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Annurcoic acid: A new antioxidant ursane triterpene from fruits of cv. Annurca apple

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Abstract

'Annurca' is a variety of apple produced in the south of Italy. The phytochemical study of the ethereal extract of the reddened fruits led to the isolation of a new ursane triterpen, as well as five known compounds, which were identified by spectroscopic techniques. The new compound was identified as the acid, 1α,19α-dihydroxyursan-28-oic, and named annurcoic acid. Antioxidant activities of all the isolated compounds were assessed by measuring their ability to scavenge 2,2′-diphenyl-1-picrylhydrazyl (DPPH) radicals and to inhibit the autoxidation of methyl linoleate (MeLo) in vitro.

Keywords: 'Annurca' apple; Triterpenes; Annurcoic acid; Spectroscopic analysis; Antioxidant activity

1. Introduction

'Annurca' is a variety of apple cultivated in Italy especially in the Campania region. Its flavour and aromas are dependent on the climatic and soil conditions of the production areas (http://www.agendaonline.it/gastronomia/prodotti/melaannurca.htm, Di Lillo).

The 'Annurca' apple is present on about 430 ha, of the national surface, producing 120,000 quintals of the product per year (http://www.bioflavour.it/La_mela_annurca.htm). It represents 60% of the Campania apple production and is considered IGP (Indicazione Geografica Protetta) by the European Union in the framework of the authentication and protection of characteristic agro-alimentary products.

The fruit is medium-to-small sized with flat shape; the skin is thick; first it is yellowish-green and has a striped brilliant red blush when it ripens; the flesh is quite firm and strong, with average juiciness. It is sweet but slightly acid, with modest aroma and good flavour characteristics (Lo Scalzo, Testoni, & Genna, 2001).

This apple undergoes a typical reddening treatment. Generally, the unreddened fruits are collected in October, and placed for 25–30 days on a layer of straw or sawdust on the soil, and day by day, sprayed with water. When the sun-exposed surface of the fruit becomes red, the apples are turned to redden on the opposite side.

In the framework of the regional research centre for the agro-alimentary productions of the Campania, we studied the chemical characteristics of the 'Annurca' apple fruit. The present study reports the isolation and characterization of a new triterpene acid, named annurcoic acid, besides other ursane triterpenes.

2. Materials and methods

2.1. General experimental procedures

NMR spectra were recorded at 300 MHz for ^{1}H and 75 MHz for ^{13}C on a Varian 300 spectrometer Fourier transform NMR in $CDCl_3$ at 25 °C. Proton-detected

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heteronuclear correlations were measured using a gradient heteronuclear single-quantum coherence (HSQC), optimized for $^{1}J_{HC} = 140$ Hz, and a gradient heteronuclear multiple bond coherence (HMBC), optimized for $^{n}J_{HC} = 8$ Hz. Optical rotations were measured on a Perkin–Elmer 141 in MeOH solution. Electronic impact mass spectra (EI-MS) were obtained with an HP 6890 instrument equipped with a MS 5973 N detector.

The preparative HPLC apparatus consisted of a pump (Shimadzu LC-10AD), a refractive index detector (Shimadzu RID-10A) and a Shimadzu Chromatopac C-R6A recorder. Preparative HPLC was performed, using a RP-8 (Luna 10 μm, 250 × 10 mm i.d., Phenomenex) column. Analytical TLC was performed on Merck Kieselgel 60 F_{254} or RP-8 F_{254} plates of 0.2 mm layer thickness. Spots were visualized by UV light or by spraying with H_2SO_4–AcOH–H_2O (1:20:4). The plates were then heated for 5 min at 110 °C. Preparative TLC was performed on Merck Kieselgel 60 F_{254} plates, of 0.5 or 1 mm film thickness. Flash column chromatography (FCC) was performed on Merck Kieselgel 60 (230–400 mesh) at medium pressure. Column chromatography (CC) was performed on Merck Kieselgel 60 (70–240 mesh).

2.2. Fruit samples collection

'Annurca' apple fruits were collected in Sant'Agata de' Goti, near Caserta (Italy), in October, 2003, when the fruit had just been harvested (green peel). The green fruits were reddened on the straw until November and then stored in a climatic cell at 0 °C and 98% dampness.

