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Antioxidant and cytoprotective potentials of Parmeliaceae lichens and identification of active compounds

Title in Spanish: *Potencial antioxidante y citoprotector de líquenes parmeliáceos e identificación de compuestos activos*

Carlos Fernández-Moriano[1], Pradeep Kumar Divakar[2], Ana Crespo[2] and M. Pilar Gómez-Serranillos[1]*

[1]Department of Pharmacology, Faculty of Pharmacy, University Complutense of Madrid, Plaza Ramón y Cajal s/n, 28040, Madrid, Spain.[2]Department of Plant Biology II, Faculty of Pharmacy, University Complutense of Madrid, Plaza Ramón y Cajal s/n, 28040, Madrid, Spain.

ABSTRACT: Lichens, symbiotic organisms with special features, are able to synthesize exclusive secondary metabolites that are attracting increasing interest in their pharmacological activities. Present study aimed to perform an initial screening of the antioxidant capacities of 29 lichens from Parmeliaceae family, and the cytoprotective potential of the most promising species in a model of central nervous system-like cells. Also, another goal was to determine the main chemical constituents of the promising lichens. After molecular identification of all lichen specimens by PCR techniques regarding the molecular marker ITS rDNA, antioxidant activity was measured in terms of free radical scavenging properties through ORAC assay. Methanol extracts of the three species with highest ORAC values (*Cetrelia braunsiana (Cb), Parmotrema saccatilobum (Ps)* and *Usnea ghattensis (Ug)*) were analyzed for phytochemical characterization through TLC and HPLC methods. We identified alectoronic acid as major metabolite in *Cb*, protocetraric acid in *Ps* and usnic, stictic and constictic acids in *Ug*. Concerning cytoprotective properties, their extracts were tested on human neuroblastoma cell line SH-SY5Y. Protection against H_2O_2- induced oxidative stress in such neuron-like model was assessed by cell viability assays, thus determining their optimal concentrations. Then, their effect on oxidative stress markers, such as intracellular ROS formation, glutathione levels and caspase-3 activity, were evaluated. In general, lichens extracts were able to reverse the oxidative damage caused by H_2O_2, and promoted neurons survival. Results obtained in this study imply that these lichen species might be used as promising sources for natural compounds with potential neuroprotective activity, suggesting future research avenues.

RESUMEN: Los líquenes son organismos simbióticos de especiales características capaces de sintetizar metabolitos secundarios únicos, con un creciente interés por sus propiedades farmacológicas. El presente estudio persiguió realizar un cribado de la actividad antioxidante de 29 especies liquénicas de la familia Parmeliaceae, y el potencial citoprotector de los más interesantes en un modelo neuronal. Otro objetivo consistió en derminar los principales constituyentes de dichas especies. Tras la identificación molecular de todos los especímenes por técnicas de PCR, la actividad antioxidante se evaluó por la capacidad captadora de radicales libres en el ensayo ORAC. Se analizaron por TLC y HPLC los extractos metanólicos de las tres especies con mayor valor ORAC (Cetrelia braunsiana (Cb), Parmotrema saccatilobum (Ps) and Usnea ghattensis (Ug)) y se identificaron como principales metabolitos el ácido alectorónico en Cb, el ácido protocetrárico en Ps y los ácidos úsnico, estíctico y constíctico en Ug. Estos tres extractos se probaron en células de neuroblastoma humano (línea celular SH-SY5Y). Se testó la citoprotección contra el estrés oxidativo inducido por H2O2 mediante ensayos de viabilidad celular, estableciendo las concentraciones óptimas. Posteriormente, se valoraron sus efectos en marcadores de estrés oxidativo, como la formación intracelular de especies reactivas de oxígeno, los niveles de glutatión y la actividad de la enzima caspasa-3. En general, los extractos liquénicos revirtieron el daño oxidativo causado por H2O2, promoviendo la supervivencia neuronal. Los resultados obtenidos de este estudio apuntan que las especies estudiadas pueden ser prometedoras fuentes de productos naturales con potencial actividad neuroprotecora, sugiriendo futuras líneas de investigación.

***Corresponding Author:** pserra@ucm.es
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1. INTRODUCTION

A lichen is a composite organism consisting of a stable, ecologically obligate, selfsupporting symbiosis between an exhabitant fungus (the mycobiont) and one or more extracellulary located inhabitant, the photoautotrophic partner (the photobiont: alga or cyanobacterium) (1). They present special characteristics; for instance, the morphology, physiology and biochemistry of lichens are very different from those of the isolated fungus and alga in culture. Lichens produce a great variety of secondary metabolites, being most of them unique to lichen-forming fungi. These lichen substances are mostly produced by the

mycobiont within the symbiosis, and they normally are compounds with a relatively low molecular weight and chemical diversity (aliphatic and aromatic) that accumulate in the cortex (such as atranorin and usnic acid) or in the medulla of the lichen thallus (such as protocetraric and physodic acids) as extracellular crystals (2, 3). Concerning pharmacological properties, lichens constitute a poorly known group of natural products in comparison to others (such as vascular plants and fungus); however, over the past 2 decades, there has been a renewed and growing interest in lichens as a source of novel pharmacologically active biomolecules (4).

Parmeliaceae (Ascomycota, Lecanorales) is the most numerous and widespread family among lichenized fungi, comprising more than 2700 species grouped in about 80 genera; in addition, it is probably the most interesting family from a pharmacological point of view (5). For instance, the representative lichen Usnea ghattensis have displayed in previous investigations interesting antimicrobial properties together with a promising antioxidant potential, as evidenced by chemical tests in vitro (6, 7). Similarly, Bugni et al. demonstrated the presence of active anti-inflammatory constituents in the lichen Parmotrema saccatilobum (8).

The lichen forming fungal family Parmeliaceae, and fungi in general, exhibit few taxonomically useful characters, many of which are homoplasious. The interpretation of morphological features is sometimes difficult to evaluate, and accurate identification of lichenized fungal species remains challenging (9, 10). In many cases, characters used for species identification may be subtle to discern, and diagnostic morphological and chemical characters could be lacking in juvenile or fragmentary samples. The traditional phenotype-based approach to species recognition in Parmeliaceae has been shown in some cases to substantially misrepresent diversity (11, 12).

In view of the previous information, and considering the existence of few studies on intracellular ROS (reactive oxygen species) modulation by lichen extracts and metabolites (13), none focused on their role as preventive compounds in nervous system-like cells under oxidative stress conditions, we carry out the present work. The aim of the study deals with the evaluation for the first time of the in vitro neuroprotective potential, based on antioxidative effects, displayed by the methanol extract of Parmeliaceae lichens; for that purpose, we initially perform an initial screening of antioxidant capacity of various molecularly identified lichen specimens. In addition, we aim to identify the main constituents of the most promising lichens.

2. MATHERIAL AND METHODS

2.1. Reagents

Cell culture products were obtained from Gibco (Grand Island, NY) and all other chemicals were from Sigma–Aldrich (St. Louis, MO).

2.2. Lichen samples

The 29 lichen sample used in the present study were collected in different countries and continents, authenticated by expert lichenologists (P.K. Divakar y A. Crespo) and deposited in the Herbarium of the Faculty of Pharmacy (MAF), University Complutense of Madrid, with the identifying data presented in Table 1. Lichenic material was examined at morphological level with a binocular loupe Nikon SMZ800. For microscopic observations, an Olympus DP11 microscope was used.

Table 1. The 19 species of Parmeliaceae lichens used in the study, together with the location of their recollection and MAF codes.

