Apoptosis-Inducing Effects of Sterols from the Dried Powder of Cultured Mycelium of *Cordyceps sinensis*

Hisashi Matsuda,[a] Junji Akaki,[b] Seikou Nakamura,[a] Yoshie Okazaki,[a] Hiroaki Kojima,[b] Makoto Tamesada,[b] and Masayuki Yoshikawa*,[a]

[a] *Kyoto Pharmaceutical University; Misasagi, Yamashina-ku, Kyoto 607–8412, Japan: and* [b] *R&D Company, Kobayashi Pharmaceutical Co., Ltd.; 1–30–3 Toyokawa, Ibaraki, Osaka 567–0057, Japan.*

Received December 2, 2008; accepted January 6, 2009; published online January 23, 2009

The methanolic extract and its ethyl acetate-soluble fraction from the dried powder of cultured *Cordyceps sinensis* mycelium showed cytotoxic effects on promyelocytic leukemia HL-60 cells. Sitosterol (1), 5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3β-ol (2), 5α,8α-epidioxy-22*E*-ergosta-6,9(11),22-trien-3β-ol (3), 5α,6α-epoxy-5α-ergosta-7,22-dien-3β-ol (4), and ergosterol (5) were isolated from the ethyl acetate fraction. Among the isolated compounds, 2—4 with a peroxide ring or an epoxide ring showed substantial cytotoxic activity with IC_{50} values of 7.3—7.8 μg/ml, but 1 and 5 showed only moderate activity. Furthermore, apoptosis of HL-60 cells was observed 24 h after treatment with 2—4 (10, 20 μg/ml) using the TdT-mediated dUTP nick-end labeling method, and their apoptosis-inducing activities are suggested to be dependent on the activation of caspases-3/7. To the best of our knowledge, this is the first report of the isolation of sterol constituents (1—5) from cultured *C. sinensis* mycelium.

Key words *Cordyceps sinensis*; cultured mycelium; sterol constituent; apoptosis; caspase-3

Cordyceps (Chinese name 冬虫夏草), mainly distributed in Qinghai, Xizang, and Sichuan provinces of China, has been used as a popular and effective antiaging and tonic folk medicine and to treat various human diseases such as hepatitis, cancer, *etc.* Various biological activities of *Cordyceps* including antitumor effects were reported.[1—3] Recently, *Cordyceps sinensis* has been cultured to fulfill medicinal needs, since *Cordyceps* is very limited in nature.[4,5] Among the chemical constituents of cultured *C. sinensis* mycelium, cordycepin (**6**), dioxopiperazines, polysaccharides, and peptides were reported.[5—7] However, sterol constituents such as 5α,6α-epoxy-5α-ergosta-7,22-dien-3β-ol (**4**) and ergosterol (**5**), which were reported to be contained in *Cordyceps*,[8,9] have not been isolated from the mycelium so far.

We previously reported the inhibitory effects of the hot-water extract of *C. sinensis* mycelium on the metastasis of B16 melanoma cells in mice.[10] As a continuing study, the methanolic extract and its ethyl acetate-soluble fraction from the dried powder of cultured *C. sinensis* mycelium showed cytotoxic effects on promyelocytic leukemia HL-60 cells. Previously, several studies on the cytotoxic and apoptosis-inducing effects of *Cordyceps* were reported.[1—3] However, the active constituents of cultured *C. sinensis* mycelium have not been investigated sufficiently. In this paper, the cytotoxic effects of the methanolic (MeOH) extract and several sterol constituents (**1**—**5**) from the dried powder of cultured *C. sinensis* mycelium on HL-60 cells are described.

Results and Discussion

To investigate the cytotoxic effects of the MeOH extract and its active constituents from the dried powder of cultured *C. sinensis* mycelium, the viabilities of test sample-treated HL-60 cells were evaluated in the WST-8 assay. The MeOH extract (yield: 18.9% from the dried power) exhibited significant cytotoxic effects (IC_{50} value=199 μg/ml). The MeOH extract was then partitioned into an ethyl acetate (EtOAc)–water (H_2O) mixture. The aqueous phase was then extracted with 1-butanol (1-BuOH), and the EtOAc- (3.8%), 1-BuOH- (5.2%), and H_2O- (9.9%) soluble fractions were obtained. The EtOAc- and 1-BuOH-soluble fractions showed cytotoxic effects (IC_{50} values of 120 and 199 μg/ml, respectively), and the H_2O-soluble fraction showed less potent effects (IC_{50} value >200 μg/ml).

