In case of ***R. roesleri*** (Table 4.11, Figure 4.12) a marked increase in seed germination over control (90%) was observed when hexane extract was applied at 1.25 µg ml^{-1} (91 %) and 0.625 µg ml^{-1} (93 %) concentration. Thus, 0.625 µg ml^{-1} is found to be the optimal concentration for growth promotion. However, the ethyl acetate and methanol extracts reduced the seed germination at all the applied concentrations. In case of methanol extract, there was only 69, 71 and 73 % seed germination at 2.5, 1.25 and 0.625 µg ml^{-1} concentrations.

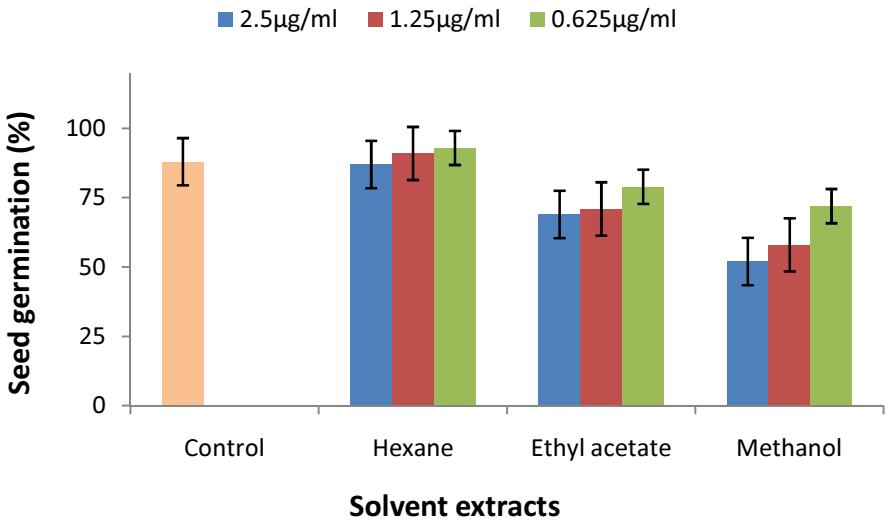


Figure 4.12: Effect of solvent extracts of *R. roesleri* on germination of wheat seeds

The above results were also statistically analyzed with Duncan Multiple Range Test, where analysis of variance gives a single overall test of difference between the groups or treatments. P values denote the significant difference between the groups at 0.01(1%) and tabulated in Table 8.

Table 4.8: Analysis of variance of % seed germination of wheat seeds

Lichen species	Source of Variation	DF	SS	MS	F	P
P. reticulata	**Between groups**	9	7130.817	792.231	604.911	< 0.001
	Residual	30	39.290			
	Total	39	7169.37			
R. roesleri	**Between groups**	9	2908.900	323.211	114.075	< 0.001
	Residual	30	85.000	2.833		
	Total	39	2993.900			

DF: degrees of freedom, SS: sum of squares, MS: mean squares, F: test statistic, P: probability, The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P < 0.001$).

Shoot length

The influence of hexane, ethyl acetate and methanol extracts of ***P. reticulata*** and ***R. roesleri*** on growth of shoot length of wheat was also studied and presented in figure 4.13 & 4.14. In case of ***P. reticulata***, shoot length of wheat showed a similar pattern as was observed for germination. There was an increase in length at lower concentration except in case of methanol extract; the length was comparable to control. In control, shoot length observed was 13.1 cm, a distinct increase in shoot length to 14.1 and 15.5 cm was observed at 1.25 and 0.625 μg ml^{-1} concentrations (Table 4.10, Figure 4.13) respectively. The ethyl acetate extract showed prominent increase in shoot length from 13.1 cm (control) to 14.7 cm at 0.625 μg ml^{-1} concentration.

In case of ***R. roesleri****,* (Table 4.11, Figure 4.14) an increase in shoot length from 13.1 (control) to 13.4, 13.8 and 14.2 cm was observed when hexane extract was applied at 2.5, 1.25, 0.625 μg ml^{-1} concentrations respectively. Increase in shoot length was found to be more at lower concentration as compared to higher concentrations. Ethyl acetate and methanol extracts showed a decrease in shoot length at a higher concentration of 2.5 and 1.25 μg ml^{-1}, while, a minor increase in shoot length was observed at lowest concentration (0.625 μg ml^{-1}). It is noteworthy, that hexane extract was showing remarkable growth at an optimal concentration of 0.625 μg ml^{-1}. The results of statistical analysis are reported in Table 4.9.

Table 4.9: Analysis of variance of shoot length of wheat seeds

Lichen species	Source of Variation	DF	SS	MS	F	P
P. reticulata	**Between groups**	9	139.446	792.231	604.911	< 0.001
	Residual	30	22.527			
	Total	39	161.974			
R. roesleri	**Between groups**	9	3031.76	323.211	114.075	< 0.001
	Residual	30	10005.41	2.833		
	Total	39	13037.17			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P < 0.001$).

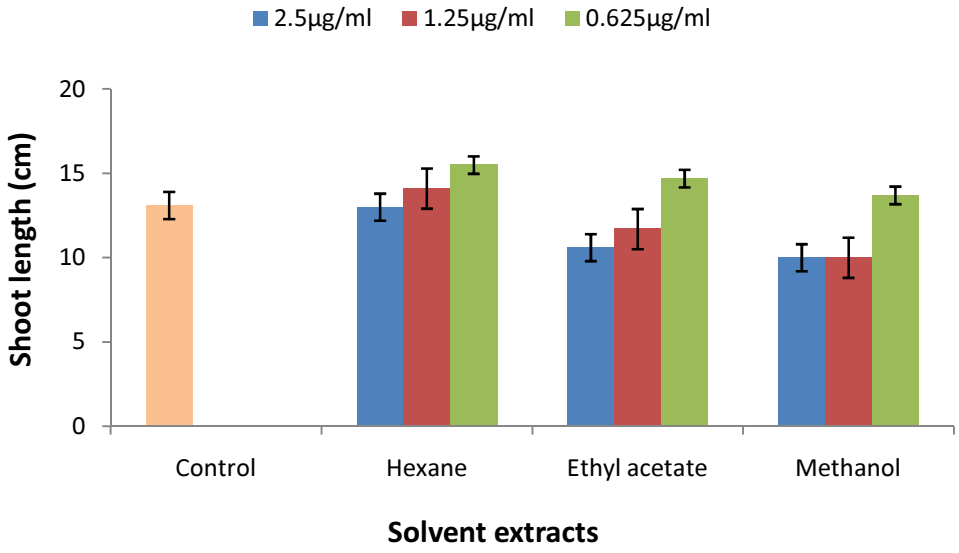


Figure 4.13: Effect of solvent extracts of *P. reticulata* on shoot length of wheat seeds

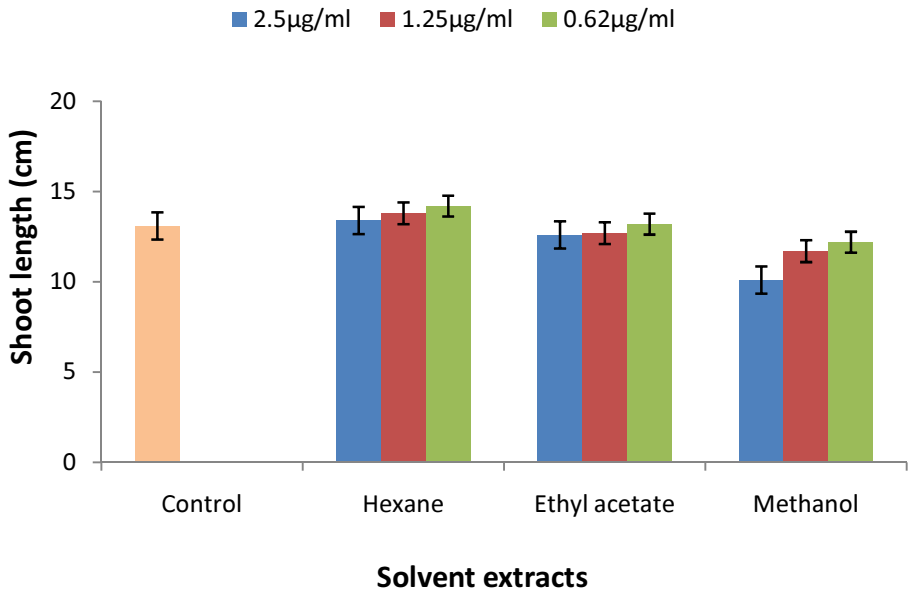


Figure 4.14: Effect of solvent extracts of *R. roesleri* on shoot length of wheat seeds

Root length

In case of ***P. reticulata***, a substantial increase in root length from 4.6 cm (control) to 8.7 cm was observed when hexane extract was applied at 1.25 µg ml^{-1} concentration (Table 4.10; Figure 4.15). With the results obtained, the optimum concentration for the hexane extract for root growth was considered as 1.25 µg ml^{-1}. A

different pattern was found, in case of ethyl acetate extract i.e., at higher concentration (2.5 µg ml^{-1}) an increase in root length from 4.6 cm (control) to 5.1 cm was observed, while at 1.25 and 0.625 µg ml^{-1} dosage, a decrease in root length from 4.6 cm (control) to 3.3 and 3.9 cm was observed respectively. However, higher concentrations of methanol extract was found to be growth retarding, and it was only at lower concentration (0.625 µg ml^{-1}) that helped in promotion of root growth (length to 5.8 cm).

Table 4.10: Allelopathic potential of solvent extracts of *P. reticulata* on wheat seeds

Extract	Concentration (µg ml^{-1})	%Germination (%Inhibition)	Shoot length (cm) (Inhibition %)	Root length (cm) (Inhibition %)
Hexane	**Control**	88 ± 1.58 (0.00)	13.1 ± 0.60 (0.00)	4.6 ±0.60 (0.00)
	2.5	87 ± 0.26 (1.13)	13.0 ± 0.17 (0.76)	6.4 ±1.50 (-39.13)
	1.25	91 ± 1.90 (-3.40)	14.1±0.26 (-18.32)	8.7 ±0.52 (-89.13)
	0.625	93 ± 1.80 (-5.68)	15.5 ±0.30 (-7.63)	6.4 ± 0.70 (-39.13)
Ethyl acetate	**Control**	88 ± 1.58 (0.00)	13.1 ± 0.60 (0.00)	4.6 ±0.60 (0.00)
	2.5	69 ± 1.32 (21.59)	10.6±1.12 (-10.68)	5.1 ± 0.26 (-10.86)
	1.25	71 ±1.35 (19.31)	11.7±0.81 (19.08)	3.3 ± 1.11 (-28.26)
	0.625	79 ±1.04 (10.22)	14.7±0.55 (-12.21)	3.9 ± 0.21 (-15.21)
Methanol	**Control**	88 ± 1.58 (0.00)	13.1 ± 0.60 (0.00)	4.6 ± 0.60 (0.00)
	2.5	52 ± 1.76 (40.90)	10.0±1.80 (23.06)	2.6 ± 0.10 (43.47)
	1.25	58 ± 1.50 (34.09)	10.0±1.65 (23.06)	2.3 ±0.62 (50.0)
	0.625	72 ± 0.36 (18.18)	13.7 ±1.57 (-4.58)	5.8 ± 0.36 (-26.08)

Mean percentage seed germination, shoot length and root length used for calculation, is an average of three replicates and their standard deviation ranged from 0.26 to 1.76, 0.17 to 1.65 and 0.10 to 1.50. Values given in parentheses indicates % Inhibition (Decrease in growth) in terms of seed germination, shoot length and root length as compared to the control.

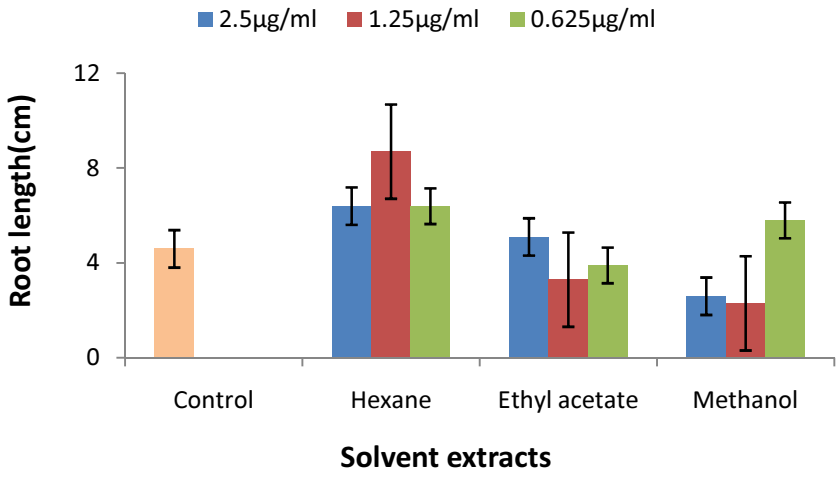


Figure 4.15: Effect of solvent extracts of *P. reticulata* on root length of wheat seeds

Table 4.11: Effect of solvent extracts of *R. roesleri* on wheat germination, shoot length and root length

Extract	Concentration (µg ml^{-1})	%Germination (% Inhibition)	Shoot length (cm) (Inhibition %)	Root length (cm) (Inhibition %)
Hexane	**Control**	90 ± 1.00 (0.00)	13.1 ± 0.60 (0.00)	4.6 ± 0.60 (0.00)
	2.5	89 ± 2.00 (1.11)	13.4 ± 0.41 (-2.29)	5.8 ± 0.36 (-26.08)
	1.25	91 ± 1.90 (-1.11)	13.8 ± 0.26 (-5.34)	6.2 ± 1.70 (-34.78)
	0.625	93 ± 1.73 (-3.33)	14.2 ± 0.60 (-8.39)	6.4 ± 0.70 (-39.13)
Ethyl acetate	**Control**	90 ± 1.00 (0.00)	13.1 ± 0.60 (0.00)	4.6 ± 0.60 (0.00)
	2.5	78 ± 1.32 (13.33)	12.6 ± 0.34 (3.81)	4.8 ± 0.30 (-4.34)
	1.25	82 ± 2.64 (1.21)	12.7 ± 1.41 (3.05)	5.4 ± 0.75 (-17.39)
	0.625	88 ± 2.64 (2.22)	13.2 ± 0.26 (-0.76)	5.9 ± 0.72 (-28.26)
Methanol	**Control**	90 ± 1.00 (0.00)	13.1 ± 0.60 (0.00)	4.6 ± 0.60 (0.00)
	2.5	69 ± 1.73 (23.33)	10.1 ± 0.30 (22.9)	3.6 ± 0.36 (21.73)
	1.25	71 ± 1.73 (21.10)	11.7 ± 0.81 (10.68)	4.1 ± 0.26 (10.86)
	0.625	73 ± 1.00 (18.80)	12.2 ± 0.34 (6.87)	5.2 ± 1.01 (-13.04)

Mean percentage seed germination, shoot length and root length used for calculation, is an average of three replicates and their standard deviation ranged from 1.00 to 2.64, 0.26 to 1.41 and 0.26 to 1.70. Values given in parentheses indicates % Inhibition (decrease in growth) in terms of seed germination, shoot length and root length as compared to the control.

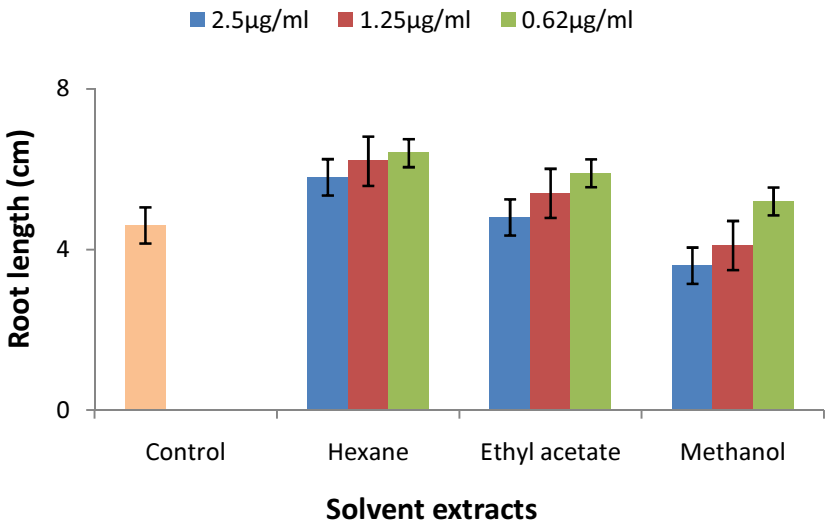


Figure 4.16: Effect of solvent extracts of *R. roesleri* on root length of wheat seeds

For all the extracts of ***R. roesleri***, 0.625 µg ml^{-1} is the optimum concentration for the growth of root length. Increase in root length was found to be more pronounced at lower concentrations as compared to higher concentrations. A significant increase in the root length to 5.8, 6.2 and 6.4 cm as compared to control (4.6 cm) was observed when hexane extract was applied at various concentrations (Table 4.11, Figure 4.16). Ethyl acetate and methanol extract increased the root length to 5.2 cm 5.9 cm at 0.625 µg ml^{-1} concentration. Above findings was also subjected to statistical analysis and is tabulated in Table 4.12.

Table 4.12: Analysis of variance for root length of wheat seeds

Lichen species	Source of Variation	DF	SS	MS	F	P
	Between groups	9	141.956	15.733	44.389	< 0.001
P. reticulata	**Residual**	30	10.660	0.355		
	Total	39	152.616			
	Between groups	9	30.480	3.387	8.038	< 0.001
R. roesleri	**Residual**	30	12.640	0.421		
	Total	39	43.120			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P < 0.001$).

(II) Test on lentil seeds (*Lens culinaris* Medikus)

Effect on seed germination

The effect of various solvent extracts of ***P. reticulata*** on seed germination of lentil seeds is shown in Table 4.13. A minor increase in seed germination from 90% (control) to 91 and 92 % was observed when the hexane extract of ***P. reticulata*** was applied at 1.25 and 0.625 μg ml^{-1} concentrations. While, ethyl acetate extract showed considerable decrease in seed germination from 90 % (control) to 87 and 70 % when applied at concentrations of 1.25 and 0.625 μg ml^{-1} respectively. Methanol extract also showed similar pattern at higher concentration of 2.5 μg ml^{-1} (95 %). The results of statistical analysis for the above findings are shown in Table 4.14.

Table 4.13: Allelopathic potential of solvent extracts of *P. reticulata* on lentil seeds

Extract	Concentration (μg ml^{-1})	% Germination (% Inhibition)	Shoot length (cm) (Inhibition %)	Root length (cm) (Inhibition %)
Hexane	**Control**	90 ±1.86 (0.00)	16.8 ± 0.07 (0.00)	8.6 ± 0.55 (0.00)
	2.5	88 ±1.58 (2.22)	15.2 ± 1.21 (9.52)	8.0 ± 1.73 (6.97)
	1.25	91 ±1.00 (7.77)	16.6 ± 0.26 (1.19)	9.2 ± 2.91 (-6.97)
	0.625	92 ± 2.08 (8.88)	18.50 ±2.45 (-10.11)	9.0 ± 1.04 (-12.79)
Ethyl acetate	**Control**	90 ± 1.86 (0.00)	16.8 ± 0.07 (0.00)	8.6 ± 0.55 (0.00)
	2.5	91 ± 1.90 (-1.11)	14.8 ± 0.40 (11.90)	7.8 ± 0.65 (9.30)
	1.25	87 ± 0.26 (3.33)	16.9 ± 1.30 (-0.59)	8.3 ± 1.68 (3.48)
	0.625	70 ± 0.32 (22.22)	17.6 ± 0.60 (-4.76)	8.8 ± 1.70 (-2.32)
Methanol	**Control**	90 ±1.86 (0.00)	16.8 ± 0.07 (0.00)	8.6 ± 0.55 (0.00)
	2.5	95 ± 1.93 (-5.55)	15.8 ± 1.40 (5.95)	8.8 ± 2.07 (-2.32)
	1.25	89 ± 1.05 (1.11)	18.8 ±1.13 (-11.90)	9.2 ± 1.15 (-6.97)
	0.625	88 ± 1.15 (2.22)	19.2 ±1.85 (-14.28)	10.1 ± 0.88 (-17.44)

Mean percentage seed germination, shoot length and root length used for calculation, is an average of three replicates and their standard deviation ranged from 0.26 to 2.08, 0.07 to 2.45 and 0.55 to 2.91 respectively. Values given in parentheses indicates % Inhibition (decrease in growth) in terms of seed germination, shoot length and root length as compared to the control

Table 4.14: Analysis of variance of % seed germination of lentil seeds

Lichen species	Source of Variation	DF	SS	MS	F	P
P. reticulata	**Between groups**	9	1668.3621	185.374	126.715	< 0.001
	Residual	30	43.887	1.463		
	Total	39	1712.250			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P < 0.001$).

Effect on shoot length

A prominent increase in shoot length was observed for all the extracts of ***P. reticulata*** when applied at 0.625 µg ml^{-1} concentrations. In case of hexane extract, the shoot length got increased to 18.5 cm at 0.625 µg ml^{-1}. However, a negligible difference from control was observed at higher concentrations (2.5 and 1.25 µg ml^{-1}). Ethyl acetate and methanol extract also showed similar increase in shoot length at lower concentrations. Methanol extract when applied at 1.25 and 0.625 µg ml^{-1} concentrations showed 18.8 and 19.2 cm shoot length respectively (Table 4.13). The results of statistical analysis for the above findings are represented in Table 4.15.

Table 4.15: Analysis of variance of shoot length of lentil seeds

Lichen species	Source of Variation	DF	SS	MS	F	P
P. reticulata	**Between groups**	9	80.785	8.976	8.118	<0.001
	Residual	30	33.170	1.106		
	Total	39	113.955			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P < 0.001$).

Effect on root length

In case of hexane extract of ***P. reticulata***, increase in root length was observed only at 1.25 µg ml^{-1} as compared to other concentrations. Ethyl acetate extract had negligible effect on the growth of root length. However, a remarkable increase in root length to 9.2 and 10.1 cm was observed with methanol extract at 1.25 and 0.625 µg ml^{-1} concentrations respectively (Table 4.13). Thus, a considerable increase in root length was observed with hexane and methanol extracts. The results of statistical analysis for above findings are shown in Table 4.16.

Table 4.16: Analysis of variance of root length of lentil seeds

Lichen species	Source of Variation	DF	SS	MS	F	P
	Between groups	9	18.900	2.100	1.235	< 0.311
P. reticulata	**Residual**	30	51.00	1.700		
	Total	39	69.900			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P < 0.311).

(III) Test on chickpea seeds (*Cicer arietinum* L.)

Effect on seed germination

In case of hexane extract of ***R. roesleri***, a significant increase in seed germination (90, 92 and 94 %) was observed at 2.5, 1.25 and 0.625 µg ml^{-1} concentrations as compared to control (88%). It was found that the ethyl acetate extract exhibited insignificant effect on seed germination, at applied concentrations (Table 4.17). However, the methanol extract when applied at 2.5 and 1.25 µg ml^{-1} concentrations showed inhibitory effect, with the decrease in seed germination to 72 and 78 % respectively. The statistical analysis of above results is shown in Table 4.18.

Effect on shoot length

Significant stimulatory effect on shoot length was recorded for hexane and ethyl acetate extract at 2.5, 1.25 and 0.625 µg ml^{-1} concentrations. While, varying effect on shoot length was recorded with methanol extract (Table 4.17). The results of statistical analysis are shown in Table 4.19.

Table 4.17: Effect of solvent extracts of *R. roesleri* on germination, shoot length and root length of chick pea seeds

Extract	Concentration (µg ml^{-1})	% Germination (Inhibition %)	Shoot length (cm) (Inhibition %)	Root length (cm) (Inhibition %)
Hexane	**Control**	88 ± 1.15 (0.00)	10.0 ± 0.30 (0.00)	3.2 ± 0.43 (0.00)
	2.5	90 ± 1.86 (-2.27)	10.1 ± 0.26 (-1.00)	3.6 ± 0.81 (-12.50)
	1.25	92 ± 1.60 (-4.54)	10.7 ± 0.26 (-7.00)	3.8 ± 0.30 (-18.75)
	0.625	94 ± 1.00 (-6.81)	11.4 ± 0.62 (-14.00)	4.1 ± 0.70 (-28.12)
Ethyl acetate	**Control**	88 ± 1.15 (0.00)	10.0 ± 0.30 (0.00)	3.2 ± 0.43 (0.00)
	2.5	80 ± 2.64 (9.09)	9.6 ± 0.30 (4.00)	2.9 ± 0.36 (9.37)
	1.25	87 ± 0.26 (1.13)	10.2 ± 0.81 (-2.00)	3.3 ± 0.81 (-3.12)
	0.625	91 ± 1.00 (-3.40)	10.8 ± 0.30 (-8.00)	3.5 ± 0.65 (-9.37)
Methanol	**Control**	88 ± 1.15 (0.00)	10.0 ± 0.30 (0.00)	3.2 ± 0.43 (0.00)
	2.5	72 ± 1.00 (18.18)	8.7 ± 0.85 (13.00)	2.6 ± 0.26 (18.75)
	1.25	78 ± 2.64 (11.36)	9.4 ± 0.43 (6.00)	2.8 ± 0.30 (12.50)
	0.625	91 ± 1.73 (-3.40)	10.6 ± 0.72 (-6.00)	3.0 ± 0.70 (6.25)

[a]Mean percentage germination, shoot length and root length is an average of four replicates and its standard error ranged from ± 0.26 to 2.64, 0.26 to 0.85 and 0.26 to 0.81 respectively. Values given in parentheses indicates % Inhibition (decrease in growth) in terms of seed germination, shoot length and root length as compared to the control

Table 4.18: Analysis of variance of % seed germination of chick pea seeds

Lichen species	Source of Variation	DF	SS	MS	F	P
	Between groups	9	1854.025	206.003	108.138	< 0.001
R. roesleri	**Residual**	30	57.150	1.905		
	Total	39	1911.175			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P < 0.001).

Table 4.19: Analysis of variance of shoot length of chickpea seeds

Lichen species	Source of Variation	DF	SS	MS	F	P
	Between groups	9	21.932	2.437	12.95	<0.001
R. roesleri	**Residual**	30	5.647	0.188		
	Total	39	27.580			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P < 0.001).

Effect on root length

The effect of hexane extract of ***R. roesleri*** on the growth of root length is significant as observed with a increase of 3.6, 3.8 and 4.1 cm compared to control (3.2 cm) at the applied concentrations. A negligible increase in root length, however, was recorded with ethyl acetate extract (Table 4.17). Hexane extract was found to be growth promoting at all the concentrations. The experimental data were statistically analyzed. The results are shown in Table 4.20.

Table 4.20: Analysis of variance of root length of chick pea seeds

Lichen species	Source of Variation	DF	SS	MS	F	P
	Between groups	9	8.0640	0.896	4.048	< .002
R. roesleri	**Residual**	30	6.640	0.221		
	Total	39	14.704			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P < 0.002).

Allelopathic potential of solvent extracts: Discussion

Allelopathic potential of lichen extracts on the sensitive dicot legume crops, lentil and chick pea and an economically important monocot crop, wheat was determined. Wheat is a cereal grain grown on more land area than any other commercial crop and is the most important staple food for humans. Globally, wheat is the leading source of vegetable protein in human food, having higher protein content than maize, rice and other major cereals. Among various pulse crops marketed as human food, chickpea and lentil are consumed second in the world. Chickpea (*Cicer arietinum* L.) and lentil (*Lens culinaris* Medikus) are the part of legume (fabaceae) family. These provide a high quality protein to the people in developing countries. Chick pea and lentil are considered environment friendly because of their reduced dependence on fertilizers; they are able to obtain much of their nitrogen requirement from the atmosphere by forming a symbiotic relationship with rhizobium bacteria in the soil.

The study revealed that solvent extracts of *P. reticulata* and *R. roesleri* are not phytotoxic to wheat, lentil and chickpea seedlings rather hexane and ethyl acetate extracts of both the species have stimulatory effect on the growth of tested seedlings. Hexane extract was found to have more stimulatory effect followed by the ethyl acetate. The effect is more pronounced on lower dosages than at higher dosages. Thus hexane and ethyl acetate extracts may contain secondary metabolites, which have growth promoting activity at low concentrations. 0.625 μg ml^{-1} of hexane extract was found to be the optimum concentration for promoting the growth of tested seeds.

Till date, few works were carried out showing the allelopathic activity of the lichens (Pyatt, 1967; Brown and Mikola, 1974; Fisher, 1979; Whiton and Lawr 1982, 1984 and Goldner *et al*., 1986). Although several former works with lichen extracts (Rondon, 1966; Dauriac and Rondon, 1976; Tolpysheva, 1984a, 1984b and Vainshtein and Tolpysheva, 1992), including the phosphate buffer extract (Vicente, 1988) observed the absence of stimulatory and inhibitory effect of these extracts on the germination rate. Those observations corroborate the idea that the chemical composition and the concentration are responsible for the biological response of the lichen. Current investigation establishes lichens as potential source of agrochemicals. Their products can be used safely as crop protectants with no detrimental effects on the major food crops.

4.2.4 Antibacterial activity

The crude extracts of lichen species exhibited versatile agrochemical activities. Thus encouraged by these results, hexane, ethyl acetate and methanol extracts of *P. reticulata* and *R. roesleri* were evaluated for antimicrobial activity against four pathogenic bacteria namely *E. coli, S. aureus*, *S. viridochromogenes* (Tü 57) and *B. subtilis* by disc diffusion method at 40 µg/disc concentration.

