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Antitumor activity of orthogonal array designed extracts from different part of *hedyotis diffusa* on human hepatocellular carcinoma HepG2

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Hedyotis diffusa, the widely applied traditional Chinese medicine have shown the inhibitive effects on different tumor cells. But the problems about how to extract antitumor products and which part was the principal anticancer extracts, are still unclear. Our current study determined the antitumor activity of extracts from different part of Hedyotis diffusa on HepG2 by MTT assay and optimized the extractive conditions by orthogonal array. After choosing the right solvent of water, the index component of p-coumaric acid in *Hedyotis diffusa* was detected by HPLC method to reflect extraction efficiencies. Then the optimal extractive conditions were got through orthogonal arraydesign and different parts of *Hedyotis diffusa* were tested by MTT assay. The results showed that fruit of *Hedyotis diffusa* not only characterized as the highest extraction efficiency of p-coumaric acid but also the highest cell inhibition rate. While the root of *Hedyotis diffusa* showed both the lower extraction efficiency and cell inhibition rate. *Hedyotis Diffusa* aqueous extraction from fruit, herb, stem, leave and roots all showed anticancer activity on HepG2. Different part of *H.diffusa* showed inhibition on HepG2 with a dose-dependent manner, and fruit showed higher inhibition rate, followed by Herb, then the root showed the lowest inhibition ratio. Fruit not only showed highest extraction efficiency of p-coumaric acid but also showed highest cell inhibition rate, probably because the active substance of fruit loss less because of its hard shell protection.

Keywords: Antitumor activity, orthogonal array, *Hedyotis diffusa*, human hepatocellular carcinomaHepG2

INTRODUCTION

Herba Hedyotis diffusa (*H. diffusa*), the dried herb of Oldenlandia diffusa (*Willd.*) Roxb (Family Rubiaceae), is of ficially listed in the Chinese Pharmacopoeia [1]. The herb mainly growing in the south of mainland China is regarded as the traditional herbal medicine for heat-clearing, detoxification and diuresis [2]. It was widely applied in the treatment of in flammations such as appendicitis, urethritis and bronchitis, due to its antibacterial activity. Then, the herb has gained increasingly attention to its usage as an antitumor herb, such as therapy in liver, lung, colon, brain, pancreas and other cancers [3]. Recently there are more and more researches about inhibitive effects of *H.diffusa* on different tumor cells [4,5]. But the problem about how to extract would have higher antitumor activity and which part of *H.diffusa* was the most effective on antitumor activity, are still unclear. Orthogonal array design (DAD) as a chemometric method for the optimization of extraction condition was adopted in this paper, which was based on three factors and four levels ($L_9 3^4$). Before all the works, the optimum solvent of water was chosen. *P*-coumaric acid for one of the active ingredients in *H.diffus*a, according to pharmacological research, showed anti-tumor and anti-cardiovascular effects [6]. What's more, *p*-coumaric acid has good stability which was naturally could be chosen as an index component of extraction process to reflect extraction efficiency and detected by High Performance Liquid Chromatography (HPLC) method.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) test has been widely used as a rapid and sensitive method for screening anticancer drugs. Which extraction from different part of *H. diffusa,* such as herbs, roots, fruits, stems and leaves, has higher inhibition rate of HepG2 were tested by MTT method. All the work aimed at elucidating the different antitumor activity in different part of herb, are very important for the use of this herbal medicine.

EXPERIMENTAL

Plant materials, Chemicals and reagents

The *H. diffusa* and adulterant of Herba Hedyotidis Corymbosae were collected from different places and all herbs were authenticated by the expert from Shan Xi Food and Drug Administration. The herbs were collected from Guangxi province and its lot number was 20111108. The adulterant was from Fuzhou city of Fujian province and its lot number was 20111026. The thin-layer plates of silica gel G was obtained from Qingdao Haiyang Chemical Co. Ltd. The standard *H. diffusa* and *p*-coumaric acid were

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purchased from Chinese food and Drug Testing Institute (Bingjing, China). The purity of *p*-coumaric acid was over 99% by HPLC-PDA analysis. Stock solution of *p*-coumaric acid was prepared in deionized water, stored at 4 °C, and freshly diluted to the desired concentrations before using. Other HPLC-grade for methanol and phosphoric acid were obtained from Tianjin Siyou Co. Ltd. (Tianjin, China). Water used for extraction was double-distilled water and deionized water was used for HPLC analysis. HepG2 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cell culture media Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and phosphate buffer saline (PBS) were obtained from Solarbio (Beijing, China). The other chemicals used, such as 3-[4,5- dimethyllthiazol-2-yl]-2,5-diphenyltetrazoliumbro-mide (MTT), trypsin and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich Chemical (St. Louis, MO).

