

Citrus Fruit Extracts Reduce Advanced Glycation End Products (AGEs)- and H₂O₂-Induced Oxidative Stress in Human Adipocytes

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Diabetes is a reactive oxygen species (ROS)-mediated pathology, with a worldwide prevalence estimated to double by 2030. A major effort has been launched to find therapeutic means to improve health conditions of diabetic patients. Recent data show that supplemental natural antioxidants represent a potential strategy as adjunct therapy. Despite the major role of adipocytes in the etiology of diabetes, little is known about the effect of natural antioxidants on adipocyte response to oxidative stress. Using a diabetes-like oxidative stress model, the potential protective effect of antioxidative flavedo, albedo, and pulp extracts of (1) tangor Elendale (*Citrus reticulata* × *Citrus sinensis*) and (2) tangelo Minneola (*C. reticulata* × *Citrus paradisis*) was investigated on human adipocytes. Besides the retardation of free-radical-induced hemolysis of human erythrocytes, non-cytotoxic concentrations of tangelo and tangor flavedo extracts significantly reduced the levels of protein carbonyls in response to advanced glycation end products (AGEs) generated by albumin glycation in SW872 cells. Flavedo extracts lowered carbonyl accumulation in H₂O₂-treated adipocytes, while tangelo and tangor flavedo, albedo, and pulp extracts suppressed ROS production in SW872 cells with or without the addition of H₂O₂. Our results clearly show that Mauritian *Citrus* fruit extracts represent an important source of antioxidants, with a novel antioxidative role at the adipose tissue level.

KEYWORDS: *Citrus*; AGEs; antioxidant; oxidative stress; adipocytes; SW872 cells

INTRODUCTION

It is now well-established that reactive oxygen species (ROS)-induced oxidation can result in cell-membrane disintegration, membrane-protein damage, and DNA mutation, leading to the initiation and development of cancers (1), diabetes (2), neurodegenerative diseases (3), aging (4), and cardiovascular diseases (5). Among the various ROS-mediated pathologies, diabetes is one of the most common endocrine disorders affecting almost 6% of the world's population (6). The incidence of diabetes is increasing, with a worldwide prevalence estimated to double by 2030, primarily because of sedentary lifestyle and obesity (7). The disease, which is a heterogeneous metabolic disorder, is characterized by hyperglycaemia consequent of defective insulin production, insulin resistance, or both. It is closely linked to obesity, with more than 70% of diabetics being overweight, and is

associated with severe complications, including cardiovascular dysfunctions, which represent the leading cause of mortality in western countries (8). One of the consequences of hyperglycemia is the excessive non-enzymatic glycation of proteins, leading to the formation of advanced glycation end products (AGEs), which have the propensity to generate ROS. Glycation and AGE modifications lead to pathological changes contributing to diabetic complications, such as cataracts, nephropathy, vasculopathy, proliferative retinopathy, and atherosclerosis (9).

A major effort has been launched to find a therapeutic means to improve health conditions of diabetic patients. Recent data show that supplemental natural antioxidants represent a potential strategy as adjunct therapy. *Citrus* is one of the most popular world fruit crops that, also providing an ample supply of vitamin C, folic acid, potassium, and pectin, contains a host of antioxidant phytochemicals that can potentially protect health (10–13). We recently reported that the *in vitro* antioxidant activities of the flavedo extracts of 21 *Citrus* fruit varieties grown in Mauritius were strongly correlated to their phytochemical composition, and in that respect, the flavedo extracts represented a significant source of natural antioxidants with potential prophylactic properties for the management of diseases (14). Further studies of

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Citrus flavanones suggest that naringenin is able to reduce glucose uptake and inhibit intestinal and renal Na⁺–glucose co-transporter (SGLT1) (15) and that both naringin and hesperidin significantly increased the glucokinase mRNA level, while naringin reduced the mRNA expression of phosphoenolpyruvate carboxykinase and glucose-6-phosphatase in the liver (16). Recently, it was reported that a *Citrus* extract of Dangyuja (*Citrus* fruit from Korea), containing high levels of flavanone glycosides, could be used to control the blood glucose level of diabetic patients by inhibiting α amylase and α glucosidase in the intestinal tract (17).

Many studies have focused on the effect of natural antioxidants on vascular and circulating cells, such as macrophages. However, despite the important role of adipocytes in the etiology of diabetes, little is known about the effect of natural antioxidants on adipocyte response to oxidative stress. The SW872 cell line has often been used in previous studies as a human adipocyte cell model (18–21). Adipocytes are known to express and secrete a variety of active molecules, so-called adipokines, which can regulate many biological processes. It has been shown that dysregulation of adipocyte functions by oxidative stress constitutes a key process leading to type-2 diabetes. It is thus not surprising that many diabetic therapies target adipose tissue [thiazolidinedione (TZD), physical activity, etc.] (22, 23). Recent experiments performed in our group showed that AGEs induce oxidative stress in adipocytes and lead to the accumulation of oxidized proteins (18, 24).

In this study, we report a comparative analysis of the polyphenolic content and *in vitro* antioxidant properties of flavedo, albedo, and pulp extracts of two varieties of *Citrus* fruits grown in Mauritius: (1) tanger Elendale (*Citrus reticulata* \times *Citrus sinensis*) and (2) tangelo Minneola (*C. reticulata* \times *Citrus paradisi*). Using a diabetes-like oxidative stress model, the potential protective effect of antioxidant *Citrus* fruit extracts on human adipocytes was evaluated. The extracts were tested on SW872 liposarcoma cells subjected or not to H₂O₂ or AGEs. Cell viability, carbonyl accumulation, free-radical formation, tumor necrosis factor α (TNF α), and apolipoprotein E (apoE) secretions were assessed in treated cells.

MATERIALS AND METHODS

Standards and Chemicals. 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) was from Analytical Rasayan, SD Fine-Chem Ltd. (Mumbai, India). 2,6-Dichloroindophenol sodium salt was from Alpha Chemika (Mumbai, India). L-Ascorbic acid was from BHD Laboratory Supplies (Poole, U.K.). High-performance liquid chromatography (HPLC) standards were from Extrasynthèse (Genay, France). Sterile Dulbecco's modified Eagle's medium (DMEM), trypsin, and L-glutamine were obtained from GIBCO (Grand Island, NY). Fetal bovine serum (FBS) was purchased from GIBCO (Grand Island, NY). Bicinchoninic acid for determination of total protein, albumin from human serum (96–99%), methylglyoxal solution (40% aq), and sterile dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich, Inc. (St. Louis, MO). Anti-DNPH antibody and 3,3',5,5'-tetramethyl-benzidine liquid substrate system for enzyme-linked immunosorbent assay (ELISA) were from Sigma. ECL anti-rabbit IgG, horseradish-peroxidase (HRP)-linked whole antibody (from donkey) was purchased from GE Healthcare Ltd. (U.K.). 2,2'-Azobis(2-methylpropanamide) dihydrochloride (AAPH) and 2',7'-dichlorofluorescein-diacetate (DCFH-DA) were from Sigma-Aldrich, Inc. (St. Louis, MO).

Plant Material. Two varieties of *Citrus* fruits, namely, tanger Elendale (*C. reticulata* \times *C. sinensis*) and tangelo Minneola (*C. reticulata* \times *C. paradisi*) were obtained from La Compagnie Agricole de Labourdonnais, situated at Mapou, in the north of Mauritius. Fruits were harvested at the mature stage when they were ready to be placed on the market or ready for processing. After harvest, the fruits were rapidly processed on the same day. They were carefully washed under running tap water and patted dry. The flavedo, followed by the albedo, of at least 10 fruits of each variety, was carefully removed with a manual peeler and cut into small pieces. The pulp

was homogenized in a Waring Commercial blender (Dynamics Corporation, New Hartford, CT). Weighed portions of flavedo, albedo, and pulp of pooled samples of each variety were lyophilized for 48 h, and the freeze-dried weight was determined. Samples were ground into a fine powder in a coffee grinder and stored in airtight containers at –4 °C until analyzed.