Hexane extract of *P. reticulata* (Table 4.21) was found to be highly effective against *E. coli* and *S. aureus* with inhibition zone of 21 mm and 20 mm respectively, whereas no activity was observed against *S. viridochromogenes* and *B. subtilis.* Ethyl acetate and methanol extract did not show any inhibition against all the tested bacteria.

Table 4.21: Antibacterial activity of solvents extracts of *P. reticulata*

Pathogenic bacteria	Inhibition zone (mm) at 40 µg/disc		
	Solvent extracts		
	Hexane	Ethyl acetate	Methanol
Control	--	--	--
Escherichia coli	+++	--	--
Staphylococcus aureus	+++	--	--
Streptomyces viridochromogenes Tü	--	--	--
Bacillus subtilis	--	--	--

(+) 0-10 mm: weakly active, (++) 11-20 mm: active, (+++) 20 mm and above: highly active

Table 4.22: Antibacterial activity of solvent extracts of *R. roesleri*

Pathogenic bacteria	Inhibition zone (mm) at 40 µg/disc		
	Solvent extracts		
	Hexane	Ethyl acetate	Methanol
Control	--	--	--
Escherichia coli	--	+	--
Staphylococcus aureus	+++	--	--
Streptomyces viridochromogenes Tü	--	++	--
Bacillus subtilis	++	--	+

(+) 0-10 mm: weakly active, (++) 11-20 mm: active, (+++) 20 mm and above: highly active

Among various extracts of *R. roesleri* (Table 4.22), hexane extract was found to be active against *S. aureus* and *B.subtilis* with inhibition zone of 20 and 14 mm respectively. Whereas ethyl acetate extract was found moderately active against *E. coli* and *S. viridochromogenes* with an inhibition zone of 10 and 14 mm respectively. Methanol extract was found slightly active against *B. subtilis* and inactive against *E. coli, S. aureus* and *S. viridochromogenes* (Table 4.22).

The present study confirmed the presence of antibacterial substances in the extracts of *P. reticulata* and *R. roesleri*. Among all the extracts, hexane extract was found to exhibit maximum activity. Similar results were obtained by Sati *et al*., 2011 on *Parmelia nilgherrense* where, non polar extracts were found effective against bacteria by showing a marked zone of inhibition while, the polar aqueous extracts was found ineffective. The probable reason for low activity of polar extracts may be attributed to the poor solubility of lichen metabolites in water.

4.3 Isolation and characterization of chemical constituents from lichens

Bioassay guided approach was followed to isolate the compounds present in crude extracts. Biologically active, hexane and ethyl acetate extracts were subjected to various separation techniques. Initially, preliminary analyses of the extracts were achieved by reversed phase high performance liquid chromatography (RP-HPLC) and liquid chromatography-Mass (LC MS) analysis to know the abundance and nature of metabolites present.

4.3.1 Preliminary Analysis

4.3.1.1 HPLC analysis of crude extracts

Hexane and ethyl acetate extracts of all the four lichen species were subjected to RP-HPLC. Each of the extract showed the presence of a number of metabolites which were further separated by column chromatography. Figures 4.17, 4.19, 4.21, 4.23 shows the elution profile of the hexane extracts of *P. reticulata*, *R. roesleri*, *U. longissima* and *S. himalayense* respectively which shows the retention time and UV spectrum of different metabolites present while, figures 4.18, 4.20, 4.22 and 4.24 shows the elution profile of the ethyl acetate extracts of *P. reticulata*, *R. roesleri*, *U.*

longissima and *S. himalayense* respectively which shows the retention time and UV spectrum of various metabolites present.

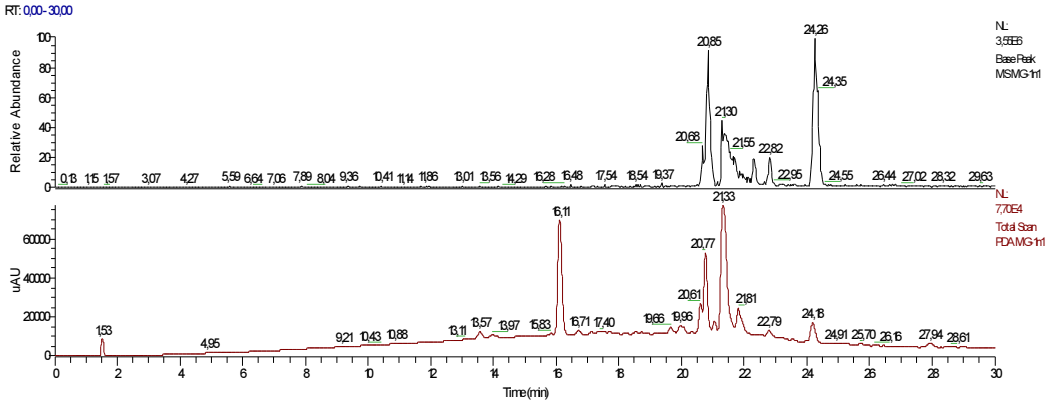


Figure 4.17: HPLC chromatogram of hexane extract of *P. reticulata*

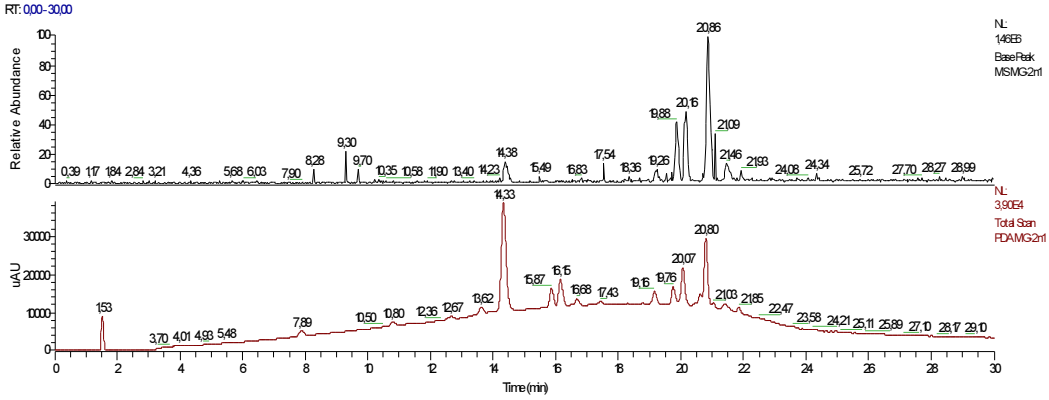


Figure 4.18: HPLC chromatogram of ethyl acetate extract of *P. reticulata*

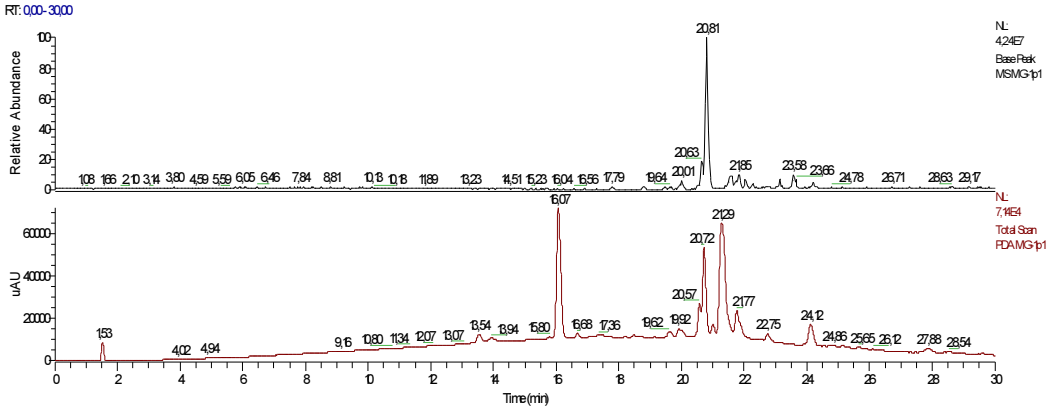


Figure 4.19: HPLC chromatogram of hexane extract of *R. roesleri*

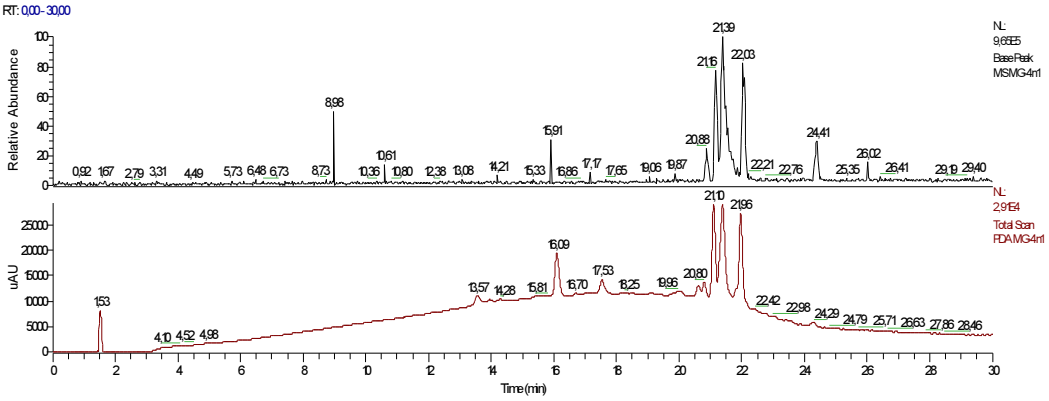


Figure 4.20: HPLC chromatogram of ethyl acetate extract of *R. roesleri*

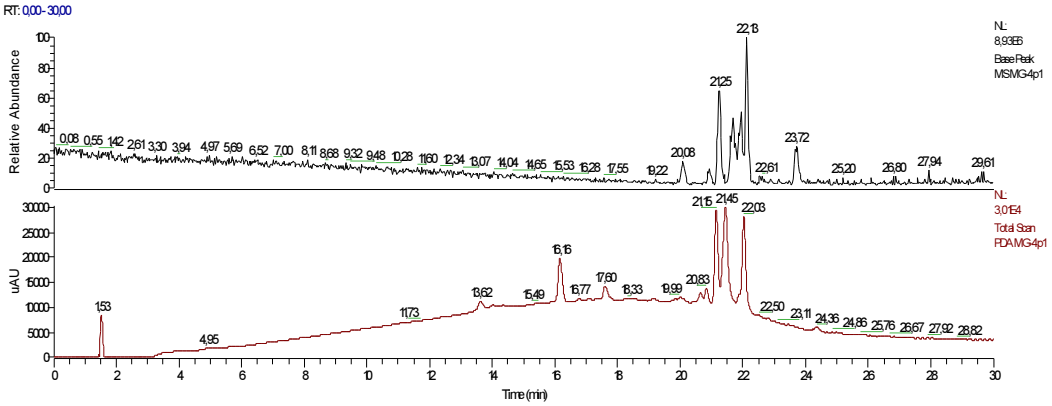


Figure 4.21: HPLC chromatogram of hexane extract of *U. longissima*

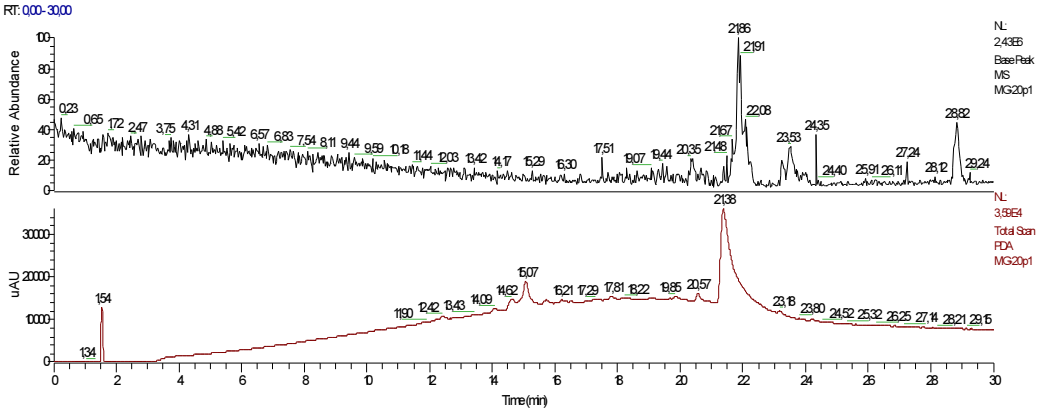


Figure 4.22: HPLC chromatogram of ethyl acetate extract of *U. longissima*

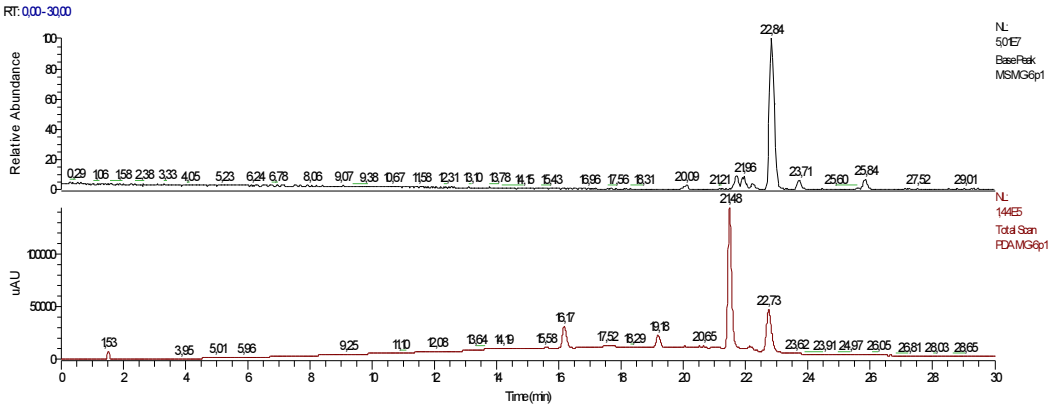


Figure 4.23: HPLC chromatogram of hexane extract of *S. himalayense*

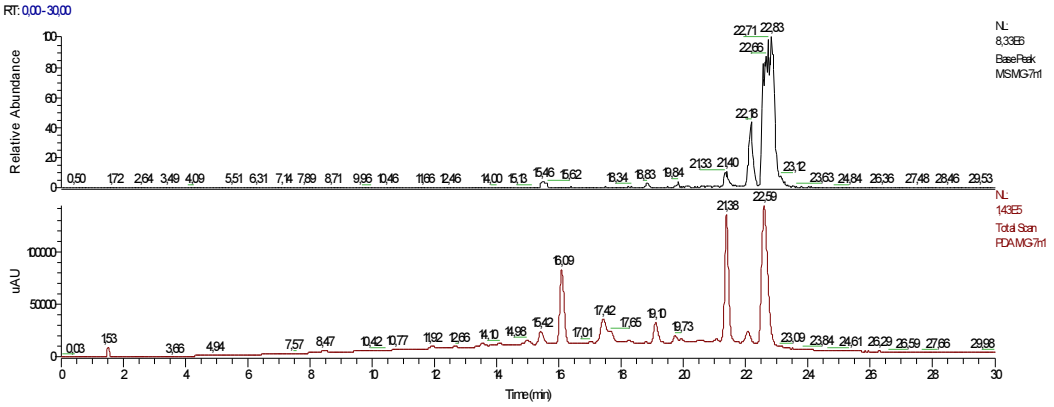


Figure 4.24: HPLC chromatogram of ethyl acetate extract of *S. himalayense*

4.3.1.2 LC-MS analysis of crude extracts

The hexane extracts of *P. reticulata* and *R. roesleri* were further subjected to the LC-MS analysis. LC-MS analysis was carried out at a concentration of 1mg/ml of the extract employing an increasing gradient solvent system of methanol and water. Figure 4.25a shows a LC chromatogram and two dimensional plots of UV scan (Figure 4.25b) describing the relative abundance of compounds with respect to their retention time. The 3D plot (Figure 4.25c), represents the retention time, relative abundance and mass range of chemical constituents (metabolites) in X, Y and Z axes respectively, present in the hexane extract of *P. reticulata*. Similarly, Figure 4.26 represents elution profile and the mass spectrum of the hexane extract of *R. roesleri.* Figure 4.26a represents the LC chromatogram, 4.26b represents the two dimensional plots of UV scan and 4.26c represents the 3D plot with the retention time, relative

abundance and mass range of chemical constituents (metabolites) in X, Y and Z axes respectively.

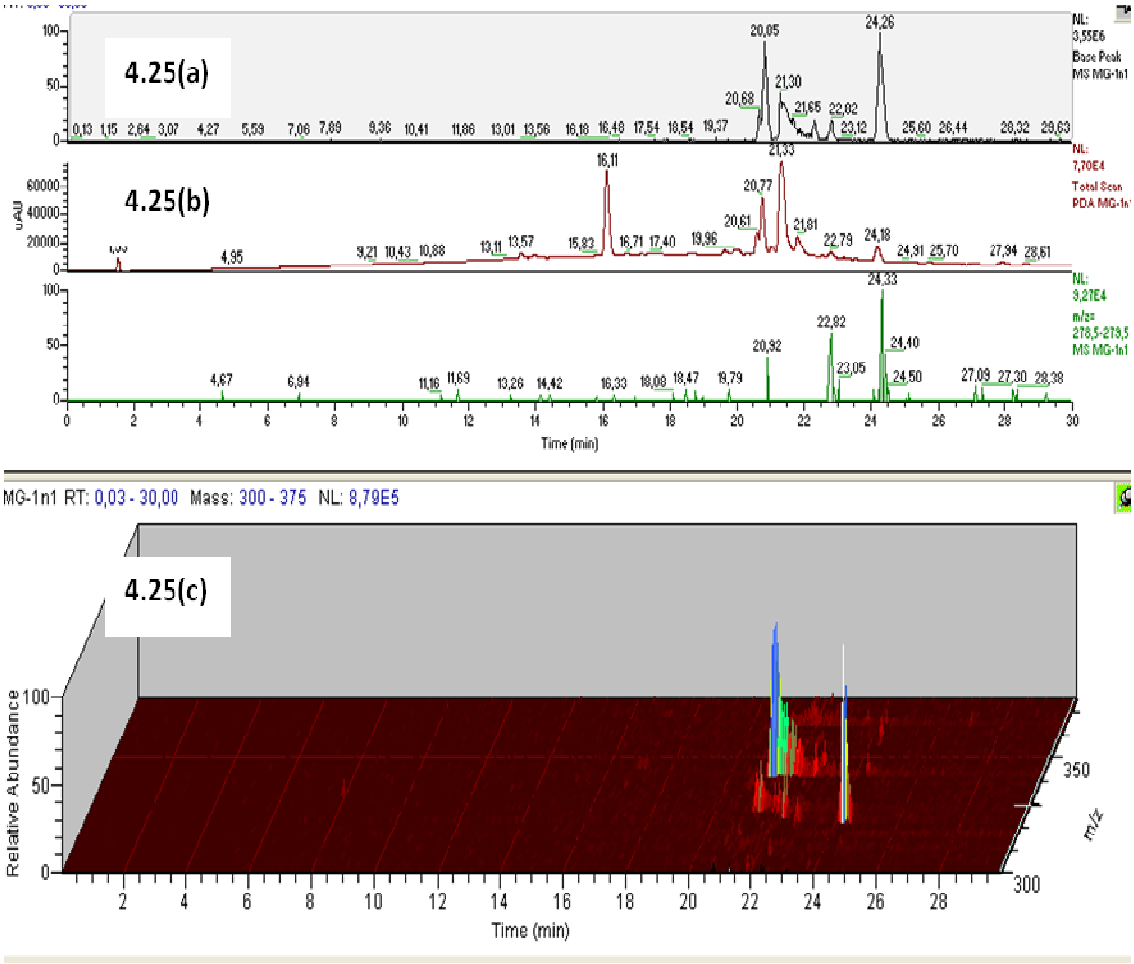


Figure 4.25: LC-MS analysis of the hexane extract of *P. reticulata* employing an increasing gradient solvent system of methanol and water. Where 4.25(a) represents the LC chromatogram, 4.25(b) represents the two dimensional plots of UV scan and 4.25(c) represents the 3D plot

A more detailed analysis of the LC chromatogram of both the species, revealed that retention time of most of the metabolites were in the range of 20-30 min, suggesting that the metabolites present have non polar characteristics. It is also worth mentioning that the secondary metabolites of hexane extracts have lower molecular weight range, *m/z* 150-500.

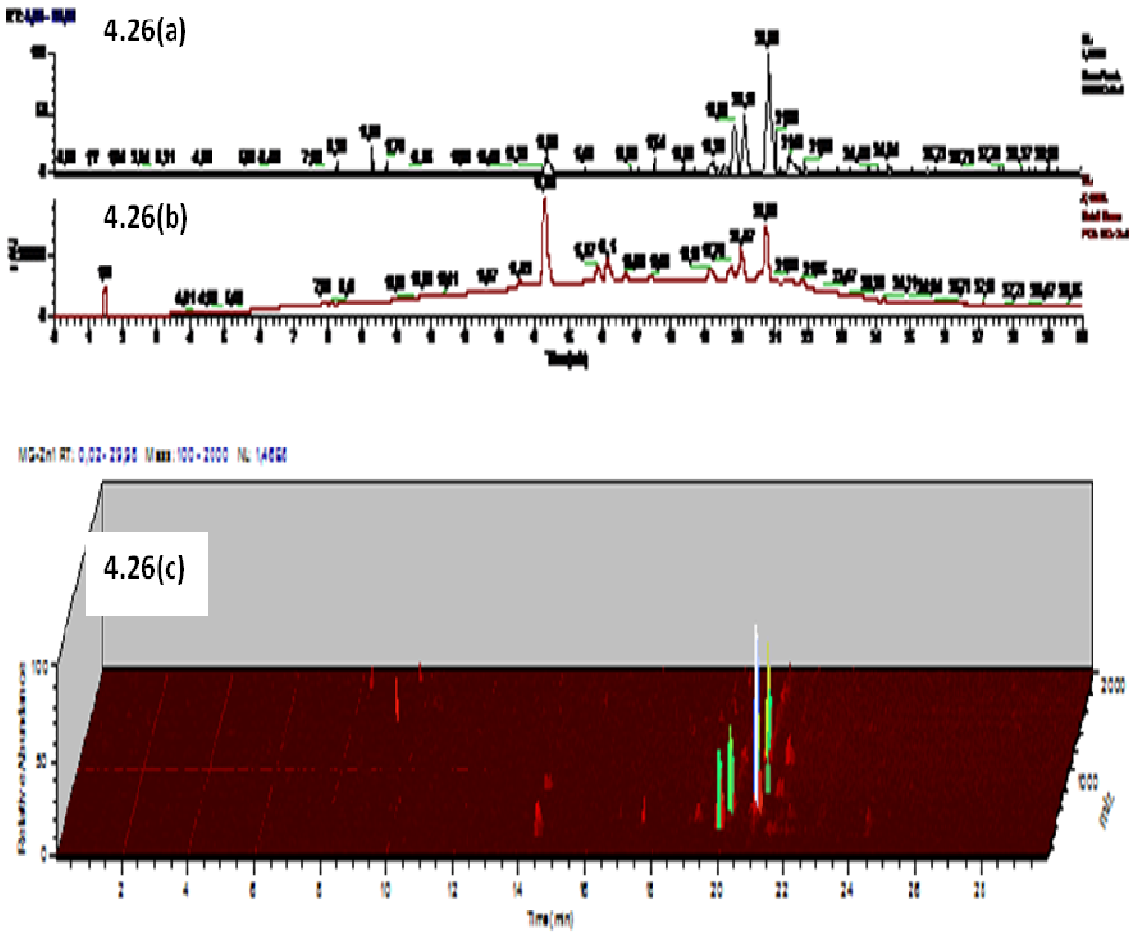


Figure 4.26: LC-MS analysis of the hexane extract of *R. roesleri* employing an increasing gradient solvent system of methanol and water, where 4.26(a) represents the LC chromatogram, 4.26(b) represents the two dimensional plots of UV scan and 4.26(c) represents the 3D plot.

4.3.2 Column chromatography: Isolation of chemical constituents

The hexane and ethyl acetate extracts of all the lichens were subjected to column chromatography. The mobile phase was gradually varied from non-polar to polar eluent. Various compounds isolated, were further purified and characterized. A detailed approach for the isolation and characterization of various constituents from the extracts is discussed below:

4.3.2.1 Isolation and identification of chemical constituents from solvent extracts of *P. reticulata*

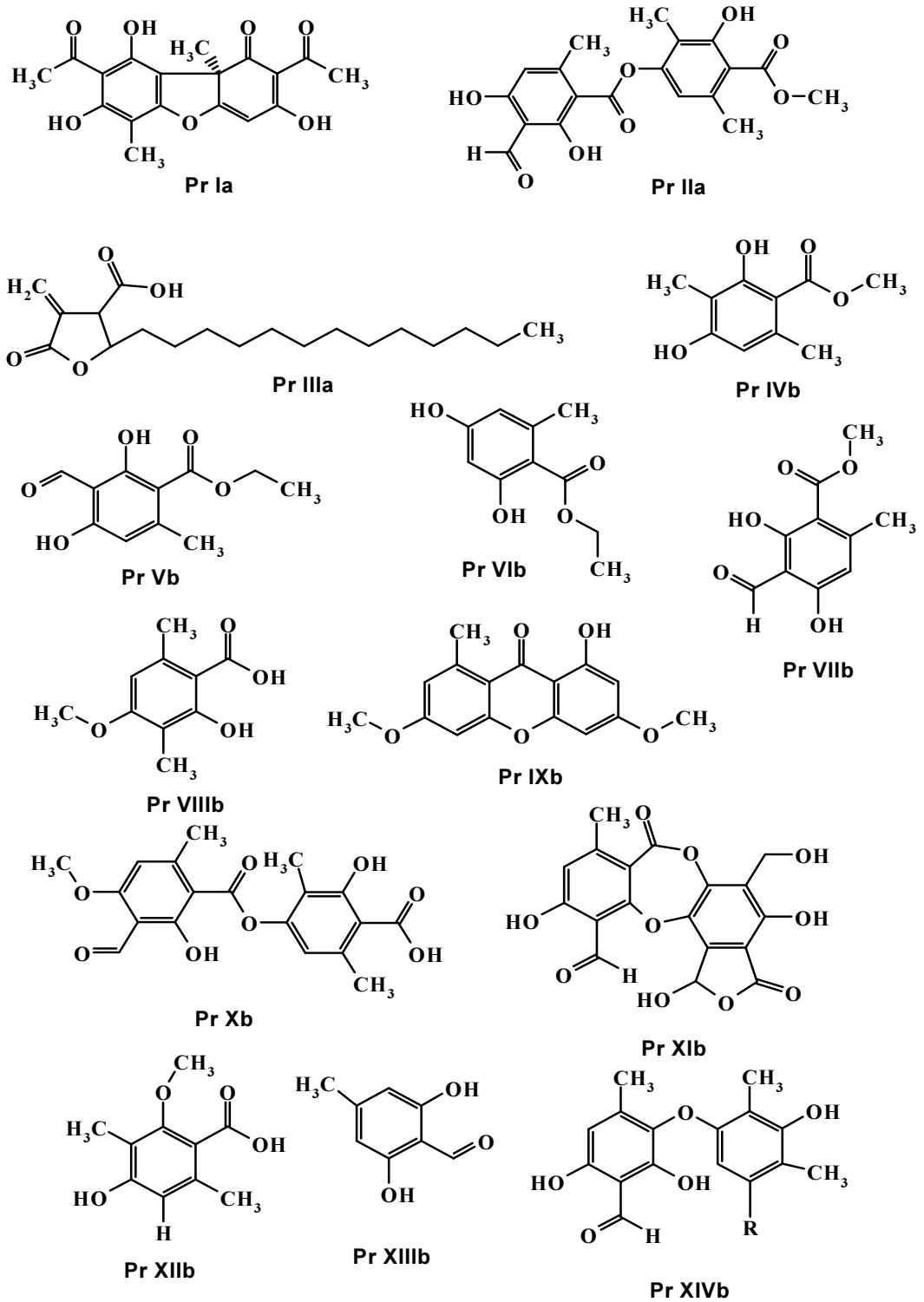


Figure 4.27: Chemical constituents isolated from *P. reticulata*

Identification of chemical constituents from hexane extract

Pr Ia: Elution of column with hexane: dichloromethane (1:1, 200 ml), gave an amorphous powder, which on crystallization from hot acetone resulted in pale yellow needle shaped crystals. $\mathbf{R_f}$ = 0.81 [TLC solvent system, toluene:acetic acid (170:30 v/v]. **m.p**. 200-206 ^{o}C. **^{1}H NMR ($CDCl_3$) δ**: 18.84 (s, 1H, OH), 13.29 (s, 1H, OH-8), 11.01 (s, 1H, OH-10), 5.96 (s, 1H, H-4), 2.68 (s, 3H, H-18), 2.65 (s, 3H, H-15), 2.09 (s, 3H, H-16), 1.75 (s, 3H, H-13). **^{13}C NMR ($CDCl_3$) δ**: 201.6 (CO, C-14), 200.1 (CO, C-17), 197.9 (CO, C-1), 191.5 (C_q, C-3), 179.2 (C_q, C-5), 163.8 (C_q, C-8), 157.4 (C_q, C-10), 155.1 (C_q, C-6), 109.3 (C_q, C-9), 105.2 (C_q, C-2), 103.9 (C_q, C-11), 101.5 (C_q, C-7), 98.3 (CH-4), 59.1 (C_q-12), 32.2 (CH_3-13), 31.3 (CH_3-18), 27.9 (CH_3-15), 7.6 (CH_3-16). **(-) ESI-MS**: *m/z* 343 {$[M-H]^-$, 60}. **(+) HR ESI-MS**: *m/z* 367.07882 $[M+Na]^+$ (calculated for $C_{18}H_{16}O_7Na$).

