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# 20-Hydroxyecdysone from *Dacrycarpus imbricatus* bark inhibits the proliferation of acute myeloid leukemia cells

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ABSTRACT

**Objective:** To investigate the anti-proliferative effects of 20-hydroxyecdysone isolated from the bark of *Dacrycarpus imbricatus* (Blume) de Laub.

**Methods:** Column chromatography was used for isolation of compounds from plant material. The structure of the isolated compound was identified by mass spectrometry and nuclear magnetic resonance techniques, including HSQC, HMBC, NOE-difference experiments. The isolated compound was tested for its anti-proliferative activity in acute myeloid leukemia (AML) and OCI-AML cells.

**Results:** Compound **1** was isolated from the ethyl acetate fraction of *Dacrycarpus imbricatus* barks by column chromatography. Its chemical structure was identified as 20-hydroxyecdysone (20HE), a cholestane-type ecdysteroid, by a combination of mass spectrometry and nuclear magnetic resonance spectrometric analyses. Our goal was to test the anti-proliferative activity of 20HE using the OCI-AML cell line. 20HE significantly decreased OCI cell number at a concentration of 1 mg/mL, whereas lower concentrations were ineffective. Moreover, this decrease was due to partial blockage of the $G_1$/S phase of the cell cycle, with a reduction of cells in the $G_2$M phase, not due to increased apoptosis.

**Conclusions:** This indicates that 20HE significantly decreases the number of cells in the $G_1$/S phase of the cell cycle in human AML cells. This is the first time that the anti-proliferative activity of 20HE against a human tumor cell line has been reported.

## 1. Introduction

*Dacrycarpus imbricatus* (*D. imbricatus*) (Blume) de Laub. (Podocarpus kawaii Hayata) is a coniferous tree in the Podocarpaceae family with red-brown bark that may grow up to 40 m tall. It is mainly distributed in West Pacific islands near China and Vietnam [1]. Its timber has been used in construction for furniture, as firewood, and is cultivated as an ornamental tree in some areas. Despite the large number of chemical studies conducted on the Podocarpaceae family, few studies have been conducted on *Dacrycarpus* species. Furthermore, to date, there is only one report on the chemical constituents of *D. imbricatus*. Two phenolic diterpenes, totarol and lambertic acid, have been isolated and identified from its wood [2]. As part of our research on the coniferous species of Vietnam, this paper describes the isolation and structural elucidation of 20HE (**1**) from the bark of *D. imbricatus* and its effects on OCI-AML cell proliferation *in vitro*.

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## 2. Materials and methods

*D. imbricatus* was collected in Lam Dong province, Viet Nam (August, 2012) and identified by Dr. Nguyen Tien Hiep (Vietnam National Museum of Nature). A voucher specimen (CPC 4709) was deposited in the Vietnam National Museum of Nature, Vietnam Academy of Science and Technology (VAST).

HR ESI MS spectrum was obtained on QStar Pulsar (Applied Biosystems). $^{1}$H-NMR (500.13 MHz) and $^{13}$C-NMR (125.77 MHz) spectral data were measured in methanol-$d_4$ ($CD_3OD$) on a Bruker Avance 500 NMR spectrometer at 25 °C. Chemical shifts were reported as $\delta$ values with reference to TMS as internal standard for $^{1}$H ($\delta$ = 0 ppm) and $CD_3OD$ for $^{13}$C ($\delta$ = 49.00 ppm). 2D-NMR experiments were recorded using standard Bruker programs. Chemical shifts were expressed in $\delta$ (ppm) and coupling constants were reported in Hertz (Hz). The purity of compound **1** was estimated to be greater than 95% by integration in $^{1}$H- and $^{13}$C-NMR.

Cell line and methods for evaluating the effect in AML cells as previously described [3].

## 3. Results

### 3.1. Extraction, isolation and structure characterization

The dried ground barks of *D. imbricatus* (900 g) were extracted with ethanol-water (90: 10 w/w, overnight, ×4 times) at room temperature. After concentration under reduced pressure, the crude extract was suspended in water and sequentially partitioned with *n*-hexane, ethyl acetate and *n*-butanol. The organic solvents were evaporated to yield the corresponding extracts (1.0 g, 5.1 g and 8.2 g, respectively). The ethyl acetate residue (5.1 g) was subjected to silica gel column, eluting with solvent system $CH_2Cl_2$–MeOH (gradient from 98:2 to 80:20) to yield 25 fractions (F1–F25). The fraction 22 (F22) was repeatedly separated on Sephadex LH-20 column eluting with MeOH and then purified by chromatography on RP18 (MeOH–$H_2O$) to afford compound **1** (Figure 1) as a white solid (27 mg).

