EVALUATION OF HEAVY METALS AND PHYTOCHEMICAL COMPOSITION OF COMMERCIAL AND HOMEMADE LEBANESE POMEGRANATE MOLASSES

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Abstract

Pomegranate *(Punica granatum* L.*)* is one of the oldest known edible fruits that grow in the Mediterranean region, Southeast Asia, and Himalayas .Pomegranate is believed to have a high antioxidant activity because of its richness in active secondary metabolites such as phenolic acids, tannins and flavonoids, including anthocyanins, flavanols, and flavonols. Pomegranate molasses is a traditional condiment that can be obtained by boiling of pomegranate juice without the further addition of sugar or other additives. It is commonly used to improve the taste and aroma characteristics of salads and various dishes in different cultures. Pomegranate molasses is sold either as a commercial processed product, with a variety of brands, or as a traditional homemade product. In our study we evaluated and compared the phytochemical composition of all brands of commercial Lebanese pomegranate molasses; in addition to some homemade samples. The mean TPC in commercial samples was found to be lower than that in homemade samples (25.55 ± 14.22 mg GAE/g, 37.27 ± 10.78 mg GAE/g respectively, p value = 0.126). The mean TFC in commercial samples was also found to be lower than that in homemade samples (75.29 ± 61.9 mg QE/g, 124.21 ± 24.68 mg QE/g respectively, with p value = 0.141 > 0.05). Alkaloids were not detected in any of the samples. The mean TT in commercial samples was found to be lower than that in homemade samples (53.67 ± 23.09 mg TA/g, 104.31 ± 47.59 mg TA/g respectively, with p value = 0.001 < 0.05). Lead and Cadmium were not detected by AAS in any of the samples. Homemade samples were found to be more concentrated than the commercial samples in arsenic (0.197 ±0.13 mg/kg) > 0.1571 ± 0.063 mg/kg) chromium (7.96 ± 2.74 mg/kg > 5.51 ± 3.69 mg/kg), and copper (0.902 ± 1.09 mg/kg > 0.35 ± 0.54 mg/kg). All samples were found to have similar mercury content (0.005 mg/kg). Only 1 homemade sample and 5 commercial pomegranate molasses samples tested in our study showed comply with arsenic Codex (ML) maximum limit of 0.1mg/kg ; however, the other 3 homemade samples and the 23 commercial samples showed to have an arsenic concentration exceeding 0.1mg/kg. On the other hand, all the samples were found to comply with the ML of Cd and Pb respectively (0.05 mg/Kg; 0.1 mg/Kg). 14 commercial and 1 homemade sample were shown to have copper concentration less than the maximum allowable limit reported by GSO (0.21 mg/Kg), while the other 3 homemade samples and the 14 commercial samples showed to have copper concentrations greater than 0.21 mg/Kg with a maximum of 2.46 mg/Kg. All the tested pomegranate samples were found to have Cr concentrations exceeding the maximum allowable limit in fresh vegetables defined by the Food Safety Standards in China (0.5 mg/Kg) with a mean of 5.817 ± 3.646 mg/Kg. The overall results showed that the difference between the Total phenolic content (TPC), Total flavonoid content (TFC), and heavy metal composition in commercial and homemade samples was not statistically significant. Only total tannins (TT) showed a significant difference between the two groups. This could be due to the difference of pomegranate fruit, soil, climate, extraction, processing techniques, and the types of additives used.

Keywords

Pomegranate – Phytochemicals – Molasses – Total Phenols – Total Flavonoids – Total Tannins - Heavy metals

Introduction

Pomegranate *(Punica granatum* L.*)* is one of the oldest known edible fruits that grow in the Mediterranean region, Southeast Asia, and Himalayas (Langley, 2000).The use of pomegranate fruit in therapy dates back to Biblical times (Aviram & Dornfeld, 2001) and preparations of different parts of the plant have been used in folk medicine for many centuries as an important source of bioactive compounds to treat a variety of health conditions (Langley, 2000). The pomegranate fruit is featured in the coat of arms of several medical associations and it has been held sacred by many of the world's major religions including Buddhism, Judaism and Islam (Langley, 2000).

Pomegranate has gained widespread popularity as a functional food. The health effects of the whole fruit, its juices, and extracts, have been studied in relation to many diseases. Pomegranate is believed to have a high antioxidant activity because of its high phenolic content, specifically punicalagins, punicalins, gallagic acid, and ellagic acid (Johanningsmeier & Harris, 2011). Pomegranate juice has been shown to protect against cardiovascular diseases where it was shown to attenuate atherosclerosis (Aviram &Dornfeld, 2001). Pomegranate extracts from the seeds, fruit, roots, bark, pericap and flowers were shown to be effective agents in the prevention of diabetic complications, by reducing oxidative stress and lipid peroxidation (Banihani *et al*., 2013), and growth of breast, prostate, colon and lung cancer cells (Adhami *et al.*, 2009). The latter biological actions of the pomegranate fruit were attributed to its high phenolic content and vitamin C (Lansky& Newman, 2007). Moreover, recent studies showed that pomegranate extracts possess antimicrobial and antiviral activities (Mccarrell *et al*., 2008; Viuda-Martos, *et al*., 2010).

Pomegranate molasses is believed to have a significant effect on arteriosclerosis, hypercholesterolemia and cancer prevention due to the antioxidant capacity of the pomegranate fruit itself (Akpinar-Bayizit *et al*., 2016). A study done on mice showed that pomegranate molasses possesses a powerful antioxidant activity and a weight loss effect (Chalfoun-Mounayar *et al.*, 2012).

Pomegranate molasses is a concentrated product of pomegranate juice which is used as a condiment in different types of foods in various cultures. Even though the processing steps of molasses include cleaning, crushing, extraction, filtration, clarification, and boiling, studies showed that they still have high mineral and phenolic contents that could contribute to the total intake of these constituents in the human diet (Yilmaz *et al*., 2007; Incedayi *et al.*, 2010). However, the chemical compositions of the pomegranate fruits and their products depend on several factors such as cultivar, climatic circumstances, maturity of the fruit, manufacturing system and cultural practices (Akpinar-Bayizit *et* a*l*., 2016).

In the Lebanese market, pomegranate molasses is sold either as a commercial processed product, with a variety of brands sold in shops, supermarkets or malls, or as traditional homemade product. The composition of the pomegranate products may be affected by the method of production and the ingredients added, if any. Few studies have been done on the chemical composition of some brands of the pomegranate molasses sold in the Lebanese market including Darna, Yamama and Chtoura. These studies assessed total phenolic content, total flavonoid content, total alkaloid content, total tannins, total saponins, total proteins, minerals content and some heavy metals (iron, calcium, magnesium, lead, copper, cadmium, chromium, manganese and

zinc). To our knowledge, our study will be the first to assess the presence of pesticide residues colorants and some heavy metals (Arsenic, Copper, Lead, Cadmium, Chromium, and Mercury) in all brands of commercial Lebanese pomegranate molasses in addition to homemade samples. Therefore, the objectives of this study are:

1) To compare the phytochemical composition of all the commercial brands available in the Lebanese market and some homemade products of pomegranate molasses
2) To evaluate the presence of heavy metals in Lebanese pomegranate molasses.

Chapter 1: Generalities

1.1 Distribution and description of pomegranate *(Punica granatum* L.*)*

Pomegranate (*Punica granatum* L.) is considered one of the ancient mystical fruits used for thousands of years by many cultures and civilizations for its beneficial effects on health, fertility, longevity and rebirth (Akpinar-Bayizit *et al.*, 2012). Pomegranates have been cherished by most religions through history (Bhandari, 2012). Pomegranates represent life, regeneration, and marriage in Greek mythology. Pomegranate seeds symbolize sanctity, fertility, and abundance in Christianity. Also, pomegranate was used as an icon of renaissance and eternal life in Christian art. In Buddhism, pomegranate is considered to be a sacred fruit that symbolizes the essence of favorable influences. An Islamic legend holds that each pomegranate contains one seed that has come down from paradise. Pomegranate plays a distinct role as a fertility symbol according to the Bedouins and Hindu. The genus, Punica, was the Roman name for Carthage, where the best pomegranates were known to grow (Jurenka, 2008). Pomegranate fruit is native to Iran but is mainly grown in Tunisia, Turkey, Spain,

Egypt, Morocco, USA, China, India, Argentina, Israel and South Africa (Kahramanoglu & Usanmaz, 2016).

The pomegranate tree is known to grow typically to 12 to 16 feet in height and it is considered long-lived where there are some specimens in France known to be 200 years old (Jurenka, 2008). It has many spiny branches, glossy leaves, large red, white or variegated flowers, and red-brown bark which turns gray as the tree ages (Jurenka, 2008). The ripe pomegranate fruit can be up to 12.5 centimeters wide covered with a red leathery skin and is crowned by the pointed calyx. It contains many seeds (arils) separated by white, membranous pericarp, and each is surrounded by small amounts of red juice (Lansky & Newman, 2007).

Pomegranate can be consumed as fresh, fruit juice, fermented fruit juice, jam, jelly, wine, vinegar, paste, and used in flavoring products. Pomegranate molasses, which is concentrated pomegranate juice, is a traditional condiment commonly used to improve the taste and aroma characteristics of salads and various dishes.

Pomegranate molasses, a thick, dark red liquid, can be obtained by boiling of pomegranate juice without the further addition of sugar or other additives. The traditional method of pomegranate molasses production usually starts with washing and cleaning the raw fruit and then crushing and pressing it to obtain fresh juice, which is filtered and evaporated in open vessel or under vacuum to obtain the molasses (Akpinar-Bayizit *e*t *al*., 2016).

1.2 Phytochemical constituents of pomegranate *(Punica granatum)*

Studies showed that the numerous remedial properties of pomegranate are due to its richness in a variety of phytochemicals (Johanningsmeier & Harris, 2011). Pomegranates have been shown to contain 124 different phytochemicals, including their derivatives, extracted from

different parts of the pomegranate tree (Sharma & Maity, 2010). According to the literature, pomegranate parts are mostly rich in phenolic compounds (phenolic acids, flavonoids, tannins) (Figure 1) and other substances including tocopherols, triterpenoids, amino acids, alkaloids, non-conjugated fatty acids and sterols.

1.2.1 Phenolic compounds

The most common extracted phenolic compouds from pomegranate parts include punicalagins, punicalins, gallagic acid, ellagic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavones. Phenolic compounds are secondary metabolites widely found in fruits and comprise a diverse group of molecules that have a large range of structures and functions that may vary from a simple phenolic molecule to a complex high-molecular mass polymer. Phenolic compounds have an aromatic ring bearing one or more hydroxyl groups and they can be classified into water-soluble compounds (phenolic acids, phenylpropanoids, flavonoids and quinones) and water-insoluble compounds (condensed tannins, lignins and cell-wall bound hydroxycinammic acids) (Haminiuk *et al.*, 2012).