The ^{1}H NMR revealed three downfield singlet at δ 11.01, 13.29 and 18.84, which correspond to three intra-molecular hydrogen bonds, two singlets at δ 2.68 and 2.65 for the two $COCH_3$ group protons and two singlets for the CH_3 group protons at δ 2.09 and 1.75. The spectra also showed a singlet at δ 5.96 for an aromatic proton. The ^{13}C NMR spectrum revealed the presence of eighteen carbon atoms; four methyl carbons, thirteen non-protonated unsaturated carbons and one protonated unsaturated carbon. It also revealed the presence of three carbonyl carbons at δ 201.6, 200.1 and δ 197.9. (-)ESI-MS spectrum showed molecular ion peak at *m/z* 343.0 $[M-H]^-$ while the HR-ESI MS *m/z* gave a value at 367.0788 $[M+Na]^+$ corresponding for the molecular formula $C_{18}H_{16}O_7Na$. The ^{1}H NMR and mass spectral data for the Pr Ia were identical to those of ***iso*-usnic acid** (Kutney *et al*., 1976) and thus was identified as **2,9-diacetyl-8,10-dihydroxy-7,12b-dimethyl-1,3-(2H,12bH)-dibenzofurandione** (Figure 4.27, Pr Ia).

Pr IIa: Further elution of column with hexane: dichloromethane (1:1, 100 ml) gave a colorless crystalline compound, which was purified by recrystallization with toluene. $\mathbf{R_f}$ = 0.75 [TLC solvent system, toluene:acetic acid (170:30 v/v)]. **m.p.** 193-195 ^{o}C. **^{1}H NMR ($CDCl_3$) δ**: 12.53 (s, 1H, 4-OH), 12.48 (s, 1H, 2-OH), 11.93 (s, 1H, 2'-OH), 10.33 (s, 1H, H-8), 6.49 (s, 1H, H-5'), 6.38 (s, 1H, H-5), 3.96 (s, 3H, H-10'), 2.67 (s, 3H, H-9), 2.52 (s, 3H, H-9'), 2.07 (s, 3H, H-8'). **^{13}C NMR ($CDCl_3$) δ**: 193.6 (CO, C-8), 172.0 (CO, C-7'), 169.5 (CO, C-7), 168.9 (C_q, C-2), 167.3 (C_q, C-4), 162.7

(C_q, C-2'), 152.3 (C_q, C-6), 151.9 (C_q, C-4'), 139.8 (C_q, C-6'), 116.7 (C_q, C-3'), 115.9 (CH, C-5'), 112.8 (CH, C-5), 110.2 (C_q-1'), 108.5 (C_q-3), 102.8 (C_q-1), 52.3 (CH_3-10'), 25.6 (CH_3-9), 24.1 (CH_3-9'), 9.4 (CH_3-8'). **(+)HR-ESI MS**: *m/z* 397.08939 $[M+Na]^+$ calculated for $C_{19}H_{18}O_8Na$.

^{1}H NMR spectrum displayed one singlet at **δ** 3.96 for methoxy (-OCH_3) protons, three singlets at **δ** 2.67, 2.52 and 2.07 for protons of three methyl groups. The spectrum also showed an aldehyde proton at **δ** 10.33, three hydroxyl protons at **δ** 12.53, 12.48 and 11.93. Additionally, the spectrum also revealed deshielded singlet signals corresponding to two aromatic protons (**δ** 6.39 and **δ** 6.50). The ^{13}C NMR spectrum revealed carbonyl ester groups at **δ** 172.0 and 169.5 as well as an aldehydic carbonyl group at **δ** 193.6. It also showed twelve quaternary sp^2 carbon signals, three methylene (-CH-) carbons (**δ** 112.8, 115.9 and 193.8) and four methyl (-CH_3) carbons (**δ** 9.4, 24.0, 25.6 and 52.3) consistent with the data obtained by ^{1}H NMR analysis. The chemical shifts of quaternary sp^2 carbons {***δ*** 102.8, 108.8, 110.3, 116.7, 139.9, 152.0, 152.4, 162.8, 167.5, 169.1, 169.5 (C=O), and 172.0 (C=O)} indicates the presence of two distinct aromatic rings probably having five substituent and a free ring hydrogen atom {***δ*** 6.50 (s) and 6.39 (s)} each.

To further confirm the structure, heteronuclear multiple bond correlations (HMBC) experiment was carried out (Figure 4.28 a, b) and it showed $^{2,3}J_{CH}$ long-range correlations between the 3H-9' (δ_H 2.52) and carbons C-5' (**δ_C** 139.9, ^2JCH), CH-6' (δ_C 115.9, $^3J_{CH}$) and C-4' (δ_C 110.3, $^3J_{CH}$); between 3H-8' (δ_H 2.07) and C-2' (δ_C 116.7, $^2J_{CH}$), C-3' (δ_C 162.8, $^3J_{CH}$) and C-1' (δ_C 152.0, $^3J_{CH}$); and between H-6' (δ_H 6.50) and C-1' (δ_C 152.0, $^2J_{CH}$), (C-2' (δ_C 116.7, $^3J_{CH}$) and C-4' (δ_C 110.3, $^3J_{CH}$). Therefore, the two methyl groups -CH_3-8' and -CH_3-9' are positioned at the same ring. The other cross peaks are also in agreement with the location of one methoxy carbonyl and one hydroxyl group in the same aromatic ring (Figure 4.28 b). The observed long-range correlation involving the remaining methyl group CH_3-9 (δ_C 25.6) and the aromatic hydrogen H-5 (δ_H 6.39) led to the assignments of C-1 and C-6 carbons.

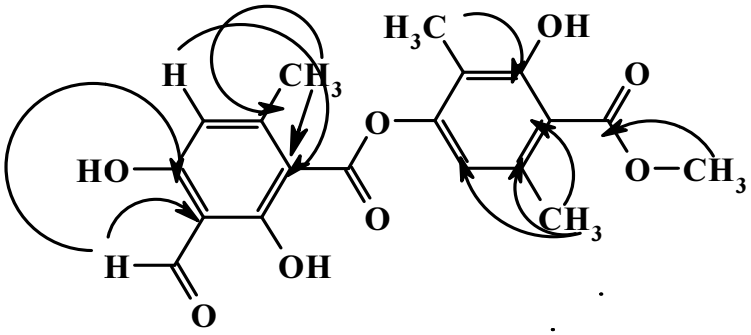


Figure 4.28 a: HMBC correlations of atranorin (Pr IIa)

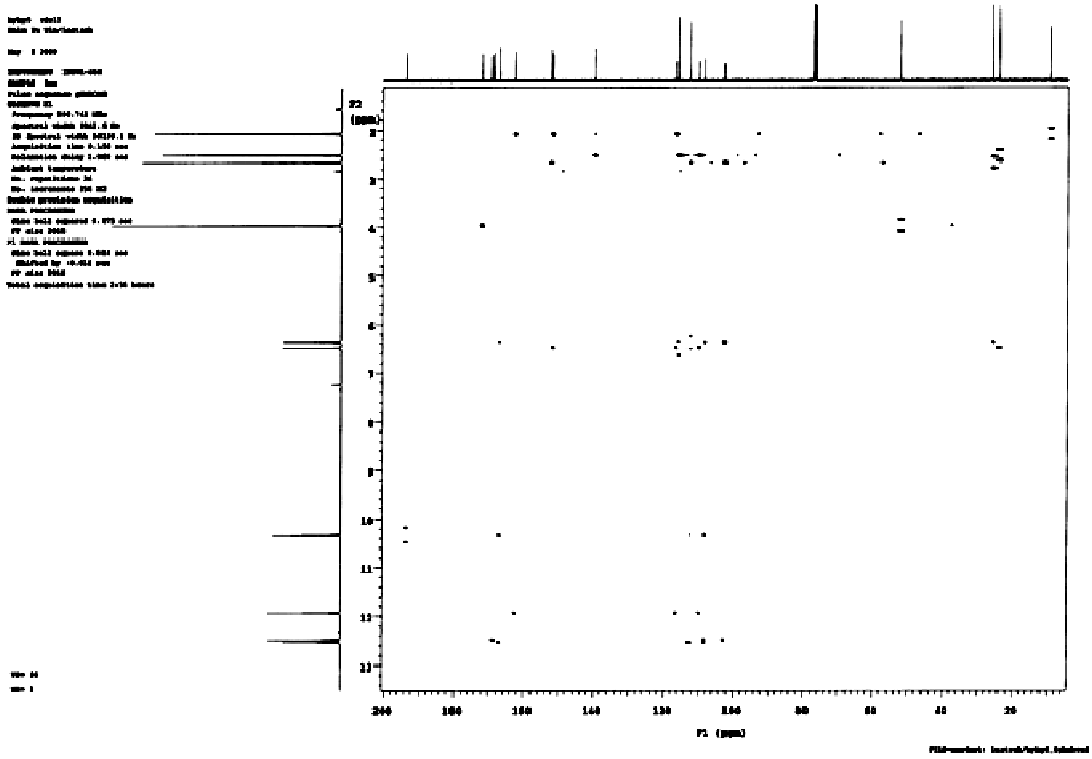


Figure 4.28 b: 2D HMBC NMR spectrum of atranorin (Pr IIa)

These deductions and the peaks at *m/z* (abundance %): 196 (100 %), 194 (90 %) and 179 (95 %) observed in the mass spectrum (Figure 4.28 c) are in agreement with the presence of a methyl 2-hydroxy-3, 6-dimethylbenzoate moiety. The mass spectrum showed molecular ion peak at *m/z* 397.1 $[M+Na]^+$, and 771.2 $[2M+Na]^+$. (+) HR-ESI MS gave a value at *m/z* 397.0893 corresponding to the formula $C_{19}H_{18}O_8$.

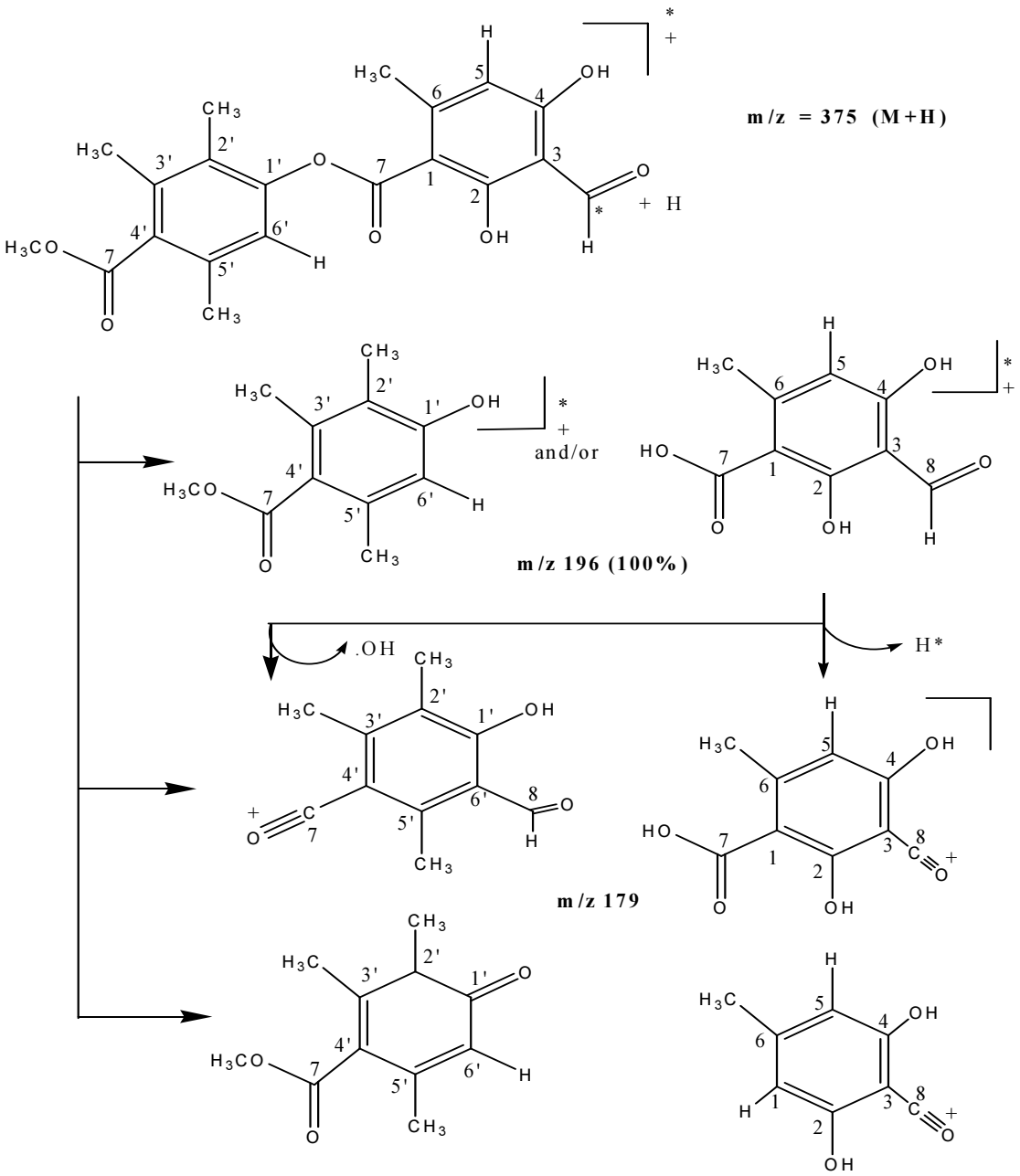


Figure 4.28 c: Mass fragmentation pattern of atranorin (Pr IIa)

Based on ^{1}H NMR, ^{13}C NMR and mass spectrum analysis, Pr IIa was found to be **3-hydroxy-4-(methoxycarbonyl)-2,5-dimethylphenyl-3-formyl-2,4-dihydroxy-6-methyl benzoate (atranorin)** (Figure 4.27, Pr IIa). Its structure was further confirmed by comparison of its ^{1}H NMR and ^{13}C NMR spectra with literature report (Gabriele *et al*., 1999).

Pr IIIa: When the column was further eluted with dichloromethane (100ml), it afforded a compound in the form of needles, which was obtained by recrystallization from hexane. $\mathbf{R_f}$ = 0.32 [TLC solvent system, toluene: acetic acid

(170:30 v/v)]. **m.p.** 90-95 ^{0}C. **^{1}H NMR ($CDCl_3$) *et al*:** 11.0 (s, 1H), 6.43 (d, 1H), 6.03 (d, 1H), 4.81 (q, 1H), 1.68 (m, 2H, 1H), 1.3-1.2 (m, 22H), 0.89 (t, 3H). **^{13}C NMR ($CDCl_3$) *δ***: 173.8 (CO, C-1), 163.1 (CO, C-4), 132.3 (C-3), 125.9 (CH_2, C-1′), 78.8 (CH, C-5), 49.4 (CH, C-2), 35.7 (CH_2, C-12′), 31.9 (CH_2, C-11′), 29.7, 29.6 , 29.6, 29.5, 29.4, 29.4, 29.0, 29.2, 24.7, 22.7 (CH_2, C-2′-C-10′) and 14.2 (CH_3, C-14′). **(+) ESI-MS**: *m/z* 325.2 $[M+H]^+$, (-) **HR-ESI MS**: *m/z* 323.2227 $[M-H]^-$ calculated for $C_{19}H_{31}O_4$

The ^{1}H NMR spectrum revealed a hydroxyl proton at **δ** 11.01, methylene protons attached to the double bond at **δ** 6.43 and 6.03 as doublets, a triplet at **δ** 0.89 for CH_3 and a multiplet for 22 protons at **δ** 1.2-1.4 indicating the presence of a long alkyl chain. ^{13}C NMR showed the presence of an acidic carbonyl at **δ** 173.8, carbonyl group exocyclic to the five member ring at **δ** 163.1. Carbon attached to the double bond showed a downfield shift at **δ** 132.4. The (-) HR ESI-MS spectrum showed a molecular ion peak at *m/z* 323.2227 $[M-H]^-$ calculated for $C_{19}H_{31}O_4$. Based on spectral data, Pr IIIa was identified as **tetrahydro-4-methylene-5-oxo-2-tridecyl-3-furancarboxylic acid (protolichesterinic acid)** (Figure 4.27, Pr IIIa). Its structure was further confirmed by comparison of its ^{1}H NMR and ^{13}C NMR with literature report (Ing′olfsd′ottir *et al*., 1998). Protolichesterinic acid has already been reported from *Cetraria islandica* and *Parmelia nepalensis Tayl.* by Aysen Özdemir *et al*. in 2003. It is the first report showing the presence of protolichesterinic acid in *P. reticulata.*

Identification of chemical constituents from ethyl acetate extract

Pr IVb: Ethyl acetate extract on elution with hexane: dichloromethane (3:1, 200 ml) gave a crystalline compound with a characteristic smell. **R_f** = 0.54 [TLC solvent system, toluene: acetic acid (170:30 v/v)]. **m. p.** 142-144 ^{0}C. **^{1}H NMR (CD_3OD) *δ***: 6.17 (s, 1H, H-5), 3.84 (s, 3H, OCH_3), 2.36 (s, 3H, H-8), 1.98 (s, 3H, H-7). **^{13}C NMR (CD_3OD) *δ***: 173.7 (CO, C-1'), 163.9 (C_q, C-2), 161.2 (C_q, C-4), 140.7 (C_q, C-6), 111.4 (CH, C-5), 109.8 (C_q, C-3), 104.9 (C_q, C-1), 51.9 (OCH_3), 24.3 (CH_3-8), 8.0 (CH_3-7). **(+) EI-MS**: *m/z* (abundance %) 196 ($[M]^+$ 26), 164 (88), 136 (100), 107 (16).

The ^{1}H NMR spectrum revealed the presence of an aromatic proton at ***δ*** 6.17, two methyl groups at **δ** 2.36 and 1.96, one methoxy at **δ** 3.84. ^{13}C NMR

spectral data showed the characteristic carbonyl carbon at **δ** 173.7. The mass spectrum showed a molecular ion peak at *m/z* 196, with fragment ion peaks at *m/z* (abundance %) 164 (88), 136 (100), 107 (16). The ^{1}H NMR and ^{13}C NMR data of the above compound are comparable to those reported earlier (Huneck *et al*., 1999). Based on spectral data, Pr IVb was identified as **methyl 2, 4-dihydroxy-3, 6-dimethyl benzoate (Evernyl)** (Figure 4.27, Pr IVb). This compound is known for a characteristic smell (smell of oak moss) since long time and is used in perfumery and cosmetic industry (Huneck *et al*., 1999).

Pr Vb: A further elution of column with hexane:dichloromethane (1:1, 200ml) gave another colorless compound which was purified by crystallization from hexane. $\mathbf{R_f}$ = 0.65 [TLC solvent system, toluene: acetic acid (170:30 v/v)]. **^{1}H NMR ($CDCl_3$) *δ***: 12.96 (s, 1H, 4-OH), 12.38 (s, 1H, 2-OH), 10.31 (s, 1H, CHO), 6.26 (s, 1H, CH-5), 4.40 (quartet, 2H, CH_3 -3'), 2.52 (s, 3H, CH_3, C-5'), 1.42 (t, 3H, CH_3-4'). **^{13}C NMR ($CDCl_3$) *δ***: 193.7 (CO, C-1'), 171.4 (CO, C-2', 168.2 (C_q, C-4), 166.3 (C_q, C-2'), 152.3 (C_q, C-6), 112.0 (CH, C-5), 108.4 (C_q, C-1), 103.9 (C_q, C-3), 61.9 (O-CH_2, C-3'), 25.4 (C-5'), 14.3 (CH_3, C-4'). **(+) EI-MS**: *m/z* (abundance %) 224 $[M]^+$, 195 (24), 177 (26), 149 (100), 121 (10), **(+) HR ESI-MS**: *m/z* 247.0757, $[M+Na]^+$ calculated for $C_{11}H_{12}O_5Na$.

^{1}H NMR revealed the presence of two hydroxyl protons at **δ** 12.96 and 12.38, a characteristic aldehyde proton at **δ** 10.31, an aromatic proton at **δ** 6.26, a quartet at **δ** 4.40 due to the splitting of -CH_2 protons because of adjacent methyl protons. The ^{13}C NMR revealed aldehyde carbon at **δ** 193.7. The mass spectrum showed a molecular ion peak at *m/z* 224 $[M^+]$. The high resolution mass spectrum reveals its molecular formula to be as $C_{11}H_{13}O_5$. Based on ^{1}H NMR, ^{13}C NMR and mass spectrum analysis, Pr Vb was identified as **ethyl-3-formyl-2, 4-dihydroxy-6-methyl benzoate (ethyl hematommate)** (Figure 4.27, Pr Vb).

Pr VIb: A colorless compound was isolated with dichloromethane (200 ml). $\mathbf{R_f}$ = 0.41 [TLC solvent system, toluene:acetic acid (170:30, v/v)]. **m.p.** 135-136 ^{0}C. **^{1}H NMR ($CDCl_3$) *δ***: 11.78 (s, 1H, 2-OH), 6.24 (s, 1H, H-5), 6.20 (S, 1H, H-3), 5.05 (s, 1H, 4-OH), 4.38 (quart, 2H, H-8), 2.44 (s, 3H, H-6), 1.39 (t, 3H, H-9). **^{13}C NMR ($CDCl_3$) *δ***:171.5 (CO, C-7), 165.3 (C_q, C-2), 159.9 (C_q, C-4), 143.9 (C_q, C-6), 111.1 (CH, C-5), 110.5 (CH, C-3), 105.83 (C_q, C-1), 51.8 (CH_2, C-8), 24.1 (CH_3, C-9),

14.3 (CH_3, C-10). **(+) EI-MS**: *m/z* (abundance %) 196, 150 (100), 164 (15), 136 (10), 122 (25).

The ^{1}H NMR revealed the presence of CH_3CH_2O protons, aromatic hydroxyl and methyl protons. ^{13}C NMR showed carbonyl of ester at ***δ*** 171.5, carbon attached to the hydroxyl at ***δ*** 165.2 and 159.9. Mass spectrum showed a molecular ion peak at *m/z* 196 $[M]^+$, with fragment ion peaks at *m/z* (abundance %) 164 (15%) $\{[M]^+ - CH_3\}$ and *m/z* 150 (100 %) $\{[M]^+ - CH_2CH_3\}$. HMBC spectral data clearly showed that an ethyl ester of benzoic acid is proximal to the carbon bearing the hydroxyl group (Figure 4.29 a, b). Based on ^{1}H NMR and ^{13}C NMR, it was characterized as **ethyl-2, 4-dihydroxy-6-methyl-benzoate (ethyl orsellinate)** (Figure 4.27, Pr VIb).

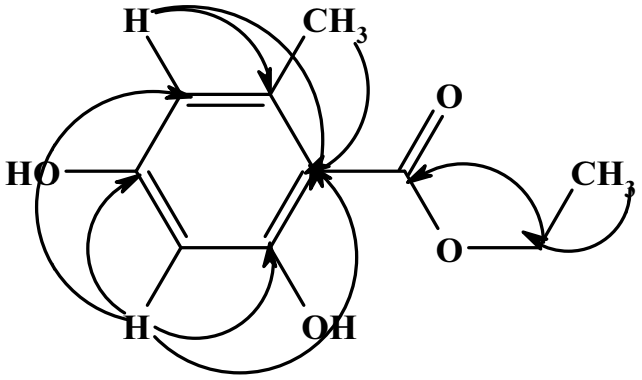


Figure 4.29 a: HMBC correlations of ethyl orsellinate (Pr VIb)

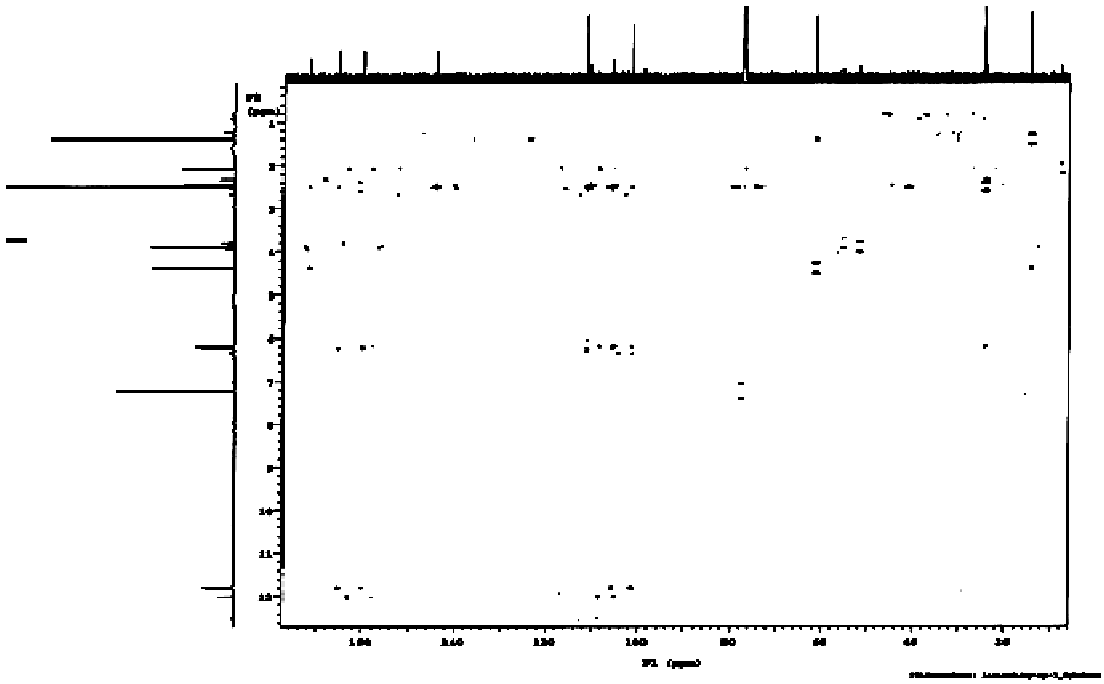


Figure 4.29 b: 2D HMBC NMR spectrum of ethyl orsellinate (Pr VIb)

Pr VIIb: Continuing with the same mobile phase as hexane: dichloromethane (1:1, 200 ml) a colorless crystalline compound was obtained with $\mathbf{R_f}$ = 0.60 [TLC solvent system, toluene: acetic acid (170:30 v/v)]. The melting point of the above compound was in the range 143-144 ^{0}C. **^{1}H NMR (CD_3OD) δ**: 12.86 (s, 1H, 2-OH), 12.39 (s, 1H, 4-OH), 10.31(s, 1H, C-3') 6.26 (s, 1H, CH-5), 3.93 (s, 3H, O-CH_3), 2.50 (s, 3H, CH_3-6). **^{13}C NMR (CD_3OD) δ**: 193.7 (CO, C-3'), 171.8 (CO, C-1'), 168.1 (Cq, C-4), 166.5 (Cq, C-2), 152.2 (Cq, C-6), 112.0 (Cq,C-5), 108.3 (Cq, C-3), 103.8 (Cq, C-1), 52.3 (O-CH_3, C-2'), 25.3 (CH_3, C-6). **(-) ESI-MS**: *m/z* 209.1 $[M-H]^-$. **(-) HR ESI-MS** *m/z* 209.0455 $[M-H]^-$ calculated for $C_{10}H_{10}O_5$.

^{1}H NMR was similar to Pr Vb except it showed the presence of methyl ester instead of ethyl ester. A formyl proton absorbed at (**δ** 10.33) with associated carbonyl (**δ** 193.67) along with another carbonyl ester linkage (**δ** 171.83), a methoxy group (**δ** 3.96, δ 52.33) and a methyl group (**δ** 2.53). The ^{13}C NMR spectrum also showed signals corresponding to Ar-OH (**δ** 168.1, 166.5), Ar-H (**δ** 152.2, 112.0, 108.3, 103.9) and Ar-Me (**δ** 25.3). Mass spectrum showed molecular ion peak at *m/z* 209.1 for $[M-H]^-$. High resolution mass analysis gave a value at 209.0455 corresponding to the molecular formula $C_{10}H_{10}O_5$. Based on ^{1}H NMR and ^{13}C NMR and mass spectral data, it was identified as **methyl-3-formyl-2, 4-dihydroxy-6-methyl-benzoate (methyl hematommate)** (Figure 4.27, Pr VIIb).

Pr VIIIb: Elution of column with dichloromethane (200 ml) also gave an orange colored compound, which was recrystallized from ethyl acetate. $\mathbf{R_f}$ = 0.55 [TLC solvent system, toluene: acetic acid (170:30 v/v)]. **m.p.** 186-188 ^{0}C. **^{1}H NMR ($CDCl_3$) δ**: 13.53 (1H, OH), 6.86 (2H, d), 3.90 (s, 3H, O-CH_3), 3.91 (s, 3H, O-CH_3), 2.95 (s, 3H, CH_3). **^{13}C NMR ($CDCl_3$) δ**: 182.4 (CO, C-9), 165.8 (Cq, C-3), 163.7 (Cq, C-13), 163.8 (Cq, C-6), 159.4 (C-OH, C-1), 156.9 (Cq, C-11), 143.4 (Cq, C-8), 115.4 (CH, C-7), 112.9 (C-10), 104.1 (C-12), 98.4 (CH-5), 96.7 (CH-4), 98.7 (CH-2), 55.7 (O-CH_3), 55.6 (O-CH_3), 23.5 (C-8, CH_3). **EI-MS**: *m/z* 196 $[M]^+$

^{1}H NMR revealed the presence of two singlet signals due to three proton each at **δ** 2.70 and at 2.30 for aromatic methyl (-CH_3) along with the singlet for three methoxy (-OCH_3) protons at **δ** 3.90. ^{13}C NMR showed the presence of carbonyl

carbon of the acid group at **δ** 172.6. Distortionless enhancement by polarization transfer analysis (DEPT) showed the presence of six quaternary and three methyl carbons. Based on the spectral data, Pr VIIIb was identified as **2-hydroxy-4-methoxy-3, 6-dimethyl benzoic acid** (Figure 4.27, Pr VIIIb).

