Natural Product Sciences
19(3) : 231-235 (2013)

Antiproliferative Phenolics from *Eclipta prostrata* in the Activated Hepatic Stellate Cells

Eun Ju Jeong[1], Mi Kyeong Lee[2], Young Choong Kim[3], and Sang Hyun Sung[3,*]

[1]*Department of Agronomy & Medicinal Plant Resources, Gyeongnam National University of Science and Technology, Jinju 660-758, Korea*
[2]*College of Pharmacy and Research Institute of Pharmaceutical Science, Seoul National University, Seoul 151-742, Korea*
[3]*College of Pharmacy, Chungbuk National University, Cheongju 361-763, Korea*

Abstract – Activity-guided isolation to search for antifibrotic compounds from natural products using HSC-T6 cells afforded nine flavonoids or phenolics, luteolin (**1**), 3'-O-methylorobol (**2**), acactin 7-rutinoside (**3**), sedelolactone (**4**), 4-methoxyphenol (**5**), 4-hydroxyaldehyde (**6**), 4-hydoxyaldehyde (**7**), 4-hydroxy-3-methoxybenzoic acid (**8**), and ferulic acid (**9**) from the methanolic extract of aerial parts of *Eclipta prostrata* L.. Among the isolated compounds, luteolin (**1**) significantly inhibited the proliferation of HSCs in dose- and time-dependent manners. Antifibrotic activity of *E. prostrata* and its phenolic compounds might provide potential therapeutical choice in the treatment of hepatic fibrosis.
Keywords – *Eclipta prostrata*, Antifibrotic activity, HSC-T6, Hepatic stellate cells

Introduction

Hepatic fibrosis, a consequence of chronic liver tissue damage caused by infection with hepatitis viruses, autoimmune, chronic alcohol abuse, metabolic agents, is characterized by the activation of the hepatic stellate cells (HSCs). In response to liver damage, HSCs undergo phenotypic transformation from vitamin A-storing quiescent cells into myofibroblast-like proliferative, fibrogenic associated with increased proliferation, and/or excessive production and accumulation of ECM components, which is the major pathological feature of hepatic cirrhosis (Friedman, 2003; Li and Friedman, 1999; Tsukada *et al*., 2006). Chronic liver diseases frequently lead to scarring, which is often accompanied by progressive loss of liver function, hence, antifibrogenic therapy which suppresses the activation of HSCs has been preferentially considered as an attractive target to prevent the pathological progression to cirrhosis in chronic liver diseases (Wu and Zern, 2000; Bataller and Brenner, 2005).

There have been growing efforts to search for antifibrotic compounds from natural products. Diverse skeleton including flavonoids, alkaloids and terpenoids have been suggested to have antifibrotic activity (Chen *et al*., 2005; Uyama *et al*., 2003; Chen and Zhang, 2003; Sakata *et al*., 2004; Lin *et al*., 2006).

E. prostrata has been used for the treatment of hepatic diseases, hyperlipidemia or snake venom poisoning in folk medicine (Bae, 2000; Ma-Ma *et al*., 1978). Recent pharmacological studies reported diverse biological activity of *E. prostrata* including hepatoprotective, anti-inflammatory, antihemorrhagic, antihyperlipidemic and antihyperglycemic activities (Wagner *et al*., 1986; Saxena *et al*., 1993; Melo *et al*., 1994; Kumari *et al*., 2006). In the previous study in our lab, we reported that the methanolic extract of aerial parts of Eclipta prostrata L. showed significant inhibitory activity on HSCs proliferation. Though activity-guided fractionation, five echinocystic acid derivatives were isolated from *E. prostrate* (Lee *et al*., 2008). Continuous to the previous study identifying hepatoprotective constituents from *E. prostrate*, in the present study, we further isolated nine phenolic compounds from $CHCl_3$ and *n*-BuOH soluble fractions of *E. prostrata* and evaluated the antiproliferative activities of the isolated compounds in HSC-T6 cells.

