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HPLC isolation of antioxidant constituents from *Xanthoparmelia* spp.

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ABSTRACT

A chromatographic method is described for the purification and characterization of secondary lichen substances with biological activity. A simple reversed-phase high-performance liquid chromatography method with gradient elution has been developed that allows the determination and isolation of salazinic, usnic and stictic acids from lichen samples in a single run and the quantification of every acid in the tested extracts. The antioxidant activity of both the isolated compounds and the respective lichen belonging to *Xanthoparmelia* genus was determined by the Oxygen Radical Absorbance Capacity (ORAC) assay; their effect as free radical scavengers, effect on cell survival by the 3(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium reduction assay and 2′,7′-dichlorofluorescin diacetate method were tested on U373 MG human astrocytome cell line. Both lichens extracts and all isolated compounds protected U373 MG cells from hydrogen peroxide-induced damage, suggesting that they could act as antioxidant agents in those neurodegenerative disorders associated with oxidative damage, such as Alzheimer's disease and Parkinson's disease.

1. Introduction

Lichens or lichenized fungi are symbiotic organisms consisting of, at least, one *fungi* and one photosynthetic partner (alga or cyanobacterium). This association is successful as lichens inhabit most ecosystems, from deserts to the highest mountains. Lichens also contain a set of unique chemical compounds that are a source of metabolites with biological activity for human use [1].

The genus *Xanthoparmelia* belongs to the Family *Parmeliaceae*, Order Lecanorales [2]. *Xanthoparmelia* is one of the larger genera of lichenized *fungi* with more than 800 species [3]. The species of this genus are saxicolous and mainly inhabit arid climates [4]. The genus *Xanthoparmelia* is distributed worldwide with a high diversity in the Southern Hemisphere [5]. This genus produces at least 92 secondary metabolites with a diverse chemical structure (phenolic compound such as depsides, depsidones, antraquinones and monocilic compounds, as well as aliphatic acids) [5,6]. Some of these compounds have been studied from a pharmacological point of view as antioxidants [7], antibiotics [8], antiproliferative [9] or antiHIV [10] agents.

From a taxonomical point of view, lichen secondary metabolites are most often studied by thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC) [6,11]. The latter technique is also used for quantification [12] and allows a better separation and identification of the lichen compounds [13]. The first attempts to analyse lichens by HPLC applied an isocratic elution of the mobile phase (constant composition of mobile phase during the analytical run) [14,15]; although good results were obtained, later approaches with a gradient elution (mobile phase proportion changes during the analytical run) yielded even better results for the HPLC analysis of lichen extracts, as they frequently contain metabolites of wide-ranging hydrophobicity whose elution is improved with this variation in the mobile phase composition [11,13]. The easiest method for the isolation of lichen substances was the collection of those spots separated by TLC, followed by acetone suspension, filtration and evaporation [1].

Numerous reports suggest that oxidative stress is implicated in the pathogenesis of many diseases including neurodegenerative disorders, cancer, diabetes or cardiovascular diseases, as well as in aging processes. Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS) such as hydrogen peroxide, superoxide anion or hydroxyl radical, and the ability of enzymatic and non-enzymatic biological systems to protect against their injury. As a consequence of oxidative stress, oxidation of proteins, lipids and DNA can occur that leads to cell degeneration and even cell death. Different therapeutic strategies have been proposed for the prevention and treatment of ROS-mediated diseases, with special emphasis on antioxidant therapy [16–19].

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Astrocytes are attracting interest from many investigators as support cells for neuronal energy metabolism. Recently, a comprehensive new concept has been proposed; i.e. astrocytic accumulation of glutamate release during neuronal activation triggers an increase in astrocytic glycolysis and the generated lactate is released from astrocytes and taken up by neurons, where it is oxidatively metabolized to fuel neuronal energy requirement [20,21]. In addition, astrocytes play wide range of roles in the survival neurons. Consequently, the mechanism of cell damage of astrocytes is now an important target for investigation.

Extracts and different isolated compounds from lichens have shown antioxidant activity. To date, the activity of isolated compounds from *Xanthoparmelia* spp. and has not been established for neurodegenerative disorders induced by oxidative processes. In this work, the antioxidant activity of *Xanthoparmelia* spp. extracts and the isolated compounds usnic acid, stictic acid and salazinic acid has been determined by the Oxygen Radical Absorbance Capacity (ORAC) assay. The antioxidant ability of both extracts and every compound has also been assayed in an oxidative stress model induced by hydrogen peroxide in U373 MG human astrocytoma cell line using the 3(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium (MTT) reduction assay and the 2′,7′-dichlorofluorescin diacetate (DCFH-DA) method.