Pr IXb: When the polarity of the mobile phase was increased with dichloromethane: acetone (95:5, 200ml) the column gave a colorless solid upon recrystallization from methanol as needle shaped crystals. $\mathbf{R_f}$ = 0.72 [TLC solvent system, toluene: acetic acid (170:30, v/v)]. **^{1}H NMR ($CDCl_3$) *δ***: 12.30 (1H, C-OH), 6.50 (1H, CH-5), 5.40 (1H, C-1' (OH)), 3.90 (s, 3H, O-CH_3), 2.70 (s, 3H, CH_3), 2.30 (s, 3H, CH_3). **^{13}C NMR ($CDCl_3$) *δ***: 172.6 (CO, C-1'), 163.1 (C-2, C-OH), 158.0 (Cq, C-4), 140.2 (Cq, C-6), 110.6 (C-3), 108.5 (Cq, C-1), 105.2 (CH-5), 51.9 (O-CH_3, C-4'), 24.1 (C-2', CH_3), 7.7 (C-3', CH_3). **(+) ESI-MS** *m/z*: 287.1 $[M+H]^+$ (+) **HR ESI-MS** *m/z* 287.0909 corresponding to the molecular formula $C_{16}H_{15}O_5$ $[M+H]^+$.

^{1}H NMR revealed the presence of one hydroxyl proton at **δ** 13.53, two aromatic protons at **δ** 6.86 and 6.28, a singlet of six protons at **δ** 3.90 for two methoxy (-OCH_3) and a three proton singlet at **δ** 2.95 for aromatic methyl proton (-CH_3). The mass spectrum showed a molecular ion peak at *m/z* 287.1 $[M+H]^+$ and high resolution mass 287.0909 corresponds to the molecular formula $C_{16}H_{15}O_5$. Thus, Pr IXb was identified as **1-hydroxy-3, 6-dimethoxy-8-methyl-xanthen-9-one (lichexanthone)** (Figure 4.27, Pr IXb), which is reported earlier from *P. formanosa*, *Pertusaria sulphurata* and *Anthocleista djalonensis* (Sundholm, 1978).

Pr Xb: Further elution of column with dichloromethane: acetone (9:1, 200ml) gave a white amorphous solid, recrystallized from aqueous acetone as colorless plates. $\mathbf{R_f}$ = 0.87 [TLC solvent system, toluene:acetic acid (170:30, v/v)]. **m.p.** 220-222 ^{0}C(dec.). **^{1}H NMR (DMSO-d_6) *δ***: 11.55 (s, 1H, 2-OH, 2'-OH), 10.58 (s, 1H, 7'-OH), 10.21 (s, 1H, H-8), 6.45 (s, 1H, H-5'), 6.38 (s, 1H, H-5), 3.88 (s, 3H, H-9), 2.35 (s, 3H, H-9'), 2.04 (s, 3H, H-8'), 1.94 (s, 3H, H-10). **^{13}C NMR (DMSO-d_6) *δ***: 193.4 (CO, C-8), 171.5 (CO, C-7'), 169.5 (CO, C-7), 164.3 (Cq, C-4), 163.6 (Cq, C-2'), 161.7 (Cq, C-2), 151.2 (Cq, C-4'), 148.6 (Cq, C-6), 138.4 (Cq, C-6'), 116.1 (CH, C-5'), 115.5 (Cq, C-1'), 103.9 (CH, C-5), 114.9 (Cq, C-1), 52.2 (CH_3, C-9), 23.3 (CH_3-

9'), 21.1 (CH_3-10), 9.2 (CH_3-8'). **(+) ESI-MS**: 397.1$[M+Na]^+$, 771.2 $[2M+Na]^+$, **(+) HR ESI MS**: *m/z* 397.08938 $[M+Na]^+$ (calculated for $C_{19}H_{18}O_8Na$).

It was observed that Pr Xb was insoluble in most of the solvents, but soluble in DMSO. The ^1H NMR spectrum showed the presence of two hydroxyls at **δ** 11.55 and 10.58, an aldehydic proton at **δ** 10.21, two aromatic protons at **δ** 6.45 and 6.38, one methoxy protons at **δ** 3.88 and the three methyl protons at **δ** 2.35, 2.04, 1.94. ^{13}C NMR revealed the presence of an aldehyde carbon at **δ** 193.4, carbonyl carbon of the carboxylic acid at **δ** 171.5 and the carbonyl carbon of ester at **δ** 169.5. High resolution mass showed a molecular ion peak at *m/z* 397.0895 $[M+Na]^+$ calculated for $C_{19}H_{18}O_8Na$. A comparison of the spectral data of Pr Xb with data reported earlier (Manojlovi *et al*., 2010*)*, confirmed that it as **baeomycesic acid** (Figure 4.27, Pr Xb).

Pr XIb: Ethyl acetate extract on evaporation gave a brown amorphous solid, which was insoluble in most of the organic solvents. It was purified by crystallization from acetone: water (80:20). $\mathbf{R_f}$ = 0.13 [TLC solvent system, toluene: acetic acid (170:30, v/v)]. **m.p.** 249-251 ^{0}C. **^{1}H NMR (DMSO-d_6) δ**: 12.04 (1H, s, C-2', OH), 10.45 (2H, s, C-3 and C-6', CHO), 8.30 (1H, s, C-4, OH), 6.86 (1H, s, C-3', OH), 6.80 (1H, s, H-5), 4.64 (2H, s, 13 CH), 2.44 (3H, s, C-6, CH). **^{13}C NMR (DMSO-d_6) δ**: 192.7 (C-3, CHO), 165.8 (C-2), 164.0 (C-4), 163.6 (C-7', CO), 160.3 (C-7, CO), 152.8 (C-6 or C-2'), 152.3 (C-6 or C-2'), 148.2 (C-4'), 138.1 (C-5' or C-6'), 137.3 (C-5' or C-6'), 123.5 (C-3'), 117.4 (C-5), 112.0 (C-1), 110.7 (C-3), 109.6 (C-1), 94.9 (C-6', CHO), 52.7 (C-3', CH-OH), 21.4 (C-6, CH) **(-)ESI-MS**: *m/z* 387.0 $[M-H]^-$, **(-) HR ESI-MS**: *m/z* 387.03577 calculated for $C_{18}H_{12}O_{10}$ (Figure 4.30).

The ^1H NMR showed the presence of phenolic OH, an aldehydic proton, aromatic methyl and benzylic proton. ^1H NMR spectral data are comparable to that of salazinic acid reported earlier by Huneck and Linscheid 1968. The mass spectrum coupled with the chromatogram showed a molecular ion peak at *m/z* 387.0 $[M-H]^-$. Thus it was characterised as **1,4,10-trihydroxy-5-hydroxymethyl-8-methyl-3,7-dioxo-1,3-dihydro-7H-benzo[e]furo[3,4,] benzo[1,4] dioxepine-11-carbaldehyde (salazinic acid),** which has been isolated from *P. saxatilis* (Nolan and Keane, 1933) (Figure 4.27, Pr XIb).

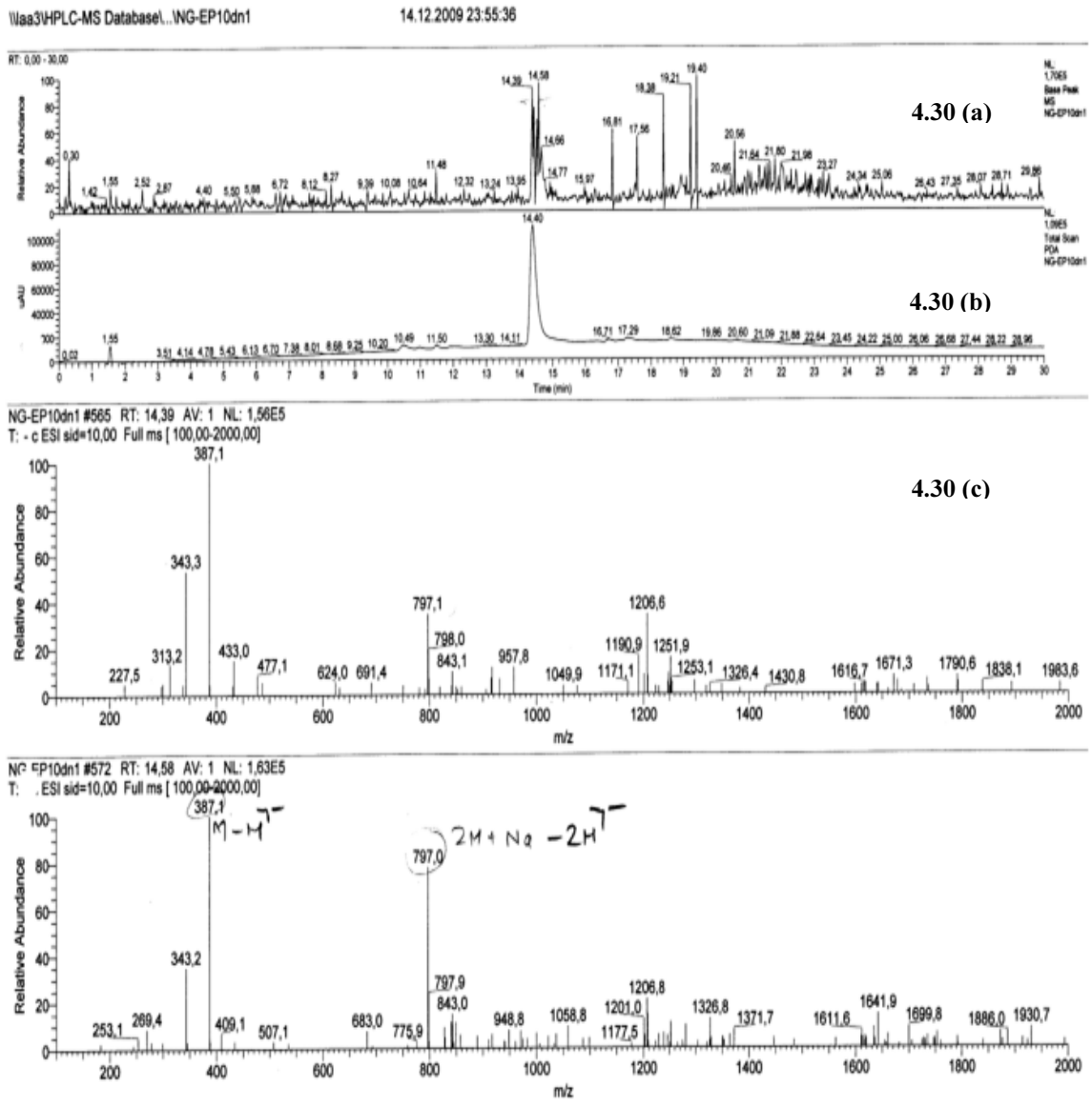


Figure 4.30: The LC-MS chromatogram of salazinic acid (Pr XIb), where 4.30(a), (b) and (c) represent the LC chromatogram, UV scan and Mass spectrum respectively

Pr XIIb: Further elution of column with dichloromethane: acetone (8:2, 200ml) gave a white solid, which was recrystallized from acetone. **Rf**= 0.50, [TLC solvent system, toluene: acetic acid (170:30, v/v)]. **^{1}H NMR (DMSO-d$_6$)** ***δ***: 11.68 (s, 1H, OH), 6.30 (s, 1H, C-3), 3.80 (s, 3H, OCH_3), 3.56 (s, 3H, OCH_3), 2.56 (s, 3H, CH_3), 2.18 (s, 3H, CH_3). **^{13}C NMR (DMSO-d$_6$)** ***δ***: 172.3 (CO, C-1'), 162.1 (Cq, C-6), 160.5 (Cq, C-2), 110.9 (Cq, C-5), 108.6 (Cq, C-1), 104.4 (CH, C-3), 52.4 (OCH_3, C-3', C-5'), 23.9 (CH_3, C-4′), 8.5 (CH_3, C-2').

The ^{1}H NMR spectra displayed two singlets at **δ** 2.65 and 2.14 of three protons each for aromatic methyl (-CH_3) and one singlet of three protons at **δ** 3.90 for

methoxy (-OCH_3). ^{1}H NMR also revealed the presence of two hydroxyl protons at **δ** 10.07 and 11.56 and an aromatic proton at **δ** 6.46. ^{13}C NMR spectra displayed carbonyl carbon of an acid group at **δ** 172.3. A methoxy carbon was determined at **δ** 52.2. Methyl carbons were detected at **δ** 24.5 (C-9) and 8.0 (C-10). Six aromatic carbons are also observed between **δ** 163.52 and 105.62. Based on the above spectral data, Pr XIIb was confirmed as **2-methoxy-4-hydroxy-3, 6-dimethyl benzoic acid** (Figure 4.27, Pr XIIb).

Pr XIIIb: Column on elution with dichloromethane gave white crystalline solid, which was purified further by recrystallisation from methanol as colorless crystals. **Rf**= 0.64, [TLC solvent system, toluene: acetic acid (170:30, v/v)]. **^{1}H NMR ($CDCl_3$) *δ***: 10.22 (s, 1H, CHO), 6.22 (s, 2H, H-4, H-6), 2.20 (s, 3H, CH_3-8). **^{13}C NMR ($CDCl_3$) *δ***: 193.3 (CO, C-1), 150.7 (C-OH, C-3), 150.8 (C-OH, C-7), 108.6 (Cq, C-5), 108.5 (CH-4), 108.5 (CH-6), 108.4 (Cq, C-2), 22.4 (CH_3, C-8). **(+) ESI-MS**: *m/z* 175 $[M+Na]^+$, **(+)HR ESI MS**: *m/*z 175.0366 was calculated for $C_8H_7O_3Na$.

^{1}H NMR revealed the presence of two hydroxyl protons at ***δ*** 12.96 and 12.38, a characteristic aldehydic proton at **δ** 10.31, an aromatic proton at **δ** 6.26 and a three proton singlet at **δ** 2.30 for aromatic methyl group. The ^{13}C NMR spectra revealed the presence of eight carbon atoms. It revealed an aldehydic carbonyl group at **δ** 193.3. It showed six quaternary sp^2 carbon signals at **δ** 150.8, 150.7, 148, 112, 110 and 109 indicating four substituted aromatic carbons and two hydrogen atoms. It also showed the presence of one methyl group at **δ** 22.4. High resolution mass spectrum showed *m/z* 175.0366 corresponding to the molecular formula $C_8H_8O_3Na$. Based on the ^{1}H NMR, ^{13}C NMR and mass spectral data Pr XIIIb was identified as **2, 6-dihydroxy-4-methyl benzaldehyde (Atranol)** (Figure 4.27, Pr XIIIb).

Pr XIVb: A colorless compound was separated on a sephadex column with methanol. **m.p.** 180-182 ^{0}C. **^{1}H NMR ($CDCl_3$) *δ***: 12.86 (s, 1H), 12.38 (s, 1H), 12.00 (s, 1H), 10.31 (s, 1H, CHO), 6.26 (s, 1H), 6.18 (s, 1H), 3.93 (s, 3H, OCH_3), 3.89 (s, 3H, OCH_3), 2.50 (s, 3H, CH_3), 2.43 (s, 3H, CH_3), 2.07 (s, 3H, CH_3), 1.22 (m, 12H, CH_2), 0.85 (t, 3H, CH_3) (Figure 4.31a). **^{13}C NMR ($CDCl_3$) *δ***: 193.7 (CO, CHO), 172.4 (CO) 171.8 (CO), 168.1 (C-OH), 166.5 (C-OH), 163.0 (CO), 157.9 (C-OH), 152 (Cq), 140.0 (Cq), 123.2 (Cq), 112.0, 110.4, 108.4, 105.1, 103.8 (Figure 4.31b).

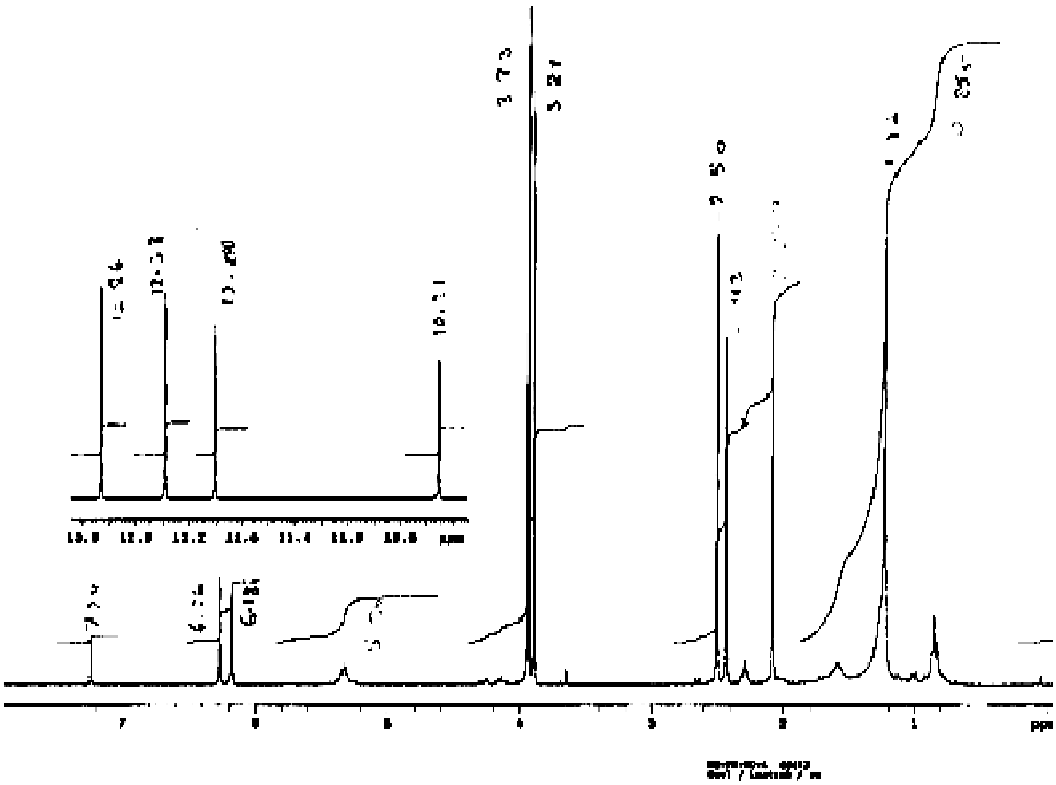


Figure 4.31a: The ^{1}H NMR spectra of Compound Pr XIVb

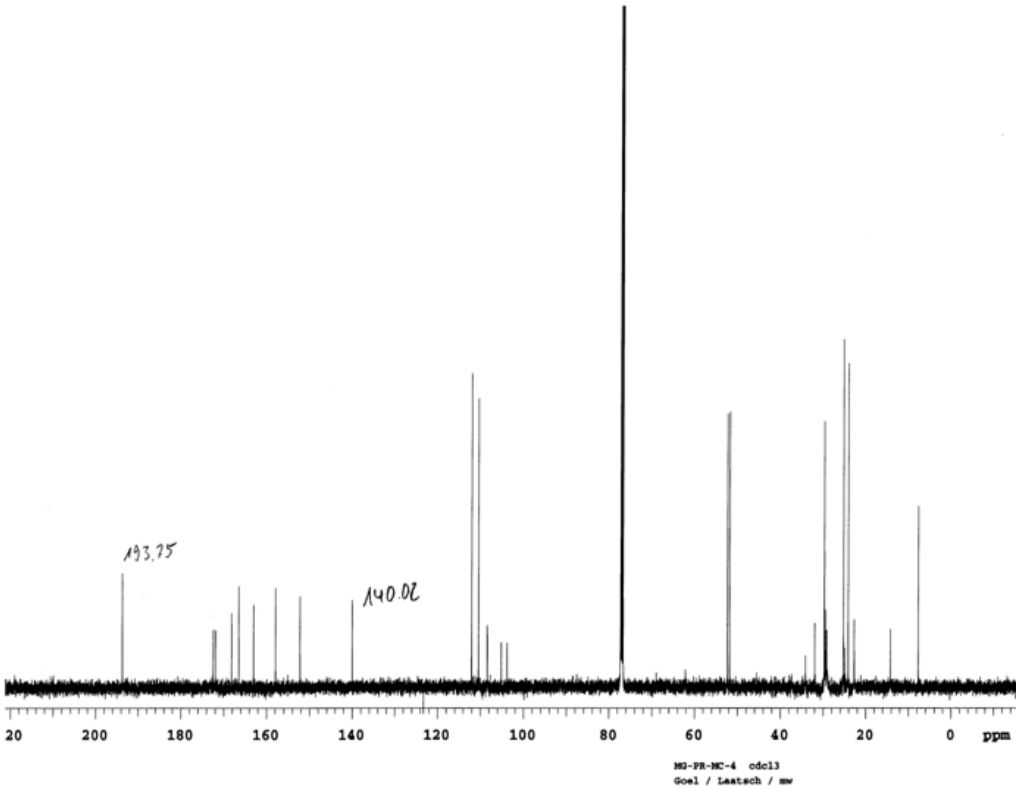


Figure 4.31b: The ^{13}C NMR spectra of Compound Pr XIVb

The ^{1}H NMR spectrum displayed two singlet at δ 3.93 and 3.89 for protons of two methoxy (-OCH_3) groups and three singlets at δ 2.50, 2.43 and 2.07 for three aromatic methyl (-CH_3) groups. It also showed an aldehydic proton at δ 10.33, three hydroxyl protons at δ 12.86, 12.38 and 12.00. Singlet at δ 6.38 confirmed the presence of two aromatic protons. The ^{13}C NMR spectrum revealed carbonyl ester groups at δ 172.0 and 171.8 and an aldehydic carbonyl group at δ 193.7. It also showed twelve quaternary sp^2 carbon signals, three CH (δ 112.8, 115.9 and 193.8) carbons and four CH_3 (δ 9.4, 24.0, 25.6 and 52.3) consistent with the data obtained by ^{1}H NMR analysis. The chemical shifts of quaternary sp^2 carbons were used to propose two aromatic rings with five substituent (*vide infra*) and one hydrogen atom [δ 6.50 (s) and 6.39 (s)] each. HMBC correlations showed hydrogen of the aldehyde group with 1J, 2J and 4J coupling. Two hydroxyls groups are presenting adjacent to the aldehydic carbon (Figure 4.27, Pr XIVb) (Figure 4.31 c, d).

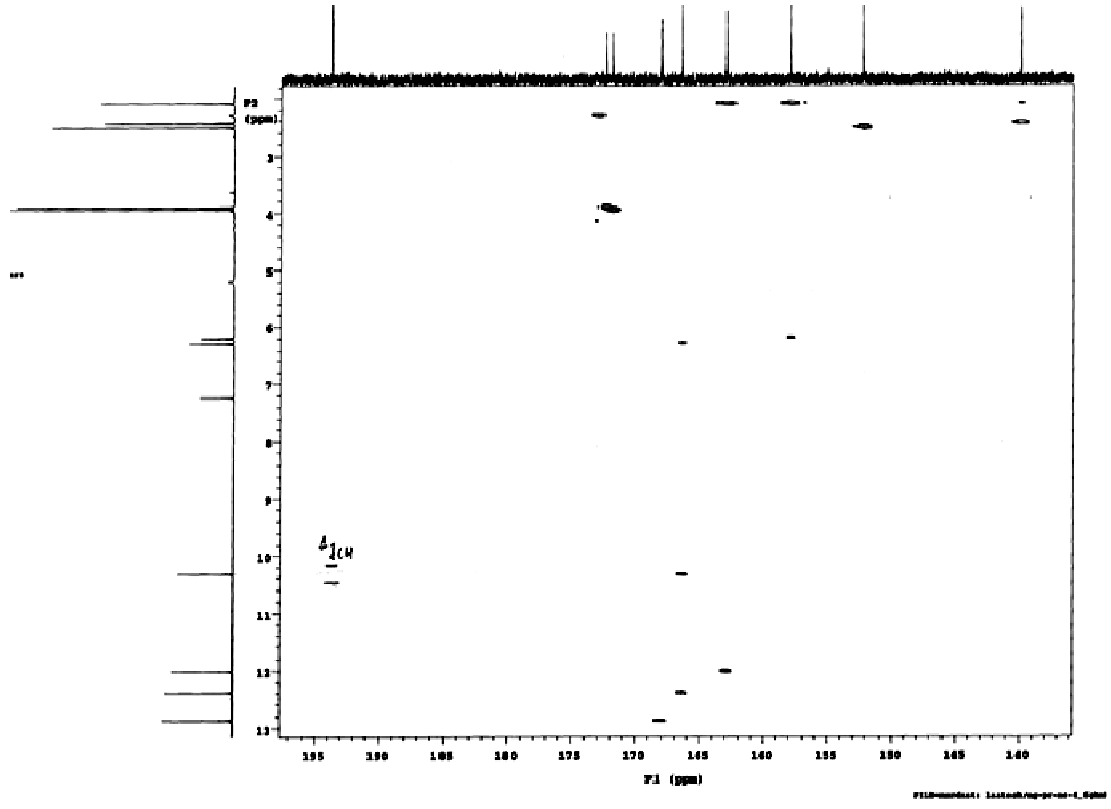


Figure 4.31c: 2D HMBC NMR spectrum of Compound Pr XIVb

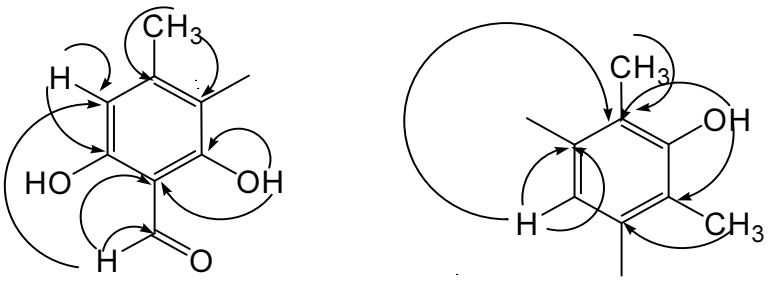


Figure 4.31d: HMBC correlations of both the rings of compound Pr XIVb

Based on the spectral data, the compound was found to be 3-(5-substituted-3 hydroxy-2, 4-dimethyl phenoxy)-2,4dihydroxy 6 –methyl benzaldehyde.

4.3.2.2 Isolation and identification of chemical constituents from crude extracts of *R. roesleri*

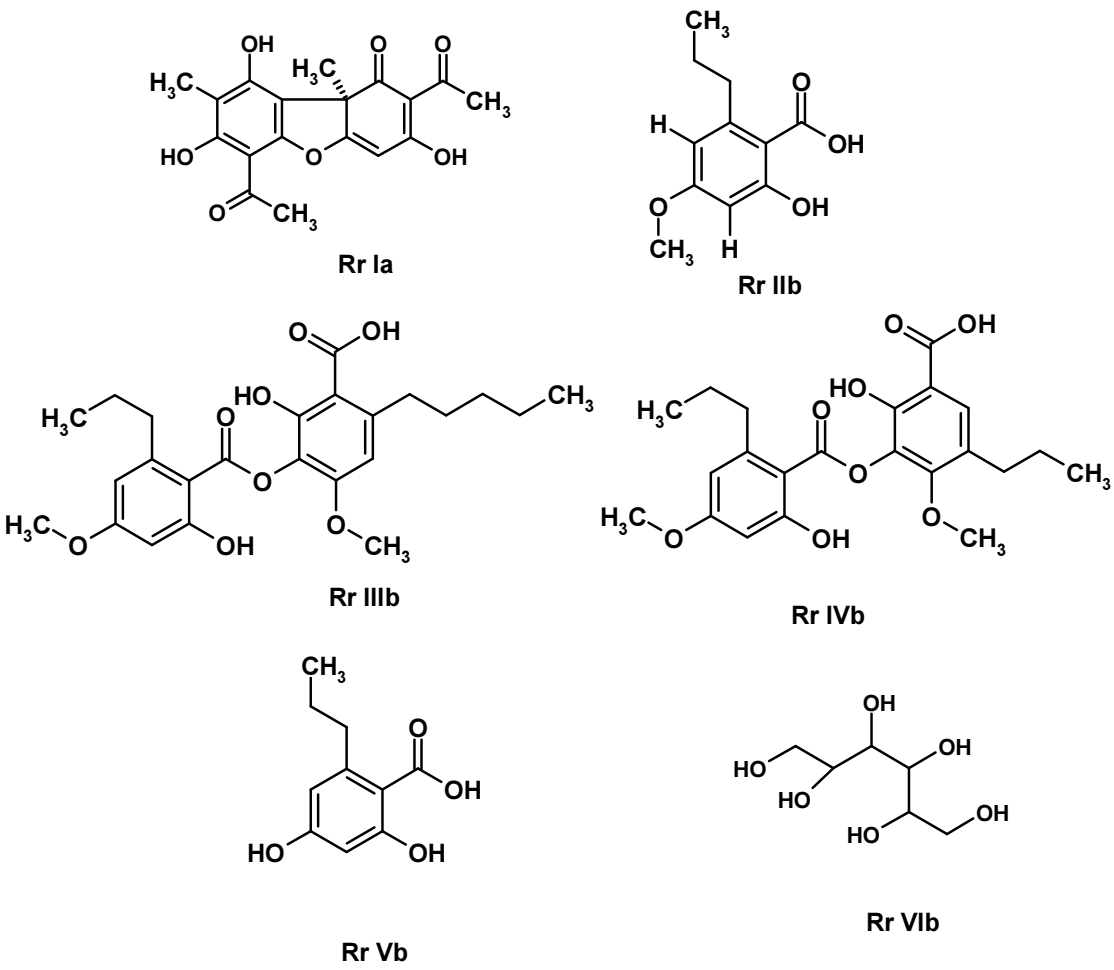


Figure 4.32: Chemical constituents isolated from *R. roesleri*

Identification of chemical constituents from hexane extract

Rr Ia: Elution with hexane:dichloromethane (50:50, v/v) gave a yellow crstalline compound, which was recrystallised from hot methanol, as yellow needle shaped crystals. $\mathbf{R_f}$ = 0.78, [TLC solvent system, toluene: acetic acid (170:30, v/v)]. **m. p**. 203 ^{0}C. **^{1}H NMR ($CDCl_3$) δ**: 1.75 (3H, s), 2.09 (3H, s), 2.66 (3H, s), 2.68 (3H, s), 5.98 (1H, s), 11.03 (1H, s), 13.3 (1H, s), 18.9 (1H, s). **^{13}C NMR ($CDCl_3$) δ**: 198.5 (C-1), 105.7 (C-2), 202.2 (C-2, -$OCCH_3$), 28.3 (C-2, -$OCCH_3$), 192.1 (C-3), 98.4 (C-4), 179.8 (C-4a), 101.9 (C-6), 200.8 (C-6, -$OCCH_3$), 31.7 (C-6, -$OCCH_3$), 155.6 (C-6a), 164.3 (C-7), 109.7 (C-8), 7.9 (C-8, -CH3), 157.9 (C-9), 105.7 (C-9a), 59.5 (C-9b), 32.6 (C-9b, -CH_3). **(+)ESI MS** *m/z* 755 $[2M+HCOO]^+$, 389 $[M+HCOO]^+$. **(+)HR ESI-MS** *m/z* 367.07883 $[M+Na]^+$.

