Comparative Evaluation of Antioxidant Potential of Alaternin (2-Hydroxyemodin) and Emodin

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The antioxidant activities of alaternin (2-hydroxyemodin) and emodin were compared for their respective potentials to inhibit lipid peroxidation in the linoleic acid system by the thiocyanate method, to inhibit total reactive oxygen species generation in kidney homogenates using 2′,7′-dichlorodihydrofluorescein diacetate, to inhibit peroxynitrite formation by the 3-morpholinosydnonimine system, which generates superoxide radical and nitrogen monooxide, and to scavenge authentic peroxynitrites. Both alaternin and emodin were found to inhibit the peroxidation of linoleic acid by the thiocyanate method in a dose-dependent manner. Whereas the former shows inhibitory activities in reactive oxygen- and nitogen-mediated reactions, the latter does not. These results indicate that alaternin is a potentially effective and versatile antioxidant and can be used to protect biological systems and functions against various oxidative stresses.

Keywords: *Alaternin; emodin; anthraquinone; peroxynitrite; lipid peroxidation; antioxidant; Cassia tora*

INTRODUCTION

Much attention has recently been focused on the development of safe and effective antioxidants because toxic free radicals play a role in the etiology of many diseases. The dried or roasted seeds of *Cassia tora* L. (Leguminosae) are used to improve vision in Chinese herbal medicine and are reputed to have medicinal value as an asperient, antiasthenic, and diuretic agent (Namba, 1980). The roasted *Cassia* seeds are frequently used to brew a tea in Korea. We have previously reported that the methanolic extract of the seeds of *C. tora* possess a radical scavenging activity on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and an inhibitory activity against aflatoxin B_1 induced mutagenicity tested in the *Salmonella typhimurium* assay (Choi et al., 1993, 1997). From this methanolic extract, alaternin (2-hydroxyemodin) was isolated as one of the active principles, together with the inactive emodin (Choi et al., 1994, 1998) on DPPH radical. Emodin was isolated as an antioxidative component from the same plant parts by Yen et al. (1998). The exceptional antioxidant characteristics of alaternin and its extensive human consumption in the form of tea in Korea prompted us to examine its further effect on antioxidant activities. In this study, the antioxidant activities of alaternin and emodin were compared by using the thiocyanate method in the linoleic acid system, the inhibitory activity against total reactive oxygen species (ROS) generation in kidney homogenates was investigated using 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA), and inhibitory and scavenging activities on authentic peroxynitrite and peroxynitrite formation were studied with the 3-morpholinosydnonimine (SIN-1) system, which generates superoxide radical and nitrogen monooxide. Further studies are planned that will attempt to identify the active sites of the anthraquinone components.

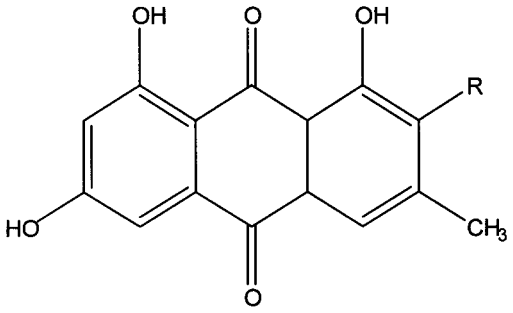

Figure 1. Structures of alaternin and emodin.

MATERIALS AND METHODS

Chemicals. Ammonium thiocyanate, ferrous chloride, linoleic acid, L-ascorbic acid, 2,6-di-*tert*-butylhydroxytoluene (BHT), and SIN-1 were purchased from Sigma Chemical Co. (St. Louis, MO). Dihydrorhodamine 123 (DHR 123) and peroxynitrite were of high quality and were purchased from Molecular Probes (Eugene, OR) and Cayman (Ann Arbor, MI), respectively.

Isolation of Alaternin and Emodin. Alaternin and emodin were isolated from *C. tora* L. as described previously (Choi et al., 1994). The chemical structures of alaternin and emodin are illustrated in Figure 1.

