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Protective effects of amide constituents from the fruit of *Piper chaba* on D-galactosamine/TNF-α-induced cell death in mouse hepatocytes

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Abstract—The methanolic extract from the fruit of *Piper chaba* (Piperaceae) was found to have a hepatoprotective effect on D-galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced liver injury in mice. From the ethyl acetate-soluble fraction, a new amide constituent named piperchabamide E together with twenty known amide constituents (e.g., piperine, piperchabamides A–D, and piperanine) and two aromatic constituents were isolated as the hepatoprotective constituents. With regard to structure–activity relationships, the amide moiety and the 1,9-decadiene structure between the benzene ring and amide moiety were suggested to be important for strong inhibition of D-GalN/tumor necrosis factor-α (TNF-α)-induced death of hepatocytes. Furthermore, a principal amide constituent, piperine, dose-dependently inhibited increase in serum GPT and GOT levels at doses of 2.5–10 mg/kg (p.o.) in D-GalN/LPS-treated mice, and this inhibitory effect was suggested to depend on the reduced sensitivity of hepatocytes to TNF-α.

The Piperaceae plant, *Piper chaba* HUNTER (syn. *P. retrofractum* VAHL.), is widely distributed in southeast Asia. The dried fruit of this plant, which is called 'Dee Plee' in Thailand, has been used as an anti-flatulent, stomachic, expectorant, antitussive, antifungal, uterus-contractile drug, sedative-hypnotic, appetizer, and counterirritant in the traditional medicine of Thailand.[1] In the course of our study of hepatoprotective constituents from medicinal plants,[2] the 80% aqueous acetone extract (12.5–50 mg/kg, p.o.) was found to have an inhibitory effect on the increase in serum GOT and GPT levels induced by D-galactosamine hydrochloride (D-GalN)/lipopolysaccharide (LPS)-induced liver injury in mice and on cell death induced by D-GalN/tumor necrosis factor-α (TNF-α) in primary cultured mouse hepatocytes (Tables 1 and 2).

Infection of hepatitis C virus and chronic consumption of alcohol are still major causes of liver injury, cirrhosis, and hepatocellular carcinoma in the world. TNF-α mediates a number of forms of organ injury through its induction of cellular apoptosis. In the liver, the biological effects of TNF-α have been implicated in hepatic injury induced by hepatic toxins, ischemia/reperfusion, viral hepatitis, and alcohol.[3] Therefore, TNF-α is considered as an important target in research to discover hepatoprotective agents.

D-GalN/LPS-induced liver injury is recognized to have two aspects. First, depletion of uridine triphosphate and an increase in sensitivity to TNF-α in hepatocytes induced by D-GalN. Second, the release of pro-inflammatory mediators, such as TNF-α, from LPS-activated macrophages (Kupffer's cells). Apoptosis of hepatocytes evoked by TNF-α is reported to be important roles in D-GalN/LPS-induced liver injury.[4] We, therefore, investigated protective constituents from *P. chaba* against D-GalN/TNF-α-induced cell death in primary-cultured mouse hepatocytes. In addition, several structural requirements and the mode of action of piperine (**5**) for the hepatoprotective effect are discussed.

Keywords: *Piper chaba*; Piperine; Piperchabamide E; Amide constituent; Hepatoprotection; TNF-α.
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Table 1. Inhibitory effects of the 80% aq acetone extract and piperine (**5**) on D-GalN/LPS-induced liver injury in mice

Treatment	Dose (mg/kg, p.o.)	*n*	s-GOT (Karmen Unit)	Inhibition (%)	s-GPT (Karmen Unit)	Inhibition (%)
Normal (vehicle)	—	7	86 ± 5**	—	28 ± 6**	—
Control (D-GalN/LPS)	—	9	5016 ± 560	—	6373 ± 928	—
The 80% aq acetone ext.	25	8	2598 ± 616**	49.0	3692 ± 1301	42.3
	50	8	1422 ± 437**	72.9	1448 ± 524**	77.6
	100	8	577 ± 35**	90.0	304 ± 12**	95.7
Normal (vehicle)		5	95 ± 5**	—	19 ± 1**	—
Control (D-GalN/LPS)	—	8	9126 ± 1477	—	9830 ± 1605	—
Piperine (**5**)	2.5	4	9098 ± 1195	0.3	7555 ± 796	23.2
	5	7	4545 ± 1259*	50.7	4817 ± 1489*	51.1
	10	8	450 ± 145**	96.1	575 ± 196**	94.3
Hydrocortisone	10	7	627 ± 262**	94.2	247 ± 123**	97.7

