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Phenolic Compounds as Arginase Inhibitors: New Insights Regarding Endothelial Dysfunction Treatment

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Key words
vascular disease, endothelial nitric oxide synthase, nitric oxide, arginase, natural products, polyphenols

received August 5, 2017
revised December 9, 2017
accepted December 31, 2017

Bibliography
DOI https://doi.org/10.1055/s-0044-100398
Published online January 17, 2018 | Planta Med 2018; 84: 277–295 © Georg Thieme Verlag KG Stuttgart · New York | ISSN 0032-0943

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ABSTRACT

Endothelial dysfunction is characterised by the low bioavailability of nitric oxide with a relevant negative impact on the nitric oxide/cGMP pathway. The loss of nitric oxide/cGMP signaling may be caused by an increased arginase activity. Plant-derived substances, especially polyphenols, are compounds that have the potential to inhibit arginase activity and they may represent an attractive therapeutic option to combat clinical outcomes related to endothelial dysfunction. An extensive review was carried out using all available data published in English in the Pubmed database, and without restriction regarding the year of publication. Despite the increased number of new substances that have been tested as arginase inhibitors, it is rare to find a compound that satisfies all the toxicological criteria to be used in the development of a new drug. On the other hand, recent data have shown that substances from plants have great potential to be applied as arginase inhibitors, most of which are polyphenols. Of the relevant mechanisms in this process, the inhibition of arginase by natural products seems to act against endothelial dysfunction by re-establishing the vascular function and elevating nitric oxide levels (by increasing the amounts of substrate (L-arginine, and endothelial nitric oxide synthase activation and stabilisation) as well as decreasing the generation of reactive species (formed by uncoupledendothelial nitric oxide synthase). This review summarises several topics regarding arginase inhibition by natural substances as well as indicating this pathway as an emergent strategy to elevate nitric oxide levels in disorders involving endothelial dysfunction. In addition, some aspects regarding structural activity and future perspectives are discussed.

Introduction

Several diseases have been linked to the development of ED, such as atherosclerosis, hypercholesterolaemia, coronary disease, erectile dysfunction, asthma, renal failure, rheumatoid arthritis, periodontitis, psychiatric disorders, and cancer, as well as diseases with a high prevalence, such as diabetes (types 1 and 2) and SAH [1–9].

The preservation of the endothelium is fundamental in maintaining the physiology of the vascular system (in the regulation of its tonus, the development of immune, structural, and proliferative functions, and interaction with other cellular types) and also in the prevention of the development/aggravation of diseases [10–12].

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ABBREVIATIONS

ABH	amino-2-borono-6-hexanoic acid
ADME	absorption, distribution, metabolism, and excretion
AII	angiotensin II
ARG	arginase
BAEC	bovine aortic endothelial cell arginase
BEC	S-(2-boronoethyl)-l-cysteine
b-ARG 1	bovine liver arginase
DFMO	alpha-difluoromethylornithine
DM1	type 1 diabetes mellitus
ED	endothelial dysfunction
EGFR	epidermal growth factor receptor
E_{max}	maximum effect
eNOS	endothelial nitric oxide synthase
HUVEC	human endothelial cell culture
EDRF	endothelium-derived relaxing factor
iNOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
NOHA	Nω-hydroxy-L-arginine
NO	nitric oxide
NOS	nitric oxide synthase
$O_2^{\bullet -}$	superoxide anion
OH·	hydroxyl
$ONOO^-$	peroxynitrite
oxLDL	oxidised low-density lipoprotein
PG	piceatannol-3′-O-β-D-glucopyranoside
ROS	reactive oxygen species
SMC	smooth muscle cell
SAH	systemic arterial hypertension
TDF	(2S)-5,2′,5′-trihydroxy-7,8-dimethoxy flavanone
THSG	2,3,5,4′-tetrahydroxystilbene-2-O-β-D-glucoside

For example, in SAH, vascular oxidative stress can precede the onset of elevated blood pressure, which, associated with conditions of hyperlipidaemia, can lead to the rapid proliferation of endothelial cells. However, the cellular division capacity is limited, which is caused by a cycle arrest of the endothelial cells. As a consequence, these senescent cells undergo morphological changes that are responsible for the increased production of reactive species, which leads to a decrease in the production of NO and increased sensitivity to apoptotic stimulus. Such events lead to progressive impairment in vascular responses, with an intensification of ED [10, 13, 14].

Thus, mechanisms such as oxidative stress, eNOS uncoupling, induction of endothelium-dependent contractile responses, and reduced endothelium-dependent hyperpolarisation can be related to a decrease in vascular response [15, 16]. However, it is worth noting that although there are several factors involved in ED, it is strongly marked by the low bioavailability of NO and, therefore, damage in the NO/cGMP pathway configures one of the most important causes of vascular impairment [6, 10, 11].

In addition, NO is responsible for vascular smooth muscle relaxation, the inhibition of adhesion and aggregation of neutrophils and platelets, participation in neurotransmission and memory processes, the immune system and gene regulation as well as cell cycle regulation and apoptosis. Due to these important effects, NO deficiency receives much attention and ED has already been mentioned in more than 20 000 scientific studies, since both represent risk factors, especially in relation to cardiovascular diseases (▶ **Fig. 1**) [6, 17, 18].

The best-characterised endothelium-derived relaxing factor (NO) is synthesised by NOS from the amino acid L-arginine, while another enzyme, L-arginine-urea hydrolase arginase, or simply ARG, is responsible for regulating the production of this biological mediator through substrate competition [6, 18].

A decrease in the formation of NO is a key point in the development of ED because there is competition for the common substrate, which raises interest in the modulating role of ARG in decreasing NO levels.

Such a modulating function may culminate in a number of vascular changes, which are characterised by impairment of vasodilatory response, increased inflammation, vascular remodelling (collagen deposition and smooth muscle tissue growth), altered platelet aggregation, and cellular apoptosis (▶ **Fig. 2**) [6, 25, 26].

It has been suggested that the inhibition of ARG activity may result in increased NOS substrate availability and, consequently, NO production. This hypothesis has been confirmed by *in vitro* and *in vivo* studies [17, 18, 27–30].

In this context, research regarding the pharmacological inhibitors of ARG as options for the development of new molecules to treat metabolic, respiratory, infectious, and cardiovascular disorders is promising. However, few substances are available for this purpose, and problems related to the pharmacokinetic and toxicological factors of these substances have not yet been resolved [25–27, 31].

Recently, plant extracts and active plant metabolites have emerged as potential alternatives for therapeutic application in several diseases that affect humans. For example, the polyphenolic extract of *Camellia sinensis* (L.) Kuntze (Theaceae) was approved by the U.S. Food and Drug Administration in 2007 for the treatment of genital warts, and in 2012, ingenol mebutate, which is a tigliane diterpenoid, started to be used in the treatment of actinic keratosis [32, 33]. Ethnopharmacological studies are currently being conducted in order to identify ARG inhibitory substances for future clinical use in relation to ED, specifically those related to cardiovascular alterations.

With regard to the latter point, special attention has been paid to substances belonging to the class of polyphenols [26, 29, 34–38]. Therefore, this article presents recent research regarding the search for new ARG inhibitors derived from medicinal plants with a potential therapeutic application in the fight against diseases related to the development of ED, as well as seeking to increase interest in the development of promising drugs in this field.

Methods

This systematic review of the literature was based on scientific material that has already been published in the English language, which was collected from the Pubmed (US National Library of Medicine – National Institutes of Health) database without restric-

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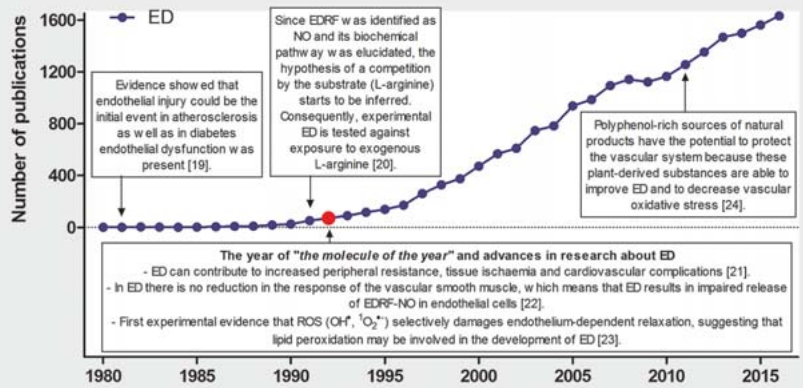
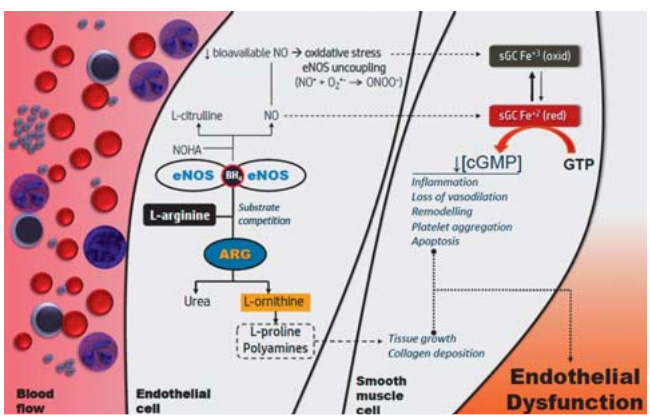


▶ **Fig. 1** Number of scientific publications regarding ED, including its causes and consequences, as well as recent interest in the application of plant-derived substances in the prevention of its complications. The Pubmed database (December 2016) was used to obtain the data and "endothelial dysfunction" was applied as the search term. EDRF: endothelium-derived relaxing factor, ED: endothelial dysfunction, ROS: reactive oxygen species.



