# *Gymnanthemum extensum* extracts exerted dose-dependent cytostatic and cytotoxic effects on A549 human lung carcinoma cells


**Siriporn Keeratichamroen[1], Kriengsak Lirdprapamongkol[1,2], Sanit Thongnest[3], Jutatip Boonsombat[3], Pornsuda Chawengrum[4], Thiwaree Sornprachum[1], Jitnapa Sirirak[5], Chris Verathamjamras[1], Narittira Ornnork[1], Somsak Ruchirawat[2,4,6], Jisnuson Svasti[1,7]**

[1]Laboratory of Biochemistry, Chulabhorn Research Institute, Bangkok, Thailand; [2]Center of Excellence on Environmental Health and Toxicology (EHT), Ministry of Education, Bangkok, Thailand; [3]Laboratory of Natural Products, Chulabhorn Research Institute, Bangkok 10210, Thailand; [4]Program in Chemical Sciences, Chulabhorn Graduate Institute, Chulabhorn Royal Academy, Bangkok, Thailand; [5]Department of Chemistry, Faculty of Science, Silpakorn University, Nakhon Pathom, Thailand; [6]Laboratory of Medicinal Chemistry, Chulabhorn Research Institute, Bangkok, Thailand; [7]Program in Applied Biological Sciences, Chulabhorn Graduate Institute, Chulabhorn Royal Academy, Bangkok, Thailand



Lung cancer remains one of the primary cancer-related causes of death in both men and women worldwide due to drug resistance and disease recurrence. The limited efficiency of current conventional chemotherapies necessitates the search for new effective anticancer agents. The present study demonstrated anti-proliferative effect and apoptosis-inducing activity of three sesquiterpene lactones isolated from *Gymnanthemum extensum*, including vernodalin (VDa), vernolepin (VLe) and vernolide (VLi), on A549 human lung cancer cells. Treatment with sub-cytotoxic doses (cell viability remaining >75%) of VDa, VLe and VLi, arrested progression of A549 cell cycle in the G0/G1 phase, while cytotoxic doses of the three compounds induced G2/M phase arrest and apoptosis. Mechanistic studies revealed that VDa, VLe and VLi may exert their anti-tumor activity through the JAK2/STAT3 pathway. Molecular docking study confirmed that VDa, VLe and VLi formed hydrogen bonding interactions with the FERM domain of JAK2 protein. The overall finding of the present study highlights the potential therapeutic value of VDa, VLe and VLi to be further developed as anticancer agents for treatment of lung cancer.


## INTRODUCTION

Gymnanthemum extensum (*G. extensum*) (syn. Vernonia extensa var. amygdalina, Asteraceae), locally known as 'Phim Phai Lin', is the only member of its genus found in Thailand (1). It is a shrub or subshrub that will typically reach 2-6 m in height. This species has been used in Thai folk medicine for reducing blood pressure, blood sugar and blood lipids, as well as for the treatment of skin abscess (2,3). Although considerable progress has been made in natural product research towards the isolation, structural determination and biological activity of the isolated compounds, little information is available on the molecular mechanisms by which these bioactive constituents mediate their effects. Lung cancer, especially of the non-small cell lung cancer (NSCLC) subtype, is one of the most serious health concerns in Thailand. Therefore, the aim of the present study was to investigate the molecular mechanisms and biological targets of vernodalin (VDa), vernolepin (VLe) and vernolide (VLi), three cytotoxic sesquiterpene lactones isolated from *G. extensum* (4), in A549 human NSCLC cells.

## METHODS AND RESULTS

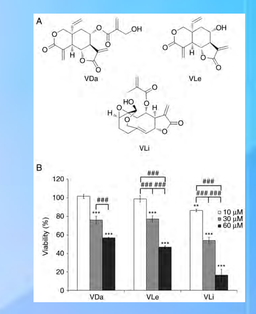


Figure 1. The cytotoxic effects of VDa, VLe and VLi (Fig. 1A) on the A549 human lung cancer cell line were evaluated by MTT assay at three concentrations (10, 30 and 60 μM). After 24 h of incubation, VDa, VLe and VLi decreased cell viability in a dose-dependent manner, with VLi being the most potent (Fig. 1B). Comparison of cytotoxic activities indicated that treatments with 30 μM VDa, 30 μM VLe or 10 μM VLi were only marginally cytotoxic (~80% viability) and may therefore be considered as sub-cytotoxic doses, while high doses (60 μM for VDa and VLe, and 30 μM for VLi) were cytotoxic (~50% viability).

