Free Radical Scavenging Activity and Antiulcer Activity of Garcinol from *Garcinia indica* Fruit Rind

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Garcinol, a polyisoprenylated benzophenone derivative, was purified from *Garcinia indica* fruit rind, and its free radical scavenging activity was studied using electron spin resonance (ESR) spectrometry. In the hypoxanthine/xanthine oxidase system, emulsified garcinol suppressed superoxide anion to almost the same extent as DL-α-tocopherol by weight. In the Fenton reaction system, garcinol also suppressed hydroxyl radical more strongly than DL-α-tocopherol. In the H_2O_2/NaOH/DMSO system, garcinol suppressed superoxide anion, hydroxyl radical, and methyl radical. It was thus confirmed that this derivative is a potent free radical scavenger and able to scavenge both hydrophilic and hydrophobic ones including reactive oxygen species. Orally administered garcinol prevented acute ulceration in rats induced by indomethacin and water immersion stress caused by radical formation. These results suggested garcinol might have potential as a free radical scavenger and clinical application as an antiulcer drug.

Keywords: *Garcinol; Garcinia indica; reactive oxygen species; electron spin resonance; antiulcer activity*

INTRODUCTION

Reactive oxygen species (ROS) have been shown to play a critical role in many diseases such as cancer (Muramatsu et al., 1995), arteriosclerosis (Steinberg et al., 1989), gastric ulcer (Das et al., 1997), and other conditions (Oliver et al., 1987; Babizhayev and Costa., 1994; Busciglio and Yankner., 1996; Brown et al., 1996; Smith et al., 1996). The intake of antioxidants such as polyphenols in tea and red wine has been seen as very attractive in the prevention of these diseases (Vinson et al., 1995; Teissedre et al., 1996; Leanderson et al., 1997; Wiseman et al., 1997; Lotito and Fraga, 1998; Cohly et al., 1998; Cao et al., 1997). Epidemiological studies suggested that the intake of polyphenols from red wine in particular reduces the risk of cardiovascular disease and arteriosclerosis (Renaud et al., 1992).

We have been studying the ROS scavenging activity of grape seed extract and its pharmaceutical properties (Yamaguchi et al., 1999; Saito et al., 1998; Yamakoshi et al., 1998; Arii et al., 1998). Grape seed extract is a water-soluble ROS scavenger, and therefore we screened oil-soluble antioxidants in other natural sources. Garcinol is a polyisoprenylated benzophenone derivative from *Garcinia indica*. The dried fruit rind of *G. indica* (cv. Kokam) is used as a garnish for curry and in some of the folklore medicine in India and contains 2−3% garcinol (Krishnamurthy et al., 1981, 1982). The chemical structure proposed by Sahu et al. (1989) is shown in Figure 1. Although it is known to be a yellow pigment (Krishnamurthy et al., 1987) and an antibiotic reagent (Bakana et al., 1987; Iinuma et al., 1996), its other biological activity is not well-known.

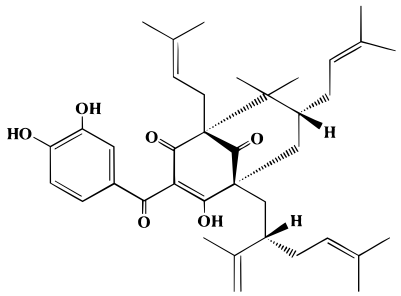

Figure 1. Chemical structure of garcinol.

Krishnamurthy (1988) reported that garcinol had no antioxidant action. However, we speculated that garcinol might exhibit antioxidative activity because the molecule had phenolic hydroxyl groups and a β-diketone structure, which were expected to contribute to the activity. Then we reconfirmed the antioxidative activity of garcinol in an emulsified system (Yamaguchi et al., 2000).

In this study we show a model of free radical scavenging activity of garcinol using the electron spin resonance (ESR) spin trapping method. As a clinical application of this free radical scavenging compound, the antiulcer activity was examined using an indomethacin-induced and water immersion stress-induced model in rats concerning radical formation (Das et al., 1997, 1998).

