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Studies on the Biologically Active Constituents of Lichens

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DEDICATED TO
MY
BELOVED PARENTS

DECLARATION

I hereby declare that the entire work submitted as a thesis towards the fulfillment of the requirements for the degree of Doctor of Philosophy in Institute of Biological Sciences at the University of Rajshahi is the result of my own investigation. The thesis contains no material which has been accepted for the award of any other degree or diploma elsewhere, and, to the best of my knowledge, the thesis contains no material previously published or written by another person, except where due reference is made in the text.

Shahina Begum Rekha

June 2008
Rajshahi University

(Shahina Begum Rekha)

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রাজশাহী-৬২০৫, বাংলাদেশ

University of Rajshahi
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সংখ্যা .....

Dated 23 June 2008

CERTIFICATE

This is to certify that the thesis entitled "Studies on the biologically active constituents of lichens" is a bonafide original research work of Shahina Begum Rekha.

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Shahina Begum Rekha

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LIST OF ABBREVIATIONS

#U	=	Number of insects used
% kill	=	Insects killed per cent
+ve	=	Positive
µg	=	microgram
µl	=	micro liter
$CHCl_3$	=	Chloroform
cm^2	=	centimeter square
Cr %	=	corrected mortality percent.
df	=	degree of freedom.
E. Pr	=	Empirical Probit.
et al.,	=	and others (author)
EtOAc	=	Ethyl Acetate
Ex Pr	=	Expected Probit
F Pro	=	Final Probit
Fig.	=	Figure
fr	=	factor (s)
h	=	hour (s)
HPLC	=	High Perform Liquid Chromatography
i.e.	=	that is
KI	=	Number of insects killed
LC_{50}	=	concentration required to kill 50% of test organisms
LD_{50}	=	dose required to kill 50% of test organisms
LDose	=	Log dose
MeOH	=	Methanol
mg	=	milligram (s)
ml	=	milliliter
mm	=	millimeter
mp	=	melting point
nm	=	nanometer
NMR	=	Nuclear Magnetic Resonance
PDA	=	Potato Dextrose Agar
Rf	=	Retention factor
TLC	=	Thin Layer Chromatography
-ve	=	Negative
Weight	=	Weighting coefficient
Wk Pro	=	Working probit
χ^2	=	Chi-squared

Abstract

In the antimicrobial activity test majority of the thirteen lichen extracts (named h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11, h12 and h13) offered antibacterial (against *S. aureus, B. megaterium, B. subtilis, B. cereus, S.-β- haemolyticus, S. lutea, S. typhi, S. dysenteriae, S. shiga, S. sonnei, S. boydii* and *E. coli*), as well as antifungal (against *A. flavus, A. fumigatus, C. albicans, Mucor* sp. and *F. vasinfectum*) activity; while against *E. coli* no efficacy was traced with the same dose maintained for the bacterial isolates. The dose was set for the antibacterial activity test as 200 µg/disc for all the extracts and the standard Ciprofloxacin was used 10µg/disc for comparison. The highest activity was found in case of sample h5, where the inhibition zones were 24-, 20-, 18-, 20-, 20-, 22-, 23-, 18-, 20-, 28-, 26- and 0 mm respectively, followed by the sample h10 where the inhibition zones were 24, 20, 15, 19, 15, 21, 23, 0, 18, 13, 25 and 0 mm respectively for above mentioned bacteria and the lowest activity was recorded for the sample h9 where the inhibition zones were 0-, 0-, 0-, 7-, 0-, 7-, 0-, 0-, 0-, 0-, 12- and 0 mm respectively; while the inhibition zones for the comparison standard were 35-, 35-, 40-, 42-, 38-, 38-, 40-, 42-, 42-, 38-, 38- and 42 mm respectively.

The dose was set for antifungal activity test as 500 µg/disc for all the extracts and the standard Nystatin was used 50µg/disc for comparison. The highest antifungal activity was found in case of sample h5, where the inhibition zones were 6-, 21-, 10-, 7- and 16 mm respectively, followed by the sample h10 where the inhibition zones were 11-, 20-, 12-, 7- and 15 mm respectively. The lowest activity was recorded for the sample h9 where the inhibition zones were 10-, 9-, 10-, 12- and 9 mm respectively; while the inhibition zones for the comparison standard were 33-, 32-, 33-, 32- and 7 mm respectively. The minimum inhibitory concentration (MIC) values for both the bacteria and fungi agents were measured. The MIC values of the crude extracts against the bacteria for the lichen sample h5 and h10 ranges between 64- to 16 µg/ml and 128- to 8 µg/ml respectively in the broth medium. The MIC values of the crude extracts against the fungi for the lichen sample h5 and h10 ranges between 1280- to 10 µg/ml and 1280- to 80 µg/ml respectively in the broth medium.

The lichen samples were subjected to cytotoxicity test and the LC_{50} values were 14.245-, 8.568-, 16.260-, 9.419-, 8.994-, 2.333-, 9.386-, 6.871-, 17.124-, 11.611-, 38.604-, 18.776- and 20.529 ppm respectively for 24h of exposure and according to the intensity of efficacy the lichen extracts could be arranged in a descending order of the samples h6> h8> h2> h5> h7> h4> h10> h1> h3> h9> h12> h13> h11. All the lichen samples were tested for repellent activity against *T. castaneum* adults. All the lichen extracts showed repellent activity, of which extracts of the sample h4 and h5 highly effective ($P<0.001$), sample h2, h6 and h8 positively effective $P<0.01$) and the rest showed repellency at $P<0.05$ level of significance.

In the toxicological profile a selected extract was considered to apply against the larvae of the red flour beetle, *T. castaneum.* The chloroform extract of the lichen sample h10 was applied through dose-mortality assay to give the LD_{50} values 372.375- 362.327- and 664.662 mg/g for the 2nd, 3rd and the 4th instar larvae respectively. While the same extract against the larvae of *M. domestica* gave the LD_{50} value 71.766 mg/g for 24 h for the 3rd instar larvae.

Four bioactive compounds have been isolated and purified from h4 (one/ named LMI-1), h10 (one/ Named LBF-2) and h13 (two/ named LMI-3 and LM-4) of which the LMI-1 and LBF-2 were subjected to NMR and relevant analyses for the determination of structures. Compounds LMI-1and LBF-2 is the same compound, even though they are isolated from different sources. Due to insufficiency in amount other two LMI-3 and LMI-4 were not possible to go through NMR analysis. Identification of lichens has also been declined, but biological activity tests of the crude extracts as well as the purified compounds have been successfully done. Anyway, the inhibiting activity of the lichen extractives (crude and pure) against the human and plant pathogenic bacteria and fungi have an immense value through pharmacological/ pharmaceutical, medicinal, toxicological and agricultural/ pesticidal point of view. The reason for decreasing activity of the pure compounds (in comparison to their respective crude extracts) has to be investigated.

The findings of this work along with the findings of the previous researchers triggered a hope for further progress in research with these promising sources (lichens) towards a molecular level investigation of its pharmacological, agricultural/ pesticidal or other effective but environment friendly potentials for a happy leading of human life.

Chapter 1

INTRODUCTION

1.1. Background information on the source material
1.2. Background information on the test organisms
1.3. Techniques for the isolation and purification of bioactive compounds
1.4. Bioassay with the purified compound(s)
1.5. Aim and objectives of this work

Chapter 1 Introduction

1. Introduction

In of search of new and more environment friendly pesticides and medicinal ingredients, scientists are leaving no proverbial stone unturned. Historically, a large portion of the world's medicine has been derived from plants and fungi. Salicylic acid is the active ingredient in aspirin and this acid is found in the genus *Salix*. Important antibiotics, such as penicillin are of course derived from fungi. Lichens are another type of organism that may hold the potential for medical exploration. In the past, Native Americans used lichens for ancient medicine and ceremonial practices. Aborigins of Australia and common people of Africa still use lichens in their indigenous system of medicine. Modern science has given a foundation for exploration of lichen species and their chemical constituents. Lichens produce protective secondary metabolites that serve to deter herbivores and colonization by pathogens. Usnic acid, stictic acid, and vulpinic acid are a few of the 700 plus secondary compounds that are produced by lichens. Interest in the antibiotic potential of lichen compounds was extremely high during the post-World War II era through the end of the 1950's. A secondary compound that generated a high amount of interest and considerable research was usnic acid. In the 1970's, usnic acid was reported to have potential as an anti tumor drug. Once again there is an interest in the potential uses of antibiotics derived from lichens as may be it is a valuable source of antibiotics for the pharmaceutical industry in future.

1.1. Background information on the source material

Lichens are symbiotic organisms consisting of a fungal, an algal and/or a cyanobacterial partner. They are well known for their ability to produce large amounts and a great variety of secondary metabolites (Elix, 1996; Huneck and Yoshimura, 1996). For more than 150 years these so-called lichen products have been used for identification and taxonomy (Culberson and Culberson, 1970; Hawksworth, 1976; Lumbsch, 1998; Nylander, 1866), and therefore lichens constitute one of the chemically best studied groups of organisms today (Schmitt *et al.*, 2005).

Within the lichen thallus carbohydrates move from the photobiont to the mycobiont (Ahmadjian, 1993). The photosynthetic partners excrete sugars (cyanobacteria) or different types of polyols (green algae), which are used by the mycobiont to form, for example, aromatic or aliphatic polyketides. These crystalline substances are typically deposited extracellularly on the fungal hyphae (Honegger, 1986). There are only few reports of secondary metabolites from lichen cyanobionts (Oksanen *et al.*, 2004; Yang *et al.*, 1993; Schmitt *et al.*, 2005).

Lichens are colorful organisms owing to numerous combinations of algal and fungal pigments (Rikkinen, 1995) and the color is often used in taxonomic studies to aid species identification but it is sometimes over-used as a characteristic to discriminate related genera such as, *Xanthoparmelia* and *Neofuscelia* (Poelt and Leuckert, 1993). The distinct colors of many lichens are due to the massive accumulation of diverse secondary compounds, the 'lichen substances'. These represent comparatively small, but also chemically complex molecules. Beside the externally visible crystallized and non-crystallized pigments that are deposited in the upper surface layers of the lichen's vegetative body, also colorless substances are common, which are predominantly found in internal parts of the thalli (Boustie and Grube, 2005).

However, color variations within species can be significant (Solhaug *et al.*, 2003) because of spatial (Gauslaa and Solhaug, 2001; Gauslaa and Ustvedt, 2003) and temporal variations (Gauslaa and McEvoy, 2005) in environmental factors (McEvoy *et al.*, 2006). These are found in the hottest desert, the coldest tundra, and the wettest rain forest. Some lichens are able to tolerate salt spray on coastal cliffs and periodic inundation by fast-moving streams; however, no lichen is truly aquatic. Lichens grow on soil, woody debris, rocks, tree bark, tree leaves, and on other lichens. In combination, the lichen symbionts produce a growth form that is unlike either fungi or algae growing alone. The association produces an undifferentiated plant called a thallus. Three growth forms are easy to recognize: crustose (crust-like), adhering tightly to the substrate by their entire lower surface, but some endolithic lichens are embedded in their rock substrate; foliose (leaf-like) with a distinct upper and lower surface, attached to their substrate only by small root-like structures, rhizines and fruticose (shrub-like), pendulous strands or hollow stalks called podetia, usually attached to the substrate at the base or holdfast.

Lichens are also named according to the fungal partner, and with over 20,000 different species of lichens sharing a much smaller number of photobiont species (McCune and Goward, 1995; Weber and Whitman, 1990) and as a successful alliance between these two organisms, lichens live as a single organism. Both inhabit the same body, or thallus, with each doing what it does best and thriving as a result of the mutual cooperation. Lichens have a long generation time, which is one reason why are considered sensitive to disturbances such as logging (Gilbert 1977; Seaward 1982). Many lichens are sessile organisms (Jahns and Ott, 1997) forming canopies that are often inhabited by numerous small herbivores (Gerson and Seaward 1977; Seaward, 1988; Gauslla *et al.*, 2006). Lichens are traditionally classified as a life-form of fungi. As an apparently successful form of fungal symbioses, the lichen-forming habit is maintained by one-fifth of all fungi, which includes more than ca 40% of ascomycetes, but only a few basidiomycetes. About 18,500 different lichen species have been described all over the world. They may grow under rather diverse and sometimes extreme ecological conditions.

Most lichen-forming fungi are members of the ascomycetes, the fungal group that includes the destructive bread molds, the edible morels, and the commercially important baking and brewing yeast. A few are members of the basidiomycetes, which also include the typical mushrooms. The algae may be either members of the chlorophyta, which includes the green algae such as most plankton, or the cyanobacteria, the blue-green algae that often form pond scum. The algae may be either single cells or filaments, chains of cells. Some lichens have more than one type of alga. Some lichens have more than one type of alga.

1.1.1. Social utilities of lichen

The lichen was said to heal impetigo and other skin diseases 'cal lichen' and this is the origin of the plant name, later defined 'Icela lichen', as the Icelanders and the Lapps (the inhabitants of the Lappland) used to eat it. The plant was very common among them; they used to eat it by night or during rainy days and after macerating it in water so that it would lose its typical bitter taste, the during famine periods.

It is well known that air pollution can compromise the productivity and biodiversity of natural ecosystems (Hutchinson and Meema, 1987; Olson *et al.,* 1992). Analysis of biological indicators can be an efficient, inexpensive alternative to air-quality monitoring with permanent instrumentation (Nimis and Purvis, 2002).

Lichens are also important nesting material for birds and mammals (Richardson and Young, 1977; Helle and Helle, 1989; Hayward and Rosentreter, 1994). For the northern flying squirrel *(Glacomys sabrinus),* in particular, differences in lichen diversity and abundance may explain differences in squirrel abundance among vegetation types and be a chief correlate of source habitats (Zabel and Waters, 1997; Campbell and Coxson, 2001; Lehmkuhl, 2004).

1.1.2. General components of lichens

Lichens are unusual and intriguing organism. Ramagni *et al.*, (2004) say that of the more than 20,000 known lichen species, only a few have been analyzed and identified as containing biologically active compounds. These natural compounds typically arise from the secondary metabolism of the fungal part of the lichen. They are deposited on the surface of threadlike hyphae-the tiny filaments that connect fungi to a food source or host-rather than compart-mentalized in the cells. As a simple model, lichens are like gardens. The alga is similar to a plant that under optimal conditions produces simple sugars from atmospheric carbon dioxide and water by photosynthesis. The fungus, as the gardener, tends the alga, providing shelter and protection from the extremes of the environment. As a consequence the alga produces enough additional sugars to supply the fungus with nourishment. With some of these sugars, the fungus makes organic compounds that possibly protect both partners from herbivores, a type of natural pesticide.

Lichens produce a wide range of organic compounds that can be divided into two groups called primary metabolites and secondary metabolites (Elix, 1996). Primary metabolites are proteins, lipids, carbohydrates, and other organic compounds that are essential to the lichen's metabolism and structure. Some of these metabolites are produced by the lichen's fungal partner and others by the lichen's algal or cyanobacterial partners. Secondary metabolites are produced by the fungus alone and secreted onto the surface of lichen's hyphae either in amorphous forms or as crystals. If these substances are only found in lichens, then they are called lichen substances (Öztürk *et al.*, 1999).

Lectins constitute a heterogeneous group of glycoproteins of non-immune origin with non-catalytic binding sites, which are capable of recognizing and reversibly binding to specific saccharide moieties (Goldstein *et al.*, 1980). Since the nineteenth century, these proteins have been described mainly in dicot (Kamemura *et al.*, 1993; Mandal *et al.*, 1994), but also in monocot (Kilpatrick and Yeoman, 1978; Crowley *et al.*, 1984), bacteria (Heerze *et al.*,

1992; Heerze and Armstrong, 1993), animals (Thiel, 1992), yeasts (Viard *et al.*, 1993), mushrooms (Yoshida *et al.*, 1994) and several symbiotic associations such as *Rhizobium*-legume, mycorrhizae (Díaz *et al.*, 1989) and lichens (Petit *et al.*, 1983, Bubrick *et al.*, 1985). The first report using lichen material showed that these glycoproteins were involved in recognition mechanisms (Lockhart *et al.*, 1978; Bubrick *et al.*, 1985). Lectins have also been considered as recognition like-proteins in other symbiosis (Díaz *et al.*, 1989, Giollant *et al.*, 1993). However, these proteins have also been related to parasitic mechanisms (Saikawa, 1982; Elad *et al.*, 1983) in attack-defense processes on the basis of their antiviral (Kumar *et al.*, 1993) or antifungal (Viard *et al.*, 1993) activity, which is a defense against animal attack (Hoedemaeker *et al.*, 1993), etc. Biochemical studies revealed that these lectins are very conserved glycoproteins with a high degree of homology (Chrispeels and Raikhel, 1991). Chrispeels and Raikhel (1991) suggested that these proteins, encoded by tandem genes, presumably arose through duplication and divergence of an ancestral gene. They also take part of a protein family involved in plant defense mechanisms (Molina and Vicente, 2000).

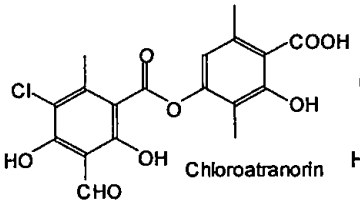
According to their chemical structures, most lichen substances are phenolic compounds (orcinol and β-orcinol derivatives), dibenzofuranes and usnic acids, depsides (barbatic acid), depsidones (salazinic acid), depsones (picrolichenic acid), lactones (protolichesterinic acid, nephrosterinic acid), quinones (parietin), and pulvinic acid derivatives (vulpinic acid). Lichens apparently evolved diverse biosynthetic pathways to produce this diversity of compounds: mainly polymalonate, shikimic acid and mevalonic acid pathways.

Apart from compounds derived from these common pathways, which are found throughout all major lichen groups, there are also some unusual compound classes among these organisms; for example, arthogalin, a cyclic depsipeptide (Huneck and Himmelreich, 1995), and other amino acid-derived compounds such as the cytotoxic scabrosin esters isolated from

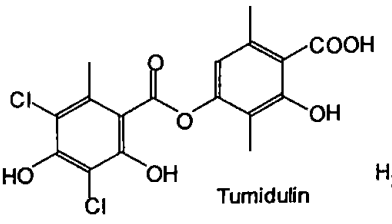
Xanthoparmelia scabrosa (Ernst-Russell *et al.*, 1999b). Uncommon features are also detected in residues of common substance classes, in the form of other intramolecular arrangements, or in the binding with other compounds such as sugars. Recently, Rezanka *et al.* (2004) described many unusual compounds as brominated depsidones (Rezanka and Gushina, 1999), brominated acetylenic fatty acids (Rezanka and Dembitsky, 1999) and monotetrahydrofuranic acetogenin derivatives (Rezanka *et al.*, 2004). Series of g-lactonic aliphatic acid glycosides were also identified (Rezanka and Gushina, 2000, 2001a, b), some of them forming a macrolactone ring (gobienins: Rezanka and Gushina, 2001c). Glycosides are not frequently encountered in lichens, yet, recent reports revealed the presence of xanthone glucosides (umbilicaxanthosides: Rezanka *et al.*, 2003) and the mycosporine collemin A from *Collema cristatum* (Torres *et al.*, 2004).

1.1.3. Constituents of lichens already reported

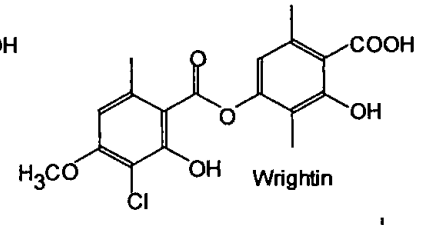
The mycobiont part of the lichens synthesize a large variety of secondary compounds (Hunek and Yoshimura, 1996) and many hypotheses concerning their biological role have been proposed (Lawrey, 1986; Fahselt, 1994; Huneck, 1999) as mentioned by Asplund and Gauslaa (2007). However, the photobiont (microalga or a cyanobacteria) part might produce certain compounds comparatively less than the mycobiont (a fungus of Ascomycetes) part. Lichens generate a number of biologically active molecules among which a noticeable role is played by the halogenated phenol compounds. A small list of the lichen compounds is given here:



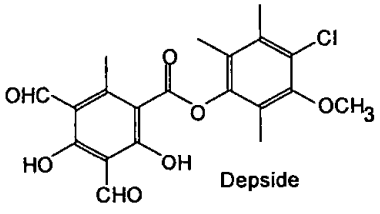
Chloroatranorin



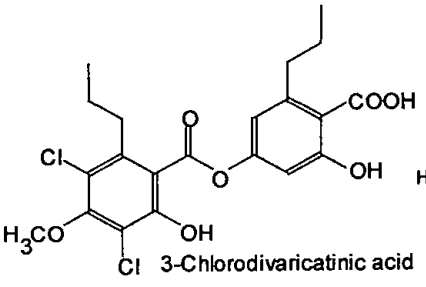
Tumidulin



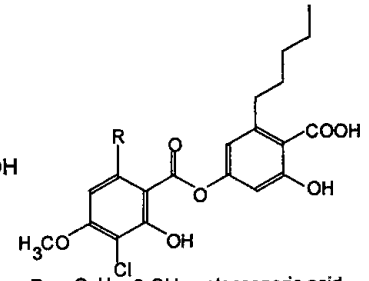
Wrightin



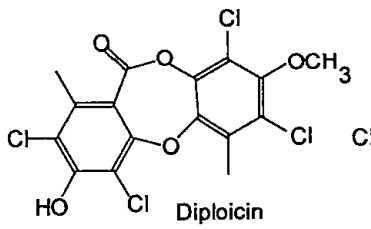
Depside



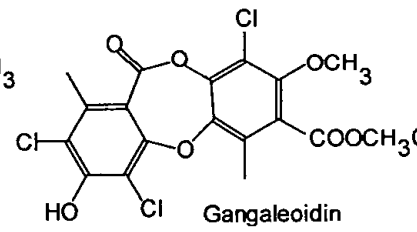
3-Chlorodivaricatinic acid



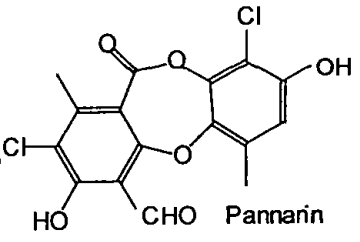
R= n-C_3H_7, 3-Chlorostenosporic acid
R= n-C_5H_{11}, 3-Chloroperlatolic acid



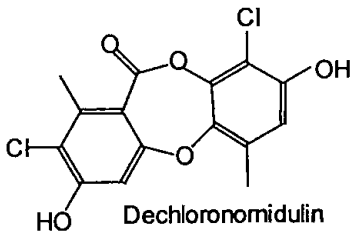
Diploicin



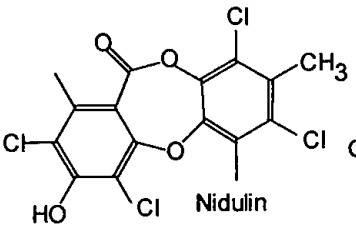
Gangaleoidin



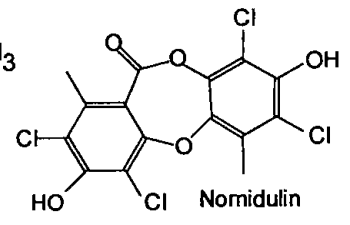
Pannarin



Dechlororonomidulin



Nidulin



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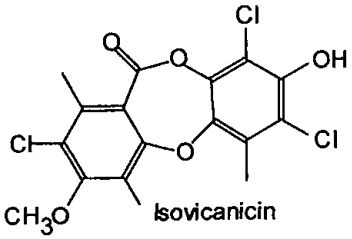
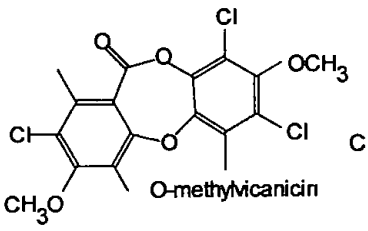


Isovicanicin



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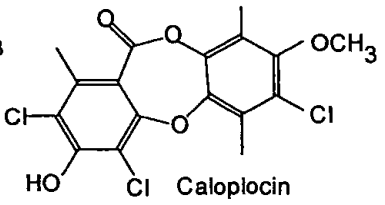

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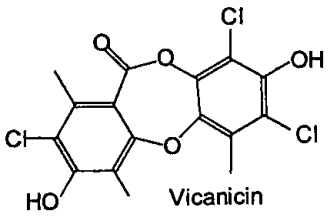

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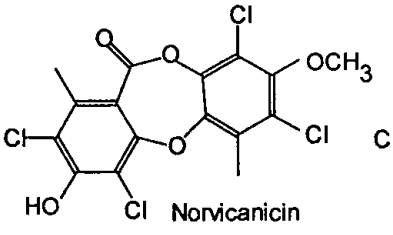

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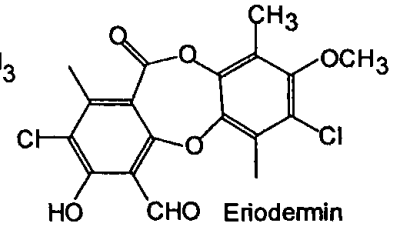
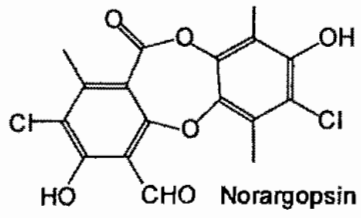


Eriodermin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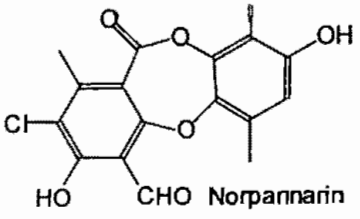
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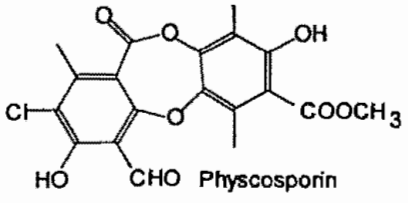
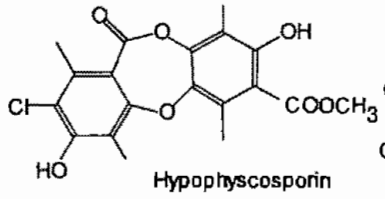

Physcosporin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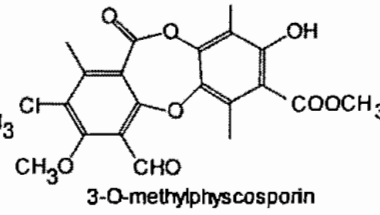
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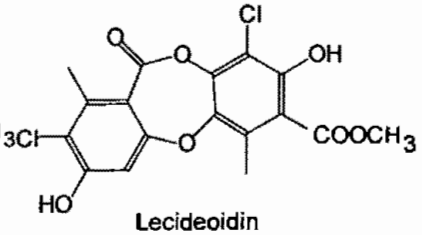
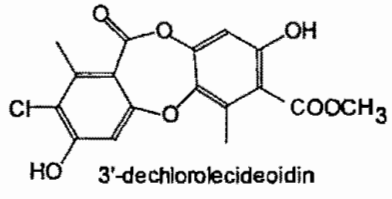

Lecideoidin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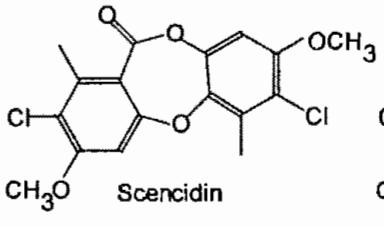
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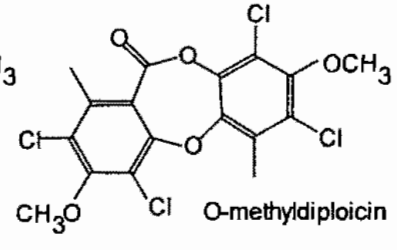
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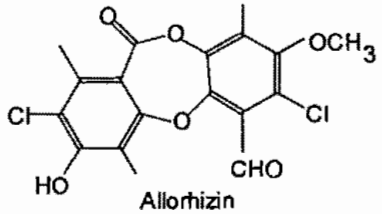
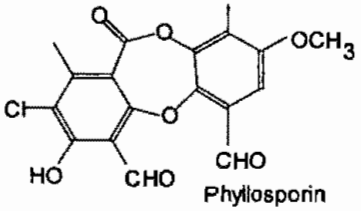
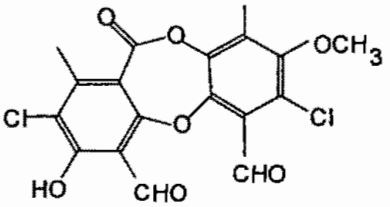
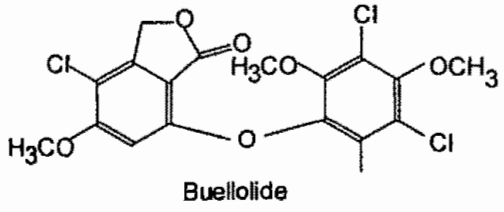
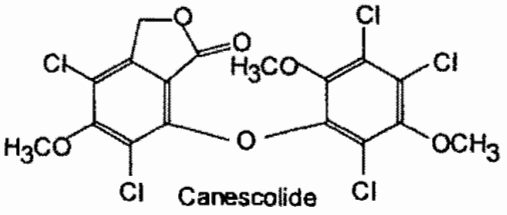

Allorhizin


Phyllosporin


Chlorophyllosporin


Buellolide


Canescolide

1.1.4. Bioactive and pharmacologically important principles from lichens reported

Lichen secondary metabolites are known to inhibit various animal consumers and pathogenic microorganisms. Nevertheless, many obligate fungal pathogens have evolved a tolerance to these inhibitory lichen compounds (James *et al.,* 1999).

The lichen-forming fungi produce antibiotic secondary metabolites that provide protection from most animals and pathogenic microorganisms (Vartia, 1973; Rundel, 1978; Lawrey, 1984, 1986). Nevertheless, certain obligate fungivorous animals (Lawrey, 1983) and fungal parasites (Lawrey, 1995) consume lichens, which suggests that tolerance to certain lichen compounds may play a role in the ecology of these organisms. Indeed, there is some evidence that lichen parasites are generally more tolerant of lichen compounds than nonlichenicolous fungi (Lawrey, 1997). There is also evidence that the enzymes produced by lichenicolous fungi are more tolerant of certain lichen compounds than others, which may explain the host ecologies of these fungi (Torzilli and Lawrey, 1995; James *et al.,* 1999).

Following the discovery of penicillin production by the fungus *Penicillium notatum*, a number of lichens, which consist of a symbiotic association between an algal and a fungal partner, were screened for antibacterial activity between 1940 and 1950 (Vartia, 1973). Several lichen compounds were found active against mycobacteria and gram-positive organisms (Stoll *et al.*,1950 and Vartia, 1973). The lichen *Cetraria islandica* (L.) Ach., commonly known as Iceland moss, has been used in European traditional medicine for treatment of minor ailments such as throat irritation and cough, but also for tuberculosis, asthma, and gastrointestinal conditions such as gastritis (Kartnig,1987). In Iceland the plant has furthermore been used for symptomatic relief of gastric and duodenal ulcer (Ingólfsdóttir *et al.*, 1997).

The wide variety of biological activities of lichens is generally correlated to their special ecological circumstances (Lawrey, 1986, 1995; Rikkinen, 1995), with production of metabolites involved in antimicrobial actions and deterrent properties (sometimes highly toxic to certain animals). Some review articles recently underlined the pharmaceutical potential of these substances (Huneck, 1999; Yamamoto, 2000; Müller, 2001). For centuries, lichens have also been used in folk medicine (Richardson, 1988; Schindler, 1988; Hawksworth, 2003). Commercial preparations based on the presence of usnic acid are available in some countries for local antiseptic activity and many properties have been described for this compound (Ingólfsdóttir, 2002). Usnic acid, like many other lichen compounds, causes allergic reactions, but a recent incident of some hepatotoxicity was attributed to usnic acid due to its inadequate use in nutraceuticals (about 500mg a day taken orally to lose weight; Durazo *et al.*, 2004; Neff *et al.*, 2004).

Lichens are still part of folk and alternative medicines in Asian countries (e.g. Benalu tehw) with various indications (Saklani and Upreti, 1992), and in the *European Pharmacopoiea* (2005), Iceland moss (= *Cetraria islandica*) is included and indicated uses correspond to a cough remedy and to a *tonicum amarum. Lobaria pulmonaria* has also been used as a cough remedy based on its lung-shaped appearance, and is still used in homeopathy (as *Sticta pulmonaria*). Together with recent studies on the antifungal activities of some Indian lichens (Shahi *et al.*, 2000, 2001, 2003), considerable information is available on ethnobotanical utilization of lichens by the different ethnic groups in India (Brij Lal and Upreti, 1995; Brij Lal *et al.*, 1985; Brij Lal, 1988; Singh *et al.*, 2000; Kumar and Upreti, 2001). The pharmacological and other biological activities of lichens and lichen substances can be divided into the following categories based on current knowledge.

Lichens have been used for medical purposes since ancient times and are known to produce unique secondary metabolites, a number of which have considerable biological activities such as antimicrobial, antiherbivore, and antibiotic (Vartia, 1973; Richardson, 1988; Lawrey, 1989; Elix, 1996). Secondary metabolites in lichens are produced by the fungus alone and secreted onto the surface of lichen's hyphae in amorphous forms or crystals. Up to now about 350 secondary metabolites are known from lichens and approximately 200 have been characterized. Papers, dealing with the biological activity of lichens and lichen substances started to appear after World War II. Lists of the antibacterial and antifungal activities of lichen compounds and lichens against bacteria and fungi can be found in a review and a book (Huneck, 1999, 2001). Among the lichen substances, the most widely distributed and the most extensively investigated one, without doubt, is usnic acid.

However, several species of lichens with antibiotic properties are also scattered in many parts of Nigeria and are used locally in the Eastern region of the country for the treatment of certain infections (Esimone and Adikwu, 1999). The antibiotic properties of lichen substances have long been known (Bustinza, 1951; Bérdy, 1982; Ingólfsdóttir *et al.*, 1985; Correché *et al.*, 1998). However, none of these studies have involved lichens obtained from the tropics, neither have there been any studies on the effect of these lichen substances on clinical isolates of microorganisms obtained from this or any part of Africa (Esimone and Adikwu, 2002).

A preliminary investigation has shown that the ethanol, water, chloroform and *n*-hexane extracts of the lichen had antibacterial and antifungal properties (Esimone and Adikwu, 1999). We had also previously established that fractions RF1 and RF2 were depsides and depsidones respectively and that they were bioactive (Esimone *et al.*, 1999). Bioactivity was assessed according to the brine shrimp lethality assay technique (McLaughlin *et al.*, 1991; Esimone and Adikwu, 1999).

Some lichen substances have been partially investigated for antitumour and antimutagenic activity: (-)-usnic acid, a dibenzofuran with an antitumoral effect against Lewis Lung carcinoma and P388 leukaemia (Kupchan and Kopperman, 1975; Takai *et al.,* 1979) was found to be involved in mitosis inhibition (Huovinen and Lampero, 1989; Cardarelli *et al.,* 1997) and apoptotic induction (Be´zivin *et al.,* 2004). The butyrolactone, protolichesterinic acid, was also found active as an antiproliferative against leukaemia cells K-562 (IC50 = 20mg/ml) and against Ehrlich solid tumour, while nephrosteranic acid derivatives have a poor activity (Hirayama *et al.,* 1980). Polyporic acid (a terphenylquinone) and derivatives (Cain, 1961, 1966), a depsidone, physodalic acid (Shibamoto and Wei, 1984), lichen glucans (Nishikawa *et al.,* 1969, 1979; Hirayama *et al.,* 1980; Nishikawa and Ohno, 1981; Hirabayashi *et al.,* 1989) including lichenin derivatives (Demleitner *et al.,* 1991) have also been investigated in this way. A review was recently published on structure features and bioactivities of lichen polysaccharides (Ólafsdóttir and Ingólfsdóttir, 2001). Concerning new lichen secondary metabolites that exhibit relevant cytotoxic activities against cancer cell lines, original N-containing complex structures (scabrosin esters) isolated from *Xanthoparmelia scabros*

Some anthraquinonic compounds, which are also encountered in higher plants (emodin and derivatives) have shown *in vitro* antiviral activity considered of interest against Human Cytomegalovirus (Wood *et al.,* 1990). Seventeen depsides and depsidones have been examined for their inhibitory activity against HIV integrase and pharmacophores derived from virensic acid allowed selection of various potent inhibitors (Neamati *et al.,* 1997). The butyrolactone, protolichesterinic acid, isolated from *Cetraria islandica,* was also found to inhibit HIV-RT (Pengsuparp *et al.,* 1995). Four depsides and above all (+)-usnic acid were found active against EBV activation and could therefore constitute valuable candidates as antitumour promoters (Yamamoto *et al.*, 1995). Concerning high molecular weight compounds, a sulphate (GE-3-S) prepared by chlorosulphonic acid treatment of GE-3, a partially acetylated (1→6) glucan from the lichen *Umbilicaria esculenta*, inhibited the cytopathic effect of HIV *in vitro* (Hirabayashi *et al.*, 1989).

