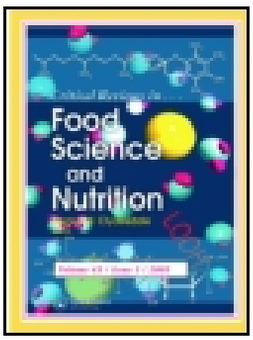

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Health benefits of walnut polyphenols: An exploration beyond their lipid profile

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ABSTRACT

Walnuts are commonly found in our diet and have been recognized for their nutritious properties for a long time. Traditionally, walnuts have been known for their lipid profile which has been linked to a wide array of biological properties and health-promoting effects. In addition to essential fatty acids, walnuts contain a variety of other bioactive compounds such as, vitamin E and **polyphenols**. Among common foods and beverages, walnuts represent one of the most important sources of polyphenols, hence, their effect over human health warrants attention. The main polyphenol in walnuts is **pedunculagin**, an ellagitannin. After consumption, ellagitannins are hydrolyzed to release ellagic acid, which is converted by gut microflora to urolithin A and other derivatives, such as urolithins B, C and D. Ellagitannins possess well known antioxidant and anti-inflammatory bioactivity and several studies have assessed the potential role of ETs against disease initiation and progression, including cancer, cardiovascular and

neurodegenerative diseases. The purpose of this review is to summarize current available information relating to the potential effect of walnut polyphenols in health maintenance and disease prevention.

Keywords: Walnuts, Polyphenols, Ellagitannins, Disease Prevention, Health

1. INTRODUCTION

Walnuts (*Juglans regia* L.) have been long consumed as a highly nutritious food in many parts of the world and they are an important component of the Mediterranean diet (Bulló, Lamuela-Raventós, & Salas-Salvadó, 2011). Recently, they have been gathering attention for their health-promoting properties, which have been reported to improve lifestyle-related diseases. The health benefits of walnuts are attributed to several of their nutrients, such as, ω-3 fatty acids (Ros et al., 2004), vitamin E (Maguire, O'Sullivan, Galvin, O'Connor, & O'Brien, 2004), and dietary fiber. In addition to these nutrients, walnuts are rich in plant sterols and especially in polyphenols, as it has been reported recently (Vinson and Cai, 2012; Regueiro *et al.*, 2014). The implications of polyphenols in human health are numerous and include beneficial effects over several disease states including: cardiovascular system dysfunction and damage (Estruch *et al.*, 2013), metabolic syndrome (Murase *et al.*, 2011), diabetes (Li *et al.*, 2011), various inflammation-related pathologies (Konstantinidou *et al.*, 2010), and cancer (Thangapazham *et al.*, 2007; Nkondjock, 2009). In addition, polyphenols have been recently described as potentially beneficial compounds for neuroprotection, (Granzotto and Zatta, 2014) and against aging (Granzotto and Zatta, 2014; Peng *et al.*, 2014) . Ellagitannins, polyphenols typically found

in walnuts, and their derived metabolites possess a wide range of biological activities which suggest that they could have beneficial effects on human health (Espín *et al.*, 2013). The purpose of this review is to summarize the available information about the health-promoting effects of walnut polyphenols, mainly pedunculagin and its metabolites, ellagic acid and urolithins.

2. CHEMISTRY AND METABOLISM OF WALNUT POLYPHENOLS

Vinson & Cai (2012) reported that walnuts represent the **seventh largest** source of total polyphenols among common foods and beverages based on their serving size. A handful of walnuts, about 50g, has significantly more total polyphenols than a glass of apple juice (240 mL), a milk chocolate bar (43g) or a glass of red-wine (150mL) which are all common food sources of polyphenols (Anderson et al., 2001). Moreover, walnut's total polyphenols were significantly higher compared to other nuts such as almonds, hazelnuts, pistachios, and peanuts (Abe, Lajolo, & Genovese, 2012; Vinson & Cai, 2012). Indeed, the total polyphenol **content reported range from** 1,576mg to 2,499mg per 100g of walnuts (Vinson and Cai, 2012). In addition, walnut polyphenol extracts also exhibited high antioxidant potential, with an antioxidant capacity of 21.4 ± 2.0mmol TE/100g and 25.7 ± 2.1mmol TE/10g measured by ABTS+ and DPPH assays, respectively (Regueiro *et al.*, 2014) .

It is well documented that the most abundant polyphenols in walnuts are ellagitannins, mainly **pedunculagin (Figure 1)** (Cerdá *et al.*, 2005; Regueiro *et al.*, 2014). Ellagitannins exhibit structural diversity according to food source. Although regardless of food source, ellagitannins are characterized by one or more hexahydroxydiphenoyl moieties esterified to a polyol (Regueiro *et al.*, 2014). Previous studies of rat intestinal contents showed that

ellagitannins could be hydrolyzed to ellagic acid at the pH found in the small intestine and cecum (Daniel *et al.*, 1989; Garcia-Muñoz and Vaillant, 2014). The presence of free ellagic acid in human plasma could be due to its release from the hydrolysis of ellagitannins, enabled by physiological pH and/or gut microbiota. Ellagic acid is further metabolized by gut flora to form urolithins, mainly **urolithin A and B** (Landete, 2011), which are probably synthesized in the colon (Garcia-Muñoz and Vaillant, 2014) (Figure 1). These urolithins circulate in blood and can reach many of the target organs where the effects of ellagitannins are observed (Larrosa, Tomás-Barberán, *et al.*, 2006). The occurrence of ellagitannins and ellagic acid in the bloodstream is almost negligible, but their derived metabolites, urolithins, can reach a concentration at micromolar levels in plasma (Larrosa, González-Sarrías, *et al.*, 2006). It is important to note that urolithin concentration in plasma and urine after ingestion of ellagitannin-rich foods can differ between individuals due to individual differences in gut microbiota (Garcia-Muñoz and Vaillant, 2014). In addition to urolithin concentration, specific urolithin production can vary between individuals. A recent study identified **three urolithin-producing phenotypes**, related to the type of urolithins produced after consumption of ellagitannin food sources (Tomás-Barberán *et al.*, 2014).