The present research describes a method for the separation, purification and characterization of secondary lichen substances that is a substantial modification of previously published works [13]. We propose an easy method for isolation of lichen substances by high-performance liquid chromatography with ultraviolet–vis detection (HPLC-UV–vis) with a better yield than TLC [1]. The method delivers fast and reproducible results. A reversed-phase high-performance liquid chromatography with a gradient elution has been developed that allows the determination and quantification of salazinic, usnic and stictic acids in a single run.

2. Experimental

2.1. Chromatographic analysis

2.1.1. Chemical and reagents

Distilled water was obtained from a Millipore water purification system (Millipore, Bedford, MA, USA). Methanol (MeOH, HPLC quality), orthophosphoric acid, toluene, acetic acid, ethyl acetate and formic acid (analytical grade) were purchased from Panreac Quimica (Barcelona, Spain).

Every solvent was degassed by ultrasonic treatment and filtered through a membrane filter (0.45 μm) before use in the HPLC system.

2.1.1.1. Standards. Usnic and stictic acids were isolated from *Xanthoparmelia conspersa* (Ach.) Hale. Usnic and salazinic acids were isolated from *Xanthoparmelia camtschadalis* (Ach.) Hale. Every isolation process followed the method described in Section 2.1.3.

2.1.2. Instrumentation

2.1.2.1. Thin layer chromatography. Samples were run on Merck silica gel 60 F254 pre-coated glass-backed TLC plates (0.25 mm) 20 cm × 20 cm with solvent systems toluene/acetic acid (170:30, v/v) toluene/ethyl acetate/formic acid (139:83:8, v/v/v) [6].

2.1.2.2. High-performance liquid chromatography apparatus. HPLC analysis was performed on a Spectra-Physics SP 8800/8810LC pump coupled to a Spectra-Physics SP8490, UV–vis detector set at 254 nm and a Clarity Lite™ data processor.

2.1.2.3. Analytical run. Mobile phase consisted on 1% orthophosphoric acid–MeOH in gradient elution from A 70% to A 30% after 15 min and then 10% A at 45 min. Initial conditions were recovered at 65 min. Flow rate was 0.5 ml/min and 10 μl of test and reference solutions, were injected. The analysis was performed on a Mediterranea-Sea C-18 (5 μm) column, 250 mm × 4.6 mm (Teknochroma, Spain), at 25 °C.

2.1.2.4. Semipreparative analysis. HPLC analysis was performed on a Spectra-Physics SP 8800/8810LC pump coupled to a Varian 9065 polychrom diode-array detection system. Mobile phase composition and elution were the same than for the analytical run. Flow rate was 2 ml/min and 500 μl of test solution were injected on a Spherisorb 5 ODS 2 (5 μm) column, 250 mm × 7 mm (Teknochroma, Spain), at 25 °C.

2.1.3. Methods

2.1.3.1. Lichen material. Samples of *Xanthoparmelia conspersa* (Ach) Hale and *Xanthoparmelia camtschadalis* (Ach.) Hale were collected in Segovia and Teruel (Spain), respectively. Herbarium samples were authenticated by a taxonomist and a specimen was deposited in the Herbarium of the Faculty of Pharmacy, Universidad Complutense de Madrid, with accession numbers MAF-Lich 15588 and MAF-Lich 15589, respectively. The first species was used for extraction and isolation of usnic and stictic acids, and the second for usnic and salazinic acids.

2.1.3.2. Thin layer chromatography. The substances were first identified by their R_f and isolated by TLC as previously reported [1]. The isolated substances identity was confirmed using the computer program Wintabolites [22] and they were further analyzed by HPLC to confirm the identity of each isolated peak according to their retention times and absorption spectra [13].

2.1.3.3. High-performance liquid chromatography. 15 mg of lichen material was extracted in 1 ml of methanol for 60 min [13]. Peaks of salazinic, usnic and stictic acids were identified based on their retention times with respect to the reference substances previously isolated by TLC and run in an analytical HPLC. This procedure also confirmed the purity of the substances isolated by HPLC compared with those isolated by TLC. Isolated substances were collected from the column according to their corresponding retention time.

2.1.3.4. Solvent evaporation. The solvent was removed using a rotary evaporator at 40 °C and pressure gradient between 70 and 500 mmHg for 60 min.