The method described by Tiegs et al.[17] was modified and used for the experiment. Male ddY mice weighing 25–30 g were used. After 20 h of fasting, a mixture of D-galactosamine hydrochloride (D-GalN) and LPS (from *Salmonella enteritidis*, Sigma) dissolved in saline was injected intraperitoneally at a dose of 350 mg/kg and 10 μg/kg to produce liver injury. Each test sample was given orally 1 h before the D-GalN/LPS was injected.[18] Blood samples were collected from the infraorbital venous plexus 10 h after the administration of D-GalN/LPS, and serum GOT and GPT levels were determined by the Reitman–Frankel method (S.TA-test Wako, Wako Pure Chemical Industries). Hydrocortisone was used as a reference compound. Each value represents mean ± SEM. Significantly different from each control, $^{*}p < 0.05$, $^{**}p < 0.01$.[19]

Table 2. Effects of the 80% aq acetone extract and constituents from the fruit of *P. chaba* on D-GalN/TNF-α-induced cell death in primary cultured mouse hepatocytes

Compound		Inhibition (%)				
	Conc. (μg/mL):	0	3	10	30	100
The 80% aq acetone extract		0 ± 1	22 ± 2**	57 ± 4**	66 ± 4**	105 ± 9**
		Inhibition (%)				
	Conc. (μM):	0	1	3	10	30
Piperonal (**1**)		0 ± 4	—	20 ± 1**	23 ± 5**	39 ± 2**
Methyl piperate (**2**)		0 ± 2	—	18 ± 3	29 ± 2*	21 ± 5
Piperchabamide A (**3**)		0 ± 4	—	29 ± 1**	32 ± 2**	70 ± 7**
Piperanine (**4**)		0 ± 2	—	31 ± 5*	50 ± 2**	53 ± 2**
Piperine (**5**)		0 ± 3	—	23 ± 5**	46 ± 4**	68 ± 3**
Piperoleine B (**6**)		0 ± 10	20 ± 3*	29 ± 3**	33 ± 1*	64 ± 7**
Pipernonaline (**7**)		0 ± 4	—	17 ± 6	31 ± 3*	48 ± 2**
Piperchabamide B (**8**)		0 ± 2	—	63 ± 6**	74 ± 3**	78 ± 5**
Piperundecalidine (**9**)		0 ± 2	27 ± 6*	40 ± 3**	49 ± 8**	58 ± 5**
Piperchabamide C (**10**)		0 ± 3	—	42 ± 5**	52 ± 1**	68 ± 5**
Dihydropiperlonguminine (**11**)		0 ± 6	34 ± 12	43 ± 9*	43 ± 5**	66 ± 6**
Piperlonguminine (**12**)		0 ± 6	27 ± 4	50 ± 6**	54 ± 6*	66 ± 3**
Piperchabamide E (**13**)		0 ± 2	—	34 ± 2**	71 ± 2**	98 ± 6**
Retrofractamide C (**14**)		0 ± 3	30 ± 13	51 ± 2**	31 ± 6**	37 ± 2**
Retrofractamide A (**15**)		0 ± 3	40 ± 4**	32 ± 5**	35 ± 5**	60 ± 5**
Piperchabamide D (**16**)		0 ± 1	—	57 ± 3**	77 ± 3**	37 ± 4**
Retrofractamide B (**17**)		0 ± 3	32 ± 5	54 ± 4**	51 ± 3**	26 ± 2**,[a]
Guineensine (**18**)		0 ± 2	—	26 ± 2**	33 ± 3**	12 ± 1**,[a]
Brachystamide B (**19**)		0 ± 3	—	22 ± 3**	11 ± 2*	−6 ± 2
N-Isobutyl-(2*E*,4*E*)-decadienamide (**20**)		0 ± 7	—	19 ± 3*	25 ± 3**	42 ± 3**
N-Isobutyl-(2*E*,4*E*)-dodecadienamide (**21**)		0 ± 3	12 ± 2	21 ± 3**	31 ± 6**	44 ± 4**
N-Isobutyl-(2*E*,4*E*)-octadecadienamide (**22**)		0 ± 5	15 ± 10	37 ± 2**	41 ± 1**	43 ± 5**
N-Isobutyl-(2*E*,4*E*,14*Z*)-eicosatrienamide (**23**)		0 ± 4	10 ± 5	3 ± 2	19 ± 12	27 ± 6

