JBUON 2014; 19(2): 406-411
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

N-butanol fraction of *Entada phaseoloides* ethanol extract inhibits hepatocellular carcinoma HepG2 cell proliferation by inducing apoptosis

Li Zhang[1], Ling Huang[1], Qiang Liu[1], Shaoyi Kuang[1], Qiaoqin Xu[1], Xianjing Qin[1], Shituo Fu[1], Qian Wu[1],Qibing Liu[1], Yu Zeng[2]

[1]*Department of Pharmacology, College of Pharmacy, Hainan Medical University, Haikou, P.R. China;* [2]*College of Management, Hainan Medical University, Haikou, P.R. China*

Summary

Purpose: *To screen for substances with inhibitory effects on the proliferation of hepatocellular carcinoma (HCC) HepG2 cell line from extracts of traditional Chinese medicinal plants including Heliciopsis lobata (Merr.) Sleum, Bidens pilosa, Entada phaseoloides, Plantago major, and Smilax, and unveil their mechanism of action.*

Methods: *3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to assess the inhibition of HepG2 cell proliferation by plant extracts. Cell apoptosis was evaluated by Hoechst 33342 staining and mitochondrial transmembrane potential (ΔΨm) dissipation was measured using JC-1 probe by fluorescence microscopy.*

Results: *Heliciopsis lobata, Bidens, Plantago, and Smilax extracts showed reduced inhibitory effects on HepG2 cell proliferation compared with Entada phaseoloides (all $p<0.05$). The n-butanol fraction of Entada phaseoloides ethanol extract exhibited the highest inhibition rate. Treatment of HepG2 cells with 500, 250, and 100 μg/ml n-butanol extract resulted in 89.92±0.58%, IC_{50} 81.66±0.42%, 68.85±0.57% decrease in cell viability, respectively, indicating an IC_{50} of 9.27 μg/ml. In the presence of 100 μg/ml entada phaseoloides n-butanol extract for 24h, apoptotic nuclei and hyperchromatic, dense fluorescent massive granules were observed in the cytoplasm, effects that increased with extract concentrations in HepG2 cells. Finally, we showed that Entada phaseoloides n-butanol extract induced depolarization of mitochondrial membrane potential.*

Conclusions: *Entada phaseoloides n-butanol extract inhibits HepG2 cell proliferation by inducing cell apoptosis likely through mitochondrial apoptotic pathway. This extract is therefore a potential natural source towards the discovery for a new drug-candidate for the treatment of HCC.*

Key words: *apoptosis, Entada phaseoloides, hepatocellular carcinoma, mitochondria, n-butanol extract, traditional Chinese medicine*

Introduction

HCC is the most common primary malignancy of the liver and the 6th most common malignancy worldwide [1,2]. Unfortunately, the incidence of HCC is increasing in many countries with an estimated number of new cases annually over 500,000, and a yearly incidence between 2.5 and 7% of patients with liver cirrhosis [2]. For patients chronically infected with hepatitis B virus (HBV) or hepatitis C virus (HCV), antiviral treatment is the only option to prevent or defer HCC occurrence [3]. In addition to liver transplantation, most radical treatment options for HCC including surgical resection, embolization, ablation, and chemotherapy are also important therapeutic methods but limited to a significant extent by toxicity, significant resistance to available chemotherapeutic agents, side effects and complexity of the procedures [4-6].

A possible way to increase the efficacy of anticancer drugs while decreasing toxicities is to

Correspondence to: Yu Zeng, PhD. Hainan Medical University, No 3 College Road, Longhua District, Haikou City, Hainan Province, Postal code 570 000, P.R. China. Tel/Fax: +86 898 66893760, E-mail:zengyu_hmu@163.com
Received: 28/11/2013; Accepted: 11/12/2013

