Assessing the Efficacies of Phenolic Compounds in Pomegranate Juice Using Thin-Layer Chromatography

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Summary. Pomegranates (*Punica granatum*) have been known for centuries for their healing properties. The phenolic components of pomegranate are believed to be responsible for their antioxidant activities, hence playing a major role in reducing oxidative stress-related disease, such as cardiovascular diseases, cancer, and neurodegenerative diseases. In this study, thin-layer chromatography (TLC) was used to, first, separate and identify the phenolic constituents of pomegranate juice and, second, to assess the antioxidant efficacies of the identified compounds. Different oxidant and hydroxyl radical generating systems (Fe$^{3+}$, Cu$^{2+}$, H$_2$O$_2$, Fe$^{2+}$–H$_2$O$_2$, and Cu$^{2+}$–H$_2$O$_2$) were used in assessing the efficacies of phenolic compounds found in pomegranate juice. A 10 × 10 cm and 20 × 20 cm sized silica gel 60 F 254 TLC plates with toluene–ethyl acetate–formic acid (60:40:10 v/v/v) as a mobile phase were used for the chromatographic separation. Two compounds, ellagic acid and gallic acid, were separated and identified. When pomegranate juice was challenged with the oxidant systems, it was observed that the phenolic compounds slowly disappeared in a concentration- and time-dependent manner. From the results, it was concluded that gallic acid had a higher antioxidant efficacy than ellagic acid. TLC has been applied for the first time to outline the antioxidant profile of pomegranate juice and assess the efficacies of phenolics using different oxidant systems, including redox-active metals and H$_2$O$_2$.

Key Words: pomegranate juice, thin-layer chromatography, hydrogen peroxide, metal ions, antioxidants

Introduction

Pomegranates (*Punica granatum*) have been viewed as an important fruit for many centuries in several different cultures and religions [1]. Its curative properties have been discussed in one of the prehistoric medical texts, *Ebers papyrus* [2]. In Judaism, it is believed that each pomegranate has 613 seeds representing the 613 commandments of the Torah [1]. Current research on pomegranate has attempted to identify the basis of its healing properties, with a considerable number of studies conducted to uncover the antioxidant [3], antimicrobial [4], and anti-malarial [5] activities.
Pomegranates contain a wide range of phenolic compounds which are considered to be responsible for its antioxidant, anti-cancer, anti-inflammatory, and antimicrobial properties [6]. The most common polyphenols present in pomegranates and their juices are anthocyanins, such as delphinidin 3,5-diglucoside, cyaniding 3-glucoside, and delphinidin 3-glucosides; hydrolysable tannins, such as ellagitannins, and gallotannins; hydroxybenzoic acids, such as gallic acid; and hydroxycinnamic acids, such as caffeic acids [7–9]. The antioxidant properties of these compounds are attributed to a key role in the prevention of oxidative stress-related diseases, including cardiovascular diseases, different cancer types, diabetes, and neurodegenerative diseases, such as Parkinson’s and Alzheimer’s diseases [10].

Oxidative stress is a state where there is an imbalance between production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) on one hand and their removal and repair of damaged complex molecules (e.g., proteins or DNA) through the natural antioxidant defence system on the other [10]. Superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (•OH), nitric oxide (NO•), and peroxynitrite anion (ONOO•) are considered as some of the most common reactive oxygen and nitrogen species (RONS) in the body [10, 11].

Many studies have been carried out to investigate the presence of metal ions in a variety of different beverages and food products [7, 12]. In the presence of redox-active metal ions, especially free copper or iron ions, hydroxyl radicals can be generated by two different chemical reactions. First, H$_2$O$_2$ can produce a hydroxyl radical (•OH) by removing an electron from the participating metal ion. Second, when superoxide radicals are involved, metal ions are regenerated so that they would be available again to react with H$_2$O$_2$. These two chemical reactions together seem to be responsible for most of the hydroxyl radical generation in biological systems [11]. This explains why some metals, such as iron and copper cause oxidative stress, and RONS induced damage to cells. The natural antioxidant protective enzymes, such as superoxide dismutase (SOD), catalase, and glutathione peroxidise, are involved in the removal of RONS. SOD catalyzes the removal of superoxide radicals, whereas catalase and glutathione aid the removal of H$_2$O$_2$. The phenolic components of pomegranates and their juices are non-enzymatic antioxidants which exert their efficacies by acting as scavengers of RONS, through hydrogen donation and metal ion-chelating activities to prevent hydroxyl radical formation [12].

