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POTENTIAL ACTIVITIES OF *ARTEMISIA VULGARIS* METHANOL EXTRACT AGAINST NTERA-2 CANCER STEM CELLS

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Summary

Potential Activities of *Artemisia vulgaris* Methanol Extract Against NTERA-2 Cancer Stem Cells

Cancer stem cells are the major cause of tumor initiation and metastasis, which can lead to drug resistance and recurrence in cancer patients. The search for natural chemicals that can inhibit the development of cancer stem cells is of interest to many scientists. *Artemisia vulgaris* L. (in Vietnamese named Ngai cuu) is known for traditional medicine to treat diseases such as diabetes, antiseptic, stomach aches, and cancer prevention. This study aims to evaluate the biological effect of methanol extract from *A. vulgaris* on the NTERA-2 cancer stem cell line. The activity of the extract was illustrated through cytotoxicity assay using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) method on monolayer and three-dimension cell culture models and *in vitro* migration assay. The extract of *A. vulgaris* exhibited inhibitory activity on NTERA-2 cells with an IC_{50} value of 18.31 µg/mL and reduced spheroid formation at the concentration of 500 µg/mL. Besides, this extract significantly reduced cell migration ability at the concentration of IC_{50} and $2xIC_{50}$. Consequently, *A. vulgaris* crude methanol extract presented obvious effects on NTERA-2 cancer stem cells, providing a new direction for investigating natural compound ingredients of this plant for more efficient cancer treatment.

Keywords: *Artemisia vulgaris, NTERA-2, Cytotoxicity, Tumorsphere formation assay, Scratch assay.*

1. Introduction

Cancer stem cells (CSCs) are a subpopulation of tumor cells with characteristics of both stem cells and cancer cells: proliferation, self-renewal, and differentiation. CSCs are thought to be associated with tumor recurrence and metastasis. Although chemotherapy and radiation can suppress large cancer populations and shrink tumors, those are not sufficient to eliminate thoroughly CSC populations. Therefore, residual CSCs would initiate and develop new tumors. The most effective strategy for cancer treatment was to combine drugs that target non-CSC and CSC tumor cells, respectively [1]. The discovery of new chemotherapy drugs to prevent tumor recurrence is necessary to overcome drug resistance caused by CSCs.

Artemisia vulgaris L. *(*AV*)*, a popular species belonging to the *Artemisia* genus from the Asteraceae family, distributes widely in natural habitats worldwide, particularly in tropical countries such as China, Indonesia, Vietnam… [2]. This plant contains various rich chemical compositions such as sterols, flavonoids, phenolic acids, and coumarins which are responsible for many biological activities. In ancient times, *Artemisia vulgaris* L. has been traditionally used to treat menstruation- and pregnancy-related ailments, heal wounds, gout, leg tiredness, and fever [3]. At present, a lot of scientific researchers have proved that this species possesses antioxidant [4], antihypotensive [5], anticancer [6], anti-inflammatory [7], antifungal [8], and antibacterial activities [9].

However, no reports exhibit the biological effect of the crude methanol extract from *Artemisia vulgaris* L. (VAM) on cancer stem cells. Therefore, in this project, we study the potential effects of VAM against proliferation, stemness, and migration properties of NTERA-2 pluripotent

human embryonal carcinoma *in vitro*.

2. Materials and methods

2.1. Research materials

The pluripotent human embryonal carcinoma cell line NTERA-2 was kindly provided by Prof. P. Wongtrakoongate, Mahidol University, Thailand. The plant was collected in Que Phong district, Nghe An province, Vietnam in April 2021. The plant identification was performed by Dr. Nguyen The Cuong, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST). The AV01 voucher sample was deposited at the Institute of Biotechnology, VAST. The fresh aerial parts of the plant were shadow-dried, chopped, and grinned. The 100 grams of dried powder was crudely extracted using 99.8% methanol at 50°C under sonicated conditions. The solvent was then thoroughly removed by a rotary evaporator to obtain 68.2 grams of VAM extract.

2.2. Cell culture

NTERA-2 cells were cultured using DMEM medium (Dulbecco's modified Eagle's medium), supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin, 1% MEM non-essential amino acids, 1% HEPES 1M in 37°C, 5% CO_2 incubator.

