JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Antiproliferative Terpenoids from Almond Hulls (*Prunus dulcis*): Identification and Structure–Activity Relationships

VINCENZO AMICO, VINCENZA BARRESI, DANIELE CONDORELLI, CARMELA SPATAFORA, AND CORRADO TRINGALI*

Dipartimento di Scienze Chimiche, Università di Catania, Viale A. Doria 6, 95125 Catania, Italy

Bioassay-guided fractionation of the EtOAc crude extract from Sicilian almond hulls, a waste material from *Prunus dulcis* crop, allowed identification of 10 constituents, isolated as pure compounds (**1**−**5**, **7**, and **10**) or unseparable mixtures (**5** + **6** and **8** + **9**). All compounds were subjected to spectroscopic analysis and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide bioassay on MCF-7 human breast cancer cells. In addition to the main components oleanolic (**1**), ursolic (**2**), and betulinic (**3**) acids, the 2-hydroxy analogues alphitolic (**4**), corosolic (**5**), and maslinic (**6**) acids, as well as the related aldehydes, namely, betulinic (**7**), oleanolic (**8**), and ursolic (**9**), were identified. From a more polar fraction, the β-sitosterol 3-*O*-glucoside (**10**) was also identified. A sample of commercially available betulin (**11**) was also included in bioassays as further support to a structure−activity relationship study. Betulinic acid showed antiproliferative activity toward MCF-7 cells (GI_{50} = 0.27 μM), higher than the anticancer drug 5-fluorouracil.

KEYWORDS: Almond hulls; *Prunus dulcis* sin. *amygdalus*; terpenoids; antiproliferative; MCF-7 cells; structure−activity relationship study

INTRODUCTION

Vegetable wastes, which are particularly rich in polyphenols with antigermination properties (*1*) and other bioactive substances (*2*), may cause ground or water pollution if simply dumped and, in many cases, are unsuitable for animal feeding due to low digestibility or bitter taste. Even maceration for composting or incineration may cause problems due to the formation of "off-odors" (*3*). Nevertheless, vegetable waste materials are also a promising source of compounds with interesting nutritional, pharmacological, or technological properties (*3*). At present, only a minimum amount of agroindustrial wastes are up-graded or recycled. This is particularly true in Europe, where biomasses of agroindustrial origin are generally dumped or used for animal feed or compost, without any pretreatment. On this scenario, as a continuation of our previous research aimed at the isolation and structure elucidation of bioactive natural products (*4*), we have recently started to study vegetable wastes largely available in the Mediterranean basin as sources of high added value products potentially useful for pharmaceutical, cosmetic, or food industry (*5*). We now report the first results of a chemical study on hulls of a commercially relevant almond cultivar from Sicily.

Almond [*Prunus dulcis* (Mill) D. A. Webb, sin. *amygdalus* Batsch] is a popular tree largely grown in the United States and Europe. Almond nuts are used as snacks or ingredients for processed foods (bakery, confectionery, and others). The world production reached 1 530 000 metric tons (Mt) in 2004 (*6*). The United States is the main producer (735 000 Mt), while European production was estimated at 241 000 Mt, mainly located in Spain, Italy, and Greece. Italian production is concentrated in the South and particularly (70%) in Sicily (*7*). Sicilian almonds are reputed of good quality and are appreciated by the confectionery industry. Almond production may afford different waste products: hulls and shells, coming from the first steps of production of shelled almonds, and skins, if peeled almonds are produced. A global amount of 600 000 Mt/year (*8*) has been reported for hulls. At present, almond crop wastes are not valorized (*9*): The common use of the shells is fuel material, while hulls and skins are generally discharged or deposited for composting.

Almond consumption is reputed to afford health benefits (*10*). Among the promising properties of almond nuts or oil, low-density lipoprotein (LDL) cholesterol-lowering (*11*, *12*) and anticancer (*13*) activities have been reported. Recent investigations on almond nuts showed the presence of an unusual diterpene glycoside (amygdaloside) (*14*), a sphingolipide, and other known constituents (β-sitosterol, daucosterol, uridine, and adenosine) (*15*). A flavanone (persicogenin 3-*O*-glycoside) has been obtained from stem bark (*16*). Recently, the main flavonoids from almond skins have been identified and their bioavailability and antioxidant capacity in vitro have been established; they act synergistically with vitamins C and E in LDL protection against oxidation (*17*). Further chemical analysis of almond skins allowed identification of several flavonoids, flavonoid glycosides, and other phenolic compounds (*18*).

* To whom correspondence should be addressed. Tel: (++39)095-7385025. Fax: (++39)095-580138. E-mail: ctringali@unict.it.

