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A New Proanthocyanidins from *Arbutus unedo* L. Stems

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Proanthocyanidins extraction using a water/methanol/acetone mixture of the *Arbutus unedo* L. was followed by structural study, indicating the presence of (+)-catechin, (+)-afzelechin and of the unknown (3,4-dihydroxy-phenyl)-5,7-dihydroxychroman-3-yl 4-hydroxybenzoate.

Key Words: Proanthocyanidins, (+)-Catechin, *Arbutus unedo* L., (+)-Afzelechin.

INTRODUCTION

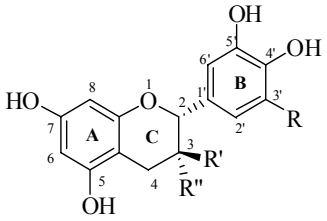
The *Arbutus unedo* L. is a large shrub that belongs to the Ericaceae family and originates in America. Yet, it is found in the garrigue, the hills of the Mediterranean areas, as well as in the Middle-East and Transcaucasia[1,2].

Arbutus unedo L. is a small tree of 4 m height, sometimes called strawberry tree due to the resemblance of its red and warty fruits to strawberries. The fruits are spherical with an approximate 2 cm diameter. The leaves are persistent and the flowers generally pinkish or white, joined together in small buckets, which makes it decorative. Fruits with yellow pulp are comestible and generally consumed fresh in frozen or in liquor form[3]. The fruits of the *Arbutus unedo* L. are used in traditional medicine as antiseptics, diuretics and laxatives. Moreover, the leaves and stems are used as antiseptics, diuretics, urinaries, astringents, depuratives and hypotensors[4].

Catechin and epicatechin (Fig. 1) are known members of the proanthocyanidin family, a term used by the international scientific community to designate a broad family of oligomers and polymers belonging to the flavonoïds class. It is also synonymous with condensed tannins.

In fact, the procyanidins are polymers of catechin and of epicatechin, while prodelphinidines consist of gallocatechin and epigallocatechin. They differ in the degree of hydroxylation on the B core, respectively dihydroxylated and trihydroxylated[5].

The flavanols units and more particularly the epicatechin can be esterified by gallic acid at the carbon 3 position. The inter-monomeric bond is of B type connecting the C4 carbon of the first monomer to the C6 or C8 position of the second monomer[6].



Catechin: **R**=H, **R'**=OH, **R''**=H
Gallocatechin: **R**=OH, **R'**=OH, **R''**=H
Epicatechin: **R**=H, **R'**=H, **R''**=OH
Epigallocatechin: **R**=OH, **R'**=H, **R''**=OH
Epicatechin 3-*O*-gallate: **R**=H, **R'**=H, **R''**=*O*-galloyl

Fig. 1. Constitutive monomeric units of the proanthocyanidins

Phytochemical research on leaves and bark of the *Arbutus unedo* L. led to the isolation of the monotropeinal iridoïds[7], unedide[8], unedoside[9], stilbericoside[10] and geiposide[7]. The isolation of anthocyanes, glucolic acid and carotenoids from the fruits of this plant was also reported in the literature[11]. The fruit contains *ca.* 14 % of carbohydrates and 150 to 280 mg of vitamin C[12].

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

EXPERIMENTAL

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

General experimental procedures: Melting points were determined on a Kofler block and are uncorrected. Optical rotations were measured on a Small Universal polarimeter. 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. IR spectra (KBr) were recorded on a Pye Unicam spectrophotometer. Mass spectra were recorded on a standard LCQ Thermo ion-trap spectrometer, operating on an ESI mode. The index of refraction was measured using Abbe refractometer 1T/4T ATA GO' S Itabshi K, Tokyo, Japan in 1% ethanol solution. Elementary analysis was performed on a LECO CHN-900 at 1000 °C, using 2.1 mg of sample under oxygen and helium atmosphere. Merck silica gel (230-400 mesh) was used for column chromatography. Merck 5554 Kieselgel 60 F254 0.25 mm thickness plate were used for TLC analysis. The chromatogram reading was done in daylight and under UV light (λ = 350 nm) before and after staining using a 1% $FeCl_3$ solution.