Lichen specie	Origin	MAF code
Bulbothrix meizospora (Zahlbr.)	India, W. Himalaya, Dugenbilta Mahhu Valley	MAF-LICH 16929
Cetraria aculeata (Schreb.)	Spain, Canary Islands, Gran Canarias, Valleseco	MAF-LICH 16927
Cetraria canadensis (Muell.Arg.)	USA, California	MAF-LICH 15621
Cetraria nigricans (Muell.Arg.)	India, North sikkim, Chubuk	MAF-LICH 16940
Cetrelia braunsiana (Muell.Arg.)	India, W. Himalaya, Kuachula Khark	MAF-LICH 16943
Cetrelia cetrarioides (Muell.Arg.)	India, North sikkim, Chubuk	MAF-LICH 16941
Cetrelia olivatorum (Muell.Arg.)	India, North sikkim, Yumthang	MAF-LICH 16942
Evernia prunastri (L.Ach.)	England, New Forest	MAF-LICH 16930
Flavoparmelia citrinescens (L.Ach.)	Argentina, Rio Negro, Missuti	MAF-LICH 16946
Flavoparmelia euplecta (L.Ach.)	Australia, New South Wales	MAF-LICH 15375

Flavoparmelia haysonii (L.Ach.)	Australia, capital territory	MAF-LICH 7535
Flavoparmelia rutidota (L.Ach.)	USA, California	MAF-LICH 16949
Hipotrachyna execta (Schaer.)	India, W. Himalaya, Kuachula Khark	MAF-LICH 15518
Lichen specie	Origin	MAF code
Myelochroa entotheiochroa (Nyl.)	Japan, Honshu, Prov. Kai, Nishizawa Valley	MAF-LICH 16937
Myelochroa irrugans (Nyl.)	Japan, Honshu, Prov. Kai, Nishizawa Valley	MAF-LICH 16931
Parmelia saxatilis (Nyl.)	England, New Forest	MAF-LICH 16932
Parmotrema abessinicum (Krempelh.)	Kenya, Rift Valley, lake Naivasha	MAF-LICH 16938
Hipotrachyna execta (Schaer.)	India, W. Himalaya, Kuachula Khark	MAF-LICH 15518
Parmotrema austrosinense (Zahlbr.)	Japan, Honshu, Prov Hitachi, Tsukuba	MAF-LICH 16934
Parmotrema perlatum (Nyl.)	England, New Forest	MAF-LICH 16938
Parmotrema praesorediosum (Nyl.)	Brazil, Pernambuco, Catimbau National Park	MAF-LICH 16945
Parmotrema reticulatum (Taylor)	Japan, Honshu, Prov. Musashi, Chichibu	MAF-LICH 16935
Parmotrema saccatilobum (Taylor)	Japan, Honshu, Prov. Kai, Nishizawa Valley	MAF-LICH 16928
Usnea arizonica (Mot.)	USA, California	MAF-LICH 16947
Usnea aurantiacoatra (Mot.)	Chile, Ambarino	MAF-LICH 15686
Usnea contexta (Mot.)	Chile, Ambarino	MAF-LICH 15710
Usnea filipendula (Zahlbr.)	USA, California	MAF-LICH 16948
Usnea ghattensis (Zahlbr.)	India, Tamil Nadu, Ghat, Nilgiri Hills	MAF-LICH 16944
Usnea sp. (Mot.)	Japan, Honshu, Prov. Musashi, Saitama	MAF-LICH 16936
Xantoparmelia coreana (Gyeln.)	Japan, Honshu, Prov. Hitachi, Tsukuba	MAF-LICH 16933

2.3. Mollecular identification

Total genomic DNA was extracted from freshly collected material and/or herbarium specimens younger than 5 years. To reduce contamination by fungal endophytes, samples were carefully prepared and visible symptoms of secondary fungal growth were removed. Small pieces (ca. 2 mm2) were carefully separated, washed in acetone for two hours to remove potential secondary metabolites and dried overnight. Samples were ground with sterile pestles into liquid nitrogen and later into the lysis buffer at 65 °C, incubated at 65 °C for two hours and later kept at room temperature overnight. DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions but with slight modifications (14).

PCR amplifications of the ITS gene fragment was performed using fungal specific primers ITS1-LM (15) and ITS2-KL (16). Genomic DNA (1–10 ng) was used for PCR amplifications of the ITS regions. The 25 µL PCR reactions contained buffer 1x (containing 10 mM Tris pH 9.0, 2.5 mM MgCl2, 50 mM KCl, 0.1% TritonX-100), 0.2 mM each dNTP, 0.5 µM each primer, 1.25 units Taq DNA polymerase (Applied Biosystems) and 1–10 ng genomic DNA extract. PCR amplifications were carried out in a Techne R TC-3000 thermal cycler under the following conditions: initial heating step of 5 min at 94°C, followed by 30 cycles of 1 min at 94°C, 1 min at 54°C and 1.5 min at 72°C. A final extension step of 5 min at 72°C was added, after which the samples were kept at 4°C. Amplification products were viewed on a 1% agarose gel stained with SYBR Safe DNA (Life Technologies Corporations, Grand Island, New York, U.S.A.), and purification was performed by adding 2 µL of ExoSAP-IT™ (Exonuclease 1-Shrimp Alkaline Phosphatase) to 10 µL of PCR products, followed by a heat treatment of 15 min at 37°C and 15 min at 80°C. Both complementary

strands were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, California, USA) with the same primers used in the amplification step and with following settings: initial denaturation at 94°C for 3 min; 25 cycles at 96°C for 10 s, 50°C for 5 s, 60°C for 4 min.. Sequencing reactions were electrophoresed on a 3730 DNA analyzer (Applied Biosystems) at the Unidad de Genómica (Parque Científico de Madrid).

Sequence fragments generated for this study were assembled and edited using the program SeqMan v.7 (Lasergene R, DNASTAR, Madison, Wisconsin, USA). The sequences were blasted against GenBank database following the methodology outlined in Sayers et al. (17) to approach the samples identity. The arbitrary ≥ 97% threshold value for assigning specimens to a closely related congeneric species-group was selected as criterion for assessing successful species identification.

2.4. Preparation of lichen extracts

Methanol extract of the 29 lichen specimens were prepared by using 1 mL of metanol per mg of lichen thallus. They were maintained for 1 hour in a sonicator (Branson) and then filtered. After evaporation of methanol at room temperature, dry extracts were kept at 4ºC in the fridge and later re-dissolved in methanol or PBS for the following experiments.

2.5. Evaluation of antioxidant activity

The Oxygen Radical Antioxidant Capacity (ORAC) assay was carried out as previously described (18). Dilutions of samples and Trolox (reference antioxidant, water soluble vitamin E analogue) were incubated in opaque 96-wells plates for 10 min at 37°C with fluorescein. After this period, the azocompound AAPH (2,2´-azobis(2-methylpropionamidine) dihydrochloride) was added to the mixture. Fluorescence is read every 56 seconds for 98 minutes using a FLUOstar Optima fluorimeter (BMG Labtech) (λexc 485nm and λem 520 nm). Area under the curve (AUC) was calculated for each sample and compared with AUC of Trolox. ORAC values are expressed as µmol Trolox equivalents (TE)/mg samples.

2.6. Cytoprotective activities

2.6.a. Neurons cultures and treatments

Neurons from SH-SY5Y (human neuroblastoma) cell line were maintained in DMEM supplemented with 10% FBS and 0.5% gentamicin in a humidified atmosphere with 5% CO2 and 37ºC. H2O2 was used as oxidative stress inductor, at a concentration of 0.1 mM for 30 min. Cells were treated with lichen extracts at different concentrations for 24 h. Extracts and H2O2 were dissolved in PBS for corresponding dilutions.

2.6.b. Assessment of cell viability (MTT assay)

Mitochondrial integrity and activity, as cell viability indicators, were determined by using MTT assay (19) with minor variations. Cells were plated at a density of 5×104 cells/well in 96-well plates overnight and then treated with different concentrations of lichens extracts (ranging from 0.5 to 250 µg/mL) for 24 h. Finally, MTT (2 mg/mL) was added and plates were incubated for 1 h at 37ºC. After removing medium, DMSO was added in order to dissolve the dark blue formazan crystals. Absorbance was then measured at 550 nm using a microplate reader Digiscan 340 (ASYS HitechGmbH, Eugendorf, Austria).

