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## ASSAYS FOR OXIDATIVE STRESS AND ANTIOXIDANT STATUS

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### ABSTRACT

Oxidative stress is a factor in many human diseases, as either cause or effect. A convenient biomarker of oxidative stress is the extent of oxidation of bases in DNA (although measures of lipid or protein oxidation may be equally informative). 8-Oxo-7,8-dihydroguanine or the corresponding nucleoside is most often measured, either chromatographically (gas chromatography-mass spectrometry, HPLC with electrochemical detection, or HPLC-tandem mass spectrometry) or enzymically, with the use of the enzyme formamidopyrimidine DNA glycosylase to convert 8-oxo-7,8-dihydroguanine to DNA breaks, which are detected with alkaline elution, alkaline unwinding, or the comet assay. Estimates of background levels of 8-oxo-7,8-dihydroguanine in normal human cells vary 1000-fold, depending on the technique used. Gas chromatography-mass spectrometry is particularly prone to oxidation of samples during derivatization, whereas HPLC suffers from this artifact to a lesser degree. In a recent interlaboratory study that measured the same samples of human cells, median values obtained with HPLC with electrochemical detection and with formamidopyrimidine DNA glycosylase differed by ~10-fold. There are still questions regarding the actual level of damage, but it is probably approximately one 8-oxo-7,8-dihydroguanine residue per 10<sup>6</sup> guanines. Assays for antioxidant protection against oxidative damage generally depend on measurements of decreases in a marker of oxidation. Potential dietary antioxidants can be screened with in vitro antioxidant assays or tested in cell culture systems. The best test, however, is in humans. The total antioxidant capacity of plasma is generally insensitive to dietary supplementation with antioxidants or antioxidant-rich foods. An increase in the resistance of lymphocyte DNA to oxidation in vitro is commonly seen, however, and a decrease in endogenous oxidation of DNA may be detected, especially after prolonged supplementation.

**Keywords:** Antioxidant, Biomarker, HPLC, Formamidopyrimidine, oxidation.

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## INTRODUCTION

Oxidative stress is widely recognized as a factor in many degenerative diseases, as either a cause or effect. The plaques that obstruct arterial flow and cause cardiovascular disease are laid down by macrophages engorged with oxidized LDL (foam cells). Oxidized bases in DNA are potentially mutagenic and so are implicated in the process of carcinogenesis. Diabetes mellitus is associated with oxidative damage to biomolecules, and any inflammatory condition inevitably leads to an increased oxidative burden, because the release of reactive oxygen species by macrophages is part of the body's defense mechanism.[2]

The role of antioxidants obtained from the diet in protection against disease is a topic of continuing interest and some controversy. Long-term intervention trials with disease as the outcome have yielded generally disappointing results, with increases in rates of lung cancer and cardiovascular disease in 2 large trials in which subjects took a  $\beta$ -carotene supplement (compared with placebo) and no effect in 2 other trials with  $\beta$ -carotene or a mixture of antioxidants.[3] An alternative, the molecular epidemiologic approach, has several advantages, notably the small number of subjects needed and the short time scale. Its success depends, however, on the use of reliable validated biomarkers, which are rare. A notable example involves chromosomal aberrations, which were shown in prospective studies to be good indicators of future risk of cancer. DNA damage is clearly the ultimate cause of cancer, because DNA base changes can be mutagenic, but, except for some special cases, DNA damage is probably better regarded as a marker of exposure to genotoxic agents than as an indicator of the likelihood that cancer will occur in an individual. In assessments of the effectiveness of particular antioxidants or antioxidant-rich foods. [4]

These experimental approaches depend on the precise measurement of oxidative damage. 8-Oxo-7,8-dihydroguanine in cellular DNA is one of the most commonly measured indices of oxidative damage. The serious problem of an oxidation artifact that affects some of the methods used to measure 8-oxo-7,8-dihydroguanine was highlighted by the activities of a consortium of laboratories known as European Standards Committee on Oxidative DNA Damage (ESCODD)[7]. It is more than likely that other biomarkers, such as lipid oxidation and protein oxidation, are subject to analogous difficulties. The following account of attempts to optimize the assay of 8-oxo-7,8-dihydroguanine should therefore be seen as a cautionary tale, with implications that reach beyond the estimation of DNA damage.

