

CYTOTOXIC COUMARIC ACID DERIVATIVES FROM *PREMNA SERRATIFOLIA* L. ON HUMAN COLON CANCER CELL DLD-1

ĐỘC TÍNH TẾ BÀO CỦA CÁC DẪN XUẤT ACID COUMARIC TỪ CÂY *PREMNA SERRATIFOLIA* L. ĐỐI VỚI DÒNG TẾ BÀO UNG THƯ RUỘT KẾT DLD-1 Ở NGƯỜI

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ABSTRACT

The dried leaves (2 kg) of *Premna serratifolia* L. (Verbenaceae) were exhaustively extracted in methanol then filtered and evaporated under reduced low pressure. The crude extract was subjected to column chromatography (CC) with gradient elution (petroleum ether, chloroform, and butanol). The petroleum ether extract has been studied in chemical constituents and biological activity. Three compounds, namely, methyl trans-4-methoxycinnamate (**1**), methyl trans-4-hydroxycinnamate (**2**) and methyl caffeate (**3**) have been isolated from this extract by using column and thin layer chromatographies. The chemical structures of pure compounds were elucidated by using 1D and 2D-NMR spectroscopies in comparison with published data. The cytotoxicity of these compounds was evaluated on human colon cancer cell line DLD-1. The results showed a significant cell inhibition. Among the tested substances, methyl caffeate (**3**) exhibited the strongest cytotoxicity toward DLD-1 ($IC_{50} = 66 \mu M$).

Keywords: *Premna serratifolia* L.; Verbenaceae; acid caffeic; cinnamate.

TÓM TẮT

Lá khô (2 kg) của cây vọng cách (*Premna serratifolia* L.), họ cỏ roi ngựa được trích kiệt với dung môi metanol, sau đó dung dịch metanol được lọc và đuổi dung môi. Tiến hành sắc ký cột đối với cao metanol, giải ly với các dung môi có độ phân cực tăng dần (eter dầu hỏa, cloroform và butanol). Cao eter dầu hỏa được nghiên cứu thành phần hóa học và hoạt tính sinh học. Ba hợp chất gồm methyl trans-4-methoxycinnamate (**1**), methyl trans-4-hydroxycinnamate (**2**) and methyl caffeate (**3**) được cô lập từ cao eter dầu hỏa bằng các kỹ thuật sắc ký cột và sắc ký lớp mỏng. Cấu trúc hóa học của các hợp chất được xác định bằng phổ cộng hưởng từ hạt nhân một chiều và hai chiều. Độc tính tế bào của các hợp chất này được thử nghiệm trên dòng tế bào ung thư ruột kết DLD-1 ở người. Trong số các hợp chất được thử nghiệm, hợp chất methyl caffeate (**3**) có hoạt tính mạnh nhất với IC_{50} là $66 \mu M$.

Từ khóa: Cây vọng cách (*Premna serratifolia* L.); họ cỏ coi ngựa; acid caffeic; cinnamate.

1. INTRODUCTION

The genus *Premna*, comprised of 200 species, is distributed in tropical and

subtropical countries Asia, Africa, Australia and Pacific islands [1]. The plant *Premna serratifolia* L. (Figure 1) is widely distributed

in Vietnam, Laos and Cambodia. In Vietnamese traditional medicine the leaves of this plant are used to treat indigestion, dysuria and dysentery. The roots are also employed against indigestion, stomachache and fever [2]. Previous phytochemical investigations on the *Premna* species reported the isolation and structural identification of a number of iridoid glycosides, polyphenols, flavonoid, coumaric acid and its derivatives, while there is no study on the bioactive constituents of *P. serratifolia* L [3, 4]. Here, in this study, chemical constituents of *P. serratifolia* and its cytotoxicity on human colon cancer cell DLD-1 are described.



Figure 1. Leaves of *Premna serratifolia* L.

2. EXPERIMENTAL

2.1 Materials

The leaves of *Premna serratifolia* L. were collected in District 12 in Ho Chi Minh City in September 2016. The plant was identified by Tran Thi Thanh Huong, Department of Biology, Nong Lam University.

2.2 Chemicals and Equipments

^1H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were recorded on either a Bruker Avance 500 spectrophotometer, using TMS as internal standard. Thin layer chromatography (TLC) was performed on silica gel (Merck, Kialselgel 60 F₂₅₄, 250 μm). Column chromatography (CC) was carried on silica gel (40 – 63 μm , Merck). Spots were

visualized by spraying 10% H_2SO_4 UV (254 and 365 nm).