2.6.c. Protection against H2O2- induced toxicity

For evaluating a possible protective effect against oxidative stress inductors, cells were exposed to 0.1 mM H2O2 for 30 min after treatments with the extract (20), and MTT assay was later conducted as previously described.

2.6.d. Intracellular ROS production assay

ROS production was assayed using the DCFH-DA method (21), with some modifications. In brief, after cellular treatments, 50 µL DCFH-DA/25 mL PBS–glucose was loaded for 30 min. After this time of incubation at 37ºC, cells were washed twice with PBS– glucose. ROS generation was examined for 2 h (λexc 480 nm and λem 510 nm) in a Microplate Fluorescence Reader FLx800 (Bio-Tek, Instrumentation, USA).

2.6.e. Determination of caspase-3 activity

Inhibition of H2O2-mediated apoptosis by extracts was assessed through the caspase-3 activity, by measuring the cleavage of a fluorogenic substrate (22). After treatments, 100 µL of cell lysates were collected and added in a 96-well plate. Well volume was completed with 50 µL reaction buffer (20 mM HEPES, 10% glycerol, 2 mM DTT, pH of 7.5) and 2 µL of caspase-3 substrate solution (1 mg/mL in DMSO) in the end. Substrate cleavage was measured fluorometrically every hour using a FLx800 (λexc 360/40 nm and λem 480/20 nm).

2.6.f. Glutathione levels

GSH and GSSG levels determination was performed according to the method described by Hissin and Hilf (23). Reaction mixture for the determination of GSH contained 50 µL of the lichen sample, 150 µL of 0.1 M sodium phosphate buffer (pH 8.0) and 20 µL of o-phthalaldehyde (1 mg/mL in methanol); instead, reaction mixture for estimating of GSSG contained 50 µL of the sample and 3 µL of N-ethylmaleimide and was maintained for 5 min in darkness in this point before adding 150 µL of 0.1 N NaOH (pH 12) and 20 µL of o-phthalaldehyde solution. Finally, both preparations were incubated for 15 min at room temperature in the dark, and fluorescence was measured at λexc 528 nm and λem 485 nm with a microplate fluorescence reader. Ratio GSH/GSSG was obtained by dividing the nmols per mg of protein of GSH and GSSG.

2.7. Phytochemical analysis

2.7.a. Thin Layer Chromatography (TLC)

TLC analyses were performed based on Culberson methods (24-26). Methanol extracts of lichen thallus were charged on the silicagel plate and run with a mobile phase composed of toluene/acetic acid (85:15). Once the plate had been saturated with the solvent within a glass cuvette, the plate was removed and dried at room temperature. It was then exposed and observed under UV light and revealed by spreading a sulfuric acid solution along the plate, which was placed on a heater.

2.7.b. High Performance Liquid Chromatography (HPLC)

HPLC analysis was performed in a SPECTRA-PHYSICS SP 8800/8810LC coupled to a UV-vis detector, measuring at a wavelength of 254nm. The column employed was a C18 Mediterranea Sea (150 x 4.6 mm, 5 µm particle size; Teknokroma) and the mobile phase consisted of A (acetonitrile / methanol (20:80 v/v)) and B (4 % acetic acid solution). Elution was performed isocratically at a flow of 1 mL/min and at room temperature. Reference chromatograms were used from lichens with known composition (Neofuscelia glabrans, N. parviloba, Xanthoparmelia exemplaris) and analyzed in the same conditions (27).

2.8. Statistical analysis

Results are expressed as means ± SD of at least three independent experiments. Statistical differences were tested using one-way ANOVA followed by Tukey's test for multiple comparisons, using the Statgraphics Centurion XVI software. A value of $p<0.05$ was considered statistically significant.

3. RESULTS

3.1. Identification of lichen species

For the molecular identification of the lichen specimens, fragments of ITS rDNA sequences obtained were analyzed through BLAST function of NCBI (National Center for Biotechnology Information) GenBank database (04 March 2015), thus attributing to a certain sequence a percentage of overlapping with the most similar by comparative analysis (see Table 2).

Table 2. Genbank code assigned to the 29 lichen species after comparison with the most similar appearing in BLAST.

Lichen specie	Genbank Code
Bulbothrix meizospora	EF 092102
Cetraria aculeata	EU 409758
Cetraria canadensis	GU 994635
Cetraria nigricans	AF 494383
Cetrelia braunsiana	GU 994636
Cetrelia cetrarioides	AF 254630
Cetrelia olivatorum	GU 994638
Evernia prunastri	DQ 923634
Flavoparmelia citrinescens	GU 994641
Flavoparmelia euplecta	HM 010928
Flavoparmelia haysonii	HM 014233
Flavoparmelia rutidota	DQ 084148
Hipotrachyna execta	GQ 919258
Myelochroa entotheiochroa	EF 042917
Myelochroa irrugans	EF 092128
Parmelia saxatilis	AF 391137
Parmotrema abessinicum	HM 017025
Parmotrema austrosinense	DQ 912360
Parmotrema perlatum	AY 586580
Parmotrema praesorediosum	DQ 912316
Parmotrema reticulatum	DQ 084184
Parmotrema saccatilobum	AB 627399
Usnea arizonica	AF 297732
Usnea aurantiacoatra	EF 179798
Usnea contexta	AJ 249573
Usnea filipendula	FR 799076
Usnea ghattensis	EF 179806
Usnea sp. (Mot.)	JF 794062
Xanthoparmelia coreana	AF 262010

3.2. Evaluation of antioxidant activity

The chemoluminescence induced by the peroxyl radical generation, initiated by AAPH in ORAC assay, decreased following the addition of several lichen extracts. As shown in Table 3, there was a vast variation in ORAC values of the 29 lichen species studied; the higher the ORAC value, the better antioxidant activity. Some lichen extracts exerted negligible antioxidant activity, such as Cetraria aculeata and Cetrelia nigricans, whereas other species demonstrated higher potential for scavenging peroxyl radicals, such as Cetrelia braunsiana (3.21 µmol TE/mg sample), Parmotrema saccatilobum (4.38 µmol TE/mg sample) and Usnea ghattensis (4.74 µmol TE/mg simple).

Table 3. ORAC values in µmol TE/mg sample (expressed as Mean ± SD) obtained for the 29 species.

Samples	ORAC values
Bulbothrix meizospora	1.14 ± 0.11
Cetrelia braunsiana	3.21 ± 0.17
Cetrelia cetrarioides	0.13 ± 0.02
Cetrelia nigricans	0.03 ± 0.01
Cetrelia olivatorum	1.00 ± 0.04
Cetraria aculeata	0.01 ± 0.01
Cetraria canadensis	2.52 ± 0.17
Evernia prunastri	2.21 ± 0.19
Flavoparmelia citrinescens	0.24 ± 0.02
Flavoparmelia euplecta	0.62 ± 0.09
Flavoparmelia haysomii	1.40 ± 0.05
Flavoparmelia rutidota	0.15 ± 0.02
Hipotrachyna execta	1.67 ± 0.07
Myelochroa entothiochroa	0.60 ± 0.04
Myelochroa irrugans	0.52 ± 0.01
Parmelia saxathis	1.22 ± 0.23
Parmotrema abessicum	0.48 ± 0.24
Parmotrema austrosinense	0.46 ± 0.18
Parmotrema perlatum	0.25 ± 0.10
Parmotrema praesorediosum	0.23 ± 0.02
Parmotrema reticulatum	0.07 ± 0.02
Parmotrema saccatilobum	4.38 ± 0.18
Usnea arizonica	0.50 ± 0.04
Usnea aurantiacoatra	0.25 ± 0.02
Usnea contexta	0.20 ± 0.04
Usnea filipendula	1.67 ± 0.04
Usnea ghattensis	4.74 ± 0.09
Usnea sp	1.83 ± 0.05
Xantoparmelia coreana	0.71 ± 0.08

At this point, we selected the three species showing higher antioxidant activity in ORAC assay (as highlighted above) for the following experiments.