### **Assessing oxidative stress**

#### **Oxidative DNA damage as a biomarker [11]**

The popularity of 8-oxo-7,8-dihydroguanine as an indicator of DNA oxidation is probably attributable to the ease with which it can be measured. However, estimates of background levels of 8-oxo-7,8-dihydroguanine in normal human cells can vary by 3 orders of magnitude, depending on the method used. Typically, researchers using gas chromatography-mass

spectrometry (GC-MS) have reported amounts of 8-oxo-7,8-dihydroguanine as great as hundreds of residues per  $10^6$  guanines. HPLC with electrochemical detection (ECD) generally yields lower values, ~5–50 residues per  $10^6$  guanines. A nonchromatographic approach makes use of the bacterial repair enzyme formamidopyrimidine DNA glycosylase (FPG) to break the DNA at sites of 8-oxo-7,8-dihydroguanine (plus some other oxidized forms of purines). The breaks are then measured with the comet assay, alkaline elution, or alkaline unwinding. This approach typically leads to 8-oxo-7,8-dihydroguanine estimates of ~0.5 residue per  $10^6$  guanines. The chromatographic methods are prone to oxidation of guanine during isolation and hydrolysis of the DNA, and antioxidants and chelators are used in attempts to control the artifact. The ESCODD was formed to develop reliable methods for measuring 8-oxo-7,8-dihydroguanine, but comparative chromatographic analyses of standard solutions of 8-oxo-7,8-dihydroguanine, calf thymus DNA, oligonucleotides with defined 8-oxo-7,8-dihydroguanine contents, and pig liver have confirmed the intractable nature of the problem of spurious oxidation, with wide variations among participating laboratories.

Recently, identical samples of HeLa cells were sent to all ESCODD partners, including those using the enzyme methods. The median value for 8-oxo-7,8-dihydroguanine measured chromatographically was 5.2 residues per  $10^6$  guanines, with a range of 1.84–214 residues per  $10^6$  guanines. The enzymic methods yielded a median value of 0.8 residue per  $10^6$  guanines (range: 0.06–5.0 residues per  $10^6$  guanines). To test the accuracy of different methods, we introduced different amounts of additional 8-oxo-7,8-dihydroguanine by treating HeLa cells with a photosensitizer plus visible light. Seven of 8 laboratories using HPLC-ECD were able to detect the dose-response relationship for 8-oxo-7,8-dihydroguanine. Furthermore, when regression lines were calculated, all except one had virtually identical slopes, indicating a high accuracy. [GC-MS and HPLC-tandem mass spectrometry methods were unable to detect the dose-response relationship.] However, the intercepts of the regression lines with the  $y$ -axis, representing background damage, varied over a 75-fold range. It seems that, despite adoption of standard protocols and the use of precautions to prevent adventitious oxidation, this remains a serious problem. The enzymic approach also has some potential problems, ie, the possibility that FPG is detecting lesions other than 8-oxo-7,8-dihydroguanine, leading to overestimation, and the fact that the method relies on indirect calibration. However, the approach does seem to be less prone to adventitious oxidation. (Refer Figure 1).

8-Oxo-7,8-dihydroguanine dose-response relationship. HeLa cells were treated with different concentrations of the photosensitizer Ro 19–8022 and light to induce 8-oxo-7,8-dihydroguanine in the DNA. Linear regression lines are shown for different laboratories that used a variety of methods to determine 8-oxo-7,8-dihydroguanine levels, ie, HPLC-ECD (solid lines), GC-MS (long dashes), or HPLC-tandem mass spectrometry.

In the final stage of ESCODD evaluations, lymphocytes were collected from volunteers in the members' own countries. The median 8-oxo-7,8-dihydroguanine value (based on the means for each set of volunteers) was 4.2 residues per  $10^6$  guanines with HPLC-ECD, compared with 0.3 residue per  $10^6$  guanines with enzymic methods. Although there is still a discrepancy between the 2 approaches, it is less than in the past, and there is (at least within the ESCODD) a

consensus that 8-oxo-7,8-dihydroguanine levels in many published studies are seriously overestimated.

