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Unusual Immuno-Modulatory Triterpene-Caffeates in the Skins of Russeted Varieties of Apples and Pears

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ABSTRACT: Three triterpene-caffeates have been isolated from skins of a russeted apple cultivar "Merton Russet" and identified by LC−MS and NMR as betulinic acid-3-*cis*-caffeate, betulinic acid-3-*trans*-caffeate, and oleanolic acid-3-*trans*-caffeate. Betulinic acid-3-*trans*-caffeate and oleanolic acid-3-*trans*-caffeate were also found in russeted pear skins. These compounds have not been previously reported in apples or pears, or in any other foods. Their presence was related to suberized tissue as they were only found in russet portions of the partially russeted apple cultivar "Cox's Orange Pippin" and were not detected in the waxy apple cultivar "Royal Gala". High concentrations of betulinic acid-3-*trans*-caffeate were found in the bark of both "Merton Russet" and "Royal Gala" trees. The three triterpene-caffeates showed anti-inflammatory activity in vitro, inhibiting NF-κB activation with IC_{50}'s of 6−9 μM. Betulinic acid-3-*trans*-caffeate, the predominant compound in the apples, was immuno-modulatory at around 10 μM in the in vitro and ex vivo bioassays, boosting production of the pro-inflammatory cytokine TNFα in cells stimulated with bacterial lipopolysaccharides.

KEYWORDS: *apple, pear, russet, triterpene, betulinic acid, betulinic acid-3-O-caffeate, inflammation, NF-κB, TNFα*

INTRODUCTION

Apple (*Malus X domestica* Borkh.) is one of the most popular fruits around the world,[1] and epidemiological studies have linked the consumption of apples with reduced risk of various diseases.[2] Apple skin is known to contain large amounts of triterpenes (up to 60 mg per apple).[3] Although these triterpenes occur in apples at concentrations similar to those of phenolic compounds,[3] their likely contribution to the health benefits associated with apple consumption is often ignored or underestimated. Pentacyclic triterpenes have been associated with numerous pharmacological properties, including modulation of inflammation and anticancer effects in vitro and in vivo.[4−7] The potential of some triterpenes as therapeutic anticancer agents is currently being evaluated in clinical trials.[5,6]

Triterpenes are terpenoids produced in plants by cyclization of 2,3-oxidosqualene,[8] with approximately 20 000 triterpenes identified to date. The ursane (ursolic acid, uvaol, α-amyrin), oleanane (oleanolic acid, erythrodiol, β-amyrin) and lupane (betulinic acid, betulin, lupeol) series of pentacyclic triterpenes derived from α-amyrin, β-amyrin, and lupeol, respectively, are the most widely distributed pentacyclic triterpenes in plants.[9] They occur particularly in the waxy coating of leaves and fruits, along with long chain fatty acids and additional secondary metabolites, such as sterols and phenolic compounds.[10] Triterpenes are included in the epicuticular and intracuticular wax (embedded in cutin polymer) of the plant cuticle.[10]

Apple skin with its cuticle plays an important functional role in the protection of the fruit, acting as the interface with the external environment. The waxy cuticle protects the plant from water loss and bacterial and fungal attacks[10−12] and provides a physical and chemical barrier against pests.[13] The skin of apples from most cultivars is composed of several layers including an outer cuticular wax layer and one or more underlying layers of epidermal cells.[14] However, some old heritage cultivars such as "Merton Russet" or "Egremont Russet" present a russeted skin (pear-type skin), characterized by a thinner wax layer and the presence of cork (suberin) in epidermal cells as a reaction to the death of epidermal tissue.[14,15] Although it appears naturally on those varieties, russeting is seen as a defect in most commercial cultivars. It results in significant economic losses for the growers, as russeted fruit is downgraded during packing and marketing because consumers prefer smooth- or waxy-skinned apples.

In our previous study on the triterpene concentrations (ursolic, oleanolic, and betulinic acid) in 109 apple cultivars,[3] we noted that all of the russeted cultivars had a different triterpene pattern from their waxy counterparts, characterized

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by a higher proportion of betulinic acid (BA). We now report on the triterpene profile of russeted apple skin in detail, with the discovery, for the first time in apples, of three triterpene-caffeates whose identities were confirmed by NMR spectroscopy. The predominant component was betulinic acid-3-*trans*-caffeate (BA-transC). After the preparation of a larger sample by chemical synthesis, we showed that BA-transC had anti-inflammatory and immuno-modulatory properties in two in vitro assays and one ex vivo assay.