2.3. Extraction and isolation

The reddened 'Annurca' apple fruits (5.21 kg) were sliced, frozen in liquid nitrogen, powdered in a mortar and infused, first in ethanol for seven days, and then in diethyl ether for seven days. After removal of the ether, a crude organic extract (4.2 g) was obtained.

2.4. Organic extract fractionation

The diethyl ether extract was chromatographed on silica gel, eluting with chloroform and ethyl acetate solutions, to obtain three fractions I–III.

Fraction I was re-chromatographed by column chromatography (CC) on silica gel, eluting with Et_2O–Petroleum ether (1:4), to obtain a fraction which, purified by HPLC [SiO_2, MeCOEt–Hexane (2:23)], gave compounds **5** (3 mg) and **1** (5 mg).

Fraction II was chromatographed by flash column chromatography (FCC) with Me_2CO–Hexane (1:17) to give a fraction which was purified by RP-8 HPLC, eluting with MeOH–MeCN–H_2O (7:2:1), to give compound **4** (13 mg).

Fraction III was chromatographed on RP-18 silica, eluting with MeOH–MeCN–H_2O (5:4:1) to obtain pure ursolic acid **2** (610 mg) and a fraction which was purified by HPLC [SiO_2, MeCOEt–Hexane (1:4)] to give **6** (4 mg) and pomolic acid **3** (6 mg).

Compound **6** was 1α,19α-dihydroxy-3-oxours-12-en-28-oic acid (annurcoic acid): $[\alpha]_D + 14.58$ ($c = 0.08$, MeOH). EI-MS: m/z 486 $[M]^+$, m/z 471 $[M–CH_3]^+$, m/z 468 $[M–H_2O]^+$. ^{1}H and ^{13}C NMR data: Table 1.

2.5. Radical-scavenging activity

The scavenging activity of metabolites is measured according to the method of Brand-Williams, Cuvelier, and Berset (1995). The method is based on the reduction of methanolic 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH·) in the presence of a hydrogen-donating antioxidant.

DPPH· solution shows an absorption band at 515 nm, appearing as intense violet-coloured. Adsorption and colour were lowered when DPPH· was reduced by an antioxidant compound.

The remaining DPPH· corresponds, inversely, to the radical-scavenging activity of the antioxidant (Kulisic, Radonic, Katalinic, & Milos, 2004).

Two milligrams of DPPH· were dissolved in 54 ml of MeOH. Investigated triterpenes were prepared by dissolving 0.11 mg of each compound in 1 ml of MeOH. Then, 38 μl of solution containing each compound were added to 1.462 ml of DPPH· solution at room temperature (Sroka & Cisowski, 2003). The absorbance at 515 nm was measured in a cuvette at 5′ and 30′ vs. blank (38 μl MeOH in 1.462 ml of DPPH· solution) using a UV-1601 Shimadzu spectrophotometer. The results were expressed as a percentage of reduction of the initial DPPH· adsorption by test compounds.

Percentage reduction of the initial DPPH· adsorption $= A_{DPPH(t)} - A_{sample(t)}/A_{DPPH(t)} \cdot 100$, where $A_{DPPH(t)}$ is the absorbance of DPPH· at time t and $A_{sample(t)}$ absorbance of sample at same time t.

2.6. Inhibition of autoxidation of methyl linoleate

The oxidation of methyl linoleate results in conjugated diene (CD) hydroperoxides. The CD products absorb UV light in the wavelength range of 230–235 nm and can be determined spectrophotometrically by their maximum absorbance at 234 nm. An increase in the absorbance at 234 nm began almost immediately when methyl linoleate was kept at 60 °C. The increase was initially rapid until a maximum was obtained after 48 h (Tilbury and Miller, 1999).

The antioxidant activity of each isolated compound was measured by its inhibition of the methyl linoleate

