# CYTOTOXIC AND IMMUNOSUPPRESSIVE ACTIVITIES OF METHYL CAFFEATE ISOLATED FROM *BALANOPHORA LAXIFLORA*

## HOẠT TÍNH GÂY ĐỘC TẾ BÀO VÀ ỨC CHẾ MIỄN DỊCH CỦA CHẤT METHYL CAFFEATE PHÂN LẬP TỪ LOÀI NGỌC CẨU (*BALANOPHORA LAXIFLORA)*

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**ABSTRACT**

Methyl caffeate, a major component, was isolated by column chromatography from *Balanophora laxiflora* growing in Tuyen Quang. The cytotoxic activity of methyl caffeate has been evaluated using A549, T24, Huh-7, 8505c and SNU-1 cell lines. Results showed that methyl caffeate exhibited cytotoxic activity against all five tested human cancer cell lines (A549, T24, Huh-7, 8505 and SNU-1) with the $IC_{50}$ values ranging from 28.83μg/mL to 50.19μg/mL. In addition, the immunosuppressive capacity of methyl caffeate through inhibition of IL-2 expression in macrophage cells (RAW264.7) was assessed for the first time. The results exhibited that methyl caffeate had a strong inhibitory effect on IL-2 cytokine secretion on RAW264.7 cells at a concentration of 100μg/mL ($P < 0.01$). These results suggest that methyl caffeate could possess immunosuppressive effects *in vitro*.

***Keywords:*** *Balanophora laxiflora*, *methyl caffeate, cytotoxicity, RAW264.7, interleukin, immunosuppressive effect*

**TÓM TẮT**

Methyl caffeat, một trong các chất chính, được phân lập bằng phương pháp sắc ký cột từ loài Ngọc cẩu (*Balanophora laxiflora*) mọc ở Tuyên Quang. Methyl caffeat được đánh giá hoạt tính gây độc tế bào đối với năm dòng tế bào ung thư người: A549, T24, Huh-7, 8505c và SNU-1. Kết quả cho thấy methyl caffeat thể hiện hoạt tính gây độc tế bào trên tất cả các dòng tế bào được thử nghiệm (A549, T24, Huh-7, 8505 và SNU-1) với giá trị $IC_{50}$ trong khoảng từ 28,83μg/mL đến 50,19μg/mL. Ngoài ra, đây là lần đầu tiên khả năng ức chế miễn dịch của methyl caffeat thông qua ức chế biểu hiện interleukin (IL-2) trong tế bào đại thực bào (RAW264.7) được đánh giá. Kết quả cho thấy methyl caffeat có tác dụng ức chế mạnh sự bài tiết cytokine IL-2 trên tế bào RAW264.7 ở nồng độ 100μg/mL ($P < 0,01$). Những kết quả này cho thấy methyl caffeat có thể có tác dụng ức chế miễn dịch *in vitro*.

***Từ khóa:*** *Ngọc cẩu, methyl caffeate, gây độc tế bào, RAW264.7, interleukin, tác dụng ức chế miễn dịch.*

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## 1. INTRODUCTION

Natural phenolic compounds have potential health benefits, and caffeic acid derivatives (CAFDs) have been predicted to act as chemopreventive agents and emerging adjuvants for cancer chemotherapy [1]. Caffeic acids (CA) are one of the abundant plant-based polyphenols, commonly found in almost plant species and popular foods such as vegetables, fruits, coffee, tea, wine. These derivatives are known to have many effects such as antioxidant, anti-inflammatory, anticarcinogenic, antiviral, immunomodulatory, and inhibited the the growth of many cancer cell lines, including liver, lung, colon cancer *[2,3]*. *In vitro* and *in vivo* studies have demonstrated the anticarcinogenic activity of caffeic acid esters (CAE) against hepatocarcinoma, a cancer causing considerable mortality across the world *[2-4]*. Methyl caffeate (MC, **1**) is a natural methyl ester had better solubility than CA. MC is known to have potent cytotoxic properties against MCF-7, A549, COLO320, HepG-2 cells and various biological activities *[4,5]*.