The conclusions from the ESCODD were as follows. GC-MS is not suitable for measuring basal 8-oxo-7,8-dihydroguanine levels in biological material. HPLC-mass spectrometry has yet to yield convincing results. HPLC-ECD can measure induced damage accurately but still suffers from adventitious oxidation during sample preparation. Enzymic methods are less susceptible to this artifact, but calibration and standardization of methods require attention. The best estimate of background oxidation in normal human cells is 0.3-4 residues of 8-oxo-7,8-dihydroguanine per  $10^6$  guanines.

### **Chemical assays for antioxidant activity [29]**

Various assays have been described in which the putative antioxidant is added to a reaction mixture in which free radicals are generated. An example is the 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) assay, in which ABTS is oxidized by 2,2'-azobis(2-amidopropane). Antioxidants delay the appearance of the colored product of this reaction. The ferric ion-reducing ability (FRA) assay was devised to assess FRA of plasma. It is a colorimetric assay based on the reduction of a colored ferric complex. It has recently found a different application, in the testing of different foodstuffs. A wide range of fruits, vegetables, berries, cereals, and nuts were tested, after a simple standard extraction with methanol/water, and an extremely wide range of values were obtained. Some representative FRA values are given in Table 1↓. The FRA values for extracts from 100 g vary widely, from 0.04 mmol/L for foods such as carrots (generally regarded as being rich in antioxidant carotenoids) to 21 mmol/L for walnuts, 11.3 mmol/L for pomegranates, and 39.5 mmol/L for rosehips (at the top of the list). The FRA value of a food component does not tell us how valuable it would be as an antioxidant within the body, because bioavailability is not addressed, but it does provide useful information on which to base the choice of foods for testing in more elaborate assays. (Refer table 1).

A variety of phytoestrogens were assessed for antioxidant capacity with 3 tests, ie, the ABTS and FRA assays and electron spin resonance spectroscopy, to determine the ability of the phytoestrogen to quench the stable galvinoxyl radical (18). Results from the electron spin resonance and FRA assays correlated extremely well and showed that most of the phytoestrogens tested had low antioxidant capacity, compared with the standard trolox. The ABTS assay results did not correlate with those for either of the other 2 methods.

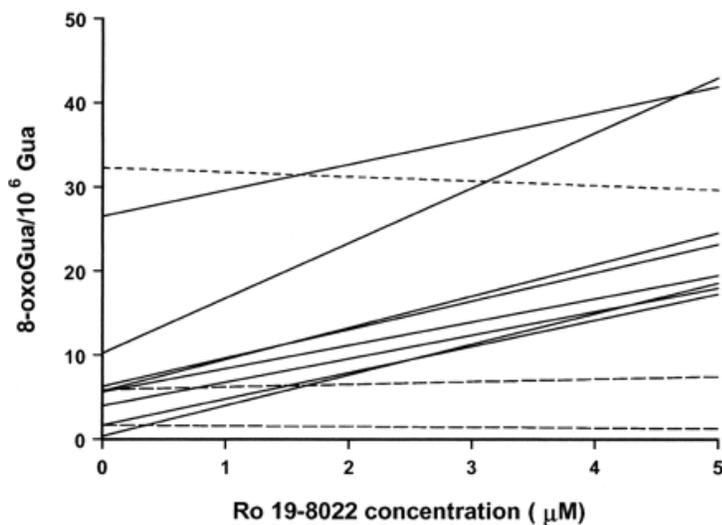
We have described a simple in vitro antioxidant assay in which the target molecule for oxidative damage is DNA. This is a modified version of the comet assay, in which cells embedded in agarose on a microscope slide are lysed with Triton X-100. The nucleoid DNA present after lysis is particularly sensitive to oxidative attack by  $H_2O_2$ , perhaps because endogenous antioxidants (such as catalase and glutathione) have been leached out of the nucleus, whereas the DNA is still associated with  $Cu^{2+}$  ions at the nuclear matrix, which can catalyze production of  $\cdot OH$  radicals through the Fenton reaction. Antioxidant activity is

assessed as the decreased induction of DNA breaks by H<sub>2</sub>O<sub>2</sub> after pretreatment of nucleoids with the antioxidant. Vitamin C demonstrated concentration-dependent protection against DNA damage. The mixture of antioxidants present in a simple extract of kiwifruit was effective over a wide range of dilutions. This assay has not yet been applied to a variety of different foods; direct comparison with the FRA assay would be a useful exercise. (Refer Figure 2).

