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Characterization of California olive pomace fractions and their *in vitro* antioxidant and antimicrobial activities

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ABSTRACT

Olive oil production yields a massive amount of byproduct, olive pomace (OP). Hexane-defatted Arbequina olive pomace from California, United States, was extracted with water and loaded to a preparative C18 chromatography. Phenolic desorption was applied by acidified methanolic-water gradients. Phenolic compound profiles and antioxidant/antimicrobial activities were determined. Results showed that the total phenolic contents of the fractions increased with the increase of the percentage of methanol in water gradients; however, the polar phenolic compound profiles generally decreased, while less-polar phenolic compound profiles increased. Oleuropein-aglycone-di-aldehyde (3,4-DHPEA-EDA) detected in water extract was not found in the acidified 35 mL/100 mL and acidified 70 mL/100 mL methanol fractions, but there was a new peak tentatively assigned as 3,4-DHPEA-EDA dimer. The *in vitro* antioxidant activities of water fractions were higher than that of higher methanolic fractions when they were compared at the same level of gallic acid equivalents; the same trend was observed for the antimicrobial activities evaluated using non-Shiga toxin-producing *Escherichia coli* O157:H7 and *Listeria innocua*. This study provides knowledge as data foundations for the practical valorization and industrial food applications of olive pomace extracts.

1. Introduction

Olive pomace (OP) is the major byproduct that accounts for up to 80–95 g/100g of semisolid mass from the olive oil industry (Klisović, Novoselić, Režek Jambrak, & BrkićBubola, 2021). Olive pomace is derived from the separation of olive oils from olive malaxation paste using traditional and classic pressure systems or modern continuous centrifugation systems(Pantziaros, Trachili, Zentelis, Sygouni, & Paraskeva, 2021). However, there are ~12–15 g/100g remaining oils presenting in dried olive pomace(Cravotto et al., 2022; Zhao, Avena-Bustillos, & Wang, 2022), therefore the olive pomace oil is recovered by solvent extraction(Chanioti & Tzia, 2019). The defatted oive pomace can be utilized for food or other applications due to its bioactive phytochemical and phenolic substances. Compared to toxic and flammable hexane, supercritical carbon dioxide has been used for OP defatting as a more environmentally friendly method (Katsinas et al., 2021). The upcycling utilization of the olive pomace includes but is not limited to directly applying the whole olive pomace as an antioxidant agent of asphalt paving materials (Zhang, Zhao, & Wang, 2022), compost (Filipović et al., 2020; Milanović et al., 2019), animal feed (de Oliveira, Roll, Medeiros Gonçalves, Lopes, & Xavier, 2021; Fathy, Mahmoud, Rashad, Ezz, & Mohammed, 2018) and extracting the phenolic portion to be used as food preservatives (Bouarab Chibane, Degraeve, Ferhout, Bouajila, & Oulahal, 2019) and cosmetics additives (Galanakis, Tsatalas, & Galanakis, 2018). It has been determined that there are ~30–52 mg phenolics/g defatted dry olive pomace in either gallic acid equivalents(GAE) (Gómez-Cruz, Cara, Romero, Castro, & Gullón, 2020, 2021; Zhao, Avena-Bustillos, & Wang, 2022), or caffeic acid equivalents(CAE) (Aliakbarian et al., 2018). Olive pomace phenolics include compounds that may benefit human health, such as hydroxytyrosol, tyrosol, oleuropein, and oleuropein aglycone among others (Sefrin Speroni et al., 2021). It has been found that olive pomace contains ~1 mg/g hydroxytyrosol and 2.3–3.5 mg/g oleuropein on a dry basis (Habibi, Mohammadi, Farhoodi, & Jazaeri, 2018), while other studies reported that oleuropein aglycones, such as 3,4-DHPEA-EDA (Zhao, Avena-Bustillos, & Wang, 2022) and 3,4-DHPEA-DEDA(Katsinas et al., 2021) possibly derived from the unstable oleuropein during the oil processing could be the major compounds in olive pomace with

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concentrations up to 3,4-DHPEA-DEDA 23 mg/g in dry basis.

Given the potential antioxidant capacity for food and cosmetics applications, significant research interests have been focused on the isolation, extraction, and purification of natural phenolic compounds (NPCs) from various waste and byproduct streams from olive oil production in Europe, such as olive mill wastewater (OMWW) from Italy (Romeo, De Bruno, Imeneo, Piscopo, & Poiana, 2020), Greece (Galanakis et al., 2018) and Spain (Sánchez-Arévalo, Jimeno-Jiménez, Carbonell-Alcaina, Vincent-Vela, & Álvarez-Blanco, 2021; Tapia-Quirós et al., 2022), the olive leaf from Spain (Talhaoui et al., 2016) and Slovenia (Borjan, Leitgeb, Knez, & Hrnčič, 2020) as well as olive pomace from Greece (Chanioti & Tzia, 2018), Italy (Balli, Cecchi, Innocenti, Bellumori, & Mulinacci, 2021; De Bruno et al., 2018) and Portugal (Nunes et al., 2021). Phenolic substances profiles found in wastewaters or olive pomace have different polarity, resulting in different solubility in water or lipid systems, therefore the extracts may contain different phenolic substances and concentrations depending on the solvent used (Romeo, De Bruno, Imeneo, Piscopo, & Poiana, 2019).

The potential utilization of the bioactive compounds in olive byproducts as natural antioxidants, preservatives, and antimicrobials has been far less investigated in the United States than in European countries. Furthermore, most of the research focused on the full phenolic spectrum of purified extract of phenolic compounds using macroporous resin (Frascari et al., 2016; B. Liu, Liu, Huang, Pei, & Di, 2020; Zhao, Avena-Bustillos, & Wang, 2022) and membrane filtration (Foti et al., 2022; Sedej et al., 2016) technologies. Goldsmith, Vuong, Stathopoulos, Roach, and Scarlett (2018) reported that the antioxidant activities of dry olive pomace extracted by water were 31.23 mg (0.125 mmol) Trolox equivalents (TrE)/g dry olive pomace by 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay and 73.54 mg (0.234 mmol) TrE/g dry olive pomace by cupric reducing antioxidant capacity (CUPRAC) assay. Our previous study(Zhao, Avena-Bustillos, & Wang, 2022) presented similar antioxidant activities of aqueous extracts of dry olive pomace that were 0.233 mmol TrE/g dry olive pomace by DPPH assay and 0.214 mmol TrE/g dry olive pomace by ferric reducing antioxidant power (FRAP), but the extracts by use of 70 mL/100 mL alcohol solvents generally had higher antioxidant activities which were 0.329 mmol TrE/g dry olive pomace by DPPH assay and 0.432 mmol TrE/g dry olive pomace by FRAP. It has also been found that phenolics from OMWW and olive oils demonstrated antimicrobial activities (Nunes et al., 2021; Yakhlef et al., 2018). Khadim, Khadur, Abdul-Jabar, and Yaseen (2020) measured the inhibition zones for representing the antimicrobial activities of olive pomace extracts against selected pathogenic bacteria. Friedman, Henika, and Levin (2013) reported bactericidal activities (BA50) values of the extracts of olive pomace and olive juice powder, against four major foodborne pathogens including *Escherichia coli* O157:H7, *Salmonella enterica*, *Listeria monocytogenes*, and *Staphylococcus aureus*. However, very limited studies reported antimicrobial activity data of olive pomace extracts in a standardized way such as minimum inhibitory concentration (MIC) values per gallic acid equivalents (GAE). Standardized data reports are important because extracts from different studies may have different phenolic purities and effective bio-active contents. In other words, without data standardization based on GAE contents, it is difficult for researchers to compare the antimicrobial activities among studies using olive pomace extracts from different geographical origins, cultivars and extracting processes.

In addition, these previous studies focused on the antimicrobial activities by use of the full phenolic spectrum of olive phenolics. Commercial reverse phase C18 (octadecylsilyl groups, ODS) vacuum cartridges on an analytical scale separation apparatus, for example, the 'Waters® Sep-Pak C18 Vac Cartridges' with sorbents from 0.1 g to 10 g, have been widely employed for obtaining specific fractions with narrow phenolic spectrum from crude extracts of diverse natural phenolic compounds. Xu et al. (2014) applied C18 cartridges to obtain methanol-water gradient phenolic fractions of Muscadine grapes and found different fractions have different phenolic compound profiles, and antioxidant/antimicrobial activities. Obtaining relatively precise phenolic fractions using a preparative scale (Kammerer, Carle, Stanley, & Saleh, 2010) methanol gradient chromatography with 100–1000 mL C18 gel would help to reveal the mechanism actions and fraction contributions to their antioxidant and antimicrobial activities, and to provide compositional information on crude extracted natural phenolic compounds. Jiang et al. (2013) employed preparative scale C18 chromatography and found that approximately 75 g/100g of extracted anthocyanins were from the 20 mL/100 mL and 30 mL/100 mL methanolic fractions from Zijuan tea. Ben Saad, Jerbi, Khlif, Ayedi, and Allouche (2020) used a 94.2 mL C18 column to acquire phenolic fractions from OMWW using 20–100 mL/100 mL acetonitrile/water gradients and reported that different fractions contained different phenolic compounds profiles, and hydroxytyrosol was mainly in the low acetonitrile fraction. Nevertheless, currently there is limited information about olive pomace phenolics separation by the use of either analytical or preparative scale C18 chromatography. The characteristics of C18 chromatographic fractions have yet to be comprehensively investigated.

