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Swarm motility inhibitory and antioxidant activities of pomegranate peel processed under three drying conditions

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ABSTRACT

During processing of ready-to-eat fresh fruits, large amounts of peel and seeds are discarded as waste. Pomegranate (*Punicagranatum*) peels contain high amounts of bioactive compounds which inhibit migration of *Salmonella* on wet surfaces. The metabolic distribution of bioactives in pomegranate peel, inner membrane, and edible aril portion was investigated under three different drying conditions along with the anti-swarming activity against *Citrobacter rodentium*. Based on the multivariate analysis, 29 metabolites discriminated the pomegranate peel, inner membrane, and edible aril portion, as well as the three different drying methods. Punicalagins (~38.6–50.3 mg/g) were detected in higher quantities in all fractions as compared to ellagic acid (~0.1–3.2 mg/g) and punicalins (~0–2.4 mg/g). The bioactivity (antioxidant, anti-swarming) and phenolics content was significantly higher in peels than the edible aril portion. Natural anti-swarming agents from food waste may have promising potential for controlling food borne pathogens.

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1. Introduction

Pomegranates (*Punicagranatum* L.) are cultivated and consumed globally. India is the largest producer of pomegranate, followed by Iran and China. United States is among the top ten producers of pomegranate (World pomegranate market, 2015). According to the most recent census of agriculture (2012), pomegranates were grown on over 30,000 acres, with California producing over 90 percent of the pomegranates within the United States (AgMRC, 2015). The production total in US was over 280,000 tons with a value of $115 million. The peels and the seeds of the pomegranate are not commonly consumed and are known to contain a higher quantity of bioactive phytochemicals.

Pomegranate is one of the important fruit with high therapeutic value for humans. Pomegranate has been consumed for centuries in many countries for the prevention and treatment of a wide range of health issues namely, inflammation, diabetes, diarrhea, bacterial infections, cardiovascular disease, anti-cancer, antiviral and immunosuppressive activities (Larrosa, Tomas-Barberan, & Espin, 2006; Lee et al., 2008; Aqil et al., 2012; Lin et al., 2013; Orgil, Spector, Holland, Mahajna, & Amir, 2016). Recently, Wu, Ma, and Tian (2013) reported that the pomegranate extracts inhibits the fatty acid biosynthesis by inactivating acetyl/malonyl transferase and β-ketoacyl synthase domains. The purported health benefits are primarily due to the presence of different type of phytochemicals, namely phenolic acids, anthocyanidins and tannins (Gil, Tomas-Barberán, Hess-Pierce, Holcroft, & Kader, 2000; Poyrazoglu, Gökmen, & Nevzat, 2002; Mena et al., 2011). The seeds and surrounding pulp (arils) are the edible portion of the pomegranate and are commonly used for the preparation of juice, syrup, jelly, food seasoning and colouring agents. The peel (outer thick skin or rind) provides a rich source of punicalins (PC), punicalagins (PG) and ellagic acids (EA). Punicalagins are reported for their beneficial effects against dysentery, hemorrhage, helminthiasis, diarrhea and acidosis (Miguel, Neves, & Antunes, 2010).

Recently, Ayala-Zavala et al. reviewed the agro-industrial potential of food byproducts as a source of antioxidants (reducing browning and lipid oxidation), antimicrobial, flavouring and natural colorants (Duman, Ozgen, Dayisoylu, Erbil, & Durgac, 2009). Certain plant-derived bioactive compounds are not biocidal, but inhibit critical processes, such as quorum sensing, motility and attachment. Suppression of these activities is expected to reduce the survival potential of food-borne pathogens. Prior *in vitro*

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studies have demonstrated the capacity of bioactive compounds to damage cell-membrane of yet another food-borne pathogen *Listeria monocytogens* (Li et al., & Xia, 2014; Xu et al., 2015). Swarming motility of *Citrobacter rodentium* which is a mouse pathogen that reproduces disease progression in mice similar to *Escherichia coli* O157:H7 infections in humans (Smith & Bhagwat, 2013; Smith, Yan, Chen, Dawson, & Bhagwat, 2016).

The antimicrobial activity of the pomegranate extracts have been reported by multiple groups, however, there is limited number of publications on anti-swarming activity (Ayala-Zavala et al., 2011; Betanzos-Cabrera, Montes-Rubio, Fabela-Illescas, Belefant-Miller, & Cancino-Diaz, 2015). Microorganisms have the ability to move on a variety of surfaces which enables them to search for favourable growth habitats, avoid stress conditions, colonize and form biofilms (Harshey & Matsuyama, 1994; Harshey & Partridge, 2015). All these processes contribute towards survival of food-borne pathogens in the environment. Recently, Mahadwar et al. (2015) examined several fruit rinds and peels for their anti-swarming properties using *Salmonella enterica*serovar *Typhimurium*as a model system. The results indicated that pomegranate peel extracts exhibited high anti-swarming activity (~85% inhibition). However, there are no satisfactory and economical animal-model systems to study *Salmonella* infections. On the other hand, *Citrobacter rodentium* is a mouse pathogen that mimics several aspects of gastrointestinal infections of humans by pathogenic *E. coli*. Infection with foodborne pathogens, such as diarrheagenic *E. coli* strains, causes a disturbance in the microbial niche followed by gastrointestinal inflammation and sometimes life-threatening conditions (Croxen & Finlay, 2010). Similar to diseases in humans, *C. rodentium* colonizes the gastrointestinal tract of mice and induces the same characteristic attachment and effacing lesions and mucosal inflammation in mouse models (Vallance, Deng, Jacobson, & Finlay, 2003).