▶ **Fig. 2** Mechanisms underlying ED, highlighting the substrate competition between nitric oxide synthase and arginase. ARG – arginase, eNOS – endothelial nitric oxide synthase, BH_4 – tetrahydrobiopterin, NOHA – Nω-hydroxy-L-arginine, NO – nitric oxide, $O_2^{\bullet -}$ – superoxide anion, $ONOO^-$ – peroxynitrite, sGC Fe^{+2} (red) – reduced soluble guanylate cyclase, sGC Fe^{+3} (oxide) – oxidised soluble guanylate cyclase, GTP – guanosine triphosphate, cGMP – cyclic guanosine monophosphate.

tion regarding the year of publication. The search terms that were used included "endothelial dysfunction" and "arginase" or "arginase inhibition", "nitric oxide" and "arginase" or "endothelial dysfunction", and "arginase inhibition" and "plant derived" or "natural compound" or "natural product" or "polyphenol".

The research publications that were included provided *in vitro* or *in vivo* results (human or rat/mouse) as well as revisions related to the proposed theme. The following were excluded: *in vivo* research with species of animals other than those mentioned above (it is important to note that studies using natural compounds such as inhibitors of *Leishmania* sp. ARG were not considered), unpublished studies, studies with incomplete information regarding references, and studies that were not in the format of a scientific article. The chemical names of the molecules presented in the course of this review are in agreement with those presented in the original references that were cited, and the scientific names of the plant species that are mentioned are in accordance with those mentioned in The Plant List (www.theplantlist.org).

For the *in silico* analysis, the oral bioavailability and distribution volume data were collected from ACD/I-Lab (https://ilab.acdlabs.com/iLab2/index.php). The ADME investigation, drug-likeness, and toxicity prediction were obtained through the PreADMET web programme (https://preadmet.bmdrc.kr/). The MDL molfiles of substances were loaded in these databases for calculations.

Arginase: an overview

ARG (L-arginine-urea hydrolase, or amidinohydrolase – EC 3.5.3.1) is a metalloenzyme that was first described in 1904 by Kossel and Dakin in mammalian liver samples [25]. Each active unit of the trimer is essentially two Mn^{+2} ions [6, 39]. The structure and stability of these ions are required for the full catalytic action of the enzyme [40].

During its catalytic cycle, the guanidine grouping of L-arginine undergoes a nucleophilic attack from a complex formed by Mn^{+2} and hydroxide ions from water molecules, forming a neutral, intermediate tetrahedral, and releasing L-ornithine and urea [6, 27, 40].

Since 1965, different ARG isoforms have been reported in human tissues [41–43]. In mammals, two of these isoforms are most prominent and, therefore, they are reported more frequently in the scientific literature, namely, ARG 1 and ARG 2 (▶ **Table 1**) [11, 41].

ARG isoforms are encoded by homologous genes that are mapped in distinct chromosomes (ARG 1 in chromosome 6q23 and ARG 2 in 14q24) [27, 36, 53–56]. A genetic sequencing study that was performed with human kidney tissue detected that the ARG 2 sequence was 58% homologous to that of ARG 1 [49], whereas human and mouse ARG 1 have 87% of the sequence in common [27]. This information is important because it points to-

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▶ **Table 1** Some human characteristics of ARG 1 and 2.

	ARG 1	ARG 2
Amino acids	322	354
Weight	105 kDa	129 kDa
K_m	0.08 at pH 8.5	4.8 at pH 7.4
Tissue distribution	Endothelial cells, nephritic glomeruli, macrophages, liver, erythrocyte, coronary arteries, corpora cavernosal, brain, retinal glia, polymorphonuclear neutrophils, and saliva.	Smooth muscle cells, endothelial cells, normal glomeruli, macrophages, kidney, gastric cancer tissue, corpora cavernosal, brain, retina, and horizontal cells at heart, placenta, lung, skeletal muscle, pancreas, and prostate.
Inducers	LPS, TNFα, hyperglycaemia, nitric oxide, AII, IL-1, and glucocorticoids.	IL-1, IL-4, IL-13, hypoxia, LPS, TNFα, thrombin, oxLDL and haemodynamic forces.
Comments	Is highly expressed in the cytosol of hepatocytes – catabolic function to convert L-arginine in ureia (ureia cycle).	Is located within the mitochondrial matrix. Has widespread tissue localisation and a relatively low specific activity (in general, anabolic functions).
References	[26, 27, 39, 41, 42, 44–52]	

The presented data does not consider other animal species. LPS: lipopolysaccharide, TNFα: tumor necrosis factor-alpha, AII: angiotensin II, IL: interleukin, oxLDL: oxidised low-density lipoprotein

wards the identification of isoforms in human samples and makes it possible to investigate enzymatic induction under normal or pathological conditions.

In eukaryotic organisms, when they are active, both ARG isoforms take the homotrimeric form (105 kDa – ARG 1 and 129 kDa – ARG 2) [6, 40, 42]. At this point, the maximum activity of ARG is about 1000 times greater than that of NOS, however, its affinity for L-arginine (K_m 1–5 mM) is lower when compared to the same enzyme (K_m 2–20 μM) [2, 57].

ARG 1 is the largest fraction of the total ARG expressed in the organism [26]. It is present in the cytosol of liver cells, where it is an integrated part of the urea cycle (conversion of the L-arginine substrate to L-ornithine and urea) as well as other enzymes [N-acetylglutamate synthase (NAGS), carbamoylphosphate synthetase (CPS1), mitochondrial ornithine transporter (OTC), ornithine transcarbamylase (ASL) and argininosuccinatesynthetase-1 (ASS1)] [2, 47, 53]. ARG 2 is mitochondrial and can be found in several tissues, mainly in the kidney. This isoform has several roles that have not yet been fully defined, including participation in the synthesis of polyamines as well as the formation of proline, creatine, glutamate, agmatine, and γ-amino-butyric acid (GABA) [27, 41, 47, 57].

Both ARG 1 and ARG 2 can be expressed in the vascular endothelium [31]. Despite some controversy about the expression of isoforms in the adjacent smooth muscle cell layer [2, 58], it has been shown that aortic smooth muscle tissues in rats express ARG 1 [59]. On the other hand, smooth muscle cells of human lung tissue express both isoforms [26, 55, 59]. In general, ARG expression can be modulated in different sites, depending on the stimulus that is applied [7, 41, 55, 60].

Furthermore, it has been demonstrated that iNOS-derived NO can nitrosate the sulphur of the cysteine residue 303 of ARG, activating the enzyme [61]. However, reduction in the levels of L-arginine caused by ARG activity may cause decreased iNOS activity [62]. These data suggest a bidirectional relationship between ARG 1 and iNOS that could play an important role in vascular diseases [2].

In vitro and *in vivo* studies have demonstrated that LPSs increase the mRNA of ARG 1/ARG 2 and iNOS in different tissues, such as the lung, heart, liver, and endothelial cells of rats [41]. In parallel, other substances, such as THF-α, high glucose concentrations, oxidised low-density lipoprotein, hydrogen peroxide or peroxynitrite, and thrombin may induce increased ARG expression (▶ **Fig. 3**) [63]. Thus, inflammatory mediators modulate the expression of iNOS and ARG, depending on the cellular system that is involved [41, 64, 65].

Interestingly, Nelin et al. [71] showed that an increase in ARG expression, whilst not affecting NOS levels, can result from the activation of the EGFR (expressed in endothelial cells). Likewise, it has been demonstrated that AII led to an increase in ARG expression and activity in the mouse aorta [51]. Furthermore, the increased expression and stimulation of AII receptors is associated with alterations in the activity of ARG [72].

Other conditions, such as hypertension, ischemia-reperfusion, intima layer hyperplasia, and aging, can elevate ARG levels, which is expressed *in vivo* in endothelial tissue [63]. Thus, in addition to its interaction with iNOS, ARG is also closely related to the maintenance of the functions of eNOS, which is an important enzyme isoform for the preservation of vascular homeostasis because the eNOS-derived NO acts to inhibit the vascular tonus, platelet aggregation, and inflammation [1, 2]. Consequently, any alteration of the system orchestrated by NO may cause what is known as ED, and although the main effect of this disorder is damage to vasodilation mechanisms, it has also been reported that local inflammation, lipoperoxidation, SMC proliferation, deposition of extracellular matrix, and platelet and thrombotic activation can occur (▶ **Fig. 2**) [10].

