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The evaluation of antioxidant and anti-inflammatory activities of *Parmotrema hypotropa* lichen extract

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Abstract

Lichens are used since antiquity for multiple therapeutic purposes, treating infections, fever, convulsions, and wounds. This study was designed to evaluate the antioxidant and anti-inflammatory effects of the lichen *Parmotrema hypotropa (NYL.) Hale*. The total phenolics and flavonoids content were determined in ethanol extract; the antioxidant activity was assessed by different chemical assays DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), β-carotene-linoleic acid, reducing power, and Cupric reducing antioxidant capacity (CUPRAC). The lichen extract was further tested *in vitro*, for its anti-inflammatory effect through the bovine serum albumin denaturation assay; also the inflammatory response was investigated on the LPS-stimulated human keratinocyte cell line (HaCaT) by measuring intercellular ROS (Reactive Oxygen Species) and TNF-α (Tumor necrosis factor-alpha) production; the amount of usnic acid in the extract was determined by High-Performance Liquid Chromatography (HPLC). The results showed that the tested extract contained high levels of total phenolics than flavonoids, and had an antioxidant effect; it prevented the protein denaturation in a dose-dependent manner, also reduced the intracellular ROS and TNF-α production by HaCaT cells. Furthermore, it did not affect the viability of HaCaT cells; the extract contained small amounts of usnic acid. Based on the different findings, *P.hypotropa* can constitute a potential source of bioactive compounds with antioxidant and anti-inflammatory effects.

Keywords: Lichen; *Parmotrema hypotropa*; antioxidant activity; anti-inflammatory effect; HaCaT cells; usnic acid.

1. Introduction

Lichens are symbiotic organisms associating fungi with algae or cyanobacteria. They are considered among the earliest settler of terrene habitats [1]. Lichens can grow on different types of surfaces including rocks, inside bark of woody plants, on soil, mosses, and other surfaces like plastics, glass, and metals [2]. Some lichens are edible and they are prepared in salads, dishes, soups, and used as food flavor enhancers [3]. They are also the source of many products used in cosmetics and perfumery industries [4,5]. The importance of these organisms and the great interest they have aroused in recent years is related to their ability to synthesize various secondary metabolites of medical interest; more than 1000 metabolites of extreme originality, exerting a wide variety of biological activities such as antimicrobial, anti-inflammatory, anti-tumor, anti-haemolytic, antitussive have been determined [6,7]. Usnic acid is one of the most studied secondary metabolites which is produced by the mycobiont and stored in the cortex of lichens; usnic acid has been known for its multiple activities like antimicrobial, antioxidant, anticancer, and larvicidal effects, it was also known for its strong ability to absorb ultraviolet rays [7].

The skin is the first immune barrier against environmental aggressors and can be affected by many inflammatory agents. Keratinocytes represent the main epidermal cells; they play an important role in the skin's protection mechanisms against biological, chemical, and physical threats [8]. They also act as an immunomodulator in managing inflammation and wound healing through their ability to produce a large number of anti-microbial and inflammatory mediators [9,10]. However, any

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alteration in the expression and production of these mediators lead to the development of inflammatory skin disorders [11]. Furthermore, keratinocytes have a crucial role in initiating the early pathogenic events in chronic skin diseases such as psoriasis and dermatitis. High levels of TNF-α, cationic antimicrobial peptides, and chemokines CXCL1, CXCL2, CXCL8, CCL2 have been reported in psoriasis [12,13], whereas high levels of TNF-α, IL-1, IL-6, GM-CSF (granulocyte-macrophage colony stimulating factor) were described in contact dermatitis [14]. Reactive oxygen species (ROS) are molecules involved in different signaling, inflammatory and metabolic processes. It is well established that the overproduction of these species leads to the loss of cellular redox and over time causes chronic diseases such as cancer, atopic dermatitis, and various metabolic disorders such as diabetes and cardiovascular disease [15]. ROS secreted during inflammation are essential for the signaling pathways of various inflammatory cytokines, NF-κB, nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) [16,17]. Therefore, the inhibition of various overproduced inflammatory mediators (ROS and cytokines) through controlling their synthesis can be an important strategy to limit skin inflammations [18,19].

This study aims to investigate the antioxidant and anti-inflammatory effects of *Parmotrema hypotropa* lichen extract. Firstly, the antioxidant activity of the lichen extract is assessed by chemical methods. Then, the anti-inflammatory effect of the extract is tested through the protein denaturation assay and on ROS and TNF-α production by LPS-stimulated HaCaT cells.

2. Material and methods

2.1. Lichen extract

The lichen was collected from the region El kala in the extreme Northeast of Algeria, in September 2019. The specimen was identified by Dr. Philippe Clerc (Conservatory and Botanical Garden of the city of Geneva, Chambésy, Switzerland) as *Parmotrema hypotropa (Nyl.) Hale*. The lichen was dried and ground in a grinder. Then, the powdered sample (12g) was extracted with ethanol (250 mL) for 24 h at room temperature. After that, the supernatant was filtered and concentrated through a rotary evaporator [20]. The obtained extract was stored at -18 °C until the next use.

2.2. Total phenolic content (TPC)

Total phenolic content was determined by the Folin-Ciocalteu reagent according to the method of Müller *et al.* [21]. Briefly, in 96-well microplate 20 µL of extract diluted in DMSO (1mg/mL) were added to 100 µL of Folin-Ciocalteu reagent and 75 µL of Sodium carbonate solution (at 75%). After 2 hours of reaction in darkness at room temperature; the absorbance was measured at 765 nm by a microplate reader (Perkin Elmer, Enspire). The total phenolic content was expressed as µg gallic acid equivalent /mg of extract (µg GAE/mg).

2.3. Total flavonoid content (TFC)

Total flavonoid content was determined according to the method described by Topçu *et al.* [22] with some modifications. In a 96 well plate, a volume of 50 µL of lichen extract diluted in DMSO (1mg/mL) was added to 10 µL of aluminum nitrate (Al (NO3)3, 9H2O at 10%), 10 µL of aqueous potassium acetate 1M (CH_3COOK) and 130 µL of DMSO. After 40 min at room temperature, the absorbance was read at 415 nm. The total Flavonoid content was expressed as µg quercetin equivalent /mg (µg QE/mg).

