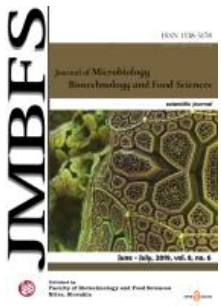

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CHEMICAL COMPOSITION, ANTIOXIDANT ACTIVITY AND MINERAL CONTENT OF *ARBUTUS UNEDO* (LEAVES AND FRUITS)

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ABSTRACT

Arbutus unedo is widely used in traditional medicine and is an easily accessible source of natural antioxidants to replace synthetic antioxidants. The bioactive compounds and antioxidant activity of the leaves and fruits extracts (after maceration and sonication) were studied. The mineral analyses exhibited large quantities of many essential minerals in fruits and leaves, especially interesting were the levels of macronutrients (K and Ca) in fruits and micronutrients (Fe) in leaves. The GC–MS analysis of essential oils of leaves and fruits led to the identification of 32 and 35 compounds, respectively. The major components in leaves were camphor, cymenene, and bornyl acetate, and in fruits, camphor, ethyl palmitate and bornyl acetate. The extraction method significantly affected the contents of total phenolics and total flavonoids, with the maceration method rendering the best results in leaves and the sonication in fruits. All maceration extracts had the highest antioxidant activity (assayed by 3 methods DPPH$^{\bullet}$, ABTS^{+} and FRAP; besides, *A. unedo* leaves showed higher activity than fruits. As a conclusion, it can be stated that *Arbutus unedo* is a plant rich in polyphenols, volatile compounds, and minerals, and that it can be used as a potential source of bioactive compounds, especially from the leaves.

Keywords: Camphor, Ericaceae family, essential oil, iron, maceration, potassium, sonication

INTRODUCTION

The study of new sources of natural antioxidants is constantly increasing because of the growing consumer demand for natural products and the interest for medicinal and nutritional plants to avoid the use of synthetic antioxidants because of their carcinogenic effect, among other factors. Medicinal plants are the source of natural antioxidants thanks to their main secondary metabolites, mainly polyphenols and essential oils (**Bursal and Köksal, 2010; Aidi Wissem *et al.*, 2010**).
The biological effects of phenolic compounds found in fruits are very wide and include antiallergic, antibacterial, anticarcinogenic, antiinflammatory, antithrombotic, antiviral, cardioprotective, hepatoprotective, and vasodilatory effects. Essential oils are very important in plant defense processes and, also have proved health benefits. In addition, they are use as raw materials or ingredients in many fields, such as the agro-food, cosmetics, and pharmaceutical industries. The beneficial effects of both polyphenols and essential oils have been mainly attributed to their antioxidant capacity which acts controlling two reaction types i) the transfer of hydrogen atoms and ii) the transfer of a single electron (**Benhammou et al., 2009; Zuzarte and Salgueiro, 2015**).
Arbutus unedo L. is a plant from the *Ericaceae* family, which is an evergreen shrub or small tree (**Isbilir et al., 2012**), and diverse medicinal properties have been attributed to its fruits, leaves, roots, and/or bark. Some of the uses of its leaves are as follows: i) the leaves of *A. Unedo* are used as antihypertensive, urinary antiseptic, astringent, depurative, against blennorrhagia and as antidiarrheal; and, ii) a tea prepared from its leaves is also used as diuretic. However, the use of the fruits of *A. Unedo* is very limited, despite its high contents of nutrients and bioactive compounds (**Orak *et al.*, 2011**).
Consequently, the objectives of this study were first to determine the profiles of the essential oils of *Arbutus unedo* (leaves and fruits) and their mineral content, and secondly to study the influence of the different extraction methods on polyphenol content and antioxidant activity of the extracts.

MATERIAL AND METHODS

Plant material

The leaves and fruits of *A. Unedo* were collected in November of 2015, in the Natural Forest of Tiaret (west of Algeria). The plant material was stored at room temperature in a dry place until used. The dried leaves and fruit were ground to get a fine powder. All analyses were run in triplicate.

Mineral content analysis by atomic absorption–emission spectrometry

The contents of mineral elements (Ca, Mg, K, Na, Cu, Fe, Mn and Zn) in dried leaves (Ar-L) and fruits (Ar-Fr) of *A. Unedo* were analyzed using Unicam Solaar 969 atomic absorption spectrometer (Unicam Limited, Cambridge, UK), after the digestion with concentrated HNO_3 (**Garcia-Garcia *et al.*, 2013**).

