Original Article

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Quantitation of β-carboline and quercetin in alligator weed (*Alternanthera philoxeroides* (Mart.) Griseb.) by LC-MS/MS and evaluation of cardioprotective effects of the methanol extracts

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Summary **Plant invasion is one of the major threats to natural ecosystems. The alligator weed grows rapidly within a small span of time and is easily available all over the world. β-Carboline and quercetin are considered as excellent bioactive components of the alligator weed. In our study LC-MS/MS methods were performed for the detection and determination of the bioactive constituents, β-carboline and quercetin in leaves, in multiple reaction monitoring (MRM) mode. The effects of methanol extract on cardiomyocyte apoptosis induced by doxorubicin using H9c2 cells were evaluated by MTT assay and Annexin V-FITC/PI staining assay. A sensitive and selective liquid chromatography tandem mass spectrometry was developed and validated for the determination of β-carboline and quercetin in this plant. According to *in vitro* cell evaluation experiments, methanol extracts significantly prevented cardiomyocyte apoptosis induced by doxorubicin.**

Keywords: *Alternanthera philoxeroides* (Mart.) Griseb., β-caroline, quercetin, MRM, cardiomyocyte apoptosis

1. Introduction

Plant invasion is a worldwide problem and considered to pose as an environmental and economic threat to human beings (*1*). *Alternanthera philoxeroides* (Mart.) Griseb. is an invasive plant, which although originated in South America, can now be found in many parts of the world (*2*). The weed can grow rapidly in a short period of time and generally form a dense tangled mat. Due to its prevalence, it shades the aquatic vegetation from sunlight and reduces water flow. The alligator weed can also cause death of fish and native plants and give favorable habitat for mosquitoes. Though it is a hazardous weed, its abundance all over the world is exceptional (*3*). Thus in this work, we have tried to find some benefits of this invasive plant. Previous chemical investigation on this species revealed a number of compounds (*4-6*). Among those compounds, β-carboline and quercetin are of great interest due to their diverse biological activities (*7,8*). It is reported they have antioxidant, antimicrobial activity as well as cardioprotective effects. In the present study, we will focus on the study of its cardioprotective effects against doxorubicin (DOX).

As it is known, doxorubicin, sold under the trade names adriamycin among others, is a chemotherapy medication used to treat cancer including bladder cancer, breast cancer, Kaposi's sarcoma, lymphoma, and acute lymphocytic leukemia (*9*). It is often used together with other chemotherapy agents. However, their clinical use is markedly hampered by a major risk of cardiotoxicity that may lead to dilated cardiomyopathy and congestive heart failure (*10*). Prevention of this cardiotoxicity remains a critical issue in clinical oncology. Based on that, we wonder maybe we can utilize the compounds of the alligator weed and develop its cardiomyocyte apoptosis prevention induced by doxorubicin.

Firstly we have developed specific methods for the quantitative analysis of the β-carboline and quercetin

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in the *A. philoxeroides* using LC-MS/MS. We also investigated the effects of methanol extract (ME) from *A. philoxeroides* on cardiomyocyte apoptosis induced by doxorubicin. The results demonstrated that ME of the alligator weed was able to reduce DOX-induced H9c2 cell apoptosis.

2. Materials and Methods

2.1. *Reagents*

Standards were purchased from Sigma-Aldrich (Sigma-Aldrich, China). The chemicals acetonitrile (ACN, LC-MS grade) was purchased from Sigma-Aldrich. Formic acid additive for LC-MS was from Sigma-Aldrich. All aqueous solutions, including the HPLC mobile phase, were prepared with water purified using a Milli-Q system (Millipore).

2.2. *Preparation of β-carboline and quercetin standards*

Stock solutions of β-carboline and quercetin (1 mg/mL) were prepared in methanol, and working standard solutions were prepared by adequate dilution of stock solutions with methanol. Solutions were stored at 4°C in the dark. The stability of the stock and standard solutions under these conditions were checked and demonstrated for at least 2 weeks.

2.3. *Sample collection and extraction*

Leaves of *A. philoxeroides* were collected in the Jinan, China, on July, August September and October 2012, from eight different individual plants (numbered AP01-AP08). Ten young leaves and ten old leaves were harvested from each plant. The young and old leaves from each plant were freeze-dried, mixed and ground to a fine powder. The eight powdered samples were then stored in a cold room (−20°C) in the dark until analysis.

