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Antioxidant Activity of Extracts and Astilbin from the Root of *Smilax glabra* of Vietnam

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Astilbin, a main component of *Smilax glabra* Roxb roots of Vietnam was isolated. The structure of astilbin was elucidated by NMR and mass spectra. The astilbin in extracts was analyzed by HPLC, with the content of 21.8% in ethanol extract and 41.5% in ethyl acetate extract. The antioxidant capacity of extracts was evaluated by methods: 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) assay, thiobarbituric acid-reactive sepecies (TBARS) assay for inhibition of lipid peroxidation via TBARS formed from Fenton system-injured mouse brain, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay for hepatoprotective effect via H_2O_2-injured mouse hepatocytes. The results indicated ethyl acetate extract with highest content of astilbin showed the most antioxidant capacity in DPPH (SC_{50}: 24.9 µg/ml), TBARS (IC_{50} 9.45) and MTT (ED_{50}: 25.25 µg/ml).

Key words: *Smilax glabra* Roxb; astilbin; antioxidant activity; DPPH; MDA; MTT; lipid peroxidation.

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The reactive oxygen species (ROS) including free radicals ($^{\cdot}OH$, $^{\cdot}O_2^{-}$, $HO_2^{\cdot}$, etc.) and non-radical molecules such as H_2O_2, O_2, and HOCl can damage and kill cells. Mechanisms responsible for the ROS-mediated injuries mainly include lipid peroxidation, DNA, and protein oxidation. They are the cause leading many serious diseases as a cancer, cardiovascular disease, and Alzheimer′s disease. Thus, the antioxidants were used to prevent the production of free radicals or to capture the free radicals and to inhibit other oxidation reactions. The use of antioxidants in pharmacology is intensively studied. Synthetic antioxidants have been in use as food additives for a long time, but reports on their involvement in chronic diseases have restricted their use in foods. Therefore, the present researches focused on natural antioxidants from plant sources [1, 2].

The *Smilax glabra* Robx. also called as “Tho Phuc Linh” is widely distributed in Vietnam. Its roots have been used as traditional folk medicine for the treatment of diseases such as rheumatism, diarrhea, ulcers, detoxication, diuretic, dermatitis, syphilis, flatulence… [3]. The chemical investigation of this plant confirmed the presence of flavonoids, phenyl propanoids and phenolic acids, and astilbin was found as the main component. The compounds showed anti-inflammatory, immunomodulatory, hepatocyte protection, anti-tumor, antibacterial, and antioxidant activities [4–7]. Moreover, the studies on antioxidant activity of compounds and extracts from roots of *S. glabra* indicated the total content of phenolics and flavonoids correlation with antioxidant properties [5–8]. However, an overview on literature indicated that this plant of Vietnam was not intensively studied for its chemical components and biological properties [9, 10]. Herein, we present the preparation and antioxidant valuation of the extracts and astilbin from *S. glabra* Roxb. of Vietnam.

EXPERIMENTS

Materials and Instruments

Materials 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH), malondialdehyde (MDA), dimethyl sulphoxide (DMSO), 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), thiobarbituric acid (TBA), ascorbic acid, and curcumin were purchased from Sigma Chemical Co.

NMR spectra were recorded on a Bruker AM500 FT-NMR. Spectrometers were operated at 500.13 MHz and 125.76 MHz for ^{1}H NMR and ^{13}C NMR spectra, respectively. ESI-MS mass spectrum was measured on an Agilent 6310 ion trap. HPLC analysis was perfomed on an Agilent 1200.

Plant Sample

The roots of *Smilax glabra* Roxb. (RSG) were collected in Tuyen Quang, September, 2013 and identified by Prof Tran Huy Thai, Institute of Ecology and Biological Resource, VAST. The vouche specimen (SG1309) was deposited at the Hebarium of the Institute of Marine Biochemistry, Vietnam Academy of Science and Technology.

