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Anti-Inflammatory Procyanidins and Triterpenes in 109 Apple Varieties

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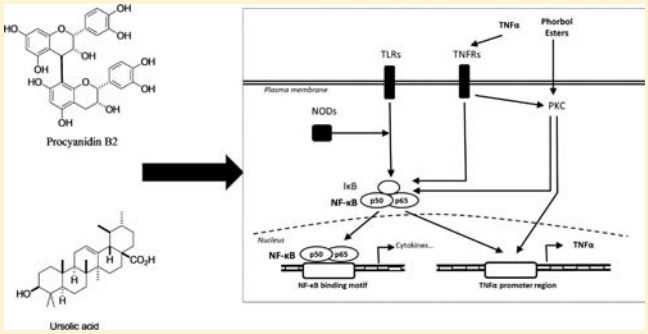
Supporting Information

ABSTRACT: We evaluated the potential of apple to reduce inflammation. Phenolic compounds and triterpenes were analyzed in 109 apple cultivars. Total phenolics ranged from 29 to 7882 μg g^{-1} of fresh weight (FW) in the flesh and from 733 to 4868 μg g^{-1} FW in the skin, with flavanols including epicatechin and procyanidins as major components. Ursolic (44.7 to 3522 μg g^{-1} FW) and oleanolic (47.2 to 838 μg g^{-1} FW) acids dominated the skin triterpene profile. Five chemically contrasting cultivars were fractionated and their immune-modulating activity measured using two cell-based assays targeting key points in the inflammation process. Cultivars exhibiting high contents of procyanidins were the most potent at inhibiting NF-κB while triterpene-rich fractions reduced the promoter activity of the gene of TNFα. This study provides new insights into how apple genetic diversity could be used to alleviate inflammation.



KEYWORDS: *apple, triterpene, procyanidin, nutrigenomics, inflammation*

INTRODUCTION

Apple (*Malus X domestica* Borkh) is among the most highly consumed fruits worldwide.[1] Numerous epidemiological studies have linked the consumption of apples with reduced risk of some cancers, cardiovascular diseases, and asthma (recently reviewed in ref 2). Apple or apple components may also have beneficial effects on Alzheimer's disease, cognitive decline of normal aging, diabetes, weight management, bone health, and gastrointestinal protection from drug injury.[2] These health-promoting properties are thought to be the result of the large amounts of phenolic compounds[3,4] and triterpenes,[5,6] which are two major groups of secondary plant metabolites,[7,8] found in apples. In plants, they are known to have a wide range of physiological activities related to protection against various forms of environmental stresses.[9,10] In humans, they have been associated with numerous biological properties such as anticancer and anti-inflammatory effects.[7,11] Most phenolic compounds are produced through the phenylpropanoid pathway[8] and encompass a wide range of chemical structures. Apples contain flavanol monomers (mainly epicatechin; see Figure S1,Supporting Information, for key structures) or oligomers (mainly procyanidin B2), chlorogenic acid and small quantities of other hydroxycinnamic acids, two glycosides of phloretin (dihydrochalcone), several quercetin glycosides (flavonols), and anthocyanins in the skin of red varieties.[12] The concentrations of these phenolic compounds depend on many factors, such as cultivar, growing conditions, storage conditions, and processing of the apples.[4,13] Pentacyclic triterpenes are notably present in plant surfaces such as stem bark, leaves, and fruit waxes, where they protect the plant from biotic and abiotic stress factors.[6] The cuticular wax of apple skin represents an important food source of these phytochemicals, with ursolic and oleanolic acids being the major components (Figure S1,Supporting Information).[6] However, little is known about the extent of variation of triterpene contents within the apple germplasm with most studies focusing on a limited range of cultivars.[14−16]

In this study, we focused on the potential of apple components to reduce inflammation. We postulated that the wide genetic diversity among the apple germplasm may be expressed as considerable variability of chemical profiles and, in

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turn, in a range of inflammation-modulating or gene-targeted properties. Some studies have shown the ability of triterpenes and certain phenolic compounds to modulate inflammation processes.[17,18] Over the past two decades, the incidence of chronic inflammatory diseases such as inflammatory bowel disease (IBD) has increased, mainly in developed countries.[19]

Inflammation can arise through the activation of the transcription factor nuclear factor-kappaB (NF-κB) via a complex and not yet fully understood cross-talk with toll-like receptors (TLRs)[20] (Figure S2,Supporting Information). NF-κB is a pivotal transcription factor in chronic inflammatory diseases, inducing the expression of numerous inflammation related-genes such as those for tumor necrosis factor α (TNFα), interleukin-1 β (IL-1β), and IL-6 or IL-8.[21,22] NF-κB can be further activated by various intra- and extra-cellular stimuli including cytokines like TNFα (Figure S2,Supporting Information). The activation of NF-κB in patients with active IBD[23] makes it a very attractive target for bioactives.

TNFα is an important pro-inflammatory cytokine. The TNFα promoter has several polymorphisms, including the −308 G/A single nucleotide polymorphism (SNP) which has been associated with increased cytokine expression and progression of symptoms in IBD.[24] Concentrations of TNFα are elevated in the intestinal mucosa, stool, and blood of patients with IBD.[25,26] Monoclonal antibodies against TNFα have been developed and used for treating IBD.[26] However, long-term use of TNFα antibodies may be linked to an increased risk of developing cancer[27] or developing infections in the elderly.[28] Finding TNFα inhibitors from food is therefore important as human physiology may be better adapted to these natural chemicals.

