

## CYTOTOXIC PHENOLICS FROM THE FLOWERS OF *HIPPEASTRUM VITTATUM*

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أدت تجزئة وتنقية الخلاصة الكحولية للأزهار الطازجة لنبات الهبيسترم فيتاتم (العائلة النرجسية) المنزرع في مصر إلى فصل ثلاثة مركبات وهي حمض الكافيك ( ) استرات الميثيل لحمض ثنائي هيدروكافيك ( ) وذلك بالإضافة إلى البانكراتستاتين ( ). وقد تم التعرف على المركبات المفصولة باستخدام دراسات الرنين النووي المغناطيسي البروتوني والكر بوني أحادي وثنائي الأحداثيات بانواعها وكذلك قياس مطياف الكتلة. وقد تم دراسة الفعالية ضد الخلايا السرطانية لهذه المركبات.

*Bioassay-guided fractionation and purification of the ethanolic extract of the fresh flowers of *Hippeastrum vittatum* (Amaryllidaceae) cultivated in Egypt yielded three compounds viz. caffeic acid (1), dihydrocaffeic acid methyl ester (2), together with the polyhydroxylated alkaloid pancratistatin (3). The structures of the isolated compounds were determined on the basis of extensive 1D ( $^1\text{H}$  and  $^{13}\text{C}$ ) and 2D (COSY, HMQC, and HMBC) NMR studies, and mass spectral measurements. The cytotoxic activity of compounds 1-3 is presented and discussed.*

### INTRODUCTION

The Amaryllidaceae alkaloids have been found to possess a wide range of pharmacological properties, particularly antiviral<sup>1,2</sup> and antitumor activities.<sup>3-5</sup> In the course of our ongoing search for drug leads from the Egyptian Amaryllidaceae plants,<sup>6-12</sup> we have investigated the flowers of *Hippeastrum vittatum* Herbert. Previous studies on the plant have led to the isolation of a variety of alkaloids belonging to different classes.<sup>7,13-21</sup>

We have investigated the fresh flowers of *Hippeastrum vittatum* and found that the ethyl acetate fraction of an ethanolic extract of the fresh flowers possesses cytotoxic activity against HeLa cells. Bioassay-guided fractionation and purification of the active fractions led to the isolation of three compounds, namely caffeic acid (3,4-dihydroxycinnamic acid) (1), dihydrocaffeic acid methyl ester (3,4-dihydroxydihydrocinnamic acid methyl ester) (2) and pancratistatin (3). Pancratistatin and caffeic acid were found to be potent cytotoxic against HeLa cells.

The structure determination of the isolated compounds was established using different spectroscopic techniques including MS, and 1D ( $^1\text{H}$  and  $^{13}\text{C}$ ) and 2D ( $^1\text{H}$ - $^1\text{H}$  COSY, HMQC, and HMBC) NMR studies.

This paper deals with the isolation, structural elucidation, and the cytotoxic activity evaluation of compounds 1-3.

### EXPERIMENTAL

#### General experimental procedures

Melting points were uncorrected. Optical rotations were measured on JASCO DIP-1000 digital polarimeter. UV spectra were recorded on a Hitachi 300 spectrometer.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Varian Unity 300 spectrometer at 300 MHz for  $^1\text{H}$  and 75 MHz for  $^{13}\text{C}$ , respectively. EIMS data were obtained with a JEOL JMS-700T mass spectrometer. HPLC was performed on semipreparative (ARII Cosmosil, 250x10 mm) C18 column (Waters) with a UV detector at 220 nm and flow rate of 2.5 mL/min. Pre-coated silica gel 60 F<sub>254</sub> plates (E. Merck) were used for TLC.

### Plant material

The fresh flowers of *Hippeastrum vittatum* were collected in April 2002 from the cultivated plants at the campus of Suez Canal University. The plant material was kindly identified by Prof. Dr. A. Fayed, Professor of plant taxonomy at Assiut University. A voucher specimen was deposited in herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Suez Canal University under the registration number HV1.

