



Chemical composition, antioxidant and antibacterial activities of extracts obtained from the roots bark of *Arbutus andrachne* L. a Lebanese tree.

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Abstract

Context and purpose of the study: The leaves, fruits, barks and roots of *Arbutus andrachne* L. (*A. andrachne*), have been adopted to have high therapeutic value resulting from the presence of antioxidant compounds such as flavonoids, phenolic and tannins. In the present work, three extracts obtained from *A. andrachne* roots bark were evaluated for their antioxidant and antibacterial activities. The total phenolic content, flavonoid, condensed tannins and anthocyanins were determined in order to correlate them with the antioxidant activity of extracts.

Main findings: The highest amounts of phenolic and tannins were found in the ethyl-acetate, while the anthocyanins ones were highly observed in the methanol-water extract. The lowest IC₅₀ values for DPPH (0.6 µg/mL), and metal chelating assay (13.45µg/mL) were recorded in the ethyl-acetate extract and the methanolic one respectively. Gram positive bacteria (*S. aureus* and *E. faecalis*) were more susceptible to the antimicrobial potential of the methanol extract, while *E. coli* and *P. aeruginosa* as Gram negative bacteria turned out to be more resistant to the same extract. The ethyl-acetate extract was more effective on *E. faecalis* than on *S. aureus*; while *E. coli* and *P. aeruginosa* were the most resistant to this extract.

Brief summary and potential implications: An appropriate dose of antioxidants derived from *A. andrachne* bark of the roots extracts in the human diet can help to avoid the risk of contracting diseases where ROS are involved in the pathogenesis. In fact, phenolic compounds in these extracts are among the natural antioxidants being studied by the scientific community due to their biological properties, e.g., antioxidant and antimicrobial activities.

Keywords: *Arbutus andrachne* L., Ericaceae, roots bark extracts, antioxidant activity, antibacterial activity

Introduction

Nowadays, growing literature has emerged regarding reactive oxygen species (ROS) and free radicals [1]. Several studies have discussed the deleterious consequences of ROS and their implications in diseases [2]. Fortunately, living systems can protect themselves against the harmful effects of ROS and free radicals via natural defense mechanisms such as enzymes and antioxidants [3]. The antioxidant beneficial effect can be explored in food nutrition by using plants as a source of antioxidants [4]. Plants contain many natural antioxidant compounds such as carotenoids,

vitamins, phenols, flavonoids, and endogenous metabolites which have been identified as free radicals or active oxygen scavengers [5]. Phenolic compounds are among the natural antioxidants being intensively studied by the scientific community due to their antioxidant and antimicrobial activities [6].

Flavonoids frequent components of the human diet, and exhibit pharmacological effects that are linked to their known biological functions in cancer and cardiovascular diseases as well as their role as antioxidants and anti-inflammatory components [7]. In the Mediterranean region, the Ericaceae family has two evergreen *Arbutus* L., species with edible fruits: *A. andrachne*, and *Arbutus unedo* L. (*A. unedo*). *A. andrachne* also called the strawberry tree,



is well known for its biological properties and has traditionally been used as an alternative medicine, [8, 9]. The fruit of this tree has antiseptic, diuretic and laxative effects, where the leaves have astringent, urinary tract antiseptic, anti-diarrheal and depurative properties [10]. This effect is likely mediated by its antioxidant activity. Previously, the antioxidant activity and the total phenolic content was determined only for the fruit part of *A. andrachne* [11]. Given the traditional use of *A. andrachne* and the poorly characterized phytochemical contents of this tree, it is of great importance to investigate the biological activities of different extracts obtained from other parts of the plant. Interestingly, the elevated levels of phenolic compounds in the *A. andrachne* fruits suggests that an antimicrobial effects could also be displayed. In this study we at: i) determining the phenolics, flavonoids, total tannins and total anthocyanins in extracts obtained from the bark of the roots of *A. andrachne*; ii) evaluating the antioxidant and antibacterial activities of the previously mentioned constituents and iii) comparing the obtained results with those previously described for *A. unedo*.

