Investigation on anti-proliferative effect of gyrophoric acid from the lichen *Umbilicaria muhlenbergii* on cancer cells

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DEDICATION

To my beloved family

ABSTRACT

Natural products, including primary and secondary metabolites, are small molecules produced by any organism. As important sources of drug components, natural products may be isolated to small fractions with different biological activities and chemical structures. Although lots of natural metabolites have been screened and yielded pharmaceutical properties, many potential sources of these products still need to be investigated, including lichens. As symbiotic fungi and algae organisms, lichen produces variety of unique secondary compounds and has been used as ingredients in ancient medicine for decades. The demonstrated biological activities of the lichens include antibiotics, anti-proliferative, antioxidants, and anticancer. The bioactivities of the crude extracts, fractions obtained, and the types of compounds depend on biosynthetic pathways in different areas and environments. Although North America is one of the areas to find variety of the lichen species, there is limited research to study the lichens' potential biological activities. In our present work, the study of the lichen *Umbilicaria muhlenbergii* crude extract and active fraction would provide important information about their potential uses.

The goal of this dissertation research was to identify the main bioactive fraction of the lichen *Umbilicaria muhlenbergii* and to investigate its biological role as a promising anticancer compound on different human and murine cancer cells. The anticancer screening of the crude extract demonstrated an inhibitory effect against MCF7. With the aid of bioassay-guided isolation protocol, the study has isolated three compounds from the methanolic extract, which demonstrated a potency anticancer against MCF7 cells. The main isolated fraction was found to be more effective through bio-screening assay and identified as gyrophoric acid with the help of MS, ^{1}H NMR, ^{13}C NMR, and 2D NMR techniques. Using MS, we demonstrarted the molecular ion at (m/z) 467.0 is gyrophoric acid. The FTIR and ultraviolet absorbance confirmed characteristic peaks of the gyrophoric acid as a depsides metabolite. In this thesis, to screen the anticancer effect of this compound and growth inhibitory effect, we tested the crude extract and gyrophoric acid on human breast cancer (MCF7), human osteosarcoma (U2OS), normal human mesenchymal stem cells (MSCs), and murine chondrogenic cell line (ATDC5). We found that the crude extract and pure compound gyrophoric acid showed distinct cytotoxic effects on human and murine cancer cells, while MSCs were more resistant to the treatment in time and dose-dependent manner. The findings of this study demonstrated that these compounds decreased proliferation and influence the cells to undergo apoptosis. Gyrophoric acid appeared more promising on U2OS cells in primary cell

viability screening. The apoptotic was assessed by fluorescence microscopy and demonstrated cell death up to 50% in human and murine cells. Gyrophoric acid pronounced more cytotoxicity effect on U2OS than MCF7 cells and the percentage of the alive cell was calculated 46.6% in MCF7 and 38.70% in U2OS. Finally, Western blot analysis showed that the increased cytotoxicity caused by the crude extract or gyrophoric acid was associated with an increased protein expression level of p53 and p21 in MCF7 and U2OS cancer cells. Mechanistically, we established that gyrophoric acid induces protein expression of p53/TP53 and p21/CDKN1A, which represent principal components of the cellular DNA damage response. Induction of p53 and p21 protein proceeds via post-transcriptional regulation because gyrophoric acid does not influence the corresponding TP53 or CDKN1A mRNA levels.

We also observed the higher growth inhibitory activity in treated murine ATDC5 cells with gyrophoric acid than the crude extract. A decreased level in the expression of NF-κB and Erk1/2 was observed in ATDC5 cells due to exposure to gyrophoric acid.

The selectivity of the cytotoxic properties of gyrophoric acid for two human cancer cell types over normal diploid MSCs and murine ATDC5 cells was observed *in vitro* will encourage future *in vivo* studies and to assess the promising antitumor activity of gyrophoric acid.

Keywords: lichens, *Umbilicaria muhlenbergii*, chemical characterization, anti-proliferative effect, cell staining, p53 signaling pathways

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ABBREVIATIONS

AMV	Avian myeloblastosis virus
APS	Ammonium persulphate
ATCC	American Type Culture Collection
BCA	Bicinchoninic acid assay
ºC	Celsius degree
CC	Column Chromatography
CoA	Coenzyme A
Ct	Cycle threshold
DAPI	4′,6-diamidino-2-phenylindole fluorescence
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
DTT	Dethiothreitol
EAE	Enzyme-assisted Extraction
ECL	Enhanced chemiluminescence
EDTA	Ethyl diamine Tetra Acetic acid
EEF1A1	Eukaryotic translation elongation factor 1 alpha 1
Erk1/2	Extracellular signal-regulated kinase 1/2
ESI	Electro Spray Ionization
EthD	Ethidium homodimer
EtOAc	Ethyl acetate
EtOH	Ethanol
FBS	Fetal Bovine Albumin
FTIR	Fourier Transform Infra-Red
g	Gram
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas Chromatography
h	Hour
H_2O_2	Hydrogen peroxide
HMBC	Heteronuclear Multiple Bond Correlation
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
HRP	Horseradish peroxidase
HSQC	Heteronuclear Single Quantum Coherence spectroscopy
IC50	Inhibitory Concentration of 50%
IGPAL CA 360	Octylphenoxypolyethoxyethanol
IR	Infra-Red
kDa	kilo Dalton
LDH	Lactate dehydrogenase
M	Molar
MAE	Microwave-assisted Extraction
MCF7	Michigan Cancer Foundation 7
MeOH	Methanol
min	Minute

mg	Miligram
µg	Microgram
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor
mL	Mililiter
mM	Milimolar
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)
MS	Mass Spectrometry
MSCs	Mesenchymal stem cells
MW	Molecular weight
NMR	Nuclear Magnetic Resonance
NaCl	Sodium chloride
NAD	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide plus Hydrogen
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
OD	Optical Density
PCR	Polymerase Chain Reaction
PH	Negative logarithm of hydrogen ion concentration
PLE	Pressurized Liquid Extraction
ppm	Part per million
PVDF	Polyvinylidene difluoride
q-PCR	Quantitative Polymerase Chain Reaction
RIPA	Radioimmunoprecipitation
R_f	Retention front
ROS	Reactive oxygen species
RNA	Ribonucleic acid
rpm	Rotation per minute
R_t	Retention time
SDS	Sodium dodecyl sulfate
SFE	Supercritical Fluid Extraction
SGCC	Silica Gel Column Chromatography
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SYBR green	Green fluorescent cyanine dye
TBS	Tris-buffered saline
TEMED	Tetramethylethylendiamine
TLC	Thin Layer Chromatography
TRIzol	Total RNA Isolation
UAE	Ultrasound-assisted Extraction
UV	Ultraviolet

Chapter 1: Introduction

1.1 The significance of this study

Today, numerous health problems remain untreatable and complicated, despite effective drugs have been discovered for many diseases. These health problems include different types of cancer, viral and fungal infections as well as cardiovascular, inflammatory, and allergic diseases [1]. For decades, natural products have been utilized in both traditional and modern medicine for treating diseases. The search for novel therapeutic agents continues and requires the understanding of the structural of the molecules, which lead to medicinal targets. In this way, the study of chemical, biological and pharmacological properties of the natural products in ancient medicine are used in modern medicine as therapeutic agents [1,2]. In addition, traditional remedies are applied around the world, and natural products have a large portion of current-day pharmaceutical agents, especially in the areas of antibiotic and cancer treatment [2]. These products as potential therapeutic sources have to go through multiple stages of development including, extraction, isolation, characterization, elucidation, and biological evaluations [3,4]. In general, natural product research is a great approach for discovering biologically active compounds with unique structures and mechanisms of action.

Among all sources of natural products, lichens are subject of many studies due to their secondary metabolites and anti-cancer potential effects.

This dissertation will focus on the isolation and characterization of the most dominant bioactive fraction of the lichen *Umbilicaria muhlenbergii* identified from MS after isolation. It is a polyphenolic secondary metabolite of the lichen *U.muh* and is categorized in depside class of the lichens. The aim of the current study was to screen the anticancer potential of active compounds on different human cancer cell types and human normal mesenchymal stem cells. The cell lines are in vitro model systems, which are widely used in basic cancer research and drug discover. They are important source of biological material for experimental purposes. Working with different sources of cancer cell lines makes it possible to explore the molecular and cellular mechanisms, cell-drug response, and gene and protein expression profiles in each cell lines. Therefore, we have decided to choose two human cancer cells and normal cells to evaluate the effect of active compound-induced apoptosis on these examined cancer cell lines by apoptosis-related gene expression. In this way, the application of the compound as a promising anticancer agent is evaluated on breast cancer (MCF7), osteosarcoma cell (U2OS) and human mesenchymal stem cell (MSCs). The murine chondrogenic cells (ATDC5) cells were also used to evaluate the antiproliferative potential of the crude extract and active compound. It is suggested that the *in vitro* experiment on murine cells can also identify potential anti-tumor agent through an anti-proliferative assessment in ATDC5 cells. The growth inhibitory effect of the compound was determined by MTT/MTS assay. Tis study was also designed to look into the effects of the active compound on apoptotic gene expression on these human and murine cell types.

1.2 Natural product

The natural product can be broadly defined as any thing which is produced by living organisms, including biomaterials, wood and silk, body fluids (e.g. plant extracts), and other natural materials that can be found in living organisms [1,2]. The natural products and their derivatives are used as food additives in the form of spices and herbs [3], antibacterial and antioxidant to protect the foods and dying textile and cosmetics [4]. To be more specific, a natural product can be described as an organic compound that is produced by plants, bacteria, fungi, and marine, andincludesiprimary and secondary metabolites with biological activities and different chemical structures [2]. These molecules have been utilized in novel medical purposes as anti-microbial, antifungal, antibiotic and anti-tumor agents [5]. More than 60 % of anticancer drugs originate from natural products [6]. Historically, terrestrial plants have been used as primary sources of natural product, and plant-derived agents have a significant role in the anticancer research [7]. Some of these natural compounds are obtained from marine organisms [8] and displaying potential to decrease cancer cell growth *in vitro* and *in vivo* [9]. Another source of natural product is originated from fungi, and among them lichen as a symbiotic organism between algae and fungi which represent nearly one-fifth of all known fungal species. Lichens as a potential natural source produce biologically active secondary metabolites and screened for their anti-cancer activities [10–12].

1.3 Pimary and secondary metabolites

The plants, fungi and many other organisms contribute to producing chemically-diverse bioactive secondary metabolites through different pathways that are different from primary products in biosynthesis pathways and molecule structures [5,8,13]. Primary metabolites are defined as carboxylic acids, amino acids, fats, proteins and nucleic acids, which are all necessary for survival of the organisms [14]. On the other hand, secondary metabolites are not essential for life, however, they have significant contributions in microorganism's living [15,16]. They have a role in producing colors, flavors, and smells [15], and considerably related to phytochemical substances found in medicinal plants [17]. Their unique biochemical pathways are more specialized for the particular family or genus of the microorganism [18]. These secondary metabolites have uncommon structures and mixtures of closely related chemical groups [8,9] with a variety of biological activities [4,10].

1.4 Importance of biological activity of natural products

As mentioned earlier, for many years natural products have been used as herbal medicines in the form of crude extracts without the isolation of the active components [19]. In the development of drug discovery, a number of important new commercialized drugs have been obtained from isolated natural sources rather than crude extracts [20]. These derived substances are used as potential metabolites against different types of cancer, viral and infectious diseases [16, 17,18]. The biological screening of the isolated natural products is represented by several biochemical assays in order to reveal the effective compound. At the end of biological and toxicological tests, we are able to detect the most potent isolated fraction(s). In addition, these bioactivity assays are inexpensive, effective and widely used in natural product investigation [21]. The approaches to discovery of biologically active products can be categorized into chemical approach, bioassay-guided approach and combination of chemical and biological strategy [22]. The combined method works especially well when the active compounds are present in a high concentration and used for new isolated compounds when the extract has shown bioactivity [23].

1.5 Lichens- natural source as a potential anti-cancer agent

Lichens as symbiotic species associated with algae and fungi are found in many regions of the world with different ecosystems and identified for their unique biological activities [24]. Over the years, lichens have been used for different purposes in cosmetics, dyes, air pollution indicators, as well as dietary and medicinal purposes [25, 26]. Because of their ability to undergo different growth conditions and their symbiotic communication, lichens have significant contribution in primary and secondary compound production [12]. The primary metabolites as basic components of metabolic pathways are produced in green algae partner and include sugar alcohols such as sorbitol and polyols [27,28], while the majority of the lichen secondary metabolites are produced in the fungi part [29] and contain phenols, flavonoids, saponins and alkaloids [28,29]. The biological activities of crude extract, and the type of isolated compounds largely depend on their biosynthetic pathways and the secondary metabolites that they have produced in different environments [24,29,30].

To date, it is estimated that over 1050 of these secondary lichen metabolites have been identified [31,32] and demonstrated therapeutic potency as anti-bacterial, anti-inflammatory, anti-cancer, antiviral and antioxidant activity [10,33–40]. Many studies have been published studying the

taxonomical [41], phylogenetic [42,43], and ecological aspects of lichens [44,45]; however, the biological roles of lichens have been poorly studied.

The present work is focused on the extraction, isolation and technical applications in the elucidation of the compounds from the lichen *Umbilicaria muhlenbergii*. The bioactivity guided isolation and cytotoxicity effects on different cancer cells are also presented.

The lichen *Umbilicaria muhlenbergii* species used in this study can be attractive as a bioactive material in pharmaceutical research.

1.6 Novelty of this project:

1. Critical review on the lichen species, extraction and identification techniques as well as the biological effect on variety of cancer cells
2. Investigation on extraction, isolation and characterization of the major compound of the lichen *Umbilicaria muhlenbergii*
3. Investigation on the bioactivity of the lichen and its main fraction on different human and murine cell lines

1.7 The objectives of this thesis:

In this thesis, to obtain crude extract and isolate the most effective fraction, we have used maceration for extraction followed fractionation by Silica gel column chromatography (SGCC). Further steps for primary identification were involved color test, phytochemical screening, and UV to detect absorbance range of the secondary metabolites. FTIR was also aiming to determine functional groups. Mass spectroscopy and 1D and 2D NMR have been performed for structure elucidation and identification of the compounds.

Moreover, to investigate whether this species induce apoptosis and to confirm any positive findings in cancer cell lines cell viability assay (MTS), cytotoxicity assay and apoptosis assay (cell staining) have been performed. We have then used Western blotting technique to detect protein expression levels by applying specific primary antibodies. Lastly, q-PCR has been performed to determine gene expression levels.

1.8 Logical outline of this study

This study is consisted of two main parts, chemical analysis and biological activity studies. The chemical part included selecting lichen species, acetone extraction, preliminary phytochemical screening for major classes of phytochemicals, fractionation and spectroscopic analysis. The second part was bio-screening of isolated fractions to find the most effective compound followed by further biochemical assays to evaluate proliferation, apoptosis, and expression levels of proteins and genes of interests. The outline scheme has presented in Figure 1.1.

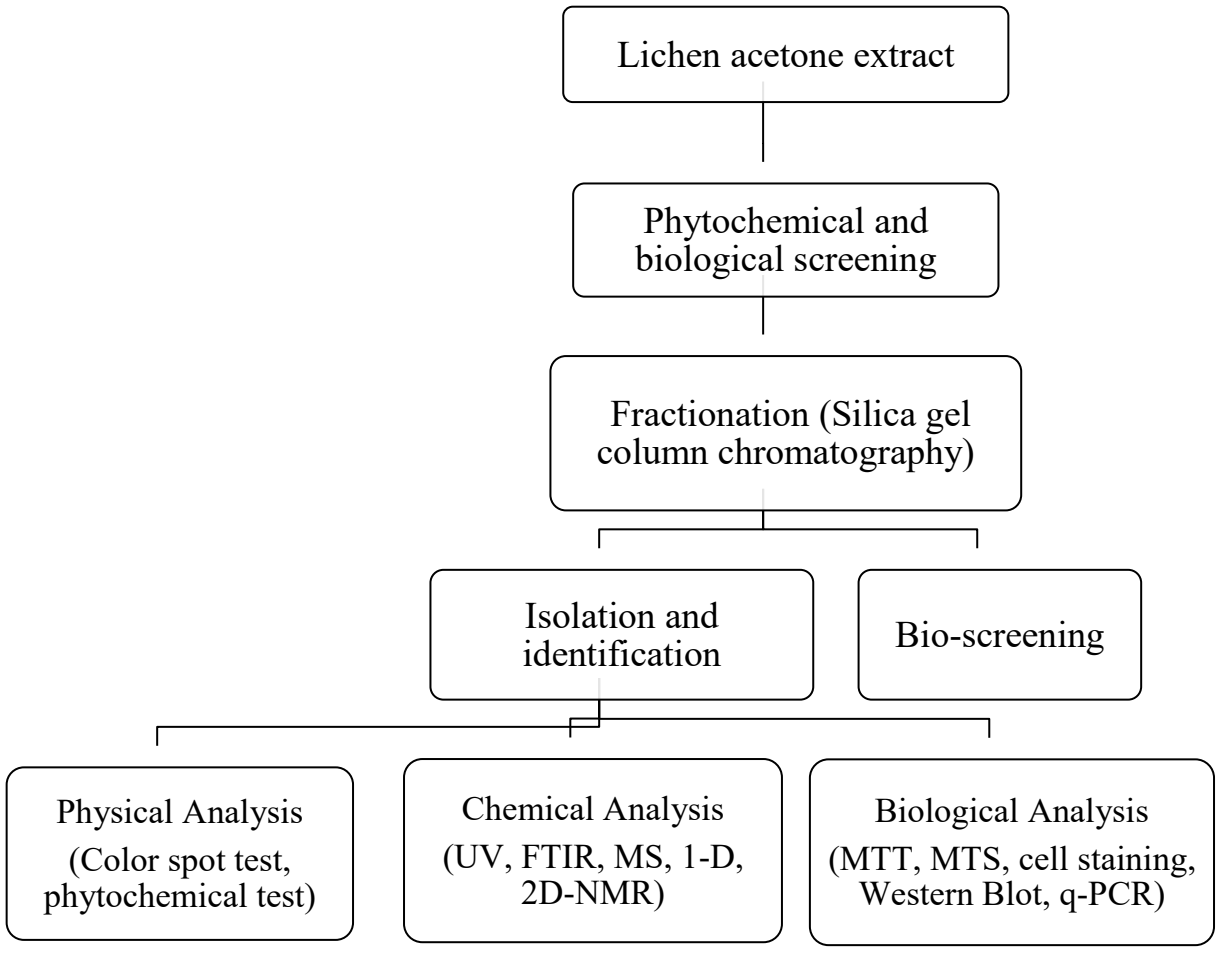

Figure 1.1: Logical outline approach of the study

1.9 Structure of this thesis

This work is divided into 6 chapters. First, a brief introduction discusses natural products including lichen. It reviews the current study and its purposes and establishes the research areas.

Chapter 2, presents the literature relevant to this project, including methods of extraction and isolation. It also discusses analytical techniques in identification of the lichen compounds, and lastly the bioactivity of a variety of lichen species against different cancer cells.

In Chapter 3, the research methodology applied in this project will be presented in detail. In this chapter, the theoretical background and the experimental details are provided.

Chapter 4, specifically focuses on the extraction and isolation of the lichen extract and continues with molecular characterization of the main compound.

Chapter 5 presents the bio-screening fractionation and biological approaches to determine the anticancer effect of the main fraction on breast cancer (MCF7), osteosarcoma (U2OS), and human mesenchymal stem cells (MSCs). The effect of lichen and bioactive fraction investigated against murine ATDC5 cells has also evaluated. This chapter also includes the effect of isolated fraction on the expression levels of genes.

Chapter 6 covers the conclusions and suggestions for future research.

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Chapter 2: Literature Review

Lichenochemicals: Extraction, purification, characterization, and application as potential anticancer agents

Expert opinion on drug discovery (Accepted in February 2020, Manuscript number ID EODC-2019-ST-0180)

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2.1 Abstract

Introduction: To date, over 1,000 lichen secondary metabolites have been identified. Despite their promising cytotoxic properties, the number of literature reports on anticancer evaluation of lichenochemicals is limited. As cancer prevalence among the human population increases, feasibility study of the lichen secondary metabolites with anti-cancer effect is growing.

Areas covered: The lack of significant progress in lichen anticancer research is due to the low levels of cytotoxic compounds contained in lichens, the technical difficulties associated with their isolation and characterization, and the insufficient understanding of their mechanism of action on different cancer cell lines. In this review, the authors have discussed these challenges and provided systematically organized information on the limitations and advantages of commonly used and newly developed methods for lichen exploration and as a promising anticancer source.

Expert opinion: Secondary lichen metabolites hold great potential for anticancer drug discovery. A systematic and multidisciplinary approach is required to advance lichen research and improve our understanding of the mechanisms responsible for the potent cytotoxic properties of lichenochemicals. More effort needs to focus on screening and discovery of new lichen-derived compounds with unique anticancer properties.

Keywords: Anticancer agents, antiproliferation, apoptosis, cancer cell lines, cytotoxicity, DNA fragmentation, extraction, lichens, lichenochemicals, secondary metabolites

2.2 Introduction

The search for effective chemotherapeutic agents has been focused on the isolation of new agents derived from natural sources ranging from a variety of organisms such as tropical plants, aquatic cyanobacteria, fungi, algae and marine organisms [1]. There are a multitude of compounds isolated and identified from natural sources for their chemotherapeutic efficacy with some of them having reached the clinic stage. For example, chemotherapeutic agents such as paclitaxel, vinca alkaloids and etoposide [2] have been derived from plants and are among the most effective drugs currently available. Cytarabine, trabectedin, and eribulin [3] are examples of chemotherapeutic agents isolated from marine organisms which are currently approved for use in humans, with

several other compounds under investigation. Rapamycin, carfilzomib and midostaurin are chemotherapeutic agents derived from bacteria and fungi which are currently in clinical use [2]. More recently, research has expanded to include secondary metabolites produced by lichens [4]. Lichens are a symbiotic association of algae and fungi that have been used historically as dyes, perfumes and home remedies in folk medicine [5,6]. The "Indian Medicinal Plant" (1984) describes the medicinal properties of lichens used for the treatment of blood and heart diseases, leprosy, bronchitis, bleeding pile, asthma, inflammation, liver and stomach diseases. The lichen species are extremely environmentally adaptive and can be found in the arctic or thermal vents, on rocks, non-fertile soils, as well as on various plants and organisms [7]. Based on worldwide lichens catalogue presently, 13,500 species have been recognized [8,9]. Lichens are a known source of over 1,000 unique secondary metabolites [10], which are produced by the fungus and secreted onto the hyphae surface. The multitude of compounds present in lichens provides us with the opportunity to discover new therapeutic agents. However, only a limited number of these metabolites have been screened for their bioactivities [11,12]. This lack of screening is mainly attributed to the low levels of active compounds contained in lichens and the technical difficulties associated with their isolation, identification and characterization. The application of lichen-derived natural products in drug development is constrained by several factors, such their physicochemical characteristics, mode of action, and degree of purity [4]. The chemical purity of the natural bioactive compounds is dependent on the extraction and purification processes used for their isolation. Conventional techniques such as maceration and Soxhlet extraction have been used for many years but non-conventional and modern techniques like microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE) are also in use nowadays and gaining prominence. Choosing the appropriate extraction method requires prior knowledge of the chemistry and selectivity of the extraction solvents used. Also, analytical techniques such as Thin-Layer Chromatography (TLC), Column Chromatography (CC), High Performance Liquid Chromatography (HPLC), Ultraviolet (UV) and Infrared (IR) Spectroscopy, Fourier Transform Infrared (FTIR) Spectroscopy, Mass Spectroscopy (MS), Nuclear Magnetic Resonance (NMR) and determination of melting point are all useful in natural product identification and characterization. Challenges in product identification arise from the fact that crude extracts may contain hundreds of compounds, and each compound may be present in a

different concentration [13]. In most cases, the biological activities of the lichen secondary metabolites produced are specific to the particular organisms and conditions of their growth [14]. Lichen extracts have shown bioactivities against various diseases such as bacterial, fungal and viral infections. Of particular interest is the potent anti-cancer activity of lichen compounds. Many studies have emphasized the importance of better understanding and deciphering the biological mechanisms of cytotoxicity and cell growth inhibition [15–17] or gene overexpression [10]. Considering the past and present trends of increased cancer prevalence among the human population, there is growing interest in bioprospecting alternative natural sources, including lichens and their secondary metabolites, for potential anti-cancer drug development. Although the lichen secondary metabolites have been reviewed previously [4,18–22], a comprehensive source of systematically organized information on their extraction, purification, characterization, and application as potential anticancer agents is lacking.

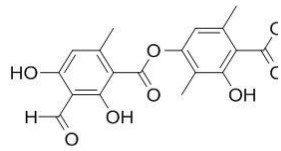
The objective of this review is to describe the principles, highlight the importance, and critically assess the limitations of commonly used and newly developed state of the art methods for exploration of lichens as a valuable source of bioactive molecules and their potential for use in traditional and novel therapeutic applications with emphasis on medical diagnosis and treatment of cancer. The advantages and disadvantages of conventional and non-conventional extraction techniques of lichen substances and their identification using basic and advanced analytical techniques are systematically reviewed. *In vitro* and *in vivo* applications of bioactive lichenochemicals on various cancer types are described and discussed.

2.3 Structure, classification, and synthesis of lichenochemicals

Lichenochemicals as potential anticancer agents represent a diverse group of compounds with different physicochemical properties and concentrations [3–25] The bioactive lichenochemicals represent an array of different chemical structures as shown in Figure 2.1 .]29-26[These structures vary with the lichen species and environmental factors including light, temperature [6,29,30], UV exposure [31,32], altitude [33,34] and seasonality [34,35]. Lichenochemicals include, but are not limited to chemical families such as flavonoids and terpenoids [36], tridepsides [9,37], orsinol tridepsides, orcinol tetradepsides, aphthosin [15], and phenolic compounds [34]. The structural identification of lichenochemicals is carried out using analytical methods such as TLC, HPLC, UV, IR, NMR, MS and X-ray crystallography [38]. For example, lichen compounds reported for *Umbilicaria* species include compounds with different aromatic, aliphatic and cyclic structures,

such as lecanoric acid, gyrophoric acid, umbilicaric acid, and norstictic acid [39], parietin [40], myristic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid, linoleic acid and linolenic acid [41], to mention just a few.

Figure 2.1: Chemical structures of anticancer lichenochemicals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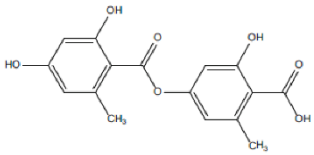
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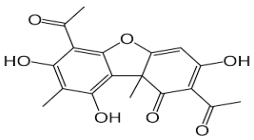
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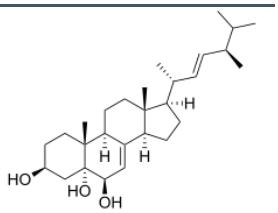
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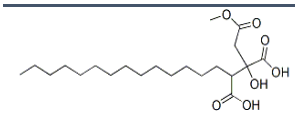
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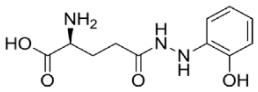
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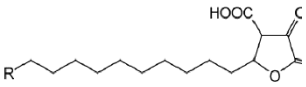
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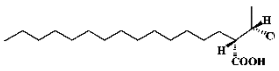
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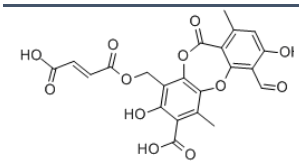
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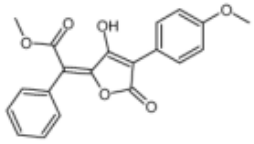
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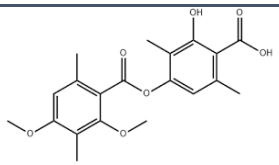
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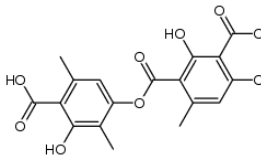
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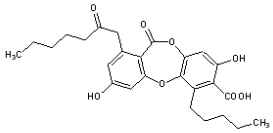
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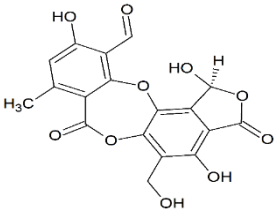
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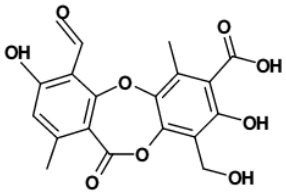
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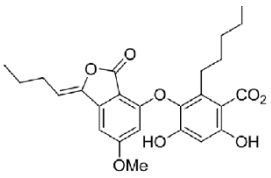
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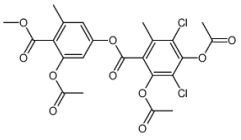
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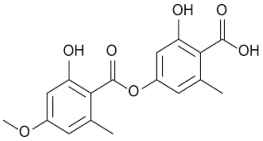
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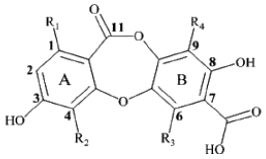
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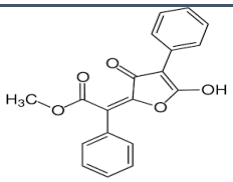
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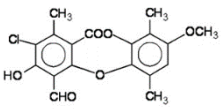
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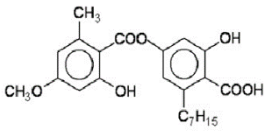
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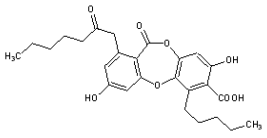
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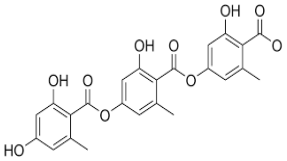
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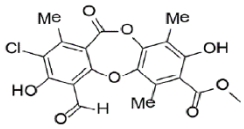	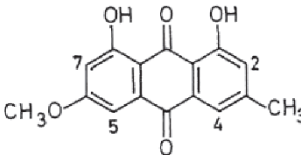	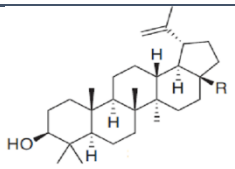	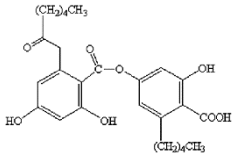
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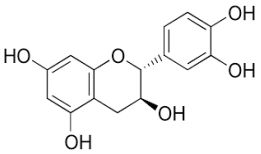	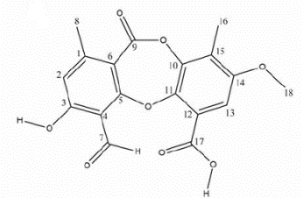	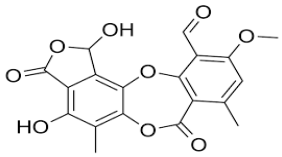	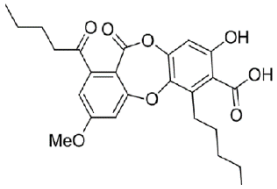
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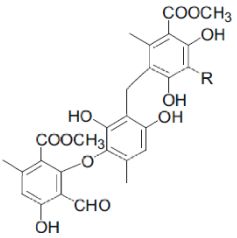	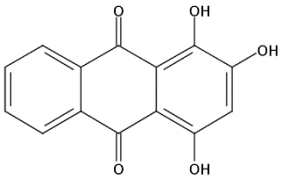	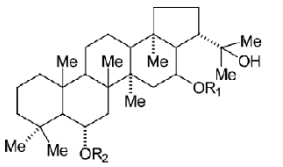	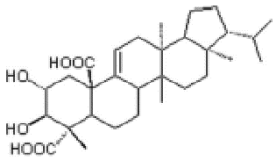
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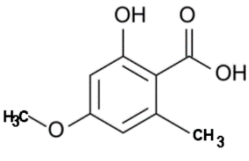	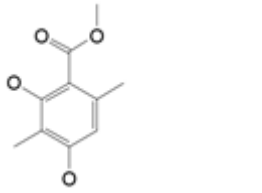	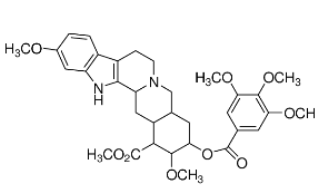	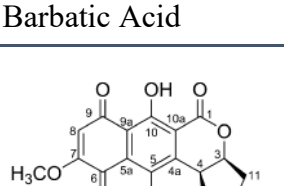
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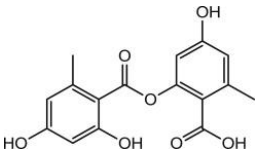	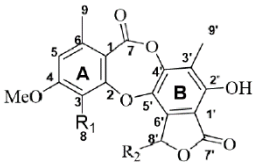	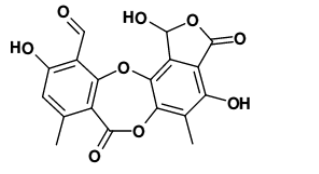	
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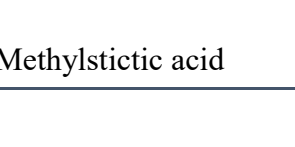	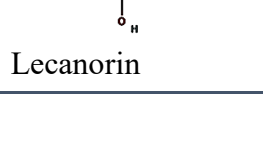		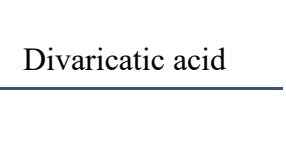
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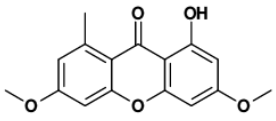
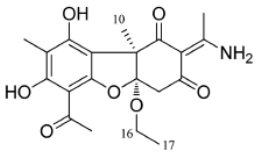


Lichexanthone



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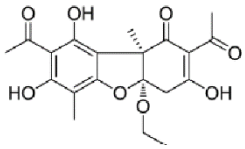

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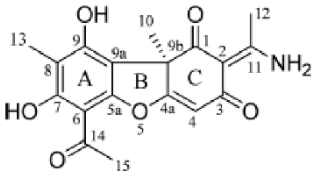
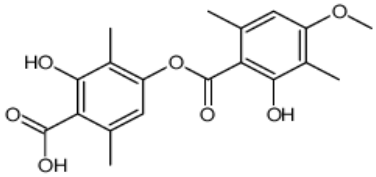
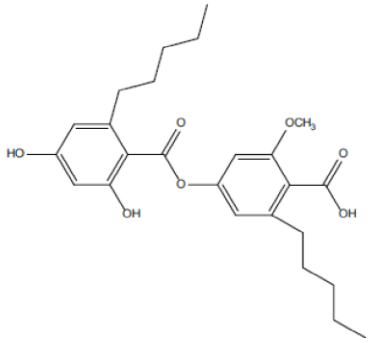


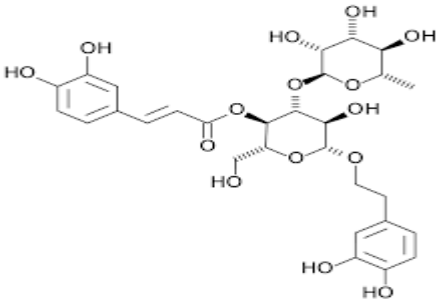
Usenamine A



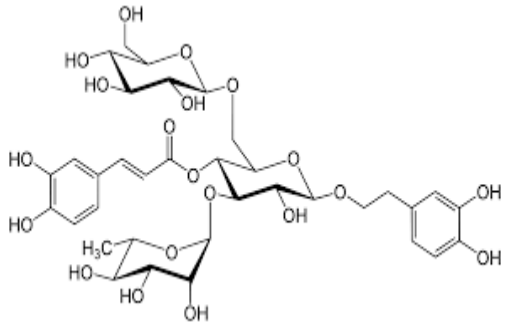
2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid



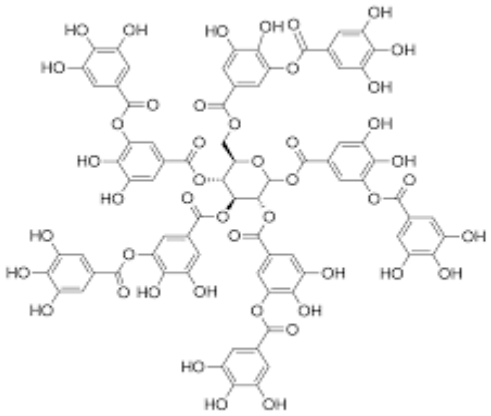
2-O-Methyl anziaic acid



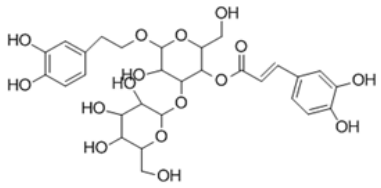
Verbascoside



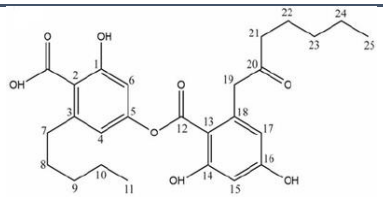
Echinacoside



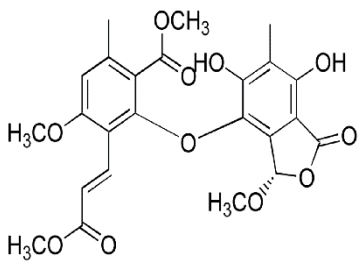
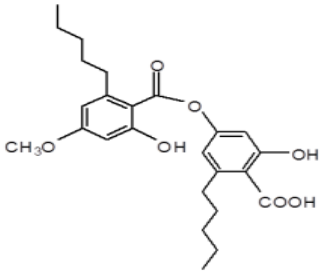
Tannic acid



Plantamoside



Olivetoric acid

	
Lobariethers	Perlatolic acid

Lichenochemicals can be classified into three groups based on their biosynthesis pathways [42,43]**.** Most of the lichen secondary constituents are generated through the polymalonate (polyketide) pathway which is associated mainly with the fungal part (mycobiont) of the lichen [44,45]. This pathway comprises a series of reactions accelerated by polyketide synthase enzymes (46) and generates chemical compounds such as depsides [6], usnic acid and its derivatives, dibenzofurans and xanthones [47], orcinols, aromatic compounds and depsidones including physodic acid, lobaric acid, norlobaridone, grayanic acid, alectoronic acid, diploicin, 4-*O*-methylphysodic acid, α-collatolic acid, lividic acid and variolaric acid [48]. These poliketides are linked by ether, aster and C-C bonds [42]. For example, in depsides, atranorin, fumarprotocetraric acid and gyrophoric acid are the major biosynthesis products obtained through this polyaromatic pathway [49]. The shikimic acid pathway is related mainly to pulvinic acid and terphenylquinone compounds [50]. Studies have demonstrated that the yellow color of pulvinic acid and calycin in the shikimic acid pathway can serve as an indicator for the presence of secondary metabolites [46,48]. Biosynthesis of lichenochemicals such as terpenes, steroids and carotenes following the mevalonic acid pathway has been reported for *Physcia aipolia* [6,48]. The photosynthetic pathway is associated mainly with production of sugar alcohols, monosaccharides and polysaccharides [47]. The algal part of the lichen (phycobiont) is involved in photosynthesis of carbohydrates that are then taken up by the lichen mycobiont [51]. The lichen photosynthesis is dependent on factors such as moisture, light, temperature, salinity and inorganic nitrogen fertilization [52].

