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Characterization and Identification of the Components Extracted from 28 Lichens in Tunisia by High Performance Thin-Layer Chromatography (HPTLC), Morphologic Determination of the Species and Study of the Antibiotic Effects of Usnic Acid

Karima Tabbabi* and Tijani Karmous

Laboratory of Natural Substances, Faculty of Sciences of Bizerte, University of Carthage, Bizerte, Tunisia

Abstract

This aim of this work is to study the isolation of usnic acid found in many lichen species and the evaluation of its antibacterial activity. The study began with a morphological determination of lichens harvested in Tunisia. The corresponding analysis identified 28 species belonging to the following families: *Xanthoria, Parmelia, Caloplaca, Ramalina, Diploschistes, Usnea*. A chromatographic study of the chemical composition of these lichens highlighted the presence of many compounds belonging to various chemical categories: depsides and depsidones, xanthones, anthraquinones, dibenzofurans, etc. Special attention was given to the components of this last category and particularly to usnic acid which is therapeutically very interesting. This component plays a very important role in fighting the bacteria responsible for numerous urinary and pulmonary infections and also in fighting viruses responsible for certain tumors. Antibacterial activity tests showed that the *Staphylococcus aureus* and *Enterococci* strains have certain sensitivity to usnic acid extracted from the *Usnea hirta* lichen. Given that various fields, and especially medicine, are currently showing considerable interest in vegetable extracts, this study will be continued in the future with the aim to isolate other components with efficient therapeutic effects.

Keywords: Lichen; *Usnea*; Chromatographic analysis; Chemical composition; Usnic acid; Antibacterial activities

Introduction

The diversity of the geographical and climatic conditions of Tunisia favors the development of a flora rich in aromatic and medicinal plants. The use of these plants attracts much attention especially given the increasing interest of many sectors such as the cosmetic and food [1-3] industries, and herbal medicine [2,4,5]. These industries are keen to incorporate natural compounds considered non-polluting and harmless in their formulations.

The exploitation of this natural wealth essentially involves the isolation of bioactive compounds such as alkaloids, flavors, essences, essential oils, etc. [6,7].

In order to contribute to the promotion of medicinal and aromatic plants a physico-chemical and morphological study was performed of a poorly-exploited but abundant plant species: lichens. This plant results from a symbiotic association between fungi and algae and grows on various media: soil, rocks, tree barks, etc. [8]. The lichen constituents are astonishing for many reasons. Some of them provide highly-sought-after fragrances [9,10] while others are very effective as antibiotic drugs [2,7]. Others are the main food of reindeer in Scandinavia.

In addition to their dyeing properties [11-13], all lichens are pollution indicators [14-17] which is sometimes the reason that prevents them from surviving.

This study focuses on Tunisia lichens which contain active principles. These are characterized by a dibenzofuran structure. Usnic acid is the most widely known bioactive compound [18-21] and so will be isolated in order to peRform an antibacterial activity test.

Tunisia has a batch of 395 lichens. Many works predict the existence of other species not yet detected in southern Tunisia. The appearance of this flora is essentially Mediterranean and most lichens belong to the squamulose and fruticose families [22-26].

Hundreds of samples were collected and most were subjected to morphological and chemical analyses through specific and chromatographic tests. Lichens rich in usnic acid were studied. The latter was isolated and a study of its antibacterial effect was conducted.

Usnic acid has been recognized as having significant activity against bacteria which are the cause of many ailments. It is characterized by its activity against *Streptococcus mutans* [27] responsible for dental lesions; against *Staphylococcus aureus* [28,29] which secretes toxins and enzymes that cause necrosis states and sepsis and *Trichomonas vaginalis* [30] that causes urinary tract infections.

We believe it is important to focus on the morphology of lichens since it is decisive in the identification of lichen substances [22]. Throughout the study, the specific terms relating to the botanical description of lichens are given extra importance given that they play a role in the understanding of the morphological study [23]. The nature and component structures characterizing the lichens are also mentioned due to their utility in the interpretation of results.

Materials and Methods

The lichen material

The lichens used in this study were collected from different regions

***Corresponding author:** Karima T, Laboratory of Natural Substances, Faculty of Sciences of Bizerte, University of Carthage, Zarzouna 7021, Bizerte, Tunisia, Tel: 0021652800776; E-mail: tabbabikarima@yahoo.fr

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around Tunisia; the north of the country: Sejnane, Nefza, Djebel Ichkeul, Hammam-Lif, Ras Jebel, Al Alia; the central region: Makthar, Siliana and Zaghouan; and the south: Gabes, Mareth.

The lichens corresponding to these samples are fixed on various media: soil and rocks (limestone, granite, etc.), shrubs (Rosemary, retama, etc.) and trees (Fig tree, olive, eucalyptus, Aleppo pine, carob tree, etc.).