MATERIALS AND METHODS

Chemicals. Solvents of analytical or high-performance liquid chromatography (HPLC) grade were obtained from Thermo Fisher Scientific (Auckland, New Zealand). Ursolic acid (UA) and oleanolic acid (OA) were purchased from Sigma-Aldrich (St. Louis, MO). Betulin and betulinic acid (BA) were obtained from ExtraSynthese (Genay, France). Betulinic acid-3-*trans*-caffeate(BA-transC) was prepared from caffeic acid and BA as described by Tanachatchairatana et al.[16] Compound purity was >95% by HPLC, and identity was confirmed by ^{1}H and ^{13}C NMR spectra matching published data for BA-transC.[17]

HPLC-DAD Analysis. Analysis was carried out on an Agilent HP1100 using a Phenomenex C18 (2) Luna (250 × 4.6 mm i.d.; 5 μm particle size) (Torrance, CA, USA) column. The mobile phase was MeOH:H_2O:phosphoric acid (88:11.95:0.05, v/v/v). Injection volume was 20 μL. Triterpenes were eluted isocratically at a flow rate of 1 mL min^{-1} and a column temperature of 35 °C. Identification and quantification of triterpenes was performed as described in Andre et al.[3] UA, OA, BA, as well as BA-transC were identified by their retention time and spectral data as compared to authentic standards and were quantified at 210 nm (UA, OA, and BA) and 320 nm (BA-transC) using five-point calibration curves. Excellent linearity (R^2 > 0.99) was obtained in the concentration range 100−6.25 μg mL^{-1} for all compounds. BA-cisC and OAC were quantified at 320 nm as BA-transC equivalent.

LCMS Analysis. Analysis was performed using a LCQ Deca ion trap mass spectrometer fitted with an atmospheric pressure chemical ionization interface (ThermoQuest, Finnigan, San Jose, CA) and coupled to a Surveyor HPLC instrument. A 20 μL aliquot was injected onto a Phenomenex Prodigy ODS (3) column (150 × 2.0 mm i.d.; 5 μm particle size). The mobile phases were (A) H_2O with 0.1% formic acid and (B) MeCN with 0.1% formic acid. The flow rate was 0.2 mL min^{-1}, and the column temperature was 35 °C. The 37 min gradient was as follows: 0−20 min, 80−100% B; 20−22 min, 100−80% B; 22−37 min, 80% B, re-equilibration time. Spectra were recorded in negative ion mode between 150 and 2000 atomic mass units.

Semipreparative HPLC. Separation was performed using a Waters instrument with a Phenomenex Luna C18 semipreparative column (250 × 10 mm i.d.; 5 μm particle size) equipped with a LiChrospher C18 guard column (25 × 10 mm). The mobile phase was MeOH:H_2O:phosphoric acid (88:11.95:0.05, v/v/v). The flow rate was 4.5 mL min^{-1}, and the column temperature was 25 °C.

NMR Analysis. ^{1}H and ^{13}C NMR spectra were acquired for acetone-d_6 solutions on a Varian AR 500 spectrometer.

Plant Material. Apple cultivars "Merton Russet", "Royal Gala", and "Cox's Orange Pippin" were grown in Hawke's Bay (New Zealand), and fruits were harvested between March and April 2010 as they matured on the tree. Each fruit was then cut into quarters and four slices (~0.5 cm at the skin edge) were cut off, avoiding the core and seeds. Each segment was peeled, and the skin and the flesh were separately frozen in liquid nitrogen. Samples were stored at −80 °C until analysis. Branches (~1 cm diameter) of apple trees ("Merton Russet" and "Royal Gala") were collected in Hawke's Bay in May 2010. The outer bark was removed and powdered under liquid N_2, then kept at −80 °C until analysis.

Three pear cultivars, "Taylors Gold", "Doyenne du Comice", and selection P267R223T070, were selected as they presented full, partial, or no russeting, respectively. They were grown in Nelson (New Zealand), and fruits were harvested in May 2010. Pears were sampled as described for apple cultivars.

