



Mini review

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Pomegranate's Ellagitannins: Metabolism and Mechanisms of Health Promoting Properties



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Abstract

The health benefits of pomegranate consumption have been known since the antiquity. More recently, the biological activities of pomegranate, including its anti-bacterial, antioxidant, anti-inflammatory and anticarcinogenic properties, have raised great interests in the scientific community and they have been the subject of a large number of scientific studies. Several phenolic compounds are present in various parts of the fruit, of which ellagitannins, hydrolysable tannins considered responsible for most health benefits of pomegranate. Despite all these efforts, the actual effectors remain unidentified and the mechanisms of action only partially elucidated. This is probably due to the complex catabolic pathway of these molecules that takes place in the gastrointestinal tract, involving both microbial and cellular modifications before entering the bloodstream. The diversity of the intestinal microbiota in the human population leads to the production of different metabolites in individuals, contributing to the heterogeneity of the response to dietary pomegranate assumption. This complex situation determines the need for special attention in designing studies on the action of ellagitannins on both cell cultures and animal models.

Keywords: Pomegranate; Ellagitannins; Ellagic acid; Punicalagin; Urolithins; Human health; Inflammation

Abbreviations: EA: Ellagic Acid; HO-1: Heme Oxygenase 1; Keap1: Kelch-like ECH-Associated Protein 1; MDF: Mucin-Depleted Foci; Nrf2: Nuclear Factor Erythroid 2-related Factor 2; Uros: Urolithins

Introduction

Pomegranate (*Punica granatum L.*) is a fruit native to Asian countries that grows mainly in the Mediterranean region. Since ancient times, pomegranate has been known for its medicinal properties [1]. Most of those beneficial effects have been attributed to the pomegranate metabolites such as polyphenols, with a particular focus on hydrolysable tannins [2]. They can be classified as gallotannins and ellagitannins, which are present in the pericarp of the fruit [3]. The difference in these two categories lies in the different phenolic groups that are esterified to hydroxyl groups of glucose molecules, namely gallic acid in gallotannins and hexahydroxydiphenic acid in ellagitannins [4]. Commercial pomegranate juice is enriched with ellagitannins because it is obtained from the whole fruit. More than 60 hydrolysable tannins have been found in the fruit peel, arils and membranous walls [5]. In particular, punicalagin is the predominant form of the hydrolysable tannins present in pomegranate [2] and is responsible for more than 90% of antioxidant bioactivity contained in pomegranate juice [6]. The amount of punicalagin present in the juice contributes from 5 to 30% of the total phenolic compounds content. Its concentration strongly depends on the geographical origin of the fruit, the type of cultivar (organic or conventional) and the treatment process used for preparation.

Normally present at concentrations varying between 100 to 700mg/l, the concentration in industrial processed extracts can reach 1900mg/L [6]. Other hydrolysable tannins present in pomegranate juice include ellagic acid (EA), gallic acid, punicalin and punicafofin, the latter two being particularly present in leaves [7]. The antioxidant properties of pomegranate are of particular interest for a potential therapeutic application in several pathologies characterized by oxidative stress. Partly thanks to their antioxidant activity, pomegranate juice and extracts can attenuate inflammatory processes, inhibit and prevent carcinogenesis, improve diabetes, promote wound healing and be active in many other situations [7-9].

The metabolism of ellagitannins in the gastrointestinal tract is rather complex, with a first hydrolysis of ellagitannins forming EA in the stomach and other decarboxylation reactions carried by the intestinal microbiota in the colon [10]. The diversity in microbiota composition is reflected in the production of different sets of metabolites in individuals. These metabolites are absorbed and chemically modified by enterocytes, before entering the bloodstream and reaching the internal organs. The analysis of the activity of the different metabolites revealed a loss of bioactivity after modification

in the enterocytes, raising several questions about the physiological relevance of studies addressing direct effects of ellagitannins.

The aim of this mini review is to summarize the knowledge on catabolism, tissue distribution of ellagitannins metabolites and in signaling pathways triggered by these molecules to stimulate a cellular response, integrating recent knowledge.

