Physiochemical Characteristics and Antioxidant Activity of a Locally Manufactured Pomegranate Fruit Juice in North Cyprus

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ABSTRACT

Phytochemical and antioxidant properties of pomegranate juice PJ produced from the Wonderful pomegranate cultivar (*Punica granatum* L.) grown locally in North Cyprus was undertaken. The objective of this research study was to determine the biologically active components present, antioxidant activity, total anthocyanin content etc. of PJ and compare it with the unprocessed Pomegranate fruit juice PF. Antioxidant activity of both samples was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay while pH differential method was used to determine the anthocyanin content.

Results obtained from the qualitative phytochemical screening of locally manufactured PJ confirmed the presence of several phytochemicals such as; tannin, phenol, saponin, flavonoids, in our sample. The pH of the juice increased only slightly when compared to the unprocessed juice with both samples having similar results for titratable acid. Antioxidant activity of PJ was high (94.60%) though slightly lower than that of the PF (95.20%) which shows that PJ possess good antioxidant activity. Total anthocyanin content (10.12 mg cyanidin-3-glucoside / 100 g), flavonoid (58.52 mg quercetin/ 100 g) and calcium (27.81 mg/kg) content was found to be higher in the PJ than the fruit (2.46 mg cyanidin-3-glucoside/ 100 g; 53.44 mg quercetin/ 100 g and 12.46 mg/kg) while the Vitamin C (67.30 mg/kg), total sugar (148.46 g/kg) and potassium (250.04 mg/100g) content was higher in the fruit than the juice (18.70 mg/kg; 139.24 g/kg and 236.14 mg/100g).

Keywords: antioxidant activity, pomegranate, phytochemical screening, colour, anthocyanins

ÖZ

Kuzey Kıbrıs'ta yerel olarak yetiştirilen Wonderful nar çeşidinden (Punica granatum L.) üretilen PJ nar meyvesinin fitokimyasal ve antioksidan özellikleri incelenmiştir. Bu araştırmanın amacı, PJ'nin varolan biyolojik olarak aktif bileşenlerini, antioksidan aktivitesini, toplam antosiyanin içeriğini vb. belirlemek ve PF işlenmemiş Nar meyve suyuyla karşılaştırmaktır. Her iki numunenin antioksidan aktivitesi DPPH (2,2-difenil-1-pikrilhidrazil) tahlili ile belirlenirken, antosiyanin içeriğini belirlemek için pH diferansiyel yöntemi kullanılmıştır. Yerel olarak üretilen PJ'nin nitel fitokimyasal taramasından elde edilen sonuçlar, birkaç fitokimyasal maddenin varlığını doğrulamıştır; Bu maddeler arasında tanen, fenol, saponin, flavonoidler bazılarıdır. Meyve suyunun pH değeri, her iki numune için de benzer sonuçlar göstermiştir. PJ'nin antioksidan aktivitesi, PF'den (% 95.20) biraz daha düşük olmasına rağmen yüksek (% 94.60) bulunmuştur. Bu da PJ'nin iyi antioksidan aktiviteye sahip olduğunu göstermektedir. PJ'de toplam antosiyanin içeriği (10.12 mg siyanidin-3-glukosid / 100 g), flavonoid (58.52 mg kerercin / 100 gr) ve kalsiyumun (27.81 mg / kg) içeriği meyve oranından (2.46 mg siyanidin- C vitamini (67.30 mg / kg), toplam şeker (148.46 g / kg) ve potasyum (250.04 mg / 100 gr) içeriği, C vitamini içeriğinde daha yüksektir. (Meyve suyu (18.70 mg / kg; 139.24 g / kg ve 236.14 mg / 100 g)).

Anahtar Kelimeler: antioksidant aktivite, nar, fitokimyasal tarama, renk, antosiyaninler

DEDICATION

I would like to dedicate this thesis to my parents and siblings for all their care and support during my stay in North Cyprus. They have been a solid presence behind me and pushed me into achieving everything I have achieved till date. It’s a blessing having you all in my life.

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INTRODUCTION

1.1 Background of thesis study

The use of plants termed medicinal plants to cure numerous ailments have been in existence since times past, with many developing countries still relying on these so called medicinal plants for curing different diseases (Wannes and Marzouk, 2016). Research has now proven that the bioactive compounds called phytochemicals which are the secondary metabolites of plants are responsible for the therapeutic and medicinal properties displayed by medicinal plants (Wadood et al., 2013).

Punica granatum L. popularly called pomegranate falls into the category of medicinal plants since it contains similar phytochemicals and has displayed numerous health benefits such as antibacterial, antioxidant, anticancer activities etc. Previous research studies have also shown that all parts of the plant including the bark, leaves, fruit etc. can serve one medicinal purpose or another (Rahmani et al., 2017). A careful survey of previous literature available on pomegranate carried out showed that there was not much work done on commercially produced fruit juices as compared to the unprocessed fruit juice itself (especially in relation to North Cyprus), to check the impact of production and storage on the medicinal properties of the fruit.

Pomegranate is widely grown in North Cyprus because of the climate suitability which is perfect for the fruit development. Hence, our study focused on the phytochemical and antioxidant activity of pomegranate juice manufactured by Alnar Narcilik Ltd. called Alnar Pomi in North Cyprus.

Figure 1: Pomegranate fruit and processed fruit juice

1.2 Medicinal plants

Medicinal plants display therapeutic properties and exhibit beneficial pharmacological impacts on humans (Motaleb, 2011). The use of medicinal plants and its products for curing different ailments in ancient times have been widely reported. In fact, about 70-80% of people in the world today rely mainly on traditional herbal medicine to meet up with their healthcare needs (Wannes and Marzouk, 2016). Medicinal plants have always been a source of raw materials for drug production (Shakya, 2016). An increase in the side effects of synthetically produced drugs and the resistance shown by some of these diseases to already existing drugs has led to a renewed interest in the use of medicinal plants by man either directly or as a source for new drugs (Awad and Awaad, 2017).

1.3 Phytochemicals

The medicinal properties displayed by plants are related to the presence of phytochemicals in them. Phytochemicals are non-nutritive chemical compounds

present in plants that protect them from microbial infections and pest infestations. These phytochemicals occur naturally in medicinal plants and are useful in healing as well as curing several human ailments (Wadood et al., 2013). Over 5000 estimated phytochemicals have been identified till date though a large amount still remain unknown (Hiu, 2003).