2.4. Evaluation of the antioxidant activity of *P.hypotropa* extracts

2.4.1. Scavenging activity of DPPH radicals

The scavenging potential of the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined according to the method of Gali and Bedjou [23]. Briefly, 160 µL of DPPH solution (1mM) added to 40 µL of the tested extract diluted in DMSO (4mg/mL) or standards at different concentrations (12.5, 25, 50, 100, 200, 400, 800 µg/mL), after 30 min of incubation in darkness at room temperature, the absorbance was measured at 517 nm.

The scavenging capability of DPPH radical was calculated using the following equation:

$$\textbf{DPPH scavenging effect}(\%) = \frac{A_{Control} - A_{Sample}}{A_{Control}} \times \mathbf{100} \quad \textbf{(1)}$$

The results were given as IC_{50} value (µg/mL) corresponding the concentration of 50% inhibition. The results were compared to the standard antioxidants BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and ascorbic acid.

2.4.2. ABTS scavenging activity

ABTS scavenging activity was measured following Mazouz *et al.* [24]. The ABTS•+ solution was prepared by mixing ABTS (7mM) with Potassium persulfate (2.45 mM). The solution was stored in the dark at room temperature for 16 hours. ABTS•+ solution was then diluted to get the absorbance of 0.700 at 734 nm. Thereafter, a volume of 160 µL of this solution was added to 40 µL of the standards and tested sample in DMSO (4mg/mL) at different concentrations (12.5, 25, 50, 100, 200, 400, and 800 µg/mL). The absorbance was measured at 734 nm after 10 min of incubation at room temperature. The results were given as the IC_{50} (µg/mL), which was

calculated according to the formula **(1)**. BHA, BHT, and ascorbic acid were used as standards.

2.4.3. *β*-carotene linoleic acid bleaching assay

The extract was assessed for its ability to protect the oxidation of the carotenoid β-carotene through hydrogen-based transfer mechanism [25]. Briefly, β-carotene/linoleic acid emulsion was prepared by dissolving 0.5mg of β-carotene in 1 mL of chloroform, then 25 µL of linoleic acid and 200 mg of tween-40 were added. The chloroform was then evaporated using a rotary evaporator. Then, 50 mL of hydrogen peroxide (H_2O_2) was added with vigorous shaking and the absorbance of the solution was adjusted by hydrogen peroxide H_2O_2 to 0,8 - 0,9 at 470nm. A volume of 160 µL of β-carotene solution was added to 40 µL of the tested sample or standards (4mg/mL) at the concentrations 12.5, 25, 50, 100, 200, 400, and 800 µg/mL. The absorbance was measured immediately at 470 nm (t=0 min) and after incubation for 120 min at 45 °C. BHA and BHT were used as standards.

The percentage of inhibition (A%) was calculated according to the following formula:

$$\mathbf{A}\,(\%) = [\mathbf{1} - (\mathbf{A(t=0)} - \mathbf{A(t=120)}) / (\mathbf{AC(t=0)} - \mathbf{AC(t=120)})] \times \mathbf{100}$$

Where A (%) is the percentage of inhibition, $A_{(t=0)}$: the absorbance value of the tested sample at 0 min, $A_{(t=120)}$: the absorbance value of the tested sample after 120 min of incubation and $AC_{(t=0)}$ is the absorbance value of the negative control (methanol without sample) at 0 min. $AC_{(t=120)}$: the absorbance value of the negative control at 120 min.

2.4.4. Reducing power assay

The reducing power activity was determined by the reduction of $Fe^{3+}(CN-)_6$ to $Fe^{2+}(CN-)_6$ as described before [26]. Briefly, 10 µL of extract or standards (4mg/mL) at the concentrations 12.5, 25, 50, 100, 200, 400, and 800 µg/mL were added to 40 µL of 0.2 M sodium phosphate buffer (pH 6,6) and 50 µL of potassium ferricyanide (1%). The solution was incubated for 20 min at 50 °C. After cooling, 50 µL of tri-chloroacetic acid (10%) was used to acidify the mixture, then 10 µL of ferric chloride $FeCl_3$ (0.1%) and 40 µL of distilled water were added. The absorbance was immediately read at 700 nm. $A_{0.5}$ values were calculated from the absorbance curves. The results were compared to BHA, BHT, and ascorbic as positive antioxidant standards.

2.4.5. Cupric reducing antioxidant capacity (CUPRAC)

Cu^{2+} ion reduction antioxidant potential was evaluated using the modified CUPRAC method [27]. In 96-well microplate 50 µL of CuCl2 (10 mM), 50 µL of neocuproine (7.5 mM), and 1 M Acetate ammonium (60 µL, pH 7.0) solutions were mixed with 40 µL of the tested sample or standards at different concentrations (12.5, 25, 50, 100, 200, 400, and 800 µg/mL). The absorbance was measured at 450 nm after 1 hour of incubation at room temperature. BHA, BHT, and ascorbic acid were used as standards. $A_{0.5}$ values were calculated from the absorbance curves.

2.5. Evaluation of the anti-inflammatory effect of *P.hypotropa* extract:

2.5.1. Inhibition of denaturation of bovine serum albumin (BSA)

The anti-inflammatory effect of the *P.hypotropa* extract was evaluated in vitro by testing its effect on BSA denaturation following the method of Kandikattu *et al*. [28]. Briefly, 500 µL of extract or standard (Diclofecan sodium) at different concentrations (1000.500.250.125 µg/mL) were added in tubes to 500 µL of the solution of BSA (0.2% prepared in Tris buffer saline pH 6.6). A tube with 500 µL of BSA and 500 µL of methanol was also prepared as control. Then, the mixture was incubated at 37 °C during 15 minutes, and heated at 72°C during 5 min. After cooling the absorbance was measured at 660 nm in a UV-visible spectrophotometer.