3. BIOLOGICAL EFFECTS OF WALNUT POLYPHENOLS AND THEIR DERIVED METABOLITES

Effects on oxidative stress

Oxidative stress can be defined as an imbalance between free radical and reactive metabolites, such as reactive oxygen species (ROS), production and their elimination. This lack

of balance can lead to cell damage potentially creating an impact in an organism as a whole (Ďuračková, 2010). Inflammation can be caused as a consequence of oxidative stress, and although excess in ROS is not the only cause, inflammation resulting from oxidative stress is the origin of many diseases (Martinon, 2010). Typical examples are dyslipidemia (Hopps *et al.*, 2010), thrombosis (Leopold and Loscalzo, 2009), metabolic syndrome (Hopps *et al.*, 2010), type 2 diabetes (Kaneto *et al.*, 2010), nonalcoholic steatohepatitis (NASH) (Hijona *et al.*, 2010), macular degeneration (Augustin and Kirchhof, 2009), neurodegenerative diseases such as Alzheimer's (Candore *et al.*, 2010) and cancer (Hussain *et al.*, 2003). During inflammation, mast cells and leukocytes are recruited to the site of damage, which leads to a "respiratory burst" due to an increased uptake of oxygen, and thus, an increased release and accumulation of ROS (Hussain *et al.*, 2003). On the other hand, inflammatory cells also produce soluble mediators, such as metabolites of arachidonic acid, cytokines and chemokines, which act by further recruiting inflammatory cells to the site of damage and producing more reactive species. Over the years, epidemiologic and observational evidence has encouraged belief in the use of bioactive compounds with antioxidant potential for disease prevention (Stanner *et al.*, 2007). Considering the previously mentioned antioxidant capacity of ellagitannins it is interesting to explore the potential of these compounds in disease prevention.

UV radiation from the sun is a potent environmental risk factor in the pathogenesis of skin damage, much of the damage caused by UVA irradiation is associated with oxidative stress. Ellagic acid may be useful for the treatment of ultra-violet induced skin damage. In an *in vitro* study, Hseu et al (2012) showed that an ellagic acid pre-treatment markedly increased HaCaT human keratocyte cell viability and suppressed UVA-induced ROS generation and

malondialdehyde (MDA) formation (Hseu *et al.*, 2012). Moreover, ellagic acid pre-treatment prevented UVA-induced DNA damage and significantly inhibited the UVA-induced apoptosis of HaCaT cells. The antioxidant potential of ellagic acid was directly correlated with the increased expression of heme oxygenase 1 (*HO-1*) and superoxide dismutase (*SOD*), which was followed by the down regulation of kelch-like ECH-associated protein 1 (*Keap1*) and the augmented nuclear translocation and transcriptional activation of nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*) with or without UVA irradiation (Hseu *et al.*, 2012**).** These results support the role of ellagic acid in activating a response against oxidative stress **(Figure 2)**.

Ellagic acid has also shown a protective role against nicotine-induced toxicity in rat peripheral blood lymphocytes (Sudheer *et al.*, 2007). Lymphocytes incubated with nicotine showed a significant increase in the levels of lipid peroxidation index, severity in DNA damage and micronuclei number. Those effects were modulated by ellagic acid treatment. Antioxidant status was also significantly depleted in nicotine-treated group, which was effectively restored by ellagic acid incubation (Sudheer *et al.*, 2007).

Anti-inflammatory effects

The protective role of ellagitannins and its metabolites against acute inflammation has been explored in *in vivo* and *in vitro* models (Umesalma and Sudhandiran, 2010; Ahad *et al.*, 2014; El-Shitany *et al.*, 2014) (Figure 3). Ellagic acid exhibited a potent anti-inflammatory effect against carrageenan-induced inflammation (El-Shitany *et al.*, 2014). The mechanisms by which ellagic acid protected against inflammation could be linked to the reduction of inflammatory molecules such as, nitric oxide (NO), malondialdehyde (MDA), interleukin-1 beta (IL-1β), tumor

necrosis factor alpha (TNF-α), cyclooxygenase 2 (COX-2) and nuclear factor-κB (NF-κB) expression, and the induction of glutathione (GSH) and IL-10 production (El-Shitany *et al.*, 2014). Urolithins have also been linked to cause a protective effect in acute inflammation. Ishimoto and colleagues investigated the anti-inflammatory role of urolithin A in carrageenan-induced paw edema in mice (Ishimoto *et al.*, 2011). The volume of paw edema was reduced 1 h after oral administration of urolithin A. In addition, plasma exhibited significant oxygen radical antioxidant capacity (ORAC) scores in treated mice, correlating with high plasma levels of the unconjugated form 1 h after oral administration of urolithin A (Ishimoto *et al.*, 2011). These studies show that both ellagic acid and its bioactive metabolite urolithin A exert anti-inflammatory effects by several mechanisms, including the reduction of oxidation and inflammatory cytokines.

Of particular interest is the role of ellagic acid and their metabolites on colon inflammation, which can help elucidate the potential role of ellagitannin-containing foods, such as walnuts, on preventing gut inflammatory diseases. Colon fibroblasts CCD18-Co cells were exposed to a mixture of urolithins A and B and ellagic acid, at concentrations comparable to those found in the colon (Giménez-Bastida *et al.*, 2012)**.** The effects on fibroblast migration and monocyte adhesion were also determined. The mixture of polyphenol metabolites significantly inhibited colon fibroblast migration by about 70% and monocyte adhesion to fibroblasts by about 50%. These effects were parallel with a significant down-regulation of inflammatory markers such as prostaglandin E2 (PGE2), plasminogen activator inhibitor 1 (PAI-1), and IL-1β, as well as other key regulators of cell migration and adhesion (Giménez-Bastida *et al.*, 2012)**.** The results show that a combination of the ellagitannin metabolites at concentrations achievable in

the intestine after the consumption of ellagitannin-containing foods, such as pomegranate or walnuts, was able to moderately improve the inflammatory response of colon fibroblasts and suggest that consumption of ellagitannin-containing foods has potential beneficial effects on gut inflammatory diseases. Additionally, a recent study further confirms the protective effect urolithins have over intestinal inflammation using RAW 264.7 murine macrophages as a model (Piwowarski *et al.*, 2015). Urolithins A, B and C decreased NO production via inhibition of the iNOS protein, and expression of IL-1β, TNF-α and IL-6 (Piwowarski *et al.*, 2015).

Inflammatory processes are associated to several pathological conditions. Available data indicate that the development of diabetic nephropathy, a serious complication confronted by diabetic patients, is also linked to inflammation. A study performed in type 2 diabetic Wistar albino rats evaluated the nephro-protective effects of ellagic acid (Ahad *et al.*, 2014). Ellagic acid treatment for 16 weeks after induction of diabetes significantly attenuated renal dysfunction and oxidative stress. It also significantly inhibited renal NF-kB activation, lowered renal pathology, suppressed transforming growth factor-beta (TGF-β) and fibronectin expressions in renal tissues. Moreover, ellagic acid significantly reduced the serum levels of pro-inflammatory cytokines, IL-1β, IL-6 and TNF-α (Ahad *et al.*, 2014) . Thus, these authors concluded that ellagic acid exerted a renal protective effect in type 2 diabetic rats by a multifactorial approach through anti-hyperglycemic, anti-glycative, antioxidant and anti-inflammatory effects. Taking this evidence into account, the anti-inflammatory properties of ellagitannins potentially exert a protective effect against a wide range of pathologies and their complications.

