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Identification of Phenolic Compounds and Antimicrobial Activity of Roots of *Arbutus unedo* L.

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The roots of *Arbutus unedo* L. were extracted with a water/methanol/acetone mixture. Two major compounds, (+)-catechin and (+) catechin gallate were identified by NMR spectroscopy. A number of phenolic compounds were also identified by gas chromatography couples to mass spectrometry (GC-MS) as benzeneacetic acid 4 hydroxy, caffeic acid, gallic acid, protocatechic acid and *bis*(2-ethylhexyl) phthalate. Analysis of the non-volatile and thermolabile phenolic compounds by GC-MS presupposes their conversion into volatile and thermotolerant derivatives. Preliminary investigations on microbiological activity showed that examined aqueous and alcoholic extracts from *Arbutus unedo* L. root have weak activity against gram-negative and gram-positive organisms as well as a weak antifungal activity.

Key Words: *Arbutus unedo* L., Phenolic compounds, Catechin, Gallic acid, Antimicrobial activity.

INTRODUCTION

Arbutus unedo L. (Ericaceae) is a widely spread species in the mediterranean basin and used in traditional medicine as an astringent, diuretic and urinary antiseptic[1]. In Algeria, many patients used beside the conventional treatment, the medicinal plants as an alternative therapy for several diseases. Recently, a special attention is paid to the bioactive elements extracted from plants in order to discover new drugs toward the treatment of several pathologies[2].

In traditional folk medicine, the species (*Arbutus unedo* L.) is supposed to have antiseptic, diuretic and laxative effects and is used to treat arterial hypertension. This later claim has been recently demonstrated in the literature, as the high tannin content of the leaf extract produces an *in vitro* inhibition of platelet aggregation[3].

The leaves of *Arbutus unedo* L. are used as a urinary antiseptic, against diabetes, an antidiarrheal adstringent, depurative, antioxidant and as an antihypertensive[4,5]. Chemical investigations of leaves and fruits indicated the presence of the essential oil, flavonoids, proanthocyanidins, iridoids glucosides, sugars non-volatile and phenolic acids and tocopherol[4-8].

Arbutus unedo L. is widespread in Terni forest, located in the proximity of Tlemcen city, west of Algeria. Its broad use in traditional medicine prompted us to isolate the roots tannins, suspected to possess antioxidant, antimicrobial and anti-hypertensive activities.

The plant (poly) phenols are a diverse group of higher secondary metabolites, possessing an aromatic ring bearing one or more hydroxy substituents, derived from the shikimate pathway and phenylpropanoid metabolism[9,10]. They include mainly simple phenols, phenolic acids, coumarins, tannins and flavonoids. These compounds usually occur in the form of glycosides or esters in plants, because of their highly water-solubility[11]. A number of analytical methods have been proposed for the separation and determination of these compounds. Most of these protocols are based on ^{1}H, ^{13}C NMR and capillary gas chromatography, coupled with mass spectrometry (GC-MS). The both methods have been used for the analysis of phenolic extracts. Analysis of the non-volatile and thermolabile phenolic compounds by GC-MS presupposes their conversion into volatile and thermotolerant ones by chemical derivatization.

The aim of this study is an analysis of the chemical composition of the *Arbutus unedo*L. roots. Identification and quantitative analysis of phenolic compounds by NMR and GC-MS (phenolic acids and tannins) in these roots was carried out. An estimation of the antimicrobial activity of the aqueous and alcoholic extracts was also performed.

EXPERIMENTAL

The roots of *Arbutus unedo* L. were collected in the Terni forest (at about 20 km south of Tlemcen) at various vegetative stages. A portion was preserved at 4 °C for further studies. The rest was air shade-dried, far from any humidity and cut into small pieces then carefully stored. Samples were deposited and identified at the Botanical Laboratory of Tlemcen University.

NMR spectra were recorded on a Bruker AMX 300 apparatus, operating with 300 MHz for ^{1}H spectra and with 75 MHz for ^{13}C spectra. Chemical shifts are given in ppm, by comparison to the acetone-d_6 signal. Merck silica gel (230-400 mesh) was used for column chromatography. Merck 5554 Kieselgel 60 F_{254} 0.25 mm thickness plate was used for TLC analysis. Mass spectra were recorded on a standard LCQ thermo ion-trap spectrometer, operating on an ESI mode. Gas chromatography coupled with mass spectrometry (GC-MS) analysis were performed using a Hewlett Pakard Autosystem XL mass spectrometer coupled to a Hewlett Pakard Turbo mass detector (quadrupole). Separation of the analytes was achieved using a HP 5MS capillary column, (30 m × 0.32 mm i.d., 0.25 mm film thickness). Helium was used as carrier gas with a linear velocity of 1 mL/s. The temperature was increased from 40-270 °C with 4 °C min^{-1} ramp.

