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Prospective, randomized, double-blind, placebo-controlled study of oral pomegranate extract on the microbiome and skin biophysical properties

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PROSPECTIVE, RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED STUDY OF ORAL POMEGRANATE EXTRACT ON THE MICROBIOME AND SKIN BIOPHYSICAL PROPERTIES

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DEDICATION

I would like to dedicate this work to my family and research team at Integrative Skin Science and Research.

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I would like to extend my gratitude toward Dr. Sivamani, who has been such a wonderful mentor to me since I joined his lab in August of 2020. I would also like to thank my colleagues at Integrative Skin Science and Research for cheering me on throughout the study recruitment, data collection, analysis, and writing process for my research thesis. Finally, I would like to thank my family for their support, especially my sisters Milisha, Milinda, and Meghan, and my friend Ervin Irimpan for being such a positive and motivating force in my life.

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ABSTRACT

For several centuries, *Punica granatum L.* has been utilized in traditional medicine to treat various ailments including diabetes, diarrhea, and parasitic and microbial infections. Current scientific research suggests that the unique phytochemical composition of the botanical is largely responsible for the range of biological activities demonstrated by the different components of *P. granatum* L. The objective of this study was to evaluate the effects of the fruit extract of *P. granatum* L. on the following parameters of skin health: facial wrinkle severity, sebum production, transepidermal water loss, melanin index, and erythema index. The hypothesis was that oral pomegranate supplementation improves the facial appearance of wrinkles and the biophysical properties of the skin because of the well-studied antioxidant and anti-inflammatory properties of the phytochemicals in the fruit extract.

A prospective, double-blind, placebo-controlled study was conducted in which subjects were supplemented daily with a punicalagin-enriched oral pomegranate extract for four weeks. The study was performed at Integrative Skin Science and Research (Sacramento, California, USA) and included 18 healthy males and females, aged 25–55 years, who were asked to restrict the intake of other polyphenolic-rich foods. A facial image collection and analysis system was used to assess the facial appearance of wrinkle

severity, and standardized, noninvasive tools were used to collect information on facial sebum production, transepidermal water loss, melanin index, and erythema index. In addition, self-reports of quality of life and digestive health were collected from the subjects. These parameters were assessed at pretreatment baseline and after four weeks of oral supplementation.

The results showed statistically significant reductions in the melanin index of the cheeks ($p < 0.01$), transepidermal water loss from the stratum corneum of the cheeks ($p < 0.01$), and facial appearance of average wrinkle severity ($p < 0.01$). Furthermore, a significant reduction was found in facial transepidermal water loss among females of the intervention group ($p < 0.01$). No adverse effects were reported over the four weeks of the study.

In conclusion, this study demonstrated an improvement in several biophysical properties of the skin with oral pomegranate extract supplementation. As a result, oral pomegranate extract could be used as an effective agent for the maintenance of skin appearance and skin health.

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LIST OF ABBREVIATIONS

ABTS•+..... 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate)

BCE.....before the common era

CLA..... conjugated linoleic acid

CLnA..... conjugated linolenic acid

DPPH 1,1-diphenyl-2-picrylhydrazyl

HHDP.....hexahydroxydiphenoyl

HPLC .....high performance liquid chromatography

MMP-1.....matrix metalloproteinase 1

MPO..... myeloperoxidase

NADPH..... nicotinamide adenine dinucleotide phosphate

P. granatum.....*Punica granatum*

P. protopunica.....*Punica protopunica*

PA ..... punicic acid

PE..... pomegranate extract

POP ..... pomegranate peel

PPOP..... polysaccharides of the pomegranate peel

PSO .....pomegranate seed oil

PSP001..... galactomannan polysaccharide

TEWL .....transepidermal water loss

TNFα.....tumor necrosis factor alpha

CHAPTER 1: INTRODUCTION

Pomegranates are the fruit of a long-lived deciduous shrub or small tree that belongs to the botanical family *Punicaceae*. *Punicaceae* gives rise to a singular genus, *Punica*, which comprises two distinct species, *Punica granatum* L. *(P. granatum* L.)and *Punica protopunica (P. protopunica). P. granatum* L. is the predominant species that bears the pomegranate fruit and is native to Iran and the Himalayas of Northern India (Ge et al., 2021; Karimi et al., 2017; Viuda-Martos et al., 2010). *P. granatum* L. is also cultivated in many other parts of the world including the Mediterranean, West Indies, tropical Africa, China, Japan, and the arid to semi-arid regions of Southeast Asia, the United States, and Russia (Viuda-Martos et al., 2010). *P. protopunica,* an isolated species endemic to the Socotra archipelago, produces fruits that are smaller in size and less sweet than the pomegranate (Guerrero-Solano et al., 2020). Because of the significant morphological differences between the fruits produced by the two species, this paper only examines the components of *P. granatum* L. in detail.

Since ancient times, *P. granatum* L. was well known for its healing properties and was regarded as a symbol of well-being, fertility, and immortality among various cultures (Braga et al., 2005; Ge et al., 2021; Karimi et al., 2017). In fact, its first documented use appears in the Ebers' papyrus in 1500 BCE as an Egyptian remedy for parasitic infections (Braga et al., 2005). In addition, different components of *P. granatum* L. were commonly used across traditional Chinese, Persian, Ayurvedic, and Mongolian medicine to treat acidosis, diarrhea, diabetes, hemorrhage, and parasitic and microbial infections (Ge et al., 2021; Karimi et al., 2017). Although the positive health effects of *P. granatum* L. were

well regarded in different parts of the world, the scientific evidence for their application in medicine was still lacking for a significant period of time.

With the advancement of scientific analytic techniques, the phytochemical composition of *P. granatum* L. was soon determined. As a result, an abundance of bioactive compounds such as amino acids, fatty acids, minerals, alkaloids, polyphenols, and vitamins were identified in different regions of the plant (Table 1). Shortly thereafter, scientific studies began to suggest an association between the phytoconstituents of the botanical and its highly touted health benefits. This finding was of significant relevance, especially in the past decade, when there appeared to be a growing interest in the utility of nutraceuticals and functional foods for the maintenance of health and the prevention of disease (Dable-Tupas et al., 2020). As more studies supported the efficacy of these alternative modalities in the management of disease, including minimal side effects, global research efforts steadily rose to identify and study more of these substances. This led to the eventual establishment of *P. granatum* L. and, most important, the pomegranate as a potent functional food and nutraceutical (Dable-Tupas et al., 2020; Sreekumar et al., 2014).

Because many studies have established an association between the observed health benefits of *P. granatum* L. and its phytoconstituents, the next section of this paper evaluates this relationship in detail. Specifically, the phytochemical composition of the fruit of *P. granatum* L. is analyzed. The pomegranate fruit is further divided into the peel, arils, and seeds. It is also important to note that there may be significant differences in the reported phytochemical composition of the botanical in this paper. This may be due to

differences in growing, cultivation, extraction, and storage practices among *P. granatum* L. cultivars around the world (Singh et al., 2018; Viuda-Martos et al., 2010).

Table 1

Phytochemical Composition of the Pomegranate Peel, Arils, and Seeds

Component	*Phytochemicals*	*Reference*
(1) Peel	*(A) Phenolic Acids* *Chlorogenic acid, caffeic acid, syringic acid, sinapic acid, p-coumaric acid, ferulic acid, vanillic acid, ellagic acid, cinnamic acid* *(B) Flavonoids* *Catechin, epicatechin, quercetin, rutin, myricetin, kaempferol, luteolin, apigenin, anthocyanins, procyanidins* *(i) Anthocyanins* *Cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, pelardonidin-3-glycoside, cyanidin 3-glucoside, cyanidin-3-rutinoside, delphinidin 3-glucoside, delphinidin-3,5-diglucoside, cyanidin hexoside, cyanidin pentoside, cyanidin 3-pentoside* HHDP-hexoside, pedunculagin I, pedunculagin II, bis-HHDP-gluconic acid (lagerstannin A), castalagin derivative, galloyl-bis-HHDP-hexoside (casuarinin) derivative, punigluconin, lagerstannin C, HHDP-hexoside *(D) Minerals* Potassium, nitrogen, calcium, phosphorous, magnesium, sodium	*Ge et al., 2021; Mayasankaravalli et al., 2020; Mirdehghan & Rahemi 2007; Jahfar et al., 2003; Singh et al., 2018; Zhai et al., 2018*

Component	Phytochemicals	Reference
	(E) Alkaloids Piperidine and pyrrolidine alkaloids (*F) Simple and Complex Polysaccharides* Glucuronic acid, galactouronic acid, glucose, mannose, ribose, rhamnose, xylose, galactose, arabinose, fucose	
(2) Arils	*(A) Sugars* Fructose, glucose, pectin *(B) Organic acids* Ascorbic acid, citric acid, malic acid *(C) Flavonoids* Quercetin, catechin *(i) Anthocyanins* Cyanidin-3-glucoside	Duman et al., 2009; Ghasemnezhad et al., 2015; Ricci et al., 2006; Viuda-Martos *et al*., 2010
(3) Seeds	*(A) Fatty acids* Punicic acid, phytosterols (beta-sitosterol, campesterol, stigmasterol), α-elostearic acid, catalpic acid, oleic acid, palmitic acid, conjugated linoleic acid, stearic acid, arachidic acid, eicosenoic acid	Boussetta et al., 2009; Rosenblat et al., 2013; Spilmont et al., 2013; Topkafa et al. 2015; Vroegrijk et al., 2011; Zarfeshany et al., 2014

Peel

The pomegranate peel (POP) comprises half of the total weight of the fruit, with the remaining weight distributed to the seeds (40%) and the arils (10%) (Viuda-Martos et al., 2010). Until recently, the POP was predominantly considered a waste product because it is part of the inedible portion of *P. granatum* L. (Malviya et al., 2014; Singh et

al., 2018). With the rise in research on the therapeutic potential of the different components of *P. granatum*, it was discovered that the POP is especially rich in compounds known as polyphenols (Karimi et al., 2017; Orak et al., 2012; Singh et al., 2018).

Polyphenols are compounds that largely consist of the secondary metabolites of plants and, to a lesser extent, animals (Pandey & Rizvi, 2009). They are more commonly known as the naturally occurring micronutrients found in green leafy vegetables, fruits, tea, coffee, dark chocolate, and red wine (Scalbert et al., 2005; Singh et al., 2018; Tsao, 2010). Structurally, polyphenols are loosely classified as compounds with phenol groups. Phenol groups have the molecular formula C_6H_5OH and are made up of an aromatic ring containing a hydroxyl group that is attached to a carbon backbone (Arts & Hollman, 2005). Despite this categorization, polyphenols encompass a wide range of compounds of varying structural and functional properties, with more than 8,000 distinct compounds identified today (Pandey & Rizvi, 2009; Tsao, 2010). More important, polyphenols are of considerable interest because of their ability to influence or directly participate in biological activities.

Many in vitro and in vivo studies have demonstrated polyphenolic antioxidant and pro-oxidant activity (Pandey & Rizvi, 2009; Scalbert et al., 2005; Tsao, 2010). The antioxidant potential of polyphenols is evidenced by their ability to scavenge free radicals, which are highly reactive compounds formed from endogenous metabolic events or from exposure to harmful exogenous agents such as environmental pollutants and industrial chemicals (Lobo et al., 2010). Unchecked levels of free radicals in the human

body may accelerate cell aging or lead to an accumulation of cell damage and progression to cancer. Polyphenols are also known to directly participate in or modulate biosignaling pathways which result in lower total oxidative stress and enhanced cell survival. On the other hand, the pro-oxidant activity of polyphenols is associated with the initiation of controlled cell death, or apoptosis, which has shown promising results in the reduction of tumor growth (Scalbert et al., 2005). In addition, a polyphenolic-rich diet has been associated with a lower risk of chronic illnesses such as cancer, diabetes, osteoporosis, and various cardiovascular and neurodegenerative diseases (Arts & Hollman, 2005; Scalbert et al., 2005; Tsao, 2010). Because of the numerous health benefits associated with these micronutrients, the polyphenolic contents of the POP, together with other components of the *P. granatum* L. peel, are described in detail in the remainder of this section.

