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# Hepatoprotective Constituents from Zedoariae Rhizoma: Absolute Stereostructures of Three New Carabrane-type Sesquiterpenes, Curcumenolactones A, B, and C

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**Abstract**—New carabrane-type sesquiterpene lactones, curcumenolactones A, B, and C, were isolated from the 80% aqueous acetone extract of Zedoariae Rhizoma (Zingiberaceae), together with 41 sesquiterpenes and two diarylheptanoids. The absolute stereostructures of curcumenolactones A, B, and C were determined on the basis of physicochemical evidence, which included nuclear Overhauser effect (NOE) and circular dichroic (CD) spectroscopic analyses. Curcumenone, a principal carabrane-type sesquiterpene from Zedoariae Rhizoma, was found to show potent protective effect on D-galactosamine/lipopolysaccharide-induced acute liver injury in mice. In addition, curcumenolactones A and B and the other constituents showed protective effect on D-galactosamine-induced cytotoxicity in primary cultured rat hepatocytes. 

## Introduction

Zedoariae Rhizoma, *Curcuma zedaria* ROSCOE (Zingiberaceae), have been used as a Chinese and Japanese herbal medicine, which is listed in the Japanese Pharmacopoeia XIII as aromatic stomachic, emmenagogus, or for the treatment of 'Oketsu' syndrome caused by blood stagnation. Furthermore, Zedoariae Rhizoma also have been used as an important fragrance and spice in Asian countries. As chemical constituents of this plant, many sesquiterpenes, such as furanogermenone,[1] germacrone (**11**),[2] and (+)-germacrone 4,5-epoxide (**14**),[3] have been isolated from Zedoariae Rhizoma, and these sesquiterpenes have been reported to exhibit anti-hepatotoxic and anti-ulcer effects.[1,4]

In the course of our studies on bioactive constituents of natural medicines and medicinal foodstuffs,[5] we have already reported that the sesquiterpene constituents from Zedoariae Rhizoma exhibited potent vasorelaxant activity. In addition, absolute stereostructures of carabrane-type sesquiterpenes, curcumenone (**4**), 4*S*-dihydrocurcumenone (**5**), and curcarabranols A (**6**) and B (**7**), were determined on the basis of physicochemical and chemical evidence.[6] To the best of our knowledge, this paper is a first report for the absolute stereostructure of carabrane-type sesquiterpene. Furthermore, we also communicated hepatoprotective activity of the 80% aqueous acetone extract and several known constituents from Zedoariae Rhizoma and their plausible mechanisms of action.[7] In this paper, we describe the structural elucidation of three carabrane-type sesquiterpene lactones termed curcumenolactones A (**1**), B (**2**), and C (**3**) from Chinese Zedoariae Rhizoma, and protective effect of a principal carabrane-type sesquiterpene, curcumenone (**4**), on D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced acute liver injury in mice. In addition, we also describe protective effects of 35 sesquiterpene and two diarylheptanoid constituents against D-GalN-induced cytotoxicity in primary cultured rat hepatocytes.

## Results and Discussion

### Isolation of sesquiterpene and diarylheptanoid constituents from Zedoariae Rhizoma

Zedoariae Rhizoma (cultivated in Szechwan province, China, purchased from Tochimoto Tenkaido Co. Ltd., Osaka) were extracted with 80% aqueous acetone at room temperature. The aqueous acetone extract was partitioned in an ethyl acetate and water mixture to give an ethyl acetate-soluble portion and an aqueous phase. The aqueous phase was further extracted with 1-butanol to

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give a 1-butanol-soluble portion and a water-soluble portion. The ethyl acetate-soluble portion was subjected to silica-gel, silver nitrate-treated silica gel,[8] and ODS column chromatography and finally HPLC to furnish 35 sesquiterpenes, curcumenolactones A (**1**, 0.00030% from the natural medicine), B (**2**, 0.00013%), and C (**3**, 0.00015%), curcumenone (**4**,[6,9] 0.041%), 4*S*-dihydrocurcumenone (**5**,[6] 0.0011%), curcarabranols A (**6**,[6] 0.00030%), B (**7**,[6] 0.00030%), furanodiene (**8**,[7,10] 0.0012%), isofuranodienone (**9**,[11] 0.00056%), zederone (**10**,[3b,7] 0.0052%), germacrone (**11**,[2,7] 0.0085%), 13-hydroxygermacrone (**12**,[7,12] 0.0033%), glechomanolide (**13**,[13] 0.00057%), (+)-germacrone 4,5-epoxide (**14**,[3,7] 0.0019%), curdione (**15**,[7,14] 0.042%), neocurdione (**16**,[7,14] 0.00090%), dehydrocurdione (**17**,[7,15] 0.0055%), curcumenol (**18**,[7,16] 0.057%), isocurcumenol (**19**,[7,17] 0.0031%), procurcumenol (**20**,[18] 0.00021%), isoprocurcumenol (**21**,[7,19] 0.00090%), alismoxide (**22**,[20] 0.00025%), 7α,11α-epoxy-5β-hydroxy-9-guaiaen-8-one (**23**,[21] 0.00090%), aerugidiol (**24**,[7,22] 0.0045%), (+)-*ar*-turmerone (**28**,[23] 0.012%), bisacumol (**29**,[24] 0.00019%), β-eudesmol (**31**,[25,26] 0.00061%), β-dictyopterol (**32**,[26] 0.00032%), curzerenone (**33**,[11] 0.028%), curcumadione (**34**,[19a] 0.00085%), zedoarofuran[27] (0.00014%), 4-epicurcumenol[27] (0.00062%), neocurcumenol[27] (0.0014%), and gajutsulactones A[27] (0.00019%), and B[27] (0.00023%) and a diarylheptanoid, curcumin (**35**,[7,28] 0.0096%). From the 1-butanol-soluble portion, seven sesquiterpenes, **24** (0.00016%), zedoarondiol (**25**,[7,29] 0.0024%), isozedoarondiol (**26**,[29] 0.00034%), zedoalactone B (**27**,[30] 0.0037%), bisacurone (**30**,[24,31] 0.00016%), zedoarolides A[27] (0.00020%), and B[27] (0.00067%), and a diarylheptanoid, bis(4-hydroxycinnamoyl)methane (**36**,[28] 0.0044%), were isolated by silica-gel and ODS column chromatography and finally HPLC (Chart 1).

