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Antioxidant Activity of *Myristica malabarica* Extracts and Their Constituents

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The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay of the ether, methanol, and aqueous extracts of the spice *Myristica malabarica* (rampatri) revealed the methanol extract to possess the best antioxidant activity. Column chromatography of the methanol extract led to the isolation of a new 2-acylresorcinol and four known diarylnonanoids of which the diarylnonanoid, malabaricone C, showed the maximum DPPH scavenging activity. Malabaricone C could prevent both Fe(II)- and 2,2′-azobis(2-amidinopropane)dihydrochloride-induced lipid peroxidation (LPO) of rat liver mitochondria more efficiently than curcumin. The anti-LPO activity of malabaricone C was attributed to its better radical scavenging and Fe(II) chelation capacities. The superior activity of malabaricone C was rationalized by a systematic structure−activity correlation of the results obtained with the structurally related diarylnonanoids and curcumin. Malabaricone C also prevented the γ-ray-induced damage of pBR322 plasmid DNA in a concentration-dependent manner. The radioprotective activity was found to correlate with its •OH radical scavenging property, which matched well with that of D-mannitol.

KEYWORDS: Antioxidant; active principle; malabaricone C; *Myristica malabarica*

INTRODUCTION

Oxidative stress has been implicated in the etiology of a number of human ailments (*1*, *2*). The reactive oxygen species (ROS) are known to cause oxidation of biological molecules with severe consequences. ROS-mediated oxidation has been proposed to induce a variety of pathological events such as atherogenesis (*3*), carcinogenesis (*4*), and aging and immune dysfunction (*5*). Hence, compounds, especially from natural sources, capable of protecting against ROS-mediated damage may have potential application in the prevention and/or curing of these diseases (*6*, *7*). Medical and nutritional experts have noted the antioxidant property of food constituents for this purpose. Spices are extensively used as natural food additives for flavoring, seasoning, coloring, and antiseptic properties. Hence, they appear to be promising candidates in this regard. Literature in traditional medicine also describes their potential role as domestic remedies for various human disorders (*8*). Several spices are also known to possess antioxidative activity (*9*, *10*).

The fruit rind of the plant *Myristica malabarica* (Myristicaceae) (popularly known as rampatri, Bombay mace, or false nutmeg) is used as an exotic spice in various Indian cuisines. This is credited with hepatoprotective, anticarcinogenic, and antithrombotic properties and is found as a constituent in many Ayurvedic preparations such as pasupasi. However, most of the medicinal attributes of the spice have not been substantiated adequately. Recently, the superoxide-scavenging and prolyl endopeptidase inhibitory activities of the methanol extract of *M. malabarica* have been reported (*11*). The phenolic compounds present in the resin of *M. malabarica* seeds were also found to prevent the oxidation of various edible oils and fats more efficiently than butylated hydroxytoluene (*12*). Given that the medicinal values of many of the plant extracts have been attributed to their antioxidant action, the possible antioxidant properties of *M. malabarica* constituents may, at least, partially explain its therapeutic properties. To this end, the antioxidant activities of the methanol extract and its constituents were evaluated through a series of in vitro experiments.

MATERIALS AND METHODS

Materials. Dry fruit rinds of *M. malabarica* were purchased from the local market. Ascorbic acid, ferrous ammonium sulfate, 2-thiobarbituric acid (TBA), and 2-deoxyribose were obtained from Himedia Laboratory (Mumbai, India). H_2O_2 (35%) was purchased from Lancaster (Morecambe, U.K.), whereas iron(III) chloride and trichloroacetic acid (TCA) were from Thomas Baker (Mumbai, India). Other materials used were EDTA (Sarabhai Chemicals, Baroda, India), 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) (Aldrich, Milwaukee, WI), 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma, St. Louis, MO), curcumin (Aldrich), and pBR322 plasmid DNA (Bangalore Genei, Bangalore, India). All solutions including the stock solution of ferrous ammonium sulfate were freshly prepared in triply distilled water. Stock solutions of test fractions and curcumin (3 mg/mL) were prepared in ethanol or 5 mM aqueous NaOH solution followed by neutralization with aqueous HCl. Appropriate blanks were used for the assay. The concentrations of the reagents and test samples for all of the biological

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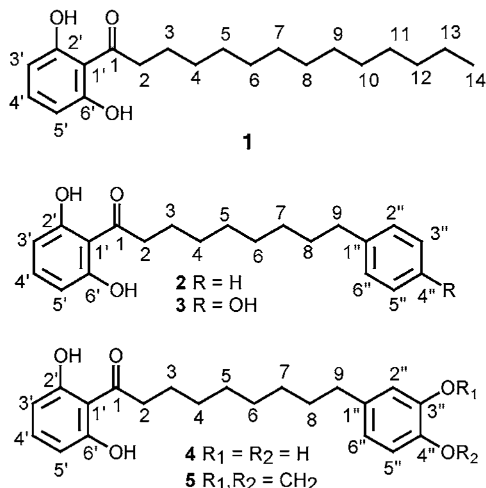

Figure 1. Chemical structures of compounds **1**–**5** isolated from the methanol extract of rampatri.

assays indicate final concentrations. Column chromatography was carried out over silica gel (SRL, Mumbai, India) using AR grade solvents. The studies were carried out using the compounds isolated by us from the extracts of the spice.

