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Screening of Antitumor and Hepatoprotective Activity Components from *Hedyotis diffusa*

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Hedyotis diffusa is a traditional Chinese medicine used for the treatment of tumor and liver injury in clinic. But what are active ingredients that play the significant role in anticancer and liver protection are not yet know. The aim of this study was to screening for the effective components which had both antitumor and hepatoprotective activity in *Hedyotis diffusa*. The ethanol extract, ethyl acetate extract, total flavonoids and total triterpenoids from *Hedyotis diffusa* were prepared, and the antitumor activity and hepatoprotective effects of four extracts were screened by SRB method. The experimental results showed that four extracts could inhibit the proliferation to hepatoma carcinoma HepG-2 cells, and the total flavonoids had the strongest inhibitory effect on HepG-2. The inhibition rate of HepG-2 cells by 1000 μg/mL total flavonoids could reach 84.9 ± 2.7%. Only total flavonoids and total triterpenoids had protective effects on normal human hepatic HL-O2 cells, and the protective ratio of total flavonoids to HL-O2 was higher than that of total triterpenoids at the same concentration. In further experiments, five components including amentoflavone, quercetin, naringenin, kaempferol and rutin were selected from total falvonoids and used to screen for the antitumor activity and hepatoprotective effects. Furthermore, the amentoflavone and quercetin, which have both anticancer and hepatoprotective effects, were selected for further study. Finally, the selectivity of amentoflavone to liver cancer HepG-2 and normal human liver HL-O2 cells is higher than quercetin. Therefore, it is concluded that the main active components of *Hedyotis diffusa* against cancer and protect liver are total flavonoids and amentoflavone.

Keywords: Screening, antitumor activity, hepatoprotective activty, *Hedyotis diffusa*

INTRODUCTION

Hedyotis diffusa is an annual herb belonging to the genus Rubiaceae, mainly distributed in the south of China and some Asian countries, such as Indonesia, Japan, Malaysia, Nepal, Philippines, Sri Lanka, Thailand [1], and so on. *Hedyotis diffusa* is a traditional Chinese medicinal with long history. It was first reported that the *Hedyotis diffusa* has the effects of clearing heat, detoxifying, anti-inflammatory, anti-tumor [2-4], liver protection [5], antioxidant [6], and immune regulation [7] in "Guangxi Traditional Chinese Medicine". It was widely used for the treatment of tumor and liver injury in clinic up to now [8-10]. The liver is the most important metabolic organ of the human body [11]. When the liver was continuously exposed to alcohol, viruses, fat, harmful molecular metabolites and other factors that could cause liver damage and then it will lead to inflammation and liver function decline. Long-term liver damage can develop into chronic liver disease, which ultimately leads to liver cancer. The occurrence and development of liver disease were faced with a great threat to the maintenance of normal human activities. On the other hand, China's tumor morbidity and mortality have shown a clear upward trend according to statistics, and cancer had became a serious public health problem. Due to the harmfulness of these two types of diseases (tumor and liver injury), the development of both anti-tumor and liver protective effects drugs had become a hot spot in human research. *Hedyotis diffusa* contains various active ingredients which simultaneously has the anti-tumor and the liver protection efficacy, including terpenoids, flavonoids, anthraquinones, alkaloids, organic acids, poly-saccharides, etc. The aim of this study was to find active ingredients with both anti-tumor and hepatoprotective activity in *Hedyotis diffusa*. The ethanol extract, ethyl acetate extract, total flavonoids and triterpenoids from *Hedyotis diffusa* were prepared through solvent extraction method, and the antitumor activity and hepatoprotective effects of four extracts were screened by Sulforhodamine B (SRB) method.

SRB test has been used as a common method for detecting cell proliferationand the inhibition rate of HepG-2 and the protection rate of HL-O2 by four different extracts including alcohol extract, ethyl acetate extract, total flavonoids and total triterpenoids. Screening out active ingredients with anti-cancer and liver-protecting activities from *Hedyotis diffusa*, which will be instructive for future mechanism of action work.