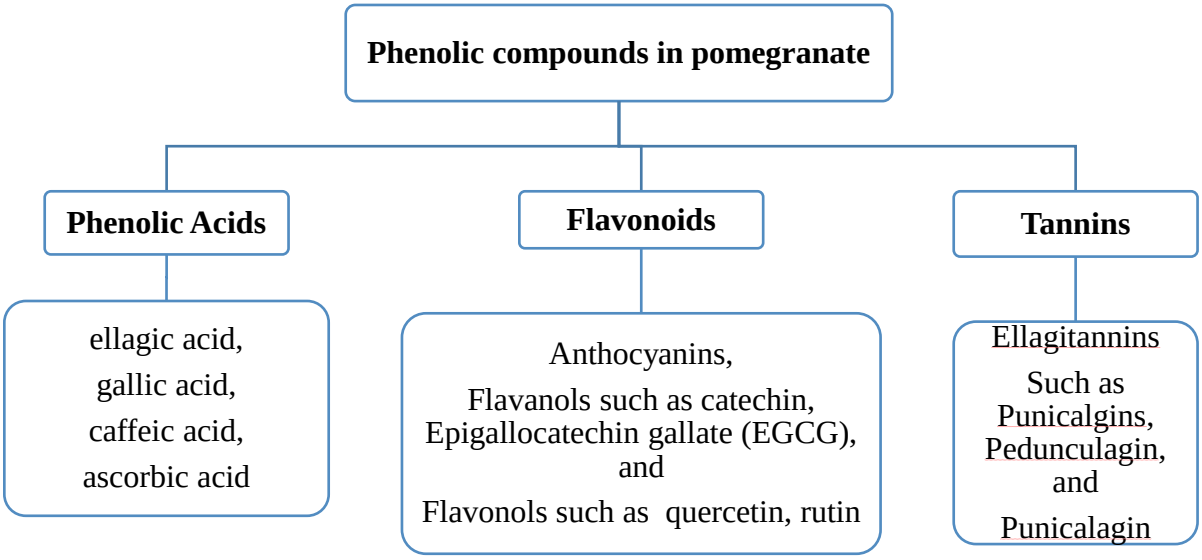


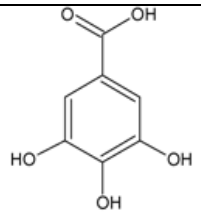
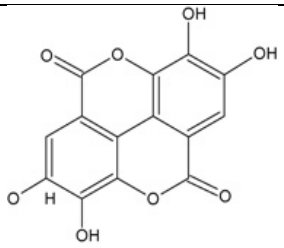
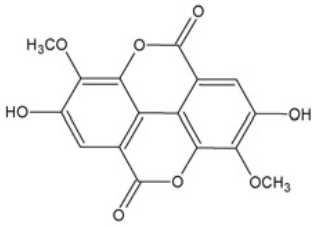
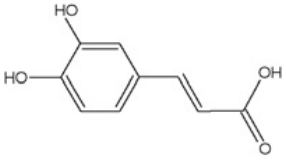
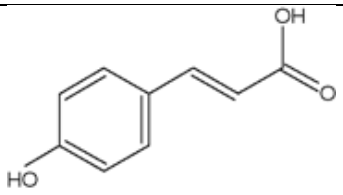
Figure 1. Major Phytochemicals in pomegranate

1.2.1.1 Phenolic acids

Phenolic acids are present in a variety of plant-based foods and their basic chemical structure includes a phenol ring attached to a carboxylic acid (-COOH) which gives them an acidic character in contrast to other phenolic compounds (Haminiuk *et al.*, 2012). Phenolic acids consist of two subgroups, the hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids have the C6–C1 structure in common and include gallic, p-hydroxybenzoic, protocatechuic, vanillic and syringic acids, which. Hydroxycinnamic acids on the other hand have a three-carbon side chain (C6– C3) in common and include caffeic, ferulic, p-coumaric and sinapic acids (Ignat *et al.*, 2011). The juice of pomegranate is rich in anthocyanins and potent antioxidant flavonoids that provide pomegranate juice with its bright color and its effective therapeutic effects (Lansky and Newman, 2007). Both flavonoids and tannins are shown to be abundant in the peels pomegranate.

Table 1 summarizes the most abundant phenolic acids in different parts of pomegranate plant including the fruit (juice), pericap (peel, rind), leaf, flower, seed and roots.

Table 1: Some major selected phenolic acids in Pomegranate (*Punica granatum)* parts

Compound Name	Chemical class	Compound Structure	Plant part (Juice, leaf, pericap, flower, seed, root)	Reference
Gallic acid	Hydroxybenzoic phenolic acids		Juice Pericap Flower	Huang et al. (2005b)
Ellagic acid	Hydroxybenzoic phenolic acids		Juice Pericap Seed	Amakura et al. (2000b), Wang et al. (2004)
3,3'-Di-*O*-methylellagic acid	Hydroxybenzoic phenolic acids		Seed	Wang et al. (2004)
Caffeic acid	Hydroxycinnamic phenolic acids (phenylpropanoids)		Juice Pericap	Artik (1998), Amakura et al. (2000a)
p-Coumaric acid	Hydroxycinnamic phenolic acids (phenylpropanoids)		Juice Pericap	Artik (1998), Amakura et al. (2000a)

1.2.1.2 Flavonoids

Flavonoids are the main bioactive compounds found in fruits that account for two-thirds of the dietary phenols and have a C_6-C_3-C_6 carbon (Ring A, C, and B) backbone or, more precisely, phenylbenzopyran functionality (Marais *et al.*, 2006).They are distributed into six subclasses (Figure 2) due to various substitutions of the C ring: flavonols, flavanones, isoflavones, flavan-3-ols, flavones and anthocyanins (Robbins, 2003).

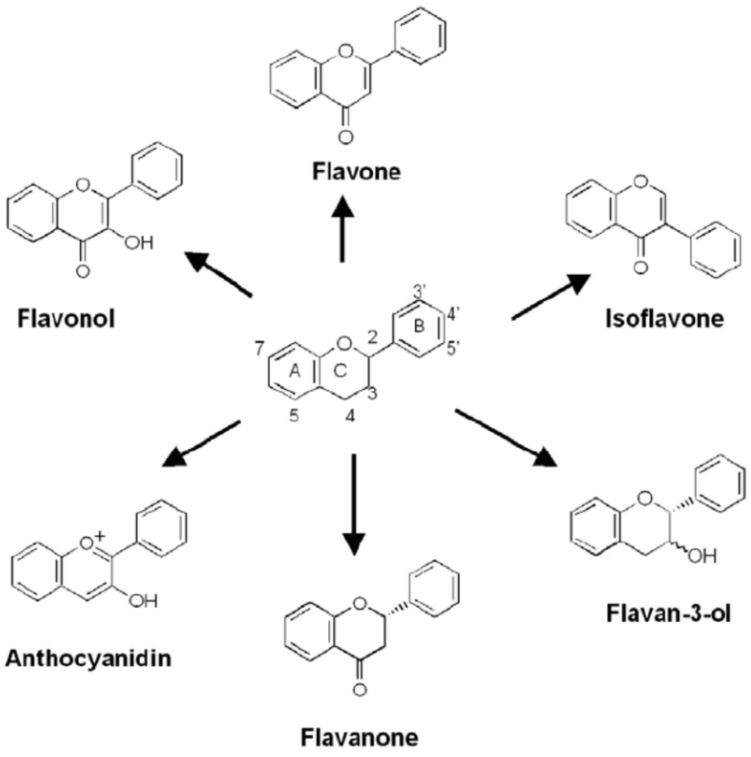


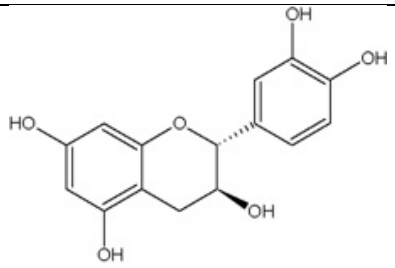
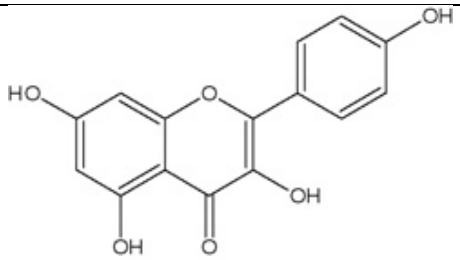
Figure 3. The chemical structure of the six subclasses of Flavonoids

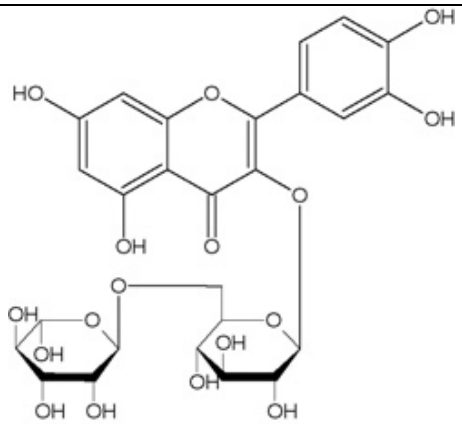
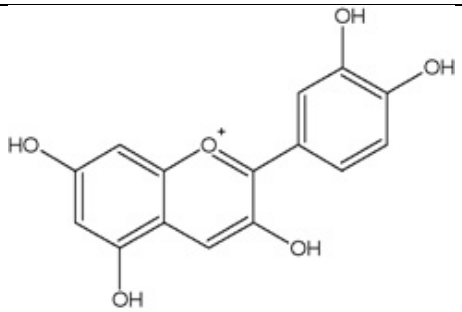
When flavonoids are linked to one or more sugar molecules they are known as flavonoid glycosides, with the degree of glycosylation directly affecting the antioxidant capacity of flavonoids, and when they are not they are called aglycones (Haminiuk *et al.*, 2012). Flavonoids were found to be very effective antioxidants, thus possessing a broad

spectrum of biological activities and when consumed regularly by humans, they have been associated with a reduction in the incidence of diseases such as cancer and heart disease (Ignat *et al.*, 2011). Furthermore, they have shown variety of health benefits such as acting as anti-inflammatory, anti-diabetic, anti-allergic and anti-platelet agents (Singh *et al.*, 2018).

