

# Anti-Inflammatory Potential of Compounds Isolated from Tunisian Lichens Species

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The lichen's special symbiotic structure enables it to produce bioactive substances. They have historically been recognized for their aesthetic and medicinal benefits. Furthermore, in recent years, they have performed in various fields, including perfumery, dyeing, and pharmacology due to their rich secondary metabolites. From our study, four compounds were isolated from organic extracts of *Parmotrema hypoleucinum*, *Roccella phycopsis*, and *Xanthoria parietina* and identified by spectroscopic investigation as atranorin, (+)-iso-usnic acid, methyl orsellinate, and parietin, respectively. The anti-inflammatory effects of lichens extracts, and pure compounds were evaluated on RAW 264.7 macrophages cells at different concentrations. At 25 µg/mL all treated samples did not show any effect on cell viability. Atranorin and (+)-iso-usnic acid showed an inhibitory effect on nitric oxide (NO) levels in lipopolysaccharide (LPS)-stimulated macrophages. Nitric oxide (NO) production was measured using Griess reagent, atranorin and (+)-iso-usnic acid showed a high anti-inflammatory potential (75.99% and 57.27% at 25 µg/mL). On the other hand, methyl orsellinate and the organic extracts of three lichens showed good anti-inflammatory activity ranging from 29.16% at 25 µg/mL to 86.91% at 100 µg/mL.

**Keywords:** Lichens, anti-inflammatory activity, atranorin, (+)-iso-usnic acid, methyl orsellinate, parietin.

## Introduction

Nature, the great designer of molecules, has created an arsenal of chemicals that constitute an infinite resource for the discovery of new compounds for the development of different effective drugs for a wide variety of diseases.<sup>[1]</sup> However, the inflammatory process is the defense reaction of the organism to stimuli, and it is these reactions that repair the damage suffered by the organism and protect it from further damage. Some diseases, such as diabetes, obesity, and

cancer, are the results of continued inflammation, which can damage tissues and affect their normal function.<sup>[2]</sup>

Natural products have been the basis of the health care system all over the world. The use of natural resources, such as plants, animals, microorganisms, and marine organisms, is a traditional way of treating various diseases.<sup>[3]</sup>

A fungus and one or more autotrophic photosynthetic species (algae or cyanobacteria) form a symbiotic relationship known as lichens. The fungus in this bond provides structural support to the thallus (lichen corpus), promotes the availability of nutrients, water, and bio-elements, and is responsible for synthesizing the lichen's quasi-totality of secondary

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metabolites. The photosynthetic organism is responsible for delivering nutrients and preventing lichen from drying out, allowing it to live in high-light conditions.<sup>[4]</sup>

In addition, they can grow in diverse and extreme environmental conditions, such as in very cold or very dry regions, at polar latitudes, or extreme altitudes.<sup>[5]</sup> Therefore, a great deal of attention has been devoted to the phytotherapeutic property because of its richness in terms of bioactive compounds that can protect organisms against various problems. Lichen substances have been attracting attention for more than 100 years.<sup>[6]</sup> They produce more than 1000 secondary metabolites exclusive to the lichens that have been described. These include aliphatic acids, anthraquinones, benzyl esters, depsidones, depsides, dibenzofurans, pulvinic acid derivatives, terpenoids, uric acids, and xanthenes.<sup>[7]</sup> The ability to synthesize a wide range of compounds helps to explain the fact that lichens have also been used as sources of dyes, perfumes, foods, as well as pharmaceuticals.<sup>[8]</sup> Lichens are important organisms for a variety of reasons. They have been known since ancient times for their medical importance.<sup>[6]</sup> The application of lichens is traditional throughout the world, and several studies have shown that they have promising medicinal as well as antibacterial, antifungal, antioxidant, cytotoxic, and anti-inflammatory properties.<sup>[6,9–10]</sup>

Nguyen et al.<sup>[11]</sup> has evaluated that lichens have anti-inflammatory properties. *Lobaria pulmonaria*, *Cladonia clathrate*, and *Teloschistes flavicans* extracts have been tested for anti-inflammatory activity.<sup>[12–14]</sup> Moreover, lichen substances exhibit anti-inflammatory activity,<sup>[11]</sup> and investigate that nearly 70 lichen substances have potential against inflammation, such as atranorin, diffractaic acid, and (+)-protolichesterinic acid isolated from *Parmelia* sp.<sup>[15]</sup>

Tunisia contains a list of 644 taxa of lichens.<sup>[16]</sup> However, most studies have been conducted to explore their diversity.<sup>[16]</sup> Besides, the focus of the studies has been on their use as bioindicators of air

pollution.<sup>[17]</sup> However, studies on the chemistry of lichens from Tunisia, which present a rich diversity, are limited. Therefore, we are interested in lichens, which are rich in secondary metabolites, and we have previously worked on crude extracts.<sup>[9–10,18–21]</sup>

In this context, the objective of this work is to evaluate the anti-inflammatory effects of crude extract as well as those of the main compounds isolated: atranorin, (+)-iso-usnic acid, methyl orsellinate, and parietin from *Parmotrema hypoleucinum*, *Roccella phycopsis* and *Xanthoria parietina* extracts.