Lichens also show enzyme inhibitory activities. Lichen acids, mainly atranorin, evernic, physodic and usnic acids, are powerful inhibitors of some metabolic enzymes, interestingly related to polyamine metabolism, such as arginase, arginine decarboxylase, ornithine decarboxylase, etc. (Legaz *et al.*, 1983, 2001; Planelles and Legaz, 1987; Okuyama *et al.*, 1991; Matsubara *et al.*, 1997, 1998; Kinoshita *et al.*, 2002). Inhibition of lipoxygenase has also been demonstrated for some lichen extracts and compounds (Ingólfsdóttir *et al.*, 1985, 1994, 2002; Ögmundsdóttir *et al.*, 1998). The inhibition of prostaglandin biosynthesis (Sankawa *et al.*, 1982) as well as the inhibition of leukotriene B4 biosynthesis depicted by Kumar and Müller (1999) can be related to the anti-inflammatory activity, analgesic and antipyretic activities (Okuyama *et al.*, 1995) and local anaesthesic effect (Correia Da Silva, 1981) of some lichen compounds.

1.1.5. Lichens used in this investigation

Different types of lichens are found on the same plant, as well as the same lichen may also take place on different plants, however there may have a difference of the types of fungi, as well as of algae just depending on their symbiotic interaction. Morphological features, such as shape of the colony, coloration, etc. have considered as the attributes to differentiate lichens present on the stem bark of a tree, and collections were made accordingly. Proper identification has been attempted with no positive result, while identification of lichens by professional centers has been hardly affordable and thus declined so far.

In this investigation lichens were selected for collection based on the name of host plants. The lichen(s) collected were mentioned according to their host plants' names and were marked as well.

Table 1. List of the host plants and ID number of the lichen samples

Sl.	Name of the host plant	ID
1.	*Areca catechu* [সুপারী]	h1
2.	*Amoora rohituka* W. & A. (=*Aphanamixis polystachya* (Wall.) [পীতরাজ]	h2
3.	*Artocarpus heterophyllus* Lamk [কাঁঠাল]	h3
4.	*Morus indica* L. [তুঁত]	h4
5.	*Anthocephalus chinensis* (Lam.) Rich.(=*A. cadamba* Miq.) [কদম]	h5
6a.	*Litchi chinensis* Sonn. (=*Nephelium litchi* Camb.) [লিচু]	h6
6b.	*Litchi chinensis* Sonn. (=*Nephelium litchi* Camb.) [লিচু]	h7
7.	*Diospyros peregrina* L. [গাব]	h8
8.	*Stereospermum chelonioides* [কামরাঙা]	h9
9.	*Borassus flabellifer* L. [তাল]	h10
10.	*Citrus grandis* (L.) Osb. [বাতাবী লেবু]	h11
11.	*Lannea coromandelica* (Houtt.) Merr. [জিগা]	h12
12.	*Mangifera indica* L. [আম]	h13

Note: Identification of the lichens has been attempted and was not successful, however the specific lichens have been marked with their morphological features, i.e. coloration, shape and size of the colony, forms of mycelia, etc. and then photographs have been made as standard samples for the repeated collections.

1.2. Background information on the test organisms

The whole project has been designed to carry on screening of the crude extracts of the test plant species on several test organisms for the detection of biological activity and isolation, purification and characterization of the bioactive compounds through chromatographic techniques, keeping an option to show extent of activity by analyzing the data statistically that read on various parameters during the course of the work. The following test steps have been taken into consideration:

Table 2. List of the test agents

Test agents	Types of tests
1. Microorganisms	Antibacterial activity test
	Antifungal activity test
2. *Artemia salina*	Cytotoxicity test
3. *Tribolium castaneum* (Hbst.)	Repellent activity tests
	Larvicidal/ Insecticidal
4. *Musca domestica*	Larvicidal activity test

1.2.1. Agents for antimicrobial activity tests

It is very important to determine whether the crude chloroform extracts are active against various types of test organisms or not and thus a preliminary antibacterial and antifungal screening of the crude extract was very much necessary. Therefore, screening was done against various pathogenic bacteria and fungi by disc diffusion assay (Bauer *et al.*, 1966; Barry, 1976) method. The fungal and bacterial isolates were cultured and used in the experiments carried out at the Molecular Biology Laboratory of the Institute of Biological Sciences, Rajshahi University, Rajshahi-6205, Bangladesh. Among the collected isolates 6 were Gram positive and 6 were Gram negative bacteria.

1.2.1.1. List of the test pathogenic bacteria

Table 3. List of the test pathogenic bacteria and their pathogenicity

Sl. No.	Name of test organism/ Strain #	Pathogenicity
Gram positive		
1	*Staphylococcus aureus* ATCC-259233	Wound infection, abscesses, endocarditis, septicaemia, osteomyelitis and food poisoning-Pallen, 2008.
2	*Bacillus megaterium* QL-38	White blotch in wheat
3	*Bacillus subtilis* QL-40	Dysentery
4	*Bacillus cereus*	Rapidly blinding forms of endophthalmitis, Callegan *et al.*, 2006; food poisoning- Drobnewski, 1993; Granum and Lund, 1997; causative agent od gastrointestinal and in nongastrointestinal infections-Kotiranta *et al.* 2000.
5	*Streptococcus-β-haemolyticus* CRL	Sinusitis, Pneumonia/ Otitis- Ulrich Glück and Jan-Olaf Gebbers, 2003; Pharyngitis; septicemia and meningitis in neonate with septicemia in the mother; rheumatic fever and glomerulonephritis- Pallen, 2008.
6	*Sarcina lutea* QL-166	Cause disease to human stomach
Gram negative		
7	*Salmonella typhi-*	Enteric (typhoid) fever
8	*Shigella dysenteriae* AL-35587	Fluid accumulation in the intestine, bacterial dysentery
9	*Shigella shiga* ATCC-26107	Shigellosis or bacterial dysentery, an acute inflammation of the intestinal tract
10	*Shigella sonnei* AJ-8992	Shigellosis or bacterial dysentery, an acute inflammation of the intestinal tract, Urinary tract infection,
11	*Shigella boydii* AL-17313	Shegellosis / bacillary dysentery; Abdominal pain; cramps; diarrhea; fever; vomiting; blood, pus, or mucus in stools; tenesmus (Fecally contaminated water and unsanitary handling by food handlers are the most common causes of contamination)
12	*Escherichia coli* FPFC-1407	Lower respiratory tract infections, skin and skin-structure infection, urinary tract infection, gyneocologic infections, intra-abdominal infections, bacterial septicemia

1.2.1.2. Test fungi used for the study

The fungal strains used in the sensitivity test are given below and pure cultures of the strains were collected from the Plant Pathology Laboratory, Department of Botany, Rajshahi University, Rajshahi-6205.

Table 4. List of the test pathogenic fungi

Serial No.	Name of test organism	Pathogenicity
1.	*Fusarium vasinfectum*	Fusarium wilt of cotton- Skovgaard *et al.*, 2001; root rot of *Pennisetu* and *Sorgham* sp. Mukerjee and Bhasin, 1986.
2.	*Aspergillus fumigatus*	Aspergillosis (of the lung) in birds and various mammals- Millner *et al.*, 1980; Summerbell *et al.*, 1992. Brian *et al.*, 2001; fruit rot of *Lagenaria*, *Malus* and *Pyrus* sp. plants. Mukerjee and Bhasin, 1986.
3.	*Aspergillus flavus*	Fruit rot in *Litchi*, *Punica* and *Lycopersicon* sp. plants; Mould of grain and seed rot *Triticum* sp. Mukerjee and Bhasin, 1986. Dermatomycoses.
4.	*Mucor* sp.	Fruit rot *Pyrus*, *Nephelium*, and stem necrosis in *Solanum* sp.- Mukerjee and Bhasin, 1986.
5.	*Candida albicans*	Candidosis- Haynes, 2001; Calderone and Fonji, 2001. Dematomycoses, Moniliasis

1.2.2. Brine shrimp (*A. salina*) nauplii

Brine shrimp lethality bioassay is a recent development in the bioassay for the bioactive compounds, which indicates cytotoxicity, as well as, a wide range of pharmacological activities (e.g. anticancer, antiviral, pesticidal, anti-AIDS, etc.) of the compounds. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simply pharmacology at a higher dose. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, anti-microbial and pharmacological activities of natural products. Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. Here *in vivo* lethality of a simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening a fractionation in the discovery of new bioactive natural products. Generally, the median effective dose (ED_{50}) values for cytotoxicity are one tenth (1/10) of median lethal dose (LC_{50}) values in the brine shrimp test.

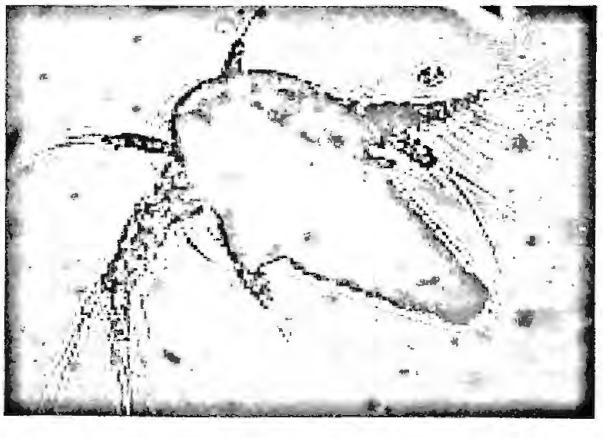


Plate 1. *A. salina* (Brine shrimp) nauplius

The *A. salina* belong to a genus of very primordial crustacean (crawfish - crayfish) the *Anostraca* (Fairy Shrimps). Crawfish of this genus just have a divided exoskeleton made of Chitin enhanced protein, no usual crust of chitin (escutcheon) as other crawfish have. There are many species within the genus of *Anostraca*, but the *A. salina* are very nice to grow, since the rate of successful hatches is very high. To carry on toxicity tests of certain materials these nauplii are very easy to grow from its marketed cysts and to set experiments thereby.

1.2.3. *T. castaneum*

The rust-red flour beetle, *T. castaneum* (Herbst) is one of the most serious pests of stored products. It is commonly known as 'red flour beetle' (Coleoptera: Tenebrionidae). Mouthparts of this pest insect are not adapted to feed on hard whole grains and they are thus found in almost any kind of flour, cracked grains etc. listed the specific food of *T. castaneum,* which includes whole-wheat flour, bran, rice flour, cornmeal, barley flour and oatmeal. It also feeds upon dried fruits, dried plant roots, nuts, chocolates, drugs, snuff, cayenne pepper, pulses and prepared cereal foods such as corn flakes (Metcalf and Flint, 1962). Not only pulses and millets, cereals are also been attacked by this beetle (Purthi and Singh, 1950).

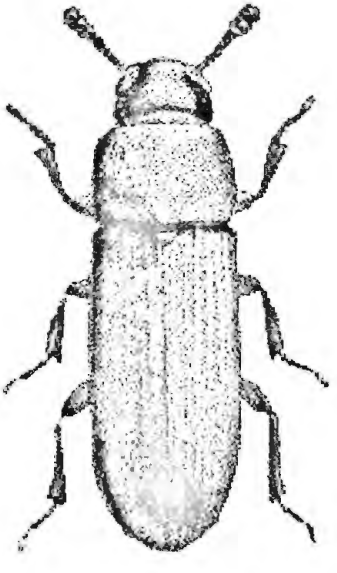


Plate 2. *T. castaneum* (Hbst.) adult

They are found in great numbers on infested materials and caused serious losses and considerable damage to flour and grains that have previously been attacked by other pests. Much of the damage done by *T. castaneum* is directly to kernels (germ and endoplasm). In case of severe infestation flour or other materials invaded may have a characteristics pungent odor as a result of the gaseous secretion exuded by the beetle. In severe infestation, the flour turn grayish and moldy and has a pungent, disagreeable odor making it unfit for human consumption (Good, 1936). Infested material will show many elongate reddish brown beetles, about 1/7 inch long crawling over the material when it is disturbed and brownish white (somewhat flattened) six-legged larval bedding on the inside of the grain kernels and crawling over the infested seeds. They are generally known among millers as "bran bugs". *T. castaneum* contaminates more than they consume.

Both the larva and adults cause damage. The young larva is yellowish white and measures 1 mm in length. As it matures, it turns reddish yellow, becomes hairy and measures over 6 mm in length. Its head, appendages and the last abdominal segment are darker. The adult is a small reddish-brown beetle, measuring about 3.5 mm in length and 1.2 mm in width. Its antennae are bent and bear a distinct club formed by the three enlarged terminal joints. The last antennal segment is transversely rounded. This insect is now widely distributed all over the world mainly through commerce. Control of these insects relies heavily on the use of synthetic insecticides and fumigants, which has led to problems such as disturbances of the environment, increasing costs of application, pest resurgence, pest resistance to pesticides and lethal effects on non-target organisms in addition to direct toxicity to users (Jembere *et al.*, 1995). Thus, repellents, fumigants, feeding deterrents and insecticides of natural origin are rational alternatives to synthetic insecticides.

1.2.4. *M. domestica*

The house fly *M. domestica* was chosen for dose-mortality studies because it is easy to rear, it has a short development time (about 12 days at 26.5°C) and it has economic and medical significance. The strategy for housefly as a model system to investigate genetic methods might also be applied to others species (Wagoner *et al.*, 1973).

Plate 3. *M. domestica*

The fly that is typical of the Muscoidea is the best known and most notorious of all flies, *M. domestica* L., the housefly, or just the fly to millions of people (Oldroyd, 1964). Flies are abundant species that can be found almost everywhere. They usually occur in such large numbers as to constitute a nuisance. Muscidae also include the little housefly, face fly, stable fly and horn fly, all of which are pests of livestock. Houseflies are important pest from the family Muscidae (Diptera). They are considered a nuisance and vectors of human and animal diseases. Houseflies transmit several diseases and pathogens that are harmful to livestock (Cumming and Cooper, 2000). These are commonly found where people work or live because of the warmer environment and ready supply of food. Eggs of houseflies are laid in moist or rotting matter, household rubbish compost of manure and once hatched the flies reach maturity in anything from two weeks in warmer weather. Flies are difficult to control because of their rapid rate of reproduction. One female fly

can produce thousands offspring in a single breeding season. Flies also have the ability to develop in small quantities of food. Flies also have the typical insect ability to enter through and hide in openings the size of a pinhead (Snyder, 1991).

M. domestica is a well-known cosmopolitan pest of both farm and home. This species is always found in association with humans or activities of humans. It is a highly versatile insect and suits its behavior of the local conditions as it finds them (Oldroyd, 1964). It is reported to transmit more than 20 human and animal diseases (Hicking 1974). Mastitis, pinkeye, anthrax, typhoid fever, amoebic dysentery, tuberculosis, cholera, Newcastle disease and *Salmonella* are some of the diseases affecting man and animals that can be transmitted by these flies. Pathogenic organisms are picked up by flies from garbage, sewage and other sources of filth and then transferred on their mouthparts and other body parts through their vomits, feces and contaminated external body parts to human and animal food (Sanchez-Arryo, 1998).

1.3. Techniques for the isolation and purification of bioactive compounds

The key to any successful program involving the investigation of biologically active plant constituents is the availability and choice of chromatographic techniques for the separation of pure substances. The aim is to have maximum yield with minimum effort (to reduce the time and cost of the separation procedure). Preparative separation techniques can be tedious and time consuming, especially when complex mixtures, such as, crude plant extracts have to be resolved. Over the past decade or so, several new techniques have been introduced, leading to the acceleration and simplification of different separation problems (Hostettmann *et al.*, 1986; Marston and Hostettmann, 1991; Hostettmann *et al.*, 1991). However, there is no universal technique capable of solving every isolation problem. All methods have advantages and limitations, so much, so that the best results are often obtained by a combination of two or more of these.

The most important preparative separation techniques employed in the isolation and purification of plant constituents are as follows:

Preparative separation methods for lichen constituents

Solid phase chromatography

Paper chromatography

Preparative TLC, Centrifugal TLC

Open-column chromatography

Vacuum liquid chromatography

Pressure column chromatography, flash chromatography

Low-pressure liquid chromatography (LPLC)

Medium-pressure liquid chromatography (MPLC),

High-pressure/High-performance liquid chromatography (HPLC)

Liquid liquid chromatography

Craig distribution

Droplet countercurrent chromatography (DCC)

Rotation locular countercurrent chromatography (RLCC)

Centrifugal partition chromatography (CPC)

Of the methods the solid phase category, column chromatography is very popular and used extensively. It can include non-exchange resins, polymeric columns, gel-filtration, and chromatography over silica-gel or chemically modified silica-gel. Open column chromatography has a high load capacity but the separation time is long and the resolution is respectively low.

According to the laboratory set up and the availability of essentials in the Department of Zoology, Rajshahi University and certain other neighboring laboratories there were limitations in choosing out the preparative separation methods, while the thin layer chromatography and the open column chromatography were used simultaneously in this investigation for the isolation and purification of the bioactive lichen compounds.

1.4. Bioassay with the purified compound(s)

Bioassay with the purified compounds has been a major target in this investigation, however, the test organisms used in this activity guided investigation for isolation and purification were also considered for this bioassay. Since the crude extracts didn't show any insecticidal activity the pure lichen compounds were subjected to antimicrobial tests to evaluate their efficacy.

1.5. Aim and objectives of this work

1. To trace presence of bioactive potentials in lichen by primary screening:
    - Using chloroform extracts against plant and human pathogenic bacteria and fungi;
    - Using *A. salina*, the recognized test agent for cytotoxic effect to evaluate cytotoxicity of the extracts by establishing LC_{50} values;
    - Using *T. castaneum* adults to evaluate efficacy of the extracts through repellent activity test;
    - Using the stored product pest *T. castaneum* to evaluate efficacy of the extracts through dose-mortality tests by establishing LD_{50} values and to evaluate efficacy of the extracts on their biology.
    - Using *M. domestica* larvae to evaluate larvicidal activity of the extracts;
2. To isolate, purify and characterize the active compounds from the promising extract(s) and to evaluate efficacy of the purified compounds against selected test agents using any suitable assay.
3. To comment on the future perspectives of the test plant depending on the achieved results.

Chapter 2

MATERIALS AND METHODS

2.1. Selection of test materials

2.2. Selection of test organisms

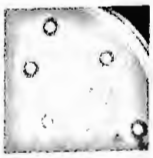
2.3. Bioassays for the activity of lichen extracts

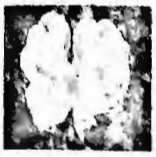
2.4. Chromatographic techniques used in this investigation

2.5. Preparative separation techniques

2.6. Isolation pathway (flow chart) of the compounds purified



Chapter 2 Materials and Methods

2. Materials and Methods

2.1. Selection of test materials

For the extraction fresh lichen materials were collected from some selected host plants and again the selection of host plants was just depending on the frequency of occurrence of lichens on their barks. Lichens were mentioned here through their ID numbers h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11, h12 and h13 (Table 1). Only crustose types of lichens have been found. No fruticose or foliose type of lichen was got so far. Necessary photographs were taken for each type of lichen.

2.1.1. Collection of lichen materials

Fresh and fair lichen patches were selected for collection. The lichen was scrapped with the help of a bland knife and the dust was collected on a paper marked with the type of the lichen.

2.1.2. Preparation of lichen materials for extraction

After collection the lichen samples were checked thoroughly to eliminate out the foreign particles as bark tissues of the host plant with the help of a set of forceps and dust or other things were waived out with the help of a camel hairbrush. The materials then ground to powder. The amount was weighed and labeled.

Plate 4. Scrapping of lichen from the bark of a host plant

Plate 5. Collected lichen materials

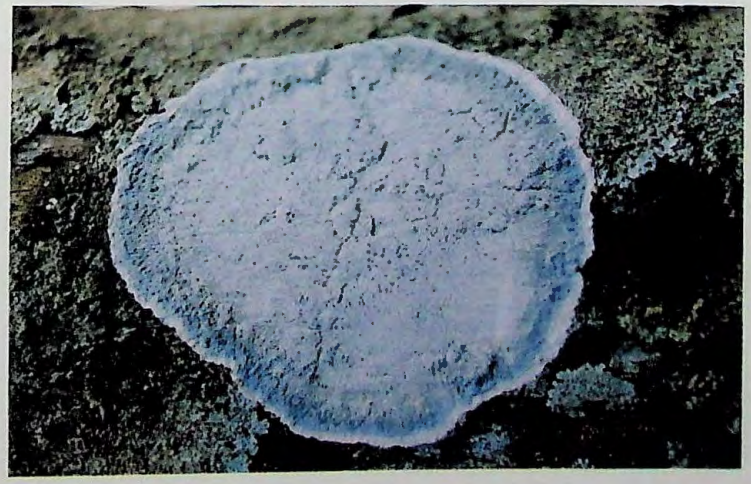


Plate 6. Lichen material h1 from *A. catechu* stem-bark

Plate 7. Lichen material h2 from *A. rohituka* stem-bark

Plate 8. Lichen material h3 from *A. heterophyllus* stem-bark

Plate 9. Lichen material h4 from *M. indica* stem-bark

Plate 10. Lichen material h5 from *A. chinensis* stem-bark

Plate 11. Lichen material h6 from *L. chinensis* stem-bark

Plate 12. Lichen material h7 from *L. chinensis* stem-bark

Plate 13. Lichen material h8 from *D. peregrina* stem-bark

Plate 14. Lichen material h9 from *S. chelonioides* stem-bark

Plate15. Lichen material h10 from *B. flabellifer* stem-bark

Plate16. Lichen material h11 from *C. grandis* stem-bark

Plate 17. Lichen material h12 from *L. coromandelica* stem-bark

Plate 18. Lichen material h13 from *M. indica* stem-bark

2.1.3. Chemical extraction of the lichen samples

There are basically two methods for extracting compounds from test materials. Which one to choose, depends on whether the aim is to extract the more polar compounds (especially glycosides), which are present in the cell vacuoles, or to obtain the less polar aglycones present on the surface or in the aerial parts. In this investigation chloroform was selected to extract all the lichen samples.

For the extraction each lichen material was air-dried and ground with a mortar and pestle. The ground lichen material was set for extraction in sufficient amount of chloroform (for 1 g of dust about 50 ml of solvent) and sealed in conical flask to keep on a shaker overnight before filtration and it was repeated thrice for one sample for a single solvent. The extracts were then fitted one after another with a round bottom flask to a vacuum rotary evaporator. The output extracts were removed to glass vials and preserved in a refrigerator at 4°C with proper labeling. Process of extraction and collection of extracts from the lichen materials have been shown in Fig. 1 and Plate 19.

Extraction of lichen materials

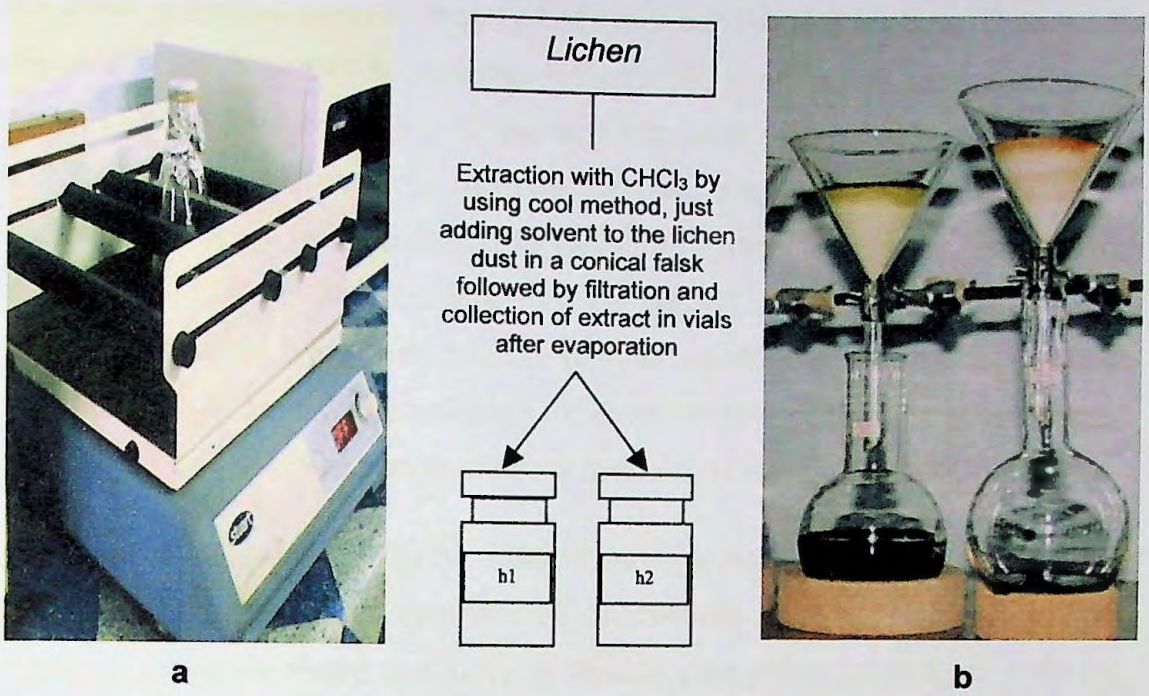


Fig. 1. a) Collection of extracts in chloroform from lichens, **b)** filtration

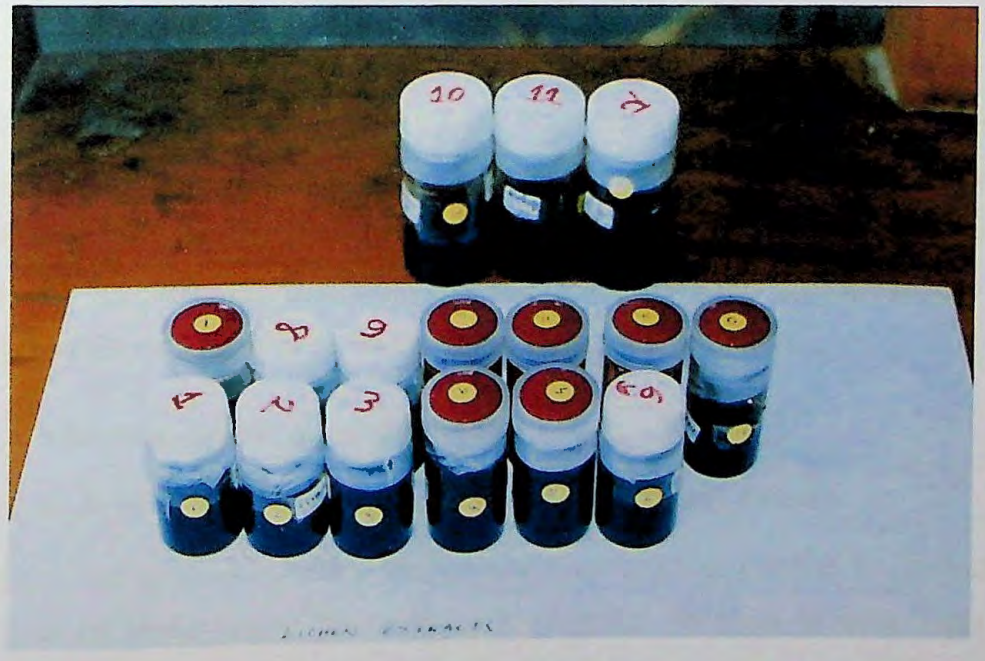


Plate 19. Lichen extracts

2.2. Selection of test organisms

To trace biological activity of any substance any microorganism could be a tool since it gives a chance to see whether or not the test material shows any effect on a single unit of living thing. A bacterium is of course similar to that of a single cell, a fungal hypha is similar to a thread of several cells. So, bacteria and fungi could be used to mitigate the test requirements. A number of bacteria and fungi were selected to carry out further efficiency tests of the extractives (Table 3 and Table 4). To be certain about efficacy of the test substance against living body another minute organism could also be used as a test agent. *A. salina* was selected for cytotoxicity test since it is very much easy to get and set in experiments as a model test agent (Table 2).

To carry on repellent activity, insecticidal/ larvicidal activity tests of the extractives of the lichen samples *T. castaneum* and *M. domestica* were selected, because these are easy cultivable and noble laboratory animals. Moreover, they are important stored grain pests in a wide variety of cereal products. The life histories made these insects as popular choice as test insects for biological studies. They are also easy to culture in large number and require no sophisticated equipments for their maintenance.

2.2.1. Collection of test insects

The brine shrimp cysts were collected from aquarium shops of Kalabagan, Dhaka. The test insects *T. castaneum* and *M. domestica* used in the present investigation were collected from the stock cultures of the Crop Protection and Toxicology Laboratory, University of Rajshahi, Rajshahi-6205, Bangladesh and reared as subcultures to be used in the experimentation.

2.2.2. Collection of test agents/insects

2.2.2.1. Culture of test agent *A. salina* (brine shrimp) nauplii

There are many species within the genus of *Anostraca*, but the *A. salina* are very nice to grow, since the rate of successful hatches is very high. To conduct cytotoxicity test the brine shrimp nauplii were used because of its

easy hatching and use in the experiment. The eggs (cysts) were collected from aquarium shops. For their easy hatching and use the requirements were as follows:

- Salt water: 1.5 - 3 tablespoons of marine salt every liter of water;
- Temperature: 26-28°C (80-82°F); 25-30°C (77-86°F);
- Light: The beaker was placed near a window with sunlight before hatching;
- Aeration: Picking up some water carefully with a spoon and let it drop back into the beaker once a day [but a small aquarium pump with a little air-stone is better];
- Helpful Hint: Brine shrimp egg is sometimes very bouyant. Swirl the water to knock down eggs;

The cysts absorb water and if the sun is shining (a signal for growing algae and other plankton) they hatch after 24-48 hours, depending on their environment. Freshly hatched *A. salina* called nauplii and have a size of just 0.25mm (0.01inch). They molt like any other crawfish when they grow to adult they molt about 17 times. If the breeding temperature is about 26-28°C (80-82°F), a nauplius hatches within 24-48 h, gets pubescent in 8-14 days and lives - depending on the concentration of salt -up to 4-5 weeks. The more salt, the less the life expectancy. Freshly hatched nauplii were used in this experiment.

2.2.2.2. Culture of test insect *T. castaneum*

Mass cultures were maintained in plastic containers (1200ml) and sub-cultures in beakers (1000ml) with the food medium. The beakers were kept in an incubator at $30^0C \pm 0.5^0C$ without light and humidity control. Each container and beaker contained 250g and 150g of food respectively. About 200 adults in each container and 100 adults in each beaker were introduced. The cultures were checked in regular intervals and eggs and larvae were separated to increase properly. A crumpled filter paper was placed in side

each container and beaker for easy movement of the beetles. The containers and beakers were covered with pieces of muslin cloth tightly fixed with the help of rubber bands to avoid possible escape of the beetles.

2.2.2.2.1. Preparation of food medium

The whole-wheat flour was used as the food medium for the insect species. The flour was sterilized at 60^0C for 36 hours in an oven. A standard mixture of whole-wheat flour with powdered dry yeast in a ratio of 19:1 (Park and Frank, 1948; Park, 1962; Zyromska-Rudzka, 1966) was used as food medium throughout the experimental period. Both the flour and the powdered dry yeast were sterilized at 60^0C for six hours in an oven. Food was not used until at least 15 days after sterilization to allow its moisture content to equilibrate with the environment (Khan, 1981).

2.2.2.2.2. Collection of eggs

About 500 beetles were placed in a 500ml beaker containing food medium. The beaker was covered with a piece of cloth and kept in an incubator at 30^0C $\pm$ 5^0C. In regular interval the eggs were collected by sieving the food medium by two sieves of 500 and 250 mesh separating the adults and eggs respectively following the methods of Khan and Selman (1981). Eggs were then transferred to petri dish (90mm in diameter) and incubated at the same temperature.

2.2.2.2.3. Collection of newly hatched larvae

After 3-5 days, larvae hatched out in described conditions. Newly hatched larvae were then collected with a fine pointed camel-hair brush and then shifted to the fresh food medium for culture. The larvae are yellowish white in colour and long cylindrical shape. It appears 1 mm in length after hatching and become 6-7mm at maturation.

2.2.2.2.4. Collection of mature larvae

Most larvae had six instars as reported by Good (1936). The larval instars were determined by counting the number of exuviae (larval skin) deposited in the food medium according to Good (1936). Two days-old larvae were considered as first instar larva while second, third, fourth, and fifth instar larvae were considered on fourth, seventh, tenth and thirteenth day from hatching respectively. Depending on these days according to larval instar sixteen days old larvae have been considered as mature larvae. Larval cultures were maintained in an incubator in the same procedure at $30^0C \pm 5^0C$ without light and humidity control. The food medium was replaced by thirty days interval to a fresh one to avoid conditioning by the larvae (Park, 1934).

2.2.2.2.5. Collection of adults

A huge number of beetles were thus reared to get a regular supply of the newly formed adults. When sufficient adults produced in the sub-cultures, they were collected from the food medium. For this purpose some pieces of filter paper were kept inside the beaker on the food. Adults crawled upon the paper and then the paper was taken out with a forceps. Beetles were then collected in a small beaker (100 ml) with the help of a fine camel-hair brush.

2.2.2.3. Culture of test insect *M. domestica*

The housefly (local stain) adults were collected from a 'Butcher's shop' of Binodpur Bazar, Rajshahi, to make stock cultures, maintained in the 'Insect Genetics Laboratory' Genetics & Breeding Department, Rajshahi University, Rajshahi, for the continuous supply of test insects to carry on the scheduled experiments in this investigation. Collected adults were released in 10 mesh cages measuring approximately 20 x 12 x 16 $inch^3$, made up of wood and fine ware nets with a door provided with muslin cloth for the easy collection of flies during experiments.

2.2.2.3.1. Food for larvae and adults

The feeding for larvae was provided in plastic pots (10 cm deep) containing 9 g of milk powder with 5 g fresh yeast (dissolved in 100 ml of water) and 100 g of wheat bran; then thoroughly stirred. The mixture put into the pots leaving 3 cm from the top. The pots were placed in inside the cages when the adults were engaged in mating and about to go laying eggs and the pots were kept for 24 hours in that state.

2.2.2.3.2. Collection of larvae

The female files (after mating) laid eggs in batches, each of which contained 100-150 eggs, which were separated out and transferred to similar pots contained food mixture (also called rearing medium) covered with cloth-net, wrapped with paper and shielded with plastic lids. These pots are then placed in a box covering with net, within 6-8 days after eggs have been hatched out to produce larvae. After a certain period of time the larvae have been transformed into pupae and ultimately emerged as adults. The third instar larvae were used as test organisms in this investigation.

2.3. Bioassays for the activity of lichen extracts

The availability of suitable bioassays for monitoring is very much important to any investigation of plants with biological activities. In order to cope with the number of extracts a high sample throughput is necessary. The test systems should ideally be simple, rapid, reproducible, and inexpensive. If active principles are only present at low concentration in the crude extract then bioassay is to be high enough sensitive for their detection. Another factor of special relevance to plant extracts is the solubility of the sample. Finding a suitable system can pose problems.

For the selection of bioassays to employ in research on plant constituents, the first step is to choose suitable target organisms. The complexity of the bioassay has to be designed as a function of the facilities and resources available. A list of bioassays taken in this investigation is shown bellow:

Table 5. List of test types

	Types of test
1. Antimicrobial	i) Antibacterial
	ii) Antifungal
2. Cytotoxicity	
3. Repellent activity	
4. Dose mortality	i) Larvicidal assay on *T. castaneum*
	i) Larvicidal assay on *M. domestica*

2.3.1. Antimicrobial tests

The antimicrobial screening of an agent is essential to ascertain its spectrum of activity against various types of pathogenic organisms. Antimicrobial activity of any lichen, plant or plant parts can be detected by observing the growth response of various microorganisms to the lichen, plant or plant part extracts. In this case lichen extracts have been screened a number of pathogenic bacteria and fungi.

2.3.1.1. *In vitro* antibacterial screening

In general antimicrobial screening *in vitro* is undertaken in the following two steps:

(i) Primary assay

It is essentially a qualitative or semiqualitative test that indicates the sensitivity or resistance of microorganisms to the compound. However, this technique cannot be used to distinguish between bacteriostatic and bactericidal agents (Reiner, 1980). The primary assay can be done in three ways such as-

A. Diffusion method
B. Dilution method and
C. Bioautographic method

Among these methods the disc diffusion method (Bauer *et al.* 1966; Reiner, 1982) is widely acceptable for the preliminary evaluation of antimicrobial activity. It uses different concentrations of the agents absorbed on sterile filter paper discs. There is no standardized method for expressing the results of antimicrobial screening (Ayafar *et al.* 1982). Some investigators use the diameter of the zone of inhibition or the minimum weight of extract that inhibits

the growth of a microorganism. Disc diffusion is essentially a qualitative or semiquantitative test indicating the sensitivity or resistance of microorganisms to the test material. No distinction between bacteriostatic and bactericidal activity can be made by this method (Reiner, 1982). However *in vitro* antibacterial activity tests were done by disc diffusion method.

(ii) Secondary assay

It quantifies the relative potency such as minimum inhibitory concentration (MIC). The lowest concentration of an antimicrobial agent required to inhibit the growth of the microorganisms *in vitro* is referred to as minimum inhibitory concentration (MIC). It is done by serial dilution technique (Reiner, 1980). The MIC measurement was done by dilution technique in this experimentation.

Principle of diffusion method

Diffusion assay (Barry, 1976) is based on the ability of antibiotics to diffuse from a confined source through the nutrient agar gel and create a concentration gradient. If the agar is seeded or streaked with a sensitive organism, a zone of inhibition will result where the concentration exceeds the minimum inhibitory concentration (MIC) for the particular organism.

In this method, measured amount of the test samples are dissolved in definite volumes of solvent to give solutions of known concentrations (μg/ml). The sterile (BBL, Cocksville, USA) filter paper (diameter 5 mm) disc are impregnated with known amounts of the test substances and dried. These test material discs are placed on plates containing a suitable medium (nutrient agar) seeded with the test organisms. These plates are kept at low temperature (4°C) for 24 hours to allow maximum diffusion. A number of events take place simultaneously which includes-

i) the dried discs absorb water from the agar medium and the material under test is dissolved;

ii) the test material diffuses from the discs to the surrounding medium according to the physical law that controls the diffusion of molecules through agar gel; and

iii) there is a gradual change of test material concentration in the agar surrounding each disc.