McCoy's 5A medium containing glutamax I (a stabilized form of L-glutamine), calcium-free and magnesium-free phosphate-buffered saline (PBS), trypsin, versene, gentamicin were obtained from Gibco BRL (In Vitrogen, Taastrup, Denmark). Fetal bovine serum (FBS) was obtained from Seromed (Biochrom KB, Berlin, Germany). Resveratrol (RV), dimethylsulfoxide (DMSO), sulforhodamine B (SRB), trichloroacetic acid (TCA) and propidium iodide were purchased from Sigma-Aldrich. Tris-base and acetic acid were purchased from Merck.

2.3 Extraction and Isolation

The dried leaves (2 kg) were extracted in methanol under reflux in three hours, then filtered and evaporated. The crude extract was subjected to column chromatography and eluted with petroleum ether, chloroform and butanol. The petroleum ether, chloroform, and butanol extracts were evaporated.

The petroleum ether extract (60 g) was subjected to column chromatography, eluted with different solvents, yielded ten fractions (E.1-E.10). From the E.2 fraction (1 g), methyl *trans*-4-methoxycinnamate (**1**) (20 mg) was isolated (20 mg). From the E.3 (500 mg) and E.4 fractions (300 mg), methyl *trans*-4-hydroxycinnamate (**2**) (25 mg) and methyl caffeate (**3**) (40 mg) were isolated, respectively.

2.4 Biological activity test

Cell lines

Human colon cancer cell line DLD-1 was obtained from American Type Culture Collections and cultured in McCoy's 5A medium supplemented with 10% FBS and 0.5ml gentamicin. Cells were subcultured by

trypsination when the cell density reached approximately 80 - 90% and incubated at 37°C in a 5% CO₂ humidified incubator. The cell line was subcultivated no more than 30 passages. DMSO was used as solvent, and all the cells were exposed to the same concentration in each experiment (< 0.1%, which is a noncytotoxic concentration).

Sulforhodamine B assay (SBR assay)

The DLD-1 cells were diluted by medium at a density of 10⁵ cells/ml and incubated in 96 well microplates in a volume of 100 µl/well. All outer wells received 100 µl/well PBS. Plates were left in the incubator for approximately 24 hours so that cells were able to attach to the bottom of the wells before exposing test samples. Stock solution (100 mM) was diluted to have a range of concentrations e.g. 120 µM, 60 µM, 30 µM, 15 µM, 7.5 µM etc. Resveratrol solution (60 µM), a known cytotoxic agent, was used as a positive control. The note for preparing test sample solutions was to double the test concentration needed due to culture medium didn't remove prior to adding stock solution.

A day after cells adhered to the bottom of wells, cells were exposed with 100 µl of each concentration of test sample and continued to be incubated in humidified and CO₂ incubator for 24 hours, 48 hours and 72 hours. At the end of assay, plates were removed from incubator and added with 50 µl of cold (4°C) 50% TCA to each well of the plate to fix the cellular protein of cells to the bottom of well. Plates were placed in the refrigeration (4 °C) for a minimum of 1 hour. After one hour of refrigeration, plates were removed the liquid from the wells by inverting over a plastic pan, then washed five times in distilled water and allowed to dry in the air. The drying process at room temperature took approximately 12 - 24 hours. The fixed cellular protein attached to

the plate was stained with SRB (Sulfohodamine B). 100 µl 0.2% (w/v) sulfohodamine B in 1% acetic acid solution was added to each well and left at room temperature for 20 min. SRB was removed and then the plates were washed 5 times with 1% acetic acid before air drying. Bound SRB was solubilized with 100 µl, 10 mM Tris-base solution and plates were left on a plate shaker for at least 20 min. Absorbance was read in a 96-well plate reader at 492nm subtracting the background measurement at 620 nm. KC4 ver 4.0 software was used for collecting data and export data from UV-Vis [8, 9].

3. RESULTS AND DISCUSSION

Compounds **1**, **2** and **3** were isolated from the petroleum ether fraction from leaves of *P. serratifolia* L. by a combination of chromatographic methods. The structures of these compounds were elucidated by spectroscopic measurements ¹H-NMR, ¹³C-NMR, DEPT and comparison with literatures. Chemical structures of compounds (1-3) from leaves of *P. serratifolia* L. was given in Figure 2.