The ^{1}H NMR spectrum displayed signals for four methyl groups at δ 1.75, 2.09, 2.65 and 2.66, a proton attached to the aromatic ring at δ 5.98 and three downfield singlets at δ 18.90, 13.29 and 11.01 corresponding to the three hydroxyl groups. The ^{13}C NMR spectrum revealed the presence of eighteen carbon atoms, four methyl groups, thirteen non-protonated unsaturated carbons and one protonated unsaturated carbon. ^{13}C NMR showed characteristic carbonyl carbon absorptions at **δ** 201.6 and 200.1. The carbons attached to the hetro atom, i.e. C-1, C-3, C-5, C-8, C-10, C-6 absorbed at δ 197.9, 191.5, 179.2, 163.8, 157.4, and 155.1. (+)HR ESI-MS was 367.07882 corresponding for the molecular formula $C_{18}H_{16}O_7Na$. The HMBC spectrum correlations peaks were observed between the following protons and carbons; δ 5.98 (1H, s, H-4) correlated with C-4a (δ 179.8) and C-9b (δ 59.5); δ 2.68 (3H, s, CH_3CO-6) with C-6 (δ 101.9) and CO (δ 200.8); δ 2.67 (3H, s, CH_3CO-2) with C-2 (**δ** 105.7) and CO (**δ** 202.2); δ 1.77 (3H, s, CH_3-9b) with C-9b (δ 59.5), C-4a (δ 179.8), C-8 (δ 109.7) and C-1 (δ 198.5); δ 2.14 (3H, s, CH_3-8) with C-8 (δ 109.7), C-9 (δ 157.9) and C-7 (δ 164.3); δ 11.10 (1H, s, OH-9) with C-8 (δ 109.7) and C9 (δ 157.9) (Figure 4.33). Based on ^{1}H NMR and ^{13}C NMR it was identified as **usnic acid**. The structure was further confirmed in comparison with literature data (Rashid *et al*., 1999; Ingólfsdóttir, 2002) (Figure 4.32, Rr Ia).

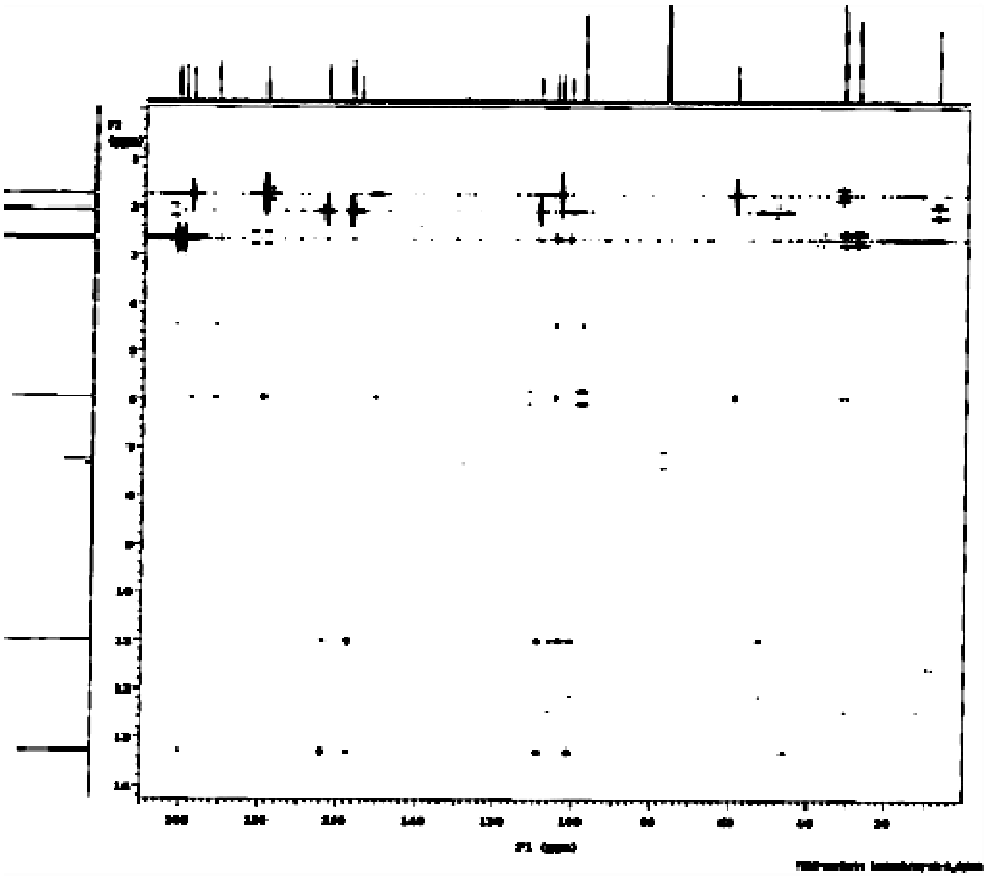


Figure 4.33: 2D HMBC spectrum of usnic acid (Rr Ia)

Identification of chemical constituents from ethyl acetate extract

Rr IIb: Elution of column with hexane: dichloromethane (40:60, v/v) gave colorless solid, which was purified by recrystallization from methanol. $\mathbf{R_f}$ = 0.78, [TLC solvent system, toluene: acetic acid (170:30, v/v)]. **m.p.** 157 ^{0}C. **^{1}H NMR ($CDCl_3$)** $\boldsymbol{\delta}$: 11.54 (s, 2H, -OH), 6.40 (dd, 2H, H-3, H-5), 3.80 (s, 3H, -OCH_3), 2.95 (t, 2H, -CH_2-1'), 1.63-1.50 (quartet, 2H, CH_2-2'), 0.90 (t, 3H, CH_3-3'). ^{13}C NMR ($CDCl_3$) $\boldsymbol{\delta}$: 173.9 (CO, C-4'), 165.9 (C-OH, C-2), 163.6 (C-OCH_3, C-4), 148.5 (Cq, C-6), 109.9 (CH, C-5), 104.7 (Cq, C-1), 98.6 (CH, C-3), 56.0 (-OCH_3, C-5'), 38.4 (-CH_2, C-1'), 24.7 (-CH_2, C-2'), 14.1 (-CH_3, C-3') (Figure 4.34). **EI MS** *m/z* 210.0

The ^{1}H NMR spectrum revealed the presence of a hydroxyl proton at a downfield value of $\boldsymbol{\delta}$ 11.54, two protons in the aromatic ring showed a doublet at $\boldsymbol{\delta}$ 6.40, the methoxy proton showed a chemical shift at $\boldsymbol{\delta}$ 3.80, Methyl of the propyl chain showed a triplet at $\boldsymbol{\delta}$ 0.90 and a multiplet of the methylene protons in the range

of δ 1.50-1.63. ^{13}C NMR spectral data showed the characteristic carbonyl carbon at δ 173.9. Carbon attached to the hetro atoms in the ring gave a shift of δ 165.9 and δ 163.6 respectively. DEPT (135^0) spectra showed a peak of methyl carbon at δ 24.7 followed by methylene carbon at δ 38.4. Its mass spectrum showed molecular ion peak at EI MS *m/z* 210 $[M]^+$. Based on the spectral data, Rr IIb was identified as **2-hydroxy-4-methoxy-6-propyl benzoic acid (Divaricatinic acid)** (Figure 4.32, Rr IIb).

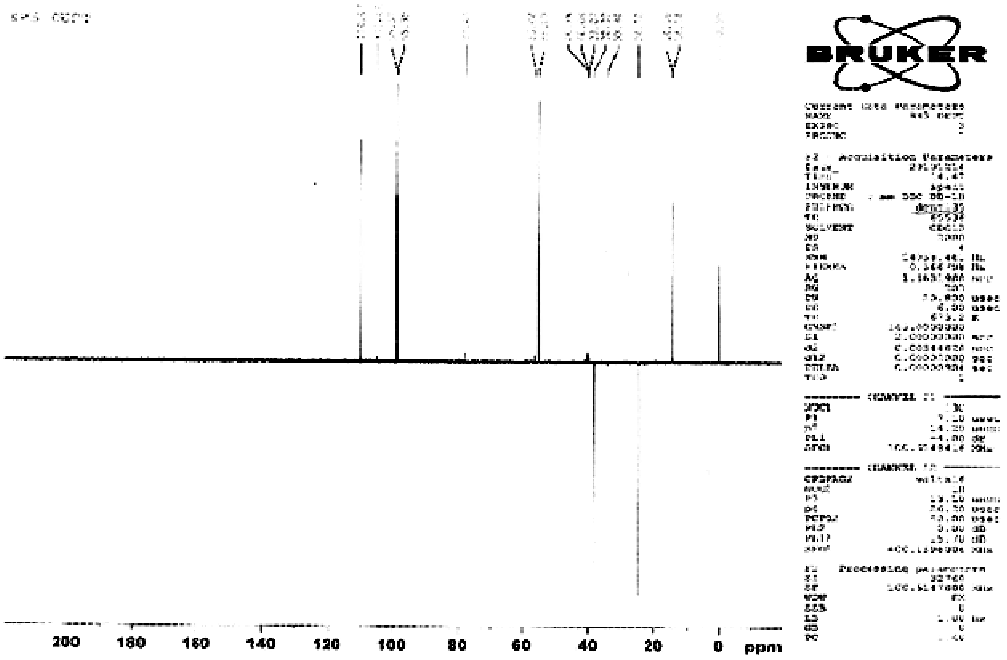


Figure 4.34: ^{13}C NMR (DEPT 135°) spectrum of Divaricatinic acid (Rr IIb)

Rr IIIb: Elution of column with hexane; dichloromethane (30:70, v/v) gave a colorless powder, which was crystallized from hexane. $\mathbf{R_f}$ = 0.45, [Solvent system, dichloromethane: methanol (9:1)]. **m. p.** 133-137 ^oC. **^{1}H NMR (CD_3OD) δ**: 6.52 (s, 1H, C-5), 6.37 (s, 2H, C-3', C-5'), 3.79 (s, 3H, -OCH_3) 3.84(s, 3H, O-CH3), 2.96 (t, 2H, C-6), 2.94 (t, 2H, C-6'), 1.69 (m, 2H, C-7, C-7'), 1.36 (m, 2H, C-8), 1.36 (m, 2H, C-9), 0.91 (t, 3H, CH_3-10, 9'). **^{13}C NMR (CD_3OD) δ**: 174.2 (CO,C-1),169.6 (CO, C-12), 165.5 (Cq, C-2'), 157.1 (Cq, C-4, C-4'), 156.4 (Cq, C-2), 147.4 (Cq,C-6'), 147.1(Cq, C-6), 125.8 (Cq, C-3), 111.3 (Cq, C-5'), 107.2 (Cq, C-1'), 106.7 (Cq,C-3'), 106.1 (Cq, C-1), 56.4 (OCH_3), 55.8 (OCH_3), 39.7 (CH_2, C-6'), 37.7 (CH_2, C-6), 33.2 (CH_2, C-7), 33.1 (CH_2, C-8), 26.4 (CH_2,C-7'), 22.5 (CH_2,C-9), 14.6 (CH_3, C-8'), 14.4

(CH_3, C-10). **(-) ESI MS *m/z*** 445.19 was found for $[M-H]^-$. (-) **HR ESI-MS** *m/z* 445.18680 $[M-H]^-$ corresponding to the molecular formula $C_{24}H_{29}O_8$.

The ^1H NMR spectrum displayed no hydroxyls as the spectrum was recorded in methanol. The two protons showed chemical shift at ***δ*** 6.37 and ***δ*** 6.52 are due to the protons of the two aromatic rings. A multiplet for 12 methylene protons (Six - CH_2) ranging from ***δ*** 1.36 - 2.96 indicates the presence of an alkyl chain and a triplet at ***δ*** 0.91 for methyl groups. ^{13}C NMR showed the presence of an acid carbonyl at ***δ*** 174.2 and the carbonyl carbon of the ester linkage between the two rings showed absorption at ***δ*** 169.6. Carbon attached to the hydroxyl groups in the two rings showed a downfield shift at ***δ*** 165.5 and ***δ*** 156.4.Cabons attached to the methoxy groups also showed a downfield shift at ***δ*** 157.1. Carbons bearing the two methoxy groups of the two aromatic rings showed a chemical shift at ***δ*** 56.4 and 55.8. Methylene carbons showed absorptions ranging from ***δ*** 39.7-26.4. HR ESI-MS spectrum showed a molecular ion peak at *m/z* 445.18680 $[M-H]^-$ corresponding to the molecular formula $C_{24}H_{29}O_8$. Based on spectral analysis, it was identified as **2-Hydroxy-3(2-hydroxy-4-methoxy-6-propyl-benzoyloxy)-4-methoxy-6-pentyl-benzoic acid (Homosekikaic acid)** (Figure 4.32, Rr IIIb). Its structure was further confirmed by comparison of its ^1H NMR and ^{13}C NMR with literature report (Culberson., 1969). Homosekikaic acid has already been reported from *Cladonia dissimilis, C. nemoxyna, C. pityrea, C. submulriformis, C. Subpityrea* (Huneck, 1996). This is the first report showing the presence of Homosekikaic acid in *R. roesleri*.

Rr IVb: Further elution of column with dichloromethane: ethyl acetate (90:10, v/v) gave a colorless solid, which was further purified by recrystallization from ethanol. $\mathbf{R_f}$ = 0.48, [TLC solvent system, dichloromethane: methanol (9:1)]. **m.p.** 150-151^oC. **^1H NMR (DMSO-d_6) *δ***: 12.30 (s, 2H, 2-OH, 2'-OH), 10.40 (s, 1H, -OH), 6.59 (s, 1H, H-5'), 6.38 (s, 1H, H-3), 6.38 (s, 1H, H-5), 3.81 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3,C-4'), 2.85 (m, 4H, 2 x CH_2), 1.65 (m, 2H, -CH_2-1''), 1.63 (m, 2H, -CH_2-1'''), 0.98 (t, 6H, 2 x CH_3). **^{13}C (DMSO-d_6) *δ*:** 173.7 (CO, C-7'), 169.3 (CO, C-7), 165.9 (Cq, C-OCH_3, C-4), 165.3 (Cq, C-OH, C-2), 157.3 (Cq, C-OH, C-2'), 156.4 (C-OCH_3, C-4'), 149.0 (Cq, C-6), 147.8 (Cq, C-6'), 125.6 (Cq, C-3'), 111.4 (CH, C-5), 109.5 (Cq, C-1), 106.9(CH, C-5'), 106.3 (Cq, C-1'), 58.2 (-OCH_3), 57.8 (-OCH_3), 39.2(-CH_2, C-1''), 39.2 (-CH_2, C-1'''), 26.0 (-CH_2, C-2''), 26.0(-CH_2, C-2'''), 15.6(-

CH_3, C-3'''), 14.6(-CH_3, C-3'') (Figure 4.35). **EI-MS** *m/z* 418.43 was observed for the molecular ion peak $[M]^+$.

^{13}C NMR spectra showed the presence of carbonyl at ***δ*** 173.7, carbonyl of the ester linkage between the two rings gave a chemical shift at ***δ*** 169.3. Carbon bearing the methoxy group showed absorbance at ***δ*** 165.9. Carbons bearing the hydroxyl group showed a downfield shift at ***δ*** 165.3 and 157.3. Based on NMR data and comparison with literature, Rr IVb was identified as **sekikaic acid**. (Figure 4.32, Rr IVb) (Huneck, 1996).

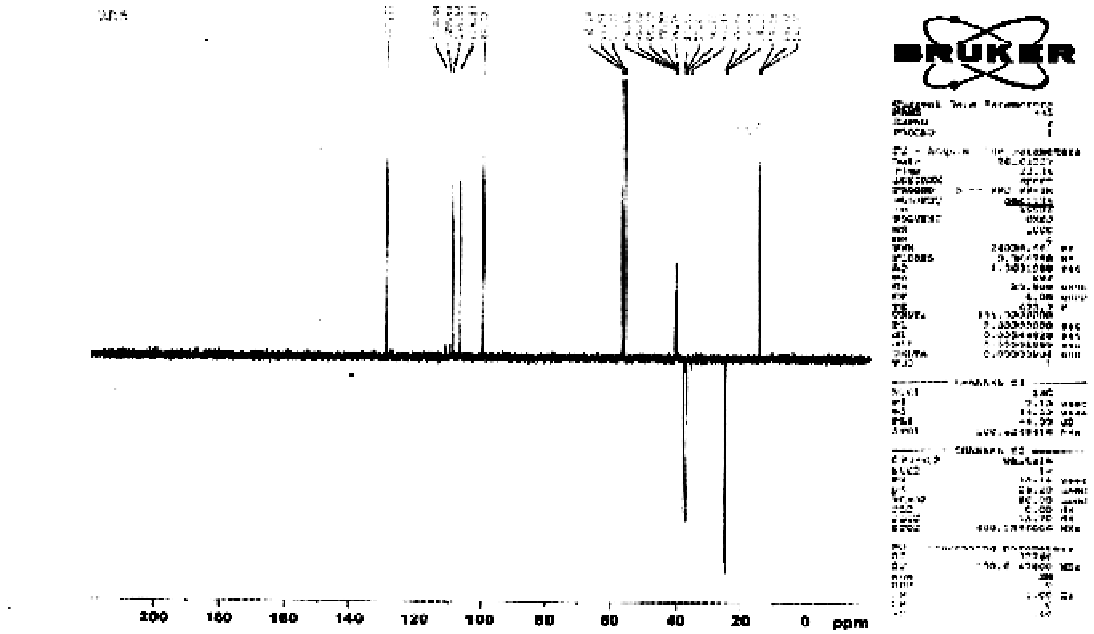


Figure 4.35: ^{13}C NMR (DEPT 135°) spectrum of Sekikaic acid (Rr IVb)

Rr Vb: Further elution of column with dichloromethane:ethyl acetate (80:20, v/v) gave a colorless solid. $\mathbf{R_f}$ =0.54 [Solvent system, Chloroform: methanol (9:1)] **m.p.** 157-159 ^oC. **^{1}H NMR (DMSO-d_6) *δ*** : 6.13 (d, 1H, H-3), 6.10 (d, 1H, H-5), 2.73 (t, 2H, -CH_2-1′), 1.52 (m, 2H, -CH_2-2′), 0.90 (t, 3H, CH_3-3′). **^{13}C NMR (DMSO-d_6) *δ*** : 173.2 (CO, C-4′), 164.1 (C-OH, C-2), 161.8 (C-OH, C-4), 147.3 (Cq, C-6), 110.3 (CH, C-5), 106.0 (Cq,C-1), 101.0 (CH, C-3), 39.2 (-CH_2, C-1′), 24.9 (-CH_2, C-2′), 14.5 (-CH_3, C-3′) **EI MS** *m/*z 196.2.

^1H NMR revealed the presence of two hydroxyl protons at ***δ*** 12.96 and 12.38, a characteristic hydroxyl proton attached to the carbonyl of acid at ***δ*** 10.31, an

aromatic proton at ***δ*** 6.26, a quartet at ***δ*** 4.40 due to the splitting of methylene (-CH_2-) protons because of adjacent methyl protons. The ^{13}C NMR revealed carbonyl carbon of the acid group at ***δ*** 173.2. The mass spectrum showed a molecular ion peak at *m/z* 196 $[M]^+$ with fragment ion peak peaks at *m/z* (abundance %) 178 (100), 150 (19), 121 (34) corresponding to the formula $C_{10}H_{12}O_4$. Based on ^1H NMR and ^{13}C NMR and mass spectrum it was identified as **2,4-dihydroxy-6-n-propyl benzoic acid (Divaric acid)** (Figure 4.32, Rr Vb).

Rr VIb: Further elution of column with dichloromethane: ethyl acetate (70:30, v/v) gave a colorless solid, which was recrystallized from acetone. **^{1}H NMR (DMSO-d_6)** ***δ****:* 3.60 (m, 2H, C-1, CH_2), 3.57 (m, 1H, C-2, CH_2), 3.40 (m, 1H, CH-3), 4.07 (s, C-3, -OH), 4.26 (C-1, -OH), 4.34 (d, 1H, C-2, -OH). **^{13}C NMR (DMSO-d_6)** ***δ****:* 71.25 (C-2, CH), 69.65, 63.73 (C-1, CH_2), 69.6 (C-3, CH). **(+) ESI-MS** *m/z* 205 $[M+Na]^+$. **(+) HR ESI-MS** *m/z* 205.0682

^1H NMR revealed the presence of three hydroxyl protons at ***δ*** 4.34, 4.26 and 4.07. Mass spectrum showed a molecular ion peak at *m/z* 205.0682 calculated for the molecular formula $C_6H_{14}O_6Na$. Based on spectral data and a search in dictionary of natural products (DNP) Rr VIb was identified as **hexane-1, 2, 3, 4, 5, 6-hexaol (ido hexitol**) (Figure 4.32, Rr VIb). Thus from *R. roesleri,* the above mentioned secondary metabolites were isolated and identified. Similar aromatic compounds were isolated from the genus *Ramalina*. usnic and sekikaic acid were isolated from Indian *R. tayloriana* growing on sandal trees (Seshadr and Subramanian,1949).

4.3.2.3 Isolation and identification of chemical constituents from crude extracts of *Usnea longissima*

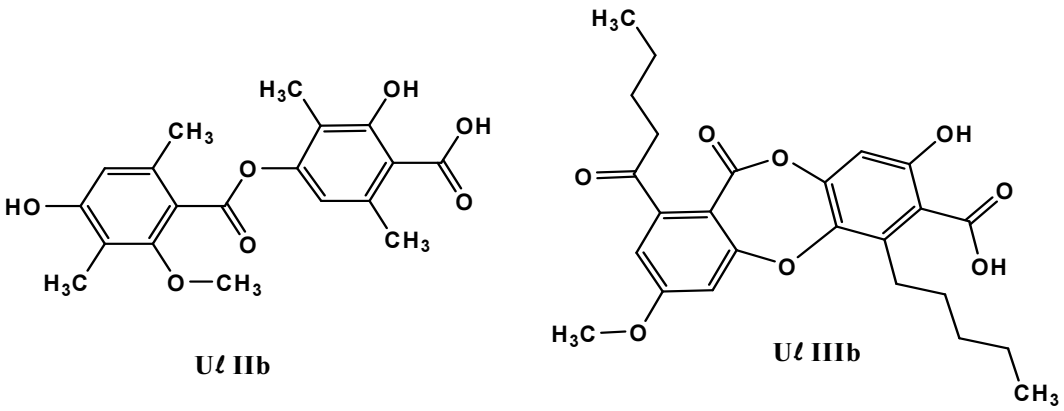


Figure 4.36: Chemical constituents isolated from *U. longissima*

Identification of chemical constituents from hexane extract

Uℓ Ia: Undissolved part of hexane extract gave a yellow crstalline compound, which was on crystallisation gave yellow needle shaped crystals. **m.p.** 203 ^{o}C. **^{1}H NMR ($CDCl_3$) δ**: 1.75 (3H, s), 2.09 (3H, s), 2.66 (3H, s), 2.68 (3H, s), 5.98 (1H, s), 11.03 (1H, s), 13.3 (1H, s), 18.9 (1H, s). **^{13}C NMR ($CDCl_3$) δ**: 198.5 (C-1), 105.7 (C-2), 202.2 (C-2-$OCCH_3$), 28.3 (C-2-$OCCH_3$), 192.1 (C-3), 98.4 (C-4), 179.8 (C-4a), 101.9 (C-6), 200.8 (C-6-$OCCH_3$), 31.7 (C-6-$OCCH_3$), 155.6 (C6a), 164.3 (C-7), 109.7 (C-8), 7.9 (C-8-CH_3), 157.9 (C-9), 105.7 (C-9a), 59.5 (C-9b), 32.6 (C-9b-CH_3). **(+)HR ESI-MS**: *m/z* 367.07882 $[M+Na]^+$ was calculated for $C_{18}H_{16}O_7Na$. Based on ^{1}H NMR and ^{13}C NMR it was identified as **usnic acid**. The structure was further confirmed in comparison with literature data (Rashid *et al*., 1999; Ingólfsdóttir, 2002) (Figure 4.32, Rr Ia).

Identification of chemical compounds from ethyl acetate extract

Uℓ IIb: Elution of column with ethyl acetate: methanol (90:10) gave an off white color powder compound. **Rf**= 0.55, [Solvent system, dichloromethane: methanol (9:1)]. **^{1}H NMR (CD_3OD) δ***:* 3.89 (s, 3H, -OCH_3-8), 2.67 (s, 3H, CH_3-9'), 2.56 (s, 3H, CH_3-9), 2.03 (s, 3H, CH_3-8'), 1.98 (s, 3H, CH_3-10). **^{13}C NMR (CD_3OD) δ** : 171.3 (CO, C-7'), 164.7 (CO, C-7), 163.5 (C-OH, C-2'), 163.4 (C-OH, C-4), 161.5 (Cq, C-2), 153.3 (Cq, C-4'), 142.0 (C-CH_3, C-6), 141.4 (C- CH_3, C-6'), 117.1 (CH, C-5'), 116.9 (Cq, C-1), 111.8 (Cq, C-1'), 111.1 (CH, C-5), 109.6 (C- CH_3, C-3), 107.5 (C- CH_3, C-3'), 56.1 (-OCH_3, C-8), 25.0 (-CH_3, C-9'), 24.3 (-CH_3, C-9), 9.5 (-CH_3, C-8'), 7.9 (-CH_3, C-10). **(+) ESI-MS** *m/z* 381.3 was observed for $[M+Na-2H]^+$, (-) **HR ESI-MS** *m/z* 383.1101 calculated for $C_{19}H_{20}O_7Na$.

^{1}H NMR spectra revealed the presence of three methyl groups at δ 1.98, 2.03 and 2.56. The aromatic proton of one of the ring showed a peak at δ 6.25, while the other two protons of the other ring showed a peak at δ 6.75. ^{13}C NMR spectra revealed carbonyl of the acid at δ 171.3, carbonyl carbon of the ester linkage between the two rings showed a peak at 164.7, carbons bearing the two hydroxyls at C-4 and C-2′ absorbed at δ 163.5 and 163.4 respectively. Among various aromatic ring carbons, C-1, C-1′, C-5, C-5′ showed absorbance at δ 116.9, 111.8, 111.1 and 117.1 respectively. High resolution mass spectra gave a molecular ion peak at *m/z* 383.1101

corresponding for the molecular formula $C_6H_{14}O_6Na$. Based on spectral data Uℓ IIb was identified as **4-O-Dimethyl Diffaractic acid** (Figure 4.36, Uℓ IIb).

Uℓ IIIb: Further elution with ethyl acetate: methanol (90:10) gave colorless flakes soluble in methanol. **Rf**= 0.57, [Solvent system, dichloromethane: methanol (7:3)]. **m.p.** 133-137 ^oC. **^{1}H NMR (CD_3OD) δ**: 6.83 (s, 1H, C-2), 6.82 (s, 1H, C-4), 6.69 (s, 1H, C-3'), 3.90 (s, 3H, -OCH_3), 3.16 (s, 2H, CH_2-8'), 2.78 (4H, m, CH_2-2', 4'), 1.66 (6H, m, CH_2 -9', 10', 11'), 1.25 (4H, m, CH_2 -3', 5'), 0.91 (t, 6H, CH_3-5', 12'). **^{13}C NMR (CD_3OD) δ** : 205.5 (CO, C-1'), 165.7 (CO, C-7'), 164.1 (Cq, C-3), 163.5 (CO, C-7), 162.7 (Cq, C-5), 150.0 (C-OH, C-2'), 148.3 (Cq, C-4'), 142.3 (Cq, C-5'), 140.7 (Cq, C-1), 134.1 (Cq, C-6'), 123.0 (Cq, C-1'), 112.4 (Cq, C-2), 112.1 (Cq, C-6), 107.6 (Cq, C-4), 107.0 (Cq, C-3'), 56.8 (-OCH_3), 42.9 (-CH_2, C-2''), 33.3 (-CH2, C-9''), 32.0 (-CH_2, C-8''), 30.6 (-CH_2,C-10''), 28.9 (-CH_2, C-2'') 23.4 (-CH_2, C-11''), 23.0 (-CH_2, C-4''), 12.6 (-CH_3, C-12''), 12.7 (- CH_3, C-5''). **(-) ESI MS**: *m/z* 455.2 $[M-H]^-$, **(-) HR ESIMS**: *m/z* 457.18564 $[M-H]^-$, calculated for the molecular formula $C_{25}H_{29}O_8$.