Estrogenic and androgenic interactions

There has been increased interest in studying the activity of phytoestrogens due to their potential health benefit. The modulation of hormone receptors by phenolic dietary components has been widely studied, many polyphenols, including isoflavones, flavanones and stilbenes have shown phytoestrogenic activity (Mantena *et al.*, 2006; Thangapazham *et al.*, 2007; Tu *et al.*, 2011; Wang *et al.*, 2011). Dietary polyphenols may also be precursors of bioactive compounds with estrogenic activity and ellagitannins, particularly their metabolites urolithins A and B, can be included in this group (Figure 4). A study has assessed the potential activity of urolithins as hormone-disruptive molecules, exerting both estrogenic and anti-estrogenic activity (Larrosa, González-Sarrías, *et al.*, 2006)**.** This work performed structure analyses, which revealed that urolithins A and B exhibited structural characteristics that made these molecules able to bind with the α- and β-estrogen receptors. An estrogen receptor (ER) competitive binding assay in MCF-7 breast cancer cells showed that both urolithins had affinity for ERα and ERβ receptors, although urolithin A bound more effectively than urolithin B, and had higher affinity for the ER α than for the ERβ receptor. Urolithins showed weaker estrogenic activity than other phytoestrogens, such as daidzein, genistein and enterolactone, but they both displayed slightly higher anti-estrogenic activity than the previously mentioned phytoestrogens, dose-dependently antagonizing the growth-promotion effect of 17-β-estradiol. In addition, our group also explored the hormone-related activity of urolithins A and B using LNCaP androgen-dependent prostate adenocarcinoma cells. Considering the reported anti-androgenic activity of other phytoestrogens such as isoflavones and the phytoestrogenic effect of urolithins observed by Larrosa and colleagues (Larrosa, González-Sarrías, *et al.*, 2006), we hypothesized that urolithins A and B

could potentially interact with the androgen receptor. We observed a significant decrease in androgen receptor (AR) mRNA and protein levels after treatment with urolithins at several time points. Electrophoretic mobility shift assays also revealed that urolithins decreased AR binding to androgen response elements, which in turn resulted in a decreased expression of AR regulated genes such as *klk3* which encodes for prostate-specific antigen (PSA) (Figure 4) (Sánchez-González *et al.*, 2014)**.** Considering the aforementioned evidence it is possible to conclude that urolithins can play a key role in the modulation of hormone and hormone-receptor dependent diseases, such as, breast and prostate cancers.

4. HEALTH BENEFITS OF WALNUT POLYPHENOLS AND THEIR DERIVED METABOLITES

Cancer

Polyphenols exert their anticancer effects by several mechanisms, such as, the reduction of pro-oxidative effect of carcinogenic agents (Duthie & Dobson, 1999; Owen et al., 2000), modulation of cancer cell signaling and of cell cycle progression (Corona et al., 2007; Corona et al., 2009; Khan & Mukhtar, 2013), promotion of apoptosis (Fabiani *et al.*, 2002; Mantena *et al.*, 2006), and modulation of enzymatic activities (Adams *et al.*, 2010). Polyphenols have also been shown to act on multiple targets in pathways not only related to cellular proliferation and death (Fini *et al.*, 2008), but also in inflammation (Kang *et al.*, 2011), angiogenesis (Granci *et al.*, 2010), and drug and radiation resistance (Garg *et al.*, 2005). In particular, the effect walnut polyphenols have on cancer prevention has been studied widely, showing promising results (Spaccarotella *et al.*, 2008; Reiter *et al.*, 2013; Hardman, 2014; Sánchez-González *et al.*, 2015).

Both *in vitro* and *in vivo* studies that assess the role of ellagitannins in several molecular pathways related to cancer initiation, development and progression have been performed.

Prostate

Prostate cancer is the second most frequently diagnosed cancer and the sixth leading cause of cancer death among men worldwide (American Cancer Society, 2011). An important target in prostate cancer is the androgen receptor, which is required for the development and progression of prostate carcinogenesis. As previously mentioned, our research group has assessed the effect that urolithins have on the modulation of the androgen receptor, which is fundamental in prostate cancer progression. We were able to determine an inhibition in both AR and PSA gene expression and a decrease in protein levels after incubating androgen-dependent LNCaP prostate adenocarcinoma cells with urolithins A and B. In addition, we were able to determine that urolithins inhibited the binding of AR to androgen response elements, which are transcription factors necessary for the transcription of genes such as PSA. An induction of apoptosis and a decrease in the anti-apoptotic protein BCL-2 were also observed (Sánchez-González *et al.*, 2014**).** In addition, after further study using a functional genomics approach we also determined CDKN1A, which encodes for p21 protein, as a node gene of urolithin A activity in a prostate cancer cell model. Upon validation, a significant increase in anti-proliferative p21 mRNA and protein levels was observed. In addition, increased activity of the apoptotic enzymes, caspases 3 and 7 was seen (Sánchez-González *et al.*, 2015). Other authors have also observed anti-apoptotic effects in other prostate cancer cells such as PC3 and DU 145, which are androgen independent, demonstrating that walnut polyphenols negatively affect prostate cancer cell viability via different mechanisms (Vicinanza *et al.*, 2013**).**

Another established target in prostate cancer chemoprevention is a cytochrome P450 enzyme, CYP1B1. Compounds inhibiting CYP1B1 activity are contemplated to exert beneficial effects at three stages of prostate cancer development, that is, initiation, progression, and development of drug resistance. Urolithins, especially urolithin A, were found to decrease CYP1B1 activity and protein expression in 22Rv1 prostate cancer cells (Kasimsetty *et al.*, 2009). Furthermore, both walnut extracts and ellagitannins have also been linked to suppressing prostate cancer cell proliferation and inducing apoptosis (Losso *et al.*, 2004; Alshatwi *et al.*, 2012; Vicinanza *et al.*, 2013; Naiki-Ito *et al.*, 2015) in several models. These cell-based assays allow for the identification of molecular pathways where walnut polyphenols may exert their activity.