2.1.3.5. Calibration graphs. Reference solutions of decreasing concentrations were obtained by dilution with eluent of the requisite standard solution. These solutions were analyzed and the corresponding peak areas plotted against the concentration of acid injected. The concentrations of the components in the analyzed samples (*Xanthoparmelia* extracts) were calculated from the chromatogram peak areas using the normalization method. The identification of the different compounds was achieved by comparison of both t_R and the absorption spectra obtained for each diluted peak with those obtained for the standards.

The data obtained were statistically processed by using a standard non-parametric variance analysis method (ANOVA) for determining significant intergroup differences. Data are presented as the mean ± standard deviation (SD) of three separate experiments performed on different samples.

2.1.3.6. Linearity, precision and accuracy. The linearity of the detector responses for the prepared standards was assessed by means of a linear regression analysis on the amounts of each standard (measured in μg) introduced in the chromatographic system and the area of the corresponding peak on the chromatogram. Accuracy and

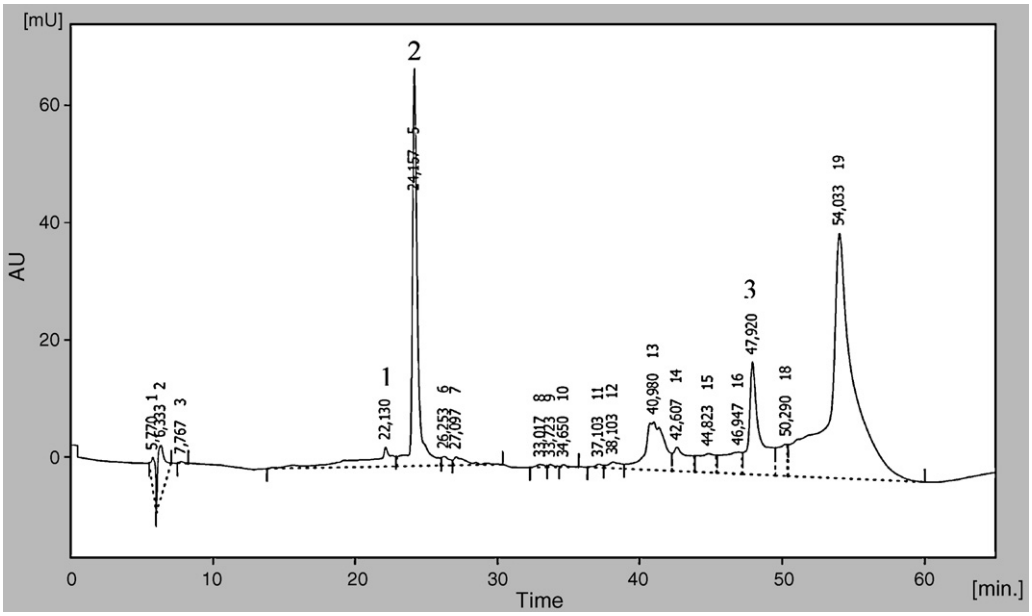

Fig. 1. HPLC-chromatogram of the standard solution containing salazinic (1), stictic (2) and usnic (3) acids.

precision were evaluated by adding increasing known amounts of each standard to a solution of a known concentration whose analysis was replicated three times. The precision was expressed as relative standard deviation (RSD) and the accuracy as the amount found.

2.2. Evaluation of neuroprotective effect

2.2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS) and gentamicin (10 mg/ml) were purchased from Gibco (Invitrogen Corporation, Paisley, UK). Hydrogen peroxide, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide, 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX), 2,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), fluorescein sodium salt and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) were obtained from Sigma–Aldrich (St Louis, MO, USA). Dimethyl sulphoxide (DMSO) was supplied by Panreac (Barcelona, Spain). Astrocyte cell line U373 MG was obtained from the European Collection of Animal Cell Cultures (ECACC, Salisbury, Wiltshire, UK).

2.2.2. Astrocyte cultures

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 0.5% gentamicin (10 mg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37 °C.

2.2.3. Cell treatments

Cells were treated with different concentrations of *X. conspersa* and *X. camtschadalis* extracts and with the isolated compounds usnic acid, stictic acid and salazinic acid for 24 h. 1 mM hydrogen peroxide (H_2O_2) was used as oxidative stress inductor (30 min). Extracts and compounds isolated from lichens as well as H_2O_2 were dissolved in phosphate-buffered saline (PBS).

