







Phenolic Acid Analogues from *Balanophora laxiflora* Inhibit Proliferation of *In Vitro* Acute Myeloid Leukemia Cells

Trinh Thi Thuy ^{1,2**}, Ba Thi Cham ^{1,2}, Nguyen Thi Thuy Linh ^{1,2},
Phan Thi Ngoc Bich ^{1,2}, Sabrina Adorisio ^{3*}
and Domenico V. Delfino ^{3,4*}

¹Department of Chemistry, Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Viet Nam

²Department of Natural Products Research, Institute of Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Cau Giay, Hanoi, Viet Nam

³Foligno Nursing School, Department of Medicine and Surgery, University of Perugia, Perugia, Italy

⁴Section of Pharmacology, Department of Medicine and Surgery, University of Perugia, Piazzale Severi, S. Andrea delle Fratte, 06132 Perugia, Italy

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Abstract: Four phenolic analogues isolated from *Balanophora laxiflora*; methyl gallate (**1**, MG), 4-hydroxy-3-methoxycinnamaldehyde (**2**), 3-methoxycinnamic acid (**3**), and methyl caffeate (**4**, MC) were shown to have antiproliferative activity on acute myeloid leukemia (OCI-AML3) cells due to impaired cell cycle progression. Three of these compounds (**1**, **3**, and **4**) caused increased apoptosis and decreased proliferation in cells. Interestingly, MC (compound **4**) significantly decreased the percentage of cells in S phase and G2/M phase, and significantly increased apoptosis in cells at low concentrations. Treatment with these compounds increased Fas ligand production and caspase-8/3, but not caspase-9-dependent, apoptosis. These results suggest that dosing with these compounds decreases proliferation and increases apoptosis in cell lines derived from hematological cancers.

Keywords: *Balanophora laxiflora* Hemsl. ex Forbes & Hemsl.; phenolic acid analogues; antiproliferative activity; caspase-8; acute myeloid leukemia cell (OCI-AML); human cancer cell lines. © 2021 ACG Publications. All rights reserved.

1. Plant Source

In the course of phytochemical studies of medicinal plants from Viet Nam, we investigate *Balanophora laxiflora* [1-5].

* Corresponding authors: Email: domenico.delfino@unipg.it; Phone: +39 75 585 8328/+39 75 585 8115 (D.V. Delfino) thuy@ich.vast.vn (T.T. Thuy); Phone: +84 978987562.

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Balanophora laxiflora Hemsl. ex Forbes & Hemsl. was collected at Na Hang district, Tuyen Quang province, Viet Nam in December, 2018. The species was identified by botanist Dr. Do Huu Thu, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology, Hanoi, Vietnam (VAST). A voucher specimen is deposited at the Herbarium of Institute of Ecology and Biological Resources, VAST, Hanoi, Viet Nam.

2. Previous Studies

Balanophora is a genus belonging to the family Balanophoraceae, which contains approximately 120 species worldwide. *Balanophora laxiflora* Hemsl. ex Forbes & Hemsl. (*B. laxiflora*) is a parasitic herb growing in Taiwan and northern Vietnam. *B. laxiflora* has been used in Vietnamese folk medicine as a tonic with antipyretic and antidotal properties as a treatment for hemorrhoids, stomach ache, hemoptysis, and to improve sexual performance [1-3]. Previous phytochemical studies of *B. laxiflora* led to the isolation of phenolic compounds and lignin [1-5]. Lignan compounds have been proven to possess DPPH radical and scavenging properties with anti-inflammatory activities [6-8]. Phenolic compounds, especially caffeic acid analogues, have been reported to have antinociceptive, anticancer, antiproliferative, proapoptotic and anti-angiogenesis activities [9-14]. Previous studies have shown that treatment with MC (compound **4**) has shown potential apoptotic activity on MCF-7 cells [11] and human breast cancer cells [12]. Recently, our group has extensively studied the effect of compounds isolated from a Vietnamese *B. laxiflora*. As part of our study, we identified four phenolic compounds (**1-4**) from this plant, namely methyl gallate (**1**, MG), 4-hydroxy-3-methoxycinnamaldehyde (**2**), 3-methoxycinnamic acid (**3**), and methyl caffeate (**4**) [1]. Natural phenolic compounds have potential health benefits, and these caffeic acid derivatives have been predicted to act as chemopreventive agents and emerging adjuvants for cancer chemotherapy [15-16].

