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Chemical composition and antioxidant activity of two lichens species (*Pseudevernia furfuracea L* and *Evernia prunastri L)* collected from Morocco

N. Aoussar[1]*, R. Manzali[2], I. Nattah[3], N. Rhallabi[1], P. Vasiljevic[4], M. Bouksaim[2], A. Douira[3], N. Manojlović[5], F. Mellouki[1]

[1] *RU Microbiology, Hygiene & Biomolecule, Laboratory of Virology, Microbiology, Quality & Biotechnology / Ecotoxicology and Biodiversity, University Hassan II Casablanca, FSTM. P.O. Box 146, Mohammedia 20650, Morocco*
[2] *RU Food Technology, INRA, RCAR-Rabat, P.O. Box 6570, Rabat Institutes, 10101, Rabat, Morocco*
[3] *Laboratory of Botany and Plant Protection, University Ibn Tofail, Faculty of Sciences, P. O. Box 133, 14000 Kenitra, Morocco*
[4] *Department of Biology and Ecology, Faculty of Sciences and Mathematics, University of Nis, Nis 18000, Serbia*
[5] *Department of Pharmacy, Faculty of Medical Sciences, University of Kragujevac, 34000 Kragujevac, Serbia*

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N Aoussar
noura.aoussar@gmail.com
+2120602374391

Abstract

The present study focused on the determination of phenolic compounds and screening of antioxidant activity of dichloromethane, acetone and methanol extracts of *Evernia prunastri L* and *Pseudevernia furfuracea L* growing in Morocco. For the identification of phenolic compounds, a HPLC–UV method was used. The antioxidant activity was performed *in vitro* by DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging method and Ferric Reducing Antioxidant Power assay. Total phenolic compounds and total flavonoid content of all the extracts were also determined. The predominant phenolic compounds were physodalic acid and evernic acid mainly in the acetone extracts of *Pseudevernia furfuracea* and *Evernia prunastri*, respectively and the highest antioxidant activity was obtained from the first one. A significant relationship between the antioxidant capacities and total phenolic contents was found. This is the first report of the chemical composition and antioxidant activity of *Pseudevernia furfuracea* and *Evernia prunastri* collected from Morocco. These Moroccan lichens might be used as potential sources of natural compounds with antioxidant activity suggesting other future research.

1. Introduction

Molecular oxygen is crucial to the life of aerobic organisms and cellular metabolism can produce normally Reactive Oxygen Species (ROS) [1], which are very useful for body and play important roles in various physiological processes such as signal transduction in a variety of cellular processes [2]. However the overproduction of ROS beyond the antioxidant capacity of biological systems leads to an oxidative stress involved in the onset of many diseases including cardiovascular diseases, cancer, diabetes, rheumatoid arthritis, or neurological disorders such as Alzheimer or Parkinson diseases [1-3].

To escape the consequences of the oxidative stress, the restore of the balance oxidant / antioxidant is important to preserve the physiological performance of the body by the use of antioxidant synthetic compounds [1,3]. The risks and harmful effects of such synthetic antioxidants have been reproved in recent years and nowadays their restricted use necessitate their replacement by natural antioxidants which are of great interest. Among the natural antioxidants, phenolic compounds have been studied as promising non-toxic antioxidants in various biological systems. Phenolic compounds, such as flavonoids, are commonly found in plants and have increasingly value thanks to their antioxidant effects and to their potential role in preventing and treating diseases related to the oxidative stress [3-5].

Lichens are fungi (mycobiont), usually ascomycetes or rarely basidiomycetes living in symbiotic association with algal partners (photobiont) namely a green alga or a cyanobacterium. Lichens produce a large number of specific secondary metabolites, about 1050 “lichen substances” have been identified [6]. These metabolites are produced by mycobiont partner and accumulate on the outer surfaces of hyphae [7-9]. Previous studies have shown that such metabolites exert a wide variety of pharmacological activities such as antibiotics, antiviral, anti-inflammatory, analgesic, antipyretic, anti-proliferative and cytotoxic [10,11].

