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RESEARCH MANUAL

VIQUA®

Synergistic bio-compound for skin and body health improvement

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ABSTRACT

VIQUA® is a natural bio-compound formulated from pomegranates selected for their unique composition of polyphenols. This variety, from the south of Spain, is grown on the sea shore in Alicante using exclusively sea water for its irrigation. This natural stressor environment allows plants to develop polyphenols more stables, and efficient actives even at low dosage comparing with normal fruits.

VIQUA® complex rich in antioxidants such as punicalagin and ellagic acid helps reduce free radicals production, reduce inflammation and glycation, promote cardiovascular health, improve micro-circulation and maintain healthy skin condition.
To assure the best delivery, the technology ADS is used so the bio-actives reach directly to the site they are effective in the body.

A placebo-controlled double-blind ingestion study of VIQUA® supplement was performed in 30 subjects. Several participants parameters improved after 1 month of ingestion: Hydration (40%), Skin roughness (31%), Skin Smoothness (27%), Water content (51%), Depth in wrinkles (-26%) and Reduction in melasma (57%). Other properties such a depigmentation effect, estrogen-like biological activity, reduction of inflammation and anti-oxidative stress have also been studied in the present document.

1. TITLE CLINICAL TRIAL

VIQUA® Oral supplement of pomegranate extract effects in body and healthy skin.

2. **PROTOCOL NUMBER:** 09-SR-OD-VIQ A clinical randomized double-blind trial comparing VIQUA® vs. Placebo.

3. SPONSOR: AXIALYS INNOVATIONS

4. LABORATORY : Instituto de Biología Molecular y Celular, Universidad Miguel Hernandez 03202 Elche (Alicante) Spain – Dr Vicente Micol

5. PRODUCT INFORMATION

Product code:	VIQ919
	No selective extract
Origin:	France
	Using ADS® Nano-technology
Appearance:	Powder form
Color:	Brown
Taste:	Characteristic

6. EXPERIMENTAL AND CONTROL DRUG

1. Experimental drug
   VIQUA® (pomegranate extract)
2. Dosage form and administration experimental drug
   Oral administration. Daily dose 0.5ml of VIQUA® added in 500 ml of water.
3. Treatment group
   2 different groups of 30 women aged 35 and 55 years
4. Drug control. Not applicable.

7. STUDY OBJECTIVE

The objective of this research is to demonstrate the antioxidant effects of the supplement VIQUA® and the properties of its compounds.
The research of this active is made in base of different essays to determinate the power of its effect. In vivo and in vitro test have been made to achieve the conclusions showed in the present document.

8. VARIABLE VALUED

Several parameters related to skin have been monitored using the face as test area. Analysis of the skin surface after treatment (VISIOSCAN), Corneometer CM 825 - Visioscan VC 98 – Skicon

9. TOTAL NUMBER OF PATIENTS

30 women aged 35 and 55 years participated in a double-blind, placebo-controlled trial testing the efficacy of a proprietary oral supplement for skin nutrition (VIQUA®) for improvement of skin rejuvenation, elasticity and appearance.

10. DURATION OF TREATMENT AND DOSAGE

Ingestion of VIQUA® supplement and Placebo during 30 days of treatment (4 weeks) of treatment with a dosage 250 mg/day twice 125 mg.

CONTENTS

1. GENERAL INFORMATION

VIQUA® is a synergistic bio-compound from pomegranate fruits which have been formulated to improve body and skin health.

VIQUA® was developed in accordance to 3 important factors:

- **Safety**

VIQUA® has been formulated with pomegranate fruits grown in Alicante farms in Spain with strict quality control.

Pharmaceutical testing is realized for all our products to always keep the safety under control.

- **Bioavailability**

VIQUA® is highly bio-available due to its ADS (Advanced Delivery System) technology which helps delivering the actives to the good location. The ingredients lead directly to the site where they are really effective.

- **Efficacy**

VIQUA® combines the bioactive compounds of the different parts of the pomegranate fruit. Using the whole fruit is essential as synergic action is only possible when all components are together.

Multifactorial effects and chemical synergy are shown in the action of multiple compounds from pomegranate extract.

VIQUA® characteristics and composition

Common name: Pomegranate
Family: Punicaceae
Scientific name: Punica granatum
Principal Nutrients: Vitamins B1, B2, C and Niacin, Potassium, Glutanic acid, Aspartic acid, tannins punicalagins ellagitannins, Flavonoids, Ellagic acid

1.1. VIQUA® CLAIMED BENEFITS

VIQUA® offers three main benefits:

▸ **Antioxidant at low dosage**

Inhibition of peroxide lipid formation / Superoxide dismutase effect

▸ **Whitening effect**

Modulation of the overall skin tone to a lighter shade by inhibiting Tyrosinase, Melanin and Pigmentation.

Anti-inflammation and anti-glycation of collagen

▸ **Anti-ageing : Dermis protection**

Inhibition of Collegenase, Elastase and Hyaluronidase

1.2. CLINICAL TRIALS AND TESTINGS

The effective action of **VIQUA®** was confirmed in a clinical study of a group of women who were observed before, during and after the treatment.

The effects of pomegranate extracts on molecular and cellular mechanisms have been researched on the systemic level, where benefits for cardiovascular, prostate, dental, and metabolic health have been shown in the clinical literature [1].

Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. Pomegranate juice has shown considerable anti-atherosclerotic, anti-hypertensive, antioxidant, and anti-inflammatory effects in human subjects and mouse models [2].

The pomegranate is a highly celebrated medicinal food plant chosen as the symbol of medicine for the 2000 UK Millennial Festival of Medicine.

1.3. POMEGRANATE ANTIOXIDANTS

This ability to deliver active molecules to specific skin sites has been demonstrated with VIQUA®, a concentrate of pomegranate antioxidants encapsulated through the ADS nanotechnology.
Numerous studies have shown the potential whitening and anti-ageing benefits of pomegranate antioxidants for the skin.

However, finished products containing pomegranate extracts have so far been limited in their effects due to a lack of stability in one of the fruit's main active molecules, punicalagin.

VIQUA® is a new type of nano-engineered antioxidant that is made up of a pomegranate extract titrated in 40% punicalagins and then encapsulated by ADS[1] nanotechnology. The encapsulating carrier uses phospholipids which have been extracted from pomegranate seed oil, making VIQUA® a 100% pomegranate extract product.
Dramatically improved stability of the pomegranate antioxidants is the first benefit of the encapsulation process. When heated to 100°C for one hour, the antioxidant content of VIQUA® now remains almost unchanged. Additional tests showed the ingredient to be completely safe in oral as well as topical applications.

In vitro the encapsulated pomegranate extract shows a dose-dependent, SOD[2]-like antioxidant activity which is superior to most common antioxidant plant extracts. Additionally, VIQUA® demonstrated the ability to inhibit the destructive action of the skin enzymes collagenase and elastase, thus potentially protecting the skin from the effects of premature ageing.

The real breakthrough of VIQUA® however is its ability to specifically transport the pomegranate antioxidants into the skin cells mitochondria, believed to be only possible through ADS nanotechnology.

[1] ADS, Advanced Delivery System.
[2] SOD, Super Oxyde Dismutase.

1.4. A HIGH STANDARD OF DELIVERY

When used in beauty products, nanotechnology-based delivery systems address three key issues.

Firstly, they aim to increase the bioavailability of their encapsulated active molecules en route to the target site. This optimises efficacy levels across the relevant body barriers – the skin for topical application and the digestive tract for oral application [3].

Secondly, consumer safety is paramount. All nano-based actives should be based on safe, biodegradable materials and should also limit the efficacy to a specific organ, tissue or even type of cell.

Finally, nanotechnology-based delivery systems should simplify the formulations of finished products. This incorporates aspects such as stability with a range of temperatures, pHs and common ingredients, reduced colour and smell, and extended shelf-life. These factors help in retaining a formulation's active molecule content, which in turn helps to avoid customer disappointment. Cosmetics that use antioxidant plant extracts often fall short of their claims, for example because they require special packaging, has shorter shelf-lives and an overall limited degree of marketability. Nanotech engineered delivery systems should improve the stability, safety and bioavailability of an active ingredient to the point where it can be used in a wide variety of oral and topical applications. Reaching this level allows many 'in and out' treatments to be offered from a common core active molecule [4].

As a new generation of nano delivery systems, ADS satisfies all of the above requirements. It gives finely targeted delivery to sites currently unreachable with other methods and offers a superior level of safety plus an outstanding degree of encapsulated active stability. A nano-size liposome of amino phospholipids, ADS is incorporated with patented Nano Active Receptors (NARs) from fenugreek. When embedded in a membrane of other bio-active ingredients, it then plays the role of a guiding device to deliver the encapsulated active molecules to a pre-defined target site. This allows for targeted,

innovative and controlled release of active compounds which will only reach the skin, regardless of whether the active is administered orally or topically.

This new delivery system offers the following benefits:

a. Made from 100% vegetable ingredients
b. Sustained delivery over a long period of time
c. Targeted delivery, in time as well as space
d. Lower required dosage of the administered actives – avoiding the risk of overdose
e. Protection against external aggression and heat resistance
f. High concentration of actives ADS can be tailored to fit specific needs.

Modifying the composition and size of the nano carrier affects the speeds of degradation and content delivery. It is also possible to increase the targeting accuracy by changing its composition or electrical charge, or by adding receptors or adhesion factors to its outer surface. Thanks to its outstanding stability and safety, it allows the same active ingredients to be delivered both orally and topically, increasing its marketable potential.

2. OBJECTIVES

The objective of this research is to demonstrate the antioxidant effects of the supplement VIQUA® and properties of its compounds. The research of this active is made in base of different essays to determinate the power of its effects. In vivo and in vitro test have been made to achieve the conclusions we show you in the present document.

The process of extraction from Pomegranate plant occurs by nano-technology under a patented method. The results are a brown powder which contains all the interesting components.

The present study shows the several properties of this compound and its advantages for the skin [5].

3. MOLECULES STRUCTURE

VIQUA® pomegranate extract is made with the whole fruit: peel, seeds and juice are involved in the manufacturing process so all the following properties detailed are inside the extract.

The quality of the extract is linked to the quality of the fruit which is recollected in the right period to achieve the better benefits.

3.1. BIOACTIVE COMPOUNDS

Pomegranate juice shows potent antioxidant and anti-atherosclerotic properties attributed to its high content of polyphenols including EA[3] in its free and bound forms [as ETs[4] and EA glycosides (EAGs)], gallotannins and anthocyanins (cyanidin, delphinidin and pelargonidin glycosides), and other flavonoids (quercetin, kaempferol and luteolin glycosides) [6].

Plant component	Constituents
Pomegranate juice	anthocyanins; glucose, ascorbic acid; ellagic acid, gallic acid, caffeic acid; catechin, EGCG; quercetin, rutin; numerous minerals, particularly iron; amino acids
Pomegranate seed oil	95% punicic acid; ellagic acid; other fatty acids and sterols
Pomegranate pericarp (peel, rind)	phenolic punicalagins; gallic acid and other fatty acids; catechin, EGCG; quercetin, rutin, and other flavonols; flavones, flavonones; anthocyanidins
Pomegranate leaves	tannins (punicalin and punicafolin); and flavone glycosides, including luteolin and apigenin
Pomegranate flower	gallic acid, ursolic acid; triterpenoids, including maslinic and asiatic acid; other unidentified constituents
Pomegranate roots and bark	ellagitannins, including punicalin and punicalagin; numerous piperidine alkaloids

Table 1. Pomegranate Fruit Parts and Constituents**.**

Over the past decade, significant progress has been made in establishing the pharmacological mechanisms of pomegranate and the individual constituents responsible for them. Extracts of all parts of the fruit appear to have therapeutic properties, and some studies report the bark, roots, and leaves of the tree have medicinal benefit as well. Current research seems to indicate the most therapeutically beneficial pomegranate constituents are ellagic acid ellagitannins (including punicalagins), punicic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones.[7] (see **Table 1)**

All of showed components are present in high quantity in VIQUA®.

[3] EA, Ellagic Acid
[4] Ets, Ellagitannins

3.1.1 Punicalagin

Punicalagin (see **Fig 1**) belongs to the polyphenols group and it is the major antioxidant polyphenol ingredient in pomegranate juice (over 50% of the antioxidant activity of pomegranate) and the most abundant in this specie.

It exists in two different isomers and it is located in most proportion in arils and skin of pomegranate fruit. Punicalagin is water soluble.

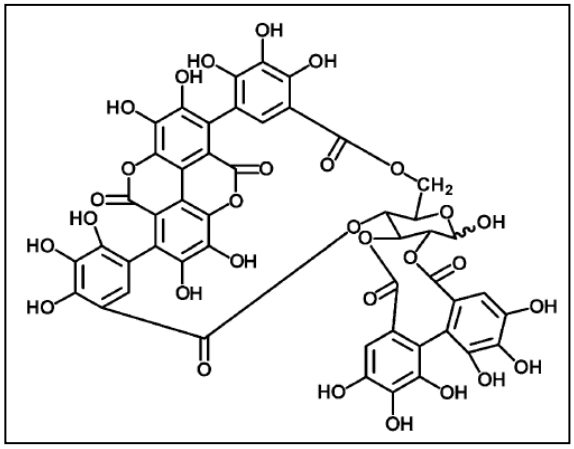

Fig 1.Punicalagin molecule structure.

A significant reduction in feed consumption and body weight could be related to high levels of it in the extract in many tests.

Pomegranate extract contents environ 6% punicalagin anomers.

Punicalagin presents a very strong lipid peroxidation inhibitory activity in a liposome model system with an EC50 value of 54.2±0.9. Antioxidant action of punicalagin is expressed not only through its scavenging reactions but also by its ability to form metal chelates. It could happen that the ability of punicalagin to inhibit lipid peroxidation was exerted by complexing iron in a catalytically silent form. This activity must be due to chain termination by scavenging the peroxyl radicals by donating electrons.

It was shown potent DPPH (2,2-diphenil-1-picrihidracilo) free radical scavenging activity, with an EC50 value of 16.7 lg/ml.

The protective effects of antioxidants in biological systems are ascribed mainly to their capacity to scavenge free radicals, chelate metal catalysts, activate antioxidant enzymes and inhibit oxidases. The radical scavenging ability of punicalagin is due for the multiple phenolic hydroxyl groups which increase the antioxidative activity by additional resonance stability and o-quinone or p-quinone formation.

3.1.2 Resveratrol

Resveratrol (see **Fig 2**) is a phytoalexin used by plants to protect themselves from fungi. This molecule has a strong anti-inflammatory effect.
Resveratrol is a potent inhibitor of copper initiated LDL[5] oxidation even more effective than flavonoids.

Resveratrol was found to inhibit growth and induce apoptosis in human cancer cells through both CD95-dependent and –independent mechanisms [8].

Several studies within the last few years have shown that resveratrol inhibits ribonucleotide reductase and DNA polymerase, induces nitric oxide production, and suppresses cell growth by arresting cells at the S and G2 phases of the cell cycle in a number of animal cells [9].

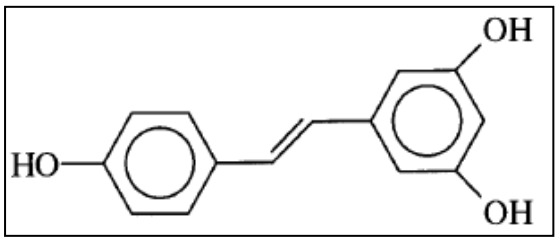

Fig 2. Resveratrol molecule structure.

The studies of immunomodulatory activity of resveratrol have shown that at the concentrations of 25-50 μM it suppressed significantly mitogen-, IL-2-, or alloantigen-induced proliferation of splenic lymphocytes and the development of antigenspecific

[5] LDL, low density lipoprotein.

cytotoxic T lymphocytes. It inhibited cytokine production by splenic lymphocytes and peritoneal macrophages. The Poliphenolic inhibited proliferation of cultured keratinocytes and fibroblasts, presumably, by inactivation of arachidonic acid cascade.

The free radical scavenging by flavonoids is highly dependent on the presence of a free –OH which makes an easy reaction by the single hydroxyl group in the B ring which apparently makes little contribution even in the presence of the conjugated double bond system and the 3-OH group [10].

The planar resveratrol structure makes easy the conjugation with others. Planarity permits conjugation, electron dislocation, and a corresponding increase in flavonoid phenoxyl radical stability.

Due to the presence of three hydroxyl groups resveratrol offers several possibilities to attack. Resveratrol present successfully high values of inhibition of lipid peroxidation.

3.1.3 Anthocyanins

Anthocyanins (see **Fig 3**) are flavonoids, a type of poliphenols bioactive compounds. It is a natural pigment responsible for red, purple, and blue coloration in plants and the largest and most important group of polar-soluble pigments in nature.

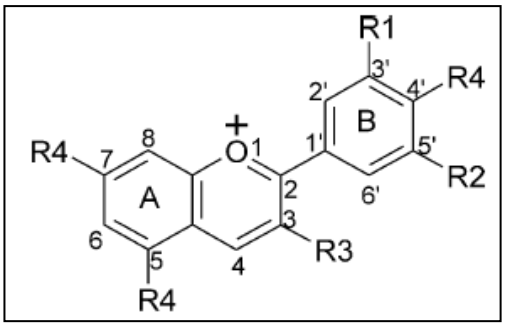

Fig 3. Anthocyanin molecule structure.

All the effects of the anthocyanins listed can be explained by several antioxidant mechanisms, including hydrogen donation, metal chelation, and protein binding. Anthocyanins can also prevent the oxidation of ascorbic acid caused by metal ions through chelating the metal ions, and forming ascorbic (copigment)-metalanthocyanin complex. It is found that anthocyanins were absorbed in their unchanged glycated forms in elderly women [11].

There are several explanations for the antioxidant mechanisms of anthocyanins. Firstly, anthocyanin with 2-benzopyran core structure is a kind of conjugate structure. The unpaired electron is not fixed in the oxygen atom, but is close to the benzene ring, thus weakening the hydrogen bond. Thus the activity of the hydrogen atom of the hydroxyl is increased, and is easily lost to become a hydrogen donor [12].

The antioxidant capacity of anthocyanins is related to the number of phenolic hydroxyls on the B-ring. Since phenolic hydroxyl is the functional group of antioxidation, then the more phenolic hydroxyls the stronger the activity. In addition, some researchers have thought that anthocyanins can chelate metal ions, as well as forming complexes with protein molecules.

Anthocyanins are able to reduce capillary permeability and fragility, so they could be the key component in red wine that protects against cardiovascular disease. The anthocyanin pigment prevented the generation of free oxygen radicals, and decreased the peroxidation of lipids.
It has been suggested that anthocyanins have the ability to stabilize DNA triple-helical complexes.

Anthocyanins could be located in the flowers (pelargonidin 3,5-diglucoside and pelargonidin 3-glucoside) and also in arils [13].

3.1.4 Ellagic acid

Eleganic acid (see **Fig 4**) belongs to polyphenols group more specifically to tannins, is a dimeric derivative of gallic acid. Ellagic acid has antioxidant, anti-inflammatory, and anticarcinogenetic properties [14].

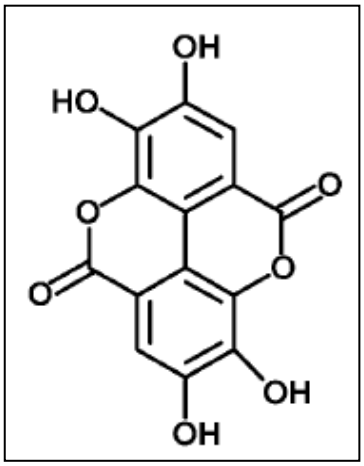

Fig 4. Ellagic acid molecule structure.

Anticarcinogenic effect such an induction of cell-cycle arrest and apoptosis, as well as the inhibition of tumor formation and growth in animals [15].
Extract contents 1,3% Ellagic acid [16].

Ellagic acid (EA) in pomegranate fruit is low and may increase in juice and supplements as a result of the hydrolysis of ellagitannins [17].

EA that is metabolized by the human microflora to yield bioavailable derivatives

Ellanic Acid was detected in human plasma at a maximum concentration after 1h postingestion but was rapidly eliminated by 4 h. Therefore, the presence of free EA in human plasma could be due to its release from the hydrolysis of ellagitannins (ETs), facilitated by physiological pH and/or gut microbiota.

Antioxidant properties can be attributed to the ET structures characterized by the presence of several hydroxy functions in ortho, which exhibit a greater ability to donate a hydrogen atom and support the unpaired electron, as compared to those of low molecular weight. EA has been shown to inhibit IL-1beta- and TNF-alpha induced activation of activator protein-1 and mitogenactivated protein kinases in activated pancreatic stellate cells in vitro which support its anti-inflammatory properties.

They can act as antioxidants, phytoalexins or as antibacterial agents are related to the treatment of illnesses involving tissue inflammation or capillary fragility [18].

3.2. MODE OF ACTION

Although pomegranate's wide-ranging therapeutic benefits may be attributable to several mechanisms, most research has focused on its antioxidant, anticarcinogenic, and anti-inflammatory properties.

The superior bioactivity of pomegranate compounds is illustrated the multifactorial effects and chemical synergy of the action of multiple compounds compared to single purified active ingredients.

Pomegranate juice and extracts have been shown to have potent in vitro antioxidant and in vivo anti-atherosclerotic properties, attributed to its high content of polyphenols including Ellagic Acid [19]

It is shown that pomegranate juice and its purified polyphenols are potent antioxidants which may be a mechanism whereby they inhibit cancer cell proliferation and induce cancer cells to undergo apoptosis. Antioxidants may act in various ways, by scavenging the radicals, by decomposing peroxides and by chelating metal ions. Therefore, the antioxidant activity can and must be evaluated with different tests for different mechanisms [20].

Sample of lipid peroxidation inhibition:

Initiation $R^* + LH \rightarrow RH + L^*$
Propagation $L^* + O_2 \rightarrow LOO^*$
$LOO^* + LH \rightarrow LOOH + L^*$
$L^* + O_2 \rightarrow LOO^*$

Termination
$L^*/LOO^* \rightarrow$ nonradical products

Antioxidant action:
$ArOH + LOO^* \rightarrow ArO^* + LOOH$

These substances have the ability as an antioxidant, which plays an important role in inhibiting the oxidation of chemical reactions, which can damage macro-molecules and can cause various health problems. The process of aging and degenerative diseases such as cancer, cardiovascular disease, blood vessel blockage that includes hiperlipidemik, atherosclerosis, stroke and high blood pressure and

disrupted his body's immune system can be caused by oxidative stress [21].

Studies in rats and mice confirm the antioxidant properties of a pomegranate by-product (PBP) extract made from whole fruit minus the juice, showing a 19% reduction in oxidative stress in mouse peritoneal macrophages (MPM), a 42% decrease in cellular lipid peroxide content, and a 53% increase in reduced glutathione levels. In vitro assay of a fermented pomegranate juice (FPJ) extract and a coldpressed seed oil (CPSO) extract found the antioxidant capacity of both are superior to red wine and similar to green tea extract. A separate study in rats with CCl4-induced liver damage demonstrated pretreatment with a pomegranate peel extract (PPE) enhanced or maintained the free-radical scavenging activity of the hepatic enzymes catalase, super oxide dismutase, and peroxidase, and resulted in 54% reduction of lipid peroxidation values compared to controls

Research in humans has shown a juice made from pomegranate pulp (PPJ) has superior antioxidant capacity to apple juice. Using the FRAP assay (ferric reducing/antioxidant power),it was found that 250 mL PPJ daily for four weeks given to healthy elderly subjects increased plasma antioxidant capacity from 1.33 mmol to 1.46 mmol, while subjects consuming apple juice experienced no significant increase in antioxidant capacity. In addition, subjects consuming the PPJ exhibited significantly decreased plasma carbonyl content (a biomarker for oxidant/antioxidant barrier impairment in various inflammatory diseases) compared to subjects taking apple juice. Plasma vitamin E, ascorbic acid, and reduced glutathione values did not differ significantly between groups, leading researchers to conclude pomegranate phenolics may be responsible for the observed results.

3.2.1. Mitochondria Penetration

VIQUA® can be shown to penetrate skin cells intact. In an in-vitro test, human 3T3-L1 adipocytes were cultured for three hours with VIQUA®, into which had previously been incorporated a fluorescent red marker. After incubation, fluorescent microscope pictures revealed that VIQUA® is specifically located inside the adipocytes, and more specifically in the zones surrounding the lipid droplets, known to be extremely abundant in mitochondria [**Annexe 2**]

VIQUA® is not only located inside the cells either, but also performs its antioxidant action from there. In a second test, adipocytes (both cultured with VIQUA® and not) were subjected to elevated oxidative stress, created by the addition of H_2O_2.

VIQUA® cultured adipocytes show a dose dependant ability to reduce oxidative stress, thus proving that the ADS powered antioxidants which are located inside the cells are active.

A further study demonstrated that VIQUA® specifically targets the cell mitochondria. In this study, the same adipocytes which were previously cultured with red fluorescent VIQUA® were also marked by a specific MitoTracker Green FM probe stained in fluorescent green; this compound is known to accumulate exclusively inside cell mitochondria. As a result, the MitoTracker's position on a microscope [**Annexe 2**] shows the exact location of the mitochondria inside the observed cells. As can be seen, the green and the red fluorescence appear on the same sites inside the cell. It can therefore be concluded that VIQUA® is located at the same spots as the MitoTracker –inside the cell mitochondria.

As the main site of free radical production, and origin of the cell's oxidative stress, the mitochondrion is a key target for any antioxidant treatment. By encapsulating pomegranate extracted antioxidants with ADS nanotechnology, VIQUA® is expected to specifically target the mitochondria of skin cells and perform antioxidant action from inside them, resulting in considerable benefits for the skin.

4. CLINICAL TRIALS

4.1. TEST IN VITRO

4.1.1. SOD-LIKE ACTIVITY

VIQUA® showed a radical scavenging activity like the Superoxide Dismutase present in cells of our body [22].

Superoxide Dismutase reactions:

$M^{(n+1)+}$-SOD + O_2^- → M^{n+}-SOD + O_2
M^{n+}-SOD + O_2^- + $2H^+$ → $M^{(n+1)+}$-SOD + H_2O_2.

4.1.2. INHIBITION OF COLLAGENASE AND ELASTASE. ANTI-AGEING BENEFITS.

Collagenase is an enzyme involved in the degradation of collagen, a protein found in connective tissues, bones, tendons, cartilage and skin, a constituent of membranes that surrounds and separates groups of cells and organs. Elastase is an enzyme involved in the degradation of elastin, the protein responsible for the elastic properties of vertebrate tissues.

Collagenase and elastase are put in the culture isolated from human tissue. After this procedure the trial is realized in this medium to study their activity in presence of proteins. The results is a decreasement of the action to break up proteins structure and it is to modulate its natural activity.

The test have confirmed the action of VIQUA® extract to inhibit collagenase and ellastase action throught several visible factors in skin.

4.1.2.1. Anti-elastase test

This enables the direct inhibiting action of VIQUA® on elastase activity of fibroblasts in human derma to be demonstrated. This assessment allows comparison of the fibroblast elastase activity after 72h treatment of the cells with different concentrations of VIQUA®.

Cells were washed free of phosphate-buffered saline, resuspended in DME medium supplemented with 10% (v/v) foetal-calf serum, and cultured at a final density of 1.5 x 107-2 x 107 cells per T75 flask.

Culture techniques.

Approx. 1.5 x 107 exudative cells can be obtained from animal tissue. A complete enzyme purification protocol required medium conditioned by about 3 x 101 cells.

Cells can be allowed to adhere for 2-4h, them washed twice with Hanks' balanced salt solution. To each flask we then added 10ml of DME-LH medium containing 2,µM-colchicine. All media contained penicillin (50units/ml) and streptomycin (50pg/ml). Cells can be cultured for 48 h, after which the conditioned medium can be collected and frozen at -20°C. The conditioned medium is replaced with fresh DME-LH medium + 2,uM-colchicine. This process could be repeated until 500-600ml of conditioned medium to be obtained.

Elastase activity can be measured by determining the amount of radioactivity released after incubation of enzyme preparations at 370C with 200,g of [3Hlelastin in 100mM-Tris/HCl buffer, pH7.8, containing 5 mM-CaCl2 and 0.02% NaN_3. Final reaction volume was 300,ul. Working stock solutions of [3Hlelastin (2mg/ml in 3-fold-concentrated assay buffer) to be stored at 40C for 1-2 weeks. At the end of incubation, assay tubes are centrifuged for 3 min in a Beckman Microfuge. The radioactivity remaining in 100,ul of supernatant is measured by liquid-scintillation' spectrometry. One unit of elastase activity was defined as the solubilization of 1.0,ug of elastin/h at 370°C.

One elastase unit is defined as the quantity of enzyme liberated by 1 µmol 4- nitroanilide/min. The elastase activity is then expressed as mU/mg protein.

5 concentrations were tested:

1 mg/ml, 5 mg/ml, 10 mg/ml, 25 mg/ml, 50 mg/ml.

Ethanol controls were carried out simultaneously at the same concentrations.

- **Results**

VIQUA® is capable of inhibiting, in dose dependant fashion, the catalytic activity of elastase, by direct effect at doses of 1mg/ml (15% inhibition) 5mg/ml (22% inhibition), 10 mg/ml (45% inhibition) 25 mg/ml (58% inhibition) and 50 mg/ml (78% inhibition) [**Annexe 3**].

The results for each concentration are expressed as the variation in optical density per minute calculated from the regression lines at 405 nm.

4.1.2.2. Anti-collagenase test

This is the demonstration of the inhibiting capacity of VIQUA® on the collagenase activity in human derma. This assessment allows comparison of the fibroblast collagenase activity after 72h treatment of the cells with different concentrations of VIQUA®.

Culture techniques.

Explants can be cultured in disposable flasks (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) in Dulbecco's Modified Eagle's medium in an atmosphere of 02-C02 (95: 5, v/v). Culture medium can be harvested daily for 10 days and examined for collagenase activity as well as for immunologic evidence of collagenase, a2-macroglobulin, and a1-antitrypsin.

Media having collagenase activity are pooled, dialyzed against several changes of distilled water at 4°C, and lyophilized. Crude enzyme powder can be stored at - 20°C. Medium from the first 24 h. of culture is dialyzed and lyophilized separately from that of the remaining days.

Enzyme purification. Collagenase powder can be dissolved in 0.05 M Tris-HCl (pH 7.5) containing 0.005 M CaCl2 to a concentration of approximately 20 mg protein per ml. Ammonium sulfate fractionation to be carried out at pH 7.0 and 0°C to a final saturation of 60%. The 60%o precipitate dissolved in 0.05 M Tris-HC1 (pH 7.5) with 0.005 M CaCl2 and dialyzed against large volumes of the same buffer. Gel filtration could be performed using reverse flow at 40C on a column 1.2 X 100 cm of Sephadex G-150 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), equilibrated with 0.05 M Tris-HCl (pH 7.5) containing 0.005 M CaCl2 and 0.2 M NaCl in a manner similar to that described by Harris, DiBona, and Krane. Fractions having collagenase activity are pooled, dialyzed against 0.05 M Tris-HCl (pH 7.5) with 0.005 M CaC12, and concentrated by pressure dialysis. The broad peak of enzyme activity obtained from Sephadex G-150 was further fractionated by gel filtration on a column (0.9 X 60 cm) of Sephadex G-75 which has been equilibrated with the same buffer in a manner similar to that used for human skin collagenase. The collagenase concentrations (ng/ml) were then corrected according to the cell protein levels (µg/ml).

5 concentrations were tested:
0.1 mg/ml, 0.5 mg/ml, 1 mg/ml, 5 mg/ml, 10 mg/ml.

Ethanol controls were carried out simultaneously at the same concentrations.

Collagenase activity is expressed in ng of collagenase free per µg cellular proteins.

- **Results**

VIQUA® anti-collagenase activity is dose dependant up to:

5 mg/ml, this concentration is capable of reducing cell collagenase activity by 47%.

10 mg/ml, this concentration is capable of reduction cell collagenase activity by 60%.

The mean of optical densities obtained at 630 nm (collagenase dosage) for each concentration were calculated.[**Annexe 3**]

The appearance of wrinkles is related to collagenase and elastase natural action in skin. After the test, VIQUA® seems to be able for inhibing action of collagenase and elastase. Every subjet shows an increment in attenuation of wrinkles (58% improvement) and skin hydration (78% improvement), both factors are related to the structure proteins so it is an index of collagen and

elastine present in sking tissue (skin tone, skin roughness and skin wellness are factor also improved in the test).

4.1.2.3. Anti-hyaluronidase test

This demonstrates the inhibiting capacity of VIQUA® relative to the hyaluronidase activity of MDCK[6] epithilial cells.

This assessment allows comparison of the MDCK epithilial cell hyaluronidase activity after 72h treatment of the cells with different concentrations of VIQUA®.

The activity is assessed by the rate at which groups of N-acetyl glucosamine derived from degradation of hyaluronic acid are freed. Measurement of the N-acetyl glucosamine (GNAc) levels present in the various concentrations is made using a colorimetric reaction with 4- dimethyl aminobenzaldehyde at 585nm.

One unit of hyaluronidase is defined as being the quantity of enzyme freed by 1µmole N-acetylglucosamine per min, the catalytic concentration expressed in units of hyaluronidase per mg cellular proteins (U/mg prot.) was calculated for each concentration.

5 concentrations were tested :
0.1 mg/ml 0.5 mg/ml 1.1 mg/ml 5.0 mg/ml 10.0 mg/ml

Ethanol controls were carried out as comparison at the same concentrations.

- **Results**

This study showed that VIQUA® is capable of reducing cell hyaluronidase activity. The maximum effect is reached with a concentration of 5 mg/ml.

No direct inhibiting action on the isolated enzyme probably means that the VIQUA® acts on synthesis of the enzyme rather than by inhibiting hyaluronidase activity.

[6] MDCK, Madin-Darby Canine Kidney

4.1.3. STABILITY TEST

The pyrolysis of VIQUA® does not occur at a normal food processing temperature for 60 minutes.

The bioestability is an Important factor which can allow the biodisponibility at several conditions

4.1.4. BIO-EFFICACY with ADS

Intracellular localization of VIQUA® by fluorescence microscopy using lipophilic probes.

The method used a fluor marker to know VIQUA® penetration and movements. Adypocites are able to receive VIQUA® compound and encapsulate the antioxidant ingredient into the cell.
After the preparation of adypocites cells, it is realized an incubation of human cells with the labeled VIQUA®. Adipocytes were incubated in the presence of VIQUA® containing a red-labeled lipid probe.
The probe locates at the surrounding area of lipid droplet.

High-pressure freeze-substitution electron microscopy of intact lipid droplets within 3T3-L1 adipocytes.

The contact area between lipid droplets and adipocyte cytoplasm reveals an electron-dense material surrounding the lipid droplet which concentrates several proteins (TIP47, adipophilin, perilipin and S3-12) the so-called “lipid droplet cortex”. Embedded within this material are numerous mitochondria.

Antioxidant capacity of VIQUA® in a cellular system (adipocytes).

Differentiated adipocytes or human breast adenocarcinoma MCF-7 cells were subjected to oxidation with 5 _M H2O2 in the presence and in the absence of VIQUA®.
_ Malondialdehyde (MDA) is an end-product of lipid peroxidation and a biomarker for oxidative stress which may oxidize proteins and DNA.
_ MDA was determined in cellular supernatants by HPLC coupled to fluorescence detection [23].

Co-localization of VIQUA® and mitochondria in adipocytes

MitoTracker Green FM probe is essentially non fluorescent in aqueous solutions and only becomes fluorescent once it accumulates in the lipid environment of mitochondria. (InvitroGen).

_ MitoTracker Green FM probe preferentially accumulates in mitochondria regardless of mitochondrial membrane potential (InvitroGen) [**Annexe 2**].

4.1.5. RESULTS AND CONCLUSIONS

VIQUA® is successfully stained by using a fluorescent red-labeled rhodamine phospholipid probe for cellular localization studies.
When 3T3-L1 murine adipocytes are incubated with red-labeled VIQUA®, red staining localizes around adipocyte lipid droplets.

1. It has been reported that mitochondria are located at the lipid droplet cortex of 3T3-L1 adipocytes.
2. MitoTracker Green FM probe preferentially accumulates in mitochondria.
3. MitoTracker Green FM probe co-stains with red-labeled VIQUA® at the intracellular space in adipocytes.
4. VIQUA® diminishes the amount of MDA in 3T3-L1 murine adipocytes oxidized in the presence of H2O2.
5. VIQUA® may act as source of antioxidants in adipocyte mitochondria environment.

In vitro test shown in 3 days the following effects:

1. Up to **60%** reduction in the activity of collagenase
2. Up to **74%** reduction in the activity of elastase

4.2. SAFETY

Pomegranate and its constituents have safely been consumed for centuries without adverse effects. Studies of pomegranate constituents in animals at concentrations and levels commonly used in folk and traditional medicine note no toxic effects. Toxicity of the polyphenol antioxidant punicalagin, abundant in pomegranate juice, was evaluated in rats. No toxic effects or significant differences were observed in the treatment group compared to controls, which was confirmed via histopathological analysis of rat organs.

Research in 86 overweight human volunteers demonstrated the safety of a tablet PFE[7] in amounts up to 1,420 mg/day (870 mg gallic acid equivalents) for 28 days, with no adverse events reported or adverse changes in blood or urine laboratory values observed.

Another study in 10 patients with carotid artery stenosis demonstrated PJ consumption (121 mg/L EA equivalents) for up to three years had no toxic effect on blood chemistry analysis for kidney, liver, and heart function.

4.2.1. TOXICITY TEST – FIXED DOSE PROCEDURE TRIAL

Objective: this study is realized to determine the toxicity level of VIQ-919 compound in mice.

Dose tested:
300 mg/Kg b.w and 2000 mg/Kg b.w
Globally Harmonised System (GHS) Classification:
5 / Unclassified
Starting Date of test:
9/7/2008
Observation:
14 days
Laboratory:
Instituto de Biología Molecular y Celular
Universidad Miguel Hernández
Elche 03202 (Alicante). SPAIN

[7] PFE , Pomegranate Fruit Extract

- **Test Substance**

Denomination: VIQ-919. Pomegranate fruit concentrate
Colour: Red
Appearance: Viscous uniform liquid, cloudy
Taste: Characteristic

- **Vehicle**

The VIQ-919 was diluted in 0.9% NaCl water solution, pH 7.4. Administered total volume was 0.220 ml in the treated and control groups.

- **Test animals**

Adult ICR female mice with 7-8 weeks were used for the assessment of the acute toxicity. Females, nulliparous and non-pregnant, were randomly assigned to control and treated groups (5 animals per group). The animals were kept in their cages 7 days prior to start of dosing to allow for acclimatisation.
Literature surveys of conventional LD50 tests show that usually there is little difference in sensitivity between the sexes but, in those cases where differences were observed, females were generally slightly more sensitive. Therefore the use of a single sex (females) is acceptable and contributes to a further decrease in the use of animals in testing [24].

4.2.1.1. Test conditions and observation

Mice received 300 mg/Kg b.w or 2000 mg/Kg b.w of aqueous suspension of VIQ-919 by oral administration using a gastric tube. Control group received is volumetric amount of the 0.9% NaCl water solution.

After the VIQ-919 was administered, food was withheld for 1 hour. After that, animals received food (Harlan Teklad Global diet) and water and libitum.

The temperature of the animal room was 23-25ºC. Lighting was artificial, with 12 hours of light and 12 in dark.

Mice were observed individually during the first 24 hours for the onset of any immediate toxic signs, with special attention during the first 4 hours, and daily during the 14 days observation period to record any delayed acute effects. All animals were killed after day 14th. The animal observation included, among others, changes in skin and fur, eyes, respiration and somatomotor activity, with especial attention to the presence of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Individual weights of mice were determined before the test substance administration and at least 4 days a week. At the end of the study the surviving animals were weighted before humanly killed.

- **Ethical issues**

Conditions of breeding and work with laboratory animals followed the regulations of Council Directive 86/609/EEC about Good Laboratory Practice (GLP) on animal experimentation.