Plant phytochemicals are secondary metabolites that are produced as end products of primary metabolism. These secondary metabolites as compared to the primary ones which include; lipids, protein, carbohydrates etc. are not essential for plant survival since they are not required by plants for growth and development. Based on their biosynthetic origin, they are classified broadly as terpenoids, phenolic metabolites and nitrogen containing compounds while their main functions in plants are to; protect the plants, attract pollinators and seed dispersing animals using colour, odour and taste (Irchhaiya et al., 2015).

A classification of the main components of phytochemicals present in plants based on their chemical composition according to (Sharkya, 2016) are;

1. Alkaloids: heterocyclic nitrogen containing compounds. Examples include caffeine, morphine, codeine etc.
2. Glycosides: carbohydrate and non-carbohydrate molecules e.g. cinnamyl acetate, polygalin, amygdalin etc.
3. Polyphenols: flavonoids and phenolic tannins are found in this category. They are aromatic aliphatic rings that contain phenols. Examples are quercetin, flavones, gallic acid, ellagic acid etc.
4. Saponins: sugar attached to triterpene or steroid aglycone. Examples are diosgenin and hecogenin.

5. Terpenes (steroids, carotenoids): long unsaturated aliphatic chains which include compounds like α and β-carotene, lutein, lycopene etc.
6. Anthraquinones: derivatives of phenolic and glyosidic compounds. Examples are luteolin, Rhein, salinos poramide etc.

A brief study of these phytochemicals and their uses is discussed below.

1.3.1 Phenolic compounds

Phenols are compounds that have at least one or more hydroxyl groups attached to an aromatic ring. More than 8000 phenolic structures have been identified in plants (Irchhaiya et al., 2015). These phenolic compounds exhibit numerous biological activities (Kahkonen et al., 1999). They are also called free radical scavengers because of their ability to act as antioxidants. The mechanism of action of these phenolic antioxidants is depicted below (Shahidi and Ambigaipalan, 2015a);

$$R^{\cdot}/RO^{\cdot}/ROO^{\cdot} + AH \rightarrow A^{\cdot} + RH/ROH/ROOH$$

$$RO^{\cdot} / ROO^{\cdot} + A. \rightarrow ROA/ROOA$$

$$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot}$$

1.3.1.1 Flavonoids

Flavonoids are a class of polyphenols that are responsible for; providing colour in plants to attract pollinators, protecting the leaves of plants from UV radiation and fungal pathogens, photosynthesis and respiration control in plants, sex determination etc. (Cushnie and Lamb, 2005), They are divided into subclasses based on their biosynthetic origin; flavones, chalcones, isoflavones, flavanols, flavanones, flavonols and anthocyanidins (Seleem et al., 2017). The basic structure of flavonoid compounds (Figure 2) consists of two benzene rings (A and B) called a flavane nucleus (2-phenyl-benzo[α]pyrane) linked via a heterocyclic pyrane ring (C) (Cushnie and Lamb, 2005). Flavonoids have been reported to display anti-inflammatory, inhibition of enzymes, antioxidant, antimicrobial, antifungal, antiviral

activity etc. (Middleton and Chithain, 1993; Harborne and Baxter, 1999; Li et al., 2000; Harborne and Williams, 2000). Figure 2 shows these subclasses of flavonoids (Tomas-Barberan and Gil, 2008).

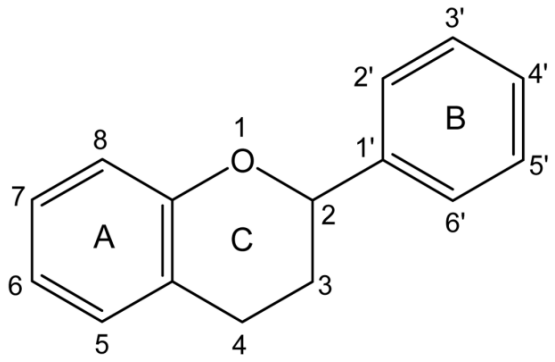

Figure 2: Main structure of flavonoids

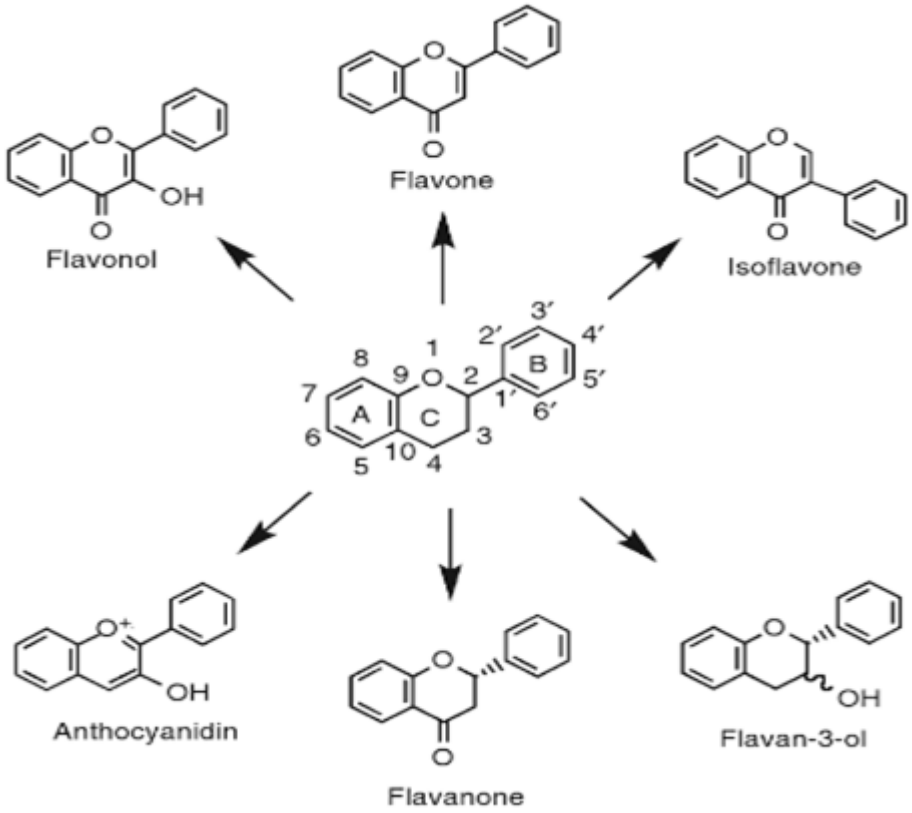

Figure 3: Subclasses of flavonoids based on biosynthetic origin

1.3.1.2 Tannins

Tannins are water soluble polyphenols that are synthesized in high concentrations in the leaves of plants (Chung et al., 1998). They have high molecular weight (from 500 Da to more than 3000 Da) and are divided into two major classes; non-hydrolysable or condensed (also called proanthocyanidins) and hydrolysable tannins (Hassanpour et al., 2011; Barbehenn and Constabel, 2011).

