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Phenolic Constituents of Pomegranate Peels (*Punica granatum L.*) Cultivated in Oman

Amani S. Al-Rawahi[1*], Giles Edwards[2], Mohammed Al-Sibani[2], Ghanim Al-Thani[2], Ahmed S. Al-Harrasi[3] and Mohammed Shafiur Rahman[1]

[1]Department of Food Science and Nutrition, College of Agricultural and Marine Sciences, Sultan Qaboos University, P. O. Box-34, Al-Khod-123, Muscat, Oman.
[2]DARIS, Centre for Scientific Research and Technological Development, University of Nizwa, P.O. Box-33, Birkat Almouz-611, Nizwa, Oman.
[3]College of Arts and Science, University of Nizwa, P.O. Box-33, Birkat Almouz-611, Nizwa, Oman.

Authors' contributions

Author ASA designed the study, performed the experimental work, wrote the protocol, and wrote the first draft of the manuscript. Author GE supervised the undertaking of the determination of polyphenols by ESI-MS/MS. Author MA managed the analyses and identification of polyphenols. Author GA assisted in the preparation of the extract and the analysis of polyphenols. Author AA helped in conceptualizing and forming the manuscript. Author SR had supervised the whole project. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: This study was undertaken to analyze total phenolics and total flavonoids contents; and total antioxidant capacity of pomegranate peel extract and to identify the major functional components in the extract.
Study Design: The extract was subjected to ESI-MS/MS.
Place and Duration of Study: Department of Food Science and Nutrition, Sultan Qaboos University and DARIS Research Center, University of Nizwa, between December 2011 and August 2012.
Methodology: Pomegranate peel extract was analyzed using a Waters Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation, Manchester, UK)

**Corresponding author: Email: amanisms@gmail.com;*

equipped with electro-spray ionization (ESI) source. Instrument control and data acquisition were performed using Mass Lynx ver. 4.1 software. The instrument was calibrated for nominal resolution for MS1 and MS2 up to 1200 *m/z* using the sodium caesium iodide standard calibration solution.
Results: Results revealed high contents of total phenolics (64.2 mg Gallic acid equivalent/ g dry solids) and total flavonoids (1.4 mg Catechin equivalent/ g dry solids) respectively. Total antioxidant capacity ranged from 42.3 – 461.2 µmolTrolox equivalent/ g dry solids. The analysis revealed the presence of 61 different polyphenols in the extract among which 12 hydroxycinnamic acids, 14 hydrolysable tannins, 9 hydroxybenzoic acids, 5 hydroxybutanedioic acids, 11 hydroxy-cyclohexanecarboxylic acids and 8 hydroxyphenyls. Major compounds were tannins and flavonoids such as; illogic acid, gallic acids, punicalin, and punicalagin.
Conclusion: A wide variety of phytochemicals present in pomegranate peel extract were identified. These functional compounds in pomegranate peels could be utilized by the food industry and pharma/nutraceutical's industry. Further work should be done to isolate and quantify major functional compounds of pomegranate peels such as ellagic acid.

Keywords: Antioxidant activity; flavonoids; tannins; phenolic acids; pomegranate peel.

1. INTRODUCTION

Polyphenols are secondary metabolites that are widespread throughout the plant kingdom with more than 8000 phenolic compounds [1]. Fruits and other products, such as tea, red wine, cereals, chocolate and legumes, are regarded as major dietary sources of polyphenols [2]. While polyphenols have been regarded as waste products or nonnutritive compounds and are not directly involved in the plant's growth and reproduction, they contribute to numerous important functions in plants such as protection from UV radiation, defense against invading pathogens and attraction of pollinators and seed dispersers as well as the plant's characteristics such as taste and color [3-5]. Polyphenols are known for their pharmacological properties, for example as antioxidants, anti-inflammatory, anti-mutagenic, anti-carcinogenic, and antimicrobial [6-10].

Many fruits and natural products, including pomegranates, have been analyzed for their antioxidant activity, flavonoids, and polyphenols content. The pomegranate, *Punica granatum*L*.,* which is one of most important crops cultivated in Oman, has been used since antiquity as a medicinal plant. From historical to modern times, different parts of pomegranate have been used for different purposes such as in cuisines (e.g., juices, jams, jellies, dressings, marinating, and wine), or as religious symbolism (e.g., righteousness, fullness, fertility, abundance), or for its medicinal values. Pomegranate peels are known for their high nutrient composition such as, vitamins A, B_6, C, E, folate, potassium and oxalic acid [11].

In addition to their nutritional value, pomegranate peels were used since ancient times as anti-thelmintic, anti-tracheobronchitis, for healing wounds, ulcers, bruises, stomatitis, diarrhea, vaginitis, and against excessive bleeding [12]. In recent years, more medicinal values of pomegranate peel have been investigated such as abortifacient, analgesic, anti-ameobic, antibacterial, anticonvulsant, antifungal, antimalarial, anti-mutagenic, antiviral, antispasmodic, diuretic, hypoglycemic, hypothermic, and antioxidant activities [13]. The major class of pomegranate phytochemicals is the polyphenols that are predominant in the fruit and includes flavonoids (flavonols, flavanols, and anthocyanins), condensed tannins

(pro-anthocyanidins) and hydrolysable tannins (HTs) (ellagi-tannins and gallo-tannins) [14]. These tannins are highly susceptible to both enzymatic and non-enzymatic hydrolysis. The hydrolysis products include glucose and ellagic acid or gallic acid. Additional phytochemicals present in pomegranate peel include organic and phenolic acids, sterols and triterpenoids, and alkaloids [15]. The ellagi-tannins present in the pomegranate peel accounts for approximately 92% of the total antioxidant activity of pomegranate fruit [16]. Therefore, the health benefits of pomegranate peel are accredited for the pharmacological activities exhibited by bioactive phytochemicals like polyphenols.

