

Research Article

Pomegranate Seed Oil (*Punica Granatum* L.): A Source of Punicic Acid (Conjugated α -Linolenic Acid)

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Abstract

The pomegranate seed oil (PSO) presents a typical fatty acid profile which includes high percentages of the punicic acid (PA) – a conjugated isomer of α -linolenic acid. Conjugated α -linolenic acids (CLnAs) are a collective term for the positional and geometric isomers of octadecatrienoic acid (C18:3) with conjugated double bonds. Recently, conjugated fatty acids have attracted significant attention due to reports of its health benefits in a variety of models of metabolic diseases and chronic inflammatory diseases. However, some work is controversial and there is still no consensus in the literature regarding their effects on animal and human organisms. This article presents a review under the pomegranate seed oil and the potential effects of conjugated α -linolenic acid to health.

INTRODUCTION

The numerous reported functional properties of pomegranate (*Punica granatum* L.) make it a unique fruit [1]. For instance, the great antioxidant activity of the fruit, as well as its juice, has been reported to have beneficial health effects and has also encouraged further studies into its nutraceutical potential and its applications in the food industry [2]. Its abundant seeds, normally waste products from pomegranate processing, are also of great interest, once their oil has a particularly rich composition [3].

A high content of phytosterols, tocopherols and a unique fatty acid composition, mainly consisting of punicic acid (55 %), were observed by Melo [4] when investigating the composition of pomegranate seed oil. Punicic acid, also known as trichosanic acid, is an omega-5 long chain polyunsaturated fatty acid and an isomer of conjugated α -linolenic acid (CLnA) with structural similarities to conjugated linoleic acid (CLA) and α -linolenic acid (LnA) [5], such as carbon composition, atomic arrangement and the number of carbon double bonds. These conjugated fatty acids have increasingly attracted scientific interest because of their several potential health benefits [6] including their antioxidant, antitumor, immunomodulatory, anti-atherosclerotic and serum lipid-lowering activities [7]. This article presents a review under the pomegranate seed oil and the potential effects of conjugated α -linolenic acid to health.

POMEGRANATE

Pomegranate has been consumed in many parts of the world for thousands of years. The use of the fruit dates back to biblical times and reports of its therapeutic qualities have echoed throughout the millennia. Its seeds were believed to have resurrecting powers by the Babylonians, to grant invincibility in battle by the Persians and to symbolize longevity and immortality by the Chinese [8].

Pomegranate (*Punica granatum* L.), member of the family Punicaceae, are one of the most ancient edible fruits. They are widely grown in Mediterranean regions (including Iran) and India, but sparsely cultivated in the USA, China, Japan and Russia. Its edible parts (corresponding to 55 - 60 % of the whole fruit weight, of which 75 - 85 % consists of juice and 25 - 15 % consists of seeds) are mostly consumed fresh; however, they are also commercially available as processed foods and drinks, such as juices, wines, liqueurs, jams and canned fruit [3,9]. In addition, the fruit is widely used in therapeutic formulations, cosmetics and food seasonings [8]. Sugars, vitamins, polysaccharides, polyphenols and minerals are found in the edible parts of pomegranate; however, their content in the fruit may vary according to several factors, such as cultivars, environmental growth conditions, ripening stage, postharvest handling and storage conditions, which, in turn, may affect the quality of the fruit as well as the extent of its beneficial health effects [10,11].

Pomegranate, known for its high antioxidant capacity, has been used for medicinal purposes for centuries [12]. Syed et al. [12] investigated their potential antiproliferative, anti-invasive and pro-apoptotic activities against various human cancer cell lineages and in animal models. To date, over 50 substances showing phytoestrogenic and antioxidant activities have been isolated from the seeds, juice and peel of the fruit and from the leaves and flowers of the tree. Dried peel from ripe pomegranate is used in the treatment of stomach-ache and has proved to be effective in preventing lipoperoxidation. Fruit extracts have succeeded in inhibiting herpes and influenza viruses as well as in suppressing the proliferation of human breast and prostate cancer cells. Reductions in bone erosion and in the severity of depression in ovariectomized rats were observed after administering pomegranate-juice concentrate and a seed extract [2,13]. Pomegranate products have also been reported to possess, among others, anti-inflammatory, antimicrobial and immunosuppressive activities and protective effects on liver function and lipid and glucose metabolism [10,14]. According to Al-Muammar and Khan [15], the routine supplementation of pomegranate juice or extracts may prevent or even correct obesity, diabetes, and cardiovascular diseases. As indicated in their review, decreasing energy intake, the intestinal absorption of dietary fats by inhibiting pancreatic lipase, and oxidative stress and inflammation might be important mechanisms for the antiobesity effects of pomegranate food as a whole [15].

The fruit can be divided into three parts: seeds, accounting for about 3% total weight and containing 20% oil; juice, accounting for approximately 30% total weight and pericarp, including skin and inner membranous walls and accounting for approximately 67% total weight [1].

