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DNA-Breaking Versus DNA-Protecting Activity of Four Phenolic Compounds *in vitro*

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Given the paradoxical effects of phenolics in oxidative stress, we evaluated the relative pro-oxidant and antioxidant properties of four natural phenolic compounds in DNA nicking. The phenolic compounds differed dramatically in their ability to nick purified supercoiled DNA, with the relative DNA nicking activity in the order: 1,2,4-benzenetriol (100% nicking) > gallic acid > caffeic acid > gossypol (20% nicking). Desferrioxamine (0.02 mM) decreased DNA strand breakage by each phenolic, most markedly with gallate (85% protection) and least with caffeic acid (26% protection). Addition of metals accelerated DNA nicking, with copper more effective (~5-fold increase in damage) than iron with all four phenolics. Scavengers revealed the participation of specific oxygen-derived active species in DNA breakage. Hydrogen peroxide participated in all cases (23–90%). Hydroxyl radicals were involved (32–85%), except with 1,2,4-benzenetriol. Superoxide participated (81–86%) with gallic acid and gossypol, but not with caffeic acid or 1,2,4-benzenetriol. With 1,2,4-benzenetriol, scavengers failed to protect significantly except in combination. Thus, in the presence of desferrioxamine, catalase or superoxide dismutase inhibited almost completely. When DNA breakage was induced by Fenton's reagent (ascorbate plus iron) the two catechols (caffeic acid and gossypol) were protective, whereas the two triols (1,2,4-benzenetriol and gallic acid) exacerbated damage.

Keywords: phenolic compounds; DNA breakage; copper; iron; ascorbate; Haber-Weiss reaction; prooxidant; antioxidant; free radical

INTRODUCTION

Phenolic compounds such as caffeic acid, gallic acid and gossypol (Fig. 1) are widely distributed in plants[1], where, with other redox active compounds they are currently under scrutiny as "phytochemicals". Such phenolics are present in many foods and beverages, and others arise through metabolic hydroxylations. Many of these compounds have substantial biological effects, but it is still not known to what extent a given phenolic is harmful or beneficial[2]. The inability to come to clear conclusions reflects their paradoxical actions in diverse test systems or reaction conditions.

In some circumstances, phenolics exhibit pro-oxidant and genotoxic effects. The phenolics selected in the current studies have each been implicated as pro-oxidant and genotoxic. Caffeic

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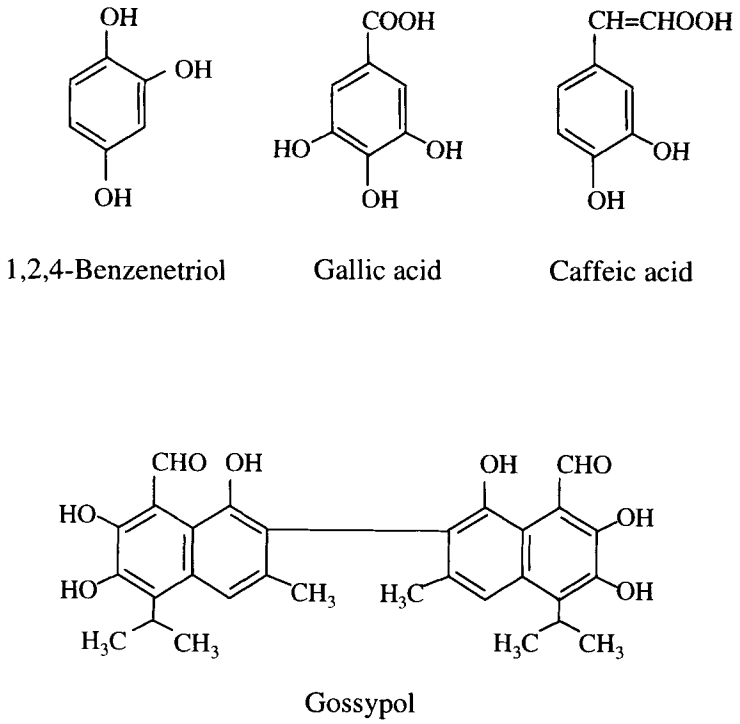


FIGURE 1 Structures of phenolic compounds investigated

acid and gallic acid, prevalent in coffee and teas, can induce DNA breakage in mammalian cells[3]. Gossypol, found in cottonseed oil and of interest as a possible male contraceptive, generates superoxide radicals in the presence of liver microsomes[4] or Fe^{3+}-EDTA[5], mediates oxygen-dependent DNA degradation in the presence of 2-mercaptoethanol and metal ions[6], and increases the frequency of DNA-strand breaks in human leukocytes[7]. 1,2,4-Benzenetriol, a metabolite of benzene and component of tobacco smoke, is a well-established pro-oxidant, genotoxin, and probable carcinogen[8–10].

Metals can accelerate the reduction of oxygen by redox-active phenolic compounds and increase their deleterious effects, in part by catalyzing Haber-Weiss type reactions[11]. Thus, copper and iron enhance formation of hydroxyl radicals, chromatid exchange and breakage by caffeic acid and gallic acid[12,13], and DNA degradation by gossypol[6].

Paradoxically however, plant phenolics also display antioxidant properties, and sometimes *protect* against genotoxins. Caffeic acid and gallic acid, for example, suppress chromosomal breakage and sister-chromatid exchange induced by some carcinogens [e.g., aflatoxin-B_1[14], N-methyl-N'-nitro-N-nitrosoguanidine[15], benzo[a]pyrene[16] and 7,12-dimethylbenz[a]anthracene[17]], or ultraviolet-light[18], in mammalian cells and bacteria. Such protection may be related to their antioxidant properties since *in vitro* these phenolic compounds can scavenge radicals[19] and inhibit pro-oxidant enzyme activity[20]. Consistent with its antioxidant activity, caffeic acid scavenges superoxide, and decreases production of lipid peroxides in mouse liver microsomes[21]. Gossypol inhibits microsomal lipid peroxidation and lipoxygenase activity induced by a NADPH generating system *in vivo*[22, 23].