### Extraction and isolation

The fresh flowers (4.53 kg) were crushed into small pieces and macerated in ethanol 70% (3x7 liters) at room temperature. The combined extracts were evaporated in vacuo under reduced pressure. The brown viscous residue was dissolved in 300 mL water and was successively extracted with n-hexane (3x200 mL) (3.20 g), CH<sub>2</sub>Cl<sub>2</sub> (3x200 mL) (1.85 g), ethyl acetate (3x200 mL) (2.23 g), and n-butanol (3x200 mL) (7.11 g). Preliminary cytotoxicity assay on the crude fractions showed that the ethyl acetate fraction possesses cytotoxicity against HeLa cells. The ethyl acetate residue (2.23 g) was subjected to flash chromatography on ODS column (35x2.5 cm) starting with H<sub>2</sub>O 100% through pure MeOH (each 300 mL). The fractions eluted with 20 and 30% MeOH showed moderate cytotoxicity (IC<sub>50</sub> = 2.0 µg/mL) against HeLa cells. The residue of these fractions (0.9 g) was flash chromatographed on ODS column (35x3.0 cm) starting with H<sub>2</sub>O 100% through pure MeOH (each 300 mL). The fractions eluted with 25% MeOH through 30% MeOH showed similar TLC patterns and were cytotoxic (IC<sub>50</sub> = 1.0 µg/mL) to HeLa cells. The residue of these fractions (325 mg) was flash chromatographed on ODS column (30x1.5 cm) starting with 10% MeCN in H<sub>2</sub>O through pure MeCN (each 300 mL). The fraction eluted with 20% MeCN was potent cytotoxic (IC<sub>50</sub> = 0.1 µg/mL) to HeLa cells. This fraction (85 mg) was finally purified on a semi-preparative C18 HPLC column (ARII Cosmosil, 250x10 mm, Waters) using 25% MeCN in H<sub>2</sub>O to give **1** (15.0 mg), **2** (11.7 mg), and **3** (4.3 mg).

**Caffeic acid (1):** Yellowish solid. m.p 220° (decomposition). EIMS: *m/z* 180 [M]<sup>+</sup>. NMR data: see Table 1.

**Dihydrocaffeic acid methyl ester (2):** Yellowish oil. EIMS: *m/z* 196 [M]<sup>+</sup>. NMR data: see Table 2.

**Pancratistatin (3):** White solid. m.p 323-325°.  $[\alpha]_D^{25} = +48.6^\circ$  (DMSO, *c* = 0.1). UV (MeOH)  $\lambda_{max}$ : 280, 237 nm. Positive HRFABMS: obsd *m/z* 326.0879 [M + H]<sup>+</sup> (calcd for C<sub>14</sub>H<sub>16</sub>NO<sub>8</sub>, 326.0876). IR (KBr)  $\nu_{max}$ : 3419, 1672, 1629, 1468, 1358, 1083, 1047 cm<sup>-1</sup>. <sup>1</sup>H and <sup>13</sup>C NMR data are previously listed in ref.(6).

## RESULTS AND DISCUSSION

### Compound 1

Compound **1** (Figure 1) was purified as yellowish solid. The EIMS showed a molecular ion peak at *m/z* 180, which is in consistent with the molecular formula of C<sub>9</sub>H<sub>8</sub>O<sub>4</sub>. The <sup>1</sup>H NMR spectrum of **1** (DMSO-*d*<sub>6</sub>) revealed resonances for eight protons (Table 1) including five signals in the aromatic/olefinic region together with signals for three exchangeable protons (2 OH and COOH). Interpretation of the <sup>1</sup>H-<sup>1</sup>H COSY and HMQC experiments showed the existence of two coupling spin systems. The first system (ABX system) is formed of the protons H-2 ( $\delta$  7.00, d, 2.0 Hz), H-5 ( $\delta$  6.74, d, 8.1 Hz), and H-6 ( $\delta$  6.94, dd, 8.1 and 2.0 Hz). The trans-coupled protons H-7 and H-8 (*J*<sub>7,8</sub> = 15.8 Hz) constitute the second coupling system (AB system) (Table 1).

