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ORIGINAL PAPER

Phytochemical constituents, antioxidant and antistaphylococcal activities of *Evernia prunastri* (L.) Ach., *Pseudevernia furfuracea* (L.) Zopf. and *Ramalina farinacea* (L.) Ach. from Morocco

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Abstract

The purpose of this work was to assess chemical composition, antibacterial activity against *Staphylococcus aureus* isolates from catheter-associated infections and antioxidant activity of methanol extracts of three lichens collected from Morocco. The phytochemical analysis of the methanol extracts of these lichens was performed by HPLC–UV method, the predominant phenolic compounds were evernic acid, physodalic acid and usnic acid for *Evernia prunastri, Pseudevernia furfuracea* and *Ramalina farinacea,* respectively. Total phenolic compounds and total flavonoid content of all extracts were also determined. As a result, *Pseudevernia furfuracea* extract had the strongest effect and the highest phenolic compounds content. All extracts showed antibacterial activity against all tested strains (MIC values ranging from 0.078 to 0.625 mg/mL), the strongest inhibition was obtained with the extract of *Evernia prunastri.*

Keywords Antioxidant activity · Antibacterial activity · *Evernia prunastri* · *Pseudevernia furfuracea* · *Ramalina farinacea* · *Staphylococcus aureus*

Introduction

Nosocomial infections are one of the most severe problems in medicine and are the fifth leading cause of death in the world (Sydnor and Perl 2011). Most of these infections in hospitals are linked to the use of urinary and intravascular catheters or mechanical ventilators. These infections are caused by many strains belonging to the hospital flora predominantly with strains of methicillin-resistant *Staphylococcus aureus* (MRSA) (Vincent et al. 1995; Brun-Buisson 2005; Sydnor and Perl 2011).

Pathogenic bacteria represent a growing threat to patient health and the increase of antibiotic-resistance make patient care practices more and more complex and pose significant challenge to researcher community (Frieri et al. 2017). Hence, there is an urgent need for new potent antibacterial agents with a new mode of action, among them bioactive phytochemicals, such as phenolic compounds, present a great interest and could find use in alternative and/or complementary therapy for staphylococcal infections.

In addition, the harmful effects of oxidative stress caused by the overproduction of reactive oxygen species (ROS) in human organism, expressed by the emergence of various chronic diseases such as cancer, cardiovascular, neurodegenerative disease and inflammation, have become a serious problem to public health (Mena et al. 2009). For this reason, it is necessary to restore the oxidant/antioxidant balance to preserve the physiological performance of the body through the use of natural antioxidant compounds such as phenolic phytochemicals.

Lichens, symbiotic organisms between a photobiont partner, a mycobiont partner (Elix and Stocker-Wörgötter

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2012; Chollet-Krugler et al. 2013) and bacteria communities (Selbmann et al. 2010; Bates et al. 2011) have been used for medical purposes since centuries to treat renal, respiratory and hepatic infections, and also as an antiseptic (Martínez-lirola et al. 1995; Bown 2001; Podterob 2008). The use of lichens in traditional medicine has been justified by the fact that they contain unique and varied bioactive molecules which are produced by fungal partner, or by the bacterial community and accumulated as crystals on the upper surface of hyphae (Cardinale et al. 2006; Dharmadhikari et al. 2010; Komaty et al. 2016). Indeed, about 1050 lichen secondary metabolites have been discovered (Dharmadhikari et al. 2010), including usnic acid, depsidones, and depsides which shown having an important pharmacological interest (Dharmadhikari et al. 2010; Komaty et al. 2016; Solárová et al. 2020).

This work represent a continuous investigation on lichens (Aoussar et al. 2017) while aiming to evaluate the chemical constituents and the in vitro antioxidant activity and for the first time, the antibacterial properties of the methanol extracts of three lichens from Morocco: *Evernia prunastri*, *Pseudevernia furfuracea* and *Ramalina farinacea* against *Staphylococcus aureus* strains isolates from catheter-associated infections.

Materials and methods

Lichen collection and identification

Lichens were harvested in the province of Khenifra (Middle Atlas, Morocco). Identification of the lichen species was achieved by Pr. Allal Douira from University Ibn Tofail, Faculty of Sciences, Kenitra, Morocco, using the relevant key and monographs (Van Haluwyn et al. 2009).