10.1021/jf052812q CCC: $33.50 © 2006 American Chemical Society
Published on Web 01/14/2006

Previous work on almond hulls led to the identification of volatile constituents (*19*, *20*), the triterpenoid acids betulinic, oleanolic, and ursolic (*8*), phenolic acids, and sterols (*21*), and to the isolation of a sesquiterpene lactone (amygdalactone) (*22*) and a prenylated benzoic acid derivative (*23*). On the basis of the present interest of betulinic acid as an antitumor and anti-HIV agent (*24*, *25*) as well as of the biological properties of oleanolic and ursolic acids (*26*), we judged Sicilian almond hulls worthy of chemical investigation, aimed to quantify the above triterpenoid acids and identify minor related constituents with antiproliferative properties. Thus, we used a cytotoxicity bioassay-guided methodology in order to isolate compounds with potential usefulness as cancer chemotherapeutic or chemopreventive agents. Crude extracts, chromatographic fractions, and purified compounds were tested with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) bioassay against MCF-7 human breast cancer cells (*27*), previously reported as sensitive to betulinic acid (*28*).

MATERIALS AND METHODS

General Methods. ^{1}H NMR spectra were measured on a Varian Inova operating at 500 MHz whereas ^{13}C NMR spectra were run at 125 MHz. Multiplicities of ^{13}C NMR resonances were determined by DEPT experiments. All NMR experiments were performed at constant temperature (27 °C) using software supplied by the manufacturer, employing deuteriochloroform, deuteriomethanol, or pyridine-d_5 as solvent on the basis of solubility of the sample and literature data. Electron impact mass spectra were recorded on a Kratos MS 50. Thin-layer chromatography (TLC) was carried out using glass-backed precoated silica gel F_{254} plates (Merck) and cerium sulfate as a spray reagent. LiChroprep Si-60 (Merck) was used as a stationary phase for flash column chromatography. Optical rotations were measured at 25 °C on a Jasco 135 instrument in chloroform, methanol, or pyridine, on the basis of solubility of the sample and literature data.

Material and Extraction. Almond hulls (*P. dulcis*, Pizzutella variety) were obtained from a private cultivation near Belpasso (Catania), on the slopes of mount Etna (September 2004). The fresh material was partially air-dried on the collection site and subsequently freeze-dried to obtain a completely dry material. This was finely ground and stored at −20 °C until use. An aliquot of the powdered material (50 g) was extracted with EtOAc (250 mL) for 24 h with continuous stirring, and the residue was then extracted with EtOH (250 mL) for 24 h. Both extracts were dried over Na_2SO_4 and taken to dryness yielding 0.745 and 0.768 g, respectively (1.49 and 1.54% of dried material). These extracts were used for bioassays.

For preparative purposes, a larger amount (987 g) of ground almond hulls was extracted with ethyl acetate (2 L × 3, total time 24 h) with continuous stirring. After evaporation of the solvent, an oily EtOAc residue (13.59 g) was obtained. The majority of this crude extract (12.51 g) was applied to a 30 cm × 5 cm silica column and subjected to flash chromatography under light nitrogen pressure, eluting first with an increasing gradient of ethyl acetate in petroleum ether (5−100%) and subsequently with ethanol. The eluates, analyzed by TLC, were pooled in six fractions (A−F).

Fractions A (573.7 mg), B (703.6 mg), and C (193.3 mg) were not analyzed in the present study. Fraction D (307.1 mg) was submitted to flash chromatography on a 30 cm × 3 cm silica column and eluted with Et_2O in petroleum ether (from 10 to 50%) to yield five subfractions: D_1−D_5. Fraction D_2 was identified as betulinic aldehyde (**7**, 8.6 mg); fraction D_4 was identified as a mixture (ratio 3:1) of oleanolic/ursolic aldehydes (respectively, **8** and **9**, 13.3 mg) (**Figure 1**).

Fraction E (6.884 g) was submitted to flash chromatography on a 28 cm × 4 cm silica column with a gradient of increasing percentage of $CHCl_3$ in petroleum ether (from 70 to 100%) and subsequently with MeOH in $CHCl_3$ (from 5 to 50%) to yield four subfractions: E_1−E_4. Fraction E_2 (2.610 g) was subjected to further chromatography on a 24 cm × 4 cm silica column, with increasing amounts of $CHCl_3$ in petroleum ether (from 70 to 100%), and subsequently with MeOH in $CHCl_3$ (from 5 to 10%) to yield eight subfractions (E_{21}−E_{28}). Fraction E_{22} was identified as betulinic acid (**3**, 154.5 mg). Fraction E_{23} (0.794 g) after further chromatography on a 32 cm × 3 cm silica column, eluted with Et_2O in petroleum ether (from 35 to 100%), gave four subfractions (E_{231}−E_{234}). Fraction E_{232} (280.9 mg) was identified as a mixture (ratio 8:2) of oleanolic/ursolic acids (respectively, **1** and **2**); fraction E_{233} (298.5 mg) was identified as ursolic acid (**2**). Fraction E_{27} (344.6 g) was submitted to further flash chromatography on a 28 cm × 3 cm silica column, eluting with EtOAc in petroleum ether (from 30 to 100%) to yield five subfractions (E_{271}−E_{275}). Fraction E_{272} was identified as alphitolic acid (**4**, 18.0 mg). Fraction E_{273} (176.5 g), submitted to chromatography on a 28 cm × 2 cm silica column, eluting with MeOH−$CHCl_3$ (from 1 to 10%), afforded four subfractions (E_{2731}−E_{2734}). Fraction E_{2732} (48.9 mg) was identified as a mixture (ratio 7:3) of corosolic/maslinic acids (respectively, **5** and **6**); E_{2733} (67.0 mg) was identified as corosolic acid (**5**).

