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Rapid identification of main antibacterial components from New Zealand 'Hass' avocado peel hexane extract

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ABSTRACT

'Hass' avocado (*Persea americana* Mill) is a very popular tropical fruit that contains several nutrients, including vitamins, proteins, minerals, and unsaturated fatty acids. 'Hass' avocado peel is one of the by-products obtained from the New Zealand (NZ) avocado processing industry. The current study focused on the antibacterial components from hexane 'Hass' avocado peel extract. The aim was to perform microbiological screening of NZ hexane 'Hass' avocado peel extract using *Staphylococcus aureus*. We used thin- layer chromatography (TLC) with direct microbiological detection to isolate the individual components that showed antibacterial activity. Mass spectrometry (MS) analysis characterized the isolated components from hexane peel extract. (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid, (±)9-HpODE, and NP-011548 are the three fatty acids that have been tentatively identified. Present findings provide new bioactive components from avocado peel and suggest potential applications for this extract as a natural antibacterial additive in soaps, and detergents.

1. Introduction

Avocado (*Persea americana* Mill.) is rich in vitamins, proteins, unsaturated fatty acids, minerals, and fiber [1]. 'Hass' is the most dominant cultivar of avocado grown in New Zealand (NZ), and the avocado industry of NZ is based on this cultivar of avocado. In NZ, other avocado types are usually used as pollinators for 'Hass' [2]. After industrial avocado oil processing, there are a large amount of by-products (peel and seed) generated as residues [3]. Avocado peel is largely considered to be waste after the fruit has been industrially processed. It is rich in natural compounds such as triterpenes, phytosterols, and polyphenols. Avocado peel can reach 13 % of the whole avocado's weight. These tons of peel by-products have to be discarded and are generally not utilized, except as animal feed [1,4,5]. Avocado peel has been shown to possess good anti-hypertensive, hypolipidemic, fungicidal, larvicidal, and antioxidant activities [3]. In addition, research has shown that avocado peel extracts show good antibacterial activity against many bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Proteus vulgaris*, and *Staphylococcus aureus*) [6,7].

Originally, the enzymatic tetrazolium salts reduction method was applied in eukaryotic cell research [8]. 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) is now one of the common tetrazolium salts that are widely used as an indicator to measure enzymatic activity of bacteria [9]. MTT can be applied in antibacterial compound quantification [10], bacterial resistant determination [11–13], and biofilm formation evaluation [14]. An improved TLC-bioautography method was used in the current study for measuring antimicrobial activity. Thin layer chromatography (TLC) is a widely used method to separate plant extract compounds. TLC can be used as a 'fingerprint' to identify the characteristics of plant extracts [15]. Plant extracts can be examined under UV light (254 or 366 nm) [16,17], which can aid in visualizing compounds with a chromophore. TLC is an easy chromatographic technique for separating a mixture of natural compounds [18]. This method does not need expensive or complicated apparatus. TLC can be used to help determine solvents with different polarity that will be used in the column or flash chromatographic separation [19]. The TLC direct bioautography method can directly locate the antibacterial activity from crude plant extracts on a developed chromatogram. It is a convenient and quick method to explore and detect the antibacterial phytochemicals in complex plant extracts [20, 21]. In the initial direct bioautography method, TLC plates were used for substance separation before it is covered by agar gel [22]. More recently, several TLC-bioautography experiments have shown that the developed TLC plates can be directly immersed into bacterial broth. The broth

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medium is the main nutrient source for test the bacteria, which can grow in the silica particles of TLC plates [20,23].

Staphylococcus aureus is a common gram-positive bacterial human pathogen. It was discovered in the 1880 s [24]. *S. aureus* has been used in several antimicrobial activity studies involving plant extracts [25–27]. The aim of this current study was two-fold. First, it aimed to test multiple screenings of anti-bacterial compounds in hexane avocado peel extract using *S. aureus*. Second, it aimed to determine the potential anti-bacterial compounds of hexane avocado peel extract form 'Hass' avocados grown in New Zealand (NZ). This is the first study to report the antibacterial activity of NZ hexane 'Hass' avocado peel extract.