The morphological identification of the lichens collected required a microscopic examination of the fragments or the apothecial sections. Certain thallus characteristics led to similar observations, especially when looking for the cellular cortex.

Analytical methods

Morphological analysis: In order to determine the different species, a microscopic examination of the fragments or the apothecial sections was required. These were mounted between the microscope cover slip and the slide [8]. In any morphologic characterization, the observation of the cellular cortex, the number, color and shape of the spores per ascus are the features of interest.

In order to examine the sections, a Carl Zeiss Jena microscope Med 2, which can perform magnifications of up to x 1000, was used. To observe the whole thallus, a Bruogg WLDM3 camera with a magnification power of up to 160 x was used.

Color tests: Color reactions occur during the determination of lichen species and when identifying lichen substances. These reactions can be observed by the naked eye and involve various tests, with each test using a suitable reagent.

These tests call for the following reactants:

- **Test C** employs a saturated aqueous solution of calcium hypochlorite Ca $(OCl_2)_2$ or sodium NaOCl
- **Test K** employs a potassium hydroxide KOH aqueous solution of about 10%
- **Test PD** uses a paraphenylenediamine ethanoic solution of between 1 and 5%
- **Test KC** uses the reagents of tests C and K applied successively.

The reagent for each test is either applied using a micropipette onto the lichen thalli studied or sprayed onto chromatographic plates developed to reveal the eluted lichen substances.

The appearance of color immediately after the execution of a test indicates the suitability of said test and is noted as K^+, C^+, PD^+, KC^+ depending on the reagent used, otherwise it is noted as K^-, C^-, PD^-, KC^-.

Analysis by high performance thin-layer chromatography: The best method for the identification of lichen substances is the thin-layer chromatography (TLC) described by Culberson and Kristensson [24] (a standardized technique which later underwent certain modifications). It involves the use of three solvent systems, silica plates ready for use and two control substances (atranorin and norstictic acid). The control substances are chromatographed simultaneously with the studied lichen extract.

High performance thin layer chromatography (HPTLC) [25], which can be used to study lichen substances [26], is used in this study. This technique has several advantages over TLC: a faster elution, it is more sensitive, a smaller amount of solvent is required and there is the possibility to elute double the number of samples [27].

This method was applied while using the experimental conditions recommended by Culberson and Ammann [28] for TLC, namely three solvent systems and two control substances.

Each substance is identified by its retention index (Rf) in three respective eluents. For every eluent, the comparison of the sample Rf with those of the controls allows to define the Rf class for the corresponding eluent.

The characterization of the classes is as follows: the starting line defines class 1; the displacements of the norstictic acid (N) and atranorin (A) determine classes 4 and 7 respectively. The distances between these classes, divided into two, yield two new classes: 2 and 3 between 1 and 4; and 5 and 6 between 4 and 7. Beyond class 7, class 8 is found which reaches up to the front of the eluent.

Thus, a class is assigned for each lichen compound chromatographed in each of the three elution systems. These attributions are also based on comparisons made with reference control samples extracted from specific lichens such as usnic acid extracted from *Usnea barbata*, lecanoric acid from *Parmelia glabratula*, etc. as well as the two controls recommended by the method described by Culberson et al.

Aliphatic acids are revealed by their thickness which makes spotting them easy through the appearance of big marks at the bottom of the plate, moistened with water, just after their development. Identification is achieved by comparing their Rf parameters with those of the reference products extracted with acetone from specific lichens, each containing a known aliphatic acid: caperatic acid in *Plastimatia glauca*, rangiformic acid in *Cladonia rangiformis*, bourgeanic acid in *Cladonia conista* and roccellic acid in *Roccella tinctoria*.

Other parameters intervene in the identification of an analyzed substance, such as its color, its fluorescence under UV before and after revelation, the results of the coloring tests with potassium hydroxide (test K), bleach (test C), and the two previous reactants applied successively (test KC), paraphenylenediamine (test PD) as well as the evolution of color with time [29].

According to these data, tables proposed by Culberson and Kristensson [24], Culberson [26], Culberson [30], White and James [31] allow to select a number of identification possibilities for the substance being considered.

The samples were prepared, processed and chromatographed according to the description given in the following preparation section:

Extraction of lichen substances: A minimum volume of (0.4 ml) of acetone (Merck for analyses) was added to 70 mg of dry lichen, corresponding to approximately a surface of 1 cm^2.

Control substances: the norstictic acid and atranorine contained respectively in lichens *Parmelia acetabulum* and *Plastismatia glauca*. These lichens each contain a single unique major component.

