Review article

Biotechnology-based Profiling of Lichens and Their Metabolites for Therapeutic Applications

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Abstract

Lichens have been inadequately studied for their medicinal value though recent studies have established them as potential sources of bioactive compounds that show antimicrobial, anticancer, antioxidant and neuroprotective properties. Ethnobotanical studies have shown that *Pseudocyphellaria aurata, Usnea bismolliuscula, Usnea longissimi, Xanthoparmelia conspersa, Sulcaria sulcate* and *Solorina crocea* have been used by humans since ancient times as a part of their folk wisdom. Recent studies have shown that metabolites from lichens show promising bioactive properties. This review focuses on the necessity for utilizing a modern biotechnology-based approach for elucidating the role and unrealized potential of lichens. Technologies such as genomics, metagenomics, and proteomics have been applied to a far lesser extent in lichens, but the limited studies have revealed the unrealized potential of lichens in modern therapeutics. We attempt to provide a broad overview of the known and unknown in lichen research – ranging from the metabolite production pathways to the genomics and metagenomics, and further to the proteomics and transcriptomics of lichens, the threads of which need to be pieced together to provide a roadmap for further studies.

1. Introduction

The symbiotic relationship established between algae or cyanobacteria and fungi form a composite organism known as lichens, which has properties different from its individual parent organisms, and possesses plant-like characteristics. Morphologically, lichens may be fruticose-having small leafless branches, foliose-leaf like flat structures, crustose-flake like structure, leprose-powder like appearance, or other forms. The environmental requirements for lichen growth are variable. Favored

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environments include extreme habitats and lichens may be found attached to plant surfaces such as leaves, bark, mosses, and on the surface of other lichens. Lichens can also be epiphytes. Reports have estimated that these organisms occupy 6% of the total earth surface. To date, 20,000 species of lichens have been identified [1]. The secondary metabolites produced by lichens can provide protection against most of the stress which may either be biotic such animal predators as or abiotic such as oxidative and UV stress [2]. The production of secondary metabolites via complex metabolic pathways involves acetyl-polymalonyl, shikimic acid, and mevalonic acid pathways. To date, thousands of secondary metabolites with important medicinal properties and therapeutic effects have been isolated and characterized from lichens [3].

Antimicrobial resistance due to rampant prescription of antibiotics and consumption of the same without prescriptions over a long period of time, especially in developing countries with little regulatory overview, has led to persistent and severe infections, incurable in most cases and leading to fatalities. Several products from microbial and plant resources are currently being extensively evaluated for their antimicrobial properties. The findings from research that included *E. coli* and *Klebsiella* spp. along with other gram-negative bacteria indicated that median total hospital cost increased by an amount of US$ 38,121 for patients infected with multi drug resistant bacteria [4].

Many pharmacological and chemical properties of lichen metabolites have been reported, the first report being in 1896 by Zopf [5]. The development of advanced analytical techniques such as magnetic resonance spectroscopy (MRI), X-ray crystallography, spectroscopy by both ultraviolet and infra-red light high-performance liquid chromatography (HPLC) and chromatographic and hyphenated mass spectrometry analyses have facilitated the identification of a large number of metabolites from lichens, many of which have promising medicinal properties. *Pseudocyphellaria aurata*, *Usnea bismolliuscula*, *U. longissimi*, *Xanthoparmelia conspersa*, *Sulcaria sulcata* and *Solorina crocea* have been used by humans since ancient times as a part of their traditional medicine systems. Whilst still largely underexplored, lichens possess considerable medicinal value. Recent studies have shown that lichens have great potential as important sources of bioactive compounds with antimicrobial, anticancer and other bioactivities. This review describes the importance and significance of these unique organisms and the therapeutic activities exhibited by their metabolites. It also gives an insight into the molecular mechanisms of metabolite production in lichens and the tools and state-of-the-art techniques such as genomics, metagenomics, transcriptomics, and proteomics that are used for the profiling of lichen metabolites and their sources.

2. Bioactive Lichen Metabolites

2.1 Antimicrobial activity

Lichens have significant global medicinal importance. They are commonly utilized amongst indigenous groups as an external disinfectant or antihemorrhagic agent in dressing wounds. They have also shown promise in treating obstetrics, gynecological problems, sexually transmitted diseases, and urinary tract infections [6]. Traditional use of *Cladonia arbuscula* to treat infections, pustules, injuries, pulmonary tuberculosis, dizziness, and hypertension was reported [6]. In India, *Cladonia mitis* and *C. rangiferina* have been used for the treatment of hepatic disorders and kidney stones, respectively. In Indonesia, *Usnea barbata* is utilized as an aphrodisiac and as a treatment for sexually transmitted diseases [7, 8].

Lichens produce a variety of bioactive metabolites like salazinic acid, protolichesterinic acid, emodin, lichenan, atranorin, gyrophoric acid, which possess pharmaceutical value. Various lichen metabolites such as usnic acid isolated from *Flavoparmelia caperata*, physodic acid obtained from

Hypogymnia physodes, gyrophoric acid purified from *Umbilicaria polyphylla*, atranorin from *Physcia aipolia*, protocetraric acid from *Flavoparmelia caperata*, fumarprotocetraric acid from *C. furcata*, stictic acid from *Parmelia conspersa* and lecanoric acid from *Ochrolechia androgyna*, have shown strong antimicrobial properties against pathogens that are active against humans as well as plants. Of these, usnic acid was found to have the greatest potential as an antimicrobial [9, 10]. The antifungal and antibacterial activity of one diaryl ether, eight depsides and ten depsidones, isolated from lichens of Chile region, were evaluated. The results obtained stated that at a concentration of 250 $mgmL^{-1}$, these compounds showed inhibitory activity against yeast as well as filamentous fungi. However, lobaric acid, divaricatic acid and difractaic acid showed a lesser degree of activity against skin infection causing dermatophytes such as *Trichophyton mentagrophytes, Epidermophyton floccosum, Microsporum gypseum* and *T. rubrum*. The results of antibacterial assay stated that depsidones, depsides and diaryl ether were inactive against gram-negative bacteria whereas perlatolic, epiforelic-1, α-collatolic acid gave a positive result against gram-positive bacteria [11].

Emodin, parietinic acid and fallacinol are anthraquinones that were isolated from lichen *Caloplacaschaereri*. These compounds were evaluated for antimicrobial activity against organisms like *Pseudomonas fluorescens, Penicillium verrucosum, Trichoderma harzianum, Escherichia coli, Staphylococcus aureus, Candida albicans, Aspergillus niger* and *Bacillus subtilis* [12]. The results from this study affirmed that the anthraquinones demonstrated a significant potent antibacterial activity against *P. fluorescens, S. aureus* and *B. subtilis* at a minimum inhibitory concentration (MIC) ranging between 20-320 μgmL^{-1}, but a potent inhibition of *E. coli* was demonstrated only by parietinic acid with a MIC of 160 μgmL^{-1}.

