Invited Review

Polyphenols and health: Moving beyond antioxidants

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Abstract. Diets in which plant foods, including berries, provide a relevant portion of caloric intake are associated with a reduced risk of certain degenerative diseases like cancer and atherosclerosis. As known, vegetables and fruits such as berries are rich in polyphenols, which are products of secondary metabolism. In the past few years, research on polyphenols has remarkably expanded and is constantly reporting interesting biological activities of these compounds. Due to the participation of oxidative processes in the onset and development of degenerative diseases, much attention has been paid to the antioxidant properties of polyphenols. Alas, the discovery of their low bioavailability – especially when compared to the concentrations of endogenous antioxidants – is questioning the actions of polyphenols as mere antioxidants.

In this review we critically discuss the current limitations of polyphenol research and we contend that, in addition to their putative antioxidant action, several biochemical and physiological processes might be influenced by polyphenols.

Keywords: Polyphenols, cardiovascular disease, microbiota, biomarkers

1. Introduction

The very vast majority of epidemiological studies yields unequivocal results: consumption of foods rich in plants' secondary metabolites, namely polyphenols such as flavonoids is associated with a reduced risk of atherosclerosis [1], cancer [2], and neurodegenerative diseases. In the vain attempt to come up with a unified hypothesis, several mechanisms have been proposed to biologically explain how polyphenols protect from degenerative diseases. The most publicized mechanism is that polyphenols act as antioxidants by scavenging free radicals or limiting their formation [3]. As reviewed here, this view is being challenged by recent research and more complex actions are being investigated.

In addition to the most biochemical theories, we need to take into consideration that high-polyphenol food consumption might actually be healthful "simply" because it allows limiting the intake of other potentially noxious foods such as those rich in animal protein and saturated fat. In synthesis, it might not be plants and their polyphenols that provide protective effects, but, rather, the exclusion or strong limitation in the intake of other foods, namely those rich in animal protein that would actually increase the risk of disease. Even though this is disputable, we should not overlook the fact that – in addition to polyphenols - plants contain polyunsaturated fats whose actions (once food is

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ingested) intermingle with those of polyphenols, resulting in an overall benefit on human health that is difficult to ascertain to individual components [4, 5]. Still within the realm of animal protein, their substitution with vegetable proteins, e.g. those of whole grain and soy (foods which are also rich in polyphenols) is associated with cardioprotection [6–8]. However, randomized trials with polyphenols do exist and are strongly suggestive of beneficial effects. In summary, it is difficult to disentangle and accurately estimate the contribution of individual plants' components such as polyphenols, especially within a complex diet such as the Western one. Furthermore, it is difficult to ascertain the exact mechanism of action of polyphenols; the widespread notion that these molecules act merely because they are antioxidant appear to be naïve and in contrast to the complexity of biological systems.

The question to be asked, in turn, is if really plant (including berries) polyphenols are endowed with the pharmacological activities everyone is talking about and, if so, what are the mechanisms of action responsible for such healthful activities. In this article we will briefly review the biological activities of polyphenols, challenge the notion that they act as antioxidant *in vivo*, and discuss the most advanced research in the area of polyphenols and human health.

2. The role of polyphenols in human health

2.1. Why so much emphasis on antioxidant activity?

The participation of excessive reactive oxygen species (ROS) production in the origination of several diseases is now supported by a wealth of experimental data. A paradigmatic example is the formation of oxidatively-modified human low-density lipoprotein (LDL), which renders these particles highly atherogenic. *In vivo* evidence of the formation of oxidized LDL includes their presence in human atherosclerotic plaques and in the bloodstream (where they circulate as negatively charged); further, their circulating levels have been positively correlated with the progression of carotid lesions. Moreover, many epidemiological studies have correlated a high dietary intake of antioxidants (e.g. tocopherols, carotenoids, flavonoids, and polyphenols) with a lower incidence of cardiovascular disease. It is noteworthy, though, that the implication of ROS in the above mentioned diseases has often been suggested on the basis of indirect observations, i.e. antioxidant supplementation contributed to alleviate certain diseases, almost exclusively in animal models. Furthermore, negative results of clinical trials have been published, dampening the enthusiasm and calling for a more cautious approach to antioxidant therapy [9, 10]. Intrinsic uncertainties in the selection of appropriate markers of ROS-mediated processes, related to diseases as well as in the dosage and duration of treatments, make it difficult to plan interventional studies and to evaluate the results.