Experimental

General experimental procedures – The ^1H and ^{13}C NMR measurements were carried out in a Bruker AMX

*Author for correspondence
Sang Hyun Sung, College of Pharmacy, Chungbuk National University, Cheongju 361-763, Korea
Tel: +82-2-880-7859; E-mail: shsung@snu.ac.kr

400 spectrometer operating at 300 and 100 MHz, respectively. Solvent signals were used as internal standards. ^{1}H-^{1}H COSY, HMQC, and HMBC NMR experiments were performed on the same spectrometer. EI-mass spectra were obtained on a JEOL JMS 700 spectrometer with a 70 eV ionizing potential. TLC and column chromatography (CC) were carried out on precoated silica gel F_{254} plates (art. 5715, Merck), RP-18 F_{254} plates (art. 15423, Merck), silica gel 60 (230 - 400 mesh, Merck), Sephadex LH 20 (18 - 110 μm, Pharmacia Co. Ltd.) and Diaion HP-20 (250 - 850 μm, Mitshubishi Chemical).

Plant material – The aerial parts of *E. prostrata* were purchased from Kyung-dong Market, Seoul, Korea in June 2004, and identified by Dr. Jong Hee Park, a professor of the College of Pharmacy, Pusan National University. A voucher specimen (SNUPH-EP2004-06) has been deposited in the Herbarium of the Medicinal Herb Garden, College of Pharmacy, Seoul National University.

Extraction and isolation – The aerial parts of *E. prostrata* (9 kg) were extracted 3 times with 80% MeOH, which yielded the methanolic extract (831 g). The methanolic extract was suspended in distilled water and partitioned successively with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH. The $CHCl_3$ fraction (77 g) was subjected to CC over silica gel eluted with *n*-hexaneEtOAc step gradient to give 11 fractions (C1-C11). C5 was subjected to CC over Sephadex LH-20 eluted with *n*-hexane-CH_2Cl_2-MeOH (5 : 5 : 1) mixture to afford 6 fractions (C5-1 to C5-6). Compounds **5** (7 mg) and **6** (6.1 mg) were obtained from C5-5 and C5-6, respectively. C6 was subjected to CC over Sephadex LH-20 eluted with *n*-hexane-CH_2Cl_2-MeOH (5 : 5 : 1) mixture to afford 6 fractions (C6-1 to C6-6). Compound **7** (4.5 mg) was obtained from C6-6. C9 was subjected to CC over Sephadex LH-20 eluted with CH_2Cl_2-MeOH (1 : 1) mixture to afford 3 fractions (C9-1 to C9-3). Compounds **2** (10 mg) and **8** (8.3 mg) were obtained from C9-3. C10 was subjected to CC over silica gel eluted with n-hexane-CH_2Cl_2-MeOH step gradient to give 13 fractions (C10-1 to C10-13). C10-13 was subjected to CC over Sephadex LH-20 eluted with CH_2Cl_2-MeOH (1 : 1) mixture to afford 3 fractions (C10-13-1 to C10-13-3). Compound **9** (7.4 mg) was obtained from C10-13-3. C11 was subjected to CC over Sephadex LH-20 eluted with *n*-hexane-CH_2Cl_2-MeOH (5 : 5 : 1) mixture to afford 3 fractions (C11-1 to C11-3). Compound **4** (80 mg) was obtained from C11-3. The *n*-BuOH fraction (138 g) was subjected to CC over HP-20 eluted with 0%, 20%, 40%, 60%, 80% and 100% MeOH to give 6 fractions (B1-B6). B1 was subjected to CC over silica gel eluted with $CHCl_3$-MeOH-H_2O step gradient to give 19 fractions (B1-1 to B1-19). B1-19 was subjected to CC over Sephadex LH-20 eluted with MeOH to give 8 fractions (B1-19-1 to B1-19-8). Compound **1** (101.4 mg) was obtained from B1-19-8. B3 was subjected to CC over silica gel eluted with $CHCl_3$-MeOH-H_2O step gradient to give 11 fractions (B3-1 to B3-11). Compound **3** (117.1 mg) was obtained from B3-8.

Culture of HSC-T6 hepatic stellate cells – An immortalized rat hepatic stellate cell line, HSC-T6 was kindly provided by Prof. SL Friedman (Columbia University, New York). HSC-T6 cells were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air-5% CO_2.