Sample Preparation. Powdered fresh material (100 mg) was mixed with EtOAc:hexane (1 mL, 50:50 v/v) (ratio 1:10). This mixture was then homogenized using a vortex for 30 s and shaken for 1 h at room temperature. After centrifugation at 10 000*g* for 15 min, the supernatant was collected and evaporated to dryness using a centrifugal vacuum evaporator. The pellet was re-extracted using EtOH:H_2O (1 mL, 80:20 v/v), homogenized and shaken for 2 h at room temperature and centrifuged as above. The supernatant was collected, combined with the lipophilic dried extract, and evaporated to dryness. Triterpenes were resuspended in EtOH (1 mL) and filtered (0.45 μm) before HPLC analysis. Two subsamples (biological replicates) of skin and bark were taken. For each of these subsamples, duplicate extractions and HPLC analyses (technical replicates) were done. Therefore, each reported concentration is the average of four values.

Isolation of Triterpene-Caffeates. Freeze-dried "Merton Russet" skin (50 g) was extracted with EtOAc:hexane (500 mL, 50:50) for 2 h, filtered, and dried. The marc was further extracted with EtOH:H_2O (500 mL, 95:5, v/v) overnight. The EtOH extract was then filtered and added to the EtOAc−hexane extract. The combined extract was coated onto C18 octadecyl-functionalized silica gel (5 g) by rotary evaporating at 30 °C and then applied to preconditioned C18 Isolute SPE cartridges for fractionation.[3] Elution with 2 × 10 mL each of H_2O, EtOH:H_2O (1:4), EtOH:H_2O (1:1), EtOH:H_2O (4:1), EtOH, and EtOAc gave twelve 10 mL fractions. Triterpene-rich fractions (fractions 8, 9, and 10) were combined and separated by column chromatography over silica gel, eluting with 0−50% MeOH in $CHCl_3$ to give 40 fractions. These were checked for the presence of triterpene-caffeates by TLC (using ferric chloride) first, then by HPLC-DAD. Fractions 6−8 (12 mg) contained caffeates BA-cisC and BA-transC, and fractions 18−21 (8 mg) contained OAC. Final purification was by semipreparative HPLC to give betulinic acid-3-*cis*-caffeate (retention time 16.78 min, 3 mg), registry number [103942-05-8]; betulinic acid-3-*trans*-caffeate (17.83 min, 3 mg) [80832-44-6]; and oleanolic acid-3-*trans*-caffeate (18.57 min, 1 mg) [97534-10-6] identified by the comparison of ^{1}H and ^{13}C NMR data with published data.[17−20]

Cell Culture. NF-kB/SEAP HEK 293 cells (Imgenex, San Diego, CA) were propagated in standard MEM medium (Invitrogen, Auckland) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) and 500 μg/mL G418 (Sigma-Aldrich, St. Louis, MO) at 37 °C in a humidified 5% CO_2 atmosphere. Cells were passaged three times a week.

Human monocytes THP-1 cells (Invitrogen, Auckland, NZ) were obtained from the American Type Culture Collection. They were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) at 37 °C in a humidified 5% CO_2 atmosphere. Media was replaced every 2−3 days and cells subcultured to a density of 2−3 × 10^5 cells mL^{-1} when cell density reached ~8 × 10^5 cells mL^{-1}.

NF-κB Assay. The assay was performed as described in Andre et al.[3] Briefly, HEK 293 cells were stably transfected with a reporter plasmid expressing the secreted alkaline phosphatase (SEAP) gene under the control of an NF-κB-inducible promoter. Cells were exposed to triterpene-caffeates (six-point concentration curve, from 1.56 to 50 μM) for 1 h before being stimulated with TNFα (final concentration of 10 ng mL^{-1}). Cells were then incubated with both compounds and TNF-α for a further 6 h before 20 μL of cell supernatant was transferred into a 96-well plate containing 100 μL of Quanti-Blue reagent (Invitrogen, San Diego, CA) for the detection of SEAP production. Data were expressed as percentage inhibition of NF-κB activation by TNFα, using cells treated with TNFα only as control. In the range of tested concentrations, cell viability was not affected for all tested compounds (data not shown), confirming that the observed effects were due to the anti-NF-κB activity and not to cell death.

