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Antioxidant Lignans from *Machilus thunbergii* Protect CCl_4-injured Primary Cultures of Rat Hepatocytes

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Abstract

Eleven lignans (**1–11**) were isolated from the CH_2Cl_2 fraction of the bark of *Machilus thunbergii* Sieb. et Zucc. (*Lauraceae*). These were identified as (−)-acuminatin (**1**), (−)-isoguaiacin (**2**), *meso*-dihydroguaiaretic acid (**3**), (+)-galbacin (**4**), (−)-sesamin (**5**), (+)-galbelgin (**6**), machilin A (**7**), machilin G (**8**), licarin A (**9**), and nectandrin A (**10**) and B (**11**).

Primary cultures of rat hepatocytes were co-incubated for 90 min with the hepatotoxin CCl_4 and each of the 11 lignans (50 μM). Hepatoprotective activity was determined by measuring the level of glutamic pyruvic transaminase released into the medium from the primary cultures of rat hepatocytes. (−)-Acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid all significantly reduced the level of glutamic pyruvic transaminase released. Further investigation revealed that these three compounds significantly preserved the levels and the activities of glutathione, superoxide dismutase, glutathione peroxidase and catalase. (−)-Acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid also ameliorated lipid peroxidation as demonstrated by a reduction of malondialdehyde production.

These results suggest that (−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid exert diverse hepatoprotective activities, perhaps by serving as potent antioxidants.

In Korean traditional medicine the bark of *Machilus thunbergii* is used for the treatment of leg oedema, abdominal pain and abdominal distension (Chung & Shin 1990). (±)-9,9′-*O*-Diferuloylsecoisolariciresinol, (±)-syringaresinol, machilin A–I, nectandrin A and B, licarin A and B, *meso*-dihydroguaiaretic acid, ssioriside, nudiposide, lyoniside, *l*(−)-*N*-norarmepavine, *dl*-*N*-norarmepavine, quercetin, afzelin, guijaverin, quercitrin, rutin, (±)-aromadendrin, trifolin, kaempferol, (±)-naringenin, scopoletin, scopolin, essential oils and mucilage have all been previously reported as components from *M. thunbergii*. In the course of screening for hepatoprotective compounds from natural products, using CCl_4-injured primary cultures of rat hepatocytes as the testing system, we found that the CH_2Cl_2 fraction of the bark of *M. thunbergii* Sieb. et Zucc. (*Lauraceae*) showed significant hepatoprotective activity against CCl_4-induced toxicity when co-incubated with the hepatotoxin. As such, we decided to isolate and identify the hepatoprotective constituents of *M. thunbergii* using our screening system.

In this study, we isolated eleven lignans (**1–11**) from the CH_2Cl_2 fraction of the bark of *M. thunbergii*. The lignans were identified as (−)-acuminatin (**1**) (El-Feraly et al 1982), (−)-isoguaiacin (**2**) (King & Wilson 1964), *meso*-dihydroguaiaretic acid (**3**) (Shimomura et al 1988), (+)-galbacin (**4**) (Achenbach et al 1987; Hada et al 1988), (−)-sesamin (**5**) (Ajaneyulu et al 1977), (+)-galbelgin (**6**) (Holloway & Scheinmann 1974), machilin A (**7**) (Shimomura et al 1987), machilin G (**8**) (Shimomura et al 1988), licarin A (**9**) (Achenbach et al 1987), nectandrin A (**10**) (Shimomura et al 1988) and B (**11**) (Shimomura et al 1988). Among the lignans described, (−)-acuminatin and (−)-isoguaiacin are reported as components of *Lauraceae*

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for the first time. (+)-Galbacin, (−)-sesamin and (+)-galbelgin have not been previously reported from this plant as far as we have been able to ascertain. For this study CCl_4 was chosen as the toxicant because it is a well-known method for inducing liver injury and its mechanism of toxicity is well established (Recknagel et al 1991).

Hepatoprotective activity was determined by measuring the level of glutamic pyruvic transaminase released into the medium from primary cultures of rat hepatocytes co-incubated with the hepatotoxin, CCl_4, plus the compounds tested. Among the compounds tested, (−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid significantly reduced the level of glutamic pyruvic transaminase. Therefore, to clarify the hepatoprotective mechanisms involved, we determined the effects of these three lignans ((−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid) on the levels of glutathione, malondialdehyde and the enzymatic activities of catalase, superoxide dismutase, glutathione-S-transferase (GST) and glutathione reductase (GSSG-R), all of which are involved with cellular defence mechanisms against oxidative stress.

