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Isolation and Structure Elucidation of Avocado Seed (*Persea americana*) Lipid Derivatives That Inhibit *Clostridium sporogenes* Endospore Germination

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ABSTRACT: Avocado fruit extracts are known to exhibit antimicrobial properties. However, the effects on bacterial endospores and the identity of antimicrobial compounds have not been fully elucidated. In this study, avocado seed extracts were tested against *Clostridium sporogenes* vegetative cells and active endospores. Bioassay-guided purification of a crude extract based on inhibitory properties linked antimicrobial action to six lipid derivatives from the family of acetogenin compounds. Two new structures and four compounds known to exist in nature were identified as responsible for the activity. Structurally, most potent molecules shared features of an acetyl moiety and a *trans*-enone group. All extracts produced inhibition zones on vegetative cells and active endospores. Minimum inhibitory concentrations (MIC) of isolated molecules ranged from 7.8 to 15.6 μg/mL, and bactericidal effects were observed for an enriched fraction at 19.5 μg/mL. Identified molecules showed potential as natural alternatives to additives and antibiotics used by the food and pharmaceutical industries to inhibit Gram-positive spore-forming bacteria.

KEYWORDS: *acetogenins, avocado, Clostridium sporogenes, endospores, antimicrobial*

■ INTRODUCTION

The genus *Clostridium* represents a group of ubiquitous anaerobic spore-forming microorganisms of great concern in the medical and food industries. *Clostridium difficile* is known to be related to postantibiotic and hospital-acquired infections.[1,2] *Clostridium botulinum* and *Clostridium perfringens*[3] are important food-poisoning agents, whereas *Clostridium sporogenes* causes food spoilage.[4] In this regard, nitrites are the most widely used food additives to control spore-forming bacteria in processed meats.[5] However, nitrites can produce *N*-nitroso compounds, most of which are known potent carcinogens.[5] Substitutes to nitrites as antimicrobial additives, which do not present the same functionality on meat flavor and color, include nisin and ethyl lauroyl arginate, each compound with its own limitations such as high costs of production,[6] limited solubility, and bitter taste.[7] Therefore, there is a growing consumer and industrial demand for new antimicrobials from natural sources.

Avocado (*Persea americana*; Lauraceae) is a tropical fruit rich in biologically active phytochemicals.[8] Industrially, avocados are processed into oil and paste, which leaves 21−30% of the fruit weight to be discarded.[9,10] However, avocado seeds and peels have bioactive compounds contents comparable to or even greater than that of the pulp;[9,10] therefore, these waste products represent a potential source of molecules with applications in the food, pharmaceutical, and cosmetic industries.

Previous reports indicated that crude extracts from different avocado tissues exhibited antimicrobial activity against yeast,[11,12] fungal spores,[10,14] fungal vegetative cells,[13] and bacteria vegetative cells.[10,11,15−20] It has been hypothesized that the antimicrobial activity may be attributed to β-sitosterol,[12] phenolics, and fatty acids.[10] Antimicrobial properties of isolated fatty alcohol derivatives[11,19,20] and acetogenins[11,14,20] have been characterized. However, there are no studies regarding effects of crude avocado extracts or pure compounds (with known chemical identity) on bacterial endospores.

Our group reported in a preliminary antimicrobial screening that an acetone extract from avocado seed exhibited significant inhibitory properties against vegetative cell growth and endospore germination of *C. sporogenes*.[21] This microorganism was used as a surrogate of proteolytic *C. botulinum*[22] and as a representative of anaerobic spore-forming bacteria. Therefore, in this work, we described the bioassay-guided purification of a crude avocado seed extract against *C. sporogenes* vegetative cell growth and endospore germination by the disk diffusion assay, which led to the discovery of the chemical nature of the

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Table 1. ^{1}H and ^{13}C NMR (500 MHz) Spectroscopic Data for Persediene (2) and Persenone-C (3) in $CDCl_3$[a]

	persediene (2)		persenone-C (3)	
position	δ_C	δ_H	δ_C	δ_H
1	67.1	4.09 (dd, 11.4, 3.8)	67.1	4.12 (dd, 11.4, 4.1)
		4.03 (dd, 11.4, 6.3)		4.07 (dd, 11.4, 6.3)
2	65.9	4.27 (m)	66.0	4.32 (m)
3	45.0	2.60 (m)	42.1	2.75 (m)
4	210.5		199.5	
5	43.4	2.42 (t, 7.2)	130.0	6.09 (d, 15.8)
6	23.4	1.55 (m)	149.3	6.87 (dt, 15.8, 6.6)
7	~29	1.24 (m)	32.3	2.21 (q, 6.9)
8	~29	1.24 (m)	27.7	1.45 (m)
9	~29	1.24 (m)	~29	1.29 (m)
10	~29	1.25 (m)	~29	1.25 (m)
11	~29	1.25 (m)	~29	1.25 (m)
12	~29	1.25 (m)	~29	1.25 (m)
13	~29	1.25 (m)	~29	1.25 (m)
14	28.9	1.36 (m)	~29	1.29 (m)
15	32.4	2.07 (m)	32.3	1.94 (m)
16	135.3	5.68 (dt, 15.1, 6.6)	129.2	5.39 (m)
17	130.8	6.02 (dd, 15.1, 10.4)	131.6	5.40 (m)
18	137.2	6.29 (dt, 17.0, 10.4)	25.3	1.98 (m)
19	114.3	4.92 (d, 10.4)	13.8	0.94 (t, 7.6)
		5.06 (d, 17.0)		
$\underline{C}(=O)CH_3$	170.9		170.9	
$C(=O)\underline{CH_3}$	20.7	2.08 (s)	20.6	2.09 (s)

[a]Chemical shift (δ) in ppm, coupling constant J in hertz.

bioactive molecules. Inhibition properties of an enriched extract and purified compounds were further characterized against microbial growth from activated *C. sporogenes* endospores by determination of the minimum inhibitory and minimum bactericidal concentrations.