Through bioassay-guided isolation,[11] sitosterol (**1**, 0.00025%), 5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3β-ol (**2**, 0.0028%),[12] 5α,8α-epidioxy-22*E*-ergosta-6,9(11),22-trien-3β-ol (**3**, 0.0045%),[12] 5α,6α-epoxy-5α-ergosta-7,22-dien-3β-ol (**4**, 0.00012%),[9] and ergosterol (**5**, 0.00024%) were obtained from the EtOAc-soluble fraction by ordinary- and reversed-phase silica gel column chromatography, preparative TLC, and HPLC (Fig. 1). To best of our knowledge, this is the first report of the isolation of sterol constituents (**1**—**5**) from cultured *C. sinensis* mycelium, not from the insect body.

Next, the cytotoxic effects of the isolated sterol constituents **1**—**5** on HL-60 cells were examined and compared with those of cordycepin (**6**), which is a well-known antitumor constituent of *Cordyceps*.[13,14] Consistent with previous reports, cordycepin (**6**) exhibited significant cytotoxic effects against HL-60 cells. Sterol constituents **2**—**4** also exhibited significant cytotoxic effects (IC_{50} values=7.3—7.8 μg/ml), but sitosterol (**1**) and ergosterol (**5**) showed less potent effects than **2**—**4** (Table 1).

Apoptosis plays an important role in the maintenance of tissue homeostasis by the selective elimination of excess cells. On the other hand, the induction of apoptosis of carcinoma cells is also recognized to be useful in cancer treatment, since cytotoxic drugs (*e.g.*, etoposide, cisplatin, and paclitaxel) used in chemotherapy for leukemia and solid tumors are known to cause apoptosis in target cells.[15—18] Therefore, the apoptosis-inducing effects of the extract, fractions, and isolated constituents in HL-60 cells were examined using the TdT-mediated dUTP nick-end labeling (TUNEL) assay (Apoptosis Screening Kit *Wako*, Wako Pure Chemical

* To whom correspondence should be addressed. e-mail: myoshika@mb.kyoto-phu.ac.jp

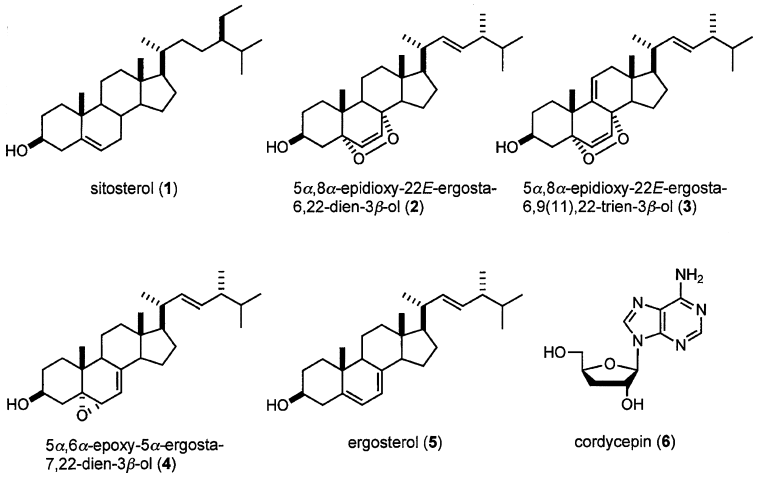

Fig. 1. Chemical Structures of Sterol Constituents **1**—**5** from the Dried Powder of Cultured Mycelium of *C. sinensis* and Cordycepin (**6**)

Table 1. Cytotoxic Effects of Sterol Constituents **1**—**5** and Cordycepin (**6**) on HL-60 Cells