3. Present Study

In the present study, compounds **1-4** were isolated from aerial part of *B. laxiflora* by a modified method previously described [1], and detail described in the supplementary material. The structures of compounds **1-4** (Figure 1) were identified as methyl gallate (**1**), 4-hydroxy-3-methoxycinnamaldehyde (**2**), 3-methoxycinnamic acid (**3**), methyl caffeate (**4**) by extensive spectroscopic analysis including 1D and 2D NMR and HR-ESIMS experiments as well as by comparison with reported data [1] (see supporting information for spectral data). The presence of compounds **1** and **4** in the ethanolic extract from the same plant material proves that they are also natural constituents. The purity of MC exceeded 96 % as determined by HPLC, and its chromatogram is shown in the supporting information (Figure S1). However, little is known about the antiproliferative activity of these compounds in acute myeloid leukemia. This study characterizes the antiproliferative, apoptotic, and cell cycle arrest effects of the phenolic compounds on acute myeloid leukemia cells (OCI-ALM3).

We characterized the effect of compounds **1-4** on the growth of OCI-AML3 cells by evaluating cell counts, apoptosis, and cell cycle progression. Among the four compounds, compound **4** was the most powerful and it was analyzed in depth. Compounds **1** and **3** at 150 and 300 $\mu\text{g/mL}$ (Figure S6A) and **4** at 15.62 and 31.25 $\mu\text{g/mL}$ (Figure 2A) significantly decreased the number of OCI cells, respectively, whereas compound **2** (that was toxic at higher concentrations) did not demonstrate any activity (Figure S6A). The decrease in cell numbers could be due to increased apoptosis, decreased proliferation, or both. Apoptosis of OCI-AML3 cells was examined using propidium iodide flow cytometry analysis, gating out necrotic cells by size (forward light scatter) [18-20]. Results show that treatment with compounds **1** and **3** significantly increased the occurrence of apoptosis at a concentration of 150 and 300 $\mu\text{g/mL}$, respectively, (Figure S6B), whereas compound **4** was the most powerful since it significantly increased apoptosis at 15.62 $\mu\text{g/mL}$ (Fig. 2B and C). Compound **2** was ineffective (Figure S6B). Thus, the decrease in OCI cell numbers was caused at least in part by increased cellular apoptosis.

In the cell cycle analysis, compounds **1**, **2**, **3** (Figure S7) and **4** (Figure. 3A and B)

significantly increased the percentage of cells in G₀/G₁ phase, although compound **3** did it only at a 300 µg/mL. Compounds **1**, **2**, **3** (at 300 µg/mL) (Figure S7) and **4** (Figure. 3A and B) decreased the percentage of cells in S phase, whereas only compounds **3** (at 300 µg/mL) (Figure S7) and **4** (Figure. 3A and B) were able to decrease the percentage of cells in G₂/M phase. We hypothesize that all compounds (**1**, **2**, **3**, and **4**) impaired the cell cycle by a similar mechanism [21]. Since MC (**4**) had the lowest MEC, it was chosen to further investigate its role in the caspase cascade, the hallmark of apoptotic cell death. Caspase activity was measured before and after treatment with compound **4** by western blot analysis. Cells treated with compound **4** activated caspase-3 and caspase-8, but not caspase-9 (Figure 4A). Thus, compound **4** promoted OCI cell apoptosis, as demonstrated by the activation of caspase-3 via the caspase-8-dependent extrinsic pathway.

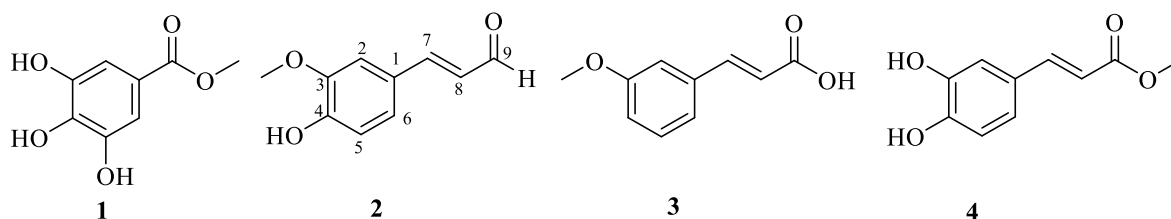


Figure 1. Structures of isolated compounds **1** - **4** from *Balanophora laxiflora*