Therefore, ARG is a regulator of the bioavailability of NO by competing with eNOS for the L-arginine substrate, and an increase in ARG activity and a consequent decrease in NO bioavailability are linked to the development of ED and its complications in

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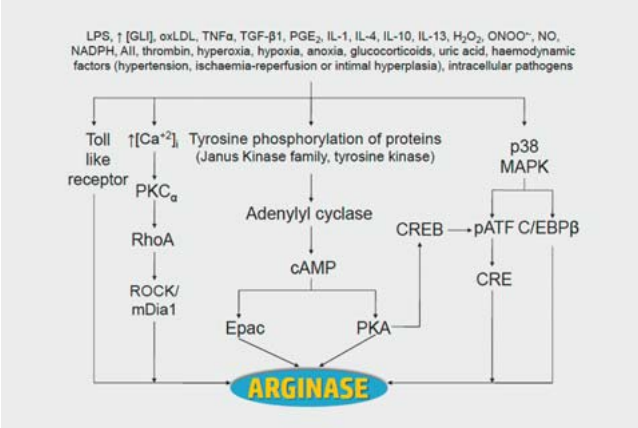

▶ **Fig. 3** Pathways involved in arginase expression. Several humoral and haemodynamic factors, including intracellular pathogens and ROS, are part of the mechanism of ARG activation [26, 41, 46]. Intracellular pathogens (e.g., *Mycobacterium tuberculosis*) induced ARG expression through the toll-like receptor pathway [66]. The formation of pores in the endothelium and hyperpermeability in the lungs (as occurs in severe pneumonia) can increase intracellular calcium concentration, activating protein kinase C (PKCα), which activates RhoA/ROCK to elevate ARG expression [67]. Similarly, the atherogenic stimulus oxLDL acts via the RhoA effectors ROCK and mDia1 to activate L-arginine catabolism by augmenting ARG levels [50]. Microgravity conditions activate the p38 MAPK (mitogen-activated protein kinase)-C/EBPβ pathway [68]. Furthermore, the induction of tyrosine phosphorylation of proteins, like the Janus kinase family (JAK1, JAK2) and tyrosine kinases (TyK-2), leads to adenylyl cyclase activity through a cAMP (cyclic adenosine monophosphate)/PKA or Epac pathway [45, 69]. The ability of p38 MAPK to phosphorylate the activation transcription factor (pATF) suggests that p38 MAPK may modulate the expression of cAMP – responsive-elements (CRE). Furthermore, CRE-binding protein (CREB) can be activated by PKA and bind to pATF as a heterodimer to facilitate ARG transcription via CRE [70]. LPS – lipopolysaccharide, GLI – glucose, TNFα – tumor necrosis factor alpha, TGF-β – transforming growth factor beta, PGE_2 – prostaglandin E_2, IL – interleukin, H_2O_2 – hydrogen peroxide, $ONOO^{\bullet-}$ – peroxynitrite, NO – nitric oxide, NADPH – dihydronicotinamide-adenine dinucleotide phosphate, AII – angiotensin II.

the various diseases in which it is present. This emphasises why ARG has become the subject of studies regarding the development of inhibitors as new pharmaceutical tools [17, 18, 61].

As previously mentioned, changes in NO bioavailability constitute the key event in the development of ED. Many mechanisms are involved in the decompensation of the NO supply, especially its inactivation due to oxidative stress (mitochondrial respiration, arachidonic acid cascade, cytochrome p450 complex, xanthine oxidase, NADH/NADPH oxidase, iNOS, peroxidases, and haemoproteins), which is associated with eNOS uncoupling and a decrease in the expression of this same enzyme, with or without a shortage of enzymatic or substrate cofactors (L-arginine) [13, 73].

Several studies have shown that blocking the advancement of ED is a powerful tool in reducing cardiovascular risks and, thus, many strategies have been investigated in order to prevent the development of ED or complications associated with it [16].

Compounds of natural origin, especially polyphenols with antioxidant activity, have been successfully tested in relation to ED [12, 74–77]. Dal-Ros et al. [35] showed that the consumption of polyphenols in red wine protected against aging-related ED by normalising the oxidative stress that was induced in the animal model that was tested. Similarly, natural products have a recognised stabilising or stimulating effect on eNOS, which promotes an increase in NO levels, which are lower in ED [12, 30, 65, 78, 79].

Another strategy that has been evaluated in studies regarding the treatment of diseases associated with ED is an attempt to provide physiological supplementation with L-arginine substrate, although this has produced controversial results that are related to limiting factors such as the consumption of this amino acid via alternative metabolic pathways, rapid metabolisation after oral administration, the need to screen patients who would clinically require L-arginine replacement, and the difficulties in determining individual levels of active ARG [4, 6, 55]. A controlled study of oral L-arginine supplementation conducted with patients with a history of myocardial infarction had to be discontinued because of the excessive mortality rate of the participants [6, 25]. It has also been observed that the exposure of cell cultures to arginine may even precipitate endothelial senescence [15].

Furthermore, it should be taken into account that the intracellular level of arginine is higher (more than 800 µM) than the extracellular level (50–200 µM). Given that the affinity of eNOS purified by this substrate is in the micromolar range of $K_m = 2.9$ µM, it is suggested that eNOS operates below its saturation concentration, and therefore would not respond to changes in the concentration of circulating L-arginine, which would theoretically refute the alternative of supplementation with the semi-essential amino acid against ED [6, 12, 80].

In fact, the chronic intake of L-arginine offers minimal therapeutic outcomes in vascular disease, showing that this substance is probably not a limiting factor regarding NO production. The exception may be when ARG is more active, reinforcing the competition with eNOS for the common substrate [15].

Thus, because a decrease in the bioavailability of NO has a central role in the mechanism of ED, and due to the fact that competition between eNOS and ARG for L-arginine can intensify this process, scientific efforts were concentrated in order to better investigate the role of ARG in this mechanism.

Scientific evidence began to emerge in the 2000s that ARG activity limited NO production by NOS, and that this was closely related to the depletion of endothelium-dependent vasodilation [81]. These results revealed the importance of ARG as a regulator of the process of the development of ED and transformed it into a new issue of interest for the scientific community regarding the search for new ways to block the degradation caused by ED in the various diseases in which it occurs (▶ **Fig. 4**) [82, 83].

In the period 1990–2011, more than 500 patents were registered in the field of new synthetic ARG inhibitors (425 were registered in the USA), most of which were boronic derivatives. Nevertheless, this constitutes a vast field of research, since many of the patented products still present problems related to pharmacodynamic and kinetic action (factors such as the lack of selectivity in relation to ARG 1 and ARG 2 isoforms, short half-life, loss of potency in physiological pH, and intrinsic toxicity) [6, 25, 27, 63, 84].

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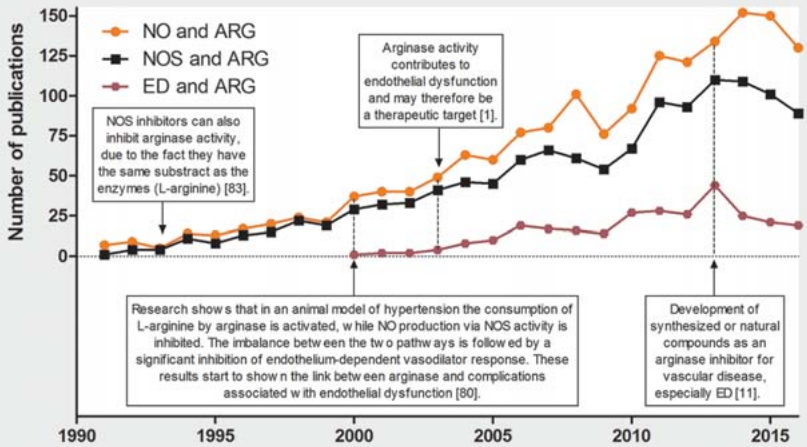

▶ **Fig. 4** Development of research regarding the following: the involvement of arginase (ARG) in endothelial dysfunction (ED) (purple line), the perception of the existence of competition between nitric oxide synthase (NOS) and ARG (black line), and the consequent reduction of the bioavailability of NO (orange line). This resulted in increased interest in the research and development of ARG inhibitors with therapeutic appeal in relation to ED. The Pubmed database (December 2016) was used to obtain the data and "nitric oxide and arginase", "nitric oxide synthase and arginase", and "endothelial dysfunction and arginase" were applied as search terms.

In 2003, the U.S. Food and Drug Administration gave approval for a representative of boronic acid derivatives (bortezomib) to be used to treat multiple myeloma and mantle cell lymphoma [85, 86]. However, toxicological tests on rats and monkeys have indicated haematological, lymphoid, cardiac, renal, gastrointestinal, and neurological problems linked to its use, and data on its genotoxicity have not yet been published [6, 25].

Thus, plants are a resource that is still little explored, but which have great potential. Research into new agents of natural origin has been gaining prominence as a source of interesting substances that can be used to develop new therapeutic options with low NO bioavailability [5, 11].

Plants as a new source of arginase-inhibiting molecules: *in vitro* and *in vivo* evidence

Different methods have been developed to study the inhibition of natural products in relation to ARG activity. *In vitro* techniques include a micro-immobilised enzyme reactor (IMER), which uses ARG that is covalently bound to an ethylenediamine monolithic convective interaction media disk submitted to an HPLC system. Using this procedure, a procyanidin-enriched extract of the stem bark from *Ficus glomerata* Roxob was assessed by simultaneous injection with an enzyme substrate (nitro guanidine benzene). As a result, the enzyme K_m values did not change, but the V_{max} decreased due to a high quantity of polymers that affected the enzyme proximity and orientation. This demonstrated, for the first time, the direct action of plant-derived compounds on ARG activity and the modifications induced on it [58].

Interestingly, the hypothesis of a molecular interaction effect between isolated substances, or plant-derived extracts and ARG, has been little explored in the literature. From this point of view, polyphenolics have an important role to play due to their ability to alter the active conformation of enzymes by destabilising the bonds between hydrogen bonds and water molecules [27].