## Results

The extracts obtained from the thalli of *Parmotrema hypoleucinum*, *Roccella phycopsis*, and *Xanthoria parietina* were collected in Tunisia and indicated as *Ph-Ex*, *Rp-Ex*, and *Xp-Ex* were analyzed by TLC using chromatographic conditions typical of lichen metabolites.<sup>[22]</sup> The extracts were purified by CC and PTLC using different eluent systems as reported in the experimental section. The main isolated metabolites were identified by their spectroscopic data (see *Supporting Information*) compared with those reported in the literature. Atranorin and (+)-iso-usnic acid,<sup>[23]</sup> were isolated from *P. hypoleucinum*, methyl orsellinate from *R. phycopsis*,<sup>[23]</sup> and parietin,<sup>[24]</sup> was identified as the main metabolite present in the extract of *X. parietina* (Figure 1).

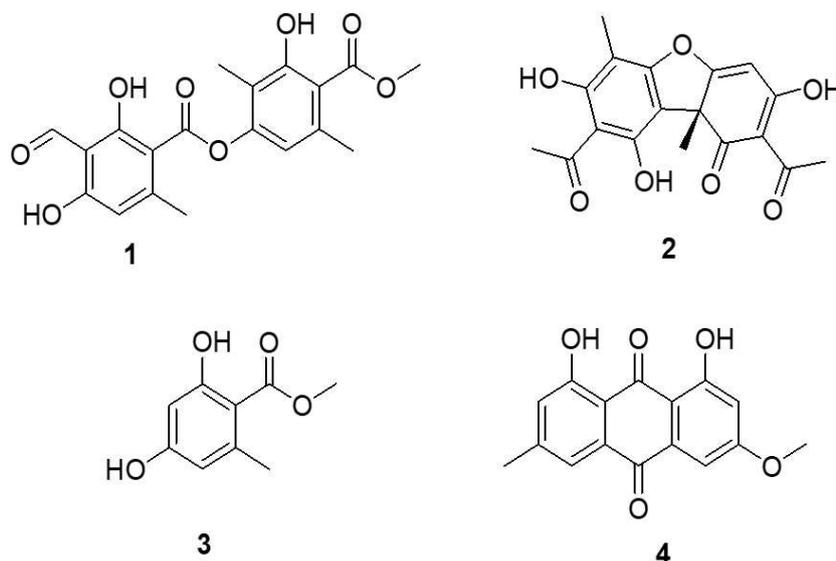
### Evaluation of the Cytotoxicity

The crude extracts and pure compounds were screened for their cytotoxicity against RAW 264.7 macrophage cells. *Table 1* shows that atranorin, parietin, and (+)-iso-usnic acid reduced the percentage of cell viability as the concentration of the extract increased. In fact, methyl orsellinate cell viability increased with the increasing concentration of the extract. As shown in *Table 1* significant differences ( $P <$

**Table 1.** Cell viability assay cytotoxic effects on RAW 264.7 cells of atranorin, (+)-iso-usnic acid, methyl orsellinate, parietin, extracts of *Parmotrema hypoleucinum*, *Roccella phycopsis*, and *Xanthoria parietina*.

µg/ mL	Atranorin (1)	(+)-Isousnic acid (2)	Methyl orsellinate (3)	Parietin (4)	<i>Parmotrema hypoleucinum</i> Extract ( <i>Ph-Ex</i> )	<i>Roccella phycopsis</i> Extract ( <i>Rp-Ex</i> )	<i>Xanthoria parietina</i> ( <i>Xp-Ex</i> )
0	100 ± 00	100 ± 00	100 ± 00	100 ± 00	100 ± 00	100 ± 00	100 ± 00
25	100 ± 00	85.24 ± 0.86	87.23 ± 2.30	100 ± 00	100 ± 00	99 ± 0.92	95.61 ± 0.82
50	81.93 ± 3.92	77.65 ± 2.01	88.88 ± 2.35	100 ± 00	93.91 ± 0.43	94.22 ± 0.13	83.80 ± 3.18
100	49.20 ± 2.33	57.15 ± 1.00	94.34 ± 2.66	86.85 ± 0.23	100 ± 00	100 ± 00	88.71 ± 5.51

Different concentrations of them (25–100 µg/mL) were used for cell treatment. Values are shown as means ± standard error of the mean. Means of three replicated analyses of the same sample (coefficient of variation < 5%).



**Figure 1.** Structures of atranorin (**1**), (+)-iso-usnic acid (**2**), methyl orsellinate (**3**), and parietin (**4**) main compounds isolated from crude extracts of *Parmotrema hypoleucinum*, *Roccella phycopsis*, and *Xanthoria parietina*, respectively.

