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ReviewArticle

PROGRESSION IN INSTRUMENTATION FOR PHYTOCHEMICAL ANALYSIS OF LICHENS

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ABSTRACT

Lichens are complex organisms have symbiotic associations between fungi and algae. Due to symbiosis lichens produce various unique extracellular lichen substances known as secondary metabolites. These chemical compounds present within the thalli and typically form crystals on the surface of the fungal hyphae. Secondary metabolites of lichens have many medicinal properties, such as antimicrobial, antioxidant, antiviral, anticancer, antigenotoxic, anti-inflammatory, analgesic and antipyretic activities. Lichens are also identified on the basis of presence of various chemicals and these chemicals are detected by different methods and instruments. Various advanced technologies for quick identification of chemicals and their extraction were developed easily. Various methods and instruments were discovered by different scientist and they evolved the procedures day by day to detect the useful chemical compounds comfortably and undoubtedly to avail their tremendous bioactive benefits. Hence, the present study wasundertaken to explain the growth of different technologies used for detection of lichen substances.

Keywords: Lichens, Phytochemical analysis, Analytical methods, Instrumentation

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INTRODUCTION

Lichens are the association between fungi (mycobiont) and algal partner (Photobiont), in some lichens algal partner is replaced by cynobacteria. Eucaryotic photobionts are called phycobionts (90 % of lichens), while cyanobacterial photobionts are termed as cyanobionts (10 % of lichen) [16]Mycobiont provides shelter, absorbs water and minerals for algal partner which are photoautotrophic, produce food in presence of sunlight[42]. Mycobiont is unique and dominates the association therefore classification of lichens is done on the basis of lichenised fungi[16].The fungal partners are mostly (98 %) Ascomycota[18, 15]and the others belong to the Basidiomycota and anamorphic fungi[16].Due to symbiotic relation lichens have complex structure which allows lichens to live in unusual environments[2].Lichens are able to survive in extreme ecological conditions such as extreme temperatures, drought, wetlands, salinity, polluted areas and nutrient-poor, highly nitrified environments[28], but some lichens are highly sensitive to change in their environment; this property of lichens is used in detection of pollution and environmental changes, therefore lichens are also known as natural biomonitors[35]. The reason behind adaptation to specific conditions in which lichens live is the production of different chemical compounds known as metabolites which provides good protection against various negative physical and biological influences. Chemical compounds synthesized by lichens are divided into two groups: primary and secondary metabolites[23].The primary metabolites known as intracellular compounds they include proteins, amino acids, carotenoids, polysaccharides and vitamins[16]. Primary metabolites are generally soluble in water and can be easily isolated from the lichens by boiling water. Some of the primary metabolites are produced by fungi and some by algae. Most of these metabolites are non-specific and also found in free-living fungi, algae and higher plants. The majority of organic compounds found in lichens are secondary metabolites, most are unique to these organisms and only a small minority occurs in the other fungi or higher plants. These substances are the crystals deposited on the surface of the hyphae, which are not soluble in water, and usually can be isolated from the lichens by organic solvents[3]. Secondary metabolites of lichens have tremendous bioactive properties, such as antimicrobial, antioxidant, antiviral, anticancer, antigenotoxic, anti-inflammatory, analgesic and antipyretic activities[39,38]. Several families of lichen products are used as antiviral or antiretroviral[48,32], anti-inflammatory[45,14]and analgesic activities[31]. Natural chemicals derived from lichens are well known as natural pesticides because they have no harmful effect on environment as synthetic chemicals have[5, 12]. Various studies on secondary metabolites of lichens have been carried out but there were some problems which scientists were facing in fast and correct identification of lichens. Traditional identification and detection techniques needs collection of lichens in large amount which results overexploitation, as the growth of lichens is very slowso the overexploitation was not good for conservation point of view. Therefore advanced techniques for identification, detection and isolation of compounds are developed which give results by utilizing minimum amount of samples correctly and on time. Constantly new and advanced techniques are designed by scientist to find more specific and interesting compounds. The aim of this review is to define or present successive analytic methods which are developed regularly to know the specific and abundant chemicals present in lichens. Study of lichen chemicals was raised from spot tests used at the beginning in lichen taxonomy approaches (identification of lichens on the basis of chemicals present in them), Thin-layer chromatography (TLC) is still extensively used and its standardized protocols were established. Crystallography of lichen chemicals was well developed from crystal shapes of chemicals to accurate measurements of lichen metabolites by X-rays.