About 18 500 different lichen taxa have been described all over the world [10,12]. In Morocco, according to Nattah et al. [13], the first work on lichens has been discussed by Braun-Blanquet and Wilczek (1923), in (1931) Gattefosse and Werner have published a catalog of 542 species and in (1996) Egea has published a bibliographical catalog of 210 genera and 1,100 species [13]. Despite the potential importance of lichens for chemical applications, in Morocco, lichens have never been studied for their chemical composition nor for their biological activities even if some species present an economic value e.g. Oak Moss extracts used in perfumery. For the best of our knowledge, this is the first study describing the evaluation of Moroccan lichens species as a source of natural antioxidant. The aim of our work was to identify the main phenolic compounds by HPLC-UV, to evaluate the antioxidant capacity and the total phenol and flavonoid contents of the dichloromethane, acetone and methanol extracts of two lichen species growing in Morocco: *Evernia prunastri* and *Pseudevernia furfuracea*.

2. Experimental details

2.1. Collection and identification of lichens

Lichen specimens were collected on trunks and branches of Cedar *(Cedrus atlantica* L*)* and Oak trees (*Quercus ilex* L) in October 2015 from the province of Khenifra in Middle Atlas of Morocco, between 32 ° 56 '50.3 N and 05 ° 28' 25.6 W about *Pseudevernia furfuracea* and 32 ° 56 '00.7 N and 05 ° 30' 09.3 W about *Evernia prunastri*. The lichen harvesting was done under supervision of and with the logistic help of the Provincial Direction of Water and Forests and the Fight against Desertification of Khenifra.

The identification of collected lichens was firstly performed in the field by observing the general morphological characteristics such as shape, color, type of branching of thallus and colorful reactions by conventional chemical reagents (potassium hydroxide 10% and sodium hypochlorite). The botanical identification was then confirmed later by a photonic microscopic study of the form of asci and size of the ascospores. The determination of the lichens species was performed based on the descriptions presented by identification keys [14,15].

2.2. Preparation of the lichen extracts

The ground dried samples (10 g) were extracted sequentially with 250 mL of dichloromethane, acetone and methanol at room temperature for a period of 24 hours. The extracts were filtered and then concentrated to dryness under reduced pressure at 40°C using a rotary evaporator (Heidolph G1, Germany). The yield of respective extracts was calculated as:

$$\text{Yield (\%)} = (\text{dry weight of extract/dry weight of samples}) \times 100$$

The obtained dried extracts were then kept in sterile tubes and stored at -20 °C.

2.3. High performance liquid chromatography (HPLC) analysis

HPLC analytical method and standard compounds preparation were carried out as described by Huneck and Yoshimura [16]. The lichen extracts were redissolved in 500 µL of acetone and analyzed on an HPLC (Agilant Technologies, 1200 Series) instrument with C18 column (25 cm x 4.6 mm, 10µm) using UV spectrophotometric detector. Methanol-water-phosphoric acid was used (90:10:0.9, v/v/v) as solvent. The sample injection volume was 10 µL and the flow rate was 1.0 mL/min. The standards used were obtained from the following lichens species: methyl lecanorate (MLE) was isolated from *Pseudocyphellaria crocata*, physodalic acid (PHY), atranorin (ATR) and chloratranorin (CHR) were isolated from *Hypogymnia physodes*, usnic acid (USN) from *Usnea barbata* and evernic acid (EVE) from *Ramalina fraxinea*.

2.4. Determination of DPPH free radical scavenging activity

The ability of *P. furfuracea* and *E. prunastri* extracts to scavenge DPPH free radicals was estimated by the reduction of the reaction color between DPPH solution and sample extracts. For this purpose, the method used by Kosanic´ et al. [17] was adopted. 2 mL of 0.12 mM solution DPPH in methanol was added to 1 mL of various concentrations of each extract (50 - 1000 µg/mL) to be tested. After 25 min at room temperature, the absorbance of the reaction mixture was measured at 517 nm using a spectrophotometer (UNICO, USA). Ascorbic acid and Trolox (2- 20 µg/mL) were used as positive controls. The scavenging activity was calculated as follows:

$$I\% = ((A\ \text{Control} - A\ \text{Sample}) / A\ \text{Control}) \times 100$$

Where A Control is the absorbance of blank sample (t= 0 min) and A Sample is the absorbance of test extract or standard (t= 25 min). Tests were carried out in triplicate. The IC_{50} values (concentration in µg/mL required to

inhibit DPPH radical by 50%) were estimated from the percentage inhibition versus concentration plot, using a Regtox software. The data were presented as mean values ± standard deviation (n = 3).