A review on tannins and its impact on human health reported the ability of tannins to form complexes with proteins that made them to be regarded as antinutrients since they reduce protein utilization which results in reduced protein digestibility, feed intake, growth rate in animals etc. though further research was advised to determine the kind and dosage of tannins that is beneficial to human health since tannins were also found to have antioxidant, antimicrobial, anticarcinogenic and antimutagenic activity (Chung et al., 1998; Okuda, 2005).

1.3.2 Saponins

Saponins are important secondary metabolites occurring in a wide variety of plant species that consist of a glycosyl residues (polar sugar molecules) attached to either a triterpene or steroid aglycone (non-polar aglycone called sapogenin) in its chemical structure (Singh et al., 2017). They have a bitter taste and are characterized by their foaming abilities (soap like foams) in aqueous solutions (Haralampidis et al., 2002). Saponin was previously considered to be an undesirable antinutrient present in legumes but recent studies have reported several health benefits (such as; to lower blood cholesterol, inhibit growth of cancer cells and its potential to act as an antifungal and antibacterial agent) associated with it (Shi et al., 2009). Industrial and commercial applications of plant derived saponins include; as food additives, in fire

extinguishers, photographic emulsions, for producing steroid hormones etc. (Balandrin, 1996).

1.3.3 Alkaloids

Alkaloids are naturally occurring organic bases that are predominantly found in plants but can also be found in bacteria, fungi and animals. The first alkaloid discovered was nicotine which was isolated from opium in 1803 by Dersosne. This paved the way for the rapid discovery of more alkaloids from plants (Woolley, 2001). At present, there are more than 18,000 different alkaloids known to man (Dembitsky, 2005). Examples of alkaloids include; caffeine, nicotine, morphine, codeine, quinine etc. Alkaloids protect plants from infection, against toxic by-products of photosynthesis and via inhibition of trehalose and glycosidase metabolism deter herbivores animals (Cushnie et al., 2014). Figure 4 below shows the structure of 2 well known alkaloids; morphine and nicotine.

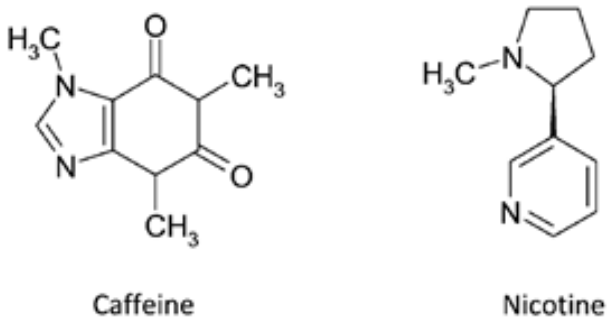

Figure 4: Chemical structure of two alkaloids

There is no single taxonomical principle used to classify alkaloids but they are usually classified according to their origin (either natural or biochemical) or chemical structure (heterocyclic and non-heterocyclic alkaloids) (Hesse, 2002). Even though some alkaloids have been found to be highly toxic and resulted in human poisoning which may cause illness, injury or even death, they have also been found to display

numerous pharmacological properties such as; antimalaria, antitumor, antihypertension, central nervous stimulant etc. (Beyer et al., 2009; Cushnie et al., 2014).

1.3.4 Terpenoids / steroids

Terpenoids are the largest and most diverse family of plant secondary metabolites with about 40,000 identified compounds existing in nature (Haque et al., 2016). Depending on the value of n in their general formula $(C_5H_8)n$, they are classified either as mono, di, oligo and polyterpenes (Sharma et al., 2017). Table 1 below shows the classification of terpenoids based on the number of isoprene units (five carbon structure) forming the parent terpene scaffold (Zhang and Liu, 2015).

Terpenoids have been found to exhibit antifungal (Haque et al., 2016), anticancer activity e.g. drug Taxol, registered name for paclitaxel a diterpenoid (Bohlmann and keeling, 2008; Huang et al., 2012) etc. They have also been used as natural flavouring agents in the food industry, to produce pesticides and disinfectants, in the pharmaceutical industry and as fragrances for the cosmetic industry (Caputi and Aprea, 2011; Bohlmann and keeling, 2008).

Terpenes $C_{10}H_{16}$, a major constituent of essential oils and plant resins are a mixture of terpenoids while Steroids are also subclass of terpenoids and are found in nature either in their free form or as glycosides. Plant steroids are required for plant growth and development. They are also responsible for regulating several traits such as seed germination and yield, flowering time etc. and controlling cell elongation, division and differentiation (Vriet et al., 2015).

Table 1: Classification of terpenoids

Isoprene Units	Name
C_5	Hemiterpenoids e.g. isoprene, methylbutenol etc.
C_{10}	Monoterpenoids e.g. myrcene, limonene, (-)-menthol etc.
C_{15}	Sesquiterpenoids e.g. artemisinin
C_{20}	Diterpenoids e.g. triptolide, andrographolide, pseudolaric acid B etc.
C_{25}	Sesterterpenoids e.g. Ceroplastol, gascardic acid, ophiobolin A etc.
C_{30}	Triterterpenoids e.g. celastrol, cucurbitacins, alisol etc.
C_{40}	Tetraterpenoids e.g. carotenoids,
$> C_{40}$	Polyterpenoids

1.3.5 Glycosides

Glycosides are compounds present in plants that have one or more sugar molecules attached through a glycosidic linkage with a non-sugar moiety or aglycone (usually termed as genin). These aglycone can be a phenol, a complex molecule or alcohol (Hollman, 1985). They are classified based on the aglycone present in the structure. They include but are not limited to; cardiac and cyanogenetic glycosides, alcoholic, anthraquinone, phenolic, saponins etc (Patel, 2016). Some medical benefits previously reported for glycosides are; antimalaria, antiviral, antioxidant, anticancer etc have been reported for different types of glycosides (Iyer et al., 2010; Niu et al., 2016; Hu et al., 2016; Liu et al., 2007).