Table 1.FRA of extracts from 100 g of various foods [1]

Category	Food	FRA
		<i>mmol/L</i>
Cereal	Oat	0.59
Roots and tubers	Carrot	0.04
Vegetables	Onion	0.67
Fruits	Kiwi	0.91
	Pomegranate	11.3
Berries	Rose hip	39.5
	Raspberry	3.06
Pulses	Broad bean	1.86
Nuts and seeds	Walnut	21.0

Figure 1



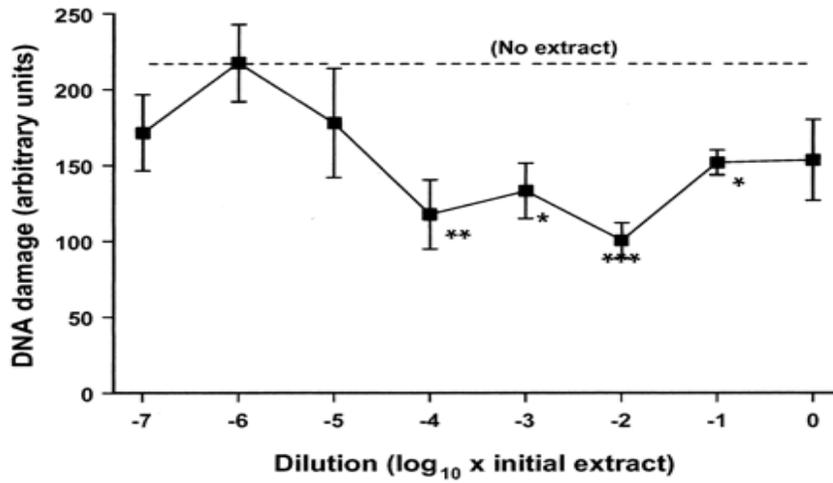


Figure 2

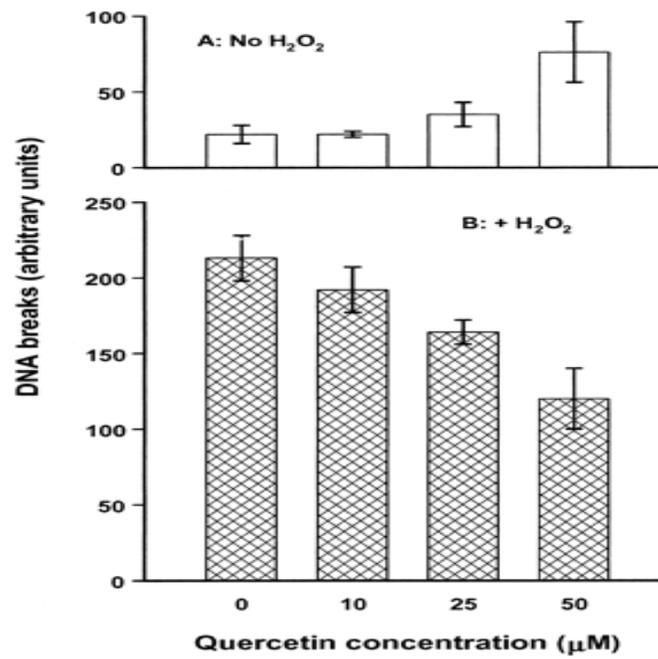


Figure 3

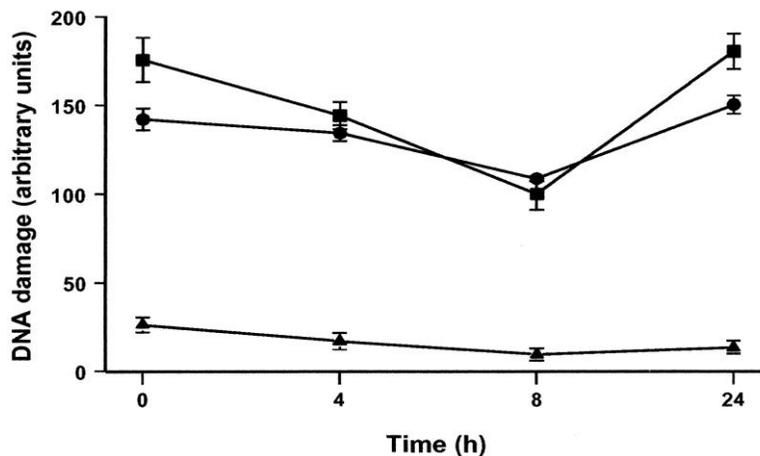


Figure 4

Antioxidant activity of kiwifruit extract analyzed with a modified comet assay. The extract, suitably diluted, was added to the nucleoids in agarose after lysis with Triton X-100, just before addition of  $H_2O_2$  to induce DNA breaks. The level of breakage in nucleoids treated only with  $H_2O_2$  is indicated by the horizontal line, and significant differences from this line ( $P < 0.05$ ) are indicated with asterisks. Bars indicate SEM.

Lipid oxidation is the basis for another method of assessing antioxidant activity. LDL particles isolated from blood have a certain amount of associated  $\alpha$ -tocopherol (vitamin E). LDL particles incubated with macrophages in a suitable medium (containing iron) are initially protected from oxidation by the vitamin E; once this is consumed, however, lipid hydroperoxides accumulate. Alternatively, chemical oxidation of LDL can occur in the presence of Cu(II) alone. If a flavonoid such as quercetin is present in either of these systems, then it delays the appearance of the lipid oxidation products. The flavonoid either scavenges free radicals, thus protecting the  $\alpha$ -tocopherol and LDL, or acts at a later stage, regenerating  $\alpha$ -tocopherol from oxidized  $\alpha$ -tocopheroxyl radicals. Red wine, which is rich in polyphenols, inhibited LDL oxidation by macrophages even when diluted 1000-fold.

The ability of phytoestrogens to inhibit peroxidation of lipids in microsomes from vitamin E-deficient rats was compared with other antioxidant assays. The phytoestrogens were relatively ineffective, compared with vitamin E. This assay and the FRA assay yielded qualitatively similar results.[31]