2.5. Ferric Reducing/Antioxidant Power (FRAP) assay

The reducing powers of the studied lichen extracts were determined according to the method described by Oyaizu [18]. Various concentrations of lichen extracts (50 to 1000 µg/mL) were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide solution (1%). After incubation in water bath at 50°C for 30 min, 2.5 mL of 10% trichloroacetic acid was added to the mixture to stop the reaction, and the mixture was centrifuged at 3000g for 10 min. The supernatant (2.5 mL) was mixed with 2.5 mL distilled water and 0.1% $FeCl_3$ (0.5 mL) and then the absorbance was measured at 700 nm using a spectrophotometer (UNICO, USA). Higher absorbance of the reaction mixture indicates greater reducing power. As positive control, ascorbic acid and trolox were used. All tests were carried out in triplicates to ensure reproducibility.

2.6. Determination of total phenolic compounds content

The total phenolic content (TPC) in lichen extracts was determined using the Folin-Ciocalteu assay reagent according to the method of Slinkard and Singleton [19]. 0.1 mL of extracts (1 mg/mL) were transferred into test tubes and their volumes made up to 4.6 mL with distilled water. After addition of 0.1 mL Folin-Ciocalteu reagent, 0.3 mL of Na_2CO_3 (2%) solution was added after 3 min. After 1h30 min incubation at room temperature the absorbance of the mixture was recorded against a blank containing extraction solvent. Gallic acid was used as the standard and TPC in lichen extracts was expressed as milligram of Gallic Acid Equivalents (GAE) per gram of the dry extract averaged from 3 parallel measurements.

2.7. Determination of total flavonoid content

Total flavonoid content (TFC) was determined according to the method used by Zilic et al. (2011) [20]. Briefly, 0.075 mL of 5% $NaNO_2$ was mixed with 0.5 mL of the sample (1 mg/mL). After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and the mixture was allowed to stand at ambient temperature for 5 min. Then, 0.5 mL of NaOH (1 M) was added, and the volume was made up to 2.5 mL with distilled water. The absorbance was measured at 510 nm using a spectrophotometer (UNICO, USA), against the blank containing the extraction solvent instead of the sample. The TFC was calculated using a standard calibration of catechin solution and expressed as micrograms of Catechin Equivalent (CE) per gram of dry extract. All tests were achieved in triplicate.

2.8. Statistical analyses

The obtained experimental data of the TPC, TFC and antioxidant activity assays were expressed by a mean and standard deviation. To evaluate statistical differences, One-way ANOVA and student's t-test were used. The comparison between the averages is performed through the Duncan test. Correlation coefficient of antioxidant properties was determined by the Pearson test, using SPSS software. $p \leq 0.05$ values are considered statistically significant.

2.9. Chemicals

All chemical reagents and solvents were of analytical grade and obtained from Sigma-Aldrich.

3. Results and discussion

3.1 Lichen identification

Morphological characteristics of the lichen species determined by macroscopic and microscopic studies of the thallus and on the basis of colorful reactions by chemical reagents allowed us to identify two species: *Pseudevernia furfuracea* (L.) Zopf (Figure 1) and *Evernia prunastri* (L.) Ach (Figure 2).

P. furfuracea (L.) Zopf had fruticose thallus measuring 8 cm in height and in the form of strips dichotomously branched (Figure 1 A). The upper side was gray with cylindrical isidia, the underside was white with black spots (Figure 1 C). The upper cortex was hyaline to yellow, measured 16 µm with gonidiale layer measured 43 µm, and the lower cortex was yellow and measured 16 µm (Figure 1 D). The thallus became yellow under the effect of potassium hydroxide and indifferent to sodium hypochlorite (Figure 1 B). Apothecia were present on the surface of the thallus, brown, measuring 1 to 3 mm, (Figure 1 E).

Figure 1: *Pseudevernia furfuracea* (L.) Zopf. **(A)** Thallus attached to the cedar. **(B)** Effect of KOH on the thallus. **(C)** Thallus showing isidia (is). **(D)** Transverse section of thallus showing anatomical features. upper cortex (uc), gonidiale layer (gl), medulla (m), lower cortex (lc). **(E)** Thallus showing apothecium (ap).