### Absolute stereostructures of curcumenolactones A (1), B (2), and C (3)

Curcumenolactone A (**1**) was isolated as colorless oil with positive optical rotation ($[\alpha]_D^{25}$ +131.9°). The electron impact (EI)–MS of **1** showed a molecular ion ($M^+$) peak at *m*/*z* 248 in addition to fragment ion peaks at *m*/*z* 230 ($M^+-H_2O$) and *m*/*z* 43 (base peak). The molecular formula $C_{15}H_{20}O_3$ of **1** was determined from the molecular ion peak observed in the EI–MS and by high-resolution MS measurement. The IR spectrum of **1** showed absorption bands ascribable to α,β-unsatulated γ-lactone, carbonyl, and olefin functions at 1759, 1715, and 1690 $cm^{-1}$, while its UV spectrum indicated the presence of an α,β-unsatulated γ-lactone chromophore form the absorption maxima at 222 nm (log ε 3.95). The $^1H$ NMR ($CDCl_3$) and $^{13}C$ NMR (Table 1) spectra of **1** showed signals assignable to a cyclopropane (δ 0.19 (dt, *J* = 6.2, 7.3 Hz, 1-H), 0.70 (ddd, *J* = 0.6, 6.2, 7.0 Hz, 5-H)), three methyl (δ 1.16 (s, 14-$H_3$), 1.77 (3H, dd, *J* = 1.8, 1.8 Hz, 13-$H_3$), 2.15 (s, 15-$H_3$)), and a methine bearing a oxygen function (δ 4.68 (qdd, *J* = 1.8, 7.7, 11.3 Hz, 8-H)) together with four methylenes (2, 3, 6, 9-$H_2$), and five quaternary carbons (4, 7, 10, 11, 12-C).

The plane structure of **1** was constructed on the basis of $^1H$–$^1H$ correlation spectroscopy (H–H COSY) and heteronuclear multiple bond correlation (HMBC) experiments. Thus, the H–H COSY experiment on **1** indicated the presence of two partial structures shown by thick lines in Figure 1: from C-1 to C-3, from C-1 to C-5, 6 and from C-8 to C-9. In the HMBC experiment, long-range correlations were observed between the following protons and carbons of **1** (3-H, 15-$H_3$ and 4-C; 14-$H_3$ and 5-C, 9-C, 10-C; 6-$H_2$, 8-H and 7-C; 13-$H_3$ and 7-C, 11-C, 12-C), so that the connectivities of the quaternary carbons (4, 7, 10, 11, 12-C) in **1** were clarified. The above-mentioned evidence led us to confirm the skeleton of curcumenolactone A (**1**) as 4-oxo-7(11)-carabren-12,8-olide.

The $^1H$ NMR nuclear Overhauser effect spectroscopy (NOESY) experiment on **1** showed NOE correlations between the signals of following proton pairs (1-H and 6α, 9α-H; 8-H and 6β, 9β-H; 14-$H_3$ and 5, 9β-H), as shown in Figure 1. On the basis of these findings, the relative stereostructure of **1** was elucidated.

Curcumenolactone B (**2**) was isolated as colorless oil with negative optical rotation ($[\alpha]_D^{27}$ −52.6°). The EI–MS of **2** showed a molecular ion peak at *m*/*z* 248 ($M^+$) and 230 ($M^+-H_2O$) and the molecular formula $C_{15}H_{20}O_3$, which is the same as that of **1**, was determined by high-resolution MS measurement. The IR spectrum of **2** showed absorption bands ascribable to α,β-unsatulated γ-lactone, carbonyl, and olefin functions at 1750, 1717, and 1684 $cm^{-1}$. In the UV spectrum of **2**, an absorption maximum was observed at 217 nm (log ε 3.78), suggestive of an α,β-unsatulated γ-lactone function. The $^1H$ NMR ($CDCl_3$) and $^{13}C$ NMR (Table 1) spectra of **2** were also found to be similar to those of **1** and indicated the presence of the same functional groups: a cyclopropane (δ 0.58 (br dd, *J* = ca. 5, 7 Hz, 5-H), 0.64 (dt, *J* = 5.2, 7.3 Hz, 1-H)), three methyl (δ 1.13 (s, 14-$H_3$), 1.77 (3H, dd, *J* = 1.7, 1.8 Hz, 13-$H_3$), 2.17 (s, 15-$H_3$)), and a methine bearing a oxygen function (δ 4.65 (qdd, *J* = 1.7, 6.4, 11.9 Hz, 8-H)) together with four methylenes and five quaternary carbons. The connectivities of the $^1H-^1H$ and the quaternary carbons in **2** was clarified by H−H COSY and HMBC experiments as shown in Figure 2. In addition, the relative stereostructure of **2** was elucidated by a NOESY experiment, which showed NOE correlations between the following proton pairs (1-H and 6α, 8, 9α-H; 14-$H_3$ and 5, 9β-H), as shown in Figure 2. On the basis of this evidence, the stereostructure of **2** was characterized to be 8-isomer of **1**.