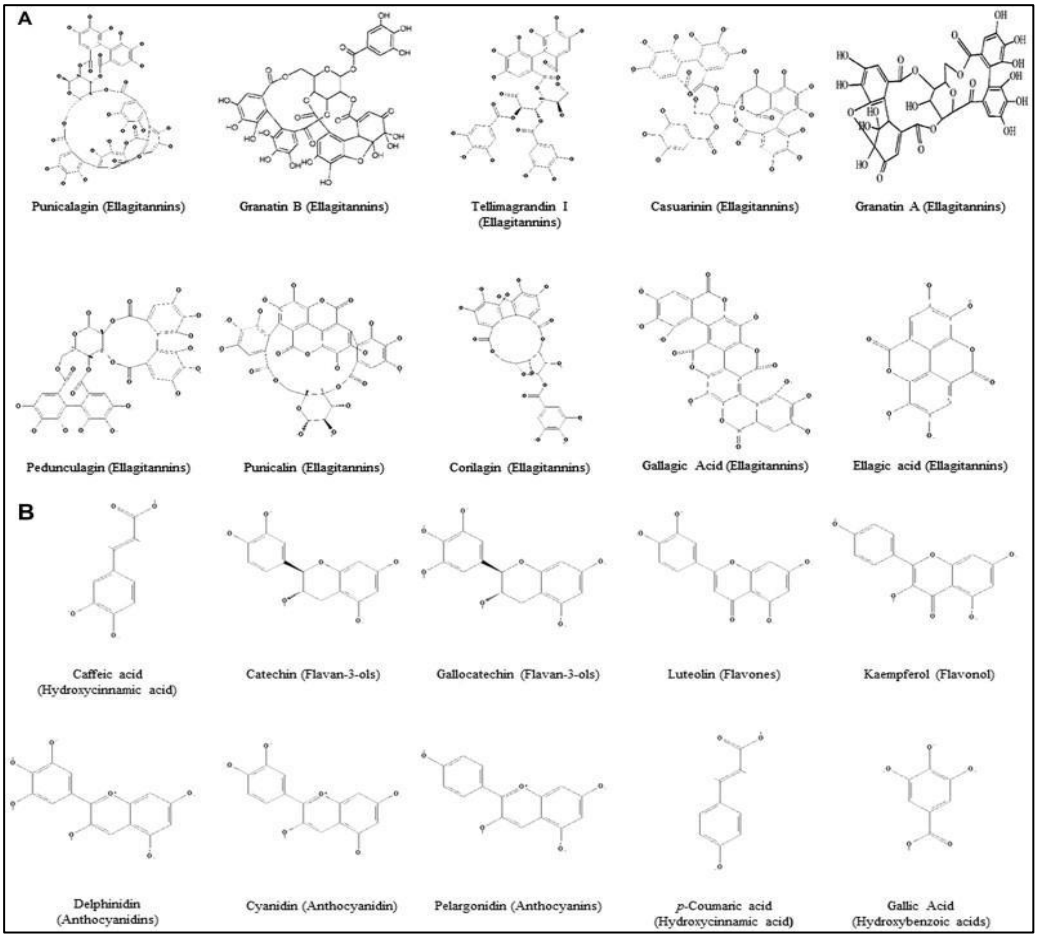
Previous studies have suggested that the POP contains the largest source of polyphenols compared with the other anatomical regions of *P. granatum* L. (Karimi et al., 2017; Orak et al., 2012; Singh et al., 2018). The POP is composed of three major classes of polyphenols: phenolic acids, flavonoids, and tannins. Phenolic acids are structurally classified as low-molecular-weight compounds with simple structures (Zanotti et al., 2015). They contain a phenol ring fused to a carbon backbone and are the derivatives of benzoic acid and cinnamic acid (Tsao, 2010; Zanotti et al., 2015). The phenolic acids found in the POP include chlorogenic acid, caffeic acid, syringic acid, sinapic acid, *p*-coumaric acid, ferulic acid, vanillic acid, ellagic acid, and cinnamic acid (Table 1.1A, Figure 1B).

Flavonoids are another class of polyphenols that contain two phenol rings fused to a carbon backbone with a C6-C3-C6 general structure (Tsao, 2010). Flavonoids are further divided into anthocyanins, flavan-3-ols, flavones, and flavonols based on differences in the attachment point of the phenol ring to the carbon backbone and in the hydroxylation patterns. The flavonoids found in the POP include catechin, epicatechin, quercetin, rutin, anthocyanins, and procyanidins (Table 1.1B, Figure 1B).

Anthocyanins are an important subset of flavonoids because they are responsible for the red pigmentation of the POP and seeds. Previous studies have reported that the POP contains 30% of the total anthocyanins found in *P. granatum* L. (Azarpazhooh et al., 2019; Singh et al., 2018). Interestingly, a diet rich in anthocyanins is associated with anti-inflammatory, antioxidant, and chemoprotective effects (de Pascual-Teresa & Sanchez-Ballesta, 2008). Other studies have also elucidated their antimicrobial properties (Silva et al., 2017). Nevertheless, additional studies must be conducted before a definitive relationship can be established between the anthocyanins isolated from the POP and different markers of health. The common anthocyanins found in the POP include cyanidin 3,5-diglucoside, pelargonidin 3,5-diglucoside, pelargonidin-3-glucoside, cyanidin 3-glucoside, cyanidin-3-rutinoside, delphinidin 3-glucoside, delphinidin-3,5-diglucoside, cyanidin hexoside, cyanidin pentoside, and cyanidin 3-pentoside (Table 1.1Bi, Figure 1B).

Figure 1

Chemical Structures of the Major Polyphenols Found in Pomegranate Peel



Note. This figure shows the chemical structures of the major polyphenols found in pomegranate peel: (A) tannins and (B) phenolic acids and flavonoids. From "Pomegranate Peel and Peel Extracts: Chemistry and Food Features," by S. Akhtar, T. Ismail, D. Fraternale, and P. Sestili, 2015, *Food Chemistry*, *174*, p. 419 (https://doi.org/10.1016/j.foodchem.2014.11.035). Copyright 2014 by Elsevier Ltd.

Tannins are the third class of polyphenols in POP. They are characterized as high-molecular-weight polyphenols weighing from 500 to 3000 Da (Smeriglio et al., 2017).

Tannins are readily detectable by taste receptors and are responsible for the slightly acidic or bitter taste of certain fruits and vegetables such as the pomegranate. The number of hydroxyl groups within the compound can be a predictor of the strength of its astringency. Tannins are structurally divided into hydrolysable tannins, such as the esters of gallic acid and ellagic acid, and condensed tannins, such as proanthocyanidins (Rauf et al., 2019). Unlike hydrolysable tannins, condensed tannins are not readily hydrolysable, lack a sugar moiety, and share structural features with flavonoids (Rauf et al., 2019; Smeriglio et al., 2017). It is important to note that hydrolysable tannins, such as ellagitannins, and ellagic acid derivatives, such as punicalagin, punicalin, and pedunculagin, are among the most abundant polyphenols found in the POP (Karimi et al., 2017; Singh et al., 2018). Interestingly, the POP was utilized to treat diarrhea for centuries and is still currently prescribed for this ailment in traditional Chinese medicine and the Dai and Miao medicines of Southern China, primarily because of the health benefits associated with this specific class of polyphenols (Ge et al., 2021). The common hydrolysable tannins found in the POP include punicalagin (hexahydroxydiphenoyl [HHDP]-gallagyl-hexoside), HHDP-gallagyl-hexose isomers (α-and β-punicalagin), punicalin (gallagyl-hexoside), ellagic acid hexoside, ellagic acid-deoxyhexoside, ellagic acid pentoside, granatin A, granatin B, corilagin, gallagyldilactone, tellimagrandin, galloyl-HHDP-hexoside, pedunculagin I, pedunculagin II, bis-HHDP-gluconic acid (lagerstannin A), castalagin derivative, galloyl-bis-HHDP-hexoside (casuarinin) derivative, punigluconin, lagerstannin C, and HHDP-hexoside (Table 1.1C, Figure 1A).

The POP also contains various minerals such as potassium, nitrogen, calcium, phosphorous, magnesium, and sodium (Table 1.1D); alkaloids such as piperidine and pyrrolidine alkaloids (Table 1.1E); and simple and complex polysaccharides (Table 1.1F). A 2018 study established that the predominant sugars found in the POP were glucuronic acid, galacturonic acid, glucose, and xylose (Zhai et al., 2018). These sugars exhibited in vivo 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS•+), and reactive oxygen species scavenging activity. In another study, the antioxidant potential of the polysaccharides of the POP (PPOP) was associated with a decrease in carbon tetrachloride-induced oxidative damage to the liver of in vivo mouse models (Joseph et al., 2012. These results broadly suggest hepatoprotection by the POP.

Joseph et al. (2013) also isolated galactomannan polysaccharide (PSP001) from the POP and demonstrated its free radical scavenging and anticancer and immunomodulatory activities in animal models. In regard to its anticancer properties, PSP001 exhibited the ability to stimulate apoptosis in cancer cells without inducing in vivo toxicity, a characteristic which makes it a great therapeutic candidate in the treatment of cancer. Furthermore, a 2014 study concluded that different PPOP, especially arabinose and xylose, demonstrated a high glucose retardation index (Hasnaoui et al., 2014). The PPOP, subsequently, can be utilized to induce a lower insulin response in humans. These study findings may also provide a rationale for the prescription of the POP as an antidiabetic agent in traditional Unani and Chinese medicine (Hasnaoui et al., 2014; Singh et al., 2018). Although these results are promising, future studies should

evaluate the different activities of the PPOP in human studies to expand the current understanding of the therapeutic potential.

The wound-healing properties of the POP have also been widely reported. For example, a 2011 study described improvements in several markers of the cutaneous wound-healing process, such as re-epithelization, wound contraction, and tensile strength, with the topical application of an ointment containing 5% methanolic extract of the POP in guinea pigs (Hayouni et al., 2011). This study also reported the presence of anthocyanins and ellagitannins using high-performance liquid chromatography (HPLC) fingerprint analysis. In another study, the topical application of a gel containing polyphenols derived from an ethanol extract of the POP resulted in the acceleration of epithelialization, fibroblast proliferation, and subsequent collagen deposition in the cutaneous wound-healing process in diabetic rats (Yan et al., 2013). The penetration of gallic acid, a phenolic acid, was reported to be 36.95% over a period of 24 hours. In both studies, it is important to note that the results were partially attributed to the potent antioxidant and anti-inflammatory activities of the polyphenols contained within the topical formulations.

In conclusion, although the POP is largely considered a waste product, recent studies have demonstrated its status as a phytochemical reservoir and provided compelling evidence for its clinical application for various conditions.

Arils

The pomegranate fruit contains around 600 arils which are the edible seed pods that encapsulate the seeds (Zarfeshany et al., 2014). The arils are used to prepare pomegranate juice and are made up of 85% water, 10% sugars, and 1.5% organic acids (Viuda-Martos et al., 2010). More specifically, the arils contain sugars such as fructose, glucose, and pectin; organic acids such as ascorbic acid, citric acid, and malic acid; and flavonoids such as quercetin, catechin, and anthocyanins such as cyanidin-3-glucoside (Ghasemnezhad et al., 2015; Viuda-Martos et al., 2010). The phytochemicals of the pomegranate arils are summarized in Table 1.2A–C.

A 2006 study established the total antioxidant capacity (measured by DPPH and superoxide free radical scavenging activity) and suppressive effects of different extracts of pomegranate arils on lipid peroxidation (Ricci et al., 2006). Moreover, the arils of *P. granatum* L. exhibited potent antimicrobial and antifungal activities. For example, in a study by Duman et al. (2009), a pomegranate aril extract created inhibition zones measuring 13–26 mm for seven bacteria (*Bacillus megaterium*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Corynebacterium xerosis*, *Escherichia coli*, *Enterococcus faecalis*, and *Micrococcus luteus*) and for three fungi (*Kluyveromyces marxianus*, *Rhodotorula rubra*, and *Candida albicans*). The observed in vitro antimicrobial and antifungal activities were associated with the antioxidant activity of the polyphenols constituting the pomegranate arils. Because there is a shortage of scientific studies evaluating the biological activities of pomegranate arils, future studies should examine the therapeutic potential of this component of *P. granatum* L. in more detail.

