December 30, 2019

PharmacologyOnLine

Archives • 2019 • vol.3 • 28-35

EVALUATION OF ANTIOXIDANT CAPACITY AND ANTIFUNGAL ACTIVITY OF THE TOTAL ETHANOLIC EXTRACT AND FRACTIONS FROM *Teloschistes exilis* (Michx.) Vain.

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Abstract

*Teloschistes exilis (*Michx.) Vain is an underexplored lichen. In the last decades, there has been a growing interest for the study of lichenic biodiversity, as a source of new natural products, because these produce potential secondary metabolites for pharmacological and industrial uses, highlighting their antioxidant and antifungal properties. For that reason, this study evaluated the antioxidant capacity with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical (ABTS*+ assay) and the antifungal activity of the total ethanolic extract (EtOH) and different polarity fractions from *Teloschistes exilis* (Michx.) Vain against *Penicillium digitatum* and *Aspergillus niger* using disk diffusion technique. According to antioxidant activity, the best-obtained value was for the EtOH with 37.58 mg/L MeOH, with ascorbic acid. In terms of antifungal activity, for equivalent sensitivity, against *Penicillium digitatum*, for each mg of ketoconazole, 213.4 mg of Petroleum ether fraction were required, while for each mg of fluconazole, 228.3 mg of Petroleum ether fraction were required. Regarding to antifungal activity against *Aspergillus niger,* for each mg of ketoconazole, 465.6 mg of Petroleum ether fraction are required, while for each mg of fluconazole, 174.6 mg of Petroleum ether fraction are needed. The highest polarity solvents have a better relative antioxidant activity in relation with standard solutions, whereas susceptibility tests applied for *Aspergillus niger* and *Penicillium digitatum* showed the best antifungal activity for the low polarity fraction of Petroleum ether for both controls. Considering that are fractions, the antioxidant and antifungal potential of *Teloschistes exilis (*Michx.) Vain should be elucidated in posterior studies.

Keywords: lichen, secondary metabolites, *Teloschistes exilis* (Michx.) Vain, antioxidant, antifungal.

Introduction

*Teloschistes exilis (*Michx.) Vain is a lichen specie, that belongs to Teloschistaceae family, placed between 2.400 and 2.900 meters above sea level, registered in Colombia [1], Argentina [2], Ecuador [3], Brazil [4], as well as other neotropics like Cuba [5], Mexico [6] and Costa Rica [7]. This lichen exhibits beard and horsehair talus, terete and small, of 2-3 cm of diameter approximately, with brilliant lacinias and orangey in the ends due to the presence of anthraquinones in cortex. When they are fertile, branches are narrow and rounded, forming apothecians of intense orange color, without marginal fibers nor cilia and soredios [2]. Lichens are defined as obligated self-supporting mutualism organisms between an exhabitant fungus (the mycobiont from *Ascomycota or Basidiomycota*) and one or more extracellularly located inhabitants, which can be either unicellular or filamentous photoautotrophic partners (the photobiont: alga or cyanobacterium) [8,9]. They biosynthesize a wide spectrum of secondary metabolites and polysaccharides because of his chemical structure, which exhibits biosynthetic pathways with derivatives as acetyl polymalonyl and mevalonic as well as shikimate [10]. Most of the secondary metabolites have biological activity as antibiotics, antifungal, antiviral, anti-inflammatory, analgesics, antipyretics, anti-proliferative and cytotoxic, even as antioxidant [11-13]. For example, treatment with usnic acid and zinc sulfate foster the reepithelialization and reduce the recurrence of human papilloma virus [14], while antifungal properties of *Leptogium cyanescens, Physcia americana* and *Pyxine aff. Cocoes* in potato fungi have been reported, reaching 100% inhibition of *Phytophthora Nicotiana* with *Physcia americana* [15]. There are studies from Teloschistaceae family, using *Caloplaca* species, finding wide spectrum antifungal and antibacterial properties against pathogens that affect plants and humans like *Bacillus, Staphylococcus aureus, Candida albicans, Trichophyton mentagrophytes, Epidermophyton floccosum* and *Trichoderma harzianum,* with better results with anthraquinones purification, suggesting that inhibitory activity is related to them [16]. Søchting et al., studied numerous secondary metabolites identifying 150 specimens from 29 species of *Telochistes,* detecting 7 anthraquinones: emodin, telochistin, falacinal and parietinic acid, also vicanicin depsidones, caloploicin and isofulgidine [17]. Furthermore, polyketides, usnic acids, chromones, depsides and dibenzofuranes have been detected too [18]. For these properties, there is a growing interest for the study of this underexplored organisms as source of new natural products [19].

