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Liquid chromatography coupled with time-of-flight tandem mass spectrometry for comprehensive phenolic characterization of pomegranate fruit and flower extracts used as ingredients in botanical dietary supplements

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Abstract

Pomegranate (*Punica granatum* L.) fruit and flower extracts, which are rich sources of bioactive phenolics, are widely utilized as ingredients in botanical dietary supplements. While the phenolic characterization of extracts of pomegranate fruit have been previously studied by LC-MS/MS, there is lack of similar data for pomegranate flowers. Herein, LC-TOF-MS/MS was utilized to comprehensively characterize the phenolics present in two pomegranate extracts, previously studied for their *in vitro* and *in vivo* biological effects, namely, a patented commercial pomegranate fruit extract (Pomella®), and a pomegranate flower extract. Seventy-one phenolics were characterized in the pomegranate fruit extract with the vast majority identified in the flower extract. However, only the pomegranate fruit extract contained tannin-glucuronides and two punicalagin isomers (a characteristic pomegranate phenolic) were identified in this extract while four were identified in the flower extract. The previously reported compounds, pomellatannin and punicatannins A/B, were identified as unique chemical markers in the pomegranate fruit and flower extracts, respectively. This study will aid in the quality control, authentication, and standardization of these botanical ingredients to evaluate their potential health benefits in future planned pre-clinical and clinical studies. Also, this is the first phenolic characterization of a pomegranate flower extract using LC-MS/MS.

Keywords

Phenolics; Pomegranate; *Punica granatum*; Tandem mass spectrometry

1 INTRODUCTION

Botanical dietary supplements are widely consumed worldwide for a variety of potential human health benefits and biological effects. However, the industry is commonly plagued by

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflicts of interest.

lack of comprehensive characterization of botanical extract ingredients which is critical to ensure authentication of study materials used in pre-clinical and clinical studies designed to evaluate their potential health benefits [1]. In addition, the identification of potential unique chemical markers present in various botanical extracts would aid in ensuring product integrity and prevent economic adulteration and counterfeiting of study materials. This has led to the utilization of analytical techniques, including, HPLC and LC-MS/MS analyses, for the characterization of botanical ingredients [1–5].

The pomegranate (*Punica granatum* L.) fruit, a rich source of bioactive phenolics, especially ellagitannins, is highly regarded for its cardiovascular, antidiabetic, antimicrobial, antiviral, anticancer, anti-inflammatory, and neuroprotective properties [6–13]. Pomegranate fruits are not only widely consumed as a food, as its edible arils and squeezed fruit juice, but also as extracts which are widely used as ingredients in oral botanical dietary supplements [7]. However, apart from its fruits, other parts of the pomegranate plant, including its flowers, which have been used as a traditional medicine for treating various ailments such as diabetes [14], are also being investigated for botanical ingredient applications. Whilst the phenolic constituents of various parts of the pomegranate fruit have been previously characterized by LC-MS/MS [15–21], there is lack of similar data on pomegranate flower extracts. Moreover, there is limited data available on the utilization of LC-MS/MS methods for the comprehensive phenolic characterization of commercial pomegranate fruit extracts used as ingredients in botanical dietary supplements. This is unfortunate considering that the economic adulteration of commercial pomegranate extracts with lower-cost exogenous ingredients has been noted as a significant problem in the botanical dietary supplement industry [7]. This has led to the recent issuance of a pomegranate laboratory guidance document for the Botanical Adulterants Program by the joint non-profit organizations, the American Botanical Council, the American Herbal Pharmacopoeia, and the University of Mississippi's National Center for Natural Product Research [22].

A variety of pomegranate extracts are available in the botanical dietary supplement markets, some containing high concentrations (> 90%) of ellagic acid, indicating potential adulteration to enhance the perceived value of the product [22]. This is because ellagic acid, formed from the hydrolysis of ellagitannins, can be obtained from various plant sources, or by chemical synthesis, and is not a unique chemical marker of the pomegranate fruit although methods for its purification from pomegranate peel has been reported [23]. Therefore, beyond ellagic acid alone, pomegranate whole fruit extracts should be standardized to characteristic pomegranate phenolics, such as the ellagitannin isomers, punicalagins A/B, the absence of which may indicate potential adulteration [22]. The use of analytical methods, such as LC-MS/MS, to comprehensively characterize the phenolics in pomegranate extracts is an additional and more definitive measure to ensure the integrity of study materials especially when they are consumed as botanical ingredients in oral dietary supplements for human health benefits.

Pomella® is a patented commercial pomegranate fruit extract that has been studied by our group and others for a wide range of biological effects using *in vitro* models as well as in animals and human subjects [13, 24–39]. While we have previously reported on the isolation and structure elucidation (by NMR) of twenty-one phenolic compounds, including a new

ellagitannin, named pomellatannin, from this pomegranate fruit extract [13], it has not yet been comprehensively characterized by LC-MS/MS. Furthermore, while we have previously reported on the isolation and structure elucidation (by NMR) of fifteen phenolic compounds, including a pair of new ellagitannin isomers, punicatannin A/B, from a pomegranate flower extract [40, 41], it has also not yet been characterized by LC-MS/MS. Therefore, the objectives of this study were to conduct comprehensive phenolic characterization of these two aforementioned pomegranate extracts, using LC-MS/MS, and identify potential unique chemical marker compounds present within them that can be used for their quality control, authentication, and standardization. This is the first reported phenolic characterization of a pomegranate flower extract and of Pomella® using LC-MS/MS.

