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A battery of translational biomarkers for the assessment of the *in vitro* and *in vivo* antioxidant action of plant polyphenolic compounds: The biomarker issue

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Abstract

Over the last decades, the scientific findings stressing the beneficial health implications of plant-derived compounds (i.e., plant extracts rich in polyphenols) have been increased significantly. Assessing the properties of such compounds throughout the redox continuum is a common practice for the evaluation of their dynamics in reinforcing the antioxidant and antitoxic defence of tissues and, even, whole organisms or in the refinement of free radical-related disease onset. In the present contribution we propose a battery of translational biomarkers for measuring the antioxidant, antimutagenic and antitoxic capacities of plant compounds in three levels. The level 1 is the characterization of plant-derived compounds *in vitro* by detecting their antiradical and reducing capacity and their antimutagenic and anticarcinogenic properties. In the level 2, the tested agents should be administered in diverse cell lines in order to be examined for their antitoxic and antioxidant capacities. Although cell lines are considered as an *in vitro* system, it is undisputable that they are an *in vivo*-like environment and, thus, a link to *in vivo* settings. The level 3 is the administration of plant compounds *in vivo*, in experimental animals or humans either individually or as food supplements. The biomarkers for oxidative damage, antioxidant capacity and reducing/oxidative potential analyzed here seem to be suitable surrogate endpoints in the effort to extrapolate *in vitro* evidence to *in vivo* models. It is recommended that the aforementioned three lines of research (i.e., *in vitro*, cell culture environment and *in vivo*) should be applied in order to holistically reveal the biological action of plant polyphenolic compounds in the frame of redox biology and toxicology.

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1. Introduction

Plant-derived compounds are plant extracts or isolated polyphenols that are abundant in diverse diets worldwide the Mediterranean diet being among them [1]. Due to their strong biological potential they are considered as major sources of antioxidants [2]. Indeed, numerous research studies of our group over the last 15 years have reported that polyphenols and extracts originating from coffee [3], legumes [4–7], grapes [8–12], sage, mint and mountain tea [13] possess potent antioxidant and antimutagenic properties *in vitro*. The antioxidant action of extracts generated from coffee [14], olive oil [15], grapes [16–20] and legumes [21] has also been demonstrated in various cell lines, a model that is considered as an *in vivo*-like environment being a link to *in vivo*.

1.1. Influence of polyphenolic compounds on disease and toxicity

A considerable body of literature supports the notion that polyphenolic compounds exert remarkable health beneficial effects. Indeed, it has been demonstrated that they are pivotal preventive agents against disease-related toxicity reducing the risk of chronic pathologic conditions, such as cardiovascular and neurodegenerative diseases as well as cancer [22]. Cancer is a heterogeneous pathology that depends on diverse factors and, thus, extreme therapeutic regimes are needed. Chemotherapy is a common approach. However, due to the wide range of cells that chemotherapeutic agents affect they consist a considerable toxic hazard even for healthy cells. To this end, new anticancer drugs based on

natural polyphenols have been developed and it has been shown that they are able to inhibit the toxic effects of regular chemically based drugs [23]. Vitamin C is a crucial antioxidant agent that its potential contribution to anticancer therapy has been well studied during the last 40 years. Although it is not a polyphenol, its antioxidant mechanism is almost identical to that of polyphenolic compounds, therefore it is worth it to examine the remarkable evidence reported by high prolific researchers. Nobel Prize winner Linus Pauling and his colleague Ewan Cameron published two groundbreaking articles in the middle 70s stressing out that high doses of vitamin C employed as supportive treatment of cancer reinforce the effectiveness of chemotherapy and are able to prolong survival time of patients [24,25]. Recent relevant studies have also shown that vitamin C not only alleviates chemotherapy side effects but also, intriguingly, hinders the progression of the disease [26]. It has been proposed that vitamin C, similarly to polyphenols, is "sacrificed" in order to dysregulate the normal function of cancer cells [27]. Given that cancer cell metabolism is altered due to the disease, high concentrations of redox active transition metals, such as Fe are generated. Vitamin C reacts with them converting into the relatively stable ascorbyl free radical and gives birth to free radicals and reactive species (e.g., hydrogen peroxide, H_2O_2), which weaken cancer cells and make them susceptible to chemotherapy [27]. We have to keep in mind that half life of vitamin C in human blood is as short as 2 h. Therefore, its potential effects as a therapeutic factor can only be pronounced when administered in quite high doses in order to achieve much higher blood concentration than the 70 μM normally observed in adults [27]. The knowledge acquired has been leveraged in order to partly comprehend the biological base of cancer and its putative treatment in association with polyphenolic compounds. Other experimental evidence published in high ranking journals, though, clearly challenges the correctness of the antioxidant approach for the amelioration of symptoms in certain cancer types [28]. Indeed, it has been demonstrated that administration of the potent antioxidant N-acetylcysteine (NAC) increases lymph node metastasis in mice and, similarly to vitamin E, it promotes the migration and invasive properties of cultured human melanoma cells [29]. On the same page with this elaborative study, NAC and vitamin E have also been found to markedly increase tumor progression and reduce survival of mice suffering from lung cancer [30]. Hence, it becomes evident that there is a need for a shift towards a new paradigm according to which cancer cells tend to be resistant to oxidative insults because antioxidants in some cases fortify their defensive mechanisms [28]. A difference of redox status between the tumor niche and the niches of healthy organs seems desirable, therefore much caution is required in order the chemotherapeutic strategy to be effective.