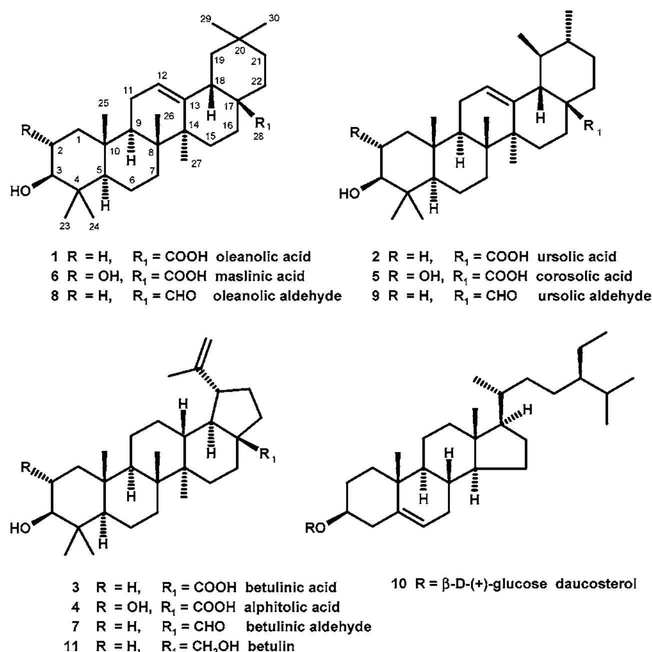

Figure 1. Structures of compounds isolated from almond hulls.

Fraction F (3.5807 g) was submitted to further flash chromatography on a 23 cm × 4 cm silica column eluting with increasing amounts of $CHCl_3$ in petroleum ether (from 70 to 100%) and subsequently with MeOH in $CHCl_3$ (from 2 to 50%) to yield five subfractions (F_1−F_5). Fraction F_3 (453.6 g) was submitted to chromatography on a 27 cm × 3 cm silica column, eluting with MeOH−$CHCl_3$ (from 3 to 12%) to give four subfractions (F_{31}−F_{34}). Fraction F_{33} was identified as a β-sitosterol 3-*O*-glucoside (**10**, 11.6 mg).

Constituents **1**−**10** were identified from ^{1}H, ^{13}C NMR, MS data, and $[\alpha]_D$, in agreement with those reported in the literature (*29*−*35*). To determine the total amount of the major constituents **1**−**3**, we carried out further chromatography of subfractions obtained from fractions E and F, employing the above-reported, or very similar, elution systems. Chromatography of fractions E_1, E_3, and F_1 and subfractions E_{21} and E_{24} afforded a total amount of 540 mg of betulinic acid (**3**). Chromatography of fractions E_3, E_4, and F_1 as well as of subfractions E_{24}−E_{26} afforded a total amount of 2980 mg of oleanolic/ursolic acids (**1** + **2**). NMR analysis of these mixtures clearly indicated **2** as the main component (approximately 65% of the mixture). Thus, the calculated total amount in the crude EtOAc extract (13.59 g) was as follows: **1**, 1365 mg (10.1%); **2**, 2514 mg (18.4%); and **3**, 721 mg (5.3%). From 1 kg of dried hulls, 703 mg of betulinic acid and a total amount of 3930 mg of the mixture oleanolic/ursolic acids can be obtained.

MTT Bioassay. *MCF-7 Cell Culture*. Human mammary adenocarcinoma cells (MCF-7) were grown in Dulbecco's minimum Eagle's medium (DMEM), 1.0 g/L D-glucose supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-alanyl-L-glutamine, and

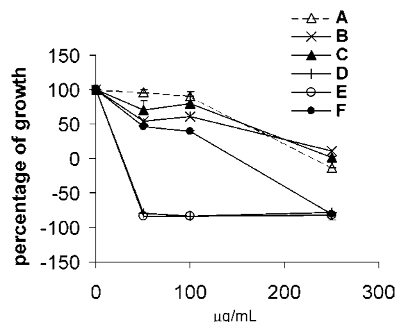

Figure 2. Effects of treatment with different concentrations of fractions A–F from EtOAc extract on MCF-7 cell growth evaluated by MTT assay. Final concentrations in culture medium are indicated in the graph. Each value is the average ± SD of four wells.

penicillin−streptomycin (50 units−50 μg for mL) and incubated at 37 °C in a humidified 5% CO_2 incubator. The culture medium was changed twice a week.