*Balanophora laxiflora* Hemsl. *ex* Forbes & Hemsl is growing in Northern Vietnam. Recently, we reported that four phenolic compounds isolated from *B. laxiflora* possess antiproliferative activity on acute myeloid leukemia (OCI-AML3) cells [6,7]. Interestingly, MC (**1**), one of the major components from this plant, significantly decreased the percentage of cells in S, G2/M phases and increased apoptosis in OCI-AML3 cells at low concentrations (15.62µg/mL) [6]. CAE were reported to inhibit inflammation by increasing leukocyte apoptosis and decreasing leukocyte concentration in exudate and it directly inhibited the interaction of NF-κB with DNA. Furthermore, Adem and his group reported that CAFDs possible effectively reduce the SARS-CoV-2 viral load, inhibit and shorten the infectious period by calculation using Molegro Virtual Docker software *[8]*. These results suggest that CAE could have immunomodulatory effects and as a potential substance to fight against COVID-19 *[8]*. However, hitertho the effects of MC on the immunomodulatory and anticancer activities still remain unknown. Therefore, the present study was carried out to assess the possibility of immunosuppression through inhibition of IL-2 expression in macrophage cell line (RAW264.7) and anticancer properties against A549, T24, Huh-7, 8505 and SNU-1 cell lines of isolated methyl caffeate from *B. laxiflora*.

## 2. EXPERIMENTAL

### 2.1. Plant materials

Plant material was collected in Na Hang district, Tuyen Quang province, Vietnam in December, 2019. The species, *Balanophora laxiflora* Hemsl. *ex* Forbes & Hemsl, was identified by botanist Dr. Do Huu Thu. A voucher specimen (No. BL-ICH 2019) was deposited in the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST), Hanoi, Viet Nam.

### 2.2. General experimental procedures

ESI MS spectrum was obtained on an Agilent 1100 LC-MSD Trap spectrometer. FT-ICR-MS model 910 TQ- FTMS, Agilent USA. HPLC-MS Agilent 1100 USA. $^{1}$H NMR (500.13 MHz) and $^{13}$C NMR (125.77 MHz) spectral data were measured on a Bruker Avance III NMR spectrometer, Switzerland. Chemical shifts were expressed in δ (ppm) downfield from TMS as an internal standard and coupling constants were reported in Hertz. Solvents $CDCl_3$ ($^{1}$H $\delta_H$7.26; $^{13}$C $\delta_C$77.0). Silica gel 60 F-254 (0.25mm, Merck); CC: Silica gel 60 (230 - 400 mesh, Merck) for the first column, silica gel 60, 40 - 63µm (Merck) and Sephadex LH-20 for the following columns.

The purity of compound MC was determined by HPLC-DAD (HPCHEM) using XDB-C18 column (150 × 4.6mm I.D, 5µm) - CUD-4, flowrate 1.0mL/min, isocratic elution (30% methanol in distilled water +0.1% formic acid, MeOH-$H_2O$, 30:70), UV spectrum at 225, 290, 330nm. temp. 25°C. Detection was carried over 35 min at a flow rate of 1mL/min. Detection was carried out at 230 and 280nm. The injection volume was 5µL.

### 2.3. Extraction, isolation and structure characterization

Extraction and separation of compound **1** from *B. laxiflora* was modified from a method described previously [6, 7]. Its chemical structure was analysed and identified by HR ESI MS and NMR techniques. The purity of MC (≥ 98%) was determined by HPLC and its chromatogram is shown in (Figure 2).

### 2.4. *In vitro* cell culture

All experimented cell lines were cultured in plastic culture flasks in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% PSF (penicillin/streptomycin/fungizone) solution under 5% $CO_2$ at 37°C. Cells were cultured with Trypsin - EDTA (0.05%) every 3 days of culture.

### 2.5. *In vitro* cytotoxic evaluation

Five human cancer cell lines including A549 (lung carcinoma), T24 (urinebladdercarcinoma), Huh-7 (hepatocarcinoma), 8505 (undifferentiated thyroid carcinoma) and SNU-1 (gastric carcinoma) were kindly provided by Prof J. M. Pezzuto, Long-IslandUnivercity, USAand Prof. Chi-Ying Huang, Yang-Ming National University, Taiwan. The cytotoxic activity of the sample was determined by *Sulforhodamine B (*SRB) assay [9-12]. Briefly, cells were seeded in the 96-well plates at a concentration of $3 \times 10^4$ cells/mL and treated with different concentrations of sample for 48 hours in the incubator at 37°C and 5% $CO_2$. Assay sample was initially dissolved in DMSO 100% and serially diluted to appropriate concentrations with a culture medium right before the assay. Subsequently, cells in each well, which had been incubated for 24 hours as described above, were treated with 10µL of samples to reach final concentrations as 100µg/mL; 20.0µg/mL; 4.0µg/mL; 0.80µg/mL and 0.16µg/mL. The plates were further

incubated for 48 hours. After that, cells were fixed with 20% *trichloroacetic acid (*TCA), washed under tap water and stained with SRB 0.4% for 30 minutes. The protein-binding SRB dye, which is related with cell viability, was dissolved in Tris-base buffer. The optical density was determined at 540nm by ELISA reader (Tecan GENios Plate Reader). $IC_{50}$ values were calculated based on the percent inhibition of cellgrowth using Tablecurve 2Dv4 software. The values reported for the compound **1** are presented as averages of three determinations. Ellipticine was used as a positive control.