2.4 Extraction of lichenochemicals

Extraction is the first step in the isolation of lichenochemicals. The choice of a suitable extraction method determines the success of the following steps of identification and characterization of

bioactive lichen compounds [5,53,54]. The extraction efficiency and stability of extracted lichenochemicals are also dependent on the extraction technique. The crude extracts, obtained using different extraction methods [55–57], are further chromatographically fractionated, and the purified compounds undergo compositional and structural characterization followed by biological activity assessment on different cancer cell lines [58–60]. Although most of the extraction processes utilized to date are conventional, such as maceration and Soxhlet extraction, more recently, green extraction techniques have been employed in order to minimize the use of environmentally-unfriendly organic solvents and to produce higher yields of bioactive compounds. Extraction of bioactive compounds from lichen samples follows suitable highly-selective solvent-based protocols that are rapid, reliable, reproducible and independent of variations in the sample matrix [61]. The sections below review conventional and non-conventional extraction techniques that are currently in use for lichenochemicals, as shown in Table 2.1.

2.4.1 Conventional extraction techniques

Most of the extraction techniques are based on the extraction efficiencies (polarity, concentration, heating, and mixing) of different solvents. Conventional extraction techniques include maceration, Soxhlet extraction and hydrodistillation.

2.4.1.1 Maceration

Maceration is the popular technique for small-scale inexpensive preparation of many herbal medicines. Lichen maceration includes soaking, grinding, solvent mixing, and filtration of extracts [62]. In principle, the solvent penetrates the sample and selectively dissolves certain lichen substances. Maceration facilitates the diffusion and surface release of bioactive compounds, which results in increased concentration and extraction yield. The extraction time is a function of the mass transfer coefficient and is dependent on the experimental conditions and sample composition. Factors affecting maceration are choice of solvent, solvent polarity and maceration time. The smaller particle size increases surface area and leads to improved solvent diffusion and extraction. In one study, *Xanthoria parietina* lichen was blended with 100% acetone and treated at room temperature for 3 days. The crude acetone extract showed anticancer property on MCF7 and MDA-MB231 breast cancer cells [63]. In another study, the dried thalli of 10 lichens of *Parmeliaceae* were extracted in methanol by a vortex-shaking maceration for 2 hours of periodical shaking (1

min every 30 min) with an extraction yield of 2.17-14.31 wt% [64]. Maceration has been used for extraction of bioactive compounds from various lichen species.

2.4.1.2 Soxhlet extraction

Soxhlet extraction is used mainly for lichen extraction with hot solvents and a reflux unit called a Soxhlet apparatus. The method is simple, inexpensive and suitable for the recovery of thermostable compounds [65] in larger quantities, which saves energy, time and cost [66,67]. The most commonly used solvents include acetone, petroleum ether, hexane, ethanol, and methanol [58,68–73]. Solvents are chosen based on their polarity, and normally a combination of solvents with a gradual change in polarity (for example, from non-polar to polar) is used for quantitative extraction of lichen metabolites. Due to the use of elevated temperatures for Soxhlet extraction, thermal decomposition of lichen substances may occur. Other disadvantages include the need for considerable amounts of solvent and the long extraction times [66]. The most common lichenochemicals isolated using Soxhlet extraction include gyrophoric acid, usnic acid, lecanoric acid and atranorin.

2.4.1.3 Hydrodistillation

Hydrodistillation is a physicochemical process of aqueous diffusion, hydrolysis of plant or microbial cell substances, and their decomposition by heat. Hydrodistillation can be carried out as water distillation, water and steam distillation, or direct steam distillation. Water distillation is the soaking of lichen material followed by boiling the resulting mixture. Hot water releases essential oils from oil glands whereas steam distillation extracts steam-volatile essential oils by passing vaporized steam through the lichen sample. The main disadvantage of this technique is the loss of heat-labile compounds at a high-temperature distillation. Hydrodistillation was used for extraction of essential oils with potent bioactivities from the two lichen species *Evernia prunastri* and *Ramalina farinacea* [74].

2.4.2 Non-conventional extraction techniques

Although conventional extraction methods are commonly used for isolation of extracts from natural sources, there have been some challenges associated with them such as extended extraction times, process economics, use of high-purity solvents, need for solvent recycling, low extraction

selectivity of solvents, and loss of heat-sensitive compounds [66]. The above problems have prompted the development of new, non-conventional extraction techniques. The non-conventional techniques, also called "green" techniques because of their contribution to environmental protection, include MAE, UAE, SFE, pressurized liquid extraction (PLE), and enzyme assisted extraction (EAE). The advantages of these techniques are the use of safe solvent auxiliaries, less hazardous product degradation and byproduct formation, energy efficiency, time saving, and higher yields.

2.4.2.1 Microwave-assisted Extraction (MAE)

MAE facilitates the diffusion of compounds from the sample to the solvent through microwave radiation that interacts with the solvent to produce heat. The amount of heat generated depends on the solvent's polarity or dielectric constant which results in increased permeability of the solvent into the sample [75,76]. Consequently, heat is transferred by the solvent to the sample via conduction and then absorbed by the water contained in the sample, which improves the extraction efficiency [77]. The microwave frequencies of MAE range from 0.3 to 300 GHz.; however, to avoid interference with radio communications, domestically-operated microwaves generally operate at 2.45GHz. Compared to maceration and Soxhlet extraction, MAE is a more rapid process [78] with higher extraction efficiency at lower energy input [79]. It requires lower amounts of solvent and results in a higher purity-lower heat decomposition product [77,80]. Factors affecting MAE are the particle size and size distribution profiles of extracts. Smaller particles have greater mass transfer coefficients and higher surface areas available for solvent-sample interactions. The particle sizes from 100 µm to 2 mm [81], require a precise solvent selection and extraction time. Ethanol, acetone, and methanol have been the commonly used solvents for MAE. Ethanol is less expensive and less toxic but has a lower extraction potential than acetone and methanol [79]. All three solvents exhibit increased polarity when exposed to higher temperatures [82]. Ionic liquids can serve as an alternative solvent for MAE and reportedly have higher extraction efficiencies than conventional solvents (83). For example, 1-alkyl-3-methylimidazolium salt with a different anion and alkyl composition extracted 90 wt% norstictic acid from the lichen *Pertusaria pseudocorallina* applying a microwave power of 100W and extraction time of only 7 min. Other solvents used for MAE include *n*-hexane, diethyl ether, acetone, tetrahydrofuran, sodium hydroxide, and water (83). A lichen extract from a finely ground *Pseudevernia. furfuracea* was obtained at a 15.2 wt% yield

using four solvents (n-hexane, diethyl ether, acetone and tetrahydrofuran) and a microwave power of 20W at 75°C for 15 min (14). The MAE of the lichen *P. furfuracea* was completed in 15 min as compared to 24 h when an oil bath was used for extraction [84]. Considering the lower consumption of solvent, reduced extraction time and increased extraction yield, MAE is viewed as a proper alternate extraction technique for medicinal plants [77]. However, it should be noted that volatile and non-polar substances and solvents are less responsive to MAE [77], and undesirable heat decomposition of thermo-sensible compounds may occur [85].

2.4.2.2 Ultrasound-assisted Extraction (UAE)

The UAE method makes use of the mechanical impact created by the ultrasound to improve the solvent penetration into extracted plant material by considering parameters such as temperature, solvent and ultrasonication [86]. Ultrasound-intensive waves of 20-2,000 kHz are passed through a sample to create compression-expansion effects that lead to the formation of voids or cavitation bubbles, which ultimately improve the mass transfer and compound extraction [76,77,87]. The UAE operating temperatures and times are typically 55-67°C and 30-60 min, respectively [65]. The main advantages of UAE are reduced cost, time, energy and solvent use (88). A limitation of this method is the extraction of heat-sensitive compounds [89]. Factors affecting UAE are the sample moisture content, particle size, sample to solvent ratio and physical parameters, such as pressure, temperature, sonication time and frequency. Because of the high cell wall permeability of ultrasound, UAE can be used on plant samples that have tough and hard cell walls [77]; however, the high-ultrasound energy may have a destructive effect on bioactive substances [75].

2.4.2.3 Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE) is used for chemical extraction under conditions that are above the solvent's critical temperature and pressure. The supercritical fluids (SFs) have low viscosity and high mass transfer coefficient that could be used for both separation and extraction purposes [65]. Carbon dioxide (CO_2) is the most common SF, sometimes modified by co-solvents such as ethanol or methanol. The property of the SF can be altered by varying the pressure and temperature, allowing selective extraction [90]. Factors affecting SFE are temperature, pressure, particle size, sample moisture, choice of fluid, flow rate, extraction time, solvent to feed ratio and solubility [88,91]. The advantages of SFE include higher diffusivity or penetration of SF into the

sample, high rate mass transfer, shorter extraction times, high selectivity, extraction of thermostable compounds at low quantities, and low solvent usage. The method is environmentally friendly with minimum waste generation as SF is recycled and reused. The SFE is scalable and can extract from a few mg to tons of material [92,93]. However, SFE is an expensive technique with challenging operating conditions that limit its applicability. The lichen *Usnea barbata* was extracted with CO_2 as a SF, operated at a 3 kg/h flow rate, 30 MPa and 40°C (Table 2.1). The SFE yields of phenolic and usnic acid from *U. barbata* exceeded those obtained from the same lichen using maceration and Soxhlet extraction [57].

2.4.2.4 Pressurized Liquid Extraction (PLE)

This method is applied for extraction of sensitive components, also known as pressurized fluid extraction (PFE), enhanced solvent extraction (ESE), accelerated fluid extraction (AFE), and high-pressure solvent extraction (HSPE) [94]. The application of high pressure keeps solvents in the liquid state beyond their normal boiling point. The extraction temperature can promote higher solubility and mass transfer rates, and decrease viscosity and surface tension of solvents, thus improving the extraction rate. Advantages include faster extraction times and low solvent usage at high extraction yields [91]. PLE is performed in static, dynamic or static/dynamic modes [65]; however, it is time and labor consuming [95].

2.4.2.5 Enzyme-assisted Extraction (EAE)

EAE is a modern, more specialized technique for extraction of bioactive compounds (96) in cases where traditional solvents are inefficient. For example, compounds of interest for EAE may be bound to polysaccharides or proteins via hydrogen or hydrophobic bonds, and the highly-specific and selective action of enzymes is required as a pre-treatment step before solvents can be used for extraction. Hydrolytic enzymes such as proteases, cellulases, pectinases, and chitinases can assist in enhanced recovery of bioactive compounds. The use of chitinases is justifiable as lichenochemicals are concentrated in the mycobiont that has chitin-containing walls. In one approach, the lichen *Parmotrema praesorediosum* was collected and sprayed with a solution of pectinase: cellulase (2:1) and then exposed to MAE for a low-cost extraction [97]. Depending on the operating conditions, a combination of extraction methods may be more effective in obtaining high-purity secondary lichen metabolites.

Table 2.1: Conventional and non-conventional methods of extraction of anticancer lichenochemicals

Lichen	Lichen (g)	Method	Solvent	Condition	Yield%	Compound(s)	Ref.
Conventional Methods							
Parmeliaceae Lichens	50 g	Maceration	Methanol	2 ml, 2 h, 20-22℃	2.17-14.31%	Protocetraric acid, usnic acid	[64]
Lobothallia alphoplaca	100 mg	Maceration	99.5% ethanol, acetone or ethyl acetate	24h, 37^0C	NA	Crude extract	[98]
Lobaria orientalis	1.4 kg	Maceration	Methanol	40 L, RT	NA	Lobarientalone A, lobarientalone B, lobariether A, lobariether B, lobariether C, lobariether D, lobariether E	[34]
Pleurosticta acetabulum	2 g	Maceration	Acetone	2 x20 ml, RT	NA	Cytochalasin E	[35]
Evernia prunastri Xanthoria parietina		Maceration	Water Methanol Ethanol	0:1 ml/g, 60 min, Dark, RT	NA	Crude extract	[99]

Usnea barbata	93.91 g	Maceration	Ethanol	600 ml	NA	Usnic acid	[57]
Xanthoparmelia somloensis	800 g	Maceration	Acetone	2x12 L	NA	Usnic acid salazinic acid	[100]
Myelochroa aurulenta	12.53 g	Maceration	Acetone	24 h	NA	16-O-Acetyl-leucotylic Acid	[101]
Pseudeverni a furfuracea		Maceration	Acetone		NA	Olivetoric acid	[102]
Cetraria aculeata	10 g	Maceration	Acetone	100 ml	NA	Crude extract	[54]
Everniastrum vexans		Maceration	Acetone	48 h, RT	NA	Atranorin	[32]
Lichina pygmaea	270 g	Maceration	Ethyl acetate	3 x1.5L, 2h, RT	NA	para-hydroxyl isomer	[103]
			Methanol	3 x1.5L, 2h, 45ºC			
			Methanol -50% in water	3 x1.5L, 2h, 45ºC			
Xanthoparmelia chlorochroa	5 g	Maceration	Acetone	50 mL, 2h, Twice	0.5-5%	Usnic, salazinic, constictic, norstictic acids	[104]
Tuckermannopsis ciliaris	5 g		Acetone	50 ml, 2h, Twice	0.5-5%	Protolichesterinic acid. ciliaris	[104]

Parmotrema dilatatum	20-40 g	Maceration	Hexane Acetone	RT	NA	Atranorin protocetraric acid	[105] [105]
Usnea subcavata						Usnic and diffractaic acids from *U. subcavata*	
Dirinaria aspera						Divaricatic acid	
Xanthoria parietina	NA	Maceration	Acetone	15Ltr, 3 days, RT	NA	Fractions A-E	[63]
Lethariella zahlbruckneri	5 g 5 g	Maceration	Acetone Methanol	100 ml, 40 ºC 3 h, 65ºC	NA	Crude extract	[106]
Lobaria pulmonaria	70 g	Maceration	Chloroform Chloroform: methanol (1:1) Methanol Methanol :water (1:1)	500 ml each, 1 day each, RT	NA	Stictic acid	[107]
Parmelia arseneana	100 g	Soxhlet	Acetone	NA	NA	Stictic acid, norstictic acid,	[9]

						usnic acid, atranorin	
Acarospora fuscata	100 g				NA	Gyrophoric acid, stictic acid, norstictic acid, usnic acid, atranorin, chloroatranorin	
Melanelia subaurifera	50 g	Soxhlet	Acetone	NA	NA	Lecanoric acid	[108]
Melanelia fuliginosa						O-methyl anziaic acid	
Cetraria islandica		Soxhlet	Petroleum ether	16 h	NA	Protolichesterinic acid	[58]
Platismatia glauca *Cladonia convoluta*	15 g	Soxhlet	n-hexane, diethyl ether, methanol	200 ml each	NA	Crude extracts	[11]
Hypogymnia physodes	15 g	Soxhlet	Methanol	150 ml, 24 h, RT	NA	Crude extract	[109]
Cladonia furcata, Cladonia pyxidata Cladonia rangiferina	100 g	Soxhlet	Acetone	NA	NA	Atranorin from *C.furcata* fumarprotocetraric acid from *C.rangiferia*	[9]

Parmelia caperata	100 g	Soxhlet	Acetone	NA	NA	Protocetraric, usnic acids	[71]
Usnea barbata	5 g	Soxhlet	Ether	NA	2.04	Usnic acid	[57]
			Ethanol		6.71		
Pseudevernia furfuracea	150 g	Soxhlet	Ethanol	250 ml, 5 days, 80ºC	20.26	Olivetoric acid, physodic acid	[110]
Rhizoplaca melanophthalma			Acetone		17.46	Psoromic acid	
Non-conventional Methods							
Hypogymnia physodes	4 g	Sonication	Acetone	400mL, 30min, 35 ºC	10.98	Physodic acid	[111]
Lobaria scrobiculata	156 g	Accelerated solvent extraction (ASE)	Dichloromethane	1. 1000 psi, 44 °C	NA	Stictic acid, usnic acid, m-Scrobiculin, 2,3,4-Trihydroxy-6-propylbenzoic acid, ergosterol, endoperoxide	[112]
			Methanol	1000 psi, 67 °C			
Protoparmeliopsis muralis, Caloplaca pusilla Xanthoria parietina	NA	Ultrasound	Water	30 min, RT	NA	Crude extract	[113]

Usnea barbata	NA	Supercritical CO_2	CO_2	Drug:extract ratio 62–100:5	NA	Usnic acid	[57]
Umbilicaria tornata	NA	Reflux extraction	Hot water	2 h at 85°C	2.64%	Crude polysaccharides	[60]

2.5 Separation, identification and characterization of lichenochemicals

Lichen extracts contain various bioactive compounds with different molecular masses, structures and properties (e.g., affinity, polarity); hence their separation and identification is challenging. The most common methods for separation and characterization of bioactive lichenochemicals can be grouped into: 1) chromatography methods (CC, TLC, HPLC); 2) spectroscopy methods (UV, FTIR, MS, NMR); and 3) physical methods (melting point, micro-crystallization). These methods will be reviewed briefly in the following sections.

2.5.1 Chromatography methods

2.5.1.1 Column Chromatography (CC)

CC separates compounds based on the affinity or polarity of stationary (solid) and mobile (solvent) phases. It is the most effective technique for separation of bioactive compounds with complex structures such as terpenoids, steroids, fatty acid derivatives, polyketides, alkaloids, polyphenols and peptides [114]. Lichenochemicals with anticancer potential that have been separated with CC from various lichen species. Lichens were first extracted in various polar (methanol, ethanol, ethyl acetate, chloroform), neutral (acetone), and/or non-polar (diethyl ether, hexane) solvents, and then fed onto differently sized chromatographic columns. Silica gel 60 with different particle and mesh sizes has been the most commonly applied stationary phase, although microporous resins have also been used [115]. The separation of lichenochemicals by CC depends primarily on the polarity of the mobile solvent phase. The general trend of separation and elution of lichenochemicals is based on gradient elution from non-polar to polar solvents with different solvent ratios. It should be noted that the particular order of solvents used as a solvent system plays a vital role in good separation. For example, solvents with lower polarity (in descending order acetone > methanol >

ethanol) are more efficient in extracting phenolic compounds from *Usnea* species [20]. Factors affecting CC separation are the initial extraction solvent, column (packing material, particle size, mesh size, length), mobile phase (solvent, polarity, gradient ratio), number of fractions, flow rate, and type of lichenochemicals.

Table 2.2: Column chromatographic specification of anticancer potential lichenochemicals

Lichen species	**Solvent**	**Column**	**Mobile phase**	**Fr.No**	**Lichen substance**	**Ref.**
Hypogymnia physodes (L.) Nyl.	Acetone	Silica gel (2.5 x 50 cm)	Gradient of a solvent system of hexane: ethyl acetate (75:25), toluene: hexane (75:35, 90:30) and toluene: ethyl acetate (75:30)	107	Physodic acid	[111]
Cladonia lichens	Acetone	Silica gel column (0.149 - 0.074 mm)	Hexane:ethyl acetate (6:1, 3:1 and 1:1)	15	Atranorin,	[9]
Cladonia lichens	Acetone	Silica gel column (0.149 - 0.074 mm)	Hexane:ethyl acetate (4:1)	10	Fumarprotocetraric acid	[9]
Xanthoria parietina	Acetone	Silica gel 60 (6 × 30 cm; 70–230 mesh)	*n*-hexane:Ethyl acetate100:0 to 0:100	40	Parietin	[63]

Endocarpon pusillum	Acetone	Reversed-phase flash column	Methanol:water (20:80,40:60, 50:50,60:40, 70:30, 80:20, and 100:0)	7	myA, myB, myC	[31]
Pseudevernia furfuracea (L.) Zopf	Ethanol	Silica gel (70–230 mesh)	*n*-hexane:ethyl acetate(90:10, 80:20,70:30, 60:40,50:50, 40:60,30:70, 20:80, 10:90 and 0:100) and ethyl acetate:methanol (90:10,80:20, 60:40 and 40:60)	NA	Olivetoric acid (OA), and physodic acid	[110]
Lagotis brevituba Maxim	80% Ethanol	Macroporous resin (D101)	Gradient mobile phase ethanol: water (0, 20, 40, 60% ethanol)	NA	Echinacosid, lagotioside, glucopyranosyl (1–6) martynoside	[115]
Lobaria orientalis	Methanol	Silica gel 60 (0.040−0.063 mm	n-hexane and gradient of ethyl acetate and methanol (stepwise, 10:0, 9:1, 8:2, and 5:5) and methanol	NA	Lobarientalones A and B, Lecanorin, Isolecanoric acid, Lobariethers	[34]
Roccella montagnei	Methanol	Silica gel (60–120 mesh)	Hexane; ethyl acetate	500	Everninic acid, Roccellic acid	[116]

			(9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5, 7:3)			
Lobariella pallida (Hook. f.) Moncada	Methanol	Si-gel 60 (0.069 - 0.200 mm)	Gradient of n-hexane: toluene (1:0 to 0:1), toluene: ethyl acetate (1:0 to 0:1), ethyl acetate: methanol (1:0 to 0:1) mixtures	148	Lobariellin, methyl orsellinate	[117]
Stereocaulon strictum var. compressum (Nyl.) I.M. Lamb	Methanol	Si-gel 60 (0.069 - 0.200 mm)	Gradient of n-hexane: chloroform (1:0 to 0:1), chloroform: ethyl acetate (1:0 to 0:1) and ethyl acetate - methanol (1:0 to 0:1) mixtures	84	Methyl haematommate, methyl-β-orcinolcarboxylate and atranorin	[117]
Stereocaulon strictum	Methanol	Si-gel 60 (0.069 - 0.200 mm)	Gradient of chloroform: ethyl acetate (1:0 to 0:1) and ethyl acetate: methanol (1:0 to 0:1) mixture	111	Porphyrilic acid	[117]

Parmotremat savoense	Methanol	Silica gel	n-hexane: ethyl acetate (9:1) followed by second silica gel elution with increasing polarity, n-hexane: ethyl acetate (8:2, 7:3, 6:4, 5:5, and 4:6)	5	Meta-depsidones	[118]
Parmelia erumpens	Chloroform	Silica gel glass column (2 cm x 75 cm)	Mixer of hexane, hexane dichloromethane, dichloromethane and ethyl acetate in different ratios.	29	2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid	[119]
Cladonia kalbii	Chloroform	Silica gel (70–230 mesh)	Chloroform: hexane (80:20)	NA	*Atranorin*	[70]
Ophioparma ventosa	Chloroform	Silica gel	cyclohexane: ethyl acetate: dichloromethane: methanol gradient system (1:0:0:0 to 0:0:1:1 via 0:1:0:0)	7	Ophioparmin, 4-Methoxyhaemoventosins, 4-Hydroxyhaemoventosin, Anhydrofusarubin lactone, Haemoventosin	[120]

Everniastrum cirrhatum	Hexane, Ethyl acetate	Silica gel (particle size 60-120 mesh) (3.0 cm and length of 72.0 cm)	Hexane	NA	methyl-3-orcinol carboxylate	[121]
Gyrophora esculenta	Hexane, Diethyl ether, Ethyl acetate	Silica gel 60	Hexane: ethyl acetate (1:9)	4	Orsellinol orsellinic acid methyl ester	[122]

2.5.1.2 Thin Layer Chromatography (TLC)

TLC is a rapid, inexpensive and qualitative method for separation and identification of lichen compounds. Finding suitable solvent(s) is central to the establishment of a successful separation process. To increase the separation accuracy, polar and non-polar solvents as well as solvent systems with multiple solvents have been used [39]. Acetone, methanol and chloroform are frequently used as single solvents whereas toluene: 1,4-dioxane: acetic acid, toluene: acetic acid, and ethyl acetate: formic acid at variable solvent ratios appear as the most popular solvent systems, as shown in Table 2.3. The l ichenochemicals identified by TLC included atranorin]14[parietin [123], lobarientalones [34], and a new glycolipid β-galactosyl-ceramide isolated from the lichen Stereocaulon ramulosum (β-GalCer-lich) [124]. Most of these compounds appear colorless on the white background of a TLC plate, hence "visualization" is necessary after elution. The visualization methods can be either non-destructive (compound is unchanged after the process) or destructive (compound is converted into a new chemical). For instance, viewing a TLC plate under UV light is non-destructive, while using a chemical stain is destructive. A UV lamp at short-wavelength of 252/254 nm or long-wavelength of 366 nm UV light can be used on TLC plates

[14,34,104,125]. Commercial TLC plates may contain a fluorescent material (e.g., zinc sulfide), so the background of the plate appears green when viewed using a short-wave UV light. If a compound absorbs at 252 nm or 254 nm, the plate appears dark as the compound prevents the fluorescent material from receiving UV light. TLC is most useful for visualizing aromatic compounds and highly conjugated systems, as they strongly absorb in the UV range. Most of the functional groups do not absorb UV light and will not appear dark under the UV lamp even though they are still there. Since the compounds remain unchanged after viewing with UV light, further visualization techniques can be applied afterwards on the same plate. The most common destructive method to visualize the TLC spots is 10-25% sulfuric acid as a spraying agent, followed by heating at 110-120 $^{\circ}$C for 10-30 min [8,37,119,125]. Other spraying agents such as 10% hydrochloric acid (104), 5% vanillin [34] and 0.5% orcinol [124] were also used. Another TLC-based technique is the High-Pressure-TLC (HPTLC), which is a sensitive method with high detection levels [38,65]. HPTLC requires short processing times and small volumes of solvent [44]. Excellent resolution and precise separation are other advantages of this technique [126]. Like other analytical techniques, TLC has some limitations in the use of solvents and data interpretation which makes the subsequent compound identification more critical. Observation and detection of lichen substances based on a single R_f value is one of the challenges of this approach [38] which requires access to a R_f database of known compounds.

Table 2.3: Column chromatographic purification of potential anticancer lichenochemicals

Lichen	**Extract**	**Solvent system**	**Spot visualization**	**Compound**	**Ref.**
Xanthoria parietina	Acetone	A-Toluene: 1,4-dioxane: acetic acid (180:45:5)	Spraying with 10%sulfuric acid and heating for 30 min at 110 ^{0}C	Parietin	[37]
Umbilicaria hirsute		B-Hexane: methyl tert-butyl ether:		Gyrophoric acid	[37]

		formic acid (140:72:18) C-Toluene: acetic acid (170:30)			
Tuckermannopsis ciliaris *Xanthoparmelia chlorochroa*	Acetone	Toluene: ethyl acetate: formic acid (139:38:8)	UV light (254 and 366 nm), sprayed with 10% HCl, and heated at about 110 °C	Protolichesterinic acid Usnic acid, Salazinic acid, Norstictic acid, Constictic acids	[4]
Teloschistes chrysophthalmus	Acetone	Dichloromethane: ethyl acetate : acetic acid (90:8:2)	NA	Parietin	[123]
Parmotrema tinctorum nylanderiana Everniastrum cirrhatum Lobaria pulmonaria Usnea rubicunda. *Usnea glabrescens, Usnea baileyi Usnea ceratina*,	Methanol	Toluene: acetic acid (200:30)	NA	Flavonoid, Phenolic compounds	[127]

Usnea fulvoreagens, *Usnea nipparensis*, *Usnea fammea*, *Usnea rubrotincta*, *Usnea pectinate*, *Usnea schadenbergiana*					
Dermatocarpon vellereum *Umbilicaria vellea*, *Rhizoplaca chrysoleuca* *Rhizoplaca melanophthalma* *Pleopsidium flavum*., *Xanthoparmelia mexicana*, *Acarospora badiofusca* *Xanthoria elegans*, *Lecanora frustulosa*, *Lobothallia alphoplaca*,	Hexane, Methanol Water	A-Toluene: dioxane: acetic acid (180: 60: 8) B-Hexane: diethyl ether: formic acid (130: 100: 20) C-Toluene: acetic acid (200: 30)	NA	Proanthocyanidin, Flavonoid, Polyphenol	[33]

Physconia muscigena., *Melanelia disjuncta*, *Xanthoparmelia stenophylla*, *Peccania coralloides*.					
Everniastrum cirrhatum Parmotrema reticulatum	Ethyl acetate,	Toluene: dioxane: acetic acid (180:45:5)	NA	Polyphenol, Polysaccharide, Protein	[128]
Parmelia erumpens	Chloroform	Toluene: acetic acid (85: 15)	Spraying with 10% sulfuric acid and heating for 10 min at 110 ^{0}C	Atranorin, Usnic acid, 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid	[119]
Everniastrum cirrhatum	Ethanol	Benzene: acetone (98: 2)	Exposing to iodine fume	Methyl-3-orcinol carboxylate	[121]
Lobaria orientalis	Methanol	A-Toluene: dioxane: acetic acid (180:45:5) *B*-*n*-hexane: diethyl ether: formic acid (130:80:20)	Visualized by UV, and sprayed with 5% vanillin in acidic aqueous solution followed by heating	Lobarientalones A and B, Lecanorin, Isolecanoric acid, Lobariethers	[34]

		C-Toluene–acetic acid (170:30)			
Cetraria aculeata	Acetone	Toluene; dioxane: glacial acetic acid (36:9:1)	NA	Lichestrerinic acid, Protolichesterinic acid	[54]
Cladonia salzmannii	Diethyl ether, Chloroform, Acetone	Toluene: dioxane: acetic acid (45:12.5:2)	Visualized under UV light wavelengths (254 nm and 366 nm) and spraying with 10% sulfuric acid and heating for 10 min at 50 ^{0}C on a hot plate	Barbatic acid, Thamnolic, D-thamnolic acids	[125]
Stereocaulon ramulosum	Hexane, Chloroform Methanol	Chloroform: Methanol: Acetic acid: Water (100:20:12:5).	Spraying with 0.5% orcinol reagent and heating for 5 min at 120 ^{0}C	β-GalCer-lich	[124]
Xanthoria parietina	Acetone	Petrol/ethyl acetate (1:1)	Spray with Ceric sulfate in sulfuric acid	Parietin	[63]

2.5.1.3 High Performance Liquid Chromatography (HPLC)

HPLC is a well-known and practiced analytical technique for separation and identification of natural products and has been widely-used for several decades [65]. Separation is based on the difference in the affinity of molecules to the mobile phase (liquid) and the stationary phase (solid adsorbent). The HPLC separation of lichenochemicals depends mainly on the type of mobile phase and its compatibility with the column. Factors affecting HPLC separations are flow rate, column length, particle diameter, particle size distribution, eluent type and composition, stationary phase type, temperature and sample concentration. Anticancer lichenochemicals have been analyzed using C18 columns of different specifications in terms of length of the column, inner diameter, particle size, and inlet pressure, as shown in Table 2.4. To achieve a good separation, different combinations of solvents are mixed at different flow rates under either isocratic or gradient mode. Each compound has a specific peak based on the type of solvent, flow rate, detector, temperature and time of elution. Peaks are visualized at a specific wavelength using a UV detector.

Table 2.4: HPLC operating condition for analysis of anticancer potential lichenochemicals

Lichen species	**HPLC**	**Column**	**Mobile phase**	**Flow rate (ml/min)**	**λ (nm)**	**Lichen substance**	**Ref.**
Usnea barbata	Agilent Technologies, 1200 Series HPLC	Zorbax Eclipse XDB-C18 (100 x 4.4 mm, 1.8µm)	Solvent A: 99% Water, 1% Orthophosphoric, Solvent B: Acetonitrile	Gradient, 0.1	NA	Usnic acid	[57]
Parmelia caperata		C18 (250 × 4.6 mm, 10 m)	Methanol: Water: Phosphoric acid (75:25:0.9)	Gradient, 1.0	NA	Usnic acid, Protocetraric acid	[71]
Parmelia saxatilis						Salazinic acid	
Cladonia subulata, Cladonia furcata						Hypoprotocetraric acid, fumarprotocetraric acid	[71]
Cladonia rangiferina						Fumarprotocetraric acid, Atranorin	
Cladonia fimbriata						Hypoprotocetraric acid, Fumarprotocetraric acid, Usnic acid, Atranorin	

Cladonia foliacea						Fumarprotocetraric acid, Usnic acid	
Cetraria islandica *Vulpicida canadensis*	Agilent Technologies, 1260 Series HPLC	Reversed-phase Mediterranea Sea18 column (150 mm × 4.6 mm, 3µm)	1% Orthophosphoric acid in milli-Q water (A): methanol (B)	Gradient, 0.6	190 & 400	Fumarprotocetraric acid Usnic, Pinastric, Vulpinic acids	[64]
Usnea longissima	Dionex, 3000 Ultimate	Phenomenex Luna-C18 (250 × 21.1 mm, 5µm)	Acetonitrile: Water: Formic acid (90:10:0.01)	Gradient, 5.0	210	Protolichesterinic acid	[58]
Chapter 1 ladonia arbuscula (Wallr.)	Dionex HPLC system	Hypersil Gold C18 (250 × 4.6 mm)	Methanol: Water: Phosphoric acid (80:20:0.9)	Gradient, 1.0	240	Usnic acid, Atranorin	[8]
Alectoria samentosa, Flavocetraria nivalis, Alectoria ochroleuca, and *Usnea florida*	Shimadzu HPLC-LC-20A	YMC-Pack ODS-A C18 ((150 x 3.9 mm I.D, 5 µm, 12 nm).	Methanol: Water: Phosphoric acid (80:20:1)	Gradient, 1.0	190 & 400	Usnic acid	[129]

Flavocetraria cucullata						Usnic acid, Salazinic acid, Squamatic acid, Baeomycesic acid, D-Protolichesterinic acid, Lichesterinic acid	[34]
Parmelia erumpens Kurok	Shimadzu LC 10AT	C18 (250 x 4.6 mm, 10 m)	Methanol: Water: Phosphoric acid (70 : 20 : 0.9)	Isocratic 1.0	NA	Atranorin, Usnic acid, 2-Hydroxy-4-methoxy-3,6-Dimethylbenzoic acid	[119]
Parmotrema reticulatum	Shimadzu HPLC RF10 AXL	Z1C-HILIC	Acetonitrile and 0.5 mM Ammonium acetate in water	Gradient 1.0	254	catechin, Purpurin, Tannic acid, Reserpine	[130]
Bryoria capillaris *Cetraria islandica*	VWR-Hitachi LaChrom Elite HPLC	Venusil XBP C18 (250 x 4.6 mm, 5µm)	Methanol: Water: Phosphoric acid (90:10:0.9)	Gradient 1.0	254	Usnic acid Fumarprotocetraric acid	[131]

Cladonia Salzmannii Nyl.	HITACHI model 655A-11	MicroPack MCH-18 reverse phase column (250 × 4.6 mm)	Methanol: Water: Acetic acid (80:19.5:0.5)	Isocratic	254	Barbatic acid	[125]
Lichina pygmaea	Semi-preparative reversed-phase HPLC	Hypersil BDS (250 x 10mm, 5 µm)	0.1% Acetic acid in Water for 45 min and Methanol/ Water (10% for 17 min,50 for 22 min, 100% for 15 min)	Nonlinear gradient, 1.0	254	Pygmeine	[103]
Lagotis brevituba Maxim	Semi-preparative HPLC	HC RP-C18 (250 × 20 mm, 5 µm)	Water: Acetonitrile, (82:18)	15	254	Echinacosid, Lagotioside, Glucopyranosyl (1–6) martynoside, Plantamoside, Verbascoside	[115]
docarpon pusillum	RP-HPLC	Pennomenax Luna C18	Solvent A: 0.1% trifluoric acid in water; Solvent B: 0.1%	Isocratic 2.0	254	myA, myB, myC	[31]

Trifluoric
acid in
Acetonitrile

2.5.2 Spectroscopy methods

2.5.2.1 Ultraviolet spectroscopy (UV)

The UV spectra of lichen secondary metabolites have been obtained using a UV spectrophotometer sometimes in conjunction with another separation technique such as liquid chromatography LC [132,133], or MS [134]. In fact, the UV-LC is currently a very common analytical method to identify new natural compounds from lichen extracts and fractions. The absorption of visible and UV radiation correlates with the excitation of atoms and molecules, from lower to higher energy levels. The UV spectra of natural organic molecules is represented by three bands, each of them a Gaussian function of energy: local-excitation, benzenoid and electron-transfer bands. These bands are recognized as three types of electronic transitions used typically for identification of aromatic compounds. At wavelengths from 200 - 400 nm, an adequate fit to the spectrum, can be obtained by consideration of only the benzenoid and electron-transfer bands [135]. Rezanka and Dembitsky [136] used UV spectroscopy for identification of brominated aliphatic compounds from seven lichens species. Posokhov [137] reported on the UV/VIS spectral properties of three novel natural products from the Turkish lichens *Pseudevernia furfuracea, Evernia prunastri*, and *Letharia vulpine* [137].