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EXPERIMENTAL

Plat materials, Chemicals and reagents

Hedyotis diffusa was collected from Yangshuo, Guangxi and certified by Professor Tianai Gao from Shanxi Provincial Food and Drug Inspection Institute. Rutin (20140719), ursolic acid (20130321), kaempferol (20140710) and quercetin (20140716) were obtained from Tianjin Shilan Technology Co., Ltd. Amentoflavone (JZ15030111), naringenin (JZ15011710) were obtained from Nanjing Jingzhu Biotechnology Co., Ltd. Macroporous adsorbent resin AB-8 and polyamide resin were purchased from Chengdu Grecia Chemical Technology Co., Ltd. Cell culture media Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), PBS and pancreatin were obtained from Wuhan Dr. Bioengineering Co., Ltd. DMSO and Tris were purchased from Solarbio (Beijing, China). SRB and hydrogen peroxide were purchased from Sigma Aldrich Chemical. All the other reagents used were of high quality.

Preparation of alcohol extract and ethyl acetate extract

The *Hedyotis diffusa* was cut into small pieces about 5 mm and each 50 g of them was refluxed by 800 mL of 70% ethanol. The extraction solution was freeze-dried to obtain alcohol extracts. And then the alcohol extracts were made into an aqueous solution and extracted with ethyl acetate. After freeze-drying, ethyl acetate extracts were obtained.

Preparation of total flavonoids

The *Hedyotis diffusa* was cut into small pieces about 5 mm and each 50 g of them was refluxed by 800 mL of 70% ethanol about 2 hours. The filtrate of alcohol extract from *Hedyotis diffusa* was obtained by hot suction filtration. After cooling to room temperature, extracts with petroleum ether until the their layer was colorless. The aqueous layer solution was concentrated by rotary evaporator (DL-400, zhenzhou changcheng Co., Ltd) at 60°C under reduced pressure. The prepared aqueous solution was purified by macroporous adsorption resin [12-13] and polyamide resin (Chengdu grecia chemical technology co. LTD), and finally the total pure flavonoid extracts of *Hedyotis diffusa* were obtained.

Preparation of total triterpenoids

Extraction with 70% ethanol reflux (v / v, 1 : 10) was performed for 3 times for each 50 g of *Hedyotis diffusa*. The pH of extraction to 12 was adjusted with sodium hydroxide solution and extracted with petroleum ether until the ether layer was nearly colorless, the water layer was taken. The pH of the water layer was adjusted from 2 to 3 with hydrochloric acid solution, and extracted with ethyl acetate until the ethyl acetate layer was nearly colorless, and the ethyl acetate layer was taken [14]. After purification by macroporous adsorption resin, the triterpene extracts of *Hedyotis diffusa* could be obtained.

Cell Lines and Cell Culture

The human cell lines used in this study were liver cancer hepatocellular carcinomas (HepG-2) cells and normal human hepatocyte cell line (HL-O2) cells, which were purchased from Wuhan Boster Biological Engineering Co., Ltd. (Wuhan, PR China). HepG-2 and HL-O2 cells were cultured in DMEM added with 10 % heat-inactivated FBS, 100 $U \cdot mL^{-1}$ penicillin and 100 $\mu g \cdot mL^{-1}$ streptomycin in a constant temperature incubator with 5% CO_2 at 37°C. Passage was digested with 0.25% trypsin. According to cell growth, subculture was carried out for 2 to 3 days to ensure that all cells used for the experiment were in a logarithmic growth phase.

SRB assay

The inhibitory rate of liver cancer HepG-2 cells and the protection rate of normal human liver HL-O2 cells were determined by SRB method to compare the effects of different extracts. HL-O2 and HepG-2 cells in logarithmic growth phase were digested with trypsin and seeded in 96-well plates at a density of 2×10^5 cells per well until cells attachment. The cells were treated with different extracts from *Hedyotis diffusa* (ethanol extracts, ethyl acetate extracts, total flavonoids and total triterpenoids) at 0, 62.5, 125, 250, 500 and 1000 μg/mL or five compounds in total flavonoids (amentoflavone, quercetin, naringenin, kaempferol, rutin) at 31.25, 62.5, 125, 250 and 500 μmol/L for 24 h, respectively. The cells were fixed with 50 μL of 30% trichloroacetic acid at 4°C for 1 h. The supernatant was discarded and the wells were rinsed 5 times with deionized water. After air drying, the cells were cultured in 100 μL of 0.4% SRB dissolved in 1% acetic acid for 30 min, washed with