Pomegranate seeds are usually waste products from the fruit processing. Their content may range from 40 - 100 $\frac{g}{kg}$ fruit weight and varies according to cultivars [3]. The seeds have significant antioxidant capacity and a rich chemical composition (sugars, polyunsaturated fatty acids, vitamins, polysaccharides, polyphenols and minerals) [12]. On, average, pomegranate seeds grown in Brazil, carbohydrate content was found to account for 43.97 %, followed by moisture content 38.30 % and high lipid content 14.06 % [16]. Given the fact that great quantities of pomegranate seeds are mostly wasted and that they can be processed to produce an attractive amount of a chemically-rich oil, which may be of great interest to the food industry as food or a nutraceutical ingredient.

Pomegranate seed oil (PSO)

Thanks to its novelty appeal, good customer acceptance, availability at low prices and rich phytochemical composition, the oil from pomegranate seeds has attracted increasing attention as a functional ingredient [17].

PSO represents between 12% and 20% of the seed total weight and consists chiefly of conjugated octadecatrienoic fatty acid. Punicic acid (C18:3-9*cis*,11*trans*,13*cis*), an isomer of this fatty acid, is found in high amounts in the oil and is synthesized *in situ* from linoleic acid (a non-conjugated octadecadienoic fatty acid), present in lower amounts (about 7%) in PSO [1].

Other isomers of conjugated linolenic acid (CLnA), such as α -eleostearic acid (C18:3-9*cis*, 11*trans*, 13*trans*) and catalpic acid (C18:3-9*trans*,11*trans*,13*cis*) occur at lower concentrations [18]. However, fruit genotypes, cultivation sites, harvesting time, climatic conditions, among others, have a major effect on the oil content of seeds as well as on its fatty acid composition [3]. Using thin layer chromatography (TLC), Yuan et al. [19] found that the total lipids in pomegranate seed oil consisted mainly of triglycerides (TG). Likewise, Lansky and Newman [1] reported significantly high levels of fatty acids (over 95%) in PSO, practically all in the form of triglycerides (99%). The triglyceride composition of PSO is varied and the most important patterns are CLnA-CLnA-CLnA and CLnA-CLnA-P [20].

Certain minor compounds, such as sterols, steroids and cerebroside: this last one being a key component of the myelin sheath in mammals, have also been found in pomegranate seed oil

[1]. Phytosterols are present at high concentrations ($4.1 - 6.2 \frac{mg}{kg}$) in PSO, mainly as β -sitosterol, campesterol and stigmasterol; tocopherols also occur, mainly as α -tocopherol ($161 - 170 \frac{mg}{100g}$) and γ -tocopherol ($80 - 93 \frac{mg}{100g}$) [20].

Table 1 shows the lipid profile of pomegranate seed oil from different pomegranate cultivars reported in the literature. Fatty acid composition is expressed as a percentage of the total fatty acids.

The unique composition of PSO has encouraged specific studies into its health benefits, including weight control, skin repair and positive alterations in plasma lipid profiles in hyperlipidaemic individuals [2]. According to Koba et al. [23], since pomegranate abounds in puniceic acid, their seed oil represents a suitable source for the investigation of the physiological roles of this CLnA isomer.

The chemical formula of puniceic acid (PA), an omega-5 long chain polyunsaturated fatty acid and a positional and geometric isomer of α -linolenic acid (LnA; C18:3-9*c*,12*c*,15*c*), is C18:3-9*c*,11*t*,13*c* [24,25]. Both chemical structures are represented in Figure 1. Theoretically, PA features 66 % *cis*-type double bonds and 33 % *trans*-type double bonds [26]. Also, it is structurally similar to conjugated linoleic acid (CLA) and α -linolenic acid (LnA), which have been reported to have numerous health benefits [6]. Therefore, conjugated linolenic acids (CLnAs) have also been investigated for their potential beneficial effects *in vivo* and *in vitro*.

Health effects of PSO

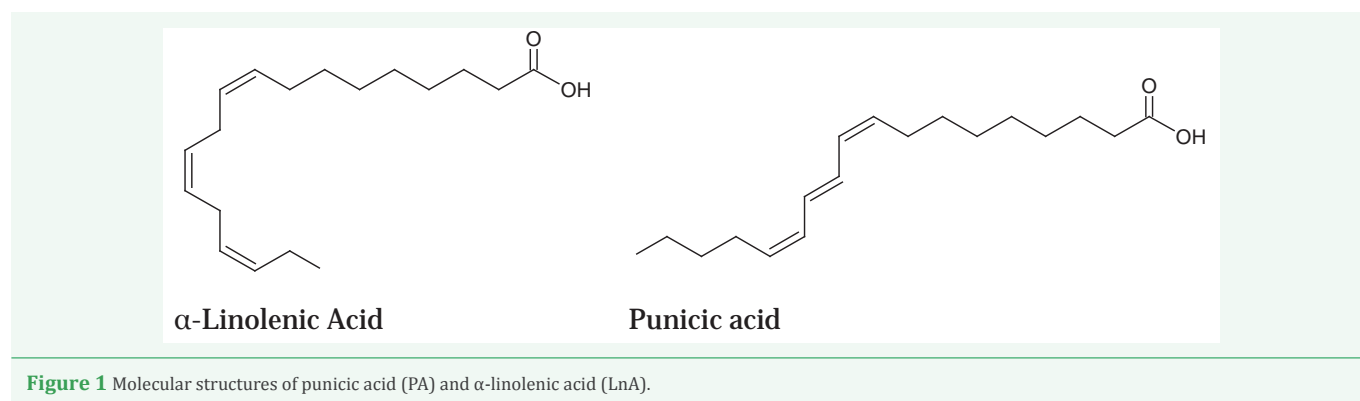
Pomegranate juice has been widely studied for their potential antioxidant and anti-inflammatory activities; in the same manner, PSO has also been reported to have beneficial effects [17,27]. For instance, they have been reported to promote epidermal tissue regeneration [27], boost the immune system *in vivo*, reduce the accumulation of hepatic triglycerides and display chemopreventive activity against hormone-related (prostate and breast) and colon cancers [17].