2.2.4. Cell viability

Cell viability was determined according to the MTT method of Mossman [21] with minor modifications. Cells were plated at a density of 5×10^4 cells/well in 96-well plates overnight. Then, cells were treated with different concentrations of lichens extracts (25, 50, 100 and 250 μg/ml) and isolated compounds (5, 10, 25 and 50 μg/ml) for 24 h. In addition, to evaluate a possible protective effect against stress oxidative inductors, cells were exposed to 1 mM H_2O_2 for 30 min. Finally, MTT (2 mg/ml) was added and plates were incubated for 1 h at 37 °C. The medium was removed and 100 μl DMSO was added to each well in order to dissolve the dark blue formazan crystals. Absorbance was measured at 550 nm using a microplate reader Digiscan 340 (ASYS Hitech GmbH, Eugendorf, Austria). Results were expressed as the percentage cell viability, setting that the optical density of untreated cells to 100%.

2.2.5. Evaluation of antioxidant activity

ORAC assay. Antioxidant activity was measured by the ORAC assay as previously described [22]. This method is based on the oxidation of fluorescein as consequence of peroxyl radicals produced by the addition of the AAPH radical inductor. Antioxidant activity quantification was determined using "area under curve" (AUC) and results were compared to a standard curve of water soluble vitamin E analogue Trolox. ORAC values are expressed as μmol Trolox equivalents (TE)/mg samples.

Intracellular ROS production assay. ROS production was assayed using the DCFH-DA method [23]. Cells were plated at a density of 5×10^4 U373 MG cells/well in 96-well plate overnight. Then, cells were incubated in PBS-glucose (PBS-G) containing 50 μl DCFH-DA for 30 min at 37 °C. Finally, cells were washed twice with PBS-G and treated with 25 and 50 μg/l of lichens extracts and 5 and 10 μg/ml of each isolated compound; these concentrations had been previously selected from the MTT assay results. Fluorescence was measured for 2 h in a Microplate Fluorescence Reader (FLx800, Bio-Tek, Instrumentation, USA) with excitation wavelength of 480 nm and emission wavelength of 510 nm.

2.2.6. Statistical analysis

Results were 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. A p value < 0.05 was considered statistically significant.

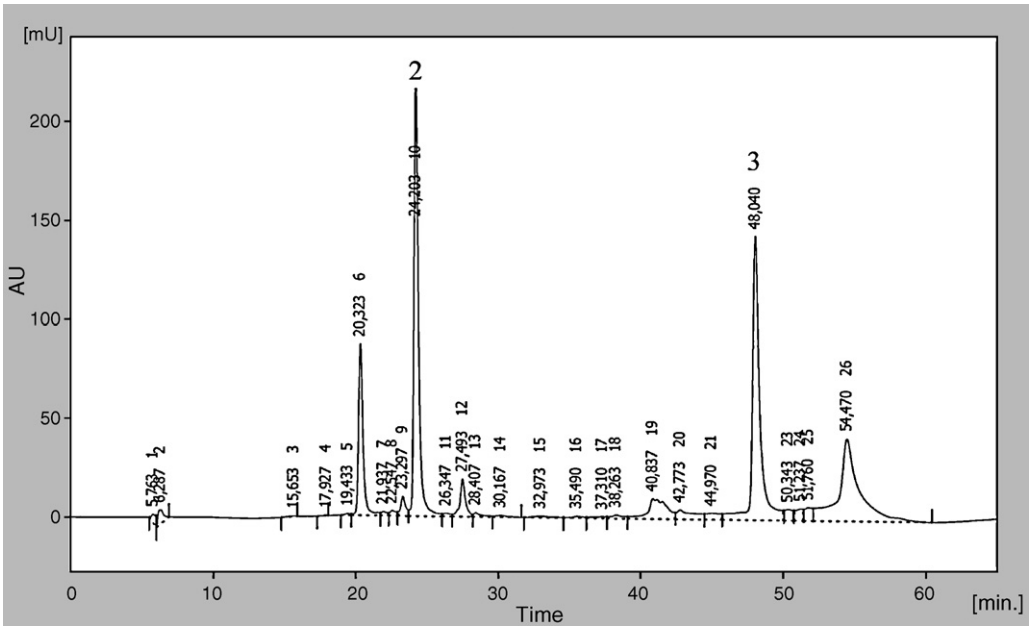

Fig. 2. HPLC-chromatogram of *Xanthoparmelia conspersa* methanolic extract containing stictic (2) and usnic (3) acids.

3. Results

3.1. Analytical

A chromatographic trace of the standard solution containing salazinic, stictic and usnic acids on the C_{18} column is shown in Fig. 1. Very good resolution of chromatographic peaks and baseline separation of all compounds was obtained after optimization of separation on the reversed-phase in 65 min. Table 1 shows the retention time of the examined lichen substances and their substance class. Retention time data (t_R) of each substance and R_f were confirmed using Wintabolites database [24].

