INVESTIGATION OF ANTIOXIDANT, ANTIMICROBIAL AND TOXICITY ACTIVITIES OF LICHENS FROM HIGH ALTITUDE REGIONS OF NEPAL

SUBMITTED TO
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IN BIOTECHNOLOGY

BY
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DECLARATION

Thesis entitled "**Investigation of Antioxidant, Antimicrobial and Toxicity Activities of Lichens from High Altitude Regions of Nepal**" which is being submitted to the Central Department of Biotechnology, Institute of Science and Technology (IOST), Tribhuvan University, Nepal for the award of the Master's degree in Biotechnology, is a research work carried out by me under the supervision of Prof. Dr. Tribikra Bhattrai, Central Department of Biotechnology, Tribhuvan University and co-supervised by Prof. Dr. Hari Datta Bhattarai, Central Department of Botany, Tribhuvan University at the laboratory work space provided by Research Institute for Bioscience and Biotechnology (RIBB). This research is original and has not been submitted earlier in part or full in this or any other form to any university or institute, here or elsewhere, for the award of any degree. All sources of information have been specifically acknowledged by reference to the author(s) or institution(s).

Baidyanath Kumar Jha

RECOMMENDATION

This is to recommend that **Baidyanath Kumar Jha** has carried out research entitled "**Investigation of Antioxidant, Antimicrobial and Toxicity Activities of Lichens from High Altitude Regions of Nepal**" for the award of **Master's degree in Biotechnology** under our supervision. To our knowledge, this work has not been submitted previously for acquiring any other degree. He has fulfilled all the requirements laid down by the Institute of Science and Technology (IOST), Tribhuvan University, Kirtipur for the submission of the thesis for the award of degree.

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LETTER OF APPROVAL

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CERTIFICATE OF ACCEPTANCE

This thesis work submitted by Baidyanath Kumar Jha entitled **"Investigation of Antioxidant, Antimicrobial and Toxicity Activities of Lichens from High Altitude Regions of Nepal"** has been accepted as a partial fulfillment for the requirements of **Master's Degree in Biotechnology**. To my/our knowledge, this work has not been submitted previously for acquiring any other degree.

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Baidyanath Kumar Jha

Abbreviations

ACA: Annapurna conservation area

ASL: Above sea level

BHA: Butylated hydroxyanisole

DCM: Dichloromethane

DPPH: 2, 2-Diphenyl-1-picrylhydrazyl

I: Inactive

IC_{50}: 50% inhibition concentration

M: Moderate

mM: Millimolar

NA: Nutrient agar

NB: Nutrient broth

PDA: Potato dextrose agar

RF: Retention factor

S: Strong

TLC: Thin layer chromatography

UV: Ultraviolet

W: Weak

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Abstract

Background: Several lichen species are reported to be used traditionally in many therapeutic practices. Many lichen species are reported as sources of several bioactive natural compounds. Several lichen species of Nepal are so far chemically unexplored.

Methods: The morphological, anatomical and phytochemical characteristics of lichens were compared for the taxonomic identification of the species. Methanol- water extract of lichens were sub fractionated into hexane, dichloromethane and methanol fractions for bioactivity assays. Antimicrobial activities of extracts were evaluated against pathogenic bacteria and fungal species. DPPH test was used for antioxidant potential evaluation. Brine shrimp test was performed to evaluate toxicity of the extracts.

Results: A total of 84 lichen specimens were collected and identified from Annapurna Conservation Area (ACA) Nepal. The specimens were identified as belonging to 19 genera and 47 species. Methanol fractions of 16 specimens and dichloromethane (DCM) fractions of 21 lichens specimens showed antioxidant activities comparable with commercial standards (BHA, Butylated hydroxyanisole, IC_{50}= 4.98 ± 0.9 μg/mL) even at crude extract level. Similarly, the DCM fraction of 17 lichens showed potential antimicrobial activity against a Gram-positive bacterium (*Staphylococcus aureus* KCTC3881) and DCM fractions of 45 lichens showed antimicrobial activity against a Gram-negative bacterium (*Klebsiella pneumoniae* KCTC2242). DCM fractions of three lichens showed antifungal activity against the yeast, *Candida albicans* KCTC 7965. Likewise, methanol fractions of 39 lichens and DCM fractions of 74 lichens showed strong toxicity against brine shrimp nauplii with more than 80% mortality.

Conclusion: IC_{50} of *Peltigera polydactyla* from Chame were determined as 5.7 ± 0.02 μg/ml for methanol fraction and 5.56 ± 0.02 μg/ml for DCM fraction, which indicates high antioxidant properties. Such biological activity-rich lichen specimens warrant further research on exploration of natural products with antioxidant, antimicrobial and anticancer (toxic) potential.

Keywords: Antimicrobial, Antioxidant, DPPH, Lichen, Thin layer chromatography

Chapter 1 - Introduction

1. Introduction

1.1 General Overview

Lichens are composite organisms consisting of symbiotic association of a fungus (the mycobiont) with a photosynthetic partner (the photobiont or phycobiont), usually either a cyanobacteria or green alga. Lichens are unique in nature and physiology because they look and behave quite differently from their component organisms (Srivastava et al., 2013). They are cosmopolitan in distribution from arctic to tropical regions and from plains to the highest mountains and some survive in the extreme environment of deserts (Paudel et al., 2012) and can be found in various surfaces ranging from soil, hard rock surfaces to barks and tree trunks and shrubs. Lichens produce secondary metabolites that fall into various chemical classes, which are, as a group, distinct from those produced by higher plants. These include: diterpene, triterpene, dibenbenzofuran, dibenzopyranone, depside, depsidones, anthraquinone, xanthones, usnic acids and pulvinic acids (Dayan and Romagni, 2001). Lichen secondary metabolites exhibit numerous biological activities including: anti-mycobacterial (Ingolfsdottir, Chung, Skulason, Gissurarson, and Vilhelmsdottir, 1998), antiviral (Neamati, Hong, and Mazumder, 1997), antioxidant (Hidalgo, Fernandez, and Quilhot, 1994), analgesic (Okuyama, Umeyama, and Yamazaki, 1995), cytotoxic, antimicrobial, fungicidal, herbicidal, repellant, photosystem inhibitory (Dayan and Romagni, 2001).

Natural products are proposed as a therapeutic alternative to conventional antimicrobial treatment, whose effectiveness is often limited by the resistance that the infectious agents have developed against the antibiotics (Ali et al., 1999; Nimri et al., 1999). Pathogenic microbes pose serious threats to human and plant health and are increasing in prevalence in institutional health care settings (James and Fred., 1997). New alternatives for combating the spread of infection by antibiotic resistant microbes in future are necessary tools for keeping pace with the evolution of ‘super’ pathogens. The most successful antibiotics that have been applied to combat disease are small molecule, secondary metabolites, including penicillin derivatives that were originally isolated from fungi (Paudel et al., 2008).

Reactive oxygen species (ROS) are harmful or lethal for living organism (Davies, 1995). ROS are ions or very small molecules which include free radicals, oxygen ions and peroxides. ROS are formed inside the living organisms as natural byproducts of the normal metabolism of oxygen and help in cell signaling. Thus, living organisms accumulate various ROS through both normal metabolic processes and exogenous sources like environmental stresses such as UV light, cigarette smoke, environmental pollutants, γ-radiation. These ROS are highly reactive and unstable due to the presence of unpaired electron in their outer shell. They have been associated with several diseases such as inflammation, cardio vascular diseases, cancer, aging-related disorders, metabolic disorders, and atherosclerosis (Ames, Shigenaga, Hagen, 1993). ROS react with various cellular components including DNA, proteins, lipids, fatty acids. Thus, the reaction between ROS and cellular components lead to damage DNA, attack unsaturated fatty acids and cause membrane lipid peroxidation decreases in membrane fluidity, loss of enzyme receptor activities, and damage to membrane proteins, ultimately leading to cell inactivation (Dean and Davies, 1993). Many lichen extracts have been reported for antioxidant properties due to their phenolic content. Antioxidant agents inhibit and prevent reactive oxygen species, which can cause degenerative diseases. Natural antioxidants are preferred over many synthetic antioxidants, which can be toxic, for therapeutic applications (Zambare and Christopher, 2012).

Some lichen substances like usnic acid, cristazarin, protolichesterinic acid, polyporic acid, depsidone and lichenin have been investigated for antitumor effects on tumor cells. In vitro anticancer activities of lichen extracts have been evaluated according to the cell proliferation assay (Tokiwano et al., 2009) in three cancer cell lines: human pancreatic (PANC-1) (Ingolfsdottir et al., 2002), prostate (DU-145) (Russo et al., 2006) and breast (MCF7) (Bogo et al., 2010) cancer cell lines (Zambare and Christopher, 2012).

1.2 Rationale and Scope of the Study

Nepal is a mountainous country which contains a diverse geographic locations ranging from 60 m to 8848 m above sea level. The International Workshop on Lichen Taxonomy - held in Kathmandu in 1994 - estimated that around 2,000 lichen species are likely to occur in Nepal (Sharma, 1995). This figure represents about 10% of the expected global lichen flora

(Baniya, 1996). As of 2010 a provisional checklist shows that lichens from 792 taxa in 187 genera have been recorded from Nepal (Olley and Sharma, 2013). There are very few literatures regarding the biochemical studies of the lichens of Nepal. In this report, we present the biochemical study of various species of lichens found around the hills of Annapurna Conservation Area (ACA).

In the present scenario, scientist community is concerned to reduce the load of hazardous synthetic chemicals and to develop environmentally benign strategies for protection of human as well as crops from diseases, current investigation has established the lichens as potential source of bioactive agents.

1.3 Objectives

General Objectives

- To study different biological activities of different lichens from hills of the Annapurna Conservation Area (ACA)

Specific Objectives

- To determine the Antimicrobial activity of the lichen extracts
- To determine the Antioxidant property of the lichen extracts
- To check for the toxicity property of the lichen extracts using Brine Shrimp Lethality test.

Chapter 2 – Literature Review

2. Literature Review

2.1 Lichens of Nepal- An overview

Lichens are commonly called Jhayaoo or Tare in Nepal. Lichens are symbiotic organisms combined of a fungal partner (mycobiont) with one or more photosynthetic partners (photobiont). The photobiont can be green algae, cyanobacteria, or both. Usually a species of lichen has the same species of alga. Cyanobacteria are symbiotic to living in nitrogen poor environments as the cyanobacteria can fix nitrogen into a form useable by the lichen. This unique symbiotic relationship among such diversified organisms makes them able to grow in some of the extreme environmental conditions on earth, they can adapt to extreme temperatures, drought, inundation, salinity, high concentrations of air pollutants, nutrient poor and highly nitrified environments (Müller, 2001; Nash, 2008).

Nepal lies in latitudinal range from 26°22' to 30°27' N and longitudinal ranges from 80°14' to 88°12' E, with an area of 1,47,181 sq. Km i.e. only 0.03% of the global land area but has huge altitudinal variation. Within the width of 130 Km the altitudinal range is from 68 m, Mahottari to 8,848 m, Mt. Everest. Such huge coverage of altitude variation has made our country rich in natural resources. It is reported that more than 80% of the rural Nepalese people depend on traditional remedies that involve the use of local plants in various forms and combinations (Rajbhandary and Bajracharya, 1994). There are 1792 medicinal plants in Nepal (Baral and Kurmi, 2006) out of which 49% are herbs, 29% trees, 14% shrubs, 8% climbers (Bhattarai and Ghimire, 2006). Traditionally, there is the use of about 1000 species of medicinal plants by various communities in Nepal for their primary healthcare system (Chaudhary, et al., 1998).

Lichens are found worldwide covering about 8% of the earth's surface and their habitat ranges from arctic to tropical regions (Sancho et al., 2007). Due to diverse geographical location and environmental conditions of Nepal several species of lichens have been recorded. There are about 300 genera and 18000 species of presently identified lichens globally (Galun, 1988). A total of 792 species belonging to 187 genera of lichen-forming fungi have been reported from Nepal (Oley et al., 2013) but many lichen species have yet to

be discovered. Baniya et al. (2010), study of elevation gradients of lichen species richness in Nepal to know their distribution pattern based upon the altitude. A total number of 525 lichens were compared including endemic species (55 spp.) showed humped relationships with elevation. Their highest richness was observed between 3100–3400 and 4000–4100 m, respectively. Almost 33% of the total lichens and 53% of the endemic species occurred above the tree line (>4300 m). These findings show the outstanding scope of the study of lichens from higher altitudes of Nepal.

Lichens of Nepal also have a huge market potential. Devkota et al. (2017), have documented that lichens of West Nepal are mainly used in international trade, while those in East Nepal they are locally used for food. A total of 20 commercially important species of lichen were identified from five trade centers and one local market. During 2000-2011, Nepal legally exported 2020 tons of lichens worth NRs. 25,293,305 (USD 240,000). The average annual turnover was 168 tons, though it is estimated that much was exported illegally. Since, the Forest Act, Forest Regulations and its amendment in 2011, the collection of lichens for harvest, trade and export in any crude or processed form was banned. This market potential shows the importance of Nepali lichens in international market for various uses. Thus intensive research is required to be done on lichens of Nepal.

2.2 Identification of Lichens

Lichens are identified based upon its morphological and chemical characteristics. There are four basic growths from the lichen based on growth form of the thallus (vegetative body of the lichen) one is the Leaf-like or Foliose, with flat sheets of tissue not tightly bond, that allows removal from the substrate. The other is fruticose, freestanding branching tubes with no upper or lower layers. Crustose are firmly attached to the substrate. One must remove a portion of the substrate to remove the lichen intact. Crustose lichens have no lower layer of the thallus. The Squamulose are tightly clustered and slightly flattened pebble-like units, with small scale-like lobes, (Bordo et al., 2001). Each of the lichen has its own distinct species of fungus, but all lichens share just a small number of algae species, thus lichens are classified based on the fungus and fungal features. Fungus can have two different forms if paired with two different "photobionts". It is uncommon but happens sometimes.

Some lichenologists use common household chemicals (bleach and iodine) and other chemicals to test the color reaction of the unique compounds found in the structure of the lichen, as well as using a lichen key to distinguish between species. However, an expert is needed to identify the lichens properly.