2.5.2.2 Fourier Transform Infrared (FTIR)

By means of FTIR, the absorption and emission of the IR-spectra of bioactive compounds in a solid, liquid or gas state are obtained. The IR spectroscopy measures the bond vibrations of various functional groups such as carbonyl, ketone, hydroxyl, and aromatic ring at different wavelengths [65]. FTIR is a specific method for primary determination of functional groups in molecules. This method makes use of spectra-correlation charts for IR frequencies in a particular region of a chemical compound [138]. Solid lichen compounds are detected in solutions of carbon

tetrachloride (CCl_4) or chloroform ($CHCl_3$) and can be incorporated into a solid potassium bromide pellet (KBr) [38]. The IR spectrum can provide information on the type of carbon-hydrogen bonds with stretches between 3,200-3,500 cm^{-1}, carbonyl band of five-member lactone at 1,735-1,750 cm^{-1}, carbonyl band of six-membered lactone at 1,760-1,780 cm^{-1}, five-membered anhydrides at 1,770 cm^{-1},844 cm^{-1}, and six-membered anhydrides appears at 1,758-1,790 cm^{-1}. These values can be used as references to identify the presence of the functional groups [38]. Several studies have used FTIR for identification of associated groups in ethanol and methanol extracts of the lichen *Parmotrema perlatum* [139] or functional groups in lichenochemicals, such as barbatic acid from *Cladonia salzmannii* Nyl [125], usnic acid from *Cladonia substellata* [140], water soluble polysaccharide from *Umbilicaria esculenta* [36], isocoumarins and benzofuran from *Pyrenulasp* [141], pigments and bio-degradative calcium oxalate from *Acarospora spp*. [29], and usnic acid from *Cladonia arbsucula, C. sulphurina, C. uncialis* [142]. FTIR spectra of the lichens *Pyrenula japonica* and *Pyrenula pseudobufonia* have been analyzed for identification of secondary metabolites [143].

2.5.2.3 Mass Spectroscopy (MS)

MS is a fast, modern and simple tool for structure identification of lichen substances [144]. Many compounds and functional groups such as depsides, depsidones, dibenzofurans and diphenyl butadiene have been identified using MS [47]. For example, the MS-based identification of lichen-derived anticancer agent cyaneodimycin from *Lichina confinis* was done using a Prevail C18 column and a combination of mobile phases of A (0.1 % formic acid in water) and B (0.1 % formic acid in acetonitrile) with a gradient flow of 1 ml/min [145]. Likewise, some glycosides and alkaloids from *Parmelia perlata* were identified using MS [146]. These studies involved use of MS or MS-GC instrumentation. MS successfully identified lichenochemicals used in various cancer cell treatments such as usnic acid derivatives [25,55], sphaerophorin, pannarin and epiphorellic acid [147], and tumidulin [31]. MS was also used in combination with other instrumentation: Time of Flight (TOF)/MS - for characterization of secondary metabolites in *Hypogymnia physodes* [109]; LC-MS - for identification of lichenochemicals in the lichen *Lobaria scrobiculata* [112]; MS/MS - for detection of protolichesterinic acid in the lichen *Cetraria islandica* [148].

2.5.2.4 Nuclear Magnetic Resonance (NMR)

NMR spectroscopy is used widely to determine the structure of organic molecules [38]. The resonance frequency of a particular simple substance is usually directly proportional to the strength of the applied magnetic field, and the NMR signal patterns are related to the nature and position of the nuclei in the molecule [65]. Moreover, NMR demonstrates which atoms are present in neighboring groups. Ultimately, NMR can provide information on how many atoms are present in each of these environments [149]. Notably, lichen substances are mostly dissolved in $CDCl_3$ or acetone-d6 or DMSO-d6 for 400-500 MHz ^1H and 75-125 MHz ^{13}C NMR, although other solvents have also been used as presented in Table 2.5.

Table 2.5: NMR operating parameters for analysis of anticancer potential lichenochemicals

Lichen species	Instrument	NMR	Solvent	Lichen substance	Ref.
Pseudevernia furfuracea (L.)	NA	^1H NMR and ^{13}C NMR	$CDCl_3$	Olivetoric acid, physodic acid	[110]
Rhizoplaca melanophthalma (DC.) Leuckert	NA	^1H NMR and ^{13}C NMR	$CDCl_3$	Psoromic acid	
Platismatia glauca, Cladonia uncialis, Parmelia sulcata, Hypogymnia physodes, Hypocenomyce scalaris	NA	^1H NMR and ^{13}C NMR		Atranorin, lecanoric acid, squamatic acid) and depsidones (physodic acid, salazinic acid), caperatic acid	[16]

Lobaria orientalis	Bruker Avance III spectrometer	500 MHz ^{1}H NMR and 125 MHz ^{13}C NMR	$CDCl_3$	Lobarientalones, lobariethers, 1,10-di-o-methylstictic acid , lecanorin, isolecanoric acid	[34]
Parmelia erumpens	Bruker DRX 500 NMR	500 MHz ^{1}H NMR and 125 MHz for ^{13}C NMR	$CDCl_3$	Atranorin, Usnic acid, 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid	[119]
	Bruker Avance 400 or Varian 400-MR or Varian 400S spectrophotometer	400 MHz ^{1}H NMR, or 75 or 125 MHz ^{13}C NMR	$CDCl_3$	Lobariellin	
Lobaria orientalis	Bruker Avance III spectrometer	500 MHz ^{1}H NMR and 125 MHz for ^{13}C NMR	$CDCl_3$	16β-acetoxyhopane-6α,22-diol	[34]
Parmotrema dilatatum	Bruker DPX-300 spectrometer	^{1}H NMR	$CDCl_3$	Atranorin	[105]
		^{1}H NMR	DMSO-d6	Protocetraric acid	
Usnea subcavata		^{1}H NMR	$CDCl_3$	Usnic acid	
		^{1}H NMR	acetone-d6	Diffractaic acid	
Parmotrema lichexanthonicum		^{1}H NMR	DMSO-d6	Lichexanthone	

Dirinaria aspera		^{1}H NMR	acetone-d6	Divaricatic acid	
Cladonia confusa		^{1}H NMR	$CDCl_3$	Perlatolic acid	
Usnea sp.		^{1}H NMR	DMSO-d6	Psoromic acid	[150]
Ramalina sp.		^{1}H NMR	DMSO-d6	Norstictic acid	
Cladonia salzmannii Nyl.	Varian Unity Plus spectrometer	300 MHz ^{1}H NMR and 75 MHz ^{13}C NMR	DMSO-d6	Barbatic acid	[125]
Hypogymnia physodes	Bruker Avance 400	^{1}H NMR and ^{13}C NMR	acetone-d6	Physodic acid	[111]
Pseudocyphellaria coriacea	NA	^{1}H NMR and ^{13}C NMR		Physciosporin	[28]
Myelochroa aurulenta	Jeol ECP-400 and Bruker DRX-500	^{1}H NMR and ^{13}C NMR	CDCl3, C6D6	leucotylic Acid	[101]
Lobaria scrobiculata	Bruker Avance 400	^{1}H NMR	NA	Stictic acid Usnic usnic m-Scrobiculin Methyl divarate Ergosterol endoperoxide	

2.6 Physical methods

2.6.1 Melting point

The melting point of lichen substances can be determined visually by monitoring the temperature at which melting of solid crystals occurs or by using a differential scanning calorimetry (DSC). DSC is a thermoanalytical technique that measures heat as a function of temperature and defines the different amount of heat required to increase the temperature of a sample against a reference. Both the sample and the reference are maintained at the same temperature throughout the experiment. The melting points of lichenochemicals with anti-cancer potential has shown in Table 2.6.

However, two different melting points were reported for atranorin (201.14 and 203.27)ºisolated *Parmelia erumpens* and *Parmotrema dilatatum*, and for usnic acid (193.12 and 197.45ºC), isolated from *Parmelia erumpens* [119] and *U. subcavata* [105]. This emphasizes the need to use additional analytical techniques, as melting point alone does not provide sufficient information to determine the identity of an unknown compound.

Table 2.6: Melting point of potential anticancer lichenochemicals

Lichen species	Lichochemical	Melting point (ºC)	Ref.
Parmelia erumpens	Atranorin	201.14-203.27	[119]
	Usnic acid	193.12-197.45	
	2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid	184.4 – 187.1	
Lobariella pallida	Lobariellin	90 - 92	[117]
Stereocaulon strictum	Porphyrilic acid	280 - 282	
Parmotrema dilatatum	Atranorin	196 – 198	[105]
	Protocetraric acid	244 - 250	
Usnea subcavata	Usnic Acid	202 – 203	

	Diffractaic acid	194 – 195
Parmotrema lichexanthonicum	Lichexanthone	188 – 190
Dirinaria aspera	Divaricatic acid	137 – 138
Cladina confusa	Perlatolic acid	106 – 108
Usnea sp.	Psoromic acid	264 – 265
Ramalina sp.	Norstictic acid	285 – 287

2.6.2 Microcrystallization

Crystallization is a process in which a solid state of a molecule is formed, where the atoms or molecules are highly organized into a structure known as a crystal. In lichen studies, crystallization aids the isolation of lichenochemicals of pharmaceutical interest [103]. The lichen compounds are characterized based on the physical properties of their crystals, which is useful in chemotaxonomic studies [38]. Microcrystallization was used successfully in the isolation of lecanoric acid crystals from a methanol solution of *Melanelia subaurifera* extracts under vacuum [108]. Recently, amorphous crystals from cytochalasin E with promising anticancer activity on colorectal cancer cells were obtained from the lichen *Pleurosticta acetabulum* [35].

2.7 Lichenochemicals in cancer research

One of the approaches for finding possible antitumor drugs is the testing of different naturally synthesized compounds. The biological part of this review relates the use of lichen/isolated compounds for anticancer purpose. As mentioned, these products have been used in folk medicine and recent screening tests with lichens/lichen compounds demonstrated the incidence of their antitumor activity. Based on that, these secondary metabolites can be considered candidates for evaluating their growth inhibitory effects on various cancer cells. Although a small number of studies have explored the mechanisms of action of these molecules, some studies have shown the effects on apoptosis, cell cycle arrest and anti-proliferation on a variety of cancer cells that will be discussed later. Anticancer research using lichen-derived secondary metabolites with antitumor activities has been carried out on a number of cancer cell lines [40,70,109,151] and demonstrated

apoptosis or anti-proliferation as the mode of action of lichenochemicals.Of lichenochemicals, usnic acid and atranorin on breast and colon cancer [12,107], prostate [147] and other cancer cell types [64,99,103,104] and revealed possibility of antitumor activity on these cancerous cells. It is worth noting that the lichen-based anticancer research has the objective of searching for lichenochemicals that have minimum side effects and toxicity [153]. Clearly these findings need developing research for those compounds that have promising results. In this section, evidence of the anticancer property and possible mechanism(s) by which lichen/lichenochemicals act on different cancer cell lines are presented. Most of these studies have performed to reveal apoptosis and anti-proliferation effects of the interest compound(s). It has been found that some of these secondary metabolites have decreased numbers of viable cells in a dose-dependent manner. Some compounds increase cytotoxicity and play a significant role in deregulation of signaling pathways. The mechanisms of action against cancer cells are measured using a variety of biological assays. For example, the cytotoxicity of lichen/lichen-derived compounds is determined by trypan blue exclusion assay, or cell viability is mostly evaluated using MTT assay. In order to understand the mechanism of apoptosis, cell cycle kinetics and gene pattern expressions which are associated with cancer- mainly p53 are studied**.** Any decrease or increase in the gene expression is evaluated by specific biomarkers and assays [64,102,121,127, 131,132]. Taken together, cell viability assays, cytotoxicity, cell cycle arrest and the tumor suppressor gene p53 evaluation are mainly considered. As described below, breast cancer is the most commonly diagnosed cancer in women [156] and colon cancer has the highest reported occurrence [106] in comparison with other cancer types that have been investigated using mainly lichen/lichen compounds.

In this section, evidence substantiating the chemotherapeutic properties and possible mechanism(s) by which lichenochemicals act on different cancer cell lines is discussed.

2.7.1 Breast cancer

The effectiveness of lichenochemicals against breast cancer has been studied on several breast cancer cell lines. The major focus in this research was on cancer cell viability, mechanism(s) of action of lichenochemicals and molecular targets, including inhibition of cell cycle, induction of apoptosis and autophagy, regulation of angiogenesis and modulation of several transcription factors. For example, the acetone extract and the secondary metabolite parietin from the lichen *Xanthoria parietina* inhibited proliferation and induced apoptosis in the MDA-MB231 cell line.

Both effects were accompanied by modulation of expression of cell cycle regulating genes for protein products such as p16, p27, cyclin D1 and cyclin A [63]. Overexpression of fatty acid synthase (FASN) and human epidermal growth factor receptor-2 (HER2) was seen in presence of protolichesterinic acid (PA), a lichen-isolated compound which induced apoptosis as well as inhibition of ERK1/2 and AKT pathways in SK-BR-3 cells. However, no changes were reported in T-47D cells as PA had no effect on the Erk1/2 and AKT signaling pathways [58]. FASN is an important enzyme responsible for catalyzing long chain fatty acids in mammalian cells [154] which controls the expression of HER2, a transmembrane receptor tyrosine kinase. Both HER2 and FASN are overexpressed in breast cancer. Any target drug that inhibits FASN expression can also be effective in inhibition of HER2 [155]. Exposure of different breast cancer cell lines to usnic acid and rapamycin caused a pronounced proliferation, migration, and invasive inhibition [156]. DNA damage and induction of p53 tumor suppressor function was observed with usnic acid on MCF7 cells [151,157,158]. Further, a combination of usnic acid and the chemotherapeutic drug bleomycin increased toxicity and caspase-3 and 8 activities, enhancing apoptosis and the p53/p21 pathways [27]. Lichenochemicals from *Hypogymnia physodes* induced apoptosis and cell growth inhibition in MCF7 breast cancer cell line, but no significant effects were observed in MDA-MB-231 cells [109]. Interestingly, a study has reported no significant morphological and microtubular changes in MCF7 cells following treatment with usnic acid [26], which may be related to the effective concentration of usnic acid. The lichenochemicals used in breast cancer studies, their IC50 values and their lichen sources are presented in Table 2.7. The different anticancer effects and IC_{50} values of the lichenochemicals are most likely related to their chemical structure that determines their anticancer mode of action on cancer cells. In case of lichen extracts, factors such as solvents used to produce the extracts and extract solubility may also play a role.

2.7.2 Colon cancer

The lichen extracts and lichenochemicals that were studied on different colon cancer cell lines had different IC_{50} values, which reflects the impact of the physicochemical properties of lichen compounds and their concentrations on targeted cell lines. For example, Methyl-β-orcinolcarboxylate (1.5 μg/ml) and usnic acid (2.3 μg/ml), together with the acetone extract from *Endocarpon pusillum* (1.84 μg/ml), had the lowest IC50 values; however, direct comparison is difficult as different colon cancer cell lines were used. All cell lines showed induced degrees of

apoptosis (cell death) and arrest in the different phases of the cell cycle. Treatment of HCT116 with lobaric acid and lobastin resulted in cell morphology changes from polygonal to circular, which is an apoptosis indicator. Moreover, PARP cleavage and under expression of Bcl-2 were observed in both [30]. Using cytofluorometric analysis with propidium iodine, Kosanic [9] reported antiproliferative activity and decrease in G2/M cell population as the two major effects of atranorin and fumarprotocetraric acid on LS174 cell line. Furthermore, atranorin was more effective than fumarprotocetraric acid in accumulation of cells in the sub-G1 phase. Acetone extract of *Lethariella zahlbruckneri* induced apoptosis in HT-29 cell line and triggered caspase-8 and caspase-9 activities [106]. An increased expression in Bax and a decrease in Bcl-2 proteins was evident from the western blot analysis. Research on two other colorectal cell lines (HCT116 and DLD-1) has confirmed signalling inhibition in dose and time dependency using different lichen compounds. Among them, lecanoric acid showed a negligible effect on Axin2 expression in HCT116 cells in comparison to physodic acid. Also, caperatic acid decreased Axin2 expression in both cell lines [16]. Ramalin, another lichen secondary metabolite, revealed cell cycle arrest of G2/M as a sign of apoptosis on HCT116 cells. Moreover, an increase in the TP53 protein expression and a decrease of CDK1 and CCNB1 has been observed [12]. Another promising anti-colorectal agent, tumidulin, displayed a reduced spheroid formation in CSC221, DLD, and HT29, mRNA expression and cancer markers, such as aldehyde dehydrogenase-1, CD133, CD44, and Lgr5. Moreover, tumidulin prevented transcriptional activity of glioma linked to oncogene (Gli1 and Gli2) [31], as presented in Table 2.7.

2.7.3 Skin cancer

The melanoma cell lines UACC-62, B16-F10, FemX, B16, HTB-140 (Hs 294T) and HaCaT (keratinocyte form adult skin) have been all used to study the anticancer effects of lichenochemicals (Table 2.7). These compounds exhibited bioactivity against skin cancer (melanoma cell line) by either apoptotic cell death or DNA fragmentation. Studies showed that treatment of melanoma A375 cell line with physodic acid at concentrations of 6.25-50 μM resulted in inhibition and apoptosis. It was proposed that physodic acid causes a decrease in gene expression of heat shock protein Hsp70 [15], a 70 kDa protein localized in solid tumors such as malignant melanoma cells [159]. In another study, two lichen compounds atranorin and usnic acid have significantly decreased cell proliferation and actin cytoskeleton in melanoma cells HTB-140 [8].

Usnic acid and the lichen *U. barbata* extract effected apoptosis and autophagy in B16 cell line cancer cells, which consequently resulted in morphology changes in the acidic cytoplasmic vesicles that facilitate vesicular transportation in the organelles and endocytic systems [57]. Two lichen metabolites, sphaerophorin and pannarin, were reported to induce apoptosis in cell line M14 and cause DNA fragmentation with considerable caspase-3 activity along with induction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [17]. Treatment with usnic, gyrophoric, and diffracteic acid revealed cytotoxicity in human keratinocytes cells [158]. It was found that usnic acid had a high toxicity against MM98 malignant mesothelioma, A431 cells, human epidermoid carcinoma, and HaCaT keratinocyte cells [100].

2.7.4 Lung cancer

The lung cancer cell lines A549, NCI-H460, NCI-H292, and 3LL cell lung carcinoma have been tested to investigate the antitumor activity of various lichen extracts and lichenochemicals (Table 2.7). As an example, physciosporin, a lichen secondary metabolite, produced a significant effect on migration and invasion of lung cancer cells by decreasing the protein and mRNA levels of N-cadherin, while increasing the expression of tumor suppressor gene KAI1. Also, physciosporin inhibited activities of the cell division control protein 42 (Cdc42) and Rac1 in lung cancer cells A549, H1650 and H1975 [28]. Another study [129] showed that tested non-small lung cancer cells by usnic acid in a dose-dependent response alleviated the β-catenin protein and oncogene metastasis-enhancing KITENIN-mediated AP-1 activity which upregulated in cancers. Usnic acid also reduced the mRNA levels of CD44, Cyclin D1 and c-myc in these cells. Usnic acid at low concentrations (6.25 and 12.5ug/mL) induced a mitochondria inhibitory effect by increasing the ROS which led to cell death [160]. In a similar fashion, examined cell line A549 with atranorin decreased the β-catenin and KITENIN-mediated AP-1 activity and the Rho family GTPase protein which are both the signal transducers and activators of transcription (STAT) [32]. A dose-dependent growth inhibition has also been observed in this cell line and caused cell accumulation at the G0/G1 cell cycle phase by XD8, a chemically synthesized derivative of the natural lichenochemical xanthone XD8 [161]. Antineoplastic activity with low toxicity on normal cells have been seen in squamous lung cancer cells treated with the lichen *Cladonia aggregata* extract and its secondary metabolite barbatic acid [150].

2.7.5 Liver cancer

Lichen secondary metabolites and lichen crude extracts have demonstrated significant anticancer reflect on human liver carcinoma cells HEPG2, C3A and WRL-68 (Table 2.7). An approach described the cytotoxicity effects of tested concentrations of *Umbilicaria tornata* and its two metabolites UTP-1 and UTP-2 on HEPG2 [31]. Lichen-derived compounds interrupted the endothelial tube production [102] and reduced the mRNA levels of N-cadherin protein in the epithelial-mesenchymal transition (EMT) [28].

Table 2.7: Lichenochemicals as promising anticancer on the predominant cancer types

Lichen	Extract/ Lichenochemical	Cancer cell line	IC_{50}	Ref.
Breast Cancer				
Cladonia convoluta	Diethyl ether fraction	MCF7	8.8 µg/ml	[11]
Parmelia caperata	*n*-hexane fraction	MCF7	13.4 µg/ml	[11]
Lobaria orientalis	16*β*-acetoxyhopane-6*α* 22-diol, Retigeric acid B, and Cerevisterol	MCF7		[34]
Cetraria islandica	Methanol extract (Fumarprotocetraric acid)	MCF7	19.51 µg/ml	[64]
Vulpicida canadensis	Methanol extract (Usnic,pinastric and vulpinic acids)	MCF7	148.42 µg/ml	[64]
Parmotrema reticulatum	Tannic acid, Catechin Purpurin and Reserpine	MCF7	103.31, 255.79 560.11 and 46.78 µg/ml	[130]
Cladonia Salzmannii Nyl.	Barbatic Acid	MCF7	18.28 µg/ml	[125]
Parmotrema tsavoense	Parmoether B	MCF7	22.32 µM	[118]

Roccella montagnei	Everninic acid and Roccellic acid	MCF7	NA	[115]
Lobaria orientalis	Lobarientalones, lobariethers, 1,10-di-o-methylstictic acid , lecanorin, isolecanoric acid	MCF7	NA	[34]
Usnea flammea Stirt	Methanol extract	MCF7	34.27mg/ml	[162]
Lobariella pallida	Methanol extract	MCF7	>67 µg/ml	[162]
Anaptychia ciliaris, Bryoria capillaris and *Cetraria islandica*	Acetone extract	MCF7	>200 µg/ml	[131]
Parmelia sulcata Taylor	Methanol extract	MCF7 and MDA-MB-231	39.1 and 16.5 µg/ml	[152]
Xanthoria parietina	Acetone extract and parietin	MCF7 and MDA-MB-231	NA	[63]
Hypogymnia physodes (L.) Nyl.	Physodic acid	MCF7, MDA-MB-231, and T-47D	93.9, 72.4, and 75.4 µM	[111]
Hypogymnia physodes (L.) Nyl.	Acetone extract	MCF7, MDA-MB-231, and T-47D	46.2, 110.4 and 60 µM	[111]
Stereocaulon alpinum Laur.	Lobaric acid	T-47D and ZR-75- 1	14.5 and 44.7 µg/ml	[163]
Cetraria islandica L. (Ach.)	Protolichesterinic acid	T-47D and ZR-75- 1	1.1 and 24.6 µg/ml	[163]
Cetraria islandica L. (Ach.)	protolichesterinic acid	SK-BR-3 and T-47D	10.8 and 11.7 µM	[58]

Colon Cancer				
Lobaia pulmonaria	Salazinic acid	LS174	35.67 μg/ml	[71]
Toninia candida	Protocetraric acid	LS174	60.18 μg/ml	[164]
Evernia prunastri	Usnic acid	LS174	2.3 μg/ml	[164]
Cladonia furcata	Atranorin	LS174	24.63 μg/ml	[9]
Cladonia rangiferina	Fumarprotocetraric acid	LS174	41.23 μg/ml	[9]
Cladonia foliacea, Cladonia furcata, Cladonia fimbriata, Cladonia subulata, Cladonia rangiferina	Acetone extracts	LS174	28.98 to 140.13 μg/ml	[9]
Cladonia furcata, Lecanora atra, Lecanora muralis	Acetone extract	LS174	40.22, 10.29, 12.23 μg/ml	[165]
Stereocaulon alpnum	Lobaric acid, lobarstin	HCT116	NA	[30]
Platismatia glauca, Cladonia uncialis, Parmelia sulcata, Hypogymnia physodes, Hypocenomyce scalaris	Salazinic acid, caperatic acid	HCT116	NA	[16]
Lagotis brevituba Maxim.	Echinacoside, lagotioside, glucopyranosyl (1–6) martynoside, plantamoside, verbascoside	HCT116	>100 μM	[115]

Everniastrum cirrhatum	Ethyl acetate extract	HCT116	250 μg/ml	[128]
Roccella montagnei	Everninic acid, roccellic acid	DLD-1, SW-620	NA	[115]
Platismatia glauca, Cladonia uncialis, Parmelia sulcata, Hypogymnia physodes, Hypocenomyce scalaris	Atranorin, lecanoric acid, squamatic acid, physodic acid, salazinic acid, caperatic acid	DLD-1	Na	[16]
Flavocetraria cucullata	Acetone extracts	HT29	10.9 mg/mL	[53]
Endocarpon pusillum	Acetone extract	CT26	1.84 μg/ml	[31]
Lobothallia alphoplaca, Melanelia disjuncta, Xanthoparmelia stenophylla	Methanol extract	RKO	222.22, 98.77 μg/ml	[33]
Everniastrum cirrhatum	Methyl-β-orcinolcarboxylate	Caco-2	1.5 μg/ml	[121]
Skin Cancer				
Parmotrema dilatatum	Atranorin	UACC-62, B16-F10, 3T3	147.2, >250, >250	[105]
Parmotrema dilatatum	Protocetraric acid	UACC-62, B16-F10, 3T3	0.52, 24, 48.5	[105]
Usnea subcavata	Usnic and	UACC-62, B16-F10, 3T3	31.5, 47.7, 41.4	[105]

Usnea subcavata	Diffractaic acids	UACC-62, B16-F10, 3T3	24.7, 25.4, 31.2	[105]
Parmotrema lichexanthonicum	Lichexanthone	UACC-62, B16-F10, 3T3	>250 each	[105]
Ramalina sp.	Norstictic acid	UACC-62, B16-F10, 3T3	32.9, 62.4, >250	[105]
Claddonia confusa	Perlatolic acid	UACC-62, B16-F10, 3T3	3.3, 18, 26	[105]
Dirinaria aspera	Divaricatic acid	UACC-62, B16-F10, 3T3	2.7, 4.4, 14.5	[105]
Parmelia erumpens	2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid	B16F10	2.53	[117]
Cladonia furcata	Atranorin	FemX	20.91	[9]
Cladonia rangiferina	Fumarprotocetraric acid	FemX	30.67	[9]
Lobaia pulmonaria	Salazinic acid	FemX	39.02	[71]
Toninia candida	Protocetraric acid	FemX	58.68	[164]
Evernia prunastri	Usnic acid	FemX	12.72	[164]
Cladonia furcata, Lecanora atra, Lecanora muralis	Acetone extract	FemX	23.52, 8.51, 9.58	[165]
Usnea barbata	Supercritical CO2 extract	B16	31.21	[57]
Ophioparma ventosa	Ophioparmin, haemoventosin	B16	>10, 2.4#	[120]
Stereocaulon ramulosum	β-GalCer-lich	B16	NA	[124]
Lichina confinis	Cyaneodimycin	B16 and HaCaT	27, 47#	[145]
Lobariella pallida, Stereocaulon strictum	Methanol extract	HaCaT	>67, >68	[117]

Ophioparma ventosa	Ophioparmin, haemoventosin	HaCaT	>10, 14.2#	[120]
Parmelia nepalensis Tayl., Parmelia tinctorum Nyl.	Gyrophoric acid, usnic acid, diffractaic acid	HaCaT	1.7, 2.1, 2.6 #	[158]
Bacidia stipata, Ochrolechia deceptionis, Hypogymnia lugubris	Atranorin, gyrophoric acid, physodic acid	A375	6.25-50#	[15]
Cladonia arbuscula (Wallr.). Ruoss subsp. squarrosa	Usnic acid	HTB-140 (Hs 294T)	NA	[8]
Lung Cancer				
Parmelia erumpens	2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid	A549	60.19 µg/ml	[117]
Parmotrema reticulatum	Tannic acid, catechin, purpurin, reserpine	A549	>600, 65.29 µg/ml	[130]
Cladonia foliacea, Cladonia subulata, Cladonia rangiferina, Cladonia furcata, Cladonia fimbriata	Acetone extracts	A549	13.58 to 70.45 µg/ml	[9]
Lagotis brevituba Maxim.	Echinacoside, lagotioside, glucopyranosyl(1–6)martynoside, plantamoside, verbascoside	A549	39.82>100 µM	[115]

Alectoria samentosa, Flavocetraria nivalis, Alectoria ochroleuca, Usnea florida	Usnic acid	A549	65.3 µM	[31]
Cetraria aculeata	Acetone extract	A549	500 µg/ml	[54]
Parmotrema tsavoense	Parmoether B	NCI-H460	36.48µM	[118]
Lobaria orientalis	Lobarientalones, lobariethers, 1,10-di-o-methylstictic acid, lecanorin, isolecanoric acid	NCI-H460	NA	[32]
Cladonia aggregata (Sw.) Nyl.	Barbatic acid	NCI-H292	12 µg/ml	[150]
Cladonia convoluta	Diethyl ether fraction	3LL	1 and 1.2 µg/ml	[11]
Parmotrema reticulatum	Tannic acid, catechin, purpurin, reserpine	WI-38	>600, 44.364 µg/ml	[130]
Liver Cancer				
Parmelia erumpens	2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid	HepG2	NA	[117]
Cetraria islandica	Methanol extract (Fumarprotocetraric acid)	HepG2	181.05 µg/ml	[64]
Vulpicida canadensis	Methanol extract (Usnic,pinastric and vulpinic acids)	HepG2	58.02 µg/ml	[64]
Parmotrema tsavoense	Parmoether B	HepG2	44.72µM	[118]

Xanthoria elegans	Water extract	HepG2	333.33 µg/ml	[33]
Lobothallia alphoplaca	Methanol extract	HepG2	333.33 µg/ml	[33]
Lobaria orientalis	Lobarientalones, lobariethers, 1,10-di-o-methylstictic acid , lecanorin, isolecanoric acid	HepG2	NA	[34]
Lagotis brevituba Maxim.	Echinacoside, lagotioside, glucopyranosyl(1–6) martynoside, plantamoside, verbascoside	HepG2	>100 µM	[115]
Usnea longissima	Usenamine A, usenamine B, isousone	HepG2	6.0−53.3 µM	[166]
Anaptychia ciliaris, Bryoria capillaris, Cetraria islandica	Acetone extract	HepG2/C3A	>200 µg/ml	[131]
Everniastrum cirrhatum	Methyl-β-orcinolcarboxylate	WRL-68	1 µg/ml	[121]

2.7.6 Cervix cancer

Caski and HeLa cervical carcinoma cell lines have been tested with extracts and lichenochemicals from various lichen species (Table 2.8). Death of cervical cancerous cells was caused by apoptosis due to proliferation inhibition of 2-hydroxy-4-methoxy 3,6 dimethylbenzoic acid, as evidenced from the cell morphology, i.e. nuclear shrinkage and chromatin condensation [119].The secondary depsidone metabolite pysodic acid from *H. physodes* markedly decreased viability and proliferation of HeLa cells [167]. Purified protolichesterinic acid had a pro-apoptotic effect, activated caspase-3, 8 and 9 and increased cytotoxicity alone and in combination with doxorubicin

after 24 hours of exposure to HeLa cells [157]. Lobaric acid and lobarstin from the antarctic lichen *Stereocaulon alpnum* demonstrated apoptosis by down-regulation of Bcl-2, upregulation of PARP, and cell cycle arrest at G2 phase [30]. Altogether, lichenochemicals show the ability to inhibit cancer cell growth and may have the implications for future development of therapeutic purposes

2.7.7 Gastric cancer

Studies have reported anticancer efficacy of lichen extracts and lichenochemicals on gastric carcinoma cell lines such as, AGS, MGC-803, AGS, and TMK1 (Table 2.8). The anti-proliferative effect of plantamoside on MGC-803 cells exhibited induction of apoptosis which was observed using annexin-V FITC/PI staining method [115]. Acetone extracts from *Endocarpon pusillum* activate apoptosis in AGS human gastric cancer cells due to deregulation of the Bcl2 protein expression and caspase activity [176]. A study demonstrated inhibition of cell movement activity and decreased levels of epithelial mesenchymal transition on tested gastric AGS cancer cells in concentrations of usnic acid [53].

2.7.8 Bone marrow cancer

Various lichen species reflected apoptosis activity against leukemia with different IC50 values (Table 2.8). K562, HL-60 and Jurkat leukemia cell lines were studied as different types of leukemia, such as erythro-leukemia, leukemia, and T cell leukemia. The anti-proliferation impact of isolated compounds from *Myelochroa aurulenta* [101] as well as induction apoptotic by usnic acid [37,54,101] were demonstrated on leukemia HL-60 cells. Anti-proliferative and cytotoxic effects related to the 5-lipoxygenase inhibitory activity were reported for protolichesterinic acid and lobaric acid [163]. On the other hand, work on the combination of doxorubicin and protolichesterinic acid did not reflect synergetic cytotoxic effects against leukemia K562. It was found that the combination of these two has no synergic effect on this cell line [157]. However, two depside components, norlobaridone and physodic acid, inhibited the M-phase phosphoprotein 1 (MPP1) in leukemia K562 cells [24]. Methanol extracts from 14 lichen species exhibited cytotoxicity against leukemia MO-91 cells, with IC50 values ranging from 10.50 to 50 µg/ml. These extracts induced normal peripheral blood mononuclear cell (PBMC) proliferation after 48 h of treatment at 25 µg/ml [127]. The glycolipid β-GalCer-lich, isolated from the lichen

Stereocaulon ramulosum, was shown to exhibit potent anticancer activity and activate invariant natural killer T cells (iNKT) when tested on bone marrow-derived dendritic cells (BMDCs) [124].

2.7.9 Kidney and brain cancer

Papillary renal cell carcinoma (PRCC) and U87MG were used as cell lines to study kidney and brain cancer, respectively (Table 2.8). Kidney and brain cancerous cells were treated with olivetoric acid, physodic acid and psoromic acid that revealed their anti-oxidative activity on these cell lines [110]. Human embryotic kidney HEK293T cells exposed to usnic acid derived from *Flavocetraria cucullata* resulted in apoptosis and decreased the levels of epithelial mesenchymal transition (EMT) and cell movement [53].

2.7.10 Bone cancer

The bone cancer cell line MG63 was studied with crude extracts from the lichens *Rocella montagnei* and *Hypogymnia physodes* with significant reduction in cancer cell proliferation due the action of lichen bioactive compounds was observed in cancer-induced Albino Wistar rats [168]. This study also suggested that the cells exposing *R. montagnei* to usnic acid might affect cell death in the G2 phase and inhibit cell proliferation and migration in the MG63 cell line, presented in Table 2.8.

2.7.11 Prostate cancer

A hexane extract from *Parmelia caperata* [11] and usnic acid from *Cladonia arbuscula* [169] were examined for their anticancer property on DU 145 prostate cancerous cells. The lichen xanthone derivatives were found to induce caspase 3-7 activity in the PC-3 cell line along with cell accumulation in G2/M and G0/G1 [170]. Sphaerophorin, pannarin and epiphorellic acid-1, isolated from *Sphaerophorus globosus*, *Psoroma* sp., and *Cornicularia epiphorella*, respectively, enhanced the caspase-3 activity and DNA fragmentation in DU-145 cells [147]. The lichen *Caloplaca pusilla* inhibited growth and reduced cell viability of PC-3 human prostate cancer cells [113].