Ellagitannins Metabolism

Dietary absorbed ellagitannins are first hydrolyzed to EA in the small intestine at physiologic alkaline pH [11]. EA is then transformed in the intestine into catabolic intermediates by a series of decarboxylation reactions carried out by bacteria of the intestinal microbiota [10], producing derivatives of dibenzopyran-6-one, also called urolithins (Uro). The complexity of this microbiota, which actively participate in the metabolism of ellagitannins, makes it more difficult to find metabolic pathways of ellagitannins. A two-way interaction exists between ellagitannins and intestinal bacteria. Indeed, polyphenols can modulate the intestinal microbial population [12], itself involved in the transformation of EA into catabolic intermediates [10]. The composition of the bacterial population in the intestine is not homogeneous between individuals and must constantly adapt to changes in the environment caused, for example, by the type of assimilated diet [13,14]. Some pathologies can also have influence or are triggered by imbalances in the intestinal bacterial population (dysbiosis). This diversity is the cause of inter-individual differences in the set of ellagitannins metabolites produced, which may explain the differences in the response observed after consumption of ellagitannins, in terms of health benefits. A study on the ability of individuals to synthesize different Uros after ingestion of walnuts or pomegranate extract capsules revealed the existence of at least three different phenotypes (metabotypes) among the population [15]. This classification is based on the type of Uros present in the urine 24 hours after ingestion. The Uros-content of urine in the three groups is defined as follows: metabotype 0 includes those unable to produce Uros; metabotype A includes those able to synthesize only Uro A (Uro-A) and, finally, metabotype B includes those able to synthesize Uros A, B (Uro-B) and isourulithin A (Iso-Uro_A). 60-70% of the cohorts belonged to the metabotype A, 20-30% to metabotype B and the rest to metabotype 0 [15,16]. The identification of intestinal microbiota is far from being complete, but the abundance of two *Clostridium* strains, *C. leptum* and *C. coccoides* has been associated to the ability to produce some Uros [17]. Moreover, intestinal bacterial species from Eggerthellaceae family have been shown to be able to produce some Uros intermediates [18]. As mentioned above, several factors can influence the metabotype. In people with intestinal dysbiosis, the distribution of these three metabotypes was changed [15]. Aging has also been described as one of the major factors influencing Uros-producing

microbiota. Until the age of 20, the A metabotype is largely predominant (70-80%) and then declines to values between 50 to 65%. The B and 0 metabotypes are less represented in young people (about 10% each). After the age of 20, the occurrence of B metabotype increases to stabilize around 35 to 45%, while the occurrence of 0 metabotype is less likely to change over life course and remains limited to 10% [19].

The descending colon has recently been identified as the primary site of ellagitannins catabolism by intestinal microbiota, using a gastrointestinal simulation model (TWIN-SHAME) coupled with absorption studies with colon adenocarcinoma-derived cell line Caco-2 [20]. Once formed, Uros are absorbed by the enterocytes and undergo a complex process of phase II-modification including methylation, sulfation and glucuronidation, which make Uros molecules more soluble and suitable to enter bloodstream and to reach internal organs where they can exert different effects [10]. The distribution of ellagitannin-metabolites in tissues was first analyzed in Iberian pigs fed with food containing ellagitannins. Thirty-one different metabolites were detected in plasma and tissues, 26 of which were conjugated. Uro-A was the only metabolite found in feces [11]. In human patients affected by colorectal cancer, the presence of Uros after dietary pomegranate juice absorption was detected in normal intestinal tissue sections of patients with colorectal cancer. Lower concentrations of Uros were also detected in malignant tissue sections [21]. Finally, concentrations of Uro-A glucuronide, Uro-B glucuronide and dimethyl EAs were also measured in human prostate gland of patients with benign prostate hyperplasia and patients with prostate cancer [22]. More recently, in normal and malignant tissues from patients with breast cancer ingesting capsules containing pomegranate extract, only phase II-conjugates Uros have been detected [23]. This complex metabolic pathway suggests that the action of pomegranate on systemic tissues is mediated by conjugated metabolites. However, recent studies showed that conjugation process weakens the bioactivity of these molecules [23,24]; an observation, which raises questions about the mediator of the effects of ellagitannins action and which also raises some concerns on the physiological relevance of in vitro studies directly applying ellagitannins or its unconjugated metabolites on cells. A possible explanation of this paradox may be based on the observation that an increase in glucuronidase activity occurs in systemic tissues under inflammatory conditions [25]. This process of reversal of conjugation has been described for Uro-A, suggesting that an increase in plasma levels of glucuronide-conjugated Uro-A may nevertheless result in a local release of free Uro-A as a consequence of glucuronidase action [25], thus allowing metabolites to exert their bioactivity. A long-term action of conjugated metabolites on cancer cells metabolism is also not excluded.