2 MATERIALS AND METHODS

2.1 Chemicals

Pomegranate (*Punica granatum* L.) flowers were collected and authenticated by matching their macroscopic and microscopic characteristics to internal monograph and botanical reference standards at Verdure Sciences (Noblesville, IN, USA) as previously reported [40]. The whole pomegranate fruit extract (Pomella®) was provided by Verdure Sciences (Noblesville, IN, USA) and was of the same lot number as the pomegranate fruit extract previously reported by our group to show neuroprotective effects against Alzheimer's disease in *Caenorhabditis elegans* and in an aged transgenic Alzheimer's disease animal model [13, 24]. LC-MS grades of acetonitrile and formic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2 Sample preparation

Finely powdered pomegranate flower (1.0 g) was extracted by sonication with methanol (10 mL) for 1 hour under room temperature. Pomella® (100 mg) was dissolved in methanol (10 mL). The solution was stored at 4 °C and filtered through a 0.22 µm filter before analyses.

2.3 Liquid Chromatography Mass Spectrometry Conditions

LC-TOF-MS/MS analyses were performed on a SHIMADZU Prominence UFLC system (Marlborough, MA, USA) consisting of two LC-20AD pumps, a DGU-20A degassing unit, SIL-20AC auto sampler, CTO-20AC column oven and CBM-20A communication bus module. Chromatographic separation was performed on a 150 mm × 4.6 mm i.d., 5 µm, Prodigy ODS column (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid/acetonitrile (A) and 0.1% aqueous formic acid (B) with a gradient elution of 5% A from 0 to 5 min, 5–12% A from 5 to 12 min, 12–17% A from 12 to 27 min, and 17–35% A from 27 to 45 min. The column temperature was 40 °C, flow rate was 0.50 mL/min, and injection volume was 4.00 µL. Mass spectrometry was performed using a Triple time-of-flight (TOF) 4600 system from Applied Biosystems/MDS Sciex (Framingham, MA, USA) coupled with an electrospray ionization (ESI) interface. Nitrogen was used in all cases. In this study, the parameters were optimized as follows: ESI voltage, –4500 V; nebulizer gas, 40 psi; auxiliary gas, 50 psi; curtain gas, 25 psi; turbo gas temperature, 450 °C; declustering potential, –80V; collision energy, 50 eV. Instrument calibration was carried

out according to the manufacturer's instructions. The mass range was set from m/z 100 to 1150. The data were acquired and processed using Analyst TF 1.7 Software.

3 RESULTS AND DISCUSSION

Accurate mass data were acquired in the full scan analysis, and product ion mass data were acquired via the information-dependent acquisition method. A Prodigy ODS column was employed to provide increased chromatographic resolution and the mobile phase contained formic acid (0.1%) which was added to alleviate peak tailing and produce better shapes. The acidic conditions did not appear to significantly affect the ionization efficiency of compounds in negative mode. A total of seventy-one phenolic compounds, including sixty four tannins, were tentatively characterized. The total ion chromatograms profiles are shown in Figure 1. Accurate mass measurements, retention time, formula, errors and main MS/MS product ions for all phenolic compounds are summarized in Table 1. All of the compounds were observed by their $[M - H]^-$ ions in negative ESI mode, which was helpful for their elemental composition by accurate mass measurement (Table 1). For ease of discussion, the phenolics are characterized into different sub-categories as described below.

3.1 Characterization of galloyl hexosides

Compounds **2** (t_R = 3.92 min), **5** (t_R = 4.99 min), **6** (t_R = 5.47 min), **7** (t_R = 6.17 min), **11** (t_R = 7.20 min) and **18** (t_R = 10.93 min) all gave the same $[M - H]^-$ ions at m/z 331, with molecular formula $C_{13}H_{16}O_{10}$ provided by TOF-MS. Their MS/MS fragmentation ions at m/z 125 (typical fragment ion for gallic acid due to loss of CO_2) and m/z 169 $[M - H - 162]^-$ indicated the existence of a galloyl moiety and the loss of a hexose moiety, respectively. Thus, compounds **2**, **5**, **6**, **7**, **11** and **18** were tentatively identified as galloyl-hexosides.

Compounds **26** (t_R = 15.02 min), **27** (t_R = 15.34 min), **31** (t_R = 16.15 min), **32** (t_R = 16.44 min), **35** (t_R = 17.06 min), **37** (t_R = 17.53 min) and **38** (t_R = 17.79 min) were tentatively identified as digalloyl-hexosides based on their $[M - H]^-$ ions at m/z 483 and MS/MS fragment ions at m/z 331 $[M - H - 152]^-$ due to the loss of a galloyl group, and m/z 169 $[M - H - 152 - 162]^-$ due to the loss of a hexose moiety. The typical MS/MS fragmentation ions of gallic acid at m/z 169 and m/z 125 were also observed.

Compounds **39**, **46**, **49**, **51**, **54**, **56**, and **59**, which eluted at retention times (t_R) of 18.02, 19.92, 21.01, 22.20, 22.94, 23.81 and 25.62 min, respectively, were tentatively identified as isomers of trigalloyl-hexosides. These compounds all had identical m/z 635 $[M - H]^-$ ions with MS/MS product ions at m/z 483 $[M - H - 152]^-$ (loss of galloyl moiety), m/z 465 $[M - H - 170]^-$ (loss of gallic acid), and m/z 313 $[M - H - 170 - 152]^-$ (sequential losses of a gallic acid and a galloyl moiety). They also exhibited typical galloyl moiety fragment ions at m/z 125 and m/z 169.