Reference controls: Reference products extracted with acetone from specific lichens each containing a known lichen substance: erythrin in *Dirina stenhammari*, fumarprotocetraric acid in *Cladonia coniocracea*, protocetraric acid in *Ramalina farinacea*, glomelliferic acid in *Parmelia loxodes*, salazinic acid in *Parmelia reticulata*, lecanoric acid in *Parmelia glabratula*, gyrophoric acid in *Ochrolechia androgyna*, psoromic acid in *Schismatomma niveum*, arthothelin in *Ochrolechia inversa*, divaricatic acid in *Haematomma ventosum*, stenosporic acid in *Ramalina stenespora*, diploïcin in *Buellia canescens*, usnic acid in *Usnea barbata*, parietin in *Caloplaca ferruginea*, gangaleoïdin in *Lecanora*

ganglaeoïdes, zeorin in *Cladonia coccifera*, 2-o-diméthylpsoromic acid in *Scherophyton circumscriptum*, granulosin in *Buellia granulosa*, 4,5-dichloronorlichexanthon in *Lecanora straminea* and chloroatranorin in *Parmelia physodes*.

Chromatographic plates: Silica gel plates 60 F over 20 × 10 cm (Merck) glass dried at 50° C for 5 min just before development. These plates differ from those used in TLC in the thickness of the adsorbent (0.20 mm instead of 0.22 mm) and the particle size of the silica gel (4 to 8 microns).

Deposit: deposits of analytes, spaced 5 mm from each other, formed 5 mm from the two edges along the length of the plate.

Three-solvent system:

A: Toluene-dioxane- ethanoic acid (18/06/0.8 by vol).

B: n-hexane-diethylether- methanoic acid (13/10/2 ml by vol).

C: Toluene- ethanoic acid (20/3 by vol).

10 ml were used for conditioning the plate and 4 ml for its development.

Developing chamber: Development was performed in a horizontal developing chamber (Camag) for HPTLC.

Elution: before eluting, the plate was placed in the developing chamber and saturated in the corresponding solvent vapors for 5 minutes. During migration, the solvent moved in the opposite direction starting from the edges. The elution was stopped when the two fronts met.

Revelation: three revelations were applied [32]:

- The examination of the plates before and after development was carried out under visible light and under UV light (λ=360 nm) of the Camag lamp.
- The impregnation of the plates with 10% sulfuric acid followed by heating at 100-110°C for 10 min allows to specifically stain the separated marks.
- Visualization by spraying the color test reagents.

The aliphatic acids were identified by their bold type immediately after their elution without any revelation; this led to their disappearance.

Extraction and analysis of usnic acid

Species containing usnic acid: The study of lichens collected by high-performance thin-layer chromatography identified usnic acid along with other components in the following 8 lichens: *Cetraria aurescens* (L_5), *Cladonia foliacea* (L_6), *Evernia divaricata* (L_{12}), *Lecanora muralis* (L_{13}), *Lecanora campestris* (L_{14}), *Parmelia caperata* (L_{19}), Parmelia *somlesciens* (L_{21}), *Squamarina cartilaginea* (L_{26}). The chromatographic analyses confirmed the presence of usnic acid as the single component in *Usnea hirta* (L_{25}). This species is used to extract usnic acid to be used in the study of antibacterial activity (Figures 1 and 2).

Extraction of usnic acid: A Soxhlet apparatus was used for the extraction of usnic acid by solvent from lichens rich in this therapeutic principle. *Usnea hirta* (L_{25}) fragments were collected and dried in air. 100 g of this lichen were heat-treated with 150 ml of hexane in a Soxhlet apparatus. The extraction time was 6 h and slurping was done every 10 minutes. The mixture collected was concentrated under vacuum and the residue was subjected to purification on a chromatographic column filled with silica gel. The elution used the chloroform -n- hexane solvent system (80/100 by volume). At the end of this process, an amount of 930 mg of usnic acid was obtained with a yield of 0.93%.

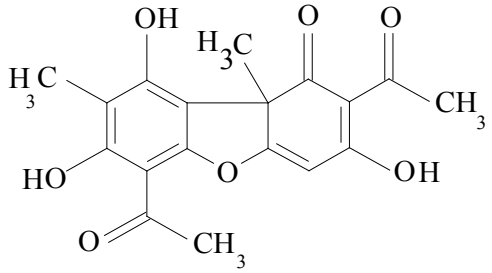

Figure 1: Usnic acid.

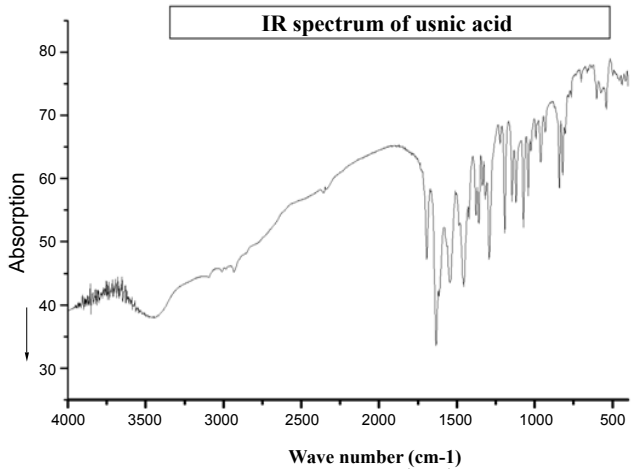

Figure 2: IR spectrum of usnic acid.

