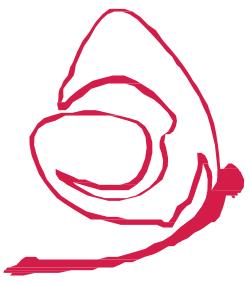


MIREI Pharma
México



Be Free®





Origen

La longevidad de la mujer japonesa

En la isla de Okinawa se encuentran las mujeres con la longevidad más alta del mundo. Durante más de 20 años, la Dra. Mizuho Nasu estudió el porqué y descubrió que su gran salud se debe a la nutrición basada en diversos alimentos fermentados, especialmente la granada, lo que les ha permitido desarrollar una microbiota óptima para el equilibrio hormonal.



La Granada

La Dra. Mizuho Nasu analizó la granada, que es la base de la salud de las mujeres en Okinawa, y descubrió que los probióticos que crecen en la flor de esta fruta son los responsables del bienestar de estas mujeres. Además, es la responsable de su piel sana, su gran vitalidad y corazón saludable.

Entre las propiedades de este antioxidante natural, podemos decir que ayuda a modular los receptores de estrógeno, disminuir la grasa corporal, reducir el colesterol y la presión arterial, prevenir la osteoporosis y ser fuente de urolitinas, que son fuente de la microbiota del colon.



Be Free es una línea de suplementos alimenticios de origen japonés hechos a base de granada fermentada y otros ingredientes NATURALES que actúan sobre tu microbiota intestinal promoviendo el balance hormonal.



MIREI Pharma
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Be Free W1 - VC

Be Free™

Es un suplemento alimenticio a base de extracto de Satsuma mandarina y extracto de Granada que te ayudará a reducir las várices.

El activo principal, la Satsuma Mandarina, es una fruta japonesa que aporta una gran cantidad de vitamina C que ayuda a la fabricación de colágeno, nutriente necesario para la reparación de los vasos sanguíneos.

Beneficios:

- Aumenta el flujo sanguíneo
- Reduce la inflamación
- Alivia el dolor, la sensación de pesadez y calambres en las piernas
- Mejora la microcirculación

Modo de Uso:



2 cápsulas al día, se toman con agua, antes del desayuno



Precio Público \$550



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Be Free W2 - MB

Be Free™

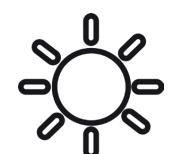
Es un suplemento alimenticio a base de extracto de espirulina y extracto de Granada que te ayudará con el control adecuado del peso, equilibrio metabólico y balance hormonal.

El activo principal, la espirulina, es un alga que favorece la pérdida de peso al actuar como inhibidora del apetito y ayuda a reducir el tejido adiposo debido a que inhibe una enzima que actúa en el proceso de la producción de los ácidos grasos en el organismo y a su acción antiinflamatoria.

Beneficios:

- Reduce selectivamente la grasa visceral
- Conserva el tejido magro
- Mejora la saciedad
- Quemador de grasa
- Reduce los síntomas climatéricos

Modo de Uso:

 1ampolla al día, diluida con 200 ml de jugo, después del desayuno.

Precio Público \$650



Be Free W3 - OE

Es un suplemento alimenticio a base de extracto de alga marina y extracto de Granada que te ayudará a fortalecer el tejido óseo y promover el balance hormonal.

El activo principal, las algas marinas, son consideradas un super alimento ya que entre sus principales propiedades se encuentra su riqueza en vitaminas, minerales y fibra.

La gran cantidad de minerales que contiene como son el yodo, calcio, hierro y fósforo, ayudan a fortalecer los huesos y dientes.

Beneficios:

- Aumenta la densidad mineral ósea
- Regenera la fuerza del hueso
- Reduce el riesgo de fracturas
- Previene la osteoporosis
- Preserva el cartílago
- Ayuda a la regeneración del tejido óseo
- Reduce la inflamación

Modo de Uso:



1 stick combinado con 200 ml de agua, antes de dormir



Precio Público \$600

Be Free[®]

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Be Free W4 - SC

Es un suplemento alimenticio a base de extracto de alga marina, extracto de yuzu y extracto de Granada que te brindara protección cardiovascular y mayor balance hormonal.

El activo principal, el Yuzu, es un cítrico híbrido asiático que aporta una gran cantidad de vitaminas entre las que destaca la vitamina C la cual ayuda a producir estrógenos provocando la reducción de algunas molestias de la menopausia.

Beneficios:

- Reduce los bochornos
- Previene enfermedades cardiovasculares
- Promueve la humedad natural de la vagina
- Aumenta la libido
- Desintoxica el intestino

Modo de Uso:



1 stick combinado con 200 ml de agua, antes de dormir



Precio Público \$650

Be Free™



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The Pomegranate and “Calidad y Seguridad Alimentaria - CSA”.



Research and technology undoubtedly help the development of a country and, in the specific case of the agricultural sector, make it possible for high-quality, safe food products to be produced. To do this it is necessary to protect and monitor the entire food chain, from raw and processed materials to the needs and demands of consumers. Nowadays, “traceability” is a concept of the utmost importance in our diets and should make it possible to track a food product back from its point of sale to its production and raw materials. Spain is a country with highly-developed technology, and both research centres and universities are becoming increasingly involved with companies from the agricultural sector. This close and growing cooperation means that these companies have a very promising future.

The research group “Calidad y Seguridad Alimentaria, CSA” (Food Quality and Safety) of the Agricultural Technology Department of the Higher Technical College of Orihuela (Miguel Hernández University, Elche) has extensive experience in studies concerning pomegranates, both at research level and in the assessment of the quality and functionality of pomegranates and their derived products, and in advising companies that produce and market these products. The subjects researched for these companies include an estimate of the useful life of products, the development of new products and the carrying out of affective studies with consumers.

The CSA group, in collaboration with its national (**Centre for Soil and Applied Biology Segura of the Spanish National Research Council - CEBAS-CSIC**) and international partners such as **Kansas State University** (USA) and the Wroclaw University of Environmental and Life Sciences (Poland), has become a leading group in studies into quality (nutritional, sensory and functional) and the acceptance of pomegranate products in international markets.

Its research work is evidenced in over 20 international articles concerning this subject, since the group started to study pomegranates in 2009. The researchers from this group have given plenary conferences about the benefits of pomegranates in numerous countries such as Turkey, Poland, Slovakia, Mexico and Spain, amongst others.



The “**Mollar de Elche**” pomegranate variety is undoubtedly the most cultivated in Spain, and is the variety for which Spain is known internationally. Mollar de Elche fruits have two main advantages: **(I)** an intense sweetness and **(II)** a very soft woody aril part (the edible part). However, it also has disadvantages: **(I)** a fairly weak colour, which worsens significantly after the thermal treatment of the juices, and **(II)** quite a simple sensory profile, dominated by sweetness and with fruity, aromatic notes that are generally not intense. On the other hand, we can state that the fruit of other non-indigenous varieties, such as Wonderful, are complementary to those of the Mollar de Elche variety.

This complementary nature is based on the fact that Wonderful pomegranates have a complex sensory profile and a very strong claret red colour. However, it also has clear disadvantages, which are that it is extremely acidic and the woody part of its arils is very hard and persistent (Vázquez-Araújo et al., 2014).

One of the latest products to be developed by the “CSA” group for the company “**Antioxidantes Naturales del Mediterráneo**” is a pomegranate concentrate based on a fusion of the Mollar de Elche and Wonderful varieties. This combination has led to the obtaining of a product called Granatum Plus Pomegranate Fusion Concentrate, which includes the advantages of both varieties and that neutralises their sensory disadvantages and includes a high content of punicalagins - 232 mg of punicalagin in the recommended daily dose (30 mL of the product).

CONCLUSIONS OF THE RESEARCH WORK OF MIGUEL HERNÁNDEZ UNIVERSITY

One of the latest research studies was focused on the comparison of the content of total punicalagins and polyphenols in a total of 50 commercial products based on pomegranates (sold in the European Union, including Spain). The products studied were divided into two large groups: (I) capsules, ampoules and extracts, and (II) juices of direct extraction, juices from concentrates and concentrates. The contents of punicalagin found in the first group were between the following values: not detected - 308 mg of punicalagin per gram of product. For group two the values ranged from no detection of punicalagins to 10.4 mg of punicalagin per gram of product. The results obtained after the analysis showed that the "Granatum Plus" products were of higher quality, in terms of the composition of the aforementioned compound (α - and β -punicalagin).

The pomegranate extract capsules and the Granatum Plus Pomegranate Fusion Concentrate were the products in which high contents of punicalagin were found (308 and 7.76 mg of punicalagin per gram of product).

Based on the research studies carried out on over 50 products during the period from October 2016 to April 2017 we can conclude that the Granatum Plus products contain optimum values of punicalagin A+B, as well as the highest values in the organoleptic tests. It has also been determined that the value for money score of its products is much higher than most of the products analysed.

Regarding the products described as eco-friendly, it has been determined that they do not have a higher content of bioactive components than the conventional Granatum Plus products.

In view of the above, we can conclude that the juices, concentrates and extracts of Pomegranate Granatum Plus fully comply with the requirements of functional food, meeting the needs of the most demanding consumer.

1. Introduction

In order to move forwards towards the future, we often need to look first at our past. A clear example of this is one of the first crops to be domesticated by humans, the pomegranate. The presence of this fruit in Spanish culture and history is so ubiquitous that it even appears in coats of arms such as that of the Kingdom of Granada during the time of the Catholic Kings.

Another example highlighting the relationship between the pomegranate, Spain and research is the emblem of the Spanish National Research Council (CSIC), which incorporates a pomegranate tree (**Figure 1**).



Figure 1. A pomegranate orchard and the emblem of the Spanish National Research Council.

The aim of the present document is to highlight the importance of this crop in Spain, which is due both to the high volume of production and the benefits for human nutrition provided by this fruit and its derived products.

1.1. The origins of the pomegranate

The pomegranate (*Punica granatum* L.) has been cultivated since antiquity. It is one of the biblical crops, together with grapes, olives and dates. According to Nikolai Vavilov, the pomegranate evolved in the 4th Centre of Origin, the Middle East (Asia Minor, Transcaucasia, Iran and the highlands of Turkmenistan).

Its systematic classification is as follows:

Division: Magnoliophyta

Class: Magnoliopsida.

Subclass: Rosidae.

Order: Myrtales.

Family: Lythraceae

Genus: *Punica*.

Specie: *P. granatum*.

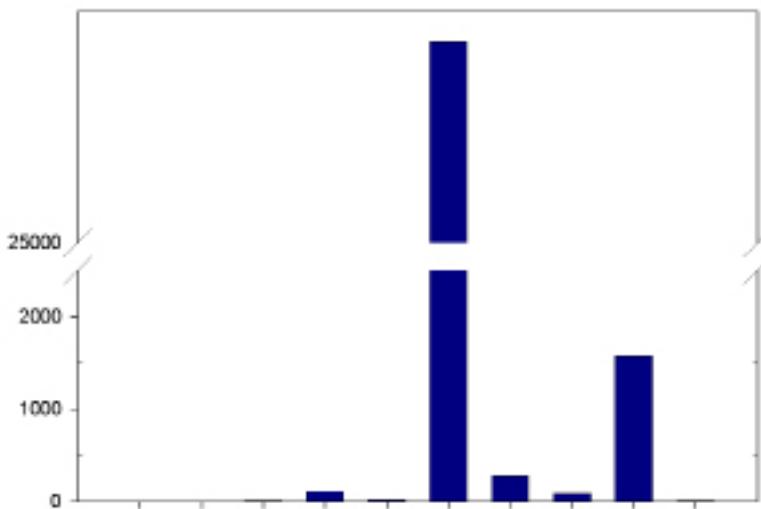


The pomegranate (*Punica granatum* L.) is a small deciduous tree that grows to a maximum of 8 metres high in the wild. It is a very valuable fruit tree in many regions of the world, especially those which are arid and semiarid, since it is capable of adapting to different areas where other, currently more widely grown fruit trees would not yield a profitable crop (Melgarejo and Salazar, 2003).

1.2. The Economic Importance of the pomegranate

The pomegranate is currently cultivated in a variety of countries, including Spain, the United States, Iran, Turkey, India, Israel, China and countries along the north coast of Africa, among others. Spain ranks as the largest producer in Europe, with an annual production of 22,311 tons (Spanish Ministry of the Environment and Rural and Marine Affairs, 2010).

Production is centred in Valencia, Andalusia and Murcia (**Figure 1**), although 90% of this is in the province of Alicante. In turn, production in Alicante is mainly concentrated in three municipalities, Elche, Albatera and Crevillente, in order of importance. This high level of concentration clearly indicates the enormous socio-economic importance of the pomegranate for these three municipalities and their surroundings.



Graph 1. Pomegranate producing communities in Spain.

1.3. The Mollar de Elche pomegranate

The pomegranate has traditionally been a highly prized and esteemed fruit in many civilizations. Together with the palm tree, the pomegranate tree is the most characteristic tree in Campo de Elche, and has been known from time immemorial. In Spain, the Mollar de Elche pomegranate (Figure 2) is by far the most popular variety and is unquestionably the most widely grown in this country.



Figure 2

The most important characteristics of the Mollar de Elche pomegranate are as follows:

- Large or very large sized fruit.
- Vigorous, fast growing tree.
- Large, dark red seed casings (arils) with very small and soft seeds.
- Maturing between October and November.
- It is more productive and of better quality and higher calibre than the Valencian group pomegranates, which rank second in Spanish production.

2. Functional products derived from the pomegranate and integral

The study of the pomegranate's bio-active components and their benefits for human health is a highly topical and important field of research. Numerous scientific studies have shown that both the pomegranate and its derived products contain many components that contribute to preventing disease and maintaining health (Larrosa et al., 2006; Sartippour et al., 2008; Koyama et al., 2010).

The pomegranate is usually consumed fresh. However, a large proportion of the crop does not present sufficient visual quality to be destined for fresh consumption, since its acceptance by consumers is very low. However, the quality of the edible part, the arils, is similar to that of those specimens with good acceptance for fresh consumption. It is therefore necessary to find an alternative commercial use for this proportion of the crop unsuitable for fresh consumption, based on industrial processing.

The most important industrial products derived from the pomegranate



- Pomegranate juice: widely marketed in the USA, and with great potential in Spain
- Ready to eat arils
- Jams.
- Wines, vinegars and liqueurs.
- Dehydrated arils
- Nutraceuticals made from peel extract
- Food condiments.
- Cosmetics: creams, oils, gels, etc.

2.1. Chemical composition of the pomegranates

The peel, white pulp membrane, arils and seeds of the pomegranate all contain many chemical compounds of high biological value (Figure 3). The most important product derived from the pomegranate is its juice, which is unquestionably the most research element, with many articles published in both the Spanish and international scientific literature.

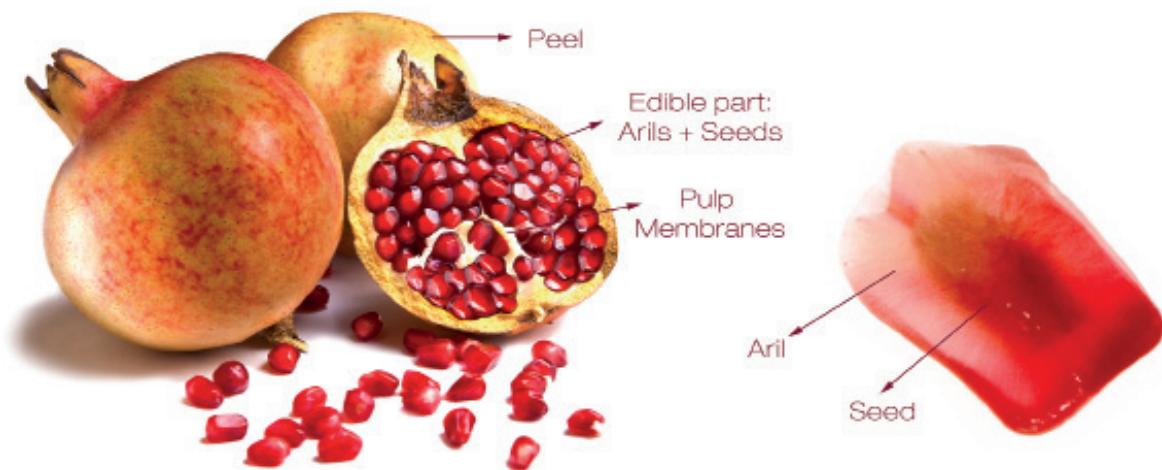


Figure 3. The different parts of the pomegranate.

About 50% of the total weight of the pomegranate corresponds to the peel and white pulp membranes, which are a major source of bio-active compounds such as polyphenols, flavonoids, ellagitannins, proanthocyanidins and minerals, mainly potassium, nitrogen, calcium, phosphorus, magnesium and sodium. Consequently, if processed correctly, nutraceutical products and food condiments made from peel and membrane extracts may provide an important source of these compounds.



The edible part of the pomegranate represents about 50% of the total weight of the fruit, of which the fleshy pulp of the arils accounts for 80% and the woody seeds contain 20%.

Pomegranate arils are composed of 85% water, 10% sugar (mainly fructose and glucose), 1.5% organic acids (principally ascorbic, citric and malic acids) and bioactive compounds such as polyphenols and flavonoids (mainly anthocyanins).

Pomegranate arils are also a major source of lipids, since fatty acids comprise between 12% and 20% of the total dry weight of the seeds.



The fatty acid profile is characterised by a high content in unsaturated fatty acids, including linolenic, linoleic, punicic, oleic, stearic and palmitic acids.

Table 1. Nutritional composition of the edible part (USDA, 2007).

Nutrient	Unit	Value per 100 g
BASIC FOOD SUBSTANCES		
Water	g	80.97
Energy	kcal	68
Protein	g	0.95
Fat	g	0.30
Carbohydrates	g	17.17
Dietary fibre	g	0.6
Total sugars	g	16.57
VITAMINS		
Vitamin C (ascorbic acid)	mg	6.1
Vitamin A	IU	108
Vitamin E (α -tocopherol)	mg	0.60
Vitamin K (phylloquinone)	μ g	4.6
OTHER		
Phytosterols	mg	17
Cholesterol	mg	0
α -Carotene	μ g	50
β -Carotene	μ g	40

Table 2. Mineral content of the edible part (USDA, 2007) and in pomegranate juice with pulp (Andreu-Sevilla et al., 2008).

	(mg/L)	(mg/kg)		
Calcium	4.6	18014	74.7	30
Magnesium	65.8	57	65.7	30
Potassium	933	3093	940	2590
Sodium	25.9	0	25.8	30
Iron	3.0	1499	8.8	3.0
Copper	2.1	661	4.7	0.7
Manganese	1.9	47	2.1	-
Zinc	4.4	0	4.4	1.2

The beneficial effects of fruit and vegetables arising from their high content in bioactive compounds are now widely accepted. The presence of the compounds listed above (**Table 2**) indicates the important nutritional value of the pomegranate.

2.2. Phenolic compounds

2.2.1. Low molecular weight phenolic compounds

Phenolic compounds can be divided into simple molecules and polymers of these with a higher molecular weight. The flavonoids are the most numerous compounds in the first subgroup, mainly represented by anthocyanins, which are responsible for the characteristic colour of the pomegranate. The principal low molecular weight phenolic compounds are the phenolic acids, which include gallic acid and ellagic acid (**Figure 4**).

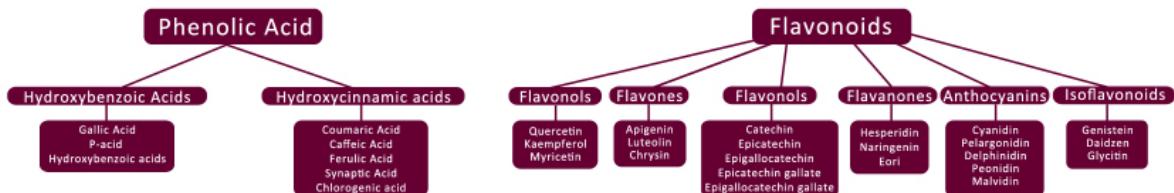


Figura 4. Low molecular weight phenolic compounds

2.2.2. High molecular weight phenolic compounds

Tannins are the most characteristic high molecular weight polyphenols. Pomegranate peel is rich in hydrolysable tannins, mainly punicalin, pedunculagin and punicalagin (**Figure 5**).

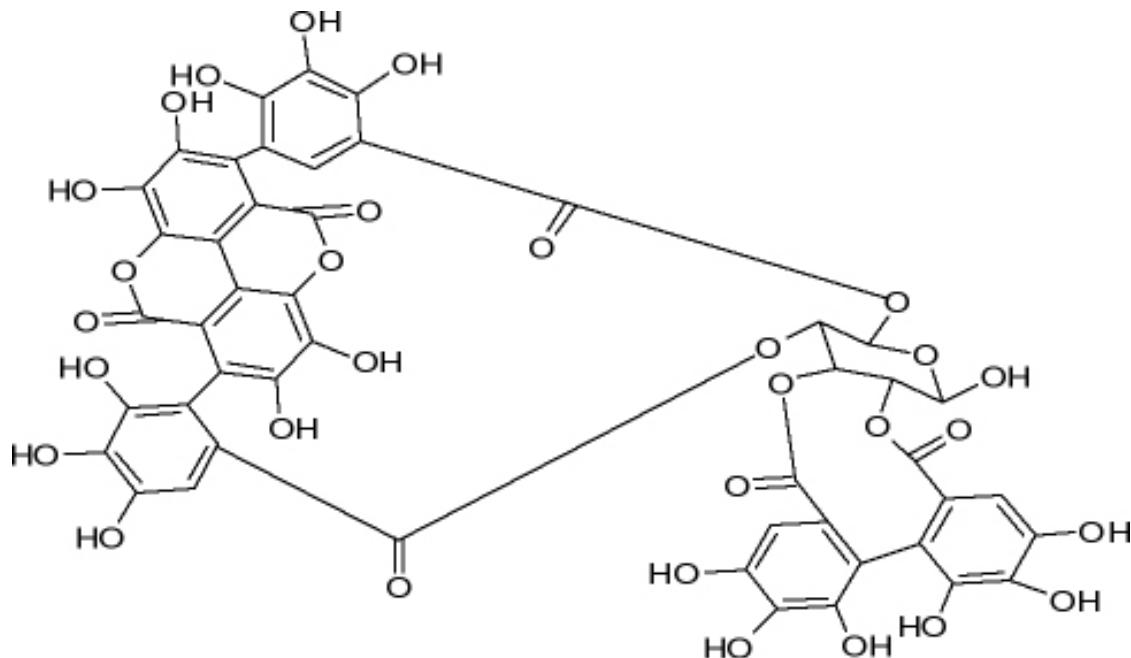


Figure 5. Molecular structure of punicalagin

2.3.The pomegranate as a functional food

The concept of functional food is complex and can refer to whether or not the components are nutrients, whether they have a positive effect on the body, or whether they have a physiological or psychological effect beyond the purely nutritional (Viuda-Martos et al., 2011a).



Functional foods include: (i) those containing certain minerals, vitamins, fatty acids or dietary fibre, (ii) foods to which biologically active substances, such as phytochemicals or other antioxidants, have been added, and (iii) probiotic foods containing beneficial live microorganisms.

Thus, given the results of various studies on the chemical composition of the pomegranate and more recently on its health benefits, the pomegranate can be considered a functional food (Melgarejo, 2010).

Anthocyanins are the compounds considered responsible for the red colour of pomegranate arils. The importance of these phenolic compounds lies in their antioxidant action, which protects against free radicals and retards the cell aging process.

The free radical scavenging activity of these flavonoids has been demonstrated in various studies, such as that conducted by Espín et al. (2000). An estimated 10% of the antioxidant action of pomegranate juice is due to the presence of these polyphenols, the anthocyanins (Gil et al., 2000).

The antioxidant capacity of pomegranate juice is three times that of red wine or green tea (Gil et al., 2000).



Its content in essential fatty acids (linoleic, linolenic and arachidonic acids) is of great importance, particularly due to the content in polyunsaturated fatty acids. These play an important role in the prevention of cardiovascular disease and some other heart problems, because this type of fatty acid significantly reduces HDL-cholesterol levels (bad cholesterol). Punicic acid has anti-atherogenic effects.

The ellagitannins can be transformed into urolithins; urolithin A may be the most active anti-inflammatory compound related to consumption of the pomegranate. Anti-inflammatory processes in the colon may be due to the unmetabolised fraction of ellagitannins (Larrosa et al., 2010).

Punicalagin, the polyphenol with the highest known molecular weight, is hydrolysed into ellagic acid and metabolised in the intestinal tract to generate urolithins. Punicalagin compounds present very high antioxidant or free radical scavenger capacity and are responsible for approximately 50% of this activity in pomegranate juice, followed by other hydrolysable tannins (33% of total activity) and, to a lesser extent, ellagic acid (3%) (Gil et al., 2000; García-Viguera et al., 2004).

The main functional properties of the punicalagins are: (Sánchez et al., 2009)



- Powerfull antioxidant effect.
- Anticancer action.
- Protective effect on the cardiovascular system.

2.4. Oxidation versus Antioxidation

Living organisms need energy, and this is provided by the basic food substances (carbohydrates, lipids and proteins).

This energy is obtained through chemical reactions which may or may not involve oxygen. Thus, we distinguish between anaerobic and aerobic metabolism.

Cells obtain more energy from aerobic metabolism. With oxygen, cells obtain more ATP from the basic nutrients (carbohydrates, lipids and proteins). Without oxygen, they obtain 20% less ATP (energy).



These oxidation reactions take place in the mitochondria, structures which are present in the cell cytoplasm. Basically, the glucose molecule (6 carbon atoms) splits into two pyruvic acid molecules (3 carbon atoms) which oxidise and release electrons and protons. These are eventually absorbed by the oxygen, converting it into water and carbon dioxide and storing energy in the form of tri-phosphate bonds (ATP).



The molecules derived from the oxidation of glucose continue to be oxidised and oxygen is reduced as it absorbs the electrons and protons; each oxygen molecule absorbs four electrons and four protons, thereby forming two water molecules. This is what is known as tetra-reduction.

However, the reaction does not always occur in exactly this way, and it has been calculated that an estimated five percent of reactions are mono- and bi-reductions

that instead of generating water and CO₂, which are easily and naturally eliminated by the excretory organs (kidneys, lungs, skin), generate harmful reactive species derived from oxygen (Reactive Oxygen Species, ROS) that are detrimental to our health because they perpetuate the oxidation of healthy tissue, leading to pathologies.

This 5% could be likened to the “soot” from a “metabolic chimney”; if it is not eliminated or neutralised, over time we become ill or age faster. Those body systems most vulnerable to attack are the circulatory, nervous and immune, or defensive, systems.

The reactive oxygen species produced in cells include hydrogen peroxide (H_2O_2), hydroxyl radical ($-OH$) and superoxide ($O_2\cdot-$).

With the emergence of oxygen on the Earth, those species that were not equipped for oxidation disappeared, whilst those that could withstand the impact of oxygen survived, because they managed to develop a system that would protect them: the antioxidant system.

Oxidation is defined as the “theft” of electrons from the outer electron layers of atoms or molecules, converting them into charged ions. The substances that remove these electrons are called oxidising agents, and when oxidised, they are reduced. If these “oxidised” ions are not neutralised by another element (a reducing agent) that offers its own electrons or protons (H^+), they are converted into free radicals and continue moving through the body until they can replace these electrons by removing them from other elements, the most vulnerable of which are the membranes that make up cells.

“Uncontrolled” oxidation in our body tissues is responsible for aging, degeneration and, of course, disease. We must fight against it if we want to survive.

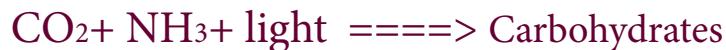
The normal function of our cellular antioxidant enzyme system is to control this excess of free radicals or reactive oxygen species produced by our own bodies:

- Superoxido dismutasa (SOD)**
- Catalasa (CAT)**
- Glutathione peroxides... and**

These three enzymes form our strongest anti-radical defence in cells. We should bear in mind that the consequence of an excess of free radicals (oxidising agents) or the inability on the part of our enzymatic defences to counter such an excess, is the risk of developing a multitude of pathological processes, and in particular, of degenerative diseases, such as Alzheimer's, Parkinson's, arthritis, etc.

Aging is no more than an imbalance in favour of oxidation mechanisms due to weak or inefficient antioxidant defence systems.

However, due to the pace of modern life, we must add many more "oxidative" attacks from our environment, which overwhelm our aforementioned innate antioxidative enzyme defence system: pollution, tobacco, radiation, countless preservatives in our food etc. But we can protect ourselves with substances that help us fight against oxidation: hydro-soluble vitamins (vitamins B1, B6, B12 and C), fat-soluble vitamins (Vitamins E and A), bio-carotenoids and polyphenols. Reactive oxygen species are also produced in plants during photosynthesis, the process whereby plants obtain energy from the sun.



Like us, plants also need to defend themselves in order to be able to survive the intense light which produces oxidation. This is the function of carotenoids, bioflavonoids and other substances that protect plants from the oxidation generated.

Everyone knows that if tomatoes, broccoli, oranges or apples did not contain antioxidant substances, they would not keep, they would simply rot. If we include these nutrients in our diet, they stimulate our antioxidant system and reduce what is known as oxidative stress.

Anti-oxidant help is always necessary, especially when our metabolism has been weakened, for example, by physical over-exertion (pregnancy, growth, sports competitions, etc.), or when our bodies are trying to recover from an infection or an operation, or when we are simply entering a transitional stage (andro- and menopause).

This search for antioxidative nutrients may be why those fruits and vegetables which are most resistant to the impact of light energy from the Sun are also those to which we are most attracted.

The concentration of antioxidants provided by a fruit or vegetable is highest at maturity, which is when we should consume them. Their attractive colours are a clear sign of the high concentration of substances they contain with strong antioxidant properties such as carotenoids, polyphenols, resveratrols, etc.

Here, special mention should be made of the pomegranate, because it contains more anti-oxidants than other fruits which are thought to be antioxidant-rich, such as citrus fruits or bilberries, and more even than green tea or red wine.



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3. Pomegranate and health

The pomegranate (*Punica granatum* L.), an ancient, mystical and distinctive fruit, was praised in antiquity in various works, such as the Bible, the Jewish Torah and the Babylonian Talmud, as a sacred fruit which promoted fertility, abundance and good luck. It also appeared in Egyptian and Greek ceremonies, art and mythology, and was the personal emblem of the Roman Emperor Maximus.



In addition to these historical antecedents, the pomegranate is used by several types of medicine for the treatment of a variety of diseases. In Ayurvedic (traditional Indian) medicine, the pomegranate is used to treat parasites, diarrhoea and ulcers and is considered to have depurative properties. The pomegranate also serves as a remedy for diabetes in Unani medicine (also practiced in India).

The great interest shown nowadays in the medicinal and nutritional benefits of the pomegranate began in 2000, and since then, more than 200 articles have been written describing the beneficial health properties of the pomegranate and its derived products. In contrast, in the period between 1950 and 1999, only about 25 scientific papers were published on this topic.

The pomegranate has a broad range of potentially therapeutic uses, including treatment and prevention of cancer, cardiovascular disease, Alzheimer's disease, inflammatory disease, oral and skin disease, obesity, erectile dysfunction and diarrhoea.

Below, details are given of the main results of a review of the scientific literature until the year 2011, describing the various therapeutic applications of the pomegranate mentioned above.



3.1. Anti-cancer and anti-tumour properties

Numerous studies have been conducted to assess the efficacy of the pomegranate and its derived products, which have been shown to have a potent antioxidant action as an anti-proliferative, anti-invasive and pro-apoptotic agent in diseased cells and in animal models (Lansky and Newman, 2007; Syed et al., 2007; Hong et al., 2008; Hamad and Al-Momene 2009).

Hong et al. (2008) demonstrated that pomegranate juice and extracts are potent inhibitors of cell growth, even more potent than some polyphenols when considered in isolation, suggesting the existence of a synergistic effect with the phytochemicals present in the pomegranate and its extracts.

A pomegranate extract applied as a topical pre-treatment reduced the incidence of tumours in mice from 100% to 30%, and also increased the latency in tumour development from 9 to 14 weeks (Afaq et al., 2005). Albretch et al. (2004) studied the effects of pomegranate oil, the polyphenols in the peel and membranes and the polyphenols from fermented juice, on prostate cancer. All of these agents individually prohibited the proliferation *in vitro* of tumour cells in human LNCaP, PC-3 and DU 145 cells, demonstrating that pomegranate-derived products have a clear anti-tumour action in the case of prostate cancer.

Kohno et al. (2004) demonstrated that the administration of pomegranate seed oil in the diet inhibited the incidence and proliferation of colon adenocarcinomas in rats.

The inhibition of colon tumours by the seed oil is associated with an increase in conjugated linoleic acid in the colonic mucosa and liver. There is scientific evidence showing that pomegranate juice suppresses the expression of COX-2 induced by TNF- α , the NF- κ B pathway and activation of Akt.

Certain bioactive components present in pomegranate juice, such as anthocyanins and flavonols, may be responsible for reducing the proliferation of cancer cells (Adams et al., 2006). Seeram et al. (2005b) described the powerful anti-proliferative action of pomegranate juice against various tumour cell lines, with a large inhibition effect of between 30% and 100%.

Pomegranate juice, ellagic acid and punicalagin induced apoptosis (genetically regulated cell death) of HT-29 colon cells, but in HCT116 colon cells, only ellagic acid and punicalagins contributed to apoptosis whereas pomegranate juice did not (Seeram et al., 2005b).

Therefore, pomegranate peel extract, which is rich in these compounds (punicalagins and ellagic acid) may have potential for the treatment of colon cancer in the future. Lansky et al. (2005b) reported that certain components present in the pomegranate significantly inhibited cancer cell invasion of the prostate in vitro (PC-3 cells).



Fjaeraa and Nanberg (2009) showed that ellagic acid induced apoptosis by measuring DNA breakage and alteration in the cell cycle. González-Sarrías et al. (2009) suggested that ellagic acid and its metabolites, such as urolithins A and B, may contribute to the prevention of colon cancer. Hong et al. (2008) showed that pomegranate juice and its extracts have a potent ability to stop proliferation and stimulate apoptosis in prostate cancer cells. More recently, Koyama et al. (2010) demonstrated that the use of pomegranate extracts with stabilised ellagitannin content (punicalagin) to treat LAPC4 prostate cells inhibited proliferation by 37% and led to apoptosis. From the foregoing, we can conclude that the pomegranate and its derived products have a beneficial effect against cancer and tumours due to the high content of compounds such as anthocyanins, ellagic acid and punicalagins. Furthermore, the cases studied have demonstrated that pomegranate products and extracts have different effects, as has administration of the compounds responsible individually or in isolation. Therefore, the use of pomegranate and its derived products is highly dependent on the type of clinical condition. It is important to emphasise that all the cases mentioned above refer to the prevention and treatment of cancer, and never to a cure for cancer or tumours. Due to their phytochemical composition, the pomegranate and its derived products are highly recommended for the prevention and treatment of cancer.

A summary is given below of the main actions or anti-tumour effects of the pomegranate and its products against different cancers (breast, colon, prostate, etc.).

Table 4. Main antitumoral effects of pomegranate fruit.

- **Antiproliferative: Stopping tumor growth.**
- **Induces apoptosis: induced cell death (suicide).**
- **Inhibits kB nuclear factor (NF-kB): regulates expression of more than 200 genes**
(immune system, cell proliferation, tumor invasion, metastasis).
- **Anti-angiogenesis: new blood vessel formation.**

Source: Dr. Gilberto E. Chéchile Toniolo (2011). II Symposium Internacional sobre el Granado, Madrid, España.

3.2. Prevention of cardiovascular disease

One of the major risk factors for developing coronary heart disease is dyslipidemia, which is characterised by elevated levels of low-density cholesterol (LDL) and/or low levels of high density cholesterol (HDL) (Esmaillzadeh and Azadbakht, 2008). Cholesterol is divided into two types: low density cholesterol (LDL), or bad cholesterol, and high density lipoprotein (HDL), or good cholesterol. Good cholesterol (HDL) is so called because it is thought to help reduce cholesterol levels in the blood; high density cholesterol is produced naturally by the body itself and removes cholesterol from the artery walls, returning it to the liver. Bad cholesterol (LDL) accumulates on the artery walls, forming plaques which hinder the flow of blood to the heart. Therefore, excessively high levels of LDL cholesterol will increase the risk of cardiovascular disease. It is thought that LDL oxidation contributes to atherosclerosis and cardiovascular diseases (Heinecke, 2006).



Several in vitro studies have been conducted with animals and humans to analyse the effect of various pomegranate-related products on the prevention and mitigation of atherosclerosis and LDL oxidation (Aviram et al., 2000; Sezer et al., 2007; Basu and Penugonda 2009; Davidson et al., 2009; Fuhrman et al., 2010). Aviram et al. (2000) analysed the effect of pomegranate juice consumption in healthy men on LDL oxidation and found that LDL levels were reduced and HDL activity was increased by around 20%. Seezer et al. (2007) compared the total polyphenol content and antioxidant action of pomegranate wine and red wine.

Both polyphenol content and antioxidant action were higher in pomegranate wines than in red wines. Both wines produced a reduction in LDL; however, due to its higher antioxidant ability, the decrease induced by pomegranate wine was greater than that caused by red wine, namely by 24% for pomegranate wine and 14% for red wine. Esmaillzadeh et al. (2006) administered 40 g of concentrated pomegranate juice to diabetic and hyperlipidemic (elevated cholesterol and triglyceride levels) patients for 8 weeks. By the end of the study, triglyceride and HDL levels had not changed. However, total cholesterol level was reduced by 5.43%, LDL by 9.24%, total cholesterol/HDL ratio by 7.27% and the LDL/HDL ratio by 11.76 %.

Basu and Penugonda (2009) summarised the main antiatherogenic mechanisms of pomegranate juice as follows:



- **It increases the antioxidant action of blood serum, reducing plasma lipids and lipid peroxidation**
- **It reduces LDL oxidation**
- **It reduces the size of atherosclerosis lesions**
- **It reduces systolic blood pressure**

Thus, pomegranate juice intake has a beneficial effect on the progression of atherosclerosis and consequently on the risk of developing coronary heart disease.

Dr. Aviram conducted numerous experiments with healthy and hypertensive patients, to whom he administered pomegranate juice for different periods of time. As a result of these studies, it was concluded that blood pressure was reduced by up to 36% after two weeks of treatment with pomegranate juice. This reduction has been attributed to the high antioxidant power of pomegranate polyphenols (Aviram and Dornfeld, 2001; Aviram et al. 2004).

3.3. Inflammatory properties

Inflammation, the human body's first physiological defence, can protect us from lesions caused by wounds and poisoning. This defence system can eliminate infectious microorganisms, eradicate irritations and maintain normal physiological functions. However, overexposure to inflammation can cause physiological dysfunctions such as asthma and arthritis (Lee et al. 2010). There is considerable scientific evidence demonstrating the anti-inflammatory action of the pomegranate and its derived products (Lansky and Newman, 2007; Shukla et al., 2008; Larrosa et al., 2010, Lee et al., 2010).

Some pomegranate extracts, particularly the extract of cold-pressed seeds, inhibit the action of cyclooxygenase and lipoxygenase enzymes in vitro. Cyclooxygenase is an important enzyme in the conversion of arachidonic acid to prostaglandins, which are in turn important mediators of inflammation. This latter, therefore, is significantly inhibited by the ingestion of pomegranate extracts. Lipoxygenase mediates the transformation of arachidonic acid into leukotrienes, which also mediate inflammation, and thus inflammation is also inhibited by pomegranate seed extract (Thomas-Barberán, 2010).