Given the rapid transformation of ellagitannins into EA in

the gastrointestinal tract, the relevance of direct application of ellagitannins, such as punicalagin, in *in vitro* enterocyte cultures may also be questioned. Nevertheless, in rats receiving punicalagin as dietary supplement, punicalagin was detected in plasma and feces, suggesting that residual punicalagin may be present in feces and be absorbed by enterocytes or exert its action on enterocytes [26]. The observation that direct ellagitannins treatment can induce apoptosis, a reduction of oxidative stress and an increase in glutamate uptake following upregulation of EAAT3 glutamate-transporter gene expression [27-30], support this hypothesis. Interestingly, cell cycle arrest has also been described for proliferating Caco-2 exposed to EA, Uro-A or Uro-B [24], suggesting that ellagitannins and their metabolites may stimulate similar signaling pathways in enterocytes.

Signaling Pathways involved in Bioactivity of Ellagitannins and their Metabolites

The anti-inflammatory and anticarcinogenic effects of pomegranate ellagitannins are directly related to the antioxidant properties of this fruit [31], but the mechanisms of pomegranate interference with these processes are yet not fully understood.

Activation of NF- κ B, a redox-regulated transcription factors, is a key target for ellagitannins anti-inflammatory activities. Under normal non-inflammatory conditions, NF- κ B is complexed by I κ B protein and retained in the cytoplasm. In response to inflammatory stimuli, such as an increase in reactive oxygen species, mitogen-activated protein kinases (MAPK) are activated. MAPKs phosphorylates another kinase, IKK α / β which in turn phosphorylates I κ B. I κ B phosphorylation releases the two subunits of NF- κ B transcription factor, p50 and p65 which can be activated by phosphorylation and can translocate to the nucleus where they exert their action inducing the gene expression of several inflammatory genes [32]. Pomegranate peel extracts has been shown to inhibit the MAPKs-NF- κ B pathway in a rat model of diabetic nephropathy, an end-stage renal disorder [33]. A direct effect on NF- κ B activation has also been shown in a rheumatoid arthritis rat model in which dietary supplementation with pomegranate rind extract inhibited synovial NF- κ B activation, reducing expression of inflammatory cytokines and infiltration of inflammatory cells [34].

Another target for the antioxidative, anti-inflammatory action of pomegranate extracts is the Nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor [35,36]. As for NF- κ B, Nrf2 is normally retained in the cytoplasm by its negative regulator: Kelch-like ECH-associated protein 1 (Keap1). Inactivation of Keap1 results in nuclear translocation of Nrf2, which can then bind to the ARE: Antioxidant Responsive Element, present in the regulatory region of several antioxidant genes including that of heme oxygenase 1 (HO-1) [37]. Punicalagin has been shown to induce HO-1 gene

expression via activation of PI3K/Akt/Nrf2 pathway in LPS-stimulated macrophages [38]. *In vivo*, it has been reported that pomegranate decreased lipid peroxidation in rats with carbon tetrachloride-induced liver-damage by an activation of AMP-activated protein kinase (AMPK)-Nrf2 signaling pathway [39-41]. In spontaneously hypertensive rats, it reduced mitochondrial superoxide anion levels improving mitochondrial function and it increased HO-1 expression by the same mechanisms [35]. AMPK-Nrf2 pathway was also involved in pomegranate extract-mediated reduction of the oxidative stress in the paraventricular nucleus relieving hypertension [35]. Interestingly, a cross talk between NF- κ B and Nrf2 has been previously described. Specifically, the cytoplasmic p65 subunit of NF- κ B protein can physically associated and retain Keap1, liberating Nrf2 [42]. This provides a "tool" for amplifying protective action of pomegranate polyphenols by the activation of the anti-inflammatory transcription factor Nrf2 and simultaneous inhibition of the pro-inflammatory transcription factor NF- κ B.

