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ANTIOXIDANT PROPERTIES OF PHENYL STYRYL KETONES

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Phenolic and non phenolic derivatives of phenyl styryl ketones were synthesized and evaluated as *in vitro* inhibitors of iron and cumene hydroperoxide dependent lipid peroxidation in rat brain homogenates. The compounds were also tested for antioxidant activity in phosphatidylcholine liposomes. Phenyl 3,5-di-*tert*-butyl-4-hydroxystyryl ketone was found to be the most potent inhibitor of peroxidation among all the compounds tested. It was found to be more active than vitamin E. It also reduced the stable free radical 1,1-diphenyl-2-picrylhydrazyl to an appreciable extent.

KEY WORDS: Phenyl styryl ketone, antioxidant, peroxidation inhibitor.

INTRODUCTION

Lipid peroxidation is thought to be an important pathological event in a variety of diseases, and drug toxicities. Its involvement in aging,[1] and in several disease states like arthritis,[2] infection with malaria or other parasites,[3] neurological damage,[4] diabetic cataract,[5] atherosclerosis,[6] and in ischemia/reperfusion injury[7,8] is very well known.

Our earlier studies showed that dehydrozingerone, a simpler analog of curcumin (Figure 1), is a good antiinflammatory agent[9] with a significant oxygen free radical scavenging activity.[10] It was also capable of inhibiting lipid peroxidation in various models.[11-13] In an effort to develop more potent antioxidants, we have synthesized and studied a number of phenyl styryl ketones. These are structurally similar to dehydrozingerone and are more hydrophobic due to the presence of an additional phenyl ring (Figure 1). These compounds also possess potent antiinflammatory activity in various models.[14-16]

The present study describes the antioxidant properties of some of the phenyl styryl ketones. Out of the compounds tested, one of the compound with sterically hindered phenolic group (compound 5) was found to be a more potent inhibitor of lipid peroxidation than vitamin E in various models.

MATERIALS AND METHODS

Thiobarbituric acid (TBA), cumene hydroperoxide (CuOOH), butylated hydroxy-toluene (BHT), (±)-α-tocopherol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), soya-

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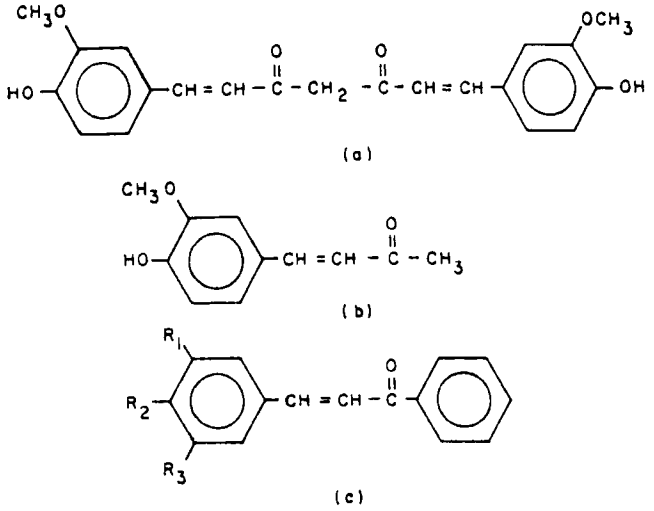

FIGURE 1 Structure of a) curcumin, b) dehydrozingerone and c) substituted phenyl styryl ketone.

Compound No.	R_1	R_2	R_3
1	OCH_3	OH	H
2	H	OH	H
3	H	OCH_3	H
4	OCH_3	OCH_3	H
5	C_4H_9	OH	C_4H_9

bean phosphatidyl choline were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Butan-1-ol was of spectroscopic grade and all other reagents were of the highest grade commercially available. U74389F was a generous gift from the Upjohn company, Michigan, U.S.A.

Synthesis

The compounds were synthesized by the method reported earlier.[15] All the compounds are known in the literature.[14–16] Structures were confirmed by melting point determination and by UV, IR, NMR spectra. The purity of the compound was tested by TLC and elemental analysis (C, H) of the compounds showed less than 0.4% variation.