^{1}H NMR spectrum revealed the presence of proton attached to the aromatic ring at δ 6.83, 6.82 and 6.82. Methyl of the alkyl chain absorbed at δ 0.91. Methylene protons absorbed at δ 3.16, 2.78, 1.66 and 1.25 with splitting patterns because of the presence of adjacent protons. High resolution mass spectra gave a peak at *m/z* 457.18564 $[M-H]^-$, corresponding to the molecular formula $C_{25}H_{29}O_8$. Based on spectral data, Uℓ IIIb was identified as **lobaric acid** (Figure 4.36, Uℓ IIIb).

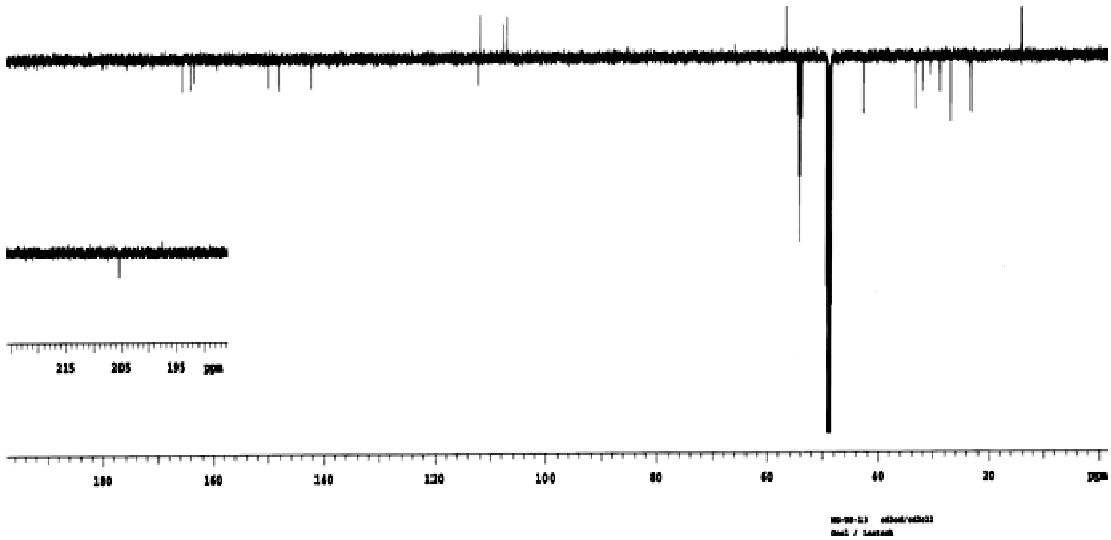


Figure 4.36a: APT spectrum of lobaric acid (Uℓ IIIb)

4.3.2.4 Isolation and identification of chemical constituents from crude extracts of *Stereocaulon himalayense*

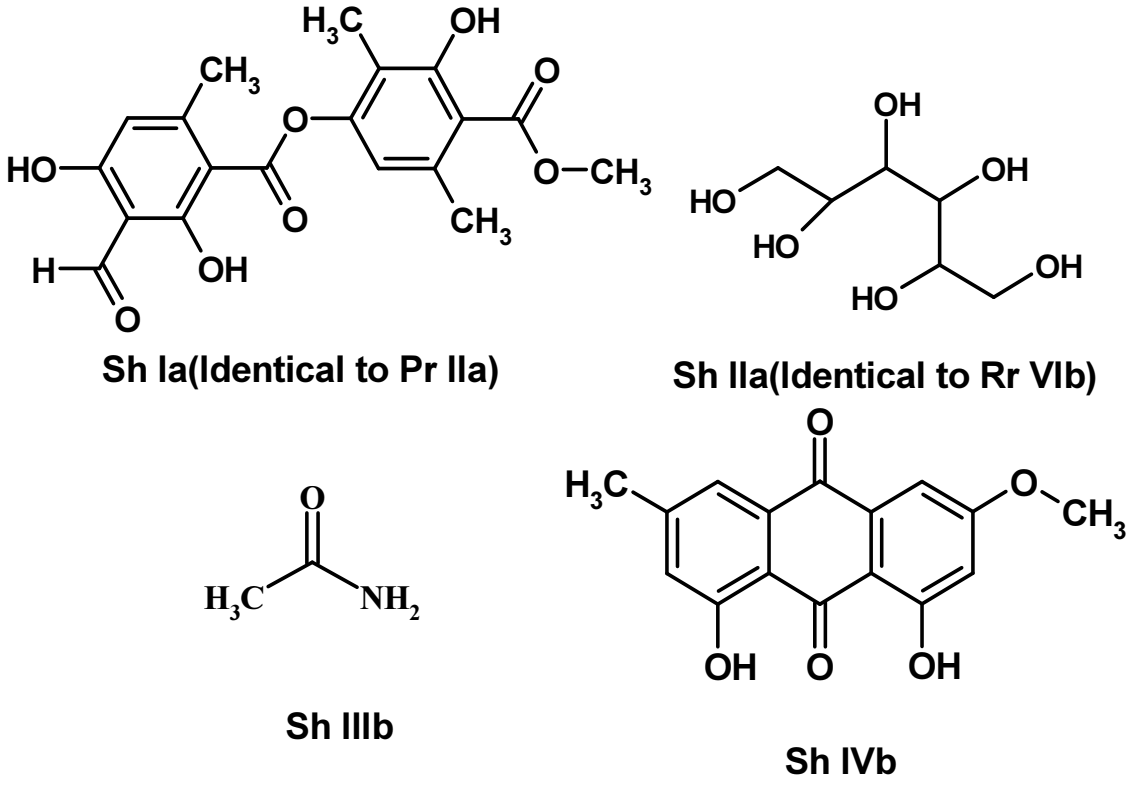


Figure 4.37: Chemical constituents isolated from *S. himalayense*

Identification of chemical constituents from hexane extract

Sh Ia: Elution of column with hexane: dichloromethane (1:1, 100 ml) gave a colorless crystalline compound. $\mathbf{R_f}$ = 0.75 [TLC solvent system, toluene:acetic acid (170:30 v/v)]. **m.p.** 193-195 ^{o}C. **^{1}H NMR ($CDCl_3$) δ**: 12.53 (s, 1H, 4-OH), 12.48 (s, 1H, 2-OH), 11.93 (s, 1H, 2'-OH), 10.33 (s, 1H, H-8), 6.49 (s, 1H, H-5'), 6.38 (s, 1H, H-5), 3.96 (s, 3H, H-10'), 2.67 (s, 3H, H-9), 2.52 (s, 3H, H-9'), 2.07 (s, 3H, H-8'). **^{13}C NMR ($CDCl_3$) δ**: 193.6 (CO, C-8), 172.0 (CO, C-7'), 169.5 (CO, C-7), 168.9 (C_q, C-2), 167.3 (C_q, C-4), 162.7 (C_q, C-2'), 152.3 (C_q, C-6), 151.9 (C_q, C-4'), 139.8 (C_q, C-6'), 116.7 (C_q, C-3'), 115.9 (CH, C-5'), 112.8 (CH, C-5), 110.2 (C_q-1'), 108.5 (C_q-3), 102.8 (C_q-1), 52.3 (CH_3-10'), 25.6 (CH_3-9), 24.1 (CH_3-9'), 9.4 (CH_3-8'). **(+)HR-ESI MS**: *m/z* 397.08939 $[M+Na]^+$ calculated for $C_{19}H_{18}O_8Na$. Spectral data was found identical to Pr IIa, thus it was identified as **atranorin** (Figure 4.27, Pr IIa).

Sh IIa: This compound was obtained with dichloromethane: ethyl acetate (70:30, v/v) as colorless solid, which was recrystallized from acetone. **^{1}H NMR (DMSO-d_6)** ***δ:*** 3.60 (m, 2H, C-1, CH_2), 3.57 (m, 1H, C-2, CH_2), 3.40 (m, 1H, CH-3), 4.07 (s, C-3, -OH), 4.26 (C-1, -OH), 4.34 (d, 1H, C-2, -OH). **^{13}C NMR (DMSO-d_6)** ***δ:*** 71.25 (C-2, CH), 69.65, 63.73 (C-1, CH_2), 69.6 (C-3, CH). **(+) ESI-MS** *m/z* 205.1 $[M+Na]^+$. Spectral data was found identical to Rr VIb , thus, Sh IIa was identified **as hexane-1, 2, 3, 4, 5, 6-hexaol (ido hexitol)** (Figure 4.32, Rr VIb).

Identification of chemical constituents from ethyl acetate extract

Sh IIIb: This was obtained as insoluble solid which later developed into white flakes on drying. **^{1}H NMR (CD_3OD)** ***δ***: 1.98 (s, 3H, CH_3). **^{13}C NMR (CD_3OD)** ***δ***: 180.3 (CO, C-1), 24.3 (CH_3, C-2). **EI MS** *m/z* 59 $[M^+]$. Based on spectral data Sh IIIb was identified as **acetamide.** (Figure 4.37, Sh IIIb).

Sh IVb: Elution of column with ethyl acetate: methanol (9:1) gave a orange colored compound on drying. **Rf**= 0.38, [Solvent system, dichloromethane: methanol (1:1)].**^{1}H NMR ($CDCl_3$)** ***δ***: 12.30 (s, 1H, 8-OH), 12.08 (s, 1H, 1-OH), 7.60 (s, CH-5), 7.32 (s, 1H, CH-4), 7.15 (s, 1H, CH-7), 6.60 (s, CH-2), 3.90 (s, 3H, -OCH_3), 2.50 (s, 3H, CH_3). **^{13}C NMR ($CDCl_3$)** ***δ*** : 190.8 (CO, C-10), 182.0 (CO, C-13), 166.5 (Cq,C-OCH_3, C-3), 165.2 (C-OH, C-1), 162.5 (C-OH, C-8), 148.4 (Cq,C-CH_3, C-6), 135.2 (Cq, C-12), 133.2 (Cq, C-14), 124.5 (CH, C-7), 121.3 (CH, C-5), 113.7 (Cq, C-9), 110.2 (CH, C-4), 108.2 (CH, C-2), 56.1 (-OCH_3, C-1'), 22.1 (-CH_3, C-2□). **(+) ESI MS**: *m/z* 307.1 $[M+Na]^+$, 591.1 $[2M+Na]^+$. **(+)HR ESI MS** *m/z* 307.0577 calculated for $C_{16}H_{12}O_5Na$. Based on spectral data Sh IVb was identified as **1, 8-dihydroxy-3-methoxy-6-methyl-anthraquinone,** commonly called as **Physcion** (Figure 4.37, Sh IVb).

Table 4.23a summerizes the total compounds isolated from the test lichen species.

Table 4.23a: Isolated compounds from the test lichen species

Compounds code	Name
Pr I a	Iso Usnic acid
Pr IIa	Atranorin
Pr IIIa	Protolichesterinic acid
Pr IVb	Evernyl
Pr Vb	Ethyl hematommate
Pr VIb	Ethyl orsellinate
Pr VIIb	Methyl hematommate
Pr VIIIb	2-Hydroxy- 4-methoxy- 3, 6-dimethyl benzoic acid
Pr IXb	1-Hydroxy-3, 6-dimethoxy-8-methyl-xanthen-9-one
Pr Xb	Baeomycesic acid
Pr XIb	Salazinic acid
Pr XIIb	2-methoxy-4-hydroxy-3, 6-dimethyl benzoic acid
Pr XIIIb	Atranol
Pr XIVb	3-(5-substituted-3-hydroxy-2,4-dimethylphenoxy)-2,4 dihydroxy-6-methyl benzaldehyde
Rr Ia	Usnic acid
Rr IIb	Divaricatinic acid
Rr IIIb	Homosekikaic acid
Rr IVb	Sekikaic acid,
Rr Vb	Divaric acid
Rr VIb	Ido Hexitol
Uℓ Ia	Usnic acid
U ℓ IIb	Diffaractic acid
U ℓ IIIb	Lobaric acid
Sh Ia	Atranorin
Sh IIa	Ido Hexitol
Sh IIIb	Acetamide
Sh IVb	1,8-dihydroxy-3-methoxy -6-methyl-anthraquinone

Where Pr, Rr, Uℓ, Sh stands for lichens *Parmelia reticulata*, *Ramalina roesleri*, *Usnea longissima*, *Stereocaulon himalayense.* a and b stands for isolation of compounds from hexane and ethyl acetate extracts respectively

Various compounds isolated from lichens species can be classified as follows:

Table 4.23b: Different class of compounds obtained from the test lichen species

Class	Lichen species	Solvent	Compound
Aliphatic acid	*P. reticulata*	Hexane	Protolichesterinic acid
Monocyclic phenols	*P. reticulata*	Ethyl acetate	Evernyl, Methyl hematommate, Ethyl hematommate, Ethyl orsellinate, Atranol
Benzoic acid derivatives	*P. reticulata*	Ethyl acetate	2-Hydroxy- 4-methoxy- 3, 6-dimethyl benzoic acid,
	R. roesleri	Ethyl acetate	2-methoxy-4-hydroxy-3,6-dimethyl benzoic acid
			Divaricatinic acid, Divaric acid
Depsides	*P. reticulata*	Hexane	Atranorin,
	P. reticulata	Ethyl acetate	Baeomycesic acid,
	R. roesleri	Ethyl acetate	Sekikaic acid, Homosekikaic acid
	U. longissima	Ethyl acetate	Diffaractic acid
Depsidones	*P. reticulata*	Ethyl acetate	Salazinic acid,
	U. longissima	Ethyl acetate	Lobaric acid
Dibenzofurans	*P. reticulata*	Hexane	Iso Usnic acid,
	R. roesleri	Hexane	Usnic acid
	U. longissima	Hexane	
Anthraquinones	*S. himalayense*	Ethyl acetate	1,8-dihydroxy-3-methoxy-6-methyl-anthraquinone
Sugar	*S. himalayense*	Hexane	Ido Hexitol
Xanthones	*P. reticulata*	Ethyl acetate	1-Hydroxy-3, 6-dimethoxy-8-methyl-xanthen-9-one
Amide	*S. himalayense*	Ethyl acetate	Acetamide

4.4 Bioactivities of chemical constituents

4.4.1 *In vitro* antifungal activity

The antifungal activity of isolated chemical constituents was screened against pathogenic fungi namely *S. rolfsii, R. solani, R. bataticola, F. udum, P. apanidermatum* and *P. debaryanum* at various concentrations and their calculated ED_{50} values were reported in Table 14 and Figure 4.38. Isousnic acid (Figure 4.27, Pr Ia) exhibited 72 to 87 % inhibition against all the test fungi at 250 µg ml^{-1} concentration with ED_{50} values ranging from 62.30 to 70.98 µg ml^{-1} (Table 4.24, Figure 4.38). Atranorin (Figure 4.27, Pr IIa) exhibited 100, 89 and 67 % inhibition zone against *S. rolfsii* at 250, 125 and 62.5 µg ml^{-1} concentrations respectively with ED_{50} value of 39.70 µg ml^{-1}. It was also able to inhibit 89, 78 and 56 % growth of *P. debaryanum* at 250, 125 and 62.5 µg ml^{-1} concentration respectively (ED_{50} = 44.79 µg ml^{-1}) (Table 4.24, Figure 4.38). These results are similar to earlier reports by Keonig and Wright, 1999; Huneck, 1999, showing antifungal activity of atranorin against another fungi *Colletotrichum gloeosporioides.*

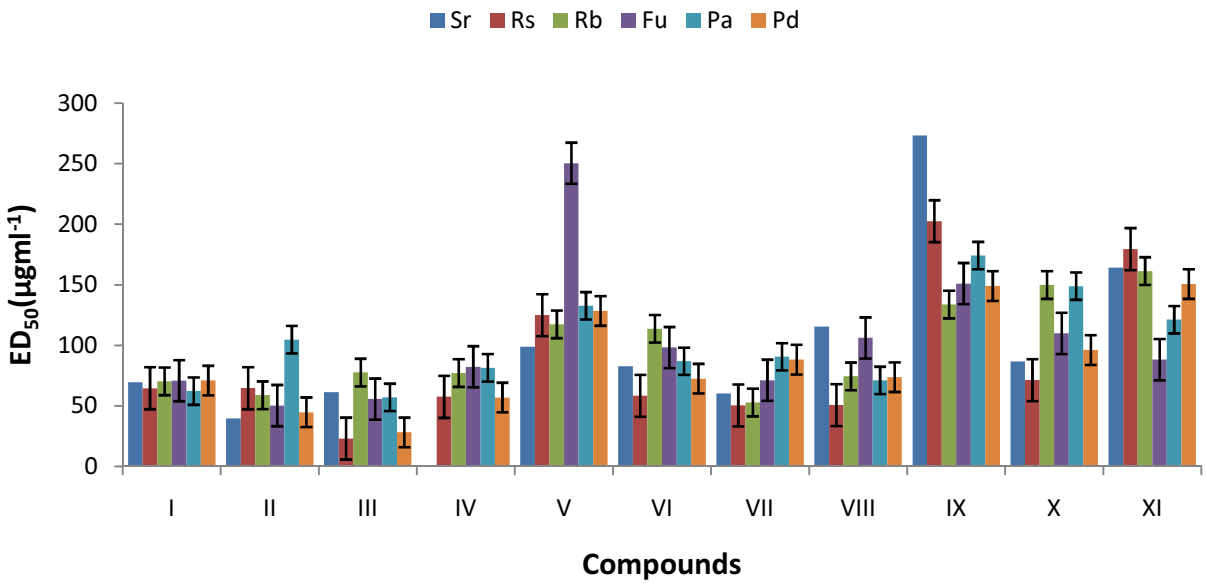


Figure 4.38: Antifungal activity of chemical constituents isolated from *P. reticulata*

Antifungal activity of protolichesterinic acid (Figure 4.27, Pr IIIa) was found to be similar with the commercial fungicide, hexaconazole against *P. debaryanum*. It showed 100, 90 and 78 % inhibition at 250, 125 and 62.5 µg ml^{-1} concentration with ED_{50} value of 16.07 µg ml^{-1}, whereas hexaconazole showed 59.2, 26.3 and 11.3 %

inhibition at 31, 15.5 and 7.25 µg ml^{-1} concentrations with ED_{50} value of 25.92 µg ml^{-1} (Table 4.24 and Figure 4.38). Protolichesterinic acid also showed 83, 77 and 67% inhibition against *R. solani* at 250, 125 and 62.5 µg ml^{-1} concentrations respectively (ED_{50} = 23.09 µg ml^{-1}) (Figure 4.38, Table 4.24).

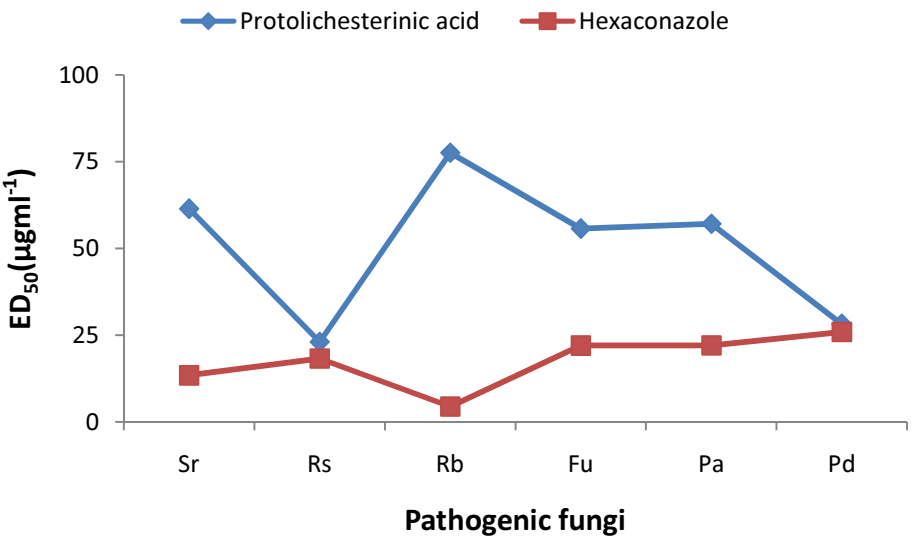


Figure 4.39: Comparison of antifungal activity of protolichesterinic acid with commercial fungicide hexaconazole

Evernyl (Figure 4.27, Pr IVb) was found to be moderately active against *R. solani* and *P. debarynum* with calculated ED_{50} of 57.50 and 56.96 µg ml^{-1} respectively. The activity of isousnic acid and evernyl was equally effective against all the test pathogens (Figure 4.38). It has been reported earlier that evernic acid severely inhibited growth of *Phytophthora infestans*, while the growth of *P. ultimum* was merely inhibited by evernic acid (Lauterwein *et al*, 1995). Ethyl hematommate (Figure 4.27, Pr Vb) was found to be less active as compared to methyl hematommate (Figure 4.27, Pr VIIb) (Table 4.24, Figure 4.40). The methyl hematommate showed 89 to 67 % inhibitions against all the test fungi as compared to ethyl hematommate, which showed 61 to 27 % inhibitions at 250 µg ml^{-1} concentration. It is noteworthy, that a change in the carbon chain length (from methyl group to ethyl group), has a detrimental effect towards antifungal activity. Methyl hematommate (Figure 4.27, Pr VIIb) showed moderate antifungal activity against all the test fungi with ED_{50} ranging from 50.52-90.59 µg ml^{-1} (Figure 4.38) while ethyl orsellinate (Figure 4.27, Pr VIb) and 2-hydroxy-4-methoxy-3, 6-dimethyl benzoic acid (Figure 4.27, Pr VIIIb) showed maximum antifungal activity against *R. solani* with ED_{50} values of 58.33 and 50.65 µg ml^{-1} respectively. Lichenxanthone (Figure 4.27, Pr IXb) showed minimum inhibition against all the test fungi (ED_{50} 133.76-273.33 µg ml^{-1}). Baeomycesic acid (Figure

4.27, Pr Xb) was found to be moderate active against *S. rolfsii* and *R. solani* with ED_{50} values 86.65 and 71.28 µg ml^{-1} respectively as compared to other test fungi. Salazinic acid (Figure 4.27, Pr XIb) was found moderately active against *F. udum* with ED_{50} value of 88.20 µg ml^{-1}. It was found less active against other test fungi.

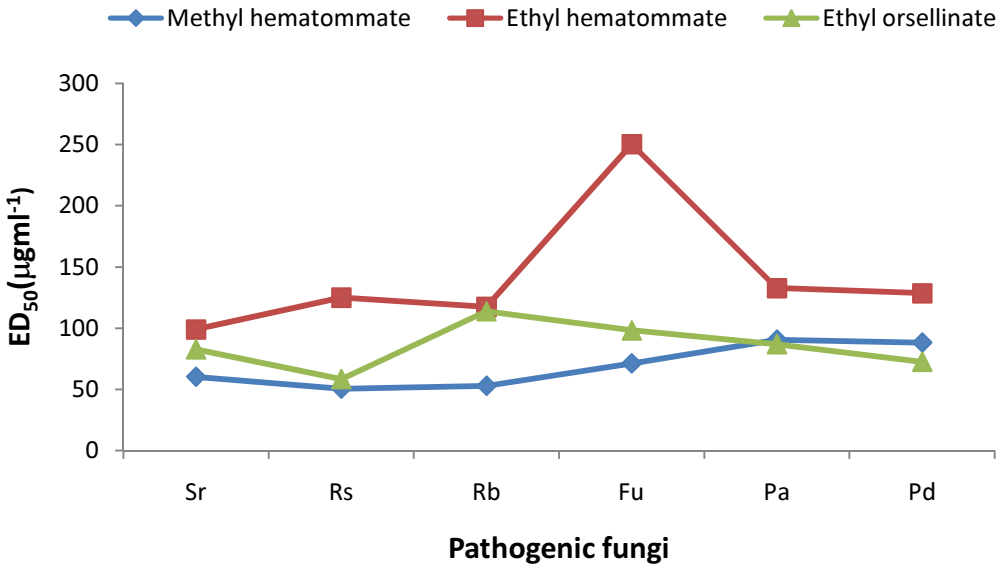


Figure 4.40: Comparison of antifungal activity of methyl hematommate, ethyl hematommate and ethyl orsellinate

Of all the chemical constituents from ***R. roesleri***, Usnic acid (Figure 4.32, Rr Ia) inhibited 74.44 to 86.4% growth of all the test pathogenic fungi when tested at 250 µg ml^{-1} concentration (Table 4.24) with ED_{50} values ranging from 12.34 to 25.69 µg ml^{-1} (Table 4.24, Figure 4.41). It was able to inhibit 58.30, 56.00 and 57.78 % growth of *R. solani*, *R. bataticola* and *P. debaryanum* respectively at 31.25 µg ml^{-1} concentration. It was found to be very active against *R. bataticola* (ED_{50} = 12.54 µg ml^{-1}), *P. debaryanum* (ED_{50} = 12.34 µg ml^{-1}) and *R. solani* (ED_{50} = 15.99 µg ml^{-1}) (Figure 4.41). The antifungal activity of usnic acid was found to be more against *R. solani* and *P. debaryanum* than commercial fungicide hexaconazole (Figure 4.42).

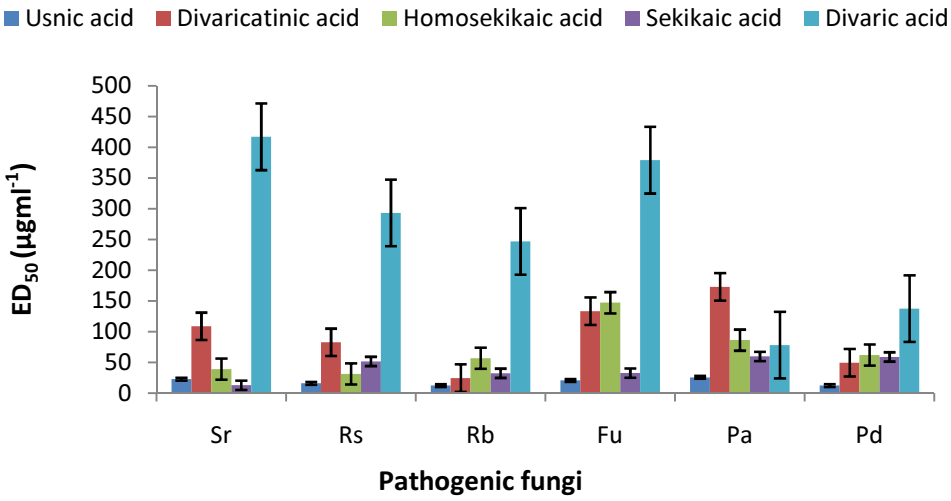


Figure 4.41: Antifungal activity of chemical constituents isolated from *R. roesleri*

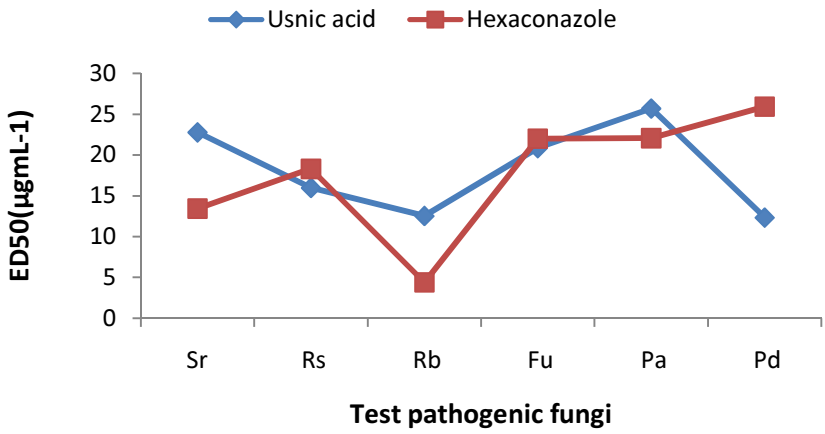


Figure 4.42: Comparison of antifungal activity of usnic acid with commercial fungicide hexaconazole

Divaricatinic acid (Figure 4.32, Rr IIb) inhibited 74, 67 and 60 % growth of *R. bataticola* at 250, 125, and 62.5 µg ml^{-1} concentrations respectively with an ED_{50} value of 24.67 µg ml^{-1}. It was also found active against *P. debaryanum* with ED_{50} value of 49.64 µg ml^{-1}. Whereas, it was found to be less active against *P. aphanidermatum* (ED_{50} = 173.01 µg ml^{-1}, Table 4.24), *F. udum* (ED_{50} = 133.52 µg ml^{-1}) and *S. rolfsii* (ED_{50} = 108.81 µg ml^{-1}) in comparison to *R. solani* (ED_{50} = 82.83 µg ml^{-1}), *P. debaryanum* (ED_{50} = 49.64 µg ml^{-1}) and *R. bataticola* (ED_{50} = 24.67 µg ml^{-1}) (Figure 4.41, Table 4.24). Homosekikaic acid (Figure 4.32, Rr IIIb) was found to be highly active against *S. rolfsii* and *R. solani* with ED_{50} values

of 39.14 and 31.50 µg ml^{-1}, respectively, in comparison to other test pathogenic fungi. It inhibited 79, 70 and 58% growth of *S. rolfsii* at 250, 125 and 62.5 µg ml^{-1} respectively and 78, 72 and 60 % growth inhibition against *R. solani* at 250, 125 and 62.5 µg ml^{-1} concentrations respectively. It was found to be moderately active against *R. bataticola* and *P. debaryanum* with an ED_{50} value of 56.90 and 61.98 µg ml^{-1} respectively. However, against *F. udum* it was found to be least active (ED_{50} = 147.18 µg ml^{-1}) (Table 4.24). Sekikaic acid (Figure 4.32, Rr IVb) showed maximum activity against *S. rolfsii* with 92.3 % inhibition at 250 µg ml^{-1}. Its ED_{50} was calculated to be 13.00 µg ml^{-1} which is similar to a standard fungicide hexaconazole (Table 4.24). It was also found active against *F. udum* (ED_{50} = 32.85 µg ml^{-1}) and *R. bataticola* (ED_{50} = 32.28 µg ml^{-1}). It was more active than homosekikaic acid (Figure 4.43).

