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EFFECT OF ANTHRAQUINONE DERIVATIVES ON LIPID PEROXIDATION IN RAT HEART MITOCHONDRIA: STRUCTURE-ACTIVITY RELATIONSHIP

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ABSTRACT.—Lipid peroxidation was induced in rat heart mitochondria with $FeSO_4$ and the inhibitory effects of various anthraquinone derivatives were compared. Oxygen consumption and malondialdehyde formation were used to quantitate the amount of lipid peroxidation. Emodin [**2**], alizarin [**13**], and alizarin complexone [**14**] significantly inhibited lipid peroxidation; their potency as inhibitors of lipid peroxidation was higher than that of α-tocopherol. Structure-activity analysis showed that two hydroxyl groups arranged in either the ortho- or meta- position in the C ring of the anthraquinone nucleus are required for such derivatives to inhibit lipid peroxidation. The diphenyl-*p*-picrylhydrazyl test showed that alizarin [**13**] and alizarin complexone [**14**] are free-radical scavengers while emodin [**2**] is not. The mechanism for emodin [**2**] to inhibit lipid peroxidation is most likely due to inhibition on the propagation of lipid peroxyl radicals in the mitochondrial membrane.

Lipid peroxidation defines the biological damage caused by free radicals that are formed under oxidative stress (1), and the heart is one of the target organs for such an injury (2,3). Several studies have demonstrated that lipid peroxidation in cardiac mitochondria may play an important role in the pathogenesis of cardiac dysfunction (4). In a previous study on Chinese medicinal herbs, the roots of *Polygonum multiflorum* Thunb. (Polygonaceae) (5) exhibited a prominent inhibitory effect on lipid peroxidation in rat heart mitochondria (6). Subsequent study showed that the inhibitory effect of the roots of *Polygonum cuspidatum* Sieb. et Zucc. (Polygonaceae) (7) was more potent than that of the roots of *P. multiflorum*. Several anthraquinone derivatives have been isolated as active components from *P. cuspidatum* roots and some contain phenolic groups. Since polyphenolic compounds are known to have antioxidant activity, the present study was undertaken to see if the anthraquinone derivatives purified from *P. cuspidatum* roots could inhibit lipid peroxidation in rat heart mitochondria. A structure-activity relationship for the antioxidant effect of these anthraquinone derivatives has been determined.

RESULTS AND DISCUSSION

Among the anthraquinone derivatives tested (Table 1), only emodin [**2**], alizarin [**13**], and alizarin complexone [**14**] significantly inhibited lipid peroxidation. Their potency as inhibitors of lipid peroxidation was higher than that of α-tocopherol. Table 2 shows the mol wts and IC_{50} values of these compounds in inhibiting oxygen consumption and malondialdehyde (MDA) formation. Figures 1 and 2 show the concentration response curves for their inhibitory effects. All other test compounds did not inhibit oxygen consumption and MDA formation to 50% of the control value at concentrations up to 0.3 mM.

Figure 3 shows the concentration response curves for **2**, **13**, **14**, and α-tocopherol needed to decolorize diphenyl-*p*-picrylhydrazine (DPPH) after incubation for 90 min.

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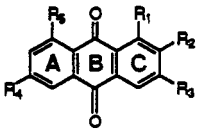
TABLE 1. Structures of the Anthraquinone Derivatives Evaluated.



Code	Compound	R_1	R_2	R_3	R_4	R_5
1	Chrysophanol	OH	H	H	CH_3	OH
2	Emodin	OH	H	OH	CH_3	OH
3	Physcion	OH	H	OCH_3	CH_3	OH
4	Emodin-1-*O*-D-glucoside	O-glc	H	OH	CH_3	OH
5	Physcion-1-*O*-D-glucoside	O-glc	H	OCH_3	CH_3	OH
6	Anthraquinone	H	H	H	H	H
7	Anthraflavic acid	H	OH	H	OH	H
8	Anthrarufin	OH	H	H	OH	H
9	Anthraquinone-2-carboxylic acid	H	COOH	H	H	H
10	2-Ethylanthraquinone	H	CH_2CH_3	H	H	H
11	1-Aminoquinone	NH_2	H	H	H	H
12	2-Aminoquinone	H	NH_2	H	H	H
13	Alizarin	OH	OH	H	H	H
14	Alizarin Complexone	OH	OH	$CH_2NHCH(COOH)CH_2COOH$	H	H

