EFFECT OF ACCELERATED AGING ON TOTAL PHENOLIC CONTENT, ANTIOXIDANT AND ANTI-DIABETIC ACTIVITIES OF LEBANESE HOMEMADE POMEGRANATE MOLASSES WITH AND WITHOUT ADDITION OF GLUTATIONE

A Thesis

presented to

the Faculty of Nursing and Health Sciences

at Notre Dame University-Louaize

In Partial Fulfillment

of the Requirements for the Degree

Master in Food Safety and Quality Management

---

by

DIMA BOU AJRAM

SPRING 2021

© COPYRIGHT

By

Dima Bou Ajram

2021

All Rights Reserved

Notre Dame University - Louaize

Faculty of Nursing and Health Sciences

Department of Nursing and Health Sciences

We hereby approve the thesis of

DIMA T. BOU AJRAM

Candidate for the degree of **Master of Food Safety and Quality Management**

__Jocelyne Boumosleh______________________ [Signature]
Dr. Full Name Co-Supervisor

__Leina Al Hosri______________________ [Signature]
Dr. Full Name Co-Supervisor

_Elias Bou Maroun______________________ [Signature]
Dr. Full Name Committee Member

Abstract

The pomegranate fruit is considered a functional food because of its bioactive compounds mainly phenolic compounds. Pomegranate molasses (PM) is a key ingredient in the Lebanese and Middle Eastern cuisine. The effects of accelerated shelf life testing (ASLT) on the total phenolic content (TPC), total flavonoid content (TFC), the antioxidant and antidiabetic activities (% DPPH scavenging activity, DPPH IC_{50}, % Fe^{2+} chelating activity, and α-glucosidase and α-amylase IC_{50} antidiabetic inhibitory activity) of the Lebanese homemade PM have never been evaluated. Moreover, the effect of glutathione (GSH), an antioxidant, on the preservation of PM has never been tested. During ASLT, as time in the incubator increased, the TPC increased in samples without added GSH (TPCs at T_0, T_6, and T_{10} were 46.83, 55.63, and 63.61 mg GAE/g PM, respectively), where T_6 and T_{10} represent half and one year on shelves at ambient temperature of 23ºC respectively. After the addition of 200 µg/L GSH, TPC_0 was 38.85 mg GAE/g PM which was lower than the TPC_0 of samples without added GSH at day 0. GSH addition did not contribute to the TPC. The TPC was decreased after the addition of GSH not only at T_0 but at T_1 to T_{10} as well At T_0, the % DPPH scavenging activity increased in a concentration-dependent manner from 21.5% to 47.71% as concentration of the PM extract without added GSH increased from 20 to 100 µg/ml and the Fe^{2+} chelating activity of 38% at 1000 µl/mg. The mean DPPH IC_{50} at T_4 to T_{10} (151.34 ± 9.75 to 208.98 ± 12.40 mg GAE/g PM, respectively) was found to be statistically significantly greater than the mean DPPH IC_{50} at T_0 (114.25 ± 9.04 mg/g PM) in PM samples without GSH; this means that there will be a gradual significant decrease in antioxidant activity (AA) of PM, reaching approximately 1.83 folds lower than initial AA, after approximately 100 days on the shelf. Unexpectedly, however, the mean DPPH IC_{50} of PM samples with added GSH was higher than those without added GSH at ecah of the different times in the incubator (T_0 to T_{10}), (for e.g., at T_0, the mean IC_{50} of samples with added GSH vs. that of samples without added GSH: 141 mg GAE/g vs. 114.25 mg GAE/g, respectively), meaning that PM without added GSH has higher AA than PM with added GSH over one year on the shelf. In addition, at T_0, PM without added GSH exerted an inhibitory activity of IC_{50} of 0.443 ± 0.05 mg/ml for α-glucosidase and IC_{50} of 1.21 ± 0.4 for α-amylase which is comparable to those exerted by acarbose, a pharmaceutical drug used for treatment of diabetes which was used as the reference standard with IC_{50} values of 0.277 mg/ml and 0.42 mg/ml for α-glucosidase and α-amylase, respectively. Meaning that PM can be considered as a potent antidiabetic natural food. Finally, a significant strong positive correlation was found between TPC and IC_{50} DPPH before and after addition of GSH ($\rho = 0.879$, $p < 0.01$) ($\rho = 0.547$, $p > 0.05$), respectively), meaning that TPC alone does not contribute to the total AA of PM and the addition of GSH improves the AA. The latter correlation, however, did not reach statistical significance. This could be due to the small number of samples in our study and therefore warrants investigation in larger studies in the future.

Keywords

Pomegranate molasses • Total phenolic content • Flavonoid content • antioxidant activity • DPPH radical scavenging • ferrous ion chelating • accelerated shelf-life testing • anti-diabetic activity • α-Amylase inhibitory assay • α-Glucosidase inhibitory assay • Glutathione

Acknowledgements

I would like to sincerely thank my advisors, Dr. Leina El Hosry and Dr. Jocelyne Bou Mosleh, for their guidance, encouragement, and expertise. Fulfillment of this research would not have been possible without your support, patience, and wealth of knowledge. Thank you for all of your instruction, mentorship, and counsel. I would like to express my gratitude to the faculty and staff over the course of my higher education. Next, I would like to extend my heartfelt gratitude to my family and friends who have loved and supported me unconditionally during my education. Last but not least, I would like to thank Rabih. Through the ups and downs, you have been inspiring, understanding, and everything I could ever ask for in a husband. I would not have made it this far without your support.

List of Abbreviations

PM	Pomegranate molasses
PJ	Pomegranate juice
GSH	Glutathione
TPC	Total phenolic content
EtOH	Ethanol
ETs	Ellagitannins
DPPH	1,1-diphenyl-2-picrylhydrazyl radical scavenging assay
FIC	Ferrous ion chelating
AA	Antioxidant Activity
TPC	Total Phenolic Content

List of figures

Figure 1: Production Flow Chart of Commercial Pomegranate Molasses…………...…………**15**

Figure 2: Step by Step Process of Homemade Pomegranate Molasses …................................**16**

Figure 3: presents the total phenolic content (TPC) for PM samples, over the course of 40 days in an incubator at 55 °C……………………………………………………………………... **57**

Figure 4:% of scavenging activity using DPPH Test at T_0 of PM sample without added GSH...**60**

List of tables

Table 1 Total phenolic contents, total anthocyanins, and antioxidant activities of juices and molasses from some pomegranate cultivars worldwide ……………………………………….**22**

Table 2: Summary of cancer preventive activity of pomegranate and its active constituent on various cancer cell lines ……………………………………………………………………….**30**

Table 3: Summary of *in vivo* studies done on humans to study effect of pomegranate on CVDs…**40**

Table 4: Schedule of removal of samples from incubator ……………………………………**49**

Table 5: DPPH scavenging activity of PM samples using different concentration of GSH (50, 100, 200 mg/L) in the absence and presence of oxygen ……………………………………………**62**

Table 6: mean IC_{50} at different time for PM samples without addition of GSH studied under ASLT using DPPH radical scavenging assay ………………………………………………………..**63**

Table 7: Correlation between TPC and IC_{50} DPPH with and without addition of GSH (200µg/L)……………………………………………………………………………...…**68**

Outline

Contents

Introduction and Background 10
Chapter 1: Preparation of Pomegranate Molasses 13
1.1 Glutathione 17
Chapter 2: Chemical Composition and Antioxidant Activity of Pomegranate 18
2.1 Oxidation and antioxidants 18
2.2 Phytochemical Composition of Pomegranate 19
2.3 Total Phenolic Content, Total Anthocyanins, and Antioxidant Activity 20
Chapter 3: Health Benefits of pomegranate 25
3.1 Antioxidant Properties 25
3.2 Antidiabetic Properties 26
3.3 Cancer 29
3.3.1 Prostate Cancer 32
3.3.2 Breast cancer 33
3.3.3 Colorectal Cancer 35
3.3.4 Leukemia 37
3.3.5 Bladder cancer 38
3.3.6 Hepatocellular carcinoma (HCC) 38
3.4. Cardiovascular Diseases (CVDs) 39
3.5. Irritable Bowel Disease 43
3.6. Alzheimer Disease 44
3.7. Male Infertility 45
3.8. Erectile dysfunction 46
Chapter 4: Materials and Methods 47
4.1 Pomegranate molasses preparation 47
4.2 Chemicals 48
4.3 Accelerated shelf-life testing (ASLT) procedure 48
4.4 Determination of total phenolic content 49
4.5 Determination of total flavonoid content 50
4.6 *In vitro* antioxidant assays 50

4.6.1 DPPH radical scavenging assay 50
4.6.2 Ferrous ion chelating (FIC) assay 51
4.7 In vitro inhibitory antidiabetic assays 51
4.7.1 In vitro inhibitory alpha amylase assay 51
4.7.2 In vitro inhibitory alpha glucosidase assay 52
Statistical analysis 53
Chapter 5: Results and Discussion 53
5.1 Determination of total phenolic content 53
5.2 Determination of total flavonoid content (TFC) 57
5.3 *In vitro* antioxidant assays 57
5.3.1 In vitro AA of PM sample using DPPH radical scavenging assay and Ferrous ion chelating (FIC) assay at T_0 without added GSH 57
5.3.2 AA of PM samples without GSH and with addition of GSH at different concentrations (50, 100, 200 mg/L) at time T_0 with and without oxygen saturation 61
5.3.3 Antioxidant Activity (AA) of PM samples without addition of GSH studied under accelerated aging (ASLT) tested using DPPH radical scavenging assay. 62
5.3.4 Antioxidant Activity (AA) of PM samples with addition of 200 µg/L GSH studied under accelerated aging (ASLT) tested using DPPH radical scavenging assay. 64
5.4 *In vitro* antidiabetic activity of PM 65
5.5 Correlation between phytochemical constituents and antioxidant activity. 67
Chapter 6: Conclusion 71
References 73

Introduction and Background

The common name of the pomegranate (*Punica granatum L.*) is derived from Latin words ponus and granatus, a seeded or granular apple (Ismail *et al.,* 2012). Pomegranate is a tree that belongs to the family "Punicaceae". It has been cultivated and naturalized since ancient times throughout the entire Mediterranean region (Atilgan *et al.*, 2014) and has spread to North Africa, Asia, Middle East (Tehranifar *et al.*, 2010; Hmid *et al.*, 2018) Afghanistan, Iran, China and the Indian sub-continent (Li *et al.*, 2015). During modern times, pomegranate cultivation stretched through the Mediterranean region to the Turkish, European borders and American southwest, California and Mexico ((Ismail *et al.,* 2012; Heber *et al.*, 2006). This wide distribution is due to the ability of pomegranate trees to adjust to variable climatic conditions (Holland *et al.*, 2009; Stover *et al.*, 2007)

Pomegranates are processed into different products: juice, jams, grenadine syrup, molasses, sauce, and wine (Tehranifar *et al.*, 2010; Patel *et al,* 2008) which have long been used in different cuisines. Nowadays, products made from pomegranate are commercially booming and well appreciated throughout the world because of their desirable taste and aroma, flavor and interesting nutritional features (Ferrara *et al*., 2011, Viuda-Martos *et al*., 2010). Pomegranate juice is a popular drink in the Middle East, and is also used in Iranian, Indian and Greek cuisines to prepare traditional recipes. The seeds of the pomegranate are used to make curries in India and Pakistan. In Lebanon, Syria, Palestine, and Turkey, pomegranate molasses (PM) is used to marinate meat, to garnish desserts and as salad dressing. Pomegranate wine is popular in Armenia (Tehranifar *et al.*, 2010; Patel *et al,* 2008).

In addition to its use in different cuisines, pomegranate is known to be the oldest medicinal plant known to humanity. It has been used in folk medicine for centuries and it has been called “nature’s power fruit”. In 2000, Lansky *at al*., reviewed the usage of pomegranate in folk medicine. In their review, the ancient Egyptians, according to Eber’s papyrus (ca. 1550 BCE), used its extracts to treat intestinal tapeworm infection. Hippocrates (400BCE) used it to reduce skin and eye inflammation, and as a digestive aid. Other traditional medicinal uses of pomegranate products included contraception and treatments for snakebite, diabetes, leprosy (Lansky *et al*., 2000).

The composition of pomegranate has become of great interest in modern medicinal uses. The fruit can be divided into three parts: the seeds (aril), the juice, and the peel. Extracts of all parts of the fruit appear to have therapeutic properties (Danesi and Ferguson, 2017). They possess strong antioxidant anti-atherogenic effects, anti-tumorigenic (Kalaycıoğlu and Erim, 2017), anti-inflammatory, anti-hepatotoxic, neuroprotective (Rahmani *et al*., 2017), antimutagenic (Cano-Lamadrid *et al.*, 2016), cancer prevention and cancer treatment (Lansky and Newman, 2007). They also improve oral health, skin health and sperm quality and help prevent Alzheimer’s disease (Zarfeshany *et al.,* 2014; Atilgan *et al.*, 2014; Caligiani, 2016)

Pomegranate Molasses (PM) is a thick syrup obtained from pomegranate juice concentration. It is a slightly astringent, sweet-sour condiment made from boiled down pomegranate juice (Yilmaz *et al.*, 2007) that gained a great interest especially in Lebanese cuisine (El Darra *et al.,* 2017). PM is referred to as “Debs El Remman” in Arabic. It is used to improve the taste of and aroma to a vast array of traditional salads and dishes such as “sfeeha” and “lahm b ajeen”, “fattoush” salad, braised “makanek” and meat etc. (El Darra *et al.,* 2017).

PM is usually prepared to be used from season to season and sometimes stored for 2 to 3 years (El Darra *et al.,* 2017). Degradation rates of principal phytochemicals are increased during storage due

to different factors such as light, oxygen, and temperature (Fischer *et al.*, 2017). The oxidation of these compounds would presumably lead to changes in the levels of antioxidants in pomegranate, as a consequence of changes in the redox equilibrium (Fischer *et al.*, 2017).

To determine and measure the chemical, microbiological and physical changes that might occur during time, the food manufacturers store the product under typical storage conditions over a specified time interval until the product becomes unacceptable for consumer consumption; after that, they can estimate expiry date. However, food manufacturers are under constant pressure to launch new products in shorter time frames and often do not have sufficient time for real-time shelf-life testing. This is why they primarily depend on accelerated shelf-life testing (ASLT), an indirect method of measuring and estimating the stability of a product by storing the product under controlled conditions, similar to normal storage conditions, that increase the rate of degradation in PM in an accelerated manner.

Today, about 3000 food additives have been approved to improve food quality, appeal, storage, nutritional value, processing, preparation and safety. Glutathione (GSH) is a strong antioxidant. GSH is widely used as a pharmaceutical compound and has the potential to be used as a food additive and in the cosmetic products. GSH is considered as a nutraceutical; therefore its presence in the food matrix will no doubt increase the nutritional value.

Although data on the chemical composition of pomegranate are available, there is little information on the influence of storage duration and temperature on the chemical composition of PM, especially PM Polyphenols, and its antioxidant activity. Moreover, studies on the effect of addition of glutathione (GSH), an antioxidant, to preserve PM are very scare. The objectives of the present study are to (i) determine the total phenolic and flavonoid content of Lebanese homemade PM; (ii) assess its antioxidant activity using DPPH radical scavenging as well as ferrous ion chelating

assays; (iii) examine its anti-diabetic activity using α-Amylase and α-Glucosidase inhibitory activities before and after accelerated shelf-life testing and (vi) determine the effect of GSH addition on the stability of PM during accelerated shelf-life testing.

Chapter 1: Preparation of Pomegranate Molasses

Pomegranate is a tropical and seasonal fruit, and its production occurs during August and September. The pomegranate can be processed into products like minimally processed fresh arils, canned arils, juice, squash, beverage, molasses, juice concentrates, frozen seeds, jam, jelly, marmalades, grenadine, wine, seeds in syrup, pomegranate spirits, vinegar, paste pomegranate powder, pomegranate rind powder, confectionery, pomegranate seed oil, etc. (Akpinar-Bayizit *et al.*, 2016; Dhinesh and Ramasamy, 2016).

Pomegranate contains 48 to 52 per cent of edible part on the whole fruit basis, which comprises of 78 percent juice and 22 percent seed (Dhinesh and Ramasamy, 2016). The high percentage of juice makes them susceptible to enzymatic and microbial deterioration reactions due to the high enzymatic and microbial activities in water (Dhinesh and Ramasamy, 2016). Thus, extracted juice is concentrated for long-term usage, storage, and easier transportation (Maskan 2006).

Concentration of fruit juices is a major unit operation in fruit processing which plays a critical role in determining the quality of final products in terms of color, flavor, aroma and nutritional properties (Maskan 2006). Manufactures extract the juice in two different methods as shown in figure 1. Dhinesh and Ramasamy (2016) reviewed in their article the different pomegranate processing methods. The first method includes washing the fruits then rolling them on a hard surface to weaken the seed sacks within the fruit. Then the extraction of juice involves the cutting

the fruit, separating the seeds, and pressing arils in screw press or basket press. While in second method, the fruits are quartered and crushed or the whole are pressed in hydraulic press and juice is strained out. Once the whole fruit is pressed, the high molecular weight phenolics called as tannins pass from the rinds into juice, causing astringency and bitterness in the juice. Therefore, the hydraulic extraction of juice should be at a pressure less than 100 psi (pound per square inch) to avoid undue yield of tannins from the rind. Pomegranate seed extractor is then used to separate grains and rind from the juice. The phenolic constituents are also responsible for the formation of cloudy appearance of fruit juices during concentration and storage. Therefore, clarification or fining is one of the most important steps in fruit juice processing. Clarification is used by both methods to remove active haze precursors and thus decrease the potential for haze formation during storage. The clarification of extracted juice is achieved by addition of either gelatin, bentonite, clay, etc. at 4°C overnight and then the juice is filtered at -30°C. The centrifugation method may also be employed for the clarification of the fruit juices. After clarification, the juice is then filtered using ultrafiltration method. Vacuum evaporation, microwave evaporation and atmospheric evaporation are widely used techniques to concentrate various juices (Maskan 2006). The concentrated juice further boiled to obtain pomegranate molasses of concentration 50.1 to 77.3 ° brix (Yılmaz et al. 2007; Vardin et al. 2008) for 23 to 190 minutes (Maskan 2006).

Traditional methods are still being used to produce PM. Figure 2 shows the traditional process of homemade PM production. The process involves collection of sour pomegranate fruits mainly September, washing, separating arils from pericarp, crushing, extraction, filtration, evaporation and concentration of pomegranate juice. The concentration is done by simply boiling it in an open vessel without the further addition of sugar or other additives until the total volume of juice is

reduced by around 80% (Akpinar-Bayizit *et al*., 2016; İncedayi *et al*., 2010; Yilmaz *et al*., 2007).

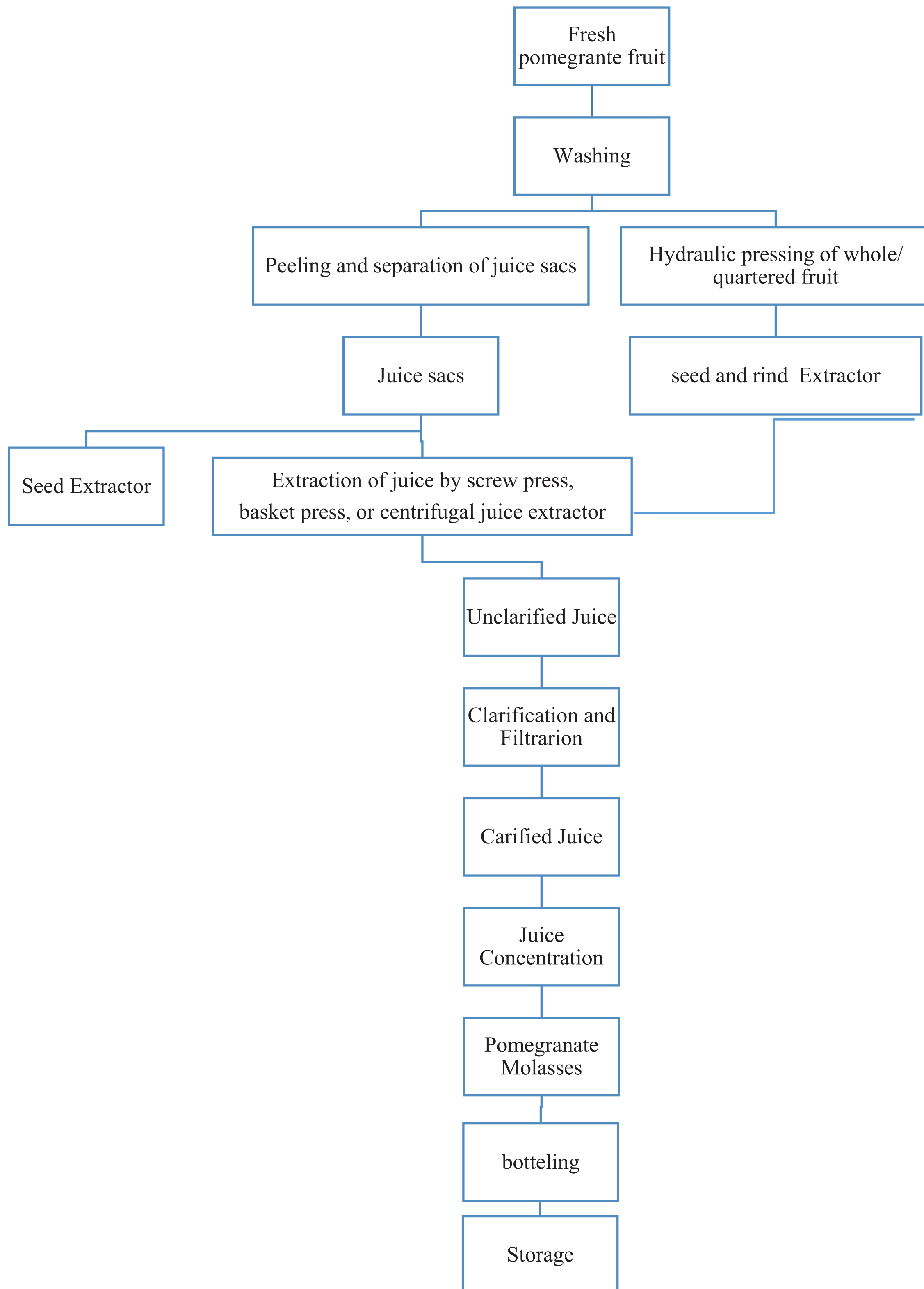

Figure 1: Production Flow Chart of Commercial Pomegranate Molasses

Step 1: Collection of Fresh Sour Pomegranate Fruit

Step 2: the pomegranate fruits are seeded

Step 3: Crushing of arils

Step 4: Filtration of Juice

Step 5: Filtration of Juice

Step 6: transfer to open vessel

Step 7: Boiling and concentrating of

Step 8: Bottling and storing

Figure 2**.** Step by Step Process of Homemade Pomegranate Molasses

1.1 Glutathione

Antioxidants are often added to foods to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and propagation step leading to the termination of the reaction and delay of the oxidation process (Shahidi *et al*. 1992; Gu¨lc¸2006).