2.7.12 Nasopharyngeal, head and neck cancer

Two isolated compounds, roccellic acid and evernic acid from the lichen *Roccella montagnei,* exhibited anticancer activity along with inhibition of the cyclic dependent kinase CDK-10 in FaDu

head and neck cancer cells [116] (Table 2.8). The bioactivity of this lichen was expressed in a significant antiproliferation effect (64% growth inhibition). The nasopharyngeal squamous cell carcinoma cell line KB was tested with barbatic acid isolated from the lichen *Cladonia aggregata*. A dose-dependent antineoplastic activity and antiproliferation, with a reported IC50 value of 6.25 μg/ml [150], and a mild synergic interaction in combination with protolichesterinic acid was observed at 20-40 μM concentrations [171].

2.7.13 Larynx cancer

Research on genotoxicity of secondary metabolites such as barbatic acid revealed changes in the genetic material of the larynx tumor cells [125]. Cytotoxic effects and inhibitory growth of larynx HEp-2 cells were reported for barbatic acid and an ether extract of the lichen *Cladonia aggregata* [150]. Lecanoric acid also produced toxic effects on larynx HEp-2 cells, with a two-fold lower IC50 value (7.2 μg/ml) than barbatic acid (15.79 μg/ml). The bioactivity of lichen/lichen-derived displays against HEp-2 tumor cell lines could lead to the development of drugs against larynx cancer cell growth, as shown in Table 2.8.

2.7.14 Neuroblastoma

The nasopharyngeal squamous cell carcinoma cell line KB was tested with barbatic acid isolated from the lichen *Cladia aggregate* showed dose dependent antineoplastic activity and antiproliferation with a reported IC50 of 6.25 μg/ml [150] and a mild synergic interaction was observed in combination with protolichesteric acid in 20-40 μM concentraions [170]. The primary study of four secondary lichen metabolites (atranorin, perlatolic acid, physodic acid and usnic acid) demonstrated an acetylcholinesterase inhibitory effect on mouse neuroblastoma (Neuro 2A). In addition, perlatolic acid enhanced the acetyl histone H3 and H4 levels [172]. These findings have encouraged further investigations into drug-like lichenochemicals on neurodegenerative diseases.

2.7.15 Ovarian cancer

Lichen derivatives such as usnic acid caused cytotoxicity in murine and human ovarian cancer cells [11]. Human ovarian carcinoma cells A2780 were treated with parietin and gyrophoric acid [37], isolated from *Xanthoria parietina* and *Umbilicaria hirsuta*, respectively, Table 2.8 .Th-β-

orcinol carboxylate was found to be particularly effective on -1 ovary cancer cells, with a IC_{50} of 0.25 µg/ml. Usnic acid, which is now commercially available, and atranorin showed programmed cell death on these cancer cells [10], whereas retigeric acid A and B exhibited cytotoxicity and growth inhibition of 3AO ovarian cells [170].

Table 2.8: Lichenochemical as potential anticancer agent on other types of cancer

Lichen	**Extract/ Lichochemical**	**Cancer type**	**Cancer cell lines**	**IC_{50} (µg/ml)**	**Ref.**
Parmelia erumpens	2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid	Cervix	Caski	2.69	[117]
Cladonia furcata, Cladonia foliacea, Cladonia rangiferina, Cladonia fimbriata, Cladonia subulata	Acetone extracts	Cervix	HeLa	11.69 to 63.68	[9]
Lobariella pallida, Stereocaulon strictum	Methanol extract	Cervix	HeLa	>67, >68	[117]
Cetraria aculeata	Acetone extract	Cervix	HeLa	200	[54]
Flavocetraria cucullata	Acetone extracts	Gastric	AGS	11.6*	[53]
Lagotis brevituba	Echinacoside, lagotioside, glucopyranosyl (1–6) martynoside, plantamoside, verbascoside	Gastric	MGC-803	37.09->100#	[115]
Endocarpon pusillum	Acetone extract	Gastric	AGS, TMK1	1.98, 6.64	[31]

Cladonia convoluta	*n*-hexane fraction	Erythro-leukemia	K562	18.6	[11]
Stereocaulon alpinum	Lobaric acid	Erythro-leukemia	K562	24.5	[163]
Cetraria islandica	Protolichesterinic acid	Erythro-leukemia	K562	8.4	[163]
Lobariella pallida, Stereocaulon strictum	Methanol extract	Erythro-leukemia	K562	>67 and >68	[117]
Lobaria scrobiculata	Usnic acid and m-scrobiculin	Leukemia	HL-60	1.7, 7.6[#]	[112]
Myelochroa aurulenta	leucotylic Acid	Leukemia	HL-60	21	[101]
Cornicularia aculeata	protolichesterinic acid,	Leukemia	K562	NA	[157]
Lichina confinis	Cyaneodimycin	Tcell leukemia	Jurkat	18.5[#]	[145]
Pseudevernia furfuracea	Olivetoric acid	Kidney, brain	PRCC, U87MG	125.71, 17.55*	[110]
Pseudevernia furfuracea	Physodic acid	Kidney, brain	PRCC, U87MG	698.19, 410.72 *	[110]
Rhizoplaca melanophthalma	Psoromic acid	Kidney, brain	PRCC, U87MG	79.4, 56.22*	[110]
Flavocetraria cucullate	Usnic acid	Darby canine kidney	MDCK	133.04	[53]
Rocella montagnei	Crude extract	Bone	MG63	100	[168]
Hypogymnia physodes	Crude extract	Bone	MG63	12.72 to 24.63	[168]

Parmotrema tinctorum, Sticta nylanderiana, Everniastrum cirrhatum, Lobaria pulmonaria, Usnea rubicunda, Usnea glabrescens, Usnea baileyi, Usnea ceratina, Usnea fulvoreagens, Usnea nipparensis, Usnea flammea, Usnea rubrotincta, Usnea pectinate, Usnea schadenbergiana	Methanol extract	Blood, Bone marrow	MO-91	10.50 to 50*	[127]
Parmelia caperata	*n*-hexane fraction	Prostate	DU145	7.9	[11]
Cladonia arbuscula	Usnic acid	Prostate	DU 145, PC-3	NA	[8]
Sphaerophorus globosus, Psoroma, sp., *Cornicularia epiphorella*	Sphaerophorin, pannarin, epiphorellic acid-1	Prostate	DU-145	NA	[147]
Cladonia salzmannii	Barbatic Acid	Larynx	HEp-2	15.79	[125]
Cladia aggregata	Barbatic acid	Nasopharyngeal	KB	6.25	[150]
Parmotrema tinctorum	Lecanoric acid	Larynx	HEp-2	7.2	[173]
Cladonia kalbii	Atranorin	Neuroblastoma	SH-SY5Y	NA	[70]

Cornicularia aculeata	protolichesterinic acid	Neuroblastoma	SH-SY5Y		[157]
Everniastrum cirrhatum	Methyl-β-orcinol carboxylate	Oral	KB 403	0.04	[121]
Xanthoria parietina	Antraquinone parietin	Ovarian	A2780	NA	[10]
Umbilicaria hirsuta	Tridepside gyrophoric acid	Ovarian	A2780	NA	[10]
Everniastrum cirrhatum	Methyl-β-orcinol carboxylate	Ovarian	PA-1	0.25	[121]
Lobaria kurokawae	Retrigeric acid A Retrigeric acid B	*Ovarian*	*3AO*	61.47 27.57	[174]

2.8 Conclusion

Lichens are widespread in different climatically extreme ecosystems, from arctic to desert areas. Due to their unique ability to adapt and grow in different climate zones and environments, lichens produce diverse bioactive metabolites. Recent studies have shown that the lichen secondary metabolites have multiple biological activities of potential pharmacological interest that require detailed examination. To date, more than 1,000 lichen secondary metabolites have been described, and that number continues to grow in the search for new natural compounds with unique anticancer properties. This necessitates the development of novel, more advanced extraction methods and analytical techniques for extraction, purification, characterization and application of lichenochemicals as potential therapeutic agents for novel drug development. The principles, advantages, and limitations of commonly used conventional and newly developed non-conventional methods for exploration of lichens as a valuable source of potent cytotoxic chemicals have been systematically reviewed. The identification of bioactive lichenochemicals using basic and advanced analytical techniques, and their *in vitro* and *in vivo* traditional and novel chemotheurapetic applications to various cancer types have been described and discussed. Collated

information of various lichens, lichen-derived secondary metabolites with their antitumor activities, their IC_{50} values and mechanism of action as well as assay methods for various cancer types are presented. Lichenochemicals can induce intrinsic and extrinsic apoptosis pathways, activate caspases and inhibit viability of different cancer cells. Also, the lichenochemical metabolites can promote overexpression of tumor suppressor genes, with significant effects in cell cycle checkpoint arrest. The cytotoxicity of lichen secondary metabolites, such as usnic acid, gyrophoric acid, lecanoric acid and atranorin, to name a few, are of particular pharmacological interest that justifies their further evaluation as promising candidates for chemotherapeutic purposes. This review can serve as a source of valuable and systematically organized information on lichen species and their lichenochemicals with notable anticancer potency. The extraction methods for isolation of lichenochemicals and analytical techniques for their characterization and potential use as anticancer agents may provide useful guidelines to researchers in the field.

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Chapter 3: Research methodology

This chapter gives an outline of research methods that were followed in this study. It provides information on the materilas and supplies used in the chemical and biological experiments of this project. It is also described the instruments and tools used for data collection. This chapter also includes the full details of procedures and techniques that were applied to conduct this study. The experimental conditions and analysis are also discussed.

3.1 Materials for chemical assays

3.1.1 Buffers and reagents used in extraction, color tests, fractionation, and sample preparation

Acetone (ACS Grade, concentration 99.9%), ethyl acetate, EtOAc (ACS grade, concentration 99.9%), formic acid (ACS Grade, concentration 98%), hexane (ACS grade, concentration 99%), methanol (ACS Grade, concentration 99.9%), toluene (ACS grade 99.7%), acetic acid (ACS, concentration 99.7%), diethy ether (ACS grade, concentration 99%) and chloroform (ACS grade, concentration 99%) were purchased from Fisher Scientific. Sodium hydroxide and ferric chloride were purchased from Sigma-Aldrich. MiliQ H_2O was used in all experiments. Iodine solution, potassium iodine, sulfuric acid (ACS grade, 99.99%), glacial acetic acid (ACS grade, > 99%), and Benedict's reagent (Analytical/chromatography quality level 100%) were obtained from Sigma-Aldrich.

3.1.2 Buffer solutions for spectroscopic methods

Methanol was obtained from Sigma-Aldrich (99.8%) and used in sample preparation for Mass Spectrometry. Dimethyl sulfoxide (DMSO-d6) was purchased from Sigma-Aldrich and used as a solvent for Nuclear Magnetic Resonance (NMR).

3.2 Lichen Umbilicaria muhlenbergii species

The lichen *Umbilicaria muhlenbergii* samples were collected around Tamblyn Lake in Thunder Bay, which is located in Northwestern Ontario, shown in Figure 3.1.

Figure 3.1: The lichen *Umbilicaria muhlenbergii*

3.3 Lichen morphology and anatomy

The thallus is foliose, umbilicate (attached to the substrate with a single point), mono-phyllous (single leaf), or polyphyllous (multiple leaves), often rigid, and usually less than 6-7 cm in diameter. It is white, gray, or brown, smooth or ridged. Its medulla is white and loose to dense with single-celled green algae as photobiont part. The lower surface is white, brown, gray, or black, smooth or solid. It has thick-walled asci with brown and uniform ascosporous and fruiting body, flask-shaped, uni- or multi-chambers, with branched, septate conidiophores. (The conidia are hyaline, single ovoid, or bacilliform with 2-6 µm long in dark brown, and simple or multi-cellular, ovoid, or irregular in shape. The spores are released from lower cortex or roots, shown in Figure 3.2 [1]. The secondary metabolites are produced in either the upper cortex with atranorin or brown pigments, or in the medulla with some combination of orcinol or ß-orcinol depsides or tridepsides such as gyrophoric acid, ß-orcinol depsidones, or aliphatic acids [2]. The Latin name of the species is *Umbilicaria muhlenbergii*, with the source of Fungi. It is originated from the *Umbilicariaceae* family, and *Umbilicaria* genus [3].

This species is found in climates that vary from warm to cold areas all around the world [2,4]. The constituents in *Umbilicaria* genus are mainly gyrophoric acid with the amount of lecanoric acid, methyl gyrophoric, and hiascic acid [5–8]. It was used broadly in pharmaceutical research [2,9–11]. The anti-tumor and anti-fungal effects were assessed in the lichen *Umbilicaria* genus [12,13].

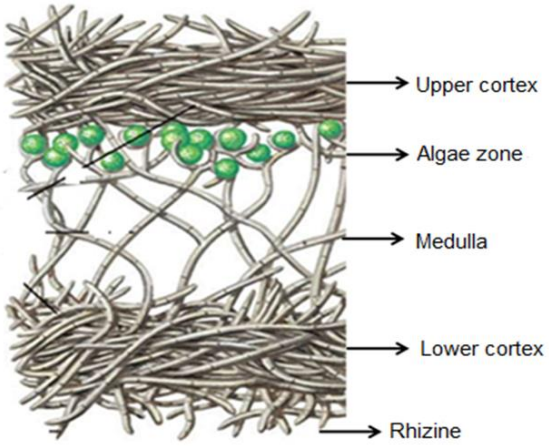

Figure 3.2: Lichen structure [1]

3.4 Preparation and extraction of lichen (*Umbilicaria muhlenbergii)*

Generally, the method for the extraction of the crude lichen extracts from the species was cold extraction and maceration [14]. As described earlier, the advantage of using maceration is to extract more materials from the original source because the thermostable components of the material are preserved under those conditions [15]. Despite a large amount of solvent needed and a longer time for Soxhlet extraction, maceration is beneficial for extracting more compounds from biomass. This method, in turn, can facilitate analysis for identifying activities of new compounds [16].

In this project, the lichen was cleaned, air-dried, and ground using mortar and pestle. The crude extract of lichen *U.muh* was produced as follows: 10 grams of dried lichen was soaked into 100 mL of acetone and placed in a shaker (Innova44, New Brunswick Scientific, USA) at room temperature for 24 hour. The extracts were then concentrated. Afterward, the extracts were filtered by glass Whatman filter NO.1 (90 mm diameter) and concentrated under reduced pressure using a rotary evaporator (BUCHI, Rotavapor, R-120) to dry completely. This process was performed using acetone as a solvent at 370 mbar for 30 ˚C [3]. The temperature was kept below 40 ˚C to minimize the degradation of thermosensitive components. The yield (14.88% w/w) was calculated using equation 3.1.

$$\text{Yield} = 100 \times \frac{\text{dry weight of the extract}}{\text{dry weight of the sample}} \qquad \text{Eq.3.1}$$

U.muh powder was stored at 4˚C for further experiments (chemical and biological).

3.5 Chromatography methods used for isolation

3.5.1 Thin-layer chromatography (TLC)

The TLC experiment was performed on silica plate (20 x 20 cm) (silica gel 60, Merck, Germany). The extract and isolated fractions were loaded and developed using solvent systems of EtOAc, MeOH, and diethyl ether and acetic acid with different ratios. The plates were then sprayed with 10% sulfuric acid reagent heated to 100˚C until spots appeared and observed under UV light at the wavelenght 365 nm.

3.5.2 Classical silica gel column chromatography

The lichen extract was isolated by silica gel column chromatography with consecutive elution. Silica gel chromatography is a very common method for the purification of natural products including lichen species based on polarity [17,18]. In this experiment, 80 g of silica gel, average pore diameter 60 °A (Sigma-Aldrich) was weighed out and the solvent hexane (1.5 times as much as the weight of silica gel) was added to the sample. The silica gel slurry was allowed to stand for 15-20 min and stirred occasionally. The column was packed carefully with no air bubbles before loading the lichen extract. After packing, 100 mg of crude extract was added to the column.
The consecutive solvents (each solvent 50 mL) were 100 vol% hexane as starting solvent, the gradient of 100% ethyl acetate, ethyl acetate: methanol (90/10 v/v), ethyl acetate: methanol (80/20 v/v), ethyl acetate: methanol (50/50 v/v), and 100 vol% methanol [12]. The fractions were collected, concentrated with a rotary evaporator (Rotavapor BUCHI, USA), and completely air dried for primary bio-screening and identification.

3.6 Structure elucidation of isolated compounds

3.6.1 Mass Spectroscopy

We pursued mass spectroscopy studies to determine the chemical composition of the fractions [19]. Initial experiments were performed using Bruker amaZon SL ion trap (Fremont, USA) by direct infusion in a negative mode. Each fraction was dissolved in 1 mL methanol (ACS, concentration 99.99%) and injected directly into the capillary tip with electrospray voltage 4500 v at the flow rate of 180 µL/h. The nitrogen gas (dry) was applied at the rate of 5.0 L/min. Ion trap gas was helium at 200˚C and 10 psi.

3.6.2 Ultraviolet spectroscopy (UV)

Most of the organic compounds and functional groups have transparency in the ultraviolet (UV) and visible regions of 190 nm to 800 nm [20,21]. A spectrophotometer can perform a wide range of end-point and kinetic applications with spectral scanning from low UV to infrared wavelengths. In this experiment, X-mark Bio-Rad microplate spectrophotometer (CA, USA) was used in the range of 200-600 nm and provided the wavelength range of the depsides in line with reported data in lichen substances [22]. The analysis revealed the presence of the aromatic group in a wavelength range for the compounds of interest.

3.6.3 Fourier transformed infrared spectroscopy (FTIR)

The FTIR is an absorption spectroscopy technique that, unlike dispersive techniques, uses a broad band infrared light source [23]. In this experiment, Bruker Tensor 37 FTIR Spectrophotometer (Fremont, USA) in mid-IR range and transmission mode was used to generate the absorbance spectra. The air-dried sample was placed in the sample compartment and measurement was performed at room temperature. Each data spectrum was acquired in 32 scans with a frequency range of 4000-600 cm^{-1}.

3.6.4. NMR Spectroscopy

The chemical structure of natural compounds can be analyzed by a nuclear magnetic resonance (NMR) [19]. Principally, the atom nuclei with the odd number of nuclides (for example, carbon 13 and hydrogen 1) have an intrinsic non-zero magnetic spin moment [21]. These nuclei, when present in an oscillating magnetic field, can absorb the energy on certain frequencies and become excited. By detecting these absorption frequencies, the isotopes present in the molecules can be identified. In this experiment, the samples were dissolved in DMSO-6 at room temperature. They were then filtered and the spectrum was recorded using Bruker NMR spectrometer (^{1}H at 500 MHz, 16 scans, ^{13}C at 125 MHz, 16 scans). All 1D and 2D NMR spectra were obtained using standard Bruker software.

3.7 Biological assays

3.7.1 Bioactivity-guided fractionation using MTT assay

The most active fraction of the lichen extract was determined using a bioassay-guided fractionation strategy [24]. Briefly, bioassay-guided fractionation was followed for the fractions eluted from silica gel chromatography and tested on MCF7 breast cancer cells. In this study, MTT assay was used as a colorimetric technique, which is described below. Afterward, the compounds were analyzed by spectrometry techniques (as described above) to identify the molecular structure of the active compound.

3.7.1.1 Cell lines

The adherent epithelial human osteosarcoma cells (U2OS), human mesenchymal stem cells (MSCs), and adherent epithelial human breast cancer cells (MCF7) were purchased from American Type Culture Collection (ATCC). Chondrogenic murine ATDC5 cells were provided by ATCC. All cells were provided by Mayo Clinic (Rochester, MN, USA).

3.7.1.2 Cell Culture Materials

Dulbecco's Modified Eagle's medium (DMEM) 1X (high glucose 4500 mg/L, 2 mM L-glutamine), Trypsin-EDTA (0.25%) phenol red and Fetal Bovine Serum (FBS), (endotoxin level< 10 EU/mL, hemoglobin level< 25 mg/dL) were obtained from Thermo Fisher Scientific. Standard cell culture flasks (T75) and multi-well plates (6, 12, 96 well) were obtained from Corning (Tewksbury, MA) company. Polymerase chain reaction (PCR) 384 well plates were purchased from Bio-Rad (Berkeley, CA, USA).

3.7.1.3 Reagents

Reagents for live/dead, MTT, and MTS assays were purchased from Promega (Madison, WI, USA). Also, 4′,6-diamidino-2-phenylindole fluorescence (DAPI) dye was provided by Mayo Clinic (Rochester, MN, USA). The 4% Bis-Tris agarose gel, protein ladder, and BCA protein assay kit were obtained from Sigma-Aldrich. Transfer buffer, running buffer, acrylamide 40%, and Western blot stripping reagent were obtained (for removing the protein-bound and re-probing the blot) from Thermo Scientific (CA, USA). Enhanced chemiluminescence (ECL) for Western buffer

was also obtained from ThermoFisher Scientific. The reagent Tween 20 (MW=1228 kDa) used for the prevention of protein-protein interaction in Western blotting, the catalyst tetramethylethylenediamine (TEMED), which was used for the polymerization of acrylamide and bis-acrylamide in gel electrophoresis, 40% acrylamide solution and 2% bis-acrylamide solution were provided by Bio-Rad (Berkeley, CA, USA). Hydrogen peroxide (H_2O_2) was obtained from (McKeen, USA). The ammonium persulphate (APS, MW=228.19, purity ≥ 98.0%) was purchased from Sigma-Aldrich.

3.7.1.4 Primary and secondary antibody supplies for Western Blotting

Two primary antibodies of p53 (SC-126, MW=53 kDa, original source: mouse, dilution ratio 1:1000) and p21 (Sc-6246, MW=21 kDa, original source: mouse, dilution ratio 1:500) were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). The antibody extracellular-signal-related kinas (Erk1/2, Sc-514302, MW=44 kDa, original source: mouse, dilution ratio 1:1000) and the antibody NF-κB (Sc-8008, MW=65 kDa, original source: mouse, dilution ratio 1:1000) were provided by Santa Cruz Biotechnology (Santa Cruz, USA). The antibody glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH, D16H11, MW=37 kDa, dilution ratio 1:5000) was purchased from Cell Signalling Technology (Danvers, MA, USA). The anti-rabbit IgG (dilution ratio 1:10000) and anti-mouse IgG (dilution ratio 1:3000) secondary antibodies were provided by Biocompare (USA).

3.7.1.5 Preparation of buffer solutions

The lysis Radioimmunoprecipitation (RIPA) buffer for protein extraction was prepared using 150 mM NaCl, 1.0% (w/v) IGEPAL CA-630. 0.5% (w/v) sodium deoxy cholate, 0.1% (w/v) sodium dodecyl sulfate(SDS) and 50 mM Tris; pH 8.0. For SDS-PAGE gels, running buffer was made using 25 mM Tris,190 mM glycine, and 0.1% SDS. Transfer buffer (Western Blotting) contained 25 mMTris, 190 mM glycine, and 20% methanol (by volume). Tris-Buffered Saline (TBS) was prepared using 150 mM NaCl and 50 mM Tris; at pH 7.6. The immunoblot blocking buffer was made of 5% (w/v) non-fat milk in tris-buffered saline (TBS) with 1% (v/v) Tween 20 surfactant.

3.7.2 Cell culture preparation

3.7.2.1 Recovery of frozen cell lines

All cell stocks were kept in a 0.5 mL aliquot of cell suspension in a medium containing 10% (v/v) concentration of DMSO at a -80 ˚C freezer or in liquid nitrogen (-196 ˚C), which is suitable for long run storage. Before the regular culturing procedure, the cells were recovered from the frozen condition and prevented from the toxic effect of DMSO. It should be noted that at a concentration of more than 4%, DMSO causes monolayer disruption. Accordingly, cell aliquots were quickly thawed in a 30˚C water bath after removal from -80 ˚C. Immediately after the ice crystal has melted, the cell suspension was transferred into a flask containing pre-warmed medium. By doing so, the containing DMSO was diluted to a lower concentration that is tolerable for the cells. The cell suspension was then incubated at a 37 ˚C incubator with 5% CO_2 for a minimum of one passage to allow the cells to recover and achieve the optimum conditions for the anti-cancer tests by MTT/MTS assay, protein extraction, and ribonucleic acid (RNA) extraction.

3.7.2.2 Cell culture procedure

Three human cell types breast cancer (MCF7), human osteosarcoma (U2OS), human mesenchymal stem cell (MSCs), and chondrogenic murine ATDC5 cells were selected for the anti-cancer test of the crude extract and active fraction. The MCF7, U2OS, and ATDC5 cells were cultured in a medium containing DMEM, high glucose, L-glutamine (500 mL), 10% (v/v) fetal bovine serum, FBS (50 mL), and the MSCs were cultured in DMEM and high glucose (4500 g/L, by adding 1% heparin). To prevent bacterial infection during cell culturing, 50 IU/mL penicillin, and 50 IU/mL streptomycin (5 mL) were added into the culture media. Additional reagents or materials needed for performing cell culture or anti-cancer test of *U.muh* crude extract and active fraction were sterilized by autoclave, Tuttnauer 2540 M, 6 gal, 120 v (Denver, USA) or filtered through a sterile 0.2 μm filter. The cells were cultured in 75 cm^2 sterile flasks and incubated at 37 ˚C, in an atmosphere of 95 % air and 5% CO_2. All procedures during cell culture were performed in class II biological safety cabinet. All safety protocols were carefully taken into account to avoid the occurrence of contamination.

3.7.2.3 The optimal condition of cell culture

The cells were monitored regularly to ensure cell viability and any sign of contaminants. The color and turbidity of media as well as cell morphology and cell density were monitored. In conditions where the cell population was too dense, the medium environment became acidic, the nutrient was depleted, and cell culture grew that resulted in cell detachment and death. Therefore, when the cell suspension had reached 70-80% confluency, the medium was changed to replace the nutrient supply and preserve the optimum conditions for cell growth.

To maintain the healthy growth of cells, the culture required regular subculture and was split into a fresh medium. Generally, the subculture of the cells was performed every three days to ensure that the cells did not overgrow. At this stage, cells were ready for further biological assays.

3.7.2.4 Sub-culturing adherent cell lines

Adherent cell lines grow by adhering to a plastic wall of the culture flask, which acts as the substrate and proliferate as a monolayer of cells. As the first step of the subculture, the old medium was removed from the flask, which was followed by the subsequent washing of the interior surface of the flask to remove the remaining medium and non-viable cells. Sterile phosphate buffer saline (PBS) was used in this step. A sterile 2 mL trypsin solution at 0.25% (w/v) concentration was added to the flask, which was then incubated in a 37 ˚C incubator for 2-3 minutes. Trypsin is a proteolytic enzyme that breaks the cell-substrate and cell-cell bonds, which yields cell detachment and creates a single-cell suspension. Subsequently, 6 mL of fresh medium was added into the flask and this eventually diluted the enzyme and stopped the proteolytic activity. At this stage, healthy cells were ready for seeding for further MTT/MTS assays, protein extraction, and RNA extraction (All the assays will be described later). Although the cell types used in this study had different rates of cell growth, as a general rule, a split ratio of 1:4 was used for routine culture of MCF7 and, due to the different rates of cell growth, the 1:10 ratio was used for U2OS, MSCs and ATDC5 cells.

3.7.2.5 Cryopreservation of cell stocks

The cell stocks were stored at -80˚C to maintain the cell viability. For adherent or monolayer cells, the cultured cells were detached by trypsinization, which was followed by the addition of fresh

media to inactivate trypsin activity. Subsequently, the adherent cell suspension was centrifuged at 1000 rpm and 4˚C for 5 minutes, then a freeze-cold medium containing 5% (v/v) DMSO was added to re-suspend the cell's pellet. DMSO acts as a cryoprotective agent, which prevents the formation of ice crystal and cell membrane breakage. The suspension of cells stock was then aliquoted in several cryovials, which were stored in a -80 ˚C freezer or transferred to liquid nitrogen tank.

3.8 MTT colorimetric assay

MTT assay is a colorimetric assay for measuring cell viability and is used as a method for cell proliferation and cytotoxicity assay [25,26]. The basic principle of this assay is based on the ability of living and metabolically active cells to reduce MTT (3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) salt to formazan product. MTT is soluble in water and, after being cleaved by the activity of mitochondrial enzymes, is converted to an insoluble crystal-form purple formazan by the cleavage of the tetrazolium ring [27]. The cells were sub-cultured at least once and subsequently reached 70-80% confluency for the MTT assay. The cell density of the single-cell suspension for adherent cells was adjusted to a final cell density of 10^4 cells/well. 100 µL cell suspension was added to each well of the 96-well microplate. The cells were then incubated at a temperature of 37 ˚C with 5% CO_2. After 24 hour incubation, cells were examined under the microscope to ensure that there were no signs of contamination and that the cells were healthy. Subsequently, the isolated lichen fractions were weighed, dissolved in 0.5% v/v DMSO, and diluted in culture media to obtain desired concentrations, then added to each well. Hydrogen peroxide (H_2O_2) was used as positive control and the cells in culture media without lichen compounds used as negative control. The plates were incubated to allow cell proliferation with or without the presence of the tested compounds. After incubation, the plates were taken out from the incubator, and 20 µl of MTT solution at a concentration of 5 mg/mL was added into each well. The plates were then kept in the incubator for the 1-4 hour until the purple crystals of formazan salt were formed, and the media were removed, and subsequently 50 µL DMSO was added into each well to dissolve the formazan crystal. The wells were then shaken for 10 min on the shaker, and the absorbance of the formazan solution in the microplate reader (Bio-Rad xMARK, USA) was measured at the wavelength of 490 nm and reference wavelength at 650 nm. The results were expressed as the value of optical density generated from 490 nm subtracted from 650 nm, which showed the amount of the purple formazan crystal formation after the elimination of the

background reading generated from the DMSO. The cell viability was calculated using equation 3.2 [22] and plotted on a graph.

$$\%\ \text{cell viability} = 100\frac{(\text{absorbance of treated sample} - \text{absorbance of blank})}{(\text{absorbance of the control} - \text{absorbance of blank})} \quad \text{Eq.3.2}$$

3.9 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a cytoplasmic enzyme involving in the conversion of pyruvate to lactate with a reduction of Nicotinamide Adenosine Dinucleotide ^{+}H (NADH) to Nicotinamide Adenosine Dinucleotide $(NAD)^{+}$ in glycolysis, which is reversible in the Krebs cycle in mitochondria. Therefore, increasing LDH is a sign of malignancy in cancer cells and can be a good target in chemotherapy. One of the common cell cytotoxicity assays based on the enzyme activity is lactate dehydrogenase assay that determines the cytotoxicity effect of the treated cells, regardless of cell type [28]. Similar to cell viability assay, cytotoxicity assay is applied to evaluate the effect of compounds on cancer cells. The assay typically is used for the detection of membrane leakage of lactate dehydrogenase in damaged cells after exposure to test substances. This method is used as a quantitative technique for cell viability and late-stage apoptosis. This experiment was performed according to the manufacturer's instructions, (Thermo Scientific, Pierce LDH assay kit), the cells were treated with 10 µL respective concentrations (300, 325, 350, 375, 400, 450, and 500 µg/mL) of the active compound for 48 hours. The absorbance was then measured at wavelengths of 490 and 680 nm using a microplate reader and percentage of cytotoxicity was calculated as follows (Eq.3.3):

$$\text{Cytotoxicity}\ \% = 100\frac{\text{Experimental LDH release}}{\text{Maximum LDH release}} \quad \text{Eq.3.3}$$

3.10 Live-Dead Assay

The apoptosis effect of the lichen fraction was evaluated using a live-dead assay. In this assay, the cells (MCF7, U2OS, and MSCs and murine ATDC5 cells) at 80% confluency seeded in 96 well plate and treated. After an appropriate time of incubation, the medium was discarded and 50 uL of the mixed reagents of Calcein AM and Ethidium homodimer (EthD) were added to each well

including the control samples. The cells were incubated for 10 minutes at room temperature. After incubation, the absorbance of live and dead cells at 460 nm and 545 nm, respectively, were measured using TECAN microplate reader (Magellan 7, USA). Subsequently, the cells were observed under a fluorescence microscope (ZEISS, ZEN Blue, and Version 2.3).

3.11 MTS Assay

Cell viabilitywas determined by MTS assay. The crude extract and active compound, gyrophoric acid were examined on the cell lines of MCF7, MSCs, and U2OS, as well as murine ATDC5 cells and the anti-proliferation effect was determined as described below.

The cancer cells were trypsinized and seeded at the appropriate cell density of MCF7 1×10^4, U2OS 1×10^3, and MSCs 1×10^3. The ATDC5 cells were also plated at 1×10^3 cells/well. The cells were treated with the crude extract and gyrophoric acid at desired concentrations. The concentrations were prepared freshly in DMSO (less than 0.5 vol %) and further diluted with culture media. All tests were included in positive (1mM H_2O_2) and negative control (cells plus media) and culture media as background reference. The cells were incubated for 72 hours at 37 ˚C. Afterward, the media were discardedand the cells were washed with PBS. Then, 100µL of MTS solution was added to each well. The cells were incubated for 0.5-4 hours and checked for color change. The absorbance was measured at 490 nm using a microplate reader (Bio-rad, USA). The blank was subtracted, and the results were expressed as a percentage of cell viability and plotted the graphs.

3.12 Protein extraction

All cell lines were plated in 6 well plate at confluency and treated with crude extract and gyrophoric acid. DMSO was used as a control. After incubation, the cells were rinsed with PBS and lysed in RIPA buffer on ice. The protein lysates were then collected and mixed completely, centrifuged, and stored at -80 ˚C for the next step (protein quantification).

3.12.1 Protein Quantification

Protein quantification is an important and essential part to determine the protein content of the sample. Before protein detection, the proteins should be quantified for all collected cells. In this test, proteins were quantified using bicinchoninic acid assay (BCA). In this assay, peptide bonds in the protein reduce Cu^{2+} to Cu^{+} proportional to the amount of protein, then the bicinchoninic acid binds to Cu^{+} and a complex is formed, which can absorb the wavelength at 650 nm [29]. Protein

quantification was performed using BCA kit according to the manufacturer's protocol. The absorbance of the bovine serum albumin as a standard with concentrations of 0.5-4 mg/mL and 5 µL of protein lysate of each sample were measured at 490 nm using microplate spectrophotometer (Bio-Rad, USA). Microsoft Excel was used to create a standard curve from the BSA standards, and the equation of the line of best fit was used to calculate the sample protein concentrations. All sample concentrations were calculated in duplicates. These samples were made ready for the Western blotting experiment.

3.13 Western blotting

Western blotting is an important technique in molecular biology to detect the protein of interest from protein mixture. In this technique, proteins were separated by size, transferred to a membrane, targeted using primary and secondary antibodies, and finally visualized and identified [30]. In this study, the effect of the effect of crude extract and the bioactive fraction was tested using antibodies for the determination of the expression of p53 and p21 proteins, which have important roles in cancer progression. Also, the effect of the crude extract and the active fraction was examined on ATDC5 cells using specific primary antibodies NF-κB and Erk1/2.

3.13.1 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) preparation

Proteins were separated by gel electrophoresis on 11%SDS-polyacrylamide gels. First, 5 µL of loading buffer containing ß-mercaptoethanol (Sigma-Aldrich) was added to each lysate (obtained from protein extraction), deionized water was also added to provide a total of 10 µL per sample for each loading. Samples were then placed in a 95 ˚C heat block for 5 min and then on ice. The amount of 10 µL per well lysates was quick-spun, sonicated, and loaded into 11% sodium dodecyl sulfate-polyacrylamide gels. The Precision Plus protein standard (Bio-Rad) was loaded alongside the samples wells as a reference, shown in Figure 3.3. The electrophoresis apparatus was filled with a running buffer. Gels were run at 120 V for 1 hour or until the samples were migrated to near the end of the gel, as indicated by the protein standard. After finishing, the gels were transferred to the membrane. (All samples loaded in triplicate).

Figure 3.3: Assembled SDS-PAGE gel for Western blotting

3.13.2 Transfer membrane

After completion of gel electrophoresis, gels were removed from the electrophoresis assembly, their edges were cut and then dripped in 1×transfer buffer for equilibration. Polyvinylidene difluoride (PVDF) transfer membrane (GE Healthcare Life Sciences, Piscataway, NJ) was soakedfor 1-2 minutes in methanol, deionized water, and transfer buffer, respectively. The transfer cassette was assembled as per the manufacturer's instructions, Trans-Blot Turbo Transfer System (Bio-Rad, USA), and the transfer membrane was run at 2.5 A and 25 V for 7 minutes. After being run, the membranes were disassembled from the apparatus for the next step.