Compounds **13** and **14**, like α-tocopherol, were free-radical scavengers while **2** was not. A concentration-dependent inhibitory effect on conjugated diene formation for **2** was not found at concentrations ranging from 1 to 300 μM.

This study showed that **2**, **13**, and **14** protected cardiac mitochondria against $FeSO_4$-induced lipid peroxidation. Their potencies in inhibiting lipid peroxidation were higher than that of α-tocopherol. All three inhibitors contain two hydroxyl groups in ring C of the anthraquinone nucleus. These two hydroxyl groups are arranged in the meta positions (R_1 and R_3 in Table 1) in **2**; while in **13** and **14**, they are arranged in the ortho positions (R_1 and R_2 in Table 1). Anthraquinone derivatives which have no hydroxyl groups (such as anthraquinone [**6**], anthraquinone-2-carboxylic acid [**9**], 2-ethylanthraquinone [**10**], 1-aminoquinone [**11**], and 2-aminoquinone [**12**]) or only one hydroxyl group (such as physcion-1-*O*-D-glucoside [**5**]) did not inhibit lipid peroxidation. Those with two hydroxyl groups affixed to different rings (such as chrysophanol [**1**], physcion [**3**], emodin-1-*O*-D-glucoside [**4**], anthraflavic acid [**7**], and anthrarufin [**8**]) also did not inhibit lipid oxidation. From these data one can conclude that two hydroxyl

TABLE 2. Molecular Weights and Medium Inhibitory Concentrations (IC_{50} Values) for Anthraquinone Derivatives and α-Tocopherol in Inhibiting Oxygen Consumption and MDA Formation in Rat Heart Mitochondria.[a]

	Mol Wt	IC_{50} Values (μM)	
		O_2 consumption	MDA formation
Emodin [**2**]	270	12.5	16.8
Alizarin [**13**]	240	63.9	49.5
Alizarin complexone [**14**]	421	45.9	42.2
α-Tocopherol	404	102	94.7

[a]Each IC_{50} value was calculated from the best-fitting line obtained by the linear regression method.

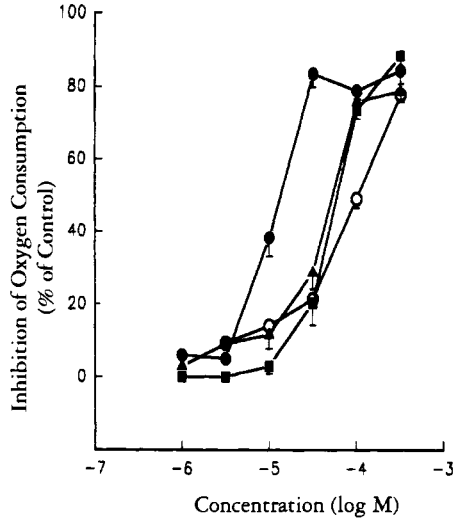


FIGURE 1. Concentration-response curves for emodin [**2**] (●), alizarin [**13**] (■), alizarin complexone [**14**] (▲), and α-tocopherol (○) in inhibiting $FeSO_4$-induced oxygen consumption in rat heart mitochondria. Each data point is the mean±s.e.m. for three different samples.

groups arranged at either the meta- or ortho- positions are required for an anthraquinone derivative to inhibit lipid peroxidation in the rat heart mitochondrial system.

