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Insulin Secretion and Cyclooxygenase Enzyme Inhibition by Cabernet Sauvignon Grape Skin Compounds

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Bioassay-guided isolation and purification of hexane and ethyl acetate extracts of Cabernet Sauvignon grape skin yielded nine compounds (**1**−**9**), which were identified as β-sitosterol-6′-linolenoyl-3-*O*-β-D-glucopyranoside (**1**), β-sitosterol (**2**), β-sitosterol-3-*O*-β-D-glucoside (**3**), oleanolic acid (**4**), oleanolic aldehyde (**5**), resveratrol (**6**), (+)-ε-viniferin (**7**), (−)-catechin (**8**), and 1-triacontanol (**9**). The structures of these compounds were established by spectroscopic methods. The compounds were assayed for insulin production using an INS-1 cell assay. In a dose−response study, compound **4** stimulated insulin production of INS-1 cells by 20.23, 87.97, 1.13, and 6.38 ng of insulin/mg of protein at 6.25, 12.5, 25, and 50 μg/mL, respectively. This trend was similar to the dose-dependent insulin production of INS-1 cells by glucose. Compound **5** also showed a dose-dependent insulin production in this assay. The isolated compounds were also assayed for cyclooxygenase-1 and -2 (COX) enzyme inhibitory activities. At 100 μg/mL, compounds **2**, **3**, and **4** inhibited the COX-2 enzyme by 11, 12, and 10%, respectively, but did not show activities on the COX-1 enzyme. Compounds **6**, **7**, and **8** at 100 μg/mL inhibited the COX-1 enzyme by 98, 99, and 98%, respectively, and the COX-2 enzyme by 0, 47, and 72%, respectively. This is the first report of β-sitosterol-6′-linolenoyl-3-*O*-β-D-glucopyranoside (**1**) from grape skin and insulin secretion activities of compounds **4** and **5**.

KEYWORDS: ***Vitis vinifera*; INS-1 cell; insulin production; cyclooxygenase enzyme; β-sitosterol-6′-linolenoyl-3-*O*-β-D-glucopyranoside; oleanolic acid**

INTRODUCTION

Diabetes, a disease in which the metabolism of carbohydrates and lipids is not regulated properly by insulin, is a serious health care issue worldwide. Insulin is secreted by pancreatic β-cells in response to elevated plasma glucose, with many factors modifying its secretion (*1*). It is widely accepted that diabetes is a trigger for vascular inflammation. Clinical evidence has confirmed the pathogenetic role of inflammation in the onset of diabetes by showing that anti-inflammatory agents prevent or delay the onset of diabetes in high-risk subjects (*2*−*4*). In one of the inflammatory processes, two distinct isoforms of cyclooxygenase enzyme, COX-1 and COX-2, convert arachidonic acid to prostaglandins (*5*). The inducible COX-2 enzyme is associated with inflammatory conditions, whereas extensively expressed COX-1 enzyme is responsible for the cytoprotective effects of prostaglandins (*6*, *7*).

For years grapes have been the subject of investigation, which highlighted the remarkable differences in the chemical composition of the cultivars and varieties examined. Stilbenes, anthocyanins, and procyanidins (*8*, *9*) from grape skin, seeds, and wines were shown to exhibit anti-inflammatory and antioxidant activities (*10*). Red wines are considered to be superior in terms of antioxidant activities than white wines. This may be due to the fact that the grape skin is in contact with the grape juice for a longer period of time prior to the fermentation of red wines. The longer contact time allows the extraction of grape skin components. However, a high proportion of active components remains in the vinification residue (*11*). Also, seeds and skins are generally discarded by the wine and grape juice industries. This unfermented waste product is a valuable raw material for the extraction of bioactive compounds. Recently, the wine industry is focusing on the isolation of antioxidant polyphenols from grape waste (*12*). In our continued interest in finding new phytoceuticals and value-added products from fruits and vegetables, we have evaluated compounds in grape skin for COX enzyme inhibition and insulin production activities. In this paper, we report the isolation and characterization of a number of compounds isolated from Cabernet Sauvignon and their in vitro insulin production and COX enzyme inhibitory activities.

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MATERIALS AND METHODS

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on Varian INOVA 300 and 500 MHz spectrometers. Compounds were dissolved in $CHCl_3$, acetone-d_6, or DMSO-d_6 and are reported in δ (parts per million) based on δ residuals of $CHCl_3$, acetone-d_6, and DMSO-d_6 at 7.24, 2.04, and 2.50, respectively, for ^{1}H NMR and 77.0, 206.7, and 39.5, respectively, for ^{13}C NMR. Coupling constants, *J*, are in hertz. Silica gel (30−60 μm particle sizes) used for medium-pressure liquid chromatography (MPLC) was purchased from Merck. Optical rotations were obtained in MeOH at 20 °C on a Perkin-Elmer 341 polarimeter (Shelton, CT). TLC plates and Prep-TLC (GF Uniplate, with binder, 500 μm) were the products of Analtech, Inc., Newark, DE. Vioxx tablets and Celebrex capsules used in the cyclooxygenase inhibitory assay as positive controls were physician's professional samples provided by Dr. Subash Gupta of Sparrow Pain Center, Sparrow Hospital, Lansing, MI. All organic solvents used were of ACS regent grade (Aldrich Chemical Co., Inc., Milwaukee, WI).