Extraction of the essential oil

The essential oils of the dried samples of leaves and fruits of *A. Unedo* were extracted by hydrodistillation using the Deryng apparatus, as previously used in thyme (**Calín-Sánchez *et al.*, 2013**). Approximately 30 g of sample were extracted and vapors containing the volatile fractions were concentrated in 1 mL of cyclohexane. After 30 min of extraction, the solvent containing the volatile compounds was recuperated and stored at 4°C until further analyses were conducted.

Chromatographic analyses

The volatile composition of the *A. Unedo* essential oils were analyzed by GC-MS using a Shimadzu GC-17A gas chromatograph (Shimadzu Corporation, Kyoto, Japan), coupled with a Shimadzu GC-MS QP-5050A mass spectrometer detector. A TRACSIL Meta.X5 column was used and the chromatographic conditions were those previously reported (**Calín-Sánchez *et al.*, 2013**). Identification was based on retention indexes, retention time of authentic standards, and mass spectra (authentic chemicals and Wiley 229 spectral library), and was considered

tentative when only based on comparison with mass spectra (**Hojjati *et al.*, 2013**).
After the identification, each volatile compound was quantified (relative abundance %) in a Shimadzu 2010 gas chromatograph equipped with a flame ionization detector (FID); the same column and similar chromatographic conditions to those previously reported for GC/MS were used.

Polyphenol extraction

Extraction by a hydro-alcoholic mixture. Approximately 1 g of plant material was mixed and macerated in a hydro-alcoholic solution (methanol/water at a ratio of 80/20 v/v) for 24 h at darkness and continuous stirring. The macerate was filtered and the solvent evaporated under vacuum at 40°C. Later, hexane was used to remove any traces of non-polar compounds (pigments, lipids, etc.). Finally, phenolic compounds were extracted using ethyl acetate containing ammonium sulfate (20%) and orthophosphoric acid (2%). The extraction was repeated 3 times to ensure complete removal of the phenolic compounds and the organic phase was evaporated using the previously described conditions. Finally, the residue was dissolved in 10 mL of methanol and stored at 4°C (**Amiot *et al.*, 1986**).
Extraction by sonication. Briefly, 0.5 g of plant material were extracted using 10 mL of 80% aqueous methanol containing 1% HCl. This mixture was sonicated twice for 20 min, and left for 24 h at 4°C. Then, the extract was centrifuged and the supernatant was collected and used for the analyses of total phenolic content and antioxidant capacity (**Nuncio-Jáuregui *et al.*, 2015; Chong *et al.*, 2013**).

Total phenolic content (TPC)

The total phenolic content (TPC) in the leaves and fruit extracts of *A. Unedo* was quantified using the Folin-Ciocalteu reagent and saturated sodium carbonate. The mixture was incubated at room temperature under dark conditions and absorbance at 765 nm was measured. A calibration curve was prepared using gallic acid (Figure 1A), and TPC was expressed as mg gallic acid equivalents, GAE/g dry weight, dw (**Benhammou *et al.*, 2009**).

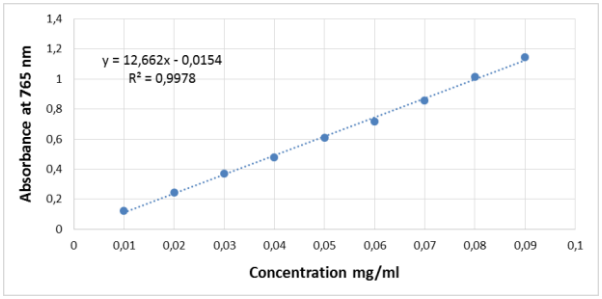
Total flavonoid content (TFC)

The Dowd method as adapted by **Djabou *et al.* (2013)** was used to quantify the total flavonoid content (TFC), and used aluminum trichloride in ethanol; the absorbance at 430 nm was recorded. A calibration curve was prepared using rutin (Figure 2B), and TFC was expressed as mg rutin equivalents, RE/g dw.

Antioxidant capacity

The DPPH$^{\bullet}$ Radical Scavenging Assay was run according to **Brand-Williams *et al.* (1995)** with slight modifications. The absorbance was measured at 517 nm, and vitamin C and BHA were used as control standards. The antioxidant capacity was expressed by the IC50 value in mg/mL; that is, the concentration of the sample required for a 50% inhibition, with lower IC50 values indicating higher antioxidant capacity.
The $ABTS^{+}$ Radical Scavenging Assay was measured according to **Re *et al.* (1999)**. The absorbance was measured at 734 nm, and results were expressed as Trolox Equivalent Antioxidant Capacity, TEAC; that is, the Trolox concentration necessary to obtain the same radical ABTS+ inhibition than 1 mg/mL of the extract under study (**Montoro *et al.*, 2006**).
The Ferric Reducing Power was measured according to **Gülçin *et al.* (2010)**, which is based on the direct reduction of $Fe^{3+}(CN^{-})_6$ to $Fe^{2+}(CN^{-})_6$. The absorbance at 700 nm was monitored, expressed as TEAC, and vitamin C and BHA were used as controls (**Gursoy *et al.*, 2009**).