To understand the phytochemistry of pomegranate peel to associate them with biological and potential health promoting properties upon their consumption by humans, more studies need to be conducted. In addition to that, phytochemicals consumed through dietary sources usually undergo considerable processing before being consumed. The effect of drying and other similar processing also becomes important in order to ascertain the stability and availability of these functional phytochemicals in processed foods and food products [17]. In Oman, peels of pomegranate are traditionally used after sun dried to heel intestinal wounds and for weight loss. No scientific evidence has been established yet on peel of Omani pomegranate to prove this. The effect of different drying methods on the retention of total phenols of peels of Omani pomegranate has been studied. Results showed that the drying methods significantly reduced the total phenols in pomegranate peel except the freeze drying which retained the highest phenolic content when compared to other drying methods (43.9 mg GAE/g dry solids) [18]. In order to identify polyphenols in peels of pomegranate, different methods have been tested. Phenolic compounds from pomegranate peel, mesocarp, and aril have identified and quantified using the HPLC-DAD-ESI/MS^n[15]. In total, 48 different compounds were detected. The rapid large scale purification of ellagitannins from pomegranate husk using the HPLC and tandem LC-ES/MS was investigated and was successful to purify some functional components such as punicalagin, ellagic acid, punicalin, and ellagic acid glycosides [14]. Other studies on the identification of phenolics from pomegranate peel extracts have been using different methods such as: LC-BCD-MS, HPLC, HPLC-DAD-ESI/MS, HSCCC [19-21]. To our knowledge, this is the first study identifying the chemical constituents of peels from Omani pomegranate. The objectives of this preliminary study were to analyze the total phenolics, total flavonoids, total antioxidant capacity, and to identify the major phytochemical constituents present in the extract of freeze dried pomegranate peel from '*Hellow'* variety cultivated in Oman. The outcome of this work will assist in isolating and quantifying the active components of pomegranate peel extract and will provide its potential for health benefits.

2. MATERIALS AND METHODS

2.1 Sample

Fresh pomegranate (*Punica granatum* L.) fruits of var. '*Hellow'* (literally; *sweet*) were purchased from local farms in Al-Jabal Al-Akhdar, Oman. The fruits were transported to the Food Science Laboratory in an electric cooler box maintained at 9˚C. Fruits were cut into four portions and arils were separated manually from peels. Peels were packed in polyethylene bags (500 g) and stored at -40°C until used further. All chemicals used were purchased from Sigma (St. Louis, MO, USA).

2.2 Drying of Pomegranate Peels

Peels were cut into small pieces (i.e. 3 cm × 3 cm), placed into plastic containers (50 ml), and were kept in a freezer at -40°C for at least 16 hours. The frozen peels were freeze-dried (i.e. freeze drying started from -40 °C and ended at 20 ^{o}C) for 96 hours using Edwards K4 Freeze Dryer (Crawley, England). Dried peels were then stored in a desiccator for one week before being ground into powder.

2.3 Extraction

Dried pomegranate peels were ground into powder using a KMF grinder (KIKA Werke, Wilmington, USA) at 9676.8 g. Powders were kept in air tight plastic containers and stored at -40°C until used for extraction. Methyl alcohol, ethyl alcohol and water were commonly used to prepare extracts [15,19,22]. However initial experimental trials showed weak peaks for ESI/MS in the cases of ethyl alcohol, methyl alcohol and water extracts, while ethyl acetated extract was capable of recovering many compounds thus ethyl acetate was chosen for the extraction of functional components from pomegranate peels [20]. Ethyl acetate (100 mL) was added to 0.5 g of dried sample in conical flasks and was stirred for 3h at room temperature (20°C). To avoid light exposure the flask was covered with aluminum foil. Mixture was then centrifuged for 30 minutes at 9676.8 g at 3°C using Harrier 18/80 refrigerated centrifuge (SANYO, MSE, UK). The supernatant was filtered using Whatman filter paper (No 1, Ø 155 mm). Extract was then stored at 4 °C until used for analysis.

2.4 Total Phenolics Content

Total phenolics content was analyzed using the Folin-Ciocalteu method [23]. In this method, 70 µL of pomegranate peel extract was placed into a 10 mL test tube and 250 µL of Folin-Ciocalteu reagent and 750 µL of 1.9 M sodium carbonate were added. The total volume was made up to 5 ml by adding distilled water, and was mixed by vortex for one minute and then incubated for two hours in dark. Subsequently, the absorbance was measured at 765 nm using a UV-visible spectrophotometer (ThermoSpectronic, Surrey, England). An appropriate calibration curve was prepared using standard solutions of gallic acid. Results were expressed as gallic acid equivalents (GAE) in mg/g dry solids.

2.5 Total Flavonoids Content

Total flavonoids content of the freeze dried peel extract was determined as the method used by Zhishen et al. [24]. Briefly, 1 mL of extract was added into a 10 mL test tube containing 4 mL of distilled water. At zero time, 0.3 mL of 5% $NaNO_2$ was added to the test tube. After 5 min, 0.3 mL of 10% $AlCl_3$ was added into the test tube. At 6 min, 2 mL of 1 M NaOH was added to the mixture. Immediately, the reaction tube was diluted with the addition of 2.4 mL of distilled water and thoroughly mixed. Absorbance of the pink colored mixture was read at 510 nm and water was used as a blank. An appropriate calibration curve was prepared using different concentrations of catechin solutions. The final results were expressed as mg catechin equivalent (CE) per g dry solids.