The thallus of *E. prunastri* (L.) Ach was fruticose measuring 3.5 cm in height, fruticose, dichotomously branched, with the thongs until 5 mm wide and were grey-green above and white below (Figure 2 A). Round soralies were present along the margins around the edges of strips (Figure 2 C). The thallus becomes yellow under the effect of KOH (Figure 2 B), K + yellow cortex on both sides (Figure 2 D). The thallus didn't show any color reaction with sodium hypochlorite (C -) with absence of apothecia.

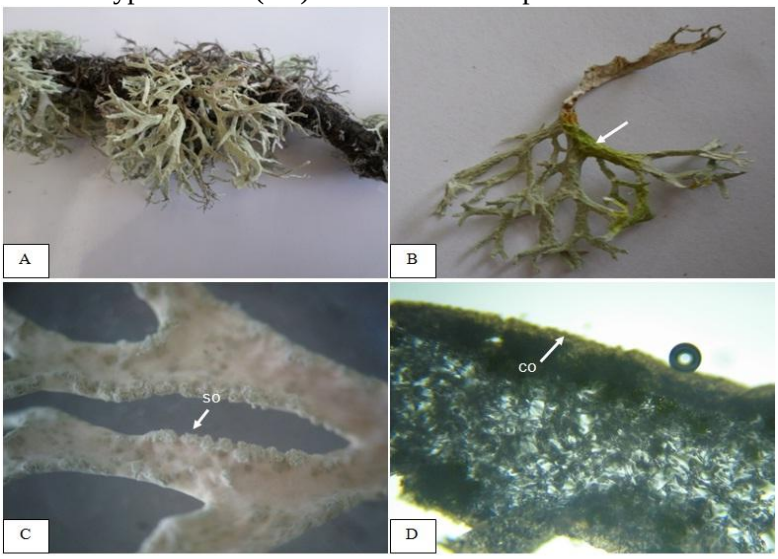

Figure 2: *Evernia prunastri* (L.) Ach : (**A)** : Thallus on a branch of oak three, (**B)** : Colorful reactions of thallus with KOH, (**C)** : soralies (so) and (**D)** : cortexe (co).

Dichloromethane, acetone and methanol extracts were prepared from *P. furfuracea* (respectively P.F D, P.F A and P.F M) and from *E. prunastri* (respectively E.P D, E.P A and E.P M) to examine their chemical composition, their TPC, TFC concentrations and antioxidant activity. The yield of all extracts obtained from 10 g of dry material was measured for each extract (Table 1). The yield of the obtained extracts was found to be in the order of: P.F M > E.P M > P.F D > E.P D > E.P A > P.F A.

3.2. Yield of extracts

Table 1: Yields (% w/w) of dried extracts from *P. furfuracea* (P.F D, P.F A, and P.F M respectively dichloromethane, acetone and methanol extracts) and *E. prunastri* (E.P D, E.P A, and E.P M respectively dichloromethane, acetone and methanol extracts)

Extracts	P.F D	E.P D	P.F A	E.P A	P.F M	E.P M
Yield (%)	4.3%	3.8%	1.8%	3.7%	10.1%	8%

3.3. High Performance Liquid Chromatography (HPLC) analysis

The HPLC–UV analysis of dichloromethane, acetone and methanol extracts of *P.furfuracea* and *E.prunastri* was used to identify their major phenolic compounds. Lichen substances were identified by comparing their retention times (t_R) with the database of the authentic substances previously isolated from lichens (Figure 3).