A



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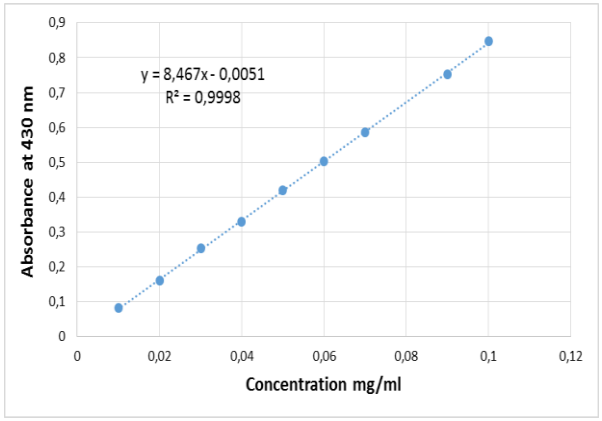

Figure 1 Calibration curves of A: gallic acid (TPC, total phenolic content), and B: rutin (TPC, total flavonoid content).

Statistical analyses

One-way analysis of variance (ANOVA) and Tukey's multiple range test were performed to compare experimental data and determine significant differences among treatments ($p<0.05$).

RESULTS AND DISCUSSION

Minerals content

Among the macro-nutrients, K (1743 and 2045 mg/100 g) and Ca (1299 and 1797 mg/100 g) predominated in both leaves and fruits, respectively (Table 1). On the other hand, the values of Mg were low, as compared to those of K and Ca, reaching values of 186 and 58.6 mg/100 g in leaves and fruits, respectively. The "fruits" of *A. Unedo* have attained the highest concentrations of most macronutrients except Mg. However, the leaves had higher contents of 3 of the micro-nutrients (Fe, Zn, and Mn) than fruits, and besides Fe was the most abundant micro-nutrient in both leaves and fruits. Results from the current study were higher than the values reported in Spanish (**Ruiz-Rodríguez *et al.*, 2011**), Turkish (**Özcan and Haciseferogullan, 2007**) and Moroccan (**Mrabti *et al.*, 2017**) samples of this same tree. In all scientific references (**Ruiz-Rodríguez *et al.*, 2011; Özcan and Haciseferogullan, 2007; Mrabti *et al.*, 2017**), K was the main macro-element followed by Ca in fruits and leaves of *A. Unedo*; regarding the micro-elements, Fe predominated in all samples from the different countries.

Table 1 Mineral contents of leaves (Ar-L) and fruits (Ar-Fr) of *Arbutus Unedo*, expressed as mg/100 g dried weigh (dw).

	Ar-L	***Ar-Fr***
Macro-elements (mg/100 g dw)		
Ca	1298 ± 96[†] b[‡]	1797 ± 53 a
Na	24.7 ± 4.9 b	43.5± 2.5 a
Mg	186 ± 4 a	58.6± 3.4 b
K	1743 ± 14 b	2045 ± 119 a
Micro-elements (mg/100 g dw)		
Fe	26.8 ± 0.5 a	7.02 ± 0.2 b
Zn	3.8 ± 0.1 a	1.9 ± 0.1 b
Cu	traces	1.5 ± 0.1
Mn	1.1 ± 0.1 a	0.3 ± 0.1 b

[†]Values are expressed as mean ± standard deviation of 3 replicates. [‡]Values followed by the same letter, within the same row, were not significantly different ($p<0.05$), according to Tukey's least significant difference test.

Table 2 Chemical composition of the leaves oil of *Arbutus unedo* L.