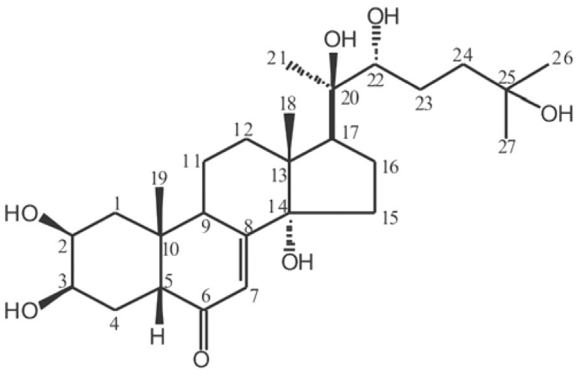


**Figure 1.** Structure of 20-hydroxyecdysone (20HE, **1**) isolated from the barks *of D. imbricatus*.

### 3.2. Effect of 20HE (1) on OCI cell number

The possible impact of 20HE (**1**) on acute myeloid leukemia OCI cells was tested. Table 1 shows that after 24 h of treatment with 20HE (**1**), the number of OCI cells was significantly decreased compared to the vehicle (DMSO) at concentrations of 1 mg/mL. Thus, 1 mg/mL 20HE (1) and a 24-h incubation time were used in further experiments unless otherwise noted.

Number of acute myeloid leukemia cells from OCI cell line was counted by hemocytometer.

### 3.3. Effect of 20HE (*1*) on cell death and cell cycle progression

The 20HE (**1**)-induced decrease in cell number could have been due to increased cell death, decreased proliferation, or both. Staining of nuclei with PI and subsequent flow cytometry analysis were applied to investigate both frequency of cell death and the cell cycle (indicator of the proliferation status of the cells) in 20HE (**1**)-treated and non-treated cells. Table 1 shows that, when cell death was analyzed, 20HE (**1**) treatment did not significantly increased occurrence of cell death. Thus, the decrease of OCI cell number was not caused by 20HE (**1**)-induced by cell death.

### 3.4. Effect of 20HE (1) on cell cycle progression

As shown, we saw a significant increase of cells in $G_0/G_1$ phase and a decrease of cells in $G_2$/M phase, whereas no significant differences were seen in the S phase of the cell cycle (Table 1). The 20HE (**1**)-dependent decrease in OCI cell number was due, at least partly, to blockage of cell entry in DNA synthesis (S phase) and consequent decrease of mitotic cells that undergo to cellular division. Thus, the decrease of OCI cell number was caused by 20HE (1)-induced cell division arrest without an augmentation of cell death.

**Table 1**

Number of acute myeloid leukemia OCI cells, evaluation of cell death, evaluation of $G_0/G_1$ phase of the cell cycle on OCI cells, and evaluation of S phase of the cell cycle on OCI cells.

| Substance concentration (mg/mL) | Acute myeloid leukemia OCI cells (*n*) | | Cell death (%) | | $G_0/G_1$ phase of cell cycle (%) | | S phase of cell cycle (%) | |
|---|---|---|---|---|---|---|---|---|
| | Control | 20HE (1) | Control | 20HE (1) | Control | 20HE (1) | Control | 20HE (1) |
| 1.000 | 0.54 ± 0.01 | 0.27 ± 0.03*** | 11.70 ± 0.90 | 11.86 ± 0.91 | 76.53 ± 2.28 | 80.90 ± 0.029* | 10.00 ± 0.52 | 7.63 ± 1.30* |
| 0.500 | 0.61 ± 0.03 | 0.56 ± 0.12 | 8.63 ± 1.08 | 7.90 ± 0.68 | 61.73 ± 2.80 | 67.06 ± 0.19 | 13.16 ± 2.77 | 11.23 ± 2.70 |
| 0.250 | 0.59 ± 0.08 | 0.50 ± 0.18 | 6.83 ± 2.02 | 6.46 ± 0.80 | 55.53 ± 1.04 | 56.43 ± 0.60 | 14.96 ± 4.02 | 15.20 ± 1.38 |
| 0.125 | 0.58 ± 0.10 | 0.58 ± 0.11 | 5.83 ± 1.15 | 6.20 ± 0.73 | 53.50 ± 1.01 | 52.90 ± 0.87 | 16.56 ± 4.06 | 16.60 ± 3.80 |