Material and methods

Chemicals

All chemicals used were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant materials

Fresh bark(s) from the roots of the Lebanese *A. andrachne* were collected in September 2013 in the region of Deir Al Kamar (at about 850m in the Mount Lebanon region). Voucher specimens were deposited in the herbarium of the Faculty of Pharmacy (voucher n°. EADR13Aar).

Preparation of the extracts

The barks from the roots were shade dried and powdered using an electric blender. The obtained mass as well as the extraction yield for the different extracts are shown in Table 1.

Methanolic extract (E1)

82 g of powdered barks of roots were placed into the extractor of a Soxhlet. The extraction was carried out by using methanol. At the end of the extraction the methanol extract was concentrated by evaporation, frozen and then lyophilized.

Total Oligomeric flavanols (TOF) extract (E2)

The TOF extract was prepared by maceration of 100 g of ground root bark in 500 ml of an acetone-water mixture (2/3) (v/v). The product of the maceration was then filtered and cleared of acetone by evaporation under reduced pressure until an aqueous solution was obtained and saturated by NaCl (400g/l) upon being agitated for overnight. The obtained precipitate was eliminated by filtration on sintered glass which allows passage of a crystal clear liquid containing TOF. The following experimental step consists of the extraction of TOF by using ethyl acetate in which the TOF are soluble and can be easily extracted. The various ethyl acetate extracts were combined, dehydrated by agitation with the anhydrous sodium sulfate, and then filtered, concentrated under reduced pressure to a final volume of approximately 20 ml. For the TOF precipitation, 5 volumes of chloroform were added to the concentrated extract under agitation. A fluffy precipitate was formed followed by filtration on a sintered glass and kept to dry under the hood.

Hydromethanolic extract (E3)

The extract (E3) was obtained from a liquid-liquid extraction of (E1) Extract: For this purpose, 82 g of root bark were extracted similarly to (E1). After evaporation, the methanolic extract was subjected to a liquid-liquid extraction by adding an equal volume of distilled water. Immediate separation of a precipitate was observed, and then the (E3) extract was filtered, concentrated and dried in vacuum.

Determination of total phenols, flavonoids, condensed tannins and anthocyanins

Total phenols, flavonoids, condensed tannins and anthocyanins quantifications in the different extracts were determined according to Singleton and Rossi [12], colorimetric assay of Kim et al [13], the method of Sun et al [14] and a modified pH differential method previously described [15] respectively. All experiments were carried out in triplicate and gallic acid, quercetin, catechin and anthocyanidins respective equivalent values were reported as $X \pm$ SD of triplicates.

Antioxidant activity

Ferric Reducing/Antioxidant Power (FRAP) assay

The reducing power was determined according to the experimental procedure described by Berker et al [16]. The absorbance was calculated at 700 nm and compared with ascorbic acid as positive control (higher absorbance readings indicate higher reducing power). The data are reported as the average of three measurements given as \pm SEM.



Diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity

The DPPH is a stable free radical characterized by its maximum absorption at 517 nm. A control was prepared for each sample by using methanol instead of the DPPH solution and Butylated hydroxytoluene (BHT) was used as compound reference [17]. Antioxidant activity was expressed as a percent inhibition of DPPH radical. The reduction of the DPPH radical was measured by continuously monitoring the decrease of absorption at 517 nm against a blank containing the same concentration of extract without DPPH radicals. Inhibitions of free radicals formation in percent (1%) were calculated as follow:

$$1\% \text{ scavenging effect} = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$$

Where A_{blank} is the absorbance of the control reaction which contains all reagents except the solution of the extract sample. A_{sample} is the absorbance value of the extract sample. IC50 values were determined from the graphs of scavenging activity against the concentration of the extracts. These values are defined as inhibitory concentration of the extract necessary to decrease the initial DPPH radical concentration by 50% and are expressed in $\mu\text{g/ml}$. Triplicate measurements were carried out and data presented are given as \pm SEM for three replications.