The percentage of inhibition of protein denaturation was calculated according to the following equation:

$$\%\ \mathbf{Inhibition\ of\ denaturation} = \frac{\boldsymbol{A_{Control} - A_{Sample}}}{\boldsymbol{A_{Control}}} \times \mathbf{100}$$

A is the absorbance.

2.5.2. Cell culture

HaCaT cell line (Human immortalized keratinocytes) was purchased from CLS Cell Lines Service GmbH,Germany. HaCaT cells were cultured, at 37 C° and 5% CO_2 at atmosphere in the incubator (New Bruswick Scientific, CO-150 CO_2 incubator), in Dulbecco's modified Eagle's medium (DMEM) high glucose, 10% fetal bovine serum (FBS), 1% Penicillin (100 U/mL) and streptomycin (100 mg/mL).

2.5.3. Cell viability assay

The effect of the extract on cell viability was assessed by the MTT assay as previously reported [29]. HaCaT cells (5x 10^3 cells/well) were plated in 96 well microplate, and then treated for 24, 48, and 72 hours in the presence of different concentration of lichen extract (50, 100, 200 µg/mL). Thereafter, 25 µL of MTT (5mg/mL) were added to each well and incubated for 3 hours. After farmazon crystal

formation the cells were incubated for 18 hours with 100 µL of cell lysate solution (50% N,N-dimethylformamide and 20% (w:v) SDS at pH= 4,5). The optical density (OD) was measured at 620 nm with the spectrophotometer (Titertek Multiskan MCC/340-DASIT, Cornaredo, Milan, Italy).

The cell viability percentage (V%) was calculated as the following equation:

$$\mathbf{V\%} = \mathbf{OD} / (\mathbf{OD} \times \mathbf{DMSO}) \times \mathbf{100}$$

OD = absorbance of the extract at each concentration
DMSO = average of the absorbance of DMSO in the different concentrations

2.5.4. Intracellular reactive oxygen release (ROS) production

Intercellular ROS formation was measured using the H_2DCF-DA probe (2',7'-dichlorofluorescein-diacetate) as depicted [30]. HaCaT cells were plated in 24-well plates and treated with different concentration of lichen extract (50, 100, 200 µg/mL), in the presence of the bacterial lipopolyscharride "LPS" (5 µg mL^{-1}). After 24 hours, the supernatants were separated and conserved at -18 °C for other uses, and the cells were collected, washed and incubated for 15 min at 37C° in the presence of H_2DCF-DA (10µM). The cell fluorescence was evaluated through the fluorescence-activated cell sorting (FACS scan; Becton Dickinson, NJ, USA) and elaborated with Cell Quest software (San Diego, CA, USA).

2.5.5. TNF-α release

The supernatants from the previous experiment (ROS) were used to measure TNF-α levels in LPS-stimulated HaCaT cells (5 µg mL^{-1}). TNF-α levels were determined using the Diaclone human TNF-α Elisa kit following the manufacturer's instructions. Results were expressed as pg/mL.

2.6. HPLC analysis for usnic acid content

A quantitative determination of +(-)usnic acid in the extract was done through a High-Performance Liquid Chromatography (HPLC).

HPLC grade from Sigma-Aldrich, Germany were used as solvents and chemicals in this experiment.

2.6.1. Preparation of sample

0.05 g of *P.hypotropa* extract was added to 10 ml of DMSO at room temperature, the solution was filtered before the analysis by 0.45 µm filters, then 20 µL of it was injected into the HPLC system.

2.6.2. Preparation of samples and analytic condition

The solution of +(-)usnic acid (1mg/mL) was prepared with acetone then seven samples of different dilutions in acetone of the solution were used to obtain calibration curves using linear regression analysis.

The analysis was performed on Aglient 1220 infinity Lc system equipped with a diode array detector, a reverse phase C 18 column was used. For the mobile phase A , a mixture of methanol and pH 7.4 phosphate buffer (70:30 v/v) was used with a flow rate of 0.8 ml/min to detect usnic acid at 245 nm by the comparison of the retention times with pure standard [31].
Each analysis was carried out in triplicate.

2.7. Statistical analysis

All experiment were performed in triplicate and expressed as means ± SD. The difference between experimental conditions and controls were analyzed using Tukey and Bonferroni's tests ($P<0,05$ was considered significant). Statistical analyses were carried out by *GraphPad Prism 5* software.

3. Results

3.1. Total phenolic and flavonoids contents of *P.hypotropa* extract

The ethanolic extract reported to contain both polyphenols and flavonoids, and it had high levels of phenolics than flavonoids. The values were shown in Table 1.

Table 1. Total phenolic and flavonoids contents in ethanol extract of *P. hypotorpa*

	TPC (µg GAE/mg)	TFC (µg QE/mg)
Ethanol	159±3.74	4.10±0.12

Values were expressed as means ± SD (n=3). GAE: Gallic acid equivalent, QE: Quercetin equivalent.

3.2. Antioxidant activities of *P.hypotropa* extract

The antioxidant and scavenging potential of the tested extract of *P. hypotropa* was evaluated through multiple methods (Table **2**). *P.hypotropa* extract showed a weak ability to reduce DPPH radical and bleaching β-carotene. In contrast, it exerted a high scavenging activity of the radical ABTS (IC_{50}= 96.18±1.07 µg/mL). The capacity of the sample to reduce metallic ions such as iron and copper was also assessed and the results demonstrated that it is more effective in reducing copper ions ($A_{0.5}$ = 39.37±1.46 µg/mL) than iron ions ($A_{0.5}$ > 200 µg/mL). Notably, the tested extract has low antioxidant activity compared to the standards.

