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CYTOTOXIC AND ANTI-MIGRATORY ACTIVITIES FROM HYDROALCOHOLIC EXTRACT OF *Euphorbia lactea* Haw. AGAINST HN22 CELL LINE

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ABSTRACT

Euphorbiaceae is a botanical family of evolutionarily and morphologically distinct group of plants. Many euphorbias have been associated with diverse pharmacological properties including anticancer activity. *Euphorbia lactea* Haw. can be found naturally throughout Thailand; however, its anticancer activity remains largely unexplored. Here, we screened hydroalcoholic extract of *E. lactea* for its anticancer potentials, focusing on cytotoxic and anti-migratory activities using a migratory competent head and neck cancer cell line HN22 as an in vitro model. We detected statistically significant and dose-dependent cytotoxic activity of *E. lactea* hydroalcoholic extract against HN22 cells through MTT assay. Flow cytometry analysis of HN22 cells treated with the extract revealed an increase in sub-G1 population, suggesting that the cytotoxic effect of *E. lactea* hydroalcoholic extract likely involved an initiation of apoptosis in this cell line. Additionally, through wound-healing assays, we unprecedentedly reported an anti-migratory activity against HN22 cells from *E. lactea* hydroalcoholic extract. Together, exhibiting two attractive attributes i.e. cytotoxic and anti-migratory activities toward HN22 cells, our results identified *E. lactea* as a promising candidate medicinal plant for further investigations to unlock its anticancer potentials.

Keywords: cytotoxic, anticancer, antimetastatic, *Euphorbia lactea*

Introduction

Cancer is a group of complex medical conditions caused by accumulated genetic and epigenetic abnormalities which enable altered cells to manifest cancerous characteristics including uncontrolled proliferation and metastasis or the spread of cancerous cells from their original site to distant organs.[1] Despite the advancement of modern medicine, cancers remain a major health concern and a leading cause of mortality and morbidity globally. To date, treatment options for cancer include surgical removal and pharmacological treatment utilizing classical and targeted therapy. Despite the expanding repertoire of effective anticancer drugs, serious side-effects associated with certain anticancer medications[2] and the emergence of drug-resistance[3,4] highlight the need for the development of novel anticancer agents.

At the time of diagnosis, metastases to regional lymph nodes or distant regions are detected in a significant proportion of cancer patients, ranging from 20% to as high as 80% depending on cancer types.[5] Metastasis is a complex process which relies on several key steps including (1) enabling migration through an alteration of cell-cell/cell-extracellular matrix (ECM) adhesion, (2) local invasion by proteolysis, (3) intravasation, (4) dissemination through the circulatory systems, (5) extravasation and (6) colonization at secondary sites.[6,7] Given that metastases are often associated with advance stages of cancers and high mortality rate[6], inhibition of this malicious pathway holds great potentials for cancer management.

As natural sources of molecularly-diverse, biologically active compounds, the plant kingdom has been a valuable resource for the discovery and the development of novel biologically active leads.[8] Indeed, many active principles with plant origin have reached clinical trials or been approved by USFDA for the treatment of various diseases including cancer.[9,10] Notably, naturally-derived anticancer agents have been estimated to constitute up to 60% of currently used anticancer drugs.[11] At present, effective, routinely prescribed anticancer agents with plant origin include the vinca alkaloids - vinblastine and vincristine from the Madagascar periwinkle *Catharanthus roseus*, podophyllotoxins - the natural precursor of etoposide from the wild mandrake *Podophyllum peltatum*, irinotecan from *Camptotheca acuminata* and taxol from *Taxus brevifolia*.[10,11]

Comprising over 2000 species, euphorbia is a large group of flowering plants in the botanical family euphorbiaceae or the spurge family. Traditional medicinal records from diverse cultures and modern phytochemical research indicate that euphorbias possess a broad range of attractive pharmacological properties including antimicrobial, anti-inflammatory and anticancer activities.[12-14] *Euphorbia lactea* Haw. is a succulent plant which can be found abundantly throughout Thailand; however, the information of its pharmacological properties is largely limited. A previous report indicates that the latex of *E. lactea* contain tirucallol, a triterpenoid compound with an anti-inflammatory activity.[15] Additionally, the cytotoxic activity of *E. lactea* against hepatocellular carcinoma cell line HepG2 and colorectal cancer cell line HCT116 has been reported.[16] However, to our knowledge, antimetastatic activity of *E. lactea* has not been described.