The benefits derived from polyphenolic compounds include also the alleviation of the neutrophil mediated toxic effects during inflammation, whose dysregulation is a common outcome of toxicity [31,32]. It is worth mentioning that polyphenols are protective compounds against toxicity induced by pesticides as well [33]. Moreover, epicatechin, lycopene and proanthocyanidins act protectively against toxicity induced by metals, such as lead and mercuric chloride in rats [34,35]. Hydroxytyrosol, a polyphenol present in olive oil prevents reactive species production and inhibits apoptosis induced by mercury in human erythrocytes and neuroblastoma cells [36]. This finding is of major importance because mercury has been correlated with autism and polyphenols seem to be an effective defensive mechanism [36]. The studies mentioned here are a few indicative examples that polyphenolic compounds are able to reverse the harmful effects (e.g., oxidative stress, inflammation, diverse pathologic conditions) of exposure to toxic agents. Such pathologies are related to the biology of reactive species and polyphenolic compounds act as oxidant scavengers *in vivo* fortifying the cellular antioxidant mechanism of defence [37].

1.2. *In vitro* vs *in vivo* activity of plant-derived compounds

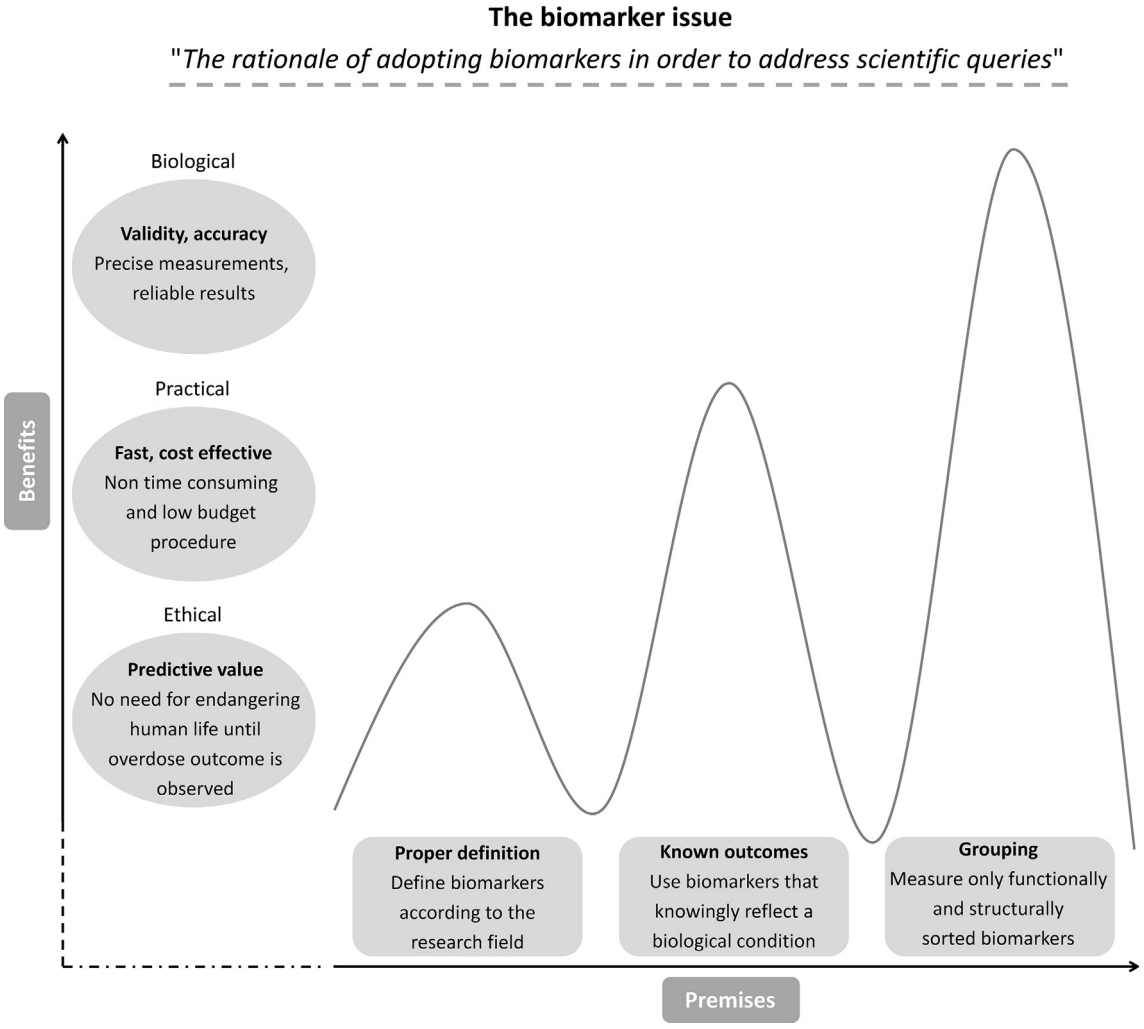
Several *in vivo* nutritional studies have also stressed the advantageous role of polyphenolic compounds. The supplementation of olive oil extracts to piglets [38], broiler chickens [39–41], sheep [42] and lambs [43] as well as grape pomace to piglets [44] and lambs [45] enhances antioxidant defence and improves other beneficial, biological processes. It is noteworthy, though, that the *in vitro* activity of a polyphenolic compound does not necessarily translate accordingly *in vivo* [46]. To this direction, Veskoukis et al. (2012a) [12] have demonstrated that a grape extract with potent *in vitro* antioxidant properties exhibits prooxidant action when administered in rats before exercise. There is accumulating evidence substantiating this idea, that is when the concentration of polyphenolic compounds exceeds an upper, yet unknown threshold, confers adverse effects through enhancement of their prooxidant nature establishing a double-edged sword in antioxidant supplementation policy [47,48]. It is now accepted that polyphenolic antioxidants in high concentrations and pH values or in the presence of iron can initiate the auto-oxidation process and