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Cytotoxicity and induction of the apoptotic activity of hirsutinolide series/sesquiterpene lactones from *Vernonia cinerea* on human colorectal cancer cells (COLO 205)

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Abstract

Hirsutinolide series/sesquiterpene lactones from *Vernonia cinerea*, were investigated for cytotoxic effects and induction of the apoptotic activity on human colorectal cancer cells (COLO 205). The results showed that hirsutinolide series significantly inhibited cell viability of COLO 205 with IC_{50} less than 1.5 μM. Flow cytometric analysis indicated that hirsutinolide series (compounds 7, 10, 11, and 22) inhibited DNA synthesis as evidenced by a decline of DNA content in S, G2/M, and G0/G1 phases of the cell cycle. Furthermore, hirsutinolide series provided anticancer activity by inducing apoptosis on COLO 205 cells through the downregulation of anti-apoptotic genes; *Bcl-2* and *Bcl-xL*.

Keywords**:** *COLO 205, cytotoxicity, hirsutinolide series/sesquiterpene lactones, induction of apoptotic activity, Vernonia cinerea*,

1. Introduction

Colorectal cancer (CRC) is the third most diagnosed cancer in both males and females. In 2017, it was forecasted that CRC would be diagnosed with 95,520 new cases worldwide and 39,910 cases in the United States (Siegel, Miller, & Jemal, 2017). Even though there are various treatments for CRC such as chemotherapy, targeted therapy, and immunotherapy, treatment failure for CRC still occurs. Therefore, development of novel active compounds in CRC has led to improved survival and cure rates. Plants have been used for several human diseases as folk medicine. Specifically, *Vernonia cinerea* Less (Asteraceae) is a plant primarily scattered in the tropical areas of Thailand and China (Haque et al., 2012). Many parts of the plant such as stem, barks, roots, leaves, and flowers have been traditionally used for several diseases for instance, skin inflammation, infections, smoking cessation, cancer, and liver diseases (Leelarungrayub et al., 2010). They also are known to be rich sources of sesquiterpene lactones and flavonoids (Youn et al., 2012). The biological activity of methanol extracts from the aerial parts of the *Vernonia* plant exhibited antidiarrheal (Ganesh, Kumar, & Kumar, 2011), antibacterial activity (Rizvi, Biswas, Arif, & Zeeshan, 2011), and chemoprotective effects during cyclophosphamide treatment Pratheeshkumar & Kuttan, 2010). *Vernonia cinerea* (Vc) has been tested in mice and has significantly increased catalase, superoxide dismutase, glutathione, glutathione peroxidase, and glutathione-*S*-transferase levels in the blood and liver (Pratheeshkumar & Kuttan, 2009). Wongwiwatthananukit, Benjanakaskul, Songsak, Suwanamajo, & Verachai (2009) illustrated the efficacy of the whole Vc plant for smoking cessation. Zhang, Won, Ong, & Shen (2005) concluded that sesquiterpene lactones composed a

functional structure, an α-methylene lactone group, which had thiol reactivity on the cell signaling pathways NF-κB and mitogen-activated protein kinase (MAPK) with regards to their potential in an anticancer drug. A study in 2014 showed hirsutinolide series inhibited human glioma cancer cell (U251MG) viability (Youn et al., 2014). In 2015, Miklossy et al. reported that the hirsutinolides and the semisynthetic analogues inhibited abnormal signal transducer and activator of transcription (Stat3) activity in the U251MG glioblastoma cancer cell and MDA-MB-231 breast cancer cells when compared to normal NIH-3T3 mouse fibroblast cells. Hirsutinolides repressed tumor cell phenotypes *in vitro* and downregulated the expression of c-Myc, Bcl-2, Mcl-1, and Bcl-xL proteins which together represent part of the mechanisms for antitumor activities both *in vitro* and *in vivo*. Latha, Darah, Jain, & Sasidharan (2010) tested the toxicity of a Vc methanol extract of and reported that the median lethal dose (LD50) of the extract was higher than 2000 mg/kg using mice. However, no studies have been performed with Vc hirsutinolides on cell cycle analysis and the apoptotic activities on human colorectal cancer cells.