Material and Methods

Samples, animals and reagents

The bark of *M. thunbergii* Sieb. et Zucc. was purchased from a commercial supplier in Seoul, Korea, and identified by Dr Dae Suk Han, an emeritus professor of the College of Pharmacy, Seoul National University.

Male Wistar rats (200–250 g) were provided by the Laboratory Animal Center, Seoul National University.

Waymouth's MB 752/1 medium, supplemental materials for cell culture and other reagents used in the evaluation of enzyme activities were obtained from Sigma (St Louis, MO). Chemicals for the isolation and purification of lignans were obtained from Duksan (Seoul, Korea) and were of the highest purity available.

Extraction and isolation

The dried bark of *M. thunbergii* (7·5 kg) was ground into a powder and extracted with 80% CH_3OH using ultrasonic homogenization. The CH_3OH extract (800 g) was suspended in distilled water and extracted with CH_2Cl_2. The CH_2Cl_2 fraction (90 g) which showed significant hepatoprotective activity was repeatedly fractionated by step gradients from vacuum silica gel column chromatography. Eleven lignans (**1–11**; Figure 1) were isolated and identified by comparison with previously reported spectroscopic data.

Culture of hepatocytes and exposure to CCl_4

Isolated hepatocytes were prepared from male Wistar rats by the collagenase perfusion technique and cultured as described by Lee et al (1995). One day after plating, the cultured hepatocytes were exposed for 1·5 h to a medium containing 5·0 mM CCl_4 with or without the compound to be tested. The test compounds were dissolved in dimethylsulfoxide with a final concentration of 0·01%. The culture medium and cells were collected for the determination of the activities of glutamic pyruvic transaminase and various antioxidant enzymes, and the levels of malondialdehyde and glutathione (Sung et al 1997; Kim et al 1999).

Determination of glutamic pyruvic transaminase activity

The activity of glutamic pyruvic transaminase in the culture medium was determined by the method of Reitman & Frankel (1957).

Assays for the activity of antioxidant enzymes

Mitochondrial fractions were prepared from primary cultures of rat hepatocytes as described elsewhere (Gibson & Skelf 1988). The activity of superoxide dismutase was determined according to the method of McCord & Fridovich (1969) by xanthine–xanthine oxidase reaction. Catalase activity was determined according to the method of Beers & Sizer (1952) based on H_2O_2 decomposition. GSSG-reductase activity was measured according to the method of Carlberg & Mannervik (1975) based on the reduction of GSSG by GSSG-R and NADPH. Glutathione peroxidase activity was determined by quantifying the rate of oxidation of glutathione to GSSG by cumene hydroperoxide, a reaction catalyzed by glutathione peroxidase (Flohe & Gunzler 1984). GST activity was determined spectrophotometrically by measuring the rate of formation of the conjugate of glutathione and 1-chloro-2,4-dinitrobenzene according to the method of Habig et al (1974).

Determination of the levels of glutathione and malondialdehyde

Total glutathione (GSH + GSSG) was measured by the method of Tietze (1969). To measure GSSG,

