Phenolic composition, antioxidant, cytotoxic activities and cardioprotective effect of hydroalcoholic extract from aerial-parts of *Hypericum attenuatum* Fisch. ex Choisy

Du-Xin Jin, Jun-Fang He, Ke-Qin Zhang, Nan-Yi Zhang

**Abstract:** This study investigated phenolic metabolites, antioxidant, cytotoxic and cardioprotective effects of the hydroalcoholic extract from the aerial parts of *Hypericum attenuatum* Fisch. ex Choisy. The total phenolic and flavonoid contents of the extract were 132.40 ± 2.06 mg GAE/g and 101.46 ± 1.47 mg QE/g respectively. The extract exhibited antioxidant activities with an EC$_{50}$ value against DPPH radical of 0.099 ± 0.03 mg/mL and a FRAP value of 1.22 ± 0.086 mmol/L Fe$^{2+}$. The extract could protect H9c2 cardiomyoblasts from the injury of H$_2$O$_2$, while it restored the H9c2 cell viability to 82.69 ± 2.33% at 100 μg/mL. The extract possessed cytotoxicity on MGC803, C666-1 and SW620 cells with IC$_{50}$ values of 69.77 ± 2.43 μg/mL, 74.97 ± 1.08 μg/mL and 58.91 ± 1.81 μg/mL, respectively. Moreover, it could promote apoptosis of the tested cancer cells. This research provided useful information for the utilization of *H. attenuatum* as herbal medicine.

**Experimental**

**Material**

The aerial parts of *H. attenuatum* were collected in July 2020 from Jilin college of Agricultural Science and Technology (geographical coordinates: 43.96°N latitude and 126.49°E longitude, the altitude of 188.3 m). The species was authenticated by professor Ke-qin Zhang from Jilin college of Agricultural Science and Technology, and the specific authentication process was as follows: Firstly, the morphological characteristics (including leaf, flower, stem, fruit and seed) of the plant were carefully observed and microscopic examination was conducted, and a detailed record of its morphological characteristics in botanical terms was made before indexing with appropriate search table. To confirm the accuracy of the retrieved result, the characteristics of the plant were further checked and compared with the morphological
description in the flora (Makovetska, 1997). Then, a voucher specimen (III-2-20200829) has been deposited at Jilin Agricultural University in August 2020. Human gastric carcinoma cells (MGC803), human nasopharyngeal carcinoma cells (C666-1), human colorectal cancer cells (SW620) and rat cardiomyoblasts (H9c2) were acquired from Shanghai Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Solarbio Science and Technology Co. (Beijing, China). Cisplatin was purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). All the standard compounds (purities ≥ 98.0%) for UPLC analysis were purchased from National Institutes for Food and Drug Control, China. Annexin V-FITC apoptosis detection kit was purchased from Sungene Biotech Co., Ltd (Tianjin, China). Acetonitrile and formic acid of chromatographic grades were purchased from Concord Technology Co., Ltd (Tianjin, China). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco BRL (Grand Island, NY, USA). All other reagents were of analytical purity.

**Preparation of H. attenuatum hydroalcoholic extract**

*H. attenuatum* hydroalcoholic extract was prepared by ultrasonic assisted with soaking method according to our previous study (Jin et al., 2019). Briefly, the air-dried and powdered plant material (500 g) was ultrasonically extracted with 62% ethanol/water (v/v) at a liquid to solid ratio of 37:1 (v/w) in an ultrasonic power of 500 W for 32 min, followed by soaking in 95% ethanol for 24 h. The extract was then centrifuged, condensed and freeze-dried. The final crude extract (35.29 g) was stored at -20 °C for further analysis.

**UPLC-Triple-TOF/MS analysis of phenolic metabolites in H. attenuatum**

Phenolic metabolites in *H. attenuatum* hydroalcoholic extract were characterized by UPLC-Triple-TOF/MS, which is equipped with a ZORBAX-SB C18 column (4.6 × 100 mm, 1.8 µm). The mobile phase consisted of two solvents: water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B). The elution gradient was as follows: 5% B to 50% B for 25 min, 50% B to 95% B for 10 min and maintained
at 95% B for 2 min at a flow rate of 0.8 mL/min. The injection volume was 5 μL and the detection wavelength was monitored at 254 nm. All the analyses were performed at 30 °C.

The mass spectrometry was carried on the AB Triple-TOF 5600 plus system (AB SCIEX, Framingham, MA, USA) equipped with an ESI (electrospray ionization source) system. MS parameters were set as follows: All the measurements were performed in negative ion mode with an acquisition mass ranging from 100 m/z to 1500 m/z. The source voltage and temperature were 4.5 kV and 550 °C, respectively. The pressure of gas 1 (air) and gas 2 (air) was set to 50 psi. The pressure of curtain gas (N₂) was set to 35 psi. Maximum allowed error was set to 2 ppm. The exact mass calibration was performed automatically using the automated calibration delivery system before each analysis.

