

3D cytotoxic and histone deacetylase (HDAC) inhibitory activities of triterpenoids isolated from leaves of *Viburnum sambucinum*

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Received 14 June 2017; Accepted for publication 29 December 2017

Abstract

The plant *Viburnum sambucinum* has been known under the local name “Vót dạng com cháy” belongs to *Viburnum* genus, a member of the Caprifoliaceae family. Many plants of *Viburnum* genus are well known for their medicinal properties and are used in the folk medicine for the treatment of various diseases. A variety of structures have been reported from the plants of the genus, such as diterpenes, triterpenes, iridoids, sesquiterpenes, flavonoids, etc. In this study, we describe 3D cytotoxic and histone deacetylase (HDAC) inhibitory activities of two nordammarane triterpenoids hupehenol D (**1**) and 12 β -hydroxy-3,15-dioxo-20,21,22-23,24,25,26,27-octanordammanrane (**2**) isolated from the leaves of *Viburnum sambucinum*. The result showed that (**1**) and (**2**) exhibited a strong activity against 3D models of LLC cell line with the IC₅₀ value of 25.89 μ M and 15.58 μ M, respectively. Compound **1** weakly inhibited activity of enzyme HDAC with the IC₅₀ of 20.78 \pm 2.86 μ M while compound **2** exhibited no effect on HDAC at 25 μ M.

Keywords. *Viburnum sambucinum*, Caprifoliaceae, hupehenol D (**1**), 12 β -hydroxy-3,15-dioxo-20,21,22-23,24,25,26,27-octanordammanrane (**2**), 3D cytotoxic, HDAC inhibitory activity.

1. INTRODUCTION

Cancer is one of the leading cause of death and globally the numbers of new cases of cancer are increasing gradually [1]. Current estimates from the American Cancer Society indicate that 7 million deaths worldwide, these numbers are expected to double by 2030 [2]. Thus, there is an increasing need for new and effective drugs for the disease treatment.

Plants have a long history of use in the treatment of cancer and it is significant that over 60 % of currently used anticancer agents are either natural products or directly derived therefrom [3].

The genus *Viburnum* (Caprifoliaceae) comprises about 200 species of shrubs and trees, which are widely distributed in the temperate or subtropical zones, from Southern America to Asia [4]. Many species of the genus *Viburnum* are employed as folk medicine to treat different diseases, such as cough, diarrhea, rheumatoid arthritis, and tumefaction. Recently, much attention has been paid to *Viburnum* genus and their chemical constituents because of their multifaceted activities. Extensive studies of *Viburnum* genus have led to the identification of

many compounds, such as diterpenes, triterpenes, iridoids, monoterpenes, sesquiterpenes flavonoids, lignans, etc. Especially, vibsan diterpenoids and triterpenoids are dominant and interesting bioactivities compounds [5-7]. In a previous study, we described chemical structure and 2D cytotoxic activity of 10 compounds isolated from the leaves of *Viburnum sambucinum*, among them, two nordammarane triterpenoids hupehenol D (**1**) and 12 β -hydroxy-3,15-dioxo-20,21,22-23,24,25,26,27-

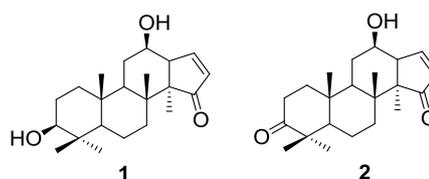


Figure 1: Chemical structure of hupehenol D (**1**) and 12 β -hydroxy-3,15-dioxo-20,21,22-23,24,25,26,27-octanordammanrane (**2**)

octanordammanran (**2**) exposed potential cytotoxic activity on several human cancer cell lines with IC₅₀ value of 4.71 \pm 0.03-5.35 \pm 0.04 μ M, respectively

[8]. Therefore, in this present study, these compounds were used to investigate the 3D cytotoxicity and histone deacetylase (HDAC) inhibitory activity on cancer cells.

2. MATERIALS AND METHODS

2.1. Cancer cell lines

MCF-7 (HTB - 22TM): Human breast carcinoma was purchased from American Type Culture Collection (ATCC); LLC (Murine Lewis lung carcinoma) was kindly provided by Prof. Jeanette Maier, Milan University, Italia. Those cancer cells were continuously cultured in Department of Applied Biochemistry, Institute of Chemistry, Vietnam Academy Science and Technology.