In this investigation, we proposed to study the cytotoxic effects, induction of apoptotic activity, and cell cycle analysis of four hirsutinolide compounds (7, 10, 11, and 22) (Figure 1) on human colorectal cancer cells (COLO 205). COLO 205 cells were found to contain a mutation of *BRAF* gene. In colorectal cancer, BRAF gene mutation contributes to carcinogenesis by promoting the anti-apoptotic function of the RAS/RAF/MEK/ERK pathway. Furthermore, this *BRAF* gene alteration is associated with significantly reducing apoptosis in tumour cells (Ikehara et al., 2005). Therefore, hirsutinolide compounds might play a significant role in anticancer activity through the induction of apoptosis in COLO 205 cells.

2. Objectives

To investigate the cytotoxicity and induction of apoptosis and cell cycle analysis of four hirsutinolide compounds (7, 10, 11, and 22) on human colorectal cancer cells (COLO 205).

3. Materials and methods

3.1 Plant material

The leaves and stems of Vc were collected in Lampang Province, Thailand. The plant materials were identified by Dr. Thanapat Songsak, (College of Pharmacy, Rangsit University). A voucher specimen (No. VCW02) was deposited at the Natural Products Chemistry Laboratory, The Daniel K. Inouye College of Pharmacy, University of Hawaii at Hilo. The isolation, structural elucidation, and the characterization of 8α-tigloyloxyhirsutinolide-13-O-acetate (compound 7), vernolide-B (compound 10), hirsutinolide-13-O-acetate (compound 11), 8α-Hydroxy-13-O-tigloyl-hirsutinolide (compound 22) have been previously reported (Youn et al., 2012, Youn et al., 2014).

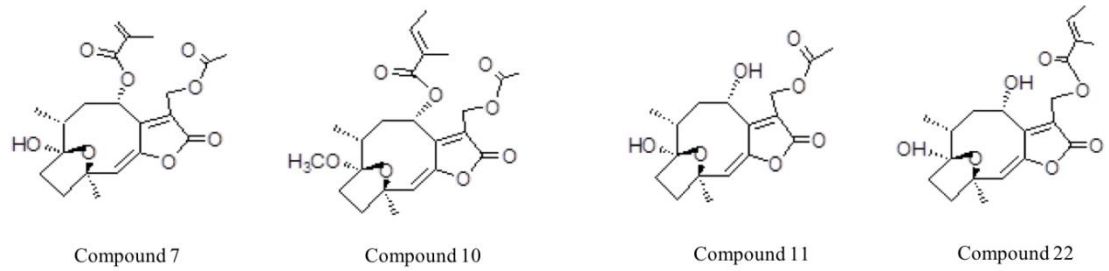

Figure 1 Structure of hirsutinolide compounds 7, 10, 11, and 22

3.2 Preparation of COLO 205 cell line

Colorectal cancer cells (COLO 205), which maintain the characteristics of colon epithelial cells derived from ascites metastatic site in culture, were routinely incubated with Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL, NY, USA) containing 2 mM L-glutamine and phenol red, supplemented with 10% (v/v) fetal bovine serum (FBS), 0.1% penicillin-streptomycin and HEPES (complete RPMI). The cell cultures were incubated at 37 °C in humidified 5% CO_2.

3.3 Cytotoxicity analysis

Compounds were tested for cytotoxic activity using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay as described by Berridge, Herst, & Tan (2005). MTT was purchased from Sigma-Aldrich (St Louis, MO, USA). The samples were prepared in 0.2% DMSO. Briefly, COLO 205 cells were seeded into each 96-well plate (5,000 cells/well) and incubated for 24 h. Cells were treated with concentrations of 0.2, 0.4, 0.8, 1.6, and 3.2 µM with hirsutinolides 7, 10, 11, and 22 for 48 h. After that, 10 µL of MTT solution (5 mg/mL) was added and incubated for 4 h at 37 °C. The absorbance of formazan dye at 570 nm was measured and compared with the negative control (0.2% DMSO). 5-fluorouracil, 5-FU (Sigma-Aldrich, St Louis, MO, USA) was used as a positive control. The cytotoxic activity was expressed as 50% inhibited cell growth (IC_{50}). The calculation of the percentage cytotoxicity as shown below:

$$100 - \frac{\text{Mean absorbance obtained from treated cells}}{\text{Mean absorbance obtained from untreated cells}} \times 100$$

3.4 Evaluation of apoptosis

Nexin (Annexin) reagent for apoptosis analysis was purchased from Merck Millipore, USA. COLO 205 cells were treated with hirsutinolides 7, 10, 11, and 22 at 0.5 µM and incubated for 24 h in 24-well plates. Culture media was then removed and placed in 15-mL tubes. Cells were washed with 1X phosphate buffer saline (PBS). Cell suspensions 100 µL ($5x10^5$ cells) were stained with 100 µL of Guava Nexin Reagent (Merck Millipore, USA) and incubated for 30 min in the dark prior to flow cytometry analysis. The reaction was measured by Guava Nexin Software. Acquired data were shown as flow cytograms (Daigle et al., 2011). The negative control was 0.2% DMSO.