Glutathione (GSH) is the most abundant non-protein thiol compound present in living organisms. GSH is a tripeptide composed by glutamic acid, cysteine and glycine, and it's naturally present in many plants and food. It is used as a pharmaceutical compound and can be used in food additives and the cosmetic industries (Li *et al.*, 2004).

Glutathione is important because it serves several functions in the body. Glutathione is an antioxidant, necessary for the neutralizing of reactive oxygen species or free radicals (Wang *et al*., 2014). It is necessary for optimal detoxification or the removal of toxic substances and carcinogens from the body. In addition, glutathione is important for immune function as well as the regeneration of other antioxidants such as vitamins C and E (Kern *et al*., 2011). The maintenance of tissue levels of GSH is critical for maintaining health preventing diseases and age-related biological disorders (Richie *et al*. 2015). Even partial GSH depletion impairs immune function and increases susceptibility to a wide range of xenobiotics and oxidative damage (Richie *et al*. 2015). Low GSH levels are associated with increased risks of numerous diseases including cancer, cardiovascular diseases, arthritis and diabetes (Richie *et al*. 2015). Many studies showed that oral GSH supplementation represents one such strategy for enhancing tissue GSH levels to counteract the disorders associated with GSH depletion such as reversing of age-associated decline in immune responsiveness (Furukawa *et al*., 1987), reducing oxidative damage of colonic mucosa (Loguercio *et al*., 2003), reducing helicobacter gastric pathologies (De Bruyne *et al*., 2016), improving the bovine embryo development after in vitro fertilization (Sun *et al*., 2015), etc.

GSH plays an important role in the food industry. It has been used as an additive in wine. The thiol group of the cysteine is the site active responsible for its biochemical properties to prevent the oxidation of wine (Lavigne *et al*., 2007). The presence of GSH in must and wine exerts a protective effect on many desirable wine aromas, limits the formation of off-flavors (Kritzinger *et al*., 2013) and the formation of browning pigments during accelerated aging (El Hosry *et al*., 2009)

Chapter 2: Chemical Composition and Antioxidant Activity of Pomegranate

2.1 Oxidation and antioxidants

Oxidation, a process that represents an essential part of aerobic life and our metabolism, is the transfer of electrons from one atom to another, with oxygen being the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP (Gülçin, 2012). However, problems may arise when the electron flow becomes uncoupled (transfer of unpaired single electrons), generating free radicals (Gülçin, 2012).

Antioxidants are compound that acts as a reducing agent and, therefore, has the ability to scavenge oxygen (McGregor and Biesalski, 2006). It binds with an unpaired electron in the free radical and forms stable molecule; it also chelates metal ions (McGregor and Biesalski, 2006). Some antioxidants, such as glutathione, ubiquinol, and uric acid, are produced during normal metabolism in the body. Others, however, must be supplied by the diet (Lobo *et al.,* 2010).

Free radicals, such as reactive oxygen species and reactive nitrogen species, are generated by the body's various endogenous systems, exposure to different physiochemical conditions such as smoking, pollution, stress, etc. or pathological states. A balance between free radicals and antioxidants is necessary for proper physiological function. If free radicals overwhelm the body's

ability to regulate them, a condition known as oxidative stress ensues. Free radicals thus adversely alter lipids, proteins, and DNA and trigger a number of human diseases including atherogenesis, neurodegeneration, inflammation, cancer, and ageing, among others (Shahidi & Zhong, 2015; Gülçin, 2012; Deng *et al.,* 2011; Lobo *et al.,* 2010).

Synthetic and natural food antioxidants, are used routinely in foods to protect the food especially those containing oils and fats, against oxidation (Lobo *et al.,* 2010). Synthetic antioxidants are recently reported to be dangerous to human health due to their high volatility and instability at elevated temperatures (Lobo *et al.,* 2010). As a result, there has been a growing interest in research that aims at identifying and examining the role of alternative natural and safe sources of antioxidants, especially of plant origin, in and assessing their effect on human health (Gülçin, 2012). Pomegranate phytochemicals can be a great source of natural antioxidants that can be used as additives in food processing or medicine.

2.2 Phytochemical Composition of Pomegranate

The composition of the pomegranate fruit varies with type, growing conditions, climate, maturity and storage conditions. The edible part of pomegranate contains significant amounts of acids, sugars, vitamins, polysaccharides, polyphenols and minerals (İncedayi *et al.*, 2010). The major class of pomegranate phytochemicals that predominates in the fruit is the polyphenols, which consist of phenolic rings bearing multiple hydroxyl groups. Pomegranate polyphenols include flavonoids (flavonols and anthocyanins), condensed tannins (proanthocyanidins) and hydrolysable tannins (ellagitannins (ETs) and gallotannins). The flavonoids include flavonols such as luteolin, quercetin, and kaempferol found in the peel extract and anthocyanins found in the arils. Anthocyanins are the water-soluble pigments responsible for the bright red color of PJ. Pomegranate anthocyanins include pelargonidin-3-glucoside, cyanidin-3-glucoside, delphinidin-

3-glucoside, pelargonidin 3,5-diglucoside, cyanidin 3,5-diglucoside, and delphinidin 3,5-diglucoside (Heber *et al.,*2006). Hydrolysable tannins (HTs) are found in the peels (rind, husk, or pericarp), membranes and piths of the fruit (Akpinar-Bayizit *et al.*, 2016). These compounds are also found abundantly in the pomegranate juice (Karaali *et al.*, 2006). The predominant pomegranate ellagitannins HT is punicalagin (Heber *et al.,* 2006). Punicalagins include gallotannins, ellagic acid tannins and gallagyl tannins (Akpinar-Bayizit *et al.*, 2016). Other phytochemicals identified from the pomegranate are organic and phenolic acids, sterols and triterpenoids, fatty acids, triglycerides, and alkaloids (Heber *et al.,* 2006).

2.3 Total Phenolic Content, Total Anthocyanins, and Antioxidant Activity

The antioxidant activity of a food compound is generally correlated with their content of phenolic compounds. Total phenolic content and total anthocyanin content are accepted measures of antioxidant activity (Kalaycıoğlu and Erim, 2017). The most commonly used methods for *in vitro* determination of the antioxidant activity of a food constituent are Folin-Ciocalteu reducing capacity assay, 2,20-azinobis-(3-ethylbenzothiazoline- 6-sulphonate) radical scavenging (ABTS) assay, 1,1-diphenyl-2-picrylhydrazyl radical scavenging assay (DPPH), ferric reducing antioxidant power assay (FRAP), cupric ions reducing power assay (CUPRAC), oxygen radical absorbance capacity assay (ORAC), inhibition of lipid peroxidation in linoleic acid system, total radical-trapping antioxidant parameter assay (TRAP), peroxyl radical scavenging, superoxide anion (O_2^-) radical scavenging, hydrogen peroxide (H_2O_2) scavenging, hydroxyl radical ($^{\bullet}OH$) scavenging, singlet oxygen ($^1O2$) quenching assay, and nitric oxide (NO) radical scavenging assays (Gülçin, 2012).

Table 1 contains findings of studies published between 2007 and 2019 on the total phenolic content, total anthocyanins, and antioxidant activity of pomegranate juice and PM. In all these

studies, researchers used Folin-Ciocalteu reagent assay to determine the total phenolic content. The total phenolic content was reported as Gallic acid equivalents (GAE) and total anthocyanins was reported mostly as Cyanidin-3-glucoside equivalents (Cy-3-glu). In the studies shown in table 1, pomegranate possessed high antioxidant activity, independent on the antioxidant test assayed and generally with significant linear correlation between total phenolic content and antioxidant capacity. The most commonly used methods to measure antioxidant activity in pomegranates in these studies were ABTS, DPPH, ORAC and FRAP.

The reported levels of the total phenolic content in the literature reviewed varied due to the type, growing conditions, climate, maturity, storage conditions of pomegranates, and preparation methods of PM (Orak, 2009; İncedayi *et al.*, 2010).The reported levels of the total phenolic content in pomegranate juice in these studies were 2960 – 9850 mg GAE/Kg (Tehranifar *et al.,* 2010), 1134.14 – 1153.64 mg GAE/L (Vegara *et al.*, 2013), 1136.2 – 3581.1 mg GAE/L (Vegara *et al.*, 2014), 2674 – 4210 mg GAE/L (Nuncio Jáuregui *et al.*, 2014), 3150 – 7430 mg GAE/L (Li *et al.*, 2015), 220 -1266.8 mg GAE/L / 942.9 -2931.5 mg GAE/L (Akhavan *et al.*, 2015), 1385 -9476 mg GAE/L / 1284 - 8295 mg GAE/L (Hmid *et al.*, 2016), 900 – 1450 mg GAE/L (Legua *et al*., 2016), 4242 – 7419 mg GAE/L (Di Stefano *et al.*, 2019) and 79.49 mg GAE/L (Chalfoun-Mounayar *et al.*, 2012). As for the PM, the reported levels of total phenolic content in the few studies mainly done in Turkey and Lebanon were 118.28 – 828.15 mg GAE/g (Akpinar-Bayizit *et al.*, 2016), 551.61 – 9695.17 mg GAE/Kg (İncedayi *et al.*, 2010), 52.56 mg GAE/g (Yilmaz *et al.*, 2007), and 252.28 mg GAE/L (Chalfoun-Mounayar *et al.*, 2012), 90 – 179.5 mg GAE/g (Nasser *et al.*, 2017). From the literature reviewed the TPC ranged between 1134 – 9476 mg GAE/L in PJ and 1.16 – 9.695 mg GAE/g in PM.

Table 1 Total phenolic contents, total anthocyanins, and antioxidant activities of juices and molasses from some pomegranate cultivars worldwide

Cultivars/ country	*Properties Studied*	*Total Phenolic Content*	*Total Anthocyanins*	*Antioxidant Activity Evaluated*	*Reference*
Twenty cultivars/ Iran	**Total Phenolic content, antioxidant activity, total anthocyanins**, pH, titratable acidity, total sugars, & ascorbic acid.	mg GAE/Kg PJ 2960 – 9850	mg cy-3-glu/Kg PJ 55.6 – 301.1	**DPPH** (%) 15.59 – 40.72	Tehranifar *et al., 2010*
One cultivar raw and cloudy fresh PJ, clarified PJ vs pasteurized PJ/ Spain	**Total phenolic contents, antioxidant activity**, co-pigmented, monomeric and polymeric anthocyanins, total monomeric anthocyanins.	mg GAE/L PJ raw, cloudy PJ 1134.14 clarified PJ 1153.64		**ORAC** (mmol TE/L) 13.94 Clatified PJ (mmol TE/L) 18.68	Vegara *et al.*,2013
Eighteen different commercial samples of pomegranate juice/ Spain	**Total phenolic contents**, **antioxidant activities**, relative densities, titratable acidity, organic acids, sugars, minerals, anthocyanins, ellagitannins and amino acids, and influence of storage on antioxidant activity, TPC, individual anthocyanins and ellagitannins	mg GAE/L PJ 1136.20 - 3581.10	mg Cy3G/L 60.43 mg Cy3,5dG/L 39.13 mg Dp3,5dG/L 26.65 mg Dp3G/L 21.59 mg Pg3G/L 9.06 mg Pg3,5dG/L 2.19	**ABTS** (mmol TE/L) 17.60–30.50 **FRAP** (mmol Fe2+/L) 32.10–56.70; (mmol TE/L) 10.40–15.80 **ORAC** (mmol TE/L) 8.84–11.81	Vegara *et al.*,2014
Three cultivars/Spain	**Total phenolic contents**, **antioxidant activity**, total soluble solids, titratable acidity, pH and maturity index, profiles of organic acid, sugars, amino acid proline, and the external and internal color of cultivars at three ripening stage	mg GAE/L PJ 2674–4210		**DPPH** (mmol TE/L) 6.35–8.63	Nuncio Jáuregui *et al.,* 2014
Nine cultivars/ Spain	**Antioxidant** activities and major derivatives of ellagic acid			**DPPH** (mmol TE/kg DM) 2541 (Thinning fruits, mean) 1245 (Ripe fruits, mean) **ABTS** (mmol TE/kg DM) 3603 (Thinning fruits, mean) 2177 (Ripe fruits, mean) **ORAC** (mmol TE/kg DM) 664–924 (Thinning fruits, mean) 338–582 (Ripe fruits, mean) **FRAP** (mmol TE/kg DM) 3977 (Thinning fruits, mean) 683 (Ripe fruits, mean)	Nuncio Jáuregui *et al.*, 2015
Ten cultivars/ China	**Total Phenolic content, antioxidant activity,** total flavonoids, tannins &	mg GAE/L PJ 3150 – 7430	mg cy-3-glu/L PJ 4 – 160	**DPPH** (mg GAE/L)	Li *et al.*, 2015

	anthocyanins concentration, soluble solid content, reducing sugar content, titratable acidity, and sugar-acid ratio.			80 – 270 (mg AAE/L) 100–250 **TRC** (mg GAE/L) 50 – 200 (mg AAE/L) 100–480 **ABTS** (mg GAE/L) 250-2000 (mg AAE/L) 1400–5900 **O_2^-** (mg GAE/L) 2400-6000 (mg AAE/L) 2500–6200	
Ten cultivars/Iran	**Total phenolic contents, antioxidant activities**, the main phenolic, the major and minor anthocyanins of juices from arils and whole pomegranate	mg GAE/L Aril juices: 220.0–1266.8 Whole PG juices: 942.9–2931.5		**DPPH** (%) Aril juices: 18.8–46.8 Whole PG juices: 36.6–71.3 **ABTS** (mg AAE/L) Aril juices: 1171–6200 Whole PG juices: 4962–16401	Akhavan *et al.* 2015
Eighteen cultivars/ 10 Morocco, 3 USA, 2 Spain/ 2 Tunisia/ 1 China	**Total Phenolic content, antioxidant activity, total anthocyanins**, condensed tannins, hydrolysable tannins.	mg GAE/L PJ 1385 – 9476 *(Moroccan)* mg GAE/L PJ 1284 – 8295 *(others)*	mg Cy-3-glu/L PJ 64.16 – 188.7 *(Moroccan)* mg Cy-3-glu/L PJ 56.68 – 178.79 *(others)*	**DPPH** (%) 31.16 – 66.85 *(Moroccan)* **DPPH** (%) 45.65 – 76.3 *(others)*	Hmid *et al.*, 2016
Nineteen cultivars/ Spain	**Total Phenolic content, antioxidant activity,** color, Analysis of organic acids and sugar profile.	mg GAE/L PJ 900 – 1450	Cy-3-glu (µM) 8.89 – 119	**ABTS** (mg TE/L) H-AA 700 - 1720 L-AA 160 - 290	Legua *et al*. 2016
Nine cultivars/ Spain	**Total phenolic contents, antioxidant activities,** total soluble solids, pH, titratable acidity, maturity index, monomeric anthocyanin pigment, flavonoids, hydrolyzable tannins, and vitamin C		mg Cy 3-glu/L 43.4 - 293.5	Total reducing capacity 97.7 – 581 GAE/100ml	Fernandes *et al., 2017*
Five genotypes/ Italy	**Total phenolic contents, antioxidant activities,** fruit characterization, identification & quantification of individual phenolic compounds	mg GAE/L PJ 4242 - 7419	Cyanidin-3,5-O-diglucoside 97.64 mg/L PJ pelargonidin-3,5-O-diglucoside 40.29 mg/L PJ	ABTS (µmol TE/100 mL) 36.73 - 221.5	Di Stefano et al., 2019
One local brand of Pomegranate Molasses (n=3)/ Turkey	**Total phenolic contents**, dry matter, ash and protein contents, pH, specific gravity, color values, viscosity, mineral elements (K, Na, Ca, Mg, Mn, Cu, Zn, Fe and P)	mg GAE/g PM 52.56		-	Yilmaz et al., 2007
one distributor/ Turkey	**Total phenolic contents, total anthocyanin content, antioxidant**	µg GAE/mL PJ 3246	mg Cy 3-glu/L PJ 492.9	Inhibition of lipid peroxidation (%)	Orak, 2009

	activities, chemical and physical analysis of PM and PJ (color, Total acidity, pH, Glucose, fructose and total inverted sugar, dry matter content (brix), minerals, HMF.	µg GAE/mL PM 9870		79.06% PM 85.91% PJ	
Seven different market brands of Pomegranate Molasses/ Turkey	**Total phenolic contents, antioxidant activities**, water soluble dry matter, viscosity, the presence of glucose syrup, total acidity, pH, HMF, protein, invert and total sugars, and some minerals (Fe, K, Ca, Mg)	mg GAE/Kg DM PM 551.61-9695.17		DPPH (%) 0 - 46.31	İncedayi et al., 2010
Lebanon	**Total phenolic contents, antioxidant activities,** *in vivo* experiment on mice (Biochemical assays, histological observation, weight measurement)	mg GAE/L PM 252.28 PJ 79.49			Chalfoun-Mounayar et al., 2012
Sweet pomegranate from local market/ turkey	**Total phenolic contents, total monomeric anthocyanin content, antioxidant activities**, pH, total titratable acidity, HMF, individual organic acids, individual phenolic compounds.	mg GAE/100g raw PJ: 296 clarified PJ: 222 concentrated Juice by three methods: 222	mg Cy 3-glu/L raw PJ: 346 clarified PJ: 294 concentrated Juice by three methods: 247 – 274	ABTS (µmol TE/ mL) raw PJ: 19.6 clarified PJ 16.5 concentrated Juice by three methods: 14.5 – 16.6	Onsekizoglu, 2013
Nine different market brands of Pomegranate Molasses/ Turkey	**Total phenolic contents, antioxidant activities**, Chemical Analysis of PM (Total acidity, pH, Invert and total sugars, dry matter content (brix), Viscosity)	mg GAE/g PM 118.28 – 828.15		DPPH (%) 16.11 - 75.22	Akpinar-Bayizit et al., 2016
Six commercial brands of pomegranate molasses/ Lebanon	**Total phenolic contents, antioxidant activities,** estimation of total tannins, total saponin, humidity content, ash proportion, minerals content, total protein, total lipids	mg GAE/g PM 90 – 179.5		DPPH (%) 66.1 – 90.6	Nasser *et al.*, 2017
Unavailable cultivar/ Iran	**Total Phenolic content, antioxidant activity, total anthocyanins,** radical scavenging activity (RSA), effect of different thermal processing on the PJ AA	mg GAE/g white pomegranate Molasses 1.16 – 1.42 Red pomegranate molasses 1.49 – 1.70			Firuzi et al., 2019
Unavailable cultivar/ Turkey	**Total Phenolic content, antioxidant activity, total anthocyanins,** effects of evaporation methods on pH, titratable acidity and colour values, antioxidant activity (AA), total monomeric anthocyanin (TMA), total phenolic content (TPC), hydroxymethylfurfural (HMF) and invert sugar content of pomegranate juice concentrates	g GAE/ kg PM in dry basis 6.81-9.40	0.65-0.76 g (Cyn-3-glu) /kg sample 1.61-1.86 g Cyn-3-glu/ kg in dry basis	DPPH (µl/ml) 7.03- 9.10	Sabanci *et al.,* 2019

AA: ascorbic acid; AAE: ascorbic acid equivalent; ABTS: 2,20-azinobis-(3-ethylbenzothiazoline-6-sulphonate); ALPA: anti-lipid peroxidative activity; CE: catechin equivalent; CUPRAC: cupric ions reducing power assay; DPPH: 2,20- diphenyl-1-picrylhydrazyl; DM: dry matter; FRAP: ferric reducing antioxidant power; FW: fruit weight; GAE: Gallic acid equivalents; H-TAA: hydrophilic total antioxidant activity; L-TAA: lipophilic total antioxidant activity; I:Inhibition; ORAC: oxygen radical absorbance capacity; PE: pyrogallol equivalents; PJ: pomegranate juice; TAC: total antioxidant capacity; TE: trolox equivalent; TEAC: trolox equivalent antioxidant capacity; TPC: total phenolic content; TOSC: total oxyradical scavenging capacity assay; TRC: total reducing capacity; H-AA: Hydrophilic antioxidant activity; L-AA lipophilic antioxidant activity; hydroxymethylfurfural HMF

Chapter 3: Health Benefits of pomegranate

Epidemiological studies have demonstrated an inverse association between intake of fruits and vegetables and mortality from age-related diseases, such as coronary heart disease and cancer, which may be attributed to the presence of bioactive compounds such as phenolic acids, flavonoids and tannins and their antioxidant properties (Akpinar-Bayizit *et al.*, 2016). Therefore, antioxidants are considered important nutraceuticals on account of their many health benefits and they are widely used in the food industry as potential inhibitors of lipid peroxidation (Deng *et al.,* 2011).