Curcumenolactone C (**3**) was also isolated as colorless oil with positive optical rotation ($[\alpha]_D^{24}$ +44.4°). Here again, the molecular formula $C_{15}H_{20}O_4$ of **3** was determined from the EI–MS (*m*/*z* 264 ($M^+$)) and by high-resolution MS measurement. The IR spectrum of **3** showed absorption bands ascribable to hydroxyl, α,β-unsatulated γ-lactone, carbonyl, and olefin functions at 3482, 1750, 1717, and 1700 $cm^{-1}$. In the UV spectrum of **3**, an absorption maximum was observed at 216 nm (log ε 3.86), suggestive of an α,β-unsatulated γ-lactone function. The $^1H$ NMR ($CDCl_3$) and $^{13}C$ NMR (Table 1) spectra of **3** showed similar signals of **1** and **2**, expect for lacking a methine bearing a oxygen function: a cyclopropane

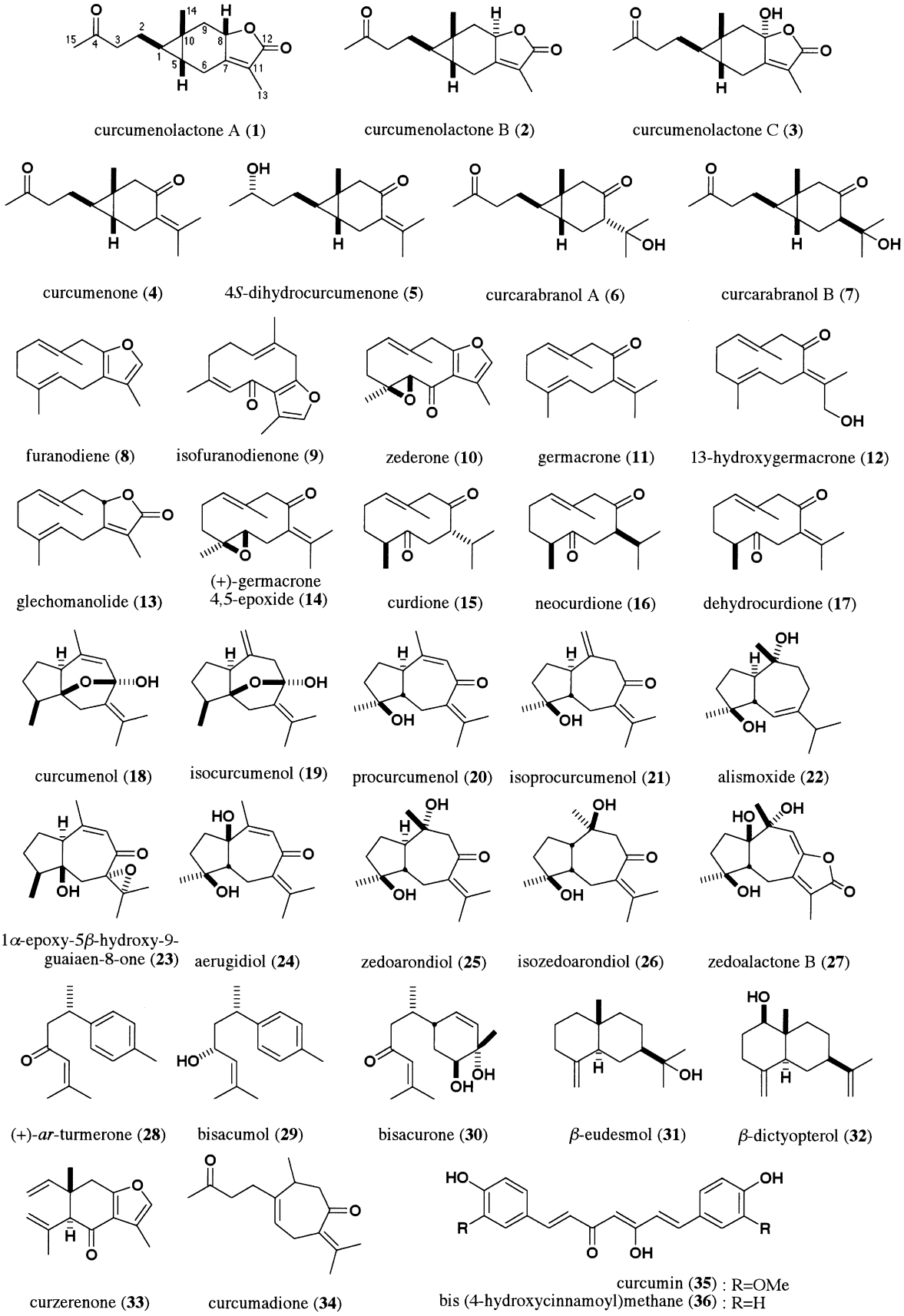
curcumenolactone A (1)
curcumenolactone B (2)
curcumenolactone C (3)
curcumenone (4)
4S-dihydrocurcumenone (5)
curcarabranol A (6)
curcarabranol B (7)
furanodiene (8)
isofuranodienone (9)
zederone (10)
germacrone (11)
13-hydroxygermacrone (12)
glechomanolide (13)
(+)-germacrone 4,5-epoxide (14)
curdione (15)
neocurdione (16)
dehydrocurdione (17)
curcumenol (18)
isocurcumenol (19)
procurcumenol (20)
isoprocurcumenol (21)
alismoxide (22)
7α,11α-epoxy-5β-hydroxy-9-guaiaen-8-one (23)
aerugidiol (24)
zedoarondiol (25)
isozedoarondiol (26)
zedoalactone B (27)
(+)-ar-turmerone (28)
bisacumol (29)
bisacurone (30)
β-eudesmol (31)
β-dictyopterol (32)
curzerenone (33)
curcumadione (34)
curcumin (35) : R=OMe
bis (4-hydroxycinnamoyl)methane (36) : R=H

**Chart 1.**

**Table 1.** $^{13}C$ NMR data for curcumenolactones A (**1**), B (**2**) and C (**3**)

| | 1 | 2 | 3 |
|---|---|---|---|
| C-1 | 32.1 | 25.7 | 30.7 |
| C-2 | 23.4 | 23.5 | 23.4 |
| C-3 | 43.5 | 43.6 | 43.6 |
| C-4 | 208.5 | 208.4 | 208.7 |
| C-5 | 26.0 | 23.2 | 26.4 |
| C-6 | 24.9 | 24.8 | 23.6 |
| C-7 | 162.0 | 159.9 | 158.5 |
| C-8 | 77.2 | 78.0 | 103.0 |
| C-9 | 42.3 | 37.3 | 46.7 |
| C-10 | 18.4 | 22.3 | 18.6 |
| C-11 | 121.9 | 120.7 | 124.3 |
| C-12 | 174.7 | 174.2 | 171.8 |
| C-13 | 8.4 | 8.5 | 8.3 |
| C-14 | 21.1 | 20.1 | 21.5 |
| C-15 | 30.0 | 30.0 | 30.0 |

The spectra were taken in $CDCl_3$ at 125 MHz.