MATERIALS AND METHODS

Preparation of Garcinol. Garcinol was prepared from *G. indica* rind. In brief, *G. indica* dried fruit rind (cv. Kokam, purchased from Indo World Trading Co., New Delhi, India)

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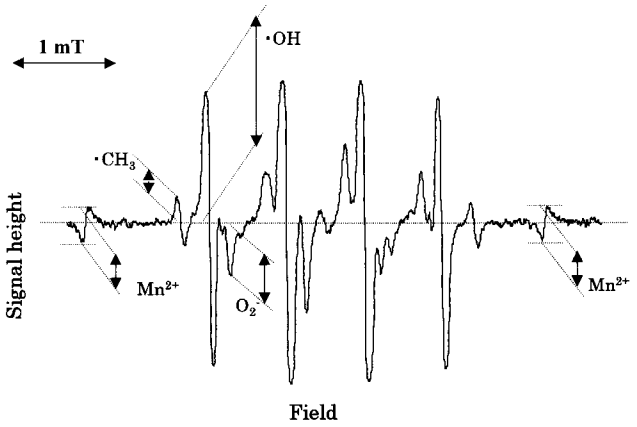

Hyperfine coupling constants of spin adducts

adducts	Hfcc, mT		
	a^N	a^N_β	a^N_γ
DMPO-OH	0.143	0.117	0.013
DMPO-OOH	0.149	0.149	
DMPO-CH_3	0.164	0.224	

Figure 2. Typical ESR spectrum in H_2O_2/NaOH/DMSO system. ESR conditions were described in the text. The scanning was started at 10 min after the mixing of all reagents. Each free radical derived signal was assigned, and its signal height was calculated at double-ended arrows in the figure. Each signal was assigned, and its hyperfine parameters were described in the figure. The signal intensity of the hydroxyl radical−DMPO adduct was so strong that the second and third peaks of the quartet signals were out of the range on the display of ESR apparatus. If the amplification rate of the apparatus is adjusted to obtain the proper spectrum of the hydroxyl radical−DMPO adduct (1:2:2:1), the signal of the superoxide anion−DMPO adduct becomes so small that it is difficult to show both signals in the same figure.

was extracted with ethanol, and the extract was fractionated by ODS column chromatography eluted stepwise with 60−80% aqueous ethanol. The fractions containing garcinol were concentrated and dried in vacuo. The residue was dissolved in hexane, and the solution was cooled at <5 °C for 2 days. Yellow amorphous precipitate was collected from the solution and washed with cold hexane on a glass filter. After drying in a vacuum desiccator, the amorphous material was solubilized in hot acetonitrile and recrystallized at room temperature. Pale yellow needle crystals were obtained from the solvent, which were identified as garcinol (Krishnamurthy et al., 1981; Sahu et al., 1989) from the following spectral data: mp 121 °C; $[\alpha]_D^{30}$ −135 ($CHCl_3$); UV λ_{max}^{EtOH} (log ϵ) 363 (3.94) and 250 (4.09) nm; IR ν_{max}^{KBr} 3200−3500, 1730, 1640 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.95 (1H, dd, J = 9.0 and 2.0 Hz), 6.91 (1H, d, J = 2.0 Hz), 6.60 (1H, d, J = 9.0 Hz), 4.96, 5.06, 5.10 (1H each, t, J = 5.0 Hz), 4.40 (d, J = 15.0 Hz), 2.80−1.46 (m, 12H, methylene and methyne), 1.78, 1.74, 1.69, 1.62, 1.59, 1.56, 1.21, 1.05 (3H each, s); EI-MS, m/z 602 $[M]^+$, 533, 465, 341.

Chemicals. 5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was obtained from Labotec Co. (Tokyo, Japan). Other chemicals were obtained from Wako Pure Chemicals, Ltd. (Osaka, Japan) unless otherwise mentioned.