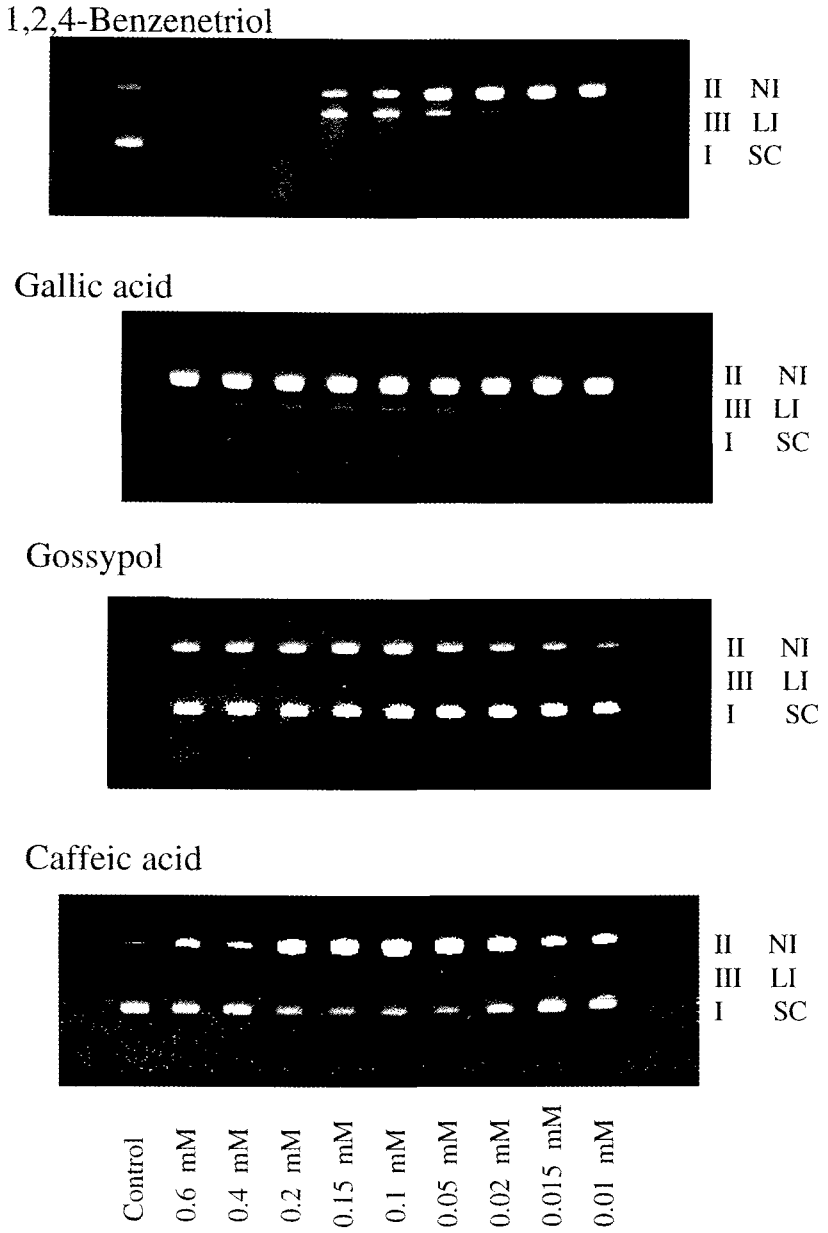


FIGURE 2 DNA breakage induced by phenolic compounds. The photograph shows fluorescence of gels stained with ethidium bromide. pdBPV-1 DNA (0.02 μg/10 μl reaction mixture) was exposed to phenolic compounds in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 30 minutes. Control means no treatment by phenolic compounds. Because two groups ran on a single gel, gallic acid and 1,2,4-benzenetriol shared the control in the 1,2,4-benzenetriol group. Similarly gossypol and caffeic acid shared the control in the caffeic acid group. The notations used: I SC, supercoiled DNA; II NI, nicked circular DNA; III LI, linear DNA. The relative amounts of each DNA form were determined by densitometry. The gels shown are representative of experiments done in duplicate

The aim of the present work is to evaluate the relative pro-oxidant and antioxidant actions of these selected plant phenolic compounds on DNA strand breakage under different reaction conditions, and to compare them to a phenolic compound, 1,2,4-benzenetriol, that is strongly genotoxic *in vivo*[24,25] and *in vitro*[26]. We have investigated the extent to which DNA nicking by these plant phenolic compounds reflects their prooxidant activity, and describe the effects of transition metal ions (copper and iron), a metal chelator (desferrioxamine) and scavengers of oxygen-derived active species. To investigate their ameliorating or exacerbating influence under a common pro-oxidant stress, we tested the effects of these phenolic compounds on DNA strand breakage induced by ascorbate plus iron.

MATERIALS AND METHODS

Reagents

1,2,4-Benzenetriol and gallic acid were purchased from Aldrich Chemical Co. (Milwaukee, WI). Caffeic acid and L-ascorbate were purchased from Sigma Chemical Co. (St. Louis, MO). Cupric sulfate, cuprous chloride, ferric chloride and ferrous sulfate were purchased from Fisher Scientific Co. (Fairlawn, NJ). Gossypol was supplied by Peking Union Medical College, China. Superoxide dismutase (4050 U/mg) was from DDI Pharmaceutical Inc., (Mountain View, CA). Catalase (65,000 U/mg) was from Boehringer Mannheim Co. (Dorval, PQ). Desferrioxamine was a gift from Ciba-Geigy Pharmaceutical Co. (Summit, NJ).

Preparation of plasmid pdBPV-1 (142–6) DNA

Supercoiled plasmid pdBPV-1 DNA was prepared according to Maniatis et al.[27]. Briefly, E. coli containing the plasmid was grown overnight in standard LB medium (10 gm bacto-tryptone, 5 gm bacto-yeast extract and 10 gm NaCl per litre of deionized H_2O), supplemented with ampicillin (40 mg per litre of LB medium). Bacteria were recovered by centrifugation. The bacteria were lysed by digestion with lysozyme and treatment with alkali. The lysate was precipitated by isopropanol and recovered by centrifugation. The plasmid DNA was selectively precipitated with polyethylene glycol (PEG). The supercoiled plasmid DNA and linear DNA (including the chromosomal DNA from bacteria) were separated by centrifugation in a CsCl-ethidium bromide gradient, and the purified supercoiled plasmid DNA was recovered by ethanol precipitation.

DNA concentration was determined by measuring the absorbance at 260 nM using a Lambda 3 UV/VIS spectrophotometer. Based on the molar extinction coefficient for typical DNA, one unit of absorbance corresponds to 50 μg/ml double-stranded DNA. DNA was stored in 10 mM Tris-HCl/1mM EDTA buffer, pH 8.0, at –20°C.

Preparation of ^{3}H-labelled PM2 DNA

^{3}H-labeled supercoiled bacteriophage PM2 DNA was prepared as described by Espejo and Canelo[28,29] and modified by Tsang[30]. Pseudomonas Bal-31 was grown at 28–30°C in 500 ml Bal-broth [10 ml of 10 mM Tris-HCl (pH 7.5), 12 gm magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 26 gm sodium chloride, 8 gm bacto-nutrient broth, 10 ml of 1 M calcium chloride and 3.5 ml of 20% potassium chloride made up to one litre with deionized water]. When the bacteria reached a density of 3×10^8/ml, 50 mg 2′-deoxyadenosine (Sigma) was added. Five minutes later, the bacteria were infected with $1–2 \times 10^{12}$ bacteriophage PM2 particles. After five minutes, 0.5 mCi of methyl-^{3}H thymidine (25 Ci/mmol, Amersham International) was added. The culture was incubated overnight. Phage particles were purified by cesium chloride density equilibrium centrifugation. The phage particles were dialyzed against 1.2 litre buffer (0.02 M Tris-HCl, pH 7.5,

