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# ANTIOXIDANTS AND THE COMET ASSAY

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**ABSTRACT**

It is widely accepted that antioxidants, either endogenous or from the diet, play a key role in preserving health. They are able to quench radical species generated in situations of oxidative stress, either triggered by pathologies or xenobiotics, and they protect the integrity of DNA from genotoxicants. Nevertheless, there are still many compounds with unclear or unidentified prooxidant/antioxidant activities. This is of concern since there is an increase in the number of compounds synthesized or extracted from vegetables to which humans might be exposed. Despite the well-established protective effects of fruit and vegetables, the antioxidant(s) responsible have not all been clearly identified. There might also be alternative mechanisms contributing to the protective effects for which a comprehensive description is lacking. In the last two decades, the Comet assay has been extensively used for the investigation of the effects of antioxidants and many reports can be found in the literature. The Comet assay, a relatively fast, simple, and sensitive technique for the analysis of DNA damage in all cell types, has been applied for the screening of chemicals, biomonitoring and intervention studies. In the present review, several of the most well-known antioxidants are considered. These include: catalase, superoxide dismutase, glutathione peroxidase, selenium, iron chelators, melatonin, melanin, vitamins (A, B, C and E), carotenes, flavonoids, isoflavones, tea polyphenols, wine polyphenols and synthetic antioxidants. Investigations showing beneficial as well as non-beneficial properties of the antioxidants selected, either at the *in vitro*, *ex vivo* or *in vivo* level are discussed.

## INTRODUCTION

The current scientific consensus holds that significant amounts of oxygen (O<sub>2</sub>) first appeared in the Earth's atmosphere some 2.4 billion years ago due to the photosynthetic activity of blue-green algae [1]. Oxygen, besides being indispensable for the production of metabolic energy in most eukaryotes, is also a life-threatening agent. DiGuissepe and Fridovich put forward a hypothesis which attempted to explain the toxicity of O<sub>2</sub> in a chronological order [2]. Initially, O<sub>2</sub> toxicity was thought to be due to the inactivation of enzymes, mainly the thiol group of cysteine residues. Later, toxicity was also attributed to the effects of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Ultimately, molecular biology techniques established that the toxic effects of O<sub>2</sub> are directly linked to its reactive forms, the radical oxygen species (ROS), acting on cellular components. The link between O<sub>2</sub> toxicity and many pathologies, e.g. pulmonary diseases, is very well-established [3], and its effect on swelling of the blood-gas barrier [4], retinal defects [5], bowel disease [6], neurodegeneration [7], cancer [8] and ageing [9]. However, the progressive increase of the O<sub>2</sub> content in the atmosphere and the evolution of species on Earth implied that organisms dependent on aerobic metabolism required biochemical defences in the form of antioxidants for their survival. These protectants against O<sub>2</sub> have also evolved to prevent potential excessive oxidation of cellular constituents [10,11] and contribute to the complex and integrated biological antioxidant defence system.

The Comet assay or single cell gel electrophoresis (SCGE) is a widely used technique for measuring and analysing DNA breakage in individual cells which can be applied to *in vitro*, *ex vivo* and *in vivo* systems. The methodology developed in the mid-eighties was originally introduced by Östling and Johanson [12], and later, Singh [13]

1  
2  
3  
4 modified it by including unwinding under alkaline conditions. Small numbers of cells  
5  
6 that have been exposed to a physical or chemical agent are embedded in a thin  
7  
8 agarose gel on a microscope slide. The cells are lysed and the DNA subsequently  
9  
10 allowed to unwind under different pH conditions. By choosing different pH  
11  
12 conditions for electrophoresis and the preceding incubation, different levels of  
13  
14 damage and sensitivity can be assessed. The degree of DNA migration can be  
15  
16 correlated to the extent of DNA damage occurring in each single cell. *In vitro*  
17  
18 investigations can be carried out virtually with any cell type; however, the cell-type-  
19  
20 of-choice in biomonitoring is the lymphocyte because blood is easily collected and  
21  
22 lymphocytes have proved to be good surrogate cells. For instance, lymphocytes  
23  
24 exhibited genotoxicity caused by anticancer agents targeting several different organs  
25  
26 [14].  
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30  
31 The Comet assay is a useful tool for examining issues related to oxidative stress in  
32  
33 human lymphocytes [15]. With regard to its ability to detect oxidative stress *in vivo*, it  
34  
35 has been used as a biomarker of pathologies [16], diet [17], occupational exposure [18]  
36  
37 and environmental pollution [19]. The use of particular antioxidants has allowed the  
38  
39 elucidation of the mechanism of DNA damage exerted by a broad variety of agents  
40  
41 [19-22]. Further, the Comet assay has revealed the prooxidant/antioxidant effects of  
42  
43 various endogenous and exogenous compounds [23].  
44  
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46

47 The Comet assay has successfully assessed interactions of antioxidants with  
48  
49 genotoxicants [24] and it has also proved a valid technique to evaluate whether  
50  
51 antioxidant/micronutrients are able to protect the integrity of the genetic material [25-  
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53 27]. This review will focus on selected examples of extensively investigated  
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55 compounds by the Comet assay with antioxidant or potential antioxidants properties.  
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## THE COMET ASSAY ON ENDOGENOUS ANTIOXIDANTS

### Catalase

Catalase is present in all major body organs, but is particularly concentrated in the liver. Catalase activity within the cell is largely located in peroxisomes [28]. It catalyses the reduction of  $\text{H}_2\text{O}_2$  to  $\text{O}_2$  and  $\text{H}_2\text{O}$ . At high concentrations of  $\text{H}_2\text{O}_2$ , catalase displays the capability of reducing it. In fact, it is very difficult to saturate the enzyme. By contrast, it decreases its efficiency at low concentrations of  $\text{H}_2\text{O}_2$ . The reason for this is that catalase requires the reaction of two  $\text{H}_2\text{O}_2$  molecules to carry out its reduction and this is more unlikely to occur as the concentration of the substrate falls. In the Comet assay, catalase has been used together with a large variety of compounds to determine whether the mechanism underlying DNA damage is mediated by  $\text{H}_2\text{O}_2$ . For instance, the presence of catalase prevented DNA damage produced by ozone *in vitro* [29]. For metals such as chromium (Cr), catalase decreased the extent of DNA damage induced by Cr(VI) but not that induced by Cr(III) *in vitro* [30]. The addition of catalase *in vitro* revealed that DNA damage exerted by oestrogenic compounds was mediated by  $\text{H}_2\text{O}_2$  in lymphocytes from healthy donors [20,21]. The brain, heart, skeletal muscle and spermatozoa contain lower levels of catalase [31]. Taking this into account, it was interesting to find out whether the addition of catalase supplied *in vitro* to low catalase cell types could prevent DNA damage induced by ROS. It was observed that catalase exerted a protective effect on sperm incubated with oestrogenic compounds [22]. The detrimental effect of oxidative stress is found in Alzheimer's and Parkinson's disease [32]. Mimicking these conditions *in vitro*, fresh isolated mouse brain cells and cultured astrocytes were exposed to  $\text{H}_2\text{O}_2$  and xanthine combined with xanthine

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4 oxidase. No DNA damage was observed when catalase was present [33]. Catalase was  
5  
6 co-incubated with disinfection by-products of drinking water such as the highly  
7  
8 genotoxic iodoacetic acid achieving a 42% reduction in the DNA damage *in vitro* [34].  
9  
10 Blood from vitiligo patients who had already undergone treatment was collected. The  
11  
12 lymphocytes were isolated and co-incubated with catalase *in vitro*. A reduction in  
13  
14 their basal levels of DNA damage was observed. In the presence of H<sub>2</sub>O<sub>2</sub>, catalase  
15  
16 prevented genetic damage to healthy volunteers and vitiligo treated patients [35]. For  
17  
18 the previous investigations, catalase was used at concentrations ranging from 100 to  
19  
20 500 IU/ml which consistently showed, in our laboratory, reduction or abolition of  
21  
22 DNA damage levels in the presence of H<sub>2</sub>O<sub>2</sub>. Since 100 to 500 IU/ml has been a range  
23  
24 of concentrations repeatedly used in our research, we also investigated catalase on its  
25  
26 own in preliminary experiments. Catalase on its own did not generate DNA damage  
27  
28 up to a concentration of 500 IU/ml, which excluded any prooxidant activity.  
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### 36 **Superoxide dismutase**

37  
38 There are different types of SOD, named in accordance with the ions they contain.  
39  
40 Copper-zinc-SODs are stable enzymes present in the cytosol, more particularly in  
41  
42 lysosomes and the nucleus. Manganese-SODs are more present in mitochondrias of  
43  
44 yeast and animals whereas iron-SODs have not been found in animal tissues. The  
45  
46 discovery of SOD enzymes provided much of the basis of the knowledge of  
47  
48 antioxidant defence systems, since it led to the superoxide theory of oxygen toxicity  
49  
50 [36]. This proposed that O<sub>2</sub><sup>-</sup> (superoxide radical) is a major factor in O<sub>2</sub> toxicity and  
51  
52 that the role of SOD as a scavenger of O<sub>2</sub><sup>-</sup> by coupling and converting it to H<sub>2</sub>O<sub>2</sub> is of  
53  
54 utmost importance. The superoxide theory of O<sub>2</sub> toxicity can be proved by the  
55  
56 following investigations. *Escherichia coli* with functional SOD genes replaced by  
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4 defective ones and *Pseudomonas aeruginosa* lacking SOD genes did not succeed in  
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6 growing in an aerobic environment [37,38]. Transgenic mice incorporating human  
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8 copper-zinc-SOD, in addition to mice copper-zinc-SOD, showed increased resistance  
9  
10 to O<sub>2</sub> toxicity [39]. By contrast, knockout mice with an absence of SOD genes  
11  
12 presented a variety of pathologies from which they died [40]. We investigated  
13  
14 whether SOD could quench the DNA damage generated by oestrogenic compounds *in*  
15  
16 *vitro* on human lymphocytes [20]. The SOD concentrations, 50 and 150 IU/ml, were  
17  
18 selected since they have been proved to reduce the levels of DNA damage in some  
19  
20 systems in our laboratory. However, the efficiency and consistency in reducing the  
21  
22 DNA damage levels can not be compared to catalase. SOD did not reduce the  
23  
24 response generated by the compounds diethylstilboestrol (DES) (100 μM), 17β-  
25  
26 oestradiol (50 μM), nonylphenol (50μM) and H<sub>2</sub>O<sub>2</sub> (80 μM). Conversely, it decreased  
27  
28 to some extent the DNA damage in at least one out of two independent experiments  
29  
30 when co-incubated with phytoestrogens like equol (250 μM), genistein (250 μM) or  
31  
32 daidzein (250 μM). An explanation for this might be the fact that higher  
33  
34 concentrations were used for phytoestrogens with regard to DES, 17β-oestradiol,  
35  
36 nonylphenol and H<sub>2</sub>O<sub>2</sub>, being more suitable for the kinetics of disproportionation of  
37  
38 the two O<sub>2</sub><sup>-</sup> carried out by SOD [20]. In other words, the rate of production of O<sub>2</sub><sup>-</sup> for  
39  
40 the non-phytoestrogenic compounds is slower than their conversion to further H<sub>2</sub>O<sub>2</sub>.  
41  
42 The fact that SOD accelerates H<sub>2</sub>O<sub>2</sub> generation in the cellular environment obligates  
43  
44 this enzyme to work in conjunction with enzymes that eliminate H<sub>2</sub>O<sub>2</sub> e.g. catalase or  
45  
46 glutathione peroxidase.  
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54 A contradictory scenario was observed with the active metabolite of the vasodilator  
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56 drug molsidomine (SIN-1 or 3-morpholinopyridone) which mediates DNA  
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58 damage via formation of peroxynitrite. It was found that catalase abolished its toxic  
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4 effect [41]. However, manganese-SOD and copper-zinc-SOD were cytotoxic. The  
5  
6 latter displayed a biphasic dose response being more pronounced at concentrations  
7  
8 ranging from 10-100 IU/ml. Moreover, SOD did not prevent DNA damage generated  
9  
10 by SIN-1 and two other nitric oxide donors S-nitrosoglutathione (GSNO) and  
11  
12 Roussin's black salt (RBS) in the Comet assay where catalase abolished DNA damage  
13  
14 [42]. Catalase combined with SOD showed no effect on responses greater than  
15  
16 catalase on its own. More recently, an increase in survival has been reported when  
17  
18 SIN-1 was incubated with SOD plus catalase in TK6 cells [43]. SOD and catalase,  
19  
20 individually, were ineffective in reducing lethality. Thus, it seems that the  
21  
22 functionality of SOD is highly dependent on the sensitivity to the agent causing  
23  
24 genotoxicity, which could be linked to the fact that the different types of SOD differ  
25  
26 in their metal binding ability and their distribution in cell compartments [44]. Clear  
27  
28 protective outcomes by SOD have been found against the antineoplastic drugs  
29  
30 (metronidazole and dimetridazole) [45], the carcinogenic aromatic amine benzidine  
31  
32 [46] and hyperbaric oxygen (HBO) [47].

