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Chemical composition and antioxidant capacity of Lebanese molasses pomegranate

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ABSTRACT

Recently, nutritional and pharmaceutical benefits of pomegranate have raised a growing scientific interest. Our present work aimed, for the first time, to investigate the importance of the Lebanese pomegranate molasses. For that, we determined the chemical composition of six samples of molasses provided from different sources to know their contents in secondary metabolites having medical importance. Then the antioxidant capacity has been evaluated for the six samples. Chemical composition of pomegranate molasses was determined using a standard phytochemical screening. Total phenolic and total flavonoids contents have been evaluated using the Folin-Ciocalteau reagent and the aluminum chloride method respectively. Finally, free radical scavenging activity of molasses was evaluated using DPPH assay. The obtained results of the phytochemical screening of molasses pomegranate indicated the presence of various secondary metabolites such phenols, flavonoids, resins, and saponin in the different studied molasses. Also, all samples showed a higher antioxidant capacity reaching 90 %. Considering properties of the identified major compounds with the higher antioxidant power of the studied samples, molasses pomegranate could be used in the medicine field including the food, pharmaceutical and cosmetic industry.

Keywords*:* Molasses pomegranate; antioxidant capacity; phytochemical screening.

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INTRODUCTION

Pomegranate (*Punica granatum* L.) is an ancient edible fruit whose cultivation has been in high demand recently due to and it being increasingly commercialized for its antioxidant and nutritional values that are important for human health [1, 2]. This fruit has antioxidant and antitumoral activity because it is a rich source of anthocyanins, tannins and other phenolic compounds [3, 4]. Moreover, recent studies showed its effective antimicrobial [5], antiviral and anti-inflammatory activities [6]. Pomegranate juice has also important clinical implications, and it has been recommended in the treatment of AIDS [3]. The potential antioxidant capacity of pomegranate can be used in the treatment and prevention of colon cancer [7] and cardiovascular disease [8, 9]. Other potential applications include male infertility, arthritis, infant brain ischemia, Alzheimer's disease, and obesity [10–13] The medicinal importance of a plant is due to the presence of some special substances like alkaloids, glycosides, resins, volatile oils, gums and tannins [14].

The pomegranate has been cultivated over the semi-arid, mild-temperate to subtropical climates in the main growing areas including most of Mediterranean countries [15] where pomegranate trees are well adapted to growing in saline and poor soils [16]. The composition of pomegranate fruits is strongly dependent on the cultivar type, growing area, climate, maturity and cultural practices [2]. In Lebanon, the fruit maturity occurs in general between August and October.

The pomegranate molasses is commonly used in salads and many dishes in Turkey, Iran and Mediterranean countries such as Lebanon and Syria [17–20]. Traditional methods are still being used to produce pomegranate molasses. The product is concentrated simply by boiling, without the addition of further sugar or other additives. Whereas the commercial methods require cleaning, crushing, extraction, filtration, clarification and evaporation in open vessel or under vacuum [21]. These different methods in the molasses fabrication could be the cause in the presence of different phytochemicals composition.

Till now, no published data was found on the chemical composition and on the biological properties of Lebanese pomegranate molasses. Therefore, the main objectives of this study were to compare the chemical composition and the antioxidant property of artisanal pomegranate molasses with three different companies producing commercial molasses.

MATERIAL AND METHOD

Collection of samples

Artisan pomegranate molasses (produced on 15/08/2015) and commercial pomegranate molasses from 3 different companies: Darna (15/05/2015 & 01/04/2016), Yamama (26/05/2015), Chtoura

(01/10/2014) have been used to realize this study. It should be mentioned that all pomegranate molasses produced industrially contain dates in its production; that is why we also worked on dates molasses. Artisanal molasses was made from acid grenades.

Samples preparation

All the samples were prepared just before each analysis and in the same manner: diluting 0.5 g of molasses extract in 1 mL of distilled water. The samples were stored at 4 °C and used for qualitative and quantitative phytochemical screening.

Phytochemical screening

Qualitative phytochemical screening

In order to study the chemical composition of the different molasses samples, qualitative tests have been conducted to detect the presence of primary and secondary metabolites as shown in Table 1 [22]. These tests are useful to estimate some biological activities that might be detected due to the presence of some secondary metabolites in molasses of the studied samples.