Seeds

Punicic acid (PA) is a polyunsaturated fatty acid which constitutes 64% to 84% of PSO (Vroegrijk et al., 2011; Zarfeshany et al., 2014). A 2009 study elucidated the potent anti-inflammatory effects of PA as it demonstrated the capacity to inhibit tumor necrosis factor alpha (TNFα)-induced phosphorylation of neutrophil cytosolic factor 1 on phospho-progesterone receptor and cytokinin-specific binding protein in neutrophils (Boussetta et al., 2009). This effectively reduced the priming of reactive oxygen species production by neutrophil nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. This same study showed that PA inhibited the TNFα-induced release of myeloperoxidase (MPO), an enzyme found in the azurophilic granules of neutrophils. The inhibition of MPO release by PA decreased the production of toxic compounds such as hydrochloric acid and chloramines, which are the products formed in the reaction between hydrogen peroxide and halides. Because TNFα-induced phosphorylation events and the release of MPO by neutrophils act synergistically to increase inflammation and because other fatty acids such as linoleic acid also act synergistically with TNFα as pro-inflammatory mediators, PA is unique in that it effectively inhibits inflammatory events at the molecular level. This finding has also been corroborated by the work of Spilmont et al. (2013) in which mice were fed a diet with 5% PSO. The results demonstrated the suppressive effects of PA on the expression of pro-inflammatory markers such as monocyte chemoattractant protein 1, interleukin-1, and interleukin-6. This suppressive capability of PA led to a minimized oxidative stress which was observed in an overall reduction of bone loss in the mouse models.

Furthermore, PSO contains other isomers of CLnA such as α-eleostearic acid and catalpic acid (Vroegrijk et al., 2011). Less than 5% of PSO consists of oleic acid, stearic acid, and palmitic acid, and less than 1% consists of arachidic acid and eicosenoic acid (Topkafa et al., 2015). In addition, PSO contains phytosterols which are steroid compounds that share similar structural features with cholesterol. The three phytosterols found in PSO are β-sitosterol, campesterol, and stigmasterol. The β-sitosterol may contribute to the overall health benefits associated with PSO. This component decreased cholesterol biosynthesis and cellular oxidative stress in murine macrophage cells when used in conjunction with a statin and punicalagin, a polyphenol found in pomegranates (Rosenblat et al., 2013). The phytochemicals of pomegranate seeds are summarized in Table 1.3A.

Pomegranate Fruit Extract

Pomegranate fruit extract is made from the pomegranate peel, arils, and seeds. Because of the significant health benefits associated with the fruit of *P. granatum* L. and its status as a functional food and nutraceutical, there is interest in understanding the effects of oral pomegranate extract (PE) supplementation on various parameters of skin health. To gain insight into these effects, this study assesses the effect of a four-week supplementation of PE on various biophysical properties of the skin, such as facial sebum production, transepidermal water loss, melanin index, erythema index, and pigmentation changes. In addition, this work examines the effect of the four-week supplementation on self-perceived quality of life and digestive health. Because of the abundance of

phytochemicals in the fruit that demonstrate potent antioxidant and anti-inflammatory effects, the four-week oral supplementation should result in the improvement of the biophysical properties of the skin compared with pretreatment baseline values.

SPECIFIC AIMS

The specific aims of this study are:

1. To evaluate changes in the appearance of facial wrinkle severity after four weeks of oral pomegranate extract supplementation.
2. To assess changes in the biophysical properties of the skin through facial sebum production, transepidermal water loss, melanin index, and erythema index after four weeks of oral pomegranate extract supplementation.
3. To investigate the effects of oral pomegranate extract supplementation on self-reports of quality of life and digestive health.

CHAPTER 2: MATERIALS AND METHODS

Study Participants, Study Design, and Intervention

This study was performed between November 2020 and March 2021 as a four-week, double-blind, placebo-controlled clinical trial. The study was conducted with the approval of the Institutional Review Board (IntegReview Ltd., Austin, Texas, USA) and registered at www.clinicaltrials.gov (NCT04596722). All participants provided written informed consent prior to participation in the study. Males and females living in the Sacramento region, aged 25–55 years, were recruited from a local dermatology clinic and through the use of social media advertisements. Individuals were screened for eligibility at Integrative Skin Science and Research (Sacramento, California, USA). Twenty-eight subjects met inclusion criteria and were randomized and preallocated into two groups using a computer-based randomization generator with blinded sealed envelopes. The PE group received an oral supplement (Pomella, Verdure Sciences, Noblesville, Indiana, USA) containing 75 mg of punicalagin per capsule for daily consumption for four weeks. The active ingredient of the oral supplement was extracted from the fruit of *P. granatum* L. which was cultivated in India. The presence of punicalagins in the oral supplement was confirmed by data provided by HPLC. The control group received an oral placebo, similar in appearance to the supplement provided to the PE group but with no active ingredient, for daily consumption for four weeks.

Individuals with a known allergy to the study agents were not included in the study. Subjects were asked to discontinue the use of other nutritional supplements,

including prebiotics and probiotics, one month prior to the baseline visit and throughout the study. The use of topical antibiotics and benzoyl peroxide were not permitted one month prior to the baseline visit and throughout the study. Individuals who had used systemic, injected, or oral antibiotics within six months of the baseline visit or those who had started a new diet like the ketogenic diet within one month of the baseline visit were excluded from the study. Current smokers and individuals who had smoked within the past one year or had a five pack-year history of smoking tobacco were excluded from the study. Individuals with a history of the following conditions were also screened from the study: malignancy; cancer (excluding nonmetastatic cancer); gastrointestinal inflammatory diseases; epilepsy; and immunologic or infectious diseases such as hepatitis, tuberculosis, human immunodeficiency virus, acquired immunodeficiency syndrome, lupus, and rheumatoid arthritis. Other exclusion criteria included individuals currently using medications which alter blood lipids, like statins and antihyperlipidemic medications; individuals with a body mass index higher than 35 kg/m^2; individuals who adhere to the vegan diet; individuals who refuse to shave facial hair which may interfere with image collection and assessment; individuals who are participating in or have participated in an intervention-based facial study prior to two weeks of the baseline visit; women who have been pregnant in the last three months or are currently pregnant, preparing to be pregnant, or lactating; and prisoners and adults who are unable to provide consent on their own.

Subjects enrolled in the study were also requested to adhere to a set of dietary restrictions to reduce the confounding effects of polyphenolic-rich foods. These

individuals were asked to reduce intake to at most one eight-ounce cup of coffee, one cup of berries, and one cup of fermented dairy products per week throughout the duration of the study. This diet also included the complete restriction of chocolate, alcohol, tea, and pomegranates or pomegranate-containing drinks for the four weeks of the study. Subjects were asked to completely avoid self-tanning, spa tanning, sun tanning, artificial tanning, dry or wet sauna treatments, and swimming daily. This was done to reduce the confounding effects of environmental or exogenous agents on the appearance and health of the skin.

Facial Imaging and Measurements of the Biophysical Properties of the Skin

All measurements were collected after subjects had adjusted to ambient conditions for 15 minutes in a climate-controlled room. The appearance of facial wrinkles was assessed using high-resolution facial photographs captured and analyzed by the BTBP 3D Clarity Pro Facial Modeling and Analysis System (BrighTex Bio-Photonics, San Jose, California, USA). The "average severity" of wrinkles was calculated by measuring the depth and the width of the wrinkles. The following biophysical properties of the skin on the forehead and cheeks were measured: facial transepidermal water loss (TEWL) using the Vapometer (Delfin Technologies Ltd., Kuopio, Finland) and facial sebum production by the Sebummeter SM 815 (Courage and Khazaka, Cologne, Germany). The SkinColorCatch (Delfin Technologies Ltd., Kuopio, Finland) was used to measure the facial erythema index and melanin index of the skin. These devices for

assessing the biophysical properties of the skin were noninvasive instruments which contacted the skin for at most 30 seconds.

Subjective Assessments of Quality of Life and Digestive Health

Self-assessments of quality of life and digestive health were collected in separate questionnaires. Both questionnaires were administered at pretreatment baseline and after four weeks of oral supplementation.

The following questions were asked in the Quality of Life Questionnaire (answer choices: 0 = Not at all; 1 = A little bit; 2 = Moderately; 3 = Quite a bit; 4 = Extremely):

(A) How confident in your skin were you before taking the study intervention?

(B) How confident in your skin are you now since taking the study intervention?

(C) Do you feel more confident in social situations since taking the study intervention?

(D)Do you feel more energized since taking the study intervention?

(E)Have you noticed more glowing skin since taking the study intervention?

(F) If you could continue using the study agent after the study, how likely is it that you would?

One additional question was asked in the Quality of Life Questionnaire (answer choices: 1 = Fair; 2 = Good; 3 = Very good; 4 = Excellent):

In general, how would you define your health?

The questions and answer choices for the Digestive Health Questionnaire were as follows:

(A) Over the past month, how many times per day or per week do you usually have a bowel movement? 1 = Every few days; 2 = Every other day; 3 = Once a day; 4 = More than once a day; 5 = Don't know.

(B) Please look at the Bristol Stool Chart and choose a number that corresponds to your usual or most common stool type. 1 = Type 1; 2 = Type 2; 3 = Type 3; 4 = Type 4; 5 = Type 5; 6 =Type 6; 7 =Type 7; 8 = Don't know.

(C) In the past month, did you take any laxatives or stool softeners, such as Ex-Lax, Metamucil, or Fiberall, to help your bowel movements? 1 = No; 2 = Yes; 3 = Don't know.

(D) If you answered yes to the question above, how many times, ON AVERAGE, have you taken laxatives or stool softeners in the past 30 days? 0 = Never; 1 = Less than 3 times; 2 = Once a week; 3 = Most days; 4 = Don't know.

(E) How would you describe your hunger over the last 30 days? 1 = Mild, can generally easily skip meals; 2 = Irregular, varies from meal to meal; 3 = Generally strong, cannot skip meals.

(F) Over the last 30 days, how long has it taken you to feel hungry after a meal? 1 = A few hours, sometimes I forget to eat; 2 = Irregular, varies from meal to meal; 3 = Soon after, I can't skip meals.

(G) Over the last 30 days, what is the amount of food you have noticed that you could eat at one time? 1 = Small, I get full easily; 2 = Varies from meal to meal; 3 = Large, I do not get full easily.

(H) How would you describe your weight over the last year? 1 = It is difficult for me to gain weight; 2 = Can maintain normal weight even with fairly large food intake; 3 = I easily gain weight.

(I) Over the last 6 months, what has been your energy level? 1 = Low; 2 = Variable; 3 = High.

Statistical Analysis

All parametric data results were presented as the mean ± standard deviation. All nonparametric data results were presented as the median ± 95% confidence interval. Statistical analysis for parametric data was performed using the Student's *t*-test to assess within-group (two-tailed, paired) and between-group (two-tailed, unpaired) differences. A chi-square test with Yates's correction was performed to analyze nonparametric data. Yates's correction was applied to reduce the overestimation of statistical significance for the survey results which were composed of responses that had an expected count smaller than 5. Values of $p < 0.05$ were considered statistically significant. Subjects served as their own controls with values reported at four weeks compared with baseline values. All data were analyzed including subjects who were enrolled in the study and those who had received study intervention.

CHAPTER 3: RESULTS

Of the 32 subjects who were screened, 28 individuals met the inclusion criteria and were randomized into either the PE supplementation group or the control supplementation group. Of the 28 subjects who were randomized, 10 subjects in the PE group and 8 subjects in the control group completed the study. In addition, for the female subjects, a subanalysis was conducted to assess facial sebum production and TEWL. This was done to reduce the confounding effects of gender-based differences in testosterone on the biophysical properties of the skin.