To determine the most optimal concentration of lichen extract to be used in this study, sterile 7.5 mm filter paper disks were treated with 200 µl chloroform only (used as a control), and 200 µl of each lichen extract. The bacteria were inoculated on full-strength Nutrient Agar (Qualigens Fine Chemicals Prod # 58673) by suspending loops of bacteria in sterile de-ionized water. The bacterial suspension was then smeared on agar plates with a sterile glass-rod to ensure the entire surface of the agar had an even coating of the bacterial suspension. Plates were divided into several areas and one filter paper disk was placed in each area so that each plate had one disk of each treatment (control, 200 µl of lichen extract). Effects of the lichen compounds on bacterial growth were quantified by measuring the diameter of the zones of inhibition less the size of the treated filter paper disks.

The plates are then kept in an incubator (37°C) for 12-18 hrs. to allow the growth of the organisms. If the test material has antimicrobial activity, it will inhibit the growth of microorganisms, giving a clear, distinct zone called 'Zone of Inhibition' Effects of the lichen compounds on bacterial growth were quantified by measuring the diameter of the zones of inhibition in term of mm. The size of the inhibitory zones depends principally on the following factors:

i) Intrinsic antimicrobial sensitivity of the test sample,

ii) Growth rate of the test microorganisms,

iii) Diffusion rate of the freshly seeded test organisms,

iv) Concentration of the freshly seeded test organisms,

v) Amount of test sample on disc,

vi) Thickness of the test medium in the Petri dishes,

vii) Composition of the culture medium,

viii) Inoculum size,

ix) Incubation time,

x) Temperature of incubation,

Test materials used for the study

i) Crude extracts of chloroform
ii) Ciprofloxacin, (10 μg/disc) as standard discs.

Apparatus and reagents

i) Blank sterilized filter paper discs (diameter 5 mm)
ii) Petri dishes (diameter 120 mm)
iii) Test tubes
iv) Inoculating loop
v) Spirit burner and a match box
vi) Sterile forceps
vii) Sterile cotton
viii) Laminar air flow unit (BIOCRAFT & SCIENTIFIC INDUSTRIES, INDIA)
ix) Micropipette (10 μl-100 μl)
x) Autoclave (ALP Co. Ltd. KT- 30L, JAPAN)
xi) Incubator (OSK- 9639A, Japan)
xii) Refrigerator (ARISTON, ITALY)
xiii) Punch machine
xiv) Beaker
xv) Nutrient agar media (DIFCO)
xvi) Solvent (methanol and chloroform) and
xvii) Vials
xviii) Rectified spirit
xix) Alcohol (95%)

2.3.1.1.1. Test organisms used for the antibacterial activity test

Twelve pathogenic bacteria were selected for the test, six of which were Gram negative and the remaining were Gram positive. These organisms of pure culture were collected from the Department of Microbiology, University of Dhaka; ICDDRB, Dhaka and Institute of Nutrition and Food Science (INFS), University of Dhaka and were further cultured at the Molecular Biology Laboratory, Institute of Biological Sciences, Rajshahi University, Rajshahi-6205. The bacterial strains used for this investigation are listed in the Table 3.

2.3.1.1.2. Sterilization procedures

The antibacterial screening was carried out in a laminar airflow unit and all types of precautions were highly maintained to avoid any type of contamination during the test. UV light was switched on for half an hour before working in the laminar hood to avoid any accidental contamination. Petri dishes and other glass-wares were sterilized in the autoclave at 121°C temperature and a pressure of 15 lbs./sq. inch for 15 minutes. Micropipette tips, culture media, cotton, forceps, blank discs, etc were also sterilized.

2.3.1.1.3. Culture media

A number of culture media are available to demonstrate the antibacterial activity. These are-

i) Nutrient agar medium
ii) Nutrient broth medium
iii) Mueller-Hinton medium
iv) Tryptic Soy broth (TSB) medium
v) Trypticase Soy agar medium
vi) *Staphylococcus* defined medium
vii) Adams and Roe medium
viii) NTH agar or broth medium.

Table 6. Composition of nutrient agar medium

Ingredient	Amount
Bactopeptone	0.5 gm
Sodium chloride	0.5 gm
Bactoyeast extract	1.0 gm
Bactoagar	2.0 gm
Distilled water	100 ml
pH	7.2±0.1 at 25°C

For demonstrating the antibacterial activity and subculture of the test organisms the nutrient agar media (DIFCO) was used.

2.3.1.1.4. Preparation of the nutrient agar (DIFCO) medium

The instant nutrient agar (DIFCO) medium was weighed and then reconstituted with distilled water in a conical flask according to specification (2.3% w/v). It was then heated in a water bath to dissolve the agar until a transparent solution was obtained.

2.3.1.1.5. Preparation of fresh culture of the pathogenic organisms

The nutrient agar medium was prepared and dispersed in a number of clean test tubes to prepare slants (5 ml in each test tube). The test tubes were plugged with cotton and sterilized in an autoclave at 121°C and 15 lbs./sq. inch pressure for 15 minutes. After sterilization, the test tubes were kept in an inclined position for solidification. These were then incubated at 37.5°C to ensure sterilization. The test organisms were transferred to the agar slants from the supplied pure cultures with the help of an inoculating loop in an aseptic condition. Burning the loop after each transfer of microorganism was done to avoid contamination very carefully. The inoculated slants were then incubated at 37.5°C for 24 hours to assure the growth of test organisms. These fresh cultures were used for the sensitivity test.

2.3.1.1.6. Preparation of the test plates

The test plates were prepared according to the following procedure.

(i) The nutrient agar medium prepared in the previous section was poured in 15 ml quantity in each in the clean test tubes and plugged with cotton.

(ii) The test tubes and a number of petri dishes were sterilized in an autoclave at 121°C and 15 lbs/sq. inch pressure for 15 minutes and were transferred into laminar airflow unit and then allowed to cool to about 45°C to 50°C.

(iii) The test organism was transferred from the fresh subculture to the test tube containing 15 ml autoclaved medium with the help of an inoculating loop in an aseptic condition. Then the test tube was shaken by rotation to get a uniform suspension of the organism.

(iv) The bacterial suspensions were immediately transferred to the sterile petri dishes in an aseptic area. The petri dishes were rotated several times, first clockwise and then anticlockwise to assure homogenous distribution of the test organisms. The media were poured into petri dishes in such a way as to give a uniform depth of approximately 4 mm.

(v) Finally, after medium was cooled to room temperature in laminar airflow unit, it was stored in a refrigerator (4°C).

2.3.1.1.7. Preparation of discs containing samples

For the preparation of discs containing samples, following procedure was utilized:

(a) Sample discs

Sterilized filter paper discs (5 mm in diameter) were taken by the forceps in to the plates. Sample solutions of desired concentrations were applied on the discs with the help of a micropipette in an aseptic condition. These discs were left for a few minutes in aseptic condition for complete removal of the solvent.

(b) Standard discs

These were used to compare the antibacterial activity of the test material. In the present study, Ciprofloxacin discs containing 10 μg/disc of antibiotic Ciprofloxacin were used as standard discs for comparison purpose.

2.3.1.1.8. Placement of the discs and incubation

For the placement of the discs, the following procedure was utilized:

(i) By means of a pair of sterile forceps, the sample impregnated discs were placed gently on the solidified agar plates seeded with the test organisms to ensure contact with the medium.

(ii) The plates were then kept in a refrigerator at 4°C for 24 hours in order to provide sufficient time to diffuse the antibiotics into the medium.

(iii) Finally, the plates were incubated at 37.5°C for 24 hours in an incubator.

Precaution: The discs were placed in such a way that they were not closer than 15 mm to the edge of the plate and for enough apart to prevent overlapping the zones of inhibition.

2.3.1.1.9. Measurement of the zones of inhibition

After incubation, the antibacterial activities of the test samples were determined by measuring the diameter of inhibitory zones in term of mm with a transparent scale.

2.3.1.2. *In vitro* antifungal screeing

The increasing incidence of opportunistic systemic mycoses in man and the associated therapeutic difficulties require the search for new antifungal drugs. Plants derived compounds may offer potential leads for novel agents against systemic fungal diseases (Hufford and Clark, 1988).

2.3.1.2.1. Test materials used for the study

Chloroform extracts of 13 lichen samples were used for the investigation of antifungal activity. The extracts were dissolved in their solvent of extraction and used at a concentration of 500 μg/disc. For a better correlation of the antifungal activities Nystatin 50 μg/disc was used as a standard.

2.3.1.2.2. Test organisms used for the study

The fungal strains used in the sensitivity test are given Table 4, while the pure cultures of the strains were collected from the Institute of Biological Science (I.B.Sc.), University of Rajshahi, Rajshahi- 6205, Bangladesh.

2.3.1.2.3. Culture media

Potato dextrose agar (PDA) medium was used to perform the antifungal activity test and for subculture of the test organisms. The composition of the medium is as follows:

Table 7. Composition of PDA medium

Ingredient	Amount
Potato	20.0 gm
Dextrose	2.0 gm
Agar	1.5 gm
Distilled water	100.0 ml

2.3.1.2.4. Preparation of the medium

The constituents of the medium was accurately weighed and dispersed in a conical flask with distilled water. It was heated in water bath to dissolve the ingredients until a transparent solution was obtained. The pH of the medium was adjusted to 5.6. The volume was adjusted by adding distilled water and sterilized in an autoclave at 121°C and 15 lbs/sq-inch pressure for 15 minutes.

2.3.1.2.5. Preparation of the test plates

The test plates were prepared according to the following procedure:

(i) About 10 ml in quantity of distilled water was poured in several clean test tubes and plugged with cotton.

(ii) The test tubes, a number of Petri dishes, glass rods, a piece of cotton and the medium were sterilized by autoclave and then transferred to the laminar air flow cabinet.

(iii) About 6 ml of the medium was poured carefully in the medium sized Petri dishes in each. The petri dishes were rotated several times, first clockwise and then anticlockwise to assure homogenous thickness of the medium and allowed to cool and solidify at about 30°C.

(iv) The test tubes containing distilled water were inoculated with fresh culture of the test fungi and were shaken gently to form a uniform suspension of the organism because of their high prevalence sporulation process.

(v) A piece of cotton was immerged in the test tubes with the help of individual glass rod and then gentle rubbed the medium and the cotton was discarded.

(vi) Finally, the plates were stored in a refrigerator (4°C) for overnight.

2.3.1.2.6. Preparation of sample containing discs

For the preparation of discs containing crude extracts of chloroform, following procedure were utilized.

(a) Sample discs

Sterilized filter paper discs (5mm in diameter) were taken to the plates with the help of forceps. Crude extracts of chloroform (500 µg/disc) were applied on the discs with the help of a micropipette in an aseptic condition. These discs were left for a few minutes in aseptic condition for complete removal of the solvent.

(b) Standard discs

These were used to compare the antibacterial activity of the test material. In the present study, ready-made Nystatin 50 µg/disc were used as standard disc for comparison purpose.

2.3.1.2.7. Placement of the discs and incubation

i. By means of a pair of sterile forceps, the dried crude extract discs and standard disc were placed gently on the solidified agar plates seeded with the test organisms to ensure contact with the medium.

ii. The plates were then kept in a refrigerator at 4°C for 24 hours in order to provide sufficient time to diffuse the antibiotics into the medium.

iii. Finally, the plates were incubated at 37.5°C for 24 hours in an incubator.

Precaution: The discs were placed in such a way that they were not closer then 15 mm to the edge of the plate and for enough apart to prevent over lapping the zones of inhibition.

2.3.1.2.8. Measurement of the zones of inhibition

After incubation, the antifungal activities of the test samples were determined by measuring the diameter of inhibitory zones in term of mm with a transparent scale. Preparation of the discs, test samples, standard sample and placement of the discs, diffusion, incubation and measurement of zone of inhibition procedures were almost the same with the antibacterial screening. Here, only the incubation period was for 48 hours at the room temperature.

2.3.1.3. Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) is the lowest concentration of the test sample or drug at which it shows the highest activity against the growth of the pathogenic microorganisms. There are two methods for determining the MIC. They are as follows:

a. Serial tube dilution technique or agar turbidimetric assay, and
b. Paper disc plate technique or agar diffusion assay.

Here 'Serial tube dilution technique' (Reiner, 1982) was followed using nutrient broth medium and potato dextrose medium to determine the MIC values of extracts against the following pathogenic bacteria and fungi.

2.3.1.3.1. Principle of serial tube dilution technique

The tubes of broth medium, containing graded doses of extracts are inoculated with the test organisms. After suitable incubation, growth will occur in those inhibitory tubes where the concentration of test samples is below the inhibitory level and the culture will become turbid. Therefore, growth will not occur above inhibitory level and the tube will remain clear through the large number of microorganisms present in the tubes.

2.3.1.3.2. Preparation of inoculum

Fresh cultures of the test organisms were grown at 37°C for overnight on nutrient agar medium. Bacterial suspensions were then prepared in sterile nutrient broth medium in such a manner so that the suspension contains 10^7 cells/ml, which was confirmed by O.D. (0.5) measurement. The suspension was used as the inoculum.

2.3.1.3.3. Preparation of the sample solution

Each of the extracts (chloroform extract) was taken in different vials at a fixed amount (2.048 mg). Then broth medium (2 ml) was added to each of the vials and agitated well to make sample solution whose concentration becomes 1024 μg/ml. The standard antibiotic Ciprofloxacin solution (Reiner, 1980) was made in the same procedure as the concentration became 512 μg/ml.

2.3.1.3.4. Procedure of experiments

i. Twelve autoclaved test tubes were taken, of which nine were marked 1, 2, 3, 4, 5, 6, 7, 8, 9 and the rest then were assigned as C_M (medium), C_S (medium + sample) and C_I (medium + inoculum).

ii. Nutrient broth medium (1 ml) was poured to each of the 12 test tubes.

iii. These test tubes were cotton plugged and sterilized in an autoclave for 15 minutes at 121°C temperature and 15 lbs/sq. inch pressure.

iv. After cooling, 1 ml of this content was transferred to the 1st test tube and mixed well and then 1 ml of this content was transferred to the second test tube.

v. The content of the second test tube was mixed well and again 1 ml of this mixture was transferred to the third test tube. This process of serial dilution was continued up to the ninth test tube.

vi. 10 μl of properly diluted inoculum was added to each of the nine test tubes and mixed well.

vii. To the control test tube C_S 1 ml of the sample was added, mixed well and 1 ml of this mixed content was discarded to check the clarity of the medium in presence of diluted solution of the sample.

viii. Inoculum (1 μl) was added to the control test tube C_1, to observe the growth of the medium.

ix. All the test tubes were incubated at 37°C for 18-24 hrs.

2.3.2. Cytotoxicity test

2.3.2.1. Cytotoxicity test through brine shrimp lethality bioassay

Test samples are prepared by the addition of calculated amount of DMSO (dimethyl sulfoxide) for obtaining desired concentration of test sample. The nauplii are counted by visual inspection and are taken in vials containing 5 ml of sea-water. Then samples of different concentrations are added to the premarked vials through micropipette. The vials are left for 24 hours and then the nauplii are counted again to find out the cytotoxicity of the test agents and compared to the results with positive control.

2.3.2.2. Experimental design

Test materials

(i) *A. salina* Leach (brine shrimp eggs)
(ii) Sea salt (non ionized NaCl)
(iii) Small tank with perforated dividing dam to hatch the shrimp
(iv) Lamp to attract the nauplii
(v) Pipette (1 ml and 5 ml)
(vi) Micropipette (10-200 µl adjustable)
(vii) Glass vials (5 ml)
(viii) Magnifying glass

2.3.2.3. Preparation of simulated sea water (brine water) and hatching of brine shrimp nauplii

Since the lethality test involves the culture of brine shrimp nauplii, i.e. the nauplii should be grown in the sea water and the sea water contains 3.8% of sodium chloride. Accordingly 3.8% sodium chloride solution was made by dissolving sodium chloride (38 gm) in distilled water (1000 ml) and was filtered off. The P^H of the brine water thus prepared was maintained between 8 and 9 using $NaHCO_3$.

2.3.2.3.1. Hatching of brine shrimp

Brine water was taken in a small tank and shrimp eggs (1.5 gm/l) were added to one side of the perforated divided tank with constant oxygen supply. Constant temperature (37°C) and sufficient light were maintained to give the sufficient aeration. After 48 hours, matured shrimp as nauplii (larvae) was collected and used for the experiment.

2.3.2.4. Preparation of sample solution

Chloroform extracts of the lichen samples h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11, h12 and h13 were taken into consideration. For each of the samples 320 μg were initially dissolved in 100 μl of pure dimethyl sulfoxide (DMSO) to make them hydrophilic before adding 1.9 ml of water to get a concentration of 320 μg/ml for each, which was used as stock solution-A. Then a series of following concentrations was made from the stock solution-A of each of the samples: 160 μg/ml, 80 μg/ml, 40 μg/ml, 20 μg/ml and 10 μg/ml. From the stock solution A, 1ml was taken (that contains 160μg) and diluted with up to 2 ml of brine water to obtain a concentration of 80 μg/ml and this is indicated a stock solution-B from this stock solution-B different concentrations of each of the samples were made by serial dilution method.

2.3.2.5. Preparation of the control group

For each concentration, one vial containing the same volume of DMSO diluted up to 10 ml with sea-water and 30 shrimp nauplii was used as negative control group. It was used to verify the validity of the test. When the nauplii in the control showed a rapid mortality, then the test is considered to be invalid as the nauplii might die due to reasons other than the cytotoxicity of the compounds.

2.3.2.6. Application of doses to the brine shrimp nauplii

In each of the five vials, 10 ml brine shrimp solution (3.8%) was taken, containing 30 brine shrimp nauplii with the help of a micropipette specific volume of each sample were transferred from the stock solution-B to the respective vials to get, final concentrations of 10 -, 20 -, 40 -, 80 and 160 µg/ml. The volumes of DMSO in these vials should not exceed 10 µl/ml of the brine solution, because above this concentration toxicity due to DMSO may arise.

2.3.2.7. Observation of mortality

After 24 hours, the vials were observed. The number of survived nauplii in each vial was counted and the results were noted. From this, percentage of mortality of brine shrimp nauplii was calculated at each concentration for each sample and the results are given in Table 14 in the 'Chapter 3 Results'.

2.3.2.8. Analysis of data

The dose mortality data were analyzed statistically by Probit analysis. The effectiveness or the dose mortality relationship (concentration-mortality relationship) of plant product is usually expressed as a median lethal concentration (LC_{50}) value. This represents the concentration of the chemical that produces death in half of the test subjects after a certain exposure time.

2.3.3. Experiments for repellent activity of the extracts

In many areas of Africa and Asia, locally available plants and minerals are being widely used to protect stored products against damage by insect infestation, as an alternative to synthetic pesticides (Golob and Webley, 1980: Su *et al.*, 1972; Ahmed and Koppel, 1985; Khalique *et al.*, 1988.). Recently, other resources have been targeted for the investigation of bioactive compounds, such as, fungi, algae, lichens, epiphytic plants, etc. In this case lichens have been taken as a source of functional biomolecules. The repellency test used was adopted from the method (No. 3) of McDonald *et al.*, (1970) with some modifications by Talukder and Howse (1993, 1994).

2.3.3.1. Preparation of doses with the crude extracts for the repellency test

A general concentration for each of the extracts was selected as stock dose for repellent activity test application to make other successive doses by serial dilution to give 314.54-, 157.27-, 78.63-, 39.32-, 19.66- and 9.83 $\mu g/cm^2$ concentrations for lichen extract, however, all these doses were not applicable for all the test samples h1 to h13.

2.3.3.2. Application of doses in the repellency test

Half filter paper discs (Whatman No. 40, diameter 9 cm) were prepared and selected doses of all the $CHCl_3$ extract separately applied onto each of the half-disc and allowed to dry out as exposed in the air for 10 minutes. Each treated half-disc was then attached lengthwise, edge-to-edge, to a control half-disc with adhesive tape and placed in a petri dish (diameter 9 cm). The orientation of the same was changed in the replica to avoid the effects of any external directional stimulus affecting the distribution of the test insects. Twenty adult insects were released in the middle of each filter-paper circle. Each concentration was tested five times. Insects that settled on each half of the filter paper disc were counted after 1 h and then at hourly intervals for 5 h. No significant difference was detected between the repellency of only solvent impregnated and untreated filter papers in tests designed to check for any possible influence of $CHCl_3$. The average of the counts was converted to percentage repellency (*PR*) using the formula of Talukder and Howse (1993, 1995):

$$PR = 2(C - 50),$$

Where, C is the percentage of insects on the untreated half of the disc. Positive values expressed repellency and negative values for attractant activity.

2.3.3.3. Observation and analysis of repellency data

Repellency was observed for one-hour interval and up to five successive hours of exposure, just by counting the number if insects in the treated and non-treated part of the filter paper spread on the floor of the 90mm petri dish. The values in the recorded data were then calculated for percent repellency, which was again developed by arcsin transformation for the calculation of ANOVA.

2.3.4. Dose mortality tests on insect larvae

2.3.4.1. Dose mortality test on *T. castaneum* larvae

Effect of toxicity of the lichen sample h10 (host plant *B. flabellifer*) against *T. castaneum* larvae was assessed by observing their chronic action on any stage of the beetles' life span. The selected food medium (1 g of whole wheat flour in a vial for each dose) was treated with different doses of the extracts to release selected number of larvae in each of the units. Changes in all the developmental stages were observed from time to time, and mortality if occurred was recorded. Any sort of anomaly in their growth was also taken into consideration.

2.3.4.2. Dose mortality test on *M. domestica* larvae

The experiment for larvicidal test on *M. domestica* larvae is not the same since the feeding is different. To test the chloroform extracts of the lichen samples against the larvae of *M. domestica* of same age were used provided with its food as a unit of volume-by-volume measurement. The food was prepared with 6.25 g of wheat bran and 0.5 g of milk powder (Red Cow) and 12 ml of water as a total of 19.5 g. For lichen extracts 250 mg was dissolved in 1 ml of solvent (chloroform) and mixed with the prepared food, however, being volatile the solvent was evaporated out shortly. To have a dose-effect to calculate toxicity by probit analysis 4 other successive doses were prepared and applied with a serial dilution of ½ successively. Thus the concentration of the extract in the food medium was calculated as 250-, 125-, 62.5-, 31.25 and 15.615 mg/g. For each of the tests ten larvae were released in the treated food medium and 3 replications were set for each of the doses. The data was read after 24 h of exposure.

2.3.4.3. Statistical analysis of the dose-mortality effects on *T. castaneum* and *M. domestica* insect larvae

The mortality records of the dose mortality experiments done on the larvae of *T. castaneum* and on *M. domestica* was corrected by the Abbott's (1925) formula:

$$P_r = \frac{P_o - P_c}{100 - P_c} \times 100$$

Where,

P_r = Corrected mortality (%)

P_o = Observed mortality (%)

P_c = Control mortality (%), sometimes called natural mortality (%).

Then mortality percentages were subjected to statistical analysis according to Finney (1947) and Busvine (1971) by using software developed in the Department of Agricultural Environmental Science, University of Newcastle upon Tyne, U.K. The dose-mortality relationship was expressed as a median lethal dose (LD_{50}).

2.4. Chromatographic techniques used in this investigation

2.4.1. Chromatography on TLC plates

Thin layer chromatography is a very convenient technique for finding the separation slurry along with its stationary phase. The mixtures of the compounds were well separated from each other and resolved by preparative thin layer chromatographic technique. This tool is considered to be one of the most helpful methods of the detection of organic compounds, which involves an adsorbent (using silica gel) as stationary phase and a solvent system as a mobile phase. Due to the differential rate of adsorption on the adsorbent, the components in a mixture migrate differentially along with he TLC plate. In other words due to the difference in mobility of the components often depend on their polarity and that of the solvents used.

To select the solvent system for the run of the open column separation was made on the preparative thin layer chromatographic plates. For the normal phase chromatography silica gel G60 F_{254} on Al sheets (Merck) were used. Ten mg/ml of he sample in the solvent extraction offered 100µl/spot by spotting 10µl for each of he sample extracts. The chromatograms then developed within a conventional camber with the following solvent systems: Pet. ether-EtOAc (1:1). All chromatograms were observed under UV at 254 and 366 nm and marked with a pencil.

2.4.1.1. Detection of the compound on TLC by Godin revelation

The properly developed plates were dried and viewed visually under UV light and Godin reagent (Godin, 1954) spray were used and number of compounds separated.

Visual detection: The development chromatogram was examined visually to detect the presence of colored compound.

I) UV light (254 and 366 nm): After development and drying the chromatogram was examined under UV light to detect fluorescent compound and the glowing spots, which were then marked.

II) Godin reagent spray: Equal volumn of 1% ethanolic solution of vanillin and 3% aqueous solution of perclorie acid was mixed sprayed on to the prepared chromatogram and 10% ethanolic solution of H_2SO_4 was also sprayed afterwards and allowed the plate to dry out at 100°C by using a hair dryer. Revelation was observed in different colors for different compounds (Godin, 1954).

III) Measurement of R_f values: The R_f values of he separated compounds were calculated on the developed chromatogram using the pre-established solvent system. The R_f values were calculated by the following formula.

$$R_f = \frac{\text{Distance traveled by the compound}}{\text{Distance traveled by the solvent}}$$

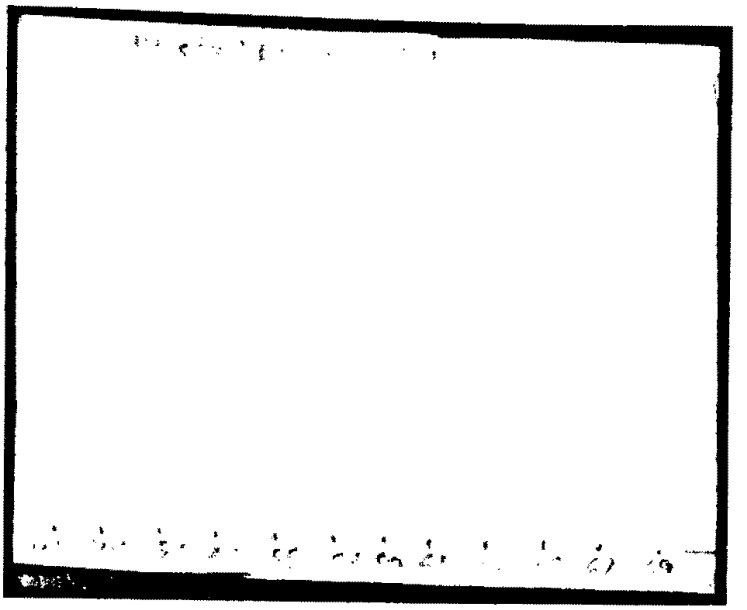


Plate 20. Revelation of compound spots by reagent spray

2.4.2. Open column chromatography

Of the methods in the solid phase category, column chromatography is very popular and used extensively. It can include non-exchange resins, polymeric columns, gel-filtration and chromatography over silica gel or chemically modified silica gel. Open column chromatography has a high load capacity but the separation time is long and the resolution is respectively low.

The stationary phase for the open column chromatography was silica gel Si60 (70-230 mesh and 230-400 mesh) (Merck) and glass column of different size (2.5×28 cm, 2.5×32 cm, 3×35 cm, 3.25×48 cm, etc) were used. Cotton pads washed with acetone, chloroform and methanol was used at the base of the gel column. A similar cotton cloud was used at the top of the column (after application of the sample and the solvent) to protect destruction of the sample layer (Plate 21). Selected solvent system were used as eluents and the elution rate was 1 ml/min. Fractions were collected carefully.

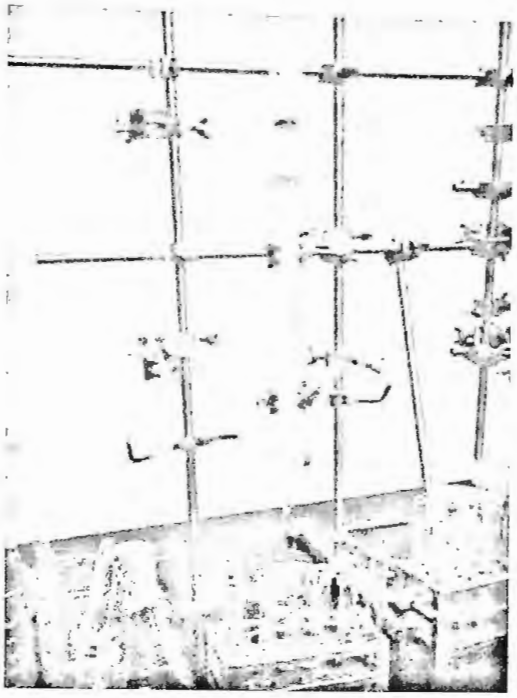


Plate 21. Open column used in the experiment.

2.4.3. Gel filtration:

Open column were used to apply sephadex LH-20 (Pharmacia) for the chromatography of exclusion. For methanol soluble samples MeOH (100%) and for $CHCl_3$ soluble samples $CHCl_3$-MeOH (1:1) systems were used. The eluent allowed about 0.5 ml/min.

2.5. Preparative separation techniques

Chromatography is an analytical technique for separating compounds on the basis of differences in affinity for a stationary and mobile phase. The separation of pure constituents from plant materials chromatography is a popular technique. The aim of choice any technique for separation is to have maximum yield with minimum effort to reduce the time and cost of the separation procedure. Preparative separation techniques can be tedious and time consuming, especially when complex mixtures, such as crude plant extracts have to be resolved. In the present study for isolation of pure compounds from lichens were done mainly by open column chromatography, while thin layer chromatography (TLC) and gel filtration were used as supporting tools.

2.5.1. Selection of extracts for fractionation

All the lichen extracts were subjected to bioassay experiments to trace presence of bioactive potentials if present there in. A number plant pathogenic bacteria and fungi (Table 3 and 4) were taken in this regard. The test samples were made using all the lichen extracts concentration of 10 mg/ml to give a concentration through which it was possible to diversified doses from 10 μg/μl. Paper discs treated with 200 μg of these samples were used to detect biological activity of the extracts. Potato dextrose agar (PDA) medium was used to perform these antimicrobial activity tests through disc diffusion method. Clear zones were observed against a dark background that had been produced by the fungus itself. The bioassay results indicated promising biological activity in the chloroform extracts of the lichen samples h4, h10 and h13; however there were other active extracts relatively weaker in activity against these bacterial and fungal isolates.

2.5.2. Selection of slurry (solvent system) for respective extracts

Aluminiun backed precoated preparative thin layer chromatographic (TLC) plates (20x20cm) with silica gel GF_{254} with 0.5mm thickness and active in the usual manner (Merck, Germany) were used. The sample was applied on the activated plates with the help of a gradient micropipette as a narrow band at 1cm above the lower edge of the plate to make sure that the sample was washed away when the plates were placed inside the TLC chamber with the solvent system. The plates were then developed in the usual manner.

After development, the chromatograms were air dried and observed visually under UV light (254nm) and sprayed with Godin reagent (Godin, 1954). The distinct bands were expected and by changing the solvent system with increase of either the polar or the apolar one. After having a better separation the selected solvent system was applied on the open column for isolation the compounds by fractionation. Small pieces of aluminium backed TLC plate was taken to spot the target extracts and run with a mixture of a relatively polar and relatively apolar solvent (1:1). For the better separation on he TLC with a

known stationary phase the amount of both solvents were increased or decreased and applied accordingly. The combination given a better separation was selected for that extract for fractionation on the open column. However, no choice was for the gel filtration (LH_{20}) if it was chloroform soluble property then $CHCl_3$-MeOH (1:1) was applied. If any fraction found soluble only in MeOH then 100% MeOH was the eluent for that fraction.

2.5.3. Isolation of the lichen compounds

2.5.3.1. Isolation of the lichen compound LMI-1

For the first fractionation the extract of the lichen sample h4 (host plant *M. indica*) was taken into consideration for gel filtration, since through this process the compounds get separated depending on the molecular size. However, the sample was found partially soluble in this slurry $CHCl_3$ - MeOH 1:1, only the soluble part was applied onto the sephadex column with the slurry mentioned above. Three fractions were found as Fr. I (T-1-25), Fr. II (T26-38) and Fr. III (T39-52). The insoluble part of the extract was then subjected to fractionation by using $CHCl_3$ (100%) on a glass column of 17.5 × 2 cm packed with silica gel (60-200 mesh/ Sigma). The elution time was adjusted to yield 1ml/min to yield seven fractions, Fr. IV (T1-21), Fr. V (T23-36), Fr. VI (T37-63), Fr. VII (T64-85), Fr. VIII (T86-96), Fr. IX (T97-98) and Fr. X (T99-120). Biological assay revealed Fr. V to contain bioactive components and it was hen subjected to fractionation. Petroleum Benzine and $CHCl_3$ (1:1) was applied on a glass column of 17.5 × 2 cm packed with silica gel (60-200mesh/ Sigma). The elution was kept similar to that of the previous one and it yielded three sub fractions SFr. I (T1-109), SFr. II (T110-146) and SFr. III (147-175) and the second fraction SFr. II gave yellow crystal needles as a pure compound, which was tested on TLC under UV and Godin reagent spray to give Golden color. The compound was named as **LMI-1.**

2.5.3.2. Isolation of lichen compound LBF-2

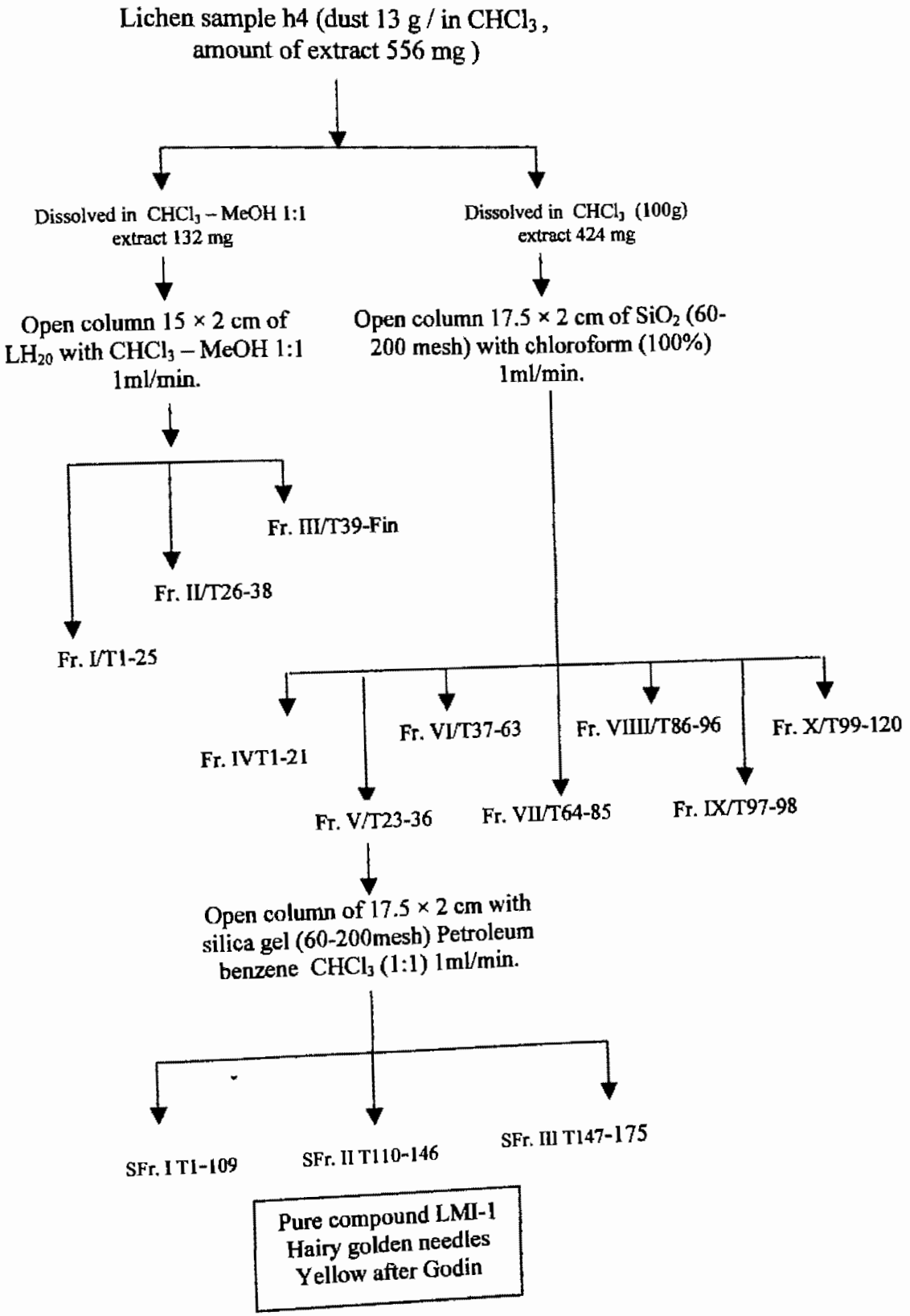
The lichen sample h10 (host plant *B. flabellifer*) was taken into consideration for gel filtration. For this fractionation LH_{20} (pharmacia) was used as the stationary phase and $CHCl_3$-MeOH (1:1) was the eluent on a glass column of 39x3 cm with an elution time 1 ml/min to give six fractions, Fr. I (T/1-15), Fr. II (T/16-35), Fr. III (T/36-60), Fr. IV (61-75), Fr. V (T76-80) and Fr. VI (T81-89). Biological assay with bacteria indicated Fr. VI for the presence of bioactive constituents there in and it was again subject to fraction. Petroleum benzene and $CHCl_3$ (1:1) was applied on a glass column of 2.5×32 cm packed with silica gel (70-230 mesh/ Sigma). This fractionation yielded three sub fractions SFr I (T/1-34), SFr. II (T/35-62) and SFr. III (T/63-90) and the SFr. II gave golden crystal needles as a pure compound which was tested on TLC under UV and Godin reagent spray to give golden color as compound LBF-2.