Extraction and Isolation of Compounds from Grape Skin. *Vitis vinifera* var. Cabernet Sauvignon grapes were harvested from Michigan State University (MSU) Horticulture farm on October 25, 2000, by Dr. Stan Howell, Viticulture and Enology, Department of Horticulture, MSU, and kept at −20 °C until extraction. The frozen grapes (14.8 kg) were thawed at room temperature and then squeezed by hand, and the juice was filtered through cheesecloth. The skin and the seeds were then suspended in distilled water and separated to yield the skin (2.8 kg, w/w) and seeds (420 g, w/w). The grape skin was then blended with methanol (4 L × 4), and the solvent was evaporated under reduced pressure. The methanol crude extract thus obtained (344 g) was suspended in distilled water, partitioned successively with hexane (1 L × 6) and ethyl acetate (500 mL × 6), and yielded 36.5, 7.0, and 300.5 g of hexane, ethyl acetate, and water soluble extracts, respectively.

The hexane-soluble portion (14 g) was subjected to silica gel MPLC and eluted with hexane (400 mL), hexane/acetone (8:1, 400 mL), hexane/acetone (4:1, 400 mL), hexane/acetone (2:1, 400 mL), and acetone (100%, 400 mL), respectively. The fractions collected were 15 mL aliquots for all solvent systems. Fractions with similar TLC profiles were combined to yield fractions A (oily residue, 11.28 g), B (1.05 g) C (1.02 g), D (405 mg), and E (268 mg). Fraction B was dissolved in chloroform, and the white precipitate formed was filtered to yield compound **9** (42 mg). Fraction C was dissolved in chloroform/methanol (1:1) and kept at 4 °C overnight. The white crystalline material precipitated was filtered off and recrystallized from ethanol to yield compound **2** (178 mg) as white needle-like crystals. Similarly, fraction D was dissolved in methanol and the solution was kept at room temperature overnight; compound **4** (68 mg) was obtained as needle-like crystals. The precipitate from fraction E was recrystallized from a methanol/chloroform (1:1) mixture and yielded a pale white solid, compound **3** (65 mg). The mother liquor obtained from the recrystallization of compound **2** (840 mg) was subjected to a silica gel MPLC and eluted with hexane/EtOAc (98:2, v/v, 400 mL), hexane/EtOAc (95:5, v/v, 400 mL), hexane/EtOAc (9:1, v/v, 400 mL), and hexane/EtOAc (8:2, v/v, 400 mL), respectively. The hexane/EtOAc (95:5) fraction was evaporated under reduced pressure, and the residue was dissolved in chloroform/methanol (1:1) and kept at room temperature overnight. The white needle-like crystals formed were filtered off to yield compound **5** (33 mg).

The ethyl acetate extract (5.6 g) was redissolved in 50 mL of EtOAc and filtered to afford an ethyl acetate soluble fraction (5.48 g). This fraction was subjected to silica gel MPLC and eluted with hexane (200 mL), hexane/acetone (4:1, 800 mL), hexane/acetone (2:1, 800 mL), hexane/acetone (1:1, 800 mL), hexane/acetone (1:2, 800 mL), and acetone (100, 400 mL), successively. The fractions collected were in 15 mL aliquots. The fractions were combined on the basis of TLC and yielded fractions 1−11. Fraction 4 was dissolved in chloroform/methanol (1:1) and kept at 4 °C overnight to afford white needle-like crystals. It was filtered off and recrystallized from methanol to yield compound **4** (1.8 g) (**Figure 1**). Fraction 5 was purified by silica gel preparative TLC using hexane/ethyl acetate (6:4) as the developing solvent. The band at R_f 0.41 was removed and eluted with chloroform/methanol (1:1), and the solvent was evaporated under reduced pressure.