Akanni et al. [87] tested the effects of the methanolic extracts of the African species *Artocarpus altilis* (Parkinson ex F.A.Zorn) Fosberg (stem bark), *Ficus exasperata* Vahl (leaves), *Kigelia africana* (Lam.) Benth. (fruits), and catechin in relation to samples of cardiac ARG. The *in vitro* results indicated that *F. exasperata* and *K. africana* were not effective, whereas *A. altilis* and catechin (both tested at 500 and 700 µg/mL) inhibited enzymatic activity in 63, 67, 42, and 52% of cases, respectively, when compared to the control.

The rhizomes of ginger [*Zingiber officinale* Roscoe (Zingiberaceae)] and saffron, which is better known as red ginger [*Curcuma longa* L. (Zingiberaceae)] (2 and 4%) were included in a diet that was rich in cholesterol (2%) that was given to rats for 14 days. At the end of the trial period, it was shown that there was a significant reduction in the ARG activity measured in the plasma and liver of the treated animals when compared to the control. In addition, the presence of gallic acid, catechin, caffeic acid, epicatechin, rutin, quercetin, quercetrin, campherol, luteolin, and curcumin in samples of the rhizomes was noted, and the results that were obtained were attributed to these substances because an inverse correlation was observed between the consumption of phenolics (flavonoids) and the total concentration of plasma cholesterol [17, 88].

These results have contributed to the study of the application of new ARG inhibitors in cardiovascular alterations, since the ED involved in these situations would be impeded by the inhibition of the enzyme, resulting in a greater blood supply (NO-mediated vasodilatation) to the tissues. Spontaneously hypertensive rats showed low pressure rates and improved endothelial function when submitted to ARG inhibition [58].

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Other studies have also tested dietary supplementation with plant extracts to inhibit *in vivo* ARG activity. Wistar rats (male, adults) were sprayed with 400 µL (200 mg/Kg) of the aqueous extract of *Yucca schidigera* Roezl ex Ortgies (Asparagaceae) (Mohave yucca) and the fractions were obtained by the partition of the extract with *n*-butanol. At the end of the 76 days of the experiment, a significant decrease in hepatic ARG activity was observed in the animals treated with the total aqueous extract of *Y. schidigera* and with its *n*-butanolic fraction ($p = 0.03$) [89].

Similarly, Schnorr et al. [29] performed a study regarding the action of a cocoa drink that was either poor (< 90 mg) or rich (985 mg) in flavanols. This mixture provided (−)-epicatechin (0.1 µM) and catechin (0.4 µM) as well as the metabolites epicatechin-7-*β*-glucuronide (0.25 µM), 4′-*O*-methyl-epicatechin (0.2 µM), and 4′-*O*-methyl-epicatechin-7-*β*-glucuronide (1.7 µM) (values of plasma concentration measured after 2 h of consumption of 200 mL of cocoa beverage that provided 2.6 µM of flavonoids) in healthy humans (2 days). A protein diet containing 0 or 4% cocoa powder was provided to male rats (28 days). As a result, in the samples of erythrocytes taken 24 h after the end of the experiment, those that belonged to the flavonoid-rich cocoa beverage group showed a decrease in the active ARG portion. A reduction in the enzymatic activity of the renal ARG in the rats was also observed.

Corroborating this, *in vitro* testing of ARG inhibition in HUVEC cells shows that both (−)-epicatechin and its mixture of flavanol metabolites exhibited effects, suggesting that after metabolisation, polyphenols can retain anti-ARG activity (at least under controlled conditions) [29].

Taken together, these results show that the *in vivo* inhibition of both isoforms of the enzyme is possible, which is represented by the previously cited results regarding ARG 1 and ARG 2 obtained in different tissues, where each of them are mostly expressed and active. Furthermore, this demonstrates that at this level it is important to understand the biological effects of low levels of enzymatic activity and its correlation with the responses that are obtained. On the other hand, research regarding ARG activity using *in vitro* techniques is still valuable because it makes it possible to predict behaviours and mechanisms for the models on which therapeutic applications are based.

The ethyl acetate extract of the lignum of *Caesalpinia sappan* L. (Leguminosae), which is used in Asian culture to promote improved circulation and also to prevent blood stasis, was evaluated in relation to ARG 2 of the kidney lysate of C57BL/6 mice as well as in HUVEC cells. As a result, residual activity in ARG 2 was observed (31%) at the highest concentration of the extract used (50 µg/mL), and the calculated IC_{50} was 36.82 µg/mL. In the other experiment that was conducted, after 18 h of incubation with 20 µg/mL of the extract, a significant decrease in enzymatic activity was observed when compared to the untreated control [90].

The aforementioned study also demonstrated that with the inhibition of the ARG in the HUVEC cells there was a dose-dependent increase in NO production, with a maximum level of 130% at 50 µg/mL. This data highlights the relationship between decreased levels of active ARG and increases in NO, which serves as a basis for ethnopharmacological applications of *C. sappan*, given the antithrombotic and provascular properties of NO [90].

A further two published studies that evaluated the use of the aqueous extract of Korean red ginseng [*Panax ginseng* C. A.Mey (Araliaceae)] to improve endothelial function impairment associated with age (in atherosclerosis models) reached similar conclusions; the extract (10–20 mg/mouse/day during 4–6 weeks) inhibited ARG activity in a nonselective manner, causing an increase in eNOS dimerisation and a consequent increase in NO levels, which strengthened the vasodilatation dependent on this mediator. Moreover, active components of Korean red ginseng (ginsenoside Rb1 and Rg3) have been linked to increased NO production in endothelial cells by the activation of the phosphoinositide 3-kinase (PI3K)/PKB intracellular pathway (also known as Akt, which is a serine/threonine-specific protein kinase) [91, 92].

Concrete evidence supports the involvement of ARG 1 and ARG 2 in the pathophysiology of erectile dysfunction. Because NO serves to relax the smooth muscles of the corpus cavernosum, inhibition of ARG, at this time, is useful for increasing the supply of the substrate to the action of eNOS [93].

Oboh et al. [38] found that extracts of the leaves of *Moringa oleifera* Lam. inhibited ARG from rat penis homogenates in a dose-dependent manner (IC_{50} of 159.59 µg/mL). In the aforementioned study, the authors identified the polyphenol composition of the extract (gallic acid, catechin, chlorogenic acid, ellagic acid, epicatechin, rutin, quercitrin, isoquercitrin, quercetin, kaempferol), which, in their opinion, contributed greatly to the mechanism of action against erectile dysfunction.

Of the secondary metabolites that have been isolated from plants, polyphenols have been extensively tested against ARG as a tool to control diseases attributed with the advancement of ED [11, 25, 27, 29, 36, 38].

Using an indirect technique (the quantification of urea produced), Reis et al. [94] found that at a concentration of 1 mM, the polyphenols (−)-epigallocatechin-3-gallate, (+)-catechin, (−)-epicatechin, and gallic acid were able to inhibit the activity of ARG isolated from rat liver by 29, 26, 22, and 20%, in that order.

Nelin et al. [71] used immunoblotting and Real-Time PCR methods in relation to ARG 1 (bovine pulmonary arterial endothelial cells) and ARG 2 respectively, to demonstrate that the induction of the expression of these enzymes by a mixture of LPS/TNF-*α* partially depended on the activity of the EGFR, and that the flavonoid genistein acted indirectly on the expression of ARG as an EGFR inhibitor.

Using a low-cost *in vitro* colorimetric technique with commercially available b-ARG 1, Bordage et al. [34] determined the ARG inhibitory potential of a range of polyphenols. Other studies, which used some changes in this technique, also evaluated the anti-ARG action of several phenolics *in vitro* (▶ **Table 2**).

As can be seen in ▶ **Table 2**, the most active phenolics were chlorogenic acid and piceatannol, and the efficacy was similar to the positive control that was used, with E_{max} values of 81 and 98% for the phenolics respectively, and an E_{max} of 97% for the BEC. It was also observed that there was competitive inhibition behaviour between these phenolics and b-ARG 1.

In relation to the study of the activity structure relationship, according to the IC_{50} data obtained in two recent studies, the caffeoyl (3,4-dihydroxycinnamoyl) group appears to be essential, since both chlorogenic acid and piceatannol have this substituent

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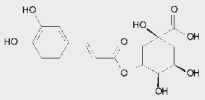
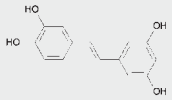
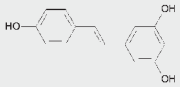
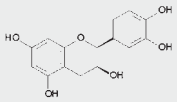
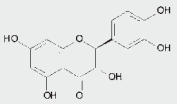
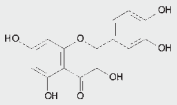
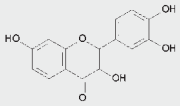
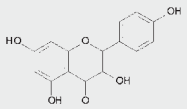
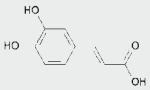
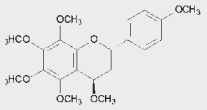
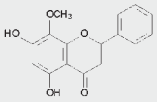
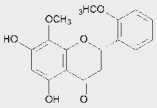
▶ **Table 2** ARG inhibition of important polyphenols from a medicinal chemistry point of view.