3.5 Cell cycle analysis

Cells were treated with hirsutinolides 7, 10, 11, and 22 at 0.5 µM for 24 h in 12-well plates, then harvested, washed with 500 µL of PBS and placed separately into 15-mL tubes, and processed for cell cycle analysis. The cell density was adjusted to 1×10^6 cells/mL prior to fixation using ice-cold 70% ethanol. Fixed cells (200 µL) were centrifuged at 450 x g for 5 minutes. The cells were then washed with 1X PBS and centrifuged at 450 × g for 5 minutes. The cells were resuspended in 200 µL Guava Cell Cycle Reagent (Merck Millipore, USA) and incubated at room temperature for 30 minutes in the dark. The cell cycle distribution of cells for each sample was determined using the Guava instrument equipped with InCyte™ software. The negative control was 0.2% DMSO (Daigle et al., 2011).

3.6 qRT-PCR analysis

Cells were treated with hirsutinolides 7, 10, 11, and 22 at 0.25, 0.5, and 1.0 µM for 24 h in 6-well plates. RNA was extracted from the cells after incubation using Trizol reagent. Total RNA was reverse transcribed into complementary DNA (cDNA) by ImProm-IITM Reverse transcription system, the resulting cDNA was used as templates for subsequent real-time PCR amplification using primers specific for *Bcl-2* (forward; 5′-TCCTTGTCTACGCTTTCCACG-3, reverse; 5′-GGTCGCATT GTGGCCTTT-3′) and *Bcl-xL* (forward;5′-GCTGAGTTACCGGCATCC-3′, reverse; 5′-TTCTGAAGGGAGAGAAAGAGATTC-3′). *GADPH* (forward; 5′-CAAGGTCATCCATGACAACTTTG-3′, reverse; 5′-GTCCACCACCCTGTTGCTGTAG-3′) was used as an internal control.

3.7 Data and Statistical Analysis

Results were reported as means and standard deviations (SD). Data were analyzed and graphed with Microsoft Excel 2013 and GraphPad Prism, version 6.0. Statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by a Tukey test for further comparison. The level of significance was set at $p \leq 0.05$.

4. Results

4.1 Cytotoxicity of hirsutinolides 7, 10, 11, and 22 on COLO 205 cells

Hirsutinolides 7, 10, 11, and 22 at concentrations of 0.2, 0.4, 0.8, 1.6, and 3.2 µM were used for treatment. IC_{50} was calculated from the dose-response curve. The results showed that there was a decrease in the mean percentage of cell viability in a dose-dependent manner (Figure 2) by using turkey's multiple comparisons. The IC_{50} of hirsutinolides 7, 10, 11, and 22 were 1.01±2.42, 1.32±3.38, 1.03±2.55, and 1.00±1.07 µM, respectively. Whereas, the IC_{50} of 5-FU was 6.30±3.14 µM.