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) was prepared in 150 mM KCl and centrifuged at 800 g for 10 min. The supernatant was immediately used for the study of *in vitro* lipid peroxidation.[17]

Preparation of Liposomes

Liposomes were prepared as follows: Soyabean phosphatidylcholine (50 mg) in chloroform (2 ml) was dried under vacuum in a rotary evaporator to give a thin homogenous film, and further dispersed in normal saline (5 ml) with a vortex mixer. The mixture was sonicated to get a homogenous suspension and further diluted with normal saline to get the required concentration of lipid.[18-19]

Lipid Peroxidation

The incubation mixture contained in a final volume of 1 ml, brain homogenate (500 μl) or liposomes (0.5 mg in 500 μl normal saline), KCl (150 mM) and ethanol (10 μl) or test compounds dissolved in ethanol. Peroxidation was initiated by adding, to give the final concentration stated, $FeSO_4$ (200 μM), $FeCl_3$ (200 μM) or CuOOH (100 μM) in case of brain homogenate and $FeCl_3$ (200 M)-ascorbic acid (25 μM) in liposomal system. After incubating for 20 min (unless indicated otherwise) at 37°C, the reaction was stopped by adding 2 ml ice cold 0.25η HCl containing 15% trichloroacetic acid, 0.38% thiobarbituric acid and 0.05% BHT. Following heating at 80°C for 15 min, samples were cooled, centrifuged at 1000 g for 10 min and the absorbance of the supernatant was measured at 532 nm. The pink color chromogen in the liposomal system was extracted with 3 ml of butan-1-ol and A_{532} of the upper organic layer was measured.[20] 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.*[21] Another identical experiment was performed in the absence of any inducing agents to find out the amount of TBARS due to spontaneous peroxidation and the value obtained was subtracted from TBARS values obtained in the presence of inducing agents. Percent inhibition of TBARS formed was calculated by comparing with vehicle only control experiments. Iron solutions were prepared fresh in distilled water and used immediately. Since most buffers trap hydroxyl radical or interfere with iron interaction,[21] the reactions were unbuffered and carried out in 150 mM KCl. Results are the means of experiments conducted in triplicate.

Reactivity with DPPH

The solution of test compound in 95% ethanol was added to DPPH (100 μM) in ethanol. After 20 min, the absorbance was measured at 517 nm.[22] The difference in absorbance between the test and the control was taken and expressed as percent scavenging of DPPH radical.

Reduction of Ferric Ions

The reaction mixture containing o-phenanthroline (0.5 mg), ferric chloride (200 μM) and test compound dissolved in 200 μl ethanol in a final volume of 5 ml was incubated for 10 min at ambient temperature. The absorbance at 510 nm was measured.[23] In another experiment sodium dithionite (300 μM) was added instead of the test compound and the absorbance obtained was taken as equivalent to 100% reduction of all the ferric ions present.

RESULTS AND DISCUSSION

Lipid peroxidation in rat brain homogenate was stimulated by the addition of Fe^{2+} (200 μM), Fe^{3+} (200 μM) or CuOOH (100 μM). Ferric ions were more effective, compared to Fe^{2+} or CuOOH, in stimulating peroxidation. The amount of TBARS formed at the end of 20 min incubation was 19.5, 86.3, and 5.7 nmoles/ml of the tissue homogenate when stimulated by Fe^{2+}, Fe^{3+} and CuOOH respectively. In unstimulated control experiments the amount of TBARS formed was 2.1 nmoles/ml. In case of the liposomal system, the amount of TBARS formed was 11.8 nmoles/mg lipid when stimulated with Fe^{3+} (200 μM)-ascorbate (25 μM) for 20 minutes. However in the absence of the inducing agents, the TBARS formed was negligible. 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.[21] Control experiments showed that none of the test compounds affected the measurement of TBARS (omission of the brain homogenate or liposomes from the reaction mixture abolished chromogen formation). Also none of the compounds interfered with the TBA test, since they did not alter the color development if they were added at the end of the incubation, but before heating.[24]

Both Fe^{2+} and Fe^{3+} stimulate lipid peroxidation through various mechanisms, like generation of hydroxyl radical,[25] decomposition of lipid peroxides,[26] or by forming perferryl or ferryl species.[27] Ferric ions also undergo reduction by endogenous reducing substances, a process necessary for the initiation of lipid peroxidation.[28] 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.[29] 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.[30] The amount of TBARS formed in cumene hydroperoxide stimulated system was lower than the iron stimulated system. The TBARS value given are means of one representative individual experiment conducted in triplicate. The variation within the experimental set was low. But, the day-to-day variation was much greater (about 15%), although, the trends observed were consistent.