In recent years there is significant consumer demand for natural products. This has resulted in increased research in the field of natural product for developing novel therapeutic agents, natural pest control agents, natural dietary supplements, and other personal care products (Shahidi & Ambigaipalan, 2015). In addition, agricultural industry has also seen remarkable growth in developing new value-added products with wide range of applications (Braithwaite et al., 2014). As a part or our ongoing research, we investigated the effect of processing (three drying methods) on bioactive ellagitannins (punicalins, punicalagins, and ellagic acid) present in pomegranate arils (edible part), inner membrane, and peels. In addition, we evaluated the anti-swarming activities of differently processed extracts and fractions against *C. rodentium,* a mouse pathogen. Multivariate analysis of the LC-MS data was also performed to determine profile changes of pomegranate samples processed under different temperatures.

2. Materials and methods

2.1. Chemicals and reagents

Punicalins (α and β), punicalagins (α and β), ellagic acid (EA) and 2,2-Diphenyl-1-picrylhydrazyl (DPPH), and Folin Ciocalteu's reagent were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). All solvents and chemicals were obtained from Fisher Chemicals (Fair Lawn, New Jersey, USA). All chemicals and solvents were either analytical reagent or HPLC grade solvents and were used directly without further purification. Deionized water (DI, 18Ω) was prepared using a Millipore Milli-Q purification system (Millipore Corp., New Bedford, Massachusetts, USA). Polyvinylidene difluoride (PVDF) syringe filters, with pore size 0.45 μm, were purchased from National Scientific Company (Duluth, Georgia, USA).

2.2. Samples

Pomegranate samples were obtained from a local grocery market (Beltsville, Maryland, USA) and different parts of the fruit, such as peels (outer thick layer or husk or skin), inner membrane (soft layer inside the husk) and the edible aril part (seed surrounded by edible pulp), were separated. It is difficult to totally separate the inner membrane from peels. The samples were dried under three different temperature conditions (−80 °C, ambient temperature ~25 °C and 50 °C) to investigate the impact of processing on metabolic content and bioactivity. The lyophilized samples (−80 °C) are designated as LyP (peel), LyM (membrane) and LyS (aril edible part). Similarly the ambient temperature (~25 °C) dried samples were named as RtP (peel), RtM (membrane) and RtS (aril edible part), the oven dried (50 °C) samples were named as OtP (peel), OtM (membrane) and OtS (aril edible part). The dried samples were ground in a coffee grinder and stored at −80 °C until used for the extraction and analysis.

2.3. Extraction and analysis of pomegranate samples

The pomegranate samples from different drying process (100 mg) were weighed separately and extracted three times with 1 ml of methanol:water (80:20 v/v) by sonication (10 min), followed by centrifugation (5000 rpm) for 10 min. The collected supernatants were pooled together and evaporated to dryness using a SpeedVac. The collected pellet was re-suspended in 1 ml of methanol:water (80:20 v/v) and the mixture was mixed on a vortex for 1 min, then filtered through a PVDF syringe filter (pore size 0.45 μm). The filtered supernatant extract was used for analysis.

The metabolite analysis were performed using an Agilent HPLC (1290) coupled with Agilent MSD (1956B) and diode-array detectors (Agilent Technology, Santa Clara, California, USA). Phenomenex C-18 column (150 × 4.60 mm, Torrance, California, USA) was used for the metabolic separation of analytes. Water and methanol acidified with 1% formic acid were used as mobile phase A and B. The gradient flow was set as follows: 1% B at 0 min and reach 20% at 35 min. 40% at 45 min, 60% at 50 min, 80% at 55 min, 95% at 60 min and reduced to 1% B at 65 min. The flow rate was 0.5 ml/min and the injection volume was 20 μl. Mass spectra were obtained using electrospray ionization in positive and negative ions mode within a mass range setting between 300 and 1200 amu. The operating parameters were as follows: needle voltage is 3500 V; capillary voltage, 70 V; drying gas temperature, 350 °C; drying gas pressure, 30 psi (nitrogen). All extraction and analyses were carried out in six replicates.

For the multivariate analysis, the LC-MS (*.xms) data files were converted to netCDF (*.cdf) using Vx Capture (version 2.1; Adron systems, Adron, USA) software and were aligned using metAlign software (http://www.metalign.nl). The partial least squares discriminant analysis (PLS-DA) was performed by using SIMCA-13 (Umetrics, Umea, Sweden) software. Heatmap for the metabolites comparison was performed using MultiExperiment Viewer (MeV, v4) program and the log values of the corresponding metabolites were used.

