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# Antioxidant and anti-diabetic activities in commercial and homemade pomegranate molasses in Lebanon

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## Abstract

Pomegranate fruit and its derived products are rich sources of bioactive compounds. They have many biological activities. Pomegranate molasses (PM) is a thick, traditional Middle Eastern syrup used in many recipes for Lebanese and international cuisines. It is a highly nutritious product which makes it of great interest. The purpose of this study was to assess the phytochemical composition, antioxidant and anti-diabetic properties of homemade and commercial PM consumed in Lebanon using samples collected from households in rural areas (n=4) and the market (n=28). The objectives of this study were to determine their total phenolic content using the Follin-Ciocalteu method, their total flavonoid content using aluminum chloride method, their antioxidant activity using DPPH radical scavenging as well as ferrous ion chelating assays and their anti-diabetic activity using  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activities with acarbose, a known anti-diabetic drug, used as the standard reference. Homemade PM samples exhibited a higher antioxidant activity than commercial samples, with the most active homemade PM sample having IC<sub>50</sub> values of 0.09 mg/mL (DPPH radical scavenging assay) and

46.78% ferrous ion chelating assay). Homemade PM samples also showed higher anti-diabetic activity than commercial samples, with the most active homemade PM sample having an IC<sub>50</sub> of 0.63 mg/mL ( $\alpha$ -amylase inhibitory activity) and of 0.41 mg/mL ( $\alpha$ -glucosidase inhibitory activity) and being almost as active as acarbose with both enzymes (1.5 times).

Significant inverse strong/moderate correlations were observed between total phenolic content/total flavonoid content and the IC<sub>50</sub> value of DPPH radical scavenging assay, indicating positive associations between total phenolic and flavonoid contents and antioxidant activity. In addition, significant negative moderate correlations were observed between total phenolic/flavonoid contents and  $\alpha$ -amylase inhibitory activity, indicating positive associations between total phenolic and flavonoid contents and anti-diabetic activity.

## **Keywords**

Pomegranate molasses; antioxidant activity; ferrous ion chelating assay; anti-diabetic activity;  $\alpha$ -amylase inhibitory assay;  $\alpha$ -glucosidase inhibitory assay

## **1. Introduction**

Fruits are rich sources of bioactive compounds with antioxidant potentials associated with diverse medicinal properties and health benefits (Li et al., 2006). Recent studies have tackled those health benefits along with the composition and biological activities of fruits such as pomegranate and its derived products (Derakhshan et al., 2018).

Phytochemicals and antioxidants abundantly present in fruits including flavonoids, tannins, and phenolic acids are known to help prevent several life-threatening diseases such as cardio and cerebro-vascular diseases and different types of cancers ( Li et al., 2006; Derakhshan et al., 2018). Bioactive compounds with antioxidant capacities dispel free radicals from cells to prevent lipid peroxidation reactions and oxidative damage in order to preserve the cell structure and function also to prevent food deterioration (Zou et al., 2016).

Oxidative stress is the underlying cause of many chronic diseases, in particular *diabetes mellitus*, as it is associated with its onset and progression (Rains & Jain, 2011). *Diabetes mellitus* (types 1 and 2) is a metabolic syndrome characterized mainly by hyperglycemia due to defects in the secretion and/or action of insulin with type 2 *diabetes mellitus* being the most common type spread worldwide. It is estimated that the number of type 2 *diabetes mellitus* patients in the World will increase from 415 million patients in 2015 to 642 million patients in 2040 (Khajebishak et al., 2019). Since type 2 diabetes is mainly linked to unhealthy dietary habits and obesity, consuming more of the natural bioactive compounds found in fruits and vegetables can prevent it's development (Rains & Jain, 2011; Li et al., 2019).

Pomegranate (*Punica granatum* L.) is an ancient fruit that belongs to the family Punicaceae and is widely grown in tropical and subtropical regions of the World. It is cultivated in Iran, India and Mediterranean countries such as Turkey, Tunisia, Egypt, Morocco and Lebanon. It is commonly consumed in this region with its derived products such as pomegranate molasses (Incedayi et al., 2010; Erdrich et al., 2015; Faour-Klingbeil & Todd, 2018).

Since ancient times, pomegranate has been broadly used as a “healing food” in many cultures, being part of folk medicine, to treat parasites and worms infections, reduce fever and heal ulcers, diarrhea, aphthae, acidosis, hemorrhage, dysentery, respiratory pathologies and microbial

infections (Akpınar-Bayızit et al., 2016). It has also been used as traditional remedy for other illnesses like cancer, dental problems, bacterial infections, skin damage caused by ultraviolet radiations male infertility, Alzheimer's disease, arthritis, obesity and infant brain ischemia (Jurenka, 2008). In Lebanon, pomegranate extract has also traditionally been used in the treatment of *diabetes mellitus* (Raafat & Samy, 2014).

Due to the quick spread of cultivation of the pomegranate fruit across the World and its widespread use in folk medicine, there has been a growing interest in identifying the active secondary metabolites and biological activities of the different parts of this fruit. Studies showed that the most common identified metabolites in the pomegranate fruit are ursolic acid and punicalic acid in pomegranate seeds, hydroxycinnamic acids and ellagitannins (punicalin and punicalagin) in pomegranate juice and peels, apigenin in the fruit leaves and maslinic acid in the flowers; in addition to other phenolic compounds, anthocyanins, hydrolysable tannins and flavonoids (Lansky & Newman, 2007; Incedayi et al., 2010; Derakhshan et al., 2018). Pomegranate molasses is a highly nutritious product since it is made by boiling down fresh pomegranate juice into a thickened syrup and would, therefore possess higher mineral and antioxidant content than fresh pomegranate juice (Yilmaz et al., 2007). Pomegranate fruit and its parts were shown to have antioxidant, anti-diabetic, anti-inflammatory, anti-carcinogenic, anti-microbial, anti-diarrheal and neuro-protective activities (Elfalleh et al., 2009; Hontecillas et al., 2009; Endo et al., 2010; Sturgeon & Ronnenberg, 2010; Bekir et al., 2013; Zhao et al., 2018). Very few studies were conducted to determine the antioxidant activity of pomegranate molasses, where this activity was associated to its high phenolic content and only one study was done to reveal its neuro-protective capacity (Derakhshan et al., 2018; Hussein et al., 2018).