Several studies have been carried out to investigate the antioxidant activities of pomegranates with the peel/mesocarp/arils and variants of the juices being assessed using antioxidant assays, such as the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, reducing power assay, 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assay and H$_2$O$_2$...
scavenging activity assays [3]. Thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) have also been used to investigate the phenolic profiles of pomegranates and their products [3, 7–9]. However, there have been no reports of the application of TLC to rank the different phenolic compounds identified in pomegranates using challenge with varied oxidant systems.

The aims of this study were, first, to separate and identify the phenolic compounds in pomegranate juice using TLC and, second, to observe the reaction upon challenge with metal ions, hydrogen peroxide, and hydroxyl radical generator model systems (Fe^{2+}, Fe^{3+}, Cu^{2+}, H_{2}O_{2}, Fe^{2+}-H_{2}O_{2}, Cu^{2+}-H_{2}O_{2}) in order to be able to assess the antioxidant efficacies of pomegranate juice.

**Experimental**

**Chemicals and Materials**

Analytical grade toluene, formic acid, ethyl acetate, chlorophorm, methanol, and acetic acid; 10 × 10 cm and 20 × 20 cm TLC glass-backed silica gel 60 F_{254} (0.25 mm in thickness); ferrous chloride tetrahydrate; ferric chloride dihydrate; cupric chloride dihydrate; hydrogen peroxide 30% (w/w) in water; DPPH; phenolic standards (gallic acid and ellagic acid); 2-aminoethyl diphenyl-borinate and polyethylene glycol were bought from Sigma-Aldrich (Poole, UK). Pomegranates grown and cultivated in California, United States, were used for the pomegranate juice purchased from a local supermarket.

**Pomegranate Juice Sample and Phenolic Standards**

Pomegranate juice (2 mL) was stirred in a glass test tube using a vortex mixer prior to spotting on the plates. The pomegranate juice was kept at −4°C before analysis, and the sample was used within 4 days after the bottle had been opened for the first time. Phenolic standard solutions were prepared with a concentration of 1.0 mg in 10 mL of methanol and were stored in glass sample tubes [13]. The standards were applied as 2.0-μL spots using Gilson micropipettes in duplicate on both sides of the central pomegranate juice sample to prevent parallax error. After chromatographic separation, gallic acid and ellagic acid were sprayed with NP/PEG and viewed under long wave UV were observed as dark blue and light blue, respectively. There $R_F$ values were taken from a mean of three replicate TLC plates (Table I).
TLC and Identification of Phenolic Compounds in Pomegranate Juice

The pomegranate juice sample was applied in triplicate in the middle of the plates as 2-, 4-, and 6-μL spots. This helped in visualizing some of the antioxidants clearly and also acted as a replicate spot on the same plate. The phenolic standards were applied as 2-μL spots on both sides of the pomegranate juice spots. The TLC plates were developed in room temperature using an all-glass TLC tank. Toluene–ethyl acetate–formic acid (60:40:10 v/v), a modified version of Bagul et al. [14], was used as a mobile phase. The mobile phase was left to equilibrate for an hour in the developing tank. The 10 × 10 cm plates were run to a distance of 8.5 cm, and the 20 × 20 cm plates were run to a distance of 18.5 cm.