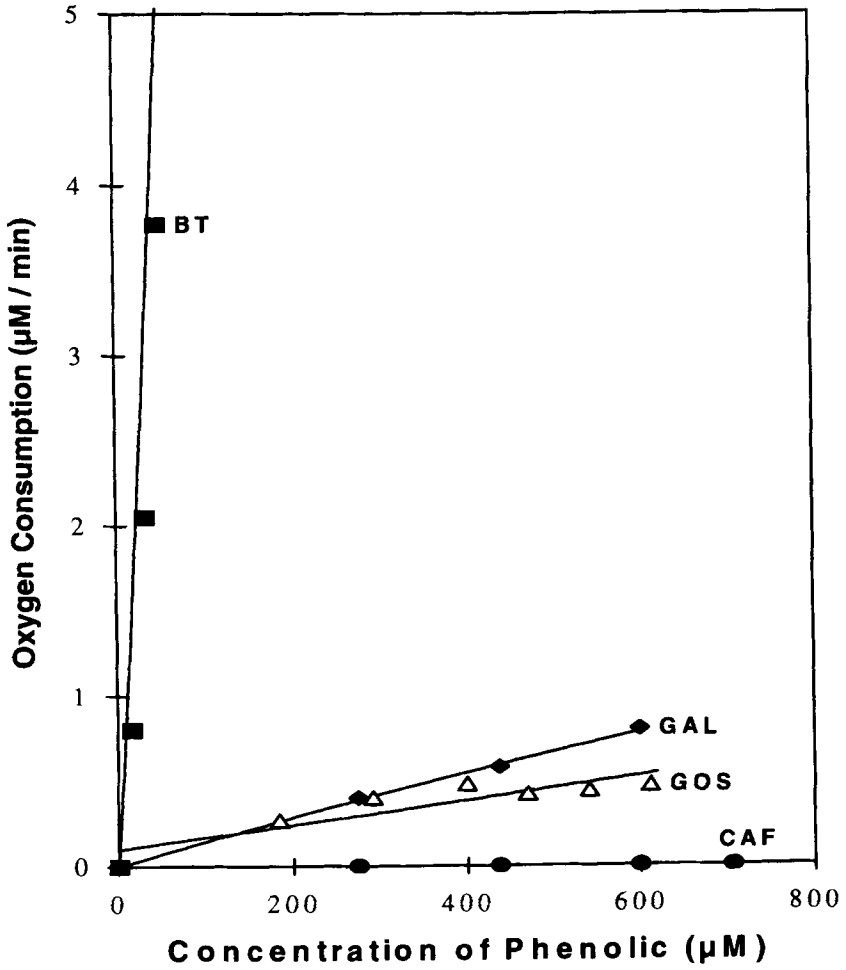
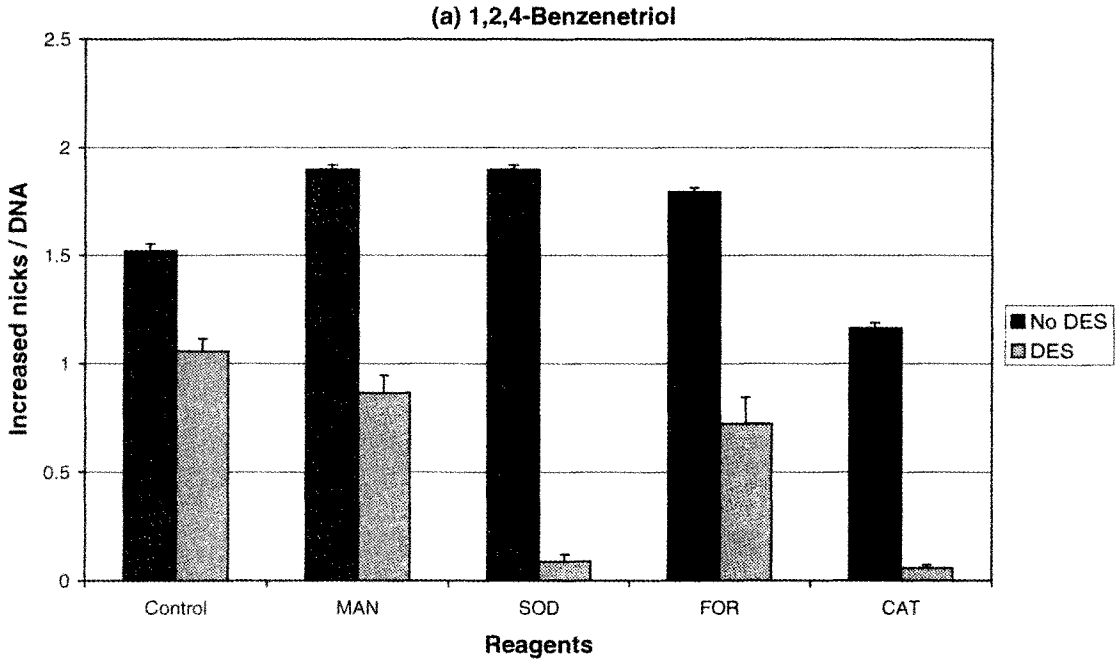


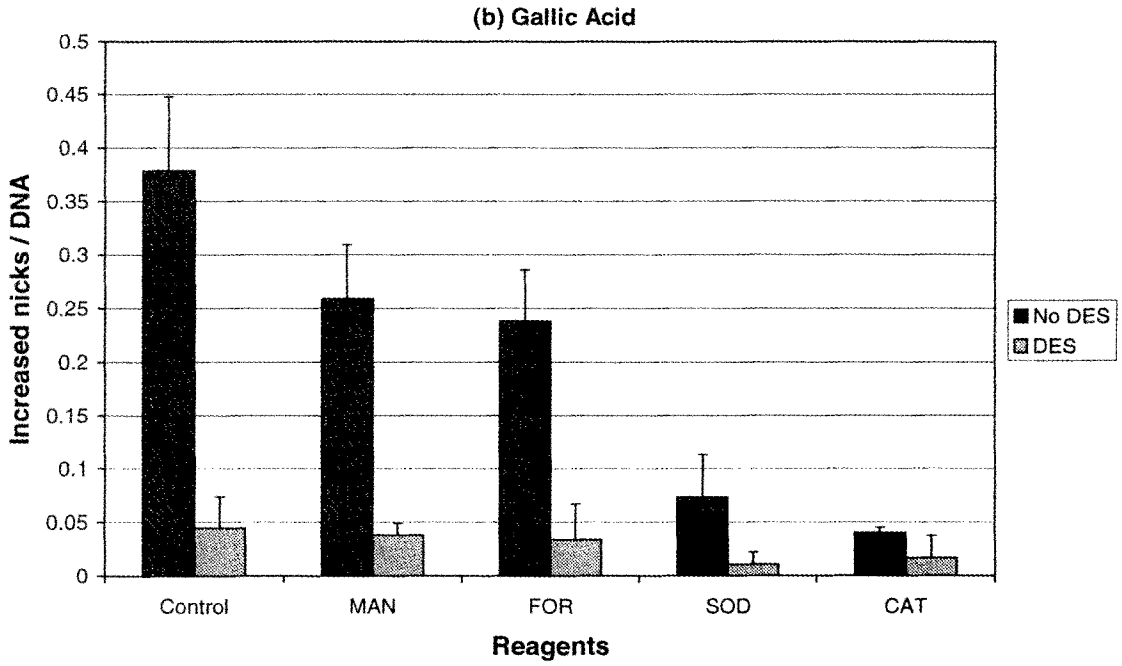
FIGURE 3 Comparison of oxygen consumption by phenolic compounds: Velocity / concentration relationship. The graph shows rates of oxygen consumption measured polarographically over a range of concentrations of each phenolic compound. Reactions were carried out in air-saturated (246 ± 6 µM O_2) 50 mM sodium phosphate buffer, pH 7.40–7.44 at 25°C. The reaction was initiated by injection of the phenolic compound into the chamber. Squares represent 1,2,4-benzenetriol (BT); diamonds represent gallic acid (GAL); triangles represent gossypol (GOS); circles represent caffeic acid (CAF). Lines are drawn smoothly through the points by an arbitrary least squares spline function having no mechanistic significance

0.1 M NaCl, 1 mM EDTA) at 4°C and lysed by 10% sodium dodecyl sulfate. Phage DNA was extracted from the aqueous phase by phenol.