Preparation of the Extracts and Isolation of Astilbin

The RSG (10.0 kg) was dried and ground then extracted with ethanol 96% (3 × 60 l) to obtain ethanol extract (EE, 1.4 kg) after removing solvent in reduced pressure. The EE was suspended in EtOH/H_2O (1/1, v/v) (2.8 l) then partioned with *n*-hexane (3 × 3 l) and ethyl acetate (4 × 3 l). The extracts were concentrated under reduced pressure to dryness to get HE residue (60 g) and EAE residue (300 g), respectively.

The EAE (10.0 g) was subjected to column chromatography (CC) on silica gel (*n*-hexane/EtOAc, 2/1, v/v) to obtain astilbin (92%). This was sequently purified by recrystallization from MeOH/H_2O (1/1, v/v) and C18 reversed phase CC to afford astilbin (4.47 g). The astilbin was accessed above 97% by HPLC analysis.

Astilbin. It is a white amorphous powder, ESI-MS *m/z*: 486 [M-2H_2O]$^-$; ^{1}H-NMR (CD_3OD, 500 MHz): δ_H 6.98 (1H, d, J = 2.0 Hz, H-2′), 6.86 (1H, dd, J = 2.0, 8.0 Hz H-6′), 6.83 (1H, d, J = 8.0 Hz H-5′), 5.94 (1H, d, J = 2.0 Hz, H-6), 5.92 (1H, d, J = 2.0 Hz, H-8), 5.10 (1H, d, J = 10.5, H-2), 4.60 (1H, d, J = 10.5 Hz, H-3), 4.28 (1H, dq, J = 6.0, 9.6 Hz, H-5″), 4.07 (1H, d, J = 1.5 Hz, H-1″), 3.68 (1H, dd, J = 3.0, 9.6 Hz, H-3″), 3.56 (1H, dd, J = 1.5, 3.0 Hz, H-2″), 3,30 (1H, m, H-4″), 1.21 (3H, d, J = 6.0 Hz, CH_3-6″); ^{13}C-NMR (CD_3OD, 125 MHz): δ_C 195.9 (C-4), 168.5 (C-7), 165.5 (C-5), 164.1 (C-8a), 147.3 (C-4″), 146.5 (C-3′), 129.2 (C-1′), 120.5 (C-6′), 116.3 (C-5′), 115.5 (C-2′), 102.5 (C-4a), 102.1 (C-1″), 97.4 (C-6), 96.2 (C-8), 83.9 (C-2), 78.5 (C-3), 73.8 (C-4″), 72.1 (C-3″), 71.7 (C-2″), 70.5 (C-5″), 17.8 (C-6″).

HPLC Analysis

The HPLC analysis was performed on an Agilent 1200 Series HPLC System, equipped with a Zorbax eclipse XDB C_{18} (4.6 mm × 150 mm, 5 µm) column, a C_{18} guard column and Zobax extend C_{18} (4.6 mm × 250 mm, 5 µm) column and maintained at 25°C. All the samples were dissolved in metanol at 5 mg/ml and filtered using 0.45 µm filter. The mobile phase was MeOH(A)/H_2O/0.3% acetic acid) (B), (30/70, v/v) at a flow rate of 0.5 ml/min for 40 min with a gradient program as follows: 0–5, 30% A; 5–10 min 30–40% A; 10–20 min, 40–50% A; 20–25 min, 50–100 A; 25–30 min, 100% A, 30–40 min, 30% A. The injection volume was of 5 µl, the DAD acquisition wavelength was set at 291 nm. Astilbin in extracts was identified by the retention times and the peak areas were used to characterize the relative contents in the study.