MTT Colorimetric Assay. MCF-7 cells (5 × 10^3/cells/0.33 cm^2) were plated in Nunclon TM Microwell TM 96 well plates (Nunc, Roskilde, Denmark) and were incubated at 37 °C. After 24 h, cells were treated with test compounds. Cells treated with 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 with colorimetric assay based on the use of tetrazolium salt MTT. The results were read on a multiwell scanning spectrophotometer (Multiscan reader), using a wavelength of 570 nm. Each value was the average of four wells. The GI_{50} value was calculated according to NCI (National Cancer Institute, Bethesda, Maryland): Thus, GI_{50} is the concentration of test compound where $100 \times (T - T_0)/(C - T_0) = 50$ (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 time zero, 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 T_0 value with the following formula: $100 \times (T - T_0)/(T_0)$.

RESULTS AND DISCUSSION

Almond hulls obtained from a *P. dulcis* crop in East Sicily on the slopes of Mount Etna were partially air-dried on the collection site and subsequently freeze-dried to obtain a completely dried material suitable to grinding and solvent extraction. The powdered material was extracted first with ethyl acetate and subsequently with ethanol, thus affording the EtOAc and EtOH crude extracts, respectively. Both extracts were subjected to the MTT bioassay with MCF-7 human breast cancer cells. The EtOAc sample proved to be cytotoxic (−60% at 1000 μg/mL), whereas the EtOH sample was inactive. On this basis, we carried out a preparative extraction of almond hulls with ethyl acetate. The crude EtOAc extract was fractionated by silica gel flash chromatography, and subfractions were pooled in six groups (A−F) on the basis of their TLC profile. These were submitted to MTT bioassay at three concentrations (50, 100, and 250 μg/mL). Results are reported in **Figure 2**. The less active fractions were A, B, and C. Fraction F was moderately active, while fractions D and E produced a pronounced cytotoxicity (−80%) even at the lowest concentration tested (50 μg/mL). On this basis, we decided to analyze in detail fractions D, E, and F. We examined first the most active fraction E, which was also the most abundant. Through repeated chromatography on silica gel, the main constituent, showing a one-spot TLC profile, was resolved into two different compounds, which were submitted to spectroscopic analysis (MS, ^{1}H NMR, ^{13}C NMR, and $[\alpha]_D$) and literature search and identified as the triterpenoids oleanolic acid (**1**) and ursolic acid (**2**) (*29*); the latter was the major component of the mixture. From a less polar subfraction, a further triterpenoid constituent was identified as betulinic acid (**3**) (*30*). At this point, we concentrated our efforts toward the minor constituents of fraction E. From the more polar subfractions of this fraction, we could isolate compounds **4** and **5** and an inseparable mixture of two constituents, **5** and **6**. The MS spectrum of **4** showed a molecular peak at *m/z* 472, 16 amu higher than that of the major acids **1**−**3**. The ^{1}H and ^{13}C NMR spectra of compound **4** clearly indicated a close relationship with betulinic acid and the presence of a further hydroxylic function. On the basis of literature search, compound **4** was identified as alphitolic acid (*31*), a 2-hydroxy analogue of betulinic acid. Preliminary MS and NMR analysis of the mixture **5** + **6** showed a close similarity with the couple oleanolic/ursolic acids. Through a literature search, compound **5** was identified as corosolic acid (*32*). By analysis of the spectroscopic data of the mixture, in comparison with those of **5**, compound **6** was unambiguously identified as maslinic acid (*32*). Also, **5** and **6** are the 2-hydroxy analogues of the corresponding major acids, **1** and **2**.

Table 1. Growth Inhibition (GI_{50}) of Compounds **1**–**11** and 5FU against MCF-7 Cells[a]

compound	GI_{50} (μM ± SEM)	compound	GI_{50} (μM ± SEM)
1	263 ± 25	**7**	181 ± 20
2	178 ± 15	**8** + **9**	202 ± 19
3	0.27 ± 0.03	**10**	239 ± 15
4	324 ± 30	**11**	17.00 ± 2
5	309 ± 28	5FU	5.34 ± 0.4
5 + **6**	296 ± 28		

[a] Mammary carcinoma cell line, Michigan Cancer Foundation (MCF).

A preliminary TLC analysis of fraction D showed a different profile with respect to fraction E and careful chromatography of this fraction to obtain a pure constituent **7** and an unseparable mixture of two compounds **8** and **9** to be obtained. Spectroscopic analysis of **7** showed a close relationship with **3** but also the presence of an ^{1}H NMR aldehydic signal at δ 9.68 and immediately allowed identification as betulinic aldehyde, as confirmed by literature search (*33*). A careful analysis of the ^{1}H NMR spectra of the mixture **8** + **9** and the presence of aldehydic signals at δ 9.33 and δ 9.41 (ratio 3:1) indicated that these constituents were the couple oleanolic/ursolic aldehydes (respectively, **8** and **9**); a literature search corroborated this assumption (*34*, *35*). TLC of fraction F indicated the presence of the main constituents **1**−**3** as well as more polar minor constituents. Repeated chromatography on F fractions afforded a further, very polar constituent **10**, whose ^{1}H NMR spectrum did not show the typical features of the co-occurring triterpenoids, instead suggesting a steroid glycoside structure. On the basis of MS, ^{1}H and ^{13}C NMR data, and reference to literature, **10** was identified as β-sitosterol 3-*O*-glucoside, known also as daucosterol, a metabolite previously reported from almond nuts (*15*).