A role for antioxidants in the prevention and treatment of CVD has gained support mostly because of their widespread availability and ease of supplementation.

Indeed, the human body cannot synthesize lipid- and water-soluble vitamins (including the antioxidant ones E and C) that, therefore, must be derived from food; moreover, plants' secondary metabolism (in particular the shikimate and acetate pathways) generates products that are currently grouped under the rubric of "phytochemicals", of which the most popular ones are the polyphenols [11]. These molecules (at least those with ortho-diphenolic structures) are endowed with potent *in vitro* antioxidant activities. As oxidative damage appears to be central in cardiovascular aging, antioxidants such as polyphenols may provide significant protection. Notably, the studies advocating the use of antioxidants suggest that intake should be well above the general levels of consumption, shifting the focus from dietary consumption to pharmacological treatment. If the animal results that show improved conditions following antioxidant supplementation are true, an increase of antioxidant intake in the population could reduce disease rates, improve treatment, and reduce hospital stays. Thus, it is conceivable that dietary interventions with antioxidants could provide an effective means to improve or maintain myocardial function in the elderly (who often do not consume antioxidants in adequate amounts) or in the malnourished. As mentioned, despite this strong theoretical rationale, there is currently conflicting evidence to suggest that individual antioxidant supplements actually lessen risk for CVD in the population.

When we address polyphenols, it must be underscored that their simple antioxidant activity - which is the frequent subject of several lay press proclamations – has been almost exclusively proven *in vitro*. In this regard, there is much controversy as to whether polyphenols retain their antioxidant features *in vivo*, following ingestion, due to their low

bioavailability [12–14]. While there are a number of papers showing increased plasma antioxidant capacity following the intake of polyphenol-rich food items [15], many investigators suggest that polyphenols' bioavailability is too low to allow significant contributions to the endogenous antioxidant machinery [12, 14]. In addition, human cells already contain several layers of antioxidants, some of which enzymatic in nature, e.g. superoxide dismutase and catalase. Intracellular antioxidants often reach millimolar concentrations, whereas polyphenols' circulating concentrations normally do not exceed the low micromolar range. In synthesis, the real contribution of polyphenols to the overall antioxidant activity appears to be negligible, at least from a theoretical viewpoint. Moreover, whether the observed increase in antioxidant capacity is really due to polyphenols or is it just an epiphenomenon is as yet to be ascertained [12]. In the end, even though circumstantial evidence does suggest an antioxidant role played by polyphenols *in vivo*, the jury is still out and most of the current marketing claims are unsubstantiated.

2.2. If not [just] antioxidants then what?

In the past few years, research on polyphenols has remarkably expanded and is unveiling several nutrition-pharma biological activities of these compounds, most of which extend beyond antioxidant activity [16, 17]. Unfortunately, the marketing departments of food and pharma industries are jumping ahead of solid scientific evidence; as a consequence, unsubstantiated claims are being made and whole foods or fortified, enriched, or enhanced foods are being created and sold as "functional foods", "nutraceuticals" or "designer foods" based on the sole antioxidant ability. In this respect, we are witnessing a "race" toward to most antioxidant extract or single compound. Notably, these claims are made on the bases on *in vitro* techniques (usually the ORAC) that, though providing suggestive information, cannot translate into proven *in vivo* actions. Much more research is needed and several myths are to be disproven.