The GC injector temperature was 250 °C and the transfer line temperature was held at 280 °C. The mass spectrometer parameters for EI mode were: ion source

temperature: 230 °C; electron energy: 70 eV; filament current: 34.6 mA; electron multiplier voltage: 1200 V, injection volume, 0.2 µL. Peaks related to complex phenols with high molecular masses were also identified by the electronic libraries. However, their mass spectral fragmentations have been fully interpreted in the literature[12].

Extraction and isolation of compounds: 200 g of roots were extracted with 850 mL of water, 360 mL of methanol and 650 mL of acetone at room temperature during 24 h. After filtration, acetone and methanol were removed inder vacuum. The aqueous phase was extracted twice with 150 mL of methylene chloride in order to eliminate pigments and lipids and then lyophilized. The dry powder obtained was extracted 4 times with 150 mL of ethyl acetate. The combined organic layers were subsequently dried on $MgSO_4$. Filtration and concentration in vacuum yielded in 2.24 g of a red solid (1.12 % yield). The solid was dissolved in 50 % aq. EtOH and fractionated on column chromatography through silica gel (65 g). Elution with EtOH-HOAc-H_2O (3:1:1) gave four fractions Fr_1, Fr_2, Fr_3 and Fr_4 (Table-1).

TABLE-1
CHROMATOGRAPHIC FEATURES OF PROANTHOCYANIDINS

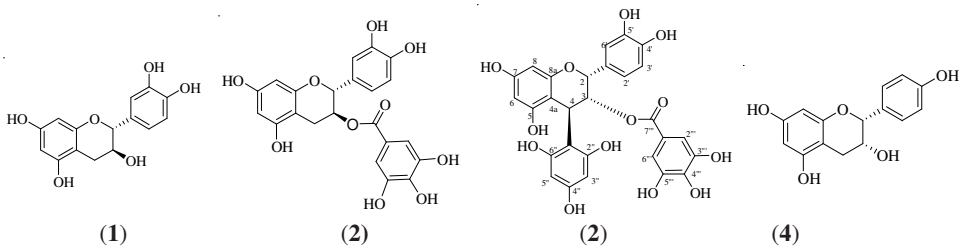
Physical aspect	Sample weight (g)	Column chromatography			
		Compound	Weight (g)	Yield (%)	R_f
Red solid 1.5	1.5	**Fr_1**	0.16	10.66	Mixture
		Fr_2	0.59	39.33	Mixture
		Fr_3	0.38	25.33	0.81 059
		Fr_4	0.37	24.66	0.43 Mixture

A more detailed study was then performed on the **Fr_3** fraction (0.5 g). Column chromatography through silica gel (65 g) of **Fr_3** was monitored by TLC using solvent. The column was washed exhaustively with MeOH-H_2O (1:1) containing 0.1 % v/v/v acetic acid. Three compounds **1**, **2** and **3** with different masses were observed. The corresponding data are summarized in Table-2.

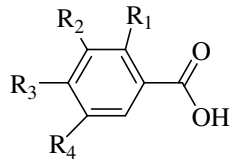
TABLE-2
SEPARATION OF THE Fr_3 FRACTION USING COLUMN CHROMATOGRAPHY

Physical aspect	Sample weight (g)	Column chromatography			
		Compound	Weight (g)	Yield (%)	R_f
Red solid	0.50	**1**	0.09	18	0.84
		2	0.26	52	0.60
		3	0.13	26	Mixture

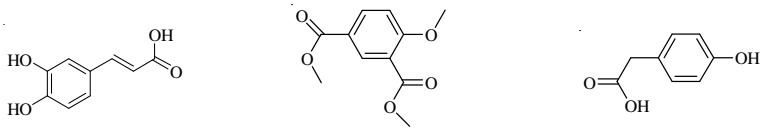
The structures of the isolated roots components are reported in Fig. 1.



(**1**) (**2**) (**2**) (**4**)



(**5**) Gallic acid: R_1=H, R_2=OH, R_3=OH, R_4=OH
(**6**) Gentisic acid: R_1=OH, R_2=H, R_3=H, R_4=OH
(**7**) Protocatechic acid: R_1=H, R_2=OH, R_3=OH, R_4=H



(**8**) (**9**) (**10**)

Fig. 1. Formulae: (+)-catechin (**1**), (+) catechin gallate, (**2**), (+)-catechin-(4α→2) phloroglucinol (**3**), (-)-epicatechin (**4**), gallic acid (**5**), gentisic acid (**6**), protocatechic acid (**7**), caffeic acid (**8**), benzoic acid, 4-(acetyloxy)-3-methoxy-, methyl ester (**9**) and 4-hydroxy phenyl acetic acid (**10**)

The following represent the obtained data for each of those two compounds.