turn into prooxidants [49] or even induce toxicity [50]. Moreover, factors such as dose, passage and time of administration, concentration and bioavailability play key roles in the manifestation of the *in vivo* behavior of polyphenolic compounds [51]. Another crucial factor allowing to extrapolate the *in vitro* findings to *in vivo* recontextualizing the obtained knowledge is to select the group of biomarkers that are considered appropriate for the biological evaluation of the plant-derived material [46].

1.3. Definitions of "redox biomarkers" and the "biomarker issue"

The term "biomarker", a portmanteau of "biological marker", has been defined by the Biomarkers Definitions Working Group (2001) [52], as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". This is a broad definition, which encompasses both normal and pathogenic biological processes and, also, therapeutic interventions that, according to our point of view, could readily be replaced by nutritional, toxicological or every putative redox altering treatments. *Sensu stricto* we define the term "biomarker" in the field of redox biology (i.e., *redox biomarker*) as follows: *a biological entity that can be accurately and reproducibly measured and that might be: i) an antioxidant molecule (e.g., glutathione, catalase), whose concentration, activity and/or structure are modified following interaction with reactive species, ii) the products of the detrimental impact of reactive species on biomolecules (e.g., protein carbonyls, malonyldialdehyde), iii) the reactive species per se*.

The "biomarker issue", that could be defined as "*the rationale of adopting biomarkers in order to address scientific queries*", is of utmost importance. Apart from certain premises, there are, also, undisputable benefits as presented in Figure 1. In order to magnify its translational potency a biomarker should be properly defined according to the research discipline. Moreover, it is essential that a study should focus on biomarkers, which knowingly reflect a biological condition or phenomenon and, subsequently, to group them according to their function reinforcing their ability to provide mechanistic answers. Validity and accuracy as well as being fast and cost effective are major advantages of the biomarker issue. There are also striking ethical problems that can be overcome. For instance, as it has been insightfully

Figure 1



A conceptual model of the benefits from and the premises for the biomarker issue.