Extraction and isolation of compounds: 300 g of stems were crushed and extracted with 850 mL of water, 360 mL of methanol, then 650 mL of acetone at room temperature during 24 h. After filtration, acetone and methanol were removed *in vacuo*. 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 vacuo* yielded in 1.65 g of a yellowish solid (0.55 % yield). A part (1.50 g) of the ethyl acetate extract was subjected to column chromatography through silica gel (65 g). Elution with methanol/water (1/1) containing 0.1 % (v/v) acetic acid gave three fractions F1, F2 and F3, all being mixtures (Table-1).

TABLE-1
CHROMATOGRAPHIC FEATURES OF PROANTHOCYANIDINS

Fractions	Fluorescence	
	Before $FeCl_3$	After $FeCl_3$
F1	Yellowish	Blue green
F2	Yellowish	Blue green
F3	Yellowish	Blue green

Our efforts subsequently turned to the study of the F1 fraction (1.0 g). The two dimensional TLC performed on silica gel using hexane/acetone/water (1/2/1) containing 0.1 % (v/v/v) acetic acid yielded three compounds **1**, **2** and **3** with different masses. The following data thus represents the results obtained after such study (Table-2).

TABLE-2
SEPARATION OF THE F1 FRACTION USING A COLUMN CHROMATOGRAPHY

Physical aspect	Sample weight (g)	Column chromatography				
		Compd.	Weight (g)	Yield (%)	m.p. (ºC)	R_f
Yellowish solid	1.0	**1**	0.38	38	240.0	0.84
		2	0.26	26	247.0	0.60
		3	0.18	18	248.6	0.34

It should be duly noted that out of the 1.0 g, a total of 0.82 g were obtained in the form of the three compounds, while 0.18 g was lost in the process. The structures of the isolated stem components are shown in Fig. 2.