The objective of this study is to compare the antioxidant and antimicrobial activities of phenolic fractions extracted from the US California Arbequina olive pomace with different phenolic profiles using preparative chromatography columns of C18 gel by rinsing gradient methanol-water mobile phase. We hypothesize that olive pomace phenolics with different phenolic compounds and polarities have effects on their antioxidant and antimicrobial activities. Individual phenolic compound profiles were analyzed and investigated, along with antioxidant activities of the phenolic fractions, such as using 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH) assay, ferric reducing antioxidant power (FRAP), iron chelation by Ferrozine assay and *in vitro* antimicrobial activities by non-Shiga toxin-producing *Escherichia coli* O157:H7 (ATCC 700728) and *Listeria innocua* (ATCC 33090). The study reported antioxidant and antimicrobial activities of olive pomace phenolics based on total phenolic content (TPC) in gallic acid equivalents (GAE) which is a standardized unit and would be directly comparable for future research on olive pomace extracts.

2. Materials and methods

2.1. Materials and chemicals

Fresh Arbequina olive pomace (OP) was collected from an olive oil processor in northern California, during the 2019 harvest season and was processed as described in our previous publication (Zhao, Avena-Bustillos, & Wang, 2022). Analytical grade of chemicals of 2, 2′-diphenyl-1-picrylhydrazyl radical (DPPH), Trolox®, 2,4,6-tripyridyl-*s*-triazine (TPTZ), and ferrous sulfate heptahydrate were all purchased from Fisher Scientific (Waltham, MA, USA). 12N hydrochloric acid (HCl), liquid-chromatography (LC) grade chemicals of hexane, water, acetic acid, sodium acetate, methanol, acetonitrile, tryptic soy broth (TSB), tryptic soy agar (TSA), phosphate-buffered saline (PBS; pH 7.4), and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Waltham, MA, USA). Ferrozine® iron reagent was bought from Sigma-Aldrich (St. Louis, USA). Phenolic compound standards of gallic acid, hydroxytyrosol (HT), tyrosol, 4-hydroxyphenylacetic acid (4-HPA), vanillic acid, vanillin, *o*-coumaric acid, oleuropein, pinoresinol, cinnamic acid, caffeic acid, p-coumaric acid, ferulic acid, apigenin-7-glucoside, apigenin, luteolin-7-glucoside, luteolin, and resazurin sodium salt were all purchased from Sigma-Aldrich (St. Louis, MI, USA). Verbascoside was bought from HWI group (Ruelzhelm, Germany). Rutin was bought from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). C18 gel powders were manually separated from Sep-Pal Vac 20 cc (5 g each) cartridges (Waters Corporation, Milford, MA, USA).

2.2. Isolation and purification of phenolic compounds from olive pomace

2.2.1. Isolation of extractable phenolics

It has been reported that defatting prior phenolics extraction facilitated extracting three times more phenolics (Senit et al., 2019) because lipids and other less-polar components, such as fat-soluble vitamins and pigments, may interfere with the following phenolic extraction and quantification (De Bruno et al., 2018). Therefore, fat removal and extraction are usually performed with hexane at room temperature (Walters, Lima Ribeiro, Hosseinian, & Tsopmo, 2018; Xu, Zhang, Wang, & Lu, 2010). To 200 g of pitted, drum-dried olive pomace in a 2000 mL beaker, 800 mL of hexane was added with gentle stirring every 30 min and stayed in a fume hood in the dark with a cover for 2 h. Then the supernatant hexane was decanted for removing oil and fat, some of the small particles of OP inevitably outflowed from the beaker with the hexane, and the hexane extractions were repeated two more times (Cecchi et al., 2023; Kim, Tsao, Yang, & Cui, 2006). Then the beaker was covered with a paper towel for overnight drying to obtain ~160 g defatted olive pomace (DOP).

Although combing water and alcoholic solvents is more effective to extract phenolics from olive pomace than water(Zhao, Avena-Bustillos, & Wang, 2022), our preliminary experiment (data not shown) of this study has found that water extract has more antimicrobial potency than methanol, ethanol, and acetone extracts. Therefore, water was used for the phenolic extraction. To 60 g DOP in a 1000 mL beaker, 300 mL of deionized (DI) water was added and sonicated for 10 min. The beaker was placed in a fume hood in the dark for 1 h with gentle stirring every 30 min. Then the supernatant was decanted and collected, and the extraction was repeated two times more to obtain ~900 mL extracts. The water extract (WE) was vacuum-filtered by double-layer Whatman filter papers. For the solid content of water extract, five mL of WE in triplicate were added into 5 cm diameter aluminum pans in an isotherm oven (Thermo Fisher Scientific, Waltham, MA, USA) at 105 °C for 48 h till constant weight.

2.2.2. Preparation of freeze-dry powders of olive pomace phenolic fractions by preparative chromatography of C18 (octadecylsilyl groups, ODS)

Two hundred and twenty g of dry C18 gel were preconditioned (Xu et al., 2014) by use of 600 mL ethyl acetate and 1000 mL methanol, then 262.5 mL (1-bed volume, BV) C18 gel was loaded with methanol into a glass column and rinsed by 2000 mL 0.5 mL/100 mL HCl acidified DI water at 4 BV/h by a MasterFlex Easy-Load peristaltic pump (Gelsenkirchen, Germany). Preparative C18 chromatography operation referred to the instruction of Jiang et al. (2013). The ~900 mL extract at ambient temperature was loaded into the 262.5 mL (1-bed volume, BV) C18 gel column (diameter* height of gel = 5 cm* 13.4 cm) at 4 BV/h to obtain loading eluted syrup (ES) fraction. Subsequently, the C18 column was rinsed with 1000 mL of 0.5 mL/100 mL HCl acidified DI water at 4 BV/h to acquire acidified water (AW) fraction. Then the C18 column was rinsed with 1000 mL of 0.5 mL/100 mL HCl acidified 35 mL/100 mL at 4 BV/h to acquire acidified 35 mL/100 mL methanol fractions (35 ME); then rinsed with 1000 mL of 0.5 mL/100 mL HCl acidified 70 mL/100 mL methanol at 4 BV/h to acquire acidified 70 mL/100 mL methanol fractions (70 ME), respectively. Finally, each of the fractions was concentrated by rotary evaporation at 40 °C to fully remove methanol and decrease the volume to ~100 mL, with a Rotavapor® R-300 (BÜCHI, New Castle, DE, USA) and these concentrated fractions were freeze-dried to obtain powders of olive pomace phenolic fractions.

2.3. High-performance-liquid-chromatography (HPLC)-diode-array detector (DAD) and HPLC-quadrupole-time of flight-mass spectrometry (QTOF-MS) analysis

Identification of individual phenolic compounds was respectively implemented by an Agilent 1290 high-performance-liquid-chromatography (HPLC) system (Santa Clara, CA, USA) with an Agilent 1290 diode-array detector (DAD) and an Agilent 1290 HPLC coupled to an Agilent 6530 quadrupole time-of-flight mass spectrometer (Q-ToF-MS) which referenced method of Zhao, Avena-Bustillos, and Wang (2022). The collision energy (CE) was 0, 15, and 30 eV. The phenolic compounds were identified according to the MS^n fragments compared to the reports of Peralbo-Molina, Priego-Capote, and Luque de Castro (2012) and Zhao, Avena-Bustillos, and Wang (2022).