Extraction. The extraction procedure used was adapted from Franke et al. and Chun et al. (25, 26). Powdered freeze-dried *Citrus* tissues were exhaustively extracted in 80% aqueous methanol at 4 °C for 3 consecutive days. After centrifugation at 3260g for 15 min, supernatants of all three extractions were pooled and stored at –20 °C until used for the determination of total phenolics and total flavonoids and for the cell-free antioxidant assays. For use in the hemolysis test and cell line model system, the solvent was evaporated from aliquot volumes under reduced pressure. The residue obtained was dissolved in a known volume of DMSO and stored at –20 °C.

Total Phenolic Content. The Folin–Ciocalteu assay was used for the determination of total phenolics present in the *Citrus* fruit extracts (27). To 0.25 mL of diluted extract, 3.5 mL of distilled water was added, followed by 0.25 mL of Folin–Ciocalteu reagent (Merck). After 3 min, 1 mL of 20% sodium carbonate was added. The whole mixture was incubated at 40 °C for 40 min. A deep blue coloration developed, whose absorbance was read at 685 nm. Results are expressed in micrograms of gallic acid per gram of fresh weight (FW) of plant material.

Total Flavonoid Content. Total flavonoids were measured using a colorimetric assay adapted from Zhishen et al. (28). A total of 150 μ L of 5% aqueous NaNO₂ was added to 2.5 mL of extract. After 5 min, 150 μ L of 10% aqueous AlCl₃ was added. A total of 1 mL of 1 M NaOH was added 1 min after the addition of aluminum chloride. The absorbance of the solution was measured at 510 nm. Flavonoid contents are expressed in micrograms of quercetin per gram of FW of plant material.

Quantitative Analysis of Flavanone, Flavonol, and Flavone Glycosides in the *Citrus* Extracts by HPLC. Chromatographic conditions were adapted from Mouly et al. (29). HPL1100 series HPLC equipped with a vacuum degasser, quaternary pump, autosampler, thermostatted column compartment, diode array detector, and HP Chemstation for data collection and analysis was used. After filtration on Millipore (0.22 μ m), 30 μ L of extract was injected on a Waters Spherisorb ODS-2 column (5 μ m particle size, 80 Å pore size, 4.6 mm inner diameter \times 150 mm). The solvents used were A, water/acetonitrile (90:10, v/v; pH 2.35), and B, acetonitrile. The gradient profile was as follows: 0–12 min, 0–8% B; 12–43 min, 8–34% B; 43–44 min, 34–70% B; 44–59 min, 70% B; and 59–60 min, back to 0% B. The diode array detector was set at 280 nm for the quantitative determination of flavanone glycosides and at 330 nm for flavone and flavonol glycosides. The column temperature was 25 °C, and the flow rate was fixed at 0.7 mL/min. The identification and quantification of the flavonoids investigated were determined from retention time and peak area in comparison to the standards used, namely, poncirin, rhoifolin, didymin, naringin, rutin, diosmin, isorhoifolin, neohesperidin, hesperidin, neeroiocitrin, and narirutin.

Trolox Equivalent Antioxidant Capacity (TEAC) Assay. The free-radical-scavenging capacity of the extracts was measured by the TEAC assay according to the method of Campos and Lissi (30). A total of 0.5 mL of diluted plant extract was added to 3 mL of the ABTS^{•+} solution generated by a reaction between 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt (ABTS, 0.5 mM) and activated MnO₂ (1 mM) in phosphate buffer (0.1 M, pH 7). Decay in absorbance was monitored at 734 nm for 15 min. TEAC values are expressed in micromoles of trolox per gram of FW.

Ferric-Reducing Antioxidant Power (FRAP) Assay. The reducing power of the extracts was assessed using the method of Benzie and Strain (31). A total of 50 μ L of sample was added to 150 μ L of distilled water, followed by 1.5 mL of FRAP reagent (40 mM HCl and 20 mL of 20 mM ferric chloride in 200 mL of 0.25 M sodium acetate buffer at pH 3.6). The absorbance was read at 593 nm after 4 min of incubation at 37 °C. Results are expressed in micromoles of Fe²⁺ per gram of FW.

Hypochlorous Acid (HOCl) Scavenging Assay. The ability of the extracts to scavenge HOCl was assessed essentially as described by Neergheen et al. (32). Results are expressed as IC₅₀ values (milligrams of FW per milliliter).

Copper–Phenanthroline Assay. The ability of the copper–phenanthroline complex to degrade DNA in the presence of a reducing agent can

be used to assess the antioxidant propensities of dietary biofactors (33). The assay was conducted essentially as described by Ramful et al. (14). Results are expressed in terms of IC₅₀ (milligrams of FW per milliliter able to inhibit 50% of DNA damage).

Iron(II) Chelating Activity. The method of Dorman et al. (34) was adapted to assess the iron(II) chelating activity in *Citrus* extracts. The reaction mixture containing 950 μ L of extract serially diluted with 80% methanol and 50 μ L of 0.5 mM FeCl₂·4H₂O was incubated for 5 min at room temperature. A total of 50 μ L of 2.5 mM ferrozine was then added and allowed to equilibrate for 10 min at room temperature. The purple coloration formed was read at 562 nm. The chelating activity was calculated according to the following equation:

$$\text{chelating activity (\%)} = \left(\frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100$$

Results are expressed as mean IC₅₀ (milligrams of FW per milliliter).

Preparation of AGEs. Human serum albumin (HSA, 96–99%) was used for the preparation of HSA glycoated by incubation with 10 mmol/L methylglyoxal (HSA_{MGO}) to be used as AGEs. A solution of 40 g/L HSA was prepared in phosphate-buffered saline (PBS, pH 7.4) and was incubated with methylglyoxal (MGO) (10 mM final concentration) at 37 °C for 7 days. PBS was added instead of MGO in the control HSA solution (HSA_{G0}). After incubation, HSA_{G0} and HSA_{MGO} were dialyzed against PBS for 3 days, sterile-filtered with 0.2 μ m Millipore filters, aliquoted in 2 mL Eppendorf tubes, and stored at –20 °C.

Inhibition of Free-Radical-Induced Hemolysis of Human Erythrocytes. The ability of the *Citrus* fruit extracts to inhibit free-radical-induced hemolysis of human erythrocytes was assessed according to Prost (35). Human blood samples were obtained from the Biochemistry Department of the CHR Felix Guyon Hospital of La Réunion and were taken on the ethylenediaminetetraacetic acid (EDTA) substrate as anticoagulant. Plasma was removed by centrifugation, and erythrocytes were washed with an isotonic solution (0.15 M NaCl). Each well of a 96-well plate was filled with 100 μ L (2×10^7 erythrocytes, 80 000 cells/ μ L final concentration) of a diluted red blood cell solution ($1/50$ in 0.15 M NaCl). A total of 100 μ L of *Citrus* extracts serially diluted in 0.15 M NaCl, 100 μ L HSA_{G0}, or 100 μ L HSA_{MGO} were then added in triplicates. Hemolysis was initiated by adding 40 μ L of 0.5 M AAPH in each well. Turbidity at 450 nm was recorded every 10 min using a 37 °C thermostatted microplate reader. Results are expressed as 50% of maximal hemolysis time (HT₅₀, in minutes), which represents the total defense against free radicals in human and animal models subjected to oxidative stress (36). The measurement of HT₅₀ is very reproducible: intra- and interassay coefficients of variation are reported to be of the order of 1.32 and 3.85%, respectively (35, 37).