Inhibition of fungal growth was shown by parietinic acid against the target fungi at a MIC ranging from 20-80 μgmL^{-1}, while parietin showed antifungal activity against *A. niger*, *C. albicans, and P. verrucosum* with MIC values of 20, 80 and 40 μgmL^{-1}, respectively. The MIC values for emodin as a standard ranged from 20-40 μgmL^{-1} against *P. verrucosum, T. harzianum* and *A. niger*. A specific type of depside was reported in *Parmelia cetrata*. It demonstrated complete inhibition of a gram-negative bacteria *Vibrio fischeri* at a concentration of 100 µM [13]. Similar antimicrobial activities were reported against *Agrobacterium tumefaciens, Pseudomonas aeruginosa, Streptococcus mutans*, *Escherichia coli,* and *Staphylococcus aureus* by the extracts of *Usnea longissima* and *Cetrelia braunsiana* at varying concentrations of 2.5 $mgmL^{-1}$ to 20 $mgmL^{-1}$ [14]. The results from these varied studies on different lichens suggests that the diverse compounds produced by lichens have activities against a wide range of bacteria and fungi, including those infecting plants, and thereby can be a valuable resource of antimicrobial metabolites for human pathogens. The compounds may be used to tackle agricultural losses caused by phytopathogens.

2.2 Anticancer activity

The use of lichen secondary metabolites as anticancer compounds can be tracked back as early as the late 1960s in a study which evaluated the anti-tumorigenic potency of polysaccharides from lichen against tumor cells [15, 16]. In a similar manner, another study reported that the lifespan of mice treated with usnic acid isolated from *Cladonia* sp. increased by 35-32% and these mice showed tumor inhibitor activity against Lewis lung carcinoma [17].

Another study demonstrated the anticancer properties of ramalin against the HCT116 cell line, which is used for evaluating human colorectal cancer cell activities, by inducing apoptosis. The study also demonstrates that ramalin arrests the cell during the G2/M phase and modulates the regulatory aspects of corresponding genes such as cyclin-dependent kinase inhibitor 1A, and cyclin-dependent kinase 1, cyclin B1 and tumor protein p53 [18]. Lobaric acid, obtained from the lichen *Stereocaulon alpinum*, was observed to significantly decrease the proliferation of treated and malignant Hela and HCT116 cells by downregulating the DNA repair regulators and apoptosis [19].

A study was conducted on PRCC cultures and human brain GBM cell line (U87MG) obtained from infant Sprague–Dawley rats to demonstrate the anticancer activity of olivetolic acid and psoromic acid isolated from lichens. The results stated that the compounds have good cytotoxic activity, and this property can be exploited in order to treat the patients suffering from glioblastoma multiforme (GBM) [20]. Three lichen metabolites, physodic acid, atranorine and gyrophoric acid were investigated for their ability to inhibit melanoma cancer cell line (A375). The data obtained from the experiments stated that physodic acid and depsidone have cytotoxic properties that can be linked with apoptosis [21].

The data obtained from another study indicates that atranorin, lichen secondary metabolite, inhibits tumor formation in human lung cancer cells by RhoGTPase-suppressing activity, altering the activity of Wnt and STAT signaling [22]. Another group of researchers evaluated the efficacy of vulpinic acid as an anticancer compound and demonstrated that the growth of cells in treated cell lines (L929, HepG2, CaCo2, Vero, and Wehi, Hep2C, and RD) was inhibited by the secondary metabolite. At the cellular level, vulpinic acid modifies the levels of mRNA of corresponding p53, Bcl-2 and Bax genes at its IC50. This study was the first to report the apoptotic effect of vulpinic acid on the levels of mRNA [23]. Table 1 lists the different lichens with promising results of anti-cancer activity against cancer cell lines. These studies provide an indication that *in vitro* results of lichen metabolites against cell lines are promising and warrant further studies on potential toxicity against normal cells and then onto clinical trials.

Table 1. Some of the reported anti-cancer activities of metabolites from lichens

Lichen Species	Type of Cancer	References
Lobaria kurokawae	Prostate cancer	[24]
Psoromadi morphum	Prostate cancer	[25]
Psoroma pallidum	Colon cancer	[26]
Cladonia lepidophora	Cervix cancer	[26]
Snea hirsute	Breast cancer	[27]
Umbilicaria hirsute	Colon cancer	[27]
Ochrolechia deceptionis	Cervix cancer	[26]
Usnea barbata	Colorectal cancer	[28]
Hypogemnia physodes	Glioblastoma	[29]

2.3 Antioxidant activity

Antioxidant activity is largely attributed to the phenolics present in the plants. A study reported the presence of phenolics including methyorsenillate, atranorin and ordenellic acid in *Parmotrema stuppechhaum (Nyl) Hale*, a species of Parmeliaceae. These phenolics have medium antioxidant capacity [30]. In lichens, these phenolic compounds are crucial for growth and development under stress. *In vitro* studies have also reported that these phenolic compounds are more effective antioxidants than Vitamin C and E at molar concentrations [31]. Another group evaluated the antioxidant activities of *Dermatocarpon intestiniformis, Bryoria fuscescens, Pseudevernia furfuracea* and *Peltigera rufescens*. Extracts of *P. rufescens* demonstrated the highest antioxidant activity among the group [32].

A similar study of the antioxidant activities of *Cladia, Bulbothrix, Ramalina* and *Parmotrema* species of Malaysia demonstrated that the free radical scavenging activities of the extracts increased with the increase in the concentration of the extracts [33]. Another study on

phenolic compounds showed a better antioxidant activity in the presence of non-phenolic compound proteins or carbohydrates. This suggested that the activity might be due to synergistic effects on the antioxidant activities of the compounds present in the extract [32]. The lichen metabolites both synergistically or when isolated showed antioxidant properties to variable extents and if explored for their full potential, lichens can act as a rich source of antioxidant compounds. This is important considering that antioxidant compounds have been suggested as potential treatments to prevent onset of cancer [34].