2.6 Total Antioxidant Capacity

Total antioxidant capacity was determined using Biovision Total Antioxidant Capacity (TAC) Assay Kit (Catalog #K274-100, Milpitas, CA, USA). Cu^{2+} reagent, assay diluent, and protein

mask were kept at room temperature. The lyophilized Trolox Standard (1µmol) was dissolved in 20 µL of pure DMSO by mixing on a vortex, and then 980 µL of ddH_2O was added and mixed well, generating a 1 mM solution. Reconstituted standard was aliquoted and stored at -20°C for later use. To measure the antioxidants; a Trolox standard curve was calibrated by adding 0, 4, 8, 12, 16, 20 µL of the Trolox standard to individual wells. The total volume was adjusted to 100 µL with ddH_2O to give 0, 4, 8, 12, 16, 20 nM of Trolox standard. Sample volumes between 0 -20 µL were assayed per well and were done in triplicate. All well volumes were adjusted to 100 µL with ddH_2O. To prepare the working solutions; one part of Cu^{2+} reagent was diluted with 49 parts of assay diluent. 100 µL Cu^{2+} working solution was added to all standard and sample wells. The plate was then covered and incubated at room temperature for 1.5 hours. The absorbance was read at 570 nm using the plate reader. The standard curve was plotted as a function of Trolox concentration. Sample antioxidant Trolox equivalent concentrations were determined using the absorbance data. Results were then expressed in µmol/g dry solids.

2.7 Determination of Polyphenols ByEsi-Ms/Ms

The pomegranate peel extract was analyzed using a Waters Quattro Premier XE tandem quadrupole mass spectrometer (Waters Corporation, Manchester, UK) equipped with electro-spray ionization (ESI) source. Instrument control and data acquisition were performed using MassLynx ver. 4.1 software. The instrument was calibrated for nominal resolution for MS1 and MS2 up to 1200 *m/z* using the sodium caesium iodide standard calibration solution. The sample was introduced by direct infusion at a flow rate of 40 µL/min and was run in both positive and negative ionization mode for the preliminary screening (full scan mode) from 50 *m/z* to 1200 *m/z.* The compounds of interest were tuned by optimizing the sample cone voltage (20-50 V) and a capillary voltage to 3.5 KV in order to observe the bioactive substances most amenable to electrospray ionization. The parent ions of the compounds of interest were then subjected to tandem MS analysis using the daughter scan function using the following tuning parameters: (ESI^-), capillary voltage set between 3.3 to 3.6 KV, cone voltage (30 V), extractor (3 V), RF Lens (0.2 V), source temperature (100°C), dissolvation temperature (150°C), multiplier (650 V), the collision energy was optimized in the range from 20 to 45 eV. Most of the daughter peaks were identified by matching the fragmentation patterns obtained from the daughter scan to those theoretically and experimentally documented in literature [24-35].

3. RESULTS AND DISCUSSION

3.1 Total Phenolics Content, Total Flavonoids Content and Total Antioxidant Capacity

It is well known that plant phenolics and flavonoids are highly effective free radical scavengers and antioxidants. Table 1.illustrates the total phenolics and total flavonoids in the extract of freeze dried pomegranate peel. The peel extract exhibited a high content of total phenolics and total flavonoids i.e. 64.2 mg GAE/g dry solids and 1.4 mg CE/g dry solids, respectively. The flavonoids accounted for a small amount of total phenolics in the pomegranate peel extract. In the present study, total phenolics were higher than a similar study done on Egyptian pomegranate peel extract in which the extract had6.2 mg GAE/g dry solids [11]. On the contrary, in the cases of pomegranate varieties from India and from Kashmir district, higher phenolic contents (124.3 - 249.4 mg GAE/g dry solids) and flavonoid contents (49.1-59.4 mg CE/g dry solids) in pomegranate peel extract were reported [22,34].

A Yamani cultivar was found to have high total phenolics (91.2 mg GAE/g dry solids) with no detection of total flavonoids [36]. This variation of total phenolics and total flavonoids could be associated with the difference in cultivars, methods of extraction and environmental conditions such as relative humidity and temperature of extracts. It was suggested that the high amounts of bioactive compounds are available in non-edible part which could be used for different purposes in the food industry such as enrichment or development of new products [34].

Table 1. Analysis of the total phenolics and total flavonoids of pomegranate peel extracts

	Total phenolics[a]	Total flavonoids[b]
Extract	64.2±0.50	1.4±0.03

a (mg GAE/g ds), b (mg CE/g ds).
GAE: gallic acid equivalents; CE: catechin equivalent.
Values are expressed as means of triplicate determinations ± SD

The results in Table 2 of total antioxidant activity revealed *Punica granatum* peel extract is a potential source of antioxidant. Similar correlation of antioxidant activity and phenolic contents has also been shown in other studies [28]. The reducing power method (TAC assay) is based on the reduction of Cu^{2+} to Cu^{1+} by antioxidants. This method is simultaneously cost-effective, rapid, stable, selective and suitable for a variety of antioxidants regardless of the chemical type or hydrophilicity. In addition, it was reported that the results obtained from *in vitro* cupric ions (Cu^{2+}) reducing measurements might be more efficiently extended to possible *in vivo* reactions of antioxidants. The observed antioxidant potential for these extracts can be related to the presence of various functional groups, such as hydroxyl and carbonyl groups [35,36]. Studies have confirmed that the antioxidant activity of plant extracts depended on the concentration of phenolic compounds, and the antioxidant power of pomegranate peel extract has been found to linearly increase with the concentration of peel phenolics up to the level of 400 mg/g [32,33]. In contradiction with our results, the ethyl acetate fraction had the lowest antioxidant potential comparing to some other fractions used e.g. water and methanol fractions [34].