**HPLC analysis of hyperforin and hypericin**

Hyperforin and hypericin were analyzed on an Agilent 1200 HPLC system equipped with an Intersil C8 (250 × 4.6mm, 5 μm) column. All the samples including hyperforin, hypericin and *H. attenuatum* hydroalcoholic extract were dissolved in 1 mg/mL, and were filtered through a 0.22 μm filter membrane before HPLC analysis with an injection volume of 10 μL. The flow rate was 1 mL/min. For hyperforin, the elution process was performed with 70% acetonitrile (v/v) in an isocratic mode, and the detector was monitored at 270 nm. For hypericin, the elution was conducted in an isocratic mode with a mobile phase composed of 50% acetonitrile, 40% methanol, 8% water and 2% phosphoric acid (v/v/v/v), and the detection wavelength was monitored at 590 nm. The components in *H. attenuatum* hydroalcoholic extract was identified via comparing their retention times with those of standards chromatographed under the same conditions.

**Total phenolic content**

Total phenolic content of *H. attenuatum* hydroalcoholic extract was determined by Folin-Ciocalteu colorimetric method according to the method of Singleton and Rossi (1965) with minor modifications. 100 μL of the extract (50 mg/mL) was mixed with 400 μL of sodium carbonate (7.5%, w/v) and 500 μL of Folin-Ciocalteu reagent. After
reacted in dark for 30 min, the absorbance of solution was measured at 765 nm using a microplate reader. Gallic acid was used instead of the extract to produce the calibration curve. Total phenolic content was expressed as mg of gallic acid equivalents per gram (mg GAE/g) of *H. attenuatum* extract.

### Total flavonoid content

Total flavonoid content was determined using the method of Meda et al. (2005) with minor modifications. Briefly, 0.5 mL of the extract (50 mg/mL) was mixed with 4.5 mL of 30% ethanol solution, 0.3 mL of 5% NaNO₂ solution and 0.3 mL of 10% AlCl₃ solution for 6 min. Then, 2 mL of 10% NaOH was mixed with the reaction for 15 min before reading the absorbance at 500 nm. Quercetin was used as standard to quantify the total flavonoid content in the extract as it is a ubiquitous flavonoid that exist in many plants. The results were expressed as mg of quercetin equivalents per gram (mg QE/g) of *H. attenuatum* extract.

### Antioxidant activities

#### DPPH radical scavenging activity

The measurement of 2,2’-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity was performed following the method of Saeed et al. (2012) with minor modifications. 0.2 mL of the extract was mixed with 0.6 mL of methanol and 0.2 mL of 0.15 mmol/L DPPH methanol solution, vortexed and incubated in dark for 30 min. Then, the absorbance was read at 517 nm. Methanol was added instead of the extract in the reaction as control group. Ascorbic acid was used as positive sample. The scavenging activity was calculated as \((\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})/\text{OD}_{\text{control}} \times 100\%\).

#### ABTS radical scavenging activity

The 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging capacity was measured by the method of Re et al. (1999) with slight modifications. An ABTS stock solution was prepared by mixing 7 mmol/L ABTS solution with 2.45 mmol/L *K₂S₂O₈* in a ratio of 9:1 (v/v), and was subjected to storing in dark for 12 h before diluting with 5 mmol/L PBS (pH 7.4) until its absorbance reached 0.7 ± 0.02 at 734 nm. Then, 10 μL of the extract was mixed with 290 μL of
ABTS working solution and reacted at 37 °C in dark for 30 min. The results were read at 734 nm with a microplate reader (BioTek Instruments, Inc., USA). PBS or ascorbic acid instead of the extract was added as control or positive sample. The scavenging activity was calculated as \((\text{OD}_{\text{control}} - \text{OD}_{\text{sample}})/\text{OD}_{\text{control}} \times 100\%\).

**Ferric reducing antioxidant power (FRAP) assay**

The FRAP index was measured according to the method described by Benzie and Strain (1996) with slight modifications. Briefly, FRAP reagent was prepared via mixing 1 mL of 10 mmol/L 2,4,6-tri-(2-pyridyl)-1,3,5-triazine (TPTZ) solution (dissolved in 40 mmol/L HCl), 1 mL of 20 mmol/L FeCl$_3$·6H$_2$O solution and 10 mL of 0.3 mol/L acetate buffer (pH 3.6). Then, 20 μL of the extract was reacted with 280 μL of FRAP solution at 37 °C for 5 min. The absorbance was measured at 593 nm. Ascorbic acid instead of the extract was used as positive sample. The FRAP value was calculated from a calculation curve given by FeSO$_4$·7H$_2$O standard solutions and expressed as mmol/L Fe$^{2+}$.