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38 Overall, the importance of SOD in the body is undisputable. For instance, the SOD  
39  
40 malfunction in the body is a common denominator in a variety of degenerative  
41  
42 processes, diseases and syndromes namely atherosclerosis, myocardial infarction,  
43  
44 Parkinson's disease, Alzheimer's dementia amongst others [48].

### 45 46 47 48 49 **Glutathione peroxidase and selenium**

50  
51  
52 Glutathione peroxidase (GPx) is widely distributed in animal and human tissues. GPx  
53  
54 levels are high in the kidney, liver and whole blood, moderate in lens and erythrocytes  
55  
56 and low in alveolar lining plasma and blood plasma [31]. Its electron donor, the  
57  
58 reduced form of glutathione (GSH), is found at intracellular concentrations that are  
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4 often in the millimolar range [28]. GPx is considered the major peroxide removing  
5  
6 enzyme found in human tissue, being highly specific for GSH but low for H<sub>2</sub>O<sub>2</sub> [49].  
7  
8 GPx reacts with H<sub>2</sub>O<sub>2</sub> as well as peroxides. Selenium, found to be a component of  
9  
10 GPx [50], maintains GPx activity. Thus, selenium-GPx plays an important metabolic  
11  
12 regulatory role. The deficiency of selenium intake is clearly demonstrated by its low  
13  
14 levels in plasma which may be associated with certain forms of heart disease [51,52].  
15  
16 In a recent meta-analysis, selenium supplementation was observed to be associated  
17  
18 with reduced cancer incidence in men but not in women [53]. The literature agrees on  
19  
20 the protective effect of selenium evaluated with the Comet assay towards a variety of  
21  
22 chemical or physical toxic agents. *In vitro* investigations found that selenium impeded  
23  
24 DNA damage from H<sub>2</sub>O<sub>2</sub> in murine lymphoma cells [54], sodium selenate prevented  
25  
26 UVA-mediated DNA damage in human skin fibroblasts [55], sodium selenite and  
27  
28 selenomethionine protected keratinocytes from UV-induced oxidative damage [56]  
29  
30 and sodium selenate quenched potassium dichromate in human lymphocytes [57].  
31  
32 Such preventive effects have also been corroborated in supplementation studies with  
33  
34 selenium compounds in the Comet assay. Beagle dogs with prostatic cancer  
35  
36 supplemented with selenomethionine or high-selenium yeast lowered the basal DNA  
37  
38 damage when compared to a non-supplemented group [58]. Mice supplemented with  
39  
40 κ-selenocarrageenan (0.25 µg/ml) showed protective effects against lead acetate in  
41  
42 mice [59]. Selenium has also been suggested to be a DNA repair promoter [60,61].  
43  
44 Seo and collaborators confirmed by means of the Comet assay that selenomethionine  
45  
46 induces DNA repair in normal human fibroblasts *in vitro* after a challenge with UV-  
47  
48 radiation [62]. Nevertheless, it remains inconclusive which is/are the most suitable  
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50 selenium compound/s to prevent DNA damage.  
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4 Prooxidant responses of selenium compounds have also been reported. We observed  
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6 DNA damage induced by sodium selenate, sodium selenite and selenous acid on their  
7  
8 own on human lymphocytes [57]. Results obtained with the Comet assay allowed the  
9  
10 conclusion that selenite induced oxidative stress and apoptosis and these effects were  
11  
12 significantly attenuated by SOD, catalase and deferoxamine [63].  
13  
14

### 17 **Iron chelators**

18  
19 The regulation of iron and copper in the body plays a key role as a control in the  
20  
21 generation of ROS. During evolution, humans have developed a complex system of  
22  
23 transport and storage proteins to ensure that these metals are rarely allowed to roam  
24  
25 free, hence being harmful [64]. Iron promotes the formation of the highly reactive  
26  
27 HO<sup>•</sup> leading to free radical DNA damage. In order to avoid this, the iron entering the  
28  
29 circulation in the intestine is bound to transferrin, which also accepts iron released in  
30  
31 hemolysis in the spleen, with an iron turn over of about 10 times/day [31]. Most  
32  
33 intracellular iron is stored complexed with the ubiquitous protein ferritin which acts as  
34  
35 a natural iron chelator. There must be a regulation of the cellular iron balance which  
36  
37 involves iron-regulatory proteins being triggered by special base sequences in the  
38  
39 mRNAs of ferritin and transferrin receptor proteins [65]. The importance of such a  
40  
41 mechanism of defence against ROS has been observed by exposing lymphocytes to  
42  
43 HBO which induces DNA damage [66]. However, it also leads to an adaptive  
44  
45 response. HBO-exposed lymphocytes showed a small but reproducible increase in  
46  
47 cellular ferritin levels, which might indicate that the underlying protective mechanism  
48  
49 is based on an induction of ferritin, which may act in an antioxidant manner [67].  
50  
51 Blood transfusions are required in thalassaemia patients but if carried out without the  
52  
53 presence of iron chelators they might develop into iron overload pathology. The  
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4 effectiveness of two exogenous chelating agents (deferoxamine and deferiprone) was  
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6 evaluated in the Comet assay in healthy and thalassaemia patients whose blood was  
7  
8 treated *in vitro* with several different iron compounds, thus, simulating a post-  
9  
10 transfusional iron overload. The iron compounds (ferric chloride, ferrous chloride and  
11  
12 ferrous sulphate) generated DNA damage in healthy subjects and patients but not  
13  
14 hemosiderine. Deferoxamine and deferiprone showed a reduction of DNA damage  
15  
16 depending on the iron compound used. However, for a thalassaemia patient both  
17  
18 chelating agents prevented DNA damage induced by all iron compounds [68,69].  
19  
20

21  
22 A tool not to be neglected when investigating the effects of antioxidants is the  
23  
24 intervention study. It has to be borne in mind that normal human plasma contains  
25  
26 numerous high and low molecular mass active redox components that are able to react  
27  
28 rapidly with organic and inorganic oxygen radicals, which has led to their  
29  
30 classification as important biological antioxidants [70]. Such molecules are  
31  
32 caeruloplasmin, albumin (the protein itself and possibly also albumin-bound  
33  
34 bilirubin), transferrin, haptoglobin, and hemopexin [71]. The physiological parameters  
35  
36 obtained for these plasma proteins might be necessary in order to interpret the results.  
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## 42 **Melatonin**

43  
44 Melatonin is a hormone which varies in the diurnal cycle. It is characterised as a  
45  
46 powerful antioxidant which, unlike others, does not undergo redox cycling [72]. A  
47  
48 single melatonin molecule is reported to scavenge up to 10 ROS/RNS being  
49  
50 documented that the free radical scavenging capacity of melatonin extends to its  
51  
52 secondary, tertiary and quaternary metabolites [73] and it is likely to be carried out by  
53  
54 the donation of a hydrogen of its –NH group [31]. Furthermore, melatonin has been  
55  
56 shown to regulate on the one hand antioxidant enzyme activity and on the other  
57  
58 cellular mRNA levels for glutathione peroxidase, superoxide dismutases and catalase  
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4 both under physiological and under conditions of elevated oxidative stress [74]. Its  
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6 precursor, serotonin, is reported to be a lipid peroxidation inhibitor [75].  
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8  
9 In the Comet assay, it has demonstrated clear antioxidant activity against ROS  
10  
11 generators like the anticancer drugs adriamycin in rats [76], idarubicin in  
12  
13 lymphocytes and cultured cancer cells [77] or the penicillin derivative amoxicillin  
14  
15 [78]. Melatonin has also reduced the effects of  $\gamma$ -radiation in human blood exposed *in*  
16  
17 *vitro* [79]. The *in vivo* Comet assay exhibited the protective effects of melatonin on  
18  
19 rat brain cells against ionizing radiation [80] and magnetic fields (50 Hz) on rat  
20  
21 lymphocytes [81]. Basal DNA damage in patients diagnosed with Graves' disease  
22  
23 diminished when lymphocytes were exposed to melatonin (100  $\mu$ M, 4h) *in vitro* [82].  
24  
25 Conversely, melatonin on its own (0.1-1 mM) has also been shown to generate a  
26  
27 slight increase in DNA damage in mammalian cells *in vitro*, a slight reduction when  
28  
29 co-incubated with H<sub>2</sub>O<sub>2</sub> and no effect with bleomycin [83]. Clastogenic effects have  
30  
31 been corroborated on its own at a concentration of 100  $\mu$ M [84].  
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### 38 **Melanin**