(δ 0.15 (dt, $J = 5.8$, 7.0 Hz, 1-H), 0.76 (ddd, $J = 1.0$, 5.8, 5.8 Hz, 5-H)), and three methyl (δ 1.19 (s, 14-$H_3$), 1.77 (3H, d, $J = 2.1$ Hz, 13-$H_3$), 2.15 (s, 15-$H_3$)). The structure of **3** was elucidated on the basis of H–H COSY and HMBC experiments as shown in Figure 2. The plane structure of **3** was characterized to be 8-hydroxy-4-oxo-7(11)-carabren-12,8-olide. Furthermore, the relative stereostructure of **3** was clarified by a NOESY experiment, which showed similar NOE correlations to those of **2**, except for the NOE correlations due to the 8-proton (1-H and 6α-H; 5-H and 6β-H, 14-$H_3$; 9β-H and 14-$H_3$).

Finally, the absolute stereostructures of **1**–**3** were determined by the circular dichroic (CD) spectrum on α,β-unsatulated γ-lactone moiety.[32] Namely, the CD spectrum of **2** showed the characteristic Cotton curve (Δε −5.45 (220 nm), +1.73 (245 nm)) for the 8*R* configuration in *endo*-α,β-unsatulated lactones. On the other hand, the CD data of **1**, the 8-isomer of **2**, showed a Cotton curve (Δε +4.55 (230 nm)) and its absolute stereostructure was determined to be *S*. The CD spectrum (Δε −7.88 (225 nm), +6.06 (250 nm)) of **3** was similar to that of **2**, so that the absolute stereostructure of **3** was determined to be a 8*R* configuration.

## Protective effects of curcumenone (4) and curcumin (35) on liver injury induced by D-GalN/LPS in mice

The principal carabrane-type sesquiterpene, curcumenone (**4**), at doses of 25 or 50 mg/kg (po) showed a potent inhibitory effect on increase of serum GOT (s-GOT) and serum GTP (s-GTP) induced by D-GalN/LPS in mice (Table 2). Inhibitory effect of **4** on this injury was stronger than that of curcumin (**35**), which was reported to inhibit liver injury induced by $CCl_4$.[33]

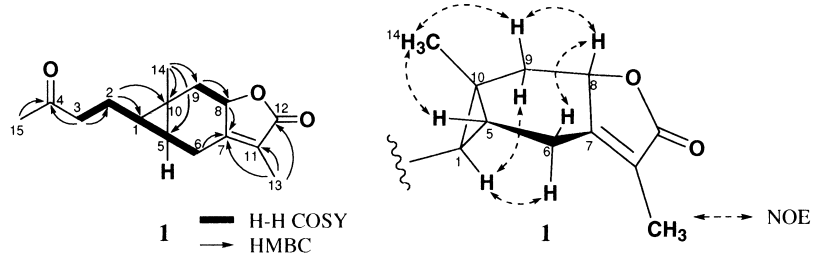


**Figure 1.** H–H COSY, HMBC, and NOE correlations of **1**.

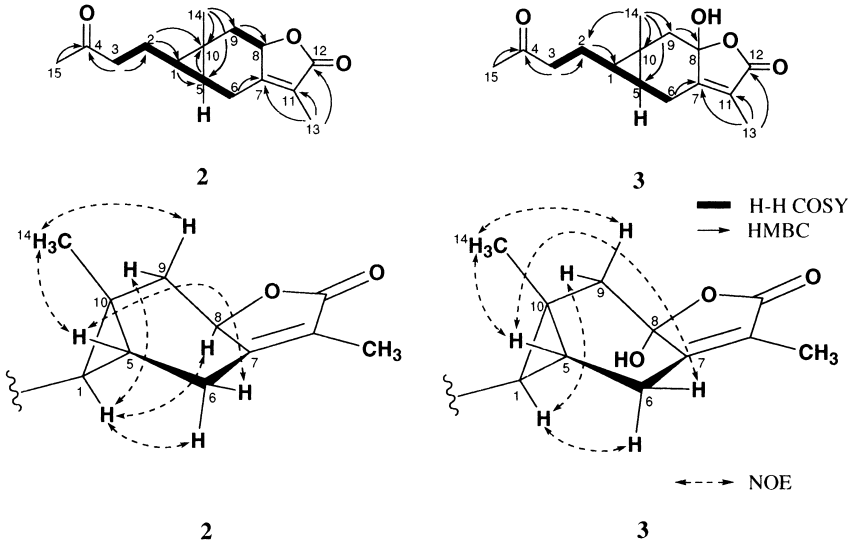


**Figure 2.** H–H COSY, HMBC, and NOE correlations of **2** and **3**.

**Table 2.** Protective effect of curcumenone (**4**) and curcumin (**35**) on liver injury induced by D-GalN/LPS in mice

| Sample | Dose (mg/kg, po) | N | Karmen unit | |
|---|---|---|---|---|
| | | | s-GPT | s-GOT |
| Untreated control | — | 10 | 17±1** | 55±5** |
| Treated control (D-GalN/LPS) | — | 11 | 4021±1050 | 4817±1510 |
| Curcumenone (**4**) + D-GalN/LPS | 12.5 | 9 | 2455±766 | 2139±743 |
| | 25 | 9 | 1625±821 | 1419±638* |
| | 50 | 9 | 413±35** | 590±36** |
| Hydrocortisone + D-GalN/LPS | 20 | 5 | 82±11** | 133±11** |
| Treated control (D-GalN/LPS) | — | 10 | 6605±1985 | 6033±1647 |
| Curcumin (**35**) + D-GalN/LPS | 12.5 | 10 | 5024±1189 | 4770±1218 |
| | 25 | 10 | 3253±981 | 3177±979 |
| | 50 | 9 | 1916±483* | 2220±563* |

Each value represents the means ± SEM Significantly different from the control: $*p < 0.05$, $**p < 0.01$.