To understand the mechanism for the antioxidant effects of **2**, **13**, and **14**, the decrease in optical absorption at 517 nm was monitored following the trapping of the

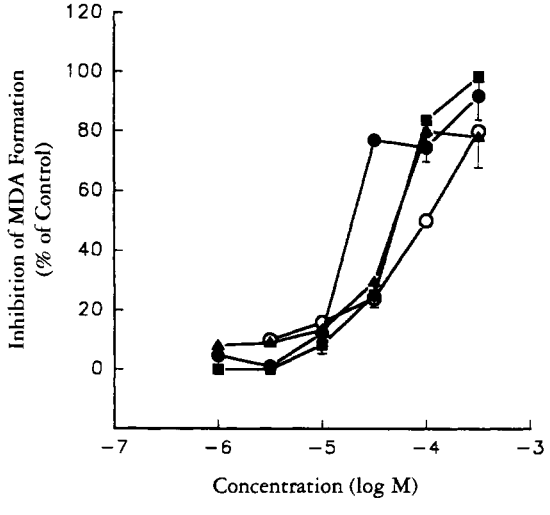


FIGURE 2. Concentration-response curves for emodin [**2**] (●), alizarin [**13**] (■), alizarin complexone [**14**] (▲), and α-tocopherol (○) in inhibiting $FeSO_4$-induced MDA formation in rat heart mitochondria. Each data point is the mean±s.e.m. for three different samples.

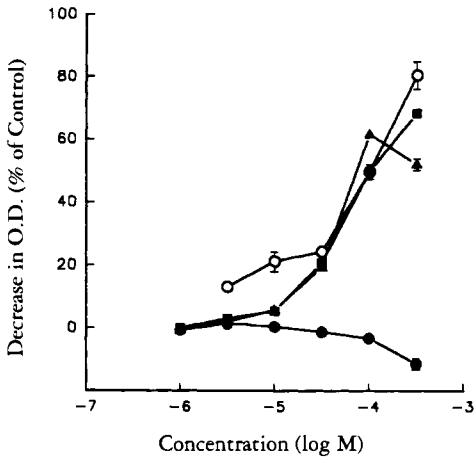


FIGURE 3. Concentration-response curves for emodin [**2**] (●), alizarin [**13**] (■), alizarin complexone [**14**] (▲), and α-tocopherol (○) in scavenging DPPH. The scavenging activity was monitored with a decrease in optical density (O.D.) at 517 nm. Each data point is the mean ± s.e.m. for three different samples.

unpaired electron of DPPH. A positive DPPH test suggested that **13** and **14** are free-radical scavengers. However, **2** was found to have no free-radical scavenging activity. It is possible that the quinoid structure of **2** could be activated to a semiquinone radical intermediate (8). In spite of the fact that **2** is a free-radical generator while **13** and **14** are free-radical scavengers, all three of these anthraquinone derivatives inhibited lipid peroxidation. Disparity among the effects of **2** on oxygen consumption, MDA formation, and the DPPH assay is most likely due to the fact that the DPPH test is a chemical reaction in which mitochondria are not involved. Because the formation of a conjugated diene, an intermediate product of lipid peroxidation, was not inhibited by **2**, the mechanism for this compound to inhibit lipid peroxidation could be the interruption of the propagation of lipid peroxyl radicals in the mitochondrial membrane. For **2**, hydroxyl groups are arranged in the meta- position, while for **13**, they are arranged in the ortho- position. This could be the reason for their differential effects in scavenging DPPH.

In traditional Chinese medicine, the dried roots of *P. cuspidatum* have been used widely for the treatment of rheumatism, icterus, menostasis, coughs, burns, trauma, and ulcers (9). In a previous study in this laboratory, a H_2O extract of the dried root of *P. multiflorum* was found to inhibit $FeSO_4$-induced lipid peroxidation in rat heart mitochondria. At 2 mg/ml, both oxygen consumption and MDA formation were reduced to 50% of control values (6). However, when the effects of their EtOAc extracts were compared, *P. cuspidatum* was found to be more potent than *P. multiflorum*; the IC_{50} values for *P. cuspidatum* (EtOAc extract) in inhibiting oxygen consumption and MDA formation were 0.073 and 0.81 mg/ml, respectively. In contrast, at 1 mg/ml, the EtOAc extract of *P. multiflorum* inhibited lipid peroxidation only partially.