Instrumentation. The IR spectra were recorded as films on a Nicolet FT-IR model Impact 410 spectrometer. The ^{1}H NMR spectra were recorded in MeOH-d_4 with a Bruker AC-200 (200 MHz) spectrometer, and the data are provided in δ scale (ppm). The coupling constant (J) values are expressed in hertz. GC analysis was carried out with a Chemito (Mumbai, India) gas chromatogram instrument using a 30 m × 0.25 mm i.d. 5% DB-5 splitless capillary column (Chemito), 2 mL/min He as the carrier gas, and a temperature program of 80−240 °C at 4 °C/min and a flame ionization detector. The absorbance spectrophotometry was carried out at 25 °C using a Jasco V-550 UV−vis spectrophotometer. Wavelength scans and absorbance measurements were made in 1 mL quartz cells of 1 cm path length.

Preparation of Plant Extracts. The dry fruit rinds (20 g) of *M. malabarica* were powdered with a grinder and extracted successively with ether, methanol, and water (60 mL × 4 days with each solvent) at room temperature. The supernatants in each case were decanted. The entire process was repeated three times, and each of the combined supernatants was filtered through a nylon mesh and evaporated at <40 °C in vacuo to obtain the respective extracts. These were designated rampatri ether (RE; 7.30 g, 36.5%), methanol (RM; 5.77 g, 28.9%), and aqueous extracts (RW; 0.42 g, 0.02%), respectively, and stored in a vacuum desiccator.

Isolation of Chemical Constituents from RM. A part of RM (3.7 g) was subjected to partial fractionation using a silica gel (25 g) column and eluting with hexane to collect five 300 mL fractions. These on evaporation in vacuo gave the subfractions F1−F5, which were assayed for their antioxidant activity by the DPPH method. The antioxidant activity was primarily confined to F2, which was subjected to rigorous column chromatography (silica gel, 0−20% EtOAc/hexane) to isolate five compounds, **1**−**5**. These were fully characterized using IR and ^{1}H NMR spectroscopic data. Of these, compound **1** was isolated for the first time from the plant, whereas the presence of compounds **2**−**5** was earlier reported (*13*). The structures of the isolated compounds are shown in **Figure 1**. The yields reported for compounds **1**−**5** are with respect to the weight of the dry fruit rinds.

1-(2′,6′-Dihydroxyphenyl)tetradecan-1-one, 1. Yield, 0.39%; GC, t_R = 44.8 min (single peak); ^{1}H NMR (MeOH-d_4) δ 0.78 (dist. t, 3H, CH_3−), 1.17 (bs, 18H, 9 × −CH_2−), 1.45−1.60 (m, 4H, 2 × −CH_2−), 2.99 (t, J = 7.46 Hz, 2H, −$COCH_2$−), 6.21 (d, J = 8.24 Hz, 2H, H-3′, H-5′), 7.07 (t, J = 8.24 Hz, 1H, H-4′); MS (m/z) 41 (21.2), 55 (15.9), 123 (6.6), 137 (100), 138 (8.1), 152 (22.3), 165 (22.8), 176 (5.7), 189 (15.6), 302 (3.9), 320 (M^+, 2.4). Anal. Calcd for $C_{20}H_{32}O_3$: C, 74.96; H, 10.06%. Found: C, 75.19; H, 10.18%.

1-(2′,6′-Dihydroxyphenyl)-9-phenylnonan-1-one (Malabaricone A), 2. Yield, 1.2%; ^{1}H NMR (MeOH-d_4) δ 1.21 (s, 8H, 2 × H4−H7), 1.42−1.61 (m, 4H, 2 × H3 and H8), 2.47 (t, J = 7.32 Hz, 2H, $ArCH_2$−), 2.99 (t, J = 7.30 Hz, 2H, −$COCH_2$−), 6.22 (d, J = 8.22 Hz, 2H, H-3′, H-5′), 7.0−7.14 (m, 6H, aromatic).

1-(2′,6′-Dihydroxyphenyl)-9-(4″-hydroxyphenyl)nonan-1-one (Malabaricone B), 3. Yield, 2.0%; ^{1}H NMR (MeOH-d_4) δ 1.31 (s, 8H, 2 × H4−H7), 1.43−1.58 (m, 4H, 2 × H3 and H8), 2.47 (t, J = 7.6 Hz, 2H, $ArCH_2$−), 3.08 (t, J = 7.54 Hz, 2H, −$COCH_2$−), 4.78 (bs, 1H, −OH), 6.32 (d, J = 8.22 Hz, 2H, H-3′, H-5′), 6.66 (dd, J = 8.48, 2.2 Hz, 2H, H-2″, H-6″), 6.95 (dd, J = 8.48, 2.2 Hz, 2H, H-3″, H-5″), 7.17 (t, J = 8.22 Hz, 1H, H-4′).