39  
40 Melanin is a pigment mainly found in skin and hair. Epidemiological data suggests  
41  
42 that the incidence of solar radiation-related skin cancer is higher in individuals with  
43  
44 genetically determined poor ability of the skin to tan and low pigmentation [85]. In  
45  
46 cultured melanocytes, melanin offered protection against induction of major DNA  
47  
48 lesions by UVB [86]. It has been postulated that the ability of melanin to quench  
49  
50 excited states of photosensitising dye molecules and singlet O<sub>2</sub>, and scavenge reactive  
51  
52 radicals is an important factor in the protective action of melanin against oxidative  
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54 damage and this could take place by the interaction of melanin with oxidising and  
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56 reducing radicals by the hydroquinone and quinone nature of its subunits, which can  
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4 act as efficient electron donors and acceptors, respectively [87]. The same authors  
5  
6 also state that whether or not a relatively low influx of ROS expected in melanotic  
7  
8 systems under typical irradiation conditions will have significant biological impact  
9  
10 depends predominately on the melanotic system and more particularly its antioxidant  
11  
12 capacity and susceptibility to oxidative damage [87].  
13  
14

15 It has been investigated in the Comet assay in order to evaluate the extent of its  
16  
17 protective effects against UV. Recent investigations conclude that melanin protects  
18  
19 against UVA-DNA damage [88] and acts as a radioprotectant when cysteine and GSH  
20  
21 are depleted [89]. Furthermore, depending on the concentration of carotenoid  
22  
23 mixtures, protection against melanogenic intermediates and/or exogenous DNA  
24  
25 damage can be achieved *in vitro* [90]. By contrast, earlier studies showed that melanin  
26  
27 [91] and 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [92], a melanin precursor,  
28  
29 increased DNA damage in the presence of UVA.  
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## 37 **THE COMET ASSAY ON DIETARY ANTIOXIDANTS**

### 38 **Vitamins**

39  
40  
41 The antioxidant status of blood plasma is directly linked to diet and is defined by  
42  
43 components such as vitamins and flavonoids. It is postulated a role of ascorbic acid in  
44  
45 the prevention of cancer, heart disease and the augmentation of immune function such  
46  
47 as in the prevention of cold [93-95]. *In vivo*, vitamin C is able to quench ROS  
48  
49 efficiently; thus, reducing DNA damage to protooncogenes and tumour suppressor  
50  
51 genes which might explain its anticancer properties [96]. Within the cell, vitamin C is  
52  
53 also utilised as an electron donor as part of the interaction between iron and ferritin  
54  
55 [97]. Outside the cells, vitamin C acts in conjunction with vitamin E, present in lipid  
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4 membranes, to quench free radicals and prevent lipid peroxidation [98]. In this  
5  
6 manner, vitamin C may assist in preventing the oxidation of low density lipoprotein,  
7  
8 which is thought to be a major initiating event in atherosclerosis [99]. In the Comet  
9  
10 assay, vitamin C, 2-4 h after intake, has been observed to provide significant  
11  
12 protection to the DNA of isolated lymphocytes when challenged with H<sub>2</sub>O<sub>2</sub> [100].  
13  
14 Further evidence of protection was seen against the effects of H<sub>2</sub>O<sub>2</sub> when vitamin C  
15  
16 was present at low concentrations (up to 1 mM); by contrast, there was exacerbation  
17  
18 at higher doses (5 mM) [15,23,101]. Results were inconclusive when oestrogenic  
19  
20 compounds were co-incubated with vitamin C (0.5 and 1 mM) in isolated  
21  
22 lymphocytes showing no common pattern in the responses [20]. There was no  
23  
24 evidence of a protective effect of vitamin C against the damage caused by bleomycin  
25  
26 [23]. In sperm, for instance, addition of vitamin C to preparations did not affect  
27  
28 baseline DNA integrity but did provide sperm with complete protection against H<sub>2</sub>O<sub>2</sub>-  
29  
30 induced DNA damage [102]. In intervention studies, supplementation of 100 mg/day  
31  
32 to 50-59 year-old men led to a decrease in oxidative base damage to non/smokers and  
33  
34 enhanced resistance against oxidative damage [17]. The antioxidant effect of vitamin  
35  
36 C in a long-term study has been observed, measuring oxidative DNA damage and  
37  
38 DNA repair in blood cells with the Comet assay. Male smokers were given vitamin C  
39  
40 (2 x 250 mg) daily in the form of plain or slow release tablets combined with plain  
41  
42 release vitamin E (2 x 91 mg), or placebo for 4 weeks. The outcome of this study  
43  
44 suggested that long-term vitamin C supplementation at a high dose, i.e. 500 mg,  
45  
46 together with vitamin E in moderate dose, i.e. 182 mg, decreased the steady-state  
47  
48 level of oxidative DNA damage in lymphocytes of smokers [103].  
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56 Therefore, the fat-soluble vitamin E ( $\alpha$ -tocopherol) also plays a role in preventing free  
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58 radical damage by disrupting the chain reaction of lipid peroxidation [104]. Vitamin E  
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4 works in conjunction with vitamin C since the latter is able to regenerate  $\alpha$ -tocopherol  
5  
6 from the tocopherol radical formed by the reaction with ROS. Its ability to trap  
7  
8 peroxy radicals and singlet  $O_2$  has also been stated [105,106]. In recent years, vitamin  
9  
10 E has been utilised *in vitro* to protect from genotoxicants and this has been evaluated  
11  
12 with the Comet assay. For instance, it conferred protective effects against complex  
13  
14 mixtures of organic compounds adsorbed onto ambient air particles [107], bleomycin  
15  
16 [108], benzopyrene [24], acrylamide [109], antibiotics like ciproflaxin [110] or  
17  
18 streptozotocin [111] and anaesthetic gases [112]. Moreover, Trolox (a vitamin E  
19  
20 analogue) displayed a protective effect against  $H_2O_2$  in a modified Comet assay with  
21  
22 buccal cells [113].  
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26 Vitamin A as well as retinol, its equivalent in animals, are important antioxidants.  $\beta$ -  
27  
28 carotene, can either be converted in the intestinal mucosa to two identical molecules  
29  
30 of retinal, or vitamin A, depending on the presence of the enzyme  $\beta$ -carotene 15,15'-  
31  
32 dioxygenase [114]. However, most carotenoids are not able to generate vitamin A.  
33  
34 The full range of antioxidant activities of vitamin A remains incomplete. Moreover,  
35  
36 both, harmful and quenching effects have been observed in the Comet assay. Vitamin  
37  
38 A is capable of reducing the genotoxic effects of N-nitrosomorpholine in human  
39  
40 hepatoma cells HepG2 [115]. It decreased the extent of DNA damage evoked by the  
41  
42 anticarcinogenic compound imanitib [116] and, when administered at high doses  
43  
44 (50000 IU/ml), prevented DNA damage in rats treated with the hepatocarcinogen p-  
45  
46 dimethylaminoazobenzene [117]. Conversely, retinol supplementation (7  $\mu$ M, 24 h)  
47  
48 induced DNA breaks in V79 cells *in vitro* [118].  
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52

53 Vitamin B belongs to a family of chemically distinct vitamins that play important  
54  
55 roles in cell metabolism. Vitamin B<sub>1</sub> (thiamine) possesses antioxidant properties that  
56  
57 protect against the damaging effects of alcohol, smoking and ageing [119]. One  
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4 investigation showed that after an intervention of low and middle-dose vitamin C and  
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6 thiamine in mice added concomitantly with lead acetate, DNA damage measured in  
7  
8 the Comet assay was lower than that of groups exposed only to lead acetate [120].  
9  
10 Elevated vitamin B<sub>2</sub> (riboflavin) levels have been reported to provide protection  
11  
12 against damage caused by oxidative injury [121]. One study evaluated the antioxidant  
13  
14 capability of riboflavin in the Comet assay, which was demonstrated to exert a  
15  
16 protective effect against argemone oil/sanguinarine in mice [122]. In contrast, vitamin  
17  
18 B<sub>3</sub> (niacin) does not protect DNA integrity from genotoxicants. In fact, it inhibits  
19  
20 DNA repair and this has been confirmed in the Comet assay [123-125]. Although  
21  
22 some vitamins can not be classified as antioxidants, their roles as dietary factors must  
23  
24 be highlighted. For instance, Vitamin B<sub>9</sub> (folic acid), vitamin B<sub>12</sub> (cobalamin) and  
25  
26 zinc play key roles in DNA metabolism and repair [126]. Folic acid is essential in the  
27  
28 synthesis of purine nucleotides and pyrimidine nucleoside thymidine [127]. Thus, its  
29  
30 presence at precise levels is indispensable to maintain DNA stability when ROS or  
31  
32 other genotoxic compounds reach the DNA.  
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38 Coenzyme Q10 (ubiquinone, ubidecarenone, or CoQ<sub>10</sub>) is a benzoquinone and it is  
39  
40 involved in the production of energy in the mitochondria. Coenzyme Q10 is the only  
41  
42 lipid soluble antioxidant synthesised endogenously and inhibits, overall, lipid but also  
43  
44 protein and DNA oxidation when it is in its reduced form (ubiquinol) [128]. Its  
45  
46 antioxidant features have been confirmed *in vitro* where its supplementation enhances  
47  
48 DNA resistance towards H<sub>2</sub>O<sub>2</sub>-induced oxidation but does not inhibit DNA strand  
49  
50 breaks formation as observed in the Comet assay [129]. The formation of 8-  
51  
52 hydroxydeoxy-guanosine (8-OHdG), a marker of oxidative stress, has been analysed  
53  
54 by the Comet assay after coenzyme Q10 (3 mg/kg/day) supplementation to human  
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56 probands [130]. During supplementation, delayed generation of 8-OHdG in  
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4 lymphocyte DNA was observed. This effect was long-lasting and could be observed  
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6 even 12 weeks after supplementation stopped.  
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## 10 **Carotenes**