3.3. Cytoprotective activities

3.3.a. Assessment of cell viability and protection against H2O2- induced cell death

Nine different concentrations ranging from 0.25 to 100 µg/ml were tested for each extract in order to determine the effects of single extracts in the viability of SH-SY5Y cells through the MTT assay. Results obtained for the effects of Cb, Ps and Ug are shown in Figure 2, and expressed as the percentage of cell viability, considering the optical density of untreated control cells as 100% (triton X-100 5% was used as a negative control). A significant cell viability loss was observed for Cb from 25 µg/ml and higher concentrations; however, from 10 µg/ml of Ps and Ug cell viability appeared diminished.

Therefore, the concentrations of the extracts that compromised cell viability were discarded, and five/six concentrations of each extract were chosen for assessing their protection against oxidative stress and cellular toxicity exerted by hydrogen peroxide. H2O2 decreased cell viability to approximately 55% of control, but Cb 0.25-0.5 µg/mL, Ps 0.25 µg/mL and Ug 0.25-0.5 µg/mL significantly reversed that effect and protected neurons viability. The concentrations displaying highest protection against H2O2 were then chosen for each extract (0.5 µg/ml for Cb and Ug and 0.25 µg/mL for Ps) and assayed in the following experiments.

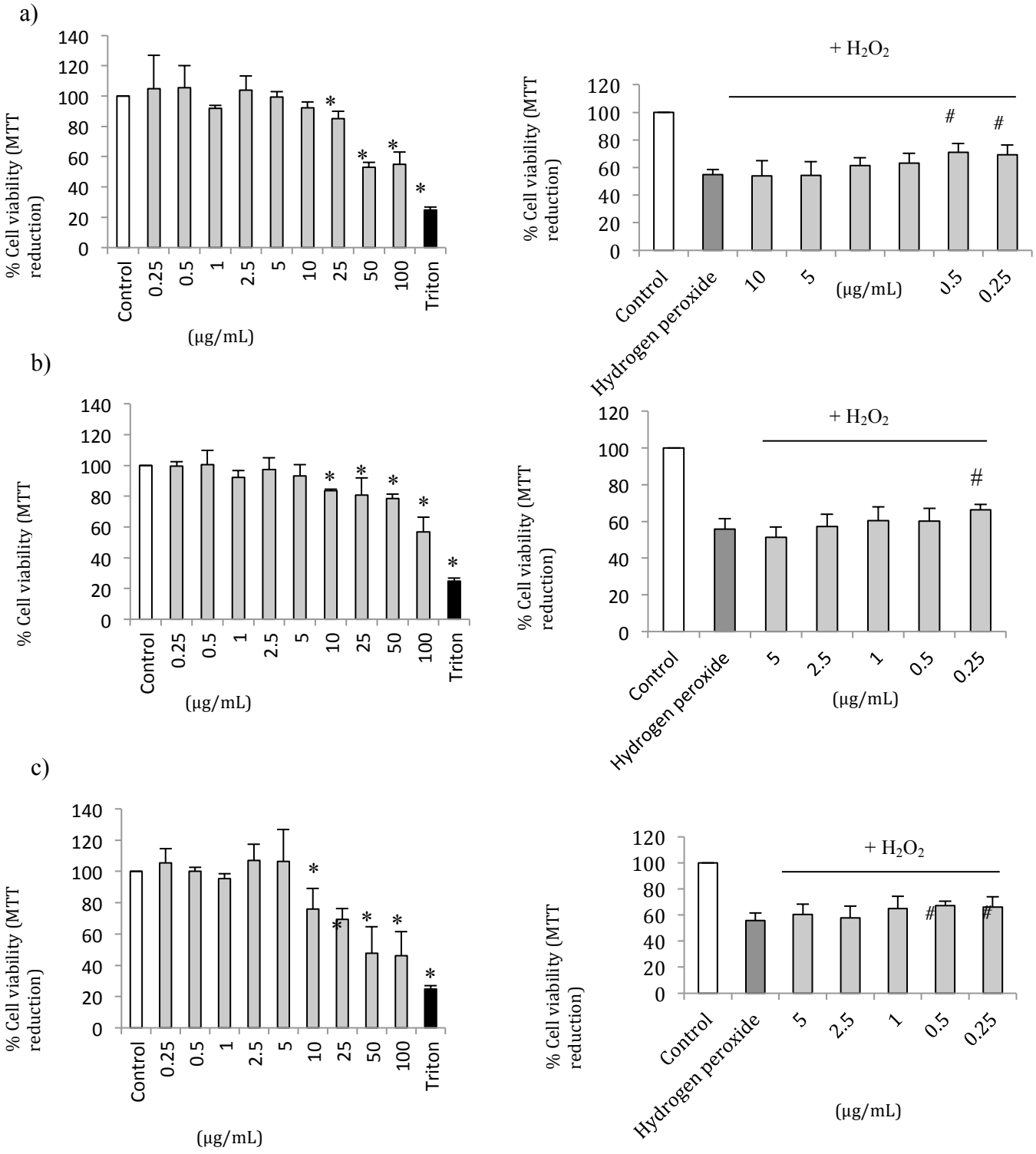


Figure 2. Effects of extracts *Cb* (a), *Ps* (b) and *Ug* (c) on the cell viability of SH-SY5Y cells evaluated by MTT assay. Initially, cells were treated only with the extracts in a range of concentrations from 0.25 to 100 µg/ml (24 h) (graphics on the left) and certain concentrations were later tested for protection against H_2O_2 (0.1 mM, 30 min) (graphics on the right). Means ± SD, *p < 0.05 *Vs* control; # p < 0.05 *Vs* H_2O_2.

3.3.b. Intracellular ROS production assay

Figure 3 shows that SH-SY5Y cells exposed to H_2O_2 presented intracellular ROS levels significantly increased to approximately 20% in comparison to control cells (100% ROS generation) throughout the experiment. Moreover, none of the lichen extracts caused by themselves remarkable intracellular ROS production when compared to control cells (data not shown). However, pretreatments with the chosen concentrations of the three extracts displayed a significant inhibitory activity against H_2O_2- induced ROS production within the neuron-like cells.

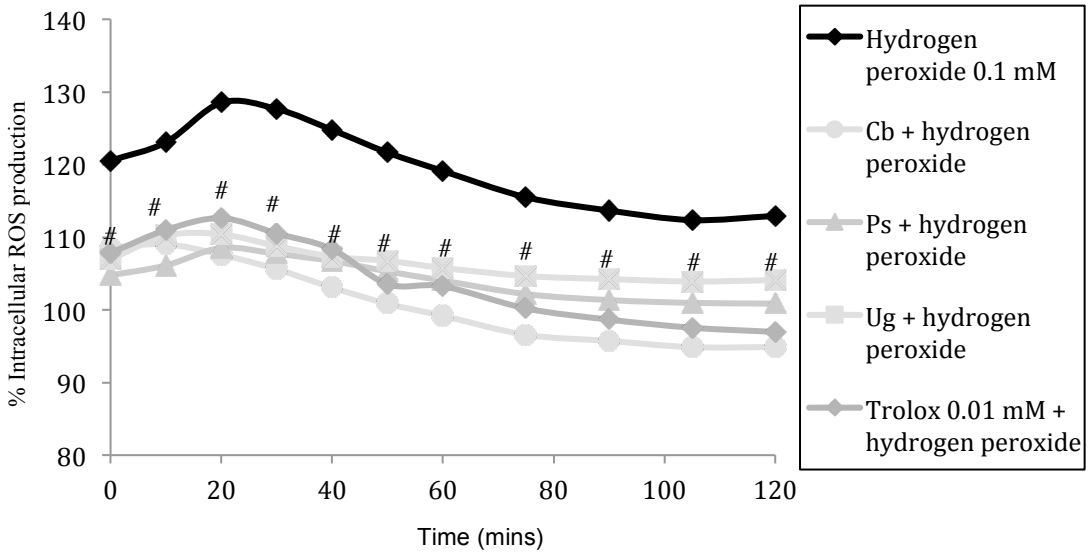


Figure 3. Effects of pretreatments with *Cb*, *Ps*, *Ug* and trolox on H_2O_2- induced ROS generation in SH-SY5Y cells (concentrations: 0.5 µg/mL for *Cb* and *Ug* and 0.25 µg/mL for *Ps*).. Mean values, # p < 0.05 *Vs* H_2O_2, and affects all points below.