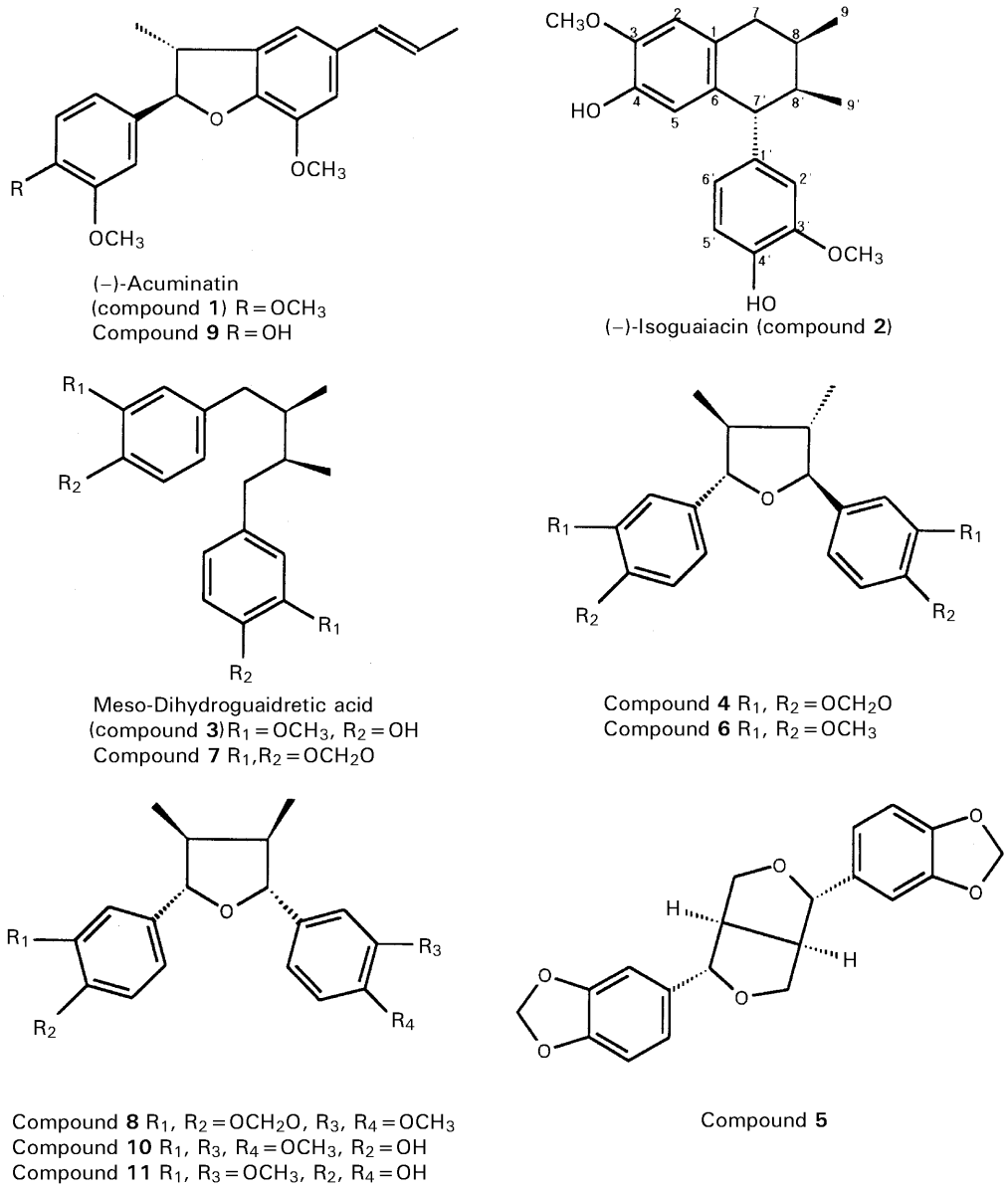

Figure 1. Chemical structures of compounds **1**–**11** from the CH_2Cl_2 fraction of *M. thunbergii*.

GSH was removed by reacting it with 4-vinylpyridine. The level of malondialdehyde was determined by the modified thiobarbituric acid method (Ohkawa et al 1979).

Protein assay

Protein content was measured by the method of Lowry et al (1951) with bovine serum albumin as a standard.

Statistical analysis

All data are expressed as the mean ± s.d. The evaluation of significance was determined by one-way analysis of variance. $P < 0.05$ was considered to be statistically significant.

Results

Isolation and identification of compounds **1**–**11**

Compounds **1**–**11** (Figure 1) were isolated from the CH_2Cl_2 fraction of *M. thunbergii* and identified as (−)-acuminatin (**1**), (−)-isoguaiacin (**2**), *meso*-dihydroguaiaretic acid (**3**), (+)-galbacin (**4**), (−)-sesamin (**5**), (+)-galbelgin (**6**), machilin A (**7**), machilin G (**8**), licarin A (**9**), nectandrin A (**10**) and B (**11**). The ^{13}C NMR data for (−)-isoguaiacin are

Table 1. Effects of compounds **1**–**11** on the activity of glutamic pyruvic transaminase released from CCl_4-injured primary cultures of rat hepatocytes.