Cell Culture of SW872. Human SW872 liposarcoma cells (ATCC HTB-92) obtained from the laboratory of Dr. Lise Bernier (Clinical Research Institute of Montreal, Montreal, Quebec, Canada) were cultivated in DMEM with 10% fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (100 units/mL), and streptomycin (100 units/mL). Cells were grown in a 5% CO₂ incubator at 37 °C. Following trial experiments using different dilutions, cells were plated in triplicate for each condition, at 200 000 cells/500 μ L per well in a sterile 24-well plate (for the following ELISAs: protein carbonyl, apoE, and TNF α) and at 32 000 cells/200 μ L per well in a sterile 96-well plate (for cytotoxicity analyses and the DCFH–DA assay).

Cytotoxicity Measurement by the Dimethylthiazol Diphenyl Tetrazolium (MTT) Assay. The effect of *Citrus* extracts on SW872 cell viability was assayed by the MTT assay. The reduction of tetrazolium salts is widely accepted as a reliable method to examine cell viability/proliferation (38). At 24 h after seeding of SW872 cells in 96-well plates, culture medium was replaced by a solution containing 180 μ L of DMEM (1% FBS) with 20 μ L of different concentrations of *Citrus* extracts (0.01–1%) and allowed to incubate for 24 h. At 3 h before the end of incubation, 20 μ L of the MTT dye (5 mg/mL) was added into each well. Medium was then carefully removed, and 100 μ L of DMSO was added to each well. The plate was left under gentle agitation in the dark, at room temperature for 1 h, to dissolve the dark blue formazan crystals. The plate was read using a microplate reader (Fluostar, BMG France) at 595 and 690 nm (background absorbance). Absorbance of the sample was obtained by subtracting the background absorbance from the optical density (OD) at 595 nm. The

proliferation rate was expressed as a percentage of control cells treated only with 1% (v/v) DMSO.

Quantification of Carbonylated Proteins by ELISA. Protein carbonyls are formed by a variety of oxidative mechanisms and are sensitive indices of oxidative injury. A total of 5 μ L of cell lysates (3–5 μ g of protein) was denatured by 5 μ L of 12% sodium dodecyl sulfate (SDS) for 10 min at room temperature. The protein carbonyls present in the samples were then derivatized to 2,4-dinitrophenylhydrazine (DNP) hydrazone with 5 mM DNP in 2 M HCl for 20 min at room temperature and neutralized. DNP is a chemical compound that specifically reacts and binds to carbonylated proteins. Samples were diluted in coating buffer, adsorbed to wells of an ELISA plate (NUNC Maxisorp) for 3 h at 37 °C, blocked with PBS/Tween (0.1%)/bovine serum albumin (BSA, 1%) overnight at 4 °C, and probed with an anti-DNP antibody (3 h at room temperature) (Sigma D9656), followed by a HRP conjugated secondary antibody (1 h at room temperature) (ECL anti-rabbit IgG, HRP-linked whole antibody from donkey). The tetramethylbenzidine (TMB) substrate was then added and allowed to oxidize for 15 min at room temperature, leading to the formation of a sapphire blue complex. The reaction was stopped by the addition of 2 M HCl, and absorbance was measured at 450 nm. Results are expressed as a percentage of the absorbance compared to control cells.

Determination of Intracellular ROS Production. DCFH–DA was used for the determination of intracellular ROS production. In principle, DCFH–DA is able to readily penetrate the cell membrane, whereas the diacetate esteric form can be rapidly de-esterified by the membrane-bound enzyme, esterase, to yield the DCFH-free form. The latter is the reduced form of the fluorescent dichlorofluorescein (DCFH). Upon reaction with ROS, DCFH is oxidized to yield the fluorescent DCF, whose intensity can be correlated with the amount of ROS formed *in situ*.

Cells were seeded in 96-well plates, incubated for 24 h, and washed with PBS. DMEM (1% FBS) containing *Citrus* extracts (0.05 and 0.1%) or DMSO (0.1%) was added, and following an overnight incubation, the medium was removed and cells were washed with PBS and DMEM (1% FBS) containing 10 μ M DCFH–DA. After 30 min of incubation at 37 °C, cells were washed with PBS, 200 μ L of 0.1 mM H₂O₂ or PBS was added, and the fluorescence of the oxidized form of DCF–DA (DCF) was monitored in a microplate reader at 492 nm (excitation) and 520 nm (emission) every 5 min for 1 h. Results are expressed as a percentage of fluorescence of cells compared to control cells (SW872 cells incubated with DMSO).

apoE and TNF α ELISA. After media collection, cells were washed 2 times with PBS, then lysed in lysis buffer [25 mM Tris (pH 8), 2 mM EDTA, 1% Triton, and 1% protease inhibitor mixture (GE Healthcare)], and spun for 10 min at 10000g. A total of 100 μ L of culture medium was used for apoE quantification by ELISA, as described in ref 39. TNF α was quantified using a human TNF α ELISA kit from eBioscience.

Statistical Analysis. Simple regression analysis was performed to calculate the dose–response relationship of the standard solutions used for calibration as well as test samples. Unicam Vision 32 software (Unicam, Ltd., U.K.) was used to evaluate initial and final antioxidant rate values for the TEAC assay. Data are expressed as the mean \pm standard deviation (SD) from at least two independent experiments performed in triplicates. Statistical significance was determined using one-way analysis of variance (ANOVA) (followed by Dunnett's multiple comparison test) for multiple comparisons, with a *p* value of less than 0.05 required for significance, using Prism software, version 4.0 (GraphPad Software, San Diego, CA).

RESULTS

Phenolic Content of *Citrus* Fruit Extracts. The total phenolic and flavonoid compositions of the *Citrus* extracts are presented in Table 1. Tangor and tangelo flavedo and albedo extracts contained 6–7-fold total phenolics compared to the pulp extracts. The amount ranged from 6343 \pm 100 to 7667 \pm 57 μ g/g of FW in the following increasing order: tangor albedo < tangelo flavedo < tangelo albedo < tangor flavedo. Highest levels of total flavonoids were obtained in tangelo albedo extracts (4207 \pm 51 μ g/g of FW), followed by its flavedo extracts (3171 \pm 54 μ g/g of FW).

Table 1. Total Phenolic and Flavonoid Contents of Flavedo, Albedo, and Pulp Extracts of *Citrus* Fruits Analyzed^a

	tangelo			tangor		
	flavedo	albedo	pulp	flavedo	albedo	pulp
total phenolic (μg of gallic acid/g of FW)	6339 \pm 131	7203 \pm 86	1062 \pm 10	7667 \pm 57	6343 \pm 100	1009 \pm 6
total flavonoids (μg of quercetin/g of FW)	3171 \pm 54	4206 \pm 51	625 \pm 4	2863 \pm 54	2282 \pm 78	445 \pm 10

^a Data represent mean \pm SD of two independent experiments each performed in triplicates.