4.2.2. RESULTS AND CONCLUSIONS OF THE TEST

There were no deaths of mice administered with 300 mg/Kg b.w or with 2000 mg/Kg b.w of VIQ-919 within short and long-term outcome [**Table 1** in **Annexe1**].
Body weight gain during the observation period among the two treated groups was comparable to their respective controls [**Table 2** in **Annexe1**]. No significant differences were noticed on weight gain between treated and control groups at the end of the study [**Table 3** in **Annexe1**].

The oral administration of VIQ-919 did not cause any appreciable alterations in water and food intake in both treated groups during the observation period of time.

No behavioural signs of toxicity were observed in both treated groups (300 mg/Kg or 2000 mg/Kg b.w) with VIQ-919 including tremors, convulsions, diarrhea, lethargy, etc. Two and three of the five treated mice with 300 and 2000 mg/Kg b.w of VIQ-919, respectively showed low mobility in the first 5-15 minutes after oral administration. A rapid increase

in their activity was evident after this short period of time in most of the animals, but in the 300 mg/Kg b.w group two mice showed low mobility 20 min after administration, even one of them showed nauseas.

However in the following hour any signs of abnormal behaviour were evident [**Table 3** in **Annexe1**].

- **Post examination. Clinical observation**

Once the assay was completed, two individuals from each group of treatment (300 and 2000 mg/Kg) with VIQ-919 were sacrificed and compared with their respective controls. Observation comprised examination of the external surface of the body (skin), all orifices, mucous membranes and the cranial, thoracic and abdominal cavities and their contents.

The post-mortem analysis of the individuals subjected treated did not show abnormalities on vital organs such as brain, heart, lungs, liver, spleen, kidneys or intestines. Pancreas, liver and spleen showed normal size and colour. Digestive system (stomach, duodenum, ileum, etc.) was also completely normal compared to controls [see **pictures 1, 2** in **Annexe 1**].

No particular dosage-related effects were observed, since neither toxicity nor abnormal behavior were noticed in 300 or 2,000 mg/Kg b.w groups of VIQ-919.

- After postmortem examinations of the sacrificed individuals were carried out, it can be confirmed through gross observations the complete absence of abnormalities in the vital organs examined: brain, heart, lungs, liver, spleen, kidneys or intestines, in the mice group treated with 2,000 mg/Kg b.w of VIQ-919.

- Mice administered with 300 or 2,000 mg/Kg b.w of VIQ-919 did not develop any clinical signs of toxicity either immediately or during the post treatment period. No mortality occurred in any of the treatment groups.

Taking these results into account and following the OECD Guideline, the VIQ-919 should be classified by the Globally Harmonised System (GHS) as unclassified or very low toxicity substance (GHS 5).

- The LD50 of VIQ-919 is considered to be higher than 2,000 mg/Kg b.w. because no death occurred in the mice group treated with 2,000 mg/Kg b.w. For a person of an average of 70 Kg, the toxicity level could be equivalent to a value higher than an intake of 140g of VIQ-919.

- As recommended by OECD Guideline Nº 420 for testing of chemicals, the use of a fixed dose level higher than 2,000 mg/Kg should be considered exceptionally and only when is justified by specific regulatory needs. For reasons of animal welfare concern, testing on animals in GHS Category 5 ranges (2,000-5,000 mg/Kg) is discouraged and should only be considered when there is a strong likelihood that results of such a test have a direct relevance for protecting human or animal health or the environment, or some evidences of the possible toxicity of the compound have been previously pointed out.

4.3. TEST IN VIVO

The phytochemistry and pharmacological actions of all *Punica granatum* components suggest a wide range of clinical applications for the treatment and prevention of cancer, as well as other diseases where chronic inflammation is believed to play an essential etiologic role.

4.3.1. ABSORPTION, METABOLISM, AND ANTIOXIDANT EFFECTS IN HEALTHY HUMAN VOLUNTEERS

4.3.1.1. Introduction

Several studies have been performed in the assessment of absorption of polyphenols from beverages and dried extracts.

Overall, very little information is available regarding effective amounts of antioxidant dietary supplements.

The potent bioactivity of punicalagins and other ellagitannins can be explained by its ability to hydrolyze into ellagic acid (EA) and other smaller polyphenols in vivo and across the mitochondrial membrane in vitro.

Due to the health benefits now attributed to pomegranate consumption and the rising popularity of pomegranate botanical supplements, human intervention studies using pomegranate extract supplements are crucial for determining the efficacy of pomegranate extracts in the prevention of chronic diseases and establishing science-based dosing recommendations. The current study is the first to investigate pharmacokinetic parameters, absorption, metabolism, and ex vivo biological activities of a whole-fruit extract of pomegranate standardized to punicalagins in healthy human volunteers [25].

4.3.1.2. Method

Standardized pomegranate extract in capsules form. Each capsule contained 400 mg of pomegranate extract. The 800 mg of extract used in this study contained 330.4 mg of the major ellagitannins punicalagins and 21.6 mg of EA as shown in the HPLC-PDA profile.

4.3.1.3. Instruments used

The HPLC system (Agilent Technologies, Waldbronn, Germany) was equipped with a diode array detector (DAD) and mass detector in series with a binary pump and autosampler and controlled by software (v. A08.03) from Agilent Technologies (Waldbronn, Germany). The HPLC column was a C18 LiChroCART column (25 _ 0.4 cm, particle size 5 m, Merck) and the solvent system consisted of a gradient system with water (5% formic acid, v/v)

The HPLC system included a LC-10ATVP pump, Rheodyne-7725i sampler, and SPD-10AVP detector with Winchrome software and a Luna C-18 column (4.6 _ 150 mm). The mobile phase, solvent A (2% glacial acetic acid in water) and solvent B (2% aqueous acetic acid in water), was used in binary linear gradient conditions as follows: 0-5 min, 99% A in B; 5-20 min, 99-40% A in B; 21-30 min, 40-10% A in B with a flow rate of 1.0 mL/ min. The wavelength was monitored at 378 nm for punicalagins and 366 nm for EA.

4.3.1.4. Subjects selection

30 healthy male and nonpregnant/ nonlactating female subjects (BMI = 32.6 ± 0.98, Age = 37.6 ± 3.6) were asked to abstain from polyphenol-containing foods, such as tea, wines, berry fruits, etc., for 3 days according to a detailed list they were given, after approval from the University of Florida Institutional Review Board. Moreover, subjects were asked not to consume large amounts of alcohol and antioxidant supplements, not to exercise excessively, and to sleep at least 6-8 h during the night before the study day.

4.3.1.5. Variables assessed

HPLC-MS Analysis of Plasma Samples

EA and its in vivo generated metabolites were measured by LC-MS/MS methods. In addition, ex vivo antioxidant activity of plasma (ORAC assays) was determined. Punicalagins were not detected in plasma as previously reported for animals. However, consistent with previous studies involving consumption of ellagitannins, EA and EA-derived metabolites were detected as follows: 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one glucuronide (urolithin A, M-H m/z 389), hydroxy urolithin A (M-H m/z 243), urolithin A-glucuronide (M-H m/z 403), 3-hydroxy-6H-dibenzo[b,d]pyran-6-one glucuronide (urolithin B; m/z 227), and dimethyl ellagic acid-glucuronide (M-H m/z 505). Urolithins A and B were detected after 8 and 24 h in altogether 3 of the 11 subjects, whereas urolithin A-glucuronide was detected in 6 of the 11 subjects. Hydroxyl-urolithin A was found in 3 subjects at several time points from 2 to 24 h. Also urolithin A-glucuronide was found over a period of 2-24 h in 6 of the subjects. Dimethyl ellagic acid-glucuronide was detected in 2 subjects after 8 and 24 h. Urolithins have been proposed as microbial metabolites that are biosynthesized in the colon and can circulate in plasma up to 24 h after the intake. The presence of these metabolites in early plasma samples (<8 h) in

our study may be attributed to either the action of colonic microflora on ellagitannins previously consumed by the subjects or may be due to plasma enzymatic action on circulating ellagic acid, although the latter possibility remains to be investigated (see **Fig.5**).

Pharmacokinetic Analysis.

High interindividual variability was observed, as the metabolism of polyphenols is known to vary in human subjects. For several subjects a complete data set was not available due to concentrations being below the lower limit of quantification of 1.6 ng/mL. For this reason pharmacokinetic parameters were estimated using the mean for all subjects.

Three of the subjects had extraordinarily high concentrations of EA in their plasma after 24 h. These values were eliminated, as noncompliance was assumed. The observed mean C_{max} and t_{max} for EA in plasma were determined as 33.8 ± 12.7 ng/mL at 1 h.

Antioxidant Capacity (ORAC Assay)

After the consumption of the pomegranate extract, the antioxidant capacity of plasma was significantly increased after 0.5 h (31.8%). The individual increase in antioxidant capacity was up to 2.55-fold after 0.5 h, up to 1.62-fold after 1 h, and up to 1.43- fold after 2 h. A second peak in antioxidant capacity was determined after 6 h (31.7%). Subjects were allowed to consume food after the 4 h blood draw; therefore, this second increase in antioxidant capacity is likely to be influenced by the consumption of food or potentially by more slowly absorbed antioxidant compounds from the pomegranate extract. Since t_{max} of EA was observed after 1 h, the peak effects of antioxidant capacity in plasma may not have been caused by EA but rather by other phenolic compounds, metabolites, or a combination of these with EA.

Overall, the pomegranate extract administered in this study potentially caused an increase in plasma antioxidant potential, characterized by a high intersubject variability. A more comprehensive study including a placebo group is required to confirm the antioxidant effects of the pomegranate extract.

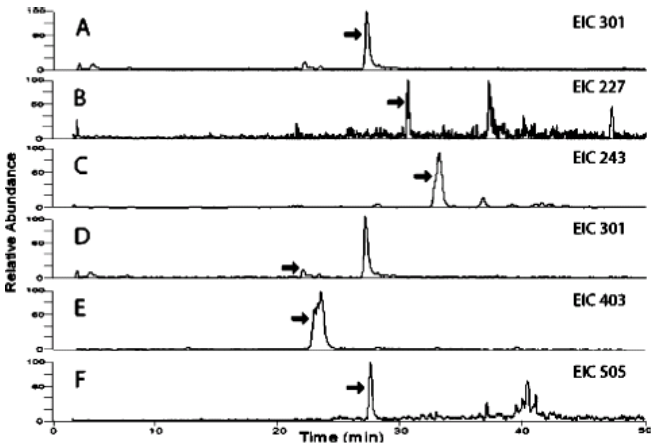

Fig. 5. HPLC-MS extracted ion chromatograms showing the presence of ellagitannin metabolites in human plasma.

4.3.2. EFFECTS OF ORAL ADMINISTRATION OF ELLAGIC ACID-RICH POMEGRANATE EXTRACT ON ULTRAVIOLET-INDUCED PIGMENTATION IN THE HUMAN SKIN

4.3.2.1. Introduction

Pomegranate has been extensively used in traditional medicines in many countries. The Chinese, for example, have used pomegranate as a traditional product antibacterial, anti-inflammatory and hemostasis applications.

Pomegranate is rich in phenolic compounds, and very rich in ellagic acid which is a natural occurring phenolic compound found in many natural sources like pomegranates and other red fruits. It has been reported that ellagic acid has a high affinity for copper at the active site of tyrosinase and inhibits its activity. It is thought that ellagic acid has a topically effect due its application, it suppressed UV-induced skin pigmentation of humans.

In the present study, it is performed a double-blind, placebo-controlled trial to clinically evaluate the protective and ameliorative effects of ellagic acid in pomegranate extract on pigmentation in the skin

after ultraviolet ray (UV) irradiation, using female subjets in their 20s to 40s.

4.3.2.2. Method

The study was a placebo-controlled double blind study during 4 weeks of ingestion of the supplement twice a day and after breakfast

30 subjects per group were randomly assigned to the high-dose pomegranate extract (200 mg/d extract), low-dose pomegranate extract (100 mg/d) and placebo control.

The test food used was a round tablet (300 mg in weight and 9 mm in diameter) containing a 90% of ellagic acid.

The control food was a placebo tabled containing dextrin, cellulose, maltitol and sucrose esters of fatty acid and caramel colour.

4.3.2.3. Instruments used

A spectro-colorimeter (Minolta Co., Ldt.) was used for measuring L values, and a Mexameter for measuring melanin and erythema values.

4.3.2.4. Subjects selection

39 female volunteers between 20 and 40 years old were enrolled in the study. Exclusion criteria:

- Those who routinely used medical product and/or cosmetics with whitening effects, such as ellagic acid and vitamin C.
-Those who had atopic dermatitis or other skin disorders.
- Those who had severe deseases such as diabetes mellitus, liver disorders, renal disorders or cardiovascular deseases.
- Those who had a food allergy
- Those who were pregnant, breast-feeding or expected to become pregnant during the study period.
-Those who had a menstrual disorder or whose skin condition was substancially changed by menstruation.
-Those who were already enrolled in another study at the start of this study.

The dropout and discontinuation criteria for test subjects were as follows:

1) Those with a low intake rate of the test food (less than 80%)

2) Those detracting frm the reliability of study results by acts such as missing recording in the diary and others.

3) Those who became unable to continue the study due to personal reasons.

No new volunteers were recluted to replace dropout subjects.

Informed consent for the study was obtained from all subjects in accordance with the objectives of the Helsinki Declaration.

4.3.2.5. Variables assessed

Each subject took orally the two tablets prescribed together with cold or warm water, taking care not to masticate the tablets, after breakfast for 4 weeks.

Measurement of whitening effects (protective or ameliorative effects on UV-induced pigmentation): On the day before the start of ingesting the test food, each subject received UV irradiation on the inside of upper left arm and on the next day (at the start of the test food intake), a minimum erythema dose (MED was measured). Before ingesting the test food, each subject received 1.5 MED of UV irradiation on the inside of the upper right arm. Luminance (L), melanin and erythema values were determined before the start of the test food intake and at 1, 2, 3 and 4 week after the start of the test food intake.

The skin color around the region irradiated with UV rays was also measured, for correcting the values, because the trial was performed during summer.
A questionnaire consisting of 20 items were conducted regarding the state of the skin before and after the test food intake period. Subjects checked

"yes" or "no" for questionnaire items before the test food intake, and evaluated the state of the skin in five grades (improved, slightly improved, no change, slightly worsened, worsened) after the end of the test food intake period.
Subjects enter the state of taking the test food, physical condition and other information every day in their diary.
The statistical analysis was a paired t-test used to asses intergroup differences of the changing rate of values determined before and at 1,2,3 and 4 week after the start of test food intake.

- **Evaluation of whitening effects**

The inhibitory and ameliorative effects of the test food on UV-induced pigmentation in the skin were evaluated by intergroup comparison of the values before and at 1, 2, 3 and 4 week after the start of the test food intake, as well as the data of questionnaires before and after the test food intake period, between the test food groups and control group.
The measures with the spectro colorimeter expressed in L (luminance) values for all groups showed a decrease in first measure [26].

Melasma is a topical disorder manifesting a dark color distributed in spots in the skin. The treatment with pomegranate extract can demonstrate a visible amelioration, due the measures of luminescence showed in this experiment.

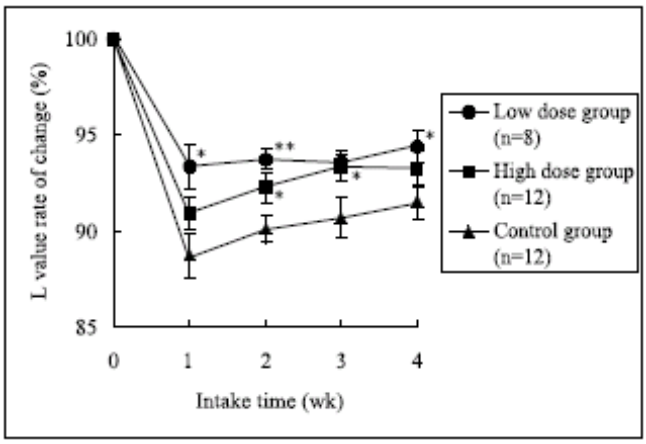

Fig 6. Change in L values in the UV-irradiated region on the inside of the upper arm in the three groups. Rate of change in L value at each measuring time point versus the baseline values considered as 100% are shows. Data are means±SE *p<0.05 versus control group, **p<0.01 versus control group.

In only 1 week the test group showed an inhibition in melanin production up to 1.73% of inhibition compared to Placebo group.
Both, group high dose pomegranate extract and low dose show minimum pigmentation during the study (see **Fig.6**).

It is known that ellagic acid present in pomegranate extract inhibits melanin formation by acting on tyrosinase, wich is the main enzyme-producing melanin.

Pomegranate extract orally administered to humans has a protective effect on slight sunburn caused by UV irradiation even at such low doses as were used in the study.

- **Evaluation of erythema and melanin**

The change in melanin value (measured with Mexameter) was evaluated in test groups. A tendancy to inhibit the increase in melanin production was observed after 1 week of intake.

Changes in erythema were observed and measured. A tendancy to inhibit the increase in the erythema values was observed after 1 week of intake.

- **Results of questionnaire**

The items answered concerning the state of skin conducted at the end of the test food intake period and were analized using Willcoxon's paired test, indicating improvement in "brightness of the face" and "stains and freckles" larger in test groups.

4.3.3. INGESTION OF VIQUA® SUPPLEMENT AND STUDY IN SKIN CONDITIONS

4.3.3.1. Study description

A placebo-controlled double-blind ingestion study of VIQUA® supplement was performed. A double blind, placebo controlled clinical study on 30 volunteers with orally administered. Clinical trial was

realized in base on the dose for the effect achievement 250 mg/day (125 mg twice a day).

The evaluated parameters were: Hydration, Tonicity, Inflammation, Desquamation, and Elasticity. The subjects were asked to keep in the rest in the room for at least 30 minutes. Further, with respect to the make up at the measurement site, it was in principle prohibited to put on makeup from 60 minutes before the inspection.
During the study, subjects were asked to give a self-assessment of their visual skin appearance using a Visual Numerical Scale (0-10).

4.3.3.2. Method

The study was a placebo-controlled double blind study of 4 weeks ingestion of VIQUA® supplement containing pomegranate extract and Placebo (same appearance of test supplement). The ingestion period was 4 weeks in both groups.

The treatment consisted in 2 tablet of 125 mg (product or placebo) taken twice a day, so the total diary intake is 250 mg orally per day.

4.3.3.3. Instruments used

*Corneometer CM 825 - Visioscan VC 98 – Skicon

Several parameters related to skin have been monitored using the face as test area.

4.3.3.4. Subjects selection

Individuals who showed signs of skin ageing (facial lines, wrinkles, dark spots, loss of skin elasticity) were recruited. 30 individuals were evaluated (female: 30 – Between 30 and 55 years) skin phototype I-IV. Before execution of the study, physicians fully explained the purpose of this study to the subjects, and written consents were obtained.

The subjects treated with VIQUA® always expressed a higher satisfaction score compared with those who were given the placebo Pomegranates are nature's secret gift to youthful, beautiful looking skin.

VIQUA® follows where nature leads, in this case powered by the innovative ADS nano-delivery system made of pomegranate itself, VIQUA® concentrates the beautifying essence of pomegranate in its most powerful and bioavailable form.

The result is a whitening, anti-ageing and anti-inflammatory beauty product with clinically proven efficacy. Its perfect stability and safety allows use in a diverse range of food and cosmetic applications.

This new generation nano delivery system demonstrates how advancement in nanotechnology exploits the best of nature and science to raise the efficacy and safety of active ingredients to a new level.

4.3.3.5. Variables assessed

- **Skin hydration:**

Skin hydration improved significantly under treatment, whereas the placebo group showed a decrease.

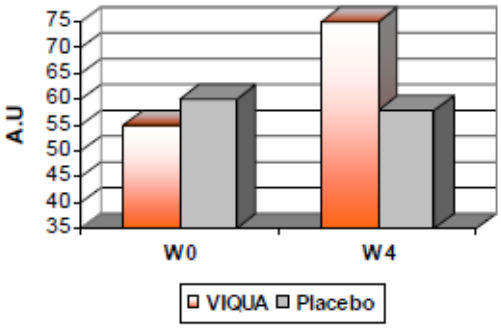

Fig 7. Comparing skin hydration condition before and after applying Viqua / Placebo.

Re-establish the natural healthy balance of the skin. Improve dry skin by boosting the health and strength of the layers of the skin so that it naturally keeps hydration locked in.

Skin hydration improved significantly under treatment (+40%), whereas the placebo group showed a decrease (-0,3%) (See **Fig 7**).

- **Skin roughness:**

The skin roughness, measured with Visiocan VC98, has significantly decreased only after the 0 active treatments.

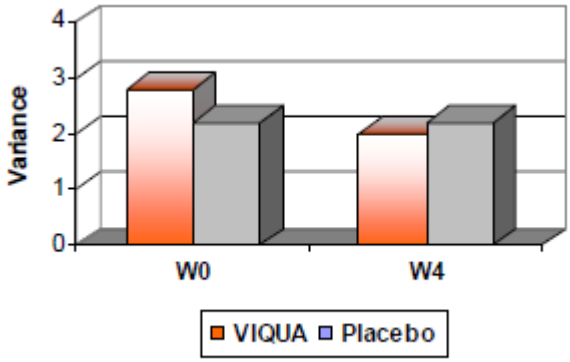

Fig 8. Comparing decrease of skin roughness before and after applying Viqua / Placebo.

The skin roughness, measured with Visioscan VC98, has significantly decreased only after the active treatment (-31%)(see **Fig 8**).

- **Skin smoothness:**

The surface is a parameter measured with Visioscan VC98, which calculates the size of the wavy skin surface, is an indirect measurement of skin smoothness. High smoothness will lead to decreased surface In this case; the surface has significantly decreased only after the active treatment. The skin smoothness has improved only after the active treatment.

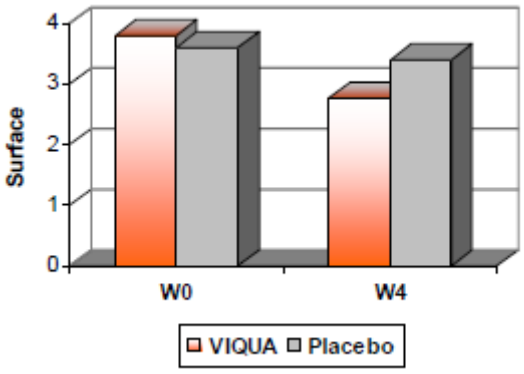

Fig 9. Comparing surface parameter of skin before and after applying Viqua / Placebo.

The active treatment increased the health and strength of the dermis, epidermis and supporting structures, resulting in healthier, younger looking skin.

Skin smoothness improved, the surface parameterhas significantly decreased only after the active treatment (-27%)(see **Fig 9**).

- **Depth of wrinkles:**

The volume parameter, measured with Visioscan VC98, is indicator for the depth of wrinkles. In this study, the volume has significantly decreased only with the active treatment (see **Fig 10**).

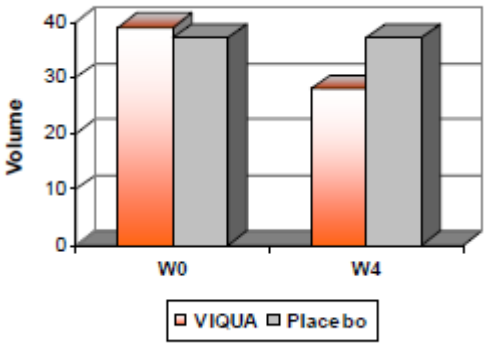

Fig 10. Comparing depth of fine lines and wrinkles before and after applying Viqua / Placebo.

A significant decrease in depth of fine lines and wrinkles was recorded (volume -26%).

- **Water content:**

The water content of stratum corneum, Measured by Skicon, has significantly increased only after treatment.

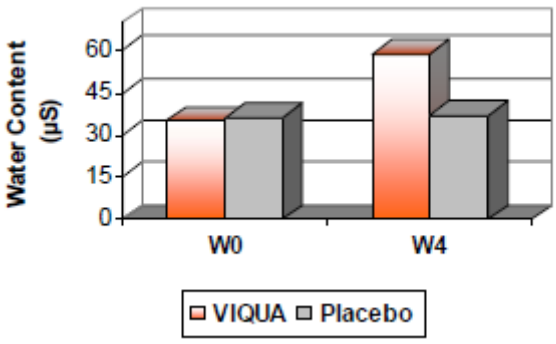

Fig 11.. Comparing water content of skin before and after applying Viqua / Placebo.

The water content of the skin's upper layers particularly improved with VIQUA® (51%)

Water content of stratum corneum has significantly increased only after the active treatment whereas in placebo group it is decreased. (see **Fig 11**).

- **Skin inflammation:**

Source of inflammation, dark spots have disappeared only after the active treatment. Skin inflammation has been significantly improved.
Source of inflammation, dark spots have disappeared only after the active treatment.

Significant inflammation inhibition has been noticed through blood vessels remodeling

- **Blood circulation:**

At Week0 it was observed high permeability of bloods vessels.

Blood vessel remodeling suggested significant inhibition of inflammation processes.

At Week4 the permeability was significantly reduced and blood circulation increased [**Annexe 1**]

The self-assessment test shows evident perception's differences of all the estimated parameters that are correlated to the product's efficacy. The subjects assuming the active treatment have always expressed a higher score compared with the one's assuming the placebo.

The active treatment has proved much better than placebo. The clinical study confirms the results from the biophysical measurements.

The treatment was well tolerated and there were no reported side effects.

- **Action in Melasma**

Melasma (or chloasma) is a common disorder of cutaneous hyperpigmentation predominantly affecting sun-exposed areas in women. The pathogenesis of melasma is not fully understood and treatments are frequently disappointing and often associated with side effects.

Melasma tends to appear during pregnancy (mask of pregnancy) and in women who take oral contraceptives, although it can occur in anyone. The disorder is most common in sunny climates and among people with darker skin. Irregular, patchy areas of dark colour appear on the skin, usually on both sides of the face. Rarely, melasma appears on the forearms.

In a clinical trial to test melasma evolution with antioxidants treatment has be realized. Factors like relationship to pregnancy, hormonal therapy, sun exposure and cosmetic use has to be taken. Patients are asked about their previous use of hydroquinones and family history of melasma.

Melasma may be due to blood circulation, which may cause the formation of patches.

For the melasma area, the diameter of the melasma area was measured by a ruler at three different places and the mean values were determined. The pigmentary intensity was rated using the national standard colour chart [27].

The benefits showed in melasma are:

Skin tone became generally brighter and dark spot/blemish color attenuated. The responsible are anthocianins which can enhance blood circulation in cells signifying a Melasma reduction of 80%.

4.3.4. PHYTOESTROGENS IN POMEGRANATE EXTRACT. ESTROGEN-LIKE BIOLOGICAL ACTIVITY

Japanese women are reported to have a low frequency of hot flushes compared with postmenopausal western women, in part attributed to their high phytoestrogen consumption. Whether this is valid has been recently challenged.

Evidence from several human studies demonstrates that certain dietary phytoestrogens can produce mild estrogenic effects in the postmenopausal woman, including estrogen-like effects on vaginal cytology

and reductions in hot flushes. The variations in response may depend on populations studied; products used, and study design, particularly with respect to duration of exposure, variability in response, and nonresponse in some postmenopausal women to phytoestrogen supplementation.

The use of certain plants in traditional medicine and folklore may be ascribed to their estrogenic properties. The pomegranate is associated with fertility.

It is also well known that the dried pomegranate seeds contain oil, which has been shown to contain not only the steroidal estrogen estrone, but also the isoflavonic phytoestrogens genistein and daidzein and the phytoestrogenic coumesten, coumestrol. When injected, a hydrolyzed concentrate of the oil produced increased uterine weight in immature rabbits, and cornification of vaginal epithelium in ovariectomized adult mice, both indicative of estrogenic action.

Finally the tremendous recent explosion of interest in phytoestrogenic compounds in medicine for potential prophylaxis and treatment of menopausal vasomotor phenomena, osteoporosis, estrogen depletement cardiovascular disease and cancer (Knight and Eden, 1996) raises the possibility that pomegranate seed oil and extracts might be employed in menopausal women as external and internal phytoestrogen medicaments, as a possible alternative or supplement to conventional hormone replacement therapy (HRT)

Phytoestrogen supplementation like VIQUA® may benefit some women in alleviating menopausal symptoms, however it is difficult to make specific recommendations of formulation and dosage.

4.3.5. RESULTS AND CONCLUSION

In the VIQUA® group, significant decrease was confirmed in the SEsm value and the SEsc value at the poll, whereby it was found that the smoothness of skin was recovered, the dryness of corneum was reduced and the scale was decrease and confirmed in every value.

The treatment was well tolerated and there were no reported side effects.

- Visibly smoother and softer skin
- Diminished appearance of fine lines & wrinkles
- Increased skin hydration and suppleness
- Balanced skin tone
- Skin wellness & youthful appearance

Participants' comments were very positive, with good tolerance and no side effects [**Annexe 4**].

Skin inflammation: Cold pressed pomegranate seed oil in VIQUA® has been shown to inhibit both cyclooxygenase and lipoxygenase enzymes in vitro.

Significant inflammation inhibition has been noticed through blood vessels remodeling; the active treatment has proved much better than placebo. The clinical study confirms the results from the biophysical measurements.

Whitening effect: In vitro studies using normal human epidermal keratinocytes and PFE demonstrate PFE incubation with cell cultures ameliorates ultraviolet A and B radiation induced cell damage in a dose- and time-dependent manner, providing evidence at a cellular level that PFE may be an effective photo-chemopreventive agent.

A double-blind, placebo-controlled trial evaluated the protective and ameliorative properties of **pomegranate extract** and its PFE constituent on UV-induced skin pigmentation.

The analysis of the questionnaire results demonstrated a trend toward amelioration of UV-induced damage in both Pomegranate Extract groups compared to placebo.

Method: 30 patients (skin types IV to VI) received a 6 weeks treatment (30 pills of 250mg). Both VIQUA® and Placebo pills had the same characteristics during 6 months.

The patients presented a depigmentation improvement of 67% with no side effects.

VIQUA® improves skin appearance by decreasing skin pigmentation and aging spots as Ellargic acid play important role in inhibiting tyrosinase, which is responsible for skin pigmentation.

Anti-oxidative stress: Oxidative stress, a major contributor to cardiovascular diseases, is associated with lipid peroxidation in arterial macrophages and in lipoproteins Pomegranate was indeed shown recently to possess impressive ant oxidative properties due to its polyphenolic, tannins and Anthocyanins, LDL oxidation is a key factor in the formation of plaque in the arteries, also called atherosclerosis.

Pomegranates are composed of alkaloids and soluble polyphenols, tannins, and anthocyanins, which scavenge free radicals and help prevent DNA damage. The main active constituent in VIQUA® extract is a phenolic compound known as ellagic acid which has high antioxidant and anti-viral benefits, and potent anti-cancer effects.

Visible results: 4 weeks after the end of the study, a significant improvement of the following parameters has been detected in the active treated groups:

- Skin hydration improved significantly under treatment in +**40%**, whereas the placebo group showed a decrease (-0,3%).
- The skin roughness, measured with Visioscan VC98, has significantly decreased only after the active treatment in -**31%**.
- Skin smoothness improved, the surface parameter has significantly decreased only after the active treatment in -**27%**.
- A significant decrease in the depth of fine lines and wrinkles were recorded -**26%**.
- Water content of stratum corneum has significantly increased only after the active treatment in + **51%.**
- Melasma reduction can be rise the **68%** as global score in only 30 days.

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ANNEXE 1. DATA COLLECTION

a) TOXICITY TEST

ACUTE ORAL TOXICITY – FIXED DOSE PROCEDURE
OECD GUIDELINE 420 (17-09-2001)

Test substance: **VIQ-919**

Sponsor: AXIALYS INNOVATIONS

Dose tested: 300 mg/Kg b.w and 2000 mg/Kg b.w

Globally Harmonised System (GHS) Classification: 5 / Unclassified

Starting Date of test: 9/7/2008

Observation: 14 days

Laboratory: Instituto de Biología Molecular y Celular
Universidad Miguel Hernández
Elche 03202 (Alicante). SPAIN

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0. OBJECTIVE

The general objective of this study is to determine the toxicity level of VIQ-919 in mice.

1. TEST SUBSTANCE

Identification of the test product: Information concerning the test product: Pomegranate fruit concentrate

2. VEHICLE

The VIQ-919 was diluted in 0.9% NaCl water solution, pH 7.4. Administered total volume was 0.220 mL in the treated and control groups.

3. TEST ANIMALS

Adult ICR female mice with 7-8 weeks were used for the assessment of the acute toxicity. Females, nulliparous and non-pregnant, were randomly assigned to control and treated groups (5 animals per group).

The animals were kept in their cages 7 days prior to start of dosing to allow for acclimatisation.

Literature surveys of conventional LD50 tests show that usually there is little difference in sensitivity between the sexes but, in those cases where differences were observed, females were generally slightly more sensitive (Lipnick RL et al 1995). Therefore the use of a single sex (females) is acceptable and contributes to a further decrease in the use of animals in testing.

4. TEST CONDITIONS AND OBSERVATIONS

Mice received 300 mg/Kg b.w or 2000 mg/Kg b.w of aqueous suspension of VIQ-919 by oral administration using a gastric tube. Control group received isovolumetric amounts of the 0.9% NaCl water solution. After the VIQ-919 was administered, food was withheld for 1 hour. After that, animals received food (Harlan Teklad Global diet) and water ad libitum. The temperature of the animal room was 23-25ºC. Lighting was artificial, with 12 hours of light and 12 in dark.

Mice were observed individually during the first 24 hours for the onset of any immediate toxic signs, with special attention during the first 4 hours, and daily during the 14 days observation period to record any delayed acute effects. All animals were killed after day 14th. The animal observation included, among others, changes in skin and fur, eyes, respiration and somatomotor activity, with especial attention to the presence of tremors, convulsions, salivation, diarrhoea, lethargy, sleep and coma.

Individual weights of mice were determined before the test substance administration and at least 4 days a week. At the end of the study the surviving animals were weighted before humanly killed.

5. ETHICAL ISSUES

Conditions of breeding and work with laboratory animals followed the regulations of Council Directive 86/609/EEC about Good Laboratory Practice (GLP) on animal experimentation.

6. RESULTS

There were no deaths of mice administered with 300 mg/Kg b.w or with 2000 mg/Kg b.w of **VIQ-919** within short and long-term outcome (Table 1).

Body weight gain during the observation period among the two treated groups was comparable to their respective controls (Table 2). No significant differences were noticed on weight gain between treated and control groups at the end of the study (Table 3).

The oral administration of **VIQ-919** did not cause any appreciable alterations in water and food intake in both treated groups during the observation period of time. No behavioral signs of toxicity were observed in both treated groups (300 mg/Kg or 2000 mg/Kg b.w) with **VIQ-919** including tremors, convulsions, diarrhea, lethargy, etc.

Two and three of the five treated mice with 300 and 2000 mg/Kg b.w of **VIQ-919,** respectively showed low mobility in the first 5-15 minutes after oral administration. A rapid increase in their activity was evident after this short period of time in most of the animals, but in the 300 mg/Kg b.w group two mice showed low mobility 20 min after administration, even one of them showed nauseas.

However in the following hour any signs of abnormal behavior were evident (Table 3).

6.1 MORTALITY

Table 1. Mortality and approximate lethal dose (LD) in mice treated orally with VIQ-919

Sex	Dose (mg/Kg)	N° of animals	N° of accumulated dead animals														Mortality* (%)	Approximate LD_{50} (mg/Kg)
			Days of treatment															
			Short-term			Long-term												
			0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
Female	Control	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	> 2000 mg/Kg
	300	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	
	2000	5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

* (Number of dead animals / Number of treated animals)*100

6.2 BODY WEIGHT CHANGES

Table 2. Body weight (g) changes in female mice treated orally with VIQ-919

Dose (mg/kg)	Animal Number	Days after treatment										
		0	1	2	5	6	7	8	9	12	13	14
0	Control 1	27	29	30	29	30	30	30	29	30	30	30
	Control 2	27	26	26	27	28	29	28	27	27	27	28
	Control 3	27	27	28	30	31	30	29	29	29	29	30
	Control 4	26	26	27	29	29	30	30	30	29	29	30
	Control 5	26	25	26	26	26	27	26	27	28	28	28
	Mean	**26.60**	**26.60**	**27.40**	**28.20**	**28.80**	**29.20**	**28.60**	**28.40**	**28.60**	**28.60**	**29.00**
	SD	**0.55**	**1.52**	**1.67**	**1.64**	**1.92**	**1.30**	**1.67**	**1.34**	**1.14**	**1.14**	**1.15**
300	Treatment 1	26	27	27	27	27	28	29	29	30	30	30
	Treatment 2	25	26	26	27	27	27	28	28	28	28	28
	Treatment 3	26	28	27	30	30	31	30	30	31	30	30
	Treatment 4	25	25	25	26	26	27	27	27	27	27	27
	Treatment 5	26	25	25	25	26	27	27	27	29	28	28
	Mean	**25.60**	**26.20**	**26.00**	**27.00**	**27.20**	**28.00**	**28.20**	**28.20**	**29.00**	**28.60**	**28.60**
	SD	**0.55**	**1.30**	**1.00**	**1.87**	**1.64**	**1.73**	**1.30**	**1.30**	**1.58**	**1.34**	**1.34**

Dose (mg/kg)	Animal Number	Days after treatment										
		0	1	2	5	6	7	8	9	12	13	14
2000	Treatment 1	27	27	28	28	28	30	30	31	31	30	30
	Treatment 2	27	27	28	29	29	30	30	30	29	28	29
	Treatment 3	28	29	28	29	29	29	29	28	30	30	30
	Treatment 4	25	27	25	25	24	26	26	26	27	28	28
	Treatment 5	27	28	29	30	30	29	30	29	30	31	31
	Mean	**26.80**	**27.60**	**27.60**	**28.20**	**28.00**	**28.80**	**29.00**	**28.80**	**29.40**	**29.40**	**29.60**
	SD	**1.10**	**0.89**	**1.52**	**1.92**	**2.35**	**1.64**	**1.73**	**1.92**	**1.52**	**1.34**	**1.14**

Table 3. Effect of VIQ-919 on weight gain in mice

Group	Mean initial weight (g)	Mean final weight (day 14)	Mean weight changes (day 14)
Control	26.60 ± 0.55	29.00 ± 1.15	2.60 ± 1.14
VIQ-919 300 mg/Kg	25.60 ± 0.55	28.60 ± 1.34	3.00 ± 1
VIQ-919 2000 mg/Kg	26.80 ± 1.10	29.60 ± 1.14	2.80 ± 0.84

Figure 1. Body weight changes in female mice orally treated with 300 mg/ Kg b.w of VI Q-919

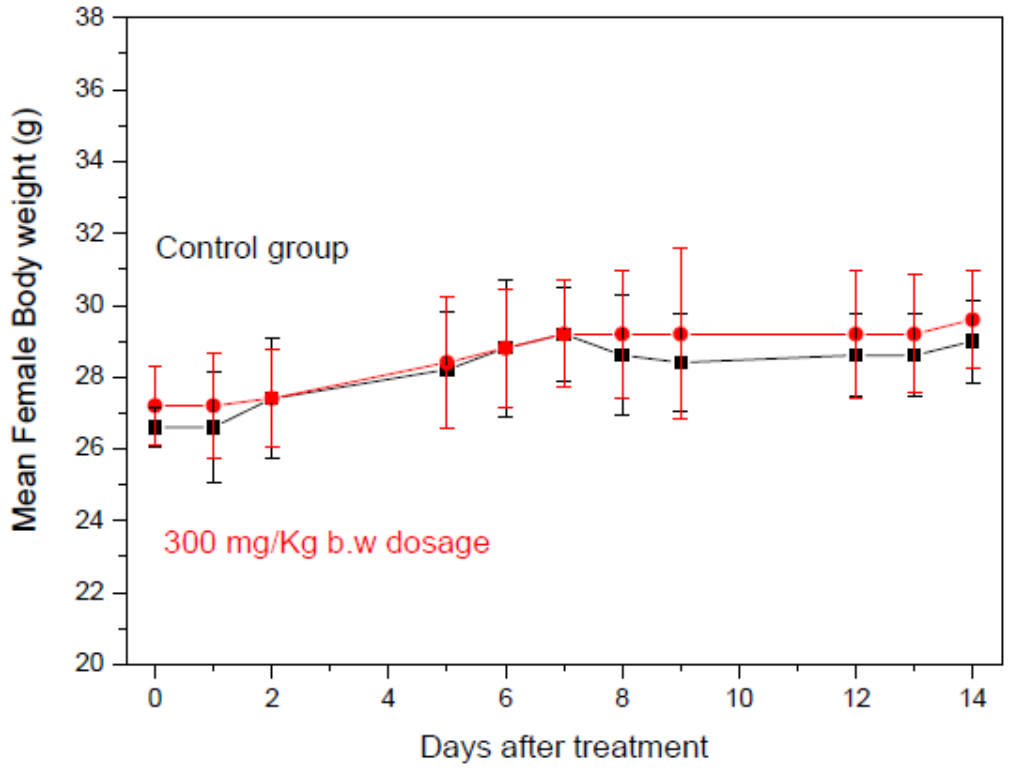
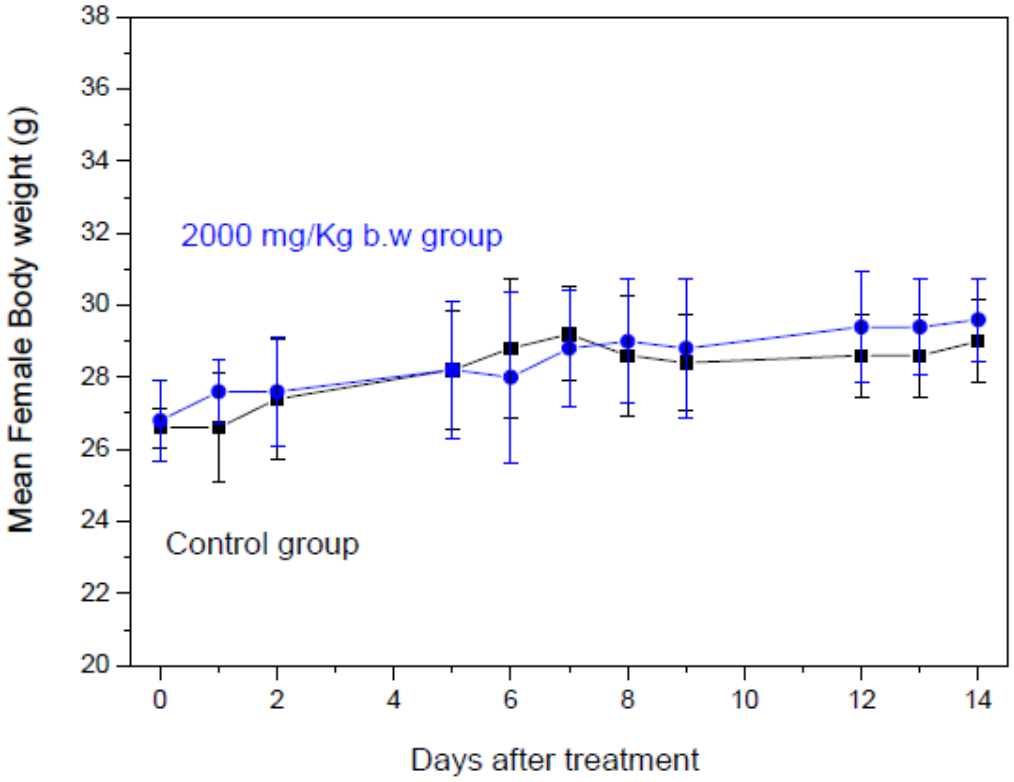


Figure 2. Body weight changes in female mice orally treated with 2000 mg/ Kg b.w of VIQ-919



7. POSTMORTEM EXAMINATION. MACROSCOPIC OBSERVATIONS

Once the assay was completed, two individuals from each group of treatment (300 and 2000 mg/Kg) with VIQ-919 were sacrificed and compared with their respective controls. Observation comprised examination of the external surface of the body (skin), all orifices, mucous membranes and the cranial, thoracic and abdominal cavities and their contents.