The pomegranate fruit is considered a functional food because it has bioactive compounds mainly polyphenols, present in most of its parts and its derivatives such as juice, seed oil, and peel powder (Viuda-Martos *et al.* 2010). The bioactive compounds can act as antioxidant (C¸ am and others 2009), as antitumoral (Hamad and Al-Momene 2009) or antihepatotoxic (Celik *et al.,* 2009) agents, and improve cardiovascular health (Davidson *et al.,* 2009). They have been seen to have antimicrobial (Duman *et al.,* 2009), antiinflammatory (Lee *et al.,* 2010), antiviral (Haidari *et al.,* 2009) and antidiabetic (Xu *et al.,* 2009) properties, and they can improve oral (Di Silvestro *et al.,* 2009) and skin (Aslam *et al.,* 2006) health. They help prevent Alzheimer's disease (Subash *et al.,* 2014) and improve sperm quality (Türk *et al*., 2007) and erectile dysfunction in male patients (Forest *et al*., 2007).

3.1 Antioxidant Properties

Pomegranate juice (PJ) is a powerful antioxidant drink which can be explained by the highest concentration of polyphenols in PJ as compared to other fruit juices (Hmid *et al.,* 2016); it has a higher antioxidant activity than many fruit juices, such as blueberry, cranberry, grape, apple, orange, red wine and green tea (Akpinar-Bayizit *et al.*, 2016). Akpinar-Bayizit *et al.* (2016)

demonstrated that pomegranate juice and seed extracts have 2-3 fold more *in vitro* antioxidant capacity than red wine or green tea. This makes pomegranate molasses (PM) a highly nutritious product with strong antioxidant activity due to the presence of concentrated polyphenols (İncedayi *et al.*, 2010).

3.2 Antidiabetic Properties

Diabetes type 2 is the most common metabolic disease in the world and is still increasing. According to the World Health Organization, diabetes type 2 is the third most prevalent disease after cardiovascular and oncological disorders. The International Diabetes Federation mentioned that approximately 463 million adults worldwide aged between 20 & 79 years were living with diabetes in 2019 and this number is expected to rise to 700 million adults by 2045 (www.idf.org). In the MENA Region there were 55 million in in 2019 and this figure is expected to increase to 108 million adults by 2045 (www.idf.org). In Lebanon, the prevalence of diabetes type 2 in the Lebanese population was reported to be 12.9% in 2019 (WHO, 2019). Since pomegranate fruit and its derivatives are rich in powerful antioxidant agents (polyphenols), these agents attack free radicals thereby, reducing oxidative stress and inhibiting digestive enzyme such as alpha amylase and alpha glucosidase that lead to lowering postprandial glucose levels (Kamtekar *et al.,* 2014), making pomegranate fruit and its derivatives natural antidiabetic agents (Kamtekar *et al.,* 2014).

Numerous studies have described the antidiabetic activity of different parts of pomegranate; such as seeds, flowers, and juice. Jafri *et al.* (2000) reported that oral administration of an aqueous-ethanolic (50%, v/v) extract of pomegranate flowers had a significant blood glucose lowering effect in normal, glucose-fed hyperglycemic and alloxan- a compound used to induce diabetes in experimental animals, induced diabetic rats. The effect of the extract was maximum at 400 mg/kg. Das *et al.* (2001) investigated the hypoglycemic activity of methanolic seed extract of pomegranate

in rats made diabetic by streptozotocin- a compound used to induce diabetes in experimental animals. After 12 hours of oral feeding with pomegranate seed extract (300 and 600 mg/kg), streptozotocin-induced diabetic rats experienced a significant reduction in blood glucose levels of 47% and 52%, respectively. Li *et al.* (2005) investigated the effect and action mechanism of a methanolic extract from pomegranate flower (PGF) on hyperglycemia *in vivo* and *in vitro* Zucker diabetic fatty (ZDF) rats- a genetically modified popular obese, type 2 diabetes research model of rat- under fasting and non-fasting conditions. Oral administration of PGF extract (500 mg/Kg) markedly lowered plasma glucose levels in non-fasted ZDF rats by 43.1%, whereas it had little effect in the fasted animals, suggesting that it affected mainly postprandial hyperglycemia in type 2 diabetes. In another test, Li and his colleagues the effect of PGF extract on plasma glucose levels of mice that were increased after oral loading of sucrose or glucose. PGF extract dose-dependently (250–1000 mg/kg) inhibited the increase of plasma glucose level in sucrose-loaded mice by 13.9%, 19.7%, and 20.6%, respectively, whereas it showed little effect in glucose-loaded mice. PGF showed similar results to Acarbose (300 mg/kg) - an anti-diabetic drug used to treat diabetes mellitus type 2 – which caused 10.6% reduction of plasma glucose level in sucrose-loaded mice but not in glucose loaded mice. *In vitro*, PGF extract demonstrated a potent inhibitory effect on intestinal α-glucosidase activity (IC_{50}: 1.8 μg/ml). Kam *et al.* (2013) compared the inhibitory effects of extracts (0.25–1 mg/ml) from different pomegranate parts against mammalian type dietary carbohydrate digestive enzymes. Their results demonstrated that the methanolic pomegranate flower extract strongly inhibited porcine pancreatic α-amylase enzyme activities in a concentration-dependent manner, with an IC_{50} value of 653 μg/ml. The other pomegranate parts (dried crude juice, seed and peel) showed weak or no inhibitory effects against α-amylase. Moreover, the methanolic extract of both pomegranate flower and peel inhibited α-glucosidase

enzyme activities, with IC_{50} values of 187 μg/ml and 835 μg/ml, respectively. In particular, the flower extract inhibited α-glucosidase in a concentration-dependent manner. However, pomegranate seed extract and dried crude juice showed weak or no inhibitory effects against α-glucosidase. This further supports that pomegranate flower and peel exhibits greater medicinal benefits in diabetes in comparison to the juice and seed. They also showed that both fractions of flower extract polyphenols, gallic acid and ellagic acid, selectively inhibited α-glucosidase activities, with gallic acid showing greater potency than ellagic acid. Banihani *et al.* (2020) aimed to investigate the effects of fresh PGJ on levels of melatonin- a hormone that regulates the sleep–wake cycle, insulin- a hormone that regulates of blood glucose levels, and fasting blood glucose in people with impaired fasting glucose (IFG). The study was a randomized clinical trial in which 28 participants (10 males, 18 females) with IFG were recruited. Blood specimens from each participant were collected before (−5 min), and 1 and 3 hr. after PGJ administration at 1.5 ml/kg of the body weight, and melatonin, insulin, and glucose were measured. People with IFG, but not healthy individuals, had significant antihyperglycemic response ($p < .0001$) to PGJ 3 hr. after ingesting the juice. This response was not correlated with the age of participants ($p = .4287$). In addition, homeostatic model assessment of insulin resistance was significantly decreased ($p < .0001$) among people with IFG 3 hr. after ingesting the juice. Moreover, 1 hr. after PGJ administration, decreases in melatonin and increases in insulin were significantly observed among healthy ($p = .0284$, $p = .0017$) and IFG ($p = .0056$, $p = .0007$) individuals, respectively. Banihani and his colleagues concluded that fresh PGJ lowers melatonin, increases the level of insulin, and ameliorates insulin resistance in people with IFG.

Banihani *et al.* (2013) reviewed the potential mechanisms of hypoglycemic effect of pomegranate extracts *in vivo* are: 1) the increase in the function of β-cell; 2) a significantly lower activity of α-

amylase and α-glucosidase carbohydrate digestive enzymes; 3) affects glucose homeostasis by modulating the activity of certain transcriptional factors (e.g., peroxisome proliferator-activated receptor γ that regulates fatty acid storage and glucose metabolism, and nuclear factor κB (NF-κB), which is an ubiquitous transcription factor that is involved in inflammatory and immune responses, as well as in regulation of expression of many other genes related to cell survival, proliferation, and differentiation. 4) protection of pancreatic tissue; and *in vitro* are: 1) an increase in insulin secretion; 2) inhibition of the enzyme α-glucosidase and dipeptidyl peptidase-4 (DPP4) that play a major role in glucose metabolism, in a similar way to common hypoglycemic drugs, however, determining the health benefits of polyphenols found in the pomegranate fruit, in particular the hypoglycemic action of the juice, and its prompt pharmaceutical incorporation, is challenging because of presence of multiple confounders in the relationship including the type of crop, the harvest, the variety of the fruit, the parts of the fruit used to extract the juice and the method of extraction; the individual characteristics of the organism; the dose administered and the bioavailability of these compounds.

3.3 Cancer

Cancer is a pathological condition described by excessive and abnormal cell growth that may metastasize and affect the functions of various body parts (Panth *et al.,* 2017). The chemotherapeutic agents that are used to control and destroy cancer cells are proven to be themselves possible candidates for cancer generation as they can kill normal cells. So, therapeutic approach for cancer treatment and prevention is weighed in terms of benefit to risk ratio (Panth *et al.,* 2017). As a result there has been an increased interest for the search for herbs and fruits rich in powerful antioxidants possessing anti-cancer activities to prevent the adverse effects generated by the use of existing chemotherapeutic regimens (Panth *et al.,* 2017). Table 2 highlights the

potential health benefits of pomegranate fruit and the underlying mechanism of its inhibition of cancer progression. The studies summarized below showed that pomegranate has demonstrated anti-proliferative, anti-metastatic and anti-invasive effects on various cancer cell lines *in vitro* as well as *in vivo* animal models or human clinical trials.

Table 2: Summary of cancer preventive activity of pomegranate and its active constituent on various cancer cell lines

Type of cancer	*Cancer cell line/model*	*Main result*	*Reference*
Prostate Cancer	PC3, DU145, and LNCaP	- Pomegranate seed oil, juice polyphenol and pericarp polyphenol inhibited proliferation, xenograft growth and invasion.	Albrecht *et al.*, 2004
Prostate Cancer	PC-3 cells.	- cell growth was inhibited by pomegranate fruit extract - apoptosis of extremely aggressive human prostate carcinoma PC-3 cells	Lansky *et al.,* 2005
Prostate Cancer		- PFE inhibits cell growth - Induces apoptosis via modulation of proteins regulating apoptosis	Malik *et al.,* 2006
Prostate Cancer	Androgen dependent LNCaP prostate tumor Cells	- Pomegranate juice consumption lowered LNCaP cell proliferation by 12% - Increased apoptosis by 17% - Raised serum NO by 23% - Showed a marked decrease in oxidative state and sensitivity to lipid peroxidation.	Pantuck *et al.*, 2006
Prostate Cancer	LAPC-4 cells	- Ellagic acid and urolithin A from pomegranate suppressed LAPC-4 xenograft growth in immunodeficient mice.	Seeram *et al.*, 2007
Prostate Cancer	LAPC4 xenograft Model	- Pomegranate extract halted cell viability, proliferation and NF-kB activation. - Induced apoptosis.	Rettig *et al.*, 2008
Prostate Cancer	22Rv1	- Inhibits cytochrome P450 enzyme and CYP1B1 activity. - 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced CYP1B1 inhibitory activity in the 22Rv1 prostate cancer cell line.	Kasimetty *et al.,* 2009
Prostate Cancer	DU-145, PC-3	- EA from pomegranate caused cell arrest, and reduced cyclin B1 and cyclin D1. - Urolithin A induced G2/M arrest, raised cyclin B1 and cdc2 phosphorylation and caused apoptosis in both cell lines.	Vicinanza *et al.*, 2013
Breast cancer	MCF-7	- Pomegranate polyphenols obtained from fermented juice, aqueous pericarp and seed oil inhibited aromatase activity by 60–80%.	Kim *et al.*, 2002
Breast Cancer	MCF-7 and MB-MDA-231	- PFJ polyphenols effected a 42% reduction in the number of lesions compared with control - HPLC peak separated from FPJ (Peak B) and pomegranate seed oil each effected an 87% reduction	Mehta *et al.*, 2004
Breast cancer	MB-MDA-231 and MDA-ERα7	- Punicic acid inhibited breast cancer proliferation by 92 and 96% for MDA-MB-231 and MDA-ERα7 cells,	Grossmann *et al.,* 2010

		respectively compared to untreated cells by 40 μM punicic acid. - Punicic acid induced apoptosis in the MDA-MB-231 and MDA-ERα7 cells by 86 and 91%, respectively compared to untreated control cells - Disrupted cellular mitochondrial membrane potential.	
Breast cancer	MCF-7	- Ellagitannin-derived compound obtained from pomegranate possessed anti-aromatase activity - prevention of estrogen responsive tumors	Adams *et al.,* 2010
Breast cancer	MCF-7	- Pomegranate fruit skin extract inhibited MCF-7 cell growth. - Induced apoptosis and downregulated MRE11, RAD50, NBS1, RAD51, BRCA1, BRCA2 and RCC3 gene responsible for DNA strand break repair.	Shirode *et al.,* 2014
Colorectal cancer	HT-29, HCT116, SW620	- Punicalagin, EA, TPT and PJ caused apoptosis in HT-29 colon cells. - In HCT116 cell line, PJ could not induce apoptosis.	Seeram *et al*., 2005
Colorectal cancer	HT-29	- PJ, TPT and punicalagin reduced TNF-α mediated COX-2 protein expression and reduced binding to the NF-kB response element - PJ also reduced activation of AKT stimulated by TNF-α.	Adams *et al.,* 2006
Colorectal cancer	HT-29 cells	- Urolithins caused reduction in cell proliferation and clonogenic effectiveness.	Kasimsetty *et al.,* 2010
Leukemia	HL-60	- PE showed differentiation promoting activity because of the high content of flavonoid and ellagitannin compounds.	Kawaii and Lansky, 2004
Leukemia	K562	- Pomegranates peels extract suppressed growth of K562 cell line via cell cycle arrest at G2/M phase and by activation of apoptosis.	Joseph *et al*., 2012
Leukemia	4 lymphoid cell line (Jurkat, CCRF-CEM, MOLT-3, SUP-B15), 4 myeloid leukemia cell lines (HL-60, THP-1, K562, KG1a)	- PJE caused apoptosis and reduced cellular proliferation via S phase arrest. - PJE caused greater extent of apoptosis in lymphoid cells and two of the myeloid cell lines (HL-60 and KG-1a) as compared with no tumor control cells.	Dahlawi *et al*., 2012
Leukemia	CCRF-CEM, MOLT-3, HL-60 and THP-1	- Acetonitrile fraction of pomegranate fruit extract showed apoptosis, cell cycle arrest and inhibition of proliferation towards leukemia cell.	Dahlawi *et al.,* 2013
Bladder cancer	UBUC T24 and J82 Cell	- Stimulation of ER is the prime apoptotic mechanism of PEE-induced inhibition of bladder cancer cell besides death receptor signaling and mitochondrial damage pathway.	Lee *et al*., 2013
Bladder cancer	Human bladder cancer cells (EJ cell)	- Polyphenols in pomegranate rind extract suppressed bladder cancer cell EJ proliferation through p53/miR-34a axis.	Zhou *et al.,* 2015
Hepatocellular carcinoma (HCC)		- Pomegranate emulsion reduced the occurrence, multiplicity, number, volume and size of hepatic nodules and precursors of HCC, as well as decreased lipid peroxidation in liver and oxidation of protein.	Bishayee *et al*., 2011
Hepatocellular carcinoma (HCC)	Dietary carcinogen diethylnitrosamine (DENA)-induced rat hepatocarcinogenesis	- Pomegranate phytochemicals showed chemoprevention of hepatic cancer by anti-proliferative and pro-apoptotic method by Wnt/β-catenin signaling mechanism.	Bhatia et al., 2013

PFE: Pomegranate fruit extract, EA: ellagic acid, TPT: total pomegranate tannin, PFJ: pomegranate fermented juice, PJE: pomegranate juice extracts, PE: pomegranate extract, NF-κB: nuclear factor-kappa B

3.3.1 Prostate Cancer

Prostate cancer is the most common invasive malignancy and the second leading cause of cancer-related deaths among U.S. males with a similar trend in many Western countries (Malik *et al.,* 2006) where 30% of men over 50 and 80% of men over 80 years of age are reported to have some histological prostate abnormalities (Bell and Hawthorne, 2008). Thus, the identification of novel targets for the prevention and treatment of prostate cancer has become an urgent challenge. Table 2, presents the effects of various parts of pomegranate fruit and its extracts on the proliferation of prostate cancer cells through different mechanisms.

Albrecht et al (2004) extracted crude polyphenols from pomegranate oil, seed oil, fermented juice polyphenols and pericarp of the pomegranate fruit and found that proliferation and invasion of LNCaP, DU145 and PC3 prostate cancer cells *in-vitro* could be significantly reduced in the presence of specific pomegranate phytochemicals that are punicalagin, ellagic acid (EA) and anthocyanins at doses ranging from 20 to 100 μg/mL. Lansky et al. (2005) and Malik *et al.* (2006), both reported that cell growth was inhibited and followed by apoptosis of extremely aggressive human prostate carcinoma PC-3 cells by utilizing pomegranate fruit extract. Malik *et al.* (2006), explains that the inhibition of cell growth and induction of apoptosis was via modulation of proteins regulating apoptosis which may delay prostate cancer progression.

In 2005, Pantuck and his colleagues conducted an open-label, single-arm, phase II, Simon two-stage clinical trial to determine the clinical and laboratory effects of treatment with 8 oz. of pomegranate juice by mouth daily on patients with prostate cancer. *In vitro* assays comparing pretreatment and posttreatment patient serum on the growth of LNCaP, human prostate adenocarcinoma cells, showed a significant 12% decrease in cell proliferation and a 17% increase in apoptosis. Similar results were found by Seeram *et al.* (2005) pomegranate phytochemical

ellagic acid and its gut-microbial metabolite urolithin A reduced human prostate cancer cells (LAPC-4) xenograft growth in immune-deficient mice that were injected subcutaneously with human CaP cells (LAPC-4). Rettig *et al.* (2008) identified other mechanisms to regulate apoptosis and to delay prostate cancer progression. The pomegranate extract (PE) was administered to animals in doses based on the amount of ellagitannins in a single serving of PJ (240 mL single strength juice, 80 mg of ellagitannins, as punicalagins). PE inhibited NF-κB and cell viability of prostate cancer cell lines. PE-induced apoptosis was based on PE-mediated NF- κB. PE slowed the emergence of LAPC-4 androgen-independent xenografts in a mice model via prevention of proliferation and induction of apoptosis. Also, PE reduction raised NF-κB activity during the transition from androgen dependence to androgen independence in the LAPC-4 xenograft model. Another mechanism was explained by Kasimetty *et al.* (2009). The study showed that pomegranate ellagitannins (ETs.) and their microbial metabolites (Urolithins, punicalins and punicalagins) by inhibiting the cytochrome P450 enzyme and CYP1B1 activities seem to exert beneficial effects on three stages of prostate cancer development. Urolithins, punicalins and punicalagins were also tested for their 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced CYP1B1 inhibitory activity in the 22Rv1 prostate cancer cell line. Urolithins were also studied for their cellular uptake and inhibition of TCDD-induced CYP1B1 expression. Vicinanza *et al* (2013), found that ellagic acid from pomegranate caused cell arrest, and reduced cyclin B1 and cyclin D1. While urolithin A induced G2/M arrest, raised cyclin B1 and cdc2 phosphorylation and caused apoptosis in both cell lines.

3.3.2 Breast cancer

Breast cancer is most common cancer a leading cause of cancer death in women worldwide (Althuis *et al*., 2005). Several studies were done on pomegranate and explained its anti-aromatase

activity and its chemopreventive efficacy in breast cancer via different mechanisms. Pomegranate phytochemicals such as ellagitannins and its derivatives are thought to work as anti-aromatase agents work as inhibitors by blocking the enzyme aromatase, which turns the hormone androgen into small amounts of estrogen in the body. This means that less estrogen is available to stimulate the growth of hormone-receptor-positive breast cancer cells (Adams *et al.*, 2010). Some of these studies are summarized in table 2.