Preparation of the lichen extracts

Lichen extraction was achieved as described by our previous study (Aoussar et al. 2017), the dried powdered lichen materials were placed in an erlenmeyer flasks soaked in the methanol for 24 h at ambient temperature and under continuous shaking. Finally, the extracts were filtered and then concentrated using the rotary evaporator (Heidolph G1, Germany) at 45 °C. The extraction yields were calculated for each species.

High Performance Liquid Chromatography (HPLC) analysis

HPLC analytical method and standard compounds preparation were carried out as described by Huneck and Yoshimura (1996). The Phytochemical analysis of the methanol extracts of lichens was determined by HPLC (Agilant Technologies, 1200 Series) with Column C18 (250 mm × 4.6 mm, 10 µm) with the mobile phase (solvent) of methanol/water/phosphoric acid (90/10/0.9:v/v/v) at flow rate of 1.0 mL/min in the presence of a detector of UV spectrophotometric (254 nm). The reference compounds used were isolated from the various lichens species: methyl lecanorate (MLE) from *Pseudocyphellaria crocata*, physodalic acid (PHY), atranorin (ATR) and chloratranorin (CHR) from *Hypogymnia physodes*, usnic acid (USN) from *Usnea barbata* and evernic acid (EVE) from *Ramalina fraxinea* and were analyzed in the same chromatographic conditions. The constituents of the methanol extracts were determined by comparison of the retention times (t_R) and absorption spectra (200–400 nm) of the corresponding peaks between chromatograms of test and reference solutions.

Antioxidant activity

The antioxidant ability of the lichen extracts was evaluated by the determination of DPPH radical scavenging activity and by the evaluation of ferric reducing power according to the method described by Kosanić et al. (2014), and by Oyaizu (1986), respectively. Ascorbic acid and Trolox were used as standards.

The total phenolic content (TPC) in tested extracts was estimated as gallic acid equivalents using the Folin-Ciocalteu method (Slinkard and Singleton 1977). The total flavonoid content (TFC) was quantified as µg of (QE)/mg of dry extract using the method described by Zilic et al. (2011).

Antibacterial activity

Eight *S. aureus* strains were selected for antibacterial activity including one reference strain (ATCC 25923) and seven clinical isolates with three Methicillin Resistant *Staphylococcus aureus* (MRSA) obtained from patients with catheter related infections treated at the IbnRochd University Hospital of Casablanca, Morocco. All strains were identified as *S. aureus* and biofilm producers as described by Achmit et al. (2016).

The minimum inhibitory concentration (MIC) values were determined in 96-well microplates using the microdilution assay according to Satyajit et al. (2007) with slight modifications. All the wells of microplates were filled with 100 µL of Mueller–Hinton broth, the first row of microtiter plate was filled with 100 µL of extracts in dimethylsulfoxide (DMSO) then two fold dilutions were achieved to obtain serially descending concentrations from 5 to 0.002 mg/mL. The inoculum (5×10^6 CFU/mL) was added to all wells and resazurin solution (0.015%) was used as an indicator of microbial growth. DMSO solution was used as negative control and the plates were incubated at 37 °C for 24 h. The

MIC corresponds to the lowest concentration of the tested extracts without color change of the resazurin (McNicholl et al. 2006).

After determining the MIC, streak subculturing of the wells without change of the resazurin color was performed on nutrient agar in Petri dishes. The minimum bactericidal concentration (MBC) was the lowest concentration that didn't show bacterial growth after incubated for 24 h at 37 °C.

Statistical analysis

All results were expressed as mean ± standard deviation of three parallel measurments. The statistical analysis was performed using SPSS software 22.

Results and discussion

Obtained methanol extracts of *E. prunastri, P. furfuracea* and *R. farinacea* with yields of 9%, 14% and 8%, respectively, were analyzed qualitatively by HPLC–UV. For the identification of their major lichenic acids we have compared their retention times (t_R) with the standard substances previously isolated from lichens (Fig. 1 and Table 1).