Measurement of TNFα Production. *In Vitro Using THP-1 Cells.* THP-1 cells were first differentiated into macrophages to produce detectable amounts of TNFα.[21] To maturate, cells were suspended at a concentration of 2 × 10^5 cells mL^{-1}, and 5 nM PMA was added. Cells

were plated at 1 mL per well in a 24-well plate and incubated at 37 °C for 3 days. Media was then removed and replaced with 0.5 mL of media containing an appropriate concentration of BA-transC for 1 h. Five micrograms milliliter^{-1} LPS was then added to the mixture for 4 h. Supernatants were collected and stored at −80 °C for TNFα analysis. The levels of TNFα were measured by enzyme-linked immunosorbent (ELISA) assay (BD Biosciences, San Jose, CA), following the manufacturer's instructions. Absorbance at 450 and 650 nm (reference wavelength) was measured using a microplate reader. TNFα concentrations were determined using a six-point calibration curve from 0 to 1000 pg TNFα mL^{-1}. Cytotoxicity was not detected for any of the tested compounds (data not shown) using the resazurin reduction test.[22]

Ex Vivo Using Whole Blood. Blood samples were collected in heparinized tubes from four healthy volunteers (two men and two women, aged between 30 and 45 years). The blood was processed immediately and transferred to 96 deep well plates. In each well, 200 μL of blood and 260.8 μL of RPMI 1640 medium or medium containing appropriately diluted triterpene samples were added, and the plates were incubated for 30 min at 37 °C in a humidified 5% CO_2 atmosphere. LPS (1 μg mL^{-1}; 51.2 μL in media; LPS from *Escherichia coli* 0111:B4, Invitrogen, San Diego, CA) or LPS-free media (51.2 μL) was then added and mixed by gentle pipetting. Plates were incubated for 18 h at 37 °C with 5% CO_2. Plates were then centrifuged at 900*g* for 10 min, and the supernatants were collected. TNFα concentrations were evaluated by ELISA assay as described above. For this experiment, ethical approval was given by the New Zealand Northern Y Regional Ethics Committee (reference NTY/06/09/082), and written informed consent was obtained from each subject.

Statistical Analysis. The significance of differences between mean measurements was evaluated using a Student *t* test. The software Minitab was used for all analyses.

RESULTS AND DISCUSSION

Discovery of Triterpene-Caffeates in Apples. The triterpene profiles of the skins of most waxy apple cultivars are dominated by ursolic and oleanolic acid.[3] In our previous study,[3] russeted varieties from both New Zealand and Luxembourg revealed a different triterpene chemical pattern from that of their waxy counterparts (Table 1), characterized by (i) a higher proportion of betulinic acid and (ii) the presence of unknown russet-specific compounds (Figure 1A) in an HPLC screen. In the present study, we investigated the identity of those compounds in the skin of the cultivar "Merton Russet" pictured in Figure 2 and confirmed their identity by LCMS and NMR.

Table 1. Triterpene and Triterpene-Caffeate Concentrations in Skins of "Merton Russet" and "Royal Gala" Apples[a]

		triterpene concentration (nmol g^{-1} FW)	
compounds	abbreviations	"Merton Russet"	"Royal Gala"
betulin (**1**)[b]	BE	87.8 ± 19.3	nd[e]
betulinic acid (**2**)[b]	BA	264.5 ± 67.4	100.8 ± 43.8
betulinic acid-3-*cis*-caffeate (**3**)[c]	BA-cisC	24 ± 3.9	nd
oleanolic acid (**5**)[b]	OA	103.3 ± 21.4	1157.5 ± 409.7
ursolic acid (**4**)[b]	UA	97.9 ± 18.0	2974.8 ± 1583.9
betulinic acid-3-*trans*-caffeate (**6**)[d]	BA-transC	130.6 ± 30.4	nd
oleanolic acid-3-*trans*-caffeate (**7**)[c]	OAC	12.8 ± 3.3	nd

[a]Data are expressed in nmol g^{-1} of fresh weight (FW). For each compound, the mean value represents apple fruit skin analyses from two different trees. Numbers in bold refer to the peaks in Figure 1. [b]Identity was confirmed by LC−MS and a commercial standard. [c]Identity was confirmed by LC−MS and NMR. [d]Identity was confirmed by LC−MS, NMR, and a chemically synthesized standard. [e]Not detected.