Boussetta et al. (2009) showed that the punicic acid and conjugated fatty acid present in pomegranate seed oil has a proven anti-inflammatory effect in vivo and therefore limits lipid peroxidation. Lee et al. (2010) analysed four hydrolysable tannins, among which were punicalagin and punicalatin, all isolated from the pomegranate. Each of these compounds at different doses significantly inhibited the production of nitrogen monoxide (NO) in vitro, producing an anti-inflammatory effect.

De Nigris et al. (2007) demonstrated that administration of pomegranate juice and pomegranate extracts to obese rats significantly reduced the expression of certain genetic markers which influence cardiovascular inflammation.



Subsequently, Romier-Crouzet (2009) obtained similar results with pomegranate juice and pomegranate extracts, and observed inflammatory prevention due to high ellagic acid content. Lastly, Larrosa et al. (2010) found that administration of pomegranate extracts reduced prostaglandin levels in colonic mucosa, again due to the high levels of ellagic acid present in the pomegranate.

3.4. The anti-diabetic properties of the pomegranate

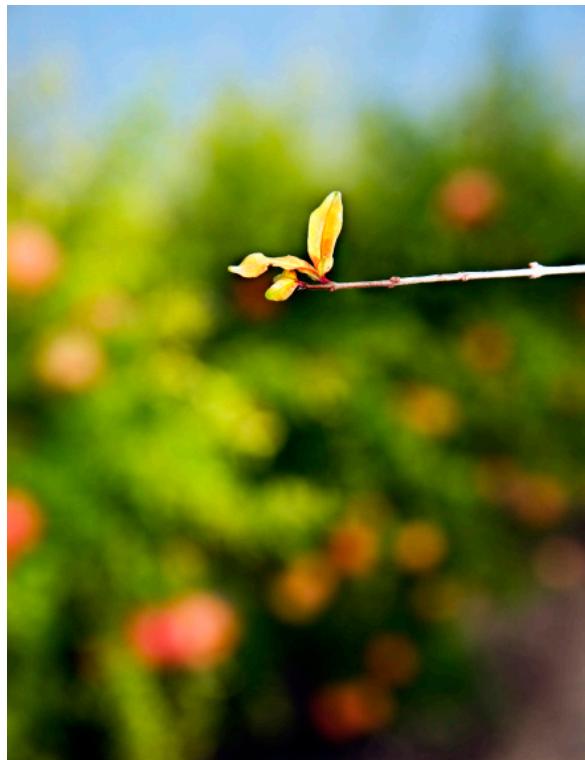


Diabetes is the most common metabolic disease in the world, affecting millions of people. According to the International Diabetes Federation, the estimate for 2025 is that this disease will affect about 333 million people. Diabetes ranks third in prevalence after cardiovascular disease and cancer.

This is where the pomegranate and its derived products can play a key role, and there is a significant body of scientific evidence supporting the anti-diabetic properties of this fruit (Huang et al., 2005; Li et al., 2005; Katz et al., 2007; Parmar and Kar, 2007; Li et al., 2008; Bagri et al., 2009).

Diabetes is associated with high levels of oxidative stress and the development of atherosclerosis. It seems clear that the antioxidant compounds present in the pomegranate can exert a significant effect on diabetes.

For example, Katz et al. (2007) demonstrated the hypoglycemic action of pomegranate flowers, seeds and juice. The mechanisms by which the pomegranate and its derived products exert this effect remain unknown. However, although there are numerous hypotheses about these mechanisms, all seem to suggest the inhibition of certain genetic markers and certain compounds that induce oxidative stress. For example, Li et al. (2005) suggested that the anti-diabetes mechanism of pomegranate flower extract was inhibition of the α -glucosidase enzyme. Pamar and Kar (2007) demonstrated that administration of pomegranate peel extract normalised the adverse effects of a compound that induces diabetes in mice.



Mcfarlin et al. (2009) studied the effect of pomegranate seed oil on fat accumulation in mice and observed an improvement in insulin sensitivity.

All these findings, together with those related to cardiovascular disease, suggest that the pomegranate and its derived products have a beneficial effect on diabetes and on cardiovascular disease in diabetic patients, since their effects on heart disease have also been established.

The main components with anti-diabetic properties are the polyphenols; these compounds affect blood glucose through numerous mechanisms, including the inhibition of glucose absorption through the gut or via peripheral tissues.

The most probable anti-diabetic mechanism is inhibition of the α -glucosidase enzyme. Other mechanisms may include the reduction of blood sugar level due to absorption by peripheral tissues rather than through the intestine (Scalbert et al., 2005).

3.5. Prevention of oxidative deterioration

Oxidative deterioration is a very topical issue and a clear example of this is that the action of fruit and vegetables against oxidative deterioration (high content of antioxidant compounds) is one of the properties or characteristics most highly valued by consumers. Generally, an antioxidant can be defined as a natural or artificial substance with the ability to protect a biological system by neutralising free radicals such as oxygen, nitrogen and lipid radicals (Cano and Arnao, 2004).

These antioxidant properties endow fruit and vegetables with beneficial health properties, protecting against or reducing the risk of certain degenerative diseases (Brandt et al., 2004, Chen et al., 2007). Consequently, antioxidant content has become an important quality parameter of fruit and vegetables in recent years.

Compounds with antioxidant properties include anthocyanins and other phenols (Espin et al., 2007; Dorais et al., 2008), carotenoids (Perera and Yen, 2007) and vitamins A, C and E (Hoursome et al., 2008).

The compounds responsible for the potent antioxidant action of the pomegranate and its derived products have been studied by many researchers, using both *in vitro* and *in vivo* models. The antioxidant action *in vitro* of the pomegranate and its derived products has been assessed in several studies (Naveena et al., 2008; Cam et al., 2009; Mousavinejad et al., 2009; Tezcan et al., 2009).

Tzulker et al. (2007) determined that the high antioxidant capacity of the pomegranate and its derived products is due to the presence of punicalagins, and not of anthocyanins as previously thought.

The mechanisms of antioxidant action in vivo remain unclear, although it is known that these mechanisms act on biological matrices in a very complex way. Madrigal-Carballo et al. (2009) suggested that the pomegranate's phenolic compounds undergo a redox reaction, since the hydroxyl groups of the phenolic molecules donate a hydrogen molecule to reducing agents. Other researchers (Amarowicz et al., 2004) have reported that the antioxidant action of phenolic compounds is due to their ability to capture free radicals and chelating metal cations.

3.6. Prevention of skin damage

The process of photoageing includes molecular and structural damage of the skin, such as inflammation, decreased collagen synthesis, thickening or proliferation of the epidermis (surface of the skin), incomplete degradation of collagen fragments and protein oxidation.

All these changes translate clinically into a thin skin with wrinkles, yellowish discolouration, round or oval white spots or irregular dark spots and telangiectasia (visible blood vessels), among others.

These are also accompanied by the appearance of benign lesions such as seborrheic keratoses or lentigines (coffee-coloured lumps or spots), sebaceous hyperplasia and premalignant lesions such as actinic keratoses.

Skin damage occurs as a result of natural aging. However, exposure to the sun also induces major damage to the skin. Prolonged exposure to ultraviolet rays can cause numerous problems, such as skin cancer.

Studies conducted with different pomegranate extracts (Aslam et al., 2006) suggest that pomegranate peel extracts promote regeneration of the dermis, while seed oil extracts regenerate the epidermis.



Pacheco-Palencia et al. (2008) described the protective properties of pomegranate extracts against UVA and UVB radiation due to a reduction in the generation of reactive oxygen species (ROS). Afaq et al. (2009) suggested that UVB radiation-induced skin damage can be reduced by the intake of products derived from pomegranate peel and seeds.

All these scientific results demonstrate the excellent ability of pomegranate peel and seed extracts to protect the skin.

3.7. Antimicrobial properties of the pomegranate and its derived products

Many food preservation technologies, some of which have been in use for a long time, protect food from alteration by microorganisms. Thus, microorganisms can be inhibited by refrigeration, reduced water activity, acidification, modified atmosphere packaging, by non-thermal treatments or by addition of antimicrobial compounds.

Antimicrobial products for food use consist of chemical compounds which are added or already present in food that kill or retard the growth of microorganisms, thus increasing resistance to alterations in quality or safety. The main targets of antimicrobial agents are the microorganisms that cause food poisoning (infectious agents and toxin producers) or that alter foods and whose metabolic end products (catabolites) or enzymes cause bad odours, unpleasant flavours, texture problems, changes in colour and/or health risks (Davidson and Zivanovic, 2003).



The use of synthetic and chemical agents with considerable antimicrobial properties as microbial growth inhibitors is one of the oldest techniques on earth for the control of microbial growth, and is therefore a suitable preservation technique (Viuda-Martos et al., 2008).

At present, there is a tendency to replace these chemicals with possible natural treatments by using agents present in fruits, vegetables and herbs. The principal natural antimicrobial agents are the essential oils obtained from herbs and spices. Essential oils derived from plants are known for their high antimicrobial action against a broad range of bacteria and fungi. In addition, they can enhance the antioxidant action of the treated products themselves (Ayala-Zavala et al., 2005).

The antimicrobial action of the pomegranate and its derived products has been demonstrated in numerous studies which have reported the inhibition of the activity of numerous microorganisms (Reddy et al., 2007; McCarrell, 2008; Al-Zoreky 2009; Choio et al., 2009; Gould et al., 2009).

Reddy et al. (2007) found that different pomegranate extracts in different solvents (water, ethanol, etc.) presented a significant antimicrobial action against *E. coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Cryptococcus neoformans* and *S. aureus*. Al-Zoreky (2009) showed that the growth of *Listeria monocytogenes*, *S. aureus*, *E. coli* and *Yersinia enterocolitica* is significantly inhibited by pomegranate peel extract. Choi et al. (2009) investigated the *in vivo* and *in vitro* effect of the application of different concentrations of pomegranate peel extract on skin to inhibit the growth of *Salmonella*, and found that the minimum dose was 62.5 mg/L.

In general, the potent inhibitory action of the pomegranate and its derived products is attributed to the high concentration of compounds such as polyphenols, tannins and anthocyanins. Very recent studies have found that the use of derivatives and by-products as food additives not only improves antioxidant capacity but also ensures total safety due to the considerable ability of the pomegranate and its extracts to inhibit the activity of micro-organisms that cause food deterioration (Navarro et al., 2011; Viuda-Martos et al., 2011b).

3.8. Effects of the pomegranate on oral health

Maintaining optimal dental health is not only important to preserve the appearance and function of teeth, but also to protect against cardiovascular disease. Science now recognises that chronic inflammatory periodontal disease is closely related to the worsening of cardiovascular disease (Dumitrescu, 2005).

Di Silvestro et al. (2009) showed that a mouthwash based on pomegranate extracts effectively reduced the amount of dental plaque microorganisms. This beneficial effect is mainly attributed to the clear action of polyphenol and flavonoid compounds against the development of gingivitis. Gingivitis is a bacterial oral disease that produces inflammation and bleeding of the gums, and is caused by food debris caught between the teeth.

Menezes et al. (2006) studied the effect of a pomegranate extract on dental plaque microorganisms. They found that it was highly effective, reducing the number of microorganisms by 84%.

Sastravaha et al. (2005) demonstrated the effectiveness of a gel containing pomegranate extracts as an additional, complementary treatment for standard periodontal therapy. Badria and Zidan (2004) showed that pomegranate flavonoids possess an antibacterial action in vitro against the microorganisms responsible for gingivitis.

Fewer studies have been conducted on the effect of the pomegranate and its derived products on oral disease compared to research on diseases such as cancer or cardiovascular disease. The cases cited above are the most recent examples of research on this question.

Consumption of pomegranates, either as fresh fruit, derived products or even in extract form, is also enjoyable because of the delicious flavour, making the pomegranate a perfect solution for proper oral health.

Table 5 summarises some of the most relevant studies.

Study model	Clinical condition	Part of the plant	Dose	Time (days)	Effect	Reference
In vivo	Diabetes	Flowers	250 mg/(kg d)	21	Reduces total cholesterol, triglycerides, LDL cholesterol and increases HDL cholesterol	Bagri <i>et al.</i> 2009
In vivo	Diabetes	Peel	20 mg/(kg d)	28	Increases activity of enzymes involved in diabetes, liver and kidney	Alyhunibat <i>et al.</i> 2010
In vivo	Healthy	Peel	50 mg/(kg d)	28	Antioxidant protection from numerous enzymes	Murthy <i>et al.</i> 2002
In vivo	Healthy	Juice	-	28	Antioxidant protection from numerous enzymes	Faria <i>et al.</i> 2007
In vivo	Healthy	Ellagic acid	60 mg/(kg d)	45	Reduces cholesterol, fatty acids, triglycerides, and phospholipids	Devipriya <i>et al.</i> 2008
Humans	Healthy	Juice	250 mL/d	28	Reduces lipid and LDL cholesterol oxidation	Guo <i>et al.</i> 2008
Humans	Healthy	Fruit	100 g	10	Increases the antioxidant capacity of plasma	Hajimahmoodi <i>et al.</i> 2009

Table 5. in vivo studies conducted to evaluate the beneficial effects of the pomegranate on laboratory animal and human health.

Study model	Clinical condition	Part of the plant	Dose	Time (days)	Effect	Reference
In vivo	Healthy	Peel	500 mg/kg	36 h	Anti-inflammatory properties against oedema and granulomas	Al Yahya (2005)
Humans	Healthy	Ellagic acid extract	100 mg/day	28	Inhibits ultraviolet radiation damage to pale skin	Kasai <i>et al.</i> , (2006)
Humans	Healthy	All Parts	-	-	Inhibits allergic reactions	Park <i>et al.</i> , (2008)
In vivo	Diabetes	Flowers	400 mg/kg	45	Reduces lipid oxidation and glucose levels	Manoharan <i>et al.</i> , (2009)
In vivo	Cancer	Fruit	-	240	Reduces carcinogenic lung tumours	Khan <i>et al.</i> , (2007)
In vivo and humans	Cancer	Fruit	50-150 µg/ml	3	Inhibits growth of lung tumours	Khan <i>et al.</i> , (2006)
Humans	Diabetes	Juice	40 g	56	Reduces LDL cholesterol levels	Esmailzadeh <i>et al.</i> , (2006)

Study model	Clinical condition	Part of the plant	Dose	Time (days)	Effect	Reference
In vivo	Healthy	Peel and leaves	20 mg/ml	16	Faster wound healing	Soni <i>et al.</i> , (2011)
In vivo	Cancer	Fermented juice	-	-	Retards metastasis of mammary cells	Khan <i>et al.</i> , (2007)
In vivo	Healthy	Juice	-	45	Inhibits damage to kidney cells and oxidative stress	Ilbey <i>et al.</i> , (2009)
In vivo	Diarrhoea	Peel	100-400 mg/kg	-	Reduces gastroenteritis	Onais <i>et al.</i> , (2007)
Humans	Healthy	Fruit	-	1	Antibacterial activity against dental plaque microorganisms	Menezes <i>et al.</i> , (2006)
In vivo	Atherosclerosis	Juice	-	60	Powerful anti-atherosclerosis action	Kaplan <i>et al.</i> , (2001)
Humans	Healthy	Juice	-	2	Presence of urolithin in human urine	Seeram <i>et al.</i> , (2006)

Study model	Clinical condition	Part of the plant	Dose	Time (days)	Effect	Reference
In vivo	Diabetes	Seed oil	-	-	Weight loss and reduction in type 2 diabetes	McFarlin et al., (2008)
Humans	Healthy	Juice	300 ml/d	14	Reduces systolic pressure	Carpenter et al., (2009)
Humans	Metabolic syndrome	Juice	240 ml	30	Improves endothelial function	Hashemi et al., (2010)
In vivo	Diabetes	Flowers	500 mg/kg	42	Reduces cardiac fibrosis	Huang et al., (2005)
In vivo	Cancer	Seed oil and fermented juice	-	-	Seed oil is better than fermented juice against breast cancer	Mehta et al., (2004)
In vivo	Cancer	Fruit	50-150 mg/ml	3	Inhibits cancer cell markers	Khan et al., (2007)
In vivo	Cirrhosis	Peel	50 mg/kg	28	Prevents cirrhosis, has anti-oxidative properties and reduces gallbladder	Toklu et al., (2007)

Study model	Clinical condition	Part of the plant	Dose	Time (days)	damage		Reference
					Effect		
In vivo	Diabetes	Peel	600 mg/kg	1	Beneficial effects against diabetes		Najafzadeh et al., (2011)
In vivo	Cancer	Peel extract and arils	0.8 mg	1	Prevents multifocal development and survival of prostate cancer cells		Sartippour et al., (2008)
In vivo	Influenza	Seed extract	175 mg/KG	28	Considerably reduces the concentration of the influenza virus		Figueroa et al., (2006)
In vivo and humans	Cardiovascular disease	Juice	-	-	Inhibits the development of atherosclerotic lesions due to the protection of LDL against oxidation		Aviram et al., (2002)
In vivo and humans	Healthy	Juice	-	98	Anti-atherogenic effects in humans and anti-atherosclerotic effects in mice		Aviram et al., (2000)
Humans	Hypertension	Juice	50 ml/day	14	Inhibits oxidative stress		Aviram et al., (2001)
In vivo	Diabetes	Peel and seed	200	14	Powerful anti-diabetic action		Das et al., (2009)

Study model	Clinical condition	extract	mg/kg/day			Effect	Reference
		Part of the plant	Dose	Time (days)			
Humans	Carotid artery stenosis	Juice	-	3 years		Reduces systolic blood pressure	Aviram et al., (2004)
In vivo	Healthy	Peel	100-1000 mg/kg/day	35		Inhibits the proliferation of melanocytes and melanin	Yoshimura et al., (2005)
In vivo	Healthy	Flowers	50 mg/kg	1 h		Powerful analgesic effect	Guno, C (2008)
In vitro and In vivo	Salmonella	Peel	3.9-2000 mg/ml	4		Powerful anti-bacterial action of ethanol extracts	Choi et al., (2011)
Humans	Healthy	Ellagic acid	100-200 mg/day	28		Oral administration lightens skin affected by UV radiation	Kasai et al., (2006)
In vivo	Healthy	Peel	50 mg/kg/day	20		Powerful protective effect against radiation therapy	Toklu et al., (2009)
In vivo	Healthy	Flowers	50-100	-		Powerful antihistamine action and	Barwal et al., (2009)

Study model	Clinical condition	Part of the plant	mg/kg/day		potential role against asthma	Reference
			Dose	Time (days)		
In vivo	Diarrhoea	Peel	10-400 mg/kg/day	4 hours	Anti-diarrhoea effect and powerful cytotoxic action	Hasan et al., (2009)
In vivo	Cancer	Seed oil	5%	140	Effective anti-cancer agent for skin	Hora et al., (2004)
Humans	Cancer	Extract	-	-	Induces apoptosis when combined with genistein	Lous Jeune et al., (2005)
Humans	Cancer	Juice	-	28	Prevents pro-carcinogenic activation	Faria et al., (2007)
Humans	Cancer	Peel	25-300 µg/ml	3	Reduces the proliferation of breast cancer cells	Dikmen et al., (2011)
Humans	Erectile dysfunction	Juice	-	28	Suggests that long-term treatment is effective	Forest et al., (2007)
In vivo	Obesity	Leaf extract	800 mg/kg	35	Inhibits development of obesity	Lei et al., (2007)

Study model	Clinical condition	Part of the plant	Dose	Time (days)	Effect	Reference
In vivo	Healthy	Seeds	250-500 mg/kg/day	21	Cognitive deficits induced by scopolamine	Kumar <i>et al.</i> , 2009
In vivo	Healthy	Polyphenol extract	4.8 mg/day	1	Visibly reduces neonatal brain damage	West <i>et al.</i> , 2007
In vivo	Myocardial Infarct	Juice	100-300 mg/kg/day	21	Alleviates cardiotoxic effects and may be valuable for the treatment of micro strokes	Mohan <i>et al.</i> , 2010
Humans	Heart disease	Juice	240 ml/day	90	Daily consumption of pomegranate juice improves acute myocardial Ischaemia	Summer <i>et al.</i> , (2005)
In vivo	Menopause	Juice and extract	-	14	Anti-depressant action and less bone loss	Mori-Okamoto <i>et al.</i> , (2004)
In vivo	Healthy	Juice	1 ml	49	Significant improvement in the quality of semen	Türk <i>et al.</i> , (2008)

3.9. Other health-related properties of the pomegranate

3.9.1. Effects of the pomegranate against diarrhoea

Only two studies have been conducted recently that demonstrated the effect of pomegranate peel extract in the prevention of diarrhoea. Both experiments were conducted on laboratory rats; after the administration of a pomegranate peel extract, it was observed that both the number of bowel movements and the mass of the same were reduced. The studies were conducted by Qnais et al. (2007) and Olapour et al. (2009). The dose proposed by the latter for the treatment of this condition was 400 mg per kg of body weight.

3.9.2. Effects of the pomegranate on sperm quality and erectile dysfunction

The basic purpose of semen is reproduction, since it acts as a “vehicle” for transporting sperm to the female reproductive tract. Although orgasm and sexual pleasure accompany the ejaculation of semen, erection and orgasm are controlled by independent mechanisms, thus the emission of semen is not essential for the enjoyment of sex.

Türk et al. (2008) found that consumption of pomegranate juice produced an increase in the concentration of sperm in the epididymis, an increase in mobility and a greater density of spermatogenic cells; it also reduced the amount of poor quality semen compared to the reference or control group.

In a more recent study, this same research group suggested that ellagic acid has a protective effect on testicles and sperm. This effect may be related to the potent action of ellagic acid against oxidative stress (Türk et al., 2010).

Erectile dysfunction is the repeated inability to develop or maintain an erection which is firm enough for successful sexual intercourse. In a study carried out by Forest et al. (2007), it was found that after four weeks of consumption of pomegranate juice, patients showed better erectile function than other patients who had been given a placebo.

3.9.3 Effects of the pomegranate on obesity

Obesity is a chronic disease of multifactorial origin that is characterised by the excessive accumulation of fat or general hypertrophy of adipose tissue in the body. Obesity, therefore, refers to a situation where the natural energy reserve of humans and other mammals, stored as body fat, increases to a point where it is associated with multiple complications, such as certain health conditions or diseases and increased mortality.

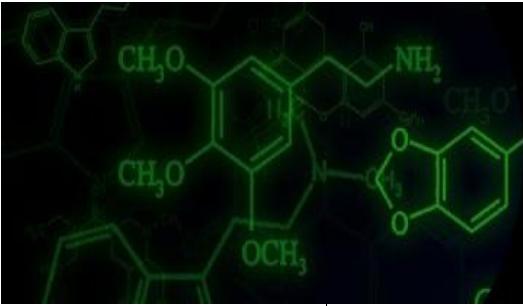
The World Health Organisation's (WHO) definition of obesity is when the Body Mass Index (BMI), which is a calculation based on an individual's height and weight, is equal to or greater than 30 kg/m^2 . Another sign of obesity is an abdominal perimeter greater than or equal to 102 cm in men and 88 cm in women.

Obesity forms part of the metabolic syndrome, and is a known risk factor. In other words, it increases the risk of developing various diseases, particularly cardiovascular disease, type 2 diabetes mellitus, sleep apnoea, stroke, osteoarthritis, and some forms of cancer and dermatological and gastrointestinal ailments.

Although obesity is an individual clinical condition, it has become an increasingly serious public health problem, and the WHO believes that "obesity has reached epidemic proportions worldwide, and at least 2.6 million people die each year because of obesity or overweight. Although previously considered a problem confined to high income countries, obesity is now also prevalent in low and middle income countries".

<i>in vivo</i> studies	Part of the plant	Time (days)	Effect	Reference
Rats	20% pomegranate extract (6% punicalagin)	37	Weight loss at baseline	Cerdá <i>et al.</i> 2003
Mice	Leaves	-	On a high fat diet, leaf extract reduced the development of	Lei <i>et al.</i> 2007

Table 6. Studies to assess the effect *in vivo* of the pomegranate or its extracts on obesity.



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A review on proactive pomegranate one of the healthiest foods

Mahaveer Suman and Prerak Bhatnagar

Abstract

Pomegranate a bioactive fruit being grown in tropical climatic conditions has various therapeutic benefits due to bioactive compounds in it. It is now being utilized in nutrition as well as medicinal industries due to its strong potential. It has potent nutritional values and immense health benefits. Pomegranate fruits, seeds and peels are intensively used in traditional medicine as a natural therapy. It contains plethora of valuable ingredients such as flavonoids, ellagitannin, punicalagin, ellagic acid, vitamins and minerals. The principal active constituents including punicalagins and ellagitannin are responsible for immeasurable health benefits due to its strong antioxidant activity. The pomegranate has been used since time immemorial in natural and holistic medicine therapies to treat sore throats, coughs, digestive disorders, urinary infections, skin disorders, arthritis, and to expel tapeworms. The health benefits reported word wide suggested that pomegranates might be useful in treating such serious conditions as different types of cancer, osteoarthritis, diabetes and Alzheimer. This is because pomegranates have the potential to thin the blood, increase blood flow to the heart, reduce blood pressure, and plaque in the arteries and ability to reduce bad cholesterol while increasing good cholesterol. Pomegranate juice can lower C-reactive proteins and reduce the inflammation of the lever. Juice of flower is used to treat nose bleeds. The fruit pulp and the seed are stomachic. Dried, pulverized flower buds are employed as a remedy for bronchitis. Research domain in this area is expanding rapidly and gaining popularity because of an understanding of the fact that naturally available phytonutrients along with polyphenols and antioxidants offer the available best protection against many diseases and disorders. Pomegranate protects our body in a cellular level. The objective of this review was to present a deep thought of the multi-functional, nutraceutical, bio-active components and medicinal effects of pomegranate in combating various human diseases, disorders and human ailments has been duly supported by various researchers in medicine, nutrition, biochemistry, physiology and horticultural sciences.

Keywords: PJ (Pomegranate juice), *Anti-oxidants, Punicalagin, Ellagic acid*

Introduction

Pomegranate (*Punica granatum L.*) is a shrub or small multi stem tree that grows approximately 16 to 26 feet tall. Pomegranate has multiple long-lived branches and its leaves are 2 cm wide and 3-7 cm long and flowers have 3-7 petals which are red in color. Fruit is berry like with a leathery rind (husk or peel) enclosing many seeds surrounded by juicy arils and seeds may vary from about 200 to 1400 in number. The husk is composed of two parts: pericarp and mesocarp (albedo). Pomegranate tree is one of the oldest domesticated tree for its countless health benefits, known even before the 21st Century. Folk medicines make use of all parts of this tree. Various researchers have identified about 153 phytochemicals including their derivatives in pomegranate. Polyphenols are the major class of phytochemicals, extracted from almost all parts of pomegranate tree, but are most abundant in fruits. Flavonoids, hydrolysable tannins and condensed tannins are the major pomegranate polyphenols. Anthocyanins, which impart red colour to the arils, are most abundant and responsible for potential health benefits. Pomegranate contain more than 18 hydrolysable tannins in leaves, bark and fruits, among which gallotannins, ellagitannins, punicalagin and punicalin have attracted most attention among researches, and which are pomegranate's most powerful antioxidants. Other phytochemicals reported in pomegranate include catechin and procyanidins, organic acids, phenolic acids, sterols, terpenoids, fatty acids, triglycerides, alkaloids and some other compounds. The prophylactic and curative potential of these bioactive compounds has been proved against cardiovascular diseases, hypertension, all types of cancers, inflammations, hyperlipidemia, diabetes, ageing, Alzheimer's disease, etc., and are, in addition, antibacterial, antifungal, antiviral, anthelmintic, vermicidal and molluscidal agents.

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Pomegranate juice contains 3-fold more antioxidants than green tea and red wine as well as several common fruits like apples, grapes, etc. Researchers have used various technologies to extract, purify and analyse these phytochemicals for chemical characterization and evaluation of their antioxidant capacities.

Nutritional value of pomegranate

Pomegranate is a rich source of these bioactive compounds. Nutrients content in a fruit may change during the development of the tree, during fruit maturation, under different environmental and cultivation conditions and between pomegranate cultivars. It is a diet full of nourishment for growing children, ageing/elderly or sick persons and pregnant women. It has the power to cure hunger, quench thirst and refresh the drunkard. The pomegranate fruits are consumed fresh or used for the preparation of fresh juice, jelly, jam, and paste and also for flavoring and coloring the culinary preparations. The fruit has been regarded as a "healing food" with numerous beneficial effects in several diseases. Pomegranate peel comprises about 50% of the total fruit weight and is an important source of minerals especially potassium, calcium, phosphorus, magnesium, and sodium; complex polysaccharides and high levels of diverse range of bioactive compounds such as phenolics, flavonoids, proanthocyanidin compounds and ellagitannin (ETs), as well as punicalagins and its isomers, as well as lesser amounts of punicalin, gallagic acid, ellagic acid, and ellagic acid glycosides. Pomegranate seed oil (PSO) contains an exceptional conjugated fatty acid called punicic acid (trienoic acid) that makes up approximately 65% to 80% of the oil from pomegranate seeds. Punicic acid is also referred as a super conjugated linolenic acid whose effect is even more potent than that of an ordinary conjugated linolenic acid. Seeds also contain protein, crude fibers, vitamins, minerals, pectin, sugars, polyphenols, isoflavones, the phytoestrogens, coumestrol and the sex steroid, estrone. The edible part of the pomegranate fruit (50%) consists of 40% arils and 10% seeds. The fresh juice contains 85% water, 10% total sugars, 1.5% pectin, ascorbic acid and polyphenolic flavonoids (El-Nemr *et al.* 1990)^[6]. The major sugars as reported by Ozgen (2008) were fructose (6.4 g/100 ml) and glucose (6.8 g/100 ml); the major acids were citric (1.78 g/100 ml) and malic (0.12 g/100 ml). They further reported that pomegranate is a rich source of Vitamin C, Vitamin K, Vitamin B₆ and pantothenic acid (Vitamin B₅). It also consists of Vitamin A, Vitamin E, thiamin and riboflavin in small amounts. In addition to these, niacin and folate are present in traces. Pomegranate is a rich source of minerals like potassium and copper and is very low in sodium, hence useful in the control of hypertension. Iron is present in small quantity, while traces of magnesium, phosphorus, zinc and selenium are also there. Ascorbic acid (vitamin C) content varies with varieties. The quality studies reported ascorbic acid content to vary between 52.8 to 72.0 mg/100 g fresh weight (fw) for arils and 76.8 to 118.4 mg/100 g fw for peels and it was found significantly higher in peels than the arils, with differences ranging from 24.4 to 97.0% depending on variety (Opara *et al.* 2009)^[14]. PJ is considered beneficial for diabetics, despite the juice containing significant sugar concentrations. The explanation given in support of this is that, "In most juices, sugars are present in free and harmful forms but in PJ, however, the sugars are attached to unique antioxidants – the polyphenols forming a complex, which actually make these sugars protective against atherosclerosis" (Rock *et al.* 2008)^[16]. Rozenbers *et al.*

(2006)^[17] concluded from their study that PJ consumption by diabetic patients does not worsen their diabetic parameters but contributes to serum paraoxonase-1 stabilization, increased association with HDL cholesterol, and enhanced catalytic activities, leading to retardation of atherosclerosis development in diabetic patients. The fruit is low in fats (< 0.12 g/100 g arils) and sweet varieties have almost half the fat content than that of bitter/ sour varieties (Hernandez *et al.* 2001)^[7].

Health benefits

Although many phytochemicals have positive effect on health, very many can be toxic and harmful, however, plants containing the most harmful phytochemicals are usually not treated as foods. The major health benefits of pomegranate are because of its anthocyanin contents, which are present in appreciably large quantities and have antioxidant properties. Andreu *et al.* (2008)^[3] recently reviewed composition of pomegranate fruit juice, beneficial health effects, including association with anti carcinogenicity and anti-inflammatory properties.

Antioxidant properties

Pomegranate possesses punicalagins which are extremely potent antioxidants found in pomegranate juice and peel. They are of so high potency that pomegranate juice has been found to be three times the antioxidant activity of red wine and green tea and eight times higher levels than those dictated in grapes, grapefruit, and orange juices, respectively. The pomegranate extract and powder is typically made from peel due to its high antioxidant and punicalagin content. The antioxidant agents reduce oxidative damage (damage due to oxygen) such as that caused by free radicals, which are highly reactive chemicals and attack molecules by capturing electrons and thus modifying chemical structures. Oxidative damage (oxidation) to the cells is partly responsible for the effects of ageing and certain diseases. Cells produce free radicals that are deficient in electron. In order to stabilize these free radicals react by taking electrons from certain key components in the cell and in the process, they damage these cells. Antioxidants divert this damage by donating electrons to the free radicals and stabilize them, thus, saving cell components from the scavenging effect of free radicals. The environment is also a source of free radicals caused by ultraviolet radiation or airborne pollutants, such as cigarette smoke. It is a well known fact that most free radical damage is repaired naturally; a fraction may however, remain unrepairs. This free radical damage may reduce or take over the body's natural defense. Punicalagin as a core element supports human health by protecting cells from being damaged by UV radiations by keeping more cells out of harm's way. Increase in cell damage with lapse of time leads to ageing and diseases like cancers, cardiovascular problems and some other problems. Polyphenols and vitamins, which have antioxidant properties, in one's diet help in countering some of the damage. Polyphenols especially anthocyanins and vitamins, present in appreciably large quantities in pomegranate can play an important role in benefiting human health.

Cancer cells

Cancer is caused as a result of disfunctioning of immense system due to multi-factors and numerous diseases involved in this process. In this vista, pomegranates fruits, seed and peels illustrate cancer preventive role and mechanism which

seems to be due to rich source of antioxidants. Pomegranate juice can hinder the proliferation of cancerous cells in human body. It can block the flow of blood towards the tumors resulting in reducing its size and ultimately its destruction. Breast and prostate cancer cells have significant evidence of slowing down the activities of malignant cells. Inhibition of cancerous cells causing prostate cancer in adults has been well recognized. Pomegranate extract also stops the proliferation of these dangerous cells to other parts of the body. Many phytochemicals have an anti- carcinogenic (anti-cancer) action as they slow cell proliferation (division) by interfering with the cell cycle, induce apoptosis (cell suicide) inhibit phase 1 enzymes – that convert harmless substances into carcinogens and induce phase 2 enzymes – enzymes that can attach carcinogens to molecules that facilitate speedy excretion (Best 2006)^[4]. The pomegranate extract has shown to induce programmed cell death and to inhibit tumor invasion, proliferation and angiogenesis. The fruit juice, peel and oil possess anticancer activities, including interference with tumor cell proliferation, cell cycle, invasion and angiogenesis (Lansky and Newman 2007)^[11]. A wide variety of plants produce phytoestrogen that are secondary metabolites. Controversies on use of pharmacological hormone replacement therapy (HRT) for the treatment of menopausal symptoms necessitates further studies and identification of natural sources of estrogen (Jordan 2003)^[8]. Pomegranate was identified the best known source for curing it. In fact, pomegranate tree is one of the only plants in nature known to contain estrone (Van Elswijk *et al.* 2004)^[21]. Because of its unique composition, seed-oil is a powerful health-benefiting agent, due to its antioxidative, anticancer, and antilipidemic properties. The estrogenic compounds luteolin, quercetin and kaempferol have been identified in pomegranate (Van Elswijk *et al.* 2004)^[21]. Other major components ellagic acid, caffeic acid and punicalic acid found in pomegranate fruit are having known anticancer activities.

Breast cancer

Extracts of pomegranate have anti estrogenic properties. Hormone levels in serum are well controlled by pomegranate extracts by inducing beneficial effects in blood plasma. The extracts from peel, seed, the seed oil and fermented/unfermented fruit juice all have suppressive effect on human breast cancer cells (Settheetham *et al.* 1995)^[19]. Bingham *et al.* (1998)^[5] had reported that diets rich in phytoestrogens protect against breast, prostate, colon cancer as well as cardiovascular disease and osteoporosis. The whole pomegranate seed oil is more chemopreventive for breast cancer and avoids the use of chemical agents to slow the development of cancer (Mehta and Lansky 2004)^[13]. The fermented PJ polyphenols in comparison to fresh juice recorded approximately twice the antiproliferative activity and inhibited cancerous lesion formation induced by a popular carcinogen (Kim *et al.* 2002)^[9]. Bioavailability and maximal oral intake of pomegranate supplements juice extracts for getting protein response in breast cancer. Tanner *et al.* (2008)^[20] in a study found that PJ may be a useful nutrient-based, non-chemotherapeutic treatment alternative for the inhibition of estrogen receptor negative breast cancer cell proliferations of feline and human breast cancer cell types. It can delay the onset of breast cancer by blocking aromatase enzymes and cancer cell differentiation.

Colon cancer

PJ, ellagic acid, punicalagin and pomegranate tannins show

apoptosis in specific colon cell and get rid of unneeded or abnormal cells. (Larrosa *et al.* 2006)^[12]. Punicalgin is freely bioavailable, body readily breaks down and as an antioxidant, it helps to neutralize free radicals. Pomegranate seed oil which is composed of 70% of conjugated linolenic acid, suppress colon carcinogenesis hence, dietary pomegranate seed oil significantly inhibits incidence of adenocarcinomas (Kohno *et al.* 2004)^[10]. In a study by Saruwatari *et al.* (2008)^[18], the results indicated that the inhibition of sulfotransferase activity by punicalagin in Caco-2 cells was responsible for the reductions seen in 1-naphthyl sulfate accumulation. They also suggested that constituents of PJ, most probably punicalagin, impair the enteric functions of sulfoconjugation and that this might have positive effects upon the bioavailability of drugs and other compounds present in food and in the environment. These effects might be due to the anticarcinogenic properties of PJ. Punicalgin is an ellagitannin, a type of phenolic compound which is responsible for more than 50% of the juice potent's antioxidant activity. The maximum levels of ellagic acid are present in pomegranate and anticarcinogenic properties of ellagic acid has been found the probable anti-inflammatory role in the treatment of lacerative colitis as to prevent the development of colon cancer.

Skin cancer

Exposure to ultraviolet (UV) radiation has been associated with several acute and chronic conditions. The UV-B component may cause sunburn, hyperpigmentation, edema, hyperplasia, immunosuppression, photo ageing, and skin cancer whereas UV-A may be responsible for tumor formation. The biochemicals delphinidin, cyanidin and pelargonidin, (anthocyanidins), Punicalin, pedunculagin, punicalagin and gallagic and ellagic acid esters of glucose (hydrolysable tannins) in pomegranate are strong antioxidants and antiinflammatory agents these compounds which account for 92% of the antioxidant of the whole fruit (Afaq *et al.* 2005)^[2], protect from ultraviolet radiation. Pomegranate fruit extract contains antioxidants and anti-inflammatory phytochemicals that can treat human epidermal keratinocytes. Fruit extracts provided protection against Ultraviolet A mediated activation of signal transducers and activators of transcription.

Cholesterol level

The phytosterols in pomegranate both lower the existing LDL cholesterol and cease the production of bad cholesterol. Pomegranate is rich in major antioxidants like anthocyanins and tannins that may help to block the buildup of cholesterol in arteries which in return protect heart damage. Juice of this fruit may help to reduce the concentration of low density lipoproteins from the body which may protect the body from stroke attack. Energy and body HDL levels decreased showing beneficial effects of leaf extract of pomegranate. Pomegranate juice decreased cholesterol absorption, increased faecal excretion of cholesterol, had a favourable effect on enzymes concerned in cholesterol metabolism, drastically reduced LDL cholesterol, and improved LDL/HDL cholesterol and total/HDL ratios (Esmaillzadeh *et al.*, 2006)^[1]. Pomegranate juice consumption decreased LDL susceptibility to aggregation and retention and increased the activity of serum paraoxonase. Pomegranate juice improve cholesterol profiles and treat the atherosclerotic plaque that are responsible for many heart attacks and many strokes. All these evidences suggest the potential cardioprotective effect of pomegranate fruit.

