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...
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% CO₂ at 37 ºC 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 CO₂ and CO₂ modified with 1%, 5%, and 10% ethanol (v/v) at 60 ºC 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 10⁴ 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 controls. 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 and DMSO were added to fully dissolve purple crystals by shaking for 10
Entada phaseoloides in hepatocellular carcinoma

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 experimental group – OD blank control group) / (OD negative control group – OD blank control group)] × 100%. Data are expressed in % inhibition as means±standard deviation (SD) of three independent experiments. *p< 0.05 vs JBUON 2014; 19(2): 408

### Results

#### Table 1. Inhibition of HepG2 proliferation by various extracts

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>Water extract of H. lobata</th>
<th>70% ethanol extract of H. lobata</th>
<th>90% ethanol extract of H. lobata</th>
<th>Water extract of Bidens</th>
<th>Water extract of Smilax</th>
<th>70% ethanol extract of Plantago</th>
<th>70% ethanol extract of E. phaseoloides</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>32.6±0.4*</td>
<td>65.1±0.5*</td>
<td>63.1±0.4*</td>
<td>30.9±0.6*</td>
<td>10.9±0.6*</td>
<td>45.2±0.6*</td>
<td>96.2±0.6*</td>
</tr>
<tr>
<td>250</td>
<td>27.6±0.4*</td>
<td>55.3±0.5*</td>
<td>51.4±0.4*</td>
<td>19.6±0.3*</td>
<td>9.7±0.4*</td>
<td>5.2±0.5*</td>
<td>95.2±0.3</td>
</tr>
<tr>
<td>100</td>
<td>26.1±0.4*</td>
<td>41.4±0.5*</td>
<td>45.6±0.3*</td>
<td>47.0±0.3*</td>
<td>68.8±0.6*</td>
<td>5.2±0.6*</td>
<td>85.2±0.7</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>54.2</td>
<td>38.9</td>
<td>24.9</td>
<td>35.6</td>
<td>49.3</td>
<td>48.3</td>
<td>9.3</td>
</tr>
</tbody>
</table>

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

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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).

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 Helicopsis 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 Helicopsis lobata and Bidens water extracts. Plantago ethanol extract showed almost no

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

<table>
<thead>
<tr>
<th>Concentration (μg/ml)</th>
<th>70% ethanol extract</th>
<th>Petroleum ether</th>
<th>Chloroform</th>
<th>Ethyl acetate</th>
<th>N-butanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>500</td>
<td>28.5±0.4*</td>
<td>18.5±0.4*</td>
<td>45.1±0.5*</td>
<td>54.9±0.5*</td>
<td>89.9±0.6</td>
</tr>
<tr>
<td>250</td>
<td>27.2±0.4*</td>
<td>35.2±0.3*</td>
<td>21.4±0.4*</td>
<td>49.6±0.3*</td>
<td>81.7±0.4</td>
</tr>
<tr>
<td>100</td>
<td>18.5±0.4*</td>
<td>11.4±0.5*</td>
<td>43.6±0.3*</td>
<td>47.0±0.3*</td>
<td>68.8±0.6</td>
</tr>
<tr>
<td>IC_{50}</td>
<td>24.2</td>
<td>28.9</td>
<td>29.2</td>
<td>15.6</td>
<td>9.3</td>
</tr>
</tbody>
</table>

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

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-1 probe (magnification ×200). Depolarization of mitochondrial transmembrane potential is specifically indicated by a decrease in the red-to-green fluorescence intensity ratio.
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<sub>50</sub> 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**