Test sample	Conc. (μg/ml)	Cell viability (% of control)	IC_{50} (μg/ml)
Sitosterol (**1**)	0.0	100.0±9.3	26.7
	10.0	98.6±3.7	
	20.0	77.7±4.3**	
	40.0	17.3±1.6**	
	80.0	5.7±4.8**	
2	0.0	100.0±2.1	7.8
	1.9	99.7±1.8	
	2.5	88.8±4.8**	
	5.0	71.0±0.5**	
	7.5	53.2±4.7**	
	10.0	44.6±2.3**	
3	0.0	100.0±4.9	7.5
	1.9	90.2±1.6*	
	2.5	80.9±6.1**	
	3.8	78.1±1.7**	
	5.0	70.1±3.5**	
	7.5	49.8±1.9**	
	10.0	37.3±3.1**	
4	0.0	100.0±2.9	7.3
	1.9	94.7±4.0	
	2.5	98.4±2.0	
	3.8	93.1±2.1*	
	5.0	93.1±0.8*	
	7.5	38.1±3.9**	
	10.0	22.2±2.4**	
Ergosterol (**5**)	0.0	100.0±10.6	23.3
	10.0	107.7±11.6	
	15.0	99.6±6.8	
	30.0	17.3±12.4**	
	40.0	3.5±1.4**	
Cordycepin (**6**)	0.0	100.0±16.5	3.8
	1.8	92.6±8.0	
	2.5	64.5±5.2**	
	3.5	56.8±3.8**	
	7.0	36.9±6.9**	
	10.0	30.9±9.6**	

After the incubation of HL-60 cells (1×10^4 cells/100 μl/well) with test compounds in RPMI 1640 medium supplemented with 10% FBS in 96-well microplates for 48 h, 10 μl of WST-8 solution was added to each well. After a further 1 h of culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader at 450 nm. Values represent the means±S.D. ($n=3$ or 6). Significantly different from the control group, * $p<0.05$, ** $p<0.01$.

Industries, Osaka, Japan).

Previously, the cytotoxic effects of **2**—**4** against several tumor cell lines were also reported,[9,12,19—21] but their apoptosis-inducing activities have not been studied sufficiently. As shown in Fig. 2A, the absorbance at 492 nm was increased 24 h after coculture with **2**—**4** and cordycepin (**6**), similar to that seen after coculture with actinomycin D, but sitosterol (**1**) and ergosterol (**5**) did not show such effects. Among the active sterols **2**—**4**, **3** showed the most potent activity. These results indicate that DNA fragmentation, which is characteristic of apoptotic cells, occurred after coculture with **2**—**4** and **6**.

Caspases play a central role in the apoptotic signaling pathway and contribute to the overall apoptotic morphology by cleavage of various cellular substances. Caspase-8 is the initial caspase activated in response to receptors with a death domain that interacts with the fas-associated death domain. The mitochondrial stress pathway begins with the release of cytochrome *c* from mitochondria, which then interacts with Apaf-1, causing self-cleavage and activation of caspase-9. The effector caspase-3 is downstream from the activator caspases and cleaves various cellular targets.[22,23] On the other hand, a caspase-independent cell death program has been reported recently.[24] Therefore, to confirm that caspases are involved in the apoptosis-inducing activities of **2**—**4**, we examined the enzyme activity of caspases-3/7 in HL-60 cells after coculture with **2**—**4** using a commercial kit (Caspase-Glo 3/7 Assay, Promega KK, Madison, U.S.A.). As shown in Fig. 2B, the intensity of luminescence was markedly increased after coculture with **3**, and compounds **2** and **4** also showed greater activity than **6** at the concentration of 20 μg/ml. Compounds **1** and **5** showed less potent activity. Recently, the apoptosis-inducing effects of **1** through the activation of caspase-3 and alteration of the Bax/Bcl-2 ratio in leukemic U937 cells have been reported.[25] However, the apoptosis-inducing activity and increase in caspase-3/7 activity after coculture with **1** were very weak under our experimental conditions. These findings suggest that the apoptotic cell death of HL-60 cells induced by **2**—**4** is largely dependent on the activation of caspases-3/7, possibly caspase-3, although the effects of **1**—**6** on DNA fragmentation and caspase 3/7 activity do not completely accord with their cytotoxic effects. The