Study of usnic acid antibacterial activity: The laboratory of the Bizerte Regional Hospital helped in the study of the antibacterial activity of usnic acid on bacteria responsible for the infectious state of some patients.

The *in vitro* evaluation of the antibacterial activity was performed using the liquid medium dilution technique. The method involves the preparation of a series of usnic acid solutions with decreasing concentrations from a stock solution having a concentration of 5 mg/ml. The same amount (2 ml) of bacterial suspension was added to each solution containing germs whose behavior towards the usnic acid was to be studied.

The mixtures thus prepared were incubated at a suitable temperature (37°C) for 24 h. Once incubation was complete the solutions were inspected with the naked eye. Solutions with disorders corresponded to the non-inhibition of the strain with usnic acid. The first tube of the prepared series, where no disorder was observed, would provide the minimum inhibitory concentration (MIC).

Results and Discussion

Morphology

The *Xanthoria parietina* species (L_{27}) was identified and which is dominant lichen in the Mediterranean climate and highly resistant to pollutants (fluorine, lead, etc.). It is extracted in areas located along the coast (Tunis, Cap Bon, Sahel, etc.) and inland (Kef, Makthar, etc.).

Growing on various supports, (soil, tiles, trees, stone) it belongs to the foliose family. The thallus has a circular shape, limits which are slightly detached and an orientation-dependent color. Sunshine promotes orangey-green lichens while grayish-green lichens are favored by shade.

This lichen is characterized by its color reaction K^+ purple-red and for being rich in parietin. The morphological results also showed the *Parmelia* (*Parmelia cetrata* L_{18}, *Parmelia caperata* L_{19}, *Parmelia pulla* L_{20}, *Parmelia somlasciens* L_{21}) and *Ramalina* (*Ramalina lacera* L_{23}) families, foliose and fruticose lichens respectively, widespread in regions where rainfall is abundant (Sejnane, Djebel Ichkeul). Almost all of these lichens are arboreal but calcifuges.

The *Parmelia* can develop usnic acid accompanied with other substances belonging to the depside, depsidone and anthraquinone classes. The divaricatic acid is the main substance of *Ramalina*.

Most *Cladonia* (*Cladonia foliacea* L_6, *Cladonia rangiformis* L_7, *Cladonia cartilaginea* L_8) lichens identified were squamulose. This species is ubiquitous in Tunisia. These lichens are mostly soilborne and calcifuges [8], growing on tree trunks. Many substances developed by the *Cladonia* play an important role in the determination of species because of the color reactions which they give rise to. The *Cladonia* can develop usnic acid whose presence depends on the medium (support, sunshine, humidity, etc.). The morphological study led to the identification of two groups of very wide-spread crustaceous lichens in regions of high humidity (littoral, north). The first group is represented by *Diploïcia* L_9, *Lecanora* L_{13}, L_{14} and *Lecidella* L_{16}, all growing exclusively on trees. *Diploschistes* L_{10} and *Caloplaca* (*Calplaca saxicola* L_2), occurring in soil and rocks, form the second group. These crustacean lichens can develop substances belonging to different chemical families: parietin, atranorin, etc.

It must be noted that during this characterization, lichen belonging to the Usnea genus was found. This was *Usnea hirta* (L_{25}) collected in the Sahel area. This terricolous lichen is rich in usnic acid. Confirmation of this hypothesis was provided by a chromatographic analysis. Results are presented in the Table 1.

High-performance thin-layer chromatography (HPTLC)

The analysis of Tunisian lichens by HPTLC using similar conditions to those for the TLC method recommended by Culberson and Ammann [31] led to the results shown in Table 2.

The analysis of each lichen studied by HPTLC consisted in carrying out a comparison of Rf obtained by the three elution systems with control chromatographic data atranorin and norstictic acid. Moreover, the allocation was based on the results of the action of the various indicators used (UV, H_2SO_4, potassium hydroxide, hypochlorite, etc.) and on the values of the retention indices indications measured compared with those of the reference products.