Group	Protection (%)
Control	100 ± 5
CCl_4	0 ± 10
CCl_4 + (−)-acuminatin (**1**)	50 ± 12**
CCl_4 + (−)-isoguaiacin (**2**)	53 ± 15*
CCl_4 + *meso*-dihydroguaiaretic acid (**3**)	56 ± 10**
CCl_4 + compound **4**	28 ± 10
CCl_4 + compound **5**	25 ± 10
CCl_4 + compound **6**	22 ± 5
CCl_4 + compound **7**	10 ± 3
CCl_4 + compound **8**	7 ± 3
CCl_4 + compound **9**	9 ± 4
CCl_4 + compound **10**	0 ± 2
CCl_4 + compound **11**	9 ± 2
CCl_4 + silybin (100 μM)	65 ± 5**

A primary culture of rat hepatocytes was exposed to 5 mM CCl_4 with or without one of the compounds **1**–**11** (50 μM). The activities of glutamic pyruvic transaminase were measured as described in Materials and Methods. The glutamic pyruvic transaminase values of control and CCl_4-injured were 24·98 ± 3·42 and 109·62 ± 10·34 int. unit L^{-1}, respectively. They were expressed as a percentage of the control. Silybin (100 μM) was used as a positive control. Each value represents the mean ± s.d. (n = 3). *$P < 0.05$, **$P < 0.01$ compared with Cl_4.

presented here for the first time as far as we are able to determine.

(−)-Isoguaiacin (**2**). ^{13}C NMR ($CDCl_3$) δ: 16·09 (CH_3), 16·13 (CH_3), 29·55 (CH_2CHCH_3), 35·58 (CH_2), 40·89 ($CHCH_3$), 50·72 ($CHCHCH_3$), 56·06 (OCH_3), 110·84 (CH), 111·77 (CH), 114·02 (CH), 116·30 (CH), 122·30 (CH), 127·89, 131·12, 139.29, 143·75 (COH), 143·93 (COH), 145.23 ($COCH_3$), 146·50 ($COCH_3$).

Hepatoprotective activity of (−)-acuminatin, (−)-isoguaiacin and meso*-dihydroguaiaretic acid on CCl_4-induced toxicity*

To evaluate the hepatoprotective activities of the 11 lignans, the compounds at a concentration of 50 μM were added to primary cultures of rat hepatocytes in the presence of CCl_4 (Table 1). Among the eleven compounds tested, only (−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid, significantly decreased the level of glutamic pyruvic transaminase released from CCl_4-injured primary cultures of rat hepatocytes into the medium at concentrations ranging from 10 to 100 μM (Figure 2).

Effect of (−)-acuminatin, (−)-isoguaiacin and meso-dihydroguaiaretic acid on GSH/GSSG content

The levels of total glutathione and reduced glutathione were decreased in CCl_4-injured hepatocytes, but were partially restored by concomitant treatment with (−)-acuminatin, (−)-isoguaiacin or *meso*-dihydroguaiaretic acid, at a concentration of 50 μM. (−)-Isoguaiacin and *meso*-dihydroguaiaretic acid increased significantly the ratio of GSH/GSSG, an index of cellular oxidative status (Table 2).