According to Asahina (1937), the lichen substances can be classified into the following groups: Aliphatic lichen substances (includes acids and polyhydric alcohols) and aromatic lichen substances (includes pulvinic acid derivatives, depsides, depsidones, Anthraquinones, xanthone derivatives). Lichens secondary metabolites are usually insoluble in water and can be extracted into organic solvents (such as ethanol, methanol, ether, hexane, DCM). The secondary metabolite content of lichen is generally 0.1% and 10% of the dry weight of the thallus.

2.3 Antimicrobial Activity of Lichens

Antimicrobial studies are done to identify the potential of particular specimen, extracts and their compounds against pathogenic species of microbes for new drug discoveries. Disc-diffusion assay has been the preliminary procedure for detecting the antimicrobial properties. Lichens and their products have been used in traditional medicines for centuries and still is subject of interest as endogenous treatments in various parts of the world (Richardson, 1991).Traditionally; lichen is used in the treatment of constipation, kidney disease, infection, and others. The ethnic people living in the ruler area have been using lichen to cure a series of ailments, for food, fodder, spice. The extract from the lichens is also used in dyes (purple pigment from *Roccella sp.* and brown pigment from *Parmelia, Ochrolechia* and *Evernia sp.* (Muggia et al., 2009), perfumes (from *Evernia prunastri)*, and others.

Devkota et al. (2017) studied that, lichens are being used in several ways by different communities of Nepal. They recorded the ethnic use of seven species of lichens belonging to four families (Parmeliaceae, Physciaceae, Ramalinaceae and Usneaceae) and six genera (*Heterodermia, Everniastrum, Parmotrema, Ramalina, Thamnolia* and *Usnea*) among the Limbu, Sherpa, Lama, Gurung, Rai, Dalit, Tamang, Chhetri and Brahman communities. The study revealed six use values namely; Medicinal value (*Heterodermia diademata* to treat

wounds and to stop bleeding after an injury), food value, ritual and spiritual value, aesthetic and decorative value, bedding value and ethno-veterinary value (EVV) from different parts of Nepal. Three lichen species, *Everniastrum cirrhatum, E. nepalense* and *Parmotrema cetratum* are consumed by the Limbu and Rai communities.

Structures of more then 1000 different lichen substances has been determined to date and many of them are pharmaceutically relevant (Muggia et. al., 2009). Secondary metabolites are products of polyketide pathway, mainly monocyclic and/or bicyclic phenols joined by an ester bond (depsides), both ester and ether bonds (depsidones) or furan heterocycle (dibenzofurans and usnic acid), antraquinones, xanthones, chromones and secondary aliphatic acids and esters (Stojanović et al., 2011).

Interest on lichens in the field of pharmacology is due to their unique and varying biologically active substances mostly present in their secondary metabolites. The intensive use of antibiotics has accounted for antibiotic resistance factors and evolution of multiply drug resistant microorganisms. Lichen metabolites exert a wide variety of biological actions including antibiotic, anti-mycotic, antiviral, anti-inflammatory, analgesic, antipyretic, anti-proliferative, and cytotoxic effects (Molnár and Farkas, 2010). Even though lichen biologically active compound and their biological activities still remain unexplored to a great extent.

Ranković et al. (2010) conducted a disc-diffusion assay to know the antimicrobial property of aqueous, acetone and methanol extracts (50 mg/ml) of the lichens (*Lecanora frustulosa* and *Parmeliopsis hyperopta*) against several Gram-positive bacteria (*Bacillus mycoides, Bacillus subtilis* and *Staphylococcus aureus*), Gram-negative bacteria (*Klebsiella pneumoniae and E. coli)* and fungi(*Aspergillus flavus, Candida albicans, Fusarium oxysporum etc.).*With acetone and methanol extract zone of inhibition against fungi was within the range 13-17 mm (with MIC 3.12-12.5 mg/mL) similarly for bacteria zone of inhibition was in the range of 11-21 mm (with MIC 0.78-3.12 mg/mL), while the aqueous extracts showed no inhibition atoll. Zone of inhibition of *Lecanora frustulosa* against *Staphylococcus aureus, Klebsiella* and *pneumonia, Candida albicans* were in the range of 18-21 mm, 19-23 mm and 14 mm respectively. Where as zone of inhibition for *Parmeliopsis hyperopta* extracts against

Staphylococcus aureus, Klebsiella pneumonia and *Candida albicans* were as 15 mm, 18-21 mm and 20-22 mm respectively. Methanol extracts showed better results than the acetone extracts.

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

In a research conducted by Güvenç (2012), DCM extract and ethanol fractions of *Pseudevernia furfuracea* showed strong anti-inflammatory activity on carrageenan-induced hind paw edema model without inducing any visible acute toxicity or gastric damage. Also, external application of the ointment prepared with methanol extract and ethanol fraction onto the wounds protruded promising wound healing activity. Moreover, the results showed that nonpolar fractions of the extract have significant antimicrobial activity against especially *Candida* species and polar fractions (especially MeOH) display antioxidant, anti-inflammatory, anticipative and wound healing activities.

The wide scale screening of antimicrobial activity of lichen extracts by several researchers shows that microbial inhibitions can vary within the lichen extract depending upon the type of solvent used for extraction and microbes tested, thus it is very critical to choose the solvent depending upon the intent of the research. Inappropriate choice of the solvent may interfere with the out come of the research and provide irrelevant data.

2.4 Antioxidant Assay

Antioxidants are chemical compounds which can delay the start or slow the rate of lipid oxidation reaction in food systems. By definition, antioxidants are a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides. Huang et al. (2005), defines it more relevantly as, biological antioxidant is a synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. In biochemistry and medicine, antioxidants are enzymes or other organic substances, such as vitamin E or β-carotene that are capable of counteracting the damaging effects of oxidation in animal tissues. In a chemical industry, antioxidants often refer to compounds that retard autoxidation of chemical products such as rubber and plastics. In food chemistry, antioxidants include components that prevent rancidity of fat, a substance that significantly decreases the adverse effects of reactive oxygen species on the normal physiological function of human being. Biological antioxidants include enzymatic antioxidants (e.g., Superoxide dismutase, catalase and glutathione peroxidase) and nonenzymatic antioxidants such as oxidative enzyme (e.g., cycloxygenase) inhibitors, antioxidant enzyme cofactors (Se, Coenzyme Q10), ROS/RNS scavengers (Vitamin C and E), and transition metal chelators.

The oxidation induced by Reactive Oxygen Species (ROS) may result in cell membrane disintegration, membrane protein damage and DNA mutations which play an important role in aging and can further initiate or propagate the development of many diseases, such as arteriosclerosis, cancer, diabetes mellitus, liver injury, inflammation, skin damages, coronary heart diseases and arthritis (Khemani, 2012).

According to Gupta (2017), free radicals are broadly divided into two types: Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). ROS includes both oxygen radicals and certain radicals that are oxidizing agents or can easily converted into radicals. RNS is also a collective term including nitric oxide and nitrogen dioxide radicals as well as non radicals like nitrous acid, N_2O_3, $ONOO^-$ are also included.

Generally two types of antioxidant assays are widely used for different antioxidant studies. One is associated with lipid peroxidations, including the thiobarbituric acid assay (TBA),

malonaldehyde/high-performance liquid chromatography (MA/HPLC) assay, malonaldehyde/gas chromatography (MA/GC) assay, beta-carotene bleaching assay, and conjugated diene assay. Other assays are associated with electron or radical scavenging, including the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, ferric reducing/antioxidant power (FRAP) assay, ferrous oxidation-xylenol orange (FOX) assay, ferric thiocyanate (FTC) assay, and aldehyde/carboxylic acid (ACA) assay, (Moon et al., 2009).

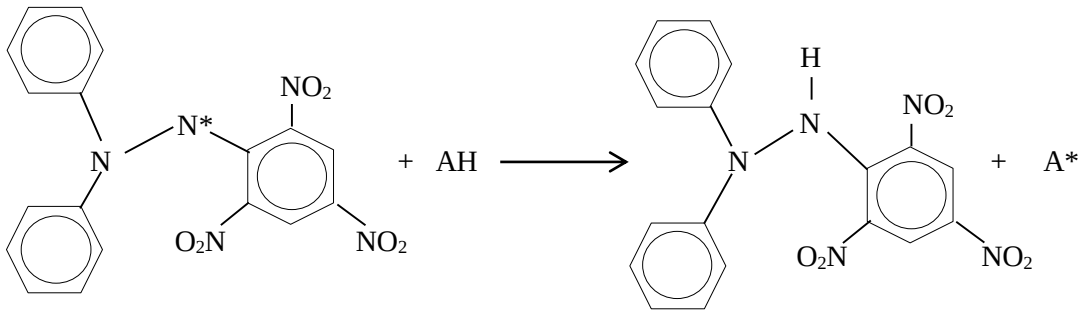
Madhavi et al. (1995), defines antioxidant capacity or antioxidant activity as an oxidant specific terms like “peroxy radical scavenging capacity”, “superoxide scavenging capacity”, “ferric ion reducing capacity” etc. Jayaprakasha and Rao (2000) examined antioxidant capacities of lichen compound, methyl orsellinate, atranorin, osellinic acid and lecanoric acid with high antioxidant capacity.

Antioxidant capacity assays are divided into hydrogen atom transfer (HAT) reactions based assays and single electron transfer (ET) reactions based assays. The ET based assays is carried out by one redox reaction with the oxidant which leads to change in color as an indicator at reaction endpoint. Most HAT based assays monitor competitive reaction kinetics, and the quantification is obtained from the kinetic curves. HAT based methods generally are composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant. HAT and ET based assays are intended to measure the radical (or oxidant) scavenging capacity (Huang et al., 2005)

It is estimated that most used synthetic antioxidants have environmental toxic and carcinogenic effects. So, there is a growing interest towards finding new antioxidants from natural resources origin which may not show any adverse effect. Several in vitro studies on plants, micro- and macroalgae, macromycetes and lichens verifies the fact that their product with antioxidant capacity are cable of exhibiting protective effects against oxidative stress in biological systems. Thus, it is necessary to utilize natural antioxidants for their protective effect against oxidative stress and physiological dysfunctions. To find novel natural antioxidant sources, our prime interest is focused on lichens.

2.4.1 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

Reaction mechanism of DPPH **(**Fig. 2.1) shows the structure of the violet color is a stable radical owing to stabilization by delocalization on to aromatic rings. $DPPH^{\cdot}$ can trap other radicals easily but does not dimerize. Because of an unpaired electron a strong absorption band is centered at about 517 nm, the solution of DPPH radical form is deep violet in color and it becomes colorless to pale yellow after reduction upon reaction with hydrogen or electron donating antioxidant. Color is the product of electronic transitions in atoms or molecules and is an indicator of the physical properties of chemical substances at the atomic level. A change in the electronic transitions results in a change in the light absorbed by the molecules and subsequently, causes a change in color. The colored complex formed in antioxidant assays is called a charge–transfer (CT) complex or electron–donor–acceptor complex. A CT complex is the association of two or more molecules, or different parts of one molecule, in which a fraction of electronic charge is transferred between the molecular entities (i.e., the radical and antioxidant). This transfer results in an electrostatic force of attraction between the radical and antioxidant providing a stabilizing force for the CT complex (Nabeelah et al., 2020).The decrease in absorbance depends linearly on antioxidant concentration. Trolox, ascorbic acid, butylated hydroxyanisole (BHA), α-tocopherol are commonly used standard antioxidant.



2,2-Diphenyl-1-picrylhydrazyl (DPPH, Violet color)

2,2-Diphenyl-1-picrylhydrazine (DPPH-H, yellow/colorless)

Fig. 2. 1 : Reaction mechanism of DPPH free radical scavenging.

In the research conducted by Marijana et al. (2010), antioxidant activity of the acetone, methanol and aqueous extracts of the lichens *Cladonia furcata, Hypogymnia physodes, Lasallia pustulata, Parmelia caperata* and *Parmelia sulcata* has been screened in vitro by using DPPH radical scavenging. Among the lichens tested, *Lasallia pustulata* had powerful antioxidant activities. Acetone, methanol and aqueous extracts of this lichen showed 90.93, 69.87 and 65.08% DPPH radical scavenging activities respectively. Those various antioxidant activities were compared to standard antioxidants such as ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol.

Antioxidant capacities of the ten *Parmeliaceae* species were determined by evaluating the free radical scavenging activities (ORAC and DPPH assays) and the ferric reducing power (FRAP assay) of their methanol extracts. With regard to DPPH method, *Myelochroa irrugans* (EC_{50} = 384 μg/ml) and *Flavoparmelia euplecta* (EC_{50} = 582 μg/ml) presented the strongest DPPH radical scavenging activity. On the other hand, the lowest free radical scavenging effectiveness was displayed by the methanol extract of *Flavoparmelia caperata* (EC_{50} = 3216 μg/ml) and *Lethariella canariensis* (EC_{50} = 2894 μg/ml) (Fernández-Moriano et al., 2016)

Kosanić (2011), observed DPPH scavenging activity of the *C. aculeata* extracts. For the methanol extract IC_{50} value was 51.65 ± 1.38 μg/ml, where as for the ethyl acetate extract was 41.4 ± 0.94 μg/ml. These findings show the potential of DPPH to determine the antioxidant capacity of the compounds present in the samples.

2.5 Toxicity Test

Toxicity tests examine the effects of biotic and abiotic factors on living organisms under controlled laboratory conditions. It is generally conducted using fish, crustaceans, insects, birds, shrimps, algae, invertebrates, rodents, aquatic and terrestrial plants. Acute toxicity are short-term tests (mostly, 48-96 hours of exposures) that measures growth, reproduction and mortality to determine the lethal dose (LD_{50}) for terrestrial species and lethal concentration (LC_{50}) for species, the dose at which 50% of the exposed test population dies. Species with short generation times, reproductive endpoints are generally used to determine acute toxicity.

Chronic toxicity tests are conducted for longer periods (generally 9-30 months) and measures growth, reproduction and mortality to determine sub lethal endpoints. Chronic toxicity tests may take time for the entire life-cycle of the organism (i.e., from zygote to age of first reproduction). Chronic tests are infeasible due to long period of the research, which leads to huge expense.