2.4. *Analysis sample and extract preparation*

A precise amount of 50 mg of each sample was weighed and extracted with 100 mL of methanol by ultrasound for 0.5 h, and macerated in dark overnight. The extracts were filtered and reported to the correct volume used for extraction. 200 g of the whole plant powder was extracted with 1 L methanol for 24 hours at 25°C. After 24 hours, the mixture was filtrated and the filtrate was evaporated to dryness. The weight of the extract was 13.2 g.

2.5. *LC-MS/MS conditions*

Chromatographic analysis was performed using a Shiseido HPLC system (Kyoto, Japan) equipped with pump (3202), auto-injector (5100), and column heater (3004). Chromatographic analysis was achieved with an Agilent Zorbax SB-C18 (150 × 4.6 mm, i.d.) with a particle diameter of 5 μm. Temperature of the column oven was set at T = 25°C during all experiment, and samples were held at a constant temperature of 4°C by the autosampler thermostat. The mobile phase was a mixture of water acidified with 0.1% of formic acid (A) and ACN (B). The elution of analyte was performed using an isocratic method of 90% of B. The flow rate was set at 0.5 mL/min. The injection volume was 2 μL.

An AB SCIEX QTRAP®5500 tandem mass spectrometer (AB, USA) was connected to the LC system though an electrospray ionization (ESI) interface. The ESI source was operated in positive ionization model for β-carboline and negative ionization model for the qucercetin. Quantification was performed using multiple reaction monitoring (MRM) method with the transitions of the precursor ions to the product ions of m/z 169.0 → 115.1 for β-carboline, and m/z 301.0 → 150.9 for quercetin, respectively. The ionization source conditions were see as follows: Entrance Potential (EP) 10 V; curtain gas (CUR) 30 psi; collision gas (CAD): Medium; ion spray voltage (IS): 5500 V for β-carboline and − 4200 V for quercetin; temperature (TEM) 550°C; nebulizer gas (GS1) 55 psi; heater gas (GS2) 55 psi. Compound dependent parameters were shown in Table 1. System control and data analysis were performed by AB Sciex Analyst software (version 1.6.2).

2.6. *Method validation*

Standard solutions of two representative compounds at a constant concentration were injected into the LC-MS/MS system. The procedure was carried out in triplicate for each concentration. The analyte areas obtained were plotted against the corresponding concentrations of the analyte (expressed as ng/mL) and the calibration curves were constructed by means of the least-square method (*11*).

The limits of quantification (LOQ) and limits of detection (LOD) were evaluated at the concentration in which the quantifier transition presented a signal to noise (S/N) ratio of > 10 and > 3 respectively.

Precision assays were carried out on standard solutions and leaf extracts. Standard solutions of the

Table 1. Multiple reaction monitoring (MRM) optimised parameters for the quantitation of β-carboline and quercetin in *A. philoxeroides* leaves

Analytes	Compounds	
	β-Carboline	Quercetin
Precursor ion	169.0	301.0
Product ion	115.1	150.9
Collision energy	35.0	− 24.2
Declustering potential	140	− 120
Entrance potential	10	− 10
Collision cell exit potential	13	13

selected compounds at three different concentrations (low, middle, and high) were analyzed six times within the same day to obtain intra-day precision and six times over six different days to obtain inter-day precision, both expressed as percentage relative standard deviation (RSD%). Similarly, leaf extracts were analyzed six times within the same day to test intra-day precision and six times over six different days to obtain inter-day precision.

Method accuracy was tested by means of percentage recovery assays, adding known amounts of standard solutions of the analytes leaf extracts, which had been already analyzed. The added concentrations corresponded to the lower limit, an middle value and a high value (*12*).

2.7. *Protective effects of methanol extract on doxorubicin-induced apoptosis in H9c2 cells*

Rat cardiac H9c2 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin and 5% CO_2 at 37°C. Cells were plated at an appropriate density according to each experimental design. H9c2 cells were incubation with ME (10, 20, 40, 80, 160 mg/mL) for 24 h. After incubation with 10 μM doxorubicin for another 24 h, a modified MTT assay was used to determine cell viability (*13*).

2.8. *Annexin V-FITC/PI staining assay*

Rat cardiac H9c2 cells seeded in 6-well plates (1.5×10^5 per well) were exposed to ME (10, 20, 40, 80, 160 mg/mL) for 24 h. After incubation with 10 μM doxorubicin for another 24 h, cells were harvested and washed with cold PBS. The cell surface phosphatidylserine in apoptotic cells was quantitatively estimated by using Annexin V-FITC/PI apoptosis detection kit according to the manufacturer's instructions (Becton Dickinson, USA). The analysis was performed on a FACScan flow cytometry.