Thiocyanate Method in the Linoleic Acid System. Autoxidation of linoleic acid was carried out by using the

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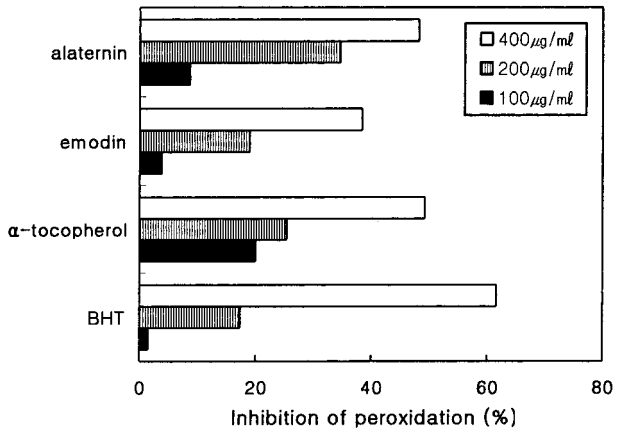

Figure 2. Antioxidant activities of alaternin, emodin, α-tocopherol, and BHT. The activity was determined according to the thiocyanate method.

method of Mitsuda et al. (1966). Different amounts of samples dissolved in 0.1 mL of EtOH (100, 200, and 400 μg/mL) were added to a reaction mixture in a screw-cap vial. Each reaction mixture consisted of 2.5 mL of 0.02 M linoleic acid in EtOH and 2.0 mL of 0.2 M phosphate buffer (pH 7.0). The vial was incubated in an oven at 40 °C. At regular intervals during incubation, a 0.1 mL aliquot of the mixture was diluted with 4.0 mL of 75% EtOH, which was followed by the addition of 0.1 mL of 30% ammonium thiocyanate. Precisely 3 min after the addition of 0.1 mL of 20 mM ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance at 500 nm was measured. The solutions without added samples were used as blank samples. The test were run twice and averaged.

Assay for Free Radical Generation. Rat kidney homogenates prepared from the kidneys of freshly killed male Wistar rats weighing 150−200 g were mixed with or without a suspension of anthraquinones, alaternin, and emodin and then incubated with 12.5 μM DCHF-DA at 37 °C for 30 min. Phosphate buffer (50 mM) at pH 7.4 was used. The fluorescence intensity of oxidized DCF was monitored on a spectrofluorometer (Bio-Tek Instruments, Inc., Winooski, VT), with excitation wavelength at 460 nm and emission wavelength at 530 nm (Label and Bondy, 1990).

Measurement of Peroxynitrite Scavenging Activity. Peroxynitrite scavenging was measured by monitoring the oxidation of DHR 123 by modifying the method of Kooy et al. (1994). DHR 123 (5 mM) in dimethylformamide, which was purged with nitrogen, was stored at −80 °C as a stock solution. This solution was then placed in ice and was not exposed to light prior to the study. The buffer used consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride at pH 7.4, and 100 μM diethylenetriaminepentaacetic acid (DTPA), each of which was prepared with high-quality deionized water and purged with nitrogen. The final concentration of DHR 123 was 5 μM. The background and final fluorescent intensities were measured 5 min after treatment with and without the addition of authentic peroxynitrite. Oxidation of DHR 123 by SIN-1 gradually increased. However, DHR 123 was oxidized rapidly by authentic peroxynitrite, and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of oxidized DHR 123 was measured with a microplate fluorescence reader (FL 500, Bio-Tek Instruments) at the excitation and emission wavelengths of 480 and 530 nm, respectively. Results were expressed as means ± SE ($n = 3$) for the final fluorescence intensity minus background fluorescence. The effects were expressed as the percent inhibition of oxidation of DHR 123.

RESULTS

Figure 2 shows the antioxidant activity of alaternin and emodin in the linoleic acid peroxidation system as measured by the thiocyanate method, compared with those of commercial antioxidants α-tocopherol and BHT. The antioxidant activity of these samples became more pronounced with increasing concentration up to 400 ppm. Alaternin was more effective than α-tocopherol, emodin, and BHT when the concentration was 200 ppm.