1.3.6 Vitamin C and mineral content

Vitamin C also known as ascorbic acid, is a water soluble vitamin that possesses antioxidant properties since it reacts directly with reactive oxygen species (ROS) present in the body and controls free radical mediated tissue damage (Koc et al., 2017). Deficiency of vitamin C in the body could result in scurvy, fatigue, gum inflammation, malaise etc. The main source of vitamin C include; fruits (such as orange, pomegranate, strawberry etc.) and vegetables. The amount of vitamin C present in fruits and beverages is considered a quality factor hence, it is of vital importance to monitor the impact of postharvest treatment on the vitamin C content of fruits and beverages since it can easily be destroyed by heat treatment making it highly sensitive to chemical and enzymatic oxidation (Mditshwa et al., 2017).

Minerals on the other hand are essential nutrients that the human body requires but cannot produce. These inorganic substances are found in food and they include; calcium, potassium, magnesium, phosphorus, Iron, zinc, sodium etc. Calcium is an essential nutrient since it is essential for building strong bones and teeth, regulating blood pressure and cholesterol level etc. while potassium helps to break down and use carbohydrates, control electrical activity of the heart, maintain blood pH balance and support normal growth (Soetan et al., 2010).

1.4 Antioxidant activity

High concentration of free radicals in the body causes oxidative stress which is responsible for several health issues such as; diabetes, chronic obesity, cancers, aging, atherosclerosis, cardio vascular diseases etc. associated with humans (Ogutu and Mu, 2017). These radicals which are highly unstable and reactive are produced by enzymatic and non-enzymatic reactions in the cell and also from normal metabolic processes that occur in the human body (Bagchi and Puri, 1998; Liu et al.,

1999). Antioxidants on the other hand are chemical compounds that have the ability to protect cells from free radicals by donating an electron to the free radical so as to neutralize its impacts (Lobo et al., 2010). Most medicinal plants have been shown to possess antioxidant activity due to polyphenolic compounds found in them (Kahkonen et al., 1999). Depending on their mechanism of action, antioxidants can be classified as primary or secondary antioxidants. Primary antioxidants inhibit oxidation chain reaction by donating hydrogen or acting as free radical acceptors and forming more stable radicals while secondary antioxidants prevent oxidation by suppression of oxidation promoters (Shahidi and Zhong, 2015b). Chemical assays used to determine/monitor the antioxidant activity of samples are tabulated in Table 2 below.

Table 2: Assays to determine antioxidant capacity

Chemical Assay	
Radical/ROS (reactive oxygen species) scavenging methods.	1. Oxygen radical absorbance capacity (ORAC) assay 2. Chemiluminescence assay 3. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay 4. Trolox equivalent antioxidant capacity (TEAC) assay

Non-radical redox potential based methods.	1. Ferric reducing antioxidant power (FRAP) assay. 2. Cupric reducing antioxidant capacity (CUPRAC) assay. 3. Cyclic voltammetry
Metal chelating capacity	
Total phenolic content TPC	

1.5 Pomegranate fruit (*Punica granatum* L.)

Pomegranate *Punica granatum* L. a fruit bearing deciduous shrub belongs to the Punicaceae family. The tree is primarily grown in mild-temperate and subtropical regions (e.g. Mediterranean parts) of the world. To date, there are more than 1,000 known cultivars of the fruit (Lansky and Newman, 2007). The edible part of the fruit (50% of the fruit weight) which includes the arils and seeds is either consumed fresh or used to produce other products such as; juice, jam, paste, canned beverages etc. (Fadavi et al., 2005). Pomegranate juice PJ is also used in the food and beverage industry as flavouring and colouring agents and in the cosmetic and healthcare industries as a dye (Al-Said et al., 2009). Research is now focused on having a good knowledge of the fruit characteristics from different cultivars grown in different regions with particular attention paid to the edible parts. This information can be useful for cultivar classification to determine cultivars that can be used in the processing industries and for fresh market consumption (Hasnaoui et al., 2011).

Pomegranate fruits can be classified as; sweet, sour sweet and sour varieties depending on the maturity index MI values (Martinez et al., 2006). The maturity index is determined by dividing the Total soluble solids (TSS reported in °Brix) by the total acidity of the sample expressed as amount of citric acid. Table 3 below shows the MI range used in this classification.

Table 3: MI classification of pomegranate juice

Pomegranate Variety	Maturation Index (TSS/TA)
Sweet	31-98
Sour sweet	17-24
Sour	5-7

Numerous medical benefits and antioxidant activity associated with the consumption of pomegranate fruit and its products have made it become one of the most studied fruit in the world today. The fruit, peel, seeds and leaves of pomegranate tree possess several bioactive compounds that have therapeutic roles in the cure for many diseases (Rahmani et al., 2017). Orgil et al. found that the so called non-edible parts of the fruit and tree including the bark, leaves, seeds, peel etc. contained more biologically active compounds than the edible fruit itself with higher antiproliferative activity (Orgil et al., 2014). These important pharmacologic compounds which are responsible for the medicinal properties shown by pomegranate including their chemical class, compound name and structure can be found in a review by (Lansky and Newman, 2007). This confirms that pomegranate falls into the category of medicinal plants.

1.5.1 Physical characteristics and medicinal benefits of pomegranate fruit

Bioactivities displayed by pomegranate fruit has been attributed to the high amount of antioxidant polyphenols present (Fawole et al., 2011). Physiochemical characteristics, phytochemical analysis, pharmacological actions, morphology, antioxidant, antibacterial, antimicrobial studies etc. of pomegranate has been investigated by many researchers. A brief literature review will be undertaken in the preceding paragraphs to access the previous studies undertaken and the results obtained from each research work.

Many studies have reported several variations observed in the physiochemical composition of pomegranate cultivars studied to date. These variations could be related to factors such as; storage conditions, climate, region where it is grown and cultural practices (Aarabi et al., 2008).