Table 2. HPLC Data of Flavanone, Flavone, and Flavonol Glycoside Levels in Tangelo and Tangor Flavedo, Albedo, and Pulp Extracts^a

	tangelo			tangor		
	flavedo	albedo	pulp	flavedo	albedo	pulp
poncirin	5.61 \pm 0.35	8.26 \pm 0.18	0.71 \pm 0.02	4.73 \pm 0.30	2.85 \pm 0.04	0.35 \pm 0.01
rhoifolin	ND ^b	ND	0.56 \pm 0.01	7.86 \pm 0.06	ND	ND
didymin	5.26 \pm 0.20	16.67 \pm 0.57	3.60 \pm 0.10	13.94 \pm 0.41	44.74 \pm 0.31	4.86 \pm 0.02
naringin	ND	ND	ND	ND	ND	ND
rutin	11.71 \pm 0.27	ND	0.73 \pm 0.01	10.62 \pm 0.17	5.72 \pm 0.18	0.74 \pm 0.01
diosmin	5.66 \pm 0.12	ND	ND	5.91 \pm 0.21	ND	ND
isorhoifolin	5.13 \pm 0.06	3.42 \pm 0.08	0.41 \pm 0.01	8.90 \pm 0.31	2.62 \pm 0.13	0.69 \pm 0.01
neohesperidin	10.33 \pm 0.30	10.18 \pm 0.32	ND	5.67 \pm 0.19	4.87 \pm 0.05	0.73 \pm 0.01
hesperidin	163.21 \pm 1.71	217.67 \pm 8.32	17.04 \pm 0.12	234.08 \pm 3.47	301.18 \pm 3.48	26.98 \pm 0.07
neorierocitrin	15.75 \pm 0.39	ND	0.56 \pm 0.02	8.43 \pm 0.19	47.22 \pm 4.35	1.34 \pm 0.04
narirutin	13.99 \pm 0.32	60.77 \pm 2.28	20.91 \pm 0.10	20.03 \pm 0.22	177.80 \pm 4.19	ND

^a Data expressed in mg/g of FW as mean \pm SD of two independent experiments each performed in duplicates. ^b ND = not detected.

Table 3. Antioxidant Activities of Tangelo and Tangor Flavedo, Albedo, and Pulp Extracts as Measured by the TEAC, FRAP, HOCl, Copper–Phenanthroline, and Iron(II) Chelation Assays^a

	tangelo			tangor		
	flavedo	albedo	pulp	flavedo	albedo	pulp
TEAC (μmol of trolox g^{-1} of FW)	43.1 \pm 0.4	61.1 \pm 1.7	7.1 \pm 0.4	46.1 \pm 1.4	36.6 \pm 1.5	6.2 \pm 0.4
FRAP (μmol of Fe^{II} g^{-1} of FW)	55.0 \pm 0.9	48.9 \pm 0.5	7.8 \pm 0.2	57.8 \pm 0.9	49.5 \pm 1.2	9.2 \pm 0.1
HOCl (IC_{50} , mg of FW mL^{-1})	5.2 \pm 0.1	5.6 \pm 0.1	53.6 \pm 0.7	4.4 \pm 0.1	7.5 \pm 0.1	99.8 \pm 1.7
copper–phenanthroline (IC_{50} , mg of FW mL^{-1})	6.3 \pm 0.5	71.7 \pm 2.8		7.5 \pm 0.6	67.7 \pm 2.5	
iron(II) chelating activity (IC_{50} , mg of FW mL^{-1})	10.8 \pm 0.2	132.1 \pm 3.7		9.1 \pm 0.2	167.1 \pm 9.5	

^a Data expressed as mean \pm SD of two independent experiments each performed in triplicates. Pulp extracts of tangelo and tangor fruits were unable to inhibit DNA damage in the copper–phenanthroline assay and chelate iron(II) ions at the maximum concentrations used in this experiment.

Tangor flavedo and albedo contained lower levels of flavonoids. The pulps were relatively poor in flavonoids.

The flavonoid profile of flavedo, albedo, and pulp extracts of tangelo and tangor was analyzed by HPLC, and the flavonoid glycoside levels in the extracts are shown in **Table 2**. Flavonoid glycosides were higher in flavedo and albedo extracts compared to pulp extracts. Naringin was not detected in any of the *Citrus* extracts, while poncirin, didymin, isorhoifolin, and hesperidin were present in all of them. Hesperidin was present at highest concentrations in both *Citrus* varieties, and its content ranged from 301 \pm 3 mg/g of FW (tangor albedo) to 17 \pm 0.1 mg/g of FW (tangelo pulp). Narirutin, which ranked second after hesperidin, was present in highest concentrations in albedo extracts of both tangelo and tangor.

Antioxidant Activity of *Citrus* Fruit Extracts in Cell-Free Systems. The TEAC, the FRAP, the HOCl scavenging activities, the ability to protect DNA against damage (copper–phenanthroline assay), and the iron(II) chelating activity of tangelo and tangor extracts are given in **Table 3**. Flavedo extracts of both *Citrus* varieties had the highest antioxidant potentials in all of the test systems, except in the TEAC assay, where tangelo albedo was more potent. The TEAC values ranged from 61.1 \pm 1.8 $\mu\text{mol/g}$ of FW (tangelo albedo) to 6.2 \pm 0.4 $\mu\text{mol/g}$ of FW (tangor pulp). Although the *Citrus* albedo extracts showed good scavenging and reducing abilities in the TEAC, FRAP, and HOCl assays, they had low activities in the cuphen (IC_{50} , 71.7 \pm 2.8 and 67.7 \pm 2.5 mg

of FW mL^{-1}) and iron chelation (IC_{50} , 132.1 \pm 3.7 and 167.1 \pm 9.5 mg of FW mL^{-1}) assays. Pulp extracts exhibited very low antioxidant activities in the TEAC ($< 10 \mu\text{mol}$ of trolox g^{-1} of FW), FRAP ($< 10 \mu\text{mol}$ of Fe^{II} g^{-1} of FW), and HOCl (IC_{50} , > 50 mg of FW mL^{-1}) assays and did not show any ability to protect DNA from damage or chelate metal ions.

The ability of the *Citrus* extracts to retard free-radical-induced hemolysis of human erythrocytes was investigated, and typical hemolysis curves are illustrated in **Figure 1a**. Tangelo flavedo extracts increased the hemolysis time of red blood cells, compared to the control, in a dose-dependent manner. The hemolysis half-times (HT_{50}) of tangelo and tangor extracts (flavedo, albedo, and pulp), at 0.01 and 1%, are shown in panels **b** and **c** of **Figure 1**. There were significant increases in HT_{50} [$(*) p < 0.05$ and $(**) p < 0.01$], compared to the control, in erythrocytes treated with the *Citrus* extracts (0.01 or 1%). These increases in HT_{50} reached 100 and 50% when red blood cells were incubated with flavedo and albedo extracts, respectively. It is interesting to note that tangelo pulp extract at 1% did not differ significantly from the control ($p > 0.05$) in its capacity to delay red blood cell hemolysis.

The effect of HSA on free-radical-induced erythrocyte hemolysis was also investigated, and the results are presented in **Figure 1d**. The free radical scavenging property of native human serum albumin (HSA_{G0}) is illustrated by the significant increase in HT_{50} compared to the control ($p < 0.05$). The antioxidant activity of glycated albumin (HSA_{MGO}), on the

