Journal of Medicinal Plants Research Vol. 5(7), pp. 1066-1071, 4 April, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Inhibitory effect on B16/F10 mouse melanoma cell and HT-29 human colon cancer cell proliferation and cordycepin content of the butanol extract of *Paecilomyces militaris*

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Accepted 21 May, 2010

The cultured mycelium of strain *Paecilomyces militaris* CMG 01 (Pm) was extracted sequentially by hexane, ethyl acetate (EtOAc) and butanol (BuOH). Among them, the BuOH extract exhibited potent anti-proliferative activity on B16/F10 mouse melanoma and HT-29 human colon cancer cells. Active chemical fractions of the Pm BuOH extract led to the isolation of a nucleoside compound, which structure was identified as cordycepin on the basis of ^{1}H, ^{13}C NMR and MS data. The further evaluated on cordycepin showed that cordycepin significantly inhibited B16/F10 and HT-29 cells proliferation and its content in BuOH extract was 68.89 mg/g. our results suggest that the BuOH extract of Pm is a potential source of natural anticancer agents.

Key words: *Paecilomyces militaris*, B16/F10 mouse melanoma cell, HT-29 human colon cancer cell, cordycepin.

INTRODUCTION

Cordyceps sinensis (Cs) known as "Dong Chong Xia Cao" in folk is a well-known traditional medicine, *Cordyceps militaris* (Cm) has medicinal properties similar to Cs and is widely used as a substitute for Cs in health supplements. Various biological activities such as anti-tumor (Liu et al., 1997; Yoo et al., 2004), anti-inflammatory (Yu et al., 2004; Won and Park, 2005), anti-oxidative (Yu et al., 2006) and antifibrotic (Nan et al., 2001) activities of Cm have been reported.

Naturally occurring Cm is not easily available for food in large amounts for its high production cost, because of its rarity and outstanding curative effects, several mycelia strains have been isolated from natural Cm and manufactured by fermentation technology and are commonly sold as health food products. After many researchers have successfully isolated Cm related fungi from natural Cm, they focus on investigating their fermented biological activity and component (Schimidt et al., 2002; Schimidt et al., 2003; Zhao et al., 2006; Hu et al., 2009).

Our recent study has shown that the butanol (BuOH) extract of the cultured mycelium of strain *Paecilomyces militaris* CMG 01(Pm), isolated from natural fresh Cm collected in Yunnan province, China, exhibited potent anti-proliferative activity on B16/F10 mouse melanoma and HT-29 human colon cancer cells. Activity guided chemical fractions of this extract led to the isolation of a nucleoside compound; the compound structure was identical with cordycepin on the basis of ^{1}H, ^{13}C NMR and MS data. Many anticancer activities of cordycepin tested by various cancer cells have been reported (Yoshikawa et al., 2004; Thomadaki et al., 2005; Nakamura et al., 2006; Wu et al., 2007; Yoshikawa et al., 2007). In the present study, we evaluated the cytotoxic effect on B16/F10

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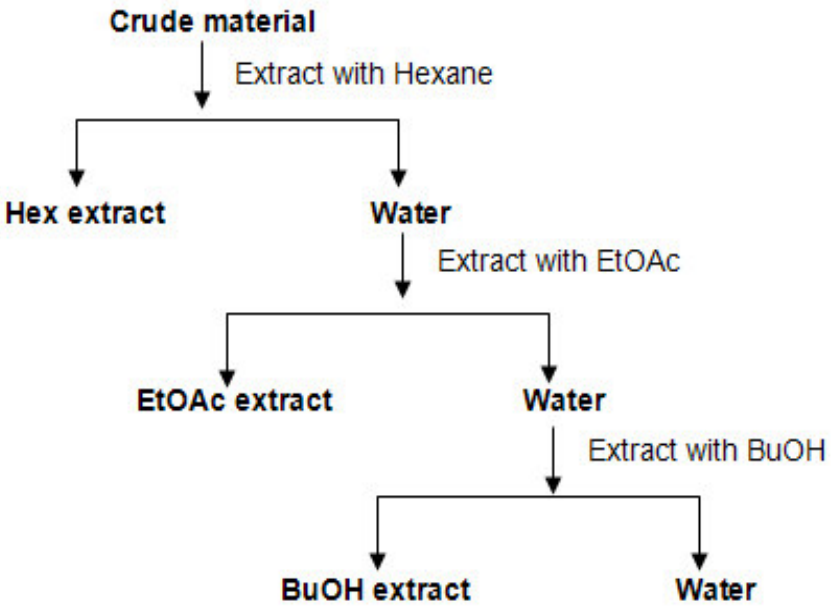