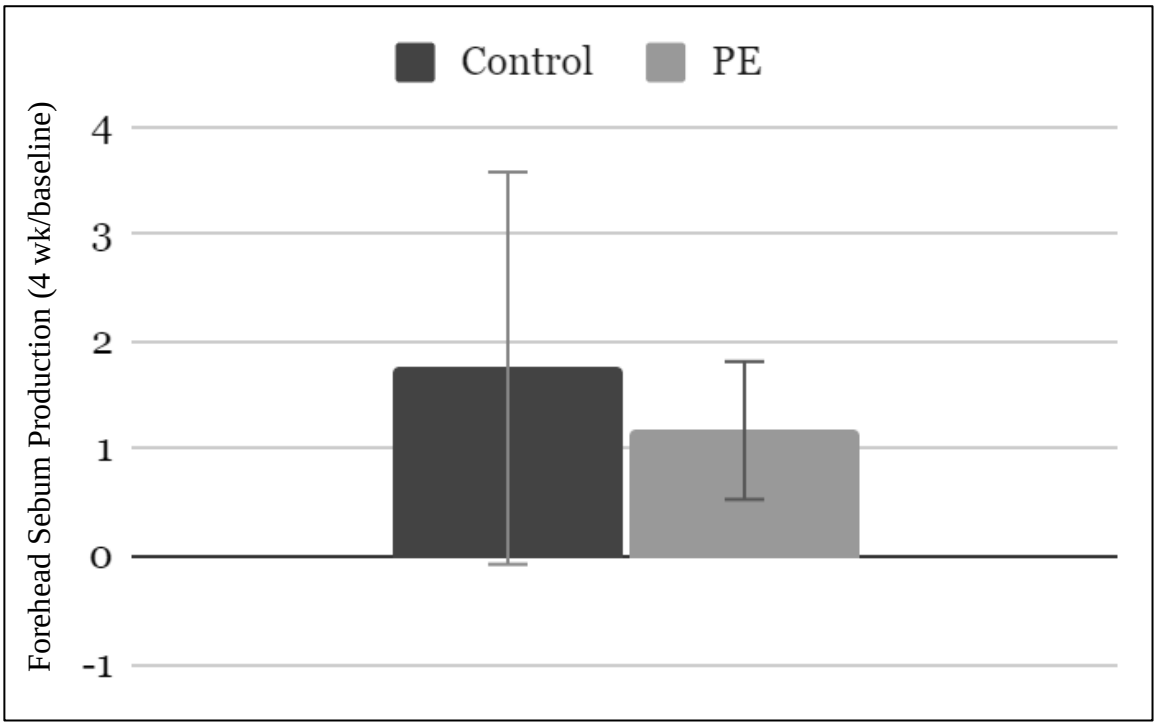
The results of the study are plotted as bar graphs in Figures 2–14. Since subjects served as their own control, individual baseline values were calculated as baseline/baseline, or 1. Individual values at the 4 weeks were calculated as 4 weeks/Baseline. Subsequently, the mean and standard deviation of the change from baseline was calculated for each cohort. This is represented by the *y*-axis in each figure.

Facial Sebum Production

There were no statistically significant differences in the forehead sebum production (Figure 2) and the cheek sebum production (Figure 3) between subjects in the control group and the PE group compared with pretreatment baseline. In addition, the observed differences within both cohorts were not statistically significant.

Figure 2

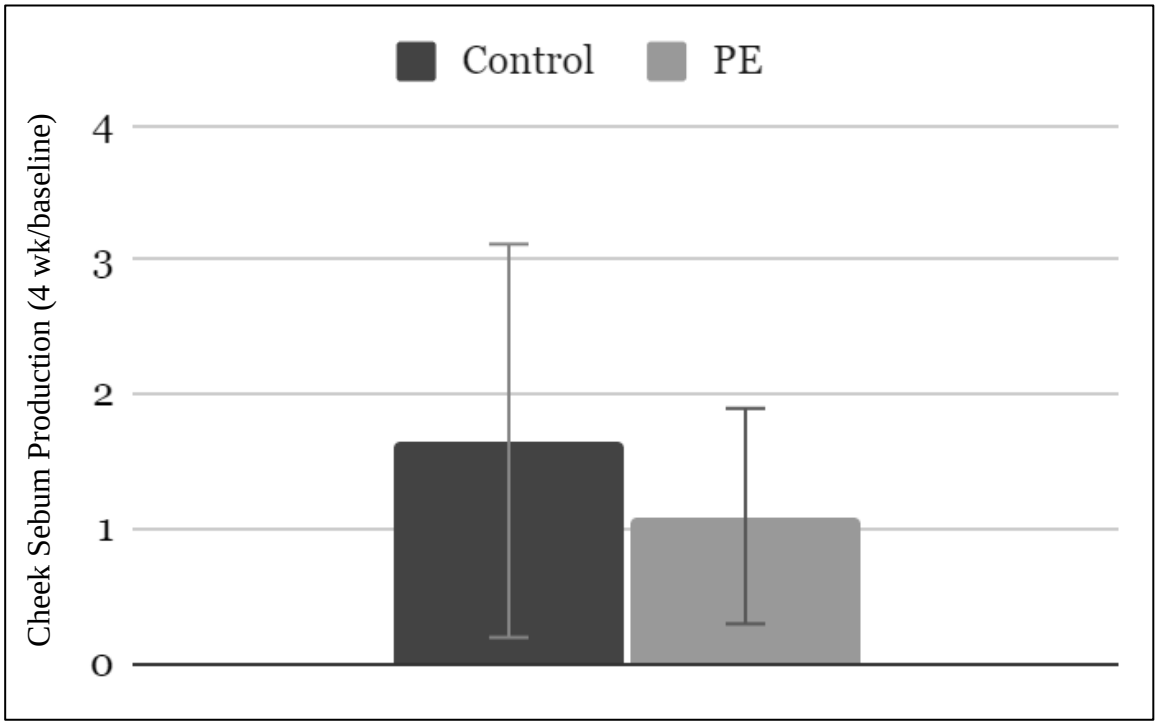
Mean Forehead Sebum Production of Control and PE Groups Relative to Pretreatment Baseline



Note. This bar graph shows the mean changes in forehead sebum production of the control group and the PE group after four weeks. Results are not statistically significant ($p < 0.05$). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

Figure 3

Mean Cheek Sebum Production of Control and PE Groups Relative to Pretreatment Baseline



Note. This bar graph shows the mean changes in cheek sebum production of the control group and the PE group after four weeks. Results are not statistically significant ($p < 0.05$). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

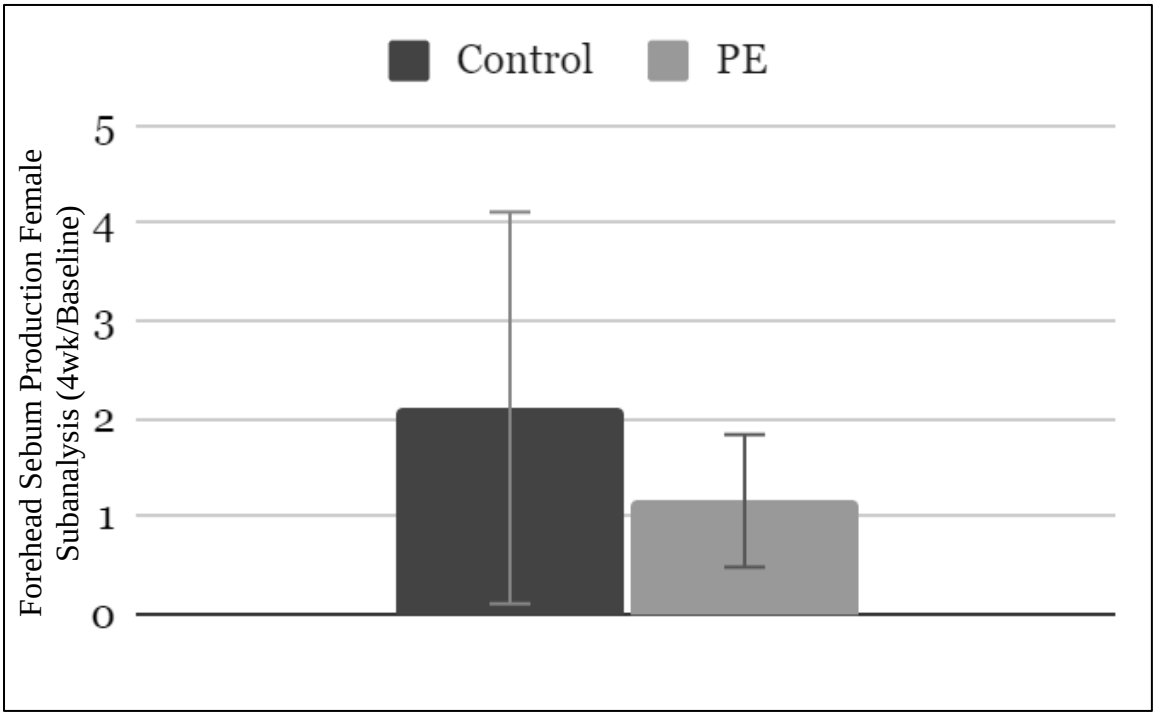
Facial Sebum Production (Female Subanalysis)

In the female subanalysis, there were no statistically significant differences in the forehead sebum production (Figure 4) and the cheek sebum production (Figure 5) between subjects in the control group and the PE group compared with pretreatment

baseline. The observed differences within the control group and the PE group were also not statistically significant.

Figure 4

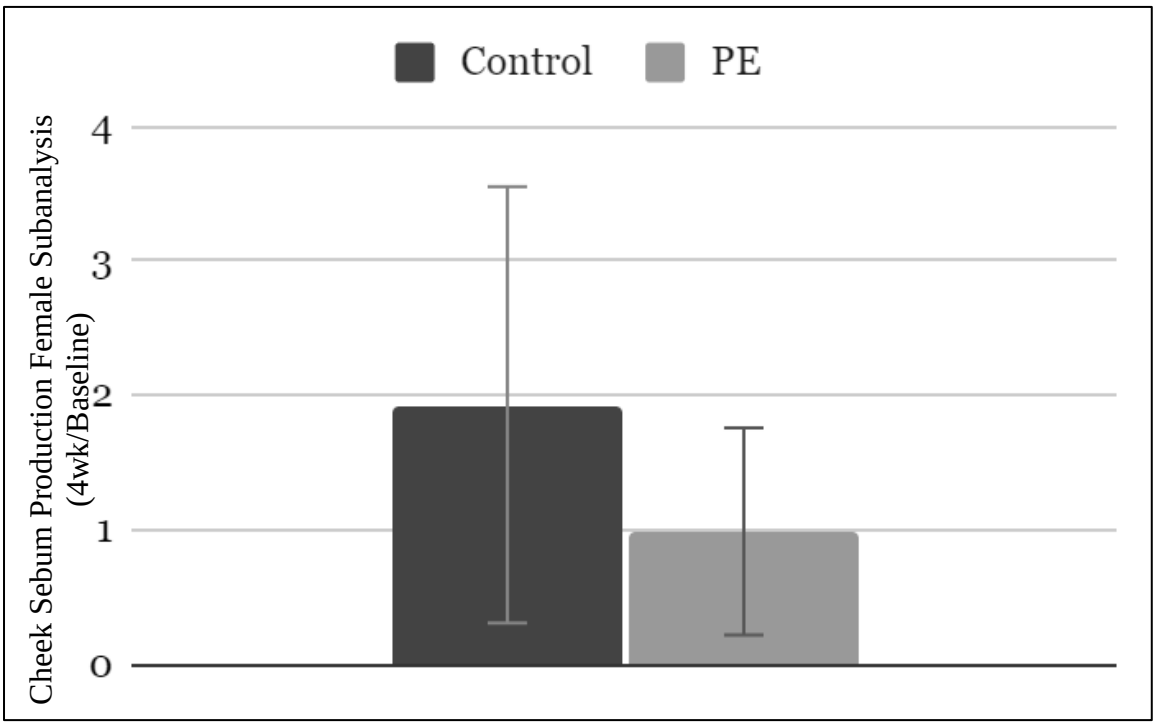
Mean Forehead Sebum Production of Control and PE Groups Relative to Pretreatment Baseline (Females Subanalysis)



Note. This bar graph shows the mean changes in forehead sebum production of the females in the control group and the PE group after four weeks. Results are not statistically significant ($p < 0.05$). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

Figure 5

Mean Cheek Sebum Production of Control and PE Groups Relative to Pretreatment Baseline (Females Subanalysis)



Note. This bar graph shows the mean changes in cheek sebum production of the females in the control group and the PE group after four weeks. Results are not statistically significant ($p < 0.05$). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