0.05) were observed. In addition, parietin showed a percentage of cell viability more than 100% for 25 and 50  $\mu\text{g/mL}$ . Also, atranorin has cell viability of more than 100% for 25  $\mu\text{g/mL}$  and 81.93% for 50  $\mu\text{g/mL}$ . However, methyl orsellinate showed high cell viability for 100  $\mu\text{g/mL}$  with 94.34%. While the (+)-iso-usnic acid was lower than all other extracts tested. In the methanolic extracts of the three species, the viability of the cells varied as a function of concentration and species. Indeed, *P. hypoleucinum* and *R. phycopsis* showed a percentage of cell viability of more than 100% for 25 and 100  $\mu\text{g/mL}$ . On the other hand, *X. parietina* showed lower cell viability (Table 1).

#### Anti-Inflammatory Activity

The anti-inflammatory activity of lichen extracts and pure compounds was evaluated using the Griess test to measure the level of NO produced by activated RAW 264.7 murine macrophages. During stimulation with lipopolysaccharide (LPS), macrophages express inducible nitric oxide synthase (iNOS) and produce large amounts of NO. Therefore, the anti-inflammatory activity of atranorin and (+)-iso-usnic acid were tested at different concentrations (6.5  $\mu\text{g/mL}$ , 12.5  $\mu\text{g/mL}$ , 25  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , and 100  $\mu\text{g/mL}$ ) and parietin, methyl orsellinate, and *Ph-Ex*, *Rp-Ex*, *Xp-Ex* were tested at 25  $\mu\text{g/mL}$ , 50  $\mu\text{g/mL}$ , and 100  $\mu\text{g/mL}$ . Significant differences were recorded across matrices ( $P < 0.05$ ) (Figures 2 and 3). To measure the effect of the extract of the teased lichens and their isolated compounds on

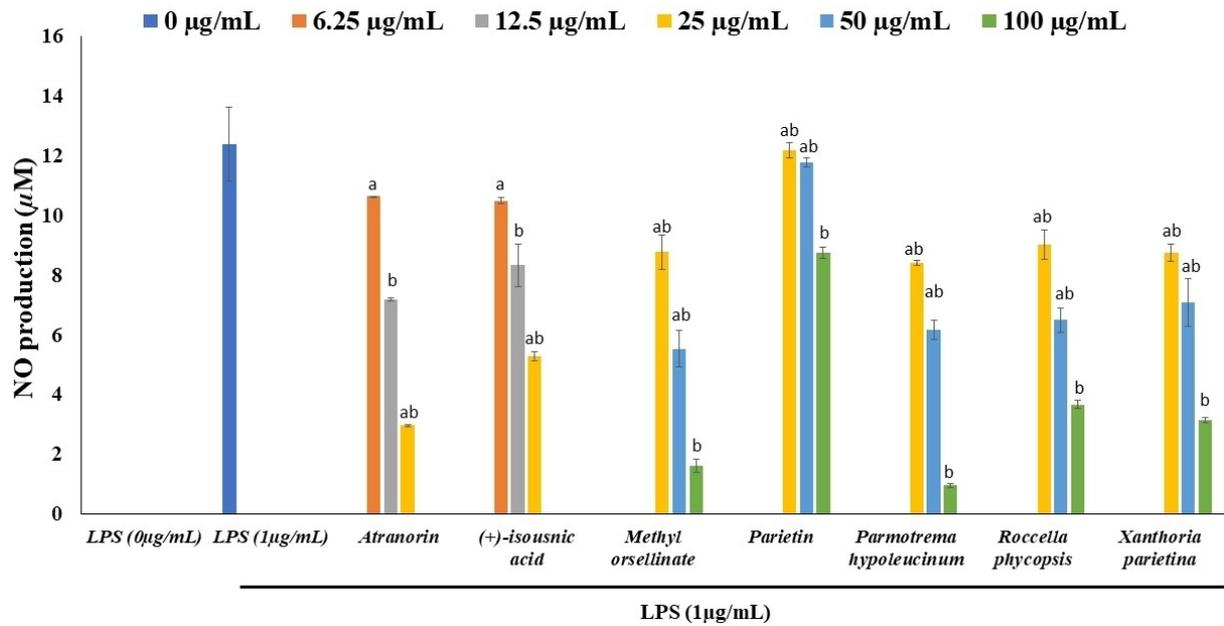
NO production, we estimated the amount of nitrite in the medium using the Griess reaction. As shown in Figure 2, the different compounds inhibited LPS-stimulated NO production in a concentration-dependent manner. A Pearson's correlation coefficient was performed to assess the relationship between the anti-inflammatory activity and the lichens and their compounds levels. The result showed that there was a positive correlation with  $r = 0.628$  (Table 2).