Table 1
NMR data of compound **6** in CD_3OD

Position	DEPT	^{13}C NMR	^{1}H NMR	HMBC
1	CH	70.4	4.58 dd (12.3, 6.6)	H-2, H-5
2	CH_2	50.5	2.30 dd (12.3, 6.3)	H-1, H-25
			1.17 overlapped	H-25
3	C	217.1	–	H-2, H-23, H-24
4	C	49.1	–	H-23, H-24
5	CH	58.9	1.18 overlapped	H-2, H-23, H-24, H-25
6	CH_2	20.4	1.56	H-5
			1.32	
7	CH_2	33.9	1.61 m	H-26
			1.22 m	
8	C	41.2	–	H-26, H-27
9	CH	48.3	1.76 m	H-25, H-26
10	C	38.8	–	H-1, H-2, H-5, H-25
11	CH_2	24.8	2.07 m	H-9
			1.76 m	
12	CH	128.9	5.30 m	H-18
13	C	140.2	–	H-11, H-18, H-27
14	C	42.7	–	H-12, H-18, H-26, H-27
15	CH_2	29.6	1.81 m	H-16, H-27
			2.09 m	
16	CH_2	26.6	2.58 dd (16.5,11.7)	H-15, H-18
			1.52 overlapped	
17	C	48.9	–	H-16, H-18
18	CH	55.1	2.51 s	H-22, H-29
19	C	73.6	–	H-18, H-29, H-30
20	CH	43.1	1.36 ddd (9.8, 6.6, 3.9)	H-18, H-29, H-30
21	CH_2	27.3	1.71 m	H-30
			1.25 m	
22	CH_2	39.0	1.65 m	H-30
			1.74 m	
23	CH_3	25.4	1.12 s	H-24
24	CH_3	22.1	1.11 s	H-23
25	CH_3	16.4	1.30 s	H-2, H-5, H-9
26	CH_3	17.7	0.86 s	H-9
27	CH_3	24.8	1.32 s	H-15
28	C	182.3	–	H-16, H-18
29	CH_3	27.0	1.18 s	H-18, H-30
30	CH_3	16.6	0.92 d (6.6)	H-20, H-21

autoxidation in the bulk phase (Ly, Shimoyamoda, Kato, & Yamauchi, 2003). Methyl linoleate (0.1 mmol), containing investigated metabolites, or α-tocopherol (0.025 μmol; 0.025 mol% based on methyl linoleate), used as reference, were placed in a test tube (1.5 cm in diameter) and incubated at 60 °C in the dark. After 48 h of incubation, each sample (1 μl) was withdrawn and dissolved in 1.0 ml of ethanol. The formation of hydroperoxides was monitored by measuring the formation of conjugated diene hydroperoxides spectrophotometrically at 234 nm. The amount of conjugated diene hydroperoxides was calculated using molar absorptivity of 26,000 (Fishwick & Swoboda, 1977).

The antioxidant activity was expressed as the percentage formation of methyl linoleate conjugated diene hydroperoxides (100%) after 48 h of oxidation (Kähkönen et al., 1999).

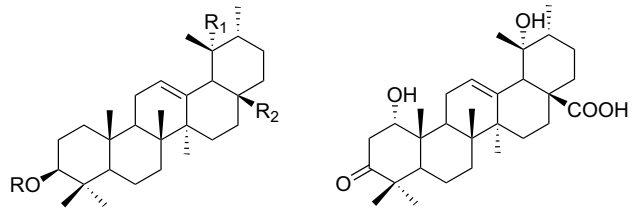
3. Results and discussion

From the organic extract of reddened 'Annurca' apple fruits, six ursane triterpenes (**1**–**6**) were isolated (Fig. 1). All the compounds were characterized on the basis of their spectroscopic data and the ^{13}C NMR shown in Tables 1 and 2.

Compounds **1** and **2** were identified as the ursolaldehyde (Hota & Bapuji, 1994) and ursolic acid (Seo, Tomita, & Tori, 1975), respectively. Compound **3** was characterized as pomolic acid by comparison of its spectral data with those reported in the literature (Dong-Liang & Xiao-Ping, 1992).

Compound **4** was characterized as uvaol, already reported by Siddiqui, Hafeez, Bergum, and Siddiqui (1986), as a constituent of *Nerium oleander*.

Compound **5** showed NMR data similar to those recorded by the previous compound. The differences



1 R = H R_1 = H R_2 = CHO
2 R = H R_1 = H R_2 = COOH
3 R = H R_1 = OH R_2 = COOH
4 R = H R_1 = H R_2 = CH_2OH
5 R = Ac R_1 = H R_2 = CH_2OH

6

Fig. 1. Compounds isolated from the ethereal fraction of cv. 'Annurca' apple.

