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Solvent Effect on Total Phenolic Content, Total Flavonoids Content, and the Antioxidant Activity of *Ramalina lacera* and *Evernia prunastri* Lichens Collected from the Trunks of *Argania spinosa* L.

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ABSTRACT

The study was aimed to evaluate the impact of extraction solvent on the phenolic content, total flavonoids content, and the antioxidant activities of acetonic, methanolic, and aqueous extracts of two lichen species: *Evernia prunastri* and *Ramalina lacera* collected from trunks of *Argania spinosa* using the ultrasound assistance extraction. Various in vitro antioxidant assays were utilized such as 2,2-diphenly-picryly-hydroxyl free radical (DPPH) assay, and ferric reducing antioxidant power (FRAP) assay. All tested samples exhibited a good antioxidant activity, for the DPPH assay, the inhibition percentage ranged from 85±0.2% to 27±0.01%, the phenolic content ranged from 13.17±0.5 mgGAE/g DW to 3.31±0.3 mgGAW/g DW, and flavonoids ranged from 5.84±0.03 mgRE/g DW to 0.01±0.03 mgRE/g DW. This study demonstrates that the extraction solvent has a significant influence on lichens phenolic compounds and on their antioxidant activity, also showed that flavonoids contents are significantly correlated to antioxidant activity of studies lichens; moreover, it shows that ultrasound extraction in a good method to extract the lichens compound. This study suggests that lichens *Ramalina lacera* and *Evernia prunastri* could be utilized as natural antioxidant source.

Keywords: lichens, lichens compounds, antioxidant activity, extraction solvent, extraction.

INTRODUCTION

Research on new sources of bioactive compounds could be guided to the elaboration of new drugs with possible biological activities have been rising in the last decade (Newman et al. 2008) Plants and other species like bacteria, fungus, lichens are one of the sources that contributed significantly to the development of bioactive compounds with many biological activities (Atanasov et al. 2021, Khairan et al. 2021).

Argan ecosystem of oust-south of Morocco, encompass a flora of great importance and diversity, besides the Argan trees, aromatic and medicinal plants (Msanda et al. 2021), there is another species that get less attention and their investigation is still limited like lichens. Lichens are non-flowering plants well-known formulating from the symbiotic association among a fungus and a green alga or cyanobacterium (Zhao et al. 2021). Lichens could be found worldwide from the tropical to the polar regions (Ranković and Kosanić 2021). Lichens produce large compounds of secondary metabolites (Adenubi et al. 2022). Those substances are unique to lichens-forming fungi, and it also, called lichens substances(Nayaka and Haridas 2020). Most of these substances belong to phenolic compounds; dibenzofurans, depsides, depsidones, pulvinic acid derivatives, lactones, and quinines (Yusuf 2020). According to several studies these substances have impressive biological actions including: antioxidant, antibacterial, antifungal, anti-inflammatory, and antitumor properties. (Mohammadi et al. 2020,

Solárová et al. 2020). Oxidative stress causes a variety of pathological conditions comprising cardiovascular dysfunction, drug toxicity, inflammation, and neurodegenerative (Pisoschi et al. 2021). As reported by many studied lichens phenolic compounds have a good capacity to scavenge toxic free radicals (Emsen et al. 2021), so they could be a source of natural antioxidant agents. The current study objectives were to determine the influence of extraction solvent on the total phenolic content, flavonoids content, and antioxidant activity of *Evernia prunastri* and *Ramalina lacera,* in order to contribute to valorization of those species because to the best of our knowledge there is no detailed studies about the phytochemistry, and antioxidant activity of lichens growing in Argan ecosystem.

MATERIALS AND METHODS

Solvents and reagents

2,2-diphenly-1-picryl-hydroxyl, (sigma-Aldrich), sodium carbonate (sigma-Aldrich), Gallic acid (sigma-Aldrich), Potassium hexacyanoferrate (III) (sigma-Aldrich), Rutin (rutoside), trichloroacetic acid, ascorbic acid (sigma-Aldrich), Folin-Ciocalteau reagent (sigma-Aldrich), Methanol, and acetone (sigma-Aldrich).