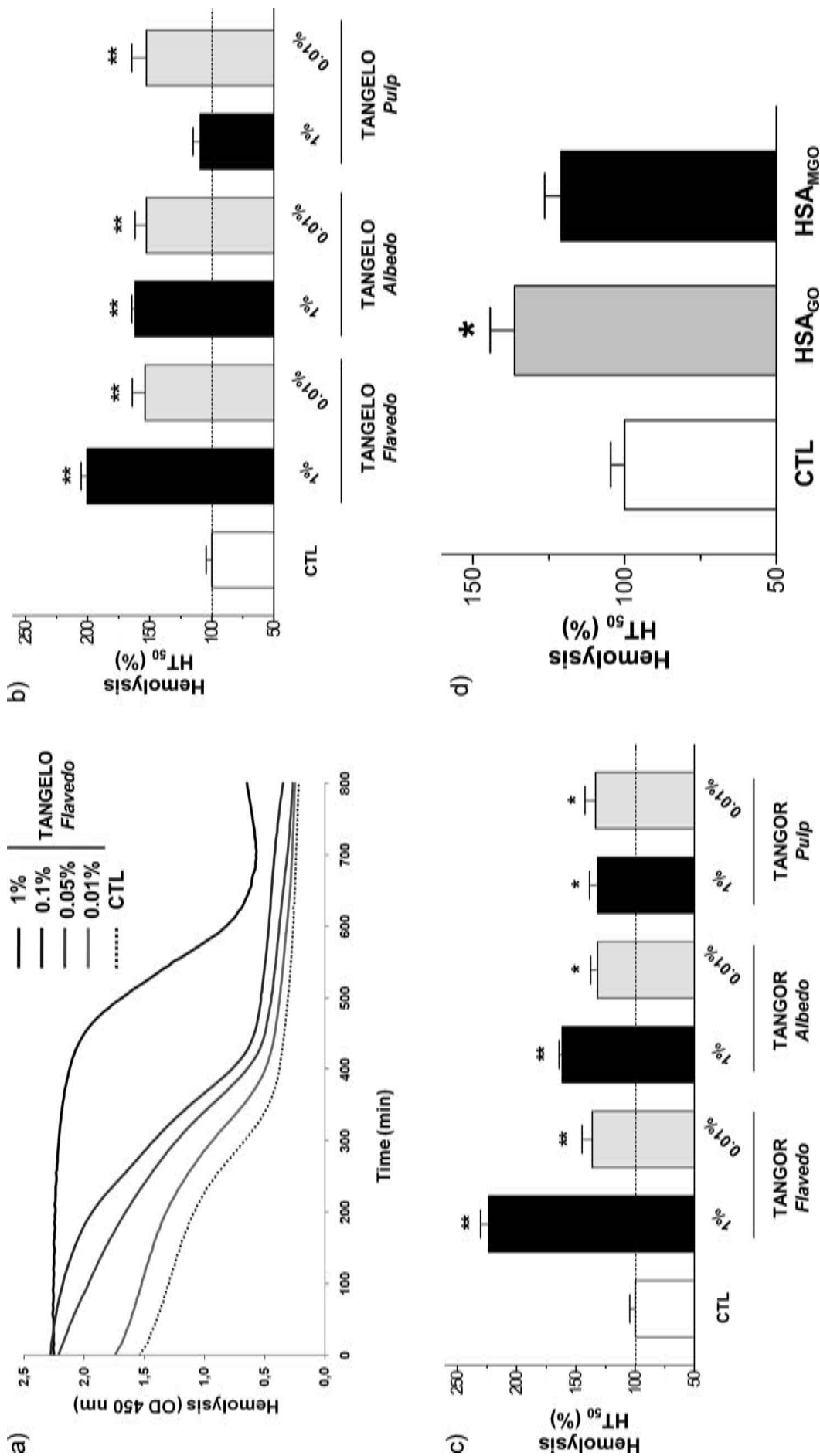


Figure 1. Effects of Citrus extracts and MGO-modified HSA on the free-radical-induced blood hemolysis test. Blood was prepared as described in the Materials and Methods and incubated with free radicals generated from 52.4 mmol/L AAPH at 37 °C. Hemolysis was monitored by spectrophotometry at 450 nm, and data were curve-fitted by computer analysis. (a) Curves illustrating the antioxidant effect of increasing concentrations of tangelo flavedo extracts compared to the control (1% DMSO). Results are expressed as 50% of maximal hemolysis time (HT₅₀, in minutes). (b) Histograms showing the antioxidant effects of two different concentrations of tangelo extracts on free-radical-induced blood hemolysis. Hemolysis half-times (HT₅₀) were expressed as a percentage of the control (DMSO). Results are expressed as mean ± standard error of the mean (SEM) of three independent experiments performed in triplicate using one-way ANOVA; (***) $p < 0.01$ versus the control. (c) Histograms showing the antioxidant effects of two different concentrations of tanger extracts on the free-radical-induced blood hemolysis. Results are expressed as a percentage of the control (DMSO) and represent mean ± SEM of three independent experiments performed in triplicate using one-way ANOVA; (*) $p < 0.05$ and (***) $p < 0.01$ versus the control. (d) Histograms showing the effects of native (HSA_{GO}) and methylglyoxal-modified HSA (HSA_{MGO}) on the free-radical-induced blood hemolysis test. Results are expressed as a percentage of the control (PBS) and represent mean ± SEM of three independent experiments performed in triplicate using one-way ANOVA; (*) $p < 0.05$ versus the control.

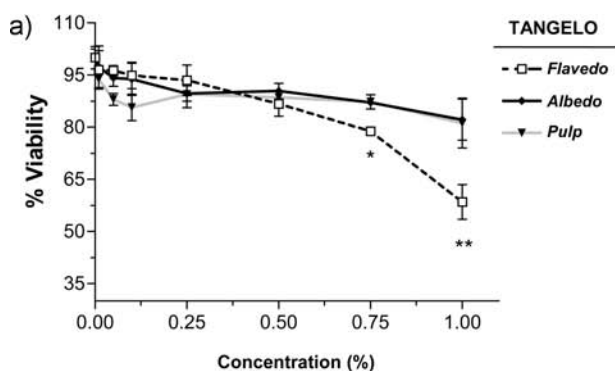
other hand, was strongly impaired, with no significant difference from the control.

Antioxidant Activity of Citrus Fruit Extracts in the SW872 Human Liposarcoma Cell Line System. *Effect of Citrus Extracts on SW872 Cell Line Viability.* The MTT test was used to evaluate the effect of Citrus extract pretreatment on cell viability. SW872 cells were treated with different concentrations (0.01–1%) of tangelo and tangor flavedo, albedo, and pulp extracts, respectively, for 24 h. The concentrations of Citrus extracts used for the cell treatments (% v/v) with the corresponding phenolic contents in gallic acid equivalent ($\mu\text{mol/L}$) are given in Table 4. Panels a and b of Figure 2 show that the flavedo extracts decreased cell proliferation in a dose–response manner, with significant reductions at 0.75 and 1%. Albedo and pulp extracts had no adverse effects on cell viability, even at the highest concentration tested (1%). In subsequent experiments, cells were thus incubated in the presence of low concentrations of flavedo, albedo, and pulp extracts (0.05 and 0.1%), for which no cytotoxic effect was noted.

Effect of Flavedo Extracts on Carbonyl Accumulation in AGE- and H_2O_2 -Treated Cells. Adipocytes and monocytes have previously been shown to accumulate protein carbonyls in response to AGEs generated by albumin glycation (18, 24, 40). The effect of different concentrations of HSA_{G0} and HSA_{MGO} on the carbonyl content of SW872 cells is shown in Figure 3a. HSA_{G0} and HSA_{MGO} (0.015 mM) had no effect on carbonyl accumulation. Increasing the concentration of native and glycated albumin to 0.03 mM resulted in an increase in the carbonyl content of the cells compared to the control. However, carbonyl accumulation was significantly higher ($p < 0.05$) in cells treated with HSA_{MGO} compared to cells treated with HSA_{G0}. HSA_{G0} (0.05 mM) reduced the carbonyl content of cells by 20% of the control, whereas the same concentration of HSA_{MGO} increased carbonyl accumulation by 10% ($p < 0.05$).