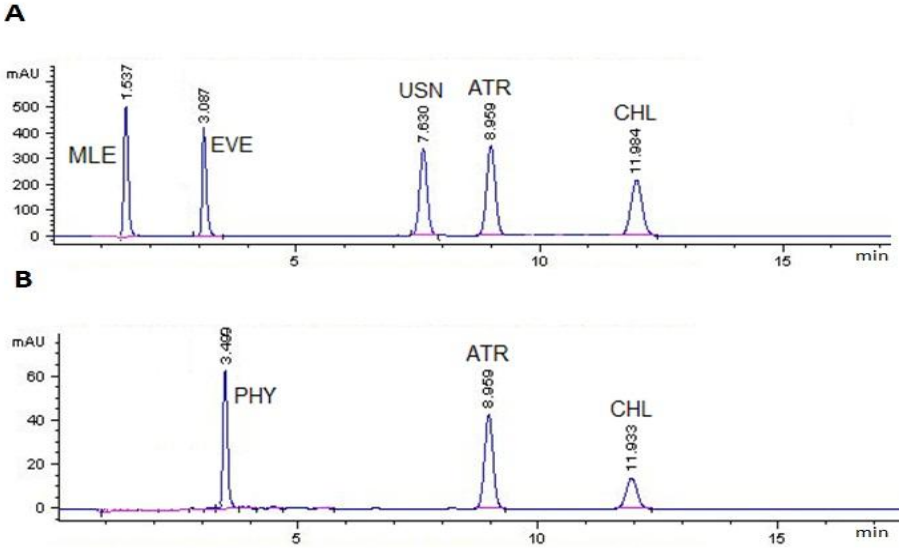

Figure 3: HPLC chromatograms at 254 nm of the standards used for identification of the phenolic compounds present in *Evernia prunastri* **(A)** and *Pseudevernia furfuracea* **(B).**
MLE = Methyl lecanorate, EVE=Evernic acid, USN=Usnic acid, ATR=Atranorin, CHL=Chloratranorin, PHY=Physodalic acid

Obtained data confirmed that the main compounds in dichloromethane and acetone extracts of *P. furfuracea* are PHY (t_R = 3.495 ± 0.017 min), ATR (t_R = 8.942 ± 0.023 min) and CHL (t_R = 11.958 ± 0.036 min). PHY is the most abundant substance in acetone extract. In methanol extract, the chromatogram contains only a small amount of PHY (Figure 4).

As it is evidenced in the chromatograms of *E. prunastri* (Figure 5), EVE (t_R = 3.065 ± 0.039 min), USN (t_R = 7.574 ± 0.035 min), ATR (t_R = 8.935 ± 0.026 min) and CHL (t_R = 11.944 ± 0.038 min) are present in the dichloromethane and acetone extracts, and EVE is the most abundant compound in acetone extract. In methanol extract, EVE and USN are also detected but the most abundant compound is MLE (t_R = 1.535 ± 0.002 min).

3.4. DPPH radical scavenging activity

The DPPH radical scavenging activity of the studied lichens is given in figure 6. The results showed a statistically significant difference between studied extracts ($p < 0.05$) and between studied extracts and positive controls ($p < 0.05$). The antioxidant activity was increased from 2.11 ± 0.007% to 86.94 ± 0.010% in accordance with the increase of the concentration of the extracts from 50 to 1000 µg/mL of *P. furfuracea* and *E. prunastri***.** Among the tested extracts, the acetone extracts showed largest DPPH radical scavenging activity: 86.94 ± 0.010% and 22.13 ± 0.002% with 1000 µg/mL of extract from *P. furfuracea* and *E. prunastri*, respectively.

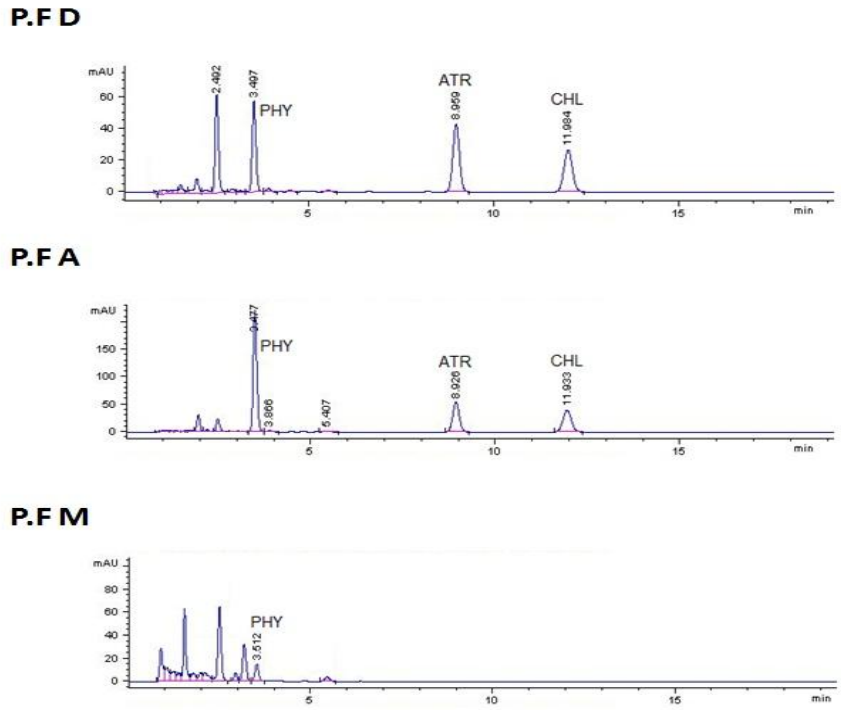

Figure 4: HPLC chromatograms at 254 nm of the dichloromethane (P.F D), acetone (P.F A) and methanol (P.F M) extracts of *Pseudevernia furfuracea.*
PHY= Physodalic acid, ATR = Atranorin, CHL = Chloratranorin