2. Method

2.1. Materials

TLC silica gel 60 F254 plates (20 cm × 10 cm) were purchased from Merck (Darmstadt, Germany). The 'Hass' avocado peel in this study was provided from GROVE (Avocado Oil New Zealand Ltd, NZ) in September 2021. *Staphylococcus aureus* ATCC 25923 was used in the study. The tested strains were obtained from School of Food & Advanced Technology microbiology laboratory of Massey University, cultured in Brain heart infusion (BHI) broth (Hi-media, M002) at 37 °C, and stored in BHI agar slants at 4 °C.

2.2. Chemicals and reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) was purchased from AK Scientific Inc. (Union City, CA, USA). Sulfuric acid (H_2SO_4), vanillin (≥99 %), acetone, hexane, and methanal were purchased from Sigma-Aldrich Company Ltd. (St. Louis, MO, USA). BHI broth was purchased from Remel (Lenexa, KS, USA). All the chemical reagents were of analytical grade or higher purity.

2.3. Preparation of the extracts

The Soxhlet extractor was used to extract the freeze dried (–80 °C) avocado peel grounded powder, as reported previously [28]. Eighty-gram avocado samples were extracted with 1000 mL of methanol for 6−8 h at 64 °C. An electric heater (Zhengzhou Keda Machinery and Instrument Equipment Company Ltd, Hainan, China) was used to maintain the temperature. Hexane was used to separate the polyphenol and oil mixture extract through the solution static layering system in a separatory funnel. The final hexane oil extract from the avocado peel was concentrated on the rotary evaporator (Buchi, Flawil, Switzerland) at 50 °C. Then, the concentrated hexane oil was transferred to a glass vial for storage at 22 °C.

2.4. Thin layer chromatography

In the antimicrobial assay, aluminum-backed silica gel 60 F254 plates (Merck, Darmstadt, Germany) were used to locate the main compounds of the hexane peel extract. The TLC plates were cut into 5 cm × 10 cm rectangles. A series of hexane 'Hass' avocado peel extracts (1 mg/mL) were directly deposited as spots (2.4 – 9.6 μL) on the TLC plates. In all bioautographic assays, the solvent systems prior to testing the hexane 'Hass' avocado peel spotted extract was hexane: acetone (6:4). The developed TLC plates were removed from the chamber after the solvent had travelled 8.0 cm from the starting baseline. Two TLC plates were developed for each sample: one for the antibacterial test and one for a reference. The chemical components on reference TLC plates were detected through a vanillin/H_2SO_4 reagent and visualized under a UV lamp at 254 nm.

The vanillin/H_2SO_4 reagent was prepared as a combination of the vanillin stock solution and sulfuric acid stock solution in a ratio of 1:1. The vanillin stock solution was made by mixing 2 g of vanillin (99 %) with 50 mL of absolute ethanol. The sulfuric acid stock solution was made by mixing 10 mL of H_2SO_4 (98 %) with 40 mL of absolute ethanol. These two stock solutions were stored at room temperature (22 °C) in the dark before using.

2.5. Bioautography

Staphylococcus aureus was used for the antibacterial testing. The bacteria were incubated in 500 mL of BHI broth at 37 °C for overnight growth. The final concentration of the microorganism was approximately 10^8 cell/mL. The prepared avocado peel extract TLC plates were soaked in the prepared bacterial BHI broth for 10 s. Then the bacteria-TLC plates were incubated at 37 °C for 24 h under humid conditions. Finally, the bacteria-TLC plates were sprayed with the MTT aqueous solution (5 mg/mL) for 30 min and incubated at 37 °C. The biological activity of the hexane avocado peel extract was assayed by observing clear white inhibition zones and a purple background in the remaining area.