develop complementary and alternative medicine [6]. *Heliciopsis lobata, Bidens, Entada phaseoloides, Plantago,* and *Smilax* are natural medicinal plants found in the Hainan province (China) and widely used in the treatment of various diseases in Hainan Li region. *Heliciopsis lobata* (Merr) Sleum (Proteaceae) is widely distributed in central and southern mountainous areas of Hainan where the root bark of which is used as medicine especially for the treatment of mumps, dermatitis as well as cancer [7]. *Bidens pilosa* Linn (Asteraceae) grows in eastern, central, south, and southwest provinces of China and all the parts of this bitter and non-toxic plant, with a variety of pharmacological effects, are used as medicines [8]. *Bidens pilosa*, either as a whole plant or different parts, has been reported to be useful in the treatment of more than 40 disorders such as inflammation, immunological disorders, digestive disorders, infectious diseases, cancers, metabolic syndrome, wounds, and many others [9]. PMII is a pectic polysaccharide fraction isolated from *Plantago major* L. (Plantaginaceae) leaves, a plant used in traditional medicine to aid the healing of wounds [10]. In addition, it was shown that *Plantago major* extracts inhibited the growth of Balb/C mice Ehrlich ascites tumors [11]. Smilax china Linn dispels dryness and dampness, detoxifies and dissipates blood stasis, and was found to possess anticancer compounds [12,13]. *Entada phaseoloides* (L.) Merr (Leguminosae), mainly found in Hainan, Taiwan, Fujian, Guangdong and Guangxi provinces of China, was shown to have antiinflammatory effects and is believed to have also anticancer properties [14,15]. In addition, a new triterpene saponin termed Phaseoloideside E, was isolated from *E. phaseoloides* seeds with strong cytotoxic activity against various malignant cells and apoptotic effects in Ec-109 cells [16].

Despite these unique properties, n-butanol extracts from these plants have not been evaluated for their therapeutic properties, especially their capacity to inhibit proliferation of HepG2 HCC cells. Therefore we aimed to carry out extraction and separation of the medicinal plants mentioned above in order to select the extracts with pronounced antitumor activity.

Methods

Cell culture

The human hepatoma cell line HepG2 was provided by the Institute of Pharmacology in Sun Yat-Sen University. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) purchased from Boster (Wuhan, China), and supplemented with 10% fetal bovine serum (Thermo Fisher Scientific Inc., USA) and 100 U/ml penicillin/streptomycin. Cells were incubated in a humid environment containing 5% CO_2 at 37 °C and treated in logarithmic growth phase with extracts at different concentrations.

Medicinal plant materials

H. lobata, Bidens, E. phaseoloides, Plantago, and *Smilax* were collected and characterized by Prof Kang Shengli of the Department of Natural Medicines in Hainan Medical College.

Plant material extraction

Supercritical fluid extraction (SFE) was performed on an ISCO (Lincoln, NE, USA) model SFX3560 supercritical fluid extractor equipped with two syringe pumps using pure CO_2 and CO_2 modified with 1%, 5%, and 10% ethanol (v/v) at 60 °C and 34.0 MPa. 50 g powder of ground medicinal materials was used for ethanol or water extraction. According to our previous experiments, *H. lobata* was extracted respectively with 250 ml water, 90% ethanol, and 70% ethanol by heating under reflux for 2h three times. The extracts were combined, concentrated and lyophilized to obtain water extracts and ethanol extracts. *Bidens* and *Smilax* samples were extracted with water while *E. phaseoloides* and *Plantago* materials were extracted with 70% ethanol. Then, *E. phaseoloides* ethanol extracts were suspended in 100 ml water and successively extracted with petroleum ether, chloroform, ethyl acetate, and n-butanol. The resulting fractions were concentrated and lyophilized to obtain the extracts.

Two hundred mg of water or ethanol extract obtained from each medicinal plant material and various *E. phaseoloides* ethanol extract fractions were dissolved in 20 μl dimethyl sulfoxide (DMSO) and diluted 500-fold with DMEM to obtain 20 μg/ml solution for each extract. Each sample solution was then diluted with DMEM to achieve final concentrations of 500 μg/ml, 250 μg/ml, and 100 μg/ml, respectively. We used 0.05% DMSO and 20 μg/ml cisplatin as negative and positive controls, respectively.

MTT assay

MTT assays were carried out as previously described [17]. 1 x 10^4 HepG2 cells in logarithmic phase were seeded in 96-well plates in 200 l medium, in triplicate. Media with no cells were added into blank control wells. After 12h incubation, 20 μl of the different test articles (extracts and controls) were added to cultures followed by 48h incubation. Then, 20 μl of 5 mg/ml MTT (Gracia Chemical Technology Co, Ltd., Cheng) were added into each well and incubated for 4h. After careful removal of culture media, 150 μl DMSO were added to fully dissolve purple crystals by shaking for 10

min. The absorbance in each well was measured at 490 nm with an automatic microplate reader (ThermoFisher Instrument Co., Ltd, Shanghai, China). Results were recorded to calculate inhibition rates of tumor cells and IC_{50} of each extract, allowing evaluation of their preliminarily pharmacodynamic effects. The inhibition rate of tumor cells was calculated as follows: inhibition rate of tumor cells (%) =[1-(OD $_{\text{experimental group}}$ – OD $_{\text{blank control group}}$)/(OD $_{\text{negative control group}}$ – OD $_{\text{blank control group}}$)]× 100%