Kim et al (2002) examined and found the polyphenols, obtained from pomegranate fermented juice, seed oil and pericarp extract to possess great anti-aromatase activity against MDA-MB-435 and MCF-7 breast cancer cell line; these polyphenols inhibited aromatase activity up to 60–80% and suppressed 17-beta-hydroxysteroid dehydrogenase type 1 up to 34 to 79%. Mehta *et al.* (2004) evaluated the potential chemopreventive efficacy of a purified chromatographic peak of fermented pomegranate juice, (Peak B), made mainly of phenolic compounds with potent chemoprotective properties, and also of whole pomegranate seed oil, comprised mainly of punicic acid and trienoic acid, pomegranate phytochemicals, with anticarcinogenic properties and at very low doses (1 μg/ml in organ culture), in mouse mammary organ culture. The treatment caused 42% reduction in the number of lesions compared with control while peak B and pomegranate seed oil each resulted in an 87% reduction. The results highlighted enhanced breast cancer preventive potential both for the purified compound peak B and for pomegranate seed oil, both greater than that previously reported results for pomegranate fermented juice polyphenols. Adams *et al* (2010) examined six compounds from ellagitannin-derived compounds of pomegranate, including urolithin B, and found these compounds to have anti-aromatase activity, with urolithin B being the most effective, and to prevent estrogen responsive tumors in breast live cell assay via multiple pathways. Grossmann *et al* (2010) investigated the potential ability of 40 μM punicic acid to

interfere with growth of both an estrogen insensitive breast cancer cell line (MDA-MB-231) and an estrogen sensitive breast cancer cell line developed from the MDA-MB-231 cells (MDA-ERα7). Proliferation was inhibited 92% and 96% for MDA-MB-231 and MDA-ERα7 cells, respectively compared to untreated cells. Furthermore, punicic acid induced apoptosis in the MDA-MB-231 and MDA-ERα7 cells by 86% and 91%, respectively and disrupted cellular mitochondrial membrane potential compared to untreated control cells. Such inhibition of proliferation was due to lipid peroxidation of cells and activation of protein kinase C. Shirode et al., (2014) showed that pomegranate extract can halt the abnormal multiplication of as well as induce apoptosis in MCF-7 breast cancer cells. The inhibition of MCF-7 cell multiplication was found to occur by stimulation of cell cycle arrest at G2/M phase, leading to apoptosis. Furthermore, genes linked with cell division, DNA replication, chromosome organization, DNA repair and RNA processing were downregulated by pomegranate extract; whereas genes, responsible for regulation of cell proliferation and apoptosis, were upregulated.

3.3.3 Colorectal Cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and the second leading cause of cancer-related deaths worldwide , with nearly 1.85 million new cases and 880,792 deaths in 2018 (Kinany *et al.,* 2019). Table 2, summaries studies done on effect of pomegranate and CRC.

Seeram and his colleagues (2005) evaluated the synergistic and/or additive contributions of purified polyphenols extracted from PJ, punicalagin, EA, total pomegranate tannin (TPT), as compared to PJ as a whole against colon cancer cell line (HT-29, HCT116, SW480, SW620) apoptosis. PJ showed greatest anti-proliferative activity against all cell lines at treatments between 12.5 and 100 μg/ml. PJ inhibited proliferation from 30% to 100%. However, in HCT116 cell line, PJ could not induce apoptosis. SW480 non-metastatic colon cancer cells showed sensitivity to

pomegranate polyphenols with EA inhibiting cell proliferation from 49% to 76%, punicalagin from 1% to 65% and TPT from 1% to 67%. In SW620 metastatic colon cancer cells, EA inhibited proliferation from 14% to 35%, punicalagin from 0% to 57% and TPT from 0.02% to 40%. Proliferation of HT-29 colon cancer cells was inhibited from 0% to 21% by EA, from 1% to 55% by punicalagin and from 2% to 71% by TPT, and in HCT116 colon cancer cells, EA induced inhibition of proliferation from 53% to 87%, punicalagin from 0% to 72% and TPT from 13% to 87%. The trend in antioxidant activity was PJ<TPT<punicalagin<EA. The superior bioactivity of PJ compared to its purified polyphenols illustrated the multifactorial effects and chemical synergy of the action of multiple compounds compared to single purified active ingredients. The effectiveness of PJ inhibiting cancer cell proliferation and apoptosis through the modulation of cellular transcription factors and inflammatory cell signaling proteins was investigated by Adams et al. (2006). PJ, at a dose of 50 mg/L, reduced tumor necrosis factor-α (TNF-α) –mediated cyclooxygenase-2 (COX-2) protein expression by 79%, while TPT and punicalagin decreased it by 55 and 48%, respectively. Furthermore, PJ, TPT and punicalagin reduced binding to the NF-κB response element (DNA binding) by 6.4-fold, 10-fold and 3.6-fold, respectively whereas, EA did not reduce binding to the NF-κB response element. Moreover, PJ also reduced activation of AKT stimulated by TNF-α, useful for NF-κB activity. Therefore, pomegranate might be beneficial for balancing inflammatory signaling pathway in colon cancer cells. Kasimsetty *et al.* (2010), showed that pomegranate juice containing ETs and its metabolites, urolithins, suppressed 2,3,7,8-tetrachlorodibenzo-pdioxin, resulting in CYP1- dependent ethoxy resorufin-O-deethylase activity *in vitro* causing reduction in cell proliferation of HT-29 cells. Kasimsetty explained that the suppression of cell proliferation was caused via cell cycle arrest in the G0/G1 and G2/M steps of the cell cycle, followed by initiation of cell death.

3.3.4 Leukemia

Kawaii and Lansky (2004) studied the effects of flavonoid-rich PEs obtained from fresh, fermented juice and pericarp of pomegranate on differentiation of HL-60 human leukemia cells and found that both pericarp and fermented juice extracts were strong promoters of cellular differentiation and prohibited proliferation in HL-60 cell whereas, fresh juice extract showed only a relatively mild differentiation-promoting effect. The effectiveness is contributed to the high content of flavonoid and partly due to the presence of ellagitannin compounds. Joseph et al., 2012 evaluated the anticancer properties of the polysaccharide, PSP001, obtained from the pomegranate fruit, in relation to MCF-7 (breast cancer), KB (nasopharyngeal carcinoma) and K562 (leukemia). The study showed *in vitro* growth stimulatory effect on isolated normal lymphocytes, and growth suppression response on leukemia cell lines indicating immunomodulatory activity. Dahlawi *et al.,* (2012) investigated the potential effect of pomegranate juice extract (PJE) on induction of apoptosis and inhibition of cellular proliferation in eight leukemia cell lines; four lymphoid cell lines: Jurkat (peripheral blood T cell leukemia), SUP-B15 (acute lymphoplastic leukemia), MOLT-3, CCRF-CEM and four myeloid leukemia cell lines: HL-60, THP-1, K562 and KG1a (acute myelogenous leukemia) and non-tumor hematopoietic stem cells (control cells). PJE significantly induced apoptosis in all cell lines, including non-tumor control cells, however, lymphoid cells and 2 of the myeloid cell lines (HL-60 and KG-1a) were affected the most. Furthermore, PJE induced cell cycle arrest. Then, in 2013 Dahlawi and his colleagues investigated further the effects of specific fractions obtained from PJ by solid phase extraction, on four leukemia cell lines. Solid phase extraction demonstrated that only acetonitrile fraction showed apoptosis, cell cycle arrest and inhibition of cell proliferation towards leukemia cell lines CCRF-CEM (acute lymphoblastic leukemia), MOLT-3 (acute lymphoblastic leukemia patient released following chemotherapy),

HL-60 (human promyelocytic leukemia) and THP-1 (acute monocytic leukemia). Liquid chromatography mass spectrometry identified that the acetonitrile fraction of pomegranate contains high amount of ETs and comparatively less amount of anthocyanins.

3.3.5 Bladder cancer

Bladder cancer (BC) is one of the most common genitourinary malignancies. Pomegranate could be a promising in inhibiting proliferation of bladder cancer cells as explained by studies summarized below.

The anticancer activity of Taiwanese pomegranate fruit ethanol extract (PEE) on urinary bladder urothelial carcinoma (UBUC), and its mechanism was examined by Lee et al. (2013). PEE reduced UBUC T24 and J82 cell proliferation and involved apoptosis dose dependently. PEE effectuates S-phase arrest in UBUC T24 cells and causes de-regulation of cyclin A as well as CDK1, the complex that is useful for S to G2/M transition. PEE administration might trigger the apoptosis in T24 cells through death receptor signaling and mitochondrial damage pathway. Zhou et al. (2015) treated human bladder cancer cells (EJ cell) with 100 µg (GAE)/mL pomegranate rind extract (PRE). After 48 h PRE evoked poor cell viability and caspase-dependent pro-apoptosis appearance. PRE also elevated p53 protein and triggered miR-34a expression. The c-Myc and CD44 were confirmed as direct targets for miR-34a in EJ cell apoptosis induced by PRE. Polyphenols in PRE were shown to inhibit bladder cancer cell EJ proliferation via p53/miR-34a axis.

3.3.6 Hepatocellular carcinoma (HCC)

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related death worldwide, and chemoprevention represents a viable approach in lowering the mortality of this disease (Bishayee

et al. 2011). Oxidative stress is a precipitating factor of hepatocellular carcinoma (HCC), one of the most lethal cancers (Bhatia *et al.*, 2013). Bishayee et al (2011) and Bahitia et al (2013) both examined the apoptosis-inducing, anti-proliferative and Wnt/β-catenin pathway-modulatory activity of pomegranate emulsion in diethyl nitrosamine (DENA) –induced hepato-carcinogenesis in rat. In both studies, 1 or 10 g/kg of pomegranate emulsion was given 4 weeks before and after dietary carcinogen DENA introduced. Bishayee et al (2011) showed that pomegranate emulsion increased protein and messenger RNA expression of the hepatic nuclear factor E2-related factor 2 (Nrf2). Their study suggest that pomegranate constituents can be chemopreventive for hepatocarcinogenesis possibly through potent antioxidant activity achieved by upregulation of several housekeeping genes under the control of Nrf2 without toxicity. Bahitia et al (2013) study also revealed chemopreventive activity of pomegranate manifested by reduced incidence, number, multiplicity, size, and volume of hepatic nodules. This effect was mediated by pomegranate antioxidant activity and inhibition of NF-κB which is considered a potent stimulant of Wnt/β catenin signaling which is involved in cell proliferation, cell survival, and apoptosis.

3.4. Cardiovascular Diseases (CVDs)

Many clinical studies investigating the effects of pomegranate on CVDs were performed in the last two decades. These studies consistently demonstrated the protective properties of pomegranate products. Table 3, summarizes some of clinical studies done on humans to investigate the effect of pomegranate juice, molasses, or juice extract on CVDs. In hypertensive patients, daily consumption of pomegranate juice for 2 weeks reduced the activity of angiotensin converting enzyme (ACE) by 36% as well as decreased systolic blood pressure (SBP) by 5% (Aviram and Dornfeld, 2001). Another study done by Aviram *et al.* (2004), reported that consumption of PJ for

long periods (for 3 years) by patients with carotid artery stenosis significantly reduced intima-media thickness, by up to 30%, after 1 year. The patients' serum paraoxonase 1 (PON 1) activity was increased by 83%, whereas serum LDL and systolic blood pressure were reduced after 1 year of PJ consumption and were not further reduced during the remaining 2 years of PJ consumption. Whereas, serum lipid peroxidation was further reduced by up to 16% after 3 years of PJ consumption.

Studies done on diabetic patients and hemodialysis patients showed that pomegranate improves symptoms of CVDs. Consumption of PJ for several weeks and months can cause reduction in total cholesterol, LDL, TG, and an increase in HDL (Aviram *et al.*, 2004; Esmaillzadeh *et al.,* 2004; Parsaeyan *et al.* 2012; Shishehbor *et al.*, 2016; Sohrab *et al.,* 2017; Barati Boldaji *et al., 2020*). Also, Pomegranate can have a positive effect on blood pressure. Several studies showed that significant decrease in both systolic and diastolic blood pressure (Aviram & Dornfeld, 2001; Lynn et al.2012; Parsaeyan *et al.* 2012; Asgary *et al.* 2014; Shema-Didi *et al.,* 2014; Stockton *et al.,* 2017; Moazzen *et al.,* 2017; and Barati Boldaji *et al., 2020*). Other effects were increase in serum total antioxidant capacity (Aviram *et al.*, 2004; Sohrab *et al.* 2015; Shishehbor *et al.*, 2016; Barati Boldaji *et al., 2020*), decrease in malondialdehyde and interleukin-6 (Barati Boldaji *et al., 2020;* Shishehbor *et al.*, 2016; and Sohrab *et al.* 2015).

Table 3: Summary of *in vivo* studies done on humans to study effect of pomegranate on CVDs.

In vivo Studies		Sample	Part of Plant	Dose	Vehicle of administration	Duration	Effect of pomegranate	Reference
Human	**Experimental**	hypertensive patients, nonsmokers aged 62 to 77 yrs (n=10)	PJ	50 ml/day (equivalent to 1.5mmol of total polyphenols)	PJ	2 weeks	- 36% decrement in serum ACE activity - 5% reduction in SBP - dose-dependent inhibitory effect (31%) of PJ on serum ACE activity was observed also in vitro	Aviram & Dornfeld 2001
Human	**Randomized clinical trial**	non-smokers, with asymptomatic severe carotid	PJ	PJ- treated group: 50 ml/day (equivalent	PJ	Phase 1: 1year (n=19)	- significant IMT reduction by 30% - serum PON1 activity increased by 83%	Aviram *et al.*, 2004

		artery stenosis aged 65-75 yrs (n = 19) 5 women and 14 men		to 1.5mmol of total polyphenols		Phase 2: 3 years (n=10)	- LDL decreased - serum levels of antibodies against oxidized LDL were decreased by 19% - serum TAS was increased by 130% - SBP was decreased by 21% - Serum lipid peroxidation was decreased	
				Control group: placebo	Placebo drink			
Human	**quasi-experimental**	Diabetic patients with hyperlipidemia Women (n= 14) & men (n=8)	CPJ	CPJ-treated group 40 g/day	CPJ	8 weeks	- Significant reduction in total cholesterol - Significant reduction low-density lipoprotein (LDL)-cholesterol LDL - Significant reduction cholesterol/ high-density lipoprotein (HDL)-cholesterol - Significant reduction total cholesterol/HDL-cholesterol - No significant changes in serum triacylglycerol and HDL-cholesterol concentrations	Esmaillzadeh *et al.,* 2004
Human	**randomized, placebo-controlled, double-blind study**	45 patients (avg. 69 yrs) who had CHD and myocardial ischemia. Pomegranate Juice (n=26) Placebo (n= 19)	PJ	PJ-treated group: 240 ml/day	PJ	3 months	- After 3 months, the extent of stress-induced ischemia decreased in the pomegranate group	Sumner *et al.*, 2005
				Control: 240 ml/day	Placebo (modified sports beverage)			
Human	**randomized, double-blind, parallel trial**	Men (45 to 74 yrs.) & women (55 to 74 yrs.) with moderate coronary heart disease risk. PJ-treated group (n= 146) Control (n= 143)	PJ	PJ-treated group: 240 ml/day	PJ	18 months	- No significant effect on the overall CIMT progression rate of subjects who were at risk for moderate coronary heart disease. - Slowed CIMT progression in subjects with increased oxidative stress and disturbances in the TG-rich lipoprotein / HDL axis	Davidson *et al.* 2009
				Control: 240 ml/day	Placebo drink			
Humans	**open-label intervention**	healthy, non-smoking volunteers aged 30 to 50 years (n=51) Female =35 Male =16	PJ	PJ: 330 ml/day	PJ	4 weeks	- no effect of the intervention on PWV - significant fall in SBP, DBP, and mean arterial pressure	Lynn *et al.*2012
				Control drink :330 ml/day	Lemonade			
Human	**quasi-experimental interventional study**	Diabetic type II patients (n=50)		200 ml/daily	PJ	6 weeks	- Significantly decreased concentration of fasting blood sugar, total cholesterol, LDL-C and MDA - Significantly increased paraoxonase and arylesterase activity of PON1 after the intervention - No significant changes in serum triglyceride and HDL-C.	Parsaeyan *et al.* 2012
Human	**double-blind, placebo-controlled,**	Hemodialysis patients (HD) (n= 35 control	PJ	PJ-treated group: 100 cc x 3 times/week	PJ	1 year	- Anti-hypertensive - Anti- hyperlipidemia among HD patients.	Shema-Didi *et al.,* 2014

	randomized, clinical trial	& 66 experimental)		Control: 100 cc 3 times/week	Placebo drink			
Human	**single-blind clinical trial**	Hypertensive patients aged 30–67 years (n= 10 control & 11 experimental)	PJ	PJ-treated group: 150 ml/day Control: 150 ml/day	PJ Drinking water	2 weeks	- Effective hypotensive - Improve endothelial function by decreasing serum concentrations of VCAM-1 - PJ as a beneficial supplement for hypertensive subjects	Asgary *et al.* 2014
Human	**randomized double-blind clinical trial**	Diabetic type II patients, aged 40-65 years (n = 25 control & n = 25 experimental)	PJ	PJ-treated group: 250 ml/day Control: 250 ml/day	PJ Placebo juice	12 weeks	- 30% significant decrease in plasma IL-6 in PJ group - 32% significant decrease in hs-CRP in the PJ group	Sohrab *et al.* 2014
Human	**randomized clinical trial**	Diabetic type II patients aged 37-60 (n = 85) & control healthy volunteers aged 30-60 (n=50)		PJ-treated group DMII n=85: 1.5 ml/BW PJ-treated group: DMII n=23 1.5 ml/BW Control: n=50 1.5 ml/BW	PJ Tap water Tap water	1 week	After 3 hours of PJ consumption: - Decreased FSG among DMII - Increased β-cell function among DMII - Decreased insulin resistance among DMII	Banihani *et al.* 2014
Human	**randomized double-blind clinical trial**	Diabetic type II patients aged 56±6.8 years. PJ-treated group (n=22) control (n=22)		PJ-treated group: 250 ml/day Control: 250 ml/day	PJ Placebo juice	12 weeks	- Increased plasma total antioxidant capacity after 12 weeks of intervention - Decreased malondialdehyde after 12 weeks of intervention - Decreases lipid peroxidation - Delays onset of DMII complications related to oxidative stress. - No significant differences were observed in plasma concentration of CML and pentosidine between the two groups.	Sohrab *et al.* 2015
Human	**Quasi-Experimental Study**	Diabetic patients 25-60 yrs (n=40)	CPJ	CPJ-treated group 50 ml/L daily	CPJ	6 weeks	- Significant increase in both total and HDL-C (4.7% and 3.9%, respectively) from baseline. - significant reduction in serum IL-6 - increase in serum TAC by ~ 75% - No significant change in TG, LDL-C, FBG, and BP - No change TNF-α and hs-CRP	*Shishehbor et al.*, 2016

Human	**Randomized crossover controlled trial**	individuals suffering from metabolic syndrome (n=30)	PJ	PJ-treated group 500 ml/day Control Placebo	PJ	1 week 1-week washout period, then placebo for 1 week	- PJ lowered the level of SBP, DBP, & hs-CRP in patients with metabolic syndrome - However, PJ increased TG & VLDL-C.	Moazzen *et al.,* 2017
Humans	**Exploratory randomized, double-blind, placebo-controlled trial**	Seven males and 22 females age: 34.5 ± 13.7 years	PE	PE (Pomanox, Pomegreat) daily placebo capsule daily	capsule	8 weeks	- significant decrease in DBP - insignificant decrease in SBP - no change in body fat percentage, lean body mass, waist circumference and WHR	Stockton *et al.,* 2017
Human	**randomized crossover trial**	Hemodialysis patients 18 – 65 yrs (n=41) Assigned randomly into 2 groups	PJ	PJ-treated group100 ml/L 3 times/wk After dialysis session Control (usual care)	PJ	8 weeks then 4-week washout period was established and then the role of the groups was exchanged	In PJ-treated group: - TG decreased, HDL-C increased, SPB & DPB significantly decreased, LDL-C, decreased serum TAC increased In control group - MDA and IL-6 Increased while decreased in PJ treated group	*Barati Boldaji et al., 2020*

intima-media thickness (IMT), necrosis factor-α (TNF-α), high-sensitivity C-reactive protein (hs-CRP), interleukin-6 (IL-6), total antioxidant capacity (TAC), triglycerides (TG), blood pressure (BP) systolic blood pressure (SBP), diastolic blood pressure (DBP), waist:hip ratio (WHR), pulse wave velocity (PWV), angiotensin converting enzyme (ACE), total antioxidant status (TAS), malondialdehyde (MDA)
.

3.5. Irritable Bowel Disease

Shah *et al*. (2016) tried to evaluate the effect of PJ and purified punicalagin (PW) on nuclear factor kappa B (NFκB), which is an important pharmacological target in inflammation, and the signaling pathways leading to its expression in colon inflammation. Male Sprague–Dawley rats were divided into six groups: positive and negative control, vehicle (50 % ethanol), standard chow (100 mg/kg, b.w.), PJ (400 mg/kg, b.w.), PW (4 mg/kg, b.w.). Colitis was induced with 2,4-dinitrobenzene sulfonic acid (DNBS) and animals were euthanized on 18th day. Pretreatment with PJ and PW significantly lowered the disease extent and severity as indicated by reduction in CMDI and DAI when compared with DNBS-treated rats. Gene expression studies showed decreased mRNA levels of TNF-α, IL-18, and IL-1β in PJ and PW-treated groups. NFκB mRNA levels were found to be reduced by 84% and 64 % in PJ and PW-treated groups, respectively. These results suggest that

PJ is more biologically active over punicalagin alone and can be potentially used for the treatment of inflammatory bowel disease.