Instruments. ESR spectra were recorded on a JEOL JES-RE1X spectrometer using a flat quartz cell designed for aqueous solution.

ESR Conditions. Conditions of ESR spectrometry were as follows: magnetic field, 336.3 ± 5 mT; power, 8.0 mW; modulation frequency, 100 kHz; frequency, 9.425 GHz; modulation amplitude, 0.063 mT; gain, 500; time scan, 1min.; time constant, 0.03 s.

Superoxide Anion Scavenging Assay in Hypoxanthine/Xanthine Oxidase System. Superoxide scavenging activity assay in the hypoxanthine/xanthine oxidase system was based on the method of Mitsuya et al. (1990) and slightly modified. Fifty microliters of 100 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazyl]ethanesulfonic acid (HEPES) buffer (pH 7.4), 2 mM hypoxanthine, and sample solution were mixed and followed by the addition of 5 μL of DMPO. Fifty microliters of xanthine oxidase (0.4 unit/mL in 100 mM HEPES buffer) was then added to the reaction mixture. One minute after the enzyme addition, scanning was started at room temperature. The signal height of the superoxide anion−DMPO adduct in the lowest magnetic field was measured, and the height was represented as the ratio to Mn^{2+} reference.

Garcinol was suspended in water at 1% (w/v) with 3% (w/v) sodium dodecyl sulfate (SDS) and diluted with 3% SDS solution. The garcinol−SDS suspensions were added to the reaction mixture.

Hydroxyl Radical Scavenging Assay in Fenton Reaction System. Hydroxyl radical scavenging activity assay in the Fenton reaction system was based on the method of Ogawa et al. (1994) and slightly modified. In brief, 50 μL of 2 μM ferrous sulfate in 1.1 μM diethylenetriaminepentaacetic acid (DTPA), 100 mM HEPES buffer (pH 7.4), and sample solution were mixed followed by the addition of 5 μL of DMPO, and 50 μL of 0.01% of hydrogen peroxide solution was then added. After 1 min, scanning was started at room temperature. The signal height of hydroxyl radical−DMPO adduct in the lowest magnetic field was measured and was represented as the ratio to that of Mn^{2+} as reference.

Garcinol was suspended in water at 1% (w/v) with 3% (w/v) SDS and diluted with 3% SDS solution. The garcinol−SDS suspensions were added to the reaction mixture.

Generation of Reactive Oxygen Species in the H_2O_2/NaOH/DMSO System. Fifty microliters of dimethyl sulfoxide (DMSO), the same volume of 25 mM NaOH, and sample solution (aqueous) were mixed in a disposable plastic tube, followed by the addition of 5 μL of DMPO and 50 μL of 30% hydrogen peroxide. In the case of an oil-soluble antioxidant, the sample was dissolved in DMSO and an equal volume of water prepared by a Milli-Q SP reagent water system (Millipore) and 25 mM NaOH were mixed, followed by the addition of 5 μL of DMPO and 50 μL of 30% hydrogen peroxide.

The reaction mixture was put into the quartz flat cell and set in the ESR apparatus, and then scanning was begun at 10 min after the addition of hydrogen peroxide at room temperature. The signal height was calculated using a radical analyzer program attached to the instrument. The calculation was done for the positive signal height of methyl radical−DMPO adducts and hydroxyl radical−DMPO adducts and for the negative signal height of superoxide−DMPO adducts in the lowest magnetic field (Figure 2). The signal height was represented as the ratio to that of Mn^{2+} as reference.

Animals. Male Wistar/Crj rats (Charles River Japan, Atsugi) weighing 170−190 g were used in in vivo studies. The animals were fasted but were allowed free access to water for 24 h before the experiments.