Table 1. Inhibitory Effect of Alaternin, Emodin, and Trolox on Total ROS in Kidney Homogenates

compd	concn (μM)	kidney homogenates[a] (fluorescence/min)
control		360.5 ± 7.1
alaternin	2	344.7 ± 10.9 (4)
	10	238.7 ± 9.7 (34)
	50	159.3 ± 9.4 (56)
emodin	2	365.0 ± 4.0 (−)
	10	353.0 ± 13.0 (−)
	50	341.3 ± 21.9 (−)
Trolox	2	298.7 ± 17.4(17)
	10	209.7 ± 10.7 (42)
	50	127.0 ± 4.4 (65)

[a] Kidney homogenates were incubated in serum-free media and preparated with various concentrations of each sample. After preincubation for 1 h, 12.5 μM DCHF-DA was added and change in fluorescence was measured. Values are means ± SD of three experiments. Figures in parentheses indicate percent of control.

The DCHF-DA system is useful to measure changes of ROS such as $O_2^{\bullet -}$, $OH^{\bullet}$, and H_2O_2. Liposoluble DCHF-DA becomes ″DCHF as a result of the activities of mitochondrial esterase or hydrolysis in kidney (or liver) homogenate and is subsequently oxidized by ROS to a highly water-soluble fluorescent 2′,7′-dichlorofluorescein (DCF). Alaternin and water-soluble α-tocopherol analogue Trolox were found to inhibit free radical generation in kidney homogenates in a concentration-dependent manner (Table 1). The effectiveness of inhibition of alaternin was similar to that of Trolox. On the other hand, emodin was found to be inactive at the same concentration.

The ability of the anthraquinones (alaternin and emodin) from *C. tora* to inhibit peroxynitrite formation by SIN-1 was determined. SIN-1, the concomitant product of the superoxide anion radical and nitric oxide, raises the possibility that a significant amount of peroxynitrite may be formed from the combination of these two radicals. Because of this, SIN-1 is frequently used as a standard compound for the continuous formation of peroxynitrite (Hogg et al., 1992; Muller et al., 1997). The results (Figure 3) indicate that the aromatic ortho-dihydroxylated alaternin was a potent inhibitor of peroxynitrite formation by SIN-1. Emodin without an aromatic ortho-dihydroxylated catechol group, however, showed a low contribution. The alaternin was further investigated by comparison with penicillamine as an effective scavenger of peroxynitrite in vitro (Chung et al., 1998). Alaternin and penicillamine efficiently inhibited peroxynitrite, the concentrations necessary for 50% inhibition being 12.5 ± 0.30 and 1.56 ± 0.61 μM, respectively. Thus, alaternin was as efficient as penicillamine in inhibiting peroxynitrite formation. The ability of the anthraquinones to scavenge authentic peroxynitrite was also determined. The results obtained (Figure 4) were similar to the inhibitory effects on peroxynitrite formation by SIN-1. The most specific inhibitory effect of alaternin was further investigated by comparison with penicillamine, and both of these compounds efficiently scavenged peroxynitrite. Alater-

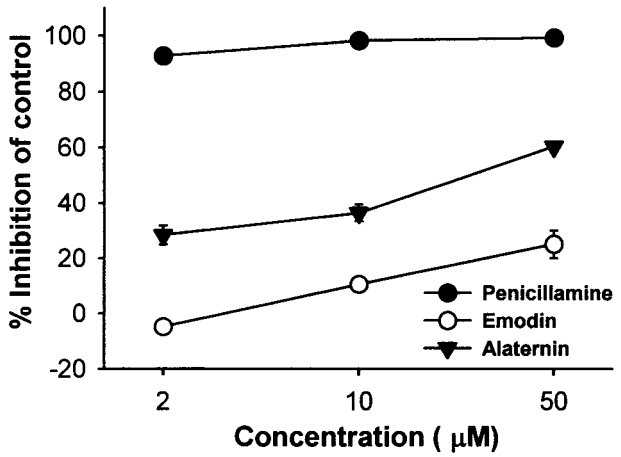

Figure 3. Dose−response curve of penicillamine (●), emodin (○), and alaternin (▼) on peroxynitrite formation by SIN-1.

