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Isolation and Biological Activities of an Alkaloid Compound (3-methylcanthin-5, 6-dione) from *Picrasma quassiodes* (D. Don) Benn.

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Abstract – An alkaloid, 3-methylcanthin-5, 6-dione, was isolated from the stem of *Picrasma quassioides* (D. Don) Benn. and characterized by comprehensive analyses of its 1D and 2D NMR spectra. It was also evaluated for its cytotoxic activity *in vitro* against three human cancer cell lines (MDA-MB-231, HT-29 and NCI-N87), using MTT assays. We found that 3-methylcanthin-5, 6-dione exhibited significant anti-inflammatory activity via inhibiting NO production induced in LPS-stimulated murine macrophage RAW264.7 cells. The antioxidant activity of 3-methylcanthin-5, 6-dione was measured by DPPH free radical scavenging assays, hydroxyl radical scavenging assays and reducing power assays. Our results showed that 3-methylcanthin-5, 6-dione has significant biological activities.

Keywords – Alkaloid, anti-inflammatory activity, antioxidant activity, antiproliferative activity, 3-methylcanthin-5, 6-dione, *Picrasma quassioides*

Introduction

Picrasma quassioides (*P. quassioides*) is a medicinal herb belonging to the family of Simaroubaceae and used as a tumefaction, furuncle, vermifuge or insecticidal agent. The crude extracts of bark and fruit have been investigated intensively for these pharmacological properties and various compounds have been isolated: quassinoids (Yang *et al*., 1979), tirucallanes, ionone (Sugimoto *et al*., 1979) and β-carboline (Koike *et al*., 1990) alkaloids. Although many compounds were isolated from the bark and fruit of *P. quassioides*, their bioactivities have not yet been investigated.

Alkaloids are widespread in the plant kingdom and naturally occurring chemical compounds containing basic nitrogen atoms and are also present in plants used in medicine (Silva *et al*., 2008). Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, and animals, and are part of the group of natural products (Rumalla *et al*., 2008). Many alkaloids can be purified from crude extracts by acid-base extraction and are toxic to other organisms (Su *et al*., 2008). They often have pharmacological effects and are used as medications and recreational drugs.

In the present study, an alkaloid, 3-methylcanthin-5, 6-dione, was isolated from *P. quassioides*. This alkaloid has been identified (Ohmato and Koike, 1985), and Love (Love, 2006) reported that it has anticancer activity against human lung cancer cells (PC-6 cells). However, there have been no papers reporting its biological activities. Therefore, it is worthwhile to investigate the antioxidant, anti-inflammatory and anticancer activities of 3-methylcanthin-5, 6-dione using a variety of biochemical and cell-based assays.

Experimental

Plant material – The stems of the plant (*P. quassioides*) were purchased from Chuncheon, South Korea, in 2006. A voucher specimen was deposited at Kangwon National University.

Extraction and isolation – The air-dried and powdered stems of *P. quassioides* (4.50 kg) were extracted with MeOH (w : v = 1 : 20) three times for 3 h at 70 °C. The filtrate was concentrated by vacuum evaporation to obtain a crude extract (504.27 g), which was suspended in distilled water and then partitioned with CH_2Cl_2, EtOAc and *n*-BuOH to afford a CH_2Cl_2 layer (87.45 g), an

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EtOAc layer (6.34 g), an *n*-BuOH layer (31.67 g) and a water layer (335.00 g), respectively. Each fraction was tested for its antioxidant activity in a DPPH free radical scavenging assay and for its anticancer activity against human colon cells (Yin and Wang, 2007). The *n*-BuOH fraction exhibited the strongest activity. Therefore, the condensed *n*-BuOH fraction was further subjected to isolation procedures to isolate a single compound.

The *n*-BuOH fraction (31.67 g) was subjected to chromatography on an MCI gel column eluting with H_2O/MeOH (1 : 0, 3 : 2, 2 : 3, 0 : 1) as a step gradient mixture. The eluant afforded 4 fractions (PM1-PM4). A mixture of fractions 2, 3 and 4 (PM2-4) (8.62 g) was subjected to silica gel column chromatography eluting with CH_2Cl_2/MeOH (15 : 1 - 0 : 1) as a step gradient. The eluant afforded 12 fractions (PM2-4Si1-12). Fraction PM2-4Si4 (1349.50 mg) was subjected to chromatography on a Sephadex LH-20 gel column using MeOH as the eluant to afford 4 subfractions (PM2-4Si4S1-4). Fraction PM2-4Si4S2 (1140.40 mg) was subjected to silica gel column chromatography using a step gradient of CH_2Cl_2/MeOH (20 : 1 - 0 : 1) as the eluant. It afforded 9 fractions (PM2-4Si4S2Si1-9). Fraction PM2-4Si4S2Si9 (710.40 mg) was subjected to RP-18 gel column chromatography using MeOH/water (7 : 3 - 1 : 0) as eluant to afford 4 fractions (PM2-4Si4S2Si9RP1-4). Finally, an alkaloid compound (20.12 mg) was isolated.