The IC_{50} values of **2** for oxygen consumption inhibition and MDA formation inhibition were 19.4 and 20.2 μM (5.2 and 5.5 μg/ml), respectively. Our laboratory recently reported that magnolol and honokiol purified from the root bark of *Magnolia officinalis*, a Chinese medicinal herb, inhibited lipid peroxidation in rat heart mitochon-

dria at 0.1 μM (10). The potency of **2** is therefore less than those of magnolol and honokiol. The effects of tannins on lipid peroxidation on rat heart mitochondria have also been compared (11). The IC_{50} values of pedunculagin, epicatechin-3-*O*-gallate, procyanidin B_2, epigallocatechin-3-*O*-gallate, and gallocatechin-3-*O*-gallate for oxygen consumption inhibition were 7.8, 8.4, 11.0, 12.2, and 14.0 μM, respectively, while for MDA formation inhibition, the IC_{50} values were 12.0, 5.4, 9.0, 9.4, and 8.6 μM, respectively. The potency of **2** is closer to those of the above-mentioned tannins than to magnolol.

Anthraquinone derivatives are widely present in plants. Some of them have exhibited antitumor (12) or antiviral (13) activity. Emodin [**2**] was found recently to be a vasodilator with an immunosuppressive effect (8,14). Structure-activity analysis of various anthraquinone derivatives showed that a free hydroxyl group at the β-position of the anthraquinone nucleus plays an important role in their immunosuppressive effects (8). Anthraflavic acid [**7**], an anthraquinone derivative that contains a hydroxyl group in ring C, inhibited thymidine uptake in phytohemagglutinin-stimulated human mononuclear cells. Since this compound did not inhibit lipid peroxidation in our experiments, the immunosuppressive effect of anthraquinone derivatives could be mediated through a mechanism different from the inhibition of lipid peroxidation. The therapeutic relevance of anthraquinones, however, is questionable because they exhibit toxic potential, such as mutagenicity.

EXPERIMENTAL

TEST COMPOUNDS.—Certain anthraquinone derivatives, including anthraquinone [**6**], anthraflavic acid [**7**], anthrarufin [**8**], anthraquinone-2-carboxylic acid [**9**], 2-ethylanthraquinone [**10**], 1-aminoquinone [**11**], 2-aminoquinone [**12**], alizarin [**13**], and alizarin complexone [**14**] were purchased from Aldrich Chemical Co. (Milwaukee, WI).

PLANT MATERIAL.—The roots of *P. cuspidatum* were purchased from a market in Taichung, Taiwan, and were identified by staff at the National Research Institute of Chinese Medicine, Taipei, where a voucher specimen is deposited.

EXTRACTION AND ISOLATION.—Chrysophanol [**1**], emodin [**2**], physcion [**3**], emodin-1-*O*-D-glucoside [**4**], and physcion-1-*O*-D-glucoside [**5**] were purified from the roots of *P. cuspidatum*. A dried MeOH extract of the plant was re-extracted with *n*-hexane. The insoluble residue was then successively extracted with EtOAc and *n*-BuOH. After evaporation of the solvent, the concentrated extract of the EtOAc-soluble fraction was applied to a Si gel flash cc system and eluted repeatedly with a gradient of $CHCl_3$/MeOH mixtures ranging from 100:0 to 75:25. The anthraquinone derivatives were further purified by prep. tlc and their structures identified using ir, uv, ^{1}H-nmr, ^{13}C-nmr, and ms, and by comparison of spectral data with reported values (15–20).