2.4 Anti-neurodegenerative properties of lichens

Neurodegenerative disorders result in slow and gradual loss in the structure or function of neurons, and therefore affect the ability of the brain to control specific functions properly. The status of identifying the anti-neurodegenerative compounds from lichens is poor. Figure 1 shows a list of lichens or their compounds that have been demonstrated to have negative effects on neural degeneration. A study on various lichen species identifies us with 31 lichen species of *Graphidacea*e, whose extracts or metabolites possess anti-neurodegenerative properties including the inhibition of tyrosine kinase [35]. Amongst the six evaluated species, *Phaeographopsis indica, Graphina glaucorufa, G. salacinilabiata, G. multistriata, Graphis assamensis* and *G. nakanishiana* demonstrated moderate to high inhibition of tyrosine kinase. Another study demonstrated that methanolic extracts of *Heterodermia podocarpa, Parmotrema tinctorum* and *Arthothelium awasthii* possessed tyrosine kinase inhibitory activity [36]. It was also demonstrated that acetone extract of *Umbilicaria crustulosa* had the potential to activate cholinesterase [37]. As per the results of these studies, many lichens and their compounds have demonstrated the potential to prevent neurodegeneration. In the current scenario where the geriatric population in many countries has increased disproportionately in comparison to the younger age groups, the onset of neurodegeneration and associated functional capabilities is a serious concern because of the lack of standard modes of treatment except for palliative care. Further in-depth evaluation is needed to exploit the full potential of lichens in the field of these conditions.

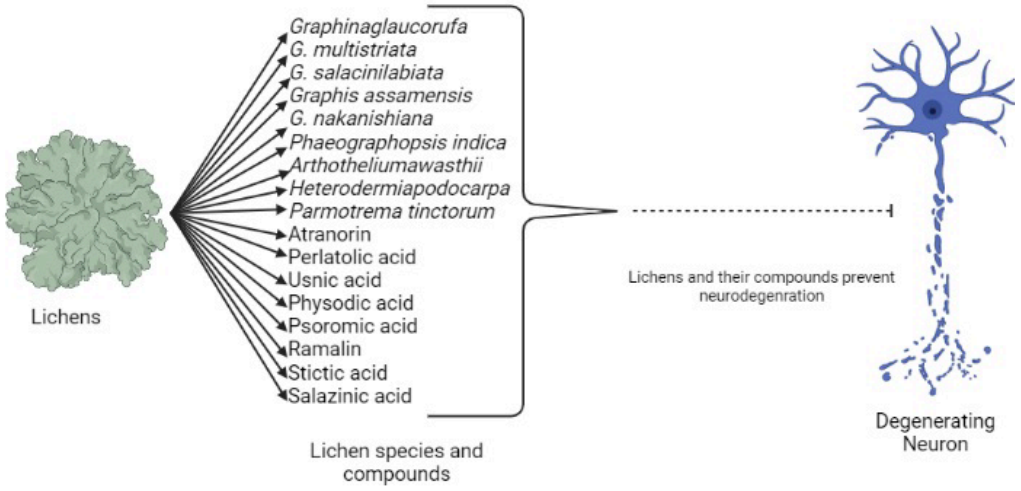

Figure 1. Anti-neurodegenerative lichen species and compounds

3. Important Pathways in Metabolite Production

Culberson and Elix in 1999 classified the lichen secondary metabolites on the basis of their structure and origin. The three major pathways involved in lichen metabolite production are the shikimate pathway, the acetyl-malonate pathway, and the mevalonate pathway. As seen in Figure 2, the algal partner provides glucose and mannitol/other sugars to the fungal partner, which in turn plays a vital role in the production of the important secondary metabolites such as despone and usnic acid.

3.1 Shikimate pathway

The shikimate pathway acts as a way for the production of aromatic acids and their derivatives. This pathway is present in both plants and microorganisms but absent in the case of animals. The pathway is initiated by an aldol reaction between D-erythrose 4-phosphate that was produced in the pentose phosphate cycle, and phosphoenolpyruvate (PEP) obtained from glycolysis. Phosphate is eliminated and another aldol reaction occurs leading to the formation of 3-dehydroquinic acid. Shikimic acid 3-phosphate reacts with PEP leading to the formation of an intermediate where 1, 2 phosphoric acid present in the side chain is eliminated and this is followed by the formation of chorismic acid. Claissen rearrangement occurs which converts chorismic acid to prephenic acid by transfer of the side chain, allowing it to directly attach to the carbocycle. Further decarboxylation, aromatization and dehydroxylation occurs in phenylpyruvic acid and L-Phenylalanine (also known as C6-C3 building blocks). The two C6-C3 building blocks combine to produce terphenyl quinones which later leads to the formation of pulvinic acid derivatives. The latter and erphenyl quinones are the ones most often formed in lichens via the shikimate pathway.

The production of shikimic acid derivatives like rhizocarpic acid, stictic acid and psoromic acid has been successfully demonstrated in the mycobiont isolated from *Rhizocarpon umbilicatum.* Methods to increase the metabolite production in the lichens *in vitro* can be via changing the nutrient composition of the media by playing around with the various carbon sources, which can in turn, improve the production ratios of the metabolites [38].

3.2 Mevalonate pathway

Multiple C5 isoprene units join in a head to tail manner to form the structurally diverse class of the terpenoids. Claissen condensation between two molecules of acetyl coenzyme A leads to the formation of acetoacetyl-CoA, and here a third acetyl coenzyme A molecule is added with the help of a stereospecific aldol addition reaction which gives the compound a branched chain ester. This compound is later reduced with the help of aldehyde and hemithioacetal leading to the formation of mevalonic acid.

A series of chemical reactions take place on the 6 carbon mevalonic acid, converting it into five-carbon phosphorylated isoprene units. Phosphorylation of the primary alcohol group starts the reactions. Two different ATP dependent enzymes lead to the formation of mevalonic acid diphosphate. The latter undergoes decarboxylation and dehydration leading to the formation of isopentenyl pyrophosphate. Cyclization of geranyl pyrophosphate (GPP) results in the formation of limonene. Two isoprene units react in succession with GPP forming farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), respectively. Triterpenes are formed from squalene, containing six isoprene units. Steroids are formed by the cyclization of squalene. This pathway is mainly responsible for the production of steroids (brassicasterol, ergosterol), terpenes (leucotylin, zeorin, and leucotylic acid) and carotenoids (astxantin, zeaxantin, and canthaxanthin).