### Protective effects of chemical constituents from Zedoariae Rhizoma on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes

The inhibitory effects of sesquiterpenes (**1**–**34**) and diarylheptanoids (**35**, **36**) on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes were examined. Curcumenolactones A (**1**) and B (**2**), germacrone (**11**), glechomanolide (**13**), curdione (**15**), neocurdione (**16**), 7α,11α-epoxy-5β-hydroxy-9-guaiaen-8-one (**23**), and β-dictyopterol (**32**) showed potent inhibitory activity as shown in Table 3. On the other hand, 13-hydroxygermacrone (**12**), aerugidiol (**24**), zedoarondiol (**25**), and isozedoarondiol (**26**) were found to strengthen the cytotoxicity by D-GalN, despite they showed little cytotoxic effect to the hepatocytes in the absence of D-GalN (viability at 100 μM: >90%). As to structural requirements of carabrane-type sesquiterpene for the activity in vitro, γ-lactone moiety seems to be important, since **1** and **2** having γ-lactone moiety showed the activity and curcumenolactone C (**3**) having γ-lactone moiety with the 8-OH group and **4**–**7** did not.

Although curcumenone (**4**) and curcumin (**35**) showed inhibitory effect on D-GalN/LPS-induced liver injury in mice, they did not show cytoprotective effect against D-GalN-induced cytotoxicity in vitro. They oppositely showed cytotoxic effects on the hepatocytes using MTT assay. Viabilities of **4** and **35** at 100 μM were 75.2±1.9 (%) and 3.7±0.2 (%), respectively. In addition, weak cytotoxicity was observed in the hepatocytes treated with furanodine (**8**) [viability at 100 μM: 72.4±3.6 (%)], and zederone (**10**) exhibited strongest cytotoxic effect [viability at 100 μM: 6.7±0.6 (%)] among the sesquiterpene constituents.

## Experimental

The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter ($l = 5$ cm); UV spectra, Shimadzu UV-1200 spectrometer; CD spectra, JASCO J-720WI spectropolarimeter; IR spectra, Shimadzu FTIR-8100 spectrometer; EI–MS and high-resolution MS, JEOL JMS-GCMATE mass spectrometer; FAB–MS and high-resolution MS, JEOL JMS-SX 102A mass spectrometer; $^1$H NMR spectra, JNM-LA500 (500 MHz) spectrometer; $^{13}$C NMR spectra, JNM-LA500 (125 MHz) spectrometer with tetramethylsilane as an internal standard.

The following experimental conditions were used for chromatography: ordinary-phase silica-gel column chromatography, Silica-gel BW-200 (Fuji Silysia Chemical, Ltd, 150–350 mesh); reversed-phase silica-gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd, 100–200 mesh); TLC, pre-coated TLC plates with Silica-gel $60F_{254}$ (Merck, 0.25 mm) (ordinary phase) and Silica-gel RP-18 $60F_{254}$ (Merck, 0.25 mm) (reversed phase); reversed-phase HPTLC, precoated TLC plates with Silica-gel RP-18 $60WF_{254S}$ (Merck, 0.25 mm); detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous $H_2SO_4$ and heating.

### Extraction and isolation

Dried Zedoariae Rhizoma (9.0 kg, cultivated in Szechwan, China and purchased from Tochimoto Tenkaido Co. Ltd., Osaka) were finely minced and extracted with 80% aqueous acetone (20 L) at room temperature for three times. Evaporation of the solvent under reduced pressure gave the aqueous acetone extract (320 g). The aqueous acetone extract (291 g) was partitioned into a AcOEt–$H_2O$ (1:1) mixture, and the aqueous layer was further extracted with 1-BuOH. Removal of the solvent under reduced pressure from the AcOEt-, 1-BuOH-, and water-soluble portions yielded 198, 96, and 7 g of residues, respectively.

The AcOEt-soluble portion (96.5 g) was subjected to ordinary-phase silica gel column chromatography (3.0 kg, *n*-hexane→*n*-hexane–AcOEt (40:1→20:1→10:1→5:1→3:1→1:1→1:2)→AcOEt→acetone→MeOH) to afford thirteen fractions (Fr. 1 (3.6 g), Fr. 2 (6.9 g), Fr. 3 (5.2 g), Fr. 4 (2.3 g), Fr. 5 (2.7 g), Fr. 6 (4.0 g), Fr. 7 (12.3 g), Fr. 8 (6.4 g), Fr. 9 (2.3 g), Fr. 10 (7.6 g), Fr. 11 (4.9 g), Fr. 12 (20.5 g), Fr. 13 (17.8 g)). Fraction 1 (3.6 g) was further subjected to silver nitrate-treated silica gel column chromatography (*n*-hexane–AcOEt (50:1→AcOEt) to furnish furanodiene (**8**, 54 mg). Fraction 2 (1.2 g) was further subjected to reversed-phase silica gel column chromatography (MeOH–$H_2O$ (70:30→80:20→90:10)→MeOH) and silver nitrate-treated silica gel

**Table 3.** Protective effect of chemical constituents from Zedoaria Rhizoma on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes[a]