Al.maiman and Ahmad carried out a study to determine the change in the physical and chemical properties of pomegranate fruit during maturation. The study revealed that seed content, pH, glucose and fructose content of the fruit increased while titratable acidity, ascorbic acid and polyphenol content reduced during maturation (Al.maiman and Ahmad, 2002).

Aarabi et al checked the impact of twenty-five different cultivars and storage on the organic acid composition of pomegranate fruit in Iran. Eleven major acids (citric, malic, succinic, tartaric, acetic, oxalic, shikimic, maleic, fumaric, tartaric and ascorbic) were identified using HPLC while significant differences were obtained in the organic acid content of the cultivars. Also, after a 60 days storage period at 4 °C of the juice obtained from three out of the twenty-five cultivars studied, the total

amount of organic acids in all three cultivars decreased which would have an impact on the organoleptic properties of the pomegranate juice during storage (Aarabi et al., 2008). Similar research carried out on the sugar and organic acid content of pomegranate fruits in Tunisia found a strong correlation between the citric acid content and sourness in taste of pomegranate (Hasnaoui et al., 2011).

Characterization of twenty pomegranate cultivars grown in Spain was undertaken to determine if the fruit could be consumed directly, processed industrially or used for medicinal purposes. The physiochemical and mineral analysis, total phenol content and antioxidant activity of all samples showed that they are significant differences in the cultivars studied. The researchers concluded that cultivars that could be used consumed directly had soft seeds; hard seeds with intense red colour for juice production while those with high antioxidant activity and total phenol content, crude fiber, minerals like potassium could serve as functional foods in cosmetic or medical industries (Alcaraz-armol et al., 2017).

Morphological, biochemical characteristics and antioxidant activity of eighteen Moroccan pomegranate juice was done to determine the quality of the fruits produced locally and compare them with foreign cultivars. All local cultivars showed variation in their morphology and high antioxidant activity (4577.12 ± 29.73 mg/ L of juice) which was due to the high phenolic content (1384.85-9476.32 mg gallic acid equivalent GAE/L) when compared with foreign cultivars. This could be related to the variability in climatic conditions (Hmid et al., 2016).

Previous study by Gil and colleagues have shown pomegranate juice to display higher antioxidant activity than red wine and green tea which are well known

antioxidant. This study was also able to relate the antioxidant activity of the juice to the presence of different phenolic compounds (anthocyanin, ellagic acid, hydrolysable tannins and punicalagins). Arrangement of antioxidant capacity with respect to the phenolic compounds shows that; punicalagins > hydrolysable tannins > anthocyanin > ellagic acid (Gil et al., 2000). Several other studies have also reported the antioxidant activity of different cultivars of pomegranate (Barzargani-Gilani et al., 2014; Fawole et al., 2011).

The antimicrobial activity of fresh pomegranate juice was examined against clinical strains of *Staphylococcus epidermidis*. Results showed that the juice had a minimum inhibitory concentration MIC of 100% at concentration of 20% which was attributed to its antioxidant capacity and high phenol content (Betanzos-Cabrere et al., 2015). The arils of six pomegranate varieties from turkey was also tested against seven different bacteria and three fungi. All arils of pomegranate studied had antimicrobial effects on all microorganisms under study which confirmed the antimicrobial potential of pomegranate fruit (Duman et al., 2009). Zoreky also tested the antimicrobial activity of various extracts of pomegranate peels towards several food-borne pathogens and found some positive results (Zoreky, 2009).

Kim et al., researched the potential of pomegranate juice and oils (mainly the polyphenols present) in treating human breast cancer. Results obtained showed the potential of this fruit to exert multiple suppressive effects in vitro on breast cancer cells (Kim et al., 2010). Pomegranate fruit can also be used to prevent and treat prostate cancer (Bell and Hawthorne, 2008). Other studies have also been reported to show the anti-cancer properties exhibited by pomegranate fruits and its derivatives (Hora et al., 2003; Lansky et al., 2005; Malik and Mukhtar 2006).

Other reported uses of pomegranate include; treatment of type 2 diabetics (Banihani et al., 2013), cardiovascular diseases (Sestili et al., 2002), enhancing spermatogenic cell density and sperm quality (Turk et al., 2008), atherosclerosis (Al Jarallah et al., 2013) etc.

1.6 Aim of Research study

Much work has been focused on the analysis of pomegranate fruit and its cultivars with only few research related to the commercially produced and sold pomegranate juices. The aim of this thesis is to fill this knowledge gap by focusing on the phytochemical screening and antioxidant activity of a locally produced pomegranate fruit juice in North Cyprus. The phytochemical analysis of our sample will be used to determine the presence of several biologically active compounds such as tannins, saponins, phenols etc. present in the sample. A comparism will also be done to evaluate the impact of thermal pasteurization of pomegranate fruit on the total acidity, pH, antioxidant activity, colour and mineral content of the locally produced pomegranate juice.

1.7 Research outline

The first chapter of this thesis work will focus on the introduction, literature review and aim of undertaking this study of locally manufactured pomegranate juice from a juice producing factory in North Cyprus. Chapter 2 will contain the experimental section where all chemicals and experimental procedures used in our analysis will be outlined. The third chapter will focus on the results obtained from all analysis carried out and discussion. Finally, Chapter 4 concludes the whole study and gives recommendations based on results obtained.

Chapter 2

EXPERIMENTAL

2.1 Materials and methods

Pomegranate juice used for all analysis was kindly supplied to us by Alnar Narcilik Ltd. (ALNAR POMI), North Cyprus. Reagent grade chemicals; Iron (III) Chloride, sodium hydroxide, hydrochloric, acetic and sulfuric acid, chloroform, iodine was all supplied by Sigma Aldrich while Potassium iodide, sodium carbonate was purchased from Alfa Aeser was used for all analysis carried out. The pH value of the sample was measured at room temperature using a wtw–intolab-ph/conductivity 720 meter. All chemicals were used without further purification, solutions were prepared using distilled water while experiments were conducted in triplicates with the average results/observations reported.