Chemical structure of the alkaloid compound that was isolated – Orange-red powder; The results of NMR: ^{1}H-NMR (400 MHz, DMSO-d_6): δ 8.46 (1H, d, J = 8.00 Hz, H-8), 8.23 (1H, d, J = 7.75 Hz, H-11), 8.07 (1H, d, J = 6.90 Hz, H-2), 7.70 (1H, td, J = 7.75, 1.00 Hz, H-10), 7.55 (1H, td, J = 8.00, 1.00 Hz, H-9), 7.51 (1H, d, J = 6.90 Hz, H-1), 6.02 (1H, s, H-4), 3.91 (3H, s, H-1"); ^{13}C-NMR (100 MHz, DMSO-*d*6): δ 170.8 (C-5), 157.6 (C-6), 140.9 (C-11c, 139.8 (C-7a), 137.1 (C-2), 130.5 (C-10), 126.1 (C-9), 125.3 (C-3a), 125.0 (C-11a), 123.5 (C-11), 116.6 (C-8), 104.7 (C-1), 93.8 (C-4), 42.2 (C-1").

Cell culture maintenance – Colon adenocarcinoma (HT-29), stomach carcinoma (NCI-N87) and breast carcinoma (MDA-MB-231) cell lines were maintained as exponentially growing cultures in RPMI 1640 cell culture medium, and murine macrophages (RAW264.7 cells) were grown in DMEM cell culture medium, supplemented with 10% fetal bovine serum. All cell lines were cultured at 37 °C in an air/carbon dioxide (95 : 5) atmosphere.

DPPH free radical scavenging assay – Two milliliters of various concentrations of sample or water (control) was added to 2 mL DPPH solution (0.02 M in MeOH). Blanks contained 2 mL distilled water and 2 mL of sample solution. The mixture was shaken immediately after adding DPPH and allowed to stand at room temperature in the dark. The decrease in absorbance at 517 nm was measured after 30 min until the reaction reached a steady state. All experiments were done in triplicate. DPPH radical scavenging activity was calculated as follows : Scavenging effect (%) = $(1 - (A_i - A_j) / A_0) \times 100\%$; where A_0 is the A_{517} of DPPH without sample (control), A_i is the A_{517} of sample and DPPH, and A_j is the A_{517} of sample without DPPH (blank). The EC_{50} value (the concentration at which 50% of the radicals were scavenged by the test sample) were determined. The lower the EC_{50} value, the higher the antioxidant activity.

Hydroxyl radical scavenging assay – The Fenton reaction mixture consisted of 200 μL of $FeSO_4 \cdot 7H_2O$ (10 mM), EDTA (10 mM) and 2-deoxyribose (10 mM). A 200 μL sample and 1 mL of 0.1 M phosphate buffer (pH 7.4) were mixed and the total volume was brought to 1.4 mL. Thereafter, 200 μL of 10 mM H_2O_2 was added and the reaction mixture was incubated at 37 °C for 4 h. After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added and the mixture was placed in boiling water for 10 min. It was centrifuged (5 min, 300 rpm) and the absorbance was measured at 532 nm. Each assay was performed in triplicate. The hydroxyl radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = (1 - (A_1 - A_2) / A_0) \times 100\%$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of sample, A_2 was the absorbance without 2-deoxyribose (blank). EC_{50} values were calculated from the dose response curves.

Reducing power assay – Samples were mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was then placed in a 50 °C water bath for 30 min. Samples were kept at room temperature and 2.5 mL of 10% trichloroacetic acid was added to the mixture. The mixture was centrifuged at 3000 rpm for 10 min, and 2.5 mL of the upper layer solution was mixed with 2.5 mL distilled water and 0.5 mL of 0.1% ferric chloride. The absorbance of the reaction mixture was measured at 700 nm with a spectrophotometer.

Cell growth inhibition assay – 3-methylcanthin-5, 6-dione was tested at different concentrations (10 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL); each experiment was replicated three times. 3-methylcanthin-5, 6-dione was dissolved in DMSO and further diluted with cell culture medium containing different concentrations of DMSO. Tumor cells were seeded in a 96-well plate at a

concentration of 2×10^5 cells/mL. After 4 h (at 37 °C, in a humidified atmosphere of 5% CO_2), samples were added to the wells at a final concentration of 10 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL, respectively. The cells were then incubated for an additional 72 h at 37 °C. MTT stock solution (50 μL; 2 mg/mL in PBS) was added to each well for a total reaction volume of 250 μL. After incubating for 4 h in a humidified atmosphere of 5% CO_2 at 37 °C, the supernatants of each well were removed. The formazan crystals in each well were dissolved in 150 μL of DMSO. The amount of purple formazan was determined by measuring the absorbance at 550 nm after 15 - 20 min with an ELISA microplate reader (Athukorala *et al*., 2006). IC_{50} values (concentration that induce 50% inhibition of cell growth) were calculated from the dose response curves.