5% acetic acid to remove the uncombined SRB colorant, air dried and dissolved in 10 mmol/L trisbase solution (pH 10.5). After blending, the absorbance (OD) values were measured at 515 nm by using a microplate reader (Thermo Scientific, USA). Cell inhibitory rates were calculated as the following formula: Inhibitory rates % = (absorbance of untreated cells-absorbance of treated cells) / absorbance of untreated cells × 100%.

Statistical Analysis

The dates were expressed in standard deviation (X ± SD). *T*-test analysis was used to compare the differences between different groups. When $p<0.05$, there was a statistical difference. All experiments were repeated three times in parallel.

RESULTS AND DISCUSSIONS

Result of total flavonoid purity determination

With UV full wavelength scanning from 400 to 800 nm, rutin has maximum absorption at 501 nm showed in Fig1. The standard curve was prepared with rutin as the standard. The linear regression equation was $Y = 10.88\ X + 0.0043$, r = 0.9984.

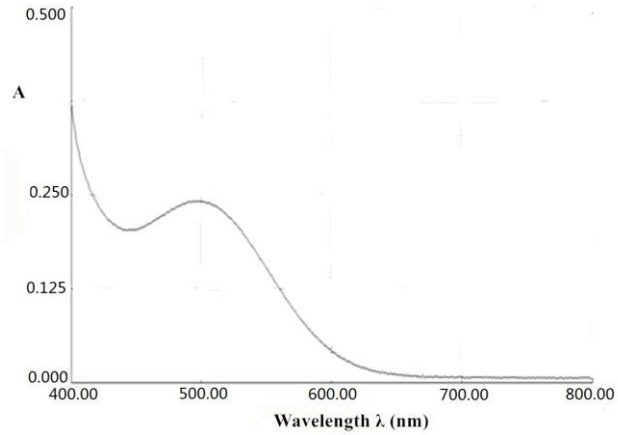

Fig.1. UV wavelength scanning for rutin

Calculate the total flavonoid purity according to the following formula [15]: Total flavonoids purity (%) = $DY / W \times 100\ \%$, D represents dilution multiple and W is sample quantity. The results were shown in Table 1, the purity of total flavonoids determined is 65.6 %.

Table 1. Determination of the purity of total flavonoids

No.	sample quantity (mg)	Absorbance	Purity (%)	Average purity (%)	RSD(%)
1	15.0	0.429	65.0		
2	15.0	0.437	66.3	65.6	0.66
3	15.0	0.432	65.5		

Result of total triterpenoid acid purity determination

With UV full wavelength scanning from 400 to 800 nm, ursolic acid has maximum absorption at 548 nm showed in Fig.2. The standard curve was prepared with ursolic acid as the standard. The linear regression equation was $Y = 5.9203\ X - 0.01$, r=0.9994.

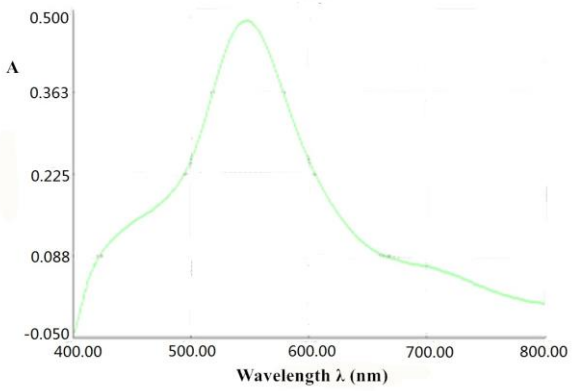

Fig.2. UV wavelength scanning for ursolic acid

Calculate the purity of total triterpenoid acid according to the following formula: Total triterpenoid acids purity (%) = $DY / W \times 100\ \%$, D represents dilution multiple, and W is sample quantity. The results are shown in Tab.2, the purity of total triterpenoid acids determined was 22.0 %.