Compound 1: ^{1}H NMR (300 MHz, acetone-d_6) δ: 2.41-2.94 (m, H-4), 3.87 (ddd, H-3), 4.42 (d, H-2), 5.74 (d, H-6), 5.89 (d, H-8), 6.67 (dd, H-2', H-3'), 6.77 (d, H-6'), 9.60 (4H, m, exchangeable proton), signals that disappear after adding a D_2O drop. ^{13}C NMR (75 MHz, acetone-d_6) δ: 28.87 (C-4), 68.18 (C-3), 82.77 (C-2), 95.46 (C-8), 96.18 (C-6), 100.73 (C-4a), 115.29 (C-2'), 145.66 (C-5'), 120.10 (C-6'), 132.25 (C-1'), 115.76 (C-3'), 145.73 (C-4'), 156.96 (C-5), 157.23 (C-7), 157.76 (C-8a); EIMS 70 eV, m/z 290 (M^+, rel. int. 100).

Compound 2: ^{1}H NMR (300 MHz, acetone-d_6) δ: 2.76-2.82 (m, H-4), 3.96 (ddd, H-3), 4.42 (d, H-2), 5.75 (d, H-6), 5.90 (d, H-8), 6.65 (dd, H-2', H-3'), 6.77 (d, H-6'), 7.02 (s, H-2'''), 7.02 (s, H-6'''). ^{13}C NMR (75 MHz, acetone-d_6) δ: 31.15 (C-4), 69.85 (C-3), 84.51 (C-2), 97.20 (C-8), 97.86 (C-6), 102.42 (C-4a), 117.03 (C-2'), 147.30 (C-5') 121.85 (C-6'), , 133.96 (C-1'), 111.86 (C-3'),147.40 (C-4'), 158.70 (C-5), 158.99 (C-7), 159.52 (C-8a), 123.96 (C-1'''), 173.84 (C-7'''), 104.05 (C-2'''), 147.67 (C-3'''), 140.41 (C-4'''), 147.77 (C-5'''), 104.65 (C-6'''); EIMS 70 eV, m/z 442 (M^+, rel. int. 100). Used symbols: (d) doublet; (dd) doublet of doublet; (m) multiplet.

Preliminary investigations on microbiological activity

Microbial strains, inoculation preparation and media: The strains were isolated from the medical reanimation department at the university-hospital of Tlemcen. It concerns two gram (-): *Echerichia coli*, *Pseudomonas aeruginosa* and one gram (+): *Staphylococcus aureus*. *Candida albicans* used throughout this study were isolated at the Dermatology Department (University-Hospital of Tlemcen).

Paper disc diffusion: The agar plate containing the appropriate medium was spread with the inoculums previously adjusted to the microbial densities cited above. Extracts were dissolved in sterile DMSO at 10 mg mL^{-1}. Sterile filter paper discs (6 mm diameter) were aseptically put on agar surfaces and immediately impregnated with 10 µL (100 µg) of prepared extracts. Similarly, 10 µL of DMSO were used as a blank. Antibiotics discs (6 mm in diameter) of piperacilline (PIP: 100 µg) and amphotericin B (AmB: 100 µg) were also used as positive controls. After incubation, the diameters of inhibition zones and the sensitivity were measured with a calliper.

Concentrated aqueous and alcoholic extracts from *Arbutus unedo* L. roots were the subject of microbiological examination. The diameters of inhibition zones of extracts growth of four reference strains of microorganisms were determined[13-15].

RESULTS AND DISCUSSION

In order to identify these two compounds, a comparative study of their spectral data with those of (-)-epicatechin-3-O-galloyl-(4β→2)phloroglucinol (**3**)[16] and (-)-epiafzelechin (**4**)[17] was made (Tables 3 and 4).