Next, the percentage inhibition of NO production is calculated compared to cells treated with LPS alone (Figure 2). The results showed that atranorin had the best percentage inhibition of NO in a dose-dependent manner at concentrations vary from 6.5  $\mu\text{g/mL}$  (14.10%) to 25  $\mu\text{g/mL}$  (75.99%) followed by (+)-iso-usnic acid, which had a similar inhibition of 15.31% at 6.5  $\mu\text{g/mL}$  and 57.27% at 25  $\mu\text{g/mL}$ . While the concentration of parietin, methyl orsellinate, and three crude extracts varied from 25 to 100  $\mu\text{g/mL}$ . Indeed, methyl orsellinate and the three lichen extracts demonstrated

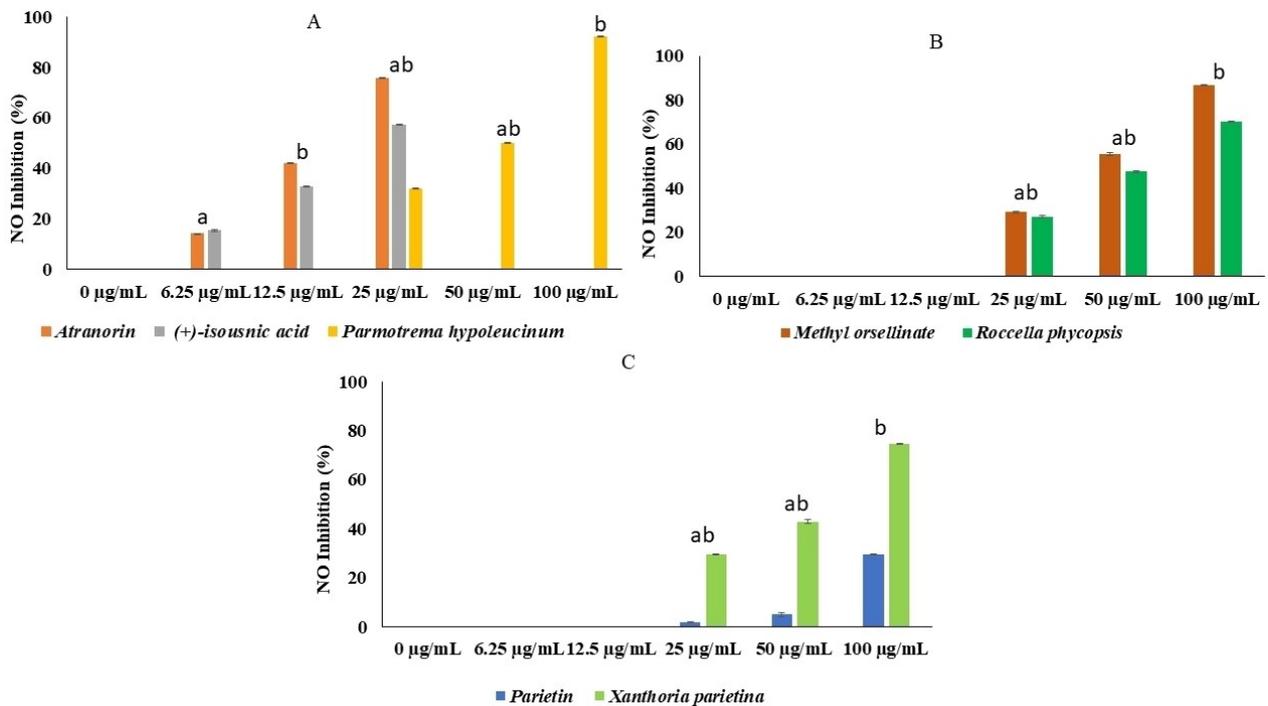
**Table 2.** Pearson's correlation between the anti-inflammatory activity and atranorin, (+)-iso-usnic acid, methyl orsellinate, parietin, and the extracts of *Parmotrema hypoleucinum*, *Roccella phycopsis*, and *Xanthoria parietina* levels.

Anti-inflammatory activity	Compounds and lichens extract levels
	0.628**

\*\* Correlation is significant at the 0.01 level (2-tailed).



**Figure 2.** Inhibitory effects of atranorin, (+)-iso-uscnic acid, methyl orsellinate, parietin, crude extracts of *Parmotrema hypoleucinum*, *Rocella phycopsis*, and *Xanthoria parietina*, on nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells. Cells were pretreated with the indicated concentrations of all extracts for 1 h before incubation with LPS (1 µg/mL) for 24 h.