Given the similarities among CLAs, LnAs and puniceic acid,

Table 1: Fatty acid composition of pomegranate seed oil (PSO). Results are expressed as average (percentage of total fatty acids) \pm standard deviation.

Fatty Acids	Reference (Number of cultivars studied)				
	1 (25)	2 (21)	3 (15)	4 (6)	5 (1)
14:0	0.7 \pm 1.5	0.18 \pm 0.21		0.35 \pm 0.10	
16:0	5.7 \pm 4.1	5.07 \pm 1.30	2.45 \pm 0.19	4.00 \pm 0.76	4.04 \pm 0.34
18:0	2.1 \pm 3.1	4.20 \pm 1.56	1.52 \pm 0.26	2.92 \pm 0.56	2.30 \pm 0.21
18:1 (ω -9)	9.0 \pm 5.6	7.86 \pm 2.25	4.19 \pm 0.61	5.68 \pm 1.69	5.29 \pm 0.25
18:2 (ω -6)	10.8 \pm 6.9	8.36 \pm 2.36	4.49 \pm 0.49	4.08 \pm 1.04	6.05 \pm 0.53
18:3 (9c11t13t)		10.70 \pm 4.44	6.41 \pm 0.27		
18:3 (9t11t13t)		8.78 \pm 5.16	1.03 \pm 0.16		
18:3 (9t11t13c)		15.24 \pm 6.17	3.48 \pm 0.34		
18:3 (9c11t13c)	71.5 \pm 17.9	36.98 \pm 10.12	74.11 \pm 1.55	81.22 \pm 2.15	58.14 \pm 2.10
20:0		0.69 \pm 0.14	0.39 \pm 0.04	0.53 \pm 0.18	0.50 \pm 0.04
20:1		1.65 \pm 1.85	0.61 \pm 0.09		0.61 \pm 0.05
22:0	0.1		0.18 \pm 0.02		
24:0		0.97 \pm 0.94		1.00 \pm 0.24	

1. [21] - India; 2. [20] - China and Tunisia; 3.[3] - Turkey; 4. [22] - Georgia, USA; 5. [16] - Petrolina, Brazil - 23.05% unidentified fatty acids).



a CLnA found in high amounts in pomegranate seed oil (PSO), research into the health benefits of PSO has focused on body weight and serum lipid reducing effects; however, to date, results have been inconsistent among them. For example, Koba et al. [23], using different seed oils (pomegranate, bitter gourd, Chinese catalpa and pot marigold), reported that only the rats fed PSO showed a considerable reduction in perirenal adipose tissue weight compared with the control group (rats fed α-linolenic acid-enriched linseed oil). In a similar study, Koba et al. [28] suggested that punicic acid may have played an important role in the dose-dependent reductions observed in perirenal and epididymal adipose tissue weights in rats fed a basal diet containing 10% rapeseed oil and 0 %, 0.25 % or 0.50 % punicic acid. Yuan et al. [19], however, observed no change in adipose tissue weight in mice fed an experimental diet supplemented with 1 % PSO for six weeks.

Dietary PSO, rich in punicic acid, attenuates hepatic triglyceride accumulation in obese, hyperlipidemic rats [29]. Statistically significant, dose-dependent increases in serum triglyceride and phospholipid levels were observed in rats fed experimental diets containing PSO when compared with mice in the control group. Although higher serum total cholesterol levels were also

detected, their increase showed no statistical significance [30]. Yuan et al. [19], in contrast, reported significantly lower liver triglyceride levels in mice fed a diet supplemented with PSO than those in rats in the control group and in rats fed CLA-enriched diet; however, no significant changes in phospholipid and total cholesterol levels were observed.

To date, there have been few studies researching PSO effects on humans, and the results have also been inconsistent among them. Recently, after a four-week treatment with PSO, no changes in body composition and serum cholesterol and LDL-cholesterol levels were detected in hyperlipidemic patients, whereas a decrease in triglyceride levels took place [31]. Yuan et al. [24,25] observed no significant changes in any serum lipid levels when healthy subjects were given seeds of *Trichosanthes kirilowii*, another natural source of punicic acid (three grams per day, for 28 days).