1-(2′,6′-Dihydroxyphenyl)-9-(3″,4″-dihydroxyphenyl)nonan-1-one (Malabaricone C), 4. Yield, 6.7%; ^{1}H NMR (MeOH-d_4) δ 1.20 (s, 8H, 2 × H4−H7), 1.33−1.47 (m, 2H, 2 × H3), 1.49−1.65 (m, 2H, 2 × H8), 2.32 (t, J = 7.24 Hz, 2H, $ArCH_2$−), 2.99 (t, J = 7.54 Hz, 2H, −$COCH_2$−), 6.17 (d, J = 8.22 Hz, 2H, H-3′, H-5′), 6.35 (dd, J = 7.98, 2.0 Hz, 1H, H-6″), 6.48 (d, J = 2.0 Hz, 1H, H-2″), 6.53 (d, J = 7.98 Hz, 1H, H-5″), 7.07 (t, J = 8.22 Hz, 1H, H-4′).

1-(2′,6′-Dihydroxyphenyl)-9-(3″,4″-methylenedioxyphenyl)nonan-1-one (Malabaricone D), 5. Yield, 8.1%; ^{1}H NMR δ 1.31 (s, 8H, 2 × H4−H7), 1.55−1.67 (m, 4H, 2 × H3 and H8), 2.49 (t, J = 7.2 Hz, 2H, $ArCH_2$−), 3.09 (t, J = 7.14 Hz, 2H, −$COCH_2$−), 5.85 (s, 2H, methylenedioxy), 6.22 (d, J = 8.12 Hz, 2H, H-3′, H-5′), 6.38 (dd, J = 8.01, 2.1 Hz, 1H, H-6″), 6.47−6.55 (m, 2H, H-2″, H-5″), 7.24 (t, J = 8.12 Hz, 1H, H-4′).

DPPH Scavenging Assay. An ethanolic solution of DPPH (100 μM) was incubated with an ethanolic solution of each of the test samples (3.5 and 7.0 μg/mL), and the absorbance was monitored spectrophotometrically at 517 nm. The percentage of reduction of the DPPH absorbance gave the DPPH scavenging activity of the test samples and was used as the marker of their antioxidant activities. The concentration ($IC_{0.2000}$) of the test compound that induced a decrease of 0.20 in absorbance during a 30-min observation was also calculated with compound **4** and curcumin by carrying out concentration-dependent studies (up to 17.0 μg/mL) (*14*). Ethanol solutions of the respective test samples were used as the blank, whereas curcumin was the positive control. The time-dependent studies were carried out for 1−5 min using a fixed concentration (4.0 μg/mL) of compound **4** and curcumin.

Estimation of Total Phenolic Contents. The method (*15*) down-scaled to 1 mL final volume was followed to determine the amounts of total phenolics in the test samples. The test samples (each 100 μL) were mixed with 500 μL of 1:10 Folin−Ciocalteau reagent followed by the addition of Na_2CO_3 (400 μL, 7.5%). After incubation of the reaction mixture at 24 °C for 2 h, the absorbances at 765 nm were recorded. Gallic acid monohydrate was used as the standard. The total phenolic contents of the test samples are expressed as gallic acid equivalents (μg of GAE)/mg of dry weight of the samples.

Lipid Peroxidation Assay. Lipid peroxidation of rat liver mitochondria was carried out as reported earlier (*16*) with minor modifications. The mitochondrial lipid peroxidation was initiated by addition of Fe(II) (50 μM) and ascorbic acid (500 μM) or AAPH (50 mM) to the reaction mixture, which also contained the following components at the final concentration stated: mitochondrial fraction (4.0 mg of protein/mL) and test samples in potassium phosphate buffer, pH 7.4 (50 mM). The mixture was incubated at 37 °C for 30 min with the Fe(II)−ascorbic acid system and for 1 h with the AAPH system, TCA−TBA−HCl (15% TCA, 0.375% TBA, 0.25 M HCl) solution was added, and the mixture was heated for 10 min on a boiling water bath. After cooling, the precipitate formed was removed by centrifugation at 1000g for 10 min, and the absorbance of the supernatant at 532 nm was read.

Lipid Hydroperoxide Assay. This assay was carried out essentially according to the known FOX2 method (*17*). Briefly, small unilammelar liposomes were prepared from egg lecithin at 10% concentration in 10 mM Hepes, pH 7.4, buffer. In the reaction mixture (0.5 mL) containing Tris buffer, pH 7.4 (125 mM), KH_2PO_4 (1 mM), the liposome (1× from the 10× stock) with or without compound **4**, was added Fe(II) (200 μM) and ascorbic acid (200 μM), and the mixture was incubated at 37 °C for 30 min. At the end of the incubation a 50 μL aliquot was taken to which 0.5 mL of FOX2 reagent was added. The tubes were vortexed and the absorbance at 560 nm read after 30 min.

Iron Chelation Study by Compound 4. The iron chelation study was carried out by recording the UV−vis spectra (190−800 nm) of a

solution of **4** (25 μM) in water as such and after the addition of aliquots of ferrous ammonium sulfate (0−150 μM) solution. The chelation capacity of compound **4** was evaluated from the change and/or shift of the absorbance (*18*).