3.13.3 Immunoblotting and antibodies

Transfer membranes were washed with TBS-Tween 20, 3×5 min to equilibrate the blots. Then, the blots were blocked with 5% milk solution for 1 hour at room temperature while being shaken. Blocked membranes were incubated with primary antibodies in 5 % milk on a shaker overnight at 4 ˚C. The loading reference antibody glyceraldehyde 3-phosphate dehydrogenase (GAPDH), p21 and p53 primary antibodies were added to the membranes for MCF7, U2OS and MSCs cells. Also, the loading reference antibody glyceraldehyde 3-phosphate dehydrogenase (GAPDH), NF-κB and Erk1/2 antibodies were added to the membranes for ATDC5 cells. All the membranes were then washed in TBS-Tween 20, 3×5 min. Following this step, anti-rabbit from (R&D Systems, Minneapolis, MN) and anti-mouse (Pierce Antibody Products, Rockford, IL) IgG secondary

antibodies in 5% milk solution were prepared and added to the membranes on a shaker for 1 hour at room temperature. The membranes were then washed and analyzed.

3.13.4 Chemiluminescence Imaging and data analysis

Standard enhanced chemiluminescence (ECL) method was performed to detect protein-banding patterns on the immunoblots by horseradish peroxidase (HRP) substrate (ThermoFisher Scientific, USA). The blots were detected using the ChemiDoc MP imaging system (Bio-Rad, Berkeley, CA, USA), and bands on the images were normalized and statistically analyzed using Image Lab software Bio-Rad (Berkeley, CA, USA) with reference to GAPDH.

3.14 Sample preparation for RNA extraction

The cell lines of MCF7, U2OS, MSC, and ATDC5 were seeded in 12 well plate and treated with gyrophoric acid and crude extract. DMSO was used as control. After an appropriate time of incubation, the cells were rinsed with PBS, and then 500 uL of TRIzol (Invitrogen Life Technologies) was added to isolate the cells. The RNA concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) and samples were stored at -80 ºC for the next step (cDNA synthesis).

3.14.1 cDNA synthesis

cDNA was synthesized with isolated RNA samples (described above) by adding random hexamer primers and oligo-primers as a mixture for reaction 1. The mixture incubated for 5 min at 65˚C and placed on ice for 1 min. Then, the mixture for reaction 2 containing dithiothreitol (DTT), AMV Reverse Transcriptase, and 5X buffer (Promega, Madison, USA) was added, spun down, and run for 1.5 hour (according to the manufacturer's instruction). After completion of the reaction, cDNA samples were diluted with RNase free water (Fisher Scientific, USA) and stored at -20 ˚C for further step (quantification q-PCR).

3.14.2 Quantitative Polymerase Chain Reaction (q-PCR)

For analysis of gene expression levels, the RNA has to be transcribed into cDNA using a reverse transcriptase [31]. In this work, we used a two steps reaction. The RNA was first reverse transcribed into cDNA as previously described in the cDNA synthesis section. Then, an aliquot of the reverse transcription reaction mixture was used for the q-PCR experiment. The reaction

mixture contained the primers, SYBR green dye, and cDNA. The gene-specific primers were designed through GENBANK and ordered by Invitrogen Science Technologies (Carlsbad, USA). SYBR green dye (Applied Biosystems, USA) was used as a fluorescent dye, binds to cDNA, and emits light at the wavelength 490 nm [32].The reaction mixture was then added to 384 well microplate (GA, USA), each well was 10 µL, and run in a thermo-cycler real-time PCR machine CFX384 TOUCH (Bio-Rad, Hercules, CA). During the reaction, more dye binds to new synthesized DNA. When the DNA denaturing happens, the dye molecules are eventually released and fluorescence signals will have fallen. At the end of each PCR cycle, fluorescence measurement is performed to determine the amplification of each gene in comparison with the reference gene.

3.14.3 Primer design

The primer design is essential for accurate and specific q-PCR quantification. They are synthesized chemically by joining nucleotides together. In this study, two housekeeping genes of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Elongation factor 1-alpha 1(EEF1A1) as well as target genes TP53 and CDKN1A/P21 were used). Primer design was performed through GenBank with some considerations, such as the annealing temperature (58–60˚C), and base pairs (bp) length of the PCR product. Primer design must be carried out for all primers including endogenous or housekeeping genes, which are not affected by chemotherapeutic drugs or other chemicals. Forward and backward primers are needed for q-PCR reaction based on the manufacturer's design.

In real-time quantitative PCR (q-PCR) experiments, breast cancer cells (MCF7), osteosarcoma cells (U2OS), and normal diploid mesenchymal stem cells (MSCs) were treated with the crude extract and the active fraction. As mentioned, the reference genes of GAPDH and EEF1A1 were used according to the protocol, and samples were prepared for q-PCR. In this method cycle treshold (C_t), the cycle for each gene was considered and used in the calculation to reach out to the threshold.

3.14.4 Analysis of q-PCR

In this work, the levels of expression of TP53 and CDKN1A/P21 target genes were evaluated in comparison with the GAPDH and EEF1A1 reference genes, which were equally expressed in all cells. Raw data from real-time PCR was analyzed using Excel (Microsoft Version 2013, USA).

The mRNA levels were normalized with the reference genes and measured using the delta cycle threshold (Ct) method.

3.15 Statistical analysis

MTS assays, life/dead staining and western blot analyses were all performed in triplicates (n =3) and evaluated using standard statistical applications (Microsoft Excel and GraphPad Prism), $p<0.05$. Differences between groups were also assessed using Student's t-testing.

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Chapter 4: Extraction and identification

Lichens produce a large diversity of bioactive compounds with several biological effects. Approximately 1000 lichen secondary metabolites with a variety of biological activities are known so far. The secondary metabolites are classified by their biosynthetic origins and chemical structures. Dibenzofurans, depsidesand depsidones, naphthoquinones, anthraquinones, xanthones, and other specific class compounds showed promising anticancer potential against variety of cancer cell types [1,2]. One of the most studied lichen polyphenolic compounds with high biological activity, including anti-proliferative effect, are depsides and depsidones [3,4]. Studies showed anti-proliferative activity and induction of apoptosis mediated by well-known depsides atranorin and gyrophoric acid [5], protolichesterinic acid [6], olivetoric acid [7], and atranorin acids [8] on different cancer cell lines. The anticancer activity of *Umbilicaria* species was confirmed by bio-screening tests on different tumor cells [8–11]. The bioactivity of lichen *Umbilicaria muhlenbergii* extract was also assessed against breast cancer MCF7 [12,13]. Gyrophoric acid as depside secondary metabolite is a characteristic compound of the lichen genus *Umbilicaria* [14–16]. The role of anti-proliferative effect of gyrophoric acid on different cancer cells has been previousely demonstrated in several studies [5,15,17,18]. In this chapter, we mainly focused on the extraction, isolation, and chracterization of the active derived fraction of the lichen *Umbilicaria muhlenbergii* species. As preliminary identification steps, the lichen *U.muh* was collected, extracted, and subjected to microchemical color tests and phytochemicals analysis to reveal the major constitents. Then, the crude extract was subjected to silica gel column chromatography. The fractions obtained were further tested for the bioactive anticancer effects. The active and inactive fractions were also characterized based on the bioassay-screening. The data would be accompanied by a series of spectroscopy analyses, including NMR, MS, UV, and FTIR aiming to identify the compound responsible for the anticancer effect.

4.1 Extraction

Finely ground dry thalli of the lichen *U.muh* (10 g) was extracted using acetone. The sample was then filtered and concentrated using a rotary evaporator. The concentrated extract (yield of 14.88%) was kept at 4˚C for further use. The physical appearance of the lichen extract was dry green powder. Figure 4.1 shows each step of the extraction procedure.

Figure 4.1: Extraction of the lichen Umbilicaria muhlenbergii. The maceration was started with the ground lichen, which yielded a yellow-green dry extract.

4.2 Primary identification of the lichen substances

The thallus of the lichen *U.muh* and its crude extract were primarily verified with microchemical color test and phytochemical screening analysis. This simple color reaction provides a useful hint for functional groups in the lichen species [16,19]. According to Huneck (1999), changing the color by adding the reagent can be an initial sign in identifying the lichen substance due to the presence of the functional groups. In this study, the color test reaction was used for detecting the depsides class in the lichen *U.muh* [16,20].

4.2.1 Microchemical color test

The typical color test reagents were potassium hydroxide (K), sodium hypochlorite (C), and the combination of K and C. According to Huneck (1999), reagent C was used specifically for the depsides group of secondary metabolites. Any color observation was denoted by a positive sign. In the KC test, reagent K was applied and then reagent C was added. Figure 4.2 shows the results for lichen and its crude extract, respectively.

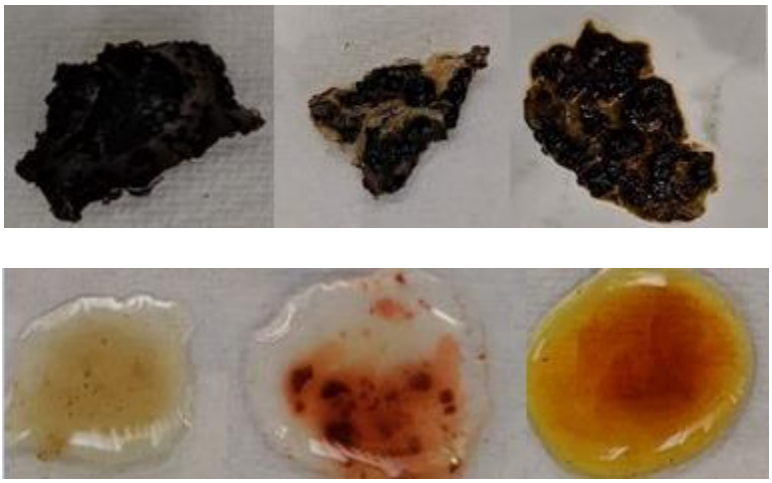

Figure 4.2: Spot test results for the lichen *Umbilicaria muhlenbergii*, from left to right: potassium hydroxide (K), sodium hypochlorite (C), the combination of K and C, Top: Color reaction for the thallus of *U.muh,* Bottom: Color reaction for lichen acetone extract

As observed in the results obtained for the thallus and crude extract, both samples showed positive color changes in response to the reagents. This can be a primary qualitative characterization of the secondary metabolite of gyrophoric acid in *U.muh* [16]. According to Huneck (1999) and Orange (2011), the orange-red color change can be related to the presence of the depsides class of metabolite in *U.muh* extract [21,22].

In the next experiment, chemical tests were carried out on the crude extact using standard procedures to identify the chemical constituents of the *U.muh* crude extract.

4.2.2 Preliminary qualitative phytochemical screening

It is found that lichen contains the different classes of phytochemicals with biological activities [23]. These metabolites contain alkaloids, terpenoids, flavonoids, and phenolic compounds [8,24]. Several studies have proven the bioactivities of phenolics and flavonoids in both natural extracts and secondary compounds such as anticancer, anti-inflammatory, and antibacterial activities [25–27]. In this experiment, phytochemical screening reagents were added, causing the change in color according to existing phytochemicals in the extract [27,28]. The results are shown in Figure 4.3.

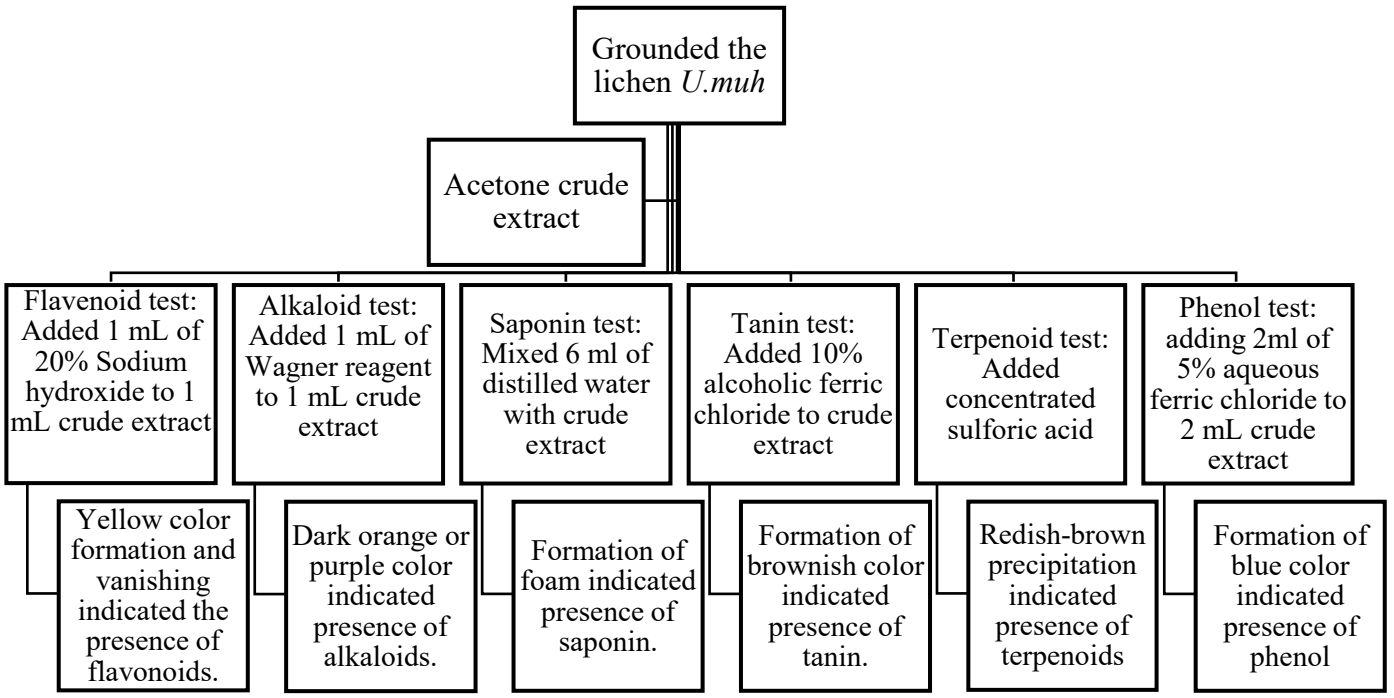

Figure 4.3: Test methods for the screening of phytochemicals in the *Umbilicaria muhlenbergii* crude extract

Phytochemical screening confirmed the presence of phyto-constituents such as alkaloids, flavonoids, terpenoids, saponin, and phenols in the *U.muh* crude extract. As presented in Table 4.1, the results revealed the crude extract exhibited higher phenolic and alkaloid contents, which is consistent with the previous findings [29–32].

Table 4.1: Major phytochemical classes in *Umbilicaria muhlenbergii*

Plant component	First experiment	Second experiment
Flavonoid	-	-
Saponin	-	-
Alkaloid	+++	++
Terpenoid	-	-
Tannin	++	+
Phenol	+++	++

+++ Highly present, ++ moderate present, + low present, - absence

4.3 Fractionation by silica gel column chromatography (SGCC)

The repeated silica gel column chromatography with polar-nonpolar gradient solvents was used as the separation process, aiming to obtain the fractions containing compounds of similar polarities or/and molecular sizes. The polarity of the solvent, which is passed through the column, affects the relative rates of the compounds passing through the column. The polar solvents are more effective on the polar molecules of a mixture and solvate the polar compounds. Consequently, a highly polar solvent will move highly polar molecules through the column [19,33]. For this purpose, the dried acetone extract (loaded 400 mg crude extract) was passed through a gravity silica gel column in ambient pressure and temperature. Completely non-polar hexane and polar methanol did not show to generate any residue during the fractionation process. The solvents system was optimized to obtain a high polar fraction as the main fraction (fraction 1), and the less polar fractions 2 and 3 were acquired while less polar solvents were passing through the column. Subsequently, the fractions were tested against MCF7 breast cancer cells for primary bio-screening (The details are described in Chapter 5). These eluents were then subjected to a rotary evaporator to remove the solvent, yielding more polar fraction 1 (15.80 mg), and less polar fractions 2 (2.05mg), and fraction 3 (1.43 mg). We further examined the fractions by the TLC experiment using the solvent diethyl ether: acetic acid and ^{1}H NMR. The fraction 1 had the Rf (0.42) value indicated high polarity, whereas fraction 2 as a trace compound showed less polarity with higher Rf. Fraction 3 did not move through the solvent in the TLC plate due to smear separation, and no spot appeared. After the initial detection and bio-screening of the obtained fractions, the bioactive compound was selected and used for further identification purposes.

As mentioned above, the fractions were submitted to bio-screening (using MTT assay); an interactive approach between the chemical fractionation and characterization [34,35]. The primary bio-screening exhibited the most bioactive fraction. Based on these results, fraction 1 was considered the main bioactive compound, and its structure would be further elucidated and confirmed. Fraction 2 was evaluated as minor compound due to less bioactivity effect; however, fraction 3 remained unknown due to lack of the Rf and insufficient residue. These preliminary results are consistent with previous findings showing potential anti cancer properties of the lichen-derived compound gyrophoric acid [36–38].

4.4 Characterization and identification of the compounds

Known compounds from previously reported data were used to identify the compounds. Isolated compounds (active and inactive) were subjected to mass spectroscopy and ^{1}H NMR analysis. Due to limited quantities of the purified compound 2, only the ^{1}H NMR data was informative. Consistent with previous studies, a complete NMR data set (^{1}H, ^{13}C, HMBC, and HSQC) was obtained for the potential active compound of gyrophoric acid. Moreover, FTIR and UV analyses were performed to disclose the main functional groups of these substances.

4.4.1 NMR and Mass spectroscopy Analysis

Compound 1 was isolated as an amorphous solid. It has UV absorbance at the λ_{max} of 210, 240, 310, 340 nm, which was corresponded to the UV absorbance of the depsides class in the lichens [7,27]. The main peak in the mass spectrum showed the deprotonated molecular ion $[M^{-1}]^{-}$ at m/z 467, suggesting the molecular formula of $C_{24}H_{20}O_{10}$ with the exact molecular mass of 468.40 g/mol [14,19,39,40], as shown in Figure 4.10. Its molecular weight was also confirmed by previous MS data [39,41]. As presented in Figure 4.4, the ^{1}H NMR spectrum of this compound revealed that signal protons attached to carbon atoms, such as methyl groups and the peaks appeared in the range of 0.8 to 2.4 ppm. Moreover, the peaks in the range of 6-8 ppm were associated with aromatics [31], supporting aromatic rings in this compound. The signals at 3-4.5 ppm indicated ester functional group or oxygenated carbon [14,42]. The peaks were observed at 2.35, 2.36, and 2.49 ppm associated with the signals of (3H, s) and methyl groups [15,19,43]. The peaks at 10.06 ppm (H, s, HO), 10.35 ppm (1H, s, HO), and 10.52 ppm (1H, s, HO) can be assigned to hydroxyl groups [15,39,43,44]. The ^{13}C NMR spectrum showed typical peaks at 19.35, 21.28, 22.12, 100.54, 107.22, 108.49, 109.84, 140.20, 156.30, 160, 161.12, and 170 ppm [15]. The solvent (DMSO-d6)

peak appeared at 40 ppm. In the ^{13}C NMR spectrum, the chemical shifts in the range of 15-40 ppm were associated with carbons in the methyl groups, as shown in Figure 4.4. The carbons' peaks for R-CO-OR and R-COOH were also observed in the range of 155-170 ppm [15,42,44]. The peaks in the range of 19.35 to 22 ppm were assigned to methyl groups at the carbon positions C-8, C-8', and C-8' in the ^{13}C NMR spectrum, shown in Figure 4.5.

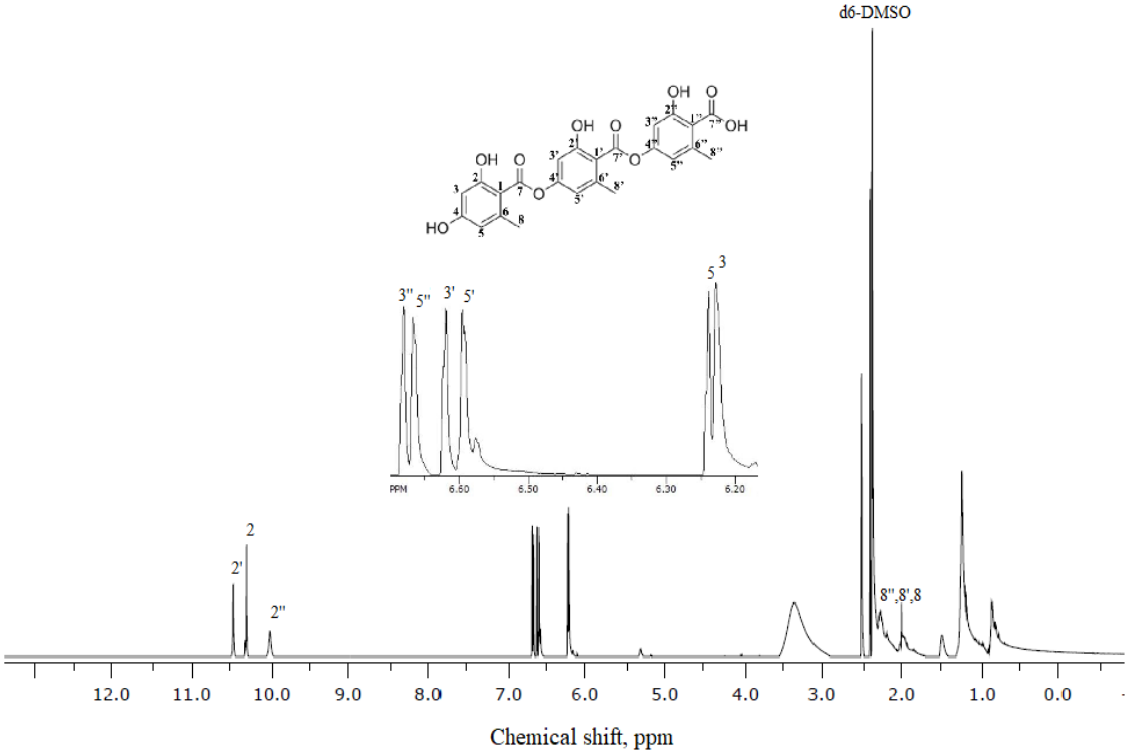

Figure 4.4: ^{1}H NMR for compound 1, 500 MHz, DMSO-d6

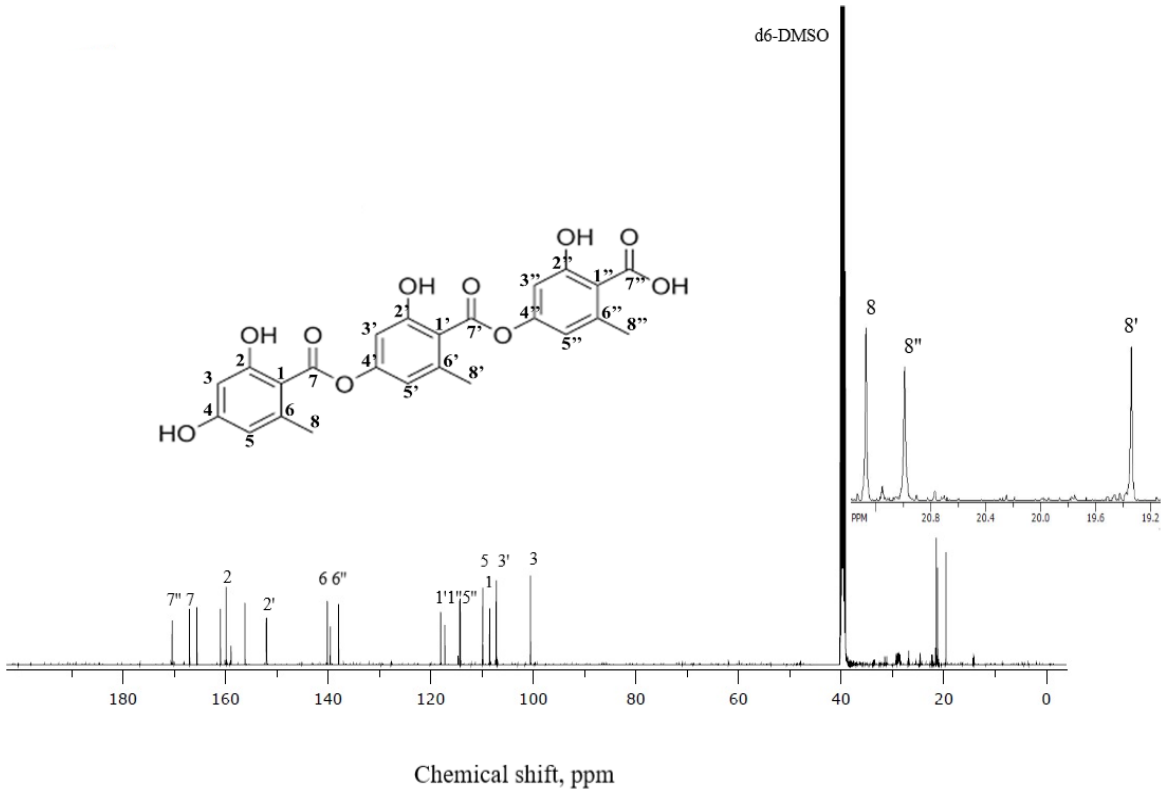

Figure 4.5: ^{13}C NMR for compound 1 in DMSO-d6

The correlations between carbon and proton signals were detected by HSQC and HMBC NMR. The HMBC spectra showed the carbon chemical shifts at the higher ppm region were correlated to the protons attached to the aromatic rings. The HMBC spectra also indicated the correlations between carbons at 19.35, 21.28, and 22.12 ppm, and the methyl group protons at the 2.35, 2.26, and 2.49 ppm, respectively [14,15,19]. The HMBC experiment also revealed a correlation between carbons at 109, 113, 114 ppm to the protons at 6.66, 6.49, and 6.69 ppm in aromatic rings [15,44], shown in Figure 4.6.

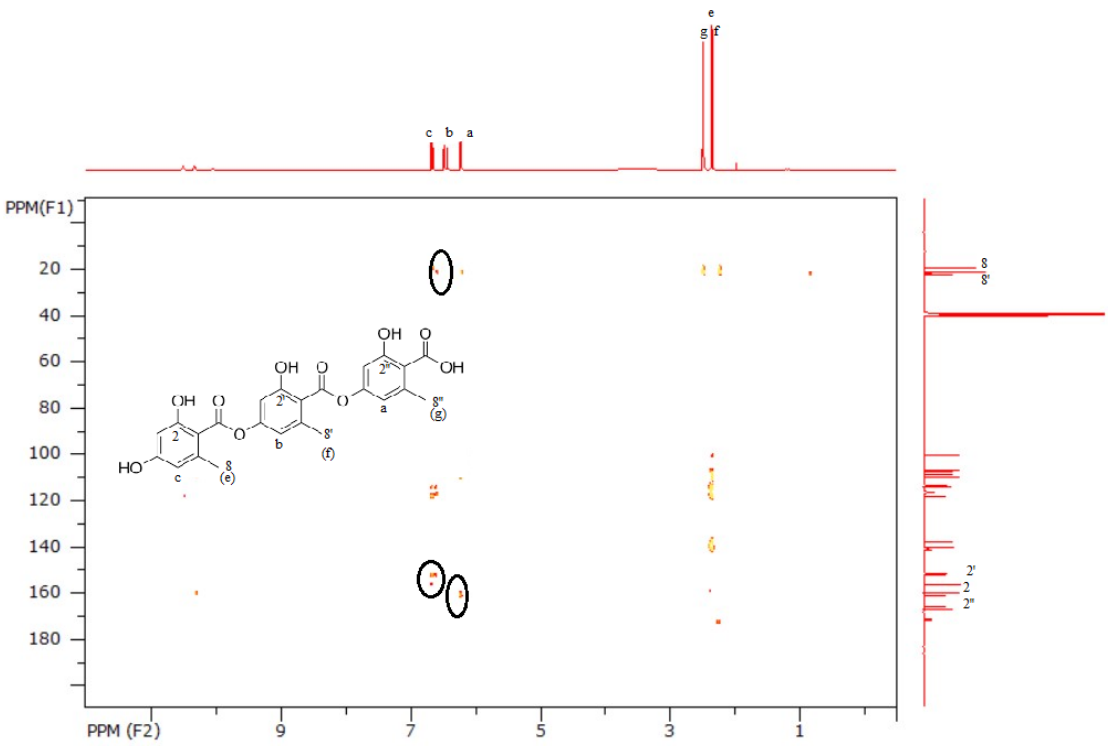

Figure 4.6: HMBC NMR spectra of compound 1

Consistent with previous findings, the HMBC correlations between carbon chemical shift at 170 ppm and protons at 6.49, 6.44 ppm can be assigned to -COOH group [14,15], Figure 4.7. The correlation signals for hydroxyl groups observed between carbons at 160, 156.30, 162.44, and 161 ppm and proton signals at 2.35, 2.36, 2.49, 6.25 ppm [15]. The HSQC spectra showed the direct correlation between carbon at 167.18 ppm and the proton signal at 6.66 ppm confirmed the aromatic ring [15,45]. The significant carbon-hydrogen correlations are shown in 2D HMBC and HSQC spectra (Figures 4.6 and 4.7).

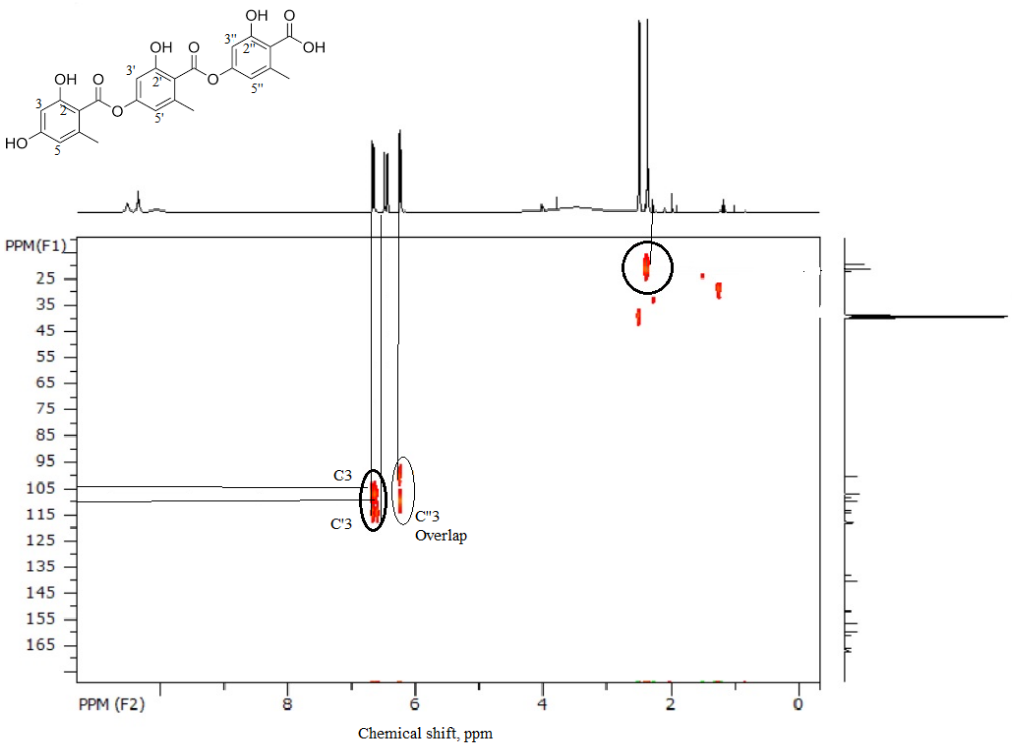

Figure 4.7: HSQC NMR spectra of compound 1

A comparison of the NMR data with previous studies confirmed the chemical structure of compound 1 as gyrophoric acid [32–35]. The proposed structure of gyrophoric acid is depicted in Figure 4.8.

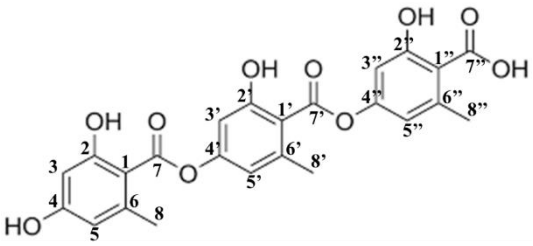

Figure 4.8: The proposed structure for compound 1 (gyrophoric acid)

Compound 2 was isolated as an amorphous powder. This compound showed less bioactivity effect against cancer cells and was considered a minor compound [14,15]. The UV absorbance was observed at λmax 240, 270, and 305 nm and confirmed as a depsides metabolite [7,40]. Ion trap mass spectroscopy showed pseudo molecular ion peak at m/z 316.9 $[M^{-1}]^-$ with the exact mass at m/z 318.40, and the molecular formula was determined as $C_{16}H_{14}O_7$ from MS data [7,27,34], as

presented in Figure 4.12. The ^{1}H NMR spectrum of this compound displayed resonances with a wide signal (region of 1.13-3.97 ppm) corresponding to methyl groups [7,41]. The proton resonances, including the aromatic protons, were detected in the range of 6.16 ppm and 6.26 ppm [7,31], as shown in Figure 4.9. We assumed the presence of the carboxylic acid in this compound and looked for a singlet in a region of 10-13 ppm. This characteristic peak was observed at 11.61 ppm, indicating a specific group in depsides class in lichens [27,33,42]. This compound can be identified as lecanoric acid, which is a known depside compound in the lichen *Umbilicaria* genus and always is accompanied with gyrophoric acid as a trace compound [14, 15].

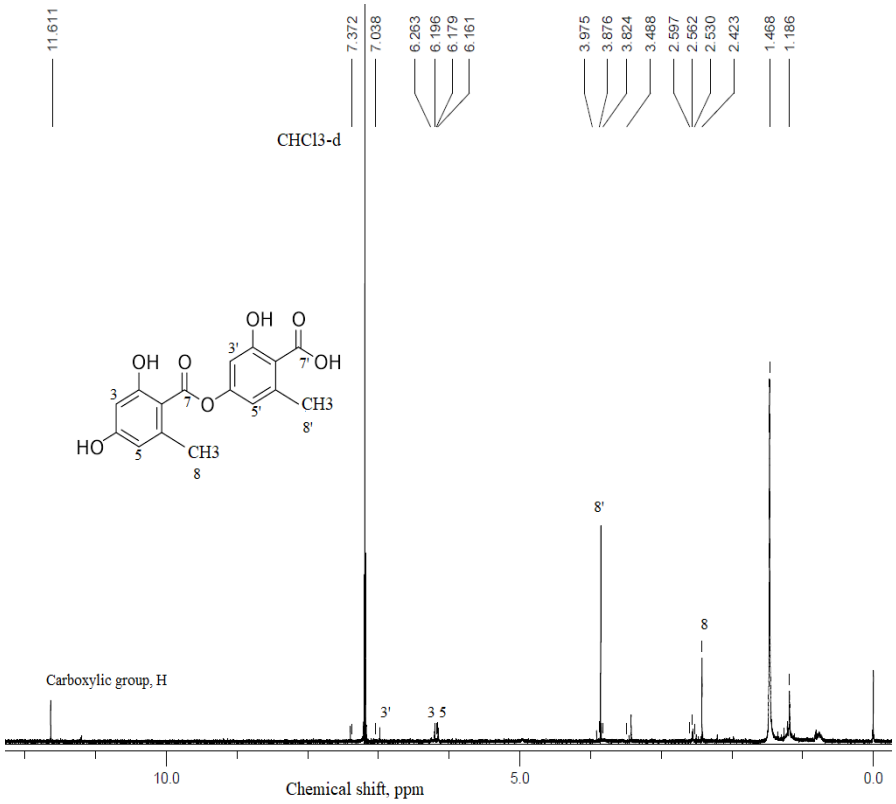

Figure 4.9: ^{1}H NMR spectrum of compound 2

The NMR results for compounds 1 and 2 have been earlier confirmed by MS data, as shown in Figures 4.10 and 4.12, repectively. As observed in MS spectrum (Figure 4.10), a negative ion signal at m/z 467.0 with a monoisotopic peak $[M^{-1}]^{-}$ was identified as gyrophoric acid. It was also produced a fragment at m/z 316.9, consistent with reported studies [41,46,47].