2.3. Anti-proliferative assay

The inhibitory effect of the extract on NTERA-2 was evaluated by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. NTERA-2 cells ($3x10^4$/mL) were pre-cultured in 96-well plates for 24 h, then treated with VAM extract at various concentrations of 0.8, 4, 20, and 100 µg/mL for a further 48 h. The cells added with DMSO 0.5% were the negative control (NC). Subsequently, 20 µL MTT dye (5 mg/mL) was added to each well and incubated for 4 h in a CO_2-humidified incubator. Then, the medium was removed, and the formazan was dissolved in DMSO. The absorbance was read by a microplate reader (BioTek, Elx800) at 540 nm. The data were analysed and shown as the percentage of cell survival, and the IC_{50} value was calculated by TableCurve2Dv4. The percentage of cell survival was determined by the following formula:

$$\%\ \text{Cell viability} = \frac{\text{OD(treated sample)}}{\text{OD(untreated sample)}}\ \text{x}\ 100\%$$

2.4. Tumorsphere assay

In 96-well ultrathin surface plates, cells were cultured to form tumorspheres. A total of 5000 cells/well were grown in DMEM medium throughout two days to form a stable tumorsphere. After 48h, the spheres were treated with the VAM extract at a concentration range from 0.8 to 500 µg/mL and incubated at 48h at 37°C. Tumorsphere size was observed under a microscope and analysed by using ImageJ software. Then, NTERA-2 cell proliferation in spheres was evaluated through MTT assays.

2.5. Migration assay

NTERA-2 cells (10^5 cells/well) were cultivated in a 24-well plate in DMEM medium and incubated at 37°C and 5% CO_2 overnight. A sterilized 20-200 µL pipet tip was used to scratch each well. Detached cells were removed by washing with PBS buffer, then adding medium with VAM at several concentrations ($0.5xIC_{50}$, IC_{50}, and $2xIC_{50}$) to each well. The scratch area was taken image at day 0 (0 h) via microscope. After the 24-hour-incubation, the closure area was observed under the microscope to acquire the images at 24 h. The results were determined and analysed by using ImageJ software. The percentage of closure was calculated by the following formation: % Closure = ($Area_{0h}$ - $Area_{24h}$)/$Area_{0h}$) *100.

2.6. Statistical analysis

The data was calculated by GraphPad Prism 5.0 software, which utilized the unpaired t-test and one-way analysis of variance to determine statistical significance.

3. Results

3.1. The effect of the crude methanol extract from Artemisia vulgaris L. *on NTERA-2 cell viability*

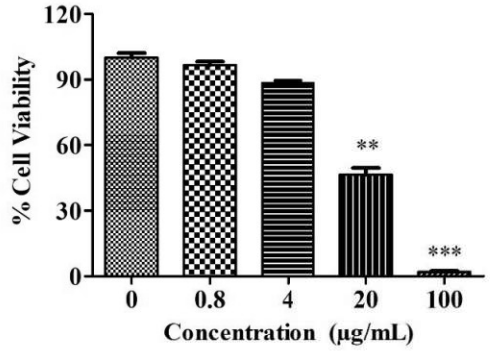

Fig. 1. Anti-proliferation effect of VAM extract at several concentrations on NTERA-2 cells. **P < 0.01, ***P < 0.001

In Fig. 1, there was a reduction in the percentage of cell viability in a dose-dependent manner when NTERA-2 cells were treated with VAM extract. After 48 h of incubation, VAM extract at concentrations of 20 µg/mL, and 100 µg/mL significantly inhibited cell survival with a percentage of inhibition of about 53.58 ± 3.13% and 99.04 ± 0.86%, respectively. In contrast, treatment with 0.8 and 4 µg/mL did not significantly suppress NTERA-2 cell proliferation. The value of half maximal inhibitory concentration (IC_{50}) was calculated at 18.31 µg/mL.

3.2. Reducing NTERA-2 tumorsphere

VAM extract was further evaluated for inhibitory effect on 3-dimensional tumorspheres (3D) of NTERA-2 with five concentrations of 500, 100, 20, 4, and 0.8 µg/mL. Treatment with 500 µg/mL of VAM extract for 48h destroyed NTERA-2 tumorspheres. In addition, NTERA-2 cell viability in spheroid form was significantly reduced by treating VAM extract at 100 and 500 µg/mL.