Compounds **62** (t_R = 29.52 min), **65** (t_R = 30.89 min) and **68** (t_R = 32.23 min) all had the same $[M - H]^-$ ions at m/z 787. Based on the MS/MS daughter ions formed by sequential losses of gallic acid/galloyl moieties at m/z 617 $[M - H - 170]^-$, m/z 465 $[M - H - 170 - 152]^-$ and m/z 313 $[M - H - 170 - 152 - 152]^-$, as well as the typical daughter ions at m/z

169 and m/z 125, compounds **62**, **65**, and **68** were tentatively identified as isomers of tetragalloyl-hexoside. Compound **70** (t_R = 35.61 min) displayed $[M - H]^-$ ions at m/z 939 and was tentatively identified as a pentagalloyl-hexoside based on the MS/MS daughter ions formed by sequential losses of gallic acid/galloyl moieties at m/z 787 $[M - H - 152]^-$, m/z 635 $[M - H - 152 - 152]^-$, m/z 465 $[M - H - 152 - 152 - 170]^-$ and m/z 313 $[M - H - 152 - 152 - 170 - 152]^-$, as well as the typical daughter ions at m/z 169 and m/z 125 indicating the existence of galloyl moiety.

3.2 Characterization of hexahydroxydiphenoyl (HHDP) derivative tannins

Compounds **1** (t_R = 3.80 min), **3** (t_R = 4.40 min) and **4** (t_R = 4.88 min) all had the same $[M - H]^-$ ions at m/z 481, with a molecular formula of $C_{20}H_{18}O_{14}$ provided by TOF-MS. Their MS/MS fragmentation ion at m/z 301 $[M - H - 180]^-$ suggested the loss of a hexose moiety. The daughter ions at m/z 275, 257 and 229 were also observed, suggesting the formation of ellagic acid and supporting the existence of a hexahydroxydiphenoyl (HHDP) moiety. Thus, compounds **1**, **3**, and **4** were tentatively identified as HHDP-hexosides. Compounds **15** (t_R = 9.60 min), **19** (t_R = 11.86 min), and **30** (t_R = 15.83 min) had the same $[M - H]^-$ ion at m/z 783. The product ions at m/z 481 $[M - H - 302]^-$ and m/z 301 $[M - H - 302 - 180]^-$ indicated the loss of a HHDP group and a hexose. Diagnostic daughter ions at m/z 301, 275, 257, and 229 suggested the formation of ellagic acid and confirmed the presence of the HHDP moiety. Therefore, compounds **15**, **19**, and **30** were tentatively identified as isomers of bis-HHDP-hexoside.

Compounds **9**, **14**, **22**, **25**, **29**, **33**, **40**, **44**, and **50**, which eluted at t_R 6.82, 9.10, 13.80, 14.94, 15.47, 16.57, 18.34, 19.27 and 21.49 min, respectively, were identified as galloyl-HHDP-hexosides due to their deprotonated ions $[M - H]^-$ ion at m/z 633 with MS/MS fragment ions of m/z 481 (loss of a galloyl moiety $[M - H - 152]^-$, HHDP-hexoside), m/z 463 (loss of a gallic acid $[M - H - 170]^-$), and m/z 301 (loss of galloyl hexose $[M - H - 152 - 180]^-$). Diagnostic daughter ions at m/z 301, 275, 257 and 229 suggested the formation of ellagic acid and the existence of the HHDP moiety. Compounds **36** (t_R = 17.15 min), **47** (t_R = 20.69 min), **52** (t_R = 22.52 min), **58** (t_R = 24.73 min) and **64** (t_R = 30.68 min), had the same $[M - H]^-$ ion at m/z 785 and were identified as digalloyl-HHDP-hexosides. In the MS/MS spectra, diagnostic daughter ions at m/z 301, 275, 257, and 229 suggested the formation of ellagic acid and the existence of the HHDP moiety. The fragment ion at m/z 483 ($[M - H - 302]^-$, loss of 302 Da) was due to the loss of the HHDP moiety. Fragment ions at m/z 615 ($[M - H - 170]^-$, loss of a gallic acid) and m/z 463 ($[M - H - 170 - 152]^-$, loss of a gallic acid and a galloyl moiety), as well as the diagnostic daughter ions at m/z 169 and 125, indicated the existence of two galloyl moieties. The fragment ion at m/z 331 $[M - H - 302 - 152]^-$ was due to the loss of HHDP and a galloyl moiety, and the fragment ion at m/z 313 $[M - H - 302 - 170]^-$ was due to the loss of HHDP and gallic acid. Compound **69** (t_R = 33.97 min), with an $[M - H]^-$ ion at m/z 937, was identified as trigalloyl-HHDP-hexoside. Its typical fragment ions at m/z 785 $[M - H - 152]^-$, m/z 635 $[M - H - 302]^-$, and m/z 483 $[M - H - 152 - 302]^-$, represented digalloyl-HHDP-hexoside, trigalloyl-hexoside and digalloyl-hexoside, respectively. It also gave fragment ions at m/z 767 $[M - H - \text{gallic acid}]^-$, m/z 597 $[M - H - \text{gallic acid} - \text{gallic acid}]^-$, m/z 445 $[M - H - \text{gallic acid} - \text{gallic acid} - \text{galloyl}]^-$. Compound **43** (t_R = 19.04 min) had a $[M - H]^-$ ion at m/z 935 and was

tentatively identified as galloyl-bis-HHDP-hexoside. It gave typical MS/MS fragment ions at m/z 783 ($[M - H - 152]^-$), m/z 633 ($[M - H - 302]^-$) and m/z 481 ($[M - H - 152 - 302]^-$) suggesting bis-HHDP-hexoside, galloyl-HHDP-hexoside, and HHDP-hexoside, respectively. It also gave diagnostic MS/MS fragment ions at m/z 301, 257, 169 and 125, representing HHDP and galloyl moieties.