A brief history about analysis of lichen chemical compounds

The chemistry of lichens comes in existence since the early times of organic chemistry. W. Zopf and O. Hesse., the two names which were known from classical period of lichen chemistry. Zopf was a botanist from Germany, he published various papers on lichen substances and summarized in the well-known book Die Flechtenstoffe in chemischer, botanischer, pharmakologischer and technischer Beziehung, published in 1907. O. Hesse was a chemist born in 1835 at Saxony, Germany; he published 18 papers on lichen substances from 1861 to 1905 and also wrote an article on Flechtenstoffe in 1912 edition of Abderhalden's Biochemisches Handlexikon[19].Vulpinic acid[41] and lecanoric acid[17] were the first lichen substances which were known for their structure and was synthesized by E. and H. Fischer in 1913[19]. The structure of most lichen substances remained unknown before the beginning of the outstanding work of the famous Japanese chemists Y. Asahina and S. Shibata in 1921. Asahina and Shibata published classical chemistry of Lichen Substances, in 1954. Both scientists and their coworkers not only elucidated the structure of numerous compounds, but also described their synthesis and

introduced the determination of lichen substances by microcrystallization, without thin layer chromatography and high performance liquid chromatography it was a remarkable progress at that time. Between 1920 and 1940 Koller, Pfau, S. Shibata Robinson and Schopf were also done important work on the chemistry of lichen substances. Publication of Chicita F. Culberson's Chemical and Botanical Guide to Lichen Products was the milestone in the field of lichen substances published in 1969[19]. Two supplementary volumes (C.F. Culberson 1970; C.F. Culberson et al. 1977)[7,10]together summarizes the data of about 430 lichen substances and their occurrence known up to 1976. The chemical analysis of lichens became routine after the introduction of thin layer chromatography in the 1960s and of high performance liquid chromatography later. Number of scientists of different laboratories has been working on the chemistry of lichens from many years; few of them are listed in Table No.1.

S.No.	Name of Scientists	Countries	Chemical compounds/Techniques used
1.	K. Aghoramurthy	India	Depsidones
2.	B. Akermark	Sweden	Aliphatic acids, Dibenzofuranes
3.	F.W. Bachelor	Canada	Depsides
4.	B. Bodo and L. Molho	France	Aliphatic acids
5.	T. Bruun	Norway	Chromones, Triterpenoids
6.	J.D. Connolly	Great Britain	Aromatic compounds
7.	R.E. Corbett	New Zealand	Triterpenoids
8.	C.F. Culberson and W.L. Culberson	USA	Depsides, TLC, Chemotaxonomy
9.	B. Czeczuga	Poland	Carotenoids
10.	Dembitsky	Israel	Lipids
11.	H. Erdtman	Sweden	Depsones and Dibenzofuranes
12.	A.G. Gonzalez and J.B. Barrera	Spain	Phenolic compounds
13.	K. Huovinen	Finland	HPLC
14.	M.F. Keogh	Venezuela	Depsidones, Aliphatic compounds
15.	J.P. Kutney	Canada	Usnic acid
16.	B. Lindberg	Sweden	Carbohydrates
17.	W.S.G. Maass	Canada	Depsides
18.	A. Mathey	France	Quinones
19.	O.B. Maximov and N.P. Mishchenko	Russia	Depsidones, Quinones
20.	K. Moosbach	Sweden	Biosynthesis
21.	S. Neelakantan	India	Depsidones
22.	T.J. Nolan	Ireland	Depsidones
23.	G. Quinkert	Germany	Usnic acid, Aspicilin
24.	B. Renner	Germany	Dibenzofuranes
25.	J. Santesson	Sweden	Depsides, Xanthones, TLC
26.	M.V. Sargent	Australia	Depsidones, Dibenzofuranes
27.	T.R. Seshadri	India	Depsides
28.	Y.J. Solberg	Norway	Aliphatic compounds, Steroids
29.	W. Steglich	Germany	Quinones
30.	R. Tabacchi	Switzerland	Synthesis of Depsides and Depsidones
31.	K. Takahashi	Japan	Usnic acid
32.	Wachtmeister	Sweden	Dibenzofuranes
33.	A.L. Wilkins	New Zealand	Triterpenoids
34.	Y. Yamamoto	Japan	Culture of lichens
35.	Yoshimura	Japan	HPLC, Culture of lichens
36.	Yosioka	Japan	Triterpenoids
37.	J.A. Elix	Australia	Depsides and Xanthones