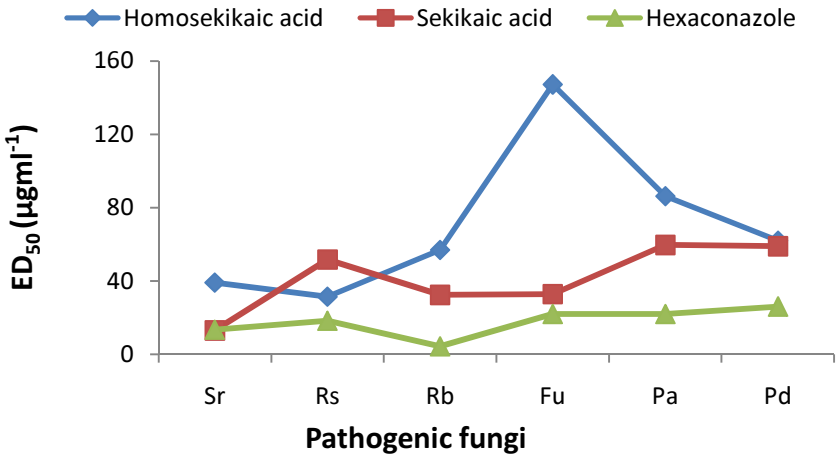


Figure 4.43: Comparison of antifungal activity of homosekikaic acid, sekikaic acid with fungicide hexaconazole

Divaric acid (Figure 4.32, Rr Vb) was found to be less active against most of the test fungi in comparison to usnic acid, divaricatinic acid and homosekikaic acid (Table 4.24, Figure 4.41). However, it was found moderately active against *P. aphanidermatum* with ED_{50} value of 78.20 µg ml^{-1}. This is first report of the antifungal activity of divaric acid. Similarly, hexitol (Figure 4.32, Rr VIb) isolated from *S. himalayense* was found to inactive against all the tested fungi.

Among various lichen metabolites, majority of compounds belongs to the class of phenolics. Accumulation of phenolics in lichen thallus is typical characteristic of lichen defense mechanism. *In vivo* studies revealed that these hydroxy benzoic acid

derivatives are rapidly hydrolyzed to the corresponding acid, which is then conjugated and excreted, thus their toxicity is generally low (Wilson *et al*,. 1998). Therefore, it is not surprising that lichen derived mononuclear phenolic compounds such as methyl and ethyl orsellinate, methyl β-orsellinate (Fujikawa *et al*., 1970; Ingólfsdóttir *et al*., 1985), and methyl hematommate (Hickey *et al*., 1990) display antibiotic activity against a variety of microorganisms. The present study evaluated the activity of these hydroxy benzoic acid derivatives obtained, against plant pathogenic fungi. The present study also supports the observations by earlier workers.

Detailed study of the activity exhibited by isolated metabolites from lichens revealed that the protolichesterinic acid (Figure 4.27, Pr IIIa), an aliphatic α-methylene-α-lactone and usnic acid (Figure 4.32, Rr Ia) showed maximum antifungal activity against all the test fungi. Activity of protolichesterinic acid was comparable to the hexaconazole, a commercial fungicide against *P. debarynum* and *R. solani* and activity of usnic acid was comparable to the hexaconazole against *P. debaryanum* and *F. udum* (Table 4.24; Figure 4.38, 4.42). The antifungal response of the protolichesterinic acid towards the tested pathogens may be attributed to the presence of the furan moiety with a long alkyl chain attached to it. Usnic acid, isolated from *R. roesleri* and *U. longissima* exhibited the broad spectrum activity towards the tested fungi. Studies have shown that the intramolecular hydrogen bonds responsible for the lipophilic nature of usnic acid are responsible for its diverse activities. These findings are comparable to the results obtained by Proksa *et al*., who reported antifungal activity of usnic acid against the plant pathogens *Penicillium frequentans* and *verticillium albo-atrum*.

Thus, it can be inferred that the presence of protolichesterinic acid in hexane extract of *P. reticulata* and usnic acid in hexane extracts of *R. roesleri* and *U. longissima* may be responsible for the better antifungal activities of these extracts. It is also noteworthy to mention that the differences in the activity of these compounds against various fungi indicate that these metabolites act in a species-specific fashion.

Table 4.24: Antifungal activities of chemical constituents isolated from solvent extracts of *P. reticulata* and *R. roesleri* against pathogenic fungi.

Compound No.	Compound Name	Concentration (µg ml^{-1})	Inhibition (%)					
			Rb	Rs	Fu	Pa	Pd	Sr
Pr Ia	Iso usnic acid	250	87.17±1.25	86.37±1.02	81.00±1.34	75.40±2.95	72.10±1.60	84.00±1.41
		125	69.10±0.88	73.13±0.35	63.33±0.94	53.16±1.73	66.23±1.60	66.10±2.78
		62.5	29.30±3.90	35.20±1.10	33.40±2.77	46.10±3.10	35.13±0.90	32.80±0.87
		ED_{50}	**70.36**	**64.62**	**70.86**	**62.30**	**70.98**	**69.63**
Pr IIa	Atranorin	250	80.00±2.64	81.33±1.73	90.00±1.00	60.33±2.30	89.67±1.50	100±1.00
		125	67.67±1.22	62.00±0.41	81.34±0.61	43.00±0.81	78.00±0.60	89.67±1.08
		62.5	44.33±0.94	40.00±0.85	50.00±0.17	22.00±0.60	56.33±0.75	67.34±1.33
		ED_{50}	**58.82**	**64.63**	**50.32**	**104.73**	**44.79**	**39.70**
Pr IIIa	Protolichesterinic acid	250	89.33±0.75	83.33±0.40	90.33±1.13	80.33±1.89	100±1.66	90.00±0.91
		125	50.00±0.10	77.00±0.72	78.00±0.45	72.33±1.20	90.00±1.58	72.16±0.23
		62.5	28.33±0.66	67.00±0.20	44.33±0.80	44.33±0.85	78.32±1.04	39.33±0.94
		ED_{50}	**77.64**	**23.09**	**55.68**	**57.10**	**16.07**	**61.41**
Pr IVb	Evernyl	250	90.34±2.90	80.33±1.20	80.46±0.47	88.30±1.04	89.33±1.03	74.32±0.75
		125	50.33±1.04	67.00±0.52	50.00±0.00	50.00±0.20	72.33±0.96	50.00±1.49
		62.5	28.33±1.04	46.00±0.62	26.67±0.50	22.33±0.66	44.00±0.92	33.00±1.11
		ED_{50}	**77.21**	**57.50**	**82.31**	**81.50**	**56.96**	**80.21**
Pr Vb	Ethyl hematommate	250	53.36±1.68	55.47±2.00	27.40±0.72	50.40±0.43	56.47±1.90	61.00±0.62
		125	42.80±0.79	33.53±0.70	13.46±2.77	32.60±2.21	26.30±0.70	47.50±3.34
		62.5	27.40±2.80	15.30±4.64	6.40±1.01	13.06±2.64	15.13±0.41	28.40±0.70
		ED_{50}	**117.35**	**124.98**	**250.36**	**132.69**	**128.50**	**98.97**
Pr VIb	Ethyl orsellinate	250	67.16±0.85	90.36±0.81	79.43±1.72	83.30±1.83	91.36±0.83	78.00±0.43
		125	28.00±2.20	68.06±1.58	32.46±1.13	50.36±2.21	61.23±1.16	50.50±1.30
		62.5	16.36±0.86	44.36±0.92	17.33±0.85	18.50±2.52	28.33±2.95	28.30±0.10
		ED_{50}	**113.75**	**58.33**	**98.20**	**86.92**	**72.51**	**82.69**

contd.

Compound No.	Compound Name	Concentration (μg ml^{-1})	Inhibition (%)					
			Rb	Rs	Fu	Pa	Pd	Sr
Pr VIIb	Methyl hematommate	250	78.20±3.21	81.70±5.31	67.33±1.70	69.26±0.41	71.33±3.31	89.16±1.19
		125	61.43±0.58	72.60±3.06	50.46±0.75	46.36±2.70	47.26±1.67	66.60±0.65
		62.5	53.13±0.95	50.63±3.31	46.46±0.70	24.43±1.80	34.00±1.35	44.21±1.24
		ED_{50}	**52.90**	**50.52**	**71.22**	**90.59**	**88.20**	**60.33**
Pr VIIIb	2-Hydroxy-4-methoxy-3,6-di methyl benzoic acid	250	68.00±0.17	82.00±1.30	58.00±0.10	79.33±2.30	83.83±0.87	53.00±1.41
		125	56.40±2.80	70.60±2.21	46.46±0.70	67.66±0.70	61.40±1.31	44.30±4.64
		62.5	39.40±1.82	50.00±0.85	23.66±0.35	28.36±0.64	33.00±1.70	28.36±2.27
		ED_{50}	**74.48**	**50.65**	**106.13**	**71.11**	**73.8**	**115.56**
Pr IXb	Lichexanthone	250	50.00±0.45	41.00±0.72	53.33±1.04	42.66±1.80	42.03±1.27	37.33±0.80
		125	30.26±0.41	29.36±3.30	24.53±0.70	23.40±2.80	28.40±0.70	23.20±3.21
		62.5	11.43±0.75	19.63±1.20	20.33±0.94	13.03±0.20	9.03±0.95	16.67±0.68
		ED_{50}	**133.76**	**202.50**	**151.03**	**174.14**	**148.95**	**273.33**
Pr Xb	Baeomycesic acid	250	49.13±3.44	81.63±0.75	57.86±0.20	44.40±2.00	77.14±1.25	72.40±0.62
		125	28.40±2.77	61.40±2.36	47.13±0.94	29.30±0.45	38.23±2.90	44.43±1.73
		62.5	17.10±0.36	33.40±0.91	33.46±0.94	11.36±2.66	18.30±1.04	32.20±0.36
		ED_{50}	**149.87**	**71.28**	**109.85**	**148.90**	**143.50**	**86.65**
Pr XIb	Salazinic acid	250	41.20±1.24	45.20±1.10	67.00±1.34	61.00±1.35	43.46±1.13	38.20±0.36
		125	26.80±0.60	30.20±2.00	50.13±2.66	25.50±0.87	11.06±0.61	21.03±1.26
		62.5	9.00±1.91	22.10±1.17	31.00±0.36	28.06±0.15	11.93±1.87	5.93±0.15
		ED_{50}	**161.35**	**179.47**	**88.20**	**121.22**	**150.67**	**164.10**

contd.

Compound No.	Compound Name	Concentration (µg ml^{-1})	Inhibition (%)					
			Rb	Rs	Fu	Pa	Pd	Sr
Rr Ia	Usnic Acid	250	86.4±0.17	78.1±0.65	82.22±0.17	74.44±0.62	74.44±0.26	80.7±0.67
		125	76.11±0.46	72.11±0.11	74.44±0.22	64.44±0.67	68.89±0.01	72.7±0.67
		62.5	63.9±0.61	63.3±0.46	57.78±0.72	56.67±1.02	64.44±1.03	65.1±0.32
		ED_{50}	**12.54**	**15.99**	**20.86**	**25.69**	**12.34**	**22.78**
Rr IIb	Divaricatinic acid	250	73.8±1.02	72.2±1.20	57.78±0.63	54.44±0.33	72.22±0.36	61.60±1.03
		125	67.78±1.03	67.7±1.13	47.78±1.02	45.56±0.11	63.33±0.44	53.27±0.96
		62.5	60.00±0.29	39.20±0.12	42.22±0.91	40.00±0.27	53.33±0.47	41.26±0.38
		ED_{50}	**24.67**	**82.83**	**133.52**	**173.01**	**49.64**	**108.81**
Rr IIIb	Homosekikaic acid	250	68.60±1.32	78.89±0.57	62.50±0.29	62.60±0.76	67.20±0.17	78.89±0.62
		125	57.20±0.07	72.22±0.62	46.30±0.10	53.40±0.99	56.60± 0.69	70.00±0.63
		62.5	52.30±0.15	60.00±0.93	29.60±0.39	46.70±0.22	51.0±0.88	57.78±0.92
		ED_{50}	**56.94**	**31.50**	**147.18**	**86.32**	**61.98**	**39.14**
Rr IVb	Sekikaic acid	250	81.76±0.22	76.32±0.27	86.7± 0.25	90.1± 0..66	79.3±0.35	92.3 ± 0.17
		125	67.30±0.11	61.98±0.18	74.6± 0.59	73.1±0.98	67.3±0.20	89.2 ± 0.92
		62.5	62.60±0.29	54.64±0.37	64.20± 0.60	52.2± 0.20	51.0± 0.88	77.5 ± 0.48
		ED_{50}	**32.28**	**51.68**	**32.85**	**59.69**	**58.96**	**13.00**
Rr Vb	Divaric acid	250	60.0± 0.27	47.2± 0.10	53.3± 0.27	76.6±0.55	70.3± 0.79	53.4±0.44
		125	52.4± 0.21	40.4± 0.58	44.5± 0.21	69.2± 0.10	62.1± 0.15	41.0±0.70
		62.5	45.5± 0.26	39.0± 0.56	39.6± 0.10	61.5± 1.30	50.3± 0.58	33.3±0.32
		ED_{50}	**246.9**	**293.2**	**379.0**	**78.2**	**137.6**	**417.0**
Standard Reference	Hexaconazole	31.00	86.96±0.36	72.56±0.30	70.00±0.79	65.26±0.56	59.23±0.30	90.66±0.47
		15.50	73.23±0.41	38.20±0.70	23.66±0.26	32.73±0.15	26.30±0.43	45.30±0.50
		7.25	60.13±0.30	20.60±0.20	18.56±0.11	14.06±0.38	11.13±0.49	28.22±0.28
		ED_{50}	**4.40**	**18.30**	**22.01**	**22.06**	**25.92**	**13.43**

Rb=Rhizoctonia bataticola; Rs=Rhizoctonia solani; *Fu=Fusarium udum;Pa=Pythium aphanidermatum*;*Pd=Pythium debarynum; Sr=Sclerotium rolfsii.* [a]Mean percentage inhibition, used for ED_{50} calculation, is an average of four replicates and its Standard error ranged from ±0.2 to 3.4, ED_{50} values (µg ml^{-1}) calculated from mean. Degrees of freedom = 3

4.4.2 Herbicidal potential

Metabolites namely usnic acid, homosekikaic acid and divaricatinic acid were tested at 50, 25 and 12.50 µg ml^{-1} concentrations for Herbicidal potential on the growth of *P. minor* seeds.

Seed Germination

Usnic acid (Figure 4.32, Rr Ia) at different concentrations viz. 50, 25 and 12.5 µg ml^{-1} significantly affected germination of *P. minor* (Table 4.25, Figure 4.44). There was only 19.7 % germination of seeds at 50 µg ml^{-1} concentration as compared to the control with 90 % germination. It was observed that rate of germination is inversely dependant on concentration. There was only 52.7 and 39.5 % seed germination at 12.5 and 25 µg ml^{-1} concentrations respectively. The extent of inhibition increased with increasing concentration of usnic acid. Similar observation was also recorded with homosekikaic acid and divaricatinic acid. Homosekikaic acid was found to be less effective as compared to usnic acid. There was only 45.5, 58.7 and 64 % seed germination in comparison to usnic acid with 19.7, 39.5 and 52.7 % at 50, 25 and 12.5, µg ml^{-1} concentrations respectively. The seed germination with divaricatinic acid was found to be 52.2, 72.7 and 79.2 % at 50, 25 and 12.50 µg ml^{-1} concentrations respectively. Among three compounds tested above for seed germination, maximum inhibition was recorded with usnic acid followed by homosekikaic acid and divaricatinic acid (Table 4.25; Figure 4.44).

Shoot length

Usnic acid at all the concentrations showed the decrease in growth of shoot length (Table 4.25). It was observed that there was only 1.8, 3.6 and 4.8 cm shoot length at 50, 25 and 12.5 µg ml^{-1} concentrations respectively as compared to control with 10.7 cm of shoot length. At 50 µg ml^{-1} concentration, 83.18 % root inhibition was observed with usnic acid (Figure 4.45). Likewise, a significant decrease in shoot length from 10.7 cm in control to 4.2, 5.0 and 6.9 cm was recorded when homosekikaic acid applied at 50, 25 and 12.5 µg ml^{-1} concentrations respectively. There was only 30.9, 27.1 and 24.3 % inhibition of shoot length when divaricatinic acid was applied at 50, 25 and 12.5 µg ml^{-1} concentrations, respectively (Table 4.25, Figure 4.45). Maximum inhibition of shoot length was recorded with usnic acid followed by homosekikaic and divaricatinic acid.

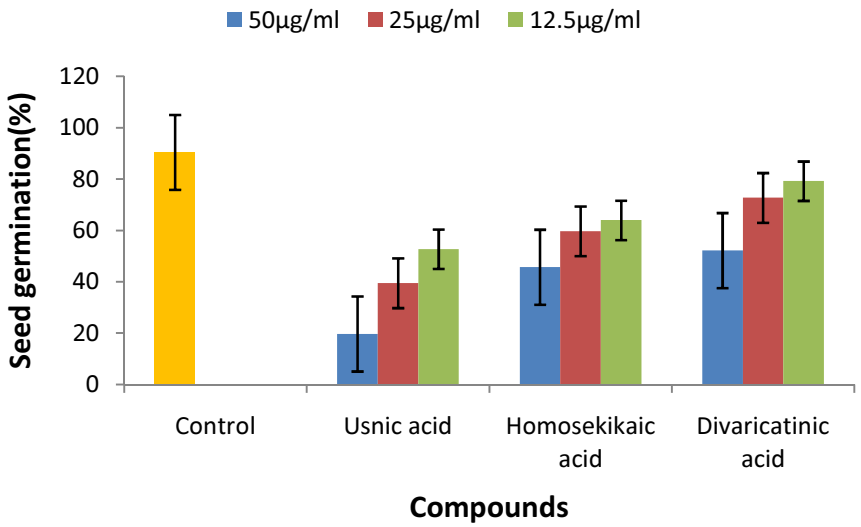


Figure 4.44: Comparison of seed germination of usnic acid, homosekikaic acid and divaricatinic acid.

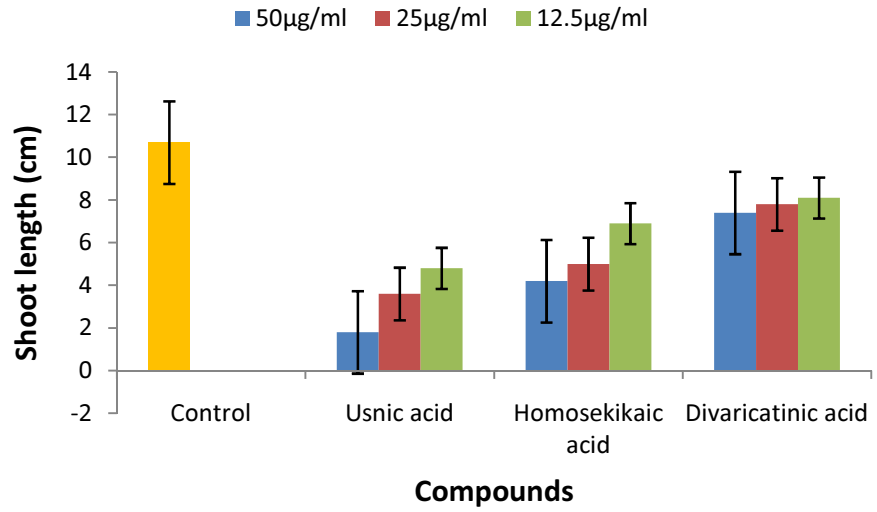


Figure 4.45: Comparison of shoot length of usnic acid, homosekikaic acid and divaricatinic acid

Root length

Similar to the effect of usnic acid and homosekikaic acid towards the shoot length, reduced root length was also recorded at all the concentrations applied. There were only 0.6, 1.7 and 1.9 cm root length observed at 50, 25 and 12.50 µg ml^{-1} concentrations of usnic acid respectively as compared to control (3.4 cm). Similarly, significant decrease in root length from 3.4 cm to 1.6 cm was recorded when homosekikaic acid was applied at 25 µg ml^{-1} concentration. Homosekikaic acid showed maximum inhibition at 25 µg ml^{-1} concentration than at 50 and 12.5 µg ml^{-1} concentrations. There were only 20.1, 11.7 and 8.8 % inhibition of root length at 50,

25 and 12.5 µg ml^{-1} concentrations respectively with divaricatinic acid. Maximum inhibition in root length was observed with usnic acid followed by homosekikaic acid and divaricatinic acid (Figure 4.46, Table 4.25).

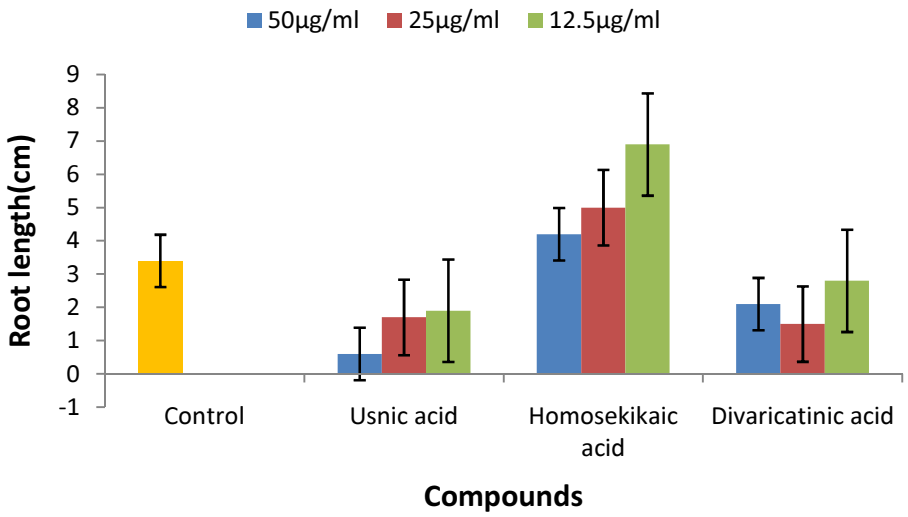


Figure 4.46: Comparison of root length of usnic acid, homosekikaic acid and divaricatinic acid

Discussion

The present study has revealed the herbicidal activity of usnic acid against *P. minor.* The results indicated that the effect of these metabolites on seed germination is concentration dependant. The treatments with 50 and 25 µg ml^{-1} of metabolite concentration significantly reduced the germination rate, whereas treatments with 12.5 µg ml^{-1} concentration had a low inhibitory effect. Cao *et al*., 2007, however reported a contrary effect that the germination of two pastures (*Galium verum* and *Lilium pumilum*) decreased with increasing aqueous extract concentration of *S. chamaejasme*. The potential role of lichen metabolites in allelopathic interactions has previously been reviewed (Lawrey, 1995; Dayan and Romagni, 2002). The phytotoxic effect of certain lichen metabolites may deter the growth of other lichen populations as well as that of competing seedlings for a particular ecological niche. The herbicidal activity of other depsides barbatic acid, lecanorin and the tridepside gyrophoric acid as PSII inhibitors has been reported earlier. This is the first report for the herbicidal activity of the depside homosekikaic acid.

Table 4.25: Effect of secondary metabolites isolated from *R. roesleri* on germination, shoot length and root length of *P. minor*

Compounds	Concentration (µg ml^{-1})	%Germination (Inhibition %)	Shoot length in cm (Inhibition %)	Root length in cm (Inhibition %)
Usnic acid	Control	90.5 ± 1.29 (0.00)	10.7 ± 0.08 (0.00)	3.4±0.16 (0.00)
	50	19.7 ± 1.71(78.18)	1.8 ± 0.26 (83.18)	0.6 ± 0.29 (82.35)
	25	39.5 ± 1.29 (56.34)	3.6 ± 0.40 (66.26)	1.7 ± 0.80 (50.00)
	12.5	52.7 ± 1.71 (41.71)	4.8 ± 0.71 (54.20)	1.9 ± 1.48 (44.00)
Homosekikaic acid	Control	90.5 ± 1.29(0.00)	10.7 ± 0.08 (0.00)	3.4 ± 0.16 (0.00)
	50	45.7 ± 2.22 (49.45)	4.2 ± 0.88 (60.75)	2.1 ± 0.33 (38.20)
	25	59.7 ± 1.50 (34.14)	5.0 ± 0.86 (53.27)	1.5 ± 0.77 (55.89)
	12.5	64.0 ± 1.83 (29.28)	6.9 ± 0.74 (35.52)	2.8 ± 0.93 (17.68)
Divaricatinic acid	Control	90.5 ± 1.29(0.00)	10.7 ± 0.08 (0.00)	3.4 ± 0.16(0.00)
	50	52.2 ± 2.22 (42.27)	7.4 ± 0.92 (30.92)	2.7 ± 0.54 (20.18)
	25	72.7 ± 1.71 (19.67)	7.8 ± 0.56 (27.10)	3.0 ± 0.51 (11.77)
	12.5	79.2 ± 1.26 (12.45)	8.1 ± 1.33 (24.30)	3.1 ± 0.42 (8.82)

Each value is the mean ± S.D. of three repetitions. Values in parenthesis indicate inhibitory percentage in terms of % germination, shoot and root length as compared to control.

4.4.3 Algaecidal activity

Microalgae are the first organisms emerged over 2 billion years ago and first to carry out photosynthesis and produce food on their own. Algae are of particular interest for studies because they inhabit wide range of ecological habitats and have remarkable adaptability to varying environmental conditions. Although, algae don't influence the agriculture directly but may hinder the agricultural yield in many indirect ways. By covering the water surface, disturbs the irrigation requirements of any nearby field. Sometimes, they alter the life cycle of aquatic flora and fauna by depriving them from respiration by affecting the amount of dissolved oxygen in water. Thus many chemicals and inorganic salts are applied in the water surfaces to avoid the overgrowth of algae in fresh water. This in turn, disturbs the natural aquatic ecosystem because of the continuing widespread water pollution. Thus, a bioassay

was carried out against the growth of three microalgae namely *Chlorella vulgaris C. Sorokiniana* and *Scenedesmus subspicatus* at 40µg/disc.

Among isolated chemical compounds tested for algaeicidal activity, isousnic acid and protolichesterinic acid were found to be very active against *Chlorella vulgaris* and *C. sorokiniana,* whereas they were found inactive against *Scenedesmus subspicatus* (Table 4.26). Evernyl was found to be very active against *Chlorella vulgaris* and inactive against *C. sorokiniana* and *S. subspicatus.* Among other metabolites ethyl orsellinate was found to be very active against *C. sorokiniana* and weakly active against *S. subspicatus*. Usnic acid and homosekikaic acid were found to inhibit the growth of *C. vulgaris* and *C. sorokiniana*. Whereas, no growth inhibition were observed when these algae were treated with divaricatinic acid and ido hexitol. Sekikaic acid was able to inhibit the growth of *C. vulgaris* only. All the metabolites were found to be inactive against *S. subspicatus* except divaric acid, which was found to be moderately active against *S. subspicatus* (Table 4.26). Usnic acid and protolichesterinic acid showed potent inhibitory activities against *E. coli* and *S. aureus.* This is the first report algaeicidal activity of metabolites isolated from lichen species against these microalgae.

4.4.4 Antibacterial activity

Preliminary biological screening against human pathogenic bacteria

Encouraged by the findings of these lichen metabolites which shows strong activities against various plant pathogens, the hexane, ethyl acetate and methanol extracts of *P. reticulata* and *R. roesleri* were evaluated for antimicrobial activity. In order to find out the metabolite responsible for activities, the isolated metabolites were evaluated for antimicrobial activity by disc diffusion assay against pathogenic bacteria namely *E. coli, S. aureus*, *S. viridochromogenes* (Tü57) and *B. subtilis* at a concentration of 40 µg/disc.

Iso usnic acid (Figure 4.27, Pr Ia) was found to be very active against *S. aureus* and *E. coli* (Table 4.27), whereas, it was to found be feebly active against *S. viridochromogenes* and inactive against *B. subtilis*. Atranorin (Figure 4.27, Pr IIa) was found to be inactive against *E. coli* and *B. subtilis* while weakly active against *S. aureus.* Perry and coworkers (1999) have also reported atranorin (60 µg/disc concentration) to be inactive against these microorganisms. Protolichesterinic acid (Figure 4.27, Pr IIIa) was found to be most active against *E. coli* and *S. aureus* (Table

4.27). The protolichesterinic acid, isolated from Iceland moss has been found to possess antibacterial properties against *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, and *Staphylococcus aureus* (Stoll *et al*, 1950).