The substance classes can be distinguished by their UV spectra together with their t_R value. Depsidones have highly variable hydrophobicities and show t_R values around 23 min. Usnic acid (usnic acids class) shows the highest t_R value (47 min, approximately).

The detector response was linear in the range between 0.2 and 25 μg per 10 μl samples, and the chromatographic system was able to detect 0.2 μg of usnic acid in the 10 μl injected. The recovery rate for usnic acid was 91.8 ± 1.9% (n = 15).

HPLC isolated acids show higher purity than the same reference substances isolated by TLC, as seen by comparison of the TLC chromatographies for both obtained standards (data not shown).

Figs. 2 and 3 show the HPLC-chromatogram of *X. conspersa* and *X. camtschadalis*, respectively, under the analytical conditions above described.

X. camtschadalis contents were 9.285 ± 0.47 and 0.432 ± 0.06 mg of salazinic and usnic acids, respectively while *X. conspersa* showed 1.763 ± 0.08 and 0.437 ± 0.07 mg of stictic and usnic acids, respectively (% w/w).

Table 1
Retention time of the examined lichen substances and their substance class. Values are the means of three determinations ± SD.

Compound	Retention time (t_R ± SD) (min)	Substance class
Salazinic acid	23.15 ± 0.06	Depsidones
Stictic acid	24.11 ± 0.10	Depsidones
Usnic acid	47.67 ± 0.14	Usnic acids

3.2. Evaluation of neuroprotective ability

3.2.1. Antioxidant activity

The antioxidant activity determined by ORAC assay for lichens extracts and isolated compounds is shown in Table 2. Lichens had a higher antioxidant capacity than the isolated compounds in the following rank order: *X. camtschadalis* > *X. conspersa* > usnic acid > salazinic acid > stictic acid. However, the ORAC assay did not reflect the antioxidant actions of these lichens extracts and isolated compounds in biological systems.

3.2.2. Cell viability

The results obtained for the effect of *X. conspersa* and *X. camtschadalis* extracts, and usnic, salazinic and stictic acids on U373 MG cell viability and proliferation are shown in Fig. 4. A significant cell viability loss was observed for *X. conspersa* at 250 μg/ml (63.0 ± 7.1) and 100 μg/ml (69.6 ± 4.1) and for *X. camtschadalis* at 250 μg/ml (71.3 ± 7.5). The isolated compounds, usnic and stictic acids significantly reduced cell viability at 50 and 25 μg/ml (76.7 ± 1.13, 82.8 ± 0.5 and 72.1 ± 8.0, 82.1 ± 5.2, respectively) whereas salazinic acid was toxic only at 50 μg/ml (82.8 ± 1.6). In every case, triton was used as negative control because it causes cell lysis.

When U373 MG cells were treated only with hydrogen peroxide, cell viability decreased nearly 40% compared with control cells. However, pretreatment with both *Xanthoparmelia* spp. extracts for 24 h significantly increased cell viability (10% and 16% for *X. conspersa* and 18% and 19% for *X. camtschadalis* at 50 and 25 μg/ml compared with H_2O_2-treated cells). Pretreatment with usnic, stictic and salazinic acids also improved cell viability. Usnic acid showed

Table 2
ORAC values for *Xanthoparmelia conspersa* and *Xanthoparmelia camtschadalis* extracts and isolated usnic, salazinic and stictic acid. Results are expressed as micromol Trolox equivalents per milligram ± SD obtained from three independent experiments.

X. camtschadalis extract	8.80 ± 0.9
X. conspersa extract	4.98 ± 0.3
Usnic acid	2.95 ± 0.1
Salazinic acid	2.74 ± 0.2
Stictic acid	2.32 ± 0.2