**Figure 3.** Percentage of NO inhibition in LPS-stimulated RAW 264.7 macrophages. Cells were treated with different concentrations of atranorin, (+)-iso-uscnic acid, and *Parmotrema hypoleucinum* extract (A). Cells were treated with different concentrations of methyl orsellinate and *Rocella phycopsis* extract (B). Cells were treated with different concentrations of parietin and *Xanthoria parietina* extract (C). Cells were treated with the indicated concentrations of all extracts for 1 h before incubation with LPS (1 µg/mL) for 24 h. Values are shown as means  $\pm$  standard error of the mean. Means of three replicated analyses of the same sample (coefficient of variation < 5%).

good anti-inflammatory activity, ranging from 29.16% at 25  $\mu\text{g/mL}$  to 86.91% at 100  $\mu\text{g/mL}$ . However, parietin has a lower NO inhibition. As shown in Figures 2 and 3 the three lichen extracts have a higher anti-inflammatory potential for Ph-Ex (29.16% at 25  $\mu\text{g/mL}$  and 92.26% at 100  $\mu\text{g/mL}$ ).

The nitrite levels in the culture medium were measured by the Griess reaction. Values are shown as means  $\pm$  standard error of the mean. Means of three replicated analyses of the same sample (coefficient of variation  $<5\%$ ) were compared to the cells treated with LPS alone.

## Discussion

Natural has remained the main new source of structurally significant chemicals that lead to the development of new active compounds.<sup>[3]</sup> Lichen is one of the resources producing several compounds. Which can use them in dyes and perfumes, as well as in pharmaceuticals.<sup>[11]</sup> Recently, more thousand lichens compound are known.<sup>[7]</sup>

However, depsides, depsidones, ethers of diphenyl, and dibenzofurans have been reported as major bioactive compounds found in lichens. The lichens compounds have many applications such as biopharmaceutical applications.<sup>[25]</sup> In addition to its value as a source of natural compounds, several biological activity studies have been conducted on lichens and these studies show that it has bioactivities, including anti-inflammatory, antioxidant, anti-tumor, and cytotoxic effects.<sup>[2,10–11,26–27]</sup>

The objective of this work is to provide information on the potential role of these organisms as a natural source of molecules that could be exploited as anti-inflammatory agents.

The literature search reveals that few research groups have already worked on the chemical screening of Tunisian lichen<sup>[9–10,18–21]</sup> and reported the isolation and use of their compounds.<sup>[28]</sup> Therefore, given the growing interest in the phytochemical composition of lichens. As a result, this work is the first Tunisian study screening the anti-inflammatory activity of lichens.

However, studies on *Parmotrema hypoleucinum* are limited.<sup>[29]</sup> Where we first isolated secondary compounds from methanol extract of *P. hypoleucinum* and we estimated the inhibitory activities to produce inflammatory factors, including NO, in LPS-stimulated macrophages.

In the current study, we were interested in finding an anti-inflammatory agent from a natural product derived from lichens. Therefore, we evaluated the toxicity of the compounds on RAW 264.7 macrophage cells to valorize them as an anti-inflammatory agent.

To achieve this objective, we isolated four compounds: atranorin, (+)-iso-usnic acid, methyl orsellinate, and parietin. The structures of all compounds were determined by the analysis of their spectroscopic data.<sup>[22–24]</sup> Then we investigated the cytotoxic effects of lichens extract (*P. hypoleucinum*, *R. phycopsis*, and *X. parietina*) and the four compounds by measuring the cell viability of RAW 264.7 macrophages cells. Cell viability of RAW 264.7 macrophages was unaffected with a concentrated extract of 25  $\mu\text{g/mL}$  by the presence of atranorin, parietin, and (+)-iso-usnic acid, and methyl orsellinate in a less important. While (+)-iso-usnic acid and atranorin decreased cell viability compared to other extracts at 50 and 100%. Because of the cell viability test, we evaluated the anti-inflammatory potential of the four compounds and the extracts of *P. hypoleucinum*, *R. phycopsis*, and *X. parietina*. The tested compounds and the lichen extracts show a relatively strong anti-inflammatory effect.

RAW264.7 is a functional macrophage line used to study the anti-inflammatory properties of drugs.<sup>[30]</sup> In addition, the LPS-stimulated macrophages test is a mostly reported test used to evaluate the anti-inflammatory activity of numerous natural products and synthetic compounds.<sup>[31]</sup>

Besides, the methanol extracts of *P. hypoleucinum*, *R. phycopsis*, and acetone extract of *X. parietina* showed very little cytotoxicity and reduced NO production in LPS-stimulated RAW 264.7 cells in a dose-dependent manner. Our results corroborate those illustrated by Hong et al.<sup>[27]</sup> indicating that the concentration of compound and extract could influence the anti-inflammatory activity.

The obtained results are also conformed with those previously by Kim et al.<sup>[32]</sup> anti-inflammatory effects of methanol extract from *Amandinea* sp. in LPS-stimulated raw 264.7 macrophages. Also, Mun et al.<sup>[31]</sup> investigate the anti-inflammatory effects in LPS-stimulated RAW264.7 cells of the *Heterodermia hypoleuca* and their secondary metabolite atraric acid. Poornima et al.<sup>[33]</sup> evaluate the anti-inflammatory potential of the acetone extract of *Parmotrema austrosinense*.