2.2. Histone deacetylases (HDAC) activity colorimetric kit

Histone deacetylases enzyme (HDAC) inhibit activity was determined by HDAC Activity Colorimetric assay Kit (BioVision-US) on MCF7 cancer cell lines. Kit components: HDAC substrate (Boc-Lys(Ac)-pNA, 10 mM), 10x HDAC Assay buffer, Lysine Developer, HDAC inhibitor (Trichostatin A, 1mM), Hela nuclear extract (5 mg/ml), Deacetylated Standard (Boc-Lys-pNA, 10 mM).

2.3. 3D cytotoxic activity assay

3D LLC cell line culture method:

LLC cancer cells was monolayer maintained in medium DMEM supplemented 2 mM L-glutamine, 10 mM HEPES, 1.0 mM sodium pyruvate, 10 % fetal bovine serum-FBS (GIBCO). Cells were transferred with ratio 1:3 every 3-5 days and incubated in a humidified atmosphere of 5 % CO₂ at 37 °C. Using trypsin-EDTA to detach cells and then resuspended in culture medium. Using Fisher scientific hemacytometer cat 0267110 (Hauser, USA) to calculated cells. Then, multicellular tumor-spheroids (MCTSs) were cultured on plate with agar medium. 190 µl cell solution (5000 cells/well) were plated in DMEM medium with 10 % FBS. After 3-4 days' culture at 37 °C in an atmosphere containing 5 % CO₂, the spheroids were formed and ready to used [9].

Invitro 3D cytotoxic activity assay

The 3D cytotoxicity of samples on LLC cell lines were determined by the MTT assay [10]. After 72 hours incubation, the cell reaches the confluence.

Then, cells were incubated in the presence of various concentrations of the samples in 0.1 % DMSO for 48 h at 37 °C/5 % CO₂. Ellipticine was used as reference. 20 µl/well of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) were added. After 4 h incubation, removed medium, 100 µl DMSO were added/well and shake 5-10 minutes. Viable cells were determined by microplate spectrophotometer at 490 nm.

Statistical analysis

A percent inhibition was calculated using the following formula:

$$\% \text{ inhibit} = \frac{\text{OD (sample)} \times 100}{\text{OD (control)}}$$

All the experimental results were as mean ± standard deviation (SD) of three parallel measurements. The data was entered into Microsoft Excel[®] database and analyzed using Table Curve giving IC₅₀.

2.4. HDAC inhibitory activity assay

Principle of assay

Inhibition of histone deacetylases (HDACs) has been implicated only two simple steps. HDAC catalyze the removal of ε-acetylated lysine group of substrate Boc-Lys(Ac)-pNA (step 1). So that, the second step, substrate Boc-Lys(Ac)-pNA was continuously removed by Lysine Developer to release yellow pNA (p-nitroaniline) can be easily analyzed using ELISA plate reader at 400-405 nm.

Procedure

HDAC inhibitory activity of samples was measured with HDAC Colorimetric Assay Kit (BioVision- US).

MCF7 cell line was grown in medium DMEM supplemented 2 mM L-glutamine, 10 mM HEPES and 1.0 mM sodium pyruvate, 10 % FBS (GIBCO) and incubated in a humidified atmosphere of 5 % CO₂ at 37 °C. Cells were harvested into plate and incubated overnight. After removal of medium and washing with phosphate-buffered saline (pH 7.4), cells were harvested and using Nuclear/Cytosol Fractionation KIT (Biovision) to obtain nuclear extract. Samples were mixed with Hela nuclear extract that contains a variety of HDAC enzymes and has HDAC activity. Then, cells were incubated for 24-48 h at 37 °C. HDAC colorimetric substrate was added to inhibitor and Hela nuclear extract mixture. Incubated in 37 °C for 1h. 10 µl Lysine Developer was added to stop the reaction and incubate for 30 minutes in 37 °C. A color is developed after 1hour treatment with the lysine developer. Absorbance at 400-405 nm was measured

by microplate reader equipment. Trichostatin A was used as a reference.

Statistical analysis

A percent HDAC inhibition was calculated using the following formula:

$$\% \text{ inhibit} = 100\% - \frac{\text{OD sample} \times 100}{\text{OD}(\text{control})}$$

The data was expressed as mean \pm SD from three independent experiments. The data was entered into Microsoft Excel[®] database and analyzed using Table Curve 2Dv4 giving IC₅₀.

