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Bioassay-Guided Isolation of Antiproliferative Compounds from Grape (*Vitis vinifera*) Stems

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The fractionation, guided by cell-growth inhibition assay, of the EtOAc crude extract from grape stems of the Sicilian *Vitis vinifera* variety 'Nerello Mascalese' allowed identification of ten constituents, isolated either as pure compounds (**1**, **3**–**5**, **7**–**10**) or inseparable mixtures (**2a-d** and **6a-e**). The pure constituents were: two triterpenoid acids, oleanolic (**1**) and betulinic acids (**5**); daucosterol (**7**); a stilbenoid, *E*-resveratrol (**3**) and its dimer *E*-ε-viniferin (**4**); the simple phenol gallic acid (**8**); and the flavanols catechin (**9**) and gallocatechin (**10**). Four 6'-*O*-acyldaucosterols (**2a-d**) and five 1,2-di-*O*-acyl-3-*O*-β-D-galactopyranosyl glycerols (**6a-e**) were also identified as inseparable mixtures. All the isolated compounds were subjected to spectroscopic analysis and MTT bioassay on MCF-7 human breast cancer cells. The majority showed growth-inhibitory activity, **5** being the most active (GI_{50} = 0.57 μM). Compounds **3**–**5** were also tested on HT-29, U-87-MG and U-373-MG cell lines.

Keywords: *Vitis vinifera*, grape stems, growth-inhibitory activity, terpenoids, polyphenols, galactopyranosyl glycerols.

The vegetable/beverage industry will have to sustain increasing costs for treating solid and liquid wastes: their use for animal feed or fertilizer without pre-treatments is complicated by animal intolerance to some waste components [1], and the known germination inhibition properties of many polyphenols [2]. On the other hand, maceration for composting or incineration frequently causes formation of 'off-odors' as well as ground or water pollution [1]. Thus, there is a growing interest in possible exploitation of biomasses of agro-industrial origin as a source of added-value constituents, that is compounds with interesting nutritional, pharmacological or technological properties [1] for potential use in the pharmaceutical, cosmetic and food industries. Up to recent times, vegetable wastes have been scarcely addressed for up-grading or recycling, and this is particularly true in southern Europe, an area rich in agro-industrial production, with special reference to some fruit crops, like citrus, grape and olive. Vegetable wastes from these cultivations are rich in bioactive compounds and antioxidative polyphenols [3-5], and various applications of crude extracts and isolated compounds from these sources have been recently proposed [6-8].

Grape is one of the world's largest fruit crops, and production in Europe reaches approximately 30 million tons per year, cultivated mainly as *Vitis vinifera* for wine production. The estimated amount of by-products from grape cultivation is approximately 20% of the fresh harvest [1]. In modern practice of winemaking, freshly collected grapes are first de-stemmed and a high fraction of waste material is constituted by these stems. After de-stemming, grapes are pressed to obtain must, and grape pomace (mainly skins and seeds) is accumulated in this step. Grape pomace is frequently destined for the distillation and production of alcoholic beverages (grappa). Conversely, there is no real utilization for stems except for composting.

In the frame of our recent research work focused on the valorisation of vegetable wastes through identification of high added-value natural compounds [9,10], we planned a chemical study of grape stems from Sicilian production of red wine. We have recently carried out a study of the main constituents of de-stemmed grape pomace from various Sicilian cultivars, and in particular of 'Nerello Mascalese' (NM), widely cultivated in Sicily and affording a

crude methanolic extract with a high antioxidant activity in comparison with other such extracts examined [10,11]. Pursuing this effort and in view of a possible exploitation of both grape pomace and stems obtained from winemaking of the same cultivar, we have now examined a crude lipophilic extract of NM stems. Chemical studies specifically focused on grape stems are, to the best of our knowledge, quite rare and mainly addressed to polyphenols [12-14].

In this study, we used a bioassay-guided methodology in order to isolate, firstly, compounds with cell-growth inhibition properties and potential usefulness as either cancer chemotherapeutic or chemopreventive agents. In fact, there is an increasing interest in the search for natural products obtained from foods or vegetable materials for countering cancer occurrence and development. If such compounds could be obtained from grape stems, this would afford a further valorisation of winemaking by-products. We have recently used this approach in the search for antiproliferative compounds from almond hulls, employing MTT bioassay on MCF-7 human breast cancer cells as a guide to fractionation of the extract [15]. In a similar way, we tested the crude grape stem extract, chromatographic fractions, and purified compounds on MCF-7 cells. Here we report the results of this study.