Table 2
^{13}C NMR data of compounds **1**–**5** from cv. 'Annurca' apple

Position	**1**[a]	**2**[b]	**3**[b]	**4**[a]	**5**[a]
1	38.7	40.0	39.8	38.8	38.5
2	27.2	27.9	28.7	27.2	23.6
3	79.0	79.7	78.8	79.0	80.9
4	38.8	40.2	39.8	38.9	38.0
5	55.2	56.7	56.7	55.2	55.3
6	18.3	19.5	19.6	18.3	18.2
7	33.1	34.3	34.1	32.8	32.7
8	39.8	39.8	41.1	39.8	40.0
9	47.6	48.9	48.2	47.6	47.6
10	36.9	38.1	38.1	36.9	36.8
11	23.2	24.4	24.8	23.4	23.4
12	126.2	126.8	129.5	125.0	125.0
13	139.1	139.6	139.9	138.7	138.7
14	42.2	43.2	42.6	42.0	42.0
15	28.1	29.2	30.7	26.0	26.0
16	23.2	25.3	26.6	35.2	35.2
17	50.1	48.9	48.7	38.0	37.7
18	52.6	54.3	55.1	54.0	54.0
19	39.0	40.4	73.6	39.0	39.3
20	38.8	40.4	43.1	39.4	39.4
21	31.9	31.8	27.9	30.6	30.6
22	33.1	38.1	39.0	30.6	30.6
23	28.1	28.8	27.3	28.1	28.0
24	15.5	16.0	17.4	15.6	16.8
25	15.6	16.4	15.9	15.7	15.7
26	17.2	17.7	16.3	17.3	17.4
27	23.3	24.1	24.6	23.3	23.3
28	207.5	181.8	182.3	69.9	70.0
29	16.7	17.8	27.1	16.8	16.8
30	21.1	21.6	16.6	21.3	21.3
1Ac	–	–	–	–	171.9
2Ac	–	–	–	–	21.3

[a] Recorded in $CDCl_3$.
[b] Recorded in CD_3OD.

consisted in the presence in **5** of a methyl as singlet at δ 2.05 and in the downshift of the H-3 double doublet from δ 3.21 of uvaol to δ 4.51, indicating the linkage of an acetyl group to the hydroxyl positioned at C-3. This hypothesis was confirmed by ^{13}C NMR and 2D NMR data.

The new compound **6** was identified as 1α,19α-dihydroxy-3-oxours-12-en-28-oic acid and named annurcoic acid. Its EI-MS spectrum showed a molecular peak at *m*/*z* 486 and the ^{13}C NMR exhibited thirty signals in accordance with the molecular formula $C_{30}H_{46}O_5$.

The ^1H NMR (Table 1) showed a methyl, as a doublet at δ 0.92, and six methyls, as singlets, at δ 0.86, 1.12, 1.11, 1.18, 1.30 and 1.32. In addition to the aliphatic protons ranging from 2.1 to 1.0 ppm, in the spectrum, there were also present an olefinic proton at δ 5.30, a carbinol proton at δ 4.58, a double doublet at δ 2.58, a singlet at δ 2.51 and a double doublet at δ 2.30.

In the ^{13}C NMR spectrum, a carbonyl carbon at δ 217.1 and a carboxyl carbon at δ 182.3 were evident, besides twenty-four aliphatic carbons, two olefinic carbons at δ 140.3, 128.9, and two carbinol signals at δ 73.6 and 70.4, respectively.

In the TOCSY experiment, correlations were evident between the methyl doublet and the methyne at δ 1.36 which showed a cross peak with the methylene protons at δ 1.71 and 1.25 which, in their turn, were correlated with the methylene at δ 1.65 and 1.74. Heterocorrelations between the doublet methyl protons and the carbons at δ 43.1 and 73.6, those between this latter and the methyl at δ 1.18 and the singlet proton at δ 2.51 and, finally between this proton and the olefinic carbon, the carboxyl at δ 182.3 and the carbons at δ 26.6 and 48.3, allowed us to localize a hydroxyl on the C-19 carbon and the carboxyl at the C-28 carbon on an ursane triterpene skeleton.