Table 2 summarizes the most abundant flavonoids extracted from different parts of pomegranate plant including the fruit (juice), pericap (peel, rind), leaf, flower, seed and roots.

Table 2: Some major selected flavonoids acids in Pomegranate (*Punica granatum)* parts

Compound Name	**Chemical class**	**Compound Structure**	**Plant part (Juice, leaf, pericap, flower, seed, root)**	**Reference**
Catechin	Flavonoids		Juice Pericap	de Pascual-Teresa et al. (2000)
Kaempferol	Flavonoids		Pericap	van Elswijk et al. (2004)

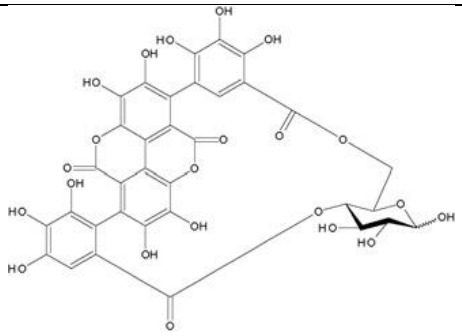
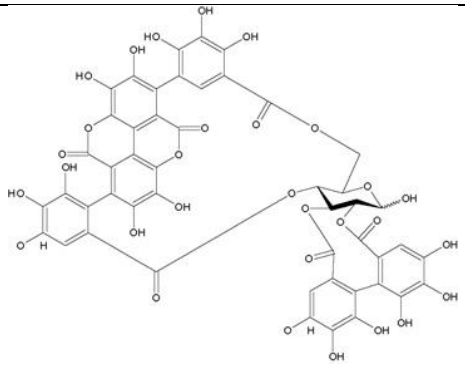
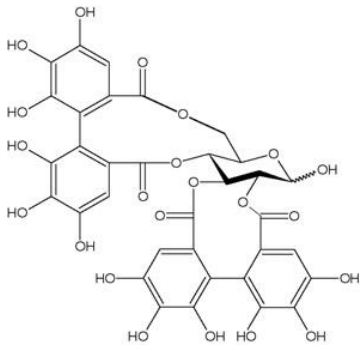
Compound Name	Chemical class	Compound Structure	Plant part (Juice, leaf, pericap, flower, seed, root)	Reference
Rutin	Flavonoids (Flavonol glycosides)		Pericap Juice	Artik (1998)
Cyanidin	Flavonoids (Anthocyanidins)		Pericap	Noda et al. (2002)

1.2.1.3 Tannins

Tannins are another important class of polyphenols that are found in fruits and are mostly present as phenolic polymers. Tannins are astringent and bitter substances of different molecular weights (Haminiuk *et al.,* 2012). They may be subdivided into hydrolysable tannins that are based on flavan-3-ols (-)-epicatechin and (+)-catechin and condensed tannins that are derivatives of gallic acid (Ignat *et al.,* 2011). Tannins have diverse effects on biological systems since they are potential metal ion chelators, protein precipitating agents and biological antioxidants (Ignat *et al.,* 2011).

Table 3 summarizes the most abundant tannins that are extracted from different parts of pomegranate plant including the fruit (juice), pericap (peel, rind), leaf, flower, seed and roots.

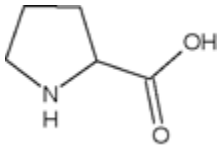
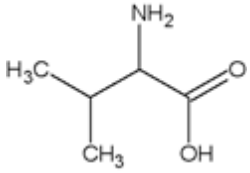
Table 3: Some major selected tannins in Pomegranate (*Punica granatum)* parts

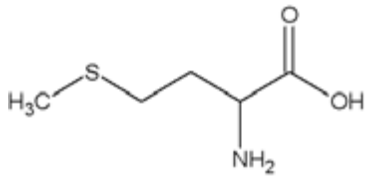
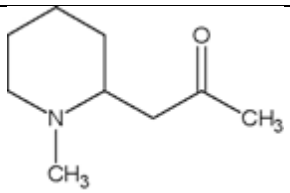
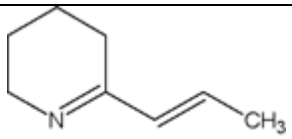
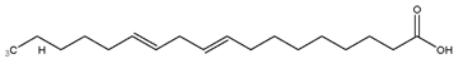
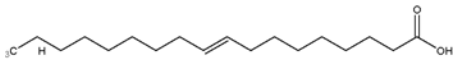
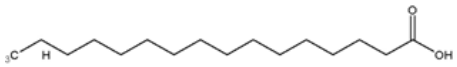
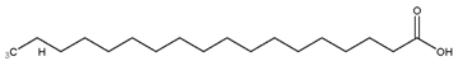
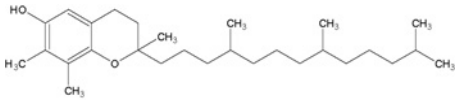
Compound Name	**Chemical class**	**Compound Structure**	**Plant part (Juice, leaf, pericap, flower, seed, root)**	**Reference**
Punicalin	Tannins (Ellagitannins)		Pericap Leaf Roots	Gil et al. (2000)
Punicalagin	Tannins (Ellagitannins)		Pericap Leaf Roots	Gil et al. (2000)
Pedunculagin	Tannins (Ellagitannins)		Pericap	Satomi et al. (1993)

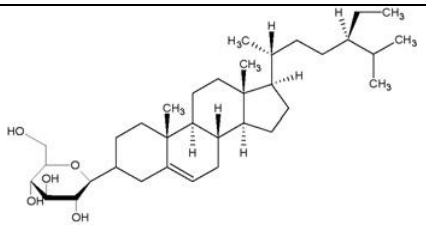
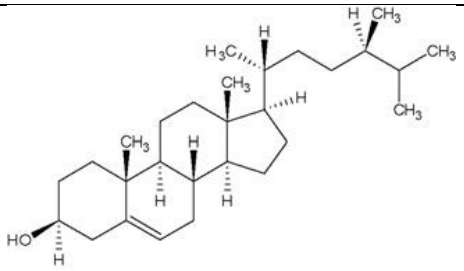
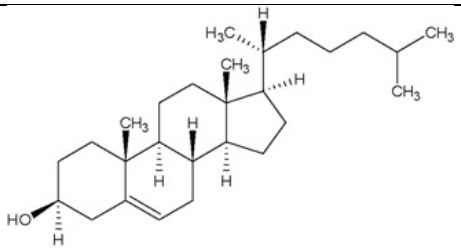
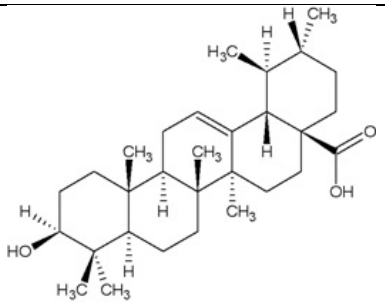
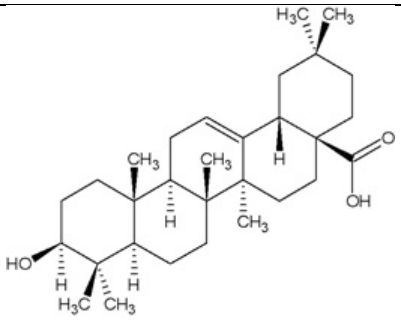
1.2.2 Other compounds

Other phytochemical classes have been shown to be found in the parts of pomegranate. Alkaloids are a major group of phytochemicals found mainly in the roots such as *N*-Methylpelletierene, Pseudopelletierene and Pseudopelletierene. Triterpenoids, which are a subgroup of Terpenes, such as ursolic acid and oleanolic acid are also found in some pomegranate parts. Some other compounds found in pomegranate parts include fatty acids, amino acids, tocopherols and sterols. Pomegranate seed oil (PSO) comprises 12–20% of total seed weight. The oil consists of approximately 80% conjugated octadecatrienoic fatty acids in addition to some minor components such as sterols and steroids (Lansky and Newman, 2007). Table 4 summarizes some other phytochemicals extracted from different parts of pomegranate plant including the fruit (juice), pericap (peel, rind), leaf, flower, seed and roots.

Table 4: Some other phytochemicals found in Pomegranate (*Punica granatum)* parts

Compound Name	**Chemical class**	**Compound Structure**	**Plant part (Juice, leaf, pericap, flower, seed, root)**	**Reference**
Proline	Amino acids		Juice	Velioglu et al. (1997)
Valine	Amino acids		Juice	Seppi Ak Franciosi (1980)

Compound Name	Chemical class	Compound Structure	Plant part (Juice, leaf, pericap, flower, seed, root)	Reference
Methionine	Amino acids		Juice	Seppi Ak Franciosi (1980)
N-Methylpelletierene	Pelletierine alkaloids		Roots	Neuhofer et al. (1993)
Hygrine	Piperidine alkaloids		Roots	Neuhofer et al. (1993)
Linoleic acid	Non-conjugated fatty Acids		Seed	Schubert et al. (1999)
Oleic acid	Non-conjugated fatty Acids		Seed	Schubert et al. (1999)
Palmitic acid	Non-conjugated fatty Acids		Seed	Schubert et al. (1999)
Stearic acid	Non-conjugated fatty acids		Seed	Schubert et al. (1999)
α-Tocopherol	Tocopherols		Seed	Kim et al. (2002)

Compound Name	Chemical class	Compound Structure	Plant part (Juice, leaf, pericap, flower, seed, root)	Reference
Daucosterol	Sterols		Seed	Wang et al. (2004)
Camesterol	Sterols		Seed	Abd El Wahab et al. (1998)
Cholesterol	Sterols		Seed	Abd El Wahab et al. (1998)
Ursolic acid	Triterpenoids		Seed Fruit	Huang et al. (2005c)
Oleanolic acid	Triterpenoids		Fruit	Huang et al. (2005a)

Chapter 2: Traditional uses of pomegranate *(Punicagranatum)*

According to Mahdihassan, 1984, ancient civilizations considered the pomegranate fruit a symbol of life, health, longevity, knowledge, femininity, fecundity, morality, immortality and spirituality. The most famous reported usage worldwide has been as a vermifugal agent in which pomegranate extracts were used as a killer and expeller of intestinal worms (Ismail et al., 2012). In the Ayurvedic medicine, an ancient system of medicine rooted in India, pomegranate bark and roots were believed to have anthelmintic properties and the peels are considered to be a powerful cure for diarrhea and oral aphthae (Naovi et al., 1991; Arseculeratne *et al.*, 1985). In ancient Egypt, an infusion of pomegranate root in water, which was strained and drunk, was used to treat infection with tape worms (Aboelsoud, 2010). In Indian Subcontinent, bleeding noses and ulcers have been treated with dried pomegranate peel, bark and flower infusions according to Ismail et al. (2012). In the Unani system of medicine that is practiced in the Middle East and India, pomegranate flowers are used as a remedy for diabetes (Bhandari, 2012). Other uses of pomegranate extracts in ancient medicine have been reported. Pomegranate peel powder has been used for the treatment of hyperacidity by orally ingesting 5–10 g of peel powder two to three times a day (Ismail et al., 2012). In addition, pomegranate peel extract was gargled as a liquid to relieve sore throat and hoarseness.