Obtained data confirmed that the main identified compounds in the methanol extract of *P. furfuracea* were physodalic acid ($t_R = 3.50 \pm 0.10$ min), atranorin ($t_R = 9.00 \pm 0.10$ min) and chloratranorin ($t_R = 12.04 \pm 0.10$ min).Their amounts in this extract were presented in the following order: PHY > ATR > CHL. In the methanol extract of *E. prunastri*, methyl lecanorate ($t_R = 1.53 \pm 0.10$ min), evernic acid ($t_R = 3.08 \pm 0.20$ min), usnic acid ($t_R = 7.64 \pm 0.10$ min), atranorin ($t_R = 9.04 \pm 0.10$ min) and chloroatranorin ($t_R = 12.09 \pm 0.10$ min) were identified with the evernic acid as the most abundant compound. While, in the extract of *R. farinacea,* usnic acid ($t_R = 7.63 \pm 0.10$ min) was the major compound, followed by evernic acid ($t_R = 3.12 \pm 0.10$ min) (Fig. 2).

Antioxidant activity of the methanol extracts of the studied lichens has been determined by the DPPH assay and data are presented in Fig. 3. The results showed that all studied extracts exhibited relatively strong DPPH

Table 1 Chemical composition of the methanol extracts of the tested lichen species (as percentages of the total absorbance of the HPLC chromatograms recorded on 254 nm)

Compound	t_R values (min)	Relative abundance (%)		
		P. furfuracea (P.F)	*E. prunastri* (E.P)	*R. farinacea* (R.F)
Methyl lecanorat	1.53	/	7.14	/
Evernic acid	3.08	/	71.43	8.57
Physodalic acid	3.49	17.01	/	/
Usnic acid	7.63	/	1.43	26.38
Atranorin	8.95	4.92	17.84	/
Chloroatranorin	11.99	0.91	2.14	/

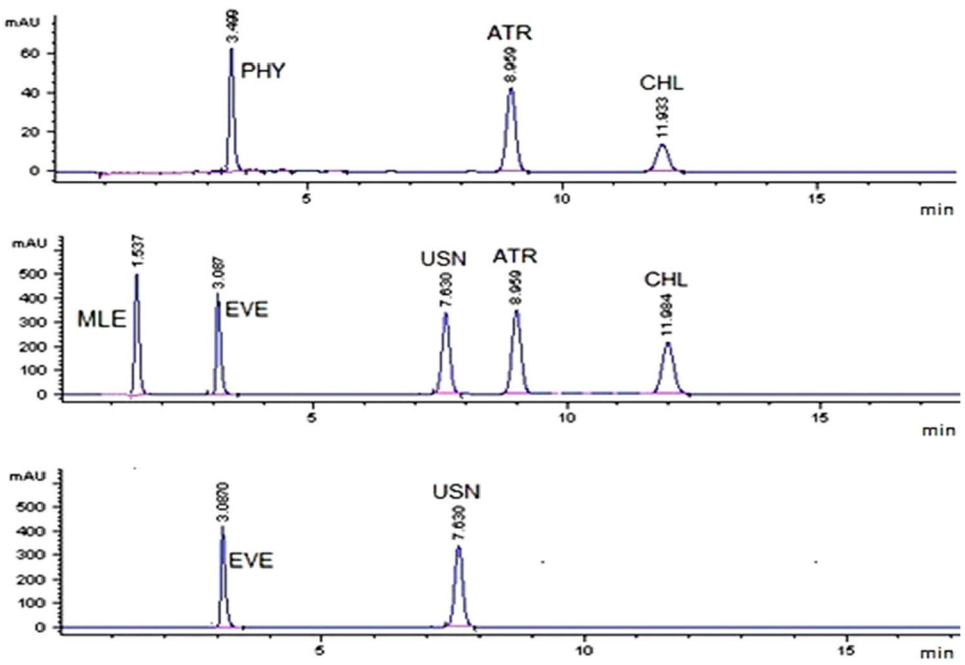

Fig. 1 HPLC–UV chromatograms at 254 nm for selected standards, Peaks: *MLE* Methyl lecanorate, *EVE* Evernic acid, *PHY* Physodalic acid, *USN* Usnic acid, *ATR* Atranorin, *CHL* Chloratranorin