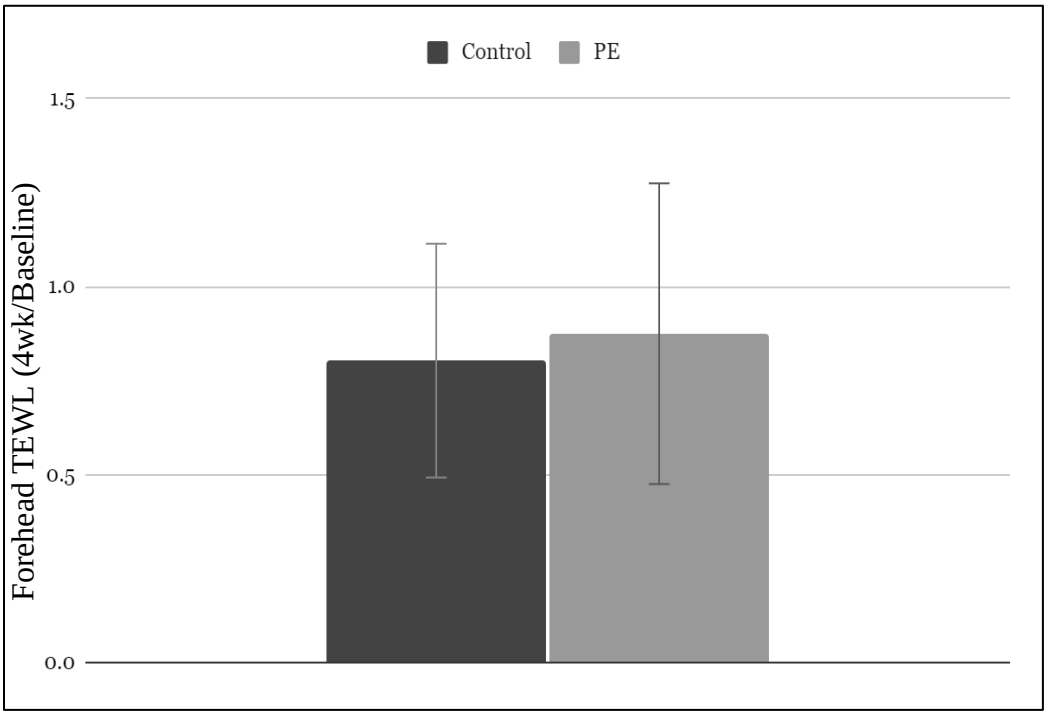
Facial TEWL

Compared with pretreatment baseline, the observed differences in the forehead TEWL (Figure 6) and the cheek TEWL (Figure 7) between subjects in the control group

and the PE group were not statistically significant. In addition, the observed within-group differences in the control group for both forehead TEWL and cheek TEWL were not statistically significant. The observed differences in forehead TEWL within the PE group were not statistically significant; however, there was a statistically significant decrease of 0.78 ± 0.19, or 22%, in the cheek TEWL of the PE cohort ($p < 0.01$).

Figure 6

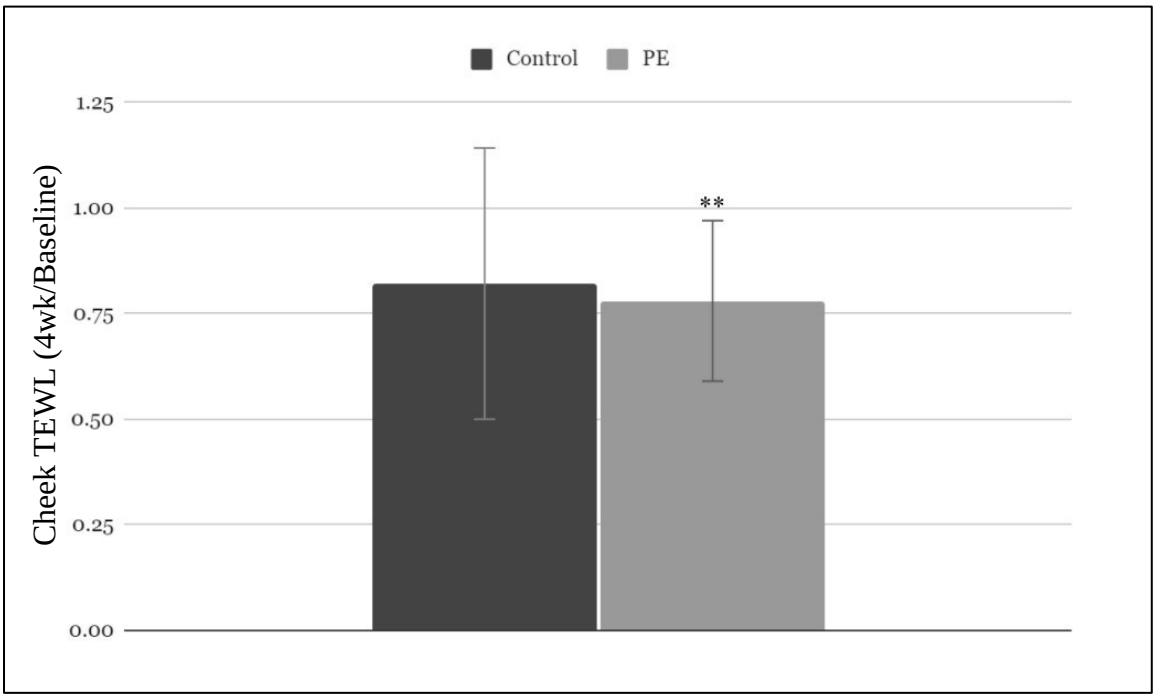
Mean Forehead TEWL Production of Control and PE Groups Relative to Pretreatment Baseline



Note. This bar graph shows the mean changes in forehead TEWL of the control group and the PE group after four weeks. Results are not statistically significant ($p < 0.05$). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

Figure 7

Mean Cheek TEWL Production of Control and PE Groups Relative to Pretreatment Baseline



Note. This bar graph shows the mean changes in cheek TEWL of the control group and the PE group after four weeks. In the PE group, the average change in cheek TEWL of was 0.78 ± 0.19, which was statistically significant (**$p < 0.01$). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

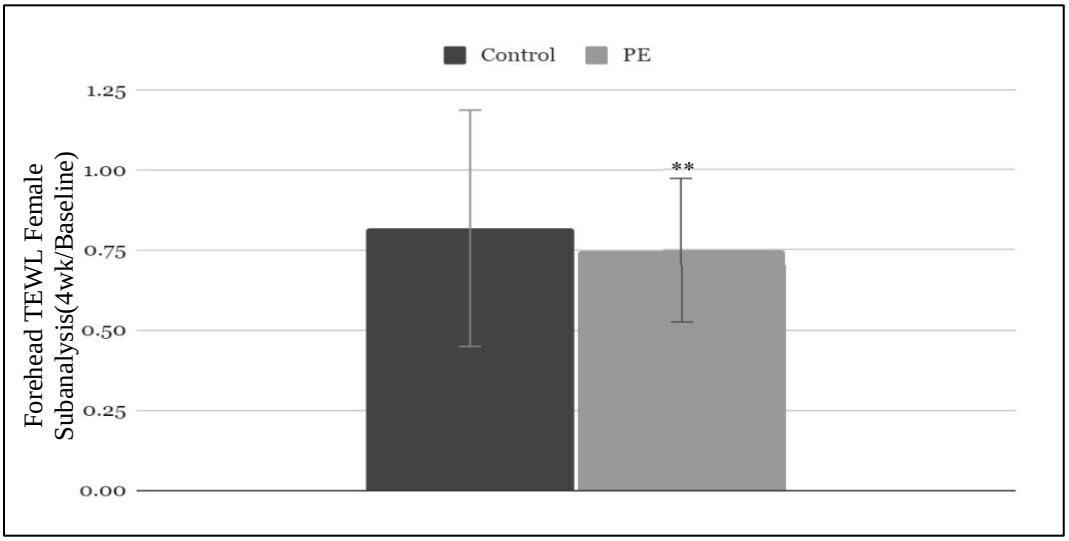
Facial TEWL (Female Subanalysis)

The observed differences in the forehead TEWL (Figure 8) and the cheek TEWL (Figure 9) between subjects of the control group and the PE group were not statistically

significant compared with pretreatment baseline. Within the control group, there were no statistically significant differences in the forehead TEWL and the cheek TEWL compared with pretreatment baseline. There was a statistically significant decrease of 0.75 ± 0.22, or 25%, in the forehead TEWL of the PE group ($p < 0.01$). There was also a statistically significant decrease of 0.76 ± 0.18, or 24%, in the cheek TEWL of the PE cohort ($p < 0.01$).

Figure 8

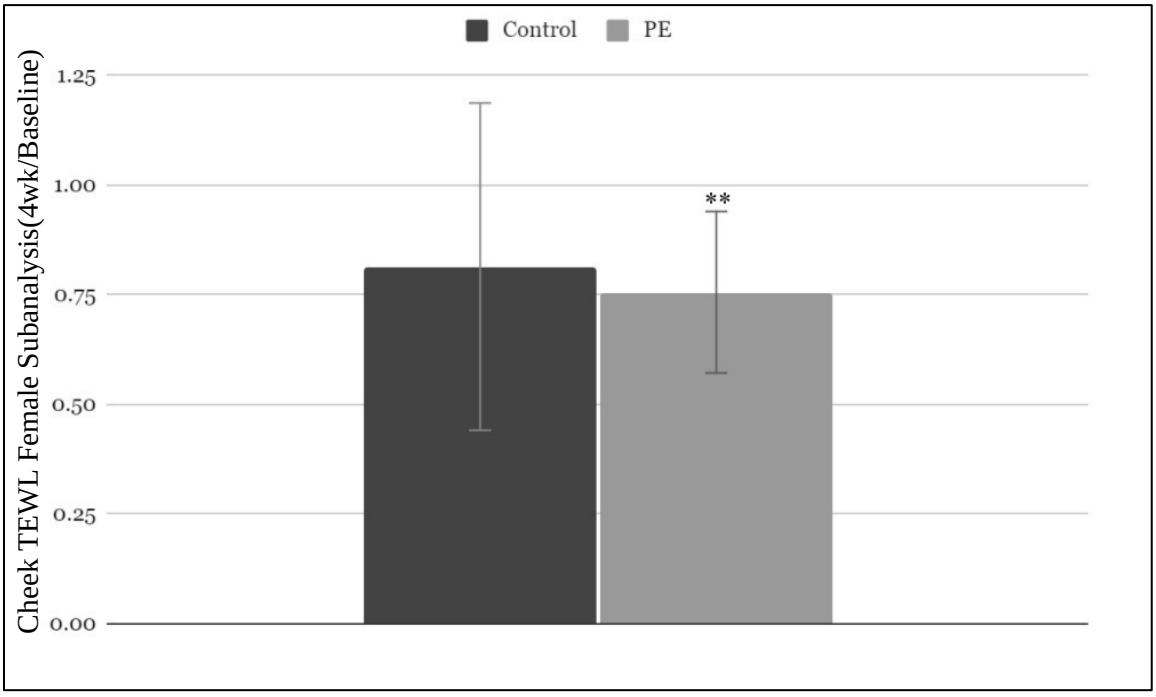
Mean Forehead TEWL Production of Control and PE Groups Relative to Pretreatment Baseline (Female Subanalysis)



Note. This bar graph shows the mean changes in forehead TEWL of the females in the control group and the PE group after four weeks. In the PE group, the average change in forehead TEWL of was 0.75 ± 0.22, which was statistically significant (**$p < 0.01$). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

Figure 9

Mean Cheek TEWL Production of Control and PE Groups Relative to Pretreatment Baseline (Female Subanalysis)



Note. This bar graph shows the mean changes in cheek TEWL of the females in the control group and the PE group after four weeks. In the PE group, the average change in cheek TEWL of was 0.76 ± 0.18, which was statistically significant (**$p < 0.01$). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