PM is a famous traditional condiment used in Lebanon with many commercial brands distributed in the market in addition to homemade pomegranate molasses made mostly in rural areas. Only few commercial brands have been evaluated in terms of total phenolic content and antioxidant activity but to the best of our knowledge, pomegranate molasses has not been tested for its flavonoid content and anti-diabetic activity yet. Therefore, the purpose of this study was to assess the phytochemical composition, antioxidant and anti-diabetic capacities of homemade and commercial pomegranate molasses. The objectives of this study were to:

- 1) Determine the total phenolic content of PM (commercial and homemade) samples using the Follin-Ciocalteu method.
- 2) Determine the total flavonoid content of PM (commercial and homemade) samples using the aluminum chloride method.
- 3) Assess the antioxidant activity of PM (commercial and homemade) samples using DPPH radical scavenging as well as ferrous ion chelating assays.
- 4) Assess the anti-diabetic activity of PM (commercial and homemade) samples using  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory activities.

## **2. Materials and Methods**

### **2.1. Chemicals**

Folin-Ciocalteu's phenol reagent, sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), gallic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, quercetin, ethylene-diamine-tetra-acetic acid (EDTA), 3-[2-Pyridyl]-5,6-diphenyl-1,2,4-triazine-4,4'-disulfonic acid monosodium salt hydrate (Ferrozine), sodium nitrite  $\text{NaNO}_2$ , aluminium chloride  $\text{AlCl}_3$ , sodium hydroxide  $\text{NaOH}$ ,  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes, potassium tartrate, sodium phosphate, sodium chloride, 4-Nitrophenyl- $\beta$ -D- glucopyranoside (P-NPG), starch and acarbose were purchased from Sigma-

Aldrich Co (Steinheim, Germany).  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  was purchased from Merck (Darmstadt, Germany). Ultra-pure water was used for ferrous ion chelating assay. All other reagents and organic solvents used were of analytical grade.

## **2.2. Raw material - Sampling**

Pomegranate molasses samples were produced in the fall of 2018 and collected in the spring of 2019. A total of twenty-eight commercial PM bottles were selected from almost all commercial PM brands available in major supermarkets in Mount Lebanon including Shouf Area, Aley, Metn and Baabda districts. The commercial brands are: Al Barakah, Al Rabih, Annabil, Al Wadi Al Akhdar, Aoun, Ashka, Baydar, Boulos, Chtoura Gardens, Chtoura Fields, Cortas, Gardenia, Houkoul Khadraa, Jana, Jeita, Kortbawi, Lumiere, Maxim's, Maymouna, Mechaalani, Mrouj Chtoura, Monzer Basha, Salloum, Spinneys, Taj, Tiba, Terrois, Yamama. There was no clear explanation on the bottles of the commercial samples about the manufacturing processes. A total of four homemade PM bottles were selected from randomly selected households in Mount Lebanon (one/household). The homemade samples were traditionally and manually produced by cleaning the pomegranate fruits, crushing it to extract the pomegranate juice, filtrating and then concentrating the juice by boiling and evaporation in open containers without adding further sugar, additives or citric acid.

All collected PM bottles were previously unopened airtight bottles. Prior to the analyses of the commercial and homemade PM samples, the collected bottles were stored between 0-4 °C for one month. At the time of the analyses, samples were taken from the stored bottles ( $n_{\text{homemade}} = 4$ ,  $n_{\text{commercial}} = 28$ ). PM samples were diluted as follows: PM was weighed in a 10 mL volumetric flask where 4 mL of ethanol were added. The mixture was vortexed for 1 min and then distilled

water was added until the 10 mL mark. These diluted PM samples were used in all the experiments.

### **2.3. Total phenol content for pomegranate molasses**

Total phenols in the diluted PM samples were assessed by a modified Folin-Ciocalteu method (Koivikko et al., 2005). Briefly, 0.5 mL of the diluted sample was mixed with 0.5 mL of 1 N Folin-Ciocalteu reagent. The mixture stood for 3 min, after which 1 mL of 20% sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) was added. Samples were incubated in the dark at room temperature for 45 min then centrifuged (5 min at 2400 g). Absorbance of the supernatants was measured at 730 nm using a Jenway 6405 UV/Vis spectrophotometer. Total phenolic content was expressed as mg of gallic acid equivalents (GAE) per 100 mg of sample. All measurements were performed in duplicate.

### **2.4. Total flavonoid content for pomegranate molasses**

Total flavonoids in the diluted PM samples were estimated using the modified aluminum chloride method (Shams Ardekani et al., 2011) where quercetin was used as a standard and the flavonoid content was measured as quercetin equivalent. An aliquot (1 mL) of sample or standard solution of quercetin (20, 40, 60, 80 and 100 mg/L) was added to 10 mL volumetric flask containing 4 mL of double distilled water. Then 0.3 mL 5%  $\text{NaNO}_2$  was added to the flask and after 5 min, 0.3 mL  $\text{AlCl}_3$  (10%) was also added. At the 6<sup>th</sup> min, 2 mL NaOH (1 M) was added, and the total volume was made up to 10 mL with double distilled water. The solution was mixed and the absorbance was measured versus prepared reagent blank at 510 nm. Total flavonoid content was expressed as mg of quercetin equivalents (QE) per 100 mg of sample. All measurements were performed in duplicates.



