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INHIBITORY EFFECT AND ACTION MECHANISM OF SESQUITERPENES FROM ZEDOARIAE RHIZOMA ON D-GALACTOSAMINE / LIPOPOLYSACCHARIDE-INDUCED LIVER INJURY

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Abstract : Hepatoprotective sesquiterpenes were isolated from the aqueous acetone extract of Zedoariae Rhizoma, the rhizome of *Curcuma zedoaria* ROSCOE (Zingiberaceae). Principal sesquiterpenes, furanodiene, germacrone, curdione, neocurdione, curcumenol, isocurcumenol, aerugidiol, zedoarondiol, and curcumenone and curcumin were found to show potent protective effect on D-galactosamine (D-GalN) / lipopolysaccharide (LPS)-induced acute liver injury in mice. Plausible action mechanisms for their hepatoprotective activity were clarified on the basis of the inhibitory effect on D-GalN-induced cytotoxicity in primary cultured rat hepatocytes, LPS-induced NO production in cultured mouse peritoneal macrophages, and D-GalN / tumor necrosis factor-α (TNF-α)-induced liver injury in mice.

Zedoariae Rhizoma, the rhizome of *Curcuma zedoaria* ROSCOE (Zigiberaceae), is mainly used as a stomachic in Japan, while, in Chinese traditional medicine, it has been prescribed for the treatment of syndrome caused by blood stagnation and for promoting menstruation in various preparations. During the course of our studies on the hepatoprotective constituents of natural medicines,[1] the aqueous acetone extract of Zedoariae Rhizoma was found to inhibit the D-galactosamine (D-GalN) / lipopolysaccharide (LPS)-induced acute liver injury (Fig. 1), which was known to be immunologically induced liver injury.[2] In this study, we described the isolation of hepatoprotective constituents from Zedoariae Rhizoma and their plausible action mechanisms for the hepatoprotective activity.

Isolation of Sesquiterpenes (1-13) and Curcumin (14) from Chinese Zedoariae Rhizoma

Chinese Zedoariae Rhizoma cultivated in Sichuan Province (purchased from Tochimoto Tenkaido, Osaka, 1996) was extracted with 80% aqueous acetone at room temperature. The aqueous acetone extract inhibited the increase of serum GOT (s-GOT) and serum GPT (s-GPT) induced by D-GalN / LPS in mice at a dose of 100 mg/kg (Fig. 1). The aqueous acetone extract was partitioned into an ethyl acetate-water mixture to furnish the ethyl acetate soluble portion and water phase. The water phase was further extracted with 1-butanol to give the 1-butanol-soluble portion. The ethyl acetate-soluble portion was subjected to ordinary-phase silica-gel, silver nitrate-treated ordinary-phase silica-gel, and reversed-phase silica-gel column chromatography and finally repeated HPLC to provide furanodiene (**1**, 0.0012% from the natural medicine), zederone (**2**, 0.0052%), germacrone (**3**, 0.0085%), 13-hydroxygermacrone (**4**, 0.0033%), curdione (**5**, 0.042%), dehydrocurdione (**6**, 0.0055%), neocurdione (**7**, 0.0009%), curcumenol (**8**, 0.057%), isocurcumenol (**9**, 0.0031%), isoprocurcumenol (**10**, 0.0009%), aerugidiol (**11**, 0.0045%), curcumenone (**13**, 0.041%), and curcumin (**14**, 0.0096%) together with thirteen minor known sesquiterpenes.[3] From the 1-butanol-soluble portion,

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zedoarondiol (**12**, 0.0024%) and four minor components[3] were isolated by similar separation methods as case of the ethyl acetate-soluble portion.[4]