For all the species chromatographed, the isolated lichen substances were thirty two. They belong to the following chemical classes:

- Xanthones represented by arthothelin, granulosine, 4,5-dichloronorlichexanthone and thiophanic acid. These substances are present in *Lecidella elaeochroma* (L_{15}) and

Lichen ref	Species of lichen	Support	Places of sampling	Description and characteristics of the species
L_2	*Caloplaca aurantia*	Limestone	Djebel Ichkeul	- Lichen orange-red around the edges characterized by whitish parts - Owns numerous apothecia orange color - Lichen nitrophile
L_6	*Cladonia foliacea*	Ground	Sejnane	- Squamulose lichen pushing especially on medium not limestone or limestone little -White thallus, xerophile non orophile - Always as on floor mats
L_7	*Cladonia rangiformis*	Ground	-Nefza -Boukornine	- Lichen soil-borne, small thallus - Has green-gray color podetions length 2 to 12 cm -Podetions branched, rigid and brittle
L_8	*Cladonia cartilaginea*	- Ground -Soft rocks -Trees	Boukornine	-Lichen squamulose or foliacea -Has long podetions of several cm -Granular thallus, cracked, sometimes mealy -Apothecia rare, yellowish or pink scarlet red, single-celled spores -Lichen usually calcifuge, and soil-borne humicole
L_9	*Diploïcia canescens*	Cork	Sejnane	- Lichen crustacean, sometimes blue-gray whitish gray, lobbed the periphery - Apothecia thin edge - Lichen nitrophile, saxicole, lignicolous or corticolous
L_{10}	*Diploschistes gypsaseus*	Ground	Sahel (Zarmdine)	- White lichen thallus, floury appearance - Apothecia not embedded in the thallus, with black discs and thallin thick edge
L_{13}	*Lecanora muralis*	Rock	Kef	- bright green crustacean lichen thallus, markedly lobed on the outskirts, fendrilled-aeroled - Attached directly to the support, owns numerous greenish-brown color apothecia - Nitrophile lichen growing on the most diverse substrates
L_{14}	*Lecanora campestris*	Eucalyptus	Sejnane	- Lichen crustacean non lobbed the periphery light gray - Thallus with brown apothecium with clear edge -Corticolous lichen
L_{16}	*Lecidella Carpathica*	Ground	Sejnane	- White lichen thallus sometimes greenish, granular, cracked - Flat or slightly convex apothecia, evergreen edge -Thallus C^-, calcifuge, nitrophile
L_{19}	*Parmelia caperata*	Cork	Sejnane	- Foliose lichen growing on the trunks, branches and old trees but rarely on rocks and soils -Greenish thallus shaped rosette, strong adhesion to the substrate -Lichen photophile, very sensitive to pollution
L_{20}	*Parmelia pulla*	Rock	Sejnane	-Foliose Lichen thallus brown or greenish brown, strong adhesion to the substrate - Apothecia always present with flat or concave disk same color to thallus
L_{21}	*Parmelia somlesciens*	Rock	Boukornine	-Foliose greenish lichen, non calcicole, just stick to the substrate, elongated or not lobes -Very common lichen
L_{23}	*Ramalina lacera*	Cork	Sejnane	-Fruticose lichen thalloid drawn shaped strips of 1 to 10 cm high, highly branched green-whitish - Growing on bark, in wood, non-limestone in very humid environments
L_{25}	*Usnea hirta*	Tree trunks	Djebel Ichkeul	-Thallus of a clear or yellowish green, very narrowed at base - loose medulla K^-, C^-, PD^- - Soralies abundant on terminal branches

Table 1: Results of the morphological determination of harvested lichens.