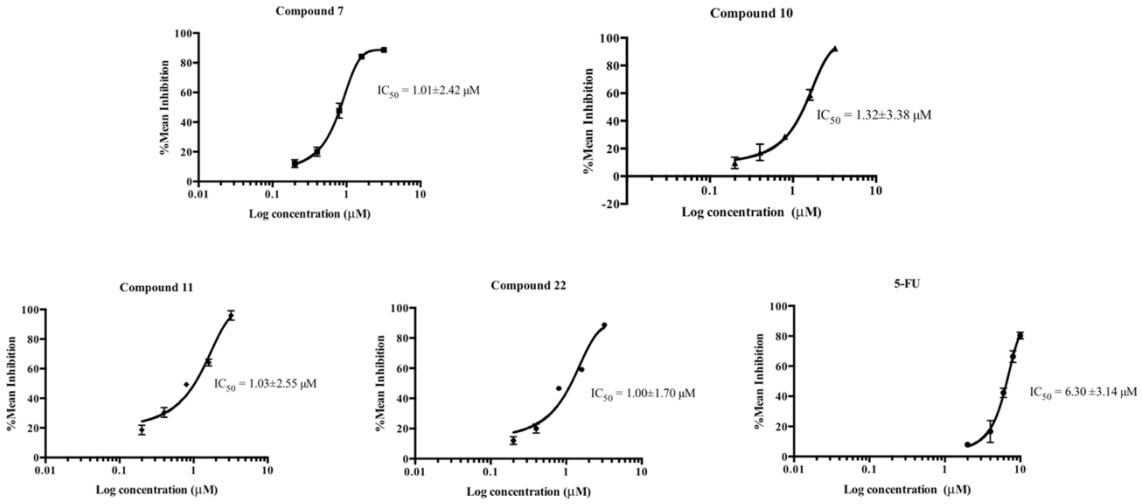

Figure 2 Log-concentration inhibition shows the mean percentage inhibition and SD of hirsutinolide compounds 7, 10, 11, and 22, and 5-fluorouracil (a positive control) against COLO 205 cells after 48 hours incubation. Data were presented as mean ± SD (n = 3).

4.2 Apoptosis induction of hirsutinolides 7, 10, 11, and 22 on COLO 205 cells

COLO 205 cells were treated with hirsutinolides 7, 10, 11, and 22 at 0.5 µM and incubated for 24 h. The concentration used was based on the concentration tested in the MTT assay that provided cytotoxic activity without causing extreme cell deaths. Three populations of cells can be distinguished in this assay. Firstly, non-apoptotic cells which are Annexin V^- and 7-AAD^-. Secondly, early apoptotic cells which were Annexin V^+ and 7-AAD^-. Finally, late-stage apoptotic and dead cells were shown as Annexin V^+ and 7-AAD^+. The proportion of cells were measured by flow cytometry and the results revealed that hirsutinolides 7 (Figure 3b), 10 (Figure 3c), 11 (Figure 3d), and 22 (Figure 3e) produced a trend of early apoptosis induction and significantly increased the mean percentage of cell populations at the late apoptotic stage compared to the control (Figure 3a).

4.3 Cell cycle assay of hirsutinolide compounds 7, 10, 11, and 22 on COLO 205 cells

The DNA was stained with propidium iodide (PI) to allow identification of cells in different stages of the cell cycle. Resting cells (G0/G1phase) contain two copies of each chromosome. In mitosis, cells synthesize DNA (S phase), so more PI was intercalated and increased the fluorescence intensity. The DNA content = doubles (G2/M phase) when all chromosomes have replicated. Thus, the cells fluoresce with double the intensity of the G0/G1 population.

The effect of compounds 7 and 22 on the cell-cycle progression in COLO 205 cells was determined by flow cytometry (Figure 4). The cell population in G_0/G_1, S, and G_2/M phases of hirsutinolide-treated cells showed a significant decrease in cell population DNA count when compared to untreated cells (Figure 4a). All four compounds showed potential to cause DNA damage to COLO 205 cells (Figures 4b-4e).

4.4 qRT-PCR analysis

Hirsutinolides 7, 10, 11, and 22 at 0.25, 0.5, and 1.0 µM can downregulate *Bcl-2* and *Bcl-xL* genes in COLO 205 cells. Therefore, hirsutinolide compounds might play a significant role in anticancer activity through the induction of apoptosis in COLO 205 cells.