2.4. Total phenolic content (TPC) and radical scavenging activity (DPPH) of the pomegranate extracts

For the TPC analysis of the different pome extracts, 20 μl of the extracts were added to 96-well plate, followed by 80 μl of saturated sodium carbonate solution. After ~3 min, 100 μl of 2 N Folin–Ciocalteu's reagent was added. The mixture was incubated at room temperature for 1 h. The absorbance was measured with SPECTRA max plus 384 (Molecular Devices, Sunnyvale, CA, USA)

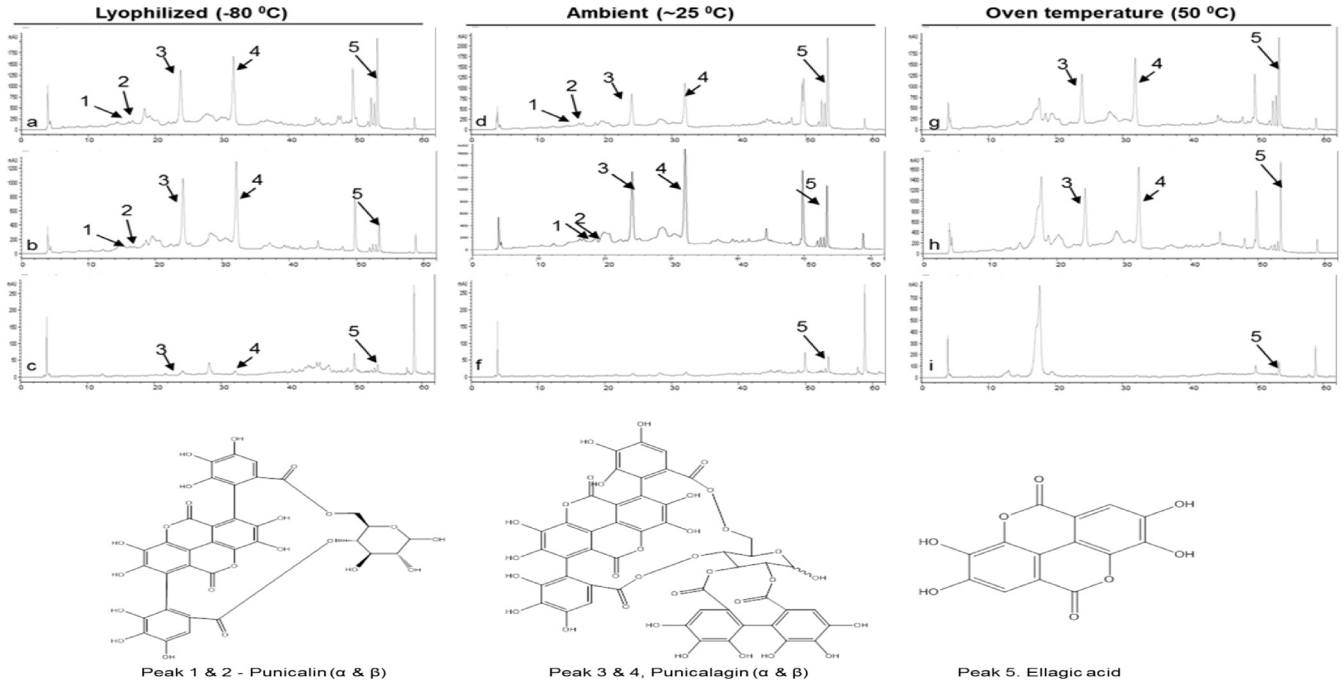


Fig. 1. LC-MS chromatogram of pomegranate peel (a, d, g), inner membrane (b, e, h) and edible portion (c, f, i) processed under three different temperature conditions. No's 1–5 were identified based on the standard compounds retention time and mass details.

Table 1
Identification of the metabolites from pomegranate peel extracts using ultraviolet and mass spectral data.

S. No	[#]Rt (min)	$[M-H]^-$	$[M+H]^+$	MW	λ-max	Name	[*]References
1	16.03 ± 0.06	781	783	782	232, 258, 372	α-Punicalin	1–3
2	16.57 ± 0.06	781	783	782	232, 258, 372	β-Punicalin	1–3
3	23.84 ± 0.06	1083	1085	1084	232, 260, 376	α-Punicalagin	1–3
4	31.80 ± 0.07	1083	1085	1084	237, 260, 378	β-Punicalagin	1–3
5	53.50 ± 0.02	301	303	302	234, 254, 366	Ellagic acid	1–3

[*] 1. Authentic standards; 2. Seeram et al., 2005; 3. Fischer et al., 2011
[#] Rt Retention time.

at 750 nm. At least triplicate analysis was carried out with each extract and the results were expressed in gallic acid equivalent mg/g (John, Jung, Lee, Kim, & Lee, 2013).

The radical scavenging activity of the pomegranate extracts were examined using DPPH assay (John et al., 2013). In brief, 200 mM of DPPH in ethanol served as a source of DPPH radical for the assay. The radical scavenging activity was performed by mixing 20 µl of pomegranate extracts with 180 µl of DPPH and were incubated for 30 min in the dark. Then, the absorbance was taken at 515 nm using 96 well microplate reader (SPECTRA max plus 384, Molecular devices, Sunnyvale, California, USA). The results were expressed in Trolox equivalents (mg/g) of the dried pomegranate fraction.

2.5. Bacterial growth conditions

The *C. rodentium* strain used is a nalidixic acid (nalr)-resistant spontaneous mutant of ATCC strain 51459 (Smith & Bhagwat, 2013). Cultures of *C. rodentium*, nalr were streaked on LB agar plates from freezer stocks, and a single colony was inoculated in LB broth (Difco Chemicals, Detroit, MI) (Bhagwat et al., 2005). For aerobic growth, a single colony of *C. rodentium* was inoculated in 10 ml LB medium in 125 ml flask and incubated in shaker incubator at 210 rpm at 37 °C for 18–20 h (Bhagwat et al., 2005), and then diluted and grown to an OD600 of 1.5.