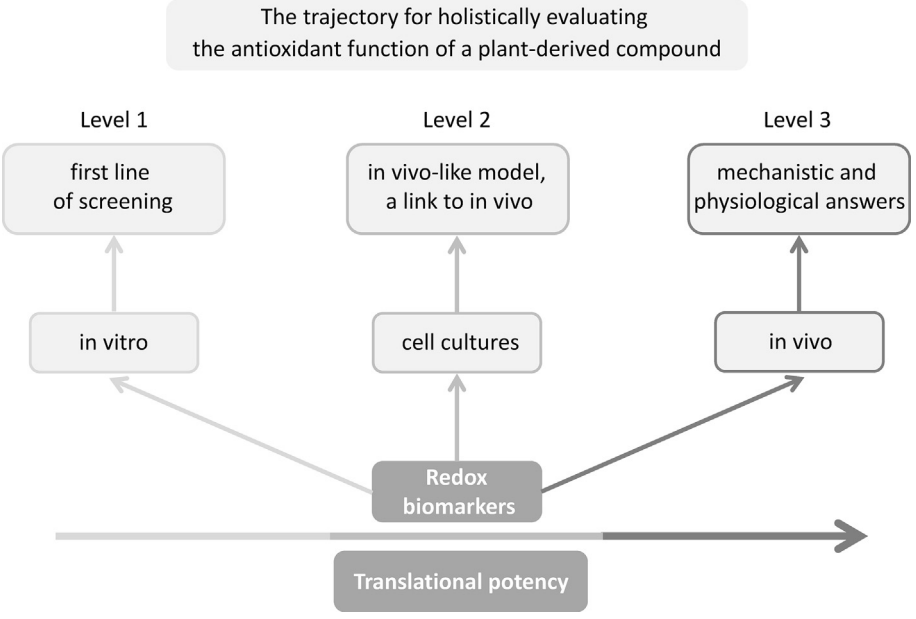
noted, it is unethical to wait for indications of liver damage due to paracetamol overdose in order to proceed to the appropriate treatment of a patient. Instead, a toxicity biomarker (i.e., paracetamol concentration in plasma) could easily predict whether treatment is required [53]. Additionally, there is no need for using excessively high number of experimental animals, thus, conforming with the concept of the three Rs (i.e., Replacement, Reduction and Refinement) [54]. This, also, applies in clinical trials. For example, a relatively small size of subjects would be needed to determine the effect of a new drug on LDL concentration. However, in the absence of biomarkers a multi-year trial would be required to evaluate the prevention of deaths from heart attack. Recontextualizing for redox biology, a relatively small size of subjects would be needed in order to determine the effect of a new drug on mitigating aging induced chronic low grade inflammation. Nevertheless, in the absence of biomarkers a multi-year trial would be required to evaluate the beneficial impact of this drug on activities of the aged population, such as walking or general well-being.

As the field of redox biology is progressing research has returned back to the biomarker issue, which seems rather unexpected. It is undisputable, though, that in the modern era of the field biomarkers gain much acceptance as tools that reveal the biological principles of redox mechanisms [55]. Indeed, the adoption of a battery/panel of biomarkers is a necessary step prior to the characterization of a polyphenolic compound as anti- or prooxidant. We need to point out, yet, that it is crucial to group biomarkers to form networks with functional and, even, structural homology [56,57] or to pick the proper biomarkers [58,59] in order to reveal their true value. Furthermore, it is preferable to cluster them on the grounds of the biological system (i.e., *in vitro*, cell culture environment, *in vivo*) they are measured, thus, escalating their translational potency (Figure 2). As it has been previously mentioned, polyphenolic compounds present in plant extracts partly contribute to the reverse of toxicity induced by pathologies and diseases. To this end, the antioxidant potency is a measure of this ability. Based on the above, the aim of the present contribution is to propose a battery of redox biomarkers that allow the holistic characterization of a plant-derived compound with respect to its antioxidant activity. The principles and the experimental procedures of the assays for the measurement of the biomarkers proposed in this paper are thoroughly described in the articles cited.