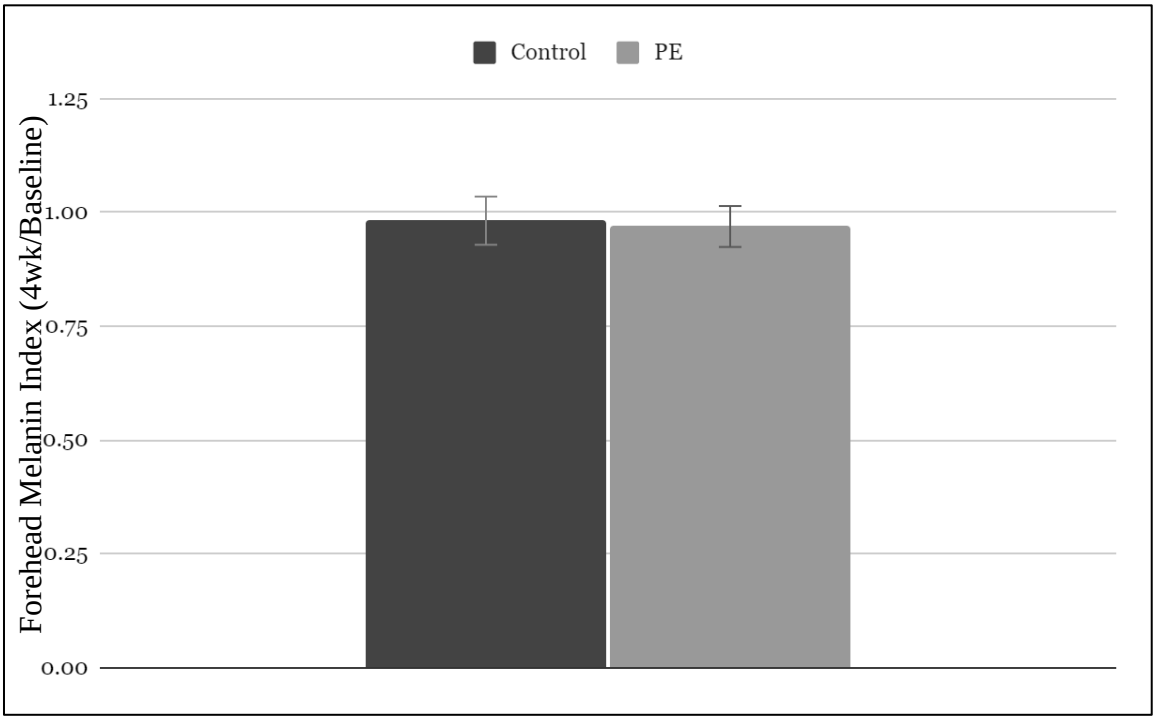
Facial Melanin Index

Compared with pretreatment baseline, the observed differences in the forehead melanin index (Figure 10) and the cheek melanin index (Figure 11) between subjects in

the control group and the PE group were not statistically significant. Furthermore, the observed differences in the forehead melanin index and the cheek melanin index within the control group were not statistically significant. Although the differences in the forehead melanin index within the PE group were not statistically significant, there was a statistically significant decrease of 0.97 ± 0.04, or 3%, in the cheek melanin index of the PE cohort ($p < 0.01$).

Figure 10.

Mean Forehead Melanin Index of Control and PE Groups Relative to Pretreatment Baseline

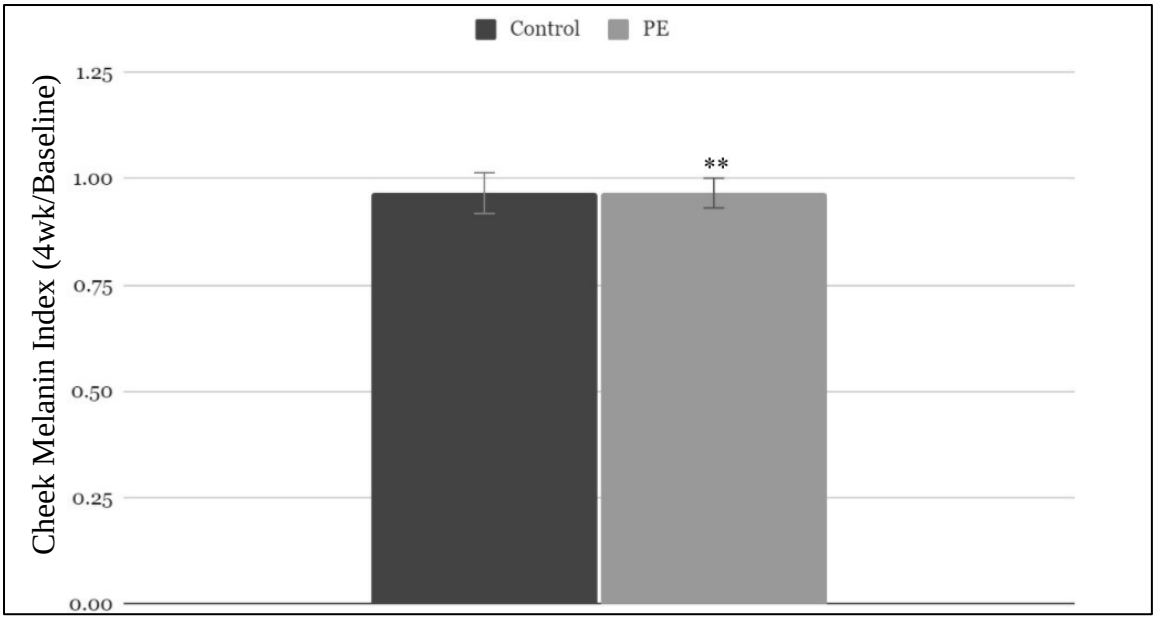


Note. This bar graph shows the mean changes in the forehead melanin index of the control group and the PE group after four weeks. Results are not statistically significant

($p < 0.05$). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

Figure 11.

Mean Cheek Melanin Index of Control and PE Groups Relative to Pretreatment Baseline



Note. This bar graph shows the mean changes in the cheek melanin index of the control group and the PE group after four weeks. In the PE group, the average change in the melanin index of the cheeks is 0.97 ± 0.04, which was statistically significant $^{**}p < 0.01$. Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

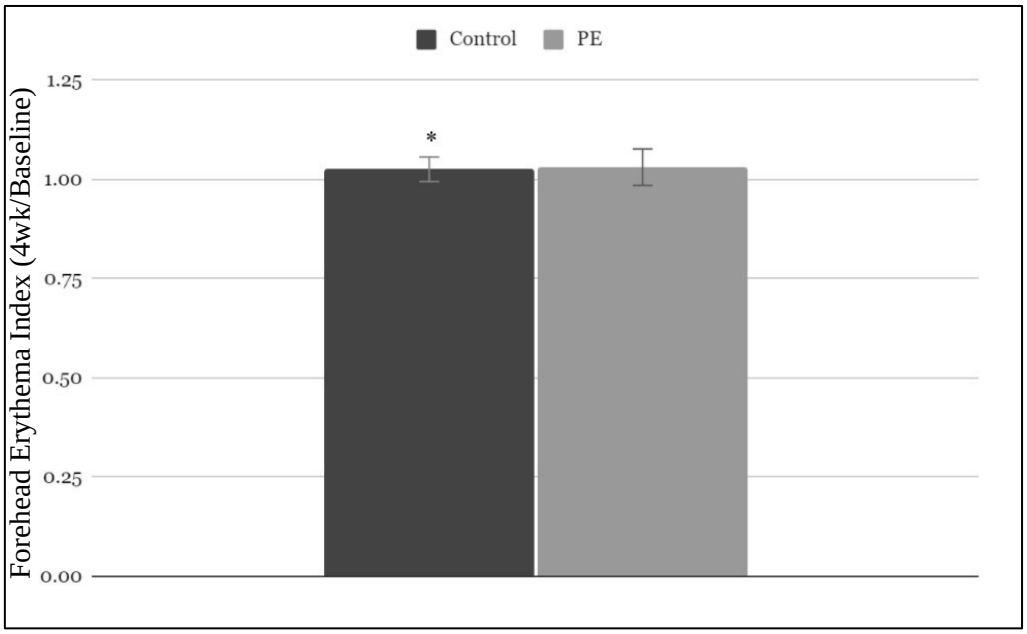
Facial Erythema Index

The observed differences in the forehead erythema index (Figure 12) and the cheek erythema index (Figure 13) between subjects in the control group and the PE group

were not statistically significant compared with pretreatment baseline values. In addition, the observed differences in the forehead erythema index and the cheek erythema index within the PE cohort were not statistically significant. However, within the control group, the increase in forehead erythema index of 1.01 ± 0.03, or 1%, was statistically significant ($p < 0.05$), and the increase in cheek erythema index of 1.02 ± 0.02, or 2%, was statistically significant ($p < 0.05$).

Figure 12.

Mean Forehead Erythema Index of Control and PE Groups Relative to Pretreatment Baseline 6

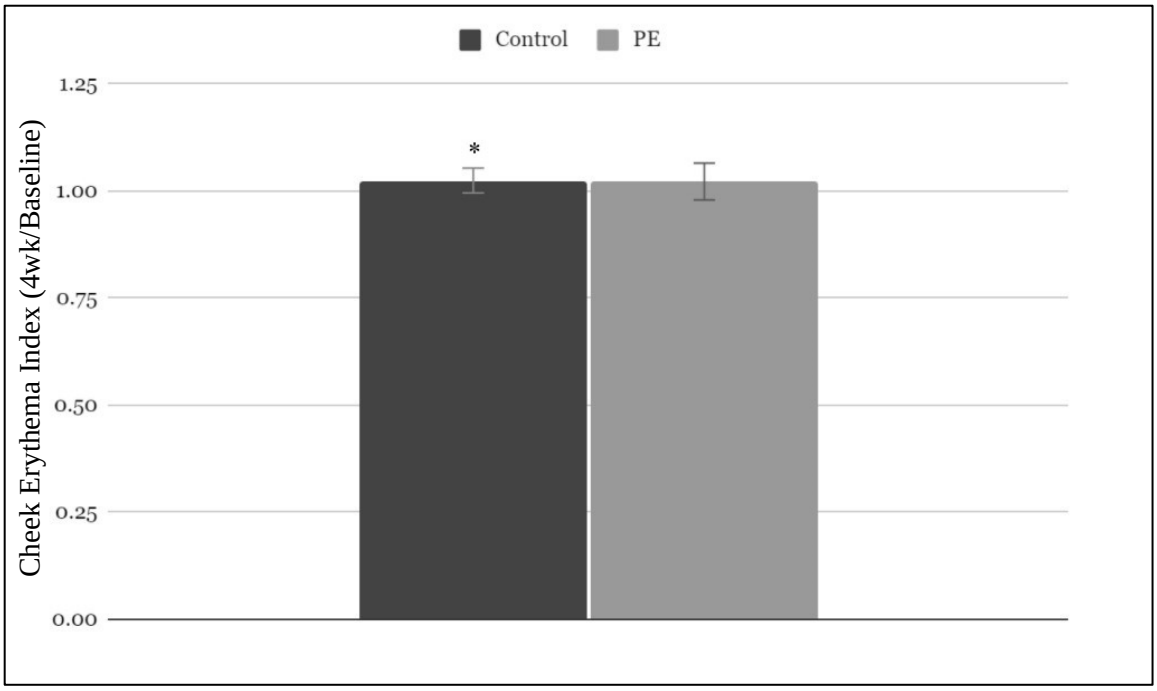


Note. This bar graph shows the mean changes in the forehead erythema index of the control group and the PE group after four weeks. In the PE group, the average change in the erythema index of the forehead is 1.01 ± 0.03, which was statistically significant *(*p*

< 0.05). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

Figure 13.