All of the above-reported compounds, as pure constituents (**1**−**5**, **7**, and **10**) or unseparated mixtures (**5** + **6** and **8** + **9**), were subjected to the MTT bioassay on MCF-7 human breast cancer cells. 5-Fluorouracil (5FU) was also tested as a positive control. A sample of commercially available betulin (**11**) was included as further support to a structure−activity relationship study. Results are reported in **Table 1** as GI_{50}. The most active compound is betulinic acid with GI_{50} = 0.27 μM. All of the other tested samples were active toward MCF-7 cells in the range 17−324 μM. Although the antiproliferative activity of **3** was predictable on the basis of previously reported data, it is

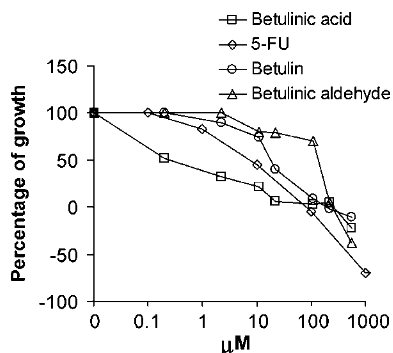

Figure 3. Effects of different concentrations of pure compounds (betulinic acid, betulinic aldehyde, betulin, and 5-FU) on MCF-7 cell growth evaluated by MTT assay. Each value is the average of four wells. SDs were lower than 10% and were omitted for the sake of clarity.

interesting to note that betulinic acid in our test against MCF-7 cells was significantly more active than the positive control 5FU (GI_{50} = 5.34 μM), a well-known anticancer agent for clinical use (*36*). Betulinic acid was first reported to be selectively cytotoxic against melanoma cells (MEL-2, ED_{50} = 1.2 μg/mL) (*37*). More recent studies report **3** being cytotoxic also for other nonmelanoma human tumor cells (*24*) and is considered a promising anticancer candidate (*38*). Antitumor properties have been previously reported also for the triterpenoid acids **1** and **2** (*24*, *26*), the 2-hydroxy analogues **4**−**6**, the aldehydes **7**−**9**, and β-sitosterol 3-*O*-glucoside (**10**) (*33*, *39*−*41*). Compounds **2** (*42*), **3** (*28*), **5** (*43*), and **10** (*44*), in particular, have been tested on MCF-7 cells. Structure−activity relationship studies have been reported for betulinic acid and some closely related compounds. The carboxylic function of these triterpenoids is reported as important for membrane permeability and consequently for bioactivity (*45*). A comparative study reports comparable B16 2F2 melanoma cell growth inhibition values for aldehydes **7**−**9** and suggests that the carbonyl function is important for bioactivity (*33*). On the basis of our data, the 2-hydroxy analogues **4**−**6**, as well as aldehydes **7**−**9** have a comparable activity so their structural differences seem to have little influence on the activity. **Figure 3** reports the dose−response curves for the betulinic acid family (compounds **3**, **7**, and **11**). It is worth noting that growth-inhibiting activity dramatically decreases in the order **3** > **11** > **7**, thus confirming the importance of the carboxylic function. Betulin (**11**), a compound easily available from white birch bark (*46*), shows approximately one-third activity with respect to 5FU against MCF-7 cells, but it is noticeably more active than the related triterpenoid compounds here examined, with the exception of **3**. Previously reported data on mouse melanoma B16 2F2 cells follow an opposite trend (*33*), thus indicating that additional studies are needed to establish unambiguous structure−activity relationships for these related triterpenoids. Betulinic acid (**3**) is confirmed as the most important member of this family of related compounds and the terminal moiety, including a five-membered ring, an isopropenyl chain, and a carboxylic function appear to be the reason for its higher antitumor activity. In addition to the above-reported activity, **3** is under active study as HIV and cancer chemopreventive principle and also displays further biological activities, among them antiinflammatory and in vitro antimalarial activity (*24*). It is worth noting here that **3** has a favorable therapeutic index and showed no toxic effects in mice at doses up to 500 mg/kg (*37*). Hepatoprotective, antiinflammatory, and antihyperlipidemic properties have been ascribed to oleanolic acid and ursolic acid, and more recently, both compounds have been reported as antitumor promotion agents (*26*). These triterpenoids have been used also in the cosmetic field (*47*, *48*).