Potential therapeutic evidence for the use of pomegranate juice or extracts to interfere with cancer pathology has been described. These findings are based on the ability of ellagitannins contained in the pomegranate to interfere with cellular proliferation and apoptotic processes [7]. Breast cancer is the second leading cause for cancer related death in women and the potential therapeutic role of phytochemicals has been intensively investigated [43]. In MCF-7 cells, a human breast cancer cell line, pomegranate extract induced the arrest of cell proliferation and promoted pro-apoptotic effects [44, 45]. EA has been shown to arrest proliferation and induce a cell cycle arrest in the G0/G1 phase in MCF-7 cells. This inhibition was mediated by mechanisms involving the activation of TGF β /Smad3 pathway that down-regulates cyclin A2 and E2, together with an up-regulation of several cyclin-dependent kinase inhibitors, including CDKN1A/p21 (cyclin-dependent kinase inhibitor 1A/p21) protein [46,47]. These mechanisms result in a stop of the cell cycle [48]. The involvement of p21 protein as a target for anticarcinogenic activity of ellagitannins has been demonstrated also using colon carcinoma-derived cell lines. Ellagitannins and/or metabolites present in pomegranate extracts such as punicalagin (used at a concentration of 100 μ mol/l) inhibit cell proliferation in undifferentiated Caco-2 and HT-29 cells, blocking cell cycle in G0/G1 and G2/S stages [27,28, 49]. Ellagitannins metabolites (EA, Uro-A and Uro-B) have also been shown to affect MAPK signaling pathways leading to a blockade of Caco-2 cell proliferation [24]. The corresponding glucuronide-conjugates failed at once to affect cell proliferation, confirming the negative effect of conjugation on ellagitannins metabolites bioactivities [50]. Cell cycle arrest in Caco-2 cells by ellagitannins metabolites is also mediated by a downregulation of the miR-224, a microRNA targeting CDKN1A/p21 mRNA and by an activation of the transcription factor TP53 [50]. MicroRNAs are endogenous RNA molecules of

20-25 nucleotides in length, which bind to the 3'-untranslated region of target mRNAs causing their degradation [51]. The same study reported a strong heterogeneity in mechanisms triggered by ellagitannins metabolites as a function of the cell line considered. Indeed, levels of TP53 expression induction were not the same in the three cell lines used (Caco-2, SW480 and HT-29) and miR-224 was only downregulated in Caco-2 cells in response to Uros treatment [50]. Moreover, in SW480 and HT-29, but not in Caco-2, another micro RNA, miR-215, was upregulated, inducing p53 and p21 accumulation [50] with a subsequent cell cycle arrest [52]. In Caco-2 and SW480 treated with single and mixed metabolites, an increase of c-MYC expression was also observed [50]. The concomitant induction of TP53 and c-MYC favors chemotherapy-induced apoptosis in colon carcinoma cells [53], thus providing evidence for a possible use of ellagitannins metabolites to sensitize tumor cells to anticancer treatment. Finally, the expression of p53 also increased in Uro-A-treated HCT116 human colon cancer cells, determining a stop in G2/M cell cycle phase transition and reducing cellular glycolytic potential [54].

The use of ellagitannins metabolites was also evaluated in prostate cancer. EA have been shown to inhibit proliferation and to induce apoptosis in androgen-dependent, but not in androgen-independent, human prostate cancer cell lines. The anti-proliferative action was mediated by the regulation of the expression of several cell-cycle-related proteins, including CDKN1A/p21, and by the upregulation of the expression of several markers of apoptosis, including caspase-3 [55]. Pro-apoptotic activity has also been described for pomegranate extracts in metastatic prostate cancer cell lines [56]. In these cells, ellagitannins induced an increase in cleavage of poly (ADP-ribose) polymerase and caspase-3, and downregulation of the level of expression of survivin protein [56], an anti-apoptotic protein associated with bone metastasis from prostate cancer [57]. This downregulation was attributed to a reduced activation of Stat3 transcription factor [56], known to bind the survivin gene promoter [58].