Compounds **8** ($t_R = 6.36$ min) and **16** ($t_R = 9.83$ min) had the same $[M - H]^-$ ions at m/z 649. The product ions at m/z 497 ($[M - H - 152]^-$) were due to the loss of a galloyl moiety. Diagnostic daughter ions at m/z 301, 275 and 229 suggested the formation of ellagic acid and the existence of HHDP. The product ions at m/z 301 ($[M - H - 152 - 196]^-$) indicated the loss of a galloyl moiety and a glucuronic acid. Thus, compounds **8** and **16** were tentatively identified as galloyl-HHDP-glucuronides. Compound **42** ($t_R = 18.69$ min) was identified as digalloyl-HHDP glucuronide due to their deprotonated $[M - H]^-$ ion at m/z 801 with MS/MS fragment ions of m/z 499 (loss of HHDP $[M - H - 302]^-$), m/z 649 (loss of a galloyl moiety $[M - H - 152]^-$), m/z 347 (loss of a galloyl moiety and HHDP $[M - H - 152 - 302]^-$), and m/z 301 (loss of digalloyl glucuronic acid $[M - H - 152 - 152 - 196]^-$).

Compounds **13**, **17**, **24**, and **34**, which eluted at t_R 9.09, 10.31, 14.39 and 16.79 min, respectively, exhibited $[M - H]^-$ ions at m/z 1083. Gil *et al.* [42] reported a quasimolecular ion at m/z 1083 $[M - H]^-$ in accordance with punicalagin (glucose + gallagyl + hexahydroxydiphenyl), a characteristic ellagitannin found in pomegranate. Punicalagin is known to form fragments at m/z 301 (residue of ellagic acid), m/z 601 (residue of gallagic moiety) and m/z 781 (loss of ellagic acid). Compounds **13**, **17**, **24** and **34** all gave typical MS/MS fragment ions at m/z 301, m/z 601 and m/z 781, and thus, they were tentatively identified as HHDP-gallagyl-hexosides (punicalagin isomers) which is consistent with a previous report of four punicalagin isomers being detected in pomegranate [16].

Compound **12** ($t_R = 7.74$ min) had a $[M - H]^-$ ion at m/z 781 and the diagnostic fragment ion at m/z 601 indicated the loss of a gallagyl moiety. Fragment ions at m/z 300 and m/z 299 were observed, due to the quinoidic and radical products of ellagic acid after the fission of the gallagic acid moiety [18]. The fragment ion at m/z 601 ($[M - H - 180]^-$) indicated the loss of a hexose moiety. Therefore, compound **12** was tentatively identified as gallagyl-hexoside. Compound **20** ($t_R = 12.08$ min) had a $[M - H]^-$ ion at m/z 933 with a daughter ion of m/z 781 equivalent to the $[M - H]^-$ ion of compound **12**. The release of this ion was caused by the loss of a galloyl moiety. The typical fragment ions at m/z 601, m/z 300 and m/z 299 of compound **12** were all observed for compound **20**. Thus, compound **20** was tentatively identified as galloyl-gallagyl-hexoside.

Compounds **28** ($t_R = 15.39$ min) and **60** ($t_R = 27.68$ min) had the identical $[M - H]^-$ ions at m/z 951. Compound **28** showed fragment ions at m/z 907 ($[M - H - CO_2]^-$) and m/z 783 ($[M - H - CO_2 - 124]^-$), indicating the existence of a valoneoyl group [43]. Further fragment ions at m/z 605 ($[M - H - CO_2 - HHDP]^-$), m/z 481 (HHDP-hexoside) and m/z 301 suggested that compound **28** was HHDP-valoneoyl glucoside. Compound **60** gave dehydrated fragment ions at m/z 933, m/z 915 and m/z 897, and showed additional loss of ellagic acid fragment ions at m/z 631 and m/z 613. Therefore, based on these fragmentation

patterns and by comparison to literature [18], compound **60** was tentatively identified as galloyl-HHDP-DHHDP (dehydrohexahydroxydiphenoyl)-hexoside.

Compound **48** ($t_R = 21.00$ min) was tentatively identified as pomellatannin, a new tannin previously isolated and identified (by NMR) from *Pomella*® [13], due to its quasimolecular ion at m/z 839 and MS/MS fragment ions. Figure 2 shows the proposed fragmentation pathways of pomellatannin. In its MS/MS spectra, fragment ions at m/z 537, 519, 479, 461, 435, 301, 257 and 229 were observed. The diagnostic product ions at m/z 301, 257 and 229 confirmed the existence of the HHDP group. The MS/MS ions at m/z 537, 479 and 435 were produced by the subsequent losses of a HHDP group (302 Da), an acetone (58 Da), and carbon dioxide (44 Da) from the precursor ion at m/z 839. The fragment ions at m/z 519 and 461 were dehydrated products.