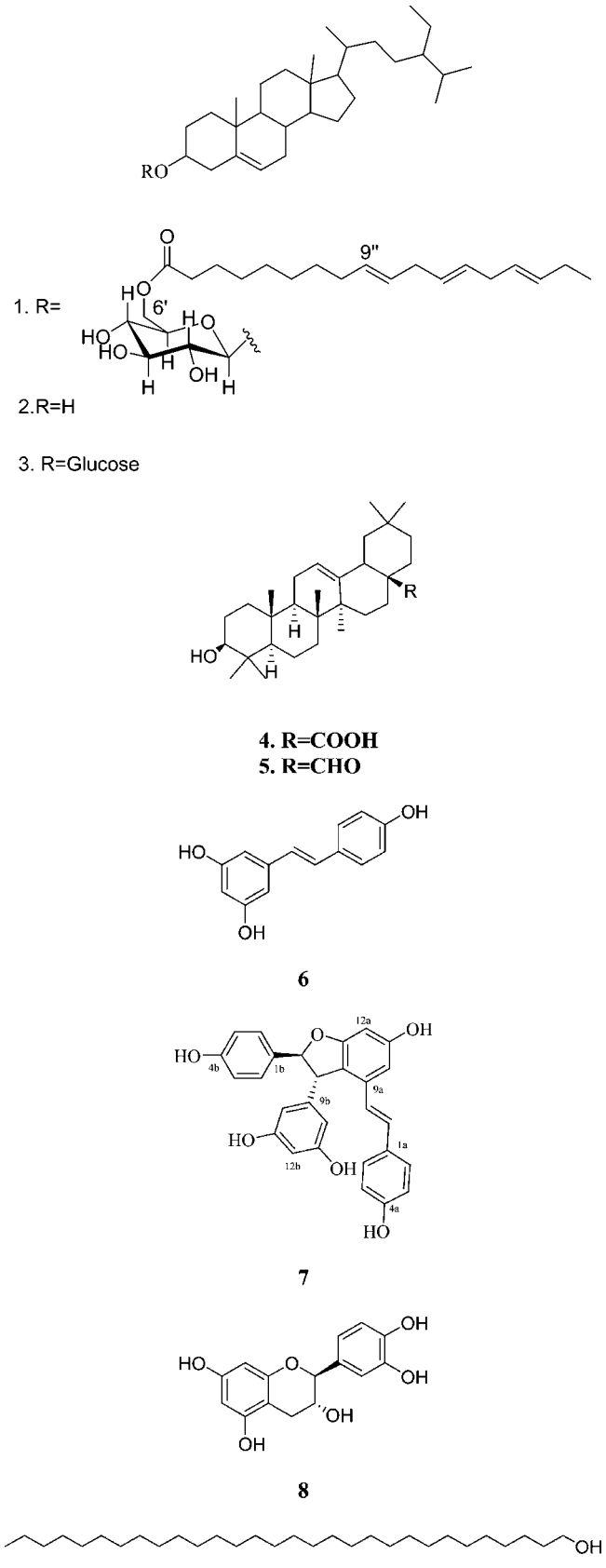

Figure 1. Structures of compounds **1**–**9**.

The residue yielded gave one spot on TLC, compound **1** (17.8 mg). Similarly, fraction 6 was purified by silica gel preparative TLC using chloroform/methanol (9:1) as the developing solvent; a blue band under UV light (254 nm) with R_f 0.55 was removed and eluted with methanol.

The solvent was removed under reduced pressure and yielded a white powder (**6**, 40.8 mg). Fraction 7 was purified by preparative TLC using hexane/acetone (1:1) as the mobile phase; a dark band under UV light (254 nm) with R_f 0.38 was collected and eluted with methanol. The solvent was removed under vacuum, and a pale yellow solid was obtained (**7**, 26 mg). Purification of fraction 10 by preparative TLC using chloroform/methanol (85:15) as developing solvent gave a dark band under UV light with an R_f 0.45. It was removed and eluted with methanol, and removal of solvent under reduced pressure afforded compound **8** (12 mg).

Compound **1**: FABMS, *m*/*z* 859 $[M + Na]^+$, 837.7 $[M + H]^+$, 397; ^{1}H NMR ($CDCl_3$) δ 0.66 (3H, s, CH_3-18), 0.79 (3H, d, $J = 7.0$ Hz, CH_3-26), 0.82 (3H, d, $J = 7.0$ Hz, CH_3-27), 0.83 (3H, t, $J = 7.5$ Hz, CH_3-29), 0.86 (3H, t, $J = 6.5$ Hz, CH_3-18″), 0.90 (3H, d, $J = 6.5$ Hz, CH_3-21), 0.98 (3H, s, CH_3-19), 2.76 (m), 3.31 (m, glc-2′), 3.37 (m, glc-5′), 3.46 (m, H-3), 3.40−3.50 (m, glc-3′ and glc-4′), 4.27 (1H, d, $J = 11.5$ Hz, glc-6′a), 4.35 (1H, d, $J = 8.0$ Hz, glc-1′), 4.39 (1H, dd, $J = 11.5$, 6.0 Hz, glc-6′b), 5.33 (5H, m, H-6, H-9″, H-10″, H-12″, and H-13″); ^{13}C NMR ($CDCl_3$) δ 174.50 (C-1″), 140.32 (C-5), 130.19, 130.00, 129.70, 128.09, 127.91, 127.51, 122.13 (C-6), 101.21 (C-1′), 79.60 (C-3), 76.05 (C-3′), 73.90 (C-5′), 73.55 (C-2′), 70.22 (C-4′), 63.31 (C-6′), 56.13 (C-17), 56.78 (C-14), 50.19 (C-9), 45.86 (C-24), 42.34 (C-13), 38.92 (C-4), 39.78 (C-12), 37.28 (C-1), 36.72 (C-10), 36.15 (C-20), 34.25 (C-2″), 33.97 (C-22), 31.93 (C-7), 31.89 (C-8), 29.2−29.7 (4″−16″), 29.19 (C-25), 28.23 (C-2), 27.23 (C-16), 26.16 (C-23), 26.16 (C-23), 24.97 (C-15), 24.30 (C-3″), 23.09 (C-28), 22.7 (C-17″), 21.07 (C-11), 19.80 (C-27), 19.35 (C-19), 19.04 (C-26), 18.78 (C-21), 14.09 (C-18″), 11.97 (C-29), 11.85 (C-18). The NMR data of compound **1** were identical to the published data of β-sitosterol-6′-linolenoyl-3-*O*-β-D-glucopyranoside (*13*).