Colombia is the richest neotropical country with 1.700 lichens and 1.396 of them are in the Andean or Cordillera region, hosting the highest concentration of biodiversity [20]. Taking advantage of this and highlighting that information about *Teloschistes exilis* (Michx.) Vain is limited, we determined the antioxidant and antifungal activity of *Teloschistes exilis* (Michx.) Vain, from EtOH and their fractions of increasing polarity for possible applications in medicine and food industry fields.

Methods

Collection of samples

The lichen material was collected in municipalities of Une and Chipaque, in the department of Cundinamarca, Colombia (N 4° .39' 30", O 74.00' 77" and N 4° 26' 58" O 74° 03' 28") respectively. Fifty grams of *Teloschistes exilis* (Michx.) Vain were collected and during a week, the specimens were at open air-drying. Then, the specimens were taken to the Cryptogams section of the Curatorship of the UDBC

Forestry Herbarium of the Colombian Lichenology Group (GCOL) at the Francisco José de Caldas District University in Bogotá, where Dr. Bibiana Moncada, Ph.D., identified them as *Teloschistes exilis* (Michx.) Vain.

Preparation of extracts and fractions

50 g of *Teloschistes exilis* (Michx.) Vain. were pulverized in a blade mill. After, it was extracted in a soxhlet extraction system with ethanol as solvent, using solid/liquid extraction with EtOH at 78.37 °C, for 8 days. Over 15 days, EtOH was fractionated by liquid/liquid method with increasing polarity solvents as Petroleum ether at 30-40 °C, Dichloromethane at 39.6 °C, Ethyl acetate at 77.1 °C and MeOH 64.7 °C. Once EtOH and fractions were obtained, they were concentrated in reduced pressure in the Rotary evaporator (IKA RV® 10 CONTROL), at 40 °C and 60 rpm. Next, the extract and the fractions were place in water bath at 40 °C until dryness. Finally, they were preserved in glass flasks in refrigeration.

Antioxidant activity

The 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) cationic radical assay ($ABTS^{*+}$) [21] was used. The standard solutions used were Ascorbic acid, Rutin and Trolox. 50 milligrams of $ABTS^{*+}$ were dissolved in 50 ml of deionized water and 2.45 mg of potassium persulfate were added. The solution was left to react at 3 °C for 48 hours without light and next, solutions were prepared till reach an absorbance of 0.750 ± 0.050 with a wavelength of 745 nm. For standard solutions, 10 mg were dissolved in 100 ml of MeOH and dilutions of 1, 2, 2.5 and 3 ppm were prepared for Ascorbic acid and Trolox while 3, 5, 10 and 20 ppm for Rutin. For standard solutions from EtOH and the fractions of *Teloschistes exilis (*Michx.) Vain, stock solutions of Petroleum ether, Dichloromethane, Ethyl acetate and MeOH were prepared, in a concentration of 1.000 mg per liter of MeOH. Of each sample, dilutions from 50 to 400 mg were prepared to get 10-95% of absorbance. Later, initial absorbance was measured each 30 seconds during 15 minutes at 754 nm adding a glass cell with volume between 950 and 600 µL of $ABTS^{*+}$ radical. After that, 50 mg per liter of MeOH neither from extract nor fraction of *Teloschistes exilis* (Michx.) Vain were taken to evaluate antioxidant activity.