Protective Activity of Compound 4 against γ-ray-Induced Strand Break in Plasmid DNA. The assay was carried out as described earlier (*19*). The samples were prepared in a final volume (14 μL) and irradiated for 2.0 min at 25 °C up to a dose of 16 Gy using a ^{60}Co source (dose rate = 8 Gy/min). In all experiments, the concentration of the supercoiled pBR322 DNA was 200 ng in a 10 mM potassium phosphate buffer, pH 7.4. Compound **4** was added as an aqueous solution to achieve the final concentration (0−1.25 mM). After irradiation, the resulting forms of the plasmid, form I (supercoiled) and form II (open circular), were separated by electrophoresis, stained with ethidium bromide, and visualized under UV light. The relative intensities of the bands were determined with a Bio-Rad gel documentation system.

2-Deoxyribose Assay with Compound 4. The reaction mixture (1 mL) contained 2-deoxyribose (2.8 mM), iron(III) chloride (20 μM), EDTA (100 μM) [EDTA and iron(III) chloride were mixed prior to the addition of 2-deoxyribose], and H_2O_2 (200 μM) without or with compound **4** or mannitol (0−2.5 mM) in a 10 mM potassium phosphate buffer, pH 7.4. The reaction was triggered by adding ascorbic acid (300 μM) and subsequent incubation of the mixture for 1 h at 37 °C. Solutions of iron(III) chloride, ascorbic acid, and H_2O_2 were prepared in deaerated water just prior to use. A solution of TBA in 50 mM NaOH (1 mL, 1% w/v) and TCA (1 mL, 2.8% w/v aqueous solution) was added, the mixture was heated for 15 min on a boiling water bath, and the amount of chromogen produced was spectrophotometrically measured (*20*) at 532 nm.

Statistics. Data are presented as mean ± standard error (SE) of five experiments. The data presented for protection against γ-ray-induced plasmid DNA damage are the mean of two experiments.

RESULTS AND DISCUSSION

Besides oxygen metabolism, various ROS can be formed in cells by transition metal [especially Fe(II)] mediated reactions (*21*) and radiation exposure (*22*) leading to deleterious effects on membrane lipids and DNA. Consequently, we aimed to study the protective activity of the active constituents from the extracts of the fruit rinds of *M. malabarica* against iron-mediated lipid peroxidation and γ-ray-induced DNA damage. For this, the plant constituents were partitioned by solvent extraction. Using the DPPH assay, the antioxidant activity was located primarily in the methanol extract (RM). This on subsequent fractionation furnished the most active formulation, F2, from which five pure compounds were isolated by column chromatography. Subsequently, the active constituent was identified using a series of in vitro methods. Comparison of the assay results with those of curcumin established the mechanism of its antioxidant action.

Isolation of the Chemical Constituents of *M. malabarica*. Different extracts (RE, RM, and RW) were prepared by extracting rampatri successively with ether, methanol, and water. Because RM showed the best activity by the DPPH assay, it was fractionated into five fractions (F1−F5) of different polarities. From the most active fraction (F2), five compounds **1**−**5** were isolated and characterized by spectroscopy. Of these, compounds **2**−**5** were reported as malabaricones A−D (*13*). Compound **1** has not been isolated so far from the spice. Its alkyl chain length was confirmed from the mass spectrum. GC analysis on a capillary column showed only a single peak revealing its chemical purity.

DPPH Radical Scavenging Activities of Different Rampatri Extracts/Constituents. DPPH provides a stable carbon-centered free radical that absorbs at 517 nm. The change in the absorbance of DPPH in the presence of a test compound provides a suitable method (*14*) for evaluating its ability to scavenge free radicals generated independent of any enzyme- or metal-based system. The DPPH assay results are known to correlate well with the lipid peroxidation inhibitory capacity of a test compound (*23*). The assay was carried out with 3.5 and 7 μg/mL of the test samples along with curcumin as the positive control.

Table 1. Comparative DPPH Radical Scavenging Abilities of Rampatri Samples and Curcumin[a]

test sample	% scavenging activity	
	3.5 μg/mL	7.0 μg/mL
RE	14.2 ± 2.6	25.5 ± 3.2
RM	23.7 ± 2.2	39.3 ± 2.3
RW		10.1 ± 1.6
F2	21.1 ± 1.5	38.2 ± 3.5
F4	3.8 ± 0.5	7.3 ± 2.1
compound **3**	4.0 ± 0.4	5.0 ± 0.6
compound **4**	31.3 ± 3.4	59.9 ± 2.8
curcumin	22.8 ± 0.9	42.3 ± 3.2

[a] Means were compared statistically by one-way ANOVA at $p < 0.05$. For RE, RM and RW, means were significantly different at both test concentrations. Means of RM and F2 were not significantly different from each other, but both were significantly different from F4 at both test concentrations. For compounds **3** and **4** and crcumin, means were significantly different at both test concentrations.

The results of the study are presented in **Table 1**. Among the plant extracts (RE, RM, and RW), the methanol extract RM showed the best result, the activity being primarily confined to its F2 and, to a significantly lesser extent, to F4 fractions. Although five phenolic compounds **1**−**5** were the major constituents of F2, compound **4** (malabaricone C) accounted for most of the radical scavenging activity. Compound **3** showed weak scavenging activity, whereas the other compounds were inactive. The order of the DPPH radical scavenging abilities of the test samples was compound **4** > RM > F2 > RE > F4 ∼ compound **3**. A concentration-dependent study with the best compound, **4**, and the positive control, curcumin, revealed their $IC_{0.2}$ values as 6.4 ± 0.6 and 8.5 ± 0.4 μM, respectively. We have also carried out time-dependent DPPH radical scavenging studies with compound **4** and curcumin, each at the final concentration of 4.0 μg/mL. It was found that the reaction was very fast. Within 1 min, compound **4** and curcumin could scavenge 26.88 ± 3.54 and 13.44 ± 2.58% of the radicals. This increased to 33.65 ± 2.93 and 16.23 ± 3.56%, respectively, in 2 min. Thereafter, the increase was less. Thus, compound **4** was found to be the best radical scavenger among the test samples including curcumin. Hence, all subsequent studies were carried out primarily with RM, F2, and compounds **3** and **4**.