Hepatocytes were isolated from male ddY mice (35–38 g) by collagenase perfusion method. A suspension of 4×10^4 cells in 100 μL William's E medium containing fetal calf serum (FCS, 10%), penicillin (100 units/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well tissue culture plate, and pre-cultured for 4 h at 37 °C under a 5% CO_2 atmosphere. The medium was exchanged with fresh medium containing D-GalN (1 mM) and TNF-α (20 ng/mL) with or without a test sample. After incubation for 20 h, the viability of the cells was assessed by the MTT colorimetric assay.[20] Each value represents mean ± SEM ($n = 4$). Significantly different from each control, $^{*}p < 0.05$, $^{**}p < 0.01$.[19]

In our previous study,[5] we isolated four new amides [piperchabamides A (**3**, 0.0029%), B (**8**, 0.0041%), C (**10**, 0.0032%), and D (**16**, 0.0037%)] together with nine known amides [piperanine (**4**, 0.42%), piperine (**5**, 2.84%), pipernonaline (**7**, 0.47%), piperlonguminine (**12**, 0.22%), retrofractamide B (**17**, 0.049%),guineensine (**18**, 0.081%), *N*-isobutyl-(2*E*,4*E*)-octadecadienamide (**22**, 0.14%), *N*-isobutyl-(2*E*,4*E*,14*Z*)-eicosatrienamide (**23**, 0.18%), and dehydropipernonaline (0.078%)] and a known aromatic constituent [methyl piperate (**2**, 0.11%)] from the fruit of *P. chaba*. In the present study, we additionally isolated a new amide constituent named

piperchabamide E (**13**, 0.0083%)[6] together with eight known amide constituents [piperoleine B (**6**, 0.0082%),[7] piperundecalidine (**9**, 0.017%),[8] dihydropiperlonguminine (**11**, 0.015%),[9] retrofractamides A (**15**, 0.0075%)[10] and C (**14**, 0.036%),[10] brachystamide B (**19**, 0.0083%),[11] *N*-isobutyl-(2*E*,4*E*)-decadienamide (**20**, 0.018%),[12] *N*-isobutyl-(2*E*,4*E*)-dodecadienamide (**21**, 0.0034%)[13] and an aromatic constituent [piperonal (**1**, 0.0047%)[14]] (Fig. 1).[15] Most of them, except for **2**, **19**, and **23**, significantly inhibited the cell death induced by D-GalN/TNF-α in the hepatocytes at 1–30 μM. Notably, compounds **8**, **12**, **14**, **16**, and **17** showed strong effects at 3 μM with more than 50% inhibition. However, several amide constituents such as **14**, **16**, and **17** exhibited no concentration-dependent inhibition at 3–30 μM, suggesting that these showed cytotoxic effects at higher concentrations. With regard to the structural requirement of the amide constituents for the activity,

Table 3. Inhibitory effects of piperine (**5**) on increase in serum TNF-α levels induced by D-GalN/LPS in mice

Treatment	Dose (mg/kg, p.o.)	*n*	TNF-α (pg/mL)	Inhibition (%)
Normal (vehicle)	—	4	4 ± 3**	—
Control (D-GalN/LPS)	—	6	233 ± 46	—
Piperine (**5**)	5	4	197 ± 52	15.7
	10	4	212 ± 27	9.2
	20	4	238 ± 71	−2.2
Hydrocortisone	10	4	22 ± 2**	92.1

After 20 h of fasting, a mixture of D-GalN and LPS dissolved in saline was intraperitoneally injected into male ddY mice (25–30 g, body weight) at a dose of 350 mg/kg and 10 μg/kg. Each test sample was given orally 1 h before the D-GalN/LPS was injected. Blood samples were collected from the infraorbital venous plexus 1.5 h after the administration of D-GalN/LPS, and the serum TNF-α was determined using an ELISA kit (GE Healthcare Sciences).[21] Hydrocortisone was used as a reference compound. Each value represents mean ± SEM. Significantly different from the control, $^{**}p < 0.01$.[19]