2.5.3.3. Isolation of lichen compounds LMI-3 and LMI-4

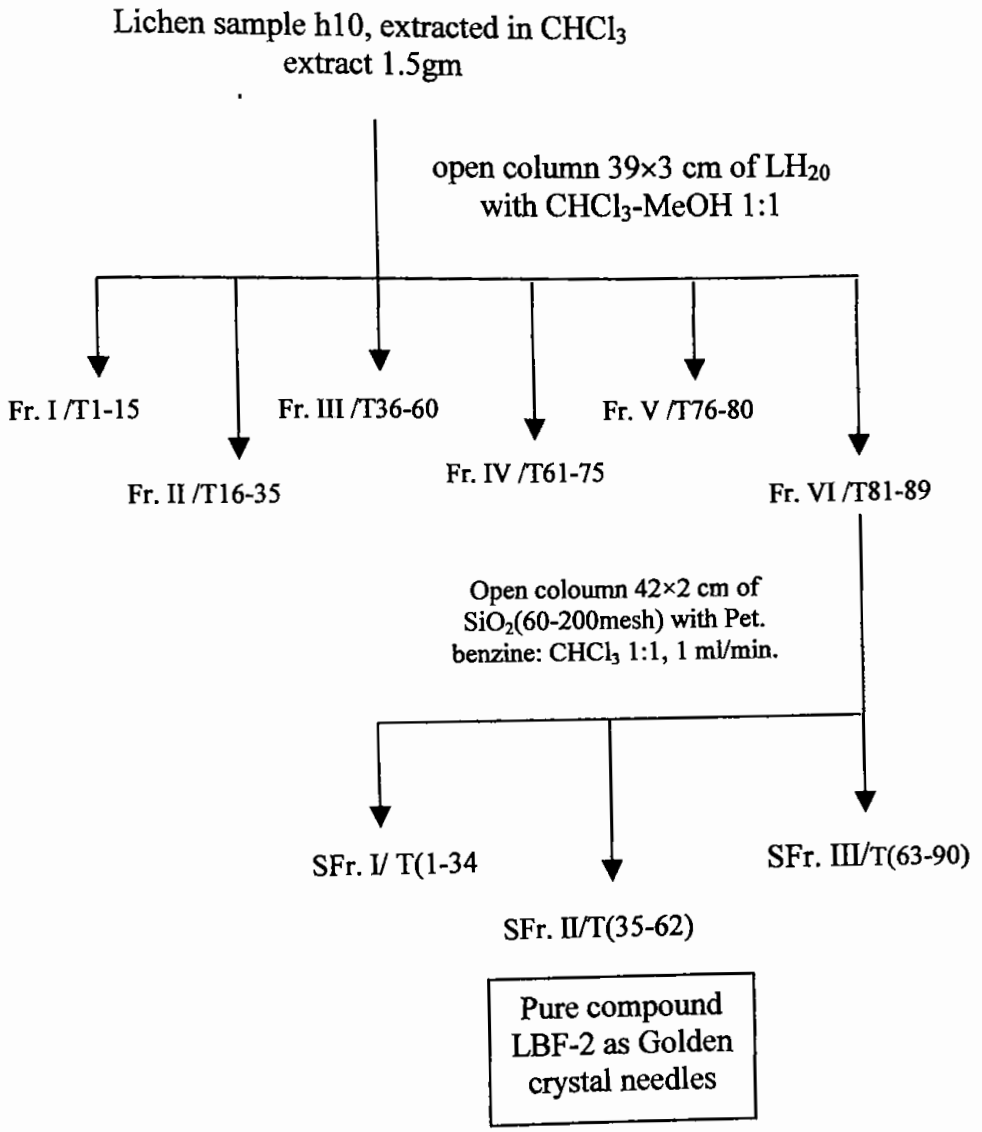
Similar to that of the above two fractionation this time for the extract of the lichen sample h13 (host plant *Mangifera indica*) an open column of LH_{20} (pharmacia) was used as the stationary phase and $CHCl_3$-MeOH (1:1) was the eluent on a glass column of 39 × 3 cm for 360 mg of the extract. Elution time was adjusted to yield 1ml/min to give three fractions Fr. I (T/1-16), Fr. II (T 17-47) and Fr. III (T/48-72). Biological assay with bacteria indicated Fr. II and Fr. III for the presence of bioactive compounds there in and those were then subjected to fractionation. The second fraction Fr. II was applied on a glass column of 42 × 2 cm packed with silica gel (60-200 mesh/ Sigma) with a slurry of $CHCl_3$: Acetone (7:3) and the elution was kept 1 ml/min. This fractionation yielded 4 fractions as Fr. I (T/1-15), Fr. II (T/16-34), Fr. III (T/35-52) and Fr. IV (T/53-58). Biological assay indicated the Fr. III for the presence of bioactive compounds and this was then subjected to fractionation with a glass column of 42 × 2 cm was packed with silica gel (60-200mesh) and eluted with acetone (100%) to give 15 tubes, of which 7 to 13th tube contained the pure compound (LMI-3) as white crystals. The solvent system $CHCl_3$-Acetone (7:3) has been used to elute the Fr. III on a column of silica gel (60-200 mesh) and the elution time was 1ml/min. This fractionation yielded five sub fractions SFr. V (T/1-7), SFr. VI (T8-10), SFr. VII (T/11-23), SFr. VIII (T/24-50) and SFr. IX (T/51-54), of which SFr. VII was a pure compound.

2.6. Isolation pathway (flow chart) of the compounds purified

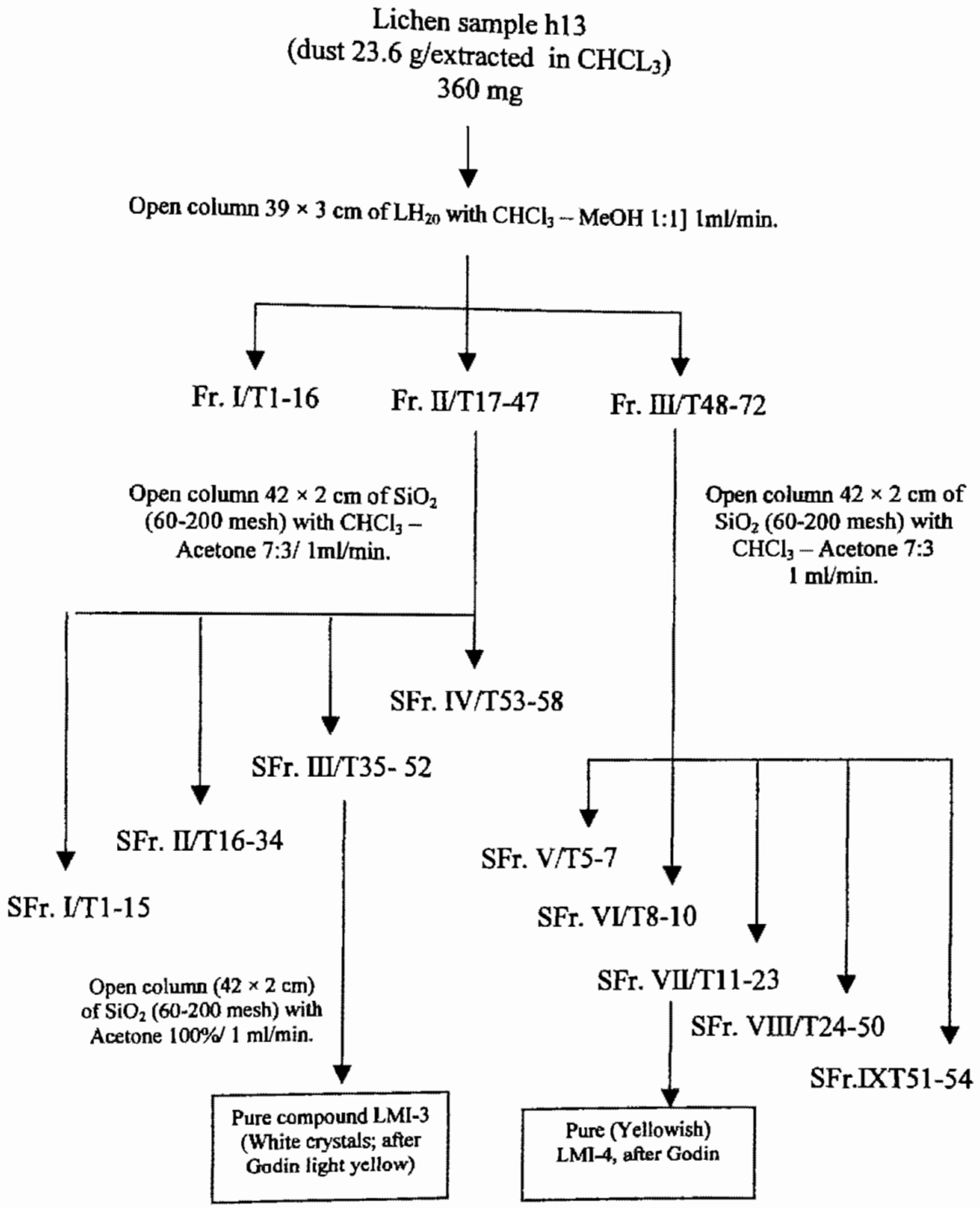
2.6.1. Pathway of isolation of the found compound LMI-1



2.6.2. Pathway of isolation of the found compound LBF-2



2.6.3. Pathway of isolation of the found compound LMI-3 and LMI-4



Chapter 3

RESULTS

3.1. Output extracts

3.2. Antimicrobial activity of the lichen extracts

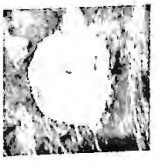
3.3. Cytotoxic effect of the lichen extracts against *A. salina* nauplii through dose-mortality assay

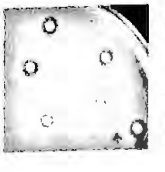
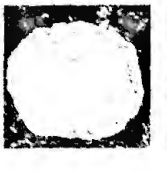
3.4. Repellent activity of lichen extracts against *T. castaneum* adults

3.5. Dose mortality effects of lichen extracts

3.6. Purification of bioactive lichen compounds






3. Results

3.1. Output extracts

Collective amounts of the lichen samples and the extracts were more or less sufficient to go through testing for biological activities and fractionation for the isolation of bioactive compounds. However, depending on biological activity of the extracts, as well as available facilities led to isolate compounds from three of the collections. An extract checklist including percent collection is given below:

Table 8. A checklist of output extracts with per cent collection

Sl. #	Lichen samples (Name of host plant)	Color	Solvent used	Wt. of dust (g)	Wt. of extract (g)	Per cent collection
1.	h1 (*A. catechu*)	Grey	$CHCl_3$	8	0.328	4.10
2.	h2 (*A. rohituka*)	Grey	$CHCl_3$	5	0.267	5.34
3.	h3 (*A. heterophyllus*)	Greenish	$CHCl_3$	9	0.339	3.77
4.	h4 (*M. indica*)	Grey	$CHCl_3$	49	0.957	1.95
5.	h5 (*A. chinensis*)	Grey	$CHCl_3$	5	0.203	4.06
6a.	h6 (*L. chinensis*)	Grey	$CHCl_3$	27.6	0.845	3.06
6b.	h7 (*L. chinensis*)	Greenish	$CHCl_3$	20.5	0.232	1.13
7.	h8 (*D. peregrina*)	Yellow	$CHCl_3$	5.5	0.171	3.11
8.	h9 (*S. chelonioides*)	Grey	$CHCl_3$	5.5	0.136	2.47
9.	h10 (*B. flabellifer*)	Yellow	$CHCl_3$	61	1.684	3.76
10.	h11 (*C. grandis*)	Grey	$CHCl_3$	10	0.270	2.70
11.	h12 (*L. coromandelica*)	Grey	$CHCl_3$	15.7	0.333	2.12
12.	h13 (*M. indica*)	Grey	$CHCl_3$	39.6	0.535	1.35

3.2. Antimicrobial activity of the lichen extracts

3.2.1. Antibacterial activity (*in vitro*) of the lichen extracts

Crude extracts of the lichen samples were subjected to screen against a number of plant and animal (including human) pathogenic Gram positive and Gram negative bacteria with the sample dose 200 μg/disc of each of the extracts and their efficacy against the test bacteria considering the size of the clear-zones were recorded in Table 9.

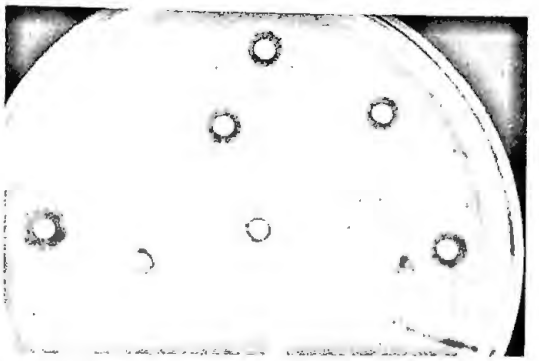


Plate 22. Antibacterial activity by disc diffusion method

From the data it is obvious that lichen h5 and h10 extracts are comparatively better effective against both Gram positive and Gram negative bacteria. However, lichens hosted on h9 and h13 showed lowest activity in general. The inhibition zones against *S. aureus* were 18-, 12-, 18-, 12-, 24-, 12-, 10-, 12-, 0-, 24-, 10-, 7-, 14-, 0- and 35 mm for the extracts of the lichen samples h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11, h12 and h13 along with the control (the only solvent chloroform) and the standard (Ciprofloxacin) respectively. Against *B. megaterium* the inhibition zones were 12-, 15-, 15-, 0-, 20-, 10-, 10-, 12-, 0-, 20-, 10-, 0-, 7-, 0- and 35 mm; against *B. subtilis* the inhibition zones were 15-, 12-, 12-, 0-, 18-, 10-, 8- 10-, 0-, 15-, 9-, 8-, 9-, 0- and 40 mm; against *B. cereus* the inhibition zones were 13-, 10-, 14-, 7-, 20-, 11-, 9-, 12-, 7-, 19-, 8-, 7-, 12-, 0- and 42 mm; against *S.- β-haemolyticus* the inhibition zones were 15-, 13-, 15-, 7-, 20-, 10-, 7-, 9-, 0-, 15-, 10-, 8-, 10-, 0- and 38 mm; against *S. lutea* the inhibition zones were 16-, 13-, 20-, 0-, 22-, 7-, 0-, 7-, 7-, 21-, 13-, 9-, 12-, 0- and 38 mm; against *S. typhi* the inhibition zones were 21-, 18-, 13-, 12-, 23-, 15-, 17-, 17-, 0-, 23-, 17-, 15-, 17-, 0- and 40 mm; against *S. dysenteriae* the inhibition zones were 17-, 14-, 14-, 7-, 18-,

10-, 12-, 12-, 0-, 0-, 11-, 15-, 10-, 0- and 42 mm; against *S. shiga* the inhibition zones were 14-, 11-, 10-, 7-, 20-, 8-, 11-, 15-, 0-, 18-, 9-, 8-, 12-, 0- and 42 mm; agaisnt *S. sonnei* the inhibition zones were 15-, 13-, 16-, 11-, 28-, 13-, 12-, 12-, 0-, 13-, 9-, 7-, 12-, 0- and 38 mm and against *S. boydii* 25-, 20-, 19-, 13-, 26-, 17-, 22-, 21-, 12-, 25-, 19-, 16-, 22-, 0- and 38 mm for the extracts in the aforesaid order, the solvent and the standard (Ciprofloxacin) respectively (Table 9); however, against *E. coli* no inhibition zones were traced with the same doses maintained.

Table 9. Efficacy of the lichen extracts against pathogenic bacteria

Sl.	Lichen samples (Sensitivity for 200µg of extracts /24h)	Gram positive bacteria						Gram negative bacteria					
		S. aureus	*B. megaterium*	*B. subtilis*	*B. cereus*	*S. β- haemolyticus*	*S. lutea*	*S. typhi*	*S. dysenteriae*	*S. shiga*	*S. sonnei*	*S. boydii*	*E. coli*
1.	h1	18	12	15	13	15	16	21	17	14	15	25	0
2.	h2	12	15	12	10	13	13	18	14	11	13	20	0
3.	h3	18	15	12	14	15	20	13	14	10	16	19	0
4.	h4	12	0	0	7	7	0	12	7	7	11	13	0
5.	h5	24	20	18	20	20	22	23	18	20	28	26	0
6a.	h6	12	10	10	11	10	7	15	10	8	13	17	0
6b.	h7	10	10	8	9	7	0	17	12	11	12	22	0
7.	h8	12	12	10	12	9	7	17	12	15	12	21	0
8.	h9	0	0	0	7	0	7	0	0	0	0	12	0
9.	h10	24	20	15	19	15	21	23	0	18	13	25	0
10.	h11	10	10	9	8	10	13	17	11	9	9	19	0
11.	h12	7	0	8	7	8	9	15	15	8	7	16	0
12.	h13	14	7	9	12	10	12	17	10	12	12	22	0
Control		0	0	0	0	0	0	0	0	0	0	0	0
Standard		35	35	40	42	38	38	40	42	42	38	38	42

(0) No activity traced; numerical value presents diameter of clear zones in mm.

3.2.1.1. Minimum inhibitory concentrations (MICs) of the lichen extracts against test bacteria

Depending on the collected amount of extracts it was hardly possible to carry out experiments for the MIC values, as well as for isolation of compounds for all the extract samples. Thus, two of the lichen samples were subjected to experimentation for the measurement of the MIC values and the results of MIC values of $CHCl_3$ extract of lichens (h5 and h10) have been shown in Tables 10 and 11.

3.2.1.1.1. MICs of lichen sample h5 extract against bacteria

The MIC values of the lichen sample h5 extract have been established as 64 µg/ml against *B. megaterium*, *B. subtilis* and *S. -β-haemolyticus*, and 32 µg/ml against *S. aureus, B. cereus* and *S. lutea*. For the Gram negative bacteria 64 µg/ml against *S. dysenteriae* and *S. shiga*, 32 µg/ml against *S. typhi* and *S. boydii* and 16 µg/ml against *S. sonnei*.

Table 10. MICs of the lichen h5 extract against bacteria

Test items	Nutrient broth medium (ml)	Diluted extract solution (µg/ml)	Inoculam added (ml)	Gram positive bacteria						Gram negative bacteria				
				S. aureus	*B. megaterium*	*B. subtilus*	*B. Cereus*	*S. -β- haemolyticus*	*S. lutea*	*S. typhi*	*S. dysenteriae*	*S. shiga*	*S. sonnei*	*S. boydii*
1	1	512	10	-	-	-	-	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-	-	-	-	-
4	1	64	10	-	-	-	-	-	-	-	-	-	-	-
5	1	32	10	-	+	+	-	+	-	-	+	+	-	-
6	1	16	10	+	+	+	+	+	+	+	+	+	-	+
7	1	8	10	+	+	+	+	+	+	+	+	+	+	+
8	1	4	10	+	+	+	+	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+	+	+	+	+
10	1	1	10	+	+	+	+	+	+	+	+	+	+	+
C_s	1	512	00	-	-	-	-	-	-	-	-	-	-	-
C_1	1	00	10	+	+	+	+	+	+	+	+	+	+	+
C_m	1	00	00	-	-	-	-	-	-	-	-	-	-	-
Results of MIC values in (µg/ml)				32	64	64	32	64	32	32	64	64	16	32

C_1= Medium (inoculum), C_m= Medium, C_s= Medium + sample

3.2.1.1.2. MICs of lichen sample h10 extract against bacteria

The MIC values of the lichen sample h10 extract have been established as 128 µg/ml against *S. aureus*, *B. subtilis* and *S. -β-haemolyicus*, 64µg/ml against *B. megaterium*, 32 µg/ml against *B. cereus* and 8 µg/ml against *S. lutea*. Against Gram negative bacteria the MIC values have been established as 128 µg/ml against *S. sonnei*, 32 µg/ml against *S. shiga*, 16 µg/ml against *S. typhi* and 8 µg/ml against *S. boydii*. From the result it is obvious that lichens have the potentials to restrain several animal (including human) and plant pathogens from causing diseases.

Table 11. MICs of the lichen h10 extract against bacteria

Test items	Nutrient broth medium (ml)	Diluted extract solution (µg/ml)	Inoculam added (ml)	Gram positive bacteria						Gram negative bacteria			
				S. aureus	*B. megaterium*	*B. subtilus*	*B. cereus*	*S. β- haemolyticus*	*S. lutea*	*S. typhi*	*S. shiga*	*S. sonnei*	*S. boydii*
1	1	512	10	-	-	-	-	-	-	-	-	-	-
2	1	256	10	-	-	-	-	-	-	-	-	-	-
3	1	128	10	-	-	-	-	-	-	-	-	-	-
4	1	64	10	+	-	+	-	+	-	-	-	+	-
5	1	32	10	+	+	+	-	+	-	-	-	+	-
6	1	16	10	+	+	+	+	+	-	-	+	+	-
7	1	8	10	+	+	+	+	+	-	+	+	+	-
8	1	4	10	+	+	+	+	+	+	+	+	+	+
9	1	2	10	+	+	+	+	+	+	+	+	+	+
10	1	1	10	+	+	+	+	+	+	+	+	+	+
C_s	1	512	00	-	-	-	-	-	-	-	-	-	-
C_1	1	00	10	+	+	+	+	+	+	+	+	+	+
C_m	1	00	00	-	-	-	-	-	-	-	-	-	-
Results of MIC values in (µg/ml)				128	64	128	32	128	8	16	32	128	8

C_1= Medium (inoculum), C_m= Medium, C_s= Medium + sample

3.2.2. Antifungal activity *(in vitro)* of the lichen extracts

The antifungal activity of the crude extracts of the lichen samples at a concentration of 500 µg/disc for each against five pathogenic fungi, *A. flavus, A. fumigatus, C. albicans, Mucor* sp. and *F. vasinfectum* along with the standard Nystatin have been presented in the Table 12. From the results it is obvious that the chloroform extract of the lichen samples h5 and h10 are more effective against the test fungi mentioned above. However, the extract of the

lichen h3 was also found promisingly effective. Against *A. flavus* the inhibition zones recorded were 9-, 9-, 11-, 9-, 6-, 6-, 11-, 10-, 10-, 11-, 7-, 6-, 0-, 9- and 33 mm, for the extracts of the lichen samples h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11, h12, h13, the control dose as the only solvent (chloroform) and the standard (Nystatin) respectively; against *A fumigatus* the inhibition zones were 17-, 17-, 11-, 11-, 21-, 13-, 12-, 13-, 9-, 20-, 17-, 12-, 14-, 9- and 32 mm; against *C. albicans* the inhibition zones were 11-, 9-, 12-, 11-, 10-, 12-, 10-, 8-, 10-, 12-, 9-, 10-, 10-, 10- and 33 mm; against *Mucor* sp. the inhibition zones were 7-, 9-, 6-, 0-, 7-, 7-, 8-, 11-, 12-, 7-, 7-, 7-, 7-, 7- and 32 mm; against *F. vasinfectum* the inhibition zones were 13-, 13-, 11-, 10-, 16-, 13-, 9-, 8-, 9-, 15-, 11-, 9-, 13-, 9- and 7 mm for the extracts in the aforesaid order of the lichen samples, the solvent and the standard (Nystatin) respectively.

Table 12. Antifungal activity of the lichen extracts against pathogenic fungi

Sl.	Lichen samples	Sensitivity against the following fungi for 500µg of extracts /24h (mm)				
		A. flavus	*A. fumigatus*	*C. albicans*	*Mucor* sp.	*F. vasinfectum*
1.	h1	9	17	11	7	13
2.	h2	9	17	9	9	13
3.	h3	11	11	12	6	11
4.	h4	9	11	11	0	10
5.	h5	6	21	10	7	16
6a.	h6	6	13	12	7	13
6b.	h7	11	12	10	8	9
7.	h8	10	13	8	11	8
8.	h9	10	9	10	12	9
9.	h10	11	20	12	7	15
10.	h11	7	17	9	7	11
11.	h12	6	12	10	7	9
12.	h13	0	14	10	7	13
	Control (Chloroform)	9	9	10	7	9
	Standard (Nystatin)	33	32	33	32	7

(0) No activity traced; numerical value presents diameter of clear zones in mm.

3.2.2.1. Minimum inhibitory concentrations (MICs) of lichen extracts against pathogenic fungi

For the measurement of MIC values the lichen samples h5 and h10 were selected depending on the amount of extracts. However, the results of MIC values have been shown in Tables 13. The MIC values of the lichen sample h5 extract were 1280 µg/ml against *A. flavus* and *Mucor* sp., 640 µg/ml against *C. albicans* and 10 µg/ml against *A. fumigatus* and *F. vasinfactum*; and for the lichen sample h10 extract were 1280 µg/ml against *Mucor* sp., 320 µg/ml against *A. flavus*, 160µg/ml against *C. albicans* and 80 µg/ml against *A. fumigatus* and *F. vasinfactum*.

Table 13. MICs of the lichen h5 and h10 extracts against pathogenic fungi

Test Items	Diluted extract solution (µg/ml)	Inoculum added (ml)	Fungi tested with h5					Fungi tested with h10				
			A. flavus	*A. fumigatus*	*C. albicans*	*Mucor sp.*	*F. vasinfectum*	*A. flavus*	*A. fumigatus*	*C. albicans*	*Mucor sp.*	*F. vasinfectum*
1	1280	10	-	-	-	-	-	-	-	-	-	-
2	640	10	+	-	-	+	-	-	-	-	+	-
3	320	10	+	-	+	+	-	-	-	-	+	-
4	160	10	+	-	+	+	-	+	-	-	+	-
5	80	10	+	-	+	+	-	+	-	+	+	-
6	40	10	+	-	+	+	-	+	+	+	+	+
7	20	10	+	-	+	+	-	+	+	+	+	+
8	10	10	+	-	+	+	-	+	+	+	+	+
9	5	10	+	+	+	+	+	+	+	+	+	+
10	2.5	10	+	+	+	+	+	+	+	+	+	+
C_s	1280	00	-	-	-	-	-	-	-	-	-	-
C_1	00	10	+	+	+	+	+	+	+	+	+	+
C_m	00	00	-	-	-	-	-	-	-	-	-	-
Results of MIC values in µm/ml			1280	10	640	1280	10	320	80	160	1280	80

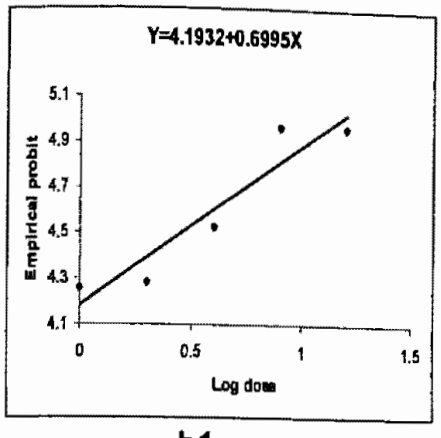
C_1= Medium (inoculum), C_m= Medium, C_s= Medium + sample

3.3. Cytotoxic effect of the lichen extracts against *A. salina* nauplii through dose-mortality assay

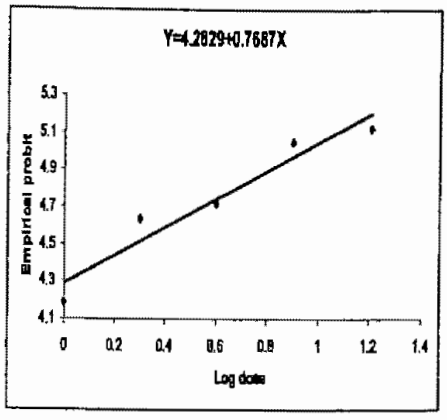
The median lethal concentration or LC_{50} values of the tested crude chloroform extracts of the lichen samples h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11, h12 and h13 were found promising against the 1 day nauplii of *A. salina* and the results have been presented in Table 14 and Appendix Tables I – XIII. The LC_{50} values for lichen extracts were 14.245-, 8.568-, 16.260-, 9.419-, 8.994-, 2.333-, 9.386-, 6.871-, 17.124-, 11.611-, 38.604-, 18.776- and 20.529 ppm for 24h of exposure respectively for the lichen extracts mentioned above; while the regression equations were Y = 4.1932 + 0.6994 X, Y = 4.2829 + 0.7687 X, Y = 3.9340 + 0.8802 X, Y = 3.9821 + 1.0451 X, Y = 3.3292 + 1.7515 X, Y = 4.6798 + 0.8703 X, Y = 3.7803 + 1.2542 X, Y = 4.3786 + 0.7424 X, Y = 3.6356 + 1.1060 X, Y = 3.8639 + 1.0670 X, Y = 3.6131 + 0.8741 X, Y = 3.2563 + 1.3691 X, Y = 3.7263 + 0.9706 X respectively and the $\chi 2$ values were 3.15, 1.59, 4.79, 3.61, 10.47, 2.74, 20.77, 1.68, 3.86, 1.25, 3.35, 3.83 and 4.55 respectively, while all for 3 degrees of freedom. Ninety five per cent confidence limits for the performance of lethality could be seen in Table 14 and Appendix Tables I – XIII; and the slopes are clarified in Figures 2 – 5. According to the intensity of efficacy the lichen extracts could be arranged in a descending order of the samples h6> h8> h2> h5> h7> h4> h10> h1> h3> h9> h12> h13> h11.

Table 14. Cytotoxic effects of lichen extracts against *A. salina* nauplii

Lichen sample	Time exposed	LC_{50} value (ppm)	95%confidence limits		Regression equation	χ^2 value
			Lower limits (ppm)	Upper limits (ppm)		
h1	24h	14.245	7.5764	26.7813	Y=4.1932+0.6994X	3.15 (3)
h2	24h	8.568	5.5023	13.3423	Y=4.2829+0.7687X	1.59 (3)
h3	24h	16.260	9.4634	27.9385	Y=3.9340+0.8802X	4.79 (3)
h4	24h	9.419	6.6866	13.2668	Y=3.9821+1.0451X	3.61 (3)
h5	24h	8.994	6.1428	13.1673	Y=3.3292+1.7515X	10.47 (3)
h6	24h	2.333	1.6342	3.3304	Y=4.6798+0.8703X	2.74 (3)
h7	24h	9.386	4.4088	19.9828	Y=3.7803+1.2542X	20.77 (3)
h8	24h	6.871	4.5437	10.3907	Y=4.3786+0.7424X	1.68 (3)
h9	24h	17.124	10.9055	26.8878	Y=3.6356+1.1060X	3.86 (3
h10	24h	11.611	7.9788	16.8959	Y=3.8639+1.0670X	1.25 (3)
h11	24h	38.604	16.2945	91.4561	Y=3.6131+0.8741X	3.35 (3)
h12	24h	18.776	12.6806	27.8021	Y=3.2563+1.3691X	3.83 (3)
h13	24h	20.529	11.7010	36.0188	Y=3.7263+0.9706X	4.55 (3)



h1



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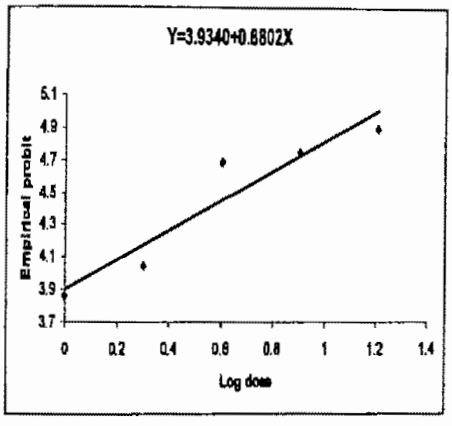

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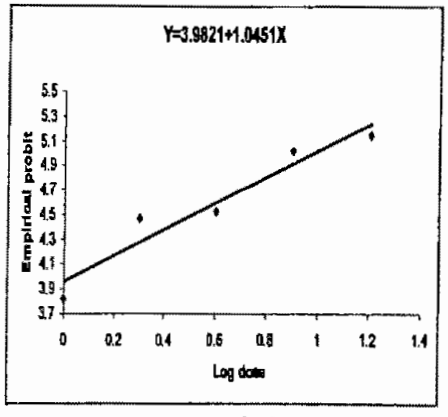
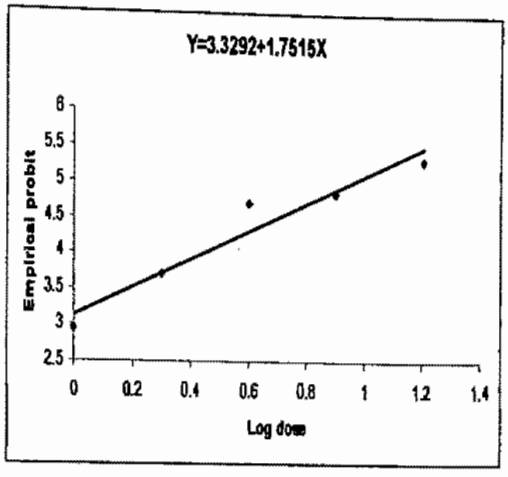


h4

Fig. 2. Probit mortality lines of the chloroform extracts of the lichen samples h1, h2, h3 and h4 against *A. salina* nauplii after 24 h of exposure.



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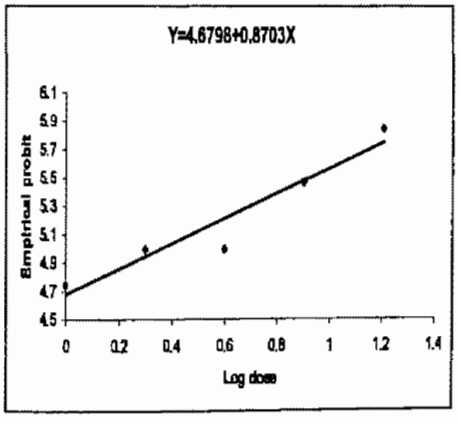

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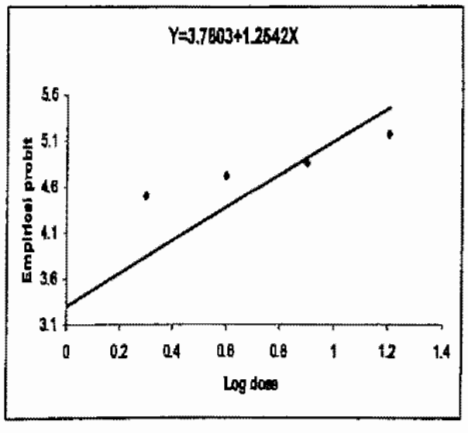
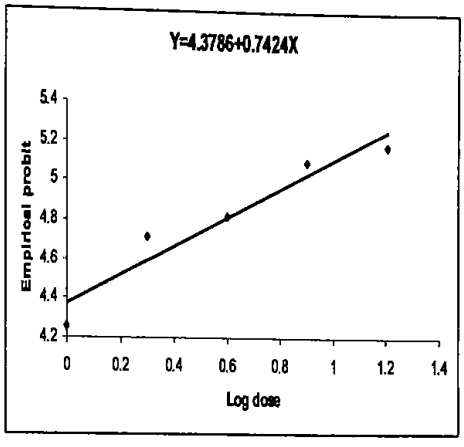


h 7

Fig. 3 . Probit mortality lines of the chloroform extracts of the lichen samples h5, h6 and h7 against *A. salina* nauplii after 24 h of exposure.