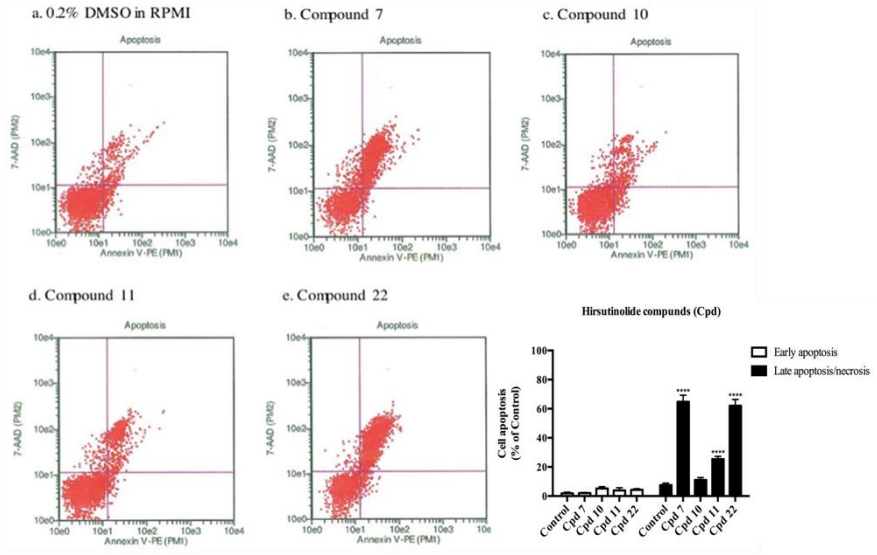

Figure 3 Apoptosis analysis of control showed viable cells at the percentage of 90.6, and early apoptosis of 1.3%. Late apoptosis and death cells were 6.6% (a). Apoptosis analysis of compound *7, 10, 11, and 22* showed no significantly apoptosis induction (b-e) and showed the percentage of late apoptosis significantly increased to 61.6% (b), 9.9% (c), 24.0% (d), and 59.0% (e), respectively. The bar chart is the comparison of the mean percentage of four hirsutinolides compounds at 0.5 µM on the induction of apoptosis of COLO 205 cells. Data presented as mean ± SD (n = 3), ****P≤0.0001 vs. control (0.2% DMSO in RPMI).

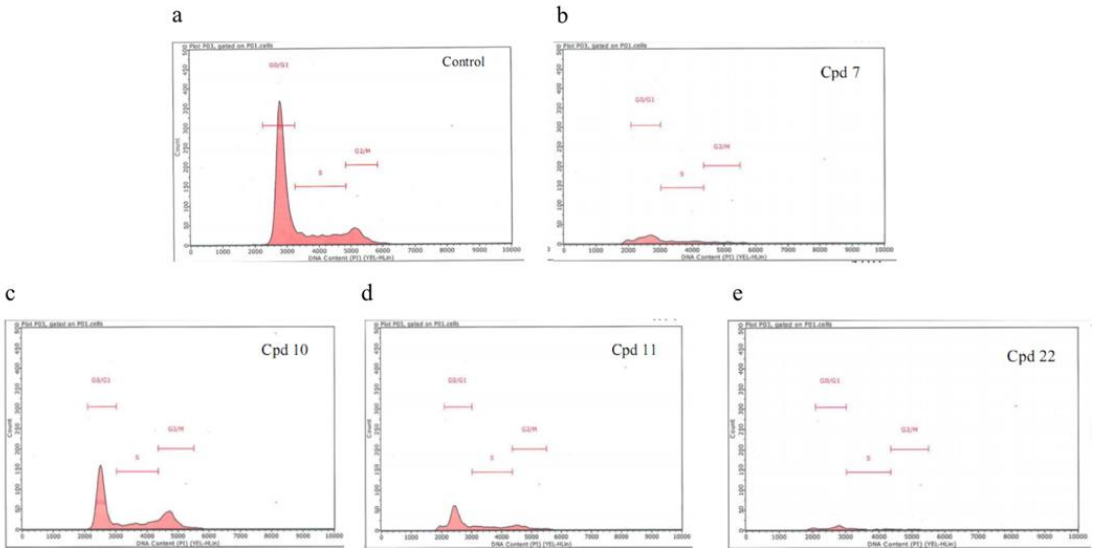

Figure 4 Cell cycle assay of control (untreated cells) on COLO 205 cells, which showed DNA contents and cell count of cells in the G0/G1phase, S phase, and G2/M phase. Compound 22 (e) and 7 (b) on COLO 205 cells at 0.5 µM which showed a significant reduction of DNA content in the G0/G1 phase, S phase, and G2/M phase whereas compound 10 (c) and 11 (d) on COLO 205 cells at 0.5 µM showed a slight reduction of DNA content and cell counts in the G0/G1 phase, S phase, and G2/M phases as compared to control.