Iron chelating effect

The ferrous ion-chelating potential was evaluated following the method described by Decker and Welch [18]. EDTA was used as reference and three replicates were made for each test sample. Chelating activity (%) was then calculated as follows:

$$\text{Chelating activity (\%)} = (1 - A_{562} \text{ of sample}/A_{562} \text{ of control}) \times 100$$

Antibacterial assay

The bark roots extracts of *A. andrachne* were individually evaluated for their antibacterial activity against four types of microorganisms: *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC27950) *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 2921). Microbial strains were provided by the department of medical resuscitation of Fattouma Bourguiba University Hospital of Monastir (Tunisia). Bacterial stock cultures were maintained at 4°C on LB agar [tryptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 1% (w/v) and agar 2% (w/v)], being sub-cultured periodically at 37°C. Bacterial inoculum (10^6 - 10^7 CFU/ml) was prepared in nutrient broth (NB) (Himedia). The bacteria were incubated at $37 \pm 2^\circ\text{C}$ for 48 hours.

Determination of minimum inhibitory concentration (MIC)

MIC value of the bark roots extracts from *A. andrachne* was evaluated by broth microdilution method [19, 20]. MIC was interpreted as the lowest concentration of the test sample which showed no visible growth after 24 hours of incubation at 37°C. Four replicates were maintained in each experiment.

Results and discussion

Extraction yields as well as total phenolic, flavonoid and condensed tannins contents of roots' extracts from *A. andrachne*.

During the isolation of phenolic compounds, the choice of the extraction procedure depends not only on the type of the compounds to be isolated but also on the nature of solvent used and polarity [21]. The extraction yields of E1, E2 and E3 of bark roots extracts were determined to be 4.25 ± 0.73 , 1.12 ± 0.21 and 2.19 ± 0.46 g respectively (Table1).

Table 1: Extraction mass and yield of extracts obtained from *Arbutus andrachne* bark roots.

Extraction solvent	Initial mass used (g)	Mass after extraction (g)	Yield (%)
Methanol (E1)	82	3.49 ± 0.42	4.25 ± 0.73
Ethyl acetate (E2)	100	1.12 ± 0.21	1.12 ± 0.21
Methanol-water (E3)	82	1.80 ± 0.37	2.19 ± 0.46

Interestingly, our results are in full agreement with previous reports [22] showing that yields of extraction with polar solvents, including methanol and water, are more important than less polar solvents, (hexane, dichloromethane).

The amounts of various phenolic compounds in the different solvents extracts (methanol, ethyl acetate and methanol-water) of the *A. andrachne* bark roots are shown in table 2.



Table 2: Total phenolic content, flavonoids, tannins, anthocyanins in the different extracts of *Arbutus andrachne* bark roots.

Extraction solvent	Total phenols ^a (mg GAE/g)	Total flavonoids ^b (mg QE/g)	Total tannins ^c (mg CA/g)	Total anthocyanins ^d (mg)
Methanol (E1)	416.15 ± 3.9	46.03 ± 1.8	126.66 ± 1.2	65.38 ± 1.5
Ethyl acetate (E2)	547.23 ± 2.1	48.81 ± 1.9	207.83 ± 1.8	73.64 ± 0.4
Methanol-water (E3)	298.65 ± 1.1	17.25 ± 0.5	48.14 ± 0.6	338.41 ± 1.2

^a mg GAE : mg gallic acid equivalent per g of dry material (dM)

^b mg QE : mg quercetin equivalent per g of dM

^c mg CE: catechin equivalent per g of dM

^d mg anthocyanins per g of dM

Each value is expressed as a mean of three values ± standard deviation.