Grape stems were obtained from mechanical de-stemming of the 'Nerello Mascalese' grapes, a typical Sicilian cultivar of *Vitis vinifera*, as the first step in the winemaking procedures. Fresh stems were freeze-dried and subsequently ground. The powdered material was extracted with ethyl acetate and this extract was subjected to the MTT bioassay towards MCF-7 human breast cancer cells and proved to be cytotoxic (-62% at 1000 μg/mL). Thus, a preparative extraction of grape stems with ethyl acetate was carried out. The crude extract was fractionated by silica-gel flash-chromatography and fractions were pooled into five groups (A-E) on the basis of their TLC profile. The pooled fractions were submitted to MTT bioassay at three concentrations (50, 100, and 250 μg/mL). Results are reported in Figure 1. Fraction A was inactive and was disregarded in further analysis. Fraction B showed only a pronounced cytostatic activity (GI_{50} < 50 μg/mL), although it was not cytotoxic up to 250 μg/mL. Fractions C and D exhibited a strong cytostatic/cytotoxic activity with GI_{50} values respectively of 70 and 105 μg/mL. Fraction C showed the highest antiproliferative activity, thus being the most cytotoxic fraction. Fraction E was not cytotoxic, but showed a moderate cytostatic activity. Therefore, we decided to analyze firstly the most active fraction C. However, the analysis of the moderately active fractions B, D and E was then accomplished in view of the possibility that antiproliferative constituents could be present as minor components in a mixture including inactive compounds. Figure 2 reports all the compounds isolated in this study (**1**-**10**).

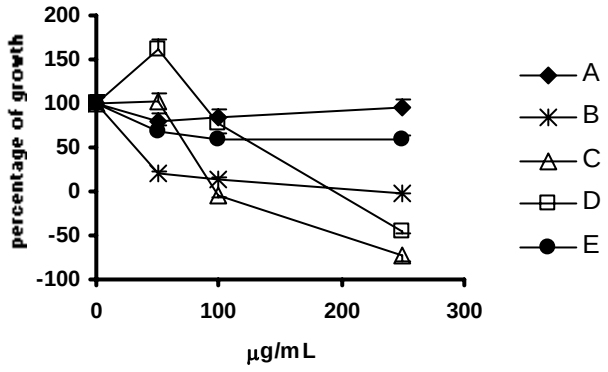

Figure 1: Effects of treatment with different concentrations of fractions A-E on MCF-7 cell growth evaluated by MTT assay. Final concentrations in culture medium are indicated in the graph. Each value is the average ± SD of 4 wells.

Flash-chromatography of fraction C gave four sub-fractions (C_1-C_4), which were submitted to the MTT bioassay affording the following results: C_1, GI_{50} = 72 μg/mL; C_2, GI_{50} = 250 μg/mL; C_3, GI_{50} = 120 μg/mL; C_4, GI_{50} = 80 μg/mL. On this basis, sub-fraction C_2 was not further analyzed. Chromatography of sub-fraction C_1 afforded, as its main constituent, compound **1**, which was identified as the triterpenoid oleanolic acid on the basis of spectral analysis and comparison with an authentic sample [15,16]. Repeated chromatography of sub-fraction C_3 afforded a product showing a single-spot TLC profile. When this product was submitted to preliminary MS, ^{1}H NMR and ^{13}C NMR spectral analysis, it clearly appeared as a mixture of acylated terpenoid glycosides bearing different fatty acid residues, globally indicated in the following as **2**. Because attempts to separate the constituents of this mixture were unsuccessful, **2** was submitted to an extensive NMR study, including 2D NMR methods (COSY, HSQC). This, aided by a careful library