Mean Cheek Erythema Index of Control and PE Groups Relative to Pretreatment Baseline



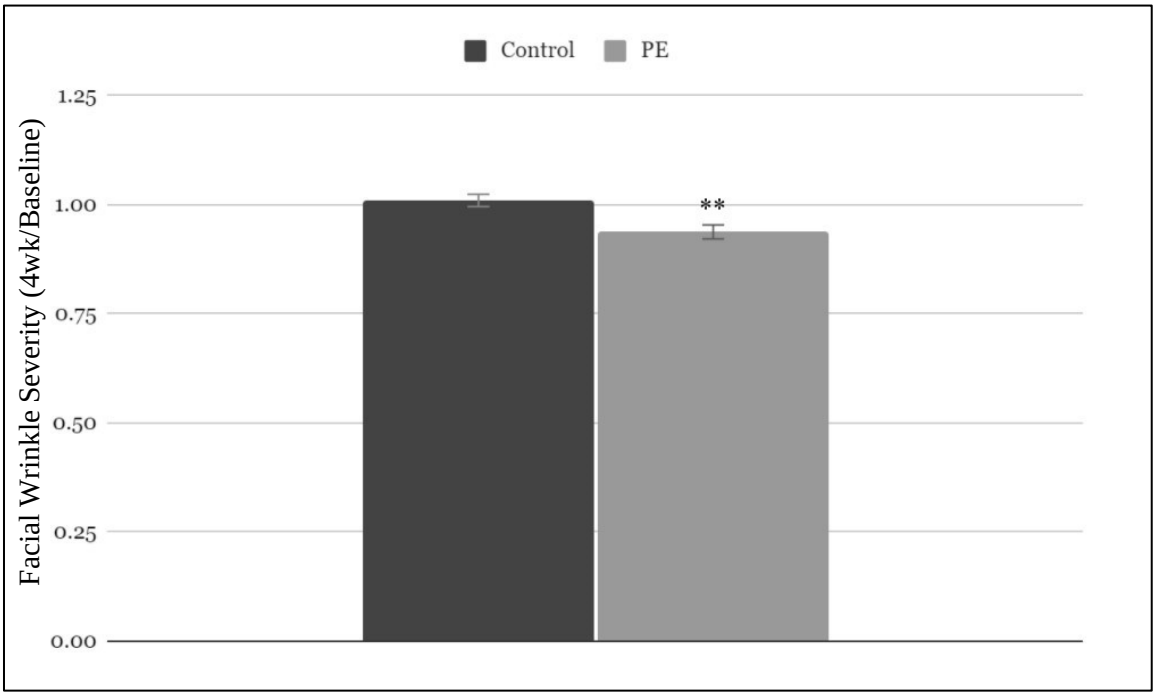
Note. This bar graph shows the mean changes in the cheek erythema index of the control group and the PE group after four weeks. In the PE group, the average change in the erythema index of the cheeks is 1.02 ± 0.02, which was statistically significant *(*p* < 0.05). Error bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

Facial Wrinkle Severity

There was a statistically significant decrease of 0.938 ± 0.014, or 6%, in the facial wrinkle severity of the PE group ($p < 0.01$). There was no statistically significant change in the facial wrinkle severity of the control group. These results are shown in Figure 14.

Figure 14.

Mean Wrinkle Severity Index of Control and PE Groups Relative to Pretreatment Baseline



Note. This bar graph shows the mean changes in the mean facial wrinkle severity of the control group and the PE group after four weeks. In the PE group, the average change in facial wrinkle severity is .94 ± 0.01, which was statistically significant *(*p* < 0.01). Error

bars represent standard deviations. Participants: control (n= 8); PE (n= 10). PE = pomegranate extract.

Quality of Life

In Group A, the median pre-treatment response to "In general, how would you define your health?" was 2.5 ± 0.642 or "Good" and "Very good." The median response after four weeks of oral supplementation was 3 ± 0.578 or "Very good." These results were not statistically significant, X^2 (3, N=8) = 0.333, p= 0.95. In Group A, the median pretreatment response to "How confident in your skin were you before taking the study intervention" was 2 ± 0.524 or "Moderately." After four weeks of oral supplementation, subjects in Group A were still moderately confident in their skin. These results were not statistically significant, X^2 (4, N=8) = 0.625, p= 0.96. After taking the oral supplement for four weeks, subjects reported feeling "Moderately" confident in their skin (2 ± 0.359). These results were not statistically significant, X^2 (4, N=8) = 6.071, p= 0.19. In Group A, subjects reported feeling "Moderately" confident in social situations since taking the study intervention (2 ± 0.823). These results were not statistically significant X^2 (4, N=8) = 3.111, p= 0.54. After four weeks of oral supplementation, subjects in Group A reported feeling "A little bit" or "Moderately" more energized since taking the study intervention (1.5 ± 1.71). These results were not statistically significant, X^2 (4, N=8) = 0.9, p= 0.92. Subjects in Group A reported "A little bit" or a "moderate" difference in the glowy appearance of their skin after four weeks (1.5 ± 0.907). These results were not statistically significant X^2 (4, N=8) = 2.528, p= 0.64. After four weeks of taking the study intervention, subjects were

"moderately" or "quite a bit" likely to continue using the agent (2.5 ± 0.975). These results were not statistically significant X^2 (4, N=8) = 4.125, p= 0.39.

In Group B, the median pretreatment baseline response to "In general, how would you define your health?" was "Good" (2 ± 0.666). After four weeks of oral supplementation, the median perception of health in Group B rose to "Very good" (3 ± 0.640). These results were not statistically significant, X^2 (3, N=10) = 1.052, p= 0.79. The median pretreatment response to "How confident in your skin were you before taking the intervention" in Group B was "Moderately" (2 ± 0.640). This response did not change after four weeks. These results were not statistically significant, X^2 (4, N=10) = 1.083, p= 0.90. After four weeks of oral supplementation, subjects in Group A reported feeling "Quite a bit" more confident in their skin 3 ± 0.523. This is distinct from Group A, where subjects only reported feeling moderately more confident in their skin after taking the study intervention. However, these results were not statistically significant, X^2 (4, N=10) = 8.51, p= 0.07. In Group B, subjects reported feeling a little bit more confident in social situations since taking the study intervention (1 ± 0.879). These results were not statistically significant, X^2 (4, N=10) = 0.583, p= 0.96. After four weeks of oral supplementation, subjects in Group B felt "a little" more energized (1 ± 1.02). These results were not statistically significant, X^2 (4, N=10) = 1.667, p= 0.80. Subjects in Group B also reported noticing more glowing skin "a little" more after taking the study intervention (1 ± 0.879). These results were not statistically significant, X^2 (4, N=10) = 1.667, p= 0.80. Subjects in Group B were also moderately likely to continue using the

study agent after four weeks of oral supplementation (2 ± 0.879). These results were not statistically significant, X^2 (4, N=10) = 0.9, p= 0.92.

Digestive Health

In Group A, subjects reported a median response of having a bowel movement once per day (3 ± 0.489). This response did not change after four weeks (3 ± 0.359). These results were not statistically significant X^2 (4, N=8) = 0.167, p= 0.99. The median stool type was Stool Type 4 from the Bristol Stool Chart at pretreatment baseline (4 ± 0.978) and after four weeks of oral supplementation (4 ± 0.741). These results were not statistically significant, X^2 (7, N=8) = 1.444, p= 0.98. In Group A, subjects reported feeling that their hunger was "Irregular, varies from meal to meal" and "Generally strong; cannot skip meals" (1.5 ± 0.614). After four weeks of oral supplementation, the median response changed to "Irregular, varies from meal to meal" (1 ± 0.359). These results were not statistically significant, X^2 (2, N=8) = 0.5, p= 0.78. In Group A, subjects also report that the time it takes them to feel hungry after eating varies from meal to meal (2 ± 0.490). This response did not change after four weeks of oral supplementation (2 ± 0.359). These results were not statistically significant, X^2 (2, N=8) = 0.167, p= 0.92. Subjects felt that the amount of food they can eat at one time varies from meal to meal at pretreatment baseline (2 ± 0.444) and after four weeks of oral supplementation (2 ± 0.444). These results were not statistically significant X^2 (2, N=8) = 0.1, p= 0.92. At pr-treatment baseline, subjects reported that they can maintain normal weight even with fairly large food intake and that they gain weight easily (2.5 ± 0.370). However, after four weeks of oral supplementation,

the median response among subjects shifted to being able to maintain normal weight even with fairly large food intake (2 ± 0.359). These results were not statistically significant, X^2 (2, N=8) = 0, p= 1. Over the last six months, subjects in Group A felt that their energy levels were variable at both pretreatment baseline (2 ± 0.444) and after four weeks of oral supplementation (2 ± 0.321). These results were not statistically significant, X^2 (2, N=8) = 0.5, p= 0.78.

In Group B, only nine of the ten subjects completed the Digestive Health Questionnaire. Overall, the subjects reported that they usually have a bowel movement once a day in the past month at pretreatment baseline and after four weeks of oral supplementation (3 ± 0.576). These results were not statistically significant X^2 (4, N=9) = 1.292, p= 0.86. Subjects reported that their stool type corresponds to stool type 4 in the Bristol Stool Chart at pretreatment baseline (4 ± 0.635). The median response shifted to stool type 3 after four weeks of oral supplementation (3 ± 0.544). These results were not statistically significant, X^2 (7, N=9) = 0.625, p= 0.99. Subjects in Group B also felt that their hunger is “Irregular, varies from meal to meal” in the past thirty days at pretreatment baseline (2 ± 0.511) and after four weeks of oral supplementation (2 ± 0.566). These results were not statistically significant, X^2 (2, N=9) = 0.167, p= 0.92. In Group B, subjects report that the time it takes them to feel hungry after eating varies from meal to meal at pretreatment baseline (2 ± 0.566) and after four weeks (2 ± 0.327). These results were not statistically significant, X^2 (2, N=9) = 1.944, p=0.38. The amount of food that subjects in Group B can eat at one time also varies from meal to meal at pretreatment baseline (2 ± 0.370) and after four weeks (2 ± 0.326). These results were not statistically

significant X^2 (2, N=9) = 0.5, p=0.79. In the past year, subjects reported that they easily gain weight at pretreatment baseline (3± 0.475). However, after four weeks of oral supplementation, subjects reported that they can maintain normal weight even with fairly large food intake (2 ± 0.436). However, these results were not statistically significant, X^2 (2, N=9) = 0.75, p=0.69. Subjects in Group B also report variable energy levels in the past thirty days at pretreatment baseline (2 ± 0.544) and after four weeks (2 ±0.327). These results were not statistically significant, X^2 (2, N=9) = 1.7, p=0.43.

CHAPTER 4: DISCUSSION

There were no observations of any statistically significant changes in facial sebum production in the control group and the PE group after four weeks compared with pretreatment baseline. However, some trends in the data were observed. After one month of oral supplementation, the facial sebum production of both groups increased compared with pretreatment baseline. Curiously, the increase in facial sebum production of the PE group was lower than in the control. For example, the average forehead sebum production of the control group and the PE group increased by 75% and 17%, respectively, from pretreatment baseline values (Figure 2). Likewise, the average cheek sebum production of the control group and the PE group increased by 65% and 10%, respectively (Figure 3). A similar trend was observed in the female subanalysis of the forehead sebum production. The average forehead sebum production increased by 111% and 16% in the control group and the PE group, respectively (Figure 4). However, the average cheek sebum production increased by 93% in the control group and decreased by 1% in the PE group (Figure 5). This suggests that there may be an excipient or placebo that was driving the increase in the sebum production rate and that this increase was suppressed by the PE. However, these findings are only statistical trends which would require an expanded cohort to assess for statistical significance.

These results are somewhat consistent with the conclusions of a review which suggested that polyphenol-enriched foods, like green tea, exert an inhibitory effect on facial sebum production (Saric et al., 2017). Similar to green tea, PE is a natural reservoir

of polyphenols (Table 1). In fact, the pomegranate peel is rich in flavonoids such as epicatechin (Table 1.1B), one of the four major polyphenols found in green tea (Mahmood et al., 2013). Because the phytoconstituents of the pomegranate peel make a significant contribution to the phytochemical composition of the PE utilized in this study, PE may be an effective agent in the treatment of excessive facial sebum production.

However, there are several limitations with this inference about PE. First, Mahmood et al. (2013) reported that green tea is rich in other flavonoids, such as epicatechin gallate, epigallocatechin, and epigallocatechin-3-gallate, which are not found in the fruit of *P. granatum* L. . As a result, the observed effects may be due to the unique phytoconstituents of green tea alone. In addition, the studies referenced by Saric et al. (2017) demonstrated a decrease in facial sebum production after the direct topical application of polyphenol-enriched ingredients to the face, whereas this study examined the effect of oral PE supplementation on facial sebum production. The supplementation period in this study of only four weeks may be too short to see a statistically significant difference in this parameter. In fact, the studies referenced by Saric et al. (2017) involved the application of the topical products for 60 days in one study and eight weeks in the other. Therefore, it would be beneficial to evaluate the long-term and short-term effects of both an oral and a topical formulation of PE on facial sebum production in future studies. This would help to determine if PE truly has the capacity to reduce facial sebum production like its polyphenol-rich counterparts and if the observed trend in this study has clinical significance. The study design would also assist in determining the length of

the supplementation period and the mode of delivery, oral or topical, which achieves the best results.