An analytical C18 column (Eclipse Plus, 4.6 mm × 250 mm, 5 μm, Agilent Technologies) was used for separation. Elution was applied using mobile phase A (3 mL/100 mL acetic acid aqueous solution) and mobile phase B (50 mL/100 mL methanol and 50 mL/100 mL acetonitrile). The following linear gradient was used: 0 min starting from 5 mL/100 mL B (while 95 mL/100 mL A, similarly hereinafter); linear increase to 30 mL/100 mL B at 25 min; to 35 mL/100 mL B at 35 min; to 40 mL/100 mL B at 40 min; to 70 mL/100 mL B at 50 min; to 100 mL/100 mL B at 55 min, then decreasing to 5 mL/100 mL B at 60 min and holding at 5 mL/100 mL B for 5 min for the column equilibrium for the next injection. The flow rate was 1.0 mL/min. The injection volume was 20 μL. The DAD was set to maximum or optimum absorbance wavelengths at 280 nm for hydroxytyrosol, tyrosol, 4-hydroxyphenylacetic acid (4-HPA), vanillic acid, vanillin, *o*-coumaric acid, oleuropein, pinoresinol, cinnamic acid; at 320 nm for caffeic acid, p-coumaric acid, ferulic acid, apigenin-7-glucoside, apigenin, verbascoside; and at 365 nm for rutin, luteolin-7-glucoside, and luteolin, respectively. Standard curves were made by each of the standard chemicals at concentrations of 10, 20, 40, 60, 80, and 100 mg/L, respectively. And the results of individual phenolic compounds were expressed as mg/g dry basis.

2.4. Total phenolic content and antioxidant activities analysis

2.4.1. Total phenolic content

The total phenolic content was determined by Folin-Ciocalteu assay based on the method of Huang et al. (2020) with slight modification. To adjust the sample concentration to a linear range, 0.02 g of the freeze-dried fraction of loading eluted syrup was dissolved in 10 mL of DI water; 0.02 g of the freeze-dried fraction of acidified water was dissolved in 10 mL of DI water; 0.02 g of the freeze-dried fraction of acidified 35 mL/100 mL methanol was dissolved in 10 mL of 70 mL/100 mL methanol; and 0.02 g of the freeze-dried fraction of acidified 70 mL/100 mL methanol was also dissolved in 70 mL/100 mL methanol, respectively; therefore the dilution factor (DF) was 10/0.02 = 500 for these C18 fractions. But 5 mL of the water extract was diluted by 5 mL DI water which rendered the DF to be 2. 30 μL of each of the aforementioned dissolved samples was added by 1.8 mL DI Water, 150 μL Folin-Ciocalteu reagent, 450 μL 20 g/100 mL Na_2CO_3, and 570 μL DI Water (the adding sequence cannot be changed). This total 3 mL solution was incubated for 30 min in dark, then 200 μL solution out of the 3 mL was pipetted into a FALCON tissue culture microplate with 96 cuvettes (Corning incorporation, Corning, USA), then determined by absorption at 725 nm in a BioTek® Synergy H1 microplate reader (Winooski, USA). 30 μL extraction solvent (i.e. 70 mL/100 mL methanol) with all the other chemical reagents was served as blank. 0.1–2.0 mg/mL of gallic acid (GA; molecular weight, MW = 170.12 g/mol) was utilized as a standard for the calibration curve (y = 0.5785x+ 0.0118, R^2 = 0.9984). And the results were expressed as gallic acid equivalents (mg GAE/g dry basis) using the calibration curve and the dilution factors as well as the solid content.

2.4.2. 2,2′-diphenyl-1-picrylhydrazyl radical (DPPH) assay

The total antioxidant activity by DPPH assay was implemented based on the method of Yuan et al. (2018) and Huang et al. (2020) with slight modifications. To adjust the sample concentration to a linear range, 2 mL of each of the dissolved fractions of acidified 35 mL/100 mL methanol and acidified 70 mL/100 mL methanol for TPC determination was added in 8 mL of 70 mL/100 mL methanol respectively, therefore the additional dilution factor (DF) was 5 which made the total DF = 2500 for

these two C18 fractions. However, extracts of loading eluted syrup, acidified water and water extract were all the same as the formation of TPC determination. 195 μL methanolic solution of DPPH (MW 394.32 Da, 4.7 mg/100 ml methanol, Abs = 0.590) was added to 5 μL of dissolved samples in the FALCON 96 cuvettes microplate. The absorbance of the remaining DPPH was determined at 515 nm after 30 min dark incubation at room temperature (RT). The percentage of inhibition of DPPH of the test sample and known solutions of Trolox were calculated by the following formula: percentage of inhibition = 100 * (A0 – A)/A0, where A0 was the absorbance of DPPH methanolic solution without any inhibition, acquired by measuring combination of 5 μL extraction solvent and 195 μL methanol solution of DPPH, and A was the final absorbance of the test sample at 515 nm. Blank was made by 5 μL extraction solvent and 195 μL methanol without DPPH. The calibration curve between percentage of inhibition and known methanol solutions of Trolox (MW = 250.29 g/mol) was then established (y = 49.649x-0.6321, R^2 = 0.9966). These solutions were mixed and allowed to react for 30 min at room temperature in dark. Trolox standard solutions were prepared at a concentration ranging from 0.1 to 1.6 μmol/mL. The radical scavenging activities of the test samples were expressed as Trolox equivalent (TrE) antioxidant capacity (mmol TrE/g dry basis, 1 mmol TrE/g equals to 1000 μmol TrE/g) on their percentage inhibitions using the calibration curve and the dilution factors as well as the solid content.

2.4.3. Ferric reducing antioxidant power (FRAP)

The FRAP assay was implemented according to the methods of D'Amato, De Feudis, Guiducci, and Businelli (2019) and Zhao, Avena-Bustillos, and Wang (2022). The sample solutions for the FRAP test were equal to those of the DPPH test. The fresh working FRAP solution was prepared by mixing: 1) 10 mmol/L 2,4,6-tripirydyl-*s*-triazine (TPTZ, MW 312.33 Da) dissolved in 40 mmol/L HCl, 2) 20 mM $FeCl_3$ (MW 162.20 Da), and 3) 300 mmol/L acetate buffer at pH 3.6, with a ratio of 1:1:10, respectively. Then it was kept at 37 °C water bath before use. 50 μL of the sample solution was added to 950 μL of FRAP solution, then the mixture was incubated at 37 °C in the dark for 30 min. And the absorbance of 0.2 mL of the mixed soltuion was measured at 595 nm in the FALCON 96 cuvettes microplate. A methanol and Trolox solution (MW = 250.29 g/mol, between 0.1 and 1.6 μmol/mL) was used for the standard curve (y = 1.1479x- 0.0113, R^2 = 0.9934). The results were expressed as mmol (1000 μmol) Trolox equivalents per g of samples (mmol TrE/g dry basis) using the calibration curve and the dilution factors as well as the solid content.

2.4.4. Iron chelation by ferrozine assay

The Ferrozine method was used for evaluating chelating activity based on the works of Karamać and Pegg (2009) and Sun et al. (2022) with modifications in acetic acid-sodium acetate pH buffer systems. Four reagents were used for the experiment including A: 0.1 mL olive phenolic solution of water extract (WE) and C18 fractions of loading eluted syrup, acidified water, acidified 35 mL/100 mL methanol, and acidified 70 mL/100 mL methanol at 0.2 mmol/L GAE, B: 0.3 mmol/L acetic acid-sodium acetate buffer (0.9 mL) of pH 6.81 or 3.87, respectively, C: 0.1 mL of 0.8 mmol/L $FeSO_4$ solution in water, and D: 0.2 mL of 5 mmol/L Ferrozine reagent. The mixing sequence was A-B (pH 6.81 or 3.87)-C-D. Each step had a 2 min interval with general shaking from the last tubes (in triplicate) to the next step using a stopwatch. The mixture of all the agents was left to stand for 10 min at ambient temperature, then 0.2 mL of the mixture was pipetted into the FALCON 96 cuvettes microplate, and absorbance readings were taken at λ = 562 nm.

Blank (minimum absorption) also known as 'full chelation' equals the scenario that no free ion can be captured by Ferrozine to show any absorption or saying that no free iron ions were left for the formation of Fe^{2+} ion-Ferrozine complex, were prepared similarly: (A) 0.1 mL of each phenolic compound solution (each of the extracts has its own blank because some of the iron-phenolic complexes also have background absorption and colors as the so-called blank) was mixed with (B) and (C), but 0.2 mL of DI water was added instead of (D) to mimic no formation of Fe^{2+} ion-Ferrozine complex.

Accurate iron content (none chelation, maximum absorption) of reagent (C) was measured by adding 0.1 mL DI water instead of phenolic compounds solution (A) therefore all the free Fe^{2+} ions in (C) solution reacted with Ferrozine to form Fe^{2+} ion-Ferrozine complex. Blank for iron content determination was prepared by adding 0.1 mL DI water instead of phenolic compounds solution (A) and adding 0.1 mL of DI water instead of (C) to test the background of the system for blank subtraction.

A standard curve of absorbance ratio versus iron concentration in the range of 0–1.0 mmol/L $FeSO_4$ was constructed (y = 1.1617x+ 0.00136, R^2 = 0.9999 at pH 6.81; y = 1.178x- 0.0046, R^2 = 0.9969 at pH 3.78). Final data were reported as mmol Fe^{2+}/mmol GAE of water extract and C18 fractions.