Cancer is the second leading cause of death globally, accounting for an estimated 9.6 million death or one in six deaths, in 2018 (WHO). Lichen substances show antitumor activities, which makes it ideal source of potential drugs for lethal malignant diseases. Usnic acid is one of the most extensively studied lichen metabolite. Usnic acid exhibited an anti-proliferative effect on human leukemia cells (K562) and endometrial carcinoma (HEC-50) cells (Krismundsdottir et al., 2002). Lichen compound depsidone pannarin inhibited cell growth and induces apoptosis in human prostate carcinoma DU-145 and human melanoma M14 cells (Russo et al., 2006, 2008). Protolichesterinic acid isolated from *Cetraria islandica* showed inhibition of growth of breast cancer cell lines and mitogen stimulated lymphocytes (Ogmundsdottir et al., 1998). Usnic acid from *Usnea diffracta* showed antipyretic and analgesic effects of lichen substances on animal studies, it inhibited acetic-acid-induced writhing in mice and raised the pain threshold in dose dependent manner (Okuyama et al., 1995). Furthermore, DCM, acetonitrile (ACN) and hexane extracts of lichen *Evernia prunastri* was prepared by Shcherbakova et al. (2016) and found that, extracts inhibit the proliferation of glioblastoma U87 cells in a concentration range of 12.6 – 35.2 μg/ml. *Evernia prunastri* was known to have compounds such as usnic acid, evernic acid, atranorin, salazinic acid. DCM extract showed in vitro the highest activity against U87 glioblastoma cells where as usnic acid was less active against U87 cells.

2.5.1 Brine Shrimp (*Artemia salina*) Lethality Assay

Brine shrimp lethality assay is a simple, rapid and convenient method for toxicity test of bioactive chemicals, medicines, pesticides, heavy metal etc. Toxicity test is performed on the basis of killing ability of test substances on a simple zoological organism such as *Artemia salina*. The results of this assay generally correlate properly with cytotoxic and antitumor properties. Brine shrimp (*Artemia salina*) lethality assay is a preliminary toxicity screening

assay used to examine the cytotoxic effect of bioactive compounds. The larvae (nauplii; singular nauplius), about 22 mm long, are big enough to be observed via naked eyes while small enough to be hatched in large amount without occupying huge spaces (Sarah, Anny, & Misbahuddin, 2017). However, choice of solvent used for extraction should be done carefully on the basis of the desired outcome of research and should be used in minimal concentration. It's a preliminary toxicity screening method, later on experimentation is carried on mammalian animal models (Wu et al., 2014).

Paudel et al. (2012, 2014), methanol extracts of twenty four lichen species belonging to six families were collected from mountainous region of Nepal and found that extracts of *Heterodermia sp.* And *Ramalina sp.* showed similar toxicity effect as commercial standard berberine chloride indicating a potent source of anticancer drugs. Also, A total of 114 plant species, including 80 species of higher plants, 19 species of lichens and 15 species of mosses, were collected from Oymyakon region of the Republic of Sakha (Yakutia), Russia and biological study of their extracts showed that one species of higher plant *Rheum compactum* and one species of lichen *Flavocetraria cucullata* showed the toxicity against Brine shrimp larvae in 100 µg/mL of concentration. Lichens from higher altitude and extreme environments produce secondary metabolites with higher biological activity.

The brine shrimp mortality of lichen *Parmelia perlata* was conducted by Mayilsamy and Geetharamananis (2016), and stated that highest brine shrimp mortality of 100% was with hexane and methanol extracts at 100 and 200 ppm, respectively. Further extracts of six lichen species collected from Brazil and Antarctica were investigated by Ravaglia et al. (2014), and found that extracts of *P. wainioi, C. cryptochlorophaea*, and *C. aggregate* were less toxic to *A. salina* than those of *P. mesotropum, P. cetratum*, and *S. alpinum* with LC_{50} in the range of 151.0 to >600 µg mL^{-1}).

2.6 Chromatographic Technique

The Russian botanist, Tswett coined the term “chromatography" in 1906. Chromatography represents a separation technique; whereas chromatograph is a system for performing chromatography. In the earlier days it was used to separate and recover the substances of a

sample, now, complete chromatography systems are often used to separate and quantify sample components. Based upon the mobile phase chromatographic technique can be grouped as gas chromatography (GC), with gas as mobile phase and liquid chromatography (LC), with Liquid as mobile phase. Thin layer chromatography, reverse phase chromatography and column chromatography with liquid solvents as mobile phase are examples of LC. TLC is most commonly used technique for its convenience.

2.6.1 Thin Layer Chromatography (TLC) of Lichen Extracts

Thin layer chromatography (TLC) is a principal chromatography method used to separate mixture of compounds, which gives quick idea about the number and type of substances in a mixture. It can be used for estimation of known and unknown compounds on the basis of Retention factor (RF) value. It can be performed on a sheet of on a sheet of glass, plastic, or aluminum foil, which is coated with a thin layer of adsorbent material, such as silica gel, aluminum oxide, or cellulose (blotter paper), which is known as the stationary phase. Once the sample has been applied on the plate, the mobile phase (solvent or solvent mixture) will be drawn up the plate through capillary action. As the compounds move up at a different rate in the plate, they get separated (Bele & Khale, 2011) . Applications of TLC, is for monitoring the evolution of a reaction, compound identification and analyze the purity of the substance.

Several studies have been conducted to analyze the biochemical properties of the lichens via TLC, which makes it a routine ideal tool. Paudel et al. (2008), performed TLC analysis of extracts of lichens collected from Antarctica, and found that majority of these separated compounds had antioxidant property. Similarly, Begora and Fahselt (2000), combined TLC with densitometry method for quantification of secondary metabolites of lichens, the compounds separated using TLC was quantified by using UV light and density dependent absorbance of the compounds. TLC has also been utilized for identification of lichens and categorization of its metabolites. Tabbabi and Karmous (2016), in their analysis identified 28 species belonging to the following families: Xanthoria, Parmelia, Caloplaca, Ramalina, Diploschistes, and Usnea. A chromatographic study of the chemical composition of these lichens showed the presence of many compounds belonging to various chemical categories such as depsides and depsidones, xanthones, anthraquinones, dibenzofuran, etc.

Chapter 3 -Material and Methods

3. Materials and Methods

3.1 Collection and Identification of Lichen Species

A total of eighty four lichen specimens (Table 2) were collected from five different geographical locations of ACA of western Nepal in the time period of January, 2014 to April, 2014. Lichens were collected by Prof. Dr. Hari Datta Bhhatarai, Mitesh Shrestha and myself on bright sunny day to avoid excess moisture which could have degraded the samples and ulter the bioactive properties of lichen substances.

Detailed map of ACA

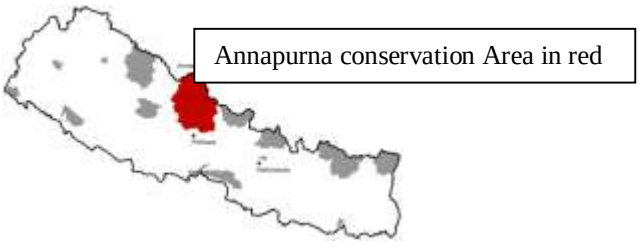


Fig. 3 1: Map of Annapurna Conservation Area (ACA) and our sample collection site shown by arrow

Lichens loosely attached to the substratum were collected with the help of sharp knife and kept in separate polythene bag punched with holes to release excess moisture, labeled and tied with rubber band. Collected samples were placed in air tight plastic container and transported to laboratory by bus. Voucher specimens of the lichen are deposited in the lichen herbarium of the Research Institute of Bioscience and Biotechnology, Kathmandu, Nepal.

3.2 Extraction

Extraction procedure was carried out according to previously described procedure by Paudel et al. (2007, 2008 and 2012). Completely freeze-dried and ground lichen samples, 1– 10 g (Table 2) were extracted separately in a mixture of 200 ml of methanol and water (90:10 v/v) and left at room temperature for an average period of 36 hours. After this, the extract was filtered using cotton and kept in a separate flask. This was designated as primary extract. To the residue, another 200 ml of 90 % methanol was added and left in the water bath at 45 °C for 6 hours. This was designated as secondary extract. The extract thus obtained was also filtered using cotton and mixed with the primary extract obtained earlier. The solvent, now 400 ml (primary and secondary extract combined), was evaporated at 38 °C under reduced pressure using rotary vacuum evaporator leaving only around 10 ml of solvent. This extraction procedure was repeated three times to ensure the complete extraction of extractable compounds. The total extract of a specimen was dissolved in distilled water (100 mL) and washed with hexane (300 mL) three times to remove pigments and fats. The water phase of extract was further washed with dichloromethane (300 mL) (DCM) thrice to remove medium polar compounds. The remaining water phase was freeze-dried, and residue dissolved in methanol. Insoluble parts of sugar and polysaccharides were removed. DCM wash was further concentrated using rotavapour and considered as DCM extract. Hexane was recycled for later use while. The DCM and methanol fractions of the extracts were then stored at −20 °C until further use.

3.3 Chemical Reagents

2,2-Diphenyl-1-picrylhydrazyl (DPPH), Butylated hydroxyanisole (BHA), Ampicillin, Amphotericin B and Berberine chloride were purchased from Sigma-Aldrich, USA. All chemicals and reagents were of analytical grade. The solvents used during extraction and

chromatography were of normal grade quality and they were distilled before use. The bacterial culture media, nutrient agar (NA) and Nutrient Broth (NB) and the fungal growth medium, Potato dextrose medium (PDA) were purchased from Difco, USA.

3.4 Antimicrobial Assay

3.4.1 Target Microorganisms and Culture Condition

Three clinical microorganisms, including one Gram-positive (*Staphylococcus aureus* KCTC3881) and one Gram-negative (*Klebsiella pneumoniae* KCTC2242) bacteria and a fungus (*Candida albicans* KCTC 7965), were purchased from Korean Collection of Type Culture (KCTC). Bacterial strains were grown on Nutrient Agar (NA) at 37 ℃ and *C. albicans* was grown on Potato Dextrose Agar (PDA) at 25 ℃.

3.4.2 Disk Diffusion Assay

Sterile paper disks (Adventic, Japan) of 6 mm size were loaded with lichen extract at a concentration of 2 mg/disk in triplicate and allowed to dry at room temperature under sterile conditions. The disks were kept on the surface of NA and PDA plates, which had been freshly swabbed with the overnight grown broth culture of the target microbial strains. Then, the plates were incubated at optimum growth temperature of each strain for 24–48 h. The zones of inhibition around the lichen extract loaded paper disks were reflective of the antimicrobial effectiveness of the extract. Paper disks loaded with methanol, the solvent used to dissolve crude extract, were used as negative controls and the paper disks loaded with Ampicillin and Amphotericin B were used as positive controls for bacteria and fungi respectively.

3.5 Antioxidant Assay

DPPH free radical scavenging assay free radical scavenging activity for the lichen extracts was estimated by using a previously described method (Blois MS. 1958). Primary screening was done by mixing one mL of DPPH solution (0.1 mM of DPPH in methanol) with 3 mL of the test extract. The mixture was incubated at room temperature (RT) for 30 min. The change in color of the solution from purple to yellow indicated the presence of antioxidant property in the extract.

After the primary screening, those extract which showed positive antioxidant property were then prepared similarly at different concentrations and their absorbance was taken at 517 nm in a UV-Visible spectrophotometer (SCINCO). The experiment was conducted in triplicate. IC_{50} of each of the positive extract was obtained by comparing with the available commercial standard. Reaction mixtures without the test extract and with Butylated hydroxyanisole (BHA) were used as negative and positive controls, respectively.

Radical scavenging activity was calculated using the following formula.

$$\% \text{ Radical Scavenging activity} = [(\text{Control absorbance} - \text{Sample absorbance}) / \text{Control absorbance})] * 100$$

Standard graph was plotted taking the concentration on the X-axis and percentage scavenging activity on the Y-axis. Based on this graph, IC_{50} value of each sample was calculated based on the formula

$$IC_{50} = (LN(conc.>50\%) - ((pi>50\% - 50)/(pi>50\% - pi<50\%) * LN(conc. >50\%/conc.< 50\%)))$$

The IC_{50} is the concentration of an inhibitor where the response is reduced by half. The IC_{50} value of the different lichen extracts was compared. The sample or extract having the lowest IC_{50} is considered to have the best antioxidant property.

3.6 Brine Shrimp Lethality Test

Brine shrimp lethality test (BST) was used to evaluate the toxicity of various lichen's crude extracts (Meyer et al., 1982) with slight modification. The eggs of *Artemia salina* were hatched in aerated seawater in light at 25 °C. The hatched active nauplii were attracted towards the direction of light. The active nauplii (about 100) were selected and treated with 1 mg/mL of the test samples in 24 well plates. The effects of the test samples were monitored after 24 h of treatment by observing the live nauplii. The mortality rate of the nauplii indicated the toxicity of the test samples. The activity was categorized into four groups-strong (80% to 100% death of nauplii), moderate (50% to 80% death of nauplii), weak (less than 50% death of nauplii) and

inactive (no death at all) (Table 2). Berberine chloride was used as positive control and sea water was used as negative control.

3.7 Separation of Chemical Constituents Using Thin Layer Chromatography

Thin layer chromatography analysis of the crude extracts that showed positive results during the primary screening for antioxidant were performed by using analytical silica gel TLC plates (Merck) to gather information regarding their concentration and polarity. An aliquot of 200 microgram lichen extract was loaded in the bottom of TLC plates (size. 10 cm X 20 cm) and run on the mobile phase of 10% methanol in DCM. The plates were observed under UV (254 nm and 365 nm) to observe the bands of separated compounds. The plates were sprayed with DPPH solution (4 mM) in methanol. The yellow color developed against purple surface background of DPPH indicated antioxidant active compounds in the extracts.

3.8 Data Analysis

All data were expressed as mean ± SD from a minimum of three replicates. 50% Inhibition concentration (IC_{50}) was calculated by using Microsoft Excel 2007.

Chapter 4 - Results

4. Results

4.1 Collection and Identification of Lichens

A total of eighty four lichen specimens were collected and identified by analyzing morphological, anatomical and phytochemical characteristics using a lichen identification check list (Awasti, 2007). These specimens on later identification with help of Dr. C. B. Baniya at Central Department of Botany were found belonging to 47 different species under 19 different genera and 12 different families (Table 1). Among others, twenty two specimens were identified up to genus level only. 11 lichen species: DAN-1 to DAN-11 were collected from Danaque (2230 m asl), 16 lichen species : JOM-1 to JOM-16 were collected from Jomsom (2760 m asl), 9 lichen species : CHA-1 to CHA-9 were collected from Chame (2713 m asl), Similarly, 21 lichen species: GHAN-1 to GHAN-21 were collected from Ghandruk (2012 m asl), while remaining 27 lichen species : SAR-1 to SAR-27 were collected from Sarangkot (1750 m asl).