The work presented here aimed at (i) determining the extent of variation of triterpenes and phenolic compounds in apple germplasm and (ii) evaluating their potential to reduce IBD-related inflammation in vitro. One hundred and nine apple cultivars grown in New Zealand and Luxembourg were first analyzed for their contents of triterpenes and phenolic compounds. Five apple cultivars were then selected on the basis of their contrasting phytochemical profiles. Their skin and flesh crude extracts were fractionated and investigated for their immune-modulating activity using two in vitro HEK 293 cell-based assays targeting key points in the inflammation pathway. More precisely, their ability to inhibit NF-κB activation and to reduce the activity of a TNFα promoter variant was examined. Pure apple compounds were also tested in these assays.

MATERIALS AND METHODS

Plant Material. The starting plant material consisted of 109 cultivars of apples, originating from both New Zealand (NZ) and Luxembourg. A total of 94 cultivars were grown in Hawke's Bay (NZ), and fruits were harvested between February and April 2009. This collection included 67 old heritage cultivars as well as 27 elite breeding selections from Plant & Food Research (PFR). For each cultivar, two samples (each comprising six mature apples from one tree) were used. Each fruit was cut into quarters, and one slice (∼0.5 cm at the skin edge) from each quarter was cut, avoiding the core/seeds. Each slice was peeled, and the skin and the flesh were separately frozen in liquid nitrogen. Samples were stored at −80 °C until analysis. Fifteen cultivars grown in Luxembourg were also included in the study. Fruits were sampled from five old heritage cultivars organically grown in the Haus vun der Natur orchards (Kockelscheuer, Luxembourg) and from 10 commercial varieties grown in different locations in Luxembourg and purchased from a supermarket. Fruits were harvested or purchased in October 2009. They were sampled as described above, analyzed for dry matter content, freeze-dried, vacuum packed, shipped to Plant & Food Research in Auckland (NZ), and stored at 4 °C prior to analysis.

The five most contrasting cultivars in terms of phytochemical profile were further investigated for their genotype-specific anti-inflammatory activity using in vitro HEK 293 cell-based assays. The cultivars were 'Eifeler Rambour' (heritage variety grown in Luxembourg), *Malus sylvestris* (heritage, NZ), 'Niagara' (heritage, NZ), 'Royal Gala' (commercial, NZ), and 'Merton Russet' (heritage, NZ). Harvest and sampling of the NZ apples were in March−April 2010, whereas the Luxembourg apples were harvested in September 2010, sampled, and sent to Auckland for analysis. All samples were freeze-dried and stored at 4 °C prior to analysis.

Chemicals. Solvents, of analytical or high-performance liquid chromatography (HPLC) grade as required, were obtained from Thermo Fischer Scientific (Auckland, New Zealand). Triptolide, phorbol 12-myristate 13-acetate (PMA), resazurin, chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, catechin, epicatechin, rutin, phloridzin, ursolic acid, and oleanolic acid were purchased from Sigma-Aldrich (St. Louis, MO). Procyanidin B2, cyanidin-3-galactoside, phloretin, and betulinic acid were obtained from ExtraSynthese (Genay, France).

Preparation of Apple Extracts. *Chemical Characterization of Apple Cultivars.* Powdered frozen apple skin samples (500 mg) were mixed with 10 mL of EtOAc/hexane (50:50, v/v), then homogenized using a vortex for 30 s and shaken for 1 h at room temperature (RT). After centrifugation at 6000*g* for 15 min, the supernatant was collected and evaporated to dryness using a CentriVap. The pellet was re-extracted using 10 mL of EtOH/H_2O (80:20, v/v), homogenized, and shaken for 2 h at RT. The resultant solution was centrifuged, and the supernatant was collected, combined with the lipophilic dried extract, and evaporated to dryness. Phenolics and triterpenes were resuspended in 1 mL of EtOH and filtered (0.45 μm) prior to HPLC analysis. Apple flesh samples were directly extracted with EtOH/H_2O (80:20, v/v) for polyphenols only as they contain very low levels of triterpenes.[29]

For each apple genotype, two subsamples (biological replicates) of both skin and flesh were taken. For each of these subsamples, duplicate extractions and HPLC analyses (technical replicates) were done. Therefore, each reported concentration in Supporting Information Table S1 is the average of four values.

Fractionation of Apple Extracts for in Vitro Assays. Finely ground dried apple skin or flesh samples (10 g) were extracted with 100 mL of EtOAc/hexane (50:50, v/v) by shaking for 2 h, then filtered. The residue was further extracted with 100 mL of EtOH/H_20 (95:5, v/v) overnight by shaking. The ethanol extract was then filtered, combined with the EtOAc/hexane extract previously dried, and stored at −20 °C. For fractionation, a 5 g C18 Isolute SPE cartridge was conditioned with 10 mL of EtOH, then 10 mL of 1:1 EtOH/H_2O, and then 10 mL of H_2O. An aliquot of extract (50 mL) was coated onto 2 g C18 (Aldrich octadecyl-functionalized silica gel) by rotary evaporation at 30 °C and applied to the preconditioned SPE cartridge. This was eluted with 2 × 10 mL each of H_2O, 1:4 EtOH/H_2O, 1:1 EtOH/H_2O, 4:1 EtOH/H_2O, EtOH, and EtOAc to give 12 10 mL fractions, which were collected into glass tubes. Aliquots (1 mL) of apple extract and fractions (AFs) were dried at 20 °C in Eppendorf safelock tubes and stored at −20 °C until analysis. For the NF-κB bioassay, AFs were resuspended in 1 mL of 1:5 DMSO/H_2O, chemically characterized, and diluted 250× in cell culture medium. For the TNFα promoter assay, AFs were resuspended in 250 μL of DMSO, and then diluted 1000× in cell culture medium. The DMSO concentration for both bioassays was maintained at 0.1%. Results from fractions 1 and 12 are not reported since they contained insignificant levels of phytochemicals, and the bioassay results were not significantly different from the control.