Table 2. Antioxidant capacity of *Punica granatum* peel extracts as Trolox equivalents (µmol/g dry solids)

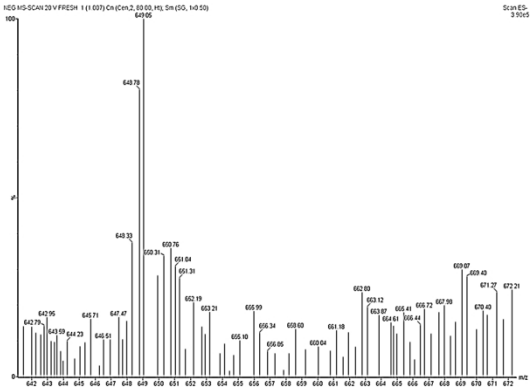
Concentration (µg/mL)	TAC
2	42.3 ± 3.1a
4	113.1 ± 2.9b
6	230.2 ± 3.5c
8	309.5 ± 2.8d
10	461.2 ± 1.8e

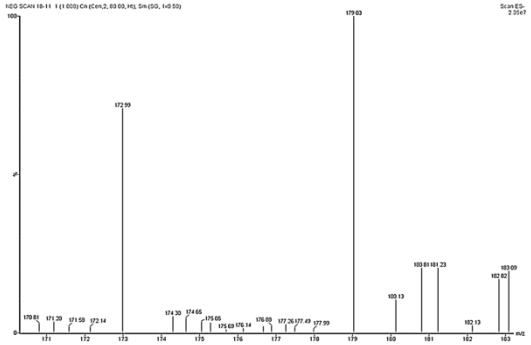
Values are expressed as means of triplicate determinations ± SD. Significant differences between values are indicated by different letters ($P < 0.05$).

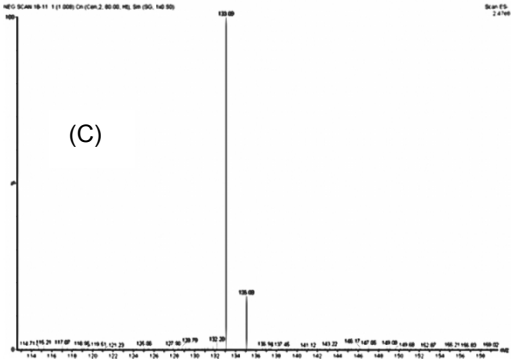
3.2 Identification of Polyphenols by Esi-Ms/Ms

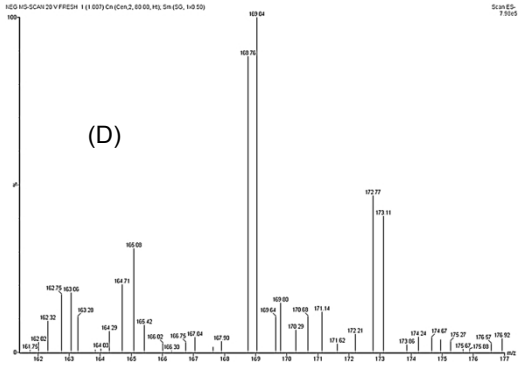
The present study demonstrated the fractionation of pomegranate peel in ethyl acetate as a solvent which was very efficient in exhibiting various compounds of polyphenols when subjected to the ESI-MS/MS analysis. ESI-MS/MS spectra of the direct infusion of *Punica* ethyl acetate fraction showed the presence of wide array of phenolic compounds. The main

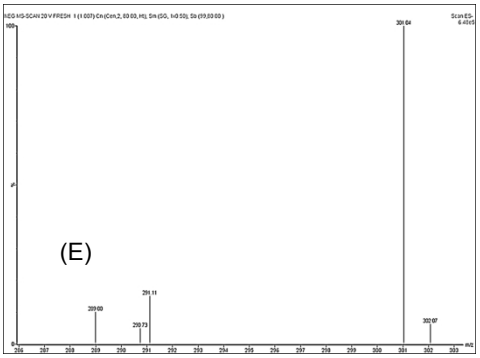
constituents of ethyl acetate fraction were monitored by diode-array and mass spectrometry. Typical direct infusion mass spectra of phenolic compounds of interest from the pomegranate peel extract are shown in Fig.1A-G. Table 3 outlines the peak list for the full scan (precursor ions) and daughter scan (product ions) mass spectra for the major compounds of interest. The profile of the extract showed 16 major phenolic compounds, 43 derivatives, and 2 un-identified compounds on the basis of their precursor and product ion mass spectra. The phenolic compounds were classified into six categories: hydroxycinnamic acids, hydrolysable tannins, hydroxybenzoic acids, hydroxybutanedioic acids, hydroxycyclohexane carboxylic acids and hydroxyphenyls. Peak identities were obtained by matching their pseudo molecular ions $[M–H]^-$ obtained by ES/MS and tandem MS with the expected theoretical molecular weights from literature.