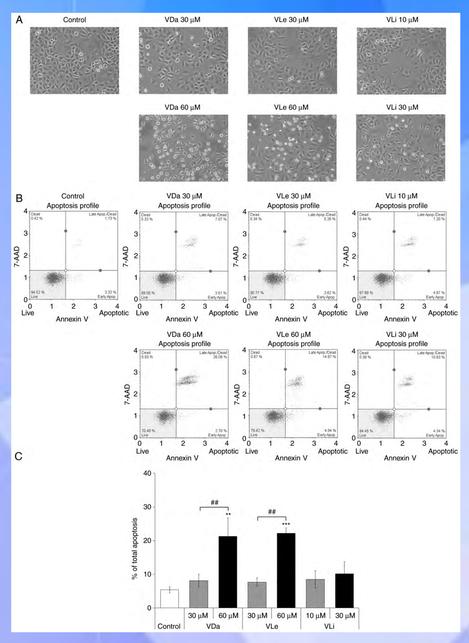


Figure 2. Morphological investigation by inverted phase-contrast microscopy revealed that treatment with VDa, VLe and VLi resulted in a decrease in cell numbers, induction of cell rounding and detachment of cells from the vessel surface (Fig. 2A). In addition, flow cytometric analysis was performed in A549 cells treated with *G. extensum*-derived compounds for 24 h. The results indicated that the number of apoptotic cells increased in cells treated with VDa, VLe and VLi compared with untreated cells, corresponding with the increase in concentration (Fig. 2B and C). In addition, necrotic cell populations were <1% following all treatment types and concentrations, suggesting that VDa, VLe and VLi were dose-dependently cytotoxic towards A549 cells through the induction of apoptosis.

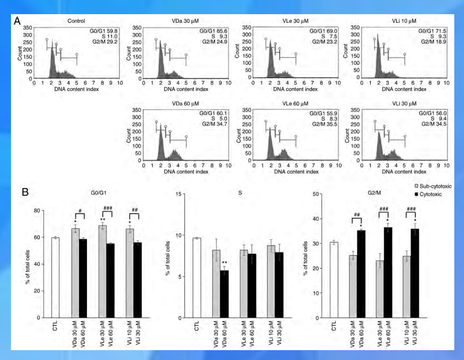


Figure 3. Cell cycle distribution after treatment with VDa, VLe and VLi for 24 h was assessed using a Muse Cell Analyzer. Treatment of A549 cells with sub-cytotoxic doses of VDa, VLe and VLi caused a significant increase in the G0/G1 population compared with untreated control cells. Consistent with this result, a decrease in the proportion of cells in the S phase and G2/M phase of the cell cycle was observed, compared with the control. However, increasing the concentration of VDa, VLe and VLi to cytotoxic doses resulted in a significant increase in the percentage of cells in the G2/M phase compared with untreated control cells. The increased number of cells in the G2/M phase was accompanied by a proportional decrease in the G0/G1 cell populations and S phase populations compared with the control group. These data suggest that VDa, VLe and VLi exerted dose-related cytostatic effects on A549 cells, resulting in inhibition of cellular proliferation.

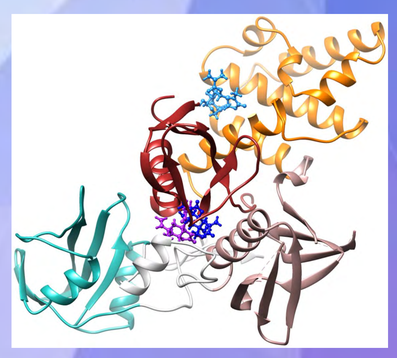

Figure 4. Binding positions of VDa (purple), VLe (deep blue) and VLi (sky blue) in the cavity of JAK2 (PDB ID: 4Z32), where the Src homology 2 domain of JAK2 is shown in green, the linker is shown in white and the F1, F2 and F3 lobes of the FERM domain are shown in red, orange and rose gold, respectively.

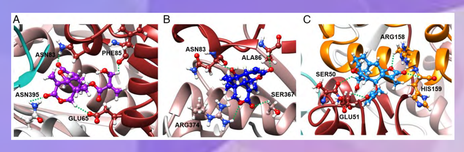


Figure 5. Hydrogen bond interactions observed in the binding of (A) VDa, (B) VLe and (C) VLi in the cavity of JAK2 (PDB ID: 4Z32).

Table I. Cell cycle progression of A549 cells treated with the JAK2/STAT3 inhibitor, CBC-I.

| | Cell cycle distribution, % | | |
|---|---|---|---|
| Treatment | $G_0G_1$ | S | $G_2$/M |
| Control | 59.9±0.9 | 9.6±0.1 | 30.5±0.8 |
| STAT3-specific inhibitor | | | |
| CBC-I 10 μM | 63.0±1.7 | 7.0±0.6 | 29.7±1.2 |
| CBC-I 50 μM | 47.5±4.1 | 8.8±0.2 | 43.0±4.0 |

A specific inhibitor of JAK2/STAT3, CBC-I, was used to confirm whether the effects of VDa, VLe and VLi on A549 cells were mediated through the inhibition of JAK2/STAT3 signaling. Cell cycle analysis indicated that the cell population tended to shift to G0/G1 and G2/M phase arrest when treated with 10 and 50 μM CBC-I, respectively, similar to that observed with sub-cytotoxic and cytotoxic treatments of *G. extensum*-derived compounds.