2. Level 1: biomarkers for the assessment of the antioxidant activity of plant polyphenolic compounds *in vitro*

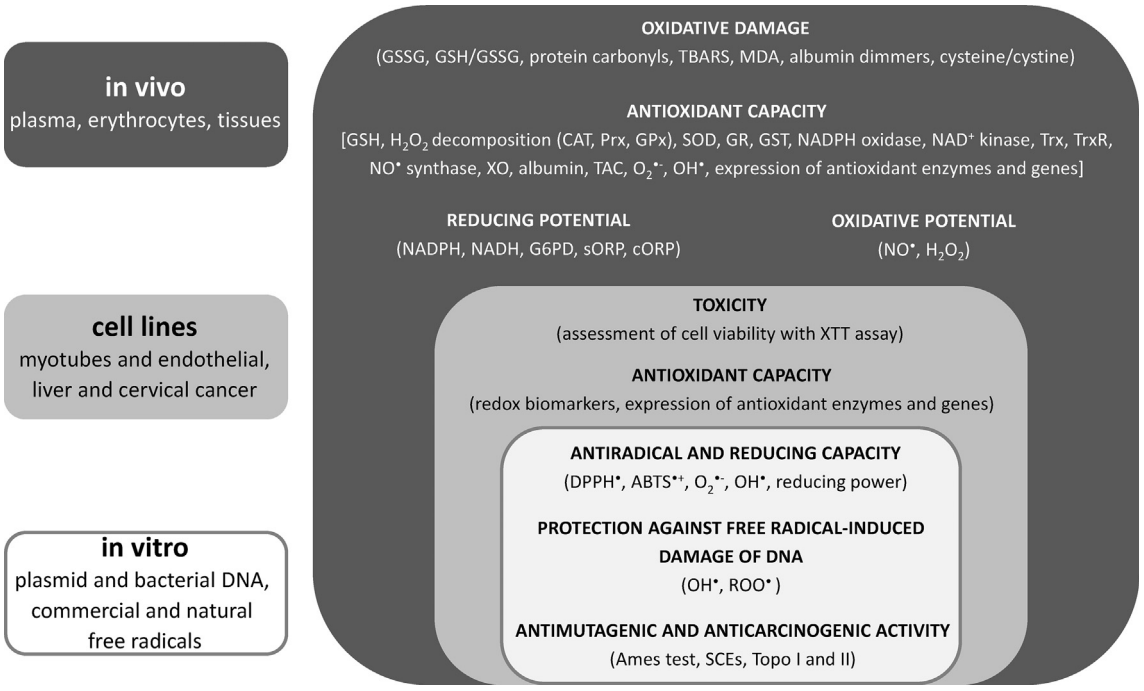
The first line of screening for assessing the potential antioxidant activity exhibited by a polyphenolic compound is comprised by evaluating the proper biomarkers *in vitro* (Figure 3). As it has been stated in the previous paragraphs the *in vitro* biomarkers should be clustered in three major categories. The biomarkers for assessing the antiradical and reducing capacities of the compounds in question belong to the first category. The antiradical capacity can be revealed spectrophotometrically by examining the scavenging ability of a compound against

Figure 2



The contribution of redox biomarkers to the holistic characterization of a plant-derived compound in terms of antioxidant action.

Figure 3



The proposed battery of translational biomarkers for the characterization of a plant-derived compound *in vitro*, in cell culture environment and *in vivo* as well, in order to holistically reveal its biological action. GSSG: oxidized form of glutathione, GSH: reduced form of glutathione, TBARS: thiobarbituric acid reactive substances, MDA: malonyldialdehyde, CAT: catalase, Prx: peroxiredoxins, SOD: superoxide dismutase, GPx: glutathione peroxidase, GR: glutathione reductase, GST: glutathione S-transferase, NADPH: nicotinamide adenine dinucleotide phosphate reduced, NAD^+: nicotinamide adenine dinucleotide oxidized, Trx: thioredoxin, TrxR: thioredoxin reductase, XO: xanthine oxidase, TAC: total antioxidant capacity, NADH: nicotinamide adenine dinucleotide reduced, G6PD: glucose 6-phosphate dehydrogenase, sORP: static oxidation-reduction potential, cORP: capacity oxidation-reduction potential, XTT: (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide), $DPPH^•$: 2,2-diphenyl-1-picrylhydrazyl radical, $ABTS^{•+}$: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) radical, SCEs: sister chromatid exchanges, Topo I and II: topoisomerases I and II.

