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DEHYDROZINGERONE AND ISOEUGENOL AS INHIBITORS OF LIPID PEROXIDATION AND AS FREE RADICAL SCAVENGERS

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Abstract—The antioxidant properties of three related compounds, dehydrozingerone, isoeugenol and eugenol, were investigated using various models. Isoeugenol was found to be the most active in inhibiting ferrous-ion-, ferric-ion- and cumene-hydroperoxide-induced lipid peroxidation in rat brain homogenates. These compounds also showed significant hydroxyl radical scavenging activity. Isoeugenol was potent in scavenging superoxide anion generated by the xanthine–xanthine oxidase system, whereas eugenol was found to inhibit xanthine oxidase. The high antioxidant activity of isoeugenol may be due to the presence of a conjugated double bond, which increases the stability of the phenoxyl radical by electron delocalization. Such electron delocalization is not possible with eugenol. In dehydrozingerone, the stability was decreased by an electron withdrawing keto group at the para position.

Dehydrozingerone and curcumin share many chemical and pharmacological properties. Both are styryl ketones with similar substitutions on the phenyl ring (Fig. 1). In addition, both have exhibited potent anti-inflammatory activity in various models [1–3]. Curcumin is a good inhibitor of lipid peroxidation [4, 5] and a scavenger of oxygen free radicals [6, 7]. Since our previous studies have shown moderate activity for dehydrozingerone as an oxygen free radical scavenger [8], we were interested in investigating whether dehydrozingerone also has the ability to inhibit lipid peroxidation.

While the present study was in progress, a report appeared in this journal about the potent inhibition of lipid peroxidation by eugenol [9]. Eugenol and dehydrozingerone have many structural similarities, such as substitution on the phenyl ring and a side chain double bond that is not conjugated with the phenyl ring (Fig. 1).

However, isoeugenol, a structural isomer of eugenol with its side chain double bond conjugated with the phenyl ring, is much more similar to dehydrozingerone (Fig. 1). Isoeugenol has not been studied thus far for its effect on lipid peroxidation. Hence, in the present study, we have investigated the structure–activity relationships among dehydrozingerone, isoeugenol and eugenol for their effect on lipid peroxidation. Further, in order to understand the mechanism of action, we have also studied their ability to scavenge free radicals and to reduce ferric ions.

MATERIALS AND METHODS

Materials. Eugenol, cumene hydroperoxide, butylated hydroxytoluene (BHT)†, xanthine, xanthine oxidase, nitroblue tetrazolium (NBT), and 2-deoxy-D-ribose were obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Isoeugenol (98% pure) was from Industrial Perfumes Ltd. (Bombay, India). Dehydrozingerone was synthesized as reported by us previously [1].

Preparation of rat brain homogenate. Albino Charles-Foster rats (180–200 g) of either sex were used for the study. Prior to decapitation and removal of the brain, the animals were anesthetized with ether and perfused transcardially with ice-cold normal saline to prevent contamination of brain tissue with blood. Tissue was weighed, and homogenates (10%, w/v) were prepared in 0.15 M KCl and centrifuged at 800 *g* for 10 min. The supernatant was used immediately for the study of *in vitro* lipid peroxidation [4].

Lipid peroxidation. The incubation mixture contained, in a final volume of 1 mL, brain homogenate (0.5 mL), KCl (0.15 M) and ethanol (10 μL) or test compounds dissolved in ethanol. Peroxidation was initiated by adding, to give the final concentration stated, ferric chloride (0.2 mM) or ferrous sulfate (0.2 mM) or cumene hydroperoxide (0.1 mM). After incubating for 20 min at 37°, the reaction was stopped by adding 2 mL of ice-cold 0.25 N HCl containing 15% trichloroacetic acid, 0.38% thiobarbituric acid and 0.05% BHT. Following heating at 80° for 15 min, samples were cooled and then centrifuged at 1000 *g* for 10 min; the absorbance of the supernatant was measured at 532 nm. The amount of lipid peroxidation was determined using the molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as thiobarbituric acid reactive substances (TBARS) as described by Braughler *et al.* [10]. An identical experiment was performed in the absence of any inducing agents to determine the amount of TBARS due to spontaneous

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† Abbreviations: BHT, butylated hydroxytoluene; NBT, nitroblue tetrazolium; and TBARS, thiobarbituric acid reactive substances.

