Natural Product Sciences
6(2) : 53~55 (2000)

Isolation and Anti-thrombotic Activity of Citric acid 1,5-dimethyl ester from *Gastrodia elata*

Mi Kyung Pyo, Kyung Mi Park and Hye Sook Yun-Choi*

Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea

Abstract – In the course of continous work for the discovery of anti-thrombotic constituents from tubers of *Gastrodia elata*, citric acid 1,5-dimethyl ester was isolated from ethylacetate soluble fraction prepared from the methanol extract. The structure of the compound was determined by the spectroscopic data. The anti-thrombotic effect of this compound was observed with prolonging the bleeding time in thrombin-induced thrombosis model of mice.
Key words – *Gastrodia elata*, citric acid 1,5-dimethyl ester, anti-thrombotic

Introduction

Gastrodia elata Blume (Orchidaceae) has been considered as one of the most important herbal medicines and used for the treatment of headaches, migraine, dizziness, childhood convulsion, epilepsy, rheumatism, neuralgia and other neuralgic and nervous affections (Bensky and Gamble, 1986, Tang and Eisenbrand, 1992). In the course of continous work for discovery of anti-thrombotic constituents from steamed and dried tubers of *Gastrodia elata*, citric acid 1,5-dimethyl ester was isolated from ethylacetate soluble fraction prepared from the methanol extract. The EtOAc fraction was one of the active subfractions (Paik *et al.*, 1995). The anti-platelet, anti-thrombotic and fibrinolytic effects were evaluated.

Experimental

General experimental procedure – Melting point was determined on a Mitamura-Riken melting point apparatus and uncorrected. IR spectrum was recorded on a Jasco FT/IR-5300 spectrometer and ^{1}H- and ^{13}C- NMR spectra were taken at 300 MHz and 75.5 MHz respectively on a Varian Gemini-2000 spectrometer with tetramethylsilane as the internal standard. Mass spectrum was taken with a VG Analytical VG 70-VSEQ. The elemental analysis was performed with a GmbH vario EL Elemental analysensystem by Seoul Branch Analytical Lab, Korea Basic Science Institute. Collagen and ADP were purchased from Chrono-Log Corp., U.S.A. and the assay kit of FDP (fibrinogen/fibrin degradation products) was obtained from Murex (England). The rats (Sprague-Dawley) and ICR mice were bred at the Animal Station of Natural Products Research Institute, Seoul National University.

Plant meterials – Steamed and dried tubers of *Gastrodia elata* were purchased from a crude drug market in Seoul and were identified by Prof. Hyung Joon Chi, Natural Products Research Institute, Seoul National University.

Isolation of citric acid 1,5-dimethyl ester – The steamed and dried tubers of *Gastrodia elata* (6 Kg) were refluxed with methanol three times for six hours each. The concentrated MeOH extract was partitioned between $CHCl_3$ and H_2O and the H_2O layer was further extracted with EtOAc to obtain EtOAc soluble fraction (40 g). 10 g of the EtOAc soluble fraction was chromatographed on a silica gel (700 g) column eluting with $CHCl_3$-MeOH (70:1) affording the colorless prisms (200 mg). mp 98-100°C (from MeOH-EtOAc), Anal. calcd. for $C_8H_{12}O_7H_2O$: C, 40.33; H, 5.94; found: C, 40.30; H, 5.92; IR ν_{max} (KBr) cm^{-1}; 3430, 1742; ^{1}H-NMR ($CDCl_3$); 3.71 (6H, s, 2×OCH_3), 2.96 and 2.84 (2H each, *d*, J = 16.5 Hz, 2×CH_2CO), ^{13}C-NMR (Acetone-d_6); δ 43.51, 51.82, 73.70, 171.00, 175.02; EI-MS *m/z*; 221 [m+1]$^+$, 171, 143 (base peak), 101, 59.

Platelet aggregation – Blood collected from rat heart after surgery using syringes containing 0.1 volume of 2.2% sodium citrate, was made to platelet rich plasma (PRP) by centrifugation at 200 g for 10

*Author for correspondence.

min. Platelet number was adjusted to 400-450×10^6/ml by mixing PRP and platelet poor plasma (PPP) with the aid of platelet counter (PLT-4, Texas International Lab., U.S.A.). The degree of platelet aggregation was measured with platelet aggregometer (Model 490-X, Chrono-Log Corp., U.S.A.). After 3 min pre-incubation, sample or vehicle was added and an aggregating inducing agent was added (ADP; 6×10^{-6} M, collagen; 3×10^{-6} g/ml) at 1 min. The reduction in turbidity of PRP was observed as the degree of aggregation.