TLC identification

Weighting dried and powdered samples (1 g) of *H. diffusa* and extracting with 10 mL ethanol under refluxing for 30 min. Then the hot extraction was filtered and ethanol was used to dissolve the residue after drying, from which we could get test solvent. At the same time, we could get the control medicinal solvent with same method. According to TLC experiment, take prepared solvent 5μL and identified simultaneously in the same thin layer plate. The mobile phase was a mixed solvent of ether (30 – 60 °C) : toluene : ethylacetate : Glacial acetic acid (20 : 40 : 14 : 1, v / v / v / v) and sprayed with 10 % sulfuric acid ethanol solution after unrolling. Then the layer was heated in 110 °C until the spots were clear.

HPLC analysis

HPLC analysis was carried out using an Agilent 1200 system (Agilent, California, America). Chromatographic separation of *p*-coumaric and other components were achieved on a Agilent TC-C18 column (4.6 × 250 mm, 5 μm) protected by a SHIMADZU LC C18 guard column (4.6 × 10mm, 5 μm). The mobile phase for HPLC analysis consisted of methanol / 0.1 % aqueous solution of phosphoric acid (30 : 70, v /v, pH 3.0) with a flow rate of 1.0 mL / min. The sample volume of 20 μL was injected. The detection wavelength was determined by UV-visible spectrophotometry detection and diode array detector (DAD). The purity of *p*-coumaric acid was also determined by HPLC-DAD analysis. When detected the purity of *p*-coumaric acid, we investigated the exclusive of chromatographic peaks. Then system suitability was studied by detecting the peak difference of *p*-coumaric acid, *H. diffusa* extraction and adulterant extraction. The component was confirmed from their retention times. For analyzing the concentration of *p*-coumaric acid in extract, a calibration curve was obtained based on the relationship of the concentration of *p*-coumaric acid and the peak-area of the chromophore by linear regression. The intra-day and inter-day repeatability were investigated for consecutive three days at the optimized extraction condition for samples. Standard addition test was performed to determine recoveries of *p*-coumaric acid. In this assay, the standard of *p*-coumaric acid was added to the samples with known content. The resultant samples were analyzed using the developed HPLC method mentioned above. The experiments were repeated three times for every sample. The precision of the method was evaluated with six sequential runs of sample solution. The ratio of measured and known amounts was used to calculate the recovery.

Orthogonal assaydesign

Before all the studies, different solvent (water, methanol and ethanol) were firstly investigated. OAD with a three-factor interaction is an efficient testing strategy in this study. The three factors including the volume of water, extraction time and the times of decoction and their level values were designated in Tab.1.

Table 1. Factors and levels of the orthogonal array design L9 (3^4) matrix

	Factors	
A(Volume of water)	B (Extraction time (h))	C(Times of decoction)
30-fold	1	1
40-fold	2	2
50-fold	3	3

The three-factor, three-level OAD for each factor, which were fixed in the probable working range, was selected to evaluate the extraction ratio of *p*-coumaric acid. Nine experiments were performed in order to estimate the best conditions for the extraction efficiency. Single factors including the volume of water, extraction time and the times of decoction were studied to provide a basis for OAD.

Extraction preparation of H. diffusa

Dried and powdered samples of *H. diffusa* were placed in flask and weighed accurately. Then the herb was refluxed by water. After cooling naturally, weighed flask again and made up loss reduction with double - distilled water. The supernate was filtered through a 0.45 μm filter to directly inject into HPLC system for *p*-coumaric acid analysis. Then the extraction was evaporated to test the cell viability.