Compound **4**: white needle-like crystals, mp 300−302 °C; ^{1}H NMR ($CDCl_3$) δ 5.27(1H, t, H-12), 3.20 (1H, dd, $J = 11.0$, 5.0 Hz, H-3), 2.81 (2H, dd, $J = 14.0$, 4.0 Hz, H-2), 0.92 (3H, s), 0.77 (3H, s), 0.75 (3H, s), 0.89 (3H, s), 0.91 (3H, s), 1.12 (3H, s), 0.97 (3H, s); ^{13}C NMR δ 38.5 (C-1), 27.3 (C-2), 79.1 (C-3), 38.8 (C-4), 55.4 (C-5), 18.4 (C-6), 32.8 (C-7), 39.4 (C-8), 47.7 (C-9), 37.2 (C-10), 23.1 (C-11), 122.7 (C-12), 143.6 (C-13), 41.7 (C-14), 27.8 (C-15), 23.5 (C-16), 46.6 (C-17), 41.2 (C-18), 46.0 (C-19), 30.7 (C-20), 33.9 (C-21), 32.5 (C-22), 28.1 (C-23), 15.6 (C-24), 15.3 (C-25), 17.2 (C-26), 26.0 (C-27), 182.5 (C-28), 33.0 (C-29), 23.6 (C-30). The NMR data of **4** were identical to the published data of oleanolic acid (*14*).

Compound **5**: white needle-like crystals, mp 169−170 °C; ^{1}H NMR ($CDCl_3$) δ 9.37 (1H, s, CHO), 5.32 (1H, t, $J = 3.5$ Hz, H-12), 3.19 (1H, dd, $J = 11.5$, 4.5 Hz, H-3), 2.60 (dd, $J = 14.0$, 4.5 Hz, H-2), 1.11 (3H, s), 0.96 (3H, s), 0.89 (3H, s), 0.88 (3H, s), 0.87 (3H, s), 0.75 (3H, s), 0.70 (3H, s); ^{13}C NMR (CD_3Cl) δ 39.4 (C-1), 27.1 (C-2), 79.0 (C-3), 38.7 (C-4), 55.1 (C-5), 18.2 (C-6), 33.1 (C-7), 40.3 (C-8), 49.1 (C-9), 36.9 (C-10), 23.4 (C-11), 123.2 (C-12), 142.9 (C-13), 41.6 (C-14), 27.7 (C-15), 22.0 (C-16), 47.4 (C-17), 45.5 (C-18), 46.0 (C-19), 30.6 (C-20), 33.1 (C-21), 28.1 (C-22), 32.7 (C-23), 17.0 (C-24), 15.3 (C-25), 17.2 (C-26), 26.7 (C-27), 207.7 (C-28), 32.7 (C-29), 23.6 (C-30). The NMR data of **5** were identical to the published data of oleanolic aldehyde (*15*).

Compound **6**: pale white powder; ^{1}H NMR (DMSO-d_6) δ 7.37 (2H, d, $J = 8.4$ Hz, H-6′), 6.91 (1H, d, $J = 16.2$ Hz, H-7), 6.78 (1H, d, $J = 16.2$ Hz, H-8), 6.74 (2H, d, $J = 8.4$ Hz, H-5′), 6.36 (2H, s, H-2, 6), 6.10 (1H, s, H-4); ^{13}C NMR (DMSO-d_6) δ 158.6 (C-3, 6), 157.2 (C-4′), 139.2 (C-1), 127.8 (C-7, 1′, 2′, 6′), 125.6 (C-8), 115.5 (C-3′, 5′), 104.2 (C-2), 101.7 (C-4). The NMR data of **6** were identical to the published data of resveratrol (*16*).