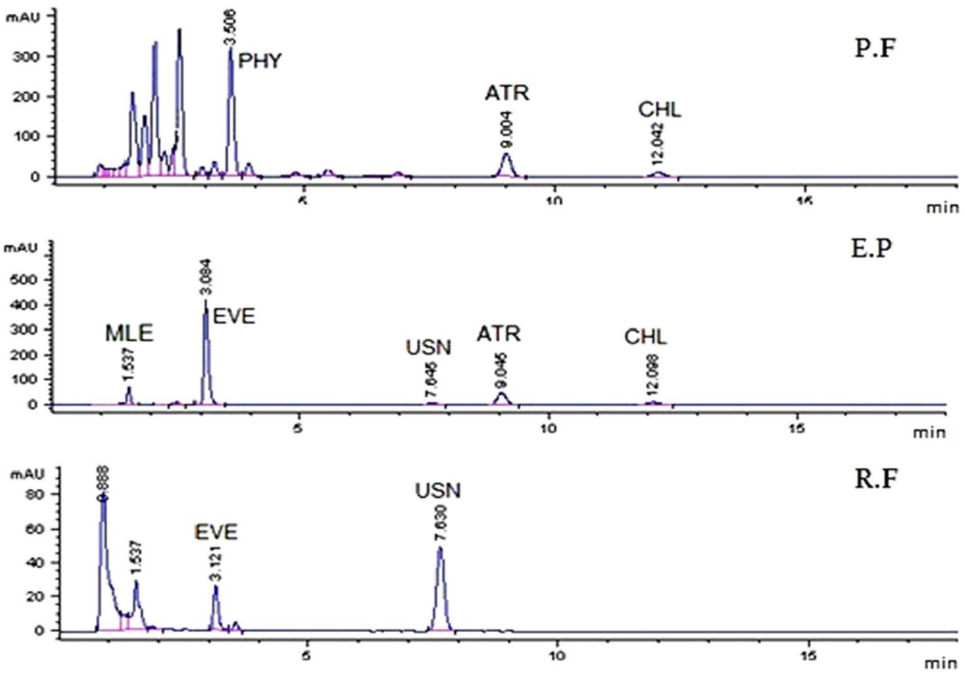

Fig. 2 HPLC–UV chromatograms at 254 nm of the methanol extracts of *E. prunastri* (E.P), *P. furfuracea* (P.F) and *R. farinacea* (R.F). *MLE* Methyl lecanorate, *EVE* Evernic acid, *PHY* Physodalic acid, *USN* Usnic acid, *ATR* Atranorin, *CHL* Chloratranorin

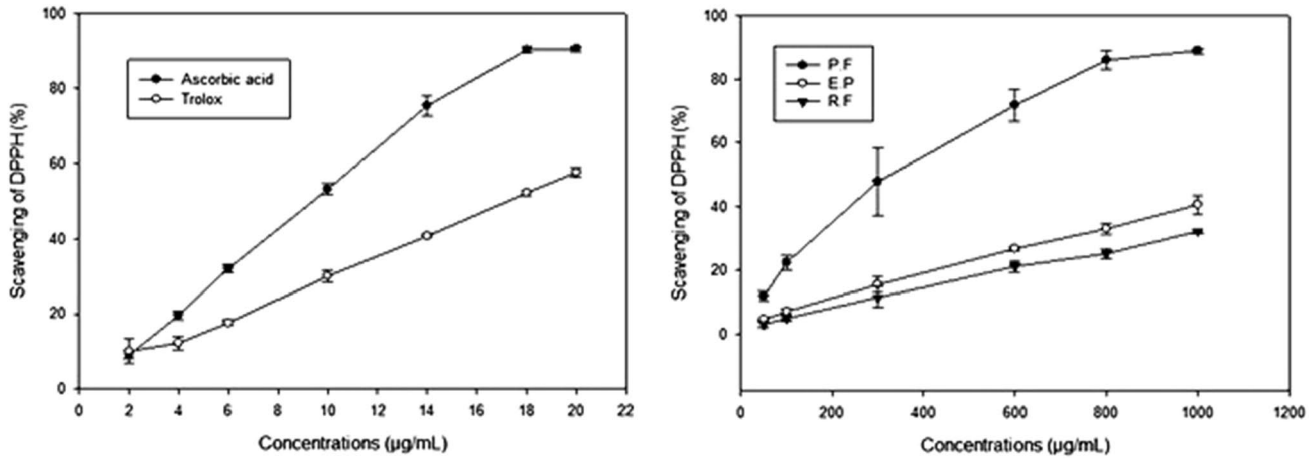

Fig. 3 DPPH radical scavenging capacity of standards (Ascorbic acid and Trolox) and the methanol extracts of *E. prunastri* (E.P), *P. furfuracea* (P.F) and *R. farinacea* (R.F)