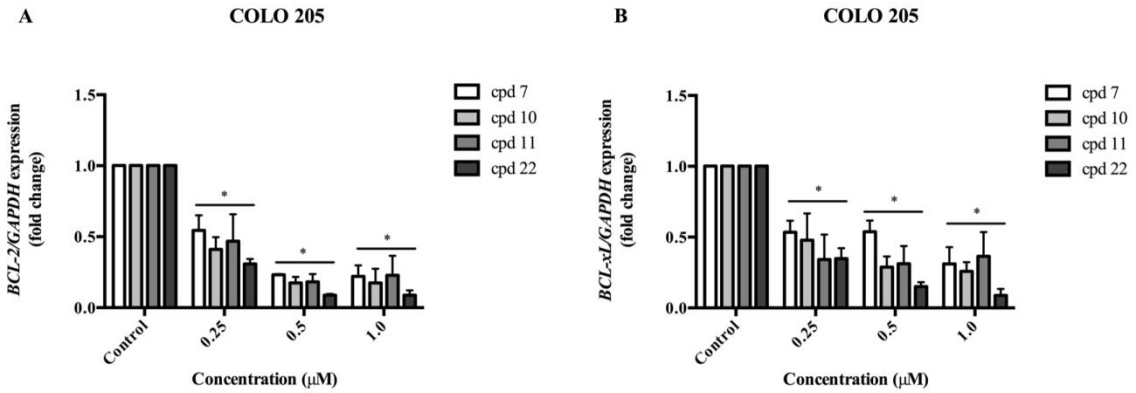

Figure 5 The inhibitory effects of compound 7, 10, 11, and 22 on apoptotic-related genes were decreased in colorectal cancer cells. The expression of *Bcl-2* (A) and *Bcl-xL* (B) gene by was determined qRT-PCR analysis of COLO 205 cells. Data are presented as mean ± SD (n = 3). **p* < 0.05vs. control (0.2% DMSO)

5. Discussion

Our study shows that among all four compounds, compound 22 had the highest potency of cytotoxicity on COLO 205 cells. This investigation indicates (note: you never really prove anything) that hirsutinolides 7, 10, 11, and 22 had significant cytotoxicity on COLO 205 cells with the maximum percentage inhibition of all compounds at 85 to 95%. They also showed the potential to damage DNA of COLO 205 cells as indicated in the reduction of DNA content. Compound 7, 11, and 22 play a significant role through the induction of apoptosis. Also, hirsutinolides 7, 10, 11, and 22 at 0.25, 0.5, and 1.0 μM COLO 205 cells can downregulate *Bcl-2* and *Bcl-xL* genes in colorectal cancer cells. Therefore, hirsutinolide compounds might play a significant role in anticancer activity through the induction of apoptosis in COLO 205 cells via the anti-apoptotic *Bcl-2* family genes. All of the compounds demonstrated non-cell-cycle specific anticancer activity, indicated by the reduction of DNA content in all cell cycle phases, which could be developed for future use in combination with cell-cycle specific anticancer agents. Notably, the anticancer activity of hirsutinolides was associated with a position 13 hydrophobic ester side chain in compounds used in this study. Therefore, it was strongly associated with the inhibition of Stat3 and NF-κB activation, which leads to cancer cell death as suggested by others (Miklossy et al., 2015).

Further studies on hirsutinolides should be confirmed by studying the change of mRNA levels and proteins involved in the cell cycle phases and apoptosis pathway. The repression of NF-κB activation and Stat3 activation in cancer cells related to the inhibition of cell proliferation, cell migration, and angiogenesis and are still required.

6. Conclusion

Presented herein are hirsutinolide natural products and their semi-synthetic analogs that inhibit Stat3 activation and function and induces anti-proliferative effects in vitro. Data suggest the decrease in cell viability and growth is a result from cell cycle arrest at the G0/G1 phase, S phase, and G2/M phase. The semi-synthetic analogs and the ability of the hirsutinolides to induce apoptosis through downregulation of anti-apoptotic genes *Bcl-2* and *Bcl-xL* genes, reduction of DNA content, and suppress colorectal cancer cells proliferation are all novel experimental results.

7. Acknowledgements

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8. References

Berridge, M. V., Herst, P. M., & Tan, A. S. (2005). Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. *Biotechnology Annual Rev*iew, *11*, 127-152. DOI: 10.1016/S1387-2656(05)11004-7

Daigle, S. R., Olhava, E. J., Therkelsen, C. A., Majer, C. R., Sneeringer, C. J., Song, J., . . . Pollock, R. M. (2011). Selective killing of mixed lineage leukemia cells by a potent small-molecule DOT1L inhibitor. *Cancer Cell*, *20*, 53-65.