Analysis of Phenolics. Identification and quantification of the phenolic compounds was performed by HPLC-DAD according to their retention time and spectral data as compared to pure standards. HPLC linked to a mass spectrometer (LC-MS) was further used to identify the unknown compounds.

HPLC-DAD Analysis. Identification and quantification of the phenolic compounds was performed using a Dionex Ultimate 3000 system (Sunnyvale, CA) equipped with a diode array detector (DAD). A 5 μL aliquot was injected onto a Dionex C18 Acclaim PolarAdvantage II column (150 × 2.1 mm i.d.; 3 μm particle size) (Sunnyvale, CA). The mobile phases were (A) H_2O with 0.1% formic acid and (B) MeCN with 0.1% formic acid. The flow rate was 0.35 mL min^{-1}, and the column temperature was 35 °C. The 42 min gradient was as follows: 0−5 min, 0−8% B; 5−10 min, 8−15% B; 10−20 min, 15−20% B; 20−27 min, 20% B constant; 27−34 min, 27−100% B; 34−36 min, 100% B constant; 36−42 min, 0% B, re-equilibration time. Simultaneous monitoring was set at 254 nm, 280 nm, 320 nm, and 520 nm for quantification. Phenolic compounds were identified by their retention time and spectral data as compared with standards and were quantified using five-point calibration curves. Furthermore, a validation standard was injected after every 10th injection.

HPLC-ESI-MS Analysis. Identification of the main phenolic compounds was performed by LC-MS using a LCQ Deca ion trap mass spectrometer fitted with an electrospray ionization (ESI) interface (ThermoQuest, Finnigan, San Jose, CA, USA) and coupled to a Surveyor HPLC instrument. Column and elution conditions were as described above for the HPLC-DAD analysis. Spectra were recorded in negative ion mode between 100 and 1000 atomic mass units (amu).[30]

HPLC-DAD Analysis of Triterpenes. Identification and quantification of ursolic, oleanolic, and betulinic acids was performed using the same Dionex Ultimate 3000 instrument. A 20 μL aliquot was injected onto a Phenomenex C18 (2) Luna column (250 × 4.6 mm i.d.; 5 μm particle size) (Torrance, CA). The mobile phase was MeOH/H_2O/phosphoric acid (88:11.95:0.05, v/v/v). Ursolic, oleanolic, and betulinic acids were eluted isocratically at a flow rate of 1 mL min^{-1} and a column temperature of 35 °C. They were identified by their retention time and spectral data as compared with authentic standards and were quantified at 210 nm using five-point calibration curves. Furthermore, a validation standard was injected after every 10th injection.

Cell Culture. The NF-κB/SEAP (Secreted Alkaline Phosphatase) HEK 293 cell line was purchased from Imgenex (San Diego, CA). A 1.4 kb human TNFα promoter fragment was PCR-isolated and subcloned into the luciferase reporter vector pGL4.14 (Promega, Madison, WI). The −308G/A variant was subsequently generated by site-directed mutagenesis (QuikChange II, Stratagene/Agilent, Santa Clara, CA) and verified by sequencing. Plasmids were stably transfected into Flp-In 293 cells (Invitrogen, Auckland) by electroporation, and isolated clones were screened for TNFα promoter-luciferase activation with PMA and inhibition with triptolide.[31] All cell lines were propagated in cell culture medium, comprising minimum essential medium (MEM) (Cat. #11095, Invitrogen, Auckland) and 10% heat-inactivated fetal bovine serum (Invitrogen, Auckland) at 37 °C in a humidified 5% CO_2 atmosphere. For routine culture maintenance, the cell culture medium was supplemented with 500 μg/mL G418 (Sigma-Aldrich, St. Louis, MO) for NF-κB/SEAP HEK 293 and with 150 μg/mL hygromycin and 100 μg/mL zeocin (Invitrogen, Auckland) for TNFα promoter clones Flp-In 293/TNFpWTLuc and Flp-In 293/TNFp-308Luc. Cells were passaged 2−3 times a week.

Cell Viability. Cytotoxicity of the AFs was determined using resazurin/Alamar Blue[32] on NF-κB/SEAP HEK 293 cells following the NF-κB assay. One hundred microliters of cell culture medium and 10 μL of 440 μM resazurin solution in phosphate buffered saline (PBS) were added to each well after treatment of the cells with AFs (7 h) and removal of culture medium. After 2 to 3 h, the formation of resorufin by cellular metabolic activity was recorded using a microplate fluorometer (excitation wavelength 544 nm, emission at 590 nm, 37 °C). A negative control with no cells was used for background subtraction. The percentage of cell viability was calculated using the nontreated cells as positive control (100% cell viability). For the highest concentrations of AFs studied, all treatments showed cell viability >70%.

NF-κB Assay. NF-κB/SEAP HEK 293 cells were stably transfected with the SEAP gene under the control of an NF-κB-inducible promoter. The NF-κB assay was performed using a standard protocol developed for screening phytochemical extracts. Cells were first seeded into 96-well plates at 5 × 10^4 cells per well and allowed to attach at 37 °C for 18 h. Cells were then exposed to AFs for 1 h before being stimulated with TNFα (final concentration of 10 ng mL^{-1}; Peprotech, Rocky Hill, NJ). Cells were then incubated for a further 6 h before 20 μL of culture supernatant was transferred into a 96-well plate containing 100 μL of Quanti-Blue reagent (Invivogen, San Diego, CA) for the detection of SEAP production. After 1 h of incubation at 37 °C, absorbance was measured at 620 nm. Data were expressed as percentage inhibition of NF-κB activation (100% NF-κB activation), using cells treated with TNFα only as the control (100% NF-κB activation).