This is the first analysis of Sicilian almond hulls. On the whole, the data reported confirm that this waste material is a rich source of bioactive compounds. Among them, the minor constituents **4**−**9** have not been previously reported from almond hulls or even from *P. dulcis*. Almond hulls may be a convenient source of betulinic acid: An amount of at least 703 mg/kg dried hulls of betulinic acid (**3**) could be obtained from this material. Although white birch bark is a good source of betulin (**11**), it contains a considerably lower level of **3** (*46*) and chemical conversion of **11** is normally required to obtain **3** (*49*). In addition, a total amount of at least 3930 mg/kg dried hulls of oleanolic/ursolic acids is obtainable from this material, with an estimated 65% of ursolic acid. In almond crop areas, like Sicily, hulls are easily available and their exploitation may contribute to reduce disposal costs for the almond agroindustry.

ACKNOWLEDGMENT

We thank Dr. Giuseppe Fortunato for his contribution to this work.

LITERATURE CITED

(1) Northup, R. R.; Dahlgren, R. A.; McColl, J. G. Polyphenols as regulators of plant-litter-soil interactions in northern California's pygmy forest: A positive feedback? *Biogeochemistry* **1998**, *42*, 189−220.

(2) Aliotta, G.; Cafiero, G.; De Feo, V.; Di Blasio, B.; Iacovino, R.; Oliva, A. Allelochemicals from rue (*Ruta* graveolens L.) and olive (*Olea europaea* L.) oil mill waste waters as potential natural pesticides. *Curr. Top. Phytochem.* **2000**, *3*, 167−177.

(3) Laufenberg, G.; Kunz, B.; Nystroem. M. Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations. *Bioresour. Technol.* **2003**, *87*, 167−198.

(4) Neri, P., Tringali, C. Applications of modern NMR techniques in the structural elucidation of bioactive natural products. In *Bioactive Compounds from Natural Sources*; Tringali, C., Ed.; Taylor & Francis: London, United Kingdom, 2001; pp 70−127.

(5) Amico, V.; Napoli, E. M.; Renda, A.; Ruberto, G.; Spatafora, C.; Tringali, C. Constituents of grape pomace from the Sicilian cultivar 'Nerello Mascalese'. *Food Chem.* **2004**, *88*, 599−607.

(6) FAO STAT Database in www.fao.org; accessed 05/12/05.

(7) http://www.euroinfosicilia.it/; accessed 05/12/05.

(8) Takeoka, G.; Dao, L.; Teranishi, R.; Wong, R.; Flessa, S.; Harden, L.; Edwards, R. Identification of three triterpenoids in almond hulls. *J. Agric. Food. Chem.* **2000**, *48*, 3437−3439.

(9) Di Blasi, C.; Tanzi, V.; Lanzetta, M. A study on the production of agricultural residues in Italy. *Biomass Bioenergy* **1997**, *12*, 321−331.

(10) Singh, R. B.; Dubnov, G.; Niaz, M. A.; Ghosh, S.; Singh, R.; Rastogi, S. S.; Manor, O.; Pella, D.; Berry, E. M. Effect of an Indo-Mediterranean diet on progression of coronary artery disease in high risk patients (Indo-Mediterranean Diet Heart Study): A randomised single-blind trial. *Lancet* **2002**, *360*, 1455−1461.

(11) Jenkins, D. J. A.; Kendall, C. W. C.; Marchie, A.; Faulkner, D.; Vidgen, E.; Lapsley, K. G.; Trautwein, E. A.; Parker, T. L.; Josse, R. G.; Leiter, L. A.; Connelly, P. W. The effect of combining plant sterols, soy protein, viscous fibers, and almonds in treating hypercholesterolemia. *Metabolism* **2003**, *52*, 1478−1483.

(12) Spiller, G. A.; Jenkins, D. A. J.; Bosello, O.; Gates, J. E.; Cragen, L. N.; Bruce, B. Nuts and plasma lipids: An almond-based diet lowers LDL-C while preserving HDL-C. *J. Am. Coll. Nutr.* **1998**, *17*, 285−290.

(13) Davis, P. A.; Iwahashi, C. K. Whole almonds and almond fractions reduce aberrant crypt foci in a rat model of colon carcinogenesis. *Cancer Lett.* **2001**, *165*, 27−33.