Agarose gel electrophoresis analysis of DNA cleavage

Supercoiled plasmid BPV-1 DNA was incubated with the reagents for the defined period, and the reaction was terminated by diluting the samples in loading buffer (0.05% bromophenol blue, and 6% glycerol). These samples were fractioned by agarose gel electrophoresis at 60 volts for 90 minutes. Each gel contained 0.7% (W/V) agarose gel prepared with a buffer that consisted of 0.04 M Tris-acetate, 0.002 M EDTA. The gels were stained with 0.5 µg/ml ethidium bromide for 30 minutes, destained in water for 2–3 minutes, and





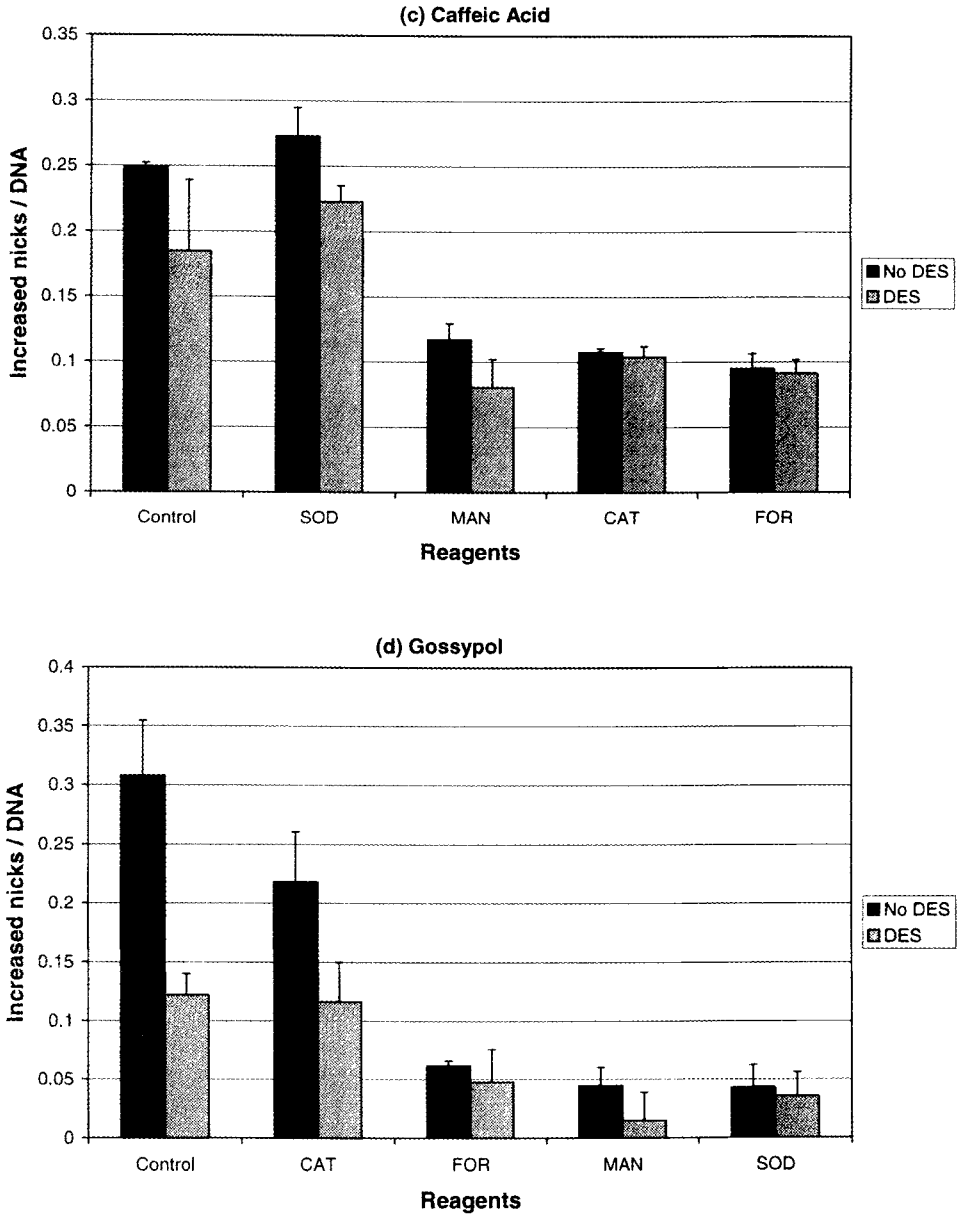


FIGURE 4 Effects of antioxidants on DNA breakage induced by phenolic compounds with or without desferrioxamine. ^{3}H-labelled PM2 DNA (0.1025 – 0.14 μg/50 μl reaction mixture) was exposed to phenolic compounds in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 15 minutes and the proportion of nicked DNA determined by the filter-binding assay. Abbreviations are as follows: FOR, formate 5 mM; MAN, mannitol 5 mM; DES, desferrioxamine (20 μM); SOD, superoxide dismutase (20 U/ml); CAT, catalase (20 U/ml). Inactivated superoxide dismutase and catalase were boiled for 40 minutes prior to addition to the reaction mixtures added prior to addition of phenolic compounds, and were ineffective (data not shown). The data show the increase in DNA nicks induced by the phenolics above background (0.17 nicks/DNA molecule). Concentrations of phenolics were used that gave a substantial increase in nicks over background. The bars represent the means of experiments done in triplicate, and error bars show the standard deviation. Graphs represent: (a) 1,2,4-Benzenetriol (18 μM); (b) Gallic acid (200 μM); (c) Caffeic acid (500 μM); (d) Gossypol (700 μM)

photographed under ultraviolet illumination. A Polaroid MP-4 camera and Polaroid 57 or 55 professional films were used for the photography.

Supercoiled circular (form I), nicked circular (form II), and linear (form III) forms of plasmid DNA migrated through 0.7% (W/V) agarose gels at different rates. Form I migrated fastest, and form III migrated between form I and II.

Analysis of DNA damage by filter-binding assay using nitrocellulose filters

The ^{3}H-labeled supercoiled PM2 DNA was incubated with test reagents. The incubation was terminated via precipitation of PM2 DNA by ethanol. PM2 DNA was resuspended in 25 or 50 µl of 1 mM sodium phosphate buffer (pH 7.4). The samples were then treated as follows, to allow strand separation of the nicked DNA molecules. A 10 µl aliquot of the DNA reaction mixture was diluted with 40 µl of 10 mM Tris-HCl/1 mM EDTA buffer, pH 8.0. To this solution, 5 µl of 100 mM Tris-HCl (pH 7.5) and 0.15 ml SE buffer (0.01% sodium dodecyl sulfate, 2.5 mM EDTA-NaOH, pH 7.0) were added. The DNA was then denatured by the addition of 0.2 ml of 0.3 M K_2HPO_4-KOH (pH 12.4) for 2 minutes at room temperature, and neutralized with 0.1 ml of 1 M KH_2PO_4-HCl (pH 4.0). The solution was diluted with 0.2 ml of 5 M NaCl and 5 ml of NT buffer (1 M NaCl, 50 mM Tris-HCl, pH 8.0), and filtered through nitrocellulose filters presoaked in NT buffer. The reaction tube was rinsed with 2 ml NT buffer, and the rinse was filtered. The filters were then washed with another 2 ml NT buffer, dried, and the radioactivity was determined by liquid scintillation counting. Total DNA was determined by measuring the radioactivity of 10 µl of reaction mixture spotted on a blank filter. By this method, the average number of nicks (N) per DNA molecule can be calculated by equation $N = -\ln P$; where P was the fraction of DNA that was not retained on the nitrocellulose filter[31].