After the TLC-bioautography analysis, a general TLC method was performed to isolate and collect the avocado peel antibacterial compound. The isolation areas were determined by the results from the vanillin/sulfuric acid reagent-sprayed TLC plates and the TLC-bioautography plates. The isolated area should have clear color response bands on the vanillin/sulfuric acid reagent-sprayed TLC plates, and clear *S. aureus* inhibition zones on the TLC-bioautography plates. Then, the target compounds were collected via acetone elution, followed by mass spectrometry for the compounds' identification. All the TLC-bioautography experiments were conducted in triplicate to ensure the reproducibility and reliability.

2.6. LC-MS analysis

The collected antibacterial compounds were analyzed by LC-MS. The LC-MS analysis was performed on a Thermo Scientific Ultimate 3000 system (Sunnyvale, CA, USA). A Thermofisher Hypersil GOLD C18 Column 100 × 2.1 mm, particle size 1.9 μM, was used. The mobile phases were: (A) water with 0.1 % of formic acid and (B) Acetonitrile; the gradient programme started with 25 % B from 0 to 2 min; 25–50 % B from 2 to 16 min; 95 % B from 16 to 25 min; 95–25 % B from 25 to 26 min; 25 % B from 26 to 28 min. The flow rate was 0.2 mL/ min. The oven temperature was set at 40 °C, and the injected sample volume was 10 μL.

For the MS detection, FullScan-MS runs were acquired with a resolving power of 70,000 and a mass range of 100–800 *m/z*, an AGC target of 1×10^6, max IT auto. The ddMS2 also runs in a mass range from 100 to 800 *m/z*. The resolving power data dependent scan mode (ddMS2) was 17,500 for all fragmentation products. The fragment data was carried out using Agilent Mass Hunter Qualitative software for negative mode analysis.

2.7. Data processing for tentative identification

The MS spectra were processed through Compound Discoverer 3.3 (Thermo Fisher Scientific, San Jose, CA, USA) to tentatively identify the target fractions from the hexane 'Hass' avocado peel extract. The raw data file was automatically analyzed by a user-defined workflow (Fig. S1). The retention time, spectra selection, blank subtraction, and relevant peaks identification (candidate comparison with the mzCloud and ChemSpider databases) were considered in this workflow.

There are four steps in this whole hexane avocado peel extract antibacterial study. Step 1: Sample extraction by Soxhlet extraction with the solution static layering system. Step 2: TLC-bioautography analysis of antibacterial fractions. Then, analysis the fractions by LC/MS in full scan + data dependent MS2 mode. Step 3: Use Compound Discoverer 3.3 to identify the antibacterial compounds in the specific fractions. Step 4: Select the identified compounds according to the best match score from mzCloud and ChemSpider. The experimental flowchart is shown in

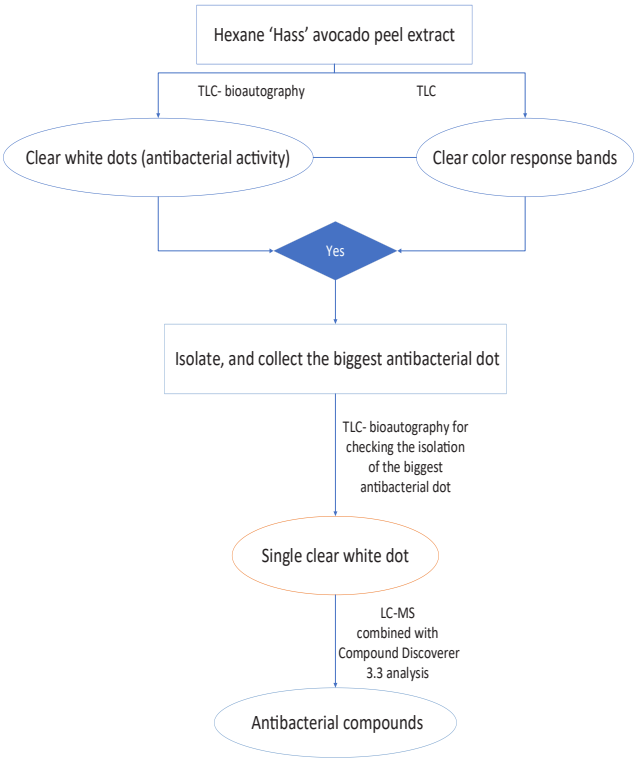

Fig. 1. The antibacterial compounds' identification flowchart of hexane 'Hass' avocado peel extract.