Antiulcer Test (Stress-Induced). A gastric injury model based upon a modification of the method described by Takagi and Okabe (1968) was induced by water immersion stress in six rats per group. Test compound (garcinol) dispersed in water containing 1% of carboxyl methylcellulose−Na (CMC−Na) was administered orally at the dose of 200 mg/kg 30 min after the animals had been immersed in the 23 °C water. Seven hours later, the animals were killed under Nembutal anesthesia. The stomachs were removed and fixed by inflation with 8 mL of 10% formalin solution in phosphate-buffered saline. They were then incised along the greater curvature, and the length of each lesion formed on the glandular portion was measured under a dissecting microscope. The sum of the length of lesions in each animal was calculated.

Antiulcer Test (Indomethasin-Induced). A gastric injury model based upon a modification of the method described by Djahanguri (1969) was induced by indomethacin. After a 24 h fast, test compound was orally administered to animals at a dose of 40−200 mg/kg. Thirty minutes later, they were given indomethacin solution containing 0.1% CMC−Na and

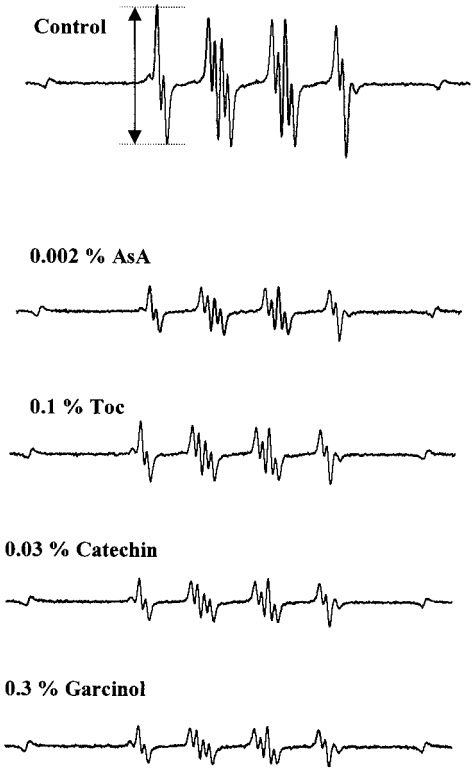

Figure 3. ESR spectra in hypoxanthine/xanthine oxidase system. The spectrum indicated as control was that of the superoxide anion−DMPO adduct in the hypoxanthine/xanthine oxidase system without antioxidant. A double-ended arrow indicates the signal height for determining the radical intensity. Other spectra are of test compounds added at the concentrations indicated. AsA, Toc, and catechin mean L-ascorbic acid, DL-α-tocopherol, and (+)-catechin, respectively.

0.1% Tween 20 at 50 mg/kg by sc and 7 h later were killed under Nembutal anesthesia. Other experimental procedures were the same as for the stress-induced antiulcer test described above.

RESULTS AND DISCUSSION

In the hypoxanthine/xanthine oxidase system, garcinol suppressed the signal of the superoxide anion−DMPO adduct on ESR charts (Figure 3), and the manner was dose dependent (Figure 4). In this system, the addition of surfactant (SDS) did not affect superoxide anion−DMPO adduct formation (data not shown). D-Ascorbic acid (AsA) was used as a representative water-soluble antioxidant and DL-α-tocopherol (Toc) as an oil-soluble antioxidant. (+)-Catechin (catechin) was used as a naturally occurring polyphenolic compound.

The superoxide anion scavenging rate of garcinol was almost the same as that of Toc and less than those of ascorbic acid and catechin at the same weight/volume concentration. Toc suspension was prepared with surfactant in the same way as garcinol. Therefore, the activity of garcinol and tocopherol might depend on their solubility in an aqueous system.