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12 Carotene is a terpene which can be found in two primary forms:  $\alpha$ -carotene and  $\beta$ -  
13 carotene.  $\beta$ -Carotene is the most common form and can be found in yellow, orange  
14 and green leafy fruit and vegetables. Carotenoids are the most potent biological  
15 quenchers of singlet  $O_2$  [131]. Carotenoids interact with singlet  $O_2$  either via a  
16 physical quenching mechanism, in which the excited energy from singlet  $O_2$  is  
17 transferred to the carotenoid and then dissipated to the surroundings as heat, or  
18 chemical quenching, in which the carotenoid is destroyed in the process by addition of  
19  $O_2$  to its double bond system [132]. The action of carotenoids as chain-breaking  
20 antioxidants has also been investigated. The reaction of  $\beta$ -carotene with a lipid radical  
21 results in the formation of a carbon-centred- $\beta$ -carotene radical intermediate. This  
22 intermediate structure has two possible fates; it can act as a prooxidant by reacting  
23 with  $O_2$  or it can react with another lipid radical to form stable products [133]. Despite  
24 being listed as an antioxidant [134], its outcomes are often confounding when  
25 investigated. In cancer prevention, the usefulness of  $\beta$ -carotene can not be confirmed,  
26 as harmful effects have been observed in clinical trials [135,136]. Some, but not all, *in*  
27 *vitro* investigations display its ability to inhibit DNA damage generated by ROS when  
28 evaluated by the Comet assay. For instance, carotenes protected DNA integrity  
29 against ROS generated through catechol-oestrogens [137].  $\beta$ -Carotene also quenched  
30  $O_2^-$  generated by xanthine plus xanthine oxidase when its concentrations ranged from  
31 1-3  $\mu$ M but not at higher concentrations [138].  $\beta$ -Carotene (0.5-1  $\mu$ M) prevented  
32 DNA damage against  $H_2O_2$  at low concentrations but ambiguous results were found at  
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4 higher concentrations [139]. Conflicting conclusions can be found in the literature in  
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6 *ex vivo* studies. Lymphocytes challenged with H<sub>2</sub>O<sub>2</sub> after supplementation with β-  
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8 carotene exacerbated DNA damage [139]. By contrast, men aged 50-59 years old  
9  
10 supplemented with vitamin C, E and β-carotene increased resistance to DNA damage  
11  
12 when lymphocytes were incubated *ex vivo* with H<sub>2</sub>O<sub>2</sub> [17]. When the Comet assay  
13  
14 was utilised to evaluate DNA repair capability, promotion of repair by vitamin C, E  
15  
16 and β-carotene after radiation-induced DNA damage in mouse leukocytes *in vitro* was  
17  
18 observed [140]. Likewise, rejoining of breaks in the first few hours appeared  
19  
20 substantially fast in lymphocytes following supplementation with β-carotene although  
21  
22 the authors concluded that while certain carotenoids appear to enhance recovery from  
23  
24 oxidative damage, this was most likely a protective antioxidant effect against  
25  
26 additional damage induced by atmospheric O<sub>2</sub>, rather than a stimulation of DNA  
27  
28 repair [141].  
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33 Lycopene, a bright red carotenoid pigment, is found in red fruits. Because of the  
34  
35 unsaturated nature of lycopene it is considered to be a potent antioxidant and a O<sub>2</sub><sup>·-</sup>  
36  
37 quencher [142]. Among all carotenes, it is considered the most efficient at quenching  
38  
39 singlet O<sub>2</sub> [143]. The daily intake of a beverage prototype (Lyc-o-Mato(R)) containing  
40  
41 a natural tomato extract was able to modify plasma and lymphocyte carotenoid  
42  
43 concentrations. It also significantly reduced DNA damage in lymphocytes subjected  
44  
45 to oxidative stress [144].  
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50 Lutein, zeaxanthin and astaxanthin are also antioxidants and may act as a filter to  
51  
52 protect the macula from potentially damaging forms of ultraviolet light. Observational  
53  
54 data suggest that high dietary intake of macular xanthophylls (lutein and zeaxanthin)  
55  
56 are associated with a lower risk of advanced age-related macular degeneration [145].  
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58 Hence, the antioxidant capacity of the macula carotene-like components has been  
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4 investigated in the Comet assay on human neuroblastoma and rat trachea epithelial  
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6 cells as models. Data obtained after challenge with UVA and radical nitric species  
7  
8 (RNS) generators show that the ability of zeaxanthin, lutein and astaxanthin to reduce  
9  
10 the DNA damage depends on the type of RNS donor, the carotenoid concentration  
11  
12 and the cell model used [146,147].  
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## 16 17 18 **Flavonoids**

19  
20 *In vitro* and animal studies have shown that flavonoids possess anti-inflammatory,  
21  
22 antioxidant, antiallergenic, hepato-protective, antithrombotic, antiviral and  
23  
24 anticarcinogenic activities [148]. In addition, flavonoids have been identified as  
25  
26 fulfilling most of the criteria to be considered as antioxidants: the flavonoids inhibit  
27  
28 the enzymes responsible for  $O_2^{\cdot\cdot}$  production [149,150]; the low redox potentials of  
29  
30 flavonoids thermodynamically allow them to reduce highly oxidising free radicals  
31  
32 such as  $O_2^{\cdot\cdot}$ ,  $RO^{\cdot}$  and  $HO^{\cdot}$  [151]; and a number of flavonoids chelate trace metals  
33  
34 [152]. Besides scavenging, flavonoids may stabilise free radicals by complexing with  
35  
36 them [153]. There is clear evidence that radical scavenging activity depends on the  
37  
38 structure of the flavonoids and the substituents of the heterocyclic rings [154]. This  
39  
40 might explain why ROS-quenching efficiency differs amongst different flavonoids.  
41  
42 Quercetin, the most abundant flavonoid in the human diet [155], was recently  
43  
44 investigated against the formation of oxidative DNA damage both *in vitro* and *ex vivo*  
45  
46 in the Comet assay. A significant dose-dependent protection by quercetin against the  
47  
48 formation of oxidative DNA damage generated by  $H_2O_2$  was observed *in vitro*. *In vivo*,  
49  
50 four weeks of quercetin-rich fruit juice intervention led to a significant increase in the  
51  
52 total antioxidant capacity of plasma. After intervention, the level of oxidative damage  
53  
54 upon *ex vivo* exposure to  $H_2O_2$  decreased, although not significantly [156]. Human  
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4 lymphocytes were pre-treated with a variety of flavonoids on their own, then  
5  
6 challenged with H<sub>2</sub>O<sub>2</sub> and, finally, measured with the Comet assay [157]. All  
7  
8 flavonoids protected against oxidative DNA damage, moreover, it was concluded that  
9  
10 free flavonoids (quercetin, luteolin, myricetin and kaempferol) displayed greater  
11  
12 protection than conjugated (quercetin-3-glucoside, quercitrin and rutin). Protection  
13  
14 against H<sub>2</sub>O<sub>2</sub> was confirmed for myricetin, quercetin and rutin in Caco-2 and HepG2  
15  
16 cells [158] and for quercetin and luteolin in murine and human leukaemia cell lines  
17  
18 [159,160]. With regard to other genotoxicants, quercetin and rutin displayed  
19  
20 antigenotoxic effects on DNA damage induced by mitomycin C, in a concentration-  
21  
22 dependent manner [161]. Additionally, rutin, quercetin and naringin protected against  
23  
24 the genotoxicity of UVA on mouse fibroblasts [162]. DNA damage in human  
25  
26 lymphocytes and sperm after treatment with four oestrogen-like compounds ( $\beta$ -  
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28 oestradiol, DES, daidzein, and genistein) and its modulation by flavonoids (quercetin  
29  
30 and kaempferol) was examined using the Comet assay. Quercetin and kaempferol  
31  
32 reduced the DNA damage produced in sperm and lymphocytes by the four  
33  
34 oestrogenic compounds and by H<sub>2</sub>O<sub>2</sub> (positive control) [163]. Flavonoids (silymarin,  
35  
36 miryctin, quercetin, kaempferol, rutin and kaempferol-3-rutinoside) were selected in  
37  
38 order to counteract the effects of food mutagens in lymphocytes and sperm measured  
39  
40 with the Comet assay. There were slightly different profiles in lymphocytes and  
41  
42 sperm, but antigenotoxic effects were observed over a similar dose range (50-550  $\mu$ M)  
43  
44 [164,165]. The previous investigations also showed that flavonoids can themselves  
45  
46 be genotoxic and can act in a prooxidant/antioxidant way over different dose ranges.  
47  
48 Prooxidant effects were observed at around 100  $\mu$ M whereas antioxidant effects  
49  
50 occurred at 500  $\mu$ M. Nevertheless, it has to be taken into account that flavonoids  
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52 preventing DNA damage in particular experimental designs might not be efficient in  
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4 other investigations. Hence, reports can be found in the literature where flavonoids  
5  
6 failed to protect against oxidative stress [155,159,160].  
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### 10 **Isoflavones**

11  
12 Isoflavones are found in soy products and they are closely related to flavonoids.  
13  
14 Isoflavones are known to interact with animal and human oestrogen receptors causing  
15  
16 effects in the body similar to those induced by the hormone oestrogen [166].  
17  
18 Therapeutic effects in cancer [167,168] and cardiovascular diseases [169] have been  
19  
20 described. The most studied isoflavones are daidzein, equol and genistein. The  
21  
22 mechanism of action of isoflavones is complex and includes several cellular pathways.  
23  
24 For instance, genistein has been reported to inhibit steroidogenesis, block several  
25  
26 protein tyrosine kinases, arrest the cell cycle, induce apoptosis and has antiangiogenic  
27  
28 and antimetastatic properties [170,171]. Their antioxidant properties have been  
29  
30 confirmed in the Comet assay. Twenty-four hours supplementation with daidzein and  
31  
32 genestein in Jurkat T-cells (2.5 to 20  $\mu\text{M}$ ) and in peripheral blood lymphocytes of  
33  
34 healthy subjects (0.01 to 2.5  $\mu\text{M}$ ) displayed a significantly increased DNA protection  
35  
36 from  $\text{H}_2\text{O}_2$  in both cell types in the Comet assay. Both, daidzein and genestein were  
37  
38 equally protective. In addition, since the protective effect was found at concentrations  
39  
40 attainable in plasma after soy consumption (less than 2  $\mu\text{M}$ ), it can be assumed that  
41  
42 the antioxidant activity of isoflavones contributes to the healthy properties of soy  
43  
44 [172]. The role for isoflavones in the prevention of male infertility was explored *in*  
45  
46 *vitro* in the Comet assay [173]. Pre-treatment with genistein or equol (a non-steroidal  
47  
48 oestrogen metabolised from daidzein) at doses of 0.01-100  $\mu\text{M}$  significantly protected  
49  
50 sperm DNA integrity after  $\text{H}_2\text{O}_2$ -mediated damage. Addition of genistein and equol in  
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52 combination was more protective than administered on their own. Therefore, the  
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4 previously described investigations open the possibility of combined effects of  
5  
6 flavonoids, which should be considered when designing studies and interpreting data.  
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8 In an ample review on the genotoxicity of phytoestrogens, Stopper and colleagues  
9  
10 report that genistein (10-400  $\mu\text{M}$ ) and daidzein (100-400  $\mu\text{M}$ ) were found to exert  
11  
12 genotoxic effects *in vitro* when assessed with the Comet assay [174]. By contrast, it is  
13  
14 also stated that most published *in vitro* studies exhibited only weak or no effects for  
15  
16 daidzein.  
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### 21 22 **Tea polyphenols**

