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Chemical studies on antioxidant mechanism of garcinol: analysis of radical reaction products of garcinol with peroxyl radicals and their antitumor activities

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Abstract—Antioxidant actions of garcinol (**1**), a polyisoprenylated benzophenone, purified from *Garcinia indica* fruit rind, are believed to contribute to its chemopreventive activity. However, the mechanisms of its antioxidant reactions remain unclear. The objective of this study was to characterize the reaction products of garcinol with peroxyl radicals generated by thermolysis of the azo initiator azo-bis-isobutyrylnitrile (AIBN). Structure elucidation of these products can provide insights into specific mechanisms of antioxidant reactions. Four reaction products (**2**–**5**) were isolated and identified. Their structures were determined on the basis of detailed high field 1D and 2D spectral analysis. The identification of these products provides the first unambiguous proof that the double bond of the isopentenyl group is a principal site of the antioxidant reaction of **1**. The induction of apoptosis in human leukemia HL-60 cells, the inhibition of NO generation, and the inhibition of LPS-induced iNOS gene expression by Western blot analysis by **1** and its four oxidation products (**2**–**5**) were investigated.

1. Introduction

Garcinol (**1**), also called camboginol, is a polyisoprenylated benzophenone derivative isolated from *Garcinia indica* and other species.[1–4] The dried rind of *G. indica* (cv. Kokum) is used as a garnish for curry and in some of the folklore medicine in India and contains 2–3% garcinol by weight.[1,2] Garcinol is structurally similar to a well-known antioxidant, curcumin (**6**), which contains both phenolic hydroxyl groups and an enol form of a β-diketone moiety. Recently, garcinol has attracted considerable interest because of its associated beneficial health properties, including antibiotic activities,[5] antiulcer activity,[6] suppressed colonic aberrant crypt foci (ACF) formation,[7] and induction of apoptosis through cytochrome c release and activation of caspases in human leukemia HL-60 cells.[8] It also showed strong antioxidant activity. In the H_2O_2/NaOH/DMSO system, garcinol suppressed superoxide anion, hydroxyl radical, and methyl radical; in the Fenton reaction system, garcinol suppressed hydroxyl radical more strongly than DL-α-tocopherol; in the hypoxanthine/xanthine oxidase system, emulsified garcinol suppressed superoxide anion to almost the same extent as DL-α-tocopherol by weight;[6] in a phenazine methosulfate/NADH-nitroblue tetrazolium system, garcinol exhibited superoxide anion scavenging activity and suppressed protein glycation in a bovine serum albumin/fructose system.[9] It also showed nearly three times greater 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity than DL-α-tocopherol by weight.[9] However, the antioxidant mechanisms of garcinol remain unclear. We recently reported the structures of two major oxidation products (**4** and **5**) of garcinol with DPPH.[10] The identification of these structures provided the first unambiguous evidence that the principal oxidation sites of garcinol are on the 1,3-diketone and the phenolic ring part. It is generally accepted that depending on how oxidation is achieved, the condensation products are different. To continue our study on the antioxidant mechanisms of garcinol, we reacted garcinol with peroxyl radicals generated by thermolysis of the initiator 2,2′-azo-bis-isobutyronitrile (AIBN). The antioxidant process of this reaction is thought to be divided into the following stages:

1. Radical generation stage

$$RN{=}NR \xrightarrow{\Delta} 2R^{\cdot} + N_2$$

$$2R^{\cdot} + 2O_2 \rightarrow 2ROO^{\cdot}$$

Keywords: garcinol; peroxyl radical; antioxidant mechanism; antitumor activities.