Antifungal activity

The Kirby – Bauer (disk diffusion) method was implemented, using Saboraud dextrose agar as growing medium. The microorganisms *Penicillium digitatum* and *Aspergillus niger* were used. The inoculum preparation was made taking a part of each fungus and then they were transferred to a test tube of 3-4 mL with saline solution 0.85% w/v., creating a settlement in the stock that must have a turbidity similar to pattern 5 in Mac Farland standard ($1{,}5 \times 10^9$ u.f.c/mL). After, obtained inoculums were sowed with a swab adding 0.1 ml in agar surface in petri dishes for *Penicillium digitatum* and for *Aspergillus niger*. Later, petri dishes were impregnate with EtOH and fractions, depending on what corresponded. To prepare them, 500 mg of extract or fraction were dissolved in 1.000 mL of Diclorometh ane and masses of 10, 20, 40 and 60 µL (5, 10, 20 and 30 mg of extract or fraction respectively) were evaluated. As positive control of the test, azoles fluconazole and ketoconazole were used, preparing 1 mg dissolved in 2 mL of DMSO, using volumes of 10 ml (0,005 mg of fluconazole and ketoconazole), 20 ml (0.01 mg of fluconazole and ketoconazole), 30 ml (0.015 fluconazole and ketoconazole) and 40 ml (0,020 mg fluconazole and ketoconazole). Finally, a reference curve was created considering the data of concentrations against inhibition zones obtained after 15 days of incubation at 22 °C.

Results

Antioxidant activity

The relative antioxidant activity (RAA) shows the performance of EtOH extract and its fractions, compared with standard solutions. The results show that, for each milligram of Ascorbic acid, the best activity was for the fraction of MeOH from EtOH extract, with 37.58 mg/L MeOH. In addition, fraction of MeOH from EtOH extract was the best compared with Rutin, obtaining 16.0 mg/L MeOH. With Trolox, RAA of MeOH from EtOH extract remains being the best with 30.2 mg/L MeOH.

Antifungal activity

In order to evaluate the inhibitory effect of total extract and fractions of *Teloschistes exilis (Michx.) Vain* against *Aspergillus niger* and *Penicillium digitatum,* data from 15 days was used.

The relative antifungal activity was determined using two commercial antimycotics, ketoconazole and fluconazole. The best relative antifungal activity (AFA) to inhibit *Aspergillus niger* and *Penicillium digitatum* found was for Petroleum ether fraction with 465.5 milligrams and 213.4 milligrams/ milligram of ketoconazole, respectively. Regarding to fluconazole, Petroleum ether was also the best AFA with 174.6 milligrams and 228.3 milligrams for *Aspergillus niger* and *Penicillium digitatum,* respectively.

Discussion

The results show that EtOH extract and fractions (with increasing polarity solvents (MeOH and Ethyl acetate) from *Teloschistes exilis* (Michx.) Vain have a better RAA compared with standard solutions. Nonetheless, the best RAA found was with ascorbic acid. According to antifungal activity against *Aspergillus niger* and *Penicillium digitatum*, low polarity fraction Petroleum ether, had the best results compared with antimycotic controls. Comparing with the evidence, a study evaluated the antioxidant activity of some lichens, including *Teloschistes chrysophthalmus,* finding that it reduces $ABTS^{*+}$ radicals but less than *Parmotrema* and similar to *Ramalina* [18]. Another study of antioxidant activity of *Teloschistes exilis* (Michx.) Vain with α, α-diphenyl-β-picrylhydrazyl (DPPH), found that total ethanolic extract had the best result of RAA with 46.6 [8] in contrast with our results with $ABTS^{*+}$ method reaching a RAA of 37.6, emphasizing that the latter method is more sensitive to measure the activity of both hydrophilic and lipophilic compounds, instead, DPPH can be only dissolved in organic medium measuring antioxidant activity of low polar compounds [22]. The results showed that increasing polarity fractions gave better results in terms of RAA for antioxidant activity, agreeing with previous investigations and suggesting that these fractions are rich in flavonoids and phenolic compounds, related with antioxidant due to the interaction of hydroxyl groups with the free radical, arresting the oxidation process [9].