Figure 1. The procedure for preparation extracts of Pm (Hex=hexane, EtOAc=ethyl acetate, BuOH=butanol).

mouse melanoma and HT-29 human colon cancer cells of BuOH extract and compared with that of ethyl acetate (EtOAc) extract of Pm; further evaluated the cytotoxic effect of cordycepin on B16/F10 mouse melanoma and HT-29 human colon cancer cells. Notably, the Pm BuOH extract contained the content of the isolated compound cordycepin more than Pm EtOAc extract.

MATERIALS AND METHODS

Cultivation and extraction of *Paecilomyces militaris* CMG 01 mycelium

Mycelium of Pm was inoculated on solid medium composed of *Dioscorea opposite, Hordeum vulgare* and wheat bran, and cultured at 25 - 28℃ for 25 days. The cultured material was ground to a fine powder with a grinder. Powder (1200 g) was extracted with 95% EtOH for three times at room temperature, combine the extract solvent and then evaporated under vacuum to remove the solvents to give a yield of 70 g crude material. The crude materials were kindly provided by institute of herb biotic resources, Yunnan University (IHBR, Kunming, China). Specimen is deposited at IHBR with the code CMG 01.

Preparation extracts of *Paecilomyces militaris* CMG 01

The crude material were dissolved with water, after remove the insoluble solid by filtration, the liquid phase was extracted sequentially from non-polar to polar solvents by hexane, EtOAc, BuOH (1:10 w/v for all solvents) as illustrated by Figure 1. The liquid-liquid phase extraction was performed in Erlenmeyer flasks shaking and concentrated by a rotary evaporator to dryness. From this procedure get hexane extract (31 g), EtOAc extract (6 g) and BuOH extract (7 g).

Cell culture

Both mouse melanoma B16/F10 cells and human colon carcinoma HT-29 cells were obtained from the American Type Culture Collection (Rockville, MD). Both cell lines were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum in a humidified incubator at 37℃ in the presence of 5% CO_2.

Anti-proliferative assay

We used the Dojindo Cell Counting Kit-8 (CCK8 kit, Dojindo Laboratories, Gaithersburg, MD), according to the manufacturer's instructions. CCK8 kit utilizes convenient assays using a tetrazolium salt, WST-8(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces an orange water-soluble formazan dye upon bioreduction by cellular dehydrogenases. Cell viability was determined by the amount of formazan, which is directly proportional to the number of living cells and measured by the absorbance at 450nm. Cells (1×10^4 cells/well) in 96-well plates were incubated with various concentrations in the absence or presence of samples for the indicated times. Cell proliferation rate (%) was calculated as the absorbance of sample-treated cells divided by the absorbance of control cells (n = 3). Cell viability of the control group was 100%.

Isolation and identification

The isolation procedure was carried out on Combiflash Companion (Teledyne Isco), TLC was performed on 25 DC-Alufolien Kieselgel 60 F_{254} (Merck KGaA, Germany). The BuOH extract was purified by column chromatography on silica gel RediSep Flash Column (Teledyne Isco) and successively eluted with a gradient of chloroform/methanol (9:1 to 4:1, v/v), Of the three fractions separated by column chromatography, the third fraction (F3) showed apoptotic activity, after recrystal F3 in MeOH, produce