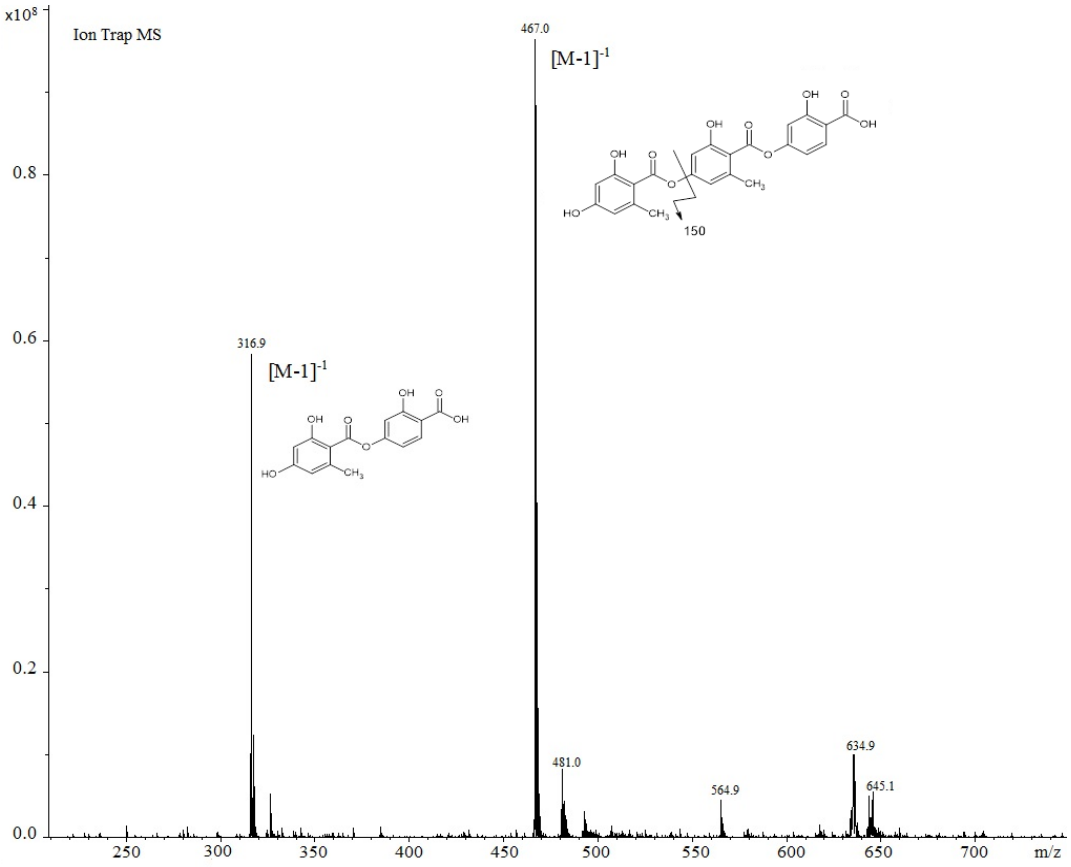

Figure 4.10: Mass spectra of compound 1

The possible fragmentation is summarized in Table 4.2. Gyrophoric acid, as a depside with two ester bonds, showed preferential ester bond cleavage to yield an ion at m/z 316.9 and verified by MS. The dissociation could be formed while the molecule is passing through the ionization chamber in the mass instrument [19,42,48]. The fragmentation pattern schematically showed in Figure 4.11, in agreement with previous studies [40,41].

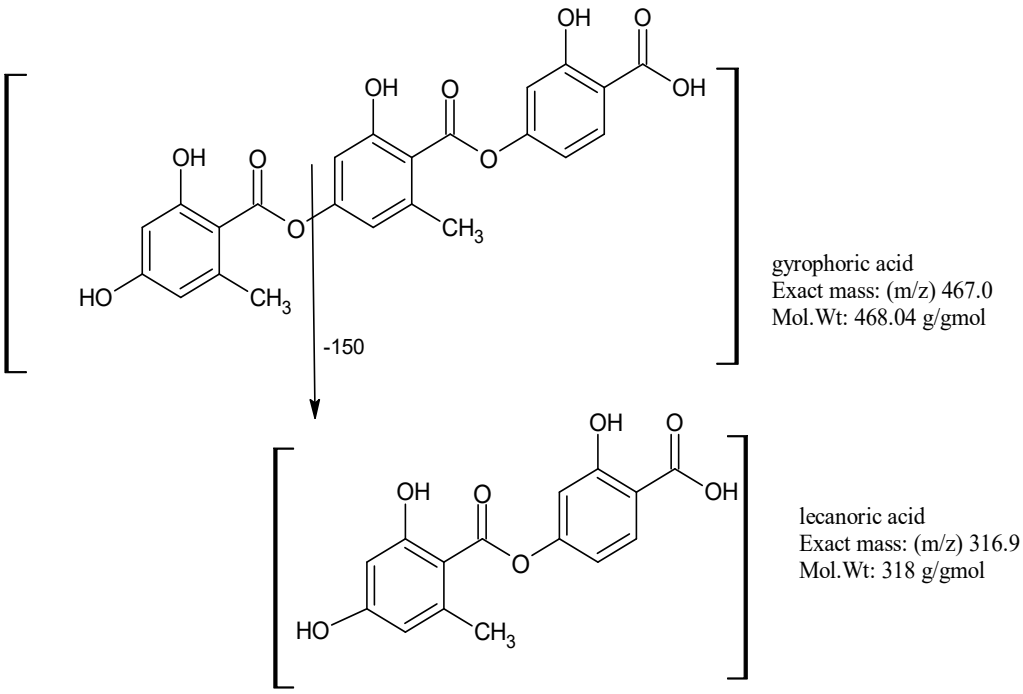

Figure 4.11: Proposed fragmentation of gyrophoric acid [7,29, 30]

Table 4.2: The observed fragment for compound 1 (gyrophoric acid) from MS data

Fragment	Accurate mass (m/z)	Exact mass (g/gmol)	Formula
Molecular ion (main fraction)	467.0	468.40	$C_{20}H_{24}O_{10}$
Fragmentation	316.9	318	$C_{16}H_{14}O_7$

Similarly, the peak with a $[M^{-1}]^-$ pseudomolecular ion at m/z 316.9 was identified as lecanoric acid [39], as shown in Figure 4.12.

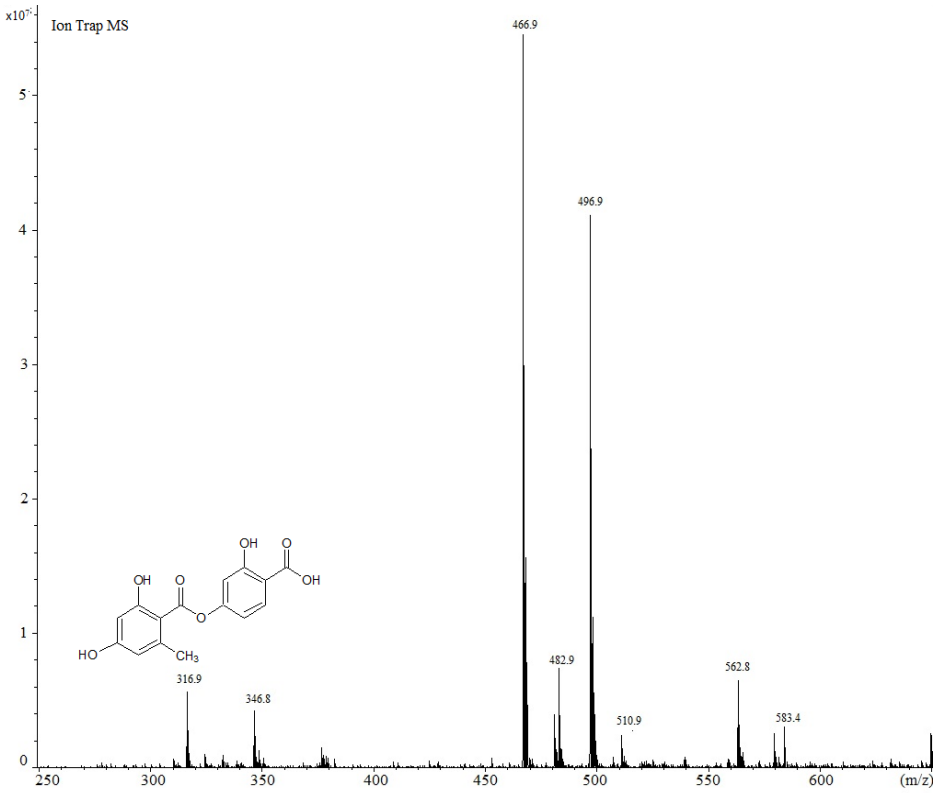

Figure 4.12: Mass spectra of compound 2

The ion mass spectrometry showed the deprotonated molecules $[M^{-1}]^-$ peaks at m/z 496.9, m/z 582.9, m/z 466.9, and m/z 316.9. A detailed analysis of the MS spectra revealed that the most intense peaks of the molecular ions were formed due to the loss of small molecules, such as H_2O, CO_2, -COOH, OCH_2, CH_3, and cleavage of the ester bond of the ions (Figure 4.13). The proposed fragmentations are presented in Table 4.3. The fragmentation patterns were similar to those reported in the lichens' depside class [7,27,29]. As gyrophoric acid and lecanoric acid belong to depside secondary metabolites, similar structures with different substitutions were observed in their molecules [20,22].

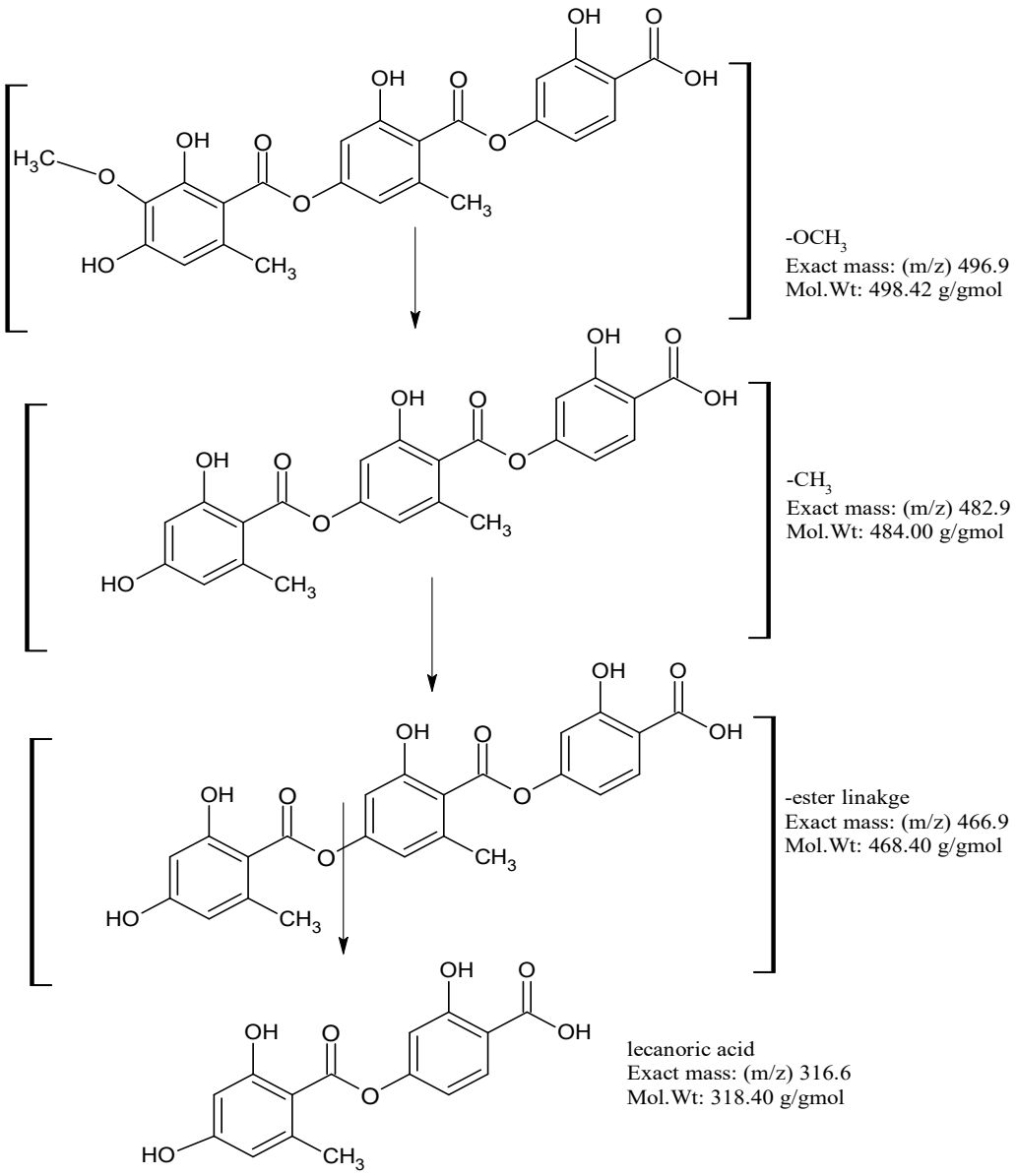

Figure 4.13: The observed molecular ions of compound 2 [46]

The observed molecular ions of MS spectra of compound 2 are presented in Table 4.3.

Table 4.3: The observed fragments of compound 2 from MS data

Fragment	Ion mass (m/z)	Exact mass (g/gmol)	Formula
Molecular ion	316.9	318.40	$C_{16}H_{14}O_7$
Fragmentation 1	482.9	484.40	$C_{20}H_{20}O_{11}$
Fragmentation 2	466.9	468.40	$C_{20}H_{20}O_{10}$

Thus from combination of NMR data with mass spectra and comparison with reported data for known compounds, the structures of these compounds could be confirmed.

4.4.2 Ultraviolet absorption

As mentioned earlier, one of the largest groups of aromatic lichen substances is depsides, which is also produced in the lichen *U.muh*. The depsides consist of orcinol and β-orcinol derivative carboxylic acids joined by an ester linkage that binds two or three rings, as shown in Figure 4.14. The orcinol depsides have a characteristic spectra of absorbance in the range of 240-330 nm. It is observed in common depside metabolites, such as gyrophoric acid and lecanoric acid [20].

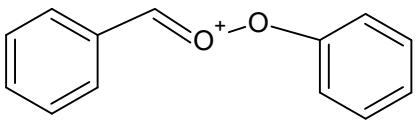

Figure 4.14: Depside

The maximum absorption occurs at about 270 and 307 nm. Any substitution on the phenyl ring, such as methoxyl in place of hydroxyl, or the alkyle substitute such as CH_3 and C_2H_5 affect the absorption pattern. As a rule, increasing the number of substituents, especially carboxyl or carbonyl on rings significantly reduce the absorption bands [49].

In our study, to confirm the depside characteristic of the compounds, the ultraviolet absorbance was recorded. The qualitative analysis of UV spectra of these compounds showed a high similarity corresponded to the β-orcinol depsides, presented in Figure 4.14. As observed, the curves of these depsides are similar, although the absorption bands are partially sharp. Both compounds, gyrophoric acid and lecanoric acid, showed absorption in the range of 211–216 nm, 234–242 nm, and 310–340 nm, summarized in Table 4.4. These absorbances are the characteristic ranges for β-orcinol depsides with carboxylic or a hydroxyl group attached to the aromatic rings [20,49]. The UV absorbance of gyrophoric acid was detected in the range of 200-340 nm in a good agreement with previous findings [19,49], indicating the conjugation effects of -OH and–CH_3 as electron-donating groups as wells as pi-bonding in this molecule [19,42,49]. Similar to gyrophoric acid, the absorbance bands were detected in lecanoric acid at 240, 270 and 305 nm, as shown in Figure 4.15. In both acids, the absorption bands at λmax= 220-250 nm confirmed the conjugated electron for R-COOH [7,37].

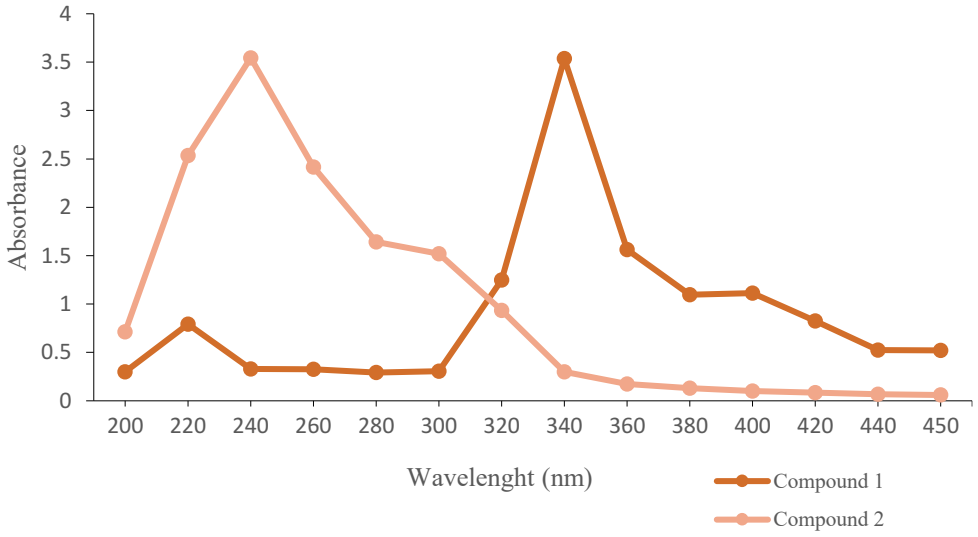

Figure 4.15: Ultraviolet spectrum of compounds 1 and 2

The UV data recorded in the present work are given in Table 4.4. It can be mentioned that the UV is somewhat sensitive to the presence of impurities in which the position of maximum and minimum getting affected.

Table 4.4: UV absorption of two depsides; compounds 1 and 2

Compound	Wavelength (nm)
Compound 1	210, 240, 310, 340
Compound 2	240, 270, 305

4.4.3 FTIR analysis

FTIR analysis was further confirmed the main structural groups of compounds 1 and 2. We have analyzed absorbance bands between wave numbers of 4000 and 600 cm^{-1} in the FTIR spectrum. The weak IR absorption around 3000 cm^{-1} suggested that the compound was likely to be unsaturated as it contained C=C or aromatic bonds [50], shown in Figure 4.16. A prominent peak in 1440-1395 cm^{-1} region appeared due to C-O bond vibration, which was assigned to the ester and carboxylic acid groups in both compounds. The bands at 670-900 cm^{-1} revealed the aromatic ring and C-H bendings [43,49]. The strong bending signals corresponding to 1580, 1200, and 1140 cm^{-1} attributed to the aromatic region [19]. The peaks at 1655 cm^{-1} (bonded C=O) and 1585-1479 cm^{-1} were assigned to OH bending [8,43]. Similar to gyrophoric acid (compound 1), the most remarkable signals in compound 2 were observable at 1300-1660 cm^{-1}, which was assigned to the aromatic region. Furthermore, the vibrational band at 1400 cm^{-1} was attributed to the CH_3 region [8,43]. The carbonyl stretch C=O of carboxylic acid appeared as an intense band in the 1760-1690 cm^{-1} region. The observed signals from 1076 to 1198 cm^{-1} illustrated the C-O stretching bands of ester [31]. Compared to available data, compound 2 can be confirmed as lecanoric acid [7,31].

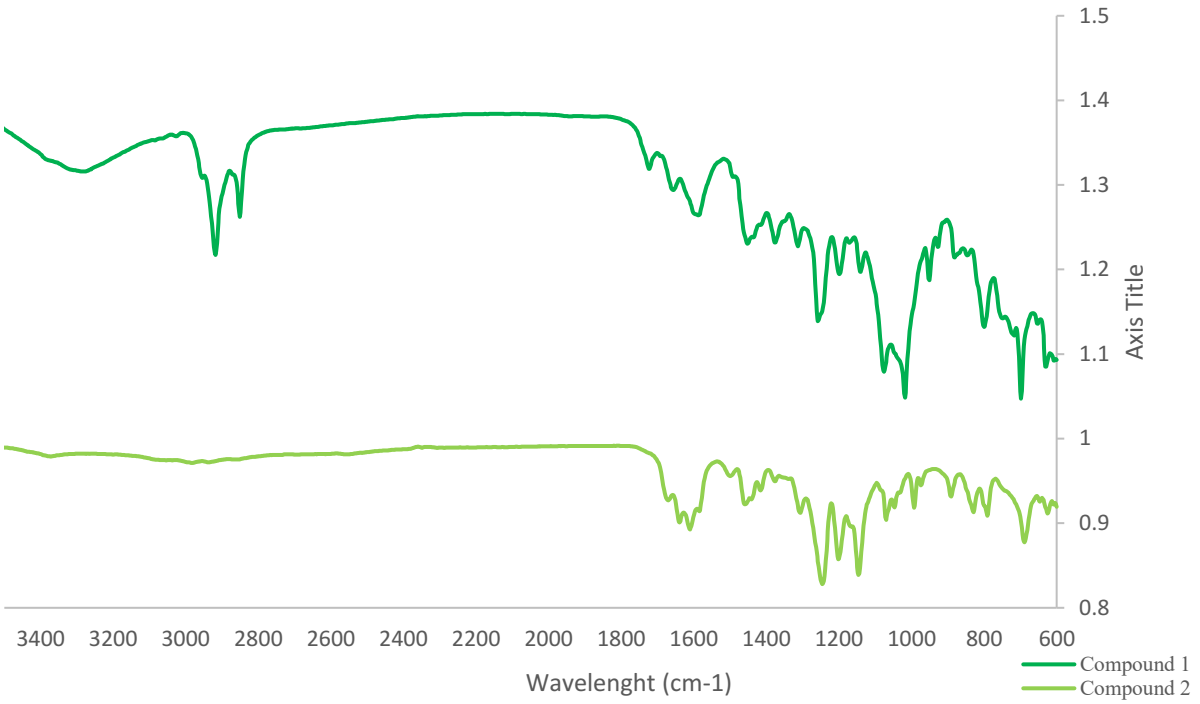

Figure 4.16: FTIR spectra of compounds 1 and 2

The assignments of major bands for compounds 1 and 2 are presented in Tables 4.5 and 4.6, respectively.

Table 4.5: Major bands and possible FTIR assignments for bioactive compound, identified as gyrophoric acid

Band (cm^{-1}) [15,19,30,32,33,46]	**Compound 1**	**Assignment**
700	685	C-H bend
740	737	aromatic, meta,
800	791	para
840	829	C-H bend
870	891	C-H bend
900	920	C-H bend
985	968	C-H bend
		C-H bend
		C-H bend
1000	1005	C=C aromatic
1050	1034	
1070	1086	
1140	1144	Ester in aromatic
1200	1202	region
1240	1244	
1310	1308	
1350	1340	
1385	1377	C=C ring
1450	1450	Aromatic C=C s
1465	1475	O=H bending
1500	1497	O=H bending
1715	1625	C=O and H bond (conjugated)
1665	1672	C=O and H bond (conjugated)
3050	2943	C-H aromatic

Table 4.6: Major bands and possible FTIR assignments for compound 2

Band (cm^{-1}) [19,45,49,50]	**Assignment**
606	C-H bend aromatic
629	C-H bend
652	C-H bend
698	C-H bend
748	C-H bend
798	C-H bend
878	C-H bend
928	C=C aromatic
951	
1018	
1076	Ester aromatic region
1136	
1198	
1246	
1313	
1375	C=C ring
1450	Aromatic C=C s
1479	O=H bending
1585	O=H bending
1655	C=O and H bond (conjugated)
1724	C=O and H bond (conjugated)
2068	C-H aromatic
2800	
3024	
3292	

*The possible assignment of the IR frequencies was performed according to Huneck (1999)

Based on the FTIR interpretation, we found clear evidence of characterization of the functional groups. The FTIR spectra revealed different vibrational bands corresponding to OH, C=O, aromatic or ester in both compounds when compared with previous studies [19,30,32,33,50].

4.5 Discussion

Natural products are produced by biosynthetic pathways and cover a wide range of organic compounds in drug discovery research due to their pharmacological or biological activities [51]. These natural molecules are found from the antarctic to tropical areas and proven to be valuable sources and diverse structures [52,53]. Among them, lichens are composite organisms consisting of fungi and green algae in symbiosis. They are known to produce a large number of secondary metabolites through a series of biosynthetic pathways [4,54]. These species are used as foods, dyes, perfumes, spices, as well as for medicininal purposes [55] and possess a wide variety of biological activities [54,56,57]. The studies demonstrated that lichens, such as *Umbilicaria* species, synthesized polyphenolic depsides and depsidone metabolites through a biosynthetic pathway with an anticancer effect [58–60]. These secondary products revealed anticancer and anti-proliferative effects against cancer cells [61].

In the current study, the lichen *Umblicaria muhlenbergii* was collected, dried, and extracted. The extraction procedure determines the quantity and quality of the crude extract obtained from the solvent [62]. We ued acetone as a solvent, which resulted in a yield of about 14.88 %, in comparison with other studies that utilized acetone (6.6%) and methanol (4.8%) [63,64]. The studies showed that the yield was high when using these polar-solvents in lichen extraction, and it was significantly changed due to the polarity of the lichen extracts [65]. The lichen species have also been studied with regard to their phytochemical constituents. The studies indicated positive results for the presence of phenols, flavonoids, alkaloids, and terpenoids using different reagents [32,66]. Among these compounds, phenols have a particular role as anticancer, antibacterial, and antibiotic agents. Moreover, the previous studies demonstrated the *Umbilicaria* genus as a rich source of phenolic compounds with anticancer activity [26,67,68]. In the present work, the phytochemical screening was carried out to compare the phytochemical content in *U.muh* crude extract. The results revealed more phenolic and flavonoid content in the crude extract.

The variation of the phytochemical content in the *U.muh* extract compared with those reported in the previous studies might be due to the different geographical locations, environmental conditionss, and temperature affecting the pathways and the extract yield [27,66]. The method of extraction might be another reason for obtaining different phytochemical contents. Lastly, different interactions between the extract and the chemical reagents might have effects on the phytochemical

contents [69]. Although, the phytochemical qualitative analysis predicted the crude extract contents and led to the primary identification of the secondary metabolites, however, phytochemical analysis cannot precisely depict the structure of chemicals.

In this project, we have further studied the isolation of *U.muh* crude extract following gravity column chromatography [68]. The metabolites may be isolated using ion-exchange chromatography (based on the charge), silica gel chromatography (polarity and solubility), or size exclusion chromatography compared with affinity chromatographyis performed based on specific binding sites of the compounds [70]. We have verified that using silica gel chromatography produced similar results to the previous findings [71,72]. The gradient of polar-nonpolar solvents was led to fractionate the phenolic compounds [36,73–75]. Although the separation of the secondary metabolites might be difficult due to their similar polarity and structures. In some cases, the structural differences can be seen in one or two methyl or hydroxyl groups or number of aromatic rings [14,45,76,77]. Anticancer activity of the fractions evaluated and one fraction exhibited induction level of MCF7 cancer cells demonstrated the presence of lichen bioactive substance. Consistent with our findings, similar result was also obtained for anticancer activity of other lichen metabolites against various cell lines [15,78]. The chemical structure of bioactive compound further elucidated using different analytical and spectroscopic techniques [79]. The ion trap mass spectroscopy in the negative mode revealed the mass characterization of the metabolites in *U.muh* species. It showed fragmentation through the breakage of ester bond [46,80]. Our findings followed previously reported studies and verified the mass formula and fragmentation pathways for gyrophoric acid as the main active compound as well as inactive compound lecanoric acid [49]. Although the fragmentation pathways of major ions weere very similar but MS data was informative. Comparing the MS fragmentation of the isolated compound with those of the known authentic samples may be sufficient to provide the structure characterization of the compounds [39,40,42]. This finding was supported by ^{1}H NMR and 2D HMBC experiments which implied protons, carbons and three methyl groups confirming the presence of gyrophoric acid. In the current study, it was also found that the isolated compounds with the same chromophore properties belong to the *Umbilicaria* genus [81]. The UV data (presented in Figure 4.15) suggested that acetone was effective in extraction of the UV-absorbing depsides compounds [49,82]. In this study, the UV results were also provided λ_{max} confirming metabolites gyrophoric acid and lecanoric acid corresponded to those reported for the lichens *Umbilicaria antarctica* and

Umbilicaria Hoffm. species [83,84]. The successful recording of FTIR spectra of obtained metabolites has been accomplished. We have already demonstrated that the selection of key features in FTIR spectra provided a suitable way of identification of the obtained substances which is comparable with reported spectral patterns. The characteristic peaks indicating ester linkage and the carbonyl were observed at the region (1780-1600 cm^{-1}) as a proof of depsides [46,85]. In line with previous studies, the FTIR spectra also demonstrated the identical signal at 1450 cm^{-1} verifying aromatic ring in both compounds [86-88].

In conclusion, we have investigated the lichen *Umbilicaria muhlenbergii*, one of the lichen varieties. The fractions were isolated from the acetone extract of *U.muh*, which one of the fractions obtained showed a profound *in vitro* growth inhibitory effect against MCF7 breast cancer cells. Moreover, we were able to identify the main bioactive compound as gyrophoric acid. The chemical data obtained will enable us to get better perspectives of this lichen-derived metabolite characteristic and contribute toward a useful anti-cancer agent entity. Therefore, as primary bio-sceening revealed that compound 1 exhibited a moderate cytotoxicity effect, making it promising for further investigation to determine its mechanism which is discussed in next chapter.

4.6 References

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Chapter 5: Evaluation of the biological activities on different cell types

Studies have shown exciting insights into the lichen species and their bioactive metabolites as natural resources with anti-proliferation and anti-tumor effects using different cell viability and cytotoxicity assays [1–7]. We also investigated the impact of the lichen *Umbilicaria muhlenbergii* crude extract and its bioactive fraction on different cancer cells. This chapter describes the bioactivity of the isolated fractions on MCF7 breast cancer cells using the cell viability MTT assay. The active fraction would be further tested for the final bioassays. We added two more cell lines, osteosarcoma cells (U2OS) and normal diploid mesenchymal stem cells (MSCs), to evaluate cell viability (using MTS assay) and apoptosis (using cell staining assay). The crude extract and active compound were also examined on murine chondrogenic cells (ATDC5). (All details are described in Chapter 3).

Our experimental approach is summarized in Figure 5.1. In the first step of the experiments, the bio-screening of the fractions was determined using MTT assay. The bioactive compound was further examined in different concentrations and prolonged treatment periods on MCF7 cells. The cytotoxicity LDH assay performed for MCF7 as an indicator of membrane breakdown [8], the lactate hydrogen peroxide released into the culture media as a sign of cell damage [9]. The fluorescence cell staining as the apoptosis indicator and cell viability MTS assays [10–12] were performed for treated MCF7, U2OS, MSCs and ATDC5 cells. Western blot analysis was conducted on human cells to determine the expression levels of p53/p21.The expression level of NF-κB and extracellular-signal-related kinase (Erk1/2) were also evaluated in murine ATDC5 cells by Western blot analysis.

Furthermore, to investigate the mRNA levels of TP53 and P21 (CDKN1A) genes, a real-time quantitative poly chain reaction (RT q-PCR) technique was used. We show some preliminary results to evaluate the anti-proliferative and apoptotic effects of the lichen *U.muh* extract and its bioactive fraction.

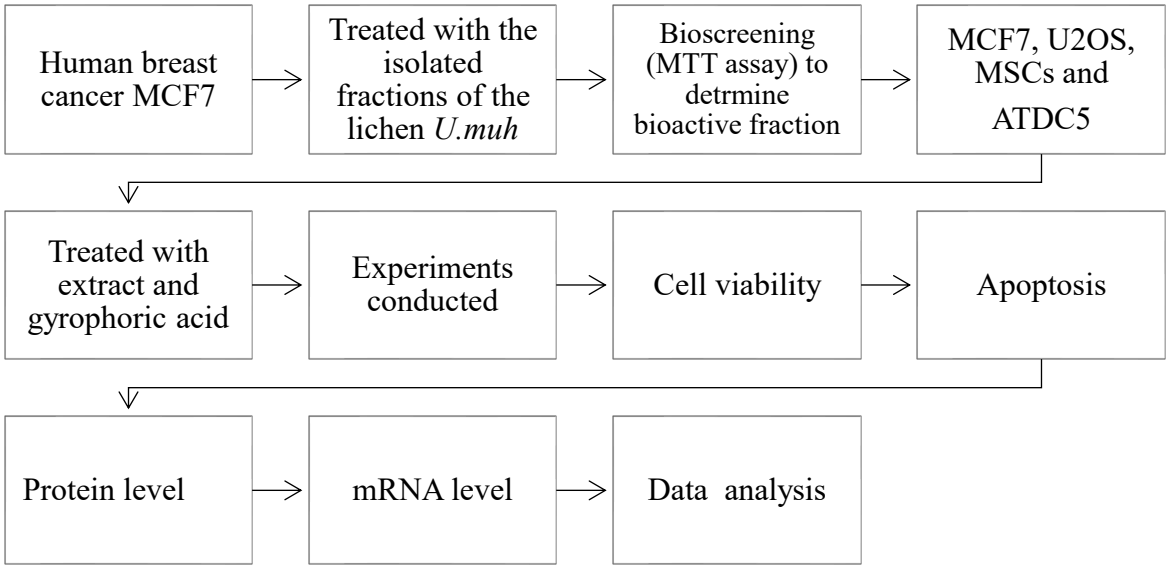


Figure 5.1: Experimental strategy of isolated fractions and bioactive substance of the lichen *Umbilicaria muhlenbergii* treatments in human breast cancer (MCF7), human osteosarcoma cells (U2OS), human MSCs, and murine chondrogenic ATDC5 cells

5.1 Bioactivity screening of the crude extract

The MCF7 cells were exposed to different concentrations of *U.muh* crude extract to determine the cell growth inhibitory effect using MTT assay. In our study, decreased in cell viability was observed in treated cells, as presented in Figure 5.2. The cell growth inhibition was prominent at higher concentrations of the crude extract.

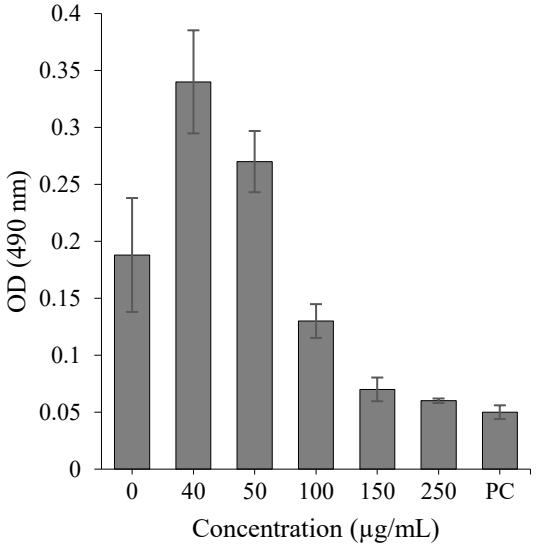


Figure 5.2: Cell viability MTT assay. MCF7 cells were tested at a range of concentrations (40, 50, 100, 150, and 250 µg/mL), PC: positive control (1mM H_2O_2). The experiment was carried out in triplicate. Data represent mean ± SD.

Figure 5.2 presents the dose response for the MTT assay employed when MCF7 cells were exposed to the crude extract (0–250 µg/mL) for 48 hours. It shows that treatd MCF7 cells were susceptible to crude extracts in a concentration-dependent manner. The above result suggests that increasing the concentration of the crude extract results in increased toxicity in MCF7. Therefore, to check the isolated fractions contain active compounds inhibiting MCF7 cell growth, we performed the MTT assay to assess toxicity of the fractions.

5.2 Bioassay fractionation using MTT assay

The anti-proliferative activity of the fractions was first assessed by MTT assay. This experiment was performed to study the cytotoxicity effect on MCF7 cells proliferation and to determine the most bioactive fraction.

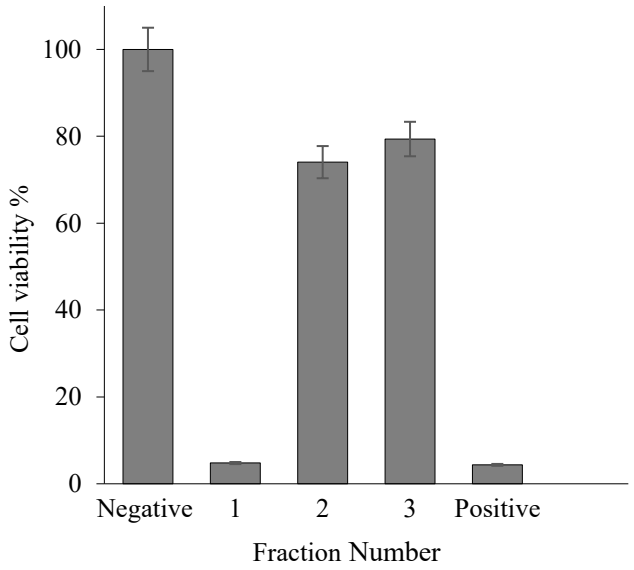


Figure 5.3: Bioassay fractionation using MTT assay for isolated fractions (1-3). The MCF7 cells were treated at a concentration 1000 µg/mL for 48 hours, positive control (1mM H_2O_2), and negative (media and cells without substances). The experiment was carried out in triplicate. Data represent mean ± SD.

The interaction between the fractions and MCF7 cells produced changes in metabolic activity. It is widely assumed that MTT is reduced by active mitochondria in living cells, while this reaction is not observed in dead cells as a response to the exposure to the fractions. A remarkable difference was observed on MCF7 after 48 hours of treatment with fraction 1. However, no significant changes were detected for other fractions compared with untreated cells shown in Figure 5.3. The reduced number of cells was probably due to the cytotoxic effect in a dose-and time-dependent manner. Therefore, this fraction was chosen for further investigations.

It should be mentioned that the spectroscopic techniques were used to identify the bioactive fraction as gyrophoric acid. (All details are described in Chapter 4).

To further obtain the insight to the role of bioactive fraction in cell growth inhibitory, we performed another anti-proliferation assay. In the next experiment, the additional MTT assay revealed cell sensitivity to the bioactive fraction as compared with the control and provided an extra view of the anti-proliferative effect of gyrophoric acid in the range of effective concentrations.