## **2.5. Bioactive potentials**

### **2.5.1. DPPH radical scavenging assay**

The scavenging activity of the PM diluted samples for DPPH radical was determined by the method of Gow-Chin and Hui-Yin with slight modifications (Gow-Chin & Hui-Yin, 1995). Serial further dilutions of the PM diluted samples were prepared in ethanol (EtOH). The basic procedure was to add an aliquot (1 mL) of test sample to 1 mL of DPPH 0.15 mM EtOH solution. The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark. The absorbance was read at 517 nm using a UV/Vis spectrophotometer, and the calculations of the scavenging activity (SA in %) was as follows: SA (%):  $[1 - (A_{\text{sample}} - A_{\text{sample blank}}) / A_{\text{control}}] \times 100$ . One mL of sample solution and 1 mL of EtOH was used as a sample blank. One mL of DPPH solution and 1 mL of EtOH was used as a negative control. Ascorbic acid was used as the positive control. Stock solutions of ascorbic acid (0.8 mg/mL) were diluted with EtOH to give concentrations ranging from 1.5 to 20  $\mu\text{g/mL}$ . All measurements were performed in triplicate.

### **2.5.2. Ferrous ion chelating assay**

The ferrous ion chelating activity was determined according to (Lim et al., 2007). Equal volumes of 0.12 mM  $\text{FeSO}_4$ , test sample of diluted PM at different concentrations, and 0.6 mM ferrozine were mixed. The solutions were allowed to stand for 10 min at room temperature, and the absorbance of  $\text{Fe}^{2+}$ -ferrozine complex was measured at 562 nm using UV/Vis

spectrophotometer. Ultra-pure water instead of sample solution was used as a negative control. Ultra-pure water instead of ferrozine solution was used as a blank, which is used for error correction because of unequal color of the sample solutions. EDTA-Na<sub>2</sub> was used as the positive control. The ability of the sample to chelate ferrous ions is calculated by using the following formula:  $A_0 - (A_{s \text{ tested}} - A_{s \text{ alone}} / A_0) \times 100$  where  $A_0$  is the absorbance of the negative control,  $A_{s \text{ tested}}$  is the absorbance of the sample tested and  $A_{s \text{ alone}}$  is the absorbance of the sample alone. All measurements were performed in duplicate.

### **2.5.3. $\alpha$ -amylase inhibitory activity**

$\alpha$ -amylase inhibitory activity of PM diluted samples was carried out according to the standard method with minor modification (Ademiluyi & Oboh, 2012). In a 96-well plate, reaction mixture containing 50  $\mu$ L phosphate buffer (100 mM, pH 6.8), 10  $\mu$ L  $\alpha$ -amylase (2 U/mL), and 20  $\mu$ L of varying concentrations of the PM diluted samples (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1 mg/mL) was pre-incubated at 37°C for 20 min. Then, the 20  $\mu$ L of 1% soluble starch (100 mM phosphate buffer pH 6.8) was added as a substrate and incubated further at 37°C for 30 min. 100  $\mu$ L of the DNS color reagent was then added and boiled for 10 min. The absorbance of the resulting mixture was measured at 540 nm using Multiplate ELISA Reader. Acarbose at various concentrations (0.1-0.5 mg/mL) was used as a standard. Without PM solutions was set up in parallel as control and each experiment was performed in triplicates. The results were expressed as percentage inhibition, which was calculated using the formula: Inhibitory activity (%) =  $(1 - A_s / A_c) \times 100$ ; where,  $A_s$  is the absorbance in the presence of test substance and  $A_c$  is the absorbance of control.

### **2.5.4. $\alpha$ -glucosidase inhibitory activity**

$\alpha$ -glucosidase inhibitory activity of PM diluted samples was carried out according to the standard method with minor modification (Shai et al., 2011). In a 96-well plate, reaction mixture containing 50  $\mu$ L phosphate buffer (100 mM, pH 6.8), 10  $\mu$ L  $\alpha$ -glucosidase (1 U/mL), and 20  $\mu$ L of varying concentrations of the diluted samples (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1 mg/mL) was pre-incubated at 37°C for 15 min. Then, 20  $\mu$ L P-NPG (5 mM) was added as a substrate and incubated further at 37°C for 20 min. The reaction was stopped by adding 50  $\mu$ L Na<sub>2</sub>CO<sub>3</sub> (0.1 M). The absorbance of the released p-nitrophenol was measured at 405 nm using Multiplate ELISA Reader. Acarbose at various concentrations (0.1-0.5 mg/mL) was included as a standard. Without test substance was set up in parallel as a control and each experiment was performed in triplicates. The results were expressed as percentage inhibition, which was calculated using the formula: Inhibitory activity (%) =  $(1 - A_s / A_c) \times 100$ ; where,  $A_s$  is the absorbance in the presence of test substance and  $A_c$  is the absorbance of control.