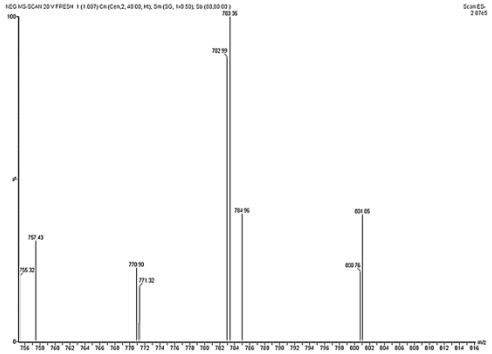












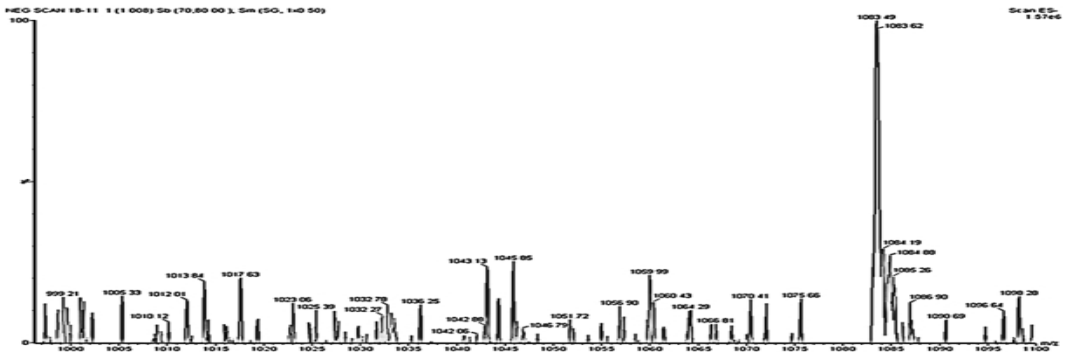


Fig. 1. ESI-MS/MS spectra of *Punica granatum* peel extract ethyl acetate fractions by direct infusion show the presence of: (A) Malic acid ([M-H]- m/z 133) , (B) Gallic acid ([M-H]- m/z 169), (C) Caffeic acid ([M-H]- m/z 179), (D) Ellagic acid ([M-H]- m/z 301), (E) Lagerstannin C ([M-H]- m/z 649), (F) Pedunculagin I ([M-H]- m/z 783), (G) Punicalagin ([M-H]- m/z 1083)

It is evident that various antioxidant substances are present in pomegranate peels. In addition, the peels contain significant amounts of ellagitannins, such as punicalin (Compound 22) and punicalagin (Compound 26) together with hexahydroxydiphenic acid (HHDP) derivatives (Compounds 23 & 24) and flavonoids, such as kaempferol and quercetin derivatives (Compounds 52 –55 & 17) as well as the ellagic acid (Compounds 14,19) and gallic acid derivatives (Compounds 27-35). Different reports demonstrated that these components possess anti-inflammatory, antitumor, and apoptotic properties [14].

Table 3. Characteristic ions of phenolic compounds of pomegranate peel extract

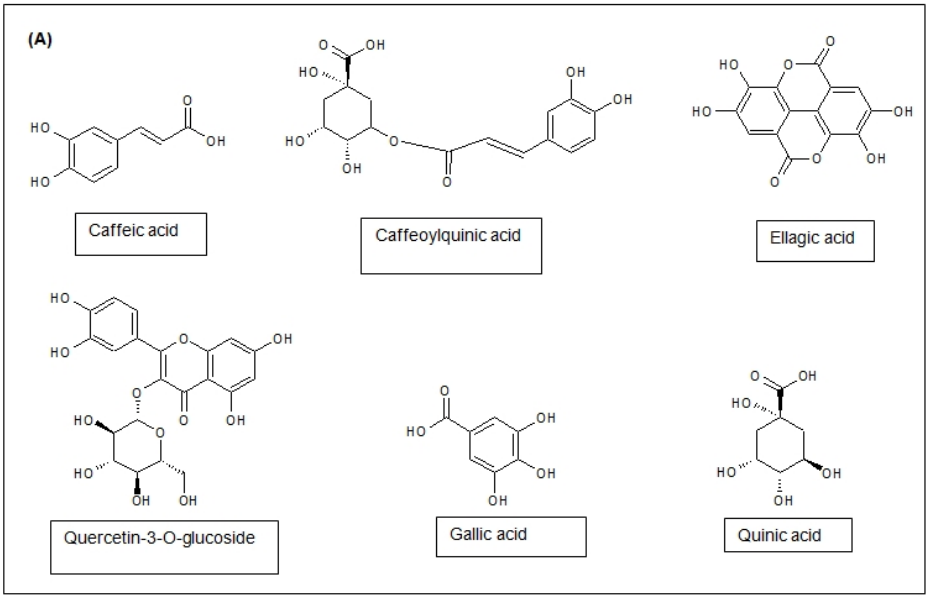
compound	assignment	Chemical formula	[M-H]- m/z	MS-MS m/z
Hydroxycinnamic acid derivatives				
C1	Caffeic acid	$C_9H_8O_4$	179	59, 71, 75,89, 135
C2	Fragment of caffeic acid	$C_8H_8O_2$	135	71, 75
C3	Fragment of caffeic acid	$C_8H_6O_4$	165	59,71, 75
C4	Caffeic acid derivative	$C_9H_{10}O_4$	181	59, 71, 89
C5	Caffiec acid derivative		215	59, 71, 89, 179, 225
C6	Caffiec acid derivative		217	59, 71, 89, 179, 181
C7	Caffiec acid derivative		219	179
C8	Caffeic acid phenethylester	$C_{17}H_{16}O_4$	283	59, 179
C9	Methyl gallate der		325	169, 183
C10	Caffeoylquinic acid	$C_{16}H_{18}O_9$	353	111, 129
C11	Caffeic acid der		457	179, 215