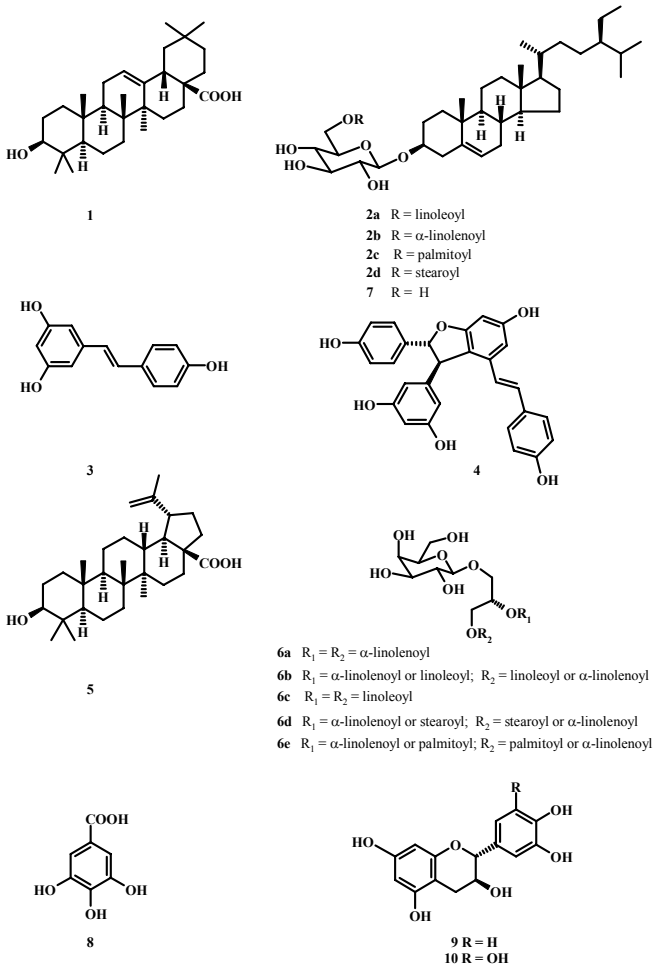

Figure 2: Structures of compounds **1**, **2a-d**, **3-5**, **6a-e** and **7-10**

search and measurement of peculiar ^{1}H NMR coupling constants of the sugar protons, established the common moiety of the mixture as daucosterol [(3β)-stigmast-5-en-3-yl β-D-glucopyranoside)] [17] and the OH group in C-6' as the acylated function. By treatment of an aliquot of **2** with NaOMe/MeOH, a mixture of fatty acid methyl esters was obtained and submitted to GC-MS analysis; this allowed the identification of four different esters, namely: methyl linoleate (36.5% of the mixture), methyl α-linolenate (33.8%), methyl palmitate (23.7%) and methyl stearate (6.0%). The structures of the constituents of the mixture were consequently determined as 3-*O*-(6'-*O*-linoleoyl-β-D-glucopyranosyl)-β-sitosterol (**2a**), 3-*O*-(6'-*O*-α-linolenoyl-β-D-glucopyranosyl)-β-sitosterol (**2b**), 3-*O*-(6'-*O*-palmitoyl-β-D-glucopyranosyl)-β-sitosterol (**2c**) and 3-*O*-(6'-*O*-stearoyl-β-D-glucopyranosyl)-β-sitosterol (**2d**).

The sub-fraction C_4 was submitted to further chromatography, affording as main constituents compounds **3** and **4**. MS and ^{1}H NMR analysis of compound **3** allowed its rapid identification as the stilbenoid *E*-resveratrol; this was confirmed by comparison of the experimental data with those reported in the literature [18] and by comparison with an authentic sample. Identification of compound **4** was less obvious and was aided by ^{13}C NMR analysis. This constituent gave a quasi-molecular ion peak at *m*/*z* 453 $[M–H]^-$ by ESI-MS. Its ^{1}H NMR spectrum showed some analogies with that of **3**, but also important differences, suggesting for **4** the structure of a stilbenoid dimer. A literature search [19] and measurement of $[\alpha]_D$ allowed unambiguous identification of **4** as the resveratrol dimer (-)-*E*-ε-viniferin [20].

Constituents of the less polar fraction B showed a TLC profile partially similar to that of sub-fractions C_1. In fact, careful chromatography allowed the isolation of oleanolic acid (**1**) and the related triterpenoid betulinic acid (**5**), identified by spectral analysis, literature search and comparison with an authentic sample [15,21].