2.2 Preparation of pomegranate juice

Ripe pomegranate fruits of the Wonderful cultivar were collected from several farms located in North Cyprus in November 2016. The arils in the fruit were separated from the skin by manually peeling off the fruit skin before extracting the juice, PF with the aid of a juice extractor. Pomegranate juice sold commercially by the company was produced by thermal pasteurization at 72 °C for 15 seconds before fast cooling to 4 °C. The pomegranate juice obtained, PJ was packaged in airtight bottles (1 Litre) and stored at 4 °C before further use.

2.3 Phytochemical analysis/screening of pomegranate juice

Different phytochemical tests were carried out to determine the presence of various biologically active chemicals present in PJ. These tests adapted from Andrianin et al. (2015) and Nwokonkwo (2014) were based on the principle that the functional groups present in the sample will either form precipitates or lead to colour changes when they come in contact with specific reagents.

2.3.1 Tannin and phenol test

This test is based on the colour change observed (black or bluish green) when tannins and phenols present in the sample react with Iron (III) salts. To carry out this test, 2 mL of the PJ in a test tube was diluted to 5 mL using distilled water. Then, about 2-3 drops of 1M Iron (III) chloride solution was added to the already prepared PJ solution.

2.3.2 Saponin test

Two separate tests were conducted to determine the presence of saponin in PJ. For the first test, 2 mL of water was added to 2 mL of PJ sample in a test tube and shaken vigorously while for the second test, same volume of PJ was mixed with 2 mL of olive oil and also shaken vigorously. The formation of froths or foams on shaking signify the presence of saponins in our sample.

2.3.3 Flavonoids test

5 mL of dilute NaOH solution was added to 1 mL of PJ in a test tube. The solution was then made acidic (verified with the use of a litmus paper) by adding slightly concentrated HCl. The formation of precipitates was used as a basis to test the presence of flavonoids since flavonoids form precipitates in acidic solutions.

2.3.4 Steroids test

To determine the presence of steroids in our sample, 2 mL of chloroform was mixed with 2 mL of our sample (PJ) in a separating funnel. 2 mL of concentrated sulfuric acid was then carefully added to this solution before shaking the separating funnel. The organic layer was removed and evaporated until it became completely dry using a water bath. 5 mL of concentrated sulfuric acid was added and the mixture was heated for about 10 minutes in the water bath and allowed to cool to room temperature. Any change in colour observed would be used to determine the presence of steroids in PJ.

2.3.5 Alkaloids test

To carry out this test, we prepared the Wagner's reagent by dissolving 0.50g of Iodine and 1.5g of Potassium Iodide in 25 mL of distilled water. 2 mL of this reagent was then mixed with 2 mL of our sample in a test tube. The presence of alkaloids is confirmed by the formation of a coloured precipitate.

2.3.6 Cardio-active glycosides test

Two separate tests (Lieberman and Salkowski Test) were used to identify the presence of Cardio-Active Glycosides in PJ based on observable colour changes in the sample solution. For the Lieberman test, 2 mL of acetic acid was added to 0.5 mL of our sample before cooling it in an ice bath. Afterwards, 3 drops of concentrated sulfuric acid was added. To carry out the Salkowski test, 2 mL of chloroform was added to the same volume of PJ used in the Lieberman test followed by the addition of 1 mL 5% sulfuric acid solution. Any colour change observed in both tests was noted.

2.3.7 Carboxylic acid test

Test was carried out using a litmus paper. Blue litmus paper was dipped into a solution of PJ and resulting colour change (to red) was observed.

2.3.8 Ester test

0.5 mL of concentrated sulfuric acid was carefully added to a warm solution of 1 mL PJ and 2 mL 95% ethanol and allowed to cool down. 5 mL of aqueous sodium carbonate was added to the ensuing mixture and transferred to an evaporating dish. The sweet-smelling odour characteristics of esters was used to determine the presence of esters in our sample.

2.4 Total anthocyanin content determination

This was determined according to a method obtained in literature by (Fawole et al., 2011) known as the pH differential method. 1 mL of already prepared PJ was mixed with 9 mL of two different buffers (KCl buffer solution; pH 1.0 and CH_3COONa buffer solution; pH 4.5) and the absorbance of both samples was taken at 520 and 700 nm with a T80+ UV-vis spectrophotometer (Beijing, version 5.0).

$$A = (A_{520}-A_{700})\ pH1.0 - (A_{520}-A_{700})\ pH4.5 \quad (1)$$

The total anthocyanin content of PJ and PF determined from equation 3 was expressed in mg/100 mL of cyanidin-3-glucoside present in the sample.

$$TAC = \frac{A*M*DF*100}{MA} \quad (2)$$

Where A, is the sample absorbance

M (449.2 g/mol) is the molecular weight

MA (26,900) represents the molar absorptivity of cyanidin-3-glucoside

DF is the dilution factor (10).

2.5 Vitamin C content determination

Vitamin C (also known as ascorbic acid) content of PJ and PF was determined by titrating the sample with an indicator dye 2,6- dichlorophenol indophenol according to a method proposed in the standard Official Method of Analysis (AOAC 2000). Vitamin C content was then expressed in mg/kg of sample.

2.6 Total flavonoid determination

The absorbance of a mixture containing 0.25 mL PJ (and PF for the fruit), 0.075 mL of 5% sodium nitrite, 0.150 mL of 10% aluminium chloride, 0.5 mL of 1M NaOH and 0.775 mL of distilled water was measured using a spectrophotometer at wavelength of 510 nm (Fawole et al., 2011). Total flavonoid content was expressed in mg of quercetin equivalent/kg (mg QE/kg sample).