The <sup>13</sup>C NMR spectrum of **1** (Table 1) showed resonances for nine carbons including five methines (C-2, C-5, C-6, C-7 and C-8), and four quaternary carbons (C-1, C-3, C-4, and C-9). Interpretation of all protonated carbons within **1** was made possible from the HMQC experiment. The assignment of the quaternary carbons was unequivocally secured from HMBC correlations (Figure 2). For example, HMBC correlations of H-2/C-4 ( $\delta$  148.0), H-5/C-3 ( $\delta$  145.5), H-5/C-1 ( $\delta$  125.6), H-7/C-9 ( $\delta$  167.8), and H-8/C-9.

The above-mentioned data are in good agreement with the reported data for caffeic acid (3,4-dihydroxycinnamic acid).<sup>22</sup> For the best of our knowledge, this is the first report of caffeic acid in the members of family Amariyllidaceae.

**Table 1:**  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift data of compound 1 (DMSO- $d_6$ ).

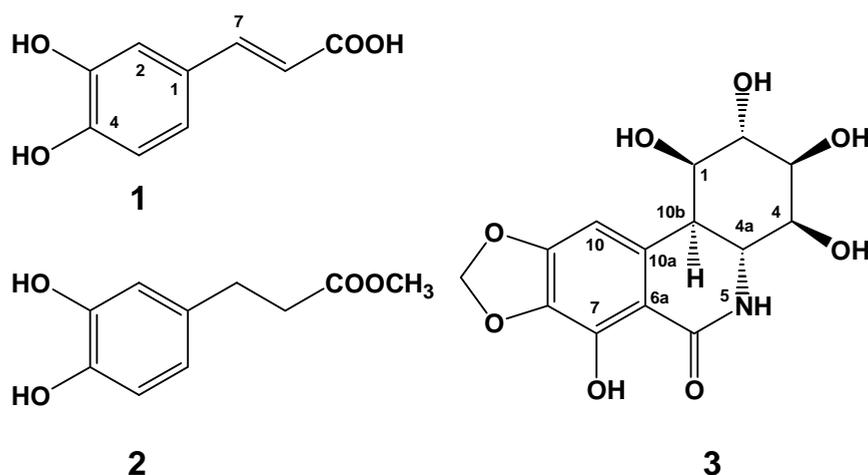
No.	$\delta_{\text{C}}$ (mult.)	$\delta_{\text{H}}$ [mult., $J(\text{Hz})$ ]	HMBC with C
<b>1</b>	125.6 (C)		
<b>2</b>	114.5 (CH)	7.00 (1H, d, 2.0)	C-4
<b>3</b>	145.5 (C)		
<b>HO-3</b>		$\sim 9.5$ (1H, br hump)*	
<b>4</b>	148.0 (C)		
<b>HO-4</b>		$\sim 9.5$ (1H, br hump)*	
<b>5</b>	115.7 (CH)	6.74 (1H, d, 8.1)	C-1, C-3
<b>6</b>	121.0 (CH)	6.94 (1H, dd, 8.1, 2.0)	C-4
<b>7</b>	144.3 (CH)	7.38 (1H, d, 15.8)	C-1, C-2, C-6, C-8, C-9
<b>8</b>	115.2 (CH)	6.15 (1H, d, 15.8)	C-7, C-9
<b>9</b>	167.8 (C)	$\sim 9.5$ (1H, br hump)*	