Analogously, fraction D showed some TLC features similar to those of sub-fraction C_4, as confirmed by the isolation of the stilbenoids **3** and **4** from the less polar sub-fractions. The more polar sub-fraction D_4 afforded a further constituent (**6**). Its ^{1}H NMR spectrum showed high field signals assignable to fatty acid acyl chains, and lower field signals suggesting the presence of a monosaccharide unit and a glycerol moiety. Further NMR analysis, including ^{13}C NMR and ^{1}H-^{1}H COSY spectra, and $J_{H,H}$ measurements, indicated the structure of 1,2-di-*O*-acyl-3-*O*-β-D-galactopyranosyl glycerol and showed that the isolated constituent was a mixture of analogues bearing different acyl chains. Also, this inseparable mixture was submitted to alkaline methanolysis both for identification of the fatty acid constituents and for determination of the absolute configuration of the stereogenic centre at C-2 of the glycerol moiety. After work-up of the reaction mixture, GC-MS analysis of the organic phase allowed identification of methyl α-linolenate as the main constituent (78.9%), accompanied by minor amounts of methyl linoleate (15.2%), methyl palmitate (4.7%) and methyl stearate (1.2%). ESI-MS, ^{1}H NMR analysis and $[\alpha]_D$ measurement of the product obtained from the water-soluble fraction demonstrated the presence of (2*R*)-1-*O*-β-D-galactopyranosyl glycerol [22], thus establishing the constituents of the mixture as (2*S*)-1,2-di-*O*-acyl-3-*O*-β-D-galactopyranosyl glycerols. Although we were

not able to isolate the single components of the mixture, the main component was established as (2*S*)-1,2-di-*O*-α-linolenoyl-3-*O*-β-D-galacto-pyranosyl glycerol (**6a**) on the basis of both the higher percentage of methyl α-linolenate (determined by GC-MS) and ESI-MS data of the mixture (*m/z* 775, $[M + H]^+$, 797 $[M + Na]^+$, 813 $[M + K]^+$). Minor components were: (2*S*)-1(2)-*O*-α-linolenoyl-2(1)-*O*-linoleoyl-3-*O*-β-D-galactopyranosyl glycerol (**6b**) (*m/z* 777 $[M + H]^+$, 799 $[M + Na]^+$, 815 $[M + K]^+$); (2*S*)-1,2-di-*O*-linoleoyl-3-*O*-β-D-galactopyranosyl glycerol (**6c**) (*m/z* 779 $[M + H]^+$, 801 $[M + Na]^+$, 817 $[M + K]^+$); (2*S*)-1(2)-*O*-α-linolenoyl-2(1)-*O*-stearoyl-3-*O*-β-D-galactopyranosyl glycerol (**6d**) (*m/z* 803 $[M + Na]^+$, 819 $[M + K]^+$); and (2*S*)-1(2)-*O*-α-linolenoyl-2(1)-*O*-palmitoyl-3-*O*-β-D-galactopyranosyl glycerol (**6e**) (*m/z* 753 $[M + H]^+$, 775 $[M + Na]^+$, 791 $[M + K]^+$).

Chromatography of fraction E firstly allowed the isolation of (3β)-stigmast-5-en-3-yl β-D-glucopyranoside (daucosterol, **7**) identified by comparison of NMR spectroscopic data and $[\alpha]_D$ value with those reported in the literature [17]. Further chromatography of the more polar eluate afforded compounds **8**-**10**. The analysis of MS, ^1H and ^{13}C NMR spectra of compounds **8** and **9**, and $[\alpha]_D$ measurement of **9**, allowed their easy identification respectively as the widespread polyphenols gallic acid [23] and (+)-catechin [24], then definitely confirmed by comparison with authentic samples. The ^1H NMR spectrum of compound **10** showed a clear analogy with that of catechin (**9**). ESIMS suggested the presence of a further OH group; this was corroborated by a literature check and $[\alpha]_D$ measurement conclusively established **10** as (+)-gallocatechin [25].

All the above reported compounds, either as pure constituents (**1**, **3**–**5**, **7**–**10**) or mixtures (**2** and **6**), were subjected to the MTT bioassay on MCF-7 human breast cancer cell. 5-Fluorouracil (5-FU) was also tested as a positive control. Results are reported in Table 1 as GI_{50} (μM). The majority of the isolated compounds showed growth inhibitory activity toward MCF-7 tumor cells (GI_{50} < 300 μM). Betulinic acid (**5**), as expected from our previous assay [15] and literature data, was confirmed to be a potent antiproliferative agent (GI_{50} = 0.57 μM) and was more active than the positive control 5-FU against MCF-7 cells. It is worth noting here that betulinic acid was not obtained from the most active fraction C, but was isolated from fraction B. A further constituent with significant antiproliferative activity was resveratrol (**3**, GI_{50} = 26 μM). Other compounds showing a mild/poor activity were ε-viniferin (**4**, GI_{50} = 99 μM), gallic acid (**8**, GI_{50} = 101 μM), oleanolic acid (**1**, GI_{50} = 213 μM) and gallocatechin (**10**, GI_{50} = 284 μM).