Table 2. Antioxidant activities of ethanol extract of *P.hypotropa*

	DPPH IC_{50} (µg/mL)	ABTS IC_{50} (µg/mL)	β-Carotene bleaching IC_{50} (µg/mL)	Reducing power $A_{0.50}$ (µg/mL)	CUPRAC $A_{0.50}$ (µg/mL)
ETH	> 800	96.18±1.07	268.62±2.44	>200	39.37±1.46
BHA	6.14±0.41	1.81±0.10	1.05±0.03	9.29±0.22	5.35±0.71
BHT	12.99±0.41	1.29±0.30	0.91±0.01	8.41±1.46	8.97±3.94
AA	4.39±0.01	3.04±0.05	NT	5.45±0.15	8.69±0.14

Values are means ± standard deviation of three parallel measurements. BHA: butylated hydroxyanisole; ETH: ethanol extract; BHT: butylated hydroxytoluene; AA: ascorbic acid; NT: not tested.

3.3. Anti-inflammatory effect of *P.hypotropa* extract

3.3.1. Effect of the extract on BSA denaturation assay

The tested extract exerted a protective effect gainst the heat-induced denaturation of BSA in a dose-dependent manner. The results showed that the extract inhibited the BSA denaturation at 38.39% at 1000 µg/mL. (Figure **1**).

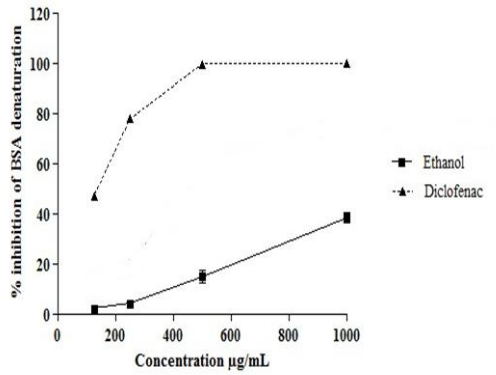

Fig. 1. Effect of the *P.hypotropa* ethanol extract on BSA denaturation.

Effect of* P.hypotropa *extract on Hacat cell line viability

The cytotoxic effect of *P. hypotropa* extract was evaluated using the MTT assay. The results showed that the extract administered at different concentrations and different times (24, 48 and 72h) had no cytotoxic effect on the HaCaT cells (Figure **2**).

3.3.2. Effect of *P.hypotropa* extract on intercellular ROS release

Measurement of intercellular ROS level in LPS-stimulated HaCaT cells (Figure **3**) demonstrated that the lichen extract significantly ($p < 0.001$) inhibited intracellular ROS formation at all the tested concentrations. The extract induced a significant ($p < 0.001$) decrease of the ROS release by 30.35±0.69,

38.17±4.67, and 52.54±1.16 % compared to the LPS-stimulated HaCaT cells at the concentrations of 50, 100, and 200 µg/mL, respectively.

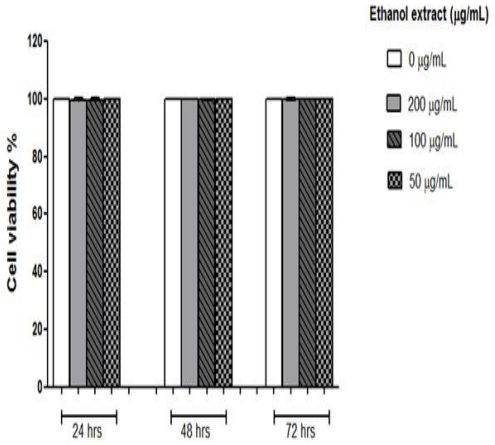

Fig. 2. Effect of ethanol extract (50-200 µg/mL) on HaCaT cells viability after 24, 48 and 72 hours.

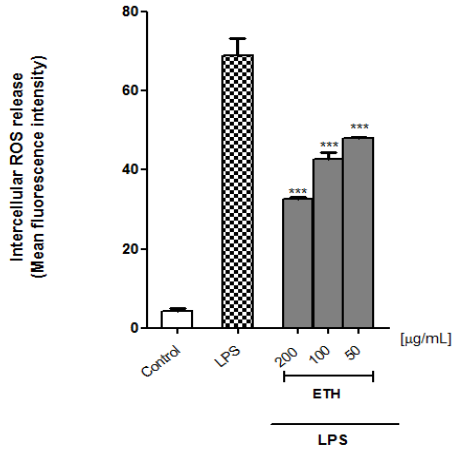

Fig. 3. Effect of *P. hypotropa* extract (50-200 µg/mL) on the intracellular ROS levels in LPS-stimulated HaCaT cells. * Significant P < 0.001 compared to LPS-stimulated cells. (*LPS: LPS-stimulated cells, ETH: ethanol extract).***

3.3.3 Effect of *P.hypotropa* extract on TNF-α secretion

The inhibitory effect of the tested extract on TNF-α secretion by LPS-stimulated HaCaT cells was evaluated using an ELISA assay. Data indicate that the extract decreased TNF-α production (Figure **4**). The inhibitory effect of the extract was significant ($p < 0.001$) at 100 and 200 µg/mL.

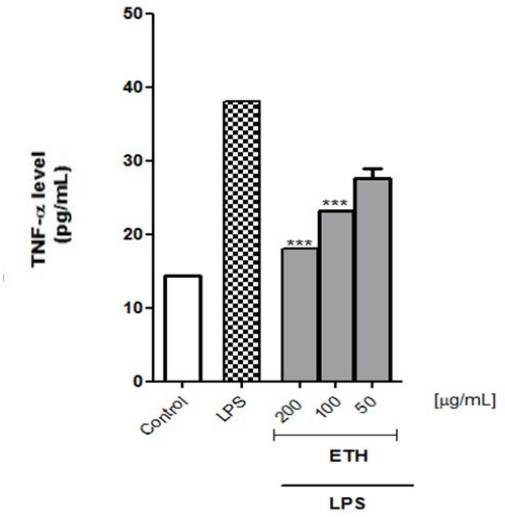

Fig. 4. Effect of *P. hypotropa* extract (50-200 µg/mL) on TNF-α levels (pg/mL) in LPS-stimulated HaCaT cells. * Significant P < 0.001 compared to LPS-stimulated cells. (*LPS: LPS-stimulated cells, ETH: ethanol extract).***

3.4. Determination of usnic acid in *P.hypotropa* by HPLC

The quantification of usnic acid in lichen extract was realized by the identification of peaks in chromatographs resulted from the HPLC analysis, through the comparison of retention times with the standard (Figure **5**).