| | Inhibition (%) | | | | |
|---|---|---|---|---|---|
| | 0 μM | 3 μM | 10 μM | 30 μM | 100 μM |
| Sesquiterpenes | | | | | |
| 1. Carabrane type | | | | | |
| Curcumenolactone A (**1**) | 0.0±5.2 | 13.9±11.3 | 16.8±4.6 | 29.7±7.6* | 65.5±5.7** |
| Curcumenolactone B (**2**) | 0.0±3.7 | −1.7±2.7 | 7.9±7.4 | 37.7±8.8** | 71.1±4.3** |
| Curcumenolactone C (**3**) | 0.0±5.2 | 12.3±9.3 | 12.5±5.4 | 15.3±9.2 | 21.5±5.0 |
| Curcumenone (**4**) | 0.0±4.5 | −4.8±2.1 | −2.3±2.3 | −10.4±3.3* | −12.2±2.6**,[b] |
| 4*S*-Dihydrocurcumenone (**5**) | 0.0±1.0 | 0.6±3.5 | 0.9±2.8 | −2.0±2.5 | −1.4±3.5 |
| Curcarabranol A (**6**) | 0.0±0.7 | 3.3±1.1 | 6.0±0.7* | 5.8±1.3 | 7.9±1.0 |
| Curcarabranol B (**7**) | 0.0±8.2 | 1.2±9.7 | −5.2±11.6 | −1.2±13.0 | 3.1±11.0 |
| 2. Germacrane type | | | | | |
| Furanodiene (**8**) | 0.0±0.2 | 0.3±0.3 | 0.1±0.2 | 1.0±0.3 | −0.5±0.2[b] |
| Zederone (**10**) | 0.0±1.2 | −2.4±1.1 | −3.4±1.2 | −6.2±0.4[a] | −7.9±0.1[b] |
| Germacrone (**11**) | 0.0±10.0 | 18.4±8.9 | 40.6±8.9** | 61.0±8.6** | 59.8±6.3** |
| 13-Hydroxygermacrone (**12**) | 0.0±2.6 | 8.3±6.5 | −7.2±3.3 | −17.0±3.5* | −21.7±3.0** |
| Glechomanolide (**13**) | 0.0±8.5 | −1.8±2.0 | 2.2±7.7 | 3.9±8.1 | 91.5±11.5** |
| (+)-Germacrone 4,5-epoxide (**14**) | 0.0±7.3 | −0.5±3.4 | 19.4±5.7* | 16.0±3.1 | 22.2±7.5* |
| Curdione (**15**) | 0.0±4.0 | 3.0±4.4 | 12.1±5.3 | 40.9±9.5* | 77.1±5.8* |
| Neocurdione (**16**) | 0.0±2.4 | −0.8±1.6 | −0.1±1.7 | 6.6±1.6 | 44.6±5.3* |
| Dehydrocurdione (**17**) | 0.0±0.9 | 3.4±3.2 | 6.0±3.1 | 3.4±1.9 | −6.3±0.3 |
| 3. Guaiane type | | | | | |
| Curcumenol (**18**) | 0.0±2.7 | 0.1±3.7 | −0.4±2.7 | 6.5±4.2 | 25.1±5.3** |
| Isocurcumenol (**19**) | 0.0±2.7 | −0.8±3.1 | 2.8±2.3 | 3.6±2.9 | 14.2±5.9 |
| Isoprocurcumenol (**21**) | 0.0±7.4 | 1.1±0.5 | 3.0±1.3 | −1.4±1.1 | −5.2±0.4 |
| Alismoxide (**22**) | 0.0±0.4 | −0.8±0.9 | 0.1±1.1 | −0.2±0.9 | 2.1±1.5 |
| 7α-11α-epoxy-5β-hydroxy-9-guaiaen-8-one (**23**) | 0.0±3.4 | 13.8±3.5** | 18.1±5.7** | 29.8±3.6** | 55.2±4.1** |
| Aerugidiol (**24**) | 0.0±12.8 | −12.9±10.1 | −25.7±14.2 | −37.5±14.1 | −41.5±8.0* |
| Zedoarondiol (**25**) | 0.0±6.0 | −5.3±10.6 | −4.1±13.8 | −8.7±12.1 | −35.6±7.9* |
| Isozedoarondiol (**26**) | 0.0±6.7 | 8.0±5.1 | −4.6±5.3 | −2.6±4.0 | −20.6±3.8* |
| Zedoalactone B (**27**) | 0.0±3.2 | 0.0±2.5 | 7.1±3.3 | 9.7±5.0 | 3.7±4.0 |
| Zedoarolide A | 0.0±6.5 | 4.2±9.6 | 18.1±9.3 | 24.0±4.2 | 14.7±8.4 |
| Zedoarolide B | 0.0±4.4 | 14.8±4.8** | 17.8±8.2** | 23.9±11.0** | 31.8±7.8** |
| 4. Bisaborane type | | | | | |
| (+)-*ar*-Turmerone (**28**) | 0.0±8.0 | −8.8±3.3 | 15.0±6.9 | −23.0±4.1* | −5.2±4.4 |
| Bisacumol (**29**) | 0.0±0.6 | 0.0±0.6 | 0.6±0.3 | 1.7±0.8 | 4.6±1.0 |
| Bisacurone (**30**) | 0.0±2.3 | 5.1±2.0 | 2.6±1.6 | 0.9±3.1 | −7.0±0.9 |
| 5. Eudesmane type | | | | | |
| β-Eudesmol (**31**) | 0.0±9.9 | 3.3±10.6 | 6.8±8.8 | 11.3±6.3 | 26.9±9.2* |
| β-Dictyopterol (**32**) | 0.0±0.9 | −6.2±11.4 | −9.0±9.5 | 22.7±17.0 | 45.9±12.1** |
| Zedoarofuran | 0.0±9.9 | −0.9±10.3 | −1.5±7.3 | 2.8±5.8 | 14.4±7.7 |
| 6. Elemane type | | | | | |
| Curzerenone (**33**) | 0.0±3.0 | 8.0±2.5 | 11.2±3.5** | 16.6±2.9** | −3.8±2.1 |
| 7. Xanthane type | | | | | |
| Curcumadione (**34**) | 0.0±2.7 | 1.2±3.2 | 5.3±3.3 | 2.8±1.9 | 11.2±1.4* |
| Diarylheptanoids | | | | | |
| Curcumin (**35**) | 0.0±3.7 | 0.1±3.8 | 1.1±2.2 | −17.7±1.3**,[b] | −44.3±0.3**,[b] |
| Bis(4-hydroxycinnamoyl)methane (**36**) | 0.0±3.1 | 2.8±2.1 | 12.6±2.3* | 17.2±3.5** | 2.7±2.5 |

[a]Each value represents the means ± SEM ($N = 4$). Significantly different from the control: *$p < 0.05$, **$p < 0.01$.
[b]Cytotoxic effect was observed.