Inhibition of NO production by LPS-stimulated RAW264.7 cells – 160 μL of the murine macrophage cell line, RAW264.7, in a 96-well plate (0.2 mL, 4×10^5 cells/mL) was incubated for 12 h at 37 °C, 5% CO_2. The cells were then treated with 20 μL of LPS (2 μg/mL, final concentration) and 20 μL 3-methylcanthin-5, 6-dione solution (5 μg/mL, 10 μg/mL and 20 μg/mL, final concentrations). After 24 h incubation, 100 μL of supernatant from each well were transferred to a new 96-well plate. We then added 50 μL of sulfanilamide solution and NED solution to each well and let the mixture stand for 10 min. Last, absorbance was measured at 550 nm. The production of NO was determined by measuring the quantity of nitrite in the supernatant from the cells cultured under different conditions using a standard curve constructed with nitrite concentrations ranging from 0 - 100 μM. L-NAME at a concentration of 5 μg/mL was used as a positive control (Chen *et al*., 2008).

Results

Reducing power, DPPH free radical and hydroxyl radical scavenging activities – In order to measure the antioxidant activity of 3-methylcanthin-5, 6-dione, DPPH free radical and hydroxyl radical scavenging activities were determined. 3-methylcanthin-5, 6-dione has very high DPPH (EC_{50}: 143 μM) and hydroxyl radical (EC_{50}: 64.40 μM) scavenging activities compared to positive controls (Table 1). Another antioxidant activity measurement assay, a reducing power assay, was also evaluated. As shown in Fig. 2, 3-methylcanthin-5, 6-dione exhibited reducing power activity similar to the positive control, á-tocopherol. This means that 3-methylcanthin-5, 6-dione has very high antioxidant activity.

Table 1. Antioxidant activity of 3-methylcanthin-5, 6-dione isolated from *P. quassioides* by DPPH free radical and hydroxyl radical scavenging tests

Sample	DPPH Scavenging Activity (EC_{50}: μg/mL)	Hydroxyl Scavenging Activity (EC_{50}: μg/mL)
3-methylcanthin-5, 6-dione	35.87 ± 1.54	16.11 ± 2.56
Positive Control		
α-Tocopherol	14.44 ± 0.89	6.66 ± 1.05
L-Ascorbic acid	3.48 ± 0.24	*ND

*ND: No Detected.

Table 2. Anti-proliferation (MTT) assay using human stomach cancer (NCI-N87), colon cancer (HT-29) and breast cancer (MDA-MB-231) cells and 3-methylcanthin-5, 6-dione from *P. quassioides*

Cell lines	Cell Growth Inhibition (IC_{50}: μM)
HT-29	54.96 ± 2.35
NCI-N87	60.88 ± 1.89
MDA-MB-231	104.08 ± 2.13

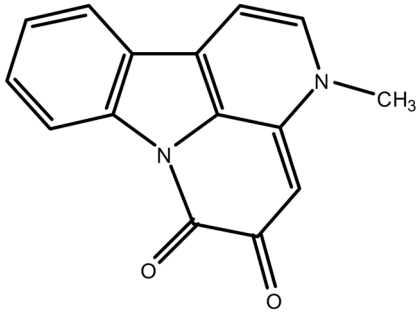

Fig. 1. Structure of 3-methylcanthin-5, 6-dione.

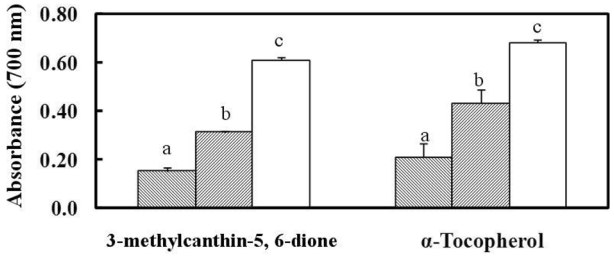

Fig. 2. Reducing power assay of 3-methylcanthin-5, 6-dione isolated from *P. quassioides* (▒: 50 μg/mL; ▨: 100 μg/mL; □ : 200 μg/mL). α-Tocopherol was used as a positive control. Vertical bars represent means of three replications ± SD. Bars labeled with different letters indicate a significant difference at $P < 0.05$.