Table 1: Detection of primary and secondary metabolites by phytochemical screening

Metabolites	**Added reagent**	**Expected result**
Alkaloids	Dragendorff reagent	Red or orange precipitate
Tanins	$FeCl_3$ (1%)	Blue coloration
Resines	Acetone + water	Turbidity
Saponines	Agitation	Formation of foam
Phenols	$FeCl_3$ (1%) + $K_3(Fe(CN)_6)$ (1%)	Green-blue coloration
Terpenoids	Chloroform + H_2SO_4 conc	Reddish brown coloration
Flavonoids	KOH (50%)	Yellow color
Carbohydrates	α-naphtol + H_2SO_4	Purple ring
Reducing sugar	Fehlings (A+B)	Brownish-red precipitate
Quinones	HCl conc	Yellow precipitate
Sterols & Steroids	Chloroform + H_2SO_4 conc	Red color (surface) + fluorescence Greenish-yellow
Cardiac glycosides	Glacial acetic acid + $FeCl_3$ (5%) + H_2SO_4 conc	Ring
Diterpenes	Copper acetate	Green color
Anthraquinones	HCl (10%) + Chloroform + Ammonia (10 %)	Pink color
Proteins & aminoacids	Ninhydrin 0.25%	Blue color
Lignines	Safranine	Pink color
Phlabotannins	HCl (1%)	Blue color
Anthocyanines	NaOH (10%)	Blue color
Flavanones	H_2SO_4 conc	Bluish-red color
Fixed oils and fats	Spot Test	Oil stain

Quantitative phytochemical screening

Estimation of total phenolic content (TPC)

The method of Folin-Ciocalteau reagent has been used to estimate the TPC in the different samples [23]. In brief, a 100 µL of each used sample was mixed with 0.5 mL of Folin-Ciocalteau's phenol reagent (1/10 dilution in water). After 5 min, 10 mL of a 7% Na_2CO_3 solution was added to the mixture. The blend was incubated in the dark, at room temperature for 30 min. The absorbance of blue-colored solution for all samples was measured at 765 nm using a Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg of gallic acid equivalent (GAE) per g of sample.

Total phenol content = GAE x V x D /m,

Where **GAE** is the gallic acid equivalence (mg/mL); **V** is the volume extract (mL), **D** is dilution factor and **m** is the weight (g) of the sample.

The blank was formed by 0.5 mL water-MeOH and 1.5 mL of Na_2CO_3 (2%).

Estimation of total flavonoid content (TFC)

The aluminium chloride method [24] was used for the determination of TFC of all the samples. Briefly, 1 mL of extract was mixed with 1 mL of methanolic aluminum chloride solution (2 %). After an incubation period at room temperature in the dark for 1 h, the absorbance of all samples was determined at 415 nm using a Gene Quant 1300 UV-Vis spectrophotometers. The results were expressed in mg per g of rutin equivalent (RE).

Flavonoids content = RE×V×D/W

Where RE is Rutin equivalent (µg/mL), V is the total volume of sample (mL), D is dilution factor, W is the sample weight (g).

The blank was formed by 1 mL MeOH and 1 mL of 2 % methanolic aluminum chloride solution.

Estimation of total alkaloid content (TAC)

The determination of alkaloids content has been done according to the method of Harborne [25]. 1 g of the sample was weighed into a 250 mL beaker and 100 mL of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 h. After that, the extract was filtrated and reduced in a water bath to 25 mL of its original volume. Concentrated ammonium hydroxide was added drop by drop to the extract until the precipitation was complete. Then the precipitates were washed with dilute ammonium hydroxide and filtered. The residue was dried in the oven at 40 ºC and weighted. The alkaloid content was determined using the following formula:

% Alkaloid = [final weight of the sample / initial weight of the extract] ×100

Estimation of total tannins

Samples (0.5 g) were added to 75 mL of distilled water and contents were heated in a boiling water bath for 1 h. Then, the mixture was centrifuged (2000 rpm, 20 min). The supernatants were collected and the volume was adjusted to 100 mL with distilled water. 20 mL of lead acetate (4%) was added to the supernatants with constant stirring, which was maintained for at least 1 h. These solutions were filtered by Whatman filter paper and the filtrates were placed in an oven at 105 °C for 1 h. The mass of the content was weighed to give a T1 and was returned again to the oven for another 1 h. The samples were transferred to a muffle furnace then heated to 550 ^{o}C for 2 h, then weighed to give a T2.