3. RESULTS AND DISCUSSION

3.1. 3D cell culture cytotoxic activity

Cell-based assays are the key tool used to assess the potential efficacy of a new compound in drug discovery. Most of the commonly used cytotoxic anticancer drugs were discovered through random high-throughput screening in cell-based cytotoxicity assays [11]. In general, cell culture modes include monolayer cells on a two-dimensional (2D) surface

and multilayer cells or aggregate clusters in a three-dimensional (3D) scaffold [12]. To date, 2D cell-based assays in multiwell plates are widely used in drug screening because of their low costs and easy operation. However, 2D cell cultures can result in errors in predicting tissue-specific responses due to the loss of native morphology and limited cell-cell and cell-extracellular matrix interactions [13]. Recently, research has found that 3D cell cultures provide a more physiologically relevant environment for cells and allow the study of cellular responses to drug treatment more closely to what occurs *in vivo* than 2D model [14-16].

In our previous report, two nordammarane triterpenoids hupehenol D (**1**) and 12 β -hydroxy-3,15-dioxo-20,21,22-23,24,25,26,27 octanordammanrane (**2**) isolated from leaves of *Viburnum sambucinum* exposed strong cytotoxic activity on 4 cancer cell lines: KB, HepG2, Lu1 and MCF7 with IC₅₀ values in range of 4.71 \pm 0.03-5.35 \pm 0.04 μ M. In this study, two of these compounds were evaluated cytotoxicity on 3D spheroid model. The results are shown in figure 2 and table 1.

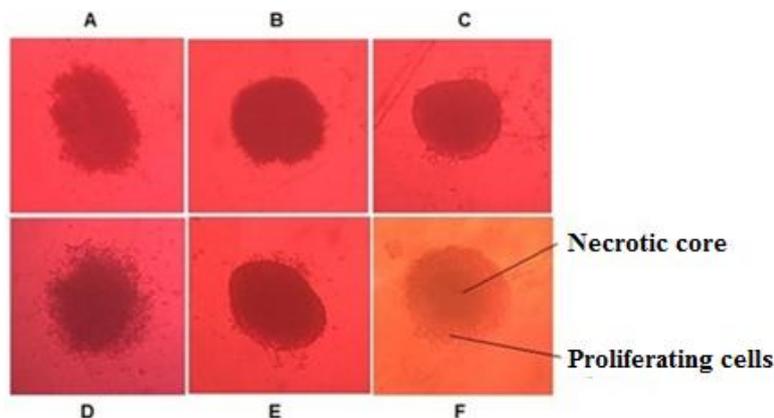


Figure 2: Images of LLC 3D spheroids after 3 days treating by **1** and **2** 3D spheroids treating by compound **1** at 100 μ g/ml (A), 20 μ g/ml (B), 4 μ g/ml (C) 3D spheroids treating by compound **2** at 100 μ g/ml (D), 4 μ g/ml (E) 3D spheroids without compounds (F)

Table 1: Cytotoxic activity of compounds (**1**) and (**2**) on 3D LLC cell culture

Test concentration (μ g/ml)	% inhibit		Test concentration (μ g/ml)	% inhibit
	Compound (1)	Compound (2)		Ellipticine
100	94.11	95.88	10	64.36
20	92.05	83.80	2	24.01
4	19.00	57.58	0.4	7.51
0.8	-3.09	2.36	0.08	-4.57
IC ₅₀	8.96 \pm 2.95 μ g/ml	5.36 \pm 1.53 μ g/ml	IC ₅₀	6.30 \pm 0.22 μ g/ml
	25.89 \pm 8.52 μ M	15.58 \pm 4.44 μ M		25.61 \pm 0.89 μ M

From figure 2, we can obviously observe the morphological parameters of 3D spheroid after being

treated with compounds **1** and **2**. At 20 μ g/ml and 100 μ g/ml concentration of compounds **1** and **2**,

external proliferating layers of spheroids were gradually darkened, unstable, indistinguishable to necrotic core and the structure of spheroids were deformed. Volume of spheroid also were decreased compare to control. Especially, tumor spheroid treated by compound **2** at 100 $\mu\text{g/ml}$ were disintegrated, highly variability to control.