Our lichen extract contained 0.032 mg of usnic acid in 5mg/mL of ethanol extract. The result obtained is showed in (Table **3**).

Table 3: Usnic acid content and retention time of *P.hypotropa* extract.

	Usnic acid content (mg)	Retention time (min)
ETH	0.032 ± 0.001	7,143±0.001

Values were expressed as means ± SD (n=3). ETH: ethanol extract of *P.hypotropa.*

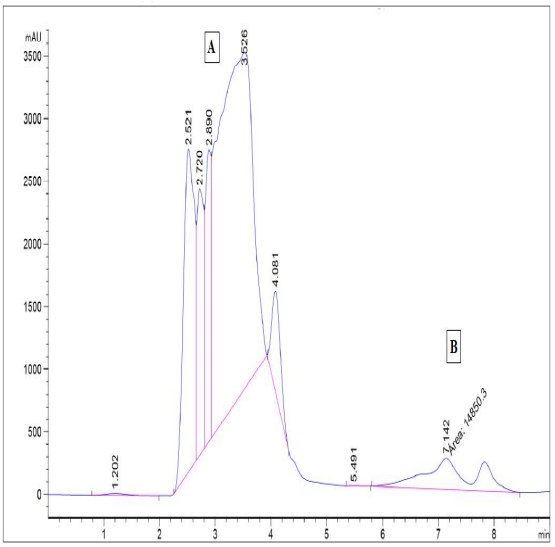

Fig. 5. HPLC analysis of usnic acid in *P.hypotropa* ethanol extract. A: solvent; B: usnic acid (retention time: 7.14 min).

4. DISCUSSION

Lichens were used in traditional medicine to cure infections, fever, diarrhea, convulsions, and skin diseases such as wounds [1]. In this work, we have tested the antioxidant and the anti-inflammatory activities of ethanol extract of lichen *P.hypotropa.* In our knowledge, the biological activity of this lichen has not already been documented. Firstly, the antioxidant activity of the extract has been evaluated using different tests. The utilization of different complementary methods is recommended when studying a crude extract composed of a mixture of bioactive compounds with diverse mode of action. Our results demonstrated the presence of total phenolic and flavonoids in *P.hypotropa* lichen

extract, similar findings on *Parmotrema* species have been reported by different works [32,33]. At the same time, the ethanolic extract exerted a moderate antioxidant activity. Similar results have been reported in the genus of *Parmotrema* with ethanol extract, while other extracts of the same genus showed a significant effect [34]. The moderate antioxidant activity of our extract could be related to the chosen solvent which can affect the concentration of secondary metabolites in lichens. Several studies confirmed the antioxidant properties of lichen [35,36] where the antioxidant effect was influenced by the extraction solvent.

In fact, a close relationship between total phenolic content and antioxidant activity has been confirmed by different studies [37–40]. The ability of phenolic compounds to scavenge the free radicals is due to their capacity to donate electrons or hydrogen atoms of their hydroxyl groups [41].

In the second part of this work, the anti-inflammatory effect of the extract was assessed by the test of protein denaturation using BSA. The protein denaturation process is one of the multiple causes inducing an inflammatory response through the degeneration, the loss of tissue functionality, and the secretion of pro-inflammatory mediators [42]. The test is based on measurement of the capacity of the extract to prevent the loss of protein structure induced by the heat. The results showed that the ethanolic extract had a protective effect against protein denaturation. A similar protective effect was reported in another extract of the lichen *Parmotrema austrosinense* [43].

Furthermore, the anti-inflammatory effect of *P.hypotropa* extract was assessed on HaCaT cells. The LPS-stimulated keratinocytes are a common model to discover new treatments for skin inflammation disorders such as atopic dermatitis and psoriasis [44,45]. The results showed that the viability of HaCaT cells was not affected by adding the extract of *P.hypotropa.* Further, the tested extract exhibited a significant reduction of intercellular ROS produced by the stimulated HaCat cells. During skin inflammations, ROS are excessively produced by keratinocytes and can oxidize the biologic molecules (lipids, proteins, and DNA) and lead to several disorders [11,46]. The inhibition of intracellular ROS by lichen extracts was reported in SH-SY5Y neuroblastomas treated by *Parmotrema perlatum* [47], and by *Cetraria islandica* and *Vulpicida canadensis* on U373-MG cells (astrocytes) [48].

On the other hand, TNF-α is a pro-inflammatory cytokine produced by several skin cells including keratinocytes following their exposition to external agents such as LPS [49]. Our results showed the inhibition of TNF-α by the extract of *P.hypotropa***,** similar findings on lichen were reported by on LPS-stimulated Raw 264.7 cells treated by *Amandinea sp* extract [50]. The anti-inflammatory properties of *P.hypotropa* extract can be ascribed to the presence of polyphenols and flavonoids content. It has been reported that polyphenols inhibit TNF-α and NF-*κ*B signaling pathway in human keratinocytes [46,51,52]. Likewise, they modulate macrophages function by inhibiting cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and TNF-α production [53]. Flavonoids, as well, are found able to inhibit the expression cytokines and chemokines like TNFα, IL-1β, IL-6, IL-8 in multiple cell types such as LPS-activated mouse primary macrophages and human peripheral blood mononuclear cells [54].

In addition ethanol extract of *P.hypotropa* found to contain usnic acid. Many studies confirm the production of usnic acid in lichens[55], Rajan et al. [33] have confirmed the presence of usnic acid in *Parmotrema sp*. Usnic acid is a secondary metabolite produced only by lichens, it is a dibenzofurans derivatives of phenolic compounds composed of a furane retrocycle bonds. Among the isolated secondary metabolites of lichens usnic acid was the most widely studied for its biological activities; it is a very active substance which plays crucial role in human health [56]. Many biological activities of usnic acid have been reported [31]. Usnic acid has strong antioxidant activities [57], it could decrease the high levels of inflammatory mediators (TNF-α, Il-6) in LPS-stimulated cell lines [32]. Therefore, we suggest that the presence of usnic acid in the studied extract seemed to be responsible for its antioxidant and anti-inflammatory activities.