Lichens samples

Lichens species: *Evernia prunastri* L. Ach and *Ramalina lacera* (With) Laundon, were collected from trunks of *Argania Spinosa* L. in March in the Aghroud region, Agadir, Morocco. The samples were identified by Pr. Allal Douira from university Ibn Toufail, Faculty Sciences, Kenitera, Morocco.

Lichens extracts

The studies lichens were air-dried at ambient temperature, and after that ground into powder using household blender. The extracts were prepared by weighing separately 2.5 g of grounded thallus, placed in 100 ml Erlenmeyer conical flask and added 50 ml of organic solvents: acetone, methanol, aqueous methanol (70:30 v/v), and distilled water, then the flasks were placed in ultrasonic bath (Tan ssinic Tl-H-15) for 45 min. the ultrasonic bath was operated a frequency of 35 khz at 25°C. The all obtained extracts were filtered using Whatman paper then dried to obtain crude extract. And then the crude extracts were storage at 4 degrees until further use.

Total phenolic content

Total phenolic contents of different extracts were evaluated using the Folin-Cioculteu assay, following method of Ben ElHadj Ali et al. (2020) with some modifications: in test tubes, 50µl of lichens extracts (2 mg/ml) was added to 450µl of Folin-Cioculteu reagent (10%), and after 5min 450µl of sodium carbonate (7.5%) was added. The tubes were incubated for 2h, and then the absorbances were readied at 765 nm using UV-1800PC UV-vis spectrophotometer. Gallic acid was utilized to execute the calibration curve. the results were expressed as means± standard deviation (SD) mg of Gallic acid equivalent per g of dry weight.

Flavonoids content

With some modifications, flavonoids contents of different samples were calculated using the method described by Kocira et al. (2018), 250 µl of samples (2 mg/ml) were added to 250 µl of aluminum chloride (10%). After 1h of incubation at ambient temperature the absorbances were measured at 450 nm using UV-1800PC UV- vis spectrophotometer Rutin is used to execute the calibration curve. The results were presented as means± SD mg of Rutin equivalent per g of dry weight.

Antioxidant capacity

Dpph scavenging activity

With some modifications Siripatrawan and Harte (2010) method was utilized to evaluate the antioxidant activity of studies lichens. 500 µl of each extract was mixed with 500 µl of methanolic DPPH solution. After 30 min of incubation in the dark the absorbances were readied at 517 nm using UV-1800PC UV-vis spectrophotometer. Ascorbic acid was used as standard and the formula below was utilized to determine the inhibition percentage (%).

$$\text{DPPH inhibition percentage}(\%) = ((A_{NC}-A_s)/A_{NC}) \times 100 \quad (1)$$

where: A_{NC} – the absorbance of negative control,
A_S – the absorbance of samples.

Ferric reducing antioxidant power assay

The test was carried out using (Metrouh-Amir et al. 2015) method with some modification. In the test tubes, 250 µl of extracts, 1.250 ml of phosphate buffer (0.2M, ph=7), and 1.250 ml of potassium hexacyanoferrate solution (1%) were mixed. After incubation at water bath at 50°C for 20 min, 1.250 ml of trichloroacetic acid (10%) was added, and then the mixture was centrifuged for 10 min at 3000 rpm, after from each tube 1.250 ml of supernatant was taken and combined with 1.250 ml of distilled water and 0.25 ml of $FeCl_3$ (0.1%). Ascorbic acid was used as reference, and the absorbances was measured at 700 nm.

Statistical analysis

Statistical analyses were executed using SPSS software (version 22). The findings were expressed as means± standard deviations (SD). In order to determine the differences in average among phenolic content, flavonoids content, ant the antioxidant activity of samples the one-way ANOVA was utilized, and the differences were considered significant at $p<0.05$. Correlation among TPC, TFC, and antioxidant activity evaluated with different assay was examined by Pearson correlation test. Also, principal component analysis (PCA) was used to examine and quantify statistical correlation between the various factors: TPC, TFC, DPPH, and FRAP assays.