2.7 Antioxidant activity determination

Free scavenging activity of the both samples was determined by the rate of inhibition of DPPH (2,2-diphenyl-1-picrylhydrazyl) radical. To carry out this procedure, 0.735 mL of methanol was used to dilute 0.015 mL of PJ under dim light before adding 0.750 mL of methanolic DPPH solution. The mixture was kept in the dark at ambient temperature for about half an hour before reading the absorbance at 517 nm. Free radical scavenging activity (antioxidant index in %) was calculated using equation 1 below and scavenging activity was also expressed as ascorbic acid equivalent per millilitre of PJ i.e. mM/mL of PJ sample (Fawole et al., 2011).

$$\text{Free radical scavenging activity (\%)} = \frac{Ac - As}{Ac} * 100 \qquad (3)$$

Where A_s is the sample absorbance and A_c is the absorbance of blank control

2.8 pH measurement and colour determination

The pH of PJ and PF was measured directly with the aid of a pH meter (wtw–intolab-ph/conductivity 720 meter) at ambient temperature. Colour determination of PJ was undertaken using a Color Quest XE spectrophotometer based on the CIELAB co-ordinates (L *, a *, b *). L is a measure of the relative lightness (white has a value of 100) or darkness (black has a value of 0) of the PJ. On the a-axis (i.e. a*), the colour runs from green to red with a negative "**a**" value indicative of how much green the sample is while positive value indicates more red. Positive b* values on the other hand signals more yellow while negative signals more blue. From these three-dimensional co-ordinates, the colour intensity or saturation C * and the hue angle H° can be calculated from equations 2 and 3 below.

$$C^* = (a^{*2} + b^{*2})^{1/2} \qquad (4)$$

$$H° = \arctan (b^*/a^*) \qquad (5)$$

2.9 Total acidity determination

10 mL of PJ was diluted using 190 mL of water and homogenized for about 2 minutes. 50 mL aliquot of this solution was then titrated with 0.1 M NaOH solution to an end-point of 8.2 using phenolphthalein as indicator (Al- said et al., 2009). Total titratable acidity was expressed as g of citric acid CA/100g of sample.

Chapter 3

RESULTS AND DISCUSSION

3.1 Phytochemical analysis

The results obtained from the physiochemical analysis of PJ is shown in Table 4 below.

Table 4: phytochemical screening of PJ

Phytochemical Analysis	Observation
Tannins	+
Saponins	+
Flavonoids	+
Steroids and triterpenoids	-
Cardio-active glycosides	+
Phenols	+
Carboxylic acids	+
Esters	+
Alkaloids	+

+: present -: not present

As seen from the table above, PJ contains several bioactive compounds such as tannins, saponins, carboxylic acids, esters, alkaloids flavonoids, phenols and cardio-active glycosides while no steroids were found. Similar bioactive chemicals are also present in the pomegranate fruit in different quantities depending on the cultivar, region, storage conditions and influence of climatic conditions (Opara et al., 2009; Al-Said et al., 2009; Aarabi et al., 2008). The presence of this bioactive compounds as said earlier discussed, has been discovered to be responsible for the medicinal

properties displayed by several plants and fruits. For example, the antioxidant and antimicrobial activity of the leaves and barks of three species of *Alnus* was related to the high amount of phenols and flavonoids present (Dahija et al., 2014). Another Study also carried out on the shoot of *Limonium delicatulum* reported that the phenolic compounds present (phenols, flavonoids and tannins) in the sample was responsible for its antioxidant and antimicrobial activity (Medini et al., 2014). Since similar compounds are present in both the PF and juice in varying amounts, hence it is expected that the both should exhibit similar characteristics. This observation has been supported by several studies as reported in our literature survey; antioxidant activity of pomegranate juice and fruit (Hmid et al., 2016; Tehranifar et al., 2010a, b), antimicrobial activity of fruit, freshly prepared and commercial juice (Duman et al., 2009; Betanzos-Cabrera et al., 2015; Al-Zoreky 2009), antimutagenic activity of pomegranate peel extract (Negi et al., 2003), pomegranate seed and oil used for therapeutic purposes to control diabetes (McFarlin et al., 2009), antibacterial (Braga et al., 2005), antimalaria (Reddy et al., 2007), anti-cancer (Kim et al., 2010) etc. Hence, consumption of PJ is beneficial to human health.

3.2 pH, total acidity and colour determination

The pH of PJ and PF was found to be 3.24 and 3.20 respectively. This reported pH of the PF just about falls into the required pH range (2.93-3.20) for edible arils of pomegranate by the US Food and Drug Administration (FDA 2007). pH values obtained in our study for both PJ and PF was within the range (2.75 - 4.14) reported by Akbarpour et al. (2009) and (2.76-4.03) by Al Said et al. (2009) for cultivars found in Iran and Oman respectively. The variation in pH observed is due to several factors like; maturity, post-harvest handling and type of cultivar (Opara et al., 2009).

Total acidity reported as the amount of Citric Acid CA (g CA / 100 g) found in both samples was 0.67 for the fruit and 0.61 for the juice respectively. This shows that there was a slight decrease in the acidic content of PJ as compared to the unprocessed fruit juice which was also reflected in the slightly higher pH value of the PJ. Values obtained fall within the range of those reported by other researchers (Tehranifar et al., 2010a; Melgarejo et al., 2000) for Iranian and Spanish cultivars but higher than that obtained by Fawole et al. (2011) for 3 cultivars grown in South Africa. Lower citric acid content in PJ implies that it should taste better than the unprocessed one (Hasnaoui et al., 2011).

Table 5: Colour determination of PF and PJ

CIELAB colour index	Unprocessed pomegranate fruit PF	Processed pomegranate Juice PJ
L*	45.57	20.12
a*	28.03	41.86
b*	32.01	32.47
C*	42.54	52.98
Hue*	48.79	37.80

Results obtained from the colour determination of processed and unprocessed fruit juices is tabulated in Table 5. As seen from the table, processing the fruit juice reduced the hue angle and lightness L* of the pomegranate indicating that the colour of the processed pomegranate juice becomes darker and tends towards more red. An increase in a* value of PJ as compared to the fruit also signifies higher red coloration while there was no significant change in the b* values of both samples. Similar results were obtained by Guo et al, when using Pulse electric field instead of the

thermal processing method to produce pomegranate juice (Guo et al., 2014). The colour intensity C* of the juice was also higher than that obtained in the fruit.

3.3 Vitamin C and mineral content

Vitamin C content of PJ was found to be 18.70 mg/kg PJ sample. This is far lower than the vitamin C content obtained for the unprocessed fruit juice (67.30 mg/kg). This could be as a result of the oxidation of the ascorbic acid present during the thermal processing of the fruit. In fact, vitamin C present in fruits is usually affected by storage time and conditions, high temperature, low relative humidity and post-harvest handling (Lee and kader, 2000). Vitamin C content obtained for both samples is still higher than that determined by Fadavi (2005) for all 10 pomegranate cultivars studied in Iran though it was lower than that found by Opara (2009) for five pomegranate varieties in Oman.