3.6. Alzheimer Disease

Although there are no proven ways to delay onset or slow progression of Alzheimer's disease (AD), studies suggest that diet can affect risk. Pomegranates contain very high levels of the powerful antioxidant- polyphenols as compared to other fruits and vegetables. Polyphenols have been shown to be neuroprotective in different model systems (Hartman *et al.*, 2006; Subash *et al.,* 2014). In 2006, Hartman and his colleagues studied the influence of dietary supplementation with PJ on behavior and AD-like pathology in a transgenic mouse model. Transgenic mice, expressing a form of the amyloid precursor protein (APP) that causes early onset familial AD, received either PJ or sugar water control from 6 to 12.5 months of age. PJ-treated Alzheimer mice had significantly less 50% accumulation of soluble amyloid-beta and amyloid deposition in the hippocampus as compared to control mice. Moreover, PJ-treated group learned water maze tasks more quickly and swam faster than controls. Another study was done in 2014 by Subash and his colleagues to investigate the effect of dietary supplementation of Omani pomegranate extract on memory, anxiety, and learning skills in an AD mouse model possessing the double Swedish APP mutation. The experimental groups of APP-Transgenic mice (Tg), starting at the age of 4 months, were fed a custom mixed diet (pellets) containing 4% pomegranate and or standard chow (control group). They assessed spatial memory and learning ability, psychomotor coordination, and anxiety-related behavior in Tg and wild-type mice at the age of 4 to 5 months and 18 to 19 months. Tg mice in the control group that were fed a standard chow diet without pomegranates showed at the age of 18 to 19 months significant memory deficits, increased anxiety-related behavior, and severe impairment in spatial learning ability, position discrimination learning ability, and motor coordination

compared with the wild-type mice on the same diet. In contrast, PJ-treated group showed significant improvements in memory, learning, locomotor function, as well as reduction in anxiety as compared with Tg mice in the control group.

These results suggest that dietary supplementation with pomegranates may slow the progression of cognitive and behavioral impairments in AD. However, further studies to validate and determine the mechanism of these effects, as well as whether substances in PJ may be useful in AD are needed.

3.7. Male Infertility

Although in the ancient medicine, the seeds and rind of the pomegranate were used by Hippocrates, Soranus, and Dioscorides to prevent conception in women; studies have shown that the pomegranate fruit improves male fertility (Türk *et al*., 2007).

In 2007, Türk and his colleagues studied the effects of PJ consumption on sperm quality, spermatogenic cell density, antioxidant activity and testosterone level of male healthy rats. Their experiment included twenty-eight healthy adult male Wistar rats that were divided into four groups; each group containing seven rats. Over seven weeks, one milliliter (mL) distilled water, 0.25 mL PJ plus 0.75 mL distilled water, 0.50 mL PJ plus 0.50 mL distilled water and 1 mL PJ were given daily by gavage to rats in the first, second, third and fourth groups, respectively. The researchers noticed a significant decrease in malondialdehyde (MDA) level and marked increases in glutathione (GSH), glutathione peroxidase (GSH-Px) and catalase (CAT) activities, and vitamin C level were observed in rats treated with different doses of PJ. Moreover, PJ consumption provided an increase in epididymal sperm concentration, sperm motility, spermatogenic cell density and diameter of seminiferous tubules and germinal cell layer thickness, and it decreased abnormal sperm rate when compared to the control group. Another study done in 2016 by Türk

and his colleagues to study whether PJ consumption has an ameliorating effect on carbon tetrachloride (CCl_4), a compound that causes sperm damage and testicular apoptosis associated with the oxidative stress, in male rats. The study comprised of four groups (groups 1–4). Group 1 received olive oil distilled water daily; group 2 was treated with 5 ml/kg PJ olive oil daily; group 3 was treated with 0.25 ml/kg CCl_4 dissolved in olive oil, weekly distilled water daily; and group 4 received weekly CCl_4 daily PJ. All administrations were performed by gavage for 10 weeks. CCl_4 administration caused significant decreases in body and reproductive organ weights, sperm motility, concentration and testicular catalase activity, significant increases in MDA level, and abnormal sperm rate and apoptotic index along with some histopathological damages when compared with the control group. However, significant ameliorations were observed in absolute weights of testis and epididymis, all sperm quality parameters, MDA level, apoptotic index, and testicular histopathological structure following the administration of CCl_4 together with PJ when compared with group given CCl_4 only. In conclusion, these studies provide a strong evidence that PJ consumption ameliorates damages in male reproductive organs and improves sperm quality, spermatogenic cell density, antioxidant activity and testosterone level.

3.8. Erectile dysfunction

PJ with its inherent antioxidant properties has been shown to have potential benefit for erectile dysfunction (ED) due to its ability to decrease fibrosis, increase nitric oxide (NO) bioavailability, reduce atherosclerotic plaque, and may play a role in smooth muscle relaxation as well (Forest *et al.*, 2007).

Azadzoi and his colleagues (2005) searched for markers of oxidative stress in cavernous ischemia and examined the effect of long-term antioxidant intake on arteriogenic ED. The rabbit model of arteriogenic ED demonstrated decreased intracavernous blood flow, erectile dysfunction, loss of

smooth muscle relaxation, decreased endothelial nitric oxide synthase (NOS) and neuronal NOS, increased inducible NOS expression, diffused cavernous fibrosis and increased cavernous levels of the oxidative product isoprostane 8-epi-prostaglandin F2α. However, long-term PJ intake of 3.87 mL increased intracavernous blood flow, improved erectile response and smooth muscle relaxation in ED and control groups while having no significant effect on NOS expression. PJ intake prevented erectile tissue fibrosis in the ED group. A randomized-controlled trial done by Forest and his colleagues (2007) to examine the efficacy of PJ in improving erections in 53 men subjects with mild to moderate ED. The crossover design consisted of two 4-week treatment periods separated by a 2-week washout period. Efficacy was assessed using International Index of Erectile Function (IIEF) and Global Assessment Questionnaires (GAQ). Of the 42 subjects who demonstrated improvement in GAQ scores after beverage consumption, 25 reported improvement after drinking pomegranate juice. Further, 17 subjects showed preference of one beverage to the other. Subjects were more likely to have improved scores when pomegranate juice was consumed. However, overall statistical significance was not achieved. This pilot study suggests the possibility that these associations in larger cohorts and longer treatment periods may achieve statistical significance.

Chapter 4: Materials and Methods

4.1 Pomegranate molasses preparation

In October 2018, a homemade PM sample was prepared according to the traditional method explained before in figure 2, collected in an air tight glass bottle, filled to the top,and was directly stored at a temperature of – 25° C in the chemistry lab freezer at NDU main campus .

4.2 Chemicals

Folin-Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), gallic acid, 2, 2-Diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid, (+)-catechin, ethylene-diamine-tetra-acetic acid (EDTA), 3-[2-Pyridyl]-5,6-diphenyl-1,2,4-triazine-4,4´-disulfonic acid monosodium salt hydrate (Ferrozine), β-carotene, linoleic acid, and Tween-40 were purchased from Sigma-Aldrich Co (Steinheim, Germany); $FeSO_4 \cdot 7H_20$ was purchased from Merck. All other reagents and organic solvents used were of analytical grade.

4.3 Accelerated shelf-life testing (ASLT) procedure

45 heat resistant glass jars with aluminum lid (each 30 ml) were each filled with 5g PM. 1 ml GSH solution (200 µg/L) was added to only 22 jars. All samples were subject to oxygen flush to initiate oxidation. Samples were done in duplicates. The glass jars were closed immediately and placed in an incubator preset at 55ºC. Samples were retrieved from the incubator on different days.

The accelerated aging time (AAT) was calculated according to the following equation:

$$\text{AAT} = \frac{Desired\ Real\ Time\ (RT)}{Q_{10}[(T_{AA} - T_{RT})/10]}$$

Where,

T_{AA}= 55 ºC (Accelerated aging temperature)

T_{RT}= 23 ºC (Ambient temperature)

Q_{10}= 2 (2 being the most common value used in accelerated adding testing)

RT= 365 days

According to the formula used, the time needed to incubate sample at 55 ºC was 40 days which is equivalent to one year on shelves at ambient temperature.

Samples were removed from the incubator according to table 4 and immediately stored in a freezer at - 25 ºC until analysis.

Table 4: Schedule of removal of samples from incubator

Time	Accelerated Aging Time (AAT) / days in incubator	Real Time (RT)/ days on shelves
T_0	0 days	0
T_1	2 days	9.19
T_2	4 days	36.76
T_3	8 days	73.52
T_4	12 days	101.09
T_5	17 days	147.03
T_6	22 days	183.79
T_7	26 days	238.93
T_8	31 days	284.88
T_9	36 days	330.83
T_{10}	40 days	367.58

4.4 Determination of total phenolic content

Total phenolic content was estimated by Folin Ciocalteu's method described by Bhalodia *et al.*, 2011 and Patel *et al.*, 2010. 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 (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 performed using distilled water instead of PM sample. Gallic acid was used as standard. The calibration curve was plotted using standard Gallic acid. The data for total phenolic contents were expressed as mg of Gallic acid equivalent weight (GAE)/ 100 g of dry mass.

4.5 Determination of total flavonoid content

Total flavonoid content was measured with the aluminum chloride colorimetric assay. 1ml of aliquots and 1ml standard quercetin solution (100, 200, 400, 600, 800, 1000 µg/ml) was positioned into test tubes and 4ml of distilled water and 0.3 ml of 5 % sodium nitrite ($NaNO_2$)solution was added into each. After 5 minutes, 0.3 ml of 10 % aluminum chloride ($AlCl_3$) was added. At 6th minute, 2 ml of 1 M sodium hydroxide was added. Finally, volume was making up to 10 ml with distilled water and mix well. Orange yellowish color was developed. The absorbance was measured at 510 nm using Jenway 6405 UV/Vis spectrophotometer. The blank was performed using distilled water. Quercetin was used as standard. The samples were performed in triplicates. The calibration curve was plotted using standard quercetin. The data of total flavonoids were expressed as mg of quercetin equivalents/ 100 g of dry mass (Patel *et al*., 2010; Pallab *et al*., 2013; Satish Kumar *et al*., 2008; Patel *et al*., 2012).

4.6 *In vitro* antioxidant assays

4.6.1 DPPH radical scavenging assay

Antioxidant activity of the PM solution was assayed using DPPH free radical a method explained by Yan and Chen (1995) with slight modifications. DPPH solution was prepared by dissolving 0.6 mg of DPPH in 100 mL ethanol and stirred well. PM solution was prepared by dissolving 50 mg of PM in 2 ml ethanol and 8 ml distilled water. Serial volumes of PM solution (20, 40, 60, 80, and

100 µl) was prepared and EtOH was added to form 1ml total volume. The basic procedure was to add an aliquot (1 mL) of test sample to 1 mL of DPPH. 1 mL of sample solution of 20, 40, 60, 80, and 100 µl volumes with added EtOH up to 2ml is used as a sample blank and DPPH solution (1 mL) plus EtOH (1 mL) is used as a negative control. All measurements will be performed in duplicate.

The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark. The absorbance was read at 517 nm using a Jenway 6405 UV/Vis spectrophotometer, and the calculations of the scavenging activity (%) (SA) is as follows:

SA (%): $[1- (A_{sample} - A_{sample\ blank}) / A_{control}] \times 100$.

4.6.2 Ferrous ion chelating (FIC) assay

The ferrous ion chelating activity was determined according to Lim *et al* (2007). Equal volumes of 0.12 mM $FeSO_4$, test sample (at different concentrations), and 0.6 mM ferrozine will be mixed. The solutions will be allowed to stand for 10 min at room temperature, and the absorbance of Fe^{2+}-ferrozine complex will be measured at 562 nm using a Jenway 6405 UV/Vis spectrophotometer. Ultra-pure water instead of sample solution will be used as a negative control. Ultra-pure water instead of ferrozine solution will be used as a blank, which is used for error correction because of unequal color of the sample solutions. EDTA-Na_2 will be used as the positive control. The ability of the sample to chelate ferrous ions will be calculated by using the formula given by the procedure. All measurements will be performed in duplicate.

4.7 In vitro inhibitory antidiabetic assays

4.7.1 In vitro inhibitory alpha amylase assay

The 500 µL of plant extract was incubated with 500 µL of α-amylase solution (enzyme solution (2 units/ml) was obtained by dissolving 0.001 g of α-amylase in 100 ml of 0.02 M sodium phosphate buffer pH 6.9 with 6.7 mM sodium chloride) at room temperature (32°C) for about 10 minutes. After incubation, 500 µL of 1 % starch solution (dissolving 1 g of potato starch in 100 ml of distilled water with boiling and stirring for 15 minutes) was added and was incubated at room temperature (32°C) for about 10 minutes. To the above, 1 ml of DNSA reagent was added to stop the reaction and was incubated in hot water bath (85°C) for 5 minutes. After 5 minutes, reaction mixture color changed to orange-red and was removed from water bath and cooled to room temperature. It was diluted up to 5 ml of distilled water. Extracts at different concentrations (2, 5, 10, 15, 20 mg/ml) were performed in triplicates. Individual blank was performed by replacing enzyme with buffer. Control was performed by replacing extract with solvent.

The inhibition percentage of α-amylase was assessed by the following formula:

The α-amylase inhibitory activity = (Ac+) – (Ac-) – (As - Ab)/

(Ac+) – (Ac-) × 100

Where,

Ac+ that absorbance of 100% enzyme activity (only solvent with enzyme)

Ac- that absorbance of 0% enzyme activity (only solvent without enzyme)

As that absorbance of test sample with enzyme

Ab that absorbance of test sample without enzyme

4.7.2 In vitro inhibitory alpha glucosidase assay

The inhibition of α-GLU was evaluated using a 96-microplate reader based on the method described by Kim *et al*. using α-glucosidase from Saccharomyces cerevisiae (Kim *et al.,* 2005). Each well contained 100 µL α-GLU (1.0 U/mL) and with 50 µL of the different concentrations of the tested or reference compounds. After preincubation for 10 min, 50 µL of 3.0 mM pNPG (4-Nitrophenyl-β- D –glucopyranosiduronic) (dissolved in phosphate buffer 20 mM, pH 6.9) was added to start the reaction, incubated at 37 °C for 20 min and stopped by adding 2 mL of 0.1 M Na_2CO_3. The absorbance was measured at 405 nm. The results of the juice on the α-GLU inhibition were expressed as percentage of enzyme inhibition.

Inhibition (%) = $[(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$.

Statistical analysis

The mean values of IC_{50} at different times (T_0 to $T1_0$) for PM samples studied under ASLT without addition of GSH using DPPH radical scavenging assay were calculated. Analysis of Variance/ Kruskal Wallis test was used to compare the mean IC_{50} values at the different times. Spearman's correlation analysis was performed to examine the correlation between Total Phenolic Content (TPC) and IC_{50} DPPH with and without addition of GSH (200µg/L). The statistical analysis was carried out using the statistical package for social sciences (SPSS) version 23.

Chapter 5: Results and Discussion

5.1 Determination of total phenolic content

The Folin-Ciocalteu method is a most common used method to determine the total phenolic in the substrate that usually is participated with gallic acids as the standard reference. The color of Folin-Ciocalteu reagent changes from yellow to blue upon the detection of phenolic in the extracts which

is normally due to the chemical reduction of tungsten and molybdenum oxides mixture in the reagent.

At T_0, the mean total phenolic content (TPC) of the tested samples without added GSH, done in triplicates, was found to be 46.83 ± 2.95 mg GAE/g PM. This finding is concordant with those of Yilmas *et al.* (2007) and Incedayi *et al.* (2010) (mean TPC: 52.56 and 55.10 mg GAE/g PM respectively). On the other hand, it is discordant with findings of other studies; specifically, the mean TPC of our tested samples without added GSH is higher than that reported in Onsekizoglu (2013), Firuzi *et al.* (2019), Sabanci *et al.* (2019), and Farahmand *et al.* (2015) (mean TPC: 2.22, 1.49 to 1.7, 6.81 to 9.40, and 6 mg GAE/g PM respectively) and lower that that reported in Akpinasr-Bayizit *et al.* (2016), Chalfoun-Mounayar *et al*. (2012), and Nasser *et al.* (2017) (mean TPC: TPC of 118.28 to 828.15 mg GAE/g PM, 252.28 ± 33.67 mg GAE/L, and 90 to 179.5 mg GAE/g PM respectively). These variations in TPC can be explained by differences in processing techniques such as pressing and juice extraction, filtration, cooking time, pressure ... (Nayak *et al.* 2015). High amounts of phenolic compounds are concentrated in peels (Farahmand *et al. 2015*) so*,* the pressing and extraction techniques are directly related to the TPC of the extract. Extracts from the whole fruit have higher TPC, and, therefore, stronger antioxidant properties than those from the juice. This difference in the activity is probably due to the presence of tannins found in the pericarp of the fruit as suggested by Gil *et al.* (2000) who studied the relationship between the different techniques of extraction and the polyphenolic contents in the pomegranate juice. The juice that is used to prepare the traditional Lebanese homemade PM is extracted from the arils mostly without pressing the whole fruit (Akpinar-Bayizit *et al*., 2016; İncedayi *et al*., 2010; Yilmaz *et al*., 2007). This is because juice extracted from the whole fruit has more bitter and astringent taste due to the presence of high amounts of hydrolysable tannins in the peel and rind. Astringency

and bitterness were significantly correlated with the polyphenol and hydrolysable tannin contents in the juice (Benjamin and Gamrasni, 2016).

Variations in TPC content due to cooking time and temperature have been reported by several studies (Dhinesh and Ramasamy, 2016; Orak, 2009; Öztan, 2006; Nayak *et al*. 2015). Several studies showed that the degradation of color, antioxidants, and antioxidant activity were more significant in conventional/ traditional methods compared to industrial methods (Yousefi *et al*, 2012) since conventional or traditional methods use higher temperatures and longer times (2- 5 hrs.) to achieve desired concentrations as compared to industrial methods. Therefore, the lower the temperature and the shorter the time used during the process of juice concentration are, the higher TPC and, therefore antioxidant content, is (Yousefi *et al.*2012) This explains the variation in TPC content among samples prepared using different traditional methods .

TPC for PM samples without added GSH, over the course of 40 days in the incubator at 55 °C are presented in figure 3. There is a positive linear increase in TPC with time (y= 0.3953 + 46.478, R^2= 0.967). During ASLT, as time in the incubator increased, the TPC values in samples showed an increase as compared to the initial values. At T_0, initial TPC_0 was 46.83 mg GAE/g PM, at T_6 (representing half year on the shelves at ambient temperature of 23 °C) TPC_6 was 55.63 mg GAE/g PM, and at T_{10} (representing one year on the shelves at ambient temperature of 23 °C) TPC_{10} was 63.61 mg GAE/g PM. Some authors have previously observed this phenomenon and reported a possible increment of polyphenolic compounds associated with the microbial growth or with reactions between oxidized polyphenols and formation of new compounds of antioxidant character during juice storage (Martinez-Flores *et al.,* 2015). Kallithraka *et al*. (2009) studied the changes in phenolic composition and antioxidant activity of white wine during bottle storage. He found that one should expect oxidation of antioxidants during storage and accelerated aging, but because

reactions between oxidized phenolic compounds may bring about formation of novel antioxidants, it would appear rather impossible to predict the antioxidant properties of wines after having spent some months in bottles For example, Kallithraka *et al*. (2009) reported considerable increase in Gallic acid concentration during accelerated aging testing. Castro-López (2016) reported similar results where they tested the changes in TPC of eight fruit juices including pomegranate juice stored for over than 20 days at 4, 8, and 11 °C. During storage, the TPC values in all samples studied by Castro-López *et al.* (2016) showed a tendency to increase starting day 12,where the samples stored between 8 and 11°C showed higher polyphenols content than the initial values. These findings support our finding of an increase in TPC associated with the continued release of phenolic compounds from the food matrix or the degradation of complex polymerized phenolic structures into simple phenolic structures as the storage temperature becomes higher and the storage time longer (Prabakaran *et al*., 2018).

After the addition of 200 µg/L GSH, TPC was also calculated. At T_0, TPC_0 was 38.85 mg GAE/g PM which was lower than the TPC_0 of samples without added GSH at day 0. GSH is a strong

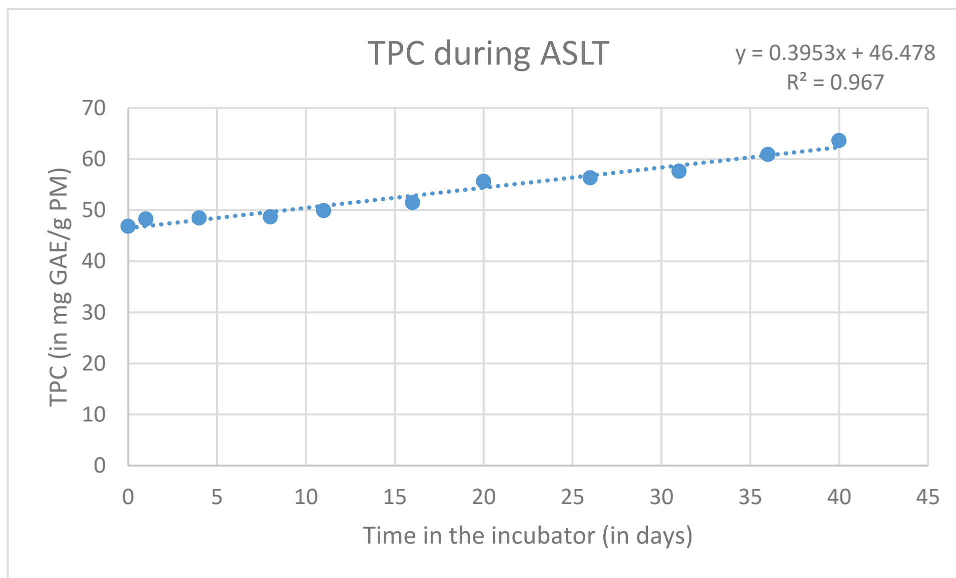

Figure 3 presents the total phenolic content (TPC) for PM samples, over the course of 40 days in an incubator at 55 °C.

antioxidant; however, its addition did not contribute to the TPC. The TPC was decreased after the addition of GSH not only at T_0 but at T_1 to T_{10} as well. Data obtained were not in accordance with our predictions. Therefore, further research should be done to explain the decrease in TPC after addition of GSH.