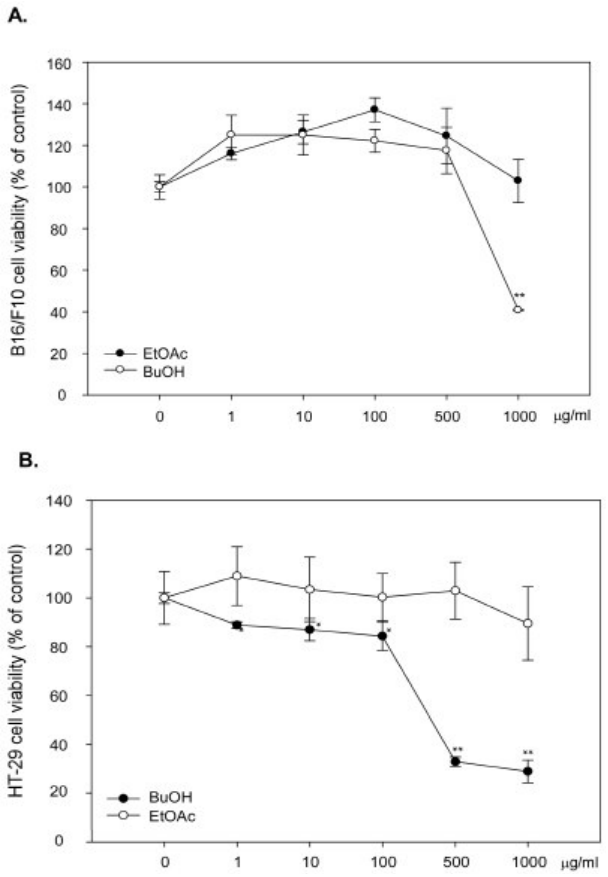

Figure 2. Effect of the BuOH and EtOAc extracts of Pm on B16/F10 mouse melanoma and HT-29 human colon cancer cell proliferation. (a) B16/F10 (1×10^4 cells/well) (b) HT-29 cells (1×10^4 cells/well) were seeded in 96-well tissue culture plate followed by treatment with the indicated concentrations of BuOH and EtOAc extracts of Pm for 72hr. Cell proliferation was determined by CCK-8 assay. Results are mean value ± SD. (n = 3). *p < 0.05, **p < 0.01.

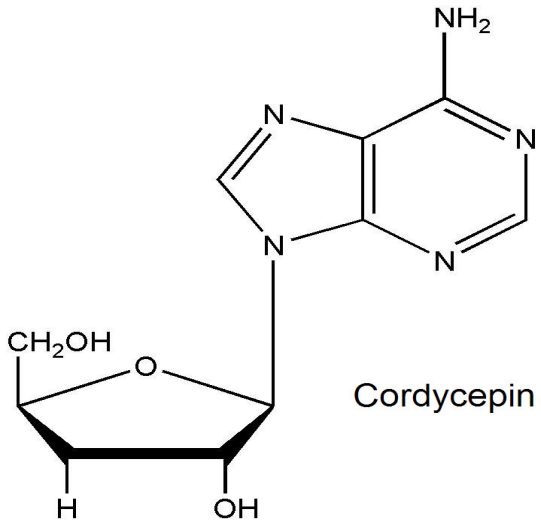

Figure 3. Structure of cordycepin.

white powder pure compound. Structural determination of the active isolated compound was made by spectroscopic analysis. NMR spectra were recorded on a Bruker Advance 400 spectrometer system (9.4 T, Karlsruhe, Germany). ESI-MS spectra were carried out on a VG Micromass Autospec (Micromass, Manchester, UK).

HPLC analysis extracts of *Paecilomyces militaris* CMG 01

1 mg of the extracts of Pm samples were dissolved in MeOH respectively and then filtered through 0.45 mm membrane before being injected into the HPLC system. The HPLC system consisted of a Gilson-305 Pump, a Soma S-3702 UV-Vis Detector, The samples were injected into an analytical ODS column (5um particle size, 4.6 × 250 mm, Gilson, American) and the mobile phase consisted of 0.025 mol/l KH_2PO_4 in $3DH_2O$ (A) and acetonitrile (B) according to an isocratic elution scheme (A: 91%, B: 9%) at a flow rate of 1.0 ml/min. All chromatographic procedures were performed at 25℃; the peaks were detected at 260 nm.

Standard solutions of cordycepin were prepared into 5.0, 10.0, 25.0, 50.0 and 100.0 μg/ml by diluting with MeOH from 200 μg/ml stock. A sample size of 10 μl was injected for the HLPC analysis. The content of cordycepin in the sample were calculated from standard curves obtained by analysis of cordycepin chemical standards (Sigma), and the correlation coefficient (r^2) were higher than 0.999 in the case.