Other potential health effects have also been suggested for PSO. For instance, reduced body weight gain and leptin and insulin levels were detected in CD-1 mice (pathogen-free) given a high-fat diet supplemented with PSO, indicating a potential risk-reducing effect on the development of type 2 diabetes [32]. Similarly, diet supplementation with PSO was found to improve

glucose tolerance and suppress obesity-related inflammation [33]. In addition, PSO was reported not to affect liver insulin sensitivity, but to improve peripheral insulin sensitivity in mice fed a high-fat diet [18]. They also found that PSO was able to increase carbohydrate oxidative capacity and concluded that it prevents diet-induced obesity and reduces insulin resistance.

Pomegranate seed oil (PSO) was also shown to exhibit *in vivo* antioxidant and anti-inflammatory activities by limiting neutrophil-activation and lipid peroxidation consequences, which may be useful in the prevention and treatment of several inflammatory diseases, such as inflammatory bowel disease, rheumatoid arthritis and coronary heart disease [34]. It led to improved renal function and reduced protein and lipid damage in rats undergone hexachloro-1,3-butadiene (HCDB)-induced nephrotoxicity [35]. The mechanisms involved, however, are yet to be elucidated.

Conjugated α -Linolenic Acid (CLnA)

Conjugated linoleic acid (CLA) was first identified in the 1980s and, since then, research into their cytotoxic effect on cancer cells as well as body fat-reducing and lipid metabolism-normalizing effects has advanced; more recently, since 2000, other fatty acids containing conjugated double bonds, CLnAs, have also attracted increasing scientific attention as novel types of functional fatty acids because of their cytotoxic and antitumor properties [36].

Conjugated α -linolenic acid (CLnA) is a collective term describing a group of octadecatrienoic fatty acid isomers with three conjugated double bonds (C18:3), which may be of *cis* or *trans* configuration at positions 9,11,13 and 8,10,12 [3,27].

Source, biosynthesis and isomers of CLnAs

CLnAs occur in plant lipids, especially seed oils [37], and represent between 40 and 80 % of total fatty acids [38, 39]. Table 2 shows the five most commonly known isomers found in seed oils from important plants: α -eleostearic acid (α -ESA), punicic acid (PA), calendic acid, jacaric acid and catalpic acid [24,25]. In fact, according to Sassano et al. [27], there are other two isomers, β -eleostearic (β -ESA; C18:3-9*t*,11*t*,13*t*) and β -calendic (C18:3-8*t*,10*t*,12*t*), making it seven in total.

Of all the CLnA-containing seeds, only *Trichosanthes kirilowii* Maxim. (TK) seeds are edible. The punicic acid content of this popular Chinese snack ranges between 32 % and 40 % of total seed weight [24,40-42].

These isomers are found in select seeds, commonly as the most abundant fatty acids. Each plant seed accumulates only one

isomer of conjugated linolenic acids (CLnAs), which is synthesized from linoleic acid through a specific conjugase enzyme [23,39].

CLnAs may also be produced during the processing of vegetable oils, as a result of isomerization and dehydration of secondary oxidation products of linoleic and α -linolenic acids [37,43]. Although alkaline isomerization is effective in mass producing conjugated linolenic acid (CLnAs), the resulting mixture comprises numerous isomers of different CLnAs, which makes identification difficult. However, these synthetic fatty acids have been reported to have some important and singular physiological effects, such as anticarcinogenic and anti-obesity [23]. Coakley et al. [44] investigated six strains of intestinal bifidobacteria for their anticancer activity and suggested that the inhibitory effect on cancer cells observed may be related to the ability of the bacteria to convert LnA to CLnAs.

Metabolism and incorporation of CLnAs

Plourde et al. [45] showed that a CLnA equimolar mixture (C18:3-9*c*,11*t*,15*c* and C18:3-9*c*,13*t*,15*c*) was apparently absorbed as efficiently as rumenic acid (C18:2-9*c*,11*t*) when ingested under nutritional and physiological conditions. Both CLnA isomers were mainly incorporated into neutral lipids. They were metabolized by the elongation/desaturation pathway similarly to LnA. The C18:3-9*c*,13*t*,15*c* isomer was converted to the 20:5 w-3 conjugated isomer while the C18:3-9*c*,11*t*,15*c* isomer was converted to the 22:6 w-3 conjugated isomer. Yuan et al. [46] reported that mice fed a six-week diet supplemented with 1% punicic acid (PA) showed a significantly higher proportion of 22:6 w-3 in liver phospholipids than that in mice given a diet supplemented with 1% α -eleostearic acid (α -ESA), which is consistent with the observations made in a study by Koba et al. [23], who also reported high levels of 22:6 w-3 in the liver of mice fed a diet supplemented with 0.5% PA-containing pomegranate seed oil. On the other hand, four-week diet supplementation with α -ESA-containing bitter melon seed oil led to increased proportion of 22:6 w-3 in rat livers [47].