2.6. Surface swarming and bioactivity of pomegranate extracts

C. rodentium cells were grown overnight and spotted on *N*-minimal media agar plates (0.6%), supplemented with 38 mM glycerol and 10 mM $MgCl_2$ (Sun-Yang, Pontes, & Groisman, 2015). Swarm agar plates were incubated in moist chambers at 37 °C for 8 h and surface swarm diameter was recorded. To test bioactivity of pomegranate fractions, all fractions were diluted in methanol: water (50: 50 v/v) and 25 µl volume was placed at the center of the swarm plate and spread over small circle (r = 1.5 cm). To estimate effect of extra moisture, control plates with 25 µl of water or 25 µl of 50% methanol were prepared. All samples and controls were tested in triplicate.

3. Results and discussion

3.1. HPLC analysis of pomegranate fractions

Fig. 1 depicts a typical chromatogram of the three pomegranate fractions (peels, inner membrane, and aril) dried at three different temperatures (freeze-dried, ambient temperature drying and oven drying at 50 °C) using diode-array detections. The five commonly determined bioactive phytochemicals (α-punicalin and β-punicalin and α-punicalagin, β-punicalagin, and ellagic acid) identified in each fraction are labelled in each chromatogram. Both

peel and inner membrane showed closed similarities in profiles of samples dried under three different conditions. However, the aril portion dried under different condition showed significant difference in the chromatographic profile. Therefore only peels and arils were used for different analysis. The lyophilized peel extracts showed trace quantity of α-punicalin and β-punicalin and higher concentration of α-punicalagin, β-punicalagin, and ellagic acid. Same pattern was observed with inner membrane which showed higher concentrations of punicalagins, ellagic acid, and lower amounts of punicalins. In case of aril portion, the punicalins were not detected and comparatively lower concentrations of punicalagins and ellagic acid were detected. Similar HPLC profile was reported in a recent study by Calín-Sánchez et al. (2013). The authors reported that the amount of punicalagins and ellagic acid were significantly greater in peels as compared to arils portion. The oven drying process significantly modified the metabolic profile. A distinct peak at retention time 17.7 min was observed. It was distinctly noticeable in the oven dried aril extracts (Fig. 1i). Careful analysis showed presence of the same metabolite in oven dried peels and inner membrane extracts (Fig. 1g and h). This peak showed similar UV–visible profile as punicalagins but had different retention time and mass spectral data as compared to punicalins, punicalagins and ellagic acid. This metabolite is currently unidentified. Detail characterization of this metabolite will be carried out after developing preparative scale procedures for purification of this metabolite.

The individual major phytochemicals namely, punicalins, punicalagins and ellagic acid were identified based by comparison with authentic commercial standards, mass and UV spectral data. The identification details are presented in Table 1. Results revealed that the identified metabolites were present in higher concentration in peel as compared to the aril portion (Fig. 2). Similar results were reported by Calín-Sánchez et al. (2013), where higher concentration of punicalagins were observed in peels as compared to the