**Cell culture**

All cells tested in this study were cultured in DMEM high glucose medium which was supplemented with 10% (v/v) inactivated fetal bovine serum (Gibco, America), 1% penicillin (100 U/mL) and 1% streptomycin (100 μg/mL) at 37 °C in a humidified cell incubator containing 5% CO$_2$.

**Cytotoxicity assay**

Cytotoxic activity of *H. attenuatum* hydroalcoholic extract against the tested cancer cell lines was analyzed by MTT assay. Different cancer cells at a density of 5×10$^3$ cells/well were plated in 96-well culture plates and incubated for overnight before treatment. The extract at different concentration (12.5, 25, 50, 100 and 200 μg/mL) were added into 96-well plates for 24 h before adding 10 μL of MTT (0.5 mol/L) for 4 h. The produced formazan precipitate was dissolved in 100 μL of DMSO after discarding the culture medium in the 96-well plate. The absorbance was measured at 490 nm. Cisplatin instead of the extract was utilized as positive sample in this assay. Cell viability was calculated as \((\text{OD}_{\text{sample}}/\text{OD}_{\text{control}}) \times 100\%\).
H$_2$O$_2$-induced injury in H9c2 cells

The H9c2 cells in the exponential growth phase were seeded into 96-well plates at a density of 5×10$^4$ cells/well and allowed to adhere for 12 h. Prior to detecting the cardioprotective potential of *H. attenuatum* hydroalcoholic extract, H$_2$O$_2$ at different concentrations (50, 100, 150 and 200 μmol/L) and the extract at different concentration (12.5, 25, 50, 100 and 200 μg/mL) were respectively added to the cells and evaluated by MTT assay to ascertain the suitable H$_2$O$_2$-stimulative concentration and the extract’s non-toxic concentration. Then, the cells were pre-treated with the extract at 37 °C for 24 h before adding H$_2$O$_2$ of suitable concentration to the cells for 24 h to induce cell injury. The surviving cells in the plate were evaluated by MTT assay. Cell viability was calculated as (OD$_{\text{sample}}$/OD$_{\text{control}}$) × 100%.

Flow cytometry analysis

The percentage of early apoptosis and late apoptosis in the detected cancer cell lines was detected by flow cytometer using Annexin V-FITC/PI apoptosis detection kit. Briefly, the different cancer cell lines were treated with 50 μg/mL of the extract for 24 h. Then, the cells were collected, washed twice with ice-cold PBS, centrifuged at 1500 r/min for 5 min before staining with Annexin V-FITC and PI in 1 × binding buffer for 15 min in dark at room temperature. The results were detected by a flow cytometer equipped with Cell Quest software.

Statistical analysis

All assays were carried out in triplicate, and the results were expressed as means ± standard deviations. Data were analyzed by one-way analysis of variance (ANOVA). *P* < 0.05 or **P** < 0.01 was considered to be statistically significant. SPSS software was used for all statistical analysis.

References:


Makovetska OY. Comparative research of quantitative morphological characteristics and productivity of some Hypericum L. species on introduction in Kyiv. Ukrayins'kyi Botanichnyi Zhurnal.


**Table S1** Phenolic and flavonoid contents in *H. attenuatum* hydroalcoholic extract

<table>
<thead>
<tr>
<th></th>
<th>Total phenolic content (mg GAE/g)</th>
<th>Total flavonoid content (mg QE/g)</th>
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<tr>
<td><em>H. attenuatum</em> extract</td>
<td>132.40 ± 2.06</td>
<td>101.46 ± 1.47</td>
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**Table S2** UPLC-Triple-TOF/MS characterization of phenolic constituents in *H. attenuatum* extract