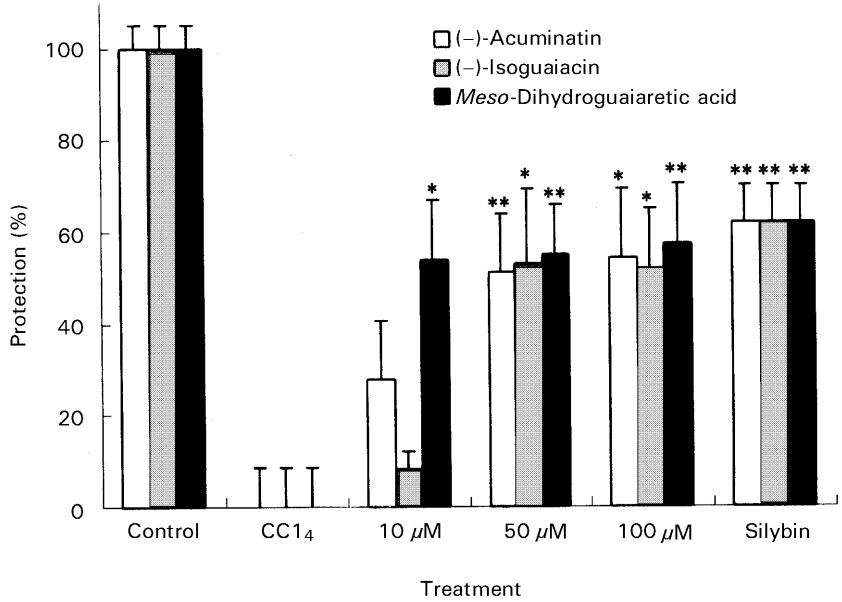

Figure 2. Effects of (−)-acuminatin (**1**), (−)-isoguaiacin (**2**) and *meso*-dihydroguaiaretic acid (**3**) on the activity of glutamic pyruvic transaminase released from CCl_4-injured primary cultures of rat hepatocytes. Values are expressed as a percentage of the control. Each value represents the mean ± s.d., n = 3. *$P < 0.05$, **$P < 0.01$ significantly different from CCl_4.

Table 2. Effect of (−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid on glutathione levels in primary cultures of rat hepatocytes injured by CCl_4.

Group	Total glutathione (nmol (mg protein)$^{-1}$)	Reduced glutathione (nmol (mg protein)$^{-1}$)	Reduced glutathione/GSSG
Control (no CCl_4)	49·9 ± 2.1	41·4 ± 2.3	5·3 ± 0.9
CCl_4	29·6 ± 5.0	17·4 ± 0.4	1·5 ± 0.1
CCl_4 + acumination	37·6 ± 0.6**	21·5 ± 0.7*	1·3 ± 0.3
CCl_4 + (−)-isoguaiacin	39·6 ± 3.3*	34·3 ± 3.0*	6·4 ± 0.4**
CCl_4 + *meso*-dihydroguaiaretic acid	42·0 ± 3.0*	33·5 ± 3.3*	3·8 ± 0.1**
CCl_4 + silybin (100 μM)	39·7 ± 4.3*	28·5 ± 3.4*	2·5 ± 0·6*

Primary cultures of rat hepatocytes were exposed to 5 mM CCl_4 with or without 50 μM (−)-acuminatin, (−)-isoguaiacin or *meso*-dihydroguaiaretic acid. The glutathione levels were measured as described in Materials and Methods. Values given represent the mean ± s.d. for three separate experiments. The controls were defined as cultures not receiving carbon tetrachloride. *$P < 0·05$, **$P < 0·01$ compared with CCl_4-injured alone.

Table 3. Effect of (−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid on the activities of hepatic antioxidant enzymes in primary cultures of rat hepatocytes injured by CCl_4.

Group	Glutathione peroxidase (μmol NADPH consumed min^{-1} (mg protein)$^{-1}$)	Catalase (μmol H_2O_2 consumed min^{-1} (mg protein^{-1})	Superoxide dismutase (units mL^{-1})	Malondialdehyde (nmol (mg protein)$^{-1}$)
Control (no CCl_4)	5·9 ± 0.8	941·7 ± 179.8	8·4 ± 0.2	1·6 ± 0.7
CCl_4	3·8 ± 0.5	541·3 ± 67.5	4·0 ± 0.3	5.2 ± 0·7
CCl_4 + (−)-acumination	4·3 ± 0.5	892·0 ± 41.9*	7·1 ± 1·1**	3·3 ± 0.6*
CCl_4 + (−)-isoguaiacin	4·4 ± 0.6	888·1 ± 80.4*	8·3 ± 1.4**	2·8 ± 0·1*
CCl_4 + *meso*-dihydroguaiaretic acid	5·1 ± 0.2*	930·1 ± 46.4*	8·9 ± 1·0**	2·3 ± 0.7*
CCl_4 + silybin (100 μM)	5·0 ± 0.5*	732·0 ± 54.2*	6·5 ± 0.6**	3·2 ± 0.4*

Primary cultures of rat hepatocytes were exposed to 5 mM CCl_4 either with or without 50 μM (−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid. The activity of each enzyme was measured as described in Materials and Methods. Values given represent the mean ± s.d. for three separate experiments. The controls were defined as cultures not receiving carbon tetrachloride. *$P < 0·05$, **$P < 0·01$ compared with CCl_4-injured alone.