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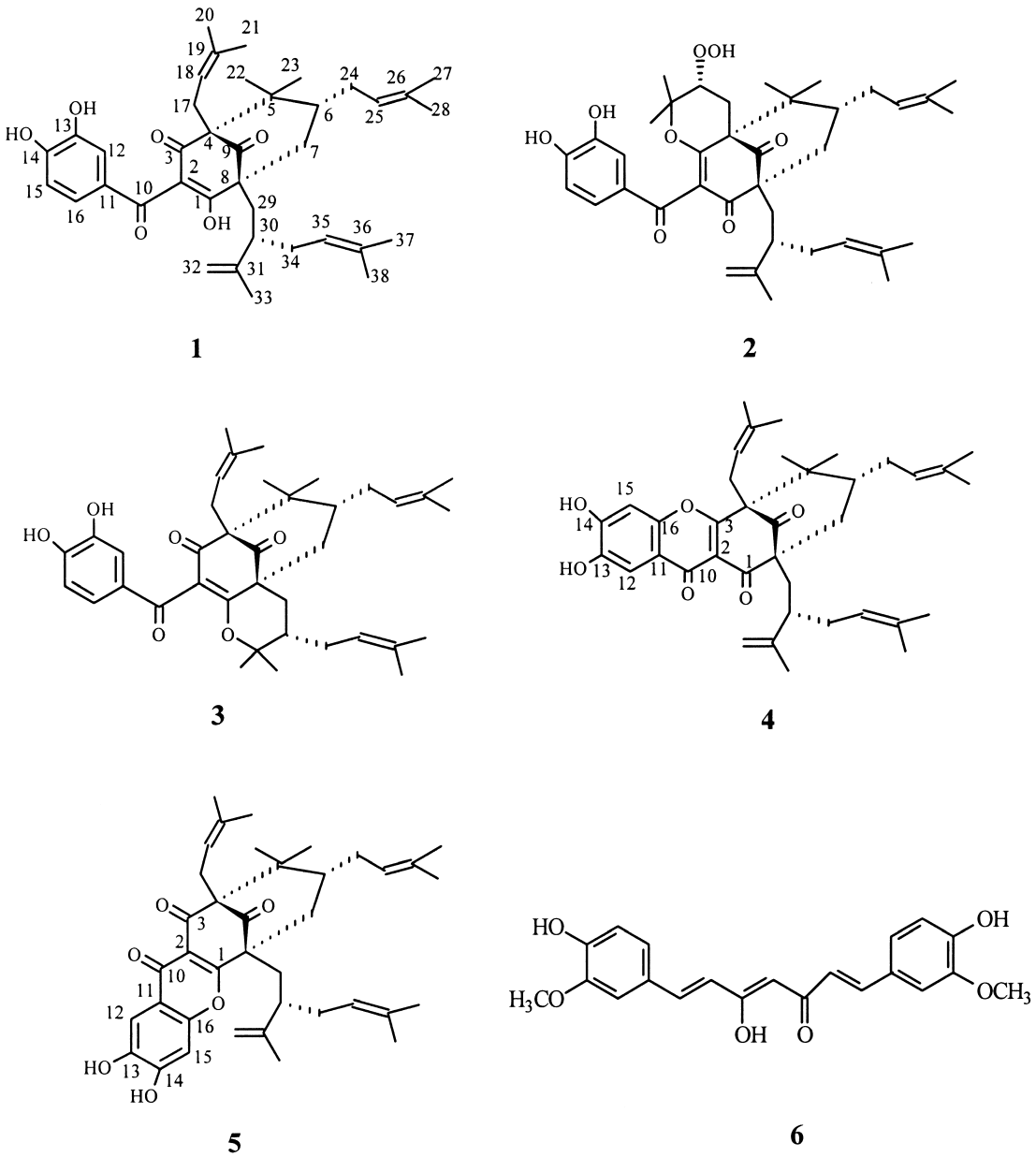


Figure 1. Structures of compounds **1**–**5** and curcumin (**6**).

2. Radical trapping stage

$$ROO^{\cdot} + AH \rightleftharpoons ROOH + A^{\cdot}$$

3. Radical termination stage

$$A^{\cdot} + X^{\cdot} \rightarrow \text{nonradical material}$$

AIBN decomposed thermally to yield alkyl radicals ($R^{\cdot}$), which react with oxygen rapidly to generate peroxyl radicals ($ROO^{\cdot}$). AH is the phenolic antioxidant, $A^{\cdot}$ is the antioxidant radical, and $X^{\cdot}$ is another radical species or the same species as $A^{\cdot}$.[11] Although the second stage is a reversible process, the third stage is irreversible and produces stable radical termination compounds. Structural information about these nonradical products would afford important contributions to antioxidant mechanism studies. In the study presented here, we have succeeded in isolating and characterizing reaction products of garcinol with alkylperoxyl radicals from AIBN in a homogeneous acetone system. Four reaction products (**2**–**5**) were isolated and identified. Their structures were determined on the basis of detailed high field 1D and 2D spectral analysis. The induction of apoptosis in human leukemia HL-60 cells, the inhibition of NO generation, and the inhibition of LPS-induced iNOS gene expression by Western blot analysis by **1** and its four oxidant products (**2**–**5**), and curcumin (**6**) were investigated.

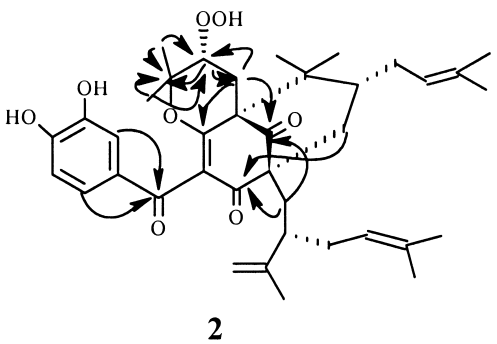
2. Results and discussion

2.1. Structural elucidation of four oxidation products (2–5)

Reaction of garcinol (**1**) with peroxyl radical was carried out

and four major oxidation products (**2**–**5**) were isolated and identified on the basis of their spectral data (Fig. 1).