In the Fenton reaction system, garcinol suppressed hydroxyl radical formation as shown in Figure 5, dose-dependently; the rate was less than that of ascorbic acid but greater than those of catechin and tocopherol (Figure 6). Actually garcinol can act as an iron chelator (Yamaguchi et al., 2000); the suppression of hydroxyl radical in the system may be caused by not only direct

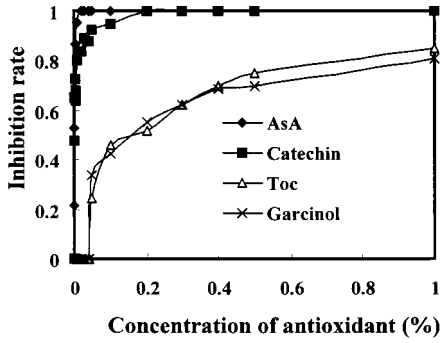

Figure 4. Dose dependency of the signal height reduction of the superoxide anion−DMPO adduct by AsA, Toc, catechin, and garcinol. The main signal height of the superoxide anion−DMPO adduct in the lowest magnetic field (indicated in Figure 3 as a double-sided arrow) was recorded as the ratio against Mn^{2+} reference. Inhibition rate was represented by the following equation: inhibition rate = [signal height (no sample added) − signal height (sample added)]/signal height (no sample added). Sample solutions were prepared at the concentrations indicated on the abscissa, and 50 μL of each was added to the reaction mixture.

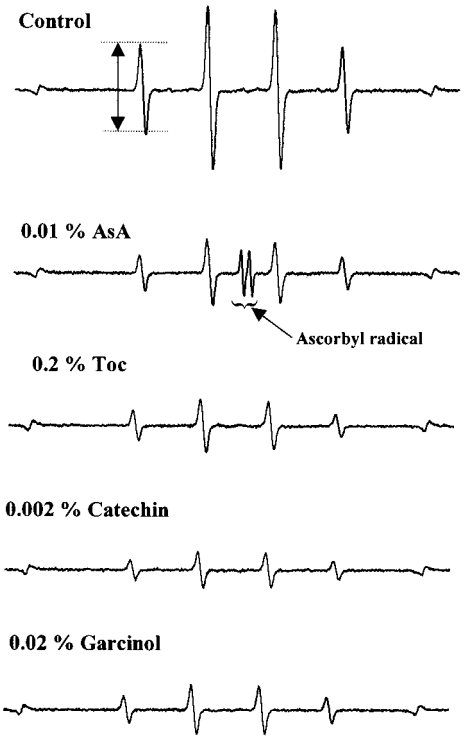

Figure 5. ESR spectra in the Fenton reaction system. The spectrum indicated as control was that of the hydroxyl radical−DMPO adduct in the Fenton reaction system without antioxidant. A double-ended arrow indicates the signal height for determination of the radical intensity. Other spectra are of test compounds added at indicated concentrations.

scavenge but also the inhibition of the Fenton reaction by Fe^{2+} chelation. Ascorbic acid was actually a potent hydroxyl radical scavenger but simultaneously formed its own radical (ascorbyl radical) (Figure 5). It is reported that an ascorbyl radical might cause oxidative reactions of other compounds and that ascorbic acid had a pro-oxidant activity (Ramanathan and Nagaratham, 1993; Podmore et al., 1998). However, garcinol did not form its own radical (Figure 5) and therefore is useful as a hydroxyl radical scavenger because there is less possibility that it may cause other oxidative stress.

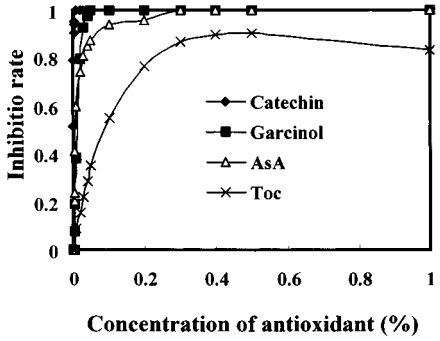

Figure 6. Dose dependency of the signal height reduction of the hydroxyl radical−DMPO adduct by ascorbic acid, tocopherol, catechin, and garcinol. The main signal height of the hydroxyl radical−DMPO adduct in the lowest magnetic field (indicated in Figure 5 as a double-ended arrow) was recorded as the ratio to Mn^{2+} as reference. Inhibition rate was the same as in the legend of Figure 4. Sample solutions were prepared at the concentrations indicated on the abscissa, and 50 μL was added to the reaction mixture.