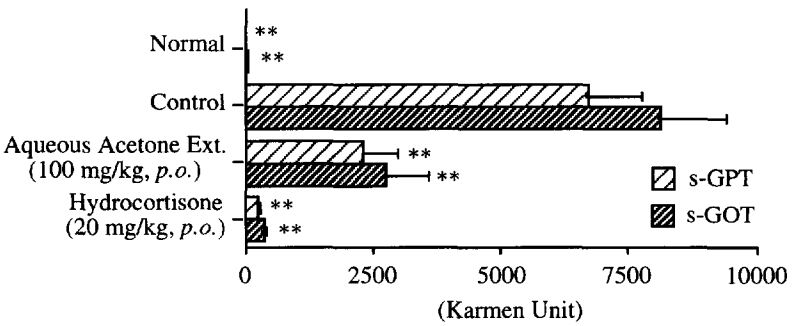
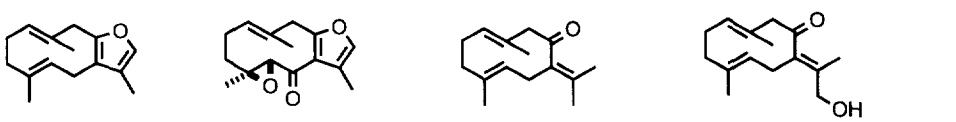
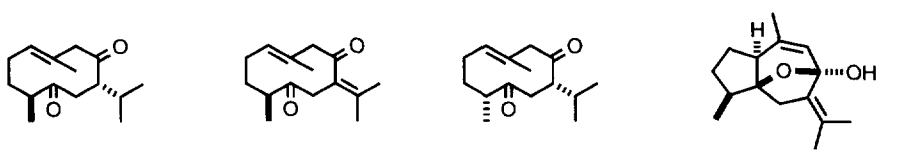


Fig.1 Inhibitory Effect of Aqueous Acetone Extract from Zedoariae Rhizoma on D-GalN / LPS-Induced Liver Injury in Mice

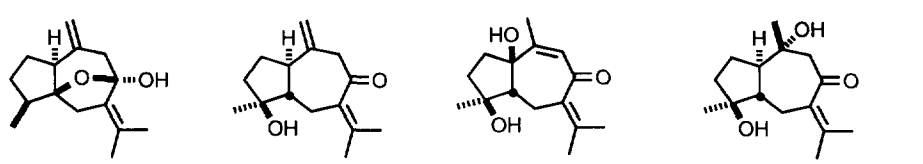
Male ddY mice weighing 25 - 27 g were used. After 20 h of fasting, D-GalN (350 mg/kg) and LPS (10 μg/kg) was injected *i.p.* to produce liver injury. Each test sample was given orally 1h before D-GalN / LPS injection. Blood samples were collected 10 h after D-GalN / LPS injection. Each column represents the mean with S.E. (n=8 - 12, $^{**}p<0.01$).



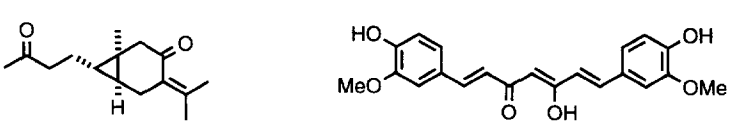
furanodiene (**1**) zederone (**2**) germacrone (**3**) 13-hydroxygermacrone (**4**)



curdione (**5**) dehydrocurdione (**6**) neocurdione (**7**) curcumenol (**8**)



isocurcumenol (**9**) isoprocurcumenol (**10**) aerugidiol (**11**) zedoarondiol (**12**)



curcumenone (**13**) curcumin (**14**)

Bioassay Methods

In Vivo Experiment :

The D-GalN / LPS (from *Salmonella enteritidis*)-induced liver damage and D-GalN / tumor necrosis factor-α (TNF-α)-induced liver damage described by Tiegs *et al.*[2] was modified and used for this experiment. Detail protocols were described in footnotes of Fig. 1 and 4. Liver samples were fixed in formalin, embedded in paraffin and stained with hematoxylin-eosin, and histological inspection was performed.