2.5. Antimicrobial activities

2.5.1. Bacterial strains

A non-Shiga toxin-producing *Escherichia coli* O157:H7 (ATCC 700728) and *Listeria innocua* (ATCC 33090) were kindly provided by Linda Harris's lab (University of California, Davis) and used as model bacterial strains for Gram-negative and Gram-positive foodborne pathogens, respectively. Briefly, each cryo-preserved stock was cultured by incubating in tryptic soy broth (TSB) at 37 °C with constant shaking at 200 rpm overnight. An overnight culture was streaked on tryptic soy agar (TSA) and incubated at 37 °C for 48 h. The TSA plate with microbial colonies was stored at 4 °C before use. Before each experiment, a single colony from the TSA plate was transferred into TSB and incubated at 37 °C overnight with constant shaking at 200 rpm to obtain the stationary phase cultures. The bacterial cultures (ca. 9.0–9.5 log Colony Forming Unit, CFU/mL) were washed twice with phosphate-buffered saline (PBS; pH 7.4) and serially diluted in TSB (ca. 5.3 log CFU/mL) and PBS (ca. 8.0 log CFU/mL) with 5 mL/100 mL dimethyl sulfoxide (DMSO) to prepare an inoculum for the microdilution assay and plate count assay, respectively.

2.5.2. Determination of the minimal inhibitory concentration (MIC) and minimal lethal concentrations (MLC) of C18 fractions

The MIC and MLC values of the C18 fractions against *E. coli* O157:H7 or *L. innocua* were determined using a resazurin-based microdilution assay (Elshikh et al., 2016) with slight modifications. In the presence of bacterial respiration, the non-fluorescent resazurin (blue-purple color) is reduced to the fluorescent resorufin (pink color) and used as an endpoint indicator in the microdilution assays. The assay was performed in a sterile 96-well microtiter plate (Costar 3596, Corning, Kennebunk, ME, USA). Briefly, 100 μL of TSB with 5 mL/100 mL DMSO were dispensed in each well except the wells in the first column of each row. A 200 μL of C18 fractions (6.8–16 mg GAE/mL in TSB with 5 mL/100 mL DMSO) was added to each well of the first column and serially diluted two-fold in the following wells within the same row. After the serial dilution of the C18 fractions, 100 μL of *E. coli* O157:H7 or *L. innocua* inoculum (ca. 5.3 log CFU/mL) was added to each well containing C18 fraction to achieve a final bacteria concentration of ca. 5.0 log CFU/mL. A negative control containing C18 fractions and TSB (5 mL/100 mL DMSO), without bacterial cells, and a positive control containing bacterial cells and TSB (5 mL/100 mL DMSO), without C18 fractions, were included in each row. The plates were incubated at 37 °C for 24 h. After the incubation for 24 h, 20 μL of resazurin solution (0.5 mmol/L) was added to each well, and the plate was incubated for an additional 3 h at 37 °C. The lowest concentration of C18 fractions that inhibited the color change of resazurin solution was considered the MIC of the C18 fraction. To determine the MLC values of the C18 fractions, bacterial suspensions in each well were streaked on TSA and incubated at 37 °C for 24 h. The lowest concentrations of the C18 fraction that resulted in no recovery of bacterial colonies on TSA were considered as the MLC of the C18

fraction.

2.5.3. Comparison of antibacterial activities of crude water extract with 35 mL/100 mL methanolic eluate

The antimicrobial activities of water extract and acidified 35 mL/100 mL methanol fraction were evaluated against *E. coli* O157:H7 and *L. innocua*, respectively. A 10 μL of the bacterial inoculum (ca. 8.0 log CFU/mL) prepared as described above was inoculated with different concentrations (0.5, 1.0, and 2.0 mg GAE/mL) of water extract and acidified 35 mL/100 mL methanol fraction, respectively. The final concentration of bacteria in these samples was ca. 6.0 log CFU/mL, and the samples were incubated at room temperature for 0, 30, and 60 min, respectively. Bacterial cells incubated with PBS (5 mL/100 mL DMSO) without water extract, nor acidified 35 mL/100 mL methanol fraction were used as control. After treatment, the bacterial suspensions of each treatment were serially diluted in PBS, surface-plated on TSA, and incubated at 37 °C for 48 h before the colonies were counted. The theoretical detection limit for the direct plating was 1.0 log CFU/mL.

2.6. Statistical analysis

Triplicate data were analyzed by multiple comparison tests with Fisher's least significant difference (LSD, $p < 0.05$) method by R software 4.1.2. Principal component analysis (PCA) was applied for the overall statistical analysis of the phenolic compound profile using R software 4.1.2. Heatmap cluster analysis and data visualization were implemented in MATLAB 2022a (The MathWorks Inc., Natick, MA, USA) based on a Three-in-One food omics analysis tool 'Ana' package (Zhao & Wang, 2022).

3. Results and discussion

3.1. Total phenolic contents in C18 fractions

Because the solid content of the water extract was determined to be 2.44 g/100 g at 105 °C oven dry, and C18 fractions were all freeze-dried, each compound was expressed in dry basis in Fig. 1. The total phenolic contents (TPC) of water extract and the C18 fractions of loading eluted syrup (ES), acidified water (AW), acidified 35 mL/100 mL methanol (35 ME), and acidified 70 mL/100 mL methanol (70 ME) were 77.05, 2.88, 29.39, 170.56 and 235.96 mg gallic acid equivalents (GAE)/g dry basis of C18 fractions, respectively (Fig. 1). Compared to the TPC in the crude extracts of olive pomace, Spizzirri, Carullo, Aiello, Paolino, and Restuccia (2021) found that the TPC of the crude extracts of olive pomace from Italy were between 0.111 and 0.280 Milliequivalents (mEq) GAE/g crude extracts (as 1 mEq = 1 mmol, the results also equal to ~19–48 mg GAE/g crude extracts) depending upon extraction conditions such as ethanol percentage, time and temperature. Our previous study found a similar order of magnitudes results of crude extracts (full phenolic spectrum) of a US California olive pomace which were between 52 and 69 mg GAE/g dry basis of crude extracts depending upon extraction media of water, 70 mL/100 mL ethanol, and 70 mL/100 mL methanol (Zhao, Avena-Bustillos, & Wang, 2022). The 70 mL/100 mL ethanol crude extract (full phenolic spectrum) from defatted olive pomace but purified by macroporous resin in our previous study was found to be 303 mg GAE/g of freeze-dried extract (Zhao, Avena-Bustillos, & Wang, 2022). Therefore, the acidified 35 mL/100 mL methanol and acidified 70 mL/100 mL methanol fractions in this study were also purified via the preparative C18 gel chromatography to increase the total phenolic contents as compared to the phenolic contents in those reported crude extracts but were less than that of the macroporous resin purified extract in our previous study.

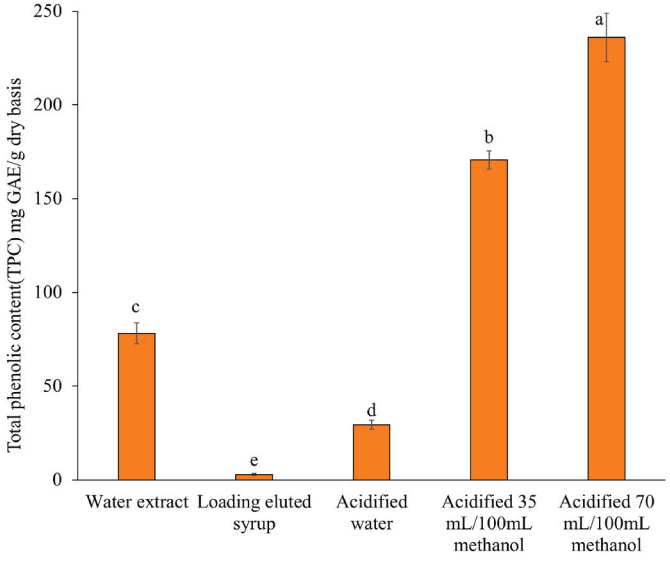

Fig. 1. Total phenolic content (TPC) expressed as gallic acid equivalents (GAE) per dry basis of water extract and C18 fractions, in triplicates, $p < 0.05$.

3.2. Individual phenolic contents in C18 fractions

Twenty-four individual phenolics of the water extract were identified using both chemical standards and mass fragments (Supporting Information, S.Fig. 1.a). The peak (19) of 3,4-DHPEA-EDA, which is also known as oleuropein-aglycone-di-aldehyde, at 31.43 min could be the major phenolics in the water extract of olive pomace, solely based on the peak area comparison. It has been reported that 3,4-DHPEA-EDA was a major phenolic compound from olive leaves (Akazawa et al., 2021; Fuccelli et al., 2018). It has also been found that decarboxymethyl oleuropein-aglycone dialdehyde (3,4-DHPEA-DEDA, 23 mg/g) in olive pomace from Spain (Katsinas et al., 2021) and 3,4-DHPEA-EDA in olive pomace from the US California (Zhao, Avena-Bustillos, & Wang, 2022) were dominant phenolic compounds, respectively. However, due to the lack of the commercially available chemical standard of 3, 4-DHPEA-EDA, it was not quantified in this study.