The different extracts of *Dirinaria consimilis* and *Ramalina leiodea* have anti-inflammatory activity.<sup>[34]</sup>

Furthermore, the results obtained are compatible with those previously published by Nguyen et al.<sup>[11]</sup>

indicating that the lichens and their metabolites have anti-inflammatory activity. Moreover, the isolated compounds (+)-iso-usnic acid and atranorin, methyl orsellinate, and parietin have an inhibitory activity on the production of inflammatory factors, including NO, in LPS-stimulated macrophages. Several studies have explored the anti-inflammatory of various lichens compounds such as lobaric acid, usnic acid, atranorin, and atraric acid.<sup>[30–31,35–38]</sup>

Lobaric acid from *Stereocaulon alpinum* influences lipopolysaccharide (LPS)-induced inflammatory responses in macrophages.<sup>[38]</sup> Furthermore, *P. hypoleucinum*, *R. phycopsis*, and *X. parietina* are promising lichens that have previously exhibited therapeutic properties.<sup>[29,39–40]</sup>

Coassini-Lokar et al.<sup>[29]</sup> demonstrated that *P. hypoleucinum* has a high concentration in usnic, norstictic, and hypostictic acids. *R. phycopsis* showed good antioxidant activity with rich total phenolic and flavonoid contents according to Aydin and Kinalioğlu.<sup>[39]</sup> Moreover, Mendili et al.<sup>[10]</sup> has shown a higher phenolic content in *X. parietina* and important antioxidant activity; this species also showed an antibacterial effect.<sup>[9]</sup> *X. parietina* is also known for its major metabolite, parietin an orange anthraquinone pigment characterized for its antibacterial, anticancer, antiproliferative, and antifungal activities.<sup>[40–41]</sup> Also, it may reduce the effect of UV radiations.<sup>[42]</sup>

(+)-Iso-usnic acid is a lichen secondary metabolite found in dibenzofurans groups.<sup>[43]</sup> Ureña-Vacas et al.<sup>[43]</sup> showed that the dibenzofurans isolated from lichens (alectosarmentin, condidymic acid, didymic acid, iso-usnic acid, isostrepsilic acid, usimines A–C, and usnic acid) were reviewed, most of which showed antibacterial, antifungal, and cytotoxic activities. Iso-usnic acid isolated from *Parmelia reticulata* also exhibits antifungal effects.<sup>[44]</sup>

Atranorin, a depside, is one of the most common secondary metabolites in lichens. It is one of the valuable bioactive secondary metabolites present in *Parmotrema austrosinense*, *P. tinctorum*, and *P. grayanum*. Also, it is found in *Cladina kalbii*, *C. furcata*, *Lethariella canariensis*, *Hypotrachyna revoluta*, and *Usnea articulata*.<sup>[45]</sup> Atranorin was found to be a good antimicrobial agent, antioxidant, anticancer, and larvicidal agent. It also has a good UV absorbing ability. However, Melo et al.<sup>[37]</sup> indicated that atranorin extracted from *Cladina kalbii* has anti-inflammatory properties.

Studzińska-Sroka et al.<sup>[46]</sup> reports on atranorin's anti-inflammatory, analgesic, antibacterial, antifungal,

cytotoxic, antioxidant, antiviral, and immunomodulatory activities.

Methyl orsellinate showed antitumor activity whereas showed their ability to inhibit the proliferative of different cell lines.<sup>[47]</sup>

Previous lichens such as *Usnea*, *Cladonia*, *Parmelia*, *Lobaria*, *Roccella*, and *Xanthoria* utilized as medicines over the years were used for inflammation.<sup>[11]</sup> Also, many studies have reported the anti-inflammatory activity of substances obtained from various lichen species.<sup>[48]</sup> Nguyen et al.<sup>[11]</sup> described nearly 70 lichen substances have an effect against inflammation including atranorin, (+)-iso-usnic acid, and methyl orsellinate. This agrees with our results where we found that they have a high inflammatory potential. Also, Varol<sup>[41]</sup> reported that parietin has pharmacological activities including anti-inflammatory activity. Ranković,<sup>[6]</sup> mentioned that *Usnea diffracta*, *Umbilicaria esculenta*, and *Usnea longissima* and their compounds have been shown anti-inflammatory activity.

We can interpret that (+)-iso-usnic acid, and atranorin inhibited the production of inflammatory NO mediators more than the methanolic extract of *P. hypoleucinum*. Which probably induced an antagonistic effect on the other compounds in the extract. In the same way, methyl orsellinate shows better inflammatory potential than *R. phycopsis*. However, parietin has a low inflammatory activity compared to the methanol extract of *X. parietina*, which proves that having a synergistic effect with the other compounds in the extract. There may be some other compounds, that are responsible for their inflammatory potential.