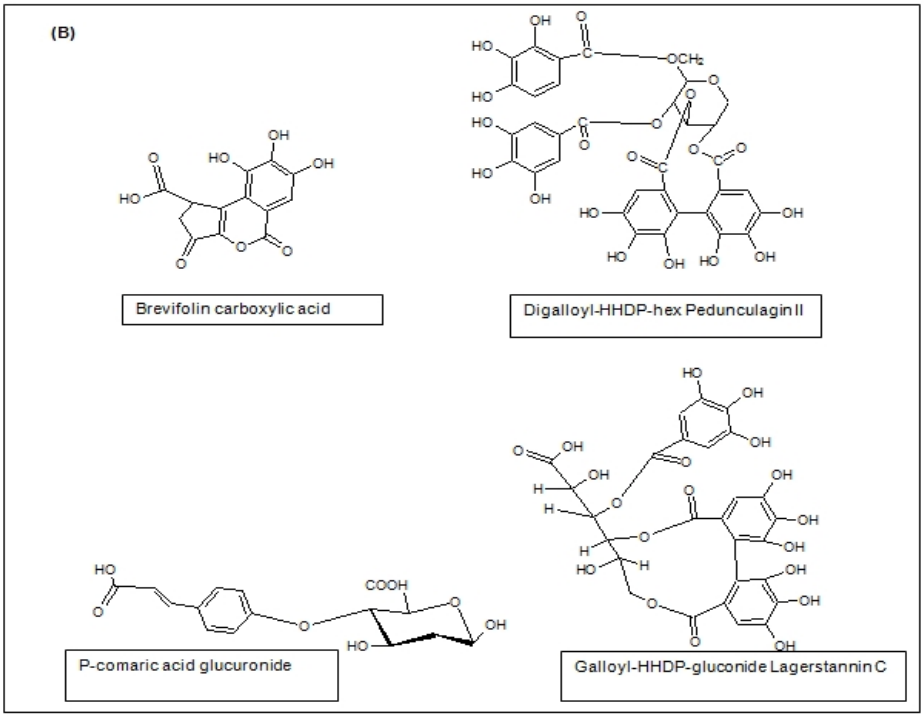
C12	P-Comaric acid glucuronide	$C_{15}H_{16}O_9$	339	163
Hydrolysable tannins				
C13	Brevifolin carboxylic acid	$C_{13}H_8O_8$	291	80, 247
C14	Ellagic acid	$C_{14}H_6O_8$	301.13	185, 229, 257
C15	Galloyl-hex (glucogallin)	$C_{13}H_{16}O_{10}$	331	125, 169
C16	Eschweilenol C	$C_{20}H_{16}O_{12}$	447.17	432, 389, 77
C17	Quercetin-3-O-glucoside	$C_{15}H_{10}O_7$	463	89, 265, 301
C18	HHDP-hex	$C_{13}H_{18}O_{14}$	481	257, 275, 301, 180
C19	Ellagic acid der		515	229, 275, 301
C20	Galloyl-HDDP-hex	$C_{27}H_{22}O_{18}$	633	275, 301
C21	Galloyl-HDDP-gluconide Lagerstannin C	$C_{27}H_{22}O_{19}$	649	301
C22	Punicalin	$C_{34}H_{22}O_{22}$	781	601, 299, 300
C23	Bis-HHDP-hex Pedunculagin 1	$C_{34}H_{24}O_{22}$	783	301, 602, 481
C24	Digalloyl-HHDP-hex Pedunculagin II	$C_{34}H_{26}O_{22}$	785	633
C25	Galloyl-HHDP-DHHDP-glucosideGranatin B	$C_{34}H_{28}O_27$	951	481
C26	HHDP-gallagylglucosidePunicalagin	$C_{48}H_{28}O_{30}$	1083	781, 601, 602
Hydroxybenzoic acids				
C27	Gallic acid	$C_7H_6O_5$	169	69, 79, 83, 97,110, 125
C28	Fragment of gallic acid	$C_6H_6O_3$	125	69, 79
C29	Gallic acid der		277	79, 97
C30	Gallic acid der		279	79, 97
C31	Gallic acid der		395	125, 169, 215
C32	Gallic acid der		397	127, 171, 181, 217
C33	Vanilic acid der		255	128, 167
C34	Protocatechuic acid der		435	79, 89, 153,171
C35	Methyl gallate der		311	169, 183
Hydroxybutanedioic acids				
C36	Malic acid	$C_4H_6O_5$	133	71, 92, 115
C37	Malic acid glucoside der		317	117, 135
C38	Fragment of malic acid	$C_4H_4O_4$	115	92

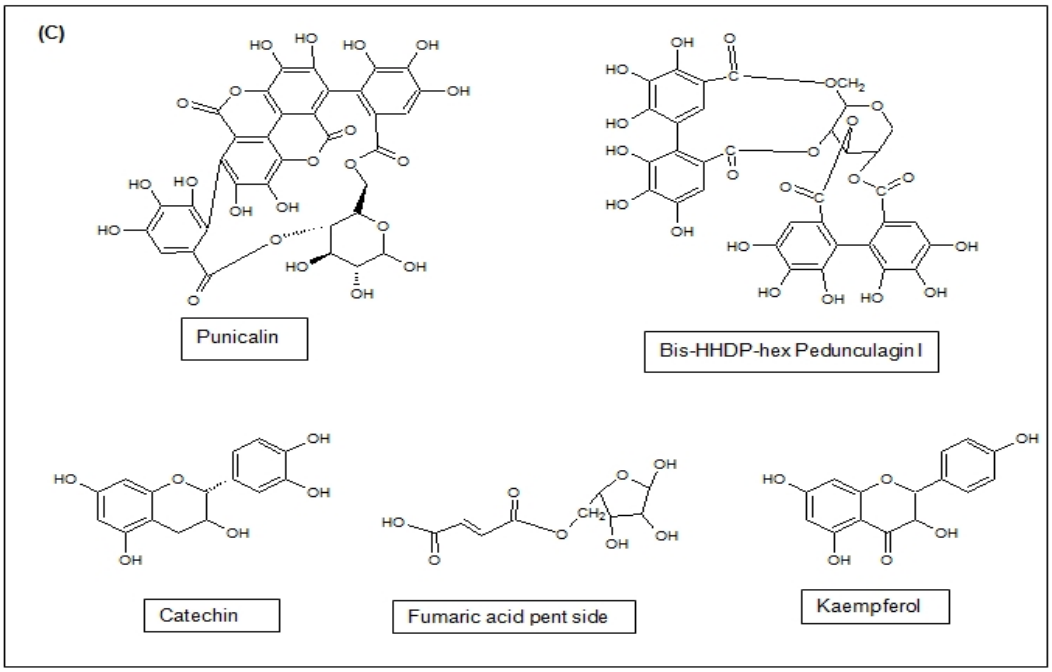
C39	Malic acid glucoside der		313	71, 115, 133
C40	Fumaric acid pentoside	$C_9H_{12}O_8$	247	115
Hydroxycyclohexanecarboxylic acids				
C41	Fragment of quinic acid		101	57, 87
C42	Fragment of quinic acid	$C_6H_8O_2$	111	67
C43	Fragment of quinic acid	$C_6H_{10}O_3$	129	67
C44	Fragment of quinic acid	$C_6H_{12}O_4$	147	57, 87, 129
C45	Fragment of quinic acid	$C_7H_8O_4$	155	67, 111
C46	Fragment of quinic acid	$C_7H_{10}O_5$	173	57, 67, 111
C47	Quinic acid	$C_7H_{12}O_6$	191	57, 67,87,111,129
C48	Quinic acid methyl ester	$C_8H_{14}O_6$	205	57,67, 87, 111, 129
C49	Quinic acid der		371	87, 111, 129, 173, 191
C50	Quinic acid der		373	173, 191
C51	Quinic acid acetyl glucoside der		577	191, 217, 287, 395
Hydroxyphenyls				
C52	Kaempferol	$C_{15}H_{10}O_6$	285	127, 181, 257
C53	Kaempferol 3-O-rutinoside	$C_{27}H_{30}O_{15}$	593.33	285
C54	Kaempferol der		575	169, 285, 291, 303
C55	Kaempferol der		573	169, 285, 303
C56	Catechin	$C_{15}H_{14}O_6$	289	179, 205, 245
C57	Gallocatechol	$C_{15}H_{14}O_7$	305.16	79, 97, 125
C58	Isorhamnetin	$C_{16}H_{12}O_7$	315	271, 115, 133
C59	HHDP- acetyl glucoside der		541	275, 301
C60	Not identified		292.12	Could not be fragmented
C61	Not identified		813	755, 194, 489