	Compound	RT[¥] (min)	RI[¥] (exp)	Leaves (%)	Fruits (%)
1	α-Fenchone	9.03	1418	17.5[†] a	1.51 b
2	Cymenene	10.02	1448	0.48	0.29
3	Linalool oxide	10.06	1450	0.72	1.30
4	Salvene	10.32	1458	0.17	0.03
5	(*Z*,*Z*)-2,4-Heptadienal	10.83	1473	0.97	0.85
6	Fenchyl acetate	11.00	1478	1.72	2.04
7	α-Ylangene	11.42	1491	0.78	0.61
8	(*E*,*E*)-2,4-Heptadienal	11.74	1501	1.61 a	0.12 b
9	Camphor	12.54	1524	43.5 a	37.6 b
10	Linalool	13.45	1550	0.71	0.40
11	1-Octanol	13.78	1560	0.41	0.26
12	Pinocarvone	14.26	1574	0.36	0.26
13	Bornyl acetate	14.69	1586	16.0 a	12.9 b
14	Caryophyllene	15.28	1603	0.16	0.13
15	Terpinen-4-ol	15.38	1606	0.87	0.80
16	Myrtenal	16.33	1632	0.47	0.23
17	(*E*)-2-Decenal	16.94	1649	0.60 a	0.19 b
18	Pinocarveol	17.24	1657	0.36 a	0.16 b
19	Myrtenyl acetate	18.53	1693	3.16	2.58
20	α-Terpineol	18.81	1701	0.52	0.44
21	Eucarvone	18.89	1703	3.16	3.12
22	δ-Carvone	20.13	1737	0.80	0.60
23	2-Methylacetophenone	21.59	1777	0.16	0.10
24	Cuminal	21.84	1783	0.17 a	0.03 b
25	Myrtenol	22.27	1795	0.50	0.38
26	Anethole	23.59	1832	nd	0.93
27	*m*-Cymen-8-ol	24.22	1849	0.55	0.45
28	*p*-Cymen-8-ol	24.36	1853	0.44	0.64
29	Neryl acetone	24.71	1863	1.03 a	0.06 b
30	β-Ionone	27.54	1943	0.21 a	0.06 b
31	Ethyl myristate	31.68	2061	nd	1.31
32	Viridiflorol	32.54	2088	0.38 a	0.07 b
33	2-Hexadecanone	34.30	2141	0.56	nd
34	Nonanoic acid	35.70	2184	0.92	0.66
35	Methyl palmitate	37.30	2234	nd	5.64
36	Ethyl palmitate	38.55	2274	nd	23.3

[†]Values are expressed as mean ± standard deviation of 3 replicates. [‡]Values followed by the same letter, within the same row, were not significantly different (p<0.05), according to Tukey's least significant difference test. [¥]RT: retention time; RI: retention index; nd: not detected.

Volatile compounds

A total of 36 compounds were found in both leaves and fruits of *A. Unedo* (32 and 35 in leaves and fruits, respectively (Table 2). The 5 major components in leaves were: camphor (43.5%), α-fenchone (17.5%), bornyl acetate (16.0%), myrtenyl acetate (3.16%), and eucarvone (3.16%). On the other hand, the 5 major compounds in fruits were: camphor (37.6%), ethyl palmitate (23.3%), bornyl acetate (12.9%), and methyl palmitate (5.64%). The chemical composition of *A. Unedo* essential oils reported here was different from those reported previously in samples from Turkey and Portugal. In the Turkish leaves, it was found that (*E*)-2-decenal and α-terpineol predominated (**Kivcak *et al.*, 2001**), while in fruits the most abundant compounds were (*Z*)-3-hexen-1-ol, 1-hexanol, hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate and hexyl acetate (**Oliveira et al., 2011**). These differences on composition may be related to different factors such as harvesting season, growth cycle of the plant, as well as region and even extraction method (**Berka-Zougali *et al.*, 2010**). The Portuguese samples were collected in the Natural Park of Monteshinho in the region of Trás-os-Montes (North-eastern Portugal), were rain is abundant and plants grow wild and without any cultural intervention (**Oliveira et al., 2011**); while the Turkish samples were collected from West Anatolia in Izmir-Cicekliköy, but not further information is provided about growing or climatic conditions (**Kivcak *et al.*, 2001**).

Total phenolic (TPC) and flavonoid (TFC) contents

It is clear from data on Table 3 that the leaves of *A. Unedo* have higher TPC (expressed as mg GAE/g) and TFC (expressed as mg RE/g) than the fruits. In leaves, the maceration method was more efficient for TPC and higher values were reached, while results in TFC were equivalent and no effect of the extraction method was found. In fruits, the sonication method was more effective for both TPC and TFC.

The sonication extraction was previously studied and was found more effective for the extraction of phenolic compounds than the classical maceration (**Balouiri *et al.*, 2014**), but this extraction under the current experimental conditions did not give good results for the leaves, although results were better for the fruits.