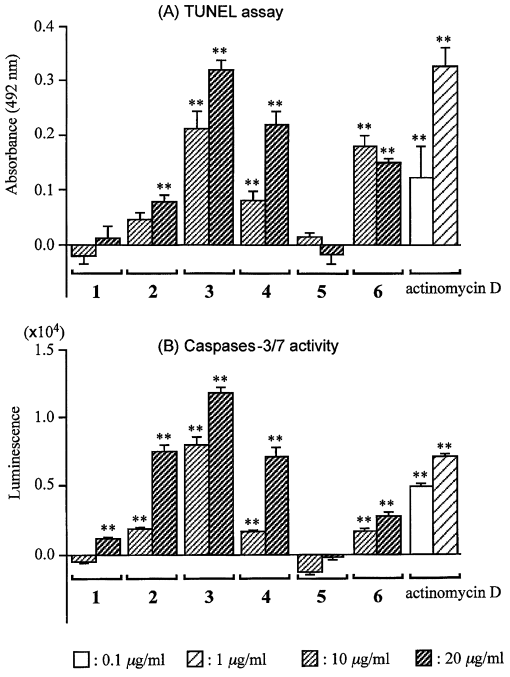

Fig. 2. Apoptosis-Inducing Effects of **1**—**6** in HL-60 Cells

HL-60 cells (1×10^4 or 2×10^4 cells/well) in 10% FBS-RPMI-1640 medium containing test samples were incubated for 24 h. (A) The apoptosis-inducing effect of the test sample was examined in the TUNEL assay. Each value of absorbance represents the difference from the control. (B) The activity of caspases-3/7 was examined using a commercial kit. Each bar represents the mean with S.D. ($n=3$ or 6). Significantly different from the control group, ** $p<0.01$.

mechanism of these active constituents should be studied further.

In conclusion, the methanolic extract and its EtOAc-soluble fraction from the dried powder of cultured *C. sinensis* mycelium showed cytotoxic effects on HL-60 cells. 5α,8α-Epidioxy-22*E*-ergosta-6,22-dien-3β-ol (**2**), 5α,8α-epidioxy-22*E*-ergosta-6,9(11),22-trien-3β-ol (**3**), and 5α,6α-epoxy-5α-ergosta-7,22-dien-3β-ol (**4**) were isolated from the EtOAc-soluble fraction and they exhibited substantial cytotoxic and apoptosis-inducing activities. The apoptosis-inducing activities of **2**—**4** are suggested to be dependent on the activation of caspases-3/7.

Experimental

General Experimental Procedures The following instruments were used to obtain physical data: specific rotations, Horiba SEPA-300 digital polarimeter ($l=5$ cm); EI-MS, JEOL JMS-GCMATE mass spectrometer; ^{1}H-NMR spectra, JNM-LA500 (500 MHz) and JNM-ECA600 (600 MHz) spectrometers; ^{13}C-NMR spectra, JNM-LA500 (125 MHz) and JNM-ECL600 (150 MHz) spectrometers with tetramethylsilane as an internal standard; and HPLC detector, Shimadzu RID-6A refractive index detector. HPLC and YMC Pack ODS-A (250×4.6 mm i.d. and 250×20 mm i.d., respectively) columns were used for analytical and preparative purposes, respectively.

The following experimental conditions were used for chromatography: ordinary-phase silica gel column chromatography, Silica gel BW-200 (Fuji Silysia Chemical, Ltd., Aichi, Japan, 150—350 mesh); reversed-phase silica gel column chromatography, Chromatorex ODS DM1020T (Fuji Silysia Chemical, Ltd., 100—200 mesh); preparative TLC and TLC (ordinary phase), precoated TLC plates with Silica gel 60F_{254} (Merck, 2.0 mm for preparative TLC, 0.25 mm for TLC); TLC (reversed phase), precoated TLC plates with Silica gel RP-18 F_{254S} (Merck, 0.25 mm); reversed-phase HPTLC, precoated TLC plates with Silica gel RP-18 WF_{254S} (Merck, 0.25 mm); and detection was achieved by spraying with 1% $Ce(SO_4)_2$–10% aqueous H_2SO_4 followed by heating.