Compound **71** ($t_R = 43.33$ min) was tentatively identified as punicatannin A/B, two new compounds previously isolated and identified (by NMR) from pomegranate flowers [40]. These are two stereoisomers which cannot be distinguished by mass spectrometry and therefore, compound **71** was identified as punicatannin A/B due to its quasimolecular ion at m/z 997 and the MS/MS fragment ions. Figure 3 shows the proposed fragmentation pathways of punicatannin A/B. In its MS/MS spectra, fragment ions at m/z 845, 827, 695, 615, 525, 463, 301, 257 and 229 can be observed. The diagnostic product ions at m/z 301, 257 and 229 clearly supported the existence of the HHDP group. The MS/MS ions at m/z 695 and 525 were produced by the subsequent losses of a HHDP group (302 Da), and a gallic acid (170 Da) from the precursor ion at m/z 997. The MS/MS ions at m/z 615 and 463 were produced by the subsequent losses of a benzofuran carboxylic acid moiety (382 Da), and a galloyl moiety (152 Da) from the precursor ion at m/z 997. The MS/MS ions at m/z 845, 827 and 381 were produced by loss of a galloyl, gallic acid and galloyl-HHDP-hexosyl moiety, respectively, from the precursor ion at m/z 997.

3.3 Characterization of other ellagic acid derivatives

Compounds **55** ($t_R = 23.14$ min), and **57** ($t_R = 24.25$ min) all had the same $[M - H]^-$ ions at m/z 463, with molecular formula of $C_{20}H_{16}O_{13}$ provided by TOF-MS. Their MS/MS fragmentation ions at m/z 301, 300, 257, and 229 indicated the existence of an ellagic acid moiety and the loss of a hexosyl moiety. Thus, compounds **55** and **57** were tentatively identified as ellagic acid-hexosides. Compounds **61** ($t_R = 29.16$ min), and **63** ($t_R = 30.45$ min) had the same $[M - H]^-$ ions at m/z 433, with a molecular formula of $C_{19}H_{14}O_{12}$ provided by TOF-MS. The MS/MS fragmentation ions at m/z 301, 300, 257 and 229 indicated the existence of ellagic acid moiety and the loss of a pentosyl moiety. Thus, compounds **61** and **63** were tentatively identified as ellagic acid-pentosides. Compound **66** ($t_R = 31.05$ min) had a $[M - H]^-$ ion at m/z 447, with a molecular formula of $C_{20}H_{16}O_{12}$ provided by TOF-MS. The MS/MS fragmentation ions at m/z 301, 300, 257, and 229 indicated the existence of an ellagic acid moiety and the loss of deoxyhexosyl moiety. Thus, compound **66** was tentatively identified as ellagic acid-deoxyhexoside.

3.4 Characterization of other phenolic compounds

Compound **10** ($t_R = 7.15$ min) exhibited an $[M - H]^-$ ion at m/z 169.0155, with a molecular formula of $C_7H_6O_5$ provided by TOF-MS. In the MS/MS spectra, it gave a characteristic product ion at m/z 125 due to the loss of CO_2 (44 Da). Thus, compound **10** was tentatively identified as gallic acid. Compound **41** ($t_R = 18.57$ min) was tentatively identified as methyl-gallic acid due to the $[M - H]^-$ ion at m/z 183 and fragment ions at m/z 168 $[M - H - \bullet CH_3]^-$ [44], m/z 140 $[M - H - \bullet CH_3 - CO]^-$ and m/z 124 $[M - H - \bullet CH_3 - CO_2]^-$. Compound **67** ($t_R = 31.82$ min) had a $[M - H]^-$ ion at m/z 300.9986, with a molecular formula of $C_{14}H_6O_8$ provided by TOF-MS. In its MS/MS spectrum, the characteristic fragment ions at m/z 257, 229, 201 and 185 were observed, which is coincident with ellagic acid [18]. Therefore, compound **67** was identified as ellagic acid.

Compound **45** ($t_R = 19.43$ min) had a $[M - H]^-$ ion at m/z 291.0152, with a molecular formula of $C_{13}H_8O_8$ provided by TOF-MS. It exhibited a decarboxylation fragment ion at m/z 247 $[M - H - 44]^-$. According to the literature, it was tentatively identified as brevifolin carboxylic acid [14]. Compounds **23** ($t_R = 14.25$ min) and **53** ($t_R = 22.68$ min) had the same $[M - H]^-$ ions at m/z 469. By comparison to the literature [19], these two compounds were tentatively identified as isomers of valoneic acid bilactone. Their fragment ion at m/z 425 and m/z 407 were produced due to sequential decarboxylation $[M - H - 44]^-$ and dehydration of valoneic acid bilactone.

Compound **21** ($t_R = 12.21$ min) had a $[M - H]^-$ ion at m/z 305 and was tentatively identified as gallo catechin/epigallocatechin by comparing the typical fragment ions at m/z 261, 221, 179, 167, 165, 137, 125 and 109 with the reported of diastereoisomers of gallo catechin (gallo catechin and epigallocatechin) [45].

3.5 LC-TOF-MS/MS comparison of pomegranate flower and fruit extracts

As shown in Table 1, LC-TOF-MS/MS analyses revealed that the pomegranate flower extract was very similar to the pomegranate fruit extract and contained the vast majority of the identified compounds except for compounds **8**, **12**, **13**, **15**, **16**, **17**, **20**, **21**, **28**, **42**, **43**, **48**, **58**, and **66**. Notably, the pomegranate fruit extract contained tannin-glucuronides which were not detected in the pomegranate flower extract. In addition, the pomegranate fruit extract contained only two punicalagin isomers while four punicalagin isomers were detected in the flower extract. Lastly, the previously reported new compounds, pomellatannin [13] and punicatannins A/B [40], were identified as unique chemical markers in the pomegranate fruit and flower extracts, respectively. However, further studies using a larger number of samples of these respective extracts, produced under different conditions, to confirm the presence/absence of these aforementioned compounds, are warranted.