Table No.1: Scientists working on the chemistry of lichens from different countries on specific lichen compounds[19]

Different Methods Used for detection and identification of lichen chemical substances

There are various methods used for the detection and isolation of chemical compounds from lichens, they are modified day by day to established new technologies to make the procedure more reliable.

Color spot test

Color spot testhas been used since nineteenth century[30].This is a preliminary test used to test the presence of chemicals on the basis of change in color by applying different reagents, in this test Color reactions should be considered as useful hints for the presence of functional groups or elements. Colour changes take place due to the presence of particular secondary metabolite in the thallus, which is termed as spotting. Spot tests are performed by placing a small drop of reagent on the lichen thallus, either directly on the upper surface (cortex) or on the medulla by scraping cortex with the help of a blade. The colour changes at the reagent application point of the thallus are noted as positive (+) or negative (-). Three main reagents used in color reactions are potassium hydroxide (commonly called K test), sodium hypochlorite (known as C test), and paraphenylenediamine (abbreviated as P or PD). K (consisting of 10 % potassium hydroxide in water) is a useful reagent to differentiate between quinones and pulvinic acid derivatives as it turns quinones into a bright red to deep purple color. It also turns yellow and then red with most orthohydroxy aromatic aldehydes. C (saturated aqueous $Ca(OCl)_2$ or commercial laundry bleach) is a reagent used for meta-dihydroxy phenols. Ethanolic solution of para-phenylenediamine (2–5 %) is highly toxic, used as macrochemical reagent which gives yellow to orange colors with aldehyde-containing depsides. In some cases application of mixture of reagents is required in which KOH is applied before having coloration with C reagent and known as KC+. KC turns red with C– depsides and depsidones which undergo rapid hydrolysis to yield a meta-dihydroxy phenolic. Blue color is obtained with dihydroxy dibenzofurans, whereas a yellow reaction appears with usnic acid[28]. This test has been used universally as rapid, non-specific means for detecting the presence of certain unspecified lichen substances. This test is most convenient and simple to perform, even under field conditions. This is particularly useful to distinguish some closely shaped species which could not be unambiguously discriminated on the basis of morphological characters. Nowdays, spot tests remain either helpful or necessary to confidently discriminate between some lichens and are still met as a very important criterion in determination keys. However, this is only a preliminary step in the process of identification of lichens or its substances. In order to identify accurately the secondary metabolite present in the lichen thallus, one has to perform more sensitive test such as TLC or HPLC[27, 21, 22,26,40].

Microcrystallography

Microcrystallization of lichen substances was mainly developed by Asahina and Shibata since 1950[37].This test work on the principal of formation of crystals, some of the secondary metabolites form characteristic crystals when a crystallizing reagent is added and gently warmed. Earlier microcrystallization was very important way to identify lichen metabolites before TLC and high-performance liquid chromatography (HPLC). The general procedure of microcrystallization is start with extraction of chemicals with the help of acetone. Acetone is the most used solvent as it extracts compounds with a wide range of polarity. A small piece of lichen sample was placed on a glass slide;few drops of acetone were added on that. It was allowed for a while so that chemical present in lichen sample eluteted out on the slide. The piece of lichen was removed and residue was allowed for drying then few drops of proper microcrystallization reagent were added on that before capping with a cover glass slip.Extra solvent was allowed to evaporate by keeping the slide upon a spirit lamp for a while. Slides were observed under the microscope for the formation of crystals. Glycerol, ethanol and glacial acetic acids are some of the chemicals used in different combination to make the microcrystallization reagent. The most commonly used combination are GAW and GE, corresponding to H2O/glycerol/ethanol 1:1:1 (v/v/v) and to acetic acid/glycerol 1:3, respectively. Microcrystallography has been largely superseded by the more sensitive and reliable method such as TLC. But the technique is still useful for a number of lichen compounds which are difficult to identify in TLC due to the same Rf class (or value) or spot colour (e.g. lecanoric acid and gyrophoric acids, barbatic and diffractaic acids). However, mixture of substances may be difficult to identify with this method and also minor substances may be undetectable [27,21,22,26,40].