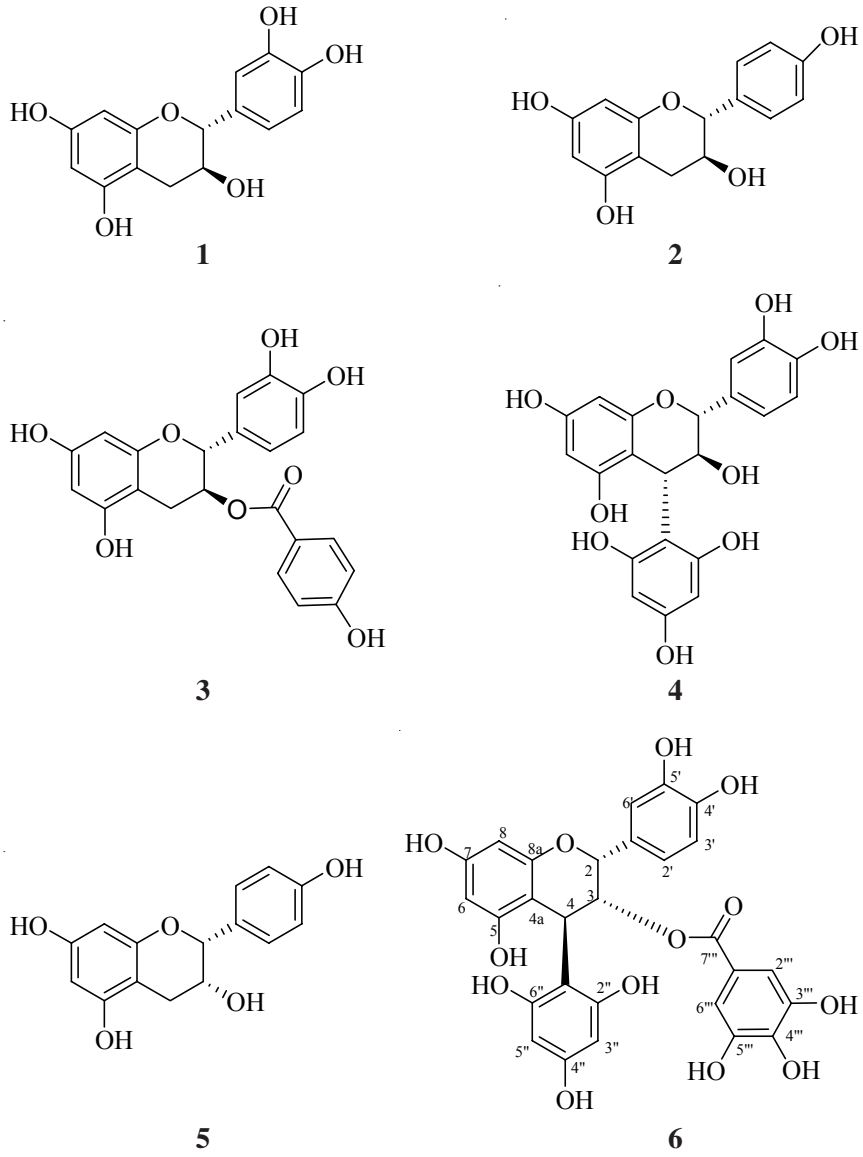


Fig. 2. Formulae: (+)-catechin (**1**), (+)-afzelechin (**2**), (+)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxychroman-3-yl-4-hydroxybenzoate (**3**), (+)-catechin-(4α→2) phloroglucinol (**4**), (+)-epiafzelechin (**5**), (-)-epicatechin-3-O-galloyl-(4β→2) phloroglucinol (**6**)

As logically presumed, the isolated stems components **1**, **2** and **3** were subsequently submitted to full identification, mostly relaying on spectral analysis. The following represent the obtained data for each of those three compounds.

Compound 1: Yellowish solid; m.p. 240 °C (lit.[13] 242 °C); $[\alpha]_D^{20}$ + 3.1° (c 0.10, MeOH) (lit.[14] $[\alpha]_D^{20}$ + 3° (c 0.10, MeOH)); n_D = 1.3420; IR (KBr, ν_{max}, cm^{-1}): 3363, 2955, 2924, 2854, 1609, 1518, 1456, 1363, 1281, 1141, 1101, 869, 818; ^{1}H NMR (300 MHz, acetone-d_6) δ: 2.52-2.85 (m, H-4), 3.98 (ddd, H-3), 4.58 (d, H-2), 5.87 (d, H-6), 5.94 (d, H-8), 6.75 (dd, H-2', H-3'), 6.85 (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: 28.50 (C-4), 68.80 (C-3), 82.84 (C-2), 95.51 (C-8), 96.30 (C-6), 100.82 (C-4a), 115.25 (C-2'), 146.20 (C-5'), 120.02 (C-6'), 132.22 (C-1'), 116.07 (C-3'), 146.23 (C-4'), 157.55 (C-5), 157.82 (C-7), 156.90 (C-8a) ; EIMS 70 eV, m/z 290 (M^+, rel. int. 100); Anal. Calcd. (%) for $C_{15}H_{14}O_6$: C 62.07, H 4.86; Found (%): C 61.98, H 4.75.

Compound 2: White needles; m.p. 247 °C (lit.[15] 248-251 °C); $[\alpha]_D^{20}$ + 9° (c 0.10, MeOH) (lit.[15] $[\alpha]_D^{20}$ + 59.3° (c 1.23, MeOH)); n_D = 1.3360; IR (KBr, ν_{max}, cm^{-1}): 3326, 2958, 2924, 2853, 1609, 1520, 1466, 1369, 1283, 1140, 1100, 974, 870, 816; ^{1}H NMR (300 MHz, acetone-d_6) δ: 2.40-2.78 (m, H-4), 3.86 (ddd, H-3), 4.42 (d, H-2), 5.74 (d, H-6), 5.89 (d, H-8), 6.75 (dd, H-2', H3') 6.65 (dd, H-5', H-6'), 9.20 (3H, m, exchangeable proton), signals that disappear after adding a D_2O drop. ^{13}C NMR (75 MHz, acetone-d_6) δ: 30.45 (C-4), 68.38 (C-3), 82.75 (C-2), 96.17 (C-8), 95.49 (C-6), 100.69 (C-4a), 120.04 (C-2'), 115.75 (C-5'), 120.10 (C-6'), 132.25 (C-1'), 115.28 (C-3'), 145.66 (C-4'), 157.23 (C-5), 157.76 (C-7), 156.95 (C-8a) ; EIMS 70 eV, m/z 274 (M^+, rel. int. 100); Anal. Calcd. (%) for $C_{15}H_{14}O_5$: C 65.69, H 5.15; found (%): C 65.58, H 5.08.