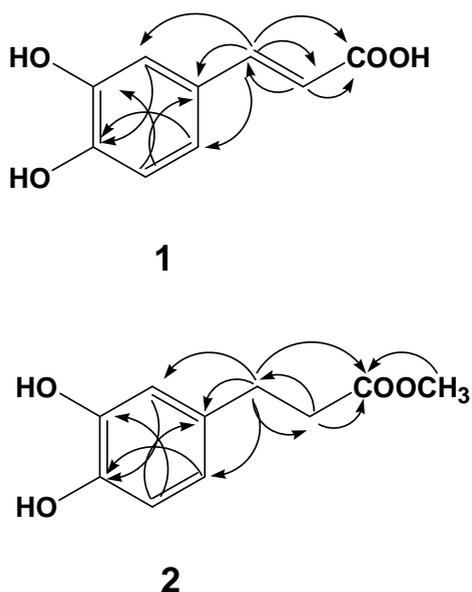
\*) Overlapped signals.

**Table 2:**  $^1\text{H}$  and  $^{13}\text{C}$  chemical shift data of compound 2 (DMSO- $d_6$ ).

No.	$\delta_{\text{C}}$ (mult.)	$\delta_{\text{H}}$ [mult., $J(\text{Hz})$ ]	HMBC with C
<b>1</b>	131.2 (C)		
<b>2</b>	115.4 (CH)	6.57 (1H, d, 2.1)	C-4
<b>3</b>	144.9 (C)		
<b>HO-3</b>		8.50 (1H, brs)*	
<b>4</b>	143.4 (C)		
<b>HO-4</b>		8.50 (1H, brs)*	
<b>5</b>	115.5 (CH)	6.62 (1H, d, 8.1)	C-1, C-3
<b>6</b>	118.6 (CH)	6.44 (1H, dd, 8.1, 2.1)	C-4
<b>7</b>	29.6 ( $\text{CH}_2$ )	2.67 (2H, t, 7.5)	C-1, C-2, C-6, C-7, C-9
<b>8</b>	35.3 ( $\text{CH}_2$ )	2.52 (2H, t, 7.5)	C-7, C-9
<b>9</b>	172.7 (C)		
<b>10</b>	51.1 ( $\text{CH}_3$ )	3.57 (3H, s)	C-9

\*) Overlapped signals.

**Fig. 1:** Structures of the isolated compounds 1-3.



**Fig. 2:** HMBC correlations of **1** and **2**.

### Compound 2

Compound **2** (Figure 1) was purified as yellowish oil with a molecular formula of  $C_{10}H_{12}O_4$  as established from the EIMS ( $m/z$  196). Its  $^1H$  NMR ( $DMSO-d_6$ ) spectrum revealed resonances for 12 protons (Table 2) including signals for three protons in the aromatic region forming an ABX system (H-2, H-5, and H-6), two two-proton triplets in the aliphatic region forming  $A_2B_2$  system ( $H_2$ -7 and  $H_2$ -8), together with three-proton singlet for the methyl ester ( $H_3$ -10). In addition, a two-proton broad singlet appeared at  $\delta$  8.50 for the OH moieties at C-3 and C-4.

The  $^{13}C$  NMR spectrum of **2** (Table 2) displayed resonances for 10 carbons including four quaternary carbons (C-1, C-3, C-4, and C-9), three methines (C-2, C-5, and C-6), two methylenes (C-7 and C-8) and one methyl (C-10). Interpretation of all protonated carbons of **2** was made possible from the HMQC experiment. The assignment of the quaternary carbons was unequivocally secured from HMBC correlations (Figure 2). For example, HMBC correlations of H-2/C-4 ( $\delta$  143.4), H-5/C-3 ( $\delta$  144.9), H-5/C-1 ( $\delta$  131.2),  $H_2$ -7/C-1,  $H_2$ -7/C-2,  $H_2$ -7/C-8,  $H_2$ -7/C-9 ( $\delta$  172.7),  $H_2$ -8/C-9, and  $H_3$ -10/C-9 secure the assignment of all carbons within **2** (Figure 2).