Paper Chromatography

Paper chromatography was first introduced for the separation and characterization of lichen substances in 1952, by Wachtmeister[46,47]. A few relationships between chemical structure and chromatographic behavior have been studied by Mitsuno in 1953. Mainly paper chromatography is used for the separation of amino acids. Cellulose fiber containing paper is used as the support or adsorbent, as the cellulose fibers have a film of moisture around them when dipped in suitable solvents this helps in separation of the components of mixtures. In this technique separation of chemicals occur on the basis of binding tendency of chemicals with cellulose membrane (stationary phase) and movement of chemicals with different solvents (moving phase). Column partition chromatography is also a technique used for the separation of chemical compounds from lichens, but in this technique large amount of sample is required for the separation then in paper chromatography. Paper chromatography is out dated after Thin Layer Chromatography (TLC) was introduced in which silica gel was used as a stationary phase. It gives more reliable results then paper chromatoghraphy[27, 21, 22,26,40].

Thin-layer chromatography (TLC)

Identification of lichen products by TLC were first attempted by Santesson who reported Rf values of 80 lichen metabolites[36,33,34]applied TLC in the field of Lichenology to separate depsides and depsidones. Different authors reported different solvent systems and chromatographic conditions[4,36]. It is a relatively simple and inexpensive technique which can be performed by anyone with access to basic laboratory facilities. Earlier the glass plates were loaded with the prepared slurry of silica gel; it was spreaded well by ensuring the uniform thickness all over the plate and maintaining the uniformity of thickness was the tedious job in this procedure. Later the pre coated aluminium plates of different pore size were used to overcome the thickness issue. In this technique lichen substances are extracted in acetone and the extract is spotted on to glass or aluminium plates coated with silica gel. The plate is placed in a sealed tank so that the base of the plate is immersed in a shallow layer of a specific mixture or organic solvents. TLC was developed in three solvent systems ("A": benzene/dioxane/acetic acid 90/25/4; "B": hexane/ethyl ether/formic acid 5/4/1; "C": toluene/ acetic acid 85/15). The different lichen substances present in the sample are separated from each other during the passage of solvent through the silica gel layer and are later made visible by spraying with sulphuric acid. The resulting spots are provisionally identified by their colour and relative position in comparison to the control sample. Culberson and Kristinsson (1970)[9] first described a TLC-based method to cope with Rf variations ensuring higher reproducibility and associating other identification methods to certain identification possibilities. In this process atranorin and norstictic acid were loaded in the form of spots along with the samples of interest, and 8 Rf classes were defined according to the relative positions of each spot compared with atranorin and norstictic acid. Another solvent system (referred to as solvent G: toluene/ethyl acetate/formic acid 139/83/8) was later introduced to separate polar compounds such as β-orcinol depsidones[11].

HPTLC

There are some draw backs of simple TLC technique some of them are- Analysis of less samples in long period of time; large amount of samples are required and mixture of samples while loading upon the plates. Later the use of high-performance thin-layer chromatography (HPTLC) in screening lichen substances was developed. HPTLC is more sensitive, allows the running of more samples in a shorter period of time, and requires smaller amounts of solvent[1]. Similar Rf value does not mean single compound, therefore, reliability to only Rf value remains a challenging and risky task. However, standardized methods listed above enable collecting valuable clues regarding chemical structures and might certain identification possibilities [27, 21, 22,26,40].

High-performance liquid chromatography (HPLC)