4 CONCLUDING REMARKS

In summary, a reliable method employing LC-TOF-MS/MS was developed for the unequivocal identification of phenolic compounds in a commercial pomegranate fruit extract (Pomella®) and a pomegranate flower extract. The data accumulated from the current study will aid in the quality control, authentication, and standardization of these botanical

ingredients to evaluate their potential health benefits in planned pre-clinical and clinical studies which will be pursued in the future. In addition, the phenolic characterization, using the LC-TOF-MS/MS methods developed herein, of various commercial pomegranate botanical dietary supplements, obtained from different manufacturers, will be pursued by our group in the future.

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Non-standard abbreviations:

HHDP	hexahydroxydiphenoyl
DHHDP	dehydrohexahydroxydiphenoyl

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Figure 1.
Total ion chromatograms of pomegranate flower (A) and fruit (B) extracts.

Figure 2.
The proposed fragmentation pathways of pomellatannin.

Figure 3.
The proposed fragmentation pathways of punicatannin A/B.

Table 1.

Characterization of phenolic compounds in pomegranate flower and fruit extracts by LC-TOF-MS/MS.

No.	t _R (min)	Molecular formula	HR-ESI(-)-MS [M-H] ⁻ m/z exptl. (theor.)	Error (ppm)	Major MS/MS ions	Identification	Presence	
							Flower	Fruit
1	3.80	C ₂₀ H ₁₈ O ₁₄	481.0631 (481.0623)	1.4983	301, 275, 257, 229	HHDP-hexoside	+	+
2	3.92	C ₁₃ H ₁₆ O ₁₀	331.0678 (331.0670)	2.2032	271, 211, 169, 125	galloyl-hexoside	+	+
3	4.40	C ₂₀ H ₁₈ O ₁₄	481.0627 (481.0623)	0.6668	301, 275, 257, 229	HHDP-hexoside	+	+
4	4.88	C ₂₀ H ₁₈ O ₁₄	481.0624 (481.0623)	0.0431	301, 275, 257, 229	HHDP-hexoside	+	+
5	4.99	C ₁₃ H ₁₆ O ₁₀	331.0680 (331.0670)	2.8073	271, 211, 169, 125	galloyl-hexoside	+	+
6	5.47	C ₁₃ H ₁₆ O ₁₀	331.0675 (331.0670)	1.2970	271, 211, 169, 125	galloyl-hexoside	+	+
7	6.17	C ₁₃ H ₁₆ O ₁₀	331.0678 (331.0670)	2.2032	271, 211, 169, 125	galloyl-hexoside	+	+
8	6.36	C ₂₇ H ₂₂ O ₁₉	649.0684 (649.0682)	0.2271	497, 301, 275, 229, 195	galloyl-HHDP-glucuronide	-	+
9	6.82	C ₂₇ H ₂₂ O ₁₈	633.0732 (633.0733)	-0.2178	481, 463, 421, 301, 275, 257, 249, 229	galloyl-HHDP-hexoside	+	+
10	7.15	C ₇ H ₆ O ₅	169.0155 (169.0142)	7.4136	125	gallic acid	+	+
11	7.20	C ₁₃ H ₁₆ O ₁₀	331.0676 (331.0670)	1.5991	271, 211, 169, 125	galloyl-hexoside	+	+
12	7.74	C ₃₄ H ₂₂ O ₂₂	781.0524 (781.0529)	-0.7637	721, 601, 575, 449, 393, 301, 300, 299, 271, 245, 229	galloyl-hexoside	-	+
13	9.09	C ₄₈ H ₂₈ O ₃₀	1083.0579 (1083.0592)	-1.2592	1065, 807, 781, 763, 721, 601, 575, 549, 341, 301, 300, 299, 275, 273, 271	HHDP-gallagyl-hexoside [*]	-	+
14	9.10	C ₂₇ H ₂₂ O ₁₈	633.0737 (633.0733)	0.5719	481, 463, 301, 275, 257, 249, 229	galloyl-HHDP-hexoside	+	+
15	9.60	C ₃₄ H ₂₄ O ₂₂	783.0684 (783.0686)	-0.3148	481, 437, 419, 341, 329, 301, 299, 275, 257, 229	Bis-HHDP-hexoside	-	+
16	9.83	C ₂₇ H ₂₂ O ₁₉	649.0684 (649.0682)	0.2271	497, 301, 284, 275, 229, 195	galloyl-HHDP-glucuronide	-	+
17	10.31	C ₄₈ H ₂₈ O ₃₀	1083.0586 (1083.0592)	-0.6129	807, 781, 763, 721, 601, 575, 549, 341, 301, 300, 299, 275, 273, 271	HHDP-gallagyl-hexoside [*]	-	+
18	10.93	C ₁₃ H ₁₆ O ₁₀	331.0675 (331.0670)	1.2970	169, 125	galloyl-hexoside	+	+
19	11.86	C ₃₄ H ₂₄ O ₂₂	783.0680 (783.0686)	-0.8257	481, 301, 275, 257, 229	Bis-HHDP-hexoside	+	+
20	12.08	C ₄₁ H ₂₆ O ₂₆	933.0622 (933.0639)	-1.8811	781, 763, 721, 601, 575, 549, 449, 393, 301, 300, 299, 271	galloyl-gallagyl-hexoside	-	+
21	12.21	C ₁₅ H ₁₄ O ₇	305.0674 (305.0666)	2.7313	261, 221, 179, 167, 165, 137, 125, 109	galocatechin/epigallocatechin	-	+
22	13.80	C ₂₇ H ₂₂ O ₁₈	633.0737 (633.0733)	0.5719	481, 463, 421, 301, 275, 257, 249, 229	galloyl-HHDP-hexoside	+	+
23	14.25	C ₂₁ H ₁₀ O ₁₃	469.0056 (469.0048)	1.5687	425, 407, 379, 300, 279, 251, 195	valoneic acid bilactone	+	+
24	14.39	C ₄₈ H ₂₈ O ₃₀	1083.0581 (1083.0592)	-1.0746	781, 721, 601, 575, 549, 301, 300, 299, 275, 273, 271	HHDP-gallagyl-hexoside [*]	+	+