Measurement of cell viability – Compounds to be tested were dissolved in dimethylsulfoxide (DMSO). Our preliminary study showed that DMSO at a final concentration of 0.1% in media did not affect the cell viability. HSC-T6 cells were treated with vehicle or compounds to be tested for 48 h or as indicated. 18β-Glycyrrhetinic acid (Sigma-Aldrich Co.) was used as a positive control. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) assay. HSC-T6 cells were incubated with 0.5 mg/ml of MTT in the last 2 h of the culture period tested. Reduction of MTT to formazan was assessed in an ELISA plate reader.

Statistical analysis – The evaluation of statistical significance was determined by the Student's *t*-test with a value of $p < 0.05$ or less considered to be statistically significant.

Results and Discussion

The methanolic extract of the aerial parts of *E. prostrata* was fractionated into *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH fractions. Among them, $CHCl_3$ and *n*-BuOH soluble fractions showed effective inhibitory activities on the proliferation of HSCs at the concentration of 100 μg/ml (50.4% and 40.1% of the control, respectively, $p < 0.01$). Activity-guided isolation of $CHCl_3$ and *n*-BuOH fractions afforded nine compounds. The structures of compounds **1-9** were identified as luteolin (**1**) (Owen *et al*., 2003), 3¢-O-methylorobol (**2**) (Dean *et al*., 2004; Hosny *et al*., 1999), acactin 7-rutinoside (**3**) (Park *et al*., 1995), sedelolactone (**4**) (Li *et al*., 2003; Wang *et al*., 2006; Emmanuel *et al*., 2001), 4-methoxyphenol (**5**) (Knuutinen

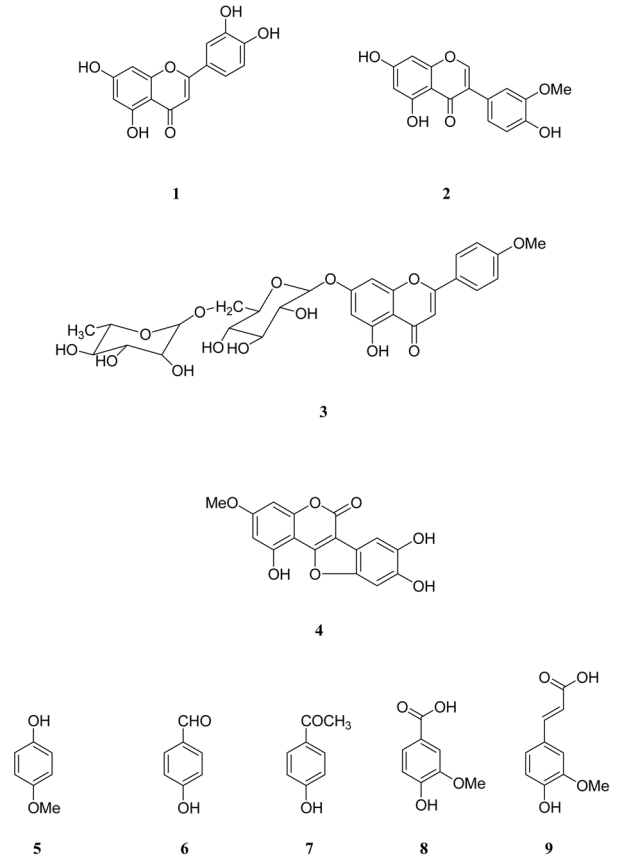

Fig. 1. The structures of compounds **1 - 9** isolated from *E. prostrata.*

et al., 1988), 4-hydroxyaldehyde (**6**) (Li *et al*., 1992), 4-hydoxyaldehyde (**7**) (Li *et al*., 1992), 4-hydroxy-3-methoxybenzoic acid (**8**) (Stalin *et al*., 2006), ferulic acid (**9**) (Anselmi *et al*., 2006), by the comparison of their spectroscopic data with those of previously reported (Fig. 1).

Antiproliferative activities of the isolated compounds **1 - 9** were measured in HSC-T6 cells, HSC-T6 cell is immortalized rat hepatic stellate cells which retain all features of activated stellate cells, including expression of desmin, α-smooth muscle actin, and glial fibrillary acidic protein, and it can esterify retinol into retinyl esters (Vogel *et al*., 2000). The activation of HSCs can be promoted by addition of serum, cytokines and other factors (Wu and Zern, 2000; Chen *et al*., 2005), and also can be induced by the specific culturing conditions *in vitro*. Culturing HSCs on uncoated plastic plates is known to cause spontaneous activation leading to myoblastic phenotype, mimicking the process seen *in vivo*. Thus, we evaluated antifibrotic activity of *E. prostrata* employing the activated HSC-T6 cells by assessment of cell viability using MTT assay.