Substance	Structure	IC_{50} (µM)
Chlorogenic acid[a]		10.6
Piceatannol[a]		12.1
Resveratrol[a]		18.2
(−)-Epicatechin[a]		19.9
Taxifolin[a]		23.2
Quercetin[a]		31.2
Fisetin[a]		82.9
Kaempferol[a]		179.1
Caffeic acid[a]		86.7
(2*R*,4*S*)-4,5,6,7,8,4′-Hexamethoxylflavan[b]		> 200
Wogonin[b]		> 200
(2*S*)-5,7-Dihydroxy-8,2′-dimethoxyflavanone[b]		25.1

continued

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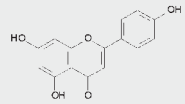
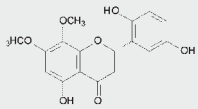
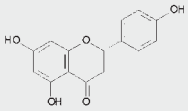
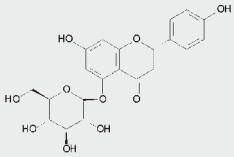
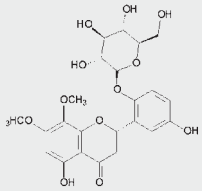
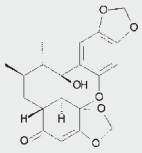
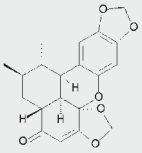
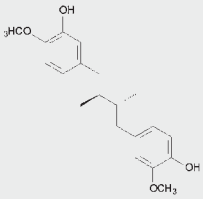
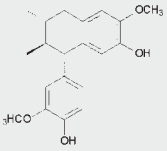
▶ **Table 2** *Continued*

Substance	Structure	IC_{50} (μM)
Apigenin[b]		> 200
(2*S*)-5,2′,5′-Trihydroxy-7,8-dimethoxyflavanone[b]		11.6
Naringenin[b]		> 200
Naringenin-5-*O*-*β*-D-glucopyranoside[b]		> 200
(2*S*)-5,5′-Dihydroxy-7,8-dimethoxyflavanone-2′-*O*-*β*-D-glucopyranoside[b]		> 200
7-Hydroxysauchinone[c]		89.6
Sauchinone[c]		61.4
meso-Dihydroguaiaretic acid[c]		> 200
Guaiacin[c]		> 200

continued

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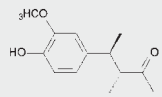
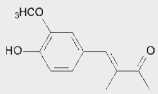
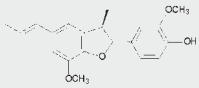
▶ **Table 2** *Continued*

Substance	Structure	IC_{50} (μM)
(7*S*,8*R*)-4-Hydroxy-3,7-dimethoxy-1′,2′,3′,4′,5′,6′,7′-heptanorlign-8′-one[c]		> 200
(*E*)-7-(4-Hydroxy-3-methoxyphenyl)-7-methylbut-8-en-9-one[c]		> 200
Licarin A[c]		> 200

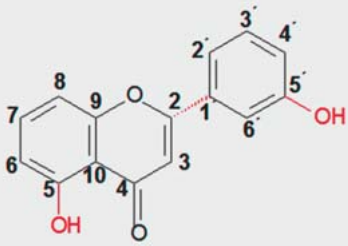
[a] [34], Mammal bovine liver arginase (b-ARG 1); [b] [36] and [c] [95], arginase 2 from the kidney of C57BL/6 mice

in their structure. This is reinforced by the fact that in isolation, caffeic and quinic acids did not present satisfactory enzymatic inhibition when compared to the whole molecule. In relation to the derivatives of the flavonoid class, whose prototype is quercetin, pertinent observations include the importance of hydroxyl in C5 for the maintenance of activity, while the presence of the carbonyl group in C4 and the unsaturation at the C2–C3 bond exerted less significant influence, as well as the fact that the substitutions of hydroxyl, glucose, or acetate at the C3, C7, C8, and C2′ positions appear to have had no positive influence on the inhibition of arginase. Furthermore, the hydroxyl in C5′ (catechol group) is essential to the inhibitory activity as well as the α bond between C2-C1′, which increases the activity (▶ **Fig. 5**) [34, 36].

Based on the *in vitro* results obtained by Kim et al. [36], who tested eight flavonoid-type substances isolated from a methanolic extract of *Scutellavia indica* L. in relation to ARG 2 from mouse kidney homogenate, another group of researchers sought to perform more in-depth *in vivo* research regarding the anti-ARG properties of the substance TDF, which had been previously isolated. In that study, the authors used a hyperlipidemia model to demonstrate that TDF inhibited both ARG 1 (IC_{50} of 12.18 μM) and ARG 2 (IC_{50} of 11.86 μM) in a noncompetitive manner, simultaneously increasing NO levels by the phosphorylation and dimerisation of eNOS, as well as indicating an improvement in vascular function in normal mice that received a standard diet, and also $ApoE^{-/-}$ mice fed on a high cholesterol diet [96].

In the study by Kim et al. [36], referred to above, PG was used as a positive control (IC_{50} of 1.0 μM).

Piceatannol (3,3′,4′,5-transtetrahydroxystilbene) is naturally found in rhubarb rhizomes [*Rheum undulatum* L. (Polygonaceae)] and can be metabolised from resveratrol through hydroxylation by the action of cytochrome P4501B1 [97]. The stilbene derivative PG was first evaluated by Woo et al. [65] and it showed antioxidant capacity and important inhibitory *in vitro* action in relation to ARG 1 and ARG 2, which was associated with the dose-dependent increase in NO levels. In the experiments, PG behaved as a nonselective ARG inhibitor in C57BL/6 mice (IC_{50} of 11.22 μM for liver lysate and IC_{50} of 11.06 μM for kidney lysate) and was able to



▶ **Fig. 5** Structure-activity relationships of flavonoid-type polyphenols as arginase inhibitors. Highlights in red lines indicate important parts of the molecule in relation to anti-arginase action; the hydroxyl group (–OH) at C5′ and C5 and the α bond between C2-C1′ are essential for the activity.

increase NO production and decrease ROS in isolated aortic fragments.

Inspired by these results regarding the potential of PG, Frombaum et al. [98] compared the behaviour of resveratrol and piceatannol in relation to BAEC. The effects were measured in BAEC that was stimulated by high concentrations of glucose (25 mM) for 24 h in order to mimic the hyperglycaemic conditions observed in the diabetes state. As a result, both resveratrol (10 μM) and PG aglycone (1 μM) were shown to produce enzymatic inhibition in the experiments; the efficacy of the latter was considered to be greater, sustaining its therapeutic potential for application in relation to ED.

The research group led by Woo et al. [99] subsequently proved that the administration of PG (~ 500 μg/mouse/day for 6 weeks) was able to improve ED in an animal model of hyperlipidaemia via ARG inhibition and, reciprocally, eNOS activation through enhanced stability of the eNOS dimer.

Based on these results, a review was published regarding the effects of piceatannol on the diversity of cardiovascular impairment, including the prevention of hypercholesterolaemia, cardiac arrhythmia, monocyte adhesion to the endothelium, proliferation

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and migration of SMCs, ED, and angiogenesis, as well as its anti-inflammatory, vasorelaxant, and antioxidant effects [97].

However, the application of piceatannol, or its derivative glucopyranoside, as a pharmaceutical product to reduce cardiovascular risks is limited due to its low oral bioavailability and a lack of studies regarding its pharmacokinetic profile [84, 97].

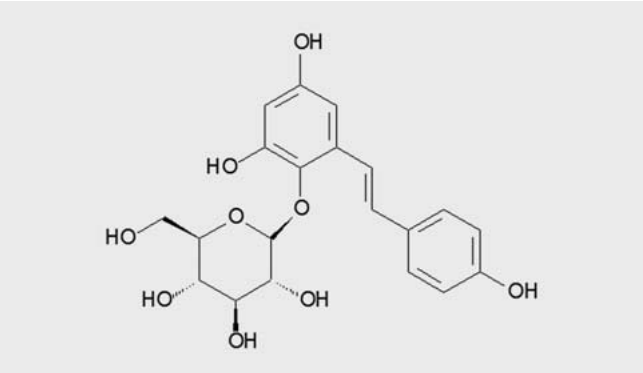
In an attempt to contribute to resolving these problems, Nguyen and Ryoo [100] proposed a study regarding the intravenous administration of piceatannol in mice with endothelial function compromised by old age. The animals (C57BL/6, male, 65 weeks) received injections of piceatannol (30 mg/Kg body weight/day) over 4 consecutive days, after which time the tissues of interest were properly treated for subsequent analysis. In conclusion, the *in vivo* potential for ARG inhibition of piceatannol as well as its ability to improve the vascular function of senescent mice was reinforced by the increase in NO production by the phosphorylation of eNOS Ser1177 and the stabilisation of its dimer, strengthening the results obtained by Woo et al. [99] with the glucuronidated form of the stilbene derivative.

Thus, according to the promising results that have been obtained with piceatannol and PG, and in view of its structural similarity to resveratrol, Yi et al. [30] identified a new substance, THSG, (▶ **Fig. 6**) from the *Polygonum multiflorum* Thunb (Polygonaceae) rhizome and tested it as an ARG inhibitor and eNOS activator. According to the authors, the mechanisms by which THSG acts are similar to those found for TDF, i.e., the restoration of vasculature function by the inhibition of ARG 1 and ARG 2 (25 and 38%, respectively, at 50 μM), the increase of NO, and the decrease in ROS formation by the phenomenon of uncoupled eNOS. In addition, it was identified that THSG presented noncompetitive inhibition in relation to ARG 2 [96].