Compound **2**, a yellow amorphous solid, was assigned the molecular formula of $C_{38}H_{50}O_8$ determined by HRFAB-MS *m/z* 635.3581 $[M+H]^+$ (calcd for $C_{38}H_{51}O_8$, 635.3584), as well as from its ^{13}C NMR data. The molecular formula indicated fourteen degrees of unsaturation, which showed that **2** had the same unsaturation as **1**. The ^1H NMR spectrum of **2** showed the presence of three AMX pattern aromatic protons at δ 7.39 (d, J=1.8 Hz), 7.26 (dd, J=1.8, 8.4 Hz), and 6.79 (d, J=8.4 Hz) ppm, respectively; one oxygenated methine at δ 4.42 ppm; two isopentenyl groups [two vinylic protons at δ 4.97, t, J=6.0 Hz, and 4.87, t, J=6.0 Hz; and four vinylic methyl groups at δ 1.64 (for two methyl signals), 1.59, and 1.57 ppm, respectively], one isopropenyl group [two singlets of 2H at δ 4.48 and 4.44, together with a methyl singlet at δ 1.44], and four methyl groups on saturated carbons [four methyl singlets at δ 1.15, 1.09, 1.08 and 1.04], in addition to vinylic and methine protons [a complex multiplet of 12H in the region of δ 2.80–1.45]. Thus the significant difference in the ^1H NMR spectrum of **2** compared to **1** was the absence of the double bond of one isopentenyl group. As mentioned above, **2** had the same unsaturation as **1**. All of these suggested that there was one more ring in **2** than in **1**. The above findings were in agreement with the ^{13}C NMR spectrum of **2**. It also showed the presence of two isopentenyl groups [two methine carbons of trisubstituted olefinic groups at δ 124.5 and 123.3, and four methyl groups at δ 18.1, 18.2, 25.9, and 26.0], one isopropenyl group [δ 112.9 for a terminal methylene carbon, and δ 18.0 for a methyl signal], and four methyl groups on saturated carbons [δ 23.9, 24.4, 26.5 and 27.3]; three methine carbons for the aromatic ring at δ 114.8, 116.8 and 124.3; two oxygen-substituted aromatic carbons at δ 143.8 and 150.5; one oxygenated quaternary carbon at δ 71.6, and one oxygenated methine at δ 92.1. Furthermore, in the ^{13}C spectral data one of the carbon atoms of the enolized 1,3-diketone in **1** was changed from δ194.0 (or 195.2) to δ 175.2 in **2**. The ^{13}C spectral data of the carbonyl (C-10) was also changed from δ 199.1 in **1** to δ 190.6 in **2**. The HMBC correlation between $C_{\delta 175.2}$ and H-17 (δ 2.68 and 2.07), $C_{\delta 194.2}$ and H-7 (δ 2.19 and 2.04), H-29 (δ 2.08 and 1.74); $C_{\delta 190.6}$ and H-12 (δ 7.39), H-16 (δ 7.26); $C_{\delta 71.6}$ and H-17 (δ 2.68 and 2.07), H-20 (δ 1.04), H-21 (δ 1.09) and $H_{\delta 92.1}$ (δ 4.42); $C_{\delta 92.1}$ and H-17 (δ 2.68 and 2.07), H-20 (δ 1.04) and H-21 (δ 1.09) (Fig. 2) suggested that δ 175.2 could be assigned to C-3, δ 194.2 to C-1, δ 190.6 to C-10, δ 71.6 to C-19, and δ 92.1 to C-18, respectively. All of these indicated that the carbonyl at C-3 was enolized and the oxygen was attached to C-19. The proton and carbon shifts at position 18 (δ_C 92.1, δ_H 4.42) were deshielded relative to those of a secondary alcohol and, when considered with the molecular formula, suggested that a hydroperoxyl group was present at this position.[12,13] Compound **2** gave a positive peroxide reaction with FeSCN.[14] The α configuration for this hydroperoxyl group was supported by the large $J_{17,19}$ coupling constant (dd, J=7.2, 9.6 Hz). Thus, the structure of **2** was deduced as shown (Fig. 1). The complete interpretation of the NMR data was based on the results of COSY, TOCSY, HMQC and HMBC.



2

Figure 2. Significant HMBC (H→C) correlations of **2**.

Compound **3**, a yellow amorphous solid, had a molecular formula of $C_{38}H_{50}O_6$ determined by APCIMS (*m/z* $[M+H]^+$ 603) as well as its ^{13}C NMR data, which was the same as the molecular formula of **1**. The ^1H NMR spectrum of **3** also showed the presence of three AMX pattern aromatic protons at δ 7.42 (d, J=1.8 Hz), 7.00 (dd, J=1.8, 8.4 Hz), and 6.66 (d, J=8.4 Hz) ppm, respectively; three isopentenyl groups [three vinylic protons at δ 5.10, t, J=6.0 Hz, 4.90, t, J=6.0 Hz, and 4.85, t, J=6.0 Hz; and five singlet methyl groups at δ 1.71, 1.65, 1.62, and 1.56 (for two methyl groups), respectively], and four methyl groups on saturated carbons [four methyl singlets at δ 1.22, 1.15, 0.97 and 0.94], in addition to methylene and methine protons [a complex multiplet of 12H in the region of δ 3.10–1.45]. Thus, the significant difference in the ^1H NMR spectrum of **3** compared to that of **1** was the absence of the double bond of the isopropenyl group. As mentioned above, **3** had the same molecular weight as **1**. This implied that there was one more ring in **3** than in **1**. The above findings were in agreement with the ^{13}C NMR spectrum of **3**. It also showed the presence of three isopentenyl groups [three methine carbons of trisubstituted olefinic groups at δ 125.3, 124.9 and 123.9, and six methyl groups at δ 17.7, 17.8, 17.9, 25.4, 25.5, and 25.7], and four methyl groups on saturated carbons [δ 21.1, 22.3, 26.6, and 28.2]; three methine carbons for the aromatic ring at δ 114.6, 119.7 and 121.4; two oxygen-substituted aromatic carbons at δ 144.9 and 150.8; and one oxygenated quaternary carbon at δ 86.8. Furthermore, in the ^{13}C spectral data, the chemical shift of one of the carbon atoms of the enolized 1,3-diketone in **1** was changed from δ 194.0 (or 195.2) to δ 171.9 in **3**. The ^{13}C spectral data of the carbonyl (C-10) was also changed from δ 199.1 in **1** to δ 194.7 in **3**. All of these indicated that the carbonyl at C-1 was enolized and the oxygen was attached to C-31. Therefore, compound **3** was identified as cambogin.[1,2,15] This was further confirmed by comparing **3** with standard cambogin by TLC plate.