### 2.6. Determine the effect of the sample on IL-2 expression

*Determination of RAW264.7 cell growth:* To determine cell survival rateunder the influence of tested sample, the MTT methodwas performed according to Mosman et al. (1983) with small modification. Specifically, RAW 264.7 cells subsequently seeded into a 96-well microtiter plate at 2.5 x $10^5$ cells/well (190µL) and incubated with sample (10µL) to reach final concentrations at 100µg/mL and 20µg/mL. Cells treated with DMSO1% are used as negative control; The blank well has only culturedmedium. At the day 3.2µL MTT (5mg/mL) was added to each well of the plate and incubated at 37°C for 4 hours; The plate were then shaken gently for 10 minutes before measuring the formazan color content at 540nm wavelength by using an ELISA plate reader (BioTek). The cell survival percentage under sample treatment will be determined by using the following formula:

$$\%\ \text{survived} = \frac{\text{OD(sample)} - \text{OD(blank)}}{\text{OD(DMSO)} - \text{OD(blank)}}$$

*Determine the effect of the sample on the interleukin 2 (IL-2) expression*

RAW 264.7 cells were placed in the experimental wells of the 96-well plate with the appropriate amount of cells ($2.5 \times 10^5$ cells/well in 190µL of medium) and incubated at 37°C overnight. Test compounds (10µL) were introduced into the cell seeded wells at different concentrations. Some wells without sample but with cells (190µL) and 10µL of 1% DMSO will be used as negative controls. After 2 hours of incubation, lipopolysaccharide (LPS) at a concentration of 1µL/mL was provided into all experimental wells. After 24 hours, cell culture supernatants were collected and assayed to evaluatethe presence of interleukin 2 (IL-2 mouse ELISA Kit) according to the kit manufacturer's instructions (R&D Systems, Minneapolis, US).

*Statistical analysis:* The data was processed using Excel, presented as the mean ± standard deviation (±SD). Differences was assessed for significance using analysis of variance (one-way ANOVA) and Student's *t*-test, F'test where probability $P < 0.05$ was considered significant.

## 3. RESULTS AND DISCUSSION

Compound **1** was isolated from aerial part of *B. laxiflora* by a modified method previously described [6]. Based on HR-ESI-MS, 1D and 2D NMR experiments as well as by comparison with reported data, its structure was identified as methyl caffeate (**1**) (Figure 1) [6,7]. The presence of compound **1** in the ethanolic extract from this plant material proves that it is rearly natural component and not an artifact produced during extraction and separation.

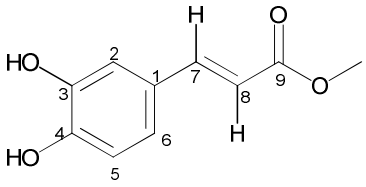


Figure 1. Structures of methyl caffeate (**1**) isolated from Balanophora laxiflora

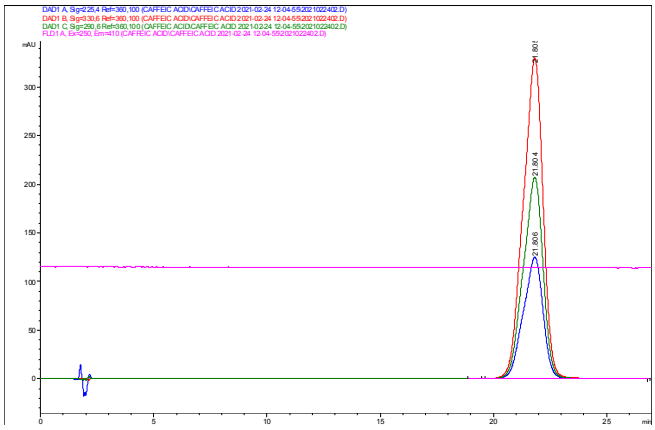

Figure 2. HPLC chromatogram of methyl caffeate (MC, **1**)

Recently, pharmacological studies have demonstrated thatcaffeate derivatives possess potentcytotoxic activities and several anti-inflammatory activities [8,13-17]. Since, MC is one of the most common in plants and the most active substances from *B.laxiflora* and easily semi-synthesized from CA, we decided to evaluate its cytotoxicity effects [8,18]. A549, T24, Huh-7, 8505 and SNU-1 human cancer cell lines were chosen for screening their inhibitory effect using SRB method [18]. Compounds **1** were initially screened at a fixed concentration of 100µg/mL and subsequently assayed at 4 concentrations (e.g., 100.0, 20.0, 4.0, 0.8) and for further $IC_{50}$ value determination. Results were presented in Table 1. The results showed that MC inhibited the growth of all tested cancer cell lines (A549, T24, Huh-7, 8505, SNU-1) at average level with $IC_{50}$ values of 28.83±2.38; 50.19±3.50; 42.15±2.13; 27.03±2.75; 34.50±4.16µg/mL, respectively.