23  
24 Green tea has attained a high reputation as a health-promoting dietary component  
25  
26 ascribed to the antioxidant activity of epigallocatechin gallate (EGCG) [175]. EGCG  
27  
28 has been demonstrated to act protectively in human cells against bleomycin [176], UV  
29  
30 light [177],  $\text{H}_2\text{O}_2$  and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [178]. The  
31  
32 protective effects of EGCG are likely to be due to a combination of several different  
33  
34 mechanisms, including modulation of expression of antioxidative systems, direct  
35  
36 scavenge of free radicals and promotion of DNA repair [176]. In addition to EGCG,  
37  
38 other tea polyphenols such as epicatechin (EC); epicatechin gallate (ECG);  
39  
40 epigallocatechin (EGC); and theaflavins (THFs) were studied in the Comet assay on  
41  
42 benzopyrene-induced DNA damage in Chang liver cells [179]. EC and ECG impeded  
43  
44 DNA damage at concentrations ranging from 10 to 100  $\mu\text{M}$ , whereas EGC, EGCG  
45  
46 and theaflavines inhibited DNA damage at concentrations ranging from 10 to 50  $\mu\text{M}$ .  
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49 Current evidence is growing that tea constituents can be cell damaging and prooxidant  
50  
51 themselves as proved by the enhanced genotoxicity of EGCG [175]. This was further  
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53 corroborated for EGCG and, additionally, for EGC and theaflavins [179].  
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## Wine polyphenols

Resveratrol is found in the skin of red grapes and as a constituent of red wine. The scope of biological interactions by resveratrol is extensive and has been listed in a recent review [180]. Resveratrol has been shown to bind to numerous cell-signaling molecules such as multidrug resistance protein, topoisomerase II, aromatase, DNA polymerase, oestrogen receptors, tubulin and F1-ATPase. It activates various transcription factors, suppresses the expression of antiapoptotic gene products, inhibits protein kinases, induces antioxidant enzymes, suppresses the expression of inflammatory biomarkers, inhibits the expression of angiogenic and metastatic gene products, and modulates cell cycle regulatory genes. Moreover, numerous studies have demonstrated that this polyphenol holds promise against cancer [181]. There is growing evidence that resveratrol can prevent or delay the onset of heart diseases, ischemic and chemically induced injuries, pathological inflammation and viral infections [182]. It has been suggested to be a chemopreventive by virtue of its ability to protect DNA as well as to induce DNA repair [183]. It has also been proposed to have beneficial effects in brain pathologies mediated by oxidative stress [184]. For instance, it has the capability to quench the effects of H<sub>2</sub>O<sub>2</sub> to a certain extent in glioma cells although resveratrol *per se* induced a slight time and dose-dependent DNA damage. Using H<sub>2</sub>O<sub>2</sub> in peripheral blood lymphocytes as a model of oxidative stress, DNA damage was not observed in the presence of resveratrol [185,186] as well as in the presence of coumarin, curcumin and vanillin [185]. Resveratrol was also efficient at quenching the chemotherapy drugs cisplatin and selenium-cisplatin [187] and the alkylating carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) [183]. By means of the Comet assay it has been shown that the anticancer mechanism of

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4 plant polyphenols, such as resveratrol, might be due to the prooxidant action induced  
5  
6 by the mobilisation of endogenous copper [188,189].  
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### 10 **Synthetic antioxidants**

11  
12 Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are chain-  
13 breaking antioxidant food additives used for the prevention of food spoilage. BHA  
14 and BHT block lipid peroxidation which can eventually lead to DNA damage. BHA  
15 and BHT have markedly contradictory effects which might be due to their phenolic  
16 nature. Precisely, its structure has been addressed as the reason why they exert  
17 anticarcinogenic effects by its ability to intercept free radicals [190]. BHA was  
18 utilised to counteract the detrimental effect of iodoacetic acid, a highly genotoxic  
19 disinfection by-product of drinking water, and showed a reduction in DNA damage  
20 measured with the Comet assay [34]. By contrast, it showed no reduction when co-  
21 incubated with MNNG [191] but significantly reduced the level of  
22 formamidopyrimidine-DNA-glycosylase plus endonucleaseIII-sensitive sites, which  
23 at least partially are caused by oxidative DNA lesions [192]. The genotoxic  
24 compound benzidine was examined for DNA damage in human lymphocytes using  
25 the alkaline Comet assay [46]. Its toxicity was highly decreased when co-treated with  
26 BHT. Other synthetic antioxidants used in animal food like salts of ethoxyquin have  
27 been screened in the Comet assay and genotoxic effects have been observed for the  
28 salts although lower than that of ethoxyquin [193]. On the other hand, ethoxyquin  
29 salts, similarly to ethoxyquin, effectively protected the cells from the oxidative effect  
30 of H<sub>2</sub>O<sub>2</sub>. Complexes of the antioxidant ethoxyquin with rutin or quercetin were  
31 studied in human lymphocytes [194]. Such complexes decreased the level of DNA  
32 damage induced by H<sub>2</sub>O<sub>2</sub> on its own.  
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## WHAT MAKES THE COMET ASSAY SUITABLE FOR RESEARCH ON ANTIOXIDANTS?

Tice and colleagues suggested several advantages of the *in vitro* Comet assay compared to other genotoxicity assays [195]: it has demonstrated sensitivity for detecting low levels of DNA damage; there is a requirement for small number of cells per sample; it has flexibility; there is a low cost and ease of application; studies can be conducted using relatively small amounts of a test substance; and a relatively short time is needed to complete an experiment. The Comet assay is commonly used for the investigation of the effects of antioxidants in intervention studies. The Comet assay can be virtually applied on any cell type, as long as a single cell suspension is obtained. While human lymphocytes remain the most popular cell type for monitoring purposes, sperm, buccal, nasal, epithelial and placental cells are also used [196]. The authors consider that it is mainly two lines of development of the Comet assay that will consolidate this assay as a very valuable tool in the research on antioxidants *in vitro*, *in vivo* and *ex vivo*. 1) Much effort is currently addressed to establish the Comet assay as a relatively high throughput technique [197]. A large number of antioxidant compounds e.g. carotenoids (over 600) [31], flavonoids or antioxidants from natural or synthetic origin already exist and the number of reports identifying new potential antioxidant compounds grows rapidly. Thus, there is the need to evaluate them quickly and reliably. 2) An avenue of investigation focuses on the link between antioxidants (and micronutrients) and DNA repair, this being an indirect mechanism to confront oxidative stress. The Comet assay can easily measure DNA repair capacity in human volunteers and, in turn, this can be used as a biomarker to evaluate the effects of dietary supplements [198].

## CONSIDERATIONS OF THE USE OF COMET ASSAY FOR RESEARCH ON ANTIOXIDANTS

The Comet assay has a range of detection limited by the structural organisation of the DNA; it is saturated when all the DNA loops are relaxed [199]. Despite its precision not being as great as chromatographic methods, it seems to be more accurate at estimating low levels of damage, this being due to the adventitious oxidation occurring during sample preparation in such methods [199,200]. Two considerations have to be taken into account before interpreting results obtained *in vitro* [23]. Firstly, the reactions involved in oxidation and reduction are exquisitely sensitive to the chemical milieu in which they occur. It is not surprising, therefore, that contradictory results with a single putative antioxidant can be obtained using *in vitro* systems based on the use of widely different media. Secondly, the choice of the agent with which to induce oxidative damage and against which the efficacy of an antioxidant can be measured, could display very diverse outcomes.

For interpretation of responses *in vivo*, the issue of confounding factors must be thoroughly addressed. There are endogenous confounding factors, such as age, gender, and genetic make-up as well as exogenous factors, including lifestyle habits (smoking, drinking, diet, vitamin supplementation, medication, type and duration of employment, stress, etc.). The correlation between biomarkers and the various factors which affect them is complex. Sometimes the variables are not completely independent of each other [196]. It also has to be borne in mind that dietary antioxidant levels in the blood are dependent on the intake of fruit and vegetables and therefore might be expected to show seasonal variation which varies for each fruit and vegetable [201]. Some authors recommend future studies to be carried out with a placebo-controlled, parallel design

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4 rather than a crossover design, based on the fact that participation in an antioxidant  
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6 intervention study may cause changes in dietary habits [202].  
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9 A glance into Comet assay literature renders many parameters by which data are  
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11 published, namely, % DNA in Tail, % DNA in Head, Olive tail moment, Tail extent  
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13 moment, arbitrary units.... In addition, there is a variety of Comet software available,  
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15 each one with its own specialities. Moreover, the staining method used in each  
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17 laboratory has to be considered. These aspects eventually lead to the issue of how  
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19 comparable are results. It is suggested that judicious selection of different parameters,  
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21 staining methods along with inter-laboratory validation and harmonisation of  
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23 methodologies will further help in making this assay more robust and widely  
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25 acceptable for scientific as well as regulatory studies [203].  
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### 33 34 **OTHER TECHNIQUES AVAILABLE FOR RESEARCH ON** 35 **ANTIOXIDANTS WITH WHICH TO COMPARE THE COMET ASSAY** 36