Ref	Lichen species	No. of pigments	Rf'/RfN'/RfA'			Rf Class		Color of the task after revelation		UV(λ)=360 nm		Color test	Nature and class of	
			A	B	C	A	B	C		Before revelation	After revelation		Nature	Class
L_1	*Caloplaca aurantia*	1	39/25,38	30/19,31	30/13,28	7	6-7	7-8	Yellow	Orange	Orange	K^+ red	Parietin	e
L_2	*Caloplaca saxicola*	4	30/25,38 39/25,38 34/25,38 32/25,38	25/19,31 30/19,31 27/19,31 23/19,31	17/13,28 30/13,28 16/13,28 25/13,28	5 7 6 6	5-6 6-7 6 5	5 7-8 5 6	Yellow Yellow Orange red Orange	Yellow Red Red Orange	Yellow Orange Red Orange	K^+ red K^+ red K^+ red K^+ red	Emodin Parietin Faccinal Teloschistine	e e e e
L_3	*Acarospora schleïcheri*	1	33/24,37	23/18,30	20/12,27	6	5	6-5	Yellow lemon	Dark red	Brown	_	Rhizocarpic acid	j
L_4	Aspicilia *calcerea*	1	2/24,37	2/18,30	1/12,27	1-2	1-2	1	Yellow	_	Dark green	C^+ red	Erythrine	a
L_5	*Cetraria aurescens*	2	35/24,37 6/24,37	30/18,30 13/18,30	26/12,27 3/12,27	6-7 2	6-7 3	6-7 2	Gray-gren _	Dark _	Olive –	KC^+ yellow K^+ yellow	Usnic Acid Caperatic Acid	f h
L_6	*Cladonia foliacea*	2	3/24,37 35 /24,37	16/18,30 30/18,30	3/12,27 26 /12,27	2 6-7	3 6-7	2 6-7	Gray Gray green	Colorless Derk	colorless Olive	PD^+ red KC^+ yellow	Fumarprotocetraric acid Usnic acid	d f
L_7	*Cladonia rangiformis*	3	4/25,39 3/25,39 39/25,39	16/20,31 14/20,31 31/20,31	4/13,28 5/13,28 28/13,28	2 1-2 7	3 3 7	2 2 7	Gray Fat Yellow orange	Colorless _ dark	Colorless _ Brown	PD^+ red _ K^+ yellow	Fumarprotocetraric acid Rangiformic acid Atranorin	d h c
L_8	*Cladonia cartilaginea*	1	4/25,39	16/20,31	4/13,28	2	3	2	Gray	Colorless	Colorless	PD^+ red	Fumarprotocetraric acid	d
L_9	*Diploïcia canescens*	3	30/25,39 39/25,39 39/25,39	27/20,31 31/20,31 31/20,31	24/13,28 28/13,28 30/13,28	6 7 7	6 7 7	6 7 7-8	Fat Yellow orange Yellow	Colorless Dark Colorless	Colorless Brown Orange	_ K^+ yellow PD^+ orange	Diploïcine Atranorin Chloroatranorin	b c c
L_{10}	*Diploschistes gypsaceus*	1	11/24,38	22/19,31	10/13,28	3	5	3	Gray (A), yellow (B, C)	Colorless	Dark green	C^+ red	Lecanoric acide	a
L_{11}	*Fulgensia fulgida*	1	38/24,38	30/19,31	30/13,28	7	6-7	7-8	Gray-green yellow	Orange	Orange	K^+ red	Parietin	e
L_{12}	*Evernia divaricata*	3	25/24,38 36/24,38 23/24,38	29/19,31 30/19,31 26/19,31	25/13,28 38/13,28 15/13,28	5 6-7 3-4	6-7 6-7 6	6 6-7 5	Orange Gray green Yellow	Gray dark _	Gray Olive Green	C^+ red KC^+ yellow _	Divaricatic acid Usnic acid Evernic acid	a f a
L_{13}	*Lecanora muralis*	6	3/24,37 23/24,37 24/24,37 29/24,37 35/24,37 37/24,37	16/18,31 19/18,31 25/18,31 22/18,31 30/18,31 31/18,31	3/13,27 11/13,27 20/13,27 20/13,27 26/13,27 27/13,27	2 3-4 4 5 6-7 7	3 4-5 5 5 6-7 7	2 4-5 5-6 5-6 6-7 7	Gray Fat Fat-brown Fat-gray Fat-gray green Fat-yellow orange	_ _ Blue-gray _ Dark Dark	Violet _ Brown Pink Olive Brown	PD^+ red _ PD^+orange PD^+ red KC^+ yellow K^+ yellow	fumarprotocétraric acid murolic acid psoromic acid Zéorine Usnic acid Atranorin	d h d i f c
L_{14}	*Lecanora campestris*	3	29/24,37 35/24,37 37/24,37	22/18,31 30/18,31 31/18,31	20/13,27 26/13,27 27/13,27	5 6-7 7	5 6-7 7	5-6 6-7 7	Gray Gray green Yellow orange	_ Dark Dark	Pink Olive Brown	PD^+ red KC^+yellow K^+ yellow	Zéorine Usnic acid Atranorin	i f c
L_{15}	*Lecidella elaeochroma*	3	21/24,38 25/24,38 37/24,38	18/19,31 19/19,31 30/19,31	11/13,28 14/13,28 25/13,28	3 4 7	4 4 6-7	3 4-5 6	_ Orange brown colorless	Orange Red Orange	Orange Red brown Red brown	_ C^+ orange –	4,5-dichloronorli-chéxanthone Arthothelin Granulosin	g g g
L_{16}	*Lecidella carpathica*	3	30/24,38 38/24,38 38/24,38	27/19,31 31/19,31 31/19,31	24/13,28 30/13,28 28/13,28	6 7 7	6 7 7	6 7-8 7	Fat Yellow Yellow orange	colorless colorless Dark	_ Orange Brown	_ PD^+ orange K^+ yellow	Diploïcin Chloroatranorin Atranorin	b c c
L_{17}	*Lobaria pulmonaria*	2	18/24,38 25/24,38	20/19,31 19/19,31	10/13,28 13/13,28	3 4	4-5 4	3 4	Yellow Yellow	_ _	Green Purple	C^+ red K^+ red	Gyrophoric acid Norstictic acid	a a