The obtained results revealed that there was a variation in the amount of total phenols ranging from 298.65 to 547.23 mg of Gallic Acid Equivalent (GAE)/g of dry material (dM). The highest amount of phenolic content was found in the ethyl-acetate E2 extracts (547.23 ± 2.1 mg GAE/g of dM), while the lowest amount of phenolic compound was found in the methanol E1 extract (298.65 ± 1.1 mg GAE /g of dM). The amount of phenolic compound in the methanol-water E3 extracts was (416.15 ± 3.9 mg/g of dM). A comparison was then carried out with a previous study in order to compare the obtained results with the ones derived from *A. unedo*. Previously, a report from Djabou et al showed that the total phenolic content of the methanolic extracts of *A. unedo* roots was 335.16 mg GAE/g [23]. The phenolic content values recorded in our study are higher than those observed by Djabou et al and much higher than those detected in *A. unedo* leaves and fruits from the northeast of Portugal which in turn fall in the range of 17-170 mg GAE/g [24]. Total flavonoids content of the three bark root extracts E1, E2 and E3 were 46.03 ± 1.8, 48.81 ± 1.9 and 17.25 ± 0.5 mg of Quercetin Equivalent (QE)/g of dM respectively (Table 2). The highest amount of flavonoid content was observed in the ethyl-acetate E2 extracts. Regarding the total tannin, the E2 extract exhibited a higher value (207.83 ± 1.8.mg of Catechin Equivalent (CE)/g of dM), than the one obtained from the E1 extract (126.66 ± 1.2 CE/g of dM) and E3 (48.14 ± 0.6 CE/g of dM) (Table 2). These lower concentrations of tannin, observed in E1 and E3 extract, than total polyphenols and flavonoids in these same extracts or condensed tannins detected in E2 extract are probably due to the fact that methanol is not the most suitable solvent for the extraction of these compounds. This probability is supported by several studies that have shown that condensed tannins are better removed by acetone / water (70/30) [25] a mix primary used for the E2 extraction mode. Overall, roots of *A. andrachne* possess high total phenolic sand. For the total anthocyanins the obtained results

seem to be different. In fact, the high value of anthocyanins (338.9 ± 0.8 mg/g of dM) was recorded in the methanol-water extract E3 (Table 2). Previous studies reveal that aqueous extracts prepared directly are richer than those preceded by extraction with methanol [26]. This could explain the high levels of polyphenols, flavonoids and tannins in E1 and E2 extracts compared to E3. It can be concluded that the extracts obtained using ethyl-acetate and methanol solvents were considerably more effective for extracting total phenols, flavonoids and condensed tannins. However, the methanol-water solvent seems to be more suitable for extracting anthocyanins.

Antioxidant activities

The antioxidant activity of the three studied extracts was assessed using three different tests: scavenging effect on DPPH free radicals, reducing power and metal chelating power.

DPPH free radical scavenging assay

The antioxidant activity of the three bark root extracts was determined by using the 2,2-diphenyl 1-picrylhydrazyl (DPPH) radical scavenging method. *A. andrachne* bark of root extracts displayed a strong free radical scavenging activity on DPPH assay at very low concentrations ranging from 1 to 10 µg/ml. As seen in figure 1A, the highest value was determined in bark root E2 extract. BHT and each of the three studied extracts were not equivalent in terms of their inhibitory effects. The *A. andrachne* extracts had a relatively very high antiradical activity compared to that of BHT as a standard antioxidant, by reporting an IC50 value much lower than the one exhibited by the BHT. The amounts of extract and BHT needed to decrease the DPPH concentration by 50% (IC50) are reported in Table (3). The three obtained extracts from the *A. andrachne* root bark had a strong antioxidant activity (E1, E2 and

E3 IC₅₀ = 0.87, 0.63 and 1.8 µg/ml respectively), which is higher than that of BHT (IC₅₀ = 13.87 µg/ml). For comparative purposes we report the results obtained by Djabou *et al.*, [23] presenting the antioxidant activity observed for ethyl acetate and methanolic extracts of *A. unedo* roots, expressed in terms of IC₅₀. This study shows that the ethyl acetate extract had the highest radical scavenging activity, corresponding to the lowest IC₅₀ value of 1.2, µg/ml and 6.9 µg/ml for the methanolic one.