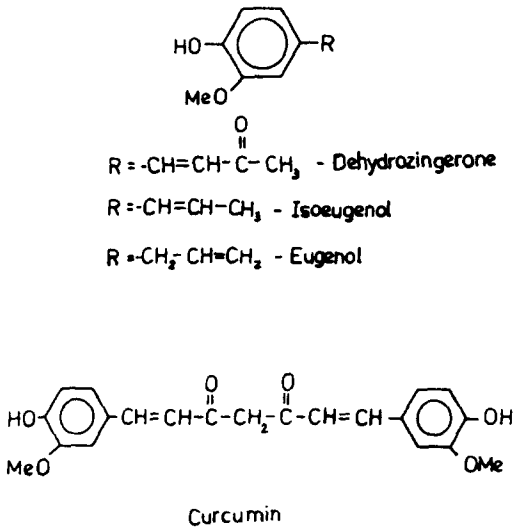

Fig. 1. Structure of curcumin, dehydrozingerone, isoeugenol and eugenol.

peroxidation, and that value was subtracted from the TBARS values obtained in the presence of inducing agents. Percent inhibition of TBARS formation was calculated by comparing diluent-only controls with drug-control experiments. Iron solutions were prepared fresh in distilled water and used immediately. Since most buffers trap hydroxyl radical or interfere with iron conversion [10], the reactions were carried out in unbuffered 0.15 M KCl. Results are expressed as the means ± SEM of triplicate experiments.

Deoxyribose degradation by iron-dependent hydroxyl radical [7]. Reaction mixtures contained, in a final volume of 1 mL, the following reagents at the final concentrations stated: deoxyribose (2.8 mM), KH_2PO_4-KOH buffer, pH 7.4 (20 mM), $FeCl_3$ (0.1 mM), EDTA (0.1 mM), H_2O_2 (1 mM), ascorbate (0.1 mM) and drug (variable concentration). The reaction mixture was incubated for 1 hr at 37°. Deoxyribose degradation was measured as TBARS, as described under lipid peroxidation. Test compounds were dissolved in 0.05 N NaOH, and the pH was adjusted to 7.4 with 0.1 N HCl. Ethanol was avoided as solvent since it interferes with hydroxyl radical determination.

Superoxide scavenging activity. Superoxide anion was generated by xanthine–xanthine oxidase and measured by the NBT reduction method [11]. To the reaction mixture in phosphate buffer, pH 7.8 (0.1 M), containing xanthine (0.1 mM), NBT (600 μM) and test compounds in ethanol (0.2 mL) was added xanthine oxidase (0.05 U/mL). After incubating for 10 min at 25°, the absorbance was read at 560 nm. Percent scavenging of superoxide was calculated by comparing the results of the test compounds with those of the control experiments (without test compounds). The test compounds were also studied for their ability to reduce NBT directly by incubating both of them together for 10 min and measuring the absorbance at 560 nm. The effects of the test compounds on xanthine oxidase activity were studied by measuring the formation of uric acid from xanthine. To the reaction mixture containing xanthine (0.1 mM) and test compound in phosphate buffer, pH 7.8 (0.1 M), was added xanthine oxidase (0.05 U/mL). After incubating for 10 min at 25°, the absorbance was measured at 295 nm and compared with the control (without test compound). The necessary corrections were made for the absorbance of test compounds.