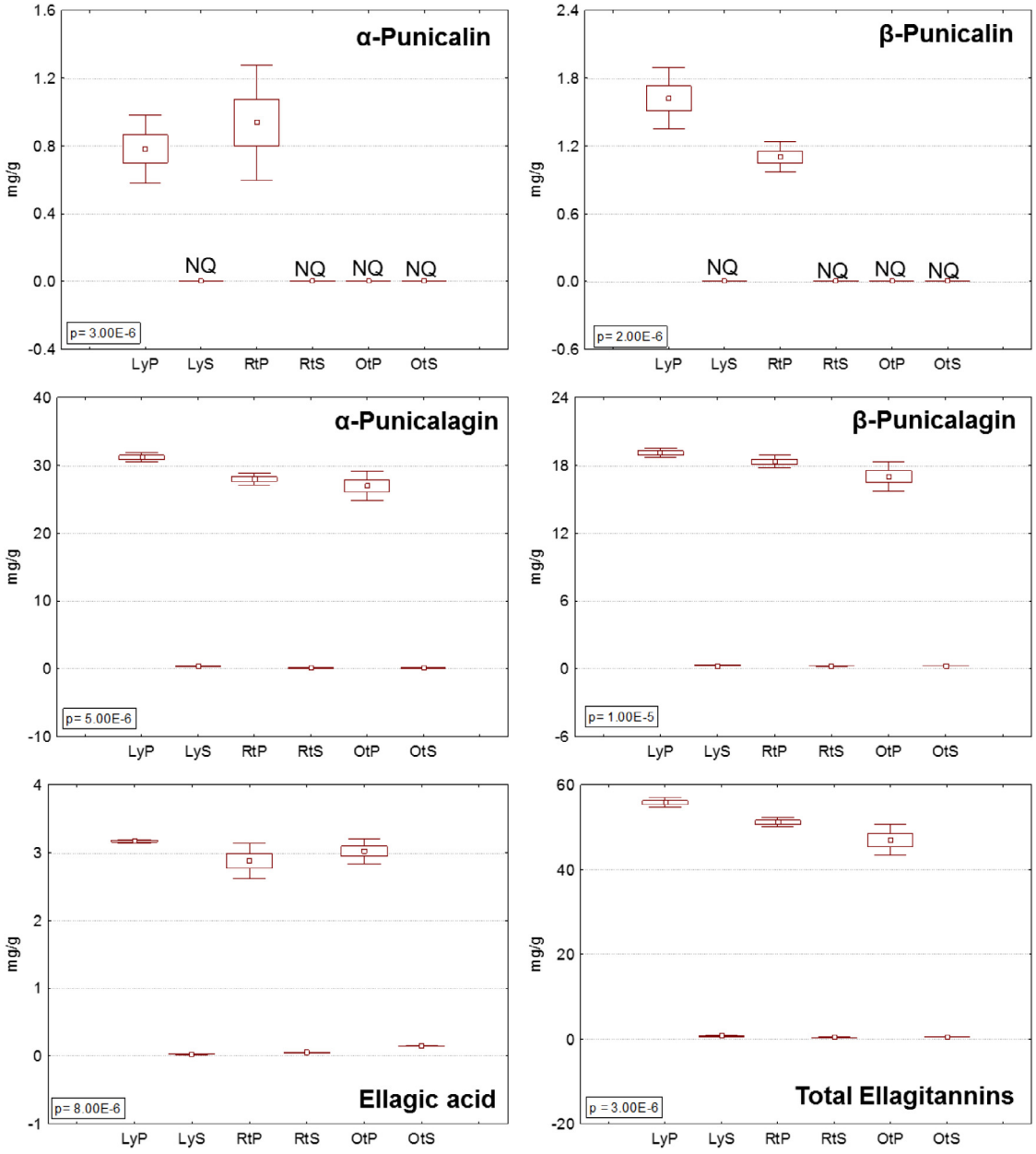


Fig. 2. Punicalins, punicalagins, and ellagic acid content in peels and aril portion of pomegranate samples dried under three different conditions.

arils. Lyophilized peels showed a higher quantity of α and β-punicalins (0.8 mg/g and 1.6 mg/g) as compared to the inner membrane (0.2 mg/g and 0.2 mg/g). In the aril portion of the oven dried samples punicalins were not detected in the quantifiable amounts. Similarly, punicalagins (~38.6–50.3 mg/g) and ellagic acid (~2.8–3.2 mg) were detected in comparatively higher concentrations in peels and the inner membrane portions of all three dried samples, whereas the concentrations of punicalagins and ellagic acids in the aril portion was significantly lower (~0.1–0.3 mg/g).

Most reports found in the literature for the analysis of pomegranate have focused on evaluating juices produced by pressing intact or peeled fruits (Mousavinejad, Emam-Djomeh, Rezaei, & Khodaparast, 2009; Qu, Breksa, Pan, & Ma, 2012), residual husks and rinds (Zhou, Wu, Li, Zhang, & Hu, 2008). Fischer, Carle, and Kammerer (2011) compared the different pomegranate juice with the peel and reported that peels contains ~40 mg/ml of punicalagin A, ~45 mg/ml of punicalagin B and ~98 mg/ml of ellagic acid, whereas the handmade juice contains only ~10 mg/ml of punicalagins and ~3 mg/ml of ellagic acids. Same trend was observed in the present study stating that the peels are rich in punicalagins and the aril portion showed very low concentration of punicalins, punicalagins and ellagic acid. Similar profile has also been recently reported by other researchers (Li, Chen, Jia, Liu, & Peng, 2016).

3.2. Multivariate analysis of pomegranate samples processed under different temperature

The PLS-DA analysis of the LC-MS data obtained from six replicate analysis of the three separated pomegranate fractions samples (arils, peels, and inner membrane) is presented in Fig. 3A. The results showed presence of four distinct clusters. The lyophilized peels and the ambient dried peels clustered as one group. Similarly, the lyophilized and the ambient dried arils and inner membrane clustered together. However, oven dried samples showed two distinct clusters separated from the ambient and the freeze-dried samples. The above results indicated that the drying process significantly influences the phytochemical composition of pomegranate samples. Fig. 3B shows the mass ions of the metabolites from the peels and the aril portion of pomegranate samples. Significant differences were observed between the peel and the aril portion.

Based on the VIP (>0.75 to <1.5) values of the loading plot, the metabolites contributing variations were investigated. Fig. 4 shows a list of 29 metabolites that showed significant variation between three fractions and drying methods. These metabolites were tentatively identified by comparing the molecular and fragment ions in positive and/or negative ion mode, UV–vis spectral data as well as retention time with available authentic commercial standards and published literature data (Seeram, Lee, Hardy, & Heber, 2005; Fischer et al., 2011). The metabolites were compared by plotting a heatmap based on the log values of the peak area obtained from the negative ion mode of the samples. Result revealed that most of the metabolites were present in higher concentration in peel and inner membrane as compared to the edible part.