Chapter 3: Pharmacological studies on pomegranate

The pomegranate fruit has attracted a lot of scientific interest lately after the exciting preliminary findings related to the therapeutic effects of pomegranate.

Studies on pomegranate extracts of different plant parts have shown to employ significant anticancer, analgesic, anti-inflammatory, neuroprotective, sexual stimulant, anti-atherogenic, hypoglycemic, antidepressant, antioxidant, hypolipidaemic, antimicrobial, antifungal, antiviral, anti-alzheimer, immunomodulatory, estrogenic, skin protective, cardioprotective, dental care, musculoskeletal , gastro-protective, hepato-protective, anti-trichomonial, anti-obesity, anti-diarrhoeal and nootropic effects (Kalshetti *et Al.,* 2015).

3.1 Cancer Treatment

Recent research has shown that consumption of pomegranate extracts from the seeds, fruit, roots, bark, pericap and flowers, selectively inhibited the growth of breast, prostate, colon, skin and lung cancer cells in culture in preclinical animal studies.

An in-vitro study conducted by Seeram *et al*. (2005) evaluated anti-proliferative and apoptotic activities of pomegranate juice , purified punicalagin and total pomegranate tannins extracted from pomegranate husk, and ellagic acid on human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620) and prostate (RWPE-1, 22Rv1) tumor cells. It showed that pomegranate juice had the greatest anti-proliferative activity compared to punicalagin ellagic acid and pomegranate tannin extract, against all cell lines by inhibiting proliferation from 30% to 100% (Seeram *et al*., 2005). In KB oral cancer cells, ellagic acid inhibited proliferation from 45% to 88%, punicalagin from 0% to 42% and TPT from 0% to 27%. In HT-29 colon cancer cells, proliferation was inhibited from 0% to 21% by EA,

from 1% to 55% by punicalagin and from 2% to 71% by TPT. In RWPE-1 immortalized prostate epithelial cells, ellagicacid inhibited proliferation from 78% to 92%, punicalagin from 64% to 94% and TPT from 44% to 88% (Seeram *et al*., 2005).

In a study done by Wang *et al.* (2011), prostate cancer cell lines that are hormone refractory and invasive (DU145 and PC3) were used to assess the involvement of pomegranate juice in prostate cancer progression. Pomegranate juice was shown to up-regulate genes involved in cell adhesion such as E-cadherin, intercellular adhesion molecule 1 (ICAM-1), down-regulate genes involved in cell migration such as hyaluranan-mediated motility receptor (HMMR) and type I collagen and significantly reduce the level of secreted pro-inflammatory cytokines/chemokines such as IL-6 and IL-12p40. This resulted in decreasing inflammation and delaying prostate cancer progression by deterring the adhesion and rearrangement of cytoskeletal elements that allow cancer cells to move by increasing cell adhesion molecules and decreasing molecules that facilitate cell migration (Wang *et al.*, 2011). In a recent study, done by Adaramoye *et al.* (2017), in which normal prostate (BPH-1) cells and cancerous prostate cells (PC-3 and LNCaP) were cultured and treated with punicalagin, a major polyphenol found in pomegranate, it was found that punicalagin inhibited viability of the cancer cells in PC-3 and LNCaP (Adaramoye *et al.*, 2017).

Pomegranate extract was also shown to have a potential effect on the induction of apoptosis and inhibition of cellular proliferation in leukemia cell lines associated with cell cycle arrest (Dahlawi *et Al.*, 2012).

The effect of pomegranate extract on colon cancer was assessed by Kasimsetty *et al.* (2010). The results of their study revealed that the release of the phytochemicals

ellagitannins and their intestinal bacterial metabolites, urolithins, in the colon upon consumption of pomegranate juice exhibited dose- and time-dependent decreases in cell proliferation and clonogenic efficiency of HT-29 cells. The inhibition of cell proliferation was mediated through cell cycle arrest in the G0/G1 and G2/M stages followed by induction of apoptosis thus potentially curtailing the risk of colon cancer development (Kasimsetty *et al*, 2010).

Also, an in-vitro study done by Mahmoudi *et al.* (2017) on human breast cancer cell lines MCF-7 and MDA-MB-468 showed that pomegranate seed oil reduced the colony formation for MCF-7 and MDA-MB-468 cell lines when compared to the control group. Cell binding for both cell lines was reduced indicating that pomegranate seed oil may inhibit the migration and invasion of human breast cancer cell lines (Mahmoudi *et al.,* 2017). Moreover, pomegranate extract has the ability to suppress breast cancer stem cells. It was shown to inhibit mammosphere formation in two different cell lines, neoplastic mammary epithelial HMLER and breast cancer Hs578T, indicating that it inhibits cancer stem cells' ability to self-renew (Nallanthighal *et al.* 2017).

On the other hand, the anti-proliferative and pro-apoptotic properties of pomegranate fruit extract on lung tumorigenesis were examined (Khan *et al.* 2007). Normal human bronchial epithelial cells and human lung carcinoma A549 cells were treated with different doses of pomegranate fruit extract for 72 h. Pomegranate fruit extract resulted in a 33, 40 and 47% decrease in the viability of lung cancer cells at the doses of 50, 100, and 150 μg/ml of pomegranate fruit extract, respectively, but had minimal effect on normal bronchial cells at these doses.

It's important to mention, however, that a synergy in the interactions of the different extracts from several parts of the pomegranate fruit (peels, juice and seeds) was found to be more potent in inhibiting prostate cancer cell proliferation, invasion and phospholipase A-2 expression than any single extract (Lansky, *et al.*, 2005).

3.2 Diabetes treatment

Pomegranate extracts have been shown to have aldose reductase inhibitory action which when combined with pomegranate antioxidant activity can be of potential therapeutic use in prevention of diabetic complications. (Karasu *et al.*, 2012). In addition, in a study done on Tricetin, a type of flavones, isolated from the pomegranate flower, it was found that tricetin exhibited the strongest α-glucosidase, α-amylase, and lipase inhibitory activities that was comparable to the anti-diabetic drug acarbose (Sheng Wu & Li Tian, 2018).

To evaluate the hypoglycaemic activity of pomegranate seed, a study was carried out by Das *et Al.*, on rats made diabetic by injecting them with streptozotocin, a compound that produces permanent diabetes with extra pancreatic lesions that mimic the pathological status found in human diabetes. The diabetic rats were then given pomegranate seed extract. The results showed a significant reduction of blood glucose levels in streptozotocin-induced diabetic rats at the end of 12 h (Das *et al.*, 2001).

3.3 Cardiovascular diseases treatment

The antioxidant properties of pomegranate juice also contribute to it having potent anti-atherogenic effects in healthy humans and in atherosclerotic mice. In a study done by

Aviram *et al.* (2000) on healthy male volunteers, 13 healthy, nonsmoking men aged 20–35 y who were taking no medication were supplemented with 50 mL pomegranate juice/day (1.5 mmol total polyphenols) for 2 weeks. The results showed decreased LDL susceptibility to aggregation and retention and significant increase the in the activity of serum paraoxonase by 20% which is bound to HDL, increasing gradually and significantly ($P < 0.01$) the resistance of HDL to copper ion–induced oxidation. In the same study, 30 atherosclerotic apolipoprotein E–deficient mice were divided into 3 groups of 10 and each of the 3 groups received 0, 6.25, or 12.5 mL PJ (equivalent to 0, 0.175, and 0.350 mmol total polyphenols) in their drinking water per mouse per day for 14 weeks. Researchers found oxidation of LDL to be reduced by up to 90% after pomegranate juice consumption. (Aviram *et al.,* 2000).

In a randomized, placebo-controlled, double-blind study, Sumner *et al.* (2005) investigated the effect of daily consumption of pomegranate juice for 3 months on myocardial perfusion in patients who had coronary heart disease and myocardial ischemia. The patients were given either 240 mL pomegranate juice or a sports beverage of similar color, flavor, and caloric content daily for three months. Results showed a reduction in myocardial ischemia and improvement in myocardial perfusion. Researchers concluded that daily consumption of pomegranate juice may improve stress- induced myocardial ischemia in patients who have coronary heart disease and the pomegranate juice not only prevented hardening of the arteries by reducing blood vessel damage, but also reversed the progression of coronary heart disease (Sumner *et al.,* 2005).

3.4 Hepatic diseases treatment

A study done by Shaban *et al.* (2013) revealed that pomegranate possess protective abilities against induced hepatic injury in rats. 45 adult male rats were divided into 7 groups and they were treated with pomegranate peel and seed extracts for 23 weeks with different doses. The results showed that these extracts improved liver functions and reduced the oxidative stress and apoptosis of the liver compared to control group (Shaban *et al.,* 2013).

In a study done by Chidambara Murthy *et al.* (2002) albino rats with chemically damaged livers demonstrated were fed with dried methanolic extract, followed by carbon tetrachloride. Results showed that that pretreatment with a methanolic extract of pomegranate peel at 50 mg/kg (in terms of catechin equivalents) followed b carbon tetrachloride enhanced the free-radical scavenging activity of the hepatic enzymes and reduced lipid peroxidation values (Chidambara Murthy *et al.,* 2002).

3.6 Antioxidant activity

The antioxidant activities of pomegranate and its products have been studied in many in-vitro and in-vivo trials and using a variety of assays for determination. Pomegranate and its products showed high antioxidant activities, independently of the antioxidant test assayed, with significant linear correlation between phenolic content and antioxidant activity (Elfalleh *et al.,* 2009).

Many cellular processes are likely to be involved along with bioactive compounds found in pomegranate, and its products, to enhance reactive oxygen species elimination and inhibit their generation thus, reducing oxidative stress. Oxidative stress can induce DNA damage and trigger redox-dependent transcription factors which lead to cancer, inflammatory,

cardiovascular and neurodegenerative diseases, and aging (Akpinar-Bayizit *et al*., 2012). It has been shown that pomegranate extracts scavenge free radicals, and decrease macrophage oxidative stress and lipid peroxidation in animals (Rosenblat *et Al*. 2006).