Animal and human studies on the potential link between walnuts and prostate cancer prevention are still limited, but the results seem promising. Reiter and colleagues tested whether a walnut-enriched diet influenced the growth of prostate cancer xenografts growing in male nude mice (Reiter et al., 2013). They found that the walnut-enriched diet reduced the number of tumors and the growth of the LNCaP xenografts. These authors hypothesized that the most likely explanation for the finding that a walnut-enriched diet forestalled the growth of prostate tumors is that the inhibitory effect was a consequence of the combined actions of several phytochemicals, among them the polyphenolic compounds. Similarly, another recent *in vivo* study found that prostate tumor weight and growth rate were reduced in the TRAMP (transgenic adenocarcinoma of mouse prostate) cancer model after treatment with a walnut diet (Davis *et al.*, 2012). Like Reiter et al. (2013), the authors stated the beneficial effects of a walnut-enriched diet probably represent the effects of multiple constituents in whole walnuts and not due to specific bioactive compounds such as, fatty acids or tocopherols.

Human studies are still lacking, and a recent study points the fact of a need to better design *in vitro* and *in vivo* approaches in order to mimic physiological conditions as closely as possible (González-Sarrías, Giménez-Bastida, *et al.*, 2010). This intervention study involving sixty-three patients with either benign prostate hyperplasia or prostate cancer divided subjects into three groups: controls, walnut intake (35g walnuts/day), or pomegranate intake (200mL pomegranate juice/day) for 3 days before prostate surgery. The main metabolite detected after consumption was urolithin A glucuronide. No apparent changes in the expression of CDKN1A, MKi-67 or c-Myc (all related to cancer cell proliferation) were found after consumption of either experimental food treatment. The results from this study demonstrated that conjugates of urolithins, specifically glucuronides, and dimethyl ellagic acid can reach and enter the human prostate gland upon consumption of ellagitannin-rich sources such as pomegranate juice and walnuts. Considering their results and the lack of changes in proliferation markers, these authors expressed the need to design better *in vitro* studies that should focus on the bioactivity and exposure time of the actual *in vivo* metabolites formed upon consumption of ellagitannins. (González-Sarrías, Giménez-Bastida, *et al.*, 2010). Another clinical trial done in healthy men showed that consuming walnuts on a regular basis did not influence serum PSA levels although it did improve biomarkers of prostate health (Spaccarotella *et al.*, 2008), this indicates a positive effect on prostate health of including walnuts as part of men´s diet.

As a conclusion, all the previously mentioned findings point out the need for studies using whole foods, such as walnuts, in human intervention trials to truly assess their effects on prostate cancer and to identify effective, food-based chemoprevention diets for prostate and other cancers.

Breast Cancer

Considerable amount of research has focused on the role food bioactive components have in the development and progression of breast cancer. In a study conducted by Hardman and colleagues (2011), female mice were fed control or walnut-containing diets, and were followed for tumor development. Compared to a diet without walnuts, their consumption significantly reduced tumor incidence, number of tumors per mouse and their size. Gene expression analyses indicated that a walnut diet altered expression of multiple genes associated with proliferation and differentiation of mammary epithelial cells. Although specific bioactive components were not tested, the results of this study indicate that walnut consumption could contribute to a healthy diet for the prevention of breast cancer (Hardman *et al.*, 2011).

It is well known that an important target in breast cancer research is the estrogen receptor since estrogens have a critic role in the development and growth of breast cancer (Clemons and Goss, 2001). As such, research addressed to study the interaction of ellagitannins against estrogen positive breast cancer models is of interest. In an *in vivo* study, the role of ellagic acid over the expression of miRNAs related to breast cancer was evaluated using an ACI rat model, which develops mammary tumors upon estrogen E2 (estradiol) exposure. Recent reports have associated several miRNAs with estrogen receptors in breast cancers. Ellagic acid reversed the deregulation of a number or miRNAs, such as, miR-375, miR-206, miR-182, miR-122, miR-127 and miR-183 detected after E2 incubation. It also modulated their target proteins which include ERα, cyclin D1, RASD1, FoxO3a, FoxO1, cyclin G1, Bcl-w and Bcl-2. These observations provide mechanistic insight into the molecular events behind the chemo-preventive action of

ellagic acid specifically in breast cancer (Munagala *et al.*, 2013). Ellagic acid also exhibited the potential to down-regulate the 17β-estradiol-induced hTERT α+β+ mRNA expression in MCF-7 estrogen sensitive breast carcinoma cells (Strati *et al.*, 2009).

As previously mentioned, the estrogen receptor represents an important target for the treatment of hormone-dependent breast cancer with anti-estrogens, due to the effect estrogen has on breast cancer cell growth and progression. The hormone-like activity reported for urolithins has been related to their interaction with the estrogen receptor in MCF-7 cells, showing a high binding affinity to this hormone receptor (Larrosa, González-Sarrías, *et al.*, 2006), and exerting both estrogenic and anti-estrogenic effects. Therefore, urolithins can potentially antagonize the response of hormone-dependent breast cancer cells to estrogens. In addition, the potential anti-aromatase activity of ellagitannin-derived compounds has been studied in MCF-7aro cells (ER-positive aromatase overexpressing cells) (Adams *et al.*, 2010). The aromatase enzyme converts androgen to estrogen and as such, plays a key role in breast carcinogenesis. They screened a panel of ellagitannin-derived compounds and identified six with anti-aromatase activity; Urolithin B was shown to most effectively inhibit enzymatic activity. Their results suggested that urolithin B likely inhibits the proliferation of MCF7aro breast cancer cells primarily through aromatase inhibition, and that anti-proliferative effects caused by the other test compounds may be caused by an aromatase-independent mechanism such as direct antagonism of estrogen receptor signaling or a combination of aromatase-dependent and independent mechanisms (Adams *et al.*, 2010). Strong anti-proliferative activity was observed after ellagic acid incubation in MCF-7 and Hs 578T cell lines (Losso *et al.*, 2004), although it is interesting to note that in this study cell lines from different cancer models were studied and the most resistant were the

breast cancer cells (Losso *et al.*, 2004). Considering all the previously mentioned studies, the role of ellagitannins and derived metabolites in breast cancer prevention is promising. Additional studies could aid in further elucidating the molecular effects ellagitannins have in breast cancer development and progression inhibition.