L_{18}	Parmelia *cetrata*	2	9/25,38 38/25,38	7/19,32 32/19,32	2/13,28 28/13,28	2 7	2 7	2 7	Orange bright yellow-orange	Colorless Dark	Orange Brown	K^+ orange K^+ yellow	Salazinic acid Atranorin	d c
L_{19}	*Parmelia caperata*	4	6/25,38 2/25,38 36/25,38 38/25,38	13/19,32 12/19,32 31/19,32 32/19,32	3/13,28 2/13,28 26/13,28 28/13,28	2 1 6-7 7	3 3 6-7 7	2 2 6-7 7	fat Dark gray Gray-green Yellow orange	– – Dark Dark	– Purple Olive Brown	K^+yellow PD^+orange KC^+yellow –	Caperatic acid Protocetraric acid Usnic acid Atranorin	h d f e
L_{20}	*Parmelia pulla*	4	18/24,38 26/24,38 25/24,38 23/25,38	20/19,31 23/19,31 30/19,31 23/19,31	10/13,27 22/13,27 26/13,27 26/13,27	3 4-5 5 5	4-5 5-6 6-7 6-7	3 6 6 6	yellow Pale yellow Orange Yellow	– Light blue Gray –	green Bleu purple Gray Purple	C^+red C^+red C^+red C^+red	Gyrophoric acid glomelliferic acid divaricatic acid stenosporic acid	a a a a
L_{21}	*Parmelia somlesciens*	4	9/24,38 25/24,38 36/24,38 33/24,38	7/19,31 19/19,31 28/19,31 31/19,31	1/13,27 13/13,27 25/13,27 27/13,27	2 4 6-7 7	2 4 6-7 7	1-2 4 6-7 7	Bright orange Bright yellow Gray green Yellow orange	– – Dark Dark	Orange Purple Olive Brown	K^+red K^+red K^+yellow K^+yellow	Salazinic acid Norstictic acid usnic acid Atranorin	d d f c
L_{22}	*Pertusaria flavida*	2	18/24,38 33/24,38	6/19,31 27/19,31	9/13,27 25/13,27	3 6	2 6	3 6	Orange Pale orange	– Red orange	Orange Brown	PD^+orange K^+yellow C^+orange	Stictic acid Thiophanic acid	c g
L_{23}	*Ramalina lacera*	2	32/24,37 25/24,37	27/18,31 30/18,31	20/13,27 23/13,27	5-6 4-5	6 6-7	5-6 6	– Orange	– Gray	– Gray	– C^+red	Bourgeanique acid Divaricatic acid	h a
L_{24}	*Roccella tinctoria*	2	2/24,37 11/24,37	2/18,31 21/18,31	1/13,27 9/13,27	1-2 3	1-2 5	1 3	Yellow Gray (A), yellow (B, C)	– –	Dark green Dark green	C^+red C^+red	Erythrin Lecanoric acid	a a
L_{25}	*Usnea hirta*	1	34/24,37	30/18,31	27/13,27	6-7	6-7	6-7	Gray-green	Dark	Olive	KC^+yellow	Usnic acid	f
L_{26}	*Squamarina cartilaginea*	3	17/24,37 24/24,37 35/24,37	18/18,31 22/18,31 30/18,31	7/13,27 18/13,27 27/13,27	3 4 6-7	4 5 6-7	2 5 6-7	Brown Dark brownGris-Green	Colorless Gray-blue dark	Yellow brown Brun Olive	– PD^+red KC^+yellow	Acide 2-O-diméthyl-psoromique Psoromic acid Usnic acid	d d f
L_{27}	*Xanthoria parietina*	1	31/24,37	28/18,31	32/13,27	7	6-7	7-8	Yellow	Orange	Orange	K^+red	Parietin	e
L_{28}	*Psora decipiens*	0	–	–	–	–	–	–	–	–	–	–	–	–

*: R_f'=Rfx100, R_fN'=RfNx100, R_fA'=RfAx100 are the respective pretensions of the factors studied pigment and witnesses (norstictic acid and atranorin).
**: Elution solvents: A=Toluene-dioxane-acetic acid (180/60/8), B=n-Hexane-ethyl ether - formic acid (130/100/20), C=Toluene- acetic acide (200/30).
***: Color tests: K (Potasse), C (Hypochlorite), PD (Paraphenylenediamine).
****: Chemical class of the pigment: a=Depside α-orsellic, b=Depsidone α-orsellic, c=Depside β-orsellic, d=Depsidone β-orsellic, e=Anthraquinone, f=Dibenzofurane, g=Xanthone, h=Aliphatic acid, i=Triterpene.

Table 2: Results of the characterization and identification of the components extracted from 28 lichens in Tunisia by high-performance thin-layer chromatography (HPTLC).

Pertusaria flavida (L_{22}). Xanthones are yellow dyeing substances.

- Anthraquinone derivatives such as parietin seen in *Xanthoria parietina* (L_{27}), *Fulgencia fulgida* (L_{15}) and *Caloplaca aurantia* (L_1). The parietin is a dye which is distinguished by its resistance to washing and light.
- The depsides (atranorin, erythrin, lecanoric acid, divaricatic, stenosporic and gyrophoric acids…) and the depsidones (salazinic, psoromic, protocetraric, fumarprotocetraric acids…) were detected in lichens L_7, L_9, L_{12}, L_{13}, L_{17}, L_{20}, L_{21}, L_{22}. The depsides and depsidones are known for their dyeing power.
- Dibenzofuran derivatives were represented in lichens L_5, L_6, L_{12}, L_{13}, L_{14}, L_{19}, L_{21} and L_{25} by usnic acid which has antibiotic properties.