DPPH Free Radical Scavenging Activity Assay

DPPH free radical scavenging activity was determined using minor modified method previously described [11, 12]. Briefly, the DMSO dissolved samples (1 ml, 200–25 mg/ml) was mixed with 0.5 mM DPPH/ethanol (96%) solution (1 ml) to get the samples at different concentrates as 12.5 µg/ml; 25.50 µg/ml, 50 µg/ml and 100 µg/ml. The absorbance or Optical Density

(OD) of the reaction mixture was determinded by ELISA Plate Reader (Bio-Rad) at 515 nm 30 min later. The DPPH radical–scavenging capacity (SC %) was calculated by the following equation:

$$SC\ (\%) = [1-(OD_{sample}-OD_{blank})/OD_{control}] \times 100\% \quad (1)$$

Where OD_{sample} is the absorbance in the presence of samples (extracts or compounds) and DPPH; $OD_{(blank)}$ is the absorbance in the presence of testing samples; $OD_{Control}$ is the absorbance in the presence of DPPH. All measurements were performed in triplicate and ascorbic acid was used as a positive standard. SC_{50} value is the concentrate of sample at that inhibition 50% of DPPH free radical.

Thiobarbituric Acid-reactive Sepecies (TBARS) Assay

TBARS assay was used to assess lipid peroxidation using the method of Lora (1997) and Manmohan (2012) with a few modifications [13, 14]. MDA was identified as a product of lipid peroxidation which reacted with TBA to give a pink coloured species that gave an absorbance at 532 nm. Briefly, the healthy BALB/c mouse brain was homogenated in phosphate buffered solution (pH = 7.4) in 1/10 (v/v). The obtained homogenate and solution of the samples (extracts and compounds were dissolved in DMSO) were used as stock solutions. The extracts/DMSO solution at different concentrations (0.2 ml) was added to phosphate buffer solution (0.8 ml) then the obtained solution was mixed with the mouse brain homogenate (1 ml). The peroxidation was initiated by the addition of the Fenton system (0.1 mM $FeSO_4$/15 mM H_2O_2 in (1/1, v/v) (1 ml). In the final reaction, concentration of samples as 6.25 μg/ml; 12.5 μg/ml; and 25.50 μg/ml and the reaction was incubated for 5 min. To finish reaction, 10% trichloacetic acid (TCA, 1 ml) was added to the reaction mixture and this was centrifuged at 3500 rpm for 10 min to remove precipitate. To the supernatant solution, 0.8% thiobarbituric acid (TBA, 1 ml) was added at 100 °C for 15 min then cooled to room temperature. The absorbance or OD of the reaction mixture was determinded by ELISA Plate Reader (Bio-Rad) at 532 nm. The inhibition percentage of the lipid peroxidation (% inhibition) was calculated using following formula:

$$\%\ Inhibition = [1-OD_{sample}/OD_{control}] \times 100 \quad (2)$$

Where OD_{sample} is absorbance in the presence of mouse brain homogenate and samples (extracts or compounds); $OD_{control}$ is absorbance in the presence of mouse brain without samples.

An IC_{50} value denotes the concentration of sample which is required 50% inhibition of lipid peroxidation. All measurements were performed in triplicate and curcumin was used as a positive standard. SC_{50} value is the concentrate of sample at that 50% inhibition for lipid peroxidation.

MTT Cell Viability Assay for Hepatoprotective Effect

The MTT cell viability assay was used to assess H_2O_2 injured-hepatoprotective effect of extracts was identified as method described by Mosmann (1983) and Chen (2006) [15, 16]. The hepatocytes were isolated from the BALB/c mouse line cultured in DMEM medium with 2 mM L-glutamine, 10 mM HEPES and 1 mM sodium pyruvate then supplemented with 10% fetal bovine serum-FBS (GIBCO), finally, incubated in a humidified atmosphere at 37°C and 5% CO_2 for 2 or 3 days before the use. The cell suspension (200 μl) was added to each well of a flat-bottom 96 well plate at a density of 1×10^4 cells/ml. Cells incubated in a humidified atmosphere at 37°C with 5% CO_2 overnight. The extracts or curcumin were added to each well at different concentrates as 6.25 μg/ml; 12.5 μg/ml; 25.50 μg/ml and incubated for 2 h, then, H_2O_2 (100 μM) was added to wells and the plate was incubated for additional 2 h. MTT (50 μl of a solution 1 mg/ml) was added to each well then incubated at 37 °C for 4 h. The supernatant