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38 Oxidative stress is a disturbance in the prooxidant/antioxidant balance in favour of the  
39  
40 former [204] as a result from diminished levels of antioxidants and/or increased  
41  
42 production of reactive species. Direct measurement of reactive species provides  
43  
44 information on the status of the antioxidant system. Most reactive species only exist  
45  
46 for a short time *in vivo* and direct measurement can be extremely difficult. Another  
47  
48 approach might focus on the measurement of the end products of the interactions of  
49  
50 reactive species with biomolecules. Alternative end products which might be  
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52 combined with the ones measurable with the Comet assay might provide more  
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54 compelling information on the efficiency of the antioxidant defence as well as the  
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56 mechanisms of the genotoxicant or pathology investigated. There is a set of  
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4 techniques commonly used on the detection of free radicals despite it is claimed that  
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6 one major obstacle for research on free radicals and antioxidants is the lack of specific  
7  
8 and sensitive methods to quantify oxidative stress *in vivo* and *in vitro* [205]. The only  
9  
10 technique that can detect free radicals directly is the spectroscopic technique of  
11  
12 electron spin resonance (ESR) or also called electron paramagnetic resonance (EPR)  
13  
14 [31]. It only detects fairly unreactive radicals since reactive species do not accumulate  
15  
16 enough to be measured; and thus, a solution is to add traps or probes [206]. Trapping  
17  
18 permits a molecule to react with a trap molecule to give a measurable stable product.  
19  
20 The method of spin trapping generates a product detectable by ESR. However, there  
21  
22 are a large number of other probes and they are listed in Halliwell and Whiteman's  
23  
24 review [206]. Berliner and colleagues state that there is no ideal probe that meets the  
25  
26 following criteria: sensitivity of the measuring device, stability of the reaction  
27  
28 products, specificity of free radical or oxidant reactions, localisation, toxicity and  
29  
30 invasiveness [207].  
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35 “Fingerprint” or “footprinting” addresses the measurement of end products of  
36  
37 oxidative damage. The main condition is that they must be specific markers and the  
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39 advantage is that there is a broad variety of targets. Lipid peroxidation can be  
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41 evaluated by measurement of their end products such as malondialdehyde [208], 4-  
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43 hydroxynonenal [209] and acrolein [210] among many others. Detection of  
44  
45 isoprostanes appears to be the most comprehensive measurement of lipid peroxidation,  
46  
47 particularly if obtained from urine and plasma. The protein carbonyl assay (PCC),  
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49 which measures the amount of protein carbonyl groups, is the most frequently used  
50  
51 biomarker of protein damage [211]. According to Chevion and co-workers, there are  
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53 several methodologies for the quantisation of PCC; in all of them 2,4-dinitrophenyl  
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55 hydrazine is allowed to react with the protein carbonyls to form hydrazone, which can  
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4 be analysed optically by radioactive counting or immunohistochemically; moreover,  
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6 using PCC as a marker, it could be demonstrated that oxidative damage to proteins  
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8 correlates well with ageing and the severity of some diseases [212]. There is not yet a  
9  
10 “gold standard” with regard to measurement of oxidative DNA damage [206].  
11  
12 Notwithstanding, DNA products of free radicals reactions can be measured by a  
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14 plethora of techniques namely, high performance liquid chromatography (HPLC)  
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16 [213], gas chromatography-mass spectroscopy (GC-MS) [214] and liquid  
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18 chromatography-mass spectroscopy (LC-MS) [215]. Measurement of 8-OHdG is a  
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20 very common method of assessing DNA damage. However, it has downsides: 1) it is  
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22 intrinsically unreliable to measure any single reaction product, including 8-OHdG, as  
23  
24 an index of oxidative DNA damage since their relative amounts are highly dependent  
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26 on reaction conditions [206,216] and 2) chromatographic techniques have proved to  
27  
28 be prone to generation of artefacts [217]. Consequently, there is no consensus as to  
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30 what the true levels of oxidised damage are in human DNA [218] and; what is more,  
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32 it might be that 8-OHdG is only a minor product of oxidative DNA damage.  
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38 The use of specific enzymes in the Comet assay has allowed this assay to detect  
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40 oxidised DNA bases. Endonuclease III excises oxidised pyrimidines.  
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42 Formamidopyrimidine DNA glycosylase excises 8-OHdG and ring-opened purines  
43  
44 resulting from oxidation. If a digestion step is included with the mentioned repair  
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46 enzymes, then the increase in strand breaks will correspond to the recognition of  
47  
48 oxidised bases. In Collin’s view, the Comet assay combined with  
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50 formamidopyrimidine is the most convenient and reliable method for monitoring  
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52 levels of 8-OHdG and for assessing oxidative stress in general, although of course it  
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54 should not be used without proper controls and calibration [219]. Halliwell and  
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59 Whiteman question whether the reliability of the Comet assay is due to a lower  
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4 baseline since artefacts have been minimised or the Comet assay simply just generates  
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6 a different kind of artefact which leads to an underestimation of DNA damage. In any  
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8 case, it seems very unlikely that all the oxidised bases in compact DNA can be  
9  
10 recognised by exogenously applied enzymes [206]. Further, the Comet assay only  
11  
12 indicates that DNA strand breaks have occurred. Breaks can arise from numerous  
13  
14 sources – direct damage to DNA, but also from DNA repair intermediates, alkali-  
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16 labile sites, overt toxicity or even apoptosis.  
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19 A very different approach is the total antioxidant capacity (TAC). TAC considers the  
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21 cumulative action of all the antioxidants present in plasma and body fluids, thus  
22  
23 providing an integrated parameter of measurable antioxidants [220]. TAC measured  
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25 *in vitro* bears no similarity to *in vivo* measurements and may not have direct  
26  
27 implication *in vivo* [221]. In most intervention trials carried out, TAC failed to  
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29 demonstrate an effect of the supplementation of antioxidants and this might be  
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31 explained by the effect of endogenous antioxidants in addition to those from dietary  
32  
33 origin [219]. Moreover, assays for TAC measurement in plasma differ in the type of  
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35 oxidation source, target and measurement used to detect the oxidised product; thus,  
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37 providing a wide variety of results [221].  
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42 At present it would appear that there is not a single reliable biomarker of antioxidant  
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44 effects on oxidative stress. Hence, complementary end points and techniques are  
45  
46 encouraged when researching the effects of antioxidants with particular emphasis on  
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48 intervention studies. Even so, the interpretation of data must be done with caution.  
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## 52 53 54 55 56 **CONCLUSION** 57 58 59 60 61 62 63 64 65



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4 Despite aerobic organisms generating ROS as a natural by-product of O<sub>2</sub> metabolism,  
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6 metazoa have evolved mechanisms of defence that cope with this reactive chemical  
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8 species. However, oxidative stress occurs in situations of imbalance. This is when  
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10 ROS levels increase and protective compounds, namely antioxidants, are  
11  
12 overwhelmed. As a result, significant damage to cell structures like lipids, proteins  
13  
14 and DNA is exerted. Thus, O<sub>2</sub> is considered a potential mutagen, clastogen and  
15  
16 teratogen that may be responsible for, or at least part of, the background genetic  
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18 instability [222]. The protective effect of antioxidants is universally accepted, either  
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20 inherent in the body or ingested. However, certain aspects such as the mechanisms of  
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22 action, undefined properties of compounds or compounds with dual behaviour  
23  
24 (prooxidant and antioxidant) remain unclear and are subjected to investigation. For  
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26 instance, the elucidation of how antioxidant properties operate *in vitro* can provide a  
27  
28 better understanding of the, sometimes confounding, *in vivo* situation. It is precisely  
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30 in the human situation where the Comet assay might assist to define supplements with  
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32 specific antioxidants which modulate the DNA damage baseline and reveal to what  
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34 extent antioxidant levels in plasma are involved.  
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40 Medicine might benefit from current investigations scrutinising the properties of a  
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42 vast number of antioxidants as well as delving into the effects of diets. Future medical  
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44 treatments could rely on co-treatments with antioxidants to minimise diseases  
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46 involving oxidative stress. Thus, the Comet assay *per se* provides a reliable and  
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48 flexible technique to address and approach these type of investigations.  
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Table 1: Descriptive listing of publications quoted in the present manuscript.

Reference number	Antioxidant (concentration)	Genotoxicant and/or disease	Target cells (Species)	Type of experiment	Antioxidant effect observed	Publication, year.
[16]	Vitamin C (0.04 – 5 mM)	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	Generated DNA damage on its own, slight reduction DNA damage at low concentrations, increase at higher concentrations.	Anderson et al, 1994.
[17]	Vitamin C (100 mg/day) + vitamin E (280 mg/day) + β-carotene (25 mg/day)	Non/Smokers +/- H <sub>2</sub> O <sub>2</sub>	Lymphocytes (non/smokers 50-59 years old men)	<i>In vivo and ex vivo</i>	Reduction DNA damage baseline, increased resistance to DNA damage (H <sub>2</sub> O <sub>2</sub> )	Duthie et al, 1996.
[20]	Catalase (100-500 U/ml)	Diethylstilboestrol/ 17β-oestradiol/ nonylphenol/ equol/ genistein/ daidzein/ H <sub>2</sub> O <sub>2</sub>	Lymphocytes/ sperm (human)	<i>In vitro</i>	Reduction DNA damage	Anderson et al, 2003.
[20]	SOD (50-150 U/ml)	Diethylstilboestrol/ 17β-oestradiol/ nonylphenol/ equol/ genistein/ daidzein/ H <sub>2</sub> O <sub>2</sub>	Lymphocytes/ sperm (human)	<i>In vitro</i>	Reduction DNA damage (equol, genistein, daidzein)	Anderson et al, 2003.
[20]	Vitamin C (0.5-1 mM)	Diethylstilboestrol/ 17β-oestradiol/ nonylphenol/ equol/ genistein/ daidzein/ H <sub>2</sub> O <sub>2</sub>	Lymphocytes/ sperm (human)	<i>In vitro</i>	Reduction DNA damage or no effect (no apparent pattern)	Anderson et al, 2003
[21]	Catalase (100-500 U/ml)	Triiodothyronine/ noradrenaline	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Djelic and Anderson, 2003.
[22]	Catalase (100-500 U/ml)	Triiodothyronine/ L-Thyroxine sodium salt/ noradrenaline	Sperm (human)	<i>In vitro</i>	Reduction DNA damage	Dobrzynska et al, 2004.
[23]	Vitamin C (0.04-5 mM)	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	Slight reduction DNA damage (up to 1 mM). Increase DNA damage (5 mM)	Anderson and Phillips, 1999.
[24]	Vitamin C (40 or 100 μM) +/- vitamin E (30 or 100 μM)	BaP	Lymphocytes (human female)	<i>In vitro</i>	Reduction DNA damage	Gajecka et al, 1999.
[39]	Catalase (20 μg/ml)	Ozone	Leukocytes (human)	<i>In vitro</i>	Prevention DNA damage	Diaz-Llera et al, 2002
[30]	Catalase (250 U/ml)	Chromium (III)/ (VI)	Lymphocytes (human)	<i>In vitro</i>	Reduction (chromium (VI))	Blasiak and Kowalik, 2000.
[33]	Catalase (250 U/ml)	H <sub>2</sub> O <sub>2</sub> / xanthine + xanthine oxidase	Cortical cells (adult mice)/ cultured astrocytes (rat)	<i>In vitro</i>	Abolition DNA damage	Cemeli et al, 2003.
[34]	Catalase (500 U/ml)	Iodoacetic acid	CHO (hamster)	<i>In vitro</i>	Reduction DNA damage	Cemeli et al, 2006.
[34]	BHA (10-100 μM)	Iodoacetic acid	CHO (hamster)	<i>In vitro</i>	Reduction DNA damage	Cemeli et al, 2006.
[35]	Catalase (250 U/ml)	Vitiligo +/- H <sub>2</sub> O <sub>2</sub> / vitiligo +/- 17β-oestradiol	Lymphocytes (human)	<i>In vitro, ex vivo</i>	Reduction DNA damage	Schallreuter et al, 2006.
[38]	SOD (200 U/ml)	SIN-1, GSNO or RBS	HIT-T15 (hamster)	<i>In vitro</i>	No effect DNA damage	Delaney et al, 1997.
[41]	SOD (144 U/ml)	Metronidazole/ dimetridazole	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Ré et al, 1997.
[42]	SOD (100 U/ml)	Benzidine	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Chen et al, 2003.
[42]	BHT (100 μM)	Benzidine	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Chen et al, 2003.