Fig. 1.

3. Results and discussion

3.1. Preliminary screening of antibacterial components

The TLC-bioautographic test was performed to assess the antibacterial activity of hexane 'Hass' avocado peel extract (Fig. 2). The hexane 'Hass' avocado peel extract showed good activity against *S. aureus* (Fig. 2). Phytochemical screening was used to reveal the different chemical components in the hexane 'Hass' avocado peel extract. Most plant chemical compounds react with a vanillin/sulfuric acid spray reagent. The different color changes in the reaction represent different compound varieties [23]. The preliminary TLC-bioautographic test focused on optimizing the separation conditions of the hexane avocado peel extract. The selected mobile phase [hexane: acetone (60:40, v/v)] separated the compounds in the hexane avocado peel extract. Most of the separated TLC bands could not be clearly visualized under UV light (254 nm) (Fig. 2B). The developed hexane peel extract TLC plate was assessed by direct bioautography against *S. aureus*. The *S. aureus* inhibition zones were visualized as white spots on a purple background (Fig. 2C).

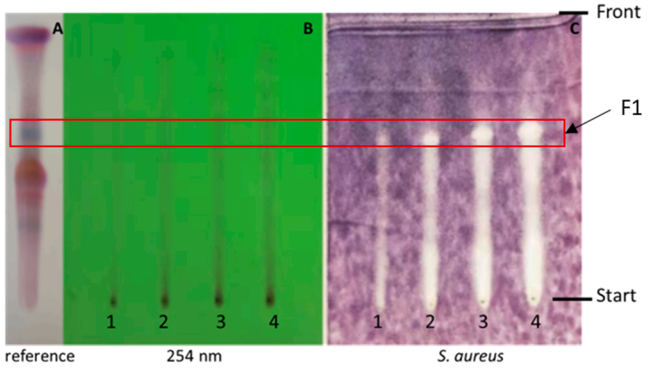

Fig. 2. Thin layer chromatograms (A) and bioautograms (C) of hexane 'Hass' avocado peel extract. Applied volume: 1, 2.4 μL; 2, 4.8 μL; 3, 7.2 μL; 4, 9.6 μL; reference: 9.6 μL. Mobile phase: hexane–acetone (60: 40, v/v). Panel (A) TLC plate after vanillin/sulfuric acid reagent treatment and documented in visible light, panel (B) TLC plate under UV 254 nm, panel (C)TLC- bioautography assay: Bioautograms using *S. aureus*.

In the vanillin/sulfuric acid reagent-sprayed TLC plate, the color response of the chemical component bands could be visualized clearly (Fig. 2A). The vanillin/sulfuric acid reagent-sprayed TLC plate showed more chemical components than the TLC plate under UV light (254 nm). We selected the chemical components that not only showed clear color response bands on the vanillin/sulfuric acid reagent-sprayed TLC plate (Fig. 2A), but also had a clear *S. aureus* inhibition zone on the TLC-bioautography plate (Fig. 2C) for the further isolation and collection. There was one main band that showed the highest antibacterial activity in the TLC-bioautography plate. This detected band (red circle in Fig. 2) was denoted as F1 for the further isolation and collection. The inhibition zone (white areas) of F1, which is against the purple background on the chromatograms, denotes the growth inhibition of *S. aureus*. There is a long tail on the TLC plate under UV light (254 nm). In addition, the extractive polarity of hexane is lower than most medium polarity solvents (dichloromethane, acetone, or isopropyl alcohol). According to this preliminary result, it is speculated that the hexane extract of avocado peel comprises fatty acids in its chemical composition.