Table 1: Lichen collected from different geographical locations of ACA and its family.

S.No.	Symbol	Name	Family
1	DAN-1	*Parmotrema cetratum*	Parmeliaceae
2	DAN-2	*Usnea pectinata*	
3	DAN-4	*Everniastrum cirrhatum*	
4	DAN-5	*Parmotrema sanoti-angelii*	
5	DAN-6	*Parmotrema reticulatum*	
6	DAN-9	*Parmotrema sp.*	
7	DAN-10	*Parmotrema tinctorum*	
8	JOM-2	*Parmotrema thomsonii*	
9	JOM-4	*Everniastrum cirrhatum*	
10	JOM-5	*Usnea sp.*	
11	JOM-8	*Parmotrema tinctorum*	
12	JOM-10	*Parmotrema reticulatum*	
13	JOM-11	*Parmelina quercina*	
14	JOM-13	*Parmotrema sp.*	
15	CHA-2	*Parmotrema sp.*	
16	CHA-3	*Parmotrema sp.*	
17	CHA-6	*Usnea sp.*	
18	GHAN-2	*Parmotrema melanothrix*	
19	GHAN-4	*Parmotrema praesorediosum*	
20	GHAN-9	*Parmotrema sp.*	
21	GHAN-12	*Parmelia sp.*	
22	GHAN-13	*Parmotrema sp.*	

23	GHAN-16	*Parmelia meiophora*	
24	GHAN-17	*Usnea baileyi*	
25	GHAN-19	*Parmotrema sp.*	
26	GHAN-21	*Hypotrachyna flexilis*	
27	SAR-2	*Parmotrema reticulatum*	
28	SAR-4	*Usnea coralline*	
29	SAR-7	*Parmotrema reticulatum*	
30	SAR-9	*Parmelaria thomsonii*	
31	SAR-10	*Everniastrum nepalenses*	
32	SAR-12	*Parmotrema reticulatum*	
33	SAR-13	*Parmotrema sp.*	
34	SAR-14	*Parmelia omphalodes*	
35	SAR-15	*Parmotrema reticulatum*	
36	SAR-16	*Parmotrema sp.*	
37	SAR-18	*Parmotrema sp.*	
38	SAR-19	*Parmotrema sp.*	
39	SAR-22	*Parmelia omphalodes*	
40	DAN-3	*Ramalina conduplicans*	Ramalinaceae
41	JOM-3	*Ramalina sp.*	
42	CHA-1	*Ramalina roesleri*	
43	SAR-5	*Ramalina conduplicate*	
44	DAN-8	*Cladonia verticillata*	Cladoniaceae
45	JOM-12	*Cladonia squamosal*	
46	CHA-5	*Cladonia verticillata*	
47	GHAN-6	*Cladonia sp.*	
48	SAR-11	*Cladonia coccifera*	
49	SAR-25	*Cladonia coccifera*	
50	DAN-11	*Peltigera polydactyla*	Peltigeraceae
51	JOM-6	*Peltigera polydactyla*	
52	CHA-8	*Peltigera polydactyla*	
53	JOM-1	*Lobaria retigera*	Lobariaceae
54	SAR-6	*Lobaria disecta*	
55	JOM-9	*Leptogium delavayi*	Collemataceae
56	JOM-16	*Leptogium delavayi*	
57	CHA-4	*Leptogium sp.*	
58	GHAN-10	*Leptogium delavayi*	
59	GHAN-14	*Collema sp.*	
60	CHA-7	*Lepraria sp.*	Stereocaubceae
61	CHA-9	*Lepraria sp.*	
62	GHAN-1	*Coccocarpia erythroxyli*	Coccocarpiaceae

63	GHAN-5	*Pertusaria leucosora*	Pertusariaceae
64	SAR-27	*Pertusaria sp.*	
65	DAN-7	*Heterodermia diademata*	Physciaceae
66	JOM-7	*Heterodermia diademata*	
67	JOM-14	*Heterodermia indica*	
68	JOM-15	*Heterodermia leucomela*	
69	GHAN-3	*Heterodermia diademata*	
70	GHAN-7	*Heterodermia diademata*	
71	GHAN-8	*Heterodermia punctifera*	
72	GHAN-15	*Heterodermia microphylla*	
73	GHAN-18	*Heterodermia leucomelos*	
74	SAR-1	*Heterodermia indica*	
75	SAR-3	*Heterodermia punctifera*	
76	SAR-8	*Heterodermia podocarpa*	
77	SAR-17	*Heterodermia speciosa*	
78	SAR-20	*Heterodermia microphylla*	
79	SAR-21	*Heterodermia microphylla*	
80	SAR-23	*Heterodermia microphylla*	
81	SAR-24	*Heterodermia microphylla*	
82	SAR-26	*Heterodermia speciosa*	
83	GHAN-11	*Lecidea sp.*	Lecideaceae
84	GHAN-20	*Pannaria complanata*	Pannariaceae

DAN, JOM, CHA, GHAN and SAR represents the sample collection sites Danaqe, Jomsom, Chame, Ghandruk and Sarangkot respectively.

4.2 Methanol and DCM Extraction

All the 84 samples of lichens collected from different geographical regions of western development region of Nepal were subjected to methanol and DCM extraction and these extracts were used to carry out various experiments. The results of these experiments are given below.

4.2.1 Antioxidant Test of Lichen Extracts

Antioxidant property of methanol extract of lichens collected from various region of Nepal was determined using the solution of DPPH (0.1 mM) and taking BHA as the pure antioxidant reference compound. IC_{50} value was calculated for each positive sample taking the concentration vs. % radical scavenging activity (517 nm). There was gradual increase in % radical scavenging activity as the concentration of the extract increased (Fig 4.1-4.4). The IC_{50} value for BHA was found to be 4.98 ± 0.4 µg/mL. Maximum IC_{50} value (87.3 ± 7.1 µg/ml) was found in methanol fraction of *Parmelaria thomsonii* collected from SAR-9 and minimum IC_{50} value (5.56 ± 0.17 µg/ml) was found in the DCM extracts of *Peltigera polydactyla* from CHA-8 (Table 2). The samples with lower IC_{50} are considered as the best antioxidants and vice versa. Table 2 shows that only 37 extracts possessed antioxidant

activities. Among them, the DCM fractions of *Parmoterma centratum, Peltigera polydactyla* and *Ramalina roesleri* and methanol fractions of *Peltigera polydactyla* and *Parmoterma sp.* Showed comparatively strong DPPH reducing activity. In the present experiment the data showed that 16 methanol fractions, 21 DCM fractions, 7 both methanol and DCM fractions, 9 only methanol fractions and 14 only DCM fractions were antioxidant active. The lowest IC_{50} was possessed by the *Peltigera polydactyla* (5.56 ± 0.17 µg/ml) collected from Chame whereas IC_{50} of standard antioxidant (BHA) was found to be 4.98 ± 0.4 µg/ml and hence considered it as the best antioxidant among other samples. The results indicated the variability of antioxidant compounds in the lichen extract.

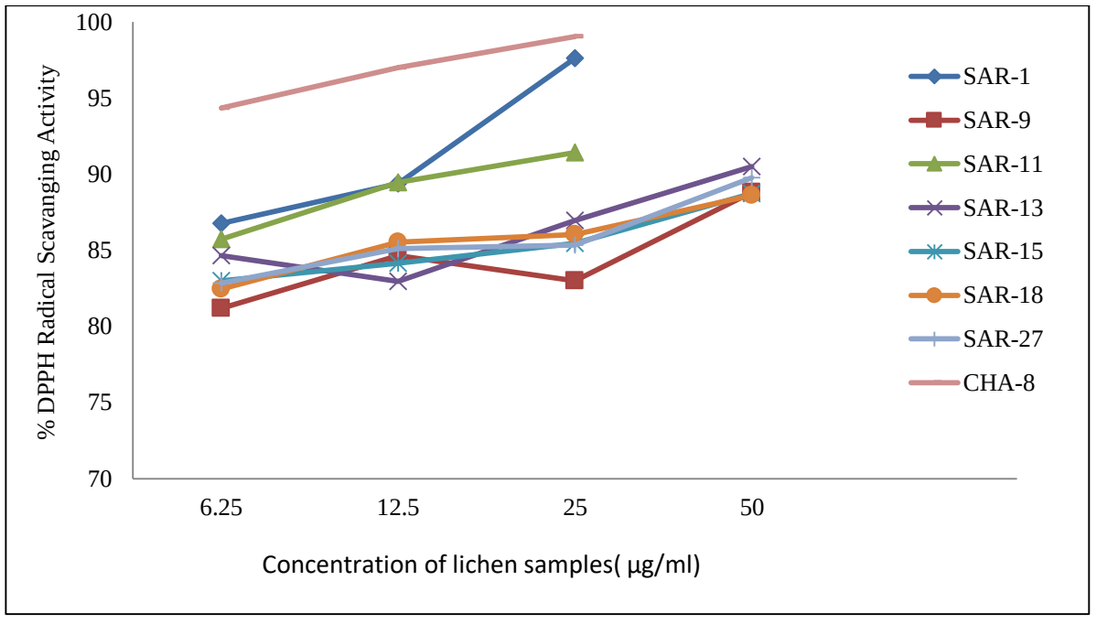


Fig. 4. 1 : % DPPH free radical scavenging capacity of lichen extracts of methanol fractions. SAR and CHA represent the sample collection sites Sarangkot and Chame respectively.

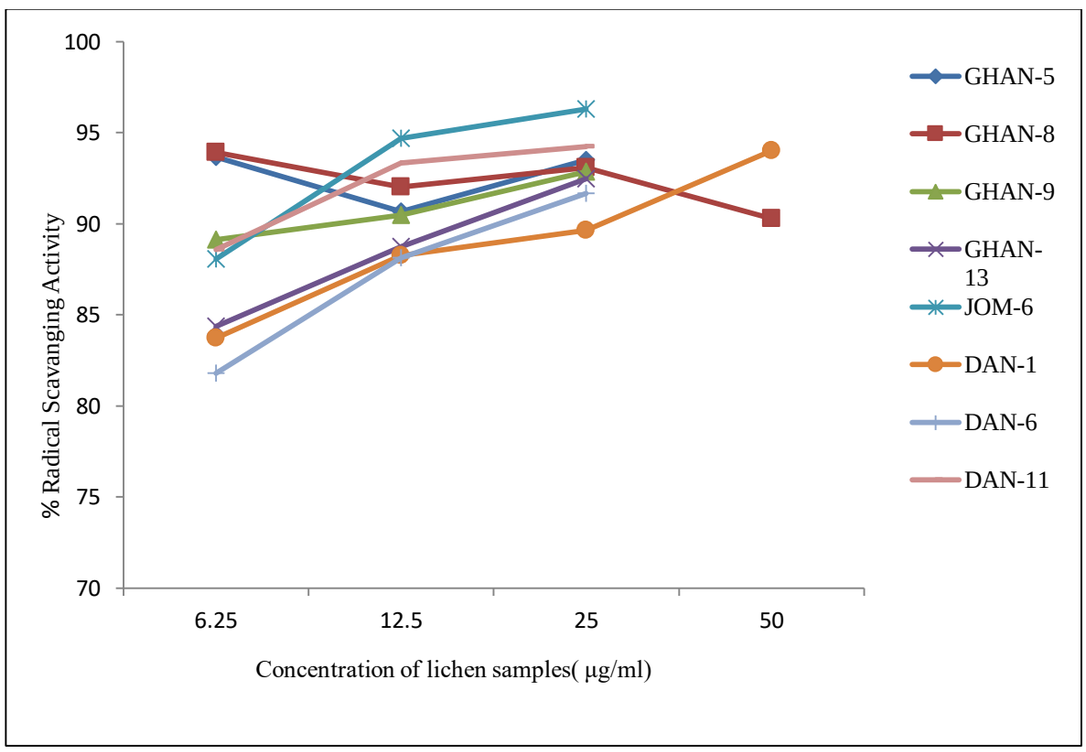


Fig 4. 2 : % DPPH free radical scavenging capacity of lichen extracts of methanol fractions. GHAN, JOM and DAN represent the sample collection sites Ghandruk, Jomsom and Danaqe respectively.

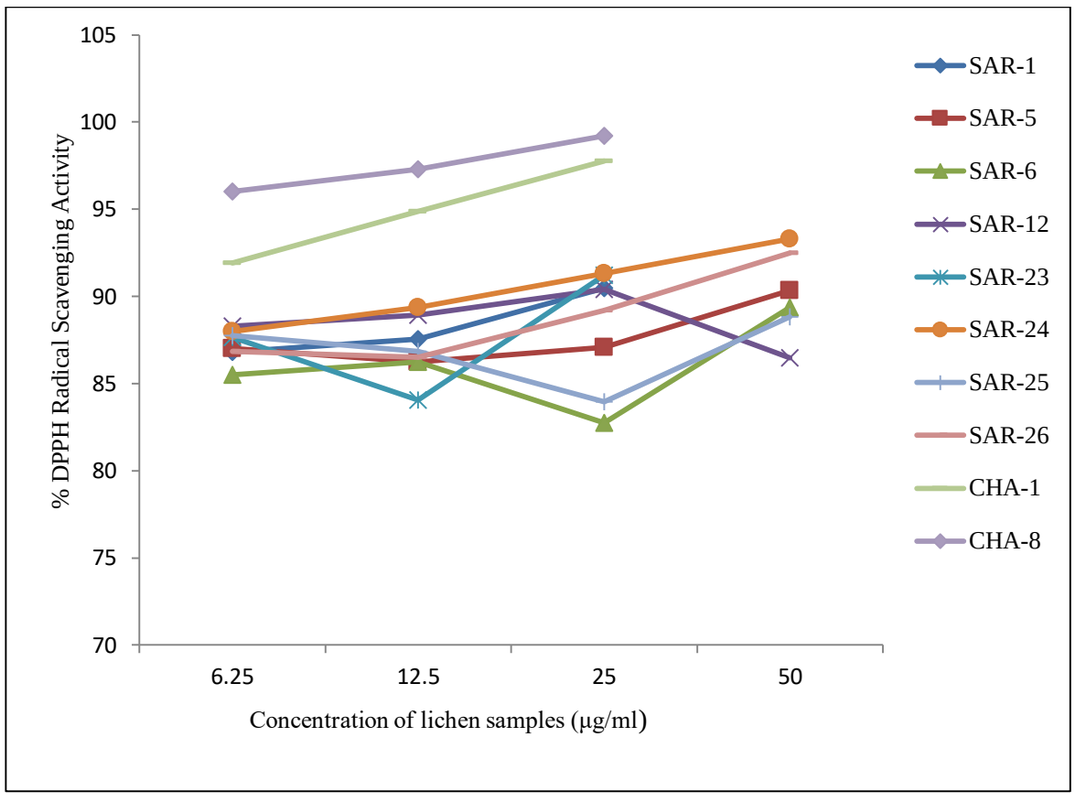


Fig. 4. 3 : % DPPH free radical scavenging capacity of lichen extracts of DCM fractions. SAR and CHA represent the sample collection sites Sarangkot and Chame respectively.