Cell culture and Cell viability assay

HepG2 cells were cultured in DMEM supplemented with 10% (v / v) heat-inactivated FBS and 1% (v / v) antibiotic/antimycotic mixture in a humidified incubator aerated with 5% CO_2 at 37°C. When cells reached 70– 80 % confluence, they were trypsinized, counted, and seeded in 96-well culture plates at a concentration of 5×10^6 cells / well. After cultured for 24 h, the medium was replaced by DMEM medium supplemented with 2 % FBS containing various doses of dry extraction. After incubation for the length of time indicated, MTT was added (final concentration 0.5 mg/mL) and kept incubating for 4 h. The formazan crystals were dissolved in 100 μL of DMSO and the plates were read at 570 nm with a microplate reader (Thermo Scientific, USA).

RESULTS AND DISCUSSIONS

Authenticate of herb

The results of TLC were showed in Fig.1.

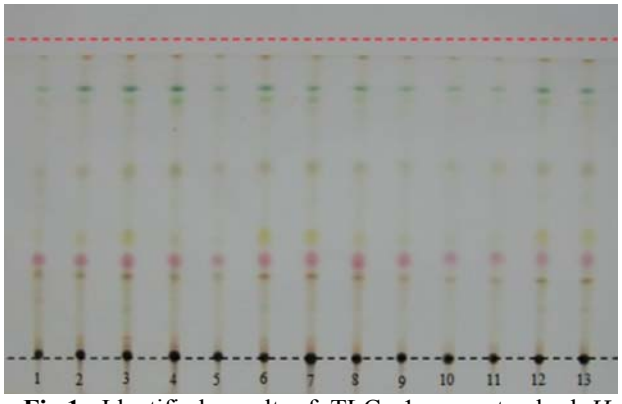

Fig.1. Identified result of TLC. 1 was standard *H. diffusa* 2 -13 was the sample *H. diffusa* of difference batches. The mobile phase was a mixed solvent of toluol (30～60°C) -petroleum ether - ethyl acetate - glacial acetic acid (20 : 40 : 14 : 1), color developing agent was 10 % sulfuric acid and ethanol solution.

After investigating the effect of difference of mobile phase, producers of thin layer plate, spotting way, evolving temperature and relative humidity (result not show), we found that the band of samples corresponding to standard *H. diffusa* was clearly observed and well separated from other components.

Result of HPLC analysis

A reversed-phase mode was used in HPLC for the separation of *p*-coumaric acid and other components for convenience of handling samples. In the proposed method, the separation was tested on C18 column and the flow rate was set at 1.0 mL/min at 25°C. With UV full wavelength scanning from 200 to 400nm, the wavelength of 224nm and 308nm simulated the optimum chromatogram (Fig.2a). When thinking about the end absorptionat 224nm, 308nm was chosen as detection wavelength. The purity of *p*-coumaric acid was over 99.0% by HPLC-DAD analysis (Fig.2b).

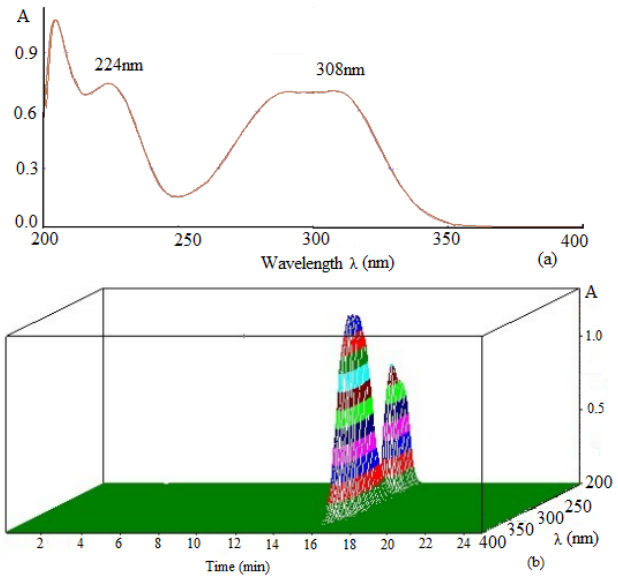

Fig.2. UV wavelength scanning. (a) Measurement results of detecting wavelength by UV spectra of UV-visible spectrophotometry detection. (b) Purity of p-coumaric acid showed in 3D chromatogram by HPLC-PAD detection.