Among the compounds tested, compounds **1**, **2** and **4** showed the inhibitory activities on HSC cells proliferation with statistical significance at a concentration of 100 μM for 48 h incubation, while the others showed weak activities (Table 1).

Table 1. Inhibitory effects of compounds **1 - 9** isolated from *E. prostrata* on the proliferation of HSC-T6 cells

	% of control	
	10 μM	**100 μM**
Control	100.0 ± 1.4	
1	75.3 ± 2.1*	24.0 ± 0.9**
2	79.0 ± 1.3*	58.2 ± 1.1**
3	80.7 ± 2.0*	82.0 ± 2.0*
4	82.4 ± 1.2*	59.0 ± 1.4**
5	95.0 ± 1.7	94.9 ± 1.9
6	91.0 ± 1.1	90.7 ± 2.0
7	92.2 ± 3.0	88.3 ± 2.5*
8	93.7 ± 2.7	94.5 ± 2.2
9	96.8 ± 1.5	94.5 ± 1.6

HSC-T6 cells were incubated with each compound tested at the concentration of 100 μM for 48 h. Cell viability was measured by the MTT assay. The percent cell viability (%) was calculated as 100 × (absorbance of compound treated/absorbance of control). Results are expressed as the mean ± SD of three independent experiments, each performed using triplicate wells. *$p < 0.01$, **$p < 0.001$ compared with control.

Among the isolated compounds, luteolin (**1**) showed the most potent activity which suppressed the viability of HSCs to 24.0 ± 0.9% of non-treated control cells. Also, luteolin (**1**) decreased the HSCs proliferation in dose- and time-dependent manners (Fig. 2). Consistent with our result, Zhao *et al*., (2002) reported luteolin as an effective therapeutic inhibiting the proliferation and collagen deposit in HSCs. It has been also reported that CCl_4-induced liver fibrosis was prevented by luteolin through promoting extracellular matrix degradation in the fibrotic liver tissue and enhancement of hepatic regenerative capability. The exact action mechanism of luteolin in the suppression of HSC activation needs to be clarified by further investigation.

In summary, we isolated nine phenolic compounds from *E. prostrata* and evaluated their antiproliferative activity in HSC-T6 cells. Among the compounds isolated, luteolin (**1**), 3'-O-methylorobol (**2**) and sedelolactone (**4**) showed antiproliferative activities. These constituents isolated from *E. prostrata* are thought to contribute hepatoprotective effects of *E. prostrata.*