Recently, the immunomodulatory and cytokine antagonist effects of caffeates have been of particular interest to scientists around the world [8]. Caffeic acid esters (CAE) have been described to exhibit positive activities regarding immunosuppressive process, and IL-2 is the major cytokine responsible for this effects [15,16]. Macrophages serve important roles in immunity and protect the body from pathogen invasion. Cytokines play key roles in cellular immune responses, and a variety of immune cell interactions are mediated by cytokines [15]. Therefore, in this study, effects of MC on the growth of RAW264.7 cells as well as on their IL-2 secretion capacity were investigated.

Table 1. Cytotoxic activity of methyl caffeate (**1**) and ellipticine[a]

| Concentration (μg/mL) | % inhibition | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MC (1)/cell line[b] | | | | | Ellipticine | | | | |
| | A549 | T24 | Huh-7 | 8505 | SNU-1 | A549 | T24 | Huh-7 | 8505 | SNU-1 |
| **100** | 81.69 | 79.56 | 89.22 | 82.32 | 85.21 | 89.08 | 87.77 | 90.32 | 92.38 | 91.03 |
| **20** | 46.33 | 27.27 | 30.89 | 41.30 | 37.62 | 71.99 | 71.89 | 78.52 | 82.38 | 80.33 |
| **4** | 15.31 | 15.26 | 11.00 | 26.49 | 18.02 | 49.36 | 48.16 | 49.99 | 51.23 | 50.72 |
| **0,8** | 10.02 | 6.83 | 5.45 | 6.75 | 7.43 | 25.56 | 22.21 | 24.65 | 23.87 | 23.56 |
| **$IC_{50}$** | 28.83 ±2.38 | 50.19 ±3.50 | 42.15 ±2.13 | 27.03 ±2.75 | 34.50 ±4.16 | 0.46 ±0.05 | 0.52 ±0.04 | 0.39 ±0.04 | 0.38 ±0.03 | 0.39 ±0.03 |

[a] *Ellipticine: positive control;*

[b] *Cell lines: A549 (lung carcinoma), T24 (urinebladdercarcinoma), Huh-7 (breast carcinoma), 8505 (undifferentiated thyroid carcinoma) and SNU-1 (gastric carcinoma).*

When determining the effect of MC on the growth of RAW264.7 cells (cell concentration is $2.5\times10^5$ cells/well), MC did not cause more than 20% cell death at high concentrations (100 and 20μg/mL).Therefore, the concentration range of 100-20-4-0.8μg/mL was used to determine the effect of the sample on the expression of cytokine IL-2. The results indicated that MC had a significant inhibitory effect on IL-2 cytokine secretion in RAW264.7 cells at a concentration of 100μg/mL ($P < 0.01$ compared with LPS control). At lower concentrations (20.0, 4.0 and 0.8μg/mL), MC exhibited the ability to inhibit IL-2-producing macrophages, but at a weaker level and unsignificant level. On the basis of these findings, we could suppose that MC might affect to T cells in the immune system.

Table 2. Effects of of methyl caffeate (**1**) on IL-2 expression and control LPS[a]

| Concentration (μg/mL) | Fold times change in IL-2 content compared to negative control |
|---|---|
| **100** | 0.52**± 0.07 |
| **20** | 0.82± 0.09 |
| **4** | 0.89± 0.09 |
| **0.8** | 0.97± 0.12 |
| **1**[a] (LPS) | 4.16 ± 0.05 |
| Negative control | 1 ± 0.01 |

[a] *LPS: Control Lipopolysaccharide*

To our knowledge, MC is very safe with $LD_{50}$ could not be determined at the highest oral doses [19]. Furthermore, this natural substance is readily obtained by semisynthetic route from caffeic acid. Thus, the compoundis a potential lead for research and application in pharmaceuticals [8,15-18].

## 4. CONCLUSIONS

In conclusion, our studies presented for the first time an indirect evidence of immunosuppressive activity through IL-2 expression of MC and reinforces this capacity in accordance with the previous reports of other caffeic acid derivatives (CAFDs). The effect on other cell-mediated immune responsesof MC is under our further investigation.

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**THÔNG TIN TÁC GIẢ**

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