3. Results

3.1. *Preliminary assays*

β-Carboline and quercetin, mass spectra were first acquired in full-scan mode (100-400 m/z) by infusion of reference solutions at 1 μg/mL. We used MRM to quantify the two compounds. The chosen MRM transitions for LC-MS/MS quantitative analysis were: β-carboline (169.0 → 115.1 for qualitative purposes); quercetin (301.0 → 150.9 for qualitative purposes) according to the spectra C and D in Figure 1 . The collision energy used to fragment the β-carboline and quercetin were optimized in the spectra E and F. Base on the data, we selected 35 V for β-carboline and − 24.2 V for quercetin to generate the sufficiently abundant fragmentation ion. The precursor ion and the product ion, together with optimized MS parameters for each compound are given in Table 1.

3.2. *Method validation*

The whole methodology was successfully validated in terms of linearity, precision and accuracy according to International Regulatory Guidelines, thus demonstrating reliability and suitability of this analytical strategy. Good linearity ($r^2 > 0.995$) was obtained over the 0.3-100 ng/mL concentration range for all the analytes. The LOQ and the LOD values were 0.3 ng/mL and 0.1 ng/mL, respectively, for the two compounds. Intra-day and inter-day repeatability were evaluated for each compound. CV% did not exceed a value of 15% for intra-day assay and 20% for inter-day assay. Mean recovery values were always higher than 95%; thus, method accuracy is satisfactory (Table 2).

3.3. *Quantitative results*

The validated method was applied to the analysis of *A. philoxeroides* leaf. Quantitative results are shown in Table 3 and each value is the mean of the results obtained from five analyses. It's shown that β-carboline and quercetin in the extracts from September are richer than those from other months.

3.4. *Cardioprotective effect*

To analyze the protective effects of methanol extract (ME) on doxorubicin-induced cytotoxicity in H9c2 cells, cell proliferation was examined after incubation with methanol extract (10, 20, 40, 80, 160 mg/mL) in the presence of doxorubicin. The result of cell viability was shown on Figure 2. The cell viability of the negative group in the presence of the DOX was 40.75 ± 4.25%, while incubation with different concentrations of ME for 24 h, the cell viability was boosted significantly. With treatment of 10, 20, 40, 80, 160 mg/mL ME, the cell viability was 38.43 ± 11.5%, 66.33 ± 6.03%, 79.00 ± 3.6%, 84.33 ± 5.5%, 83.16 ± 8.12% respectively. The pretreatment had a significant protective effect against doxorubicin-mediated cytotoxicity.

3.5. *Decrease of apoptosis induced by DOX*

Flow cytometry analysis results were shown in Figure 3. It was demonstrated that DOX induced 53.88% H9c2 cell apoptosis, while with the pretreatment of ME at the concentration of 10, 20, 40, 80, 160 mg/mL the cell apoptosis was decreased to 51.18%, 42.5%, 33.18%, 25.2%, 23.46% induced by DOX. It was illustrated that ME could decrease the cell apoptosis induced by DOX. And the protection effect of ME was relevant