The postmortem analysis of the individuals subjected treated did not show abnormalities on vital organs such as brain, heart, lungs, liver, spleen, kidneys or intestines. Pancreas, liver and spleen showed normal size and color. Digestive system (stomach, duodenum, ileum, etc.) was also completely normal compared to controls

8. CONCLUSIONS OF THE TEST

- No particular dosage-related effects were observed, since neither toxicity nor abnormal behavior were noticed in 300 or 2,000 mg/Kg b.w groups of VIQ-919.

- After postmortem examinations of the sacrificed individuals were carried out, it can be confirmed through gross observations the complete absence of abnormalities in the vital organs examined: brain, heart, lungs, liver, spleen, kidneys or intestines, in the mice group treated with 2,000 mg/Kg b.w of VIQ- 919.

- Mice administered with 300 or 2,000 mg/Kg b.w of VIQ-919 did not develop any clinical signs of toxicity either immediately or during the posttreatment period. No mortality occurred in any of the treatment groups.

Taking these results into account and following the OECD Guideline, the VIQ- 919 should be classified by the Globally Harmonised System (GHS) as unclassified or very low toxicity substance (GHS 5).

- The LD50 of VIQ-919 is considered to be higher than 2,000 mg/Kg b.w. because no death occurred in the mice group treated with 2,000 mg/Kg b.w. For a person of an average of 70 Kg, the toxicity level could be equivalent to a value higher than an intake of 140 gr of VIQ-919.

- As recommended by OECD Guideline Nº 420 for testing of chemicals, the use of a fixed dose level higher than 2,000 mg/Kg should be considered exceptionally and only when is justified by specific regulatory needs. For reasons of animal welfare concern, testing on animals in GHS Category 5 ranges (2,000-5,000 mg/Kg) is discouraged and should only be considered when there is a strong likelihood that results of such a test have a direct relevance for protecting human or animal health or the environment, or some evidences of the possible toxicity of the compound have been previously pointed out.

b) IN VIVO TEST

Pilot study: To investigate whether nutritional intervention may favorably enhance skin rejuvenation, elasticity, and appearance.

Study product: VIQUA®

Sponsor: MI INNOVATION LABO on behalf of AXIALYS INNOVATIONS

Protocol number: 09-SR-OD-VIQ

Principal investigators: Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Elche 03202 (Alicante) SPAIN

Final report date: October 19, 2009

This study was performed accordance to the declaration of Helsinki and the requirement of Good Clinical Practices.

Protocol

A clinical randomized, double-blind trial comparing VIQUA® vs. Placebo.

Method

30 women aged 35 and 55 years participated in a double-blind, placebo-controlled trial testing the efficacy of a proprietary oral supplement for skin nutrition (VIQUA®) for improvement of skin rejuvenation, elasticity and appearance.

Dosage

Daily dose 0.5ml of VIQUA® added in 500 ml of water – Placebo (same characteristics as the ingredient).

Duration

30 days treatment

Instruments used

Corneometer CM 825 - Visioscan VC 98 – Skicon

Environment before measurements

Room temperature: 18 to 20°C, humidity: 45 to 60%

The subjects were asked to keep in the rest in the room for at least 30 minutes. Further, with respect to the make up at the measurement site, it was in principle prohibited to put on makeup from 60 minutes before the inspection.

Subjects backgrounds

	VIQUA® group	Placebo group
Number of subjects	15	15
Age (years old)	42 ± 5.1	46 ± 4.8
Height (cm)	162.8 ± 6.2	162.1 ± 7.5
Body weight (kg)	53.7 ± 5.2	55.3 ± 8.1
BMI	20.3 ± 1.6	21.3 ± 2.6
Number of smokers	3	2

No significant differences were observed in any of the background subjects including other factors of skin trouble, time and regularity of sleep, time of exposure to direct sunlight (hour/day), irregular menstruation, occupation, or alcohol drinking habit.

Results of dermatological diagnosis before and after ingestion of the tested food supplement

	VIQUA® group	Placebo group
Number of subjects	15	15
Age (years old)	42 ± 5.1	46 ± 4.8
Height (cm)	162.8 ± 6.2	162.1 ± 7.5
Body weight (kg)	53.7 ± 5.2	55.3 ± 8.1
BMI	20.3 ± 1.6	21.3 ± 2.6
Number of smokers	3	2

The value represents the mean of each group. (Non parametric analysis was employed for statistical analysis. 0 (no symptoms) – 1 (mild) – 2 (moderate) – 3 (severe)

Improvement rate of each symptom

Symptoms	Tested food supplement	Total n° of subjects with symptoms	Improvement rating				Improvement rate (improved or better)
			Markedly improved	Improved	Unchanged	Aggravated	
Smoother & softer skin	VIQUA®	11	4	5	2	0	81%
	Placebo	14	0	1	13	2	-7%
Reduction of fine lines & wrinkles	VIQUA®	14	1	7	6	0	57%
	Placebo	12	0	3	9	4	-8%
Increased skin hydration & suppleness	VIQUA®	9	2	5	2	0	78%
	Placebo	11	0	0	11	0	0%
Balanced skin tone	VIQUA®	14	3	7	4	0	71%
	Placebo	13	0	2	8	3	-7%
Skin wellness	VIQUA®	15	4	9	2	0	87%
	Placebo	15	0	0	15	0	0%

Measurement results of moisture content before and after ingestion of the tested food supplement

		VIQUA® group		Placebo group	
		Before ingestion	After 30 days	Before ingestion	After 30 days
Moisture content	1 cm below the left eye	43.3 ± 5.3	56.4 ± 7.1	45.7 ± 6.1	44.6 ± 5.2
	Left forearm (3 cm above the elbow)	37.2 ± 6.3	44.3 ± 5.7	38.9 ± 5.8	38.3 ± 6.7
	Dorsal neck (3 cm below spinous process of neck)	43.3 ± 7.8	56.2 ± 6.7	45.8 ± 5.4	43.9 ± 5.6

Each value represents the mean ± S.D. – The moisture content was measured by using Corneometer CM825 (manufactured by Courage + Khazaka Electronic Gmbh). This apparatus measures the moisture content of epidermis by measuring the electronic capacity via corneal layer.

Analysis by a Microscopic three-dimensional skin surface analyzer (VISIOSCAN)

(VISIOSCAN: manufactured by Courage + Khazaka Electronic Gmbh). In use of this apparatus, the skin surface was irradiated with special ultraviolet ray source, and the image is taken by a high performance CCD camera and digitalized for evaluation. The following factors were sampled as parameters.

a) **SEsm (Skin Smoothness)**

This value is calculated from the average of the width and depth of wrinkles by the formula (i), and one of indices which show the smoothness of skin. The smaller this value is, the smoother the skin surface is.

Fmx: average width of furrows for the row analysis

Fmy: average width of furrows for the column analysis

Co: right frontier of the histogram whose calculation is based on a set-up values.

Cu: left frontier of the histogram whose calculation is based on a set-up values.

b) Ser (Skin Roughness)

This value is obtained by calculating the ratio of points darker than the set-up points in the whole image and further calculating by the following formula (ii), and one of indices which show the roughness of skin. The higher the value is, the rougher the skin is.

I: counter whose start value is 0 and which is incremented each time the gray value of the current point is smaller than the threshold issued from the set-up programs.

Nx: amount of point per row.

Ny: amount of point per column.

c) SEsc (Skin Scaliness)

Epidermolysis parts are counted to be brighter than the set-up values in the image. The SEsc value is obtained by calculating the ratio of these parts relative to the entire part by the following formula (iii), and is one of indices which show the dryness of scale (corneum). The lower the value is, the more moist and the less epidermolysis (scale).

I: counter whose start value is 0 and which is incremented each time the gray value of the current point is bigger than the threshold issued from the set-up programs.

Nx: amount of point per row.

Ny: amount of point per column.

d) SEw (Skin Wrinkles)

This value is an index which is calculated by the following formula (iv) and shows the surface texture in vertical and horizontal directions of skin or the number and width of wrinkles. The higher the value is, the more the number of wrinkle is and the wider the width of wrinkles is.

Fax: amount of furrows for the row analysis.

Fmx: average width of furrows for the row analysis.

Fay: amount of furrows for the column analysis.

Fmy: average width of furrows for the column analysis.

K: factor

e) Correction K (Kurtosis)

This value shows the smoothness of the whole skin. This value shows the quality of histogram in hue point of skin. The closer to 0 the value is, the smoother in curve the hue point is and the closer to ideal skin.

VIQUA® group		Before ingestion	After 30 days
Kurtosis (Ideal value: 0)	Below left eye	0.60	0.30
	Left upper arm	0.95	0.36
	Poll	0.81	0.34
SEsm (Ideal value: Lowest value)	Below left eye	377.7	308.6
	Left upper arm	386.3	376.7
	Poll	442.9	338.9*
SEr (Ideal value: Lowest value)	Below left eye	0.45	0.20
	Left upper arm	0.36	0.21
	Poll	0.67	0.34
SEsc (Ideal value: Lowest value)	Below left eye	239.8	132.0
	Left upper arm	343.7	195.7
	Poll	356.2	175.2
SWw (Ideal value: Lowest value)	Below left eye	35.1	27.6
	Left upper arm	32.4	26.9
	Poll	29.7	23.5

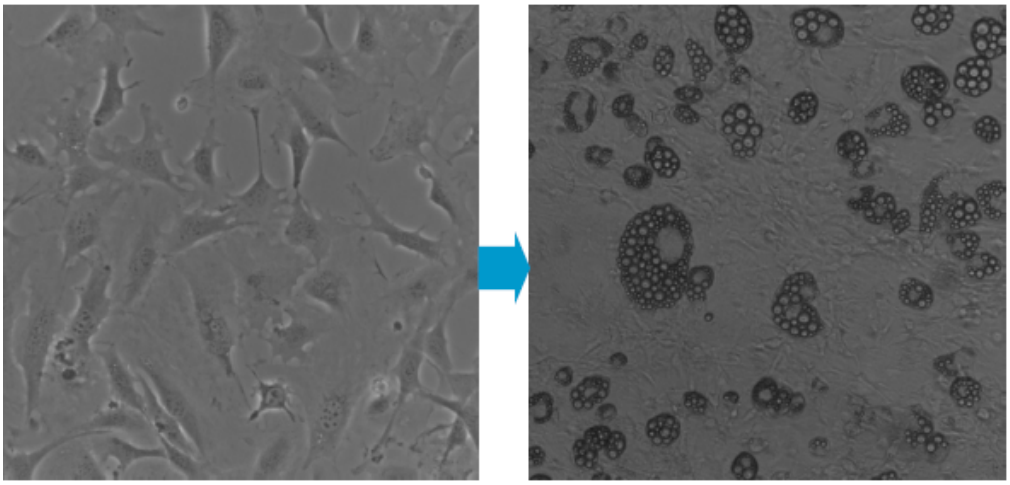
The values represent the mean in each group

VIQUA® group		Before ingestion	After 30 days
Kurtosis (Ideal value: 0)	Below left eye	0.41	0.39
	Left upper arm	0.46	0.44
	Poll	0.52	0.56
SEsm (Ideal value: Lowest value)	Below left eye	378.3	372.7
	Left upper arm	347.2	3456.6
	Poll	380.1	379.1
SEr (Ideal value: Lowest value)	Below left eye	0.27	0.23
	Left upper arm	0.18	0.19
	Poll	0.42	0.40
SEsc (Ideal value: Lowest value)	Below left eye	165.5	160.8
	Left upper arm	218.2	209.7
	Poll	238.6	216.2
SWw (Ideal value: Lowest value)	Below left eye	27.9	27.0
	Left upper arm	23.8	24.1
	Poll	28.5	29.2

The values represent the mean in each group

ANNEXE 2. VIQUA® BIO-EFFICACY with ADS®

Culture and differentiation of 3T3-L1 preadipocytes

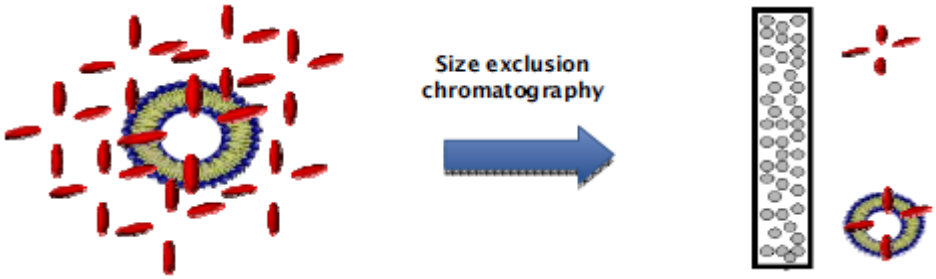


3T3-L1 preadipocytes Differentiated adipocytes

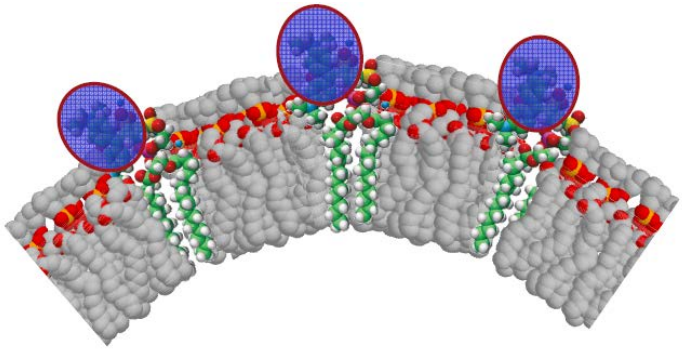
Differentiation culture media (12-15 days)

Labelling of VIQUA® nanocapsules

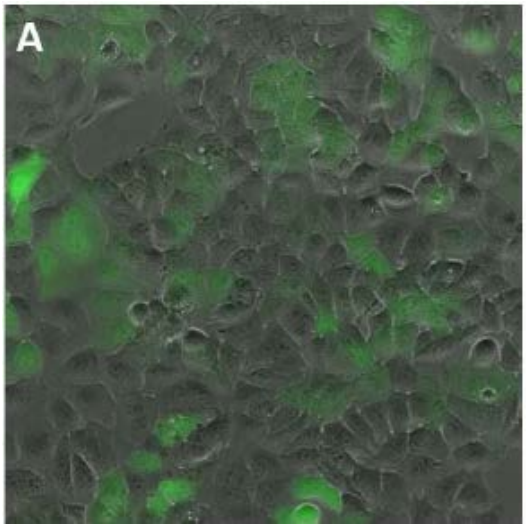
Separation of labelled VJQUA® from free probe



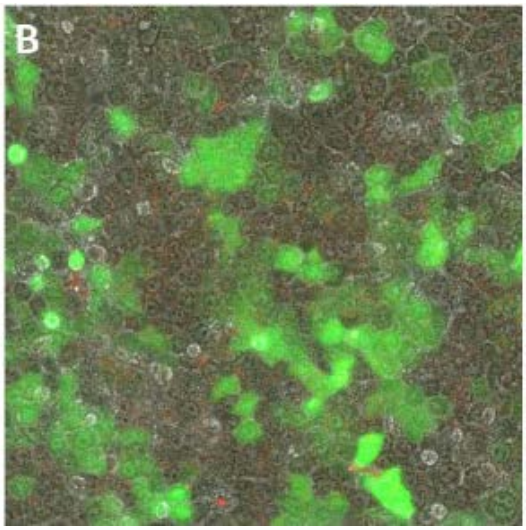
VIQUA® : nanoencapsulated antioxidant ingredientProbe: Dipalmitoyl-Phosphatidylethanolamine-N-(Lissamine Rhodamine B Sulfonyl)



Incubation of human cells with labeled VIQUA® (fluorescence microscopy)

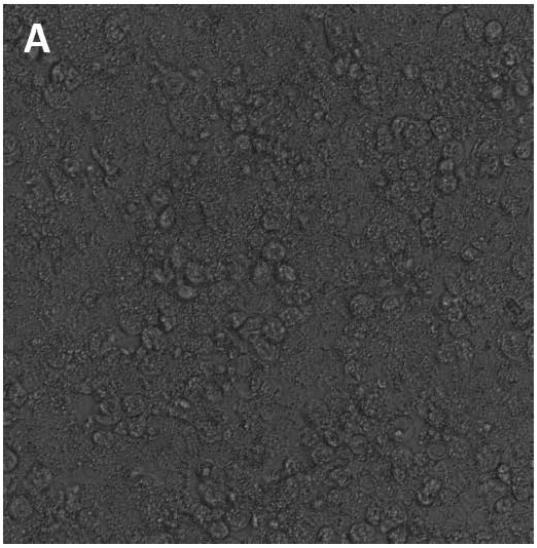


Non-labeled MCF-7 cells (green cells express GFP, green fluorescent protein)

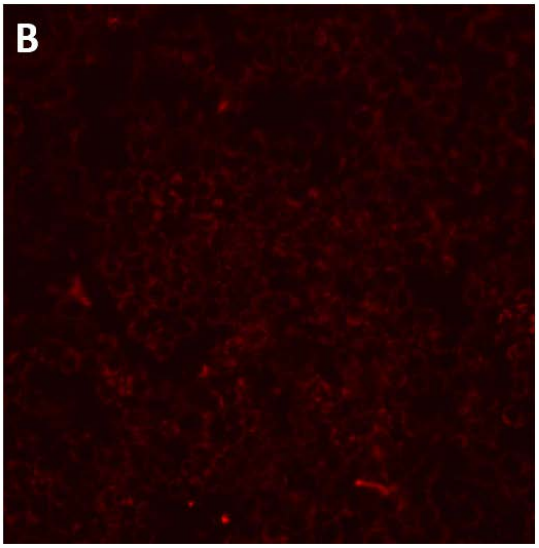


MCF-7 cells incubated with labeled VIQUA® (red labelling is VIQUA®)

Incubation of human cells with labeled VIQUA® (fluorescence microscopy)

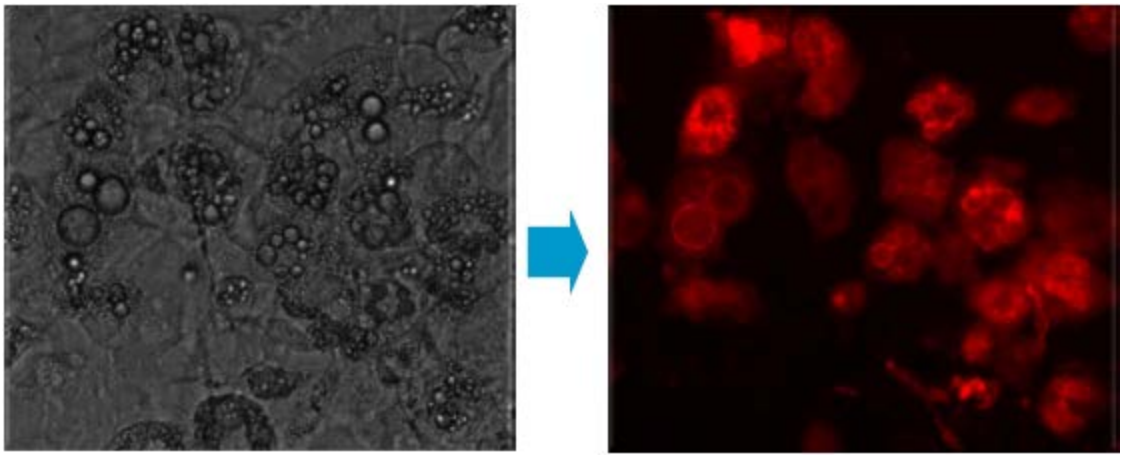


Non-labeled MCF-7 cells observed by phase contrast microscocopy.



MCF-7 cells incubated with labeled VIQUA® observed by fluorescence microscopy (red labelling is VIQUA®)

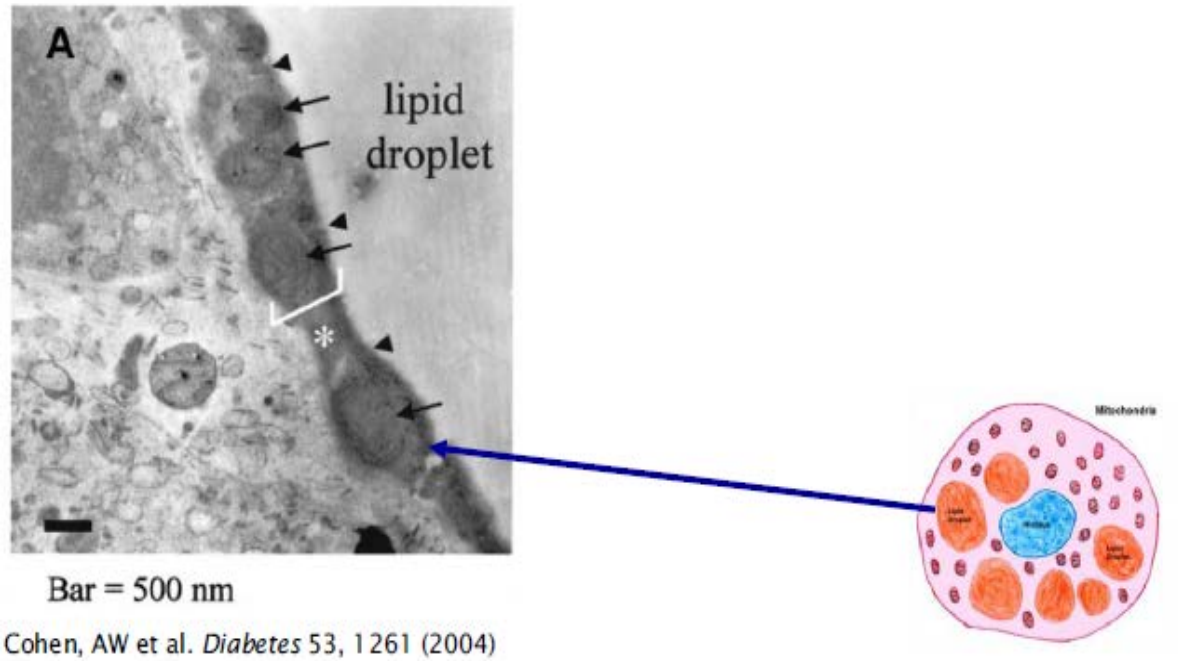
Incubation of adipocytes with labeled VIQUA® (fluorescence microscopy)



3T3-L1 differentiated adipocytes observed by phase contrast microscopy.

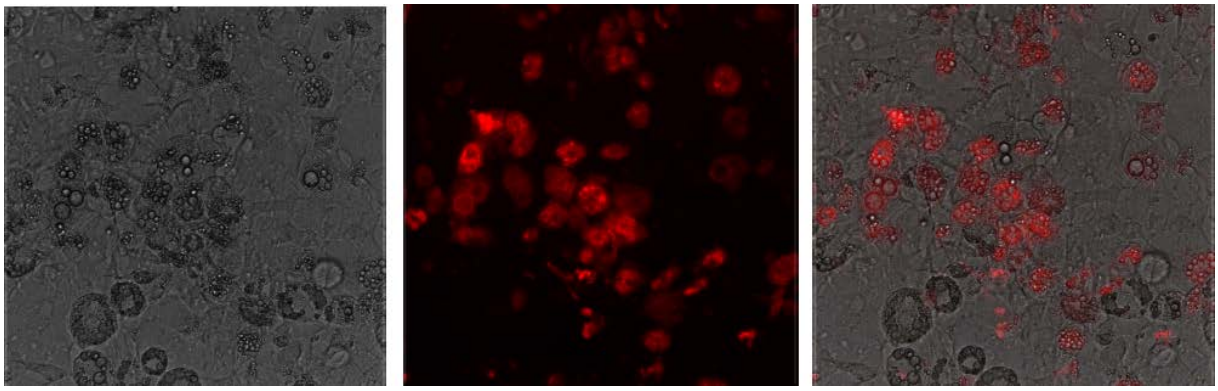
3T3-L1 differentiated adipocytes incubated with labeled VIQUA® for 3 hrand observed by fluorescence microscopy.

Mitochondria are located at the lipid droplet cortex of 3T3-L1 adipocytes



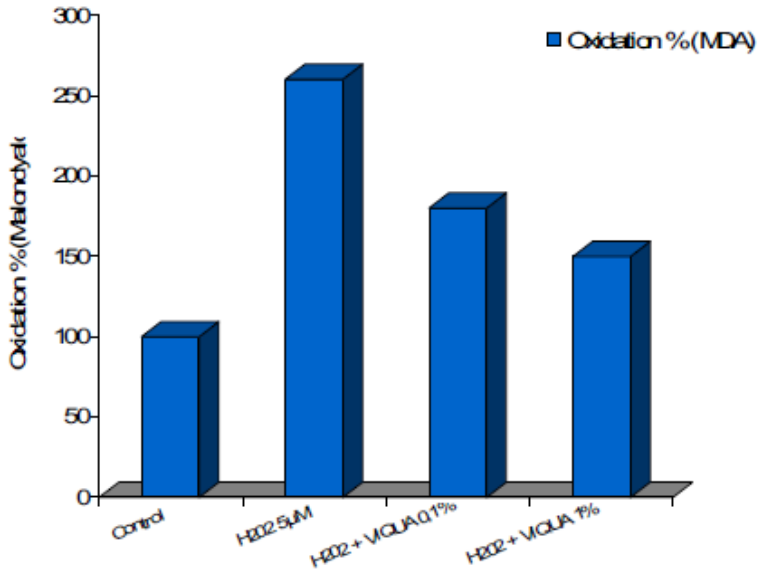
Cohen, AW et al. *Diabetes* 53, 1261 (2004)

Incubation of adipocytes with labeled VIQUA®

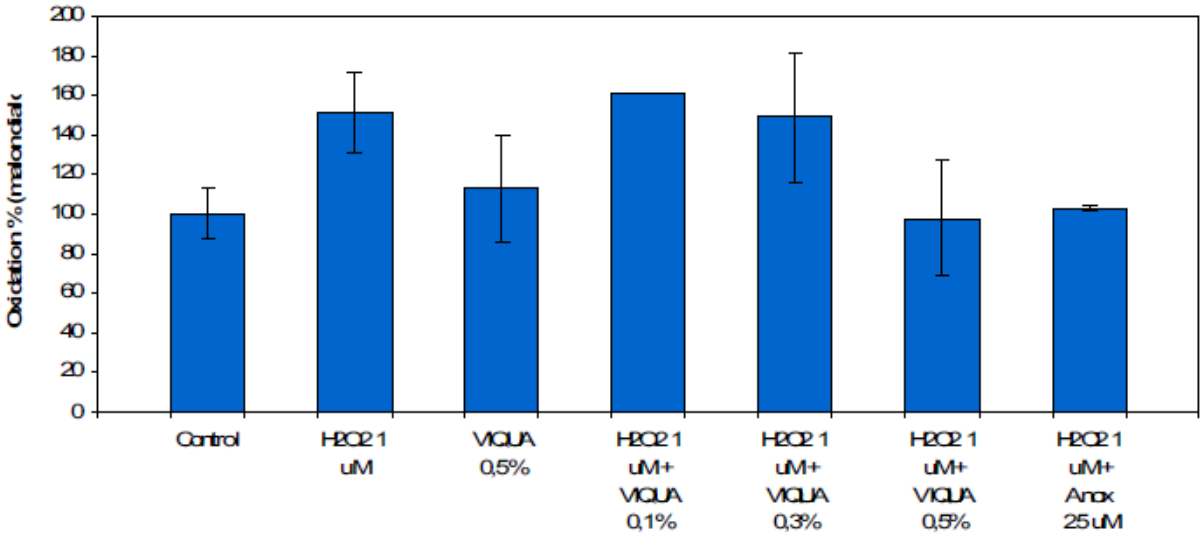


Adipocytes were incubated in the presence of VIQUA® containing a red-labeled lipid probes

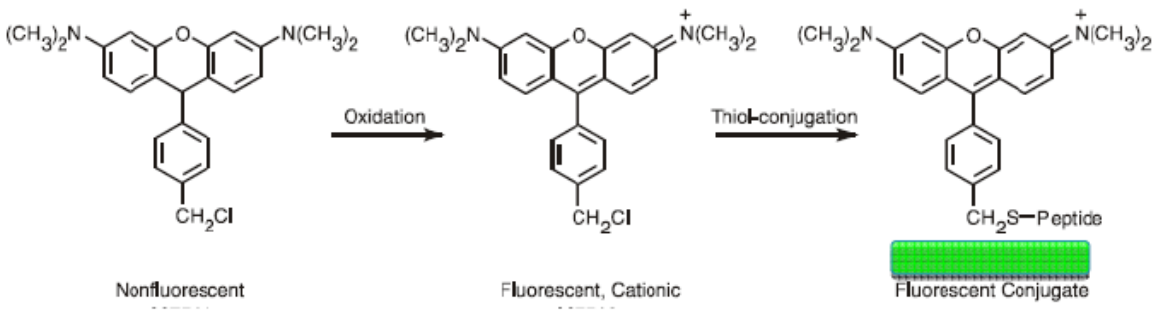
Antioxidant capacity of VIQUA® in 3T3-L1 differentiated adipocytes



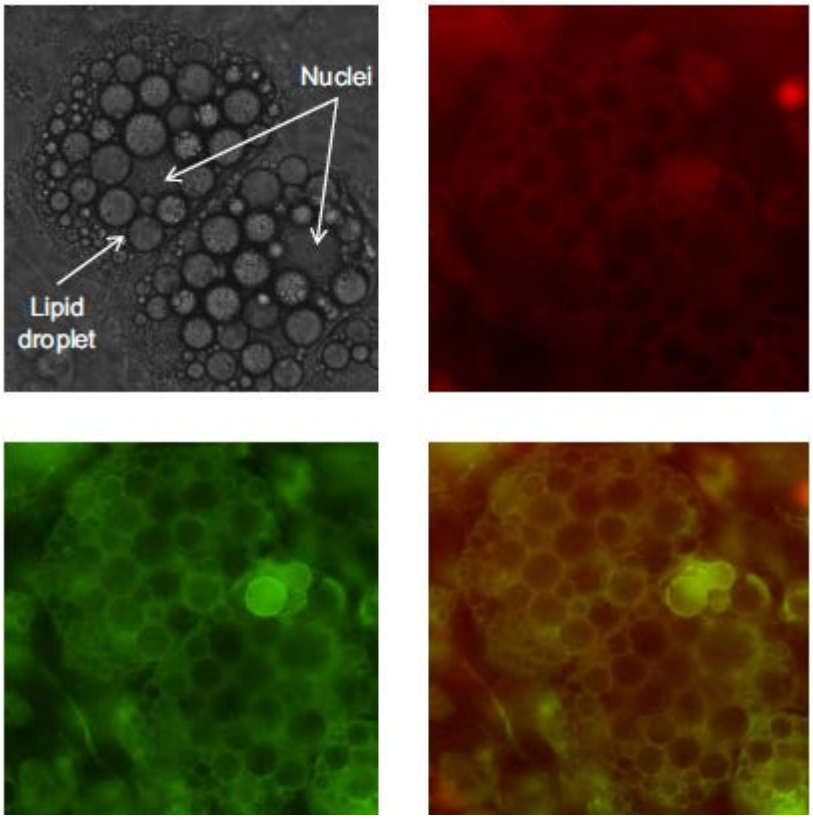
Antioxidant capacity of VIQUA® in human breast adenocarcinoma cells



Co-staining of VIQUA® phospholipids and adipocyte mitochondria



Co-staining of VIQUA® phospholipids and adipocyte mitochondria

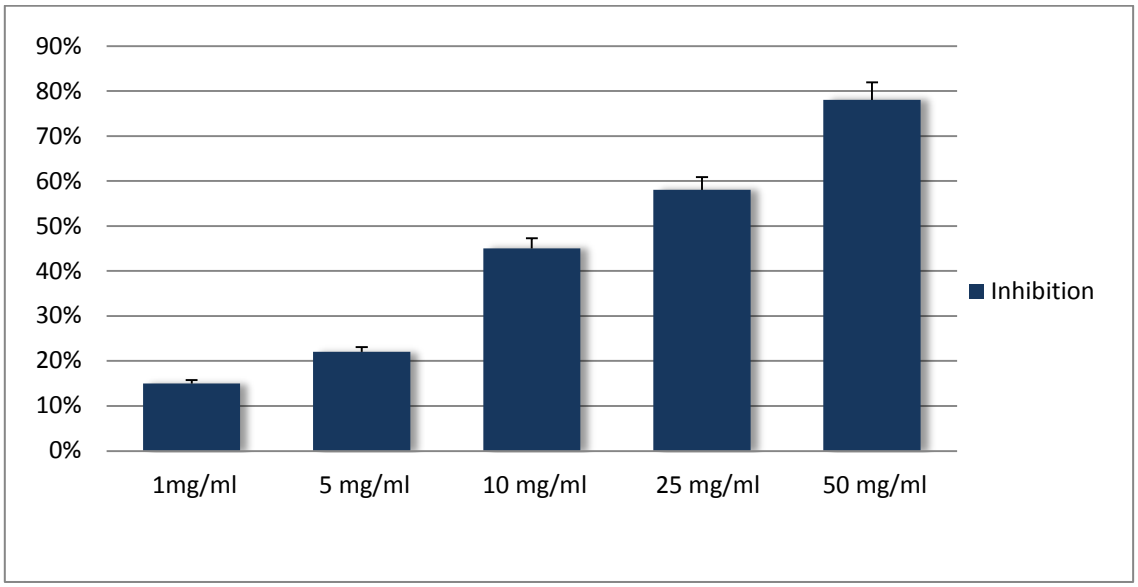


Red: Phospholipid (VIQUA®)

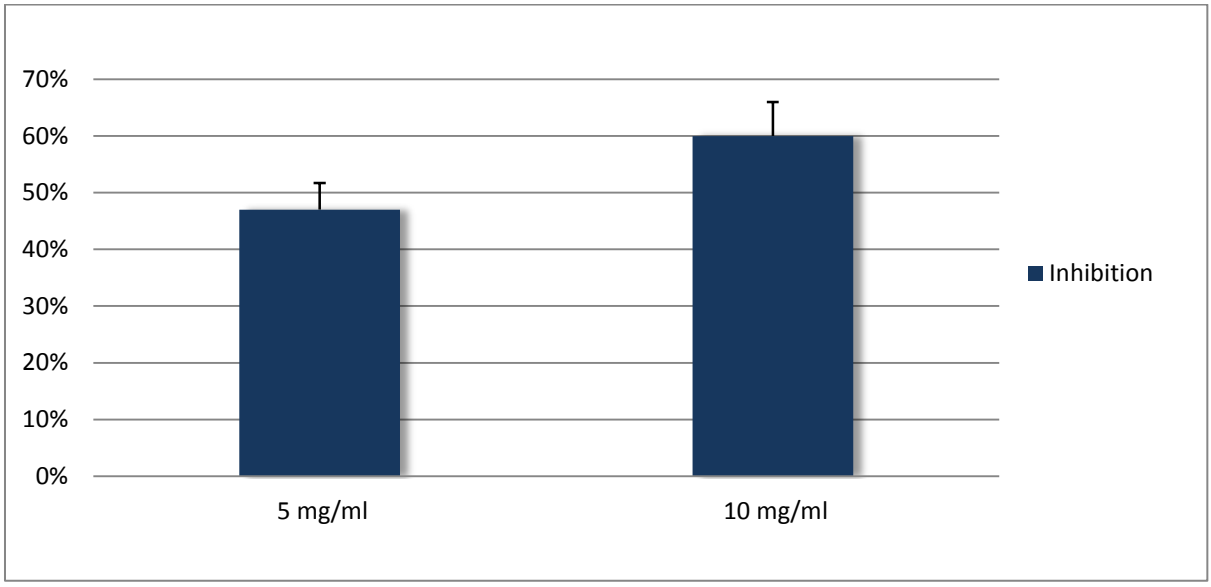
Green: Mitotracker Green

ANNEXE 3. INHIBITION OF ELASTASE AND COLLAGENASE ACTION WITH VIQUA®

- Results of elastase inhibition with VIQUA®:

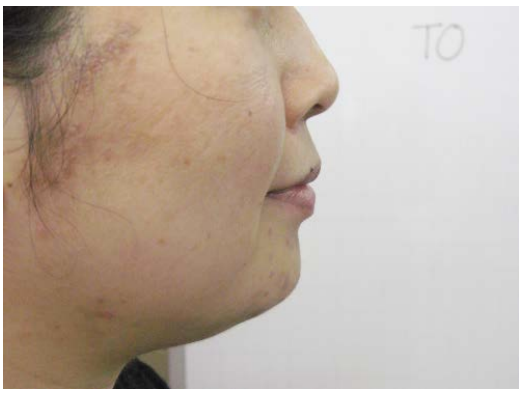


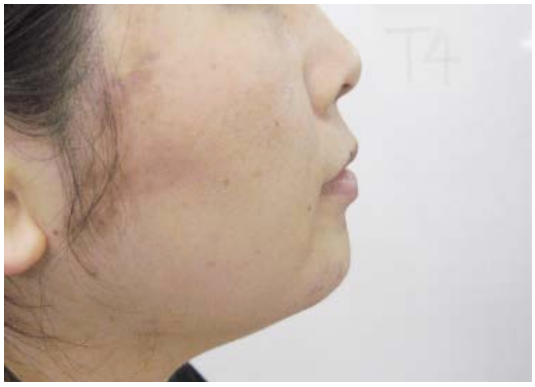
- Results of collagenase inhibition with VIQUA®:



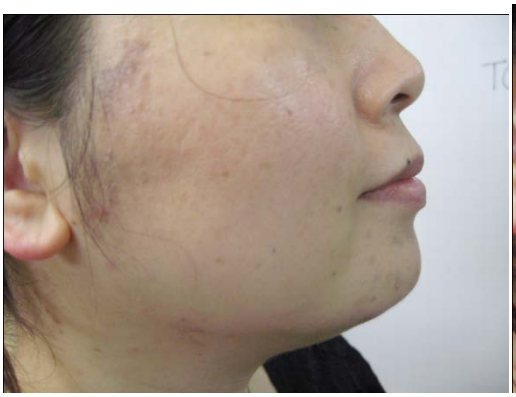
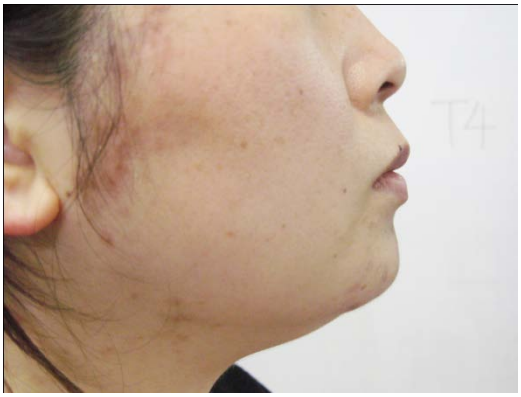
ANNEXE 4. MELASMA BEFORE AND AFTER TREATMENT

- Results of improvement in melasma with VIQUA®:

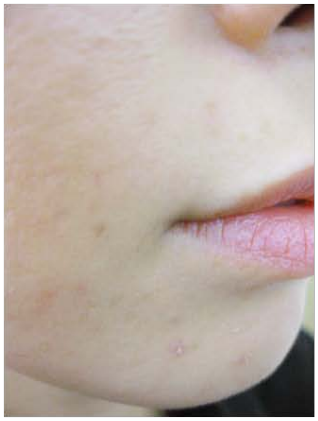
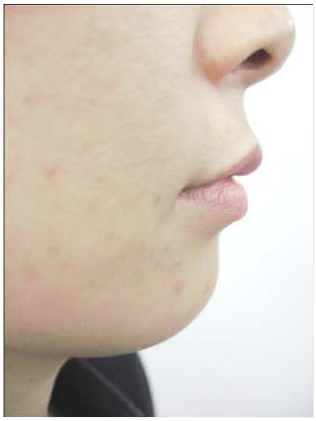




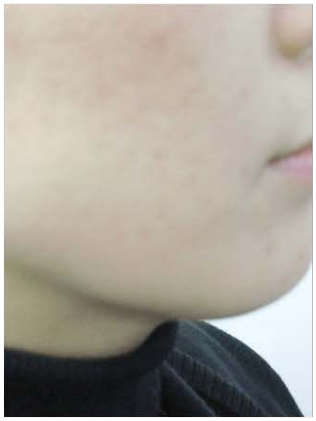
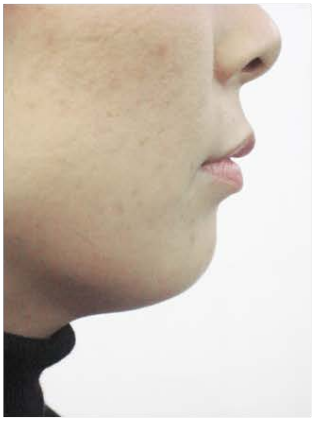
Before After

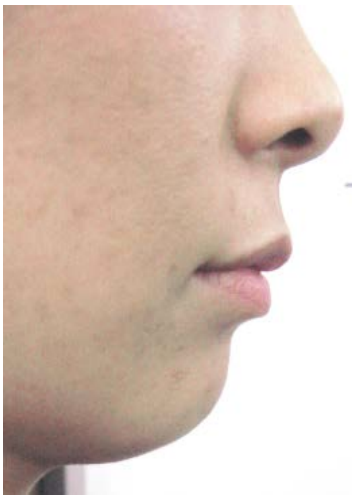
Before After

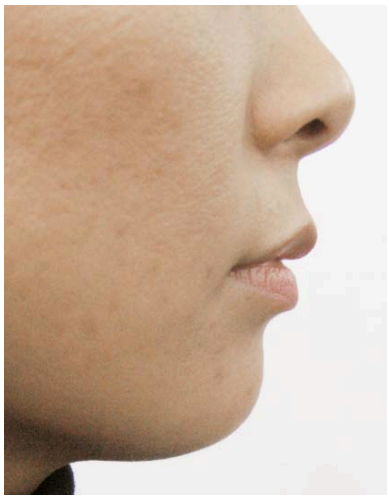
Before After

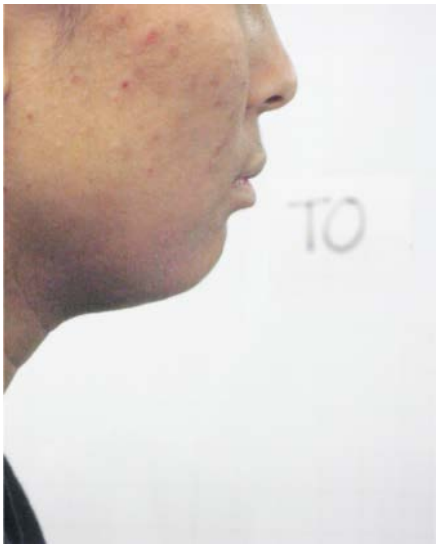
Before After



Before

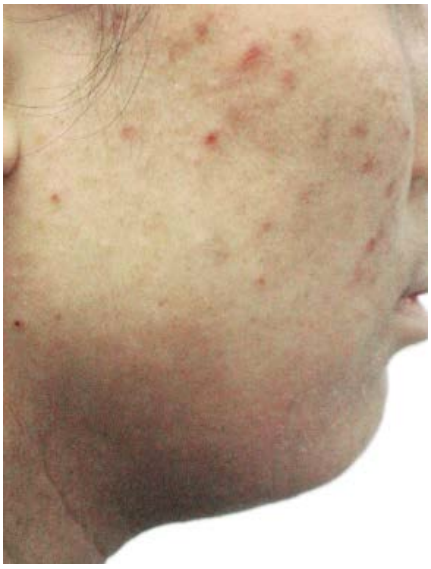


After

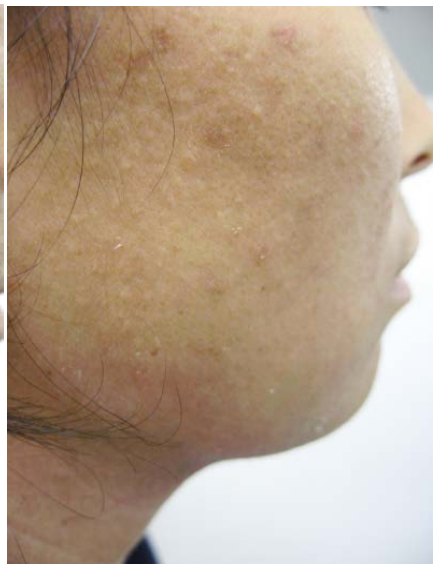


Before

After



Before



After

7. OTHER SELECTED ARTICLES

**Full-text available*
***Abstract only (free publication restricted)*

Pomegranate bio-actives properties

[1] **Jurenka JS,** *Therapeutic applications of pomegranate (Punica granatum L.): a review;* Alternative Medicine Review 2008 **13**(2), 128-44*

[2] **Johanningsmeier SD, Harris GK,** *Pomegranate as a Functional Food and Nutraceutical Source;* Annual Review of Food Science and Technology 2011 **2**, 181-201**

[3] **Xiang L, Xing D, Lei X, Wang W, Xu L, Nie L, Du L,** *Effects of Season, Variety, and Processing Method on Ellagic Acid Content in Pomegranate Leaves;* Tsinghua Science & Technology 2008 **13**(4), 460-465**

[4] **Saad H, Charrier-El Bouhtoury F, Pizzi A, Rode K, Charrier B, Ayed N,** *Characterization of pomegranate peels tannin extractives;* Industrial Crops and Products 2012 **40**, 239-246**

[5] **Qu W, Breksa III AP, Pan Z, Ma H,** *Quantitative determination of major polyphenol constituents in pomegranate products;* Food Chemistry 2012 **132**(3), 1585-1591**

Antioxidant effect

[6] **Madrigal-Carballo S, Rodriguez G, Krueger CG, Dreher M, Reed JD**, *Pomegranate (Punica granatum) supplements: Authenticity, antioxidant and polyphenol composition;* Journal of Functional Foods 2009 **1**(3), 324-329*

[7] **Wang Z, Pan Z, Ma H,1 Atungulu GG,** *Extract of Phenolics From Pomegranate Peels;* The Open Food Science Journal 2011 **5**, 17-25*

[8] **Shiban MS, Al-Otaibi MM, Al-Zoreky NS,** *Antioxidant Activity of Pomegranate (Punica granatum L.) fruit peels;* Food and Nutrition Sciences 2012 **3**, 991-996**

[9] **Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S,** *Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract;* Food Chemistry 2006 **96**(2), 254-260**

[10] **Negi PS, Jayaprakasha GK, Jena BS,** *Antioxidant and antimutagenic activities of pomegranate peel extracts;* Food Chemistry 2003 **80**(3), 393-397**

Anti-inflammatory effect

[11] **Ismail T, Sestili P, Akhtar S,** *Pomegranate peel and fruit extracts: A review of potential anti-inflammatory and anti-infective effects;* Journal of Ethno pharmacology 2012 **143**(2), 397-405**

Skin health

[12] **Afaq F, Zaid MA, Khan N, Dreher M, Mukhtar H,** *Protective effect of pomegranate derived products on UVB-mediated damage in human reconstituted skin;* Experimental Dermatology 2010 **18**(6), 553-561*

[13] **Zaid MA, Afaq F, Syed DN, Dreher M, Mukhtar H,** *Inhibition of UVB-mediated oxidative stress and markers of photoaging in immortalized HaCaT keratinocytes by pome-granate polyphenol extract POMx;* Photochemistry and Photobiology 2007 **83**(4), 882-8**

[14] **Afaq F, Khan N, Syed DN, Mukhtar H,** *Oral Feeding of Pomegranate Fruit Extract Inhibits Early Biomarkers of UVB Radiation-Induced Carcinogenesis in SKH-1 Hairless Mouse Epidermis;* Photochemistry and Photobiology 2011 **86**(6), 1318–1326**

[15] **Park HM, Moon E, Kim AJ, Kim MH, Lee S, Lee JB, Park YK, Jung HS, Kim YB, Kim SY**, *Extract of Punica granatum inhibits skin photoaging induced by UVB irradiation;* International Journal of Dermatology 2010 **49**(3), 276-82**

[16] **Bae JY, Choi JS, Kang SW, Lee YJ, Park J, Kang YH,** *Dietary compound ellagic acid alleviates skin wrinkle and in-flammation induced by UV-B irradiation;* Experimental Dermatology 2010 2010 **19**(8), 182-90**

Therapeutic applications of pomegranate (Punica granatum L.): a review

Jurenka, Julie

Alternative Medicine Review 2008 **13**(2), 128-44

Abstract

The pomegranate, Punica granatum L., is an ancient, mystical, unique fruitborne on a small, long-living tree cultivated throughout the Mediterranean region, as far north as the Himalayas, in Southeast Asia, and in California and Arizona in the United States. In addition to its ancient historical uses, pomegranate is used in several systems of medicine for a variety of ailments. The synergistic action of the pomegranate constituents appears to be superior to that of single constituents. In the past decade, numerous studies on the antioxidant, anticarcinogenic, and anti-inflammatory properties of pomegranate constituents have been published, focusing on treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, bacterial infections and antibiotic resistance, and ultraviolet radiation-induced skin damage. Other potential applications include infant brain ischemia, male infertility, Alzheimer's disease, arthritis, and obesity. (Altern Med Rev 2008;13(2):128-144)

Introduction

The pomegranate, Punica granatum L., an ancient, mystical, and highly distinctive fruit, is the predominant member of two species comprising the Punicaceae family. It was lauded in ancient times in the Old Testament of the Bible, the Jewish Torah, and the Babylonian Talmud as a sacred fruit conferring powers of fertility, abundance, and good luck. It also features prominently in the ceremonies, art, and mythology of the Egyptians and Greeks and was the personal emblem of the Holy Roman Emperor, Maximilian. Pomegranate is the symbol and heraldic device of the ancient city of Granada in Spain--from which the city gets its name. The genus name, Punica, was the Roman name for Carthage, where the best pomegranates were known to grow. Pomegranate is known by the French as grenade, the Spanish as granada, and literally translates to seeded ("granatus") apple ("pomum"). (1)

The pomegranate tree typically grows 12-16 feet, has many spiny branches, and can be extremely long lived, as evidenced by trees at Versailles, France, known to be over 200 years old. "[he leaves are glossy and lance-shaped, and the bark of the tree turns gray as the tree ages. The flowers are large, red, white, or variegated and have a tubular calyx that eventually becomes the fruit. The ripe pomegranate fruit can be up to five inches wide with a deep red, leathery skin, is grenade-shaped, and crowned by the pointed calyx. The fruit contains many seeds (arils) separated by white, membranous pericarp, and each is surrounded by small amounts of tart, red juice. The pomegranate is native from the Himalayas in northern India to Iran but has been cultivated and naturalized since ancient times over the entire Mediterranean region. It is also found in India and more arid regions of Southeast Asia, the East Indies, and tropical Africa. The tree is also cultivated for its fruit in the drier regions of California and Arizona. (2)

In addition to its ancient historical uses, pomegranate is used in several systems of medicine for a variety of ailments. In Ayurvedic medicine the pomegranate is considered "a pharmacy unto itself" and is used as an antiparasitic agent, (3) a "blood tonic," (4) and to heal aphthae, diarrhea, and ulcers. (5) Pomegranate also serves as a remedy for diabetes in the Unani system of medicine

practiced in the Middle East and India? The current explosion of interest in pomegranate as a medicinal and nutritional product is evidenced by a MedLine search from 2000 to present, revealing over 130 new scientific papers pertaining to its health effects. Between 1950 and 1999 only 25 such publications appear on MedLine. (7) The potential therapeutic properties of pomegranate are wide-ranging and include treatment and prevention of cancer, cardiovascular disease, diabetes, dental conditions, erectile dysfunction, and protection from ultraviolet (UV) radiation. Other potential applications include infant brain ischemia, Alzheimer's disease, male infertility, arthritis, and obesity.

The following abbreviations for various pomegranate extracts will be used throughout the article:
*Pomegranate juice—PJ
*Pomegranate by-product—PBP
* Fermented pomegranate juice--FPJ
* Cold-pressed seed oil--CPSO
* Pomegranate peel extract--PPE
* Pomegranate pulp juice--PPJ
* Pomegranate fruit extract--PFE
* Pomegranate flower extract--PFLE
* Hydroalcoholic extract of pomegranate--HAEP
* Gel-based pomegranate extract—GPBE

Biochemical Constituents

Over the past decade, significant progress has been made in establishing the pharmacological mechanisms of pomegranate and the individual constituents responsible for them. Extracts of all parts of the fruit appear to have therapeutic properties," and some studies report the bark, roots, and leaves of the tree have medicinal benefit as well. (3) Current research seems to indicate the most therapeutically beneficial pomegranate constituents are ellagic acid ellagitannins (including punicalagins), punicic acid, flavonoids, anthocyanidins, anthocyanins, and estrogenic flavonols and flavones. Table 1 lists the principal constituents of the Punica granatum tree and fruit. Figure 1 depicts the structure of ellagic acid.

Table 1. Pomegranate Fruit Parts and Constituents[8-21]

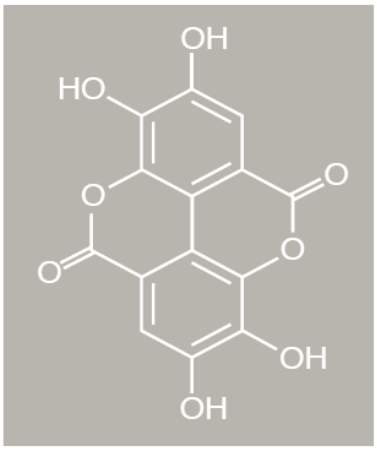
PLANT COMPONENT	CONSTITUENTS
Pomegranate juice	anthocyanins;[8] glucose, ascorbic acid;[9] ellagic acid, gallic acid, caffeic acid;[10] catechin, EGCG;[11] quercetin, rutin;[12] numerous minerals, particularly iron;[13] amino acids[7]
Pomegranate seed oil	95-percent punicic acid;[14] other constituents, including ellagic acid;[10] other fatty acids;[14] sterols[15]
Pomegranate pericarp (peel, rind)	phenolic punicalagins; gallic acid and other fatty acids;[10] catechin, EGCG;[11] quercetin, rutin, and other flavonols;[12] flavones, flavonones;[16] anthocyanidins[17]
Pomegranate leaves	tannins (punicalin and punicafolin); and flavone glycosides, including luteolin and apigenin[16]
Pomegranate flower	gallic acid, ursolic acid;[18] triterpenoids, including maslinic and asiatic acid;[19] other unidentified constituents
Pomegranate roots and bark	ellagitannins, including punicalin and punicalagin;[20] numerous piperidine alkaloids[21]

Constituent Standardization versus Synergy

The goal of many pomegranate studies has been to identify the therapeutic constituents. Commonly found in many plants, ellagic acid exhibits powerful anticarcinogenic (22) and antioxidant (23) properties, propelling it to the forefront of pomegranate research. Many commercially available pomegranate extracts are being standardized to contain 40-percent (or more) ellagic acid; however, Lansky, a prominent researcher on the medicinal properties of pomegranate, cautions against focusing on ellagic acid standardization to the exclusion of other therapeutically important pomegranate constituents. (24) Research on ellagic acid with other flavonoids such as quercetin supports his contention. (25,26) Lansky's research confirms the synergistic action of several pomegranate constituents is superior to ellagic acid in suppressing prostate cancer. (27,28) To quote Lansky, "The recent profusion onto the nutraceuticals marketplace of products standardized to 40 percent (or even higher) ellagic acid represents a cynical, lucre-driven attempt to replace the power of the pomegranate with the power of ellagic acid. The pomegranate needs no such tricks or enhancements. It is rather an extraordinary; albeit mysterious (and messy), fruit with a complete medicinal power contained within its juice, peel, and seeds." (24)

Biochemistry/Pharmacokinetics

Figure 1. Structure of Ellagic Acid



Although little is known about the metabolism and bioavailability of ellagitannins from food sources, three small human trials and one case study have investigated the bioavailability, absorption, metabolism, and in vivo antioxidant effects of pomegranate. In the case study, consumption of 180 mL pomegranate juice (PJ) by a single subject yielded 31.9 ng/mL plasma ellagic acid at one hour, with rapid plasma clearance by four hours post-ingestion. This was the first direct evidence that ellagic acid consumed from food was absorbed in humans. (29) A study of 18 healthy volunteers by the same researchers confirmed the rapid absorption and plasma clearance of ellagitannins and also confirmed urolithin metabolites excreted in the urine can persist for 48 hours after pomegranate juice ingestion, thereby suggesting an explanation of the benefits of long-term pomegranate administration. (30)

In a 13-day clinical trial involving six healthy subjects (4 men and 2 women), one liter of PJ containing 4.37 g/L punicalagins and 0.49 g/L anthocyanins was consumed by all six subjects for five days. Three pomegranate juice metabolites were detected in the plasma--urolithin A, urolithin B, and a third unidentified minor metabolite; urinalysis at 24 hours revealed six metabolites--the three found in the plasma as well as an aglycone metabolite corresponding to each of three plasma metabolites. Maximum excretion rates occurred 3-4 days after juice ingestion. Significant variability of urinary metabolite concentrations was observed among subjects and may be attributable to differences in colonic microflora, where the ellagitannins are believed to be metabolized. (31) The persistence of urolithin A and B in the urine may be responsible for pomegranate's long-term antioxidant effects, rather than the polyphenols found in the juice.

In another study, 11 healthy men and women were placed on a polyphenol- and antioxidant-free diet for three days prior to consuming pomegranate extract (plant parts used were not specified). Subjects were given 800 mg capsuled pomegranate extract daily containing 330.4 mg punicalagins and 21.6 mg ellagic acid (EA). [C.sub.max] and [T.sub.max] for plasma EA was 33.8 [+ or -] 12.7 ng/ mL at one hour post-ingestion, similar to values observed in the case study when similar amounts of

punicalagins and EA were administered. This study also demonstrated a significant increase (31.8%) in plasma antioxidant capacity 30 minutes after extract administration; one and two hours post ingestion, values were increased 1.62- and 1.43-fold, respectively. (32)

Mechanisms of Action

Although pomegranate's wide-ranging therapeutic benefits may be attributable to several mechanisms, most research has focused on its antioxidant, anticarcinogenic, and anti-inflammatory properties.

Antioxidant Mechanisms

An in vitro assay using four separate testing methods demonstrated pomegranate juice and seed extracts have 2-3 times the antioxidant capacity of either red wine or green tea. (33) Pomegranate extracts have been shown to scavenge free radicals and decrease macrophage oxidative stress and lipid peroxidation in animals (34) and increase plasma antioxidant capacity in elderly humans. (35)

Studies in rats and mice confirm the antioxidant properties of a pomegranate by-product (PBP) extract made from whole fruit minus the juice, showing a 19-percent reduction in oxidative stress in mouse peritoneal macrophages (MPM), a 42-percent decrease in cellular lipid peroxide content, and a 53-percent increase in reduced glutathione levels. (34) In vitro assay of a fermented pomegranate juice (FPJ) extract and a cold-pressed seed oil (CPSO) extract found the antioxidant capacity of both are superior to red wine and similar to green tea extract. (14) A separate study in rats with C[Cl.sub.4]-induced liver damage demonstrated pretreatment with a pomegranate peel extract (PPE) enhanced or maintained the free-radical scavenging activity of the hepatic enzymes catalase, super oxide dismutase, and peroxidase, and resulted in 54-percent reduction of lipid peroxidation values compared to controls. (36)

Research in humans has shown a juice made from pomegranate pulp (PPJ) has superior antioxidant capacity to apple juice. Using the FRAP assay (ferric reducing/antioxidant power), Guo et al found 250 mL PPJ daily for four weeks given to healthy elderly subjects increased plasma antioxidant capacity from 1.33 mmol to 1.46 mmol, while subjects consuming apple juice experienced no significant increase in antioxidant capacity. In addition, subjects consuming the PPJ exhibited significantly decreased plasma carbonyl content (a biomarker for oxidant/antioxidant barrier impairment in various inflammatory diseases) compared to subjects taking apple juice. Plasma vitamin E, ascorbic acid, and reduced glutathione values did not differ significantly between groups, leading researchers to conclude pomegranate phenolics may be responsible for the observed results. (35)

Anticarcinogenic Mechanisms

In vitro assays utilizing three prostate cancer cell lines (DU-145, LNCaP, and PC-3) demonstrated various pomegranate extracts (juice, seed oil, peel) potently inhibit prostate cancer cell invasiveness and proliferation, cause cell cycle disruption, induce apoptosis, and inhibit tumor growth. These studies also demonstrated combinations of pomegranate extracts from different parts of the fruit were more effective than any single extract. (27,37)

Several animal studies have elucidated pomegranate's potential anticancer mechanisms. Two studies in mice implanted with the prostate cancer PC-3 cell line demonstrated pomegranate fruit extract (PFE; edible parts of the fruit, excluding the peel) inhibits cell growth and induces apoptosis via modulation of proteins regulating apoptosis. (38,39)

In an open-label, phase II clinical trial in 46 men with recurrent prostate cancer, 16 patients (35%) showed a significant decrease in serum prostate specific antigen (PSA) levels (average=27%) during

treatment with eight ounces of pomegranate juice. Corresponding in vitro assays using patient plasma and serum demonstrated significant decreases in prostate cancer cell line proliferation and increased apoptosis. Nitric oxide preservation via ingestion of pomegranate polyphenols significantly correlated with lower PSA values. These results indicate pomegranate may affect prostate cancer because of antiproliferative, apoptotic, antioxidant, and possibly anti-inflammatory effects. (40)

Recent research also indicates pomegranate constituents inhibit angiogenesis via downregulation of vascular endothelial growth factor in MCF-7 breast cancer and human umbilical vein endothelial cell lines. (41)

Anti-inflammatory Mechanisms

Cold pressed pomegranate seed oil has been shown to inhibit both cyclooxygenase and lipoxygenase enzymes in vitro. Cyclooxygenase, a key enzyme in the conversion of arachidonic acid to prostaglandins (important inflammatory mediators), was inhibited by 37 percent by a CPSO extract. Lipoxygenase, which catalyzes the conversion of arachidonic acid to leukotrienes, also key mediators of inflammation, was inhibited by 75 percent by a CPSO extract. By comparison, an FPJ extract resulted in a 23.8-percent inhibition of lipoxygenase in vitro. (14)

Another in vitro study that may have far-reaching implications for those suffering from osteoarthritis (OA) demonstrated PFE has a significant and broad inhibitory effect on matrix metalloproteinases (MMPs), a subgroup of collagenase enzymes expressed in high levels in arthritic joints and involved in the turnover, degradation, and catabolism of extracellular joint matrix. In pretreated human femoral OA chondrocytes, PFE inhibited IL-1beta-induced destruction of proteoglycan, expression of MMPs at the cellular level, and phosphorylation and activation of mitogen-activated protein kinases (signal transduction molecules involved in MMP expression). The suppression of MMP expression in OA chondrocyte cultures by PFE suggests pomegranate constituents prevent collagen degradation and may inhibit joint destruction in OA patients. (42)

Other Mechanisms

A pilot study in type 2 diabetic patients with hyperlipidemia found concentrated PJ decreased cholesterol absorption, increased fecal excretion of cholesterol, had a beneficial effect on enzymes involved in cholesterol metabolism, significantly reduced total and LDL cholesterol, and improved total/HDL and LDL/HDL cholesterol ratios. (43)

PJ consumption by hypertensive patients inhibits serum angiotensin converting enzyme (ACE; a catalyst for the conversion of angiotensin I to angiotensin II, a potent vasoconstrictor) activity, thereby reducing systolic blood pressure (44) and potentially protecting against cardiovascular disease.

Animal studies have revealed three possible hypoglycemic mechanisms for Punica granatum extracts. Pomegranate flower extract (PFLE) improved insulin sensitivity and lowered glucose levels in rats as early as 30 minutes post-glucose loading. PFLE also inhibited alpha-glucosidase in vitro, thereby decreasing the conversion of sucrose to glucose. (45) PPE demonstrates significant hypoglycemic activity in diabetic rats, via enhanced insulin levels and regeneration of pancreatic beta cells. (46)

Numerous in vitro studies (3,47,48) and two human trials (49,50) demonstrate the antimicrobial activity of pomegranate extracts. The growth of Staphylococcus aureus, Streptococcus pyogenes, Diplococcus pneumoniae, Escherichia coli O157:H7, and Candida albicans was inhibited via direct bacteriocidal or fungicidal activity.

Clinical Applications

Prostate Cancer

Among males in the United States and other Western countries, prostate cancer is the second-leading cause of cancer-related death. In vitro studies show several PFEs inhibit prostate cancer cell growth, induce apoptosis of several prostate cancer cell lines (including highly aggressive PC-3 prostate carcinoma cells), suppress invasive potential of PC-3 cells, and decrease proliferation of DU-145 prostate cancer cells. (27,37,38) Lansky et al found combining equal amounts of FPJ, PPE, and CPSO extracts resulted in a 99-percent suppression of DU-145 prostate cancer cell invasion across a Matrigel matrix. CPSO extract or FPJ extract alone resulted in 60-percent suppression of invasion, and combining any two extracts induced 90-percent suppression. Studies in mice have also demonstrated PFE inhibits prostate tumor growth and decreases PSA levels. (38,39)

These promising results led some of the same researchers to conduct a two-stage phase II clinical trial in men with recurrent prostate cancer and rising PSA levels. All eligible patients had previous surgery or radiation therapy for prostate cancer, Gleason scores (a grading system for predicting the behavior of prostate cancer) [less than or equal to] 7, rising PSA value of 0.2-5.0 ng/mL, no prior hormonal therapy, and no evidence of metastases. Baseline PSA doubling times were established for 22 participants who were then started on eight ounces PJ (570 mg total polyphenol gallic acid equivalents) daily until meeting disease progression endpoints. Endpoints measured were: effect on PSA levels, serum lipid peroxidation and nitric oxide levels, in vitro induction of proliferation and apoptosis of LNCaP cells in patient serum containing pomegranate constituents, and overall safety of extract administration. (40)

Based on preliminary results achieved in phase I, 24 additional patients were enrolled and 46 patients were evaluated over 13 months in both stages of the trial. Of these, 35 percent (n=16) demonstrated decreased PSA levels, the primary trial endpoint--average decrease=27%; median decrease=18%; range 5-85%. Four of 46 patients (8.7%) met objective response criteria and exhibited > 50-percent reduction in PSA values, meeting criteria for a phase III trial. In addition, an average 40-percent reduction in serum oxidative state was observed in patients accompanied by a significant reduction in serum lipid peroxidation compared to baseline. Nitric oxide serum metabolites measured at nine months after study initiation revealed an average 23-percent increase, which significantly correlated with baseline PSA levels. (40)

An in vitro arm of the trial using patient serum investigated whether PJ consumption had any effect on growth rates or apoptosis of LNCaP prostate cancer cells in culture. Serum collected at nine months after study initiation and incubated with LNCaP decreased cell growth by an average of 12 percent in 84 percent of patients compared to baseline. An average 17.5-percent increase in apoptosis in 75 percent of patients was also noted. This study indicates PJ or PJ constituents may have promise as a therapy for prostate cancer, particularly recurrent type with rising PSA levels; phase III studies are currently underway. (40)

Other Cancer Types

Numerous in vitro studies have investigated the therapeutic effect of pomegranate extracts against several other cancer cell lines. In HT-29 colon cancer cells, cyclooxygenase-2 (COX-2) expression is increased via activation of nuclear factor kappa-B (NF[kappa]B) by tumor necrosis factor-alpha (TNF-[alpha]), an inflammatory cell signaling process that may be a cause of cancer initiation and progression. Treatment of HT-29 colon cancer cells with PJ, total pomegranate tannins, or concentrated pomegranate punicalagin induced a significant decrease in COX-2 expression. PJ treatment resulted in the highest level of COX-2 suppression (79%) compared to treatment with single constituents. The effects were attributed to synergistic activity of the bioactive constituents thought to be necessary for pomegranate's anti-inflammatory and anticarcinogenic activity. (51)

Another in vitro study investigated the effects of punicalagin, ellagic acid, total pomegranate tannins, and PJ on several cell lines. Although all preparations decreased viable cell numbers in KB and CAL-27 oral cancer cell lines, as well as in HT-29 and HCT-116 colon cancer cell lines, a higher degree of suppression was obtained with pure PJ, an affect attributed to the synergy of its bioactive constituents. (52)

Research utilizing breast cancer cell lines MCF-7 and MB-MDA-231 demonstrates pomegranate constituents effectively inhibit angiogenesis, (41) tumor growth, (53) proliferation, and invasiveness, (54) and induce apoptosis. (55) To examine the effect of FPJ and CPSO extracts, and an HPLC-isolated peak B (from the fruit extract), Mehta and Lansky used the mouse mammary organ culture, an animal model of breast cancer having [greater than or equal to] 75-percent accuracy of predicting in vivo carcinogenesis. They found cancerous glands treated with each pomegranate compound exhibited decreased lesion incidence--37 percent for FPJ, and 75-90 percent for both peak B and CPSO. Seed oil is comprised mainly of punicic acid, a trienoic acid with anticarcinogenic properties and effective at very low doses (1 [micro]g/mL in organ culture). Peak B is believed to be a phenolic compound with potent chemopreventative properties. (53)

Research in mice has shown PFE inhibits tumorigenesis in lung cancer and skin cancer models. In the lung cancer study, mice given daily oral dosages of PFE comparable to what humans could reasonably consume (exact dosages were not available) exhibited significantly less lung tumor growth than mice not receiving PFE. (56) In mice treated with skin-cancer-inducing 12-O-tetradecanoylphorbol-13-acetate (TPA), animals treated topically with PFE had significantly reduced incidence of skin tumors. In the PFE-treated group, only 30 percent of mice exhibited tumors compared to 100 percent of mice treated with TPA and no PFE. This result was attributed to suppression of inflammation (COX-2, MAPKs, NF[kappa]B) and the tumor proliferation marker ornithine decarboxylase. (57)

Lansky and Kuwaii investigated the effect of flavonoid-rich PJ and FPJ and pomegranate pericarp extracts on HL-60 human leukemia cell differentiation (the ability of cancer cells to revert to normal cells) and proliferation. Because of the structural similarity between plant flavonoids and retinoids (the latter being established pro-differentiating agents), it was hypothesized that flavonoid-rich pomegranate extracts might have a similar effect on differentiation. In vitro assays confirmed both the FPJ and pericarp extracts strongly promoted cellular differentiation and inhibited proliferation in HL-60 cell cultures; the effect of PJ on cellular differentiation was less significant. This study suggests another mechanism by which pomegranate constituents impart an anticarcinogenic effect. (58)

Atherosclerosis

In vitro, animal, and human trials have examined the effects of various pomegranate constituents on prevention and attenuation of atherosclerosis. One of the preeminent researchers in endothelial function and nitric oxide (NO) biochemistry, Louis J. Ignarro, PhD, investigated the effects of pomegranate juice and other fruit juices on endothelial function, comparing propensities to protect NO from destruction by reactive oxygen species in vitro. Results of the antioxidant portion of the study demonstrate pomegranate juice possesses significantly greater antioxidant capacity at much lower concentrations (> 1000-fold dilutions) than either grape or blueberry juice, which was attributed to the high anthocyanin flavonoid content and higher total flavonoid content in PJ than the other juices. (59)

Because impaired endothelial function is an early indicator of atherosclerosis, this study examined the effect of PJ on proliferation of rat aortic smooth muscle cells in culture. PJ proved superior to other juices, significantly enhancing NO's effect on cardiac endothelium even at 2,000-fold dilutions. PJ did not influence endothelial nitric oxide synthase (eNOS) expression, leading Ignarro et al to conclude the antioxidant properties of PJ protect NO from free radical destruction and augment the antiproliferative action of NO on rat aortic smooth muscle cells. (59)

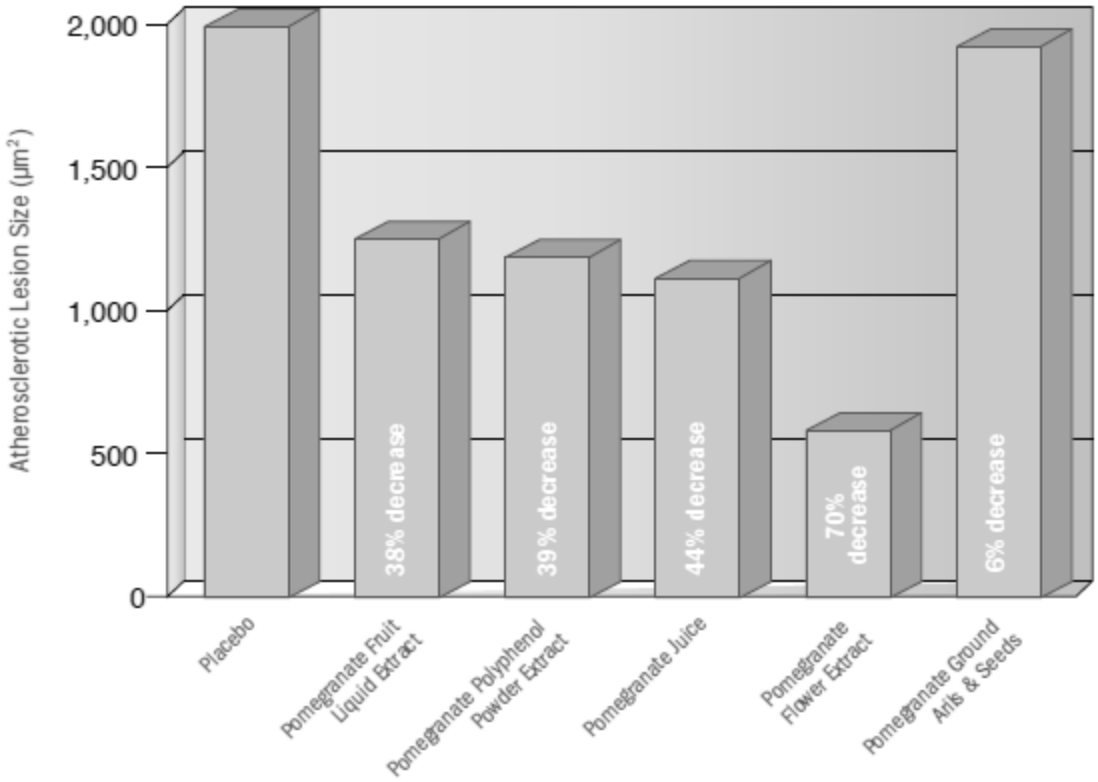
In early-stage atherosclerosis, elevated plasma cholesterol, increased oxidative stress, and increased cholesterol esterification rates are factors contributing to foam cell formation and development of atherosclerotic lesions. (60,62) Research in atherosclerotic apolipoprotein-E deficient (E[degrees]) mice by Aviram et al at the Lipid Research Laboratory in Haifa, Israel, has focused on the ability of pomegranate extracts to inhibit atherogenesis. (34,63) Two months of PJ to E[degrees] mice with advanced atherosclerosis reduced MPM lipid peroxide content by 42 percent compared with placebo-treated mice; MPM lipid peroxide content in PJ-treated mice was 20-percent lower than in four-month-old control mice. In addition, MPM harvested from PJ-treated mice exhibited 80-percent lower rates of cholesterol esterification than placebo-treated mice. In PJ-treated mice atherosclerotic lesion size in the aorta was 17-percent smaller than in the age-matched placebo group. PJ and an isolated tannin fraction from PJ were also given to young E[degrees] mice prior to development of significant atherosclerosis. Researchers found 25- and 17-percent reductions in plasma lipid peroxide concentrations with the isolated tannin fraction and PJ, respectively. (64)

Aviram et al also investigated the anti-atherosclerotic effects of a PBP extract after the juice was removed. Four-month-old E[degrees] mice with significant atherosclerosis were given PBP extract (containing 51.5 lag gallic acid equiv/kg/day) with an eight-fold higher polyphenol concentration than PJ for three months. This resulted in a significant reduction in MPM oxidative status as evidenced by a 27-percent decrease in total macrophage peroxide levels, a 42-percent decrease in cellular lipid peroxide levels, and a 19-percent decrease in peritoneal macrophage uptake of oxidized LDL. (34)

To further identify the most potent anti-atherogenic pomegranate components, Aviram et al analyzed several more pomegranate extracts from all parts of the plant. Atherosclerotic E[degrees] mice were given six different pomegranate preparations with varying amounts of total polyphenols and gallic acid content for three months. Antioxidant activity, atherosclerotic lesion size, MPM oxidative status, blood sugar, and lipid profiles were examined. Confirming earlier results, this study demonstrated PFLE more significantly affects atherosclerotic lesion size (Figure 2), lipid profiles, and blood sugar levels than other extracts tested; two PPEs demonstrated the most potent antioxidant effects. Mechanisms associated with the anti-atherogenic effects of pomegranate in this study include increased MPM uptake of oxidized LDL, decreased lipid peroxidation, and decreased cholesterol levels. (65)

The effect of PJ consumption on lipid peroxidation in plasma and HDL- and LDL-lipoproteins was examined in a double-armed human trial. In the first study, 13 healthy, nonsmoking men (ages 20-35) were given 50 mL PJ daily (containing 1.5 mmol total polyphenols) for two weeks. In the second study (duration [less than or equal to] 10 weeks), three healthy men (same age range) were given increasing doses of PJ ranging from 20-80 mL daily (0.54-2.16 mmol total polyphenols). Fasting blood samples were drawn from participants pre-study and after one and two weeks of PJ supplementation. No significant effect was observed in either study on plasma lipid profile or lipoprotein patterns. The results did show, however, for the first time in humans, that PJ has an inhibitory effect on lipid peroxidation in plasma and in lipoproteins, with the middle dose (50 mL daily) being the most effective, yielding a 32-percent decrease in plasma lipid peroxidation. PJ (in a dose-dependent manner) also demonstrated up to 90-percent inhibition of collagen-induced platelet aggregation in human platelets ex vivo. (63)

Figure 2. Atherosclerotic Lesion Size with Various Pomegranate Extracts



Aviram M, Volkova N, Coleman R, et al. Pomegranate phenolics from the peels, arils, and flowers are antiatherogenic: studies *in vivo* in atherosclerotic apolipoprotein E-deficient (E°) mice and in vitro cultured macrophages and lipoproteins. *J Agric Food Chem* 2008;56:1148-1157.