Blood pressure

Systolic blood pressure can be reduced by juice of pomegranate to a significant level. Potassium in it can prevent arteries from stiffness and atherosclerosis. It improves blood flow to the heart and reduces the incidents of heart attack. Clinical examination of improving blood pressure and endothelial functions of the body by consuming pomegranate juice have been performed. Chronic administration of pomegranate juice extract showed reduction in the mean arterial blood pressure and vascular reactivity changes to various catecholamines. The potent flavonoids such as catechus, tannic and ellagic acid, make pomegranate a stronger antioxidant than red wine and equal to or better than green tea.

Memory and mood enhancer

Flavonoids are believed to improve memory from declining and delay the onset of Alzheimer disease. These antioxidants have potential role for fighting against depression that can cause memory loss. Vinegar of pomegranate juice contains flavonoids of deep red color that helps prevent against radical formation. Pomegranate juice have natural compound Estrone that improves mood in menopausal women. It is generally believed that this compound can act as a replacement therapy for women suffering from mood disturbances.

Arthritis and Joint Pain

The foods that are rich in nutrients, minerals and antioxidants may help to neutralize rheumatoid arthritis. Pomegranate is low in fat, cholesterol and sodium but rich in nutrients, minerals and antioxidants. The studies suggested the extract of pomegranate may block production of cartilage destroying enzyme. Inflammation of joints can result in Pain and swelling in later age. Presence of Flavones in pomegranate extract has anti-inflammatory effects that may reduce collagen induced arthritis and painful swelling of joints. Cyclooxygenases and lipoxygenase enzymes inhibits by extracts present in pomegranate seed oil. These key mediators of inflammation decreases 75 percent by seed oil while pomegranate juice causes 23.8 percent reduction in these enzymes. In osteoarthritis these extracts diminishes matrix metallo-proteinases which is involved in degradation of extracellular joint matrix. It prevents collagen degradation and stops joint destruction.

Bacterial infections

Antibiotics are effective remedy in the inhibition of bacteria growth or growth of microorganism. The recent surge in multi-drug resistant bacteria and the likely chance of widespread global pandemics necessitate the need for additional therapeutic options to counteract conventional drugs. On the other side, antibiotics resistance against microorganism is one of the major problems in the use of antibiotics against microorganism. Pomegranate has millions of bioactive compounds fighting against infections like diarrhea and ulcers. Nearly every part of pomegranate plant including ingredients of seeds, flowers, stem, bark and leaves show very effective role in the inhibition of growth of pathogens. Plants are one of the good sources of secondary metabolites including tannins, terpenoids, alkaloids, flavonoids and glycosides, which confirmed antimicrobial activities. Ellagic and gallic acids as natural antimicrobial agents has been used against *Staphylococcus aureus* and *Escherichia coli* for their ability to precipitate membrane proteins and inhibit enzymes that leads to lysis of cells.

Preservation and/or enhancement of probiotic bacteria in the gut is important for maintaining gastrointestinal health.

Hypercholesterolaemia

A recent study shows that peel of pomegranate has prebiotic potential for lowering down the fat content of the body. It reduces the cholesterol of high fat diet over a period of 4 weeks and gut microbiota supports its action. Polyphenols present in pomegranate has the ability to prevent atherosclerosis and macrophage formation. This can be done by either direct combination of polyphenols with lipoproteins of the body or by indirect means through accumulation in arterial microphages. This in return blocks the synthesis of oxygen species and lipid peroxidation and lipid rich microphages. It also results in hydrolysis of lipids in atherosclerotic lesions.

Immune system

The benefits of pomegranate can be potentially utilized for the people of lesser development countries. Various health conditions related to throat and respiration can be resolved by pomegranates. As this fruit is rich in iron content it ensures normal platelet count of the body by which a person feels less fatigue symptoms. These fruits are often recommended for liver prevention. Pomegranate juice helps in the reduction of platelet aggregation and oxidative stress in humans. Pomegranate juice inhibits aggregation and oxidation of atherosclerotic lesion and attenuate platelet activation. Pomegranate exerts bactericidal activity against food and water borne pathogenic bacteria.

Tooth loss

The flavonoids present in pomegranate exerts beneficial action which are conducive to oral health. Pomegranate juice is effective against dental plaque microorganisms decreasing the CFU. Additionally, there was significant reduction in the level of dental plaque microorganisms after the rinsing with pomegranate juice. Dental plaque and tooth loss can be prevented by seeds of this fruit. It possesses anti-microbial functions which can be helpful against oral bacteria. Health of gums can be improved by its seeds. It has been demonstrated by performing a study regarding the ability of peel or pulp of pomegranate richness in antioxidants. Flavonoids and phenolic compounds were also high in peel extract that were beneficial against oxidation and atherosclerosis and can be used as a natural supplement.

Anti-diabetic effect

A range of studies evidences that medicinal plants or constituents of medicinal plants show role in the management of diabetes and its complication including Diabetic retinopathy. By various studies it has been confirmed that by giving 200 mg of peel extract according to per kg of the body weight, polyphenols present in it affects glycemic index of the body by either inhibiting the uptake of glucose to peripheral tissues or by blocking its absorption by the gut. Administration of crude powder of *Punica granatum* husk decreased the concentration of glucose, triglycerides, cholesterol, LDL cholesterol and raised the level of HDL cholesterol and hemoglobin content in the blood. The well known compounds in pomegranate like punicalagin acid, ellagic, gallic, olaenolic, ursolic and uallic acids have been recognized as having antidiabetic actions.

Pregnancy outcomes

Pomegranate is rich in antioxidants so they protect placenta from oxygen reactive species. They also contain little amount of folate that helps fight against birth defects. It can help in the purification of blood and can enhance milk production. Acidity of urine reduces by the intake of pomegranate juice and improves health of urinary bladder by preventing harmful bacteria from entering into the urinary tract. This prevents inflammation and enhances immunity of the body. The intake of pomegranate juice decreases placental oxidative stress *in vivo* and *in vitro* and may limit placental injury and thus confer protection to the exposed foetus.

Effect on reproductive system

Pomegranate shows pivotal role in the elevation of hormones linked to reproductive system. Pomegranate juice showed elevation in testosterone, luteinizing hormone and follicle stimulating hormone depleted after the injection of carbon tetrachloride (CCL4). Pomegranate extract and ascorbic acid administration reduced the deleterious effect of lead acetate on daily sperm production and epididymal sperm number. PJ consumption showed increase in epididymal sperm concentration, sperm motility and spermatogenic cell density. Long-term pomegranate juice intake increased intracavernous blood flow, improved erectile response and smooth muscle relaxation in erectile dysfunction. Pomegranate increases testosterone production which is primarily responsible for men's health both inside and outside. Pomegranate augments nitric oxide; a vital ingredient for getting an erection pomegranate tends to increase it most.

Conclusion

Pomegranate is a pivotal source of all essential nutrients and rich in polyphenols particularly Ellagitannins (ET) and ellagic acid (EA) which can be digested easily and refreshes physically or mentally tired people from regular intake. All parts of this fruit have been utilized in food industry for prevention and treatment of various diseases. It has been regarded best vis a vis green tea due to its strong antioxidant potential. The biological screening of *Punica granatum* extracts and compound have shown antioxidant, antiperoxidative, antibacterial, inflammation, antitumor, hepatoprotective, antiarthrogenic and antidiarrhoeal. It shows no toxic effects to liver and other organs of pomegranate extract and can be used as natural supplement. However, it is the dire need of the time to promote its cultivation by traditional and non-traditional means in order to gain benefits of this fruit as well as to compete in global market for its export. In addition to this more conclusive studies and experiments are needed to evaluate effects of pomegranate in treating diarrheal diseases and in preventing fungal proliferation. The immense potential of this fruit in overcoming infections can bring tremendous progress in nutritional and therapeutic industries. The available research offers substantial supplementation and evidence to enrich the diet with pomegranate juice or extract to harness the benefits for protecting us from important stresses and diseases particularly diabetics, development of cancer and improvement of oral health and skin texture. The wide array of benefits can be utilized by inclusion of pomegranate in dietary schedule.

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Review Article

Pomegranate Fruit as a Rich Source of Biologically Active Compounds

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Pomegranate is a widely used plant having medicinal properties. In this review, we have mainly focused on the already published data from our laboratory pertaining to the effect of methanol extract of pericarp of pomegranate (PME) and have compared it with other relevant literatures on *Punica*. Earlier, we had shown its antiproliferative effect using human breast (MCF-7, MDA MB-231), and endometrial (HEC-1A), cervical (SiHa, HeLa), and ovarian (SKOV3) cancer cell lines, and normal breast fibroblasts (MCF-10A) at concentration of 20–320 $\mu\text{g}/\text{mL}$. The expressions of selected estrogen responsive genes (PR, pS2, and C-Myc) were downregulated by PME. Unlike estradiol, PME did not increase the uterine weight and proliferation in bilaterally ovariectomized Swiss-Albino mice models and its cardioprotective effects were comparable to that of 17 β -estradiol. We had further assessed the protective role of PME on skeletal system, using MC3T3-E1 cells. The results indicated that PME (80 $\mu\text{g}/\text{mL}$) significantly increased ALP (Alkaline Phosphatase) activity, supporting its suggested role in modulating osteoblastic cell differentiation. The antiosteoporotic potential of PME was also evaluated in ovariectomized (OVX) rodent model. The results from our studies and from various other studies support the fact that pomegranate fruit is indeed a source of biologically active compounds.

1. Introduction

Punica granatum L. (Punicaceae) is a nutrient dense fruit rich in phytochemical compounds [1]. Plants produce low molecular weight compounds which are broadly called phytochemicals, usually as a mechanism of defence. Some plants contain distinct families of phytocompounds, which are structurally similar to steroid hormone, 17 β -estradiol (E2) and compete with the endogenous hormone for binding to estrogen receptor (ER), thus reducing the hormonal effect of endogenous estrogens [2–4]. These compounds are termed as phytoestrogens. Most of these phytoestrogens present in the diet are inactive compounds, which, on consumption, go through series of enzymatic changes in the gastrointestinal tract, resulting in the formation of compounds having structure similar to that of estrogens [5]. Phytoestrogens have captured major research and clinical attention due to its effectiveness in the prevention and treatment of perimenopausal and

menopausal symptoms, over hormone replacement therapy (HRT) [6]. They may act both as agonists and/or antagonists in a site-specific manner, similar to the hormonal action of selective estrogen receptor modulators (SERMs) [7–9]. It can also function as antioxidants and protect DNA from oxidant-induced damage [10]. Research on pomegranate is gaining momentum due to its tremendous nutritional values and medicinal uses. The current review focuses on the use of pomegranate as a phytoestrogen rich and nutraceutical fruit with emphasis to the work done in our laboratory using methanolic extract of pericarp of pomegranate (PME).

2. Chemical Constituents of Pomegranate Fruit and Tree

The chemical composition of the fruits differs depending on the cultivar, growing region, maturity, cultivation practice,

TABLE 1: Principal constituents of different parts of pomegranate tree and fruit. The different parts of pomegranate plant like peel, root, bark, flower, leaves, and so forth exhibit different phytochemicals.

Pomegranate peel	Pomegranate juice	Pomegranate root and bark	Pomegranate flower	Pomegranate leaves	Pomegranate seed
(i) Gallic acid (ii) Ellagic acid (iii) Punicalin (iv) Punicalagin (v) Caffeic acid (vi) Ellagitannins (vii) Pelletierine alkaloids (viii) Luteolin (ix) Kaempferol (x) Quercetin	(i) Simple sugars (ii) Aliphatic organic acids (iii) Gallic acid (iv) Ellagic acid (v) Quinic acid (vi) Flavonols (vii) Amino acids (viii) Minerals (ix) EGCG (x) Ascorbic acid	(i) Ellagitannins (ii) Piperidine alkaloids (iii) Pyrrolidine alkaloid (iv) Pelletierine alkaloids	(i) Gallic acids (ii) Ursolic acid (iii) Triterpenoids (iv) Fatty acids	(i) Carbohydrates (ii) Reducing sugars (iii) Sterols (iv) Saponins (v) Flavanoids (vi) Tannins (vii) Piperidine alkaloids (viii) Flavone (ix) Glycoside (x) Ellagitannins	(i) 3,3'-Di-O-methyllellagic acid (ii) 3,3',4'-Tri-O-methyllellagic acid (iii) Punicic acid (iv) Oleic acid (v) Palmitic acid (vi) Stearic acid (vii) Linoleic acid (viii) Sterols (ix) Tocopherols (x) Sex steroids
References [20–26]	References [15, 20, 26–30]	References [21, 23]	References [31–33]	References [21, 22, 34, 35]	References [36–41]

climate, and storage circumstances [11]. About 50% of the total fruit weight corresponds to the peel, which is an important source of bioactive compounds such as phenolics, flavonoids, ellagitannins, and proanthocyanidin compounds, minerals, mainly potassium, nitrogen, calcium, phosphorus, magnesium, and sodium, and complex polysaccharides. The edible part of the pomegranate fruit (50%) consists of 40% arils and 10% seeds. Arils contain 85% water, 10% total sugars, mainly fructose and glucose, and 1.5% pectin, organic acid, such as ascorbic acid, citric acid, and malic acid, and bioactive compounds such as phenolics and flavonoids, principally anthocyanins [12]. The seed cover of the fruit contains delphinidin-3-glucoside, cyanidin-3-glucoside, delphinidin-3,5-diglucoside, cyanidin-3,5-diglucoside, pelargonidin-3,5-diglucoside, and pelargonidin-3-glucoside with delphinidin-3,5-diglucoside being the main anthocyanin in pomegranate juice [13]. 12–20% of total seed weight of pomegranate comprises seed oil and is self-possessed with more than 70% of the conjugated linolenic acids. The fatty acid component of pomegranate seed oil comprises over 95% of the oil, of which 99% is triacylglycerols. Minor components of the oil include sterols, steroids, and a key component of mammalian myelin sheaths, cerebroside [14, 15]. Interestingly, punicic acid, which is a conjugated isomer unique to pomegranate oil, constitutes 70–76% of the seed oil [16]. Phenolic compounds, together with flavonoids, anthocyanins, and tannins, are the main group of antioxidant phytochemicals that are important due to their biological and free radical scavenging activities [17]. Phenolic acids, flavonoids, and tannins are present in different parts of pomegranate fruit and this may be one of the reasons why many of the studies demonstrated that combinations of pomegranate extracts from different parts of the fruit were more effective than a single extract [18]. In a comparative analysis, anthocyanins from pomegranate fruit were found to possess higher antioxidant activity than vitamin-E (α -tocopherol), β -carotene, and ascorbic acid [19]. Table 1 represents the key constituents of pomegranate fruit and tree [20–41].

3. Therapeutic Functions of Pomegranate

Extracts of all parts of the pomegranate fruit exhibit therapeutic properties [15] and target a range of diseases including cancer, cardiovascular disorders, diabetes, male infertility, Alzheimer's disease [42], aging, and AIDS [43] (Figure 1). Although pomegranate's extensive therapeutic benefits may be attributed to a number of mechanisms, most researchers have determined its antioxidant, anticarcinogenic, and anti-inflammatory properties. Various therapeutic applications of *Punica granatum* are discussed here.

3.1. Cancer. Research on breast cancer cell lines demonstrated that pomegranate constituents efficiently inhibited angiogenesis [44], invasiveness [40], growth [45], and induced apoptosis [46]. Its anti-invasive, antiproliferative, and antimetastatic effects were attributed to the modulation of Bcl-2 proteins, upregulation of p27 and p21, and downregulation of cyclin-cdk network [47]. Pomegranate constituents inhibit angiogenesis via downregulation of vascular endothelial growth factor (VEGF) in human umbilical vein endothelial and MCF-7 breast cancer cell lines [44], thereby hampering the tumor growth. Prostate cancer cells, when treated with pomegranate juice, increased adhesion and decreased the migration. Molecular analyses revealed that pomegranate juice increased the expression of cell-adhesion related genes and inhibited the expression of genes involved in cytoskeletal function and cellular migration. It would possibly affect prostate cancer because of its apoptotic, antioxidant, antiproliferative, and anti-inflammatory properties, suggesting that it may be beneficial in slowing down or preventing cancer cell metastasis [48]. The application of pomegranate extract to the skin of mice before they were exposed to a carcinogenic agent was shown to inhibit the appearance of erythemas and hyperplasia and the activity of epithelial ornithine decarboxylase [49]. An *in vivo* study in TRAMP mice model suggested that oral supplementation of

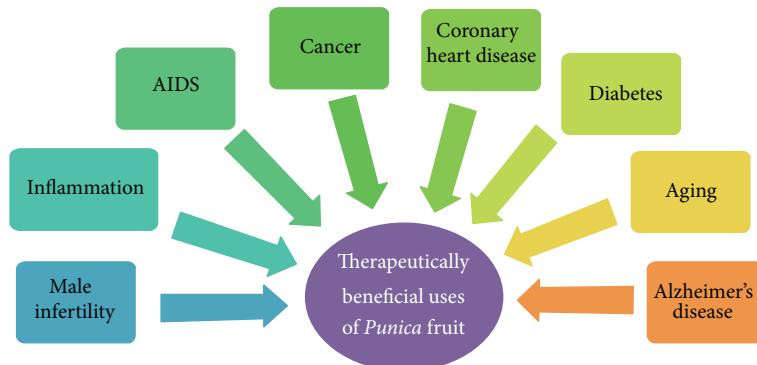
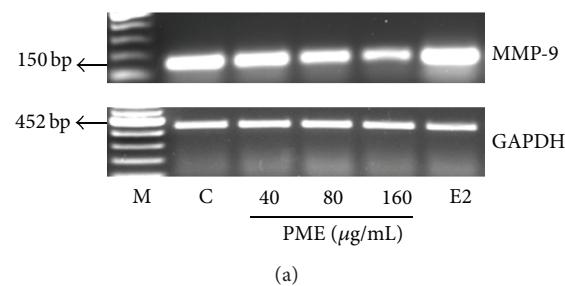
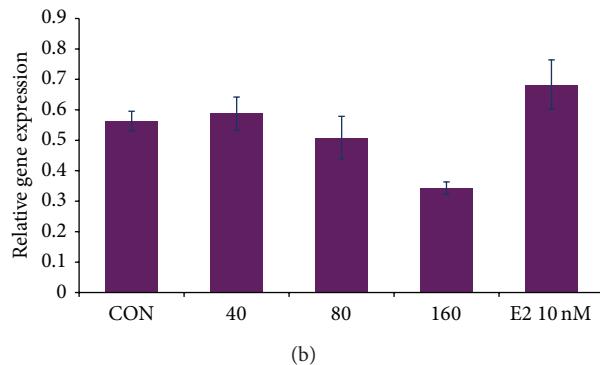


FIGURE 1: Therapeutically beneficial uses of *Punica* fruit. Pomegranate fruit has been proven to act against various diseases like cancer, cardiovascular disorders, diabetes, AIDS, and Alzheimer's disease.



(a)



(b)

FIGURE 2: Effect of PME on MMP-9 transcription in MCF7 cells. (a) MCF7 cells were incubated with PME (40, 80, and 160 μ g/mL) and E2 (10 nM) for 24 hrs and semiquantitative RT-PCR was done. (b) shows the ratio of density of MMP-9 gene expression to that of endogenous control GAPDH and it represents mean \pm SE of 3 replicates (* $P < 0.05$).

pomegranate fruit extract inhibited metastasis and increased overall survival [50].

Matrix metalloproteinases (MMPs) are good markers of tumor cell invasion and migration [51]. Phytochemicals have been shown to target the activity and secretion of MMPs in estrogen responsive cancers [52]. Constituents of pomegranate minimize tumor cell invasion into normal tissue and metastasis to distant sites and these actions develop due to the inhibition of selected metalloproteinase activity, decreased focal adhesion kinase activity, and reduced VEGF expression [15]. With semiquantitative RT-PCR, we had found out that PME downregulated the transcription of MMP-9 suggesting its possible role in the inhibition of tumor invasion (Figure 2) whereas E2 (10 nM) did not significantly affect the transcription of MMP-9 [53] which correlated with

earlier studies suggesting that estrogen stimulated MMP-9 secretion without increasing its gene transcription [54].

We had assessed the estrogenicity/antiestrogenicity of PME in a panel of *in vitro* biological assays and the expression of endogenous estrogen sensitive markers (pS2 and PR) in breast carcinoma cell lines were analyzed [53]. When MCF-7 cells pretreated with PME were treated with estrogen, the c-Myc expression was not induced as much as when treated with estrogen alone, demonstrating the effect of PME in estrogen regulated mechanism (Figure 3). ER positive cells treated with PPT (4,4',4''-(4-Propyl-(1*H*)-pyrazole-1,3,5-triyl)trisphenol) (ER α selective agonist) and DPN (Diarylpropionitrile) (ER β selective agonist) clearly showed that PPT increased the pS2 protein levels, whereas DPN did not produce any significant effect. When given in

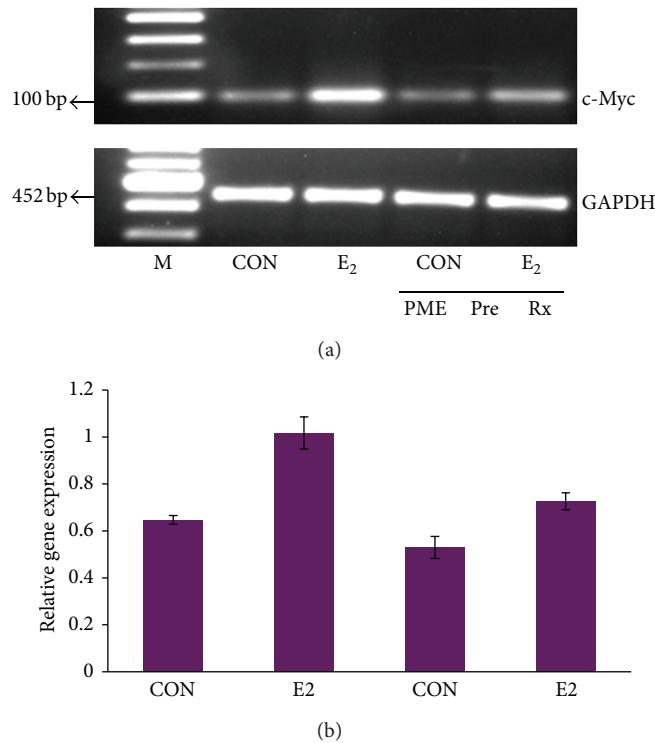


FIGURE 3: Effect of PME pretreatment on E2 induced expression of c-Myc. (a) MCF7 cells were pretreated with 100 nM E2 for 4 hrs, with or without PME pretreatment (80 μ g/mL) for 12 hrs and RT-PCR was done. (b) shows the ratio of density of c-Myc gene expression to that of endogenous control GAPDH and it represents mean \pm SE of 3 replicates (* $P < 0.05$).

combination with PPT, PME reduced the pS2 protein levels indicating the role of ER α in mediating the effects of PME on pS2 expression (Figure 4). Thus the effect of PME on expression of pS2 was mediated by ER α and not by ER β [53].

Pomegranate fruit extract was revealed to inhibit UV-B-mediated phosphorylation of mitogen-activated protein kinase (MAPK) and nuclear factor NF- κ B activation [55]. Pomegranate juice almost downregulated the TNF α induced Akt (protein kinase B) activation required for NF- κ B activity [56]. Koyama et al. [57] examined the effects of pomegranate extract (POMx) on the IGF system and found out cell growth inhibition and apoptosis. Their findings suggested that POMx treatment reduced mTOR phosphorylation at Ser2448 and Ser2481, whereas IGFBP-3 increased phosphorylation at those sites. These results suggested that POMx decreased prostate cancer cell survival by inhibiting IGF1 expression. To conclude, pomegranate fruit has anticancer properties that can be attributed to different mechanisms.

3.2. Cardiovascular Disorders. *In vitro, in vivo* and human trials had examined the effects of a range of pomegranate constituents on the prevention and reduction of atherosclerosis and LDL oxidation [58]. Evidence suggested that polyphenolic antioxidants contained in pomegranate juice can cause reduction of oxidative stress and atherogenesis through the activation of redox-sensitive genes ELK-1 and p-JUN and increased eNOS expression. Their results indicated that

proatherogenic effects induced by disturbed shear stress can be reversed by constant administration of pomegranate juice [59]. Pomegranate juice consumption for 3 years by patients with carotid artery stenosis reduced common blood pressure, LDL oxidation, and carotid intima-media thickness [60]. Azadzoi et al. demonstrated that 8-week administration of pomegranate juice concentrate daily in a rabbit model of arteriogenic erectile dysfunction significantly increased intracavernous blood flow and smooth muscle relaxation, probably via its antioxidant effect on enhanced nitric oxide preservation and bioavailability [61]. A pilot study in type 2 diabetic patients with hyperlipidemia found that concentrated pomegranate juice decreased cholesterol absorption, increased faecal excretion of cholesterol, had a favourable effect on enzymes concerned in cholesterol metabolism, drastically reduced LDL cholesterol, and improved LDL/HDL cholesterol and total/HDL ratios [62]. Aviram et al. analyzed atherosclerotic lesion size, antioxidant activity, blood sugar, peritoneal macrophages, oxidative status, and lipid profiles for 3 months after giving 6 different pomegranate preparations with varying amounts of total polyphenols and gallic acid content in atherosclerotic apolipoprotein-E deficient mice and found that pomegranate phenolics and pomegranate unique complexed sugars could mimic the antiatherogenic effects of pomegranate extracts [63]. All these evidences suggest the potential cardioprotective effect of pomegranate fruit.

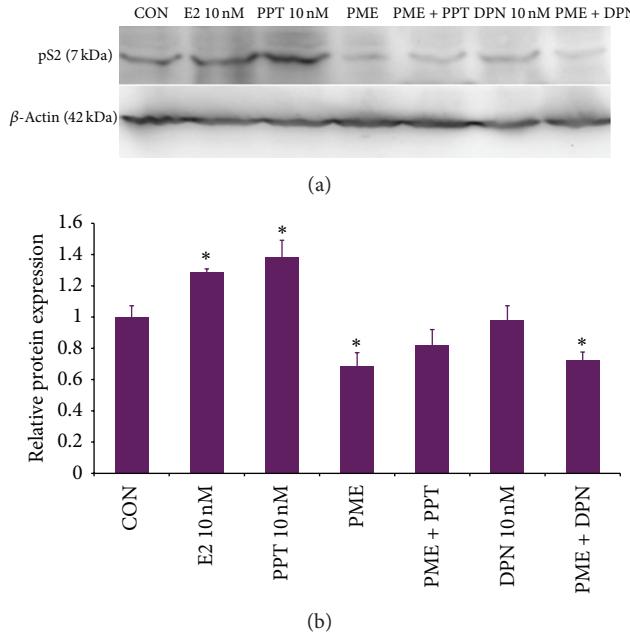


FIGURE 4: Effect of PME on pS2 protein expression in MCF7 cells. (a) Immunoblot result of MCF7 cells treated with 10 nM E2, PME (80 µg/mL), 10 nM PPT, 10 nM DPN, and PME (80 µg/mL) with 10 nM PPT or 10 nM DPN for 48 hrs. (b) Data are presented as densitometric ratio relative to nontreated control (CON) cells. The values are mean ± SE of 3 separate experiments (* $P < 0.05$).

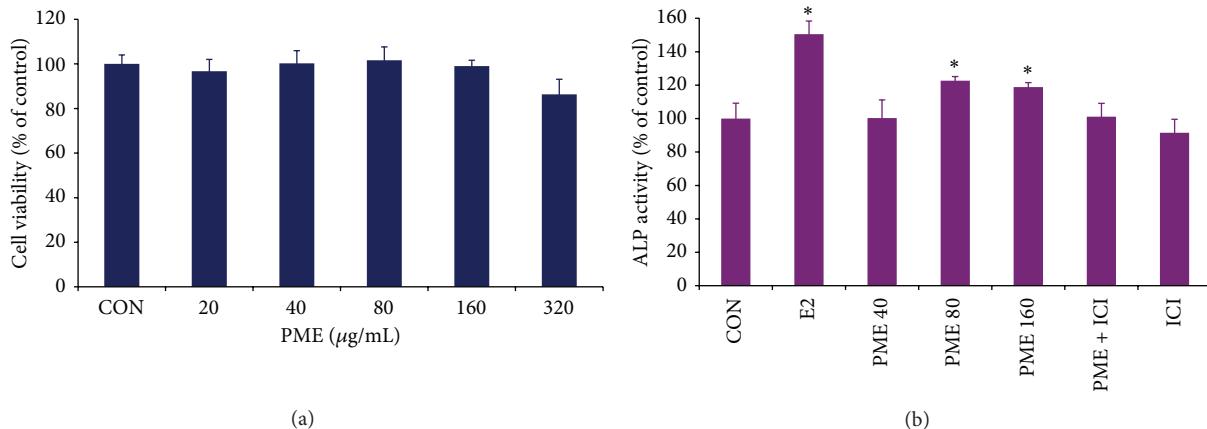


FIGURE 5: PME increased ALP activity in MC3T3-E1 osteoblast without affecting cell proliferation. (a) Dose-dependent effect of PME on cell proliferation. MC3T3-E1 osteoblasts were treated with 0 (control), 20, 40, 80, 160, and 320 µg/mL of PME for 48 hrs and the cell proliferation was determined by MTT assay. The cell proliferation was expressed as percentage cell viability over the untreated control. (b) Cells were treated with E2 (10 nM), PME (40, 80, and 160 µg/mL), ICI (1 µM) with or without PME (80 µg/mL) for 48 hrs. ALP activity was then measured and results are expressed as percentage over the untreated control. Results are expressed as mean values ± SE of five replicates. * $P < 0.05$ when compared to untreated control.

3.3. Antosteoporotic Potential. Tissue selective estrogen agonist/antagonists are currently being investigated as alternatives to estrogen in the prevention and treatment of postmenopausal osteoporosis [64–66]. Bone loss after ovariectomy is associated with high bone turnover where bone resorption rate exceeds the bone formation rate [67]. To assess the protective role of *Punica* on skeletal system, we had examined the effect of PME on a well-characterized osteoblastic cell population (osteoblastic MC3T3-E1 cells) and examined its effect on Alkaline Phosphatase (ALP),

which is a commonly used bone remodelling marker. The results (Figure 5) indicated that PME significantly increased ALP activity, supporting its suggested role in modulating osteoblastic cell differentiation [68].

Ovariectomized rodent model is a well-established system for estrogen deficiency induced bone loss and used by researchers previously [69, 70]. We had evaluated the antosteoporotic potential of the extract in estrogen deficiency induced osteoporosis in young adult mice of 6–8 weeks of age by assessing the bone turnover by serum ALP. In

TABLE 2: Effect of E2, PME, and tamoxifen on chosen markers of bone metabolism of ovariectomized mice. Serum calcium, phosphorus, and Alkaline Phosphatase (ALP) levels of sham control (SS CON) and Ovx mice exposed to 0.1% ethanol (vehicle control), E2 (1 mg/kg bwt), PME (50, 100 mg/kg bwt), and tamoxifen (TAM, 10 mg/kg bwt) for 7 days (bwt = body weight). Data are expressed as mean \pm SE ($n = 5$).

	Sham control	OVX control	E2 (1 mg/kg bwt)	PME (50 mg/kg bwt)	PME (100 mg/kg bwt)	TAM (10 mg/kg bwt)
Calcium (mg/dL)	9.46 \pm 0.313	10.94 \pm 1.18	8.188 \pm 0.7040 ^a	8.5 \pm 0.707	9.09 \pm 0.194	9.908 \pm 0.165
Phosphorus (mg/dL)	7.43 \pm 0.63	8.818 \pm 0.698	8.026 \pm 1.066	7.516 \pm 1.731	8.78 \pm 1.980	8.146 \pm 0.0680
ALP (U/L)	140.6 \pm 11.28	181.8 \pm 34.07 ^a	115.2 \pm 23.61 ^b	130.4 \pm 12.77	120 \pm 9.02 ^b	134.6 \pm 17.54 ^b

^a $P < 0.05$ versus sham control, ^b $P < 0.05$ versus ovx control.

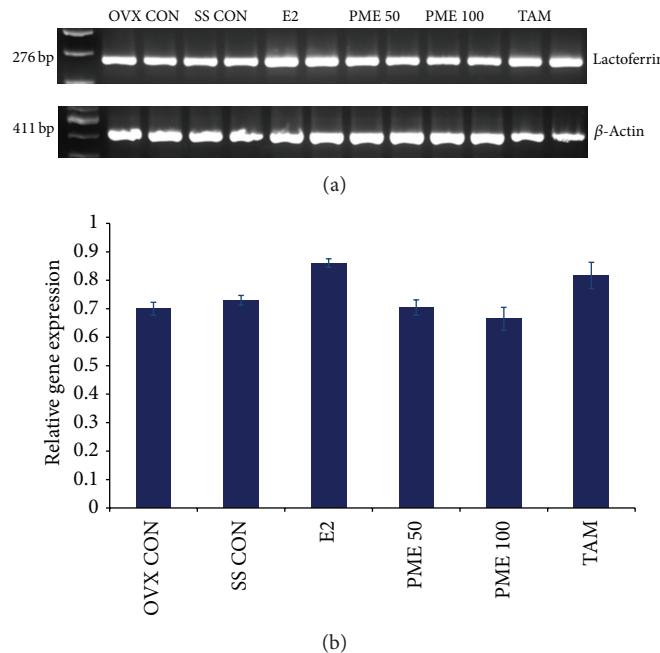


FIGURE 6: PME did not alter lactoferrin expression in murine uterus. (a) RT-PCR detection of Lactoferrin m-RNA in sham control (SS CON) and ovariectomized mice exposed to 0.1% ethanol (vehicle control, Ovx CON), E2 (1 mg/kg bwt), PME (50, 100 mg/kg bwt), and tamoxifen (TAM, 10 mg/kg bwt) for 7 days. (b) shows the ratio of density of target gene expression to that of endogenous control beta-actin and it represents mean \pm SE of three replicates.

comparison to the sham surgery (SS) control, ovariectomized (Ovx) control animals showed an increase in ALP activity indicating an increase in bone turn-over rate in these animals. PME in higher concentration was found to be effective in decreasing this bone turnover, though E2 was better in controlling the accelerated bone turnover (Table 2). The experimental model differed from aged Ovx mice wherein the osteoporosis is induced only by estrogen deficiency and not by a combination of natural bone loss due to age and ovarian hormone deficiency. An increase in bone turn-over rate was indicated by higher serum ALP level in the Ovx group compared to the SS control group. Therefore, high rate of bone turnover was well corrected by PME suggesting that it might play a protective role against ovarian hormone insufficiency related bone resorption. But E2 as well as PME was able to significantly decrease ALP levels in Ovx mice (Table 2). Serum calcium and phosphorous levels in Ovx control, PME treated, and tamoxifen treated animals were similar to that of SS control animals. Significant decrease in calcium levels

was observed in E2 treated animals in comparison to SS control (Table 2). Our findings clearly indicated that the possible bone preserving effect of PME is almost comparable with E2 [53]. Earlier studies had shown that an acute or chronic exposure to xenoestrogens or dietary phytoestrogens alters uterine expression of estrogen sensitive genes in mice [71]. So in order to check whether PME has any effect, a semiquantitative RT-PCR was done to analyze uterine mRNA levels of lactoferrin in ovariectomized mice fed with PME for 7 days. Lactoferrin is a well-known estrogen target gene and a biologically active molecule for bone regeneration [72]. The positive control E2 increased the uterine accumulation of lactoferrin mRNA in Ovx animals compared to the vehicle treated Ovx control (Figure 6). Lactoferrin expression did not differ significantly between the groups that received PME (50, 100 mg/kg bwt) and the vehicle (0.1% ethanol) treated Ovx control group, indicating the lack of estrogenicity of PME on uterine endometrium in the doses tested in our study. Tamoxifen (10 mg/kg bwt) was found to increase the

expression of lactoferrin, though not significantly [68]. As there are promising results from both *in vitro* and *in vivo* studies, we suggest evaluating the antiosteoporotic potential by clinical trials with pomegranate fruit extract that has no side effects on uterine endometrium alongside a significant decrease in bone turn-over rate.

3.4. Other Clinical Applications. *In vitro* assay showed that fermented pomegranate juice extract is better than red wine and comparable to green tea [37]. There were also reports that pomegranate juice possessed considerably greater antioxidant capacity at much lower concentrations (>1000-fold dilutions) than either grape or blueberry juice [73]. *Punica granatum* peel extract decreased lipid peroxidation in hepatic, cardiac, and renal tissues and at the same time it had a facilitatory effect on the scavenging capability of superoxide anion and hydrogen peroxide [74]. Formerly, it was shown that pomegranate peel extract supplementation alleviated oxidative damage of the liver and enhanced the hepatic structure and function in rats exposed to bile duct ligation [75]. Pretreatment of carbon tetrachloride-induced liver damage in rats with pomegranate peel extract resulted in the reduction of lipid peroxidation and at the same time, the free-radical scavenging activity of catalase, superoxide dismutase, and peroxidase were considerably enhanced [76]. Many studies had keenly explored the anti-inflammatory properties of pomegranate fruit [15, 77–79]. Studies indicated that pomegranate extract inhibited PMACI-induced proinflammatory cytokines assembly by inhibiting the gene expression. This is achieved by blocking JNK and ERK-MAPK activation and NF- κ B activation in human KU812 cells [80]. Larrosa et al. showed that pomegranate extract supplementations led to reduced prostaglandin E2 (PGE2) levels in the colon mucosa by downregulating the overexpressed COX-2 and prostaglandin E synthase (PTGES) levels owing to the action of ellagic acid [78]. *Punica granatum* extract had been found to be particularly effective for controlling oral inflammation, dental plaque, and bacterial and fungal counts in periodontal disease and *Candida*-associated denture stomatitis [81, 82]. Another study proposed that inhibition of number of signal transduction pathways and the downstream pathogenic cellular response by pomegranate extract or compounds may be a useful approach for the prevention of the onset and severity of inflammatory arthritis [77]. The dynamism of pomegranate fruit in newer areas of pharmacological effects might be delivered in the future.

4. Pomegranate Extract as a Phytoestrogen

Due to the possible adverse side effects of estrogenic stimulation (such as increase in tumor risk), many women have turned to phytoestrogens as an alternative for HRT [83]. The features that facilitate the chemicals to bind with ER are the steric and hydrophobic properties of a compound, as well as the hydrogen bonding between the phenolic hydroxyl group and the ER binding site [84]. Phytoestrogens bind to both forms of ER and showed a lower binding affinity than E2. Some of them exhibit a higher binding affinity to ER β than

to ER α which may indicate that they have different pathways for their actions and explains tissue specific changeability in phytoestrogenic action [85]. Both genomic and nongenomic mechanisms have been projected to explain phytoestrogenic effects on human health [86]. The best move towards the avoidance and handling of estrogen-dependent breast cancer is to selectively hold estrogen activity in the affected tissues without compromising its beneficial effects [87]. Regrettably, at this time, available antiestrogen such as tamoxifen used in the treatment of ER-positive breast cancer has side effects and agonism in the uterine endometrium, leading to an uncertain connection to endometrial carcinoma [88–90]. A competitive radioactive binding study was done to ascertain whether PME interacts with ER and had shown that PME binds to ER and inhibited the binding of labelled estrogen to ER in a dose-dependent manner [53, 91].