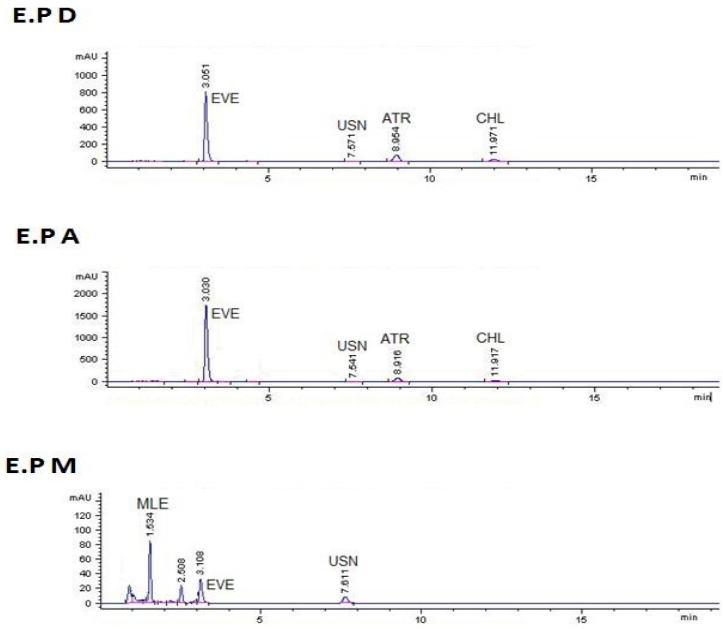

Figure 5: HPLC chromatograms at 254 nm of the dichloromethane (E.PD), acetone (E.PA) and methanol (E.PM) extracts of *Evernia prunastri.*
MLE = Methyl lecanorate, EVE = Evernic acid, USN = Usnic acid, ATR = Atranorin, CHL = Chloratranorin

According to the Duncan's test the antioxidant activities obtained for the dichloromethane and methanol extracts are included in the same group, and were lower than that measured for the acetone extract. Among the tested extracts, the acetone extract from *P. furfuraceae* showed the highest DPPH radical scavenging activity with an IC_{50}= 240.320 ±15.264 µg/mL. However, this value was lower than IC_{50} obtained for ascorbic acid and trolox (8.872±0.064 µg/mL and 17.834±0.497 µg/mL, respectively). For *E. prunastri* the IC_{50} obtained for all extracts exceeded 1000 µg/mL.