TNFα Promoter Genotype-Specific Assay. Flp-In 293/TNFpWTLuc and Flp-In 293/TNFp-308Luc cells were seeded into 96 well plates at 5 × 10^4 cells per well and incubated 18−24 h to allow attachment. Diluted AFs were added to the cells, and after 30 min, the TNF promoter was induced by the addition of 2 ng mL^{-1} PMA. After a further 6 h incubation, the cells were washed gently with PBS and were lysed with 100 μL/well 1× cell culture lysis reagent (Promega, Madison, WI). Plates with lysates were frozen at −80 °C until the time of assay. After thawing, plates were mixed for 15 min at 300 rpm and centrifuged for 15 min at 4000 rpm at room temperature. Five microliter lysate aliquots were transferred to a shallow 384 well white polypropylene plate (Cat. #267462, NUNC/ThermoFisher, Auckland) using a Biomek 3000 liquid transfer workstation (Beckman Coulter, Auckland). The luciferase assay was performed in a Fluostar Omega multiplate reader (BMG Labtech, Mornington, Victoria, Australia) using the Luciferase Assay System (Promega, Madison, WI; 5 μL substrate injection/well, 1 s count/well). Data were expressed as percentage of inhibition of TNFα promoter activation, using cells treated with PMA only as control (100% promoter activation). For log IC_{50} determination for pure compounds, a range of dilutions of compounds was assayed in duplicate, and sigmoidal inhibition curves were fitted to the data by nonlinear regression using GraphPad Prism 5.02.

Statistical Analysis. Hierarchical cluster analysis (HCA) was determined on log transformed data to evaluate relationships between variables. Principal component analysis (PCA) was performed on centered and standardized data in order to compare the phytochemical profile of the different apple genotypes. A multiple regression analysis was used to evaluate the extent to which the variation in bioactivity could be explained by the phytochemical concentrations. Data were first centered and standardized. The contributions of each of the metabolites in the final models were calculated as proportions with the type I (sequential) sums of squares as numerators and the model sums of squares as denominators. The significance of differences between mean measurements was evaluated using Student's *t* test. Minitab software was used for all analyses.

RESULTS AND DISCUSSION

Variability of Phenolic Compounds and Triterpenes in the Apple Germplasm. HPLC-DAD polyphenol profiling of the apple extracts revealed the presence of flavanols, flavonols, dihydrochalcones, and hydroxycinnamic acids, as well as anthocyanins in the skin of red cultivars. High UV-absorbing peaks were then putatively identified by LC-MS from their mass spectra and previous literature.[12,33,34] These assignments were confirmed by HPLC-DAD comparison of their UV spectra and retention times with those of authentic standards when available.

The skin and flesh total phenolic contents varied greatly among the 109 apple cultivars (Supporting Information Table S1). Total phenolic compound contents ranged from 29 to 7882 μg g^{-1} of FW in the flesh and from 733 to 4868 μg g^{-1} FW in the skin. These levels are consistent with the levels

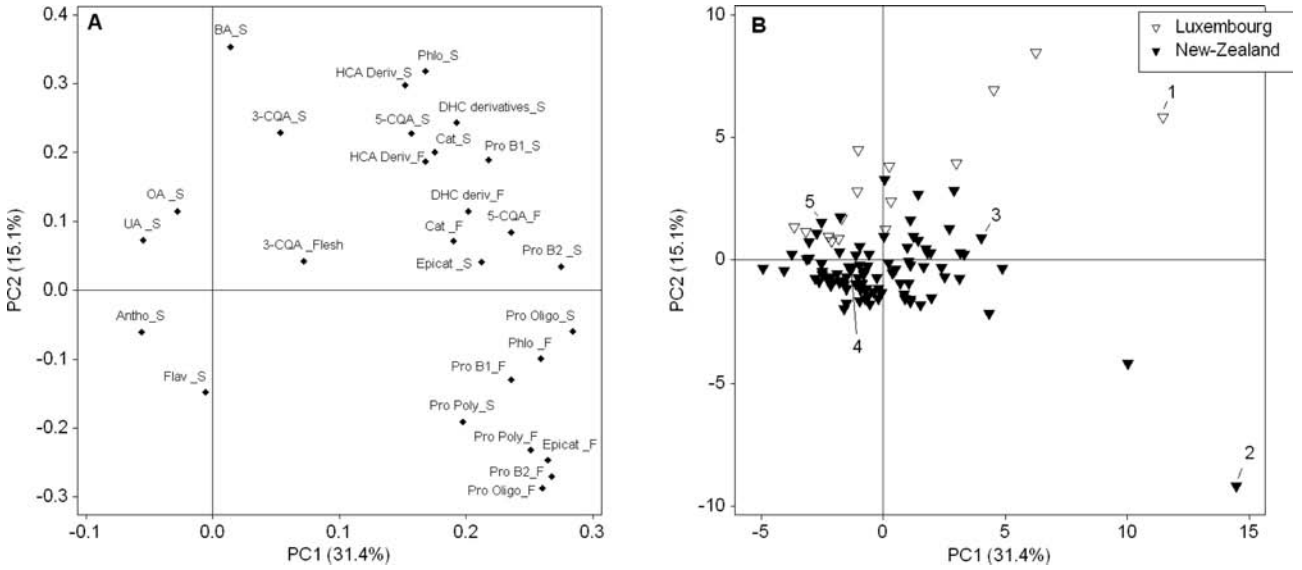

Figure 1. Principal component analysis resulting from the chemical characterization of 109 apple cultivars: (A) loading plot showing the relationships between 27 phytochemical components; (B) score plot of the 109 cultivars grown either in Luxembourg or in New Zealand. Abbreviations: Antho, total anthocyanins; Cat, catechin; Epicat, epicatechin; Pro B1, procyanidin B1; Pro B2, procyanidin B2; Pro Oligo, procyanidin oligomers; Pro Poly, procyanidin polymers; 3-CQA, neochlorogenic acid; 5-CQA, chlorogenic acid; HCA Deriv, hydroxycinnamic acid derivatives; Phlor, phlorizin; DHC, dihydrochalcone derivatives; Flavo, flavonols; OA, oleanolic acid; UA, ursolic acid; BA, betulinic acid.