## 2.6. Statistical analysis

The mean values of total phenolic content, total flavonoid content, IC<sub>50</sub> for the DPPH scavenging activity, % of inhibition of ferrous ion chelation, IC<sub>50</sub> for the  $\alpha$ -amylase inhibition activity, IC<sub>50</sub> for the  $\alpha$ -glucosidase inhibition activity were calculated for the commercial and homemade PM samples. Mann-Whitney U test was used to compare these mean values between the commercial and homemade pomegranate molasses and spearman's correlation analysis was performed to examine the correlation between each of total phenol and total flavonoid contents and IC<sub>50</sub> of DPPH scavenging capacity, % of inhibition of ferrous ion chelation, IC<sub>50</sub> for  $\alpha$ -amylase inhibition and IC<sub>50</sub> for  $\alpha$ -glucosidase inhibition. The statistical analysis was carried out using the statistical package for social sciences (SPSS) version 23.

## 3. Results and discussion

### 3.1. Total phenolic content in pomegranate molasses

The total phenolic content of the commercial and homemade PM samples ranged between 9.86-78.28 mg GAE/g PM and 21.19-44.32 mg GAE/g PM, respectively (**Table 1**). The mean value of total phenolic content of the homemade ( $37.27 \pm 10.79$  mg GAE/g PM) was higher than that of the commercial ( $26.13 \pm 14.32$  mg GAE/g PM) PM samples. However, the difference between the mean values was found to be not statistically significant ( $p$ -value 0.087). This finding might indicate that the phenolic content of homemade PM samples is more effectively preserved which is assumed to be due to the production techniques characterized by not adding any preservatives such as sugar or citric acid and crushing/squeezing the whole fruit, or/and due to fraud problems in some of the commercial brands resulting from addition of lower cost juices to pomegranate juice. For instance, some commercial brands reported adding sugar, salt and/or citric acid in the ingredients section which might have affected their phenolic content. Our findings were inconsistent with the findings of other studies. A study conducted by Incedayi et al., (2010) in Turkey showed that the range of total phenolic content in commercial PM samples, tested using the Folin-Ciocalteu method and a spectrophotometer measuring at a 765 nm, was lower than that found in our study (ranged between 0.55-9.69 mg GAE/g PM). In another study conducted in Lebanon on fresh pomegranate juice and molasses from six different sour pomegranate varieties, the mean level of total polyphenols in molasses ( $252.28 \pm 33.67$  mg GAE/L) was found to be three times higher than in pomegranate juice ( $79.49 \pm 25.25$  mg GAE/L) due to the fact that PM is a concentrate of pomegranate juice (Chalfoun-Mounayar et al., 2012). Also, Yilmaz et al., (2007) reported that total phenols content of commercial pomegranate molasses, determined according to the Folin-Ciocalteu method, was five to ten times higher than that of fresh pomegranate arils (52.6 mg GAE/g) which is also higher than that of commercial PM in our

samples. In a study conducted by Akpinar-Bayizit et al., (2016) total polyphenols content of commercial pomegranate molasses was determined spectrophotometrically at 725 nm according to modified Folin-Ciocalteu method and it varied between 118.28 to 828.15 mg GAE/g of PM which is much higher than the amounts found in our study. However, Özmert Ergin (2020) findings did not support the findings of our study, since the average phenolic content of commercial PM samples tested also using Folin-Ciocalteu method was found to be higher ( $490 \pm 278$  mg GAE/L) than that of homemade PM samples ( $24.11 \pm 4.06$  mg GAE/L); this was attributed to the lack of support and awareness about the hygienic conditions during the production stages for the traditional PM. The differences in total phenolic content of PM among these studies are expected to be due to factors such as cultivars, climate, soil and production techniques.

### **3.2. Total flavonoid content in pomegranate molasses**

Total flavonoid content of commercial and homemade PM samples ranged between 12.15-90.92 mg QE/g PM and 28.73-48.28 mg QE/g PM, respectively (**Table 1**). The mean value of the total flavonoid content of the homemade PM samples ( $40.09 \pm 8.41$  mg QE/g PM) was found to be significantly higher ( $p$ -value 0.035) than that of the commercial ones ( $29.47 \pm 17.61$  mg QE/g PM). These findings were supported by the findings of a study conducted by Nasser et al., (2017) in Lebanon, where total flavonoid content of commercial pomegranate molasses samples was determined using aluminum chloride method and ranged between 54.34 and 74 mg Rutin Equivalent/g of PM. In their study, the homemade/artisanal PM sample had the highest value of 137.74 mg Rutin Equivalent/g of PM. In another study by Kaur et al. (2014), total flavonoid content of pomegranate juice (8.54-23.99 mg QE/100g) was much less than that found in our study, since PM is a concentrate of the juice (Kaur et al., 2014). Another study done on

pomegranate juice samples of twelve varieties and five clones in Italy showed that they contain high amounts of flavonoids such as pelargonin and catechin-cyanidin-3-hexoside and that flavonoid content differs between varieties and cultivars (Gómez-Caravaca et al., 2013).

It should be added that flavonoids possess free radical scavenging and metal ion chelating capacities and bioactive potential including anti-diabetic, anti-proliferative, anti-inflammatory, anti-carcinogenic and neuro-protective effects (Hartman et al., 2006; Haminiuk et al., 2012; Akpınar-Bayizit et al., 2016; Singh et al., 2018; Zhou et al., 2018).