It has been established that prolonged sonication is not recommended for the extraction of antioxidant bioactive components, as it may lead to the degradation of the active constituents (**Annegowda *et al.*, 2010**). Thus, the in future studies, the extraction time can be optimized to get the highest possible TPC, if that is the only objective of the extraction.

The high contents of both TPC and TFC obtained in the current extracts of *A. unedo* were similar to those previously reported in other studies (**Boulanouar *et al.*, 2013; Orak *et al.*, 2011; Mendes *et al.*, 2011**), even though different extraction techniques were used. **Mendes *et al.* (2011)** confirmed that the leaves extract had 10 times higher TPC than that of the fruit extract.

Table 3 Total phenolic content (TPC, expressed as mg gallic acid equivalents, GAE/g) and total flavonoid content (TFC, expressed as mg rutin equivalents, RE/g) of leaves (Ar-L) and fruits (Ar-Fr) of *Arbutus Unedo*.

	TPC (mg GAE/g)		TFC (mg RE/g)	
Extraction method	**Maceration**	**Sonication**	**Maceration**	**Sonication**
Leaves (Ar-L)	175.4 ± 1.3 a	100.2 ± 3.0 b	65.2 ± 0.7 a	66.5 ± 1.2 a
Fruits (Ar-F)	13.2 ± 0.6 b	34.3 ± 1.9 a	0.4 ± 0.1 b	2.1 ± 0.1 b

[†]Values (mean of 3 replications) followed by the same letter, for the same plant part (leaves or fruits) and same parameter (TPC or TFC), were not significantly different (p>0.05), Tukey's least significant difference test.

Antioxidant capacity

The antioxidant capacity of the *A. Unedo* extracts was tested using three different methods (DPPH•, $ABTS^+$, and FRAP). The extract showed an interesting antioxidant activity, and the effect of the extraction method and part of the plant under study (leaves or fruits) showed similar behavior (Table 4). In the DPPH•, the leaves extract obtained by maceration led to the lowest values of the IC50, meaning it had the highest antioxidant activity. On the other hand, the lowest IC50 for the DPPH• was found in fruits after sonication. In fact, the antioxidant activity (DPPH•) of the leaves extract was equivalent to that of vitamin C and higher than that of BHA.

In the other two methods ($ABTS^+$ and FRAP), the highest antioxidant activity (highest values of TEAC) were found for the macerated extracts of the leaves of *A. unedo*. However, both standards (vitamin C and BHA) showed significantly higher activity than the plant extracts assayed. A good correlation between antioxidant activity and TPC has been widely reported (17), as is indicated by high correlation coefficients, R^2; in this particular case, values of R^2= -0.2827, 0.9118, and 0.8107 were found for DPPH•, $ABTS^+$, and FRAP, respectively. These results agreed with previous results (28) and (31).

Table 4 Antioxidant capacities of leaves (Ar-L) and fruits (Ar-Fr) of *Arbutus Unedo*, determined by radical scavenging assay (DPPH•) expressed as IC50% (µg/mL), and Trolox Equivalent Antioxidant Capacity, TEAC ($ABTS^+$) and Ferric Reducing Antioxidant Power (FRAP) expressed as TEAC.

		Antioxydant activity					
		DPPH• (µg/mL)		$ABTS^+$ (TEAC)		FRAP (TEAC)	
Extraction method		Maceration	Sonication	Maceration	Sonication	Maceration	Sonication
Leaves (Ar-L)		2.5 ± 0.1 b	52.4 ± 0.1 a	0.90 ± 0.01 a	0.30 ± 0.01 b	0.80 ± 0.01	0.10 ± 0.01
Fruits (Ar-F)		32.0 ± 0.4 b	194.2 ±0.1 a	0.10 ± 0.01 a	0.03 ± 0.01 a	0.04 ± 0.01	0.01 ± 0.01
Standards	Vitamin C	2.2 ± 0.1		1.60 ± 0.02		2.00 ± 0.30	
	BHA	5.9 ± 0.1		3.30 ± 0.02		1.00 ± 0.02	

†Values (mean of 3 replications) followed by the same letter, for the same plant part (leaves or fruits) and same parameter (DPPH•, $ABTS^+$, or FRAP), were not significantly different (p>0.05), Tukey's least significant difference test.

CONCLUSION

Fruits and leaves contained large quantities of many essential minerals and trace element. GC–MS analyses of essential oils of leaves and fruit allowed the identification of 32 and 35 compounds, respectively. Under the assayed conditions, the maceration was an effective method for the extraction of phenolic compounds. Experimental results demonstrated that *A. Unedo* leaves extracts exhibited much higher antioxidant activity than fruits.

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