5. Pomegranate as a Potential Nutraceutical

According to De Felice, who coined the term nutraceutical, it can be defined as, “a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease” [92]. It may range from isolated nutrients, herbal products, dietary supplements, and diets to genetically engineered “designer” foods and processed products such as cereals, soups, and beverages [93, 94]. Anthocyanidins (delphinidin, cyanidin, and pelargonidin) and hydrolysable tannins (such as punicalagin, pedunculagin, punicalin, gallagic, and ellagic acid esters of glucose), account for the major antioxidant activity of whole fruit [22, 95]. The peel, which is also a major part of the fruit, is an imperative source of bioactive compounds such as phenolics, flavonoids, ellagitannins, proanthocyanidin compounds [96], minerals, [97], and complex polysaccharides [98]. Aviram and others reported that systolic blood pressure was reduced, after 1 year of pomegranate juice consumption. This was believed to be related to the potent antioxidant properties of pomegranate polyphenols [60]. Hong et al. confirmed that pomegranate juice and pomegranate extracts were more potent inhibitors of cell growth than isolated individual polyphenols in cell lines, influential synergistic and/or additive effects of several phytochemicals including proanthocyanidins, anthocyanins, and flavonoid glycosides [99]. Pomegranate contains agents, particularly polyphenolic flavonoids, which exert actions that could be well conducive to good oral health, particularly in relation to gingivitis development [100]. Pomegranate juice had the greatest antioxidant potency composite index among beverages like black cherry juice, cranberry juice, grape juice, apple juice, orange juice, red wines, blueberry juice, and iced tea; and the antioxidant activity was at least 20% superior to any of the other beverages tested [101–103]. Each and every part of pomegranate provides health benefits, that is, a nutraceutical food.

6. Summary and Conclusions

The discovery that plants generate hormonally active phytochemicals has altered our understanding of the connection

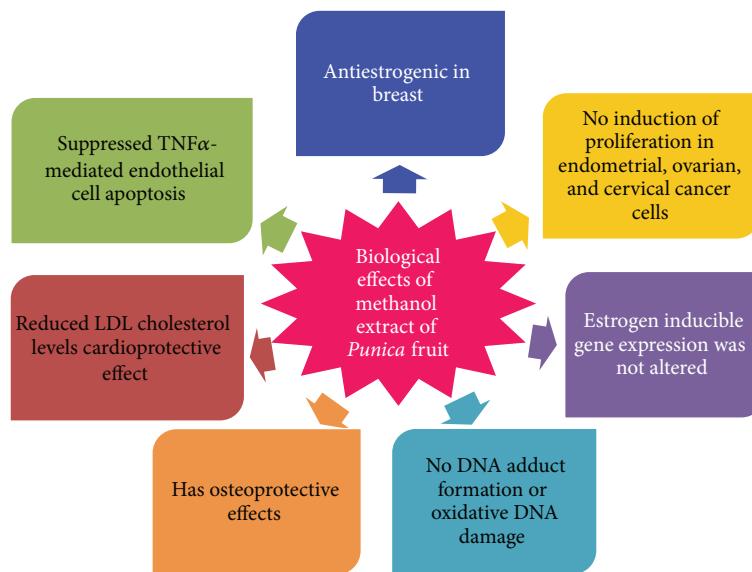


FIGURE 7: Biological effects of PME. PME was found to be antiestrogenic in breast, exhibited cardioprotective and osteoprotective effects, and had no estrogenicity in uterus. It did not induce DNA adduct formation or oxidative DNA damage and suppressed TNF α -mediated endothelial cell apoptosis.

between diet and human health. It is well established that fruit or plant extracts are a complex mixture of various constituents and, in most of the instances, it is not clear whether a single compound or a mixture of compounds is responsible for the reported effects [104]. The thought of the whole herb or multiherb preparation not only addresses multiple targets, but possibly will alleviate the toxicity and side effects of a single, isolated compound from the plant. Many *in vitro* and *in vivo* studies pointed out high nutritional and potential tissue specific action of extract of *Punica granatum*. Proofs are accumulating that compounds present in a fruit or herb extract augment each other's biological effect. For example, it has been reported that quercetin and ellagic acid (both are also present in pomegranate) together make use of a more prominent inhibitory effect against cancer cell growth than either compound alone [105]. We had found that PME has antiestrogenic effect in the mammary gland, without compromising the beneficial effects of estrogen in the cardiovascular and skeletal system and had no estrogenicity in the uterus [53]. PME could possibly be considered as an ideal SERM and further studies might demonstrate its suitability and possible application in estrogen dependent breast cancers with beneficial effects in other hormone dependent tissues. Figure 7 describes the biological effects of PME, as observed in our studies. Furthermore, it would be valuable to investigate the long-term effects of PME in the *in vivo* models of estrogen deprivation to demonstrate its suitability in HRT. To achieve this goal, a better understanding is needed regarding the orchestrated action of SERM, receptor and coregulators that contribute to distinct patterns of gene expression. Although scientific research is being carried out to study the biological activity of a lot of food phytochemicals, the health claims attributed to the final marketed nutraceutical products have normally little or doubtful scientific foundation.

This is owing to the fact that a great deal of scientific conclusion is derived from animal testing and *in vitro* assays, while human clinical trials are limited. Some key issues such as metabolism, bioavailability, toxicity, and dose/response of these food bioactive compounds or nutraceuticals themselves have not been well recognized yet. Currently, numerous clinical trials are in progress exploring the therapeutic potential of pomegranate extracts. Its potential use as a nutraceutical needs to be investigated. We may thus anticipate that many of the open issues about the biological effect of extract of *Punica granatum* will be answered in the near future.

Authors' Contribution

Sreeja Sreekumar, Hima Sithul, Parvathy Muraleedharan, and Juberiya Mohammed Azeez have equal authorship.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Influence of Oral and Gut Microbiota in the Health of Menopausal Women

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Sex differences in gut microbiota are acknowledged, and evidence suggests that gut microbiota may have a role in higher incidence and/or severity of autoimmune diseases in females. Additionally, it has been suggested that oral, vaginal, and gut microbiota composition can be regulated by estrogen levels. The association of vaginal microbiota with vulvovaginal atrophy at menopause is well described in the literature. However, the relevance of oral and gut microbiota modulation in the immune system during estrogen deficiency and its effect on inflammatory diseases is not well explored. Estrogen deficiency is a condition that occurs in menopausal women, and it can last approximately 30 years of a woman's life. The purpose of this mini-review is to highlight the importance of alterations in the oral and gut microbiota during estrogen deficiency and their effect on oral and inflammatory diseases that are associated with menopause. Considering that hormone replacement therapy is not always recommended or sufficient to prevent or treat menopause-related disease, we will also discuss the use of probiotics and prebiotics as an option for the prevention or treatment of these diseases.

Keywords: saliva, oral health, mouth diseases, gut microbiota, estrogen, menopause

INTRODUCTION

We harbor trillions of microorganisms that associate with specific tissues and are termed microbiota. This rich community of microorganisms, mostly bacteria, has co-evolved in a symbiotic relationship with humans in such a way that it is now essential for several physiological functions and controls many aspects of host physiology (Backhed et al., 2005; Backhed, 2012; Grover and Kashyap, 2014).

One of the factors that plays a pivotal role in microbiota modulation, although broadly understudied in current research, is the change in female sexual hormones throughout life. Two phases occur in a woman's life that are characterized by several physiological, metabolic and immunological changes: menarche, or the first menstruation of a woman, which occurs during adolescence between 10 and 15 years of age (Hoffmann et al., 2004), and menopause, which occurs between age 45 and 55 and includes the cessation of menstrual periods and loss of the reproductive function of the ovaries (Brotman et al., 2014). In fact, estrogen and the microbiota of a woman's body tend to be investigated more extensively during the woman's reproductive years than during menopause or the phase of estrogen decline. One exception is the vaginal microbiota,

which has been widely investigated during menopause. Here, we consider menopause or the menopausal phase, including perimenopause (before menopause), menopause and postmenopause (after menopause).

Considering that menopause can last for approximately 30 years of a woman's life (Brotman et al., 2014), the purpose of this mini-review is to highlight the importance of alterations in the oral and gut microbiota during estrogen deficiency and determine their relevance in oral infections and inflammatory diseases that are associated with menopause.

THE INTERACTION BETWEEN ORAL MICROBIOTA AND FEMALE SEX HORMONES

The oral cavity (mouth) is composed of several distinct microbial habitats, including the lips, the teeth, the gingival sulcus, the tongue, the cheeks, the palate and the tonsils, which are colonized by hundreds of different bacterial, viral, and fungal species (Dewhirst et al., 2010; Yost et al., 2015). The microbial communities associated with these structures are in symbiosis with the host (Sanz et al., 2017). However, in the presence of stressors that can perturb this homeostasis, several oral infectious diseases may appear, including dental caries and periodontitis (Almstahl et al., 2010; Dewhirst et al., 2010). Many of these disease are recognized to be caused by the consortia of organisms in a biofilm rather than a single pathogen (Jenkinson and Lamont, 2005). In addition, poor oral health and oral diseases may be associated with many systemic diseases (Seymour et al., 2007), such as cardiovascular diseases (Joshipura et al., 1996; Monteburgnoli et al., 2004; Belenguer et al., 2006), stroke (Joshipura et al., 2003), preterm birth (Offenbacher et al., 1998), diabetes (Genco et al., 2005), and pneumonia (Awano et al., 2008).

In healthy individuals, the microorganisms found in the mouth with the largest representation include *Streptococcus*, *Actinomyces*, *Veillonella*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Treponema*, *Neisseria*, *Haemophilus*, *Eubacteria*, *Lactobacterium*, *Capnocytophaga*, *Eikenella*, *Leptotrichia*, *Peptostreptococcus*, *Staphylococcus*, and *Propionibacterium* (Jenkinson and Lamont, 2005; Liu et al., 2012). The behavior of these organisms can be very dynamic and adapt to a wide range of environments and interactions with other microbial species while aggregated in biofilms over the oral surfaces.

Estrogen receptor-beta has been detected in the oral mucosa and salivary glands (Valimaa et al., 2004), and some evidence shows age-related hormonal changes in the exfoliated normal buccal mucosa of women (Donald et al., 2013). Moreover, the vaginal and buccal epithelia share some microscopic similarities. As observed by Thompson et al. (2001), the patterns of surface keratinization and the distribution and appearance of the lipid lamellae in the intercellular spaces were similar between vaginal and buccal epithelial samples of postmenopausal women. Therefore, given that many menopausal women also suffer from oral discomforts in addition to climacteric symptoms (Meurman et al., 2009), an understanding of the impact of female sex

hormones on the characteristics of the oral microbiota may be clinically relevant, especially during menopause. Some of the main complaints from women in menopause include dry mouth and tooth loss, and the existing data have focused on the salivary microbial composition and the microbiota characteristics of the gingival sulcus. Therefore, this review will explore the main findings of the relationship between the oral microbiota and menopause in saliva and periodontal support.

Saliva

Saliva plays an important role in the maintenance of oral health integrity and the protection against dental caries and other oral diseases (Marsh et al., 2016; Wang et al., 2016). The salivary microbiota is highly diverse and complex (Curtis et al., 2011).

Estrogen and menopause-related hormonal imbalances are believed to affect oral health (Cao et al., 2007). According to the literature (Meurman et al., 2009), together with climacteric complaints, various oral discomforts are reported in menopausal women. The main peri- and postmenopausal symptoms include xerostomia (subjective oral dryness) and/or hyposalivation (Mahesh et al., 2014), which may increase the occurrence of mucosal and dental diseases, such as candidiasis. Few studies have investigated the effects of hormone replacement therapy in such patients (Mahesh et al., 2014; Lago et al., 2015), although the existing results show an improvement in symptoms following such treatment (Mahesh et al., 2014; Lago et al., 2015).

The quantitative and qualitative changes in saliva may alter the regular homeostasis of oral health, subsequently leading to specific changes in the salivary bacterial composition (Nasidze et al., 2009, 2011; Belstrøm et al., 2014). However, recent findings have shown that patients with severe hyposalivation do not differ in their bacterial profiles compared with those with normal salivary flow rates (Belstrom et al., 2016), although the corresponding study did not focus on the evaluation of such differences between menopausal and non-menopausal women.

Because the salivary composition may be influenced by the presence of oral diseases, prescribed medications and general health (Belstrom et al., 2016), researchers must pay attention to the sample size and control for confounding factors when revising the existing literature to confirm the external validity of any quantitative and qualitative changes in saliva related to menopause.

Periodontal Support

The periodontium is the specialized tissue that both surrounds and supports the teeth. Periodontal disease, which includes gingivitis and periodontitis, is highly prevalent in adults, and disease severity increases with age. This inflammatory disease develops over time with the accumulation of biofilm (dental plaque), bacterial dysbiosis, the formation of periodontal pockets, gum recession, and tissue destruction (including alveolar bone loss), which can ultimately lead to tooth loss (Michaud et al., 2017).

Fluctuating female sexual hormone levels in menopausal women may represent key factors that respond to changes detected in the oral cavity (Dutt et al., 2013). Menopause is accompanied by decreased bone density, which may have

implications for oral health such as the risk of enhanced progression of periodontal infections and tooth loss (Hernandez-Viguera et al., 2016). According to the literature, sex-related hormonal changes may cause the gums to become more susceptible to plaque and create a much higher risk for gingivitis and advanced periodontitis (Suresh and Radfar, 2004).

Periodontitis is a chronic inflammatory process that occurs in response to an increase in Gram-negative bacteria in the biofilm (Ruby and Barbeau, 2002), affecting the tissues that surround and support the teeth. Specific bacterial species, such as *Porphyromonas gingivalis* and *Tannerella forsythensis*, were found to be important in the etiology of periodontitis in postmenopausal women (Brennan et al., 2007). In addition, changes in periodontal status were found to be associated with variations in sex hormone levels (Mascarenhas et al., 2003), and the occurrence of periodontitis was reported to be greater in postmenopausal women who did not receive hormone replacement than in premenopausal women (Haas et al., 2009). Therefore, from a clinical point of view, the roles of sex hormones and hormone therapy in the prevalence of subgingival bacterial infection in peri- and postmenopausal women are of great interest.

In a cohort study that included 106 women aged 50–58 years, hormone replacement therapy led to a decreased number of positive samples showing the periodontal pathogens *P. gingivalis*, *Prevotella intermedia*, and *T. forsythia* from the subgingival plaque (Tarkkila et al., 2010). Consistent with this result, a previous study found improved periodontal probing depths and tooth mobility in 190 randomized women who received hormone therapy for 1 year (López-Marcos et al., 2005). Conversely, Pilgram et al. (2002) investigated 135 women in a randomized, controlled trial who received estrogen replacement for 3 years and did not find any changes in clinical parameters such as the attachment of teeth or the bone mineral density of the lumbar spine. In mice, estrogen seems to modulate IL-1 production and participate in the resistance of females to disseminating dentoalveolar infections, leading to the enhanced localization of these infections (Youssef and Stashenko, 2017), which draws attention to the potential role of sex-related hormones in the modulation of oral mucosal infections.

Non-conventional treatment approaches for oral infections, with a particular emphasis on dental biofilm-related diseases, have gained attention in recent years. The use of probiotics and prebiotics to improve gastrointestinal health has now led to an interest in using these treatments to control oral diseases (Allaker and Ian Douglas, 2015). However, few studies have focused on recovery of the oral equilibrium by promoting beneficial microbiota. Despite differences in the composition of the gut and oral microbiota, the community types observed in the gut are predictive of the community observed in the mouth and vice versa (Ding and Schloss, 2014). Among other host factors, the oral microbiota serves as an inoculum for the intestine, and the microorganisms that find adequate conditions in the mouth give rise to distinct types of communities in the intestine. Interestingly, oral inoculation with *P. gingivalis* in experimental models leads to a change in the intestinal microbiota, which is a possible mechanism for the establishment

of diseases associated with periodontitis, such as cardiovascular diseases (Arimatsu et al., 2014). In this sense, understanding the role of health-associated microorganisms may have utility in the application of these approaches for the prevention and treatment of disease (Sanz et al., 2017).

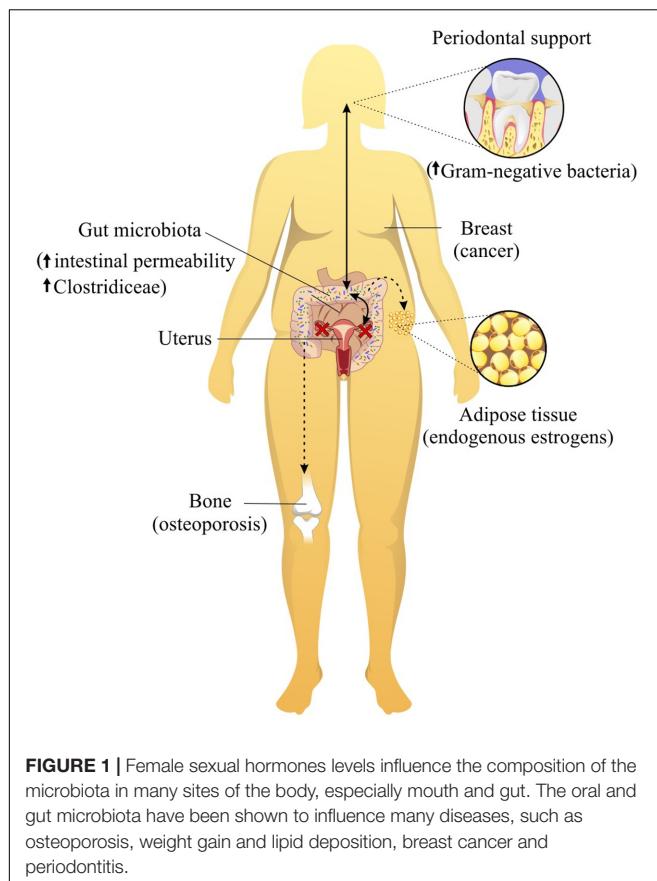
THE INTERACTION BETWEEN GUT MICROBIOTA AND FEMALE SEX HORMONES

As mentioned earlier, female sex hormones levels influence the composition of the microbiota in many sites of the body, especially the gut. Due to intimate contact with the larger gut immune system, the gut microbiota has been shown to influence many diseases outside of this organ (Figure 1). Accordingly, imbalance of the gut microbiota, called dysbiosis, has been extensively related to metabolic and immunological diseases. Interestingly, the presence or absence of estrogen may be able to alter the gut microbiota equilibrium and corresponding disease pathways. Some autoimmune diseases affect more often women than men, including systemic lupus erythematosus (Jiang et al., 2005), Sjogren's syndrome (Patel and Shahane, 2014) and rheumatoid arthritis (Oliver and Silman, 2009). Gender differences have also been reported for the outcome of microbial infections (Fischer et al., 2015). Interestingly, the onset of autoimmune diseases, asthma (Akinbami et al., 2016) and other diseases occurs after menarche or during the reproductive period of women. Experimental findings in mice have shown that the interactions among the microbiota, female sexual hormones, and immunity are associated with the development of autoimmune diseases (Yurkovetskiy et al., 2013, 2015), including type 1 diabetes (Markle et al., 2013) and rheumatoid arthritis (Wu et al., 2010). The non-obese diabetic (NOD) mouse exhibits spontaneous, immune-mediated pancreatic beta cell destruction causing type 1 diabetes (T1D) with a complex genetic and environmental etiology. The NOD T1D incidence shows a strong 2:1 female to male sex bias (Markle et al., 2013). Interestingly, germ-free NOD female mice lack this gender bias for diabetes. Additionally, after castration, males exhibit a similar microbiota composition and T1D incidence to females (Markle et al., 2013). In general, this study shows that the microbiome is a causal factor and not simply a consequence of autoimmune disease.

The Relevance of the Gut Microbiota in the Health of Menopausal Women

When the interaction between the gut microbiota and estrogen is altered due to a lack of estrogen, this relationship is restructured according to the new circumstances. However, host functional alterations, such as metabolic and immunological changes, also occur.

Obesity affects 65% of postmenopausal women and is associated with the onset of metabolic dysfunction (Leenens et al., 2017). Multiple studies have suggested that postmenopausal women exhibit increased total fat mass and abdominal fat and



decreased lean body mass compared with those of premenopausal women, regardless of aging (Aloia et al., 1995; Schreiner et al., 1996; Cordina-Duverger et al., 2016). The accumulation of abdominal fat in postmenopausal women appears to be a critical factor in the development of insulin resistance and type 2 diabetes (Lobo et al., 2014), and the relationship between the gut microbiota and a lack of estrogen is likely responsible for weight gain and lipid deposition during menopause (Figure 1). The gut microbiota can metabolize estrogen-like compounds such as isoflavonoids, which are found in soy foods, and promote the growth of some specific bacteria (Frankenfeld et al., 2014; Chen and Madak-Erdogan, 2016; Miller et al., 2017). Indeed, the administration of soy isoflavones to postmenopausal women was shown to increase the concentration of *Bifidobacterium* and suppress Clostridiaceae, which are known to be involved in inflammatory diseases (Frankenfeld et al., 2014; Nakatsu et al., 2014). This suppression of Clostridiaceae, a family of Clostridia associated with obesity (Figure 1), likely explains why diets containing phytoestrogens have been shown to improve weight gain in menopausal women.

Few studies have investigated whether prebiotics and probiotics can improve insulin sensitivity in postmenopausal women or body fat in mice. The intake of flaxseed mucilage, a prebiotic, is known to improve insulin sensitivity and alter the gut microbiota in obese postmenopausal women (Brahe et al., 2015). Thus far, the implications of the gut microbiota with low

levels or the absence of estrogen hormone in the metabolism of women have not been sufficiently studied and require further clarification.

Another link between the gut microbiome and menopausal health is related to bone. Interestingly, the gut microbiota has also been found to influence bone homeostasis. Approximately one in two women over age 50 will break a bone because of osteoporosis. A study that involved twenty postmenopausal women with a mean age of 65 years showed that the group that consumed *Lactobacillus helveticus*-fermented milk had increased serum calcium levels and reduced bone reabsorption compared with those of the control milk consumption group (Narva et al., 2004). Experimental studies have also demonstrated similar results. For instance, *L. reuteri* treatment significantly protected ovariectomized mice from bone loss and increases in bone marrow CD4+ T-lymphocytes, which promote osteoclastogenesis (Britton et al., 2014). Another study that investigated probiotic treatment for cortical bone loss found reduced expression of two inflammatory cytokines, TNF- α and IL-1 β , and increased expression of osteoprotegerin, a potent inhibitor of osteoclastogenesis, in the cortical bone of ovariectomized mice (Ohlsson et al., 2014). Additionally, sex steroid deprivation has been reported to promote intestinal permeability (Figure 1), and the oral administration of *L. rhamnosus* GG (LGG) or VSL#3 (a combination of tree probiotics) to estrogen-deficient mice significantly reinforced intestinal barrier integrity and completely protected the mice against sex steroid depletion-induced bone loss (Li et al., 2016). Importantly, to confirm the role of the gut microbiota in bone health, another experiment also showed that germ-free mice are protected against the bone loss induced by the absence of sex steroids (Li et al., 2016).

We must mention that the gut microbiota may influence the risk for breast cancer through effects on endogenous estrogens produced by adipose tissue in postmenopausal women (Figure 1) (Key et al., 2003). A cross-sectional study on 60 healthy postmenopausal women found that women with a more diverse gut microbiome and an abundance of four Clostridia taxa exhibited an elevated urinary ratio of hydroxylated estrogen metabolites to parent estrogens (Fuhrman et al., 2014), which is related to the etiology of breast cancer (Flores et al., 2012; Kwa et al., 2016). However, another study compared 48 postmenopausal breast cancer patients and 48 control patients and observed that postmenopausal women with breast cancer exhibited an altered composition and estrogen-independent low diversity of their microbiota (Goedert et al., 2015). These different findings on gut microbiota diversity and breast cancer could be explained by the fact that disease outcome or disease stage can also affect the microbiota. In this scenario, the consumption of the soy isoflavone daidzein, which is metabolized by some bacteria of the microbiota to generate equol and O-desmethylangolensin (ODMA), could represent a therapeutic strategy for breast cancer prevention. Some, but not all, studies have shown a lower risk of breast cancer associated with equol production (Hullar et al., 2014). However, only approximately 30–50% of the population can metabolize daidzein (Frankenfeld et al., 2004; Uehara, 2013; Nakatsu et al., 2014) to equol, likely

due to the host microbiota. Therefore, an investigation into the administration of the soy isoflavone daidzein together with probiotic bacteria to produce equol is warranted and could offer benefits in the prevention of breast cancer in menopausal women.

CONCLUDING REMARKS

Many chronic diseases can emerge after estrogen levels decline, which will affect a considerable part of a woman's life. Understanding the role of the microbiota in women's health at the menopausal phase could help to improve strategies for microbiota modulation and prevent dysfunction. The oral and gut microbiotas have been extensively studied in women of reproductive age, while the menopausal period has been somewhat overlooked. The use of hormone replacement is not indicated for all menopausal women, and considering that probiotics and prebiotics can affect the dysfunction of bone, adipose tissue, oral and other tissues, such treatments may constitute an important therapeutic strategy. Pro- and prebiotics can also be used in conjunction

with menopause hormone therapy and may attenuate the side effects that can arise from hormone replacement. In conclusion, the scientific findings published to date do not definitively demonstrate how non-vaginal microbiota sites influence the health of menopausal women. Thus, many questions remain unanswered and warrant further investigation to improve the quality of life of menopausal women.

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AV, PC, DR, and CF drafted and revised the manuscript.

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Urolithins: The Colon Microbiota Metabolites as Endocrine Modulators: Prospects and Perspectives

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Selective estrogen receptor modulators (SERMs) have been used in hormone related disorders, and their role in clinical medicine is evolving. Tamoxifen and raloxifen are the most commonly used synthetic SERMs, and their long-term use are known to create side effects. Hence, efforts have been directed to identify molecules which could retain the beneficial effects of estrogen, at the same time produce minimal side effects. **Urolithins, the products of colon microbiota from ellagitannin rich foodstuff, have immense health benefits and have been demonstrated to bind to estrogen receptors.** This class of compounds holds promise as therapeutic and nutritional supplement in cardiovascular disorders, osteoporosis, muscle health, neurological disorders, and cancers of breast, endometrium, and prostate, or, in essence, most of the hormone/endocrine-dependent diseases. One of our findings from the past decade of research on SERMs and estrogen modulators, showed that **pomegranate, one of the indirect but major sources of urolithins, can act as SERM.** The prospect of urolithins to act as agonist, antagonist, or SERM will depend on its structure; the estrogen receptor conformational change, availability and abundance of co-activators/co-repressors in the target tissues, and also the presence of other estrogen receptor ligands. Given that, urolithins need to be carefully studied for its SERM activity considering the pleotropic action of estrogen receptors and its numerous roles in physiological systems. In this review, we unveil the possibility of urolithins as a potent SERM, which we are currently investigating, in the hormone dependent tissues.

Keywords: urolithin, estrogen receptor, selective estrogen receptor modulators, pomegranate (*Punica granatum* L.), PhytoSERM

INTRODUCTION

Selective estrogen receptor modulators (SERMs) are non-steroidal compounds that bind to estrogen receptors and can act like estrogen or be a partial agonist or antagonist with mixed activity depending on the tissue it acts. Tamoxifen and raloxifen are the most commonly used SERMs in treating breast cancer, which is often observed to exert side effects in hormone dependent-tissues.

Tamoxifen, for instance, though osteoprotective like estrogen (1, 2), is reported to increase the uterine weight, endometrial cancer, stroke, and pulmonary embolism (3). A drug that has protective effects in estrogen-dependent tissues and prevents its deleterious effects would serve as an ideal SERM (4). Overcoming the side effects of synthetic SERMs is highly coveted for treating ER-positive breast cancer and other hormone related disorders. Hence, there has been an extensive search for alternatives from plant-based molecules with structural and functional resemblance to estrogens such as phytoestrogens. These are present in soy, grains, vegetables, and berries and are often metabolized by microbiota to form compounds, with or without having an estrogen-like activity (5). Many times, these phytoestrogens are metabolized by gut microbiota, which often have a stronger activity attributed to their higher lipophilicity, leading to a better absorption, and a higher affinity with estrogen receptors (6). These metabolites can, in turn, modulate the gut microbiota rendering a bidirectional relationship (7, 8). For instance, S-equol derived from daidzein by an intestinal bacterial metabolism also displays a profile like that of the daidzein's, and is of clinical importance (6, 9). Notably, many of the phytoestrogens like genistein, coumestrol, and liquiritigenin display more affinity toward estrogen receptor β (ER β) than to estrogen receptor α (ER α), but the implications and underpinnings of these differences remain elusive (10). It is probable that those tissues where ER β is critical, such as ovary, prostate, lung, cardiovascular, and central nervous systems (CNSs) (11), might be more influenced by these compounds.

Pomegranate has been known to have extensive medicinal properties which have been attributed to its constituents, working individually or in combination (12, 13). We have also demonstrated that pomegranate can act as SERM (4). Pomegranate is rich in ellagitannins and ellagic acid, which is further metabolized to urolithins by a specific colon microbiota. Ellagitannins and ellagic acid are also found in certain berries and nuts like walnuts and pecans. However, there is lot of inter-individual variability in the presence and abundance of ellagic acid and urolithins in plasma, urine, and feces of the individuals consuming ellagitannin-rich food, which could be primarily due to the presence, absence, or abundance of some specific microbiota (14). Urolithin family, characterized by a chemical structure containing α -benzo-coumarin scaffold, majorly include Urolithin A (UA), Urolithin B (UB), Urolithin C (UC), Iso-Urolithin A (Iso-UA), the recently discovered Urolithin M7R, Urolithin CR, and Urolithin AR (15). The main metabolites found in plasma, tissues, and excreted in urine and feces include UA, UB, and Iso-UA, which are subsequently absorbed and metabolized into their corresponding phase II conjugates (glucuronides or sulfates) and can persist in the bloodstream up to 3–4 days after the intake (13). Urolithins are understood to be actively glucuronidated in the large intestinal enterocytes before entering the bloodstream. Their maximal concentrations in the plasma can reach up to 35 μ M for glucuronide and 8-O-glucuronide, 0.745 μ M for Iso-UA 3-O-glucuronide, and 7.3 μ M for and urolithin B 3-O-glucuronide (16). Repeated consumption of ellagitannin-containing food products can

significantly increase the concentration of these conjugates in urine (16). Among the urolithins, UA and its conjugates are found at the highest concentrations in human plasma ranging from 0.024 to 35 μ M. Urolithins are detected in high concentrations in the colon and can also reach the systemic tissues such as the prostate and mammary gland (13). Direct supplementation with UA significantly increases plasma levels and provides more than six-fold exposure to UA vs. pomegranate juice (17).

Accumulating evidence suggests urolithins have extensive health benefits (18). It has been shown that urolithins have estrogenic and antiestrogenic activity (19) via competitive binding assays, proliferation assays (20), and transactivation assays (21), and is predicted to bind to ER α in a similar orientation as that of the estrogen (21). However, the conjugates of urolithins, which reach the breast tissue after the ellagitannin intake, apparently lack estrogenic and anti-estrogenic activity (22). The ellagic acid from which urolithins are derived have also been reported to exhibit estrogenic activity at low concentrations (10^{-7} to 10^{-9} M), via ER α , whereas it was a complete estrogen antagonist via ER β (23), though another investigation demonstrates absence of any estrogenicity or antiestrogenic activity in ellagic acid (21). Notably, UA and UB have been known to inhibit aromatase (24) and 17 β -hydroxysteroid dehydrogenases (25), which are the enzymes critical for estradiol synthesis. All these pointedly illustrate the ability of urolithins to have estrogenic/anti-estrogenic or SERM-like profile. Briefly, estrogenic chemicals are those that can directly activate or inhibit estrogen action or can indirectly modulate its action. These can act as endocrine disruptors, which are defined as “an exogenous agent that interferes with the production, release, transport, metabolism, binding, action, or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes” (26). The SERMs are cornerstone strategy to treat breast cancers, infertility problems, postmenopausal problems like hot flashes, osteoporosis, and for hormone replacement therapy. Hence, this implies that it is highly beneficial if urolithins can be employed as SERMs or as estrogenic compounds, with minimum side effects. Of note, urolithins are considered safe according to toxicity studies (27, 28), and, most importantly, UA has been recently recognized by food drug administration (FDA, USA) as GRAS (Generally Recognized As Safe) for its use as an ingredient. In this review, we explore the prospect of urolithins to act as endocrine modulators/disruptors by consolidating and connecting the existing body of evidence that underpins the health benefits of urolithins in hormone-dependent tissues and propose that it can act as an estrogen agonist, or antagonist or as SERMs, and detail its potential to modulate the hormone related pathways. The following sections unfold the benefits of urolithins in tissues where estrogen has a remarkable role. This includes its anti-inflammatory potential, cardiovascular benefits, breast, endometrial and prostate cancer protection, bone, muscle, and cognitive health where estrogen receptors are abundant and play a pivotal role.

COLON BACTERIA: THE COOKS WHO BROUGHT OUT THE DELICACY

Right from the mode of delivery to the kind of feeding pattern, the environment and the dietary pattern mold the gut microbiota. The response to diet depends on the type of bacteria that inhabits the gut and also interaction of the host microbe. This process is cyclic and inter-dependent (29). Similarly, the nutritional availability and, hence, therapeutic, or preventive effect of a diet can vary with microbiome features and their abundance. The inter-individual variability in the presence and abundance of ellagitannins/urolithins in plasma and urine samples after consumption of ellagitannin rich food was suggestive of their microbial origin in colon. Speculations were put to rest when results from Cerdà et al. (30) confirmed that urolithins and its types correlated with the type of the fecal bacteria. This affirmed the microbial origin of urolithins in humans and explains the difference in the therapeutic and nutritional effects of pomegranate and berries that have similar rich composition of ellagitannins. This discovery led to the active research in identification of the microorganisms which can convert ellagitannins/ellagic acid to different types of urolithins. Selma et al. identified intestinal bacterial species from human feces, namely, *Gordonibacter urolithinfaciens* and *Gordonibacter pamelaiaeae* (31), belonging to the family Eggerthellaceae, which transformed the ellagic acid into UC, and another strain CEBAS 4A4, belonging to a new genus from the same family, could produce Iso-UA (32) under anaerobic condition leading to the categorizing of individuals into three metabotypes, according to the gut microbiota composition (33). After ingestion of ellagitannin-rich food products, individuals without urolithins production belonged to metabotype 0, with UA production as the unique final product that fits into metabotype A, and UB and/or Iso-UA belongs to metabotype B (14, 18). Thus, this difference in microbiota composition would further influence the health benefits associated with ellagitannin-rich food (34). Notably, urolithins have been found in breast milk of mothers, who consume ellagitannin rich walnut, and it resembles Urolithin metabolites of the mothers as well (35). Investigations on how these bacteria can improve the metabolism of an ellagitannin-rich food, how would it further bring about health benefits, and an examination of its safety aspects are vital before considering them as a potential probiotic.

ANTI-INFLAMMATORY ACTIVITY AND CARDIOVASCULAR PROTECTION

Ellagitannins and urolithins have antioxidant (36), anti-inflammatory (37–41), and immunomodulatory properties (42). Urolithins inhibit NF- κ b in colon fibroblast (43), in osteoarthritic models (44), and in rat primary chondrocytes (45), but whether they act via estrogen receptors or membrane receptors like estrogen do, is not studied estrogens (46) is not studied. The UA and its metabolites have been shown to be protective in cardiac health (47, 48). *In vivo* studies with streptozotocin-induced type-1 diabetes rats demonstrated

that urolithins administration reduced the myocardial expression of the pro-inflammatory cytokine fractalkine, thus, improving the cardiac performance (49). Furthermore, urolithin B-glucuronide (UB-glu) could counteract trimethylamine-N-oxide-induced cardiomyocyte damage (48). Direct consumption of pomegranate has also shown the beneficial effects in the cardiovascular health (50–53). In our earlier study, we found a reduction in low density lipopolysaccharide (LDL) in the Swiss-albino mice after consumption of methanolic extract of pomegranate (PME), which was induced upon ovariectomy when compared to sham control (4).

Estrogens exert anti-inflammatory activity via the receptors like ER α , ER β , and GPR-30 that are present on cardiac cells encompassing cardiomyocytes, fibroblasts, vascular endothelial, and smooth muscle cells (54), thus, being beneficial in cardiovascular diseases such as coronary heart disease, ventricular hypertrophy, atherosclerosis, etc., via nuclear or non-nuclear pathways (55, 56). The ER β appears to have a substantial cardioprotective effect (54). However, whether the protective effects of the pomegranate components or of urolithins are caused by these receptors are not investigated. It would be worthwhile to unravel whether they show similar or varying profile and benefits in different categories such as in pre-, peri- and post-menopausal women, and whether pomegranate benefits correlate their metabotype and different hormonal phases in women.

BREAST CANCER

Our previous research showed that methanolic extract of a pomegranate peel reduced breast cancer proliferation by binding to estrogen receptor without affecting uterine weight, unlike estradiol or tamoxifen (4). A plethora of evidence points to preventive possibilities of pomegranate in breast cancer at its various stages and processes of cell survival (12). We had also reported that the PME can inhibit the proliferation induced by endogenous SERM 27 hydroxycholesterol (57). From the findings so far, urolithins are active molecules, but its availability and type are dependent on the gut microbiota and, hence, the extract may have limitations in its applicability. For the first time, Larossa et al. demonstrated in 2005 that UA and UB can act as “enterophytoestrogens,” exhibiting estrogenic activity in a dose-dependent manner without antiproliferative or toxic effects. Urolithins in combination with estrogen showed antiproliferative activity and anti-estrogenic activity and thwarted the proliferative activity of estrogen in the cell line models of human breast. The competitive binding assay showed that UA had much higher affinity to both ER α and ER β than UB. The UA had a slightly higher affinity toward ER α than ER β , with half maximal inhibitory concentration (IC50) values being at 0.40 and 0.75 μ M. Skledar et al. (21) reported a comparatively higher half maximal effective concentration (EC50) value for ER α , i.e., 5.60 μ M. However, the conjugated metabolites of urolithins lacked these activities (22, 58). Hence, it is speculated that the potential antiproliferative/cytotoxic, as well as estrogenic/antiestrogenic activities, in breast tissues would

primarily depend on the metabolite formed in the specific tissues. It is also suggested that though conjugation may hinder the direct antiproliferative activity, there is a probability of a long-term tumor-senescent chemoprevention (22).

Urolithins have been documented to inhibit aromatase and proliferation of breast cancer cells stimulated by testosterone (24). Strikingly, UA and UB inhibit 17 β -hydroxysteroid dehydrogenases (17 β -HSD1) (25), an enzyme involved in dihydrotestosterone (DHT) inactivation and in the conversion of inactive estrone (E1) to estradiol (E2), and, hence, critical for estradiol synthesis. Of note, high 17 β -HSD1 mRNA expression in patients with breast cancer correlates with a weak prognosis for breast cancer, thus, enhancing the breast cancer proliferation and invasion (59). Additionally, urolithins inhibit androgen receptor (60). These results point to its ability to act as anti-estrogenic in breast cancer. The UA also suppresses hyperactivated transglutaminase TGM2 which is one of the novel gene signatures expressed in metastatic cells that have undergone induction and reversion of epithelial–mesenchymal transition (EMT) and have induced metastasis (61). Also, urolithins can cross blood brain barrier and, hence, its potential in preventing breast to brain metastasis is worth investigating, since there is very less information on anti-estrogens or aromatase inhibitors that can cross blood brain barrier, which is a potential endocrine tissue (62, 63).

ENDOMETRIAL PROTECTION

Endometrial cancer is the sixth most commonly occurring cancer in women (64). In premenopausal women, endometrial proliferation is driven by estrogen, whilst after menopause, peripheral tissues like adipose tissue takes over estrogen synthesis, which implies that obesity increases likelihood of endometrial cancer in the postmenopausal uterus (65). In this context, it is worthwhile to mention that the pomegranate and urolithins have been shown to have preventive roles in obesity (4, 66–68). Urolithins are found to inhibit human endometrial cancer cells in an *in vitro* study (20). It also modulated the expression of ER α -dependent genes like ER β , PGR, pS2, and GREB1. The UA and UB exhibited antiproliferative activity in the human primary endometriotic cells and reduced the invasion and expression of Matrix metalloproteases (MMPs) and matrix adhesion receptor. Both UA and UB were found to decrease the viability and integrity of endometriosis spheroids (69). These studies are indicative of the ability of urolithin to have protective effects on the endometrium. Endometrial cancers are mostly hormone driven *via* estrogen receptors (70) and, hence, are often a side effect of SERMs like tamoxifen (71). Therefore, SERMs which do not activate endometrial proliferation is much desired in the present clinical context. If urolithins are further explored for their activities in hormone-dependent tissue, they can be exploited for their SERM activity. Hitherto, there are only *in vitro* evidence. It is important to consider that, within the body, the urolithins undergoes the phase-II conjugation (72) and reaches the systemic tissues. These conjugates have not been studied for antiproliferative activity in the endometrial cancer. Interestingly,

the tissue level of the deconjugation of urolithin glucuronides has also been reported (72). Indeed, more concrete studies and evidence are needed to understand and to prove the real protective capacity of these compounds and their distribution with respect to endometrium and their combinatorial behavior with other synthetic SERMs.