5.2 Determination of total flavonoid content (TFC)

Flavonoids comprise a large group of polyphenolic compounds in vegetables and fruits. The pomegranate flavonoids, present in the bark and skin responsible for the red color, have a very high antioxidant activity and are useful for blood circulation. The main flavonoids are quercetin, which, in addition to an antioxidant action, also showed antiviral and cardio-protective effects, kaempferol, with anticancer properties, and the rutin, a molecule with antithrombotic properties (Caruso *et al.,* 2020). The mean TFC, of samples done in duplicates, at T_0 was 1.6 ± 0.21 mg quercetin equivalent/g PM. Our findings were supported by a study done by Farahmand *et al.* (2015), where the TFC calculated for pomegranate molasses (PM) was 1.6 mg quercetin equivalent/g PM. Nasser et al. (2017) studied the chemical composition and antioxidant activity of four PM samples (3 commercial and 1 artisanal PM). Nasser and his colleagues found that the mean TFC was 54.34 for the three commercial molasses samples and 137.74 mg Rutin equivalent/g for the artisanal PM sample. The differences in results between our study and the study done by Nasser might be due to different factors such as types of grenade, soil, climate, extraction and evaporation techniques.

5.3 *In vitro* antioxidant assays

5.3.1 In vitro AA of PM sample using DPPH radical scavenging assay and Ferrous ion chelating (FIC) assay at T_0 without added GSH

In addition to analyzing the phenolic content of each sample, the antioxidant activity (AA) of the PM samples were studied using DDPH assay and ferrous ion chelating assay. It is very difficult to assess the AA of a product based on a single method. A single method will provide basic information about antioxidant properties, but a combination of methods describes the antioxidant properties of the sample more accurately. Moreover, there are substantial differences in sample preparation, extraction of antioxidants, selection of end points and expression of results, even for the same method, so that comparison between the values reported by different laboratories can be quite difficult (Viuda-Martos *et al*. 2013).

DPPH assay is an easy and accurate method widely accepted as a tool for estimating the radical scavenging activity of potential antioxidants (Sánchez-Moreno, 2002; Buenger *et al*., 2006). DPPH is a stable free radical characterized by a deep violet color, it is dissolved in ethanol to form a DDPH solution with a spectrophotometric absorption at about 520nm. When a solution of DPPH is mixed with a hydrogen donor such as anti-oxidative agent, it is converted to its reduced form; as a result of which, the deep violet color will fade indicating the antioxidant effect of a hydrogen donor.

The DPPH scavenging activities exhibited by PM samples were expressed as IC_{50} which is defined as the concentration of substrate that causes 50% loss of the DPPH activity. These values were determined using the regression equations obtained from concentration-activity curves. The lower the IC_{50} is the higher the antioxidant activity (Onsekizoglu, 2013).

Figure 4 shows the AA in terms of % DPPH at day zero of the accelerated aging. This figure shows that the % DPPH scavenging activity increases in a concentration-dependent manner from 21.5% to 47.71% as concentration increased from 20 to 100 μg/ml. The mean antioxidant activity for our PM samples, done in duplicates, at T_0 was higher than that for ascorbic acid that was used as a

standard (IC_{50} = 114.25 ± 9.04 μg/ml vs. IC_{50} = 30 μg/ml, respectively). These results are consistent with previously published data on PM. Akpinar-Bayizit et al. (2016) studied the antioxidant activity for nine samples of PM using DPPH. Akpinar-Bayizit and his colleagues found that AA differs from sample to sample where IC_{50} ranged between 140 to 471 μg/ml, with the Inhibition (%) increasing between in concentration-dependent manner (16.11 to 75.22%). Nasser et al. (2017) studied the chemical composition and antioxidant activity of PM. Nasser and his colleagues found that DPPH inhibition percentage varied between 66 to 75 % for the commercial samples and was 90 % for the artisanal molasses sample. İncedayi *et al.* (2010) found that AA using DPPH test was 0 to 46.31 % in molasses samples that were diluted to 14 brix.

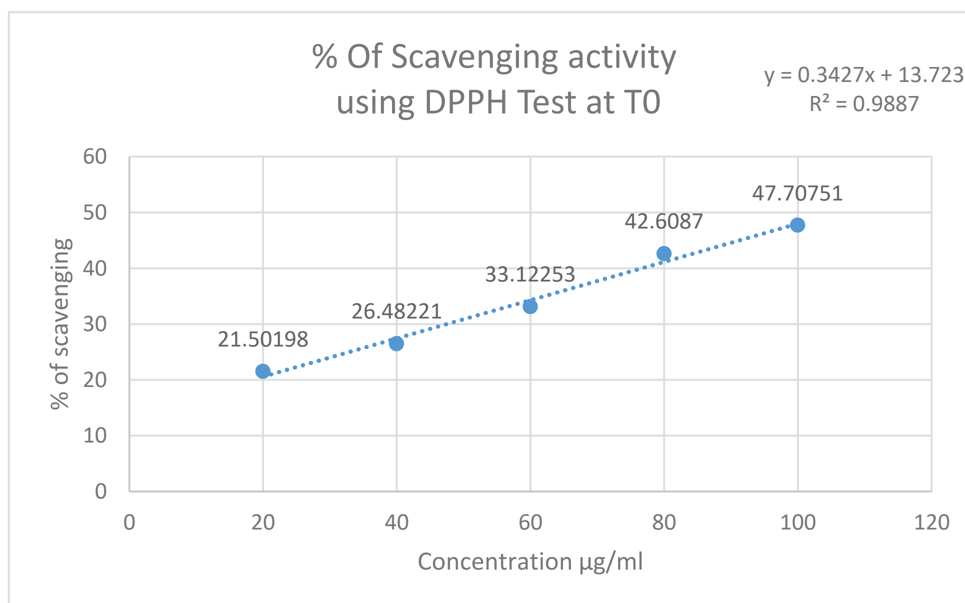

Figure 4: % of scavenging activity using DPPH Test at T_0 of PM sample without added GSH

In living system, the transition element iron has two or three unpaired electrons, and thus act as a free radical and powerful catalyst for oxidation reactions. The metal ion chelating activity of an antioxidant molecule is capable of inactivation, catalysis and inhibition of the harmful transition metal ions responsible for the generation of oxygen free radicals in living organisms. Specifically, an antioxidant, as a chelating agent, converts H_2O_2 (Hydrogen peroxide) into -OH (also called Fenton and Haber-Weiss reaction), forms low-risk redox potential complexes, decomposes alkyl peroxides to the heavy reactive alkoxyl as well as hydroxyl radicals, and prevents oxyradical generation and the consequent oxidative damage. Biochemically, during the metal ion chelating assay, the extract and standard compounds interfere with the formation of ferrous and ferrozine complexes and are able to capture ferrous ion before the formation of ferrozine due to their chelating activity. Ferrozine can quantitatively form complexes with Fe^{2+}. Thus in presence of chelating agents, the complex formation is disrupted, resulting in drop off of the pinkish red colored complex. Thus reduction of the color intensity is proportional to the metal chelating activity (Patel, 2013).

At T_0, the PM samples exhibited a Fe^{2+} chelating activity in a concentration dependent manner with the chelating percent ranging between 33 – 43 % at a concentration of 1000 µl/mg. These chelating percents were higher than that reported in a study by Farahmand *et al*. (2015) in which the samples exhibited an activity of 11% at a concentration of 1000 µl/mg. PM shows that it has lower chelating power than other extracts of pomegranate fruit such as peel and rind. For example a study done by Fawole and his colleagues in 2012, showed that pomegranate peel methanolic extracts exhibit a Fe^{2+} chelating activity ranging between 79.44 – 89.67% at a concentration of 1000 µl/mg from seven pomegranate cultivars in South Africa. Another study done by Viuda-Martos *et al*. (2013) to evaluate *in vitro* antioxidant activity of pomegranate peel powder (PPP),

obtained as a coproduct in the juice extraction process, showed in analysis of metal-chelating properties that PPP was able to chelate the ion iron and it does so in a concentration-dependent manner with values ranging between 2.47 and 47.04% and with extract exhibiting a 25.87% chelating activity at a concentration of 1000 mg/ml. The chelating activity reported in this study was higher than the chelating activity of pomegranate juice studied by Aloqbi and his colleagues (2016) who reported that the chelating activity of PJ was only 16 % at concentration 1000 µl/mg.

5.3.2 AA of PM samples without GSH and with addition of GSH at different concentrations (50, 100, 200 mg/L) at time T_0 with and without oxygen saturation

The IC_{50} values are calculated to determine the concentration of the sample required to inhibit 50% of radical. The lower the IC_{50} value, the higher the antioxidant activity of samples. Table 5 shows the AA (IC_{50} DPPH) of PM samples under several conditions (with/without added GSH and with/without oxygen saturation) in order to determine the possible concentration of GSH that will be added to the samples to be studied under ASLT. The IC_{50} DPPH calculated showed that AA is higher in all samples without oxygen saturation with and without added GSH. Our findings were supported by findings of Van Der Sluis *et al.* (2005). Var Der Sluis and his colleagues found that the effect of the presence of oxygen was clear on the degradation rates on antioxidant activity and concentration of some polyphenols in apple juice. Moreover, the addition of GSH at a concentration of 200 mg/L enhanced the AA. However, samples 2 and 3, with added 50 and 100 mg/l of added GSH respectively, showed an increase in IC_{50} reflecting a lower AA than samples without added GSH or samples with a 200 mg/L of added GSH; this result remains to be analyzed in a future study. This finding shows that glutathione appears to be a promising additive to enhance the AA of PM at a concentration of 200 mg/L and, therefore, GSH at a concentration of 200 mg/L was chosen to be added to the tested samples. The addition of GSH to enhance the quality of certain

foods was tested in different studies at different concentrations. A study done by Stebbins *et al.,* (2017) to study the effect of GSH addition on the stabilization of anthocyanins in blackberry juice used a concentration of 500 mg/L under accelerated aging at 30 $^{\circ}$C, reported that very high additive

Table 5: DPPH scavenging activity of PM samples using different concentration of GSH (50, 100, 200 mg/L) with and without oxygen saturation

Sample	IC_{50} (mg GAE/g) with O_2 saturation	IC_{50} (mg GAE/g) without O_2 saturation
Sample 1 without added GSH	170.4	82.44
Sample 2 with 50 mg/L GSH	260.7	115.16
Sample 3 with 100 mg/L GSH	218.8	117.27
Sample 4 with 200 mg/L GSH	141.6	78.22

concentrations are often needed to limit anthocyanin degradation as well as the concentration of additives in the system must be balanced in order to prevent a pro-oxidant environment. Another study done by Hosry *et al.* (2009) to study the effect of GSH addition on the browning susceptibility of white wines under accelerated aging at 55 $^{\circ}$C, reduced GSH was added to the studied wine samples at four doses: 10, 15, 20 and 30 mg/L. They noticed that samples with (10, 15 and 20 mg/L) of added GSH showed maximum TPC vs the control sample (no added GSH); however, samples with 30 mg/L of added GSH, showed a slight decrease in TPC when compared with samples with lower concentrations of added GSH. Therefore, the proper dosage of GSH that could be added depends on several factors, such as: the added dose should have no negative impact on the final product's color, taste and smell while taking into consideration the benefits and cost of addition.

5.3.3 Antioxidant Activity (AA) of PM samples without addition of GSH studied under accelerated aging (ASLT) tested using DPPH radical scavenging assay.

The antioxidant Activity (AA) of PM samples without addition of GSH was studied under accelerated aging (ASLT) tested using DPPH radical scavenging assay.Table 6 shows the mean IC_{50} at different times for PM samples. The mean IC_{50} at T_4 to T_{10} was found to be statistically significantly greater than the mean IC_{50} at T_0 (114.25 ± 9.04 mg/g PM). However, there was no statistically significant difference between the mean IC_{50} at T_1, T_2, and T_3 and initial IC_{50} at T_0. Therefore, after approximately 100 days on the shelves, there will be a gradual significant decrease in antioxidant activity of PM, reaching approximately 1.83 folds lower than initial AA after one year on the shelves. The studies that were done to evaluate the AA of different kinds of food under accelerating aging or real shelf-life testing showed different trends for AA. Van der Sluis *et al.* (2005) showed that accelerated shelf-life testing of enriched apple juice during 4 days at 80 °C showed decreases in the antioxidant activity of 20 - 40%. On the other hand, a study done by Kallithraka *et al.* (2009) to evaluate changes in phenolic composition and antioxidant activity of white wine during bottle storage versus accelerated aging test, showed that during storage of wine over nine months on shelves, the AA was significantly increased with time whereas accelerated aging did not significantly alter wine antiradical activity but it reduced the reducing power-antiradical activity, another test used to evaluate AA. A similar increase in AA in carrot juice stored over 20 days was observed by Martínez-Flores *et al*. (2015) where AA increased from 6.52 - 6.86 µmol TE/mL to 7.07 µmol TE/mL on day 10 of storage in samples of carrot juice. Another study done by Kevers and his colleagues (2007) to examine the change of AA during storage of selected fruits and vegetables showed that AA, measured with DPPH, varied by fruit/vegetable. The AA was generally stable during storage of most of studied fruits and vegetables. However, a transient increase, a rapid increase and then a plateau in the AA was observed during storage in yellow pepper/ asparagus/ plum and onion; orange and apple, respectively. On the other hand, a

decrease by 25 % and 50% in the AA was observed during storage in apricot; spinach/ banana/ broccoli and leek, respectively. The reported inconsistency in AA trends during storage can be explained by the possible degradation of some phenolic compounds or formation of byproducts of antioxidant properties during storage that may affect the antioxidant capacity.

Table 6: mean IC_{50} at different times for PM samples studied under ASLT without addition of GSH using DPPH radical scavenging assay

Time in the Incubator (T_n, where n is the number of days in the incubator)	**IC_{50} (mg GAE/g)**	**Mean Difference**
T_0	114.25 ± 9.04	
T_1	136.7 ± 0.97	-22.45
T_2	130.67 ± 1.24	-16.42
T_3	132.34 ± 1.23	- 18.09
T_4	151.34 ± 9.75	- 37.09*
T_5	157.6 ± 17.17	- 43.35*
T_6	161.42 ± 7.45	-47.17*
T_7	182.14 ± 8.13	- 67.89*
T_8	192.27 ± 1.74	-78.02*
T_9	170. 06 ± 10.9	-55.81*
T_{10}	208.98 ± 12.40	-94.73*

** The mean difference is significant at 0.05 level*

5.3.4 Antioxidant Activity (AA) of PM samples with addition of 200 µg/L GSH studied under accelerated aging (ASLT) tested using DPPH radical scavenging assay.

When examining the AA of PM samples after addition of 200 µg/L of GSH, data showed that there was no statistically significant difference in mean IC_{50} of the samples studied under

accelerated aging *(ASLT*) (T_0 to T_{10}) using DPPH radical scavenging assay. The mean IC_{50}'s of PM samples with added GSH were higher than those without added GSH from T_0 to T_{10} that were showed in table 5. For instance, the IC_{50} at T_0 with added GSH is higher than the IC_{50} at T_0 without added GSH (141 mg GAE/g vs. 114.25 mg GAE/g, respectively), meaning that PM without added GSH has higher AA than PM with added GSH over one year on shelves. This shows that AA is higher in samples without GSH. These results contradict with those we obtained from the experiment done to determine the concentration of GSH that should be added to PM samples (table 5). This inconsistency in results obtained may be due to the extractability of phenolic compounds from the matrix. Further testing should be done to further understand these contradictions. The results obtained by Van Der Sluis (2005) are in accordance with our own results. During 4 days of accelerated shelf-life testing of enriched apple juice with flavonoids at 80 °C, data showed a decrease in the antioxidant activity of 20−40%. This can be explained by the fact that mixtures of antioxidants can interact antagonistically or synergistically to neutralize free radicals (Jacobo-Velázquez and Cisneros-Zevallos; 2009). Therefore, fortification of foods with antioxidants might not lead to higher AA as predicted.

5.4 *In vitro* antidiabetic activity of PM

The inhibitors for dietary carbohydrate digestion which inhibit the activities of α-amylase and α-glucosidase are effective in delaying glucose absorption at the level of the gastrointestinal tract in humans, and hence minimizing the occurrence of postprandial spikes. Because the most commonly used α-amylase and α-glucosidase inhibitor, acarbose, a known anti-diabetic drug (for e.g., Glucobay and Precose), has been associated with significant gastrointestinal adverse effects, much effort has been put into investigations for effective inhibitors that derive from natural resources with less side effects (Kam *et al.* 2013)

The anti-diabetic activity of Lebanese homemade PM was determined using the α-amylase and α-glucosidase inhibitory assay. Results were expressed as IC_{50} and acarbose was used as the reference standard with IC_{50} values of 0.277 mg/ml and 0.42 mg/ml for α-glucosidase and α-amylase, respectively. The lower the IC_{50} value, the higher the antidiabetic of samples. Data obtained showed that PM exerted an inhibitory activity of IC_{50} of 0.443 ± 0.05 mg/ml for α-glucosidase (1.6 times higher than acarbose) and IC_{50} of 1.21 ± 0.4 for α-amylase (2.88 times higher than acarbose). Even thought, PM has lower inhibition ability than acarbose, which is a purified drug not a crude extract like PM, it can considered a potent antidiabetic food.

Les *et al.* (2018) evaluated the effects of pomegranate juice, its main polyphenols, specifically punicalagin and ellagic acid, as well as its main metabolite urolithin A, on physiological and pharmacological targets of metabolic diseases such as obesity and diabetes. They used enzyme inhibition bioassays α-glucosidase and they found that PJ exhibited an inhibition of α- glucosidase with a similar profile to acarbose. Its main polyphenols and the metabolite urolithin A also inhibited this enzyme, being more potent than PJ and acarbose. IC_{50} values were 0.0055, 0.015, 0.025, 0.38 and 1.01 mg/mL for punicalagin, urolithin-A, ellagic acid, acarbose and PJ respectively, with punicalagin and urolithin A being the best inhibitors. Our results show that PM has more inhibition ability than PJ against α- glucosidase but lower than its individual polyphenols or metabolites.

Kam *et al.* (2013) compared the inhibitory effects of extracts (0.25–1 mg/ml) from different pomegranate parts against mammalian type dietary enzymes α-glucosidase and α-amylase. Their results demonstrated that the methanolic pomegranate flower extract strongly inhibited porcine pancreatic α-amylase enzyme activities in a concentration-dependent manner, with an IC_{50} value of 0.653 mg/ml. The other pomegranate parts (dried crude juice, seed and peel) showed weak or

no inhibitory effects against α-amylase. Moreover, the methanolic extract of both pomegranate flower and peel inhibited α-glucosidase enzyme activities, with IC_{50} values of 0.187 μg/ml and 0.835 μg/ml, respectively.

5.5 Correlation between phytochemical constituents and antioxidant activity.

Correlation coefficients between the TPC and IC_{50} DPPH radical scavenging activity, the reciprocal to antioxidant activity, are presented in Table 7. Data showed that there is a strong positive correlation between TPC and IC_{50} DPPH without and after addition of GSH ($\rho = 0.879$, $p < 0.01$) ($\rho = 0.547$, $p > 0.05$), respectively, meaning that our results show that TPC is negatively correlated with antioxidant activity. Moreover, a weak positive correlation was found between TPC and IC_{50} DPPH from T_0 to T_3 without addition of GSH, = 0.312, $p > 0.05$). This shows that total phenols alone do not contribute to the total antioxidant activity. The correlation between TPC and antioxidant activity improved with the addition of GSH but the correlation did not reach statistical significance.