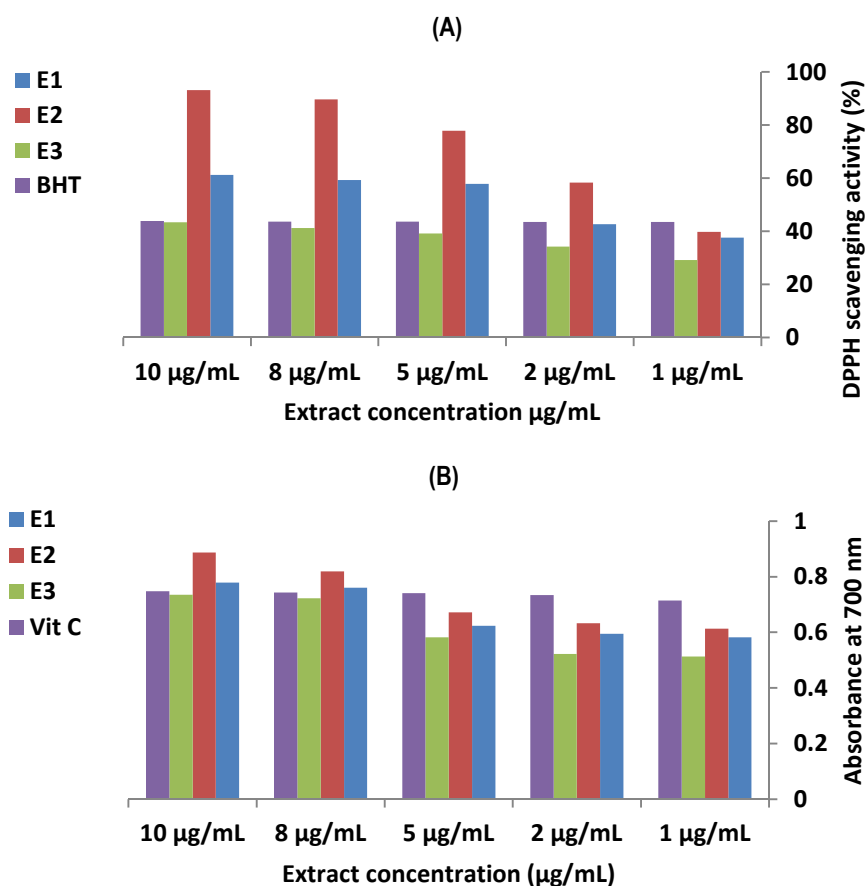
Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP chemical assay allowed us to observe the reducing power of the different extracts, which may indicate their potential antioxidant capacity. The three extracts exhibited a concentration-dependent activity for FRAP. The results are shown in figure 1B. Regarding the extracts efficiencies, high FRAP values were obtained at very low concentrations (1-10µg/mL) compared to the

vitamin C that was used as a positive control because of its high capacity to reduce oxidized molecules. Among the examined extracts the ethyl acetate E2 extract showed the highest reducing power in comparison to the methanol E1, methanol-water E3 ones and to the vitamin C which served as a positive control. The FRAP abilities of E1 and E2 extracts were comparable, but slightly higher than E3. Our results are similar to those reported by Djabou *et al.*, showing that the activities obtained in the FRAP assay for the ethyl acetate extract were stronger than the corresponding value for ascorbic acid used as a positive control [23].

Metal chelating effect

Figure 1C clearly shows that the chelating effect increases by increasing the concentrations of the extract. The calculated IC₅₀ values of the three extracts are reported in table 3.



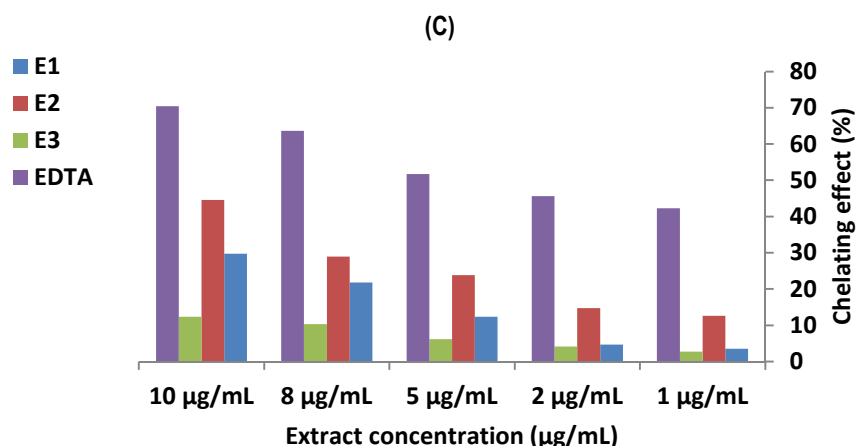


Figure 1. (A) DPPH Scavenging activity, (B) Ferric Reducing Antioxidant Power (FRAP) and (C) Metal chelating effect of the 3 extracts obtained from the bark roots of *A. andrachne* tree. Each value is expressed as mean \pm standard deviation ($n=3$).