RESULTS AND DISCUSSION

Polyphenols content

The total polyphenols content (TPC) of acetonic, methanolic, and aqueous extracts of both species showed significant difference ($p<0.05$), according to solvent used (Table 1). Nonetheless TPC ranged from 13.17±0.5 mg AGE/g of DW, to 3.31±0.03 mg AGE/g of DW and it differs among the studies species and extraction solvent used. For *Evernia prunastri* the best content of phenolic compounds was found in acetonic extract (13.10 mg AGE/g of DW), and the lowest TPC was found in aqueous extract with 6.20±0.02 mg AGE/g of dry weight. On the other hand, for *Ramalina lacera* acetone recorded a high amount of TPC followed by methanol while the lowest amount was found in the aqueous extract with 12.10±0.2 mg AGE/g of DW, 5.22±0.2 mg AGE/g of DW, and 3.31±0.03 mg AGE/g of DW respectively.

Flavonoids content (TFC)

Flavonoids content (TFC) of different lichens studied in the present study generally ranged from 5.84±0.03 mg RE/g of DW to 0.01±0.03 mg RE/g of DW. The data in Table 2 illustrates that extraction solvent has a notable effect on TFC content. In case of *Evernia prunastri* Methanolic extract had the best TFC 5.84±0.03 mg RE/g of DW although the lowest content of 0.05±0.03 mg RE/g of DW was found in the water extract. Meanwhile, the Methanolic extract of *Ramalina lacera* give better results on the flavonoids content with 3.97±0.3 mg RE/g of DW, and the lowest TFC was found in aqueous extract (0.01±0.03 mg RE/g of DW), this could be because of the high solubility of flavonoids in methanol (Ben El Hadj Ali et al., 2014) The data revealed a significant difference ($p<0.05$) among TFC of Methanolic, acetonic, and aqueous extracts, and no significant difference ($p<0.05$) between TFC obtained by acetone, and aqueous solvent was found.

Polyphenols compounds are important secondary metabolites that had a significant role in the protection from oxidative rancidity, their antioxidant potential depends on their hydroxyl groups arrangement (Ansari et al. 2020). Results obtained agreed with the literature data, found that the amount of total phenolic compounds vary from one species to another, this could be due to

Table 1. Total phenolics content of acetone, methanolic extract, and water extracts of *Evernia prunastri* and *Ramalina lacera*

Extracts	Total phenolics content mgAGE/g DW	
	Evernia prunastri	*Ramalina lacera*
Acetone extracts	13.17^{c}±0.5	12.10^{c}±0.2
Methanol extracts	7.73^{b}±0.25	5.22^{b}±0.2
Water extracts	6.20^{a}±0.01	3.31^{a}±0.03

Note: data are means ± SD, means with different letters in the same column are statistically significant at $p<0.05$.

Table 2. Total flavonoids content of acetone, methanolic extracts, Methanol-water extracts and Water extracts of *Evernia prunastri* and *Ramalina lacera*

Extracts	Flavonoïdes content mgRE/g DW	
	Evernia prunastri	*Ramalina lacera*
Acetone extracts	0.12^{a}±0.01	0.06^{a}±0.1
Methanol extracts	5.84^{b}±0.03	3.97^{b}±0.3
Water extracts	0.05^{a}±0.03	0.01^{a}±0.03

Note: data are means ± SD, means with different letters in the same column are statistically significant at $p<0.05$.

the chemical properties of those substances and the capacity of the extraction solvent used to extract the phenolic substances. Likewise, what has been reported by other authors is that the organic solvents had a high efficiently to extract the phenolic compounds from lichens(Odabasoglu et al. 2004), in the present study we found that the aqueous extracts showed the lowest TPC this could be due to the low solubility of lichens compounds is water solvent, because the most of lichens compound are insoluble in water and have to extract by organic solvent (Gandhi et al. 2022).

The antioxidant activity

DPPH radical scavenging

The data DPPH radical scavenging capacity of different lichens samples are given in Figure 1. For *Evernia prunastri*, the highest potential to scavenging DPPH radical was obtained by methanol extract with an inhibition percentage of 61.59±0.3% followed by aqueous extract 30.14±0.7% while the lowest inhibition percentage attributed to acetone extract. Statically there is no significant difference ($p<0.05$) among inhibition percentage of acetone and aqueous extracts. In case of *Ramalina lacera* among the different extracts. The findings illustrated that there was a significant difference ($p<0.05$) according to used solvent. The methanolic showed the highest radical scavenging activity 85.59±0.2%, the lowest potential to scavenging DPPH radical was obtained by water extract 27.89±0.6%. However, the radical scavenging of ascorbic acid 97.65±0.6% was greater than all tested extracts. Comparing our results with the data on antioxidant activity of *Ramalina* lichens obtained by others researchers these values are very good, for example Gulluce et al., (2006), have documented in their study that the methanolic extract of *Ramalina pollinarea* did not have any DPPH radical scavenging activity.