On the basis of the significant activity of compounds **3** and **5** against MCF-7 cells, as well as of their current interest as antiproliferative agents, we have carried out a new set of bioassays aimed at determining further growth inhibition properties of these compounds. Compound **4** was also included in this evaluation in view of its structural analogy with resveratrol. Compounds **3**, **4**, and **5** were assayed against a colon carcinoma cell line (HT-29), as well as two CNS glioma cell lines (U87-MG and U373-MG). Results are reported in Table 1. Compounds

Table 1: Growth Inhibition (GI_{50}) of compounds **1**–**10** and 5-FU against MCF-7, HT-29, U87-MG and U373-MG cells.

Compound	GI_{50}[a] (MCF-7)[b]	GI_{50}[a] (HT-29)[c]	GI_{50}[a] (U87-MG)[d]	GI_{50}[a] (U373-MG)[d]
Oleanolic acid (**1**)	213 ± 25	–	–	–
3-*O*-(6'-*O*-Acyl-β-D-glucopyranosyl)-β-sitosterol (**2a–d**)	> 300	–	–	–
E-Resveratrol (**3**)	26 ± 5.0	83 ± 8.1	22 ± 2.6	10 ± 0.7
(–)-*E*-ε-Viniferin (**4**)	99 ±12	143 ± 7.0	59 ± 6.0	17 ± 0.8
Betulinic acid (**5**)	0.57 ± 0.21	142 ± 10.9	46 ± 3.6	4.39 ± 0.5
1,2-Di-*O*-acyl-3-*O*-β-D-galactopyranosyl glycerol (**6a–e**)	290 – 312	–	–	–
(3β)-Stigmast-5-en-3-yl β-D-glucopyranoside (**7**)	> 300	–	–	–
Gallic acid (**8**)	101 ± 18	–	–	–
(+)-Catechin (**9**)	> 300	–	–	–
(+)-Gallocatechin (**10**)	284 ± 23	–	–	–
5-Fluorouracil (5-FU)	4.80 ± 0.15	42 ± 2.5	3.46 ± 0.3	3.84 ± 0.2

[a] μM ± SEM;
[b] Mammary carcinoma cell line, Michigan Cancer Foundation (MCF).
[c] Human colorectal adenocarcinoma cell line.
[d] Human astrocytoma cell lines.

3-5 were moderately active against HT-29 and U87-MG cells, whereas they showed a higher antiproliferative activity towards U373-MG cells.We have recently listed a number of literature reports on the important anti-tumor and anti-HIV properties of the triterpenoids **1** and **5** [15]. Resveratrol (**3**) is well-known as a grape phytoalexin and considered one of the main phenolic compounds in red wine with chemopreventive properties towards coronary heart disease (CHD) and cancer. A variety of important biological activities has been recently reported for this stilbenoid, among them inhibition of carcinogenesis and tumor cell cycle progression, interference with intracellular signal transduction regulating cell survival and apoptosis in various human cancer cell lines [26-28]. Its identification in parts of *Vitis vinifera* different from grapes is not frequent and this is the first report from Sicilian grape stems. In view of the biological importance and relatively high commercial cost of resveratrol, we consider it worthy of note that one Kg of dried grape stems may afford, in principle, approximately 130 mg of this metabolite. Resveratrol and its oligomer ε-viniferin (**4**) have been reported as antioxidants and inhibitors of CYP 450 [29] and have been previously tested on MCF-7 cells [30]. Compounds **6a**–**e** are known to widely occur in chloroplast membranes of plant cells. They are reported to possess potent anti-tumor-promoting activity in an animal model [31] and anti-inflammatory activity *in vitro* [32]. Compounds **2a**–**d** have been previously cited as antiproliferative principles on tumor cells (although different from MCF-7) [33] and this is the first report of these compounds from grape stems. Gallic acid **8** and the flavonols **9** and **10** are well-known as constituents of black and green tea and reputed to possess antioxidant [34] and anticarcinogenic properties [35]. On the whole, the data on the EtOAc extract of 'Nerello Mascalese' grape stems show that it may be a useful source of bioactive constituents, many of them possessing antiproliferative or anticarcinogenic properties. Selectively enriched fractions of the extract or the single purified constituents may be useful in pharmaceutical formulations or may be substrates for future chemical optimization. The antioxidative properties reported in the literature for the phenolic constituents **4** and **8**–**10** suggest also possible exploitation of grape stem extracts in food or cosmetic fields.