Most of the previous studies, on pomegranate, focus on identification of different metabolites from peels or pomegranate juice (Li et al., 2016; Mphahlele, Fawole, Makunga, & Opara, 2016; Calín-Sánchez et al., 2013). The metabolites were extracted with varying aqueous-alcohol combinations with a focus on extraction of punicalins, punicalagins, and ellagic acid. Fischer et al. (2011) lyophilized the pomegranate parts (juice, mesocarp, and peel) prior to the extraction with methanol: water (8:2, v/v). Similar metabolites were reported by the same group. In addition, the authors also investigated anthocyanins which were not studied in the present study as the focus of this study was to evaluate the anti-swarming activity of different pomegranate parts processed at three drying temperature.

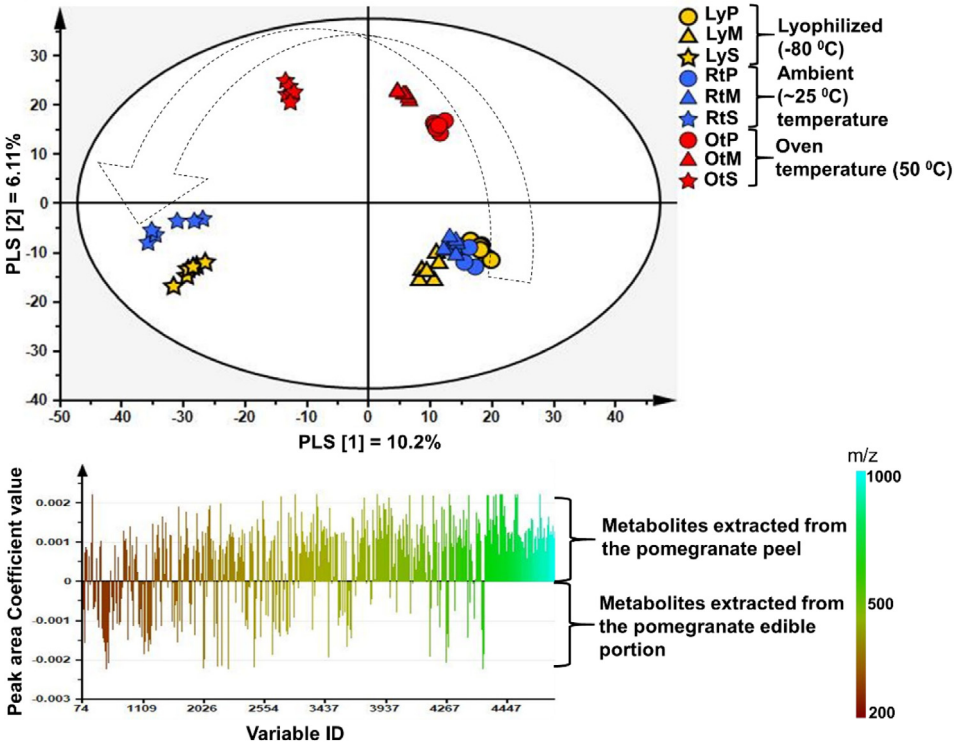
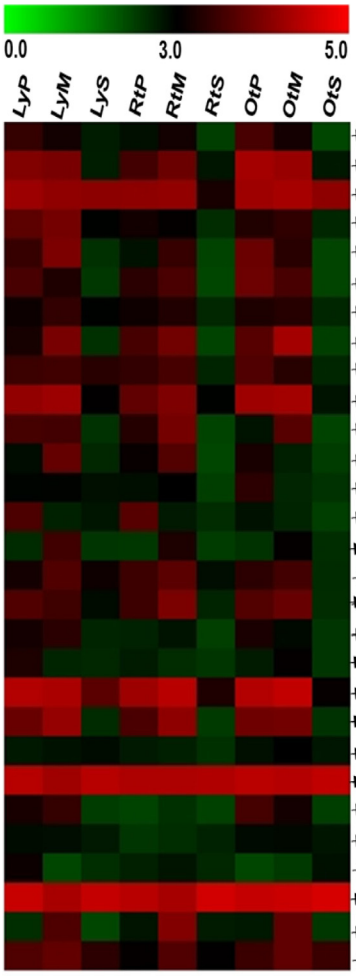


Fig. 3. PLS-DA based clustering pattern of different parts of pomegranate samples analyzed by LC-MS (a), the non-targeted metabolic coefficient plot between the pomegranate peel and edible portion (b).



Rt¥	[M-H]⁻	[M+H]⁺	VIP≠	Name∞
4.2	481	-	0.906	HHDP-hexoside
10.3	649	-	1.009	Lagerstannin C
12.1	331	333	1.325	Monogalloyl-hexoside
14.3	783	-	1.195	Pedunculagin I
16.0	781	783	1.084	α-Punicalin*
16.7	781	783	1.192	β-Punicalin*
18.1	783	-	0.831	Pedunculagin I
23.9	1083	1085	1.284	α-Punicalagin*
24.9	783	785	1.236	Bis-HHDP-hexoside (pedunculagin I)
31.4	469	471	1.075	Valoneic acid bilactone
31.8	1083	1085	0.923	β-Punicalagin*
38.9	801	-	1.153	Punigluconin
41.5	785	-	1.076	Digalloyl-HHDP-hexoside
44.1	1085	-	1.232	Digalloyl-gallagyl-hexoside
44.3	643	645	1.391	Ellagitanin
44.4	633	635	1.130	Galloyl-HHDP-hexoside
46.9	951	953	0.961	Granatin B
47.3	951	953	1.039	Ellagitannin
47.7	468	470	1.072	Valoneic acid bilactone
49.2	785	-	1.188	Digalloyl-HHDP-hexoside
49.6	463	-	0.813	Ellagic acid-hexoside dimme
49.8	463	465	1.405	Ellagic acid hexoside
51.5	953	-	1.239	Ellagitannin
52.2	433	435	0.851	Ellagic acid-pentoside
52.4	447	449	1.373	Ellagic acid-rhamnoside
53.3	301	303	0.851	Ellagic acid*
53.7	593	595	1.400	Ellagitannin
55.1	609	-	0.831	Ellagic acid-(p-coumaroyl)hexoside
55.9	507	-	1.119	Syringetin-hexoside