Table 7: Correlation between Total Phenolic Content (TPC) and IC_{50} DPPH with and without addition of GSH (200µg/L)

	N	*IC_{50} DPPH Without GSH (200µg/L)*	*IC_{50} DPPH Without GSH (200µg/L)*
TPC Without GSH (200µg/L) (time T_0 to T_{10})	16	0.879 **	
TPC Without GSH (200µg/L) (time T_0 to T_3)	9	0.312	
TPC With GSH (200µg/L) (time T_0 to T_{10})	4		0.547

*** Correlation is significant at 0.01 level (2-tailed)*

On the other hand, Anahita *et al.* (2015) evaluated the total phenolic content, total antioxidant activity (TAA), and antioxidant vitamin composition of pomegranate seed and juice. Data showed that there is a strong positive correlation between TAA and TPC (Pearson correlation $R^2 = 0.91$ at $p<0.05$). Phenolic content might act as important contributor of antioxidant activity effect in PJ. Derakhshan *et al.* (2018) evaluated chemical and antioxidant properties of arils from six pomegranate cultivars obtained from various sites from the Mediterranean region of Turkey. They studied the correlation between TPC and antioxidant activity that was estimated using two standard procedures: ferric reducing ability of plasma (FRAP) and standard trolox equivalent antioxidant capacity (TEAC) assays. They found a significant positive correlations between each of FRAP and TEAC and TPC ($R^2 = 0.93$ and 0.94, respectively) showing that phenolic compounds contribute significantly to the antioxidant capacity of fruits. Bertoncelj *et al.* (2007) studied the phenolic

content, antioxidant activity and color of Slovenian honeys. Data showed that there is a positive linear correlation between the total antioxidant activity, determined by the FRAP method, and phenolic content was observed. The high correlation coefficient (r = 0.966) indicates that phenolic compounds are one of the main components responsible for the antioxidant behavior of honey. Also, the correlation between the free radical scavenging activity and total phenolic content was statistically significant; the correlation coefficient was equal to 0.932. Baiano *et al.* (2009) studied the phenolic composition and antioxidant activity of extra-virgin olive oils extracted from several Italian varieties at production and during storage. The antioxidant activity was measured according to the following tests: radical scavenging of the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation and β-carotene bleaching method. Data showed that the phenolic content was strongly positively correlated with the antioxidant activity measured according to the β-carotene test (R^2 = 0.839) and weakly positively correlated with the radical scavenging ability (R^2 = 0.550), indicating that phenolic compounds contribute significantly to the inhibition of the lipid oxidation but on the other hand TPC do not scavenge free radicals as we concluded in our study. Jacobo-Velázquez and Cisneros-Zevallos (2009) studied the correlation between antioxidant activity and TPC of carrots stored under super atmospheric oxygen (80% oxygen and 20% nitrogen) and air for 48 h at 20 ◦C. They noticed that samples with the strongest correlation between TPC and antioxidant activity were the group of carrots stored under super atmospheric oxygen (R^2 >0.96). Under hyperoxia condition, the total phenolic and total as well as the specific antioxidant activity increased, indicating an increase in the effectiveness of the phenolic mixture after storage under super atmospheric oxygen or air to inhibit reactive oxygen species as compared to carrots before storage. On the other hand, the total phenolic content and the total antioxidant activity for air-stored carrots also increased and were higher than those in carrot samples before

storage, however, the slope of the regression line was smaller and thus the specific antioxidant activity decreased with the correlation coefficient between antioxidant activity and TPC (R^2 = 0.75). Jacobo-Velázquez and Cisneros-Zevallos (2009) concluded that the mixtures of phenolic compounds that are accumulated during air storage are qualitatively and quantitatively different and less efficient in neutralizing free radicals than those phenolic compounds present in carrots before storage.

Some studies showed results that support our study results. Samad *et al.* (2016) studied the antibacterial properties and effects of fruit chilling and extract storage on antioxidant activity, total phenolic and anthocyanin content of four date palm. The AA was determined using DPPH free radicals scavenging assay. They noticed a decrease in the antioxidant activity of extracts after extract storage was in agreement with our findings. Moreover, they reported similar observations, where higher antioxidant activity was not associated with higher amount of TPC. They found a poor linear correlation between TPC and $1/IC_{50}$ before (R^2 = 0.0449) and after (R^2 = 0.0011) extract storage

According to Jacobo-Velázquez and Cisneros-Zevallos (2009), the reactivity of phenols is dependent on the level of methylation, methoxylation or other group substituents since phenols act as proton donors. Therefore the oxidation of other antioxidants such as ascorbic acid or anthocyanins within the extracts might also result in the lower antioxidant activity. Moreover, mixtures of antioxidants can interact antagonistically or synergistically to neutralize free radicals Therefore, the typical approach used to analyze correlations between total antioxidant activity and TPC do not reflect these characteristics of phenolic compounds.

Chapter 6: Conclusion

Recent years, due to the awareness on the health benefits of pomegranate and its various products, an increase in demand was found worldwide. Pomegranate fruit has received attention because it contains many different phytochemical compounds. Pomegranates are a rich source of phenolic compounds that include hydrolysable tannins, condensed tannins, and flavonoids (flavonols, flavanols and anthocyanins) and have potent antioxidant capacity. PM is a product that can preserve these bioactive compounds made responsible for the health-promoting effects of pomegranate since is usually prepared to be used from season to season and sometimes stored for 2 to 3 years.

There has been an increased interest in consuming homemade and traditional products in Lebanon. The demand of artisanal and homemade PM has increased since it is produced without the addition of sugar or other additives. Therefore, a screening for total phenols, total flavonoids, anti-diabetic and antioxidant activities and the effect of accelerated aging with and without addition of a strong antioxidant such as GSH, was performed. Our data showed that the traditional Lebanese homemade PM has high TPC, TFC, and AA as compared with PM produced worldwide. Moreover, it showed to have a potent antidiabetic activity comparable with acarbose. Unfortunately, the addition of GSH to homemade samples did not increase neither the TPC nor the AA, as well as did not protect samples against the effect of accelerated aging as expected. Other studies might be important to explain these findings.

Finally, as recommendations, several factors should be further considered in evaluation of TPC, AA, and anti-diabetic activity of traditional homemade PM in future studies. Some of these factors are: 1) differences in content and quantities of phenolic compounds in fruit due to difference in

genotype, pre-harvest environmental conditions as well as the degree of maturity at harvest 2) extraction of whole fruit rather than arils only to increase the TPC and therefore the AA by traditional producers pf PM 3) sample preparation which plays an important role in analysis since PM is a concentrated fruit extract and very viscous in nature. So, any small variations happen in the quantity/solvent/sorbent/time can have a huge impact on the results 4) dose and effect of addition of synthetic or natural antioxidants to certain food such as GSH since antioxidants can act synergistically, additively, or antagonistically to inhibit reactive species.

References

Adams, L. S., Seeram, N. P., Aggarwal, B. B., Takada, Y., Sand, D., & Heber, D. (2006). Pomegranate juice, total pomegranate ellagitannins, and punicalagin suppress inflammatory cell signaling in colon cancer cells. Journal of agricultural and food chemistry, 54(3), 980-985.

Adams, L. S., Zhang, Y., Seeram, N. P., Heber, D., & Chen, S. (2010). Pomegranate ellagitannin–derived compounds exhibit antiproliferative and antiaromatase activity in breast cancer cells in vitro. *Cancer Prevention Research*, *3*(1), 108-113.

Akhavan, H., Barzegar, M., Weidlich, H., & Zimmermann, B. F. (2015). Phenolic compounds and antioxidant activity of juices from ten Iranian pomegranate cultivars depend on extraction. Journal of Chemistry, 2015.

Akpinar-Bayizit, A., Ozcan, T., Yilmaz-Ersan, L., & Yildiz, E. (2016). Evaluation of antioxidant activity of pomegranate molasses by 2, 2-Diphenyl-l-Picrylhydrazyl (DPPH) method. International Journal of Chemical Engineering and Applications, 7(1), 71-74.

Albrecht,M., Jiang W., Kumi-Diaka J., Lansky E.P., Gommersall L.M., Patel A., Mansel R.E., Neeman I.,

Aloqbi, A., Omar, U., Yousr, M., Grace, M., Lila, M. A., & Howell, N. (2016). Antioxidant activity of pomegranate juice and punicalagin. *Natural Science*, *8*(06), 235.

Althuis MD, Dozier JM, Anderson WF, Devesa SS, Brinton LA. (2005). Global trends in breast cancer incidence and mortality 1973–1997. Int J Epidemiol 34: 405–412.

Ammar A, Turki M, Hammouda O (2017). Effects of Pomegranate Juice Supplementation on Oxidative Stress Biomarkers Following Weightlifting Exercise. Nutrients; 9: 819.

Anahita, A., Asmah, R., & Fauziah, O. (2015). Evaluation of total phenolic content, total antioxidant activity, and antioxidant vitamin composition of pomegranate seed and juice. *International Food Research Journal, 22*(3), 1212.

Asgary, S., Sahebkar, A., Afshani, M. R., Keshvari, M., Haghjooyjavanmard, S., & Rafieian-Kopaei, M. (2014). Clinical evaluation of blood pressure lowering, endothelial function improving, hypolipidemic and Anti-Inflammatory effects of pomegranate juice in hypertensive subjects. Phytotherapy Research, 28(2), 193-199. doi:10.1002/ptr.4977

Atilgan, D., Parlaktas, B., Uluocak, N., Gencten, Y., Erdemir, F., Ozyurt, H., ... & Aslan, H. (2014). Pomegranate (Punica granatum) juice reduces oxidative injury and improves sperm concentration in a rat model of testicular torsion-detorsion. *Experimental and therapeutic medicine*, *8*(2), 478-482.

Aviram, M., Rosenblat, M., Gaitini, D., Nitecki, S., Hoffman, A., Dornfeld, L., ... & Hayek, T. (2004). Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduces common carotid intima-media thickness, blood pressure and LDL oxidation. *Clinical Nutrition*, *23*(3), 423-433.

Baiano, A., Gambacorta, G., Terracone, C., Previtali, M. A., Lamacchia, C., & La Notte, E. (2009). Changes in phenolic content and antioxidant activity of italian extra-virgin olive oils during storage. *Journal of Food Science, 74*(2), C177-C183. doi:10.1111/j.1750-3841.2009.01072.x

Banihani, S. A., Fashtaky, R. A., Makahleh, S. M., El-Akawi, Z. J., Khabour, O. F., & Saadeh, N. A. (2020). Effect of fresh pomegranate juice on the level of melatonin, insulin, and fasting serum glucose in healthy individuals and people with impaired fasting glucose. Food Science & Nutrition, 8(1), 567-574. doi:10.1002/fsn3.1344

Banihani, S. A., Makahleh, S. M., El-Akawi, Z., Al-Fashtaki, R. A., Khabour, O. F., Gharibeh, M. Y., . . . Al-Khasieb, N. J. (2014). Fresh pomegranate juice ameliorates insulin resistance, enhances β -cell function, and decreases fasting serum glucose in type 2 diabetic patients. Nutrition Research, 34(10), 862-867. doi:10.1016/j.nutres.2014.08.003

Bell, C., & Hawthorne, S. (2008). Ellagic acid, pomegranate and prostate cancer—a mini review. Journal of Pharmacy and Pharmacology, 60(2), 139-144.

Benjamin, O., & Gamrasni, D. (2016). Electronic tongue as an objective evaluation method for taste profile of pomegranate juice in comparison with sensory panel and chemical analysis. *Food Analytical Methods*, *9*(6), 1726-1735.

Bhatia, D., Thoppil, R. J., Mandal, A., Samtani, K. A., Darvesh, A. S., & Bishayee, A. (2013). Pomegranate bioactive constituents suppress cell proliferation and induce apoptosis in an experimental model of hepatocellular carcinoma: role of Wnt/β-catenin signaling pathway. Evidence-based Complementary and Alternative Medicine, 2013.

Bishayee, A., Bhatia, D., Thoppil, R. J., Darvesh, A. S., Nevo, E., & Lansky, E. P. (2011). Pomegranate-mediated chemoprevention of experimental hepatocarcinogenesis involves Nrf2-regulated antioxidant mechanisms. Carcinogenesis, 32(6), 888-896.

Blomhoff, R., Carlsen, M. H., Andersen, L. F., & Jacobs, D. R. (2006). Health benefits of nuts: Potential role of antioxidants. British Journal of Nutrition, 96(S2), S52-S60. doi:10.1017/BJN20061864

Castro-López, C., Sánchez-Alejo, E. J., Saucedo-Pompa, S., Rojas, R., Aranda-Ruiz, J., & Martínez-Avila, G. C. G. (2016). Fluctuations in phenolic content, ascorbic acid and total carotenoids and antioxidant activity of fruit beverages during storage. *Heliyon*, *2*(9), e00152.

Chalfoun-Mounayar, A., Nemr, R., Yared, P., Khairallah, S., & Chahine, R. (2012). Antioxidant and Weight Loss Effects of PomegranateMolasses. Journal of applied pharmaceutical science, 2(6), 45.

Dahlawi, H., Jordan-Mahy, N., Clench, M. R., & Le Maitre, C. L. (2012). Bioactive actions of pomegranate fruit extracts on leukemia cell lines in vitro hold promise for new therapeutic agents for leukemia. Nutrition and cancer, 64(1), 100-110.

Dahlawi, H., Jordan-Mahy, N., Clench, M., McDougall, G. J., & Le Maitre, C. L. (2013). Polyphenols are responsible for the proapoptotic properties of pomegranate juice on leukemia cell lines. Food science & nutrition, 1(2), 196-208.

Das AK, Mandal SC, Banerjee SK, Sinha S, Saha BP, Pal M. Studies on the hypoglycaemic activity of Punica granatum seed in streptozotocin induced diabetic rats. Phytother Res2001;15:628–9.

Davidson, M. H., MD, Maki, K. C., PhD, Dicklin, M. R., PhD, Feinstein, S. B., MD, Witchger, M., RD, Bell, M., . . . Aviram, M., DSc. (2009). Effects of consumption of pomegranate juice on carotid Intima–Media thickness in men and women at moderate risk for coronary heart disease. American Journal of Cardiology, the, 104(7), 936-942. doi:10.1016/j.amjcard.2009.05.037

De Bruyne, E., Ducatelle, R., Foss, D., Sanchez, M., Joosten, M., Zhang, G., . . . Flahou, B. (2016). Oral glutathione supplementation drastically reduces helicobacter-induced gastric pathologies. Scientific Reports, 6(1), 20169. doi:10.1038/srep20169

Deng, J., Cheng, W., & Yang, G. (2011). A novel antioxidant activity index (AAU) for natural products using the DPPH assay. Food Chemistry, 125(4), 1430-1435. doi:10.1016/j.foodchem.2010.10.031

Derakhshan, Z., Ferrante, M., Tadi, M., Ansari, F., Heydari, A., Hosseini, M. S., . . . Sadrabad, E. K. (2018). Antioxidant activity and total phenolic content of ethanolic extract of pomegranate peels, juice and seeds. *Food and Chemical Toxicology, 114*, 108-111.

Di Stefano, V., Pitonzo, R., Novara, M. E., Bongiorno, D., Indelicato, S., Gentile, C., ... & Melilli, M. G. (2019). Antioxidant activity and phenolic composition in pomegranate (Punica granatum L.) genotypes from south Italy by UHPLC–Orbitrap-MS approach. Journal of the Science of Food and Agriculture, 99(3), 1038-1045.

El Darra, N., Rajha, H. N., Saleh, F., Al-Oweini, R., Maroun, R. G., & Louka, N. (2017). Food fraud detection in commercial pomegranate molasses syrups by UV–VIS spectroscopy, ATR-FTIR spectroscopy and HPLC methods. Food Control, 78, 132-137. doi:10.1016/j.foodcont.2017.02.043

El Hosry, L., Auezova, L., Sakr, A., & Hajj-Moussa, E. (2009). Browning susceptibility of white wine and antioxidant effect of glutathione. International Journal of Food Science & Technology, 44(12), 2459-2463. doi:10.1111/j.1365-2621.2009.02036.x

Elfalleh, W., Hannachi, H., Tlili, N., Yahia, Y., Nasri, N., & Ferchichi, A. (2012). Total phenolic contents and antioxidant activities of pomegranate peel, seed, leaf and flower. *Journal of Medicinal Plants Research*, *6*(32), 4724-4730.

Esmaillzadeh, A., Tahbaz, F., Gaieni, I., Alavi-Majd, H., & Azadbakht, L. (2004). Concentrated pomegranate juice improves lipid profiles in diabetic patients with hyperlipidemia. Journal of medicinal food, 7(3), 305-308.

Farahmand, M., Golmakani, M., Mesbahi, G., & Farahnaky, A. (2017). Investigating the effects of large-scale processing on phytochemicals and antioxidant activity of pomegranate juice: Processing effects on pomegranate antioxidants. Journal of Food Processing and Preservation, 41(2), e12792. doi:10.1111/jfpp.12792

Fawole, O. A., Makunga, N. P., & Opara, U. L. (2012). Antibacterial, antioxidant and tyrosinase-inhibition activities of pomegranate fruit peel methanolic extract. *BMC Complementary and Alternative Medicine, 12*(1), 200-200. doi:10.1186/1472-6882-12-200

Fernandes, L., Pereira, J. A., Lopéz-Cortés, I., Salazar, D. M., González-Álvarez, J., & Ramalhosa, E. (2017). Physicochemical composition and antioxidant activity of several pomegranate (Punica granatum L.) cultivars grown in Spain. *European Food Research and Technology*, *243*(10), 1799-1814.

Firuzi, M. R., Niakousari, M., Eskandari, M. H., Keramat, M., Gahruie, H. H., & Mousavi Khaneghah, A. (2019). Incorporation of pomegranate juice concentrate and pomegranate rind powder extract to improve the oxidative stability of frankfurter during refrigerated storage.Lwt, 102, 237-245. doi:10.1016/j.lwt.2018.12.048

Fischer, U. A., Carle, R., & Kammerer, D. R. (2013). Thermal stability of anthocyanins and colourless phenolics in pomegranate (punica granatum L.) juices and model solutions. Food Chemistry, 138(2-3), 1800-1809. doi:10.1016/j.foodchem.2012.10.072

Fischer, U. A., Dettmann, J. S., Carle, R., & Kammerer, D. R. (2011). Impact of processing and storage on the phenolic profiles and contents of pomegranate (Punica granatum L.) juices. *European Food Research and Technology*, *233*(5), 797.

Furukawa, T., Meydani, S. N., & Blumberg, J. B. (1987). Reversal of age-associated decline in immune responsiveness by dietary glutathione supplementation in mice. Mechanisms of ageing and development, 38(2), 107-117.

Geldof A.A., Campbell M.J., Pomegranate extracts potently suppress proliferation, xenograft growth, and invasion of human prostate cancer cells, J. Med. Food, 7, 274 (2004).

Gil, M. I., Tomás-Barberán, F. A., Hess-Pierce, B., Holcroft, D. M., & Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food chemistry*, *48*(10), 4581-4589.

González-Ortiz, M., Martínez-Abundis, E., Espinel-Bermúdez, M. C., & Pérez-Rubio, K. G. (2011). Effect of pomegranate juice on insulin secretion and sensitivity in patients with obesity. Annals of Nutrition and Metabolism, 58(3), 220-223. doi:10.1159/000330116

Grossmann, M. E., Mizuno, N. K., Schuster, T., & Cleary, M. P. (2010). Punicic acid is an ω-5 fatty acid capable of inhibiting breast cancer proliferation. International journal of oncology, 36(2), 421-426.

Gülçin, İ. (2012). Antioxidant activity of food constituents: An overview. Archives of Toxicology, 86(3), 345-391. doi:10.1007/s00204-011-0774-2

Heber, D., Schulman, R. N., & Seeram, N. P. (Eds.). (2006). Pomegranates: ancient roots to modern medicine. CRC press.

Hmid, I., Hanine, H., Elothmani, D., & Oukabli, A. (2018). The physico-chemical characteristics of Morrocan pomegranate and evaluation of the antioxidant activity for their juices. Journal of the Saudi Society of Agricultural Sciences, 17(3), 302-309.

Holland, D., Hatib, K., & Bar-Ya'akov, I. (2009). 2 Pomegranate: Botany, Horticulture, Breeding. *Horticultural reviews*, *35*(2), 127-191.

https://www.idf.org/aboutdiabetes/what-is-diabetes/facts-figures.html

https://www.idf.org/our-network/regions-members/middle-east-and-north-africa/diabetes-in mena.html

https://www.who.int/diabetes/country-profiles/lbn_en.pdf?ua=1

Incedayi, B., Tamer, C. E., & Çopur, Ö. U. (2010). A research on the composition of pomegranate molasses. Journal of Agricultural Faculty of Uludag University, 24(2), 37-47.

Ismail, T., Sestili, P., & Akhtar, S. (2012). Pomegranate peel and fruit extracts: a review of potential anti-inflammatory and anti-infective effects. *Journal of ethnopharmacology*, *143*(2), 397-405

Jacobo-Velázquez, D. A., & Cisneros-Zevallos, L. (2009). Correlations of antioxidant activity against phenolic content revisited: a new approach in data analysis for food and medicinal plants. *Journal of Food Science*, *74*(9), R107-R113.

Jafri MA, Aslam M, Javed K, Singh S. 2000. Effect of Punica granatum Linn. (flowers) on blood glucose level in normal and alloxan-induced diabetic rats. J Ethnopharmacol 70(3): 309–14.

Joseph, M. M., Aravind, S. R., Varghese, S., Mini, S., & Sreelekha, T. T. (2012). Evaluation of antioxidant, antitumor and immunomodulatory properties of polysaccharide isolated from fruit rind of Punica granatum. Molecular medicine reports, 5(2), 489-496.

Kalaycıoğlu, Z., & Erim, F. B. (2017). Total phenolic contents, antioxidant activities, and bioactive ingredients of juices from pomegranate cultivars worldwide. *Food chemistry*, *221*, 496-507.

Kallithraka, S.,Salacha, M.I.,Tzourou,I.,2009. Changes in phenolic composition and antioxidant activity of white wine during bottle storage: Accelerated browning test versus bottle storage. Food Chemistry 113 (2), 500–505.