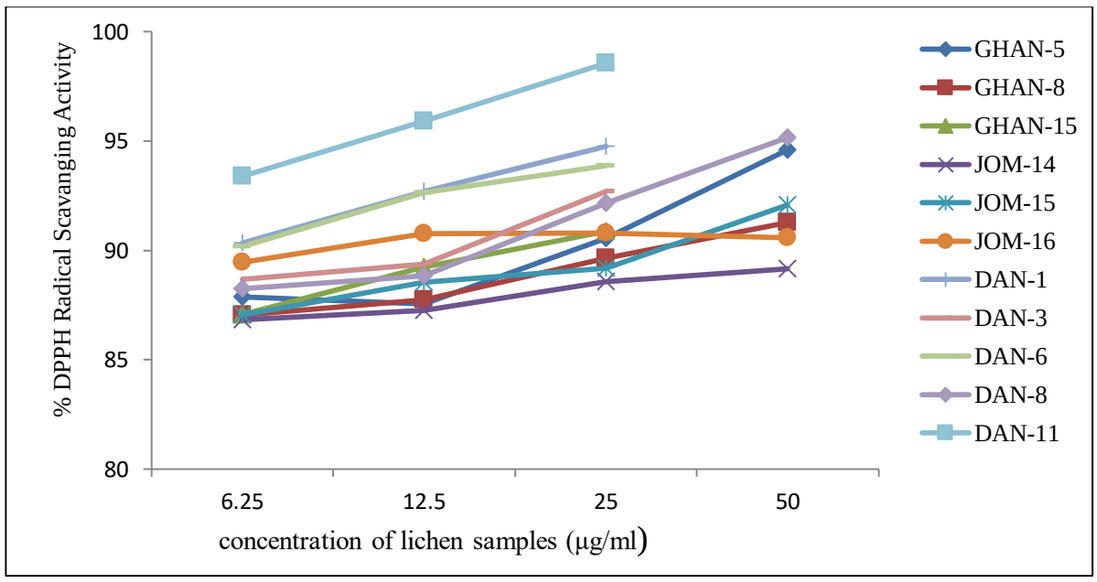


Fig. 4. 4 : % DPPH free radical scavenging capacity of lichen extracts from DCM fractions. GHAN, JOM and DAN represent the sample collection site Ghandruk, Jomsom and Danaqe respectively.

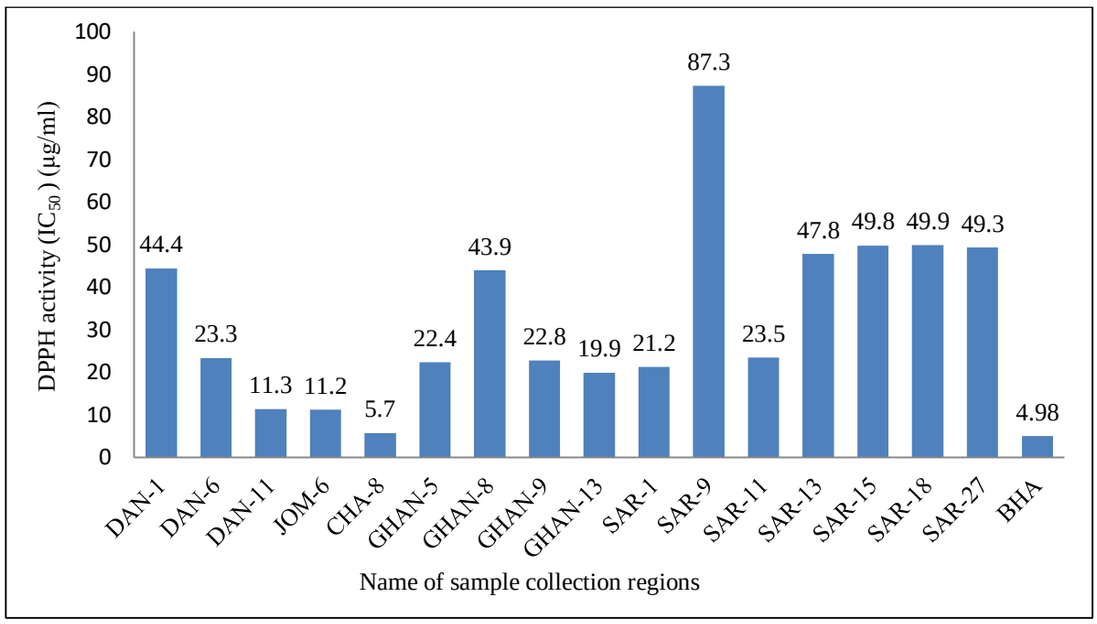


Fig. 4. 5 : IC_{50} value of standard compound (BHA µg/ml) along with lichen extracts of Methanol fraction (µg/ml) observed after 30 minutes. DAN, JOM, CHA, GHAN and SAR represent the sample collection sites Danaqe, Jomsom, Chame, Ghandruk and Sarangkot respectively.

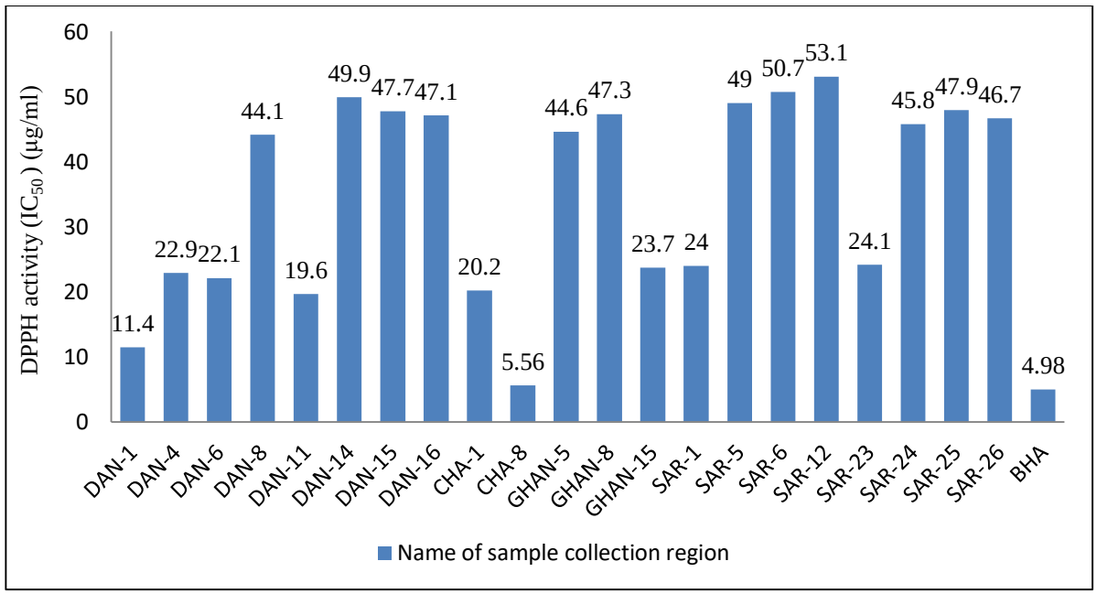


Fig. 4. 6 : IC_{50} value of standard compound (BHA µg/ml) along with Lichen extracts of DCM fraction (µg/ml) observed after 30 minutes. DAN, CHA, GHAN and SAR represent the sample collection sites Danaqe, Chame, Ghandruk and Sarangkot respectively.

4.3 Antimicrobial activities

Only DCM fraction of the lichen extracts were active against *S. aureus* and *K. pneumoniae*. The sizes of zone of inhibition of active fractions are given in Table 2. Seventeen extracts showed activities against *S. aureus* and 45 extracts showed activities against *K. pneumoniae*. Twelve extracts showed antibacterial activities against both *S. aureus* and *K. pneumoniae*. Thus, the test lichens were found to be antimicrobial active against both Gram-positive and Gram-negative bacterial strains. Only three extracts were active against *C. albicans*. The comparative study of obtained results (Table 2) showed that the antimicrobial constituents in the lichens extracts were different. Also comparative antimicrobial activity of DCM fraction of lichen against *S. aureus, K. pneumonia and C. albicans* are shown in (Fig. 4.7), (Fig. 4.8) and (Fig. 4.9) respectively.

Table 2 : Lichens collected from Annapurna conservation area and biological activities of their extracts.

S. N.	Symbol	Name	Dry wt. (gms)	DPPH activity (IC_{50}) (µg/ml) in fraction of		Anti microbial activity of DCM fraction (mm)			Brine Shrimp toxicity	
				Methanol	DCM	A	B	C	MeOH	DCM
1	DAN-1	*Parmotrema cetratum*	10	44.4±0.5	11.4±0.1	7	7	-	S	S
2	DAN-2	*Usnea pectinata*	10	-	-	-	-	-	S	S
3	DAN-3	*Ramalina conduplicans*	10	-	22.9±0.7	-	12	-	S	S
4	DAN-4	*Everniastrum cirrhatum*	2	-	-	-	11	-	I	S
5	DAN-5	*Parmotrema sanoti-angelii*	10	-	-	10	10	-	S	S
6	DAN-6	*Parmotrema reticulatum*	10	23.3±0.3	21.1±0.1	10	9	-	S	S
7	DAN-7	*Heterodermia diademata*	10	-	-	-	7	-	I	S
8	DAN-8	*Cladonia verticillata*	9.1	-	44.1±0.3	-	8	-	W	S
9	DAN-9	*Parmotrema sp.*	10	-	-	-	7	-	I	S
10	DAN-10	*Parmotrema tinctorum*	10	-	-	-	7	-	I	S
11	DAN-11	*Peltigera polydactyla*	3.6	11.3±0.2	19.6±0.1	-	-	-	I	S
12	JOM-1	*Lobaria retigera*	10	-	-	-	-	-	S	S
13	JOM-2	*Parmotrema thomsonii*	10	-	-	8	11	-	S	S
14	JOM-3	*Ramalina sp.*	10	-	-	-	-	-	S	S
15	JOM-4	*Everniastrum cirrhatum*	10	-	-	-	14	-	S	S
16	JOM-5	*Usnea sp.*	10	-	-	-	10	-	S	S
17	JOM-6	*Peltigera polydactyla*	9.2	11.2±0.1	-	-	-	-	S	S
18	JOM-7	*Heterodermia diademata*	10	-	-	-	-	12	I	S
19	JOM-8	*Parmotrema tinctorum*	10	-	-	11	7	-	S	S
20	JOM-9	*Leptogium delavayi*	10	-	-	-	-	-	W	I

21	JOM-10	*Parmotrema reticulatum*	5.9	-	-	-	-	-	S	I
22	JOM-11	*Parmelina quercina*	5.6	-	-	-	10	-	I	S
23	JOM-12	*Cladonia squamosal*	10	-	-	12	13	-	I	S
24	JOM-13	*Parmotrema sp.*	10	-	-	11	7	14	S	S
25	JOM-14	*Heterodermia indica*	2.8	-	49.9±2.4	9	8	11	I	S
26	JOM-15	*Heterodermia leucomela*	3.8	-	47.7±0.5	8	9	-	I	S
27	JOM-16	*Leptogium delavayi*	0.9	-	47.1±1.8	-	-	-	I	W
28	CHA-1	*Ramalina roesleri*	10	-	20.2±0.2	-	-	-	I	S
29	CHA-2	*Parmotrema sp.*	10	-	-	-	-	-	S	I
30	CHA-3	*Parmotrema sp.*	10	-	-	-	-	-	S	S
31	CHA-4	*Leptogium sp.*	0.8	-	-	-	-	-	I	S
32	CHA-5	*Cladonia verticillata*	10	-	-	-	-	-	I	S
33	CHA-6	*Usnea sp.*	9	-	-	-	-	-	S	S
34	CHA-7	*Lepraria sp.*	6.8	-	-	-	-	-	S	I
35	CHA-8	*Peltigera polydactyla*	10	5.7±0.02	5.56±0.2	-	-	-	I	S
36	CHA-9	*Lepraria sp.*	10	-	-	-	-	-	I	W
37	GHAN-1	*Coccocarpia erythroxyli*	3.4	-	-	-	-	-	I	I
38	GHAN-2	*Parmotrema melanothrix*	10	-	-	-	7	-	S	S
39	GHAN-3	*Heterodermia diademata*	10	-	-	-	7	-	W	S
40	GHAN-4	*Parmotrema praesorediosum*	10	-	-	-	9	-	I	S
41	GHAN-5	*Pertusaria leucosora*	10	22.4±0.9	46.6±1.0	-	13	-	S	S
42	GHAN-6	*Cladonia sp.*	10	-	-		9	-	I	S
43	GHAN-7	*Heterodermia diademata*	10	-	-	-	-	-	S	S
44	GHAN-8	*Heterodermia punctifera*	10	43.9±2.1	47.3±0.6	-	-	-	S	S