### **3.3. Antioxidant activity of pomegranate molasses using DPPH assay**

The IC<sub>50</sub> value for DPPH scavenging activity of the commercial and homemade PM samples ranged between 0.09-0.76 mg/mL and 0.09-0.26 mg/mL, respectively (**Table 2**). The IC<sub>50</sub> mean value of the homemade PM (IC<sub>50</sub> 0.15 ± 0.07 mg/mL) was lower than that of the commercial samples (IC<sub>50</sub> 0.23 ± 0.15 mg/mL); it should be noted that a lower IC<sub>50</sub> means that a lower concentration of PM is needed to achieve 50% of the enzyme inhibition and therefore indicating a stronger antioxidant activity. However, this difference was not found to be statistically significant (*p*-value 0.231). Both commercial and homemade pomegranate molasses samples exhibited an antioxidant activity close to that of ascorbic acid, a very potent antioxidant agent that is commonly used as a reference compound (IC<sub>50</sub> 0.03 mg/mL) which is logical especially that PM contains sugars. These findings are supported by the findings by Nasser et al., (2017) where the antioxidant capacity was stronger in homemade/artisanal than in commercial pomegranate molasses. In contrast, a study done by Özmert Ergin, (2020) showed that the commercial PM samples exhibited higher antioxidant activity than the homemade ones. This was attributed to the lack knowledge in the homemade PM production while, for the commercial

ones, standards should be strictly applied to prevent adulterations such as the addition of colorants, thickeners antioxidants.... A study done by Kamal et al. (2018) confirmed the presence of strong antioxidant markers in PM which was associated with the presence of vitamin C, gallic acid, rutin and ellagic acid. In a study conducted by Chalfoun-Mounayar et al., (2012) PM showed the strongest antioxidant properties *in vitro* as compared to pomegranate juice which indicated that high temperature did not affect the antioxidant activity of PM against reactive oxygen species, on the contrary, was assumed to help the cells of pomegranate fruit release polyphenols. Another study done by Incedayi et al., (2010) also showed that PM exhibited potent antioxidant activity but contrary to the previous study, long term thermal processing of pomegranate molasses samples was found to affect their antioxidant activity. In addition, the study conducted by Akpinar-Bayizit et al. (2016) showed that PM has potent antioxidant activity (560.23-1885.23  $\mu\text{mol}$  trolox equivalent per gram of sample) and the difference in its antioxidant activity between the tested samples is affected by several factors, such as cultivar and climatic conditions during fruit maturation.

#### **3.4. Antioxidant activities of pomegranate molasses using $\text{Fe}^{2+}$ chelating assay**

PM samples exhibited  $\text{Fe}^{2+}$  chelating activity in a concentration dependent manner. As shown in **Table 2**, while using the highest concentration for all samples (1 mg/mL), the percentage of inhibition of ferrous ion chelation of commercial PM samples ranged between -40.52% and 41.93% and that of homemade samples ranged between 28.47% and 46.78%. In addition, the mean percentages of inhibition of ferrous ion chelation of homemade and commercial PM samples were  $34.87\% \pm 8.24$  and  $-2.06\% \pm 17.81$  ( $p$ -value 0.03) respectively, indicating that commercial pomegranate molasses showed significantly lower chelating activity as compared to homemade ones. This finding might indicate that the commercial PM samples contained iron that

formed complexes with the antioxidants in PM, thus exhibiting no Fe<sup>2+</sup> chelating activity and power. Our findings were inconsistent with the findings of other studies. In a study conducted by Viuda-Martos et al., (2011) in Spain, ferrous ion chelating assay was performed on arils alone (AB) and pomegranate arils and peels combined (WFB) with different concentrations (10 g/L-100 g/L). At these concentrations, AB and WFB samples showed chelating activities ranging between 0-35.59% ± 0.35 and 23.71% ± 0.35-42.87% ± 0.36, respectively. These results showed that pomegranate arils and peels have a lower chelating activity than the homemade PM samples in our study (WFB, concentration of 10 g/L: 23.71% ± 0.35 vs. homemade PM, concentration of 1 mg/mL: 34.87% ± 8.24) ; this can be attributed to the variation in the concentration of phenolic compounds in the PM samples in our study. In another study done by Fawole et al., (2012), the ferrous ion chelating ability of methanolic extracts of pomegranate peel was assessed. Researchers found the average ferrous ion chelating activity to be low with the concentration of 0.01 mg/mL of pomegranate peel's methanolic extracts. The activity, however, of almost all methanolic extracts increased above 50% at a concentration of at 0.1 mg/mL, indicating that the tested pomegranate fruit peel showed a stronger chelating activity on ferrous ion than the PM tested in our study.

Correlations between the assessed phytochemical constituents (phenols and flavonoids) and both DPPH free radical scavenging activity and Fe<sup>2+</sup> metal chelating activity are shown in **Table 3**. Total phenolic content/total flavonoid content were found to have an inverse strong/moderate significant correlation with the IC<sub>50</sub> of DPPH scavenging activity ( $r_{\text{phenolic content}} -0.542$ ,  $p$ -value 0.001;  $r_{\text{flavonoid content}} -0.483$ ,  $p$ -value 0.005). These inverse correlations mean that as the total phenolic/ flavonoid content of PM increases, the IC<sub>50</sub> of DPPH free radical scavenging activity decreases, indicating that a lower concentration of PM is needed to scavenge 50% of DPPH free



radical and therefore PM has a greater antioxidant capacity. Therefore, PM was shown to have strong anti-radical scavenging activity and capacity to reduce oxidative stress.