The aim of this study is to assess anticancer potentials of *E. lactea,* focusing on cytotoxic and anti-migratory activities. Hydroalcoholic crude extract prepared from freshly-cut specimen has been tested for cytotoxic and anti-migratory activities against head and neck cancer cell line HN22 using MTT assay and wound-healing assay, respectively. Cytotoxicity manifested by the extract has been further studied through a cell cycle distribution analysis using flow cytometry.

Materials and methods

Preparation of *E. lactea* hydroalcoholic extract

The specimen (Figure 1) has been identified through a comparison of its morphology with pictures in plant databases such as http://www.eol.org and https://www.cabi.org. All specimen used in the extraction is propagated from cuttings from one mother plant and grown in our greenhouse in Bangkok, Thailand. 849 g of freshly cut stems age 3-6 months were weighted, grounded in 750 ml of 95% ethanol, allowed to stand for 2 days and then filtered through No. 4 Whatman filter paper to remove debris. The collected filtrate was then concentrated using a rotary evaporator at 50 ^{o}C and further dried on a water bath at 60 ^{o}C. Afterward, dried extract was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 100 mg/ml and store at -20 ^{o}C.

Figure 1 Photograph of *E. lactea* Haw. Live *E. lactea* Haw. plant specimen propagated from cutting and grown in our greenhouse (Bangkok, Thailand).

Cell culture

HN22 cells were kindly gifted by Professor Praneet Opanasopit, Faculty of Pharmacy, Silpakorn University. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Waltham, MA, USA) supplemented with 10% FBS (Gibco), 1% GlutaMAX (Gibco), 100 units/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). Cells were incubated at 37 ^{o}C in a humidified atmosphere with 5% CO_2.

Evaluation of cytotoxic effects by MTT assay

$1x10^4$ cells/well were seeded onto 96-well plate. Varying concentrations of *E. lactea* extract were added to the culture media. Cells were then allowed to expose to the extract for 16, 48 or 72 h. 0.5% DMSO was used as vehicle control. 5 µg/ml oxaliplatin (TTY Biopharm, Taipei, Taiwan), an anticancer agent, was used as positive control. Afterward, cells were washed with phosphate buffer saline (PBS) solution then incubated with 1 mg/ml thiazolyl blue tetrazolium bromide (Sigma-Aldrich, St. Louis, MO, USA) for 4 h. To dissolve formazan crystals, 100 µl of DMSO were added to each well and mixed. Then, absorbance at 550 nm was measured using a microplate reader (A Packard bioscience). All experiments were conducted in triplicate. The results were presented in chart as %viability normalized to control group. One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test was performed for statistical analysis using the scientific statistic software Graphpad Prism version 5 (GraphPad Software Inc., La Jolla, CA). $p < 0.05$ was considered statistically significant.

Cell cycle analysis by flow cytometry

Cells were treated with 500µg/ml of the extracts or 0.5% DMSO vehicle control for 48 h, then washed, harvested and fixed with 70% ice-cold ethanol. After fixation, cells were washed twice with ice-cold PBS and treated with 100 µg/ml of DNase-free RNase A (Bio Basic, Amherst, NY, USA) in PBS

containing 0.1% v/v Triton-X 100 (Sigma-Aldrich, St. Louis, MO, USA) for 5 minutes at room temperature. Cells were then stained with 20 µg/ml propidium iodide (Life technologies, Carlsbad, CA, USA) in PBS containing 0.1% v/v Triton-X 100 for 15 min at room temperature while protected from light. Cell cycle distribution was acquired with a flow cytometer (Facscanto, BD biosciences, San Jose, CA, USA). Data were analyzed using ModFitLT V3.0 software (BD biosciences). All experiments were done in triplicate. One-way ANOVA followed by Dunnett's multiple comparison test was performed for statistical analysis using the scientific statistical software Graphpad Prism version 5 (GraphPad Software Inc., La Jolla, CA). $p < 0.05$ was considered statistically significant.