45	GHAN-9	*Parmotrema sp.*	3	22.8±0.9	-	-	12	-	W	S
46	GHAN-10	*Leptogium delavayi*	8.5	-	-	-	-	-	W	I
47	GHAN-11	*Lecidea sp.*	10	-	-	-	12	-	S	S
48	GHAN-12	*Parmelia sp.*	10	-	-	-	9	-	S	S
49	GHAN-13	*Parmotrema sp.*	10	19.9±3.0	-	-	11	-	I	S
50	GHAN-14	*Collema sp.*	1.7	-	-	-	-	-	I	I
51	GHAN-15	*Heterodermia microphylla*	10	-	23.7±0.1	-	-	-	I	S
52	GHAN-16	*Parmelia meiophora*	6.9	-	-	-	15	-	S	S
53	GHAN-17	*Usnea baileyi*	1.8	-	-	-	-	-	S	S
54	GHAN-18	*Heterodermia leucomelos*	10	-	-	7	7	-	I	S
55	GHAN-19	*Parmotrema sp.*	10	-	-	-	11	-	S	S
56	GHAN-20	*Pannaria complanata*	10	-	-	11	10	-	S	S
57	GHAN-21	*Hypotrachyna flexilis*	10	-	-	-	9	-	I	S
58	SAR-1	*Heterodermia indica*	7.8	21.2±0.3	24.0±0.1	-	8	-	S	S
59	SAR-2	*Parmotrema reticulatum*	10	-	-	-	9	-	S	S
60	SAR-3	*Heterodermia punctifera*	7.83	-	-	-	-	-	I	S
61	SAR-4	*Usnea coralline*	9.6	-	-	-	-	-	S	S
62	SAR-5	*Ramalina conduplicate*	5.3	-	49.0±2.3	-	12	-	S	S
63	SAR-6	*Lobaria disecta*	10	-	50.7±2.2	-	7	-	W	S
64	SAR-7	*Parmotrema reticulatum*	10	-	-	-	7	-	W	S
65	SAR-8	*Heterodermia podocarpa*	10	-	-	-	12	-	W	S
66	SAR-9	*Parmelaria thomsonii*	10	87.3±7.1	-	-	10	-	W	S
67	SAR-10	*Everniastrum nepalenses*	10	-	-	-	8	-	S	S

68	SAR-11	*Cladonia coccifera*	10	23.5±0.8	-	-	-	-	I	S
69	SAR-12	*Parmotrema reticulatum*	5.7	-	53.1±6.9	-	-	-	S	S
70	SAR-13	*Parmotrema sp.*	10	47.8±1.3	-	9	-	-	S	S
71	SAR-14	*Parmelia omphalodes*	10	-	-	10	11	-	I	S
72	SAR-15	*Parmotrema reticulatum*	3.5	49.8±0.1	-	-	9	-	M	S
73	SAR-16	*Parmotrema sp.*	1.5	-	-	-	8	-	S	S
74	SAR-17	*Heterodermia speciosa*	6	-	-	7	-	-	M	S
75	SAR-18	*Parmotrema sp.*	8.6	49.9±0.3	-	-	-	-	S	S
76	SAR-19	*Parmotrema sp.*	8.4	-	-	9	-	-	S	S
77	SAR-20	*Heterodermia microphylla*	10	-	-	8	-	-	W	S
78	SAR-21	*Heterodermia microphylla*	7.3	-	-	-	-	-	I	I
79	SAR-22	*Parmelia omphalodes*	10	-	-	-	-	-	S	S
80	SAR-23	*Heterodermia microphylla*	6.4	-	24.1±0.4	-	-	-	W	S
81	SAR-24	*Heterodermia microphylla*	1.2	-	45.8±1.1	9	-	-	I	S
82	SAR-25	*Cladonia coccifera*	0.6	-	47.9±0.2	-	-	-	I	S
83	SAR-26	*Heterodermia speciosa*	1.4	-	46.7±1.3	-	13	-	I	S
84	SAR-27	*Pertusaria sp.*	0.4	49.3±2.6	-	-	12	-	I	S
I		BHA		4.98±0.4						
II		Ampicillin(10 µg)				20	19			
III		Amphotericin B (10 µg)						20		
IV		Berberine chloride (8 µg)							W	
V		Berberine chloride (12 µg)							M	
VI		Berberine chloride (16 µg)							S	

IC_{50} (50% inhibition in DPPH color) data, A-antibacterial active against *Staphylococcus aureus* (inhibition zone in mm), B-antibacterial activity against *Klebsiella pneumoniae* (inhibition zone in mm), C-antifungal activity against *Candida albicans* (inhibition zone in mm), S-strong activity (more than 80% death of brine shrimp), M-Moderate(50%–80% of death of brine shrimp), W-Weak (less than 50% of death of brine shrimp), I-inactive (no death at all).

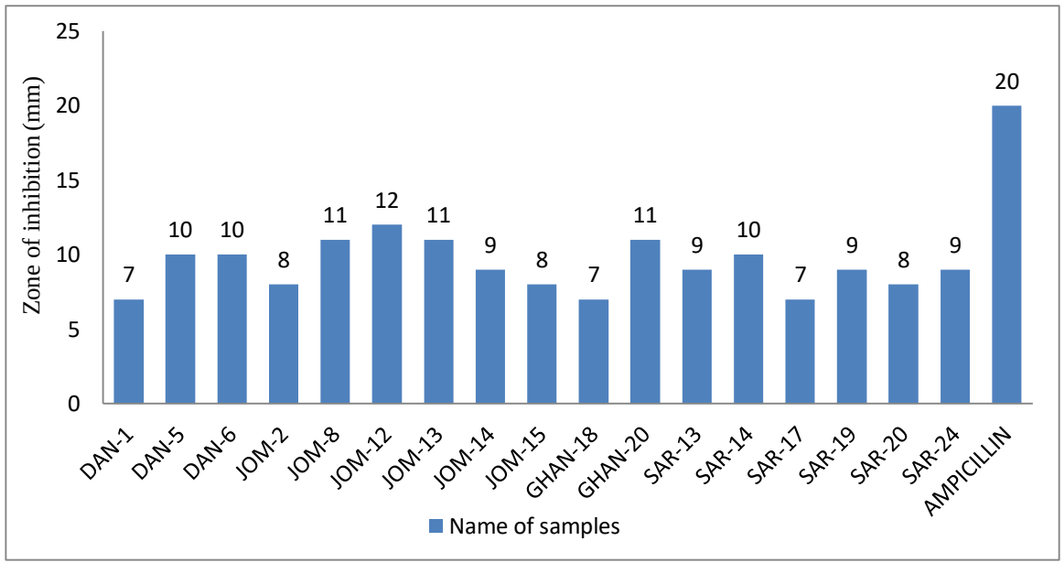


Fig. 4. 7 : Zone of inhibition of DCM extract (2 mg/disk) and standard (Ampicillin 10 µg) against *S. aureus.* DAN, JOM, GHAN and SAR represent the sample collection sites Danaqe, Jomsom, Ghandruk and Sarangkot respectively.

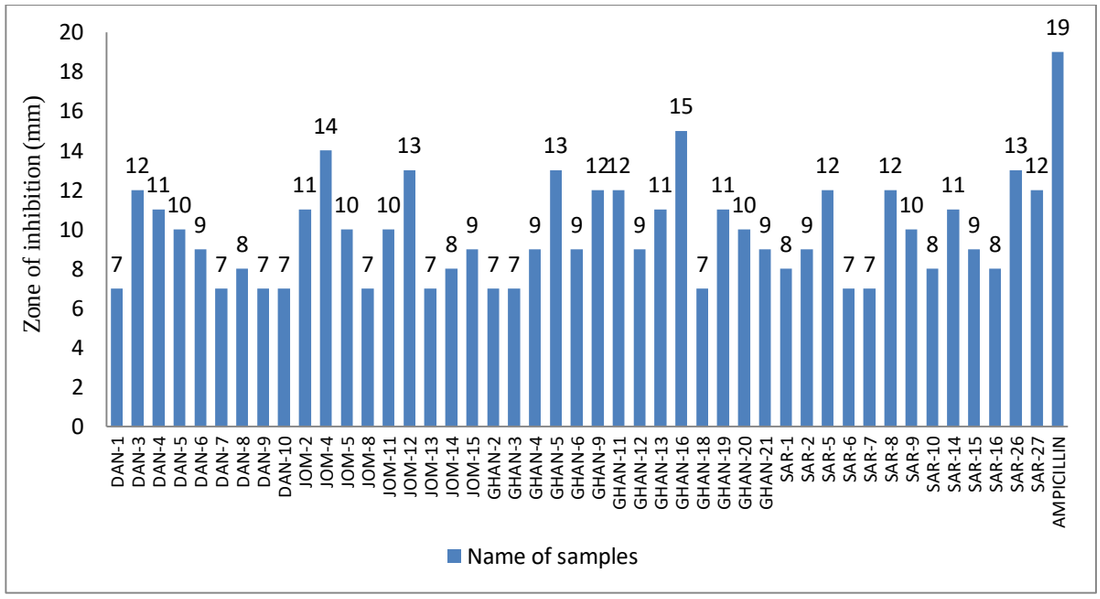


Fig. 4. 8 : Zone of inhibition of DCM extract (2 mg/disk) and standard (Ampicillin 10 µg) against *K. pneumonia.* DAN, JOM, GHAN and SAR represent the sample collection sites Danaqe, Jomsom, Ghandruk and Sarangkot respectively.

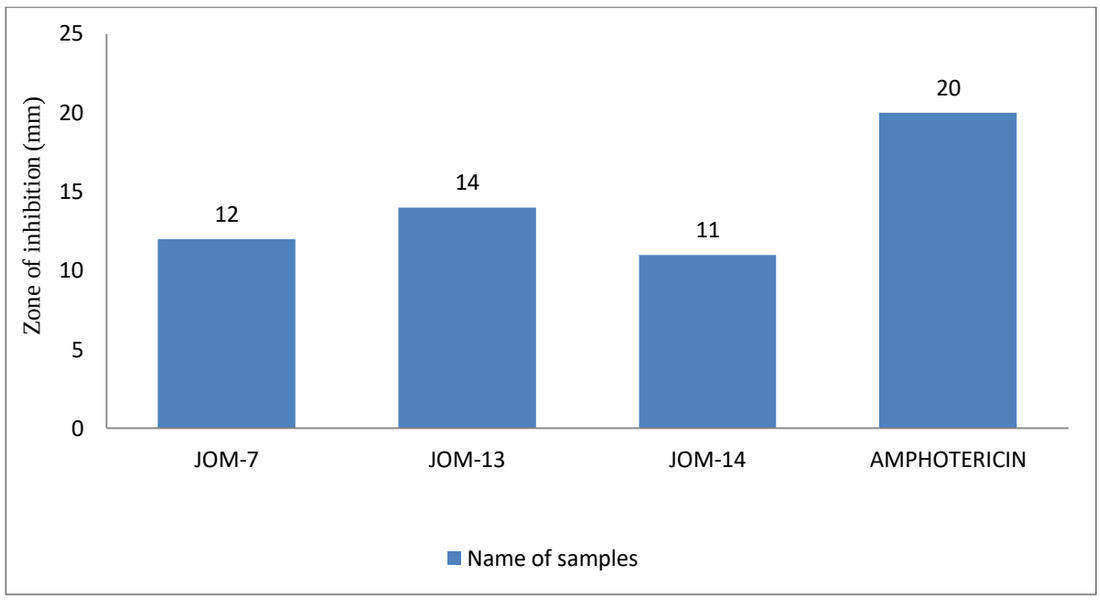


Fig. 4. 9 : Zone of inhibition of DCM extract (2 mg/disk) and standard (Amphotericin 10 µg) against *C. albicans*. JOM represents sample collection site Jomsom.

4.4 Toxicity Against Brine Shrimp (*Artemia salina*)

Hundred percent of *A. salina* nauplii were alive after 24 h of experiment in the negative control. LC_{50} of positive control sample was obtained 8.4 µg/mL. Interestingly, majority of the lichens extracts showed toxicity against Brine shrimp nauplii (Table 2). Four categories of activities have been observed: (S) strong-100-80% death of nauplii, (M) moderate-80-50% death of nauplii, (W) weak-less than 50% death and (I) inactive-no death of brine shrimp nauplii after 24 h of incubation. DCM fractions were found to be more toxic than methanol fractions. Strong activities were shown by 74 DCM fractions and 39 methanol fractions while 8 DCM fractions and 32 methanol fractions did not show any activity against *A. salina* nauplii. The lichen extracts of ACA region showed stronger toxicity against *A. salina* than the species of other region (Poudel et al., 2012).

4.5 TLC Based Antioxidant Activity Screening of Lichen Extracts

TLC based chemical screening of the bioactive fractions of the lichens showed a number of bioactive compounds contained in the extracts. As the mobile phase is less polar than the stationary phase in normal phase TLC, polar compounds will tend to have a lesser affinity for the mobile phase than nonpolar compounds. Therefore, polar compounds tend to spend less of the elution time in mobile phase than a non/low polar compound, so will travel "slower" up the plate, and have a low RF where as moderately polar compounds have a greater attraction to the mobile phase. Thus, a compound with a lower RF tends to have more polar functional groups than a compound with a higher RF (Nicolas, 2021). As expected, compounds from methanol fractions had a low RF value than those from the DCM fraction in this TLC system, which indicates that they were more polar (Fig. 4.10). TLC based phytochemical screening was performed only for the extracts which showed antioxidant activities. The extract with the highest IC_{50} was selected i.e. of *Peltigera polydactyla* as

shown in (Fig. 4.11). The number of antioxidant active bands in methanol fractions is comparatively small than in DCM fractions (Fig. 4.11). But, the intensity of color change of sprayed DPPH was much higher in the bands obtained from the methanol fractions than from the DCM fractions. The results indicated that methanol fractions contained stronger or higher concentration of antioxidant compounds than the DCM fractions. The results also indicated that the low intensity of color change of DCM fractions contained higher number of moderately active antioxidants or low concentration of antioxidant compound than the methanol fractions.