Reduction of ferric ions. The reaction mixture containing *o*-phenanthroline (0.5 mg), ferric chloride (0.2 mM) and test compound dissolved in 0.2 mL ethanol in a final volume of 5 mL was incubated for 10 min at ambient temperature. The absorbance at 510 nm was measured [12]. In another experiment, sodium dithionite (0.3 mM) was added instead of the test compound, and the absorbance obtained was taken as equivalent to 100% reduction of all the ferric ions present.

Statistical analysis. Results are means ± SEM. Statistical analysis was carried out by Student's *t*-test and analysis of variance (ANOVA test) followed by Tukey's test for multiple comparisons. $P < 0.05$ was regarded as significant.

RESULTS

Inhibition of lipid peroxidation in rat brain homogenates. Lipid peroxidation in rat brain homogenate was stimulated by the addition of ferric ion (0.2 mM), ferrous ion (0.2 mM) or cumene hydroperoxide (0.1 mM). Ferric ions were more effective than ferrous ions or cumene hydroperoxide in stimulating peroxidation. The amounts of TBARS formed were 86.3, 19.5 and 5.7 nmol/mL of the tissue homogenate when stimulated by ferric ion, ferrous ion and cumene hydroperoxide, respectively. In unstimulated control experiments, the amount of TBARS formed was 2.1 nmol/mL. In all these experiments, BHT was added after the incubation but before heating. This prevents the formation of additional TBARS during the heating due to the breakdown of the lipid hydroperoxide [10]. Control experiments showed that none of the test compounds affected the measurement of TBARS (omission of the brain homogenate from the reaction mixture abolished chromogen formation). The TBARS values given are averages from one representative experiment conducted in triplicate. The variation within the experimental set was low. However, the day-to-day variation was much greater (about 15%), although the trends observed were consistent. The effects of dehydrozingerone, eugenol and isoeugenol on lipid peroxidation induced by ferric ion, ferrous ion and cumene hydroperoxide are given in Fig. 2, a–c. In the case of ferric-ion-stimulated lipid peroxidation (Fig. 2a), isoeugenol showed the highest inhibition followed by eugenol and dehydrozingerone. Isoeugenol was less effective than BHT at concentrations below 50 μM and similar to BHT at concentrations at or above 50 μM. At concentrations below 10 μM, all test compounds were less effective than BHT. Similar results were obtained with ferrous-ion-stimulated lipid peroxidation (Fig. 2b). The order of inhibition was