column chromatography (*n*-hexane–AcOEt (30:1)→AcOEt) to furnish isofuranodienone (**9**, 4 mg), germacrone (**11**, 55 mg), (+)-*ar*-turmerone (**28**, 89 mg), and curzerenone (**33**, 214 mg). Fraction 5 (2.7 g) was separated by reversed-phase silica gel column chromatography (MeOH–$H_2O$ (70:30)→MeOH) to give curdione (**15**, 1.8 g). Fraction 7 (12.3 g) was purified by reversed-phase silica gel column chromatography (MeOH–$H_2O$ (60:40→80:20)→MeOH) and finally HPLC (YMC-pack ODS-A, MeOH–$H_2O$ (70:30) or $CH_3CN$–$H_2O$ (60:40), and YMC-pack SIL, *n*-hexane–AcOEt (8:1)) to furnish zederone (**10**, 49 mg), glechomanolide (**13**, 26 mg), (+)-germacrone 4,5-epoxide (**14**, 15 mg), neocurdione (**16**, 9 mg), dehydrocurdione (**17**, 44 mg), isocurcumenol (**19**, 143 mg), 7α,11α-epoxy-5β-hydroxy-9-guaiaen-8-one (**23**, 19 mg), bisacumol (**29**, 4 mg), β-eudesmol (**31**, 8 mg), and β-dictyopterol (**32**, 15 mg), 4-epicurcumenol (29 mg), neocurcumenol (63 mg), gajutsulactones A (9 mg), and B (10 mg). Fraction 8 (6.4 g) was subjected to ordinary-phase silica gel column chromatography (*n*-hexane–AcOEt (5:1→1:1)→AcOEt→MeOH), reversed-phase silica gel column chromatography (MeOH–$H_2O$ (70:30 or 80:20)→MeOH) to furnish curcumenone (**4**, 1.7 g) and curcumenol (**18**, 1.6 g). Fraction 10 (1.4 g) was purified by reversed-phase silica gel column chromatography (MeOH–$H_2O$ (40:60→50:50→60:40→90:10)→MeOH), and finally HPLC (YMC-pack ODS-A, MeOH–$H_2O$ (60:40)) to furnish 4*S*-dihydro-curcumenone (**5**, 9 mg), 13-hydroxygermacrone (**12**, 28 mg), and curcumadione (**34**, 7 mg). Fraction 12 (20.5 g) was subjected to ordinary-phase silica gel column chromatography ($CHCl_3$–MeOH (100:1→50:1→30:1→10:1)→MeOH), reversed-phase silica

gel column chromatography (MeOH–$H_2O$ (50:50→70:30)→MeOH), and finally HPLC (YMC-pack ODS-A, MeOH–$H_2O$ (55:40) or $CH_3CN$–$H_2O$ (30:70)) to give curcumenolactones A (**1**, 13 mg), B (**2**, 5 mg), and C (**3**, 7 mg), curcarabranols A (**6**, 12 mg) and B (**7**, 12 mg), and procurcumenol (**20**, 9 mg), isoprocurcumenol (**21**, 40 mg), alismoxide (**22**, 11 mg), aerugidiol (**24**, 25 mg), zedoarofuran (6 mg), and curcumin (**35**, 417 mg).

The 1-BuOH-soluble portion (75 g) was also subjected to ordinary-phase silica gel column chromatography (2.3 kg, $CHCl_3$–MeOH (30:1→10:1→5:1→1:1)→MeOH) to afford 13 fractions (Fr. 1 (3.4 g), Fr. 2 (1.2 g), Fr. 3 (10.9 g), Fr. 4 (3.5 g), Fr. 5 (9.2 g), Fr. 6 (4.4 g), Fr. 7 (4.4 g), Fr. 8 (1.2 g), Fr. 9 (2.2 g). Fr. 10 (4.6 g), Fr. 11 (6.7 g), Fr. 12 (2.5 g), Fr. 13 (20.8 g)). Fraction 10 (4.6 g) was further subjected to reversed-phase silica gel column chromatography (MeOH–$H_2O$ (40:60→70:30)→ MeOH), and finally HPLC (YMC-pack ODS-A, MeOH–$H_2O$ (60:40)) to furnish aerugidiol (**24**, 14 mg), zedoarondiol (**25**, 220 mg), isozedoarondiol (**26**, 31 mg), bisacurone (**30**, 15 mg), and bis(4-hydroxycinnamoyl) methane (**36**, 393 mg). Fraction 12 (2.5 g) was purified by reversed-phase silica gel column chromatography (MeOH–$H_2O$ (30:70→50:50→80:20)→MeOH), and finally HPLC (YMC-pack ODS-A, MeOH–$H_2O$ (30:70)) to give zedoalactone B (**27**, 33 mg), and zedoarolides A (18 mg) and B (60 mg). These known constituents were identified by comparison of their physical data with those of authentic samples (**22**, **28**, **31**, **35**) or with reported values.[2,3,6,7,9–26,28–31]

**Curcumenolactone A (1).** Colorless oil, $[\alpha]_D^{25}$ +131.9° (*c*, 0.1, $CHCl_3$). High-resolution EI–MS: calcd for $C_{15}H_{20}O_3$ ($M^+$): 248.1413. Found: 248.1427. CD Δε (nm, MeOH): +4.55 (230). UV (MeOH, nm, log ε) 222 (3.95). IR (film) 2924, 1759, 1715, 1690, 1082, 1023 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) δ 0.19 (1H, dt, *J* = 6.2, 7.3 Hz, 1-H), 0.70 (1H, ddd, *J* = 0.6, 6.2, 7.0 Hz, 5-H), 1.16 (3H, s, 14-$H_3$), 1.32 (1H, dd, *J* = 11.3, 13.5 Hz, 9α-H), 1.61 (2H, dt, *J* = 7.3, 7.3 Hz, 2-$H_2$), 1.77 (3H, dd, *J* = 1.8, 1.8 Hz, 13-$H_3$), 2.15 (3H, s, 15-$H_3$), 2.51 (2H, t, *J* = 7.3 Hz, 3-$H_2$), 2.55 (1H, dd, *J* = 7.7, 13.5 Hz, 9β-H), 2.78 (1H, qdd, *J* = 1.8, 7.0, 16.8 Hz, 6β-H), 2.90 (1H, br d, *J* = ca. 17 Hz, 6α-H), 4.68 (1H, qdd, *J* = 1.8, 7.7, 11.3 Hz, 8-H). $^{13}C$ NMR ($CDCl_3$) δc given in Table 1. EI–MS *m*/*z* (%): 248 ($M^+$, 5), 230 ($M^+ - H_2O$, 30), 43 (100).