Under the chromatographic condition mentioned above, *p*-coumaric acid and other components could be separated in the chromatograms ($R > 1.5$) completely, and there was no interference with the chromatographic peak of *p*-coumaric acid. Meanwhile, Herba Hedyotidis Corymbosae which is the adulterants of *H. diffus*a was also investigated,

and there was no corresponding chromatographic peak of *p*-coumaric acid (Fig.3).

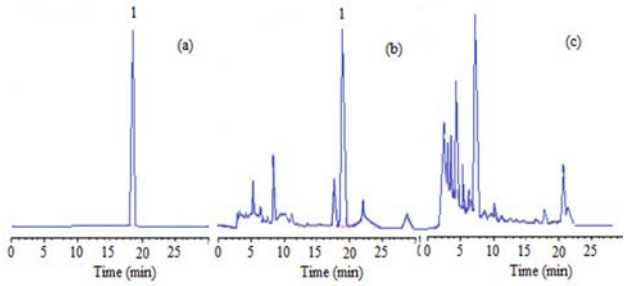

Fig.3. Represent chromatograms of *p*-coumaric acid (a) standard solutions of p-coumaric acid (32.29μg/ml); 1, p-coumaric acid. t_R = 18.327 min; (b) extracted sample (Jiangxi province, 20111023); (c) adulterant of Herba Hedyotidis Corymbosae.

The selected HPLC method was of higher specificity. The analytical regression curve of *p*-coumaric acid was A = 153.39 C– 91.59, and its linearity was in the concentration range of 4.04 to 64.58 μg/mL with a correlation coefficient of 0.9999, where C (μg/mL) was the concentration of *p*-coumaric acid determined, and A was the peak area. The intra-day and inter-day precision was determined by a sample solution of *p*-coumaric acid under the selected chromatography conditions, and the RSD was taken as a measure of the intra- and inter-day precisions, which were less than 0.1 and 0.5%, respectively. The recoveries of the investigated components ranged from 98.1% to 103.2 %, and their RSD values were less than 1.8 %. Considering the results of the recovery test, the method is accurate. In order to improve the chromatographic separation and make the peak shape better, an appropriate volume of phosphoric acid was added to the mobile phase. The effect of the *p*H value of mobile phase on the separation and peak areas of *p* - coumaric acid in the range of 3.5–7.5 was investigated. It was found that *p*H lower than 6.0 brought a serious tailing of peaks.

Result analysis of OAD

In order to achieve the higher extraction efficiency of *p* - coumaric acid, primary single factor experiments about extraction solvent, extraction method, the effect of extractiontime, times of decoction, volume of solvent added in medicines were performed before OAD experiments. The results showed that the inhibitory rate of the aqueous extraction on HepG2 cells was higher than that of methanol and ethanol extractions. The cell inhibitory rate of aqueous extraction was 28.3%, while the cell inhibitory rate of methanol and ethanol were 6.2% and 12.9%, respectively. Overall, the water was chosen as the solvent to extract. Then the reflux was chosen as an extraction method for its higher extraction rate of *p* - coumaric acid (0.87mg/g) than ultrasound (0.42mg/g). The Fig.4a showed the effect of extraction time under different conditions by changing the time from 0.5 to 3 hours, when the extraction time increased, the extraction rate of *p* - coumaric acid was improved and got to highest (1.27mg/g) at 2 h. So the extraction time of 1, 2 and 3 hour was investigated in OAD. Times at four different values (1, 2, 3 and 4) were evaluated to optimize the extraction process (Fig.4b). The extraction rate of p-coumaric acid increased when increasing the extraction times. The efficiency of p-coumaric acid increased a little at 3 times, so the extraction time of 1, 2 and 3 was investigated in OAD. The effect of the volume of water added in extraction of *H.diffusa* efficiency was shown in (Fig.4c).

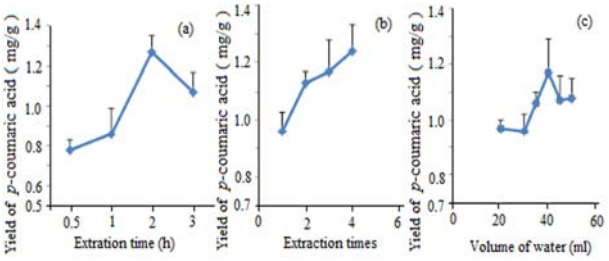

Fig.4. The results of primary single factor experiments. (a) the effect of extraction time, the highest extraction rate of *p* - coumaric acid (1.27mg/g) was got at 2h. (b) the effect of extraction times, theyield of *p* - coumaric acid was 1.17 mg/g at 3 times and 1.24 mg/g at 4 times.(c) the effect of volume of water, the maximum yield of *p* - coumaric acid value (1.17 mg/g) at 40 ml of water.