One way to trigger the extrinsic apoptotic pathway is to stimulate receptors of the TNF receptor family by their ligands. In order to see if this was the case, OCI cells with or without the addition of compound **4** were cultured and after 24 hours were harvested, their RNA extracted and a real-time has been performed to evaluate the expression of Fas ligand (FasL), TNF α (both belonging to the TNF family and causing apoptosis by extrinsic pathway) and TGF- β that can promote apoptosis due to its function as a tumor suppressor and with the involvement of the activation of SMAD proteins and of AP1 transcription factor [22]. Figure 4B shows that compound **4** significantly increased the expression of FasL but not TNF- α and TGF- β . Thus, the increased expression of FasL confirmed that the extrinsic apoptotic pathway was triggered by compound **4**.

Among these, compounds **1**, **3** and **4** decreased cell number in OCI-AML3 cells. This study verified that methyl esters (compounds **1** and **4**) displayed higher anti-leukemic effects than acidic esters (compound **3**), while aldehyde (compound **2**) had no effect on cells number. MC (compound **4**) had a significant effect on cells at a much lower concentration than MG (compound **1**), and exerted its effect by blocking cells at G₀/G₁ phase of the cell cycle although its effect in increasing apoptosis seemed to be the main mechanism since it doubled at a concentration as low as 15.62 µg/mL. Apoptosis is the target of different drugs, such as glucocorticoids, used to treat lymphohematopoietic cancer [23] and, in particular, induction of apoptosis is achieved by different drugs to treat acute myeloid leukemia, a malignancy of hematopoietic system that remain a challenge despite the advancement in cancer biology and therapy [24]. In AML, drug targets were located in both the intrinsic (mediated by caspase-9) and extrinsic (mediated by caspase-8) apoptotic pathways. In the first pathway, Bcl-2 and Mcl-1 were targeted with good results, whereas results obtained by targeting the extrinsic pathway were less impressive, although good results were obtained by targeting XIAP and cIAP molecules [24]. In our work, compound **4** induced apoptosis by significantly increasing another extrinsic apoptotic pathway molecule, Fas ligand, able to induce caspase-8-dependent apoptosis by an autocrine mechanism. Thus, FasL can be added to the extrinsic apoptotic drug targets in AML cells and compound **4** from *B. laxiflora* could be useful in triggering its production, thus promoting AML apoptosis. Further *in vivo* studies will clarify if this can be a novel possible way to treat AML.

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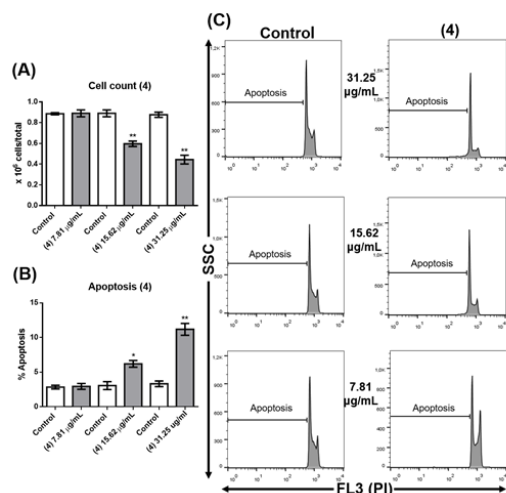


Figure 2. (A) Bars represent the number of viable cells counted after 24 h of treatment with vehicle (Control, white bars), or compound **4** at the concentrations reported on the x-axis (Gray bars). (B) Effects of **4** on induction of apoptosis in OCI-AML3 cells. Bars represent the percentage of cell death after 24 h of treatment, with control vehicle (Control, white bars) or **4** at the concentrations reported on the x-axis (gray bars). (C) Histograms from a representative experiment, in which propidium iodide (PI) staining is shown on the x-axes on the logarithmic scale (FL3). Data from five independent experiments are reported as standard error of the mean (SEM). ** $p < 0.01$, * $p < 0.05$.