**Curcumenolactone B (2).** Colorless oil, $[\alpha]_D^{27}$ −52.6° (*c*, 0.1, $CHCl_3$). High-resolution EI–MS: calcd for $C_{15}H_{20}O_3$ ($M^+$): 248.1413. Found: 248.1406. CD Δε (nm, MeOH): −5.45 (220), +1.73 (245). UV (MeOH, nm, log ε) 217 (3.78). IR (film) 2923, 1750, 1717, 1684, 1090, 1032 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) δ 0.58 (1H, br dd, *J* = ca. 5, 7 Hz, 5-H), 0.64 (1H, dt, *J* = 5.2, 7.3 Hz, 1-H), 1.13 (3H, s, 14-$H_3$), 1.36 (1H, dd, *J* = 11.9, 11.9 Hz, 9β-H), 1.65 (2H, dt, *J* = 7.3, 7.3 Hz, 2-$H_2$), 1.77 (3H, dd, *J* = 1.7, 1.8 Hz, 13-$H_3$), 2.17 (3H, s, 15-$H_3$), 2.54 (2H, t, *J* = 7.3 Hz, 3-$H_2$), 2.64 (1H, br dq, *J* = ca. 19, 2 Hz, 6α-H), 2.64 (1H, dd, *J* = 6.4, 11.9 Hz, 9α-H), 3.12 (1H, dd, *J* = 7.3, 18.6 Hz, 6β-H), 4.65 (1H, qdd, *J* = 1.7, 6.4, 11.9 Hz, 8-H). $^{13}C$ NMR ($CDCl_3$) δc: given in Table 1. EI–MS *m*/*z* (%): 248 ($M^+$, 25), 230 ($M^+ - H_2O$, 20), 43 (100).

**Curcumenolactone C (3).** Colorless oil, $[\alpha]_D^{24}$ +44.4° (*c*, 0.2, $CHCl_3$). High-resolution EI–MS: calcd for $C_{15}H_{20}O_4$ ($M^+$): 264.1361. Found: 264.1364. CD Δε (nm, MeOH): −7.88 (225), +6.06 (250). UV (MeOH, nm, log Δ) 216 (3.86). IR (film) 3482, 2926, 1750, 1717, 1700, 1034 $cm^{-1}$. $^1H$ NMR ($CDCl_3$) δ: 0.15 (1H, dt, *J* = 5.8, 7.0 Hz, 1-H), 0.76 (1H, ddd, *J* = 1.0, 5.8, 5.8 Hz, 5-H), 1.19 (3H, s, 14-$H_3$), 1.61 (2H, dt, *J* = 7.0, 7.0 Hz, 2-$H_2$), 1.74 (1H, d, *J* = 17.7 Hz, 9α-H), 1.77 (3H, d, *J* = 2.1 Hz, 13-$H_3$), 2.15 (3H, s, 15-$H_3$), 2.50 (2H, t, *J* = 7.0 Hz, 3-$H_2$), 2.54 (1H, d, *J* = 17.7 Hz, 9β-H), 2.84 (1H, br d, *J* = ca. 16 Hz, 6α-H), 2.90 (1H, qdd, *J* = 2.1, 5.8, 15.9 Hz, 6β-H), 3.65 (1H, brs, 8-H). $^{13}C$ NMR ($CDCl_3$) δc given in Table 1. EI–MS *m*/*z* (%): 264 ($M^+$, 5), 246 ($M^+ - H_2O$, 20), 43 (100).

## Bioassay

### Protective effect on D-GalN/LPS-induced liver injury in mice

The method described by Tiegs et al.[34] was modified and used for this experiment. Briefly, male ddY mice weighing about 25–27 g were fasted for 20 h before the experiment. The D-GalN (350 mg/kg) and LPS (10 μg/kg) dissolved in saline were injected intraperitoneally to produce liver injury. Test sample was given orally 1 h before D-GalN/LPS injection. Blood samples were collected 10 h after D-GalN/LPS injection. Serum GOT and GPT were determined by the Reitman-Frankel method (commercial kit, S.TA-Test Wako, Wako Pure Chemical Industries Co. Ltd, Osaka).

### Protective effect on cytotoxicity induced by D-GalN in primary cultured rat hepatocytes

The hepatoprotective effects of these constituents were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using primary cultured rat hepatocytes.[35] Hepatocytes were isolated from male Wistar rats (130–160 g) by collagenase perfusion method.[36] The cell suspension at $4\times10^4$ cells in 100 μL Williams' E medium containing calf serum (10%), penicillin (100 units/mL), streptomycin (100 μg/mL), insulin (1 μM), and dexamethasone (1 μM) was inoculated in a 96-well tissue culture plate, and precultured for 4 h at 37 °C under a 5% $CO_2$ atmosphere. The medium was exchanged with a fresh medium containing D-GalN (1 mM) and a test sample, and the hepatocytes were cultured for 44 h. The medium was exchanged with 100 μL of the fresh medium, and 10 μL of MTT (5 mg/mL in phosphate buffered saline) solution was added to the medium. After 4 h culture, the medium was removed, 100 μL of isopropanol containing 0.04 N HCl was then added to dissolve the formazan produced in the cells. The optical density (OD) of the formazan solution was measured by microplate reader at 570 nm (reference: 655 nm). Inhibition (%) were obtained by next formula.

$$\text{Inhibition}(\%) = \left[\left(\text{OD}_{(\text{sample})} - \text{OD}_{(\text{control})}\right)/\left(\text{OD}_{(\text{normal})} - \text{OD}_{(\text{control})}\right)\right] \times 100$$

Cytotoxic effects of the constituents were assessed by MTT colorimetric assay. Briefly, after 44 h incubation with a test sample in the absence of D-GalN, MTT assay was performed as described above.

## Statistics

Values are expressed as means ± SEM. Statistical significance was assessed by one-way analysis of variance following Dunnett's multiple comparison test. Probability ($p$) values less than 0.05 were considered significant.

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