Table 3: IC₅₀ values obtained from DPPH and metal chelating tests of extracts obtained from bark roots of *Arbutus andrachne* tree.

Solvent	DPPH scavenging values	Metal chelating values
Methanol (E1)	0.87 \pm 0.4	13.45 \pm 1.2
Ethyl-acetate (E2)	0.63 \pm 0.2	33.11 \pm 2.7
Methanol-Water (E3)	1.8 \pm 0.6	16.88 \pm 1.8
BHT control:	13.87 \pm 1.1	EDTA: 3.58 \pm 0.7

In fact the metal chelating power of the three extracts of *A. andrachne* root bark is good, but far from being compared with EDTA (IC₅₀ = 3.58). This may indicate that these extracts are poor in appropriate chelating cations groups. Many studies have reported that metal chelating potency plays a minor role in the overall antioxidant activities of some polyphenols, which can explain the relatively moderate metal chelating effect of our extracts compared to the extraordinary antioxidant potential correlated to their high content of polyphenols [27]. In this study, and upon considering the IC₅₀ values, total phenols of each sample and each extraction method using the spearman test of correlation, we find a significantly negative linear correlation for DPPH free radical scavenging essay ($p < 0.001$). In the same way, a significantly negative linear correlation between the total phenols content and IC₅₀ values on the iron chelating effect assay ($p < 0.001$) was established. However, a positive linear correlation was obtained between the same two tests and the total flavonoids and the total anthocyanidins contents ($p = 0.667$). Contrary to the FRAP test, a significantly positive linear correlation was obtained for total phenols and IC₅₀ values ($p = 0.667$). However, a negative linear

correlation was also obtained respectively between the total flavonoids content, the total anthocyanidins content and the same tests, respectively ($p < 0.001$). Our results can be compared with those reported by Malheiro et al, [28], who studied the antioxidant, antimicrobial activities and total phenolic content in 19 different genotypes of *A. unedo* leaves' aqueous extracts, obtained from Portugal. In fact, a correlation between the total phenols content and antioxidant activity showed that genotypes with higher antioxidant activity and lower IC₅₀ values also reported high total phenols content. Moreover, a genotype reporting the lowest antioxidant activity in both tested chemical assays is among the genotypes with lower total phenols content. When a regression analysis was performed between the IC₅₀ values obtained in the antioxidant evaluation and the total phenolic content, very negative correlations and extremely negative correlations ($P < 0.001$) were established, respectively, for DPPH and FRAP methods. The apparent conflict in the results concerning the different correlations among the different tests and studies is not surprising. In fact, in our work we use different solvents with different polarities that probably extract different classes of compounds. Various

polyphenolic phytochemicals may react with free radicals in different ways, depending on their chemical structure, and thus lead to different scavenging activities [29]. On the other hand, different plant extracts could present different behaviors according to the antioxidant evaluation methodologies. For example, FRAP activity of the methanolic and hydromethanolic extracts E1 and E2 of the root bark is probably due to their high level of catechin compounds. This proposal is consistent with a recent finding that the catechol ring is the only structure that increases the reducing power of a compound to 36% [30]. Similarly the high antioxidant activity of the ethyl-acetate extract (E2) is in agreement with the fact that flavanols are naturally occurring antioxidants found in various plant types known for their capacity to inhibit the ability of free radicals to trigger negative changes within the body chemistry.