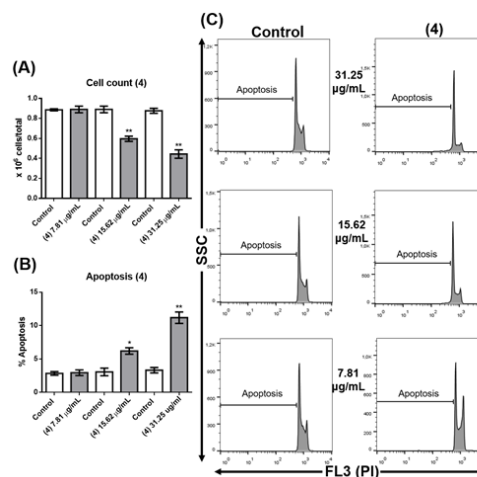


Figure 3. Effects of **4** on OCI-AML3 cell cycle progression. (A) Bars represent the percentage of cells in G0/G1, S, or G2/M phase after 24 h of treatment with control DMSO vehicle (Control, white bars) or compound **4** at the concentrations reported on the x-axes (Gray bars). (B) Panels show flow cytometry analyses of a representative experiment. Data from five independent experiments are reported as SEM. ** $p < 0.01$.

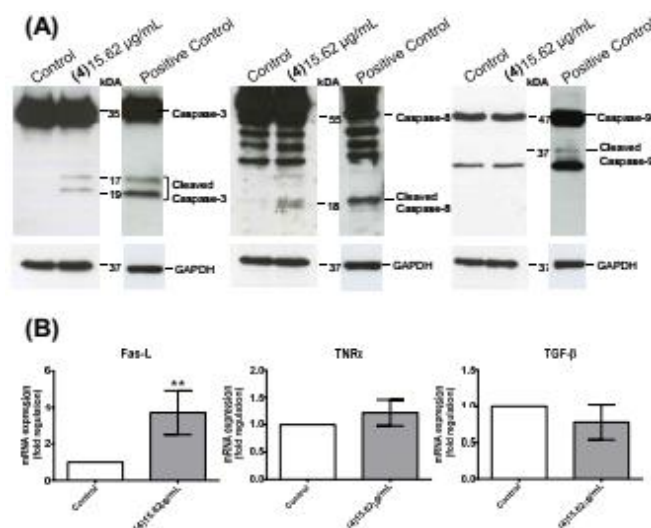


Figure 4. Effects of (**4**) on apoptotic pathways. (A) Western blot analysis of pro-caspase-3 (caspase-3), activated caspase-3 (cleaved caspase 3), pro-caspase-8 (caspase-8), activated caspase-8 (cleaved caspase 8), pro-caspase-9 (caspase 9), and activated caspase-9 (cleaved caspase 9) in OCI-AML3 cells treated with vehicle (Control) or (**4**) at 15.62 $\mu\text{g/mL}$ for 24 h. Western blots are representative of five independent experiments. (B) Real-time PCR analyses from five independent experiments (mean \pm SEM) of Fas-L, TNF- α , TGF- β , in cells treated with vehicle (Control) or 15.62 $\mu\text{g/mL}$ (**4**), ** $p < 0.01$.

In conclusion, three compounds (**1**, **3**, and **4**) decreased cell number in OCI-AML3 cells. This study verified that methyl esters (compounds **1** and **4**) displayed higher antiproliferative effects than acidic esters (compound **3**), while aldehyde (compound **2**) had no effect on cells number. MC (compound **4**) had a significant effect on cells at a much lower concentration than MG (compound **1**). Hopefully, additional bioactive studies will clarify this effect and examine its potential therapeutic use.

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Conflict of interest statement

We declare that we have no conflict of interest.

Supporting Information

Supporting information accompanies this paper on <http://www.acgpubs.org/journal/records-of-natural-products>

ORCID

Trinh Thi Thuy: [0000-0001-5568-6925](https://orcid.org/0000-0001-5568-6925)

Ba Thi Cham: [0000-0001-6690-8845](https://orcid.org/0000-0001-6690-8845)

Nguyen Thi Thuy Linh: [0000-0003-2226-6064](https://orcid.org/0000-0003-2226-6064)

Phan Thi Ngoc Bich: [0000-0002-1020-4010](https://orcid.org/0000-0002-1020-4010)

Sabrina Adorisio: [0000-0001-9310-3487](https://orcid.org/0000-0001-9310-3487)

Domenico Delfino: [0000-0003-1792-9468](https://orcid.org/0000-0003-1792-9468)

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