5.2.1 Anti-proliferative activity of gyrophoric acid on MCF7 cells

The MCF7 cells were exposed to increasing concentrations from 300 to 500 µg/mL of this secondary metabolite gyrophoric acid for 48 hours. The concentrations of less than 300 µg/mL was found to be not toxic on this cell line. To this end, we treated MCF7 cells with different concentrations (300-500 µg/mL) to find out the effective concentrations on MCF7 cells. The cell survival was then evaluated using the MTT assay and the results were compared with those of untreated cells (control). A reduction of viability was observed in MCF7 cells to the action of gyrophoric acid. Figure 5.4 shows this treatment induced loss of the cell viability in a concentration range for 48 hours of exposure in MCF7 cells.

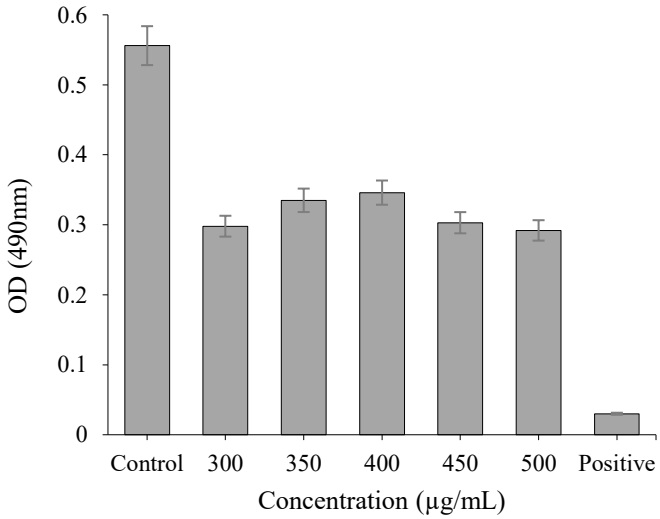


Figure 5.4: Evaluation of the anti-proliferative effect of gyrophoric acid on MCF7 cells using the MTT assay. The cells were treated at different concentrations (300-500 µg/mL) for 48 hours, positive control 1mM H_2O_2, control (media and cells without test compound). The experiment was carried out in triplicate.

This result illustrates that gyrophoric acid prohibited the stimulation in mitochondrial function leading to MCF7 cells death [13,14]. Metabolically active cells converted MTT dye to water-insoluble blue formazan by reducing the tetrazolium, while the non-viable cells were not responsive to this reaction due to cytotoxicity of the gyrophoric acid. We further evaluated/confirmed cell death in MCF7 cells induced by gyrophoric acid using lactate dehydrogenase assay (LDH) based on cell membrane damage [15].

5.3 Lactate dehydrogenase cytotoxicity assay (LDH)

The cytotoxic effect of gyrophoric acid on MCF7 cells membrane was examined by determining the percentage of LDH released into the culture medium following 48 hours of treatment. LDH is a cytosolic enzyme that is released into the extracellular space and in culture media in response to cell damage and plasma membrane breakage [8]. In this study, we assessed the membrane permeability of the MCF7 cells while treated with gyrophoric acid at concentrations (300-500 µg/mL) for 48 hours. The cell undergoes death due to exposure to gyrophoric acid, and the presence of this enzyme in the culture medium can be used as an indicator of cell death.

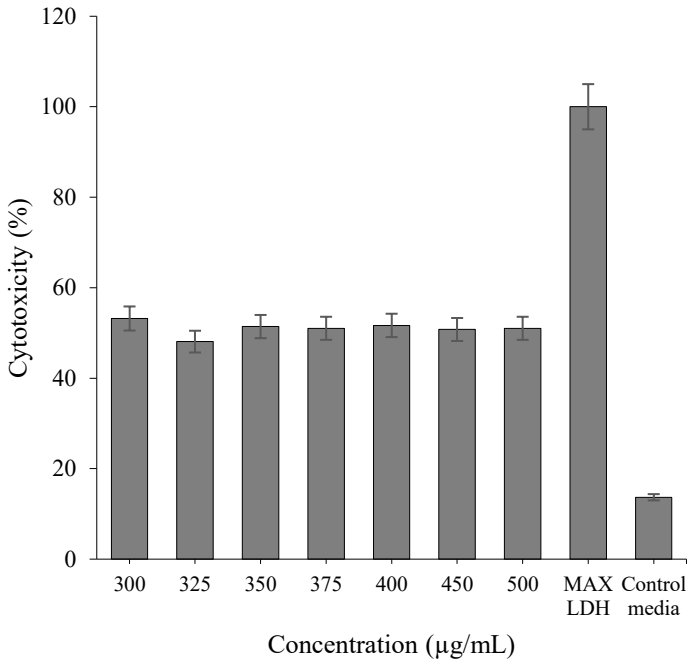


Figure 5.5: Comparison of LDH leakage assay in MCF7 cells after exposure to gyrophoric acid at concentrations (300-500 µg/mL) for 48 hours of treatment. Data presented as percentage of cytotoxicity compared with the control. The experiment was performed in triplicate.

Our result demonstrated that gyrophoric acid caused LDH released into the medium, which is consistent with previous studies [1,8,16]. Figure 5.5 shows that MCF7 cells are sensitive to gyrophoric acid as indicated by the LDH leakage assay. Compared with the control (Maximum LDH), treatment with gyrophoric acid was shown cytotoxicity effect up to 50% at the concentration range of 300 to 500 µg/mL.

We have further studied cell proliferation and apoptosis via multiple assays on different cells based on these findings. We added two other human cells, osteosarcoma cells (U2OS) and normal diploid MSCs, to evaluate the cell growth inhibitory effect. Also, to expand the understanding of bioactivity effects and to have the evidence of induction cell death, both the crude extract and gyrophoric acid were examined on these cell lines.

In the current study, in addition to human cell lines MCF7, U2OS, and MSCs, we evaluated the cytotoxicity effect of the crude extract and gyrophoric acid against the murine chondrogenic ATDC5 cells.

5.4 Cellular metabolic activity: MTS Cell viability assay

Matabolically active cells have high activity of the mitochondrial NADH-dependent dehydrogenase enzymes and these catalytic activities can be directly measured by color reaction. In the current study, we evaluated the cytotoxicity effect of the crude extract and active compound on mitochondrial enzyme activity through MTS assay. The MCF7, U2OS, MSCs, and ATDC5 cells were treated with the crude extract and gyrophoric acid at different concentrations (125, 250, 500 µg/mL). The assessment of cellular metabolic activity in MCF7, U2OS and MSCs is illustrated in Figures 5.6, 5.7, and 5.8, respectively. As observed, gyrophoric acid displayed more cell growth inhibitory effect on MCF7 and U2OS cells, while the crude extract had less activity. On the other hand, gyrophoric acid at the concentration of 500 µg/mL was more pronounced against the cell proliferation of U2OS when compared to MCF7 and MSCs after 72 hours of treatment. The results also indicated that gyrophoric acid might have suppressed cell proliferation more than the crude extract in U2OS cells at a higher concentration (500 µg/mL) Figure 5.7. In fact these cell lines showed distinct differences in sensitivity to the crude extract and gyrophoric acid treatment due to different type of the cells and interaction with the compounds.

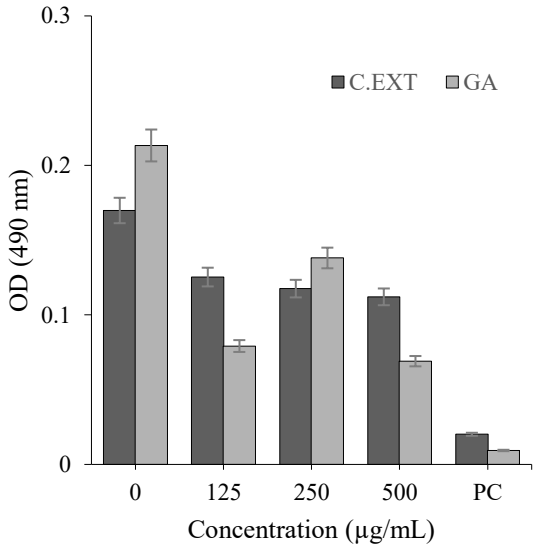


Figure 5.6: Effects of the crude extract and gyrophoric acid on cell viability on MCF7 breast cancer cells. The cells were treated with varying concentrations of the crude extract and gyrophoric acid (125, 250, 500 µg/mL) for 72 hours. PC: positive control (1mM H_2O_2), GA: gyrophoric acid, C.EXT: crude extract. The experiment was carried out in triplicate.

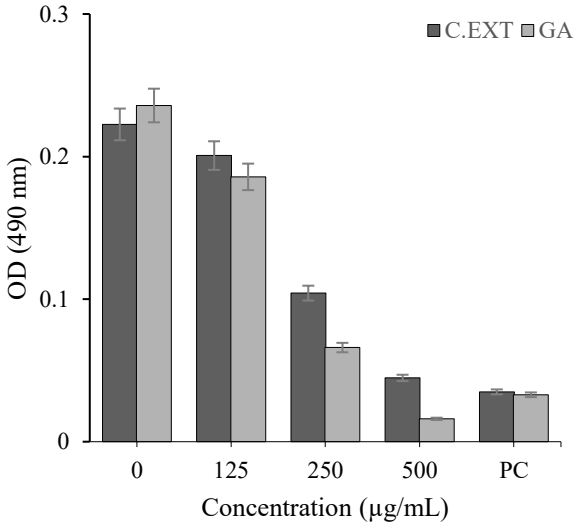


Figure 5.7: Effects of the crude extract and gyrophoric acid on cell viability on U2OS osteosarcoma cells. The cells were treated with varying concentrations of the crude extract and gyrophoric acid

(125, 250, 500 µg/mL) for 72 hours. PC: positive control (1mM H_2O_2), GA: gyrophoric acid, C.EXT: crude extract. The experiment was carried out in triplicate.

The MSCs as normal cells, showed more resistance to the crude extract and gyrophoric acid exposure, as shown in Figure 5.8. Compared to MCF7 and U2OS, the MSCs was more stable against the treatment, and no significant influence on cell viability was observed by the studied compounds even at the highest concentration.

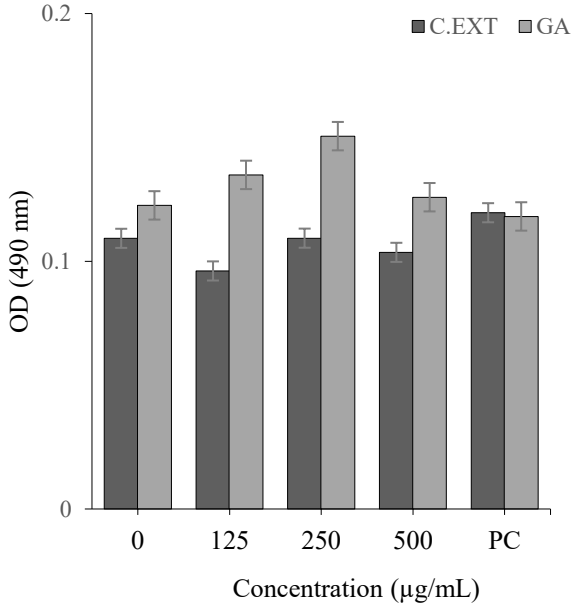


Figure 5.8: Effects of the crude extract and gyrophoric acid on cell viability on MSCs.

The cells were treated with varying concentrations of the crude extract and gyrophoric acid (125, 250, 500 µg/mL) for 72 hours. PC: positive control (1mM H_2O_2), GA: gyrophoric acid, C.EXT: crude extract. The experiment was carried out in triplicate.

The anti-proliferative effect of the crude extract and gyrophoric acid was also evaluated on murine ATDC5 cells. The cell viability remarkably decreased after 72 hours of exposure to the crude extract and gyrophoric acid at a concentration of 500 µg/mL. Interestingly, the crude extract had no significant effectat lower concentrations (125 and 250 µg/mL) compared to gyrophoric acid at

these concentrations. It revealed that gyrophoric acid treatment reduced cell viability and proliferation in ATDC5 cells. The results are shown in Figure 5.9.

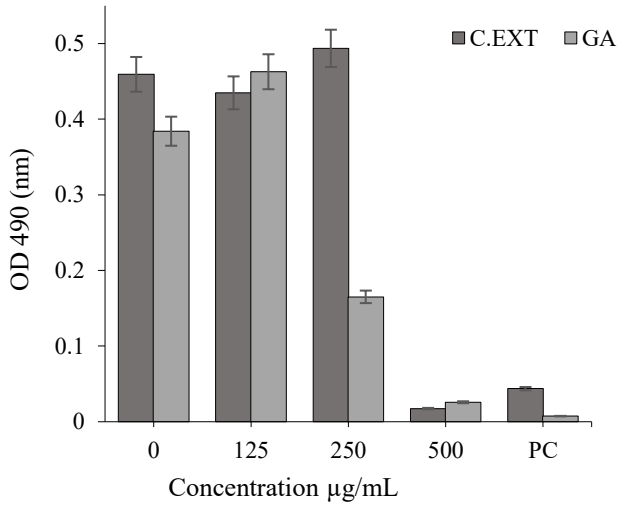


Figure 5.9: Effects of the crude extract and gyrophoric acid on cell viability on murine ATDC5 cells treated with varying concentrations of gyrophoric acid and the crude extract (125, 250, 500 µg/mL) for 72 hours. PC: positive control (1mM H_2O_2), GA: gyrophoric acid, C.EXT: the crude extract. The experiment was carried out in triplicate.

Therefore, the above results illustrate that increasing concentration results in increased toxicity and loss of viability in treated cells MCF7, U2OS and ATDC5. Generally, a significant anti-proliferative effect was found for U2OS, and gyrophoric acid reduced metabolic activity more than other studied human cell lines. To further obtain an insight to the role of the compound in apoptosis, we conducted cell staining experiment for human and murine cancer cells.

5.5 Cell staining as a confirmation of cell death

Based on MTS assay results, we evaluated the mode of cell death induced by gyrophoric acid using fluorescence dye staining. In the present study, the morphological detection of cell death was accomplished by staining treated MCF7, U2OS, and MSCs. The apoptotic effect of gyrophoric acid was also determined in ATDC5 cells. The staining with green-fluorescent Calcein-AM indicated intracellular esterase activity in live cells leading to the bright green color in alive cells.

In contrast, red-fluorescent ethidium homodimer (EthD) indicated the loss of plasma membrane integrity as a sign of dead cells [17]. These morphological features showed that gyrophoric acid caused cell growth inhibition by inducing apoptosis in MCF7 and U2OS, as shown in Figure 5.10 and 5.11, respectively. The live-cell percentage U2OS cells was also plotted and the results were compared with that of the control samples as shown in Figure 5.10B.

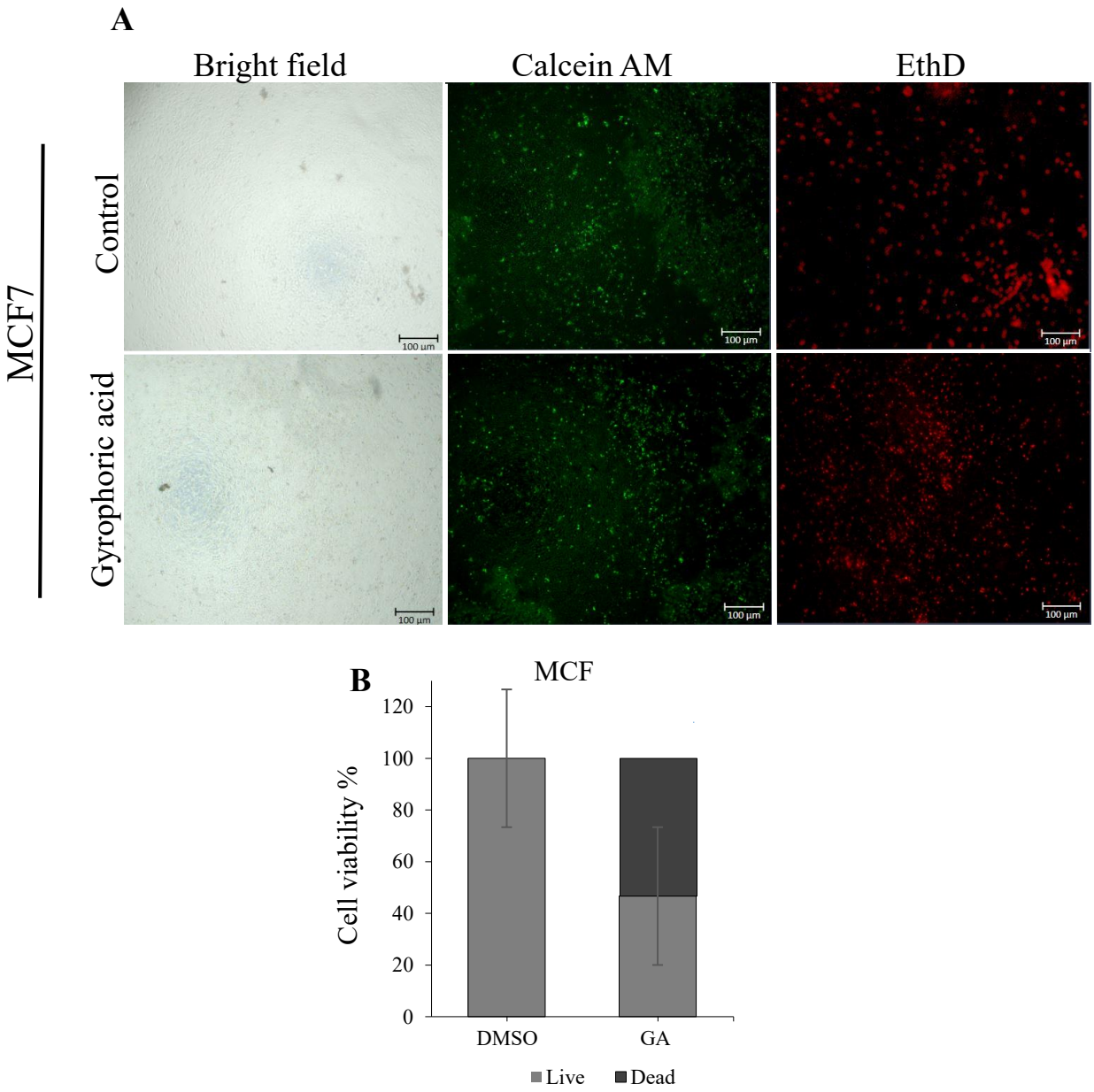
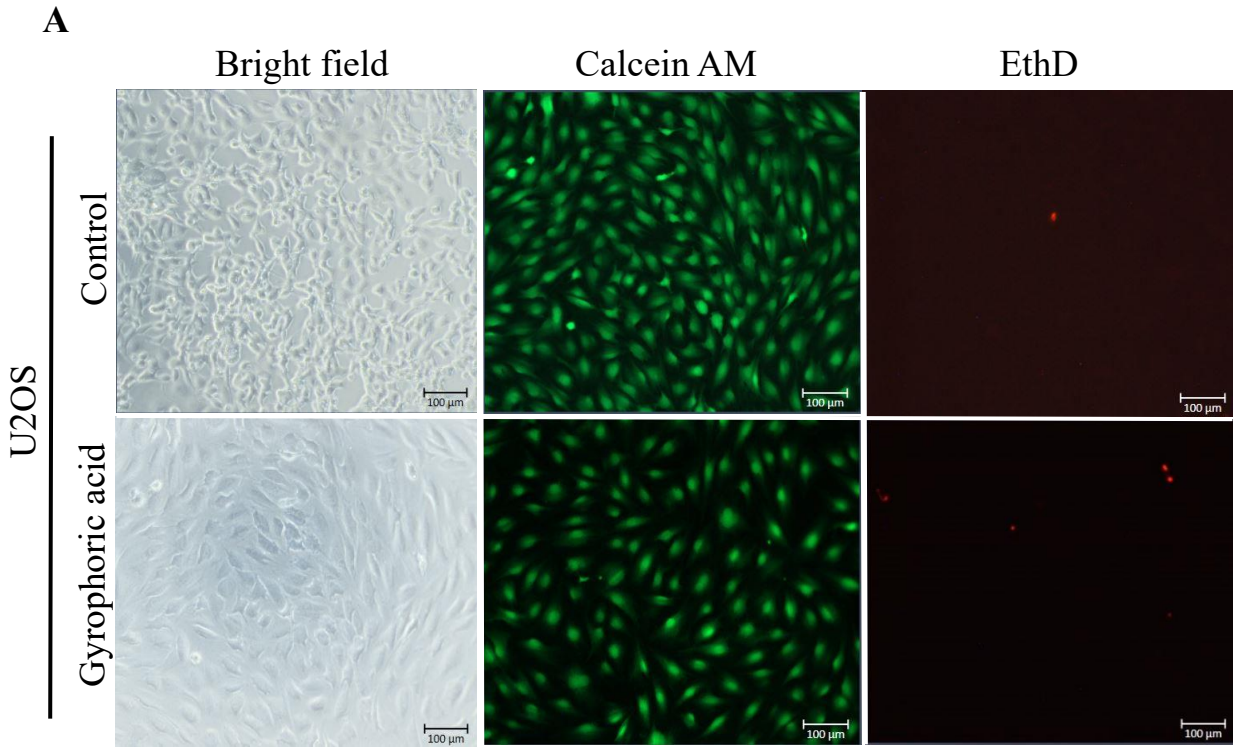


Figure 5.10: Cell viability staining assay. MCF7 breast cancer cells were treated with gyrophoric acid (700 µg/mL) for 72 hours. The stained cells in 96-well plates were examined by fluorescent microscopy imaging (A). (Zeiss, Zen Lite 3.1). The plate was then scanned with a microplate

reader to obtain quantitative data. Live/dead cell viability assay showing the comparison of live/dead cells' fluorescence intensity stained with Calcein-AM (green: live cells) and EthD (red: dead cells). The percentages of live or dead cells were calculated, and the results plotted (B). DMSO was used as control.

The morphological evaluation of U2OS by phase-contrast microscopy confirmed the apoptosis effect that previously assessed by the MTS assay, as shown in Figure 5.11A. The secondary metabolite gyrophoric acid was demonstrated an apoptotic effect on U2OS cells. The live-cell percentage U2OS cells was plotted and compared with the control as shown in Figure 5.11B.



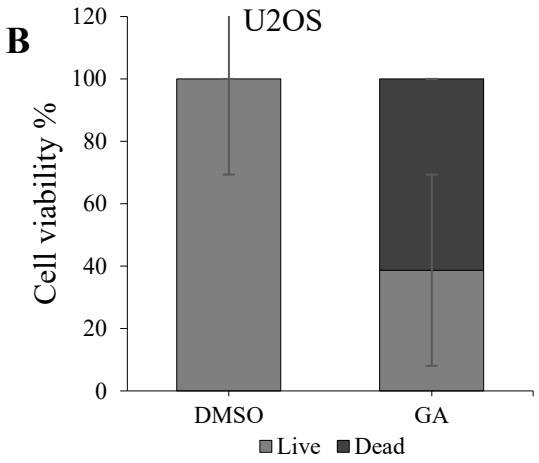


Figure 5.11: Cell viability staining assay. U2OS osteosarcoma cells were treated with gyrophoric acid (700 µg/mL) for 72 hours. The stained cells in 96-well plates were examined by fluorescent microscopy imaging (A). (Zeiss, Zen Lite 3.1). The plate was then scanned with a microplate reader to obtain quantitative data. The cell staining assay showing the comparison of live/dead cells' fluorescence intensity stained with Calcein-AM (green: live cells) and EthD (red: dead cells). The percentage of live or dead cells was calculated, and the results plotted (B). DMSO was used as control.

We tested the fluorescence staining on MSCs and the characteristics of MSCs were also compared with the control. A moderate effect was seen in MSCs as shown in Figure 5.12A. The Calcein-AM (green) and EthD (red) staining presented the live and dead cells, respectively. The percentage of live cells compared with the control shows the effect of gyrophoric acid on MSCs. The live-dead of cells following the treatment with gyrophoric acid was plotted and shown in Figure 5.12 B.

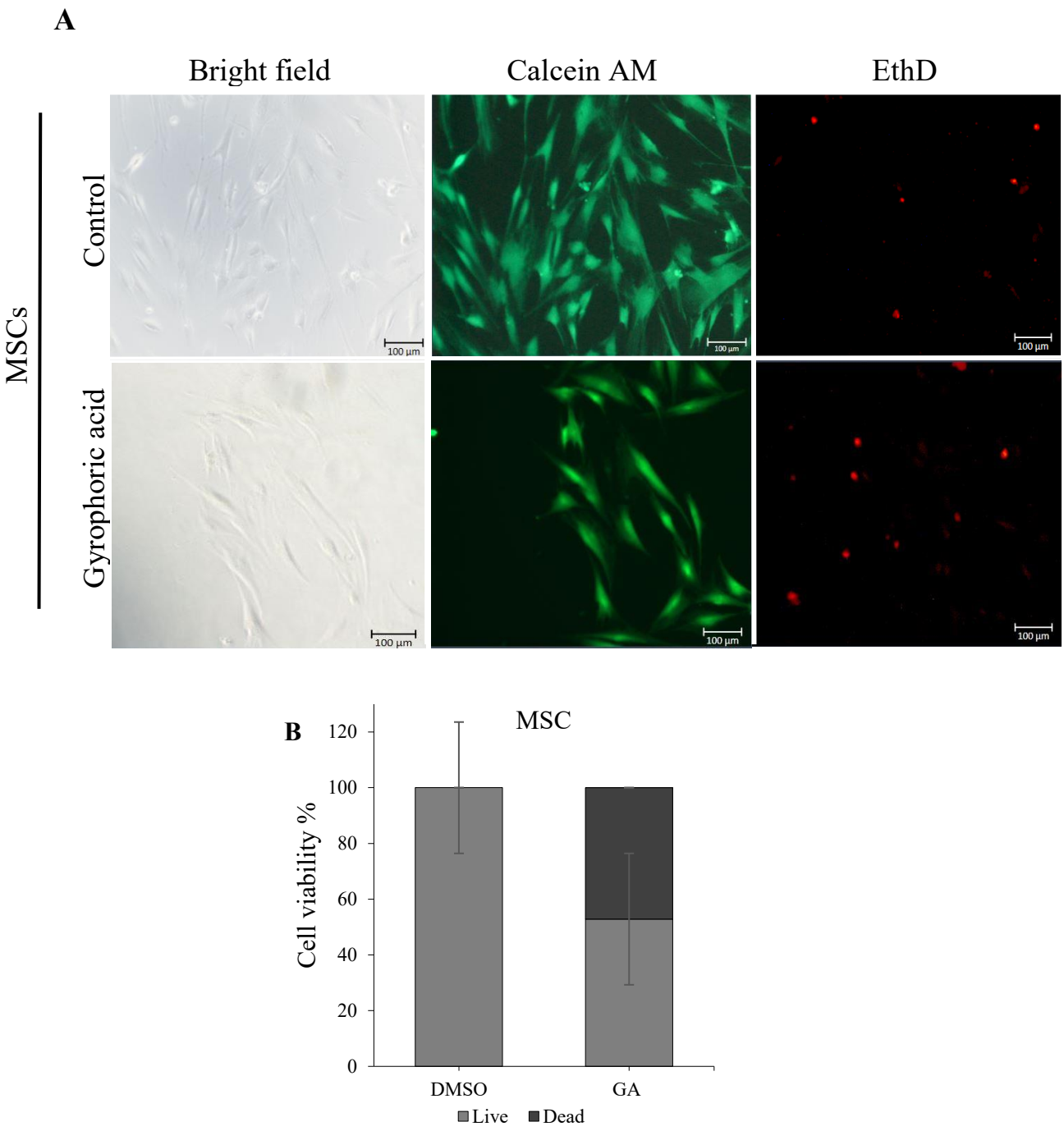
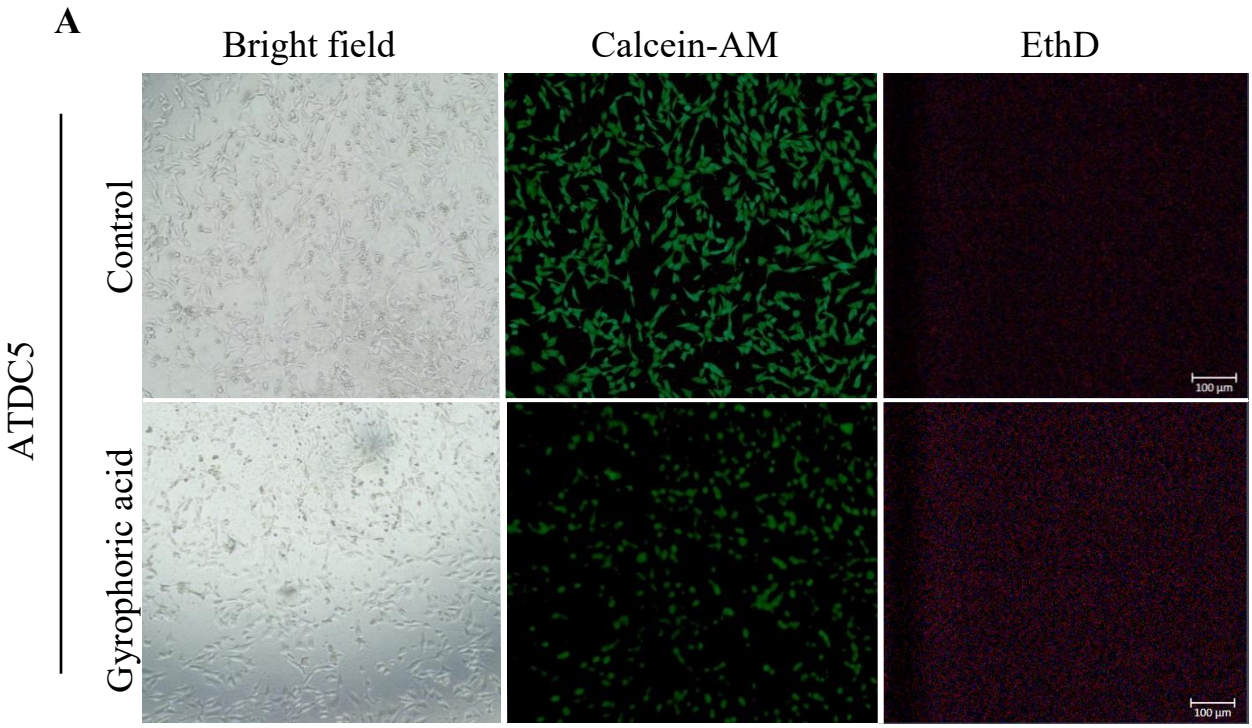


Figure 5.12: Cell viability staining assay. MSCs were treated with gyrophoric acid (700 µg/mL) for 72 hours. The stained cells in 96-well plates were examined by fluorescent microscopy imaging (top). (Zeiss, Zen Lite 3.1). The plates were then scanned with a microplate reader to obtain quantitative data. The cell staining assay showing the comparison of live/dead cells' fluorescence

intensity stained with Calcein-AM (green: live cells) and EthD (red: dead cells). The percentage of live or dead cells was calculated, and the results were plotted (B). DMSO was used as control.

Rather than evaluating the morphological changes of the treated human cell lines, we attempted to verify the growth inhibitory role of gyrophoric acid on ATDC5 cells after 72 hours of treatment. As shown in Figure 5.13 A and B, gyrophoric acid could decrease the number of live cells compared with the control. The live-cell percentage in ATDC5 cells was calculated 48.26 % after exposure to gyrophoric acid.



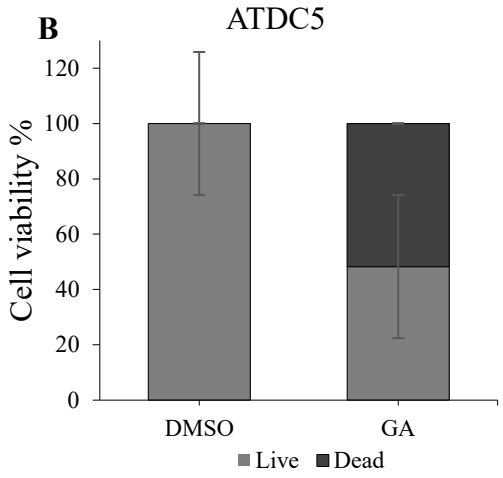


Figure 5.13: Cell viability assays using Calcein-AM/EthD staining. ATDC5 cells were treated with gyrophoric acid for 72 hours. The stained cells in 96-well plates were examined by fluorescent microscopy imaging (A). (Zeiss, Zen Lite 3.1). The plates were then scanned with a microplate reader to obtain quantitative data. The cell staining assay showing the comparison of live/dead cells' fluorescence intensity stained with Calcein-AM (green: live cells) and (red: EthD). The percentage of live or dead cells was calculated, and the results plotted (B). DMSO was used as control.

The degree of apoptosis was assessed using fluorescent dyes, and it was indicated that Calcein-AM (green dye) was able to pass cellular membranes of target cells, as demonstrated by confocal microscopy. The control cells were predominantly green without membrane changes (Figures 5.10-5.13) and indicating the cells in the control samples were alive. The assay described above provided hints to the chemosensitivity of the treated cells in response to gyrophoric acid and revealed its potential apoptosis effect. The cell viability was calculated to be 46.6% in MCF7, 38.70% in U2OS, 52.85% in MCS's, and it was observed that the viability of all cells was decreased by time of exposure to the lichen-derived metabolite. The results obtained for MCF7 were comparable with previous studies of the lichen metabolites [13,18–22]. Additionally, our finding showed the pro-apoptotic effect of gyrophoric acid on murine ATDC5 cells (48.26 %), and it may conclude that this compound can be also a successful inductor of apoptosis in ATDC5 cells.

In the next experiment, the data obtained can be confirmed by Western blot analysis. We would provide more evidence of the cytotoxicity effect of the crude extract and gyrophoric acid on MCF7,

U2OS, MSCs, and ATDC5 through Western blot analysis. The protein lysates of all these cell lines were examined with specific antibodies to show the level of expression in treated cells.

5.6 Western blot

To confirm the induction of apoptosis by lichen crude extract and gyrophoric acid, we evaluated the mechanism of the anti-proliferative effect of these two compounds on expression level of pro-apoptotic p53 and p21 (two widely used target proteins). In this experiment, we first treated MCF7, U2OS, and MSCs, and then proteins were collected for Western blot. (The details are discussed in Chapter 3). We confirmed the preliminary observation in cell cycle regulators and apoptosis using specific antibodies. The results are shown in Figures 5.14, 5.15, and 5.16 for U2OS, MCF7, and MSCs, respectively

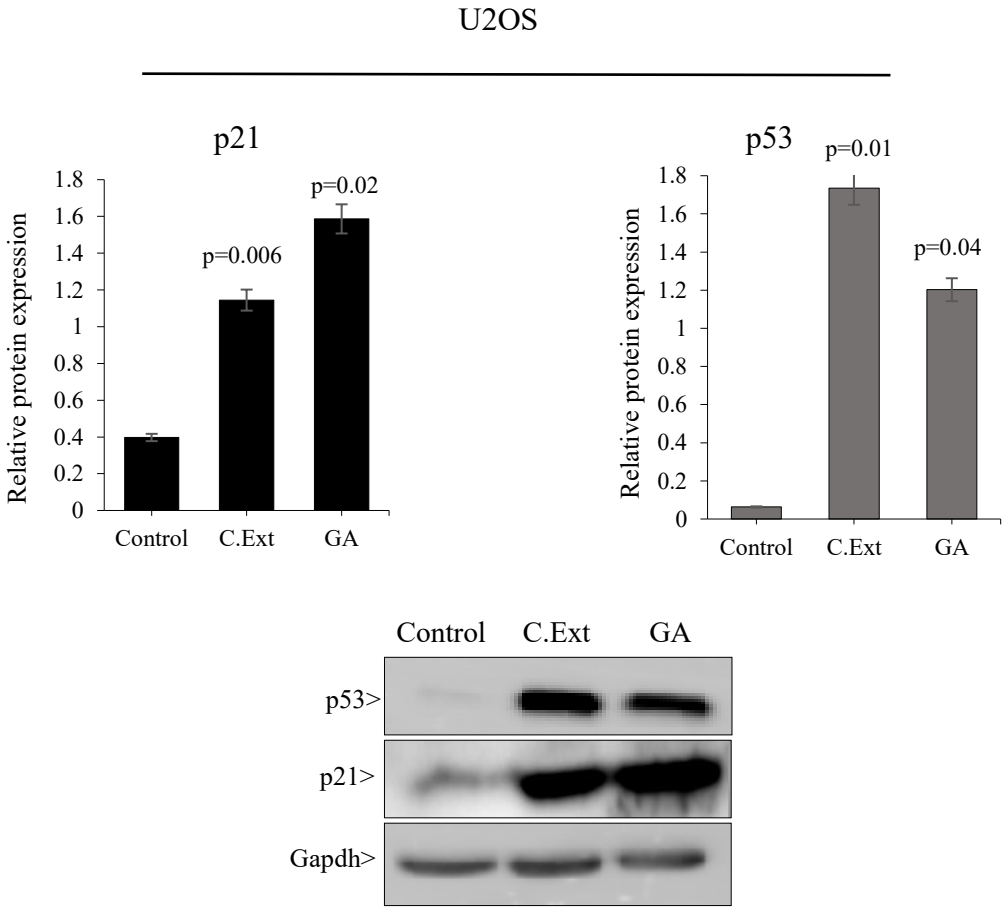


Figure 5.14: Effect of the crude extract and gyrophoric acid on the protein expression of p21 and p53 in treated U2OS cells for 72 hours. DMSO was added respective to the cell culture medium as a control, Gapdh used as a reference. Data revealed p53 and p21 protein upregulation in U2OS

cells considerably different from untreated cells (control). (n=3), p-value was calculated by Student t-test. The graphs illustrated the quantification of p53/p21 using image Lab software (Bio-Rad, USA). C.Ext: crude extract, GA: gyrophoric acid.