No.	t _R (min)	Molecular formula	HR-ESI(–)-MS [M–H] [–] m/z exptl. (theor.)	Error (ppm)	Major MS/MS ions	Identification	Presence	
							Flower	Fruit
25	14.94	C ₂₇ H ₂₂ O ₁₈	633.0737 (633.0733)	0.5719	481, 463, 421, 301, 275, 257, 249, 229	galloyl-HHDP-hexoside	+	+
26	15.02	C ₂₀ H ₂₀ O ₁₄	483.0785 (483.0780)	0.9743	423, 405, 331, 313, 271, 241, 211, 169, 125	digalloyl-hexoside	+	+
27	15.34	C ₂₀ H ₂₀ O ₁₄	483.0775 (483.0780)	–1.0956	423, 331, 313, 271, 211, 169, 125	digalloyl-hexoside	+	+
28	15.39	C ₄₁ H ₂₈ O ₂₇	951.0735 (951.0745)	–1.0724	907, 783, 605, 481, 425, 301, 275, 257, 249, 229	HHDP-valoneoyl-hexoside	–	+
29	15.47	C ₂₇ H ₂₂ O ₁₈	633.0736 (633.0733)	0.4139	481, 463, 421, 301, 275, 257, 249, 229	galloyl-HHDP-hexoside	+	+
30	15.83	C ₃₄ H ₂₄ O ₂₂	783.0676 (783.0686)	–1.3365	481, 301, 275, 257, 229	Bis-HHDP-hexoside	+	+
31	16.15	C ₂₀ H ₂₀ O ₁₄	483.0793 (483.0780)	2.6304	423, 331, 313, 271, 211, 169, 125	digalloyl-hexoside	+	+
32	16.44	C ₂₀ H ₂₀ O ₁₄	483.0785 (483.0780)	0.9743	423, 331, 313, 271, 241, 211, 193, 169, 125	digalloyl-hexoside	+	+
33	16.57	C ₂₇ H ₂₂ O ₁₈	633.0731 (633.0733)	–0.3758	481, 301, 275, 257, 249, 229	galloyl-HHDP-hexoside	+	+
34	16.79	C ₄₈ H ₂₈ O ₃₀	1083.0587 (1083.0592)	–0.5206	781, 721, 601, 575, 549, 301, 300, 299, 275, 273, 271	HHDP-gallagyl-hexoside [*]	+	+
35	17.06	C ₂₀ H ₂₀ O ₁₄	483.0785 (483.0780)	0.9743	331, 313, 271, 211, 169, 125	digalloyl-hexoside	+	+
36	17.15	C ₃₄ H ₂₆ O ₂₂	785.0836 (785.0842)	–0.8873	615, 483, 463, 419, 331, 313, 301, 275, 257, 249, 229, 169, 125	digalloyl-HHDP-hexoside	+	+
37	17.53	C ₂₀ H ₂₀ O ₁₄	483.0787 (483.0780)	1.3883	439, 331, 313, 287, 169, 125	digalloyl-hexoside	+	+
38	17.79	C ₂₀ H ₂₀ O ₁₄	483.0785 (483.0780)	0.9743	331, 313, 271, 211, 169, 125	digalloyl-hexoside	+	+
39	18.02	C ₂₇ H ₂₄ O ₁₈	635.0869 (635.0889)	–3.2877	483, 465, 423, 313, 295, 271, 235, 211, 193, 169, 125	trigalloyl-hexoside	+	+
40	18.34	C ₂₇ H ₂₂ O ₁₈	633.0737 (633.0733)	0.5719	481, 463, 421, 301, 275, 257, 249, 229	galloyl-HHDP-hexoside	+	+
41	18.57	C ₈ H ₈ O ₅	183.0313 (183.0298)	7.6650	168, 140, 124	methyl gallic acid	+	+
42	18.69	C ₃₄ H ₂₆ O ₂₃	801.0772 (801.0792)	–2.5107	649, 499, 347, 301, 275, 229, 195	digalloyl-HHDP-glucuronide	–	+
43	19.04	C ₄₁ H ₂₈ O ₂₆	935.0781 (935.0796)	–1.6098	917, 783, 633, 571, 481, 419, 329, 301, 275, 257, 249, 231, 169, 125	galloyl-bis-HHDP-hexoside	–	+
44	19.27	C ₂₇ H ₂₂ O ₁₈	633.0730 (633.0733)	–0.5338	481, 463, 421, 301, 275, 257, 249, 229	galloyl-HHDP-hexoside	+	+
45	19.43	C ₁₃ H ₈ O ₈	291.0152 (291.0146)	1.9209	247, 219, 191	Brevifolin carboxylic acid	+	+
46	19.92	C ₂₇ H ₂₄ O ₁₈	635.0886 (635.0889)	–0.6109	483, 465, 423, 331, 313, 295, 271, 211, 169, 125	trigalloyl-hexoside	+	+
47	20.69	C ₃₄ H ₂₆ O ₂₂	785.0839 (785.0842)	–0.5022	615, 483, 463, 419, 331, 313, 301, 275, 257, 249, 229, 169, 125	digalloyl-HHDP-hexoside	+	+
48	21.00	C ₃₇ H ₂₈ O ₂₃	839.0926 (839.0948)	–2.6950	537, 519, 479, 461, 435, 301, 273, 257, 229	pomellatannin	–	+
49	21.01	C ₂₇ H ₂₄ O ₁₈	635.0886 (635.0889)	–0.6109	483, 465, 313, 169, 125	trigalloyl-hexoside	+	+
50	21.49	C ₂₇ H ₂₂ O ₁₈	633.0737 (633.0733)	–0.5338	481, 463, 421, 301, 275, 257, 249, 229	galloyl-HHDP-hexoside	+	+
51	22.20	C ₂₇ H ₂₄ O ₁₈	635.0882 (635.0889)	–1.2408	483, 465, 313, 211, 169, 125	trigalloyl-hexoside	+	+
52	22.52	C ₃₄ H ₂₆ O ₂₂	785.0802 (785.0842)	–5.2181	615, 483, 463, 419, 331, 313, 301, 275, 257, 249, 229, 169, 125	digalloyl-HHDP-hexoside	+	+