Wound-healing assay

HN22 cells were plated onto a 6-well plate and allowed to grow into a confluent monolayer. Cells were then removed in three linear scratches using a p200 pipette tip and washed with PBS to remove detached cells. Fresh media containing varying concentrations of *E. lactea* extract or DMSO vehicle control were then added. Afterward, cells were incubated for 16 h. Pictures of cells at 0- and 16-hour were captured from 5 different microscopic fields. Surface areas of the wound occupied by migrated cells were analyzed using ImageJ.[17] All experiments were performed in triplicate. The relative area presented in chart is the wound area occupied by migrated cells of HN22 cells treated with *E. lactea* extract normalized to DMSO-treated control group. One-way ANOVA followed by Dunnett's multiple comparison test was performed for statistical analysis using the scientific statistic software Graphpad Prism version 5 (GraphPad Software Inc., La Jolla, CA). $p < 0.05$ was considered statistically significant.

Results

Hydroalcoholic extract of *E. lactea* (EL) exhibited a dose-dependent and statistically significant cytotoxic activity against head and neck cancer cell line HN22 exposed to the extract for 48 and 72 h (Figure2, A and B, respectively). The viability of HN22 was reduced by over 50% when treated with 500 µg/ml of the extract for 72 h, indicating that the IC_{50} of the extract is within the range of 250-500 µg/ml (Figure 2A), at least in this treatment condition. To further explore the cytotoxic activity of EL, cell cycle distribution of EL-treated HN22 was analyzed by flow cytometry (Figure 2C). Although the percentage of cells in G0/G1, S and G2/M phases were comparable to that of the control group, a significant increase in sub-G1 population was detected in HN22 treated with EL (Figure 2D, boxed), suggesting that the cytotoxic effect mediated by EL may be attributable to the induction of apoptosis in this cell line.

Next, we employed a wound-healing assay to evaluate the effect of EL against the *in vitro* migration of cancer cells using HN22 as a model. At 16-hour after cell removal, the cell-free areas of HN22 monolayer were almost completely closed (Figure 3A). Treatment of HN22 cells with EL resulted in a marked decrease in the wound-closure rate compared to the vehicle control (Figure 3A) which reflected a suppression of migration. Quantitation of the wound area indicated that treatment of HN22 with 12.5-100 µg/ml of EL resulted in a statistically significant decrease in the migration of HN22 cells compared to the control group (Figure 3B). Maximal inhibition of migration (approximately 40%) was detected at 25 µg/ml of EL. Notably, the extract within this concentration range and treatment time did not elicit any detectable change in the viability of HN22 cells (Figure 3C).