Compound **4**, a yellow amorphous solid, was assigned the molecular formula of $C_{38}H_{48}O_6$ determined by positive-ion APCI-MS ($[M+H]^+$ at *m/z* 601), as well as from its ^{13}C NMR data. The molecular formula indicated fifteen degrees of unsaturation, which showed that **4** had one more unsaturation than **1**. The ^1H and ^{13}C NMR data of **4** are identical with those of GDPPH-1 that we reported from the reaction between garcinol and the stable radical DPPH.[10] Thus, compound **4** was identified as shown (Fig. 1). This was further confirmed by comparing **4** with standard GDPPH-1 by TLC plate.

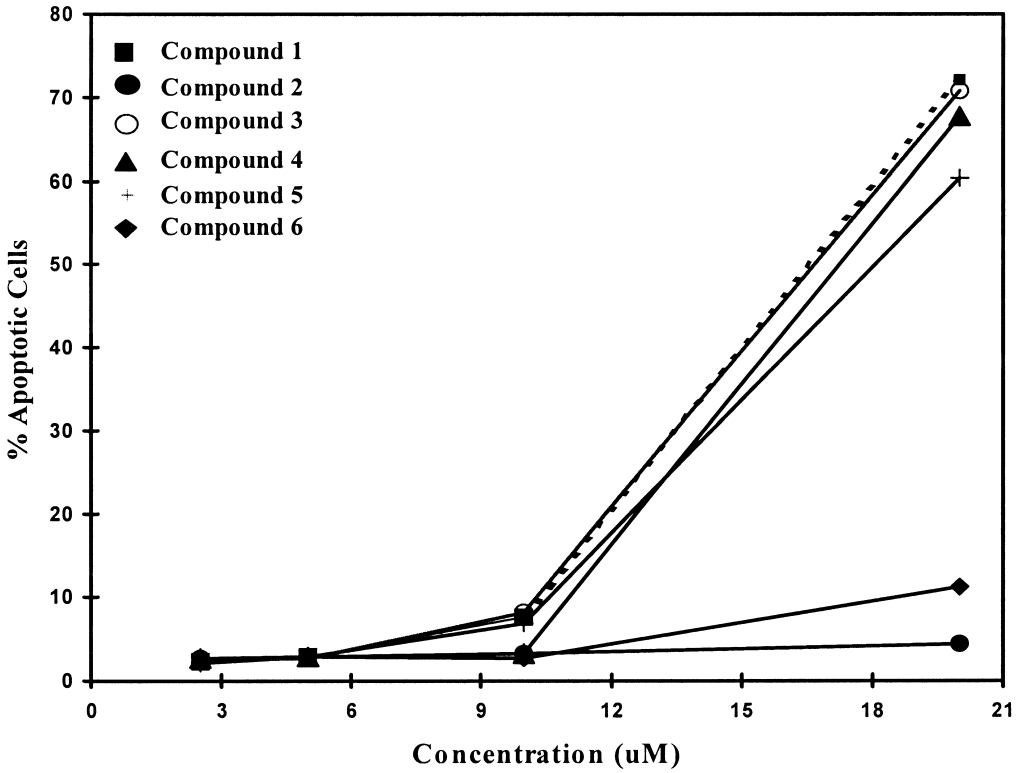


Figure 3. Induction of apoptosis by compounds **1**–**5**, and curcumin (**6**) in HL-60 cells. HL-60 cell was treated with different concentrations (2.5, 5, 10, and 20 μM) of compounds **1**–**5**, and curcumin for 24 h and apoptosis was quantified by flow cytometry. The method of flow cytometry used is described in Section 3.

Compound **5** was isolated as a yellow amorphous solid. The positive-ion APCI-MS of **5** displayed a molecular ion peak at *m/z* $[M+H]^+$ 601, supporting a molecular formula of $C_{38}H_{48}O_6$, which was the same as that of **4**. The ^1H and ^1C NMR data of **5** are identical with those of GDPPH-2 that we reported from the reaction between garcinol and the stable radical DPPH.[10] Thus, compound **5** was identified as shown (Fig. 1). This was further confirmed by comparing **5** with standard GDPPH-2 by TLC plate.