3.3.c. Determination of caspase-3 activity

We evaluated the potential inhibitory activity of lichen extracts on caspase-3 activity by a fluorimetric method. As shown in Figure 4, exposition of SH-SY5Y cells to H_2O_2 resulted in a remarkable increase by over 270 % of caspase-3 activity compared to control cells. Pretreatments with *Cb* and *Ug* were able to significantly reverse this elevation, almost to basal levels. However, *Ps* pretreatment could not diminish the induced caspase-3 up-regulation. With these results, protective effects of *Cetrelia braunsiana* and *Usnea ghattensis* extracts might be partially attributed to an inhibition of apoptosis, but not in the case of *Parmotrema saccatilobum*.

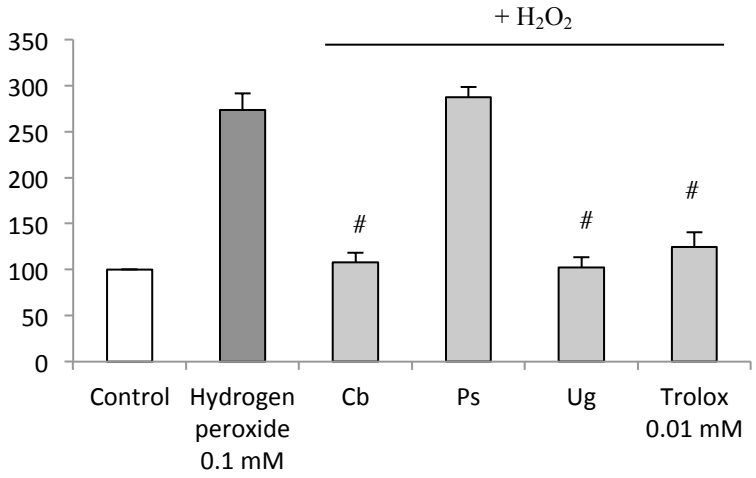


Figure 4. Effects of pretreatments with *Cb*, *Ps*, *Ug* and trolox on H_2O_2-induced caspase-3 over-activation (concentrations: 0.5 µg/mL for *Cb* and *Ug* and 0.25 µg/mL for *Ps*; 24 h). Cells were treated with lichen extracts and H_2O_2 (0.1mM; 30 mins). Means ± SD, # $p < 0.05$ *Vs* H_2O_2.

3.3.d. Gluthatione levels

GSH and GSSG levels were measured as important oxidative stress markers by using fluorometric methods. It was evidenced that glutathione is mostly found as its reduced form in control cells and, when SH-SY5Y cells were incubated with H_2O_2 (0.1 mM for 30 min), GSH levels decreased significantly (7.77 nmol/mg protein) when compared with control cells (9.47 nmol/mg protein), while the levels of the oxidized form also increased significantly (2.62 nmol/ mg protein for control cells and 9.05 nmol/mg protein for cells treated only with H_2O_2). On the other hand, pretreatments with the three lichen extracts, at the concentrations previously mentioned for each one, ameliorated antioxidant capacity in neurons by increasing GSH/GSSG ratio compared to cells treated only with H_2O_2. Methanol extract of *Usnea ghattensis* was the most effective in restoring normal GSH and GSSG levels against the oxidative damage induced by H_2O_2 and the only one showing a statistically significant difference. Results obtained for all extracts and trolox (as reference antioxidant) are expressed in Figure 5.

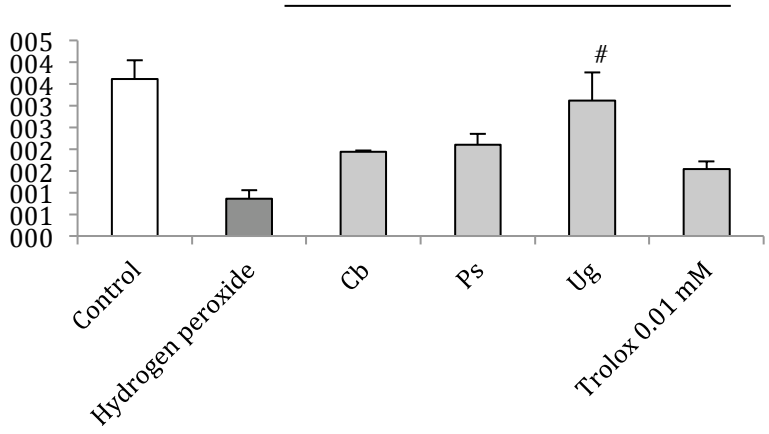


Figure 5. Effects of pretreatments with *Cb*, *Ps*, *Ug* and trolox on H_2O_2-induced changes in GSH/GSSG ratio (concentrations: 0.5 µg/mL for *Cb* and *Ug* and 0.25 µg/mL for *Ps*; 24 h). Cells were treated with lichen extracts and H_2O_2 (1mM). Means ± SD, # $p < 0.05$ *Vs* H_2O_2.

3.4.Phytochemical analysis

In view of the results of ORAC and cytoprotective assays, we carried out a phytochemical analysis for the extracts of the three species under study (*Cetrelia braunsiana, Parmotrema saccatilobum* and *Usnea ghattensis*) with the purpose to correlate the antioxidant potential with their chemical composition.

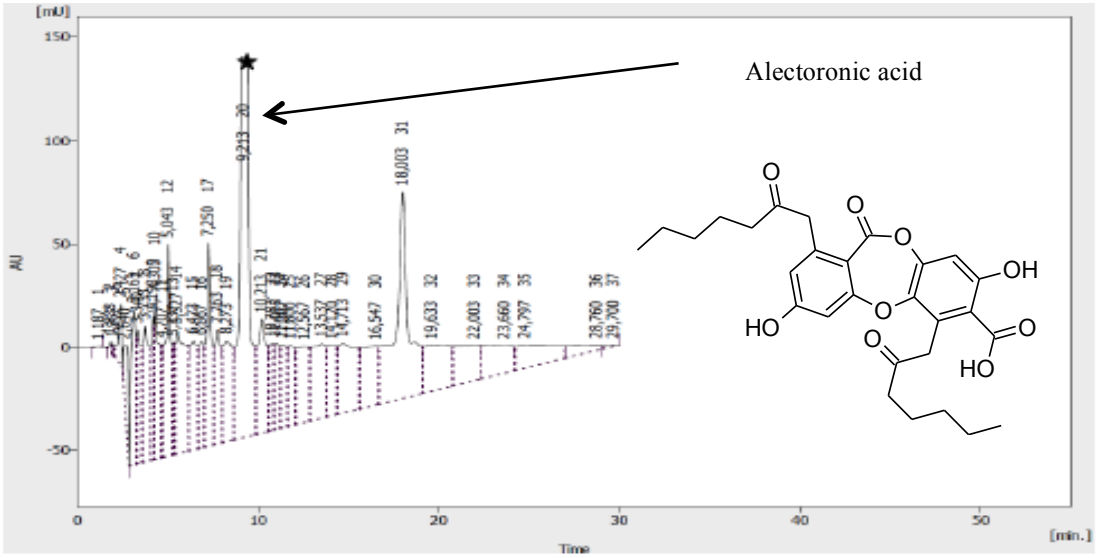
Identification of the different components was done based on the Rf values (ratio between the distance run by the compound and by the eluent) and their comparison with those values present in scientific bibliography (28). Table 4 collects the data obtained through TLC analyses with the compounds identified for each lichen specie.