Compound **1** was obtained as a white crystal. The ¹H-NMR spectrum showed the presence of two pairs of *ortho*-coupling aromatic protons [δ_{H} 7.46 (2H, *d*, *J*=8.5 Hz, H-2, H-6), δ_{H} 6.89 (2H, *d*, *J*=8.5 Hz, H-3, H-5)], two *trans* olefinic protons [δ_{H} 7.64 (1H, *d*, *J*=16.0 Hz, H-7) and δ_{H} 6.30 (1H, *d*, *J*=16.0 Hz, H-8)], two methoxy groups [δ_{H} 3.83 (3H, *s*, H-10) and δ_{H} 3.79 (3H, *s*, H-11)]. The ¹³C-NMR spectrum of **1** showed signals of 11 carbons, including three quaternary carbons, six methine carbons and two methyl carbons, detected by the DEPT experiments, including signals of carbonyl carbon [δ_{C} 167.75 (C-9)], six aromatic carbons [δ_{C} 161.4 (C-4), δ_{C} 129.7 (C-2, C-6), δ_{C} 114.3 (C-3, C-5), δ_{C} 127.1 (C-1)], two olefinic carbons [δ_{C} 144.5

(C-7) and δ_C 115.3 (C-8)], methyl groups [δ_C 55.5 (C-10) and δ_C 51.7 (C-11)]. From all the above evidence and comparison with published data [5], the structure of **1** is methyl *trans*-4-methoxycinnamate.

Compound **2** was also obtained as white crystal. The 1H NMR spectrum showed the presence of two pairs of *ortho*-coupling aromatic protons [δ_H 7.42 (2H, *d*, $J=7.0$ Hz, H-2, H-6), δ_H 6.85 (2H, *d*, $J=7.0$ Hz, H-3, H-5)], two *trans* olefinic protons [*trans* δ_H 7.46 (1H, *d*, $J=16.0$ Hz, H-7) and δ_H 6.30 (1H, *d*, $J=16.0$ Hz, H-8)], a singlet of methoxy protons [δ_H 3.80 (3H, *s*, H-10)], hydroxyl

phenol proton [δ_H 5.97 (1H, *s*)]. The ^{13}C NMR spectrum of **2** showed signals of ten carbons, including three quaternary carbons, six methine and one methoxy groups, detected by the DEPT experiments, in which, there are signals of carbonyl carbon [δ_C 168.2 (C-9)], six aromatic carbons [δ_C 157.9 (C-4), δ_C 130.0 (C-2, C-6), δ_C 115.9 (C-3, C-5), δ_C 115.0 (C-1)], two olefinic carbons [δ_C 157.9 (C-7), δ_C 127.0 (C-8)]. Based on the NMR spectrum as well as comparison with the literature [6], compound **2** was identified as methyl *trans*-4-hydroxycinnamate.

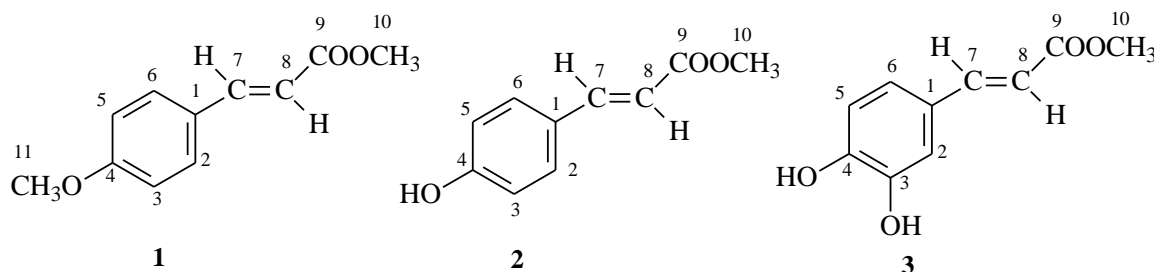


Figure 2. Chemical Structures of 1-3

Compound **3** was also obtained as white crystal. The 1H NMR spectrum showed signals of ten protons including two hydroxyl phenol protons [δ_H 8.31 (2H, *s*)], three aromatic protons of *ortho* and *meta*-coupling [δ_H 7.05 (1H, *dd*, $J = 8.0$ Hz, 2.0 Hz, H-6), δ_H 6.88 (1H, *d*, $J = 8.5$ Hz, H-5) and δ_H 7.15 (1H, *d*, $J = 2$ Hz, H-2)], two *trans* olefinic protons [δ_H 7.55 (1H, *d*, $J = 16.0$ Hz, H-7) and δ_H 6.29 (1H, *d*, $J = 16.0$ Hz, H-8)] and a singlet of methoxy protons [δ_H 3.71 (3H, *s*)]. The ^{13}C NMR spectrum of **3** showed signals of ten carbons, including four

quaternary carbons, five methine and one methoxy group, detected by the DEPT experiments, in which, there are signals of carbonyl carbon [δ_C 167.8 (C-9)], six aromatic carbons [δ_C 148.7 (C-4), δ_C 146.3 (C-3), δ_C 127.6 (C-1), δ_C 122.5 (C-6), δ_C 115.1 (C-5) and δ_C 115.3 (C-2)], two olefinic carbons [δ_C 145.7 (C-7) và δ_C 116.3 (C-8)]. Based on the NMR spectra as well as comparison with the literature [7], compound **3** was identified as methyl *trans*-3,4-dihydroxycinnamate (methyl caffeate).