Material The dried powder of cultured *C. sinensis* mycelium, which was powdered with a warm current of dry air after removal of the culture medium by centrifugal separation, was obtained from Shandong Handong Lukang Pharmaceutical Co., Ltd. (Shandong, China) (lot no. 0412012).

Extraction and Isolation The dried powder of cultured mycelium of *C. sinensis* (5.0 kg) was extracted three times with MeOH under reflux for 3 h. Evaporation of the solvent under reduced pressure gave the methanolic extract (944 g, 18.9%, antiproliferation against HL-60: $IC_{50}=199$ μg/ml). A portion (400 g) of the MeOH extract was partitioned into an EtOAc–H_2O (1 : 1, v/v) mixture, and the aqueous layer was successively extracted with 1-BuOH. Removal of the solvents *in vacuo* yielded an EtOAc-soluble fraction (77.3 g, 3.8%, $IC_{50}=120$ μg/ml), 1-BuOH-slouble fraction (112.4 g, 5.2%, $IC_{50}=169$ μg/ml), and an aqueous phase (210.3 g, 9.9%, $IC_{50}>200$ μg/ml). A portion (76.7 g) of the EtOAc-soluble fraction was subjected to ordinary-phase silica gel column chromatography [2.0 kg, *n*-hexane→*n*-hexane–EtOAc (20 : 1→10 : 1→5 : 1→1 : 1, v/v)→EtOAc→$CHCl_3$–MeOH (20 : 1→10 : 1→5 : 1, v/v)→MeOH] to give 11 fractions [fr. 1 (1.0 g, $IC_{50}=150$ μg/ml), fr. 2 (10.7 g, $IC_{50}>200$ μg/ml), fr. 3 (2.1 g, $IC_{50}>200$ μg/ml), fr. 4 (2.5 g, $IC_{50}=122$ μg/ml), fr. 5 (15.3 g, $IC_{50}=10.7$ μg/ml), fr. 6 (4.2 g, $IC_{50}=42.5$ μg/ml), fr. 7 (3.3 g, $IC_{50}=23.3$ μg/ml), fr. 8 (1.4 g, $IC_{50}=42.6$ μg/ml), fr. 9 (1.3 g, $IC_{50}=23.7$ μg/ml), fr. 10 (13.5 g, $IC_{50}=130$ μg/ml), and fr. 11 (19.1 g, $IC_{50}>200$ μg/ml)]. A portion (15.1 g) of fr. 5 was subjected to ordinary-phase silica gel column chromatography [450 g, *n*-hexane→*n*-hexane–EtOAc (20 : 1→5 : 1→1 : 1→1 : 2→1 : 4, v/v)→EtOAc] to give eight fractions [fr. 5-1 (141 mg, $IC_{50}=21.0$ μg/ml), fr. 5-2 (1.2 g, $IC_{50}=53.2$ μg/ml), fr. 5-3 (5.4 g, $IC_{50}=2.3$ μg/ml), fr. 5-4 (4.5 g, $IC_{50}=84.5$ μg/ml), fr. 5-5 (983 mg, $IC_{50}=15.4$ μg/ml), fr. 5-6 (325 mg, $IC_{50}=5.8$ μg/ml), fr. 5-7 (253 mg, $IC_{50}=3.3$ μg/ml), and fr. 5-8 (232 mg, $IC_{50}=49.6$ μg/ml)]. A portion (5.2 g) of fr. 5-3 was purified by ordinary- and reversed-phase silica gel column chromatography, preparative TLC [*n*-hexane–EtOAc (5 : 1, v/v)], and HPLC [MeOH–H_2O (90 : 10, v/v)] to give sitosterol (**1**, 5.4 mg), 5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3β-ol (**2**, 60.1 mg), 5α,8α-epidioxy-22*E*-ergosta-6,9(11),22-trien-3β-ol (**3**, 2.5 mg), and ergosterol (**5**, 4.0 mg). A portion (300 mg) of fr. 5-6 was purified by ordinary- and reversed-phase silica gel column chromatography to give 5α,8α-epidioxy-22*E*-ergosta-6,22-dien-3β-ol (**2**, 35.5 mg). A portion (230 mg) of fr. 5-7 was purified by ordinary- and reversed-phase silica gel column chromatography and HPLC [MeOH–H_2O (90 : 10, v/v)] to give 5α,6α-epoxy-5α-ergosta-7,22-dien-3β-ol (**4**, 2.7 mg).