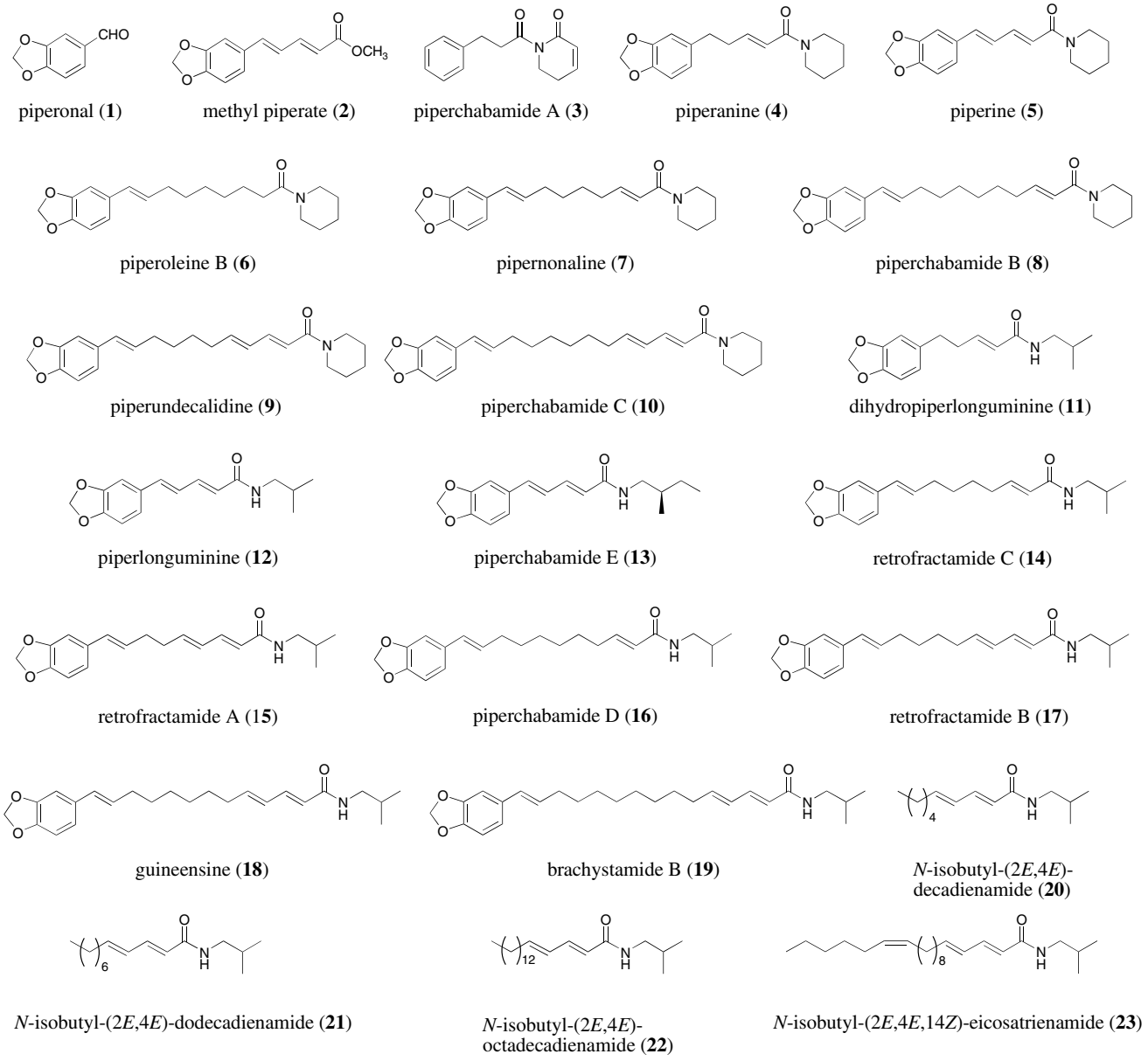

Figure 1. Chemical structures of isolated constituents from the fruit of *Piper chaba*.

Table 4. Inhibitory effects of piperine (**5**) on D-GalN/TNF-α and D-GalN-induced cell death in primary cultured mouse hepatocytes and TNF-α-induced cell death in L929 cells

Treatment	Conc. (μM)	Cell viability (%)		
		D-GalN/TNF-α (hepatocytes)	D-GalN (hepatocytes)	TNF-α (L929 cells)
Normal (vehicle)		100.0 ± 2.2**	100.0 ± 3.1**	100.0 ± 2.3**
Control	—	51.2 ± 2.2	35.1 ± 2.4	56.5 ± 1.0
Piperine (**5**)	10	72.4 ± 1.5**	40.6 ± 3.1	64.0 ± 2.3*
	30	76.1 ± 3.0**	36.5 ± 2.1	66.2 ± 0.3**
	100	84.3 ± 5.1**	29.2 ± 1.7	92.2 ± 1.0**

D-*GalN/TNF-α-induced cell death in the hepatocytes:* The experimental protocol is described in the footnote of Table 2.