No.	t _R (min)	Molecular formula	HR-ESI(-)-MS [M-H] ⁻ m/z exptl. (theor.)	Error (ppm)	Major MS/MS ions	Identification	Presence	
							Flower	Fruit
53	22.68	C ₂₁ H ₁₀ O ₁₃	469.0051 (469.0048)	0.5026	425, 407, 301, 300, 299, 271, 243	valoneic acid bilactone	+	+
54	22.94	C ₂₇ H ₂₄ O ₁₈	635.0881 (635.0889)	-1.3982	483, 465, 313, 295, 271, 211, 169, 125	trigalloyl-hexoside	+	+
55	23.14	C ₂₀ H ₁₆ O ₁₃	463.0514 (481.0518)	-0.4144	301, 300, 257, 229	ellagic acid-hexoside	+	+
56	23.81	C ₂₇ H ₂₄ O ₁₈	635.0893 (635.0889)	0.4912	483, 465, 423, 331, 313, 295, 271, 221, 211, 169, 125	trigalloyl-hexoside	+	+
57	24.25	C ₂₀ H ₁₆ O ₁₃	463.0515 (481.0518)	-0.3144	301, 300, 257, 229	ellagic acid-hexoside	+	+
58	24.73	C ₃₄ H ₂₆ O ₂₂	785.0863 (785.0842)	2.5517	615, 483, 463, 419, 331, 313, 301, 275, 257, 249, 229, 169, 125	digalloyl-HHDP-hexoside	-	+
59	25.62	C ₂₇ H ₂₄ O ₁₈	635.0885 (635.0889)	-0.7684	483, 465, 423, 313, 295, 271, 211, 169, 125	trigalloyl-hexoside	+	+
60	27.68	C ₄₁ H ₂₈ O ₂₇	951.0713 (951.0745)	-3.3855	933, 915, 897, 765, 631, 613, 463, 445, 301, 275, 257, 249, 229, 169	galloyl-HHDP-DHHDP-hexoside	+	+
61	29.16	C ₁₉ H ₁₄ O ₁₂	433.0411 (433.0412)	-0.3458	301, 300, 257, 229	ellagic acid pentoside	+	+
62	29.52	C ₃₄ H ₂₆ O ₂₂	787.0980 (787.0999)	-2.4733	635, 617, 483, 465, 447, 423, 313, 295, 249, 211, 169, 125	tetragalloyl-hexoside	+	+
63	30.45	C ₁₉ H ₁₄ O ₁₂	433.0408 (433.0412)	-1.0386	301, 300, 257, 229	ellagic acid-pentoside	+	+
64	30.68	C ₃₄ H ₂₆ O ₂₂	785.0827 (785.0842)	-2.0337	615, 483, 463, 419, 331, 313, 301, 275, 257, 249, 229, 169, 125	digalloyl-HHDP-hexoside	+	+
65	30.89	C ₃₄ H ₂₈ O ₂₂	787.0971 (787.0999)	-3.6167	635, 617, 573, 465, 313, 295, 211, 169, 125	tetragalloyl-hexoside	+	+
66	31.05	C ₂₀ H ₁₆ O ₁₂	447.0571 (447.0568)	0.4476	301, 300, 257, 229	ellagic acid-deoxyhexoside	-	+
67	31.82	C ₁₄ H ₆ O ₈	300.9986 (300.9989)	-1.2986	284, 257, 245, 229, 201, 185	ellagic acid	+	+
68	32.23	C ₃₄ H ₂₈ O ₂₂	787.0972 (787.0999)	-2.7467	617, 465, 449, 423, 313, 295, 169, 125	tetragalloyl-hexoside	+	+
69	33.97	C ₄₁ H ₃₀ O ₂₆	937.0930 (937.0952)	-2.4067	785, 767, 635, 617, 597, 483, 465, 445, 419, 313, 301, 275, 257, 249, 229, 169, 125	trigalloyl-HHDP-hexoside	+	+
70	35.61	C ₄₁ H ₃₂ O ₂₆	939.1076 (939.1109)	-3.5197	787, 769, 635, 617, 599, 465, 447, 431, 313, 295, 169, 125	pentagalloyl-hexoside	+	+
71	43.33	C ₄₃ H ₃₄ O ₂₈	997.1123 (997.1163)	-4.0966	845, 827, 695, 615, 525, 463, 445, 381, 301, 257, 229	punicatannin A/B	+	-

* punicalagin isomers