Furthermore, the DQ-COSY experiment showed cross peaks between the proton at δ 4.58, which resulted correlated with the carbons at δ 70.4 in the HSQC experiment, and the two diastereotopic protons at δ 2.30 and 1.17 which were correlated with the carbon at δ 50.5. Heterocorrelation experiments allowed the complete structural characterization: the HMBC spectrum showed correlations between the protons at δ 2.30 and 1.17 and the carbons at δ 217.1, 70.4 and 38.8 assigned to the carbons C-3, C-1 and C-10. The carbonyl carbon was also correlated with two methyl protons, H-23 and H-24, at δ 1.12 and 1.11, which were both correlated with the tetrasubstituted C-4 carbon at δ 49.1, while the C-1 carbinol showed interactions with the H-5 methine at δ 1.18 and the H-25 methyl at δ 1.30. The remaining correlation, indicated in Table 1, unequivocally confirmed the proposed structure.

A NOESY experiment showed a cross peak between the olefinic proton and the H-11 protons and the H-29 methyl and those between the H-1 and the H-2 proton at δ 2.30 and the methyl protons at δ 1.30 which were both correlated with the H-24 protons at δ 1.11. These data allowed the stereostructure of compound **6** to be assigned.

The antioxidant activity of 'Annurca' triterpenes, by measuring their capacity to scavenge the DPPH$^{\cdot}$ and

to inhibit formation of methyl linoleate conjugated diene hydroperoxide, showed that these compounds have true antioxidative properties. The standard used in both methods, was α-tocopherol, a known natural antioxidant and the results are shown in Figs. 2 and 3.

When the scavenging of DPPH radical was tested, the strongest activities were observed for uvaol and ursolic acid. Most of the remaining compounds show a lower scavenging activity. Almost all the compounds show a higher activity than α-tocopherol. Instead, pomolic acid appears as the strongest antioxidant compound in the second assay. It inhibits the autoxidation of MeLo by 75%. The tested metabolites, except for ursolic acid, **2**, exhibit more antioxidative activity than does α-tocopherol.

Triterpenes have been reported as antitumoral natural products (Kim, Ahn, & Ryu, 1998; Kim, Yoon, & Ryu, 2000) and recently pomolic acid has been tested on the human leukemic cell line K562 (Fernandes et al., 2003). In addition to inhibiting the cell growth, compound **3** was very effective in blocking the proliferation of Leucena 1, a vincristine-resistant derivative of K562 (Rumjanek et al., 2001). Ursolic acid **2**, the main component of 'Annurca' apple, has been reported to induce pleiotropic biological activities, such as antibacterial, hepatoprotective, immunomodulatory and antiproliferative activities (Liu, 1995). It is able to reduce, in vitro, growth of several cancer cell lines, such as human leukemic HL-60 cells (Simon, Najid, Chulia, Delage, & Rigaud, 1992) or human breast MCF-7 cells (Es-Saady, Simon, Jayat-Vignoles, Chulia, & Delage, 1996). Ursolic acid also induced apoptosis in other experimental cancer cell lines (Baek et al., 1997; Harmand et al., 2003).

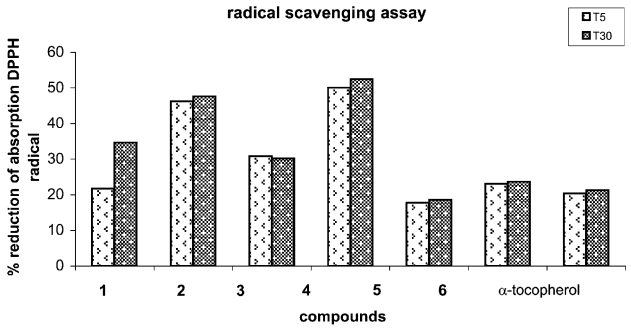


Fig. 2. Percentage reduction of DPPH absorption in the presence of the compounds isolated from cv. 'Annurca' apple at 5 and 30 min.

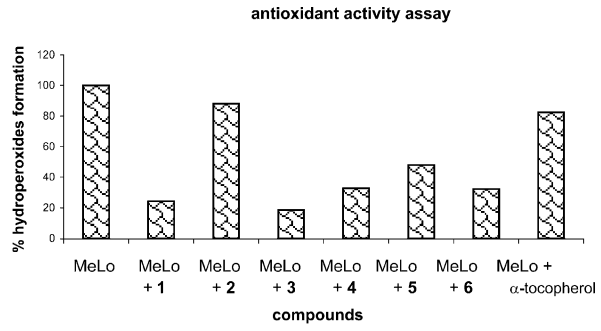


Fig. 3. Percentage hydroperoxides formation of methyl linoleate in presence of the compounds isolated from cv. 'Annurca' apple.

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