Compound **7**: pale white power, $[\alpha]_D$, +23° (methanol); FAB-MS, *m*/*z* 455 $[M + H]^+$; ^{1}H NMR (DMSO-d_6) δ 7.12 (2H, d, $J = 9.0$ Hz, H-2a, 6a), 6.66 (2H, d, $J = 9.0$ Hz, H-3a, 5a), 7.11 (2H, d, $J = 8.5$ Hz, H-2b, 6b), 6.74 (2H, d, $J = 8.5$ Hz, H-5b, 5b), 5.32 (1H, d, $J = 5.0$ Hz, H-7a), 4.39 (1H, d, $J = 5.0$ Hz, H-8a), 6.81 (1H, d, $J = 16.5$ Hz, H-8b), 6.56 (1H, d, $J = 16.5$ Hz, H-7b), 6.59 (1H, brd, $J = 2.0$ Hz, H-14b), 6.23 (1H, d, $J = 1.5$ Hz, H-12a), 6.05 (3H, br s, H-10a, 14a, 12b); ^{13}C NMR (DMSO-d_6) δ 160.7 (C-11b), 158.7 (C-11a, 13a), 158.6 (C-13b), 157.8 (C-4a), 157.5 (C-4b), 145.5 (C-9a), 134.8 (C-9b), 131.5 (C-1a), 128.9 (C-1b), 127.7 (C-2b, 6b), 127.6 (C-7b), 127.0 (C-2a, 6a), 121.8 (C-8b), 118.2 (C-10b), 115.6 (C-3b, 5b), 115.2 (C-3a, 5a), 105.4 (C-10a, 14a), 103.1 (C-14b), 101.1 (C-12a), 95.9 (C-12b), 92.4 (C-7a), 55.1 (C-8a). The NMR and optical rotation data confirmed that **7** is (+)-ϵ-viniferin (*17*).

Compound **8**: pale white power; $[\alpha]_D$, −17°; FAB-MS, *m*/*z* 291 $[M + H]^+$; ^{1}H NMR (acetone-d_6) δ 6.88 (1H, d, $J = 2.0$ Hz, H-2′), 6.77 (1H, d, $J = 8.0$ Hz, H-5′), 6.73 (1H, dd, $J = 8.0$, 2.0 Hz, H-6′), 6.02 (1H, d, $J = 2.0$ Hz, H-8), 5.87 (1H, d, $J = 2.0$ Hz, H-6), 4.55 (1H, d, $J = 7.5$ Hz, H-2), 3.98 (1H, ddd, $J = 8.0$, 7.5, 5.5 Hz, H-3), 2.91 (1H, dd, $J = 16.0$, 5.5 Hz, H-4a), 2.52 (1H, dd, $J = 16.0$, 8.0 Hz, H-4b); ^{13}C NMR (acetone-d_6) δ 82.8 (C-2), 68.3 (C-3), 28.8 (C-4), 100.6 (C-10), 157.2 (C-5), 95.3 (C-6), 157.7 (C-7), 96.1 (C-8), 156.9 (C-9), 131.8 (C-1′), 115.2 (C-2′), 146.1 (C-3′), 146.0 (C-4′), 115.7 (C-5′), 119.8 (C-6′). FAB-MS, $[\alpha]_D$, and NMR data of **8** were consistent with the published data of (−)-catechin (*18*).

Compound **9**: white power, EIMS, *m*/*z* 438 $[M]^+$; ^{1}H NMR ($CDCl_3$) δ 0.86 (3H, t, $J = 6.3$ Hz), 1.23 (s), 1.54 (t, $J = 6.9$ Hz), 3.62 (2H, t, $J = 6.6$ Hz); ^{13}C NMR ($CDCl_3$) δ 63.1, 32.8, 31.9, 30.9, 29.7, 29.4, 29.3, 25.7, 22.7, 14.1. The EIMS and NMR data of **9** were found to be identical to the published data of 1-triacontanol (*19*).

Hydrolysis of Compound 1 and Methylation of the Resulting Fatty Acids. Diazomethane was prepared by reacting *N*-nitroso-*N*-methylurea with concentrated KOH solution in ether (*20*). The diazomethane generated was stored in ether until methylation of the fatty acid. Compound **1** (1 mg) was dissolved in methanol (0.5 mL) and reacted with 1% KOH in methanol (0.5 mL) overnight. The resulting solution was neutralized with 3 M HCl and evaporated under vacuum. The residue was suspended in water and extracted with EtOAc. The EtOAc solution was evaporated to dryness and the residue reacted with diazomethane in ether until the solution became pale yellow. The resulting yellow solution was kept at room temperature for 1 h in a fume hood and evaporated. The residue obtained was dissolved in hexane and analyzed by GC-MS. Similarly, the standard samples of palmitic, stearic, oleic, linoleic, and linolenic acids (1 mg each) were methylated by reaction with diazomethane in ether. The resulting methyl esters of the fatty acids were dissolved in hexane and analyzed by GC-MS.

GC-MS Analysis. The GC-MS analyses of fatty acid methyl esters were accomplished on an HP-6890 GC-MS equipped with an HP-5MS capillary column (30 m × 250 μm × 0.25 μm) purchased from HP and an electron capture detector maintained at 250 °C. Direct (splitless) injection was carried out by a model 7673 injector at 250 °C, and the carrier gas was helium at 0.8 mL/min. The injection volume was 1.0 μL. The GC temperature program consisted of an initial temperature of 50 °C, which was held for 2 min, then raised at 10 °C /min to reach a final temperature of 250 °C, and held until the end of the analysis. The spectrum was scanned from *m*/*z* 40 to 550.