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[43]	SOD	Hyperbaric oxygen	Lymphocytes (human)	<i>Ex vivo</i>	Reduction DNA damage	Muth et al, 2004.
[47]	Sodium selenite (Culture 100 nM for 10 days)	H <sub>2</sub> O <sub>2</sub>	Leukemic lymphoblasts (mice)	<i>In vitro</i>	Reduction DNA damage	Bouzyk et al, 1997.
[48]	Sodium selenate (600 µM)	UVA	Skin fibroblasts (human)	<i>In vitro</i>	Reduction DNA damage	Emonet-Piccardi et al, 1998.
[49]	Sodium selenate (50 nM)/ selenomethionine (200 nM)	UV	Keratinocytes (human)	<i>In vitro</i>	Prevention DNA damage (with pre-treatment)	Rafferty et al, 2003.
[50]	Sodium selenate (0.5 M)	Potassium dichromate	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Cemeli et al, 2003.
[51]	Selenomethionin (6 µg/kg/day) / high-selenium yeast (6 µg/kg/day)	Elder	Lymphocytes and prostate cells (dogs)	<i>In vivo supplementation</i>	Reduction DNA damage	Waters et al, 2003.
[52]	Kappa-selenocarrageenan (0.25 µg/ml)	Lead acetate	Blood cells (Mice)	<i>In vivo supplementation</i>	Reduction DNA damage	Yuan and Tang, 2001.
[56]	Sodium selenite (10 µM)	On its own	HepG2 (human)	<i>In vitro</i>	Generation DNA damage	Shen at al, 1999.
[59]	Ferritin (intracellular levels)	Hyperbaric oxygen	Lymphocytes (human)	<i>Ex vivo</i>	Increased expression ferritin	Rothfuss and Speit, 2002.
[60]	Deferiprone (0.05-1.6 mM)/ deferoxamine (0.05-1.6 mM)	Ferric chloride or ferrous chloride	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage (deferioxamine)	Anderson et al, 2000.
[61]	Deferoxamine (0.05-1.6 mM)	H <sub>2</sub> O <sub>2</sub> + ferrous sulphate/ H <sub>2</sub> O <sub>2</sub> + ferric chloride or H <sub>2</sub> O <sub>2</sub> + ferrous chloride	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage (H <sub>2</sub> O <sub>2</sub> + ferrous sulphate)	Anderson et al, 2000.
[65]	Melatonin (10 mg/kg/ for 6 days) or (0.1-2.5 mM)	Adriamycin	(rats)	<i>In vivo and in vitro</i>	Reduction DNA damage	Kim et al, 2005.
[66]	Melatonin (50 µM)/ amifostine (14 mM)	Idarubicin	Lymphocytes healthy subjects, leukemic K562 or HeLa (human)	<i>In vitro</i>	Reduction DNA damage by melatonin. Reduction DNA damage only in lymphocytes healthy subjects by amifostine. Generation DNA damage in cancer cells by amifostine	Majsterek et al, 2005.
[67]	Melatonin (10-50 µM)	Amoxicillin	Lymphocytes, gastric mucosa cells (human)	<i>In vitro</i>	Reduction DNA damage	Arabski et al, 2005.
[68]	Melatonin (300 mg single-supplement)	Gamma-irradiation	Whole blood (human)	<i>Ex vivo</i>	Reduction DNA damage	Vijayalaxmi et al, 1998.
[69]	Melatonin (100 mg/kg)	Ionising radiation	Brain cells (rat)	<i>In vivo</i>	Reduction DNA damage	Undeger et al, 2004.
[70]	Melatonin (0.5-1 mM)	Magnetic fields + iron ions	Lymphocytes (rat)	<i>In vitro</i>	Reduction DNA damage at 0.5mM and abolition at 1 mM	Jajte et al, 2001.
[71]	Melatonin (100 µM)	Graves' disease	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Tang et al, 2005.
[72]	Melatonin (0.1-1 mM)	On its own, H <sub>2</sub> O <sub>2</sub> or bleomycin	CHO (hamster)	<i>In vitro</i>	Slight increase DNA damage (on its own). Slight reduction DNA damage (H <sub>2</sub> O <sub>2</sub> ). No effect DNA damage (bleomycin)	Festa et al, 2001.
[73]	Melatonin (100 µM)	On its own	CHO (hamster)	<i>In vitro</i>	Generation DNA damage	Anisimov et al, 2006.



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[74]	Melanin (stimulated to reach 14, 93 to 164 µg melanin / mg protein)	H <sub>2</sub> O <sub>2</sub>	Melanocytes, HaCat keratinocytes (human)	<i>In vitro</i>	Reduction DNA damage	Hoogduijn et al, 2004.
[75]	Melanin (stimulated melanin production)	Ionising radiation	Melanoma cell line (human)	<i>In vitro</i>	Reduction DNA damage	Kinnaert et al, 2004.
[76]	Melanin (stimulated melanin production, tomato extract 50 µg/ml, palm fruit 10 µg/ml, vit E 35 µM and vit C 100 µM)	UVA	Melanocytes (human)	<i>In vitro</i>	Reduction DNA damage	Smit et al, 2004.
[77]	Melanin (stimulated melanin production)	UVA	Melanocytes, fibroblasts (human)	<i>In vitro</i>	Generation DNA damage	Marrot et al, 1999.
[78]	DHICA (0.125-2 µM)	UVA	HaCat keratinocytes (human)	<i>In vitro</i>	Generation DNA damage	Kipp and Young, 1999.
[80]	Vitamin C	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human non-smokers)	<i>Ex vivo</i>	Reduction DNA damage	Panayiotidis and Collins, 1997.
[81]	Vitamin C	On its own +/- H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	Slight reduction DNA damage (low concentrations). High increase DNA damage (high concentrations).	Harréus et al, 2005.
[82]	Vitamin C (300-600 µM)	On its own +/- H <sub>2</sub> O <sub>2</sub>	Sperm (human)	<i>In vitro</i>	No effect on DNA damage (on its own), reduced DNA damage (H <sub>2</sub> O <sub>2</sub> )	Donnelly et al, 1999.
[83]	Vitamin C (2x 250 mg/day) + Vitamin E (2x 91 mg/day) 4 weeks	Non/Smokers	Lymphocytes (human)	<i>In vivo</i>	Reduction DNA damage smokers (Vitamin C 500 mg +Vitamin E 182 mg)	Møller et al, 2004.
[85]	Vitamin E (10 µM, 2h pre-treatment)/ vitamin C (0.5 mM, 1h pre-treatment)	Organic compounds adsorbed on to ambient particles, BaP or 5,9-dimeDBC	HepG2 (human)	<i>In vitro</i>	Reduction DNA damage (vitamin C). Slight reduction DNA damage (vitamin E)	Lazarová and Slameňová, 2004.
[86]	Vitamin E (20 µM)	Bleomycin	Colonic mucosa (human)	<i>In vitro</i>	Reduction DNA damage	Wozniak et al, 2004.
[87]	Vitamin E (10-25 µM)	Acrylamide	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Blasiak et al, 2004.
[88]	Vitamin E (50 µM, 4h pre-treatment)	Ciprofloxacin	Primary culture astrocytes (rat)	<i>In vitro</i>	Reduction DNA damage	Gürbay et al, 2006.
[89]	Vitamin E (10-50 µM)	Streptozotocin	Lymphocytes/ HeLa (human)	<i>In vitro</i>	Reduction DNA damage	Blasiak et al, 2004.
[90]	Vitamin E (300 mg/day) + vitamin C (500 mg/day)	Waste anaesthetic gases	Lymphocytes (human)	<i>In vivo</i>	Reduction DNA damage	Sardas et al, 2006.
[91]	Trolox (50-200 µM)	H <sub>2</sub> O <sub>2</sub>	Buccal cells (human)	<i>In vitro</i>	Reduction DNA damage	Szeto et al, 2005.
[92]	Vitamin A (10µM/24h)/ vitamin C (0.5 mM/1h)/ vitamin E (10 µM/2h)	N-nitrosomorpholine	HepG2 hepatoma (human)	<i>In vitro</i>	Reduction DNA damage	Robichová et al, 2004.
[93]	Vitamin A (5 µM)/ vitamin C (10 µM)/ vitamin E (10 µM)	Imanitib	Myelogenous leukemia K562 (human)	<i>In vitro</i>	Abolition DNA damage (vitamin A and C) and reduction DNA damage (vitamin E).	Czechowska et al, 2005.
[94]	Vitamins A (10,000-50,000	p-dimethylaminoazobenzene	Hepatocytes	<i>In vivo</i>	Reduction DNA damage (high	Velanganni et al, 2007.

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	IU/ml) + vitamin C (75-1000 mg) + vitamin E (50-500 mg) / kg body. Once a week for 6 months.		(mice)		doses each vitamin individually and low doses as mixture)	
[95]	Vitamin A (5-7 µM)	On its own	V79 lung cells (Chinese hamster)	<i>In vitro</i>	Generation DNA damage	Klamt et al, 2003.
[97]	Thiamine + vitamin C	Lead acetate	Testicular cells (mice)	<i>In vivo</i>	Reduction DNA damage (low and middle doses)	Zhang et al, 2006.
[99]	Riboflavin (50 mg/kg) +/- vitamin C (150 mg/kg). Single or multiple doses. 24h prior, during genotoxicant administration or 24h after.	Sanguinarine/ argemone oil	Bone marrow/ whole blood (mice)	<i>In vivo</i>	Reduction DNA damage (in all cases)	Ansari et al, 2006.
[101]	Nicotinamide (5 µM)	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	Inhibits DNA repair. Reverses necrosis to apoptosis	Tronov et al, 2002.
[102]	Nicotinamide (200-800 mg/kg)	+/- Ionising radiation	Tumor/ thymus/ spleen/ testis/ bone marrow/ brain (mice)	<i>In vivo</i>	Extensive DNA damage. Slowed down strand break rejoining (except for brain cells)	Zheng and Olive, 1996.
[105]	Coenzyme Q10 (100 µM)	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	No effect DNA damage. Reduction DNA oxidation	Tomasetti et al, 1999.
[106]	Coenzyme Q10 (3 mg/kg/day) 2 weeks.	On its own	Lymphocytes (human)	<i>In vivo</i>	Generation 8-OHdG (detected by Comet assay)	Niklowitz et al, 2007.
[110]	β-carotene (0.25-10 µM) + lycopene (0.25-10 µM)	Catechol-oestrogens	Naked plasmid DNA, V79 lung cells (Chinese hamster)	<i>In vitro</i>	Reduction DNA damage	Muzandu et al, 2005.
[111]	β-carotene (1-10 µM) + lycopene (1-10 µM)	Xanthine/xanthine oxidase	Colon adenocarcinoma HT29 (human)	<i>In vitro</i>	Reduction DNA damage (Up to 3 µM). Generation DNA damage (4-10 µM)	Lowe et al, 1999.
[112]	β-carotene (0.5-8 µM)/ lycopene (0.5-8 µM)/ lutein (0.5-8 µM)	H <sub>2</sub> O <sub>2</sub>	Leukemia MOLT-17 (human)	<i>In vitro</i>	Reduction DNA damage (0.5-1 µM)	Astley et al, 2004.
[112]	β-carotene/ lycopene/ lutein, (natural isolate capsules, 15 mg/d, 4 weeks)	+/- H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human males)	<i>In vivo/ ex vivo</i>	Generation DNA damage (β-carotene). No effect DNA damage on its own (lycopene, lutein). No effect after H <sub>2</sub> O <sub>2</sub> challenge (β-carotene, lycopene, lutein)	Astley et al, 2004.
[113]	β-carotene (12 mg/kg) + vitamin C (400 mg/kg) + vitamin E (200 mg/kg)	γ-radiation	Cultured lymphocytes (mice)	<i>In vitro</i>	Reduction DNA damage after 1h "liquid holding"	Konopacka et al, 1998.
[114]	β-carotene (15 mg/day)/ lycopene (15 mg/day)/ lutein (15 mg/day) 1 week. Supplementation separated by 3-week wash-out periods.	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>Ex vivo</i>	Rejoining DNA breaks (lycopene and β-carotene). No effect (lutein)	Torbergson and Collins, 2000.
[116]	Lyc-o-Mato((R)) 250 ml/daily, (provides 6 mg lycopene, 4 mg phytoene, 3 mg phytofluene, 1 mg β-carotene and 1.8 mg α-	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>Ex vivo</i>	Reduction DNA damage	Porrini et al, 2005.