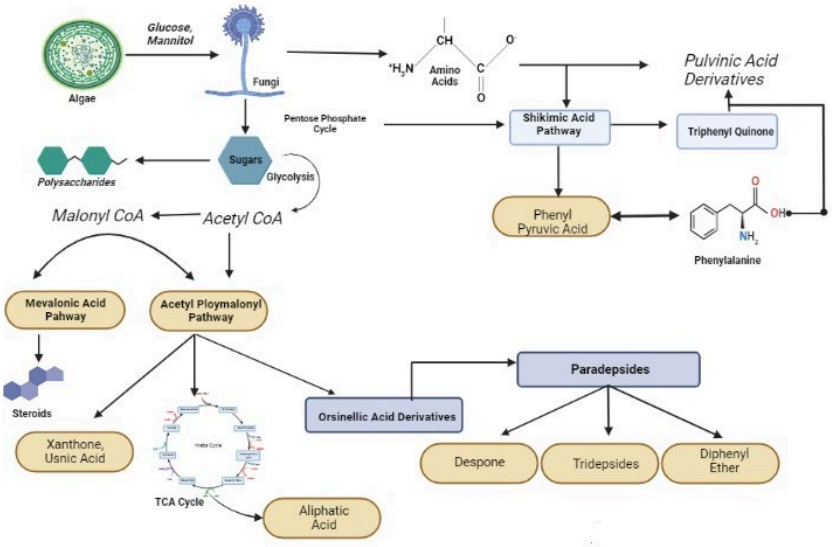

Figure 2. A summary of pathways involved in lichen metabolite synthesis

3.3 Acetyl-malonate pathway

Multiple Claissen rearrangements are responsible for the formation of polyketide chains. Acetyl CoA and multiple malonyl CoA units assemble together in different steps, which terminate with a decarboxylation reaction in each step. Orsellinic acid, an important component in the formation of depsidones and depsides, is produced in an aldol reaction followed by enolization and hydrolysis which involves a polyketide. Lecanoric acid is a depside resulting from the esterification of two molecules of orsellinic acid. The orcinolic despidone possesses an α- or a β-keto group in the side chain of the first ring, which affects and modulates ester linkage formation between the first and second rings, primarily because of the formation of enol lactones. The 2-hydroxyl component of ring A and the 5-position of ring B are conjoined due to oxidative cyclization, and this results in converting depsides into depsidones. Xanthones and chromones form a key intermediate, resulting from the Claisen reaction. This intermediate undergoes aromatization and cyclization reactions which are specifically related to the polyketides that contain five keto groups (5,7-dihydroxy-2-methylchromone).

Polyketides comprised of eight keto groups successively undergo aldol reactions. These are in turn followed by enolization, oxidation, decarboxylation, and selective methylation to produce parietin (anthraquinone group). The main classes of metabolites produced via this pathway include depsidones, depsides, anthraquinones, dibenzofurans, xanthones and chromones. Usnic acid is one of the major pharmaceutically active metabolites produced by this pathway.

The production of metabolites depends on various factors like environmental conditions and the substrate on which the lichen is growing. Variations in the quantity of usnic acid produced in lichens grown on media with differing nutrient composition have been documented [35]. Lichens grown on malt yeast extract produced double the amount of usnic acid than those grown on the water agar medium. It can be said that the amount of a particular compound produced by lichen can be altered if the optimum substrate and environment conditions are altered. In this way, we can selectively increase the production of the medicinally important metabolites.

4. Modern Omics Technologies for Understanding Lichens

4.1 Genomics

Studies involving the analysis of lichens at molecular levels mainly use genomic DNA from the mycobionts that have been grown as pure cultures. Due to the difficulties in maintaining and establishing lichens and mycobionts, the numbers of studies conducted in this field are very low. However, some of the studies conducted have dealt with reproduction in *Graphis scripta* and *Ochrolechia parella* [39], understanding and analysis of DNA methylation in *Cladonia grayi* [40], and mating type patterns and their associated genes in *Xanthoria parietina* [41, 42]. A number of experiments conducted depend on metagenomic DNA that was isolated from the entire lichen itself, followed by analysis for taxon-specific primers to obtain information about hybridization patterns or to recover the informative amplicons.

For instance, Southern hybridization on the genomic DNA isolated from lichen thalli was used for the identification of the symbionts in the lichen chimera [43]. Studies conducted on lichens based on polymerase chain reaction (PCR) analysis for polyketide synthase (PKS) genes have revealed their vital role in the biosynthesis of polyketide-derived secondary metabolites which are specific to lichens and the partnermycobiont, rarely obtained from other sources. Amplified gene fragments of the PKS genes were obtained and have helped in phylogenetic as well as ecological analysis of the lichen symbiosis [44]. As the first step in omics-based studies depends on DNA quality, the following Section focuses on some standardization steps that have been attempted to obtain good quality DNA from lichens.

Apart from the basic techniques used in DNA isolation, various modifications have been reported for more effective extraction and subsequently better-quality DNA for further amplification – not just from lichens but also from fungi. Both are rich in polysaccharide content as well as significant amounts of polyphenols and tannins that can interfere with DNA quality [43].

The main problem associated with lichen DNA isolation is the presence of a large number of inhibitors like phenolics and polysaccharides. These inhibitors are difficult to remove, and they interfere with the functioning of the different enzymes used in the process of DNA isolation. This later affects the purity of the nucleic acids that have been isolated [45].

Many research groups have used either a mixture of phenol and chloroform or chloroform after the sample had been incubated in hot lysis buffer coupled with detergents that may include SDS or CTAB. Later, the DNA was precipitated with isopropanol. Guanidine thiocyanate, a chaotropic salt, was used to denature proteins, and as mentioned in the work of different study groups, the precipitated DNA was transferred to silica particles which had been washed in ethanol to remove the polysaccharides [43, 46, 47]. The four different protocols mentioned in Table 2 were identified and standardized for the isolation of DNA from lichen thalli. They are considered as benchmark procedures. The protocol followed by Lee and Taylor [48] is nearly a 2 h 30 min procedure, of which 1 h is for incubation. It uses a Tris-HCl lysis buffer containing EDTA at a pH of 7.2 along with β-mercaptoethanol and SDS [44]. Both phenol: chloroform (1:1) and phenol: chloroform: isoamyl alcohol (25:24:1) were used for the extraction and isolation of DNA. The final precipitation of DNA was done with isopropanol and sodium acetate (pH 5.2). Another method developed used SDS lysis buffer. The key aspect of this procedure was the use of Nucleon Phytopure proprietary resin which contains free boric acid groups that attach to the polysaccharides covalently and facilitates in their removal. DNA is precipitated only by isopropanol [49].

Table 2. Comparison of the standard DNA isolation protocols from lichen thalli

Sr. No.	Lysis Buffer Preparation	Key Aspects in Protocol	Time Consumed	Disadvantages	Advantages	Ref.
1	Tris-HCl, EDTA, SDS and β-mercaptoethanol	Lichen sample incubated at 65°C for 1 h, DNA obtained treated with phenol and chloroform (1:1) first, then with phenol, chloroform and isoamyl alcohol (25:24:1); DNA can be precipitated using isopropanol and sodium acetate	2 h 30 min	Impure DNA obtained	High yield	[47]
2	Guanidinium thiocyanate (GuSCN) solubilized in Tris-HCl, EDTA, and Triton X-100	Lichen or the fungal material incubated in guanidine buffer, DNA precipitation using glassmilk and NaI	2 h 40 min	Reagents are highly toxic.	Pure DNA obtained; High yield	[46]
3	SDS lysis Buffer	Chloroform added along with Nucleon Phytopure proprietary resin, DNA precipitated with isopropanol	2 h 30 min	Impurities like proteins and tannins are not removed.	High yield	[50]
4	EDTA, Tris-HCl and SDS	Incubation at 55ºC for 12 h, DNA purification multiple times with phenol: choloroform: isoamyl alcohol, precipitation in cold absolute ethanol	16 h	Coprecipitation of polysaccharides	-	[49]