Insulin Secretion Assay. INS-1 cells used in the assay were rat pancreatic β-cells derived from the parental RINm5f lines (*21*). This cell line was maintained in the Bioactive Natural Products and Phytoceutical Laboratory. Cells were grown at 37 °C under 5% CO_2 in a humidified incubator in RPMI 1640 medium containing 11.1 mM glucose and supplemented with 10% fetal bovine serum (FBS), 1 mM pyruvate, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 50 μM 2-mercaptoethanol, 100 units of penicillin/mL, 1 mM pyruvate, and 100 μg of streptomycin/mL. Cells were passaged every week by detachment with trypsin EDTA to ensure adequate confluency. All studies were performed on INS-1 cells between passage numbers 70 and 84.

Cells were counted (0.75×10^6), plated out in a 24-well plate, and incubated. After 36 h of incubation, the cells were treated with the medium containing 4 mM glucose and incubated for another 24 h. The 10× MSS solution was prepared by adding NaCl (69.4 g), KCl (3.54 g), KH_2PO_4 (1.615 g), $MgSO_4 \cdot 7H_2O$ (2.925 g), and $CaCl_2$ (2.82 g) to 1 L of distilled water. Krebs−Ringer buffer (KRB) was prepared by combining 15 mL of 10× MSS, 0.063 g of $NaHCO_3$, and 0.357 g of HEPES. Bovine serum albumin (BSA) was added to the KRB, incubated for 30 min, and divided into incubation and conditioning buffers. Incubation buffer contained KRB and isobutyl methyl xanthine (IBMX), and conditioning buffer contained KRB and 4 mM glucose. Then cells were incubated twice, each time for 30 min in the incubation buffer. Conditioning buffer (1 mL) with aliquots of samples (10 μL)

was added to the cells and incubated for 20 min, and plates were placed on ice to stop further insulin secretion. An aliquot of the solution was withdrawn from each well to Eppendorf tubes and centrifuged; 100 μL of the supernatant was removed, diluted to 300 μL with conditioning buffer, and stored for insulin ELISA. The remaining media in each well were transferred to test tubes and centrifuged, and the pellet was mixed with NaOH (500 μL, 1 N). Similarly the cells in the wells were treated, the NaOH digests were combined, and the total protein was determined according to the Lowry method.

For ELISA, washing buffer [(Na_2HPO_4 (1.15 g), $KH_2PO_4 \cdot H_2O$ (0.2 g), KCl (0.2 g), NaCl (8.0 g), Tween 20 (0.5 mL) per liter] and sample buffer (NaFAM) [Na_2HPO_4 (4.6 g), $NaH_2PO_4 \cdot H_2O$ (1.05 g), BSA (60.0 g), NaCl (6.0 g), sodium merthiolate (0.24 g)] were prepared. The coating antibody (rabbit anti-guinea pig) (100 μL) was added to 96-well plates, refrigerated overnight, washed with washing buffer, treated with 100 μL of anti-insulin antibody per well, and kept at 4 °C (24 h). Plates were then washed with washing buffer, incubated with 300 μL of NaFAM (30 min), and washed with washing buffer after removal of NaFAM; samples (100 μL) were added to the wells (in duplicate) and incubated for 50 min. The peroxidase enzyme (100 μL) was added to each well, incubated at 37 °C for 40 min, and washed with 200 μL of washing buffer. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfuric acid) (ABTS) dye was added. The plates were kept at room temperature for 1 h, and optical density (OD) was measured at 405 nm using an EL 800 Universal Microplate Reader (Alpha Scientific Medical, Inc., La Verne, CA). Pure insulin was used to determine the standard curve in the ELISA assay. Insulin standards were assayed in triplicate to generate a linear relationship between the amount of insulin and OD.

Test samples were dissolved in DMSO and diluted with KRB to yield final concentrations of 50, 25, 12.5, and 6.25 μg/mL for each test sample with a final DMSO concentration of 0.2%. Samples were assayed in quadruplicate, and results were reported as means ± standard error of the mean. The insulin secretion per milligram of protein was calculated by Students *t* test with unequal variance using Excel (*22*).

Cyclooxygenase Inhibitory Assay. COX-1 enzyme inhibitory activity was measured by using the enzyme preparation from ram seminal vesicles purchased from Oxford Biomedical Research, Inc., Oxford, MI. COX-2 enzyme inhibitory activity was measured using enzyme prepared from HPGHS-2 cloned insect cell lysate and diluted with Tris buffer (pH 7.0) to give an approximate final concentration of 1.5 mg of protein/mL. Assays were conducted at 37 °C and pH 7 by monitoring the initial rate of O_2 uptake using an Instech micro oxygen chamber and electrode attached to a YSI 5300 biological oxygen monitor. Each assay mixture contained 0.6 mL of 0.1 M Tris buffer (pH 7), 1 mM phenol, hemoglobin (85 μg), and arachidonic acid (27 mM). DMSO solutions of test samples or DMSO alone (10 μL) were added to the assay chamber and incubated with COX enzyme for 5 min. Reaction was initiated by adding arachidonic acid into the assay chamber containing test sample and COX enzyme. The data were recorded using QuickLog for Windows data acquisition and control software (Strawberry Tree, Inc., Sunnyvale, CA), and each sample was assayed twice (*23*).