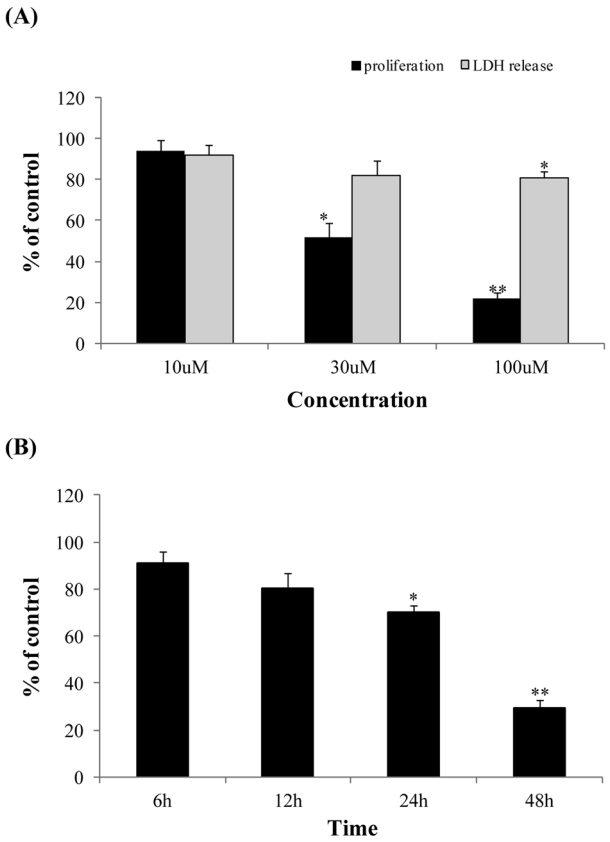

Fig. 2. Dose- (A) and Time-dependent (B) effects of **1** on the proliferation and LDH release in HSC-T6 cells. HSC-T6 cells were incubated with **1** at the concentrations of 10, 30 and 100 μM for 48 h (A); HSC-T6 cells were incubated with **1** at the concentration of 100 μM for indicated time (B). Cell viability was measured by the MTT assay. The percent cell viability (%) was calculated as 100 × (absorbance of compound treated/ absorbance of control). Results are expressed as the mean ± SD of three independent experiments, each performed using triplicate wells. $*p < 0.01$, $**p < 0.001$ compared with control.

Acknowledgements

This work was supported by Gyeongnam National University of Science and Technology Grant.

References

Anselmi, C., Centini, M., Ricci, M., Buonocore, A., Granata, P., Tsuno, T., and Facino, R.M., Analytical characterization of a ferulic acid /– cyclodextrin inclusion complex. *Journal of Pharm. Biomed. Anal*. **40**, 875-881 (2006).

Bae, K.H., *Dictionary of Korean Folk Medicine*. Kyu Hak Sa, Seoul p. 297 (2000).

Bataller, R. and Brenner, D.A., Liver fibrosis. *J. Clin.Invest*. **115**, 209-218 (2005).

Chen, A. and Zhang, L., The antioxidant (–)-epigallocatechin-3-gallate inhibits rat hepatic stellate cell proliferation in vitro by blocking the tyrosine phosphorylation and reducing the gene expression of platelet-derived growth factor-beta receptor. *J. Biol. Chem*. **278**, 23381-23389 (2003).

Chen, Y.W., Li, D.G., Wu, J.X., Chen, Y.W., and Lu, H.M., Tetrandrine inhibits activation of rat hepatic stellate cells stimulated by transforming growth factor-b in vitro via upregulation of Smad 7. *J. Ethnopharmacol*. **100**, 299-305 (2005).

Dean, W.R., Daniel, R., and Doerge, M.I., Churchwell Goncalo Gamboa da Costa M., Matilde, M., William, H.T., Inhibition of Extrahepatic Human Cytochromes P450 1A1 and 1B1 by Metabolism of isoflavones Found in Trifolium pretense. *J. Agri. Food Chem*. **52**, 6623-6632 (2004).

Emmanuel, S., Amalraj, T., and Ignacimuthu, S., Hepatoprotective effect of coumestans isolated from the leaves of Wedelia calendulacea less. in paracetamol-induced liver damage. Indian *J. Exper. Biol*. **39**, 1305-1307 (2001).

Friedman, S.L., Liver fibrosis - from bench to beside. *J. Hepatol*. **38**, S38-S53 (2003).

Geerts, A., History, heterogeneity, developmental biology, and functions of quiescent hepatic stellate cells. *Semin Liver Dis*. **21**, 311-335 (2001).

Hosny, M. and Rosazza John P.N., Microbial hydroxylation and methylation of genistein by Streptomycetes. *J. Nat. Prod*. **62**, 6909-6912 (1999).

Knuutinen, J., Autio, P., Klein, P., Kivelä, S., Virkki, L., and Lahtiperä, M., Synthesis and structure verification of chlorinated 4-Methoxyphenols, models of metabolites of chlorophenolic compounds. *Chemosphere* **17**, 1821-1829 (1988).

Kumari, C.S., Govindasamy, S., and Sukumar, E., Lipid lowering activity of Eclipta prostrata in experimental hyperlipidemia. *J. Ethnopharmacol*. **105**, 332-335 (2006).

Lee, M.K., Ha, N.R., Yang, H., Sung, S.H., Kim, G.H., and Kim, Y.C., Antiproliferative activity of triterpenoids from Eclipta prostrata on hepatic stellate cells. *Phytomedicine* **15**, 775-780 (2008).

Li, C.C., Xie, Z.X., Zhang, Y.D., Chen, J.H., and Yang, Z., Total Synthesis of Wedelolactone. *J. Org. Chem*. **68**, 8500-8504 (2003).