The concentration of two minerals (calcium and potassium) out of the several important minerals present in pomegranate was determined for both the processed and unprocessed pomegranate fruit juice. Calcium content in the processed juice (2.781 mg/100g) was found to be higher than that of unprocessed juice (1.246 mg/100g) while the reverse was observed in the case of the potassium present (236.1 mg/ 100g in PJ and 250.2 mg/100g in fruit). Potassium content of our samples was higher than that reported by Fadavi et al. though calcium content was found to be lower (Fadavi et al., 2005). High potassium content of our sample is in agreement with previous studies which identify potassium as the most abundant mineral found in pomegranate (Alcaraz-Marmol et al., 2017, Fadavi et al., 2005).

3.4 Total Anthocyanin content

The anthocyanin content of both PF and PJ was determined using the pH differential method. Studies have confirmed the antioxidant activities displayed by anthocyanins (Seeram and Nair, 2002). PJ was found to have a far higher anthocyanin content (101.20 mg cyanidin-3-glucoside/kg) when compared to that of PF (24.60 mg/kg). Stability of anthocyanin is influenced by several factors like temperature, light, pH and oxygen (Jaiswal et al., 2010). Our result showing the anthocyanin content in PJ to be higher than that for PF is similar to that obtained by Albarici and Pessoa (anthocyanin content in Açaí fruit increased by about 42%) which showed that pasteurization i.e. processing can result in higher anthocyanin content in processed fruit juices. The reason for this increase was ascribed to the evaporation of water during pasteurization and lower degradation during the thawing process (Albarici and Pessoa, 2012). Our result also agrees with what we obtained from the colour determination of both samples which showed that a* value of PJ (which is related to "more red") was higher than that of PF since the redness depends on the concentration of anthocyanin (Hernandez et al., 1999). Total anthocyanin content in PF was lower than that reported elsewhere (Fawole et al., 2011; Tehranifar et al., 2010b) while that of the juice was lower than that reported by (Bazargani-Gilani et al., 2014).

3.5 Antioxidant activity

The antioxidant activity of our PJ and PF was evaluated using the DPPH radical scavenging assay. The degree of discoloration of the mixture is indicative of the scavenging potential of PJ (Tehranifar et al., 2010b). In our study, we found that the PJ showed a high total antioxidant capacity of 94.60% which was comparable to that of the unprocessed fruit juice (95.20%). This shows that thermal treatment of the

pomegranate fruit did not have any serious impact on its antioxidant ability. The antioxidant activity of both the fruit and juice is relatively higher than that reported by (Tehranifar et al., 2010b; Hmid et al., 2016) but comparable to that of (Fawole et al., 2011) for different cultivars of pomegranate. Hence, both samples displayed good antioxidant capabilities.

3.6 Total flavonoid

Flavonoids in general have been found to exhibit several medicinal properties such as; antioxidant, anti-inflammatory, antiallergic, enzyme inhibition, anti-carcinogenic effects etc. (Cushnie and Lamb, 2005). It can also be bactericidal to some bacteria strains (Chikezie et al., 2015). The Total flavonoid content of PJ (585.17 mg quercetin equivalent/kg) was found to be higher than that of the fruit (534.40 mg/kg). Quercetin has been reported to inhibit lung cancer cell growth (Yang et al., 2006). A study carried out by Ohemeng and his colleagues showed that quercetin which is found in considerable amount in our sample can inhibit the DNA gyrase of *E. coli* hence exhibiting antibacterial properties (Ohemeng et al., 1997). Quercetin from propolis was also found to show antifungal activity by inhibiting the development of *Candida spp* (Herera et al., 2010). The presence of this specific flavonoid in the PJ shows that PJ can also possess all the above listed properties though more study will be required to isolate all the flavonoids present in PJ apart from quercetin and determine the chemical and pharmacological impact of each one of them separately.

Chapter 4

CONCLUSION

A comprehensive study was undertaken to determine the phytochemical compounds and antioxidant activity of a locally manufactured pomegranate fruit juice. Our results confirmed the presence of several bioactive compounds such as; tannins, saponins, alkaloids, flavonoids, phenols, carboxylic acids and esters in the pomegranate juice while steroids were not found. Antioxidant activity of both processed and unprocessed pomegranate juice was found to be higher than 90%. This shows that adequate consumption of both the processed and unprocessed fruit juice would display several health benefits. Similar antioxidant activity, total acidity, pH value was determined for both of the samples which showed that thermal pasteurization method used by the manufacturers did not have any major impact on the PJ. The colour intensity and redness of our processed fruit juice sample was higher than the unprocessed fruit juice which could be an indication of the increase in the total anthocyanin content of our sample. This was confirmed in the total anthocyanin test carried out which showed an increase of about 300% anthocyanin in the processed fruit juice when compared to the pomegranate fruit juice itself. The vitamin C content of the processed fruit juice (1.87 mg/100 g) was considerably lower than that of the unprocessed one (6.73 mg/100 g).

Conclusively, both samples (PF and PJ) showed good antioxidant potential and it is advisable to incorporate PJ into diet but more study is required to determine the

chemical constituents and content of the different chemically active compounds present in the processed pomegranate juice (isolate, identify and characterize) so as to be able to relate the bioactive chemicals directly with the antioxidant or medicinal properties displayed. Also, the antimicrobial, anticancer, antiviral and anti-inflammatory activity of the processed fruit juice coupled with the impact of packaging and long-term storage on the bioactive components of PJ can also be investigated.

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APPENDIX

Appendix A: Results obtained from analysis of processed and unprocessed pomegranate fruit juice

	Unprocessed fruit juice PF	Processed fruit juice
Total sugar (g/kg)	148.46	139.24
Vitamin C (mg/Kg)	67.30	18.70
Total anthocyanin (mg/Kg) (cyanidin-3-glucoside)	24.60	101.20
Total acidity (g/100g) (citric acid content)	0.67	0.61
pH	3.20	3.24
Total flavonoid (mg/Kg) (quercetin content)	534.40	585.17
Antioxidant activity (%) (0.1 g/L DPPH radical reduction ratio)	95.20	94.60
Calcium	12.46	27.81
Potassium	2502.38	2361.38
Colour		
L*	45.57	20.12
a*	28.03	41.86
b*	32.01	32.47
C*	42.54	52.98
Hu*e	48.79	37.80

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