Densitometric analysis

The relative amounts of supercoiled, nicked circular and linear DNA were estimated by densitometric scanning of the photograph negatives with a SP4100 computing integrator and Soft Laser Scanning Densitometer from Hoefer Scientific instruments, San Francisco, California.

Measurement of oxygen consumption

Deionized distilled water, 50 mM sodium phosphate buffer (pH 7.4 and 8.7), or 100 mM Na_2CO_3 buffer (pH 10.0) was bubbled with argon in sealed vials for at least 30 minutes. Vials containing solid phenolic reagents were filled with argon, and deoxygenated buffer was added to dissolve the phenol or ascorbate, with the minimum amount of alkali needed to ensure complete dissolution. Aliquots of the reagent solutions were added to 3 ml of air-saturated 50 mM sodium phosphate buffer to initiate the reactions. Final pH in the reaction solutions was between 7.40 and 7.44. Oxygen consumption was monitored using a Clark-type oxygen electrode at 25°C. Data were collected on a strip chart recorder, and the maximal rates of oxygen consumption calculated and plotted in the figures.

Statistical analysis

Standard statistical methods were used to calculate the standard errors of the means. All reactions were performed at least in duplicate and usually triplicate. The standard deviation for a population was divided by the square root of the number of replicates to estimate the standard error of the means. Significance of differences between means was examined by Student's t-test and analysis of variance. The level of confidence required for significance was set in advance to $p < 0.05$.

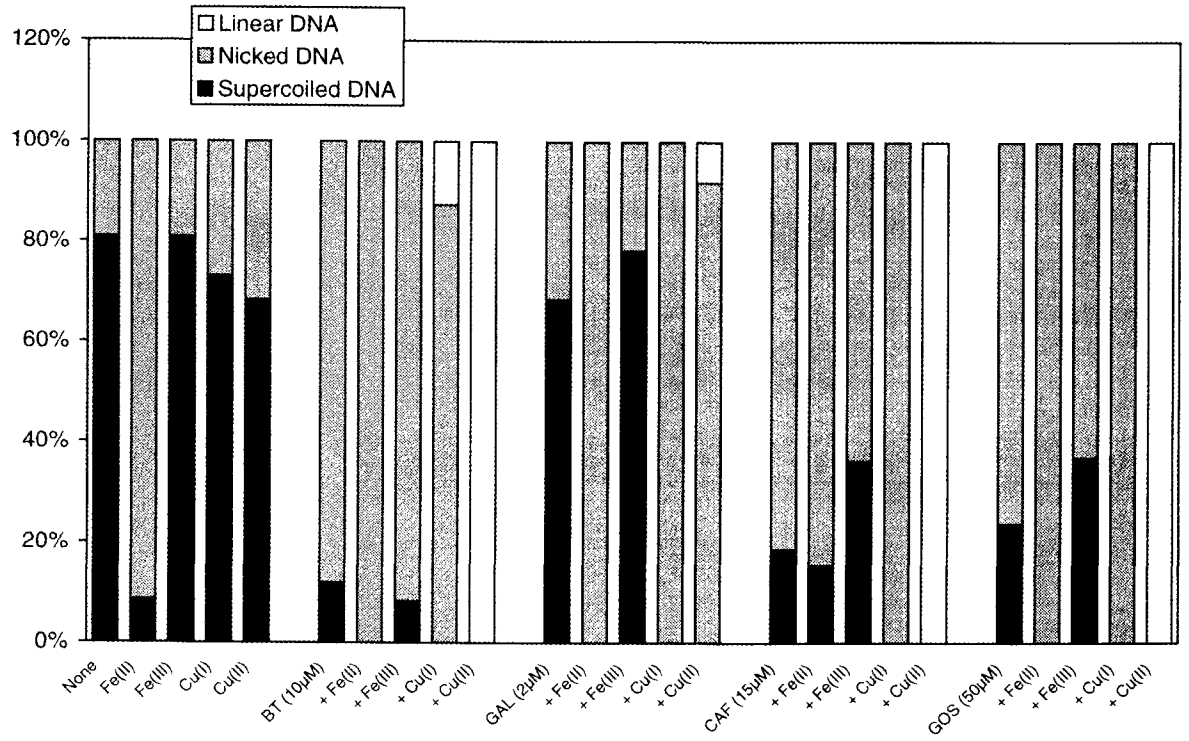


FIGURE 5 Effects of copper and iron on DNA breakage induced by phenolic compounds. pdBPV-1 DNA (0.02 μg/10 μl reaction mixture) was exposed to the phenolic compound, copper (25 μM) and iron (25 μM) in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 30 minutes. DNA breakage was evaluated by agarose gel electrophoresis and densitometer as described in Material and Methods. The bars represent the means of 2–4 experiments, and standard errors of the means were ±10% or less. Abbreviations are as follows: Fe(II), ferrous sulfate; Fe(III), ferric chloride; Cu(I), cuprous chloride; Cu(II), cupric sulfate. Phenolic compound concentrations were: 1,2,4-benzenetriol (BT), 10 μM; gallic acid (GAL), 2 μM; caffeic acid (CAF), 15 μM; gossypol (GOS), 50 μM

RESULTS

Phenolic compounds alone induce DNA breakage

Phenolic compounds induce rapid DNA breakage at low concentrations

All four phenolic compounds cleaved supercoiled plasmid pdBPV-1 DNA [form I] to nicked circular DNA [form II] or, at higher concentrations to linear DNA [form III]. The phenolic compounds differ in ability to nick DNA with damage ranging from 20 to 100% of the DNA molecules (densitometry analysis) at 0.02 mM (Fig. 2). Over the concentration range of 0.01 mM to 0.2 mM, the relative DNA breaking activity was in the order of 1,2,4-benzenetriol > gallic acid > caffeic acid > gossypol. 1,2,4-Benzenetriol at 0.01 mM rapidly converted supercoiled DNA to nicked circular DNA. At 0.05 mM 1,2,4-benzenetriol, linear DNA was formed, and at 0.2 mM, DNA was almost completely degraded to smaller heterogeneous DNA fragments. As can be seen in Figure 2, gallic acid converted supercoiled DNA to nicked circular DNA, but not to linear DNA or DNA fragments. Caffeic acid induced less DNA breakage than gallic acid, but

more than gossypol. Gossypol was the least effective in inducing DNA breakage, converting part of the supercoiled DNA to nicked circular DNA. Cleavage of DNA by caffeic acid was biphasic, decreasing at concentrations above 0.2 mM.