Compound 3: Yellowish solid; m.p. 248 °C; $[\alpha]_D^{20}$ + 132° (c 0.10, MeOH); n_D = 1.3460 ; IR (KBr, ν_{max}, cm^{-1}): 3382, 2987, 2932, 1704, 1624, 1522, 1468, 1357, 1283, 1142, 1081, 1031, 960, 869, 816, 766, 622; ^{1}H NMR (300 MHz, acetone-d_6) δ: 2.39-2.79 (m, H-4), 3.86 (ddd, H-3), 4.42 (d, H-2), 5.75 (d, H-6), 5.89 (d, H-8), 6.66 (dd, H-2', H-3'), 6.76 (d, H-6'), 7.69 (s, H-2'''), 7.75 (s, H-3'''), 7.85 (s, H-5'''), 8.03 (s, H-6'''). ^{13}C NMR (75 MHz, acetone-d_6) δ: 28.85 (C-4), 68.38 (C-3), 82.74 (C-2), 95.49 (C-8), 96.17 (C-6), 100.69 (C-4a), 115.22 (C-2'), 145.66 (C-5') 115.75 (C-6'),, 132.55 (C-1'), 115.28 (C-3'),145.73 (C-4'), 156.95 (C-5), 155.71 (C-7), 157.23 (C-8a), 126.79 (C-1'''), 157.76 (C-7'''), 120.03 (C-2'''), 120.28 (C-3'''), 140.10 (C-4'''), 120.52 (C-5'''), 120.10 (C-6''') ; EIMS 70 eV, m/z 410 (M^+, rel. int. 100) ; Anal. Calcd. (%) for $C_{22}H_{18}O_8$: C 64.39, H 4.42; found (%): C 64.28, H 4.35.

RESULTS AND DISCUSSION

In view of identifying these three compounds, a comparative study of their spectral data with those of (+)-catechin-(4α→2) phloroglucinol (**4**)[16], (+)-epiafzelechin (**5**)[15,17] and (-)-epicatechin-3-O-galloyl-(4β→2) phloroglucinol (**6**)[17] was achieved (Tables 3 and 4).

TABLE-3

^{1}H RMN DATA OF COMPOUNDS **1**, **2**, **3**, (+)-CATECHIN-(4α→2) PHLOROGLUCINOL (**4**)[16], (+)–EPIAFZELECHIN (**5**)[15,17] AND (-)-EPICATECHIN-3-O-GALLOYL-(4β→2) PHLOROGLUCINOL (**6**)[17]; CHEMICAL SHIFTS δ IN ppm AND COUPLING CONSTANT IN Hz

H	**1**	**2**	**3**	**4**	**5**	**6**	
2	4.58 (d 7.5)	4.42 (d 7.6)	4.42 (d 8.0)	4.38 (d 9.2)	4.85 (d 1.1)	5.45 (br)	C Ring
3	3.98 (ddd 9.8, 7.6, 2.3	3.86 (ddd 8.0, 5.6, 2.5)	3.86 (ddd, 8.0, 5.6, 2.4)	4.48 (dd 9.27, 7.8)	4.17 (ddd 4.3,3.5,1.1)	5.24 (m)	
4	2.52 (dd 8.1, 16)	2.40 (dd 8.8, 16)	2.39 (dd 8.4, 16.4)	4.43 (d 7.8)	3.53 (dd 4.3, 16)	4.62 (d 1.5)	
	2.85 (dd 5.4, 16)	2.78 (dd 5.6, 16)	2.79 (dd 5.6, 16.4)		3.64 (dd 3.5, 16)		
4a							A Ring
5							
6	5.87 (d 2.25)[a]	5.74 (d 2.4)[a]	5.75 (d 2.0)[a]	5.89 (d 2.3)[a]	5.98 (d 2.3)[a]	6.02 (d 2.3)[a]	
7							
8	5.94 (d 2.27)[a]	5.89 (d 2.0)[a]	5.89 (d 2.4)[a]	5.91 (d 2.3)[a]	5.94 (d 2.3)[a]	6.10 (d 2.3)[a]	
8a							
1’							B Ring
2’	6.75 (d 1.76)	6.76 (d 8.0)	6.63 (d 2.0)	7.00 (d 1.8)	7.28 (d 8.4)	6.95 (d 1.8)	
3’	6.72 (d 8.08)	6.71 (d 8.0)	6.66 (d 8.4)	6.79 (d 8.1)	6.75 (d 8.4)	6.73 (d 8.2)	
4’							
5’		6.66 (d 8.4)			6.78 (d 8.4)		
6’	6.85 (dd 8.1, 1.77)	6.62 (d 8.4)	6.76 (dd 8.4, 2.0)	6.85 (dd 8.1, 1.8)	7.28 (d 8.4)	6.79 (dd 8.2,1.8)	
1”							D Ring
2”							
3”				5.96 (brs)		7.97 (brs)	
4”							
5”				6.00 (brs)		7.97 (brs)	
6”							
1’’’							
2’’’			7.69 (s)				
3’’’			7.75 (s)			7.05 (s)	
4’’’							
5’’’			7.85 (s)				
6’’’			8.03 (s)			7.05 (s)	

[a]Assignments with the same footnote are Interchangeable.