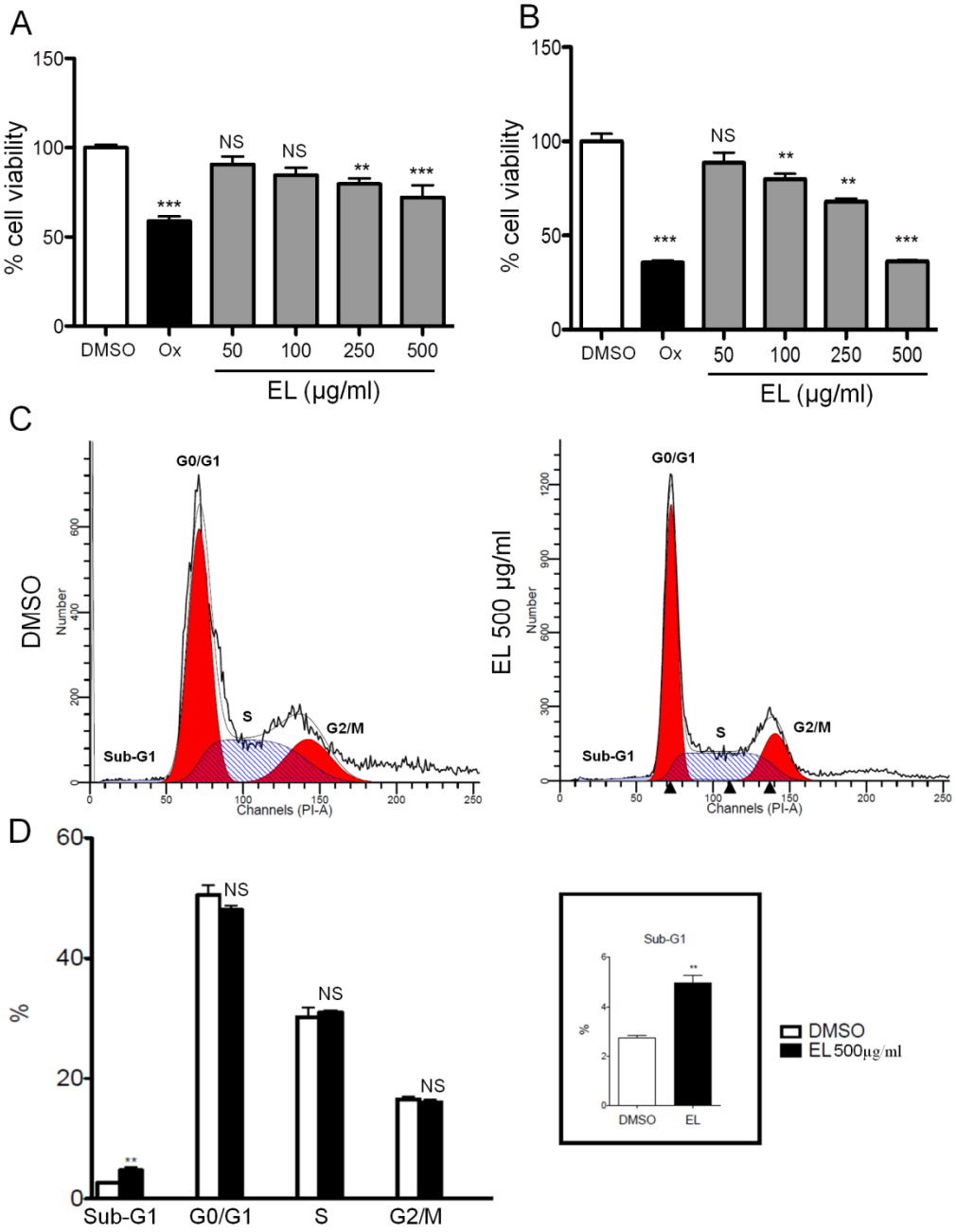

Figure 2 Cytotoxic activity and effects on cell cycle distribution of *E. lactea* hydroalcoholic extract. Representative MTT assay from three biological replicates of HN22 cells treated with various concentrations of *E. lactea* hydroalcoholic extract (EL), 0.5% DMSO as a vehicle control, or 5 µg/ml oxaliplatin (Ox) as a positive control for 48 (A) or 72 h (B). (C) A representative cell cycle distribution of HN22 cells treated with 500 µg/ml of *E. lactea* hydroalcoholic extract or 0.5% DMSO vehicle control for 48 h. (D) Quantitation of cells from (C) in sub-G1, G0/G1, S and G2/M phase of the cell cycle. All MTT and flow cytometry experiments were performed in triplicate. Bars represent average ± SD. ***, $p < 0.001$ versus control group. **, $p < 0.01$ versus control group. NS, $p > 0.05$ versus control group.