Determination of bleeding time and serum FDP level – After one hour of oral administration of vehicle or sample, thrombin (250 U/kg) was injected to one of the tail vein of mice to induce thrombosis and ketamine (200 mg/kg, *i.p.*) was injected to peritoneal cavity to anesthetize. Five min after the anesthetization, bleeding time was determined by trans-section of tail, 2 mm from the tip (Dejana *et all.*, 1982). Blood was carefully blotted every 15 seconds on filter paper until no more blood was absorbed on the filter paper. Blood was collected from heart after 1 hour of thrombin injection. Each blood sample was mixed well with soybean trypsin inhibitor and *Bothrops atrox* venom in sample collection tube and incubated at 37°C for 30 min. After centrifugation at 1200 g for 5 min twice, the supernatant serum was separated and stored in freezer over night. Thrombo-wellcotest kit (Murex Diagnostic, England) was used for FDP assay and the assay was performed semi-quantitatively, according to the procedure in the kit.

Statistical Analysis – Data were summarized as mean±SD. Statistical analysis were performed by *t*-test. Differences were considered significant when $p < 0.05$.

Results and Discussion

The EtOAc fraction prepared from MeOH extract of *Gastrodia elata* was one of the sub-fraction with anti-platelet or anti-thrombotic activity (Paik *et al.*, 1995). The EtOAc soluble fraction was chromatographed on a silica gel column eluting with $CHCl_3$-MeOH (70:1) affording the colorless prisms upon recrystallization from MeOH-EtOAc. The IR spectrum showed absortion bands for hydroxyl (3430 cm^{-1}) and ester (1742, 1253 cm^{-1}) groups. The elemental analytical result indicated that molecular formular is $C_8H_{12}O_7H_2O$. The ^{1}H-NMR spectrum showed a methoxyl peak at 3.71 (6H, s, 2×OCH_3) and methylene signals arising from the citrate moiety at δ 2.96 and 2.84 (2H each, d, J = 16.5 Hz, 2×CH_2CO). The ^{13}C-NMR spectrum showed only five peaks, two at δ 175.02, 171.00 ascribable to the carboxylic carbons, one methoxy carbon peak at δ 43.51, one methylene carbon peak at δ 51.82 and a quaternary carbon peak at δ 73.70. EI-MS spectrum exhibited a $[m+1]^+$ peak at m/z 221. The structure of this compound was identified as citric acid 1,5-dimethyl ester (Fig. 1), on the basis of the above data.

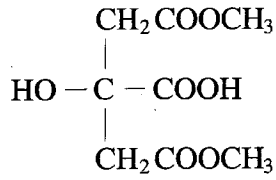

Fig. 1. Structure of citric acid 1,5-dimethyl ester.

Citric acid 1,5-dimethyl ester was synthesized as a mixture of monomethyl esters, 1,6-dimethyl ester and trimethyl ester from citric acid via controlled Fischer esterification, and separated by high-speed centrifugal countercurrent chromatography (Witherup *et al.*, 1995). However, this compound was isolated from plants for the first time and the preliminary data was previously reported by the authors (Yun-Choi & Pyo, 1999).

Although citric acid 1,5-dimethyl ester, is one of the constituents of the fraction with the anti-thrombotic effects (Paik *et al.*, 1995), this compound showed very mild inhibitory effect on rat platelet aggregation induced by collagen or ADP with the IC_{50} values of larger than 1×10^{-3} M. The effects of this compound on blood coagulation system were also evaluated. When 100 mg/kg of citric acid 1,5-dimethyl ester was orally administered to mice, the

Table 1. Effects of citric acid 1,5-dimethyl ester on bleeding time and serum FDP level

Sample	No. of mice	Bleeding time (sec)	FDP (ug/ml)
Normal	10	941.0±395.15	2.2±0.63
Control (thrombin 250U/kg, *i.p.*)	7	634.3±260.05	9.9±1.77
Citric acid 1,5-dimethyl ester (100 mg/kg, *p.o.*)	8	1038.8±370.54*	10.0±2.45

*P<0.05 from control

bleeding time increased about two-fold compared to the control (thrombin 250 U/kg, *i.v.*) (Table 1). However, the FDP level (10.0±2.45 μg/ml) in serum of the treated mice was not different from the control (thrombin treated) values. The above results are suggestive that the present citric acid 1,5-dimethyl ester shows anti-thrombotic effect with the mechanism different from the inhibitory effect against platelet aggregation induced either ADP or collagen and fibrinolysis.

Acknowledgements

This work was supported partially by the Brain Korea 21 project of Ministry of Education. The authors are grateful for the financial supports.

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(Accepted March 2, 2000)