Hyperlipidemia

Pomegranate flowers have been used in both the Unani and Ayurvedic systems of medicine as a remedy for diabetes. Based on historical use, a study in diabetic rats explored the effects of PFLE on cardiac lipid metabolism in 13-to 15-week old Zucker diabetic rats. Animals were given 500 mg/kg PFLE or placebo for six weeks, and total cholesterol, triglyceride, and nonesterified free fatty acids (NEFA) were determined prior to treatment (nonfasting), at week 4 (nonfasting), and week 5 (fasting) in both rat plasma and cardiac tissue. PFLE was shown to activate peroxisome proliferator-activated receptor (PPAR-[alpha]), a cardiac transcription factor involved in myocardial energy production via fatty acid uptake and oxidation. PPAR-[alpha] activation decreased cardiac uptake and circulation of lipids. Decreases were observed in cardiac tissue triglyceride content at the end of the study and in plasma total cholesterol and NEFA after four weeks of treatment. (66)

A pilot study involving 22 type 2 diabetic patients (8 men and 14 women) investigated the cholesterol-lowering effects of 40 g concentrated PJ for eight weeks. Statistically significant decreases were observed in total cholesterol (from 202.4 [+ or -] 27.7 mg/dL at baseline to 191.4 [+ or -] 21 mg/dL at study conclusion), LDL cholesterol (124.4 [+ or -] 31.9 mg/dL at baseline to 112.9 [+ or -] 25.9 mg/dL at study conclusion), total/HDL cholesterol ratio (5.5 [+ or -] 1.3 at baseline to 5.1 [+ or -] 1.1 at study conclusion), and LDL/HDL ratio (3.4 [+ or -] 1.2 at baseline to 3.0 [+ or -] 0.9 at study conclusion). The authors attributed these effects to decreased absorption and increased fecal excretion of cholesterol, as well as possible affects on HMG-CoA reductase and sterol O-acyltransferase, two enzymes key to cholesterol metabolism. (43)

Table 2. Antioxidant Activity of Pomegranate Juice Extract in Patients with Carotid Artery Stenosis

ANALYSIS	BASELINE	1 MONTH	3 MONTHS	1 YEAR	3 YEARS
Total Antioxidant Status (nmol/liter)	0.95 ± 0.12			2.20 ± 0.23 (⇧130%)	
Serum Antibodies against LDL Oxidation (EU/mL)	2670 ± 61	1563 ± 69	1670 ± 52	⇩19%	
AAPH-induced Serum Lipid Peroxidation (nmol/mL)	1670 ± 66			691 ± 43 (⇩59%)	⇩75%
Serum Paraoxonase 1 (PON1) Arylesterase Activity (Units/mL)	56 ± 5			97 ± 10 (⇧73%)	107 ± 10 (⇧83%)
Lipid Peroxide Content of Carotid Lesions (nmol/mg of lesion protein)			⇩61%	⇩44%	

EU – Enzyme units
AAPH – 2.2'-azobis, 2-amidinopropane hydrochloride

Hypertension

A small clinical trial demonstrated PJ inhibits serum ACE and reduces systolic blood pressure in hypertensive patients. Ten hypertensive subjects (ages 62-77; seven men and three women) were given 50 mL/ day PJ containing 1.5 mmol total polyphenols for two weeks. Two of seven patients were also diabetic and two were hyperlipidemic. Seven of 10 subjects (70%) experienced a 36-percent average decrease in serum ACE activity and a small, but significant, five-percent decrease in systolic blood pressure. (44)

Carotid Artery Stenosis

In a small, long-term study, 19 subjects (ages 65-75) with severe carotid artery stenosis (70-90% stenosis of internal carotid arteries) were randomized to receive either 50 mL PJ daily containing 1.5 mmoles total polyphenols (n=10) or no treatment (n=9) for one year; five subjects continued PJ for an additional two years. Study participants were treated with similar hypocholesterolemic and antihypertensive medications and no dietary or lifestyle changes occurred in either group. Blood samples were collected and echo Doppler analysis was performed at baseline and at 3, 6, 9, 12, 22, 28, and 36 months. Control subjects demonstrated a mean nine-percent increase in intima-media thickness (IMT) of left and right carotid arteries during the first year. Conversely, those consuming PJ had reduced IMT at 3, 6, 9, and 12 months ranging from 13 percent at three months to 35 percent at one year compared to baseline values. (67)

Most serum biochemistry parameters remained unchanged by PJ consumption over the first year, with the exception of triglyceride concentrations, which increased 16 percent but remained in the normal range. Serum lipid peroxidation in subjects consuming PJ was significantly reduced by 59 percent after one year, and levels of LDL-associated lipid peroxides were also decreased by as much as 90 percent after six months of supplementation. Body mass index did not change in treated subjects but systolic blood pressure was reduced an average of 16 percent during the three-year study. (67) In addition to previous reports of reduced systolic blood pressure (44) and inhibition of

lipid peroxidation, (63) this study demonstrated that PJ consumption (via antioxidative mechanisms) significantly reduces various aspects of IMT in patients with severe carotid artery stenosis (Table 2).

Myocardial Perfusion

In a double-blind, randomized, placebo-controlled trial, 39 patients were given either 240 mL PJ (polyphenol content not specified) (n=23) or a sports beverage of similar color, flavor, and caloric content daily for three months (n=16). Although both control and treatment patients demonstrated similar levels of stress-induced ischemia at baseline, at three months stress-induced ischemia increased in the placebo group (from 5.9 [+ or -] 4.3 to 7.1 [+ or -] 5.5) but decreased in the treatment group (from 4.5 [+ or -] 3.1 to 3.7 [+ or -] 3.7). In addition, angina episodes increased 38 percent in the placebo group but decreased 50 percent in the treatment group (a net change of 88 percent). These results demonstrate a reduction in myocardial ischemia and improved myocardial perfusion (as measured by stress-induced ischemia) in patients consuming pomegranate juice. (68)

Diabetes

In an animal model of diabetes, Huang et al demonstrated the favorable effect of PFLE on lipid profiles (66) and cardiac fibrosis's of Zucker fatty diabetic rats. Rosenblat et al investigated the effect of 50 mL/day PJ for three months on oxidative stress, blood sugar, and lipid profiles in 10 type 2 diabetic patients (history of diabetes for 4-10 years) and 10 healthy controls (ages 35-71). (69) In diabetic patients, triglyceride levels were 2.8 times greater, HDL cholesterol was 28-percent lower, and hemoglobin A1C (HbA1C) values were 59-percent higher than in control patients. Insulin was only slightly lower in patients than controls, and C-peptide (a proinsulin metabolite marker for endogenously secreted insulin) was slightly higher in diabetic patients than in healthy controls at baseline (indicating slight hyperinsulinemia). Consuming PJ for three months did not significantly affect triglyceride, HDL cholesterol, HbA1C, glucose, or insulin values, but did lower serum C-peptide values by 23 percent compared to baseline in diabetic patients--a sign of improved insulin sensitivity.

PJ consumption also significantly reduced oxidative stress in the diabetic patients as evidenced by a 56-percent reduction in lipid peroxides and a 28-percent reduction in TBARS compared to baseline serum levels. In addition, a 39-percent decrease in uptake of oxidized LDL by human monocyte-derived macrophages (an early development in foam cell formation and atherogenesis) was observed in diabetic patients after PJ consumption. Researchers concluded that despite the sugars naturally present in pomegranate juice, consumption did not adversely affect diabetic parameters but had a significant effect on atherogenesis via reduced oxidative stress. (69)

Dental Conditions

Topical applications of pomegranate preparations have been found to be particularly effective for controlling oral inflammation, as well as bacteria and fungal counts in periodontal disease and Candida-associated denture stomatitis.

Dental Plaque

A hydroalcoholic extract of Punica granatum fruit (HAEP) was investigated for antibacterial effect on dental plaque microorganisms. Sixty healthy patients (33 females/27 males; ages 9-25) with fixed orthodontic appliances were randomized to three groups of 20: (1) control group who rinsed with 15 mL distilled water; (2) a group who rinsed with 15 mL chlorhexidine, a standard antiplaque mouth rinse; and (3) a group who rinsed with a 15-mL HAEP solution. Rinsing duration was one minute and dental plaque material was collected from each patient prior to and after rinsing. Samples were diluted and plated on Meuller-Hinton agar and incubated at 37[degrees] C for 48 hours. HAEP decreased the number of colony forming units (CFU) of dental plaque bacteria 84 percent, comparable to chlorhexidine (79-percent inhibition) but significantly better than the control rinse

(11-percent inhibition). Both HAEP and chlorhexidine were effective against Staphylococcus, Streptococcus, Klebsiella, and Proteus species, as well as E. coll. The ellagitannin, punicalagin, is thought to be the fraction responsible for pomegranate's antibacterial activity. (49)

Periodontal Disease

A preliminary and follow-up study by a group of Thai researchers investigated the effect of biodegradable chips impregnated with Centella asiatica and P. granature pericarp on periodontal disease in 20 patients with gum pocket depths of 5-8 mm. A baseline exam was performed and followed by root planing and scaling of target teeth. Subgingival placement of the medicated chips (treatment group) and non-medicated chips (placebo/control group) followed, and pocket depth, attachment level, bleeding, and gingival and plaque indexes were measured at baseline and after three and six months. All treatment sites demonstrated a trend toward decreasing plaque and significant improvements were noted in pocket depth and attachment level at three months compared to placebo. (70)

In the follow-up study, 15 patients who had completed standard periodontal therapy but still had pocket depths of 5-8 mm were implanted with the same medicated chips. The same parameters were measured again at baseline and after three and six months, but researchers also measured inflammatory markers interleukin-1[beta] (IL-1[beta]) and IL-6. Significant improvement was noted in all re-measured parameters and confirmed by significant decreases in IL-1[beta] and IL-6 at three and six months compared to baseline. (71)

Denture Stomatitis

The primary etiologic factors for denture stomatitis are poor oral hygiene, inflammation from ill-fitting dentures, and Candida infection, (72,73) which manifest as swelling, pain, burning in the mouth, and aphthous ulcers. (74) In a randomized, double-blind study of 60 subjects (ages 19-62) with candidiasis confirmed via mycologic examination, the effect of a gel-based P. granatum bark extract (GPBE) was evaluated for its effect on healing of oral lesions and direct fungicidal effect. Patients were randomized into two groups of 30: one received miconazole oral gel (a standard therapy) and the other used GPBE, both three times daily for 15 days. Gels were applied to oral surfaces, dentures were removed and cleaned nightly, then brushed with the corresponding oral gels. All subjects reported an improvement in symptoms and general oral health. Clinical symptoms of those using miconazole were slightly better (27/30 satisfactory improvement) compared to GPBE (21/30 satisfactory improvement). Clearing of Candida infection was approximately the same in both groups (25/30 in the miconazole group and 23/30 in the GPBE group). (50)

Interestingly, despite randomized subject placement, there were three times more subjects with good oral hygiene scores in the miconazole group compared to the GPBE group, possibly accounting for the superior results observed by miconazole therapy. Also, because the initial step in the development of Candida denture stomatitis is adherence of organisms to dentures and the miconazole gel was stickier than GPBE, contact duration of miconazole was longer. A stickier GPBE might result in improved clinical response. (50)

Bacterial Infections

The only human trials examining the antibacterial properties of pomegranate extracts have focused on oral bacteria. (49,50,70,71) However, several in vitro assays demonstrate its bacteriocidal activity against several highly pathogenic and sometimes antibiotic-resistant organisms. Brazilian researchers evaluated the synergistic effect of a P. granatum methanolic extract with five antibiotics on 30 clinical isolates of methicillin-resistant Staphylococcus aureus (MRSA) and methicillin-sensitive S. aureus. (75) Antibiotics tested were chloramphenicol, gentamicin, ampicillin, tetracycline, and oxacillin. Although synergistic activity between the pomegranate extract and all five antibiotics was

noted in the S. aureus isolates, synergy with ampicillin was the most pronounced. A combination of the two increased the lag time to bacterial growth by three hours (over that of ampicillin alone) and was also bacteriocidal as evidenced by a 72.5-percent reduction in methicillin-sensitive organisms and a 99.9-percent reduction in MRSA. Based on earlier research (76) and the results of this study, the ellagitannin, punicalagin, is thought to be the primary constituent responsible for the observed antibacterial effects.

Another organism that can cause significant disease in humans is enterohemorrhagic Eschericbia coli (E. coli O157:H7), which can present with diarrhea, hemorrhagic colitis, thrombocytopenic purpura, and hemolytic uremic syndrome. P. granatum and seven other Thai medicinal plant extracts were tested for in vitro activity against E. coli O157:H7. An ethanolic PPE, one of the two most effective extracts against E. coli O157:H7, was shown to be both bacteriostatic and bacteriocidal, indicating PPE may be an effective adjunct treatment for E. coli O157:H7 infection.
47)

Ultraviolet Radiation

In vitro studies using normal human epidermal keratinocytes and PFE demonstrate PFE incubation with cell cultures ameliorates ultraviolet A and B radiation-induced cell damage in a dose- and time-dependent manner, providing evidence at a cellular level that PFE may be an effective photo-chemopreventive agent. (77,78)

A double-blind, placebo-controlled trial evaluated the protective and ameliorative properties of pomegranate extract and its EA constituent on UV-induced skin pigmentation. An ethanolic PPE was prepared containing 89.5 percent EA, confirmed by HPLC analysis. Thirty-nine healthy women (ages 20-49) were randomly assigned to one of three groups: (1) high-dose (200 mg/day) EA tablets; (2) low-dose (100 mg/ day) EA tablets; and (3) placebo (0 mg EA) tablets for four weeks. Prior to the first dose, subjects received a 1.5 minimum erythema dose (MED) of UV radiation on the inside upper right arm. Melanin, luminance, and erythema values were measured at baseline and at the end of each of the next four weeks. A questionnaire was completed by subjects to evaluate PPE's effectiveness on improvement of UV-induced slight sunburn. Rate of change for luminance, melanin, and erythema values was not significantly different for subjects receiving either EA dose compared to placebo or compared to baseline values. However, analysis of the questionnaire results demonstrated a trend toward amelioration of UV-induced damage in both EA groups compared to placebo. (79)

Erectile Dysfunction

A study using a rabbit model of arteriogenic erectile dysfunction (ED) measured the effect of PJ concentrate on intracavernous blood flow and penile erection. Azadzoi et al found eight weeks administration of 3.87 mL PJ concentrate (112 [micro]mol polyphenols) daily significantly increased intracavernous blood flow and smooth muscle relaxation, probably via its antioxidant effect on enhanced NO preservation and bioavailability. (80)

A randomized, double-blind, placebo-controlled, 10-week crossover trial in 53 men (mean age 46) investigated PJ's therapeutic effect on mild-to-moderate ED. Subjects with other medical conditions that might contribute to ED were excluded, and subjects were asked to refrain from taking ED medication for the duration of the study. The trial consisted of two four-week treatment periods separated by a two-week washout. During the first four weeks, subjects were given PJ (1.5 mmol polyphenols daily) or placebo beverage, followed by washout and crossover to the other group. Although assessment via the International Index of Erectile Function and Global Assessment Questionnaires demonstrated a trend toward improvements in ED, statistical significance was not achieved. This may be attributable to small sample size, short study duration, subject compliance

with beverage consumption, or may indicate the PJ dosage did not have an appreciable effect on ED. (81)

Male Infertility

Research in rats demonstrates PJ consumption improves epididymal sperm concentration, spermatogenic cell density, diameter of seminiferous tubules, and sperm motility, and decreases the number of abnormal sperm compared to control animals. An improvement in antioxidant enzyme activity in both rat plasma and sperm was also noted. (82)

Neonatal Hypoxic-Ischemic Brain Injury

Neonatal hypoxic-ischemic (HI) brain injury in severely preterm, very low birth-weight infants is a major cause of infant illness and death (83) and has been associated with an increase in reactive oxygen species. (84) Two studies in which pregnant mice were given PJ in drinking water revealed the neonatal offspring, when subjected to experimentally-induced HI brain injury, had significantly less brain tissue loss (64% decrease) and significantly decreased hippocampal caspase-3 activity (84% decrease) compared to neonates with experimentally-induced HI brain injury from dams who consumed a control beverage. (85,86) These results suggest PJ has an antioxidant-driven neuroprotective effect conferred from mother to neonate.

Alzheimer's Disease

The neuroprotective properties of pomegranate polyphenols were evaluated in an animal model of Alzheimer's disease. Transgenic mice with Alzheimer's-like pathology treated with PJ had 50-percent less accumulation of soluble amyloid-beta and less hippocampal amyloid deposition than mice consuming sugar water, suggesting PJ may be neuroprotective. Animals also exhibited improved learning of water maze tasks and swam faster than control animals. (87)

Obesity

PFLE (400 or 800 mg/kg/day) given to obese hyperlipidemic mice for five weeks caused significant decreases in body weight, percentage of adipose pad weights, energy intake, and serum cholesterol, triglyceride, glucose, and total cholesterol/HDL ratios. Decreased appetite and intestinal fat absorption were also observed, improvements mediated in part by inhibition of pancreatic lipase activity. (88)

Potential Drug Interactions

Based on pomegranate's current popularity and research suggesting its therapeutic benefit in cancer, cardiovascular disease, and other diseases treated with prescription medications, it has been of interest to determine whether pomegranate extracts have any effect on cytochrome P450-3A, the hepatic enzyme system responsible for metabolism of many prescription medications. A randomized, single-dose, crossover study in 13 healthy human volunteers demonstrated PJ pretreatment did not affect elimination half-life or distribution of intravenous midazolam (a benzodiazepine derivative with anxiolytic, amnestic, hypnotic, anticonvulsant, and muscle relaxant properties), nor did it affect the [C.sub.max] or clearance of oral midazolam. (89) This human study contradicts a rat study showing PJ has an inhibitory effect on carbamazepine pharmacokinetics, an anticonvulsant medication also metabolized by cytochrome P450-3A. (90)

Table 3. Ongoing Pomegranate Trials

CLINTRIALS.GOV IDENTIFIER	STUDY FOCUS	SPONSOR	ESTIMATED ENROLLMENT	STUDY START DATE	ESTIMATED COMPLETION DATE	STATUS
NCT00413530	Rising PSA levels in men with previous prostate cancer	M.D. Anderson Cancer Center; Houston, TX	300 subjects	December 2006	December 2008	Currently recruiting
NCT00060086	Recurrent prostate cancer	Jonsson Comprehensive Cancer Center; National Cancer Institute	29-40 subjects	March 2003	September 2004 or longer	Ongoing
NCT00433797	Prostate cancer	University of Oslo; Norwegian Cancer Society; The Research Council of Norway	102 subjects	June 2007	March 2009	Currently recruiting
NCT00381108	Benign prostatic hyperplasia	University of California, Irvine; Jarrow Pharmaceuticals	20 subjects	September 2005	March 2009	Currently recruiting
NCT00455416	Follicular lymphoma	University of Oslo, Norway	45 subjects	April 2007	December 2009	Currently recruiting
NCT00336934	Rising PSA levels in men with previous prostate cancer	Jonsson Comprehensive Cancer Center; National Cancer Institute	250 subjects	November 2005	December 2009	Currently recruiting
NCT00428532	Atherosclerosis in diabetics	HaEmek Medical Center, Israel	10 males 10 females	March 2007	August 2007	Completed; publication pending
NCT00655031	Prevention of rhino-virus infection	Pom Wonderful LLC	150 subjects	April 2008	June 2008	Currently recruiting

Safety of Pomegranate Extracts

Pomegranate and its constituents have safely been consumed for centuries without adverse effects. Studies of pomegranate constituents in animals at concentrations and levels commonly used in folk and traditional medicine note no toxic effects. (91) Toxicity of the polyphenol antioxidant punicalagin, abundant in pomegranate juice, was evaluated in rats. No toxic effects or significant differences were observed in the treatment group compared to controls, which was confirmed via histopathological analysis of rat organs. (92)

Research in 86 overweight human volunteers demonstrated the safety of a tableted PFE in amounts up to 1,420 mg/day (870 mg gallic acid equivalents) for 28 days, with no adverse events reported or adverse changes in blood or urine laboratory values observed. (93) Another study in 10 patients with carotid artery stenosis demonstrated PJ consumption (121 mg/L EA equivalents) for up to three years had no toxic effect on blood chemistry analysis for kidney, liver, and heart function. (67)

Conclusion

An explosion of interest in the numerous therapeutic properties of Punica granatum over the last decade has led to numerous in vitro, animal, and clinical trials. Pomegranate is a potent antioxidant, superior to red wine and equal to or better than green tea. In addition, anticarcinogenic and anti-inflammatory properties suggest its possible use as a therapy or adjunct for prevention and treatment of several types of cancer and cardiovascular disease. Because of pomegranate's antimicrobial properties, it may aid in preventing infection by dental pathogens, pathogenic E. coli O157:H7, and antibiotic-resistant organisms such as MRSA. Pomegranate's effect on bacterial pathogens has only been tested in vitro, however, necessitating human trials to refute or substantiate any clinical effect. The possibility that pomegranate extracts may also have an effect on several other disease processes, such as Alzheimer's disease, osteoarthritis, neonatal brain injury, male infertility, and obesity, underscores the need for more clinical research. Currently, numerous

clinical trials are in progress exploring the therapeutic potential of pomegranate extracts (Table 3). (94)

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Johanningsmeier SD, Harris GK

Pomegranate as a Functional Food and Nutraceutical Source

Pomegranate, a fruit native to the Middle East, has gained widespread popularity as a functional food and nutraceutical source. The health effects of the whole fruit, as well as its juices and extracts, have been studied in relation to a variety of chronic diseases. Promising results against cardiovascular disease, diabetes, and prostate cancer have been reported from human clinical trials. The in vitro antioxidant activity of pomegranate has been attributed to its high polyphenolic content, specifically punicalagins, punicalins, gallagic acid, and ellagic acid. These compounds are metabolized during digestion to ellagic acid and urolithins, suggesting that the bioactive compounds that provide in vivo antioxidant activity may not be the same as those present in the whole food. Anthocyanins and the unique fatty acid profile of the seed oil may also play a role in pomegranate's health effects. A more complete characterization of pomegranate components and their physiological fate may provide mechanistic insight into the potential health benefits observed in clinical trials.

Reference [3] Tsinghua Science & Technology 2008 **13**(4), 460-465

Xiang L, Xing D, Lei X, Wang W, Xu L, Nie L, Du L

Effects of Season, Variety, and Processing Method on Ellagic Acid Content in Pomegranate Leaves

Ellagic acid (EA) has aroused great interest worldwide owing to its antioxidant, anti-inflammatory, and anticarcinogenetic properties. The EA content in pomegranate leaf was measured in this study using high performance liquid chromatography to investigate the effects of season, variety, and processing method on the EA level. The results show that the EA content in 11 varieties of pomegranate from the Zaozhuang region in China range from 1.30 mg. g^{-1} to 6.46 mg. g^{-1} of dry weight in five consecutive seasons from June to October. An analysis of variance (ANOVA) shows that the EA content is significantly dependent on the season ($p<0.05$). The EA content increases significantly during the growing season to the highest level in September and October. The effect of the leaf variety on the EA content is less significant than the season. The processing methods have different effects on the EA content. Soaking for 24 hours slightly increases the EA content ($p<0.05$). Heating at 80°C or 100°C for 1 h after soaking has little influence on the EA content, while slow-fired cooking at high temperature significantly elevates the EA content ($p<0.05$). To improve quality and stability, several parameters such as leaf collection time, slow-fired cooking, and cooking time should be strictly controlled during the processing of pomegranate leaf tea and its extract.

Reference [4] Industrial Crops and Products 2012 **40**, 239-246

Saad H, Charrier-El Bouhtoury F, Pizzi A, Rode K, Charrier B, Ayed N

Characterization of pomegranate peels tannin extractives

The phenolic extractives of pomegranate peels from various cultivars were studied. The total polyphenols, the condensed and the hydrolyzable tannin contents were analyzed for 4 Tunisian pomegranate cultivars (Mekki, Chelfi, Gabsi, Jbeli). Matrix assisted laser desorption/ionization time-

of-flight (MALDI-TOF) mass spectrometry was used to examine the tannin oligomer structure. Analysis shows fluctuating rates of total polyphenols, condensed and hydrolyzable tannins between the different cultivars, but they are considered higher than those found with other plants.

Flavonoid oligomers up to pentamers and some hexamers were detected in MALDI-TOF positive ion mode analysis. In the negative ion mode analysis the majority of the oligomers have glucose residues linked to them.

Reference [5] Food Chemistry 2012 **132**(3), 1585-1591

Qu W, Breksa III AP, Pan Z, Ma H

Quantitative determination of major polyphenol constituents in pomegranate products

The anti-oxidant content and potential health benefits associated with consuming pomegranate and pomegranate-containing products has lead to increased consumer demand for this crop resulting in it becoming a high value crop. The potential health benefits and high anti-oxidant content of this fruit is attributed to the polyphenolic compounds it contains, including the ubiquitous phenolic acids, gallic acid and ellagic acid, and punicalagin A and punicalagin B, two polyphenolics unique to this fruit. A rapid HPLC–UV method targeting these four metabolites requiring minimal sample cleanup and offering runtimes half as long as existing methods was established. Within day and inter-day run-to-run variability for the four metabolites ranged from 1.9% to 6.6% and 5.3% to 11.4%, respectively. Spike recovery percentages for gallic acid, punicalagin A, punicalagin B and ellagic acid were found to be 98.5%, 92.4%, 95.5%, and 96.5%, respectively. This method was applied to the evaluation of various pomegranate products, including commercial drinks, handmade juice, and marc extracts. This method may be readily used to verify the presence of pomegranate metabolites in juices, extracts, and other products.

Pomegranate (Punica granatum) supplements: Authenticity, antioxidant and polyphenol composition

Madrigal-Carballo S, Rodriguez G, Krueger CG, Dreher M, Reed JD

Abstract

Pomegranates contain a complex mixture of gallotannins, ellagitannins, ellagic acid and anthocyanins. However, label claims on pomegranate supplements (PS) may not correlate with actual content of antioxidants, polyphenols or tannins. Nineteen PS were evaluated for their authenticity by determining ellagitannin composition by RP-HPLC and studying the relationship between total polyphenols as measured by the Folin–Ciocalteau assay and antioxidant capacity by oxygen radical absorbing capacity (ORAC), free radical scavenging properties by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and ferric reducing antioxidant power (FRAP). Only a limited number of pomegranate supplements were authentic. Product labels were inconsistent with polyphenol composition and antioxidant content. A majority of the samples ($n = 13$) contained disproportionately high amounts of ellagic acid and low or no detectable pomegranate tannins. Only six products had tannin composition that resembled pomegranates (punicalagin, punicalin, ellagitannins and gallotannins). PS-01 (natural pomegranate extract) was the most representative of pomegranate fruit polyphenols with 99% total pomegranate polyphenol and the highest antioxidant capacity across all measures. Correlations between total polyphenols and antioxidant content were high ($R^2 > 0.87$) in products that had polyphenol composition resembling pomegranates. Products that contained high amounts of ellagic acid and low or no detectable pomegranate tannins had poor correlations between total polyphenols and antioxidant content. The results indicate that reliable labeling information, better standardization, improved manufacturing practices and regulation of the market is required to assure consumers of the quality of pomegranate supplements.

1. Introduction

Improved methods for determining authenticity, standardization and efficacy of nutritional supplements are required for growth and regulation of the market. There are increasing numbers of pomegranate (Punica granatum) supplements in the retail markets in the United States. Most of these products are promoted based on their possible health benefits and antioxidant content, but their authenticity has not been evaluated and their manufacturing is not highly regulated.

Hydrolyzable tannins are the most abundant polyphenols and antioxidant compounds in pomegranates and include gallotannins, ellagitannins and gallagyl esters such as punicalagin and punicalin (Fig. 1). Pomegranate also contains oligomeric ellagitannins with two to five glucose core molecules crosslinked by dehydrodigalloyl and/or valoneoyl esters (Tanaka et al., 1986a,b; El-Nemr et al., 1990; Clifford and Scalbert, 2000; Afaq et al., 2005; Reed et al., 2005). The objective of this study was to correlate polyphenol content of PS to antioxidant content. Authenticity and quality of PS were also studied using qualitative analysis of polyphenol composition as determined by reverse phase HPLC.

2. Materials and methods

2.1. Pomegranate supplements (PS)

The products used for the study (Table 1) are available in retail stores. All products were analyzed prior to their expiration dates as stated on their packages (see Table 1 for list and codes for pomegranate supplements tested).

2.2. High performance liquid chromatography

A 5-mg sample of each PS was solubilized in 5 mL of methanol (10%, v/v), sonicated for 15 min in a FS-14 ultrasonic bath (Fisher Scientific,Waltham, MA) and centrifuged for 10 min at 1000g in a 5415 C centrifuge (Eppendorf, Westbury, NY). One hundred microlitres of supernatant were injected onto a Spherisorb ODS2, C-18 column (10 lm, 25 cm · 0.45 cm, Waters Co., Milford, MA). The solvents for elution were trifluoroacetic acid (TFA)/water (0.1%; solvent A) and methanol (solvent B). The elution profile was a series of step gradients: 100–73% A over 30 min; 73–45% A over the next 15 min, 45–0% A over 5 min. The flow rate was maintained at 2 mL/min, and the elution was monitored using a 996 diode array detector (Waters Co., Milford, MA) and Millennium software (Waters Co., Milford, MA) for collecting and analyzing three-dimensional chromatograms. The amount of each phenolic compound or group was estimated by multiplying the percent composition by the amount of total polyphenols in the supplement.

2.3. Folin–Ciocalteau assay for total phenolics (TPC)

The Folin–Ciocalteau method was used to quantify total phenolic compounds in the pomegranate nutritional supplements (PNS), using a pomegranate polyphenol standard (PPS) developed in a previous work (Martin et al., in press). Stock solutions of PPS were serially diluted and used to generate a standard curve for the estimation of total polyphenols. Water (3 mL), undiluted Folin–Ciocalteau reagent (0.2 mL, Sig- ma, St. Louis, MO) and 0.1 mL of the PPS solution (1 mg/mL; 50% (v/v) methanol) were added to tubes and the solutions were mixed and incubated at room temperature for 10 min followed by addition of 0.6 mL 20% (w/v) Na2CO3. After mixing, tubes were incubated at 40C for 20 min then rapidly cooled to room temperature in an ice bath. All samples were analyzed at 755 nm by UV–Vis spectrophotometry. A 5-mg sample of each PS was solubilized in 5 mL of 50% (v/v) methanol, sonicated for 15 min in a FS-14 ultrasonic bath (Fisher Scientific, Waltham, MA) and centrifuged for 10 min at 1000g in a 5415 C centrifuge (Eppendorf, Westbury, NY). One millilitre of the supernatant was collected and diluted with 50% (v/v) methanol such that the absorbance fell within the range of the PPS calibration curve.

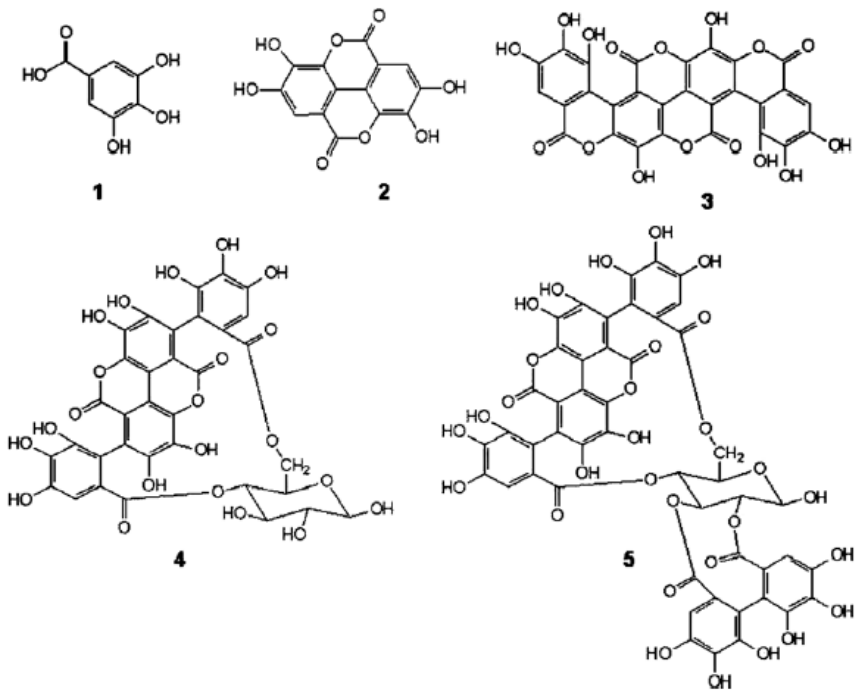

Fig. 1 – Structures of polyphenolic compounds found in pomegranate (Punica granatum). [Gallic acid (1), ellagic acid (2), gallagic acid (3), punicalin (4) and punicalagin (5).]

Table 1 – Polyphenolic composition, total phenolics (TP) and total antioxidants (TA) of pomegranate supplements.

Sample ID	Description (supplier)	Polyphenolic composition (mg PPS/g dry matter)[a]					TA & TP values[b]				Antioxidant index (%)
		Punicalin	Punicalagin isomer A	Punicalagin isomer B	Ellagic acid	Olligomers	ORAC (uM/g)	DPPH (uM/g)	FRAP (uM/g)	PPS (mg/g)	
	Pomegranate skin composition	13	100	232	13	755	–	–	–	–	
	Pomegranate arils composition	5	6	20	4	712	–	–	–	–	
PS-01	Natural pomegranate polyphenol extract 1000 mg (A)	10	51	126	33	766	3210	4485	1680	986	100.0
PS-02	Pomegranate extract 500 mg (B)	13	1	5	39	382	2175	2100	781	440	53.0
PS-03	Pomegranate extract 285 mg (whole fruit and seed powder) (C)	0	0	0	608	126	516	2170	218	734	35.1
PS-04	Ellagic acid 50 mg, anthocyanins 80 mg (D)	11	52	104	67	119	81	223	92	354	7.2
PS-05	Pomegranate fruit extract 250 mg (E)	5	6	10	358	227	2410	1360	371	605	45.8
PS-06	Fermented pomegranate powder blend 400 mg (F)	0	0	0	16	3	59	74	3	19	1.5
PS-07	Whole pomegranate fruit extract 400 mg (G)	22	13	17	32	189	898	1435	589	272	30.8
PS-08	Pomegranate 200 mg (H)	45	17	34	42	187	824	1370	485	325	29.0
PS-09	Pomegranate extract 200 mg (whole fruit and seed) (I)	0	0	0	410	89	1815	985	252	499	34.3
PS-10	Pomegranate extract 500 mg (J)	0	1	3	604	31	542	2275	158	640	34.9
PS-11	Pomegranate extract 250 mg (whole fruit) (K)	0	0	1	734	43	532	2390	210	778	37.7
PS-12	Pomegranate 250 mg (fruit conc., fruit skin and whole fruit) (K)	0	95	201	172	245	1940	2910	1043	736	64.0
PS-13	Pomegranate 200 mg (seed and fruit extract) (L)	40	16	38	120	156	910	1190	445	369	28.1
PS-14	Pomegranate 400 mg (fruit extract) (M)	13	84	175	38	473	2475	3850	1590	783	83.9
PS-15	Pomegranate 200 mg (fruit extract and seed) (N)	20	6	11	346	142	785	1170	275	526	26.6
PS-16	Pomegranate blend 440 mg (seed powder, aqueous extracts of juice, peel, leaf and flower) (O)	1	0	0	37	13	148	74	42	52	3.0
PS-17	Pomegranate fruit extract 400 mg (P)	22	6	9	582	128	1034	927	239	746	28.4
PS-18	Pomegranate 350 mg (fruit extract and seed meal) (Q)	4	1	2	307	105	392	1027	59	420	18.3
PS-19	Pomegranate extract 250 mg (whole fruit) (R)	27	2	8	122	111	675	1130	457	270	24.4
	Mean	12	18	39	246	186	1127	1639	473	503	36.1
	SD	14	30	63	241	184	924	1182	486	259	25.2
	Variation	1.1	1.6	1.6	1.0	1.0	0.8	0.7	1.0	0.5	0.8

a Polyphenolic composition by RP-HPLC is expressed as (mg PPS/g dry matter) times the polyphenolic fraction obtained from the HPLC assigned peak area integration.
b ORAC, oxygen radical absorbing capacity; DPPH, free radical scavenging properties; FRAP, ferric reducing antioxidant capacity; PPS, total phenolics as pomegranate polyphenols standard equivalents.

2.4. Antioxidant assays

All antioxidant assays were performed at Covance Analytical Laboratories, Inc. Madison, WI.

2.4.1. Total oxygen radical absorbance capacity (ORAC)

A mixture of 125 lL fluorescein (0.16 lM) and 250 lL, 2,20 -azobis-amidinopropane dihydrochloride (AAPH, 147 mM) at 37 C was combined with 250 lL of each dietary supplement sample diluted in phosphate buffer (0.2 M; pH 7.0). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards ranged from 5 to 40 lM. The decrease in fluorescence of fluorescein was determined by collecting readings at excitation 535 nm and emission 595 nm every minute for 45 min in a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA). The ORAC value was evaluated as area- under-curve (AUC) as previously reported by Cao et al. (1995).

2.4.2. Free radical scavenging capacity (DPPH)

The colorimetric DPPH scavenging capacity assay was performed according to a previously described laboratory protocol (Cheng et al., 2006). An aliquot of 500 lL of different concentrations of supplement sample dilutions in 50% (v/v) acetone was added to 500 lL of 0.208 mM DPPH solution. The initial concentration was 0.104 mM for DPPH in all reaction mixtures. Each mixture was vortexed for a few seconds and left to stand in the dark for 40 min at ambient temperature. The absorbance of each reaction mixture at 517 nm was measured against a blank of 50% (v/v) acetone using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, CA).