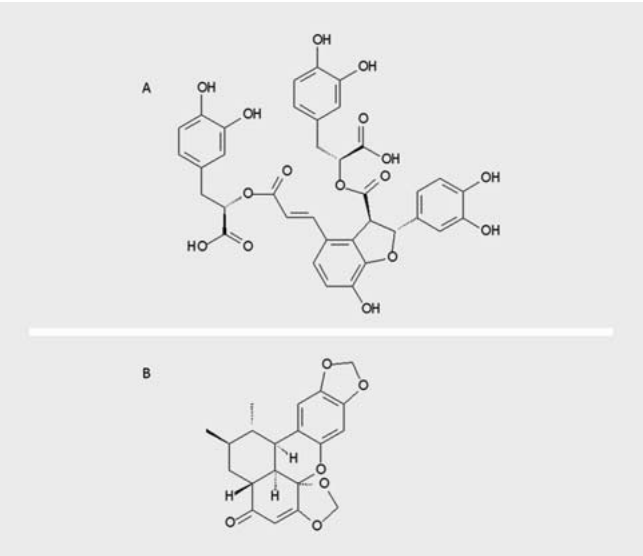
According to a survey, *in vitro* and *in vivo* research carried out in recent years supports the fact that numerous polyphenols that are derived from the most diverse plants are active in improving endothelial function by increasing NO bioavailability. In accordance with epidemiological investigations, basic and clinical research studies suggest that polyphenols demonstrate beneficial effects for the maintenance of vascular homeostasis in animal models as well as in humans [24].

Other phenolic substances have also been tested as inhibitors of ARG activity or expression with a view of developing new pharmaceutical products to be used regarding ED-related problems.

Quercetin is widely known for its multifaceted biological action and has shown promising anti-ARG results, although only in a limited fashion thus far (only one scientific publication was located). Nikolić et al. [88] induced a model of acute renal failure in adult male rats by the intramuscular injection of 50% glycerol (8 mL/Kg) with pretreatment (2 h) of subcutaneous quercetin (20 mg/Kg). As a result, the flavonoid was able to decrease levels of plasma urea and creatinine, as well as decreasing hepatic ARG activity when compared to the control group (glycerol only). According to the researchers, the established antioxidant action of quercetin, combined with the inhibition of L-arginine consumption (anti-ARG effect), may have contributed to the provision of a substrate for the synthesis of NO, whose vasorelaxant power contributed to decreasing vascular resistance and restoring renal function.



▶ **Fig. 6** Molecular structure of 2,3,5,4′-tetrahydroxystilbene-2-*O*-*β*-D-glucoside isolated from the rhizome of *P. multiflorum* Thunb. (Polygonaceae).



▶ **Fig. 7** Molecules of salvianolic acid B (**A**), and sauchinone (**B**).

Other substances with important action against ARG include the polyphenolics salvianolic acid B [isolated from *Salvia miltiorrhiza* Bunge (Lamiaceae)] [101] and sauchinone [isolated lignan from *Saururus chinensis* (Lour.) Baill. (Saururaceae)] [95] (▶ **Fig. 7**).

Both of these substances are active in inhibiting ARG, particularly salvianolic acid B, which also decreased the expression of iNOS in RAW 264.7 macrophages that were induced by LPS [84, 101].

Such decreased levels of iNOS provide protection from the toxic effects of high NO concentrations derived from this high throughput isoform and, together with reduced ARG activity, this enhances the potential of salvianolic acid B against cardiovascular diseases associated with ED.

It is also worth mentioning that ellagic acid has received special attention from researchers because of its pluripotent biological activity and the multiple molecular targets that it acts upon [102]. Based on this, an animal model of hepatocellular carcinoma

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demonstrated that the oral administration of ellagic acid (50 mg/Kg/day), 7 days before and 14 days after tumour induction (N-nitrosodiethylamine and CCl_4), provided 23.6% of inhibition of ARG activity when compared to the negative control group (healthy rats). In that particular study, the elevation of ARG levels after the injection of the tumour agent was considered a marker of disease progression, and other studies have also attributed a biomonitoring function to this enzyme in the most varied clinical conditions, such as the oxidative stress observed in pregnant, overweight women and their neonates [103, 104].

It is interesting to note that ARG activity is related to tumour progression, since the formation of polyamines and proline that are the result of enzyme action can contribute to cell proliferation and tumour growth, as shown by studies that have found a relationship of risk between the increased expression of ARG 2 and the appearance of disease [93, 105]. Thus, ARG inhibition has the potential to curb this process, which might work in favour of the action of other anticancer substances.

Stolarczyk et al. [105] studied the aqueous and ethyl acetate extracts (aerial parts) of three species of *Epilobium sp*. [*Epilobium angustifolium* L., *Epilobium parviflorum* Schreb, and *Epilobium hirsutum* L. (Onagraceae)], as well as polyphenols isolated from these species, in relation to the ARG of prostate cancer (LNcaP) cells and demonstrated that almost all the extracts (50 and 70 µg/mL) and phenolics that were tested, which included quercetin-3-*O*-glucuronide and oenothein B (20 and 40 µM), were able to significantly inhibit enzymatic action.

Furthermore, the same authors provided valuable data regarding anti-ARG research. They made an incubation of *E. hirsutum* herb extract, which contains high concentrations of oenothein B (dimeric macrocyclic ellagitannin), with human gut flora (final concentration 1.6 mg/mL) for 48 h. After this time, the metabolites urolithins A, B, and C, which can be detected in plasma (0.5–18.6 µM), were produced and then tested for anti-ARG potential in LNcaP cells. The results showed that both urolithin A (ARG activity of 39.8 ± 2.5 mUnits of urea/mg protein) and C (ARG activity of 27.9 ± 3.3 mUnits of urea/mg protein) were active as enzyme inhibitors compared with the control cells (65.2 ± 1.1 mUnits of urea/mg protein), whereas urolithin B was inactive. Thus, these data suggest that anti-ARG activity remains in metabolites as well as in its precursor compound, at least under *in vitro* conditions.

Indeed, the amount of ellagitannins in systemic circulation and tissues is virtually undetectable, whereas urolithins and their conjugates can be found in higher levels (µM). It has been reported that ellagitannin metabolites can be detected in the liver and kidneys [106], urolithins are enhanced in the prostate, intestinal tissue, and colon in mice, and urolithin A-glucuronide is the main metabolite found in the human prostate (> 2 ng/g tissue) as well as traces of urolithin B-glucuronide and ellagic acid-dimethyl ether [102, 107].

Regarding the plasma concentration, the level of polyphenols and their metabolites found *in vivo* needs to be biologically applicable and should also be taken into account. Engler et al. [108] found that the consumption of chocolate containing high levels of flavonoids improved endothelial function and increased the plasma concentrations of epicathecin (already reported as an ARG inhibitor) in healthy adults, with a marked increase after 2 weeks (204.4 ± 18.5 nmol/L).

Other studies have been performed to better characterise the absorption and metabolism of polyphenols, which would help to shed light on the pivotal relationship between the bioavailable amount and the biological effect. In an *ex vivo* experiment to measure NO-dependent vasodilation, Schroeter et al. [109] performed an incubation of preconstricted rabbit aortic rings with a mixture of flavanols and their metabolites (catechin, epicatechin, 4′-methyl-epicatechin, epicatechin-*O*-*β*-D-glucuronide, and 4′-*O*-methyl-epicatechin-*O*-*β*-D-glucuronide) in the same higher plasma concentration achieved after 2 hours of administration, resulting in relaxation (74.2 ± 14.5%).

However, there is still a lack of data about the pharmacokinetics of plant-derived compounds. Characterisation of factors such as absorption, distribution, metabolism, excretion (ADME), and toxicological parameters may help to improve the evaluation of the drug-likeness features of plant-derived substances. For this purpose, methods of drug-likeness prediction have been developed (drug database screens, knowledge-based methods, and functional group filters) and they serve as valuable tools, especially in the pharmaceutical field [110] (▶ **Tables 3** and **4**).

The potential therapeutic properties of bioactive substances depend on their bioavailability after oral administration. Therefore, matrix effects (for example, the vehicle for solubilisation or composition of the diet), the physical and chemical properties of the substance (degree of glycosylation/acylation, basic structure [benzene or flavones], conjugation with other phenolics, molecular size, degree of polymerisation, solubility/partition coefficient), interindividual variations (gastrointestinal secretions, motility, blood/lymph flow, etc.), and other interactions (alcohol or the presence of macronutrients like fat, protein, and carbohydrates) can be important factors to be considered in relation to the bioavailability of natural substances as well as the dosage used. Furthermore, gastric pH, enterocyte metabolism, digestive enzyme activity, first pass metabolism, and mechanisms of resistance (expression of apical multidrug resistance-associated proteins such as P-glycoprotein 1) should all be considered [111–114].

Aglycones, simple phenolic acids, and flavonoids can be absorbed in the stomach or small bowel mucosa. If this does not occur, the phenolic substance will be carried to the colon, which contains catalytic and hydrolytic potential that is powered by microorganisms. This colonic microflora transforms polyphenols (glycoside derivates with a hydrophilic nature and relatively high molecular weight) into more simple substances, such as phenolic acids (aglycone) [115]. In addition, bile plays a pivotal role in the adsorption of plant-derived polyphenols from the gastrointestinal tract (enterohepatic cycle) [116].

As shown in ▶ **Table 3**, all the reviewed polyphenols with anti-ARG potential are moderately or well absorbed (human intestinal absorbed and Caco2 permeability), but this inversely correlates with oral bioavailability (a minority have good parameters). It is suggested that this is due to first-pass metabolism, which extensively alters the quantities of substances in plasma.

Manach et al. [117] evaluated data from 97 studies about kinetics and the absorption of polyphenols among adults (the ingestion of a single dose of the substance). They found that gallic

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▶ **Table 3** Pharmacokinetic properties of revised polyphenol compounds with anti-ARG potential.