Inhibition of NO production by LPS-stimulated RAW264.7 cells – As shown in Fig. 3, the results indicate that 3-methylcanthin-5, 6-dione significantly inhibited NO

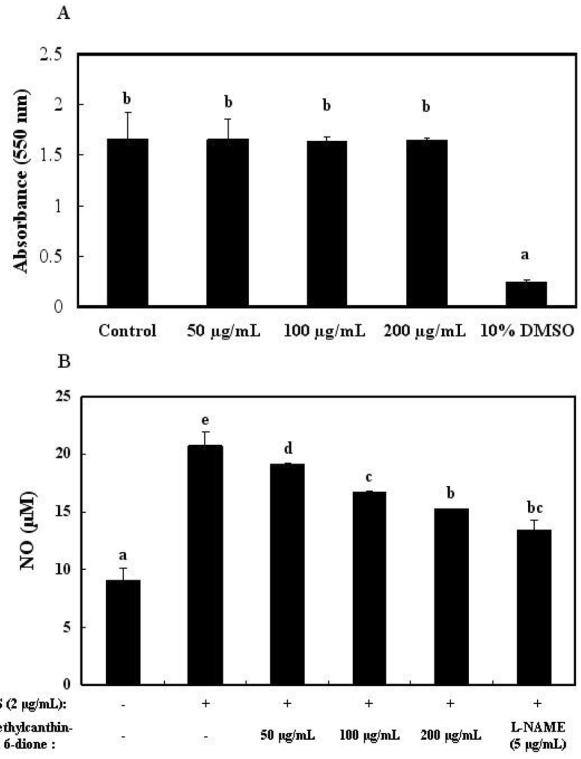

Fig. 3. Anti-inflammatory activity of 3-methylcanthin-5, 6-dione isolated from *P. quassioides*. L-NAME (5 μg/mL) was used as a positive control. Vertical bars represent means of three replications ± SD. Bars labeled with different letters indicate a significant difference at $P < 0.05$.

production by LPS-stimulated RAW264.7 cells. This result was compared to a positive control, L-NAME.

Cytotoxicity against cancer cells – 3-methylcanthin-5, 6-dione was evaluated for its cytotoxic activity. It showed strong activity against the cancer cell lines tested. The IC_{50} for growth inhibition was 0.74 mM against HT-29 cells, 0.68 mM against NCI-N87 cells, and 0.78 mM against MDA-MB-231 cells (Table 2).

Discussion

Reactive oxygen species (ROS) are ions or very small molecules that include oxygen ions, free radicals, and peroxides, both inorganic and organic (Liu *et al*., 2002). Oxidative damage caused by free radicals can theoretically contribute to chronic diseases, such as cancer, cardiovascular diseases, and age-related macular degeneration, and to aging (Prinn *et al*., 2001). 3-methylcanthin-5, 6-dione showed very high DPPH and hydroxyl radical scavenging activities (Table 1) and significant reducing power (Fig. 2). Our results thus suggest that 3-methylcanthin-5, 6-dione may be used as antioxidant or a food additive.

Inhibition of NO production may have potential therapeutic value when related to inflammation (Stichtenoth and Frolich, 1998). Furthermore, under inflammatory conditions, macrophages can greatly increase, simultaneously, their production of both NO and the superoxide anion (O_2^-), which rapidly react with each other to form the peroxynitrite anion ($ONOO^-$), thus playing a role in inflammation and also possibly in the multistage process of carcinogenesis (Cunha *et al*., 2009). In this study, NO released from LPS-stimulated murine RAW264.7 cells was analyzed. Fig. 3 shows that 3-methylcanthin-5, 6-dione induces a significant inhibition of NO production.

The ability to induce tumor cell apoptosis is an important property of a anticancer drug candidate, which discriminates between anticancer drugs and toxic compounds (Tan *et al*., 2005). The MTT assay is a novel method of quantifying metabolically viable cells via their ability to reduce a soluble yellow tetrazolium salt to blue-purple formazan crystals (Lee *et al*., 2008). The crystals are thought to be produced by the mitochondrial enzyme succinate dehydrogenase, and they can be dissolved and quantified by measuring the absorbance of the resultant solution. The absorbance of the solution is related to the number of live cells. A multiwell spectrophotometer assay can be semiautomated to process a large number of samples and provide a rapid object measurement of cell number (Kogure *et al*., 2003). Our study showed that 3-methylcanthin-5, 6-dione exerted a significant proliferation inhibitory activity against HT-29, NCI-N87 and MDA-MB-231 (Table 2).

The alkaloid, 3-methylcanthin-5, 6-dione isolated from *P. quassiodes* showed significant antioxidant, anti-inflammatory and anticancer activities. It could therefore be considered as a functional food ingredient or a pharmaceutical. However, *in vivo* experiments are needed to understand its mechanisms of action.

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