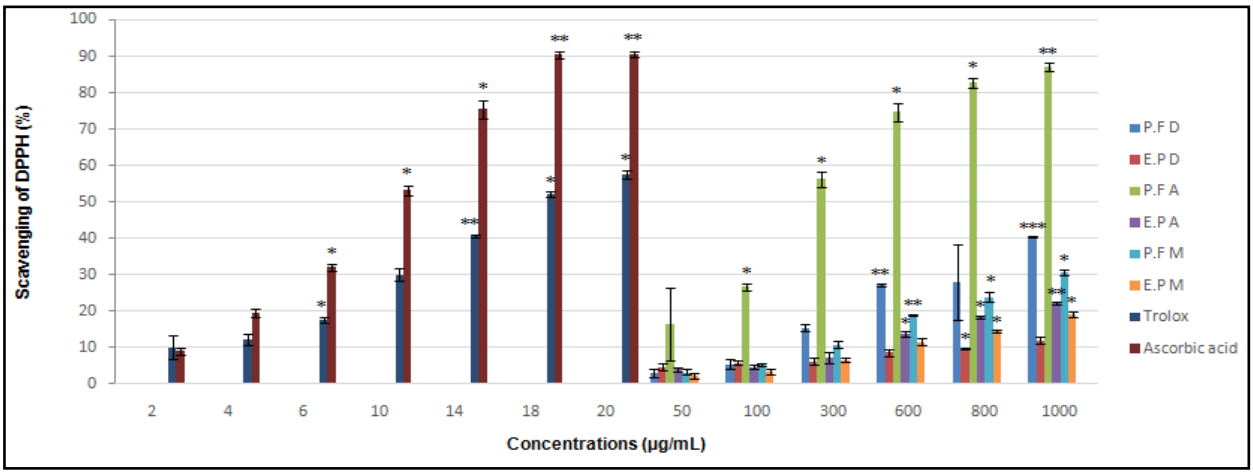

Figure 6: DPPH radical scavenging activity of extracts from *P. furfuracea* (P.F D, P.F A, and P.F M respectively dichloromethane, acetone and methanol extracts) and from *E. prunastri* (E.P D, E.P A, and E.P M respectively dichloromethane, acetone and methanol extracts).
Data are presented as mean +/- SD, n=3 experiments, p values; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

3.5. Reducing power (FRAP)

The reducing power of the studied lichen extracts are represented in figure 7. The reducing power was dose dependent and increased with increasing amounts of extracts. For *P. furfuracea*, the absorbance of the acetone extract was higher than those of other extracts and increased from 0.043 ± 0.005 to 0.890 ± 0.036.

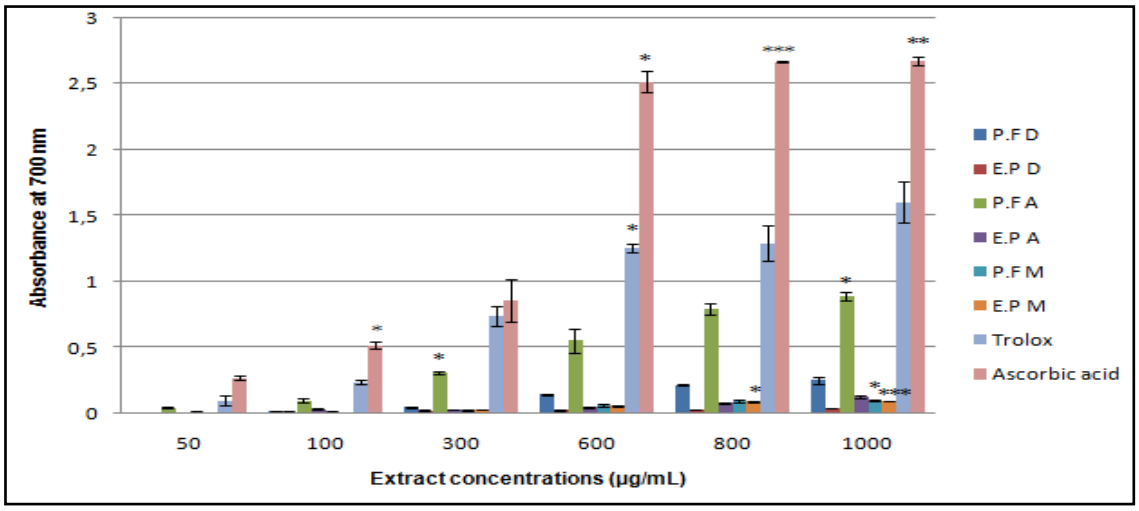

Figure 7: Reducing power of extracts from *P. furfuracea* (P.F D, P.F A, and P.F M respectively dichloromethane, acetone and methanol extracts) and from *E. prunastri* (E.P D, E.P A, and E.P M respectively dichloromethane, acetone and methanol extracts).
Data are presented as mean +/- SD, n=3 experiments, p values; *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Reducing power of the acetone extracts of *E. prunastri* was also higher than the dichloromethane and methanol extracts but lower than that obtained for *P. furfuracea*. Values of the absorbance varied from 0.007 ± 0.002 to 0.130 ± 0.011. The reducing power of standards varied from 0,270± 0,015 to 2,674± 0,035 and from 0,102± 0,036 to 1,607± 0.160 respectively for ascorbic acid and Trolox.

3.6. Determination of total phenolic and flavonoids contents

The total phenolic and flavonoids contents in extracts of *P. furfuraceae* (P.F D, P.F A, and P.F M) and *E. prunastri* (E.P D, E.P A, and E.P M) were determined as Equivalent Gallic Acid using an equation obtained from a standard gallic acid graph ($R^2 = 0.990$) for TPC and for TFC by using a calibration curve of catechin ($R^2 = 0.985$). Obtained results are given in Table 2.