In Vitro Experiment :

Protective Effect on D-GalN-Induced Cytotoxicity : 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.[5] Hepatocytes were isolated from male Wistar rats (130 - 160g) by collagenase perfusion method. The cell suspension at $4x10^4$ cells in 100 μL William's E medium containing calf serum (10%), penicilline (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 medium, and 10 μL of MTT (5 mg/mL in PBS) solution was added to the medium. After 4 h culture, the medium was removed, 100 μL of isopropanol containing 0.04N HCl was then added to dissolve the formazan produced in the cells. The optical density (O.D.) of the formazan solution was measured by microplate reader at 570 nm (reference : 655 nm). Inhibition (%) were obatined by next formula.

$$\text{Inhibition (\%)} = [(\text{O.D.}_{(\text{sample})}-\text{O.D.}_{(\text{control})})/(\text{O.D.}_{(\text{normal})}-\text{O.D.}_{(\text{control})})] \times 100$$

NO Production from Macrophages Stimulated by LPS and Cytotoxicity : Cells were collected from the peritoneal cavities of mice by washing with 6-7 mL of PBS. The cells were washed with PBS and resuspended in RPMI 1640 medium containing 10% fetal calf serum (FCS). The total cell number was counted with a hemocytometer, and the peritoneal cells were inoculated into 96-well tissue culture plate (5×10^5 cells/100 μL in a well). After incubation for 1 h at 37℃ in 5% CO_2 atmosphere, nonadherent cells were removed from some of the cells by washing with PBS. And then the cells were incubated with RPMI 1640 medium (200 μL/well) containing 10% FCS, LPS and a test sample. The cytotoxicity of a test compound to the cell was determined by using the MTT colorimetric assay after 20 h incubation of cells in the medium containing 10 % FCS and a test compound. Nitrite which accumulated in the culture medium was measured spectrophotometrically using Griess reagent with sodium nitrite as a standard.[6]

Statistical Analysis :

Each value was expressed as the mean±S.E. and the statistical significance was assessed by one-way analysis of variance following Dunnett's test.

Results and Discussion

The hepatoprotective effects of the sesquiterpene constituents (**1**-**13**) and curcumin (**14**) from the aqueous acetone extract of Zedoariae Rhizoma were examined by monitoring the inhibitory activity on the increase of serum GPT and GOT induced by D-GalN / LPS in mice (Table 1). Nine sesquiterpenes, furanodiene (**1**),

germacrone (**3**), curdione (**5**), neocurdione (**7**), curcumenol (**8**), isocurcumenol (**9**), aerugidiol (**11**), zedoarondiol (**12**), and curcumenone (**13**) and a diarylheptanoid, curcumin (**14**), strongly inhibited the increase of s-GOT and s-GPT at a dose of 50 mg/kg. Dose-response inhibitory activity of several constituents (**1**, **3**, and **5**) was shown in Fig. 2 and they were also found to reduce the remarkable necrosis induced by D-GalN / LPS in histological examination. To shed light on the action mechanism, we examined the inhibitory effect of sesquiterpenes (**1**-**13**) and curcumin (**14**) on cytotoxicity induced by D-GalN in primary cultured rat hepatocytes. Germacrone (**3**), curdione (**5**), neocurdione (**7**), and curcumenol (**8**) exhibited potent inhibitory activity as shown in Table 1, while 13-hydroxygermacrone (**4**), aerugidiol (**11**), zedoarondiol (**12**), and curcumin (**14**) were found to strengthen the cytotoxicity by D-GalN. However, **4**, **11**, and **12** showed little cytotoxicity to the hepatocytes in the absence of D-GalN by using MTT assay (survival rate : > 93%), whereas curcumin (**14**) at 10^{-4}M showed the strong cytotocity (survival rate : 4%).[7] In *in vivo* experiment, **3**, **5**, **7**, and **8** (50 mg/kg, *p.o.*) also inhibited the D-GalN / TNF-α-induced liver injury in mice (Fig. 4). This evidence revealed that **3**, **5**, **7**, and **8** exhibited hepatoprotective effects by directly acting on liver. Next, we examined the effects of the sesquiterpenes (**1**-**13**) and curcumin (**14**) on the LPS-induced NO production as an index of the activation of macrophages. Among the sesquiterpenes with the hepatoprotective effect, furanodiene (**1**), germacrone (**3**), neocurdione (**7**), curcumenol (**8**), isocurcumenol (**9**), and zedoarondiol (**12**) at 10^{-4}M were found to inhibit the NO accumulation in the medium, and they showed little cytotoxicity at this concentration by using MTT assay (survival rate : > 85%). These findings led us to suggest the following action mechanisms for the hepatoprotective effect of sesquiterpenes (**1**, **3**, **5**, **7**, **8**, **9**, **12**) ; 1) inhibition against the activation of