Among the other metabolites evaluated, evernyl (Pr IVb), ethyl hematommate (Pr Vb), ethyl orsellinate (Pr VIb), methyl orsellinate (Pr VIIb), salazinic acid (Pr XIb) and atranol (Pr XIIb) were found inactive against all the tested bacteria. Whereas 2-hydroxy-4-methoxy-3, 6-dimethy benzoate (Pr VIIIb) was found weakly active against *S. viridochromogenes* and lichexanthone (Pr IXb) against *S. aureus*. Baeomycesic acid (Pr Xb) was found weakly active against *E. coli* and *B. subtilis* and moderately active against *S. aureus.* Of the various metabolites isolated from *R. roesleri*, usnic acid was found to be very active against *S. aureus* at 40 µg/disc. However, it was found to be inactive against *B. subtilis* and active against *E. coli*. Divaricatinic acid was found to be weakly active against *S. aureus and S. viridochromogenes* and inactive against *E.coli* and *B. subtilis.* Homosekikaic acid was found to be most active against *E. coli* and *S. aureus* and inactive against *S. viridochromogenes* and *B. subtilis* (Table 4.27).

The high antimicrobial activity of usnic acid is known for a long time and our results show similar findings for the antimicrobial activity of usnic acid. Usnic acid showed a broad spectrum activity against all the test organisms. Lauterwein *et al* (1995) reported a MIC value ranging from 2 to 16 μg/ml for usnic acid against clinical isolates of *Staphylococcus aureus*. Perry and coworkers (1999) observed the antimicrobial activity of usnic acid against *Bacillus subtilis* and *Candida albicans*

Lichens are vast source of biologically active compounds, although, the mode of antibiotic action of lichen compounds not fully explored. One of the reasons according to Levin (1971), chemical resistance developed to attack by microorganisms is either constitutive or induced. Constitutive resistance is based on the accumulation and modification of normal metabolites in response to attack and is best suited to defense against bacteria, viruses, fungi and nematodes. Many lichen metabolites are produced at constant rates as the thallus enlarges regardless of the presence of grazers or microorganisms. That suggests a constitutive defense system is followed by lichens (Levin). However, significant research and development still remains an unexplored realm.

Table 4.26: Algaecidal activity of isolated chemical constituents

Compound No.	Compound Name	*Inhibition of microalgae at 40 μg/disc		
		C. vulgaris	***C. sorokiniana***	***S. subspicatus***
	Control	---	---	---
Pr Ia	Iso usnic acid	+++	+++	---
Pr IIa	Atranorin			
Pr IIIa	Protolichesterinic acid	+++	+++	---
Pr IVb	Evernyl	+++	---	---
Pr Vb	Ethyl hematommate	---	---	---
Pr VIb	Ethyl orsellinate	---	+++	---
Pr VIIb	Methyl hematommate	---	---	---
Pr VIIIb	2-Hydroxy-4-methoxy-3,6-dimethyl benzoic acid	---	---	---
Pr IXb	Lichexanthone	---	---	---
Pr Xb	Baeomycesic acid	---	++	---
Pr XIb	Salazinic acid	---	---	---
Pr XIIb	2-methoxy-4-hydroxy-3,6-dimethyl benzoic acid	+	---	+
Pr XIIIb	Atranol	---	---	---
Rr Ia	Usnic acid	+++	+++	---
Rr IIb	Divaricatinic acid	---	---	---
Rr IIIb	Homosekikaic acid	+++	+++	---
Rr IVb	Sekikaic acid	+++	---	---
Rr Vb	Divaric acid	---	+++	---
Rr VIb	Ido Hexitol	---	---	---
U*l*-IIb	4-O-Dimethyl Diffaractic acid	---	---	---
U*l*-IIIb	Lobaric acid	---	---	---

*(+) 0-10 mm: weakly active, (++) 11-20 mm: active, (+++) 20 mm and above: highly active

Table 4.27: Antibacterial activity of isolated chemical constituents

Compound No.	Compound Name	*Inhibition of pathogenic bacteria			
		E. coli	***S. aureus***	***S. viridochromogenes***	***B. subtilis***
	Control	---	---	---	---
Pr Ia	Iso usnic acid	+++	+++	+	---
Pr IIa	Atranorin	---	+	+	---
Pr IIIa	Protolichesterinic acid	+++	+++	---	---
Pr IVb	Evernyl	---	---	---	---
Pr Vb	Ethyl hematommate	---	---	---	---
Pr VIb	Ethyl orsellinate	---	---	---	---
Pr VIIb	Methyl hematommate	---	---	---	---
Pr IIIb	2-Hydroxy-4-methoxy-3,6-dimethyl benzoic acid	---	---	+	---
Pr IXb	Lichexanthone	---	++	---	---
Pr Xb	Baeomycesic acid	+	---	++	---
Pr XIb	Salazinic acid	---	---	---	---
Pr XIIb	2-methoxy-4-hydroxy-3, 6-dimethyl benzoic acid	---	++	---	---
PrXIIIb	Atranol	---	---	---	---
Rr Ia	Usnic acid	++	+++	---	---
Rr IIb	Divaricatinic acid	---	+	+	---
Rr IIIb	Homosekikaic acid	+++	+++	---	---
Rr IVb	Sekikaic acid	---	---	---	---
Rr Vb	Divaric acid	---	---	---	---
Rr VIb	Ido Hexitol	---	---	---	---
Uℓ IIb	4-O-Dimethyl Diffaractic acid	---	---	---	---
Uℓ IIIb	Lobaric acid	---	---	---	---

*(+) 0-10 mm: weakly active, (++) 11-20 mm: active, (+++) 20 mm and above: highly active.

PART II
Biochemical studies on lichens

A natural product refers to a chemical compound or substance produced by a living organism found in nature that usually has a biological activity for use in various industries like pharmaceutical drug discovery, agriculture and food industry. All organisms produce many substances by metabolism or by metabolic processes. These substances are termed as metabolites. Metabolites are the organic compounds that can be a starting material, an intermediate, or an end product of metabolism. Based on their origin and function, these metabolites are divided into two classes: The primary metabolites and the secondary metabolites

The former refers to the compounds produced through primary metabolism and are essential for the normal growth, development and reproduction of any organism. They may be as simple as a sugar molecule and may constitute the building blocks such as carbohydrates, fats, proteins and nucleic acids (RNA, DNA) which are required for the life processes. Lichens, in particular are known to produce a diversity of unique metabolites that are characteristic to them only. These metabolites are produced through secondary metabolism which usually has many important ecological functions. They are proved to be essential for the survival of organism in many indirect ways. These metabolites are formed in the living organisms following three biochemical pathways as shown in the figure 4.47.

- ❖ Shikimic acid pathway
- ❖ Mevalonicacid pathway
- ❖ Polyketide pathway

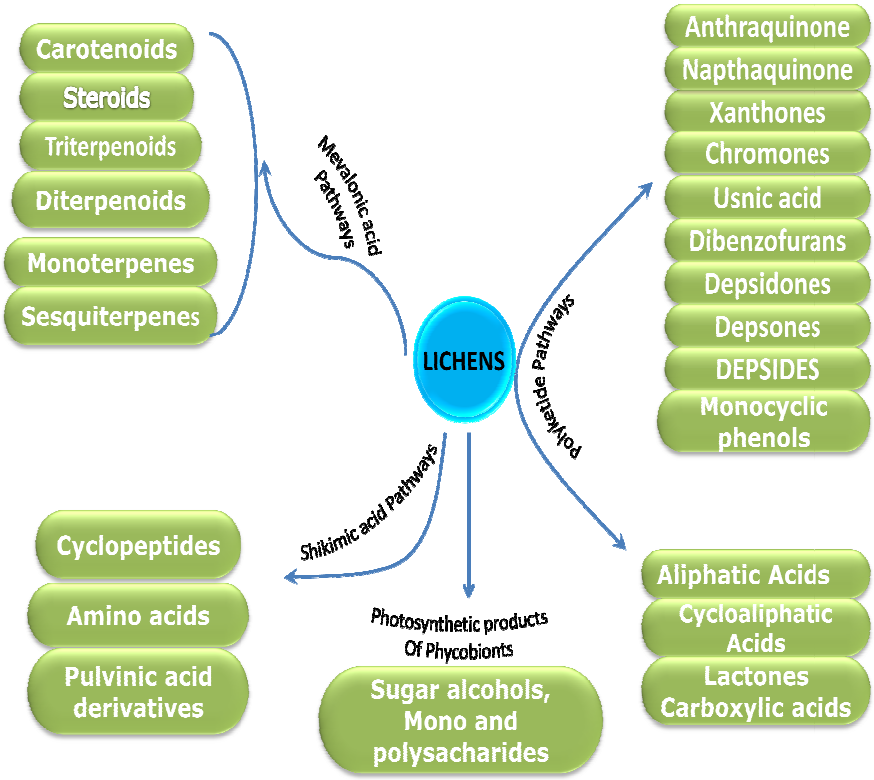


Figure 4.47: Biosynthetic pathways in lichens

Shikimic acid pathway: Several lichen metabolites are derived from shikimate. These constitute the pulvinic acids, terphenylquinones and are widely distributed. These metabolites are formed from the joining of two phenyl pyruvates.

Mevalonic acid pathway: Lichens produce mainly terpenoids from the mevalonate pathway. However, many of these are common to other organisms. Lichens screened (taken) in the present study didn't give any of the metabolites derived from the shikimic and mevalonic pathway.

Polyketide pathway: Many of the poly aromatic compounds unique to lichens are produced by polyketide pathway. Secondary metabolites produced *via* the polyketide pathway are unusual compounds. They are most likely formed from the linking of two or three aromatic units through ether, ester and carbon–carbon bonds.

Among the aliphatic acids isolated, protolichesterinic acid (Pr IIIb), an aliphatic α-methylene-α-lactone isolated from the hexane extract of *P. reticulata* is known to be formed by the polyketide pathway. The large concentrations of mainly phenolic compounds that are accumulated in the thallus are typical of lichens.

Phenolic compounds namely evernyl (Pr IVb), methyl hematommate (Pr VIIb), ethyl hematommate (Pr Vb), ethyl orsellinate (Pr VIb), atranol (Pr XIIIb) are isolated from the hexane and ethyl acetate extract of *P. reticulata*. These phenolic compounds are assumed to be originated from the fungal partner as studies with the lichen mycobionts without the algal partner have shown their ability to biosynthesize extraordinary constituents under stress conditions. Other Benzoic acid derivatives namely, 2-Hydroxy-4-methoxy-3, 6-dimethyl benzoic acid (Pr VIIIb), 2-methoxy-4-hydroxy-3, 6-dimethyl benzoic acid (Pr XIIb), divaricatinic acid (Rr IIb) and divaric acid (Rr Vb) were obtained from ethyl acetate extracts of *P. reticulata* and *R. roesleri*.

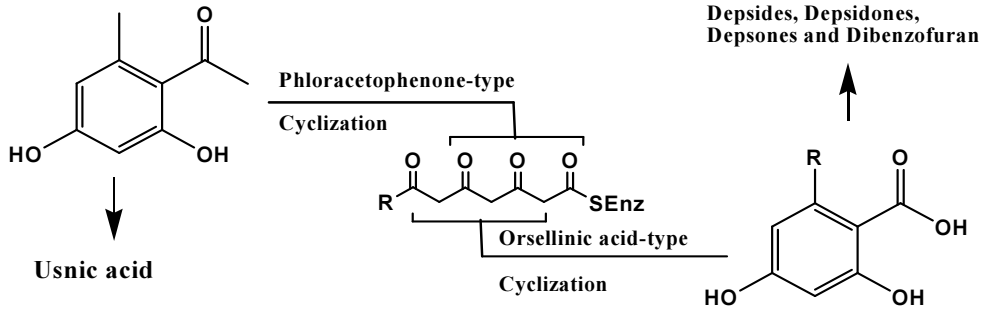


Figure 4.48: Two cyclization patterns commonly invsolved in the formation of simple tetraketides in lichens.

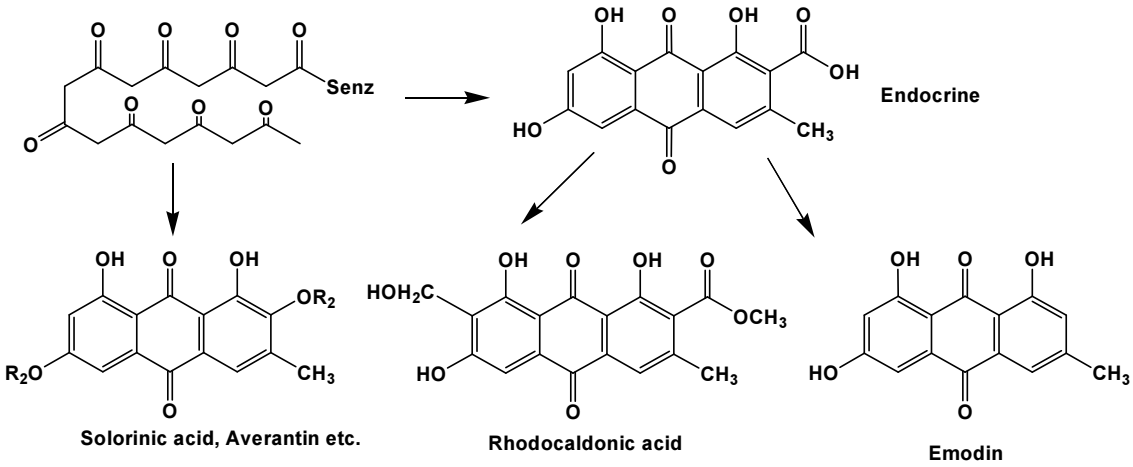


Figure 4.49: Biogenesis of anthraquinones derived from the cyclization of an octaketide chain.

One of the important classes of compounds found in lichens is depsides. These are formed by the condensation of two or more hydroxybenzoic acid molecules. It occurs via esterification of one of the carboxyl group of one molecule with a phenolic hydroxyl group of a second molecule. Among depsides, atranorin (Pr IIa), baeomycesic acid (Pr

Xb), diffaractic acid, sekikaic acid (Rr IVb) and homosekikaic acid (Rr IIIb) were isolated and originate from the linking of two orsellinic acid units with an ester bond and differ from each other in the nature and the number of substitutions on the phenyl rings. Among dibenzofurans, iso usnic acid (Pr Ia) and usnic acid (Rr Ia), are one of the most abundant lichen metabolites found in the yellow-green species of the genera *Usnea* and *Ramalina*, are derived from the phloracetophenone type cyclization (Figure 4.48) rather than the orsellinic acid-type units.

Depsidones have an ether linkage in addition to the ester linkage of the depsides, resulting in a rigid polycyclic system. Therefore, they are based on an 11*H*-dibenzo [*b*,*e*][1,4]dioxepin-11-one ring system. In the present study, salazinic acid, β-orcinol-type depsidones is isolated from *P. reticulata* and lobaric acid, an orcinol depsidone is isolated from *U. longissima*. This chemical feature has been shown to be important for the various biological activities. Lichens make several modifications to the basic structures to yield a wide range of analogues. Polyketides are formed by the sequential addition of 2 carbon units (derived from malonyl-CoA) to the suitable building blocks (usually acetyl- or acyl-CoA groups). The first three additions yield a 4-unit backbone (tetraketide) that can cyclize *via* either of two mechanisms shown in the figure 48. The metabolites obtained vary with the number of 2-carbon units that have been linked and the folding pattern of the linear polyketide. The lichexanthone obtained in the present study, is obtained from hexaketide precursor. Anthraquinones represent a large group of biologically active lichen metabolites, but these compounds are not unique to lichens only and are found in other higher plants.1,8-dihydroxy-3-methoxy-6-methyl-anthraquinone obtained from the *S. himalayense* is derived from the cyclization of octaketide (8 basic units) (Figure 4.49) (Dayan, 2001).

Thus, in the current study, most of the lichen metabolites are derived from the polyketide biosynthetic pathway (also called acetylpolymalonyl pathway), with a few originating from the shikimate and mevalonate biosynthetic pathways. The majority of lichen secondary metabolites originates from the fungal partners, but produced primarily when the organisms are in their symbiotic association. Other compounds are produced when the mycobiont is isolated from its symbiont, however, these are usually different from those produced in the symbiosis. So, it can be inferred that, some metabolites may be produced by the fungus or the alga alone, while others are exclusively produced by synergistic action of both partners in symbiosis.

Conclusions & Future Scope

Conclusions and future scope

Chemical pesticides and plant growth regulators are the essential ingredients to maximize yield in modern agriculture. Alarm resulting from the growing incidence of pesticide hazards has prompted global efforts directed toward the search for alternative pest control strategies. One of the strategies is isolation and identification of bioactive compounds of natural origin that are the chemicals from living organisms, such as plants, animals, insects, and microorganisms and are often termed as secondary metabolites. These metabolites have a proven track record in providing novel source of new agents for both the pharmaceutical and agrochemical industries (Surajit *et al.*, 2006). Their importance to the mankind can only be judged from the fact that several isolated compounds of natural origin and their synthetic analogs are currently in various stages of clinical trial for their efficacy towards many known diseases that today's world encounters (Cragg *et al*., 1997). It has been well established that the metabolites from microbes are unique in comparison to plant metabolites and provide a greater structural diversity. Also these metabolites often possess highly specific biological activity. Thus, steadily over the years, the focus of interest has been shifted from plants to the microbial metabolites and they have been the major source of new drugs.

Lichens, in this context offer a virtually untapped goldmine of compounds with diverse biological activities. A detailed survey of literature revealed that the lichens are found sensitive to any minute change in microclimatic conditions and their chemical diversity (metabolite production) depends on certain conditions like the extreme environmental conditions, high altitudes, extreme temperature variations, and varied climate. In the present scenario, when the scientific community is compelled to develop environmentally benign pest control strategies, current study was undertaken to explore these exclusive class of symbiotic microbes for their agrochemical potential. In the present study, lichen species from high altitudes of Kilberi forest ranges (Northern Himalayas) were collected and screened for various allelopathic activites. Lichens namely *Parmelia reticulata* Tayl., *Ramalina roesleri* Nyl., *Usnea longissima* Ach. Articus and *Stereocaulon himalyense* Lamb. were collected from the

bark of trees, shrubs and rocks of the forest. These samples were deposited in the Herbarium of the Department of Botany, University of Delhi, Delhi, India.

A comparative and comprehensive study was made to investigate the influence of hexane, ethyl acetate and methanol extracts of the lichen species against a wide range of soil borne phyto pathogenic fungi, a devastating weed of Indian wheat rice cropping system along with the allelopathic potential on major food crops wheat, lentil and chickpea seeds. A preliminary biological screening was carried out against microalgae namely *Chlorella vulgaris*, *C. sorokiniana* and *Scenedesmus subspicatus*.

A detailed comparative analysis of the antifungal activity exhibited by all the extracts of *P. reticulata, R. roesleri, U. longissima* and *S. himalayense* revealed that the hexane extract of *U. longissima* showed maximum inhibition against *R. bataticola*, (ED_{50}=18.9 μg ml^{-1}), *S. rolfsii* (ED_{50}=24.8 μg ml^{-1}), *P. debaryanum* (ED_{50}=28.3 μg ml^{-1}) and *P. aphanidermatum* (ED_{50}=37.0 μg ml^{-1}). In a similar manner, hexane extract of *R. roesleri* had maximum inhibition against *R. solani* (ED_{50}=27.7 μg ml^{-1}) and *F. udum* (28.4 μg ml^{-1}). *R. bataticola* was found to be sensitive towards all the three extracts of lichens tested, offering a vast range of compounds that can be used as growth inhibitors against its infestation. The good activity may be attributed to the additive effect of the metabolites present in the extract. The hexane extracts of all the lichens showed approximately equal antifungal activity against *S. rolfsii, R. bataticola* and *R. solani.* In general, the hexane extract of all the lichen species were most active, followed by the ethyl acetate and methanol extracts.

The herbicidal potential of the solvent extracts and isolated metabolites were also estimated against the weed *P. minor*. Maximum inhibition was observed at higher concentrations as compared to the lower concentrations. Maximum shoot and root length inhibition was recorded in the seedlings treated with hexane extract followed by the ethyl acetate and methanol extracts. In general, extracts of *R. roesleri* exhibited a remarkable reduction in the overall growth of *P. minor* seeds. On the basis of these observations, it can be proposed that the lichens species may contain allelochemicals that impair the growth, which may also in turn act as a potential inhibitory agent on weed.

The influence of solvent extracts of *P. reticulata* and *R. roesleri* on the growth of major food crops; wheat (*Triticum aestivum* L.), chick pea (*Cicer arietinum* L.) and lentil (*Lens culinaris* Medikus) was also evaluated. The study revealed that the hexane and ethyl acetate extracts of *P. reticulata* and *R. roesleri* have a growth stimulatory effect on tested seeds at lower dosages. Analysis of the growth of the plants under study indicated that the solvent extracts of *P. reticulata* and *R. roesleri* are not phytotoxic to the tested seedlings.

This is the first study of this kind reporting remarkable agrochemical activities of *P. reticulata, R. roesleri, U. longissima* and *S. himalayense* against various pests with no harmful effect on the sensitive dicot legume crops, lentil and chick pea and an economically important monocot crop wheat which has expressed reasonable merit for further research to be conducted aiming at the isolation and identification of chemical constituents responsible for inhibitory activities of crude extracts.

Thus, a bioassay guided approach was followed in order to understand the type of compound responsible for the biological activity. Biologically active extracts of all the lichen species yielded twenty four compounds. In total, fourteen compounds (Pr Ia- Pr IIIa, Pr IVb- Pr XIVb) were isolated and identified from *P. reticulata*, six compounds (Rr Ia, Rr IIb- Rr VIb) were isolated and identified from *R. roesleri*, three compounds (Uℓ Ia, Uℓ IIb- Uℓ IIIb) were isolated and identified from *U. longissima* and four compounds (Sh Ia- Sh IIa, Sh IIIb- Sh IVb) were isolated and identified from *S. himalayense* (Table 4.23a). Further, these compounds were evaluated for various biological activities in order to determine their agrochemical potential.

Among the isolated compounds tested for their antifungal potentiality, protolichesterinic acid (Figure 4.27, Pr IIIa) isolated from hexane extract of *P. reticulata* exhibited maximum antifungal activity against *P. debaryanum* and *R. solani*. It exhibited 100, 90 and 78% inhibition at 250, 125, and 62.5 μg ml^{-1} concentration against *P. debaryanum* with ED_{50} value of 16.07 μg ml^{-1}, whereas hexaconazole showed 59.23, 26.30 and 11.30% inhibition at 31.0, 15.5, and 7.25 μg ml^{-1} concentrations with ED_{50} value of 25.92 μg ml^{-1}. It exhibited 83, 77, 67 % inhibition at 250, 125, and 62.5 μg ml^{-1} concentration against *R. solani* with an ED_{50} value of 23.09 μg ml^{-1} . whereas hexaconazole showed 73, 38 and 21% inhibition at 31.0, 15.5, and 7.25 μg ml^{-1} concentrations with ED_{50} value of 18.30 μg ml^{-1}. Usnic

acid (Figure 4.32, Rr Ia) isolated from hexane extract of *R. roesleri* and *U. longissima* showed a broad spectrum antifungal activity against all the test fungi with ED_{50} values ranging from 12.34 to 25.69 μg ml^{-1}. Sekikaic acid is the next active compound isolated from ethyl acetate extract of *R. roesleri.* It exhibited remarkable activity against *S. rolfsii* with ED_{50} value of 13.00 μg ml^{-1}. It is worth mentioning that the inhibitory response of usnic acid is comparable to hexaconazole, a commercial fungicide against *P. debaryanum* and *F. udum* and the inhibitory response of protolichesterinic acid is comparable to hexaconazole, against *R. solani* and *P. debaryanum.* The improved antifungal activity of hexane extract as compared to ethyl acetate extracts may be due to the presence of the protolichesterinic acid and usnic acid in the extracts of the respective species. Differences in the activity of these compounds against various fungi indicate that the compounds act in a species-specific fashion.

The herbicidal activity of isolated compounds was evaluated against *P. minor* in terms of seed germination, shoot length and root length. In the current study, usnic acid was found most effective in suppressing the germination and early seedling growth of *P. minor* followed by homosekikaic acid and divaricatinic acid. The effect of the metabolites on growth of *P. minor* showed a significant concentration dependant effect. The treatments with 50 and 25 μg ml^{-1} concentrations significantly reduced the germination rate, while a low concentration treatment had low inhibitory effect.

Among isolated compounds tested against microalgae, isousnic acid and protolichesterinic acid were found to be very active against *Chlorella vulgaris* and *C. sorokiniana*. Usnic acid and evernyl were found active against *C. vulgaris* and inactive against *C. sorokiniana* and *Scenedesmus subspicatus.* Among other metabolites, ethyl orsellinate showed potent inhibition against *C. sorokiniana* and a mild inhibition against *S. subspicatus*. Homosekikaic acid was found to inhibit the growth of *C. vulgaris* and *C. sorokiniana*. This is the first report of algaeicidal activity of metabolites isolated from lichen species. Usnic acid and protolichesterinic acid showed very strong inhibitory potential against *E. coli* and *S. aureus.* The results showed that lichen extracts and isolated metabolites exhibit strong effects against the test organisms, even in relatively low concentrations. Among all the extracts, hexane extract exerts a stronger inhibitory action than the other.

In view of the aim of our investigation and results obtained, it can be concluded that investigated lichen extracts manifested strong, but varying degree of agrochemical activities. These similarities and differences in the agrochemical potentiality of extracts of different species of lichens are probably a consequence of the synergistic effect of different components present in the crude extracts. The synergistic effect is also observed for differences in the agrochemical potential of crude extacts and their respective isolated metabolites for example, hexane extract of *P. reticulata* exhibited an ED_{50} value of 25.1 μg ml^{-1} against *R. bataticola*, while one of the most active compound, atranorin, isolated from the same extract exhibited an ED_{50} value of 58.8 μg ml^{-1} when tested exclusively. Similarly, ethyl acetate extract of *P. reticulata* exhibited an ED_{50} value of 43.0 μg ml^{-1} against *R. bataticola* whereas none of the isolated compounds showed an ED_{50} value below 50 μg ml^{-1}.

Current study establishes lichens as potential source of agrochemicals. Their products can be used safely as crop protectants with no detrimental effects on major food crops. The study also highlights the plant growth promoting activity of the test lichens for the first time. The compounds isolated, in present study can also serve as leads for chemical synthesis to yield new products. Appropriate chemical modification may increase their potency. It may also be proposed that lichens can serve as potential agent to become a complement or alternative to more traditional chemical treatment for sustaining environmental health and reduce the load of hazardous synthetic chemicals. This in turn provides incentives for conservation of biodiversity and stimulates economic development. The present study can further be extended to understand the mechanism of interaction with the test organism, in order to obtain products with improved efficacy and to develop a novel class of bioactive agents. At the same time, field trials investigating the agrochemical efficacy of the samples under natural environmental stress conditions are also needed.

Scope for future research

Lichen studies are primarily limited due to the difficulties observed in the production of secondary metabolites because of their growth. Progress in repeatedly expressing such metabolites under optimized culture conditions in mycobionts could contribute to future applications of selected lichen metabolites. In this course, production of desired metabolites can be enhanced by growing lichens in stimulating

environment. As an alternative method, the transfer of genes responsible for the production of desired lichen metabolites suggested by Huneck, (1999) may considerably enlarge the access to lichen derived substances in high-throughput screening programs. Techniques such as computer-aided drug design and combinatorial chemistry may help to design and develop novel synthetic analogues of lichen metabolites. Current study also highlights the application of lichens and their metabolites still deserves consideration in order to increase the natural product pool and uses thereof as potential agrochemicals.

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Publications

Publications

Journals:

1. Mayurika Goel, Prem Dureja, Archna Rani, Prem Lal Uniyal, Hartmut Laatsch. Efficacy of major chemical constituents of Himalayan Lichen Ramalina roesleri Nyl. towards plant pathogenic fungi. *2011* (**Communicated**).

2. **Mayurika Goel**, Prem Dureja, Archna Rani, Prem Lal Uniyal, Hartmut Laatsch. Isolation, Characterization and Antifungal Activity of Major Constituents of the Himalayan Lichen Parmelia reticulata Tayl., *J. Agric. Food Chem., 2011, 59 (6), 2299-2307.*

3. **Mayurika Goel**, P. K. Sharma, P. Dureja, A. Rani, and P. L. Uniyal. Antifungal activity of extracts of the lichens *Parmelia reticulata, Ramalina roesleri, Usnea longissima* and *Stereocaulon himalayense*. *Archives of Phytopathology and Plant Protection*., *2011, 44 (13), 1-12*

Conferences:

1. Oral Presentation: Lichens: A novel source of antimicrobial agents, Ist International Conference on Antimicrobial Research (ICAR2010), Valladolid, **Spain**, 3-5 November **2010**.

2. Poster presentation: Synergism between synthetic and natural products: opportunities for environment friendly fungicides, *14th Annual Green Chemistry and Engineering Conference (ACS)*, Washington, **U.S.A**, 21-23 June **2010**.

3. Oral Presentation: Synergism between synthetic and natural products: Opportunities for environment friendly fungicides: *14th Annual Green chemistry and Engineering Conference: Inovation and application*. Washington**, U.S.A**, 21-23 June **2010**.

4. Poster presentation: Isolation and Characterization of Atranorin from hexane extract of lichen *Parmelia reticulata*, *3rd National Symposium on Analytical Sciences (NSAS)*, Department of Chemistry, Himachal Pradesh University, **Shimla**, India, 12-14 April **2010**.

5. Poster presentation: Isolation and Characterization of an antifungal metabolite Protolichesterinic acid from Parmelia reticulata, *National conference on chemistry- structure, Reaction dynamics & Spectroscopy (CSIR)*, St. Stephens College, **New Delhi**, India, 21-23 August **2008**.

6. Poster presentation: Antifungal and Plant growth regulatory activity of lichens Metabolites: *(IUPAC) 1st International conference on Agrochemicals protecting crop, Health and Natural Environment*, **New Delhi**, India, 8-11 January **2008**.