■ MATERIALS AND METHODS

Plant Material. Avocados (*P. americana* Mill cv. var. Hass) were obtained by Avomex International, S.A. de C.V. (Sabinas, CL, México) from Uruapan, Michoacán, México. Avocados were collected at full fruit expansion stage (~20% dry weight) and stored and transported unripe under controlled temperature conditions (10 °C) in containers with fresh air supply. Following commercial practices, avocados were treated with 80−100 ppm ethylene for 3 h and considered ripe after 7 days, when fruit color and softness were adequate (black skin with a firmness value of <40 N). Seeds were manually separated from the pulp, washed with water, vacuum packed, and stored at −80 °C prior to use.

Chemicals. Analytical grade methanol, heptane, ethyl acetate, hexane, and acetone were purchased from DEQ (San Nicolás de los Garza, NL, México). HPLC grade water and methanol were purchased from Fisher Scientific International (Winnipeg, MB, Canada), and isopropyl alcohol was acquired from Omnisolv (Gibbstown, NJ, USA).

Crude Extract Preparation and Partition. Unfrozen avocado seeds were ground in a colloidal mill. Ground seed compounds (2 kg) were extracted with acetone (1:2 w/v) for 24 h at 25 °C and concentrated at reduced pressure to obtain extract F001. Dry extract F001 was then partitioned in biphasic system 1 (heptane/methanol, 1:1 v/v), at an extract-to-each-phase of the solvent system of 1:1 w/v. Phases were separated and concentrated to yield extracts F002 (heptane-soluble compounds) and F003 (methanol-soluble compounds). Extract F003 was further partitioned in biphasic solvent system 2 (ethyl acetate/water, 1:1 v/v), using the previously described procedure to yield extracts F004 (ethyl acetate-soluble compounds) and F005 (water-soluble compounds). Extracts F001−F005 were adjusted with methanol to 2.5 mg/mL of solids and subjected to antimicrobial screening as described below.

Centrifugal Partition Chromatography (CPC) Fractionation of Extract F002. After assessment of bioactivity, extract F002 was further fractionated in a 1 L CPC system (Kromaton Technologies, Angers, ML, France), using heptane/methanol (1:1 v/v) as the solvent system. The CPC column was entirely filled with the upper phase of the solvent system to serve as stationary phase, and then the lower phase was pumped at a flow rate of 10 mL/min to establish hydrodynamic equilibrium. Extract F002, dissolved in 65 mL of lower phase, was then injected. Following the elution−extrusion approach,[23] the lower phase was used to elute fractions during 170 min, and then the upper phase was pumped during an additional 100 min. A total of 240 fractions were collected (10 mL/fraction), and their corresponding partition coefficients (K_D) were calculated according to the method of Berthod et al.[23] Fractions were individually concentrated to dryness, and 70 groups of fractions were composed by mixing equal weights of consecutive fractions so that each group had a final concentration of 2.5 mg/mL of solids. The bioactivity of the fractions was evaluated by the disk diffusion method as described below.

Purification and Further Characterization of Active Compounds. *Chromatographic Profile Analysis.* Antimicrobial screening identified the most potent CPC fractions against *C. sporogenes*. Bioactive fractions were then mixed together to obtain an enriched active fraction (EAF) extract. The extract was then adjusted to 1 mg/mL of solids and injected (2 μL) into two independent analytical Agilent 1100 HPLC systems (Agilent Technologies, Santa Clara, CA, USA), one of the instruments coupled to a photodiode (PDA) detector G1315D and the other to a MS-TOF detector G1969A, the latter equipped with an electrospray ionization interface (ESI). Separation was performed in a 100 × 3 mm i.d., 3.5 μm Zorbax Extend-C_{18} column (Agilent Technologies), with mobile phases that consisted of water 100% (A) and methanol 100% (B) with 0.1% formic acid when used for LC-MS. Solvent was pumped at 0.38 mL/min using a gradient of 0−2 min, 70−85% B linear; 4−22 min, 85−90% B linear; 22−24 min, 90−100% B linear; 24−26, 100% B isocratic, followed by 6 min of re-equilibration. ESI conditions included analysis in positive-ion mode; N_2 as nebulizing gas at 45 psi; drying gas flow rate and temperature of 8 L/min and 300 °C, respectively; voltage at the capillary entrance set at 2500 V; and

fragmentation, skimmer, and Oct RFV voltages at 100, 60, and 250 V, respectively. PDA detection at 220 nm produced a chromatogram with seven peaks (eluting at 7.1, 10.0, 12.1, 12.5, 13.5, 15.0, and 15.8 min, respectively) labeled as peaks A, B, C, D, E, F, and G, respectively. Peaks B−F were selected for further purification.

Preparative Isolation of Chromatographic Peaks. Initial isolation of peaks contained in EAF extract (at 50 mg/mL of solids) was achieved in a preparative Agilent 1100 HPLC system, equipped with a PDA detector G1315B and fraction collector G1364B. The column used was a 250 × 20 mm i.d., 5 μm, Phenomenex Prodigy C_{18} (Torrance, CA, USA), and the mobile phases were water 100% (A) and methanol 100% (B) at a flow rate of 20 mL/min. Solvent gradient was 0−4 min, 70−85% B linear; 4−22 min, 85−90% B linear; 22−24 min, 90−100% B linear; 32−35 min, 100% B isocratic, followed by 10 min of re-equilibration. Detection at 220 nm produced a chromatogram similar to the one obtained in the previous chromatographic profile analysis, from which peaks previously labeled as B−F were collected from each run. Peaks were independently pooled, concentrated in a stream of nitrogen, and stored at −20 °C until use. HPLC-MS analysis of the isolated peaks showed that, in most cases, further purification was needed to obtain pure compounds.