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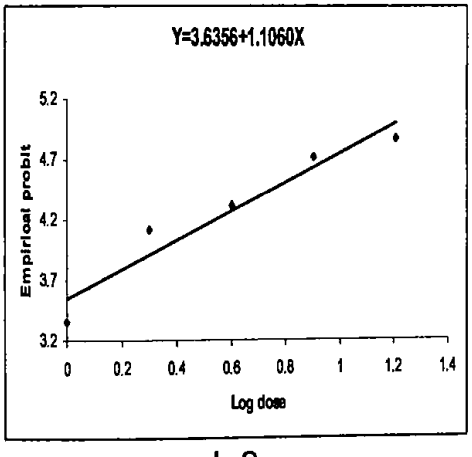
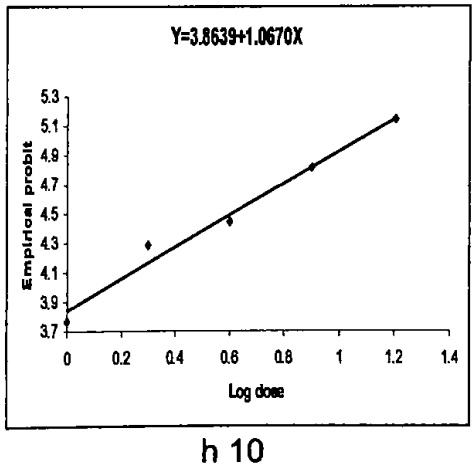
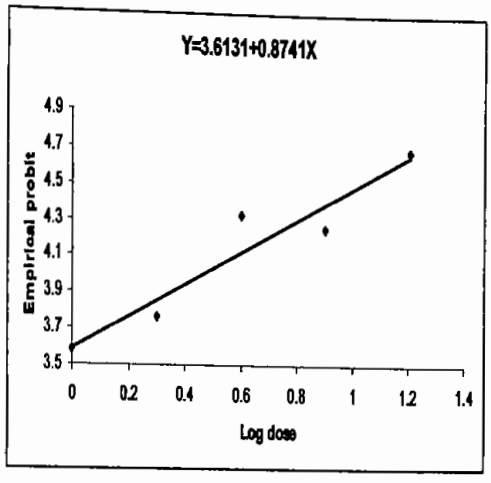


h 9

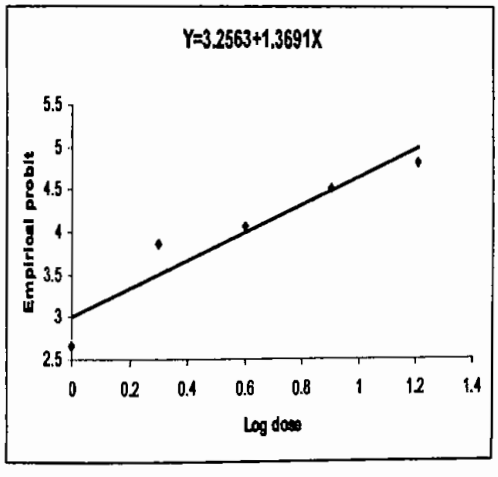


h 10

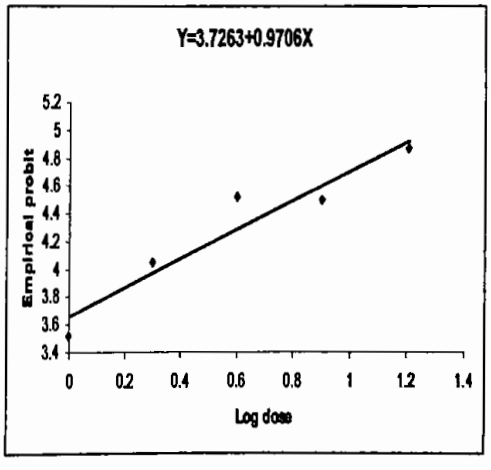
Fig. 4. Probit mortality lines of the chloroform extracts of the lichen samples h8 h9 and h10 against *A. salina* nauplii after 24 h of exposure.



h 11



h12



h13

Fig. 5. Probit mortality lines of the chloroform extracts of the lichen samples h11, h12 and h13 against *A. salina* nauplii after 24 h of exposure.

3.4. Repellent activity of lichen extracts against *T. castaneum* adults

Chloroform extracts of the lichen samples h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11, h12 and h13 were tested for repellent activity against *T. castaneum* adults. The test doses were arranged in a range from 314.54 to as less as 9.83 μg cm^{-2} (314.54-, 157.27-, 78.63-, 39.32-, 19.66- and 9.83 μg cm^{-2}) and the ANOVA results have been presented in Table 15 and Appendix Tables XIV – XXVI. The F ratios have been established as 20.75178248 for h1, 17.99056548 for h2, 18.3919282 for h3, 68.20076978 for h4, 202.1016635 for h5, 32.79777458 for h6, 10.24107941 for h7, 23.31528753 for h8, 15.14562 for h9, 8.989087839 for h10, 16.00717126 for h11, 18.50794484 for h12 and 17.12251938 for h13 to ensure that all the lichen extracts showed repellent activity, of which extracts of the sample h4 and h5 highly effective ($P<0.001$), sample h2, h6 and h8 positively effective $P<0.01$) and the rest showed repellency at $P<0.05$ level of significance. The P values found were 4.84809 E-05, 8.26754 E-08, 7.85048 E-06, 7.15065 E-12, 1.78038 E-13, 1.58496 E-07, 0.000261597, 1.65543 E-06, 2.66039 E-05, 0.000528675, 1.89 E-05, 7.53987 E-06 and 1.23796 E-05 respectively and thus the intensity of repellent activity could be arranged in a descending order of h5 > h4> h6> h8> h2> h12> h13> h11> h9> h7>h10> h3>h1 (Table 15).

Table 15. ANOVA results of repellency of *T. castaneum* adults by lichen extracts

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h1	$CHCl_3$	Between dose level	5461. 916	3	1820.639	*20.75178248	4.84809E-05
		Between time interval	43.815	4	10.954	0.12485065	0.970682483
		Error	1052.809	12	87.734		
		Total	6558.540	19			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h2	$CHCl_3$	Between dose level	23860.129	6	3976.688	**17.99056548	8.26754E-08
		Between time interval	1328.481	4	332.120	1.50251475	0.232928405
		Error	5305.031	24	221.043		
		Total	30493.6429	34			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h3	$CHCl_3$	Between dose level	4417.087	4	1104.272	*18.3919282	7.85048E-06
		Between time interval	654.661	4	163.666	2.7258867	0.066386709
		Error	960.658	16	60.041		
		Total	6032.406	24			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h4	$CHCl_3$	Between dose level	22448.208	5	4489.642	***68.20076978	7.15065E-12
		Between time interval	543.398	4	135.850	2.06364904	0.123840231
		Error	1316.596	20	65.830		
		Total	24308.202	29			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h5	$CHCl_3$	Between dose level	54041.688	4	13510.422	***202.1016635	1.78038E-13
		Between time interval	392.088	4	98.022	1.4663071	0.258630907
		Error	1069.594	16	66.850		
		Total	55503.370	24			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h6	$CHCl_3$	Between dose level	5647.007	4	1411.752	**32.79777458	1.58496E-07
		Between time interval	671.549	4	167.887	3.90034991	0.021373455
		Error	688.706	16	43.044		
		Total	7007.262	24			

to be contd.....

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h7	$CHCl_3$	Between dose level	13622.880	4	3405.720	*10.24107941	0.000261597
		Between time interval	3644.982	4	911.245	2.74013633	0.065426935
		Error	5320.877	16	332.555		
		Total	22588.739	24			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h8	$CHCl_3$	Between dose level	12559.045	4	3139.761	**23.31528753	1.65543E-06
		Between time interval	3179.379	4	794.845	5.90236983	0.004089083
		Error	2154.645	16	134.665		
		Total	17893.069	24			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h9	$CHCl_3$	Between dose level	7030.377	4	1757.594	*15.14562	2.66039E-05
		Between time interval	1768.761	4	442.190	3.81046	0.023201824
		Error	1856.742	16	116.046		
		Total	10655.879	24			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h10	$CHCl_3$	Between dose level	15238.629	4	3809.657	*8.989087839	0.000528675
		Between time interval	2847.257	4	711.814	1.679563372	0.203724848
		Error	6780.945	16	423.810		
		Total	24866.831	24			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h11	$CHCl_3$	Between dose level	2682.571	4	670.643	*16.00717126	1.89E-05
		Between time interval	2015.163	4	503.791	12.02467859	0.000106
		Error	670.342	16	41.897		
		Total	5368.076	24			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h12	$CHCl_3$	Between dose level	16857.996	4	4214.499	*18.50794484	7.53987E-06
		Between time interval	6303.434	4	1575.858	6.92037241	0.001966742
		Error	3643.407	16	227.713		
		Total	26804.837	24			

Test material	Extract	Source of Variation	SS	df	MS	F	P-value
h13	$CHCl_3$	Between dose level	3397.418	4	849.355	*17.12251938	1.23796E-05
		Between time interval	391.477	4	97.869	1.97299156	0.14735006
		Error	793.673	16	49.605		
		Total	4582.568	24			

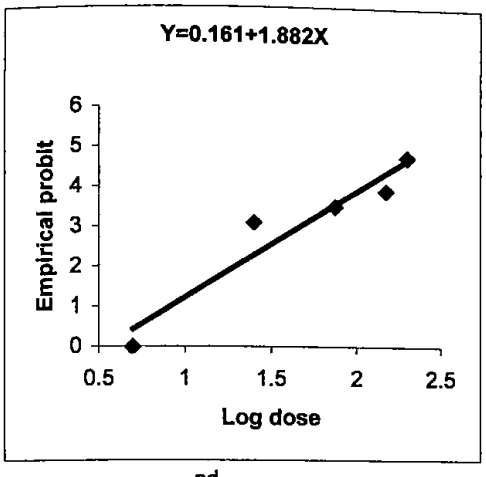
3.5. Dose mortality effects of lichen extracts

3.5.1. Dose mortality effect of lichen extracts against *T. castaneum*

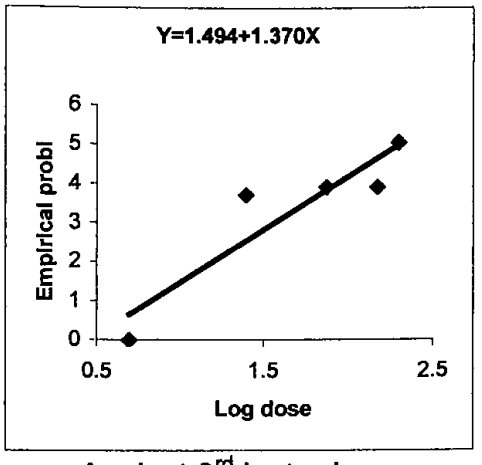
The chloroform extract of the lichen sample h10 collected from the host plant *B. flabellifer* was tested against *T. casteneum* larvae through dose-mortality assay at doses 5-, 25-, 75-, 150- and 200mg/g, where the test insects were released into the treated food to observed mortality or any sort of abnormality due to the effect of the extract in comparison to the control dose. The results have been presented in the Table 16 and Appendix Tables XXVII - XXIX and the slopes are clarified in Figure 6 for the mortality recorded. The LD_{50} value of 2nd instar was 372.375mg/g; while regression equations were Y= 0.161+1.882X; the $\chi 2$ value was 3.806 for 3 degrees of freedom and the 95% confidence limits were 169.238 to 819.336mg/g. The LD_{50} value of 3rd instar was 362.327 mg/g; while regression equations were Y=1.494+1.370X; the $\chi 2$ value was 8.548 for 3 degrees of freedom and the 95% confidence limits were 92.569 to 1418.196mg/g. And The LD_{50} value of 4th instar was 664.662mg/g; while regression equations were Y=2.638+0.837X; the $\chi 2$ value was 11.733 for 3 degrees of freedom and the 95% confidence limits were 35.948 to 12289.35 mg/g.

Table 16. Probit mortality of *T. castaneum* adults by chloroform extracts of lichen samples

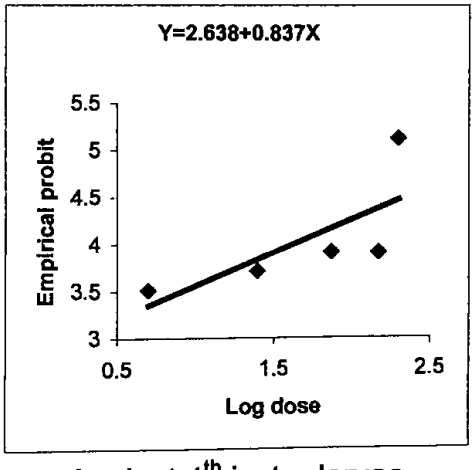
Test instar	LD_{50} value (mg/g)	95% Confidence limits		Regression equation	χ^2 Value
		Lower limits	Upper limits		
2nd instar	372.375	169.238	819.336	Y = 0.161+1.882X	3.806 (3)
3rd instar	362.327	92.569	1418.196	Y = 1.494+1.370X	8.548 (3)
4th instar	664.662	35.948	12289.350	Y = 2.638+0.837X	11.733 (3)



Against 2nd instar larvae



Against 3rd instar larvae



Against 4th instar larvae

Fig. 6. Probit mortality lines of the chloroform extract of the lichen sample h10 against *T. castaneum* larvae.

3.5.2. Dose mortality effect of lichen extracts against *M. domestica* larvae

In this case the $CHCl_3$ extracts of the lichen sample h10 has been used. The crude extract dose mortality results against larvae of *M. domestica* were found promising. The $CHCl_3$ extracts of the materials was effective against the 3rd instar larvae of *M. domestica* and the results has been presented in Appendix Table XXX and the slopes are clarified in Figure 7. The LD_{50} value was 71.766 mg/g for 24 h of exposure; while the regression equations were Y=3.2404+0.9481X; the $\chi 2$ value was 1.6417 for 3 degrees of freedom and the 95% confidence limits were 43.2178 to 119.1712 mg/g for 24 h of exposure.

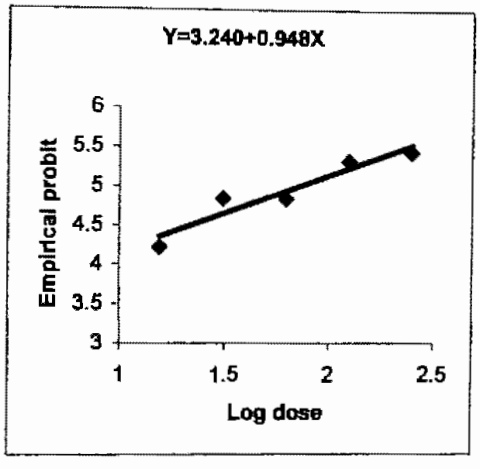


Fig. 7. Probit mortality lines of the chloroform extract of the lichen sample h10 against 3rd instar larvae of *M. domestica*.

3.6. Purification of bioactive lichen compounds

3.6.1. Physical remarks of the pure compounds

The isolated compounds and there physical stature have been presented in Table 17. Compound LMI-1 was isolated from the lichen sample h5 (*Morus indica*); compound LBF-2 was from h10 (*B. flabellifer*) and 2 compounds LMI-3 and LMI-4 were from the lichen sample h13 (*Mangifera indica*). However, the compounds from h13 were not subjected to NMR analysis, but biological activity tests have been carried out.

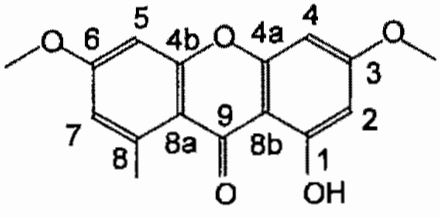
Table 17: Compounds purified from lichens

Extract	Compound/ Source	Retention factor (Rf)	Physical identity of the compounds /Godin reagent spray
$CHCl_3$	LMI-1/h5	0.62	Yellow Crystal needles/Golden
	LBF-2/h10	0.62	Yellow Crystal needles/Golden
	LMI-3/h13	0.64	White crystals/Yellow
	LMI-4/h13	0.50	White crystals powder/Yellow

3.6.2. Characterization of the purified compound(s)

3.6.2.1. Characterization of the purified compounds LMI-1

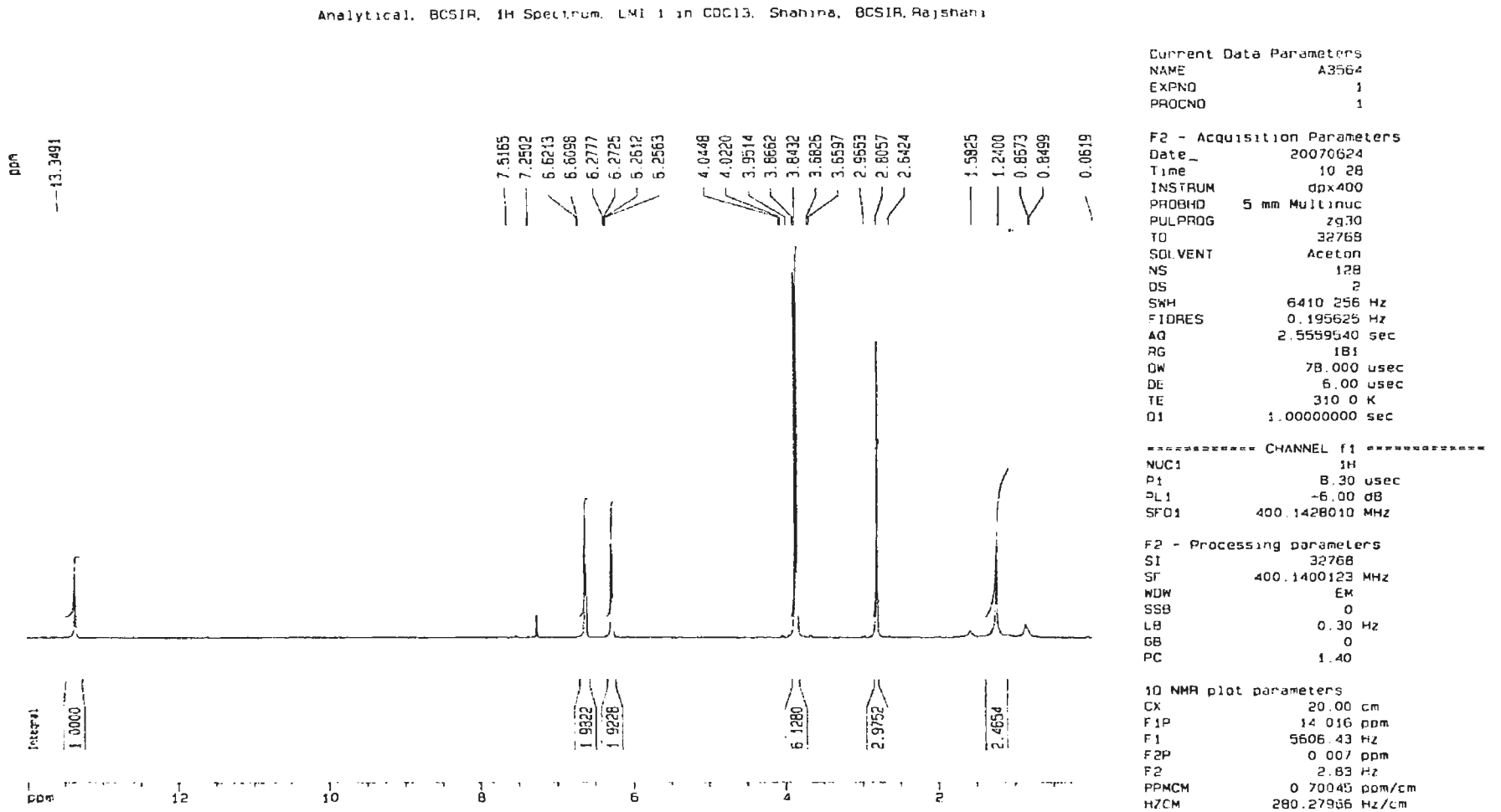
Compound LMI-1 was isolated from *Morus indica.* The samples LMI-1 and LBF-2 were subjected to NMR analyses at the BCSIR Laboratories, Dhaka, Bangladesh. The molecular ion peak (M/z=286.29) of high resolution EIMS spectrum suggested that the molecular formula should be $C_{16}H_{14}O_5$ (calcd. M/z=286.08). Yellow crystals; mp 165 - 166 °C, lit. 187 - 190°C; [3] UV λ_{max} (MeOH) nm (log e) 250 (2.50) and 312 (2.28) nm; UV, ^{1}H NMR, and 13C NMR data (recorded in CDCl3), consistent with literature values of Woodson and Schery (1965) and Garcia and Brown (1976) and the compound is a known one and named as lichexanthone. Relevant NMR data are presented in Figures 8-29.



Lichexanthone

Calderon *et al.*,2002; *J. Nat. Prod.* **65** (12): 1751 - 1753
Woodson and Schery, 1965; *Ann. Mo. Bot. Gard.* **4**: 360 - 362
Garcia and Brown, 1976; *Phytochemistry* **15**:1093 - 1095

Compounds LMI-1and LBF-2 is the same compound, even though they are isolated from different sources. May be the amount of impurities, represented by singals in proton NMR between 0-2ppm (probably grease, fat, residual solvent like petroleum ether?) are of different amount.



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PROCNO	1

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AQ	2.5559540 sec
RG	181
DW	78.000 usec
DE	6.00 usec
TE	310.0 K
D1	1.00000000 sec

========== CHANNEL f1 ==========

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PL1	-6.00 dB
SFO1	400.1428010 MHz

F2 - Processing parameters

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WDW	EM
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GB	0
PC	1.40

1D NMR plot parameters

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F1	5606.43 Hz
F2P	0.007 ppm
F2	2.83 Hz
PPMCM	0.70045 ppm/cm
HZCM	280.27986 Hz/cm