Hoechst 33342 staining

Hoechst 33342 staining detects morphological changes in apoptotic cells. The cells were cultured in 6-well plates with sterilized coverslips in the presence or absence of *E. phaseoloides* n-butanol extract for 24h, washed twice with PBS at room temperature and stained with 10 g/ml Hoechst 33342 (Sigma) diluted in PBS (15 min room temperature in the dark). Thereafter, cells were examined for nuclear changes (i.e., chromatin condensation and nuclear fragmentation) and characteristics of apoptosis under BX 60 fluorescence microscope (Olympus, Japan).

Measurement of mitochondrial transmembrane potential

Mitochondrial transmembrane potential (ΔΨm) dissipation was measured by using JC-1 probe (Beyotime, Jiangsu, China) by fluorescence microscopy. JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (525 ± 10 nm) to red (610 ± 10 nm). Mitochondrial transmembrane potential depolarization is specifically indicated by a decrease in the red-to-green fluorescence intensity ratio [18]. After treatment with different concentrations of *E. phaseoloides* n-butanol extract for 12h, cells were harvested and incubated with 10 μg/ml JC-1 for 30 min at 37 °C, and observed under a BX 60 fluorescence microscope (Olympus, Japan).

Statistics

All statistical analyses were conducted using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as means±standard deviation (SD) of three independent experiments. Statistical significance was evaluated by one-way analysis of variance (ANOVA) with Student-Newman-Keuls (SNK) test used for *post hoc* analysis. A p-value less than 0.05 was considered statistically significant.

Results

Effect of water or ethanol extracts of the medicinal plant materials on HepG2 cell proliferation

Table 1 summarizes the inhibition rates and derived IC_{50} obtained after treatment of HepG2 cells with various extracts for 48h. Compared with all the other medicinal plant extracts, 70% ethanol extract of *E. phaseoloides* showed the strongest inhibition on HepG2 cell proliferation. Indeed, E. phaseoloides ethanol extract inhibited cell proliferation in a dose-dependent manner, with 100, 250 and 500 μg/ml resulting in 85.2±0.7, 95.2±0.3, and 96.2±0.6% inhibition of HepG2 cell proliferation, respectively. The data indicated an IC_{50} value of 9.3 μg/ml for this extract, significantly lower than the values obtained for extracts from other plants. Therefore, 70% ethanol extract of *E. phaseoloides* was selected for subsequent studies. In these experiments, cisplatin treatment (20 μg/ml) resulted in 78.4% inhibition of HepG2 cell growth.

Effect of different E. phaseoloides ethanol extract fractions on HepG2 cell proliferation

The different *E. phaseoloides* ethanol extract *fractions* were tested for their inhibitory activity on HepG2 cells, and the results are summarized in Table 2. We found that chloroform and petroleum ether fractions displayed low inhibition rates on HepG2 cells even at high concentrations of 500 μg/ml (< 50%) while treatment with ethyl acetate and n-butanol fractions resulted in higher HepG2 cytotoxicity. Of the latter two, the butanol fraction was the most effective causing 89.9 ± 0.6, 81.7±0.4 and 68.8±0.6% cell death when used at 500, 250 and 100 μg/ml, respectively, a dose-dependent effect. These data indicated an IC_{50} of only 9.3 μg/ml in HepG2 cells for the n-butanol fraction. In these experiments, cisplatin treatment (20 μg/ml) resulted in 78.4% inhibition of HepG2 cell growth.

Table 1. Inhibition of HepG2 proliferation by various extracts

Concentration (μg/ml)	*Water extract of H. lobata*	*70% ethanol extract of H. lobata*	*90% ethanol extract of H. lobata*	*Water extract of Bidens*	*Water extract of Smilax*	*70% ethanol extract of Plantago*	*70% ethanol extract of E. phaseoloides*
500	32.6±0.4*	65.1±0.5*	63.1±0.4*	30.9±0.6*	10.9±0.6*	45.2±0.6*	96.2±0.6
250	27.6±0.4*	35.3±0.3*	51.4±0.4*	19.6±0.3*	9.7±0.4*	5.2±0.5*	95.2±0.3
100	26.1±0.4*	11.4±0.5*	43.6±0.3*	47.0±0.3*	68.8±0.6*	5.2±0.6*	85.2±0.7
IC_{50}	54.2	38.9	24.9	35.6	49.3	48.3	9.3