3D spheroid cytotoxic ability of agents were also reflected by the IC_{50} value. The results shown in Table 1 indicated that two compounds **1** and **2** again exhibited a strong activity against LLC with the IC_{50} value of 25.89 μM and 15.58 μM , respectively. Ellipticine showed the IC_{50} value of 25.61 μM . Compare IC_{50} between 2D and 3D cell models, the IC_{50} value of compounds **2** and **1** gained from the 3D culture model is 3-5 fold higher than those from the 2D model, respectively. This finding is in concordance with several studies showing that cultured cells on 3D are more resistant to drug than on 2D models. A number of studies have found that cells cultured in 3D models are more resistant to anticancer drugs than 2D cultures. For example, ovarian cancer cell survival and proliferation in 3D cultures after paclitaxel treatment was reduced by 40 % or 60 % in 3D cell spheroids, while the same treatment led to 80 % reduced cell viability in the 2D cell monolayer [17]. Karlsson et al. studied the drug sensitivity of colon cancer HCT-116 cells in a 3D spheroid and 2D monolayer model in response to four standard anticancer drugs (melphalan, 5-FU, oxaliplatin, and irinotecan) and two promising investigational cancer drugs (acriflavine and VLX50). The results indicated that all drugs were highly active in 2D monolayer culture but generally less active and gradually lost their activity in 3D spheroids [18]. The stronger drug resistance in 3D culture results primarily from signals from dynamic cellular interactions between neighboring cells and

extra cellular matrix input into the cellular decision-making process. The increased drug resistance in 3D culture can also be attributed to limited diffusion through the spheroid and to hypoxia, cause by multilayer cells spheroid form [14, 18].

Recently, a growing body of evidence has shown that 3D cell cultures provide a more physiologically relevant environment for cells and allow the study of cellular responses in a setting that more closely resembles *in vivo* environments. A number of studies have demonstrated that cellular behavior in 3D cultures rather than 2D culture occur more similarly to those *in vivo*. Therefore, there is a great need for *in vitro* 3D cell culture assays, which would bridge the 2D monolayer cell culture systems and the animal models [12,19].

3.2. HDAC inhibitory activity results

Histone dactylases (HDACs) are a group of enzymes that remove acetyl groups from histones which are the primary protein components of chromatin. HDACs play a major role in the epigenetic regulation of gene expression through their effects on the compact chromatin structure. In recent years, alterations in acetylate levels and over expression of various HDACs in many cancer cell lines and tumor tissues have been reported. Therefore, HDACs have become promising therapeutic target for cancer treatment [20].

In the next experiment, compounds **1** and **2** continuously were evaluated HDAC inhibit activity on MCF7 to initially explore mechanism of cytotoxicity of them. MCF7 cell line was treated with different concentration of compounds. Negative control contain cells only, no reagents. The results were displayed in table 2.

Table 2: HDAC inhibitory activity of the compounds **1** and **2** in MCF7 cells

Test concentration (μM)	% inhibit		Test concentration (μM)	% inhibit Trichostatin A
	Compound (1)	Compound (2)		
21	51.12	35.32	1	82.0
14	40.02	37.14	0.1	74.23
7	35.74	28.91	0.01	56.89
3.5	14.83	2.45	0.001	42.19
IC_{50}	$20.78 \pm 2.86 \mu\text{M}$	$> 21 \mu\text{M}$	IC_{50}	$3.1 \pm 0.5 \text{ nM}$

Histone deacetylases enzyme (HDAC) inhibitory activity of compounds **1** and **2** was determined by HDAC Activity Colorimetric assay kit (BioVision-US) on MCF7 cancer cell lines. As shown in table 2, compound **1** weakly inhibited activity of enzyme HDAC with the IC_{50} of $20.78 \pm 2.86 \mu\text{M}$ while the

reference Trichostatin A possessed the IC_{50} of $3.1 \pm 0.5 \text{ nM}$. Compound **2** exhibited no inhibition activity of enzyme HDAC at the highest testing concentration which was 25 μM . This finding suggests that anticancer mechanism of **1** and **2** do not act over the inhibition of HDAC enzymes and should be explored

in further studies.

4. CONCLUSION

Two triterpenoid hupehenol D (**1**) and 12 β -hydroxy-3,15-dioxo-20,21,22-23,24,25,26,27-octanordammanrane (**2**) derived from leaves of *Viburnum sambucinum* were evaluated 3D cytotoxic and HDAC inhibitory activities for the first time. They displayed strong 3D cytotoxic activity on LLC spheroids with IC₅₀ value of 3D cell is higher 3-5 fold than 2D cells. However, both compounds showed insignificant HDAC inhibitory activity.

Acknowledgments. *The authors would like to gracefull the Subject of Institue of Chemistry (Grant No: VHH.2017.2.8) and the French-Vietnamese Project for financial support to complete this work.*

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