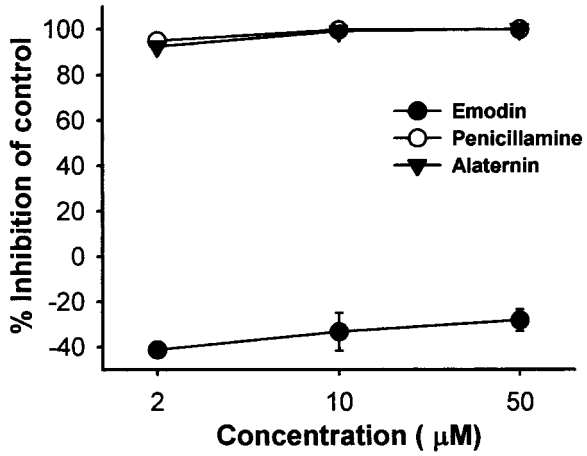

Figure 4. Dose−response curve of penicillamine (○), emodin (●), and alaternin (▼) on authenic peroxynitrite scavenging activity.

nin and penicillamine were needed at concentrations of 1.44 ± 0.05 and 1.57 ± 0.22 μM, respectively, to scavenge 50% authentic peroxynitrite, indicating that alaternin was as efficient as penicillamine.

DISCUSSION

There has been much interest in free radical scavengers because of a crucial role of free radicals on the pathogenesis of various diseases. We have recently reported a strong free radical scavenger, alaternin, on DPPH radicals (Choi et al., 1994). In contrast, emodin, without a free phenolic hydroxy group at C-2, showed no scavenging activity, even at high concentrations. The high radical scavenging property of alaternin is probably due to a superior stability of radicals derived from a catechol moiety compared to that of phenoxyl radical (Ruiz-Larrea et al., 1994). For alaternin, hydroxyl groups are arranged in both the meta and ortho positions in rings A and C, whereas for emodin, they are arranged in the only the meta position. This could be the reason for the difference in their effectiveness in scavenging DPPH radicals. The radical scavenging mechanism of alaternin is considered to be due to the *o*-dihydroxyl groups of the anthraquinone modifying to an *o*-quinone structure because the antioxidative molecular mechanism of ortho-dihydroxylated (+)-catechin is demonstrated by NMR analysis (Sawai and Sakata, 1998). However, further direct studies of the reactivity and production of the intermediates, of molecular products of interactions with oxidized species, and of the kinetics of the interaction with free radicals are required to elucidate the free radical scavenging mechanism. Although emodin is a free radical generator whereas alaternin is a free radical scavenger, the inhibitory activity of emodin on lipid peroxidation has previously been observed both in the thiocyanate method (Yen et al., 1998) and in rat heart mitochondria (Huang et al., 1995). Huang et al. (1995) also reported that two hydroxyl groups arranged at either the meta or ortho position in the rings of the anthraquinone nucleus are required for an anthraquinone derivative to inhibit lipid peroxidation in the rat heart mitochondrial system. These results prompted us to evaluate the antioxidative potential of both compounds. Using simple and cost-effective methods, the comparative antioxidant potential of alaternin and emodin has been monitored on the basis of the inhibitory activity of lipid peroxidation in the linoleic acid system by the thiocyanate method, inhibition of total ROS generation in kidney homogenates using the DCHF-DA system, inhibitory activity of the peroxynitrite formation by the SIN-1 system, which generates superoxide radical and nitrogen monooxide, and scavenging activity of an authentic peroxynitrite.

The present work showed that alaternin may act as a strong antioxidant and a radical scavenger in all kinds of tested assay systems, whereas emodin inhibits lipid peroxidation only by the thiocyanate method. Emodin as compared to alaternin did not show any inhibitory or scavenging activity in the reactive oxygen- and nitrogen-mediated reactions.

It is well established that lipid peroxidation is one of the reactions set into motion as a consequence of the formation of free radicals in cells and tissues. The one-electron reduction products of O_2, superoxide anions, hydrogen peroxide, and hydroxyl radicals actively participate in the initiation of lipid peroxidation. In the present experiments, both alaternin and emodin showed potent inhibition of lipid peroxidation in the linoleic acid system. The structure−activity relationship study of both compounds indicated that the free hydroxyl group at the β-position of the anthraquinone nucleus plays an important role in the inhibition of lipid peroxidation. Emodin, however, had no effect on ROS generation in kidney homogenates.

Antioxidative materials acting in living systems are classified into preventive antioxidants and chain-breaking ones (Halliwell and Gutteridge, 1990). Alaternin exhibited potent inhibition of ROS in kidney homogenates and also inhibited autoxidation of linoleic acid. Alaternin thus acts as a preventive antioxidant. On the other hand, emodin inhibited autoxidation of linoleic acid but had no effect on ROS generation. Emodin thus acts as a chain-breaking antioxidant.