Table 4. Gallic Acid Equivalents ($\mu\text{mol/L}$) of the Different Concentrations of Extracts Used

	tangelo			tangor		
	flavedo	albedo	pulp	flavedo	albedo	pulp
1%	717	585	213	1234	421	200
0.75%	538	439	160	925	316	150
0.5%	359	293	107	617	211	100
0.25%	179	146	53	308	105	50
0.1%	72	59	21	123	42	20
0.05%	37	29	11	62	21	10
0.01%	7	6	2	12	4	2



The effect of tangelo and tangor flavedo extracts on the carbonyl content of cells is depicted in Figure 3b. Cells incubated with native albumin, HSA_{G0}, or glycated albumin, HSA_{MGO}, had lower levels of carbonyls than control cells. A cumulative protective effect against carbonyl accumulation is observed in the presence of flavedo extracts and native or glycated albumin. The lowest level of protein carbonyls was obtained when cells pretreated with tangelo flavedo extracts were incubated with HSA_{G0} (–17%, $p < 0.05$). Figure 3c shows the effects of flavedo extracts on H_2O_2 -mediated oxidative stress in cells. Adipocyte treatment with 100 μM H_2O_2 , which did not affect cell viability, resulted in an increase in carbonyl accumulation in the cellular medium (149% higher than the control). This increase in carbonyl content, because of H_2O_2 , was significantly reduced in cells pretreated with tangelo flavedo by 50% ($p < 0.05$). A marginal reduction of carbonyl accumulation in H_2O_2 -treated adipocytes was noted when cells were previously incubated in the presence of tangor flavedo extracts.

Effect of Citrus Extracts on Intracellular ROS Production. The effect of Citrus fruit extracts (0.1%) on ROS production in SW872 cells with or without the addition of 0.1 mM H_2O_2 is shown in panels a and b of Figure 4, respectively. Under both conditions, tangelo and tangor flavedo, albedo, and pulp extracts suppressed ROS production in the cells compared to the control. Flavedo extracts were more potent in this effect, with ROS levels significantly different from the control [(**) $p < 0.01$ and (*) $p < 0.05$]. In the absence of H_2O_2 -generated stress, flavedo extracts from tangelo and tangor reduced intracellular ROS production in the adipocytes by 40% ($p < 0.01$) and 35% ($p < 0.01$), respectively. These protections were less important but still significant in cells incubated with H_2O_2 . In these latter conditions (Figure 4b), 25% ($p < 0.01$) and 20% ($p < 0.05$) reduction in intracellular ROS formation were observed in cells pretreated with 1% flavedo extracts from tangelo and tangor, respectively.

Effect of Citrus Extracts on $\text{TNF}\alpha$ and apoE Secretions by Adipocytes. $\text{TNF}\alpha$ is a pleiotropic cytokine, which at the adipocyte level has been shown to exert determinant activities, such as differentiation regulation and lipid accumulation (lipolysis) (41). The effect of Citrus extracts (0.1%) on $\text{TNF}\alpha$ secretions by SW872 cells is shown in Figure 5a. Cells incubated in the presence of 0.1 mM H_2O_2 secreted 17% higher levels of $\text{TNF}\alpha$ compared to the control ($p < 0.05$). Surprisingly, all of the Citrus extracts induced significant increases in the levels of $\text{TNF}\alpha$ secretions in the following order: flavedo > albedo > pulp.

ApoE is a component of lipoproteins, whose role at the adipocyte level is not fully understood. However, recent studies

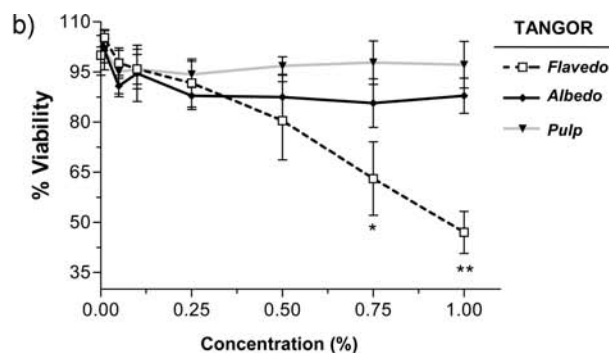


Figure 2. Effects of Citrus fruit extracts on SW872 cell viability. The relative proliferation rate of SW872 cells incubated for 24 h with different concentrations of (a) tangelo or (b) tangor extracts was determined by the MTT assay, as described in the Materials and Methods. Viability was expressed as a percentage of the corresponding control cells treated with only 1% (v/v) DMSO. Results are expressed as mean \pm SEM of three independent experiments performed in triplicate using one-way ANOVA; (*) $p < 0.05$ and (**) $p < 0.01$ versus the control.