commercial [i.e., 2,2-diphenyl-1-picrylhydrazyl ($DPPH^•$), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{•+}$)] and natural [i.e., superoxide ($O_2^{•-}$), hydroxyl ($OH^•$)] free radicals. A polyphenolic compound is considered as an antioxidant when it donates one electron to free radicals leading to their neutralization and, therefore, protects the biomolecules against detrimental oxidative modifications [3,5,12]. The evaluation of the reducing capacity of a compound is reliably monitored via the reducing power assay, which examines its ability to reduce ferrous (Fe^{+3}) to ferric (Fe^{+2}) ions, meaning that it is a strong electron donor and, hence, a reducing agent capable of protecting cells against redox perturbations [60]. It has to be noted that the presence of Fe^{+2} inside the cellular environment is not beneficial, since this transition metal can donate the extra electron to other reactive species, thereby resulting in the formation of free radicals through the Fenton reaction. Therefore, reducing power is an *in vitro*/test tube assay that is used to examine the reducing capacity of a plant extract in isolated conditions without being associated with the detrimental effects of Fe^{+2} in the cell. The second class includes biomarkers for the evaluation of the protective activity of polyphenolic compounds against free radical-induced DNA damage. Specifically, the ability of a compound to inhibit the double stranded DNA scission induced by hydroxyl ($OH^•$) and peroxyl ($ROO^•$) radicals that are artificially generated using an agarose gel is a reliable indication of its antioxidant nature, at least *in vitro* [4,5,12,16]. The evaluation of the antimutagenic and anticarcinogenic action of plant compounds by examining the inhibitory activity against bleomycin induced mutagenicity in *Salmonella typhimurium* cells (i.e., the Ames test), mitomycin C-induced sister chromatid exchanges and human topoisomerases I and II is the third group of the *in vitro* biomarkers proposed herein [10,61–63]. Experimentally, a potential antimutagenic compound prevents the formation of mutations using bacterial and peripheral blood lymphocyte DNA as experimental models, whereas it inhibits the activity of the enzymes (i.e., topoisomerases I and II) that induce DNA scissions.

As depicted in Figure 2, *in vitro* redox biomarkers are obviously the less powerful ones on the basis of

translational potency. They offer valuable, yet observational, information but, undoubtfully, they are the first line of screening for the putative beneficial action of a polyphenolic compound. The next step of the trajectory for holistically evaluating the antioxidant function of a plant compound is its introduction in cultured cell lines, which serve as a link to *in vivo* settings.

3. Level 2: biomarkers for the assessment of the antioxidant activity of plant polyphenolic compounds in cell lines

There is increasing research interest regarding the cytoprotective effects of dietary compounds in *in vivo*-like systems, specifically cell culture environment against oxidative stress aiming towards the discovery of potential therapeutic and chemopreventive agents. The physiological and cancer cell lines are quite useful tools in order to mechanistically examine the capacity of plant derived compounds to alter cell redox equilibrium and proliferation status. Among the most fundamentally used cell lines are the physiological C2C12 myoblasts and EA.hy926 endothelial cells along with the liver cancer HepG2 and cervical cancer HeLa cells (Figure 3). The C2C12 myoblasts consist a muscle-centric model that merits research since exercise and inflammation are inherently linked to skeletal muscle oxidative stress [64]. The EA.hy926 endothelial cells simulate the innermost part of blood vessels which are affected by the onset of redox related diseases. With respect to the cancer cell lines, they are a model to examine the anticancer effects of polyphenol-rich plant extracts [65] with HeLa being the most widely studied cell line. Regarding HepG2 cells they are a model for the simulation of liver metabolism and examination of xenobiotic toxicity as they preserve the morphology and the basic biological functions of hepatocytes.

The first, typical step is to investigate the putative cytotoxicity of the compound in question. The application of the XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay is used for testing cell viability and exerts tissue-specific activity [15,17,66]. Special caution is demanded, though, in the detection of the action of polyphenols in cell viability. Indeed, there is experimental evidence demonstrating that polyphenolic compounds present in plant extracts may reduce the XTT salt thereby resulting in false negative results in regards to cytotoxicity. Additionally, they may alter mitochondrial function without a loss of viability, which would also lead to false positive and negative results [67,68]. However, these potential artifacts can be partly overcome by applying a washing step with cell culture medium prior to the treatment of the cells with the plant extract in question, as it has been previously shown [69]. In order, nevertheless, to ensure that XTT reduction is a reliable biomarker for cell viability, it is impaired to use trypan blue as a reference assay. Interestingly, it has been reported that XTT and trypan blue measure cell viability accurately at 40 μM of (−)-epigallocatechin-3-gallate, which is a common polyphenol for diverse kinds of plant extracts [67]. Indicatively, previous studies of our group have reported that polyphenol-rich extracts are not cytotoxic in a concentration range between 0.5 and 20 μg/ml that, based on their composition, is translated to 0.6−25 μM [15,70]. Therefore, XTT is a reliable indicator of plant extract toxicity until a polyphenolic concentration of 40 μM. If this boundary is surpassed, then trypan blue should be adopted as a correction agent. Subsequently, non-cytotoxic concentrations are preferably administered to the examined cell lines in order to assess reduced glutathione (GSH) and reactive oxygen species (ROS) levels through flow cytometry using the fluorescent dyes mercury orange and 2,7-dichlorofluorescein diacetate, respectively. The aforementioned biomarkers reflect cell redox status and the potential alterations induced by the tested compounds [14,15,17,60,71]. Furthermore, plant-derived compounds are nuclear factor (erythroid-derived 2)-like 2 (Nrf2) inducers upregulating the expression of antioxidant and detoxification genes [72]. This is probably achieved through their interaction with kelch-like ECH-associated protein 1 (Keap1), since polyphenols in their oxidized form readily react with the sulfhydryl groups of Keap1 Cys residues leading to the derepression of Nrf2 and the concomitant upregulation of genes that harbor antioxidant/electrophile response element (ARE/EpRE) in their promoter regions. Some of the most important genes are presented in Table 1. The expression of these genes is usually investigated both at the mRNA and the protein level through real-time PCR and western blot analysis, respectively [18,19,63,73,74]. This gene network covers the majority of cellular antioxidant and detoxifying mechanisms, thus, their analysis provides a first insight concerning the action mechanism of an extract. However, in order to elucidate its effects on tissues an *in vivo* study, which is the third level of the trajectory towards the characterization of an extract, is considered necessary (Figure 3).