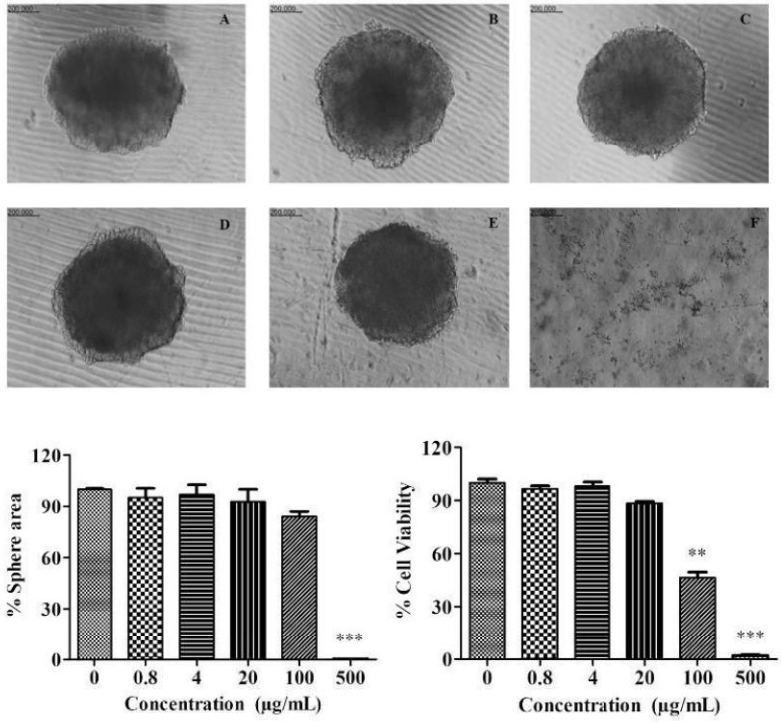

Fig. 2. The inhibitory effect of VAM extract on tumorsphere was accessed through the size of sphere area and cell viability on spheroid form with (A) DMSO 0.5% as untreated control; (B, C, D, E, F) VAM extract 0.8; 4; 20, 100, 500 µg/mL. **P < 0.01, ***P < 0.001

3.3. Preventing NTERA-2 migration

Subsequently, the impact of this extract on the migration of NTERA-2 cells was examined using a scratch assay with three concentrations: 0.5xIC_{50}, IC_{50}, and 2xIC_{50}. The outcomes of the scratch assay presented that VAM extract at concentrations of IC_{50} and 2xIC_{50} significantly prevented the cell migration with the percentage of closure area of 25.89 ± 3.03% and 0.75 ± 0.20%, compared with negative control as 34.95% after 24 hour-treatment (Fig. 3).