PROSTATE WELLNESS

In 2020, prostate cancer was the second most commonly occurring cancer in men, as well as the fifth leading cause of death among men (64). It is noteworthy to mention that prostate cancer has been reported to be driven by ER α , AR, and non-genomic estrogen signaling pathways mediated by orphan receptors like GPR30 and ERR α and, hence, phytoestrogens have been shown to have a beneficial role in prostate cancer (73, 74). Pomegranate and its metabolites, ellagic acid and urolithins, found concentrated in mouse prostate, colon, and intestinal tissues (75), prevent proliferation of prostate cancer (60, 76–79). Interestingly, it has been reported that the beneficial effects of pomegranate juice against prostate cancer may be exerted by urolithin glucuronides and dimethyl ellagic acid (38). Combinatorial treatment with urolithins and bicalutamide, a clinically used non-steroidal anti-androgen, used to treat prostate cancer (80), also showed antiproliferative effects on human prostate cancer cells. Antiproliferative effects of urolithins were more conspicuous in androgen-independent than in androgen dependent cells. Antiproliferative activity of urolithins was mediated by AR through AKT signaling pathway (81) and P53-MDM2 pathway (82). Although the possible role of estrogen receptor has been proposed in other studies (80), there is still no clear evidence. Apart from this, urolithins can strengthen muscles (83), which is an added advantage while treating prostate cancer by androgen deprivation, given that this therapy can cause muscle weakness (84). If this study proves or extends its applicability in humans, urolithins could be helpful in general wellness of prostate, as an SERM, an adjuvant, or for muscle strengthening. Pomegranate and its constituents, including its colonic metabolite, have shown propitious results in prostate cancer. These findings provide very interesting leads and points to the ability of the pomegranate metabolites for the prevention of prostate cancer recurrence (85). The chemopreventive potential of pomegranate ellagitannins, coupled with the finding that urolithin metabolites accumulate in prostate, suggests that pomegranate may be a prospective therapeutic formulation in prostate cancer. Major urolithin that accumulates in the prostate after ellagitannin consumption is UA and its metabolite Urolithin A glucuronide (UA-glu). However, the concentration of these are very low, that is, in the range of ng/g albeit urolithins reach micromolar level concentrations in the bloodstream (38). Hence, the *in vitro* evidence using supraphysiological concentrations of urolithins or unconjugated urolithins might not give a realistic data on whether these metabolites can be protective in prostate cancer. Studies should be undertaken to understand the physiological levels of urolithin, or its conjugates, that can accumulate in the prostate upon consumption of urolithins at

its safe doses, and how is it beneficial in prostate cancer at these doses.

UROLITHINS A GLIMMER OF HOPE IN BONE HEALTH

Our earlier studies have shown that pomegranate has a protective role in osteoporosis (86) using MC3T3-E1 cells and ovariectomised Swiss-albino mice. The results indicated that the PME (80 µg/ml) has significantly increased the ALP (Alkaline Phosphatase) activity, in agreement with the findings of Spilmont (87), suggesting its role in modulating osteoblastic cell differentiation. This connotes its potential as a promising nutritional supplement in management of osteoporosis associated with menopause. The UA has been shown ameliorate intervertebral disc degeneration, a common cause of back pain (88) in a needle-puncture rat tail model via c-JUN and PI3K/Akt/NF-κB pathways. The UA inhibited the inflammatory molecules and debilitated the degradation of the extracellular matrix (ECM) induced by IL-1β. Both *in vitro* and *in vivo* evidence support its protective role in osteoarthritis (44). The question whether urolithins can act *via* estrogen receptors like estrogen (89) in these cells have not yet been explored. Evidence points to the potential of urolithins in promoting bone health, giving it an edge to be an ideal SERM.

NEURODEGENERATIVE DISEASE

A plethora of studies using different models have adduced that estrogens play a vital part in protecting women against stroke and neurodegenerative diseases, though the mechanisms have not been fully elucidated. All the neural cells express estrogen receptors and the neuroprotective properties are, in part, attributed to the receptor activation in multiple cell types. Microglial cells, the major immune cells that inhabit the CNS, are regulated by estrogen, which, in turn, protects the neuronal functions and prevents neurodegeneration (90). This offers the prospect of selectively targeting estrogen receptors in the treatment of neurodegenerative conditions that comes with aging and menopause. Interestingly, PE is demonstrated to act against Alzheimer's and Parkinson's disease in many studies (91–95). Presence of urolithins in brain after consumption of pomegranate has also been reported (95). Urolithins were the only compounds, among 21 others, that is isolated from the extract that met the criteria required for the penetration of blood-brain barrier (BBB) permeability (94). The β-amyloid fibrillation was averted by urolithins in an *in vitro* study. In Alzheimer's model, UA imparts cognitive protection by protecting neurons from cell death, and by triggering neurogenesis *via* anti-inflammatory signaling. In addition, it inhibits monoamine oxidase (MAO) (96), an enzyme that inactivates monoamine neurotransmitters in neurological disorders, such as depression and Parkinson's disease (97). Although the neuroprotective effects of urolithins are reported, evidence is still weak, partly due to the lack of physiologically relevant studies using the circulating conjugated urolithins that might reach brain tissues, in a nutritional context.

In 2017, González-Sarrías et al. (98) demonstrated that UA and Iso-UA, but not UB-glu, showed a slight attenuation of the H₂O₂-induced cytotoxicity in human-derived neuroblastoma SH-SY5Y cells. Another study showed that media from lipopolysaccharide (LPS)-BV-2 murine microglial cells co-culture cell model, treated with urolithins, preserve the SH-SY5Y cell viability (99), and protect neuroinflammation, although, methylated urolithins have not been detected in circulation in humans, so far. Thus, to date, only limited studies were performed with conjugated urolithins in the context of neuronal protection. Notably, SERMs like tamoxifen and raloxifene are known to regulate the functions of astrocytes, neurons, and microglia *via* ERα and the ERβ and G-protein coupled estrogen receptor (GPR30) (100). Hence, it would be interesting to unveil the potential of the urolithins to act as estrogen receptor modulators in the neuroimmune axes.

AGING AND MUSCLE STRENGTH

Ryu et al. (83) found that feeding of *C. elegans* during entire lifetime, i.e., from eggs until death with 50 µM of UA, UB, UC, and Urolithin D (UD), has extended the lifespan by 45.4, 36.6, 36, and 19.0%, respectively, and was dose dependent. However, the treatment with ellagic acid had no effect. Mitophagy induced by urolithins in mammalian cells, *C. elegans*, and rodents culminated in the improvement of the overall health. The short duration of urolithin administration in young worms and mammalian cells reduced the mitochondrial content without affecting the maximal respiratory capacity. This proved that despite the decrease in the mitochondrial pool after UA treatment, the remaining mitochondria are robust and meet the energy requirement. A long-term UA administration in rodents induced mitochondrial biogenesis and mitophagy in the muscles of both young and old animals. The UA, at a dose of 50 mg/kg daily in aged mice which is equivalent to 4 mg/kg in humans, improved the age-related muscle decline, as well as the muscle strength suggesting its potential for treating an impaired muscle functionality. This dosing is within standard dosing regimens used for both nutrition and pharmaceutical active ingredients. Urolithins may also have different mechanisms which regulate the mitochondrial biogenesis or mitophagy, since they are known to have an estrogen receptor binding affinity. Both UA and UB regulate skeletal muscle mass also by enhancing synthesis of protein and inhibiting the ubiquitin–proteasome pathway (101). The UB can produce muscle hypertrophy and reduce muscle atrophy in mice with sciatic nerve denervation. Both urolithins, UA, and UB in different models have shown their capacity to enhance the muscle strength by different mechanisms, thus, implying its therapeutic and preventive potential in enhancing muscle strength in various pathological and age-related maladies. Given these, the ability of these molecules to act *via* estrogen receptors or how different is its action in females can be examined since estrogen deprivation, or its reduction with menopause (102) or ovarian failure, results in weakness of the skeletal muscle. Evidence points that estrogen improves the mitochondrial membrane microviscosity and the bioenergetic function in skeletal muscle (103) and in muscle proteostasis. It

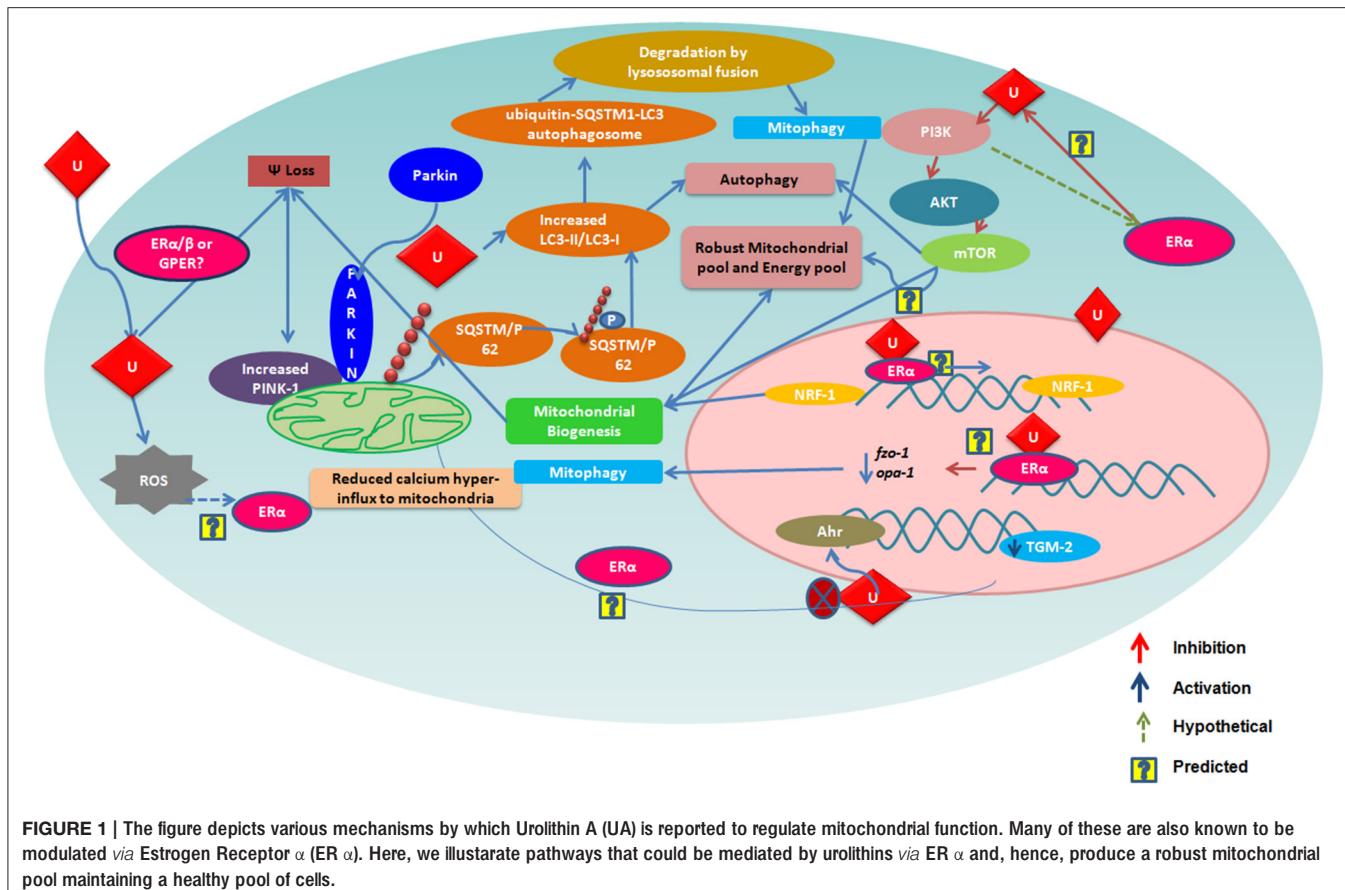


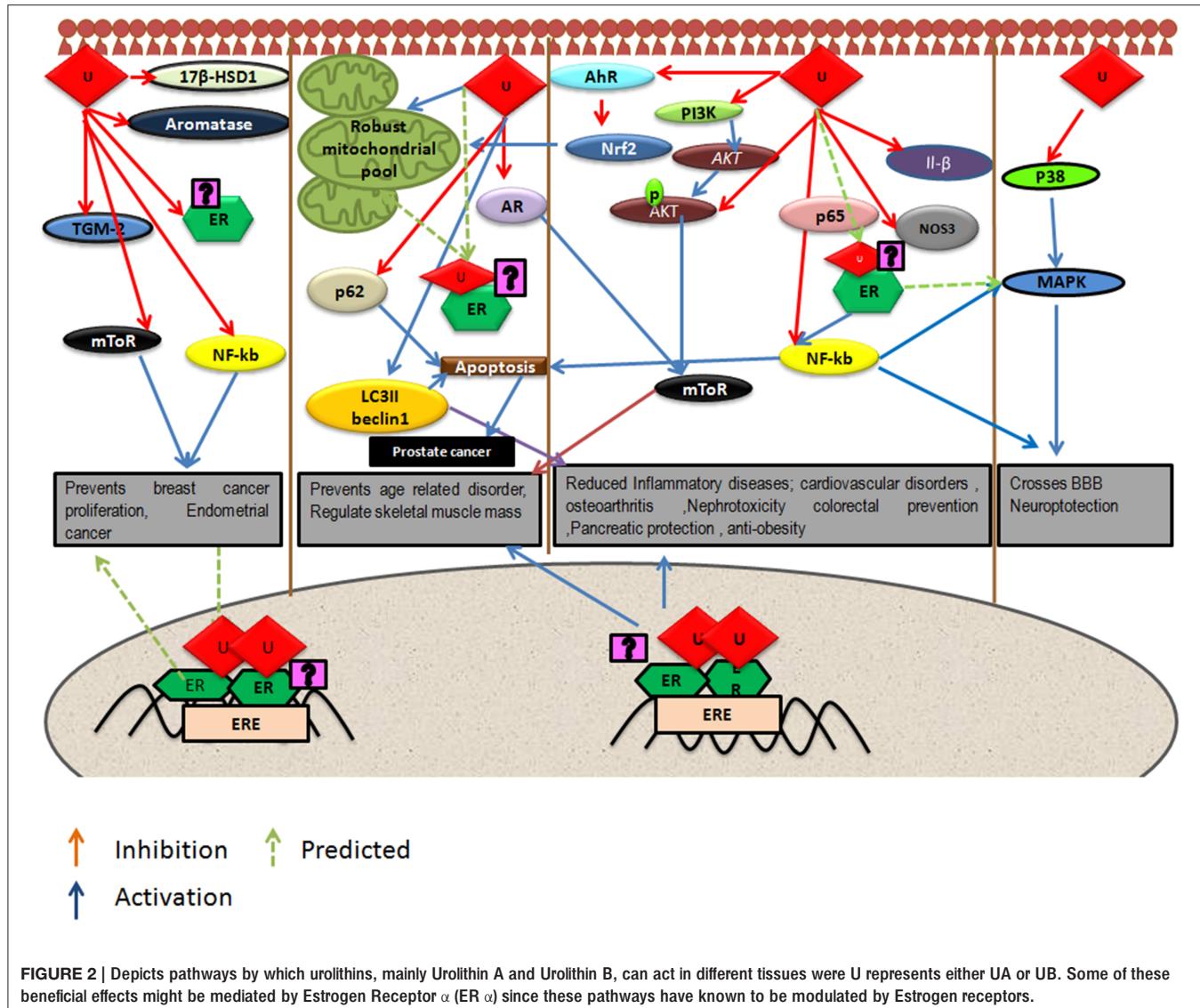
FIGURE 1 | The figure depicts various mechanisms by which Urolithin A (UA) is reported to regulate mitochondrial function. Many of these are also known to be modulated via Estrogen Receptor α (ER α). Here, we illustrate pathways that could be mediated by urolithins via ER α and, hence, produce a robust mitochondrial pool maintaining a healthy pool of cells.

also increases the collagen content of tendons and ligaments. Nevertheless, it must be noted that these benefits come at the cost of a decreased connective tissue stiffness (104). It would be intriguing to know whether urolithins can take over the function of estrogen in its absence.

MITOCHONDRIAL REGULATION BY UROLITHINS

Urolithins can induce mitophagy (83) and mitochondrial biogenesis in aged animals; the final consequence being the improvement of organismal phenotype and maintenance of maximum respiratory capacity emphasizing potential of urolithins having a dual role to maintain healthy mitochondria. The role of estrogen receptors in mediating this effect has not been investigated. The UA was found to induce PINK-1 and has increased the biomarkers for autophagy and mitophagy with ubiquitination of p62/SQSTM. Cells have developed sophisticated and elaborate mechanisms to adapt to stress conditions and alterations in metabolic demands, by regulating mitochondrial number and function by the generation of new mitochondria and by the removal of damaged or unwanted mitochondria for the maintenance of mitochondrial and cellular homeostasis (105). This implicates that urolithins could help

in preventing many pathological conditions resulting in or caused from damaged mitochondria or failed mitochondrial metabolism. It has been reported that UA exert gut barrier functions through activation of aryl hydrocarbon receptor (AhR)-nuclear factor erythroid 2-related factor 2 (Nrf2)-dependent pathways to upregulate epithelial tight junction proteins (40). The UA inhibits transglutaminase type 2 (TGM2)-mediated mitochondrial calcium influx, which alleviates high glucose-stimulated amyloidogenesis and neuronal degeneration (106), thus, regulating mitochondrial and calcium homeostasis. Tamoxifen, a SERM, is also known to target mitochondria by ER-dependent and independent pathways. Further, the tamoxifen-resistant cells are known to display altered mitochondrial pathways with increased mitochondrial content like many other cancer drugs (107). It has been seen that drug resistance can be overcome by modulating estrogen–estrogen receptor-mitochondrial pathway. Estrogen (108) via ERs is involved in the life cycle of mitochondria and controls the mitochondrial biogenesis, mitochondrial quality control, and mitophagy (108, 109). Examining whether urolithins can modulate mitochondrial pathways via estrogen receptors can help better understand its potential. The **Figure 1** consolidates the estrogen mediated mitochondrial pathways and how urolithins, through ERs could or by other mechanisms, can possibly act in a similar way. Thus, urolithins by different molecular pathways exert beneficial effects



in multiple tissues, but whether these effects involve estrogen receptors remain elusive. **Figure 2** shows the different pathways via which urolithins act and how estrogen receptor could be part of these mechanisms.

CORONAVIRUS DISEASE (COVID-19)

Clomiphene and toremifene SERMs are reported to have potent inhibitory activity in filovirus infections like EBOLA infection (110), while raloxifene hydrochloride and quinestrol inhibit flaviviruses such as Zika virus in ER-independent pathways (111). Use of SERMs in the cytokine storm and in the inflammation associated with COVID-19 is suggested as a promising pharmacological option (112). One of the main reasons that the COVID-19 is a threat to global health is due to the lack of targeted therapeutic agents. Out of the several coronavirus proteins proposed as druggable targets,

3-chymotrypsin-like protease or main protease (Mpro), a non-structural protein that breaks down the viral polyproteins to generate other non-structural proteins, the RNA-dependent RNA polymerase and helicase, is considered pivotal (113). Given this, it is interesting to note that urolithin metabolites can exert a mild anti-SARS-CoV-2 Mpro inhibition (113) at physiological relevant concentrations ($2\text{--}50 \mu\text{M}$), detectable in human colon tissues after consumption of hydrolyzable tannin-rich foods, as per clinical studies (114). Also, the pomegranate peel extract, punicalin, punicalagin, and UA have the potential to block the SARS-CoV-2 spike (S) glycoprotein receptor-binding domain (RBD)-ACE2 receptor on host cells contact, which is one of the first steps of virus infection (115). Additionally, it has been understood that SARS-CoV-2 can activate pro-inflammatory chemokines in the early stage, and this can lead to the development of either a protective immune response or an exacerbated inflammatory response

TABLE 1 | The table consolidates the relevant studies related to Urolithins and their significant outcome.

Urolithin type	Activity/ disease tested	Model	Concentration	Effects	Molecular target/pathway	Conclusion	Reference and year
UA and UB	Estrogenic/Anti-estrogenic	MCF-7	0.1–40 μ M	Proliferative, but prevents E2 induced proliferation. Binds to ER α and ER β with different affinity. UA has higher affinity	Estrogen receptors	UA and UB have estrogenic and anti-estrogenic activity	Larrosa et al., 2006 (19)
UA	Colon inflammation	Male Fischer rats induced with acute colitis by dextran sodium sulfate	UA:15 mg kg/ day	Pomegranate extract and UA decreased inflammation markers and favorably modulated the gut microbiota. UA preserved colonic architecture	UA decreased inflammation markers like iNOS, cyclooxygenase-2, PTGES, and PGE2 in colonic mucosa	UA probably the most active anti-inflammatory compound derived from pomegranate ingestion in healthy subjects, while in colonic inflammation group the effects may be by non-metabolized ellagittannin-related fraction	Larrosa et al., 2010 (37)
UA, UB, mUA, mUB	Alzheimer's disease	<i>In silico</i> , <i>C. elegans</i>	10–100 μ M 10 μ g/ml for 40 h	Urolithins passes BBB criteria, urolithin reduces A β fibrillation mUB protected <i>C.elegans</i> from A β induced neurotoxicity and paralysis	NA	Urolithins can reduce A β fibrillation	Yuan et al., 2015 (94)
UA	Prostate cancer	LNCaP cell line	40 μ M	UA increase cells in G1-phase, induction of apoptosis	UA upregulates CDKN1A	A potential chemopreventive agent for prostate cancer	Sánchez-González et al., 2016 (79)
UA and UB	Endometrial cancer	ECC1, Ishikawa cell	0.1–50 μ M	Antiproliferative, G2/M arrest, ER α modulation	Cell cycle proteins, suppresses ER α , enhances ER β , and PGR, Ps2, GREB1, down GRIP1	Antiproliferative in endometrial cancer	Zhang et al., 2016 (20)
UA, UB, UC, and UD	Lifespan extension	Bacteria: <i>E. coli</i> , C2C12 myoblast, K intestinal cell <i>C. elegans</i> , Sprague-Dawley rats C57BL/6J	25–50 μ M, 8 h 10–50 μ M, 4–24 h 50 μ M till death, 25 mg/kg/d–7days, 50 mg/kg/d–34 weeks	Mitophagy induction Improved pharyngeal pumping rate and mobility, better maintenance of muscle fiber organization, mitophagy induction, decreased mitochondrial content while maintaining maximum respiratory capacity, long term exposure induced mitochondrial biogenesis	UA lowered <i>fzo-1</i> and <i>opa-1</i> : Important in mitochondrial fusion machinery UA acted via genes <i>bec-1</i> , <i>sgst-1</i> and <i>vps-34</i> , and the mitophagy genes <i>pink-1</i> , <i>dct-1</i> and <i>skn-1</i> (<i>Nrf2</i>) homolog	UA improves mitochondrial and muscle function	Ryu et al., 2016 (83)
UA, UB, UC	Pheochromocytoma	PC12 cells	10–300 μ g/ml	UC treatment increased lactate dehydrogenase release and membrane lipid peroxidation, and induced cell apoptosis, cell cycle arrest at S phase, and Reactive oxygen species (ROS)	Apoptosis Pathway: Bcl-2/Bax caspase 9 and caspase 3	UC, showed potent cytotoxicity in PC12 cells compared to EA	Yin et al., 2017 (125)

(Continued)

TABLE 1 | Continued

Urolithin type	Activity/ disease tested	Model	Concentration	Effects	Molecular target/pathway	Conclusion	Reference and year
UA and UB	Diabetic cardiomyopathy	Wistar rats induced with type-II diabetes	2.5 mg/kg /day: IP 3 weeks	Prevented early response of cardiac cells to hyperglycemia, improved myocardial microenvironment, and maximal rate of ventricular pressure rise, recovery of cardiomyocyte contractility, and calcium dynamics	SERCA2/PLB Ratio increase and Reduced CX3CL1 when compared to diabetic group	Prevents the initial inflammatory response of myocardial tissue to hyperglycemia	Savi et al., 2017 (49)
UA and UB	Toxicity study	Human peripheral lymphocytes Wistar rats	0.0006–2.29 mg/ml 1,000 mg/kgw oral, 2.5 mg/kg bw i.v	No changes or frameshifts No gene mutations by base pair 28- and 90-day study: Non-genotoxic, no change in clinical chemistry, hematology, or urine analysis. No toxicity observed at any target organ	NA	The NOAEL was the highest dose tested, 5% UA by weight in the diet, or 3,451 mg/kg bw/day in males and 3,826 mg/kg bw/day in females	Heilman et al., 2017 (27)
UA, UA, UB	Skeletal muscle mass	C2C12 myotubes Twelve-week-old male or female C57/Bl6 J mice	15 μ M 10 μ g/day of urolithin B during 28 days	UB not UA enhances differentiation of C2C12 myotubes UB induces muscle hypertrophy, reduces muscle atrophy	Represses ubiquitin proteasome pathway. crosstalk between the AR and the mTORC1 pathway, possibly via AMPK	UB has potential for the treatment of muscle mass loss	Rodriguez et al., 2017 (101)
UA and EA	Cisplatin-induced nephrotoxicity	Male Sprague Dawley rats	50 mg/kg body weight-5 days	UA reduced creatinine and tubular apoptotic cells in Cisplatin-induced kidney damage Reduced macrophage infiltration	Reduced NF- κ b and NOS3, Iba1 induced by cisplatin in kidney	UA mitigates cisplatin-induced nephrotoxicity in rats	Guada et al., 2017 (117)
UA, UB, UC	Prostate cancer	LNCap cells	10–40 μ M	Urolithins inhibited proliferation of LNCaP prostate cancer cells. The mixtures of bicalutamide with UA and UB had additive anti-proliferative effect. Combinations of bicalutamide with UA and UB had attenuated pro-apoptotic activity	NA	The differences in activity of urolithins in prostate cancer imply health benefits and interactions will depend on the type of produced ellagittannins metabolite	Stanislawska et al., 2018 (80)
UA	Anti-inflammatory potential in macrophages	J774.1 murine macrophage HEK,293 cell lines	1–50 μ M	UA strong inhibitor of M1 (LPS) macrophage polarization, UA elevates autophagic flux in macrophages	Inhibit p65 nuclear translocation Reduced pro-inflammatory proteins and NO production Impaired Akt/mTOR signaling	Increased activity of the autophagic cellular recycling machinery aids the anti-inflammatory bioactivity of UA	Boakye et al., 2018 (118)
UA	Colorectal cancer	SW620	1–30 μ M	UA decreased cell proliferation, and cell migration, induced autophagy, and apoptosis. Suppressed cell cycle progression	Induced LC3	UA induces autophagy and inhibit CRC cell growth and metastasis	Zhao et al., 2018 (126)
UA, UB, Iso-UA, and UA conjugates	Breast cancer	MCF-7 MDA-MB-231	1–50 μ M	Alycones exerted antiproliferative and estrogenic/antiestrogenic activities but both their glucuronide and sulfate conjugates lacked these activities	NA	Antiproliferative and estrogen receptor modulatory activity in breast cancer cells	Ávila-Gálvez et al., 2018 (58)

(Continued)

TABLE 1 | Continued

Urolithin type	Activity/ disease tested	Model	Concentration	Effects	Molecular target/pathway	Conclusion	Reference and year
UA	Effect on immune cells	Murine CD4+ T cells	5–50 μM	UA regulates of Ca ²⁺ entry into CD4+ T cells leading to suppression of CD4+ T cell activation	Upregulates the expression of miR-10a-5p which in turn decreases store-operated Ca ²⁺ entry (SOCE), by downregulating Orai1 and STIM1/2 expression	UA could be used a natural immune suppressant during various inflammatory disorders including inflammatory bowel disease	Zhang et al., 2019 (121)
UA	Alzheimers disease (AD)	PPswe/PS1ΔE9 (APP/PS1) mouse model of AD	300 mg/kg	UA ameliorated cognitive impairment, prevented neuronal apoptosis, and enhanced neurogenesis, attenuated Aβ deposition, and peri-plaque microgliosis and astrogliosis in the cortex and hippocampus	UA enhanced cerebral AMPK activation, decreased P65-NF-κB activation and P38MAPK, and suppressed Bace1 and APP degradation	UA imparted cognitive protection by protecting neurons from death and triggering neurogenesis via anti-inflammatory signaling	Gong, 2019 (96)
UA	Tissue deconjugation of UA	LPS administered male Sprague-Dawley rats	26 mg / kg b.w	Tissue deconjugation of UA-glur to UA after lipopolysaccharide (LPS)-induced inflammation	NA	Tissue deconjugation of Uro-A glur to UA after lipopolysaccharide (LPS)-induced inflammation, explaining systemic <i>in vivo</i> activity of free Uro-A in microenvironments subjected to inflammatory stimuli	Ávila-Gámez et al., 2019 (72)
UA	Mitochondrial and cellular health	Healthy, sedentary elderly individuals	1,000–2,000 mg of UA delivered orally	UA has a favorable safety profile UA bioavailable in plasma modulated plasma acylcarnitines and skeletal muscle	Mitochondrial gene modulation	UA induces a molecular signature of improved mitochondrial and cellular health	Andreux et al., 2019 (123)
UA and synthetic analog UAS03	Beneficial activities at gut epithelium	HT29 bone marrow derived macrophages Male mice (C57BL/6J; 6–8 weeks old)	Oral doses 20 mg/kg at 6–24 h	Anti-inflammatory activities and enhanced gut barrier function	Activation of aryl hydrocarbon receptor (AhR) (Nrf2)-dependent pathways to upregulate epithelial tight junction proteins	Attenuated colitis in pre-clinical models by remediating barrier dysfunction in addition to anti-inflammatory activities	Singh et al., 2019 (40)
UA	Increase availability by nanoparticle encapsulation	Male Sprague/Dawley rats	Oral gavage a single dose of 50 mg plain UA, 25 mg P2Ns UA, or 10 mg or 25 mg P2Ns-GA UA	Nanoparticle encapsulated UA led to a seven-fold enhancement in oral bioavailability. It attenuated the histopathological hallmarks of cisplatin-induced AKI and reduced mortality by 63%	Nanoparticle UA therapy downregulated Nrf2 and P53-inducible genes and involved anti-apoptotic signaling	Nanoparticles greatly increase the oral bioavailability of UA leading to improved survival rates in AKI mice, in part by reducing renal oxidative and apoptotic stress	Zou et al., 2019 (124)

(Continued)

TABLE 1 | Continued

Urolithin type	Activity/ disease tested	Model	Concentration	Effects	Molecular target/pathway	Conclusion	Reference and year
UA	Type 2 diabetes	Type 2 diabetes model was induced by HFD; and streptozotocin (85 mg/kg)	UA (50 mg/kg/d) alone or UroA-chloroquine combination for 8 weeks	UA improved symptoms of diabetic mice, pancreatic function indexes. UA decreased mitochondrial swelling and myelin-like cytoplasmic inclusions	Upregulated light chain 3-II (LC3II) and beclin1, downregulated sequestosome 1 (p62), and decreased apoptotic protein cleaved caspase3 partly by (p-Akt)-p-mTOR pathway	UA protects pancreas against diabetes	Tuohetaerbake et al., 2020 (120)
UA	Osteoarthritis	Primary chondrocytes <i>Ex vivo</i> organ culture of articular cartilage	1–15 μ M 1–7 days	No UA cytotoxicity UA protected IL-1 β induced cartilage damage. UA protective in <i>ex vivo</i> organ culture of articular cartilage	UA protected chondrocytes against IL-1 β -induced injury by activating the mitogen-activated kinase (MAPK)/nuclear factor- κ B (NF- κ B) signaling pathways	UA attenuated IL-1 β -induced cell injury in chondrocytes via its anti-inflammatory action	Ding et al., 2020 (45)
UA	Obesity	Six-week-old male C57BL/6 mice 4-week-old male leptin-deficient <i>ob/ob</i> mice	30 mg/kg	UA increases energy expenditure by enhancing thermogenesis in brown adipose tissue and inducing browning of white adipose tissue	UA enhances adipose tissue production of triiodothyronine (T3), which activates thermogenic genes PGC1a and UCP-1	UA suggested as potent anti-obesity agent	Xia et al., 2020 (67)
UA	Alzheimer's disease	SH-SY5Y cells Streptozotocin (STZ)-induced diabetic mouse model	UA:100 nM UA:2.5 mg/kg/day: 8 weeks	UA prevented A β -induced mitochondrial calcium influx, mtROS accumulation, Tau phosphorylation, and cell death in neuronal cells	UA significantly reduced high glucose-induced TGM2 expression and disrupted AIP-AhR complex.	UA may prevent diabetes mellitus associated AD pathogenesis by reducing TGM2-dependent Mitochondria-associated membranes (MAM) formation and maintaining mitochondrial calcium and ROS homeostasis	Lee et al., 2020 (106)
UA	Campylobacteriosis	Abiotic IL-10 $^{-/-}$ mice infected with <i>C. jejuni</i>	0.114 mg /kg/B.W/day	UA lowered pathogen loads in ileum, but not colon. Improved clinical outcome and less inflammatory sequelae of infection. Reduced intestinal and systemic pro-inflammatory immune responses	Lowered IFN- γ , TNF- α	Oral UA administration is a promising treatment option for acute <i>C. jejuni</i> infection	Mousavi et al., 2021 (122)
UA UB urolithin glucuronides	Anti-inflammatory activity	THP-1-derived macrophages, RAW 264.7 macrophages	40 μ M	UA was the most active metabolite in terms of LPS-induced inflammatory response inhibition	Attenuate NF κ B p65 nuclear translocation, and stimulate ERK1/2 phosphorylation	UA the most potent in inflammatory response	Bobowska et al., 2021 (16)
UA mUA UB	SARS-CoV-2, main protease (Mpro) inhibitors	Assay kit consisting of recombinant Mpro	2–50 μ M	Urolithins inhibited severe acute respiratory syndrome corona virus (SARS-CoV-2) SARS-CoV-2 Mpro (by 6.6–100.0%) and bound directly to the Mpro protein	Inhibition of Mpro	Inhibitory effects of tannins and their metabolites on SARS-CoV-2 Mpro	Li et al., 2021 (113)

The UA denotes Urolithin A, UB Urolithin B and UC Urolithin C. The mUA is methylated urolithin A, while the mUB is urolithin B.

(116). The latter one may lead to cytokine storm, which is clinically manifested by acute respiratory distress syndrome and systemic consequences like intravascular coagulation (116). As said in the earlier section of this review, UA exhibits anti-inflammatory activity in various tissues (39, 45, 117–119) by modulating proteins like NF- κ b (44, 45), and other pro-inflammatory molecules like cytokine fractalkine (49, 120). Thus, UA manifests a natural immune-suppressant profile (121). It also protects *C. jejuni* infection in mice and protects other organs including lungs from inflammatory response (122). Furthermore, a recent study illustrated tissue deconjugation of UA-glu to UA in endotoxemia, thus, increasing free UA in systemic tissues, reaching relevant concentrations, and, hence, probably imparting a higher anti-inflammatory potential (72). In the light of these findings, urolithins with its immune modulatory and anti-inflammatory activity could be exploited to reduce such exacerbated inflammatory response as well.

SAFETY ASSESSMENT

Oral administration of UA has been studied at both preclinical (27, 37, 121) and clinical level (123). Both oral and intravenous administration of urolithins showed a higher prevalence of the conjugated forms of urolithins, namely glucuronidated and sulfonated forms (27). Urolithins did not induce genotoxicity in the *in vitro* assays. The no-observed-adverse-effect-level (NOAEL) was the highest dose tested, and UA was given as 5% of the diet, or 3,451 mg/kg bw/day in males and 3,826 mg/kg bw/day in females, in the 90-day oral study. In the *in vivo* studies, the clinical parameters, blood test, or hematology did not point to any toxicity. Human randomized (123), study on the safety profile of UA in the elderly and sedentary human subjects did not show adverse effects on UA consumption in any of the oral dosing regimens. Its presence was seen in plasma and skeletal muscles. The biomarkers of mitochondrial function in the skeletal muscle and plasma metabolomics were also recorded in the study. Efforts are also being made to make urolithins more bioavailable (28, 123, 124). As mentioned earlier, the UA has also been recently recognized as GRAS for its use as an ingredient by FDA, USA. The major studies performed using urolithins has been consolidated in Table 1.

CONCLUSION

To summarize what has been discussed, so far, we propose that urolithins could be beneficial in general wellness and health. Its relevance seems more pronounced in the hormone-dependent tissues, which connote its potential in hormone or endocrine-related pathogenesis. A plethora of evidence pointedly illustrate the health benefits of urolithins in cardiovascular health, muscle strengthening, bone health, breast and endometrial cancer, aging, brain related diseases, and pathologies stemming

from an inflammatory response or its consequence like in the case of COVID-19 infection. Many of these may involve the hormone receptor estrogen receptors along with the other pathways. The mechanism of action of urolithins, mediated *via* estrogen receptors, is very sparsely studied. Competitive binding studies and transactivation assays point to its ability to act as an estrogen agonist. However, it is known that estrogen receptors exhibit a complex and dynamic activity depending on the different conformation it attains according to the ligand structure and binding. It depends on the tissue it acts since the co-factors available and recruited by estrogen receptors vary between the cell types. Albeit estrogen receptor agonists, antagonists and SERMS can activate or repress unique genes, they can also trigger or repress similar subset of genes. Ergo, urolithins need to be examined for its responses in different hormone responsive tissues; its potential as estrogenic and endocrine disruptors, and whether the known health benefits involve an ER-mediated action. Urolithins, or its source, which include ellagitannin-rich food like pomegranate and the bacteria responsible for its production, could also serve as supplement as probiotics. Also, studies can be undertaken to illustrate the potential of urolithins at a clinical level on how these molecules would act in combination with an already known synthetic SERM. Nonetheless, due to pleotropic nature of estrogen receptors, it is important to consider the potential long-term merits and the adverse effects of urolithins in the estrogen receptor-dependent tissues. Taking together the recent research on urolithins, we propose this could serve as an endocrine modulator and that further investigations in this direction need attention.

AUTHOR CONTRIBUTIONS

RV and SS conceived the idea of the article. RV and VR screened and retrieved the data and prepared diagrams. RV prepared the manuscript draft. RV, AS, TS, RA, PM, and LL tabulated various studies. SS reviewed and corrected the manuscript. All authors read and approved the final manuscript.