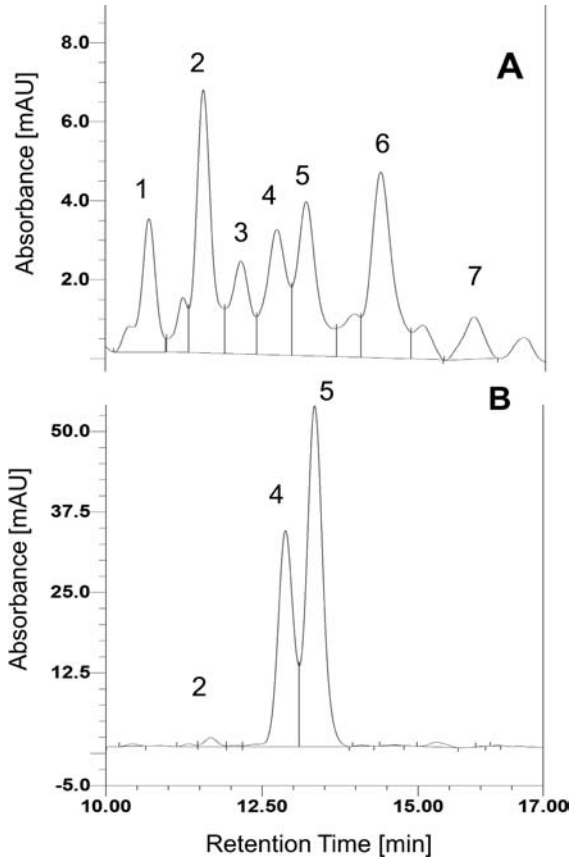

Figure 1. Triterpene HPLC profile of "Merton Russet" (A) and "Royal Gala" (B) skin extracts recorded at 210 nm. See Table 1 for peak identification.

Figure 2. Apples from the cultivars "Merton Russet" (top) and "Royal Gala" (bottom).

Peak 1 (Figure 1) was identified as betulin by LCMS on the basis of a molecular ion $[M - H]^-$ at m/z 441 and a retention time identical to that of authentic betulin. To our best knowledge, this is the first report of the presence of betulin in apple skin. Peaks 2, 4, and 5 had similar mass spectra, with molecular ions $[M - H]^-$ at m/z 455, and were identified as

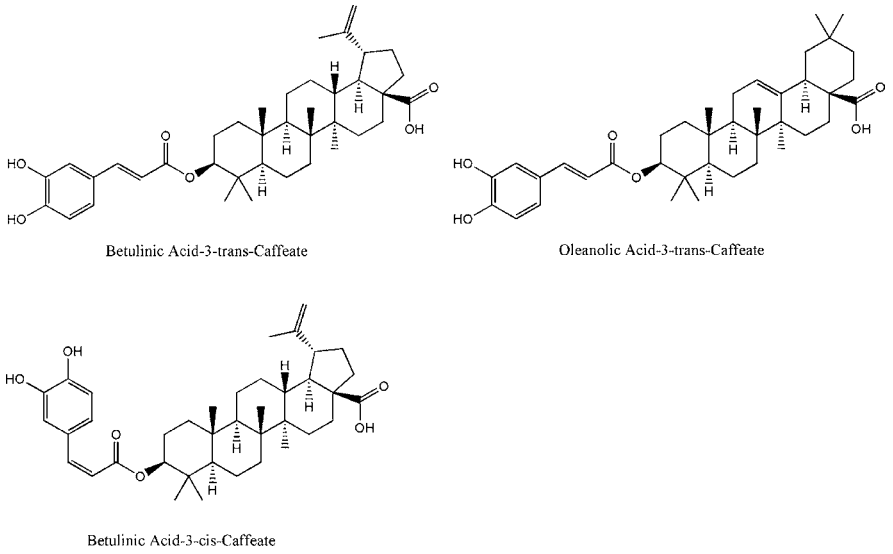

Figure 3. Structures of the triterpene-caffeates identified in the russeted skin of the apple cultivar "Merton Russet".