was removed and DMSO (100 µl) was added to each wells. Cell viability (CV) was assessed by absorbance of cell solution measured at 492 nm by micro plate spectrophotometer (Biotek, USA) and was quantified using MTT assay and was calculated by the following equation:

$$CV\ \% = \frac{[OD_{Sample} - OD_{Negative\ control}] \times 100}{OD_{Hep.} - OD_{Negative\ control}} \quad (3)$$

Where OD_{sample} is absorbance in the presence of hepatocytes (Hep.), the samples (extracts or compounds) and H_2O_2; $OD_{Negative\ control}$ is the absorbance in the presence of hepatocytes and H_2O_2; $OD_{Hep.}$ is the absorbance in the presence of the hepatocytes. All measurements were performed in triplicate and ascorbic acid was used as a positive standard. An EC_{50} value denotes the concentration of samples which protects 50% of cell viability.

RESULTS AND DISCUSSION

Astilbin and the Extracts from RSG

Astilbin (Figure 1), a flavonoid glucoside, is a main compound in RSG. Thus, the antioxidant extracts from RSG correlated content of astilbin.

Using various chromatographic methods, we isolated astilbin (**1**). The ^{1}H-NMR of **1** showed the presence of two *meta* protons in A ring at δ_H 5.92 (d, *J* = 2.0 Hz) and 5.94 (d, *J* = 2.0 Hz); three ABX system protons in B ring at δ_H 6.98 (d, *J* = 2.0 Hz), 6.86 (dd, *J* = 2.0, 8.0 Hz); 6.83 (d, *J* = 8.0 Hz), and two *trans*- protons in C ring at δ_H 5.10 (d, *J* = 10.5 Hz), 4.60 (d, *J* = 10.5 Hz), assigned to flavanonol aglycone (taxifolin); one anomeric proton at δ_H 4.07 (d, *J* = 1.5 Hz), four methin protons at δ_H 4.28 (dq, *J* = 6.0, 9.6 Hz), 3.68 (dd, *J* = 3.0, 9.6 Hz), 3.56 (dd, *J* = 1.5, 3.0 Hz), 3,30 (m, H-4″), and a secondary methyl group at δ_H 1.21 (d, *J* = 6.0 Hz), assigned to sugar moiety. The ^{13}C-NMR spectrum of astilbin exhibited the presence of 21 carbons including eight non-protonated carbons, twelve methine carbons, and

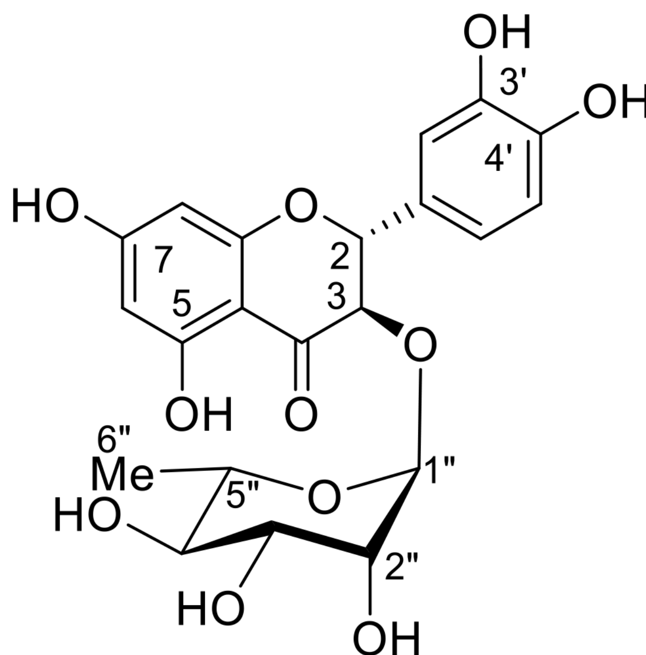

Figure 1. The structure of astilbin (**1**).

one methyl carbon. Overall consideration of ^{1}H-and ^{13}C-NMR data of **1** indicated that the structure of **1** was similar to that of astilbin [8].