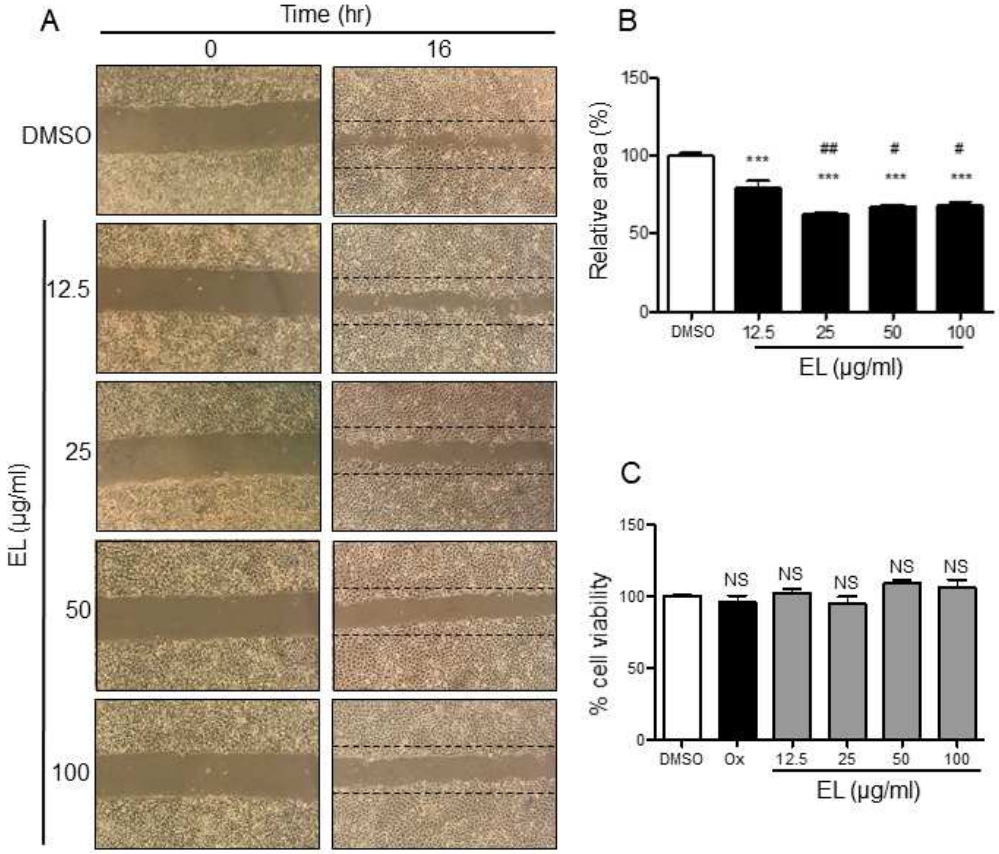

Figure 3 *E. lactea* hydroalcoholic extract inhibited migration of HN22 cells. (A) Representative images of wound-healing assay. HN22 cells were treated with varying concentrations of *E. lactea* hydroalcoholic extract (EL) or vehicle control (0.5% DMSO). Pictures were taken at 0- and 16-h after treatment. (B) Quantitation of the wound area occupied by migrated cells at 16-hour after treatment from (A) as described in materials and methods. The average values depicted were calculated from 5 different microscopic fields. Bars represent average ± SD. ***, $p < 0.001$ versus the control group. ##, $p < 0.01$ versus the group treated with 12.5 µg/ml of EL. #, $p < 0.05$ versus the group treated with 12.5 µg/ml of EL. (C) MTT assay of HN22 treated with varying concentrations of EL, the vehicle control (0.5% DMSO) or 5 µg/ml oxaliplatin (Ox) at 16-hour revealed no significant cytotoxic effect. NS, $p > 0.05$ versus control group.