The distribution of the CLnA accumulated by the Caco-2 cells, as well as that of their bioconversion products, 9*c*,11*t*-CLA and 9*t*,11*t*-CLA, into the different lipid classes was also determined. It was observed that Caco-2 cells take CLnA (α - and β -eleostearic, catalpic or punicic acid) up at different rates and then convert them at CLAs, but with varying efficiency depending on the structure of the Δ 13 double bond. The distribution of CLnA between neutral lipids (NL) and phospholipids appeared to be linked to their number of *trans* double bonds: the higher the number, the higher the accumulation in the NL fraction [48].

Table 2: Major isomers of conjugated α -linolenic acid found in plant seeds.

Plant	Fattyacid	Isomericconformation	% in theoil
Tung (<i>Aleuritesfordii</i>)	α -eleostearic	9 <i>cis</i> ,11 <i>trans</i> ,13 <i>trans</i>	70
Bittergourd(<i>Momordicacharantia</i>)	α -eleostearic	9 <i>cis</i> ,11 <i>trans</i> ,13 <i>trans</i>	60
Pomegranate (<i>Punica granatum</i>)	Punicic	9 <i>cis</i> ,11 <i>trans</i> ,13 <i>cis</i>	72
Catalpa (<i>Catalpaovata</i>)	Catalpic	9 <i>trans</i> ,11 <i>trans</i> ,13 <i>cis</i>	31
Potmarigold (<i>Calendulaofficinalis</i>)	Calendic	8 <i>trans</i> ,10 <i>trans</i> ,12 <i>cis</i>	33
Jacaranda (<i>Jacarandasp</i>)	Jacaric	8 <i>cis</i> ,10 <i>trans</i> ,12 <i>cis</i>	32

References: [7,23].

In general, *in vivo* studies have shown that CLnAs are efficiently absorbed in the organism and metabolized to CLAs [4,23,42,49], which has increased interest in CLnAs. These researchers suggest that CLnAs may be metabolized to CLAs via a $\Delta 13$ saturation reaction catalyzed by a NADPH (nicotinamide adenine dinucleotide phosphate) dependent enzyme, which is either a novel enzyme capable of recognizing conjugated trienoic acid or the enzyme active in the leukotriene B₄ reductive pathway.

In a study by Tsuzuki et al. [49], aimed at investigating the absorption and metabolism of punicic acid (PA) and α -eleostearic acid (α -ESA) in rat intestine using a lipid absorption assay in lymph from the thoracic duct, both isomers were slowly absorbed in unaltered form, and some of them were rapidly converted into CLAs. Likewise, Yuan et al. [42] observed that PA was quickly metabolized to 9*c*,11*t*-CLA in rat plasma and different rat tissues. Levels of the CLA isomer and PA were undetectable right after treatment (t=0); both were detected in rat plasma and tissues at t=4, 8, 12 and 24 h (each rat fed about 645mg PA). PA and the CLA levels in liver tissue and plasma were higher than those in the brain, heart, kidney and adipose tissues.

The accumulation of PA in all the tissues examined was observed to be considerably higher than that of α -ESA [41,46]. They also reported a higher relative rate of conversion of α -ESA to 9*c*,11*t*-CLA in liver phospholipids and triglycerides. The highest conversion rates of α -ESA was found in adipose tissue (91.8 %), spleen (91.4 %) and kidney (90.7 %), whereas the highest conversion rate of PA was observed in the liver (76.2 %). The conversion rate of both α -ESA and PA was lowest in the heart, 84.6 % and 54.5 %, respectively [46]. As regards chemical structures, PA and α -ESA only differ in terms of the configuration of the double bond at position 13, i.e. PA has a *cis*-type double bond and α -ESA has a *trans*-type double bond. Such structural feature either may affect the degree of saturation of the conversion enzyme or may make α -ESA a specific substrate for the conversion enzyme, which may explain the better conversion rate of α -ESA observed. Yuan et al. [25,42] suggested that CLnA-CLA conversion enzymes may be synthesized in the liver. These results indicate that CLnA isomers are metabolized and incorporated selectively. More research, however, is needed to fully understand the mechanisms of CLnA conversion to CLA.

Cao et al. [43] have reported the selective nature of CLnA incorporation in milk lipids in rats. Of the three CLnA isomers used to supplement the diet given to maternal rats, 9*c*,11*t*,13*c*-CLnA (one *trans*-type double bond) was found in highest levels in milk lipids, followed by 9*c*,11*t*,13*t*-CLnA (two *trans*-type double bonds) and then by 9*t*,11*t*,13*t*-CLnA (three *trans*-type double bonds). Interestingly, CLAs were detected in milk from maternal rats fed the diet supplemented with CLnAs, suggesting that they are able to metabolize CLnAs to CLAs. These results also suggest that the oxidoreductase catalyzing the reduction of the $\Delta 13$ -double bond may have exhibited greater affinity for the CLnA with only one double bond in the *trans* configuration.