Our findings were supported by the findings of other studies. A study done by Kaur et al., (2014) on pomegranate varieties in India showed a significant strong correlation between total phenolic content (DPPH  $R^2 = 0.9$ ), total flavonoids content (DPPH  $R^2 = 0.85$ ) and antioxidant activity, as assessed by three *in vitro* assays including the DPPH radical scavenging assay. These findings indicated that both total phenolic and total flavonoid contents are strong determinants of the powerful antioxidant activity attributed to pomegranate. In contrast, Özmert Ergin (2020) conducted a study on total phenolic content and antioxidant activity of pomegranate molasses in Turkey using the DPPH scavenging activity assay and found no significant correlation between phenolic content and antioxidant activity which he attributed to differences in cultivars/species of pomegranate in addition to various additives used in pomegranate molasses production especially the commercial ones which may have affected the results. An Ambigaipalan et al., (2016) study revealed that the pomegranate peel contains 79 phenolic compounds, including 16 phenolic acids and 12 flavonoids. Phenolic acids were the major phenolic compounds in pomegranate peel followed by flavonoids, hydrolysable tannins and proanthocyanidins. This study can prove how the phenolic content in pomegranate molasses is increased and its health benefits improved whenever the whole pomegranate fruit is used during pomegranate molasses production, including the peel and not only the juice part. In another study conducted by Derakhshan et al., (2018) on pomegranate peel, juice and seeds extracts, a positive significant correlation was reported between antioxidant activity tested using the  $\beta$ -carotene bleaching test and phenolic ( $r = 0.78$ ), flavonoids ( $r = 0.95$ ), and flavonol ( $r = 0.89$ ) contents in all samples, using Folin-Ciocalteu and aluminum chloride calorimetric methods, respectively.

### 3.5. Anti-Diabetic activity of pomegranate molasses using $\alpha$ -amylase and $\alpha$ -glucosidase

#### inhibitory assays

The anti-diabetic activity of commercial and homemade PM samples was determined using the  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory assays. The results were expressed as  $IC_{50}$ . Acarbose, a known anti-diabetic drug, like Glucobay and Precose, was used as the reference standard for both assays with  $IC_{50}$  values of 0.42 mg/mL and 0.28 mg/mL for the  $\alpha$ -amylase and the  $\alpha$ -glucosidase inhibitory assays, respectively. We should note that a lower  $IC_{50}$  value means that a lower concentration of PM was needed to achieve 50% of the enzyme inhibition and therefore indicating a higher anti-diabetic activity.

The  $IC_{50}$  of  $\alpha$ -amylase inhibition of the commercial PM samples ranged between 0.68 and 28.24 mg/mL and of homemade samples between 0.63 and 2.06 mg/mL (**Table 4**). Data obtained showed that the homemade PM samples ( $IC_{50}$  1.19  $\pm$  0.61 mg/mL) were more active against  $\alpha$ -amylase than the commercial ones ( $IC_{50}$  3.98  $\pm$  5.14 mg/mL) meaning that they are more effective against diabetes and this difference was found to be statistically significant ( $p$ -value 0.014). Moreover, homemade pomegranate molasses was found to be very close in activity to the reference standard acarbose. Only 1.5 to 5 times (on average  $\sim$ 2.8 times) less active than acarbose ( $IC_{50}$  0.42 mg/mL) in spite the fact that PM contains natural sugars that can interfere with this assay and activity against  $\alpha$ -amylase.

The  $IC_{50}$  of  $\alpha$ -glucosidase of commercial PM samples ranged between 0.32 and 4.05 mg/mL and of homemade samples between 0.4 and 1.83 mg/mL (**Table 4**). Results showed that the homemade PM samples ( $IC_{50}$  0.78  $\pm$  0.7 mg/mL) were more active against  $\alpha$ -glucosidase compared to commercial ones ( $IC_{50}$  1.47  $\pm$  0.77 mg/mL), meaning a higher effectiveness against

diabetes for the homemade samples, but this difference was not found to be statistically significant ( $p$ -value 0.077). Similarly, homemade PM samples were found to be very close in activity to the reference standard acarbose; only 1.4 to 6.5 times (on average  $\sim 2.8$  times) less potent than acarbose ( $IC_{50}$  0.28 mg/mL) despite the fact that PM contains natural sugars that can interfere with this assay and activity against  $\alpha$ -glucosidase.

A previous study was done by Kam et al. (2013) on seven extracts from different parts of pomegranate (juice, flower, peel and seeds) to determine their inhibitory activities against  $\alpha$ -amylase and  $\alpha$ -glucosidase enzymes. It showed that the strongest inhibitory effect on both enzymes was that of the flower extract followed by the peel extract on  $\alpha$ -glucosidase alone. These results were associated with the presence of gallic acid and ellagic acid and led to the conclusion that pomegranate flowers and peels have an anti-diabetic activity. In addition, Šavikin et al. (2018) showed that all pomegranate peel extracts/fractions revealed a better inhibition activity against  $\alpha$ -glucosidase enzyme than  $\alpha$ -amylase and this anti-diabetic activity was also attributed to the presence of high levels of gallic and ellagic acids. Another study conducted by Les et al., (2018) showed that pomegranate juice exhibited an inhibitory effect against  $\alpha$ -glucosidase enzyme that is similar to acarbose, which is a reference inhibitor of this enzyme, with polyphenols such as punicalagin, urolithin-A, ellagic acid being responsible for the inhibition of the enzyme and therefore the presence of an anti-diabetic activity.