2.3. Is there an RDA for polyphenols?

A frequent question that the lay public asks scientists and nutritionists is that of "how much" antioxidants and polyphenols should be eaten everyday, i.e. if we have an RDA for epicatechin, hydroxytyrosol, etc. It should be immediately underscored that even the RDA for antioxidant vitamins is not really based on their antioxidant actions, but, rather, on their multifaceted biological activities. In should be also mentioned that "polyphenol deficiencies" in diets are as yet to be described. In brief, there is no evidence-based indication of the lowest amount of polyphenols that should be consumed everyday to maintain optimal health. The same line of reasoning applies to antioxidants, for which there is no clear indication of how much is needed to contrast excessive ROS production. In brief, the discovery that several phenolic molecules exert interesting biological activities – some of which may even be classified as "pharmacological" [18] – calls for an evaluation of their intake and consequent correlation with incidence of degenerative diseases. Indeed, one of the current limitations in assessing the intake of polyphenols (either as a group or individually) is the scantiness of information on their content in foodstuff. This is due on one side to the developing technology that will allow precise measurement of their concentrations and on the other side to the relatively recent interest triggered by polyphenols. It is noteworthy that, in the area of diet and health, the predominant topic is still that of caloric intake and of macronutrient repartition, rather than that of micronutrients. Notably, composition databases are being built (e.g. www.phenol-explorer.eu) and metabolomic techniques are rapidly advancing [19]. Hence, we can foresee, in the near future, accurate correlations between intakes and disease incidence/prognosis. One example is that of a French population study that has given us recent information on total polyphenol intake. The SU.VI.MAX cohort consisted of 4942 middle aged participants who consumed a total of 337 polyphenols with at least half of the population consuming 258 polyphenols [20]. The estimation of intake was done by using the recent Phenol-Explorer database mentioned above. The authors reported that 98 polyphenols were consumed at levels of more than 1 mg/day and the mean total intake was 1193 mg/d (820 mg/d as aglycones). The maximum intake was 1.8 g/day and the highest contributors were non-alcoholic beverages (mainly coffee) and fruits.

2.4. There are no reliable biomarkers of antioxidant activity

When dealing with food, we face what Linus Pauling called "orthomolecular medicine" [21]. In this case we should talk about orthomolecular nutrition. Indeed, though the gap between pharmacology and nutrition is narrowing

[17], nutrition and the study of bioactive compounds face the challenge of addressing the fate of molecules that our body already contains. While drugs are almost exclusively composed of molecules that our body does not habitually contain, food provides substances whose levels are constantly modulated by their intake. These differences are underscored by two major obstacles that are as yet to be overcome. One is that of accurately measure the variations in concentrations of micronutrients that follow their ingestion. Technology is still limited, and the field of metabolomics is rapidly evolving, but it cannot – as yet – provide accurate information through the use of methods approved and shared worldwide [19]. In practice, it is quite difficult to have access to methods and techniques agreed upon by investigators. Therefore, we still cannot carry out accurate dose-response studies with polyphenols and we cannot accurately follow their absorption, distribution, metabolism, and excretion (ADME) as we routinely do with drugs (as mandated by regulatory bodies).

Another major limitation we are facing in polyphenol research is that of the scantiness of biomarkers to assess their actions *in vivo*, including the antioxidant activities that are making them famous. Some biomarkers have been shown to be influenced by polyphenols. The most notable ones are those of inflammation, which can be positively modulated by polyphenols [22] and whose consequences on cardiovascular outcome are, likely, more important than those due to antioxidant actions [23–25]. Polyphenols also influence lipid metabolism [26, 27].

As mentioned above, food components are different than drugs and their actions on human physiology are usually moderate. This translates into a difficulty to measure *in vivo* activities, because the effects of polyphenols are (and should be) of modest magnitude. One reflection is that, while medicines are habitually employed for limited timeframes and address very specific conditions, food and its macro- and micro-components are ingested throughout a lifetime, during which even modest daily effects would become noteworthy.