% Tanins = (T1 - T2 / initial weight) x 100

T1 = mass of the sample in the furnace 105 °C after 1 hour

T2 = mass of the sample in the furnace 550 °C after 2 hours

Estimation of total saponin

Samples (1 g) have been added to 100 mL of ethanol (20 %) and then heated over a hot water bath for 4 h at 45 ºC with continuous stirring. The mixture was filtered and the residue was again extracted with another 100 mL of ethanol (25 %). The combined extracts were concentrated by using rotary evaporator with 40 ºC to get 40 mL approximately. The concentrate was transferred into a 250 mL separatory funnel in which 20 mL diethyl ether were added and shaken vigorously. The ether layer was discarded while the aqueous layer was kept. The purification process was repeated. 30 mL of n-butanol was added. The combined n-butanol extracts were washed twice with 10 mL of sodium chloride (5%). The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven at 40 ºC to a constant weight and the saponin's conent was calculated [26]. Saponin's content was calculated using the following formula:

% Saponin = [final weight of sample / initial weight of extracts] × 100

Estimation of humidity content

Samples (1 g) were placed in an oven at 105 ºC for 1 h and then cooled in a dessicator for half an hour. The mass of the content was recorded and was returned again to the oven for another 1 h. After heating, it was placed again in the desiccators for half an hour. These yielded a dry powder in which its mass was recorded again in order to calculate the percentage of humidity in the sample [27].

% Humidity = [(Initial weight - final weight) / powder of sample] × 100

With:

Initial weight = Sample weight + crucible weight (before heating)

Final weight = Sample weight + crucible weight (after heating)

Estimation of ash proportion

Samples (1-2 g) were placed and burned in a furnace burning (muffle furnace) at 550 ºC for 5 h till obtaining an ovary gray color of the powders. Then, the residues have been weighted and the percentage of ash has been estimated according the essential dry weight of plant powder [28].

% Ash = (final weight / initial weight) X 100

Initial weight = Sample weight + crucible weight (before heating)

Final weight = Sample weight + crucible weight (after heating)

Estimation of minerals content

An acid digestion was carried out to determine the minerals content. 1g of samples was put in an oven at 80 ºC during 24 h. Then, 10 mL of concentrated HCl was added at 80 ºC with agitation followed by covering the beaker. From time to time, few drops of H_2O_2 (35 %) were added. The beaker was warmed for 15 h. After the evaporation of HCl, 10 mL of HNO_3 were added. Vacuum filtration was performed for the obtained mixture followed by syringe filtration [29].

The minerals: iron, calcium, magnesium, lead, copper, cadmium, chromium, manganese and zinc were determined by the atomic absorption spectrometry.

Estimation of total protein

Proteins were determined using the method of AOAC [30]. Samples (1 g) were placed into specific tube (500 mL) with a catalyst (5 g of K_2SO_4 and 0.25 g of $CuSO_4$). 12-15 mL of H_2SO_4 (96-98%) and 10 mL of H_2O_2 (30-35%) were added to the samples. The samples digestion was kept for 20 min at 100 ^oC. After cooling the tubes, distillation was carried out by automatically adding 50 mL of water and 50 mL of NaOH (35%) for 5 min. The released NH_3 was captured in an erlenmeyer flask containing 25 mL of boric acid (4%). Titration of ammonium ion was made using a solution of H_2SO_4 (0.1M) in the presence of 3-5 drops of Tashiro indicator. The protein content was calculated by multiplying the mineral nitrogen content by 6.25.

Protein content = 6.25 * volume H_2SO_4

Estimation of total lipids

Total lipids were evaluated according to the method described by Aberoumand [31]. 2 g of samples were extracted by Soxhlet apparatus containing petroleum ether (bp: 40-60 ºC) till the extraction of total lipids. After that, the extract was put in a beaker and placed in the oven at 100 ºC in order to evaporate the entire solvent. Finally, it was cooled in a dessicator and weighted.

% Lipids = [lipid weight / powder weight] x 100

Evaluation of the antioxidant activity by DPPH assay

The antioxidant activity was evaluated according to the method of Rammal *et al.* [32] using free radical DPPH. Samples with increased concentrations (0.1, 0.2, 0.4, 0.5 mg/mL) were prepared. 1 mL of each prepared dilution of each sample was added to 1 mL of DPPH (0.15 mM in methanol) reagent. The solutions were incubated in the dark at room temperature for 30 minutes and the absorbance was measured at 517 nm by a Gene Quant 1300 UV-Vis spectrophotometer. The DPPH scavenging ability of samples was calculated according to the following equation:

% Scavenging activity = [(Abs control - Abs sample)] / (Abs control) ×100

Control was prepared by mixing 1 mL DPPH with 1 mL of selected solvent. The blank was composed of 1 mL of the selected solvent. The ascorbic acid was used as a positive control. The Abs control is the absorbance of DPPH + water-methanol; Abs sample is the absorbance of DPPH radical + sample.