Seeram *et al.* (2005) stated that the antioxidant activities of pomegranate juice were significantly higher than those found in other fruit juices, such as blueberry, cranberry, and orange. Other studies demonstrated that pomegranate juice and seed extracts have 2-3 times the *in vitro* antioxidant capacity of either red wine or green tea (Akpinar-Bayizit *et al*., 2012).

Pomegranate molasses are alleged to have significant protective effects against arteriosclerosis, cholesterol levels and cancer due to the antioxidant potential of pomegranate itself (Akpinar-Bayizit *et al*., 2016; Kamal, *et al*., 2018) and because concentrated pomegranate juice products, may have two to three folds higher mineral content and antioxidant capacity than fresh pomegranate juice (Yilmaz *et al*., 2007).The Gallic Acid content of pomegranate molasses and fresh pomegranate juice is 252.28 and 79.49 mg of Gallic Acid equivalent/L respectively, which causes the pomegranate molasses to have the strongest antioxidant properties compared to pomegranate juice (4 times more active than juice) (Chalfoun-Mounayar *et al.*, 2012).

The antioxidant effect and weight loss effect of pomegranate molasses and juice were measured *in vitro* by Chalfoun-Mounayar *et al.* (2012). 3 groups of mice were supplied with water only, water with pomegranate molasses and water with pomegranate juice respectively (4 ml/l) for 11 weeks. Pomegranate molasses showed to have 4 times stronger antioxidant properties than juice and researchers found significant reduction in weight in the groups of mice consuming pomegranate juice and molasses compared to the control group of mice (no pomegranate molasses added to drinking water) (Chalfoun-Mounayar *et al.*, 2012).

Akpinar-Bayizit *et al*. (2012) evaluated the antioxidant activity of pomegranate molasses by 2,2-diphenyl-l-picrylhydrazyl (DPPH) method and the results showed that the total phenolic content, varied from 118.28 to 828.15 mg of gallic acid equivalent per gram of pomegranate molasses, and antioxidant activity was found to be between 560.23 to 1885.23 μmol trolox equivalent per gram of sample. This means that pomegranate molasses can react against reactive oxygen species, such as superoxide anion, that may attack biological macromolecules, giving rise to protein, lipid, and DNA damage, cell aging, and oxidative stress-originated diseases.

Lebanese molasses phenolic content and antioxidant activity were assessed by Nasser G *et al*. (2017) in 3 brands of commercial pomegranate molasses and 1 artisanal sample of pomegranate molasses. The obtained results indicated the presence of various secondary metabolites such as phenols, flavonoids, and saponin and all samples showed a high antioxidant capacity reaching 90 %.

Chapter 4: Possible toxicants in pomegranate and its products

The fruit peel of pomegranate (*Punica granatum*) is known to exhibit a high affinity for Cu (II), Ni(II), Cd(II) and Zn(II) ions (Rao *et al.,* 2010). Nasser G *et al*. (2017) evaluated the presence of some heavy metals (lead, copper, cadmium, chromium, manganese and zinc) in 3 commercial brands of Lebanese pomegranate molasses. The results showed that copper and lead were detected in some samples.

The residue dynamics of the pesticides difenoconazole and propiconazole on pomegranate was studied after application at the recommended and double doses of active ingredients by Mohapatra (2016). The residues of difenoconazole and propiconazole remained on the

pomegranate fruit surface and did not move to the edible part (aril) after 2 years. However, if the whole pomegranate was used in the production of molasses, pesticide residues may be transferred to molasses.

The residue level and dissipation of carbendazim in/on pomegranate fruits has been studied by Mohapatra, & S. Lekha (2016). Carbendazim has been detected on the fruit, in addition in the arils. But, the residue level of carbendazim on pomegranate whole fruits from standard dose treatment was less than the EU maximum residue limit of 0.1 mg kg−1 at harvest and the carbendazim residues were <Limit of quantification in the aril and field soil at harvest (Mohapatra, & S. Lekha, 2016)

Utture *et al.,* (2012) conducted a food safety evaluation of buprofezin, dimethoate and imidacloprid residues in pomegranate. Results showed that the residues of buprofezin and dimethoate were confined to outer rind, while residues of imidacloprid penetrated into the arils and membrane, although at less than the maximum residue limit in all samples even at double dose (Utture *et al.,* 2012).

In a study done by Tran *et al.,* (2012) to evaluate the presence of pesticides in fashionable fruit juices including pomegranate, it was shown that the pesticide residue of fludioxonil has been detected in pomegranate juice at 89.2 ng/g.

Chapter 5: Materials and Methods

5.1 Sample description and collection

The samples (n=32) include 28 commercial brands of pomegranate molasses that were bought from different Lebanese markets and 4 homemade pomegranate molasses products.

The homemade pomegranate molasses samples were collected from trusted sources where no additives and sugars are added in their preparation. Homemade samples were prepared by concentrating pomegranate juice without the further addition of sugar and other additives. Pomegranate fruits were cleaned, squeezed, and strained to obtain juice free of rind and membrane pieces. Then, the juice was boiled on medium heat until the mixture thickens up, with stirring at regular intervals to avoid the molasses burning from the bottom of the pan. The traditional spoon test was done to check if the molasses is cooked to the perfect consistency. The spoon was dipped in the molasses and a line was traced across the back of the spoon with a fingertip. If the line remained visible, it was cooked perfectly. The pomegranate molasses was left to cool down for 10 minutes and then it was transferred it to clean glass jars and refrigerated until use.

All samples were analyzed in duplicates.

5.2 Chemicals

All chemicals were analytical reagent grade and obtained from the following sources: Aluminum Chloride, Ammonium Hydroxide, Folin-Ciocalteau reagent, Folin-Denis reagent, Gallic acid, Hydrogen Peroxide, Nitric Acid, Quercetin, Sodium Carbonate, Sodium Hydroxide, Sodium Nitrite, Tannic Acid (Sigma-Aldrich Co, Steinheim, Germany); Ethanol, Sodium Chloride (Fisher Scientific, UK).

5.3 Quantitative screening

5.3.1. Total Phenolic Content (TPC)

Total phenolic content was estimated by the Folin Ciocalteu's method described by Bhalodia *et al*., 2011. 0.1 g of PM was dissolved in 2 ml ethanol and 8 ml distilled water. The solution was mixed for 2 minutes using a vortex. Then, 0.2 ml of aliquots and standard Gallic acid solutions (10, 20, 40, 60, 80, 100 μg/ml) was positioned into the test tubes and 0.3 ml of distilled water and 0.5 ml of Folin Ciocalteu's reagent was mixed and shaken. After 5 minutes, 1 ml of 20 % sodium carbonate was. It was allowed to incubate for 40 minutes in the dark at room temperature. Intense blue color was developed. After incubation, the tubes were then centrifuged (10 min at 10000 g). Absorbance was measured at 730 nm using Jenway 6405 UV/Vis spectrophotometer. The blank was prepared using distilled water instead of PM sample. Samples were analyzed in duplicates, and the Total Phenolic concentration was extrapolated from a standard curve, constructed by using Gallic acid as a standard. The results for total phenolic contents were

expressed as mg of Gallic acid equivalent weight (GAE)/ g of pomegranate molasses according to the following formula: TPC = GAE x V x D /m, where TPC is the total phenolic content in mg/g of the extracts, GAE is the concentration of Gallic acid established from the calibration curve in mg/ml, V is the volume of the extract solution in ml, D is the dilution factor, and m is the mass of the PM in g (Anahita *et al*., 2015).

5.3.2. Total Flavonoid Content (TFC)

The total flavonoid content was measured by the Aluminum chloride colorimetric method (Sahu*et al.,* 2013). 0.5 g of PM was dissolved in 2 ml ethanol and 8 ml distilled water. The solution was mixed for 2 minutes using a vortex. 1ml of aliquots and standard Quercetin solution (20, 40, 60, 80,100 µg/l) was taken into 10ml volumetric flask, containing 4ml of distilled water. 0.3ml of 5%$NaNO_2$ was added to the flask. After 5min, 0.3ml 10%AlCl3 was added to the mixture. At the 6th min 2ml of 1M NaOH was added and volume was made up to 10ml with distilled water. The absorbance was measured at 510nm using Jenway 6405 UV/Vis spectrophotometer. The blank was prepared using distilled water instead of PM sample. Samples were analyzed in duplicates, and the Total Flavonoid concentration was extrapolated from a standard curve, constructed by using Quercetin as a standard. The results for total flavonoid contents were expressed as mg of Quercetin equivalent weight (QE)/ g of dry mass according to the following formula: TFC = QE x V x D /m, where TFC is the total flavonoid content in mg/g of the extracts, QE is the concentration of Quercetin established from the

calibration curve in mg/ml, V is the volume of the extract solution in ml, D is the dilution factor, and m is the mass of the PM in g (Madaan *et al*., 2011).

5.3.3. Total alkaloid content (TAC)

Total alkaloid content was assessed using the method of Harborne: 1g of the sample was mixed with 10% acetic acid in ethanol, leaving to stand for at least 4 hr. The extract was concentrated into one-quarter of the original volume and then add concentrated NH_4OH drop by drop to precipitate the alkaloid. The extract was collected by centrifugation, washed with 1 % NH_4OH and filtered. The residue was dried in the oven at 40 ºC and weighted. The alkaloid content was calculated using the following formula: % Alkaloid = [final weight of the sample / initial weight of the extract] ×100 (Nasser G *et al*., 2017).

5.3.4 Tannins (TT)

0.1 g of PM was dissolved in 2 ml ethanol and 8 ml distilled water. The solution was mixed for 2 minutes using a vortex. 1.0 ml of the aliquots and standard solution of tannic acid (100 - 800 ug/ml) was made up to 7.5 ml with distilled water. Then 0.5 ml Folin-Denis reagent and 1 ml Na_2CO_3 solution were added. The volume was increased to 10 ml with distilled water and absorbance was measured at 700 nm using Jenway 6405 UV/Vis spectrophotometer. The total tannic acid content was expressed as mg of tannic acid equivalent per gram of extract (Padma *et al.,* 2013). The blank was prepared using distilled water instead of PM sample.

Samples were analyzed in duplicates, and the Total Tannins concentration was extrapolated from a standard curve, constructed by using Tannic acid as a standard. The results for total Tannins were expressed as mg of Tannic acid equivalent weight (TAE)/ g of dry mass according to the following formula: TTC = TAE x V x D /m, where TTC is the total tannins content in mg/g of the extracts, TAE is the concentration of Tannic acid established from the calibration curve in mg/ml, V is the volume of the extract solution in ml, D is the dilution factor and m is the mass of the PM in g.