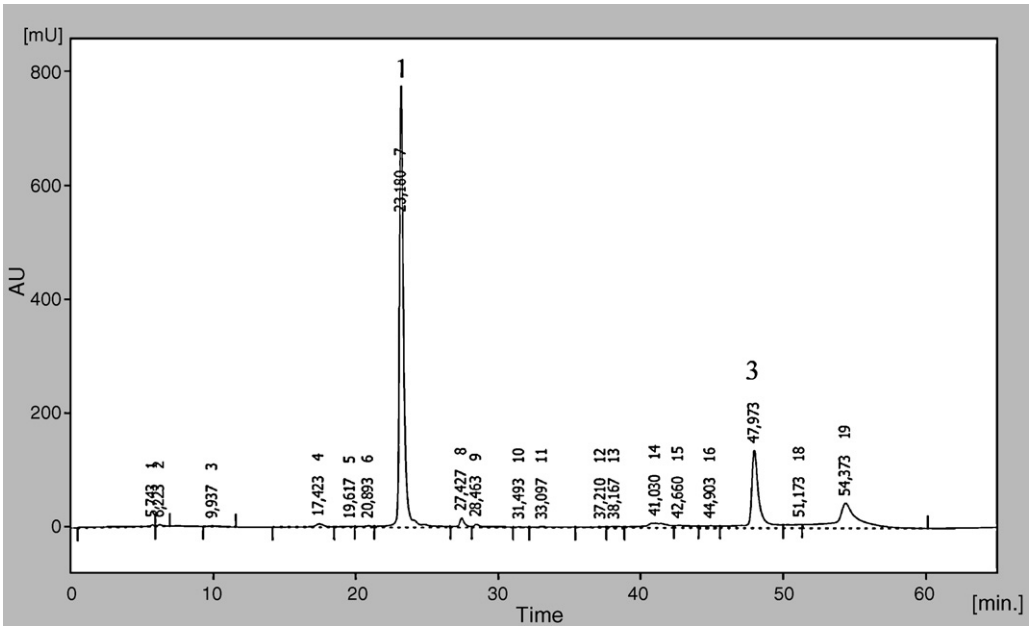

Fig. 3. HPLC-chromatogram of *Xanthoparmelia camtschadalis* methanolic extract containing salazinic (1) and usnic (3) acids.

a protective effect at all concentrations assayed, whereas salazinic acid and stictic acid did so only at 5, 10 and 25 μg/ml concentrations. All of these compounds isolated from lichens extracts were more active at 5 and 10 μg/ml concentrations and, as a result, these were the concentrations chosen for measurement of intracellular ROS production, determined through DCFH-DA method.

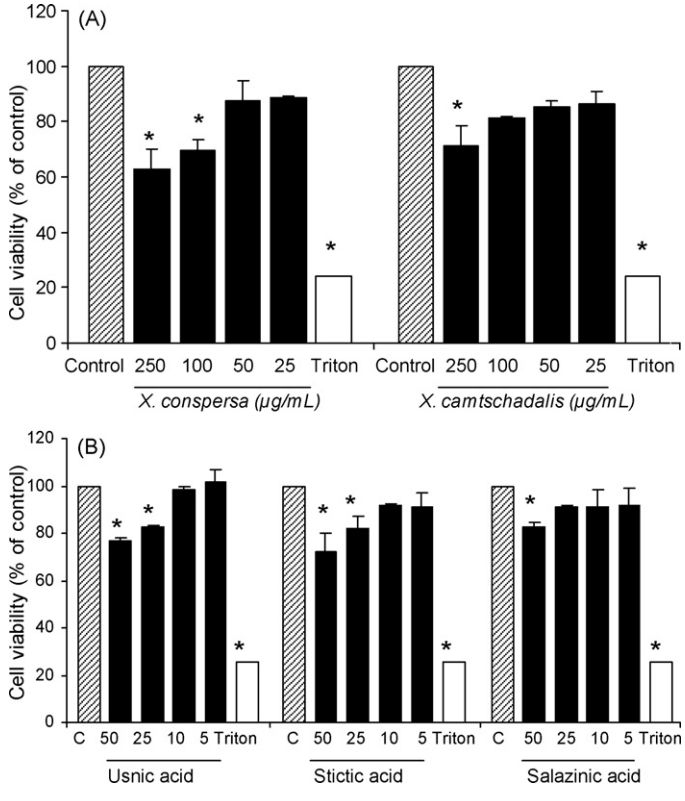

Fig. 4. Effect of lichens extracts and isolated compounds on U373 MG cell viability by MTT assay. (A) *X. conspersa* and *X. camtschadalis* (25, 50, 100 and 250 μg/ml) (B) isolated usnic, stictic and salazinic acids (5, 10, 25 and 50 μg/ml). Results are expressed as mean ± SD obtained from three independent experiments. $^{*}p < 0.05$ *versus* control group.

Both lichens extracts and lichen isolated compounds significantly decreased ROS production induced by hydrogen peroxide in U373 MG cells. *X. camtschadalis* extract showed stronger ROS scavenging activity than *X. conspersa* extract (Fig. 5). Among the lichen isolated compounds, usnic acid showed the strongest activity, followed by salazinic acid and stictic acid (Fig. 6). These results agree with those obtained in the ORAC assay.