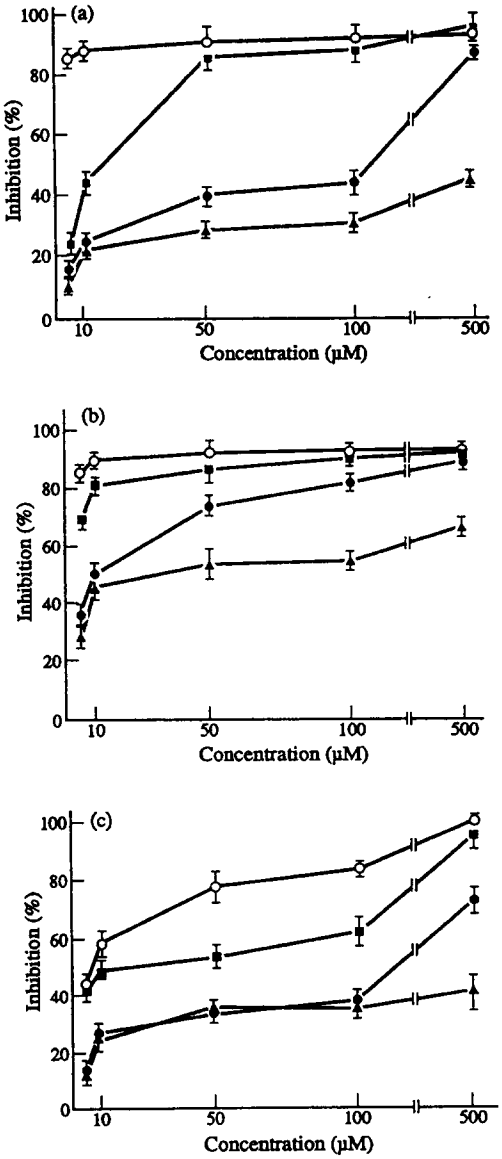

Fig. 2. Effects of test compounds on lipid peroxidation in rat brain homogenates induced by (a) ferric ion, 0.2 mM; (b) ferrous ion, 0.2 mM; and (c) cumene hydroperoxide, 0.1 mM. Key: isoeugenol (■—■), eugenol (●—●), dehydrozingerone (▲—▲), and BHT (○—○). Results are means ± SEM of experiments conducted in triplicate.

BHT, isoeugenol, eugenol and dehydrozingerone. In the case of cumene-hydroperoxide-stimulated peroxidation (Fig. 2c), the same trend was observed. Isoeugenol was more active than eugenol and dehydrozingerone and less active than BHT.

Deoxyribose degradation. The ability of each test compound to scavenge hydroxyl radical was measured by studying the competition between deoxyribose and the test compound for hydroxyl radical generated from the Fe^{3+}–ascorbate–EDTA–H_2O_2 system. The hydroxyl radicals attack deoxyribose and set off a series of reactions that eventually

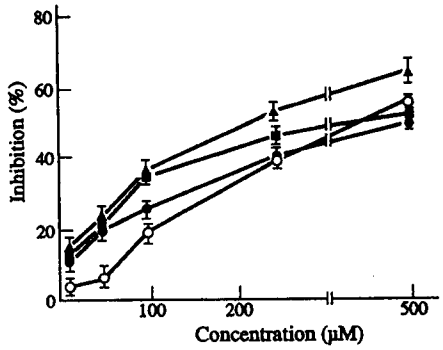

Fig. 3. Effects of test compounds on the degradation of deoxyribose mediated by hydroxyl radical. Key: isoeugenol (■—■), eugenol (●—●), dehydrozingerone (▲—▲), and DMSO (○—○). Results are means ± SEM of three experiments.

result in TBARS formation. When a molecule scavenges hydroxyl radical, it decreases TBARS formation. Figure 3 shows the inhibition of TBARS formed in the presence of test compounds. The amount of TBARS formed in uninhibited controls was 10.7 nmol/mL. Control experiments showed that none of the test compounds affected the measurement of deoxyribose degradation (they had no effect when added to the TBA reagents), nor did they react with hydroxyl radical to give TBARS (omission of the deoxyribose from the reaction mixture completely abolished chromogen formation). At all the concentrations tested, dehydrozingerone was found to be more active than dimethyl sulfoxide (DMSO), which was used as the standard for comparison. At concentrations above 200 μM, dehydrozingerone was significantly more active than isoeugenol and eugenol ($P < 0.05$). However, at concentrations below 50 μM the difference in the activities among the test compounds was not significant.

The second-order rate constant for the reaction of the scavenger with the hydroxyl radical was determined as described by Halliwell *et al.* [13], and the results are given in Table 1. The rate constants of the test compounds were similar to that of DMSO. Statistical analysis of the slopes of the regression did not show any significant difference.

The reaction mixtures containing Fe^{3+}–EDTA–H_2O_2–ascorbate generate hydroxyl radical at a rapid rate. But in the absence of ascorbate, generation is very slow. Such a system is useful for identifying compounds capable of accelerating hydroxyl radical formation [14]. The compounds were tested in such a system of slow generation of the hydroxyl radical. None of the compounds accelerated hydroxyl radical generation; in fact, all of them produced a slight reduction (data not shown). Also, none of the compounds were capable of reducing Fe^{3+}–EDTA chelate (see Table 4). Such an activity would be required in order to substitute for ascorbate as a pro-oxidant.