5.3.5. Heavy metals

5.3.5.1 Copper, Lead, Cadmium, Chromium, and Arsenic

The presence of the following heavy metals copper (Cu), lead (Pb), cadmium (Cd), chromium (Cr), and arsenic (As) was evaluated using a Thermo Scientific™ iCE™ 3500 AAS Atomic Absorption Spectrometer.

The pomegranate molasses samples were weighed (0.5 g) and transferred into a digestion vessel. The microwave digestion vessels containing the samples were placed in a fume extraction hood before adding 9ml Nitric acid and 1ml H_2O_2. The vessels were placed into ETHOS UP – Milestone high performance microwave digestion system and digested for 30 min at 200ºC, followed by cooling for 15 min until the temperature reached 30-40 ºC. The samples were transferred into 100 ml volumetric

flask, under a fume extraction hood, and the volume was brought to 100 ml by adding distilled water.

The samples were transferred into sample tubes and placed in the rack of the auto sampler of the Thermo Scientific iCE 3500 AAS.

The calibration curve of each metal was made with standard solutions, diluted by 9% HNO_3, with these concentrations: 6, 12, 16, 20 and 30 µg/L for Arsenic and Chromium, 2, 6, 8, 12, and 16 µg/L for Lead, 1, 3, 4, 6, and 8 µg/L for Cadmium, and 6, 8, 12, 16, and 20 µg/L for Copper.

5.3.5.2 Mercury

The concentration of mercury (Hg) in the samples was measured using DMA-Direct Mercury Analysis System- Milestone. No sample preparations was required. The samples were weighed (0.1 g) and analyzed directly.

5.4. Statistical Analyses

Statistical analysis were performed using SPSS software to analyze the results and compare the composition of the collected samples.

Means ±standard deviations for the pomegranate molasses commercial brands and pomegranate molasses homemade samples were calculated. Comparisons between means of pomegranate molasses commercial brands and pomegranate molasses homemade samples were made by means of independent

samples t-test. Data was analyzed using SPSS version 22 and a p-value of < 0.05 was used as indicative of statistical significance.

Chapter 6: Results and Discussion

6.1. Determination of the Phytochemical the composition

6.1.1 Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of pomegranate molasses was determined through analysis of Folin-Ciocalteau method where the absorbance of the blue color was measured at 765nm, which is the result of the redox reaction of the reduction of the Folin- Ciocalteu reagent by phenolic compounds. The total polyphenol content was calculated via the Gallic acid standard curve.

	Commercial Brands	TPC (mg/g of PM)		Commercial Brands	TPC (mg/g of PM)
1	Al Baraka	18.48	**18**	Machaalany	21.64
2	AlBaydar	22.79	**19**	Maxims	34.86
3	AlHokool AlKhadraA	15.26	**20**	Maymone	24.30
4	AlNabil	34.45	**21**	Monzer	36.80
5	AlRabih	35.32	**22**	Moroj Chtoura	20.81
6	Alwadi	22.61	**23**	Sallom	37.02
7	Aoun	22.57	**24**	Spinneys	9.86
8	Ashkar	28.24	**25**	Taj	11.88
9	Bolous	10.68	**26**	Terrois	52.46
10	Chtoura Fields	14.81	**27**	Tiba	23.84
11	Chtoura Gardens	21.98	**28**	Yamama	21.79
12	Cortas	21.85			
13	Gardenia	10.08		**Homemade**	**TPC (mg/g of PM)**
14	Jana	27.42	**29**	HM1	44.32
15	Jeita	13.47	**30**	HM2	41.60
16	Kortbawi	78.28	**31**	HM3	21.19
17	Lumiere	22.06	**32**	HM4	41.97

Table 5: Total Phenolic Content (TPC) expressed as mg of Gallic acid equivalent weight (GAE)/g of PM

	Commercial Or Homemade Sample	N	Minimum	Maximum	Mean	Std. Deviation	Sig.
Total Phenolic Content (mg/g of PM)	0 Commercial Sample	28	9.86	78.28	25.557500	14.2277209	0.126
	1 Homemade Sample	4	21.19	44.32	37.270000	10.7874588	

Table 6: Mean Values and Mean comparison of Total Phenolic Content (TPC) between commercial and homemade samples

The total phenolic content of the 28 commercial brands and the 4 homemade samples are shown in Table 5. The TPC of commercial pomegranate molasses samples varied between a minimum of 9.86 mg GAE /g for Spinneys brand and a maximum of 78.28 mg/g for Kortbawi brand having a mean of 25.55 ± 14.22 mg GAE/g (Table 6). For the homemade samples, TPC varied between a minimum of 21.19 mg GAE/ g for HM3 and a maximum of 44.32 mg GAE /g for HM1 having a mean of 37.27± 10.78 mg GAE/g (Table 6). Those findings are incompatible with the results of a research study done by Yılmaz et al. (2007), using 3 commercial pomegranate molasses samples, where TPC was reported to be 52.6 mg GAE/g. The high TPC values in pomegranate molasses reported were expected because pomegranate molasses is a concentrated product in comparison to pomegranate juice. Phenolic compounds increase in their percentage during processing, compared to pomegranate juice where the total phenolics are reported to be 9870 µg GAE / ml in pomegranate juice concentrate (Orak, 2009). On the other hand, these results are discordant with a study done on 4 Lebanese commercial pomegranate molasses samples where TPC varied between 90 and 179.5 mg GAE/ g (Nasser G *et al*., 2017). A study done on seven different market Turkish brands by Incedayı et al (2010) reported that the total amount of phenolic compounds in commercial pomegranate molasses samples ranged between 0.55 and 9.69 mg

GAE/g. Different factors such as type of pomegranate fruit, soil, climate, extraction, processing techniques, and the difference in sample size might be the reason behind these differences between our molasses samples and those of Nasser G et al (2017) and Incedayı et al (2010).

The mean TPC in commercial samples was found to be lower than that in homemade samples (25.55 ± 14.22 mg GAE/g, 37.27 ± 10.78 mg GAE/g respectively, p value = 0.126) (Table 6). But this difference is not statistically significant since p value > 0.05. These findings are not compatible with the study of Özmert Ergin (2020) on 3 commercial and 3 homemade pomegranate molasses samples, where the average phenolic content in commercial pomegranate molasses samples was found to be higher (490.22 ±278 mg GAE/L) than that in the traditional pomegranate molasses samples (24.11 ±4.06 mg GAE/L). The differences of processing techniques and the species of pomegranate used in the Lebanese and the Turkish pomegranate molasses may be the reason behind the incompatibility of our results and those of Özmert Ergin (2020).

6.1.2. Total Flavonoid Content (TFC)

The total flavonoid content (TFC) of pomegranate molasses was determined by the spectrophotometric assay based on aluminium complex formation where the absorbance was measured at 510nm. TFC was calculated based on the Quercetin curve and the standard curve equation was y= 0.5443x - 0.0004, where $R^2 = 0.9942$.

	Commercial Brands	TFC (mg/g of PM)		Commercial Brands	TFC (mg/g of PM)
1	Al Baraka	8.65	**18**	Machaalany	59.12
2	AlBaydar	6.91	**19**	Maxims	74.03
3	AlHokool AlKhadraA	7.98	**20**	Maymone	7.24
4	AlNabil	129.40	**21**	Monzer	107.35
5	AlRabih	79.28	**22**	Moroj Chtoura	83.11
6	Alwadi	42.21	**23**	Sallom	91.44
7	Aoun	90.83	**24**	Spinneys	37.27
8	Ashkar	65.23	**25**	Taj	37.95
9	Bolous	40.79	**26**	Terrois	209.83
10	Chtoura Fields	47.28	**27**	Tiba	78.48
11	Chtoura Gardens	111.79	**28**	Yamama	171.00
12	Cortas	81.70			
13	Gardenia	11.38		**Homemade**	
14	Jana	75.97	**29**	HM1 (Dima 1)	146.78
15	Jeita	39.60	**30**	HM2 (Dima 2)	122.51
16	Kortbawi	276.05	**31**	HM3 (Salwa)	90.35
17	Lumiere	64.32	**32**	HM4 (Mira)	137.20

Table 7: Total Flavonoid Content (TFC) expressed as mg of Quercetin equivalent weight (QE)/g of PM

	Commercial Or Homemade Sample	N	Minimum	Maximum	Mean	Std. Deviation	Sig.
Total Flavonoid Content (mg/g of PM)	0 Commercial Sample	28	6.91	276.05	76.2925	61.90999	0.141
	1 Homemade Sample	4	90.35	146.78	124.2100	24.68154	

Table 8: Mean Values and Mean comparison of Total Flavonoid Content (TFC) between commercial and homemade samples

The TFC in commercial pomegranate molasses samples was found to vary between a minimum of 6.91 mg QE /g for AlBaydar brand and a maximum of 276.05 mg QE /g for Kortbawi brand having a mean of 76.29 ± 61.9 mg QE /g (Table 7, 8). In homemade samples, TFC was found to vary between 90.35 mg QE /g for HM3 and 146.78 mg QE /g for HM1 with a mean OF 124.21 ± 24.68 mg QE /g (Table 7, 8). Those findings are inconsistent with the results of Nasser G et al

(2017) where the TFC in commercial pomegranate molasses was reported to range between 54.34 mg RE/g and 137.74 mg RE/g.

The mean TFC in commercial samples was found to be lower than that in homemade samples (76.29 ± 61.9 mg QE/g, 124.21 ± 24.68 mg QE/g respectively, with p value = 0.141 > 0.05) (Table 8). These findings are compatible with the findings of Nasser G et al (2017) where the artisanal molasses has the highest value of TFC (137.74 mg/g) compared to the commercial ones (68.5 mg/g).

This difference between commercial and artisanal samples might be due to different factors such as types of grenade, soil, climate, extraction and evaporation techniques (Nasser G *et al*., 2017).

6.1.3. Total Tannins

The total tannins (TT) of pomegranate molasses was determined through the analysis of Folin-Denis method where the absorbance of the blue color was measured at 700nm. The total tannins concentration was calculated via the Tannic acid standard curve shown in Figure 4, and the standard curve equation was $y = 7.9071x + 0.0445$, where $R^2 = 0.9889$.

The total tannins in commercial pomegranate molasses samples were found to vary between a minimum of 15.72 mg TA /g for Taj brand and a maximum of 99.88 mg TA /g for Cortas brand having a mean of 53.679 ± 23.096 mg TA/g (Table 9, 10). In homemade samples, the total tannins were found to vary between a minimum of 50.37 mg TA/g for Homemade sample 3 (HM3) and a maximum of

144.99 mg TA/g for Homemade sample 4 (HM4) having a mean of 104.312 ± 47.59 mg TA/g (Table 9 ,10).