Final Purification of Active Compounds. Peak B contained a single component (compound **1**); therefore, additional purification was not necessary. Purification of all the other peaks was conducted in a Phenomenex Synergi Hydro-RP column (250 × 4.6 mm i.d., 4 μm), using water 100% and methanol 100% as mobile phases (A and B, respectively) at a flow rate of 1 mL/min. Isocratic methods were optimized for each peak. Individually, peaks C−F were dissolved in methanol HPLC grade and then injected into a semipreparative Agilent 1100 HPLC system coupled to a PDA detector G1314A and fraction collector G1364C. Compounds **2**, **3**, and **4** were recovered from peaks C, D, and E, respectively, whereas peak F was resolved in two major components, designated compounds **5** and **6**, which were coeluting under the method described under Chromatographic Profile Analysis. Each isolated compound was collected separately, concentrated, and immediately stored at −20 °C in amber vials until use. Antimicrobial activity of the EAF extract and pure compounds **1**−**6** at 0.5 mg/mL of solids was evaluated as described below.

Nuclear Magnetic Resonance (NMR) Spectroscopy. The ^{1}H NMR and ^{13}C NMR, COSY, HSQC, and HMBC spectroscopic experiments for the isolated compounds were performed on a 500 MHz Avance III NMR spectrometer from Bruker (Rheinstetten, Germany). $CDCl_3$ was used as solvent, and chemical shifts were referenced to the solvent signal.

Persediene (**2**): colorless oil; $[\alpha]_D^{22}$ +1.10° (*c* 0.22, $CHCl_3$); LC-MS (ESI$^+$), *m/z* (rel int) 727 $[2M + Na]^+$ (15), 375 $[M + Na]^+$ (100), 353 $[M + H]^+$ (40), 335 $[M + H - H_2O]^+$ (15), 293 $[M + H - HOAc]^+$ (23), 275 $[M + H - HOAc - H_2O]^+$ (8); LC-MS-TOF, *m/z* 353.2706 $[M + H]^+$ (calcd for $C_{21}H_{37}O_4$, 353.2692); ^{1}H and ^{13}C NMR spectroscopic data are presented in Table 1.

Persenone-C (**3**): colorless oil; $[\alpha]_D^{22}$ +9.50° (*c* 0.22, $CHCl_3$); LC-MS (ESI$^+$), *m/z* (rel int) 727 $[2M + Na]^+$ (28), 375 $[M + Na]^+$ (67), 353 $[M + H]^+$ (100), 335 $[M + H - H_2O]^+$ (22), 275 $[M - HOAc - H_2O + H]^+$ (11); LC-MS -TOF, *m/z* 353.2708 $[M + H]^+$ (calcd for $C_{21}H_{37}O_4$, 353.2692); for ^{1}H and ^{13}C NMR spectroscopic data, see Table 1.

Quantitation of Compounds in the Enriched Active Fraction Extract. The compounds in the EAF extract were quantitated by external calibration in triplicate using the analytical HPLC-PDA method described under Chromatographic Profile Analysis. Standard curves were constructed using compounds isolated and purified in our laboratory from avocado seeds (>96% purity). Data are expressed as milligrams of individual compound per milligram of total solids, on a dry weight basis.

Determination of Antimicrobial Activity. *Preparation of Vegetative Cells and Endospore Suspensions.* Putrefactive anaerobe *C. sporogenes* PA 3679 (ATCC 7955) was obtained from the American Type Culture Collection (Manassas, VA, USA). Vegetative cell cultures were grown in trypticase−peptone−glucose−yeast extract (TPGY) medium at 37 °C for 24 h in a Difco Laboratories GasPak anaerobic jar (Sparks, MD, USA). TPGY medium consisted of (per liter of distilled water) 50 g of tryptone, 5 g of peptone, 4 g of dextrose, 20 g of yeast extract, and 1 g of sodium thioglycolate.[24] Endospore cultures were prepared by growing *C. sporogenes* in reinforced clostridial medium (RCM) at 37 °C for 14 days in an anaerobic jar. Afterward, endospores were harvested by centrifugation at 2862*g* for 20 min at 22 °C and resuspended in sterile 1× phosphate-buffered saline (PBS). The suspension received ultrasonic treatment for 15 min to remove endospores from mother cells and, by washing several times with 1× PBS, a suspension primarily consisting of endospores was obtained, which was confirmed by phase-contrast microscopy.[25] All microbiology reagents were purchased from Difco Laboratories (Sparks, MD, USA).

Disk Diffusion Method. For antimicrobial screening of avocado seed extracts at different stages of purification, *C. sporogenes* stocks of vegetative cells or endospore suspensions were adjusted to an initial optical density (OD) at 600 nm of 0.1, which corresponded to an initial average cell concentration of 8×10^6 CFU/mL. In addition, the endospore suspension received a heat-shock treatment (80 °C for 15 min) to stimulate spore germination and inactivate any remaining vegetative cells.[24] After treatment, the suspension was placed in ice before the plates were inoculated. Aliquots (100 μL) of vegetative cells or endospore suspensions were evenly spread with a sterile plastic rod on the surface of TPGY agar plates. Sterile filter paper disks (6 mm diameter) were impregnated with aliquots (5 μL) of the extracts and air-dried for approximately 2 h in a biological safety cabinet before the disks were placed on the inoculated plates. A disk impregnated with ethanol or methanol (5 μL) was used as a solvent control for the EAF extract and purified compounds, respectively. Nisaplin (Danisco, Beaminster, DO, UK), which contains 2.5% w/w of nisin, served as a positive control. A stock solution of 50 mg/mL Nisaplin in sterile water was prepared to obtain in 5 μL a residual concentration of 6.25 μg of nisin per disk. All samples were assayed in triplicates. Each plate contained six disks evenly spaced: four tested samples and the positive and solvent controls. Plates were incubated for 36 h at 37 °C in an anaerobic jar. Subsequently, diameters of inhibition zones were measured to the nearest millimeter.