Statistical analysis

The results are presented as mean ± standard deviation (SD). One way analysis of variance (ANOVA) procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, Dunnett's t-test was used for comparisons of multiple group means.

RESULTS

The effects of extracts of *Paecilomyces militaris* CMG 01on B16/F10 mouse melanoma and HT-29 human colon cancer cells proliferation

The anti-proliferative activities of Pm extracts on the B16/F10 mouse melanoma and on the HT-29 human colon cancer cells were evaluated using cell viability assay. Both cell lines were treated with EtOAc or BuOH extract at the indicated concentrations (0, 1, 10, 100, 500 and 1000 μg/ml) for 72 h. Pm BuOH extract exhibited potent anti-proliferative activity compared to Pm EtOAc extract on B16/F10 mouse melanoma and on HT-29 human colon cancer cells as shown in Figure 2. The radical scavenging activities of Pm extracts (hexane, EtOAc, BuOH and water) were measured using DPPH analysis. Among these extracts, only EtOAc and BuOH extracts of Pm were shown to have radical scavenging activities (Data not shown).

Compound structure identification

Structural determination of the isolated compound was made by spectroscopic methods including NMR and MS, and it was characterized as cordycepin (3'-deoxyadenosine) (Figure 3). The compound was

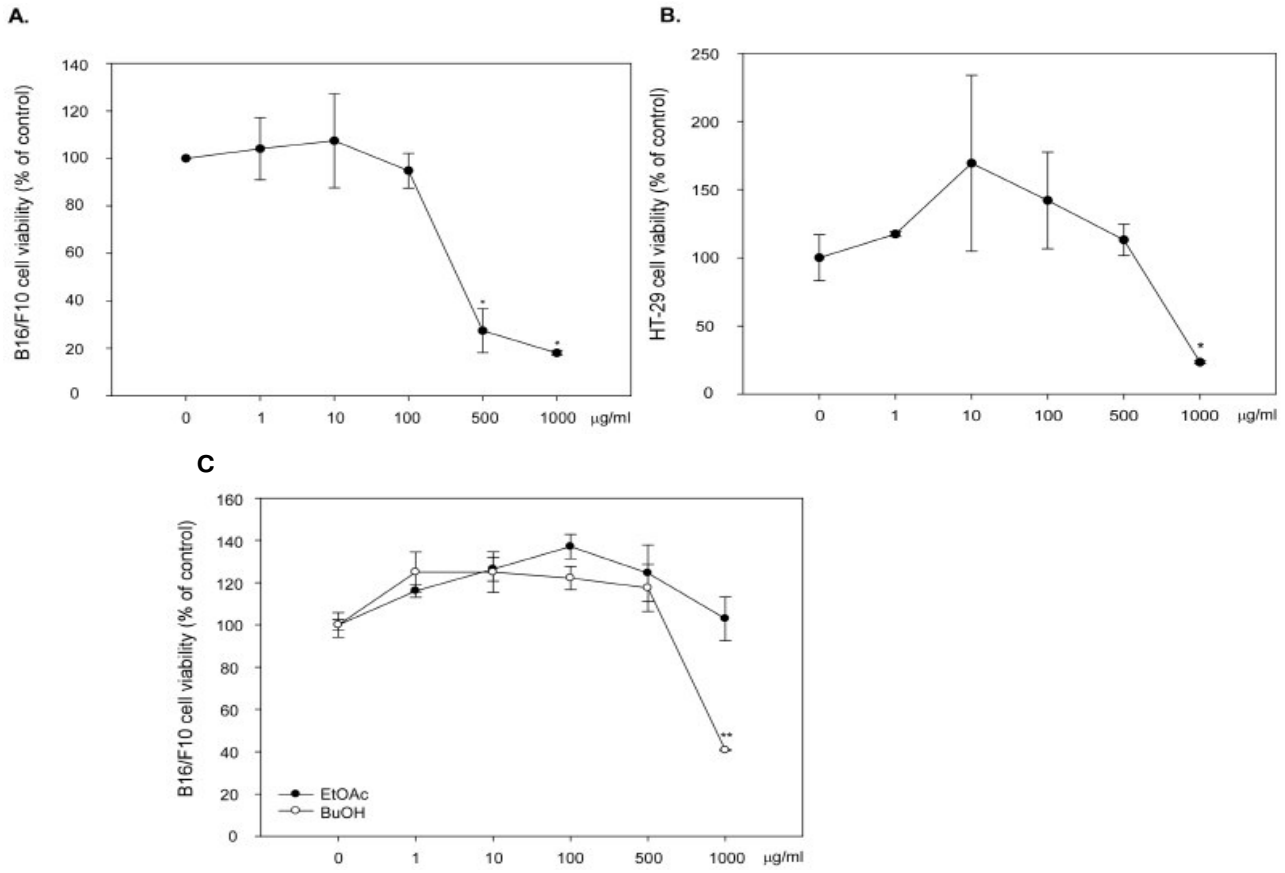

Figure 4. Effect of cordycepin on B16/F10 mouse melanoma and HT-29 colon cancer cell proliferation. (a) B16/F10 (1×10^4 cells/well) and (b) HT-29 cells (1×10^4 cells/well) were seeded in 96 well tissue culture plate followed by treatment with the indicated concentrations of cordycepin for 72 h. Cell proliferation was determined by CCK-8 assay. There was substantial decrease in proliferation of B16/F10 cells in a dose-dependent manner. Results are mean value ± SD. (n = 3). *p < 0.01.