2.4.3. Ferric reducing antioxidant capacity (FRAP) assay

A 300-lL portion of the reaction solution (2,4,6-tri[2-pyridyl-S- triazine] 10 mM TPTZ, 20 mM ferric chloride and 300 mM (pH3.6) sodium acetate buffer in a 1:1:10 volume ratio) was heated at 37 C for 10 min and then 25 mg of the dietary supplements samples were added to the reaction mixture and its absorbance was read at 593 nm in a Molecular Devices Spec- traMax M2 plate reader. Ferrous sulphate standards ranged from 100 to 1000 lM. Results were expressed as lM Fe (Fe3+ ions converted to Fe2+).

2.5. Statistical analysis

Statistical analysis was performed using commercial software. The direction and the magnitude of the correlation between the variables were calculated using analysis of variance (ANOVA test). The criteria for statistical significance were p 6 0.05 (*), p 6 0.01 (**), p 6 0.001 (***).

An overall Antioxidant Index was determined by assigning all assays an equal weight, assigning a index value of 100 to the best score for each test, and then calculating an index score for all other samples within the test as follows: Anti-oxidant Index Score = [(sample score/best score) · 100] and then averaging all the four tests for each PS for an antioxidant index. All assays were given equal weight and an overall mean index value was calculated on a normalized basis for each PS

3. Results and discussion

Qualitative analysis of polyphenol composition of PS indicated that only seven products had tannin composition that resembled pomegranates. Only sample PS-01 (natural pomegranate extract) had an ellagitannin composition that was similar to pomegranate arils and skin with 99% purity and the highest antioxidant capacity across of measurements (Table 1). The polyphenol composition of pomegranate arils and skins is characterized by a high proportion of punicalagin, punicalin,

ellagitannins and gallotannins in comparison to ellagic acid (Table 1). Pomegranate arils contain anthocyanins, in small amounts, but the rind does not (data not shown). None of the PS contained anthocyanins. However, the pomegranate anthocyanins have not been shown to correlate to antioxidant capacity (Tzulker et al., 2007). The majority of PS (n = 13) contained disproportionately high amounts of ellagic acid compared to pomegranate arils and rind, including PS (n = 6) in which low or no pomegranate tannins were detected. In PS that contained detectable amounts of punicalagin and other pomegranate tannins, the absence of anthocyanins indicates that these supplements were most likely derived from extractions of the press cake which is the by-product of pressing juice from the whole fruit and is primarily composed of rind and seeds. However, the absence of punicalagin and other pomegranate tannins in PS indicates either these products did not contain pomegranate and that ellagic acid was added or pomegranate tannins were degraded during manufacturing.

Pomegranate supplements that had similar tannin composition to rind readily dissolved in aqueous methanol to give a brownish colored solution. On the other hand, PS that contained high levels of ellagic acid did not dissolve in aqueous methanol because ellagic acid has low solubility in this solvent. These products were characterized by white suspensions when agitated but quickly precipitated. Free ellagic acid in pomegranate fruit is low and may increase in juice and supplements as a result of the hydrolysis of ellagitannins (Bala et al., 2006; Ignarro et al., 2006).

Table 2 – Correlations between total phenolics and total antioxidants of pomegranate supplements.

	Correlation coefficient values (R^a)		
	ORAC	DPPH	FRAP
Folin	0.3706***	0.6472***	0.2991***
ORAC	–	0.4983***	0.6897***
DPPH	–	–	0.7019***

*$p \leqslant 0.05$.
**$p \leqslant 0.01$.
*** $p \leqslant 0.001$.

Table 3 – Correlations and significances between total phenolics and total antioxidants for pomegranate supplements resembling pomegranate ellagitannins composition and ellagic acid.

Sample group		Correlation coefficient (R^2)		
		ORAC	DPPH	FRAP
Pomegranate resembling samples				
	Folin	0.8737***	0.9209***	0.8989***
	ORAC		0.9541***	0.9355***
	DPPH			0.9925***
EA resembling samples				
	Folin	0.0404	0.4912**	0.3293*
	ORAC		0.0083	0.6368***
	DPPH			0.0779

* $p \leqslant 0.05$.
** $p \leqslant 0.01$.
*** $p \leqslant 0.001$.

The variation among PS in content of total polyphenols (TP) and antioxidant assays was large with coefficients of variation exceeding 50%. The total polyphenol content ranged from approximately 1% to close to 100% of the sample weight (Table 1).

All of the assays for content of polyphenols and antioxidant are REDOX reactions. Polyphenolic molecules undergo REDOX reactions because phenolic hydroxyl groups readily donate hydrogen to reducing agents. The Folin–Ciocalteau reagent is a REDOX reagent used to estimate total

polyphenolic compounds. Previous studies have reported that the content of total polyphenols, as determined by the Folin– Ciocalteau reagent, is highly correlated with other antioxidant assays (Wang et al., 1996; Benzie and Szeto, 1999; Apak et al., 2007), such as ORAC, DPPH and FRAP. However, correlations among TP, ORAC, DPPH and FRAP in PS were lower ($R2 < 0.65$) than expected (Table 2). These low correlations may be a result of the low solubility of ellagic acid in the 10 supplements in which ellagic acid was essentially the only polyphenol present.

Correlations between TP and TA were high ($R2 > 0.87$) in products that had a polyphenol composition resembling pomegranates (Table 3). Meanwhile, correlations among TP and TA was low or not significantly different from 0 in products that contained high amounts of ellagic acid and low or no detectable pomegranate tannins.

4. Conclusions

Product labels were inconsistent with polyphenol composition and antioxidant activity. Two samples contained no detectable polyphenols. The majority of the samples (n = 13) contained disproportionately high amounts of ellagic acid compared to pomegranate, including samples in which low or no pomegranate tannins were detected. Only six products contained pomegranate ellagitannins (punicalagin, punicalin, ellagitannins and gallotannins).

PS-01 was most similar to the ellagitannin composition of pomegranate fruit and the highest purity and antioxidant capacity across all measures.

The correlations among total polyphenols and antioxidant capacity were high in PS that contained pomegranate ellagit- annins. However, in PS that had high levels of ellagic acid and low or no detectable pomegranate ellagitannins, the correlations among total polyphenols and antioxidant capacity were low or not significantly different from 0, which may be explained in part by the low solubility of ellagic acid. High levels of ellagic may result from addition of ellagic acid to the product and/or extensive hydrolysis of the pomegranate ellagitannins during processing. Reliable labeling information, better standardization, improved manufacturing practices and regulation of the market is required to assure consumers of the quality of pomegranate supplements.

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Extract of Phenolics From Pomegranate Peels

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Abstract: The effects of different solvents, temperature conditions, solvent-solid ratios and particle sizes on solid-solvent extraction of the total phenolics, proanthocyanidins and flavonoids herein also referred to as antioxidant from pomegranate marc peel (PMP) was studied. Water, methanol, ethanol, acetone, and ethyl acetate extraction efficiencies at extraction times of 0.17 to 10 min, extraction temperatures of 25 to 95°C, ratios of solvent/solid of 5:1 to 50:1 and particle sizes of 10 to 40 mesh were evaluated. At 40 °C, solvent/solid ratio of 15 : 1, extraction time of 240 min and particle size of 40 mesh, methanol gave the highest extract yield of the total phenolics (8.26%), followed by water (5.90%), ethanol (1.55%), acetone (0.37%), and ethyl acetate (0.18%), respectively. However, at an extraction temperature of 95°C, the total phenolics extract yield with water was 11.15% for particle size of 40 mesh, solvent/solid ratio of 15:1, and extraction time of 2 min. Despite the lowest extract yield at extraction temperature of 40 °C, solvent/solid ratio of 15:1, extraction time of 240 min and particle size of 40 mesh, ethyl acetate extraction gave the highest content of the total phenolics (20.24%), proanthocyanidins (2.65%) and flavonoids (3.92%) in the extract. The DPPH antioxidant activity of extracts had a linear relationship with the total phenolics content in the extracts (R2=0.9779). This study revealed that water extraction, which
has the economic and safety merits, can be used as an environmentally friendly method for producing antioxidants from the PMP.

Keywords: Antioxidant, pomegranate, total phenolics, extract, temperature, time, ratio of solvent/solid, particle size, marc, residues.

1. INTRODUCTION

Pomegranate (*Punica granatum L*.) is an important fruit of tropical and subtropical regions, which originated in the Middle East and India and has been used for centuries in ancient cultures for its medicinal purposes. It is widely reported that pomegranate exhibits antivirus, antioxidant, anticancer, and antiproliferative activities [1-3]. Pomegranate is consumed fresh and in processed form as juice, wines, flavors, and extracts. Commercial pomegranate juice has the highest antioxidant activities compared to other fruit juices, red wine, and green tea and currently is a high value product in the agricultural market.

The pomegranate antioxidant activity is typically higher in commercial juices extracted from whole pomegranates than in experimental juices obtained from the arils only. This can be attributed to its high content of polyphenols in peel, such as condensed tannins and anthocyanins. The processing of pomegranate juice involves squeezing juice from the fruit with the seeds and the peels together. The resulting marc on a weight basis consists of approximately 73 % peels and 27% seeds and has a high potential for value addition as a source of phenolics, proanthocyanidins and flavonoids which are herein also referred to as antioxidants.

It has been reported that the peel in particular possesses relatively higher antioxidant activity than seed and pulp and therefore might be a rich sources of natural antioxidants [4-7]. Recently, natural antioxidants have become very popular for medical and food applications and are preferred by consumers than synthesized antioxidants, such as BHA and BHT. For instance, the use of agricultural

wastes such as wine-making wastes as alternative low-cost sources of phenolics compounds has been on the increase [8-10]. Extraction is the first step in the commercial isolation of these antioxidant compounds from pomegranate. However, efficient
methods for extraction of antioxidants embedded in the pomegranate peels such as phenolics, proanthocyanidins and flavonoids and the determination of kinetic parameters which are important for designing efficient extraction process for their production from peels have not been studied.
Accordingly, the objective of this research was to evaluate solid-solvent extraction of antioxidants from the pomegranate marc peel (PMP) and further elucidate how different solvents, temperature conditions and solvent-solid ratios affect the extraction of the antioxidant compounds.

2. MATERIALS AND METHODS

2.1. Materials

Pressed pomegranate marcs (from 'Wonderful' variety of pomegranate) were kindly provided by Stiebs Pomegranate Products (Madera, CA), a pomegranate juice processor. They were collected after juicing and kept at -18°C until used. Prior to experiments, the samples were thawed at 4°C followed by hot air oven drying at 40 °C to a moisture content of about 8% (dry basis). The moisture content was determined by using oven drying at 105 °C until constant weight was achieved. The peels and seeds were manually separated.
The dried peel was ground in a mill (WBB-6, Gruendler Pulverizing Co., St. Louis, MO) equipped with a 5 mm opening sieve. The ground material was sieved through 10, 20, 30, 40 mesh screens. Five groups of different particle size samples were obtained: >10, 10~20, 20~30, 30~40, and <40 meshes.

2.2. Extraction Procedures and Effects of Different Parameters on Total Phenolics

2.2.1. Solvents

The extraction yield of antioxidant compounds from plant materials is influenced mainly by the conditions under which the process of liquid-solid extraction is carried out to separate a soluble fraction from a permeable solid [11]. In the present work, five solvents with different polarities were used to identify the most suitable one for the recovery of antioxidant components from pomegranate peel. The polarity of a solvent besides the dipole moment, polarizability and hydrogen bonding determines what type of compounds it is able to dissolve. Five types of solvents were used in this experiment: deionized (DI) water (polar solvent with a dielectric constant of 80); ethanol (polar with a dielectric constant of 24); methanol (polar with a dielectric constant of 33); acetone (polar with a dielectric constant of 21) and ethyl acetate (non polar with a dielectric constant of 6). All chemicals used were of analytical grade. For each solvent, dried and ground peel was extracted in a thermostatic water bath shaker (R/76, New Brunseick Scientific Co., Inc., Edison, NY) with a 15:1 (w/w) ratio solvent/sample (dry weight) at 40 °C for 4 h in a conical flask. The liquid extract was separated from solids by vacuum enhanced filtration through Whatman No. 1 filter paper. The filtrates were air dried in hood at room temperature and residual moisture removed in a vacuum oven at 50±2 °C. The dried extracts were weighted to analyze the total extract yield, the contents and yield of antioxidant compounds including total phenolics, proanthocyanidins and flavonoids. The reported results, as illustrated in equations 1-3, include the total extract yield (%), the yield of total antioxidant (either phenolics or proanthocyanidins or flavonoids) from the PMP (%), and the content of antioxidant (either phenolics or proanthocyanidins or flavonoids) (%) in extract respectively:

$$\text{Total extract yield}\,(\%) = \frac{\text{g dried extract}}{\text{100g PMP}} \times 100 \quad (1)$$

$$\text{Yield of antioxidant}\,(\%) = \frac{\text{g total of antioxidant}}{\text{100g PMP}} \times 100 \quad (2)$$

$$\text{Content of antioxidant}\,(\%) = \frac{\text{g total of antioxidant}}{\text{100g dried extract}} \times 100 \quad (3)$$

All reported weights and percentages are dry basis unless specified otherwise. All the extraction trials were carried out in triplicate.

2.2.2. Extraction Time and Temperature Effect

To study the effect of extraction time, samples of 3 g PMP powder (40 mesh) were mixed with 45 g DI water and extracted at 25, 60, and 95 °C for 0.167, 0.333, 0.5, 1, 1.5, 2, 4, 6, 8 and 10 min. The liquid extract was separated from solids by vacuum enhanced filtration through Whatman No. 1 filter paper. The filtrate was transferred to a 50 ml flask after filtration, and DI water was added to make the finally volume to 50 ml. After the filtrate volume adjustment, the total phenolics concentration was measured.

To determine the effect of extraction temperature on the recovery of phenolics, temperatures of 20, 40, 60, 80, 95°C were tested during a 2 min extraction. Samples (40 mesh) of 5, 3, 1.8 g were mixed with 45 ml DI water to achieve the following ratios: 9, 15, 25(w/w).

2.2.3. Solvent-Solid Effect

The effect of solvent-solid ratio on the total phenolics extraction was studied. Samples (40 mesh) were mixed with 45 g DI water at ratio of solvent-solid from 5 to 50, and extraction performed at temperature of 60 °C for 2 min. The phenolics yields were determined.

2.2.4. Particle Size Effect

The PMP samples of five particle sizes were investigated in this study: >10, 10-20, 20-30, 30-30 and <40 mesh. Extractions were conducted at 60°C for 2, 20 and 60 min. The ratio of solvent/solid used was 15 and the phenolics yields were measured.

2.3. Analysis Assay

2.3.1. Total Phenolics Content

The total phenolics content in the extract was determined by the Folin-Ciocalteu method [5]. The 0.05 g of dried extracts was dissolved in 5 ml methanol or the filtrate made up to 50 ml were used directly. Aliquots of 10 µl of samples were mixed with 2.5 ml of 10-fold-diluted Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The total volume of the mixture was adjusted to 25 ml using DI water and allowed to stand for 30 min at room temperature before the absorbance was measured at 760 nm using a spectrophotometer (Hewlett Packard 8452A, Diode Array, USA). The total phenolics content in the extract was calculated and expressed as tannic acid equivalents (TCE; g/100 g dry mass) using a tannic acid (0–0.004 mg/ml) standard curve.

2.3.2. Flavonoid Content

The flavonoids content was measured using a modified colorimetric [5]. A quantity of 0.05 g of dried extracts was dissolved in 5 ml methanol or the filtrates made up to 50 ml were used directly. A volume of 0.4 ml of the solution was transferred to a 25ml flask containing 5 ml of 30% ethanol and mixed with 0.75 ml of 5% sodium nitrite for 5 min. Then, 0.75 ml of 10% aluminum nitrate was added. After 6 min, the reaction was stopped by adding 5 ml of 1 M sodium hydroxide. The mixture was further diluted with 30% ethanol up to 25 ml. The absorbance of the mixture was immediately measured at 510 nm. The flavonoids content was calculated and expressed as rutin equivalents (RE, g/100 g dry mass) using a rutin (0~0.03 mg/ml) standard curve.

2.3.3. Proanthocyanidin Content

Determination of Proanthocyanidins was based on the procedure reported in literature [5]. A quantity of 0.05 g of dried extracts was dissolved in 5 ml methanol or the filtrates made up to 50 ml were used directly. A volume of 1 ml solution was mixed with 3 ml of 4% vanillin–methanol solution and 1.5 ml hydrochloric acid and the mixture was allowed to stand for 15 min at room temperature. The absorbance at 500 nm was measured and the Proanthocyanidins was expressed as catechin equivalents (CE, g/100g dry mass) using a catechin (0~0.08 mg/ml) standard curve.

2.3.4. Antioxidant Activity

The antioxidant activity of PMP extracts was measured in terms of hydrogen donating or radical scavenging ability, using a modified DPPH method [11]. A volume of 10µl of 0.01 g/ml of dried extract in methanol solution was added to 1 ml (500 µM) of DPPH solution and diluted to 25 ml with methanol. The solution was shaken vigorously with vortex and incubated at room temperature (25±2°C) for 20 min. The decrease in absorbance at 517nm was determined at the end of incubation period with a Spectrophotometer. The control was prepared as above without any extract and methanol was used as blank. Radical scavenging activity was expressed as the inhibition percentage (I %) and was calculated using the following formula:

$$I\% = ((A_c - A_s)/A_c) \times 100$$

where, *Ac* is the absorbance of the control reaction (containing all reagents except the test compound) and *As* is the absorbance of the test compound.

3. RESULTS AND DISCUSSION

3.1. Effect of Extraction Procedures and Different Parameters

3.1.1. Influence of Solvents

Results for the total extract yields reported as percentage of g of extract per 100g pomegranate peel on dry basis indicated that the pomegranate peel extracted with methanol gave the highest total extract yield (46.51±0.86), followed by water (43.19±2.24), ethanol (17.71±0.23), acetone (3.81±0.08) and ethyl acetate (0.88±0.08) when the extractions were done with the ratio of solvent/sample of 15:1 (w/w) at 40 °C for 4 h. It should be noted that, because of polarity differences between solvents, the solubility of the solute into the solvent is expected to be different. Water, methanol and ethanol

are polar protic solvents of dielectric constants of 80, 33 and 24 respectively, while acetone and ethyl acetate are polar aprotic and non-polar solvents of dielectric constants of 21 and 6 respectively. It has been reported that pomegranate peel extract yield (%, w/w) were 9.38, 7.53 and 1.04 for methanol, water and ethyl acetate respectively under the following experimental conditions: peel powder (25 g) extraction by mixing using a magnetic stirrer with 100 mL of the corresponding solvents at 30 °C for 1 h, filtration through Whatman No. 41, residue re-extraction with the same solvent, extract pooling and concentration under vacuum at 40 °C [11]. Our findings agree in terms of solubility trend but differ in the extracted yield.

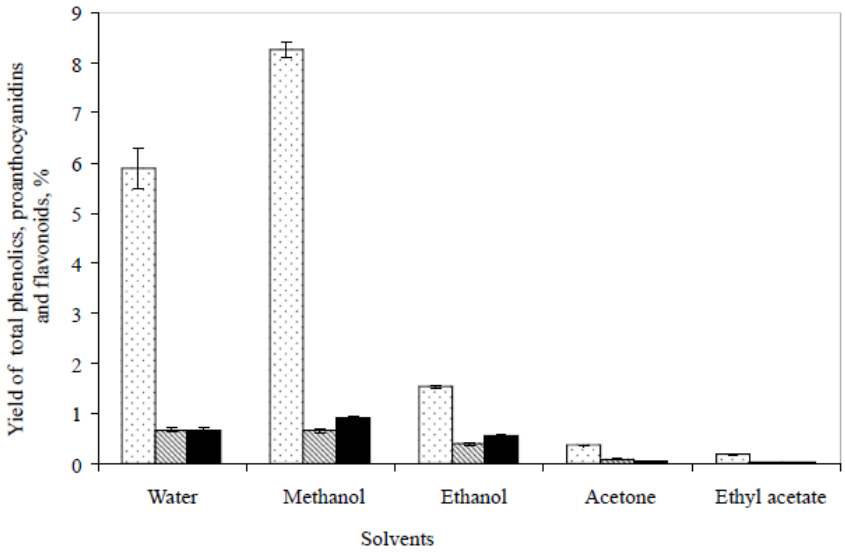

Fig. (1). The effect of different solvents on the yield of total phenolics, proanthocyanidins and flavonoids from the marc of pomegranate peels. Extraction was conducted at a temperature of 40 °C, a solvent/solid ratio of 15:1, a particle size of 40 mesh and an extraction time of 240 min: □, Total phenolics; ▨, Proanthocyanidins; ■, Flavonoids.

The effect of different solvents on the yield of total phenolics, proanthocyanidins and flavonoids from the pomegranate peels are shown in Fig. (**1**). Methanol and water gave the top two yields of all three antioxidant components, which indicate that they are more effective than ethanol, acetone, and ethyl acetate for the antioxidants' extraction from the pomegranate peel. Particularly for the phenolic content, our results are different from the result reported elsewhere in literature [12] that the phenolic content from water extraction was the lowest among ethyl acetate (EtOAc), acetone, methanol and water. In the preceeding results, the phenolic contents of EtOAc, acetone, MeOH and water extracts were found to be 16.5, 52, 46.2 and 4.8%, respectively. That withstanding, the value for the total phenolic yield obtained using MeOH is comparable to that reported by other researchers [11]. This deviation particularly in the values is likely to be due to the difference in extraction and phenolic content determination procedures [12]. For instance, the powder from pomegranate peel was extracted with a Soxhlet extractor for 4 h [12], filtered, concentrated under vacuum at 40 °C [11] and then dissolved in methanol:water (6:4 v/v) (1 mg/ml) for evaluation of antioxidant capacity; the concentration of phenolics in the extracts was determined [11] and results were expressed as (+) catechin equivalents. In our determination, the results were expressed as tannic acid equivalents (TCE; g/100 g dry mass). Despite the low yield of total phenolics, proanthocyanidins and flavonoids from the pomegranate peel (%: g total calculated weight of antioxidants/100 g PMP), the concentration of these compounds in the extracts content wise were the highest in the usage of ethyl acetate among the five different extraction solvents (Fig. **2**). The total phenolics content was

higher in methanol extract (18%) than in water extract (14%) and comparatively lower in ethanol extract (9%).

It is reported [11] that pomegranate phenolics content was 44% with methanol, 3.0% with water, and 18% with ethyl acetate. Our results, however, show that the total phenolic content in the water extract and the MeOH extract was nearly the same: 14% and 18% respectively. Factors that have been attributed to bringing variation include the method of extraction [12], mixture of different solvents [5] and use of different materials [13] among others. A possible factor for higher content of phenolics, proanthocyanidins and flavonoids (%) in extract (g phenols/100 g dried extract) is due to the higher purity of extract associated with using ethyl acetate. The use of methanol, ethanol, acetone, and water, generally yields a significant co-extraction of concomitant substances and decreases the yield of target antioxidants [14]. So whereas ethyl acetate may exhibits significant selectivity in respect of natural products, methanol and water allow for higher total extract yield (g dried extract/100g PMP). The proanthocyanidins content of ethanol and acetone
extracts were almost the same (3%), although higher than the contents of water extract (1%) and methanol extract (1%). The content of flavonoids in ethanol extract (4%) is much higher than water extract (1%), methanol extract (2%), and acetone extract (1%) (Fig. **2**). Water would be a better extracting agent than methanol when the toxicity and cost aspects are considered. Hence, in furthering studies on pomegranate total phenolics extraction, water was chosen as the best solvent.

3.1.2. Influence of Extraction Time and Temperature

Fig. (**3**) shows the kinetics curves of total phenolics yield. The parabolic shaped curve had three distinct phases. The initial phase was characteristically almost linear (up to 2 min at 25°C, 0.5min at 60 °C, 0.33min at 95°C) with higher percentage of phenolics yield increments per unit time. The second phase displayed a lower percentage of phenolics yield increments per unit time before the final asymptotic ending of the third phase. The results indicate that extraction process is sensitive to the extraction time and temperature in the early stage [14]. A similar trend has been observed using different materials [14] in a study of the kinetics of extraction of proanthocyanidins from dry grape seeds using ethyl acetate with different contents of water (10, 15 and 20%). In the study it was noted that the kinetic curves obtained were of parabolic shape, with the initial part being linear, thus reflecting a strong increase in the yield of proanthocyanidins, whereas their second parts showed a slower increase and an asymptotic ending [14]. Further, it has been identified that in
water-extracts of grape, the yield of polyphenols gently increased with the time [8]. Shorter times (preferably <8 h) were reported for grape marc phenolics extraction at 60 °C [15]. The antioxidant phenolics extraction time is 90 min from pine sawdust (*Pinus pinaster*) at 50 °C with 5:1 of liquid–solid ratio [16].

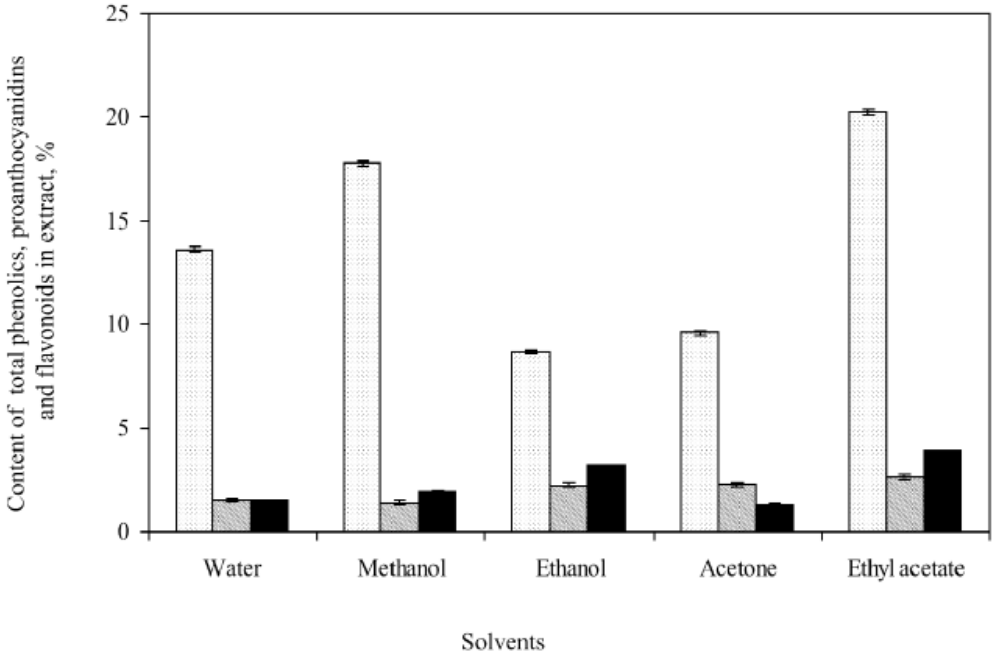

Fig. (2). The effect of different solvents on the content of total phenolics, proanthocyanidins and flavonoids from the marc of pomegranate peels (PMP) in the extract (g dried extract/100 g PMP). Extraction was conducted at a temperature of 40 °C, a solvent/solid ratio of 15:1, a particle size of 40 mesh and an extraction time of 240 min. □, Total phenolics; ▨, Proanthocyanidins; ■, Flavonoids.

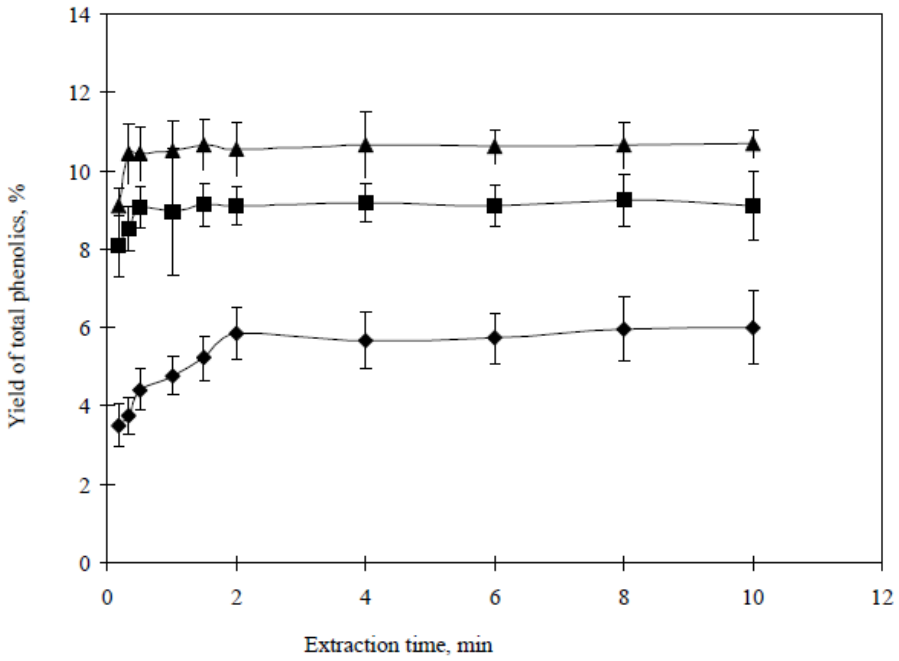

Fig. (3). The kinetics curves of water extracted total phenolics yield. The water extraction was done using a ratio of solvent to solid of 15：1 and particle size of 40 mesh. ◆, 25°C; ■, 60°C; ▲, 95°C.

The equilibrium times and concentrations were 2, 0.5, 0.33 min and 6.55, 9.14, 11.92 % at 25, 60, 95 °C respectively, as shown in Fig. (**3**). The diffusion of the dissolved solute within the solid into the solvent is the rate limiting step [17]. The short equilibrium time lies in three aspects: firstly, the fine particle sizes which enlarges the resolve surface area and shorten the mass transfer distance; secondly, the loose tissue of pomegranate peel with larger diffusion of the dissolved solute within the solid into the solvent and thirdly, the variety of phenolics.

The effect of extraction temperature on the extraction rate is shown in Fig. (**4**). An increase in temperature significantly increases diffusivity as established by the Einstein equation. The equilibrium concentration has a linear relationship with the extraction temperature. Temperature strongly influenced the total phenolics yields, but may enhance purity, probably because temperature increase favors extraction by increasing solubility and diffusion coefficient of any compounds, not only of antioxidants [10]. Extraction temperature, however, is affected by the extract mass transfer velocity and the equilibrium concentration.

3.1.3. Influence of Ratio of Solvent/Solid

The solid-solvent ratio affects the concentration gradient within the particles of raw material (Fig. **5**). The rates of extraction increased with a larger concentration gradient (Fig. **5**) in the first stage, and then reached equilibrium when most of the phenolics had been extracted out. The equilibrium ratio of solvent/solid decreased at higher extraction temperature before reaching equilibrium: ratio of 25 at 95°C and ratio of 30 at 60°C.

3.1.4. Influence of Particle Size

Particle size is also a factor to be considered during extract processing. Smaller particle size reduces the diffusion distance of the solute within the solid and increases the concentration gradient, which ultimately increases the extraction rate. Since the path of solute to reach the surface is shorter, the extraction time is reduced. In Fig. (**6**), total phenolics extract yield of smaller particle size goes up when the extraction time is 2 min. The foregoing results agree with earlier reports whereby higher yields in total phenolics and anthocyanins extraction resulted from a decrease in size of black currant juice press residues [9]. Fig. (**6**) also illustrates further the effect of extraction time on total phenolics extract.

3.2. Antioxidant Activity

The DPPH assay was employed. The DPPH assay has been widely used to determine the free radical-scavenging activity of various plants and pure compounds [18, 19]. DPPH is a stable free radical which dissolves in methanol or ethanol, and its purple color shows a characteristic absorption at 517 nm. When an antioxidant scavenges the free radical by hydrogen donation, the color from the DPPH assay solution becomes light yellow.

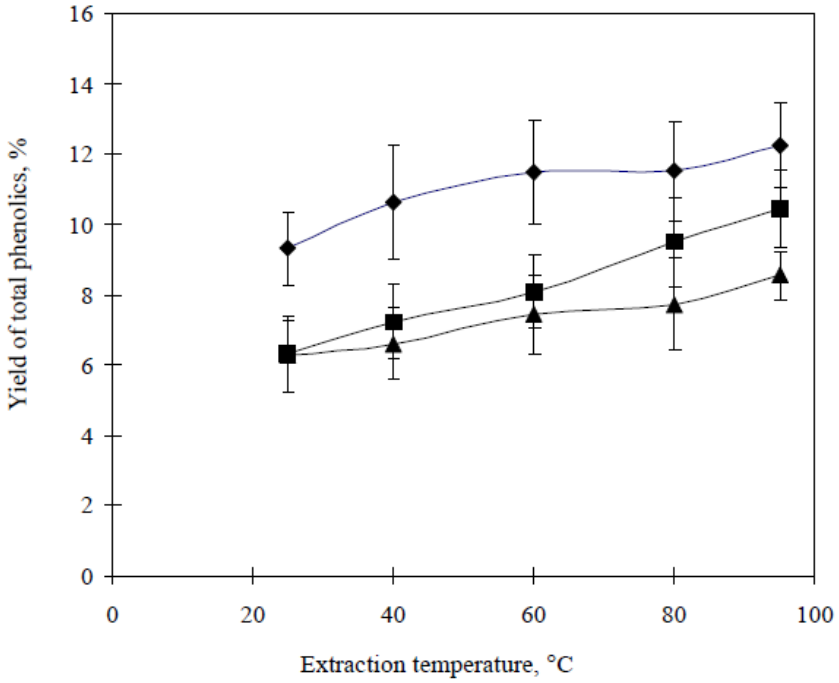

Fig. (4). The effect of extraction temperature on the yield of total phenolics for different solvent solid/ ratios. Extraction time was 2 min and the particle size of 40 mesh: ▲, 1;09; ■, 1:15; ◆, 1:25.

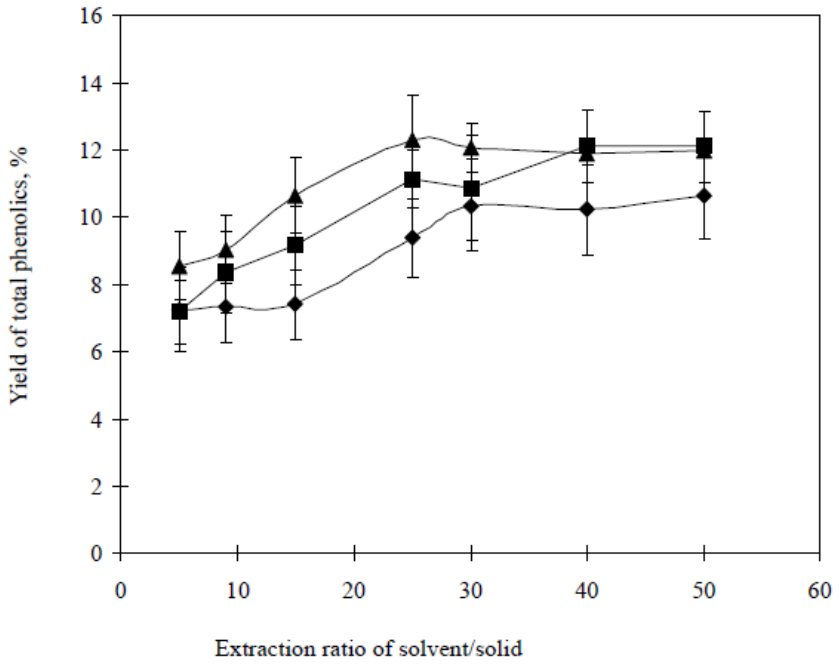

Fig. (5). The effect of the ratio of solvent /solid (gg^{-1}) used in the extraction on the yield of total phenolics. The extraction time was 2 min and the particle size of 40 mesh was used. ◆, 25°C; ■, 60°C; ▲, 95°C.

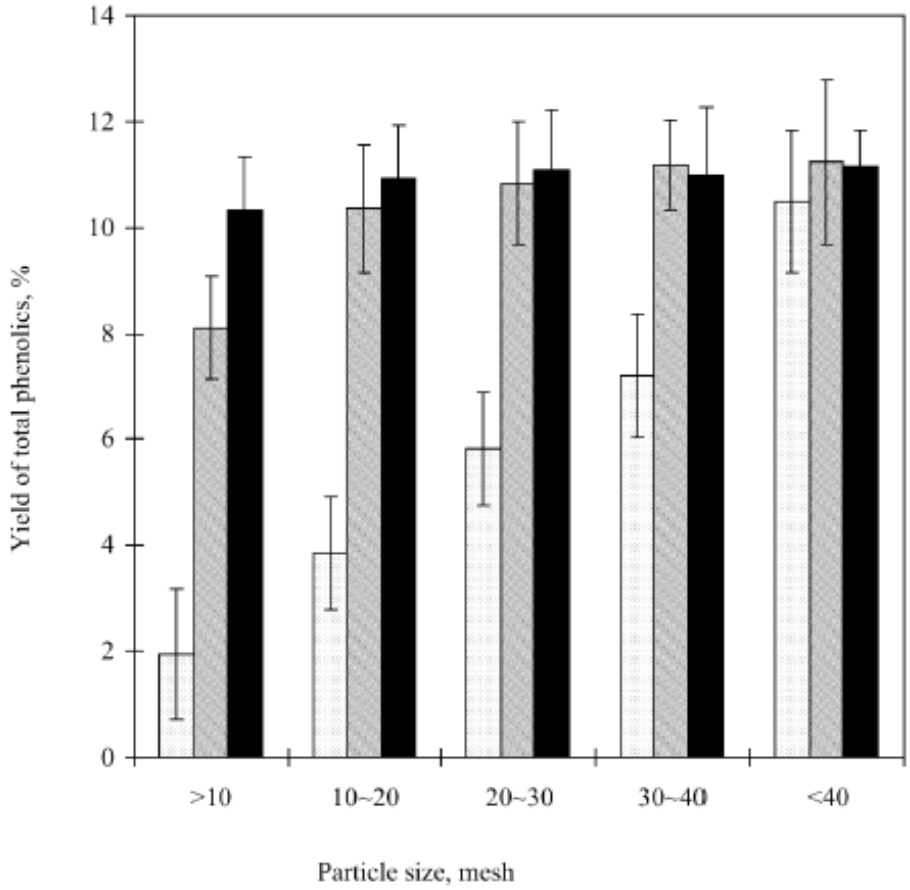

Fig. (6). The effect of particle size on the yield of total phenolics with different extraction times (2, 20 and 60 min). The extraction temperature was at 60°C. □, 2min; ▨, 20min; ■, 60min.

Our study of antioxidant activities of the extracts was carried out to investigate the correlations between the antioxidant activity and the content of phenolics, proanthocyanidins and flavonoids and results are shown in Fig. (**7**). The results indicate a strong correlation between DPPH and total phenolics (R2=0.98), but no correlation existed with proanthocyanidins (R2=0.01) and flavonoids (R2=0.05). Other studies [11, 12] also reported that the antioxidant activity of pomegranate peel correlated to the total phenolics. Therefore, the total phenolics yield should be one of the most important indicators of effective extraction process for producing high quality product. Comparing methanol with water as the solvent in pomegranate antioxidant extraction, the total extract yield (dried extract/100 g PMP) were 43.18% and 46.51%, the yield of total phenolics (g total calculated weight of phenolic/100 g PMP) were 5.90% and 8.26%, the content of phenolics (g phenols/100 g dried extract) were 13.63% and 17.78%, and the DPPH antioxidant activities were 53.74% and 65.30%, respectively.