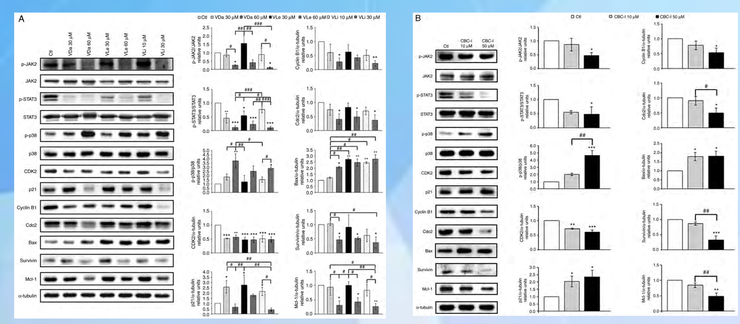


Figure 6. Western blot and quantitative analysis showing the effects of *G. extensum*-derived compounds on A549 cells. (A) Effects of VDa, VLe and VLi on the expression of JAK2/STAT3 signaling, cellular stress proteins and proteins involved in cell cycle regulation and apoptosis. (B) Effects of a specific inhibitor of JAK2/STAT3 signaling (CBC-I) on the expression of proteins related to cellular stress, cell cycle regulation and apoptosis.

## DISCUSSION

VDa, VLe and VLi, three bioactive constituents isolated from *G. extensum*, have been reported to possess various pharmacological activities. Herein, the cytotoxicity, cytostatic activity and apoptosis-inducing ability of VDa, VLe and VLi were investigated. The results demonstrated that VDa, VLe and VLi exerted cytotoxic effects by promoting apoptosis. The number of apoptotic cells increased in cells treated with VDa, VLe and VLi compared with untreated cells, corresponding with the increase in concentration. However, the percentage of apoptotic cells was lower than expected, suggesting that cell cycle arrest may have occurred, ultimately inhibiting cellular proliferation. This hypothesis is supported by the cell cycle distribution results. Cytostatic induction can be considered a relevant mechanism to inhibit, delay or reverse tumor progression (5). The present study results demonstrated that VDa, VLe and VLi possessed cytostatic effects by inhibiting cellular proliferation through cell cycle arrest, at either the G0/G1 or G2/M phase, depending on the concentrations of the compound used. VDa, VLe and VLi induced G0/G1-phase block at sub-cytotoxic doses and G2/M-phase block at cytotoxic doses, indicating that A549 cells respond to different doses of VDa, VLe and VLi through differences in the activation of signaling pathways, depending on the degree of cellular damage.

Parthenolide, a structurally related sesquiterpene lactone, has been shown to inhibit STAT3 signaling by directly interacting with JAK2 (6). Therefore, docking-based virtual screening was then performed in the current study to predict the interactions between JAK2 (PDB:4Z32) and the three compounds, and to reveal the protein-ligand complex structure. The results showed that VDa, VLe and VLi interacted with JAK2 through hydrogen bonding with amino acid residues located in the FERM domain. These findings suggested that the JAK2/STAT3 signaling may be the target of the *G. extensum*-derived compounds.

The implication of JAK2/STAT3 signaling in the cytostatic and cytotoxic effects of VDa, VLe and VLi was confirmed by the results of JAK2/STAT3 inhibitor treatment. Similar increased or decreased levels of signaling molecules associated with cell cycle arrest and apoptosis were detected upon CBC-I treatment, indicating that the JAK2/STAT3 pathway may be a critical mediator of the regulatory effects of VDa, VLe and VLi.

## REFERENCES


1. Smitinand T: Thai plant names (Botanical Names-Vernacular Names). Rong pim Buddha press, Bangkok, pp 280, 2014.
2. Egharevba C, Osayemwenre E, Imieje V, Ahomafor J, Akunyuli C, Udu-Cost AA, Theophilus O, James O, Ali I and Falodun A: Significance of bitter leaf (Vernonia amagdalina) in tropical diseases and beyond: A review. Malar Chemoth Cont 3: 120, 2014.
3. Ong KW, Hsu A, Song L, Huang D and Tan BK: Polyphenols-rich Vernonia amygdalina shows anti-diabetic effects in strepto- zotocin-induced diabetic rats. J Ethnopharmacol 133: 598-607, 2011
4. Thongnest S, Chawengrum P, Keeraticham roen S, Lirdprapamongkol K, Eurtivong C, Boonsombat J, Kittakoop P, Svasti J and Ruchirawat S: Vernodalidimer L, a sesquiterpene lactone dimer from Vernonia extensa and anti-tumor effects of vernodalin, vernolepin, and vernolide on HepG2 liver cancer cells. Bioorg Chem 92: 103197, 2019.
5. Ewald JA, Desotelle JA, Wilding G and Jarrard DF: Therapy-induced senescence in cancer. J Natl Cancer Inst 102: 1536-1546, 2010.
6. Liu M, Xiao C, Sun M, Tan M, Hu L and Yu Q: Parthenolide inhibits STAT3 signaling by covalently targeting Janus kinases. Molecules 23: 1478, 2018.


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