RESULTS AND DISCUSSION

Phytochemical screening

Due to the strong relationship between the chemical compounds and the medicinal uses of plants, a phytochemical screening was performed to find out the primary and secondary metabolites present in the different molasses samples. The results obtained by this screening (Table 2) showed the presence of resins, saponins, phenols, flavonoïds and the absence of alkaloids coumanines, terpenoïds, volatile oils, reducing sugar, sterols, steroïds, anthraquinones, protein, amino acids, fixed oils, lignins, phlabotannis, anthocyanins, flavonones, fixed oils and fats in all the different samples of pomegranate molasses. On the other hand, the results differ between the studied samples. The reducing sugars were present only in artisanal molasses and quinones were present only in Darna molasses 2015. Tannins and diterpenes were present only in Darna molasses (2016), in Date molasses and artisanal molasses. Cardiac glycosides were present only in Darna 2016, in Date and in Chtoura molasses. Whereas, carbohydrate was absent only in Chtoura molasses.

Table 2: Chemical composition of different molasses samples

	Darna 2015	Yamama 2015	Darna 2016	Dates 2016	Chtoura 2014	Craft 2015
Alkaloids	--	--	--	--	--	--
Tannins	--	--	+	+	--	+
Resines	+	+	+	+	+	+
Saponins	+	+	+	+	+	+
Phenols	+	+	+	+	+	+
Terpenoides	--	--	--	--	--	--

Flavonoids	+	+	+	+	+	+
Carbohydrates	+	+	+	+	--	+
Proteins	--	--	--	--	--	--
Quinones	+	--	--	--	--	--
Sterols	--	--	--	--	--	--
Cardiac glycosides	--	--	+	+	+	--
Diterpenes	--	--	+	+	--	--
Anthraquinones	--	--	--	--	--	--
Reducing sugars	--	--	--	--	--	+
Lignins	--	--	--	--	--	--
Phlabotannins	--	--	--	--	--	--
Anthocyanins	--	--	--	--	--	--
Flavanones	--	--	--	--	--	--
Fixed oils/fat	--	--	--	--	--	--

Total phenolic content and total flavonoid content

The TPC of pomegranate molasses samples (Table 3) varying between 90 for Darna molasses (2016) to 179.5 (mg GAE/ g of sample) for the same molasses (Darna 2015). It is important to note that Alper et al. [33] found that TPC of pomegranate molasses was lower (between 55.61 and 99.87 mg / 100g molasses). Also, date molasses has a higher content of polyphenols like commercial samples. This difference between our molasses samples and those of Alper et al. [33] might be due to different factors such as types of grenade, soil, climate, extraction and evaporation techniques.

On the other hand, the TFC of molasses samples were between 54.34 for Chtoura molasses to 137.74 (mg RE/g of sample) for the artisanal molasses. The artisanal molasses has the highest value of TFC. This difference between our molasses samples might be due to different factors such as types of grenade, soil, climate, extraction and evaporation techniques.

Table 3: Total phenolic content in different molasses samples

Samples	TFC (mg GAE/g samples)	TPC (mg GAE/g samples)
Darna 2015	74	179.5
Yamama 2015	72.45	136.6
Darna 2016	73.3	90
Date 2016	54.72	176.1
Chtoura 2014	54.34	131.1
Artisanal 2015	137.74	123.5

Total alkaloids content (TAC)

No precipitate formation was observed during the addition of concentrated ammonium hydroxide. This indicates that the pomegranate molasses doesn't contain alkaloids confirming the results of the qualitative phytochemical screening.

Total tannins, Total saponin, lipids and Protein contents

The Table below shows the percentages of tannin and saponin in various molasses samples. The percentages of tannin in the different pomegranate molasses were very similar varying between 70 and 78 %, while the molasses of date have presented a lower percentage (67.68%).

On the other hand, the percentages of saponin in 1 g of different pomegranate molasses were between 2 % for the commercial pomegranate molasses and 13.58 % for artisanal molasses having the highest value. The date molasses has presented an intermediate value (5.82 %).

The protein content was between 7.88 for the Chtoura molasses and 13.13 for the artisanal molasses (g/100g molasses), which was greater than 0.23 ± 0.06 g/100g found by Yilmaz et al. [34]. This difference might be due to the types of grenade, extraction, concentration and to the filtration of grenade juice during the commercial preparation of molasses. The date molasses has presented the highest value of protein (14.88).