### Testing antioxidant activity in cell cultures

Figure 3↓ illustrates the usefulness of (and problems associated with) cultured cell model systems for testing food components for both harmful and beneficial effects. The aim was to examine the actions of quercetin, which has been reported to have genotoxic properties, inducing mutations in the Ames test; in addition, as a polyphenol, it is known to have antioxidant properties. Quercetin, at a concentration of  $25 \mu\text{mol/L}$ , induces DNA breaks. However, in combination with the DNA-oxidizing agent  $H_2O_2$ , the same concentration of

quercetin substantially decreases the yield of damage . The biological activity of quercetin in the whole body must depend both on the concentration achieved and on the presence of reactive oxygen species. (Refer Figure 3).

Effects of quercetin on DNA damage in human lymphocytes. DNA breaks were measured with the comet assay, after 30-min incubation with quercetin at different concentrations (A) or after 30-min incubation with quercetin followed by 5-min incubation with H<sub>2</sub>O<sub>2</sub> (B). Bars indicate SEM.

### **Antioxidants in humans**

#### **Antioxidant capacity of plasma**

Various assays for total antioxidant capacity (the total radical-trapping antioxidant potential, trolox equivalent antioxidant capacity, oxygen radical absorbance capacity, and FRA in plasma assays) measure either radical scavenging or reducing capacity. They were devised, or at least have been used, to assess individual antioxidant status, as reflected in body fluids. However, in most human intervention trials in which they have been applied, they have failed to demonstrate an effect of supplementation with antioxidants. This is not at all surprising. The total antioxidant capacity is the sum of many different antioxidants, including dietary ones but also those of endogenous origin. In different assays, albumin and uric acid are major contributors. Supplementation, even if it results in a large increase in the concentration of the particular antioxidant in the plasma, can cause only a modest change in the overall antioxidant capacity.