scavenging ability with a significant difference between lichen extracts ($p < 0.01$) as it was supported by the Duncan test and also between lichen extracts and positive controls ($p < 0.001$). The methanol extract of *P. furfuracea* showed the highest antioxidant effect ($IC_{50} = 403.146 \pm 25.736$ µg/mL). The DPPH scavenging capacity obtained for the assessed methanol extracts still, however, lower than those obtained for ascorbic acid ($IC_{50} = 8.872 \pm 0.064$) and Trolox ($IC_{50} = 17.834 \pm 0.497$ µg/mL).

The results of the ferric reducing power of the studied extracts are represented in Table 2. The data show that the reducing capacity was dose dependent and increased with increasing concentration of extracts. The absorbance varied

Table 2 Reducing power of methanol extracts of *E. prunastri* (E.P), *P. furfuracea* (P.F) and *R. farinacea* (R.F)

Extract concentrations (µg/mL)	Lichen species		
	P.F	E.P	R.F
50	0.011 ± 0.001	0.002 ± 0.001	0.008 ± 0.009
100	0.028 ± 0.007	0.004 ± 0.003	0.009 ± 0.005
300	0.071 ± 0.011	0.018 ± 0.009	0.033 ± 0.015
600	0.195 ± 0.039	0.049 ± 0.011	0.087 ± 0.028
800	0.244 ± 0.043	0.097 ± 0.005	0.118 ± 0.025
1000	0.335 ± 0.051	0.098 ± 0.013	0.128 ± 0.022

from 0.002 ± 0.001 to 0.098 ± 0.013, from 0.011 ± 0.001 to 0.335 ± 0.051 and from 0.008 ± 0.009 to 0.128 ± 0.022 for E.P, P.F and R.F extracts, respectively, with a statically significant difference was observed between P.F and E.P—R.F ($p < 0.001$), but not between E.P and R.F ($p > 0.05$). *P. furfuracea* extract showed the greatest ferric reducing power. However, the absorbance of standards ranged from 0.270 ± 0.015 to 2.674 ± 0.035 and from 0.102 ± 0.036 to 1.607 ± 0.160 for ascorbic acid and Trolox, respectively.

The total phenolic compounds and flavonoids contents in *E. prunastri, P. furfuracea* and *R. farinacea* extracts were determined as GAE using a standard gallic acid curve ($R^2 = 0.990$) for TPC and for TFC, as QE using an equation obtained from calibration curve established with quercetin ($R^2 = 0.985$). Obtained results given in Fig. 4 show that TPC and TFC in the investigated extracts ranged from 74.166 ± 8.311 to 165.333 ± 6.806 µg GAE/mg of dry extract and from 17.03 ± 1.890 to 19.333 ± 3.752 µg QE/mg of dry extract, respectively. The highest level of TPC was observed for P.F extract which was significantly different from E.P and R.F ($p < 0.01$). For TFC there is no significant difference between the three lichen extracts ($p > 0.05$).

To understand the interrelationship between antioxidant activity and phytochemical contents of the studied methanol extracts of lichens, Pearson test was performed and showed no significant correlation between antioxidant activity and TFC (DPPH, $r = -0.335$; FRAP, $r = -0.407$). Whereas, a significant positive correlation ($p < 0.01$) was found between the TPC and antioxidant activity (DPPH, $r = 0.859$; FRAP, $r = 0.876$). These results indicated that lichen components (depsides, depsidones and dibenzofurans) in the studied extracts are probably responsible for the antioxidant activity, due to their ability to scavenge free radicals through the transfer of the hydrogen atom and an electron of hydroxyl group present in their structure, which are in agreement with other reported works (Kosanić et al. 2013; Manojlović et al. 2012).