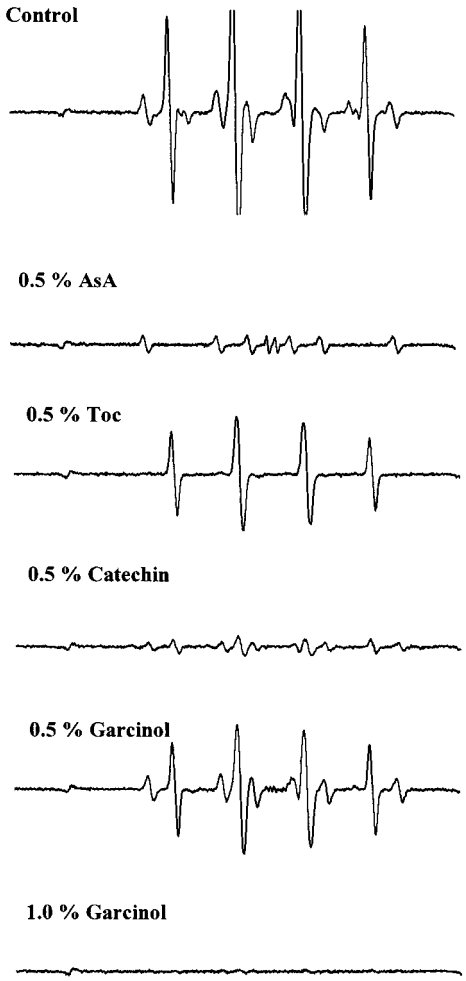

Figure 7. ESR spectra in the H_2O_2/NaOH/DMSO system. Control shows the spectrum in the H_2O_2/NaOH/DMSO system without antioxidant. Others show the spectra in test compounds added at indicated concentrations. Signal height used for radical intensity determination is indicated in Figure 2.

The H_2O_2/NaOH/DMSO system was designed to evaluate both water-soluble and oil-soluble free radical scavengers as a nonenzymatic and non-Fenton type ROS generating system by Yoshimura et al. (1999). In this system, garcinol suppressed the formation of all kinds of free radicals such as methyl radical, hydroxyl radical, and superoxide anion (Figure 7). Garcinol can suppress hydroxyl radical in this non-Fenton type ROS generating system, and therefore it was suggested that the reaction mechanism was the direct radical scavenge and not the inhibition of Fenton reaction by chelation.