Total Phenolics in Different Rampatri Extracts/Constituents. It is well-known that plant phenolics, in general, are highly effective free radical scavengers and antioxidants. Consequently, the antioxidant activities of plant/herb extracts are often explained with their total phenolic contents with good correlation. We also observed a similar correlation in the present study. The total phenolics in the extracts (RE, RM, and RW) and fractions (F2 and F4) were determined spectrophotometrically according to the Folin−Ciocalteu method and expressed as micrograms of GAE per milligram of the test samples (**Table 2**). As revealed by the data the total phenolic contents of RE and RM (130.31 ± 4.1 and 142.68 ± 4.7 μg of GAE/mg) were significantly higher than that of RW (11.04 ± 1.22 μg of GAE/mg). Overall, the order of the phenolic contents of the test samples was RM > RE > F2 > F4, which matched with their respective DPPH radicals scavenging abilities.

Anti-lipid Peroxidation (LPO) Activities of RM and Its Constituents. Owing to the high levels of unsaturation and the

Table 2. Comparative Total Phenolic Contents of Rampatri Samples

test sample	total phenolics activity[a] (μg of GAE/mg of the sample)
RE	130.31 ± 4.1
RM	142.68 ± 4.7
RW	11.04 ± 1.22
F2	134.14 ± 3.9
F4	34.10 ± 3.4

[a] Values are mean + SE for five experiments.

increased consumption of oxygen, mitochondrial lipids are susceptible to oxidative damage. Lipid peroxidation can inactivate cellular components and plays a major role in oxidative stress in biological systems. Furthermore, several toxic byproducts of the peroxidation can damage other biomolecules including DNA away from the site of their generation (*24*, *25*). Therefore, compounds possessing the anti-LPO activity are extremely important for health benefit and food preservation. Hence, the anti-LPO activities of RM, its fractions, and compounds **3** and **4** were studied.

With Iron(II) Ascorbate. The assay was carried out using rat liver mitochondria as a convenient lipid source and measuring the end products in terms of the thiobarbituric acid reactive substrates (TBARS) formed. In unstimulated experiments, the amount of TBARS was marginal, the absorption at 532 nm (A_{532}) being only 0.012 ± 0.005 ($n = 5$). In control experiments, lipid peroxidation in rat liver mitochondria was stimulated by the addition of Fe(II) (50 μM) and ascorbic acid (500 μM), and the A_{532} value increased to 0.705 ± 0.02 ($n = 5$). All of the rampatri samples and the positive control, curcumin, inhibited the lipid peroxidation in a concentration-dependent manner (**Figure 2**). At a concentration of 2 μg/mL, the percentages of protection offered were 28.2 (RM), 87.2 (F2), 10.3 (F4), 9.5 (compound **3**), 95.4 (compound **4**), and 22.4 (curcumin), respectively. The data correlated well with the respective DPPH scavenging activities of the samples. Compound **4** was the best candidate, its activity being better than that of curcumin.

The extraordinary efficacy of compound **4** in comparison to compound **3** and even curcumin might be due to its better radical scavenging and/or iron chelating abilities. Consequently, for a better understanding of the operative mechanism, the iron chelation capacity and anti-LPO activity of compound **4** against the AAPH-induced lipid peroxidation were also investigated, and the results are presented below. Although the DPPH assay results were indicative of its scavenging ability to lipid peroxyl radicals (LOO•), the study with the AAPH-mediated LPO is biologically more relevant.

With AAPH. AAPH is an efficient free radical generator and is extensively used for inducing lipid peroxidation (*26*). The alkyl peroxyl radicals produced from AAPH, which causes the lipid peroxidation, are very similar to radicals produced in biological systems (*27*). Thus, the preventive capacity of a test compound against the AAPH-induced lipid peroxidation provides a good measure of its anti-LPO activity in an iron-independent system.

The anti-LPO activities of compound **4** and curcumin were tested by carrying out the AAPH-mediated peroxidation of rat liver mitochondria. The studies were carried out using compound **4** and curcumin, each at the concentrations of 25 and 50 μM. At both concentrations, the protective activity of compound **4** was significantly higher than that of curcumin. For example, whereas compound **4** (25 and 50 μM) showed 59.84 ± 3.60 and 75.00 ± 4.90% anti-LPO activities, those with curcumin at the same concentrations were 18.93 ± 2.36 and 44.69 ± 2.68% only, respectively.