Ferric reducing antioxidant power method

For FRAP assay, as shown in Figures 2 and 3, for *Evernia prunastri* among the tested extracts, the best reducing power were showed by methanolic extract followed by acetone extracts 0.4±0.02, and 0.284±0.04 respectively. In the other hand acetone extract of *Ramalina lacera* give the highest reducing power followed by methanol

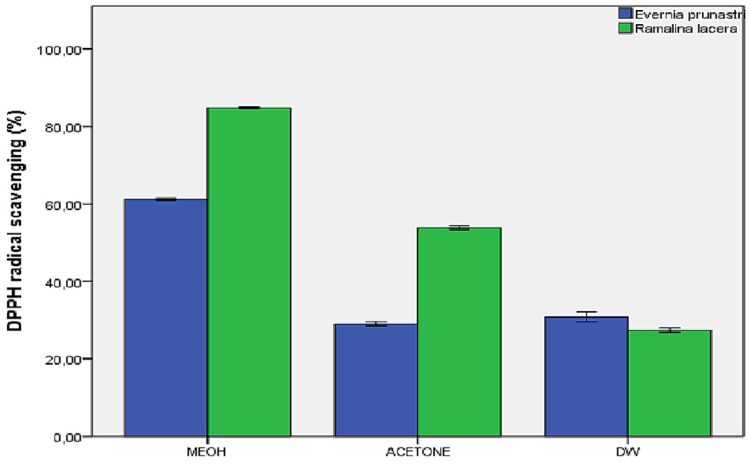

Figure 1. DPPH radical scavenging of the differents extracts of the lichens Ramalina lacerna and *Evernia prunastri*