4. Level 3: biomarkers for the assessment of the antioxidant activity of plant polyphenolic compounds *in vivo*

Several groups of biomarkers that can be evaluated in plasma, erythrocytes and tissue lysate provide evidence for the effects of plant-derived compounds on tissue oxidative stress in *in vivo* settings. To this line, the biomarkers of oxidative damage illustrate the impact of polyphenolic compounds on oxidative status of (bio) molecules. This category comprises protein carbonyls as a biomarker of protein oxidation, GSSG as the oxidized form of glutathione (GSH/GSSG is a widely accepted redox couple) and thiobarbituric acid reactive substances as a biomarker of lipid peroxidation that can be

Table 1 Antioxidant and detoxifying genes with antioxidant response elements in their promoter regions along with the functions of the encoded proteins potentially affected by plant-derived compounds.

Gene (abbreviation)	Function of the encoded protein
Catalase (CAT)	Catalyzes the decomposition of H_2O_2 to H_2O and O_2, when H_2O_2 concentration is high
Peroxiredoxins (PRDX)	Family of cysteine-dependent peroxidases that catalyze the decomposition of hydroperoxides and H_2O_2 to H_2O and O_2, when H_2O_2 concentration is low
Superoxide dismutase-1 (SOD-1)	Catalyzes the dismutation of $O_2^{\bullet-}$ to O_2 and H_2O_2
Glutathione peroxidase-1 (GPx-1)	Catalyzes the reduction of organic hydroperoxides and H_2O_2, when H_2O_2 concentration is low, with the concomitant oxidation of GSH
Glutathione reductase (GSR)	Catalyzes the reduction of GSSG generating GSH, a leading endogenous antioxidant
Thioredoxin (TRX)	Catalyzes the reduction of disulphides and peroxidases (such as peroxiredoxins) and maintains the cellular environment in a reduced state
Heme oxygenase-1 (HO-1)	Catalyzes heme degradation to CO, biliverdin and Fe^{2+}
NAD(P)H:quinone oxidoreductase (NQO-1)	Reduces quinone and endogenous antioxidants and generates NAD^+ and $NADP^+$
Glutathione S-transferase alpha 2 (GSTa-2)	Catalyzes the detoxification of electrophilic compounds (i.e., carcinogens, xenobiotics, environmental toxins and oxidative stress byproducts) via conjugation with GSH
γ-glutamate cysteine ligase catalytic subunit (γ-GCLc)	The first rate-limiting enzyme in the pathway of GSH synthesis - catalyzes the formation of γ-glutamylcycteine

GSH: the reduced form of glutathione; GSSG: the oxidized form of glutathione; NAD^+: nicotinamide adenine dinucleotide oxidized; $NADP^+$: nicotinamide adenine dinucleotide phosphate oxidized.

easily and accurately measured spectrophotometrically [56,64,75–78]. Malonyldialdehyde as a byproduct of lipid peroxidation can also be assessed through high performance liquid chromatography with diode-array detector (HPLC-DAD) increasing the credibility of the measurement [79]. Noteworthy, the oxidized form of albumin present in dimmers is, also, a significant biomarker of protein oxidative modifications as our research group has previously proposed [80,81]. The biomarkers of oxidative potential including $NO^{\bullet}$ and H_2O_2 provide extra information for tissue redox status. Interestingly, regarding H_2O_2, catalase is the main enzyme for its detoxification in erythrocytes. Still, glutathione peroxidase and peroxiredoxins play also key role in this biological process in tissues [40,43,82]. However, the exact contribution of these three enzymes in H_2O_2 detoxification still remains vague, although it is known that typically catalase, unlikely to peroxiredoxins or glutathione peroxidase, is activated when H_2O_2 concentration is high [82].