Colon Cancer

Foods rich in ellagitannins could act against colon carcinogenesis as evidenced by several studies (Losso *et al.*, 2004; González-Sarrías, Larrosa, *et al.*, 2010; Giménez-Bastida *et al.*, 2012; Qiu *et al.*, 2013). Walnut leaves, green husks, and seed extracts showed concentration-dependent growth inhibition toward human kidney and colon cancer cells (Carvalho *et al.*, 2010). Exposure of Caco-2 (colon adenocarcinoma) cells to ellagic acid and urolithins arrested cell growth at the S- and G2/M-phases, which could have been related to a decrease in the expression levels of MAPK signaling genes, tumor suppressors and genes involved in cell cycle (González-Sarrías *et al.*, 2009). Another signaling pathway known to play a pivotal role in human colon carcinogenesis is the Wnt pathway. An inappropriate activation of this signaling cascade is observed in 90% of colorectal cancers. Wnt transcriptional activation was explored in a human 293T colon cancer cell line, cells were incubated with ellagitannin extracts from several food sources, including strawberries and pomegranate, and specific polyphenols, ellagic acid (63μM) and urolithin A (39μM). All extracts, ellagic acid, and urolithin A inhibited Wnt-dependent signaling, a promising effect against colon carcinogenesis (Sharma *et al.*, 2010).

A walnut-containing diet has also been shown to inhibit colorectal cancer growth by suppressing angiogenesis. In an *in vivo* study using HT-29 cells (colon carcinoma) injected to

mice, tumor growth rate was significantly slower in walnut-fed (27%) compared with corn oil-fed animals (Nagel *et al.*, 2012). Consequently, final tumor weight was reduced by 33% versus control. Walnuts also reduced serum expression levels of angiogenesis factors, including vascular endothelial growth factor by 30% and significantly decreased angiogenesis proved by CD34 staining. In addition to the anti-proliferative effects of walnut bioactive compounds, its main metabolite, urolithin A has been shown to potentiate the effects of 5-Fluorouracil (5-FU, drug of first choice in colorectal cancer therapy) on colon cancer cells. This would suggest the need for lower 5-FU doses to achieve therapeutic effects, which could in turn reduce possible adverse effects of treatment and may indicate a role of ellagitannins as chemotherapy adjuvants (González-Sarrías *et al.*, 2015). Although, further studies are needed to confirm these findings in humans and explore underlying mechanisms in more detail, the previously mentioned studies highlight several targets modulated by EA and its metabolites in colon cancer.

Other cancers

Ellagitannins and their metabolites inhibit cancer cell growth, among other ways, through cell cycle arrest and stimulation of apoptosis. The anti-proliferative activities of ellagic acid and its metabolites have been evaluated in several human cancer cell lines, some of which have been previously mentioned (Adams *et al.*, 2010; Vicinanza *et al.*, 2013; Sánchez-González *et al.*, 2014, 2015). Other studies have also evaluated the anti-proliferative activity of ETs and other byproducts of their hydrolysis in a diverse range of cancer cell lines. These include, an *in vitro* study conducted in a human bladder cancer cell line which assessed anti-proliferative activity of four ellagitannin metabolites, urolithin A, urolithin B, 8-OMe-urolithin A, and ellagic acid. The results from this study suggested that these compounds could inhibit cell proliferation by p38-

MAPK and/or c-Jun mediated caspase-3 activation and by the reduction of oxidative stress (Qiu *et al.*, 2013). Other anti-cancer effects of ellagic acid have also been observed through PKC α gene down-regulation and decreased activity, resulting in a marked decrease in oxidative stress and cell viability in Dalton lymphoma bearing mice (Mishra and Vinayak, 2011), and in a study conducted in SH-SY5Y neuroblastoma cells ellagic acid incubation induced anti-proliferative effects including cell detachment, decreased cell viability and induced apoptosis (Fjaeraa and Nånberg, 2009).

Cardiovascular Disease

The characteristic lipid profile of walnuts has been suggested to reduce the risk of cardiovascular diseases, exerting its effect by decreasing total and LDL-cholesterol and increasing both HDL-cholesterol and the antioxidant defense system (Ros *et al.*, 2004; Nergiz-Ünal *et al.*, 2013). However, recent studies have shown the potential cardio-protection effects of ellagitannins, which could be associated to the modulation of several parameters linked to cardiovascular health (Papoutsi *et al.*, 2008; Spaccarotella *et al.*, 2008; Larrosa *et al.*, 2010). One of the strongest effects has been observed on oxidative stress and inflammation, in this sense it is interesting to explore the potential role of ETs over atherosclerosis.

The inflammatory process plays an important role in the pathogenesis of atherosclerosis through the interaction of the endothelium with immune cells (Kaneto *et al.*, 2010). Numerous signaling cascades have been elucidated and, among other functions, the inflammatory cytokine-induced adhesion molecules in the endothelium play a critical role in the inflammatory process and immune response (Han *et al.*, 2007). Adhesion molecules, namely vascular cell adhesion

molecule (VCAM)-1 and intracellular cell adhesion molecule (ICAM)-1, activated by inflammatory cytokines such as TNF-α, participate in the initiation of this interaction. Several lines of evidence support a crucial role of adhesion molecules in the development of atherosclerosis and plaque instability (Blankenberg *et al.*, 2003). Papoutsi *et al.* (2008) examined the effect of a walnut extract and ellagic acid on VCAM-1 and ICAM-1 expression in human aortic endothelial cells. Cells were incubated with TNF-α in the absence and presence of walnut extract or ellagic acid, both treatments significantly decreased TNF-α-induced endothelial expression of VCAM-1 and ICAM-1 (Papoutsi *et al.*, 2008). Authors concluded that walnut polyphenols, mainly ellagic acid, show potential as anti-inflammatory substances and provided insight into the mechanism of how walnut intake may participate in cardio-protection by improving endothelial function.

Another important intervention of walnut polyphenols as modulators of cardiovascular disease, is the protective effect they may have on the susceptibility of LDL to oxidative modification and ultimately, on atherosclerosis. In a study conducted by Anderson *et al.* (2001), polyphenol-rich walnut extracts at expected physiologic concentration were studied and compared with ellagic acid for their ability to inhibit *in vitro* plasma and LDL oxidation during oxidative stress. LDL oxidation was significantly inhibited by both ellagic acid and the walnut extract. These authors concluded that walnut polyphenols are effective inhibitors of *in vitro* plasma and LDL oxidation (Anderson *et al.*, 2001), indicating that in addition to the favorable lipid profile of walnuts, their phenolic content must also be considered as a potential contributor to the apparent anti-atherogenic effect of walnuts (Casas-Agustench *et al.*, 2011; Estruch *et al.*, 2013).

Interestingly, ellagic acid has also been associated with a protective effect on myocardial infarction induced damage. Wistar rats were used as a model in order to provide a scientific basis for the use of ellagic acid in preventing myocardial infarction (Mari Kannan and Darlin Quine, 2012). These authors also performed *in vitro* studies that confirmed the free radical scavenging and metal chelating activities of ellagic acid which could be the mechanism responsible for the protective action against mitochondrial damage in myocardial infarction.