Olive pomace phenolics are shown in the heatmap in Fig. 2 (a) and S. Table 1. Besides 3,4-DHPEA-EDA, some other major phenolics in water extract were hydroxytyrosol (HT, 4.328 mg/g dry basis, hereinafter the same for the dry basis), followed by 4-hydroxyphenylacetic acid (4-HPA, 3.565 mg/g), hydroxytyrosol-glucoside (HT-glu, 3.017 mg/g), and tyrosol-glucoside (2.375 mg/g). There are fewer phenolics in the fractions of loading eluted syrup and acidified water because the affinity interaction between phenolics and the C18 solid phase was strong when the mobile phase was water or acidified water in the C18 column. As the increment of methanol concentration in the mobile phase, the concentration of individual phenolics also generally increased, i.e. tyrosol-glucoside, tyrosol, verbascoside, and rutin in acidified 35 mL/100 mL methanol and acidified 70 mL/100 mL methanol fractions; however, this is not entirely true for hydroxytyrosol-glucoside, hydroxytyrosol, and 4-HPA, because acidified 35 mL/100 mL methanol fraction desorbed almost all these compounds, then limited amounts on C18 gel were leftover for acidified 70 mL/100 mL methanol desorption, subsequently. Specifically, loading eluted syrup and acidified water fractions had similar phenolic profiles with hydroxytyrosol (2.592 and 2.597 mg/g, respectively) and tyrosol-glucoside (0.742 and 0.737 mg/g, respectively); and acidified 35 mL/100 mL methanol fraction had hydroxytyrosol-glucoside (4.616 mg/g), hydroxytyrosol (8.969 mg/g), tyrosol-glucoside (5.029 mg/g), 4-hydroxyphenylacetic acid (4-HPA, 6.877 mg/g), verbascoside (2.601 mg/g) and oleuropein (4.274 mg/g); acidified 70 mL/100 mL methanol fraction contained hydroxytyrosol (3.741 mg/g), tyrosol-glucoside (8.564 mg/g), and tyrosol (4.415 mg/g), but generally had more less-polar compounds such as verbascoside (2.894 mg/g), rutin (2.952 mg/g), and oleuropein (4.043 mg/g) than the other fractions. It has been reported that the water solubilities are

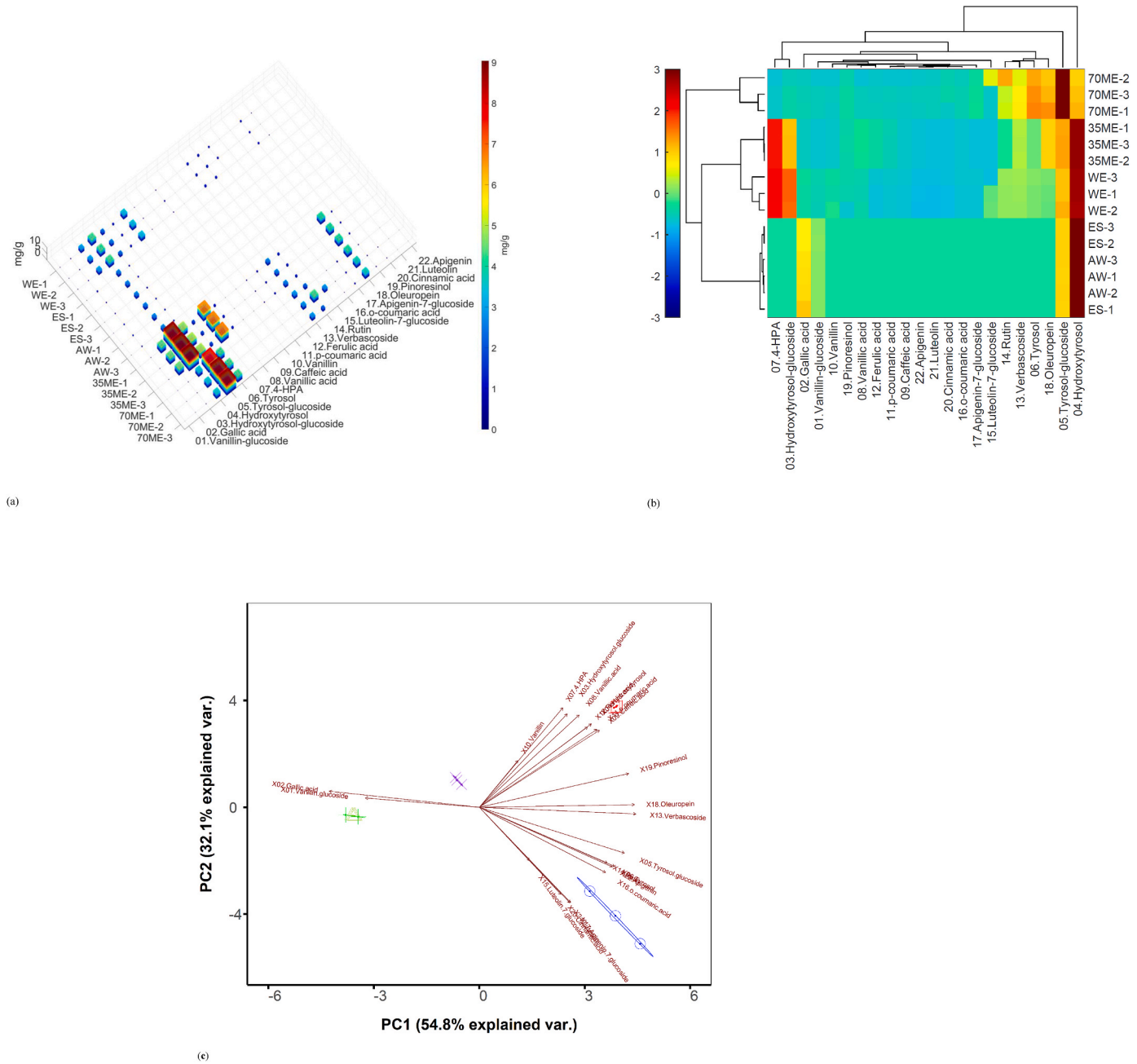

Fig. 2. (**a**) Heatmap of individual phenolic contents (mg/g dry basis) of water extract and C18 fractions (**b**) heatmap cluster analysis of individual phenolic contents; data were standardized along each row. (**c**) principal component analysis (PCA) biplot of individual phenolic contents by PC1 and PC2.
Note: Experiments were performed in triplicate. Water extract (WE✳), and the fractions of loading eluted syrup (ES+), acidified water fraction (AW△), acidified 35 mL/100 mL methanol fraction (35 ME⊞), and acidified 70 mL/100 mL methanol fraction (70 ME⊕). −1, −2, −3 represent triplicate data. The height and color code of each column in (a) represent the true concentrations of each phenolic, while the volume and size represent the relative and underestimated concentrations. Vanillin-glucoside, hydroxytyrosol-glucoside, and tyrosol-glucoside are expressed by equivalents of vanillin, hydroxytyrosol, and tyrosol, respectively. 4-HPA is 4-hydroxyphenylacetic acid. % in PCA variances is non unit fraction. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

verbascoside 0.083 mg/mL (*Verbascoside | C29H36O15 - PubChem*, n.d.), rutin 0.125 mg/mL (Frutos, Rincón-Frutos, & Valero-Cases, 2019), and oleuropein 1.734 mg/mL (*Oleuropein | C25H32O13 - PubChem*, n.d.). All of them can be considered as less-polar phenolic compounds comparing to 4-hydroxyphenyl acetic acid (4-HPA) 60.7 mg/mL(*4-Hydroxyphenylacetic Acid | C8H8O3 - PubChem*, n.d.), hydroxytyrosol 50.0 mg/mL (Napolitano, De Lucia, Panzella, & d'Ischia, 2010), and tyrosol 25.3 mg/mL (García-Salinas et al., 2020). Increasing amounts of less-polar compounds were found with the increase of methanol in the methanol-water gradients.

Foodomics enables to analyze of the entire original dataset of foodome (Jimenez-Carvelo & Cuadros-Rodríguez, 2021), and the most routinely used algorithms are heatmap cluster analysis and principal component analysis (PCA) as initial exploratory/screening strategies (Zhao & Wang, 2022). Fig. 2 (b) shows the heatmap cluster analysis of phenolic compound profiles by the standardization of the absolute values in Fig. 2 (a). Hydroxytyrosol (data in S. Table 1) was the major compound in the fractions of loading eluted syrup and acidified water,

Table 1
Minimal inhibitory concentrations (MIC) and minimal lethal concentrations (MLC) of C18 fractions against *E. coli* O157:H7 and *L. innocua*.