The first application of HPLC in the field of lichen substances was described by C.F. Culberson in 1972[8]. This method was applied to identify and quantify characteristic substances in commercially available lichen products. This technique provides a powerful complement to the established TLC method. The bonded reverse phase columns are used here, and all the aromatic lichen products are suitable for analysis with this method. Samples are dissolved in methanol and injected in to the appropriate portion column, through which an appropriate solvent or sequence of solvents is passed under high pressure. The substance separates and is detected using UV detector. The retention time (Rt or time of passage) and peak intensity are recorded by a chart recorder. HPLC is also used to measure either absolute or relative concentrations of lichen compounds, because the peak intensity (area under curve) is proportional to the concentration[27, 21, 22,26,40]. Nourish and Oliver (1976) studied the chemotaxonomy of the Cladonia chlorophaea-pyxidata complex and some allied species in Britain by HPLC. Later, C.F. Culberson and W.L. Culberson (1978), and C.F. Culberson and Ahmadjian in 1980 studied some lichen compounds by HPLC. Gradient elution methods were developed that have much better results with complex lichen extracts containing a wide range of hydrophobic compounds[6].Gradient elution method was introduced by starck[43]in lichenology that separated 13 different lichen products (depsides, depsidones, dibenzofurans, and pulvinic acids) using a reversed-phase column with a linear gradient based on a water/ methanol system. A first standardized method for separation of lichen aromatic products was described using different kinds of reversed-phase column on the basis of a methanol/orthophosphoric acid gradient system and including two internal standards: benzoic acid (low retention time) and bis-(2-ethyl-hexyl)phtalate (high retention time)[20] according to which retention indices are defined ensuring better reliability. However, the latter standard was further replaced by solorinic acid (more hydrophobic) making the method suitable for the identification of lichen extracts containing chloroxanthones or long chain depsides as well. This second standardized reversed-phase HPLC method for identification of aromatic secondary metabolites from lichens was published by Feige et al. in 1993[13] and is based on a retention index *I* calculated from the elution time of the appropriate peak compared with the standards benzoic and solorinic acids. Such a relative index corresponding to 331 lichen compounds was determined by Feige et al. in 1993[13] and this protocol was successfully applied to several genera of lichens[24]. Mietzsch et al. in 1994[25] developed a computer program used for lichen metabolites identification named Wintabolites. This software made use of HPLC-R_t values, TLC-Rf values, UV/visible colors of TLC spots, and lichen spot tests to identify molecules. The program database included 550 lichen compounds. Derivatization techniques were further published to enable detection of aliphatic lichen acids (e.g., roccellic, rangiformic acids, and lichens terinic acids) by using the same method[44].

Gas Chromatography

Generally volatile compounds are analyzed by using gas chromatography. In this method, there is a gas and a liquid phase. The

liquid phase is stationary whereas the gas phase is a mobile phase. Compounds to be analyzed are also in the mobile phase with a carrier gas which is usually helium, hydrogen or argon. The chemicals are separated depending on the migration rate into the liquid phase. Higher percentage of the chemical will lead to faster migration in the liquid phase. This is widely used in qualitative and quantitative phytochemical analysis. GC was limitedly used in detection and separation of lichen compounds because most of the lichen substances are polar and have low volatility. To solve this problem, prior derivatization is usually required in order to yield volatile enough compounds to be analyzed by GC; derivatization is a process in which a chemical compound is converted into a product of similar chemical structure. Derivatization procedures have been described by Nishikawa et al. in 1973[29] analyzed the low molecular weight carbohydrates of eight lichen species as their acetyl, trifiuoroacetyl and trimethylsilyl derivatives by GC. A hyphenated technique known as GC-MS is also used to detect the lichen chemicals. GC-MS analyzed the sample by separating the chemicals with the help of gas as mobile phase and also define the structure of the chemicals by mass spectroscopy combinedly[44].

CONCLUSION

The evaluation of secondary metabolites of lichen species can be used for the therapeutic purposes. Some innovative techniques can be used for the extracting and separating compounds for the analysis and identification of lichen compounds. The recent advanced techniques such as HPTLC, HPLC, and GC are very effective tools for the detection. The use of analytical chemistry with these given technique makes this procedure more feasible. These methods can be performed efficiently and they purify the samples in a proper way. These improved techniques pave the way for the future metabolite related studies. If we perform these techniques with the advances approach of purification, structure elucidation, and streamlining then may be the procedure of drug discovery become easy. The extracellular substance of lichens is present in various parts of lichen such as hyphae or thalli. Therefore, each part of the thallus has contained some medicinal properties against bacteria and virus including, antioxidant, antiviral, anticancer, antigenotoxic, anti-inflammatory, analgesic and antipyretic activities as well. These properties are easily identified with certain advanced techniques and give a new direction to the medicinal science.

AUTHORS CONTRIBUTIONS

All authors have contributed equally

CONFLICT OF INTERESTS

Declared none

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