5. Conclusion

The present study revealed that the lichen *Parmotrema hypotropa* has a potent antioxidant effect as examined using *in vitro* assays and possesses a significant anti-inflammatory effect through its ability to inhibit the protein denaturation, and to reduce the production of the inflammatory mediators ROS and TNF-α by keratinocytes. These important actions allow considering the lichen *P.hypotropa* a good source of new bioactive molecules for the treatment of inflammatory disorders. Nevertheless, further studies are needed to identify and purify the principal bioactive compounds responsible for *P.hypotropa*'s biological activities and to confirm their effects both *in vitro* and *in vivo*.

6. Conflicts of interest

There are no conflicts to declare.

7. Acknowledgements

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References:

[1] Shukla V, Joshi GP, Rawat MSM. Lichens as a potential natural source of bioactive compounds : a review: Phytochemistry Reviews 2010:303–14. https://doi.org/10.1007/s11101-010-9189-6.

[2] Lutzoni F and Miadlikowska J. Lichens *Current Biology* 2009;19:502–3.

[3] Zhao Y, Wang M, Xu B. A comprehensive review on secondary metabolites and health-promoting effects of edible lichen. *J Funct Foods* 2021;80:104283. https://doi.org/10.1016/j.jff.2020.104283.

[4] Joulain D. Lichen extracts as raw materials in perfumery . Part 1 : *oakmoss* 2009:49–61. https://doi.org/10.1002/ffj.1916.

[5] Zambare VP, Christopher LP. Biopharmaceutical potential of lichens *Pharmaceutical Biology* 2012;0209. https://doi.org/10.3109/13880209.2011.633089.

[6] Hong JM, Suh SS, Kim TK, Kim JE, Han SJ, Youn UJ, et al. Anti-cancer activity of lobaric acid and lobarstin extracted from the antarctic lichen stereocaulon alpnum. *Molecules* 2018;23:1–10. https://doi.org/10.3390/molecules23030658.

[7] Molnár K, Farkas E. Current results on biological activities of lichen secondary metabolites: A review. *Zeitschrift Fur Naturforsch - Sect C J Biosci* 2010;65:157–73. https://doi.org/10.1515/znc-2010-3-401.

[8] Johansen C, Rittig AH, Mose M, Bertelsen T, Weimar I, Nielsen J, et al. STAT2 is involved in the pathogenesis of psoriasis by promoting CXCL11 and CCL5 production by keratinocytes. *PLoS One* 2017;12:1–14. https://doi.org/10.1371/journal.pone.0176994.

[9] Chessa C, Bodet C, Jousselin C, Wehbe M, Lévêque N, Garcia M. Antiviral and Immunomodulatory Properties of Antimicrobial Peptides Produced by Human Keratinocytes. *Front Microbiol* 2020;11:1–15. https://doi.org/10.3389/fmicb.2020.01155.

[10] Herman A, Herman AP. Antimicrobial peptides activity in the skin. *Ski Res Technol* 2019;25:111–7. https://doi.org/10.1111/srt.12626.

[11] Albanesi C, Scarponi C, Giustizieri ML, Girolomoni G. Keratinocytes in inflammatory skin diseases. *Curr Drug Targets Inflamm Allergy* 2005;4:329–34. https://doi.org/10.2174/1568010054022033.

[12] Albanesi C, Madonna S, Gisondi P, Girolomoni G. The interplay between keratinocytes and immune cells in the pathogenesis of psoriasis. *Front Immunol* 2018;9:1–7. https://doi.org/10.3389/fimmu.2018.01549.

[13] Furue K, Ulzii D, Tanaka Y, Ito T, Tsuji G, Kido-Nakahara M, et al. Pathogenic implication of epidermal scratch injury in psoriasis and atopic dermatitis. *J Dermatol* 2020;47:979–88. https://doi.org/10.1111/1346-8138.15507.

[14] Pastore S, Mascia F, Mariani V, Girolomoni G. Keratinocytes in skin inflammation. *Expert Rev Dermatol* 2006;1:279–91. https://doi.org/10.1586/17469872.1.2.279.

[15] El Aanachi Soukaina, Gali Lynda, Rammali Said, Bensouici Chawki, Aassila Hinde & Dari Khadija. In vitro study of the antioxidant , photoprotective , anti-tyrosinase , and anti- urease effects of methanolic extracts from leaves of six Moroccan Lamiaceae. *J Food Meas Charact* 2021. https://doi.org/10.1007/s11694-020-00759-9.

[16] Forrester SJ, Kikuchi DS, Hernandes MS, Xu Q, Griendling KK. Reactive Oxygen Species in Metabolic and Inflammatory Signaling *Circulation research.* 2018:877–902. https://doi.org/10.1161/CIRCRESAHA.117.311401.

[17] Kacem M, Simon G, Leschiera R, Misery L, Elfeki A, Lebonvallet N. Antioxidant and anti-inflammatory effects of Ruta chalepensis L . extracts on LPS-stimulated RAW 264 cells. *In Vitro Cellular & Developmental Biology-Animal* 2014;8. https://doi.org/10.1007/s11626-014-9813-7.

[18] Mittal M, Siddiqui MR, Tran K, Reddy SP, Malik AB. Reactive Oxygen Species in Inflammation and Tissue Injury. *Antioxidants & redox signaling* 2014;20:1126–67. https://doi.org/10.1089/ars.2012.5149.

[19] Piktel E, Wnorowska U, Cieśluk M, Deptula P, Pogoda K, Turkowicz IM, et al. Inhibition of inflammatory response in human keratinocytes by magnetic nanoparticles functionalized with PBP10 peptide derived from the PIP2‐binding site of human plasma gelsolin. *J Nanobiotechnology* 2019:1–19. https://doi.org/10.1186/s12951-019-0455-5.