3. Mechanisms of action alternative to the antioxidant ones. Current fields of research

3.1. Activation of Phase II enzymes

It is now thought more likely that some phytochemicals, including polyphenols, are processed by the body as xenobiotics. They stimulate stress- related cell signalling pathways that result in increased expression of genes encoding cytoprotective genes. Nrf2 (NF-E2-related factor 2) is a transcription factor which binds to the Antioxidant Response Element (ARE) in cells and thus regulates enzymes involved in antioxidant functions or detoxification (e.g. thioredoxin reductase-1 and glutathione peroxidases). Polyphenols might increase gene transcription of Nrf2 mediated by such response elements. This provides grounds for the theory of hormesis, i.e. when mild stress triggers defence mechanisms. In the case of polyphenols it indicates how they could have an indirect antioxidant action.

One human example of these effects can be found in Visioli and colleagues [28], who reported a study in which 98 Chinese/Malay subjects ingested an olive preparation which was high in phenolics. After one hour, no difference in plasma antioxidant capacity was observed, but a significant increase in total plasma glutathione concentration was measured. The authors postulated that the observed effects of the olive phenols on glutathione levels might be governed by the antioxidant response element (ARE)-mediated increase in Phase II enzyme expression.

3.2. Novel targets of polyphenols: The microbiota

One often-overlooked – though being very actively investigated – aspect concerns the contribution of the intestinal microbiota to the actions of polyphenols [29, 30]. The interaction between the gut flora and polyphenols is two-ways: on one hand the ingested compounds modify the qualitative composition of the flora [31]. Most of the studies on polyphenol bioavailability and, indirectly, on their bioactivity, have focused on their absorption in the small intestine. Nevertheless, the role of the colonic microflora has emerged in the last years as paramount in the global bioavailability of polyphenols (and, therefore, their bioactivity) [32–37]. It has been estimated that only 5–10% of the total polyphenols that we ingest is absorbed in the small intestine. The remaining polyphenols (90–95% of total polyphenol intake), together with the deconjugated polyphenols excreted in the bile, reach the colon [35], where they are metabolized by the colonic microbiota [30] before being either reabsorbed [34] or eliminated [35]. Consequently, the compounds that reach our cells and tissues are chemically, biologically, and (in many instances)

functionally distinct from their dietary form [32, 38, 39]. As mentioned above, the interaction between polyphenols and the gut microbiota is bidirectional [40]. Hence, the microbiota modifies the chemical structure and, therefore, the bioavailability and bioactivity of polyphenols and these polyphenol metabolites are able to modulate gut microbiota.

3.3. Microbial metabolism of polyphenols

Only a few out of the many species of intestinal bacteria responsible for phenolic metabolism have been identified and there is scarce knowledge of the mechanisms involved in their activities [34]. The microbial community of the human colon comprises 10^{12} bacteria/g of colonic content, composed of hundreds of different species. Firmicutes and Bacteroidetes make up over 90% of the intestinal microbiota [37], although this proportion varies substantially among individuals [36, 41–43]. The microbiota metabolites of polyphenols are better absorbed in the intestine and their enterohepatic circulation ensures that the residence time in plasma for the metabolites is longer than that of their parent compounds (indeed they appear in the systemic circulation 6–8 h post-ingestion [43]) before being excreted in the urine [30, 44]. After microbial enzyme-catalyzed deconjugation of any polyphenol conjugates that reach the colon, there are two possible routes available: (a) absorption of the intact polyphenol through the colonic epithelium and passage into the bloodstream (as free or conjugated forms) or (b) breakdown of the original polyphenol structure into metabolites.