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tocopherol						
[118]	Zeaxanthin (5-100 $\mu$ M) + asthaxanthin (5-100 $\mu$ M) + lutein (5-100 $\mu$ M)	UVA	Trachea epithelial cells (rat)/ neuroblastoma cells (human)	<i>In vitro</i>	Presence carotenoids during exposure: reduction (tracheal cells) and generation (neuroblastoma) DNA damage. Addition carotenoids after exposure: generation (tracheal cells) and reduction (neuroblastoma) DNA damage	Santocono et al, 2006.
[119]	Zeaxanthin (20-40 $\mu$ M)/ asthaxanthin (20-40 $\mu$ M)/ lutein (20-40 $\mu$ M)	Reactive nitrogen species	Neuroblastoma cells (human)	<i>In vitro</i>	Reduction DNA damage	Santocono et al, 2007.
[127]	Quercetin (50 $\mu$ M)/ myricetin (1 mM)/ kaempferol and rutin (up to 10 mM)	H <sub>2</sub> O <sub>2</sub>	Caco-2 cells (human)	<i>In vitro</i>	Prevention DNA damage (quercetin and myricetin). No effect (kaempferol and rutin).	Duthie and Dobson, 1999.
[128]	Quercetin (1-100 $\mu$ M)	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Wilms et al, 2005.
[129]	Quercetin/ quercetin-3- glucoside/ quercitrin/ luteolin/ myricetin/ apigenin/ kaempferol/ rutin/ vitamin C (all of them 7.6-279.4 $\mu$ M).	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage (in all cases). Better reduction free flavonoids than conjugated ones	Norozi et al, 1998.
[130]	Quercetin/ myricetin/ rutin (10-200 $\mu$ M)	H <sub>2</sub> O <sub>2</sub>	Caco-2 cells/ HepG2 (human)	<i>In vitro</i>	Reduction DNA damage	O'Brien et al, 2000.
[131]	Quercitrin/ luteolin/ myricetin/ apigenin	H <sub>2</sub> O <sub>2</sub>	Myelogenous leukemia K562 (human)	<i>In vitro</i>	Reduction DNA damage quercetin and luteolin (20-100 $\mu$ M), rutin (100-1000 $\mu$ M). No effect apigenin.	Horváthová et al, 2004.
[132]	Quercetin/ luteolin/ apigenin/ rutin	H <sub>2</sub> O <sub>2</sub>	Leukemia L1210 (mice)	<i>In vitro</i>	Reduction DNA damage quercetin, luteolin and apigenin (600 $\mu$ M). Generation DNA damage apigenin (1.2 mM). No effect rutin.	Horváthová et al, 2003.
[133]	Quercetin (0.03-6 mM)/ rutin (0.02-3.28 mM)	Mitomycin C	Lymphocytes (human)	<i>In vitro</i>	Reduction quercetin (all concentrations) and rutin (0.02-0.82 mM). Generation DNA damage rutin (1.64-3.28 mM).	Undéger et al, 2004.
[134]	(Quercetin (10-23 $\mu$ M) +/- rutin (10-23 $\mu$ M) +/- naringin (10-23 $\mu$ M)) +/- $\beta$ -carotene (20 $\mu$ M)	UVA	C3H10T1/2 embryo fibroblast cells (mouse)	<i>In vitro</i>	Reduction DNA damage (quercetin, rutin and naringin) with/without $\beta$ -carotene. Generation DNA damage $\beta$ - carotene (on its own).	Yeh et al, 2005.
[136]	Quercetin (100-500 $\mu$ M)/ Kaempferol (100-500 $\mu$ M)	H <sub>2</sub> O <sub>2</sub> / $\beta$ -estradiol/ diethylstilbestrol/ daidzein/ genistein	Lymphocytes/ sperm (human)	<i>In vitro</i>	Reduction DNA damage (not consistently at 100 $\mu$ M but consistently at 500 $\mu$ M)	Cemeli et al, 2004.
[136]	Silymarin (100-550 $\mu$ M)/ myricetin (100-550 $\mu$ M)/ quercetin (10-500 $\mu$ M)/ kaempferol (50-500 $\mu$ M)/ rutin (100-500 $\mu$ M)/ kaempferol-3-	Food mutagens (Trp/ IQ)	Lymphocytes/ sperm (human)	<i>In vitro</i>	Generation DNA damage at lower concentrations. Reduction or abolition (quercetin) at higher concentrations.	Anderson et al, 1997.

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	rutinoside (50-500 µM)					
[137]	Silymarin (100-550 µM)/ myricetin (100-550 µM)/ quercetin (10-500 µM)/ kaempferol (50-500 µM)/ rutin (100-500 µM)/ kaempferol-3-rutinoside (50-500 µM)	Food mutagens (Trp/ IQ/ Phip)	Lymphocytes/ sperm (human)	<i>In vitro</i>	Generation DNA damage at lower concentrations. Reduction or abolition (quercetin) at higher concentrations.	Anderson et al, 1998.
[139]	Genistein and daidzein (0.01-2.5 µM in lymphocytes/24h, 2.5-20 µM in Jurkat cells/24h)	H <sub>2</sub> O <sub>2</sub>	Lymphocytes/ Jurkat T-cells (human)	<i>In vitro</i>	Reduction DNA damage in all cases.	Foti et al, 2005.
[140]	Genistein (0.01-100 µM)/ equol (0.01-100 µM)/ ascorbic acid (10-600 µM)/ α-tocopherol (1-150 µM)	H <sub>2</sub> O <sub>2</sub>	Sperm (human)	<i>In vitro</i>	Reduction DNA damage genistein and equol (0.01-100 µM), vitamin C (10-600 µM) and α-tocopherol (1-100 µM)	Sierens et al, 2000.
[141]	Genistein (10-400 µM)/ daidzein 100-400 µM)	On its own	Lymphocytes/ sperm (human)	<i>In vitro</i>	Generation DNA damage (in all cases)	Stopper et al, 2005.
[142]	EGCG (0.01-200 µM)	On its own +/- H <sub>2</sub> O <sub>2</sub>	HL60 cells (human)	<i>In vitro</i>	Generation DNA damage (in all cases)	Elbling et al, 2005.
[143]	EGCG (2 µM)	Bleomycin	Leucocytes (human)	<i>In vitro</i>	Reduction DNA damage	Glei and Pool-Zobel, 2006.
[144]	EGCG (250 µM)	UV	Lung fibroblasts/ skin fibroblasts/ keratinocytes (human)	<i>In vitro</i>	Reduction DNA damage	Morley et al, 2005.
[145]	EGCG (25-100 µM)	MNNG/ H <sub>2</sub> O <sub>2</sub>	V79 lung cells (Chinese hamster)	<i>In vitro</i>	Reduction DNA damage (in all cases)	Roy et al, 2003.
[146]	EGCG, EGC or THF (10-100 µM)	BaP	Chang liver cells (human)	<i>In vitro</i>	Generation DNA damage EGCG, EGC and THF (100 µM) when on its own. Reduction DNA damage EGCG, EGC and THF (10-50 µM) when combined with BaP	Yen et al, 2004
[147]	Resveratrol/ curcumin/ indole-3-carbinol/ ellagic acid	MNNG	V79 lung cells (Chinese hamster)	<i>In vitro</i>	Reduction DNA damage	Chakraborty et al, 2004.
[148]	Resveratrol (10-250 µM)	H <sub>2</sub> O <sub>2</sub>	Glioma C6 cells (rat)	<i>In vitro</i>	Reduction DNA damage	Quincozes-Santos et al, 2007.
[150]	Resveratrol (1.56-25 µM) / coumarin (1.56-25 µM) / quercetin (1.56-25 µM) / 7,8-dihydroxy-4-methyl coumarin (1.56-25 µM) / vanillin(1.56-25 µM) / curcumin (1.56-25 µM) rutin (2.5-50 µM)	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage quercetin, 7,8-dihydroxy-4-methyl coumarin (3.1-25 µM), curcumin, resveratrol and vanillin (6.25-25 µM). No effect rutin and 7-hydroxy-4-methyl coumarin (up to 50 µM).	Liu and Zheng, 2002.
[150]	Resveratrol (10-100 µM)/ 4-hexylresorcinol (10-100 µM)	H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Yen et al, 2003.
[151]	Resveratrol (25 µg/ml)	Selenium-cisplatin conjugated	Lymphocytes (human)	<i>In vitro</i>	Reduction DNA damage	Olas et al, 2005.
[152]	Resveratrol (50-200 µM)	On its own	Lymphocytes (human)	<i>In vitro</i>	Generation DNA damage	Azmi et al, 2006.
[153]	Resveratrol (50 µM)	Cu(II)	Lymphocytes	<i>In vitro</i>	Generation DNA damage	Azmi et al, 2005.

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[154]	BHA	MNNG	V79 lung cells (Chinese hamster)	<i>In vitro</i>	No effect DNA damage	Horváthová et al, 1999.
[155]	BHA (250 µM)	MNNG	V79 lung cells (Chinese hamster)	<i>In vitro</i>	No effect DNA damage	Slameňová et al, 2003.
[157]	Ethoxyquin-rutin (1-25 µM)/ ethoxyquin-quercetin (10-25 µM)	On its own +/- H <sub>2</sub> O <sub>2</sub>	Lymphocytes (human)	<i>In vitro</i>	Generation DNA damage (on its own). Reduction DNA damage (H <sub>2</sub> O <sub>2</sub> )	Błaszczuk and Skolimowski, 2007.