DNA nicking ability correlates with ability to reduce oxygen, except with gossypol

On the assumption that DNA-breaking activity of a phenolic compound and its ability to reduce oxygen are both determined in part by its reduction potential, we compared the rates of reduction of oxygen by these phenolic compounds. Rates of oxygen consumption were in the order 1,2,4-benzenetriol > gallic acid > gossypol > caffeic acid, with 1,2,4-benzenetriol being over 70 times as reactive as gallic acid (Fig. 3). Thus the relative reactivity toward oxygen predicts DNA-breaking ability, except that in comparison with caffeic acid, gossypol's oxygen consumption was unexpectedly high, given that it had the lowest DNA nicking activity.

Oxygen derived active species mediate DNA nicking by phenolic compounds

To clarify the roles of oxygen-derived active species in DNA breakage induced by these phenolic compounds, we examined the effects of antioxidants, in the presence and absence of desferrioxamine (Fig. 4 a-d). For qualitative comparisons, we chose concentrations of the phenolics that gave readily measurable levels of DNA damage (>0.2 nicks / DNA molecule). Scavengers were added to about ten fold excess of the level needed to remove the maximum expected level of reactive species. Desferrioxamine was used at a concentration (20 μM) ~10 times the possible concentration of adventitious metals, but at a level that would not significantly scavenge radicals. With affinities for iron and copper of Ka = 10^{31} and 10^{14}[32], desferrioxamine should compete effectively with the catecholate groups of the phenolic compounds for binding of metals (eg. Ka's of catechol for iron and copper are 10^{20} and 10^{13})[33].

Desferrioxamine alone decreased the number of nicks induced per DNA with each of the phenolics (Fig. 4 a-d), but to differing extents. This protective effect was strong with gallic acid (88%) and gossypol (60%), and mild with 1,2,4-benzenetriol (30%) and caffeic acid (26%).

In absence of desferrioxamine, catalase inhibited DNA breakage by all four phenolic compounds by 30 to 90%, least effectively with 1,2,4-benzenetriol and gossypol. Superoxide dismutase decreased DNA breakage induced by gallic acid and gossypol by 80 to 86%. However, superoxide dismutase failed to protect against DNA breakage induced by caffeic acid, and mildly (25%) stimulated DNA breakage by 1,2,4-benzenetriol. Formate and mannitol decreased DNA breakage induced by the phenolic compounds by 30–90%, except with 1,2,4-benzenetriol.

The presence of desferrioxamine enhanced or allowed inhibition by other scavengers in some cases. With 1,2,4-benzenetriol (Fig. 4a), while catalase or superoxide dismutase had relatively little effect alone, they were strongly inhibitory in the presence of desferrioxamine. With gallic acid, although desferrioxamine alone gave strong inhibition, it also further enhanced inhibition by superoxide dismutase and catalase. Desferrioxamine thus changes the mechanism of radical production and/or DNA nicking in these cases.

Summing up these actions, hydrogen peroxide and metals were the common active species in DNA breakage induced by all the phenolic compounds tested. Metals were most strongly involved in DNA nicking by gallic acid and gossypol. Superoxide was also strongly involved DNA breakage induced by gallic acid and gossypol, but not at all with caffeic acid. Superoxide was involved with 1,2,4-benzenetriol only when metals were chelated by desferrioxamine. Hydrogen peroxide was most involved with caffeic acid and gallic acid, and with 1,2,4-benzene-

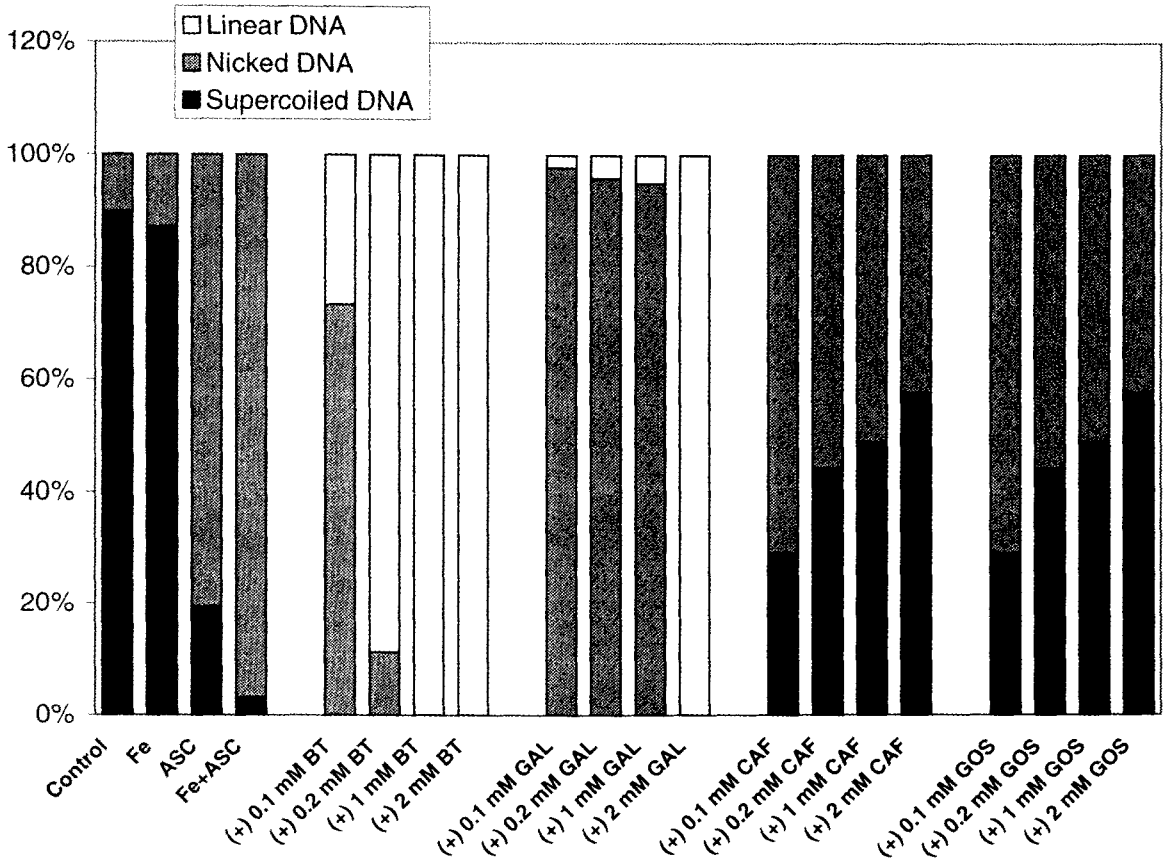


FIGURE 6 Effects of phenolic compounds on DNA breakage induced by ascorbate/Fe. pdPV-1 DNA (0.046 μg/25 μl reaction mixture) was exposed to different combinations of 0.02 mM ferric chloride (Fe), 0.2 mM ascorbate (ASC) and graded concentrations of the phenolic compound in 1 mM sodium phosphate buffer (pH 7.4) at 37°C for 30 minutes. DNA cleavage was evaluated by agarose gel electrophoresis and densitometer as described in Materials and Methods. Standard errors of the means for 2–4 experiments were ±10% or less. Phenolic compounds were added prior to ascorbate and iron. Caffeic acid (CAF); Gossypol (GOS); Gallic acid (GAL); 1,2,4-Benzenetriol (BT)

triol in the presence of desferrioxamine. Hydroxyl radicals also mediated DNA breakage induced by these phenolic compounds, most evidently by caffeic acid and gossypol.