<table>
<thead>
<tr>
<th>No.</th>
<th>Retention time (min)</th>
<th>[M-H]- (m/z)</th>
<th>Calculated molecular formula</th>
<th>MS fragments (m/z)</th>
<th>Tentative identification</th>
<th>Chemical class</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>4.45</td>
<td>329.2873</td>
<td>C(<em>{14})H(</em>{18})O(_{9})</td>
<td>167, 152, 108</td>
<td>Vanillic acid 4-O-β-D-glucopyranoside</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>2</td>
<td>5.62</td>
<td>353.3087</td>
<td>C(<em>{16})H(</em>{18})O(_{9})</td>
<td>191, 179</td>
<td>Neochlorogenic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>3</td>
<td>6.59</td>
<td>204.0375</td>
<td>C(<em>{10})H(</em>{7})NO(_{4})</td>
<td>160, 132</td>
<td>6-Hydroxykynurenic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>4</td>
<td>6.98</td>
<td>353.0872</td>
<td>C(<em>{16})H(</em>{18})O(_{9})</td>
<td>191, 179, 173</td>
<td>Chlorogenic acid</td>
<td>Phenolic acid</td>
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<tr>
<td>5</td>
<td>8.16</td>
<td>337.3093</td>
<td>C(<em>{16})H(</em>{18})O(_{8})</td>
<td>191, 163, 119</td>
<td>3-p-Coumaroylquinic acid</td>
<td>Phenolic acid</td>
</tr>
<tr>
<td>6</td>
<td>9.19</td>
<td>421.3396</td>
<td>C(<em>{10})H(</em>{18})O(_{11})</td>
<td>331, 301</td>
<td>Mangiferin</td>
<td>Phenol</td>
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<tr>
<td>7</td>
<td>10.01</td>
<td>463.3684</td>
<td>C(<em>{21})H(</em>{26})O(_{12})</td>
<td>301, 271, 255</td>
<td>Isoquercitrin</td>
<td>Flavonoid</td>
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</tbody>
</table>
Table S3 IC\textsubscript{50} values of \textit{H. attenuatum} hydroalcoholic extract against the tested cancer cell lines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC\textsubscript{50} value ((\mu)g/mL)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>\textit{H. attenuatum} extract</td>
</tr>
<tr>
<td>MGC803</td>
<td>69.77 ± 2.43</td>
</tr>
<tr>
<td>C666-1</td>
<td>74.97 ± 1.08</td>
</tr>
<tr>
<td>SW620</td>
<td>58.91 ± 1.81</td>
</tr>
</tbody>
</table>
Figure S1 Regression lines of gallic acid (A) and quercetin (B)

Figure S2 HPLC chromatograms of *H. attenuatum* hydroalcoholic extract or standards detected at different wavelengths. *H. attenuatum* hydroalcoholic extract was analyzed by HPLC at 254 nm (A), 270 nm (B) and 590 nm (D); while hyperforin and hypericin were analyzed at 270 nm and 590 nm,
respectively.
**Figure S3** MS/MS spectrum of *H. attenuatum* hydroalcoholic extract. 1 Vanillic acid 4-O-β-D-
glucopyranoside; 2 Neochlorogenic acid; 3 6-Hydroxykynurenic acid; 4 Chlorogenic acid; 5 3-p-Coumaroylquinic acid; 6 Mangiferin; 7 Isoquercitrin; 8 Quercetin-3-O-α-L-rhamnopyranosyl(1->2)-β-D-galactopyranoside; 9 Hyperoside; 10 Quercetin-3-arabinoside; 11 Apigenin-8-C-rhamnoside; 12 Astragalin; 13 Quercetin-3-O-D-glucoside-7-O-D-glucoside; 14 Quercetin-3-O-rutinoside; 15 Norathyriol; 16 Quercetin; 17 Amentoflavone.

**Figure S4** Antioxidant activity of *H. attenuatum* hydroalcoholic extract. Scavenging capacity on DPPH radical (A) and ABTS (B) radical were measured at 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL and 500 μg/mL, respectively. FRAP (C) value of the extract was determined at 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL, respectively.

**Figure S5** Protective effect of *H. attenuatum* hydroalcoholic extract against H$_2$O$_2$-induced injury in H9c2 cells. The cytotoxic effects of the extract (A) and H$_2$O$_2$ (B) on H9c2 cells were determined before the measurement. Then, the ameliorative effect of the extract against H9c2 cells injury induced by H$_2$O$_2$ was evaluated via a MTT assay (C). *P < 0.01 indicates significant compared to H9c2 cells with no treatment; *P < 0.05 or **P < 0.01 indicates significant compared to H$_2$O$_2$-induced H9c2 cells.
Figure S6 Cytotoxic effect of _H. attenuatum_ hydroalcoholic extract against three cancer cells.

Cytotoxic effects of the extract on MGC803 (A), C666-1 (B) and SW620 (C) cells were evaluated at 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL, respectively. *P* < 0.01 indicates significant compared to the cancer cell lines with no treatment.
Figure S7 Apoptosis of the detected cancer cells induced by *H. attenuatum* hydroalcoholic extract. The cells were treated with 50 μg/mL of the extract before staining with Annexin V-FITC and PI. MGC803 cells (A), C666-1 cells (C) and SW620 cells (E) with no treatment were regarded as control, while the influence of the extract on the apoptosis of the three cancer cells were shown in (B), (D) and (F), respectively.