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Activated Endospore Suspensions. Further characterization of *C. sporogenes* microbial growth from activated endospores as affected by the EAF extract and isolated compounds **3**−**5** was performed in liquid medium by determining the MIC and MBC. Serial 2-fold dilutions of the samples were prepared in test tubes with TPGY broth to a final volume of 1 mL. Each tube was inoculated (100 uL) with an endospore suspension adjusted to an initial OD at 600 nm of 0.1, as previously described. The EAF extract in ethanol was tested at concentrations of 5−2500 μg/mL and purified compounds **3**−**5** in methanol from 0.5 to 15.6 μg/mL. All concentrations were compared to the corresponding concentration of solvent. Neither alcohol alone was inhibitory unless the concentration exceeded 3.12%; therefore, results are presented for samples with solvent concentrations below this limit. A growth control (no antimicrobial added), a uninoculated tube (aseptic technique control), and a positive control (nisin) were also contemplated. After 18−24 h of anaerobic incubation at 37 °C, the MIC was defined as the lowest concentration at which no endospore outgrowth was assessed by OD at 600 nm.[25,28] To evaluate the effect of killing activated endospores suspensions (bactericidal effect), aliquots (100 uL) of cultures at and above the MIC were subcultured in antimicrobial-free medium (1 mL of TPGY broth) and incubated for 18−24 h at 37 °C in anaerobic conditions. The MBC was defined as the lowest concentration not showing endospore outgrowth determined by OD.[25,28] Turbidity was assessed against an appropriate blank because samples showed some light absorbance. Concentrations limiting OD to <0.05 unit were defined as inhibitory.

Statistical Analysis. All samples were analyzed in triplicate and data expressed as the mean ± standard deviation. Data were analyzed using JMP software version 5.0 (SAS Institute Inc., Cary, NC, USA) by one-way ANOVA, and mean comparison was performed using LSMean Student's *t* test ($P < 0.05$).

Table 2. Recovered Solids and Diameter of Inhibition Zones against *Clostridium sporogenes* PA 3679 (ATCC 7955) Generated by a Crude Avocado Seed Extract and Subfractions

extraction system	fraction label (extraction solvent)	recovered solids (%)[a,b]	diameter of inhibition zone (cm)[b,c]	
			vegetative cells	endospores
crude extract	F001 (acetone)	3.62 ± 0.15 a[d]	1.15 ± 0.07 bc	0.95 ± 0.07 bc
biphasic system 1	F002 (heptane)	0.62 ± 0.05 e	1.80 ± 0.14 a	1.35 ± 0.21 a
	F003 (methanol)	2.87 ± 0.04 b	0.95 ± 0.07 c	0.65 ± 0.07 c
biphasic system 2	F004 (ethyl acetate)	1.08 ± 0.06 d	1.43 ± 0.11 b	1.05 ± 0.07 ab
	F005 (water)	1.82 ± 0.06 c	nd	nd

[a]Data expressed as grams of solids per 100 g of ground seed. [b]Values represent the mean ± standard deviation ($n = 3$). [c]All extracts were tested at 12.5 μg/disk of solids. nd, not detected. [d]Different letters within the same column indicate significantly different values by the LSMean Student's t test ($P < 0.05$).

■ RESULTS AND DISCUSSION

Microbial control of clostridia endospore germination and subsequent outgrowth is of great concern in the medical and food industries. It has been reported that extracts of avocado fruit possess antimicrobial properties;[10−12,15,16,18] however, the effect on bacterial endospores and identity of bioactive compounds has not been fully elucidated. In this study, we identified and assessed the potential of an avocado seed extract and its partitioned subfractions to inhibit vegetative cell growth and germination of clostridia endospores.

Crude Extract Preparation and Partitioning. Crude extract F001 from avocado seed was partitioned in a liquid−liquid biphasic system to yield a heptane-soluble extract F002 and a methanol-soluble extract F003. Around 80% of F001 initial weight extract was further recovered in F003 (Table 2), indicating a high content of compounds with polar to medium-polarity properties. This observation led to the subsequent partition of F003 to obtain an ethyl acetate-soluble extract F004 and a water-soluble extract F005. However, antimicrobial screening, also shown in Table 2, indicated that the active compounds were preferably concentrated in the less polar phases (F002 and F004) and were insoluble in the water phase (F005) that showed no bioactivity. Extract F002 exhibited the largest disk inhibition zones against *C. sporogenes* vegetative cells and endospores (1.80 ± 0.14 and 1.35 ± 0.21 cm, respectively). Although extract F002 contained the least recovered solids, the inhibition zones were 1.4- and 2.1-fold larger in diameter ($P < 0.05$) than those observed for F001 and F003, respectively. Consequently, F002 was selected for further fractionation by CPC.

Centrifugal Partition Chromatography Fractionation of Extract F002. Because extract F002 was expected to contain nonpolar and medium-polarity compounds, CPC was selected for preliminary separation of these types of molecules. Subsequently, CPC fractions were regrouped on the basis of weight of recovered solids. This analysis yielded 70 different groups of fractions that were subjected to antimicrobial evaluation. As shown in Figure 1, the fraction with a partition coefficient of $K_D = 0.24$ presented the largest zones of inhibition, which averaged 2.75 ± 0.18 and 1.85 ± 0.20 cm for *C. sporogenes* vegetative cells and endospores, respectively. These values were 1.4- and 1.5-fold larger than those initially exhibited by F002 (Table 2). Additionally, the most bioactive groups presented K_D values within 0.19−0.35 (dashed zone in Figure 1), suggesting the presence of compounds with medium-polarity chemical nature, because they were displaced from the solid phase during the elution stage. These groups of fractions, with high concentrations of bioactive compounds, were mixed together to obtain the EAF extract.