HPLC Analysis

The chromatograms of astilbin, EE, and EAE were displayed in Figure 2.

The retention time of astilbin was 19.1 min at 291 nm, the contents of astilbin in the extracts (EE, EAE) were quantified by equation Y = 22642.73573X + 48.25299; (r^2 = 0.99995), where Y was the peak area, and X was the concentration of analysis (78.125 ÷ 500 µg/ml). The quantitative results are sumarised in Table 1.

Table 1. Content of astilbin in extracts.

Sample	Astilbin (mg/g)	Astilbin (%)
EE	218	21.8
EAE	415	41.5
HE	–	–

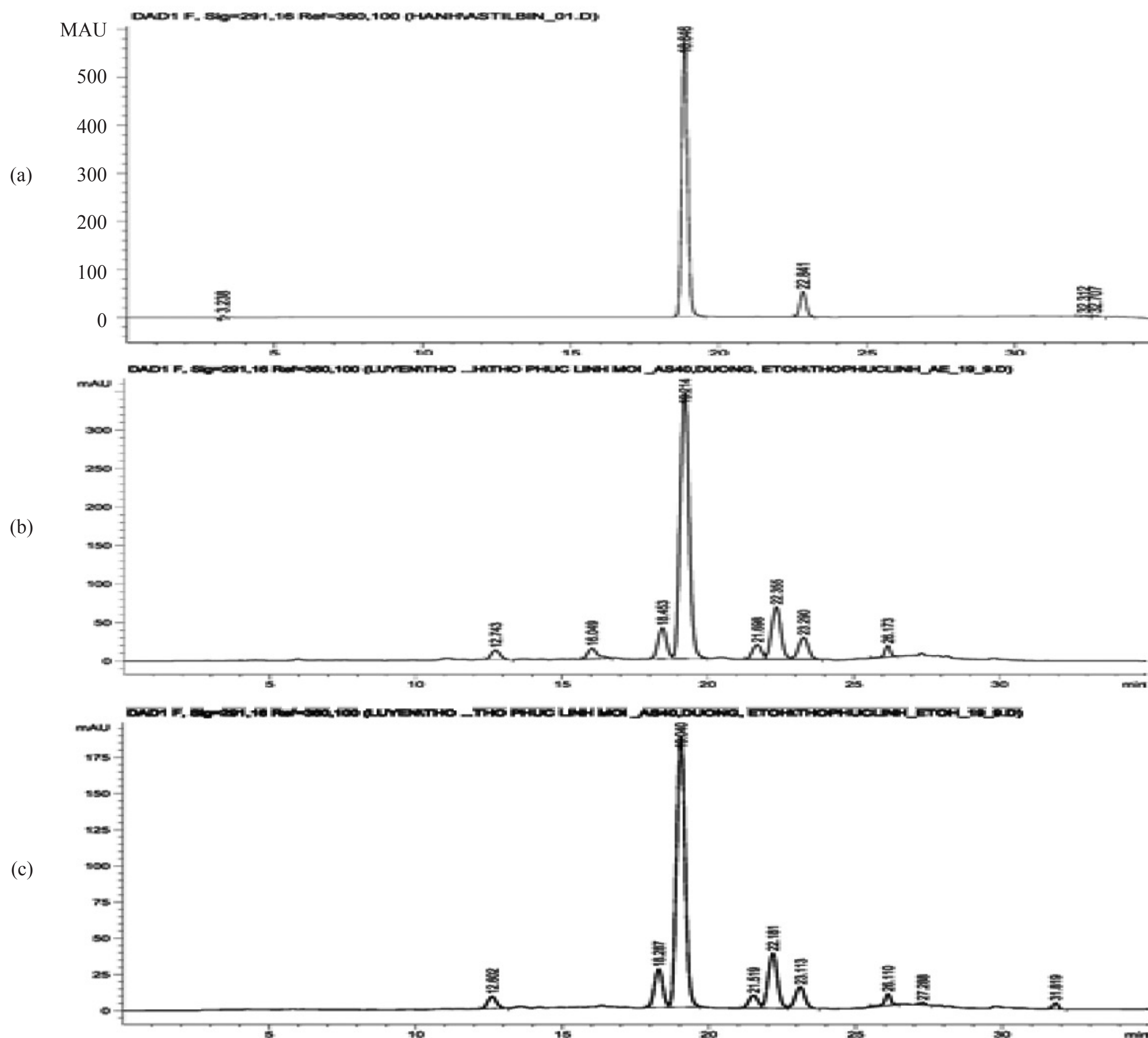

Figure 2. Chromatograms of astilbin (a); EE extract (b); EAE extract (c).

The results indicated that astilbin was one most dominant compound in RSGV extracts (Table 1). The content of astilbin in EE and EAE were 21.8% and 41.5% respectively. In addition, HE had not used HPLC analysis because the insoluble proplem of astilbin in HE. Thus, the content of astilbin was the highest in EAE.

DPPH Radical Scavenging Activity

DPPH, a stable free radical, has been widely used to evaluate the radical scavenging effectiveness of various antioxidant substansces. The flavonoids, hydrogen-donating antioxidants, can form a non-radical form DPPH-H in the reaction. The scavenging capacity of samples was described on Table 2.