Fig. 8. ^{1}H-NMR proton spectrum of the compound LMI-1

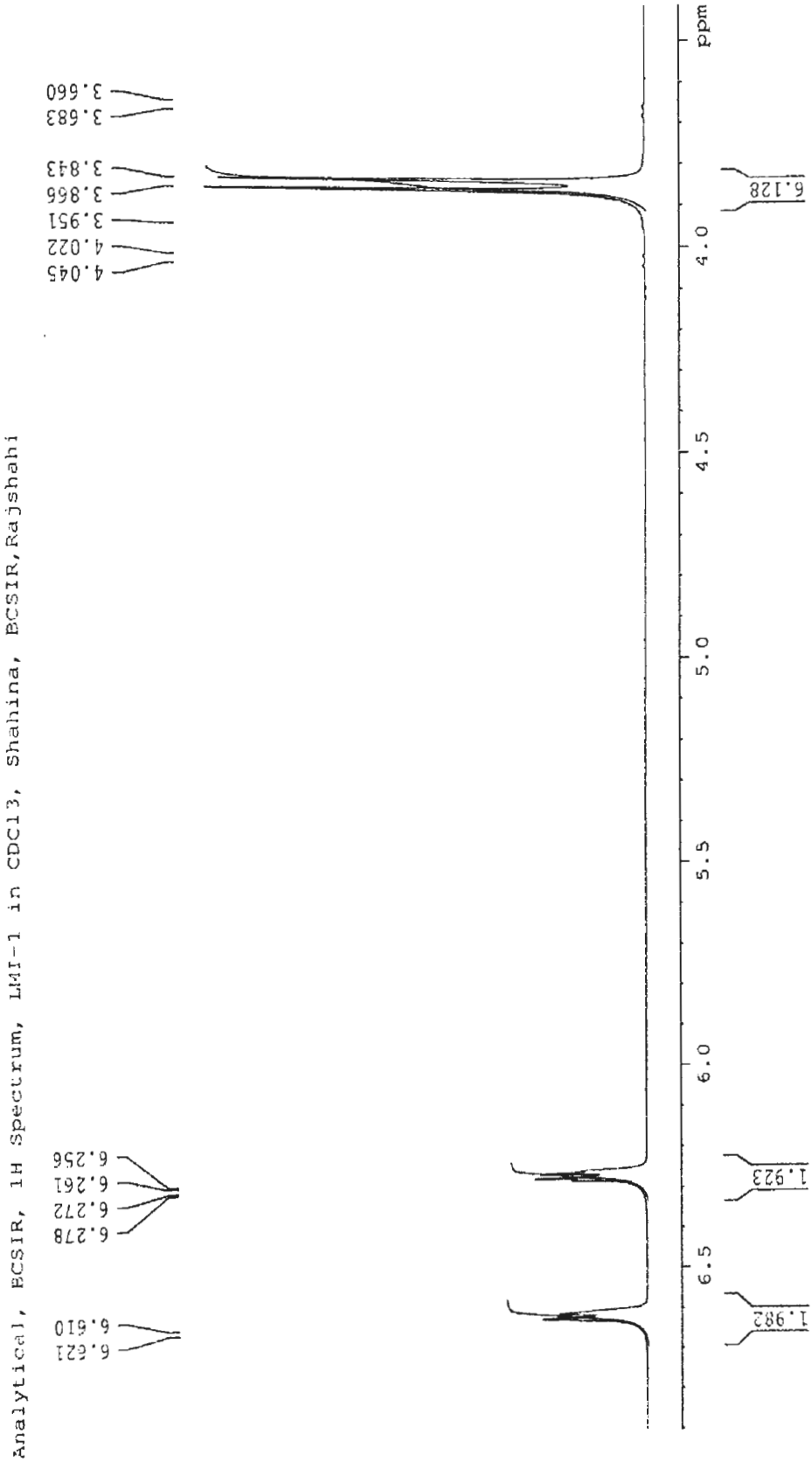


Fig. 9. ^{1}H-NMR proton spectrum of the compound LMI-1

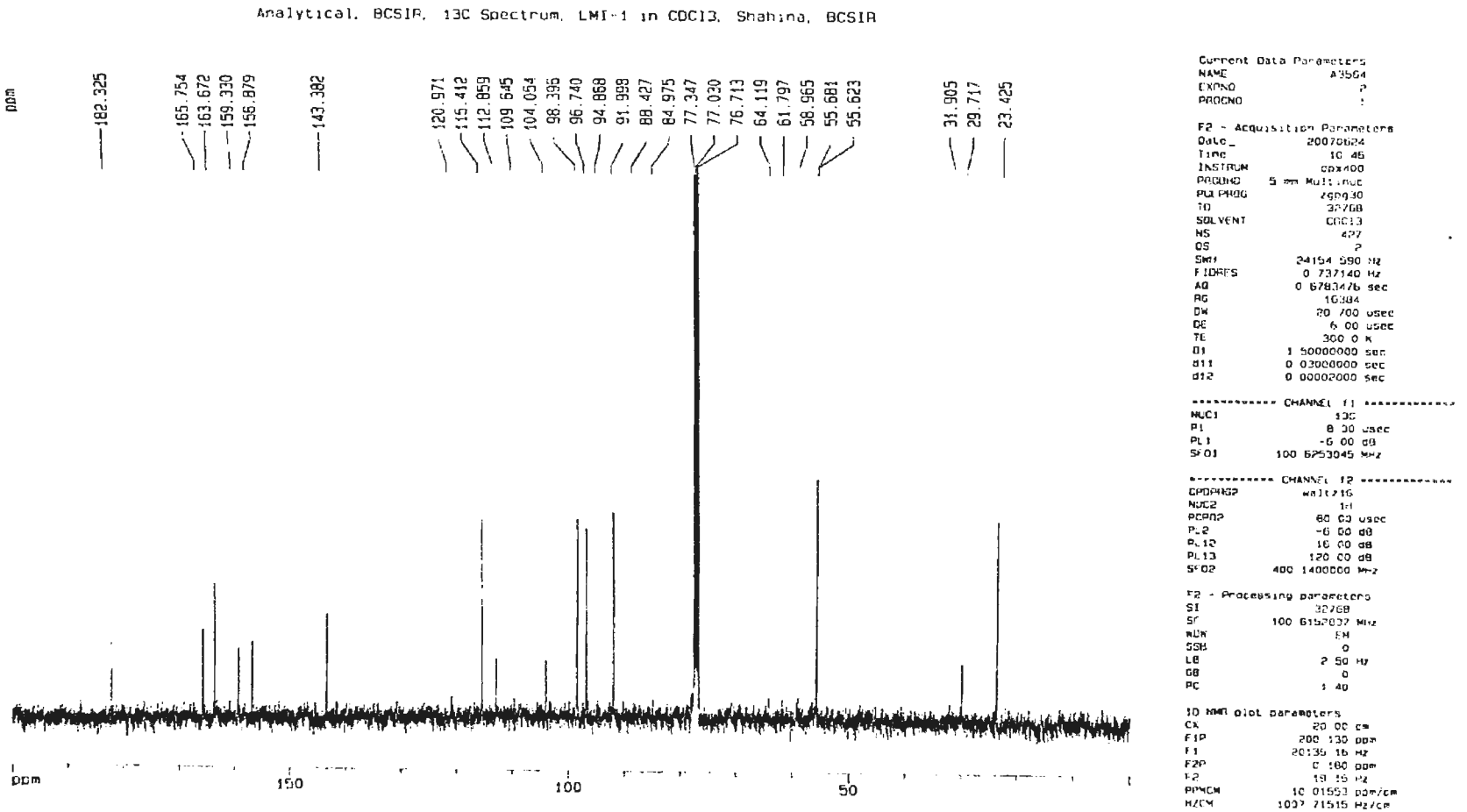


Fig. 10. ^{13}C-NMR spectrum of the compound LMI-1

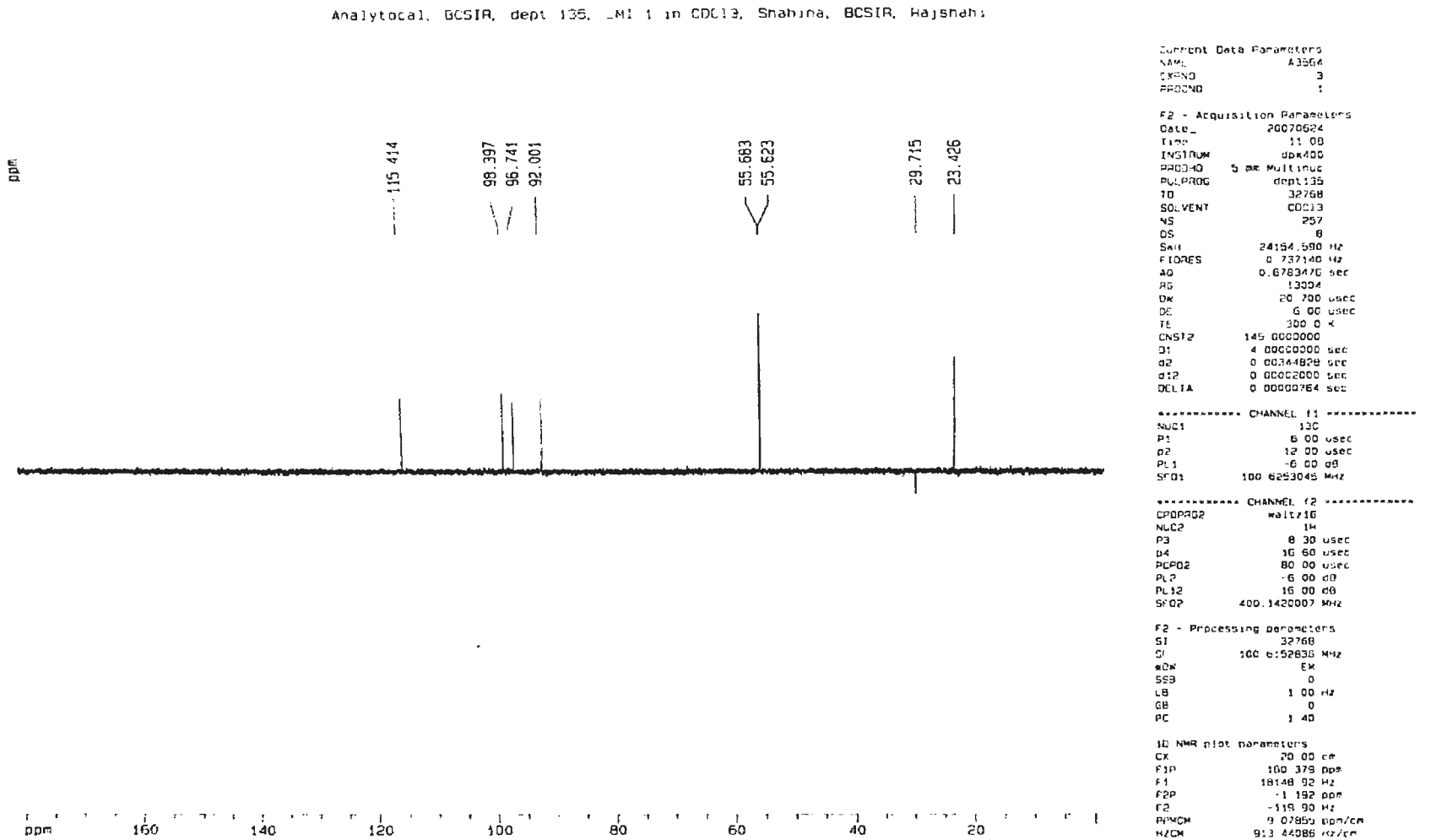


Fig. 11. ^{13}C-NMR spectrum of the compound LMI-1

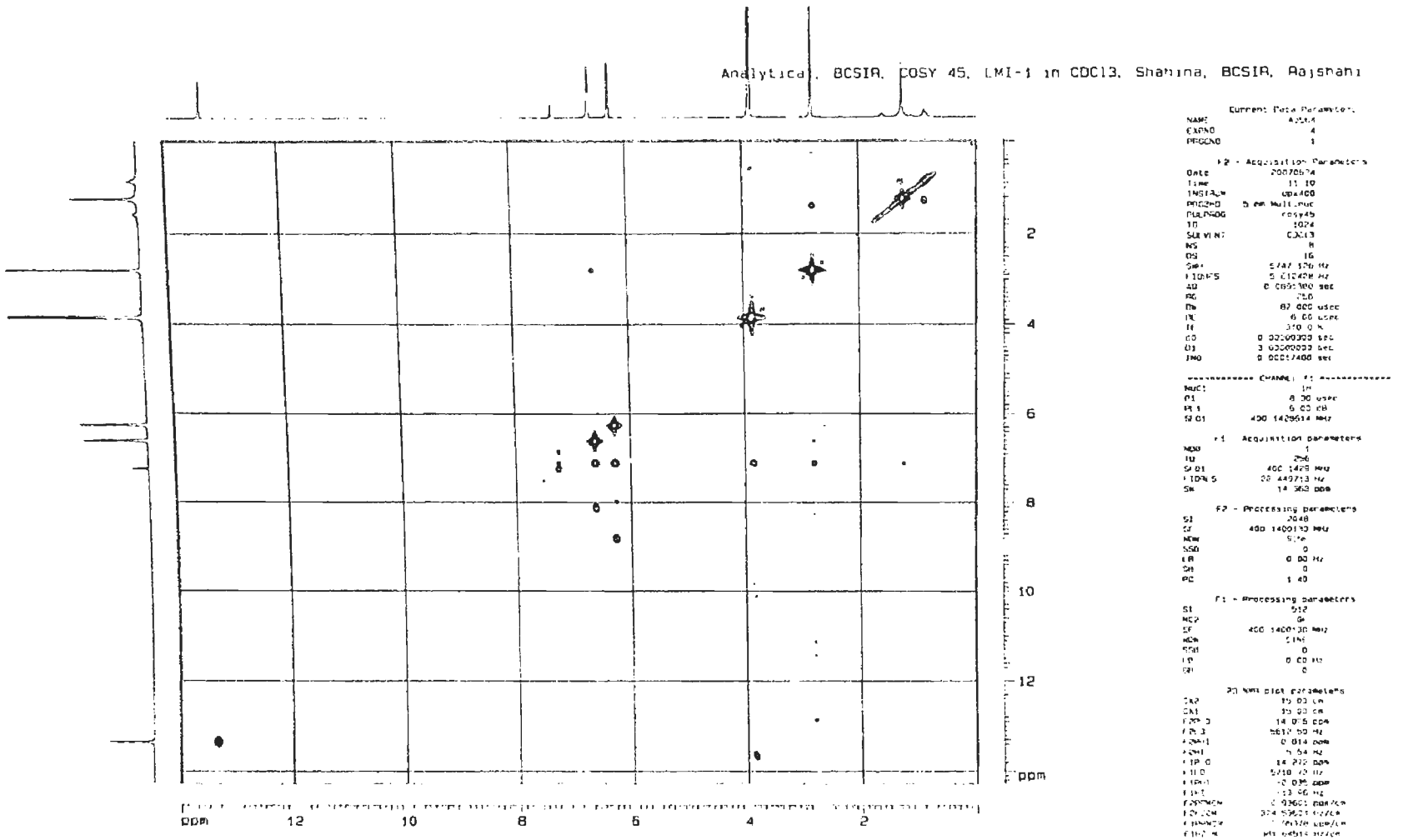


Fig. 12. ^{13}C-NMR spectrum for the COSY of the compound LMI-1

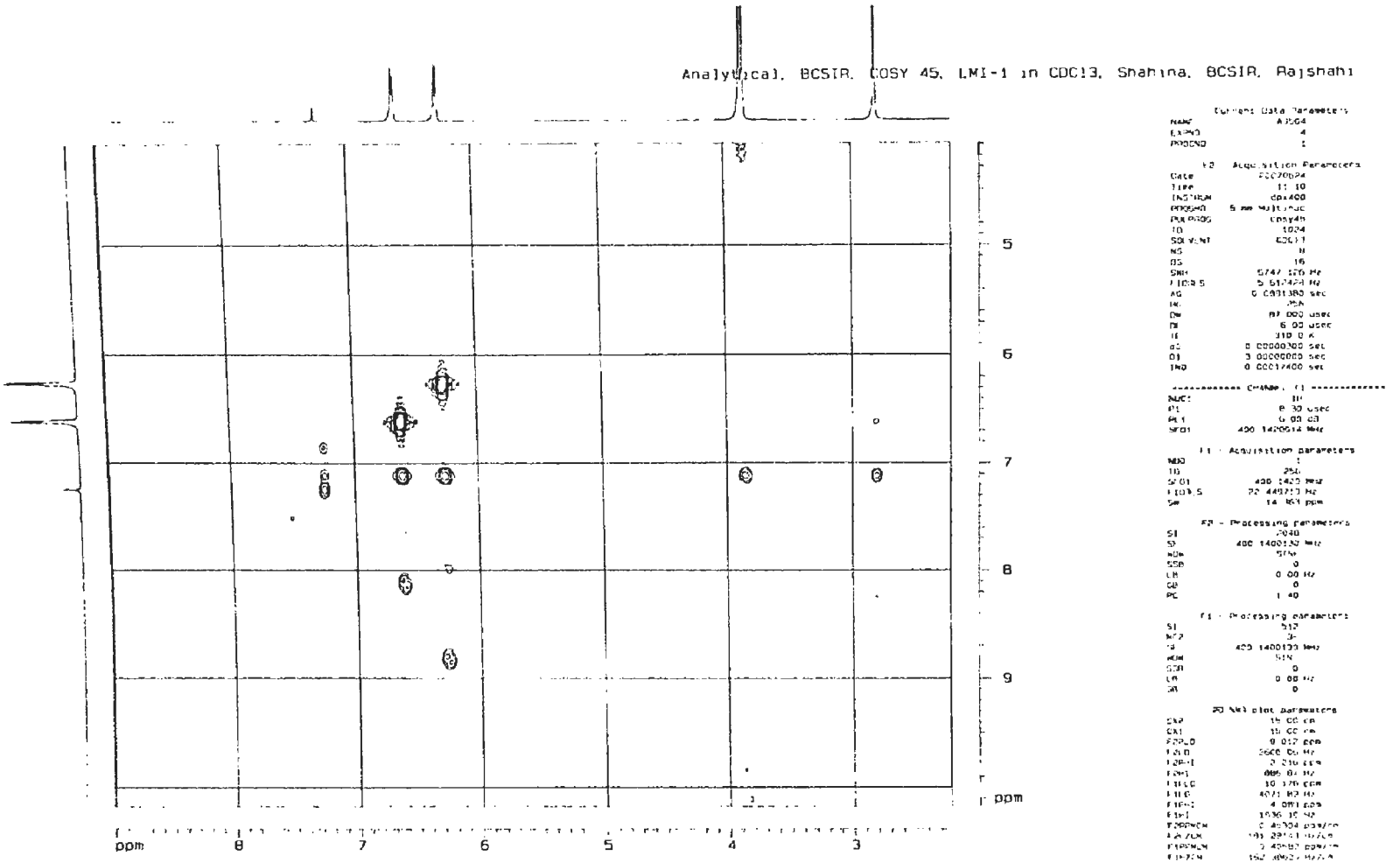


Fig. 13. ^{13}C-NMR spectrum for the COSY of the compound LMI-1

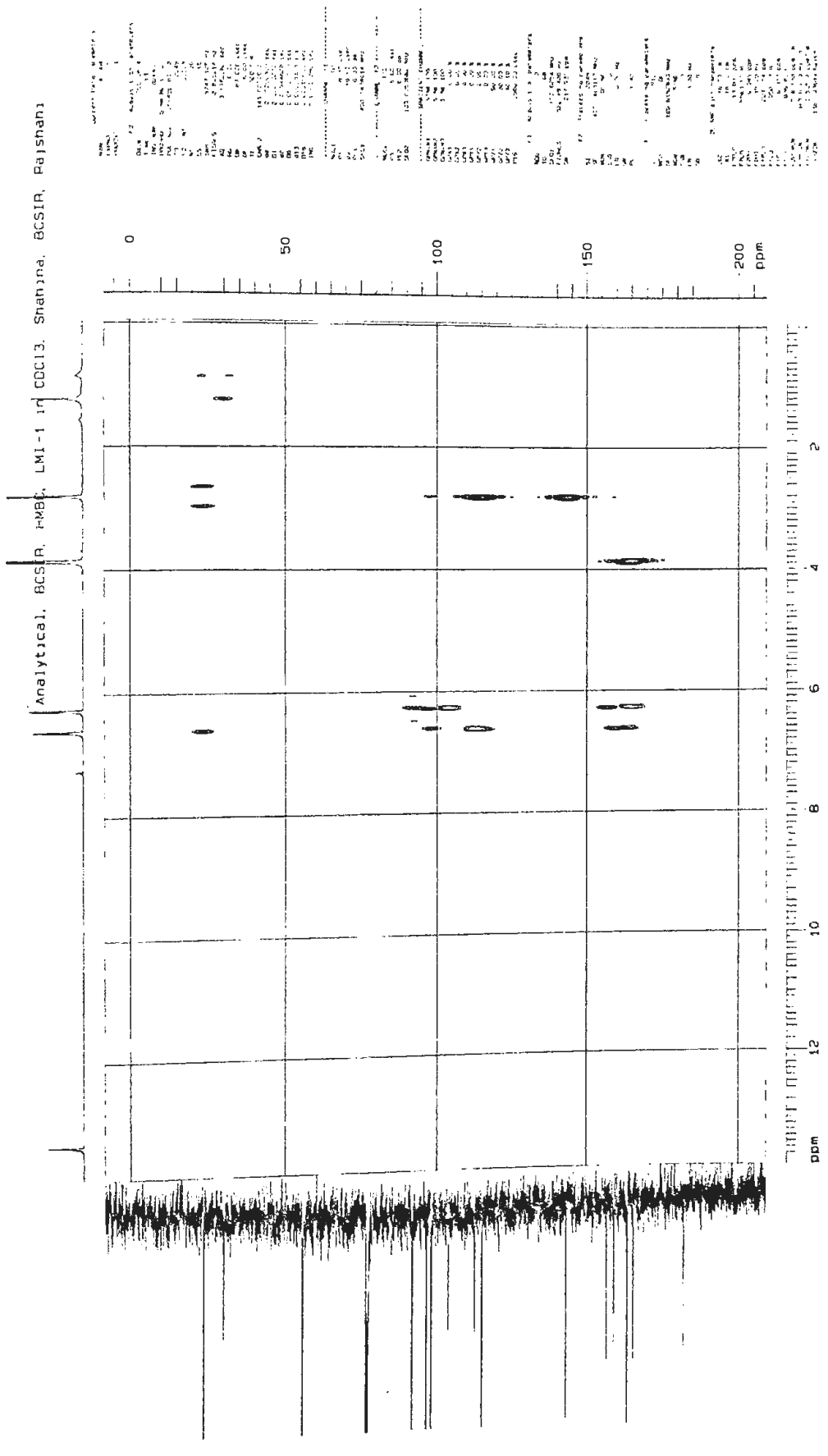


Fig. 14. HMBC spectrum of the compound LMI-1

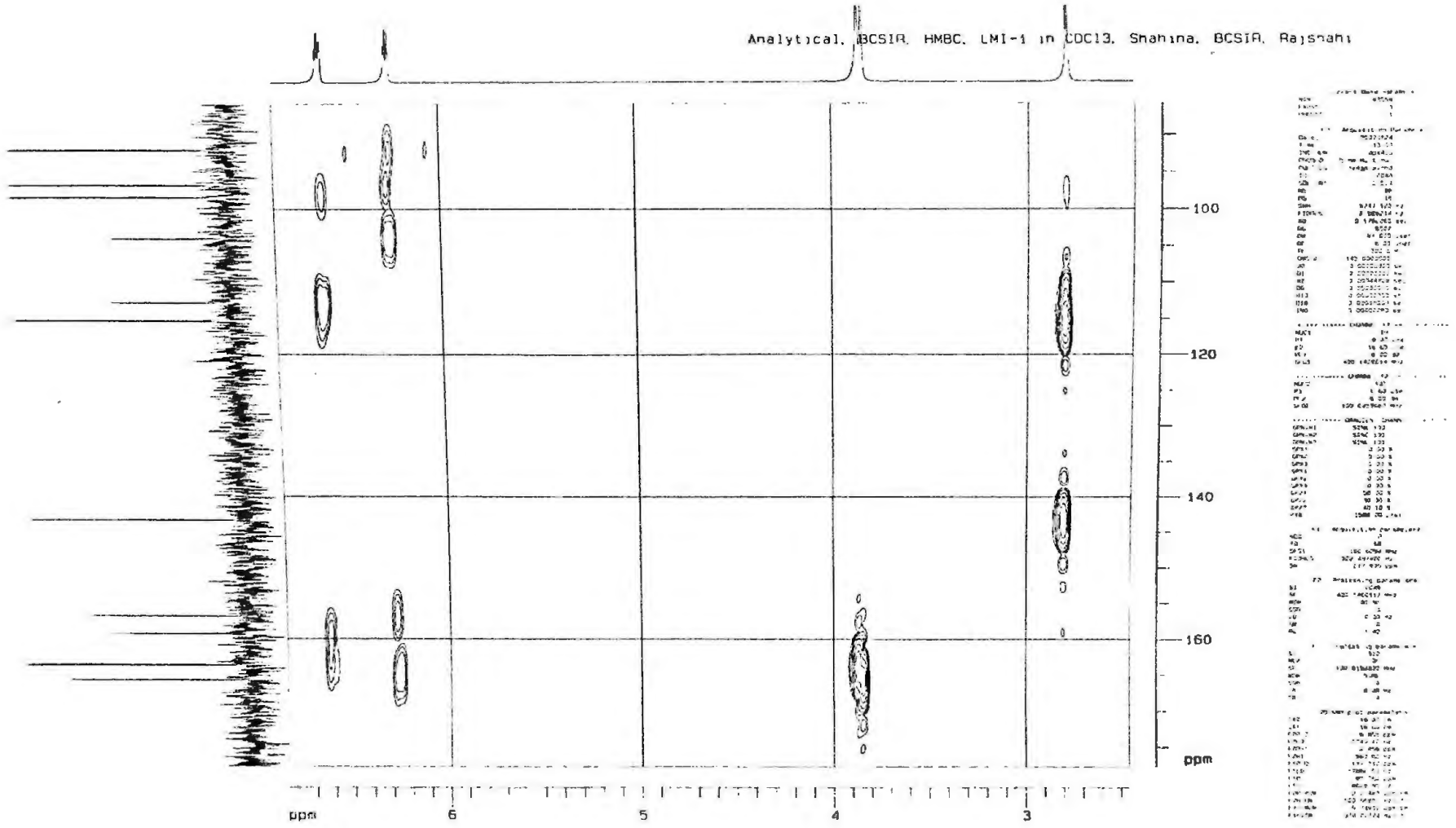


Fig. 15. HMBC spectrum of the compound LMI-1

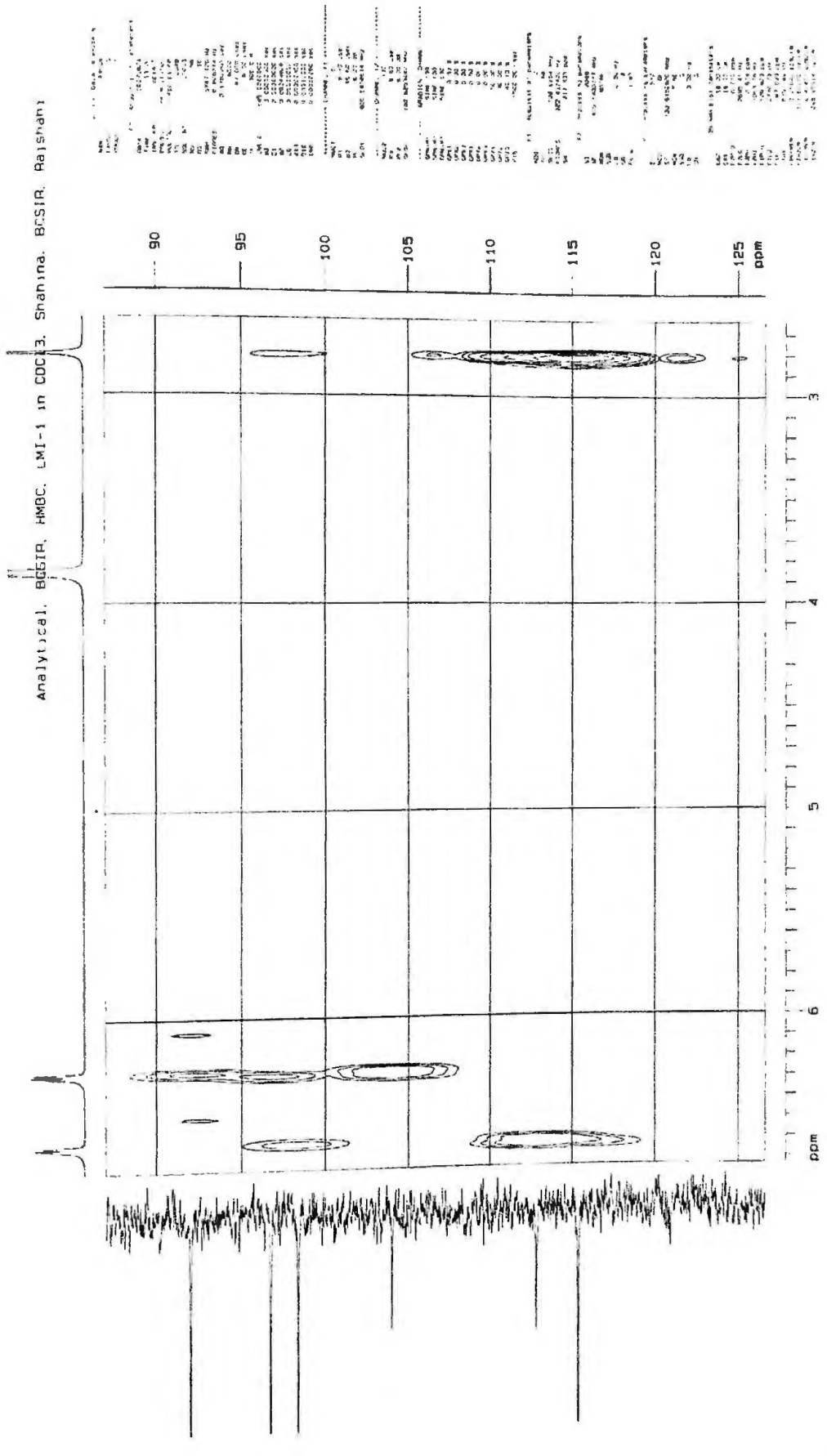


Fig. 16. HMBC spectrum of the compound LMI-1

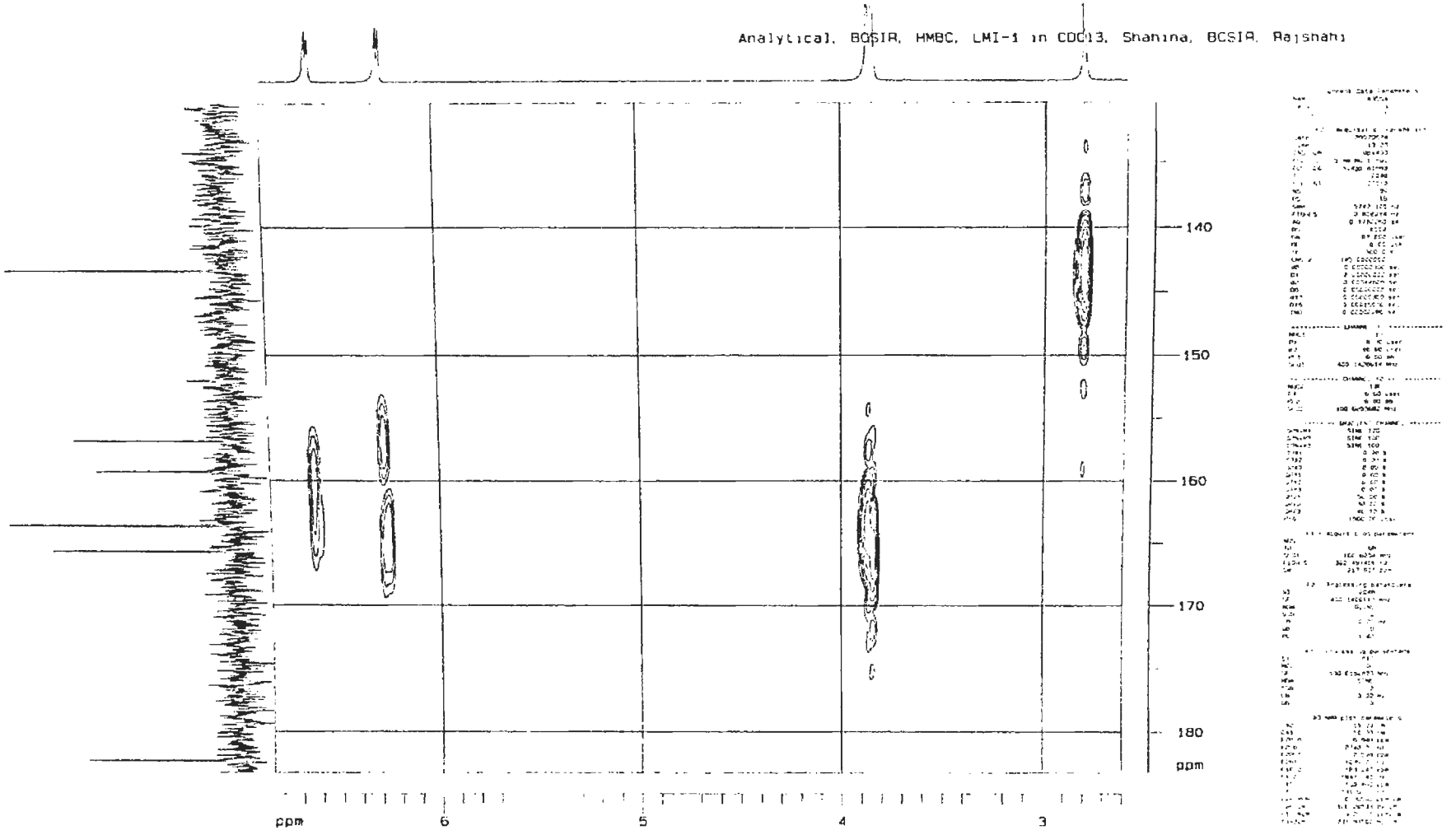


Fig. 17. HMBC spectrum of the compound LMI-1

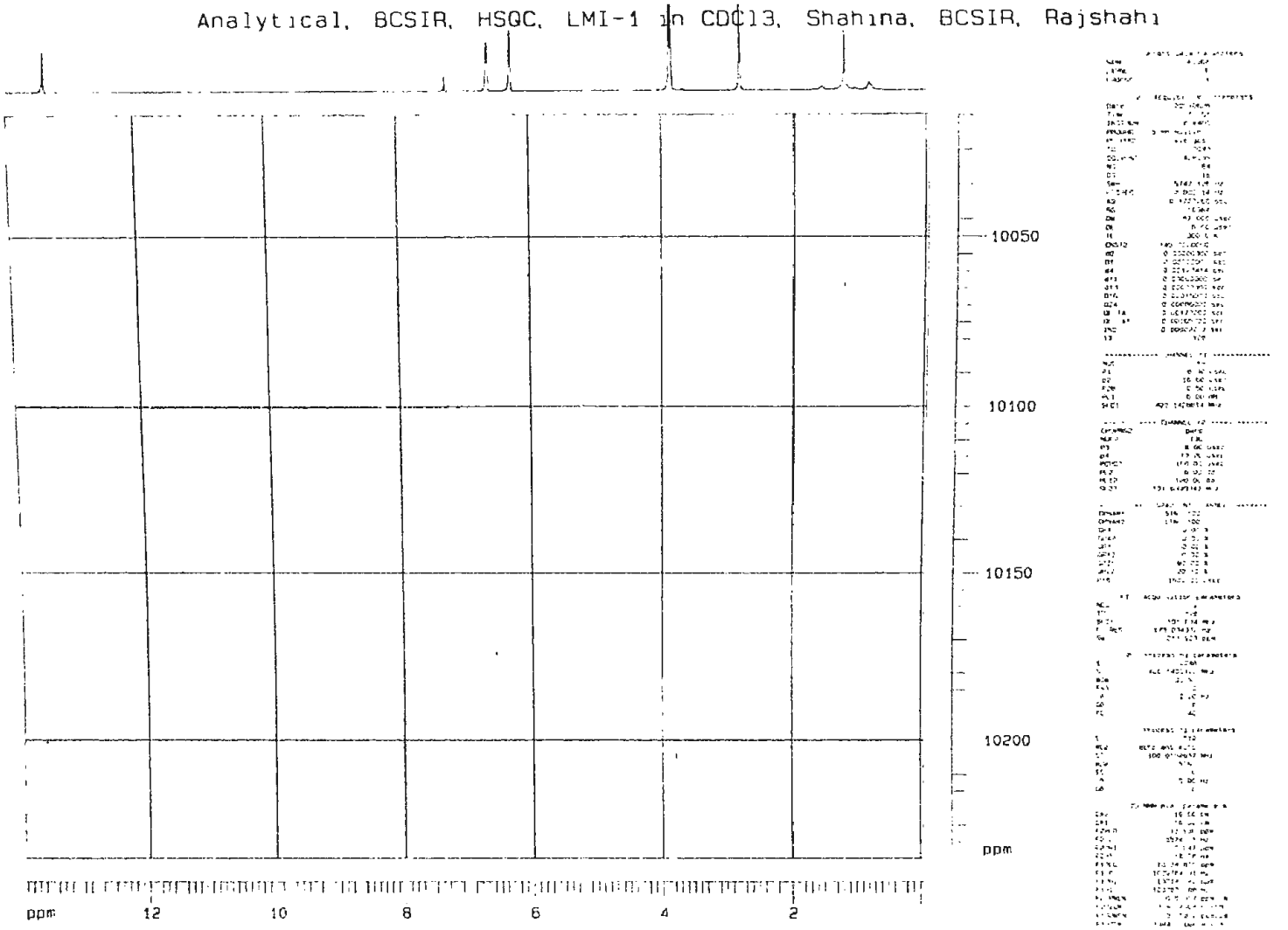
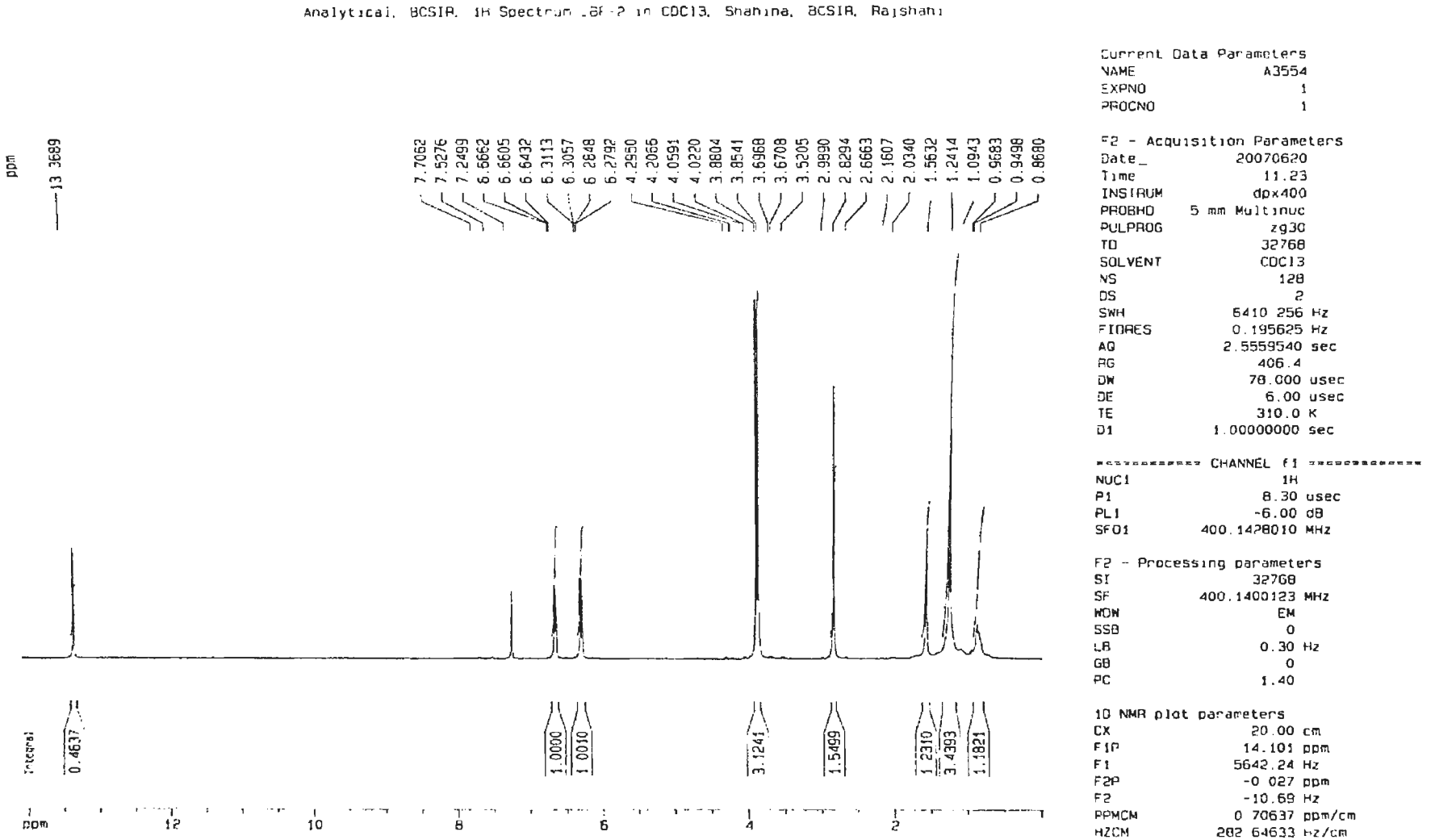


Fig. 18. HSQC spectrum of the compound LMI-1



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PROCNO 1

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Fig. 19. ^{1}H-NMR proton spectrum of the compound LBF-2