TABLE-4
^{13}C RMN DATA OF COMPOUNDS **1**, **2**, **3**, (+)-CATECHIN-(4α→2) PHLOROGLUCINOL (**4**)[16], (+)–EPIAFZELECHIN (**5**)[15,17] AND (-)-EPICATECHIN-3-O-GALLOYL-(4β→2) PHLOROGLUCINOL (**6**)[17]; CHEMICAL SHIFTS δ IN ppm

C	**1**	**2**	**3**	**4**	**5**	**6**	
2	82.84	82.75	82.74	84.70	80.00	76.20	C Ring
3	68.80	68.38	68.38	74.30	66.80	75.80	
4	28.50	30.45	28.85	39.00	29.80	35.00	
4a	100.82	100.69	100.69	106.40	100.10	101.60	A Ring
5	157.55	157.23	156.95	158.90	158.80	159.10	
6	96.30	95.49	96.17	98.10	96.70	97.30	
7	157.82	157.76	155.71	158.50	158.70	158.40	
8	95.51	96.17	95.49	97.10	95.80	96.50	
8a	156.90	156.95	157.23	158.10	157.60	158.10	
1’	132.22	132.25	132.55	133.00	131.30	132.10	B Ring
2’	115.25	120.4	115.22	116.60	129.50	115.50	
3’	116.07	115.28	115.28	116.30	115.80	116.50	
4’	146.23	145.66	145.73	146.20	158.50	146.30	
5’	146.20	115.75	145.66	146.50	115.80	146.20	
6’	120.02	120.10	115.75	121.50	129.50	119.90	
1’’				108.00		106.70	D Ring
2’’				158.60		158.90	
3’’				97.90		97.60	
4’’				159.20		159.20	
5’’				96.80		97.60	
6’’				158.60		158.90	
1’’’			126.79			122.10	
2’’’			120.03			110.90	
3’’’			120.28			146.70	
4’’’			140.10			139.90	
5’’’			120.52			146.70	
6’’’			120.10			110.90	
7’’’			157.76			168.00	

The comparison of the spectral data and especially those of ^{1}H and ^{13}C of the compounds **1**, **2** and **3** with those cited in literature[14-17] allowed us to conclude the following:

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 9.8 and J_2 7.6 Hz) (Fig. 2). As far as compound **2**, the ^{13}C NMR spectrum indicated the presence of 12 sp^2 carbons (six quaternary and six tertiary carbons). The signal deshielding of 3 out of 6 quaternary carbons showed well that these latest are all bonded

to a hetero atom. Moreover, proton NMR indicated the presence of a broad around 6.76 ppm. Taken together, the structure of **2** was determined to be the (+)-afzelechin. Its stereochemistry was also determined as being *trans* through coupling constants (J_1 8.0 and J_2 5.6 Hz) (Fig. 2).

Finally, compound **3** structure was determined on the basis of the following: first, ^{13}C NMR showed a total of 19 sp^2 carbons, ten of which are quaternary, the nine others being tertiary carbons. Secondly, signal deshielding of six out of the ten quaternary carbons showed that these ones are bonded to a heteroatom. Contrary to **1** and **2**, ^{1}H NMR of **3** indicated the presence of four protons in the form of one singlet. Finally, a multiplet at 6.66 ppm suggested three aromatic protons. Taken together, all these information suggested the structure of **3** as being 2-(3,4-dihydroxyphenyl)-5,7-dihydroxychroman-3-yl-4-hydroxybenzoate, a compound previously unknown (Fig. 2). However, its stereochemistry remains to be fully proven through further studies.

Conclusion

The work presented herein made it possible to conclude that the products resulting from the water/methanol/acetone extraction contain (+)-catechin, (+)-afzelechin and (+)-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy chroman-3-yl-4-hydroxybenzoate a compound never quoted in the literature up to now and identified for the first time in the stems of the *Arbutus unedo* L.

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