***$P < 0.001$ compared with control group; *$P < 0.05$ compared with control group. Control and 20HE (1) represent cells treated with the reported concentration of either the vehicle or the compound 20HE (1), respectively. Control and 20HE (1) represent mean ± 1 standard error from the mean (1 SEM) of three independent experiments. Data are calculated by *t*-student's test.

## 4. Discussion

Compound **1** was isolated as a white solid from the ethyl acetate extract of *D. imbricatus* barks. Its molecular formula, $C_{27}H_{44}O_7$, was deduced from combined analysis of the positive HR ESI MS at *m/z* 503.2972 $[M+Na]^+$ ($C_{27}H_{44}O_7Na$ requires 503.2985) and $^1H$-, $^{13}C$-NMR and DEPT spectra. Its HR ESI MS spectrum revealed a protonated molecular ion at *m/z* 481.3151 $[M+H]^+$ and the predominant fragments at *m/z* 371.2204 ($C_{23}H_{31}O_4$ requires 371.2222), *m/z* 445.2938 ($C_{27}H_{41}O_5$ requires 445.2954). The $^1H$-NMR spectrum exhibited five singlet methyl groups at $\delta_H$ 0.91 (Me-18), 0.99 (Me-19), 1.21 (Me-21) and 1.220 & 1.224 (each 3H, Me-26, Me-27); one olefinic methine at $\delta_H$ 5.83 (*d*, *J* = 2 Hz, H-7) and three oxymethines ($\delta_H$ 3.36, 3.86, 3.97). In addition, this structure was corroborated by three quaternary O-connected carbons and an $\alpha$, $\beta$-unsaturated ketone in the $^{13}C$-NMR spectrum.

Based on these spectroscopic data including HSQC, HMBC, NOE-difference and comparison with others in the literature [4,5], compound **1** was assigned as 20HE. This compound belongs to the phytoecdysteroid family, which is made up of well-known molting hormones, but is identified herein for the first time in this plant [6,7].

Ecdysteroids are steroid hormones that are involved in epidermal growth in arthropods, controlling cell proliferation and further differentiation of target cells [8]. 20HE is a poly hydroxylated steroid hormone that can stimulate midgut stem cell proliferation and differentiation in some insects. It was recently shown to induce osteogenic differentiation in mouse mesenchymal stem (MS) cells [9].

20HE is the most popular steroid, exhibiting low acute toxicity in mice. However, no study has described its effect on human cells. Therefore, we examined its effects on OCI-AML cell death and proliferation *in vitro*. It was added to cultures of OCI-AML cells at different concentrations. After 24 h of treatment, three parameters were evaluated: 1) cell number (using a hemocytometer); 2) cell death, and 3) cell cycle (proliferation ability) by flow cytometry. After 24 h of culture, treatment with 20HE (1 mg/mL) significantly ($P \leq 0.05$) decreased the cell number compared to untreated cells.

Because 20HE was active only at 1 mg/mL, this concentration was used to determine whether the decreased cell number was due either to increased cell death or decreased cell proliferation. Here we show that the decrease in cell number was not due to an increase in cell death because no significant differences were observed for this parameter, but rather to the inhibition of proliferation as a result of a significant block of the $G_0/G_1$ phase of the cell cycle, with no differences observed in the S phase but a significant decrease in the percentage of cells undergoing mitosis ($G_2$/M phase of the cell cycle). Thus, 20HE effectively decreased the proliferation of OCI cells although further studies are necessary to develop this initial data to determine whether this substance can be considered a candidate for future therapeutic applications.

In conclusion, we report here the isolation and identification of 20HE for the first time from *D. imbricatus*. The experiments described herein demonstrate that 20HE has a small but significant effect on human AML cells, blocking their proliferation. Although the compound has various biological activities, this is the first time it has been shown to exhibit anti-proliferative activity against human tumor cells.

## Conflict of interest statement

We declare that we have no conflict of interest.

## Acknowledgments

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