The white inhibition zones indicate that there are some effective antibacterial compounds in F1. The F1 compounds showed no fluorescence reaction under UV light (Fig. 2B). In addition, the vanillin/sulfuric acid reagent sprayed onto the plate gave a purple color reaction to F1, which covered a blue band of chlorophylls (Fig. 2A). Therefore, according to the TLC-bioautography results and the color reaction results on the TLC plates, the F1 dot of hexane avocado peel extract was isolated by TLC plates under the same separation condition (hexane:acetone (60:40, v/v)).

3.2. Separation verification of F1 dot

The isolated F1 was recovered using the same mobile phases: hexane: acetone (60:40, v/v) (Fig. 3) for the separation verification. The separated F1 could not be seen under UV light (254 nm). This means that the chemical compounds in F1 have no UV absorption (Fig. 3A). This result

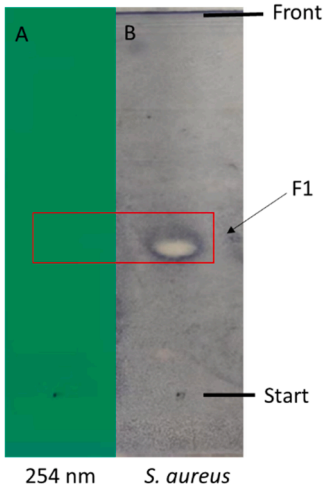

Fig. 3. Thin layer fluorogram (A) and bioautograms (B) of F1 components. Applied volume: 4.8 μL. Mobile phase: hexane–acetone (60:40, v/v). Panel (A) TLC plate under UV 254 nm, panel (B) TLC- bioautography assay: Bioautograms using *S. aureus*.

is consistent with the mark in Fig. 2B. There is only one single white dot on the TLC-bioautography plate (Fig. 3B). This result supports the idea that the activity chemical components of F1 had been effectively isolated from the hexane avocado peel extract. Next, the isolated F1 was analyzed by LC-MS.

3.3. Identification of antibacterial compounds of F1

Representative base peak chromatograms of the F1 composition obtained by HPLC-ESI-TOF-MS method are shown in Fig. S2. The mass accuracy and mass resolution combined with fragmentation spectra analysis and isotope pattern distinguished the molecular ion from the tested chemical compounds [20]. The fragments of F1 were obtained under collision energy at 30 eV. After the antibacterial fraction was analyzed on the TLC plate, the fraction MS data were processed using Compound Discoverer 3.3 software. The software automatically annotated 2149 potential chemical compounds in the F1 dot. Three compounds were found to show the highest best match score (> 80) among these potential compounds. The total ion chromatogram is shown in Fig. 4. The retention time, generated formulas, calculated *m/z*, proposed chemical compounds, mass measurement error, and chemical structure of these three identified substances are shown in Table 1. These three compounds were confidently identified and classified in the fatty acid family (Table 1).

There were a lot of phytochemical components in the crude extract. To avoid invalid identification of antibacterial compounds, we only selected chemical compounds that met the following criteria: 1) a positive match (total score) > 80 % with mzCloud and ChemSpider, and 2) exact mass accuracy (ΔMass) < 5 ppm. The isolated active F1 dot from hexane 'Hass' avocado peel extract contained a main chemical compound with *m/z* 282.5 and the generated formula $C_{18}H_{34}O_3$. It was tentatively identified as NP-011548. Moreover, two further chemical compounds were tentatively identified as (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid (*m/z* 330.5), the generated formula $C_{18}H_{34}O_3$, and