(14) Sang, S.; Li, G.; Tian, S.; Lapsley, K.; Stark, R. E.; Pandey, R. K.; Rosen, R. T.; Ho, C.-T. An unusual diterpene glycoside from the nuts of almond (*Prunus amygdalus* Batsch). *Tetrahedron Lett.* **2003**, *44*, 1199−1202.
(15) Sang, S.; Kikuzaki, H.; Lapsey, K.; Rosen, R. T.; Nakatani, N.; Ho, C.-T. Sphingolipid and other constituents from almond nuts (*Prunus amygdalus* Batsch). *J. Agric. Food. Chem.* **2002**, *50*, 4709−4712.
(16) Rawat, M. S. M.; Pant, G.; Kukreti, R.; Sakakibara, J.; Nagatsu, A. A persicogenin 3′-glucoside from the stem bark of *Prunus amygdalus*. *Phytochemistry* **1995**, *38*, 1519−1520.
(17) Chen, C.-Y.; Milbury, P. E.; Lapsley, K.; Blumberg, J. B. Flavonoids from almond skins are bioavailable and act synergistically with vitamins C and E to enhance hamster and human LDL resistance to oxidation. *J. Nutr.* **2005**, *135*, 1366−1373.
(18) Sang, S.; Lapsley, K.; Jeong, W.-S.; Lachanche, P. A.; Ho, C.-T.; Rosen, R. T. Antioxidative phenolic compounds isolated from almond skins (*Prunus amygdalus* Batsch). *J. Agric. Food. Chem.* **2002**, *50*, 2459−2463.
(19) Buttery, R. G.; Seifert, R. M.; Haddon, W. F.; Lundin, R. E. 2-Hexyl-3-methylmaleic anhydride: an unusual volatile component of raisins and almond hulls. *J. Agric. Food. Chem.* **1980**, *28*, 1336−1338.
(20) Buttery, R. G.; Soderstrom, E. L.; Seifert, R. M.; Ling, L. C.; Haddon, W. F. Components of almond hulls: Possible navel orangeworm attractants and growth inhibitors. *J. Agric. Food. Chem.* **1980**, *28*, 353−356.
(21) Takeoka, G. R.; Dao, L. T. Antioxidant constituents of almond [*Prunus dulcis* (Mill.) D. A. Webb] hulls. *J. Agric. Food. Chem.* **2003**, *51*, 496−501.
(22) Sang, S.; Cheng, X.; Fun, H.-Y.; Shieh, D.-E.; Bai, N.; Lapsley, K.; Stark, R. E.; Rosen, R. T.; Ho C.-T. New type sesquiterpene lactone from almond hulls (*Prunus amygdalus* Batsch). *Tetrahedron Lett.* **2002**, *43*, 2547−2549.
(23) Sang, S.; Lapsley, K.; Rosen, R. T.; Ho, C.-T. New prenylated benzoic acid and other constituents from almond hulls (*Prunus amygdalus* Batsch). *J. Agric. Food. Chem*. **2002**, *50*, 607−609.
(24) Cichewicz, R. H.; Kouzi, S. A. Chemistry, biological acyivity, and chemotherapeutic potential of betulinic acid for the prevention and treatment of cancer and HIV infection. *Med. Res. Rev.* **2004**, *24*, 90−114.
(25) Aiken, C.; Chen, C. H. Betulinic acid derivatives as HIV-1 antivirals. *Trends Mol. Med.* **2005**, *11*, 31−36.
(26) Liu, J. Pharmacology of oleanolic and ursolic acid. *J. Ethnopharm.* **1995**, *49*, 57−68.
(27) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(28) Urban, M.; Sarek, J.; Klinot, J.; Korinkova, G.; Hajduch, M. Synthesis of A-seco derivatives of betulinic acid with cytotoxic activity. *J. Nat. Prod.* **2004**, *67*, 1100−1105.
(29) Seebacher, W.; Simic, N.; Weis, R.; Saf, R.; Kunert, O. Complete assignments of ^{1}H and ^{13}C NMR resonances of oleanolic acid, 18α-oleanolic acid, ursolic acid and their 11-oxo derivatives. *Magn. Reson. Chem.* **2003**, *41*, 636−638.
(30) Peng, C.; Bodenhausen, G.; Qiu, S.; Fong, H. H. S.; Farnsworth, N. R.; Yuan, S.; Zheng, C. Computer-assisted structure elucidation: Application of CISOC-SEC to the resonance assignment and structure generation of betulinic acid. *Magn. Reson. Chem.* **1998**, *36*, 267−278.
(31) Lee, S.-M.; Park, J.-G.; Lee, Y.-H.; Lee, C.-G.; Min, B.-S.; Kim, J.-H.; Lee, H.-K. Anti-complementary activity of triterpenoides from fruits of *Zizyphus jujube*. *Biol. Pharm. Bull.* **2004**, *27*, 1883−1886.
(32) Taniguchi, S.; Imayoshi, Y.; Kobayashi, E.; Takamatsu, Y.; Ito, H.; Datano, T.; Sakagami, H.; Tokuda, H.; Nishino, H.; Sugita, D.; Shimura, S.; Yoshida, T. Production of bioactive triterpenes by *Eriobotrya japonica* calli. *Phytochemistry* **2002**, *59*, 315−323.