Reports on the metabolism and incorporation of CLnAs in humans are scarce in the literature. Yuan et al. [25] have investigated the metabolism and incorporation of punicic acid (PA) in healthy young subjects (n=30). Daily supplementation

with *Trichosanthes kirilowii* (TK) seeds, containing 3 g PA, for 28 days led to 0.47 % and 0.37 % increases in the proportion of PA in plasma and red blood cell membranes (RBCM), respectively. In addition, the proportion of C18:2-9*c*, 11*t* increased from 0.05 to 0.23 % in plasma and from 0.03 to 0.17 % in RBCM. Apparently, humans are able to effectively incorporate PA into plasma and RBCM and the results also suggest that PA supplementation may be associated with increased 9*c*,11*t*-CLA levels in humans, probably via a $\Delta 13$ -saturation reaction.

The potential ability of organisms to convert CLnAs to 9*c*, 11*t*-CLA has attracted increasing interest given the reported beneficial biological effects of this CLA isomer. One should consider using certain seed oils, such as *Trichosanthes kirilowii* (TK), pomegranate and bitter melon, as alternative direct sources of CLnAs and indirect sources of CLAs, taking into account their high content of CLnAs, mainly α -ESA e PA, and the moderate simplicity of purification [25,42].

Health effects of CLnAs

Recently, conjugated fatty acids have been the focus of scientific research because numerous studies have shown their beneficial effects in a variety of experimental models of metabolic and chronic inflammatory diseases [18].

Although a consensus about the health effects of conjugated fatty acids in animals and humans does not yet exist and knowledge about mechanisms is limited at present, a number of studies have reported encouraging results. For instance, in studies by Grossmann et al. [6], Igarashi and Miyazawa [50] and Yasui et al. [51], CLnAs have been shown to have cytotoxic effect on human cancer cell cultures. They have also been shown to inhibit carcinogenesis [52,53,54] and alter lipid metabolism in animals [23,30,38,55]. Grossmann et al. [6], Tsuzuki et al. [54], Igarashi and Miyazawa [56] proposed a mechanism of antitumor action of CLnAs by which CLnAs may induce apoptosis through lipid peroxidation and the protein kinase C pathway.

Interestingly, the biological activities of CLnAs may differ among isomers [57], however, their mechanisms of action remain to be elucidated. Moreover, some *in vivo* studies provided evidence that CLnAs can be metabolized to CLAs, which may be the actual compounds responsible for the health benefits attributed to CLnAs. Some of the potential beneficial effects of CLnAs are discussed below.

Effects on body composition, lipid profile and glucose metabolism

To date, studies of the effects of CLnAs on weight gain, body composition, serum lipids, and glucose metabolism and insulin resistance have yielded controversial results.

Diet supplementation with 1 % CLnAs (α -ESA and/or PA) for six weeks did not significantly affect food intake neither body and tissue weights in mice [19,24]. Yamasaki et al. [30] reported no changes in body weight and adipose tissue in C57BL/6N mice fed experimental diets containing 0.12 % and 1.2 % pomegranate seed oil (PSO), rich in punicic acid (PA) for three weeks. Similarly, Otsuka Long-Evans Tokushima Fatty (OLETF) rats fed a two-week diet supplemented with 9 % safflower oil and 1 % PSO showed no changes in abdominal white adipose tissue weights

[29]. In contrast, when investigating the effects of CLnAs on body fat, Koba et al. [58] observed a reduction in adipose tissue weight in Sprague-Dawley rats after four weeks of feeding. Also, CLnAs were found to reduce perirenal adipose tissue weight more potently than linoleic acid (LA), conjugated linoleic acid (CLA) and α -linolenic acid (LnA). Koba et al. [28] noted a dose-dependent reduction in perirenal adipose tissue weight in ICR CD-1 mice fed a diet supplemented with PSO for four weeks. Diet supplementation with calendic acid (C18:3-8t,10t,12c) led to decreased body fat content in mice [59].

Some studies reported that diet supplementation with CLnAs resulted in significant reductions in serum total cholesterol (TC) [58,60], apoB-100 [61] and liver tissue triglyceride (TG) levels [29,61]. Yang et al. [38], in contrast, noted no changes in serum total cholesterol (TC) levels neither in serum TG, HDL-C and non-HDL-C levels, but a significant decrease in liver TC levels when hamsters were fed a diet supplemented with PA or α -ESA (12.2 - 12.7 $\frac{g}{kg}$) for 42 days. Although Yamasaki et al. [30] reported no significant effects on TC in mice fed a 0.12 % or 1.2 % PSO-supplemented diet, increased serum TG and phospholipid levels were found. Koba et al. [28] observed no significant effects on serum lipids in mice given PA; however, liver TG levels were shown to be significantly lower than those in the control group, which is consistent with the observations made by Yuan et al. [19], who reported a significant reduction in liver TG levels, when compared to the control group, but no significant effects on plasma TG, TC, HDL-C and LDL-C levels when mice were given a six-week diet containing PA or α -ESA.

The effect of CLnAs on the lipid profile in humans has been studied to a limited degree. When PSO was administered to hyperlipidaemic subjects for four weeks, no favorable effects on TC and LDL-C levels, but reductions in TG levels and in the TG:HDL-C ratio were observed [31]. Further studies are required to confirm the reported effects of the intake of CLnAs on body weight gain and blood serum lipid profiles as well as elucidate their mechanisms of action.