Moreover, a report by Lee et al. (2017) described the anti-lipase activity of PE and specific tannins isolated from the fruit of *P. granatum* L. including punicalagin and punicalin. The presence of PE reduced lipase activity by 20%, whereas punicalagin and punicalin reduced lipase activity by 39.8% and 34.7%, respectively. These results are of significance as the overproduction of sebum is associated with increased bacterial lipase activity in some skin conditions such as acne vulgaris. Because the PE utilized in this study contained 75 mg of punicalagin, a similar inhibitory effect on bacterial lipase activity and the subsequent reduction in facial sebum production would be expected. Regrettably, the study design did not make a clear distinction between subjects who regularly produced high levels of sebum and those who had a confirmed diagnosis of skin conditions which involved the bacterial overproduction of sebum. Therefore, it would be worthwhile to design a study specifically examining the anti-lipase potential of PE and its isolated tannins in individuals with confirmed diagnoses of skin conditions such as acne vulgaris. Although Lee et al. (2017) studied the topical application of PE and its isolated tannins in rodent models, it would be interesting to evaluate whether similar results could be achieved with topical PE supplementation in human models.

Results from this study also showed a statistically significant ($p < 0.01$) decrease of 22% in the TEWL of the cheeks in the PE group (Figure 7). In the female subanalysis of the PE group, the reductions in the TEWL of the forehead and the cheeks by 15% and 14%, respectively, were statistically significant ($p < 0.01$) (Figures 8 and 9). Other

investigators reported that decreased TEWL from the stratum corneum of the epidermis is associated with enhanced skin barrier function and senile skin (Berardesca & Maibach, 1990; Grubauer et al., 1989). The statistically significant reduction of facial TEWL in the PE group may thus reflect an improvement in the barrier function of the skin after four weeks of oral supplementation with PE. This could be due to various factors. For example, Lin et al. 2017 suggested that although an inflammatory response and the subsequent production of reactive oxygen species and secretion of cytokines are necessary for skin barrier repair, excessive inflammation results in the disruption of skin homeostasis and the reduction of skin barrier function. It is important to note that many studies have provided compelling evidence for the antioxidant and anti-inflammatory activity of the phytochemicals in the fruit *P. granatum* L., the basis for the oral PE of this study (Boussetta et al., 2009; de Pascual-Teresa & Sanchez-Ballesta, 2008; Hayouni et al., 2011; Joseph et al., 2011; Joseph et al., 2013; Rosenblat et al., 2013; Yan et al., 2013). Free radical scavenging activity is associated with a reduction in the inflammatory response, and the phytoconstituents of the PE utilized in this study are known to directly inhibit or modulate the inflammatory response. Therefore, the oral administration of PE may be partially responsible for the reduction of facial TEWL in the PE group.

Interestingly, Azevedo et al. (2020) maintained that topical antioxidant supplementation improves the structure and function of the skin barrier. This claim was corroborated by others who reported the improvement of skin repair functions in animal models with the topical application of polyphenols from the pomegranate peel (Hayouni et al., 2011; Yan et al., 2013). Although the topical supplementation of the botanical

demonstrated positive effects on wound healing, specific parameters of skin barrier function were not evaluated by these investigators. Therefore, it would be valuable to determine if topical PE supplementation improves TEWL from the stratum corneum and subsequently maintains the integrity of the skin barrier in humans.

The current scientific literature evaluating the effectiveness of oral PE supplementation on skin barrier function appears to be in its early stages. Nonetheless, a 2012 study demonstrated that using a supplementation of pomegranate flower extract for 55 days restored decreases in skin water content and epidermal barrier function after inducing a disruption of skin homeostasis by tape stripping (Wang et al., 2012). Notably, the flowers of the pomegranate contain not only phenolic acids, such as gallic acid and ellagic acid which are found in the pomegranate peel, but also triterpenoids such as oleanolic acid and ursolic acid. Because Wang et al. (2012) did not attribute their findings to specific phytoconstituents, future studies should evaluate whether the observed results are due to the phenolic acids, the triterpenoids, or both bioactive compounds. Considering that the pomegranate peel shares similar phytoconstituents with the pomegranate flower, it would be beneficial to examine if the phenolic acids of PE have a positive effect on transepidermal water loss from the epidermis in humans.

Lin et al. 2017 also suggested that the proper functioning of the skin barrier requires adequate antimicrobial activity by the stratum corneum. This is achieved by the antimicrobial peptides housed within intracellular components of the skin and by the relatively weak acidity of the skin surface. Previous studies established the antimicrobial properties of anthocyanins which are found within the pomegranate peel and seeds (Silva

et al., 2017). In addition, the arils of the pomegranate demonstrated antimicrobial activity against various gram-positive and gram-negative bacteria (Duman et al., 2009). Because these studies did not investigate the antimicrobial activity of the different components of *P. granatum* L. on skin health, future studies should examine whether this property of these phytochemicals confers beneficial effects on facial TEWL and the barrier function of the skin.

The thinning of the epidermis is a process associated with a compromised skin barrier and higher TEWL. This structural remodeling occurs with the loss of connective tissue elements such as type 1 and type 2 collagens (Varani et al., 2000). Specifically, interstitial collagenase, or matrix metalloproteinase 1 (MMP-1), and gelatinase are key players which orchestrate these events. Notably, research by Aslam et al. (2006) concluded that the administration of 1 µl/ml of pomegranate seed oil induced a mild thickness of the epidermal layer (with normal differentiation) of human skin in organ culture. In the same work, the administration of pomegranate peel extract increased pro-collagen synthesis by dermal fibroblasts at a dose of 0.05 µl/ml and inhibited MMP-1 activity in an inverse dose-dependent manner at 0.01, 0.05, 0.1, and 0.5 µl/ml in vitro. These findings are significant because they appeared to reverse the effects of epidermal thinning and connective tissue destruction associated with a compromised skin barrier. Because pomegranate seed oil and pomegranate peel were included in the composition of the oral PE utilized in this study, the significant reduction in facial TEWL of female participants in the PE group (Figures 8 and 9) may be associated with these events occurring at the molecular level. In future studies, it would be worthwhile to include a

procedure such as a punch biopsy of the skin to determine the changes that are occurring at the cellular and tissue levels as a result of the PE supplementation.

In this study, results indicated a significant decrease in facial wrinkle severity of 0.938 ± 0.014, or 6%, in the PE group ($p < 0.01$) (Figure 14). Previous work by Choi et al. (2013) maintained that increases in water loss from the epidermis result in the remodeling of the most superficial layer of the skin from a fluid to a more rigid structure. This is associated with the thinning of the epidermis and the subsequent appearance of deeper and more spaced-out wrinkles. Because dehydrated skin is associated with higher TEWL, the restoration of the skin barrier may result in an improvement in TEWL, skin hydration, and the appearance of wrinkles. In addition, Choi et al. (2013) established a negative correlation between skin hydration and the appearance of wrinkles. Therefore, the reduction in the TEWL and the improvement in the skin barrier function of subjects in the PE group of this study may be partially responsible for the significant reduction in the severity of facial wrinkles within this cohort.

Whereas Choi et al. (2013) established the relationship between wrinkle formation and the thinning of the epidermis, Aslam et al. (2006) reported that this structural change can be reversed with the administration of 1 μl/ml of pomegranate seed oil, but not pomegranate peel extract, in human cells in organ culture. Thus, the decrease in facial wrinkle severity observed in this study (Figure 14) may be attributed to the phytoconstituents of the pomegranate seed oil. However, because the exact concentration of pomegranate seed oil in the oral PE supplement of this study was unknown, the possibility of this rationale must be further explored.

Ghimeray et al. (2015) also demonstrated a significant dose-dependent inhibition of in vitro collagenase activity with 0.5, 1, 2.5, and 5 μg/ml topical formulations of fruit extracts including *P. granatum* L. The results showed significant decreases in wrinkle area, depth, and length of 11.5%, 10.07%, and 29.55%, respectively, in the crow's feet area of the eyes with a 2% topical formulation containing fruit extracts. Although these results seem promising, it is important to remember that the formulation contained the fruit extract of other botanicals which have a distinct phytochemical composition different from *P. granatum* L. alone.

Furthermore, this study reported a statistically significant decrease of 0.97 ± 0.04, or 3%, in the melanin production of the cheek in the PE group (Figure 11). These results are corroborated by a 2014 study which found a statistically significant decrease in skin melanin production with the topical application of a microemulsion containing PE over a 12-week period with healthy Asian subjects (Parveen et al., 2014). The investigators attributed this finding to the suppressive effects of ellagic acid, a phenolic acid found in the pomegranate peel (Table 1.1A), on tyrosinase activity. Because tyrosinase is the rate-limiting enzyme in the melanin synthesis pathway, the statistically significant decrease in the melanin index of the PE group in this study (Figure 11) may be associated with the inhibitory effect of ellagic acid on melanin production.

The inhibitory effect of ellagic acid on tyrosinase was also supported by Kasai et al. (2006), who reported a statistically significant decrease in ultraviolet-induced skin pigmentation with an ellagic acid-rich oral PE supplement. Moreover, at doses of 50 and 100 μg/ml, a punicalagin-rich PE was found by Rana et al. (2013) to reduce melanin

production by 40% and 60%, respectively, in vitro. These investigators specifically reported that punicalagins isolated from PE directly inhibited melanin production by 60%, 70%, and 75% at concentrations of 20, 60, and 100 μg/ml, respectively. Because the PE administered for oral consumption in this study was rich in this specific tannin, the reduction of the melanin index in the PE group (Figure 11) may be more closely associated with the inhibitory effect of punicalagins on melanin production rather than ellagic acid. In the design of this study, subjects were restricted with the amount of sun exposure they were allowed in the four-week supplementation period. However, this was not strictly enforced, and the use of sunscreen was also not controlled. Considering that the 3% decrease in melanin production was significant but subtle, the relationship between a punicalagin-enriched PE and the facial melanin index should be further investigated with a sturdier study design.

In addition, this study showed statistically significant increases in the forehead erythema index of 1.01 ± 0.03, or 1%, and the cheek erythema index of 1.02 ± 0.02, or 2%, in the control group (Figures 12 and 13). Praveen et al. (2014), however, reported a statistically significant decrease in skin erythema after a 12-week supplementation period of a topical application of a microemulsion containing PE. These results were thought to be due to the antioxidant and anti-inflammatory properties of the phytoconstituents discussed earlier. In this study, it is important to remember that the statistically significant increase in facial erythema was unique to the control group alone. Thus, the oral PE may have had a protective effect on facial erythema in the PE group because the subjects in this group experienced no statistically significant change in facial erythema.

For the questionnaires administered in this study, there were no statistically significant changes in self-reported quality of life between both groups in the four-week supplementation period. However, some trends were observed. In the PE group, the subject's perception of health shifted from "Good" (2.00 ± 0.67) at pretreatment baseline to "Very good" (3.00 ± 0.64) after four weeks of oral supplementation, X^2 (3, N = 10) = 1.052, p = 0.79. Other investigators (Arts & Hollman, 2005; Scalbert et al., 2005; Tsao, 2010) have alluded to the positive effects of a polyphenolic-rich diet on improving various health outcomes, including risk reduction of several chronic illnesses. Therefore, in future studies, it would be valuable to include objective markers of health to supplement the self-reports collected from subjects. This could involve measurements of serum lipid profiles and vitals in addition to the research procedures performed at each visit.

There were also no statistically significant changes in the questionnaire on self-reported digestive health. However, previous studies demonstrated the prebiotic potential of PE and pomegranate juice on the gut microbiome. Li et al. (2015) specifically reported an increase in *Bifidobacterium* and *Lactobacillus* strains in stool samples of human subjects after a supplementation period of four weeks. This time interval is identical to the supplementation period in the design of this study. Therefore, it would be valuable to identify shifts occurring in the microbiome which are associated with the introduction of a polyphenol-rich agent such as PE. In future studies, this could be a good source of objective data along with self-reports of digestive health.

CONCLUSIONS

In conclusion, the four-week oral supplementation involving pomegranate fruit extract significantly improved several parameters of skin health. Specifically, the results from this study demonstrated reductions in (1) the melanin index of the cheeks, (2) the transepidermal water loss from the stratum corneum of the cheeks, and (3) the facial appearance of average wrinkle severity. In addition, significant reduction was reported in the facial transepidermal water loss among females of the intervention group. These positive effects are largely attributed to the potent antioxidant and anti-inflammatory properties of the phytochemicals which constitute the oral agent. Future studies of pomegranate fruit extract are recommended with an expanded population to improve statistical power and to evaluate the negative trend observed in facial sebum production.

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