2.2. Induction of apoptosis by compounds 1–5, and curcumin (6) in human leukemia HL-60 cells

Physiological cell death is characterized by apoptotic morphology, including chromatin condensation, membrane blebbing, internucleosomal degradation of DNA, and apoptotic body formation. In each case, nucleosomal DNA ladders, which are typical of apoptosis, were visible on agarose gel after staining with ethidium bromide. A sub-G1

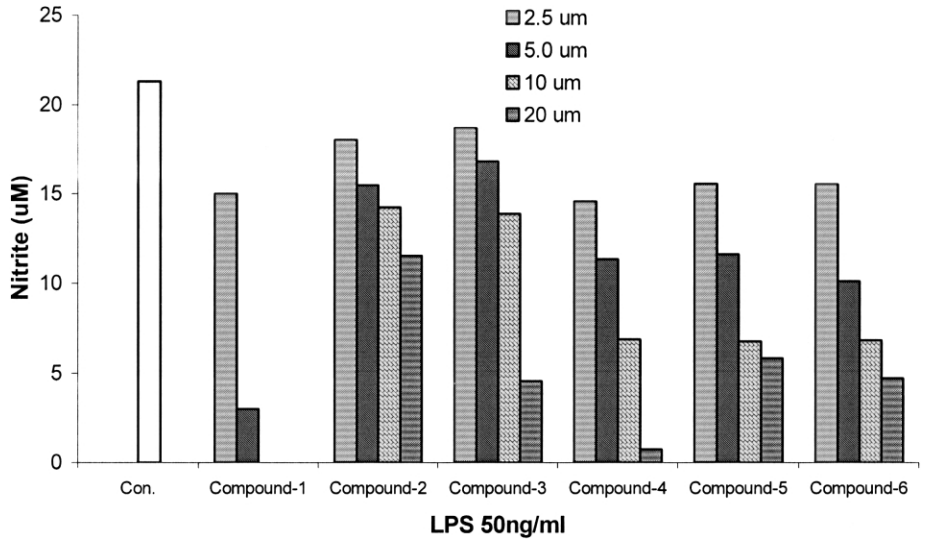


Figure 4. Effects of compounds **1**–**5**, and curcumin (**6**) on LPS-induced nitrite production in RAW 264.7 cells. The cells were treated with different concentrations of compounds and LPS (50 ng/mL) for 16 h. Nitrite was determined by Griess reaction, as described in Section 3.

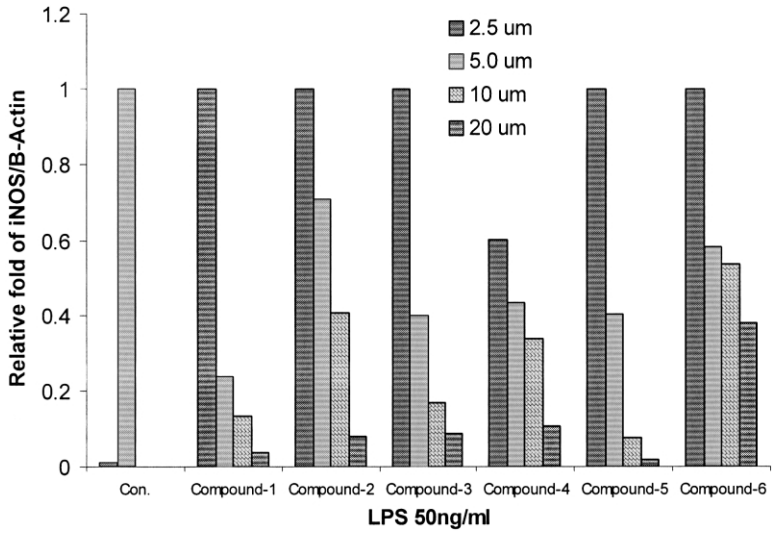


Figure 5. Western blot analysis of the inhibition of LPS-induced iNOS protein expression by compounds **1**–**5**, and curcumin (**6**). RAW 264.7 were co-treated with 50 ng/mL of LPS and 2.5 μM of compounds **1**–**5**, and curcumin for 24 h. Total protein was isolated for western blot analysis of iNOS and β-actin. Quantification of band intensities was via three independent experimental results by densitometry (IS-1000 Digital Imaging System).

(sub-2N) DNA peak, which has been suggested to be the apoptotic DNA was detected in cells that were treated with compounds *1*–**5**, and curcumin (**6**), and stained by propidium iodide. As shown in Fig. 3 the percentage of apoptotic HL-60 cells were 72.06, 4.27, 70.23, 67.6, 60.35, and 11.12% after 18 h of incubation with compounds **1**–**5**, and curcumin (20 μM), respectively. Among them, garcinol and compounds **3**–**5** appeared to be more potent and dose-dependent on the induction of cell apoptosis. When the HL-60 cells were treated with the same concentration (20 μM) of these compounds, the apoptotic potency was the same. These data are consistent with DNA fragmentation (data not shown).

2.3. Inhibition of NO generation by compounds 1–5, and curcumin (6)

Compounds **1**–**5**, and curcumin were examined to determine whether they affect NO production in macrophages activated with LPS for 16 h. Of these compounds, garcinol inhibited LPS-stimulated NO generation most strongly; however, compounds **1**–**5**, and curcumin all markedly reduced NO generation in a concentration-dependent manner (Fig. 4). The inhibitory potency was estimated as follows: garcinol>**4**>**5**>curcumin>**3**>**2**, at 10 μM. Inhibition of NO production was not due to cytotoxicity, as determined with trypan blue exclusion assay.