Transition metals potentiate DNA breakage induced by phenolic compounds

Transition metal ions, including copper and iron, potentiate damaging actions of many pro-oxidants[34–39]. Figures 4 (a-d) show that desferrioxamine alone diminished DNA nicking induced by these phenolic compounds from 27 to 89%, strongly with 1,2,4-benzenetriol and gallic acid, and weakly with caffeic acid. Traces of metal ions in solution are evidently crucial to DNA breakage. In the presence of desferrioxamine, addition of either superoxide dismutase, catalase, or hydroxyl radical scavengers further decreased DNA nicking.

Added alone, only Fe(II) induced significant DNA nicking. Cu(I), Cu(II), or Fe(III) alone were relatively ineffective. Nicking of DNA by each of the phenolic compounds however was enhanced

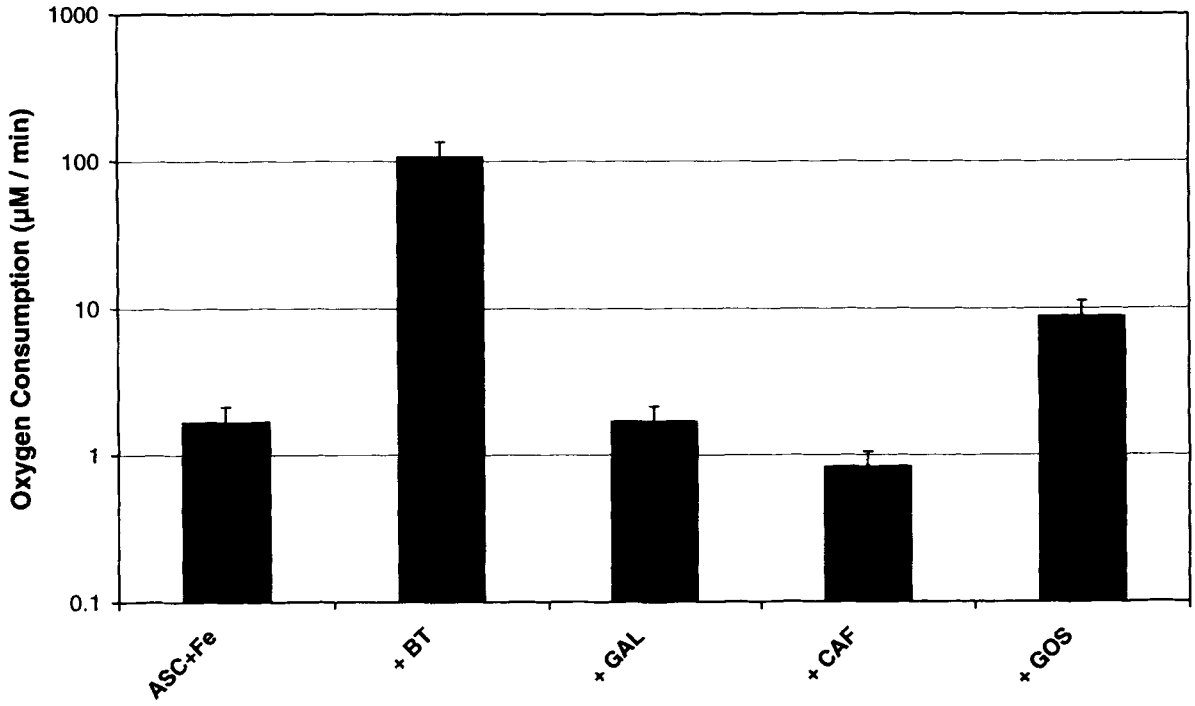


FIGURE 7 Oxygen consumption of ascorbate/Fe(III): Effects of phenolic compounds. Experimental conditions are as stated in the legend to Fig. 3. The Y axis represents oxygen consumption in logarithmic scale. Error bars represent the standard deviation of 2–4 measurements. Abbreviations are as follows: ASC, ascorbate (1.5 mM); Fe, ferric chloride (0.4 mM); BT, 1,2,4-benzenetriol (0.75 mM); GAL, gallic acid (0.75 mM); CAF, caffeic acid (0.75 mM); GOS, gossypol (0.5 mM)

notably by the presence of metal ions (Fig. 5). Copper ions were significantly more effective than iron ions in potentiating DNA-breaking activity of all of the phenolic compounds.

Phenolic compounds can inhibit or enhance DNA breakage by ascorbate/Fe(III)

Ascorbate and Fe(III) together comprise a hydrogen peroxide and hydroxyl radical generating system that readily induces DNA strand breaks[40]. Figure 6 shows the effects of phenolic compounds on DNA breakage induced by the 0.2 mM ascorbate/0.02 mM iron couple. Caffeic acid or gossypol inhibited DNA breakage induced by ascorbate in presence of Fe. This inhibition was concentration-dependent over the range of 0.1 mM to 2 mM, i.e. 0.5 to 10 times the concentration of ascorbate. DNA nicking was decreased about one third ($p < 0.05$), even when these two phenolic compounds were present at concentrations half that of ascorbate.

We concomitantly determined the actions of these phenolic compounds on oxygen consumption by 1.5 mM ascorbate in the presence of 0.4 mM Fe (III) (Fig. 7). Caffeic acid (0.75 mM) and gallic acid (0.75 mM) decreased the oxygen consumption by ascorbate/Fe(III) by 52% and 22%. Gossypol (0.5 mM) and 1,2,4-benzenetriol (0.75 mM), in contrast, were pro-oxidants, increasing oxygen consumption 5-fold and 62-fold. Atypically, gossypol accelerated oxygen consumption but was protective against DNA damage by ascorbate/Fe (Fig. 6).

DISCUSSION

Each of the phenolic compounds can act as a pro-oxidant to induce DNA breakage

Oxygen-derived active species such as superoxide, hydrogen peroxide and hydroxyl radicals mediate DNA breakage induced by the phenolic compounds tested. However, there were multiple and different mechanisms for each phenolic.

With 1,2,4-benzenetriol, desferrioxamine and catalase were the strongest inhibitors of the DNA breakage, suggesting metals and hydrogen peroxide as the major determinants in DNA breakage. However, when metals were chelated by desferrioxamine, superoxide and to a lesser extent hydroxyl radicals also played roles in DNA breakage.

In the case of gallic acid, desferrioxamine, catalase and superoxide dismutase were comparable in their inhibitory effects on DNA breakage. Although formate and mannitol were weaker in their potency, the results suggest that hydroxyl radicals also participated in the DNA cleavage reaction.

Since superoxide dismutase slightly accelerated DNA breakage induced by caffeic acid, superoxide may be a net terminator of the free radical chain reaction in autoxidation of caffeic acid. On the other hand, mannitol, catalase and formate were equipotent inhibitors. Thus hydrogen peroxide and hydroxyl radicals were important determinants in DNA breakage with caffeic acid.

With gossypol, the relative protection by the antioxidants indicated that superoxide and hydroxyl radicals were more dominant intermediates than hydrogen peroxide in the DNA breakage. Thus $O_2^{\cdot -}$ participated more directly than as a precursor to H_2O_2.