[20] Plaza CM, De Torres LED, Lücking RK, Vizcaya M, Medina GE. Antioxidant activity, total phenols and flavonoids of lichens from venezuelan andes. *J Pharm Pharmacogn Res* 2014;2:138–47.

[21] Müller L, Gnoyke S, Popken AM, Böhm V. Antioxidant capacity and related parameters of different fruit formulations. *LWT - Food Sci Technol* 2010;43:992–9. https://doi.org/10.1016/j.lwt.2010.02.004.

[22] Topçu G, Ay M, Bilici A, Sarikürkcü C, Öztürk M, Ulubelen A. A new flavone from antioxidant extracts of Pistacia terebinthus. *Food Chem* 2007;103:816–22. https://doi.org/10.1016/j.foodchem.2006.09.028.

[23] Gali L, Bedjou F. Antioxidant and anticholinesterase effects of the ethanol extract, ethanol extract fractions and total alkaloids from the cultivated Ruta chalepensis. *South African J Bot* 2019;120:163–9. https://doi.org/10.1016/j.sajb.2018.04.011.

[24] Mazouz W, Haouli NEH, Gali L, Vezza T, Bensouici C, Mebrek S, et al. Antioxidant, anti-alzheimer, anti-diabetic, and anti-inflammatory activities of the endemic halophyte Limonium spathulatum (Desf.) kuntze on LPS-stimulated RAW264 macrophages. *South African J Bot* 2020;135:101–8. https://doi.org/10.1016/j.sajb.2020.08.021.

[25] Boussadia A, Beghidja A, Gali L, Beghidja C, Elhabiri M, Rabu P, et al. Coordination properties of two new Schiff-base phenoxy-carboxylates and comparative study of their antioxidant activities. *Inorganica Chim Acta* 2020;508:119656. https://doi.org/10.1016/j.ica.2020.119656.

[26] Mamache W, Amira S, Ben Souici C, Laouer H, Benchikh F. In vitro antioxidant, anticholinesterases, anti-α-amylase, and anti-α-glucosidase effects of Algerian Salvia aegyptiaca and Salvia verbenaca. *J Food Biochem* 2020;44:1–15. https://doi.org/10.1111/jfbc.13472.

[27] Apak, R., Guclu, K.,Ozyurek, M., & Karademir. Novel Total Antioxidant Capacity Index for Dietary Polyphenols and Vitamins C and E , Using Their Cupric Ion Reducing Capability in the Presence of Neocuproine : CUPRAC Method *Journal of Agricultural and Food Chemistry* 2004:7970–81. https://doi.org/10.1021/jf048741x.

[28] Kandikattu,K, BharathRathna Kumar P, VenuPriya R, Sunil Kumar k, RanjithSingh B. Rathore.Evaluation of Anti-Inflammatory Activity of Canthium Parviflorum By in-Vitro Method *Indian Journal of Research in Pharmacy and Biotechnology* 2013;5674:2320–2.

[29] Alilou M, Marzocco S, Hofer D, Rapa SF, Asadpour R, Schwaiger S, et al. Labdane-Type Diterpenes from the Aerial Parts of Rydingia persica: Their Absolute Configurations and Protective Effects on LPS-Induced Inflammation in Keratinocytes. *J Nat Prod* 2020;83:2456–68. https://doi.org/10.1021/acs.jnatprod.0c00360.

[30] Rapa SF, Waltenberger B, Di R, Simona P, Siracusa R, Peritore AF, et al. Plumericin prevents intestinal inflammation and oxidative stress in vitro and in vivo. *The FASEB Journal* 2020:1576–90. https://doi.org/10.1096/fj.201902040R.

[31] Cansaran D, Atakol O, Halici MG, Aksoy A. HPLC Analysis of Usnic Acid in Some Ramalina . Species from Anatolia and Investigation of their Antimicrobial Activities. *Pharmaceutical Biology* 2008;0209. https://doi.org/10.1080/13880200601028503.

[32] Jain A, Bhandarkar S, Rai G, Yadav AK, Lodhi S. Evaluation of Parmotrema reticulatum taylor for antibacterial and antiinflammatory activities. Indian *J Pharm Sci* 2016;78:94–102. https://doi.org/10.4103/0250-474X.180241.

[33] Rajan VP, Gunasekaran S, Ramanathan S, Murugaiyah V, Samsudin MW, Din LB. Biological activities of four Parmotrema species of Malaysian origin and their chemical constituents. *J Appl Pharm Sci* 2016;6:36–43. https://doi.org/10.7324/JAPS.2016.60806.

[34] Ganesan A, Thangapandian M, Ponmurugan P, Sundararaj JP. Antibacterial and antioxidant activity of parmotrema reticulatum obtained from eastern ghats, Southern India. *Biomed Res* 2017;28:1593–7.

[35] Behera BC, Verma N, Sonone A, Makhija U. Antioxidant and antibacterial activities of lichen Usnea ghattensis in vitro. *Biotechnol Lett* 2005;27:991–5. https://doi.org/10.1007/s10529-005-7847-3.

[36] Poornima Shanmugam , Ponmurugan Ponnusamy , Mythili Gnanamangai Balasubramanian , Nagarjun Nagarajan AG. Evaluation of Antimicrobial, Antioxidant and Anticancer Activities of Few Macrolichens Collected From Eastern Ghats of Tamil Nadu, India. *Int Res J Pharm* 2017;8:39–43. https://doi.org/10.7897/2230-8407.080334.

[37] Hodzic Z, Srabovic M, Saletovic M. The Influence of Total Phenols Content on Antioxidant Capacity in the Whole Grain Extracts. *European Journal of Scientific Research* 2009; 28(3):471-7.

[38] Kosanić, M., Ranković, B., & Vukojević, J. Antioxidant properties of some lichen species. *Journal of Food Science and Technology*.2011;48:584–90. https://doi.org/10.1007/s13197-010-0174-2.