Bacteria	C18 fractions	MIC (mg GAE/mL)	MLC (mg GAE/mL)
Escherichia coli O157:H7	Loading eluted syrup (ES)	0.25	0.25
	Acidified water (AW)	0.5	0.5
	Acidified 35 mL/100 mL methanol (35 ME)	2.1	4.2
	Acidified 70 mL/100 mL methanol (70 ME)	1.7	3.4
Listeria innocua	Loading eluted syrup (ES)	0.25	0.25
	Acidified water (AW)	0.5	0.5
	Acidified 35 mL/100 mL methanol (35 ME)	2.1	4.2
	Acidified 70 mL/100 mL methanol (70 ME)	1.7	3.4

and the profiles of the two fractions formed a close cluster. The water extract and acidified 35 mL/100 mL methanol fraction formed another close cluster indicating similar phenolic profiles. Compared to the acidified 70 mL/100 mL methanol fraction, there are more hydrophilic phenolics such as hydroxytyrosol-glucoside, hydroxytyrosol, and 4-HPA and less hydrophobic phenolics such as verbascoside, rutin, and oleuropein in water extract and acidified 35 mL/100 mL methanol fraction. The heatmap cluster is a common data analysis method for visualizing multidimensional omics datasets. However, because it does not always represent the intrinsic connection in the dataset and may result in uninformative and/or misleading conclusions (Rajaram & Oono, 2010), it can be more informative to employ dimension-reduction algorithms PCA which would be representative of the topology inherent in omics data.

PCA is an unsupervised learning method that effectively reduces the dimensions of the original dataset and visualizes data by cluster analysis (Zhao, Zhan, et al., 2022). The PCA biplot of PC1 *vs*. PC2 is shown in Fig. 2 (c). PC1 and PC2 explained 54.8% (non unit fraction) and 32.1% (non unit fraction) of the variances respectively, resulting in the sum of the top two PCs being 86.9% (non unit fraction). The loading plot in the PCA biplot shows loading vectors of the original dimension which influence principal components. For instance, the vectors of polar compounds (gallic acid and vanillin-glucoside) are close in the vicinity and opposite from less-polar phenolics (oleuropein and verbascoside, etc.) indicating a negative correlation between the polar compounds and less-polar compounds. These vectors demonstrate that PC1 classified loading eluted syrup and acidified water into one cluster at the polar phenolics side of PC1, while acidified 35 mL/100 mL methanol and acidified 70 mL/100 mL methanol fractions into two other clusters in the different directions at the less-polar side of PC1 (Fig. 2 c). Because these C18 fractions are all derived from water extract, the water extract cluster is between the two major C18 fractions clusters.

In addition, although there was an eminent peak (19) of 3,4-DHPEA-EDA in water extract in the S.Fig. 1 (a), this peak (19) can be barely seen in all the C18 fractions. However interestingly, the area of the small peak (17) in water extract in the S.Fig. 1 (a) enormously increased in acidified 35 mL/100 mL methanol and acidified 70 mL/100 mL methanol fractions in S.Fig. 1 (d) and (e). This compound peak 'shift' indicated that the peak (17) in S.Fig. 1 (d) and (e) could derive from the peak (19) of 3,4-DHPEA-EDA in S.Fig. 1 (a). Also, based on the mass fragments analysis of HPLC-QTOF-MS for total ion chromatogram (TIC) in S.Fig. 2, the peak (17) contained fragments of MS^1 639.25 Da and MS^2 319.12 Da. Therefore, given the MS^1 319.12 Da of 3,4-DHPEA-EDA from this study and previous studies (Akazawa et al., 2021; Peralbo-Molina et al., 2012; Zhao, Avena-Bustillos, & Wang, 2022), it is further demonstrated that the peak (17) is possibly a dimer of 3,4-DHPEA-EDA because the molecular weight (MW 640.25 Da = MS^1+1 hydrogen) is exactly twice the molecular weight of the 3,4-DHPEA-EDA (MW 320.12 Da). Confirmation via nuclear magnetic resonance (NMR) may be needed to investigate the dimer formation during the C18 separation process in acidic conditions.

C18 chromatography has been widely used to obtain phenolic fractions from a wide range of plants, fruits, and vegetables. Phenolic fractions of muscadine grape skin and seeds have been acquired by analytical level cartridges with mg to g C18 gel sorbent per cartridge, and phenolic compound profiles were identified for the correlation with the antioxidant and antibacterial activities of those fractions (Xu et al., 2014). Semi-preparative C18 chromatography (~53 mL gel) has also been implemented to isolate different fractions of phenolic compounds and anthocyanins from Zijuan tea (Jiang et al., 2013). A similar technique was used to isolate phenolic fractions from olive mill waste (OMW), by use of a ~94 mL C18 cartridge (Ben Saad et al., 2020). To the best of our knowledge, this study is the first report using preparative C18 chromatography (262.5 mL gel) to investigate the phenolic profiles of olive pomace fractions. Chanioti and Tzia (2018) reported that hydroxytyrosol (up to 6.08 mg/g), rutin (up to 1.69 mg/g), and oleuropein (up to 12.86 mg/g) are major compounds that were 10–100 times higher than other minor phenolics in a Greek olive pomace extracted by natural deep eutectic solvents. Vidal, Moya, Alcalá, Romero, and Espínola (2022) found that hydroxytyrosol (16.69 mg/g), tyrosol (2.08 mg/g), verbascoside (0.46 mg/g), and oleuropein (0.54 mg/g) were major compounds in olive pomace in Spain.

3.3. *In vitro* antioxidant activities of C18 fractions

In vitro evaluation of antioxidant activities includes free radical scavenging/neutralizing and transition metal ion chelating abilities. The mechanisms of free radical scavenging include (Granato et al., 2018; D. Huang, Ou, & Prior, 2005) hydrogen atom transfer (HAT)/hydrogen donation (H•) which is normally evaluated by oxygen radical absorbance capacity (ORAC) in a water system, and single electron transfer (SET)/electron donation (•) which can be evaluated by 2,2-diphenyl-1--picrylhydrazyl (DPPH) in methanol, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) in water, and ferric reducing antioxidant power (FRAP) assay in water at pH 3.6. The mechanisms of chelation can be determined using Ferrozine-ferrous iron complex (Chelliah & Oh, 2022) or Nuclear Magnetic Resonance (NMR) (Primikyri et al., 2015). In this study, DPPH, FRAP, and Ferrozine assays were selected for rapid screening the antioxidant activities of the water extract and phenolic C18 fractions from the selected California olive pomace.

As shown in Fig. 3 (a), C18 fractions with higher total phenolic contents (Fig. 1) also had higher *in vitro* antioxidant activities of both FRAP and DPPH. And DPPH values were generally lower than FRAP values. Similar positive correlations between total phenolic content and antioxidant activities have been found in grape skin/seed phenolics (Xu, Zhang, Cao, & Lu, 2010; Zhao et al., 2021), however, it has also been reported that DPPH is not always in line with other antioxidant activities of peptides (Li et al., 2021). Contreras, Gómez-Cruz, Romero, and Castro (2021) reported the FRAP values of crude extracts (full phenolic spectrum) of olive pomace, olive leaves and residual fraction from olive pits from Spain extracted by 47 mL/100 mL ethanol were 0.542, 0.595, and 0.201 mmol TrE/g dry basis, respectively. Vidal et al. (2022) reported that the FRAP values of crude extracts (full phenolic spectrum) of olive pomace and olive leaves were 147.41 and 217.53 mg TrE/g dry basis (0.589 and 0.870 mmol TrE/g dry basis), and the DPPH values were 92.71 and 175.71 mg TrE/g dry basis (0.371 and 0.703 mmol TrE/g dry basis); therefore DPPH values were also lower than FRAP values, respectively. This study reported DPPH and FRAP values between 0.4 and 2 mmol TrE/g dry basis in a similar order of magnitude as compared with previous studies of olive wastes; however, the acidified 35 mL/100 mL methanol and acidified 70 mL/100 mL methanol fractions which were purified by the C18 column performed higher antioxidant activities between 0.5 and 2 mmol TrE/g dry basis than those previous reports.