Experimental

General methods: Column chromatography was performed by flash chromatography on LiChroprep Si 60 or LiChroprep DIOL (40-63 μm; Merck). Thin-Layer Chromatography (TLC) was carried out on Merck silica gel 60 F_{254} plates using cerium sulfate and phosphomolybdic acid as chromogenic reagents. ^{1}H and ^{13}C NMR spectra were recorded using a Varian Unity Inova spectrometer (respectively at 500 and 125.7 MHz) and performed at constant temperature (27°C) in $CDCl_3$, C_5D_5N, CD_3OD and CD_3COCD_3. Electrospray ionization mass spectrometry (ESI-MS) was carried out on a Waters Micromass ZQ2000. Optical rotations were determined on a Jasco DIP-370 digital polarimeter in the proper solvents. Gas-chromatography-mass spectrometry (GC-MS) was carried out on the Hewlett Packard gas-chromatograph model 5890 connected to a Hewlett Packard mass spectrometer model 5971A, with an ionization voltage of 70 eV, electron multiplier 1700 V, and an ion source temperature of 180°C.

Material and extraction: Grape stems (*Vitis vinifera*) were obtained in autumn 2006 from Valle Galfina Winery (Linguaglossa, on the slopes of Mount Etna). The fresh material was freeze-dried, finely ground and then stored under nitrogen at –20°C until analyzed. An aliquot of the powdered material (21.3 g) was extracted with EtOAc (150 mL x 3; total time 24 h) with continuous stirring. The extract was dried over Na_2SO_4 and taken to dryness yielding 206.5 mg (0.97% of dried material). An aliquot of this extract, used for MTT bioassay using MCF-7 human breast cancer cells, proved to be cytotoxic (-62% at 1000 μg/mL). For preparative purposes, a larger amount (510 g) of ground stems was extracted with EtOAc (3 L x 3, total time 24 h) with continuous stirring. After evaporation of the solvent, the EtOAc extract furnished a residue of 4.80 g. The majority of this crude extract was applied to a 30 x 4 cm silica column and subjected to flash chromatography under low nitrogen pressure, eluting first with an increasing gradient of EtOAc in light petroleum (from 25 to 100%), and subsequently with methanol (MeOH). The eluates, analyzed by TLC, were pooled in five fractions: A (1.112 g), B (1.061 g), C (0.501 g), D (0.240 g) and E (1.332 g), which were subjected to MTT bioassay. On the basis of the results of this test, fraction A was not further analyzed in the present study.

Fraction B was submitted to flash-chromatography on a 30 x 4 cm silica column, eluting with a gradient of diethyl ether (Et_2O) in light petroleum from 10 to 50% to yield three sub-fractions: B_1-B_3. Fraction B_3

(429.0 mg) was submitted to flash-chromatography on a 27 x 3 cm silica column eluting with a gradient of CH_2Cl_2 in light petroleum from 80 to 100% and subsequently with a gradient of Et_2O in CH_2Cl_2 from 0 to 10% to yield five sub-fractions: B_{31}–B_{35}. Fraction B_{32} (125.6 mg) was submitted to flash-chromatography on a 27 x 2 cm silica column eluting with a gradient of Et_2O in light petroleum from 20 to 100% to yield three sub-fractions: B_{321}–B_{323}. Fraction B_{322} (18.1 mg) was identified as betulinic acid (**5**, Figure 2) and fraction B_{323} (38.3 mg) as oleanolic acid (**1**, Figure 2).

As a general protocol, the isolated compounds were identified on the basis of their MS, ^{1}H and ^{13}C NMR spectra, and by comparison with literature data. In some cases, 2D NMR methods (COSY, HSQC) and/or [α] measurements were also employed.