In another method, guanidium thiocyanate was used along with Tris-HCl and EDTA coupled with NaOH and Triton X-100. It is a 2 h 40 min long procedure with a 1 h of incubation period. It uses glassmilk and NaI for precipitation of DNA [47]. Another group developed the longest procedure for DNA extraction which is of 16 h duration and had an incubation period of 12 h [45]. Similar to the method developed in 1990, it uses Tris-HCl, EDTA and SDS lysis buffers [48]. Phenol: chloroform (1:1) and phenol: chloroform: isoamyl alcohol (25:24:1) are used for DNA extraction, but the steps are followed rigorously four to five times, to ensure complete and proper extraction of DNA, after which the DNA is precipitated in cold absolute ethanol, a step which requires 2 h 30 min to complete. The best DNA concentration was seen in the protocol given by Whiting *et al*. [50].

The genes responsible for the production of secondary metabolites are present in the form of a biosynthetic gene cluster (BGC) similar to that in actinobacteria and fungi and are responsible for eliciting the production of metabolites in lichens. The size of these clusters can be up to 40 kb and may contain as many as 20 genes. Some of the genes present in a BGC are co-expressed and co-regulated [51, 52]. Usually, a BGC is identified with the name of the metabolite it produces like orcinol BGC, aflatoxin BGC, and sterigmatocystin BGC. The basic structure of a BGC comprises of a core gene which syntheses the backbone of the molecule and some accessory genes which modify the structural backbone to produce the final metabolite The core gene is responsible for defining the class of the molecules, e.g., ribosomal peptide cluster comprises of non-ribosomal peptide synthetase

(NRPS) gene which encodes for a non-ribosomal peptide from amino acids. Usnic acid is an example of a metabolite produced by NRPS system. A PKS cluster is made up of polyketide synthase (PKSs) genes where the core gene helps in the synthesis of polyketides from acyl-CoAs. The accessory gene is responsible for the modification of structural backbone or the transcription or transport of the metabolite. In most cases, BGC is responsible for coding for multiple metabolites [53].

The PKS is further divided into three classes viz., partially reducing (PR), highly reducing (HR) and the non-reducing (NR) PKSs. It has been observed that NRPKSs are involved in the production of most of the lichen metabolites. The NRPKS is made up of starter ACP transacylase (SAT), thioesterase (TE), acyl transferase (AT), ketoacyl synthase (KS), and acyl carrier proteins (ACP). KS, ACP and AT are the domains required for the synthesis of polyketide [53]. Figure 3 depicts the gene clusters involved in the synthesis of usnic acid.

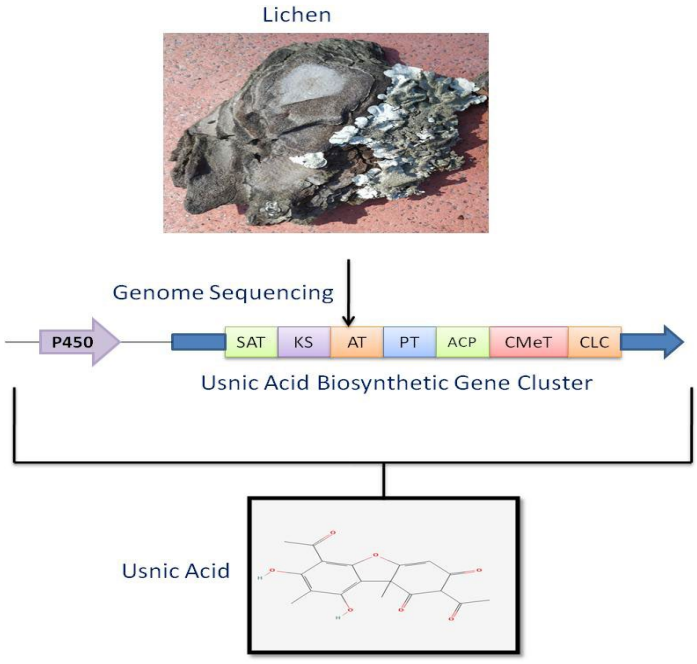

Figure 3. Gene clusters involved in usnic acid biosynthesis

The NRPKSs are classified into nine groups, out of which the functions of eight groups have been identified. Each group is responsible for the synthesis of a particular class of compounds which are mentioned in Figure 4. The group VI codes for the production of usnic acid in the lichens whereas groups IV and V code for the production of mycotoxins like aflatoxin, atrochrysone, etc. 6-methylsalicylic acid synthase (MSAS) from *Penicillium* was the first fungal PKS to be cloned in 1990 whereas first lichen PKS (PKS16) was cloned after 20 years in 2011 from *Cladonia grayi* [54]. The first lichenized fungal PKS was successfully expressed heterologously in 2021 [55]. This gap in the expression of PKS between lichenized and non-lichenized fungus has been attributed to the obligate symbiotic nature of lichens. This makes it difficult to grow the lichen mycobiont in axenic cultures.