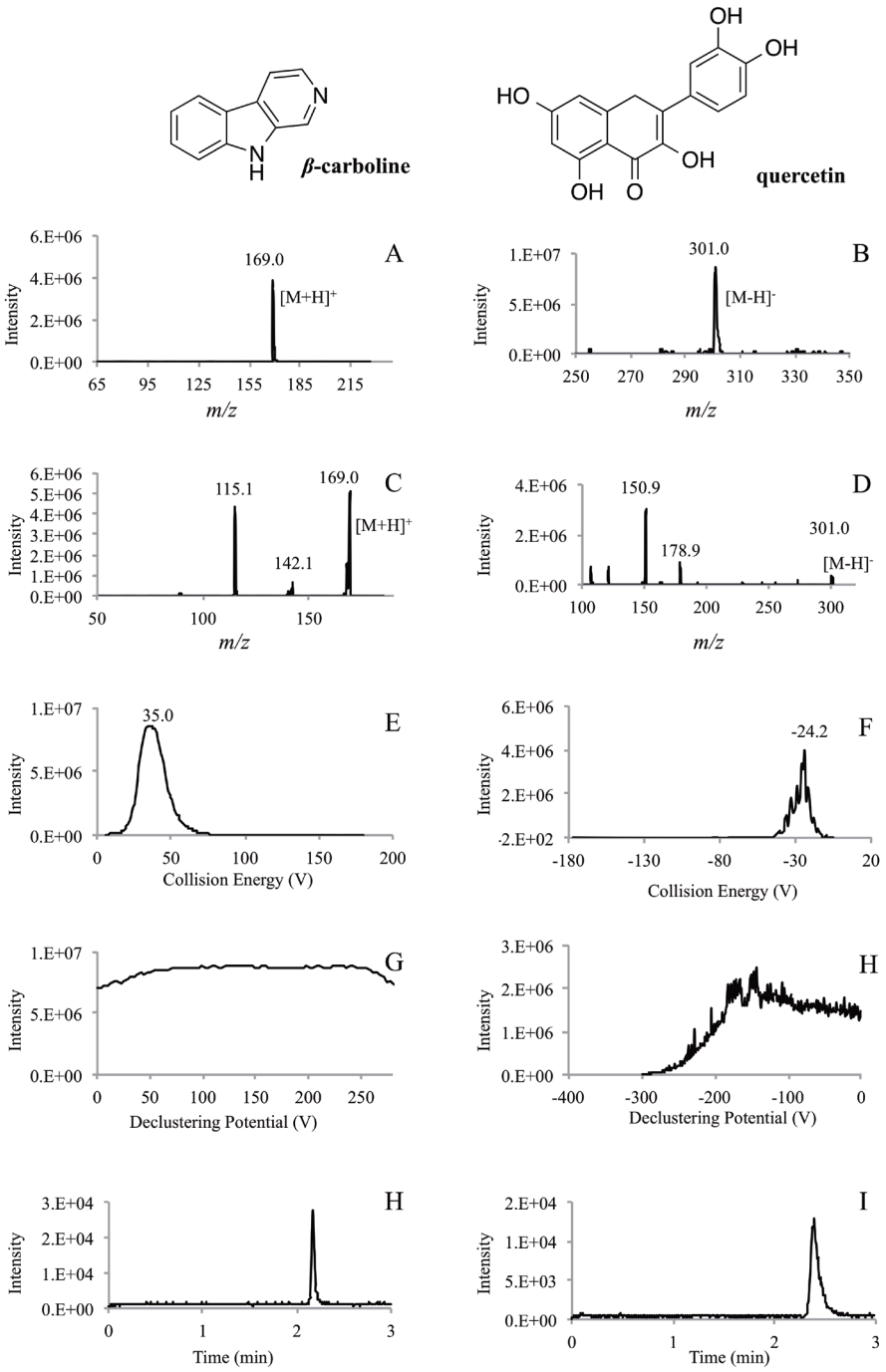

Figure 1. The MS spectra of β-carboline (A) and quercetin (B). MS-MS spectra of β-carboline **(C)** and quercetin **(D)**, obtained at the collision energy of 5 V and − 20V, respectively. According to the spectra, the precursor ions to the product ions of m/z 169.0 → 115.1 for β-carboline, and m/z 301.0 → 150.9 for quercetin were selected for the MRM. E and G were used to modify the value of collision energy and declustering potential for the MRM quantitation of β-carboline, while F and H were for quercetin. Spectra H and I were the MRM ion chromatograms of the β-carboline and quercetin, using the method described in the Material and Methods.

Table 2. Calibration parameters. Linear dynamic range, coefficient of determination (R2), limit of quantification (LOQ), limit of detection (LOD), intra-day, inter-day (CV%), and recovery (%) obtained using solutions of standards and standards addition in *A. philoxeroides* samples

Compounds	Linearity (ng/mL)	Calibration curves	r^2	Inter-day CV (%)	Intra-day CV (%)	Recovery
β-Carboline	0.3 - 100	$Y = 1.01e^5 \bullet X + 5.56e^3$	0.999	8.1%	13.4%	98.2%
Quercetin	0.3 - 100	$Y = 2.34e^5 \bullet X - 2.15e^3$	0.999	6.7%	12.5%	101.5%

to the concentration. When exposed to ME at higher concentration, there was less apoptosis cells.

4. Discussion

It has been reported that alligator weed has numbers of active compounds, such as phytosterol, flavonoid, triterpenes compounds and so on. Based on various constituents, different extractives using different solvents exhibit multiple pharmacological activities such as antinociceptive, antihyperglycemic, antioxidant, anti-microbial activities and a-glucosidase inhibitory effects. Among those compounds, β-carboline and quercetin are of great interest due to their diverse biological activities (*7,8*). For this, we analyzed the important two active components present in the leaf extract (methanolic) of *A. philoxeroides* using LC-MS/MS. Besides, the cardioprotective effect of the leaf extract was also explored by MTT and Annexin V-FITC/PI staining assay. In these two experiments Dox was explored to induced cell apoptosis. Doxorubicin (DOX) is an anti-tumor agent that is widely used in clinical setting for cancer treatment. The application of DOX, however, is limited by its cardiac toxicity which can induce heart failure through an undefined mechanism. So H9c2 cells induced by Dox is commonly used to screen cardioprotective agents. The results demonstrated that ME had obvious protective effects against doxorubicin-mediated cytotoxicity. The mechanism might be that ME could decrease the cell apoptosis induced by DOX, which was illustrated in Annexin V-FITC/PI staining assay. And the protective effects were relevant to the concentration of ME. In our experiments, only β-carboline and quercetin were determined by LC-MS/MS, but other components in ME solution might also have protective effects, which still required validation.