The comparison of the spectral data and especially those of ^{1}H and ^{13}C of the compounds **1** and **2** with those cited in literature conclude that: (i) Compound **1** is (+)-catechin with the *trans* stereochemistry at the 2-3 position. This configuration was deduced from the ^{1}H spectrum which related two great coupling constants (J_1 9.0 and J_2 6.0 Hz) (Fig. 2). (ii) Compound **2**, the ^{13}C NMR spectrum indicated the presence of 19 sp^2 carbons (12 quaternary and seven tertiary carbons). The signal deshielding of 3 out of 12 quaternary carbons showed well that these latest are all bonded to a heteroatom. Moreover, proton NMR indicated the presence of a broad around 7.05 ppm. Taken together, the structure of **2** was determined to be the (+) catechin gallate. Its stereochemistry was also determined as being *trans* through coupling constants (J_1 9.0 and J_2 6.0 Hz).

Identification of phenolic compounds by GC-MS: Silylation is an ideal procedure for the GC analysis of non-volatile and thermolabile compounds. Compared to their parent compounds, TMS derivatives are more volatile, less polar and more thermotolerant. In silylation, an active hydrogen in -OH, -COOH, =NH, $-NH_2$ or -SH is replaced by a trimethylsilyl group. Silylation is a nucleophilic substitution reaction. It is showed as a nucleophilic attack upon the silicon atom of the silyl donor, producing a bimolecular transition state. The silyl compound leaving group must be of low basicity and able to stabilize a negative charge in the transition state[18].

TABLE-3
^{1}H NMR DATA OF COMPOUNDS **1**, **2**, (-)-EPICATECHIN-3-O-GALLOYL-(4β→2) PHLOROGLUCINOL (**3**)[16] AND (+)-EPIAFZELECHIN (**4**)[17]; CHEMICAL SHIFTS δ IN PPM AND COUPLING CONSTANT IN Hz

H	1	2	3	4	
2	4.42 (d 6.0)	4.44 (d 6.0)	5.45 (brs)	4.85 (d 1.1)	C ring
3	3.87 (ddd 9.0, 6.0, 3.0)	3.96 (ddd 9.0, 6.0, 3.0)	5.24 (m)	4.17 (ddd 4.3, 3.5, 1.1)	
4	2.41 (dd 9.0, 12) 2.94 (dd 6.0, 12)	2.76 (dd 9.0, 12) 2.82 (dd 6.0, 12)	4.62 (d 1.5)	3.53 (dd 4.3, 16) 3.64 (dd 3.5, 16)	
4a					A ring
5					
6	5.74 (d 3.0) [a]	5.75 (d 2.3) [a]	6.02 (d 2.3) [a]	5.98 (d 2.3) [a]	
7					
8	5.89 (d 3.0) [a]	5.90 (d 2.3)[a]	6.10 (d 2.3)[a]	5.94 (d 2.3) [a]	
8a					
1’					B ring
2’	6.67 (d 3.0)	6.69 (d 1.8)	6.95 (d 1.8)	7.28 (d 8.4)	
3’	6.63 (d 9.0)	6.65 (d 8.2)	6.73 (d 8.2)	6.75 (d 8.4)	
4’					
5’				6.78 (d 8.4)	
6’	6.77 (dd 9.0)	6.79 (dd 8.2, 1.8)	6.79 (dd 8.2, 1.8)	7.28 (d 8.4)	
1’’					D ring
2’’					
3’’			7.97 (brs)		
4’’					
5’’			7.97 (brs)		
6’’					
1’’’					
2’’’					
3’’’		7.05 (s)	7.05 (s)		
4’’’					
5’’’					
6’’’		7.05 (s)	7.05 (s)		

d: doublet, brs: broad singlet, m: multiplet. a: Assignments with the same footnote are interchangeable.

For the preparation of the TMS derivatives, mixtures such as HMDS, TMCS and pyridine 3:1:9 (v/v/v) and BSTFA, TMCS 2:1 (v/v), have been employed. Silylation reagents are influenced by both the solvent system and the addition of a catalyst. A catalyst (*e.g.*, TMCS or pyridine) increases the reactivity of the reagent.

Our efforts subsequently turned to the study of the $\mathbf{Fr_1}$ and $\mathbf{Fr_2}$ fractions by GC-MS. In order to identify the phenolic compounds fractions the standard solutions were prepared as follows: 1 mg of each fraction was dissolved in 100 µL of TMCS and 200 µL of BSTFA. Data obtained showed excellent resolution between all compounds of interest. Retention times of silylated phenolic compounds and their molecular

TABLE-4
^{13}C NMR DATA OF COMPOUNDS **1**, **2**, (-)-EPICATECHIN-3-O-GALLOYL-(4β→2) PHLOROGLUCINOL (**3**)[16] AND (+)-EPIAFZELECHIN (**4**)[17]; CHEMICAL SHIFTS δ (ppm)