reported in the literature[4,33,35−37] (Supporting Information Table S2). For the individual phenolics compounds quantified, in both flesh and skin, the flavanols dominated in most varieties and included catechin, epicatechin, procyanidin oligomers, and procyanidin polymers (procyanidin oligomers above the hexamers). Flavanols represented on average 49% and 82% of total phenolic compounds in the skin and the flesh, respectively. The hydroxycinnamic acids included chlorogenic acid (5-*O*-caffeoyl quinic acid (5-CQA)), an isomer of chlorogenic acid (neochlorogenic acid, 3-*O*-caffeoyl quinic acid (3-CQA)), some *p*-coumaroyl quinic acids, and other hydoxycinnamic acid derivatives. They were all quantified as chlorogenic acid derivatives and constituted the second most important polyphenol class in the flesh (15% on average). High amounts of flavonols including a range of quercetin derivatives were present in the skin (41% of the sum of all phenolic compounds), while they were not detected in the flesh. Three dihydrochalcones were also identified:[33] phloridzin (confirmed by a standard), phloretin-xyloglucoside, and 3-hydroxyphloridzin. Dihydrochalcones usually represented only a small amount of total polyphenols (3.5% in the skin and 2.6% in the flesh), although in some russetted cultivars such as 'Merton Russet', dihydrochalcones represented up to 29% and 8.7% of the total phenolic compounds in the skin and in the flesh, respectively.

The elite selections from the PFR breeding program had lower average flesh phenolic contents than the heritage germplasm. Old heritage cultivars and especially the crab apple *Malus sylvestris* presented the highest concentrations of phenolic compounds, particularly procyanidins. High concentrations of dihydrochalcones such as phloridzin were also found in russetted cultivars ('Merton Russet', 'Wilmont Russet', and 'Grauapfel'). New elite cultivars contained significantly lower dihydrochalcones (flesh, 16.7 vs 26.3; skin, 46.1 vs. 94.8 μg g^{-1} FW), hydroxycinnamic acids (flesh, 76.9 vs. 157.2; skin, 63.9 vs 128.3 μg g^{-1} FW), and flesh polymeric procyanidins (128.9 vs. 253 μg g^{-1} FW) as compared with their old counterparts ($p <$ 0.05). This result is not surprising as high concentrations of those molecules are known to affect eating quality. New cultivars have been selected against bitter and astringent tastes likely caused by polymeric procyanidins or condensed tannins.[38] Furthermore, phenolic compounds and more particularly hydroxycinnamic acids such as chlorogenic acid are involved in enzymatic browning reactions,[39] which may impair fruit appearance and have probably been selected against by apple breeders.

Three major triterpene compounds were identified in the skin samples by HPLC-DAD and were compared with authentic standards (Supporting Information Table S1). These were mainly ursolic and oleanolic acids, with concentrations in the skin ranging from 44.7 to 3522 and from 47.2 to 838 μg g^{-1} FW, respectively. Betulinic acid was also present but to a lesser extent (14.3 to 212.9 μg g^{-1} FW). These concentrations are in agreement with levels reported previously.[6] The highest ranking cultivar in terms of total triterpene content was 'Niagara', a heritage variety characterized by a thick and tough waxy skin. The lowest ranking cultivar was also a heritage genotype, 'Merton Russet', characterized by a brownish skin made of suberized cells (pear skin aspect). Interestingly, the contents of ursolic and oleanolic acid in this variety were very low, whereas the betulinic acid level was among the highest (120.9 μg g^{-1} FW). All the russetted cultivars from both New Zealand and Luxembourg revealed a different triterpene pattern compared with their waxy counterparts, characterized by a higher proportion of betulinic acid.

Principal Component Analysis. PCA was performed on the 109 genotypes and the 27 compound concentrations. The first two principal components accounted for 46.5% of the total variance: PC1 31.4% and PC2 15.1% (Figure 1). Most phenolic compounds, especially monomers and oligomers of flavanols in the flesh and skin, were highly correlated with positive loadings on PC1, while skin anthocyanins, flavonols, and the triterpenes had low loadings on PC1. Betulinic acid, phloridzin, and hydroxycinnamic acids in the skin were correlated with positive