Discussions

Our result indicates that hydroalcoholic extract of *E. lactea* exhibits cytotoxic activity against head and neck cancer cell line HN22. Although the cytotoxic effect of *E. lactea* against head and neck cancer cell line HN22 has not been previously described, other group has independently reported antiproliferative activity of *E. lactea* against hepatocellular carcinoma cell line HepG2 and colorectal cancer cell line HCT116.[16] Additionally, *in vitro* cytotoxic or antiproliferative activities against head and neck cancer[18] and cancers of other organs including ovarian, breast, cervix, prostate, liver, stomach, lung, pancreas[19-27] have been detected in other euphorbia species e.g. *E. turcomanica*[28], *E. pekinensis*[19], *E. triaculeata*[24], *E. lunulata*[25], *E. lathyris*[26], *E. tirucalli*[18,27], *E. kansui*[20] and *E. supina*.[21] Together, our data suggests that *E. lactea* likely

possesses cytotoxic activity shared among euphorbias.

To further explore the underlying mechanism of cytotoxic effects mediated by *E. lactea,* cell cycle distribution of HN22 treated with the extracts was analyzed by flow cytometry. In such an assay, DNA staining fluorochromes e.g. propidium iodide are added to the cells, which enables a classification of cells into major stages of the cell cycle i.e. G0/G1, S and G2/M based on their DNA content. An altered cell cycle distribution generally indicates a blockade of the cell cycle, while an increase in sub-G1 population indicates the involvement of apoptosis due to the process-related degradation and subsequent loss of genomic DNA which diminish the stainability of DNA specific dyes.[29] HN22 cells treated with EL exhibited an increase in sub-G1 population, suggesting that the extract likely possesses a pro-apoptotic activity. In line with our findings, extracts and purified compounds from other euphorbia species, such as n-hexane extract of *E. lunulata*[30], a tetrahydroingenol diterpene DANPT from *E. erythradenia*[31], 13-oxyingenol dodecanoate (13OD) from *E. kansui*[32], lathyrane diterpenoids from the seeds of *E. lathyris*[26,33], etc. have been reported to induce apoptosis in diverse types of cancer cell lines.

Migration or cell motility is an important characteristic of metastatic cancer cells which can be monitored *in vitro* through several established tests including the wound-healing assay[34] which is employed in our study. Our result suggests that *E. lactea* exhibits an inhibitory effect against the migration of HN22 cells. To our knowledge, our data provide unprecedented evidence in support of the anti-migratory activity of *E. lactea.* Moreover, no cytotoxic effect was detected at the concentration range and the time point that the anti-migratory effects were observed, thus the interference from growth inhibition was expected to be negligible. In line with our result, previous reports by other investigators have documented anti-migratory and anti-invasive activities of other euphorbias including ethyl acetate extract from *E. humifusa*[35], polyphenols from *E. supina*[36] and a terpenoid compound Jolkinolide B from *E. fischeriana*.[37,38]

Exhibiting dual attractive properties for cancer treatment i.e. anti-migratory activity at low concentrations and cytotoxic activity at high concentrations, our results pinpoint *E. lactea* as a promising candidate medicinal plant whose anticancer potentials await further evaluation. Future research should aim to explore whether these effects of *E. lactea* are extended toward other cancer types. For anti-migratory activity, other cancer cell lines with high migratory capabilities such as lung cancer cell line A549 or breast cancer cell line MDA-MB-231 may be used as potential cell line models. Additionally, our ongoing research is underway to validate and characterize underlying mechanisms of the pro-apoptotic and antimetastatic attributes of *E. lactea*. In summary, our present data provide a fundamental insight into the potential pro-apoptotic and anti-migratory activity of *E. lactea* against head and neck squamous cell carcinoma cell line HN22 and warrant future research for the characterization of anticancer potentials of this abundant succulent plant of Thailand.

Conclusion

Here, we report cytotoxic and anti-migratory activities of hydroalcoholic extract from *E. lactea* against head and neck squamous cell carcinoma cell line HN22. MTT assay revealed that *E. lactea* extract dose-dependently inhibit the viability of HN22 cells, which was accompanied by an increase in sub-G1 population suggesting that *E. lactea* extract likely exhibited a pro-apoptotic activity. Together, our results revealed a fundamental data in support of the pro-apoptotic and unprecedented anti-migratory properties of *E. lactea* and warranted future research to further explore the anticancer potentials of this endemic flora of Thailand.

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