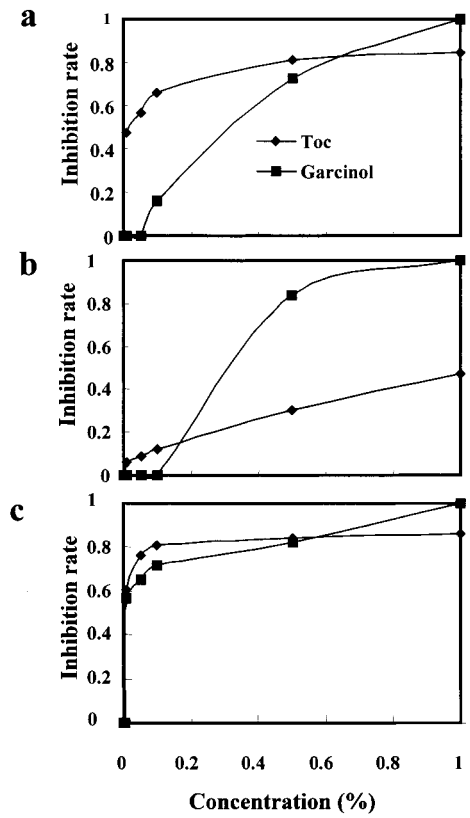

Figure 8. Comparison of DL-α-tocopherol and garcinol in radical scavenging activity in the H_2O_2/NaOH/DMSO system. DL-α-Tocopherol and garcinol were dissolved in DMSO at the concentration indicated on the abscissa, and 50 μL of each was added to the reaction mixture. Each signal height of radical−DMPO adduct in the lowest magnetic field (indicated in Figure 2) was recorded as the ratio to Mn^{2+} reference. Inhibition rate used was same in the legend of Figure 4. Sample solutions were prepared at the concentrations indicated on the abscissa, and 50 μL of each added to the reaction mixture.

The specificity against each radical species was compared with that of DL-α-tocopherol, a typical oil-soluble natural antioxidant (Figure 8). In methyl radical scavenging activity, garcinol was weaker than DL-α-tocopherol (Figure 8a), but against hydroxyl radical scavenging activity, it was stronger (Figure 8b). The superoxide anion scavenging activity of garcinol showed almost the same level of activity as Toc (Figure 8c). These tendencies were consistent with the results in the hypoxanthine/xanthine oxidase and Fenton reaction systems.

In conclusion, it was confirmed that garcinol has potent free radical scavenging activity in three kinds of free radical generating systems. Its scavenging activity against hydroxyl radical was stronger than that of Toc and its other scavenging activities were weaker. Hydroxyl radical is regarded as the most dangerous ROS, and, therefore, garcinol is expected to be useful for preventing diseases caused by that radical, such as stress-induced gastric ulcer (Das et al., 1997, 1998) and nonsteroidal anti-inflammatory drug-induced gastric ulcer (Vaananenn et al., 1991; Yoshikawa et al., 1993). A pharmacological study of garcinol in vivo on free

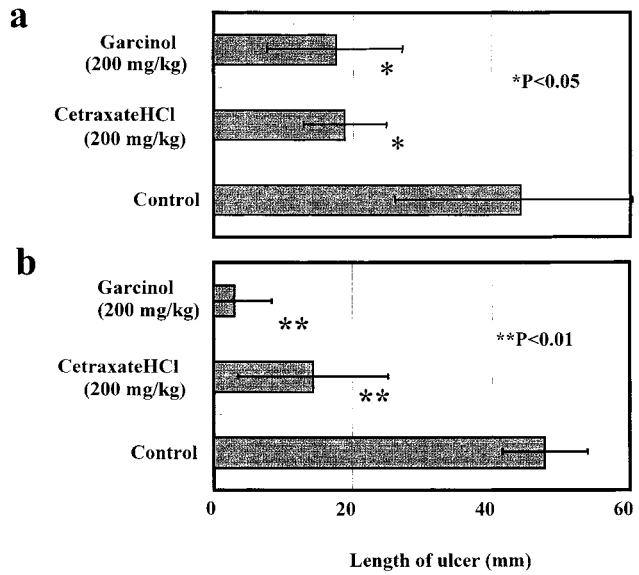

Figure 9. Inhibition of water immersion-induced gastric injury by garcinol. Error bars indicate standard deviation of six determinations. Garcinol reduced the injury significantly ($P < 0.05$) in the water immersion model (a) and also reduced it significantly ($P < 0.01$) in indomethacin-induced model (b). The significance was determined by Student's *t* test. Control was the case of only saline (containing CMC−Na) administered prior to the water immersion or indomethacin injection.

radical related diseases would be valuable. Actually, in the water immersion stress model, garcinol suppressed gastric injury formation to almost the same extent as cetraxate−HCl as a positive control (Figure 9a), and it prevents indomethacin-induced gastric injury (Figure 9b). These results suggest that garcinol, a free radical scavenger, may have potential as an antiulcer drug. Although the mechanism of its antiulcer activity is not yet understood, garcinol may scavenge reactive oxygen species on the surface of gastric mucosa, thus protecting cells from injury.

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