By calculating the Trolox equivalent (TrE) per g GAE of fractions in

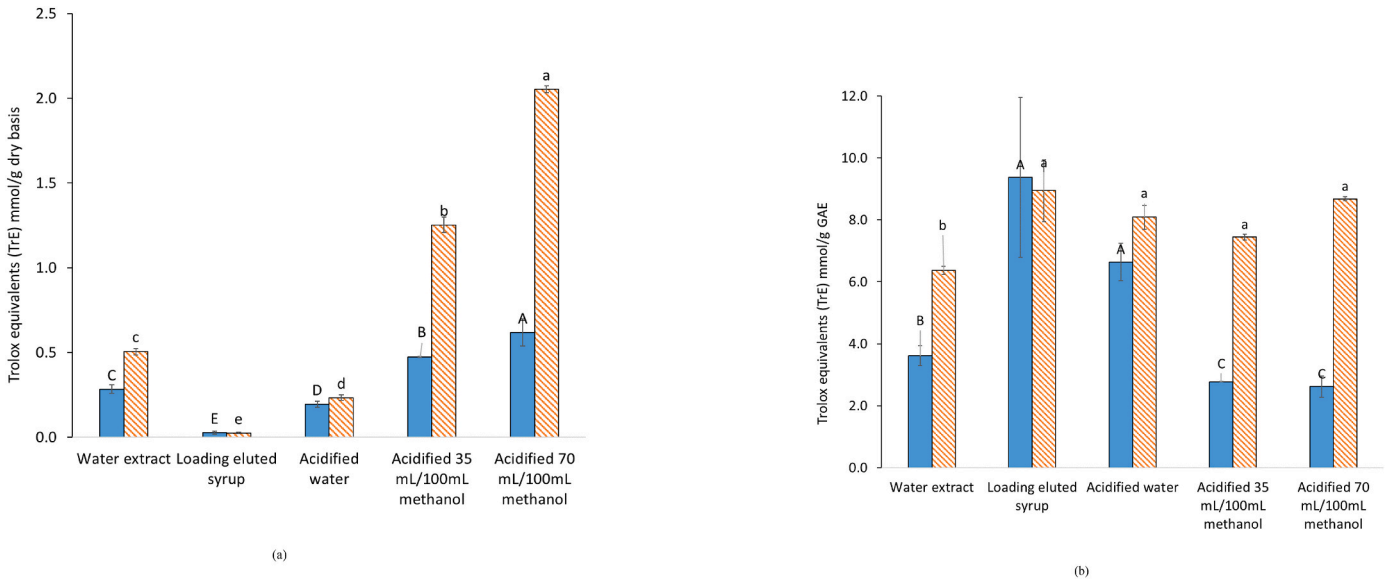

Fig. 3. Comparison of 2,2-diphenyl-1-picrylhydrazyl (■ DPPH) and ferric reducing antioxidant power (▨ FRAP) expressed by (a) Trolox equivalent (TrE) per g dry basis of C18 fractions, and by (b) Calculated Trolox equivalent (TrE) per g GAE among water extract and C18 fractions. Experiments were performed in triplicate, $p < 0.05$. Significant differences were only compared among either DPPH or FRAP.
Note: The solid content in the water extract of olive pomace was 2.44 g/100 mL, C18 fractions were freeze-dried, therefore each compound was expressed on dry basis in (a).

Fig. 3 (b), the DPPH of loading eluted syrup and acidified water fractions were significantly ($p < 0.05$) higher than that of water extract, acidified 35 mL/100 mL methanol, and acidified 70 mL/100 mL methanol fractions. Also, FRAP values of loading eluted syrup, acidified water, and acidified 70 mL/100 mL methanol fractions were higher than that of acidified 35 mL/100 mL methanol fraction, but the differences were not significant ($p > 0.05$). At pH 6.81, loading eluted syrup and acidified water fractions demonstrated significantly ($p < 0.05$) higher chelating ability than both acidified 35 mL/100 mL methanol and acidified 70 mL/100 mL methanol fractions, while the chelating ability of water extract was in between; however, at pH 3.78, the chelating ability of all fraction almost all diminished (Fig. 4) due to the low pH inhibited the deprotonation of phenolics (Rahim, Kristufek, Pan, Richardson, & Caruso, 2019).

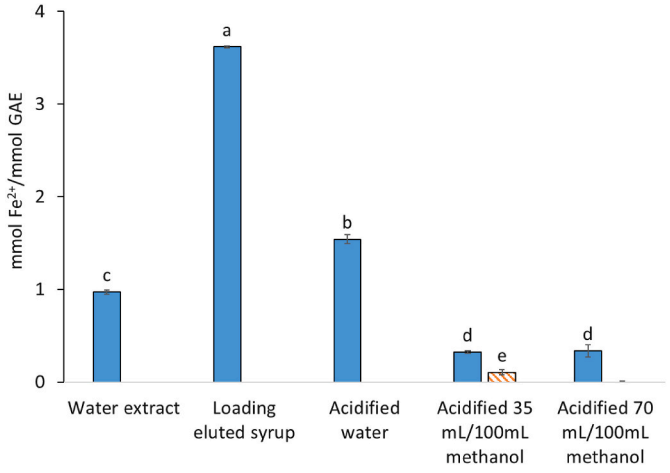

Fig. 4. Chelating ability of water extract and C18 fractions in mmol Iron (II) per mmol GAE of the fraction at ■ pH 6.85 and ▨ pH 3.78. Experiments were performed in triplicate, $p < 0.05$. Chelating abilities at pH 3.78 were almost all diminished to zero.

3.4. Antimicrobial activities of C18 fractions of olive pomace

MIC values of C18 fractions of loading eluted syrup, acidified water, acidified 35 mL/100 mL methanol, and acidified 70 mL/100 mL methanol derived from the water extract against *E. coli* O157:H7 and *L. innocua* were determined using a broth microdilution assay (Table 1). All of the tested C18 fractions showed antibacterial activities against both Gram-positive and Gram-negative bacterial strains but to different extents. Loading eluted syrup showed the lowest MIC values against both *E. coli* O157:H7 (0.25 mg GAE/mL) and *L. innocua* (0.25 mg GAE/mL), followed by acidified water fraction (0.5 mg GAE/mL, respectively), acidified 70 mL/100 mL methanol fraction (1.7 mg GAE/mL, respectively), and acidified 35 mL/100 mL methanol fraction (2.1 mg GAE/mL, respectively) fractions. Furthermore, the MIC values of the crude water extract against *E. coli* 157:H7 and *L. innocua* were measured using the same procedure but with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) dye (Wang, Cheng, Wang, Wei, & Wang, 2010) in a different batch of an experiment to compare to those C18 fractions. The MIC values of the crude water extract against *E. coli* O157:H7 and *L. innocua* were 0.79 mg GAE/mL, respectively, of which the antimicrobial activities were between the loading eluted syrup/acidified water fraction and acidified 35 mL/100 mL/70 mL/100 mL methanol fractions. The medium-range activities of the crude water extract are reasonable because all C18 fractions are derived from the crude water extract. Similar trends were observed with MLC values. Loading eluted syrup and acidified water fractions showed the same MLC values as their MIC values, however, acidified 35 mL/100 mL methanol and acidified 70 mL/100 mL methanol fractions showed two-fold higher MLC values compared to their MIC values against both tested bacteria. This indicates that loading eluted syrup and acidified water fractions are more bactericidal to the tested strains than acidified 35 mL/100 mL methanol and acidified 70 mL/100 mL methanol fractions.

MIC values of the olive pomace extracts obtained by high pressure-assisted extraction method have been reported by Nunes et al. (2021). Compared to the extracts obtained by the precedented study, our extracts showed significantly lower MIC values against the tested bacteria. For example, the loading eluted syrup fraction showed 9–20 times lower

MIC values against *E. coli* compared to those reported by Nunes et al. (2021). This indicates that our water-based extraction method and the following C18-based separation are very effective in obtaining phenolic compounds with strong antibacterial activities. Antimicrobial activities of the phenolic compounds extracted from diverse olive sources such as leaves, bark, and pulps using different extractants have been reported in previous studies (Leouifoudi, Harnafi, & Zyad, 2015; Y. Liu, McKeever, & Malik, 2017; Mehmood & Murtaza, 2018; Sánchez-Gutiérrez et al., 2021). However, the antibacterial activities of different eluates of water extract obtained from olive pomace have not been investigated so far. Interestingly, the results of the microdilution assay seemed to suggest similar trends to the results of DPPH and metal chelation assays (Figs. 3b and 4). It is possible that the higher amount of phytochemicals present in loading eluted syrup and acidified water fractions may have contributed to the enhanced antimicrobial activities of loading eluted syrup and acidified water fractions. For example, loading eluted syrup and acidified water fractions contain high concentrations of gallic acid and hydroxytyrosol (Fig. 2b) that are known to have significant antimicrobial activity (Badhani, Sharma, & Kakkar, 2015; Soni, Burdock, Christian, Bitler, & Crea, 2006; Tafesh et al., 2011).