betulinic, ursolic, and oleanolic acids, respectively, by comparison with authentic standards. Peak 3, 6, and 7 had similar UV spectra, with λ_{max} at 325 nm, supporting the presence of a hydroxycinnamoyl functionality. They all exhibited molecular ions $[M - H]^-$ at m/z 617.4 and fragment ions at m/z 573 and 161, suggesting triterpene acids esterified with caffeic acid. To identify these compounds, they were isolated by RP C18 chromatography, then silica gel column chromatography, followed by semipreparative HPLC. Betulinic acid-3-*cis*-caffeate (peak **3**, BA-cisC), betulinic acid-3-*trans*-caffeate (peak **6**, BA-transC), and oleanolic acid-3-*trans*-caffeate (peak **7**, OAC) (Figure 3) were identified by comparison of ^{1}H and ^{13}C NMR data with published data.[17−20]

Triterpene-caffeates have never been reported in apple before. The predominant compound, betulinic acid-3-*trans*-caffeate (BA-transC), has only been reported in nondietary sources such as bark and leaves.[17−20,23−27] BA-transC was chemically synthesized as described by Tanachatchairatana et al.[16] both to quantify its content in apple tissue accurately and to test its efficacy in bioassays (see below).

Triterpene-Caffeate Concentrations in Apples, Bark, and Pears. Triterpene-caffeates could not be detected in the skin of the waxy apple cultivar "Royal Gala", whereas triterpene-caffeates made up about one-quarter of the total triterpenes in the skin of "Merton Russet" (Figure 1 and Table 1). Another apple cultivar, "Cox's Orange Pippin", has skin with both waxy and russeted tissues (Figure 4A). Lower amounts of UA and OA, higher amounts of BA, and the presence of triterpene-caffeates, especially BA-transC, were detected in the russeted parts of the skin (Figure 4B). Therefore, the presence of BA-transC is tissue-dependent and could be linked to the presence of suberized cells.

To investigate this further and because BA-transC has been previously described in the bark of other trees,[17,23] we analyzed the triterpenes in tree bark from both the waxy apple cultivar "Royal Gala" and "Merton Russet". The predominant triterpene in the bark of both cultivars was BA-transC (Figure 5). We could not find any published analyses of apple bark, but the concentration of BA-transC (ca. 8 μmol g^{-1} FW, or 0.5% w/w) is about 10 times the purified yield of BA-transC reported from birch bark.[17] Because bark is highly suberized, triterpene-caffeate production is thus directly linked to tissue suberization.

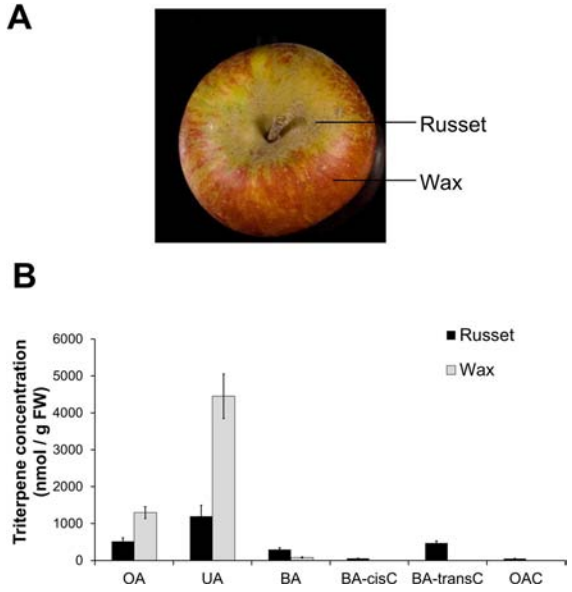

Figure 4. Apples from the cultivar "Cox's Orange Pippin" (A) and the concentrations of triterpenes (B) in the waxy and russeted skin.

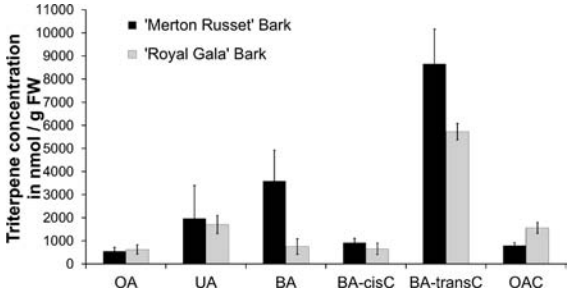

Figure 5. Triterpene concentrations in the barks of "Royal Gala" and "Merton Russet" apple trees.

An enzyme with a caffeoyl-CoA transferase activity, which would be activated in the suberization process,[28] might be involved and would need further investigation.