Antimicrobial activity

In order to evaluate the antimicrobial potential of the *A. andrachne* bark of roots, the minimal inhibitory concentration (MIC) values against the tested microorganisms were determined. The pooled extracts exhibited an antimicrobial activity against all the assayed bacteria, in a dose dependent manner for each sensitive indicator strain. Gram positive bacteria were more susceptible to the antimicrobial potential of the methanol E1 extract, with MIC values of 620 and 730 µg/ml respectively for *S. aureus* and *E. faecalis*, respectively. On the other hand, *E. coli* and *P. aeruginosa*, being Gram negative bacteria, were more resistant to the E1 extract, with MIC values of (1870 µg/ml and 920 µg/ml) respectively. However the E2 extract was more effective on *E. faecalis* than on *S. aureus* with MIC values of 310 and 620 µg/ml respectively. Gram negative strains. *E. coli* and *P. aeruginosa* were the most resistant bacteria to E2 with respective MIC values of 5000 and 4160 µg/ml. For the E3 extract, the antimicrobial activity against the four strains of bacteria was stable with MIC values of 730, 770, 780 and 860 µg/ml for *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* respectively. Overall, Gram negative bacteria were more resistant to the extracts than the Gram positive ones. Other studies reported that the different antimicrobial properties of plant extracts toward Gram positive and Gram negative bacteria may be due to the cell wall structural differences between them. Dib et al [31] had investigated the antimicrobial activities of water, methanol extracts and three phenolic fractions of the *A. unedo* roots against the same bacterial strains that we used. Water and methanol extract exhibited only a poor antibacterial activity (CMI > 800 µg/ml) against *S. aureus* or *P. aeruginosa* strains. As discussed before, these results are in concordance with our findings. However, and in contrary to our results, moderate antibacterial activity was shown by water and methanol extracts which were more effective against *E. coli* with MIC values of 200 and 600 µg/ml respectively. This difference may be due to varying constituents in different plant species, extraction solvent and time of sample collection or other geographical factors. Another study done by Afjal et al [32] has reported that Gram positive bacteria were more susceptible to the

antimicrobial potential of the methanol extract from *Sarcochlamys pulcherrima* leaves. High MIC values were recorded for the methanolic extract against *S. aureus* and *E. coli* (12.50 and 50.00 mg/ml). However, those obtained for the ethyl acetate fraction are similar to the present study (0.625 and 5 mg/ml) respectively.

Conclusion

Our results demonstrate for the first time that bark roots extracts from *A. andrachne* exhibit not only excellent free radical scavenging activities but also a potent capacity in scavenging superoxide radical. This study confirms that the roots bark of *A. andrachne* contain high amounts of polyphenol compounds which in turn have been described to have multiple biological effects including antimicrobial activities. The antibacterial as well as the antioxidant properties of the extracts from the *A. andrachne* bark of the roots, particularly against Gram-positive bacteria, is reported for the first time in the present work and represent a valuable property considering the possibility of being a source of interesting antimicrobial compounds. Overall, the obtained results could be beneficial for the development of herbal extracts from *A. andrachne* bark of the roots for pharmaceutical or nutraceutical application in order to prevent and treat illnesses and improve patients' overall health.

List of abbreviations

A. andrachne: *Arbutus andrachne*
 A. unedo: *Arbutus unedo*.
 ROS: Reactive oxygen species
 GAE: Gallic Acid Equivalent
 QE: Quercetin Equivalent
 CE: Catechin Equivalent
 dM : dry Material
 TOF: Total Oligomeric Flavanols
 NB: Nutrient Broth

Authors' contributions

EA: Have been involved in the design and the conception of the research study; have carried out the practical part of the study, made the analysis and interpretation of the data.
 JH: Have been involved in the conception of the research project subject, have collected the plant and given instructions for extractions methods.
 TH: Participated to the design of the study and performed statistical analysis and participated critically in the manuscript revision.
 FB: Participated in the running out of the antioxidant activities and the determination of the total contents of the extracts.
 MG: Participated in the running out of the antibacterial assay.



AE: Have been involved in the conception and the design of the study, have revised the manuscript for important intellectual content and given final approval for the revision to be published

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The authors report no conflict of interest.

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