Table 1. Inhibitory Effects of Constituents from Zedoariae Rhizoma on D-GalN / LPS-Induced Liver Injury in Mice, D-GalN-Induced Cytotoxicity in Primary Cultured Rat Hepatocytes, and LPS-Induced NO Production in Mouse Peritoneal Macrophages

Samples	Dose (mg/kg, *p.o.*)	D-GalN / LPS-Induced Liver Injury (*in vivo*)[a] s-GPT	s-GOT	Conc. (M)	D-GalN-Induced Hepatocytotoxicity (*in vitro*)[b]	LPS-Induced NO Production (*in vitro*)[b]
furanodiene (**1**)	50	72.9±6.7**	74.3±5.7**	10^{-4}	-0.5±0.2	96.1±1.7**
zederone (**2**)	50	39.5±12.1	35.4±14.1	10^{-4}	-7.9±0.1	-5.0±14.3
germacrone (**3**)	50	82.9±5.4**	78.1±6.8**	10^{-4}	59.8±6.3**	46.8±3.4*
13-hydroxy-germacrone (**4**)	50	36.1±14.1	21.5±19.0	10^{-4}	-21.7±3.0**	-14.6±4.6
curdione (**5**)	50	76.6±4.7**	74.6±4.7**	10^{-4}	77.1±5.8*	2.0±5.3
dehydrocurdione (**6**)	50	49.7±17.8	46.2±18.4	10^{-4}	-6.3±0.3	53.7±4.0**
neocurdione (**7**)	50	59.3±10.6**	58.4±11.1**	10^{-4}	44.6±5.3*	41.9±1.1**
curcumenol (**8**)	50	50.7±13.8*	53.3±13.4**	10^{-4}	25.1±5.3**	50.4±2.0**
isocurcumenol (**9**)	50	77.3±6.6**	80.2±5.5**	10^{-4}	14.2±5.0	36.5±5.6**
isoprocurcumenol (**10**)	50	26.7±17.2	22.3±14.7	10^{-4}	-5.2±0.4	54.5±0.4**
aerugidol (**11**)	50	88.0±2.0**	89.1±0.7**	10^{-4}	-41.5±8.0*	-8.8±3.1
zedoarondiol (**12**)	50	60.7±10.5*	54.7±12.7	10^{-4}	-35.6±7.9*	31.7±2.0**
curcumenone (**13**)	50	90.1±0.5**	88.0±0.4**	10^{-4}	-12.2±2.6	13.0±3.5
curcumin (**14**)	50	71.2±7.1*	63.4±9.1	10^{-4}	-44.3±0.3**	98.8±3.3**
hydrocortisone	20	99.0±0.1**	98.3±0.0**	10^{-7}	-	92.6±2.3**

Each value represents the mean inhibition (%) and S.E. [*p<0.05, **p<0.01, a) N=8-10, b) N=4]