It was inferred that the extraction rate of the *p* - coumaric acid increased with the increased volume of water added from 20 to 50 mL and attained the maximum yield of *p* - coumaricacid value (1.17mg/g) near 40 mL of water. The volume of water of 30, 40 and 50 mL was investigated in OAD. Since each individual extraction in OAD optimization represented multivariant combination, other factors may contribute much to the extraction efficiency. Therefore, unlike in the univariant optimization, the individual extraction cannot simply mirror the change of factors.

Finally the three factors including volume of water added in medicines, extraction time and times

of extraction were studied in OAD. Three individual factors and their level values are designated in Tab.1. The orthogonal test results of three factors were shown in Tab.2, which helpful to obtain the optimized extraction conditions.

Table 2. Orthogonal array design matrix $L_9(3^4)$ and experimental results

No.	Annotation	Factor				Peak area	Yield of *p*-coumaric acid(mg/g)
		A	B	C	D		
1	30-fold of water,1h,1time	1	1	1	1	2573.4	0.9803
2	30-fold of water,1h,1time	1	2	2	2	2789.6	1.0625
3	30-fold of water,1h,1time	1	3	3	3	2873.2	1.0943
4	30-fold of water,1h,1time	2	1	2	3	2768.3	1.0545
5	30-fold of water,1h,1time	2	2	3	1	2168.2	0.8261
6	30-fold of water,1h,1time	2	3	1	2	3352.5	1.2771
7	30-fold of water,1h,1time	3	1	3	2	3055.5	1.1636
8	30-fold of water,1h,1time	3	2	1	3	2812.6	1.0713
9	30-fold of water,1h,1time	3	3	2	1	3898.2	1.4845
k1[a]		1.0457	1.0661	1.1096	1.0970		
k2		1.0526	0.9867	1.2005	1.1677		
k3		1.2398	1.2853	1.0280	1.0734		

[a]The mean values of yield for the factors at each level with standard deviation.

Table 3. ANOVA analysis of four parameters for extraction efficiency

Source	Sum of Squares (SS)	Degrees of freedom (df)	*F* -ratio	$F_{0.05}$	$F_{0.01}$	Significance
A	0.0729	2	5.1702	19	99	>0.1
B	0.1438	2	10.1915	19	99	<0.1
C	0.0447	2	3.1702	19	99	>0.1
D	0.0141	2		19	99	

Tab.3 shows the relationship between the extraction yields of *p* - coumaric acid and the three variables. From which, it could be inferred that the Factor B (extraction time) is the most significant factor according to the R values, while the Factor A (volume of water added in medicines) is the insignificant one compared with the others. With the direct observation analysis from the results, the optimal extraction condition combination for p-coumaric acid was A3-B3-C2. Considering the Factor C (times of decoction) is little influence on extraction efficiency, and error will be increased with more times of extraction. So the optimum values of factors were: 1 times of decoction, the 50mL of water added in medicines, and extraction time 3h.

Determination results of extraction efficiency in H.diffusa

The different part of *H. diffusa* was extracted by optimized extraction condition in OAD, which was 50 mL water added in medicine to extract 3 h and 1 times. After detecting by HPLC method, the result (Tab.4) showed that there are maximum amount of *p* - coumaric acid (1.66 mg/g) in fruit, followed by leaves (1.53mg/g), while the root showed the lowest content of *p* - coumaric acid (0.86 mg / g). All of them were statistically significant ($p < 0.05$).