C	**1**	**2**	**3**	**4**	
2	82.77	84.51	76.20	80.00	C ring
3	68.18	69.85	75.80	66.80	
4	28.87	31.15	35.00	29.80	
4a	100.73	102.42	101.60	100.10	A ring
5	156.96	158.70	159.10	158.80	
6	96.18	97.86	97.30	96.70	
7	157.23	158.99	158.40	158.70	
8	95.46	97.20	96.50	95.80	
8a	157.76	159.52	158.10	157.60	
1’	132.25	133.96	132.10	131.30	B ring
2’	115.29	117.03	115.50	129.50	
3’	115.76	111.86	116.50	115.80	
4’	145.73	147.40	146.30	158.50	
5’	145.66	147.30	146.20	115.80	
6’	120.10	121.85	119.90	129.50	
1’’			106.70		D ring
2’’			158.90		
3’’			97.60		
4’’			159.20		
5’’			97.60		
6’’			158.90		
1’’’		123.96	122.10		
2’’’		104.05	110.90		
3’’’		147.67	146.70		
4’’’		140.41	139.90		
5’’’		147.77	146.70		
6’’’		104.65	110.90		
7’’’		173.84	168.00		

weights (MW) characteristic fragments in the examined fractions extracts are presented in Table-5. The phenolic compounds identified in fraction 1 (**Fr_1**) using gas chromatography mass spectrometry (GC-MS) are 4-hydroxy phenyl acetic acid (**10**), gallic acid (**5**) and protocatechic acid (**7**). Three more phenolic compounds, benzoic acid, 4-(acetyloxy)-3-methoxy-, methyl ester (**9**), gentisic acid (**6**) and caffeic acid (**8**) were identified by the present method as TMS derivatives, based upon the NIST libraries. Fraction 2 (**Fr_2**) is constitued of catechin (1), phthalylglycine and *bis*(2-ethylhexyl) phthalate.

Antimicrobial and antioxidant activity of extracts: Diameters of inhibition zones of extracts which inhibit growth of four reference strains of microorganisms were determined. The results are presented in Table-6.

TABLE-5
RESULTS AFTER GC-MS ANALYSIS OF THE DERIVATED FRACTIONS WITH BSTFA AND TMCS

Fractions	T_r (min)	Phenolic compound (M.W) of TMS derivatives		Characteristic fragments
Fr_1	24.25	Benzoic acid, 4-(acetyloxy)-3-methoxy-, methyl ester	224	195, 179, 149, 75, 45
	31.24	4-Hydroxy phenyl acetic acid	296	281, 252, 179, 133
	48.19	Gentisic acid	370	355, 281, 267, 223, 147
	48.28	Caffeic acid	396	219, 396, 381, 191
	49.53	Gallic acid	458	443, 355, 281, 179, 147
	49.87	Protocatechic acid	370	355, 311, 281, 267, 193, 147
Fr_2	26.21	Catechin	650	650, 383, 368, 267, 179, 133
	31.84	Phthalylglycine	368	262, 232, 218, 190, 160, 130, 104, 86
	43.85	*Bis*(2-ethylhexyl) phthalate	279	167, 149, 132, 113, 83, 71, 57, 41

TABLE-6
DIAMETERS OF INHIBITION ZONES OF AQUEOUS AND ALCOHOLIC EXTRACTS OF ROOTS FROM *Arbutus unedo* L.

Tested organisms	d (mm)			
	Aqueous extracts from roots	Alcoholic extracts from roots	PIP	AmB
Escherichia coli	30	15	30	Nd
Pseudomonas aeruginosa	9	i	20	Nd
Staphylococcus aureus	12	11	27	Nd
Candida albicans	15	i	Nd	22

PIP (pipéracilline; 100 µg), AmB: Amphotericin B (100 µg), Nd: not determined.

The results showed that both extracts from *Arbutus unedo* L. had weak activity against gram-negative and gram-positive organisms as well as weak antifungal activity because the diameters of inhibition zones values were small. However, the aqueous extract of roots showed high activity against *Escherichia coli* (gram-negative organisms) (30 mm).

Conclusion

The work presented herein made it possible to conclude that the products resulting from the water/methanol/acetone extraction contain (+)-catechin, (+) catechin gallate. The most phenolic compounds in fractions **1** and **2** detected by GC-MS were 4-hydroxy phenyl acetic acid, gentisic acid, hydroxy caffeic acid, gallic acid and protocatechic acid in the roots of the *Arbutus unedo* L. The aqueous extract of the roots provides an interesting antibacterial activity on *Escherichia coli*. The diameter of inhibition zones (30 mm) is comparable to that of piperacilline.

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