4. Discussion

Lichens produce a great number of different secondary metabolites, many of which occur exclusively in these symbiotic organisms. The biological role of these substances is not well known yet, although they often provide a protective function for the lichen. Nevertheless, the identification of these substances has become an integral part of modern taxonomic investigations of these organisms.

Standardized methodology and further refinements of routine analytical TLC procedures for detecting and comparing lichen metabolites have been reported [25]. Further, two-dimensional TLC has considerably improved the R_f values and the discrimination of structurally similar compounds, and has enabled the identification of minor constituents present in complex mixtures [26]. High-performance liquid chromatography has become more widely used as an effective analytical tool for the separation and identification of lichen substances. Early attempts to apply this method to lichen chemotaxonomy were made using normal-phase silica columns or reversed-phase columns with gradient elution [13].

In this work, two different isolation methods were used: typically, isolated substances (usnic, salazinic and stictic acids) were first isolated by TLC in order to help their identification (R_f values were obtained) and to be used for HPLC analysis. These isolated compounds were run under the described HPLC conditions (gradient elution, diode-array detection) so that the retention times and absorption spectra for each substance were obtained. These parameters taken altogether (R_f, t_R and absorption spectra) allowed the chemical identification of the three lichen compounds from descriptions in the literature [24,27].

Semipreparative HPLC was used to obtain the same three lichen substances once they had been chemically identified. This tech-

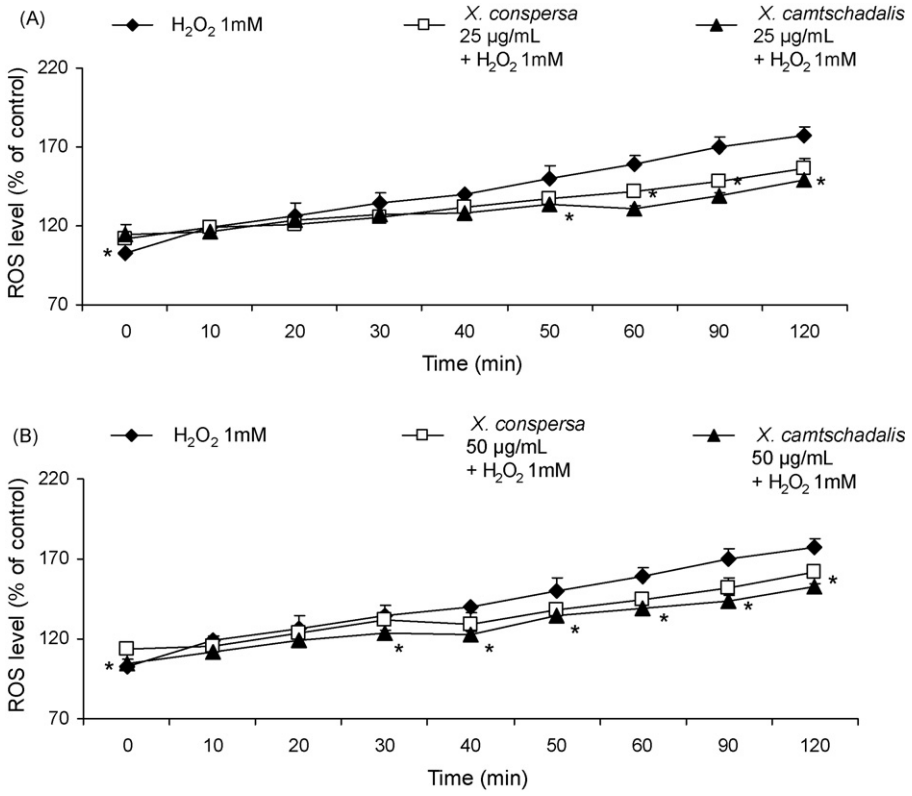

Fig. 5. Inhibitory effects of *X. conspersa* and *X. camtschadalis* extracts at 25 (A) and 50 μg/ml (B) on ROS production induced by H_2O_2 in U373 MG human astrocytoma cell line. Results are expressed as mean ± SD obtained from three independent experiments. $^*p < 0.05$ *versus* 1 mM H_2O_2.

nique allowed the extraction of purer compounds as well as giving a much better yield than the previous TLC analysis (70% *versus* 20%). These isolated compounds were then used for the biological assays (neuroprotective and antioxidant effects).

Here we propose a standardized method using gradient elution for the identification of the lichen substances (usnic, stictic and salazinic acids) that are found in a wide number of lichen genera and could be used as chemotaxonomical markers, and which also show biological activity. The lichen substances quantification in *X. conspersa* and *X. camtschadalis* confirms that usnic acid is present at nearly the same concentration in both extracts; stictic acid is predominant in *X. conspersa* whereas *X. camtschadalis* contains salazinic acid, instead. These results confirm the general chemical composition of both species as known from the literature.