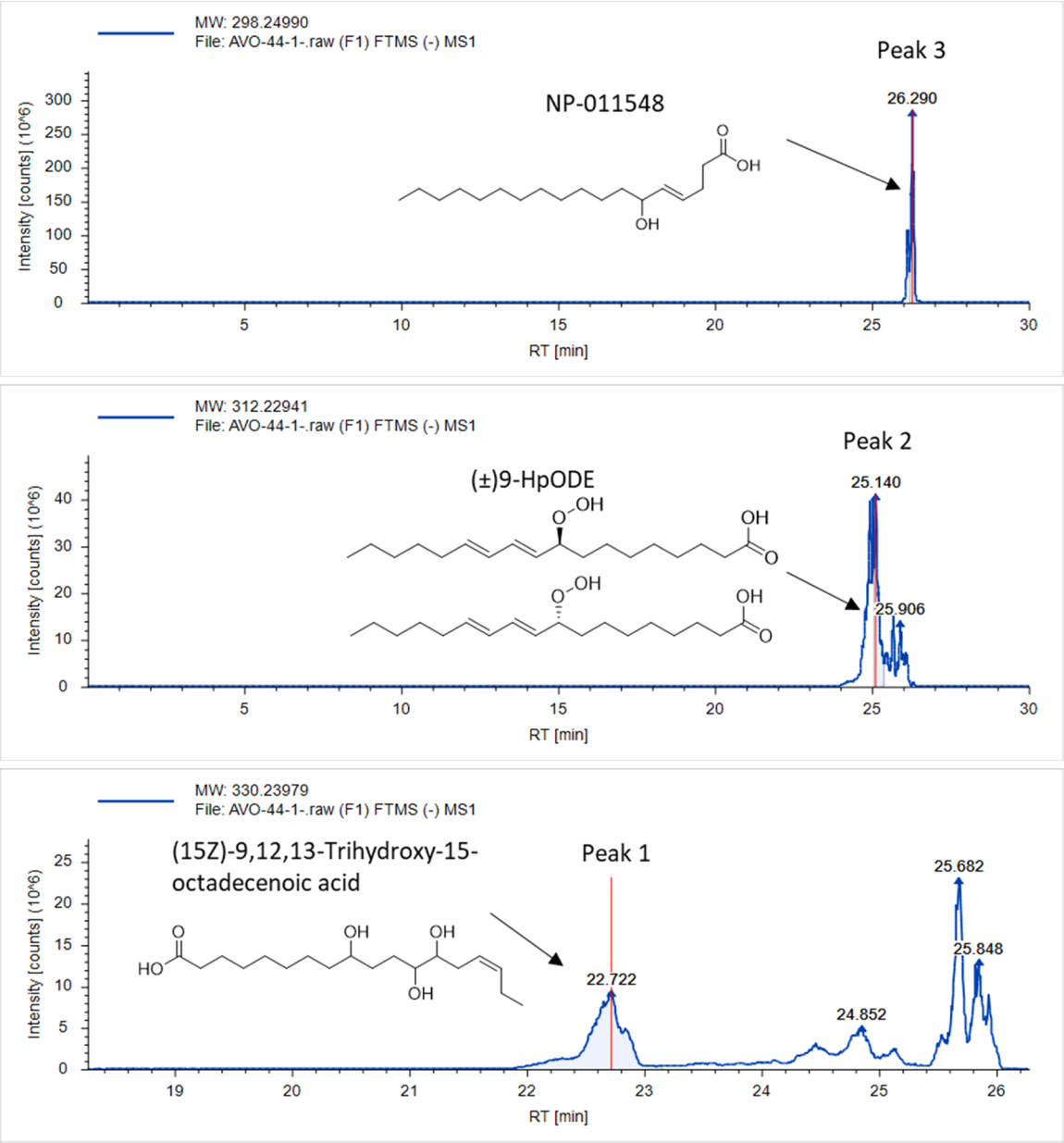

Fig. 4. Identification of (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid (peak 1), (±)9-HpODE (peak 2), and NP-011548 (peak 3) through Compound Discoverer 3.3 after chromatographic run acquisitions in FullScan mass spectra.

Table 1
Tentatively identified substances in the analyzed F1 components.