Hontecillas et al. [62] found that diet supplementation with catalpic acid (C18:3-9t,11t,13c) for 78 days increased fasting plasma glucose and insulin concentrations and improved the plasma glucose-normalizing ability following a glucose tolerance test in rats with diet-induced obesity. Hontecillas et al. [33] have reported similar results in mice fed another CLnA isomer: punicic acid (PA). McFarlin et al. [32] reported that PSO intake (average 61.79 mg per day) was associated with an improvement in insulin sensitivity in CD-1 mice, suggesting that the risk of developing type-2 diabetes may have been reduced. In addition, Vroegrijk et al. [18] observed that PSO intake improved high fat diet-induced obesity and insulin sensitivity in mice. In contrast, diet supplementation with *Trichosanthes kirilowii* Maxim. (TK) seeds in humans for 28 days had no significant effects on neither fasting serum insulin and glucose levels nor insulin sensitivity, assessed by HOMA-IR (homeostasis model assessment - insulin resistance) [24].

Antioxidant activity of CLnAs

As oxidative stress-related diseases have advanced, the

antioxidants naturally found in foods have attracted increasing interest [26]. CLnAs have been shown to suppress tumor cell growth through a mechanism involving lipid peroxidation [54,56]. Grossmann et al. [63] found that when breast cancer cells were treated with α -ESA in the presence of the antioxidant α -tocotrienol (20 $\frac{\mu mol}{L}$), cell growth inhibition and apoptosis effects of α -ESA were lost, suggesting that this CLnA isomer is able to block breast cancer cell proliferation and induce apoptosis through a mechanism that may be oxidation dependent. Although CLnAs exert their biological activities via an oxidation-related mechanism, many studies have yielded controversial results [4,7,64].

Noguchi et al. [47] reported that rats treated with BGO (bitter gourd oil), containing α -ESA, showed significant increases in plasma hydroperoxide levels and liver lipid oxidation, despite a previous study by Dhar et al. [65], who had reported that rats fed for four weeks with the same CLnA, α -ESA from karela (bitter gourd) seeds, at 0.5 % showed lowered lipid peroxidation, assessed by measuring TBARS (thyobarbituric acid reactive substances), when compared to the control group (dietary oil). Mukherjee et al. [40] investigated the effects of diet supplementation with punicic acid (PA) at different concentrations on lipid peroxidation in rats and observed maximum antioxidant activity at 0.6 % PA; however, a pro-oxidant effect was also noted at 1.2 % PA. Under the conditions described above, CLnAs may have been able to lower hydroperoxide formation by reducing the formation of free radicals and the peroxidation of PUFAs (polyunsaturated fatty acids) in erythrocyte membranes and other lipids. Alternatively, biohydrogenation or free radical addition to one of the conjugated double bonds of CLnAs may have taken place, resulting in the formation of conjugated dienes, which, in turn, may have acted as antioxidants [26,65].

When investigating the dietary effects of CLnAs on lipid peroxidation in rats with alloxan-induced diabetes mellitus, Dhar et al. [60] observed significant reductions in LDL and erythrocyte lipid peroxidation in each of the experimental groups (0.5 % CLnAs, 0.15 % α -tocopherol and 0.25 % CLnAs + 0.15 % α -tocopherol) when compared with the control group. The diet containing 0.25 % CLnAs + 0.15 % α -tocopherol also led to a greater reduction in liver lipid and membrane lipid peroxidation than the diet containing only α -tocopherol. Saha and Ghosh [66] showed that treatment of streptozotocin-induced diabetic albino rats with CLnAs (α -ESA or PA at 0.5 % total lipids), significantly lowered oxidative stress, reduced lipid peroxidation and restored the levels of the antioxidant enzymes [superoxidase dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx)], reduced glutathione and nitric oxide (NO) synthase in the pancreas, blood and erythrocyte lysate.

These same authors also investigated the antioxidant activity of two isomers of CLnAs (0.5 % total lipids given for 15 days) against sodium arsenite-induced oxidative stress. Sodium arsenite altered the activities of antioxidant enzymes in plasma, liver homogenates, the brain and erythrocytes. After administration, both CLnA isomers, α -ESA and PA, were capable of increasing the activity of SOD, CAT and GPx and lowering the activity of NO synthase to their normal levels. Although both CLnA isomers were able to successfully lower oxidative stress,

they performed differently, which can be explained by their distinct distribution of *cis-trans* double bonds, α -eleostearic, (α -ESA), featuring a greater number of *trans*-type double bonds, proved to be more effective than punicic acid (PA) [26,67]. But mixture of both isomers has shown synergistic activity and better protection than individually treated isomers at 0.5 % dose [68]. Furthermore, it has been shown that CLnA isomers (α -ESA and PA) caused amelioration of renal oxidative stress and the isomers showed synergistic activity. Administration of blended product of both the isomers caused better restoration of renal fatty acids and other altered parameters [69].