Table 3. Content of β-carboline and quercetin in *A. philoxeroides* leaves (ng/mg dry weight ± SD) (quantified by LC-MS/MS). Values shown are means (± SD) based on triplicate measurements

Samples	Collected Date	β-carboline (μg/g ± SD)	Quercetin (μg/g ± SD)
AP01	Jul 15th	13.3 ± 1.2	52.6 ± 2.7
AP02	Jul 15th	16.7 ± 2.1	45.2 ± 3.4
AP03	Aug 15th	23.2 ± 3.5	47.1 ± 5.4
AP04	Aug 15th	19.6 ± 0.8	49.5 ± 1.6
AP05	Sep 15th	35.1 ± 4.3	62.7 ± 2.5
AP06	Sep 15th	36.5 ± 2.0	70.5 ± 6.3
AP07	Oct 15th	12.7 ± 6.3	22.3 ± 2.7
AP08	Oct 15th	14.3 ± 3.3	25.6 ± 4.3

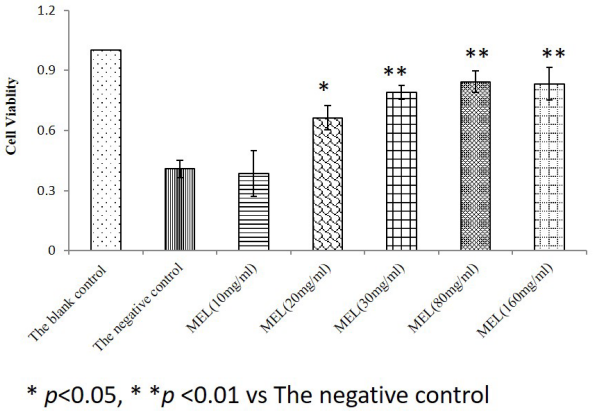

Figure 2. The cell viability results of ME exposed at different concentrations.

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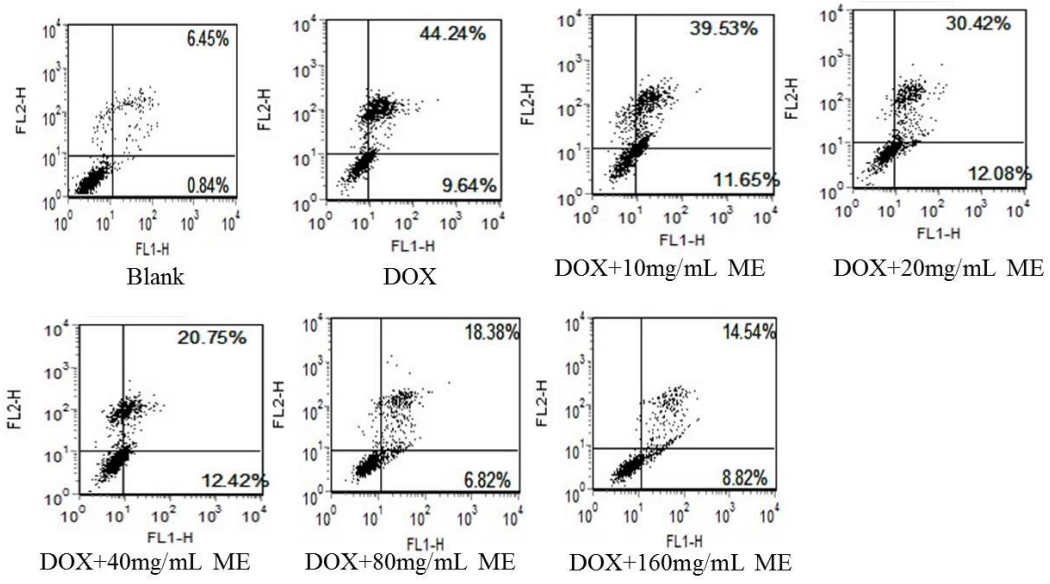

Figure 3. Flow cytometry analysis results illustrating cardioprotective effects of ME on H9c2 cells induced by Dox.

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