Kam, A., Li, K. M., Razmovski-Naumovski, V., Nammi, S., Shi, J., Chan, K., & Li, G. Q. (2013). A comparative study on the inhibitory effects of different parts and chemical constituents of pomegranate on α-Amylase and α-Glucosidase. Phytotherapy Research, 27(11), 1614-1620. doi:10.1002/ptr.491

Kamal, Y. T., Alam, P., Alqasoumi, S. I., Foudah, A. I., Alqarni, M. H., & Yusufoglu, H. S. (2018). Investigation of antioxidant compounds in commercial pomegranate molasses products using

matrix-solid phase dispersion extraction coupled with HPLC. *Saudi Pharmaceutical Journal*, *26*(6), 839-844.

Kamtekar, S., Keer, V., & Patil, V. (2014). Estimation of phenolic content, flavonoid content, antioxidant and alpha amylase inhibitory activity of marketed polyherbal formulation. *Journal of applied pharmaceutical Science*, *4*(9), 61.

Kasimetty SG, Bialonska D, Reddy MK, Thornton C, Willett KL, Ferreira D (2009). Effects of pomegranate chemical constituents/intestinal microbial metabolites on CYP1B1 in 22Rv1 prostate cancer cells. J. Agric. Food Chem., 57: 10636-10644.

Kasimsetty SG, Bialonska D, Reddy MK, Ma G, Khan SI, Ferreira D. 2010. Colon cancer chemopreventive activities of pomegranate ellagitannins and urolithins. J Agric Food Chem 58: 2180–2187.

Kawaii, S., & Lansky, E. P. (2004). Differentiation-promoting activity of pomegranate (Punica granatum) fruit extracts in HL-60 human promyelocytic leukemia cells. Journal of medicinal food, 7(1), 13-18.

Kern, J. K., Geier, D. A., Adams, J. B., Garver, C. R., Audhya, T., & Geier, M. R. (2011). A clinical trial of glutathione supplementation in autism spectrum disorders. Medical science monitor: international medical journal of experimental and clinical research, 17(12), CR677.

Kevers, C.,Falkowski,M.,Tabart,J.,Defraigne,J.O.,Dommes,J.,Pincemail,J., 2007. Evolution of antioxidant capacity during storage of selected fruits and vegetables. Journal of Agricultural and Food Chemistry 55, 8596–8603.

Kim, N. D., Mehta, R., Yu, W., Neeman, I., Livney, T., Amichay, A., Lansky, E. (2002). Chemopreventive and adjuvant therapeutic potential of pomegranate (punica granatum) for human breast cancer. Breast Cancer Research and Treatment, 71(3), 203-217. doi:10.1023/A:1014405730585

Kinany, K. E., Huybrechts, I., Kampman, E., Boudouaya, H. A., Hatime, Z., Deoula, M. M. S., Rhazi, K. E. (2019). Concordance with the world cancer research Fund/American institute for cancer research recommendations for cancer prevention and colorectal cancer risk in morocco: A large, population-based case-control study. International Journal of Cancer, 145(7), 1829-1837. doi:10.1002/ijc.32263

Kritzinger, E. C., Bauer, F. F., & Du Toit, W. J. (2013). Influence of yeast strain, extended lees contact and nitrogen supplementation on glutathione concentration in wine. Australian Journal of Grape and Wine Research, 19(2), 161-170. doi:10.1111/ajgw.12025

Lan, Y., Wu, J., Wang, X., Sun, X., Hackman, R. M., Li, Z., & Feng, X. (2017). Evaluation of antioxidant capacity and flavor profile change of pomegranate wine during fermentation and aging process. Food Chemistry, 232, 777-787. doi:10.1016/j.foodchem.2017.04.030

Lansky, E. P., Harrison, G., Froom, P., & Jiang, W. G. (2005). Pomegranate (Punica granatum) pure chemicals show possible synergistic inhibition of human PC-3 prostate cancer cell invasion across Matrigel™. Investigational new drugs, 23(2), 121-122.

Lavigne, V., Pons, A., & Dubourdieu, D. (2007). Assay of glutathione in must and wines using capillary electrophoresis and laser-induced fluorescence detection. Changes in concentration in dry white wines during alcoholic fermentation and aging. Journal of Chromatography. A, 1139(1), 130.

Lee, S., Lu, M., Chien, L., Wu, T., Huang, L., & Liao, G. (2013). Suppression of urinary bladder urothelial carcinoma cell by the ethanol extract of pomegranate fruit through cell cycle arrest and apoptosis. BMC Complementary and Alternative Medicine, 13(1), 364-364. doi:10.1186/1472-6882-13-364

Legua, P., Forner-Giner, M. Á., Nuncio-Jáuregui, N., & Hernández, F. (2016). Polyphenolic compounds, anthocyanins and antioxidant activity of nineteen pomegranate fruits: A rich source of bioactive compounds. Journal of Functional Foods, 23, 628-636.

Les, F., Arbonés-Mainar, J. M., Valero, M. S., & López, V. (2018). Pomegranate polyphenols and urolithin A inhibit α-glucosidase, dipeptidyl peptidase-4, lipase, triglyceride accumulation and adipogenesis related genes in 3T3-L1 adipocyte-like cells. *Journal of ethnopharmacology*, *220*, 67-74.

Li, X., Wasila, H., Liu, L., Yuan, T., Gao, Z., Zhao, B., & Ahmad, I. (2015). Physicochemical characteristics, polyphenol compositions and antioxidant potential of pomegranate juices from 10 Chinese cultivars and the environmental factors analysis. Food chemistry, 175, 575-584.

Li, Y., Wei, G., & Chen, J. (2004). Glutathione: a review on biotechnological production. *Applied microbiology and biotechnology*, *66*(3), 233-242.

Lipid oxidation and antioxidants in food. (2017). European Journal of Lipid Science and Technology, 119(6), 1700170-n/a. doi:10.1002/ejlt.201700170

Lobo, V., Patil, A., Phatak, A., & Chandra, N. (2010). Free radicals, antioxidants and functional foods: Impact on human health. Pharmacognosy Reviews, 4(8), 118-126. doi:10.4103/0973-7847.70902

Loguercio, C., D'Argenio, G., Delle Cave, M., Cosenza, V., Della Valle, N., Mazzacca, G., & Del Vecchio Blanco, C. (2003). Glutathione supplementation improves oxidative damage in experimental colitis. Digestive and Liver Disease, 35(9), 635-641. doi:10.1016/S1590-8658(03)00379-7

Malik, A., & Mukhtar, H. (2006). Prostate cancer prevention through pomegranate fruit. Cell cycle (Georgetown, Tex.), 5(4), 371-373.

Malik, A., Afaq, F., Sarfaraz, S., Adhami, V. M., Syed, D. N., & Mukhtar, H. (2005). Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. Proceedings of the National Academy of Sciences of the United States of America, 102(41), 14813-14818. doi:10.1073/pnas.0505870102

Martínez-Flores, H. E., Garnica-Romo, M. G., Bermúdez-Aguirre, D., Pokhrel, P. R., & Barbosa-Cánovas, G. V. (2015). Physico-chemical parameters, bioactive compounds and microbial quality

of thermo-sonicated carrot juice during storage. *Food Chemistry, 172*, 650-656. doi:10.1016/j.foodchem.2014.09.072

Martinez-Flores, H.E.,Garnica-Romo,M.G.,Bermudez-Aguirre,D.,Pokhrel,P.Mphahlele, R. R., Stander, M. A., Fawole, O. A., & Opara, U. L. (2014). Effect of fruit maturity and growing location on the postharvest contents of flavonoids, phenolic acids, vitamin C and antioxidant activity of pomegranate juice (cv. wonderful). *Scientia Horticulturae, 179*, 36-45. doi:10.1016/j.scienta.2014.09.007

Mathew, A. S., Capel-Williams, G. M., Berry, S. E. E., & Hall, W. L. (2012). Acute effects of pomegranate extract on postprandial lipaemia, vascular function and blood pressure. Plant Foods for Human Nutrition, 67(4), 351-357. doi:10.1007/s11130-012-0318-9

Mehta, R., & Lansky, E. P. (2004). Breast cancer chemopreventive properties of pomegranate (Punica granatum) fruit extracts in a mouse mammary organ culture. European Journal of Cancer Prevention, 13(4), 345-348.

Mottaghipisheh, J., Ayanmanesh, M., Babadayei-Samani, R., Javid, A., Sanaeifard, M., Vitalini, S. (2018). Total anthocyanin, flavonoid, polyphenol and tannin contents of seven pomegranate cultivars grown in Iran. Department of Agricultural and Environmental Sciences, Milan State University, Italy. Acta Scientiarum Polonorum. Technologia Alimentaria, 17(3), 211-217. doi:10.17306/J.AFS.0584

Naim, M., Schutz, O., Zehavi, U., Rouseff, R. L., & Haleva-Toledo, E. (1997). Effects of orange juice fortification with thiols on p-vinylguaiacol formation, ascorbic-acid degradation, browning, and acceptance during pasteurization and storage under moderate conditions. Journal of Agricultural and Food Chemistry, 45(5), 1861-1867.

Nasser, G., Sabbah, A., Chokeir, N., Hijazi, A., Rammal, H., & Issa, M. Chemical composition and antioxidant capacity of Lebanese molasses pomegranate.

Nayak B, Liu RH, Tang J. Effect of Processing on Phenolic Antioxidants of Fruits, Vegetables, and Grains—A Review. Crit Rev Food Sci Nutr. 2015; 55(7):887–919.

Nuncio-Jáuregui, N., Calín-Sánchez, A., Carbonell-Barrachina, A., & Hernández, F. (2014). Changes in quality parameters, proline, antioxidant activity and color of pomegranate (Punica granatum L.) as affected by fruit position within tree, cultivar and ripening stage. Scientia Horticulturae, 165, 181-189.

Nuncio-Jáuregui, N., Nowicka, P., Munera-Picazo, S., Hernández, F., Carbonell-Barrachina, Á. A., & Wojdyło, A. (2015). Identification and quantification of major derivatives of ellagic acid and antioxidant properties of thinning and ripe Spanish pomegranates. Journal of functional foods, 12, 354-364.

Onsekizoglu, P. (2013). Production of high quality clarified pomegranate juice concentrate by membrane processes. Journal of Membrane Science, 442, 264-271. doi:10.1016/j.memsci.2013.03.061

Orak HH. Evaluation of antioxidant activity, colour and some nutritional characteristics of pomegranate (Punica granatum L.) juice and its sour concentrate processed by conventional evaporation. Int J Food Sci Nutr. 2009; 60(1):1–11.

Orak, H. H. (2009; 2008). Evaluation of antioxidant activity, colour and some nutritional characteristics of pomegranate (punica granatum L.) juice and its sour concentrate processed by conventional evaporation. International Journal of Food Sciences and Nutrition, 60(1), 1-11. doi:10.1080/09637480701523306

Öztan T. Antioxidant Activities And Phenolic Substance Profile Of Purple Carrot, Its Concentrate, Shalgam Beverage, Pomegranate Juice And Sour Pomegranate Concentrate

Panth, N., Manandhar, B., & Paudel, K. R. (2017). Anticancer activity of Punica granatum (pomegranate): a review. *Phytotherapy Research*, *31*(4), 568-578.

Pantuck, A. J., Leppert, J. T., Zomorodian, N., Aronson, W., Hong, J., Barnard, R. J., ... & Heber, D. (2006). Phase II study of pomegranate juice for men with rising prostate-specific antigen following surgery or radiation for prostate cancer. Clinical Cancer Research, 12(13), 4018-4026.

Parsaeyan, N., Mozaffari-Khosravi, H., & Mozayan, M. R. (2012). Effect of pomegranate juice on paraoxonase enzyme activity in patients with type 2 diabetes. Journal of Diabetes and Metabolic Disorders, 11(1), 11-11. doi:10.1186/2251-6581-11-11

Patel, C., Dadhaniya, P., Hingorani, L., & Soni, M. G. (2008). Safety assessment of pomegranate fruit extract: acute and subchronic toxicity studies. *Food and Chemical Toxicology*, *46*(8), 2728-2735.

Patel, R. M. (2013). Ferrous ion chelating activity (FICA)-a comparative antioxidant activity evaluation of extracts of eleven naturally growing plants of Gujarat, India. *International Journal of Scientific Research*, *2*(8), 426-428.

Prabakaran, M., Lee, J., Ahmad, A., Kim, S., Woo, K., Kim, M., & Chung, I. (2018). Effect of storage time and temperature on phenolic compounds of soybean (glycine max L.) flour. *Molecules (Basel, Switzerland), 23*(9), 2269. doi:10.3390/molecules23092269

R., Barbosa-Canovas,G.V.,2015.Physico-chemical parameters: bioactive Samad, M., Hashim, S., Simarani, K., & Yaacob, J. (2016). Antibacterial properties and effects of fruit chilling and extract storage on antioxidant activity, total phenolic and anthocyanin content of four date palm (phoenix dactylifera) cultivars. *Molecules (Basel, Switzerland), 21*(4), 419-419. doi:10.3390/molecules21040419

Rettig, M. B., Heber, D., An, J., Seeram, N. P., Rao, J. Y., Liu, H., ... & Mo, D. (2008). Pomegranate extract inhibits androgen-independent prostate cancer growth through a nuclear factor-κB-dependent mechanism. *Molecular Cancer Therapeutics*, *7*(9), 2662-2671.

Richie Jr, J. P., Richie Jr, J. P., Nichenametla, S., Nichenametla, S., Neidig, W., Neidig, W., . . . Muscat, J. E. (2015). Randomized controlled trial of oral glutathione supplementation on body

stores of glutathione. European Journal of Nutrition, 54(2), 251-263. doi:10.1007/s00394-014-0706-z

Sabanci, S., Cevik, M., Cokgezme, O. F., Yildiz, H., & Icier, F. (2019). Quality characteristics of pomegranate juice concentrates produced by ohmic heating assisted vacuum evaporation. *Journal of the Science of Food and Agriculture*, *99*(5), 2589-2595.

Saric, G., Markovic, K., Major, N., Krpan, M., Ursulin-Trstenjak, N., Hruskar, M., & Vahcic, N. (2012). Changes of antioxidant activity and phenolic content in acacia and multifloral honey during storage. *Food Technology and Biotechnology, 50*(4), 434-441.

Seeram NP, Adams LS, Henning SM, et al. (2005). In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols

Seeram, N. P., Aronson, W. J., Zhang, Y., Henning, S. M., Moro, A., Lee, R. P., ... & Pantuck, A. J. (2007). Pomegranate ellagitannin-derived metabolites inhibit prostate cancer growth and localize to the mouse prostate gland. J. of Agricultural and Food Chemistry, 55(19), 7732-7737.

Shahidi, F., & Zhong, Y. (2015). Measurement of antioxidant activity. Journal of Functional Foods, 18, 757-781. doi:10.1016/j.jff.2015.01.047

Shema-Didi, L., Kristal, B., Sela, S., Geron, R., & Ore, L. (2014). Does Pomegranate intake attenuate cardiovascular risk factors in hemodialysis patients?. Nutrition journal, 13(1), 18.

Shivsharan, U., & Ravva, S. (2018). Antimicrobial Activity of Pomegranate Juice. Research Journal of Pharmacy and Technology, 11(10), 4329-4331.

Sohrab, G., Angoorani, P., Tohidi, M., Tabibi, H., Kimiagar, M., & Nasrollahzadeh, J. (2015). Pomegranate (punicagranatum) juice decreases lipid peroxidation, but has no effect on plasma advanced glycated end-products in adults with type 2 diabetes: A randomized double-blind clinical trial. Food & Nutrition Research, 59(1), 28551-6. doi:10.3402/fnr.v59.28551

Sohrab, G., Ebrahimof, S., Sotoudeh, G., Neyestani, T. R., Angoorani, P., Hedayati, M., & Siasi, F. (2017). Effects of pomegranate juice consumption on oxidative stress in patients with type 2 diabetes: A single-blind, randomized clinical trial. International Journal of Food Sciences and Nutrition, 68(2), 249-255. doi:10.1080/09637486.2016.1229760

Sohrab, G., Nasrollahzadeh, J., Zand, H., Amiri, Z., Tohidi, M., & Kimiagar, M. (2014). Effects of pomegranate juice consumption on inflammatory markers in patients with type 2 diabetes: A randomized, placebo-controlled trial. Journal of Research in Medical Sciences, 19(3), 215-220

Sohrab, G., Roshan, H., Ebrahimof, S., Nikpayam, O., Sotoudeh, G., & Siasi, F. (2018; 2019). Effects of pomegranate juice consumption on blood pressure and lipid profile in patients with type 2 diabetes: A single-blind randomized clinical trial. Clinical Nutrition ESPEN, 29, 30-35. doi:10.1016/j.clnesp.2018.11.013

Stebbins, N. B., Howard, L. R., Prior, R. L., Brownmiller, C., & Mauromoustakos, A. (2017). Stabilization of anthocyanins in blackberry juice by glutathione fortification. *Food & function*, *8*(10), 3459-3468.

Stover, E. D., & Mercure, E. W. (2007). The pomegranate: a new look at the fruit of paradise. HortScience, 42(5), 1088-1092.

Subash, S., Ph.D., Braidy, N., Ph.D., Essa, M. M., Ph.D., Zayana, A., M.Sc., Ragini, V., Ph.D., Al-Adawi, S., Ph.D. Guillemin, G. J., Ph.D. (2015). Long-term (15 mo) dietary supplementation with pomegranates from Oman attenuates cognitive and behavioral deficits in a transgenic mice model of Alzheimer's disease. Nutrition, 31(1), 223-229. doi:10.1016/j.nut.2014.06.004

Sumner, M. D., M. Elliott-Eller, G. Weidner, J. J. Daubenmier, M. H. Chew, R. Marlin, C. J. Raisin and D. Ornish (2005). Effects of Pomegranate Juice Consumption on Myocardial Perfusion in Patients With Coronary Heart Disease. American Journal of Cardiology 96: 810-814.

Sun, W., Pang, Y., Liu, Y., Hao, H., Zhao, X., Qin, T., . . . Du, W. (2015). Exogenous glutathione supplementation in culture medium improves the bovine embryo development after in vitro fertilization. Theriogenology, 84(5), 716-723. doi:10.1016/j.theriogenology.2015.05.00

Tehranifar, A., Zarei, M., Nemati, Z., Esfandiyari, B., & Vazifeshenas, M. R. (2010). Investigation of physico-chemical properties and antioxidant activity of twenty Iranian pomegranate (Punica granatum L.) cultivars. Scientia Horticulturae, 126(2), 180-185.

Thakur, N. S., Dhaygude, S., & Joshi, V. K. (2013). Development of wild pomegranate aril-in-syrup and its quality evaluation during storage. *International Journal of Food and Fermentation Technology*, *3*(1), 33-40.

van der Sluis, A. A., Dekker, M., & van Boekel, M. A. (2005). Activity and concentration of polyphenolic antioxidants in apple juice. 3. Stability during storage. *Journal of agricultural and food chemistry*, *53*(4), 1073-1080.

Vegara, S., Martí, N., Lorente, J., Coll, L., Streitenberger, S., Valero, M., & Saura, D. (2014). Chemical guide parameters for Punica granatum cv.'Mollar'fruit juices processed at industrial scale. Food chemistry, 147, 203-208.

Virgen-Carrillo, CA, Miramontes, EHV, Moreno, AGM, Mojica, L., & Castañeda-Saucedo, MC (2018). Pomegranate juice consumption (Punica granatum) and its effect on blood glucose, lipid profile and histology of the pancreas in a model of streptozotocin-induced hyperglycemia. *Latin American Nutrition Archives* , *68* (1).

Viuda-Martos, M., Fernández-López, J., & Pérez-Álvarez, J. A. (2010). Pomegranate and its many functional components as related to human health: a review. Comprehensive Reviews in Food Science and Food Safety, 9(6), 635-654.

Viuda-Martos, M., Pérez-Álvarez, J. A., Sendra, E., & Fernández-López, J. (2013). *In vitro* antioxidant properties of pomegranate (punica granatum) peel powder extract obtained as coproduct in the juice extraction process. *Journal of Food Processing and Preservation, 37*(5), 772-776. doi:10.1111/j.1745-4549.2012.00715.x

Wang, J., Wang, K., Wang, Y., Lin, S., Zhao, P., & Jones, G. (2014). A novel application of pulsed electric field (PEF) processing for improving glutathione (GSH) antioxidant activity. Food chemistry, 161, 361-366.

Wang, L. (2013). Pomegranate juice and pomegranate derived natural products as alternative treatment for cancer progression and metastasis

Wu, P. (2013). Effects of pomegranate extract supplementation on cardiovascular disease risk and physical function in patients with chronic renal failure

Yilmaz, Y., Celik, I., & Isik, F. (2007). Mineral composition and total phenolic content of pomegranate molasses. Journal of Food Agriculture and Environment, 5(3/4), 102.

Zarfeshany, A., Asgary, S., & Javanmard, S. H. (2014). Potent health effects of pomegranate.Advanced Biomedical Research, 3(1), 100-100. doi:10.4103/2277-9175.129371

Zhou, B., Yi, H., Tan, J., Wu, Y., Liu, G., & Qiu, Z. (2015). Anti-Proliferative effects of polyphenols from pomegranate rind (punica granatum L.) on EJ bladder cancer cells via regulation of p53/miR-34a axis. Phytotherapy Research, 29(3), 415-422. doi:10.1002/ptr.5267