Data are expressed in % inhibition as means±standard deviation (SD) of three independent experiments. *p< 0.05 vs *E. phaseoloides* ethanol extract

Table 2. Effect of different *E. phaseoloides* ethanol extract fractions on HepG2 cell proliferation

Concentration (μg/ml)	*70% ethanol extract*	*Petroleum ether*	*Chloroform*	*Ethyl acetate*	*N-butanol*
500	28.5±0.4*	18.5±0.4*	43.1±0.3*	54.9±0.5*	89.9±0.6
250	27.2±0.4*	35.2±0.3*	21.4±0.4*	49.6±0.3*	81.7±0.4
100	18.5±0.4*	11.4±0.5*	43.6±0.3*	47.0±0.3*	68.8±0.6
IC_{50}	24.2	28.9	29.2	15.6	9.3

Data are expressed in % as means±standard deviation (SD) of three independent experiments. *p < 0.05 vs *E. phaseoloides* n-butanol extract

E. phaseoloides n-butanol extract inhibited HepG2 cell proliferation by inducing cell apoptosis likely through mitochondrial apoptosis pathway

After Hoechst 33342 staining, HepG2 cells treated with 100 μg/ml *E. phaseoloides* n-butanol extract showed apoptotic nuclei and hyperchromatic, dense fluorescent massive granules in the cytoplasm (Figure 1). Interestingly, this effect was concentration-dependent, the fluorescence intensity increasing with the extract concentration.

In JC-1 probe detection, the red fluorescence intensity gradually decreased and the green fluorescence intensity was gradually increased, with increase in extract concentration, indicating that *E. phaseoloides* n-butanol extract was able to induce depolarization of mitochondrial membrane potential (Figure 2).

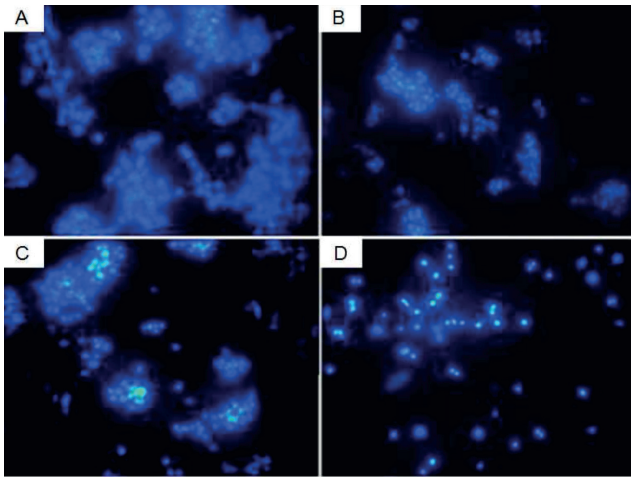

Figure 1. *Entada phaseoloides* butanol extract inhibited HepG2 cell proliferation by inducing apoptosis. HepG2 cells were treated with 100 **(B)**, 250 **(C)**, 500 **(D)** μg/ml *Entada phaseoloides* butanol extract for 24h. DMSO 0.05% **(A)** was used as negative control. The morphology of apoptotic cells was determined by Hoechst 33342 staining (magnification ×200). The cells were examined for nuclear changes (i.e., chromatin condensation and nuclear fragmentation), characteristic of apoptosis under a BX 60 fluorescence microscope.

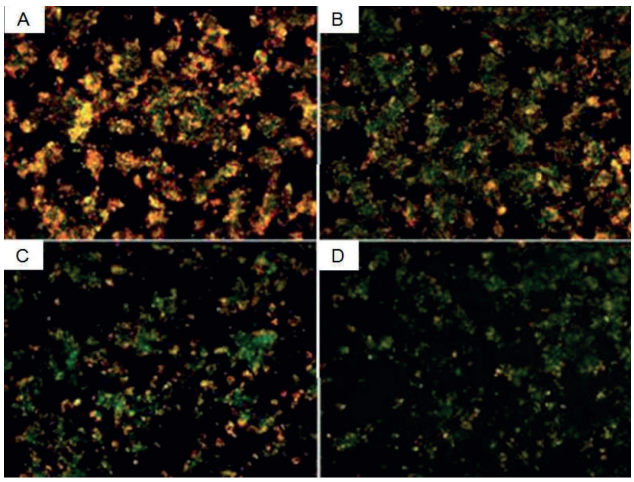

Figure 2. *Entada phaseoloides* butanol extract induced mitochondrial transmembrane potential depolarization. HepG2 cells were treated with 100 **(B)**, 250 **(C)**, 500 **(D)** μg/ml *Entada phaseoloides* butanol extract for 12h. DMSO 0.05% **(A)** was used as negative control. Mitochondrial transmembrane potential (ΔΨm) dissipation was measured by JC-1probe (magnification ×200). Depolarization of mitochondrial transmembrane potential is specifically indicated by a decrease in the red-to-green fluorescence intensity ratio.