The previously mentioned *in vitro* and *in vivo* studies provide the basis for elucidating the effect of walnut polyphenols over cardiovascular diseases. Human trials have also been performed, granting they do not directly assess the specific effect of polyphenols. Katz *et al* (2012) associated walnuts and their bioactive compounds to impact endothelial function favorably (Katz *et al.*, 2012). In a randomized controlled crossover trial, using a population of overweight individuals with visceral adiposity, the effects of daily walnut consumption on endothelial function and other biomarkers of cardiac risk were investigated. Forty-six overweight adults, with elevated waist circumference and 1 or more additional signs of metabolic syndrome, were randomly assigned to two 8-week sequences of walnut-enriched *ad libitum* diet and *ad libitum* diet without walnuts, which were separated by a 4- week washout period. The primary outcome measure was the change in flow-mediated vasodilation (FMD) of the brachial artery. In this study FMD improved significantly from baseline when subjects consumed a walnut-enriched diet as compared with the control diet. There was also a reduction in systolic blood pressure and maintenance of the baseline anthropometric values was also observed. Although specific components of walnuts were not studied specifically, these authors concluded that the daily ingestion of 56g of walnuts improves endothelial function in overweight adults with visceral

adiposity (Katz *et al.*, 2012) . This study did not assess polyphenols in particular, but it is a good example of the positive cardiovascular health effects of walnuts as a diet component. It would be interesting to assess if these effects are caused by the individual bioactive compounds of which walnuts are composed of, or if it is a result of the interaction between the various phytochemicals they contain.

Neurodegenerative Disease

Lifestyle factors greatly affect the progression of cognitive decline, with high-risk behaviors including unhealthy diet, lack of exercise, smoking, and exposure to environmental toxins leading to enhanced oxidative stress and inflammation. Although there is an urgent need to develop effective treatments for age-related cognitive decline and neurodegenerative disease, prevention strategies are underdeveloped. Thus, potential preventive effects of bioactive compounds commonly found in the diet should be explored. As previously mentioned in this review, polyphenolic compounds found in walnuts have both anti-inflammatory and anti-oxidant properties. Therefore, these compounds might reduce the oxidant and inflammatory load on brain cells, but in addition, they improve interneuronal signaling, increase neurogenesis, and enhance sequestration of insoluble toxic protein aggregates (Poulose *et al.*, 2014).

Amyloid beta-protein (Aβ) is the major component of senile plaques and cerebrovascular amyloid deposits in individuals with Alzheimer's disease. Aβ is known to increase free radical production in neuronal cells, leading to oxidative stress and cell death. Selective inhibition of Aβ oligomer formation provides an optimum target for Alzheimer's disease therapy. Several studies

have addressed the anti-amyloidogenic activities of walnuts (Lin and Salem, 2007; Muthaiyah *et al.*, 2011).

A walnut extract has been shown to reduce Aβ-mediated cell death, decrease the release of lactate dehydrogenase (a marker of membrane damage), and reduce DNA damage as evidenced by a decrease in apoptosis and a decrease in ROS generation in a concentration-dependent manner (Muthaiyah *et al.*, 2011). Although the majority of studies have not explored polyphenols specifically, it could be hypothesized that the anti-inflammatory and anti-oxidant potential in ellagitannins could be in part responsible for the neuro-protective effect of walnuts. It is worth mentioning that ellagic acid could reduce Aβ42-induced neurotoxicity toward SH-SY5Y neuroblastoma cells (Feng *et al.*, 2009), thus ellagitannins show a promise in preventing Aβ-related neurodegenerative diseases.

Another interesting effect of ellagic acid is a potential analgesic property; different animal models of pain were used to test possible mechanisms underlying systemic antinociception after ellagic acid administration with dose-dependent analgesic effects observed (Taghi Mansouri *et al.*, 2013; Mansouri *et al.*, 2015).

3. FUTURE TRENDS AND CONCLUSIONS

In view of the experimental evidence mentioned in this review, we conclude that walnut polyphenols have obvious and numerous **disease-preventive properties**. The molecular functions attributed to walnut polyphenols indicative of their capacity have been performed mainly in *in vitro* studies, which in order to accurately assess biological responses must only use

physiologically relevant concentrations. As a consequence, bioavailability and *in vivo* biological efficacy are critical issues that must be correlated before drawing any conclusions on the potential health benefits of specific bioactive compounds, including polyphenols. Nonetheless, evidence suggests that incorporating walnuts into a healthy diet could aid in the prevention and modulation of several disease states. Although, this review has focused on polyphenols, several bioactive compounds in walnuts have been linked to disease prevention, as such, it is important to incorporate walnuts as a whole, to be able to reap the benefits of the variety of compounds they are composed of. Further research, particularly human trials, are warranted, but current evidence is encouraging enough to make walnut polyphenols bioactive compounds that should be continued to explore.