Table 2 showed the DPPH cavenging effect of all samples (extracts, astilbin and ascorbic acid) and the scavenging capacity depended on concentration of the samples. At concentration of 100 µg/ml, these extracts showed inhibitory percentage to be 84.42% (EE), 89.14% (EAE) and 89.27% (HE). At concentration of 12.5 µg/ml, the scavenging activity of extract decreased with inhibitory of 19.33% (EE), 19.85% (EAE) and 26.2% (HE). Their IC_{50} values were 32.3 µg/ml (EE), 24.9 µg/ml (EAE) and 32.5 µg/ml (HE). IC_{50} value of EAE is higher than of EE and HE. Astilbin and positive control, ascorbic acid showed the most radical scavenging activity with IC_{50} values of 21.79 and 10.9 µg/ml, respectively.

TBARS Assay for Lipid Peroxidation

In these experiments, the mouse brain was used as membrane lipids which possess high concentration of polyunsaturated fatty acid. The Fenton system mainly afforded hydroxyl radicals (·OH) that oxidated mouse brain to give TBARS, for example, malondialdehyde (MDA) and derivatives of MDA. The present of antioxidant (samples as extracts, astilbin and curcumin) can effectively

Table 2. DPPH scavenging activity of samples.

Concentration (µg/ml)	Scavenging capacity (CV %)				
	EE	EAE	HE	Ast[a]	Asc[b]
200	89.27	88.9	89.27	NT	NT
100	84.42	89.14	87.89	NT	NT
50	66.25	67.5	58.71	80.17	90.94
25	39.48	51.4	41.52	54.92	84.60
12.5	19.33	30.04	26.20	27.79	58.56
6.25	NT	NT	NT	15.21	5.35
SC_{50}	32.3	24.9	32.5	21.7	10.9

[a]Ast = Astilbin; [b]Asc = Ascorbic acid; NT = Not tested.