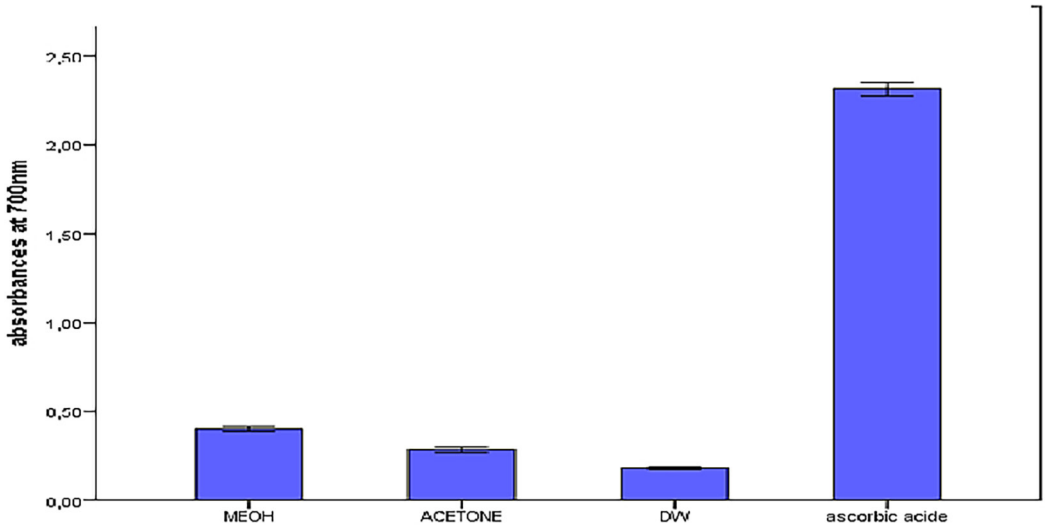

Figure 2. Reduce power of differents extracts of *Evernia Prunastri*

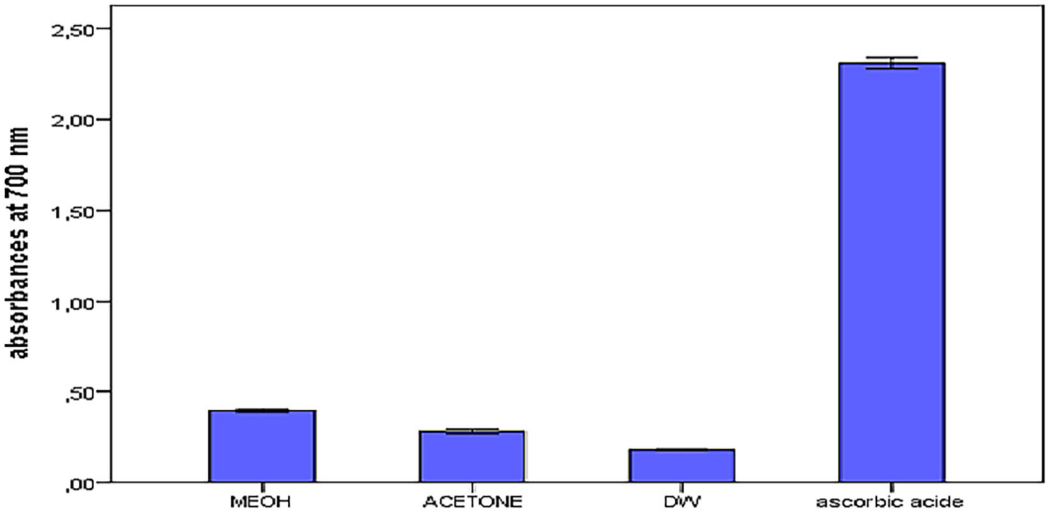

Figure 3. Reducing power of differents extracts of *Ramalina Lacera*

0.340±0.03, and 0.286±0.02 respectively. While for water extract of *Ramalina lacera* showed a very weak reducing power (0.085±0.01). However, the value recorded by the ascorbic acid still greater than all tested extracts.

When all these data obtained of this study about the antioxidant activity were taken into consideration plus the results founded in the literature, it indicated that may this antioxidant activity could be due to lichens compounds capacity of to scavenge free radicals by transferring the atom of hydrogen and an electron of the hydroxyl group present in lichens compounds structure (Fernández-Moriano et al. 2016, Nguyen et al. 2019). furthermore, we noted that the acetonic extract of *Evernia prunastri* had a good antioxidant capacity in FRAP assay but it had a moderate antioxidant activity in DPPH test, likewise the methanol extract of *Ramalina lacera* had the best inhibition percentage, but in FRAP assay had the second highest reducing power. The reason for those conflicting results between the two antioxidant assays used could be due to variation in the experimental conditions of each method. According to literature data the antioxidant activity could be different from antioxidant assay to another since the interaction of the antioxidant with other compounds plays an important role in the activity. Moreover, it should take in consideration FRAP assay reflects the presence of all reducing substances, not only polyphenolic compounds. According to Rodríguez-Roque et al., (2017), in this assay, the reducing compound (antioxidant) transfers hydrogen and electrons to oxidizing

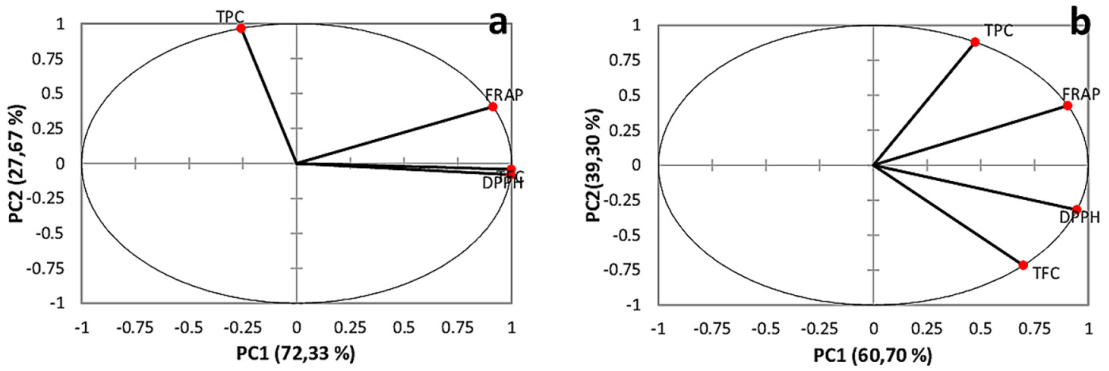

Figure 4. Principal components analysis: (a) principal components analysis of *Evernia Prunastri,* (b) principal components analysis of *Ramalina Lacera*