Effect of (−)-acuminatin, (−)-isoguaiacin and meso-dihydroguaiaretic acid on antioxidant enzymes and lipid peroxidation

Treatment with 50 μM (−)-acuminatin, (−)-isoguaiacin or *meso*-dihydroguaiaretic acid significantly preserved the activities of superoxide dismutase and catalase in primary cultures of rat hepatocytes injured with CCl_4. The activity of glutathione peroxidase was preserved by *meso*-dihydroguaiaretic acid, but not by (−)-acuminatin or (−)-isoguaiacin. All three lignans significantly reduced the production of malondialdehyde, an indicator of lipid peroxidation (Table 3).

Effect of (−)-acuminatin, (−)-isoguaiacin and meso-dihydroguaiaretic acid on the activities of GST and GSSG-R

(−)-Acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid preserved the activities of GST and GSSG-R slightly in primary cultures of rat hepatocytes injured by CCl_4, but their effects were not significant (data not shown).

Discussion

Among the eleven lignans identified, only (−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid showed significant hepatoprotective activity. To elucidate the biochemical mechanism of the hepatoprotective activity of these three lignans, we studied their effects on the cellular defence systems injured by CCl_4. In general, glutathione, which is present in large concentrations in the liver, plays a major role in the elimination of a large number of nucleophilic toxicants such as oxidative radicals. In normal cells, glutathione levels are decreased by oxidative radicals but are promptly restored to normal levels. Otherwise, administration of CCl_4 to cells rapidly and continuously decreases cellular glutathione levels and inactivates many related antioxidant enzymes including superoxide dismutase, catalase and glutathione peroxidase. Thus, the concentration of $\cdot O_2^-$ and H_2O_2 are kept high in response. This propagates lipid peroxidation leading to membrane degradation, cellular dysfunction and cell necrosis (Yasuda et al 1980).

(−)-Acuminatin preserved significantly the level of glutathione and the activities of superoxide dismutase and catalase in CCl_4-injured hepatocytes. However, (−)-acuminatin did not preserve the ratio of GSH/GSSG to a significant degree. While the compound was able to preserve only a relatively low ratio of GSH/GSSG, the fact that (−)-acuminatin inhibited significantly the release of glutamic pyruvic transaminase suggests that cellular oxidative status was not so critically affected as to damage the viability or membrane integrity of the hepatocytes. This suggestion is supported by results showing that depletion of glutathione alone did not result in a severe leakage of lactate dehydrogenose from primary cultured rat hepatocytes (Casey et al 1995). By contrast, at a concentration of 50 μM, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid not only preserved significantly total glutathione levels and GSH/GSSG ratios, but also maintained the levels of superoxide dismutase, catalase and glutathione peroxidase. They also strongly inhibited lipid peroxidation as demonstrated by a reduction in the production of malondialdehyde. As the three lignans preserved glutathione, this may lead to the scavenging of potent free radicals and the retention of the activities of superoxide dismutase, catalase and glutathione peroxidase by keeping the level of $\cdot O_2^-$ low, and lipid peroxidation suppressed. (−)-Acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid protected hepatocytes from CCl_4 by means of the activation of antioxidant enzymes and inhibition of lipid peroxidation. It is possible that as antioxidants (−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid play a role in the protection of hepatocytes from CCl_4. The hepatoprotective activities of (−)-acuminatin, (−)-isoguaiacin and *meso*-dihydroguaiaretic acid at 50 μM were as potent as that of 100 μM silybin, a positive control.

When the hepatoprotective activities of dibenzylbutane lignans (**3**, **7**) were compared, the activity of machilin A (**7**), which has two methylenedioxy moieties, was lower than that of *meso*-dihydroguaiaretic acid by 34%. Moreover, (−)-acuminatin showed stronger activity than 4-demethyl acuminatin (licarin A, **9**). These findings suggest that substitutes on the phenyl nucleus of a lignan might contribute to the expression of hepatoprotective activity. This supposition is indirectly supported by comparison to the structure–activity relationship of gomisins (Iikino et al 1984). On the other hand, tetrahydrofuranoid (**4**, **6**, **8**, **10**, **11**) and furofuranoid (**5**) lignans which have the furan ring system, showed no hepatoprotective activity against CCl_4-injured cells.

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