2.4. Inhibition of LPS-induced iNOS gene expression by Western blot analysis by compounds 1–5, and curcumin (6)

Compounds **1**–**5**, and curcumin were examined to determine whether they affect iNOS protein in macrophages activated with LPS (100 ng/mL) for 16 h. RAW 264.7 cells were treated. Inhibition of iNOS protein by these compounds was detected at 2.5, 5, 10, 20 μM. RAW 264.7 cells did not express iNOS protein when incubated in the medium without LPS for 16 h. Upon LPS treatment, iNOS protein drastically increased in these cells, and co-treatment of cells with LPS and the indicated compounds for 16 h significantly inhibited iNOS protein (Fig. 5). The inhibitory potency was as follows: garcinol>**5**>**3**>**4**>**2**>curcumin, at 10 μM. The amount of β-actin protein as an internal control remained unchanged.

2.5. Discussion

The purpose of this investigation was to isolate and characterize the reaction products of garcinol with alkylperoxyl radicals derived from AIBN in a homogeneous solution. Our previous identification of garcinol oxidation products **4** and **5**[10] provided the first unambiguous evidence that antioxidant reactions of garcinol with stable free radical DPPH involve the 1,3-diketone and phenolic ring moieties. In this current study, we identified two more reaction products (products **2** and **3**), which showed that the double bond of the isoprenyl group was also a principal site of the antioxidant reaction of garcinol. Compound **2** is a new hydroperoxy derivative of garcinol. A similar compound has been reported as a new natural product.[13] Compound **3** was identified as cambogin, also named isogarcinol, which has been reported from *G. indica*, *G. cambogia*, and other species.[1,2,4,15,16] However, ours is the first study to report the formation of cambogin as an oxidation product during the antioxidant reaction of garcinol. Cambogin has biological activities that are similar to those of garcinol. It has been claimed for use as hyaluronidase inhibitor for prevention of skin aging and as antiinflammatory and antitumor agents,[17] as a lipase inhibitor, an anti-obesity agent, and a hypolipidemic,[18] as an inhibitor for Epstein–Barr virus early antigen induction and as an antitumor agent,[19] and as an antiulcer agent.[20] Cambogin has also been reported for control of methicillin-resistant *Staphylococcus aureus*.

From our elucidation of the chemical structures of these four compounds, we proposed the antioxidant mechanism of garcinol as shown in Fig. 6. As shown in Fig. 6, garcinol has been proposed to react with peroxyl radicals by a single electron transfer followed by deprotonation from the hydroxyl group of the enolized 1,3-diketone to form a

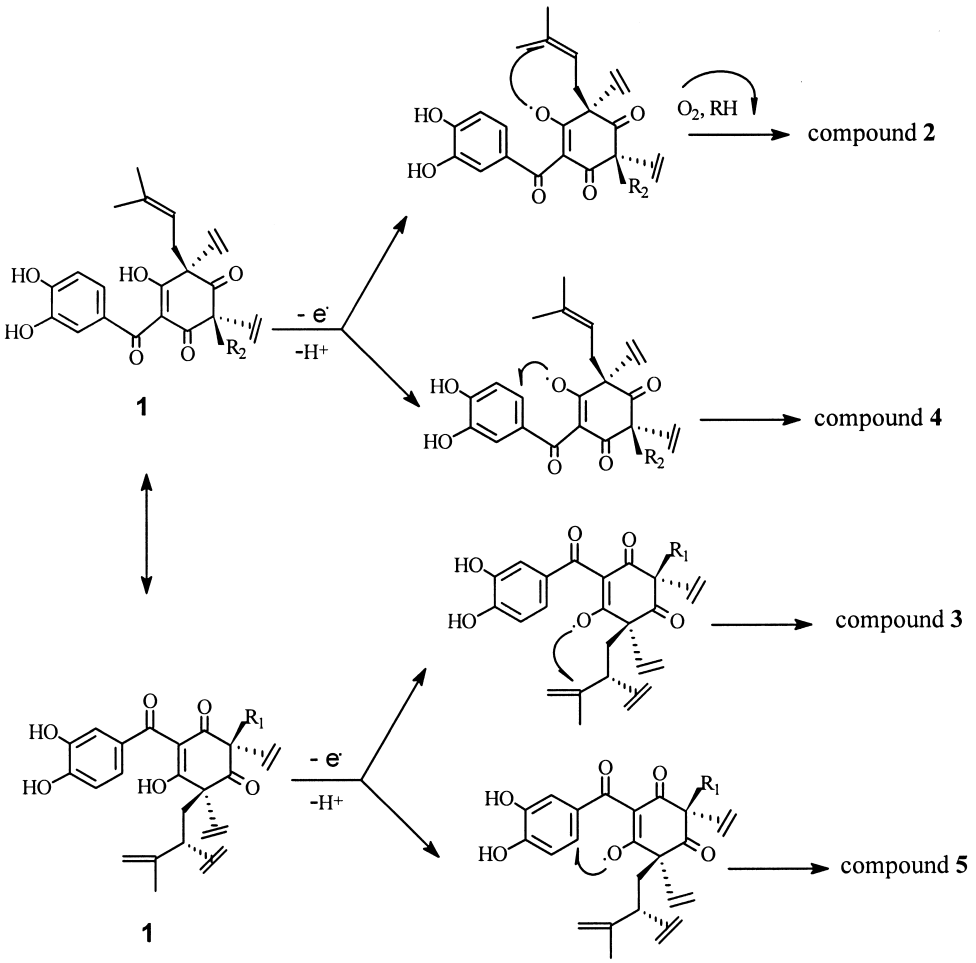


Figure 6. Proposed mechanism for the formation of oxidation products **2**–**5**.