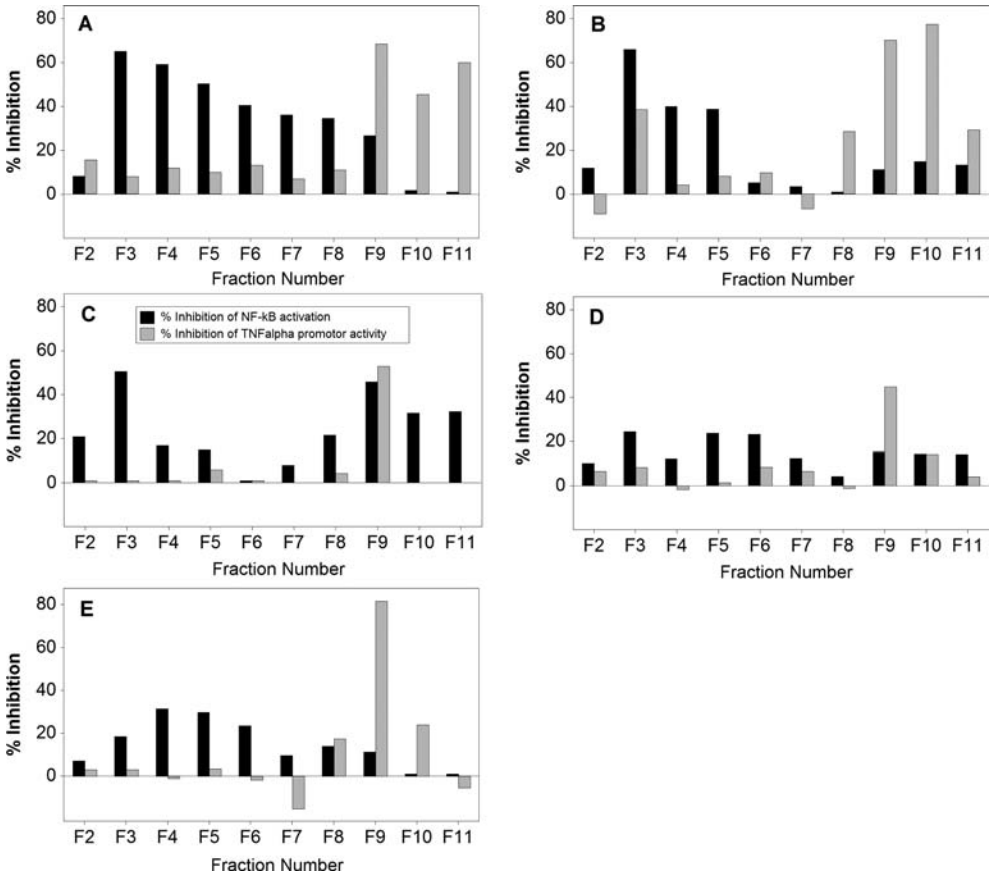

Figure 2. Bioactivities of skin fractions from 5 apple cultivars. A, 'Niagara'; B, *Malus sylvestris*; C, 'Eifeler Rambour'; D, 'Merton Russet'; E, 'Royal Gala'. Black bars represent the percentage of inhibition of NF-κB, while the gray bars show the percentage of inhibition of the mutant −308A TNFα promoter activity. See Table S3 (Supporting Information) for the phytochemical concentrations in each fraction.

loadings on PC2. In contrast, the levels of epicatechin and oligomeric and polymeric procyanidins in the flesh were negatively loaded on PC2.

PC2 could partially discriminate cultivars grown in Luxembourg from those grown in New Zealand (Figure 1B), but these differences are most likely due to the different genotypes grown in the two countries. Four cultivars were grown in both New Zealand and Luxembourg: 'Braeburn', 'Belle de Boskoop', 'Fuji', and 'Granny Smith'. When compared (Supporting Information Figure S3), cultivars grown in New Zealand presented significantly higher flesh and skin total phenolic compounds, mainly because of higher flesh flavanol content and higher skin polymeric procyanidins and flavonols. However, the level of betulinic acid was higher in the skin of Luxembourg cultivars. For flesh total polyphenolics, there was a significant level of consistency of the ranking of cultivars grown in New Zealand between our and other studies (Spearman Rank Correlation coefficient: $r_s = 0.6$ for NZ vs Volz et al.[35] data ($p < 0.05$) and $r_s = 1$ for NZ vs Tsao et al.[37] data ($p < 0.01$)), suggesting a significant impact of the genotype on flesh total phenolic content (Supporting Information Table S2). However, no significant correlation was observed for skin phenolics, suggesting a more important influence of the environment on those concentrations, likely due to the large variations in flavonols. Those observations are in agreement with the ones of Volz et al.,[35] who showed that the genetic variations in terms of phenolic content were stable across years, except for flavonols. Indeed, those phenolics are known to be particularly sensitive to light and the temperature of the environment.[40]

Bioassay Results. Five cultivars with contrasting phytochemical profiles were investigated for their immunomodulatory activity using two in vitro cell-based assays. These cultivars were selected on the basis of the principal component analysis and their contrasting chemical compositions (Table S1, Supporting Information): 'Eifeler Rambour' (1, a heritage variety grown in Luxembourg, highest epicatechin and procyanidin B1), Malus sylvestris (2, heritage NZ, highest procyanidin B2 and oligomers and polymers), 'Niagara' (3, heritage NZ, highest ursolic and oleanolic acids), 'Royal Gala' (4, commercial NZ, median in most components), and 'Merton Russet' (5, heritage NZ, lowest ursolic and oleanolic acids, lowest total polyphenolics) (numbers 1 to 5 refer to Figure 1B). Skin and flesh samples were extracted separately and then separated into 12 fractions according to polarity before being bioassayed and chemically analyzed.