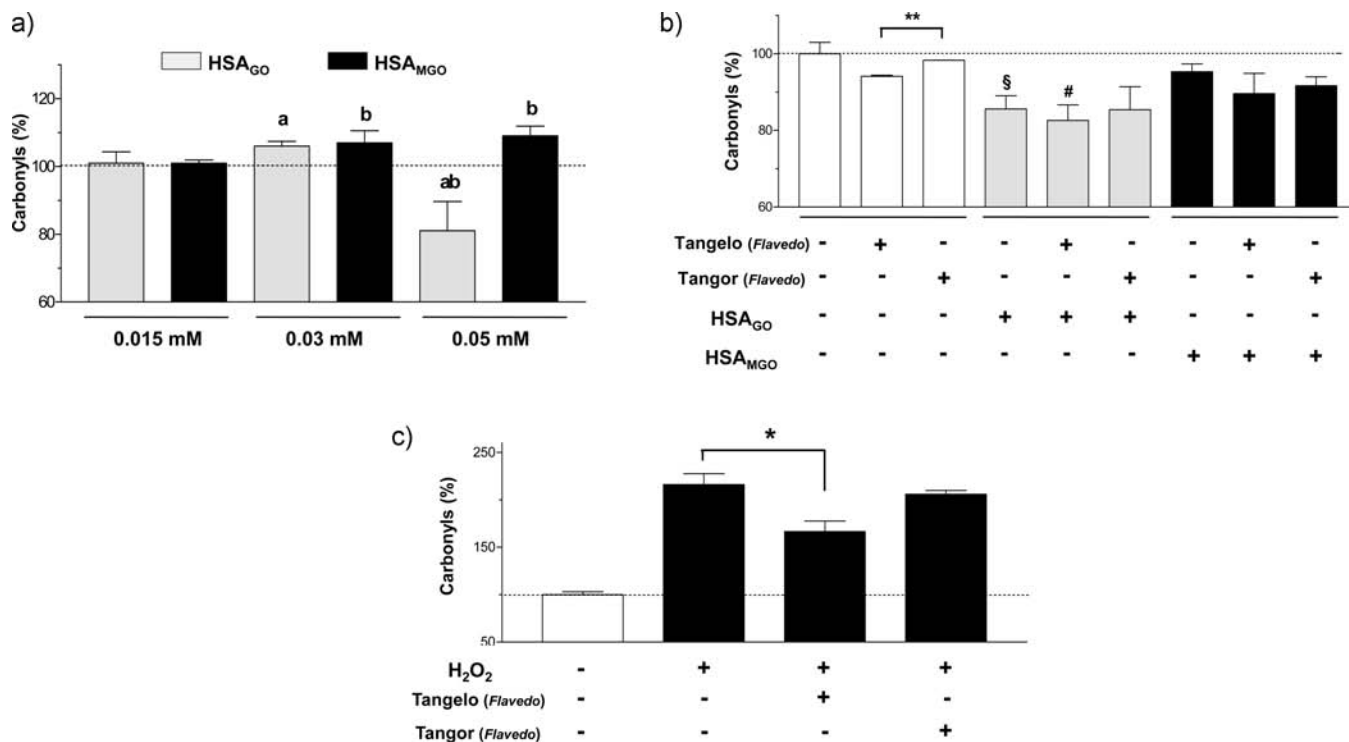


Figure 3. Effect of *Citrus* flavedo extracts on carbonyl accumulation in AGE- and H₂O₂-treated cells. Cells were seeded at a density of 100 000 cells/500 μ L per well of a 24-well plate. Cells were grown for 24 h in DMEM (10% FBS), washed with PBS (1 \times), and incubated (a) in the absence (control PBS) or with different concentrations of HSA_{GO} or HSA_{MGO} for 24 h in DMEM (1% FBS), (b) with *Citrus* extracts (0.05%) overnight, washed with PBS (1 \times), and incubated in the absence (control PBS) or with 0.05 mM HSA_{GO} or HSA_{MGO} in DMEM (1% FBS) for 24 h, (c) with *Citrus* extracts (0.05%) overnight, washed with PBS (1 \times), and incubated with H₂O₂ (100 μ M). After these treatments, cells were washed twice with PBS, and lysates were prepared. The carbonyl content in lysates was determined by ELISA, as described in the Materials and Methods. Results are expressed as a percentage of the corresponding control cells treated with only 1% (v/v) DMSO and/or PBS. Bars represent mean \pm SEM of three (a) or two (b and c) independent experiments performed in triplicate and compared using Student's *t* test for unpaired samples or one-way ANOVA. (a) Bars with identical letters are significantly different from each other by Student's *t* test for unpaired samples; (a and b) $p < 0.05$. (b) Significance using Student's *t* test for unpaired samples are (\$) $p = 0.08$ and (#) $p = 0.07$ (versus the control); (***) $p < 0.01$. (c) Significance was assessed using one-way ANOVA, followed by Dunnett's multiple comparison test; (*) $p < 0.05$.

reported a role for apoE in adipocyte differentiation, proliferation, and lipid accumulation (42). Levels of apoE secretions in control cells and cells treated with 0.1 mM H₂O₂ or flavedo extracts did not differ significantly ($p < 0.05$) (Figure 5b). Tangelo and tangor albedo and pulp extracts, on the other hand, caused the level of apoE secretions by the cells to drop significantly [from -20 to -25%; (***) $p < 0.01$ and (*) $p < 0.05$].

DISCUSSION

Oxidative stress and alterations in glucose metabolism are important risk factors of diabetes and its related complications. AGEs and their carbonyl derivatives are believed to contribute significantly to the pathogenesis of type-2 diabetes by their interaction with specific cell membrane receptors, triggering for instance the nuclear factor- κ B (NF- κ B) signaling pathway to induce the expression of pro-inflammatory mediators and elicit oxidative stress, which exacerbate diabetic complications (43). A great deal of effort has been focused on the identification of useful inhibitors of protein-AGEs to delay or prevent glycation to alleviate the phenotype of these diseases (44). Numerous AGE inhibitors, including aminoguanidine, improved diabetic complications in both animal models and clinical trials with, however, a number of adverse effects (45). It is suggested that AGE inhibitors from natural foods/dietary biofactors may reasonably serve as valuable adjuvants. The *Citrus* genus is the most important fruit tree crop in the world, bearing health-promoting fruits rich in antioxidant phytochemicals. Their role in the prevention of diseases, such as obesity, diabetes, blood-lipid-lowering and

cardiovascular diseases, and certain types of cancers have been reported (46–49). A comprehensive previous study conducted on 21 Mauritian *Citrus* species demonstrated, with established correlations, that polyphenolic-rich extracts exhibited important antioxidant propensities in various test systems (14). Two *Citrus* species, namely, tangor and tangelo, which were characterized by highest *in vitro* antioxidant activities, were chosen for evaluation of their potential protective effect on human SW872 adipocytes in this present study. Our data show that the flavedo and albedo extracts were more potent antioxidants than the pulp, with higher levels of total phenols and flavonoids. HPLC analyses confirmed the preponderance of poncirin, didymin, isorhoifolin, hesperidin, and narirutin in both extracts. These substances are widely discussed with regards to their anti-radical and anti-lipoperoxidation activities and their ability to chelate metals (50). Given that the mechanisms of action of naturally occurring antioxidants can be diverse *in vivo*, a comprehensive prediction of the antioxidant efficacy initially *in vitro* requires a multiplicity of assessing methods with various implications for molecular targets (51, 52). In this study, a number of well-established *in vitro* assays [TEAC, FRAP, HOCl scavenging, copper-phenanthroline, and iron(II) chelation assays] were used to characterize the direct antioxidant activities of *Citrus* extracts. Flavedo extracts of both *Citrus* varieties were consistently characterized by the highest activities in all test systems, except in the TEAC assay, where tangelo albedo was more potent, suggesting an influence of the variation in the chemical structures of the individual compounds (53). The extracts further showed pronounced abilities

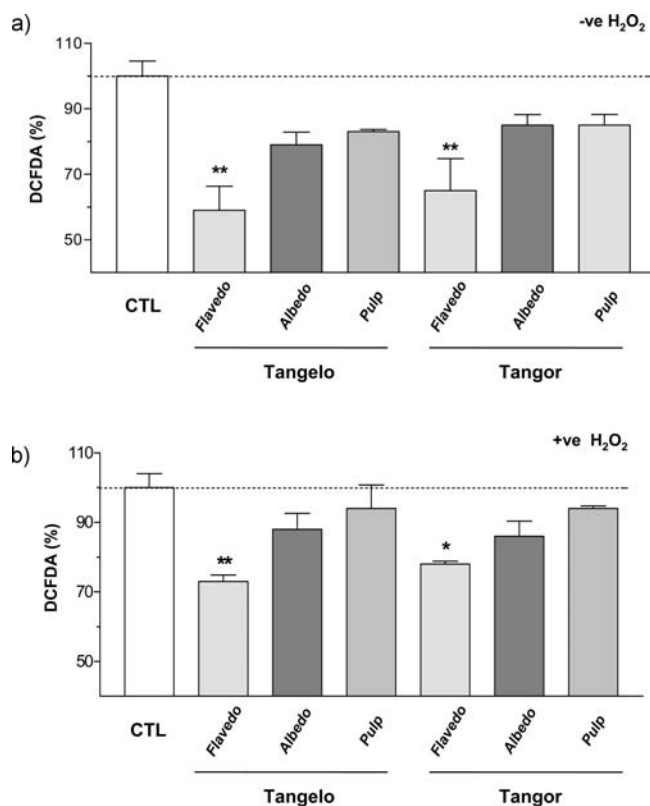


Figure 4. Comparative antioxidant activities of *Citrus* extracts in SW872 cells. Cells were seeded at a density of 160 000 cells/mL in a 96-well plate and were grown overnight in DMEM (10% FBS). Cells were washed with PBS (1 ×) and treated overnight with *Citrus* extracts at 0.1% in DMEM (1% FBS). Cells were washed, and DCFH-DA (10 mM) was added for 30 min. Medium was removed, and (a) PBS (100 μM) or (b) H₂O₂ (100 μM) was added. Fluorescence was monitored, and data were processed as described in the Materials and Methods. Results are expressed as mean ± SEM of three independent experiments performed in triplicate using one-way ANOVA, followed by Dunnett's multiple comparison test; (*) $p < 0.05$ and (**) $p < 0.01$ versus the control.

to delay free-radical-induced hemolysis in the hemolysis test, thus providing complementary evidence of their antioxidative potency. A wide range of extract concentrations were assayed, and U-shape dose-response curves were obtained with high- and low-hemolysis half-times (HT₅₀) at elevated (1%) and low (<0.1%) extract concentrations. Interestingly, significant increases in HT₅₀ were observed in the presence of very low concentrations of all tested extracts (0.01%). To our knowledge, this is the first report on the antioxidant propensity of nutritional compounds assessed by this red blood cell hemolysis test system patented in 1992 (35). The low dose-response data therefore represent favorable applicable conditions to the *in vivo* environment, without affecting cellular viability and physiology.

Despite the determinant role of adipocytes in the etiology of obesity-related disorders, there are very few reports on the effect of natural antioxidants on adipocyte response to oxidative stress.