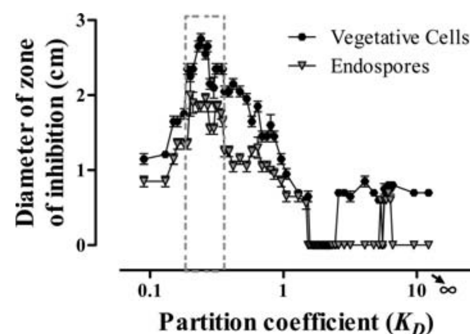

Figure 1. Diameter of zones of inhibition generated by groups of fractions, with different partition coefficients (K_D), isolated by centrifugal partition chromatography (CPC) from an avocado seed heptane-soluble extract (F002) against *Clostridium sporogenes* PA 3679 (ATCC 7955) vegetative cell growth and endospore germination. K_D values are expressed in logarithmic scale. Dashed area indicates groups of fractions (K_D = 0.19−0.35) with largest inhibition zones. Values represent the mean ± standard deviation ($n = 3$).

Purification and Further Characterization of Active Compounds. Individual chromatograms for the groups of fractions comprising the EAF extract (data not shown) consistently contained peaks B−F shown in Figure 2, whereas peaks A and G decreased in concentration as the bioactivity increased. Therefore, peaks B−F were selected for further

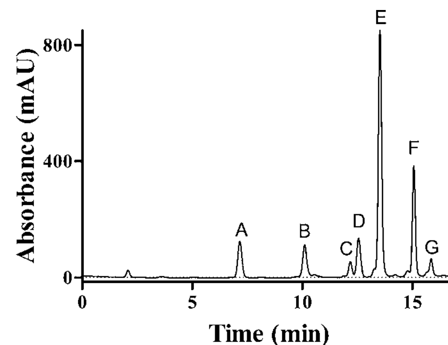

Figure 2. HPLC-PDA chromatogram at 220 nm of an enriched active fraction (EAF) extract of avocado seed with largest inhibition zones against *Clostridium sporogenes* PA 3679 (ATCC 7955) vegetative cell growth and endospore germination (dashed zone, Figure 1). Peak letters from A to G were assigned according to order of elution from a Zorbax Extend-C_{18} column.

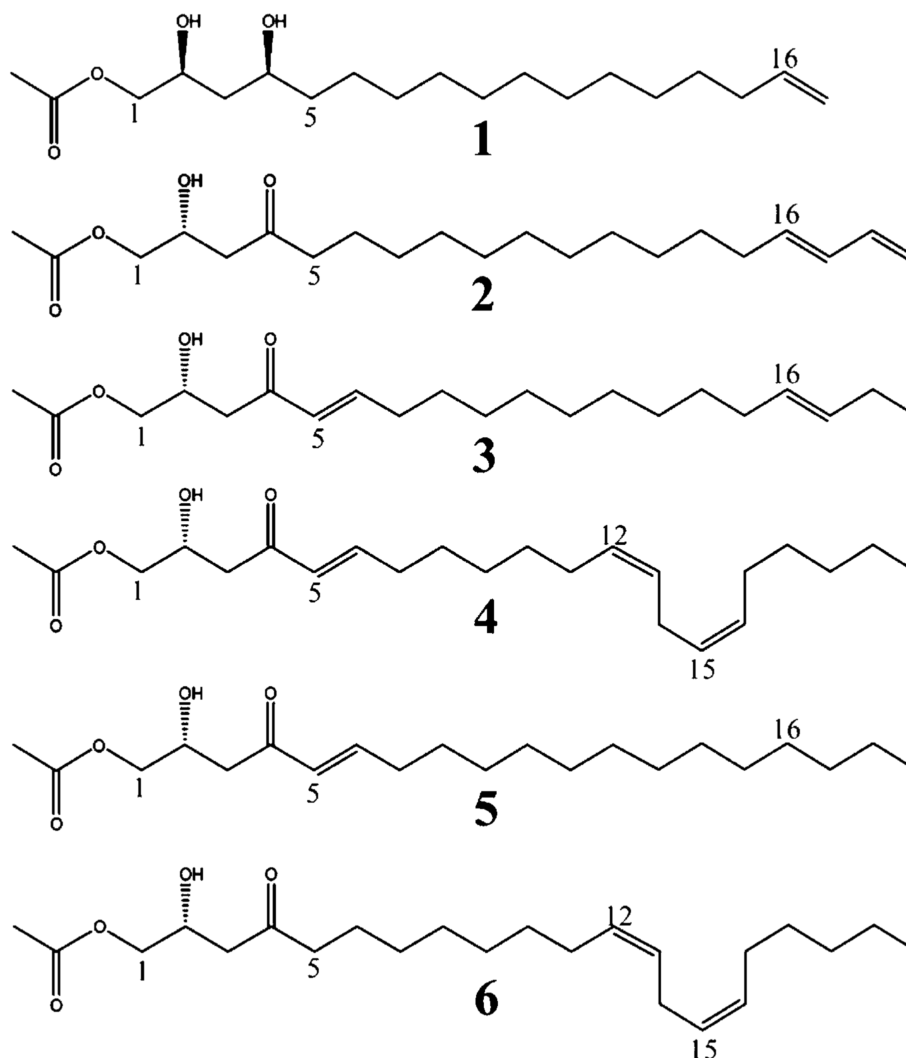

Figure 3. Chemical structures of active compounds **1**−**6** isolated from an avocado seed extract capable of inhibiting *Clostridium sporogenes* PA 3679 (ATCC 7955) vegetative cell growth and endospore germination. **1**, (2*S*,4*S*)-1-acetoxy-2,4-dihydroxy-*n*-heptadeca-16-ene; **2**, persediene; **3**, persenone-C; **4**, persenone-A; **5**, persenone-B; **6**, persin.