Concerning polyphenols, several studies have been performed on the microbial metabolism of green tea [38, 42, 45, 46] and cocoa [47–49]. They all confirm that green tea and cocoa flavanols are metabolized by the colonic microbiota into valerolactones and then phenolic acids [45, 47, 48], considered to be the main microbial metabolites derived from the biotransformation of flavan-3-ols [50]. These flavanol-derived microbial metabolites have also been identified after wine powder consumption [42], suggesting that different polyphenols are transformed by the colon microbiota into the same final metabolites. Hydroxycinnamic acid esters, quercetin [39] and polyphenols linked to rhamnose are also degraded to phenolic acids by the microbiota [41, 47]. Chlorogenic acids from coffee are poorly hydrolyzed in the stomach or small intestine. When coffee is ingested, a relatively small absorption of caffeic and ferulic acids in the small intestine and a low absorption of intact chlorogenic acids are recorded. The major absorption occurs in the colon, where dihydroferulic and dihydrocaffeic acids – products of microbial biotransformation – are the major products that have been recovered [51]. In another study, 3-hydroxyphenylpropionic and benzoic acids were the main microbial metabolites of caffeic acid and its esters, chlorogenic acid and caftaric acid. 3-Hydroxyphenylpropionic was the main metabolite of caffeic acid and its esters and accounted for 9–24% of the initial dose of the substrates [52]. Data regarding berries polyphenolic microbial metabolism have shown that protocatechuic acid is one of the quantitatively most relevant product formed from anthocyanins [51, 53, 54]. In addition, in a trial with 20 healthy volunteers who consumed thermally-processed strawberry puree, all of the volunteers produced urolithin A, but only 3 of 20 volunteers produced and excreted urolithin B from the ellagitanins. The trial confirmed that some volunteers were efficient producers of urolithins, whereas other produced much lower amounts [53]. Furthermore, punicalagins transformation of pomegranate products – in a model of colonic microbiota – yielded urolithins C and D while urolithin B was not detected [55]. As seen with urolithin B, microbial fermentation of polyphenols shows a great interindividual variability. In this regard, isoflavones from soy and prenylflavonoids from hop deserve special attention. Equol, a gut bacterial metabolite of the soy polyphenols daidzin [44] and daidzein, is more estrogenic than its precursors and posses higher antioxidant capacity than its parent molecules [39]. Notably, only ∼30–40% of the population is able to convert dadidzein into equol [33]. The inability of some subjects to produce equol is a consequence of the lack of specific components of the intestinal microflora [34].

Isoxanthohumol is a prenylated flavanone from hops and beer that has interesting biological activities, at least *in vitro*. It can be metabolized *in vivo* by the colonic microbiota to yield 8-prenylnaringenin [34], which possesses greater estrogenic activity than isoflavones [56]. As in the case of equol, subjects can be classified into poor (∼60%), moderate (∼25%) and strong (∼15%) 8-prenylnaringenin producers [37].

3.4. Modulation of gut microbiota by polyphenols

As mentioned, the polyphenol-microbiota relation is two-ways and an important aspect of polyphenol biology is their modulation of microbiota composition. The mechanisms of modulation of the gut microbiota by polyphenols