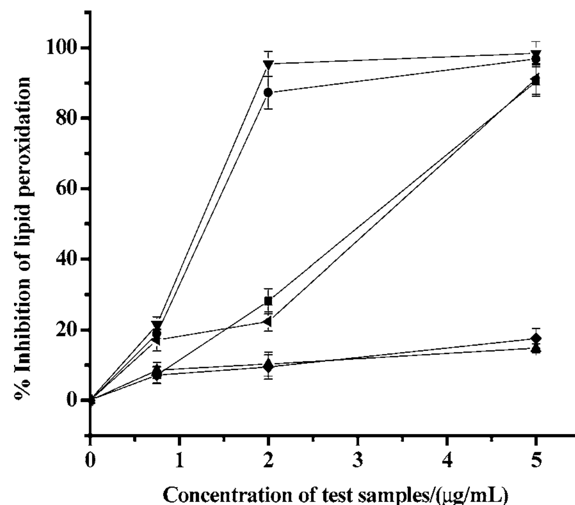

Figure 2. Comparative concentration-dependent anti-LPO activities of rampatri samples and curcumin against Fe(II)–ascorbic acid induced formation of TBARS in rat liver mitochondria: (■) RM; (●) F2; (◆) F4; (▲) compound **3**; (▼) compound **4**; (left-pointing triangle) curcumin. The values are mean ± SE ($n = 5$). Means were compared statistically by one-way ANOVA at $p < 0.05$. At 5 μg/mL, means of RM and F2 were not significantly different, but both were significantly different from that of F4. At 5 μg/mL, means of compound **4** and curcumin were not significantly different, but both were significantly different from that of compound **3**. At 2 μg/mL, means of RM, F2, and F4 were significantly different. At 2 μg/mL, means of compounds **3** and **4** and curcumin were significantly different. At 0.75 μg/mL, means of RM and F2 were not significantly different, but both were significantly different from that of F4. At 0.75 μg/mL, means of compound **4** and curcumin were not significantly different, but both were significantly different from that of compound **3**.

LOOH Assay. Considering that the TBA assay is fairly nonspecific, the anti-LPO activity of compound **4** (25 μM) was also investigated by measuring the lipid hydroperoxide (LOOH) formed in a Fenton-mediated liposomal peroxidation. In unstimulated experiments, the absorption at 560 nm (A_{560}) due to LOOH was 0.09 ± 0.02 ($n = 5$). In control experiments, the liposomal peroxidation was stimulated by the addition of Fe(II) (200 μM), and the A_{560} value increased to 0.44 ± 0.03 ($n = 5$). Compound **4** (25 μM) inhibited the iron-stimulated LOOH formation in liposomes, showing 58.72 ± 2.16% ($n = 5$) protection. Under similar conditions, the positive control, curcumin (25 μM), showed 14.12 ± 1.09% ($n = 5$) prevention of LOOH formation. Thus, the results obtained with another lipid source also confirmed the superior anti-LPO activity of compound **4** compared to curcumin.

Iron Chelation by Compound 4. Compound **4** was found to complex Fe(II) because the addition of Fe(II) in increasing concentrations (6.5−150 μM) to a fixed concentration of **4** (25 μM) led to gradual reductions in the intensities of the absorption bands (274 and 345 nm) of free compound **4** (**Figure 3**). Although the hypochromicity of the absorption bands at 345 nm was not much, the significant reduction in the intensity of the absorption bands at 274 nm clearly indicated its complexation with Fe(II). The hypochromicity was, however, observed until the Fe(II) concentration reached 100 μM, suggesting the formation of a 1:4 complex between compound **4** and Fe(II). Curcumin also formed a 1:4 complex with Fe(II) (*16*).

Molecular Basis of Superior Anti-LPO Activity of Compound 4. *Radical Scavenging Capacity.* All of the α,ω-