The chemical structures of major identified compounds are represented in Figs. 2A-D. A total of 61 $[M-H]^-$ ions were detected in the extract of freeze-dried pomegranate-peel using a tandem mass spectrometry (MS/MS) equipped with electro-spray ionization (ESI) interface. This study represents the first phenolic analysis for Omani pomegranate peel and as far as we are aware also reports the identification of some molecular ions that have not been reported before such as caffeic acid derivatives *m/z* 215, 217, 283, methyl gallat ester, quinic acid methyl ester, and hexahydroxydiphenic acid (HHDP)-acetyl glucoside derivatives. Due to the large number of detected ions and unavailability of authentic standards, identifications of all molecular ions were carried out by direct infusion analysis using the negative full scan mode and their structural information elucidated by performing a daughter scan on the

molecular ion of interest. Table 3 provides the identification details of all detected ions, with the exception of two compounds (Compounds 60 & 61 in Table 3 at *m/z* 292 and *m/z* 813) as the concentration of m/z 292 was present in a very low level and the fragmentation pattern of m/z 813 did not show any obvious structural composition. The identified compounds were classified into six categories:







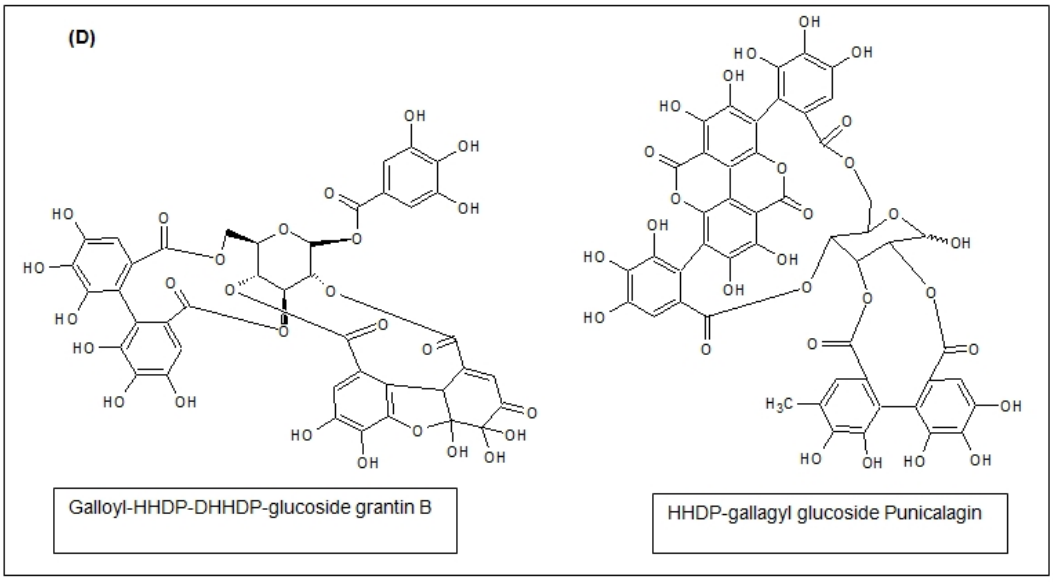


Fig. 2. (A-D) Chemical structure of major compounds found in the peel extract of *Punica granatum* using ESI-MS/MS analysis. Pages 11-15 should be in landscape position in order for the figures to fit into each page

3.2.1 Hydroxycinamic acids

Compound 1 had $[M-H]^-$ at *m/z* 179 which fragmented into MS^2 ion at *m/z* 135 (loss 44 u $[COO]^-$) and it was identified as caffeic acid. Compounds 2 -7 in Table 3 were considered

caffeic acid fragments and derivatives because they showed similar product ion spectra. The MS/MS fragmentation pathway of compound 8 at *m/z* 283 showed two fragments *m/z* 179 (loss of phenethyl alcohol group with m/z and with m/z 104 water 18 u) and *m/z* 59, so the compound was assigned as caffeic acid phenethyl ester. Recently, several studies have been focusing on this compound and results have indicated that caffeic acid phenethyl ester (CAPE) have anticarcinogenic effects and the administration of this compound maybe useful as an adjuvant therapy for prostate and other types of cancers [39]. Compound 12 at *m/z* 339 was identified as *p*-comaric acid glucuronide because its further fragmentation lost glucuronic acid moiety (176 u) yielding the fragment *m/z* 163.