is largely unknown, but polyphenols and their metabolites, in addition to their putative beneficial effect on human physiology, appear to confer added health benefits via modulation the gut microecology [30, 46]. Metabolites released into the lumen may influence the growth of the microbiota which transformed them, affecting other neighboring microflora species as well. A review of the literature suggests that polyphenol metabolites are able to enhance (or modify) some beneficial probiotic species while inhibiting the growth of non-beneficial species. In support of this notion, polyphenolic green tea extracts has been shown to have general inhibitory effects on intestinal bacteria [46], but more specifically on Bacteroides spp., Clostridium spp. (C. perfringens and C. difficile), E. coli, and Salmonella typhimurium [30]. Cocoa dietary fiber (which contains fermentable polysaccharides and free flavanol monomers, both able to modify the gut microbiota) increased the lactobacilli and Bifidobacterium spp. [49], and a pomegranate by-product and punicalagins in human fecal culture significantly inhibited the growth of the pathogenic Escherichia coli, Pseudomonas aeruginosa, clostridia and Staphyloccocus aureus. Most bifidobacteria were generally not affected, and the growth of probiotic lactobacilli and Bifidobacterium breve and Bifidobacterium infantis was significantly enhanced [55]. In another study where 25 g of wild blueberry powder in 250 mL of water were administered to healthy volunteers during six weeks, Bifidobacterium spp. increased compared to the placebo drink [54], suggesting a prebiotic effect of blueberry polyphenols. Furthermore, in a colonic model where several polyphenols were tested (caffeic acid, catechin, chlorogenic acid, epicatechin, o-coumaric acid, p-coumaric acid, phloridzin, rutin, naringenin, daidzein, genistein, quercetin and gentamicin), except rutin, all of them affected the viability of the colonic microbiota to some extent. Naringenin and quercetin were the most active molecules. In general, S. aureus was the most sensitive to polyphenols, while S. typhimurium and E. coli were comparable in their sensitivity to the treatment. The probiotic L. rhamnosus was less sensitive to the polyphenols indicating that viability of lactobacilli may be relatively unaffected by polyphenols in the gut. Generally, flavonols, isoflavones and glycosides were found to have a low antibacterial activity and phenolic acids were intermediate, while the flavanone and flavanol tested had high antibacterial activity [31]. Added to this, some phenolic acids are able to inhibit the growth of several pathogenic and non-beneficial intestinal bacteria without significantly affecting the growth of beneficial bacteria (Lactobacillus spp. and Bifidobacterium spp.), and the dihydroxylated forms (i.e. 3,4-dihydroxyphenylacetic and 3,4-dihydroxyphenylpropionic acids) efficiently destabilize the outer membrane of Salmonella. In general, non-hydroxylated and monohydroxylated phenolic acids are more potent than dihydroxylated or disubstituted phenolic acids. With regard to the saturated side chain, the order of potency, for the same benzene ring-substitution, is benzoic > phenylacetic > phenylpropionic acid [35]. Resveratrol increased Bifidobacterium and Lactobacillus counts and abolished the expression of virulence factors of Proteus mirabilis to invade human urothelial cells. Anthocyanins from berries have also proved to inhibit the growth of pathogenic Staphylococcus spp., Salmonella spp., Helicobacter pylori and Bacillus cereus. Flavonoids may also reduce the adhesion ability of L. rhamnosus to intestinal epithelial cells [30].

In summary, the interactions between polyphenols and gut microbiota are mutual, very complex, and show largely interindividual differences. Because of the biological importance of the microbial metabolites, a deeper understanding of these relationships will improve our knowledge on the health benefits of polyphenols and the factors controlling their production and whether this can be modulated advantageously for the human health.

4. Conclusions

Polyphenols exhibit a wide variety of different biological effects whose quantification *in vivo* is currently hampered by the lack of robust biomarkers. The disappointing results of antioxidant vitamins clinical trials, based on the supplementation of antioxidant vitamins in pure form [57] suggest that the interaction between the above-mentioned dietary components concomitant with a high intake of fibers, a low caloric density, and a paucity of atherogenic foods in the diet are likely – together – to effect protection from these diseases. Indeed, some trials do show positive modulation of surrogate markers of cardiovascular disease and cancer following the administration of defined amounts of polyphenols, e.g. from cocoa, olive oil, orange, etc. to human volunteers [58–61]. These healthful activities are the results of manifold and complex actions of polyphenols that do extend beyond their mere antioxidant actions. As briefly reviewed here, anti-inflammatory activities, activation of Phase II enzymes, and modulation of the gut microbiota play roles that are – biologically – more important that the purported *in vivo* scavenging of ROS or limitation

of free radical production. Therefore, future research (and, alas, advertisement) should focus on identifying an array of actions and their modulation of robust biomarkers. All of this – obviously – applies to berries and their minor components, which play multiple roles in human physiology and should not be heralded as mere antioxidants, but, rather, as multi-functional and biologically-important compounds.

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