Table 4. Determination results of p-coumaric acid in *H. diffusa*

	Peak areas	Yield of p-coumaric acid (mg/g)	Mean (mg/g)
Fruit	3604	1.39	1.66
	4493	1.73	
	4799	1.85	
Leaves	4453	1.72	1.53
	3984	1.54	
	3477	1.34	
Herb	2544	0.98	1.14
	3106	1.20	
	3248	1.25	
Stems	2426	0.94	1.05
	2552	0.98	
	3161	1.22	
Root	1950	0.75	0.86
	2515	0.97	
	2225	0.86	

Annotation: the concentration of standard *p* - coumaric acid was 4.04 μg/mL, and the peak area was 518.4, Yield of *p* - coumaric acid (mg/g) = amount of the target *p*-coumaric acid /sample mass.

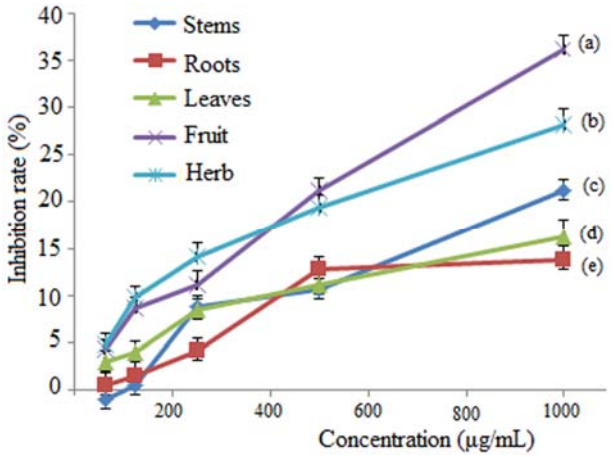

Fig.5. Result of cell viability assay, (a) result of fruit extraction (b) result of herb extraction (c) result of herb stem (d) result of leave (e) result of root

Result of cell viability assay

The Fig.5 showed the results of MTT assay, and the HepG2 cells were treated with water extractives from *H.diffusa* whose consistency were 62.5, 125, 250, 500 and 1000 μg/mL. As was shown in Fig.5,

different part of *H. diffusa* has different degrees of inhibition on HepG2 with a dose - dependent manner. The fruit showed the highest inhibition rate (36.18 ± 1.89) %, followed by herb (28.27 ± 1.14) %, then the root showed the lowest inhibition ratio (13.68 ± 1.67) %.

CONCLUSIONS

In this paper, the antitumor activity of extracts from different part of *H. diffusa* on HepG2 was investigated. The water was chosen as extraction solvent for its higher cell inhibitory rates. Based on this, water extraction condition of *H. diffusa* was optimal by OAD and the index component was detected by HPLC to reflect extraction efficiencies. The result showed that when the weight of herb was 1g, we got the optimal extract conditions which were 50 - fold of water, 1 times and 3h for each decoction. In the present study, *p* - coumaric acid showed good linear relationships (r = 0.9999) in the investigated concentration range from 4.04 to 64.58 μg/mL. The test solution also had a good repeatability (RSD＝3.6%, n = 6)) and good stability for 72 h. The recoveries, measured at six concentration levels, varied from 98.1 to 103.6%.
The results of content determination showed that there are maximum amount of *p* - coumaric acid (1.66mg/g) in fruit, followed by leaves (1.53mg/g), while the root showed the lowest content of *p* - coumaric acid. Results of cell viability assay showed that different part of H.diffusa showed inhibition on HepG2 with a dose - dependent manner, and fruit showed higher inhibition rate (36.18 ± 1.89) %, followed by Herb (28.27 ± 1.14) %, then the root showed the lowest inhibition ratio (13.68 ± 1.67) %. We found that fruit not only showed highest extraction efficiency of *p* - coumaric acid but also showed highest cell inhibition rate. Our suppositions were the active substance of fruit loss less because of its hard shell protection. While the extraction efficiency and cell inhibition ratewere not fully correspond, we think there are various antitumor constituents in H. diffusa, we just chose *p* - coumaric acid as an index component to reflect extraction efficiency of water extraction. As for root, it had lower extraction efficiency and cell inhibition rate. We think the poor growth environment determines the result. All of those will be the basis for further experiments.

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ABBREVIATIONS

H. diffusa - H. diffusa;
TLC - thin-layer chromatography;
DAD - diode array detector;
OAD - orthogonal array disign;
RSD - relative standard deviation;
DMEM - Dulbecco's modified Eagle's medium;
PBS - phosphate buffer saline;
MTT - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium;
HPLC- High Performance Liquid Chromatography;

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