Discussion

HCC, the most common primary malignancy of the liver and the 6th most common malignancy worldwide [1,2], continues to be a challenge to public health. Therefore, there is a growing body of research studies aiming to find effective antitumor drugs with low toxicity from natural traditional medicines. Humans have used plants as therapeutics for ages and indeed many plants have been shown to possess anticancer properties, including multiple examples found in Chinese medicine [19-21].

The Chinese Hainan province has a unique maritime climate environment with rich resources including the traditional Hainan and Li nationality medicines. Therefore, new antitumor drugs can be developed using these medicinal resources. In this study, some natural medicinal plants in Hainan including *Heliciopsis lobata, Bidens, Entada phaseoloides, Plantago*, and *Smilax* were assessed for their antitumor activities on HepG2 cells. We found that *E. phaseoloides* and *H. lobata* ethanol extracts showed pronounced inhibitory activity on tumor cells proliferation, and low antitumor activity for *Heliciopsis lobata* and *Bidens* water extracts. *Plantago* ethanol extract showed almost no

antitumor activity, a finding not consistent with the literature [9,10] which needs further investigation.

In popular medicine *E. phaseoloides* is mainly used for the treatment of rheumatism and gastrointestinal diseases during which no significant adverse reactions have been observed. Studies have shown that *E. phaseoloides* possesses strong antiviral and antifungal activities [22,23]. In our study it was demonstrated that *E. phaseoloides* ethanol extract displays pronounced inhibition of HepG2 cell proliferation in a dose-dependent manner, with IC_{50} of its n-butanol fraction lower than 10 μg/ml. This is likely due to the rich content in alkaloids in this fraction. Interestingly, antidiabetic properties of total saponins from *E. phaseoloides* (L.) were reported [24]. Whether the n-butanol fraction studied here possesses such compounds is to be determined. Our data demonstrated potent inhibitory effects of *E. phaseoloides* ethanol extract fractions on tumor cell proliferation. Furthermore, the butanol fraction induced apoptosis in HepG2 cancer cells in a concentration-dependent manner.

The membrane-permeant JC-1 dye is widely used in apoptosis studies to monitor mitochondrial health. In recent years, mitochondria have been given a central role in the programmed cell death control: Bcl-2 proteins are thought to maintain cell survival likely by dragging caspases into the mitochondrial membrane, or alternatively, Bcl-2 would regulate the release of some caspases activators from mitochondria [25]. In addition, mitochondria contribute to apoptosis signaling via the production of reactive oxygen species [26]. With increase in *E. phaseoloides* n-butanol extract concentration, decreased red fluorescence accompanied by increased green fluorescence intensity were observed, indicating depolarization of mitochondrial transmembrane potential by these extracts, a possible mechanism for the extract-induced apoptotic pathway.

Further studies are needed to isolate the compound(s) responsible for these effects. These findings provide a rational basis for the exploitation of *E. phaseoloides* in the treatment of HCC.

Acknowledgements

This study was supported by the Project for Modernization of Traditional Chinese Medicine of Hainan Province in 2012 (Hainan Finance and Education grant 2012, 614).