Deoxyribose undergoes "site-specific" degradation when the hydroxyl radical is generated from the

Table 1. Second-order rate constants for scavenging hydroxyl radicals

Compound	r*	Rate constant† ($M^{-1} \cdot sec^{-1}$)
Isoeugenol	0.944	1.41×10^{10}
Eugenol	0.992	1.39×10^{10}
Dehydrozingerone	0.989	2.49×10^{10}
Dimethyl sulfoxide	0.996	1.89×10^{10}
Ethanol	0.875	2.19×10^{9}

* Correlation coefficient for the regression line.

† Results are based on three tests at each of the five concentrations. Deoxyribose degradation in the presence of test compounds at five concentrations was studied by the TBARS method. The rate constant was determined from the slope of the regression line between $1/A$ and S where A is the absorbance at 532 nm in the presence of scavenger at concentration S. The rate constant, k = slope $\cdot k_{DR} \cdot (DR) \cdot A^0$, where k_{DR} was taken as 3.1×10^9 $M^{-1} \cdot sec^{-1}$ [Ref. 13], (DR) = 2.8 mM, and A^0 is the absorbance without scavenger.

Table 2. Effects of test compounds on iron-dependent "site-specific" deoxyribose degradation in the $FeCl_3$–H_2O_2–ascorbate system (without EDTA)

Compound	% Inhibition at 100 μM
Isoeugenol	40.4 ± 1.7*
Eugenol	42.0 ± 2.0*
Dehydrozingerone	40.5 ± 1.4*
Ethanol	2.5 ± 1.3

Results are expressed as means ± SEM of triplicate experiments. The amount of TBARS formed in uninhibited controls was 11.7 nmol/mL.

* Statistically significant inhibition, $P < 0.01$.

Table 3. Inhibition of xanthine oxidase by eugenol

Concentration (μM)	% Inhibition
10	7.9 ± 0.8*
50	19.1 ± 0.7*
100	36.5 ± 1.5*
250	53.3 ± 1.4*
500	81.9 ± 2.0*

Inhibition was determined by measuring the amount of uric acid generated from xanthine. About 477 nmol of uric acid was formed in the test system. Results are expressed as means ± SEM of triplicate experiments.

* Statistically significant inhibition, $P < 0.01$.

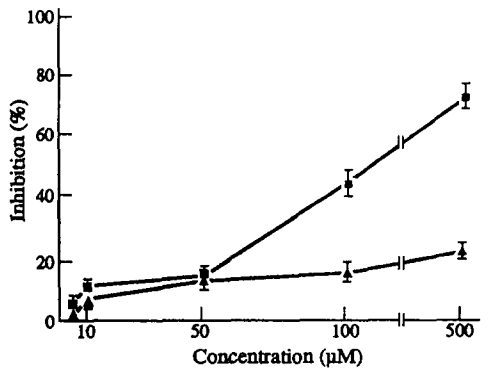

Fig. 4. Effects of test compounds on the reduction of NBT by superoxide anion generated from the xanthine–xanthine oxidase system. Key: isoeugenol (■—■), and dehydrozingerone (▲—▲). Results are means ± SEM of three experiments.

Fe^{3+}–ascorbate–H_2O_2 system in the absence of EDTA. Iron binds directly to deoxyribose in the absence of chelator (EDTA) to cause site-specific degradation [14]. Hence, the molecules that can inhibit deoxyribose degradation in the absence of EDTA are those that are capable of chelating iron, thus rendering it inactive or poorly active in the Fenton reaction [15]. The effects of the test compounds on the degradation of deoxyribose in such a system were studied (Table 2). All the test compounds inhibited the degradation of deoxyribose to a significant extent ($P < 0.01$) compared with the control, although there was no significant difference among the compounds. Ethanol, used as reference compound, was inactive.