Evidence of EA anti-cancer properties have also been obtained in vivo using different animal models. E2-induced mammary carcinogenesis ACI rats, a model of breast cancer, exhibit an aberrant miRNAs expression in breast cancer, resulting in changes in the expression of several target genes participating in the process of carcinogenesis [59]. EA dietary supplementation of E2-induced mammary carcinogenesis in ACI rats reversed aberrant miRNAs expression, reestablishing a normal expression of target genes [60]. Using Pirc rats, a genetic model of colorectal cancer spontaneously developing microscopic pre-neoplastic lesions called mucin-depleted foci (MDF) [61,62], Tortora et al. observed a 30% inhibition of the number and size of MDF after dietary supplementation with pomegranate mesocarp decoction [63]. This inhibitory effect has been attributed to the ability of Uro-A to inhibit the expression of proliferative and inflammatory markers (PCNA,

COX-2 and iNOS) and to increase the expression of apoptotic markers (caspase-3) in HT-29 cells. A similar inhibitory effect was observed in Sprague-Dawley rats injected with azoxymethane, a different animal model of colorectal cancer. In this model, the effect was exerted by an increase in the expression of the micro RNA miR-126 targeting the expression of VCAM1 and PI3Kp85βgenes [64]. Activation of PI3K/Akt - NF-κB pathway was inhibited by pomegranate juice resulting in a decreased VCAM1, COX2, iNOS and VEGF gene expression. Interestingly, these results are consistent with those of a previous study showing a significant decrease in the expression of miR-126 in colon cancer tissues and metastatic cell lines [65]. Anticarcinogenic properties of pomegranate have also been proven in animal models for prostate cancer. A transgenic rat model for adenocarcinoma of prostate also confirmed caspase-3 involvement in pro-apoptotic action of EA on prostate cancer [55]. In transgenic adenocarcinoma of the mouse prostate mice, another animal model of prostate cancer [66], pomegranate extract significantly reduced the spontaneous onset of palpable abdominal tumors and the number of metastases, in particular in lungs and liver. This inhibitory effect on prostate carcinogenesis determined an increased survival rate of the mice [67]. In the prostate tissue of these mice, clear inhibition of PI3K/Akt/mTor signaling pathway was also observed subsequently to pomegranate extract diet supplementation [67].

Finally, several clinical trials have been conducted to evaluate the therapeutic potential of pomegranate juice or extracts on human prostate cancer. These studies showed that pomegranate derivatives were safe, but their efficacy was limited to a marked increase in prostate-specific antigen concentration doubling time [8].

Conclusion

There is an important public and scientific interest in the health benefits of pomegranate fruit and its derived metabolites. However, several aspects require further investigation. The link between pomegranate consumption and the related effects is represented by ellagitannins and their metabolites. In particular EA, a phenolic lactone produced by hydrolysis of ellagitannins in the gastrointestinal tract and widespread in many fruits and vegetables. Among the many beneficial effects attributed to EA, there are the anti-inflammatory and anticarcinogenic effects, but its bioactivity is not yet well understood. It is known that different Uros can be produced by the gut microbiota starting from EA. However, the isolation and identification of bacteria strains involved in the metabolism of EA in the large intestine is far from being complete. In enterocytes, Uros undergoes an additional modification leading to a loss of bioactivity, which raises several questions on the real benefits of pomegranate consumption. Nevertheless, MAPK, PI3K/Akt, AMPK have been identified as major targets for ellagitannins and metabolites action on NF-

kB and Nrf2 transcription, two transcription factors involved, with different roles, in inflammatory processes. Control of cell proliferation and apoptosis appears as the main targets for anticarcinogenic action and it takes place mainly through the regulation of CDKN1A/p21, PCNA and caspases expressions and activations. Cell cycle control by ellagitannins metabolites also involves the expression modulation of specific micro RNAs, but this mechanism appears to be dependent on origin and specific properties of tumor cells. Despite the existing evidence on potential therapeutic action of ellagitannins and their metabolites, given the ability to elicit different cellular responses in different pathological situations, further investigations are required to confirm the real physiological relevance of these observations.

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