D-*GalN-induced cell death in the hepatocytes:* A suspension of 4×10^4 cells in 100 μL of William's E medium containing fetal calf serum (FCS, 10%), penicillin (100 units/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well tissue culture plate, and pre-cultured for 4 h at 37 °C under a 5% CO_2 atmosphere. The medium was exchanged with fresh medium containing D-GalN (1 mM) with or without the test sample. After incubation for 44 h, the viability of the cells was assessed by the MTT colorimetric assay.[20]

TNF-α-induced cell death in L929 cells: L929 cells, a TNF-α-sensitive cell line, were obtained from Dainippon Pharmaceutical (Osaka, Japan). A suspension of 3×10^4 cells in 100 μL of minimum essential medium Eagle supplemented with 1% non-essential amino acid solution (Invitrogen), FCS (10%), penicillin (100 units/mL), and streptomycin (100 μg/mL) was inoculated in a 96-well tissue culture plate. After 20 h of incubation in the medium containing actinomycin D (0.5 μg/mL) and TNF-α (20 pg/mL) with or without the test sample, the viability of the cells was assessed by the MTT colorimetric assay.[20]

Each value represents mean ± SEM ($n = 4$). Significantly different from each control, $^*p < 0.05$, $^{**}p < 0.01$.[19]

the amide structure was important [**2** (inhibition: 21% at 30 μM) <**5** (68%)], and compounds **8** and **16** which have the 1,9-decadiene structure between the benzene ring and amide moiety tended to show stronger activity (Table 2).

Next, the effect of the principal constituent, piperine (**5**), on D-GalN/LPS-induced liver injury in mice was examined. As shown in Table 1, **5** at doses of 2.5–10 mg/kg (p.o.) significantly inhibited the increase in serum GOT and GPT levels in mice. Its efficacy was near to that of hydrocortisone.

To clarify the mode of action of piperine (**5**), the effect of **5** on the increase in serum TNF-α levels induced by D-GalN/LPS in mice was examined. Furthermore, effects of **5** on D-GalN-induced cell death in the hepatocytes and TNF-α-induced cell death in L929 cells, a TNF-α-sensitive cell line,[16] were examined using the MTT assay. As shown in Tables 3 and 4, **5** at 5–20 mg/kg (p.o.) did not inhibit the increase in serum TNF-α levels apparently different from hydrocortisone. Although the compound inhibited the death of hepatocytes induced by D-GalN/TNF-α, it could not protect the cells against D-GalN. On the other hand, **5** at 10–100 μM significantly inhibited the death of L929 cells caused by TNF-α, indicating a decrease in the sensitivity of L929 cells to TNF-α. These findings suggest that the reduction in the sensitivity of hepatocytes to TNF-α is involved in the protective effect of **5**. Many compounds which inhibit cell death induced by D-GalN or production of TNF-α have been reported,[2d,j,k] but there are few reports about compounds which selectively reduce the sensitivity of hepatocytes to TNF-α.

In conclusion, from the fruit of *P. chaba*, a new amide constituent named piperchabamide E (**13**) together with twenty known amide constituents and two aromatic constituents were isolated as hepatoprotective constituents. With regard to structure–activity relationships, the amide moiety and the 1,9-decadiene structure between the benzene ring and amide moiety were suggested to be important for strong inhibition of the D-GalN/TNF-α-induced death of hepatocytes. Furthermore, a principal amide constituent, piperine (**5**), dose-dependently inhibited the increase in serum GPT and GOT at doses of 2.5–10 mg/kg (p.o.) in D-GalN/LPS-treated mice, and this inhibitory effect was suggested to depend on the reduced sensitivity of hepatocytes to TNF-α.