Our results demonstrate that the methanol extract of *P. furfuracea* showed the highest DPPH radical scavenging ability ($IC_{50} = 403.146 \pm 25.736$ µg/mL) and a greatest reducing power with a significant correlation between strong antioxidant capacity and a high amount of phenols. These results are in concordance with other studies showing that the extracts of *P. furfuracea* have a largest antioxidant activity (Kosanić et al. 2013; Mitrović et al. 2014; Aoussar et al. 2020; Hawrył et al. 2020).

The results of antibacterial effect revealed that all methanol extracts exhibited a higher antibacterial activity against the investigated *S. aureus* isolates. The determined MIC and MBC values against the tested bacteria with the type of bacterial effect are summarized in Table 3.

As it is evidenced in Table 3, all tested extracts were found to be active against all strains of *S. aureus* isolates from catheter-associated infections which including three MRSA. The MIC values varied depending to the species of lichens and ranged from 0.07 to 1.25 mg/mL, from 0.07 to 0.15 mg/mL and from 0.07 to 0.31 mg/mL for P.F, E.P and R.F, respectively. Moreover, the MIC values have not much changed for the extracts of R.F and E.P against all strains and the largest antibacterial effect was registered for *E. prunastri* extract.

The MBC determined for all extracts varied from 0.62 to 5 mg/mL.

Although the antibacterial activity of lichens and their compounds was evaluated against MRSA isolates (Lautwerwein et al. 1995; Shrestha and Clair 2013), to the best of our knowledge, no data was published concerning

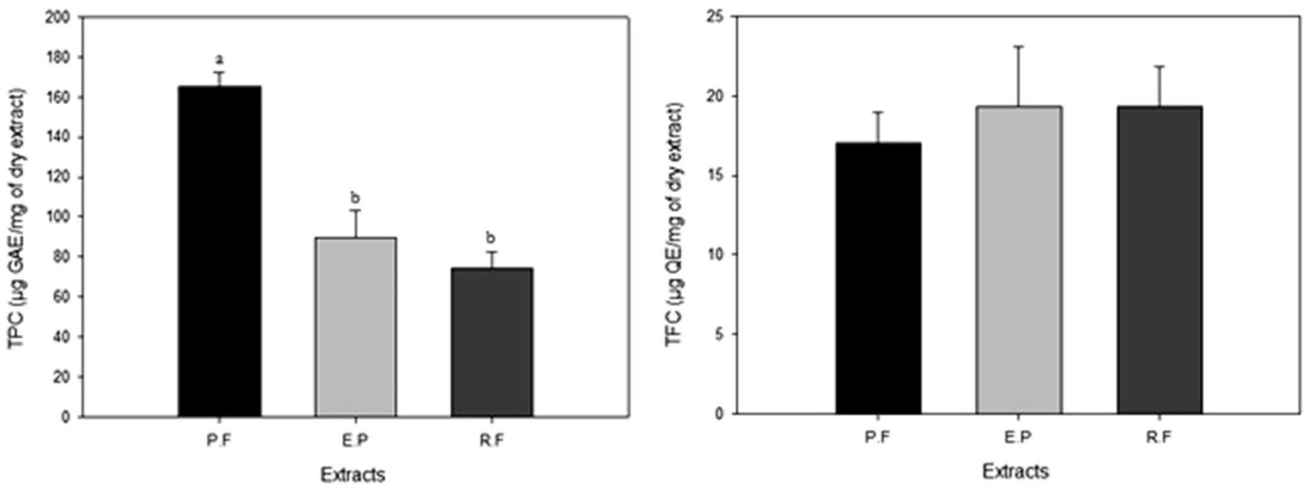

Fig. 4 Total phenolic components (left) and flavonoids contents (right) of tested lichen extracts. Values are reported to mean ($n = 3$) ± Standard Error; Values don't have the same letter differ statically at $p < 0.01$

Table 3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of methanol extracts of *P. furfuracea* (P.F), *E. prunastri* (E.P) and *R. farinacea* (R.F) against ATCC and multi-drug resistant strains of *S. aureus* clinical isolates