resonance pair.[21,22] If reaction was initiated at the hydroxyl group of C-3, **2** and **4** would be formed, and **3** and **5** would be formed if reaction was initiated at the hydroxyl group of C-1.

Cancer preventive effects have often been attributed to antioxidant actions.[22–27] In order to understand whether garcinol or its oxidation products play a functional role in biological system, the antitumor activities of garcinol and its four reaction products were individually tested. The activities of these compounds were compared with curcumin, a well-known antioxidant. These included an antitumor test of garcinol and its four reaction products (**2**–**5**) and curcumin on induction of apoptosis in human leukemia HL-60 cells, the inhibition of NO generation, and the inhibition of LPS-induced iNOS gene expression by Western blot analysis. These tests indicated that like **1**, compounds **3**–**5** showed strong inhibitory effects on these assays. Compound **2** showed very weak activity. Garcinol, and compounds **4** and **5** showed better inhibitory effects in these assays than curcumin. The potency of these compounds in apotosis-induction may vary with the different cell lines. These findings might suggest possible chemopreventive ability of garcinol and its oxidation products. Analysis of these products could provide a unique tool for assessing the contribution of antioxidant reactions to the disease preventive effects of garcinol.

It is notable that compounds **4** and **5**, the two major reaction products of our two model oxidation systems (DPPH system and peroxyl radical system), showed similar inhibitory effects on cell viability in human leukemia HL-60 cells as garcinol. Our future work will focus on whether these two reaction products of garcinol can show similar or even stronger activities in other biological systems, such as anti-inflammation, and antiulcer activities.

3. Experimental

3.1. General procedure

^{1}H (600 MHz), ^{1}C (150 MHz) and all 2D NMR spectra were run on a Varian AM-600 NMR spectrometer, with TMS as internal standard. FT-IR was performed on a Magna 550 spectrometer. The APCI MS was performed on a Fisons/VG Platform II mass spectrometer. HREI MS was run on JEOL AX-505 double focusing mass spectrometer. Thin-layer chromatography was performed on Sigma-Aldrich TLC plates (250 μm thickness, 2–25 μm particle size), with compounds visualized by spraying with 5% (v/v) H_2SO_4 in ethanol solution. CD_3OD was purchased from Aldrich Chemical Co. Ferrous ammonium sulfate and ammonium thiocyanate was purchased from Sigma Chemical Co. AIBN was purchased from Sigma Chemical Co.: ^{1}H NMR ($CDCl_3$,

600 MHz): δ 1.66 (s, CH_3); ^{13}C NMR ($CDCl_3$, 150 MHz): δ 25.3 (q, CH_3), 68.3 (s, C), 119.3 (s, C≡N). Garcinol was isolated from *G. indica* dried fruit rind.[10]

3.1.1. Oxidation of 1 and isolation of reaction products 2–5. Garcinol (**1**) (2.0 g, 3.34 mmol) was allowed to react with AIBN (6.0 g, 15.7 mmol) in 50 mL acetone incubated at 50°C for 12 h. After evaporation of the solvent in vacuo, the residue was first applied to silica gel column eluting with chloroform to get rid of the AIBN and **1** (400 mg) and then with methanol to get a mixture of four reaction products. The mixture was subjected to RP-C18 silica gel column eluting by a 90% methanol–water solvent system to give 40 mg compound **2**, 30 mg compound **3**, 200 mg compound **4** and 100 mg compound **5**.

Compound **2** was isolated as a pale amorphous substance: ^1H NMR (CD_3OD, 600 MHz): δ 7.39 (d, J=1.8 Hz), 7.26 (dd, J=1.8, 8.4 Hz), 6.79 (d, J=8.4 Hz), 4.97 (t, J=6.0 Hz), 4.87 (t, J=6.0 Hz), 4.48 (s), 4.44 (s), 4.42 (dd, J=7.2, 9.6 Hz), 1.64 (s), 1.59 (s), 1.44 (s), 1.15 (s), 1.09 (s), 1.08 (s) and 1.04 (s); ^{13}C NMR (CD_3OD, 150 MHz): see Table 1;

Table 1. δ_C (150 MHz) NMR spectral data of compounds **1**–**3** (δ in ppm, J in Hz)