Effect on NBT reduction by xanthine–xanthine oxidase generated superoxide anion. Superoxide generated by xanthine–xanthine oxidase reduces NBT to give the blue chromogen formazan. Compounds capable of scavenging superoxide can inhibit NBT reduction. Beside superoxide scavengers, compounds inhibiting xanthine oxidase activity also affect NBT reduction. Hence, all the compounds were investigated first for their effect on xanthine oxidase by measuring the amount of uric acid formed. Only eugenol was found to inhibit uric acid formation (Table 3). The inhibition was concentration dependent, and at 250 μM 53% inhibition was observed. Dehydrozingerone and isoeugenol did not interfere with uric acid formation (data not shown). However, they were capable of scavenging superoxide anion (Fig. 4). Isoeugenol was very active compared with dehydrozingerone. Control experiments showed that none of these compounds reduced NBT directly.

Reduction of ferric ions. Nagababu and Lakshmaiah [9] reported that eugenol reduces ferric ions but not Fe^{3+}–EDTA complex. Since dehydrozingerone and isoeugenol have similar aromatic substitutions, we investigated their ability to reduce ferric ions by the *o*-phenanthroline color method (Table 4). Isoeugenol was more active than eugenol, and dehydrozingerone was the least active. However, BHT was less active than dehydrozingerone. The low reactivity of BHT in reducing the Fe^{3+}–

Table 4. Reduction of ferric ions by test compounds

Compounds	% Reduction	
	0.1 mM	0.5 mM
Isoeugenol	62.0	98.0
Eugenol	45.0	98.0
Dehydrozingerone	25.7	98.2
BHT	10.3	42.9

Reduction was measured by the *o*-phenanthroline method. Sodium dithionite (0.3 mM) was added to the medium containing ferric ions (0.2 mM), and the amount of ferric ions reduced was taken as 100% for comparison. None of the test compounds could reduce ferric ions in the presence of EDTA (0.4 mM).

phenanthroline complex may be attributed to steric hindrance. The shielding of the phenolic group by an adjacent tertiary butyl moiety may hinder its reactivity towards ferric ions. A similar shielding effect due to steric hindrance has been reported for alkyl ascorbic acids [16]. All of the compounds were also tested for their ability to reduce ferric ions in the presence of EDTA. None of the compounds could reduce ferric ions.

DISCUSSION

We have studied the antioxidant properties of three structurally related compounds: dehydrozingerone, isoeugenol and eugenol. With reference to the inhibition of lipid peroxidation, isoeugenol was the most active followed by eugenol and dehydrozingerone (Table 5). Isoeugenol was a more potent scavenger of superoxide anions than was dehydrozingerone. However, in the case of the hydroxyl radical scavenging activity, dehydrozingerone was the most active followed by isoeugenol. Both ferric and ferrous ions stimulate lipid peroxidation through various mechanisms, such as the generation of hydroxyl radical [17], the decomposition of lipid peroxides [18], or by forming perferryl or ferryl species [19]. Ferric ions also undergo reduction by endogenous reducing substances, a process necessary for the initiation of lipid peroxidation [10]. Isoeugenol was very effective in inhibiting the ferrous-ion-stimulated lipid peroxidation. Its activity ($IC_{50} = 3.4\ \mu M$) was comparable to that of the standard antioxidant BHT ($IC_{50} = 2.7\ \mu M$). Also, isoeugenol was about three times more active than eugenol. Eugenol has been reported to be a potent inhibitor of Fe^{2+}-ascorbate- and Fe^{2+}–H_2O_2-induced lipid peroxidation in rat liver mitochondria [9]. In that study, eugenol was less active than BHT but more active than α-tocopherol. In the case of ferric-ion-stimulated lipid peroxidation, a similar trend was observed. Cumene hydroperoxide stimulates lipid peroxidation through the free radicals originating from the homolytic or the heterolytic fission of the O–O bond of the hydroperoxide at the level of cytochrome P450 [20]. In this case, the lipid peroxidation is completely independent of iron ions and takes place even in the presence of relatively high concentrations of chelating agents like EDTA [21]. The amount of TBARS formed in the cumene-hydroperoxide-stimulated system was lower than in the iron-stimulated system. However, the order of activity in inhibiting the formation of TBARS remained the same among the compounds tested. Isoeugenol was more active than eugenol and dehydrozingerone but less active than BHT (Table 5).