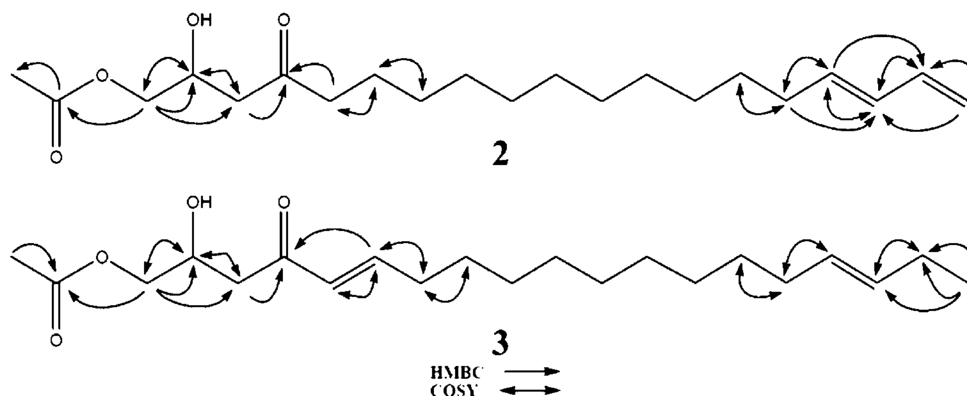

Figure 4. Key COSY and HMBC correlations for persediene (**2**) and persenone-C (**3**).

purification. Structure elucidation analyses revealed two new acetogenins (compound **2** and **3**) along with other four compounds (**1**, **4**, **5**, and **6**), known to exist in nature (Figure 3). The latter were identified by comparing their spectroscopic data with reported values as (2*S*,4*S*)-1-acetoxy-2,4-dihydroxy-*n*-heptadeca-16-ene (**1**);[14] (2*R*,5*E*,12*Z*,15*Z*)-1-acetoxy-2-hydroxy-4-oxoheneicosa-5,12,15-triene (**4**, persenone-A);[14] (5*E*)-1-acetoxy-2-hydroxy-4-oxononadeca-5-ene (**5**, persenone-B);[26] and (2*R*, 12*Z*,15*Z*)-1-acetoxy-2-hydroxy-4-oxoheneicosa-12,15-diene (**6**, persin).[27]

The new acetogenin structure was identified as persediene (**2**), and its molecular formula was established as $C_{21}H_{36}O_4$ by

LC-MS–TOF analysis. The LC-MS (ESI$^+$) analysis showed the presence of molecular ions (m/z) $[2M + Na]^+$ (727), $[M + Na]^+$ (375), $[M + H]^+$ (353), $[M + H - H_2O]^+$ (335), and $[M + H - HOAc]^+$ (293), suggesting the presence of hydroxyl and acetate groups (HOAc=CH_3COOH). The ^1H and ^{13}C NMR spectra (Table 1) indicated that assignments of C-1–C-7 of compound **2** were the same as those reported for compound **6**.[27]

Additionally, as observed for compound **1**,[14] NMR assignment of compound **2** indicated the presence of a terminal vinyl group [vinylic protons (δ_H 6.29 (1H, dt, J = 17.0 10.4 Hz, H-18), 4.92 (1H, d, J = 10.4 Hz, H_b-19), 5.06 (1H, d, J = 17.0 Hz, H_a-19)); olefinic methine δ_C 137.2 (C-18); and exomethylene δ_C 114.3 (C-19)]. An additional unsaturation located at C-16 and C-17 (δ_c 135.3 and δ_c 130.8, respectively) was confirmed. The coupling constant for H-16 and H-17 was measured at 15.1 Hz, indicative of a *trans*-geometry for C-16 unsaturation. Key COSY and HMBC correlations used to assign the structure are shown in Figure 4. The optical rotation $[\alpha]_D^{22}$ +1.10° (c 0.22, $CHCl_3$) was indicative of 2*R* stereochemistry. Therefore, the structure of persediene (**2**) was assigned as (2*R*,16*E*)-1-acetoxy-2-hydroxy-4-oxononadeca-16,18-diene.

The other novel compound, persenone-C (**3**), was established as $C_{21}H_{36}O_4$ by LC-MS-TOF analysis. The mass spectrum showed the presence of the same molecular ions as in compound **2**. The ^1H and ^{13}C NMR (Table 1) spectra indicated that assignments of **3** were very similar to that of **5**,[26] except for an additional unsaturation located at C-16 and C-17 (δ_c 129.1 and δ_c 131.6, respectively). An examination of the overlapped multiplets for H-16 (δ_H 5.39) and H-17 (δ_H 5.40) indicated a coupling constant of 15.4 Hz, dictating a *trans*-geometry for the C-16 double bond. Figure 4 presents key COSY and HMBC correlations that allowed structure assignment. The optical rotation $[\alpha]_D^{22}$ +9.50° (c 0.22, $CHCl_3$) was indicative of 2*R* stereochemistry. On the basis of the above evidence, the structure of persenone-C (**3**) was assigned as (2*R*,5*E*,16*E*)-1-acetoxy-2-hydroxy-4-oxononadeca-5,16-diene.

Antimicrobial Properties of the Enriched Active Fraction Extract and Isolated Compounds. *Disk Diffusion Method.* As shown in Table 3, the samples that showed the largest inhibition zones against *C. sporogenes* were the purified compounds **3**–**5**, although diameters of inhibition for compound **4** were not significantly different from the EAF extract values. Furthermore, compounds **3** and **5** exhibited larger diameters of inhibition for *C. sporogenes* endospores than the original heptane-soluble extract F002 (Table 2), about 18 and 22% larger, respectively, even though F002 extract was evaluated at a 5-fold higher concentration of solids. Therefore, the antimicrobial properties of the extract were significantly enhanced through the bioactivity-guided purification process, confirming compounds **3**–**5** as the main contributors of antimicrobial activity. In addition, the EAF extract and isolated compounds showed similar or up to 1.8-fold larger inhibition zones against *C. sporogenes* than nisin (Table 3), even though the commercial additive was tested at the recommended concentration for food systems, which was 2.5-fold higher than the concentration of the avocado isolated compounds (6.5 vs 2.5 μg/disk of solids, respectively).