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LEGENDS TO FIGURES

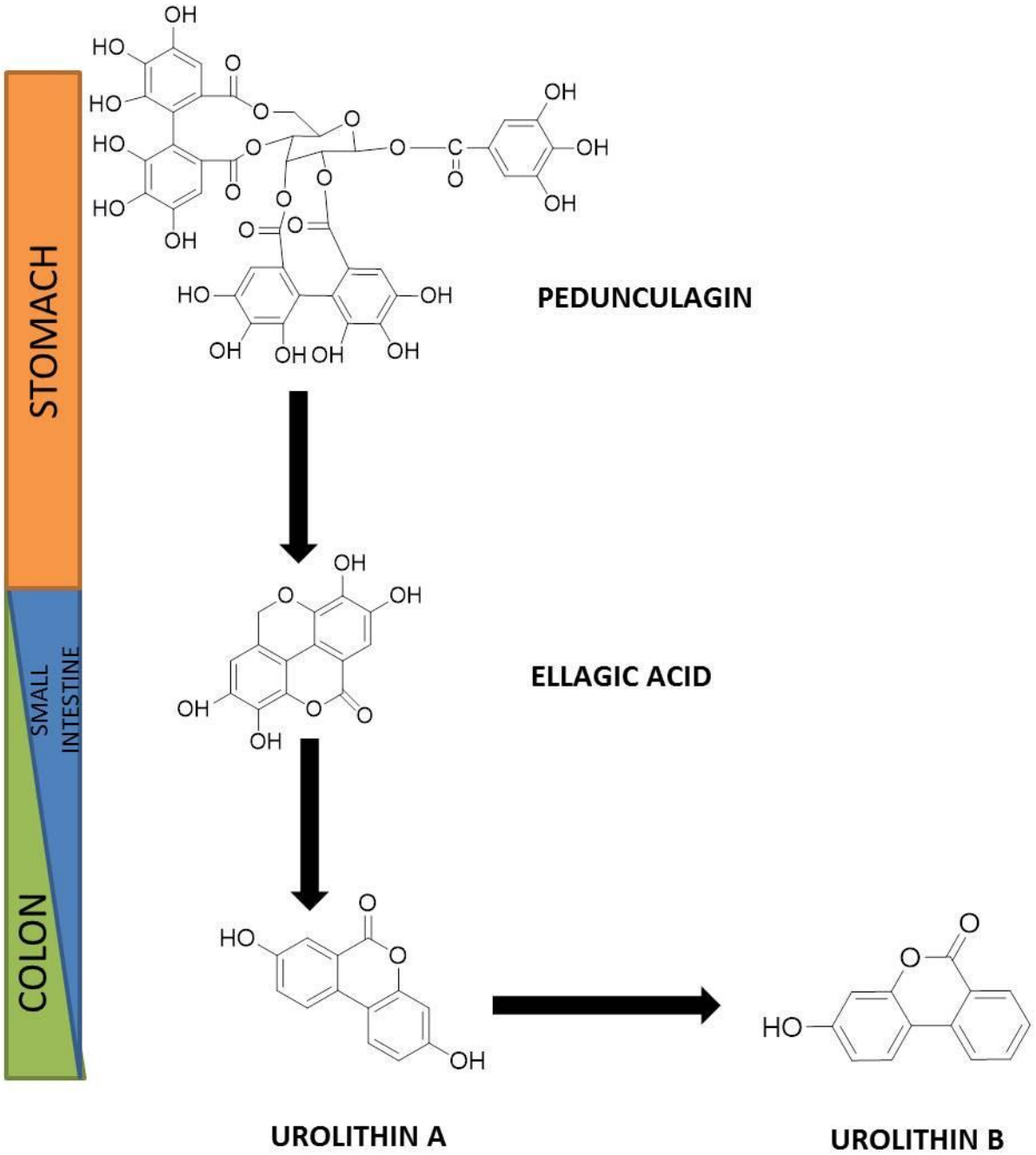

Figure 1. Graphic representation of pedunculagin metabolism.

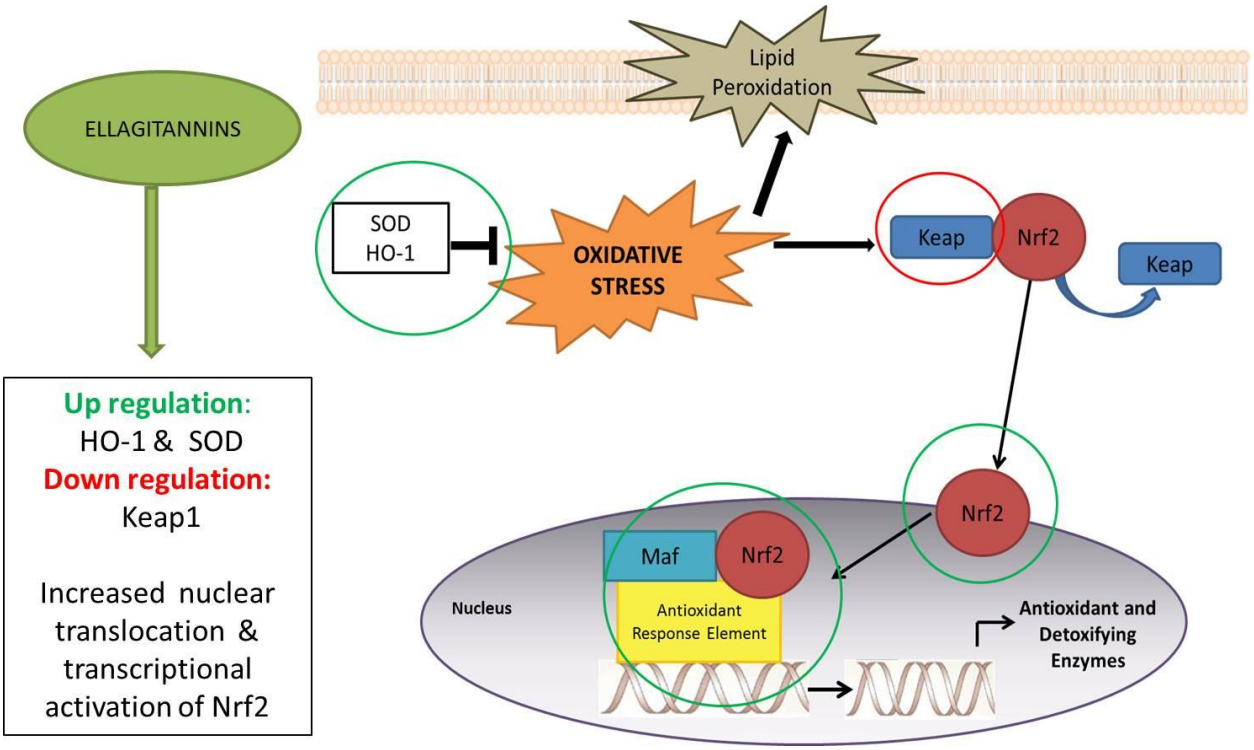

Figure 2: Scheme of the role of ellagitannins on antioxidant related processes in the cell. HO-1: heme oxygenase 1, Keap1: kelch-like ECH-associated protein 1, Maf: musculoaponeurotic fibrosarcoma oncogene, Nrf2: nuclear factor (erythroid-derived 2)-like 2, SOD: superoxide dismutase.