Table 4. Chemical composition determined through TLC analyses for the methanol extracts of *Cetrelia braunsiana* (*Cb*), *Parmotrema saccatilobum* (*Ps*) and *Usnea ghattensis* (*Ug*).

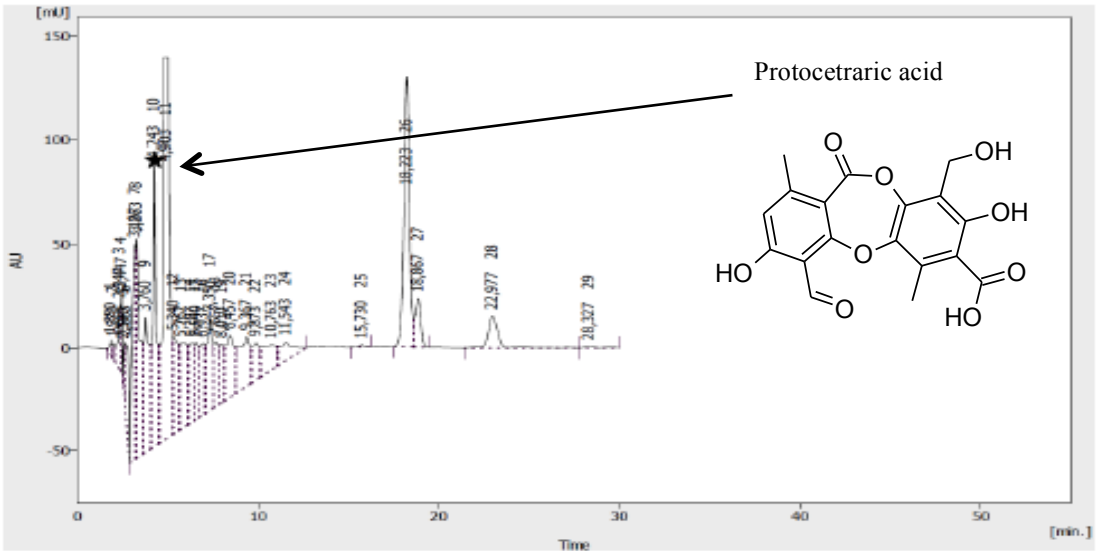
Compound	**Rf**	***Cb***	***Ps***	***Ug***
Usnic acid	0.71	-	-	+
Stictic acid	0.18	-	-	+
Constictic acid	0.20	-	-	+
Alectoronic acid	0.17	+	-	-
Atranorin	0.79	+	+	-
Protocetraric acid	0.50	-	+	-
Salazinic acid	0.40	-	-	-
Evernic acid	0.43	-	-	-

On the other hand, we performed a reverse phase HPLC analysis of the methanol extracts in order to deepen on phytochemical characterization, since it is a more sensitive and specific method. Chromatograms obtained for the three species under study were evaluated by comparing the retention times (t_R) of their main peaks to the chromatograms of the standards, analyzed under the same experimental conditions. For that purpose, *Neofuscelia glabrans* (containing alectoronic acid), *Neofuscelia parviloba* (with usnic acid) and *Xanthoparmelia exemplaris* (with protocetraric acid) were used as references. Through the HPLC method, we identified the following compounds: alectoronic acid (9.21 ± 0.20 min) in *Cetrelia braunsiana*, protocetraric acid (4.90 ± 0.11 min) in *Parmotrema saccatilobum*, and usnic acid (17.40 ± 0.23 min) in *Usnea ghattensis*. Results of retention times are expressed as mean value of three different analyses, and chromatograms obtained for the three species are collected in Figure 1.

a)



b)



c)

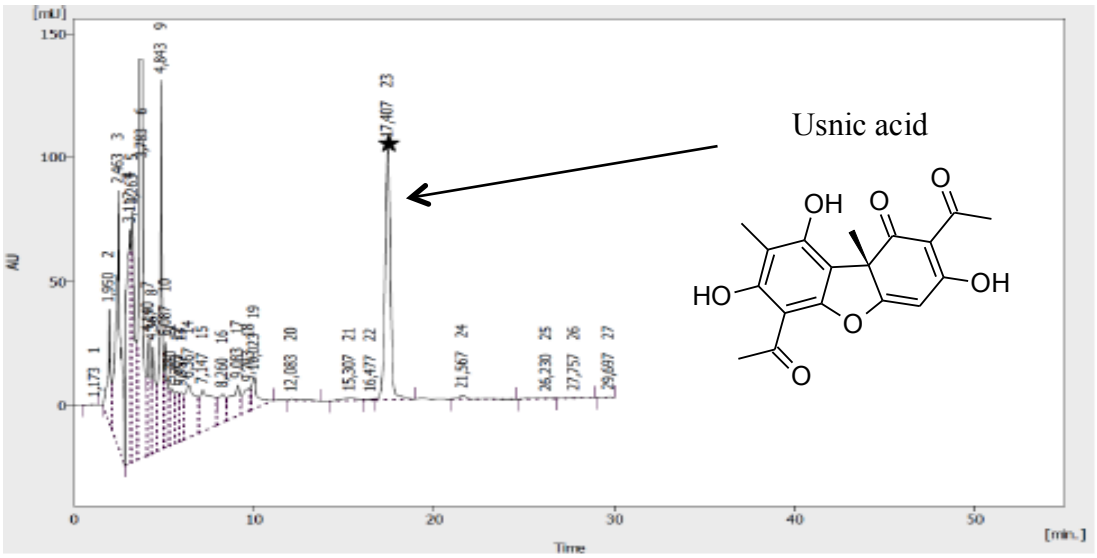


Figure 1. Chromatograms adquired at 254 nm for the metanol extracts of *Cetrelia braunsiana* (a), *Parmotrema saccatilobum* (b) and *Usnea ghattensis* (c).

4. DISCUSSION

In the lack of taxonomically significant morphological features especially at lower taxonomic level, molecular-based techniques have provided a valuable additional tool for species identification (29). The internal transcribed spacer region of the nuclear ribosomal RNA (ITS rRNA) is the most frequently sequenced fungal genetic marker, including lichenized fungi, and this locus has been recently accepted as universal DNA barcode marker for fungi (30).

The use of DNA barcoding as a major tool for identification of fungal species largely depends on the development of high-quality sequence databases that are thoroughly curated by taxonomists. A reference DNA sequence database generated from expertly identified specimens of well-circumscribed taxa may provide an effective alternative to phenotype-based identification of species by using DNA barcoding (31). In a recent study, a huge number of fungal ITS sequences especially type species lodged in Genbank have been well-crated by taxonomists (32). The National Institute of Health's (NIH) genetic sequence database, GenBank, currently provides the largest number of ITS sequences from lichen-forming fungi, especially Parmeliaceae representing a broad range of taxonomic and geographic diversity (http://www.ncbi.nlm.nih.gov/genbank/). This could provide a valuable resource for species identification through BLAST-based sequence similarity searches of DNA sequence data obtained from unidentified samples.

Considering all these points, we selected the ITS marker for our research in order to acquire correct species identification and applied BLAST-based sequence similarity searches approach to identify the 29 samples under study.