Substance	MF	MW	OB	HIA (%)	Caco2 (nm/s)	MDCK (nm/s)	PPB (%)	BBB (%)	Pgp inhibition	V_d (L/Kg)	Inhibitor (CYP)				Substrate (CYP)	
											2C19	2C9	2D6	3A4	2D6	3A4
Chlorogenic acid	$C_{17}H_{20}O_8$	352.3359	p	29.77	17.43	1.98	47.03	0.035	no	0.25	yes	yes	no	yes	no	~
Piceatannol	$C_{14}H_{12}O_4$	244.2426	m	81.95	2.37	258.17	100	1.013	no	1.58	yes	yes	no	yes	no	~
Resveratrol	$C_{14}H_{12}O_3$	228.2432	g	88.47	5.19	76.74	100	1.738	no	1.84	yes	yes	no	yes	no	no
(−)-Epicatechin	$C_{15}H_{14}O_6$	290.2680	p	66.70	0.65	44.38	100	0.394	no	1.36	yes	yes	no	yes	no	~
Taxifolin	$C_{15}H_{12}O_7$	304.2515	p	60.16	3.42	9.56	95.16	0.166	no	0.64	yes	yes	no	yes	no	~
Quercetin	$C_{15}H_{10}O_7$	302.2357	p	63.48	3.41	13.35	93.23	0.172	no	0.6	yes	yes	no	yes	no	~
Fisetin	$C_{15}H_{10}O_6$	286.2363	p	79.43	9.57	68.19	88.72	0.316	no	0.6	yes	yes	no	yes	no	no
Kaempferol	$C_{15}H_{10}O_6$	286.2363	p	79.43	9.57	29.61	89.60	0.286	no	0.61	yes	yes	no	yes	no	no
Caffeic acid	$C_9H_8O_4$	180.1574	m	82.30	21.10	109.43	40.29	0.497	no	0.31	no	yes	no	yes	no	no
(2*R*,4*S*)-4,5,6,7,8,4′-Hexamethoxylflavan	$C_{21}H_{26}O_7$	390.4269	g	98.48	55.24	1.25	85.65	0.069	yes	1.62	yes	yes	no	yes	no	yes
Wogonin	$C_{16}H_{12}O_5$	284.2634	g	93.03	4.28	152.11	90.44	0.724	no	0.64	yes	yes	no	yes	no	no
(2*S*)-5,7-Dihydroxy-8,2′-dimethoxyflavanone	$C_{17}H_{16}O_6$	316.3053	p	92.97	16.82	75.20	89.55	0.681	no	0.62	yes	yes	no	yes	no	~
Apigenin	$C_{15}H_{10}O_5$	270.2369	g	88.12	10.54	44.30	97.25	0.565	no	0.91	yes	yes	no	yes	no	no
(2*S*)-5,2′,5′-Trihydroxy-7,8-dimethoxyflavanone	$C_{17}H_{16}O_7$	332.3047	p	86.48	16.18	35.62	91.45	0.061	no	1.46	yes	yes	no	yes	no	~
Naringenin	$C_{15}H_{12}O_5$	272.2527	p	87.31	10.52	44.63	100	0.596	no	0.65	yes	yes	no	yes	no	no
Naringenin-5-*O*-*β*-D-glucopyranoside	$C_{21}H_{22}O_{10}$	434.3933	p	42.26	4.93	0.91	66.78	0.037	no	0.67	yes	yes	no	yes	no	~
(2*S*)-5,5′-Dihydroxy-7,8-dimethoxyflavanone-2′-*O*-*β*-D-glucopyranoside	$C_{23}H_{26}O_{12}$	494.4453	p	32.36	6.53	0.14	55.26	0.034	no	0.9	yes	yes	no	yes	no	~
7-Hydroxysauchinone	$C_{18}H_{16}O_7$	344.3254	m	95.35	21.78	2.54	75.16	0.475	no	1.1	no	yes	no	yes	no	~
Sauchinone	$C_{18}H_{16}O_6$	328.3160	m	98.41	38.62	27.86	87.26	1.401	no	1.28	yes	yes	no	yes	no	~
meso-Dihydroguaiaretic acid	$C_{20}H_{26}O_4$	330.4180	p	93.35	35.17	57.64	100	5.286	yes	2.49	yes	yes	no	yes	no	~
Guaiacin	$C_{20}H_{24}O_4$	328.4021	p	93.35	27.16	105.31	100	2.609	yes	2.67	yes	yes	no	yes	no	~
(7*S*,8*R*)-4-Hydroxy-3,7-dimethoxy-1′,2′,3′,4′,5′,6′,7′-heptanorlign-8′-one	$C_{13}H_{18}O_4$	238.2796	g	94.55	37.07	51.68	74.29	0.641	no	1.27	yes	yes	no	yes	no	yes
(*E*)-7-(4-Hydroxy-3-methoxyphenyl)-7-methylbut-8-en-9-one	$C_{12}H_{14}O_3$	206.2377	g	94.73	29.92	352.94	77.91	0.661	no	1.33	yes	yes	no	no	~	~
Licarin A	$C_{19}H_{20}O_4$	312.6597	p	95.65	55.84	123.41	98.77	1.206	yes	2.58	yes	yes	no	yes	no	yes

MF: Molecular formula, WM: molecular weight. OB: Oral bioavailability (p: poor, less than 30 %; m: moderate, between 30–70 %; g: good, more than 70 %). HIA: Human intestinal absorption (less than 20 %: weakly absorbed; between 30–70 %: moderately absorbed; more than 70 %: well absorbed). Caco2: *In vitro* Caco2 cell permeability (human colorectal carcinoma) (less than 4: weakly permeable; between 4–70: moderately permeable; more than 70: highly permeable). MDCK: *In vitro* MDCK cell permeability (mandin darby canine kidney) (less than 25: weakly permeable; between 25–500: moderately permeable; more than 500: highly permeable). PPB: *In vitro* plasma protein binding (less than 90 %: weakly bound; more than 90 %: strongly bound). BBB: *In vivo* blood-brain barrier penetration (C. brain/C. blood) (less than 0.1: weak penetration; between 0.1–2.0: moderate penetration; more than 2.0: high penetration). Pgp: *In vitro* P-glycoprotein inhibition. V_d: Distribution volume (less than 1: small V_d value; between 1–10: moderate V_d value). CYP: Cytochrome P-450 enzymes (~: weakly)

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▶ **Table 4** Toxicity features and drug-likeness properties of revised polyphenol compounds with anti-ARG potential.

Substance	Mutagenicity	Carcinogenity (mouse)	Carcinogenity (rat)	hERG inhibition (risk)	Lipinski's rule
Chlorogenic acid	negative	positive	negative	medium	suitable
Piceatannol	positive	negative	negative	medium	suitable
Resveratrol	positive	negative	negative	medium	suitable
(−)-Epicatechin	positive	negative	negative	medium	suitable
Taxifolin	positive	negative	positive	medium	suitable
Quercetin	positive	negative	positive	medium	suitable
Fisetin	positive	negative	positive	medium	suitable
Kaempferol	positive	negative	positive	medium	suitable
Caffeic acid	positive	negative	positive	medium	suitable
(2*R*,4*S*)-4,5,6,7,8,4′-Hexamethoxylflavan	positive	negative	positive	low	suitable
Wogonin	positive	negative	positive	medium	suitable
(2*S*)-5,7-Dihydroxy-8,2′-dimethoxyflavanone	negative	negative	positive	medium	suitable
Apigenin	positive	positive	positive	medium	suitable
(2*S*)-5,2′,5′-Trihydroxy-7,8-dimethoxyflavanone	negative	negative	positive	low	suitable
Naringenin	positive	negative	positive	medium	suitable
Naringenin-5-*O*-*β*-D-glucopyranoside	positive	negative	negative	high	suitable
(2*S*)-5,5′-Dihydroxy-7,8-dimethoxyflavanone-2′-*O*-*β*-D-glucopyranoside	negative	negative	negative	–	violated
7-Hydroxysauchinone	negative	negative	negative	low	suitable
Sauchinone	positive	negative	positive	low	suitable
meso-Dihydroguaiaretic acid	negative	negative	negative	medium	suitable
Guaiacin	positive	negative	negative	medium	suitable
(7*S*,8*R*)-4-Hydroxy-3,7-dimethoxy-1′,2′,3′,4′,5′,6′,7′-heptanorlign-8′-one	positive	negative	positive	low	suitable
(*E*)-7-(4-Hydroxy-3-methoxyphenyl)-7-methylbut-8-en-9-one	positive	negative	positive	low	suitable
Licarin A	positive	negative	positive	medium	suitable

Mutagenicity: based on the Ames test; Carcinogenity: 2-year bioassay in the mouse/rat; hERG: *in vitro* human ether-a-go-go-related gene channel inhibition; Lipinski's rule: hydrogen bond donors less than 5, hydrogen bond acceptor less than 10, molecular weight less than 500 Da; CLogP less than 5 (MlogP less than 4.15)

acid was better absorbed than other phenolic substances (the C_{max} values reached 4 µmol/L with a 50-mg dose), followed by isoflavones, catechins, flavanones, quercetin glucosides, proanthocyanidins, galloylated tea catechins, and anthocyanins [118]. Additionally, the time to C_{max} varied from approximately 1.5 h to 5.5 h, taking into account the site of intestinal absorption [117].