References

1. Fares N, Peron JM. Epidemiology, natural history, and risk factors of hepatocellular carcinoma. La Revue du praticien 2013;63:216-217.
2. Montalto G, Cervello M, Giannitrapani L et al. Epidemiology, risk factors, and natural history of hepatocellular carcinoma. Ann New York Acad Sci 2002;963:13-20.
3. Chen LP, Zhao J, Du Y et al. Antiviral treatment to prevent chronic hepatitis B or C-related hepatocellular carcinoma. World J Virol 2012;1:174-183.
4. Keane MG, Pereira SP. Improving detection and treatment of liver cancer. The Practitioner 2013;257:21-26.
5. Pons F, Varela M, Llovet JM. Staging systems in hepatocellular carcinoma. Int Hepatopancreatobil J 2005;7:35-41.
6. Wang Z, Li J, Ji Y et al. Traditional herbal medicine: a review of potential of inhibitory hepatocellular carcinoma in basic research and clinical trial. Evidence-based complementary and alternative medicine: eCAM 2013;2013:268963.
7. Liu M, Kong L, Fong WF et al. A new phenolic glucoside from the leaves of Heliciopsis lobata. Fitoterapia 2008;79:398-399.
8. Bartolome AP, Villasenor IM, Yang WC. Bidens pilosa L. (Asteraceae): Botanical Properties, Traditional Uses, Phytochemistry, and Pharmacology. Evidence-based complementary and alternative medicine : eCAM 2013;2013:340215.
9. Gupta MP, Monge A, Karikas GA et al. Screening of Panamanian Medicinal Plants for Brine Shrimp Toxicity, Crown Gall Tumor inhibition, Cytotoxicity and DNA intercalation. Pharmacol Biol 1996;34:19-27.
10. Samuelsen AB, Rieder A, Grimmer S et al. Immunomodulatory activity of dietary fiber: arabinoxylan and mixed-linked Beta-glucan isolated from barley show modest activities in vitro. Int J Mol Sci 2011;12:570-587.
11. Ozaslan M, Didem Karagoz I, Kalender ME et al. In vivo antitumoral effect of Plantago major L. extract on Balb/C mouse with Ehrlich ascites tumor. J Chin Med 2007;35:841-851.
12. Li YL, Gan GP, Zhang HZ et al. A flavonoid glycoside isolated from Smilax china L. rhizome in vitro has anticancer effects on human cancer cell lines. J Ethnopharmacol 2007;113:115-124.
13. Xu W, Liu J, Li C et al. Kaempferol-7-O-beta-D-glucoside (KG) isolated from Smilax china L. rhizome induces G2/M phase arrest and apoptosis on HeLa

cells in a p53-independent manner. Cancer Lett 2008;264:229-240.

14. Dawane JS, Pandit VA, Rajopadhye BD. Experimental evaluation of anti-inflammatory effect of topical application of entada phaseoloides seeds as paste and ointment. North Am J Med Sci 2011;3:513-517.
15. Ikegami F, Sekine T, Aburada M et al. Synthesis of entadamide A and entadamide B isolated from Entada phaseoloides and their inhibitory effects on 5-lipoxygenase. Chem Pharmaceut Bull 1989;37:1932-1933.
16. Mo S, Xiong H, Shu G et al. Phaseoloideside E, a novel natural triterpenoid saponin identified from Entada phaseoloides, induces apoptosis in Ec-109 esophageal cancer cells through reactive oxygen species generation. J Pharmacol Sci 2013;122:163-175.
17. Mosmann T. Rapid colorimetric assay for cellular growth and survival:application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.
18. Ying M, Tu C, Ying H et al. MSFTZ, a flavanone derivative,induces human hepatoma cell apoptosis via a reactive oxygen species- and caspase-dependent mitochondrial pathway. J Pharmacol Exp Ther 2008;325:758-765.
19. Dong Y, Lu B, Zhang X et al. Cucurbitacin E, a tetracyclic triterpenes compound from Chinese medicine, inhibits tumor angiogenesis through VEGFR2-mediated Jak2-STAT3 signaling pathway. Carcinogenesis 2010;31:2097-2104.
20. Efferth T, Fu YJ, Zu YG et al. Molecular target-guided tumor therapy with natural products derived from traditional Chinese medicine. Curr Med Chem 2007;14:2024-2032.
21. Efferth T, Kahl S, Paulus K et al. Phytochemistry and pharmacogenomics of natural products derived from traditional Chinese medicine and Chinese materia medica with activity against tumor cells. Mol Cancer Ther 2008;7:152-161.
22. Cos P, Hermans N, De Bruyne T et al. Further evaluation of Rwandan medicinal plant extracts for their antimicrobial and antiviral activities. J Ethnopharmacol 2002;79:155-163.
23. Olajide OA, Alada AR. Studies on the anti - inflammatory properties of Entada abyssinica. Fitoterapia 2001;72:492-496.
24. Zheng T, Shu G, Yang Z et al. Antidiabetic effect of total saponins from Entada phaseoloides (L.) Merr. in type 2 diabetic rats. J Ethnopharmacol 2012;139:814-821.
25. Mignotte B, Vayssiere JL. Mitochondria and apoptosis. Eur J Biochem 1998;252:1-15.
26. Fogg VC, Lanning NJ, Mackeigan JP. Mitochondria in cancer: at the crossroads of life and death. Chin J Cancer 2011;30:526-539.