3.2.2 Hydrolysable tannins

A number of 14 metabolites were identified in this group. The predominant compound was ellagic acid at *m/z* 301 with fragments *m/z* 257, 229, 185. The efficacy of this important compound has been considered atreatmentfor some low to mild and chronic disorders. Moreover, it has been shown to be a potential candidate as a chemo preventive agent for cancer treatment [37]. Beside its other well-known ethno pharmacological properties, ellagic acid has been demonstrated to reduce white fat deposits and triglycerides levels accumulated in the body during regular intake of high-fat diets [38]. Several studies have confirmed the cytoprotective effects of ellagic acid from pomegranate peel extracts on oxidatively injured living cells, oxidative DNA damage and depletion of the non-protein sulfhydryl pool. Higher ellagic acid concentrations are directly associated with the antioxidant activity of pomegranate peel extracts [39-41]. Compound 13 was identified as brevifolin carboxylic acid at *m/z* 291 as its MS/MS fragmentation showed a $[M-H-COO]^-$ fragment at *m/z* 247. The loss of 162 u and 18 u from *m/z* 481 to *m/z* 301 was most likely due to the breakdown of glucose and water, so this compound was identified as hexahydroxydiphenoyl - glucoside (HHDP - glucoside). Compounds 21- 26 exhibited $[M-H]^-$ ions with *m/z* 633, 649, 781, 783, 785, 951 and 1083. Their MS^2 fragmentations nearly led to the same basic structure and were all identified as Galloyl-HHDP-hex, Galloyl-HHDP-gluconidelagerstannin C, punicalin, bis-HHDP-hex pedunculagin 1, Digalloyl-HHDP-hexpedunculagin II, Galloyl-HHDP-DHHDP-glucosidegranatin B, and HHDP-gallagyl glucoside punicalagin.

3.2.3 Hydroxybenzoic acids

Four gallic acid derivatives were detected in the extract from the negative ionization mode at *m/z* 277, 279, 395 and 397 as their MS/MS fragments matched well with basic fragmentation of gallic acid. Beside gallic acid derivatives, vanilic acid and protocatechuic acid derivatives were also detected with molecular ions *m/z* 255 and 435. A compound *m/z* 311 was characterized as methyl gallate derivative; this was because it showed two fragments *m/z* 183 and 169. The 14 u difference between the two fragments was due to the loss of CH_2.

3.2.4 Hydroxybutanedioic acids

Malic acid at *m/z* 133 and malic acid glucoside derivatives at *m/z* 317 and 313 were the predominant phenolic compounds identified in this group. Compound 40 showed a $[M-H]^-$ ion at *m/z* 247, and when fragmented led to MS^2 product ion at *m/z* 115 which corresponds to fumaric acid and the loss of (132 u) corresponds to pentose moiety, so the compound was identified as fumaric acid pentoside.

3.2.5 Hydroxycyclohexanecarboxylic acids

The pseudo-molecular ion at *m/z* 191 $[M-H]^-$ and the fragments ions at *m/z* 173 $[-H_2O]^-$, 155 $[-2\ H_2O]^-$, 129 $[-COO\ -H_2O]^-$ and 111 $[-COO-2\ H_2O]^-$ suggesting that the compound was quinic acid. Three derivatives of quinic acid were detected at *m/z* 371 and 373 and 577 and all of them approximately exhibited similar fragmentation. The *m/z* 205 ion was identified as quinic acid methyl ester due to its fragmentation pathway.

3.2.6 Hydroxyphenyls

Eight others compound belonging to hydroxyphenyls group were observed in pomegranate peel extract. In accordance with MS^2 fragmentation, half of them were assigned as kaempferol derivatives with *m/z* 285, 593, 575 and 573. The other ions were *m/z* 289, 305, 315 and 541 and so they were assigned as catechin, callocatechol, isorhamnetin, and HHDP- acetyl glycoside derivatives, respectively.

Many compounds characterized by this study such as gallic acid, malic acid, quercetin, kaempferol and ellagic acid were in agreement with studies done by other researchers [15,19,25]. Other studies have identified some compounds that were not identified by this study such as gallagic acid luteolin and [19,20]. To our knowledge, (Compounds 60: m/z 292 and 61: m/z 813) have not generally been described in the pomegranate literature, thus demonstrating that the polyphenols profile of pomegranates might be more complex. The compound 61: *m/z* 813 was detected but could not been identified [41]. The aforementioned compounds may have been either overlooked in previous studies due to their low concentrations, the method of detection used or their occurrence may be cultivar dependent. Further studies should be carried out to identify the predominant phenolics responsible for the antioxidant activity of peel extract. To engage the individual phenolics of Omani pomegranate peels in nutraceuticals and or pharmaceuticals, quantification, isolation of dominant compounds, bioavailability and possible protection against some common ailments, such as cardiovascular diseases must be studied thoroughly.

4. CONCLUSION

The total phenolics, flavonoids, and antioxidant capacity analysis were performed on peel extract of Omani pomegranates. Phenolic and flavonoid contents were (64.2 mg GAE/g dry solids and 1.4 mg CE/g dry solids) respectively. Total antioxidant capacity ranged from 42.3–461.2 µmolTrolox equivalent/g dry solids for extract concentrations ranging from 2-10 µg/ml. Individual polyphenols were subjected to chromatographic analysis and results revealed the presence of 61 total compounds which include gallic acid, ellagic acid, punicalin, punicalagin, malic acid, etc. We consider that this peel extract deserves more intensive study on quantification, isolation of dominant compounds, bioavailability and possible protection against some common ailments, such as cardiovascular diseases.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors of this article have declared that no competing interests exist.

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