Effects of AFs on TNFα-Induced NF-κB Activation. Inhibitions of NF-κB activation by the skin and flesh fractions are presented in Figures 2 (black bars) and 3, respectively. Efficacies varied in a fraction- and cultivar-dependent manner. In order to explain those variations, phytochemical concentrations were measured in each skin and flesh fraction and reported in Supporting Information Tables S3 and S4, respectively. For each cultivar, the highest levels of inhibition were achieved in F3 to F5 for both flesh and skin samples. F3 from 'Niagara' and *Malus sylvestris* were particularly efficient with levels of inhibition between 60% and 80%. Most of the

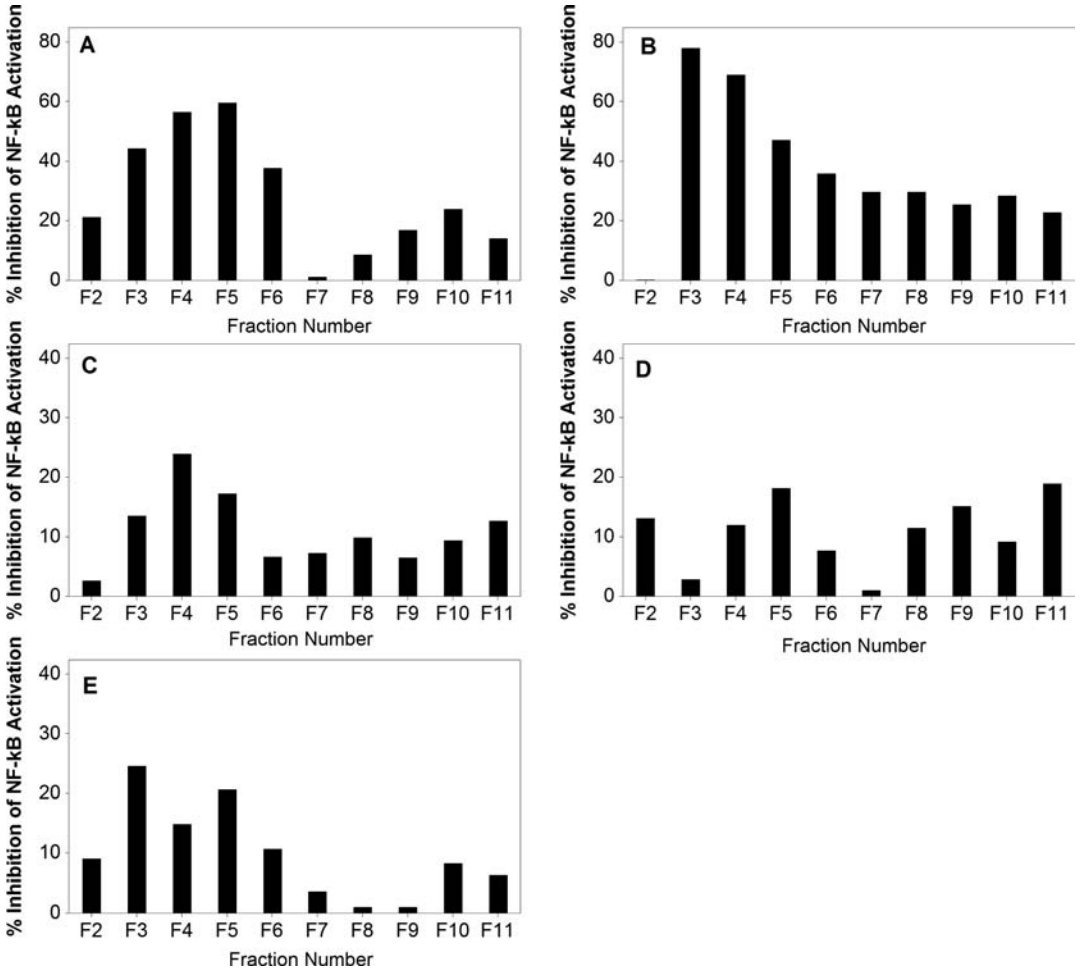

Figure 3. Inhibition of NF-κB activation by flesh fractions from 5 apple cultivars. A, 'Niagara'; B, *Malus sylvestris*; C, 'Eifeler Rambour'; D, 'Merton Russet'; E, 'Royal Gala'. Data are expressed as the % inhibition of the TNFα-induced control. See Table S4 (Supporting Information) for the phytochemical concentrations in each fraction.

phenolic compounds were eluted in F3−F5, with F3 being particularly rich in flavanols including procyanidins. Some inhibition was observed in triterpene-rich skin fractions F9−F10, particularly from Eifeler Rambour (Figure 2C), but these fractions had the lowest triterpene levels of the five cultivars (Table S3, Supporting Information).

A multiple regression analysis was used to find correlations between bioactivity and phytochemical concentrations. Most data were first grouped by family of compounds due to a high level of correlation between certain variables. Seven variables were therefore included in the analysis: total flavanols (including monomers, oligomers and polymers), total dihydrochalcones, total hydroxycinnamic acids, total flavonols, total anthocyanins, and the sum of oleanolic and ursolic acids, and betulinic acid. Betulinic acid was considered independently as it was not correlated with the other triterpenes. The first order regression model applied on both flesh and skin data together (data not shown) explained 54% of the variation of the bioactivity (adjusted R^2) and revealed a highly significant contribution of total flavanols (87%, $p < 0.0001$). This regression model assumes a linear relationship between the outcome variable (bioactivity) and the independent variables (phytochemicals), which is usually only true for a limited range of concentrations and does not take into account interactions between compounds. However, the linear regression model was the best to explain these data. The efficacy of pure compounds was then investigated (Table 1) and showed a moderate activity of procyanidin B2 (IC_{50} = 58 μM), whereas the monomers catechin and epicatechin were weaker inhibitors (IC_{50} = 82.3 μM, 90.7 μM, respectively). This suggests that the degree of

Table 1. Inhibition of NF-κB Activation and Mutant and Wild-Type TNFα Promoter Activity by Pure Compounds[a]

		TNFα IC_{50} (μM)	
compd	NF-κB IC_{50}(μM)	-308A	wild type
catechin	82.3 ± 0.7	>40	>40
epicatechin	90.7 ± 9.0	>40	>40
procyanidin B2	58.0 ± 4.3	>40	>40
phloridzin	>100	>40	>40
chlorogenic acid	>100	>40	>40
betulinic acid	11.7 ± 2.6	40.2 (−8.5, +10.9)	57.1 (−21.3, +34.0)[b]
oleanolic acid	5.0 ± 1.7	24.4 (−5.6, +7.3)[b]	19.9 (−6.0, +8.6)
ursolic acid	13.2 ± 1.6	6.1 (−1.3, +1.6)[b]	5.0 (−0.8, +0.9)[b]