To further investigate the compositional effect of the phenolic compounds on the antimicrobial activities, water extract and acidified 35 mL/100 mL methanol were selected because these two extracts showed similar phenolic profiles as illustrated in Fig. 2b. Fig. 5 shows a change in populations of *E. coli* O157:H7 and *L. innocua* cells incubated with different concentrations (0.5, 1.0, and 2.0 mg GAE/mL) of water extract and acidified 35 mL/100 mL methanol fraction at room temperature for up to 60 min. Populations of both *E. coli* O157 and *L. innocua* cells showed > 5-log reduction in 0.5 mg GAE/mL of water extract within 30 min, whereas it required four times higher concentrations of acidified 35 mL/100 mL methanol fraction (2.0 mg GAE/mL) to achieve an equivalent level of bacterial inactivation. Such results might indicate that even little compositional differences in phenolic compounds (e.g. hydroxytyrosol-glucoside, rutin, and tyrosol) and/or their interactions may have resulted in significantly different antimicrobial activities.

In addition, although the results show polar fractions such as loading eluted syrup and acidified water performed higher antioxidant and antimicrobial activities when they were compared at the same GAE level, those fractions generally have lower TPC because the high-affinity interaction between phenolics and C18 gel when the methanol gradient was low. Because these extracts showed similar phenolic profiles based

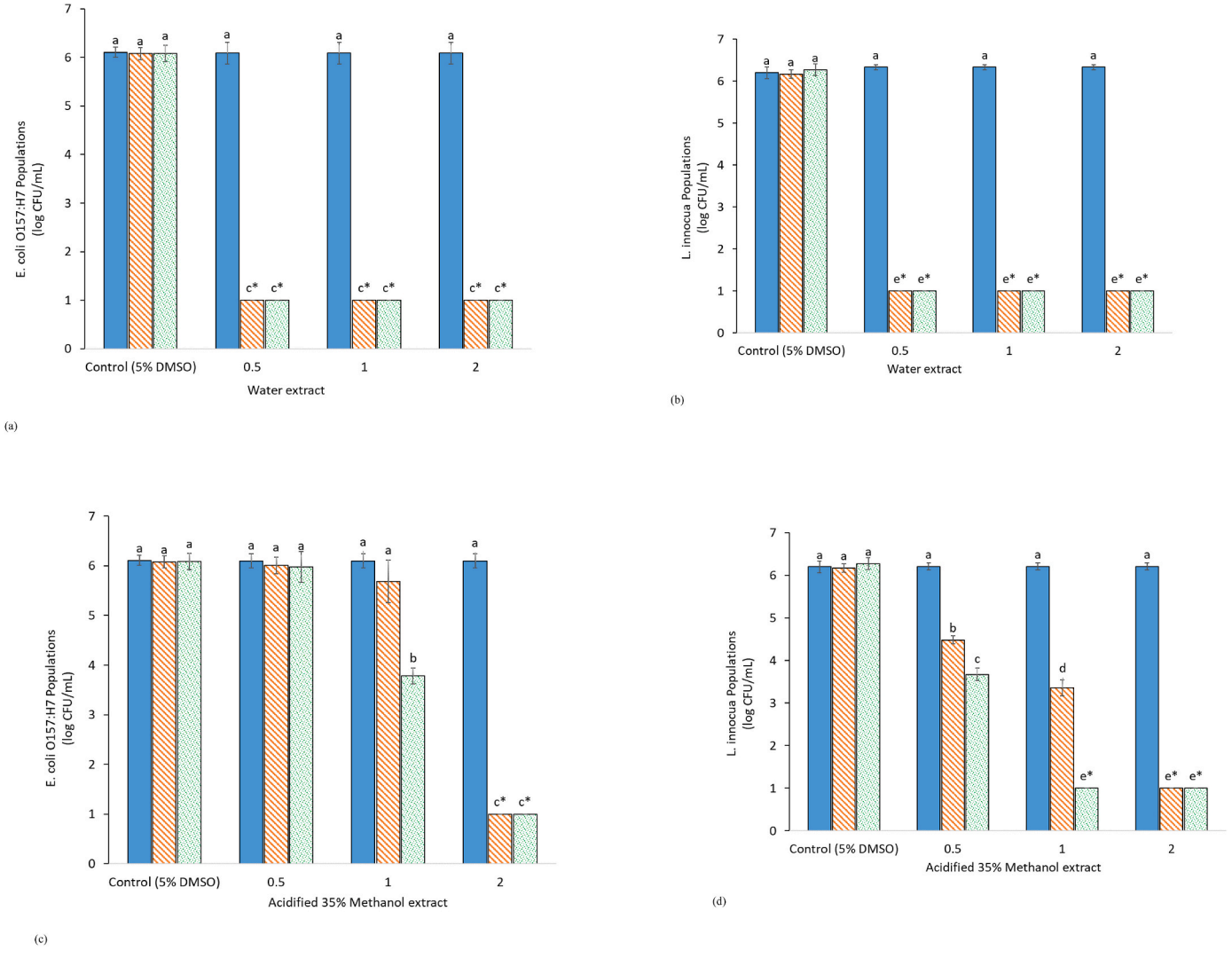

Fig. 5. Antimicrobial activities of water extract and 35 mL/100 mL methanol fraction against *E. coli* O157:H7 and *L. innocua*. Populations of (a) *E. coli* O157:H7 and (b) *L. innocua* incubated with 0.5, 1.0, and 2.0 mg GAE/mL of water extract at room temperature for ■ 0 min, ▨ 30 min and ▨ 60 min. Populations of (c) *E. coli* O157:H7 and (d) *L. innocua* incubated with 0.5, 1.0, and 2.0 mg GAE/mL of 35 mL/100 mL methanol fraction at room temperature for up to 60 min. The theoretical detection limit for direct plating was 1.0 log CFU/mL, and the asterisks* indicate that the number of viable bacteria was less than the detection limit, $p < 0.05$. Significant differences were compared among eighter *E. coli* O157:H7 (a,c) or *L. innocua* (b,d).

on the selected standards, differences in other phenolic compounds or non-phenolic components and their interactions may have contributed to the observed differences in antibacterial activities. The result of higher antimicrobial activity of lower TPC fraction could also indicate that non-phenolic compounds with antimicrobial activity may occur in those fractions. Therefore, further characterizations of other phenolic compounds, non-phenolic components and their possible synergetic interactions are needed to understand their roles in the antimicrobial activities of olive pomace.

4. Conclusions

Water extracts of olive pomace were successfully isolated to fractions containing different profiles following the increasing methanol in the methanol-water gradients by preparative C18 chromatography. The preparative operation parameters of C18 would be directedly used to scale-up the separation process; however, further studies for parameter optimizations such as the methanol gradients and volume of eluent for reducing time and solvent consumption will be needed.

Our results showed that loading eluted syrup and acidified water fractions had similar phenolic profiles with polar phenolic compounds such as hydroxytyrosol and tyrosol-glucoside. Acidified 35 mL/100 mL methanol fraction had more hydroxytyrosol and tyrosol-glucoside than loading eluted syrup and acidified water fractions; and acidified 35 mL/100 mL methanol fraction contained hydroxytyrosol-glucoside, 4-hydroxyphenylacetic acid (4-HPA), verbascoside and oleuropein. Acidified 70 mL/100 mL methanol fraction contained hydroxytyrosol, tyrosol-glucoside, and tyrosol, but generally had more less-polar compounds such as verbascoside, rutin, and oleuropein than the other fractions.

The *in vitro* antioxidant activities of water fractions were higher than that of methanolic fractions when compared at the same level of gallic acid equivalents (GAE). Loading eluted syrup showed the lowest MIC values against both *E. coli* O157:H7 (0.25 mg GAE/mL) and *L. innocua* (0.25 mg GAE/mL) which suggested higher antioxidant and antimicrobial activities, followed by acidified water fraction (0.5 mg GAE/mL), acidified 70 mL/100 mL methanol fraction (1.7 mg GAE/mL), and the acidified 35 mL/100 mL methanol fraction (2.1 mg GAE/mL). Similar trends were observed with MLC values. However, further characterizations of other phenolic compounds, non-phenolic components and their possible synergetic interactions are needed to understand their role in antimicrobial activities.

To the best of our knowledge, this is the first report on the phenolics profile, antioxidant, and antimicrobial activities of preparative C18 fractions of olive pomace from California which provides practical knowledge and references data for the valorization and industrial food applications of the olive oil byproducts.

CRediT authorship contribution statement

Hefei Zhao: Investigation, Methodology, Data curation, Formal analysis, Software, Writing – original draft. **Yoonbin Kim:** Investigation, Methodology, Data curation, Writing – original draft. **Roberto J. Avena-Bustillos:** Investigation, Methodology, Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Nitin Nitin:** Investigation, Methodology, Conceptualization, Supervision, Writing – review & editing, Funding acquisition. **Selina C. Wang:** Investigation, Methodology, Conceptualization, Supervision, Writing – review & editing, Funding acquisition.

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. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2023.114677.

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