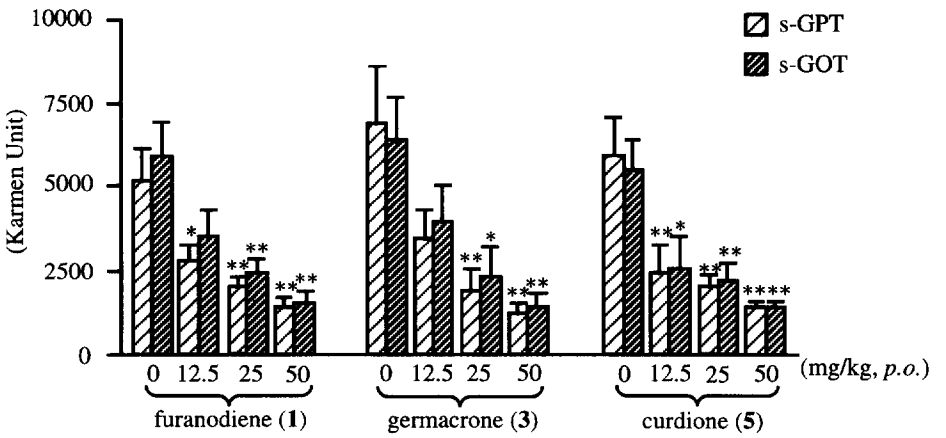


Fig. 2 Dose-Dependent Inhibitory Effects of Furanodiene (1), Germacrone (3), and Curdione (5) on Liver Injury Induced by D-GalN / LPS in Mice

Each value represents the mean with S.E. (*p<0.05, **p<0.01, N=10)

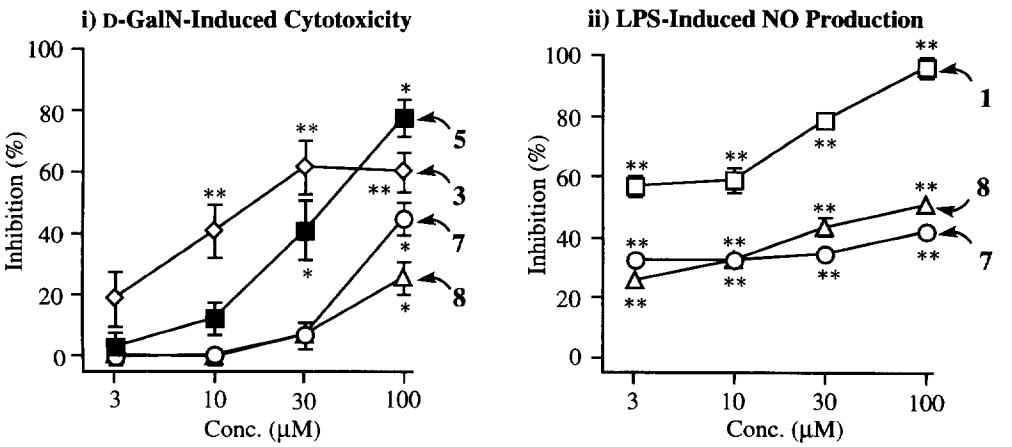


Fig.3 Concentration-Dependent Inhibitory Effects of Furanodiene (1), Germacrone (3), Curdione (5), Neocurdione (7), and Curcumenol (8) on D-GalN-Induced Cytotoxicity in Primary Cultured Rat Hepatocytes and LPS-Induced NO Production in Cultured Mouse Macrophages

Each value represents the mean with S.E. of 4 experiments (*p<0.05, **p<0.01)
□:furanodiene (1), ◇: germacrone (3), ■:curdione (5), ○:neocurdione (7), △:curcumenol (8)

macrophages by LPS [furanodiene (**1**), isocurcumenol (**9**), and zedoarondiol (**12**)] ; 2) protection of the hepatocytes against the toxicity of D-GalN [curdione (**5**)] ; 3) both effects as described above [germacrone (**3**), neocurdione (**7**), and curcumenol (**8**)]. Since overproduction of NO is well known to be a cause of inflammation, immunological responses and endotoxin-induced shocks,[8] these sesquiterpenes (**1**, **3**, **7**, **8**, **9**, and **12**) may be effective for inflammation and endotoxic shocks. On the other hand, curcumin (**14**) at 10^{-4} M also strongly inhibited the NO production, but it was deduced that this results was due to its cytotoxicity to the macrophages (survival rate : 7%). Aerugidiol (**11**) and curcumenone (**13**) lacked the inhibitory activity on NO