Fraction C was submitted to flash-chromatography on a 30 x 3 cm silica column eluting with a gradient of MeOH in $CHCl_3$ from 0 to 8% to yield four sub-fractions: C_1-C_4. Fraction C_1 (136.6 mg) was further fractionated by flash-chromatography on a 30 x 2 cm silica column eluting with a gradient of Et_2O in light petroleum from 10 to 100% to yield four sub-fractions: C_{11}-C_{14}. Fraction C_{13} (12.5 mg) was identified as **1**. Fraction C_3 (142.3 mg) was submitted to flash-chromatography on a 30 x 1 cm silica column eluting with a gradient of MeOH in $CHCl_3$ from 0 to 5% to yield six sub-fractions: C_{31}-C_{36}. Fraction C_{32} was identified as a mixture of 3-*O*-(6'-*O*-acyl-β-D-glucopyranosyl)-β-sitosterols (**2a-d**) (Figure 2). Fraction C_4 (267.9 mg) was submitted to flash-chromatography on a 28 x 2 cm silica column eluting with a gradient of MeOH in $CHCl_3$ from 1 to 8% to yield four sub-fractions: C_{41}-C_{44}. Fraction C_{42} (51.5 mg) was identified as *E*-resveratrol (**3**, Figure 2) and fraction C_{43} (26.2 mg) as (–)-*E*-ε-viniferin (**4**, Figure 2).

Fraction D was submitted to flash-chromatography on a 30 x 4 cm LiChroprep DIOL column eluting with a gradient of MeOH in CH_2Cl_2 from 0 to 25% to yield four sub-fractions: D_1-D_4. Fraction D_1 (48.1 mg) was further treated by flash-chromatography on a 40 x 1 cm LiChroprep DIOL column eluting with a gradient of EtOAc in *n*-hexane from 50 to 100% to yield three sub-fractions: D_{11}-D_{13}. Fraction D_{12} (2.9 mg) was identified as **3** and fraction D_{13} (5.7 mg) as **4**. Fraction D_4 (46.8 mg) was identified as a mixture of (2*S*)-1,2-di-*O*-acyl-3-*O*-β-D-galactopyranosyl glycerols **6a-e** (Figure 2).

Fraction E was submitted to flash-chromatography on a 30 x 3 cm silica column eluting with a gradient of MeOH in $CHCl_3$ from 0 to 20% to yield four sub-fractions: E_1–E_4. Fraction E_1 (43.3 mg) was identified as (3β)-stigmast-5-en-3-yl β-D-glucopyranoside (daucosterol, **7**, Figure 2). Fraction E_3 (185.6 mg) was further fractionated by flash-chromatography on a 30 x 2.5 cm LiChroprep DIOL column eluting with a gradient of MeOH in $CHCl_3$ (from 0 to 6%) to yield five sub-fractions: E_{31}–E_{35}. Fraction E_{32} was identified as gallic acid (**8**, Figure 2), fraction E_{33} as (+)-catechin (**9**, Figure 2), and fraction E_{34} as (+)-gallocatechin (**10**, Figure 2).

The calculated total amount of isolated compounds in the crude EtOAc extract (4.80 g) was as follows: oleanolic acid (**1**), 50.8 mg (1.06%); 3-*O*-(6'-*O*-acyl-β-D-glucopyranosyl)-β-sitosterol **2a-d**, 89.9 mg (1.87%); *E*-resveratrol (**3**), 54.4 mg (1.13%); (–)-*E*-ε-viniferin (**4**), 31.9 mg (0.67%); betulinic acid (**5**), 18.1 mg (0.38%); (2*S*)-1,2-di-*O*-acyl-3-*O*-β-D-galactopyranosyl glycerol **6a-e**, 46.8 mg (0.98 %); (3β)-stigmast-5-en-3-yl β-D-glucopyranoside (**7**), 43.3 mg (0.90 %); gallic acid (**8**), 10.2 mg (0.21%); (+)-catechin (**9**), 40.2 mg (0.84%); and (+)-gallocatechin (**10**), 14.1 mg (0.29%).