From the above spectral data and discussion, compound **2** can be assigned as

dihydrocaffeic acid methyl ester (3,4-dihydroxydihydrocinnamic acid methyl ester). For the best of our knowledge, this is the first report of dihydrocaffeic acid methyl ester from a natural source. So, this compound is considered as a new natural metabolite of *Hippeastrum vittatum*.

The existence of caffeic acid derivatives in the flowers of *Hippeastrum vittatum* is a noteworthy from the viewpoint of the chemotaxonomy of the genus *Hippeastrum* and family Amaryllidaceae.

### Compound 3

Compound **3** was purified as white solid. Compound **3** was identified as pancratistatin by comparison with an authentic sample that had been isolated recently from the flowers of *Pancratium maritimum* in our laboratory.<sup>6</sup> The complete and detailed spectral data for pancratistatin was recently reported by the author(s).<sup>6</sup>

### Cytotoxic activity of 1-3

The *in vitro* cytotoxicity of compounds **1-3** against HeLa cells were carried out according to Fukuzawa's method.<sup>23</sup> A brief description of the assay is summarized below:

#### A. Preparation of test samples

Tested samples for screening were prepared as follows. The polar crude extracts were dissolved in a mixture of  $H_2O/MeOH$  (1:1) to make a sample of 10 mg/mL. The lipophilic/organic crude extracts were dissolved in EtOH to make a solution of 5 mg/mL. Column fractions and pure compounds were prepared by dissolving in MeOH, DMSO, or water to afford final concentration of 1 mg/mL.

#### B. Cell culture

Human cervical carcinoma (HeLa) cells were maintained in adhesion on Petri dishes with Minimum Essential Medium (Gibco) supplemented with 10% fetal bovine serum, 2  $\mu g/mL$  gentamycin, 2  $\mu g/mL$  antibiotic-antimycotic, and 0.3 M  $NaHCO_3$  (adjusted to pH 7.0-7.4 with 2 M HCl). Subculture was made twice a week to maintain regular proliferation.

### C. Cytotoxicity test

HeLa cells in a 200 mL of medium (cell concentration 625 cells/mL) were plated on 96-well plates and allowed to adhere at 37° under an atmosphere of a mixture of air/CO<sub>2</sub> (95:5) for 24 hours. 2 mL of sample solution (concentration of 1 mg/mL) was added, fold serial dilution was made, and the cells were incubated for 72 h. Positive cytotoxicity control was also prepared using adriamycin (1 mg/mL in DMSO).

The cell's viability was assessed through an MTT conversion method. After 72 h, MTT (1 mg/mL, 50 mL) was added and the cells were incubated for an additional 3-4 h. The mixtures of medium and MTT solutions were removed by aspiration, and diluted with 150 mL DMSO. The optical density of each well was examined with a microplate spectrophotometer at wavelength of 510 nm. The IC<sub>50</sub> values were estimated using SOFTmax Pro3.1.1 program or plotting % inhibitions at tested concentrations on semi-log graph. Percent inhibition was calculated as  $\{(A-B)/A \times 100\}$ , where A and B were absorbance of negative control and sample, respectively.

The cytotoxicity of compounds **1-3** against HeLa cells was evaluated as IC<sub>50</sub> values and was presented in Table 3. Adriamycin was used as a positive cytotoxicity control. Caffeic acid (**1**) and Pancratistatin (**3**) showed potent cytotoxicity against HeLa cells with IC<sub>50</sub> = 0.45 and 0.06 µg/mL, respectively. Dihydrocaffeic acid methyl ester (**2**) was inactive against HeLa cells. The positive cytotoxicity control (Adriamycin) showed IC<sub>50</sub> value of 0.066 µg/mL.

**Table 3:** Cytotoxicity of compounds **1-3** against HeLa cells (IC<sub>50</sub>: µg/mL).

Comp. No.	<b>1</b>	<b>2</b>	<b>3</b> *	Adriamycin **
IC <sub>50</sub> value	0.45	> 10	0.06	0.066

\*) Data from reference 6.

\*\*) Positive cytotoxicity control.

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