## Conclusion

In this study, we successfully isolated (+)-iso-usnic acid, and atranorin from methanol extract of *Parmotrema hypoleucinum*, methyl orsellinate from methanol extract of *Roccella phycopsis*, and parietin from acetone extract of *Xanthoria parietina*. Therefore, the pure compounds and the extracts from lichens do not have a toxic effect against RAW 264.7 macrophages cells at 25 µl/mL. In addition, they inhibited the production of the inflammatory mediators NO. Moreover, our results indicate that the atranorin and (+)-iso-usnic acid showed high anti-inflammatory activity. The current study revealed that (+)-iso-usnic acid, atranorin, methyl orsellinate, and parietin could be regarded as potential sources of natural anti-inflammatory compounds. The benignity and the anti-inflammatory activity give a preview of the possibility

to valorize in pharmaceuticals application. In addition, our studies suggest that these compounds could be a potential therapeutic candidate for inflammatory diseases. Further studies are needed to establish their mechanisms of action.

## Experimental Section

### Lichen Sample

*Parmotrema hypoleucinum*, *Roccella phycopsis*, and *Xanthoria parietina* were collected from Nefza (37°02'11" N: 9°05'12" E), Ichkeul National Park (37°07'43" N: 9°39'50" E), and Bazina (36°57'36" N: 9°17'45" E) region of Tunisia in February and Mars 2019. Lichens species were identified using macroscopic observation and chemical tests. The identification was done by Dr. Ayda Khadhri assistant professor in the Faculty of Sciences of Tunisia. Voucher specimens have been deposited in the lichenological herbarium of the Department of Biology, Faculty of Sciences of Tunisia, under the registration number herb TUN-Nfz 31 (*P. hypoleucinum*), herb TUN-Ichk 043 (*R. phycopsis*), and herb TUN-Baz 12 (*X. parietina*). Lichen thalli of three species were dried in the dark at room temperature.

### Isolation and Identification of Bioactive Compounds (1–4)

#### General Experimental Procedures

Optical rotations of pure metabolites were measured in MeOH as a solvent and conducted on a Jasco polarimeter (Tokyo, Japan).

<sup>1</sup>H and <sup>13</sup>C-NMR spectra were recorded on a Bruker AMX instrument at 400 and 100 MHz, respectively. The solvents used were deuterated chloroform (CDCl<sub>3</sub>) and deuterated methanol (CD<sub>3</sub>OD).

Chromatographic column (CC) was performed on silica gel (Merck, Kieselgel 60, 0.063–0.200 mm). Analytical and preparative thin-layer chromatography (TLC and PLC) was performed on silica gel (Merck, Kieselgel 60 F<sub>254</sub>, with 2, 1, 0.25 mm film thickness) plates. Spots were visualized by exposure to UV light (254 or 365 nm) and/or by spraying with anisaldehyde/sulfuric acid reactive (ANS: anisaldehyde/acetic acid/methanol/sulfuric acid (1:14:85:1) followed by heating at 110 °C for 10 min.

### Extraction and Isolation of Atranorin and (+)-Iso-usnic Acid (1, 2) from *Parmotrema hypoleucinum*

66 g of dry powder of *Parmotrema hypoleucinum* thalli was extracted twice at room temperature with 1 L of methanol for 48 h. The suspensions were filtered, and the organic phases evaporated in a vacuum giving a brown solid residue namely *Ph-Ex*.

5.7 g of crude extract was subjected to a chromatographic purification process on a column of silica gel (CC) eluted with the following gradient of eluents: hexane: AcOEt (6:4), CH<sub>2</sub>Cl<sub>2</sub>:AcOEt (8:2), CH<sub>2</sub>Cl<sub>2</sub>:AcOEt (1:1), CH<sub>2</sub>Cl<sub>2</sub>:MeOH (8:2), CH<sub>2</sub>Cl<sub>2</sub>:MeOH (1:1), MeOH. The fractions obtained were collected both based on the usual controls by TLC and the chromatic variations of the fractions. This process yielded 14 groups of homogeneous fractions. The residue of fraction 3 (147.3 mg) was further purified on PLC on silica gel eluted with hexane: AcOEt (9:1) obtained as yellow solid compound **1** (11.0 mg, R<sub>f</sub> 0.45 in TLC on silica gel eluent hexane: AcOEt (9:1).

The fraction 8 (45.3 mg) was purified by PLC eluted with hexane: AcOEt:HCOOH (7:2.5:0.5) obtained as yellow solid compound **2** (20.1 mg, R<sub>f</sub> 0.70 in the same chromatographic condition, [α]<sub>D</sub> = +450, MeOH, c = 0.02).