4. CONCLUSIONS

The research showed that the peels from pomegranate marc are a potential resource for phenolics, proanthocyanidins and flavonoids. The antioxidant activity of pomegranate peel was attributed to the total phenolics. The pomegranate peel extracted with methanol gave the highest total extract yield, followed by water, ethanol, acetone and ethyl acetate when the extractions were done with the ratio of solvent/sample of 15:1 (w/w) at 40 °C for 4 h. Water compared well to methanol as an extracting solvent and qualifies as a better agent than methanol when toxicity and cost aspects are considered. Comparing methanol with water as the solvent in pomegranate antioxidant extraction, the total extract yield were 43.18% and 46.51%, the yield of total phenolics were 5.90% and 8.26%,

the content of phenolics were 13.63% and 17.78%, and the DPPH antioxidant activities were 53.74% and 65.30%, respectively. Shorter extraction time was needed with higher extraction temperature and smaller particle size. High yield was attainable with increased ratio of solvent/solid and was also affected by the extraction temperature. The total phenolics extract yield with water was 11.15% at the suggested extraction temperature of 95°C,
particle size of 40 mesh, ratio of solvent/solid of 15/1, and extract time of 2 min.

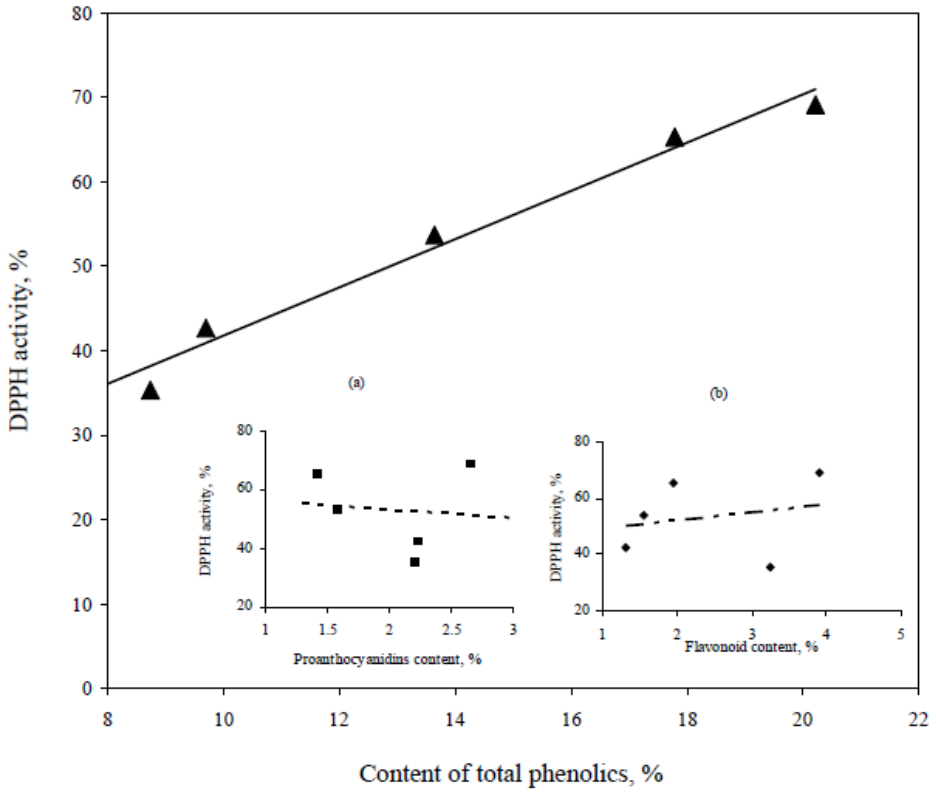

Fig. (7). The relationship between the content of total phenolics, proanthocyanidins and flavonoids of the pomegranate marc peel and the DPPH activity for the following extraction condition: extraction temperature of 40 °C, solvent/solid ratio of 15:1, extraction time of 240 min and particle size of 40 mesh. ▲, Total phenolics; _, Linear Total phenolics (y = 2.8594x + 13.183, r^2=0.9779); ■, Proanthocyanidins; ▪▪▪▪▪, Linear Proanthocyanidins (y = -2.8245x + 58.951, r^2=0.0099); ♦, Flavonoid; _, Linear Flavonoid (y = 2.8881x + 46.336, r^2=0.0511); DPPH activity (a) insert and proanthocyanidins content , DPPH activity (b) insert and flavonoid content.

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Antioxidant Activity of Pomegranate (*Punica granatum* L.) Fruit Peels

Shiban MS, Al-Otaibi MM, Al-Zoreky NS

ABSTRACT

The antioxidant activity of pomegranate fruit peels was evaluated using *in vitro* tests. 80% methanolic extracts (ME) of peels had higher yield (45.4%) and total phenolics (27.4%) than water (WE) or ether extracts (EE). The reducing power of ME was more potent ($P < 0.05$) than either WE or EE. The DPPH radical scavenging activity (%) of ME was stronger than that of *α*-catechin. Pomegranate peels contained phenolics, exhibited DPPH scavenging activity and reducing power.

Keywords: *Punica granatum*; Phenolic Compounds; DPPH Scavenging; Reducing Power

1. Introduction

There is increasing epidemiological and pharmacological evidence that plants contain biologically active components (e.g. free radical scavengers) offering health benefits and protection against degenerative diseases. In fact, oxygen radicals and lipid peroxides have been known for their alleged role in the etiology of many *in vivo* pathological reactions such as aging and cancer. In this regard, epidemiological studies have shown that consumption of fruits and vegetables is inversely associated with morbidity and mortality of cardio- and cerebrovascular diseases and certain types of cancers [1,2]. Unstable reactive oxy- gen species (ROS) react rapidly and destructively with biomolecules such as protein, lipid, DNA and RNA in the body. Uncontrolled generation of free radicals is as- sociated with lipid and protein peroxidation, resulting in cell structural damage, tissue injury or gene mutation [3,4].

The antioxidants contained in fruits and vegetables, such as ascorbic acid, flavonoids, and tannins, are sup- posed to play a very important role in the prevention of these diseases [1,5]. In biochemistry and medicine, anti- oxidants are enzymes or other organic substances, such as vitamin E or *β*-carotene, that are capable of counter- acting the damaging effects of oxidation in animal tissues and food [1]. It was stated that besides their endogenous defenses, the consumption of dietary antioxidants, such as phenolic compounds, play a vital role in protecting against ROS.

Plant phytochemicals (e.g. phenolics) have been associated with health benefits as a result of consumption of higher levels of fruits and vegetables. In fact, phenolic compounds from plants exhibit various physiological properties, such as anti-allergenic, anti-inflammatory, anti- microbial, antioxidant, anti-thrombotic, cardio-protective and vasodilatory effects [1,6,7]. Regarding food safety, one of the major causes of quality deterioration is lipid peroxidation. The oxidative deterioration of fats and oils in food products is responsible for rancidity and off flavors and thus leads to decrease in nutritional quality and safety due to the formation of secondary potentially toxic compounds [3,8,9].

In industrial practices, synthetic antioxidants have been used as food additives for more than fifty years to pre- vent peroxidation of fats and oils. Butylated-hydroxy-toluene (BHT), butylated-hydroxyanisole (BHA), tert- butylhydroquinone (TBHQ) are effective and common antioxidants preventing oxidation and off-flavor development in fats and oils. However, those chemicals are now doubted for their safety and recent literature has expressed safety concerns and health risks associated with their use in food products [5,10].

Therefore, the attention is now increasingly paid to the development and utilization of more effective, natural and non-toxic biologically-active materials including antioxidants from natural sources such as plants [5,10]. Effective antioxidants with less toxicity, especially those originating from natural plants used in folk medicine and food, are attracting the attention of medical and food scientists alike. In this regard, numerous natural medicinal plants have been evaluated for their antioxidant activities and research outcomes have shown that crude extracts or purified constituents from different medicinal plants were more effective antioxidants *in vitro* than some synthetic antioxidants. Consequently, plants could be potential sources for natural antioxidants and therefore they could be better alternatives for the synthetic ones. Natural ingredients such as antioxidants in food products could have greater application in increasing consumer acceptability and also improve stability of products. Up until now, substantial data are available on antioxidant capabilities of polyphenols from various herbs, such as green tea and rosemary [3,5,7,11]. Additionally, waste products (e.g. fruit peels) from processing of agricultural commodities could offer practical and economic sources of active antioxidants which could replace the synthetic ones [6,7,12]. Recently, the interest in the antioxidant properties of phenolic constituents from pomegranate fruits (*i.e*., arils and peels) has emerged [2,7,10,13]. The pomegranate plant (*Punica granatum* L., *Punicaceae* family) is a shrub and its fruit is a rich source of bioactive phytochemicals such as tannins and other phenolics. It is a native plant to the Mediterranean region and has been used extensively in folk medicine of some countries in Asia and other parts of the world. Interestingly, it was stated that pomegranate peels have been used since antiquity in the Middle East as colorant for textiles because of their high tannin and phenolic contents [2].

Pomegranate fruit products have been used for centuries since ancient civilizations for medicinal purposes. Stomachic, inflammation, fever, bronchitis, diarrhea, dysentery, vaginitis, urinary tract infection, and, among others, malaria have been treated using various parts of pomegranate including fruit peels [2,7,10]. Moreover, increasing numbers of pomegranate supplements and products (functional foods, therapeutic formulae and cosmetics) are also available in markets [3,7,13]. The phenolic constituents, ellagic tannins and ellagic acid, are among the potent antioxidants in peels [2,3,7,10,13,14].

Therefore, the purpose of the present investigation was to evaluate the antioxidant activity of peel extracts using *in vitro* methods.

2. Materials and Methods

2.1. Chemicals

Gallic acid monohydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and (+)-Catechin monohydrate were from Sigma (USA). Rutin was from Oxford Lab. Reagent (Mumbai, India). Other chemicals were from BDH (UK) and Fisher Scientific (USA).

2.2. Preparation of Plant Extracts

Pomegranate fruits (Yemeni varieties) at the maturity stage (17.5° Brix) were manually peeled, washed and air dried prior to extraction with solvents of different polarities [15]. Briefly, finely

powdered peels (5 g) were separately blended for 2 min (Waring blender) with 300 ml of 80% methanol, distilled water or diethyl ether. Each mixture was then left, in the dark, at room temperatures for 1 h prior to filtration (Whatman No. 1) and centrifugation (Sorvall RC-5, Dupont, USA) at 8654 g for 10 min at 5°C. When necessary, extracts of 80% methanol (ME), water (WE) and ether (EE) were kept at –20°C prior to analysis. Other sets of extracts (ME, WE and EE) were individually concentrated to dryness under reduced pressure at 40°C to determine yields (%) per original materials.

2.3. Determination of Total Phenolics

Total phenolics of peel extracts (ME, WE and EE) were determined using the method of Singleton and Rossi [16]. 200 µl portions of diluted extracts were introduced into test tubes followed by addition of 1000 µl of Folin-Cio-calteu reagent (1:10). Thirty seconds later and just prior to 8 min, 800 µl of Na2CO3 (7.5%) was added to extracts in tubes. The reaction mixtures were incubated at 24°C for 1 h prior to recording the absorbance at 765 nm against blank. Total phenolics were calculated from standard gallic acid solutions used under the same conditions, and concentrations were expressed as mg gallic acid equivalents (GAE) per g extract.

2.4. Total Flavonoids of Extracts

The amount of flavonoids in the peel extract with the highest total phenolics (ME) was determined by the AlCl3 [17]. To 1 ml of ME, 1 ml of 2% methanolic AlCl3·6H2O was added. The absorbance was measured 10 min later at 430 nm. The amount of total flavonoids was expressed as mg rutin equivalents (RE) per g ME.

2.5. Determination of Ascorbic Acid

The amount of ascorbic acid (AA) in ME was spectro-photometry determined [18] using standard solutions of AA (Sigma-Aldrich, UK).

2.6. Reducing Power of Extracts

The ferricyanide-ferric chloride method of Oyaizu [19] was adopted for evaluating the reducing power of ME, WE and EE. One ml of each extract at various concentrations (0 - 500 mg/l) was added to a test tube. One ml potassium phosphate buffer (0.2 M, pH 6.6) and freshly prepared potassium ferricyanide (1 ml, 1%) were added to extracts. The mixture was incubated in a water bath (50°C for 20 min). One ml of trichloroacetic acid (10% TCA) was added to the mixture followed by centrifugation at 5000 g for 5 min. From the upper layer of mixture, 1 ml was taken and mixed with 1 ml distilled water followed by 100 µl of freshly prepared FeCl3 (0.1 %). The absorbance (A) of samples was measured at 700 nm against blank.

2.7. DPPH· Scavenging Activity of Extracts

The DPPH radical-scavenging activity of the most active extract (ME), as revealed by both the total phenolic con- tent and reducing power, was determined following a previously published procedure [9]. WE was used for comparison purposes. Briefly, 100 µl sample at various concentrations (12.5 - 50 ppm in methanol) were distributed into different test tubes and then 3.9 ml of a DPPH solution (25 mg/l methanol) was added to each tube. The mixtures were kept, in the dark, for 30 min at room temperature. Meanwhile, (+) catechin solutions were used as references and under the same conditions. Methanol was used as blank and had no DPPH scavenging activity.

The decrease in DPPH absorbance (A) was measured at 517 nm. The DPPH radical-scavenging activity was calculated using the following formula:

DPPH radical-scavenging activity (%) = [(1 – A1/A0) × 100]; A0 is the absorbance of the control (DPPH solu- tion), and A1 is the absorbance of ME or the reference.

2.8. Statistical Analysis

Data are means of triplicate experiments, each in duplicate. The analysis of variance (ANOVA, one-way) was used to separate means at a 0.05 significant level (SPSS Version 13.0 for Windows, SPSS Inc., Chicago, IL).

3. Results and Discussion

3.1. Yield and Total Phenolics of Peel Extracts

Pomegranate peels were extracted with solvents of a different polarity. Depending on the extraction solvent, % yield significantly ($P < 0.05$) differed among extracts. As shown in **Figure 1**, 80% methanol afforded the most concentrated extract (ME) with the highest yield (45.4% ± 5.3%). Based on the solvent system, the percentage yield from pomegranate peels ranged from 1.0% to 29.2% [3,10,14]. It was pointed out that from a practical view point, a suitable extracting procedure should be developed to recover as many antioxidants as possible to produce extracts rich in natural antioxidants for potential application in health-promoting supplements for the food industry [2]. Comparing to water or ether, 80% methanol also offered extracts (ME) with the highest total phenolics (**Table 1**). Total phenolics averaged 274 ± 17 mg GAE/g, representing about 27% of ME. Reduced amounts of phenolics were present in WE or EE, 91 and 8 mg GAE/g, respectively (**Table 1**). Previous investigations reported that the phenolic concentration varied from 5 to 46% of peel extracts [2,3,14]. The variability in total phenolics among studies could be partially attributed to differences in solvents used for extracting peels, geo- graphic sources of samples and pomegranate varieties. Polyphenols are secondary metabolites which are derivatives of the pentose phosphate, shikimate and phenyl-propanoid pathways in plants [6]. They are one of the most occurring phytochemicals in plants including fruits' pericarp. In addition to their contribution to color and sensory characteristics of fruits and vegetables, phenolics also play a very important role in providing protection against *in vivo* and *in vitro* oxidation.

Table 1. Total phenolics, flavonoids and ascorbic acid of pomegranate peel extracts (means ± S.D.)

Extract[a]	Total Phenolics[b]	Total Flavonoids[c]	Ascorbic Acid (mg/g)
ME	274.1* ± 17.2	56.4 ± 2.7	2.1 ± 0.9
WE	91.2 ± 09.5	-	-
EE	08.5 ± 11.5	-	-

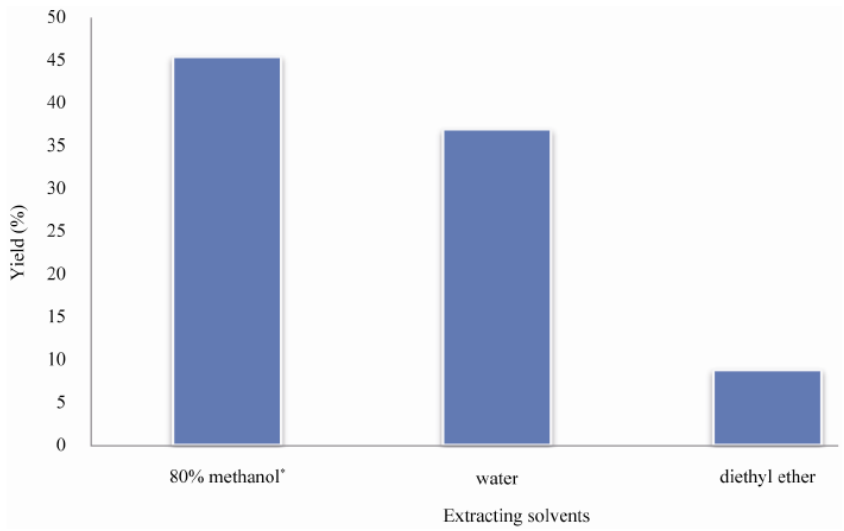

Figure 1. Extractable materials (% yield) from pomegranate fruit peels using different solvents. *P < 0.05.

Findings of the present study supported previous investigations regarding better solvents for phenolic ex- traction from plant materials. In this regard, methanol, water-methanol and acetone afforded better extracts (e.g. potent antioxidants) from pomegranate peels and other plants [3,10,15]. Diethyl ether, a less polar solvent, was not efficient in extracting phenolic constituents from peels (**Figure 1**). It is documented that phenolics are polar constituents and thus more polar solvents are better extractants of active antioxidants from plants [2,3,10,11].

Madrigal-Carballo *et al.* [13] mentioned that tannins were the major phenolics in pomegranate peels, which were more readily dissolved in 50% methanol. A mixture of methanol, ethanol, acetone and water was found to be a better extractant of active phenolics from pomegranate peels [2]. The antioxidant extracting efficiency, measured by the ferric reducing antioxidant power (FRAP), was higher in peels extracted with the solvent mixture.

3.2. Total Flavonoids and AA of Peel Extracts

Since the antioxidant activity of plants was well correlated (R2 > 0.87) with total phenolic content, including flavonoids [2,3,11,13], total flavonoids was also deter- mined in ME. ME contained 56.4 mg flavonoids (RE)/g (**Table 1**). Concentrations of both flavonoids and AA of extracted peels were comparable to those determined by Li *et al.* [2]. Flavonoids are abundant phenolics in different plant materials. This group of phenolics and AA contribute largely to the antioxidant activity of different fruits and vegetables. However, AA was only present in a small amount (2 mg/g) and thus it was unlikely to substantially contribute to the antioxidant activity of ME.

3.3. Reducing Power of Extracts

Figure 2 illustrates the reducing power of various peel extracts using the ferricyanide reduction method. The increase in A at 700 nm indicated better reducing power of test materials. In a concentration-dependent manner, the reducing power (A) increased as the amount of ME doubled in concentration. While A was 0.4 at 62.5 ppm ME, it increased to 2 at 500 ppm. At the same concentrations, ME exhibited a substantial reducing power com- pared to WE or EE (**Figure 2**). Previous studies indicated the methanolic or mixture-solvent extracts of peels had better reducing power than those of water or ethyl acetate [2,3]. The antioxidant activity has been reported to be

concomitant with the reducing power of plant materials. The higher reducing power indicated presence of reductones which are able to break free radical chains by donating hydrogen atoms and thus converting them to a more stable non-reactive species [1,3,9]. Since the reducing power was directly related to the phenolic content of peel extracts, WE and/or ME were further used in the remaining experiments.

3.4. DPPH· Scavenging Activity of Extracts

The DPPH· scavenging activity has been widely used to detect antiradical activity of different samples, due to its sensitivity to lower concentrations of active principles from natural sources. The stable radical, DPPH, has a maximum A at 517 nm and could readily undergo scavenging by antioxidants. Higher free radical scavenging activities of samples is indicated by lower A at 517 nm.
ME exhibited a significant scavenging activity ($P < 0.05$) when compared to either WE or (+)-catechin (**Ta- ble 2**). In fact, the DPPH scavenging activity of ME substantially elevated as the concentration increased from 12.5 to 50 ppm (**Table 2**). Although they were evaluated at the same concentrations, the DPPH scavenging and the above reducing power data clearly indicated that ME had superior antioxidant activity than WE. The DPPH scavenging activity as a REDOX reaction was clearly related to the total phenolics of peel extracts. The is similar to previous observations [3,20] wherein the DPPH scavenging activity of methanolic extract (rich in phenolics) of pomegranate peels was better than that of water ex- tracts. Except at 50 ppm, the scavenging activity of WE was higher than that of the reference, (+)-catechin (**Table 2**). In a different assay system, the peroxyl radical-scavenging (ROO•) activity of pomegranate peels was greater than that of fruit pulp [3]. In this regard, it was mentioned that peels contained more phenolics than did flesh tissues. Reddy *et al.* [7] stated that crude pomegranate fruit total tannins and purified constituents (e.g., ellagic acid and punicalagins) possessed antioxidant activity and strongly inhibited ROS generation with IC50 of 0.33 to 11 μg/ml. Depending on the polyphenolic composition, DPPH scavenging activity varied among products from pomegranate fruits, 74 to 4485 μM/g [13]. It was emphasized that the number of phenolic residues and hydroxyl groups substantially affected the DPPH scavenging activity of extracts from grape seeds. Additionally, glycosylated flavonols were less active than the corresponding agly- cones [21].
It is worthy to mention that crude extracts of pomegranate peels and purified fractions had no cytotoxicity for HL-60 cells [7].

Table 2. DPPH radical scavenging activity (%) of various pomegranate fruit peel extracts[a].

Concentration (ppm)	Scavenging activity (%)		
	ME*	WE	(+)-α-catechin
12.5	46.3	34.3	23.9
25	59.2	50.1	38.8
50	99.3	75.4	84.2

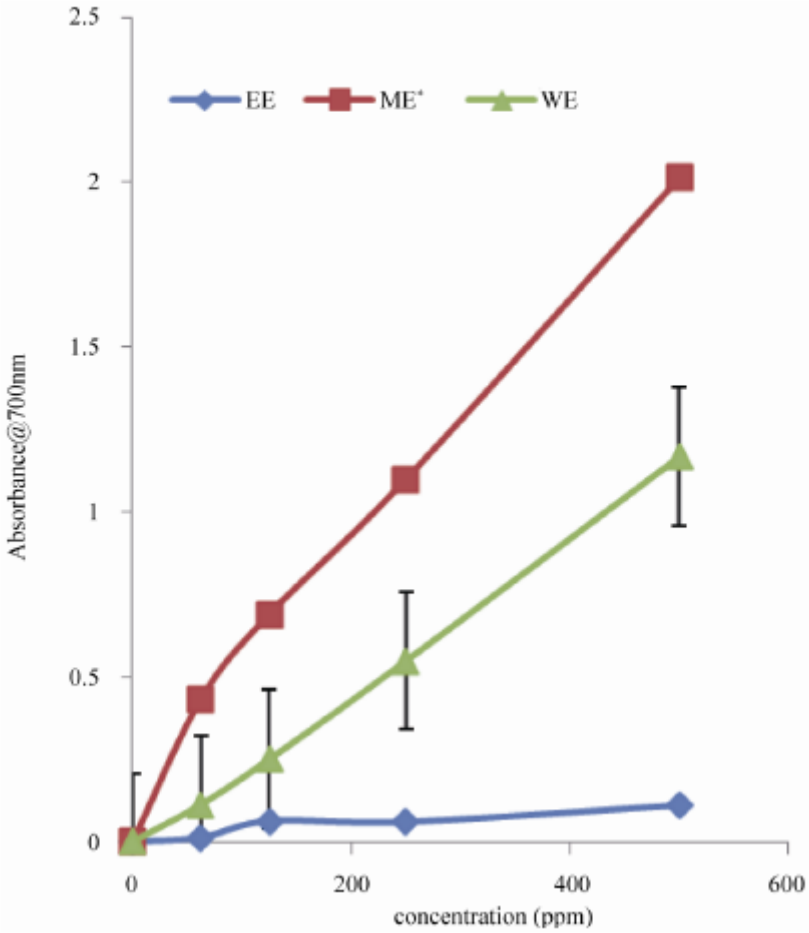

Figure 2. Reducing power (A at 700 nm) of 80% methanolic (ME), water (WE) and ether (EE) extracts from pomegranate fruit peels. *P < 0.05.

4. Conclusion

Pomegranate fruit peels are by-products of the food industry. Added-value products could be made from those wastes. Eighty percent methanol was a better solvent for extracting active constituents from peels. Phenolics from peel extracted exhibited a potent antioxidant activity as evaluated by the DPPH scavenging activity and ferric reduction tests. Crude extracts and purified fractions from pomegranate peels could provide health benefits to humans and may be employed in food preservation and pharmaceutical purposes.

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Li Y, Guo C, Yang J, Wei J, Xu J, Cheng S

Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract

Pomegranate is an important source of bioactive compounds and has been used for folk medicine for many centuries. Pomegranate juice has been demonstrated to be high in antioxidant activity and is effective in the prevention of atherosclerosis. In a previous study, we found that pomegranate peel had the highest antioxidant activity among the peel, pulp and seed fractions of 28 kinds of fruits commonly consumed in China as determined by FRAP (ferric reducing antioxidant power) assay. In this study, we extracted antioxidants from pomegranate peel, using a mixture of ethanol, methanol and acetone, and the antioxidant properties of the extract were further investigated as compared with the pulp extract. The contents of total phenolics, flavonoids, proathocyanidins and ascorbic acid were also measured. The results showed that pomegranate peel extract had markedly higher antioxidant capacity than the pulp extract in scavenging or preventive capacity against superoxide anion, hydroxyl and peroxyl radicals as well as inhibiting $CuSO_4$-induced LDL oxidation. The contents of total phenolics, flavonoids and proathocyanidins were also higher in peel extract than in pulp extract. The large amount of phenolics contained in peel extract may cause its strong antioxidant ability. We concluded that pomegranate peel extract appeared to have more potential as a health supplement rich in natural antioxidants than the pulp extract and merits further intensive study.

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Negi PS, Jayaprakasha GK, Jena BS

Antioxidant and antimutagenic activities of pomegranate peel extracts

Dried pomegranate peels were powdered and extracted in a Soxhlet extractor with ethyl acetate (EtOAc), acetone, methanol and water for 4 h each. The dried extracts were used to determine their antioxidant capacity by the formation of phosphomolybdenum complex and antimutagenicity against the mutagenicity of sodium azide by the Ames test. All the peel extracts exhibited marked antioxidant capacity, but the water extract was the lowest. The order of antioxidant capacity varied because of differential responses at four concentrations (25, 50, 75 and 100 μg/ml) in each solvent. All the extracts decreased sodium azide mutagènicity in *Salmonella typhimurium* strains (TA100 and TA1535), either weakly or strongly. At 2500 μg/plate all the extracts showed strong antimutagenicity. The antimutagenicity of the water extract was followed by acetone, EtOAc and methanol extracts. The overall results showed that the pomegranate peel extracts have both antioxidant and antimutagenic properties and may be exploited as biopreservatives in food applications and neutraceuticals.

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Ismail T, Sestili P, Akhtar S

Pomegranate peel and fruit extracts: A review of potential anti-inflammatory and anti-infective effects

ETHNOPHARMACOLOGICAL RELEVANCE: Punica granatum L. (Punicaceae) has been used for centuries in many cultures for the prevention and treatment of a wide number of health disorders such as inflammation, diabetes, diarrhea, dysentery, dental plaque and to combat intestinal infections and malarial parasites.

AIM OF THE REVIEW: This review aims at providing an up-to-date overview of the chemical constituents, traditional uses, phytochemistry, pharmacology and toxicology of Punica granatum L. Moreover, the focus of this review is the possible exploitation of this species to treat different diseases and to suggest future investigations.

MATERIALS AND METHODS: An extensive and systematic review of the extant literature was carried out, and the data under various sections were identified by using a computerized bibliographic search via PubMed, Web of Science and Google Scholar. All abstracts and full-text articles were examined. The most relevant articles were selected for screening and inclusion in this review.

KEY FINDINGS: A variety of pomegranate ethnomedical uses have been recorded. Additionally, over the last decade, there has been a dramatic increase of interest in pomegranate as a medicinal and nutritional product due to its n1ewly identified potential health effects, which include treatment and prevention of cancer and cardiovascular diseases. From the toxicological perspective, pomegranate fruit juice, extracts and preparations have been proven to be safe.

CONCLUSIONS: The ethnopharmacological relevance of pomegranate is fully justified by the most recent findings indicating the fruit is a medicinal and nutritional agent useful for treating a wide range of human disorders and maladies. Further investigations are needed to fully understand the mode of action of the active constituents and to fully exploit pomegranate's preventive and therapeutic potential.

Protective effect of pomegranate derived products on UVB-mediated damage in human reconstituted skin

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Abstract

Solar ultraviolet (UV) radiation, particularly its UVB (290-320 nm) component, is the primary cause of many adverse biological effects including photoaging and skin cancer. UVB radiation causes DNA damage, protein oxidation and induces matrix metalloproteinases (MMPs). Photochemoprevention via the use of botanical antioxidants in affording protection to human skin against UVB damage is receiving increasing attention. Pomegranate, from the tree *Punicagranatum* contains anthocyanins and hydrolyzable tannins and possesses strong anti-oxidant and anti-tumor promoting properties. In this study, we determined the effect of pomegranate derived products POMx juice, POMx extract and pomegranate oil (POMo) against UVB-mediated damage using reconstituted human skin (EpiDerm™ FT-200). EpiDerm was treated with POMx juice (1-2 µl/0.1 ml/well), POMx extract (5-10 µg/0.1 ml/well), and POMo (1-2 µl/0.1 ml/well) for 1 h prior to UVB (60 mJ/cm2) irradiation and was harvested 12 h post-UVB to assess protein oxidation, markers of DNA damage and photoaging by western blot analysis and immunohistochemistry. Pretreatment of Epiderm with pomegranate derived products resulted in inhibition of UVBinduced (i) cyclobutane pyrimidine dimers, (ii) 8-dihydro-2′-deoxyguanosine, (iii) protein oxidation, and (iv) PCNA protein expression. We also found that pretreatment of Epiderm with pomegranate derived products resulted in inhibition of UVB-induced (i) collagenase (MMP-1), (ii) gelatinase (MMP-2, MMP-9), (iii) stromelysin (MMP-3), (iv) marilysin (MMP-7), (v) elastase (MMP-12), and (vi) tropoelastin. Gelatin zymography revealed that pomegranate derived products inhibited UVB-induced MMP-2 and MMP-9 activities. Pomegranate derived products also caused a decrease in UVB-induced protein expression of c-Fos and phosphorylation of c-Jun. Collectively, these results suggest that all three pomegranate derived products may be useful against UVB-induced damage to human skin.

Introduction

Ultraviolet radiation (UV) from the sun is one of the most prominent environmental factor that has serious adverse effects including erythema, edema, hyperplastic responses, immunosupression, hyperpigmentation, premature aging of skin and majority of cutaneous malignancies (1-5). Solar UV radiation is divided into three categories: UVA or (UV 320-400nm), UVB (280-320nm) and UVC (100-280nm). Both UVB and UVA are the causative factors for sunlight-induced skin disorders (6-7). UVB radiation is the most damaging component of the solar radiation reaching the earth and acts mainly on the epidermal basal cell layer of the skin. UVB wavelength photon is absorbed by DNA, and induces DNA damage by formation of cyclobutane pyrimidine dimers (CPD) and 8-dihydro-2′-deoxyguanosine (8-OHdG) (6,8). UVB-mediated DNA damage causes mutation of oncogenes and tumor suppressor genes and is an obligatory step for progress towards skin caricinogenesis (6,8-10). UVB radiation is considered to be a complete carcinogen, and initiates a photooxidative reaction, which impairs the antioxidant status and increases the level of reactive oxygen species (ROS) accompanied by activation of signaling pathways (3,11,12). To counteract this, skin has efficient antioxidant defense mechanisms but when the generation of ROS overwhelms this defense capacity,

it impairs the ability of the skin to protect itself from the damaging effects of ROS resulting in oxidative damage of DNA, proteins and other macromolecules in the skin (6,8,11).

In recent years, botanical antioxidants have attracted considerable attention, because of their potential to quench ROS and inhibit UV-induced signal transduction pathways (1,3). Thus, it is suggested that a regular intake of antioxidants or treatment of the skin with products containing antioxidant ingredients may be a useful strategy for the prevention of UVmediated cutaneous damage (13-15). This has sparked the use of exogenous supplementation of antioxidants, notably of botanical origin in skin care products. Polyphenolics, widely distributed in botanicals with their significant amounts in vegetables, fruits and beverages, form an integral part of diet and possess strong free radical scavenging and antioxidant properties (16,17). *Punica granatum* L fruit commonly known as pomegranate is widely consumed fresh and in beverage form, as juice or wine has been used in various parts of the world as a traditional medicine and is also gaining popularity in the USA. Pomegranate is a rich source of many phenolic compounds, which include flavonoids and hydrolyzable tannins (18). Extracts from different parts of pomegranate fruit such as juice (19), seed (20) and peel (21) have been reported to exhibit strong antioxidant activity.

Studies have shown that pomegranate juice possesses antiproliferative (22), antiatherogenic (23), antiinflamatory and antitumoriogenic (22,24,25) properties. These effects of pomegranate and its derived products are attributable to its free radical scavenging and antioxidant properties (26). Recently, we have shown that treatment of immortalized HaCaT cells with POMx extract resulted in inhibition of UVB-mediated oxidative stress and markers of photoaging (27). For relevance of this work to human skin here we utilized three dimensional full thickness reconstituted human skin equivalent (EpiDerm™FT-200) that sustains differentiation and exhibit morphological, metabolic and growth characteristics similar to that of human skin *in vivo*. In the present study, we determined the effect of pomegranate derived products such as POMx juice, POMx extract and POMo against UVBmediated damage in EpiDerm™FT-200. Specifically, we determined the effect of these products on UVB-induced protein oxidation and markers of DNA damage and photoaging.

Material and Methods

Materials

Epiderm™FT-200 and EFT culture media were purchased from MatTeck Corp. (Ashland, MA). HRP-labelled antibody for CPD was purchased from Kamiya Biomedical Company (Seattle, WA). Antibody for 8-OHdG was obtained from Millipore (Billerica, MA). Antibodies for MMP-1, MMP-2 and MMP-9 were procured from Lab Vision Corporation (Fremount, CA). Antibodies for MMP-7, MMP-11 and MMP-12 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antimouse or antirabbit secondary antibody horseradish peroxidase conjugate and ECL western blotting detection reagent were purchased from Amersham Life Science (Arlington Height, IL). Protein carbonyl immunoblot kit was obtained from Cell Biolabs Inc. (San Diego, CA). Protease inhibitor cocktail set III was obtained from Calbiochem (La Jolla, CA). Novex precast tris-glycine gels were purchased from Invitrogen (Carlsbad, CA). BCA protein assay kit was obtained from Pierce Biotechnology Inc. (Rockford, IL). All other chemicals used were at least of analytical grade. POMx juice, POMx extract and pomegranate oil (POMo) were provided by POM Wonderful, Inc. (Los Angeles, CA).

Human reconstituted Skin (EpiDerm™ FT-200)

This model system consists of normal human epidermal keratinocytes (NHEK) derived from neonatal-foreskin and normal human dermal fibroblasts (NHDF) derived from neonatal skin of same donor. These were co-cultured to form a multilayered, highly differentiated model of the human skin with

functional dermis and epidermis (28-29). EpiDerm™ FT-200 consists of organized basal, spinous, granular, and cornified epidermal layers analogous to those found *in vivo* (28-30). The dermal compartment is composed of a collagen matrix containing viable NHDF. Ultrastructurally, the EpiDerm™ FT-200 closely resembles human skin, thus providing a useful *in vitro* model for study. Histochemical analysis shows the presence of basement membrane along with signaling proteins. This model also serves for *in vitro* evaluation of skin phenomenon where fibroblast-keratinocyte interactions (paracrine signaling) are important and modulate cell response, which play important role in regulating tissue homeostasis (31,32). The EpiDerm™ FT-200 was supplied as single well tissue culture plate inserts with each insert containing functionally and metabolically active reconstituted skin with surface area 1.0 cm2 shipped at 4°C on medium-supplemented, agarose gel. Upon receipt the EpiDerm was equilibrated at 37 °C, 5% CO2, in EFT media supplied along with the kit for 24 h and maintained. Through out the experiment EpiDerm™ FT-200 was maintained in 6 well culture plates at air liquid interface with lower dermal side of tissue exposed to media and the upper epidermal stratum corneum exposed to air.

POM products

POM products were provided by POM Wonderful, Inc. (Los Angeles, CA). Pomegranate fruit was sliced and squeezed for the juice and the remaining material including squeezed arils, rind and other parts were processed to remove the seeds before undergoing a series of concentration steps to produce a polyphenol rich POMx. POMx is a 70 Brix commercial grade of pomegranate extract concentrate with a polyphenol content of 135 000 p.p.m. gallic acid equivalent and ellagitannins as its major constituent. POMx juice contains anthocyanins ellagitannins and hydrolyzable tannins. The source of POMo is pomegranate seed.

Treatment and UVB irridation of EpiDerm™ FT-200

EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h. These doses of pomegranate products were decided by doing dose-response studies in terms of its photoprotective effects against UVB-mediated DNA damage (data not shown). After that, the media containing pomegranate products were removed and the skin samples were gently washed with PBS 2-3 times via gentle pipetting of the apical tissue surface to remove any non-absorbed products. A control with 0.1% DMSO in EFT medium was maintained for all experiments. The culture media were then replaced with PBS and exposed to UVB (60mJ/cm2). After UVB exposure, the culture inserts containing the skin samples were placed in fresh media and were harvested 12 h post-UVB irradiation for western blotting and RT-PCR analyses, and media was collected for gelatin zymography.

Immunostaining for CPD

For detection of CPD, EpiDerm™ FT-200 was collected 12 h post UVB irradiation, was frozen in OCT medium and sectioned. 5µm thick sections were fixed in chilled acetone for 20 min. To denature nuclear DNA, sections were treated with 70mM NaOH in 70% ethanol and neutralized for 1 min in 100mM Tris-HCl in 70% ethanol. Endogenous peroxidase was quenched by incubation in 0.3% hydrogen peroxide in methanol and washed with PBS. Nonspecific binding sites were blocked by incubating the sections with goat serum blocking solution for 1 h and incubated overnight at 4°C with anti-thymine dimer HRP-labeled antibody. After washing with PBS, the sections were incubated with DAB peroxidase substrate solution for 2 min at room temperature, washed with distilled water,

followed by counterstainig with Mayers Hematoxylin solution. Sections were rinsed in tap water, dehydrated through graded alcohol, cleared in xylene and mounted in permanent mounting medium.

Immunostaning for 8-OHdG

For detection of 8-OHdG, EpiDerm™ FT-200 was collected 12 h post UVB were fixed in 10% neutralized formalin and embedded in paraffin. Sections 5µm in thickness were deparaffinized in xylol and rehydrated, through graded ethanol solutions to 70% and washed in PBS. For antigen retrieval sections were heated at 95°C for 30 min in EDTA buffer (pH 8.0) and then cooled for 20 min and washed in PBS. To denature nuclear DNA, sections were treated with 70mM NaOH and neutralized for 1 min in 100mM Tris-HCl (pH 7.5). Endogenous peroxidase was quenched by incubation in 0.3% hydrogen peroxide. Nonspecific binding sites were blocked by incubating the sections with goat serum blocking solution for 1 h and incubated with primary antibody against 8-OHdG overnight at 4°C followed by incubation with HRP-labeled secondary antibody for 1 h at room temperature. After washing with PBS, the sections were incubated with DAB peroxidase substrate solution (Dako) for 2 min at room temperature, rinsed with distilled water followed by counterstainig with Mayers Hematoxylin solution. Sections were rinsed in tap water, dehydrated through graded alcohol cleared in xylene and mounted in permanant mounting medium.

Immunostaining for PCNA and tropoelastin

EpiDerm™ FT-200 was collected 12 h after UVB irradiation, was fixed in 10% neutralized formalin and embedded in paraffin. 5µm sections were cut, deparaffinized in xylol and rehydrated, through graded ethanol to 70% and washed in PBS. For antigen retrieval, sections were heated at 95°C for 30 min in citrate buffer (pH 6.0) and then cooled for 20 min and washed in PBS. Endogenous peroxidase was quenched by incubation in 0.3% hydrogen peroxide, for 20 min and washed in washing buffer (PBS + Tween). Nonspecific binding sites were blocked by incubating the sections with goat serum blocking solution for 1 h. Sections were incubated with primary antibody against PCNA and tropoelastin overnight at 4°C followed by incubation with specific HRP-labeled secondary antibody for 1 h at room temp. After washing in wash buffer, the sections were incubated with DAB peroxidase substrate solution for 2 min at room temperature, rinsed with distilled water followed by counterstaining with Mayers Hematoxylin solution. Sections were rinsed in tap water, dehydrated through 70-100 % graded alcohol cleared in xylene and finally mounted in permanent mounting medium.