identified on the basis of the following evidence: ESI-MS m/z: 252.2 $[M+H]^+$, 250.2 $[M-H]^-$ (calcd for $C_{10}H_{13}N_5O_3$: 251.2459). ^{1}H-NMR (DMSO) δ: 1.91 (1H, m), 2.23 (1H, m), 3.50 (1H, m), 3.66 (1H, m), 4.31 (1H, m), 4.53 (1H, m), 5.82 (1H, d, J = 2.0 Hz), 7.22 (2H, s), 8.10 (1H, s), 8.31 (1H, s). ^{13}C-NMR (DMSO) δ: 156.1(s), 152.6 (d), 148.9 (s), 139.2 (d), 119.2 (s), 90.9 (d), 80.8 (d), 74.7 (d), 62.7 (t), 34.2 (t). These data were identical with those of cordycepin (Ahn et al., 2000).

Evaluated the anti-proliferative activities of cordycepin

We further evaluated the anti-proliferative activities of cordycepin on the B16/F10 mouse melanoma and HT-20 human colon cancer cells. Both cells were treated with cordycepin at the indicated concentrations (0, 1, 10, 100, 500 and 1000 μg/ml) for 72 h. Cordycepin exerted anti-proliferative activity on B16/F10 mouse melanoma and HT-20 human colon cancer cells in a dose-dependent manner as shown in Figure 4.

Chromatogram of cordycepin of extracts of *Paecilomyces militaris* CMG 01

Peaks of cordycepin of Pm extracts were identified through comparing relative retention times (RRT) with chemical standard. The chromatograms of standard cordycepin and samples analyzed were shown in Figure 5. However, cordycepin was not detected in hexane and water extracts under the same condition. The cordycepin content of extracts of Pm was listed in Table 1.

CONCLUSION AND DISCUSSION

Cordycepin is considered to be one of the major active components of *Cordyceps* family, we compared cordycepin content between BuOH and EtOAC extracts of Pm. As shown in Table 1, the cordycepin content was 68.89 mg/g in BuOH extract and 28.66 mg/g in EtOAC extract respectively. Cordycepin content in Pm BuOH extract was higher than that in Pm EtOAC extract, which may explain that Pm BuOH extract exhibited lower 50%