Table 2: Total phenolic compounds and flavonoids contents of dichloromethane, acetone and methanol extracts of *P. furfueracea* (respectively P.F D, P.F A, and P.F M) and *E. prunastri* (respectively E.P D, E.P A, and E.P M)

Species	TPC (µg GAE/mg of dry extract)	TFC (µg CE/mg of dry extract)
P.F D	128.66 ± 3.75*	15.40 ± 0.85
E.P D	122.16 ± 30.15	15.66 ± 5.02
P.F A	237.66 ± 25.18	19.20 ± 1.15
E.P A	163 ± 26.45	17.66 ± 0.37*
P.F M	105.33 ± 13.27	14.5 ± 0.81
E.P M	94.33 ± 24.82	13.53 ± 1.07

Data are presented as mean +/- SD, n=3 experiments, p values; *: $p < 0.05$.

The determined TPC and TFC in both tested lichen species ranged respectively from 94.33 ± 24.82 to 237.66 ± 25.18 µg AGE / mg of dry extract and 13.53 ± 1.07 to 19.2 ± 1.15 µg CE /mg of dry extract and varied significantly from one extract to the other ($p < 0.05$).

The highest level of phenolic and flavonoids contents were observed for the acetone extract of *P. furfuracea* with respectively 237.66 ± 25.18 µg of GAE/mg of dry extract and 19.2 ± 1.15 µg CE/mg of dry extract. Whereas the methanol extract of *E. prunastri* showed the low content of TPC (94.33 ± 24.82µg of GAE/mg dry extract) and of TFC (13.53 ± 1.07 µg CE/mg of dry extract).

The Duncan's test confirmed that the obtained results for the dichloromethane and methanol extracts were statistically included in the same group, and were lower than those determined for the acetone extract. Otherwise, the Pearson test showed that there is a significant correlation between phenolic content and total antioxidant capacity ($R^2 = 0.786$).

In the present study, the phenolic composition and antioxidant activity of the dichloromethane, acetone and methanol extracts of *Evernia prunastri* and *Pseudevernia furfuracea* were examined. According to the recorded results, the lichen extracts prepared by acetone showed stronger *in vitro* antioxidant activity than the dichloromethane and methanol extract. The highest antioxidant activity and total phenols and flavonoid contents were obtained from the acetone extract of *P.furfuracea* characterized by physodalic acid as the most abundant phenolic compound. The evernic acid was the major phenolic compound in the acetone extract of *E.prunastri*. These results suggest that these phenolic compounds are probably responsible for the antioxidant activity of the studied extracts, they agree with published studies showing that phenolic compounds isolated from lichen species have a higher antioxidant activity than crude extracts [11,17,21,].

In our study we found a correlation between a strong antioxidant activity and a high content of phenols. This is in concordance with other reported works [11,17,21,22,23] carried out on lichen species harvested in Serbia and Turkey, which means that phenols are the main agents responsible of antioxidant activity of lichens. Indeed, it well known that the anti-radical ability of phenolic compounds is due to their capacity to trap free radicals through the transfer of the hydrogen atom then transformed into a stable molecule [24], and their reducing power is due to the presence of hydroxyl group in their structure that can serve as an electron donor [25].

Our study pointed out that the acetone extract of *Pseudevernia furfuracea* exhibited higher antioxidant activity than the acetone extract of *Evernia prunastri*. These results are similar to those reported by Kosanić et al. and Sokmen et al.[21,26], and suggest that the acetone extract of Moroccan *Pseudevernia furfuracea* can be used as precious source of natural antioxidants.

Conclusion

The antioxidant capacities, total phenolic and flavonoid contents of the dichloromethane, acetone and methanol extracts of *Evernia prunastri* and *Pseudevernia furfuracea* from Morocco were evaluated. It was found that the acetone extract of *P. furfuracea* showed the highest antioxidant capacities. A significant relationship between the antioxidant activities and the total phenolic contents was found, indicating that the phenolic compounds are the responsible of antioxidant capacities of these lichens. Based on these results, *E. prunastri* and *P. furfuracea* extracts represent an interesting source of the phenolic compounds with

antioxidative properties, that would be of interest in pharmaceutical and food industries. Further investigations are however required for isolation of these compounds and determination of their antioxidant activity.

Conflict of interests-All contributing authors declare no conflicts of interest.

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