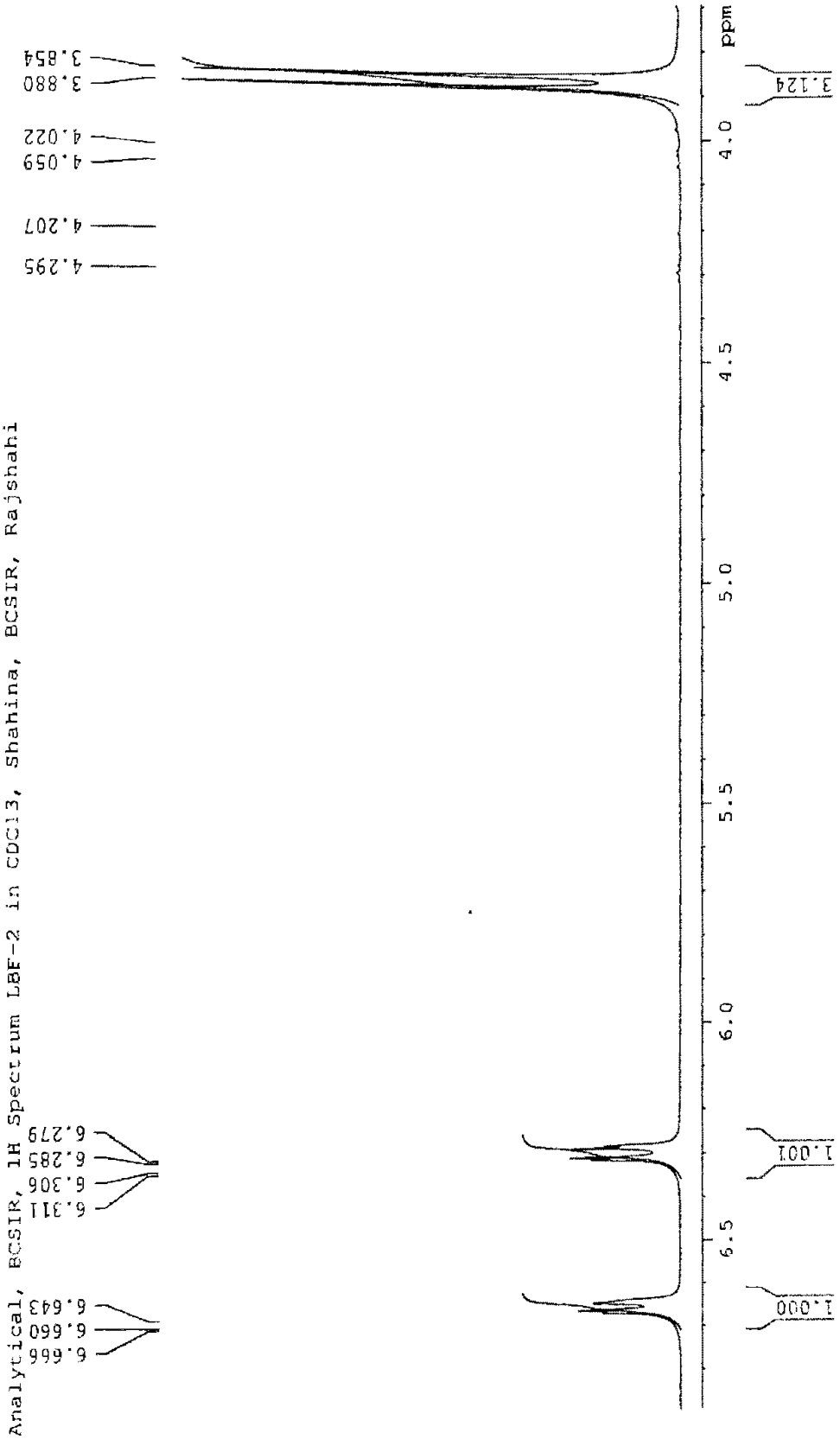


Fig. 20. ^{1}H-NMR proton spectrum of the compound LBF-2

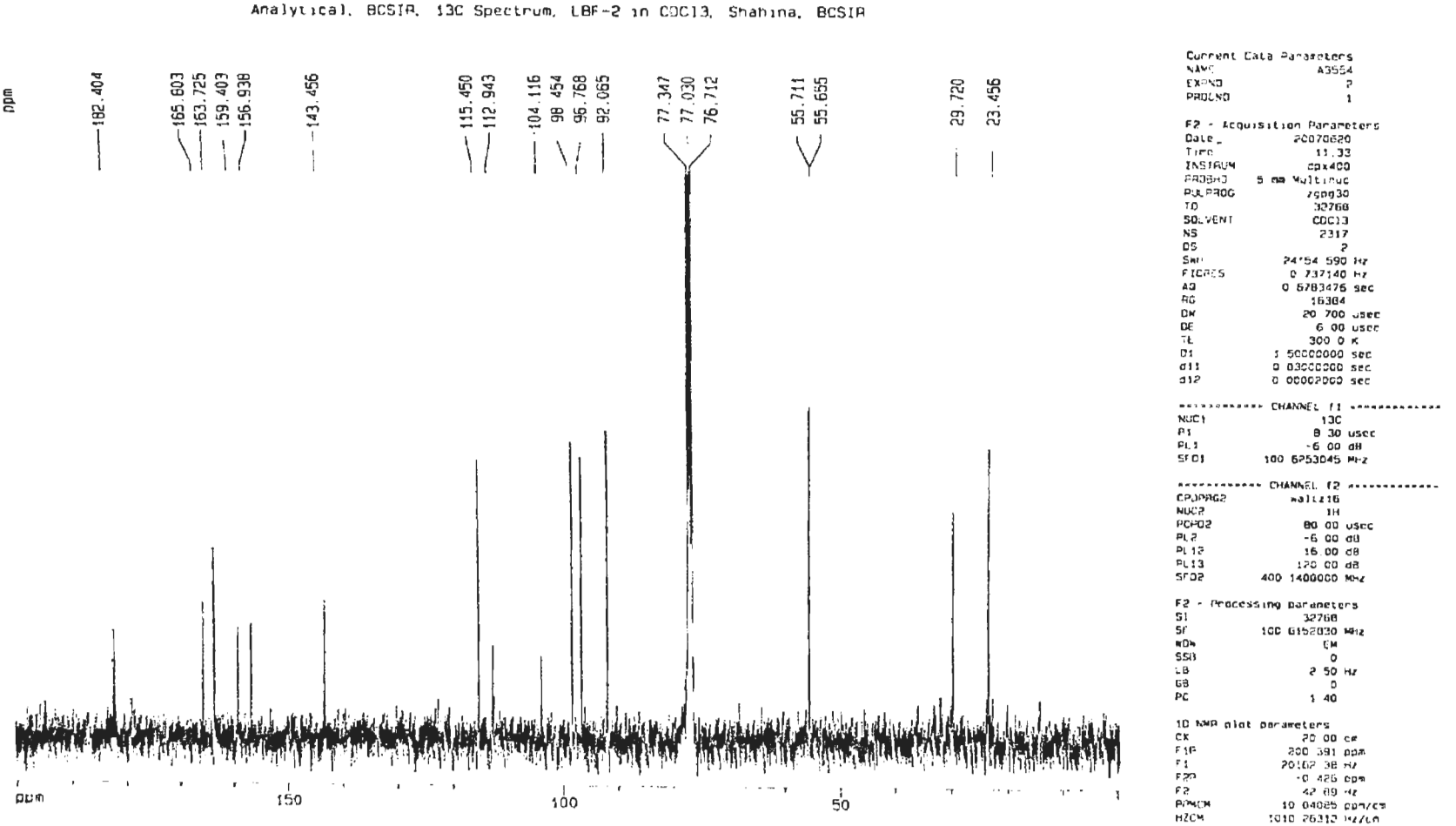


Fig. 21. ^{13}C-NMR spectrum of the compound LBF-2

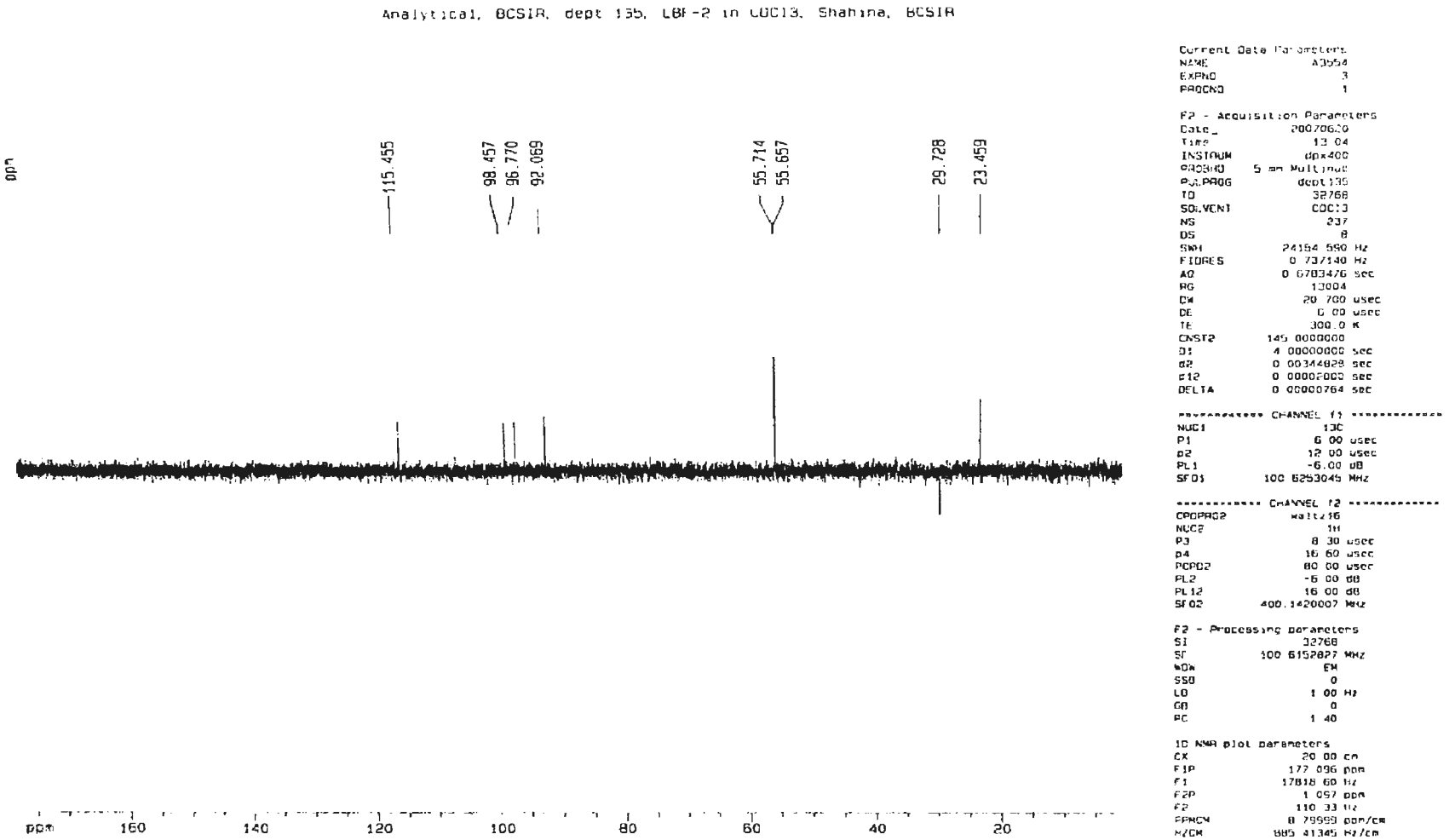


Fig. 22. ^{13}C-NMR spectrum of the compound LBF-2

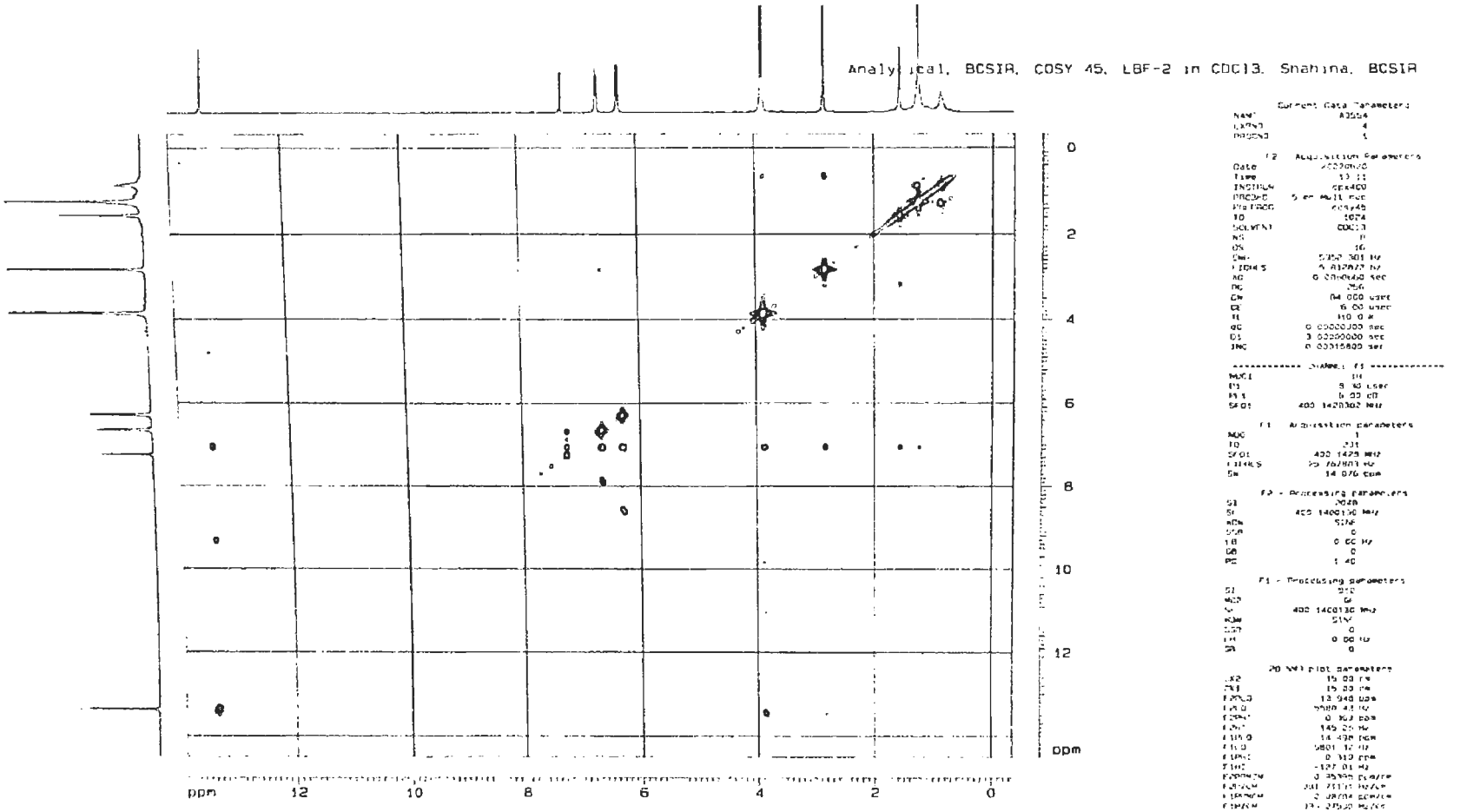


Fig. 23. ^{13}C-NMR spectrum for COSY of the compound LBF-2

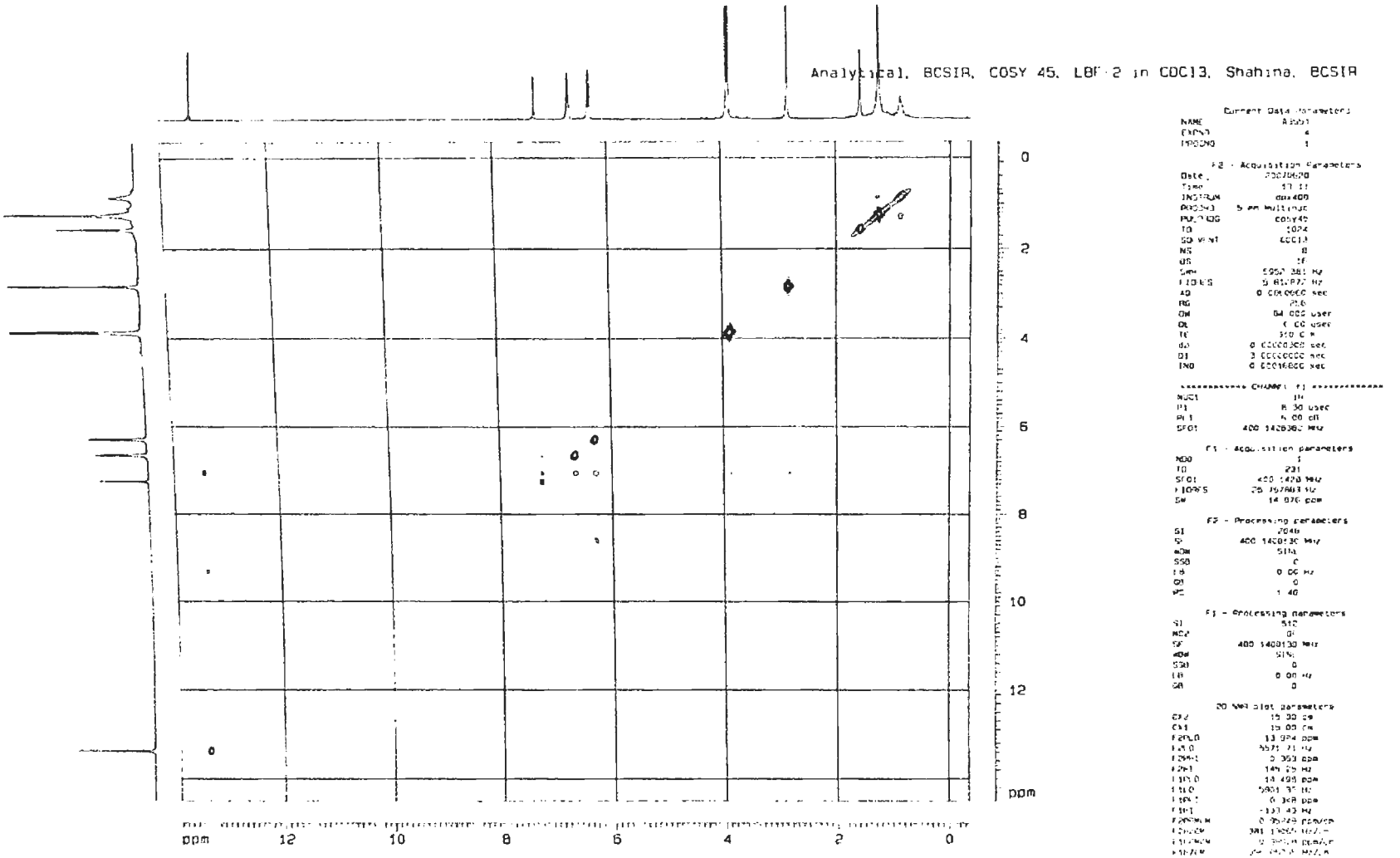


Fig. 24. ^{13}C-NMR spectrum for COSY of the compound LBF-2

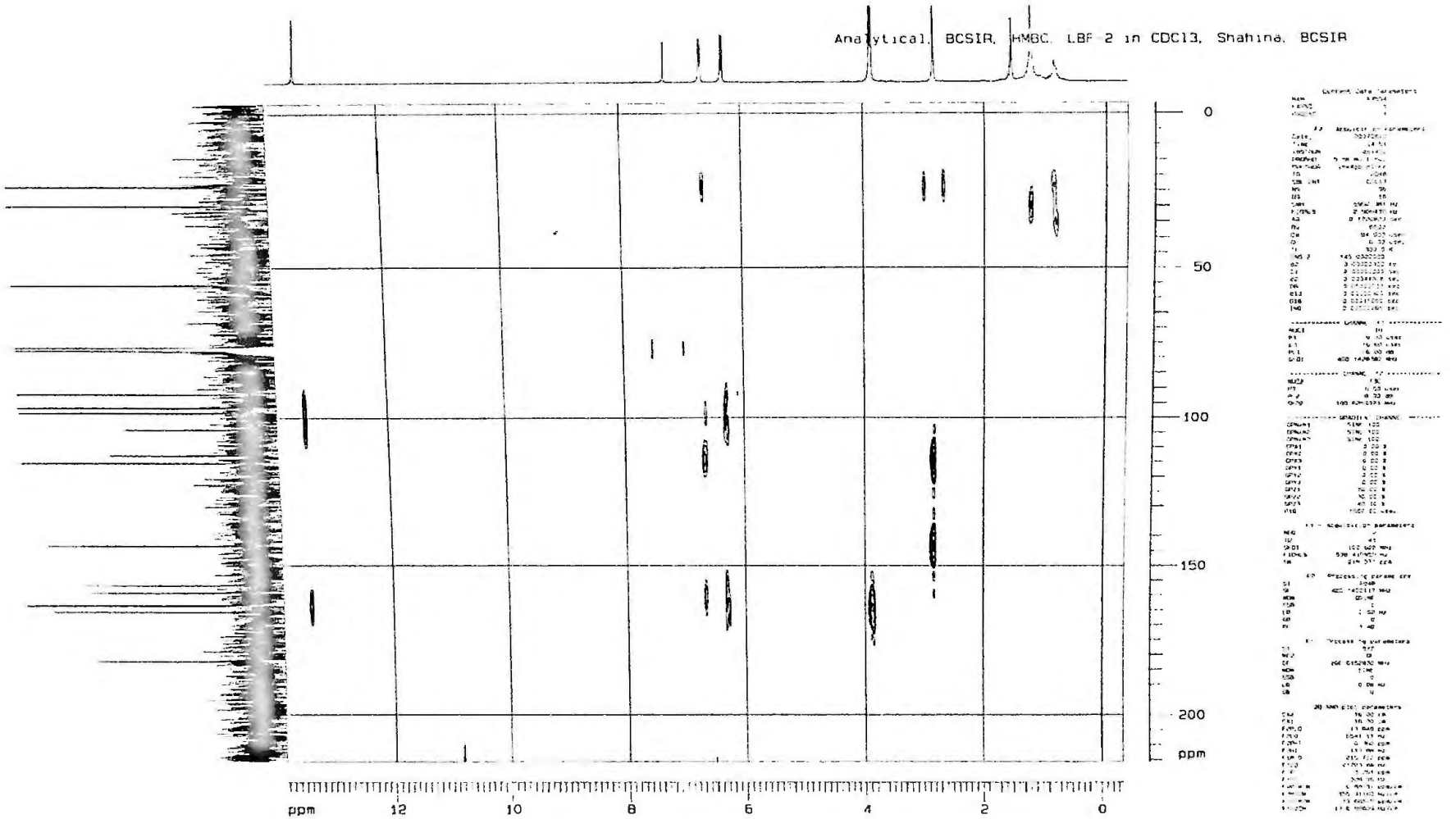


Fig. 25. HMBC spectrum of the compound LBF-2

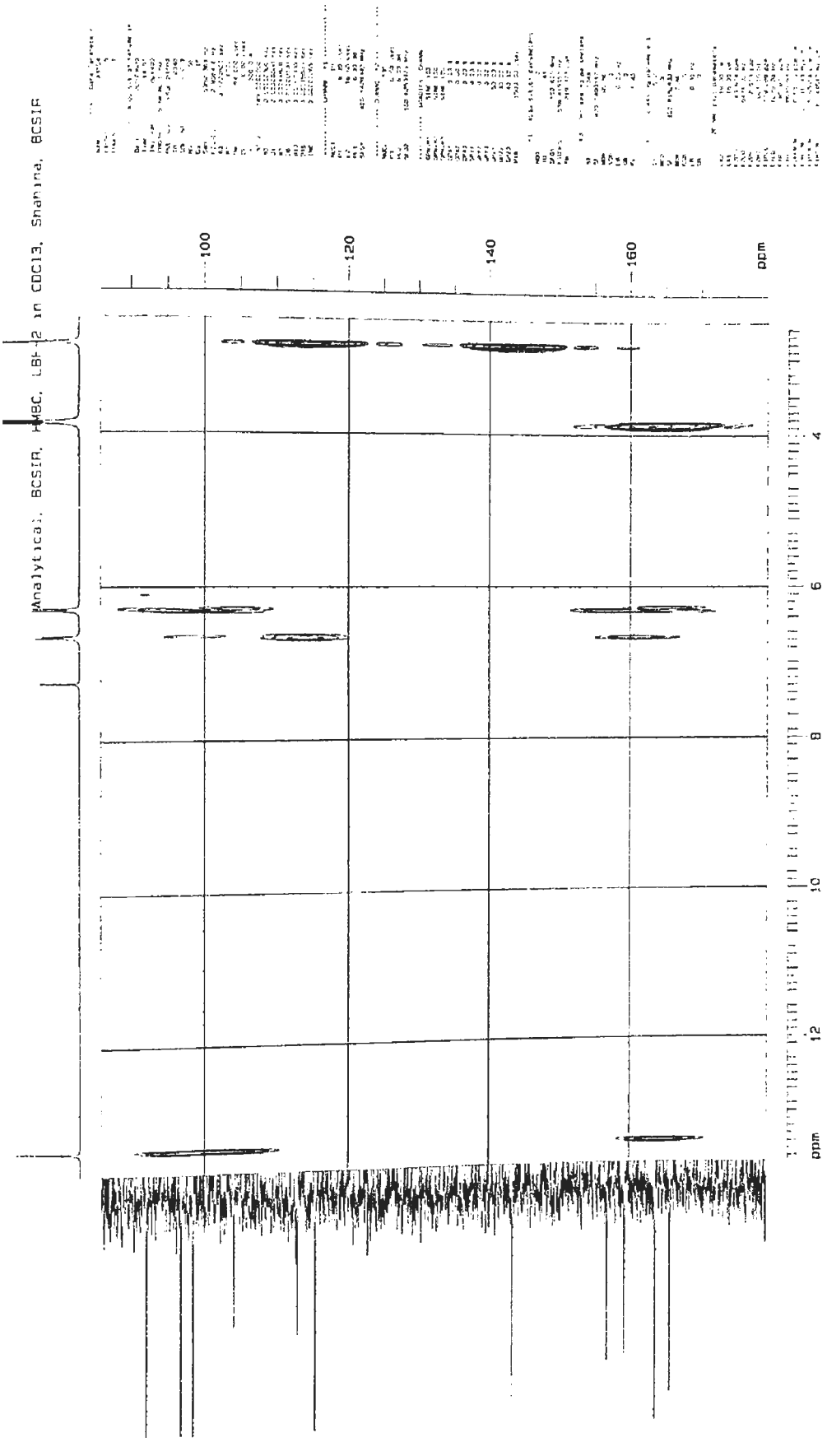


Fig. 26. HMBC spectrum of the compound LBF-2

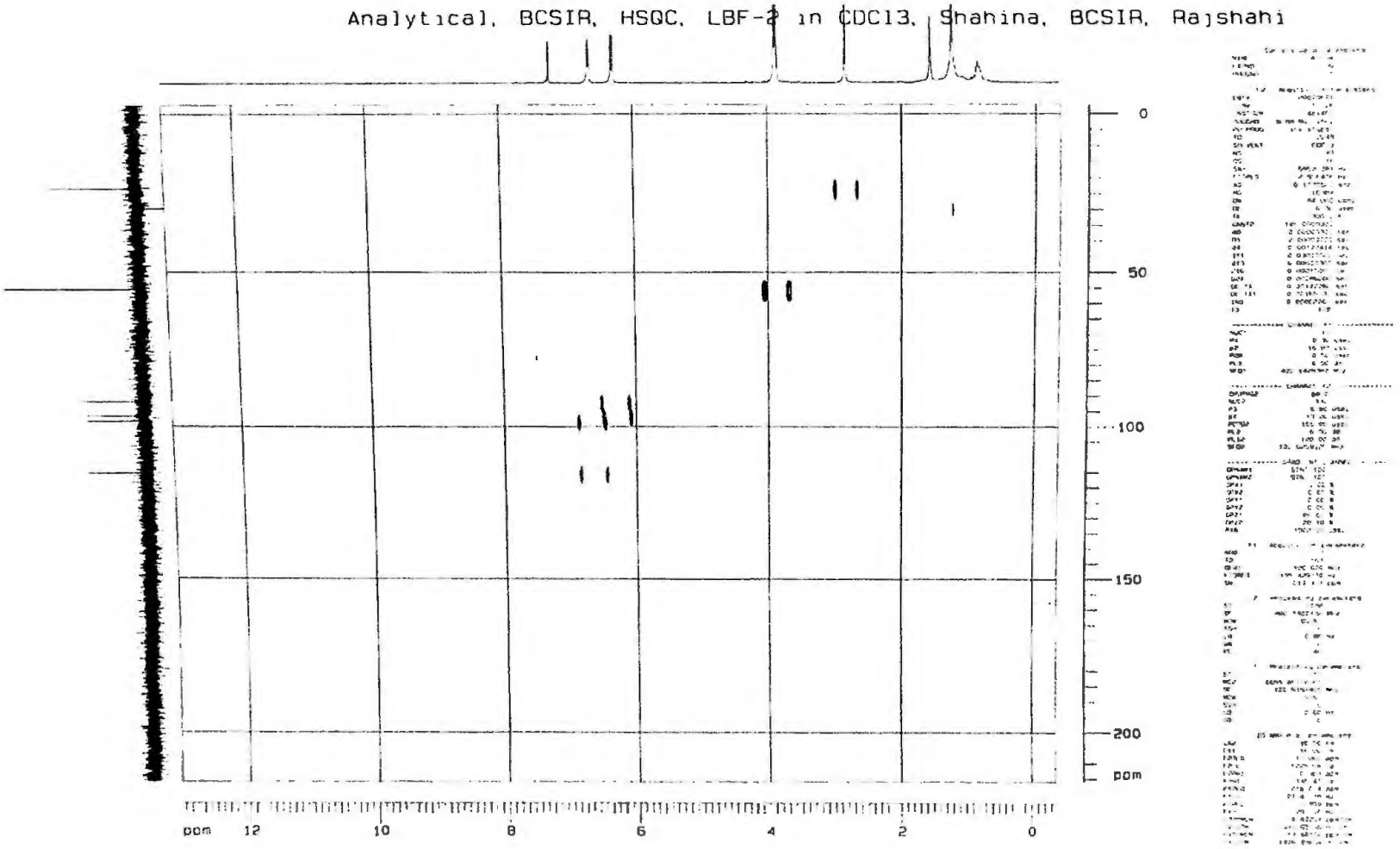


Fig. 27. HSQC spectrum of the compound LBF-2

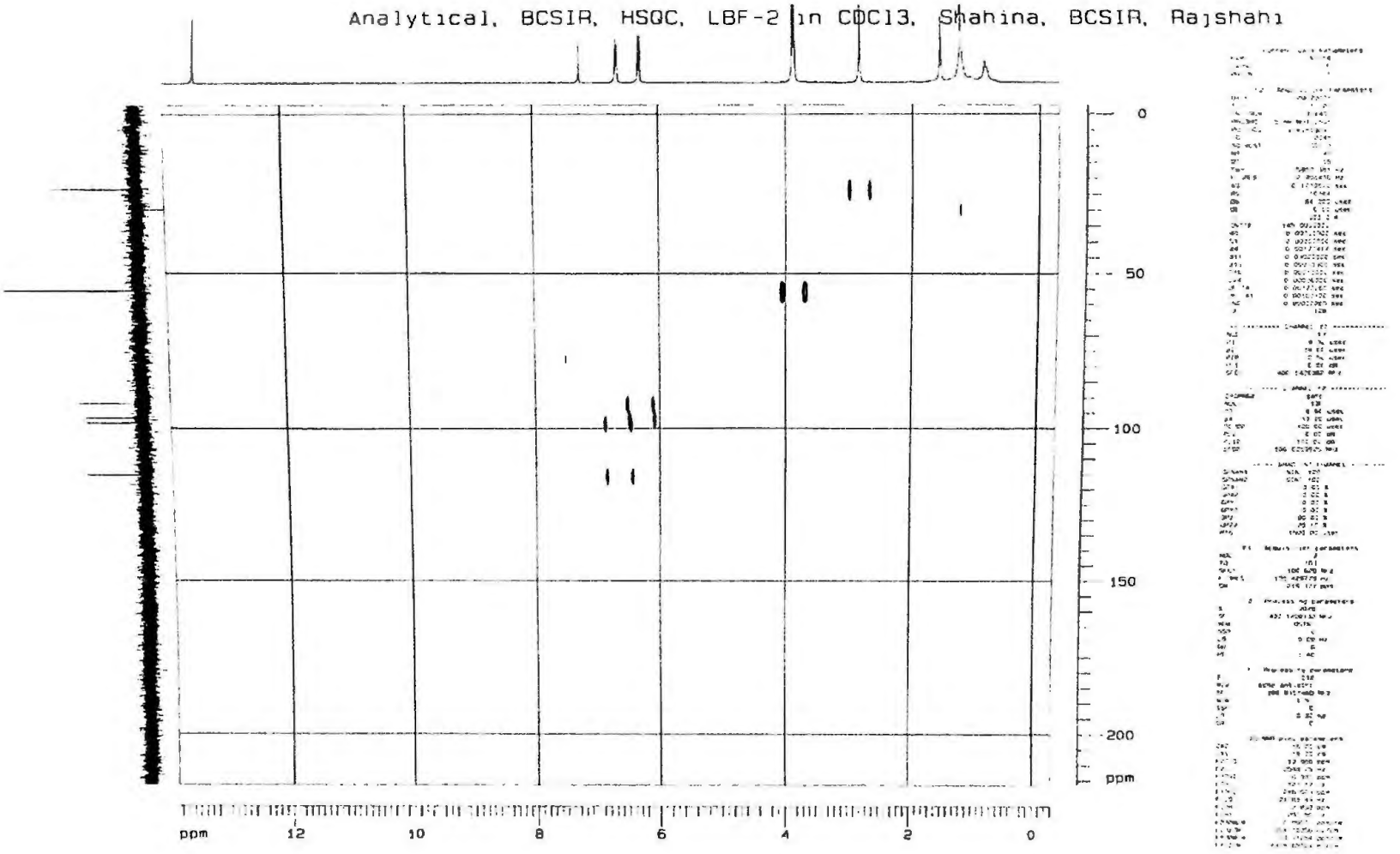


Fig. 28. HSQC spectrum of the compound LBF-2

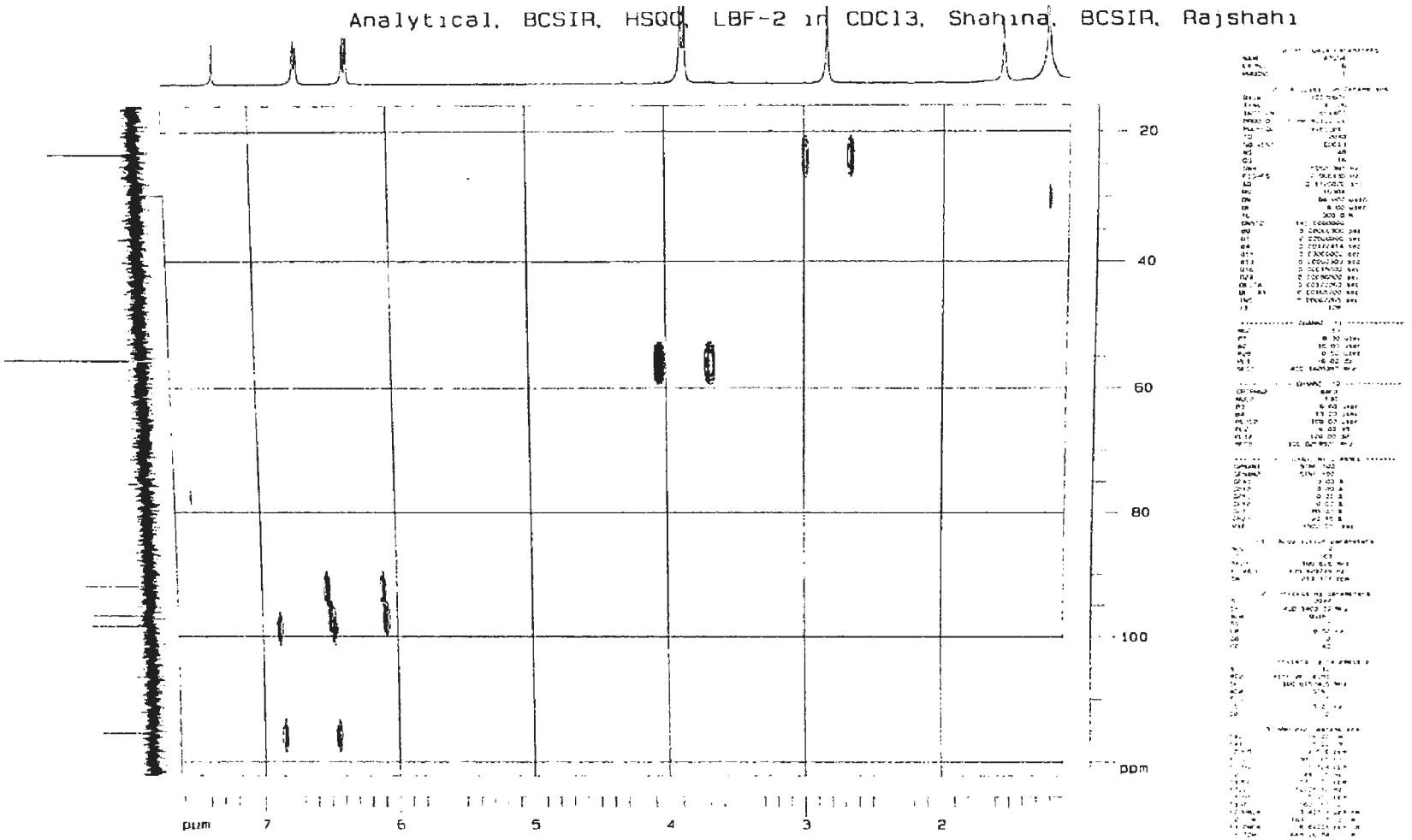


Fig. 29. HSQC spectrum of the compound LBF-2

3.7. Antimicrobial activities of the purified compounds

3.7.1. Antibacterial activities of the purified compounds

The anitbacterial activity of the pure compounds, LMI-1, LBF-2, LMI-3 and LMI-4 were tested against a number of selected test bacteria at a concentration of 200 µg/disc and the results were compared with a standard antibiotic, Ciprofloxacin 10µg/disc. The results obtained are shown in Tables 18. Among the 6 gram-positive bacteria, *B. aureus B. megaterium, B. subtilis, B. cereus, S. - β-haemolyticus* and *S. lutea* were responsive. Among them *B. aureus* was responsive to the LMI-1 pure compound with an inhibition zone of 8 mm at 200 µg/ disc application. For the LBF-2 pure compound, *B. aureus, B. megaterium, B. cereus* and *S. lutea* were responsive to give the inhibition zones 11-, 10-, 10- and 8 mm respectively. For the LMI-3 pure compound *B. aureus, B. cereus, S. - β-haemolyticus* and *S. lutea* were responsive with the inhibition zones 8-, 7-, 8- and 8 mm respectively. For the LMI-4 pure compound only *S.- β-haemolyticus* was responsive to give the clear zone of 7mm. However, the inhibition zones for the standard Ciprofloxacin 10 µg/disc were 35-, 35-, 40-, 42-, 38-, and 38 mm for the above mentioned test agents respectively.

Among the 6 gram-negative bacteria, *S typhi, S. dysenteriae S. shiga S. sonnei, S. boydii* and *E. coli* were responsive. While, only two bacteria *S. typhi* and *S. boydii* were responsive to the LMI-1 pure compound with the inhibition zones 7-and 8 mm respectively at 200 µg/disc application. For the LBF-2 pure compound, *S typhi, S. shiga* and *S. sonnei* were responsive with the inhibition zones 11-, 10- and 8 mm respectively. For the LMI-3 pure compound, *S typhi, S. shiga* and *S boydii* were responsive with the inhibition zones 10-, 7- and 9 mm respectively. For the LMI-4 pure compound, only *S. typhi* was responsive with a inhibition zone of 8 mm. The inhibition zones for the standard Ciprofloxacin 10 µg/disc were 40-, 42-, 42-, 38-, 38-, and 42 mm for the above mentioned test agents respectively.

Table 18. Activity of purified lichen compounds against pathogenic bacteria

SL.	Lichen compounds	Zone of inhibition (mm.) observed against Gram positive bacteria						Zone of inhibition (mm.) observed against Gram negative bacteria					
		S. aureus	*B. megaterium*	*B. subtilis*	*B. cereus*	*S.-β- haemolyticus*	*S. lutea*	*S. typhi*	*S. dysenteriae*	*S. shiga*	*S. sonnei*	*S. boydii*	*E. coli*
1	LMI-1	8	-	-	-	-	-	7	-	-	-	8	-
2	LBF-2	11	10	-	10	-	8	11	-	10	8	-	-
3	LMI-3	8	-	-	7	8	8	10	-	7	-	9	-
4	LMI-4	-	-	-	-	7	-	8	-	-	-	-	-
Ciprofloxacin		35	35	40	42	38	38	40	42	42	38	38	42

3.7.2. Antifungal effects of the purified compounds

The anitfungal activity of the pure compounds, LMI-1, LBF-2, LMI-3 and LMI-4 were determined at a concentration of 500 µg/disc against five pathogenic fungi. Nystatin (50µg/disc) was used as standard for comparison. The results obtained are shown in Table 19. Among the 5 pathogenic fungi, three were *A. fumigatus, C. albicans* and *F. vasinfectum* were responsive. Only *A. fumigatus* was responsive to the LMI-1 pure compound with inhibition zone 6 mm at 500 µg/disc. For the LBF-2 compound, *A. fumigatus, C. albicans* and *F. vasinfectum* were responsive with the inhibition zones 10-, 6- and 7mm respectively. For the LMI-3 compound, *A. fumigatus* and *F. vasinfectum* were responsive with the inhibition zones 8- and 6 mm respectively. For the LMI-4 compound, only *C. albicans* was responsive to give the inhibition zone 6 mm. The inhibition zones for the standard Nystatin 50µg/disc were 33-, 32-, 33-, 32- and 7 mm for the above mentioned test agents respectively.

Table 19. Activity of the purified lichen compounds against pathogenic fungi

SL.	Lichen Compounds	Clear zones (mm.) observed against pathogenic fungi				
		A. flavus	*A. fumigatus*	*C. albicans*	*Mucor* sp.	*F. vasinfectum*
1	LMI-1	-	6	-	-	-
2	LBF-2	-	10	6	-	7
3	LMI-3	-	8	-	-	6
4	LMI-4	-	-	6	-	-
5	Standard (Nystatin)	33	32	33	32	7

Chapter 4

DISCUSSION

Chapter 4 Discussion

4. Discussion

Potentiality of lichens for the production of bioactive compounds is an established truth since hundreds of lichen compounds have been introduced so far by the previous researchers. These compounds typically arise from the secondary metabolism of the fungal part of the lichen, however the compounds from the algal part is also tangible. For the symbiotic nature lichens produce glycoproteins which are related to parasitic mechanisms (Saikawa 1982; Elad *et al.*, 1983) in attack-defense processes on the basis of their antiviral (Kumar *et al.*, 1993) or antifungal (Viard *et al.*, 1993) activity, to ensure a defense against animal attack (Hoedemaeker *et al.*, 1993). Majority of the lichen substances are phenolic compounds (orcinol and *β*-orcinol derivatives), dibenzofuranes and usnic acids, depsides (barbatic acid), depsidones (salazinic acid), depsones (picrolichenic acid), lactones (protolichesterinic acid, nephrosterinic acid), quinones (parietin), and pulvinic acid derivatives (vulpinic acid), and normally the organic acids have impacts on living cells. However, these are the attributes led us to screen and isolate lichen constituents, and in Bangladesh this is still a virgin field of bioactive compound investigation from lichens.

In this investigation thirteen different host-plants have been selected for the collection of lichens for their screening and isolation of bioactive potentials. In the antimicrobial activity test majority of the collected samples h1, h2, h3, h4, h5, h6, h7, h8, h9, h10, h11, h12 and h13 found to offer antibacterial (against *S. aureus, B. megaterium, B. subtilis, B. cereus, S. β- haemolyticus, S. lutea, S. typhi, S. dysenteriae, S. shiga, S. sonnei, S. boydii* and *E. coli*) as well as antifungal (against *A. flavus, A. fumigatus, C. albicans, Mucor* sp. and *F. vasinfectum*) activity. These microorganisms are all pathogenic (to plant, animal and human being) and finding control agents for these dangerous organisms is of course interesting and something worthy. Majority of the extracts showed activity against nearly all of the test organisms, while against *E. coli* no efficacy was traced with the same dose maintained for the others. The highest activity was found in case of sample h5, where the inhibition zones were 24-, 20-, 18-, 20-, 20-, 22-, 23-, 18-, 20-, 28-, 26- and 0 mm respectively for above mentioned bacteria, followed by the sample h10 where the inhibition zones were 24-, 20-, 15-, 19-, 15-, 21-, 23-, 0-, 18-, 13-, 25- and 0 mm respectively. The lowest activity was recorded for the sample h9 where the inhibition zones were 0-, 0-, 0-, 7-, 0-, 7-, 0-, 0-, 0-, 0-, 12- and 0 mm respectively. In comparison of the crude extract activity a standard antibiotic was set against the test bacteria and the inhibition zones were 35-, 35-, 40-, 42-, 38-, 38-, 40-, 42-, 42-, 38-, 38- and 42 mm respectively. The dose was set for antibacterial activity test as 200 µg/disc for all the extracts and a standard Ciprofloxacin 10µg/disc was used for comparison. The minimum inhibitory concentration (MIC) values for both the bacteria agents were measured. The MIC values of the crude extracts against the bacteria for the lichen sample h5 and h10 ranges between 64- to 16 µg/ml and 128- to 8 µg/ml respectively in the broth medium.

Through antifungal activity tests *A. flavus, A. fumigatus, C. albicans, Mucor* sp. and *F. vasinfectum* have been used as test agents and the highest activity was found in case of sample h5, where the inhibition zones were 6-, 21-, 10-, 7- and 16 mm respectively, followed by the sample h10 where the

inhibition zones were 11-, 20-, 12-, 7- and 15 mm respectively. The lowest activity was recorded for the sample h9 where the inhibition zones were 10-, 9-, 10-, 12- and 9 mm respectively. In comparison of the crude extract activity a standard antibiotic was set against the test bacteria and the inhibition zones were 33-, 32-, 33-, 32- and 7 mm respectively. The dose was set for antifungal activity test as 500 µg/disc for all the extracts and a standard Nystatin 50µg/disc was used for comparison. The MIC values of the crude extracts against the fungi for the lichen sample h5 and h10 ranges between 1280- to 10 µg/ml and 1280- to 80 µg/ml respectively in the broth medium.

These findings are supported by many previous workers, since the lichen-forming fungi produce antibiotic secondary metabolites that provide protection from most animals and pathogenic microorganisms (Vartia, 1973; Rundel, 1978; Lawrey, 1984, 1986). Nevertheless, certain obligate fungivorous animals (Lawrey, 1983) and fungal parasites (Lawrey, 1995) consume lichens. Discovery of penicillin is a big dogma in favour of this research agenda. Stoll and his/her group (1950) introduced several lichen compounds to show activity against mycobacteria and gram-positive organisms.

Since we don't have sophisticated laboratory set up for such a critical and huge work, major active samples of the screened lichens that might have comparatively a bigger amount of extract to go through different tests were taken into consideration for thorough investigation. The sample h5 (collected from *A. chinensis*) was one of the two (h5 and h10) highly active samples, but the amount of extract was very little and was impossible to carry on with it). However, the lichen samples h4, h10 and h13 collected from *Morus indica*, *Borassus flabellifer* and *Mangifera indica* respectively were selected finally for thorough investigation. The crude extracts of these samples have been screened until the determination of the minimum inhibitory concentrations (MICs), and isolation of the bioactive compounds through activity guided fractionation and even determination of MICs for the purified compounds as well.

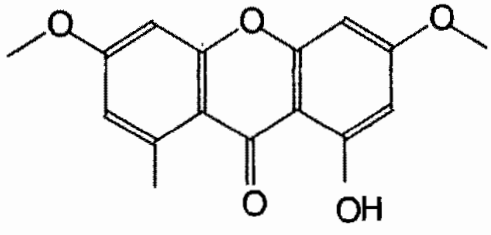
The median lethal concentration or LC_{50} values of the tested crude chloroform extracts of the lichen samples were found promising against the 1 day nauplii of *A. salina*. The LC_{50} values for lichen extracts were 14.245-, 8.568-, 16.260-, 9.419-, 8.994-, 2.333-, 9.386-, 6.871-, 17.124-, 11.611-, 38.604-, 18.776- and 20.529 ppm respectively for 24h of exposure and according to the intensity of efficacy the lichen extracts could be arranged in a descending order of the samples h6> h8> h2> h5> h7> h4> h10> h1> h3> h9> h12> h13> h11. Concerning new lichen secondary metabolites that exhibit relevant cytotoxic activities against cancer cell lines, original N-containing complex structures (scabrosin esters) isolated from *Xanthoparmelia scabros* (Ernst-Russell *et al.*, 1999b). Esimone and Adikwu (2002) reported that they had extractives from the lichen *Ramalina farinacea* (L.) were depsides and depsidones, and they were bioactive (Esimone *et al.*, 1999) and the bioactivity was assessed according to the brine shrimp lethality assay technique (McLaughlin *et al.*, 1991; Esimone and Adikwu, 1999).

Chloroform extracts of the lichen samples were tested for repellent activity against *T. castaneum* adults. All the lichen extracts showed repellent activity, of which extracts of the sample h4 and h5 highly effective ($P<0.001$), sample h2, h6 and h8 positively effective $P<0.01$) and the rest showed repellency at $P<0.05$ level of significance. The sample h4 is one of the three selected plants in this investigation. However, no previous works have been traced to be carried out to show repellent activity of the lichen extracts or lichen compounds.

The lichen extracts were subjected to insecticidal activity, however in the *Ad Hoc* experiments no insecticidal activity was traced so far. While in the toxicological profile a selected extract was considered to apply against the larvae of the red flour beetle, *T. castaneum*. The chloroform extract of the lichen sample h10 was applied through dose-mortality assay at doses 5-, 25-, 75-, 150- and 200 mg/g, where the 2nd, 3rd and 4th instar larvae were released into the treated food to observe mortality or any sort of abnormality due to the effect of the extract in comparison to the control dose. This step

offered mortality of the larvae, however no supporting works have been found in this regard. Mortality of the 3rd instar larvae of *M. domestica* was another supporting work that has been achieved against the lichen h10 extract.