Fig. 4. Metabolites contributes variations between the PLS-DA clustering patterns of pomegranate samples. ¥retention time; ≠variables imports in projection; ∞putatively identified, *based on standards.

3.3. Total phenolic content and radical scavenging activity of pomegranate extracts processed under different drying conditions

As it is difficult to totally separate inner membrane from the peels and the HPLC profiles of the inner membrane and peels showed close similarities, we compared the total phenolic content, radical scavenging activity, and anti-swarming activity of peels and the arils portion of pomegranate. The total phenolic and the radical scavenging activity of the peel and the aril parts is shown in Fig. 5. The total phenolic content of peels based on Folin-Ciocalteu's reagent varied between 80–96 mg/g gallic acid equivalents. Highest amount of total phenolic content was found in freeze-dried samples as compared to oven and air dried samples where about 15% reduction in TPC was observed. Similar range of TPC of pomegranate peels were reported by Calín-Sánchez et al. (2013) (118 mg/g gallic acid equivalents) and Fischer et al., 2011 (101.9 mg/g gallic acid equivalents). According to Legua, Forner-Giner, Nuncio-Jáuregui, and Hernández (2016), the total phenolic content of the pomegranate varieties varied from 90 to 145 mg GAE/100 ml. About 15% reduction was observed in air and oven dried samples. The radical scavenging activity by DPPH assay also showed similar trend as compared to the TPC. Highest radical scavenging activity was observed with freeze-dried peel samples. About 10–13% reduction in radical scavenging activities were observed with air and oven dried peel samples. This reduction in activity can be attributed oxidation and degradation of polyphenols in air and high temperature. It is well documented in literature that the TPC of fruits and vegetables is reduced at high temperature in presence of air (Maria John, Enkhtaivan, Kim, & Kim, 2014; Vega-Gálvez et al., 2009; Kaur & Kapoor, 2001; Asami, Hong, Barrett, & Mitchell, 2003; Calín-Sánchez et al., 2013). Mphahlele et al. (2016) recently studied the effect of drying process on the bioactive compounds, antioxidants, antibacterial and antityrosinase activity of pomegranate samples. The authors used only the pome peels for their extraction studies. Results revealed that the levels of total phenols, total tannins and total flavonoid contents were high in freeze-dried samples.

3.4. Anti-swarming activity of pomegranate fractions processed under different drying condition

C. rodentium exhibits robust swarming pattern and swarm area diameters ranged ~50–60 mm were observed for blank, 25 µl water or 25 µl methanol. Addition of water or 50% methanol had no statistically significant impact on swarm diameters. Bioactivity of pomegranate peels and aril fractions were measured in terms of maximum dilution at which half-maximal swarm activity was recorded. Both punicalagins and ellagic acid exhibited anti-swarming activity (Table 2a).

Mobility of bacterial communities on wet surfaces plays a significant role in dissemination and survival of foodborne pathogens. On moist surfaces, food-borne pathogens are capable of inducing a dimorphic hyper flagellated state to perform group migration or swarm motility (Harshey & Matsuyama, 1994; Kearns, 2010). In

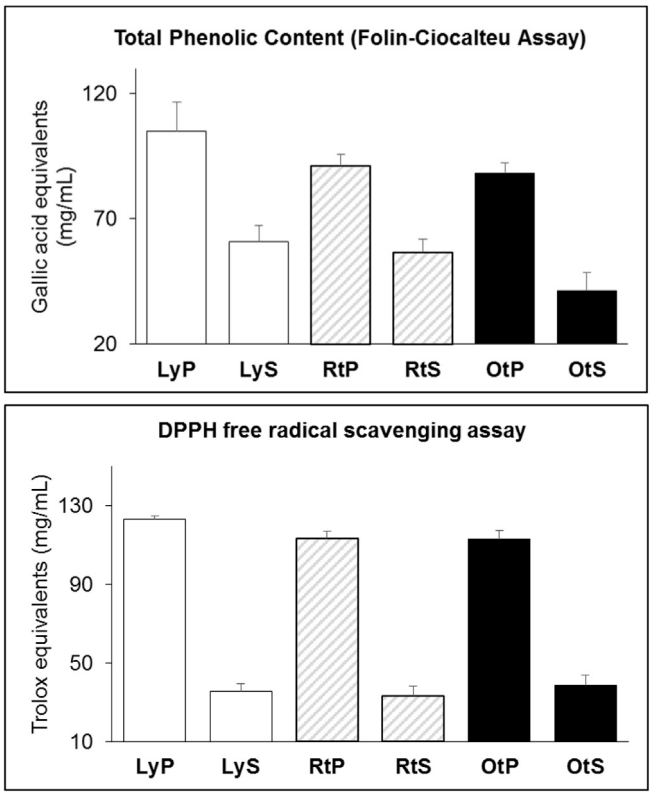


Fig. 5. Total phenolic content and antioxidant potential of pomegranate samples dried under different temperature.