Li, D. and Friedman, S.L., Liver fibrogenesis and the role of hepatic stellate cells: new insights and prospects for therapy. *J. Gastroenterol. Hepatol*. **14**, 618-633 (1999).

Li, J., Kadota, S., Kawata, Y., Hattori, M., Xu, G., Namba, Tsuneo., Constituents of the roots of Cynanchum bungei Decne. Isolation and structures of four new glucosides, bungeiside A, B, C, and D. *Chem. Pharm. Bull*. **40**, 3133-3137 (1992).

Lin, Y.L., Lee, T.F., Huang, Y.J., and Huang, Y.T., Antiproliferative effect of salvianolic acid A on rat hepatic stellate cells. *J. Pharm. Pharmacol*. **58**, 933-939 (2006).

Ma-Ma, K., Nyunt, N., and Tin, K.M., The protective effect of Eclipta alba on carbon tetrachloride-induced acute liver damage. *Toxicol. Appl. Pharmacol*. **45**, 723-728 (1978).

Melo, P.A., Nascimento, M.C., and Mors, W.B., Suarez-Kurtz, G., Inhibition of the myotoxic and hemorrhagic activities of crotalid venoms by Eclipta prostrata (Asteraceae). *Toxicon*. **32**, 595-603 (1994).

Owen, R.W., Haubner, R., Mier, W., Giacosa, A., Hull, W.E., Spiegelhalder B., and Bartsch H., Isolation, structure elucidation and antioxidant potential of the major phenolic and flavonoid compounds in brined olive drupes. *Food Chem. Toxicol*. **41**, 703-717 (2003).

Park, J.C., Lee, J.H., and Choi, J.S., A flavone diglycoside from Cirsium japonicum var. ussuriense. *Phytochem*. **39**, 261-262 (1995).

Sakata, R., Ueno, T., Nakamura, T., Sakamoto, M., Torimura, T., and Sata, M., Green tea polyphenol epigallocatechin-3-gallate inhibits platelet-derived growth factor-induced proliferation of human hepatic stellate cell line LI90. *J. Hepatol*. **40**, 52-59 (2004).

Saxena, A.K., Singh, B., and Anand, K.K., Hepatoprotective effects of

Eclipta alba on subcellular levels in rats. *J. Ethnopharmacol*. **40**, 155-161 (1993).

Stalin, T. and Rajendiran, N., A study on the spectroscopy and photophysics of 4-hydroxy-3-methoxybenzoic acid in different solvents, pH and β-cyclodextrin. *Journal of Molecular* **794**, 35-45 (2006).

Tsukada, S., Parsons, C.J., and Rippe, R.A., Mechanisms of liver fibrosis. *Clin. Chim. Acta* **364**, 33-60 (2006).

Uyama, N., Shimahara, Y., Okuyama, H., Kawada, N., Kamo, S., Ikeda, K., and Yamaoka, Y., Carbenoxolone inhibits DNA systhesis and collagen gene expression in rat hepatic stellate cells in culture. *J. Hepatol*. **39**, 745-799 (2003).

Vogel, S., Piantedosi, R., Frank, J., Lalazar, A., Rockey, D.C., Friedman, S.L., and Blaner, W.S., An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro. *J Lipid Res*. **41**, 882-893 (2000).

Wagner, H., Geyer, B., Kiso, Y., Hikino, H., and Rao, G.S., Coumestans as the main active principles of the liver drugs Eclipta alba and Wedelia calendulacea. *Planta Med*. **5**, 370-374 (1986).

Wang, W., Zhao, Y.Y., Liang, H., Jia, Q., and Chen, H.B., Coumestans from Hedysarum multijugum. *J. Nat. Prod*. **69**, 876-880 (2006).

Wu, J. and Zern, M.A., Hepatic stellate cells: a target for the treatment of liver fibrosis. *J. Gastroenterol*. **35**, 665-672 (2000).

Zhao, W., Liang, C., Chen, Z., Pang, R., Zhao, B., and Chen, Z., Luteolin inhibits proliferation and collagen synthesis of hepatic stellate cells. *Zhonghua Gan Zang Bing Za Zhi*. **10**, 204-206 (2002).

Received May 30, 2013
Revised June 20, 2013
Accepted June 25, 2013