Moreover, the best antifungal activity was obtained with Petroleum ether fraction. In contrast, a study with *Rumex crispus* with purified parietin had an antifungal activity of 10-30 µg/mL, evidencing fungicidal properties of parietin, with similar action of fenarimol and polyoxin [23]. Unlike of antioxidant activity, antifungal properties are related with low polarity compounds like terpenes, sesquiterpenes and triterpens [24].

Evidence supporting the benefits of lichens is growing [25-31]. but a limitation of the study and application of them is that secondary metabolites are synthetized in small proportions being restricted even to a specific genus or family [32]. Consequently, finding all the components and mechanisms of actions of lichens is hard. Following studies should isolate pure compounds of fractions to determine if the

compounds acts alone or together to exert the antioxidant activity [11].

Conclusions

This study showed that EtOH extract and fractions from *Teloschistes exilis* (Michx.) Vain had a better RAA compared with standard solutions. Nonetheless, the best RAA found was with ascorbic acid. With ABTS $^{*+}$ assay, the RAA outcomes comparing fractions with ascorbic acid were 53.77 mg/L for EtOH, 37.58 mg/L for MeOH and 47.06 mg/L for Ethyl acetate. With low polarity solvents RAA is low, suggesting that minimum presence of protons donor substances are related with these values.

According to antifungal activity against *Aspergillus niger* and *Penicillium digitatum*, low polarity fraction (Petroleum ether) had the best results compared with azoles controls. For *Aspergillus niger,* to get an equivalent sensibility, 465.6 mg of the Petroleum ether fraction are needed for each milligram of ketoconazole and 174.6 mg for each milligram of fluconazole. On the other hand, for *Penicillium digitatum*, to get an equivalent sensibility, 213.4 mg of the Petroleum ether fraction are needed for each milligram of ketoconazole and 228.3 mg for each milligram of fluconazole. The values are high but it is important to highlight the nature of the sample because a fraction contains a very large number of substances of unknown origin, needing studies to recognize the active ingredient.

Acknowledgments

We would like to thank to the Universidad Distrital Francisco Jose de Caldas, to Fundación Universitaria Juan N Corpas and to Universidad El Bosque

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Table 1: Relative antioxidant activity of total ethanolic extract and the fractions with ABTS $^{*+}$ assay.

Extracts, fractions and controls	IC50*	RAA** for Ascorbic acid	RAA for Rutin	RAA for Trolox
Petroleum Ether fraction	249.11	137.18	58.53	110.35
Dichloromethane fraction	354.68	195.31	83.33	157.12
Ethyl Acetate fraction	85.46	47.06	20.08	37.86
MeOH fraction	68.2	37.58	16.03	30.23
Total Ethanolic Extract	97.5	53.77	22.94	43.26
Ascorbic Acid	1.82	1.00	0.43	0.80
Rutin	4.26	2.34	1.00	1.89
Trolox	2.26	1.24	0.53	1.00

*Inhibitory concentration 50 in mg/L of MeOH.
**Relative antioxidant activity

Table 2: Relative antifungal activity (AFA) of total ethanolic extract and the fractions compared with azoles.

Extracts, fractions and controls	*Aspergillus niger*		*Penicillium digitatum*	
	Ketoconazole	Fluconazole	Ketoconazole	Fluconazole
Petroleum Ether fraction	465,6	174,6	213,4	228,3
Dichloromethane fraction	611,5	229,4	300,1	321,1
Ethyl Acetate fraction	734,4	275,5	294,2	314,8
Total Ethanolic Extract	595,9	223,5	639	683.8
Ketoconazole	1,0	0,4	1,0	1,1
Fluconazole	2,7	1,0	0,9	1,0

Antifungal activity expressed as mg extract-fraction/mg commercial antimycotic