TLC and Pomegranate Juice in the Presence of Oxidant Systems

The pomegranate juice sample was incubated for 180 min with hydroxyl radical-forming oxidant systems Fe$^{2+}$–H$_2$O$_2$ and Cu$^{2+}$–H$_2$O$_2$ at concentrations of 0–1.76 mM using TLC plates (20 × 20 cm) to compare and contrast the loss of antioxidants once challenged with Fe$^{2+}$–H$_2$O$_2$ and Cu$^{2+}$–H$_2$O$_2$. For each test sample, 0.1 mL of an equimolar H$_2$O$_2$/metal salt solutions (with the concentrations of 0, 0.22, 0.44, 0.88, 1.76, and 3.52 mM) were added to 0.1 mL of the pomegranate juice for incubation in glass sample tubes. The plates were spotted with 2 or 6 μL of test sample as indicated. After the plates had been developed, they were left to dry and were visualized under short wave UV light at a wavelength of 254 nm and long wave UV light at a wavelength of 366 nm. Before viewing the plates under long wave UV, they were sprayed with 1% methanolic 2-aminoethyl diphenyl-borinate, followed by 5% ethanolic polyethylene glycol. DPPH (0.04% methanolic solution) was also used to spray the TLC plate in order to facilitate the identification of antioxidant loss in the presence of oxidant systems.

Investigation of Metal Ions

Inductively coupled plasma mass spectrometry (ICP-MS) used an Agilent Technologies 7700 Instrument. Prior to analysis, a one in fifty dilution was made with distilled and deionized water. Measurements were taken in triplicates.
Results and Discussion

Pomegranate Juice and Phenolic Standards

The two different components separated in the pomegranate juice were identified as gallic acid and ellagic acid, using phenolic standards which are reported in previous literature [15]. These two compounds were identified using the mobile phase: toluene–ethyl acetate–formic acid (60:40:10 v/v/v). Three compounds were separated using the mobile phase: chloroform–methanol–acetic acid (50:35:15 v/v/v), one of which was identified as gallic acid, whereas the other two were unidentified despite using several different phenolic standards mentioned in previous studies (data not shown). Various different eluent systems were used with different compositions in

![Fig. 1. This chromatogram illustrates the standards after run with a large TLC plate (20 × 20 cm) and sprayed with NP/PEG. The separation can be visualized much more clearly, especially with the 6-μL PJ sample spot. Toluene–ethyl acetate–formic acid (60:40:10 v/v) was used as a mobile phase (Abbreviations: GA = gallic acid; EA = ellagic acid; PJ = pomegranate juice)
an attempt to optimize the separation of the compounds; however, the best resolution was obtained using the mobile phases mentioned above. Results for the separation and characterization can be seen in Table I and the chromatogram (Fig. 1).

**Table I.** Description and chemical structures of standards used to identify components present in pomegranate juice

<table>
<thead>
<tr>
<th>Phenolic standards</th>
<th>UV; $\lambda = 366$ nm</th>
<th>$R_f$ value</th>
<th>Structure of compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ellagic acid</td>
<td>Light blue</td>
<td>0.38</td>
<td><img src="image1" alt="Structure of Ellagic Acid" /></td>
</tr>
<tr>
<td>Gallic acid</td>
<td>Dark blue</td>
<td>0.44</td>
<td><img src="image2" alt="Structure of Gallic Acid" /></td>
</tr>
</tbody>
</table>

**Interaction of Pomegranate Juice with H$_2$O$_2$**

Both ellagic acid and gallic acid were present after a 180-min incubation at all concentrations (data not shown). This could be caused by a few different reasons. First, the incubation period could have been too short; hence, no changes in the zones were observed. Second, it could be due to the fact that H$_2$O$_2$ is a poor oxidant system as a result of lack of interaction with metal ions or, finally, it could be due to ellagic and gallic acids not being great H$_2$O$_2$ scavengers.
Seemungal et al. found that after 240-min incubation with H₂O₂ and at a concentration of 50 mM, a loss of gallic acid was seen in red wine [13]. This could be a possible explanation for not observing the loss in either of the phenolic compounds. If the incubation period had been increased to 240 min, a possible loss in gallic acid could have been observed.

**Interaction of Pomegranate Juice with Fe³⁺ and Cu²⁺**

Following 180-min incubation with Fe³⁺, the ellagic and gallic acid zones diminished gradually with increasing concentrations but did not disappear completely (data not shown). At concentrations of 40–50 mM, a near to complete loss of both compounds was seen. This fading out could possibly be as a result of metal chelating properties of gallic acid and ellagic acid.

On addition of Cu²⁺ ions, the ellagic and gallic acid zones fade out at concentrations of 30–40 mM (data not shown); however, the change is not as drastic as seen with Fe³⁺ ions. The antioxidant efficacies of ellagic and gallic acids in sequestering of Fe³⁺ are greater in comparison to Cu²⁺. When comparing the efficacies of ellagic acid and gallic acid, they both seem to have similar efficacies in the metal ion systems.