Gelatin zymography

For zymography, culture media in which EpiDerm™ FT-200 was grown after UVB exposure was subjected to substrate gel electrophoresis for detection of gelatinolytic activity. Samples were concentrated using centricon YM-30 centrifugal filter unit which retains proteins greater than 30 KD. The samples with equal protein content were mixed with non reducing sample buffer and electrophoresed in precasted 10% SDS-polyacrylamide gels containing 1% gelatin and run in Novex tris glycine SDS running buffer. To eliminate SDS content, gels were washed twice with Novex zymogram renaturing buffer for 30 min at room temperature with gentle agitation. Afterward, the gels were incubated at 37°C overnight in Novex developing buffer (50 mM Tris-HCl, pH 8, 5 mM $CaCl_2$, 1 µM $ZnCl_2$, and 0.02% NaN_3, which allows gelatinolytic enzymes to act. Gels were stained for 3 h in 40% methanol and 10% glacial acetic acid containing 0.5% Coomasie Brilliant Blue and were destained in the same solution without dye. The gelatinolytic activity of MMPs was evident as a clear band against the blue background of stained gelatin.

Whole cell lysate preparation

EpiDerm™ FT-200 lysates for western blot analysis was prepared by homogenizing the EpiDerm™ FT-200 in lysis buffer (10mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, and 1 mM EGTA) containing 0.2 mM sodium vanadate, 2 mM PMSF, 0.5% NP-40, and 0.2 U/ml aprotinin with freshly added protease inhibitor cocktail at 4°C for 15 min. The lysates were centrifuged at 13,000 rpm for 25 min at 4°C to remove cell debris. Clear supernatant was collected, and protein estimation performed by BCA method.

SDS-polyacrylamide gel electrophoresis and western blot analysis

For western blot analysis, equal amount (30-40 µg) of protein was resolved electrophoretically over 12% Tris glycine gel, and transferred to a nitrocellulose membrane. The blot containing the transferred protein was blocked in blocking buffer (5% nonfat dry milk in 20mM Tris-buffered saline, pH 7.6 containing 1% Tween 20 - TBST) for 1 h at room temperature followed by incubation with appropriate primary antibody in blocking buffer for 2 h to overnight at 4°C. This was followed by incubation with specific secondary antibody horseradish peroxidase for 2 h at room temperature and then washed 3 times, 15 min each in TBST and detected by enhanced chemiluminescence and autoradiography using Blue Lite Autorad film obtained from ISC Bioexpress (Kaysville, UT).

Determination of protein oxidation

For determination of protein oxidation, Cell Biolabs' protein carbonyl immunoblot kit was used. Briefly gel proteins were transfered to the PVDF membrane. Following the electroblotting step, PVDF membrane was immersed in 100% methanol for 15 sec, and dried at room temperature for 5 min, then equilibrated in TBS containing 20% methanol for 5 min. Membrane was washed in 2N HCl for 5 min and incubated in dinitrophenylhydrazine (DNPH) solution for 5 min. Following derivitization with DNPH membrane was washed three times with 2N HCl, and then five times with 100% methanol, 5 min each. The blot containing the derivitized protein was blocked in blocking buffer and incubated with primary antibody against DNPH for 3 h at room temperature followed by incubation in HRP labeled secondary antibody for 2 h and detected by enhanced chemiluminescence and autoradiography.

Results

POMx juice, POMx extract and POMo inhibit UVB-mediated formation of CPD and 8-OHdG in human reconstituted skin

Photodamage to epidermal DNA is considered an important factor in the development of skin cancer (6,8). It is known that both CPDs and 8-OHdG are formed in epidermal DNA after UVB irradiation and are considered as important biomarkers of DNA damage. We, therefore, performed immunohistochemical staining using antibody specific for CPD and 8-OHdG to study the effect of the pomegranate derived products on UVB-mediated formation of CPD and 8-OHdG in human reconstituted skin, EpiDerm™ FT-200. Our result show stronger and intensive staining for CPD and 8-OHdG (Figure 1) in the nuclei of UVB (60mJ/cm2) irradiated EpiDerm™ FT-200, 12 h post-UVB (60mJ/cm2) exposure compared to those of non-irradiated control. However, topical treatment of EpiDerm™ FT-200 with POMx juice (2µl), POMx extract (5 µg) or POMo (2µl) prior to UVB irradiation resulted in a significant reduction in both the number and intensity of CPD (Figure 1A) and 8-OHdG positive cells (Figure 1B). Treatment of EpiDerm™ FT-200 with POMx juice, POMx extract or POMo at the concentration used did not show any effect as compared to nonirradiated control in any of the parameter studied (data not shown).

POMx juice, POMx extract and POMo inhibit UVB-mediated increase in protein carbonyl group in human reconstituted skin

UV irradiation generates irreversible oxidation of the side chains of certain amino acids resulting in the formation of carbonyl groups on proteins (a marker of protein oxidation) (33). To study the effect of pomegranate derived products on UVB-mediated changes on generation of protein carbonyl groups, we performed immunoblot analysis after derivitization of protein carbonyl group on PVDF membrane with DNPH. Our results show that UVB (60mJ/cm2) irradiation resulted in an increase in the expression of protein with carbonyl groups whereas treatment of EpiDerm™ FT-200 topically with POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) for 1 h prior to UVB (60mJ/cm2) resulted in a decreased expression of protein with carbonyl groups (Figure 2).

POMx juice, POMx extract and POMo inhibit UVB-mediated cell proliferation in human reconstituted skin

Proliferating cell nuclear antigen is an active nuclear protein involved in both DNA damage and repair (34,35). To study the effect of pomegranate derived products on UVB-induced changes on PCNA in human reconstituted skin, EpiDerm™ FT-200, we performed immunohistochemical staining and immunoblotting using antibody specific for PCNA. Our result show that UVB (60 mJ/cm2) irradiation to EpiDerm™ FT-200 resulted in an increase in protein expression of PCNA as compared to non-irradiated control. (Figure 3A,B).

Pretreatment of EpiDerm™ FT-200 topically with POMx juice (1-2µl), POMx extract (5-10µg) or POMo (1-2µl) for 1 h prior to UVB (60mJ/cm2) diminished PCNA protein expression of EpiDerm as compared to non irradiated control (Figure 3A,B).

POMx juice, POMx extract and POMo inhibit UVB-mediated increase in tropoelastin levels in human reconstituted skin

UVB irradiation stimulates the synthesis of elastin, one of the important components of extracellular matrix (ECM) in the skin of humans and experimental animals (36-38). To study the effect of pomegranate derived products on UVB-induced changes on elastin in human reconstituted skin, EpiDerm™ FT-200, we performed immunoblotting and immunohistochemical staining for tropoelastin, a monomer precursor of elastin. Our result showed that UVB (60mJ/cm2) caused an increase in the protein expression along with immunostaning of tropoelastin as compared to non-irradiated control (Figure 4A,B). Pretreatment of EpiDerm™ FT-200 topically with POMx juice (1-2µl), POMx extract (5-10µl) or POMo (1-2µl) for 1 h prior to UVB (60mJ/cm2) decreased tropoelastin protein expression as compared to UVB (Figure 4A,B).

POMx juice, POMx extract and POMo inhibit UVB-mediated increase in the protein levels and activity of matrix metalloproteinases (MMPs) in human reconstituted skin

Exposure to UVB radiation is known to upregulate the synthesis of matrix degrading enzymes, MMPs. MMPs are a family of structurally related zinc-dependent endopeptidases, which play a role in degrading a wide variety of ECM components and play an important role in tumor invasion and photoaging (27,39). We therefore evaluated the effect of pomegranate derived products on UVB-induced MMPs activities and protein expression in human reconstituted human skin, EpiDerm™ FT-200. UVB (60 mJ/cm2) irradiation of EpiDerm™ FT-200 caused an increase in the protein expressions of MMPs-1, -2, -3, -7, -9, -11 and -12 (Figure 5A). UVB also resulted in an increase in gelatinase activity of MMP-2 and MMP-9 in the surrounding media (Figure 5B). Our data show that pretreatment of EpiDerm™ FT-200 with POMx juice (1-2µl), POMx extract (5-10µg) or POMo (1-2µl) for 1 h prior to UVB (60mJ/cm2) exposure inhibited the UVB mediated increase of MMPs-1, -2, -3, -7,

-9, -11 and -12 protein expressions (Figure 5A) and decreased MMP-2, and MMP-9 gelatinase activities (Figure 5B).

POMx juice, POMx extract and POMo inhibit UVB-induced phosphorylation of c-jun and expression of c-Fos in human reconstituted skin

Activator protein-1 (AP-1) is closely related to matrix degrading enzymes that induce breakdown of collagen. Jun proteins form homodimers or heterodimers with fos proteins to form AP-1 complexes. The transcriptional activity of AP-1 is dependent on the degree of phosphorylation of c-jun and expression of c-fos (1,3). We therefore investigated the effect of pomegranate derived products on UVB-induced phosphorylation of c-jun protein and expression of c-fos protein. Our results show that UVB (60mJ/cm2) irradiation of EpiDerm™ FT-200 increased the level of phosphorylated c-jun and c-fos proteins. Topical treatment of EpiDerm™ FT-200 with POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) for 1 h prior to UVB (60mJ/cm2) inhibited UVB-mediated phosphorylation of c-jun protein and expression of c-fos protein (Figure 6).

Discussion

Exposure of skin to solar UV radiation, particularly its UVB component, is believed to be the major cause of a variety of cutaneous disorders including photoaging and skin cancers (15,40). Studies have demonstrated that UV radiation can act as a potent inducer of ROS, which are responsible for the photooxidative damage to nucleic acids, lipids and proteins. As ROS are implicated in skin damage by UVB, scavenging of these reactive species could prevent the oxidative reactions and subsequently protect skin from the damaging effects of UVB. Therefore, the use of antioxidants to reduce the harmful effect of UV by scavenging ROS is a novel approach to prevent the damage caused by UV radiation. In recent years, considerable attention is being focused on the use of naturally occurring botanicals for their potential preventive effect against UV radiation mediated damages referred to as “photochemopreventive effects” (41). One such natural product is pomegranate, which is widely consumed fresh and in beverage forms and has been used extensively in ancient cultures for various medicinal properties (42). Pomegranate is a rich source of anthocyanins and hydrolyzable tannins and possesses potent antioxidant and anti-inflammatory properties (18,43). Studies have shown that pomegranate and other naturally occurring antioxidant-rich botanicals are effective in reducing the harmful effect of UVB-mediated skin damage (6,24). Our results show that UVB caused damage to DNA and proteins as evident from increased formation of CPD, 8-OHdG and protein carbonyl groups (Figures 1 and 2). UVB-radiation can cause DNA damage, directly by absorption of high energy photons by DNA and indirectly through ROS. Absorption of UVB energy results in DNA photoproducts such as CPD whereas ROS result in 8-OHdG formation. These photoproducts can be repaired by the nucleotide excision repair system or the base excision repair system (44). Pomegranate derived products POMx juice, POMx extract and POMo protect the EpiDerm™ FT-200 from UVB-mediated DNA damage by its strong antioxidant activity (Figure 1). It may also cause an increase in DNA repair mechanisms and therefore play a significant role in ameliorating or preventing UVB-induced DNA damage. Studies show that antioxidant such as vitamin D and EGCG inhibit DNA damage by increasing DNA repair mechanisms (45, 46).

UV irradiation, which is part of the skin damage process, causes irreversible damage to proteins by ROS generation (47). Our data (Figure 2) confirms other studies which show that UV irradiation causes oxidation of certain amino acid resulting in formation of carbonyl groups on proteins with the accumulation of oxidatively modified protein (33). The toxic effects of ROS are counteracted by antioxidants and antioxidant enzymes. Other antioxidant rich botanicals have also been reported to

protect skin cells against UV-induced oxidative damage to proteins (48). Therefore topical and/or systemic application of antioxidants could support physiological mechanisms to maintain or restore protein integrity thereby maintaining a healthy skin barrier. PCNA is an active nuclear protein involved in DNA replication, recombination and repair. UV-induced increase in cell proliferation is an early event associated with UV-mediated carcinogenesis that helps the exposed cells to proceed further into cell cycle (49), which could be prevented by arresting the cells at G1 or S phase of the cell cycle (50). Our data from immunoblot and immunohistochemical analyses revealed that UVB radiation causes a significant upregulation in PCNA, a marker of cellular proliferation, However, UVBinduced PCNA expression were decreased by pomegranate derived products pretreatment (Figure 3). These results suggest that inhibition of cell proliferation by these pomegranate derived products could be one of the mechanisms by which these agents protects damaged cells from entering the cell cycle, thereby providing damaged cells additional time for repair and in case if the damage is severe preserving their entry into apoptotic pathway (51). Solar UV damaged human skin is characterized by connective tissue damage that includes massive accumulation of abnormal elastic fibers. UVB irradiation is known to stimulate synthesis of elastin the major protein component of elastic fibers (52). Studies show that in human skin, tropoelastin the precursor monomer of elastin, are produced *in vivo* by both the epidermal keratinocytes and dermal fibroblast (53) and an interaction between epidermal keratinocytes and dermal fibroblast play a role in post-translational modification of elastin (54). Our result from immunoblot and immunohistochemical analyses show that UVB causes a significant incresed in tropoelastin level whereas pretreatment of EpiDerm™ FT-200 with POMx juice, POMx extract or POMo inhibited this UVB-mediated increase in tropoelastin levels (Figure 4). The reasons for UV induction in tropoelastin expression remain to be investigated further. It is possible however, that various cytokines and growth factors produced by inflammatory cells in photodamaged skin may play some role in the stimulation of cells to produce more tropoelastin (52). Solar radiation causes cutaneous photodamage characterized by alterations in the quantity and structure of the extracellular matrix (55). Synthesis of ECM proteins and their degradation by MMPs are part of the dermal remodeling resulting from chronic exposure of skin to UV radiation. ROS generated upon UV exposure play a major role in dermal connective tissue transformations including degradation of skin collagen (56). MMPmediated ECM damage has been shown to be a major contributor of photoaged human skin. Although UV-induced expression of MMP gene occurs predominantly in the epidermis, MMP proteins and their enzymatic activity are abundant in both the dermis and the epidermis (57,58). Our results show that UVB caused an increased in MMP-1, MMP-2, MMP-3, MMP-7 MMP-9, MMP-11 and MMP-12 protein expression along with an increase in the activity of secreted gelatinases in human reconstituted skin, whereas pretreatment with POMx juice, POMx extract or POMo abrogated this effect (Figures 5A and 5B) probably by inhibition of ROS generation due to their antioxidant activity. Therefore, inhibition of the induction of MMPs can alleviate UV-induced tumor invasion and photoaging. Studies show that UV radiation-induced generation of ROS contributes significantly to signaling events that leads to gene expression (59). The *c-jun* gene which encodes the (AP-1) sites of DNA and acts as a regulatory factor for gene transcription (60). It has been reported that activation of AP-1 participates in the UVB-driven breakdown of ECM in human skin by inducing the expression of a series of MMPs responsible for ECM degradation (61). Our results show that UVB irradiation of EpiDerm™ FT-200 increased the level of phosphorylated c-jun along with c-fos protein, whereas pretreatment of EpiDerm™ FT-200 with POMx juice, POMx extract or POMo inhibited UVB-induced phosphorylation of c-jun and c-fos protein expression (Figure 6). These results explain that inhibition of c-jun phosphorylation along with c-fos, which is known to be closely associated with AP-1 activation, may contribute to the prevention of UVB-induced AP-1, which regulates MMPs expression in human skin.

In conclusion, this study demonstrates the photochemopreventive effect of pomegranate derived products. Our data suggest that pretreatment of EpiDerm™ FT-200 with POMx juice, POMx extract or POMo inhibited UVB-mediated DNA and protein damage, increase in PCNA and tropoelastin levels along with degradation of ECM proteins. Pomegrante derived products also attenuated UVB-induced phosphorylation of c-jun and increase in cfos protein. These results suggest that all three pomegranate derived products may be useful against UVB-mediated damages to human skin. These results provide a basis for more indepth studies to asses the effectiveness of pomegranate fruit and its derived products in the prevention of UVB-mediated damage and photoaging in humans.

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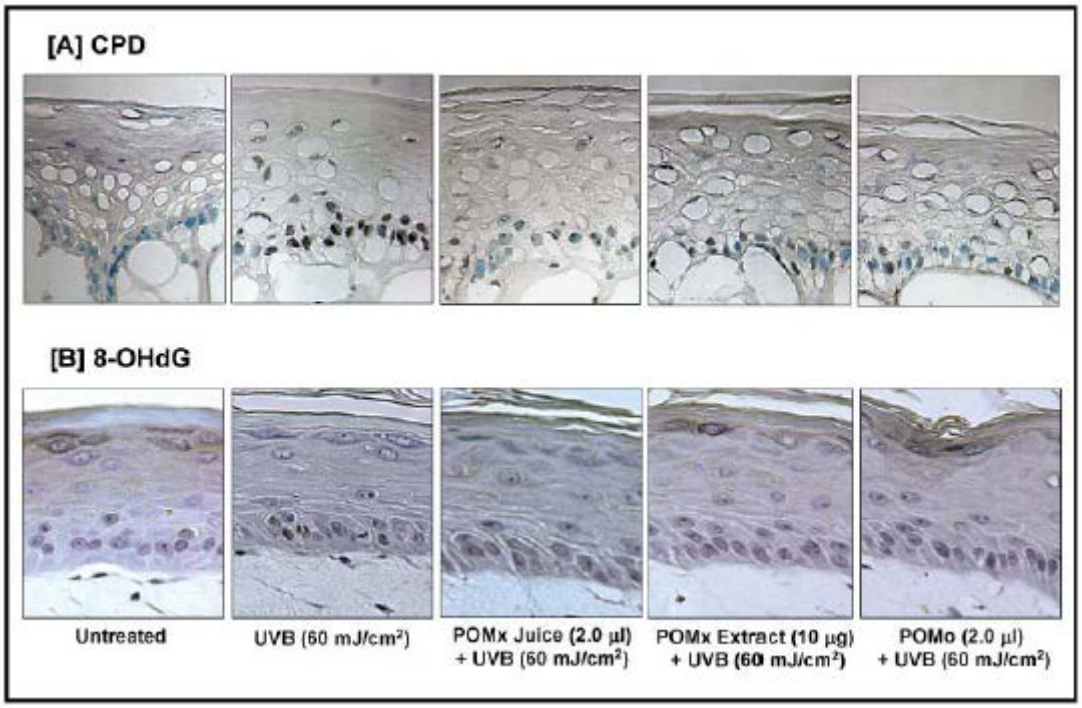

Figure 1. Effect of POMx juice, POMx extract and POMo on UVB-mediated formation of CPD in three dimensional human reconstituted skin (EpiDerm™ FT-200)

EpiDerm™ FT-200 was topically treated with or without POMx juice (2µl), POMx extract (10 µg) or POMo (2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm2). Twelve h post-UVB, skin were harvested and immunostaining was performed to detect UVB-induced DNA damage in the form of (A) CPD and (B) 8-OHdG positive cells as described in "Materials and Methods". Representative sections of immunostaining are shown from three independent experiments with similar results.

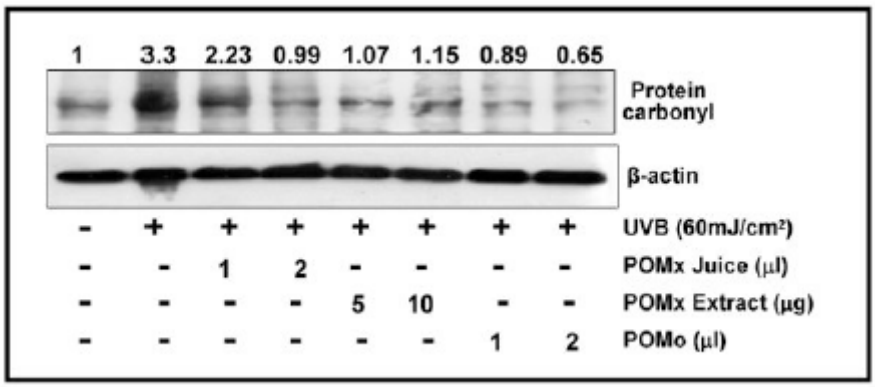

Figure 2. Effect of POMx juice, POMx extract and POMo on UVB-mediated protein oxidation in three dimensional human reconstituted skin (EpiDerm™ FT-200)

EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm2). Twelve h post UVB, EpiDerm™ FT-200 was harvested and cell lysates prepared and western blot

analysis performed after derivitization of transferred protein PVDF membrane with DNPH. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results.

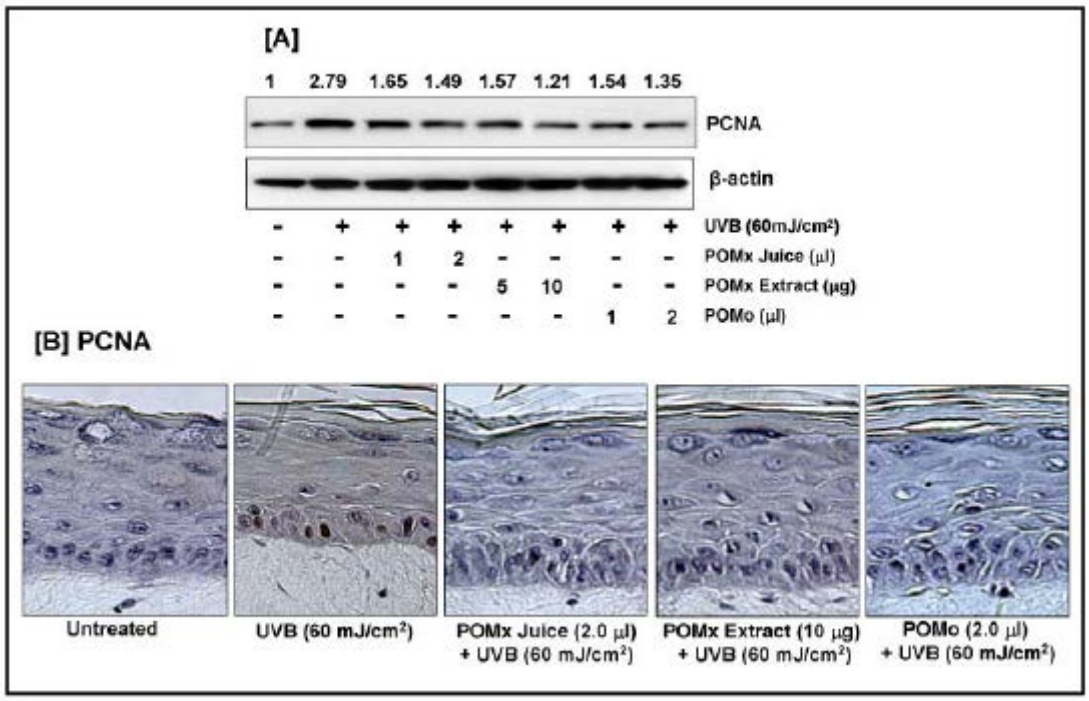

Figure 3. Effect of POMx juice, POMx extract and POMo on UVB-mediated increase in protein expression of PCNA in three dimensional human reconstituted skin (EpiDerm™ FT-200)

(A) EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2μl), POMx extract (5-10 μg) or POMo (1-2μl) diluted in EFT media (100 μl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm2). [A] Twelve h post-UVB, EpiDerm was harvested and cell lysates prepared for western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results. (B) EpiDerm™ FT-200 was topically treated with or without POMx juice (2μl), POMx extract (10 μg) or POMo (2μl) diluted in EFT media (100 μl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm2). Twelve h post-UVB, EpiDerm was harvested and immunostaining was performed to detect UVB-induced PCNA positive cells as described in “Materials and Methods”. Representative sections of immunostaining are shown from five independent experiments with similar results.

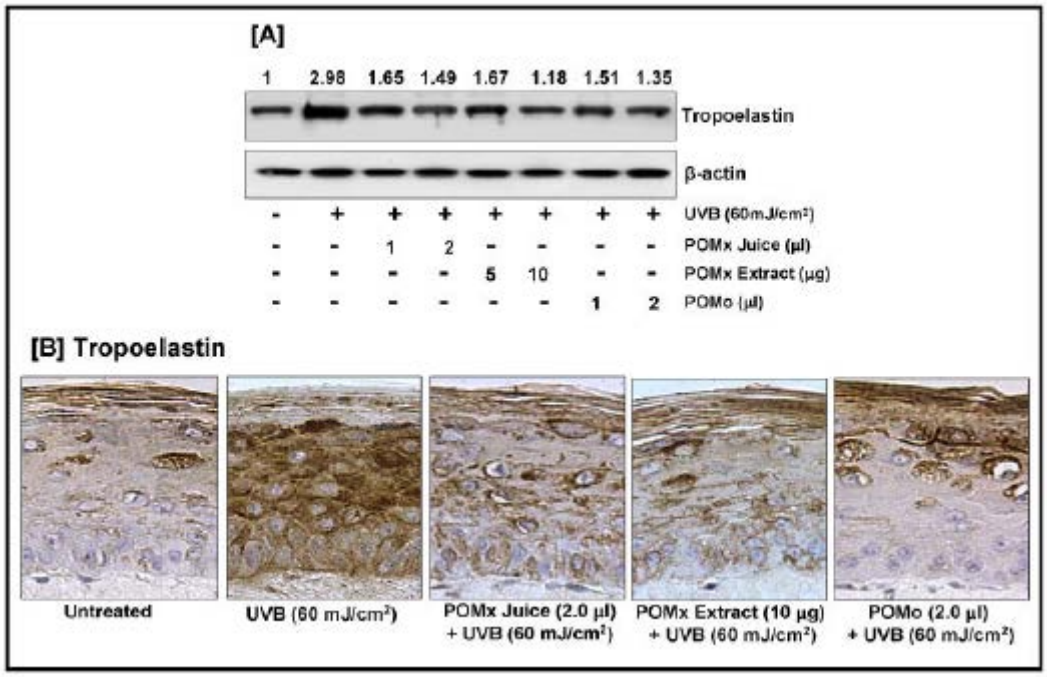

Figure 4. Effect of POMx juice, POMx extract and POMo on UVB-mediated increase in tropoelastin in three dimensional human reconstituted skin (EpiDerm™ FT-200)

(A) EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm2). [A] Twelve h post-UVB, EpiDerm was harvested and cell lysates prepared for western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results. (B) EpiDerm™ FT-200 was topically treated with or without POMx juice (2µl), POMx extract (10 µg) or POMo (2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm2). Twelve h post-UVB, EpiDerm was harvested and immunostaining was performed to detect UVB-induced PCNA positive cells as described in "Materials and Methods". Representative sections of immunostaining are shown from five independent experiments with similar results.

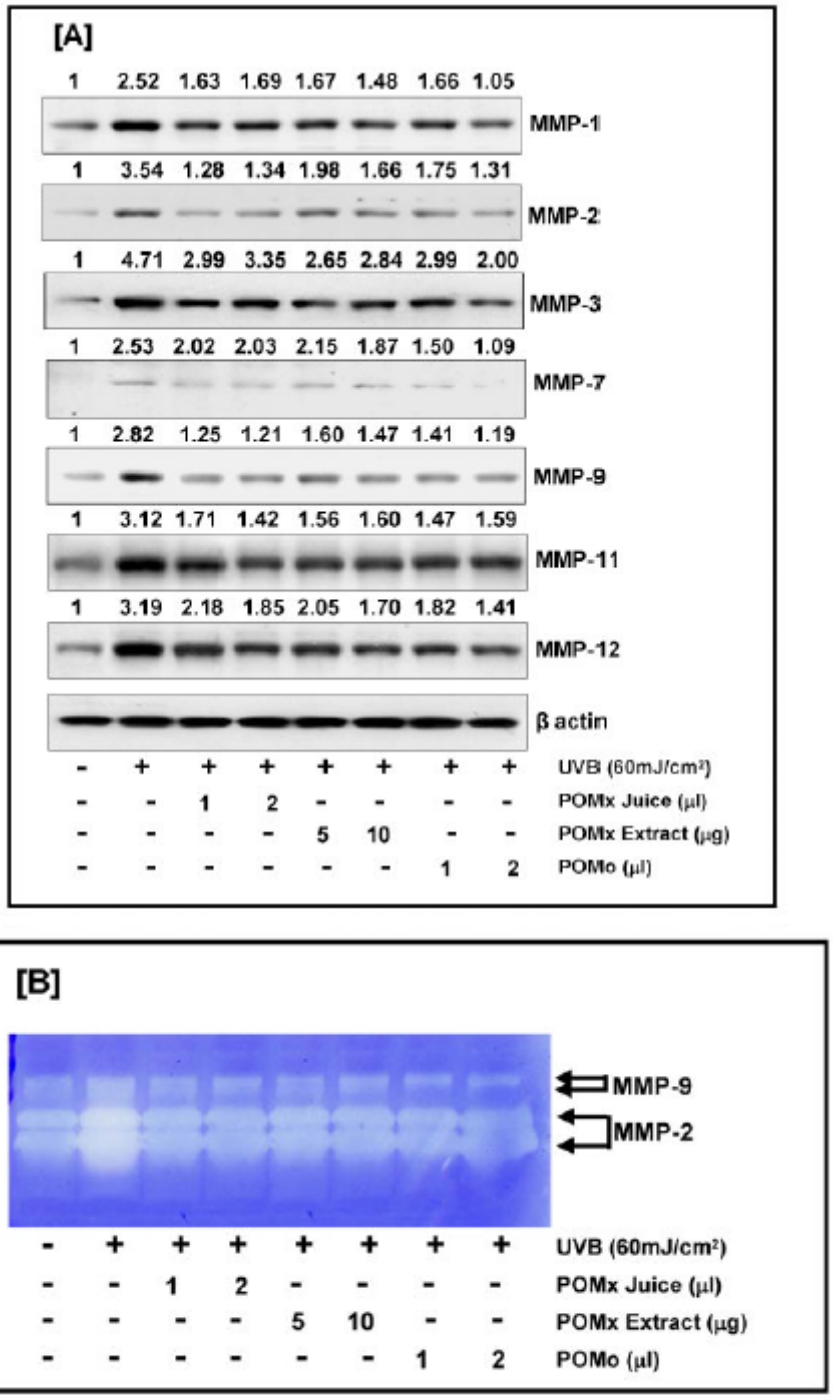

Figure 5. Effect of POMx juice, POMx extract and POMo on UVB-mediated increase in MMPs protein and gelatinase activity in human reconstituted skin (EpiDerm™FT-200)

(A) EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm2). Twelve h post-UVB, EpiDerm was harvested and cell lysates prepared for western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results. (B) EpiDerm™ FT-200 was topically treated as described above. Twelve h post-UVB, culture media was collected and gelatin zymography performed as described in "Materials and Methods". The gels shown here are representative of three independent experiments with similar results.

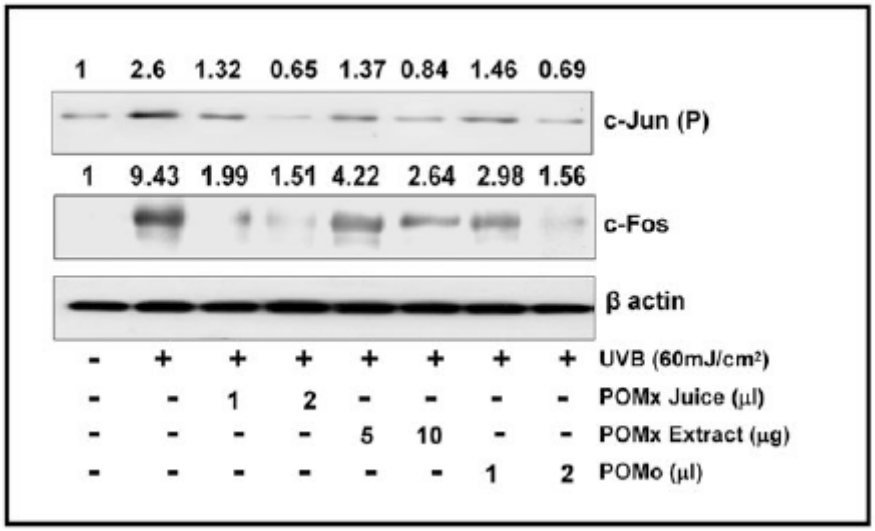

Figure 6. Effect of POMx juice, POMx extract and POMo on UVB-mediated phosphorylation of c-jun and expression of c-fos protein in human reconstituted skin (EpiDerm™ FT-200

EpiDerm™ FT-200 was topically treated with or without POMx juice (1-2µl), POMx extract (5-10 µg) or POMo (1-2µl) diluted in EFT media (100 µl/tissue/well) containing 0.1% DMSO for 1 h after which the agents were removed and EpiDerm™ FT-200 washed with PBS and exposed to UVB (60mJ/cm2). Twelve h post-UVB, EpiDerm was harvested and cell lysates prepared for western blot analysis. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The values above the figures represent relative density of the bands normalized to β-actin. The immunoblots shown here are representative of three independent experiments with similar results.

Reference [13] Photochemistry and Photobiology 2007 **83**(4), 882-8

Zaid MA, Afaq F, Syed DN, Dreher M, Mukhtar H

Inhibition of UVB-mediated oxidative stress and markers of photoaging in immortalized HaCaT keratinocytes by pome-granate polyphenol extract POMx

In recent years there has been an increase in use of botanicals with antioxidant properties as skin photoprotective agents. Pomegranate (Punica granatum L.) fruit possesses strong antioxidant and antiinflammatory properties. Recently, we have shown that pomegranate-derived products rich in anthocyanidins and ellagitannins inhibit UVB-mediated activation of nuclear factor kappa B and modulate UVA-mediated cell proliferation pathways in normal human epidermal keratinocytes. In this study, we evaluated the effect of polyphenol-rich pomegranate fruit extract (POMx) on UVB-induced oxidative stress and photoaging in human immortalized HaCaT keratinocytes. Our data show that pretreatment of HaCaT cells with POMx (10-40 microg mL(-1)) inhibited UVB (15-30 mJ cm(-2))-mediated (1) decrease in cell viability, (2) decrease in intracellular glutathione content and (3) increase in lipid peroxidation. Employing immunoblot analysis we found that pretreatment of HaCaT cells with POMx inhibited UVB-induced (1) upregulation of MMP-1, -2, -7 and -9, (2) decrease in TIMP-1, (3) phosphorylation of MAPKs and (iv) phosphorylation of c-jun, whereas no effect was observed on UVB-induced c-fos protein levels. These results suggest that POMx protects HaCaT cells against UVB-induced oxidative stress and markers of photoaging and could be a useful supplement in skin care products.

Reference [14] Photochemistry and Photobiology 2011 **86**(6), 1318–1326

Afaq F, Khan N, Syed DN, Mukhtar H

Oral Feeding of Pomegranate Fruit Extract Inhibits Early Biomarkers of UVB Radiation-Induced Carcinogenesis in SKH-1 Hairless Mouse Epidermis

Pomegranate from the plant Punica granatum L. possesses strong antioxidant and anti-inflammatory properties. Recently, we have demonstrated that treatment of normal human epidermal keratinocytes with pomegranate fruit extract (PFE) inhibited UVB-mediated activation of nuclear factor kappa B (NF-κB) and mitogen activated protein kinases pathways. Here, we evaluated the effect of PFE on early biomarkers of photocarcinogenesis employing SKH-1 hairless mice. PFE was provided in drinking water (0.2%, wt/vol) to SKH-1 hairless mice for 14 days before a single UVB (180 mJ cm(-2)) irradiation. We found that oral feeding of PFE inhibited UVB-induced: (1) skin edema; (2) hyperplasia; (3) infiltration of leukocytes; (4) lipid peroxidation; (5) hydrogen peroxide generation; (6) ornithine decarboxylase (ODC) activity; and (7) ODC, cyclooxygenase-2 and proliferating cell nuclear antigen protein expression. Oral feeding of PFE enhanced repair of UVB-mediated formation of cyclobutane pyrimidine dimers (CPDs) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG). Importantly, PFE treatment further enhanced UVB-mediated increase in tumor suppressor p53 and cyclin kinase inhibitor p21. Furthermore, oral feeding of PFE inhibited UVB-mediated: (1) nuclear translocation of NF-κB; (2) activation of IKKα; and (3) phosphorylation and degradation of IκBα. Taken together, we provide evidence that oral feeding of PFE to mice affords substantial protection from the adverse effects of UVB radiation via modulation in early biomarkers of photocarcinogenesis and provide suggestion for its photochemopreventive potential.

Reference [15] International Journal of Dermatology 2010 **49**(3), 276-82

Park HM, Moon E, Kim AJ, Kim MH, Lee S, Lee JB, Park YK, Jung HS, Kim YB, Kim SY
Extract of Punica granatum inhibits skin photoaging induced by UVB irradiation

BACKGROUND: Punica granatum (pomegranate) is kind of a fruit consumed fresh or in beverage. It has been widely used in traditional medicine in various parts of the world. In this study, we examined the efficacy of a Punica granatum (PG) extract in protecting skin against UVB-induced damage using cultured human skin fibroblasts.

METHODS: A Korean red PG sample was used, and its effects classified according to if the PG source originated from the rind, seed and fruit. The polyphenol content of PG, which is known to prevent other adverse cutaneous effects of UV irradiation, was measured by GC-MS. The protective effects of PG on UVB-induced skin photoaging were examined by determining the level of procollagen type I and MMP-1 after UVB irradiation.

RESULTS: Based on the GC-MS quantitative analysis, catechin, quercetin, kaempferol, and equol were the predominant compounds detected in PG. In the changes of expression of procollagen type I and MMP-1 in UV irradiated human skin fibroblasts treated PG, especially extract prepared from rind, the synthesis of collagen was increased and the expression of MMP-1 was decreased.

CONCLUSION: The major polyphenols in PG, particularly catechin, play a significant role in its photoprotective effects on UVB-induced skin damage.

Reference [16] Experimental Dermatology 2010 2010 **19**(8), 182-90

Bae JY, Choi JS, Kang SW, Lee YJ, Park J, Kang YH
Dietary compound ellagic acid alleviates skin wrinkle and in-flammation induced by UV-B irradiation

Ellagic acid, a polyphenol compound present in berries and pomegranate, has received attention as an agent that may have potential bioactivities preventing chronic diseases. This study examined photoprotective effects of ellagic acid on collagen breakdown and inflammatory responses in UV (ultraviolet)-B irradiated human skin cells and hairless mice. Ellagic acid attenuated the UV-B-induced toxicity of HaCaT keratinocytes and human dermal fibroblasts. Non-toxic ellagic acid markedly prevented collagen degradation by blocking matrix metalloproteinase production in UV-B-exposed fibroblasts. Anti-wrinkle activity of ellagic acid was further investigated in hairless mice exposed to UV-B, in which it attenuated UV-B-triggered skin wrinkle formation and epidermal thickening. Topical application of 10 micromol/l ellagic acid diminished production of pro-inflammatory cytokines IL-1beta and IL-6, and blocked infiltration of inflammatory macrophages in the integuments of SKH-1 hairless mice exposed to UV-B for 8 weeks. In addition, this compound mitigated inflammatory intracellular cell adhesion molecule-1 expression in UV-B-irradiated keratinocytes and photoaged mouse epidermis. These results demonstrate that ellagic acid prevented collagen destruction and inflammatory responses caused by UV-B. Therefore, dietary and pharmacological interventions with berries rich in ellagic acid may be promising treatment strategies interrupting skin wrinkle and inflammation associated with chronic UV exposure leading to photoageing.

ADS® Patent registration

PRODUCT FOR EPIDERMAL PROTECTION

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