Concerning lipids content, the obtained results showed that the total content of lipids existing in 2 g of commercial pomegranate molasses was between 7.5 % and 10 %. The highest percentage of lipids was found in artisanal molasses (15 %) and the lower percentage was in date molasses (5 %).

Table 4: Total tannins, total saponin protein and lipids contents in different molasses samples

Samples	Tannin (%)	Saponin (%)	Protein (g/100g sample)	Lipids (%)
Darna 2015	78	2.67	9.63	10
Yamama 2015	74	2.48	8.75	10
Darna 2016	76	2.94	11.36	10
Date 2016	68	5.82	14.88	5
Chtoura 2014	70	4.98	7.88	7.5
Artisanal 2015	76	13.58	13.13	15

Humidity and Ash content

The results of the humidity and ash content are shown in Table . The obtained results indicated that 1 g of commercial molasses had between 10 to 20 %. The artisanal molasses has the highest percentage of humidity 30 %.

The results of the ash content indicated that the percentage of ash in molasses sample was between 0.52 % for Chtoura molasses and 2.68 % for Yamama molasses. The artisanal molasses has a value of 1.44 %.

Table 5: Percentage of humidity and ash in different molasses samples

Samples	Humidity (%)	Ash (%)
Darna 2015	10	1.09
Yamama 2015	20	2.68
Darna 2016	10	1.1
Date 2016	20	1.5
Chtoura 2014	20	0.52
Artisanal 2015	30	1.44

Mineral content

The presence of minerals (Table) in the molasses samples has been detected using the atomic absorption spectrometry (AAS). The results showed that the artisanal molasses was much richer in calcium than in any other metal. On the other hand, Yamama molasses contained the highest value of minerals in comparison with all the other types of molasses. In addition, it is the only type that contains copper (1.6 ppm). Therefore, Chtoura molasses presented the weakest mineral value and this type does not contain cadmium while all the other molasses types do. It is important to note that artisanal molasses was the only one containing lead (0.8 ppm). The results showed the absence of chromium, magnesium and manganese.

Table 6: Mineral content in different molasses samples in mg/L (ppm = mg / kg)

Sample	Ca	Pb	Zn	Fe	Cd	Cu	Cr
Darna 2015	140.74	0	0.74	1.11	0.22	0	0
Yamama 2015	622.72	0	7.58	1.96	2.68	1.6	0
Darna 2016	49.12	0	0.88	1.27	0.23	0	0
Dates 2016	59.94	0	2.72	1.72	0.8	0	0
Chtoura 2015	6.52	0	0.38	1.11	0	0	0
Craft 2015	66.58	0.8	1.04	1.21	0.66	0	0

Biological properties

Evaluation of the antioxidant activity by DPPH assay

Free radicals and oxidants play a dual role as both toxic and beneficial compounds, since they can be either harmful or helpful to the body. An excess of those radicals generates oxidative stress, which is harmful for human beings. This process plays a major role in the development of chronic and degenerative illnesses such as neurodegenerative diseases, cardiovascular, autoimmune disorders, aging, rheumatoid arthritis and cancer [35]. The human body has several mechanisms to counteract oxidative stress by producing antioxidants, which are either naturally produced in situ,

or externally supplied through foods and/or supplements. Thus, identifying plats with potent antioxidant capacity is of a great interest. A rapid, simple and inexpensive method to measure antioxidant capacity of food involves the use of the free radical DPPH which is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate the antioxidant activity [36]. Alcoholic solutions of DPPH have a characteristic absorption maximum at 517 nm. When an electron or hydrogen atom donating antioxidant is added to DPPH, a decrease in absorbance at 517 nm takes place due to the formation of the non-radical form DPPH-H, which does not absorb at 517 nm. This reaction has been measured by the decoloration assay where the decrease in absorbance at 517 nm produced by the addition of the antioxidant to the DPPH in methanol is measured. All the molasses samples were screened for radical scavenging activity against DPPH. The antioxidant activity is represented as the percentage of DPPH radical which varies between 66 to 75 % for the commercial samples and 90 % for the artisanal molasses (Table). Date molasses had a higher antioxidant value (84 %). These results are close to those found by Karaali et al. [37] who found an antioxidant value (54.8 %) for commercial molasses. This strong antioxidant activity of pomegranate molasses could be attributed to the presence of phenolic compounds and can have future therapeutic implications by protecting cells against oxidative stress.

Table 7: Antioxidant activities of different molasses samples

	Darna 2015	**Yamama 2015**	**Darna 2016**	**Dates 2016**	**Chtoura 2014**	**Artisanal 2015**
% Scavenging activity	66.5	75.1	68	84	66.1	90.6

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