Table 3. Lipid peroxidation inhibition of the samples

Concentration (µg/ml)	Inhibition (%)				
	EE	EAE	HE	Ast[a]	Cur[b]
50	72.58	74.89	63.59	62.46	78.64
25	67.53	72.15	53.82	54.79	72.29
12.5	52.38	59.88	49.93	39.36	65.80
6.25	33.62	40.69	20.63	25.54	57.58
3.125	19.48	28.72	13.90	20.06	29.29
IC_{50}	13.26	9.45	20.95	22.99	6.48

[a]Ast = Astilbin; [b]Cur = Cucurmin.

scavenge the hydroxyl radical (·OH) by donating hydrogen atoms (H·) and electrons prevented oxidation of mouse brain. Inhibition the lipid peroxidation of extracts, astilbin, and curcumin were described in Table 3.

Table 4 showed that all samples (extracts, astilbin and curcumin) effectively exhibited lipid peroxidation and lipid peroxidation inhibition capacity depended on concentration of samples in reaction. At the concentration of 50 µg/ml, the extracts showed lipid peroxidation inhibitory capacity were 72.58% (EE) and 74.89 (EAE), similar to that of cucurmin (78.64%). The EAE showed highest lipid peroxidation inhibitory capacity with IC_{50} value of 9.45 µg/ml. HE showed the weakest inhibitory activity with IC_{50} value of 20.95 µg/ml. Thus, the extracts with higher content of astilbin have better inhibition percentage of lipid peroxidation. The scavenging capacity of extracts is stronger than its pure constituent, astilbin, which might result from the synergistic effect of different constituents in extracts [5].

MTT Cell Viability Assay for H_2O_2 Injured Hepatoprotective Effect of Extracts

The MTT cell viability assay was used to determine mouse hepatocytes viability percentage in the tested wells, in which, mouse hepatocytes were mixed with the samples (EE, EAE, HE, astilbin and ascorbic acid) then H_2O_2 was added. The cell viability percentages were described in Table 4. Table 4 showed all samples have significant hepatoprotective effect and hepatoprotective capacity depended on concentrations of samples. The sample EAE exhibited the best hepatoprotective cacpacity with EC_{50} value of 25.25 µg/ml, comparing to EE (EC_{50} value of 39.31 µg/ml) and HE (EC_{50} value of 29.43 µg/ml), astilbin (EC_{50} value of 37.53 µg/ml) and curcumin (EC_{50} value of 15.60 µg/ml).

The EE exhibited the most hepatoprotective activity may be due to astilbin and the synergistic effect of different constituents.

CONCLUSION

Astilbin, a main component of *S. glabra* roots was isolated. The structure of astilbin was elucidated by NMR and mass spectra. The content of astilbin in extracts was analyzed by HPLC, 21.8% (EE), 41.5% (EAE). These indicated astilbin as the main component in EAE.

The antioxidant capacity of extracts was evaluated by the DPPH assay methods. TBARS assay for inhibition of lipid peroxidation via TBARS was formed from Fenton system-injured mouse brain, and MTT cell viability assay for hepatoprotective effect via H_2O_2-injured mouse hepatocytes. The results exhibited that all the extracts EE, HE and EAE possessed a significant antioxidant activity and the antioxidant levels of extracts related content of astilbin in each extract. The EAE extract with highest content of astilbin showed the most antioxidant capacity in DPPH (SC_{50}: 24.9 µg/ml), TBARS (IC_{50} 9.45) and MTT (ED_{50}: 25.25 µg/ml). The EAE extract could be the candidate for preparation of an antioxidant functional food from the roots of *S. glabra.*

Table 4. Cell viability percentage of the samples.

Concentration (µg/ml)	Cell viability %				
	EE	EAE	HE	Ast[a]	Cur[b]
50	61.64	73.57	72.95	64.20	109.96
25	34.30	57.51	44.97	37.09	81.39
12.5	20.72	20.09	25.55	25.59	31.66
6.25	13.64	17.14	15.32	21.21	10.72
3.125	10.45	1.19	7.82	18.20	3.04
EC_{50}	39.31	25.25	29.43	37.53	15.60

[a]Ast = Astilbin; [b]Cur = Cucurmin.

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