The 28 species concerned differ in their composition in lichen substances except the *Xanthoria parietina* (L_{27}), *Fulgensia fulgida* (L_{11}) and *Caloplaca aurantia* species containing the same component, namely parietin. Other species also contain a single lichen component: rhizocarpic acid in *Acarospora Schleïcheri* (L_3), erythrine in *Acarospora Schleïcheri* (L_4) and usnic acid in *Usnea hirta* (L_{25}). No lichen substance is identified in Psora decipiens (L_{28}).

In the studied lichen, other components were identified; these components were fatty acids (caperatic, murolic, rangiformic, bourgeanic acids, etc.) and a triterpene (zeorine). Usnic acid was the only component of *Usnea hirta*. This lichen served as support to extract the usnic acid.

Isolation and characterization of usnic acid extracted from the *Usnea hirta* lichen

Several analytical methods have been used to identify the purified extracts obtained from the *Usnea hirta* lichen. Note that the molecule of usnic acid has the following structure:

Analysis by infrared spectroscopy: From the IR spectrum of the purified compound, the allocation of the strips collected were characterized by the following vibrations: 3433 cm^{-1}=ν_{OH}, 1683 cm^{-}

^{1}=$\nu_{(C=O)Ar}$, 1600, and 1533 cm^{-1}=$\nu_{(C=C)Ar}$, 1300-1283 cm^{-1}=$\nu_{(C-O-C)}$, a series of bands between 3083 and 2916 cm^{-1}=$\nu_{(C-H)Ar}$ et $\nu_{(C-H)Aliph}$. These vibrations and their wave numbers are similar to those reported in the literature [33] for usnic acid. All these data confirm that the isolated and purified sample is indeed usnic acid.

Analysis by ultraviolet spectroscopy: The extract of *Usnea hirta* provided a UV-visible spectrum with two bands of neighboring intensities. The first band was found at λ_{max}=230 nm and the second wavelength at λ_{max}=280 nm. This is probably a $\pi \rightarrow \pi^{*}$ transition of the conjugated system. The band at λ=280 nm can be explained by the strong combination of the system given its strong molar absorption coefficient ε. This band can only correspond to a $\pi \rightarrow \pi^{*}$ transition because if this were an $n \rightarrow \pi^{*}$ transition, a much lower ε less than or equal to 50 would be obtained. Comparing the results to bibliographic data [34], the UV spectrum of usnic acid has two bands at wavelengths λmax=230.9 nm and λmax=280.3 nm respectively. The similarity of the two spectra represents a first argument supporting that the sample under study must be usnic acid.

Results of the usnic acid antibacterial activity study extracted from Usnea lichens

The results of the antibacterial activity tests showed that the *Staphylococcus aureus*, *Escherichia coli, Klebsiella pneumoniae* and *Enterococcus faecalis* strains are very sensitive to the gentamicine antibiotic (40 mg/ ml) at dilutions of 1/256. The inhibition zones ranging from 25 to 48 mm prove the high antibacterial activity of the drug used as reference.

The tests revealed that bactericidal concentrations of usnic acid extracted from the *Usnea hirta* lichen are 1/64 for *Staphylococcus aureus*, 1/32 for *E. coli* and *Klebsiella pneumoniae* and 1/8 for *Enterococcus faecalis*. Thus, the sensitivities differ from one bacterium to another.

The inhibition zones ranged from 12 to 24 mm for *Staphylococcus aureus*, 10 to 14 mm for *E. coli* and *Klebsiella pneumoniae*. A low sensitivity was assigned to the *Enterococcus faecalis* since its zones of inhibition ranged from 3 to 7 mm.

The effect of concentration was not well proven given that the inhibition zone does not provide an increasing diameter depending on the usnic acid content. This preliminary study should also be performed on other bacterial strains.

Conclusion

In this work, lichens having the ability to develop some components with significant therapeutic effects have been studied. A morphological identification of the lichens collected from various regions of Tunisia was first made since the chemical composition varies with the plant species. This determination identified some lichen species: *Parmelia, Crustaceans, Cladonia, Diploschistes,* etc.

The chromatographic analysis revealed the existence of various compounds in these species belonging to the following chemical classes: xanthones, anthraquinones and dibenzofurans. This identification confirmed the presence of usnic acid mainly in *Usnea hirta*, a dibenzofuranic component that can have therapeutic effects. The study of the antibacterial activity revealed that three strains showed sensitivity to usnic acid. These were *Staphylococcus aureus, Escherichia coli* and *Klebsiella pneumonaea*. This result prompted us to resume the tests of this study to determine the parameters that affect the bacterial activity.

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