The compounds obtained were identified by comparison of their physical data ($[\alpha]_D$, ^{1}H-NMR, ^{13}C-NMR, MS) with reported values[9,12] or with those of authentic samples (**1**, **5**).

Bioassay. Reagents for Bioassay RPMI-1640 medium was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.); fetal bovine serum (FBS) was from Biowest (Nuaille, France); the Cell Counting Kit-8 was from Dojindo Laboratories (Kumamoto, Japan); and cordycepin (**6**) and other reagents were from Wako Pure Chemical Industries, Ltd. Ninety-six-well microplates were purchased from Asahi Glass Co., Ltd. Science Products (Tokyo, Japan), and 96-well white microplates were from Nalge Nunc International (Tokyo, Japan).

Cell Culture The human promyelocytic leukemia HL-60 cells (cell No. RCB0041) were provided by the Riken Cell Bank (Tsukuba, Japan). The cells were maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin 100 units/ml, and streptomycin 100 μg/ml.

Cytotoxicity against HL-60 Cells The proliferation of HL-60 cells was determined in the WST-8 colorimetric assay using a Cell Counting Kit-8 (Dojindo Laboratories). Briefly, after the incubation of HL-60 cells (1×10^4 cells/100 μl/well) with test compounds in RPMI-1640 medium supplemented with 10% FBS in 96-well microplates for 48 h, 10 μl of WST-8 solution (Cell Counting Kit-8) was added to each well. After a further 1 h of culture, the optical density of the water-soluble formazan produced by the cells was measured with a microplate reader (Multi Functional Microplate Reader GENios Plus, Tecan group Ltd., Männedort, Switzerland) at 450 nm. The test sample was dissolved in DMSO and diluted with the medium (final DMSO concentration less than 1%). Cell viability (percentage) was calculated using the following formula, and IC_{50} values were determined graphically.

$$\text{cell viability (\%)}=[(A-B)/(C-D)]\times100$$

A: test compound (+), WST-8 (+)
B: test compound (+), WST-8 (−)
C: vehicle (+), WST-8 (+)
D: vehicle (+), WST-8 (−)

Apoptosis-Inducing Effects After the incubation of HL-60 cells (1×10^4 cells/100 μl/well) in RPMI-1640 medium supplemented with FBS in 96-well microplates for 12 h, 100 μl of test sample solution in the medium (20, 40 μg/ml) was added to each well. After a further 24 h of culture, the

apoptosis-inducing effects of the test sample were examined using the TUNEL assay (Apoptosis Screening Kit *Wako*, Wako Pure Chemical Industries) according to the manufacturer's instructions. Values of absorbance with each test sample represent the difference from the mean of the vehicle-treated (control) group. Actinomycin D was used as a reference compound. The test compound dissolved in DMSO was diluted with the medium (final DMSO concentration less than 1%).

Activity of Caspases 3/7 HL-60 cells (2×10^4 cells/50 μl/well) were seeded into 96-well white microplates, and then 50 μl of test sample solution in the medium (20, 40 μg/ml) was added to the well. After a further 24 h of culture, the activity of caspases-3/7 was examined using a commercial kit (Caspase-Glo 3/7 Assay, Promega) according to the manufacturer's instructions. Briefly, Caspase-Glo reagent was added to the wells, and the plate was gently mixed for 30 s. After incubation at room temperature for 1 h, luminescence was measured using a microplate reader (Multi Functional Microplate Reader GENios Plus, Tecan). Actinomycin D was used as a reference compound. The test compound dissolved in DMSO was diluted with the medium (final DMSO concentration less than 1%).