Once a Genbank code had been assigned for each lichen specimen, we proceeded to evaluate the antioxidant activity through the ORAC assay. We obtained a varied range of ORAC values for the different species, what may be related to the differences in chemical composition among lichen genus and species. Among in vitro antioxidant tests, ORAC assay is classified as a hydrogen atom transfer (HAT)-based assay and, therefore, such donation of a hydrogen atom might be a plausible mechanism for explaining the antiradical properties exerted by the compounds present in the methanol extracts (33). At this point, we selected the three species with the highest ORAC values: *Cetrelia braunsiana*, *Parmotrema saccatilobum* and *Usnea ghattensis*.

Their methanol extracts were tested on a cellular model of neurons threatened by the hydrogen peroxide –induced oxidative stress; the final aim was to assess the cytoprotective properties of these lichens in nervous system–like cells, since no previous studies had dealt with the issue. Cell viability studies allowed the selection of the optimal concentration for each extract that was further used in other experiments measuring some markers of oxidative stress such as intracellular ROS levels, caspase-3 activity and concentrations of the endogenous antioxidant glutathione.

The incidence of exogenous H_2O_2 on intracellular ROS levels was evaluated by DCFH-DA fluorimetric assay. 2´,7´-diclorodihidrofluorescein-diacetate (DCFH-DA) is a non-fluorescent compound that is able to cross cell membranes and is de-acylated by intracellular esterases, turning into 2´,7´-diclorodihidrofluorescein (DCFH); DCFH, in presence of ROS like hydrogen peroxide, reacts with them and oxidizes to 2',7'-dicloroflurescen (DCF), which is already a high fluorescent compound. Intracellular fluorescence of DCF allows to quantify intracellular ROS formation and oxidative stress, since its intensity is proportional to the amount of ROS produced. Results obtained in this assay confirm that H_2O_2, under established experimental conditions, exerts induction of oxidative stress and increase intracellular ROS levels (34). The decrease in ROS generation mediated by the pretreatment with lichen extracts might suggest that oxidative stress reduction as an important mechanism underlying their cytoprotective action.

Consistence evidences demonstrate a direct relationship between cellular oxidative stress and apoptosis (35). Apoptosis is a complex process of programmed cell death in which mitochondria play a crucial role, and involves several molecular pathways including caspases activation; inhibition of apoptosis arises as a plausible mechanism for neuroprotection (36). Caspase-3 is a key enzymatic mediator in external and internal apoptosis pathways, and suppression of active caspase-3 contributes to the cellular protection against oxidative stress (37). Through a fluorimetric method, we demonstrated that caspase-3 activity was enhanced after H_2O_2 treatment, but that *Cb* and *Ug* were able to reverse such effect. Therefore, the attenuation of neuronal apoptosis may also mediate their neuroprotective effects.

Glutathione in its reduced state (GSH) is the major endogenous antioxidant in the body due to the presence of a reactive cysteine moiety. The ratio between the oxidized-type (GSSG) and reduced-type glutathione levels is often used as indicative of the cellular reducing power. As a result of a decrease in GSH levels or because of changes in GSH/GSSG ratio, cells experience greater oxidative damage (38). The measurement of the concentrations of GSH and GSSG in the different group of cells evidenced that *Ug* favors a good antioxidant status within the neurons; a pretreatment with this lichen extracts protected cells from GSH depletion induced by oxidative stress, thus promoting cell survival.

Results on chemical composition of the three species obtained by a TLC method have been confirmed through HPLC, and are in agreement with previous studies on the phytochemistry of these species (39-41). A plausible correlation between the main constituents and their potential pharmacological activities might be established. Hence, antioxidant activities exerted by methanol extracts of *Cetrelia braunsiana* (*Cb*) and *Parmotrema saccatilobum*

(*Ps*) are probably due to the presence of alectoronic acid and protocetraric acid, respectively; both extracts contain atranorin as another secondary metabolite in an importance proportion, so atranorin should also be taken into consideration when dealing with pharmacological properties. Regarding *Usnea ghattensis* methanol extract (*Ug*), usnic acid is present in similar concentration than other compounds belonging to the stictic acid complex (consictic and stictic acid), and those metabolites could also contribute to the biological activities; then, antioxidant capacity should not be only attributed to usnic acid in this specie.

5. CONCLUSIONS

Methanol extracts of *Cetrelia braunsiana*, *Parmotrema saccatilobum* and *Usnea ghattensis* have been investigated for the first time regarding their neuroprotective activities, via antioxidant actions, in a model of oxidative stress in nervous system-like cells (neuron model); for such purpose, the human astrocytoma SH-SY5Y cell line was chosen due to its extensive use as cellular model in neuroprotection experiments, in both physiological and pathological conditions (42-44).

Present study was initiated with a molecular identification of 29 lichen species collected worldwide and belonging to Parmeliaceae family. After preparation of methanol extracts from all specimens, antioxidant capacity was evaluated through ORAC assay, since antioxidation is a considered mechanism for neuroprotection. Several extracts demonstrated interesting radical scavenging actions in that assay, and the three afore-mentioned species with higher potentials were chosen for cytoprotective experiments on cellular substrate.

Through the MTT assay, optimal concentrations for each extract (0.5 µg/mL for *Cb* and *Ug*, and 0.25 µg/mL for *Ps*) were selected in view of their cytoprotective results against H_2O_2, and then tested in the experiments measuring oxidative stress markers. In general, our results indicate that these Parmeliaceae lichens are able to reverse H_2O_2-induced negative effects on redox status in neurons. *Usnea ghattensis* appears to display the best protective activity, since its extract was able to significantly decrease intracellular ROS formation, attenuate changes in glutathione system and decrease caspase-3 activity.

Concerning the active constituents in the lichens, chemical composition was defined in order to identify the major compounds in each extract. By TLC and HPLC, two largely used methods in lichens phytochemistry (45), we determined that the main constituents present in the three extracts were alectoronic acid (*Cb*), protocetraric acid (*Ps*), and usnic and stictic acids (*Ug*). All these secondary metabolites are biosynthesized through the polyketide pathway and belong to the group of structures called depsidones, except usnic acid which is a dibenzofuran compound. They might be responsible for the pharmacological properties demonstrated in the present work, and therefore, they are worthy of further investigation.

Finally, it can be stated that tested lichen species display promising neuroprotective properties, based on their antioxidative effects, in nervous system cellular model of excessive oxidative stress. Results suggest that the three species could be an interesting source of natural products with neuroprotective interest; deeper investigations may focus on the study of effects of isolated metabolites in other *in vitro* and *in vivo* models of neurodegeneration.

6. CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests.

7. ACKNOWLEDGMENTS

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8. REFERENCIAS

1. Hawksworth DL, Honegger R. The lichen thallus: a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. In: Ed. Williams MA, Plant Galls. The Systematics Association, Special Vol. 49. Clarendon Press, Oxford. 1994: pp. 77-98.
2. Huneck S. The significance of lichens and their metabolites. Naturwissenschaften 1999; 86: 559-570.
3. Molnár K, Farkas E. Current results on biological activities of lichen secondary metabolites: a review. Z Naturforsch C 2010; 65: 157-173.
4. Zambare VP, Christopher LP. Biopharmaceutical potential of lichens. Pharm Biol 2012; 50:778-798.
5. Gómez-Serranillos MP, Fernández-Moriano C, González-Burgos E, Divakar PK, Crespo A. Parmeliaceae family: phytochemistry, pharmacological potential and phylogenetic features. RSC Adv 2014; 4: 59017-59047.
6. Behera BC, Verma N, Sonone A, Makhija U. Antioxidant and antibacterial activities of lichen *Usnea ghattensis in vitro*, Biotechnol Lett 2005; 27: 991-995.
7. Behera BC, Verma N, Sonone A, Makhija U. Evaluation of antioxidant potential of the cultured mycobiont of a lichen *Usnea ghattensis*. Phytother Res 2005; 19: 58-64.
8. Bugni TS, Andjelic CD, Pole AR, Rai P, Ireland CM, Barrows LR. Biologically active components of a Papua New Guinea analgesic and anti-inflammatory lichen preparation. Fitoterapia. 2009; 80: 270-273.
9. Crespo A, Lumbsch HT. Cryptic species in lichen-forming fungi. IMA Fungus 2010; 1: 167-170.
10. Crespo A, Divakar PK, Hawksworth DL. Generic concepts in parmelioid lichens, and the phylogenetic value of characters used in their circumscription. Lichenologist 2011; 43: 511-535.
11. Divakar PK, Figueras G, Hladun N, Crespo A. Molecular phylogenetic studies reveal an undescribed species within the North American concept of