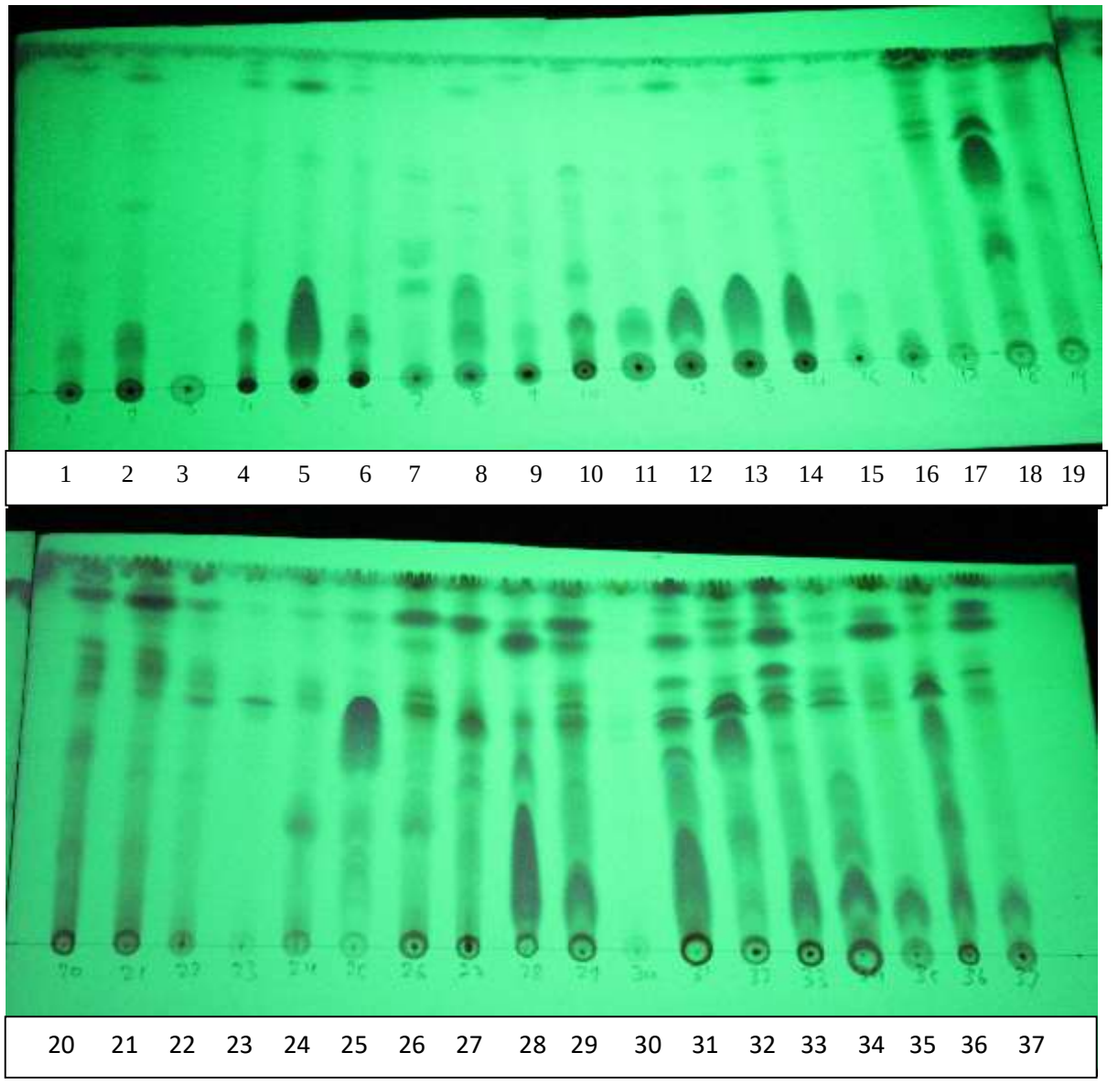


Fig. 4. 10 : TLC based chemical screening of lichen extracts**.** Plate viewed under UV (254 nm). Mobile phase for TLC development was 10% methanol in DCM. W- Methanol soluble water fraction, D-DCM fractions. The sample in the TLC plate is as follows: 1, SAR1W; 2, SAR9W; 3, SAR11W; 4, SAR13W; 5, SAR15W; 6, SAR18W; 7, SAR27W; 8, GHAN5W; 9, GHAN8W; 10, GHAN9W; 11, GHAN13W; 12, JOM6W; 13, DAN1W; 14, DAN6W; 15, DAN11W; 16, CHA8W; 17, SAR1D; 18, SAR5D; 19, SAR6D; 20, SAR12D; 21, SAR23D; 22, SAR24D; 23, SAR25D; 24, SAR26D; 25, GHAN5D; 26, GHAN8D; 27, GHAN15D; 28, JOM14D; 29, JOM15D; 23, JOM16D; 31, DAN1D; 32, DAN3D; 33, DAN6D; 34, DAN8D; 35, DAN11D; 36, CHA1D; 37 CHA8D

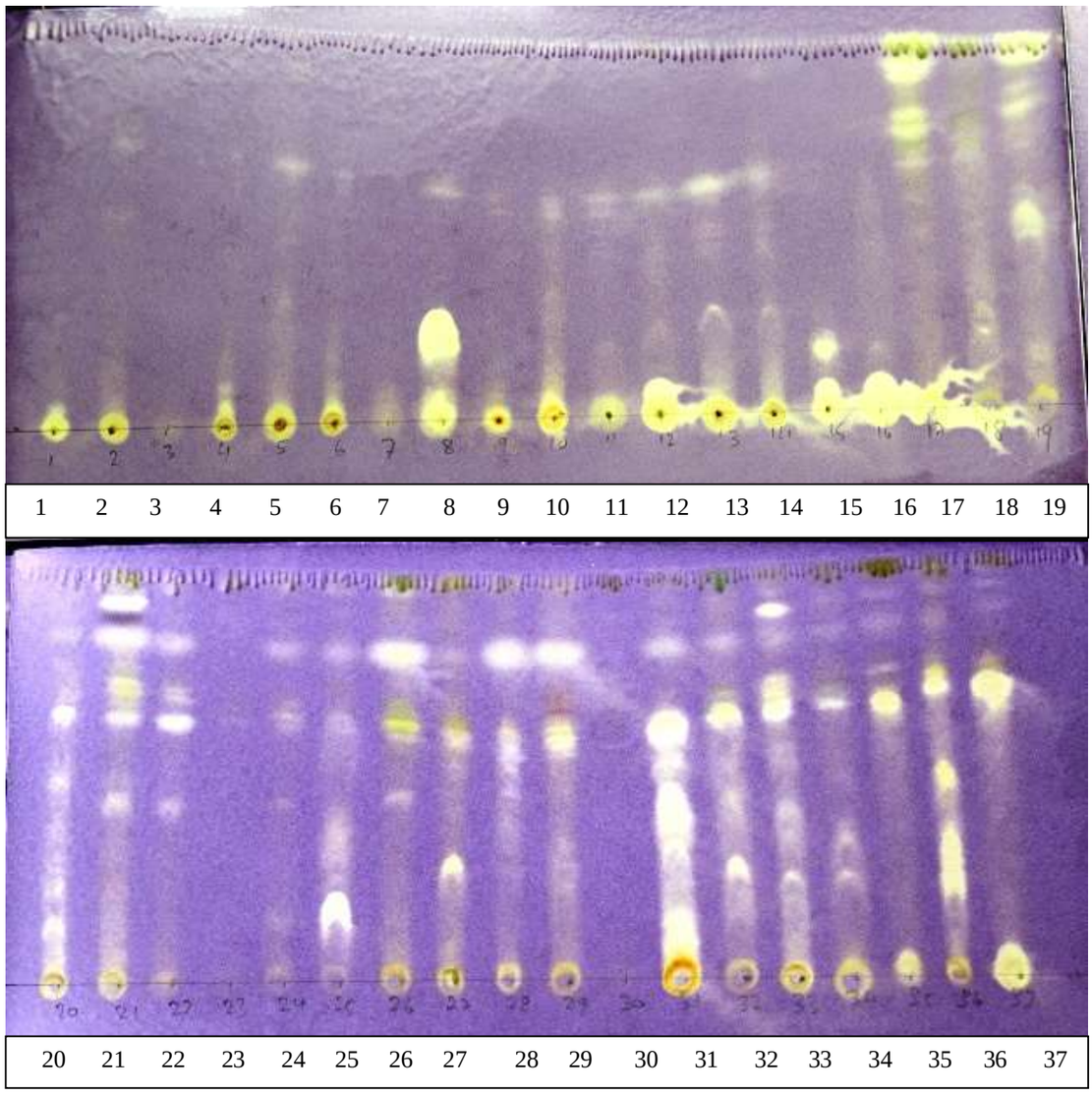


Fig. 4. 11 : TLC based antioxidant screening of the active extracts**.** Mobile phase for TLC development was 10% methanol in DCM. The purple color was from DPPH and antioxidant active fractions showed reduced in purple color to yellow. The darkness of color spots indicated the potential of antioxidant activity, the size of color spot indicated the content of antioxidant active compounds in the extracts. W-methanol soluble water fraction, D- DCM fractions of lichen extracts. The sample in the TLC plate is as follows: 1, SAR1W; 2, SAR9W; 3, SAR11W; 4, SAR13W; 5, SAR15W; 6, SAR18W; 7, SAR27W; 8, GHAN5W; 9, GHAN8W; 10, GHAN9W; 11, GHAN13W; 12, JOM6W; 13, DAN1W; 14, DAN6W; 15, DAN11W; 16, CHA8W; 17, SAR1D; 18, SAR5D; 19, SAR6D; 20, SAR12D; 21, SAR23D; 22, SAR24D; 23, SAR25D; 24, SAR26D; 25, GHAN5D; 26, GHAN8D; 27, GHAN15D; 28, JOM14D; 29, JOM15D; 23, JOM16D; 31, DAN1D; 32, DAN3D; 33, DAN6D; 34, DAN8D; 35, DAN11D; 36, CHA1D; 37, CHA8D

Chapter 5 - Discussion

5. Discussion

5.1 Collection and identification of lichens

Lichens are generally visible to necked eye in the field. However 10x hand lens is recommended. A sharp knife and small hammer are necessary tools to collect the lichens. Polythene packets, rubber band, labeling sticker, notebook, pen, rope are other necessary items for lichen collection. Mostly the lichens that are loosely attached to the substratum were collected. Lichen collection in sufficient amount is necessary to conduct detailed chemical studies. Lichen samples were air dried and placed in the herbarium after identification. In this experiment lichens were collected with help of sharp knife placed in separate polythene bags, labeled properly and closed with help of rubber band. Few quantity (Table 2) of each lichen samples were air dried, labeled properly and placed in herbarium where as some quantity of sample were freeze dried and used for extraction. However method of collection of lichens may vary according to objective of study.

Identification was done by studying their morphology, anatomy and chemistry. 'A compendium of the macrolichens from India, Nepal and Srilanka' Awasti (1991, 2007) was the main literature referred for identification of lichen. Also 'Lichen flora of Great Britain and Ireland' (Purvis et al., 1992) are commonly referred for identification of lichens. In this Research all the lichen specimens were identified by analyzing morphological, anatomical and phytochemical characteristics using a lichen identification check list (Awasti, 2007). The identification was confirmed by comparing the lichens collected at Central Department of Botany, T.U. The collected lichens were found belonging to 47 different species under 19 different genera and 12 families.

5.2 Extraction

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to extract the desired chemical components from the plant materials for further separation and characterization. Organic specific hypoallergenic substances of lichens (lichenins or lichen starch and lichen acids) that are not synthesized by other organisms are potentially promising for the search and production of new biologically active substances that can inhibit

the growth and reduce the viability of pathogenic and constantly mutating microorganisms that have adapted to already known types of antibiotic agents (Muravyova, 2002).

Various solvent have been used for isolation of active compounds in the lichen. However solvents such as acetone, ethanol, and methanol (Priya et al, 2013), water (Sarah et al, 2016), chloroform, carbon tetrachloride, acetone, hexane, petroleum ether, nefras and a mixture of the solvents (RF Patent 2317076, the Method of producing usnic acid), sodium carbonate (Grivova, 2016). In this experiment methanol was used for primary extraction.

5.3 Antioxidant Activity

Antioxidants are substances which possess the ability to protect the body from damages caused by free radical-induced oxidative stress. Free radicals (super oxide, hydroxyl radicals and nitric oxide) and other reactive species (hydrogen peroxide, hypo-chloric acid and proxynitrite) produced during aerobic metabolism in the body, can cause oxidative damage of amino acids, lipids, proteins and DNA and has been linked to majority of the systemic diseases including cancer, cardiovascular diseases, and type 2 diabetes (Phoboo et al., 2012). Acetone, methanol and aqueous extracts of this lichen showed 90.93, 69.87 and 65.08% DPPH radical scavenging activities respectively (Marijana et al., 2011). The extract of *Hypogymnia physodes* showed the most prominent effect by increasing late apoptosis (53.15%) and necrosis (32.62%) (IC_{50}= 102.40 μg/mL) TEAC value (Trolox equivalent antioxidant capacity) has been determined using the ABTS radical assay in several polar lichen species (Paudel et al., 2008; Singh et al., 2011). Antioxidant activities are compared to standard antioxidants such as ascorbic acid, butylated hydroxyanisole (BHA) and α-tocopherol.

In this experiment, in-vitro antioxidant assays based on electron transfer (ET) or hydrogen atom transfer (HAT) system- DPPH free radical was used to investigate the antioxidant activity of lichen extracts. ET-based assays measured the capacity of an antioxidant which reduces an oxidant by changing the color. Thus, the degree of color change was correlated with antioxidant potential. DPPH is commercially available stable free radical in aqueous or methanol solution and becomes a stable molecule by accepting an electron or hydrogen

radical from antioxidant compounds (Blois et al., 1958). Most of the tested lichen extracts and the commercial standard (BHA) exhibited DPPH free radical scavenging capacity in the concentration dependent manner. BHA is a strong commercial antioxidant agent and the IC_{50} of this compound was determined as 4.98 ± 0.4 µg/ml. In the present experiment, the test lichens extract showed the varying strengths to scavenge DPPH free radical. Overall, the IC_{50} of DPPH free radical scavenging capacity of active lichens extract was found between 5.6±0.2 to 87.3±7.1 µg/ml (Table 2). Among them, the DCM fractions of *Parmoterma centratum, Peltigera polydactyla* and *Ramalina roesleri* and methanol fractions of *Peltigera polydactyla* and *Parmoterma sp.* showed comparatively strong DPPH reducing activity. The methanol extract of *Parmoterma sp.* from Nepal origin showed stronger antioxidant activity (IC_{50}, 11.4 ± 0.1 µg/ml) than the same species collected from Malaysia (IC_{50}, >500 µg/ml) (Rajan et al., 2016). The plant with higher % RSA has the lower IC_{50}. The plant extract with lowest IC_{50} value is considered having better antioxidant properties. The results indicated the variability of antioxidant compounds in the lichen extracts.