Alkaline methanolysis and GC-MS analysis: A solution of the mixture of compounds **2a-d** (32.4 mg) or **6a-e** (12.4 mg) in THF (2 mL) was treated with 2.8% sodium methoxide (NaOMe) in MeOH (2 mL) and the reaction mixture was refluxed for 30 min. After neutralization with dilute HCl, the mixture was partitioned between *n*-hexane and H_2O. The fatty acid methyl esters recovered in the organic phase from both mixtures, after evaporation to dryness, were subjected to GC-MS. The following chromatographic conditions were used: Zebron DB-5 MS capillary column (30 m x 0.25 mm; 0.25 μm), helium flow rate 1.5 mL/min, injection in split mode (1:50), injected volume 1 μL, injector and detector temperatures 280°C and 300°C, respectively. The oven temperature was programmed from 80 to 280°C at 5°C/min. The organic phase of the mixture **2a-d**, when subjected to GC-MS analysis, furnished four peaks, which were identified as methyl palmitate (RT = 24.1 min, 23.7%; *m/z* 270 $[M]^+$), methyl linoleate (RT = 27.5 min, 36.5%; *m/z* 294 $[M]^+$), methyl α-linolenate (RT = 27.7 min, 33.8%; *m/z* 292 $[M]^+$), and methyl stearate (RT = 28.1 min, 6.0%; *m/z* 298 $[M]^+$). The organic phase of the mixture **6a-e**, when subjected to GC-MS analysis, furnished four peaks identified as methyl palmitate (RT = 24.1 min, 4.7%;

m/z 270 $[M]^+$), methyl linoleate (RT = 27.5 min, 15.2%; *m/z* 294 $[M]^+$), methyl-α-linolenate (RT = 27.7 min, 78.9%; *m/z* 292 $[M]^+$) and methyl stearate (RT = 28.1 min, 1.2%; *m/z* 298 $[M]^+$).

The aqueous phase obtained from the mixture **6a-e** was taken to dryness and the residue purified on LiChroprep DIOL; elution with CH_2Cl_2 and subsequently with a gradient of MeOH in CH_2Cl_2 from 0 to 10%, afforded a product identified as (2*R*)-1-*O*-β-D-galactopyranosyl glycerol.

Cell cultures: Human astrocytoma cell lines, U87-MG and U373-MG, were kindly provided by Dr Paola Dell'Albani (National Research Council - CNR, Catania, Italy), while the human colorectal adenocarcinoma (HT-29, ATCC number: HTB-38) and the human breast adenocarcinoma cells (MCF-7, ATCC number: HTB-22) were obtained from the American Type Culture Collection (ATCC, Teddington, UK). The cell lines were cultured in the growth media listed below:
- MCF-7 cells in DMEM (Cat. No. 21885-025, GIBCO, Invitrogen, Scotland, UK);
- HT-29 cells in DMEM with glutamax (4.5 g/L) (Cat. No. 31965-023, GIBCO, Invitrogen, Scotland, UK);
- U87-MG and U373-MG in RPMI 1640 (Cat. No. 61870-010, GIBCO, Invitrogen, Scotland, UK).;

Each growth medium was supplemented with 10% (v/v) heat-inactivated fetal bovine serum and penicillin-streptomycin (50 units-50 μg/mL). The cell cultures were incubated at 37°C in a humidified 5% CO_2 incubator and the culture media were changed twice a week.

MTT colorimetric assay: Each cell line (5×10^3/cells/0.33 cm^2) was plated in Nunclon TM Microwell TM 96 well plates (Nunc, Roskilde, Denmark) and was incubated at 37°C. After 24 h, cells were treated with extract (1000 μg/mL), fractions (50-250 μg/mL) or isolated compounds (0.05-300 μM). Cells treated with 0.5-1% dimethyl sulfoxide (DMSO) were used as controls. Microplates were incubated at 37°C in a humidified 5% CO_2 incubator for 3 days and then the cytotoxicity was measured using a colorimetric assay based on the use of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] [36]. To avoid the interference of the compounds under test with the tetrazolium salt, the medium was removed prior to the assay, cells were rinsed and the medium replaced with fresh containing MTT salt. The results were read on a multiwell scanning spectrophotometer (Multiscan reader), using a wavelength of 570 nm. Each value is the average of 4 wells (standard deviations were lower than 10%). The GI_{50} value was calculated according to NCI (National Cancer Institute, Bethesda, Maryland): thus, GI_{50} is the concentration of test compound where $100x\ (T-T_0)/(C-T_0) = 50$ (T is the optical density of the test well after a 72 h period of exposure to test compound; T_0 is the optical density at the beginning of the treatment, and C is the DMSO control optical density). The cytotoxicity effect was calculated according to NCI when the optical density of treated cells was lower than the T_0 value using the following formula: $100x\ (T-T_0)/T_0<0$.

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