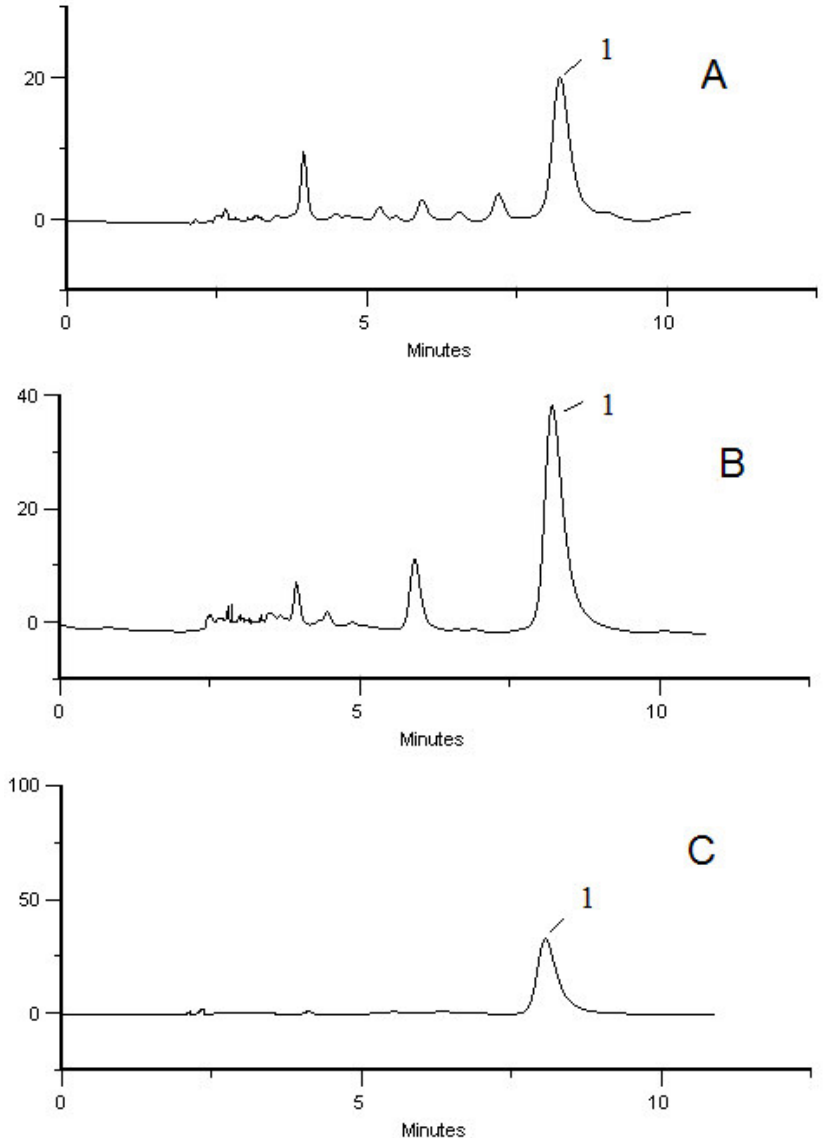

Figure 5. HPLC chromatograms of EtOAc extract (a); BuOH extract (b) of Pm and standard cordycepin (c) detected at 260 nm (1-cordycepin).

Table 1. The cordycepin content of extracts of Pm.

Cordycepin (mg/g)	Hexane	EtOAc	BuOH	Water
	-	28.66 ± 1.15	68.89 ± 1.65	-

Values represent mean ± SD, n = 3. - = not detectable, Hexane = hexane extract, EtOAc = ethyl acetate extract, BuOH = butanol extract, Water = water extract.

inhibition of growth (IC_{50}) than Pm EtOAC extract in B16/F10 mouse melanoma and in HT-29 human colon cancer cells. Moreover, our further evaluated the anti-proliferative activities of cordycepin on the B16/F10 mouse melanoma and HT-29 human colon cancer cells. Our data showed cordycepin is one of the active ingredients of Pm BuOH extract, responsible for the growth inhibition of B16/F10 mouse melanoma and HT-29 human cancer cells. It is not fully understood the bioactive compounds from the secondary metabolites of *P. militaris* at present (Hu and Li, 2007). Since each *P. militaris* collected from different environments possesses

different biological activities and components (Schimidt et al., 2002; Schimidt et al., 2003; Zhao et al., 2006; Hu et al., 2009), our results suggest that the BuOH extract of Pm is a potential source of natural anti-cancer agents.

ACKNOWLEDGMENTS

We wish to acknowledge our appreciation to Professor Yu Hong (IHBR, Yunnan University, Kunming, China) for his kindly provide strain crude materials. This work was supported by grants from the Ministry of Health and Welfare and the Agricultural R and D Promotion Center (107092-03-1-HD120), the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (MEST) (NRF-20100006815) and the Konkuk University, Seoul, Republic of Korea and the Natural Science Foundation of Yunnan Province (2008CC019).

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