Peak Number	Retention Time (min)	Molecular Formula	Molecular Weight	*m/z*	Error (ppm)	Best Match	Proposed Compound
1	22.718	$C_{18}H_{34}O_5$	330.5	330.241	-2.53	97.8	(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid
2	25.138	$C_{18}H_{32}0_4$	312.4	312.230	-2.07	89.1	(±)9-HpODE
3	26.289	$C_{18}H_{34}0_3$	282.5	298.251	-3.01	92.7	NP-011548

(±)9-HpODE (*m/z* 312.4, $C_{18}H_{32}0_4$). The mirror plots (the sample MS2 fragments (top) spectroscopically match with the mzCloud library (bottom)) of these three identified compounds are shown in Fig. S4.

TLC-bioautography is not a quantitative method to evaluate the antibacterial activities of chemical compounds [29]. However, TLC-bioautography is an ideal and suitable method in the current study for detecting antibacterial compounds. This method clearly separates antibacterial compounds and locates the positions of the hexane avocado peel extract in chromatograms. The advantages of the current TLC-bioautography method are: (1) This method avoided the solubility problem of fatty acids in a bacterial broth. (2) This method effectively found the bacteriostatic compounds effective against *S. aureus* in hexane avocado peel extract.

There are over 450 different fatty acids can be found in various plants. Some of them are unusual fatty acids [30]. These fatty acids possess unusual branching, or functional groups, such as acetylenic, epoxy, and hydroxy fatty acids [31]. Octadecenoic acids are natural products in various plant fats and oils [32]. After LC-MS and Compound Discoverer 3.3 analysis, we found that the main antibacterial components were three unusual octadecenoic acids. Desaturases, and lipoxygenase are two types of enzymes that play essential roles in the biosynthesis of unusual long-chain fatty acids [33,34]. Desaturases could introduce double bonds into the hydrocarbon chain of fatty acids [35]. These enzymes contribute to converting saturated fatty acids to unsaturated fatty acids [35,36]. Different desaturases have been reported to recognize specific fatty acids as substrates. These enzymes would introduce double bonds into the fatty acid chain at distinct positions [35]. Lipoxygenases are widely distributed in various plants. They could recognize and bind specific unsaturated fatty acids as substrates to form hydroperoxide intermediate [37]. Scientists found linoleic acid was converted from the desaturation of oleic acid by specific desaturases in plants [38,39]. Many studies showed that HpODE is the hydroperoxide intermediate that converts from linoleic acid oxidation by specific lipoxygenase [40,41]. Linolenic, linoleic, and oleic are the majority of C18 fatty acids in 'Hass' avocado [42]. The avocado peel is rich in various desaturases and lipoxygenases [43,44]. Based on the above information, we speculated that NP-011548, (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid, and (±)9-HpODE are all formed through the conversion of oleic acid. Different desaturases convert oleic acid into unsaturated fatty acids with distinct double bond positions. These unsaturated fatty acids as the specific substrates are catalyzed by different lipoxygenases, forming NP-011548, trihydroxy-15-octadecenoic acid, and (±)9-HpODE, respectively.

Many studies have already determined that long-chain unsaturated fatty acids contain antibacterial activities [29,45,46]. Antimicrobial fatty acids have been reported to penetrate bacterial cell wall layers. The cell membrane of gram-positive bacteria is thinner than that of gram-negative bacteria [46]. Therefore, the anti-gram-negative bacterial effects of long-chain fatty acids is rarely observed on the TLC plates. Enzyme activity is essential to energy production, and DNA replication of bacterium [47]. Long-chain unsaturated fatty acids could inhibit specific bacterial enzyme activity in vital cellular processes [48]. Therefore, NP-011548, (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid, and (±)9-HpODE may have abilities to interfere with specific enzyme activity of *S. aureus*. These three fatty acids could impair *S. aureus* survival on the TLC plates by disrupting the enzyme activity. The double bonds in the hydrocarbon chain of unsaturated fatty acids could disrupt the bacterial lipids packing, and promote the cell membrane fluid [49]. In addition, many studies showed that the hydroxyl group of unsaturated fatty acids could enhance the ability to disrupt bacterial cell membranes [48,50]. The hydroxyl group could increase the polarity of fatty acid molecules, facilitating unsaturated fatty acids' interaction with the lipid bilayer of the bacterial membrane [50]. In the current study, the F1 dot from the hexane 'Hass' avocado peel extract showed good antibacterial activity against *S. aureus*. This result may be because NP-011548, (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid, and (±) 9-HpODE all contain double bonds, and hydroxyl groups. The double bonds, and hydroxyl groups of these three unsaturated fatty acids could increase the cell membrane fluid of *S. aureus*. The fluidity cell membrane influences the integrity and permeability of *S. aureus* cells. As well, the membrane fluidity result in the leakage of cellular components, leading to the death of *S. aureus* [46,51].