After absorption, molecules are distributed from plasma to other compartments of the body. In relation to anti-ARG polyphenols, approximately half of them occur in free state in the circulation (weakly bound to plasma proteins) and they can reach several parts of the peripheral system to achieve their potential enzyme inhibition (V_d value). In addition, only two of these anti-ARG polyphenols have the ability to cross the blood-brain barrier, which could result in biological or toxicological effects.

For most of the polyphenols that are absorbed, the plasma concentration quickly decreases. The metabolism mainly occurs in the liver (methylation and/or conjugation with glucuronic acid or sulphate), supported by the metabolism of the kidneys and intestinal mucosa. Thus, achieving elevated levels of polyphenols in plasma requires repeated ingestion over time. However, catechins, gallic acid, and flavanones seem to have no chance to accumulate, even with sequential administrations. On the other hand, quercetin exhibits a high affinity for plasmatic albumin, which might explain its higher elimination half-life (24 h) [115, 117].

Taking this into consideration, the excretion of polyphenols occurs mainly in urine or feces (especially phenols that are resistant to microflora degradation, such as condensed tannins and those linked to macromolecules) [113], and can be expressed as MDCK cell permeability, which predicts renal excretion ability. In this context, most of the anti-ARG phenolics reviewed present moderate permeability capability, suggesting a moderate to high maintenance of these substances in the organism. Additionally, attention should be paid to those phenolics that are highly bound to plasma proteins due to the risk of toxicity from long-term use and accumulated doses [110].

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Regarding toxicity (▶ **Table 4**), plant-derived substances have a favourable spectrum in most cases, which is very important during drug development. Only one of the reviewed polyphenols presents a high risk of inhibiting hERG (a gene that encodes a potassium ion channel expressed in the heart and when inhibited can produce a long QT syndrome that results in potentially fatal arrhythmias) [119], although almost all the phenolics presented positive predictions regarding mutagenic or carcinogenic (mouse and/or rat) action. These points are relevant since they can determine the final outcome of new therapeutic approaches.

Concerning the predicted toxicological potential, the dosage must be considered because some effects only appear at higher doses. For this purpose, daily dietary reference intakes of polyphenols are required and are highly desirable, although data are currently insufficiently available to establish how to avoid upper doses with possible toxic effects [120].

Finally, completing the prediction analysis, the drug-likeness investigation of polyphenols with potential activity as ARG inhibitors showed that only one substance violated Lipinki's rule and therefore could not be recommended as an emergent drug in the management of ED.

Concluding Remarks and Perspectives

The development of new ARG inhibitors represents a very promising strategy in relation to the treatment of diseases caused by the damage involved in the production and function of NO.

The number of potential indications is broad and includes cardiovascular, pulmonary, metabolic, and neurological problems as well as erectile dysfunction and, more recently, cancer therapy since the conversion of L-arginine by ARG is a trigger for tumour promotion and progression.

Synthetic products derived from boronic acid have been extensively studied as modulators of ARG activity due to their polarisability. However, many of these prototypes have an unfavourable toxicological profile, with high potency (subnanomolar range), especially in relation to hepatocytes, which results in them being decharacterised as new inhibitors unless such obstacles are improved by means of structural, molecular, or pharmacotechnical modifications (prodrug and vector-based dosage forms). Furthermore, another issue to be addressed is the inappropriate (oral) pharmacokinetic profile presented by most of the available inhibitors, since in most cases these are substances whose structures are based on amino acids, which easily lose stability (very short half-life) and potency at physiological pH [121].

In addition, given the different expression of ARG 1 and ARG 2 in tissues, and their divergent actions depending on the pathological context, it is interesting that sufficient specific and selective inhibitors of this enzyme are available. According to the literature, specificity has not become a hindrance in relation to this issue, as opposed to selectivity for isoforms. For example, endothelial tissues express the two major isoforms of ARG, however, it is not precisely known what the role of each of these isoforms is in the evolution of ED. There remains considerable controversy about the role of the expression of ARG in different conditions like atherosclerosis and other forms of vessel inflammation. For instance, hyperglycaemia of diabetes causes ED via the activation of p38 MAPK, which produces the upregulation of ARG 1 in coronary arteries and the increased expression of ARG 2 in mesenteric arteries [44, 122].

Furthermore, the 3D comparison of ARGs has not presented significant differences (almost totally homologue), with certain portions that are considered critical to enzymatic activity [123]. Indeed, natural products have not presented enough selectivity to inhibit a specific ARG isoform, and the effects of enzyme inhibition in determined vessels cannot be generalised for all vasculatures. There is also growing evidence that ARG expression and activation can be detrimental or beneficial depending on the biological context that is analysed. For this reason, it is not yet completely understood which ARG isoform should be targeted in order to achieve better outcomes [26, 44, 84].

Thus, further in-depth studies and investigations are required regarding the consequences of ARG inhibition on essential functions of the organism, such as the processes of neuronal development, healing, and angiogenesis. This is due to the fact that the synthesis of proline and polyamines could be blocked, as well as the possibility of a possible disruption of the urea cycle in the liver. It seems contradictory that studies regarding ARG inhibition do not report significant toxic effects on the urea cycle, possibly because of high levels of ARG expression in the liver (up to one thousand times more than normal) compared to the endothelium, therefore making it unlikely that the suppression of this function can be achieved by therapeutically viable doses of the inhibitor [6, 124]. From another point of view, human ARG deficiency seems to be a disorder that is effectively treated, and acute hyperammonaemia does not represent a great risk for most patients [123].

Moreover, long-term studies of ARG inhibition have failed to observe a compensatory upregulation of the enzyme [26, 125]. Therapies utilising ARG inhibitors in systemic doses are used in the treatment of parasitic diseases without significant adverse effects [44].

A growing number of studies have demonstrated the role of ARG inhibition in functions involving cell growth and tissue repair, and such studies have produced interesting findings. In keeping with this, studies have reported that blocking the activity of ARG can prevent the reduction of angiogenesis (the maintenance of NO-induced VEGF expression), induce vascular repair in experimental ischaemic retinopathy (normalisation of NOS function and reduction of superoxide production) [126], and promote wound healing in mice (correlated with NO formation followed by reepithelialisation, since NO itself can mediate collagen synthesis) [127]. Otherwise, ARG 2 and ARG 1 knockout animals have shown conflicting results. The ARG 2 group presented diminished fertility in the males, while the ARG 1 group presented a more critical phenothype due to hyperammonaemia, which resulted in death within 10 days [123]. Thus, the extent of the effects generated by ARG inhibition *in vivo* should be better defined, even though it is probable that therapeutic doses do not cause such dramatic effects, as previously mentioned.

In the context of the study of ARG and the development of new ARG inhibitors with a focus on higher NO rates, plants are a very versatile source, given the richness of the substances that they produce (generally of low toxicity and great abundance), which can act as direct inhibitors or serve as a molecular model for the

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synthesis of semisynthetic products or products that are fully developed in the laboratory.

The data presented in this article highlights important evidence that emphasises the role of plants as a reliable source of new therapeutic agents. Promising results have been obtained in very complex pathologies, which is reinforced by the fact that from the 1940 s to 2014, of the 175 molecules that were used to treat cancer, 85 (49 %) were natural products or direct derivatives of them [33].

On the other hand, the identification of polyphenol metabolites is still a challenge requiring more in-depth studies, taking into account the innumerable factors that can influence their production, as well as the need to standardise the methodologies of identification and quantification of these compounds. In addition, efforts should be made to achieve the lower doses attained in clinically significant biological fluids and tissues to produce an effect over a suitable period of time [128]. It is also necessary to evaluate the new chemical species formed *in vivo*, compared to their original structures, to confirm either the maintenance of beneficial effects or the creation of toxic mechanisms [129].

Nevertheless, it remains unclear how after the absorption and metabolisation of polyphenols (which are mostly found in systemic circulation as glucuronidated forms following oral administration), biological activity continues occurring, since pharmacophoric sites (hydroxyl groups) are not available. Some studies have suggested that this activity might be related to a deconjugation reaction at the cellular level (this requires further investigation) or that the metabolites are still active in conjugated form [130]. Regarding the last hypothesis, quercetin glucuronides are reported to prevent cardiovascular diseases [131]. Similarly, quercetin-3′-sulfate and isorhamnetin-3-glucuronide (10 µmol/L) may prevent ED by an antioxidant mechanism, and quercetin-3-glucuronide (1 µmol/L) may prevent vascular impairment induced by endothelin-1 [132]. Furthermore, the stilbene derivative piceatannol appears to be more active as an ARG inhibitor compared to its precursor resveratrol, and for this reason it is more plausible to be applied in future clinical trials.

Thus, taking into account the foregoing, a new horizon of mechanisms related to the direct and indirect inhibition of ARG is coming into view. From another point of view, this enzyme has also become a valuable biochemical tool as a serum marker for serious diseases such as cancer.

In summary, there is substantial evidence to show the therapeutic potential of ARG inhibition in relation to the damage associated with the low bioavailability of NO. Consequently, this enzyme is highly attractive in terms of the research and development of new drugs, since such treatments can help to correct both ED and dysfunction of the adjacent smooth muscle tissue. It is important to note that secondary metabolites derived from plants with propriety to polyphenols are molecules of interest for the clinical application of ARG inhibition in relation to ED. Finally, the information compiled in this article will underpin future investigations regarding the anti-ARG activity of substances isolated from plants in order to produce reproducible and clinically relevant data in this field.

Acknowledgements

The authors are grateful to Dr. Sean Stroud for revising the English in this text.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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