As russet skin is common on pears, we investigated the triterpene profile of three pear (*Pyrus communis*) cultivars with

different degrees of russeting. The pear triterpene pattern was similar to that of apple, and again we could detect increasing amounts of BA and triterpene-caffeates with increasing russeting, that is, suberization of the skin tissue (Figure 6).

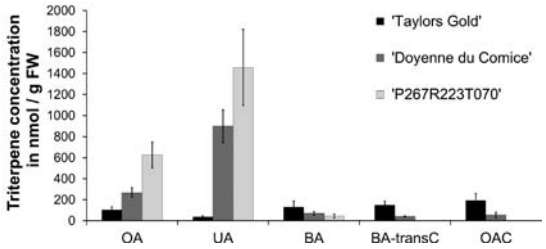

Figure 6. Triterpene concentrations in the skins of pear cultivars "Taylors Gold", "Doyenne du Comice", and the pear selection P267R223T070, presenting full, partial, or no russeting, respectively.

We could not find any previous reports of triterpene-caffeates in pears, although UA has been previously reported.[9] Interestingly, the cis form of betulinic acid-3-caffeate was not detected in pear russeted tissue.

Biological Activities of Triterpene-Caffeates. As noted above, triterpene-caffeates have never been described in apple previously, but BA-transC has been reported as contributing to the pharmacological effects of some medicinal plants. In particular, BA-transC showed anti-inflammatory properties when isolated from the leaves of *Pyracantha crenulata*,[18] from the aerial parts of *Bauhinia variegata*,[24] and from *Callistemon lanceolatus*.[25] BA-transC was also reported to have in vitro antimalarial[26] and anticancer[20,27] properties. It was the most cytotoxic compound isolated from the trunk of *Berberis koreana*[20] and the root bark of *Helicteres angustifolia*[27] against several human cancer cell lines. IC_{50} values ranged from 4.4 to 19.6 μM and were, at least, one-half those of the nonconjugated and well-studied BA, whose potential as an anticancer agents is currently being evaluated in clinical trials.[5]

The three triterpene-caffeates from "Merton Russet" skin were tested for their anti-inflammatory activity in one assay previously used on triterpene acids,[3] testing inhibition of activation of NF-kB, a key transcription factor in chronic inflammatory diseases.[29] Triterpene-caffeates were able to inhibit NF-kB activation, with IC_{50} values ranging from 6 μM (BA-cisC) to 9.1 μM (BA-transC) (Table 2). These values are similar to the IC_{50} values for triterpene acids that we previously reported: 12 μM for BA and 5 μM for OA.[3] The observed bioactivities could therefore be the result of the presence of intact triterpene-caffeates, liberated triterpenes, or other metabolites produced in the cell culture environment, as well as of complex interactions between these components. Other lupane-type triterpenes esterified with a hydroxycinnamic acid (ferulic acid) were also shown to inhibit NF-kB activity in the human hepatoma cell line HepG2, with a similar range of IC_{50} values.[31]

Table 2. Concentration Needed To Inhibit 50% of NF-κB Activation (IC_{50}) Induced by Inflammatory Cytokine TNFα[a]

compound	IC_{50} (μM)
betulinic acid-3-*cis*-caffeate	6.0 ± 1.2
betulinic acid-3-*trans*-caffeate	9.1 ± 1.0
oleanolic acid-3-*trans*-caffeate	7.1 ± 0.6

[a]Stably transfected HEK293 cells with an NF-kB/SEAP reporter gene constructs were used. Triterpene-caffeates were isolated from the skin of "Merton Russet" apple fruit. Mean IC_{50} values (±SD) were calculated from three independent experiments (n = 3).

Synthetic BA-transC was further investigated for its immunomodulatory effects using THP-1 cells. These monocytes, once differentiated into macrophages, are able to produce the pro-inflammatory cytokine TNFα when stimulated with bacterial lipopolysaccharides (LPS). Incubation of BA-transC (above 10 μM) significantly increased the amount of TNFα produced in LPS-treated THP-1 cells (p < 0.01) (Figure 7A). Similarly,