	1[a]	**2**[a]	**3**[b]
1	194.0 s	194.2 s	171.9 s
2	116.0 s	117.2 s	114.4 s
3	195.2 s	175.2 s	193.3 s
4	69.9 s	67.6 s	68.3 s
5	49.8 s	47.6 s	46.2 s
6	47.0 d	45.9 d	46.1 d
7	42.7 t	41.9 t	42.8 t
8	58.1 s	60.7 s	51.2 s
9	207.1 s	207.7 s	207.3 s
10	199.1 s	190.6 s	194.7 s
11	127.8 s	129.9 s	129.9 s
12	116.6 d	116.8 d	119.7 d
13	143.9 s	143.8 s	144.9 s
14	149.9 s	150.5 s	150.8 s
15	114.4 d	114.8 d	114.6 d
16	120.2 d	124.3 s	121.4 s
17	27.2 t	26.7 t	25.8 t
18	122.8 d	92.1 d	123.8 d
19	135.5 s	71.6 s	134.6 s
20	26.2 q	26.5 q	25.7 q
21	18.4 q	23.9 q	17.8 q
22	22.9 q	24.4 q	22.2 q
23	27.2 q	27.3 q	26.3 q
24	29.1 t	29.8 t	29.5 t
25	123.9 d	124.5 d	125.3 d
26	133.1 s	133.9 s	133.7 s
27	25.9 q	25.9 q	25.5 q
28	18.1 q	18.2 q	17.9 q
29	36.3 t	36.3 t	28.5 t
30	43.7 d	43.7 d	29.2 d
31	148.2 s	148.4 s	86.8 s
32	112.9 t	112.9 t	28.2 t
33	17.8 q	18.0 q	21.1 q
34	32.8 t	32.9 t	39.3 t
35	124.2 d	123.3 d	124.9 d
36	132.2 s	132.2 s	133.1 s
37	26.0 q	26.0 q	25.4 q
38	18.1 q	18.1 q	17.7 q

[a] Recorded in $CDCl_3$.
[b] Recorded in $CDCl_3$–CD_3OD (5:1).

negative APCI-MS m/z 633 $[M-H]^-$, HRFAB-MS m/z 635.3581 $[M+H]^+$ (calcd for $C_{38}H_{51}O_8$, 635.3584).

Compound **3** was isolated as a pale amorphous substance: ^1H NMR (CD_3OD, 600 MHz): δ 7.42 (d, J=1.8 Hz), 7.00 (dd, J=1.8, 8.4 Hz), 6.79 (d, J=8.4 Hz), 6.66 (d, J=8.4 Hz), 5.10 (t, J=6.0 Hz), 4.90 (t, J=6.0 Hz), 4.85 (t, J=6.0 Hz), at δ 1.71 (s), 1.65 (s), 1.62 (s), 1.56 (s), 1.22 (s), 1.15 (s), 0.97 (s) and 0.94 (s); ^{13}C NMR (CD_3OD, 150 MHz): see Table 1; negative APCI-MS m/z 601 $[M-H]^-$.

Compound **4** was isolated as a pale amorphous substance: ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz): the same as GDPPH-1;[10] positive APCI-MS m/z 601 $[M+H]^+$.

Compound **5** was isolated as a pale amorphous substance: ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz): the same as GDPPH-1;[10] positive APCI-MS m/z 601 $[M+H]^+$.

3.2. Preparation of ferrous thiocyanate reagent

Ferrous ammonium sulphate (0.7 g) was dissolved in 10 mL of a solution of ammonium thiocyanate (5 g) and a concentrated sulfuric acid (1 mL) in water (100 mL).[14] This reagent was prepared just before use.

3.3. Cell culture and chemicals

Human promyelocytic leukemia (HL-60) cells obtained from American Type Culture Collection (Rockville, MD) were grown in 90% RPMI 1640 and 10% fetal bovine serum (GIBCO BRL, Grand Island, NY), supplemented with 2 mM glutamine (GIBCO BRL), 1% penicillin/streptomycin (10,000 units of penicillin/mL and 10 mg/mL streptomycin). RAW 264.7 cells were cultured in RPMI-1640 (without phenol red) supplement with 10% endotoxin-free heat-inactivated fetal calf serum (GIBCO, Grand Island, NY). Lipopolysaccharide (LPS) (*Escherichia coli* 0127: B8) was purchased from Sigma Chemical Co.

3.4. Flow cytometry

HL-60 cells (2×10^5) were cultured in 60 mm Petri dishes and incubated for 12 h. Then cells were harvested, washed with PBS, resuspended in 200 μL of PBS, and fixed in 800 μL of iced 100% ethanol at −20°C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase), and incubated at 37°C for 30 min. Then 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand on ice for 30 min. Fluorescence emitted from the propidium iodide–DNA complex was quantitated after excitation of the fluorescent dye by FACScan cytometry (Becton Dickinson, San Jose, CA).

3.5. Nitrite assay

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction. One hundred microliters of each

supernatant was mixed with the same volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in water). Absorbance of the mixture at 550 nm was determined with an enzyme-linked immunosorbent assay plate reader (Labsystems Multiskan RC).

3.6. Western blotting

Total cellular extracts were prepared according to our previous papers (Pan et al., 2000), separated on 8% SDS-polyacrylamide minigels, and transferred to immobilin polyvinylidene difluoride membranes (Millipore). The membrane was incubated overnight at 4°C with 1% BSA and then incubated with anti-iNOS or anti-α-tubulin monoclonal antibodies (Transduction Laboratories). Expression of protein was detected by chemiluminescence (ECL, Amersham).

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