Table 2. Determination of the purity of total triterpenoid acids

No.	sample quantity (mg)	Absorbance	Purity (%)	Average purity (%)	RSD (%)
1	25.0	0.415	23.4		
2	25.1	0.390	21.9	22.0	1.35
3	25.1	0.369	20.7		

Concentration selection of H_2O_2 Modeling

The inhibition rates of H_2O_2 at different concentrations (50 ~ 600 μmol/L) on HL-O2 cells were shown in Fig.3. The results showed that the survival rate of HL-O2 cells decreased with the increas of H_2O_2 concentration. When the concentration of H_2O_2 was 100 μmol/L, the survival rate of HL-O2 cells was significantly lower than that of the control group ($p < 0.05$). When the concentration of H_2O_2 was 200 μmol/L , the cell inhibition rate was about 60.0%. This concentration of H_2O_2 was selected for modeling.

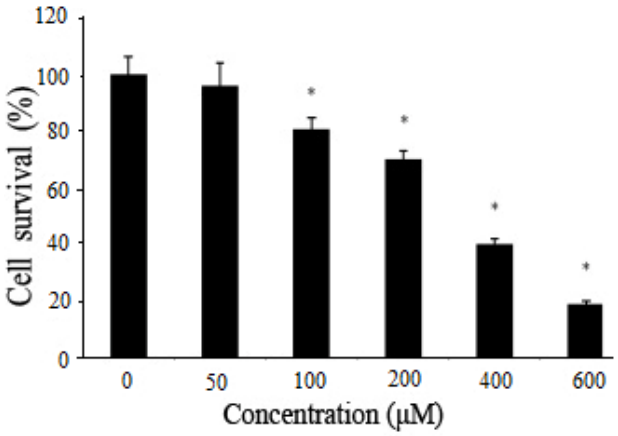

Fig.3. Cell survival rate of HL-O2 cells by different concentrations of H_2O_2. *($p < 0.05$) indicates that there is a significant difference in the survival rate of the H_2O_2 dosing group compared with the control group

Activity screening of four different extracted parts

The anticancer activities of alcohol extracts, ethyl acetate extracts, total flavonoids and total triticecarboxylic acid were shown in Fig.4 and the liver protective activitiies were shown in Fig.5. The results showed that the total flavonoids had the highest anticancer activity in the four extracts. The inhibitory rate of the total flavonoids on HepG-2 cells was 84.9±2.7%. Among the four extracts, only total flavonoids and total triterpenoid acids had hepatoprotective activities and the protective rates of 125 μg/mL and 250 μg/mL total flavonoids on HL-O2 cells were significantly higher than that of total triterpenoid acids at the same concentration ($p < 0.05$). In view of the fact that total flavonoids have the best anticancer activity and hepato-protective activity in the four extracts, total flavonoids were identified as preferred sites. Compounds in total flavonoids were selected for further screening for liver protection and anticancer activity.