Hydrogen peroxide has been implicated to trigger apoptosis in astrocytes leading to major neurodegenerative diseases. The brain is constantly exposed to ROS because of its high metabolic rate. Astrocytes are the first line of defence in the brain against neurotoxicity of ROS [28–30].

The ORAC assay showed that both lichens extracts and all the isolated compounds had antioxidant capacity. However, the ORAC assay did not reflect the antioxidant actions of these lichens extracts and isolated compounds in biological systems. As a result, their antioxidant activity in an oxidative stress model induced by hydrogen peroxide in U373 MG human astrocytoma cell line was then evaluated. First of all, their effect on cell viability and proliferation was studied in order to determine toxic concentrations and, following this, it was investigated whether *Xanthoparmelia* ssp. and isolated compounds protected U373 MG cells against H_2O_2-induced cytotoxicity. Pretreatment with both *Xanthoparmelia* spp. extracts for 24 h significantly increased cell viability; usnic, stictic and salazinic acids also improved cell viability and led to the concentrations chosen for measurement of intracellular ROS production.

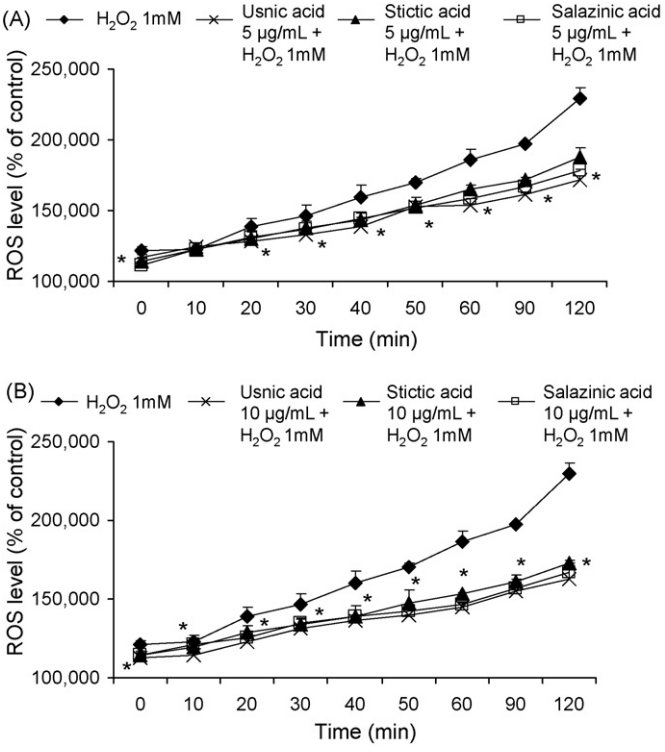

Fig. 6. Inhibitory effect of isolated compounds usnic acid, stictic acid and salazinic acid at 5 (A) and 10 μg/ml (B) on ROS production induced by H_2O_2 in U373 MG human astrocytoma cell line. Results are expressed as mean ± SD obtained from three independent experiments. $^*p < 0.05$ *versus* 1 mM H_2O_2.

We investigated whether lichens extracts and isolated compounds generated reactive oxygen species by themselves or as a result of an interaction with some component of the culture media. Subsequently, the antioxidant ability of both extracts and isolated compounds was evaluated. Both lichens extracts and isolated compounds significantly decreased ROS production induced by hydrogen peroxide in U373 MG cells and agreed with the results from the ORAC assay.

In conclusion, lichen substances are poorly studied as a new source of active metabolites. Our findings show that both lichens extracts (*X. camtschadalis* and *X. conspersa*) and the isolated lichens compounds salazinic acid, stictic acid and usnic acid induced neuroprotection through their antioxidant ability in astrocytes and probably exert a protection against oxidative stress. This suggests the possibility that they could be used as antioxidant agents in those neurodegenerative disorders associated to oxidative damage such as Alzheimer's disease and Parkinson's disease.

The development of easy-to-use analytical methods that optimize the identification and separation of lichenic substances in these samples is needed. The analyzed acids (salazinic, stictic and ucid acids) possess antioxidant activity and also serve as chemotaxonomic markers. This method, which shows good reproducibility, gives a good separation of the analyzed compounds, is easy to prepare and, with an economic mobile phase, appears to be optimal to study extracts of the genus *Xanthoparmelia* and other lichen extracts.

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