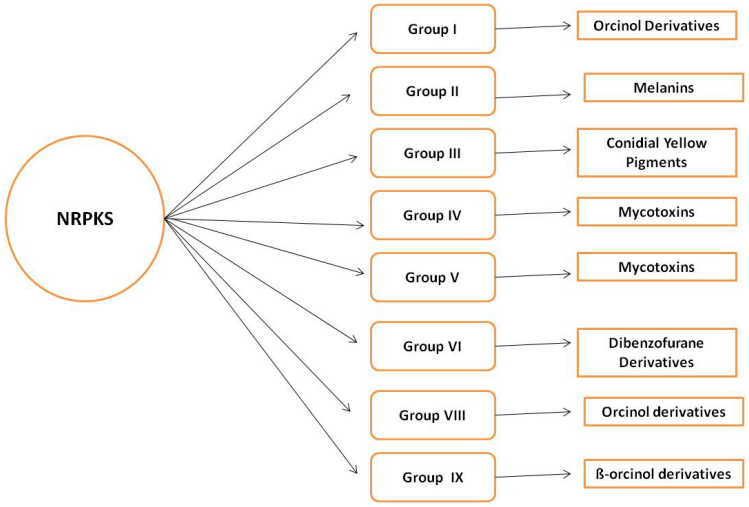

Figure 4. Classification of NRPKSs

Gene discovery based on the genome guided method has changed research on lichenized fungi. This method is based on lichen metagenome sequencing followed by the identification of fungal contigs using binning method and prediction of BGC involved in biosynthesis with the help of prediction tools like antibiotics and secondary metabolite analysis shell (AntiSMASH) [56]. The metagenomic guided linking of molecules to genes is a boon for unconventional, difficult to culture organisms like lichens. Knowledge of the structure of most secondary lichen metabolites serves as a basis for speculating on the biosynthetic pathway and the genetic information for its synthesis. Several software and algorithms are used. AntiSMASH is a BGC-identifying algorithm which predicts the clusters in the genome and classifies them as per their core gene into various clusters including NRPS cluster, terpene cluster, PKS cluster, etc. This algorithm also evaluates the BGCs in terms of the Minimum Information about a Biosynthetic Gene cluster (MiBiG) which is a BGC database used to identify most structurally and functionally similar BGCs from plants, fungi, and bacteria. This step limits the search of candidate gene/clusters to a few targets. These BGCs can further be grouped to remove divergent BGCs by clustering programs as Biosynthetic Gene Similarity Clustering and Prospecting Engine (BiG-SCAPE) and Biosynthetic Gene Cluster Families (BiG-FAM). Such phylogenetic clustering of genes provides an insight into the function of the gene cluster. This limits the search to a single most appropriate candidate gene cluster. Table 3 lists some of the lichen metabolites and their linked genes [57-59].

Table 3. Lichen metabolites and the genes responsible for their biosynthesis [60]

Sr. No.	Metabolite	Genes Encoding
1	Atranorin	atr1 (PKS23), atr2, atr3, atr4
2	Lecanoric acid	NRPKS, PKS16
3	Usnic acid	usnic acid PKS, CYP450
4	Physodic acid	PKS16, CYP450
5	Olivetoric acid	PKS16, CYP450
6	Gyrophoric acid	PKS16

With the technical advancements in the field of DNA sequencing, the genome sequencing of lichens has also gained pace. In most cases where DNA sequencing has been done, the mycobiont has been the prominent source of DNA isolation. Table 4 lists the lichen species for which genome sequencing has been performed.

Table 4. Lichen species whose genomes have been sequenced [61]

Sr. No.	Lichen Species	Source of DNA
1	*Xanthoria parietina*	Mycobiont
2	*Cladonia grayi*	Mycobiont
3	*Peltigera membranacea*	Thallus
4	*Peltigera malacea*	Mycobiont
5	*Ramalina farinacea*	Photobiont

It is evident that a large number of studies are going on globally with the renewed interest in lichens as sources of bioactive metabolites. However, we do not yet have specific databases catering to only lichens, though there is an open-source database on lichen metabolites obtained by MS/MS (https://gnps.ucsd. edu/ProteoSAFe/libraries.jsp).

4.2 Metagenomics of lichens

The mitochondrial genomes (mtDNA) have been assembled and annotated and this has become possible due to the metagenomics approach, which provides high-sequence coverage for mtDNA [62]. The mtDNAs obtained from the fungal partners of *P. membranacea* and *P. malacea* mycobionts showed the presence of DNA elements found in most of the non-lichenized fungi, but in addition to that their mtDNA also contained a gene encoding for RNAseP which is rarely found in non-lichenized fungi. Analysis of partially annotated metagenomes demonstrated the presence of an unusual set of fungal genes which code for galectin-like proteins. Further investigation by RNA-Seq showed that *rec-1* gene was only expressed in rhizines, a fungal tissue considered to be a symbiotic one due to the presence of algal as well as fungal cells [63]. *Peltigera* species have not been documented to produce lichen metabolites. Indeed, no metabolites have been reported from *P. membranacea* but a number of genes involved in the biosynthetic pathway of lichen metabolites have been reported in the metagenome of this species. Even the *Nostoc* photobiont has shown an uncommon trans-AT polyketide biosynthetic pathway which is usually seen in bacterial-eukaryotic symbiosis. Furthermore, it was demonstrated that thallus formation only occurs from the locally optimized strains which depend on the habitat specificity of species [64, 65]. Interestingly, some metagenomic studies have also shown the presence of two coexisting algal partners, which are physiologically different. *Ramalina farinacea, Trebouxia* sp. TR9 and *T. decolorans* were shown as the coexisting photobionts [66].

Pogoda *et al*. [67] used the assembled metagenomic contigs and the mapped fungal reads from lichens that have been deposited in the fungal protein database. The research has provided significant information on the fungal diversity found both as components of lichens as well as in deciduous trees. Different research concentrated on the repetitive segments that encompass the Internal Transcribed Spacer (ITS) region within the nuclear ribosomal DNA (nrDNA). Due to the limited available data regarding fungi linked with lichens, the sequencing of its short reads lacks

comprehensive coverage [68]. The nrDNA copy number ranges from a few to a thousand copies per genome in different species of fungi [69, 70]. Recent studies have shown that many kinds of organisms have symbiotic associations with lichens such as algae, non-photosynthetic bacteria and those with fungal lineage [71, 72].

Whole metagenomic analysis also plays a vital role in establishing the phylogeny of lichens. A study evaluated the metagenome of 29 species of lichens in order to obtain similarity in their genome structure. The results demonstrated that lichens contained conserved sets of genes like *nad3, nad2, nad6, nad5, nad1, nad4L, nad4, rps3, atp8, atp6, cob, cox3, cox2* and *cox1*, which are also present in other groups of non-lichenized fungi and encode for certain hypothetical proteins apart from reverse transcriptases and endonucleases. The number of genes involved in protein synthesis varied from 14 to 15 in all the species of lichens that had been evaluated. Figure 5 demonstrates the comparative genome sizes and other genome features of 29 species of lichens [73].

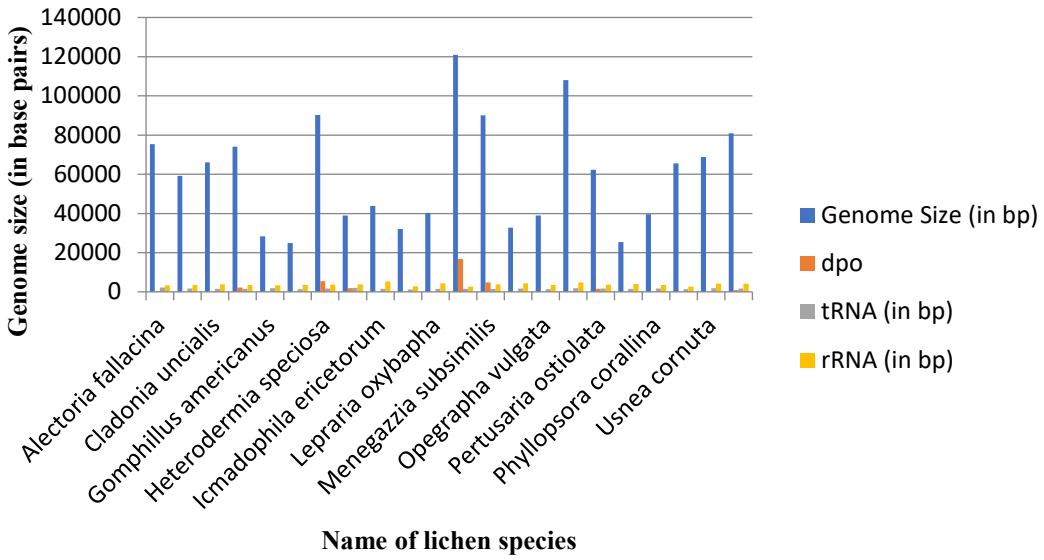

Figure 5. Comparison of genome features of lichen species [71]