[39] Rekha C, Poornima G, Manasa M, Abhipsa V, Devi JP. Ascorbic Acid , Total Phenol Content and Antioxidant Activity of Fresh Juices of Four Ripe and Unripe Citrus Fruits. *Chemical Science Transactions* 2012;1:303–10. https://doi.org/10.7598/cst2012.182.

[40] Vivek MN, Kambar Y, Manasa M, R PKT, Vinayaka KS. Radical scavenging and antibacterial activity of three Parmotrema species from Western Ghats of Karnataka , *India* 2014;4:86–91. https://doi.org/10.7324/JAPS.2014.40317.

[41] Zhu MT, Huang YS, Wang YL, Shi T, Zhang LL, Chen Y, et al. Comparison of (poly)phenolic compounds and antioxidant properties of pomace extracts from kiwi and grape juice. *Food Chem* 2019;271:425–32. https://doi.org/10.1016/j.foodchem.2018.07.151.

[42] Anyasor GN, Okanlawon AA, Ogunbiyi B. Evaluation of anti-inflammatory activity of Justicia secunda Vahl leaf extract using in vitro and in vivo inflammation models. *Clinical Phytoscience* 2019;5. https://doi.org/10.1186/s40816-019-0137-8.

[43] Poornima S, Nagarjun N, Ponmurugan P, Gnanamangai BM, Narasimman S. Toxicity and anti-inflammatory study of Parmotrema austrosinense extract against oxozalone induced intestinal inflammation in zebrafish (Danio rerio) model. *Biocatalaysis and Agricultural Biotechnolgy* 2019;21:101278. https://doi.org/10.1016/j.bcab.2019.101278.

[44] Bocheńska, K., Smolińska, E., Moskot, M., Jakóbkiewicz-Banecka, J., & Gabig-Cimińska, M. Models in the Research Process of Psoriasis. *International Journal of Molecular Sciences.* 2017; 18(12): 2514 https://doi.org/10.3390/ijms18122514.

[45] Lee, Choi, N'deh, Choi, Fan, Kim, An. Inhibitory Effect of Centella asiatica Extract on DNCB-Induced Atopic Dermatitis in HaCaT Cells and BALB/c Mice. *Nutrients* 2020; 12(2) : 411. https://doi.org/doi: 10.3390/nu12020411.

[46] Hussain T, Tan B, Yin Y, Blachier F, Tossou MCB, Rahu N. Oxidative Stress and Inflammation: What Polyphenols Can Do for Us? *Oxid Med Cell Longev* 2016;2016. https://doi.org/10.1155/2016/7432797.

[47] Sieteiglesias V, González-Burgos E, Bermejo-Bescós P, Divakar PK, Gómez-Serranillos MP. Lichens of Parmelioid Clade as Promising Multitarget Neuroprotective Agents. *Chem Res Toxicol* 2019;32:1165–77. https://doi.org/10.1021/acs.chemrestox.9b00010.

[48] Fernández-Moriano C, Divakar PK, Crespo A,

Gómez-Serranillos MP. Neuroprotective activity and cytotoxic potential of two Parmeliaceae lichens: Identification of active compounds. *Phytomedicine* 2015;22:847–55. https://doi.org/10.1016/j.phymed.2015.06.005.

[49] Cheng H, Hsiang C, Chen G, Wu S. Inhibition of Lipopolysaccharide-Induced Interleukin-1 β and Tumor Necrosis Factor- α Production by Menthone through Nuclear Factor- κ B Signaling Pathway in HaCat Cells. *Chinese Journal of Physiology* 2008;51:160–6.

[50] Kim JE, Min SK, Hong JM, Kim KH, Han SJ, Yim JH, et al. Anti-inflammatory effects of methanol extracts from the Antarctic lichen, Amandinea sp. in LPS-stimulated raw 264.7 macrophages and zebrafish. *Fish Shellfish Immunol* 2020;107:301–8. https://doi.org/10.1016/j.fsi.2020.10.017.

[51] Potapovich AI, Lulli D, Fidanza P, Kostyuk VA, De Luca C, Pastore S, et al. Plant polyphenols differentially modulate inflammatory responses of human keratinocytes by interfering with activation of transcription factors NFKB and AhR and EGFR-ERK pathway. *Toxicol Appl Pharmacol* 2011;255:138–49. https://doi.org/10.1016/j.taap.2011.06.007.

[52] Korkina LG, Pastore S, Luca C De, Kostyuk VA. Metabolism of Plant Polyphenols in the Skin : Beneficial Versus Deleterious Effects. *Current Drug Metabolism* 2008:710–29.

[53] González R, Ballester I, López-Posadas R, Suárez MD, Zarzuelo A, Martínez-Augustin O, et al. Effects of flavonoids and other polyphenols on inflammation. *Crit Rev Food Sci Nutr* 2011;51:331–62. https://doi.org/10.1080/10408390903584094.

[54] Sharma V, Mishra M, Ghosh S, Tewari R, Basu A, Seth P, et al. Modulation of interleukin-1β mediated inflammatory response in human astrocytes by flavonoids: Implications in neuroprotection. *Brain Res Bull* 2007;73:55–63. https://doi.org/10.1016/j.brainresbull.2007.01.016.

[55] Saha S, Pal A, Paul S. A Review on Pharmacological, Anti-oxidant Activities and Phytochemical Constituents of a Novel Lichen Parmotrema Species. *J Biol Act Prod from Nat* 2021;11:190–203. https://doi.org/10.1080/22311866.2021.1916596.

[56] Ranković, B., & Kosanić, M. Lichens as a potential source of bioactive secondary metabolites. *In Lichen secondary metabolites* 2019 (pp.1-29). Springer. https://doi.org/10.1007/978-3-030-16814-8.

[57] Cakmak K C, Gülçin İ. Anticholinergic and antioxidant activities of usnic acid-an activity-structure insight. *Toxicol Reports* 2019;6:1273–80. https://doi.org/10.1016/j.toxrep.2019.11.003.