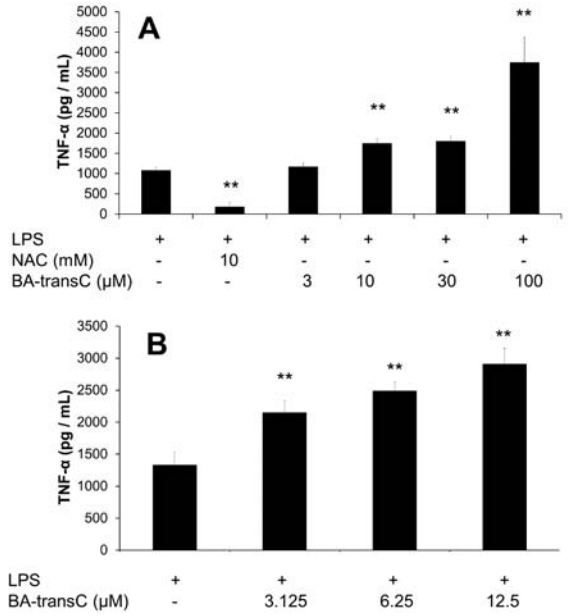

Figure 7. Production of TNFα induced by BA-transC in lipopolysaccharide (LPS) simulated THP-1 cells (A) and whole blood cells from four human volunteers (B). The symbol "**" indicates a significant difference as compared to the BA-transC-free positive control (P < 0.01). Experiments were repeated twice for the THP-1 cells and for each volunteer. Each measurement consisted of three (A) or two (B) technical replicates.

using LPS, we induced ex vivo inflammation on whole blood cells, which include, among others, leucocytes such as lymphocytes, monocytes, and macrophages. Again, we found a dose-dependent pro-inflammatory response, that is, a significant increase of TNFα secretion (p < 0.01), when adding BA-transC (Figure 7B). No significant effect of BA-transC was observed on LPS-free blood cells (data not shown). Taken together, these results suggest that BA-transC has some potential to stimulate the immune system, which may help to protect the organism from microbial or viral attacks. Immunostimulatory properties on LPS-treated human THP-1 cells and whole blood cells have also been shown for cycloartane- and lanostane-type triterpenes[32,33] and betulin.[34] On the other hand, using murine peritoneal macrophages, Rao et al.[24] showed that BA-transC had significant inhibitory effects on NO and TNFα production (IC_{50} = 10 μM), as it was described for other pentacyclic triterpenes such as maslinic acid.[30] An anti-inflammatory effect (reduction of NO production) for BA-transC was also observed in LPS-stimulated RAW264.7 murine macrophages by Jeong et al.[25]

Discrepancies between our results and some literature data, showing both anti- and pro-inflammatory effects of BA-transC, could be explained by (i) the different cell types used and their genetic background, and (ii) the different targets of the assays (for instance, NF-κB transcription versus TNFα production). While NF-κB and TNFα signaling pathways are intimately linked, their interactions are complex. There is increasing evidence that the expression of TNFα in LPS-stimulated macrophages is not directly initiated by NF-κB, although NF-κB may play a role in the maintenance or increase in TNFα expression after transcription is induced.[35] Additionally, contrasting anti- and pro-inflammatory activities have also been pointed out for UA and were suggested to be dependent on the biological status of the cells and tissues.[36]

In conclusion, in this study, we identified three triterpene-caffeates (Figure 3) in apples, and two of these in pears, that have not been previously reported in these fruits or in any other food source. Triterpene-caffeates of any sort appear to be rare in foods, although triterpene alcohol ferulates and caffeates have been found in rice bran.[37] We showed that the presence of the triterpene-caffeates was linked to suberization of the tissue, as they were detected in russeted apple skin, apple bark, and russeted pear skin, but not in waxy, nonsuberized apple skin. These compounds had immuno-modulatory properties. The bioavailability of triterpenes and their caffeate esters should be investigated, as little is known and bioavailability is essential for potential health-promoting properties through oral consumption. These results reinforce the importance of conserving old heritage apple varieties or pears as potential sources of previously unrecognized health-promoting compounds.

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Notes

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ABBREVIATIONS USED

NF-κB, nuclear factor-kappaB; TNFα, tumor necrosis factor α; IC, inhibitory concentration; PMA, phorbol 12-myristate 13-acetate; FW, fresh weight; BA, betulinic acid; OA, oleanolic acid; UA, ursolic acid; BA-cisC, betulinic acid-3-*cis*-caffeate; BA-transC, betulinic acid-3-*trans*-caffeate; OAC, oleanolic acid-3-*trans*-caffeate

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