Isolation of the bioactive compound was one of the important objectives of this work. Four compounds have been isolated and purified from h4 (one/ named LMI-1), h10 (one/ Named LBF-2) and h13 (two/ named LMI-3 and LM-4) of which the LMI-1 and LBF-2 were subjected to NMR and relevant analyses for the determination of structures. Compound LMI-1 was isolated from *Morus indica*. The samples LMI-1 and LBF-2 were subjected to NMR analyses at the BCSIR Laboratories, Dhaka, Bangladesh. The molecular ion peak (M/z=286.29) of high resolution EIMS spectrum suggested that the molecular formula should be $C_{16}H_{14}O_5$ (calcd. M/z=286.08). Yellow crystals; mp 165 - 166 °C, lit. 187 - 190°C; [3] UV λ_{max} (MeOH) nm (log e) 250 (2.50) and 312 (2.28) nm; UV, ^{1}H NMR, and 13C NMR data (recorded in CDCl3), consistent with literature values of Woodson and Schery (1965) and García and Brown (1976) and the compound is a known one and named as lichexanthone.



Lichexanthone

Compounds LMI-1and LBF-2 is the same compound, even though they are isolated from different sources. May be the amount of impurities, represented by singals in proton NMR between 0-2ppm (probably grease, fat, residual solvent, etc.) are of different amount.

The anitbacterial activity of the pure compounds, LMI-1, LBF-2, LMI-3 and LMI-4 were tested against the same set of test bacteria taken into consideration for the primary screening of the crude extracts along with same doses for both the extracts and the standard. Among the Gram-positive bacteria, *B. aureus B. megaterium, B. subtilis, B. cereus, S.- β-haemolyticus*,

and *S.lutea* were responsive. Among them *B. aureus* was responsive to the LMI-1 pure compound with an inhibition zone of 8 mm at 200 µg/ disc application. For the LBF-2 pure compound, *B. aureus, B. megaterium, B. cereus* and *S. lutea* were responsive to give the inhibition zones 11-, 10-, 10- and 8 mm respectively. For the LMI-3 pure compound *B. aureus, B. cereus, S.- β-haemolyticus*, and *S. lutea* were responsive with the inhibition zones 8- , 7-, 8- and 8 mm respectively. For the LMI-4 pure compound only *S. -β-haemolyticus* was responsive to give the clear zone of 7mm.

Among the Gram-negative bacteria, *S typhi, S. dysenteriae S. shiga S. sonnei, S. boydii* and *E. coli* were responsive. While, only two bacteria *S. typhi* and *S. boydii* were responsive to the LMI-1 pure compound with the inhibition zones 7-and 8 mm respectively at 200 µg/disc application. For the LBF-2 pure compound, *S typhi, S. shiga* and *S. sonnei* were responsive with the inhibition zones 11-, 10- and 8 mm respectively. For the LMI-3 pure compound, *S typhi, S. shiga* and *S boydii* were responsive with the inhibition zones 10-, 7- and 9 mm respectively. For the LMI-4 pure compound, only *S. typhi* was responsive with a inhibition zone of 8 mm. The inhibition zones for the standard Ciprofloxacin 10 µg/disc were 40-, 42-, 42-, 38-, 38-, and 42 mm for the above mentioned test agents respectively.

The anitfungal activity of the pure compounds, LMI-1, LBF-2, LMI-3 and LMI-4 were tested against the same set of test bacteria taken into consideration for the primary screening of the crude extracts along with same doses for both the extracts and the standard for better comparison. Among the test fungi, three were *A. fumigatus, C. albicans* and *F. vasinfectum* were responsive. Only *A. fumigatus* was responsive to the LMI-1 pure compound with inhibition zone 6 mm. For the LBF-2 compound, *A. fumigatus, C. albicans* and *F. vasinfectum* were responsive with the inhibition zones 10-, 6- and 7mm respectively. For the LMI-3 compound, *A. fumigatus* and *F. vasinfectum* were responsive with the inhibition zones 8- and 6 mm respectively. For the LMI-4 compound, only *C. albicans* was responsive to give the inhibition zone 6 mm. No activity was traced for the *A. flavus* and *Mucor* sp.

The results showed that the efficacy of the purified compounds are weaker in comparison to their respective crude extracts. However, efficacy of the purified compound (s) have been found with reduction in intensity, while it is adverse to that of expectation. It is may be because of the nature of the lichen compounds that show stronger activity in combination with other molecules. It is possible that two or more compounds work in concert with one another to inhibit growth of both the gram positive and gram negative bacteria or the test fungi.

However, according to the facilities available there in the Crop Protection Laboratory and other accessible laboratories of the Institute of Biological Sciences, Department of Biochemistry and Molecular Biology at Rajshahi University, Rajshahi-6205, Bangladesh and Bangladesh Council of Scientific and Industrial Research, Rajshahi, sophistication of this work was not possible. The main objective of this research revolves no doubt in tracing out and isolate bioactive potentials of lichens, and it offered four pure compounds LMI-1, LBF-2, LMI-3 and LMI-4 of which the LMI-1 and LBF-2 were the same even though they belong to two different lichen sources. Due to insufficiency in amount other two LMI-3 and LMI-4 were not possible to go through NMR analysis. Identification of lichens has also been declined, but biological activity tests of the crude extracts as well as the purified compounds have been successfully done. Anyway, the inhibiting activity of the lichen extractives (crude and pure) against the human and plant pathogenic bacteria and fungi have an immense value through pharmacological/ pharmaceutical, medicinal, toxicological and agricultural/ pesticidal point of view. The reason for decreasing activity of the pure compounds (in comparison to their respective crude extracts) has to be investigated.

The findings of this work along with the findings of the previous researchers triggered a hope for further progress in research with these promising sources (lichens) towards a molecular level investigation of its pharmacological, agricultural/ pesticidal or other effective but environment friendly potentials for a happy leading of human life.

Chapter 5

LITERATURE CITED

Chapter 5 Literature Cited

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Chapter 6

APPENDICES

Chapter 6 Appendices

Appendix Table I. Dose-mortality effect of lichen h1 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	25	27.778	23	4.26	4.184	4.284	42.39	4.194
2	0.301	90	26	28.889	24	4.29	4.394	4.298	47.88	4.404
4	0.602	90	33	36.667	32	4.53	4.604	4.524	54.09	4.614
8	0.903	90	47	52.222	49	4.97	4.814	4.994	56.43	4.825
16	1.204	90	47	52.222	49	4.97	5.024	4.975	57.33	5.035

Results:
Y = 4.193192 + 0.6993553 X
Chi-squared is 3.149622 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.153647
LD_{50} is 14.24448 ppm
95% Confidence limits are 7.576385 to 26.78126 ppm

Appendix Table II. Dose-mortality effect of lichen h2 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	24	26.667	21	4.19	4.288	4.184	45.27	4.283
2	0.301	90	36	40.000	36	4.64	4.517	4.628	52.29	4.514
4	0.602	90	39	43.333	39	4.72	4.746	4.714	55.44	4.746
8	0.903	90	50	55.556	52	5.05	4.975	5.04	57.06	4.977
16	1.204	90	52	57.778	55	5.13	5.204	5.15	56.43	5.208

Results:
Y = 4.282913 + 0.7686748 X
Chi-squared is 1.593212 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 0.9328882
LD_{50} is 8.568172 ppm
95% Confidence limits are 5.502341 to 13.34225 ppm

Appendix Table III. Dose-mortality effect of lichen h3 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	17	18.889	13	3.87	3.900	3.88	36.45	3.934
2	0.301	90	20	22.222	17	4.05	4.176	4.056	42.39	4.199
4	0.602	90	38	42.222	38	4.69	4.452	4.72	50.22	4.464
8	0.903	90	40	44.445	40	4.75	4.728	4.74	55.44	4.729
16	1.204	90	45	50.000	46	4.90	5.004	4.90	57.33	4.994

Results:
Y = 3.933982 + 0.8801878 X
Chi-squared is 4.785862 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.211126
LD_{50} is 16.2602 ppm
95% Confidence limits are 9.463426 to 27.93854 ppm

Appendix Table IV. Dose-mortality effect of lichen h4 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	16	17.778	12	3.82	3.960	3.832	36.45	3.982
2	0.301	90	31	34.444	30	4.48	4.281	4.490	45.27	4.297
4	0.602	90	33	36.667	32	4.53	4.602	4.524	54.09	4.611
8	0.903	90	49	54.445	51	5.03	4.923	5.015	57.06	4.926
16	1.204	90	53	58.889	56	5.15	5.244	5.176	56.43	5.240

Results:
Y = 3.982127 + 1.04506 X
Chi-squared is 3.612717 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 0.9739855
LD_{50} is 9.418581 ppm
95% Confidence limits are 6.686605 to 13.26677 ppm

Appendix Table V. Dose-mortality effect of lichen h5 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	8	8.889	2	2.95	3.350	3.042	18.72	3.329
2	0.301	90	14	15.556	10	3.72	3.870	3.720	33.3	3.856
4	0.602	90	39	43.334	39	4.72	4.3882	4.778	47.88	4.384
8	0.903	90	44	48.889	45	4.87	4.908	4.865	57.06	4.911
16	1.204	90	59	65.556	63	5.33	5.427	5.321	54.09	5.438

Results:
Y = 3.329212 + 1.751474 X
Chi-squared is 10.47137 with 3 degrees of freedom
Variance has been adjusted for heterogeneity
Log LD_{50} is 0.9539328
LD_{50} is 8.993584 ppm
95% Confidence limits are 6.142839 to 13.16729ppm

Appendix Table VI. Dose-mortality effect of lichen h6 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	40	44.445	40	4.75	4.68	4.74	54.09	4.680
2	0.301	90	48	53.333	50	5.00	4.947	4.99	57.06	4.942
4	0.602	90	48	53.333	50	5.00	5.214	5.02	56.43	5.204
8	0.903	90	63	70.000	68	5.47	5.481	5.46	54.09	5.466
16	1.204	90	73	81.111	80	5.85	5.748	5.83	47.88	5.728

Results:
Y = 4.679814 + 0.8703174 X
Chi-squared is 2.740685 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 0.367896
LD_{50} is 2.332899 ppm
95% Confidence limits are 1.634172 to 3.330382 ppm

Appendix Table VII. Dose-mortality effect of lichen h7 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	7	7.778	1	2.67	3.758	3.198	30.24	3.780
2	0.301	90	32	35.556	31	4.50	4.147	4.588	42.39	4.158
4	0.602	90	39	43.333	39	4.72	4.536	4.712	52.29	4.535
8	0.903	90	44	48.889	45	4.87	4.925	4.865	57.06	4.913
16	1.204	90	54	60.000	57	5.18	5.314	5.162	55.44	5.290

Results:
Y = 3.780301 + 1.254203 X
Chi-squared is 20.77454 with 3 degrees of freedom
Variance has been adjusted for heterogeneity
Log LD_{50} is 0.9724892
LD_{50} is 9.386188 ppm
95% Confidence limits are 4.408817 to 19.9828 ppm

Appendix Table VIII. Dose-mortality effect of lichen h8 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	25	27.778	23	4.26	4.372	4.266	47.88	4.379
2	0.301	90	39	43.333	39	4.72	4.594	4.712	52.29	4.602
4	0.602	90	42	46.667	43	4.82	4.816	4.838	56.43	4.826
8	0.903	90	51	56.667	54	5.10	5.038	5.100	57.33	5.049
16	1.204	90	54	60.000	57	5.18	5.260	5.202	56.43	5.273

Results:
Y = 4.378612 + 0.7423761 X
Chi-squared is 1.676981 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 0.8370256
LD_{50} is 6.87109 ppm
95% Confidence limits are 4.543669 to 10.39069 ppm

Appendix Table IX. Dose-mortality effect of lichen h9 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure.

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	10	11.111	5	3.36	3.556	3.365	24.21	3.636
2	0.301	90	22	24.445	19	4.12	3.9178	4.154	36.45	3.969
4	0.602	90	27	30.000	25	4.33	4.280	4.320	45.27	4.301
8	0.903	90	39	43.333	39	4.72	4.642	4.713	54.09	4.634
16	1.204	90	44	48.889	45	4.87	5.004	4.875	57.33	4.967

Results:
Y = 3.6356 + 1.106031 X
Chi-squared is 3.864468 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.2336
LD_{50} is 17.1238 ppm
95% Confidence limits are 10.90548 to 26.88781 ppm

Appendix Table X: Dose-mortality effect of lichen h10 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	15	16.667	11	3.77	3.838	3.771	33.3	3.864
2	0.301	90	26	28.889	24	4.29	4.167	4.322	42.39	4.185
4	0.602	90	30	33.333	29	4.45	4.496	4.450	50.22	4.506
8	0.903	90	42	46.667	43	4.82	4.825	4.838	56.43	4.827
16	1.204	90	53	58.889	56	5.15	5.154	5.14	57.06	5.149

Results:
Y = 3.863864 + 1.066935 X
Chi-squared is 1.251904 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.06486
LD_{50} is 11.61075 ppm
95% Confidence limits are 7.978849 to 16.89587 ppm

Appendix Table XI. Dose-mortality effect of lichen h11 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	13	14.445	8	3.59	3.590	3.596	24.21	3.613
2	0.301	90	15	16.667	11	3.77	3.859	3.771	33.3	3.876
4	0.602	90	27	30.000	25	4.33	4.128	4.36	42.39	4.139
8	0.903	90	25	27.778	23	4.26	4.397	4.266	47.88	4.403
16	1.204	90	38	42.222	38	4.69	4.666	4.686	54.09	4.666

Results:
Y = 3.613105 + 0.8741152 X
Chi-squared is 3.353846 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.586627
LD_{50} is 38.60354 ppm
95% Confident limits are 16.29454 to 91.45607 ppm

Appendix Table XII. Dose-mortality effect of lichen h12 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	7	7.778	1	2.67	3.323	2.936	18.72	3.256
2	0.301	90	17	18.889	13	3.87	3.713	3.894	30.24	3.668
4	0.602	90	21	23.333	18	4.08	4.103	4.094	42.39	4.081
8	0.903	90	33	36.667	32	4.53	4.492	4.54	50.22	4.493
16	1.204	90	42	46.667	43	4.82	4.882	4.838	56.43	4.905

Results:
Y = 3.256262 + 1.369133 X
Chi-squared is 3.830929 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.273608
LD_{50} is 18.7762 ppm
95% Confidence limits are 12.68055 to 27.80208 ppm

Appendix Table XIII. Dose-mortality effect of lichen h13 extract ($CHCl_3$) against *A. salina* nauplii after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
1	0.000	90	12	13.333	7	3.52	3.664	3.529	27.18	3.726
2	0.301	90	20	22.222	17	4.05	3.979	4.062	36.45	4.018
4	0.602	90	33	36.667	32	4.53	4.294	4.558	45.27	4.311
8	0.903	90	32	35.556	31	4.5	4.609	4.497	54.09	4.602
16	1.204	90	44	48.889	45	4.87	4.924	4.865	57.06	4.895

Results:
Y = 3.726258 + 0.9705613 X
Chi-squared is 4.553513 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.312376
LD_{50} is 20.5294 ppm
95% Confidence limits are 11.70103 to 36.01875 ppm.

Appendix Table XIV. Repellency of *T. castaneum* adults by lichen sample h1 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Areca catechu* | Sample Code: h1

Dose ($\mu g/cm^2$)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	10.00	10.00	10.00	10.00	10.00	100	100	100	100	100	90.00	90.00	90.00	90.00	90.00
		R2	10	10	10	10	10															
		R3	10	10	10	10	10															
157.27	10	R1	10	10	10	10	10	10.00	9.33	10.00	9.66	9.66	100	86.6	100	93.2	93.2	90.00	68.53	90.00	74.88	74.88
		R2	10	10	10	10	9															
		R3	10	8	10	9	10															
78.63	10	R1	10	10	10	10	10	8.66	9.66	9.33	10.00	9.66	73.2	93.2	86.6	100	93.2	58.82	74.88	68.53	90.00	74.88
		R2	7	10	10	10	9															
		R3	9	9	8	10	10															
39.32	10	R1	6	5	5	5	5	8.33	8.00	7.33	7.00	7.00	66.6	60.0	46.6	40.0	40.0	54.70	50.77	43.05	39.23	39.23
		R2	9	9	8	9	9															
		R3	10	10	9	7	7															

Appendix Table XV. Repellency of *T. castaneum* by lichen sample h2 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Amoora rohituka* | Sample Code: h2

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	10.00	10.00	10.00	10.00	10.00	100	100	100	100	100	90.00	90.00	90.00	90.00	90.00
		R2	10	10	10	10	10															
		R3	10	10	10	10	10															
157.27	10	R1	9	10	10	10	10	9.66	9.66	9.00	9.66	9.66	93.2	93.2	80.0	93.2	93.2	74.88	74.88	63.44	74.88	74.88
		R2	10	10	9	9	9															
		R3	10	9	8	10	10															
78.63	10	R1	10	10	10	10	10	9.66	9.66	9.33	9.33	9.00	93.2	93.2	86.6	86.6	80.0	74.88	74.88	68.53	68.53	63.44
		R2	10	10	8	8	7															
		R3	9	9	10	10	10															
39.32	10	R1	10	10	10	10	10	9.33	9.00	9.66	9.33	9.00	86.6	80.0	93.2	86.6	80.0	68.53	63.44	74.88	68.53	63.44
		R2	10	9	10	10	9															
		R3	8	8	9	8	8															
19.66	10	R1	9	10	10	10	9	9.33	9.66	9.33	9.66	9.00	86.6	93.2	86.6	93.2	80.0	68.53	74.88	68.53	74.88	63.44
		R2	10	10	9	9	9															
		R3	8	9	9	10	9															
9.83	10	R1	9	9	8	8	10	8.66	8.66	9.00	7.66	8.33	73.2	73.2	80.0	53.2	66.6	58.82	58.82	63.66	46.83	54.70
		R2	10	9	10	8	8															
		R3	7	8	9	7	7															
4.91	10	R1	10	6	1	1	2	8.33	6.33	4.33	4.00	3.66	66.6	26.6	-13.4	-20.0	-26.8	54.70	31.05	-21.64	-26.56	-31.18
		R2	8	8	7	6	5															
		R3	7	5	5	5	4															

Appendix Table XVI. Repellency of *T. castaneum* by lichen sample h3 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Artocarpus heterophyllus* | Sample Code: h3

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	9	10	10	10.00	10.00	9.33	9.66	9.66	100	100	86.6	93.2	93.2	90.00	90.00	68.53	74.88	74.88
		R2	10	10	10	10	10															
		R3	10	10	9	9	9															
157.27	10	R1	10	10	9	9	8	9.66	9.33	9.00	8.66	8.66	93.2	86.6	80.0	73.2	73.2	74.88	68.53	63.44	58.82	58.82
		R2	9	10	10	9	9															
		R3	10	8	8	8	9															
78.63	10	R1	10	9	10	10	10	9.66	9.33	9.00	9.33	9.00	93.2	86.6	80.0	86.6	80.0	74.88	68.53	63.44	68.53	63.44
		R2	10	10	9	10	10															
		R3	9	9	8	8	7															
39.32	10	R1	10	7	9	9	8	9.66	8.66	8.33	8.00	7.66	93.2	73.2	66.6	60.0	53.2	74.88	58.82	54.70	50.77	46.83
		R2	9	9	8	8	8															
		R3	10	10	8	7	7															
19.66	10	R1	9	5	10	10	8	7.33	5.66	8.00	7.33	7.00	46.6	13.2	60.0	46.6	40.0	43.05	21.30	50.77	43.05	39.23
		R2	8	7	7	6	7															
		R3	5	5	7	6	6															

Appendix Table XVII. Repellency of *T. castaneum* by lichen sample h4 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Morus indica* | Sample Code: h4

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
629.08	10	R1	10	10	10	10	10	10.00	10.00	10.00	10.00	10.00	100	100	100	100	100	90.00	90.00	90.00	90.00	90.00
		R2	10	10	10	10	10															
		R3	10	10	10	10	10															
314.54	10	R1	10	10	10	10	9	10.00	10.00	9.66	10.00	9.00	100	100	93.2	100	80.00	90.00	90.00	74.88	90.00	63.44
		R2	10	10	9	10	10															
		R3	10	10	10	10	8															
157.27	10	R1	0	2	4	4	6	6.66	7.33	7.33	5.66	6.33	33.2	46.6	46.6	13.2	26.6	35.18	43.05	43.05	21.30	31.05
		R2	10	10	10	8	8															
		R3	10	10	8	5	5															
78.63	10	R1	10	10	10	10	8	8.66	9.00	9.00	9.00	8.00	73.2	80.0	80.0	80.0	60.0	58.82	63.44	63.44	63.44	50.77
		R2	7	7	7	8	8															
		R3	9	10	10	9	8															
39.32	10	R1	10	10	10	10	5	9.00	9.00	8.66	8.33	6.66	80.0	80.0	73.2	66.6	33.2	63.44	63.44	58.82	54.70	35.18
		R2	9	9	9	10	9															
		R3	8	8	7	5	6															
19.66	10	R1	10	10	9	10	8	5.00	5.33	5.00	5.33	4.66	0	6.6	0	-6.80	0	0	14.89	0	14.89	15.12
		R2	0	1	1	2	2															
		R3	5	5	5	4	4															

Appendix Table XVIII. Repellency of *T. castaneum* by lichen sample h5 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Anthocephalus chinensis* | Sample Code: h5

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	10.00	10.00	10.00	10.00	10.00	100	100	100	100	100	90.00	90.00	90.00	90.00	90.00
		R2	10	10	10	10	10															
		R3	10	10	10	10	10															
157.27	10	R1	10	10	10	10	10	10.00	10.00	9.66	10.00	9.66	100	100	93.2	100	93.2	90.00	90.00	74.88	90.00	74.88
		R2	10	10	10	10	10															
		R3	10	10	9	10	9															
78.63	10	R1	10	10	8	8	9	10.00	9.33	8.33	8.33	8.33	100	86.6	66.6	66.6	66.6	90.00	68.53	54.70	54.70	54.70
		R2	10	9	8	9	8															
		R3	10	9	9	8	8															
39.32	10	R1	10	10	9	9	8	7.33	7.33	7.33	7.00	7.00	46.6	46.6	46.6	40.0	40.0	43.05	43.05	43.05	39.23	39.23
		R2	5	5	6	6	7															
		R3	7	7	7	6	6															
19.66	10	R1	0	0	0	0	2	3.00	3.00	2.33	3.00	4.00	-40.0	-40.0	-53.4	-40.0	-20.0	-39.23	-39.23	-46.95	-39.23	-26.56
		R2	4	4	5	8	8															
		R3	5	5	2	1	2															

Appendix Table XIX. Repellency of *T. castaneum* by lichen sample h6 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Litchi chinensis* | Sample Code: h6

Dose ($\mu g/cm^2$)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	10.00	10.00	10.00	10.00	10.00	100	100	100	100	100	90.00	90.00	90.00	90.00	90.00
		R2	10	10	10	10	10															
		R3	10	10	10	10	10															
157.27	10	R1	10	10	10	10	10	10.00	10.00	10.00	10.00	10.00	100	100	100	100	100	90.00	90.00	90.00	90.00	90.00
		R2	10	10	10	10	10															
		R3	10	10	10	10	10															
78.63	10	R1	10	10	10	10	10	10.00	10.00	9.66	9.00	9.66	100	100	93.2	80.0	93.2	90.00	90.00	74.88	63.44	74.88
		R2	10	10	9	9	9															
		R3	10	10	10	8	10															
39.32	10	R1	10	10	10	9	9	9.66	9.00	9.33	8.00	7.66	93.2	80.0	86.6	60.0	53.2	74.88	63.44	68.53	50.77	46.83
		R2	9	9	9	7	7															
		R3	10	8	9	8	7															
19.66	10	R1	10	10	9	9	8	8.66	9.00	8.33	7.66	7.33	73.2	80.0	66.6	53.2	46.6	58.82	63.44	54.70	46.83	43.05
		R2	7	8	8	7	6															
		R3	9	9	8	7	8															

Appendix Table XX. Repellency of *T. castaneum* by lichen sample h7 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Litchi chinensis* | Sample Code: h7

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	10.00	10.00	9.66	9.33	9.00	100	100	93.2	86.6	80.0	90.00	90.00	74.88	68.53	63.44
		R2	10	10	9	8	8															
		R3	10	10	10	10	9															
157.27	10	R1	10	9	10	7	9	9.00	9.00	9.33	8.33	8.00	80.0	80.0	86.6	66.6	60.0	63.44	63.44	68.53	54.70	50.77
		R2	8	8	9	9	8															
		R3	9	10	9	9	7															
78.63	10	R1	10	10	10	9	10	9.00	9.33	8.33	8.33	8.66	80.0	86.6	66.6	66.6	73.2	63.44	68.53	54.70	54.70	58.82
		R2	7	8	7	9	8															
		R3	10	10	8	7	8															
39.32	10	R1	6	7	9	8	8	8.00	7.33	7.66	7.33	7.66	60.0	46.6	53.2	46.6	53.2	50.77	43.05	46.83	43.05	46.83
		R2	10	8	8	9	9															
		R3	8	7	6	5	6															
19.66	10	R1	9	10	10	1	3	8.00	7.66	5.66	3.00	3.00	60.0	53.2	13.2	-40.0	-40.0	50.77	46.83	21.30	-39.23	-39.23
		R2	10	9	7	7	5															
		R3	5	4	0	1	1															

Appendix Table XXI. Repellency of *T. castaneum* by lichen sample h8 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Diospyros peregrina* | Sample Code: h8

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	10.00	10.00	9.33	9.66	9.33	100	100	86.6	93.2	86.6	90.00	90.00	68.53	74.88	68.53
		R2	10	10	9	10	9															
		R3	10	10	9	9	9															
157.27	10	R1	10	10	10	5	7	9.00	9.66	9.33	6.33	7.33	80.0	93.2	86.6	26.6	46.6	63.44	74.88	68.53	31.05	43.05
		R2	9	10	9	7	8															
		R3	8	9	9	7	7															
78.63	10	R1	9	10	8	8	9	7.00	7.00	5.66	5.33	5.00	40.0	40.0	13.2	6.6	0	39.23	39.23	21.30	14.89	0
		R2	7	5	6	6	5															
		R3	5	6	3	2	1															
39.32	10	R1	9	8	7	7	8	6.66	6.66	7.66	6.33	6.33	33.2	33.2	53.2	26.6	26.6	35.18	35.18	46.83	31.05	31.05
		R2	6	7	9	8	8															
		R3	5	5	7	4	3															
19.66	10	R1	5	5	10	5	6	6.66	6.00	7.33	4.33	5.33	33.2	20.0	46.6	-13.6	6.6	35.18	26.56	43.05	-21.64	14.89
		R2	7	8	7	4	7															
		R3	8	5	5	4	3															

Appendix Table XXII. Repellency of *T. castaneum* by lichen sample h9 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Stereospermum chelonioides* | Sample Code: h9

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	10.00	10.00	10.00	10.00	10.00	100	100	100	100	100	90.00	90.00	90.00	90.00	90.00
		R2	10	10	10	10	10															
		R3	10	10	10	10	10															
157.27	10	R1	10	10	10	10	10	9.66	10.00	9.33	9.00	9.00	93.2	100	86.6	80.0	80.0	74.88	90.00	68.53	63.44	63.44
		R2	10	10	8	8	8															
		R3	9	10	10	9	9															
78.63	10	R1	10	10	10	9	7	9.66	9.33	9.00	9.00	8.00	93.2	86.6	80.0	80.0	60.0	74.88	68.53	63.44	63.44	50.77
		R2	9	9	9	8	9															
		R3	10	9	8	10	8															
39.32	10	R1	9	10	9	10	8	8.66	7.33	7.00	7.33	7.00	73.2	46.6	40.0	46.6	40.0	58.82	43.05	39.23	43.05	39.23
		R2	8	5	5	7	7															
		R3	9	7	7	5	6															
19.66	10	R1	10	10	10	6	4	9.00	9.66	8.33	6.00	5.33	80.0	93.2	66.6	20.0	6.6	63.44	74.88	54.70	26.56	14.89
		R2	8	9	7	7	7															
		R3	9	10	8	5	5															

Appendix Table XXIII. Repellency of *T. castaneum* by lichen sample h10 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Borassus flabellifera* | Sample Code: h10

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	9.66	9.66	10.00	10.00	10.00	93.2	93.2	100	100	100	74.88	74.88	90.00	90.00	90.00
		R2	10	10	10	10	10															
		R3	9	9	10	9	9															
157.27	10	R1	6	10	10	10	9	8.00	9.66	9.66	9.33	9.00	60.0	93.2	93.2	86.6	80.0	50.77	74.88	74.88	68.53	63.44
		R2	10	10	9	9	9															
		R3	8	9	10	9	9															
78.63	10	R1	10	10	10	9	8	9.00	8.33	9.00	8.33	8.00	80.0	66.6	80.0	66.6	60.0	63.44	54.70	63.44	54.70	50.77
		R2	7	7	9	8	8															
		R3	10	8	8	8	8															
39.32	10	R1	10	10	9	10	2	8.33	8.00	5.00	6.66	3.66	66.6	60.0	0	33.2	-33.4	54.70	50.77	0	35.18	-35.30
		R2	5	6	6	8	8															
		R3	10	8	0	2	1															
19.66	10	R1	9	10	10	9	4	7.00	7.00	7.33	5.33	4.33	40.0	40.0	46.6	6.6	-13.6	39.23	39.23	43.05	14.89	-21.64
		R2	5	4	4	0	3															
		R3	7	7	8	7	6															

Appendix Table XXIV. Repellency of *T. castaneum* by lichen sample h11 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Citrus grandis* | Sample Code: h11

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	10.00	9.33	9.33	9.66	9.33	100	86.6	86.6	93.2	86.6	90.00	68.53	68.53	74.88	68.53
		R2	10	10	10	10	10															
		R3	10	8	8	9	8															
157.27	10	R1	10	10	10	10	6	9.66	9.66	9.33	9.33	7.66	93.2	93.2	86.6	86.6	53.2	74.88	74.88	68.53	68.53	46.83
		R2	10	9	9	9	9															
		R3	9	10	9	9	8															
78.63	10	R1	10	10	10	10	8	9.66	9.66	9.33	8.33	7.66	93.2	93.2	86.6	66.6	53.2	74.88	74.88	68.53	54.70	46.83
		R2	10	10	8	8	8															
		R3	9	9	10	7	7															
39.32	10	R1	10	10	9	7	7	9.66	8.00	8.00	7.66	7.33	93.2	60.0	60.0	53.2	46.6	74.88	50.77	50.77	46.83	43.05
		R2	9	7	8	8	8															
		R3	10	7	7	8	7															
19.66	10	R1	10	8	6	8	7	9.00	7.66	6.66	7.33	6.66	80.0	53.2	33.2	46.6	33.2	63.44	46.83	35.18	43.05	35.18
		R2	10	10	9	8	8															
		R3	7	5	5	6	5															

Appendix Table XXV. Repellency of *T. castaneum* by lichen sample h12 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Lannea coromandelica* | Sample Code: h12

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	10.00	9.66	10.00	9.66	9.66	100	93.2	100	93.2	93.2	90.00	74.88	90.00	74.88	74.88
		R2	10	10	10	10	10															
		R3	10	9	10	9	9															
157.27	10	R1	10	10	10	10	9	10.00	9.33	8.66	9.00	8.33	100	86.6	73.2	80.0	66.6	90.00	68.53	58.82	63.44	54.70
		R2	10	10	9	9	9															
		R3	10	8	7	8	7															
78.63	10	R1	10	6	5	5	4	8.66	7.66	5.66	5.66	5.00	73.2	53.2	13.2	13.2	0	58.82	46.83	21.30	21.30	0
		R2	8	8	7	7	7															
		R3	8	9	5	5	4															
39.32	10	R1	10	8	2	2	1	9.00	7.66	4.66	5.00	4.00	80.0	53.2	-6.8	0	-20.0	63.44	46.83	-15.12	0	-26.56
		R2	10	8	7	8	7															
		R3	7	7	5	5	4															
19.66	10	R1	10	9	8	10	9	7.33	6.00	6.00	5.33	5.33	46.6	20.0	20.0	6.6	6.6	43.05	26.56	26.56	14.89	14.89
		R2	7	7	6	6	6															
		R3	5	4	4	0	1															

Appendix Table XXVI. Repellency of *T. castaneum* by lichen sample h13 with percent repulsion and arcsin transformed data

Repellency record | Collected from the stem bark of *Mangifera indica* | Sample Code: h13

Dose (µg/cm²)	Insects used	Replication	Hourly observation					Average of hourly observation (Nc)					Percent repulsion (PR) PR = (Nc – 5) ×20%					Arcsin trasformed data				
			1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h	1h	2h	3h	4h	5h
314.54	10	R1	10	10	10	10	10	9.66	10.00	9.66	9.66	10.00	93.2	100	93.2	93.2	100	74.88	90.00	74.88	74.88	90.00
		R2	9	10	10	10	10															
		R3	10	10	9	9	10															
157.27	10	R1	10	10	10	9	8	9.66	9.66	8.66	8.66	8.66	93.2	93.2	73.2	73.2	73.2	74.88	74.88	58.82	58.82	58.82
		R2	10	10	8	9	9															
		R3	9	9	8	8	9															
78.63	10	R1	10	9	9	10	8	9.66	9.66	9.00	8.66	8.00	93.2	93.2	80.0	73.2	60.0	74.88	74.88	63.44	58.82	50.77
		R2	9	9	8	7	7															
		R3	10	10	10	9	9															
39.32	10	R1	10	9	10	9	10	8.33	8.00	8.33	8.66	8.33	66.6	60.0	66.6	73.2	66.6	54.70	50.77	54.70	58.82	54.70
		R2	8	8	9	9	7															
		R3	7	7	6	8	8															
19.66	10	R1	9	8	8	8	9	8.33	7.66	7.66	7.33	7.00	66.6	53.2	53.2	46.6	40.0	54.70	46.83	46.83	43.05	39.23
		R2	7	8	7	8	6															
		R3	9	7	8	6	6															

Appendix Table XXVII: Dose-mortality effect of lichen h10 extract ($CHCl_3$) against 2nd instar larvae of *T. castaneum* after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
5	0.698	30	1	3.333	0	0	1.413	0	0	1.476
25	1.397	30	2	6.667	3	3.12	2.765	3.379	2.28	2.792
75	1.875	30	3	10.000	7	3.52	3.688	3.529	9.05	3.690
150	2.176	30	5	16.666	14	3.92	4.271	3.946	15.09	4.256
200	2.301	30	13	43.333	41	4.77	4.512	4.768	17.43	4.492

Results:
Y = 0.1613352 + 1.882032 X
Chi-squared is 3.806461 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 2.57098
LD_{50} is 372.3745mg/g
95% Confidence limits are 169.238 to 819.336mg/g

Appendix Table XXVIII. Dose-mortality effect of lichen h10 extract ($CHCl_3$) against 3rd instar larvae of *T. castaneum* after 24 of exposure.

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
5	0.698	30	1	3.333	0	0	2.456	2.06	1.2	2.452
25	1.397	30	4	13.333	10	3.72	3.411	3.81	7.14	3.409
75	1.875	30	5	16.667	14	3.92	4.064	3.914	13.17	4.063
150	2.176	30	5	16.667	14	3.92	4.475	4	16.74	4.475
200	2.301	30	16	53.33	52	5.05	4.646	5.064	18.03	4.646

Results:
Y = 1.494461 + 1.369833 X
Chi-squared is 8.547838 with 3 degrees of freedom
Variance has been adjusted for heterogeneity
Log LD_{50} is 2.5591
LD_{50} is 362.3265mg/g
95% Confidence limits are 92.5687 to 1418.196mg/g

Appendix Table XXIX. Dose-mortality effect of lichen h10 extract ($CHCl_3$) against 4th instar larvae of *T. castaneum* after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
5	0.698	30	3	10	7	3.52	3.211	3.629	5.40	3.223
25	1.397	30	4	13.333	10	3.72	3.812	3.72	11.10	3.807
75	1.875	30	5	16.667	14	3.92	4.222	3.946	15.09	4.207
150	2.176	30	5	16.667	14	3.92	4.481	4	16.74	4.458
200	2.301	30	17	56.667	55	5.13	4.588	5.16	17.43	4.563

Results:
Y = 2.637641 + 0.8369443 X
Chi-squared is 11.73261 with 3 degrees of freedom
Variance has been adjusted for heterogeneity
Log LD_{50} is 2.822601
LD_{50} is 664.6616mg/g
95% Confidence limits are 35.94782 to 12289.35mg/g

Appendix Table XXX: Dose-mortality effect of lichen h10 extract ($CHCl_3$) against 3rd instar larvae of *M. domestica* after 24 of exposure

Dose	Logdose	Num.	Kill	% kill	Corr%	Emp probit	Expt probit	Work probit	Weight	Final probit
15.615	1.193	30	9	30	22	4.23	4.359	4.234	15.96	4.372
31.25	1.495	30	15	50	44	4.85	4.650	4.848	18.03	4.657
62.5	1.796	30	15	50	44	4.85	4.940	4.84	19.02	4.943
125	2.097	30	20	66.67	63	5.33	5.230	5.358	18.81	5.228
250	2.397	30	21	70	67	5.44	5.519	5.416	17.43	5.514

Results:
Y = 3.240421 + 0.9480911 X
Chi-squared is 1.641705 with 3 degrees of freedom
No significant heterogeneity
Log LD_{50} is 1.855917
LD_{50} is 71.76573mg/g
95% Confidence limits are 43.21784 to 119.1712mg/g