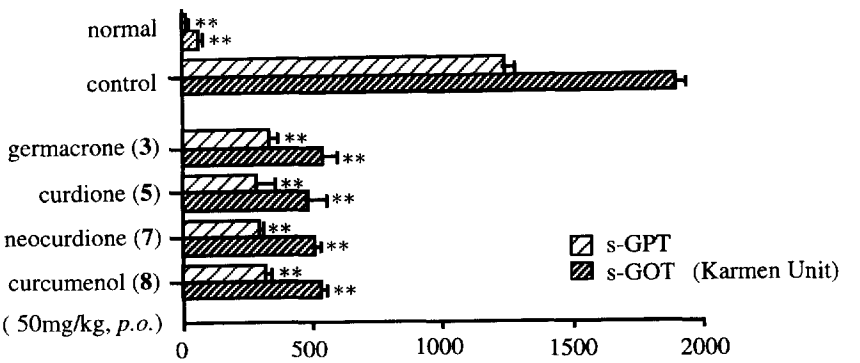


Fig.4 Inhibitory Effects of Germacrone (3), Curdione (5), Neocurdione (7), and Curcumenol (8) on Liver Injury Induced by D-GalN / TNF-α in Mice

Male ddY mice weighing 25 - 27 g were used. After 20 h of fasting, D-GalN (350 mg/kg, *i.p.*) was injected. One hour thereafter, TNF-α (10 μg/kg , *i.v.*) was given. Each test sample (50mg/kg, *p.o.*) was given orally 1 h before D-GalN injection. Blood samples were collected 10 h after D-GalN injection. Each column represents the mean with S.E. (n=7 - 10, $^{**}p<0.01$).

production and the protective activity against the cytotoxicity by D-GalN. Therefore, it was considered in case of **11**, **13**, and **14** that their metabolites might be effective for the D-GalN / LPS-induced liver injury or other mechanisms should be existent.

References and Notes

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3. From the ethyl acetate-soluble portion, (4*S*,5*S*)-(+)-germacrone 4,5-epoxide, isofuranodiene, glechomanolide, curzerenone, (+)-*ar*-trumerone, bisacumol, procurcumenol, alismoxide, 7α,11α-epoxy-5β-hydroxy-9-guaiaen-8-one, curcolone, β-eudesmol, β-dictyopterol, and curcumadione were isolated as the minor sesquiterpenes, while isozedoarondiol, zedoalactone B, bisacurone, and bis(4-hydroxycinnamoyl) methane were isolated from the 1-butanol soluble fraction.
4. a) Known compounds were identified by comparison of their physical data with those of authentic samples (**1**, **2**, **3**, **5**, **8**,**13**)[4b] or with reported values;[4c,4d] b) Shibuya, H.; Hamamoto, Y.; Cai, Y.; Kitagawa, I. *Chem. Pharm. Bull.* **1987**, *35*, 924; c) Harimaya, K.; Gao, J.F.; Ohkura, T.; Kawamata, T.; Iitaka, Y.; Guo, Y.T.; Inayama, S. *Chem. Pharm. Bull.* **1991**, *39*, 843; d) Takano, I.; Yasuda, I.; Takeya, K.; Itokawa, H. *Phytochemistry* **1995**, *40*, 1197 and references cited therein.
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7. Survival rate at 10^{-4}M : furanodiene (**1**) 73%, zederone (**2**) 7%, dehydrocurdione (**6**) 81%, curcumenone (**13**) 75%. Other constituents (**3**, **5**, **7**, **8**-**10**) at 10^{-4}M showed no hepatocytotoxicity.
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