The effect of test compounds on the lipid peroxidation is given in Table 1. All the phenolic compounds were found to be more active than the non phenolic derivatives. Introduction of a methoxy group adjacent to the phenolic group as in Compound 1 increased the activity. Compound 1 was also more active than dehydrozingerone in Fe^{3+} and CuOOH stimulated systems. Thus the introduction of a phenyl group to the side chain of dehydrozingerone results in an increased activity. Presence of bulky alkyl groups ortho to phenolic group results in sterically hindered phenols as in vitamin E, BHT, etc. Such phenols are known to be more potent antioxidants due to the stabilization of the phenoxylate radical.[31-32] Hence we synthesized compound 5 where *tert*-butyl groups are present at ortho positions to phenolic group. This resulted in further increase in the activity in all the models tested.

Compound 1 and 5, which showed considerable activity at 100 μM were tested at lower concentrations. The inhibition of lipid peroxidation by active compounds at various concentrations, in the Fe^{2+} stimulated model is shown in Figure 2. Compound 5 showed the highest activity and the activity was higher than compound

TABLE 1
Effect of test compounds on lipid peroxidation

Compound No.	% Inhibition at 100 μM		
	Fe^{2+}	Fe^{3+}	CuOOH
1	45.9	77.6	51.1
2	24.9	6.0	NA
3	29.2	1.6	NA
4	29.4	2.0	NA
5	66.8	84.2	59.3
Dehydrozingerone	48.9	29.0	34.4
Vitamin E	62.2	68.4	56.7
U74389F	64.5	50.0	42.7

Peroxidation in rat brain homogenate was induced by the addition of Fe^{2+} (200 μM), Fe^{3+} (200 μM) or CuOOH (100 μM) and estimated by TBARS method. Percent inhibition was calculated by comparing with the vehicle only control. NA: Not active

1 and vitamin E which was used as the standard. The IC_{50} values of the compounds tested in different models of lipid peroxidation is given in Table 2. Compound 5 was found to be the most potent in all the models tested. It was more potent than vitamin R and the 21-aminosteroid, commonly known as lazaroids, U74389F.

The time courses of peroxidation in the presence of test compounds in shown in Figure 3. It was observed that the compounds 1, 5 and U74389F inhibited lipid peroxidation in rat brain homogenate throughout the incubation period: there was

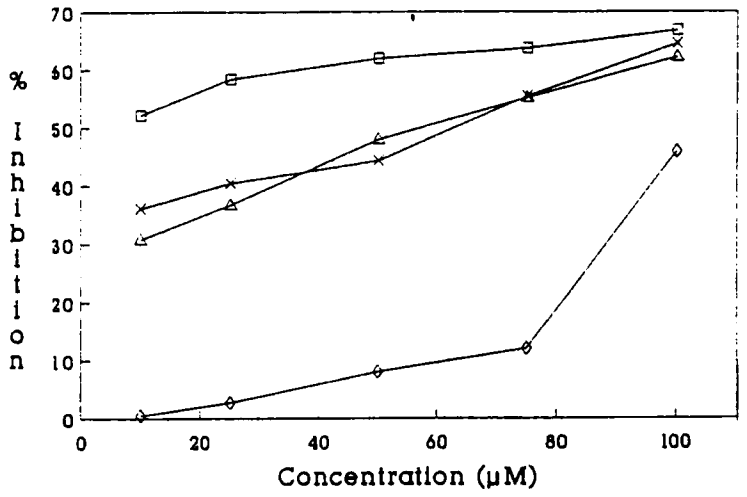

FIGURE 2 Effect of test compounds on lipid peroxidation induced by ferrous ions (200 μM) in rat brain homogenate. Compound 1 (—◇—); Compound 5 (—□—); U74389F (—X—); Vitamin E (—△—). Peroxidation was estimated by TBARS method. Percent inhibition was calculated by comparing with the vehicle only control.

TABLE 2
Lipid peroxidation inhibition by test compounds in rat brain homogenate

Compound No.	IC_{50} (μM)		
	Fe^{2+}	Fe^{3+}	CuOOH
1	>100.0	19.4	90.2
5	6.8	0.4	87.8
Vitamin E	50.7	96.7	92.6
U74389F	46.2	>100.0	>100.0

IC_{50} calculated from the regression equation based on five concentration. In some cases values for higher concentration are deleted due to deviation. But in all the cases minimum three concentration were used. Other experimental conditions are as mentioned in Table 1.

no evidence of a lag period followed by an acceleration of peroxidation to the control rate as observed in the case of vitamin E.