Table 2a
Swarm diameters of *C. rodentium*on *N*-minimal medium supplemented with 0.6% agar after 8 h incubation at 37 °C.

No	Addition to swarm media	Swarm diameter (mm)
1	None	56 ± 6.5**
2	25 µl water	59 ± 5.4**
3	25 µl 50% methanol	58 ± 7.1**
4	Punicalagin (334 µg/ml)	28 ± 7.1
5	Ellagic acid (125 µg/ml)	61 ± 6.1**

Samples were tested in triplicate, average of observed diameters (mm) with standard deviation.
** Indicate that there was no significant difference among different treatment groups ($p < 0.001$).

Table 2b
Bioactivity of various pomegranate fractions using three different extraction procedures on swarming of *C. rodentium*.

	Fruit portion	Treatment prior to extraction	Extract dilutions exhibiting 50% swarm inhibition
1	Peels		
		Lyophilization	1/20–1/40
		Ambient temperature	1/100–1/200
		Hot air (50 °C)	1/75–1/100
2	Arils		
		Lyophilization	1/5–1/10
		Ambient temperature	1/5
		Hot air (50 °C)	1/5

swarm motility bacteria can move on moist surfaces as a group of cells (Harshey & Matsuyama, 1994; Kearns, 2010) and it has been attributed to increased tolerance to stress conditions and resistance to antibiotics (Butler, Wang, & Harshey, 2010). Earlier we demonstrated that pomegranate peel extract inhibited flagella synthesis by inhibiting expression of regulatory genes belonging to Class-II and III, but not the Class-I (Mahadwar et al., 2015). The major phytochemical component classes identified to date in pomegranate fruit are anthocyanins and hydrolysable tannins, i.e. ellagitannins (Lipinska, 2014). However, the importance of ellagitannins is unclear since only a few studies have been conducted specifically testing them for antimicrobial activity (Bakkiyaraj et al., 2013; Haghayeghi, Shetty, & Labbe, 2013; Ismail, Sestili, & Akhtar, 2012; Lim, Penesyan, Hassan, Loper, & Paulsen, 2013). Consequently, large information gaps still exist with regards to isolation protocols and bioactive ingredients. Results revealed that the peel registered high anti-swarming activity as compared to the aril portion with all three drying methods. These are in agreement with the total phenolic content, radical scavenging activity as well as the total concentration of punicalins, punicalagins and ellagic acid that were quantified in higher concentration in peels as compared to the aril. Antimicrobial activity of the pomegranate metabolites were reported by various researchers in different bacterial systems. Lali Growther, Sukirtha, Savitha, and Niren Andrew (2012) reported that the pomegranate extract rich in punicalagins and ellagic acid metabolites showed high antimicrobial activity against Shiga toxin-producing *E. coli*. Glazer et al., 2012 reported negative correlation between punicalagin content against *Alternaria alternata*, *Stemphylium botryosum*, and *Fusarium* species, exposed to aqueous extracts from pomegranate peel. In general, the bioactivity of pomegranate peel depends on their ellagitannins metabolites content. Rosas-Burgos et al., (2017) revealed antimicrobial activity from three different cultivars (sweet, sour–sweet and sour) of pomegrante peels. Among the three types, the sour–sweet cultivars showed high antimicrobial activity due to the presence of high ellagic acid content. This may be due to the interaction between hydrophilic parts with the polar region of the membrane (Betanzos-Cabrera et al., 2015). Instability of the bacterial membrane may affect the substrates transportation as well as swarming of the bacteria.

The anti-swarming activity of peels dried under different drying conditions varied significantly with peels dried at ambient condition showing higher activity as compared to oven and freeze-dried samples (Table 2b). Similarly, the freeze-dried aril portion also showed lower activity as compared to air and oven dried samples. The trend for the anti-swarming activity was different from the concentrations of total phenolic content, radical scavenging activity as well as the total concentration of punicalagins and ellagic acid that were quantified in higher concentration in freeze-dried peels and arils as compared to the oven and air dried samples. These results indicated that the bioactivity may also be associated with other phytochemicals other than punicalagins and ellagic acid. Thus a detailed bioassay guided fractionation is needed to further investigate other bioactive phytochemicals present in pomegranate peels and arils.

4. Conclusions

Pomegranate peels provide a rich source of bioactive phytochemicals, total phenolic content, and radical scavenging potential as compared to the aril portion. Multivariate analysis of the LC-MS data discriminated the pomegranate peel, inner membrane, and edible aril portion, as well as the three different drying methods based on 29 metabolites. The quantity and profiles of the bioactive phytochemicals in peels are significantly influenced by the pomegranate fractions, as well as the drying method used during processing. All oven dried fractions showed the presence of unidentified peak at retention time ~17.7 min that was not present in samples freeze-dried and ambient-temperature-dried samples. The total phenolics content anti-swarming and antioxidant

activities were influenced by pomegranate fractions and drying conditions. The results warranted a need for carrying out a detail bioassay guided fractionation to identify other bioactives present in pomegranate peels.

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