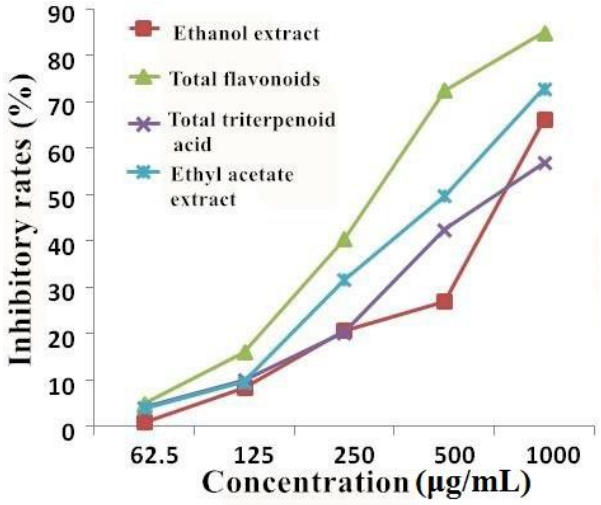

Fig.4. Inhibitory rates of four extracts on HepG-2 cells

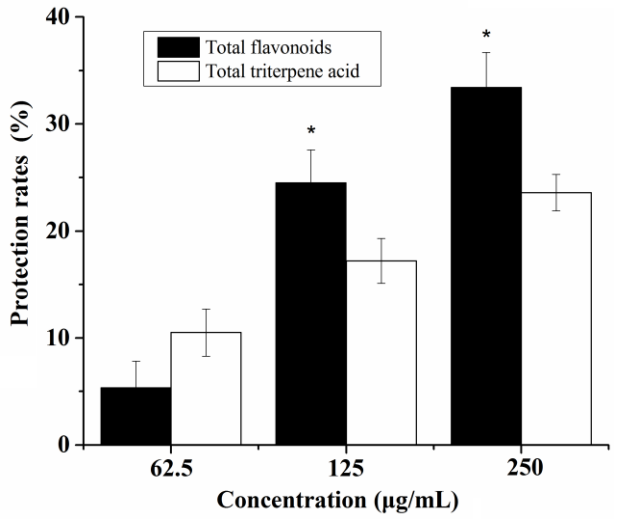

Fig.5. Protective rates of total flavonoids and total triterpenoid acids on HL-O2 cells. *($p < 0.05$) showed that there was a significant difference in the protective rate of total flavonoids compared with total triterpenoid acids

Activity screening of five different compounds in total flavonoids

Amentoflvone, quercetin, rutin, naringenin and kaempferol from total flavonoids were selected to screen for liver protection and anticancer activity. The liver protection activity was shown in Tab.3. From this results, it could be found that the compounds with better liver protective effect were amentoflavone and quercetin. The results showed that the protection rates of amentoflavone and quercetin on HL-O2 cells were higher than that

of other components. And the protection rate of 250 μmol/L amentoflavone was significantly higher than that at the same concentration of quercetin and 125 μmol/L bifendate ($p < 0.05$) (Fig.6).

Table 3. Protective effects of different flavonoids on HL-O2 cells

Compounds	Protection (%)		
	62.5μmol/L	125μmol/L	250μmol/L
Amentoflavone	6.5±1.3	19.6±3.7	40.7±2.0
Quercetin	5.0±1.8	25.1±2.3	3.0±1.2
Rutin	9.4±2.4	—	—
Bifendate		10.7±3.0	