	Commercial Brands	TT (mg/g of PM)		Commercial Brands	TT (mg/g of PM)
1	Al Baraka	66.40	**18**	Machaalany	69.77
2	AlBaydar	72.91	**19**	Maxims	69.12
3	AlHokool AlKhadraA	31.17	**20**	Maymone	57.33
4	AlNabil	59.13	**21**	Monzer	87.72
5	AlRabih	42.00	**22**	Moroj Chtoura	75.79
6	Alwadi	50.03	**23**	Sallom	31.74
7	Aoun	26.55	**24**	Spinneys	18.87
8	Ashkar	35.97	**25**	Taj	15.72
9	Bolous	32.69	**26**	Terrois	86.91
10	Chtoura Fields	17.92	**27**	Tiba	60.48
11	Chtoura Gardens	33.14	**28**	Yamama	49.45
12	Cortas	99.88			
13	Gardenia	60.27		**Homemade**	
14	Jana	83.62	**29**	HM1 (Dima 1)	78.21
15	Jeita	51.44	**30**	HM2 (Dima 2)	143.68
16	Kortbawi	72.78	**31**	HM3 (Salwa)	50.37
17	Lumiere	44.23	**32**	HM4 (Mira)	144.99

Table 9: Total Tannins expressed as mg of Tannic acid equivalent weight (TA)/g of PM

	Commercial Or Homemade Sample	N	Minimum	Maximum	Mean	Std. Deviation	Sig.
Total Tannins Content (mg/g of PM)	0 Commercial Sample	28	15.72	99.88	53.6796	23.09616	0.001
	1 Homemade Sample	4	50.37	144.99	104.3125	47.59409	

Table 10: Mean Values and Mean comparison of Total Tannins (TT) between commercial and homemade samples

The mean TT in commercial samples was found to be lower than that in homemade samples (53.67 ± 23.09 mg TA/g, 104.31 ± 47.59 mg TA/g respectively, with p value = 0.001 < 0.05) (Table 10). These findings are not consistent with the results of Nasser G et al (2017) where the TT in commercial pomegranate molasses

was reported to be very similar between the commercial samples and the homemade sample varying between 70 and 78 % of the initial weight of the sample.

Our results showed that the traditional Lebanese homemade pomegranate molasses have high total phenolic content, total flavonoid content, and total tannins as compared to those produced commercially. According to the Lebanese standard for commercial pomegranate molasses NL 813, molasses that contain more than only 5% of pomegranate juice are considered to comply with the Lebanese standards (LIBNOR, 2020). This means that the percentage of pomegranate juice in molasses varies between different brands. This directly affects the TPC, TFC, and TT and could explain our results.

6.1.4. Total Alkaloid Content (TAC)

According to the Harbone method used, Alkaloids were not detected in any of the commercial or homemade pomegranate molasses samples. No precipitate was formed during the addition of concentrated ammonium hydroxide. Wang et al. (2010) reported that the main alkaloids are abundantly found in the bark of the stem and roots of pomegranate. This might be the reason why alkaloids are not detected in molasses.

6.5. Heavy metals

The bioaccumulation of heavy metals in the food chain can be highly dangerous to human health. The presence of heavy metals in food products, even in traces, can cause serious health problems. Arsenic exposure can cause symptoms

ranging from nausea and vomiting and abnormal heart beat to the formation of skin lesions, internal cancers, neurological problems, pulmonary disease, peripheral vascular disease, and cardiovascular disease in cases of long term exposure (Smith *et al*., 2000). Chronic exposure of lead may result in mental retardation, birth defects, hyperactivity, paralysis, muscular weakness, brain damage, kidney damage and may even lead to death (Martin & Griswold, 2009). The exposure to mercury is associated with serious health effects that include inflammation, apoptosis, edema, encephalopathy and irreversible brain damage (Brochin *et al*., 2008). Long-term exposure to cadmium can result in kidney disease, fragile bones and lung damage (Bernard, 2008). Copper exposure may lead to various health effects such as nausea and vomiting, diarrhea, hepatic and/or renal complications, and even death (Agency for Toxic Substances and Disease Registry (ATSDR), 2004). Chromium [Cr(VI)] is a known carcinogen when inhaled, but when ingested some studies have shown that it induces DNA mutations and initiates tumor formations (Sun et al., 2015). Mercury, Cadmium, and Copper were shown to have a direct toxic action on steroid-producing cells in the adrenal gland (Ng & Liu, 1990).

The presence of the following heavy metals arsenic (As), lead (Pb), cadmium (Cd), chromium (Cr), and copper (Cu) was evaluated using Atomic Absorption Spectrometer. The concentrations were extrapolated from the standard curves corresponding to each metal. The standard curve equation of arsenic (As) was $y = 0.0026x + 0.0014$, where $R^2 = 0.9776$. The standard curve equation of lead (Pb) was $y = 0.0123x + 0.0097$ where, $R^2 = 0.9654$. The standard curve equation of cadmium (Cd) was $y = 0.0414x + 0.0193$ where, $R^2 = 0.9843$. The standard curve

equation of chromium (Cr) was y = 0.0113x - 0.0252, where R^2 = 0.9444. The standard curve equation of copper (Cu) was y = 0.0459x + 0.0618, where R^2 = 0.9683. The concentration of mercury (Hg) was directly analyzed in mg/Kg of Pomegranate Molasses.

	Commercial Brands	As (ppm)	Pb(ppm)	Cd(ppm)	Cr(ppm)	Cu(ppm)	Hg(ppm)
1	AlBaraka	0.05	0	0	2.53	0.64	0.0054
2	AlBaydar	0.24	0	0	4.37	0	0.0152
3	AlHokool AlKhadraA	0.08	0	0	12.96	0	0.0039
4	AlNabil	0.20	0	0	7.45	0.26	0.0057
5	AlRabih	0.12	0	0	10.22	0	0.0020
6	Alwadi	0.05	0	0	1.62	0.99	0.0094
7	Aoun	0.16	0	0	9.99	0	0.0045
8	Ashkar	0.20	0	0	1.82	0	0.0035
9	Bolous	0.16	0	0	3.38	0.25	0.0058
10	Chtoura Fields	0.20	0	0	2.14	0.31	0.0014
11	Chtoura Gardens	0.12	0	0	8.66	0.60	0.0035
12	Cortas	0.16	0	0	2.15	0.60	0.0135
13	Gardenia	0.16	0	0	5.59	0.10	0.0035
14	Jana	0.16	0	0	2.57	0.40	0.0050
15	Jeita	0.20	0	0	4.70	0.58	0.0021
16	Kortbawi	0.24	0	0	2.70	2.53	0.0041
17	Lumiere	0.20	0	0	9.72	0.11	0.0033
18	Machaalany	0.16	0	0	4.33	0	0.0066
19	Maxims	0.28	0	0	2.41	0.09	0.0077
20	Maymone	0.12	0	0	12.95	0	0.0021
21	Monzer	0.12	0	0	10.35	0	0.0046
22	Moroj Chtoura	0.16	0	0	4.23	1.39	0.0037
23	Sallom	0.05	0	0	10.28	0	0.0033
24	Spinneys	0.24	0	0	4.18	0.30	0.0039
25	Taj	0.05	0	0	1.71	0	0.0035
26	Terrois	0.24	0	0	2.53	0.36	0.0085
27	Tiba	0.12	0	0	7.06	0.46	0.0038
28	Yamama	0.16	0	0	1.70	0	0.0023
	Homemade						
29	HM1	0.08	0	0	10.21	0.28	0.0053
30	HM2	0.12	0	0	4.12	2.46	0.0044
31	HM3	0.39	0	0	9.62	0.03	0.0064
32	HM4	0.20	0	0	7.91	0.84	0.0044

Table 11: Heavy metal concentrations (ppm: mg/kg) in commercial and homemade samples

	N	Minimum	Maximum	Mean	Std. Deviation
Arsenic Concentration (ug/L)	28	.05	.28	.1571	.06399
Lead Concentration (ug/L)	28	.00	.00	.0000	.00000
Cadmium Concentration (ug/L)	28	.00	.00	.0000	.00000
Chromium Concentration (ug/L)	28	1.62	12.96	5.5107	3.69515
Copper Concentration (ug/L)	28	.00	2.53	.3561	.54693
Mercury Concentration (mg/Kg of PM)	28	.0014	.0152	.005064	.0032577
Valid N (listwise)	28				

Table 12: Heavy metals Mean Values in Commercial Brands

	N	Minimum	Maximum	Mean	Std. Deviation
Arsenic Concentration (ug/L)	4	.08	.39	.1975	.13769
Lead Concentration (ug/L)	4	.00	.00	.0000	.00000
Cadmium Concentration (ug/L)	4	.00	.00	.0000	.00000
Chromium Concentration (ug/L)	4	4.12	10.21	7.9650	2.74263
Copper Concentration (ug/L)	4	.03	2.46	.9025	1.09217
Mercury Concentration (mg/Kg of PM)	4	.0044	.0064	.005125	.0009500
Valid N (listwise)	4				

Table 13: Heavy metals Mean Values in Homemade Samples

Table 11 shows the concentrations of different heavy metals in all the tested samples. Lead and Cadmium were not detected by AAS in any of the samples. Arsenic concentration in commercial pomegranate molasses was found to vary between 0.05 mg/Kg for AlBaraka and 0.28 mg/kg for Maxims with a mean of 0.157 ± 0.063 mg/kg (Table 11, 12). In homemade samples, arsenic was found to vary between 0.08 mg/Kg for HM1 and 0.39 mg/Kg for HM3 with a mean of 0.197 ± 0.137 mg/kg (Table 11, 13). Homemade samples were found to be more concentrated in arsenic (0.197 ±0.13 mg/kg) than commercial samples (0.1571 ± 0.063 mg/kg) (Table 14). In addition, homemade samples were found to

be more concentrated in chromium (7.96 ± 2.74 mg/kg) than commercial samples (5.51 ± 3.69 mg/kg) (Table 14). In commercial pomegranate molasses, chromium concentration was found to vary between 1.62 mg/Kg for Alwadi brand and 12.96 mg/kg for AlHokool AlKhadraA brand with a mean of 5.510 ± 3.695 mg/kg (Table 11, 12). In homemade samples, chromium concentration was found to vary between 4.12 mg/Kg for HM2 and 10.21 mg/kg for HM1 with a mean of 7.765 ± 2.742 mg/kg (Table 11, 13). Homemade samples were also found to be more concentrated in copper (0.902 ± 1.09 mg/kg) than commercial samples (0.35 ± 0.54 mg/kg) (Table 14). In commercial pomegranate molasses, copper concentration was found to vary between 0 mg/Kg for 11 commercial brands (AlBaydar, AlHokool AlKhadraa, AlRabih, Aoun, Ashkar, Machaalany, Maymone, Monzer, Sallom, Taj, and Yamama) and 2.53 mg/kg for Kortbawi brand with a mean of 0.356 ± 0.546 mg/kg (Table 11, 12). In homemade samples, copper concentration was found to vary between 0.03 g/Kg for HM3 and 2.46 mg/kg for HM2 with a mean of 0.9025 ± 1.092 mg/kg (Table 11, 13). In commercial pomegranate molasses, mercury concentration was found to vary between 0.0014 mg/Kg for Chtoura Fields brand and 0.0152 mg/kg for AlBaydar brand with a mean of 0.005 ± 0.003 mg/kg (Table 11, 12). Mercury concentration was found to range between 0.0044 mg/Kg for HM2 and 0.0066 for HM3 mg/Kg with a mean of 0.005 ± 0.009 mg/kg (Table 11, 13). Homemade samples and commercial samples were found to have similar mean concentrations of mercury (0.005 mg/kg) (Table 14).