### **3.6. Correlation between phytochemical constituents and anti-diabetic activity**

Spearman correlation coefficients between total phenolic, total flavonoid contents and  $\alpha$ -amylase inhibitory activity and  $\alpha$ -glucosidase inhibitory activity are shown in **Table 5**. Significant moderate negative correlations were found between total phenolic/flavonoid contents and  $\alpha$ -

amylase inhibitory activity ( $r$  -0.436,  $p$ -value 0.013 and  $r$  -0.445,  $p$ -value 0.011 respectively). These inverse correlations mean that as the total phenolic/ flavonoid content of PM increases, the  $IC_{50}$  for  $\alpha$ -amylase inhibition decreases, indicating that a lower concentration of PM is needed to achieve 50% of  $\alpha$ -amylase inhibition and therefore, PM has a higher anti-diabetic activity. In addition, weak negative correlations were found between total phenols/flavonoids contents and  $\alpha$ -glucosidase inhibitory activity. However, these correlations were found to be not statistically significant ( $r$  -0.284,  $p$ -value 0.116 and  $r$  -0.247,  $p$ -value 0.173, respectively). This lack of statistical significance could be attributed to the small number of homemade samples. Thus, these results reveal a potential anti-diabetic activity of phenolic compounds found in PM.

#### **4. Conclusion**

The screening of commercial and homemade pomegranate molasses samples for total phenolic and flavonoids contents, antioxidant and anti-diabetic activities was performed. Homemade pomegranate molasses samples exhibited higher antioxidant activity than commercial samples against DPPH radical scavenging activity and  $Fe^{2+}$  chelating assays. Similarly, homemade pomegranate molasses samples exhibited higher anti-diabetic activity using  $\alpha$ -amylase and  $\alpha$ -glucosidase inhibitory assays, noting that homemade samples were very close in activity to the reference standard acarbose; only 1.5 to 5 times ( $\sim 2.8$  times) less potent than acarbose in activity against  $\alpha$ -amylase enzyme and 1.44 to 6.5 times ( $\sim 2.8$  times) less potent than acarbose in inhibiting  $\alpha$ -glucosidase, despite the presence and concentration of natural sugars in pomegranate molasses that might affect the results. Moreover, total phenolic and flavonoid contents were found to be correlated with antioxidant and anti-diabetic activities.

The study findings can serve as a building block for further research studies to validate these activities using other methods, quantify more classes of phytochemicals, and perform a bio-

guided fractionation to isolate and identify the bioactive molecules that are responsible for each activity. Future research studies should also aim to study a bigger number of homemade pomegranate molasses samples.

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Tables

**Table 1: Total phenols and total flavonoids contents of commercial and homemade pomegranate molasses samples**

<b>Pomegranate Brand Name</b>	<b>Total Phenols Content (mg GAE/g PM)</b>	<b>Total Flavonoids Content (mg QE/g PM)</b>
Al Barakah	18.48 ± 0.36	28.38 ± 0.51
Al Rabih	35.32 ± 1.04	26.06 ± 51.14
Annabil	34.45 ± 1.46	42.54 ± 1.86
Alwadi	22.61 ± 1.12	13.78 ± 0.00
Aoun	22.57 ± 0.35	29.82 ± 0.42
Ashkar	28.24 ± 0.56	21.37 ± 1.53
Baydar	22.79 ± 1.03	22.09 ± 0.00
Boulos	10.68 ± 0.19	13.31 ± 1.11
Chtoura Gardens	21.98 ± 0.17	36.73 ± 0.94
Chtoura Fields	14.81 ± 0.42	15.45 ± 0.60
Cortas	21.85 ± 0.10	26.81 ± 0.60
Gardenia	10.08 ± 0.16	37.38 ± 0.09
Houkoul Khadraa	15.26 ± 0.23	26.17 ± 1.28
Jana	27.42 ± 1.79	24.92 ± 0.17
Jeita	13.47 ± 0.10	12.92 ± 0.34
Kortbawi	78.28 ± 4.62	90.92 ± 0.17
Lumiere	22.06 ± 0.25	21.07 ± 0.51
Maxim's	34.86 ± 0.74	24.28 ± 0.76
Maymouna	24.30 ± 0.33	23.72 ± 0.00
Meshaalani	21.64 ± 2.80	19.36 ± 3.51
Monzer Basha	36.80 ± 1.15	35.27 ± 0.26
Mrouj Chtoura	22.74 ± 1.77	27.27 ± 2.55
Salloum	37.02 ± 1.09	30.02 ± 0.17
Spinneys	9.86 ± 0.56	12.15 ± 0.43
Taj	11.88 ± 0.31	12.37 ± 1.03
Tiba	23.84 ± 1.11	25.75 ± 1.10
Terrois	52.46 ± 4.26	69.07 ± 1.45
Yamama	36.02 ± 0.62	56.26 ± 1.37
HM Dima 1	44.32 ± 1.58	48.28 ± 1.68
HM Dima 2	41.60 ± 1.82	39.32 ± 1.17
HM Mira	41.97 ± 3.83	44.03 ± 1.17
HM Salwa	21.19 ± 0.23	28.73 ± 0.42

HM = Homemade

**Table 2: IC<sub>50</sub> of DPPH and percentage of inhibition of ferrous ion chelation of commercial and homemade pomegranate molasses samples**