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6. Piperchabamide E (**13**): colorless oil. $[\alpha]_D^{24}$ +18.7 (*c* 0.72, $CHCl_3$). High resolution EI-MS: calcd for $C_{17}H_{21}NO_3$ (M^+), 287.1521. Found: 287.1523. UV [EtOH, nm (log ε)]: 241 (4.2), 252 (4.1), 308 (4.2), 338 (4.3). IR (film, cm^{-1}): 2963, 1646, 1545, 1506, 1489, 1446, 1257, 1238, 1039, 991, 931. ^1H NMR (500 MHz, $CDCl_3$): δ 0.91 (3H, dd, J = 6.9, 7.6 Hz, 4′-H), 0.91 (3H, d, J = 6.7 Hz, 5′-H), 1.17, 1.41 (1H each, both m, 3′-H_2), 1.59 (1H, m, 2′-H), 3.15, 3.29 (1H each, both m, 1′-H_2), 5.66 (1H, br s, N-*H*), 5.93 (1H, d, J = 15.0 Hz, 2-H), 5.97 (2H, s, 12-H_2), 6.65 (1H, dd, J = 10.7, 17.7 Hz, 4-H), 6.75 (1H, d, J = 8.2 Hz, 10-H), 6.78 (1H, d, J = 17.7 Hz, 5-H), 6.88 (1H, dd, J = 1.8, 8.2 Hz, 11-H), 6.96 (1H, dd, J = 1.8 Hz, 7-H), 7.35 (1H, dd, J = 10.7, 15.0 Hz, 3-H). ^{13}C NMR (125 MHz, $CDCl_3$): δ c 11.3 (4′-C), 17.2 (5′-C), 27.1 (3′-C), 35.1 (2′-C), 45.3 (1′-C), 101.3 (12-C), 105.7 (7-C), 108.5 (10-C), 122.6 (11-C), 123.3 (2-C), 124.7 (4-C), 130.9 (6-C), 138.8 (5-C), 140.9 (3-C), 148.2 (8-C), 148.2 (9-C), 166.2 (1-C). EI-MS *m/z* (%): 287 (M^+, 48), 201 (100). Acid hydrolysis of **13** with 6 M HCl gave (+)-2-methylbutylamine which was identified with a commercially obtained compound using HPLC with an optical rotation detector. The detailed structural determination of **13** will be presented in a full paper.
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14. This compound was identified by comparison of its physical data (UV, IR, ^1H NMR, ^{13}C NMR, MS) with a commercially obtained sample.
15. The 80% (v/v) aqueous acetone extract and isolation and identification of compounds **2**–**8**, **10**, **11**, **16**–**18**, **22**, and **23** were described in our previous study.[5] The EtOAc-soluble fraction was subjected to silica gel column chromatography [*n*-hexane/EtOAc (15:1–10:1–5:1–2–1–1:1, v/v)-MeOH] to give nine fraction [Fr. 1 (7.3 g), 2 (4.0 g), 3 (3.1 g), 4 (22.0 g), 5 (3.8 g), 6 (1.8 g), 7 (11.1 g), 8 (44.2 g), and 9 (20.7 g)]. Each fraction was separated by ODS column chromatography (MeOH/H_2O) and HPLC (CH_3CN/H_2O or MeOH/H_2O) to give **1** and **2** from Fr. 2, **3** and **21**–**23** from Fr. 4, **20** from Fr. 4 and 5, **19** from Fr. 5, **16** and **18** from Fr. 5 and 6, **17** from Fr. 5–7, **10** from Fr. 6, **6**, **8**, **14**, and **15** from Fr. 6 and 7, **9**, **11**, and **13** from Fr. 7, **5** and **7** from Fr. 7 and 8, **12** from Fr. 7–9, and **4** from Fr. 8. Details of the isolation procedure will be presented in a full paper.
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18. Test samples were suspended with a 5% arabic gum solution, and the solution was administered orally at 10 mL/kg in each experiment, while the vehicle was given orally at 10 mL/kg in the corresponding control group.
19. Values were expressed as means ± SEM. One-way analysis of variance followed by Dunnett's test for multiple comparison was used for the statistical analysis. Probability (p) values less than 0.05 were considered significant.
20. MTT colorimetric assay: The medium was exchanged with 100 μL of fresh medium, and 10 μL of 3-(4,5-dimethyl-2-thiazolyl) 2,5-diphenyl tetrazolium bromide [MTT, 5 mg/mL in phosphate-buffered saline (PBS)] solution was added. After 4 h of culture, the medium was removed, and 100 μL of isopropanol containing 0.04 M 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.
21. In a pre-examination, blood samples were collected several hours after the injection of D-GalN/LPS injection, and serum TNF-α levels were determined using a commercial kit (GE Healthcare Sciences). The serum TNF-α level was maximal at 90 min after the injection, as reported previously.[2k] Therefore, serum TNF-α levels at 90 min after the injection were compared.