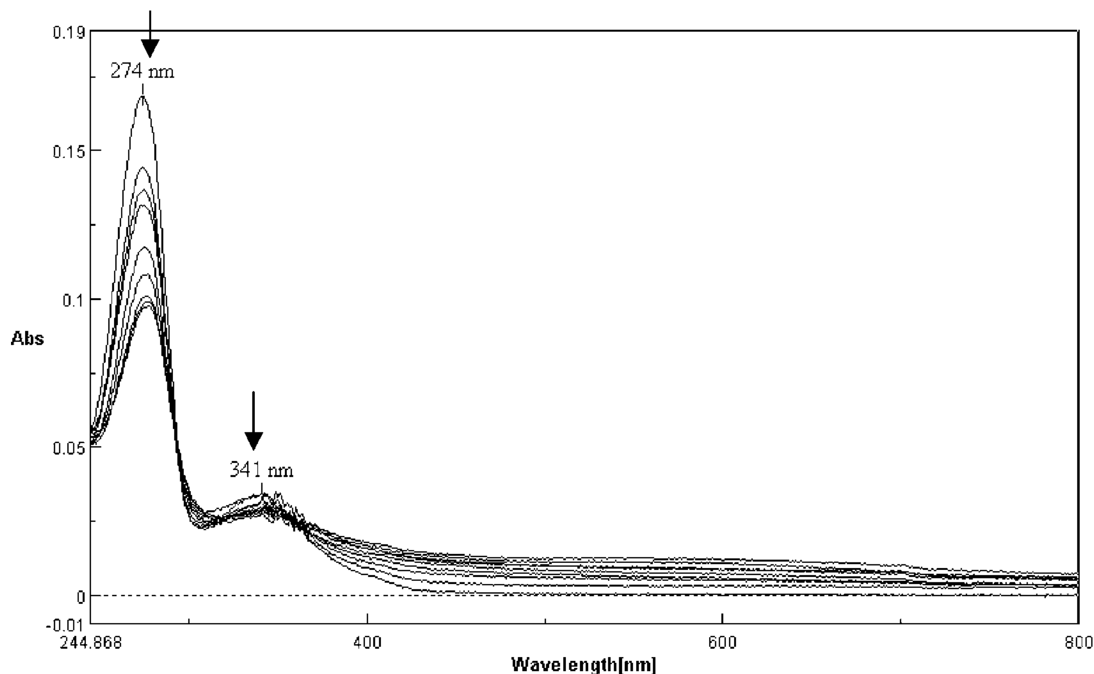

Figure 3. Fe(II) chelation capacity of compound **4**. The absorption spectrum of compound **4** (25 μM) in water was recorded in the presence of Fe(II) (0–150 μM) at 25 °C.

diarylnonanoids **2**−**5** possess a 2-acylresorcinol moiety. However, only compounds **3** and **4** containing a phenolic group in the other benzene ring were active. Evidently, the resorcinol moiety did not contribute significantly to the activity, which is also evident from the poor activity of compound **1**. The potency of substituted phenols for radical scavenging and protection against lipid peroxidation is governed by the electron-donating effect of the substituents (*28*, *29*). The presence of a substituent with a higher electron-donating capacity lowers the O−H bond dissociation enthalpy and increases the rate of H-atom transfer to peroxyl radicals (*30*). The electron-donating effect of a substituent depends on its nature and the position with respect to the phenol moiety. Thus, the activity of a strong electron-donating group, such as the hydroxyl group, at the ortho and para positions is much higher than that at the meta position. This may explain the relative order of the antioxidant potencies of compounds **3** and **4**. The results are consistent with the reported enhanced activities of the catechol compounds (*31*).

Role of Iron Chelation. The effect of iron chelation by a test compound might be either reduction in the availability of Fe(II) at specific reaction sites or generation of a less reactive complex. For example, the iron chelator 1,10-phenanthroline is known to follow the latter mechanism in its antioxidant action (*32*). Earlier, we have found that the five-membered transition metal complexes formed with catechol-containing compounds are more stable and significantly less redox-active than the six-membered complexes produced with resorcinol derivatives (*33*). Consequently, even if the resorcinol moiety of compounds **1**−**5** leads to Fe(II) complexation, its effect on the reactivity of the resultant chelate would be insignificant in the Fenton reaction. In contrast, the Fe(II)−catechol complex that is only possible with compound **4** would be more inert toward oxidation, leading to its better anti-LPO activity.

In view of its partially methylated structure, Fe(II) complexation by curcumin would be through covalent and relatively weak coordinate bonds. Consequently, despite its two catechol moieties, curcumin would furnish a more reactive Fe(II) chelate. In contrast, the Fe(II)−**4** complex will be inert because its formation involves stronger covalent bonds only. Thus, although the Fe(II)-complexing capacity of **4** qualitatively equals that of curcumin, its anti-LPO activity is anticipated to be better than that of curcumin.

Radioprotection of pBR322 DNA by Compound 4. It is well-known that radiation causes mutation and carcinogenesis, the main factor of these events being attributed to scission of DNA molecules (*22*, *34*). Due to the high concentration of water in metabolizing cells, radiation exposure of biological systems primarily leads to its radiolysis, furnishing e^{-}_{aq}, $OH^{\bullet}$, and $H^{\bullet}$. These radicals react with DNA due to the presence of various reactive sites (base and sugar) in them. For a variety of tissues, the pathophysiological importance of ROS-mediated oxidative injury caused by the exposure to radiation is widely appreciated. Hence, the protective capacity of compound **4** against γ-ray-induced DNA single-strand breaks (SSB) was also assessed.

During radiation exposure, SSB of the supercoiled (SC) DNA generates an open-circular (OC) form, which migrates more slowly than the SC form in the agarose gel electrophoresis. Exposure of pBR322 plasmid DNA to γ-radiation, as a function of dose, resulted in a significant increase in the SSBs of DNA. A linear increase in SSBs, assessed as average strand breaks per DNA molecule, was observed up to a dose of 16 Gy (data not shown), which was sufficient to convert the entire supercoiled DNA to open circular form. Hence, this dose was chosen for the studies, and the results are shown in **Figure 4**.