Statistical Analysis For statistical analysis, one-way analysis of variance followed by Dunnett's test was performed. A probability (p) value of less than 0.05 was considered to represent a statistically significant difference.

Acknowledgments This research was supported by the 21st COE Program, Academic Frontier Project, and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References and Notes

1) Paterson R. R. M., *Phytochemistry*, **69**, 1469—1495 (2008).
2) Zhang Q. X., Wu J. Y., Hu Z. D., Li D., *Life Sci.*, **75**, 2911—2918 (2004).
3) Wu J. Y., Zhang Q. X., Leung P. H., *Phytomedicine*, **14**, 43—49 (2007).
4) Park J. P., Kim S. W., Hwang H. J., Yun J. W., *Lett. Appl. Microbiol.*, **33**, 76—81 (2001).
5) Jia J. M., Ma X. C., Wu C. F., Wu L. J., Hu G. S., *Chem. Pharm. Bull.*, **53**, 582—583 (2005).
6) Mao X. B., Zhong J. J., *Biotechnol. Prog.*, **20**, 1408—1413 (2004).
7) Yu R., Wang L., Zhang H., Zhou C., Zhao Y., *Fitoterapia*, **75**, 662—666 (2004).
8) Kadota S., Shima T., Kikuchi T., *Yakugaku Zasshi*, **106**, 1092—1097 (1986).
9) Bok J. W., Lermer L., Chilton J., Klingeman H. G., Towers G. H. N., *Phytochemistry*, **51**, 891—898 (1999).
10) Wan R., Kobayashi M., Tamesada M., *J. Trad. Med.*, **23**, 83—87 (2006).
11) The IC_{50} value of each fraction is described in Experimental.
12) Kobori M., Yoshida M., Ohnishi-Kameyama M., Takei T., Shinmoto H., *Biol. Pharm. Bull.*, **29**, 755—759 (2006).
13) Kuo Y. C., Lin C. Y., Tsai W. J., Wu C. L., Chen C. F., Shiao M. S., *Cancer Invest.*, **12**, 611—615 (1994).
14) Nakamura K., Yoshikawa N., Yamaguchi Y., Kagota S., Shinozuka K., Kunitomo M., *Anticancer Res.*, **26**, 43—47 (2006).
15) Kaufmann S. H., *Cancer Res.*, **49**, 5870—5878 (1989).
16) Friesen C., Herr I., Krammer P. H., Debatin K. M., *Nat. Med.*, **2**, 574—577 (1996).
17) Fulda S., Debatin K. M., *Curr. Med. Chem. Anticancer Agents*, **3**, 253—262 (2003).
18) Sedletska Y., Giraud-Panis M. J., Malinge J. M., *Curr. Med. Chem. Anticancer Agents*, **5**, 251—265 (2005).
19) Kwon H. C., Zee S. D., Cho S. Y., Choi S. U., Lee K. R., *Arch. Pharm. Res.*, **25**, 851—855 (2002).
20) Li G., Xu M. L., Lee C. S., Woo M. H., Chang H. W., Son J. K., *Arch. Pharm. Res.*, **27**, 829—833 (2004).
21) Kim K. H., Choi S. U., Park K. M., Seok S. J., Lee K. R., *Arch. Pharm. Res.*, **31**, 579—586 (2008).
22) Earnshaw W. C., Martins L. M., Kaufman S. H., *Annu. Rev. Biochem.*, **68**, 383—424 (1999).
23) Adrain C., Martin S. J., *Trends Biochem. Sci.*, **26**, 390—397 (2001).
24) Kim R., Emi M., Tanabe K., *Oncol. Rep.*, **14**, 595—599 (2005).
25) Park C., Moon D. O., Rhu C. H., Choi B. T., Lee W. H., Kim G. Y., Choi Y. H., *Biol. Pharm. Bull.*, **30**, 1317—1323 (2007).