Overall, the further decrease of DNA breakage induced by a combination of catalase and desferrioxamine or hydroxyl radical scavengers and desferrioxamine (with 1,2,4-benzenetriol, gallic acid, and gossypol) suggests a site-specific Fenton type reaction. In these cases the presence of desferrioxamine would prevent a site-specific mechanism by removing metals from the DNA strand, and forcing outer sphere electron transfers.

The effect of scavengers on DNA damage by a phenolic can differ from their effects on oxygen reduction

As exemplified by 1,2,4-benzenetriol, the protection by scavengers against DNA damage is not simply an effect on oxygen reduction. The effects of scavengers on DNA nicking by 1,2,4-benzenetriol (Fig. 4-a) were different from their effects on the reaction of 1,2,4-benzenetriol with oxygen[41,42] in some aspects. The effects of superoxide dismutase plus desferrioxamine were similar, strongly inhibiting both autoxidation and DNA nicking. Superoxide dismutase alone however, slows the reaction of 1,2,4-benzenetriol with oxygen, but over the time of the nicking experiments mildly increased DNA damage.

Presumably, production of H_2O_2, and reduction of DNA-bound metals were the important factors in DNA nicking, and were not limited by superoxide dismutase. Also, desferrioxamine alone gives a lag, but then accelerates 1,2,4-benzenetriol autoxidation (due to accelerated reoxidation of reduced iron), but mildly inhibited DNA nicking. This mild inhibition is likely due to removal of metals from the DNA, thus decreasing site-specific damage. The most notable difference between autoxidation and DNA nicking was with the effects of catalase. Catalase has no effect on the reaction of 1,2,4-benzenetriol with oxygen, either in the presence or absence of desferrioxamine. However, catalase mildly inhibited DNA damage in the absence of desferrioxamine, and almost prevented damage in the presence of desferrioxamine. Thus metals and H_2O_2 are strongly cooperative in producing the damage to DNA, while either metals or $O_2^{\cdot -}$ propagate autoxidation.

Rates of reduction of oxygen by the phenolic compounds correlate imperfectly with DNA breakage

The ability of a phenolic compound to induce DNA breakage depends in part on the number and position of phenolic hydroxyl groups and other substituents on the benzene ring. Thus, *o*- and *p*-dihydroxyphenols readily induce DNA nicking, but *m*-dihydroxyphenols do not[2]. The replacement of a hydroxyl group by an amino group also decreases the DNA breaking activity[2]. Increased genotoxicity is often associated with increased reduction of oxygen, presumably reflecting increased ability to generate active species. The rapid reductions of oxygen by 1,2,4-benzenetriol and gallic acid are thus consistent with their strong DNA nicking ability.

The inability of caffeic acid to induce measurable oxygen consumption was consistent with its more positive reduction potential resulting from ring deactivation by alkenyl and carboxyl groups (Fig. 1). This weak ability to reduce oxygen correlated with its relatively weak ability to nick DNA. Nevertheless, since catalase and hydroxyl radical scavengers inhibited caffeic acid-induced DNA breakage substantially, one can infer that H_2O_2 and $^{\bullet}OH$ were involved. Therefore the ability to donate electrons in Fenton reactions does not necessarily reflect ability to reduce molecular oxygen. Evidently, even the slow autoxidation allows caffeic acid to generate sufficient hydrogen peroxide to nick DNA. Failure of superoxide dismutase to inhibit suggests that caffeic acid itself, or its semiquinone, rather than superoxide, serve as Fenton donors. That desferrioxamine did not sensitize to further inhibition by catalase or $^{\bullet}OH$ scavengers is evidence against site-specific metal-mediated DNA damage with caffeic acid.

The oxygen consumption of gossypol was inconsistent with its relatively low DNA nicking ability. Clearly gossypol produces oxygen-derived active species. Based on the uncharacteristically small protection by catalase, gossypol may reduce oxygen with a relatively small yield of H_2O_2.

Metals are strong stimulants of DNA breakage through redox cycling

The observation that, added alone, reduced iron was more genotoxic than reduced copper is contrary to thermodynamic considerations, since ferrous ion is a weaker reductant of oxygen. It also contrasts with the commonly observed greater genotoxicity of copper in DNA nicking[37], and base damage[38]. However, in the current studies, copper was more effective than iron when the presence of reductants allowed redox cycling (Fig. 5). On this basis, copper is more effective than iron when ability to both accept and donate electrons is required.

The phenolic compounds are anti-oxidants in relation to a more reactive species and pro-oxidants in relation to less reactive species

The actions of the phenolic compounds on ascorbate/Fe-induced DNA nicking (Fig. 6) may reflect (a) metal binding, (b) ability to generate or scavenge active oxygen species, and (c) an influence on site-specific attack on DNA by ascorbate/Fe. Since all four phenolic compounds tested include the *o*-diphenol residue, they readily bind metals. In comparison with ascorbate, 1,2,4-benzenetriol is a strong reducing agent. This is reflected in their respective rates of autoxidation [64.1 μM O_2/min for 0.75 mM 1,2,4-benzenetriol, versus 1.92 μM/min for 1.5 mM ascorbate in presence of 0.4 mM Fe(III)]. Gallic acid, gossypol and caffeic acid are weaker reductants than 1,2,4-benzenetriol, as reflected in much lower rates of oxygen consumption (Fig. 3). Addition of 1,2,4-benzenetriol increased oxygen consumption when added to ascorbate in the presence of Fe, whereas caffeic acid and gallic acid decreased oxygen consumption (Fig. 7). On this basis, with respect to ascorbate

plus Fe, 1,2,4-benzenetriol is a pro-oxidant, while caffeic acid and gallic acid are antioxidants. With regard to oxygen consumption then, it is intuitively satisfying that a phenolic species is an anti-oxidant in the presence of more reactive species, and pro-oxidant in the presence of less reactive species.

The ability of caffeic acid to inhibit DNA breakage by ascorbate/Fe(III) paralleled its ability to attenuate oxygen consumption by ascorbate/Fe(III). However, the actions of gallic acid and gossypol on DNA breakage and on oxygen consumption by ascorbate/Fe were paradoxical. Gallic acid, which slowed oxygen consumption by ascorbate in the presence of Fe, nevertheless accelerated DNA breakage. In contrast, gossypol, which increased oxygen consumption by ascorbate/Fe, protected superhelical DNA against nicking induced by ascorbate/Fe. The results show that the pattern of intermediates produced rather than the ability to reduce oxygen, is correlated with DNA damage.

CONCLUSIONS

1,2,4-Benzenetriol, caffeic acid, gallic acid and gossypol, are pro-oxidants which cleave DNA strands when they are the only active species in the reaction system. Transition metal ions aggravate DNA damage by these phenolic compounds, and generation of hydrogen peroxide is pre-eminent in causing damage. 1,2,4-Benzenetriol and gallic acid are damaging under all circumstances tested, but caffeic acid and gossypol can protect DNA against cleavage by ascorbate plus Fe. Overall the results are consistent with two long standing postulates in free radical pathology: "without metals there is no damage", and "without H_2O_2 there is no damage". The results illustrate that pro-oxidant activity is relative, and that in the presence of a common oxidative stress (ascorbate plus iron), the benzenetriols (1,2,4-benzenetriol, gallic acid) augment damage, while the catechols (caffeic acid, gossypol) moderate damage.

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