#### **Antioxidant resistance of lymphocytes and lipids to oxidation**

The principle behind this approach is that, if antioxidants are taken up by human subjects as a result of dietary supplementation, then they should reach the bloodstream and enter the blood cells, enhancing the ability of these cells, as well as the plasma lipids, to resist oxidative attack when challenged *in vitro* with a source of reactive oxygen. The comet assay has been used very successfully to monitor the antioxidant resistance of isolated lymphocytes to DNA damage induced by H<sub>2</sub>O<sub>2</sub>. After single large doses of vitamin C, vitamin E, or  $\beta$ -carotene or 20-wk supplementation with lower doses of these antioxidants, the yield of DNA breaks was significantly decreased. Real foods can also have detectable effects; a 0.5-L drink of kiwifruit juice caused a 25% decrease in breaks at 8 h . Supplementation with daily doses of 1 or 2 kiwifruits for 3 wk had a similar effect . Porrini and Riso gave female volunteers a daily dose of 25 g of tomato purée (rich in the carotenoid lycopene) for 14 d and observed DNA breaks induced by H<sub>2</sub>O<sub>2</sub> decrease by approximately one-half, compared with the level before supplementation. We examined the efficacy of a single “dose” of fried onions, which are known to be rich in flavonoids. The plasma concentrations of glycosides of quercetin and isorhamnetin were monitored during a 24-h period and reached a peak at 4 h. DNA breaks induced by H<sub>2</sub>O<sub>2</sub> were at their lowest level at 8 h. When onions were combined with tomatoes in the meal, the

protective effect of the onions was unexpectedly abolished. Very similar results were reported by Lean et al, comparing a meal of fried onions with onions plus tomato ketchup and herbs. (Refer Figure 4)

Protection of lymphocyte DNA against in vitro strand breakage by H<sub>2</sub>O<sub>2</sub>. Lymphocytes were isolated from blood samples from 6 volunteers at intervals after a meal of fried onions. Effects of 2 different concentrations of H<sub>2</sub>O<sub>2</sub>, 50 µmol/L (circles) and 200 µmol/L (squares), and the untreated control values, ie, endogenous strand breaks (triangles), are shown. Breaks were measured with the comet assay, and means for the 6 samples are shown. Bars indicate SEM.

LDL particles isolated from the blood of supplement-treated volunteers can be subjected to in vitro oxidation by macrophages or CuSO<sub>4</sub> (see above). The appearance of lipid hydroperoxides is monitored, and an increase in the lag period indicates an increase in resistance to oxidation. Fuhrman et al. reported increased resistance of LDL to oxidation when red wine was consumed with meals. Licorice extract, containing the flavonoid glycoside glabridin, was similarly protective.

## CONCLUSIONS

Assays for antioxidant status and oxidative damage are many and varied. The simplest ones are purely chemical in vitro reactions or tests in cell cultures. They can yield useful information about mechanisms of action, but extrapolation to effects of dietary antioxidants in vivo is dangerous, because uptake from the gastrointestinal tract and metabolism are not considered.

Supplementation with antioxidants in vivo seems to be the best approach, at least in principle, and experiments should be performed with human subjects if possible. (Animal experiments have not been considered here, but generally the same assays and approaches as used with human subjects can be applied.) Intervention studies are only as good as the biomarkers used to measure the effects of the intervention. Many published studies use inappropriate methods to measure oxidized bases in DNA, as ESCODD has shown; studies reporting high levels of 8-oxo-7,8-dihydroguanine (≥5 residues per 10<sup>6</sup> guanines) should be reevaluated. My personal view is that the comet assay, with FPG, is the most convenient and reliable method we have for monitoring levels of 8-oxo-7,8-dihydroguanine and for assessing oxidative stress in general, although of course it should not be used without proper controls and calibration.

Møller and Loft recently published a critical appraisal of human antioxidant supplementation trials that used biomarkers for DNA oxidation (both endogenous damage and resistance to H<sub>2</sub>O<sub>2</sub> in vitro). An important criterion was good study design, and many studies were found to be lacking. Those authors concluded that we still do not have a good understanding of the effectiveness of antioxidants in vivo and that in the future we should perhaps concentrate on groups of subjects who are in a state of increased oxidative stress (as a result of disease or smoking, for instance), for whom protective effects of antioxidants may be easier to demonstrate.

If it is important to know about lipids, then measurements of plasma malondialdehyde concentrations or LDL oxidation may yield useful data. If proteins are the main interest, then protein carbonyls can be measured. To date, however, these methods have not been subjected to the rigorous validation checks that have been applied to the markers of DNA oxidation.

Finally, antioxidant activity is not the only biologically important aspect of phytochemicals such as flavonoids. They are also likely to influence xenobiotic-metabolizing enzymes, as well as antioxidant enzymes and DNA repair pathways.

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