![TLC plate showing the interaction between PJ and Cu²⁺–H₂O₂/Fe²⁺–H₂O₂ after 180 min incubation.](image)

Fig. 2. 20 × 20 cm TLC plate showing the interaction between PJ and Cu²⁺–H₂O₂/Fe²⁺–H₂O₂ after 180 min incubation. Lanes 1–6 represent 0, 0.11, 0.22, 0.44, 0.88, and 1.76 mM concentrations of Cu²⁺–H₂O₂/Fe²⁺–H₂O₂. It can be seen that gallic acid has diminished first in both cases. Ellagic acid was lost second, as can be seen in the Fe²⁺–H₂O₂ hydroxyl radical generating system.
Interaction of Pomegranate Juice Compounds with Fe$^{3+}$–H$_2$O$_2$ and Cu$^{2+}$–H$_2$O$_2$

Figure 2 illustrates the hydroxyl radical forming systems using 20 × 20 cm TLC plates. After a 180-min incubation with Fe$^{2+}$–H$_2$O$_2$, a complete loss of gallic acid and ellagic acid can be seen at a concentration of 0.5 mM and above. At a concentration of 0.5 mM and above Cu$^{2+}$–H$_2$O$_2$, loss of gallic acid can be seen, but ellagic acid is still present at all concentrations. This suggests that gallic acid has a higher efficacy in comparison to ellagic acid. The hydroxyl radical generating model systems proved to be the best oxidant system in assessing the efficacies of the phenolic compounds identified in pomegranate juice.

Metal Ion Analysis

The metal ion analysis detected 11 different elements are shown in Fig. 3. Previous studies have found similar results confirming the presence of high concentrations of potassium and sodium [7]. The levels of metal ions reflect those previously reported for a large range of pomegranate varieties [16]. K, Ca, Mg, and Na predominated with Cu, Ni, Fe, and Mn in the range of 5–25 ppm.

Gallic Acid and Ellagic Acid

Gallic acid and ellagic acid belong to the hydroxybenzoic acid chemical class and are part of a group of different polyphenolic compounds found in various plants and fruits including pomegranates [15, 17, 18]. They exert their antioxidant efficacies by delaying, inhibiting, or preventing oxidation of compounds and hence reducing the risk of oxidative stress-related diseases. Gallic acid has three hydroxy groups (Table I) which are thought to be responsible for its high radical scavenging properties [17]. Ellagic acid has four phenolic groups and two lactones (Table I), which could possibly act as electron acceptors and hence play a role in antioxidant properties of the compound [18]. Several studies have reported beneficial properties for both compounds ranging from antioxidant, anticancer, antimicrobial to its effective properties in neurodegenerative diseases [14, 17, 18, 19].
Fig. 3. Graphs illustrating the major (top) and minor (bottom) elements present in pomegranate juice. Quantification carried out using ICP-MS with a 1 in 50 dilution of pomegranate juice made before analysis. Bars represent 95% confidence intervals.
Conclusions

In conclusion, two antioxidants were identified using TLC with toluene–ethyl acetate–formic acid (60:40:10 v/v/v) as mobile phase: ellagic acid and gallic acid. The identified compounds antioxidant efficacies were assessed using different oxidant systems. In the hydroxyl radical generating systems, it was found that gallic acid has a higher antioxidant efficacy in comparison to ellagic acid. Future research should involve the identification of the two unknown zones separated using the mobile phase chloroform–methanol–acetic acid (50:35:15 v/v/v) followed by challenging the unknowns with the different oxidant systems used to eventually be able to rank all four compounds in order of high antioxidant efficacy to low. Also, high-performance thin-layer chromatography (HPTLC) could be used to obtain better separation and resolution with the pomegranate juice sample and allow additional identification and characterization of the antioxidant efficacies. This simple yet valuable study supports the health benefits of pomegranates through their antioxidants; however, care should be taken when extrapolating these findings to complex biosystems.

Acknowledgments

We thank Julian Swinden for carrying out the metal analysis.

References


Accepted by MWH