RAT HEART MITOCHONDRIA PREPARATION.—Mitochondria were prepared from the hearts of male rats as described previously (21). Sprague-Dawley rats weighing between 200 and 300 g were decapitated, and their hearts were quickly excised, opened, and thoroughly washed with ice-cold 0.25 M sucrose. The heart tissue was finely minced with a pair of scissors into 10 volumes of SEH buffer which contained 0.25 M sucrose, 0.5 mM EGTA, and 3 mM HEPES (pH 7.2). The minced heart suspension was treated with nagarse at a concentration of 0.1 mg/ml. After 15 min incubation at 0° with occasional stirring, the supernatant was discarded and the remaining tissue washed twice with 0.25 M sucrose. The dispersed heart tissue was then homogenized with a glass pestle and diluted with sucrose solution to 10 ml per g heart tissue. The homogenates were centrifuged at 800×g for 5 min in the JA 20 rotor of a Beckman J2/21 refrigerated high-speed centrifuge. The supernatant was decanted and centrifuged at 1800×g for 5 min. After recentrifugation of the supernatant at 6000×g for 10 min, the pellet was resuspended in a KCl-TRIS solution containing 175 mM KCl and 20 mM TRIS-HCl (pH 7.4) and centrifuged again for 10 min at 12000×g. The mitochondria-rich, red-brown lower layer was suspended in 0.5 ml of KCl-TRIS solution per g heart tissue and stored at 0° for 3 days before it was used for the lipid peroxidation experiments. A cold storage period is required because freshly prepared mitochondria, like liver and myocardial homogenates (22), are resistant to lipid peroxidation. Protein concentrations of mitochondrial suspensions were determined by the Lowry method (23).

MEASUREMENT OF LIPID PEROXIDATION.—Lipid peroxidation in the rat heart mitochondria was

measured according to the method described by Sassa *et al.* (24). Peroxidation was started by adding ADP and $FeSO_4$ to the mitochondrial suspension. Final concentrations of ADP and $FeSO_4$ were 1 mM and 0.1 mM, respectively. The amount of oxygen consumed during the incubation period was monitored with a Clark-type oxygen electrode in a Gilson 5/6 oxygraph (Gilson Medical Electronics, Middleton, WI). The total volume of the assay medium in the reaction chamber was 1.6 ml.

At the end of incubation, 0.3 ml of the mitochondrial suspension in the reaction chamber was mixed with 0.1 ml of 15.2% trichloroacetic acid. Malondialdehyde (MDA) was assayed using a thiobarbituric acid technique and the level was used to represent the amount of lipid peroxides formed during incubation (25).

Although MDA is the end product of lipid peroxidation, a conjugated diene is an intermediate product. A conjugated diene formation test was performed to investigate the mechanism for **2** to inhibit MDA formation. An amount (0.3 ml) of the mitochondrial suspension in the reaction chamber was mixed with 0.7 ml EtOH and then centrifuged at 3000×g for 10 min. The uv absorption of supernatant at 234 nm was measured, with EtOH used as a blank (26).

ANTIOXIDANT EFFECTS OF TEST ANTHRAQUINONES.—For studying its antioxidant effect, an anthraquinone derivative was first dissolved in DMSO and diluted in KCl-TRIS buffer, then pipeted into the reaction chamber prior to the co-incubation of mitochondria and ADP/Fe^{2+}. Final concentrations of DMSO were 0.5%. At this concentration, DMSO showed no significant effects on lipid peroxidation. Oxygen consumption and MDA and conjugated diene formation in the rat heart mitochondria in the presence of an anthraquinone derivative were compared with controls. The concentration for an anthraquinone derivative to inhibit lipid peroxidation to 50% of the control value (IC_{50}) was plotted from the concentration-response curve.

RADICAL-SCAVENGING ACTIVITY DETERMINATION.—The radical-scavenging activity of the anthraquinone derivatives under test was determined from the reduction in the optical absorbance at 517 nm due to scavenging of the stable free radical of diphenyl-*p*-picrylhydrazine (DPPH, Sigma, St. Louis, MO). According to the method of Blois (27), 10 ml of 100 mM acetate buffer (pH 5.5), 10 ml of EtOH, and 5 ml of a 500 mM ethanolic solution of DPPH were mixed, then 5 ml of 2.5–200 mM of the test compounds were added, and the change in optical density was monitored.

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