(33) Hata, K.; Hori, K.; Takahashi, S. Differentiation and apoptosis-inducing activities by pentacyclic triterpenes on a mouse melanoma cell line. *J. Nat. Prod.* **2002**, *65*, 645−648.
(34) Hota, R. K.; Bapuji, M. Triterpenoids from the resin of *Shorea robusta*. *Phytochemistry* **1994**, *35*, 1073−1074.
(35) Zhang, Y.; Jayaprakasam, B.; Seeram, N. P.; Olson, L. K.; DeWitt, D.; Nair, M. G. Insulin secretion and cyclooxygenase enzyme inhibition by Cabernet Sauvignon grape skin compounds. *J. Agric. Food Chem.* **2004**, *52*, 228−233.
(36) Erman, M.; Baltali, E.; Karaoglu, A.; Abali, H.; Engin, H.; Ozisik, Y.; Guler, N.; Altundag, K.; Tekuzman, G.; Atahan, I. L.; Onat, D.; Sayek, I. A phase II study on the safety and efficacy of 5-fluorouracil, epirubicin, cyclophosphamide (FEC) followed by paclitaxel in the adjuvant treatment of breast cancer. *Cancer Invest.* **2005**, *23*, 215−221.
(37) Pisha, E.; Chai, H.; Lee, I. S.; Chagwedera, T. E.; Farnsworth, N. R.; Cordell, G. A.; Beecher, C. W.; Fong, H. H.; Kinghorn, A. D.; Brown, D. M.; Wani, M. C.; Wall, M. E.; Hieken, T. J.; Das Gupta, T. K.; Pezzuto, J. M. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nat. Med.* **1995**, *1*, 1046−1051.
(38) Eiznhamer, D. A.; Xu, Z.-Q. Betulinic acid: A promising anticancer candidate. *IDrugs* **2004**, *7*, 359−373.
(39) Banno, N.; Akihisa, T.; Tokuda, H.; Yasukawa, K.; Higashihara, H.; Ukiya, M.; Watanabe, K.; Rimura, Y.; Hasegawa, J.; Nishino, H. Triterpene acids from the leaves of *Prilla frutescens* and their antiinflammatory and antitumor-promoting effects. *Biosci., Biotechol., Biochem.* **2004**, *68*, 85−90.
(40) Yoshida, M.; Fuchigami, M.; Nagao, T.; Okabe, H.; Matsunaga, K.; Tarata, J.; Karube, Y.; Tsuchihahashi, R.; Kinjo, J.; Mihashi, K.; Fujioka, T. Antiproliferative constituents from umbelliferae plants VII. Active triterpenes and rosmarinic acid from *Centella asiatica*. *Biol. Pharm. Bull.* **2005**, *28*, 173−175.
(41) Kim, Y.-K.; Yonn, S. K.; Ryu, S. Y. Cytotoxic triterpenes from stem bark of *Physocarpus intermedius*. *Planta Med.* **2000**, *66*, 485−486.
(42) Es-Saady, D.; Simon, A.; Jayat-Vignoles, C.; Chulia, A. J.; Delage, C. MCF-7 cell cycle arrested at G1 through ursolic acid and increased reduction of tetrazolium salts. *Anticancer Res.* **1996**, *16*, 481−486.
(43) Aburjai, T.; Amro, B. I.; Al-Khalil, S.; Alkofahi, A. Bioactive triterpenes from *Arbutus andrachne*. *Alexandria J. Pharm. Sci.* **1999**, *13*, 115−118.
(44) Ju, Y. H.; Clausen, L. M.; Allred, K. F.; Almada, A. L.; Helferich, W. G. β-Sitosterol, β-sitosterol glucoside, and a mixture of β-sitosterol and β-sitosterol glucoside modulate the growth of estrogen-responsive breast cancer cells in vitro and in ovariectomized athymic mice. *J. Nutr.* **2004**, *134*, 1145−1151.
(45) Hu, M.; Konoki, K.; Tachibana, K. Cholesterol-independent membrane disruption caused by triterpenoid saponins. *Biochim. Biophys. Acta* **1996**, *1299*, 252−258.
(46) Galgon, T.; Hoke, D.; Drager, B. Identification and quantification of betulinic acid. *Phytochem. Anal.* **1999**, *10*, 187−190.
(47) Kuno, N.; Shinohara, G. Process for producing fat compositions containing oleanolic acid and/or maslinic acid. PCT Int. Appl. 2003, 38 pp; CODEN: PIXXD2 WO 2003029391 A1 20030410.
(48) Courtin, O. Cosmetic composition for care of sensitive skin containing oleanolic acid. Fr. Demande 2002, 13 pp; CODEN: FRXXBL FR 2811224 A1 20020111.
(49) Pichette, A.; Liu, H.; Roy, C.; Tanguay, S.; Simard, F.; Lavoie, S. Selective oxidation of betulin for the preparation of betulinic acid, an antitumoral compound. *Synth. Commun.* **2004**, *34*, 3925−3937.

Received for review November 11, 2005. Revised manuscript received December 7, 2005. Accepted December 7, 2005. This work was financially supported by Ministero della Pubblica Istruzione, Università e Ricerca Scientifica (PRIN 2003, Rome, Italy) and the University of Catania (Progetti di Ricerca di Ateneo, Catania, Italy).

JF052812Q