Table 1. The NMR data of 1-3 recorded in $CDCl_3$

N ^o	1		2		3	
	δ^1H (ppm)	$\delta^{13}C$ (ppm)	δ^1H (ppm)	$\delta^{13}C$ (ppm)	δ^1H (ppm)	$\delta^{13}C$ (ppm)
1		127.1		127.6		127.6
2	7.46 (<i>d</i> , 8.5 Hz)	129.7	7.41 (<i>d</i> , 7.0 Hz)	130.0	7.15 (<i>d</i> , 2.0 Hz)	115.3
3	6.89 (<i>d</i> , 8.5 Hz)	114.3	6.85 (<i>d</i> , 7.0 Hz)	115.9		146.3

4		161.4		157.9		148.7
5	6.89 (<i>d</i> , 8.5 Hz)	114.3	6.85 (<i>d</i> , 7.0 Hz)	115.9	6.88 (<i>d</i> , 8.5 Hz)	115.1
6	7.46 (<i>d</i> , 8.5 Hz)	129.7	7.41 (<i>d</i> , 7.0 Hz)	130.0	7.05 (<i>dd</i> , 8.0 Hz, 2.0 Hz)	122.5
7	7.64 (<i>d</i> , 16 Hz)	144.5	7.46 (<i>d</i> , 16.0 Hz)	144.8	7.55 (<i>d</i> , 16.0 Hz)	145.7
8	6.30 (<i>d</i> , 16 Hz)	115.3	6.30 (<i>d</i> , 16.0 Hz)	115.0	6.29 (<i>d</i> , 16.0 Hz)	116.3
9		167.75		168.2		167.8
10	3.83	55.5	3.80	52.0	3.71(3H, <i>s</i>)	51.5
11	3.79	51.7				
-OH			5.97	157.9	8.31(2H, <i>s</i>)	

These compounds were tested for their cytotoxicity on human colon cancer cell DLD-1 by SRB assay (Table 2). Among the tested substances, methyl caffeate (**3**) exhibited the strongest cytotoxicity toward DLD-1 (The half maximal inhibitory concentration- IC_{50} = 66 μ M) while methyl *trans*-4-methoxycinnamate (**1**) appeared to show weaker cytotoxicity after 72 hours. Comparison of the structures of these compounds showed that they have the same structure but the methoxy group at C-4 of **1** is

transferred by hydroxyl group at C-4 of **2** and two hydroxyl groups at C-3, C-4 of **3**. From the above results we found that the compounds which possess many hydroxyl groups will induce the stronger cytotoxicity and the replacement of methoxyl group for hydroxyl group seemed to attenuate antiproliferative potency of coumaric acid derivatives. Furthermore, the bioactive potential of samples also depends on the time of incubation. The more incubation time increases, the more inhibitory effect on cell proliferation increases.

Table 2. Result of the cytotoxicity test on human colon cancer cell DLD-1 of the isolating compounds. The half maximal inhibitory concentration (IC_{50}) values are reported as the mean value \pm standard deviation of there experiments.

Compounds	Time (hour)	IC_{50} (μ M)
1	24	265 \pm 0.36
	48	210 \pm 0.09
	72	135 \pm 0.64
2	24	175 \pm 0.18
	48	140 \pm 0.23
	72	97 \pm 0.06
3	24	110 \pm 0.15
	48	80 \pm 0.21
	72	66 \pm 0.03

IC_{50} of resveratrol, a positive control, is 30 μ M.

4. CONCLUSION

Three coumaric acid derivatives including methyl *trans*-4-methoxycinnamate (**1**), methyl *trans*-4-hydroxycinnamate (**2**) and methyl caffeate (**3**) were isolated from the

leaves of *Premna serratifolia* L. Their chemical structures were accomplished by using 1D and 2D-NMR spectroscopies in comparison with literatures. The compounds were evaluated the cytotoxicity on human

colon cancer cell DLD-1. Methyl caffeate (3) exhibited the strongest activity. This result showed that the presence of hydroxy groups attached directly to aromatic ring will increase the inhibitory activity.

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