S. aureus strains	Extracts	MIC (mg/mL)	MBC (mg/mL)	MBC/MIC	Effect
ATCC 25923	P.F	0.078	0.625	8.013	Bacteriostatic
	E.P	0.078	0.625	8.013	Bacteriostatic
	R.F	0.310	2.500	8.065	Bacteriostatic
S. aureus N°1	P.F	0.625	0.625	1.000	Bactericidal
	E.P	0.150	0.310	2.067	Bactericidal
	R.F	0.310	0.625	2.016	Bactericidal
MRSA N°1	P.F	0.625	5	8.000	Bacteriostatic
	E.P	0.150	5	33.333	Tolerant bacteria
	R.F	0.310	5	16.129	Bacteriostatic
S. aureus N°2	P.F	0.310	0.625	2.016	Bactericidal
	E.P	0.078	0.310	3.974	Bactericidal
	R.F	0.078	0.150	1.923	Bactericidal
S. aureus N°3	P.F	0.150	0.62	4.133	Bactericidal
	E.P	0.150	0.62	4.133	Bactericidal
	R.F	0.310	0.62	2.000	Bactericidal
MRSA N°2	P.F	0.625	5	8.000	Bacteriostatic
	E.P	0.150	0.625	4.167	Bactericidal
	R.F	0.310	1.250	4.032	Bactericidal
MRSA N°3	P.F	0.625	2.500	4.000	Bactericidal
	E.P	0.150	1.250	8.333	Bacteriostatic
	R.F	0.310	1.250	4.032	Bactericidal
S. aureus N°4	P.F	0.310	1.250	4.032	Bactericidal
	E.P	0.078	0.625	8.013	Bacteriostatic
	R.F	0.310	0.625	2.016	Bactericidal

antibacterial activity of lichen against *S.aureus* and MRSA isolated from catheter-associated infections.

According to our results, the MIC values of the majority of tested extracts were close to their MBC values, indicating that these extracts had a bactericidal effect on most of the tested bacteria at low concentrations, especially against MRSA isolates, which are becoming increasingly difficult to combat in hospitals because of emerging resistance to all current antibiotic classes by their ability to grow readily in biofilms on the surface of catheters (Haraga et al. 2017). Several studies have pointed out that the purified compounds from lichens species are more active than their crude extracts (Tay et al. 2004; Gullucea et al. 2006; Kosanić et al. 2013), suggesting that phenolic compounds are the main agents responsible for this activity. In an interesting study, Oh et al. (2018) found that the antibacterial activity against MRSA (3A048) of divaricatic acid isolated from *Evernia mesomorpha* and of vancomycin were almost the same, with MICs equal to 32 and 25 µg/mL, respectively. In addition, the antibacterial activity of divaricatic acid was reported to be higher than that of vancomycin against *Staphylococcus epidermidis*. Reported literature data indicated that usnic acid isolated from *Usnea subfloridana* presented a high antibacterial activity against eight clinical isolates of MRSA by disruption of the bacterial membrane (Gupta et al. 2012). While, Maciąg-Dorszyńska et al. (2014) demonstrated that usnic acid causes rapid and strong inhibition of RNA and DNA synthesis in *Staphylococcus aureus* strains.

Although the antibacterial activity of lichens, either as raw extracts or as purified molecules, has been widely studied, the antibacterial mechanism of these compounds has not been enough evaluated (Es-sadeqy et al. 2020).

Conclusions

The results of this research provide evidence that the methanol extracts of *E. prunastri, P. furfuracea* and *R. farinacea* exhibited potent antioxidant activity and can serve as a potential therapeutic option in damage caused by oxidative stress. The study also demonstrates that these lichens had a strong antibacterial activity against clinical strains of *S. aureus* collected from the catheter-associated infections.

To our knowledge, there are no studies that investigated the capacity of lichen extracts to effective inhibit pathogenic microorganisms isolated from catheter-associated infections. This work suggests that these studied lichen extracts might be a natural source of efficient antibiotics used in hospital settings to treat infections caused by multi-drug resistant bacteria. However, further deeping researches are necessary

to isolate the active compounds from lichen extracts and determine their biological activities in vivo and their toxical effects.

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Declarations

Conflicts of interest All contributing authors have not declared any conflicts of interest.

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