<b>Pomegranate Brand Name</b>	<b>IC<sub>50</sub> DPPH (mg/mL)</b>	<b>Inhibition of ferrous ion chelation %</b>
Al Barakah	0.19 ± 0.00	9.88 ± 1.40
Al Rabih	0.21 ± 0.00	-9.36 ± 0.67
Annabil	0.09 ± 0.00	-26.49 ± 1.40
Alwadi	0.23 ± 0.00	-5.93 ± 0.47
Aoun	0.30 ± 0.00	-2.78 ± 0.40
Ashkar	0.09 ± 0.01	9.79 ± 0.20
Baydar	0.26 ± 0.00	-18.92 ± 1.80
Boulos	0.30 ± 0.00	13.08 ± 1.92
Chtoura Gardens	0.10 ± 0.00	-26.54 ± 3.26
Chtoura Fields	0.43 ± 0.00	4.19 ± 1.06
Cortas	0.11 ± 0.00	-0.28 ± 0.47
Gardenia	0.15 ± 0.00	7.86 ± 2.26
Houkoul Khadraa	0.46 ± 0.00	-40.52 ± 1.60
Jana	0.15 ± 0.00	0.05 ± 0.13
Jeita	0.16 ± 0.00	13.13 ± 1.06
Kortbawi	0.13 ± 0.00	26.92 ± 2.33
Lumiere	0.11 ± 0.00	-16.94 ± 0.20
Maxim's	0.20 ± 0.01	-15.95 ± 1.73
Maymouna	0.10 ± 0.00	13.13 ± 4.66
Meshaalani	0.29 ± 0.00	-26.12 ± 0.27
Monzer Basha	0.34 ± 0.01	-10.49 ± 0.00
Mrouj Chtoura	0.24 ± 0.00	0.85 ± 0.21
Salloum	0.10 ± 0.00	2.16 ± 0.06
Spinneys	0.76 ± 0.10	9.74 ± 0.66
Taj	0.45 ± 0.01	11.48 ± 1.66
Tiba	0.22 ± 0.00	-4.42 ± 3.13
Terrois	0.14 ± 0.00	41.93 ± 1.20
Yamama	0.12 ± 0.00	-17.18 ± 1.73
HM Dima 1	0.09 ± 0.00	33.84 ± 7.32
HM Dima 2	0.13 ± 0.00	46.78 ± 1.26
HM Mira	0.12 ± 0.00	30.40 ± 1.40
HM Salwa	0.26 ± 0.00	28.47 ± 2.66

HM = Homemade

**Table 3: Correlation between total phenols and total flavonoids contents and antioxidant activity assays**

		<b>IC<sub>50</sub> DPPH</b>	<b>% of inhibition of ferrous ion chelation</b>
<b>Total phenols content</b>	Correlation coefficient	-0.542	0.152
	<i>p</i> -value	0.001	0.407
	N	32	32
<b>Total flavonoids content</b>	Correlation coefficient	-0.483	0.146
	<i>p</i> -value	0.005	0.424
	N	32	32

Correlation is significant at the 0.05 level (2-tailed).

**Table 4: IC<sub>50</sub> of  $\alpha$ -amylase inhibitory assay and IC<sub>50</sub> of  $\alpha$ -glucosidase inhibitory assay of commercial and homemade PM samples**

<b>Pomegranate Brand Name</b>	<b>IC<sub>50</sub> <math>\alpha</math>-amylase inhibitory assay</b>	<b>IC<sub>50</sub> <math>\alpha</math>-glucosidase inhibitory assay</b>
Al Barakah	1.38 ± 0.17	2.45 ± 0.84
Al Rabih	2.34 ± 0.01	1.44 ± 0.32
Annabil	4.74 ± 0.07	1.86 ± 0.13
Alwadi	4.11 ± 0.24	4.05 ± 0.32
Aoun	1.80 ± 0.16	2.41 ± 0.28
Ashkar	1.25 ± 0.02	0.55 ± 0.11
Baydar	2.84 ± 0.63	0.89 ± 0.07
Boulos	5.01 ± 1.98	2.01 ± 0.16
Chtoura Gardens	1.36 ± 0.83	0.82 ± 0.15
Chtoura Fields	2.26 ± 0.32	1.12 ± 0.04
Cortas	1.69 ± 0.12	1.00 ± 0.09
Gardenia	8.67 ± 2.02	0.94 ± 0.09
Houkoul Khadraa	7.96 ± 0.62	1.45 ± 0.52
Jana	5.32 ± 4.50	1.49 ± 0.49
Jeita	4.09 ± 0.37	2.02 ± 0.41
Kortbawi	1.13 ± 0.02	1.80 ± 0.19
Lumiere	3.24 ± 0.04	0.94 ± 0.02
Maxim's	28.24 ± 7.94	1.54 ± 0.44
Maymouna	2.23 ± 0.68	0.32 ± 0.10
Meshaalani	4.34 ± 3.01	1.67 ± 0.07
Monzer Basha	1.41 ± 0.27	1.21 ± 0.19
Mrouj Chtoura	3.56 ± 2.42	1.40 ± 0.08
Salloum	1.35 ± 0.54	2.61 ± 1.64
Spinneys	2.52 ± 0.13	1.10 ± 0.46
Taj	1.71 ± 1.72	1.40 ± 0.11
Tiba	3.21 ± 1.74	1.25 ± 0.12
Terrois	0.68 ± 0.03	0.45 ± 0.14
Yamama	3.04 ± 1.47	1.00 ± 0.40
HM Dima 1	0.94 ± 0.08	0.41 ± 0.00
HM Dima 2	2.06 ± 0.63	0.41 ± 0.02
HM Mira	0.63 ± 0.13	0.49 ± 0.01
HM Salwa	1.14 ± 0.00	1.83 ± 0.11

HM = Homemade



**Table 5: Correlation between total phenols and total flavonoids content and anti-diabetic activity assays**

		<b>IC<sub>50</sub> <math>\alpha</math>-amylase inhibitory assay</b>	<b>IC<sub>50</sub> <math>\alpha</math>-glucosidase inhibitory assay</b>
<b>Total phenols content</b>	Correlation coefficient	-0.436	-0.284
	<i>p</i> -value	0.013	0.116
	N	32	32
<b>Total flavonoids content</b>	Correlation coefficient	-0.445	-0.247
	<i>p</i> -value	0.011	0.173
	N	32	32

Correlation is significant at the 0.05 level (2-tailed).