The compounds were also found to inhibit Fe^{3+}-ascorbate induced peroxidation of phosphatidylcholine liposomes. Based on IC_{50} values (Table 3), compound 5 showed potent inhibition of peroxidation compared to compound 1, vitamin E, and U74389F.

Antioxidants react with the nitrogen centered radical DPPH and converts it to 1,1-diphenyl-2-picrylhydrazine. The change in the absorbance produced in this reaction has been used as a measure of the radical scavenging potential.[18,22] In our studies, only substituted phenolic compounds were found to be active (Table 4). Their activities were however less compared to vitamin E.

Dehydrozingerone is known to reduce ferric ions.[11] Hence, we also investigated

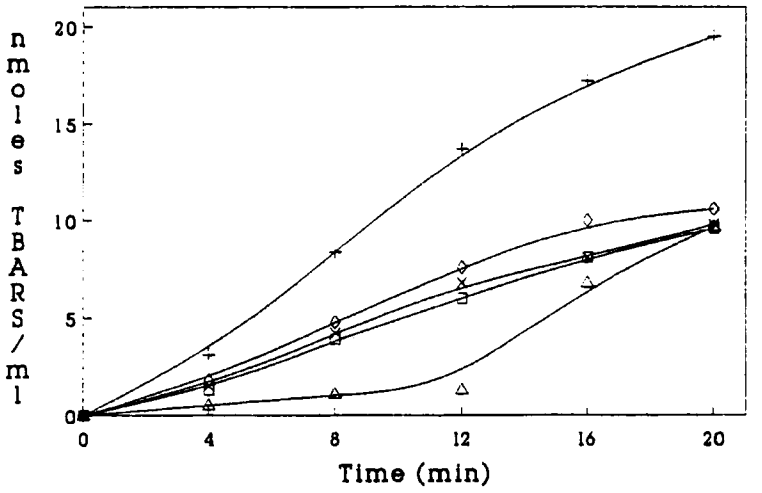

FIGURE 3 Effect of test compounds on time course of lipid peroxidation induced by ferrous ions (200 μM) in rat brain homogenate. The test compounds were added at their IC_{50} concentration except in the case of compound 1, for which 100 μM concentration was used. Other experimental conditions as in Figure 2. Compound 1 (—◇—); Compound 5 (—□—); U74389F (—✕—); Vitamin E (—△—) Control (—+—)

TABLE 3
Lipid peroxidation inhibition by test compounds in liposomes

Compound No.	IC_{50} μM Fe^{3+}-ascorbate
1	5.5
5	2.4
Vitamin E	6.1
U74389F	21.7

IC_{50} calculated from the regression equation based on inhibition of peroxidation by compounds at five different concentration. Peroxidation was estimated by TBARS method.

TABLE 4
Reduction of DPPH and ferric ions by test compounds

Compound No.	% reduction at 100 μM DPPH	Fe^{3+}
1	23.2	25.4
2	NA	19.8
3	NA	16.6
4	NA	17.9
5	24.8	31.5
Vitamin E	50.8	31.9

Reduction of stable free radical, 1,1-diphenyl-2-picrylhydrazyl (100 μM) by test compounds (100 μM) was estimated in alcoholic solution at 517 nm. Reduction of ferric ions was measured by o-phenanthroline method. To the medium containing Fe^{3+} (200 μM), sodium dithionite (300 μM) was added and the amount of Fe^{3+} reduced was taken as 100% for comparison. NA: Not active.

the ability of test compounds to reduce ferric ions by the o-phenanthroline color method (Table 4). Ortho substituted phenolic compounds were found be more active and compound 5 showed activity similar to vitamin E. Hence, these compounds may exert pro-oxidant effect by interacting with iron similar to ascorbate and gallic acid.[24,33]

On the basis of the above studies, it can be concluded that 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. Ortho substitution increases the antioxidant potency of the compounds. Many studies have shown that ortho substitution with groups like alkyl or alkoxyl increases the antioxidant properties of phenols.[31-32] This may be due to the steric crowding of the phenolic group as seen in case of BHT or vitamin E. The high antioxidant activity is further attributed to the presence of double bond in conjugation with the phenyl ring.[34] Through the double bond the stability of the phenoxyl radical is further increased by the electron delocalisation.

In conclusion, the study demonstrates that the structural features which enhances

the antioxidant properties of phenols are optimized in the phenyl 3,5-di-*tert*-butyl-4-hydroxystyryl ketone making it highly active antioxidant. Since the antioxidant therapy seems to offer protection against wide range of free radical induced diseases, this compound appears to be a promising therapeutically useful antioxidant.

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