[a]Mean IC_{50} values (± SD for the NF-κB assay and ± SEM for the TNFα promoter assay) were calculated from three experiments (n = 3). [b]Mean IC_{50} values were calculated from four experiments (n = 4).

polymerization is important for bioactivity. It is likely that other procyanidin oligomers are also active. Whereas potent inhibitory activities were shown for the individual triterpene acids, betulinic, oleanolic, and ursolic acids (IC_{50} = 11.7 μM, 5 μM, 8.2 μM, respectively; Table 1), the triterpene-rich skin fractions F9−F10 (reaching 30−40 μM in 'Niagara' and 'Royal Gala') were only weakly active. The observed inhibitory activities must be the result of various complex interactions, due to the quantified compounds, plus other nonidentified molecules, and to synergistic as well as antagonistic effects between all these components. Ursolic acid has previously been described as a NF-κB inhibitor, acting by blocking the phosphorylation of IκB and p65.[41,42]

In our assay, the cytokine TNFα was used as an inducer of the activation of NF-κB (see Figure 2S, Supporting Information, for the activation pathway), and we evaluated the potential of AFs to reduce this activation. The inhibition could happen at the molecular level inside but also outside the cell. Phenolic compounds and procyanidins in particular are well known for their binding properties to proteins[43] and may bind TNFα and inactivate it, preventing NF-κB activation. Procyanidins have been reported to inhibit the transcription factor NF-κB and the expression of NF-κB-regulated genes at multiple levels in the NF-κB activation pathway.[44−48]

Effects of AFs on −308A TNFα Promoter Activity. AFs were also tested for their ability to inhibit the activity of the −308A TNFα promoter. Only skin fractions showed activity (Figure 2) with no activity from flesh samples. The highest inhibition was from F9 and F10 for most cultivars, i.e., in the triterpene-rich fractions (Table S3, Supporting Information). The linear regression model applied on skin data could explain 47.2% of the variation of the inhibitory activity and revealed a highly significant contribution of the sum of oleanolic and ursolic acid concentrations (81%, $p < 0.0001$). The level of betulinic acid was also significantly associated with the percentage of inhibition, contributing 10% to the model. A quadratic term was added in order to explain saturation of inhibition at high inhibitor concentrations and resulted in a high increase of the fit of the model (adjusted R^2 = 77.3%), with the sum of oleanolic + ursolic acids and betulinic acid and their squared values as significant contributors. Pure individual triterpene acids showed inhibitory −308A TNFα promoter activities (Table 1), with an IC_{50} value of 24.4 μM for oleanolic acid and 40.2 μM for betulinic acid. Pure ursolic acid had the strongest inhibition (IC_{50} of 6 μM). It is worth noting that synergistic interactions between triterpene acids have been recently pointed out. Betulinic acid alone had no effect, but combinations of either oleanolic acid or ursolic acid with betulinic acid enhanced the immunomodulatory effect compared to the two separate triterpene acids.[49] For the standards, similar levels of inhibition were detected in the wild-type TNFα promoter assay as in the mutant (Table 1), indicating that apple triterpenes could be beneficial for individuals carrying either version of the TNFα promoter.

Although the NF-κB and TNFα signaling pathways are intimately linked (Figure S2, Supporting Information) and we used similar cell lines (HEK293), we obtained different results in the two bioassays. Procyanidin-rich fractions were active in the NF-κB assay but not in the TNFα promoter assay. However, Lauren et al.[30] showed that catechin and epicatechin were able to inhibit TNFα production (IC_{50} 5 μM for each; they did not test procyanidin B2) in mouse macrophages stimulated with lipopolysaccharide (LPS), presumably acting on a later stage of production or release of this cytokine. This emphasizes the complexity of interactions between NF-κB and TNFα and the multiple points where bioactive compounds may affect the activation of the NF-κB transcription factor and strength of the TNFα promoter.

Summary. We have shown the wide variability in phenolic and triterpene contents among 109 apple cultivars. Cultivars exhibiting high contents of procyanidins in flesh and skin had the potential to inhibit NF-κB activation and triterpene-rich fractions from apple skin to reduce the activity of the TNFα promoter. These results provide a solid platform for future in vivo investigations to determine whether individuals with inflammatory diseases may benefit from eating high procyanidin, high triterpene acid apple varieties or apple products.

■ ASSOCIATED CONTENT

Supporting Information

Structures of the main apple anti-inflammatory compounds; schematic overview of the possible signalling pathways; relationships between phenolic concentrations of apple cultivars grown in New Zealand and Luxembourg; polyphenol and triterpene contents in the skin and flesh of 109 apple varieties grown in New Zealand and Luxembourg (L); comparison of our total phenolic contents with literature data; polyphenol and triterpene concentrations of fractions from the skin of 5 apple cultivars as used in both in vitro bioassays; and polyphenol concentrations of fractions from the flesh of the 5 apple cultivars as used in both in vitro bioassays. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

IBD, inflammatory bowel disease; NF-κB, nuclear factor-kappaB; TNFα, tumor necrosis factor α; WT, wild type; SNP, single nucleotide polymorphism; AF, apple fraction; SEAP, secreted alkaline phosphatase; IC, inhibitory concentration; PC, principal component; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; NOD2, nucleotide-binding oligomerization domain-containing protein 2; FW, fresh weight

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