5.4 Antimicrobial Activities

The emergence of multidrug resistant bacteria has driven the need for novel antibiotics. Lichens naturally produce a wide range of unique defense chemicals and have already, historically shown medicinal efficacy. *Diploicia canescens, Pertusaria amara* and *Lepraria incana* inhibit the dermatophyte fungi (Taylor et al., 2019). *Hypogymnia physodes* demonstrated the strongest activity on Gram-positive bacteria *Sarcina lutea* (MIC and MMC values $<9.8 \times 10^{-3}$ mg/mL) and *Staphyloccocus aureus* (MIC and MMC values 3.91×10^{-2} and 7.81×10^{-2} mg/mL) (Tatjan et al., 2011). Extract from three lichen species, *Letharia Columbiana, Letharia vulpine,* and *Vulpicida Canadensis* (with MIC = 125–500 µg/ml) were effective against *Escherichia coli* (Gajendra et al., 2014). Yonghang et al. (2019) found that *Everniastrum nepalense* has potential antibacterial property against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella spp, Shigella spp, Klebsiella spp* and *Pseudomonas aeruginosa* except *E. coli.*

The antifungal activity of hexane, ethyl acetate and methanol extracts of *P. reticulata, R. roesleri, U. longissima* and *S. himalayense* against *S. rolfsii, R. bataticola,R. solani, F. udum, P. debaryanum* and *P. aphanidermatum* was evaluated using poisoned food technique at different concentrations (250, 125, 62.5, 31.2, 12.5)µg/ml (Goel et al., 2011) and the results showed *P. reticulata,* hexane extract showed maximum activity against *R. bataticola* (ED_{50} = 25.1 µg/ml). However, the ethyl acetate extract showed maximum inhibition against *F. udum* (ED_{50} = 43.7 µg/ml) and *S. rolfsii* (ED_{50} = 43.7 µg/ml). Methanol extract of *P. reticulata* also exhibited a moderate inhibition against *S. rolfsii* (ED_{50} = 51.2 µg/ml). All the three extracts were found to be equally active against *S. rolfsii.*

In this experiment only DCM fraction of the lichen extracts were active against *S. aureus* and *K. pneumoniae*. The sizes of zone of inhibition of active fractions are given in Table 1. Seventeen extracts showed activities against *S. aureus* and 45 extracts showed activities against *K. pneumoniae.* Twelve extracts showed antibacterial activities against both *S. aureus* and *K. pneumoniae*.

Thus, the tested lichens were found to be antimicrobial active against both Gram-positive and Gram-negative bacterial strains. Only three extracts were active against *C. albicans*. The comparative study of obtained results (Table. 2) showed that the antimicrobial constituents in the lichens extracts were different. The activity may be the indicative of the presence of broad spectrum antibiotic compounds or simply general metabolic toxins.

5.5 Toxicity Against Brine Shrimp (*Artemia salina*)

Artemia salina is an invertebrate of the fauna of saline ecosystems. It is used in laboratory bioassay to determine toxicity through the estimation of medium lethal concentration (LC_{50} values) which has been reported for series of toxins and plant extracts. Several naturally extracted products which had $LC_{50} < 1000\,\mu$g/mL using brine shrimp lethality bioassay (BSLB) are known to contain physiologically active principles (Pisutthanan et al., 2004). BSLB and other in vivo lethality tests have been successively employed for bioassay guide fractionation of active cytotoxic and antitumor agents (Krishnaraju and Tsay, 2006).

In the research conducted by Johnson et al. (2014), the petroleum ether, acetone, chloroform, and methanolic extracts of *A. aethiopicum* showed different mortality rate of brine shrimp which increased proportionally with the increasing concentration of the extract. The inhibitory effect of the extract might be due to the toxic compounds present in the crude extracts. The acetone extract of *A. aethiopicum* was found to be most effective against toxicity of brine shrimp nauplii with LC_{50} of 192.8 ppm and LC_{90} of 434.3 ppm. Chloroform extract showed less cytotoxicity (ppm) than other fractions. Where as 0.046 ppm of standard plumbagin showed 100% mortality of brine shrimp nauplii.

In this experiment DCM fractions were found to be more toxic than methanol fractions. Strong activities were shown by 74 DCM fractions and 39 methanol fractions while 8 DCM fractions and 32 methanol fractions did not show any activity against *A. salina* nauplii. The lichen extracts of ACA region showed stronger toxicity against *A. salina* than the species of other region (Paudel et al., 2011)

5.6 TLC Based Antioxidant Activity Screening of Lichen Extracts

TLC is performed to separate different compounds from their mixture based upon its retention factor. Retention factor of a compound varies with their polarity and depends upon the type of stationary and mobile phase used. TLC is also used to determine the concentration of compounds in the sample.

Hawrly et al. (2020) reported that, lichen extracts were analyzed using the Thin Layer Chromatography with silica gel as adsorbent and the mixture of toluene, ethyl acetate and formic acid (10/10/0.5; v/v/v, respectively) as mobile phase. Developed chromatographic plates were sprayed with Naturstoff reagent to observe the presence of some phenolic compounds. The images of plates were digitalized using TLC Analyzer software and the obtained chromatograms were exported to Excel and converted to comma-separated values (CSV) files. Next CSV files were loaded to SpecAlign program, where the smoothing, subtraction (original word misspelled) of background and normalization were performed. The chemical differences between samples were confirmed using the similarity (Pearson correlation coefficient) and distance (Euclidean distance) indices with cluster analysis and

Principal Component Analysis (PCA). Additionally, the preliminary evaluation of the antioxidant activity of the examined extracts of lichens was performed by use of the DPPH TLC test.

TLC based chemical screening of the bioactive fractions of the lichens showed a number of bioactive compounds contained in the extracts. As expected, compounds from methanol fractions had a lower RF value than those from the DCM fraction in this TLC system, which indicates that they were more polar (Fig. 4.10). The number of antioxidant active bands in methanol fractions is comparatively small than in DCM fractions (Fig. 4.11). But, the intensity of color change of sprayed DPPH was much higher in the bands obtained from the methanol fractions than from the DCM fractions. The results indicated that methanol (or water) fractions contained stronger antioxidant compounds than the DCM fractions. The results also indicated that the DCM fractions contained higher number of moderately active antioxidants than the methanol fractions.

Chapter 6 – Conclusion

6. Conclusion

A total of 84 lichen specimens including 19 genera and 47 species were collected and tested for antioxidant, antibacterial, antifungal and toxic properties against Brine shrimp nauplii. The test extract showed potent antioxidant activities. Methanol fractions were found to contain strong antioxidant compounds than DCM fractions as indicated in strong spots in TLC analysis plate after DPPH spray. The TLC analysis also indicated that DCM fractions contained the bigger number of antioxidant compounds. IC_{50} of BHA was determined as 4.98 ± 0.4 μg/ml. Overall, the IC_{50} of DPPH free radical scavenging capacity of active lichens extract was found between 5.6 ± 0.2 to 87.3 ± 7.1 μg/ml (Table 2). Among them, the DCM fractions of *Parmoterma centratum, Peltigera polydactyla* and *Ramalina roesleri* (IC_{50}: 11.4 ± 0.1, 5.6 ± 0.2 and 20.2 ± 0.2 μg/ml respectively) and methanol fractions of *Peltigera polydactyla* (IC_{50}: 5.7 ± 0.02, 11.2 ± 0.1 and 11.3 ± 0.2 μg/ml from CHA-8, JOM-6 and DAN-11 respectively) and *Parmoterma sp.* (IC_{50}: 19.9 ± 3.0 μg/ml) showed comparatively strong DPPH reducing activity. Similarly, several lichen specimens were found antibacterial active against the Gram-negative test strain *K. pneumonia and* Gram-positive *Staphylococcus aureus,* while only three lichens *Heterodermia diademata, Heterodermia indica* and *Parmotrema* sp. were antifungal active against *Candida albicans*. Most of the lichen extracts were found toxic against Brine shrimp nauplii. The results indicated that the lichens from Annapurna conservation area (ACA) warrant further research of isolation and characterization of active compounds.

Recommendation for Future Work

Based on the technical expertise developed and difficulties faced while carrying out the present work, following recommendations are made regarding the future work in this area.

1. The method used in present investigation is simple, cheap convenient and less time consuming which can be adopted for the preliminary screening of biologically active substances.

2. Further research should be carried on by using advance tools like HPLC, Mass spectroscopy, NMR etc. for quantification and identification of the compounds responsible for significant biological activity.

3. Since the crude extract of the lichen samples showed promising results of antioxidant, antimicrobial and toxicity assay compared to commercial standard and control, there is further need of research on lichen.

4. Variations in the desirable properties of samples of same species from different localities were found, also biological activity of same lichen species of ACA was recorded to be higher than documented in other countries. Thus detailed research on lichen needs to be carried out.

7. References

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Publication

Journal:

1. Jha, B. N., Shrestha, M., Pandey, D., Bhattarai, T., Bhattarai, H. D. & Paudel, B. (2017). Investigation of antioxidant, antimicrobial and toxicity activities of lichens from high altitude regions of Nepal. *BMC Complementary and Alternative Medicine, 17*, 282. https://doi.org/10.1186/s12906-017-1797-x

Appendix

1. Preparation of 0.1mM DPPH solution - 100 ml

100 ml of 0.1 mM solution of 2, 2- diphenyl-1-picryhydrazyl (DPPH) was prepared by weighing 3.943 mg of the DPPH and dissolving it in ethanol and finally maintaining the volume to 100 ml by addition of ethanol.

2. Composition of Nutrient agar (NA) media

The composition of nutrient agar media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follow.

Components	**gram/L**
Peptic digest of animal tissue	5.0
Beef extract	1.5
Yeast extract	1.5
Sodium chloride	5.0
Agar	15.0
pH	7.4 ± 0.2

3. Composition of Potato Dextrose Agar (PDA)

The composition of potato dextrose agar media (Hi Media Laboratories Pvt. Ltd, Mumbai, India) is as follow.

Ingredients	**Gm./L**
Potatoes, infusion from	200.000
Dextrose	20.000
Agar	15
Final pH (at 25°C)	5.1±0.2

Fig. : Some samples used this research

Fig. : Separation using separation funnel

Fig. : Concentrating extracts in rotavapour

Fig. : DPPH assay of some samples extracts

Fig. : Brine shrimp lethality test of some sample extracts

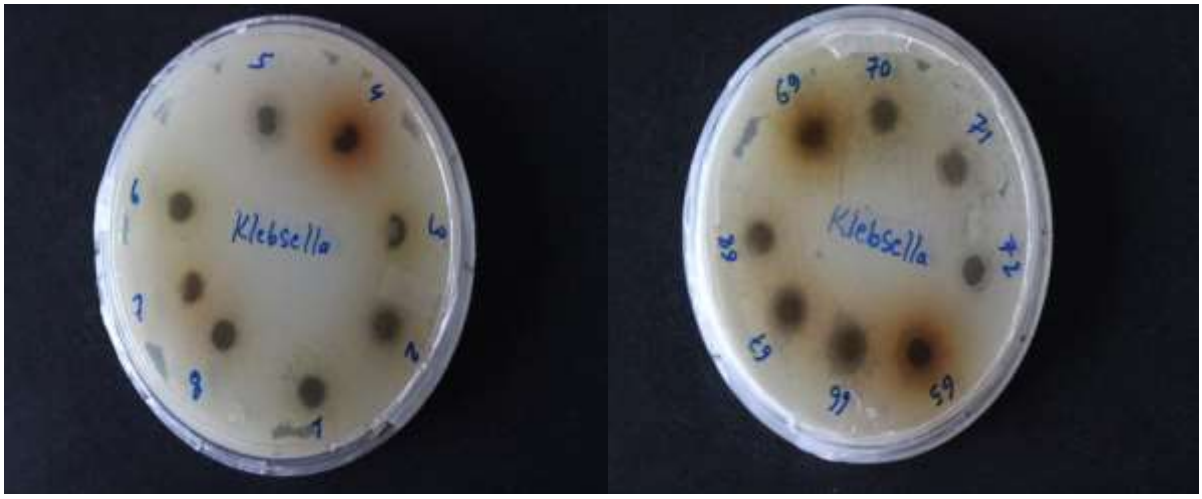

Fig. : Zone of inhibition of some sample extracts against *Klebsiella pneumoniae*

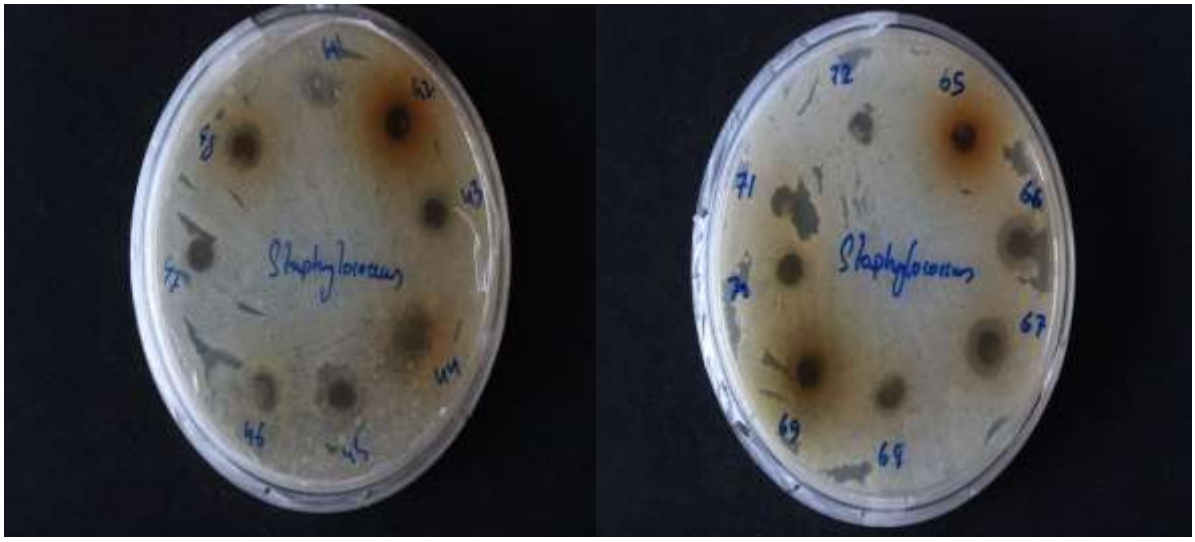

Fig. : Zone of inhibition of some sample extracts against *Staphylococcus aureus*