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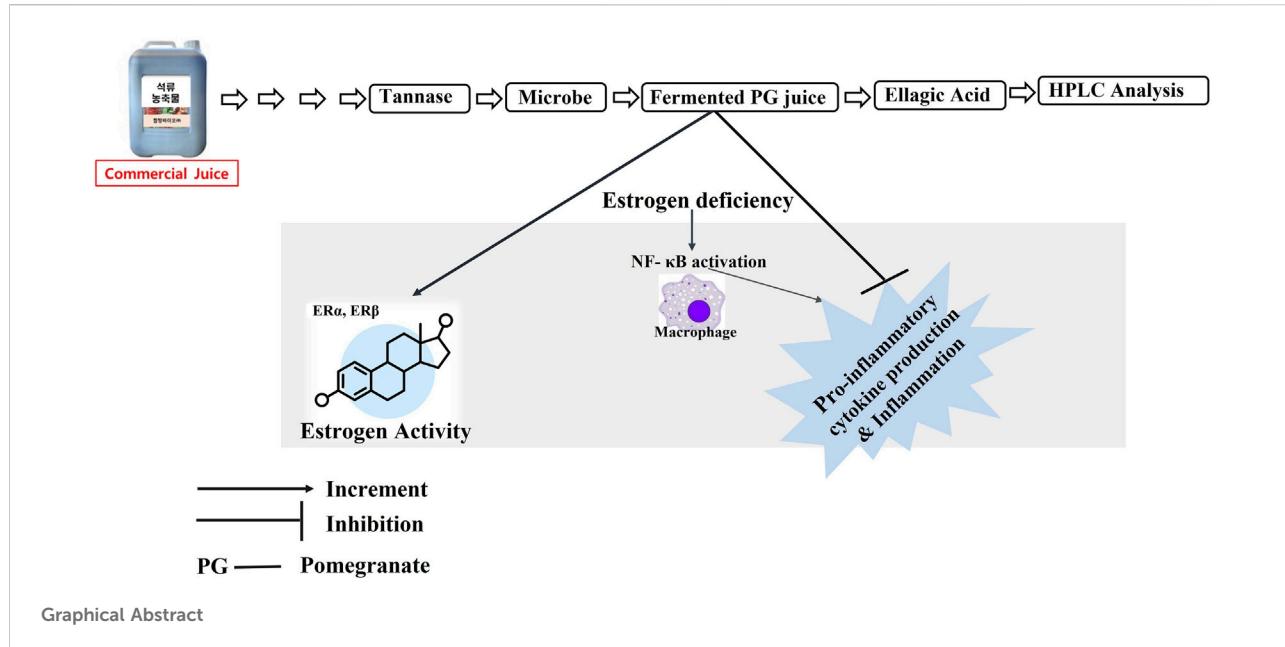
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Pomegranate juice fermented by tannin acyl hydrolase and *Lactobacillus vespulse* DCY75 enhance estrogen receptor expression and anti-inflammatory effect

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Phenolics are phytochemicals in plants, fruits, and vegetables have potential health-promoting efficacies. However, mostly available as a complex form. So, to increase the contents and nutritional value of the phenolic compounds, fermentation is most readily used in the food industry. Especially, the hydrolyzable tannins present in the pomegranate that can be liberated into monomolecular substances, which enhances biological activity. Thus, this study aims to convert hydrolyzable tannins to ellagic acid by fermentation using Tannin acyl hydrolase (TAH) and a novel bacteria strain *Lactobacillus vespulse* DCY75, respectively to investigate its effect on Estrogen receptor alpha (ER α) and estrogen receptor beta (ER β) mRNA expression along with inflammation inhibition. As a result, the fermentation enhanced the ellagic acid content up to 70% by the synergistic effect of TAH and DCY75. Furthermore, fermented pomegranate (PG-F) increased cellular proliferation as well as upregulated the gene expression of estrogen regulators such as ER α , ER β , and pS2 in breast cancer cell line (MCF-7), which commonly used to evaluate estrogenic activity. Moreover, to study the inflammation associated with low estrogen in menopause, we have analyzed the inhibition of nitric oxide (NO)/inducible nitric oxide synthase (iNOS) in RAW 264.7 cells. The PG-F juice did not exert any cytotoxicity in RAW 264.7 cells and inhibited NO production along with the downregulation of a major pro-inflammatory cytokine iNOS which indicates the anti-inflammatory potential of it. To sum it up, the fermented commercial pomegranate juice using a novel bacteria strain increased the amount of ellagic acid that the value added bioactive of pomegranate and it has significantly increased the estrogenic activity via upregulating estrogen related biomarkers expression and reduced the risk of related inflammation via



NO/iNOS inhibition. This study could be a preliminary study to use fermented pomegranate as a potential health functional food after further evaluation.

KEYWORDS

Lactobacillus vespulae, fermentation, menopause, pomegranate, inflammation

1 Introduction

Estrogens as essential steroid hormones, are secreted mainly from the ovary and placenta and have indispensable roles in women's reproductive development. It affects the proliferation, differentiation, and physiological tasks of reproductive organs, including the urinary tract, oviduct, mammary gland, and vagina (Mc Rodrigues et al., 2018). Estrogen is also responsible for performing a significant role in non-reproductive organs such as the immune, skeletal, cardiovascular, and nervous systems (Ikeda et al., 2019). Moreover, low estrogen level may lead to an irregular menstrual cycle associated with menopause or premature menopause (Talaulikar, 2022). Consequently, less ovarian estrogen production during menopause causes hot flushes, night sweats, vaginal dryness, insomnia, changes in metabolism, sexual dysfunction, and physical complications such as depression, mood swings, skin changes, etc. (Secoşan et al., 2019; Hirsch and Manson 2022). Plenty of reports claim that inflammation can increase during menopause due to declining estrogen level (Au et al., 2016; Skoczek-Rubińska et al., 2021). To minimize menopausal complications, the use of synthetic hormones (estrogen or progesterone) that imitate the function of endogenous hormones has become a priority since the 1970s (Mc Rodrigues et al., 2018). Estrogen interacts with two types of estrogen receptors (ER α and ER β) to exert its beneficial

biological effects (Kim et al., 2016). Much evidence has supported the success of estrogen replacement therapy (ERT); however, long-term use of ERT has specific side effects, including weight gain, depression, cancer development, vaginal bleeding, and stroke (Anagnostis et al., 2016; Angioli et al., 2018). Therefore, complementary remedies using herb-based phytoestrogen have become a hotspot research topic in this day and age. Phytoestrogens are plant-derived estrogens that include isoflavones, phenolic compounds, lignans, coumestans, and flavonoids. Phytoestrogen has a similar structure to human estrogen thus it can bind with ERs receptors (Chang et al., 2018). The use of synthetic estrogen may increase the risk of endometrial cancer, myocardial infarction, vaginal bleeding and invasive breast cancer in post-menopausal women (Liang and Shang 2013; Delgado and Lopez-Ojeda 2021), but such risks have not been proven while consuming plant-based phytoestrogens (Glazier and Bowman 2001; Tempfer, Froese et al., 2009). Moreover, much research has found the beneficial effect of phytoestrogen against cancer (Barnes and Peterson. 1995), cardiovascular diseases (Sirotnik and Harrath 2014), obesity, and skin diseases, including immune system (Desmawati and Sulastri 2019) though further studies are needed to specify consumption quantity or type of phytoestrogens.

Punica granatum Linn. Belongs to Punicaceae family and follows the name *Malum granatum* commonly identified as

pomegranate, which is a native fruit in the Middle East. This fruit is intensively used in the folk medicine of innumerable traditions (Li et al., 2006). *P. granatum* (PG) is distributed throughout Iran and the Himalayas in northern India, Malaysia, tropical Africa, Japan, China, Russia, and the drier parts of Southeast Asia, including some parts of the United States (Fadavi et al., 2006). PG is consumed as fresh fruit. The edible parts of pomegranate fruits are used to prepare fresh juice, canned beverages, jelly, jam, and for flavoring and coloring beverage products (Viuda-Martos et al., 2010). Different parts of this fruit are applicable for food, medicine, pharmaceuticals, cosmetics, and nanotechnology; therefore, it is considered a superfood (Putnik et al., 2019; Puneeth and Chandra 2020). According to the research conducted by Wang et al. (2018), PG is rich in hydrolyzable tannins precursor of ellagic and gallic acid, which play a crucial role in the physiological activity of Pomegranate. In addition, a previous report claimed that ellagic acid is a natural selective estrogen receptor modulator (Papoutsi et al., 2005). Bioconversion of tannin has become a hotspot of scientific research due to its commercial significance, strengthened into a glassy state with fast absorption, and scientific relevance to end products (Zhang et al., 2009). Fungal and bacterial organisms can transform hydrolyzable tannin through Tannase (Tannin acyl hydrolase), a key enzyme capable of hydrolyzing ester and depside bonds. In the contemporary study, we used a novel bacteria strain named *Lactobacillus vespulae* DCY75 introduced by HanbangBio laboratory, Kyung Hee University, for fermentation along with Tannin acyl hydrolase (TAH). *Lactobacillus* is a highly useful microbe as well as readily available. *Lactobacillus vespulae* DCY75 is a novel strain isolated from the gut of a queen wasp (*Vespa vulgaris*) by HanbangBio lab (Hoang et al., 2015), Kyung Hee University, and was used to convert PG precursors into ellagic acid. Both enzymes and bacteria are used together for the fermentation of PG juice to increase the ellagic acid content by synergistic effect.

Pomegranate has been used in medicinal systems to combat diseases such as diarrhea, ulcers, diabetes, and cancer (Saxena and Vikram, 2004, 30 Kumari et al., 2012, 31 Khwairakpam et al., 2018). Due to polyphenols high content, hydrolyzable tannins, anthocyanins, and Pomegranate have shown more excellent anti-inflammatory activity and antioxidant activity than Vitamin E, β -carotene, and ascorbic acid (Sharma et al., 2017). As fruit juices are known as a regular functional drink in the market sector, it is essential to make sure the full benefits of the juice are attributed to probiotics are to be experienced. In a previous report, the production of probiotic pomegranate juice through its fermentation by strains of lactic acid bacteria: *Lactobacillus plantarum*, *L. delbruekii*, *L. paracasei*, *L. acidophilus* was examined (Mousavi et al., 2011). However, the fermentation of PG juice using TAH enzyme and novel strain *Lactobacillus vespulae* DCY75 is not reported yet. Therefore, this study evaluated the increment of polyphenol content by fermentation along with estrogen-like effects and anti-

inflammatory activity of fermented Pomegranate (PG-F) juice *in vitro*, along with the potential mechanisms.

2 Materials and methods

2.1 Plant material

The Clear pomegranate concentrate (65 brix, pH 4.4–5.4) was obtained from Fruit Tech Natural S. A. (Murcia, Spain).

2.2 Optimum condition of tannase treatment

Tannase (Tannin acyl hydrolase, TAH) was obtained from Kikkoman Biochemifa (Nishi-Shinbashi, Japan). A 250 ml conical flask was used for tannase treatment. To prepare 5 brix pomegranate juice, the clear pomegranate juice was diluted with distilled water. The optimization of the enzyme was carried out at first. Tannase (500 unit/g) was mixed with 100 ml of 5brix pomegranate juice (pH = 5.4) to a concentration of 0.01, 0.05, 0.1, 0.25, 0.5% respectively. Then it was incubated at 37°C with shaking (150rpm) for 2 h. Secondly, the optimization of incubation time was carried out. Tannase (500 unit/g) was mixed with 100 ml of 5brix pomegranate juice to a concentration of 0.1%. And incubation was carried out at 37°C for 0.5, 1, 2, 3, 4 h. After incubation, dependent on enzyme concentration and time, tannase in the mixture was inactivated by storage in the deep freezer (-80°C). The aliquot was filtered with 0.45 μ m syringe filter into a 2 ml screw top vial before loading on HPLC system.

2.3 Inoculum preparation and culture condition of *Lactobacillus vespulae* DCY 75

The *Lactobacillus vespulae* DCY75T (KCTC 21023T) used in this study were obtained from Ginseng Bank (Suwon, Korea). For seed culture, the *L. vespulae* DCY75 was grown on MRS agar plates for 1 day. A single colony was selected from the plate and inoculated in MRS broth. The Incubation of cultured strain was carried out at 30°C for 1 day.

2.4 Fermentation of pomegranate juice

The fermentation of pomegranate juice was carried out using a 50 ml tube. The total reaction volume was 20 ml. The 0.2 ml of *L. vespulae* DCY75 (approximately 107–8 cfu/ml) was inoculated into 19.8 ml five Brix pomegranate juice (pH 5.4). Then the tannase (500unit/g) was added in the mixture to the concentration of 0.1%. The final mixtures were then incubated

TABLE 1 The conditions of the HPLC system for analyzing phenolic acids.

System/Condition	Phenolic acids (gallic acid and ellagic acid)
Flow rate	1.0 ml/min
Wavelength	260 nm
Injection Volume	5 µl
Solvents	Gradient eluent: A: Methanol B: 0.1% acetic acid in water
Column Temperature	35°C

at 30°C with shaking (150rpm) for 2 days. After incubation, DCY 75 strain and tannase in the mix were inactivated. Then the mixture was centrifuged at 8,000 rpm for 15 min, and the supernatant was filtered with a 0.45 µm syringe prior to HPLC analysis. Instead of DCY 75 strain, 0.2 ml distilled water was mixed with 19.8 ml of 5brix pomegranate juice without tannase as control, followed by the fermentation mentioned above procedure.

2.5 High-performance liquid chromatography system and condition for analyzing chemical contents

For performing the high-performance liquid chromatography (HPLC), Sun et al. (2021) method was followed with minor modification. The PG juice was filtered through a 0.45 µm syringe after centrifugation. The conditions of the HPLC system for analyzing phenolic compounds are shown in (Table 1).

HPLC system consists of Agilent 1,260 Infinity Variable Wavelength Detector (G1314F), Agilent 1,260 Infinity Standard Autosampler (G1329B), Agilent 1,260 Infinity Column Thermostat Compartment (G1316A), and the Agilent 1,260 Infinity Quaternary Pump (G1311B). ZORBAX Eclipse Plus C18 column (250 mm × 4.6 mm, 5 µm particle size) (Milford, MA, United States) was chosen as a stationary phase. For phenolic acids analysis, the eluent compositions were as follows: (0–8 min, 90%–80% B; 8–30 min, 80%–55% B; 30–60 min, 55%–30% B).

2.6 Total phenolic and total flavonoid contents determination

Total phenolics and total flavonoids of each sample were determined using the Folin-Ciocalteu method with slight modifications (Huo et al., 2021). 0.5 g of dried powdered material was extracted using 20 ml 80% methanol for 1 h with three times repetitions, then the filtrate was combined

together for evaporation. For further compound analysis, the crude extract was redissolved in distilled water. To analyze total phenolics, 0.3 ml of PG juice was added to 1.5 ml Folin-Ciocalteu reagent in corresponding wells of a 96-well microplate and incubated for 5 min after shaking thoroughly. Then 1 ml of 7.5% Na2CO3 solution was added to the microplate, and the mixture was kept in the dark for 30 min. Finally, the absorbance was quantified at 715 nm. Total phenolic content was evaluated from a standard curve using gallic acid as the standard. Results were expressed as µmol gallic acid equivalent per Gram of dry weight (µmol GAE/g DW).

Total flavonoid content was measured by using the reaction mixture containing 0.3 ml of PG juice, 0.3 ml 5% NaNO2, and 0.3 ml 10% AlCl3. The well-mixed mixture was allowed to incubate for 6 min, followed by the addition of 0.5 ml 1 N NaOH. After mixing the solution well, the absorbance was immediately measured at 510 nm. Total flavonoid content was calculated with a calibration curve based on rutin, and the results were expressed as µmol rutin equivalent per Gram of dry weight (µmol RE/g DW).

2.7 Assessment of radical scavenging activities

The free radical scavenging activity was evaluated using DPPH method with minor modifications to previous method (Akter et al., 2021). 20 µl of extract and 180 µl of DPPH solution were added to a 96 well plate and then incubated in the dark for 30 min at 25°C, followed by vigorous shaking. Afterward, the absorbance was measured at 517 nm. The percentage inhibition of the samples was assessed by using a formula mentioned below:

$$(1-\text{Absorbance of sample}/\text{Absorbance of control}) * 100.$$

The reducing power activity of the samples were determined using 100 µl of samples with 250 µL of phosphate buffer with a pH 6.6 and 250 µl of (1%) potassium ferricyanide. Then the mixture was incubated at

TABLE 2 The list of primers used for the RT-PCR.

Genes	Forward primers	Reverse primers
ERα	CCGCTCATGATCAAACGCTCTAAG	GCCCTCTACACATTTCCCTGGTT (Farabegoli, Barbi et al., 2007)
pS2	ATGGCCACCATGGAGAACAA	ATAGAACGACCAGGGGACCC (Farabegoli, Barbi et al., 2007)
ERβ	TTCCCAGCAATGTCACTAACT T ACCCAAGGTCTA	TTGAGGTTCCGCATACAGA (Kim, Kim et al., 2016) CGCACATCTCCGCAAATGTA (Ahn, Siddiqi et al., 2016)
iNOS	CGTTCAAGG	
GAPDH	AATGGGCAGCCGTTAGGAAA	GCGCCCAATACGACCAAA (Castro-Aceituno, Ahn et al., 2016)

40°C in a water bath for 20 min. Then the mixture was cooled down and 250 µl of (10%) trichloroacetic acid was added. The mixture was centrifuged at 8,000 rpm for 10 min, and supernatant was added with 100 µL distilled water and 20 µl of instantly prepared (0.1%) ferric chloride solution. The absorbance was determined at 700 nm. A blank was performed without adding PG samples. Ascorbic acid and gallic acid were applied as standards, and the results were expressed in mg of ascorbic acid and gallic acid equivalents per Gram (mg AAE/g DW or mg GAE/g DW) of the sample.

2.8 Cell culture

MCF-7, an ER-positive human breast cancer cell line, and macrophage cell line RAW 264.7 were purchased from American Type Culture Collection (ATCC). MCF-7 cells were cultured in DMEM (containing 4,500 mg/L D-glucose, L-glutamine, sodium pyruvate, and sodium bicarbonate) medium supplemented with 10% (V/V) charcoal-stripped fetal bovine serum (FBS) and penicillin-streptomycin solution. RAW 264.7 cells were cultured in DMEM with 10% FBS, and 1% p/s. The cells were grown at 37°C in a humidified atmosphere of 95% air/5% CO₂. DMEM was purchased from Welgene (Daegu, Korea), FBS and P/S were purchased from GenDEPOT, while Charcoal-Dextran was bought from Sigma-Aldrich Chemicals, United States 17β-Estradiol was purchased from Sigma (Louis, MO, United States).

2.9 Cell proliferation assay

The cell proliferation assay was performed according to the protocol reported by (Lim, Ha et al., 2011) with slight modifications. MCF-7 cells were cultured in a hormone-free medium, seeded at a density of 1 × 10⁴ cells/well in 96-well plates, and allowed to grow overnight at 37°C in a 5% CO₂ incubator. After discarding the medium, cells were separately treated with 17β-Estradiol and Pomegranate. Then the cells were incubated for 24 h, and cell viability was detected using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (20 µl/well) for 2–3 h.

Finally, the cells were stained using 100 µl DMSO to produce formazan crystal into a colored solution. The absorbance was measured at 570 nm using a microplate reader (BioTek Instruments, Inc. Winooski, VT, United States).

2.10 Determination of nitrite levels

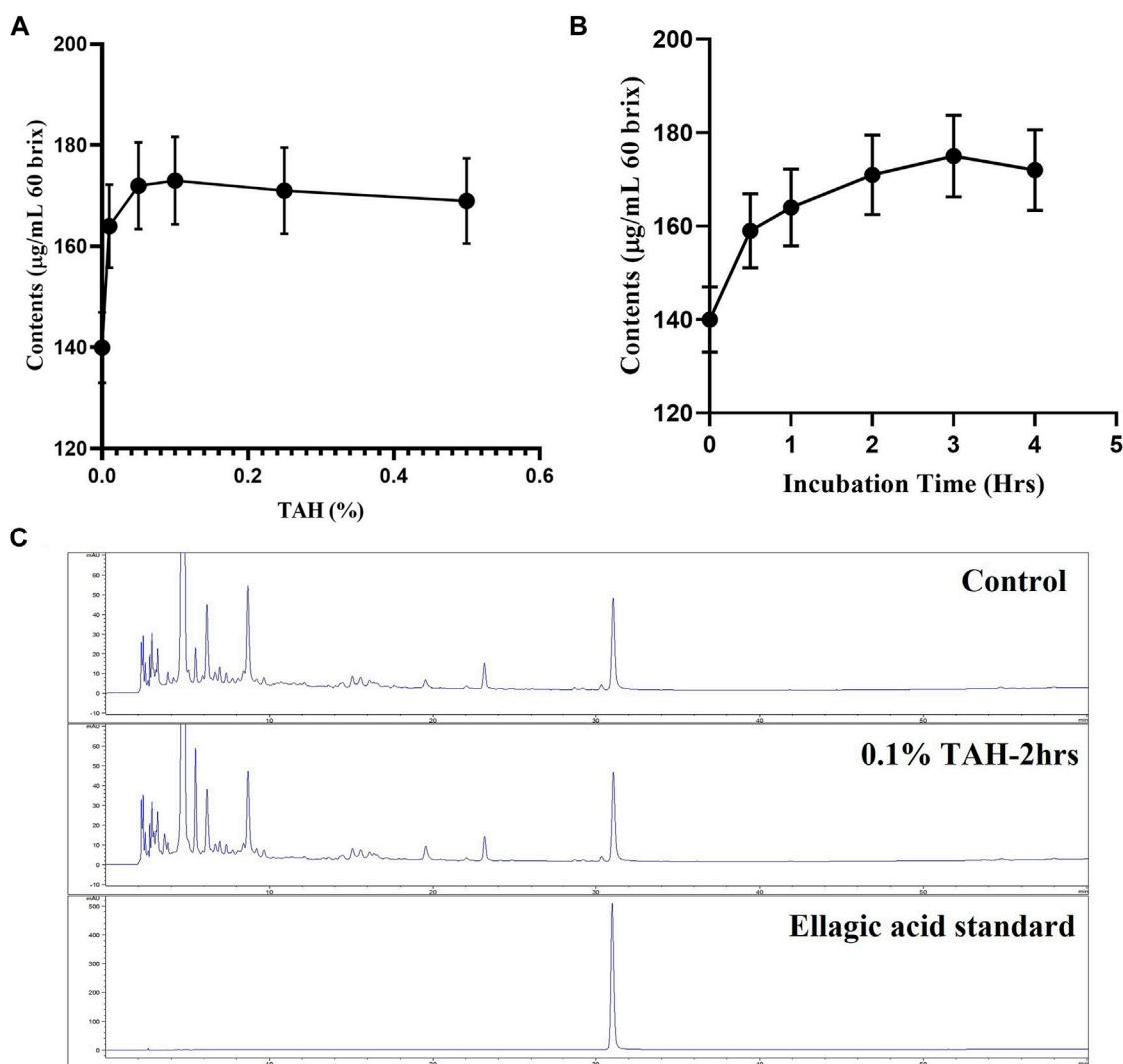
RAW 264.7 cells were used to measure cellular nitric oxide levels. For this, cells were pretreated with different concentrations of pomegranate juice, followed by the stimulation with 1 µg/ml LPS. Then the cells were kept in an incubator for 24 h. Griess reagent was used to quantify the nitrite level in the medium. Concisely, 100 µl of supernatant was mixed with 100 µl of Griess reagent. Finally, the absorbance was measured using a microplate reader (Bio-Tek Instruments, Inc.) at 540 nm (Akter et al., 2022).

2.11 Gene expression analysis

MCF-7 cells were seeded in 12 well plate at a density of 0.5 × 10⁶ cells/well and cultured as mentioned in Section 2.8. The medium was then replaced with phenol red- and serum-free DMEM with or without PM and FPM (100 µg/ml). After incubation for 24 h, cells were washed twice with PBS. To analyze the reverse transcription polymerase reaction (RT-PCR), total RNA was extracted from pomegranate-treated MCF7 cells with TriZol LS reagents (Invitrogen, Carlsbad, CA, United States). After that, cDNA was synthesized following the recommended protocol of a commercial cDNA synthesis kit (Onebio, Lithuania, EU). For cDNA synthesis, 1 µg of total RNA was used. The mentioned conditions were applied for cDNA synthesis: 42°C for 1 h and then 72 °C for 5 min.

Then the synthesized cDNA was used for amplification of the targeted gene. The list of primers used for the RT-PCR is mentioned in Table 2.

For the PCR amplifications following conditions were used: 94°C for 5 min for 1 cycle and then 94°C for 1 min, 56°C for 30 s and 72°C for 1 min for 30 cycles. Data analysis was performed with ImageJ1.30v software (Simova-Stoilova, Vaseva et al., 2010; Hazman, 2022). The relative gene expression levels were

**FIGURE 1**

Optimization of Tannase treatment. (A) Condition of enzyme percentage (B) incubation time (C) HPLC analysis for Tannase treatment.

normalized to the expression of the housekeeping gene (GAPDH).

2.12 Statistical analysis

All data were expressed as mean \pm SE of at least three independent experiments. All analyses were performed using GraphPad Prism (GraphPad software, La Jolla, CA, United States). The total variations between treated groups and untreated (control) groups were determined by Student's t-test and two-way analysis of variance (ANOVA). The significant difference was accepted at a level of $p < 0.05$.

3 Result and discussion

3.1 Optimization tannase treatment

Tannin acyl hydrolase rich is an inducible enzyme produced by various microorganisms. In current study, TAH originated from *Aspergillus oryzae* and was applied to convert active substance present as a precursor (ellagitannins) into ellagic acid. Pomegranate juice was treated with TAH for the enhancement of ellagic acid. And the condition of enzyme percentage and time used for the fermentation was optimized. The result revealed that 0.1% enzyme showed the highest ellagic acid production, whereas 2 h was the optimal time for the best ellagic acid production (Figure 1).

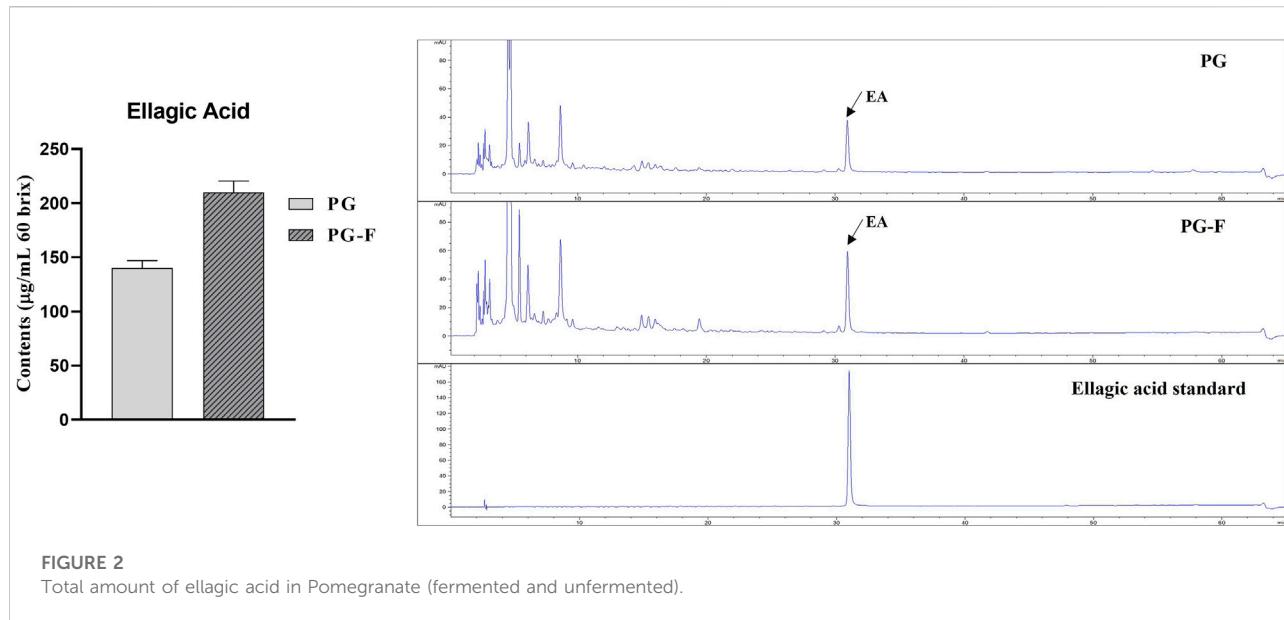


FIGURE 2
Total amount of ellagic acid in Pomegranate (fermented and unfermented).

TABLE 3 Total phenolic and total flavonoid contents.

Pomegranate	TPC	TFC
PG-Control	mg GAE/g FW 0.31 ± 0.01	mg RE/g FW 0.11 ± 0.04
PG-F	0.45 \pm 0.01	0.18 ± 0.02

3.2 Analysis of ellagic acid using High-performance liquid chromatography

Ellagic acid (EA), a phenolic phytochemical, is one of the most critical components of fruits and vegetables (Vattem and Shetty, 2005). Undoubtedly, ellagic acid is a crucial antioxidant and responsible for other pharmacological effects, including cancer, cardiovascular diseases, and inflammation (Ríos et al., 2018). Based on many reports, ellagic acid is Pomegranate's most important bioactive compound, which works against mutagen and carcinogens, heart diseases, atherosclerosis, wound healing, and skin elasticity (Seeram et al., 2005; Moccia et al., 2019). Mostly EA is present in pomegranates in a meager amount. It is mainly present as a complex form of ellagittannins, punicalagin isomers, and granatin, which can be liberated into monomolecular substances with high physiological activity; there are physicochemical and biological methods, but biotechnology using enzymes is effective (Mena et al., 2014; Garcia-Villalba et al., 2015). However, according to our results, fermentation process increased the amount of ellagic acid in Pomegranate. When PG juice was treated with TAH enzyme 0.1% for 2 days along with *Lactobacillus vespolae* DCY75, EA increased by 70% (Figure 2). Different studies have been carried

out to increase the health benefits of pomegranate juice by the fermentation process. For example, the best probiotic lactic acid bacteria were selected by evaluating the growth rate during the fermentation of pomegranate juice as a carbon source (growth factors) and the viability under low-temperature storage conditions (Mousavi, Mousavi et al., 2011) whereas our study focused on selecting a strain that promotes the production of ellagic acid, a useful ingredient in pomegranate juice. Furthermore, HPLC analysis was carried out to determine the levels of EA in the juice from the fermented and unfermented pomegranate juice. The results have shown that fermented Pomegranate has increased the ellagic acid profile compared to unfermented Pomegranate (Figure 2).

3.3 Total phenolic and total flavonoid contents

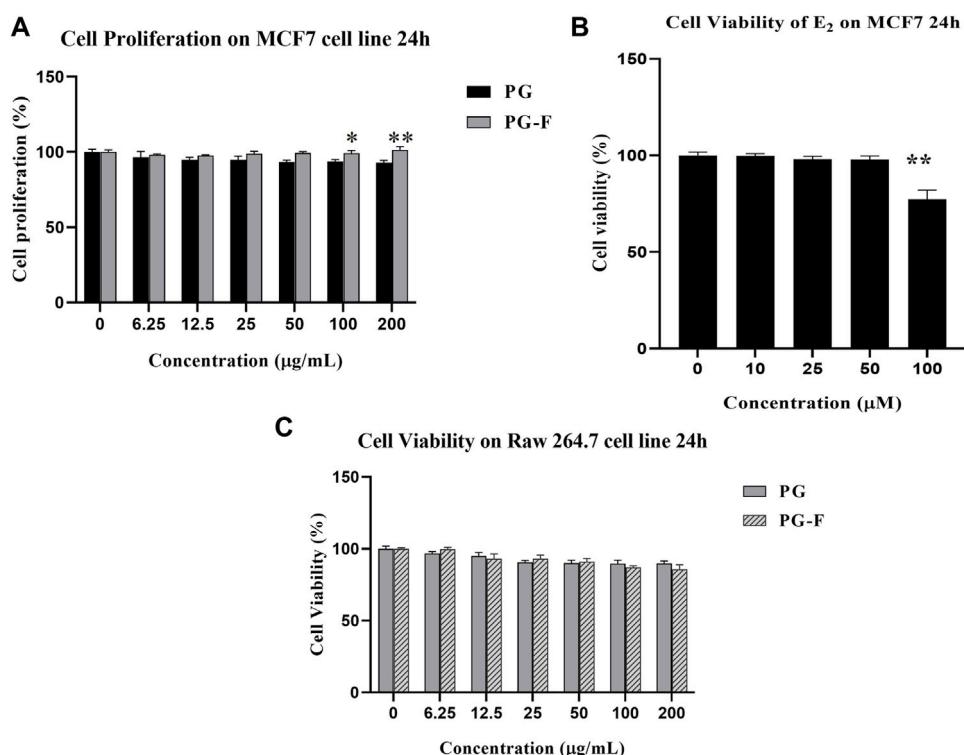
Phenolics and flavonoids are the largest phytochemicals group that possess antioxidant activity in fruits, vegetables, and plants (Ribarova, Atanassova et al., 2005). They also exhibit effects against ulcer, inflammation, tumor, depression, and cancer (Huyut, Beydemir et al., 2017). To estimate the total phenolic content (TPC) and total flavonoid content (TFC) of the pomegranate juice, the Folin–Ciocalteu and aluminum chloride colorimetric methods were carried out, respectively.

The phenolic contents vary, ranging from 0.31 ± 0.01 to 0.45 ± 0.01 mg/g, expressed as gallic acid equivalents (GAE). The flavonoid contents vary from 0.11 ± 0.04 to 0.18 ± 0.02 mg/g, expressed as rutin equivalents (RE). A previous report also reported that the TPC has increased by fermentation (Ríos et al., 2018). In this study, phenolic and flavonoid contents eventually increased in fermented PG compared to

TABLE 4 Potential antioxidant activities of Pomegranate.

Pomegranate ***In Vitro* antioxidant**

	DPPH			Reducing power		
	mg GAE/g FW	mg RE/g FW	mg AAE/g FW	mg GAE/g FW	mg RE/g FW	mg AAE/g FW
PG	1.29 ± 0.02	3.71 ± 0.04	3.71 ± 0.03	4.12 ± 0.12	12.99 ± 0.12	11.13 ± 0.05
PG-F	1.45 ± 0.03	3.92 ± 0.03	4.15 ± 0.04	4.64 ± 0.13	13.82 ± 0.02	12.45 0.08

**FIGURE 3**

Cell proliferation assessment (A) using MTT in MCF7 cells. The data shown represent the mean values of three experiments \pm SD. * $p < 0.05$, ** $p < 0.01$ as compared with the PG treated group (B) Cell viability of various concentrations of estradiol on MCF7 cells. ** $p < 0.01$, as compared with the non-treated group. (C) Cell viability measurement of RAW 264.7 cells following the incubation of various concentrations of Pomegranate for 24 h.

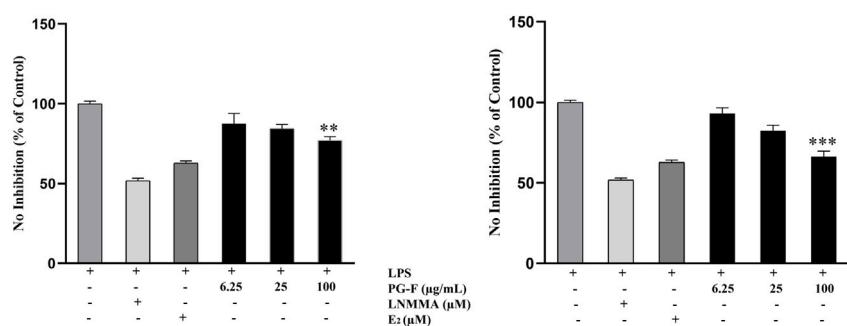
unfermented PG. The amount of TPC has significantly increased by 0.14 ± 0.01 mg/g FW in PG-F than PG (Table 3).

3.4 Antioxidant activity: DPPH and reducing power assays

Synthetic antioxidants with low cost and bland flavor have been used for decades as chemicals for food storage and the prevention of the oxidation process. Various assays are available to measure antioxidant activity. Among those, we selected DPPH and the potassium ferricyanide reducing power assay to quantify

the antioxidant activity of our samples expressed as gallic acid equivalents (GAE) and ascorbic acid equivalents (AAE). Moreover, these assays are generally used to determine the antioxidant potential of different compounds as free radical scavengers or hydrogen donors (Warinhomhoun, Muangnoi et al., 2021).

The DPPH results expressed that the antioxidant capacity of unfermented PG ranged from 1.29 ± 0.02 mg GAE/g FW to 3.71 ± 0.04 mg RE/g FW and 3.71 ± 0.03 mg AAE/g FW. The DPPH scavenging activity of fermented PG ranged from 1.45 ± 0.03 mg GAE/g FW, 3.92 ± 0.03 mg RE/g FW, and 4.15 ± 0.04 AAE/g FW, which shows PG-F has better antioxidant

**FIGURE 4**

Effects of Pomegranate on the NO inhibition. RAW 264.7 cells were pretreated with Pomegranate juice for 1 h and then stimulated with LPS (1 μ g/ml) for 24 h. The concentrations of nitrite were measured as described in the materials and methods. The data shown represent the mean values of three experiments \pm SD. ** p < 0.01, *** p < 0.001 as compared with the group treated with LPS.

activity than PG. Similarly, the result of the reducing power assay revealed that the antioxidant power of PG ranged from 4.12 ± 0.12 mg GAE/g FW, 12.99 ± 0.12 mg RE/g FW to 11.13 ± 0.05 mg AAE/g FW, and the PG-F ranged from 4.64 ± 0.13 mg GAE/g FW, 13.82 ± 0.02 mg RE/g FW to 12.45 ± 0.08 mg AAE/g FW. The result clearly shows the increase in antioxidant capacity of fermented Pomegranate (Table 4).

3.5 PG-F increased the proliferation of human MCF-7 cells

The MCF-7 cell proliferation assay assesses the cellular response in estrogenic or antiestrogenic compounds in an ER-mediated pathway (Ahn, Jeong et al., 2014). Pomegranate (PG) juice was investigated for its ability to increase the cell viability of estrogen-dependent MCF-7 cells. The MCF-7 cells were commonly used in detecting estrogen-like activity. Moreover, cells treated with estrogen-like substances can promote the proliferation of estrogen receptor-positive cells MCF-7 (Ribarova 2005).

As we know, MTT is a ubiquitously used tool to measure toxicity *in vitro* but it has some merits and demerits. According to (Ghasemi, Turnbull et al., 2021) MTT assay measurement is affected by cell number, MTT concentration, and MTT incubation time. It is essential to optimize these parameters for each cell line. Additional optimization of experiments is cost-effective, tiresome, and time killing, yet fundamental. In spite of the limitations, many previous studies have used the MTT assay to examine cell proliferation in MCF7 cells and cell viability of different cells (Hu, Hou et al., 2007; Wang et al., 2018; Tanaka, Onuma et al., 2019; Wang et al., 2021).