4.3 Proteomics and transcriptomics

Metaproteomics can help us to understand the symbiotic association at translational d levels by evaluation of proteins that have been produced by the lichens. Gel electrophoresis of proteins isolated from *Lobaria pulmonaria* were analyzed in associated with LC-MS/MS. The data obtained was searched against a protein sequence database previously reported in the UniRef100 database [74, 75]. Analysis of sequence data (including EST) obtained via next generation sequencing has provided information on eukaryotic transcriptome of the thallus obtained from *Cladonia rangiferina,* commonly known as reindeer lichen [60]. The data demonstrated that 62.8% of proteins were of fungal origin while the rest were produced by photobiont. When the wet thallus was analyzed, it was observed that the photobiont showed a higher percentage of proteins that had been identified with the help of KEGG pathways database and similar to eukaryotic result patterns [75]. The concentration of heavy metals in the lichens was analysed via lichen proteomics [76]. They further studied the accumulation of chromium in *Pseudevernia furfuracea* and its impact on suppression of enzyme

activity. The results confirmed that Cr accumulation denatured multiple proteins and hence, altered the functioning of enzymes in lichen.

Proteomics technology has been utilized to identify the microbiome present inside the lichen thallus. Researchers used 1D gel electrophoresis followed by the LC-MS/MS to identify the proteins produced by various organisms present in the lichen thallus. The study also reported the use of fluorescence *in situ* hybridization and confocal laser scanning microscopy (FISH-CLSM) to evaluate the symbiotic organisms present in *Lobaria pulmonaria* [77]. This technique helped to identify the bacterial, algal and fungal proteins and their functions during the symbiosis. A German research group also evaluated the metaproteome of *Lobaria pulmonaria*, after the generation of LC-MS/MS data; they compared the data with the NCBInr database. Distribution of phylogeny and classification of proteins on the basis of function was done with the help of Prophane 2.0 (a metaproteome analysis pipeline). The major mycobiont proteins that were identified played a vital role in metabolic pathways like glycolysis, the pentose phosphate pathway, and the tricarboxylic acid cycle. The major algal proteins belonged to photosystem I and II, underlining their role in photosynthesis of the symbionts [75]. The proteins obtained and identified are shown in Table 5.

Table 5. Proteome analysis of *Lobaria pulmonaria* [75]

Sr. No.	Proteins	Origin	Function
1	Ribulose bisphosphate carboxylase (RuBisCo)	Algal	Catalysis of Calvin cycle
2	Phosphoenolpyruvate carboxykinase (PEPCK)	Algal	Carbon assimilation
3	Porphobilinogen deaminase	Algal/Bacterial	Uroporphyrinogen-III biosynthesis
4	Nitrogenase	Bacterial	Nitrogen fixation
5	ABC transporters	Bacterial	Transport of molecules
6	Uroporphyrin III methylase	Bacterial	Siroheme and cobalamin biosynthesis
7	Protoporphyrinogen oxidase	Bacterial	Cytochrome production
8	Ferrochelatase	Bacterial	Cytochrome production
9	Oxysterol-binding related proteins (ORPs)	Fungal	Lipid metabolism
10	Eisosomes	Fungal	Transport of molecules
11	Isocitrate dehydrogenase	Fungal	Tricarboxylic acid cycle
12	Sulfite reductase	Fungal	Sulfite assimilation

4.4 Ecological genomics of lichens

The comparison of genetic sequences of cultured symbionts can help in further identification and characterization of the genes facilitating or modulating the symbiotic association between photobiont and mycobiont. Analysis of the genes regulating the secondary metabolite production, signaling, and

transport process can further increase the chances of understanding the footprints of symbiosis. A study involving the coculture of *Carex. Grayi* and *Asterochloris* sp. demonstrated that the fungal and algal genes were upregulated selectively during early developmental phases of lichens under laboratory conditions [78]. Real time PCR analyses of the expression of 41 fungal and 33 algal genes was attempted using suppression subtractive hybridization methods covering the early stages of lichen development. Matches to those fungal genes were found that encoded for proteins taking part in lipid metabolism, negative regulation of glucose repressor genes, and self and non-self-recognition. These fungal and algal genes and the proteins encoded by them have been mentioned in Table 6. The genes responsible for the production of D-arabitol reductase were also found. A chitinase-like protein, a dynein-related protein, a protein for amino acid metabolism, and arginine methyltransferase gene were found to be upregulated in the algal cells. Table 6 shows the algal and fungal proteins expressed separately during lichen symbiosis. Changes in gene expression patterns were observed when the symbionts were separated by nitrocellulose membrane, suggesting the existence of some form of extracellular communication between lichen and symbionts, even in the absence of direct cell contact.

Table 6. Algal and fungal genes responsible for protein synthesis in lichens [78]

Sr. No.	Gene	Protein	Origin
1	ARP4	Nuclear actin-related protein 4	Fungal
2	PLA2	Phospholipase 2	Fungal
3	YQE1	Dioxygenase	Fungal
4	SAM	Sterile alpha motif protein	Fungal
5	NSAR	Norsolorinic acid reductase	Fungal
6	Hsp90	Heat shock protein 90	Fungal
7	AfB1	Aflatoxin B1 aldehyde reductase	Fungal
8	oxido	D-arabinitol dehydrogenase	Fungal
9	SOD	Superoxide Dismutase	Fungal
10	amox	Amine oxidase	Algal
11	CALM	Calmodulin	Algal
12	bglu	Beta-glucosidase	Algal
13	amy	Alpha amylase	Algal
14	hydro	Bacterial cell wall associated hydrolase	Algal
15	NifU	Nitrogen fixation protein nifU	Algal
16	per	Peroxisome biogenesis factor	Algal
17	chit	Chitinase-like protein	Algal
18	flav	Flavoprotein	Algal

5. Metabolomics

Metabolomics is the branch of biology dealing with the study of the metabolite composition due to cellular reactions [63]. A metabolomics approach that involved the analysis of metabolites from the crude extract of both dry and wet thallus of *Xanthoria elegans* was developed in 2007 [79]. The study involved the analysis of polar metabolites using ^{13}C and ^{31}P NMR spectroscopy. This process helped in the detection of thirty metabolites. The analysis of data obtained revealed that conditions inducing stress affected the composition of metabolites. The results also showed enhanced concentration of the sugar alcohols ribitol and mannitol under dry conditions. This proved that sugars tend to protect cellular substances under dry conditions and help in their survival [80].