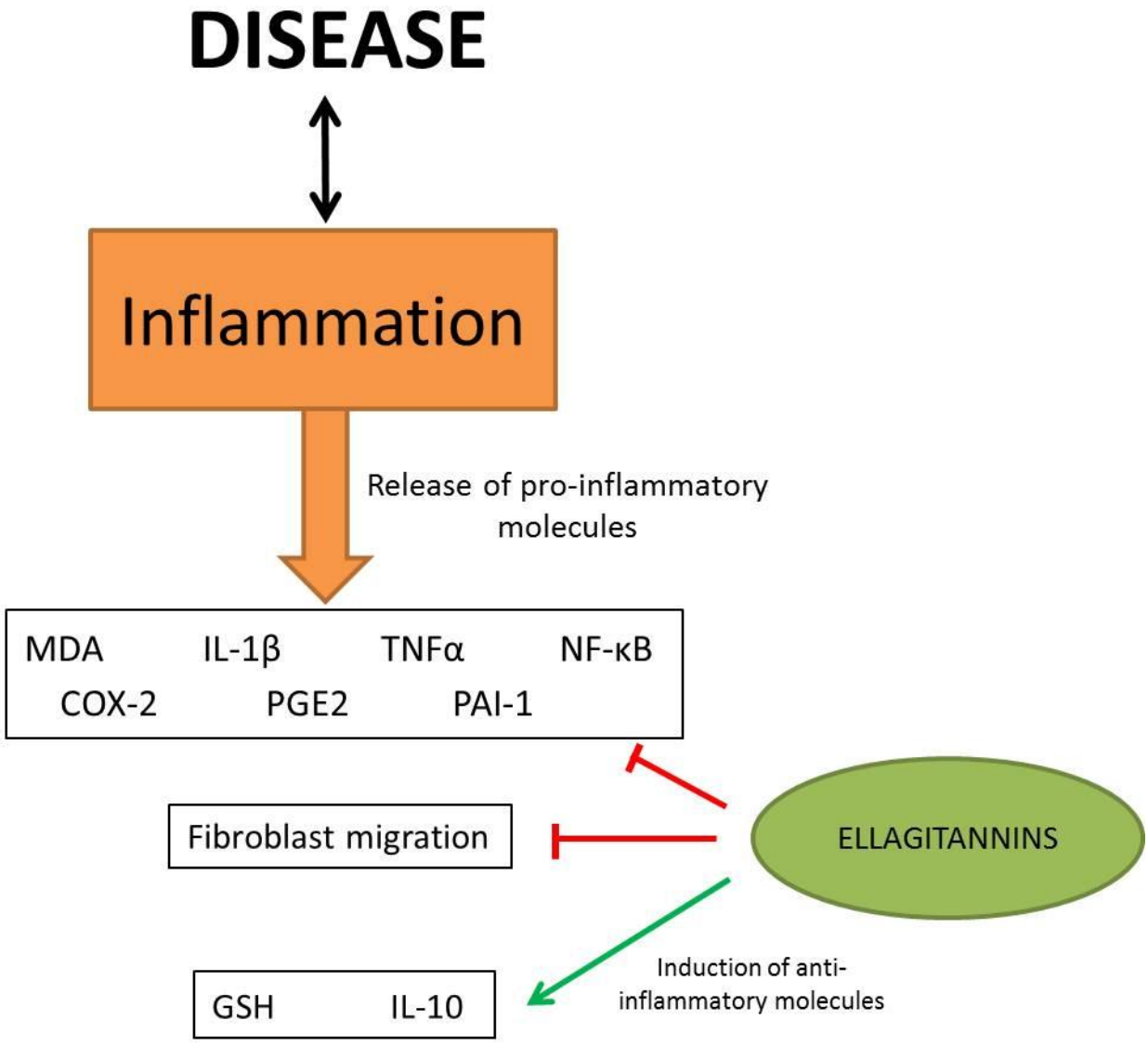

Figure 3. Schematic representation of the effect ellagitannins over the expression of inflammation-related molecules. COX-2: cyclooxygenase, GSH: glutathione, IL-10: interleukin 10, IL-1β: interleukin-1 beta, MDA: malondialdehyde, NF-κB: nuclear factor-κB , PAI-1: plasminogen activator inhibitor 1, PGE2: prostaglandin E2, TNF-α: tumor necrosis factor alpha.

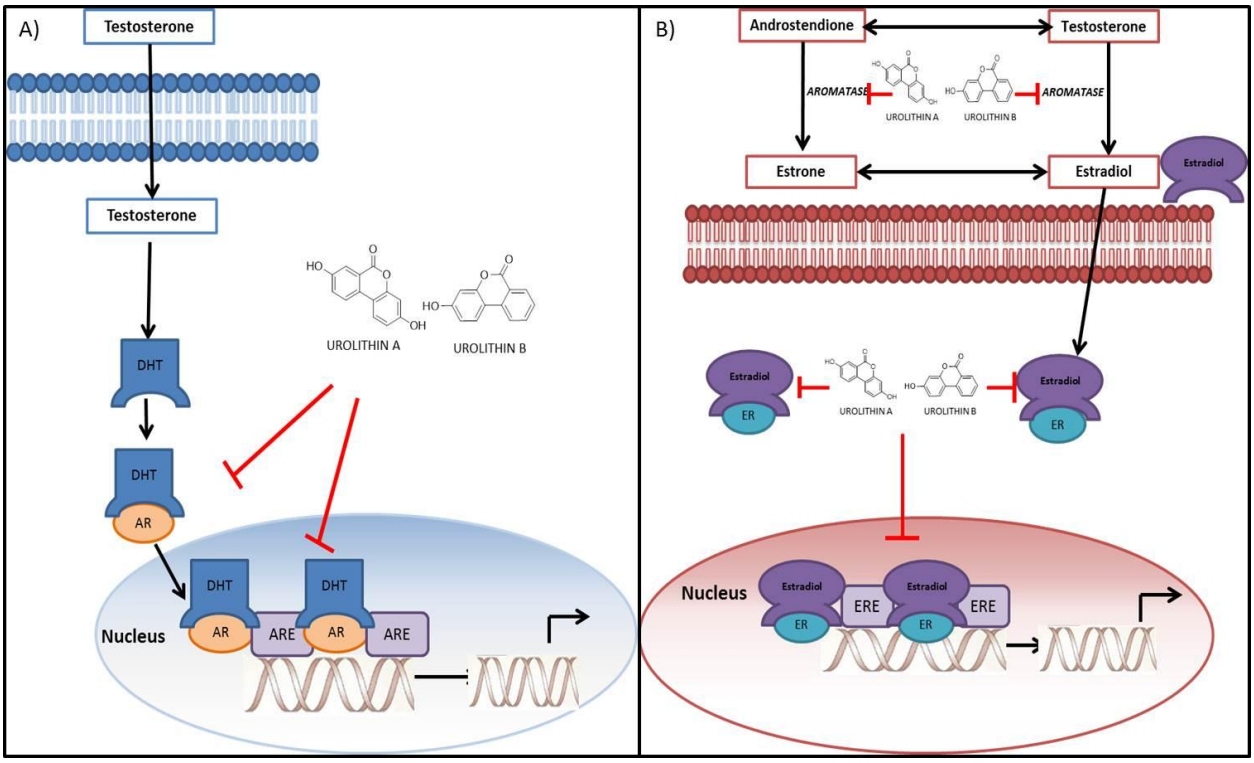

Figure 4. Proposed mechanism of the interaction between urolithins A and B with hormone receptors in A) LNCaP and B) MCF-7 cells. AR: androgen receptor, ARE: androgen response elements, DHT: dihydrotestosterone, ER: estrogen receptor, ERE: estrogen response elements.