The biomarkers of antioxidant capacity have, also, translational value and include GSH, the enzymes catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione S-transferase, glutamate cysteine ligase, NADPH oxidase, NAD^+ kinase, thioredoxin, thioredoxin reductase, peroxiredoxin, $NO^{\bullet}$ synthase and xanthine oxidase, the reduced form of albumin and the crude total antioxidant capacity (TAC) [38–40,43]. The majority of these biomarkers scavenge reactive species and are essential detoxification mechanisms against xenobiotics. Their major trait, though, is that they are part of a network the reaction cascade of which is mostly dependent on NADPH oxidation [57].

Apart from measuring the activity of the aforementioned enzymes spectrophotometrically, quantitative alterations can be measured through western blot analysis [19,42]. Furthermore, Nrf2 protein expression can also be measured by western blot and, as mentioned above, Nrf2 target genes presented in Table 1 can be estimated by real time PCR in blood and tissues as well [66]. Furthermore, glucose 6 phosphate dehydrogenase (G6PD), the enzyme responsible for NADPH generation in the pentose phosphate pathway as well as the static (sORP) and capacity oxidation-reduction (cORP) intensify the redox status of tissues that can be estimated by their capacity to scavenge superoxide ($O_2^{\bullet-}$) and hydroxyl ($OH^{\bullet}$) radicals [77,78,83].

It is worth mentioning that biomarkers of reducing potential, such as the reduced forms of nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] are usually expressed in redox couples and are key regulators of tissue redox homeostasis. Indeed, it has been reported that the disturbance of their equilibrium has been linked to the appearance of several diseases [84]. Such diseases are related to alterations in tissue redox status and generation of oxidative stress as defined in accordance to the outstanding development that the field of redox biology has met during the last 30 years. Back in 1985, Helmut Sies gave the most influential definition of oxidative stress as “a disturbance in the prooxidant-antioxidant balance in favor of the former” [85]. This definition seems unacceptable and inadequate nowadays and, thus, it has been replaced by “a disruption in redox signaling and control” [86]. This sounds as a realistic and modern way to define oxidative stress that denotes an explicit shift from the old era to

the new paradigm that reactive species are essential molecules for signal transduction [87]. Indeed, the thiol side chain of cysteine as major target of reactive species generating sulfenylation products is key regulator of protein function *in vivo* [88]. Another shift in the ideas of the field is the fact that the two most crucial redox couples, namely GSH/GSSG and cysteine/cystine are not in equilibrium with each other as it was thought until recently [86,89]. Thus, the 1985 definition of oxidative stress seems incomplete because it implies that all subcellular compartments respond identically to redox altering stimuli. However, this is not the case as it has been reported that they are independently regulated depending on which organelle or system (e.g., plasma, extracellular fluid) they are located [90]. Given that oxidative stress is also inextricably linked to signal transduction, the new definition is adequate. To this end, the cysteine/cystine redox couple, which represents the largest pool of low molecular weight thiols and disulfides in plasma is a new entry in the battery of redox biomarkers the last few years [91]. Interestingly, increased cystine levels have been associated with death related to cardiovascular disease [92]. The use of biomarkers is still a high throughput approach, though [55]. Yet, there are a lot of biomarkers for the evaluation of oxidative stress and, hence, the need for functionally grouping them still exists in order to apply their use in routine toxicological measurements [86]. By inference, the biomarkers of level 3 provide the greatest translational potency in the effort to evaluate the antioxidant power of plant polyphenolic compounds (Figure 3).

5. Concluding remarks

In the present article, we have tried to contribute to the emerging needs of the research field of redox biology by underlying the importance of biomarkers and the necessity to use them as experimental tools in the field of toxicology. The biomarkers described here represent the fundamental pillars in order to assess the role of plant polyphenolic compounds in redox biology and toxicology. Towards this direction, we have provided distinct definitions for the widely accepted term "redox biomarker" and the concept of the "biomarker issue". We also propose a battery/panel of redox biomarkers recommending their adoption in order to holistically evaluate the antioxidant function of a plant-derived compound throughout the continuum of experimental models that is *in vitro*, in cell culture environment and *in vivo*.

Conflict of interest

The authors declare no conflict of interest.

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