This experiment confirmed that the changes in the expression level of the cell cycle regulatory protein p53 induces apoptosis [22–24] in treated MCF7 and U2OS cells. It was found that the expression level of p53 and p21 in MCF7 and U2OS cells were increased in response to treatment with the crude extract and gyrophoric acid.These results suggest that tumor suppressor protein p53 facilitates the DNA damage [25,26], which initiates cell cycle arrest and induces expression level of p21 in response to the cell damage, leading to apoptosis [27].

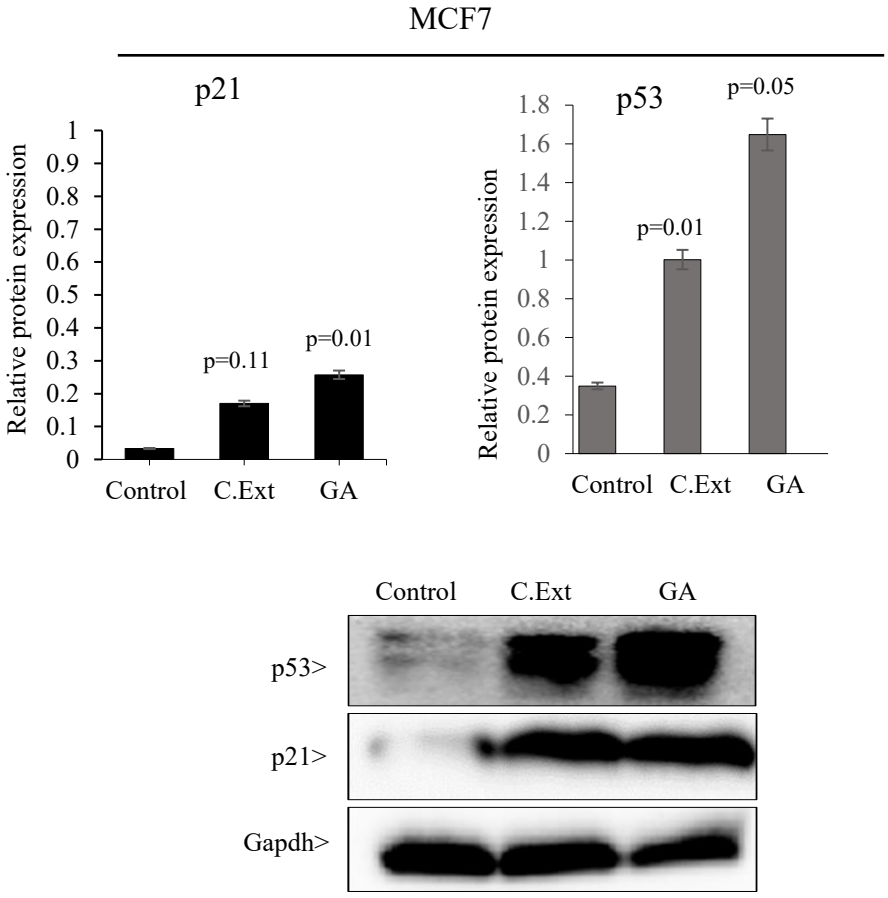


Figure 5.15: Effect of the crude extract and gyrophoric acid on the expression level of p21 and p53 in treated MCF7 breast cancer cells for 72 hours. DMSO was added respective to the cell culture medium as control, Gapdh used as a reference. Data revealed the effect of gyrophoric acid on p53

and p21 protein deregulation on MCF7 different from untreated cells (control). (n=3), p-value was calculated by Student t-test. The graphs illustrated the quantification of p53/p21 using image Lab software (Bio-Rad, USA). C.Ext: crude extract, GA: gyrophoric acid

As shown in Figure 5.16, MSCs, as normal cells, were less responsive under the crude extract and gyrophoric acid treatment. Our data suggested these compounds did not suppress mRNA expression of p53/p21 in MSCs.

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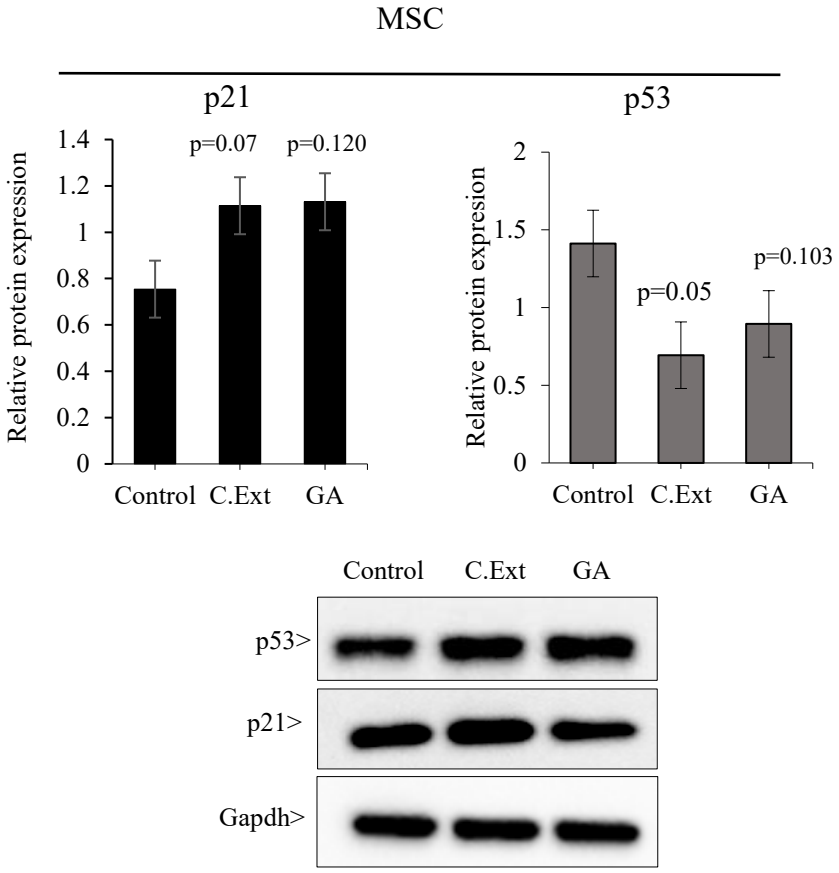


Figure 5.16: Effect of the crude extract and gyrophoric acid on the expression levels of p21 and p53 proteins in MSCs treated for 72 hours. DMSO was used as a control; Gapdh was used as a reference. Data showed no remarkable effect of the crude extract and gyrophoric acid on p53 and p21 protein deregulation on MSCs in comparison with untreated cells (control). (n-3), p-value was calculated by Student t-test. The graphs illustrated the quantification of p53/p21 using image Lab software (Bio-Rad, USA). C.Ext: crude extract, GA: gyrophoric acid.

It can be concluded that p53, as a nuclear transcription factor, pro-apoptotic, and tumor suppressor [23,26] has activated death signals and as a conclusion increased p21 expression induces cell cycle arrest and decreases cell viability [28] in these cell lines, whereas no changes in the pro-apoptotic proteins' levels were observed in normal MSCs. These obtained results are consistent with our previous findings for cytotoxicity effect of the crude extract and gyrophoric acid on MCF7 and U2OS and MSCs.

We also evaluated the effect of the crude extract and gyrophoric acid on the expression of extracellular signal-regulated kinase 1/2 (Erk1/2) and (nuclear factor) NF-κB IN ATDC5 cells. The significant decreases in the expression Erk1/2 and NF-κB were detected after 72 hours of treatment, suggesting the negative regulation in protein expression. The results are shown in Figure 5.17.

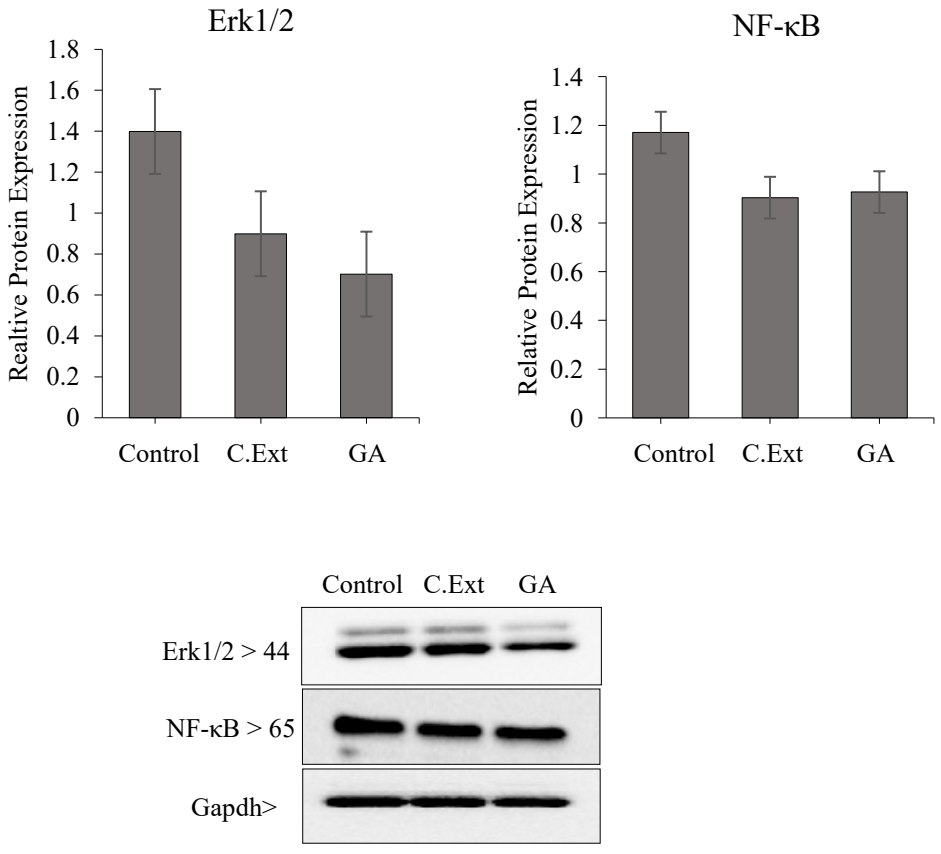


Figure 5.17: Effect of the crude extract and gyrophoric acid on the expression levels of Erk1/2 and NF-κB in treated ATDC5 cells after 72 hours of the exposure. DMSO was added respective to the

cell culture medium as control, Gapdh used as a reference. Data revealed the protein deregulation on murine ATDC5 cells considerably different from untreated cells (control) (n=3). $p < 0.05$, and $p = 0.103$ values were calculated by Student's t-test. The graphs illustrated the quantification of Erk 1/2 and NF-κβ protein level using image Lab software (Bio-Rad, USA). C.Ext: crude extract, GA: gyrophoric acid

The above findings illustrate that the crude extract and gyrophoric acid induced inhibitory effects of the Erk1/2 and NF-κB on ATDC5 cells. These compounds acted as an apoptosis inducer and deactivated NF-κB signaling pathway in this cell line. NF-κB signaling or NF-κB gene knockout inhibition was mediated by anti-tumor responses to these compounds [29]. As mentioned earlier, the inhibition of cell proliferation and induction of apoptotic cell death are two primary mechanisms by which chemotherapeutic agents kill cancer cells. We found that these compounds suppressed the cell growth in ATDC5 cells and caused apoptosis, suggesting the deactivation of NF-κB and enhances the anti-proliferative effect [30].

Similarly, Erk1/2 was downregulated in treated ATDC5 cells in response to the growth inhibitory effect of these studied compounds compared with theuntreated control cells. Ekt1/2 is translocated to the nucleus and could activate the AP-1 (activating protein-1) family of transcription factors, which has a critical role in cellular proliferation [30,31]. Since the Erk1/2 cascade activation has demonstrated to control different regulatory processes, such as cellular proliferation, migration, and cell survival [32,33], any blocking of the transfer of Erk1/2 to the nucleus affects the Erk-mediated cellular processes, such as proliferation [34,35]. Therefore, it can be concluded that both crude extract and gyrophoric acid have a role in the deactivation of the Erk1/2 pathway and remarkably reduce proliferation. We further investigated whether these compounds are able to control the expression of genes regulated transcriptionally by P53 and P21 using qRT-PCR technique.

5.7 Quantitative real-time PCR analysis for p21, p53 gene expression in human cell lines

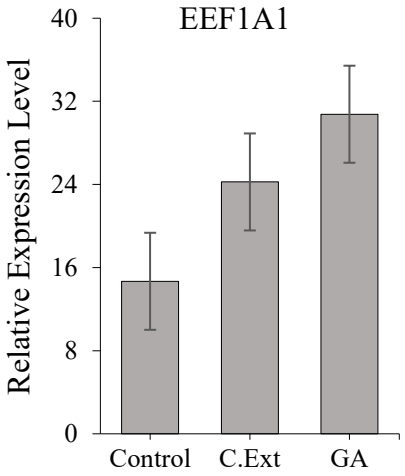
To further evaluate the effect of the crude extract and gyrophoric acid on mRNA expression, we treated MCF7, U2OS and MSCs and then determined mRNA level by qRT-PCR. In this experiment, GAPDH and EEF1A1 served as loading controls. The primers used in this study were listed in Table 5.1. All the details are described in Chapter 3. Here, we focused on mRNA analysis

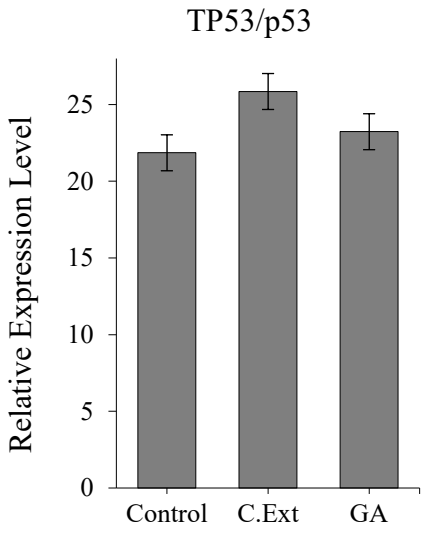
of tumor suppressor protein p53 (TP53) and its principal transcriptional target p21 (CDKN1A), as an inhibitor of cell cycle-dependent kinases. These two cell cycle regulatory genes have key roles in cancer cell proliferation and apoptosis. [26,36], as well as participate in the cellular response to DNA damage [37]. As stated earlier, the DNA damage results in the activation of p53 and induce expression p21 to trigger a growth inhibitory during the cell cycle [38]. Our results show that the levels of p53 and p21 mRNAs do not change appreciably in each of the three cell types upon treatment of near-confluent cultures for 72 hours with the crude extract and gyrophoric acid as shown in Figure 5.18A, B and C.

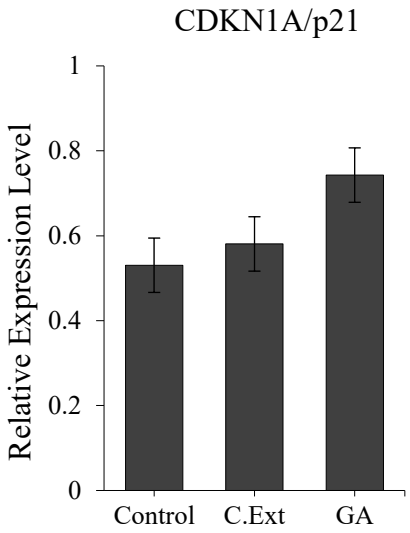
Table 5.1: Primer sequencing in q-PCR

Gene	Primer	Sequence
CDKN1A	Forward primer	CTGCCGCTTTGCAGGTGTA
	Reverse primer	CATTGTGGGCAAGGTGCTATT
TP53	Forward primer	GAGGTTGGCTCTGACTGTACC
	Reverse primer	TCCGTCCCAGTAGATTACCAC
EEF1A1	Forward primer	AAGGCTGGTTCCAAGACTGG
	Reverse primer	TGGTCGTCTCTTTCGCTCCT
GAPDH	Forward primer	GTCTCCTCTGACTTC
	Reverse primer	ACCACCCTGTTGCT

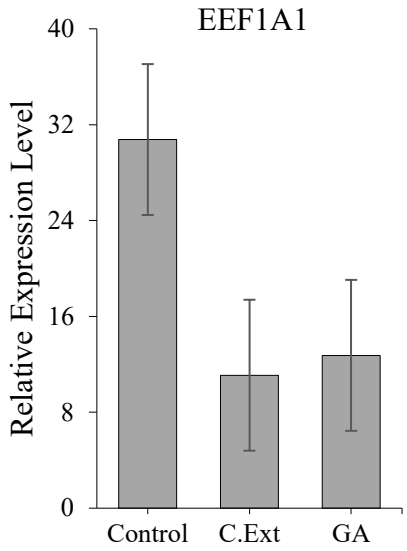
A: MCF7

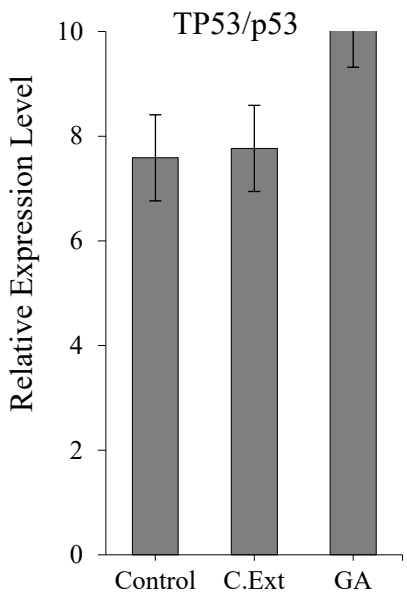

EEF1A1
Relative Expression Level
40
32
24
16
8
0
Control
C.Ext
GA

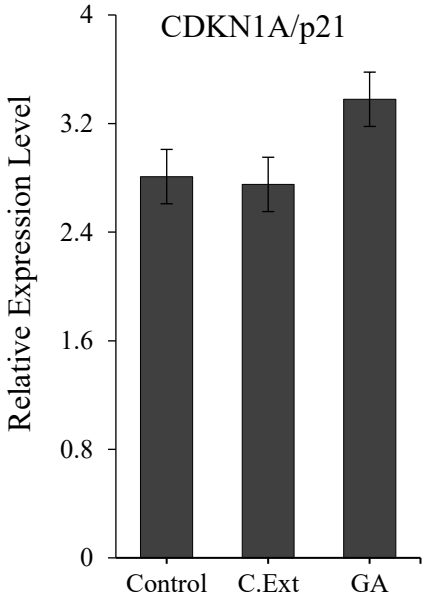

TP53/p53
Relative Expression Level
25
20
15
10
5
0
Control
C.Ext
GA


CDKN1A/p21
Relative Expression Level
1
0.8
0.6
0.4
0.2
0
Control
C.Ext
GA

B: U2OS


EEF1A1
Relative Expression Level
40
32
24
16
8
0
Control
C.Ext
GA


TP53/p53
Relative Expression Level
10
8
6
4
2
0
Control
C.Ext
GA


CDKN1A/p21
Relative Expression Level
4
3.2
2.4
1.6
0.8
0
Control
C.Ext
GA

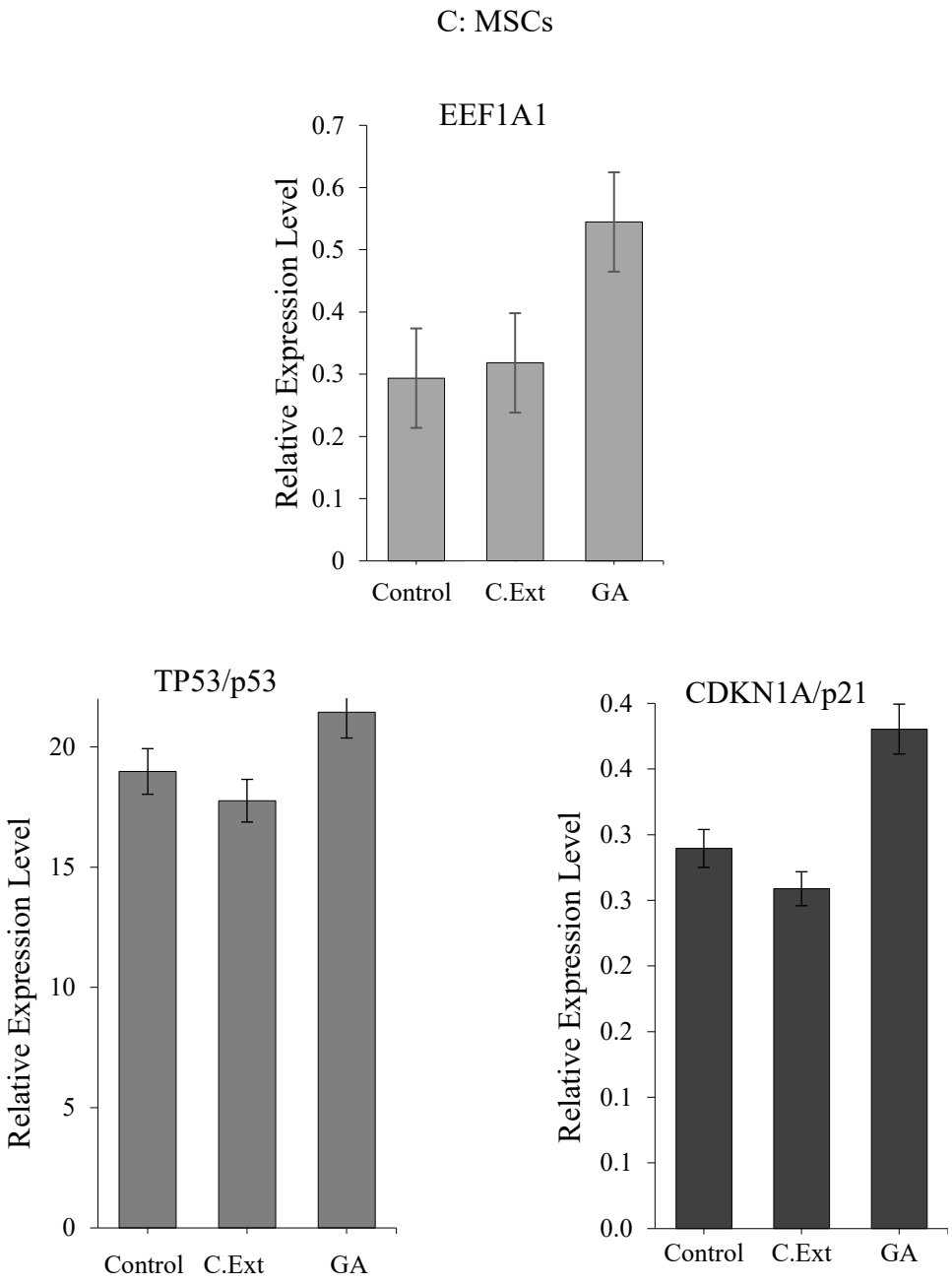


Figure 5.18: qRT-PCR analysis of the expression of p21/p53 in treated MCF7 (A), U2OS (B), and MSCs (C) with the crude extract and gyrophoric acid at concentration 700 µg/mL for 72 hours. DMSO was used as a control. The relative expression levels were normalized to GAPDH and EEF1A1 and the level of each transcript was calculated concerning cells treated with the control where expression was equal to 1: Data are expressed for two biological replicates

Altogether, these findings suggest that the treatment with gyrophoric acid as a phenolic compound had a positive influence on controlling MCF7 and U2OS cell growth by regulating apoptosis. While any remarkable changes were displayed in the MSCs as a normal cell. Consistent with previous results, *in vitro* cell viability assays have demonstrated that treatment with these compounds induced apoptosis and increased numbers of dead cells in cancer cells [38]. Also, increasing expression levels of p53 and p21 in treated human cancer cells MCF7 and U2OS showed a significant role of lichen extract and gyrophoric acid in the activation of both proteins, leading to cell cycle arrest and cell death [37,39,40]. Surprisingly, the difference of p53/p21 activation was not detected between mRNA expression and treated cells, suggesting that additional mechanism might be involved in p53/p21 activation. These results demonstrated the potential anti-tumor actcivity of gyrophoric acid in MCF7 and U2OS cells.

5.8 Discussion

Cancer cells have biological capabilities including resisting cell death, sustaining proliferative signaling pathways, growth suppressors, activating invasion, and metastasis from early to late stages [29]. The targeting of these achievements can be considered as anticancer research and investigations [30,31]. Recently, extensive research efforts focused on the ecology, taxonomy, and anticancer activity of the lichen species as a source of natural products [30,32–35]. Lichens are unique organisms that produce biologically active metabolites with a variety of effects. In the present study, we investigated the inhibitory effect of lichen substances on the cell viability of two human cancer cells as well as normal mesenchymal stem cells and murine chondrogenic cells. The crude extract of *U.muh* showed the inhibitory activity against MCF7 cells. The spectroscopic analysis identified gyrophoric acid as the main active compound of this lichen. Gyrophoric acid is the main compound and an important member of the depside group, which is found in a variety of the lichen species [36]. Gyrophoric acid has been studied as a lichen metabolite with potential anticancer properties, including, apoptotic cell death, and cell cycle arrest effects [36–39]. Although its potential anticancer activity in MCF7 has been previousely reported, however, its biological activity against human osteosarcoma cells (U2OS), human mesenchymal stem cells (hMSCs) and murine chondorgenic cells (ATDC5) has not been reported to date.

In this study, the cytotoxic effects of the crude extract and its bioactive compound gyrophoric acid were investigated in MCF7, U2OS, MSCs, and ATDC5 cells. It was found that the crude extract and gyrophoric acid have different cytotoxicity levels that might be a useful attribute for potential

anti-tumor agents. Studies reported that specific chemical structures and characteristics of the lichen extracts and their secondary metabolites may result in different biological effects of these species [41–47].

We performed cell viability assays and showed that the crude extract and the isolated metabolite exhibited an anti-growth effect against MCF7, U2OS, and murine chondrogenic cell lines (ATDC5), while had less response of normal MSCs**.** Our findings established for the first time that lichen extract and its secondary metabolite, gyrophoric acid have more prominent cytotoxic and apoptosis-inducing effects on U2OS osteosarcoma cells. In accordance with our study, this cell type has also been used to test other small molecules. For example, administration of β-elemene-paclitaxel on U2OS cells can efficiently inhibit cell growth and mediate cell cycle arrest [48]. Other small molecules such as NSC743420 (indole derivative) and MLN4924 (an AMP mimetic) are cytostatic agents that decrease U2OS osteosarcoma cell proliferation by both p53-dependent and p53-independent mechanisms [49,50].

Our results show that MCF7 cells are less sensitive to gyrophoric acid than U2OS cells. These findings complement other studies that established the anti-proliferative effects of secondary metabolites in lichen extracts (e.g., from *Parmelia sulcate*) using MCF7 cells, [50], including physodic acid and usnic acid [51,52].

In current study, as a further supporting, we used Calcein-AM and EthD staining to detect cell apoptosis and we observed that cell apoptosis rate increased with gyrophoric acid treatment in MCF7, U2OS, and MSCs and ATDC5. It was found that gyrophoric acid induced apoptosis in these cells; however, it provoked a different cell death mode. It can be suggested that this secondary metabolite was able to stimulate different cell death responses depending on the cell type. The possible reasons for this outcome may be related to the genetic and phenotypic differences between the cell lines and the different cellular level of mechanism of action and apoptotic signaling pathways (such as p53/p21, p38MAPK, Erk1/2, Akt) or oxidative streas reactions in cells [45,49,53]. Our results are consistent with the previous studies, indicating different responses of various cancer cells to the lichen extracts and secondary metabolites [54].

We also performed Western blot analysis for all cell types. Since most of the human tumors have some malfunction of the p53 signaling pathway [55], this characteristic of cancer cells was also covered in our study. The crude extract and gyrophoric acid mediated apoptosis through the activation of p53 signaling pathway [56]. The activation of p53 is led to stimulating p21 protein

synthesis, which acts as an inhibitor of cyclin Cdk2 protein and causes apoptosis [31]. We found that the treatment of MCF7 and U2OS cells with gyrophoric acid enhances the expression level of p53 and p21 based on Western blot analysis. These findings are in agreement with other studies showing that the effect of the lichen secondary metabolites ramalin, usnic acid and gyrophoric acid results in the upregulation of p53 levels in MCF7, HeLa, A2780, and HT-29 cells through the p53/p21 signaling pathway [57–63]. In accordance with these reported studies, our findings indicate that gyrophoric acid upregulates both p53 and p21 levels reflecting activation of cell cycle arrest via p53/p21 signaling pathway and induction of cell death. Unlike U2OS osteosarcoma cells and MCF7 breast cancer cells, MSCs exhibited limited toxicity in response to gyrophoric acid and did not exhibit induction of p53 and p21 protein levels. This finding clearly establishes cell type specificity of the biological effects of gyrophoric acid. The mechanistic basis for reduced cytotoxic has not been experimentally resolved and these studies are beyond the scope of the present work. Several explanations can be offered that accounts for the observation that MSCs are refractory to gyrophoric acid. Because gyrophoric acid is polyphenolic, it is predicted to cross the cell membrane independent of specific membrane transporters and thus this membrane impermeability for this compound is not likely to be an issue. Once gyrophoric acid is inside cells, it can target different enzymes, but its primary target may be DNA topoisomerase I [64].

The mRNA levels were also examined using RT q-PCR and showed that the levels of p53 and p21 mRNAs do not change noticebly in each of the three cell types upon 72 hours treatment with the crude extract and gyrophoric acid. Differently from our findings, some studies reported mRNA expression in other lichen secondary metabolites, for example, usnic acid had a role in decreasing the mRNA level of CD44, Cyclin D1, and c-myc in lung and colon cancer cells [39,65]. The different effects of the lichen compounds may be attributed to the different cell types or properties of anti-tumor agents.

In addition to these findings, we examined the effects of crude extract and gyrophoric acid on the murine ATDC5 cell line. To the best of our knowledge, it was the first *in vitro* study of anti-proliferative and apoptosis impacts of the *U.muh* crude extract and gyrophoric acid on ATDC5 murine cells. The results revealed significant cell growth inhibition by MTS and apoptosis assays in treated ATDC5 cells. The Western blotting analysis suggested that crude extract and gyrophoric acid downregulated expression of NF-κB and Erk1/2 due to the response of this cell type to the chemotherapeutic agent. Consistent with our findings, the study of usnic acid on murine

lymphocytic leukemia L1210 and atranorin on murine breast carcinoma 4T1 revealed a dose-and time-dependent apoptotic effect on these cells [51,66].

It should be considered that many of these cellular pathways were observed by experiments on cancer cells *in vitro* conditions. Even normal cells *in vitro* culture media may not show all the conditions in tissues *in vivo*. Therefore, it isn't easy to prove that a specific mechanism acts on a particular compound *in vivo* and shows the effect *in vitro*. Moreover, the exact molecular mechanisms of lichen secondary metabolites are almost entirely unknown. Further validation needs to be carried out in the future to reveal molecular mechanisms and cellular pathways of thess anti-tumor agents.

In conclusion, we have observed that cell viability of both MCF7 and U2OS cells is compromised using either unfractionated lichen extract or gyrophoric acid, with U2OS cells being more sensitive to MCF7 cells. We did not detect a significant effect of gyrophoric acid in normal MSCs. The gyrophoric acid dependent cytotoxic effects are attributable to increased levels of p53 and p21 proteins, which parallel increased cell death upon treatment with this cytotoxic compound. Our findings add new perspectives to the anticancer activities of lichen species. The selectivity of the cytotoxic properties of gyrophoric acid for two cancer cell types MCF7 and U2OS over normal diploid MSCs as observed in *in vitro* will encourage future *in vivo* studies and assess the promising but yet unproven anti-tumor activity of gyrophoric acid.

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Chapter 6: Conclusions and future works

6.1 Conclusions

The main focus of this research was to determine the main bioactive compound of the lichen *Umbilicaria muhlenbergii*. The crude extract was obtained using the acetone extraction and the primary results of the micro-chemical color test showed the presence of depsides group in the extract. The present study provided data for supporting the use of *U.muh* as a natural product and confirmed that the extract represented a significant source of phenolic compound. Based on the earlier findings, the crude extract was subjected to chromatography approaches to give some compounds. Among them, three were isolated, and one of them exhibited a significant cytotoxicity effect against MCF7 cells through bio-screening assay. The main bioactive compound was identified as gyrophoric acid by MS, UV and FTIR, and NMR data. MS spectrum showed the $[M-1]^{-1}$ molecular ion at (m/z) 467.0 with diagnostic ion at (m/z) 316.9. The FTIR spectrum showed characteristic absorption bands in the region of 1760-1690 cm^{-1} for carbonyl and the region of 670-900 cm^{-1} which was attributed to the aromatic ring. The UV spectrum had major absorption peaks at 210, 240, and 340 nm, indicating absorption regions for the depsides group of the lichen. The ^{1}H NMR and ^{13}C NMR spectra showed characteristic signals, an acidic proton signal was seen in its characteristic region as 11.67 ppm. In the HMBC spectra, carbon-proton correlations were observed between methyl and aromatic rings' carbons with their protons. The cytotoxicity effects were further evaluated on human breast cancer (MCF7) in a dose and time-dependent manner. The results revealed that the cytotoxicity effect of gyrophoric acid on tested MCF7 cells was more pronounced at concentrations of 300-500 µg/mL. The cell viability assay showed that gyrophoric acid was capable to reduce cell proliferation at specific treatment conditions. The lactate dehydrogenase assay (LDH) determined the percentage of killed cells in treated MCF7 cells at the range of concentrations 300-500 µg/mL in the appropriate exposure time. The results indicated that up to 50% of tested cells were dead and LDH released into the culture media due to loss of cell membrane integrity. These observations confirmed the promising bioactivity effect of gyrophoric acid on MCF7 cells. The human osteosarcoma (U2OS) and MCF7 were considered for our further biochemical investigations. Also, human mesenchymal stem cells (MSCs) was chosen because it was suggested as a normal cellular model in studying the morphological and biochemical changes. In the present study, the viability and apoptosis levels of treated cells

obtained by live/dead assay demonstrated a significant apoptotic effect of gyrophoric acid on both human cancer and normal cells after incubation time. The results validate the hypothesis that gyrophoric acid as a lichen secondary metabolite can be evaluated through the viability and apoptosis assays, although the sensibilities in apoptotic cells were different. U2OS cells have revealed the most sensibility to gyrophoric acid, while this compound had less cytotoxicity effect on MCF7 cells. The MSCs as the normal cell was seen more resistant to gyrophoric treatment.

The anti-proliferative effect of the active metabolite gyrophoric acid was also assessed on murine chondrogenic cells (ATDC5) and displayed the cell growth inhibitory effect in time and dose-dependent conditions. We confirmed that gyrophoric acid was capable to reduce live cells during treatment. Morphology changes were also found to be dependent on the time of exposure to gyrophoric acid, with confirmed and observable dead and live cells using the apoptotic assay. Although, the biological impact of gyrophoric acid on human and murine cancer cells is likely to be more complex and can be related to its chemical structure and cell type-dependent. Furthermore, Western blot analysis indicated different expression levels of protein p53/p21 in studied human cancer and non-cancerous cells. This compound also showed activity against murine ATDC5 cells and moderated the expression levels of NF-κB and Erk1/2 on treated cells. In conclusion, in this study, gyrophoric acid has shown cytotoxic activity at relatively higher concentrations. Altogether, our data suggest that gyrophoric acid can be considered as a promising anticancer agent.

6.2 Future work

It is essential to understand the pathways involved in apoptosis effects of the lichen compound on the cells examined. Therefore, further investigations are required to evaluate apoptosis signaling pathways and cellular-molecular level of interactions between cancer cells and the crude extract or gyrophoric acid. The lichen-derived metabolite, gyrophoric acid, as a promising anti-tumor agent could be a novel *in vitro* therapeutic approach and needs *in vivo* study. Thus, the *in vivo* treatment can be suggested as a future goal in cancer therapy.

To evaluate the synergic effect on cancer cells, it is also suggested to consider the combination therapy of gyrophoric acid and a synthetic anti-cancer drug, such as doxorubicin/paclitaxel or another secondary lichen compound. It is believed that the combination induces cellular death and proliferation.

Moreover, the cytotoxicity studies on other cancer cells should be performed to give some hypotheses about the ability of this lichen compound as a promising anti-tumor agent.