	Commercial Or Homemade Sample	N	Mean	Std. Deviation	Sig.
Arsenic Concentration (mg/kg)	0 Commercial Sample	28	.1571	.06399	0.320
	1 Homemade Sample	4	.1975	.13769	
Chromium Concentration (mg/kg)	0 Commercial Sample	28	5.5107	3.69515	0.213
	1 Homemade Sample	4	7.9650	2.74263	
Copper Concentration (mg/kg)	0 Commercial Sample	28	.3561	.54693	0.111
	1 Homemade Sample	4	.9025	1.09217	
Mercury Concentration (mg/Kg of PM)	0 Commercial Sample	28	.005064	.0032577	0.971
	1 Homemade Sample	4	.005125	.0009500	

Table 14: Mean comparison of heavy metal concentration between commercial and homemade samples

To the best of our knowledge, only a few studies determined the concentrations of some heavy metals in pomegranate molasses but with smaller samples. Nasser G et al (2017) studied the mineral composition of 4 commercial and 1 homemade sample and reported that chromium was not detected in any samples, lead was detected in the homemade sample only, cadmium was detected in 3 commercial samples (Darna 2015, Darna 2016, Yamama 2015) ranging from 0.22 to 2.68 mg/kg and in the homemade sample (0.66 mg/Kg); and copper was detected in 1 commercial sample only (Yamama 2016). Our findings are not compatible with Nasser G et al (2017) since in our study no lead and cadmium were detected in any sample, chromium was detected in all tested samples with a mean of 7.96 ± 2.74 mg/kg for homemade samples and 5.51 ± 3.69 mg/kg for commercial samples, and copper was detected in 17 commercial with a mean of 0.35 ± 0.54 mg/kg and 4 homemade samples with mean of 0.902 ± 1.09 mg/kg (Table 14). Our finding regarding the concentration of copper was incompatible with the study done by Yılmaz et al. (2007) where the mean concentration of copper detected in 3

commercial pomegranate molasses samples was reported by to be 0.61 ± 0.01 mg/kg.

According to LIBNOR standards NL 805 and NL 813 (2020), which were based on the GCC Standards for pomegranate molasses, all Lebanese pomegranate molasses should comply with the heavy metals standards stated in Codex Stand 193-1995. The Codex maximum level (ML) for arsenic (As) is 0.1 mg/kg (Codex Stand 193-1995); only 1 homemade sample (HM1) and 5 commercial pomegranate molasses samples (Al Baraka, AlHokool AlKhadraA, Alwadi, Sallom, and Taj) tested in our study showed comply with arsenic Codex standard. However, the other 3 homemade samples (HM2, HM3, and HM4) and the 23 commercial samples (AlBaydar, AlNabil, AlRabih, Aoun, Ashkar, Bolous, Chtoura Fields, Chtoura Gardens, Cortas, Gardenia, Jana, Jeita, Kortbawi, Lumiere, Machaalany, Maxims, Maymone, Monzer, Moroj Chtoura, Spinneys, Terrois, Tiba, and, Yamama) showed to have an arsenic concentration > 0.1mg/kg which may present health concerns for regular consumers of pomegranate molasses. Arsenic may accumulate in soil and irrigation water from some anthropogenic sources including manufacturing and processing facilities, waste water, dumping of sewage sludge, coal burning power plants, urban runoff, atmospheric deposition, industrial wastes, and application of arsenic-containing pesticides (Agency for Toxic Substances and Disease Registry (ATSDR), 2007). Although arsenic-containing pesticides were banned in the USA by the U.S. Environmental Protection Agency in 2009 (Bencko & Yan Li Foong, 2017), in other countries, the use of arsenic pesticides continued longer. The continued illegal use of arsenal pesticides and their accumulation in the

soil may be the cause behind the elevated levels of arsenic in the tested samples of pomegranate molasses.

Cadmium(Cd) and Lead(Pb) were not detected in any on the tested samples (0 mg/Kg), so all Lebanese pomegranate molasses in our study comply with the Codex ML for cadmium (Cd) and Lead (Pb) respectively (0.05 mg/Kg; 0.1 mg/Kg) (Codex Stand 193-1995).

The maximum allowable concentration for copper (Cu) is 0.21 mg/Kg as reported in the pomegranate molasses standard by the GCC Standardization Organization (GSO) (2015). Copper was not detected (0 mg/Kg) in 11 commercial samples (AlBaydar, AlHokool AlKhadraA, AlRabih, Aoun, Ashkar, Lumiere, Maxims, Maymone, Sallom, Taj, and Yamama). 1 homemade sample (HM3) and 3 commercial samples (Gardenia, Machaalany, and Monzer) were shown to have copper concentration less than the maximum allowable limit (0.21 mg/Kg). On the other hand, the other 3 homemade samples (HM1, HM2, and HM4) and the 14 commercial samples (Al Baraka, AlNabil, Alwadi, Bolous, Chtoura Fields, Chtoura Gardens, Cortas, Jana, Jeita, Kortbawi, Moroj Chtoura, Spinneys, Terrois, and Tiba) showed to have copper concentrations greater than 0.21 mg/Kg with a maximum of 2.46 mg/Kg for HM2. Copper is released into water and soil from municipal landfills, mining, agricultural products such as fungicides, and discarded copper products from wiring and plumbing (Agency for Toxic Substances and Disease Registry (ATSDR), 2004). The increased levels of copper in our samples could be due to soil or water contamination with copper, especially if water pipes are made of copper. Also, the use of some worn out copper products during the

preparation of pomegranate molasses could have resulted in direct contamination of the samples.

Regarding mercury (Hg) and chromium (Cr), Codex Stand 193-1995 and GCC Standardization Organization (GSO) didn't include any standard limits for their presence in pomegranate molasses (Codex Stand 193-1995; GSO, 2013). However, the maximum allowable concentration for chromium (Cr) is of 0.05 mg/L in water, as reported by WHO (2003), and 0.5 mg/Kg for fresh vegetables as defined by the Food Safety Standards in China (MHPRC, GB2762-2012-2012). All the tested pomegranate samples were found to have Cr concentrations exceeding the maximum allowable limits with a mean of 5.817 ± 3.646 mg/Kg. Although 0.05 mg/L is considered to be unlikely to give rise to significant health risks, it's used as a practical measure because of the carcinogenicity of hexavalent chromium by the inhalation route and its genotoxicity (WHO, 2003). Chromium is released into water and soil from domestic manufacturing and processing facilities, agricultural and food wastes, animal wastes, and the disposal of commercial products that contain chromium (Agency for Toxic Substances and Disease Registry (ATSDR), 2012). Pomegranates used in preparing the molasses could have high concentrations of chromium due to soil or water contaminations, which resulted in increased levels of chromium in our samples.

Our results showed that not all the tested Lebanese pomegranate molasses comply with the Lebanese Standards for heavy metals.

In our study all the homemade samples are shown to have higher concentrations of all the assessed heavy metals than the commercial ones. This

might be due to the difference in pomegranate fruit and the processing techniques used during industrial and traditional preparations of pomegranate molasses. Also, the addition of citric acid to commercial pomegranate molasses may result in the increase of some heavy metals if it was contaminated. Citric acid addition is allowed according to the Lebanese standard for commercial pomegranate molasses NL 813 (LIBNOR, 2020).

Conclusion

Due to its popular health benefits, pomegranate and its various products have gained widespread popularity as a functional food. Pomegranate molasses is a pomegranate product with flavor-enhancing effect that is believed to have high nutritional value because of its high phenolic content. Pomegranate molasses, especially homemade, has been of high demand in the Lebanese market.

This study allowed us to compare the total phenolic content, total flavonoid content, total tannins, and total alkaloid content among all the commercial brands and some homemade products of pomegranate molasses available in the Lebanese market. Also we were able to assess the presence of some heavy metals (Arsenic, Copper, Lead, Cadmium, Chromium, and Mercury). Our findings showed that the traditional Lebanese homemade PM have high TPC, TFC, TT as compared to PM produced commercially. Also, homemade pomegranate molasses have higher heavy metal concentration than the commercial ones, with some samples exceeding the maximum acceptable limits for As, Cu, and Cr. This difference could be attributed to different factors such

as type of pomegranate fruit, soil, climate, extraction, and processing techniques used while preparing pomegranate molasses.

Finally, it's recommended to take the following factors into consideration in further studies while assessing TPC, TFC, TT, and heavy metal concentrations in pomegranate molasses since they directly affect the composition of the product:

1) Differences in the type and harvest of pomegranate fruit used in molasses preparation.
2) The percentage of pomegranate juice used in preparing commercial molasses could affect the TPC, TFC, and TT directly.
3) The addition of date molasses to pomegranate molasses may affect the concentration of TPC, TFC, and TT.
4) The processing techniques while extracting the juice from pomegranate fruits could also affect the TPC, TFC, and TT, especially if different parts of the fruit, other than the arils, were included in the preparation.
5) The continued illegal use of arsenal pesticides may lead to increased levels of arsenic in pomegranate molasses.
6) The possibility of contaminations of additives such as citric acid and salt with heavy metals.
7) The type of soil where the pomegranates are planted and the quality of irrigation water. Heavy metals are known to accumulate in soil and irrigation water and may pass to plants. This may be directly related to increased heavy metal concentrations in molasses.
8) The use of equipment containing copper during pomegranate molasses preparation may lead to copper contamination.

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