Table 3. Diameter of Inhibition Zones of an Enriched Active Fraction (EAF) Extract and Isolated Compounds of Avocado Seed against *Clostridium sporogenes* PA 3679 (ATCC 7955) Vegetative Cell Growth and Endospore Germination

evaluated sample	diameter of inhibition zone[a,b] (cm)	
	vegetative cells	endospores
EAF extract	1.55 ± 0.21 b[c]	1.50 ± 0.42 ab
(2*S*,4*S*)-1-acetoxy-2,4-dihydroxy-*n*-heptadeca-16-ene (**1**)	1.05 ± 0.21 c	0.95 ± 0.07 cd
persediene (**2**)	0.95 ± 0.07 c	1.15 ± 0.07 bc
persenone-C (**3**)	1.95 ± 0.21 a	1.60 ± 0.28 a
persenone-A (**4**)	1.65 ± 0.07 ab	1.40 ± 0.14 ab
persenone-B (**5**)	1.95 ± 0.07 a	1.65 ± 0.07 a
persin (**6**)	0.90 ± 0.14 c	0.60 ± 0.14 d
vehicle control (ethanol or methanol)	nd	nd
nisaplin (6.25 μg/disk of nisin)	1.05 ± 0.07 c	0.95 ± 0.07 cd

[a]All samples were tested at 2.5 μg/disk of solids. [b]Values represent the mean ± standard deviation (n = 3). nd, not detected. [c]Different letters within the same column indicate significantly different values by the LSMean Student's t test (P < 0.05).

Determination of Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of Activated Endospores. To further evaluate the potential of molecules identified as endospore inhibitors, MIC and MBC values were determined for the EAF extract and compounds **3**–**5** against *C. sporogenes* endospore suspensions. As indicated in Table 4, compounds **4** and **5** showed the lowest MICs followed by compound **3**, with double the concentration. In accordance with disk diffusion assays, these compounds presented the largest inhibition zones (Table 3). It appears that in liquid medium the activity of compound **4** increases in reference to the EAF extract. However, all tested samples were less potent than nisin in the liquid system; they presented MICs 2–5-fold higher (Table 4).

Table 4. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Values for an Enriched Active Fraction (EAF) Extract and Compounds Isolated from Avocado Seed against *Clostridium sporogenes* PA 3679 (ATCC 7955) Endospore Germination[a]

evaluated sample	MIC (μg/mL)	MBC (μg/mL)	MBC/MIC ratio[b]
EAF extract	19.5	19.5	1
persenone-C (**3**)	15.6	–[c]	–
persenone-A (**4**)	7.8	–	–
persenone-B (**5**)	7.8	–	–
nisaplin (nisin)	156 (3.9)	312 (7.8)	2 (2)

[a]Tests were performed in triplicate. [b]Ratio MBC/MIC ≤4 indicates bactericidal activity and >4, sporostatic activity under experimental procedure described by Levison et al.[28] [c]Not determined in the range of concentrations tested (5–15.6 μg/mL).

In the range of concentrations tested, MBC values were only determined for the EAF extract and positive control (Table 4). The pooled fraction (EAF extract), which contains all purified molecules, showed the same MBC value as the MIC (19.5 ug/mL), classifying it as a bactericidal agent (MBC/MIC ratio ≤4).[28] As expected, nisin presented a lower MBC value (7.8 μg/mL) than the EAF extract but with a higher MBC/MIC ratio of 2. Purified compounds **3**–**5** showed growth when subcultured at the MIC concentration and above; however, concentrations tested (15.6 and 7.8 μg/mL) did not reach a potential scenario of a MBC/MIC ratio >4. Consequently, an exclusive sporostatic effect cannot be concluded and probable bactericidal properties of the molecules need to be tested at

higher concentrations. It is interesting that even with a higher MIC the EAF extract was bactericidal at the MIC concentration, which might suggest a synergistic effect among compounds. Further studies are needed to describe inhibition of microbial growth from endospore by these compounds alone and in combination. Also, we do not know which stage of endospore activation the compounds affect or if they could inactivate dormant spores, valuable information to elucidate mechanisms of action and develop antimicrobial control strategies.

Quantitation of Active Compounds in the Enriched Active Fraction Extract. CPC separation of the most active crude fraction (F002) allowed the removal of contaminants and enhanced the activity and purity of the resulting EAF extract (Figure 5). As observed in Figure 5, the concentration of the most potent compounds (**3**−**5**) accounted for 56% total weight of the EAF extract, and all isolated compounds (**1**−**6**) represented 90%. An additional 9% were unknown acetogenins corresponding to peaks A and G shown in Figure 2, quantitated as persenone-A equivalents. Therefore, total acetogenin concentration in the EAF extract was around 99% of its weight.

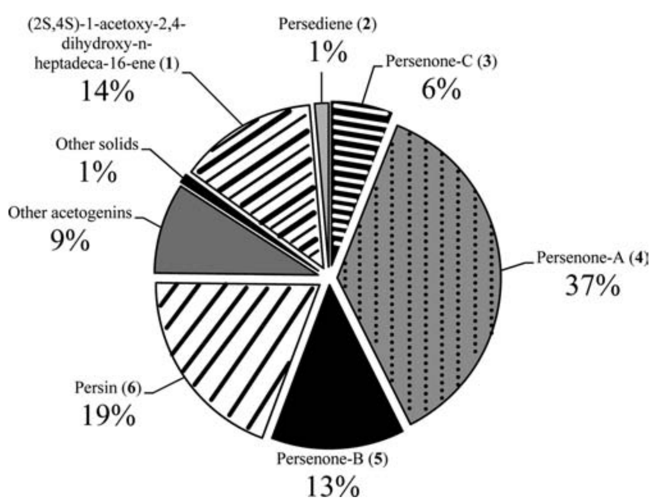

Figure 5. Relative concentrations of active compounds present in an enriched active fraction (EAF) extract from avocado seed. Percent concentration of the unknown acetogenin peaks was quantitated as persenone-A equivalents.