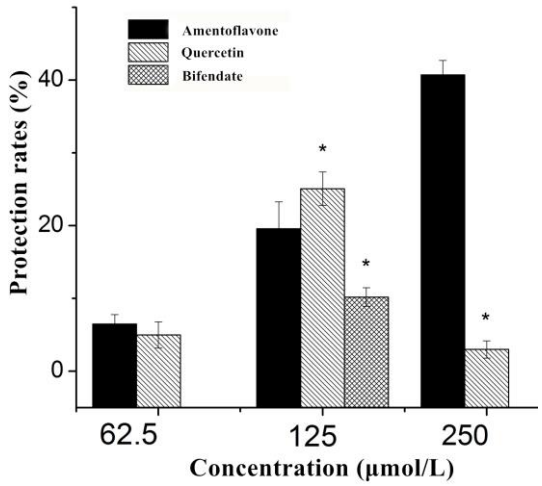

Fig.6. Protective rates of amentoflavone, quercetin and bifendate on HL-O2

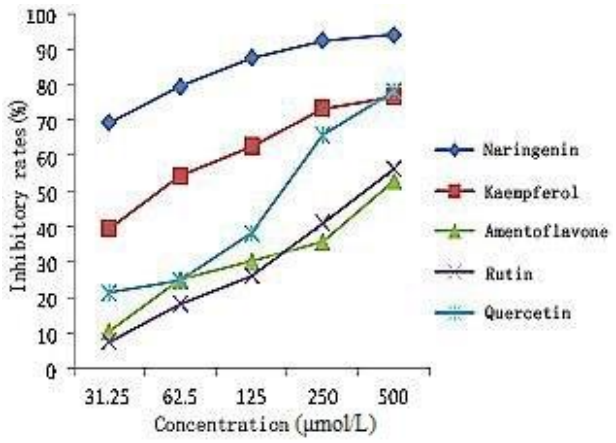

Fig.7. Inhibitory rates of five flavonoids on HepG-2 cells

Screening results of anticancer activities of Amentoflavone, quercetin, naringenin, kaempferol and rutin were shown in Fig.7. The inhibitory rates (%) of 500 μmol/L amentoflavone and quercetin on HepG-2 cells were 56.2±8.1 % and 78.0±9.3 %, respectively. It can be seen from Fig.7 and Tab.3 that the compounds with anticancer activity and liver protective activity were quercetin and amentoflavone. HL-O2 and HepG-2 cells were treated with quercetin and amentoflavone for 48 h. The cell selectivity results were shown in Fig.8. From the selectivity results, it was found that the selectivity of amentoflavone was higher than that of quercetin at 500, 250, 125 and 62.5 μmol/L. Therefore, amentoflavone was selected as the preferred compound.

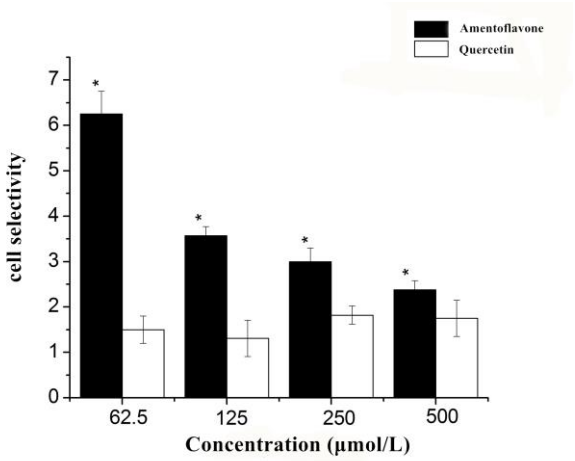

Fig.8. Results of selection of compounds. *(p<0.05) indicates a significant difference compared to quercetin

CONCLUSIONS

The inhibitory effect on HepG-2 cells and the protective effect on HL-O2 cells of different extracts from *Hedyotis diffusa* were investigated in this experiment. Ethanol extracts, ethyl acetate extracts, total flavonoids and total triterpenoids were separately prepared. The purity of total flavonoids could reach 65.6 %, and the purity of total triterpenic acids could reach 22.0 %. According to the screening results, all four extracts had inhibitory effect on HepG-2 cells, and the total flavonoids had the highest anticancer activity among the four extracts, and the inhibition rate of 1000 μg/L total flavonoids on HepG cells could be up to 84.9±2.7%. And only the total flavonoids and total triterpenoid acids in the four extracts had hepatoprotective activity, and the protective rates of total flavonoids of 125 and 250 μg/mL on HL-02

cells were significantly higher than that the same concentration of total triterpenoid acids. Since the total flavonoids had the best anticancer activity and hepatoprotective activity, total flavonoids were selected for further activity screening. Amentoflavone, quercetin, naringenin, kaempferol and rutin were selected from total falvonoids. The results proved that amentoflavone and quercetin had better hepatoprotective activity, followed by rutin, but naringenin and kaempferol had no hepatoprotective activity. What's more, the protection rate of 250 μmol/L amentoflavone was significantly higher than that in the same concentration of quercetin ($p < 0.05$). According to the screening results of anti-tumor activity, the amentoflavone and quercetin also had anticancer activity. The inhibition rates of 500 μmol/L amentoflavone and quercetin on HepG-2 cells could reach 56.2 ± 8.1 % and 78.0 ± 9.3 % respectively. From the cell selectivity results, the selectivity of amentoflavone to HL-O2 and HepG-2 cells at 500, 250, 125, 62.5 μmol/L was significantly different from that of quercetin ($p < 0.05$). Therefore, amentoflavone was screened as the preferred compound. In this experiment, the amentoflavone and total flavonoids in *Hedyotis diffusa* were screened as the active components that both had anticancer activity and liver protective activity, which provided a material basis for further research.

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ABBREVIATIONS

RSD - relative standard deviation;
DMEM - Dulbecco's modified Eagle's medium;
SRB - Sulforhodamine B;
FBS - fetal bovine serum;
HepG-2 - liver cancer cells;
HL-O2 - normal human liver cells;

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