A ^{13}C NMR study was done to analyze the polyol metabolism in *Xanthoria calcicol* [81]. A proof-of-concept of lichen symbiosis was reported with a metabolomics approach on *X. parietina* [82]. The photobiont components of *Trebouxia decolorans* were harvested in a medium containing ^{13}C-glucose, which permitted the labelling of most of the metabolites; subsequently the photobiont was combined with the mycobiont, and symbiosis re-established. The study indicates that the presence of ^{13}C-labeled fungal metabolites was due to the usage of algal biomass by the mycobiont for its own metabolite synthesis. Similarly, experiments using ^{13}C NMR of methanol extracts of the algal component alone and subsequent co-cultivation with unlabeled fungus showed differing metabolite profiles. This evidence indicates that the mycobiont utilized metabolites synthesized by the photobiont [83].

In another study, two cancer cell lines - T-47D (breast cancer cell line) and AsPC-1 (pancreatic cancer cell line) were selected. Both were sensitive to anti-proliferative effects but of different origins. Morphological changes (using transmission electron microscopy analysis) were assessed followed by metabolomic profiling and study of mitochondrial function using HPLC coupled with TOF spectrometry (based on the Agilent Seahorse XFp Realtime ATP assay). Glucose/lactate levels were studied by radiometry. Glutathione, NADP/NADPH and reactive oxygen species [ROS] were analyzed using luminescence experiments. After exposure to physodic acid (PA), both cell lines exhibited similarities in morphological and structural changes in mitochondria, which synced well with the reduction in oxidative phosphorylation and increase in glycolysis. The T-47D cell line showed decreased mitochondrial function compared to AsPC-1. The resulting decrease in cell numbers was shown to be due to the utilization of PA using the mercapturic pathway. The latter utilizes glutathione. Subsequently, after exposure to PA for 24 h, there was a concomitant increase in glutathione levels indicating enhanced synthesis. However, ROS levels were not enhanced, and the redox balance was maintained. The study concluded that PA is processed during metabolism and then excreted from the cells, thereby contributing to an increased level of glutathione. A remarkable effect was observed in the morphology and functioning of mitochondria, showing that the overall effects of PA were dependent on mitochondria [83].

A study proved the mechanism of action of usnic acid on the cancer cell lines. An apoptosis analysis kit FITC Annexin V Apoptosis detection kit 1 from BD Biosciences followed by BD FACS Aria III flow cytometer were used to score annexin V and/or PI positive cells. The results demonstrated that usnic acid was able to inhibit cell proliferation by intrinsic pathway mediated cell apoptosis [84]. The various metabolites that were evaluated for their therapeutic potential and their mechanisms of action are shown in Table 7.

Table 7. Mechanisms of action of some bioactive lichen metabolites

S. No.	Class	Metabolite	Function/Pathway	Disease Condition	Ref.
1	Dibenzofurans	Usnic acid	Cell cycle arrest, intrinsic pathway-mediated apoptotic cell death	Lung cancer	[84, 85]
			Protection against Ultraviolet B-induced damage in human lymphocytes	Photoprotective activities of ultraviolet-induced damage in cells	
2	Depside	Gyrophoric acid	Cell cycle arrest	Breast cancer	[86, 87]
			Ultraviolet A-induced damage in human keratinocytes	Photoprotective activities of ultraviolet-induced damage in cells	
		Atranorin	Cell cycle arrest and intrinsic pathway-mediated apoptotic cell death	Breast cancer	
3	Depsidone	Salazinic acid	Intrinsic pathway-mediated apoptotic cell death	Breast cancer	[86, 88]
			Ultraviolet A-induced damage in human keratinocytes	Photoprotective activities of ultraviolet-induced damage in cells	
		Vicanicin	Extrinsic pathway-mediated apoptotic cell death	Prostate cancer	[89]
		Lecanoric acid	Reduces oxidative stress on cells by interacting with superoxide radicals	Oxidative stress	[90]
		Protolichesterinic acid	Anti-inflammatory effects through inhibition of proliferative response in mitogen-stimulated lymphocytes, possibly through inhibition of 5-Lipoxygenase activity	Inflammation	[86, 89]
4	Anthraquinone	Emodin	Upregulation of MTA3 to prevent cardiac fibrosis	Cardiac fibrosis	[91, 92]
			Downregulation of Phosphoinositide 3-kinase/AKT signaling pathway	Lung carcinoma, breast cancer, cervical cancer, human hepatoma	
5	Terpenoids	Retigeric acid B	Activation of mitochondria-mediated apoptosis pathway via changes in ratio of Bax/Bcl-2 proteins	Prostate cancer	[93]
6	Glucans	Lichenan Pustulan Ths-2 Thamnolan	Anti-inflammatory effects via stimulation of dendritic cell maturation into Th2-like response	Inflammation	[94]

6. Conclusions

Bioactive metabolites derived from natural sources have gained significant attention in the research field. Lately, there has been an in-depth exploration of metabolites from lichens for therapeutic purposes, owing to the striking resemblance of their bioactivities and compounds to those obtained from plants and microorganisms. Although only a limited number of lichen-derived compounds are commercially available, thousands of known metabolites remain unexplored in terms of their potential medicinal benefits.

The objective of the review was to offer a comprehensive overview of lichen metabolites and their associated genomic, transcriptomic and metabolomic data. More than 12 lichen proteins were scrutinized using diverse proteomics technologies, such as FISH-CLSM, 1D electrophoresis, and 1D gel electrophoresis, followed by LC-MS/MS. The utilization of techniques like ITS sequencing for identifying the fungal partner and databases like BiG-SCAPE and BiG-FAM has augmented the knowledge of lichen metabolites at the molecular level to such an extent that scientists have been able to deduce the pathways and genes responsible for metabolite production. Despite their potential, lichens have not yet received the same level of attention as bacteria and fungi in terms of their bioactive natural products. Therefore, further studies utilizing omics-based approaches are necessary to gain useful insights into the synthesis of bioactive lichen metabolites. In this review, we aimed to shed light on the various techniques and technologies used for extracting and isolating lichen-based metabolites, starting from the basic workhorse technologies such as chromatography, and moving on to high end NMR, high-throughput sequencing, and metabolomic analyses. The choice of technique will depend on the aim of the experiment, which can range from simple antimicrobial activity testing to in-depth investigations of the therapeutic potential of specific lichen compounds. Overall, there is a need to harmonize and integrate these techniques into a pipeline-like format, using well-studied lichens like *Usnea* as a model for studying other lichens.

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