Compared to the un-irradiated DNA (control, lane 1), exposure of DNA to γ-radiation (16.0 Gy) led to an extensive conversion of the supercoiled form to the open circular form (lane 2). Addition of compound **4** in increasing concentrations to the DNA, prior to irradiation, progressively reduced the intensity of the band due to the open circular form (lanes 3−8). Quantification of the DNA bands revealed that compound **4** at 125 (lane 3), 250 (lane 4), 500 (lane 5), 750 (lane 6), 1000 (lane 7), and 1250 μM (lane 8) concentrations offered 18.5, 19.1, 31.6, 38.3, 40.2, and 44.8% protection, respectively, to the radiation-induced SSB formation. Compound **4** itself did not cause any DNA scission as even a 2 h incubation of DNA with it did not increase the open circular form. The radioprotection of DNA by compound **4** may be attributable to its

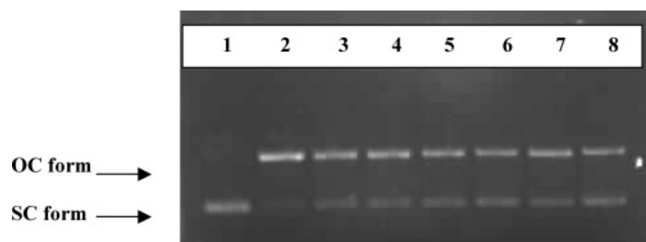

Figure 4. Gel electrophoresis pattern of pBR 322 plasmid DNA showing the open-circular (OC) and supercoiled (SC) forms after exposure to γ-rays in the presence and absence of different concentrations of compound **4**: (lane 1) DNA sample without irradiation; (lane 2) DNA sample after irradiation; (lanes 3–8) DNA samples after irradiation in the presence of 0.125, 0.25, 0.5, 0.75, 1.0, and 1.25 mM of compound **4**, respectively. Plasmid DNA (200 ng) samples in 10 mM potassium phosphate buffer, pH 7.4, in a 14 μL volume were irradiated at 25 °C up to a dose of 16 Gy using a ^{60}Co source.

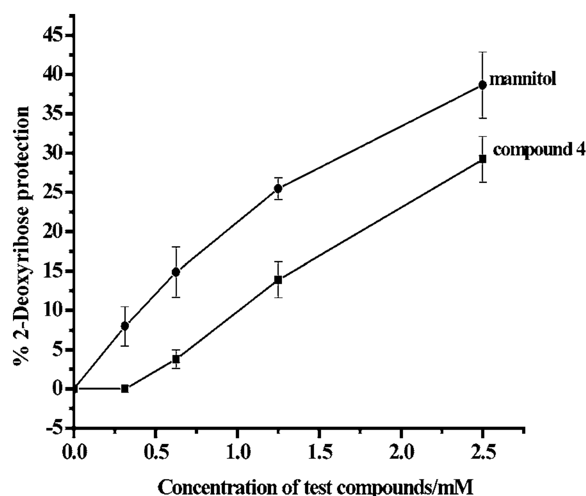

Figure 5. Comparative concentration-dependent scavenging capacities of compound **4** and mannitol for the •OH radicals. Values are mean ± SE ($n = 5$). Means were compared statistically by paired *t* test at $p < 0.05$. Means of compound **4** and mannitol were significantly different at all test concentrations.

hydroxyl radicals scavenging ability. For a better understanding of the radioprotective mechanism, the •OH radicals scavenging ability of compound **4** was also assayed.

Scavenging of •OH Radicals by Compound 4. The highly reactive •OH radicals are the predominant ROS generated endogenously during aerobic metabolism (*35*) and are believed to contribute maximally in DNA cleavage. In the 2-deoxyribose oxidation assay, compound **4** showed a high affinity for the hydroxyl radicals generated by the Fenton reaction.

In a reaction mixture containing Fe(III)−EDTA−H_2O_2 and 2-deoxyribose, very few TBARS were formed ($A_{532} = 0.09 \pm 0.03$/h, $n = 5$), whereas the addition of ascorbic acid triggered production of TBARS ($A_{532} = 2.100 \pm 0.07$/h, $n = 5$). As shown in **Figure 5**, compound **4** inhibited degradation of 2-deoxyribose very efficiently, comparable to mannitol. For example, at a concentration of 2.5 mM, compound **4** reduced the •OH radical-induced 2-deoxyribose degradation by 29.2 ± 2.9%, whereas the positive control, mannitol, showed 38.7 ± 4.2% inhibition under the same conditions.

For a variety of tissues, the pathophysiological importance of ROS-mediated oxidative injury caused by the presence of iron and other transition metal ions and exposure to radiation is widely appreciated (*21*, *22*). Iron-promoted Fenton chemistry appears to be very critical in the progression of reversible ischemic injury and acceleration of myocardial reperfusion damage (*36*). Our study using the rat liver mitochondria and plasmid DNA as in vitro model systems showed that malabaricone C (**4**) is an effective protector of lipids and DNA against both iron-promoted and radiation-induced damages, respectively, independent of DNA repair or other cellular defense mechanisms. The beneficial effects might be attributed to the radical scavenging and iron chelating abilities of malabaricone C. Malabaricone C has a strong structural resemblance to curcumin, which is credited with a diverse array of medicinal attributes (*37*). Most of the bioactivities of curcumin are believed to be due to its antioxidant activity and ability to induce apoptosis. Given that malabaricone C acts as stronger antioxidant than curcumin in the in vitro studies, the compound appears to be a promising candidate for further in vivo bioevaluation. The compound is obtained from the spice *M. malabarica*, which is extensively consumed in the Eastern world without any side effect. In view of its similar hydrophobicity to that of curcumin, the biodistribution of compound **4** is anticipated to be good. Given that the concentration of malabaricone C in rampatri is very high, it appears to be an attractive target for drug development. Incidentally, a polyherbal antitumor drug, muthu marunthu, contains *M. malabarica* as one of the ingredients (*38*).

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