Ganesh, P., Kumar, K. V., & Kumar, H. S. (2011). Antidiarrhoeal activity of methanolic extract of *V. cinerea* leaves less on female albino rats. *International Research Journal of Pharmaceutical and Applied Sciences*, *2*(5), 211-213.

Haque, M. A., Hassan, M. M., Das, A., Begum, B., Ali, M. Y., & Morshed, H. (2012). Phytochemical investigation of *Vernonia cinerea* (Family: Asteraceae). *Journal of Applied Pharmaceutical Science*, *2*(6), 79-83. DOI: 10.7324/JAPS.2012.2617

Ikehara, N., Semba, S., Sakashita, M., Aoyama, N., Kasuga, M., & Yokozaki, H. (2005). *BRAF* mutation associated with dysregulation of apoptosis in human colorectal neoplasms. *Cancer Genetics*, *115*(6), 943-950. DOI: 10.1002/ijc.20957

Latha, L. Y., Darah, I., Jain, K., & Sasidharan, S. (2010). Toxicity study of *Vernonia cinerea*. *Pharmaceutical Biology*, *48*(1), 101-104. DOI: 10.3109/13880200903046203

Leelarungrayub, D., Pratanaphon, S., Pothongsunun, P., Sriboonreung, T., Yankai, A., & Bloomer, R. J. (2010). *Vernonia cinerea* Less. supplementation and strenuous exercise reduce smoking rate: relation to oxidative stress status and beta-endorphin release in active smokers. *Journal of the International Society* of *Sports Nutrition*, *7*, 21. DOI: 10.1186/1550-2783-7-21.

Miklossy, G., Youn, U. J., Yue, P., Zhang, M., Chen, C. H., Hilliard, T. S., . . . Turkson, J. (2015). Hirsutinolide series inhibit Stat3 Activity, Alter GCN1, MAP1B, Hsp105, G6PD, vimentin, TrxR1, and importinα-2 expression, and induce antitumor effects against human glioma, *Journal of Medicinal Chemistry*, *58*(19), 7734-7748.

Pratheeshkumar, P., & Kuttan, G. (2009). *Vernonia cinerea* L. scavenges free radicals and regulates nitric oxide and proinflammatory cytokines profile in carrageenan induced paw edema model. *Immunopharmacology* and *Immunotoxicology*, *31*(1), 94-102.

Pratheeshkumar, P., & Kuttan, G. (2010). Ameliorative action of *Vernonia cinerea* L. on cyclophosphamide-induced immunosuppression and oxidative stress in mice. *Inflammopharmacology*, *18*(4), 197-207. DOI: 10.1007/s10787-010-0042-8

Rizvi, S. M. D., Biswas, D., Arif, J. M., & Zeeshan, M. (2011). In-vitro antibacterial and antioxidant potential of leaf and flower extracts of *Vernonia cinerea* and their phytochemical constituents. *International Journal of Pharmaceutical Sciences Review and Research*, *9*(2), 164-169.

Siegel, R. L., Miller, K. D., & Jemal, A. (2017). Cancer statistics. CA: A *Cancer Journal* for Clinicians, *67*(1), 7-30. DOI: 10.3322/caac.21387

Wongwiwatthananukit, S., Benjanakaskul, P., Songsak, T., Suwanamajo, S., & Verachai, V. (2009). Efficacy *Vernonia cinerea* for smoking cessation. *Journal* of *Health Research*, *23*(1), 31-36.

Youn, U. J., Park, E. J., Kondratyuk, T. P., Simmons, C. J., Borris, R. P., Tanamatayarat, P., . . . Chang, L. C. (2012). Anti-inflammatory sesquiterpene lactones from the flower of *Vernonia cinerea. Bioorganic* & *Medicinal Chemistry Letters*, *22*(17), 5559-62. DOI: 10.1016/j.bmcl.2012.07.010

Youn, U. J., Miklossy, G., Chai, X., Wongwiwatthananukit, S., Toyama, O., Songsak, T., & Chang, L. C. (2014). Bioactive sesquiterpene lactones and other compounds isolated from *Vernonia cinerea. Fitoterapia*, *93*, 194-200.

Zhang, S., Won, Y. K., Ong, C. N., & Shen, H. M. (2005). Anti-cancer potential of sesquiterpene lactones: bioactivity and molecular mechanisms. *Current Medicinal Chemistry-Anti-Cancer Agents*, *5*(3), 239-249.