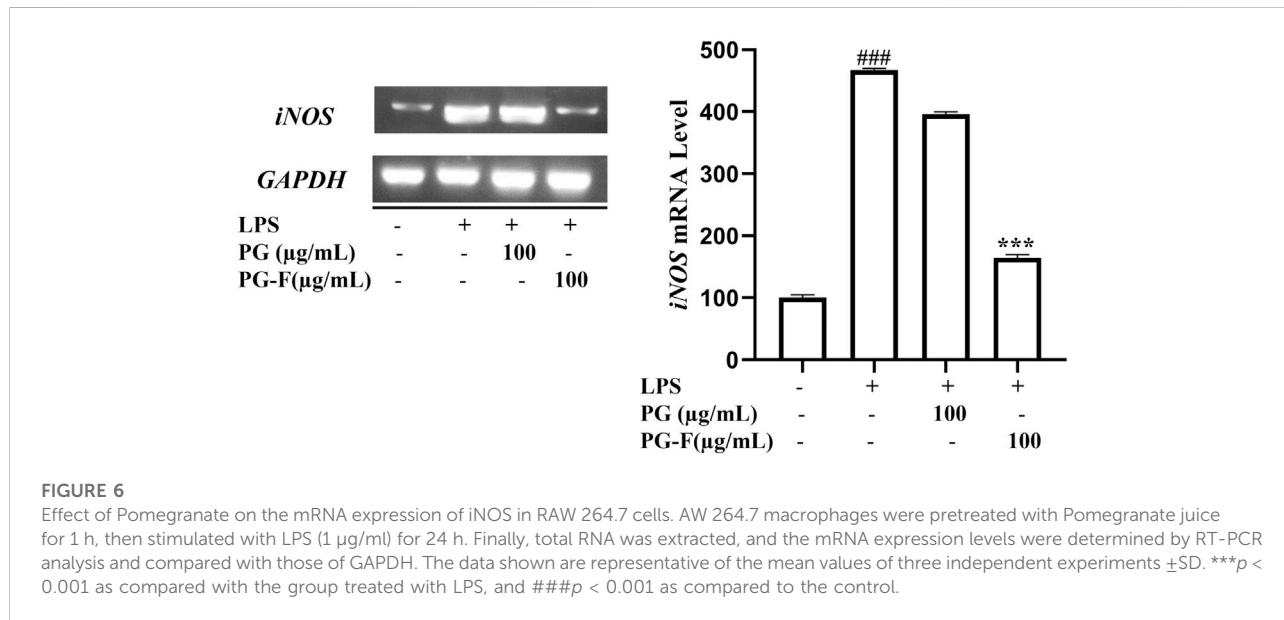
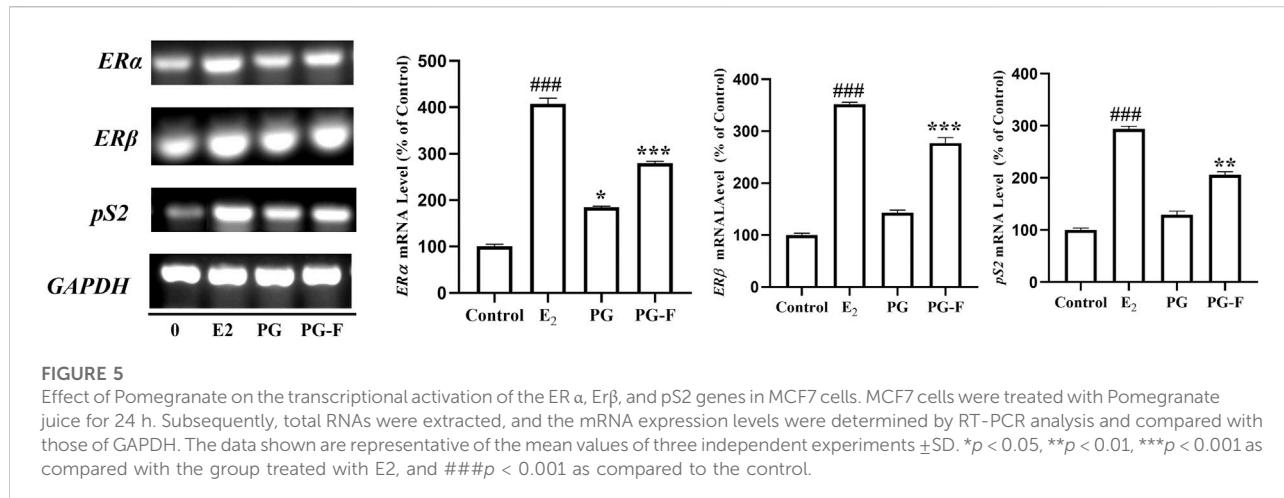
In the current study, we have used MTT assay to determine the cell proliferation in MCF7 cells. And the result showed that Pomegranate and fermented Pomegranate juice (6.25–100 μ g/ml) have also increased cell proliferation (Figure 3A). Compared

to Pomegranate, fermented Pomegranate has increased cell viability more significantly. Estradiol (E₂) was used as an ER agonist (positive control) in estrogen-derived cell proliferation in MCF-7 cells. E₂ has not shown any cytotoxicity until 50 μ M (Figure 3B) but exerted toxicity at 100 μ M. So, we selected E₂ (50 μ M) for further experiment, whereas 100 μ g/ml of juice was chosen for both the pomegranates (PG and PG-F). Besides, both PG and PG-F juice has not shown any significant change in the cell viability of Raw 264.7 cells (Figure 3C).

3.6 Effect of Pomegranate on the lipopolysaccharide induced Nitric oxide production

Macrophages play an essential role in inflammatory diseases associated with the excessive production of inflammatory mediators, such as NO, PGE2, iNOS, and COX-2 (Kim et al., 2018). The most dominant inflammatory mediator is Nitric oxide (NO), a signaling molecule which plays a key role in the pathogenesis of inflammation (Rath et al., 2014). In normal conditions, it gives an anti-inflammatory effect, but the overproduction of NO is considered as a pro-inflammatory mediator of inflammation (Bian and Murad, 2003). The overproduction of nitric oxide happens in abnormal situations such as inflammatory bowel diseases, arthritis, osteoporosis, and other inflammatory diseases of the respiratory system (Akanji et al., 2020). Consequently, subjugating NO overproduction has become an influential target in treating inflammatory disorders. Since estrogens have an anti-inflammatory role, the risks of inflammation increase with decreased estrogen levels in the postmenopausal state (Aviv, Valdes et al., 2006; Christensen and Pike 2015; Park, Lee et al., 2016).

We examined the anti-inflammatory effect of Pomegranate on Raw 264.7 cells. Cells were treated with both Pomegranates types (PG and PG-F), followed by lipopolysaccharide (LPS)



(1 μ g/ml) for 24 h. Our study used a common nitric oxide inhibitor, L-NMMA, as a positive control. As shown in Figure 3, NO production is significantly higher in LPS-treated cells, whereas in Pomegranate treated LPS-induced cells, NO production has decreased in a dose-dependent manner (Figure 4).

As we know, PG-F contains a high amount of ellagic acid that has already been proven to have a significant anti-inflammatory effect (Mousavi et al., 2011). Moreover, fermented pomegranate juice has an ample amount of total phenolic and flavonoids. Previous studies have also shown that phenolics/flavonoids can exert an anti-inflammatory effect via inhibiting NO production as well as suppressing intracellular cytokines (Zhang, Ravipati et al., 2011; Hong, Pangloli et al., 2020). Fermented Pomegranate

has shown better anti-inflammatory effects in comparison with unfermented Pomegranate juice.

3.7 Pomegranate increased the estrogen receptors mRNA expression and estrogenic activity in human MCF-7 cells and suppressed inducible isoform in lipopolysaccharide-induced RAW 264.7 macrophages

The subtypes of estrogen receptors (ER α and ER β) highly modulate the physiological functions of estrogenic compounds. ER α is found mainly in the ovary, mammary gland, uterus, male

reproductive organs, and adipose tissue. In contrast, ER β is present in the colon, the prostate, bladder, ovary, adipose tissue, and immune system (Paterni, Granchi et al., 2014). The transcriptional effects of estrogen are mediated by two key estrogen receptors (ER), ER alpha (ER α) and ER beta (ER β). These cells regulate the uterus morphological changes in response to the circulating estrogen concentrations. To determine the estrogen-like activity of Pomegranate, we measured the expression level of several genes that play an essential role in regulating the reproductive system. ER α , ER β , and the estrogen-regulated gene pS2 present in breast cancer cell line MCF7 were chosen primarily for this study. A considerable number of studies have chosen ER subtype mediated pathway to study phytoestrogenic activity of desired compounds both *in vitro* and *in vivo* (Klinge, Risinger et al., 2003; Oh and Chung 2004; Mishima, Suzuki et al., 2005; Nanashima, Horie et al., 2015; Xu, Ding et al., 2016). For good measure, the inducible isoform (iNOS) is a major downstream mediator of inflammation in various cell types including immune cells, fibroblasts, endothelial cells, and skeletal muscle cells. Besides, iNOS produces large amounts of NO as a defense mechanism (Nakazawa, Chang et al., 2017). And overproduction of NO by iNOS can inhibit energy production, cause direct injury to the mitochondrial respiratory machinery as well as causes inflammation.

The gene expressions were analyzed using RT-PCR to evaluate the estrogen-like activity of the samples. Our results revealed that both PG and PG-F increased the expression of ER α and ER β in MCF7 cells. Both of the genes expressed significantly when compared with the estradiol expression. The ER β expression was higher than that of ER α expression. In addition, both pomegranate and fermented pomegranate upregulated the expression of the estrogen-sensitive gene pS2. However, all of the genes (ER α , ER β , pS2) were more highly expressed by the treatment of PG-F than PG treatment (Figure 5). In addition, many researches have demonstrated that flavonoids/phenolics possessed biological activity as estrogens (Miksicek, 1995; Yang, Allred et al., 2012; Tungmunnithum, Thongboonyou et al., 2018).

As mentioned, PG-F has increased amount of total phenolic/flavonoids content along with high index of ellagic acid, it has shown more preferable estrogenic effect than PG via ER α , ER β mediated pathway.

Our result supports the estrogen-like activity of PG-F by promoting MCF7 cell proliferation and upregulation of ER subtypes. On the other hand, iNOS expression was suppressed by the PG-F treated LPS stimulated Raw 264.7 cells (Figure 6). This supports the lower NO production leading to the anti-inflammatory effect of our samples.

4 Conclusion

In the present study, an increased amount of EA was obtained from the fermented pomegranate juice, where the

fermentation was carried out through TAH and microbe *Lactobacillus vespulae* DCY75 for a high yield of EA. HPLC analysis has shown the difference between the EA yield in fermented and unfermented PG. The EA was significantly higher in fermented Pomegranate. On top of that, fermentation has escalated the level of total flavonoids and phenolics present in pomegranate juice. Correspondingly, the antioxidant activity of PG and PG-F was measured, and it was found that PG-F has higher antioxidant properties. While comparing both the samples, PG-F increased ER receptor expression more significantly than PG. In addition, a low level of estrogen is a crucial reason for inflammation. We measure the NO inhibition and iNOS gene expression in RAW 264.7 cell line. The fermented Pomegranate has reduced NO production dose-dependently and suppressed iNOS significantly, which possesses anti-inflammatory activity of PG-F.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Author contributions

RA performed methodology, drew figures and tables, collected data, and wrote the original draft of the manuscript. JA, JN, and ZR contributed to data. MA and ER performed editing and helped with tables and figures. S-WO and J-HO administered the project, study designed, and sample preparation. BK and DL have investigated the manuscript. DY and SK conceptualized the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author S-WO was employed by the SMART FRUIT CO., LTD. Author J-HO was employed by the Fruitycompany Co., Ltd. DL was employed by the Hanbangbio Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Review Article

Probiotic Bacteria for Healthier Aging: Immunomodulation and Metabolism of Phytoestrogens

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Age-related degeneration gives rise to a number of pathologies, many of them associated with imbalances of the microbiota and the gut-associated immune system. Thus, the intestine is considered a key target organ to improve the quality of life in senescence. Gut microbiota can have a powerful impact in the deterioration linked to aging by its nutritional and immunomodulatory activity. Reduced numbers of beneficial species and low microbial biodiversity in the elderly have been linked with pathogenesis of many diseases. A healthy lifestyle with an elderly customized diet including probiotics can contribute to reducing the chronic proinflammatory status and other age-related pathologies. Beneficial effects of probiotic lactic acid bacteria and bifidobacteria to alleviate some of these disorders based on their immunomodulatory properties as well as their capacity to produce bioactive metabolites from dietary phytoestrogens are summarized. On one hand, the preservation of gut barrier integrity and an increased ability to fight infections are the main reported immune benefits of probiotics. On the other hand, the intake of a diet rich in phytoestrogens along with the presence of selected probiotic bacteria may lead to the production of equol, enterolignans, and urolithins, which are considered protective against chronic diseases related to aging.

1. The Aging Process

The time-dependent biological complex processes that produce a gradual generalized deterioration of the anatomy and physiological functions of organisms are defined as aging. It led to weakness to environmental stress and therefore increases the risk of disease and death. Among multicellular organisms, aging is marked by a progressive decline in the function of multiple cells and tissues. Apparently, the event of aging is genetically determined and modulated by the environment, but the causes of those irreversible changes are still an unresolved challenge. Understanding aging is an important objective that may help to modify the aging process or the senescence effects. The aging rate could be determined by two major circumstances: the accumulation of damage and the effectiveness of somatic maintenance mechanisms [1]. Nine cellular and molecular hallmarks of aging have been proposed by López-Otín et al. [2], which are genomic

instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication. In human cells, the presence of telomerase suggests that cells may be programmed to undergo senescence as a mechanism to “count” cell divisions, although stress and damage accumulation are also important for the telomere shortening [3].

The main aim of aging research is to improve the quality of life. Age-related degeneration gives rise to a number of pathologies, such as osteoarthritis, atherosclerosis, lung emphysema, malignancies (gastrointestinal, prostate), and dementias. The aging process is dependent on antistress responses, which act as antiaging mechanisms. Furthermore, immunosenescence, which can be defined as a decline in the functionality of the immune system, contribute to a chronic state of basal inflammatory activity (inflammaging) [4–6].

The most studied and reproducible nongenetic intervention in aging research is dietary restriction. However, the importance of diet composition has been highlighted when applying a reduction in calorie intake to regulate the lifespan [7]. Another important factor that can play a key role in senescence is the impact of the diet on the gut microbiota composition and the chronic inflammation. Thus, age-related changes in the nutritional behaviour are associated with the imbalances of the microbiota and the gut-associated immune system. A healthy lifestyle with an elderly customized diet including probiotics can contribute to reduce the chronic proinflammatory status and other age-related pathologies [8–10].

1.1. Aging and Gut. The gastrointestinal (GI) tract is characterized by its complexity, being the main and largest site for interaction with the external environment. The GI tract is covered by a single layer of epithelial cells, which are responsible for the digestion and absorption of nutrients and electrolytes, as well as homeostasis. Moreover, the gut-associated lymphoid tissue provides an important first line of defence that controls the equilibrium between tolerance and immunity against orally acquired food and microbes. The human gut contains the enteric microbiota, whose mutualistic relationship contributes to the maintenance of health, including digestion of complex carbohydrates, intestinal homeostasis, synthesis of essential nutrients and vitamins, protection against pathogens, and stimulation of the immune system [11]. Age-associated modifications of the gut cause disorders that clearly affect the quality of life of elderly population, becoming a major cause of morbidity [12].

A distinguishing characteristic of the aging gut is the overexpression of proinflammatory cytokine IL-6, which has an effect on the intestinal barrier function and mucosal immune system [13]. Persistence of inflammaging can also facilitate cancer development and progression [6, 14]. During postmenopause/andropause periods IL-6 levels are increased. Overexpression of IL-6 might have important ramifications with regard to both impaired immunity and intestinal barrier integrity, which can downregulate innate immunity to pathogens and consequently increase the susceptibility to infections in the elderly. Moreover, those changes in the intestinal permeability could be crucial in the development of local (celiac disease, colorectal cancer, or inflammatory bowel disease) and systemic diseases (diabetes, chronic heart failure, or obesity) and even in central nervous system disorders [15, 16].

Physical and immunological impairments of intestinal barrier are correlated with age-related diseases and lifespan. The cross-talk between gut microbiota and the gut-associated lymphoid tissue has a powerful effect on the host immune response which can lead to systemic metabolic effects [17]. Thus, the intestine is a key target organ to improve the quality of life in senescence [18, 19].

1.2. Impact of Gut Microbiota on Aging. Alterations in morphology and physiological functions modify the physical environment of the elderly gut, which affect the composition of the intestinal microbiota. Moreover, antibiotics are still an

irreplaceable therapy for the elderly, which have also a huge influence on the intestinal microbiota composition. Dysbiosis is associated with various metabolic, infectious, and inflammatory disorders including malnutrition, diabetes, bowel diseases, *Clostridium difficile* infections, obesity, colon cancer, and atherosclerosis [20, 21]. An interesting clue to unravel the role of gut microbiota in some aged-related diseases is the big interindividual variations among older subjects compared to the adults [8, 22].

Gut microbiota has a strong impact in human physiology and, therefore, on the health status in the elderly and age-related diseases [23]. Its immunomodulatory properties could help in two main aspects of aging as immunosenescence and inflammaging. Aging can be considered as an immune disorder [24]. Commensal bacteria can modulate the host inflammatory response, mainly by targeting NF- κ B. It has been proposed that an increased presence of IL-6-inducing bacteria in the elderly could be associated with elevated intestinal levels of IL-6 in the gut and therefore at systemic level [14]. Thus, an aged-type microbiota shows a low microbial biodiversity, enriched in pathobionts and facultative anaerobes and depleted of *Firmicutes*, which is linked with an increase of proinflammatory signals [22, 25–27]. Another important aspect to address during the aging process is the interaction between the microbiota and the metabolism of dietary components and their potential beneficial effects in the generation of bioactive nutrients [28, 29].

Host age, health status, and environmental factors can modulate our microbiota composition. Improving the profile of the gut microbiota during human aging, mainly lifestyle factors and nutritional habits, would have an impact on human health and longevity since longevity process is associated with human gut microbiota changes [30]. The role of gut microbiota in human aging include two main aspects: immunomodulatory and nutritional (energy availability and metabolism). Dietary interventions with probiotics or fecal bacteriotherapy could be employed to rationally enrich the gut microbiota of the elderly [20, 30–33].

2. Potential Beneficial Effects of Probiotics on the Aging

Probiotics can be applied to modulate the age-related gut microbiota imbalance and to introduce strains with specific health-promoting effects. The principal claimed benefits of probiotics in elderly people are prevention of diarrheal diseases, protection against pathogens, enhancement of the intestinal barrier function, improvement of gastrointestinal motility and inflammatory intestinal disorders, immunomodulatory effects, and prevention of colon cancer [34, 35].

Probiotic intervention, with or without a specific diet composition, would help to improve the microbiota functionality in order to obtain health benefits during the old age. In this context, a diet rich in phytoestrogens can be considered an interesting therapeutic approach against aging due to their estrogenic and antioxidant actions. Here we summarize two promising beneficial effects of probiotics to alleviate

some age-related pathologies based on their immunomodulatory properties as well as their capacity to produce bioactive metabolites from dietary compounds, such as phytoestrogens.

2.1. Probiotics to Improve Immune-Health. Senescence is associated with a decline in immune function and an increase in inflammation [10]. The effects of IL-6 on intestinal permeability could increase the penetration of microbes and/or toxins into the body [10, 36]. Probiotic intervention can improve some of these age-associated modifications of the immunological features [37–39]. However, despite their promising benefits, little is known about the effect probiotics on intestinal barrier and immune function.

Probiotics can exert beneficial effects on the preservation of gut barrier integrity and function stimulating the activity and growth of beneficial bacteria and regulating the expression of tight junction proteins [40–47].

Aging process affects innate immunity, with reduced activity or number of natural killer (NK) cells, and adaptive immunity, with reduced antigen-specific IgA antibody and cellular immune responses [48]. Probiotic treatments can ameliorate some of these processes modulating cytokine production, improving distribution and function of NK cells, macrophages, granulocytes, and T cells in the circulation, and enhancing mucosal and systemic antibody responses [49–51].

Lactic acid bacteria (LAB) and bifidobacteria are commonly found in the gut of humans and other animals as well as in probiotic supplements and foods. Their immunomodulatory properties can be applied in age-related disorders. Studies carried out on mice demonstrated the potential of probiotics to palliate the effects of aging on the immune system. Administration of *Lactococcus lactis* H61 or *L. rhamnosus* MTCC 5897 improved the age-associated Th1/Th2 imbalance [52, 53]. *Bifidobacterium adolescentis* BBMN23 and *Bifidobacterium longum* BBMN68 isolated from healthy centenarians enhanced both innate and acquired immunity in mice [54]. Supplementation of aged mice with the probiotic *Lactobacillus paracasei* NCC2461 improved the specific adaptive immune response, with higher IgG2a levels after antigenic challenge [55]. The strain *L. rhamnosus* CRL1505 was able to increase the peritoneal macrophages phagocytic activity and the number of intestinal IgA⁺ cells in the intestinal mucosa of aged mice [56]. Recently, the effect of *Lactobacillus plantarum* WCFS1, *L. casei* BL23, and *Bifidobacterium breve* DSM20213 on gut barrier and immunity in accelerated aging mice was investigated. That study found that age-related decline in mucus and systemic immunity can be modulated by probiotics but also highlights the risk of translating the beneficial effects of probiotics observed in young animals or humans to the elderly [57].

Several human studies also show a higher ability to fight infections following probiotic consumption. *Bifidobacterium lactis* HN019 enhanced phagocytic activity and number of NK cells in elderly subjects [51, 58, 59]. A probiotic cheese containing *Lactobacillus rhamnosus* HN001 and *Lactobacillus acidophilus* NSFM increased the cytotoxicity of NK cells in elderly volunteers [60]. Administration of yogurt containing the probiotic strain *Lactobacillus casei* DN-114001 to elderly

people reduced the length of winter infections compared to the control group [61]. Likewise, an improvement in the nutritional and immunological status of enterally fed elderly subjects was observed by the administration of a fermented milk containing *Lactobacillus johnsonii* La1 [62].

2.2. Probiotics, Phytoestrogens, and Aging. Phytoestrogens are polyphenols present in plants or foods derived from plants foods such as soya, flaxseed, cereals, vegetables, fruit, chocolate, and tea [63–65]. Phytoestrogens such as coumestans, stilbenes, ellagitannins, lignans, and isoflavones are similar to endogenous estrogen and therefore they have both antiestrogenic and estrogenic effects [66]. Intake of these compounds may be protective against chronic diseases related to aging, such as cardiovascular and bone diseases, various cancers, menopausal symptoms, and cognitive function [67–73]. These health benefits from phytoestrogens consumption should be attributed to the bioactive metabolites produced by gut bacteria and to the modulation of the intestinal bacterial population [74, 75]. Thus, the intake of a diet rich in isoflavones (soybeans and soy derived foods), lignans (flax seeds, cereals, etc.), and/or ellagitannins (pomegranates, cherries, etc.) along with the presence of selected probiotic bacteria may ensure the production of equol, enterolignans, and urolithins in the gut, respectively [76–78] (Table 1). This approach should be considered in the prevention and improvement of aging-related pathologies.

The transformation of isoflavones, lignans, and ellagitannins by bacteria is an essential step because

- (1) equol, enterolignans, and urolithins are more bioavailable than their respective dietary phytoestrogens [79, 80] (Figure 1),
- (2) equol, enterolignans, and urolithins have more estrogenic/antiestrogenic activities than their precursors. The biological action of these derived compounds is mediated primarily by estrogen receptors [81], modulating hormone levels and expression of estrogen receptors [82, 83]. They may act as anticarcinogens through antiestrogenic actions competing with estradiol to bind estrogen receptors [84]. Equol, enterolignans, and urolithins have various estrogenic effects in postmenopausal women, such as decreased plasma levels of estrone and estradiol sulfate and changes in the metabolism of estrogen (from 16α-hydroxylation to 2-hydroxylation, a less carcinogenic pathway) [85, 86],
- (3) equol and enterolignans are more antioxidants than their precursors [80, 87], acting against DNA damage and lipid peroxidation. The antioxidant activities of enterolignans have also been suggested to contribute to the reduction of hypercholesterolemia, hyperglycemia, and atherosclerosis [88],
- (4) finally, equol, enterolignans, and urolithins have anti-inflammatory effects and exert antiproliferative and apoptosis-inducing activities [89, 90].

Although specific bacteria responsible for the equol, enterolignans, and urolithin production are still being investigated,

TABLE 1: Potential probiotic strains implicated in the metabolism of phytoestrogen.

Bacteria	Transformation/production	Reference
<i>Lb. rhamnosus</i> CRL981	Daidzin to daidzein	[100]
<i>Lb. plantarum</i> CECT 748T	Daidzin to daidzein	[80]
<i>Lactobacillus</i> sp. Niu-O16	Daidzein to dihydrodaidzein	[101]
<i>Lb. rhamnosus</i> INIA P540	Daidzin to dihydrodaidzein	[91]
<i>Ent. faecalis</i> INIA P333	Daidzin to dihydrodaidzein	[91]
<i>Lb. mucosae</i> EPI2, <i>Ent. faecium</i> EPI1, <i>Finegoldia magna</i> EPI3, and <i>Veillonella</i> sp. EP	Daidzein into equol	[110]
<i>Lactococcus garvieae</i> 20-92	Daidzein into equol	[112]
<i>B. breve</i> 15700 and <i>B. longum</i> BB536	Daidzein into equol	[113]
<i>B. adolescentis</i> INIA P784	Enterodiol production from flax seed	[78]
<i>Gordonibacter urolithinfaciens</i> and <i>Gordonibacter pamelaeae</i> DSM 19378T	Urolithin C from ellagic acid	[137]

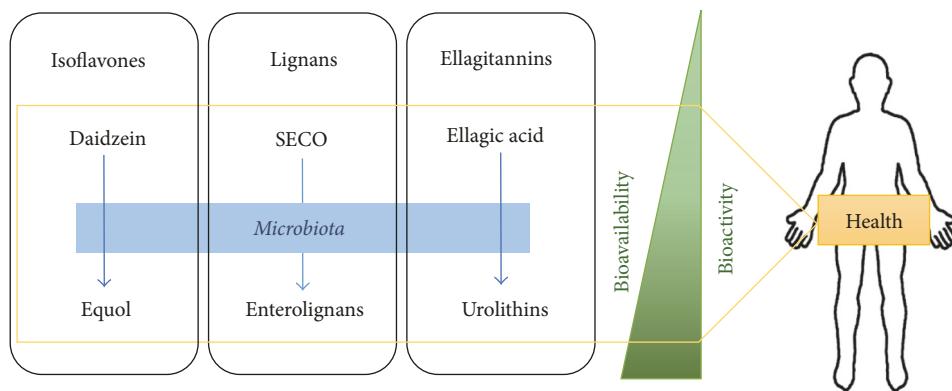


FIGURE 1: Isoflavones, lignans, and ellagitannins intake are metabolized by potential probiotic bacteria to produce equol, enterolignans, and urolithins, respectively. These compounds are more bioavailable and bioactive than their precursors.

some LAB and bifidobacteria have been involved in the metabolism of these compounds [78, 91].

2.2.1. Isoflavones, Aging, and Probiotic Bacteria. In soy and unfermented soy foods, isoflavones are as glycosides such as daidzin, genistin, or glycitin. These compounds are less estrogenic than their aglycones daidzein, genistein, and glycetein, respectively. Daidzin, genistin, or glycitin cannot be absorbed because of their higher molecular weights and hydrophilicity [92]. Then, their bioavailability requires the transformation in daidzein, genistein, and glycetein by means of β -glycosidase activities.

Benefits of soy in aging are derived from the isoflavones metabolism of bacteria, including protection against breast cancer [93], prostate cancer [94], menopausal symptoms [95], heart disease [96], osteoporosis [97], and cognitive function [98].

LAB and bifidobacteria are very important in the transformation of naturally occurring isoflavones in the form of O-glucosides, C-glucosides, or their methylated forms in the bioactive isoflavones daidzein and genistein and even

in the formation of dihydrodaidzein [91]. The capabilities of converting daidzin to daidzein have been observed in *Weissella confusa*, *Enterococcus durans* KH, and *Lactobacillus paraplantarum* KM [99], as well as in *L. rhamnosus* CRL981 [100]. *Lactobacillus* sp. Niu-O16, isolated from bovine rumen contents, converted daidzein to dihydrodaidzein [101].

Daidzein, genistein, dihydrodaidzein, and dihydrogenistein possess physiological properties of interest in healthy aging [68]. The production of daidzein and dihydrodaidzein facilitates the formation of equol and/or O-desmethylangolensin (O-DMA). Equol has enhanced effects due to its greater affinity for estrogen receptors, unique antiandrogenic properties, and superior antioxidant activity. *In vivo* and *in vitro* beneficial effects of equol have been demonstrated [102]. So, it has been possible to demonstrate *in vitro* the effect of equol against aging in skin [103] and nervous system [104]. On the other hand, the effect of equol in the improvement of menopause symptoms and in the prevention of cancers and cardiovascular diseases has been demonstrated both *in vitro* [105] and *in vivo* [106–108]. Evidence from *in vitro* studies suggests that O-DMA may have several cancer-related biological actions. However, results from human metabolic

studies and observational studies of disease risk suggest that these actions may not be physiologically relevant *in vivo* due to the amount and form (primarily glucuronide) of circulating O-DMA [109].

A mix of bacteria composed of *Finegoldia magna* EPI3, *Lactobacillus mucosae* EPI2, *Enterococcus faecium* EPI1, and *Veillonella* sp. strain EP was able to transform daidzein into equol [110]. Similarly, anaerobic incubation of *Eggerthella* sp. Julong 732 and *Lactobacillus* sp. Niu-O16 transformed dihydrodaidzein to S-equol [111], although most of equol-producing microorganisms belonging to the Coriobacteriaceae family, *Lactococcus garvieae* 20–92 [112], *B. breve* 15700, and *B. longum* BB536, were also able to produce equol [113]. LAB and bifidobacteria are also indirectly involved in the production of equol, facilitating the formation of precursor metabolites or favoring the presence of equol-producing bacteria. The administration of *Lactobacillus gasseri* influences the effect of isoflavonoids on the host, probably through changes in the gastrointestinal environment [114].

2.2.2. Lignans, Aging, and Probiotic Bacteria. Lignans, which are the major phytoestrogens occurring in Western diets, have relevant health properties [115]. However, plant lignans are not usually absorbed and must be metabolized to enterodiol and enterolactone prior to absorption [67, 116]. These compounds are the main responsible agents for the beneficial effects of lignans [117]. The transformation of plant lignans by intestinal microbiota is essential for the manifestation of these functions [118]. Enterolignans could be used in ameliorating some menopausal symptoms, protecting against atherosclerotic plaque deposition and due to their hepatoprotective effects [119–122].

Deglycosylation of the secoisolariciresinol diglucoside (SDG) present in the lignan extracts into secoisolariciresinol (SECO) is the first step towards the formation of enterolignans. The production of SECO from lignan extracts and SDG is widespread within LAB and bifidobacteria isolates [78, 123]. SDG hydrolysis is an important feature in probiotic bacteria to enhance the release of SECO, improving its bioavailability for absorption by colonic mucosa and/or the biotransformation to enterodiol and enterolactone by intestinal microorganisms [118, 124].

Nowadays, different bacteria such as *Butyribacterium methylotrophicum*, *Eubacterium callanderi*, and *Peptostreptococcus productus* and the strains *Eubacterium limosum*, *Ruminococcus productus*, *Clostridium scindens*, *Peptostreptococcus productus* SECO-Mt75m3, and *Eggerthella lenta* SECO-Mt75m2 have been involved in the production of enterolignans [65, 118]. Recently, we have described the first probiotic bacterium (*B. adolescentis* INIA P784) capable of metabolizing lignan extracts to produce enterodiol, being the first time that the production of enterolignans by a unique bacterium strain is registered [78].

2.2.3. Ellagitannins, Aging, and Probiotic Bacteria. Ellagitannins are complex derivatives of ellagic acid, which are largely metabolized by the colon microbiota of different mammals [125, 126] and humans prior to absorption [127, 128]. The

microbially mediated origin of urolithin has been demonstrated [129, 130]. Ellagittannins, ellagic acid, and urolithins exhibit anticancer properties *in vitro* and *in vivo* [69, 131]. Pomegranate extracts inhibit the growth of lung, prostate, colon, and breast cancer cells *in vitro* [132–135]. Urolithins inhibit mitogen-activated protein kinase signalling [136], which could curtail the risk of development of colon cancer by inhibiting cell proliferation and inducing apoptosis [90].

To date, only two urolithin-producing strains, *Gordonibacter urolithinfaciens* CEBAS 1/15P and *Gordonibacter pamelaeae* DSM 19378, have been identified [137, 138]. However, these strains cannot produce the downstream products urolithin A and urolithin B. Unraveling the bacterial phyla or group of bacteria responsible for production of these compounds is of great interest since they can be potentially used as probiotics [139]. Consumption of foods containing ellagic acid is also associated with health beneficial effects, and they could be mediated by the presence of urolithin-producing microorganisms [77].

Probiotics able to produce or to increase species related to the production of urolithins or other phytoestrogens such as equol and enterolignans can mean a step forward in the probiotic interventions, increasing the bioavailability of these compounds, and subsequently their therapeutic applications.

3. Conclusion

Age-related changes in nutritional behaviour and microbial diversity during aging result in a higher susceptibility to infections and diseases. Likewise, the presence of some beneficial microorganisms in the gut could help to prevent or delay some age-associated diseases by improving the immune response, or by the production of bioactive metabolites as equol, enterolignans, and urolithins. The evidence for intake of probiotics along with age specifically oriented diet to improve the health during aging is promising. However, further studies for a rational manipulation of the gut microbiota are needed to better define the role of probiotics and to assess the real potential of these interventions.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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Superfoods: Recent Data on their Role in the Prevention of Diseases

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Abstract

By the term functional food we mean food, processed or not, which on the basis of scientific studies can contribute to the achievement of specific operational objectives within the human body and play an important role in the direction of prevention degenerative diseases and health promotion. The possible beneficial properties of functional foods are due to their content in bioactive ingredients, with specific biological properties and effects within the human body. Some examples of processed functional foods are calcium - enriched milk, enriched juices with ω-3 fatty acids, yoghurt with probiotic organisms and phytosterol-enriched margarines. At the same time, constantly new scientific findings confirm the potential beneficial properties of different conventional food, such as tea, blueberries, pomegranate, berries, hippophaes and many others, which are known by the term "superfoods". Recently, the appearance of a multitude of chronic degenerative diseases such as cardiovascular disease, diabetes, obesity, osteoporosis and cancer, has led to ways of defending human health through the adoption of appropriate dietary patterns. Hence, functional foods, provided that they fit inside hygiene and balanced nutrition, are suggested as a potential solution to reinforcing the prevention strategy, avoiding the need for therapy, with the aim of promoting the health of the population. This is the reason why there is an ever-increasing trend particularly in Europe and USA. Also, improved accessibility knowledge and information from consumers, promotes an increased search for information about their beneficial properties.



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Introduction

Conceptual Approach to Superfoods

According to literature one of the categories of functional foods, conventional functional foods,

contain bioactive compounds with specific actions within the human body. In recent years many scientific studies demonstrate the importance of a non-class processed foods whose nutritional

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composition is ideal for strengthening and promoting the proper functioning of the human body.¹ These foods are known as superfoods. Conceptually superfoods are foods that are both high in nutrition value due to a high concentration of nutrients and, on the other hand, great biological value due to satisfactory bioavailability and bioactivity within the body due to a variety of bioactive ingredients they contain.² According to Wolfe (2009),³ superfoods include foods that have a dozen or more unique properties and constitute a specific set of food stuffs, natural or medium processed with numerous nutrients. They are food that according to studies they are able to increase vitality of the human body and can be a good choice for improving the overall health by strengthening the immune system.³ The most important bioactive components of superfoods which have been proven to be beneficial to human body are polyunsaturated fatty acids (ω -3, ω -6), vitamins, minerals, probiotic micro-organisms, antioxidants, essential amino acids, polysaccharides and various enzymes. Since the most important of the superfoods properties is their antioxidant activity, among the most important antioxidants of the superfoods are mostly vitamins A, C and E, flavonoids, selenium, β -carotene, zinc, lycopene, albumin, uric acid, bilirubin, coenzyme Q10 and polyphenols such as anthocyanidins.⁴ The epidemic outbreak of a multitude of degenerative diseases has increased the need to find solutions from the natural environment, with more and more people now turning to food of high nutritional value in order to improve the quality of their life and the promotion of their health. This trend is reinforced from a series of recent scientific studies that have highlighted the importance of various superfoods such as hippophaes, Goji Berries, blueberry, spirulina, kefir, royal jelly and others.⁵ Numerous research data suggest that superfoods are a very good option to improve overall health, boosting the immune system, increasing the production of serotonin and other hormones and promoting the smooth operation of the various organic systems of the human body, but only if they are included in a balanced diet and consumed in moderation and prudence.⁶ The list of superfoods is constantly increasing year by year, while tracking valuable nutrients and understanding the mechanisms of action within the human organism have activated the scientific interest, by promoting more and more scientific research studies.⁸ In

particular, the most important superfoods according to the data obtained from several studies, are the following:⁷

- Fruits: pomegranate, berries, blueberries, raspberries, strawberries, goji berry, chickpeas, grape, acai berry, hippophae.
- Dried nuts: walnuts, almonds, cereals.
- Pulses: red beans, cocoa, sweet potatoes, mastic.
- Vegetables: broccoli, spinach.
- Seaweed: spirulina, chlorella.
- Milk products: Kefir, donkey milk.
- Herbs: ginger, ginkgo biloba, tea.
- Bee products: honey, royal jelly, waxes.

Below data of the most important superfoods according to the scientific literature are presented, such as the hippophaes, maize, blueberries, tea, kefir, maca plant, acai berries, goji berries, etc., their nutritional value, and the potential beneficial actions within the human body.

Tea (*Camellia Sinensis*)

Tea is a product of the leaves of *Camellia Sinensis* plant, which belongs to the family *Theaceae*. It's the second most popular drink worldwide after water and its study is very interested due to its consumption by plenty of people around the world. Depending on the existing industrial processing, tea is categorized into three basic types: a) fermented green tea, which is produced by drying and processing with steam of the fresh leaves of the plant. In this way, the enzymes phenol oxidases are deactivated, so that the polyphenols are not oxidised. b) Oolong tea, which is produced as its leaves plants undergo a moderate fermentation prior to drying. (c) fermented black tea, which undergoes extensive fermentation prior to drying and vaporisation. This permits the action of phenol oxidases which oxidize polyphenols to various oxidized derivatives.^{8,9,10} Fresh tea leaves contain an average of about 36% polyphenols, 25% carbohydrates, 15% proteins, 6.5% lignin, 5% ash, 4% amino acids, 2% lipids, 1.5% organic acids, 0.5% chlorophyll, and carotenoids and various other substances in less than 0.1%. The polyphenols account for 18-36% of the dry weight of the tea and are either in the form of glycosides or as free aglycones. The main polyphenols found in tea are flavonoids and phenolic acids. Of the

flavonoids, catechins make up 12-24% of its dry weight, flavonols 3-4% and anthocyanidins 2-3%.⁹ The EGCG, that is, the ester of epigallocatechin with gallic acid is the most abundant catechin in tea (8-12%) followed by epigallocatechin (EGC) (3-6%) and the gallic ester of epicatechin (ECG) (3-6%).¹⁰ The most important biological role of tea, which classifies it from many scientists in superfoods, is the intense antioxidant activity within the human organism. The main mechanisms of antioxidant action of tea polyphenols within the body is the free radical scavenging activity, complexation of ions that contribute to production free radicals and engaging in pro-oxidant regulation mechanisms and antioxidant enzyme systems.¹¹ Both green tea catechins, and black tea thioflavins bind peroxide radicals by suppressing the chain reactions and retarding lipid peroxidation.¹¹ Most clinical studies show an increase in plasma antioxidant status after drinking tea, suggesting as a possible mechanism the immediate increase of the concentration of catechins and attachment to red blood cell, and various blood components, in which they exert antioxidant effects.¹² Tea has been extensively studied for its possible action on preventing and controlling carcinogenicity. His role lies primarily in the following mechanisms:(i) Antioxidant activity and free radical scavenging, (ii) Binding of activated metabolites of carcinogens. (iii) Effect on carcinogenic elimination enzymes (detoxification enzymes), iv) Prevention of the mutation mechanisms and v) Suspension of the first step of the oncogenesis mechanism.¹³ A ten year study of 8,500 people in Japan showed that volunteers who consumed 10 cups of tea a day had 3 years later cancer compared to those who consumed 3 cups.¹⁴ Other patient control studies noted that the increased consumption of tea were associated with a reduced relative risk of cancer.¹⁵ Cancer types that have been studied more extensively are cancers of the stomach, colon, skin, lung, skin, liver, prostate and breast, while most epidemiological studies have been carried out in countries of Asia where tea consumption is higher.¹⁵ The cardioprotective effect of tea has been extensively explored and seems to be confirmed by many studies. This is related to prevention of oxidation of LDL, improvement of lipid profile, prevention of haemostasis and inflammation, inhibition of atherosclerotic procedure¹⁶ and more generally through mechanisms which relate to

the action of polyphenol-cardiovascular system. Neurological diseases and aging are associated with anxiety and increase in the concentration of various ions in the cells. Recent studies in cell cultures and animal models with neuro-illnesses have shown that antioxidant and anti-inflammatory polyphenols of tea enhance the protection of neurons of the brain and prevent cell death. The tea theanine has been shown to be able to modify serotonin and dopamine levels improving memory and learning skills while improving levels of α -waves, relaxation index and proper brain function. Studies have shown that tea consumption is associated with improvement of the symptoms of neurological diseases such as Alzheimer's and Parkinson's, mainly through action mechanisms in calcium channels, oxidant stress and AGE (Advanced Glycation Endproducts) in cerebral neurons.¹⁷ In addition to antioxidant, anticancer, cardioprotective- neuroprotective and antidiabetic therapy, the antihistaminic-and anti-inflammatory effect of tea on various tissues has been studied. The tea seems to prevent histamine-induced inflammation process and is involved in preventing allergic reactions through inhibiting the release of histamine and its deactivation enzyme protein kinase. In addition, catechins have been shown to reduce the incidence of arthritis through an effect on the endopeptidases activity, while epidemiological studies have correlated tea with bone density increase and health improvement of

Table 1: Summary of some health benefits of tea (*Camellia Sinensis*)

Health benefits	Compound/s responsible for benefits
reduced relative risk of cancer	Polyphenols, green tea catechins, and black tea thioflavins,
cardioprotective effect	Green tea catechins, and black tea thioflavins
improving memory and learning skills	Theanine
reduce the incidence of arthritis	Catechins
Neurological diseases and aging	Tea polyphenols

bone and teeth.^{10,15,17} Some health benefits related to tea consumption are presented at Table 1.