RESULTS AND DISCUSSION

The Cabernet Sauvignon grapes used in this study yielded 18.9 and 2.8% of skin and seeds, respectively. The grape skin was extracted with methanol and partitioned with hexane, ethyl acetate, and water to afford three distinct fractions. The water fraction was not studied further after it was determined that it contained mainly anthocyanins, delphinidin 3-glucoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-glucoside, malvidin 3-glucoside, malvidin 3-acetylglucoside, and malvindin 3-*p*-coumarylglucoside by HPLC and LC-MS analyses. β-Sitosterol (**2**), β-sitosterol-3-*O*-β-D-glucoside (**3**), and oleanolic aldehyde (**5**) were purified from hexane extract with yields of 0.0166, 0.006, and 0.003%, respectively. Similarly, β-sitosterol-6′-linolenoyl-3-*O*-β-D-glucopyranoside (**1**), resveratrol (**6**), (+)-ϵ-viniferin (**7**), (−)-catechin (**8**), and 1-triacontanol (**9**) were purified from the EtOAc extract, and the yields were 0.0008, 0.00186, 0.0012, 0.0005, and 0.0039%, respectively. Oleanolic acid (**4**) was obtained in pure form from both hexane and ethyl acetate extracts with a combined yield of 0.07% with respect to the wet weight of the grape skin. The structures of these compounds were established by ^{1}H and ^{13}C NMR spectral experiments. Detailed explanations of structure elucidation for most compounds are not provided, but all of the supporting ^{1}H and ^{13}C NMR chemical shift values are provided under Materials and Methods.

The ^{1}H and ^{13}C NMR spectral data of compound **1** gave characteristic signals for a sterol moiety, a hexose unit, and a long-chain fatty acid moiety. The fatty acid moiety showed three double bonds as indicated by its ^{13}C NMR shifts at δ 130.2, 130.0, 129.7, 128.1, 127.91, and 127.5. Both ^{1}H and ^{13}C NMR chemical shifts of the hexose moiety indicated that it was a glucose. The doublet appearing at 4.35 ppm was assigned to the anomeric proton of the glucose and was confirmed by its correlation to the carbon at 101.2 ppm in its HMQC spectrum. The correlation of the anomeric proton of the glucose moiety to the C-3 carbon (δ_C 79.6) of the sterol in the HMBC spectrum confirmed that the glucose moiety was connected to the 3-hydroxyl group of the sterol. Similarly, the correlation of methylene protons of the glucose moiety (δ_H 4.27, and 4.39) to the carbonyl carbon (δ_C 174.6) as observed in the HMBC spectrum indicated that the long-chain fatty acid was attached to the 6′-OH of the glucose moiety. The β-sitosterol moiety in compound **1** was supported by ^{1}H and ^{13}C NMR chemical shift values and were in agreement with the published spectral data for β-sitosterol.

In addition to proton and carbon spectral data, the fatty acid functionality in compound **1** was confirmed by the GC-MS analysis of the fatty acid methyl ester derived from the hydrolysis of **1**. Compound **1** was hydrolyzed by reaction with 1% KOH in methanol, and the resulting products were methylated with diazomethane. The fatty acid methyl esters thus formed were analyzed by GC-MS and compared with the retention times of standard fatty acid methyl esters. The fatty acid moiety in compound **1** was therefore confirmed as linolenic acid. The FABMS of compound **1** gave a molecular ion at *m*/*z* 837 [59%, $(M + H)^+$]. The other significant fragment confirming the sterol moiety in the mass spectrum of **1** was at *m*/*z* 397 (20%). This is the first report of β-sitosterol-6′-linolenoyl-3-*O*-β-D-glucopyranoside (**1**) in grape skin.

Cyclooxygenase enzyme inhibitory activities of grape skin compounds were tested at 100 μg/mL. Compounds **2**, **3**, and **4** inhibited COX-2 enzyme by 11, 12, and 10%, respectively, but were inactive to COX-1 enzyme. However, compounds **6**, **7**, and **8** inhibited the COX-1 enzyme at 100 μg/mL by 98, 99, and 98%, respectively. Levels of inhibition of COX-2 enzyme by **6**, **7**, and **8** at 100 μg/mL were 0, 47, and 72%, respectively (**Figure 2b**). It is important to note that the isolated yield of compound **4** was 0.07% in the grape skin studied. Although it showed a weak but very specific COX-2 inhibitory activity, consumption of whole Cabernet Sauvignon grapes with the high concentration of **4** present may have an impact in pain relief.