Similarly to the present study, high-speed countercurrent chromatography (HSCCC) has been successfully used in combination with multiple-step column chromatography (normal, reverse phase, and size exclusion) and preparative HPLC to identify active molecules from avocado seed,[29,30] although it is relevant to mention that HSCCC differs from CPC by being a hydrodynamic technique with fast changing centrifugal force fields, whereas CPC is a hydrostatic technique that uses separation ducts.[31] Previous studies also differ from the present work in that their focus was on evaluation of the effects of hydrophilic and slightly lipophilic crude extracts and fractions on human skin cells and their physiological modulation[30] or aimed to identify molecules with antioxidant potential and low toxicity, as determined by the TEAC and DPPH assays and the "brine-shrimp" (*Artemia salina*) lethality assay that confirmed flavan-3-ols (proanthocyanidins B-1, B-2, A2-(+)-catechin, and A2-(+)-epicatechin) as the antioxidant molecules.[29] It is also relevant to discuss that in the work reported by Ramos-Jerz,[29] the fractionation of avocado seed phytochemicals, guided by the *A. salina* lethality assay, led to the association of eight acetogenin structures with a cytotoxic effect. Reported toxic acetogenins were C_{19} structures, and among them, those containing hydroxyl groups, in both C-2 and C-4 positions, exhibited the highest toxicity. As shown in Figure 3, from the acetogenins identified in the present study, only compound **1** presented those structural features. However, because similar crude extracts were also assessed in the cited work, and presented cytotoxic effects, the need for further knowledge of the toxicology of isolated molecules is still a relevant research topic.

In this work, additional HPLC-PDA purification of the EAF extract led to the discovery of two acetogenins not previously reported in any avocado tissue (compounds **2** and **3**). Together, all six compounds (**1**−**6**) were identified as responsible for the observed activity (Figure 3). Among them, compounds **3**−**5** were documented as the most potent inhibitors of *C. sporogenes* endospore growth (Tables 3 and 4). Relevant structural features shared by compounds **3**−**5** were an acetate moiety in C-1 and a *trans*-geometry unsaturation at C5−C6 conjugated to a keto group (*trans*-enone group). In contrast, Neeman et al.[11] previously observed that the presence of a hydroxyl group, instead of an acetate moiety, in the C-1 position produced higher inhibition of vegetative cell growth. They observed that compound **1** inhibited *Staphylococcus aureus*, *Candida albicans*, and *Saccharomyces cerevisiae*, whereas a naturally occurring analogue of **1**, with a hydroxyl group at C-1, additionally inhibited *Bacillus subtilis*, *Bacillus cereus*, *Salmonella typhi*, and *Shigella dysenteriae* vegetative cell growth.

The activity of avocado constituents against fungal spore germination has been previously evaluated, showing no effect on *Aspergillus niger*[10] and characterizing compound **6** as a stronger inhibitor of fungal spores from *C. gloeosporioides* than compound **4**.[14] In the present study, the inhibitory properties of compounds **4** and **6** on bacterial endospore germination were the opposite; *C. sporogenes* was inhibited approximately 2-fold more effectively by compound **4** than by **6** (Table 3). This behavior can be explained by the lack of a C5−C6 double bond in compound **6**, which is present in **4** (Figure 3).

Compounds **3** and **5** also contain the *trans*-enone group and differed only in the presence of a C16−C17 double bond. *trans*-Enone groups have also been deemed relevant for other in vitro bioactivities such as inhibition of superoxide generation[26] and cancer cell growth inhibition.[32] Structurally, for compounds **3**−**5**, the length of the aliphatic chain appears to be bioactivity related, because **3** and **5** (C_{21}) were 1.18-fold more potent than **4** (C_{23}).

The precise mechanism of inhibition against *C. sporogenes* endospore germination and vegetative cell growth remains unknown; however, inhibition of fatty acid (FA) synthesis figures as a potential mechanism.[34−36] FAs are required in several vital cell processes, such as cellular membrane synthesis of microorganisms with rapid growth rates.[33] In this sense, prior reports suggest that acetogenins in avocado fruit are structurally similar to polyunsaturated FA, such as linoleic acid,[34] with known antibacterial effect mediated by FA synthesis inhibition.[35] Furthermore, compounds **1**, **4**, and **6** have been reported as potent inhibitors of acetyl-CoA carboxylase, another key enzyme in fatty acid synthesis.[36]

In summary, results indicated that the persenone-C (**3**), persenone-A (**4**), and persenone-B (**5**) contained in avocado seed inhibited *C. sporogenes* endospore germination and vegetative cell growth and shared common structural features

of an acetyl moiety and a *trans*-enone group. Their MICs ranged from 7.8 to 15.6 μg/mL. Therefore, these identified molecules exhibit potential as natural alternatives to additives and antibiotics currently used for the inhibition of Gram-positive spore-forming bacteria. Further studies are needed to explore safe levels for human consumption as well as their efficacy and stability in more complex biological matrices.

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Notes

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■ ABBREVIATIONS USED

CFU, colony-forming units; CPC, centrifugal partition chromatography; EAF, enriched active fraction; ESI, electrospray ionization interface; K_D, partition coefficients; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration; PBS, phosphate-buffered saline; PDA, photodiode; RCM, reinforced clostridial medium; TPGY, trypticase−peptone−glucose−yeast extract

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