The inhibitory and scavenging abilities of the anthraquinones, alaternin and emodin, on peroxynitrite formation by SIN-1 and authentic peroxynitrite were closely related to those of the aromatic *o*-dihydroxyl groups (catechol). Alaternin with aromatic *o*-dihydroxyl groups had a relatively strong peroxynitrite scavenging activity. However, emodin, a compound without a catechol group, had a rather low peroxynitrite generating activity. Because both anthraquinones have common hydroxyl groups at positions 1, 5, and 7, the hydroxyl groups at these positions seemed to have little effect.

These are the first examples of anthraquinones inhibiting peroxynitrite.

The mechanism by which alaternin scavenges the peroxynitrite is yet to be established. In this study, the basic structure of anthraquinones and other structural factors turned out to be important in the scavenging mechanism. Pannala et al. (1998) suggested that there are two possible mechanisms by which to scavenge peroxynitrite: (1) nitration and (2) electron donation. Phenolic compounds can inhibit peroxynitrite-mediated nitration of tyrosine either by acting as alternative substrates for nitration, as in the case of monohydroxylated structures such as *p*-coumaric acid and ferulic acid, or by reducing reactive nitrogen species as has been demonstrated for catechol structures such as caffeic acid (Pannala et al., 1997; 1998; Kerry and Rice-Evans, 1999).

The results of this study indicate that a selective mechanism seems to exist whereby compounds susceptible to oxidation will react with peroxynitrite in preference to compounds that are substrates for nitration. Similarly, as shown here, alaternin scavenges peroxynitrite through its ability to reduce reactive nitrogen species and to form an *o*-quinone. Further research should be done to investigate the effects of the anthraquinones on the nitration of peroxynitrite and on the coupled oxidation−reduction reaction.

Finally, anthraquinone derivatives inhibited lipid peroxidation depending on structure. Although a correlation between antioxidative activities in lipid peroxidation and the ability to scavenge radicals failed, it appeared that the inhibition of lipid peroxidation by anthraquinones is based on the number and orientation of hydroxyl groups present in the molecule.

Anthraquinone derivatives are the largest class of naturally occurring quinones and are widely distributed in lower and higher plants as well as in fungi. Some of them have exhibited antioxidant (Tripathi et al., 1997), antitumor (Morreal et al., 1990; Kupchan and Karim, 1976), antimutagenic (Choi et al., 1997), immunosuppressive (Huang et al., 1992), vasorelaxant (Huang et al., 1991), and enzyme-inhibitory activities. Alaternin was found recently to have an antimutagenic activity against aflatoxin B_1 in the *Salmonella* assay system (Choi et al., 1998). Structure−activity analysis of various anthraquinone derivatives indicated that the number and position of the hydroxyl and methoxyl groups are the most critical factors for the antimutagenicity between the chemical structures of anthraquinones and their antimutagenicity (Choi et al., 1997). Furthermore, alaternin showed no cytotoxic activities in normal liver and hepatoma cell lines by MTT methods (data not shown). The exact reason alaternin shows a strong antioxidant activity compared to emodin is uncertain, but it seems that structural modification of anthraquinones (i.e., emodin to alaternin) determines the rate of metabolism of anthraquinones to semiquinone metabolites. Indeed, toxic effects of quinones correlate well with semiquinone formation rates in the xanthine oxidase system. The toxicity of naphthoquinones was greater than that of benzoquinones and much greater than that of anthraquinones (Lewis and Shibamoto, 1989). However, it must be pointed out that in the absence of semiquinone studies, the assumption is strictly theoretical.

In conclusion, the present study shows that alaternin is a novel natural antioxidant against lipid peroxidation in linoleic acid and total free radical generation of kidney homogenates and on peroxynitrite formation by SIN-1 and authentic peroxynitrite. Despite the therapeutic relevance of anthraquinones, however, their medical uses are questionable because they exhibit toxic potential, such as mutagenicity; it will be interesting to further investigate the antioxidative activity of this natural compound in preventing various radical-mediated injuries in pathological situations in vivo.

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