The insulin production by INS-1 cells grown in 4 mM glucose was initiated by isobutyl methyl xanthine (IBMX). IBMX stimulates cyclic AMP production, which in turn enhances the insulin secretion by the cells. The results from our studies with compounds from grape skin indicated that compound **4** was the most effective in stimulating INS-1 cells to secrete significant amounts of insulin (**Figure 3**). At 12.5 μg/mL, **4** increased insulin production by 87.97 ng/mg of protein in INS-1 cells compared to 4 mM glucose. A dose-dependent insulin produc-

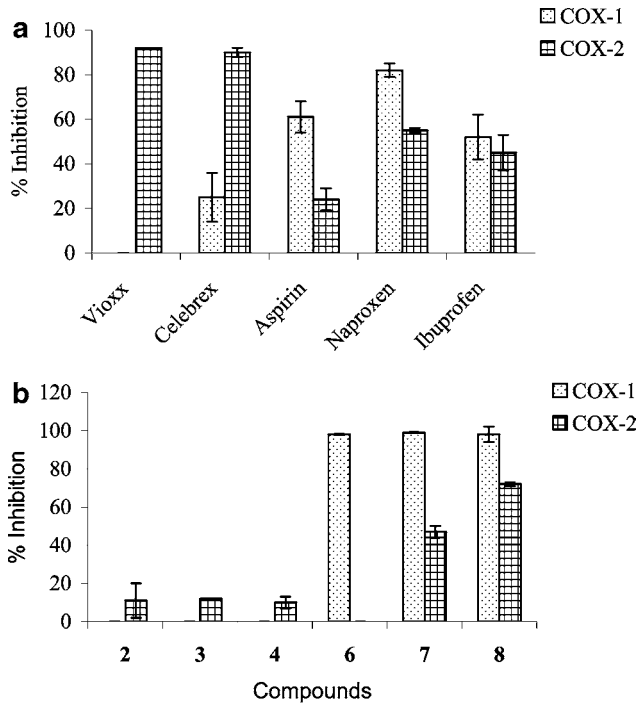

Figure 2. (**a**) Percent inhibition of COX-1 and COX-2 enzymes by Vioxx, Celebrex, aspirin, naproxen, and ibuprofen. Vioxx and Celebrex were tested at 1.67 μg/mL. Aspirin, naproxen, and ibuprofen were tested at 180, 2.52, and 2.06 μg/mL, respectively. Vertical bars represent standard deviation of each data points ($n = 2$). (**b**) Percent inhibition of COX-1 and COX-2 enzymes by compounds **2**–**4** and **6**–**8** at 100 μg/mL. Compounds **1**, **5**, and **9** were not active in this assay. Vertical bars represent standard deviation of each data points ($n = 2$).

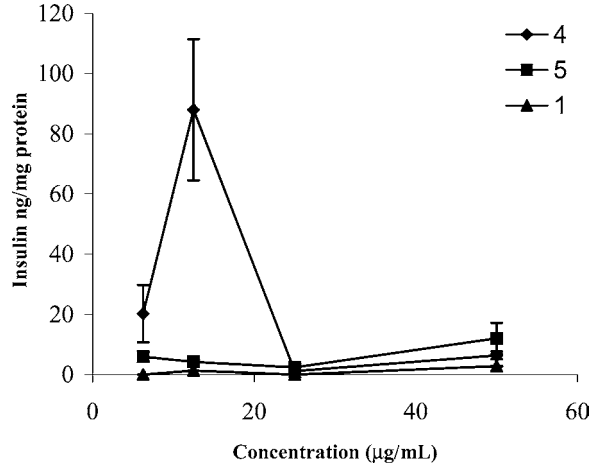

Figure 3. Insulin secretion of compounds **1**, **4**, and **5** at 6.25, 12.5, 25, and 50 μg/mL. Compounds **2**, **3**, and **6**–**9** were not active in this assay. Vertical bars represent standard deviation of each data points ($n = 4$).

tion was evident for this compound, similar to the dose-dependent insulin production by glucose in INS-1 cells. At 25 and 50 mg/mL of **4**, the secretion of insulin was reduced considerably in a dose-dependent manner (**Figure 3**). Compounds **1** and **5** also enhanced insulin secretion by 1.29 and 4.22 ng/mg of protein at 12.5 μg/mL, respectively (**Figure 3**). However, other compounds isolated from grape skin in our study did not enhance insulin secretion by INS-1 cells. Previous studies also demonstrated a positive correlation between compound **4**, oleanolic acid, and serum glucose levels in oral glucose-loaded rats (*24*−*26*). Recently, it was reported that oleanolic acid inhibited the effect of postprandial blood glucose increase in diabetic rats (*27*). Olive leaves, which contained mainly oleanolic acid, suppressed the elevation of blood glucose after oral administration of starch in human borderline volunteers with a fasting blood glucose level of 110−140 mg/dL (*27*). Our finding that oleanolic acid may increase the secretion of insulin in INS-1 cells supports the notion that grape and grape products have beneficial effects for the prevention of diabetes when consumed as part of the diet (*28*).

Our results indicate that several compounds present in grape skin or whole grapes are capable of insulin secretion as well as selectively inhibiting COX-2 enzyme. Oleanolic acid (**4**) is the most abundant compound in grape skin other than anthocyanins. In addition, it is the most effective among all grape skin compounds studied in our laboratory on the basis of insulin production and selective COX-2 enzyme inhibition. Therefore, consumption of whole Cabernet Sauvignon grapes may contribute to reduced incidence of type-2 diabetes and inflammation. Processing grape skins, a waste product of the wine and grape juice industries, may yield value-added products in this context.

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