Numerous studies have demonstrated that (±)9-HpODE possesses antimicrobial properties against various fungal and bacterial pathogens [50,52,53]. The results support the idea that long-chain unsaturated fatty acids can inhibit the growth of microorganisms [52,54]. However, there was little information on the bioactivity of (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid, and NP-011548. A lot of antibacterial research has already been done on avocado peel methanol or ethanol extracts [55,56] but not hexane extracts. Based on the literature review, this is the first study that has successfully explored and separated the uncommon fatty acids in 'Hass' avocado peel. In addition, these three octadecenoic acids have been reported for the first time in avocado peel.

TLC-bioautography is a cost effective and simple method that could be used to guide the isolation of biologically active compounds of avocado peel hexane extract. In addition, the TLC-bioautography method could provide more accurate information about the antibacterial activities of isolated fractions from hexane 'Hass' avocado peel extract. The current study combined the TLC-bioautography method, LC/MS, and Compound Discoverer 3.3 analysis to separate and tentatively identify the antibacterial components of hexane 'Hass' avocado peel extract. Three active fatty acids have been tentatively identified as (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid, (±)9-HpODE, and NP-011548 through Compound Discoverer 3.3. Nevertheless, the Compound Discoverer software is very useful in determining preliminary structure identification. It provides useful data to guide a traditional isolation and identification methods (i.e., standard identification and NMR) in the investigation of bioactive phytochemicals. Therefore, additional isolation and HPLC standard identification are needed for these three tentatively identified fatty acids from F1 dot.

4. Conclusions

The lower polarity compounds of 'Hass' avocado peel were examined for antimicrobial activity. This study is the first study to combine three methods for analyzing the hexane-extracted portion (oil-like portion) of avocado peel. The current study combined HPLC-MS data with Compound Discoverer evaluation and found that 'Hass' avocado peel contained a wide variety of fatty acids. In addition, the TLC-bioautography method showed that the hexane portion of the avocado peel had visible antibacterial activity against *S. aureus*. To the best of our knowledge, no other publication has covered antibacterial fatty acids in an avocado peel study. This study could extend knowledge about the extent of antibacterial activity of 'Hass' avocado peel.

Supplementary Materials

Fig. S1: The workflow tree from the Compound Discoverer 3.3 software. Fig. S2: The total ion chromatogram (TIC, sum of MS1 intensities over time) of the F1 fraction from hexane 'Hass' avocado peel extracts. Fig. S3: Fingerprint fragments of NP-011548, (15Z)-9,12,13-Trihydroxy-15-octadecenoic acid, and (10E,12Z)-9-Hydroperoxy-10,12-octadecadienoic acid.

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CRediT authorship contribution statement

Conceptualization, D.S., D.G.P. W.X., and M.W.; Methodology, D.S. and W.X.; Software, D.S. and W.X.; Validation, D.G.P. and M.W.; Formal Analysis, D.S.; Investigation, D.S.; Resources, D.S.; Data Curation, D.S.; Writing – Original Draft Preparation, D.S.; Writing – Review & Editing, D.G.P., W.X. and M.W.; Visualization, D.G.P.; Supervision, D.G.P.; Project Administration, D.S.; Funding Acquisition, D.G.P. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cpb.2023.100288.

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