Hippophaes (*hippophae* sp.)

Hippophaes are shrubs of about 0.5 meters height that mainly thrives on land and sandy soils. The most common type is *hippophae rhamnoides* which spreads both in Europe and China. It is consumed either fresh or dried. The fresh fruit requires immediate consumption to preserve its nutrients while there is the possibility of refrigeration to increase its shelf life. The dried fruit can be maintained for long periods of time and is the most common form encountered.¹⁸ Hippophaes is considered by the scientific community to be very important due to its high nutritional value. The fruit has high vitamin C content, ranging from 114 to 1,550 mg per 100 g with an average level of 695 mg / 100 g.¹⁹ These specific levels are up to 15 times higher than orange (45 mg / 100 g). Except for an excellent source of ascorbic acid, hippophaes is rich in other nutrients such as vitamin E, amino acids, minerals (K, Na, Mg, Ca, Fe, Zn, Se), monosaccharides, organic acids, free amino acids, volatile compounds, various flavonoids (quercetin, myricetin, kaempferol) and other phenols, fatty acids, triglycerides, waxes, glycerophospholipids, phytosterols such as β-sitosterol, esters, zeaxanthin and other carotenoids and other compounds. In total, it lists more than 190 nutrients, distinguishing vitamin C, omega-3 and omega-6 fatty acids and vitamin E.²⁰ The moderate consumption of hippophaes in a balanced diet it appears to be able to offer significant

benefits to human health, most important of which are presented below at Table 2:^{18,19,20}

Blueberries (*Vaccinium Myrtillus*)

Blueberries, (*Vaccinium Myrtillus*) come from a bush of 60-90 cm height with thick branch-foliage and translucent foliage. They can be consumed as fresh fruits or dried, the latter be the most common. The dark blue-purple color is due to the high concentration of anthocyanins, that are phytochemicals with strong antioxidant action. After numerous surveys and studies, blueberries now are classified in the category of superfoods. The plethora of nutrients contained in blueberries are presented in Table 3 which refers to 100 g of fresh fruit.²¹ More and more surveys highlight their valuable contribution in health promotion, mainly because of the containing polyphenols and especially anthocyanins. It has been shown that consumption of 120 ml of blueberry juice leads to higher levels of anthocyanins in the blood compared to red and white (2.42 mmol, 2.04 mmol and 0.47 mmol, respectively), indicating the high bioavailability of their anthocyanins. The contribution of blueberries in cerebral function seems to be associated with a reduction in the risk of declaring Alzheimer's disease and other neurodegenerative diseases by reducing symptoms such as loss of balance and coordination and prevention of memory loss. Studies have shown that a quantity of 150 g of blueberries per week may contribute to reduction in blood pressure levels, and a number of other studies have shown a potential effect on the prevention of various types of cancer,

Table 2: Summary of some health benefits of Hippophaes

Health benefits	Compound/s responsible for benefits
• Enhancement of the function of the nervous system	Vitamins of the B-complex as well as all necessary for the human body minerals and trace elements (calcium, magnesium, iron, phosphorus, copper, potassium, selenium, zinc, etc.)
• Protection against cardiovascular diseases and immune enhancement	phytosterols and unsaturated fatty acids (ω-3, ω-6 and ω-9)
• Antioxidant activity: free radical scavenging	Antioxidants: flavonoids, carotenoids
• Strong anti-inflammatory, antimicrobial, analgesic, anti-inflammatory and healing action	vitamin C, omega-3 and omega-6 fatty acids and vitamin E

such as colon cancer, due to the presence of phenolic compounds, tannins, flavones and generally antioxidant ingredients. Specific studies have shown a potential inhibitory effect of flavonoids kaempferol

and luteolin in the development of ovarian cancer. The blueberries, can be included in a balanced diet, because of their low glycemic index which can regulate blood sugar levels especially in people

Table 3: Blueberries nutrient composition per 100g fresh fruit²¹

Carbohydrates	Vitamins		
Fibers	3.6 g	Vitamin A	54.0 IU
Starch	0,0 g	Thiamine (Vit B1)	0,0 mg
Sugars	14.7 g	Riboflavin (Vit B2)	0.0 mg
Sucrose	163 mg	Niacin (Vit.B3)	0.4 mg
Glucose	7,222 mg	Pantothenic acid (Vit B5)	0,1 mg
Fructose	7,355 mg	Vitamin B6	0,1 mg
Lactose	0.0 mg	Folate (Vit B9)	6.0 mg
Maltose	0,0 mg	Vitamin C	9,7 mg
Galactose	0,0 mg	Vitamin E (α -tocopherol)	0,6 mg
Proteins (amino acids)	Vitamin K		
Tryptophan	3.0 mg	Choline	6.0 mg
Threonine	20.0 mg	Betaine	0.2 mg
Isoleucine	23.0 mg	Trace elements	
Leucine	44.0 mg	Calcium	6.0 mg
Lysine	13.0 mg	Iron	0.3 mg
Methionine	12.0 mg	Magnesium	6.0 mg
Cystine	8.0 mg	Phosphorus	12.0 mg
Phenylalanine	26.0 mg	Potassium	77.0 mg
Tyrosine	9.0 mg	Sodium	1.0 mg
Valine	31.0 mg	Zinc	0.2 mg
Arginine	37.0 mg	Copper	0.1 mg
Histidine	11.0 mg	Manganese	0.3 mg
Alanine	31.0 mg	Selenium	0.1 mg
Aspartic acid	57.0 mg	Fat and fatty acids	
Glutamic acid	91.0 mg	Total fat	0.3 g
Glycine	31.0 mg	Polyunsaturated	0.1 g
Proline	28.0 mg	Total ω -3 fatty acids	58.0 mg
Serine	22.0 mg	Total ω -6 fatty acids	88.0 mg

Table 4: Summary of some health benefits of Blueberries

Health benefits	Compound/s responsible for benefits
cerebral function and reduction of neurodegenerative diseases and blood pressure prevention of various types of cancer	polyphenols and especially anthocyanins phenolic compounds, tannins, flavones, flavonoids kaempferol and luteolin
constipation and diarrhea hepatitis C virus protection and infection of the urinary tract prevention	Dietary fibers proanthocyanidins

suffering from type II diabetes, to reduce insulin resistance and act positively on people with obesity and metabolic syndrome. The presence of fibers contributes to the constipation and diarrhea, while the antioxidants proanthocyanidins have been shown to have an effect against hepatitis C virus and can prevent a possible infection of the urinary tract.²² A summary of some health benefits of Blueberries is presented at Table 4.

Royal Jelly: High Nutritional Value Food

Royal jelly is produced by young bees excreted by their subpharyngeal glands, it has creamy texture, an acidic pH and bitter taste.²³ It is a high nutritional food as it contains high amounts of proteins. Twenty nine amino acids have been identified, with aspartic acid glutamic acid being the most abundant.²⁴ Glucose and fructose are in 90% of the total sugar content, while the remaining 10% refers to various glycosides.

The fatty acids of the royal jelly act as natural antimicrobial agents while royal jelly is a good source of metals such as K, Ca, Na, Zn, Fe, Cu and Mn, with potassium being the most abundant, and B complex vitamins (B1, B2, B3, B4, B6, B7, B8, B9 and B12).²⁵ Royal jelly contains 56% water, 17% protein, 18% sugars, 4% lipids, 3% vitamins and trace elements and 2% mineral salts. Among the important features of the royal jelly is the presence of potent peptides (jelleines) that have antibacterial action. Finally, royal jelly contains satisfactory concentration of acetylcholine.²⁶ The beneficial effects of royal jelly within the human body have been recognized by a multitude of scientific studies and this is why it is included into the most important superfoods. The following data for the bioactivity and health benefits of royal jelly is obtained from the literature²³⁻²⁶ and presented below at Table 5.

Table 5: Summary of some health benefits of royal jelly

Health benefits	Compound/s responsible for benefits
Adjustment of blood glucose levels: Royal jelly appears to reduce blood glucose levels and improves lipid profile	Organic acids with insulin-like behavior in the body.
Contribution to connective, muscle and skeletal tissue.	Royal jelly contains the amino acid
Evidence suggests that royal jelly acts as a means of protecting ligaments, muscles and skin	proline necessary for the synthesis of collagen and elastin.
Improving neurological, endocrinological and metabolic disorders	Presence of pantothenic acid. high vitamin content of the B complex vitamins and acetylcholine, which acts as a neurotransmitter.
Effect on urinary and genital system	Royal jelly consumption act as an 'adrenal regulator'. During pregnancy, some cases of swelling, high blood pressure but also eclampsia were treated with royal jelly while positive effect is also observed in amenorrhea.
Elderly disorders, insomnia, increase appetite better mental and psychological functioning of the elderly.	Royal jelly has been proven to increase hemoglobin and red blood cells, resulting in the abnormal production of red blood cells (anemia) that is observed in the elderly. Responsible compounds: the vitamin B1, the phosphorus and tryptophan contained

Spirulina (*Arthrospira plantensis*)

Spirulina is an edible seaweed of fresh water with blue-green color, due to natural pigments contained therein.²⁷ The scientific name is *Arthrospira plantensis* and is growing mainly in alkaline lakes rich in metals and metalloids. Spirulina consists

of 55-70% proteins, 15- 25% carbohydrates, 6-8% fat, 3-4% fiber, while the remaining percentage is divided into metals (iron, potassium, magnesium, etc.), trace elements and vitamins (A, B, E, K) (Table 6). Spirulina contains more than 100 nutrients and is the richest plant source of protein, it has a very

Table 6: Spirulina nutrient composition per 100g²⁸⁻³⁰

Basic Nutrients	Metals / Trace Elements
Protein (g)	62.9
Total Fat (g)	3,8
Polyunsaturated (g)	1.03
Monounsaturated (g)	2,4
Carbohydrates (g)	8,4
Sugar (g)	<0,5
Edible Fibers (g)	6.9
Aminoacids	
Isoleucine (g)	3.41
Leucine (g)	5.29
Lysine (g)	2.7
Methionine (g)	0.78
Phenylalanine (g)	2,8
Threonine (g)	2.98
Tryptophan (g)	1.16
Valine (g)	3.66
Histidine (g)	0.93
Alanine (g)	4.92
Arginine (g)	4.07
Asparagine Acid (g)	5.66
Cystine (g)	0.18
Glutamic Acid (g)	8.05
Glycine (g)	3.08
Proline (g)	2.31
Serine (g)	2.87
Tyrosine (g)	2.73
Vitamins	
Protamine A (carotene) (mg)	60.1
Vitamin B1 (thiamine HCl) (mg)	5.3
Vitamin B2 (Riboflavin) (mg)	2.44
Vitamin B3 (Niacin) (mg)	10.8
Vitamin B5 (Pantothenic Acid) (mg)	1.07
Biotin (μg)	44
Folic Acid (μg)	827
Vitamin B6 (Pyridoxine) (μg)	549
Vitamin B12 (cyanocobalamin) (μg)	182
Vitamin E (mg)	7.78
Inositol (mg)	8.24

Fatty acids

γ-Linolenic (C18: 3) (mg)	1.960.4
γ-Linolenic (C18: 3) (mg)	311.2
Linoleic (C18: 2) (mg)	138.7
Palmitic (C16: 0) (mg)	735.3
Oleic (C18: 1) (mg)	157.3
Myristic (C14: 0) (mg)	85.9
Capric (C10: 0) (mg)	61.2
Laureate (C12: 0) (mg)	59.3
Palmitoleate (C16: 1) (mg)	48.6
Stearate (C18: 0) (mg)	48.3
Arachidate (C20: 0) (mg)	42.2

good source of vitamin B12 and phytochemicals with strong antioxidants properties. Continuous studies confirm that spirulina contain high and wide range of different group of nutrients. Its characterization as superfood is due both to autonomous action of the numerous nutrients it contains (Table 7), but also to the harmonic natural synergy of these compounds.^{28,29,30}

One of the most beneficial properties of spirulina is its effect on blood glucose level. From a range of clinical studies in patients with type II diabetes mellitus, it has been proven that 2g spirulina consumption on a daily basis for four months, led to gradual reduction of glucose levels, while a similar decrease was observed in other markers, such as glycosylated hemoglobin (HbA1c).^{29,31} Another documented action of spirulina is the effect on the respiratory system. Consumption of 1 g of spirulina from patients for four months, or in combination with appropriate medication or by itself appears to contribute to substantially improve of pulmonary function and reduce of levels of immunoglobulin E (IgE). The high content of spirulina in γ -linolenic acid and antioxidants, appears to contribute to

the enhancement of immune system through the stimulation of phagocytosis, the effect of production of cytokines, chemokines and other inflammation mediators, antibody production by B-lymphocytes and HIV proliferation of T-lymphocytes. This demonstrates the regulatory role of spirulina in the functioning of the immune system by enhancing the immune response and preventing over-activity of macrophages.^{32,33,34} In other studies, a possible antiviral effect of spirulina has been demonstrated on human HCV, measles, parotitis, influenza A4, HIV, and enterovirus. The mechanism of action of the spirulina against the viruses is primarily found in preventing their penetration into the host cell via the spirulina polysaccharide spirulan.^{28,34} The antimicrobial action of spirulina in the presence of alpha-linolenic and linoleic acid, and its antioxidant action is recognized by the presence of antioxidant ingredients such as β -carotene, vitamin E, selenium and polyphenols.³⁵ All health benefits are summarized at Table 7.

Maize

Maize is an ancient cereal of high nutritional value. For thousands of years maize remained the main

Table 7: Summary of some health benefits of Spirulina

Health benefits	Compound/s responsible for benefits
effect on blood glucose level, pulmonary function	Low carbohydrate, sugars
Immune system enhancement	γ -linolenic acid and antioxidants
Antiviral effect	polysaccharide spirulan
Antimicrobial and antioxidant activity	β -carotene, vitamin E, selenium and polyphenols

Table 8: Summary of some health benefits of maize

Health benefits	Compound/s responsible for benefits
nutrients absorption and inflammation suppression	soluble proteins, fiber and monounsaturated fatty acids
immune system strengthening	lysine, a basic amino acid and rhodanine
antidepressant action	high inorganic content and especially magnesium
Vision strengthening and protection	provitamin A and vitamine E
Improvement of the lipid profile and regulation of blood sugar levels	high fiber and amino acid content

consumed cereal in Middle East and North Africa. The key attribute which distinguishes it from other cereals, such as wheat, is its very small gluten content and the different quality of it. Also, it has high levels of lysine, making products more digestible. It is probably a form of two-granule wheat (*Triticum turgidum ssp. dicoccum*), while it contains valuable nutrients with multifaceted benefits for the human organism, which characterizes it as a superfood.³⁶ Compared to wheat, it contains less saturated fatty acids, while at the same time it has higher amounts of soluble proteins, fiber and monounsaturated fatty acids. Maize due to the aforementioned composition and especially proteins, inorganic compounds and fibers it appears to contribute to the absorption of nutrients and suppression of inflammation. It contains lysine, a basic amino acid that strengthens the immune system and is important for brain function. It also contains high amounts of magnesium, copper, manganese, zinc, cobalt and others metals and trace

elements.^{37,38} The basic feature of this type of wheat is the absence of allergens and the presence of a small amount of gluten. Studies have shown that maize's inclusion in diet can offer benefits that focus on the following points:³⁶⁻³⁸ A summary of maize health benefits is presented at Table 8.

Kefir

Kefir is a fermented beverage milk, extremely refreshing, tasty, easy to digest and healthy. It is one viscous drink, foaming and sour with harsh taste. Kefir is produced by a lactic and alcoholic fermentation from a wide variety of microorganisms. Thus, it is considered superior to yoghurt that has been produced only by lactic fermentation. Russian scientists worked on the nutritional value of kefir and they have proven its beneficial properties. Kefir is superior to the other acidic milk products with regard to its action against microorganisms which enter the digestive tract with food and water, due to the presence of acetic acid producing bacteria and contained yeasts. It also shows intense hydrolysis of proteins and therefore high concentration of amino acids and peptides in the intestine, plus increased amounts of vitamin B complex.³⁹ A characteristic feature of kefir is the presence of carbon dioxide (CO₂) which contributes to the formation of a finely divided gel, therefore its components can better be in contact with digestive tracts liquids and be better absorbed. Kefir due to its special taste and its microorganisms, it promotes the secretion of enzymes from the stomach and the pancreas and thus facilitates digestion and peristaltic bowel movements and therefore the passage of food from the intestine.

The contribution of kefir to improve human health is recognized by the fact that it displays higher levels of assimilation from the human body against yoghurt, as it provides beneficial bacteria, yeasts, vitamins, minerals (Table 9) and proteins of high biological value. Kefir is a balanced superfood as it appears to boost the immune system, relieves intestinal disturbances and generally contributes to a healthy digestive system.³⁹ Kefirs' beneficial yeasts and bacteria consume most of the lactose of milk so it is an ideal food for sufferers from lactose intolerance. The increased presence of calcium, magnesium and phosphorus (Table 9) contributes to the proper growth of cells and the maintenance of

Table 9: Kefir nutrient composition (mg per 100g)⁴⁰

Vitamins and minerals (mg per 100 g)	
Calcium	120
Phosphorus	100
Magnesium	12
Potassium	150
Sodium	50
Vitamin A	0.06
Carotene	0.02
Thiamine	0.02
Vitamin B2	0.17
Vitamin B6	0.05
Vitamin B12	0.005
Phosphoric acid	0.0095
Niacin	0.09
Vitamin C	1
Vitamin D	0.08
Vitamin E	0.11
Iron	0.05
Copper	0.012
Molybdenum	0.0055
Magnesium	0.005
Zinc	0.36

good body health. From the existing research data, kefir's properties for human health seem to focus on the following points:⁴¹

- It has an effect on the treatment of pathological conditions of the organism e.g. anemia.
- It has an effect on diseases of the digestive system e.g. chronic enteritis.
- It has increased diuretic properties.
- It does not burden the human body with calories as it has low lipid content and low calories.
- It helps to the prevention of atherosclerosis and hypertension.
- It has potential anticancer effects.
- It helps reduce high blood cholesterol.
- It has strong antioxidant and antimicrobial properties.
- It strengthens the immune system.

Maca Plant (*Lepidium meyenii*)

The Maca plant (*Lepidium meyenii*) is a turnip with several similarities to that of radish. It is a herbaceous biennial or annual plant that grows at high altitudes in South America. It has short stem and lace sheets that are renewed constantly. The seeds of the plant are the only way to reproduce and its yellow flowers are converted into fruits of the order of 4-5 millimeters. Scientific studies have highlighted the production of nutrients during the metabolism of a plurality of biologically active aromatic glycosinolates. The nutritional composition of maca plant is similar to that of cereals such as maize, rice, and wheat as it consists of 60-75%

carbohydrates, 10-14% proteins, 8.5% fiber and 2.2% lipids. One hundred g dry skin contains about 250 mg of calcium, 2 g potassium and 15 mg of iron as well as significant amounts of fatty acids and 0.05-0.1% sterols. It also contains vitamins B1, B2, B12, C and E, zinc, alkaloids, tannins and saponins.⁴ Plants' composition is essentially related to its contribution to sexual function and fertility, due to the high amino acid content. The high concentration in amino acids, such as phenylalanine, tyrosine and histidine, confer to the neurotransmitter constructing factor responsible for the neurotransmitter transmission of signals to the brain. The root of the plant is consumed fresh or dried or in the form of a capsule as a dietary supplement.⁴² According to literature maca plant consumption seems to have a number of benefits for human health, which are summarized at Table 10:^{2,42}

Cranberry (*Vaccinium oxycoccus*)

Cranberries (*Vaccinium oxycoccus*), is one type of red acidic berries which are fruits of small deciduous shrubs. Cranberries are mainly found in northern Europe and America. They are consumed fresh, dried, frozen as well as dietary supplements. They are very good source of nutrients and in particular 100 g of cranberries contain 13.30 mg of vitamin C, 4.60 g of fiber, 0.36 mg of manganese, 5.10 mg of vitamin K, and 1.20 mg of vitamin E, while having a very low calorie value. Cranberries are an excellent source of antioxidant ingredients, especially phenolic compounds and in particular they contain high concentrations of proanthocyanidins, flavonoids such as flavonols, quercetin and myricetin, ellagic

Table 10: Summary of some health benefits of maca plant

Health benefits	Compound/s responsible for benefits
improvement of sexual function and increased fertility	high amino acid content
Improving the symptoms of menopause antimicrobial and detoxifying action	Amino acids such as phenylalanine, tyrosine and histidine vitamins B1, B2, B12, C and E, zinc, alkaloids, tannins and saponins
Antidepressant action Supportive in endocrine system, adrenal glands and thyroid, while promoting regulation of metabolism	Amino acids such as phenylalanine, tyrosine and histidine vitamins B1, B2, B12, C and E, zinc, alkaloids, tannins and saponins

acid and chlorogenic acid. Hence they have the potential to provide strong protection against free radicals.⁴³ Research data on the potential beneficial effects of cranberries within the organization are focusing on the following axes:

Cardiovascular System

Studies have shown that consumption of cranberries may retard the progression of the atherosclerotic process to arteries and lower LDL cholesterol levels, hence reducing the risk of developing cardiovascular disease⁴³. Clinical and animal studies indicate that the consumption of cranberry juice decreases LDL and increases HDL cholesterol. Also, cranberry consumption improved lipidemic profile in mice fed a high fat diet.⁴⁴ Favorable effects of cranberry juice on blood lipids have been shown in the population, including obese men,⁴⁵ patients with diabetes mellitus⁴⁶ and patients with low HDL and hypertriglyceridemia.⁴⁷ Additionally, an in vitro study showed that cranberry extracts inhibit the conversion enzyme and, therefore, they reduce blood pressure.⁴⁸

Urinary System

Research evidence has shown that this superfood may contribute to prevention and treatment of urinary tract infections due to high antioxidant content components and in particular proanthocyanidins, which have stalling activity against bacteria such as *E. coli*.⁴³ In a meta-analysis,⁴⁹ with data from 10 studies with a total of 1,049 participants for a period of 12 months, results showed that the consumption of cranberry decreased the overall incidence of urinary tract infection by 35%, especially for women with recurrent urinary tract infections and have reduced the calvary annual percentage of new infections by 39%.³ Possible effect on cancer pathophysiology: Although the data is not yet clear, it seems that consumption of cranberry is likely to have little inhibitory effect on carcinogenesis and may contribute to the prevention of various forms of cancer such as breast, colon, prostate and lung cancer. This seems to be due to their ellagic acid content, antioxidant with strong action that prevents DNA alteration, but also other bioactive phytochemicals.

Other Actions

Consumption of cranberry seems to protect against the appearance of dental problems (gingivitis,

plaque, periodontitis etc.). Also, data show a potential impact on acceleration metabolism, the relief of skin diseases and the improvement of mood, through the effect on hormones.⁴³

Acai berries (*Euterpe oleracea*)

Acai berries are dark blue fruits and are fruits of a palm tree type with a height of 25 meters and 3-meter leaves, thriving in Amazon forest in Brazil. The acai berries are rich in ω -3 fatty acids, amino acids, proteins, electrolytes, metals, fibers, sterols, vitamins A, B1, C and E, iron, calcium, copper, magnesium, potassium and zinc. They contain in high amounts anthocyanins, which give them important antioxidant properties. The increased protein content, even higher than the egg, in combination with its important antioxidant properties make akai berry a superfood. One hundred (100) g of dried fruit purée of acai berries contain 8.1 g of protein, 52.2 g of carbohydrates, 32.5 g of fat, traces of vitamin C, 44.2 g of fiber, 260 mg of calcium, 4.4 mg of iron, 1002 IU vitamin A, glutamate and aspartic acid.^{50,51} Acai berries are consumed either raw or dried, while widely used as a dietary supplement in various forms. The scientific data suggests that eating acai berries within a balanced diet seems to offer significant benefits for the human organism. Consumption of this superfood seems to strengthen the human immune system, exerting intense antioxidant activity and preventing cell destruction by free radicals.⁵¹ They also provide to the human body fatty acids such as ω -3 and ω -9, which improve the lipidemic profile and exert anti-inflammatory action. Additionally, it appears that

Table 11: Summary of some health benefits of Acai berries

Health benefits	Compound/s responsible for benefits
strengthening the human immune system	anthocyanins
anti-inflammatory action	ω -3 and ω -9 fatty acids
protection against cancer cells	vitamins A, B1, C and E and anthocyanins

help human body by excretion of harmful toxins. The high content of acai berry in antioxidants was proven along with its multiple benefits for health. With the participation of 12 healthy volunteers, improvements to metabolic levels and protection against cancer cells were proven. Also after taking blood and urine samples at 12 and 24 hours from the consumption of acai berries juice, a high concentration of antioxidants, mainly anthocyanins, was observed in the blood.^{51,52} A summary of acai berries health benefits is presented at Table 11 below.

Goji berries (*Lycium barbarum*)

Goji berries are endemic fruits of Tibet. The fruits are easily oxidized, and they are almost never fresh, except in the production areas. The degree of drying is differentiated depending on the species. They also called "berries of happiness" with the scientific name *Lycium barbarum*. Goji berries are one of the richest natural sources of nutrients, such as -carotene, vitamins C, E, B1 and B2, minerals, antioxidants and amino acids. Also they contain a high percentage of carbohydrates, fatty acids and fibers. Goji's fruit contains 18 amino acids, 21 trace elements, such as zinc, calcium, germanium, selenium and phosphorus, vitamins of the B complex (B1, B2, B6), more beta-carotene than carrot, more iron from spinach, vitamin E, vitamin C at concentration 500 times higher than oranges,

phytosterols, such as beta-sitosterol and beneficial fatty acids such as linoleic acid.⁵³ Goji berries are superfood with multiple benefits within the human organism. The most important action documented by many studies, is the strong antioxidant protection against the harmful free radicals present in the human body. This has the consequence of being important contributing firstly to the prevention of diseases such as cardiovascular diseases and diabetes, the pathophysiology of which is promoted in the presence of free radicals, and secondly to the strengthening of the immune system. Another action of the goji berries being studied is the possible protection against cancer, although the data is not clear. The presence of polysaccharides in the form of glycosides appears to be associated with an effect on mechanisms of carcinogenesis, while the presence of germanium and various antioxidant substances enhance the potential protection against cardiovascular action. Concerning the effect on cardiovascular prevention goji berries contribute to the reduction of LDL and lowering of blood pressure. Additionally, consumption of goji berries has been associated with the enhancement of the endogenous antioxidant system, through increased production of enzymes such as superoxide dismutase, resulting in reduction of LDL oxidation. The contribution of goji berries to the proper regulation of blood sugar concentration and prevention of insulin resistance is scientifically recognized, since these are the key factors for the prevention of type II diabetes. Research data demonstrate the beneficial effects of goji berries and the enhancement of sexual function, by increasing testosterone levels.^{53,54} Goji berries can reduce inflammation, reduce blocking of the blood vessels, while they can contribute through the antioxidants contained in the prevention of various types of cancer. Goji contribute to improved vision due to its high content of antioxidants, including compounds such as zeaxanthin, lutein, polysaccharides and polyphenolic compounds. Beta-sitosterol of Goji berries seem to significantly inhibit stomach cancer, suppressing the reproduction of cells and toxicity production of cancer cells.⁵⁴ But there are not sufficient scientific data, but only indications hence further research for safer conclusions are needed. A summary of acai berries health benefits is presented at Table 12 below.

Table 12: Summary of some health benefits of Goji berries

Health benefits	Compound/s responsible for benefits
Prevention of cardiovascular diseases and diabetes	Polysaccharides in the form of glycosides, germanium and various antioxidant substances
Reduce of inflammation and blocking of the blood vessels	Antioxidants like phenolic compounds
Stomach Cancer prevention	Beta-sitosterol
Improve vision	Zeaxanthin, lutein, polysaccharides and polyphenolic compounds

Ginger Root (*Zingiber officinale*)

Ginger comes from South Asia with its cultivation now spreading to almost all tropical countries. It comes from a herbaceous plant of the family of *Zingiberaceae*, while it consists of a fleshy rhizome with dense branches. Mainly it consists of water (80%), while it contains satisfactory quantities of potassium, zinc and polyphenols. The nutritional value of ginger per 100 g is: 0.4 g fat, 18 g carbohydrate, 2 g fiber, 2 g protein, 43 mg magnesium, 2 mg copper, 415 mg potassium, 34 mg phosphorus, 16 mg calcium, sodium 13 mg, vitamin C 5 mg, folate 11 µg.⁵⁵ The main bioactivity and health benefits following ginger root consumption, as documented by various research studies, is presented below at Table 13.^{56,57}

Pomegranate (*Punica granatum L.*)

Pomegranates are the fruit of the plant *Punica Granatum L.*, which is a deciduous shrub 2-4 meters high or small tree of 5 to 7 m high. It is cultivated all over the world and thrives in light and cool soils, and multiplied during spring. The fruit of the pomegranate in most varieties consists of 24% bark, 14% of the spores and 62% of the juice. Pomegranate is considered a popular edible fruit, while in recent years a lot of scientific studies show potential beneficial effects of the pomegranate on health promotion and advocacy from various pathologies situations, hence scientists consider it as superfood. The important properties of the pomegranate are directly related to its high content of bioactive substances, including phenolic compounds, polyphenols, ellagitannins and vitamins. Many of these phytochemicals have been shown to have significant antioxidant and

anti-inflammatory properties which promote human health. The most important pomegranate polyphenol is punicalagin which is responsible for over 50% of the strong antioxidant activity of the juice. The high content of pomegranate in polyphenols seems to be associated with the prevention of hypertension and endothelial function improvement.^{58,59} Studies have shown that consumption of pomegranate juice can lead to improved arterial blood pressure, reduced triglyceride levels and increased HDL cholesterol. Therefore, and in combination with other data, there is evidence of a significant contribution of pomegranate to slowing the atherosclerotic procedure and reducing the risk of cardiovascular disease. Also, the punicic acid, which is found in the seeds of pomegranate, has been shown to inhibit the formation of prostaglandins. Generally, several studies have concluded that pomegranate juice consumption can be beneficial to high-risk populations of atherosclerotic and cardiovascular diseases, as well as people with high risk factor for diabetes. The high content of polyphenolic components, such as anthocyanins, ellagitannins, etc., can lead to improvement of cardiovascular biomarkers, provided that pomegranate is part of a balanced diet.⁶⁰ A summary of pomegranate health benefits is presented at Table 14.

Donkey Milk

Donkey milk seems to be the best substitute for human milk due to its content of lactose, proteins,

Table 14: Summary of some health benefits of pomegranate

Table 13: Summary of some health benefits of Ginger root

Health benefits	Compound/s responsible for benefits
Cardiovascular disease prevention	polyphenols
Digestion	Inorganic compounds
Antimicrobial and anti-inflammatory activity	Vitamin C, potassium, zinc and polyphenols

Health benefits	Compound/s responsible for benefits
Antioxidant activity	phenolic compounds especially punicalagin, polyphenols, ellagitannins and vitamins
Hypertension prevention and endothelial function improvement	Punicalagin
Reducing the risk of cardiovascular disease	punicic acid, anthocyanins, ellagitannins

minerals and ω-3 fatty acids. In recent years, studies have highlighted its attributes and is considered as a superfood. The effect from colostrum and donkey milk (of the Martina Franca breed) in general has been evaluated on the functioning of the nerve cells of human peripheral blood (PBMC) to different intervals from lactation. The results showed that colostrum caused higher IgG responses, whereas donkey milk has triggered higher immunoglobulin G (IgG) responses, substances which are related to the strengthening of the immune system. Both the milk and colostrum had an effect on CD25 and CD69 of mononuclear cells that are related to the immune system via their involvement in T-cells. The ability of donkey milk to induce interleukins (IL) (IL-12, IL-1 beta and IL-10) release and tumor necrosis factor alpha (TNF α) was restricted to milk only, while colostrum lacks this ability. Finally, both colostrum as well as milk caused the release of nitric oxide (NO) with milk showing greater NO release activity, which promotes vasodilatation of the arterial endothelium. Taken together, these immunological effects are caused both from colostrum and donkey milk, can be useful in the prevention and / or treatment of human diseases associated with the immune system. Also, NO production from donkey milk can be very useful in preventing atherosclerosis, being a powerful vasodilator and an effective antimicrobial agent, as pathogens and / or their products play a strong proatherogenic role. Finally, donkey milk has been shown to have antimicrobial activity primarily against pathogenic microorganisms, hence protects against possible infections within the human body. However, more research is needed to strengthen data about the vigorous effects of donkey milk.^{61,62,63}

Summary of Superfood Properties

The Antioxidant Properties of Superfoods

Superfoods include a number of beneficial ingredients which the human organism is making use of, for the overall health improvement and the treatment of certain diseases. Superfoods when consumed even in small quantities are beneficial for the human body due to the number of beneficial substances contained. Some of the most important superfoods, such as kefir, maca plant, acai berries, goji berries, hippophaes, maize, blueberries, royal jelly, spirulina, ginger, donkey milk and pomegranate have become particularly important for the human health. Other superfoods that are reported in the literature are the aronia plant, quinoa, blackberry, and others. The most important benefit of superfoods has been shown to come from their high antioxidant content, such as carotenoids, vitamins A and E, and polyphenols. The creation of free radicals in the body is a result of normal biological processes, but the overproduction has a deleterious effect, destroying healthy cells speeding up the aging process and significantly increasing the likelihood of various diseases. At this point antioxidant components interfere and inhibit this process, scavenging the free radicals and inhibiting the resulting pathophysiological conditions associated with a variety of degeneration diseases. In a study conducted at the Department of chemistry at the National and Kapodistrian University of Athens (Proestos' unpublished work), the total antioxidant capacity (measured by the Ferric Reducing Antioxidant Power, FRAP assay) and total phenolic components (measured by Folin Ciocalteu method), after extraction with 50% aqueous methanol of various dried superfoods

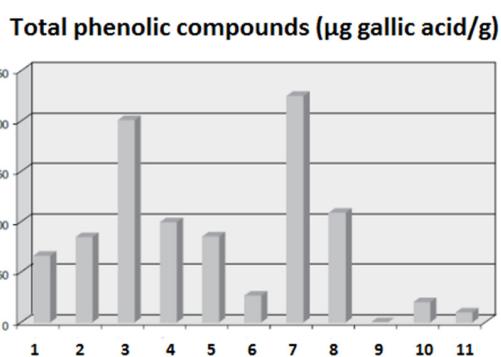


Fig. 1: Total phenolic compounds content (in µg gallic acid/g) of some superfoods, where:
1-cranberries, 2-aronia plant, 3-goji berries, 4-hippophaes, 5-blueberries, 6-quinoa, 7-raspberry,
8-acai berries, 9-ginger root fresh, 10-ginger root dried and 11-maca plant

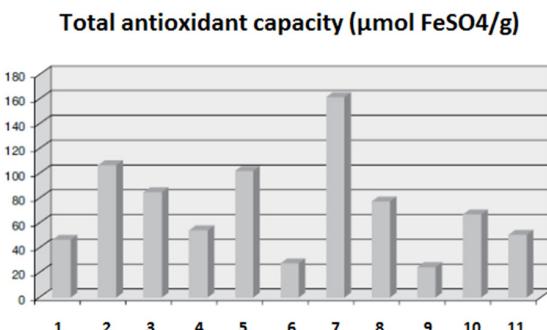


Fig. 2: Total antioxidant capacity (in $\mu\text{mol FeSO}_4/\text{g}$) of some superfoods, where: 1-cranberries, 2-aronia plant, 3-goji berries, 4-hippophae, 5-blueberries, 6-quinoa, 7-raspberry, 8-acai berries, 9-ginger root fresh, 10-ginger root dried and 11-maca plant.

was determined. The results showed high content of total phenolic compounds in goji berries, aronia plant, hippophae, blueberries, acai berries and raspberries, with goji berries and raspberry displaying the highest prices, which is explained by the high concentration of anthocyanins (Fig 1).

All of the above superfoods are rich in polyphenols, and in particular flavonoids, which have a high antioxidant activity. This also explains the high antioxidant activity observed in the same study for most of the superfoods with raspberries, aronia plant, blueberries and goji berries having the highest values (Figure 2).

The high antioxidant activity of superfoods is due to the high concentration of polyphenols on the one hand, and on the other the synergistic action of polyphenols with other antioxidants, such as carotenoids and vitamins A and E.

Superfoods within the Daily Diet

A review of the scientific data shows that superfood consumption can offer the human body a plethora of antimicrobial and antioxidant substances, fiber, plenty of vitamins (A, B, C, K, etc.), inorganic compounds but also beneficial fatty acids such as ω -3, ω -6 and other ingredients in quantities that often exceed the typical daily intake of other foods. The inclusion of superfoods in the daily diet can contribute to reduce the risk of various degenerative diseases, such as cardiovascular diseases, diabetes, metabolic syndrome, obesity, neurological conditions and cancer. So it seems that superfoods serve the basic role of conventional functional foods in

prevention, offering a high amount of bioactive compounds. At the same time, it is important that they provide a plethora of nutrients typically having low caloric content. Regardless of any recognized and scientifically documented health benefits of superfoods, it should be noted that a nutritional program should not be exclusively based in the presence of superfoods but these must be part of a healthy and balanced diet. However, continuous and fast rhythms of everyday life have led to the formation of a diet model in which certain foods which offer value nutrients are missing. This very "nutritional gap" can be covered by superfoods, by offering balanced nutrition on the one hand and significant health benefits on the other. That's the point where particular importance should be given to include superfoods in more and more nutritional standards, but not to replace the consumption of other foods that provide the human body with valuable nutrients. It is important, on the one hand, that consumers are informed by qualified scientific sources for those 'superfoods' for which there are sufficient evidence of their beneficial effects on human health to avoid the possibility of misleading, and on the other hand to understand that superfoods, which are more likely to be consumed as supplements, may have an adverse effect on their health (e.g. hypotension, pro-oxidative stress, removal from a balanced food, etc.). The continuous spread of superfoods is a fact due to the tendency to find new ways of shielding health, due to intense rhythms life of modern reality. In this context, superfoods when consumed stably and meticulously, preferably in the form of fresh or dried foodstuffs and only in special cases as supplements, always in the context of a balanced diet, can play an

important role in the direction of health promotion and the prevention of chronic diseases.

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Conflict of interest

The author declares no conflict of interest

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