Apart from inhibiting lipid peroxidation, the test compounds were also capable of scavenging free radicals such as hydroxyl radical and superoxide (Table 5). Significant variations in the trend among the compounds were observed depending on the free radical system involved. In the case of hydroxyl radicals, dehydrozingerone was the most active compound. In the case of superoxide, isoeugenol was more active than dehydrozingerone, but eugenol was found to inhibit xanthine oxidase. None of the test compounds accelerated the formation of hydroxyl radicals (data not shown), although all of them reduced ferric ions (Table 4). However, the

Table 5. Summary of antioxidant activities of test compounds expressed as IC_{50} values

System	IC_{50} (μM)			
	Isoeugenol	Eugenol	Dehydrozingerone	BHT
Lipid peroxidation				
(a) Ferric stimulated	13.2	74.2	>500.0	1.8
(b) Ferrous stimulated	3.4	11.3	29.8	2.7
(c) Cumene hydroperoxide	16.7	136.8	243.5	7.3
Free radical scavenging				
(a) Hydroxyl	563.0	966.0	265.0	763.0*
(b) Superoxide	152.6	ND†	>500.0	

The IC_{50} values were calculated from a regression equation based on five concentrations. In some cases, values for higher concentrations were deleted due to deviation. However, in all cases a minimum of three concentrations was used.

* DMSO was used as the standard for hydroxyl radical scavenging activity.

† Not done as eugenol inhibited xanthine oxidase and interfered with the test system.

test compounds were unable to reduce ferric ions in the presence of the chelating agent EDTA. This may account for the lack of a pro-oxidant effect in the Fe^{3+}–EDTA–H_2O_2 system. It is interesting to note that all three compounds inhibited "site-specific" degradation of deoxyribose to an equal extent (Table 2). This suggests the involvement of the 2-methoxyphenol moiety common to all the compounds, rather than the variable side chain.

On the basis of the above studies, the activity profile among the three test compounds appears to be isoeugenol > eugenol > dehydrozingerone. The potency depends, to a great extent, on the structure, in particular on electron delocalization in the aromatic nucleus. The antioxidant property of these compounds is essentially due to the phenolic group, which can react with a free radical to form the phenoxyl radical. The presence of a methoxy group ortho to phenol enhances the stability due to the inductive effect. In fact, many studies have shown that ortho substitution with an electron donor group like an alkyl or akloxyl group increases the antioxidant properties of phenols [22, 23]. The high antioxidant activity of isoeugenol is further attributed to the presence of a double bond in conjugation with the phenyl ring. Through the double bond the stability of the phenoxyl radical is further increased by electron delocalization. Such a possibility is absent in the case of eugenol, where, although there is a double bond, it is not conjugated and hence the resonance stabilization is not possible. This accounts for the decreased potency of eugenol compared with isoeugenol. In the case of dehydrozingerone, there is a conjugated double bond in addition to a keto group. Its antioxidant potency is least among the three compounds. This indicates the negative contribution of the keto group. The stability of the phenoxyl radical is decreased to a significant extent by the presence of an electron withdrawing group like keto at the para position. Similar results have been reported for other phenols. For example, pyrocatechuic acid was a less effective antioxidant than pyrocatechol and gallic acid less potent than pyrogallol [23].

In summary, the present study clearly demonstrates that the structural features that enhance the antioxidant potency of phenols are optimized in isoeugenol, making it a highly active antioxidant. Since antioxidant therapy seems to offer protection against a wide range of free radical-induced diseases, isoeugenol, which is also a natural product, appears to be a promising antioxidant.

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