Adipocyte cell viability was examined in the presence of different concentrations of tangelo and tangor flavedo, albedo, and pulp extracts. Only the flavedo extract produced toxic effects at high concentrations (>0.75%). The phenolic richness of the extract could contribute to this observation. Analogous reports have previously been made, whereby phenolic-rich plant extracts exerted modulatory effects. These results indicated that phenolic constituents of complex plant mixtures possess the character of a "Janus"-(anti)genotoxicant, a term used to designate compounds

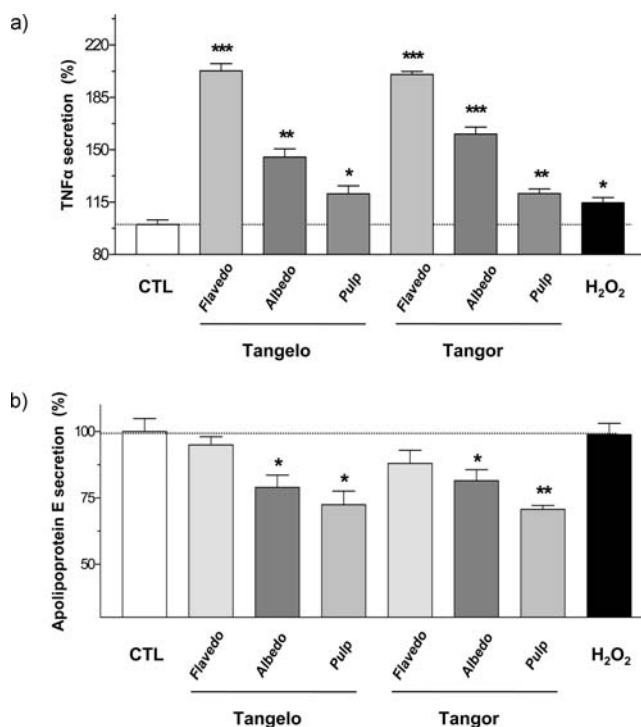


Figure 5. Effects of *Citrus* extracts on TNF α and apoE secretions in SW872 cells. About 300 000 cells were seeded in 1 mL/well of a 6-well plate. Cells were grown for 48 h in DMEM (10% FBS), washed with PBS, and treated overnight in the absence (CTL) or presence of *Citrus* extracts (0.1%) in DMEM (1% FBS). Cell supernatants were then collected and assessed for TNF α and apoE concentrations by ELISA, as described in the Materials and Methods. Results are expressed as a percentage of the corresponding control cells treated with only 1% (v/v) DMSO and/or PBS.

behaving as genotoxin or antigenotoxin, depending upon the plant extract concentrations used (54). The toxicity is suggested to be related to hydrogen peroxide formation arising from the auto-oxidation of phenolic molecules. In another work, Patil et al. showed that compounds purified from Mexican lime juice could induce apoptosis in human pancreatic cells (55), with the effects being shown to be proportionately linked to the flavonoid content.

We have previously reported that native albumin exerts strong antioxidant propensities (review in ref 56). Conversely, these activities are significantly impaired in glycated albumin (37). Our data show an increase in the half-time of AAPH-induced hemolysis in the presence of native albumin, while a significant reduction is observed with MGO-mediated glycation. Similar results were obtained on MGO-modified BSA by Faure et al. (57).

In the same vein, recent works performed by our group showed oxidative damages in adipocytes subjected to oxidative stress induced by glycated albumin (18, 24, 58). The reduction of carbonyl formation at the adipocyte level is clearly reflective of the antioxidant power of tangor and tangelo flavedos. This antioxidant propensity is reinforced with co-treatment with native albumin, while glycated albumin is devoid of antioxidant power. It is noteworthy that a small non-significant decrease in carbonyl accumulation was observed in adipocytes co-treated with MGO-modified HSA and flavedo extracts. Consistently, similar data are observed in adipocytes submitted to an oxidative stress generated by H₂O₂. A significant decrease in carbonyl formation was observed when cells pretreated with tangelo flavedo extracts were incubated in the presence of H₂O₂. Along this line, it has been reported that polyphenolics, more particularly anthocyanins,

have the ability to protect 3T3-L1 adipocytes against H₂O₂-induced insulin resistance (59). We further demonstrate that intracellular ROS formation is considerably lowered in cells pretreated with *Citrus* flavedo extracts incubated in the presence or absence of H₂O₂.

The pathological conditions of obesity are increasingly associated with an enhanced oxidative stress and inflammatory response at the adipose tissue level with elevated levels of TNF α (60, 61). However, contrary to our expectations, significant increases in TNF α secretions were measured in the supernatants of tangelo and tangor flavedo-treated cells. In a recent work, Chacón et al. showed that grape-seed flavonoids modulated IL6 and MCP1 inflammatory cytokines in human differentiated adipocytes *in vitro* with, however, no allusion to TNF α secretions (62). TNF α is described as the link between insulin resistance and obesity (63). It contributes to metabolic deregulation by impairing both adipose tissue function and its ability to store excess fuel (60). Obese adipose tissue is characterized by an enhanced infiltration of macrophages, which represent the source of TNF α production. In their work, Hirai et al. showed that tomato flavonoids had stronger inhibitory effects on inflammatory changes in macrophage–adipocyte co-cultures compared to control cultures (64). Our intriguing results about TNF α secretion by adipocytes treated by *Citrus* extracts probably suggest pro-inflammatory action, which, however, warrants further detailed investigations.

ApoE, which is a component of lipoproteins, e.g., chylomicrons, very low-density lipoprotein (VLDL), intermediate-density lipoproteins, and high-density lipoprotein (HDL), is mainly produced and secreted by the liver (65). ApoE is known to regulate both cellular and systemic cholesterol, as well as triglyceride metabolism (66, 67), and has been extensively studied for its potential role in the etiology of atherosclerosis, diabetes, and obesity. ApoE, which was shown to exhibit anti-inflammatory, anti-atherogenic, and antioxidant properties (68, 69), has been found to be highly expressed by adipose tissue and adipocytes (19, 70). However, if apoE expression by adipocytes has been known for many years, its importance in the adipose response to oxidative stress/antioxidants has never been thoroughly investigated. Significant reductions in apoE secretions were observed in albedo- and pulp-extract-treated cells. Recently, our group showed an increase in apoE secretion in SW872 cells subjected to oxidative stress induced by glucose or AAPH, a free-radical generator (67). We hypothesized that apoE may exert antioxidant effects at the adipocyte level, and its subsequent increase in expression may represent a defense response to oxidative stress (67). The decrease in apoE secretion in cells incubated with *Citrus* extract seems to be an adaptive response to the presence of this exogenous antioxidant.

In conclusion, our results show that *Citrus* extracts efficiently prevented oxidative stress in human adipocytes with no cytotoxic effects. We have characterized for the first time the beneficial effects of antioxidant *Citrus* extracts in a model of obesity-linked metabolic disorder. As such, our data convey new evidence that adipose tissue constitutes a preferential novel target for nutritional therapy. Further studies are needed to achieve a better understanding of the cellular and molecular mechanisms of nutritional antioxidants at the adipocyte level.

ABBREVIATIONS USED

AAPH, 2,2'-azobis(2-methylpropionamide) dihydrochloride; ABTS, 2,2'-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt; AGE, advanced glycation end product; apoE, apolipoprotein E; HSA, human serum albumin; HSA_{MGO}, HSA

glycated by incubation with 10 mmol/L methylglyoxal; DCFH–DA, 2',7'-dichlorofluorescein–diacetate; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FRAP, ferric-reducing antioxidant power; MTT, dimethylthiazol diphenyl tetrazolium; NF- κ B, nuclear factor- κ B; PBS, phosphate-buffered saline; ROS, reactive oxygen species; TEAC, trolox equivalent antioxidant capacity; TNF α , tumor necrosis factor α .

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