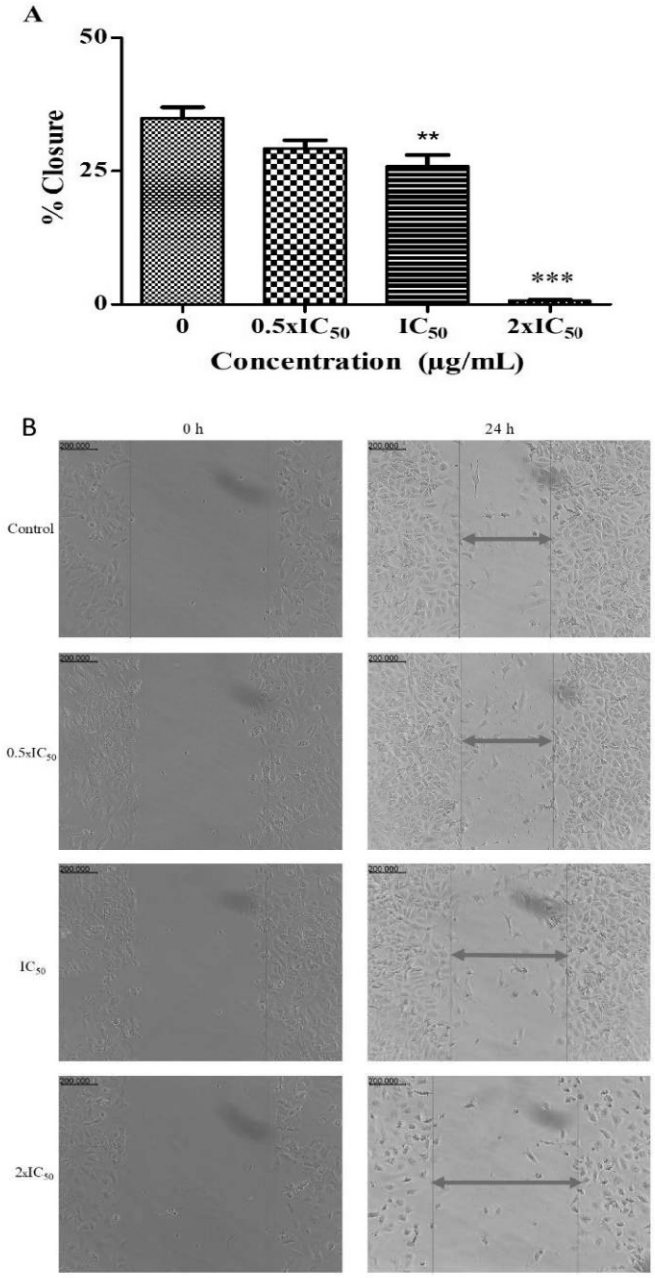

Fig. 3. (A) Evolution of the scratch closure in the control group and treated groups. The closure area was covered by cells after exposure to different concentrations of VAM extract for 24 hours. (B) The images of cell migration were taken on day 0 and after 24 h. **P < 0.01, ***P < 0.001.

4. Discussion

From this study, the data showed that the methanol extract from *A. vulgaris* suppressed NTERA-2 cancer stem cell proliferation with an IC_{50} value of 18.31 µg/mL. As reported, the extract of *Artemisia vulgaris* L. had a positive effect on HepG2 hepatocarcinomas and HaCaT keratinocytes with IC_{50} of 13.36 ± 0.45 and 27.30 ± 3.71 µg/mL, respectively [10]. In another research, *Artemisia alba*, a species belonging to the *Artemisia* genus, whose acetone extracts inhibited SW-480 colon cancer cells with IC_{50} about 240.12 ± 25.49 µg/mL [11]. In addition, the dichloromethane extract of *A. diffusa*, *A. santolina*, and *A. ciniformis* also prevented HT-29 cell growth [12]. According to Long Li *et al.*, the *Artemisia absinthium* crude extract showed a reduction in cell viability in breast cancer cells with an IC_{50} value of 25 µg/mL [13].

Besides evaluating cytotoxicity effect through

two-dimensional (2D) cultures, VAM extract was further investigated stemness-suppressing ability via tumorspheroidal assay. The results indicated that VAM reduced spheroid forming, a unique characteristic of CSCs [14]. According to the report of Tin et al., in 2013, it was also demonstrated that artemisinin, derived from sweet wormwood (*Artemisia annua*), has an inhibitory effect on the tumorspheres derived from breast cancer stem cells [15].

The process of metastasis involves tumor cells moving away from the primary tumor site, with cancer stem cells (CSCs) potentially playing a key role in enabling this movement [16]. Concerning our results, $2xIC_{50}$ of the crude extract might prevent NTERA-2 cell migration compared to the control after 24 h of incubation. Besides, the methanol extract from *A. vulgaris* also demonstrated a reduction in HCT-15 colon cancer cell migration capacity, as stated by G Lian et al., [17]. Additionally, a different study showed that certain plants belonging to the same genus, including *Artemisia dracunculus*, *Artemisia annua*, and *Artemisia absinthium*, could have wound healing potential on HaCat cell line with wound closure rates of 89.65 ± 2.18%, 98.55 ± 0.64%, and 97.84 ± 1.98%, respectively [18].

5. Conclusion

In conclusion, the crude methanol extract of *Artemisia vulgaris* demonstrated anti-cancer potential against NTERA-2 cancer stem cells by inhibiting their survival, reducing stemness characteristics by preventing tumorsphere formation as well as suppressing cell migration *in vitro*.

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