### Extraction and Isolation of Methyl Orsellinate (3) from *Roccella phycopsis*

50 g of dry powder of *Roccella phycopsis* thalli was extracted as previously reported. Crude extract, namely *Rp-Ex*, obtained as brown solid residue (14.8 g) was re-suspended in AcOEt and washed with a 2 N NaOH solution to separate neutral and acid compounds. The organic phase was dried on Na<sub>2</sub>SO<sub>4</sub>, filtered, and evaporated under reduced pressure originating a brown solid residue (1.4 g of neutral or basic compounds). This extract was subjected to purification process on CC on silica gel eluted with the following gradient of eluents: CH<sub>2</sub>Cl<sub>2</sub>, CH<sub>2</sub>Cl<sub>2</sub>:AcOEt (9:1), CH<sub>2</sub>Cl<sub>2</sub>:AcOEt (8:2), CH<sub>2</sub>Cl<sub>2</sub>:AcOEt (7:3), CH<sub>2</sub>Cl<sub>2</sub>:MeOH (9:1), CH<sub>2</sub>Cl<sub>2</sub>:MeOH (8:2), MeOH. 10 groups of homogeneous fractions were collected both based on the usual controls by TLC and the chromatic variations of the fractions. The residue of fraction 4 (204.3 mg) was purified on PLC on silica eluent CH<sub>2</sub>Cl<sub>2</sub>:AcOEt (8:2), gel yielding compound **3** as solid residue (12.2 mg, *Figure 1*).

### Extraction of Parietin (**4**) from *Xanthoria parietina*

140 g of *X. parietina* thalli were extracted twice with 1 L of acetone at room temperature for 24 h. The mixture was filtered to the separate powders and the solvent evaporated under reduced pressure, yielding 1.2 g of green solid residue. The extract was partially dissolved in chloroform. The insoluble fraction, separated by filtration and appeared as an orange solid, was identified as parietin (**4**, 350 mg, Figure 1).

### Cell Culture

The murine macrophage cell lines RAW 264.7 (American Type Culture Collection) was grown in flasks with RPMI 1640 medium (Dominique Dutscher; w/L-Glutamine), added with 10% fetal bovine serum (FBS) (Dominique Dutscher; Origin South America) and antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL). The cells were grown at 37 °C in a humid atmosphere containing 5% CO<sub>2</sub>. Before each assay, RAWs (in exponential growth phase) were plated in 24-well plates at a density of 2 × 10<sup>5</sup> cells/well. They were then incubated for 24 h to allow them to adhere.<sup>[30]</sup> Then, cells were treated with extract dissolved in DMSO.

### Cell Viability Assay

The cytotoxicity of the extracts was assessed using the resazurin assay developed by Hwang et al.<sup>[30]</sup> RAW 264.7 macrophages cells, having been adhered to in 24-well plates, were treated for 24 h with different concentrations of each sample. Following removal of the supernatant, 1 mL of a 2% resazurin solution in PBS (Dulbecco's Phosphate Buffered Saline, Dominique Dutscher) was added to each well. After incubation for 60 min, fluorescence was measured, and cell viability was calculated against the control of untreated cells using the following equation:

$$\text{Viability \%} = \frac{[\text{Fluorescence (sample)} \times 100]}{\text{Fluorescence (control)}}$$

### Anti-Inflammatory Activity (Griess Nitrite Assay)

Nitric oxide (NO) plays an important role in inflammation. Anti-inflammatory activity was based on the evaluation of the effect of the extracts and pure compounds on the level of nitrite released by the cells (RAW 264.7). The macrophages cell, adhered in 24-well

plates, were treated with four different concentrations of each extract. After 1 h of pre-treatment, the cells were treated with LPS (1 µg/mL) and incubated for 24 h at 37 °C. The supernatant was then collected and the amount of NO produced by the cells was evaluated by a colorimetric assay using the GRIESS reagent. 100 µL of cell supernatant was incubated with 100 µL of Griess reagent (0.8% sulfanilamide, 0.75% N-naphthyl ethylene diamine in 0.5 N HCl) at room temperature for 15 min. The absorbance at 540 nm was measured using the Varioskan Flash plate reader (Thermo Scientific) and the presence of nitrite was quantified from a NaNO<sub>2</sub> standard curve ( $y = 0.0115x + 0.0441$ ;  $R^2 = 0.9998$ ). The percentage of inhibition is calculated in comparison to the control-treated only with LPS. Each test was performed in triplicates.<sup>[30]</sup>

### Statistical Analyses

Statistical analyses were carried out using MS Excel 360 and SPSS software packages. Test of homogeneity of variances and test ANOVA were investigated using IBM SPSS 20, and an interesting degree of significance ( $P < 0.05$ ) was obtained. All experiments were carried out at least in triplicate, and the mean and standard deviation (SD) of replications were reported. Pearson's correlation coefficient was used to analyze the degree of association between the anti-inflammatory activity and extracts and pure compound levels.

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### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

### Author Contribution Statement

M. M. and A. K. Conceptualization, Resources, methodology, formal analysis, investigation, interpretation, data curation, writing and editing. J. M. J. a prospec-

ting visit. A. A., I. T., and M. D. analyzed data review and editing. S. A. S. supervision.

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