Table 3. Pearson's correlation coefficient

Lichens	Variables	TPC	TFC	DPPH	FRAP
Evernia prunastri	TPC	1	-0.298	-0.334	0.157
	TFC	-0.298	1	0.999	0.896
	DPPH	-0.334	0.999	1	0.878
	FRAP	0.157	0.896	0.878	1
Ramalina lacera	TPC	1	-0.300	0.167	0.804
	TFC	-0.300	1	0.890	0.326
	DPPH	0.167	0.890	1	0.721
	FRAP	0.804	0.326	0.721	1

complex this transfer is dependent on the redox capacity of FRAP, the antioxidant compound structure, and pH of the medium.

Correlation between antioxidant capacity, TPC, and TFC

Pearson linear coefficient were calculated to evaluate the impact of TPC, and TFC on the antioxidant ability of studies lichens, and data are presented in Table 3. For *Evernia prunastri* a very strong significant correlation (r=0.999; p<0.01) was found among flavonoids and antioxidant activity tested by DPPH, also a strong significant positive correlation (r = 0.896; p<0.01) among antioxidant activity tested by FRAP assay and flavonoids was found. in the other hand, a feeble negative correlation(r = -0.334; p<0.05) was found between total phenolic compound and antioxidant activity tested by DPPH assay, and feeble positive correlation (r = 0.157; p<0.05) among TPC and antioxidant activity tested by FRAP assay, and a high significant correlation (r = 0.878; p<0.01) among DPPH and FRAP was found Those correlation indicated that the flavonoids are the mainly contributing to antioxidant activity of *Evernia prunastri*, while non-flavonoids compounds may not contribute to antioxidant activity.

In the case of *Ramalina lacera* a positive and significant correlation was established among TPC and antioxidant activity tested by FRAP assay (r=0.804; p<0.01), in the other hand a very weak correlation between TPC and DPPH was recorded. The TFC was significantly correlated with antioxidant activity tested by DPPH (r = 0.890; p<0.01), however the correlation of TFC and antioxidant activity tested by FRAP was very feeble (r = 0.326; p<0.05). Several researches found a strong correlation among antioxidant activity and lichens compounds. Ranković et al. (2012), found a fort correlation between the polyphenols and antioxidant activity of *Toninia Candida* and *Usnea Burbuta*. However, some funding documented that the antioxidant activity of some lichens could due to their non-phenol substances (Odabasoglu et al., 2004b).

Principal components analysis

In the current study the principal components analysis (PCA) showed that PC1 and PC2, in total

correspond to approximately to 99% of the variation with contribution of 72.33% and 27.67% respectively (Figure 4a). The variable most contributed were TPC, TFC, and DPPH contributing 84.354, 34.504, 34.343 respectively. Variable FRAP, TFC, and DPPH were positively correlated to the PC1 axis. TPC was positively correlated PC2 whereas TFC negatively correlated to it. Variable DPPH, FRAP, and TFC showed very strong correlation. In case of *Ramalina lacera* (Figure 4b), PC1 captures 60.70% of the variance, while principal component 2 (PC2) 39.30% of the total variance, TPC is most variable contributing with 49.32% followed by DPPH 36.976%. TPC and FRAP were positively correlated to PC2 while DPPH and TFC negatively correlated to it. DPPH and TFC showed a high correlation.

CONCLUSION

Our obtained data confirmed that the lichens extracts could be a source of antioxidant compounds. In conclusion we can be started that the extraction solvent has enormous influence on the polyphenols content and the antioxidant capacity, in our study we found out that methanol is the most efficient solvent to extract total phenolic from the studies lichens and his extracts have a potential antioxidant activity, moreover our results demonstrate that ultrasound assistance extraction is an efficient method to extract lichens substances, although further study is need on the isolation and identification of those compounds.

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