Melanelixia glabra (Parmeliaceae). Fungal Divers 2010; 42: 47-55.
12. Leavitt SD, Esslinger, TL, Spribille T, Divakar PK, Lumbsch HT. Multilocus phylogeny of the lichen-forming fungal genus *Melanohalea* (Parmeliaceae, Ascomycota): Insights on diversity, distributions, and a comparison of species tree and concatenated topologies. Mol Phylogenet Evol 2013; 66: 138-152.
13. Paudel B, Bhattarai HD, Koh HY, *et al*. Ramalin, a novel nontoxic antioxidant compound from the Antarctic lichen Ramalina terebrata. Phytomedicine 2011; 18: 1285-1290.
14. Crespo A, Divakar PK, Blanco O, Schroeter B. The potential of mitochondrial DNA for establishing phylogeny and stabiling generic concepts in the parmelioid lichens. Taxon 2001; 50: 807-819.
15. Myllys L, Lohtander K, Källersjö M, Tehler A. Sequence insertions and ITS data provide congruent information on Roccella canariensis and R. tuberculata (Arthoniales, Euascomycetes) Phylogeny. Mol Phylogenet Evol 1999; 12: 295-309.
16. Lohtander K, Myllys L, Sundin R, Källersjö M, Tehler A. The species pair concept in the lichen Dendrographa leucophaea (Arthoniales): Analyses based on ITS sequences. Bryologist 1998; 101: 404-411.
17. Sayers WE, Barrett T, Benson DA *et al.* Data resources of the National Center for Biotechnology Information. Nucleic Acids Res 2011; 39: D38-D51.
18. Dávalos A, Gómez-Cordovés C, Bartolomé B. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. J Agric Food Chem 2004; 52: 48-54.
19. Mosmann T. Rapid colorimetric assay for celular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983; 65: 55-63.
20. González-Burgos E, Duarte AI, Carretero ME, Moreira PI, Gómez-Serranillos MP. Mitochondrial-targeted protective properties of isolated diterpenoids from *Sideritis* spp. in response to the deleterious changes induced by H_2O_2. J Nat Prod 2013; 76: 933-938.
21. LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2,7-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 1992; 5: 227-231.
22. Marks N, Berg MJ, Guidotti A, Saito M. Activation of caspase-3 and apoptosis in cerebellar granule cells. J Neurosci Res 1998; 52: 334-341.
23. Hissin P, Hilf RA. Fluorometric method for determination of oxidized and reduced glutathione in tissues. Anal Biochem 1976; 74: 214-226.
24. Culberson CF. A standardized method for the identification of lichen products. J Chromatography 1970; 46: 85-89.
25. Culberson CF. Improved conditions and new data for the identification of lichen products by a standardized thin-layer chromatographic method. J Chromatography 1972; 72: 113-115.
26. Culberson CF. Conditions for the use of Merck silica gel 60 F254 plates in the standardized thin-layer technique for the lichen products. J Chromatography 1974; 97: 107-108.
27. Cansaran D, Atakol O, Halici MG, Akay A. HPLC analysis of usnic acid in some extracts of the lichen *Parmelia* and investigation of their constituent. Pharm Biol 2007; 45: 77-81.
28. Orchard AA. Flora of Austarlia. Lichens-Lecanorales2, Parmeliaceae. Various authors (Eds). Australian Biological Resources Study. CSIRO Publishing. 1994: Vol 55.
29. Divakar PK, Crespo A. Molecular phylogenetic and phylogenomic approaches in studies of lichen systematics and evolution. In: Upreti DK, Divakar PK, Shukla V, Bajpal R (Eds.). Recent advances in lichenology: modern methods and approaches in lichen systematics and culture techniques. Springer, New Delhi (IN), 2015: Vol. 2, pp. 45-60.
30. Schoch CL, Seifert KA, Huhndorf S, *et al*. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. Proc Natl Acad Sci U S A 2012; 109: 6241-6246.
31. Begerow D, Nilsson H, Unterseher M, Maier W. Current state and perspectives of fungal DNA barcoding and rapid identification. Appl Microbiol Biotechnol 2010; 97: 99-108.
32. Schoch CL, Robbertse B, Robert V, *et al*. Finding needles in haystacks: linking scientific names, reference specimens and molecular data for Fungi. Database: Database (Oxford) 2014; 2014:bau061.
33. Apak R, Güçlü K, Demirata B, *et al*. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. Molecules 2007; 12: 1496-1547.
34. González-Burgos E, Carretero ME, Gómez-Serranillos MP. Nrf2-dependent neuroprotective activity of diterpenoids isolated from *Sideritis* spp. J Ethnopharmacol 2013; 147: 645-552.
35. Sinha K, Das J, Pal PB, Sil PC. Oxidative stress: the mitochondria-dependent and mitochondria-independent pathways of apoptosis. Arch Toxicol 2013; 87:1157-1180.
36. Perez-Pinzon MA, Stetler RA, Fiskum G. Novel mitochondrial targets for neuroprotection. J Cereb Blood Flow Metab 2012; 32: 1362-1376.
37. Robertson GS, Crocker SJ, Nicholson DW, Schulz JB. Neuroprotection by the inhibition of apoptosis. Brain Pathol. 2000; 10: 283-92.
38. Andersen JK. Oxidative stress in neurodegeneration: cause or consequence? Nat. Rev Neurosci 2004; 5: S18-S25.
39. Behera BC, Verma N, Sonone A, Makhija U. Experimental studies on the growth and usnic acid production in "lichen" *Usnea ghattensis in vitro*. Microbiol Res 2006; 161: 232-237.

40. Luo H, Wei XL, Han KS, Koh YJ, Hur JS. Taxonomic study on the lichen genus cetrelia (lecanorales, ascomycota) in South Korea. Mycobiology 2007; 35: 117-123.
41. Joshi Y, Wang XY, Lee YM, Byun BK, Koh YJ, Hur JS. Notes on Some New Records of Macro- and Micro-lichens from Korea. Mycobiology 2009; 37: 197-202.
42. Azmi NH, Ismail N, Imam MU, Ismail M. Ethyl acetate extract of germinated brown rice attenuates hydrogen peroxide-induced oxidative stress in human SH-SY5Y neuroblastoma cells: role of anti-apoptotic, pro-survival and antioxidant genes. BMC Complement Altern Med 2013; 13:177.
43. Venuprasad MP, Hemanth Kumar K, Khanum F. Neuroprotective effects of hydroalcoholic extract of *Ocimum sanctum* against H_2O_2 induced neuronal cell damage in SH-SY5Y cells via its antioxidative defence mechanism. Neurochem Res 2013; 38: 2190-2200.
44. Wu CR, Tsai CW, Chang SW, Lin CY, Huang LC, Tsai CW. Carnosic acid protects against 6-hydroxydopamine-induced neurotoxicity in in vivo and *in vitro* model of Parkinson's disease: involvement of antioxidative enzymes induction. Chem Biol Interact 2015; 225: 40-46.
45. Feige GB, Lumbsch HT, Huneck S, Elix JA. Identification of lichen substances by a standardized high-performance liquid-chromatographic method. J Cromatography 1993; 646: 417-427.