Significantly higher levels of 8-iso-PGF 2α , the most abundant F 2 -isoprostane and a reliable marker of oxidative stress formed *in vivo* as a result of the non-enzymatic, free radical-catalyzed peroxidation of arachidonic acid, were found in human urine after diet supplementation with TK seeds, rich in PA, suggesting that punicic acid (PA) is able to increase lipid peroxidation in humans [24].

Molecular Actions of CLnAs

The ability of natural dietary components to regulate certain molecular events represents an alternative therapeutic strategy for major health concerns worldwide, such as inflammatory bowel disease, rheumatoid arthritis, atherosclerosis and metabolic syndrome. These diseases share some features; for example, the overproduction of proinflammatory mediators (TNF α , GM-CSF, IL-1, IL-6, IL-8, leukotriene B 4 and PAF) and reactive oxygen species (ROS) and the presence of highly activated inflammatory cells, such as neutrophils, monocytes and macrophages [34]. When investigating PA effects on TNF α -induced neutrophil hyperactivation *in vitro* and on colon inflammation in rats, the same researchers observed that PA inhibited TNF α -induced priming of ROS production and MPO (myeloperoxidase) release by neutrophils, suggesting a potent anti-inflammatory effect.

Bassaganya-Riera et al. [70] provided molecular evidence *in vivo* that PA intake up regulates colonic PPAR- δ expression, the keratinocyte growth factor and the orphan nuclear receptor ROR γ expression and suppresses colonic and M1 macrophage-derived TNF- α . Also, PA was shown to increase the levels of IL-17 and IFN- γ in CD8+T cells in the mesenteric lymph nodes (i.e., mucosal inductive sites). They suggested that PA modulates mucosal immune responses and improves gut inflammation through PPAR- γ and - δ -dependent mechanisms. Likewise, dietary PA was reported to lower fasting plasma glucose concentrations, improve the glucose-normalizing ability, suppress NF- κ B activation and TNF- α expression and up regulate PPAR α - and γ -responsive genes in skeletal muscle and adipose tissue [33]. Their results demonstrate that PA can bind and robustly activate PPAR- γ and increase PPAR- γ -responsive gene expression, which will result in improved diabetic and inflammatory status. Studies of other CLnA isomers have yielded similar results. In a previous study by Hontecillas et al. [62], catalpic acid was shown to up regulate PPAR- α and its responsive genes but not to significantly affect PPAR- γ and - δ expression in white adipose tissue (WAT) in mice; suggesting that the metabolic effects of catalpic acid on glucose and lipid metabolism may be mediated through a PPAR α -dependent mechanism. Bitter melon oil, rich in 9c,11t,13t-CLnA, was reported to increase the levels of PPAR- γ mRNA and

protein in Caco-2 cells and in rats [51,53] and tung oil, containing α -ESA, was shown to activate PPAR- γ in human umbilical vein endothelial cells [52]. Recent studies show that the immune modulatory actions of α -ESA may be both PPAR- γ dependent and PPAR- γ independent to ameliorate disease activity and intestinal lesions in mice with dextran sodium sulfate induced colitis [5].

In an *in vitro* study, aimed at evaluating the effectiveness of certain CLnA isomers found in pomegranate seed oil as selective estrogen receptor modulators (SERMs), PA was found to inhibit estrogen receptors (ER) α and β at 7.2 and 8.8 μ M, respectively; α -ESA inhibited ER α and β at 6.5 and 7.8 μ M, respectively [13]. Thus, both CLnA isomers are effective as SERMs. These results indicate that PA, abundant in PSO and an effective SERM, could be used as a potential breast cancer chemopreventive agent.

Saha and Ghosh [66] showed that four week-diet supplementation with CLnAs (α -ESA and PA at 0.5 % total lipids) led to reduced inflammation in streptozotocin-induced diabetic, albino rats by significantly reducing the expression of inflammatory cytokines, such as TNF- α and IL-6, in blood and the expression of hepatic NF- κ B (p65), once higher as a result of diabetes induction.

On the other hand, one study reported that administration of 400 mg of pomegranate seed oil (rich in punicic acid) twice daily in dyslipidemic patients has no effect on serum TNF- α [71]. This result shows that although some studies indicate that CLnAs found in pomegranate seed oil and other foods may represent a novel therapeutic alternative strategy for some diseases, such as inflammatory diseases and cancer [34], additional studies are needed to prove these effects in humans.

Final Considerations

In recent years, studies worldwide have confirmed the abundant presence of punicic acid (PA), an isomer of conjugated α -linolenic acid (CLnA), in pomegranate seed oil (PSO). For this reason, PSO has been increasingly investigated as a potent functional food and/or nutraceutical ingredients in foods. But in that case, the impact of the intake of CLnAs on lipid metabolism as well as other health effects would have to be considered.

Even though numerous *in vitro* and animal studies have provided evidence of the bioactive properties of CLnAs, there have been inconclusive and/or contradictory results as well. Furthermore, human studies are scarce and some effects of CLnAs observed in animal models differ from those reported in humans. Therefore, the exact role of CLnAs in improving human health remains to be determined. Further studies are needed to fully understand the mechanisms of action as well as the physiological effects of CLnA isomers and to establish safety and recommendations regarding its usage.

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