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Original Research Article

Aerial Parts of *Selaginella doederleinii* Hieron as an Anticancer Agent against Luminal A Breast Cancer (T47D) Cell Line

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ABSTRACT

Luminal A is the most widely diagnosed breast cancer subtype worldwide. The existing luminal A therapy faces some challenges, such as drug resistance and toxicity. Therefore, new therapeutic approaches are urgently required. The application of medicinal plants, particularly aerial parts of *Selaginella doederleinii* Hieron, becomes an attempting strategy due to its abundant bioactive compounds that can target numerous signaling pathways. Therefore, this research aimed to investigate the anticancer potential and mechanism of *S. doederleinii* extract and fractions on luminal A breast cancer (T47D) cells. WST-1 assay was used to examine the cytotoxicity of the ethanolic extract and several fractions of *S. doederleinii*. Apoptosis assay, cell cycle assay, aerobic glycolysis inhibition assays, and analysis of oncogenic protein expression were conducted to investigate the anticancer effect of the most selective fraction. This study demonstrated that the ethyl acetate (EA) fraction exhibited the strongest cytotoxicity and selectivity against the T47D cells (IC_{50}= 19.31 µg/mL; Selectivity Index (SI) = 18.94). EA fraction significantly induced apoptosis, impeded the cell cycle, and diminished glucose consumption and lactate secretion, mostly starting at the concentration 2xIC_{50}in 48 h. The EA fraction also significantly downregulated the expression of p-mTOR, c-Myc, and Hexokinase 2 (HK2) proteins in modulating apoptosis, cell cycle, and aerobic glycolysis. However, HIF-1α inhibition might also contribute to the anticancer activity of the EA fraction. In conclusion, the ethyl acetate fraction of *S. doederleinii* exerted considerable anticancer effects against T47D cells. The fraction can be further developed to cure luminal A patients through more intensive studies.
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Keywords: *Anticancer, breast cancer, luminal A, Selaginella doederleinii* Hieron, *T47D cell line.*

Introduction

Breast cancer has the highest global incidence and become one of the biggest contributors to female mortality.[1,2] In 2040, the total cases of this cancer are predicted to rise in both developed and developing countries.[2,3] Breast cancer is currently being managed using various methods, including surgery, radiation, chemotherapy, and hormone therapy, depending on the subtype.[4,5] Luminal A, determined by high estrogen receptor (ER) and progesterone receptor (PR) expressions with low HER2 expression (ER+/PR+/HER2-), is the most frequently diagnosed breast cancer subtype worldwide, approximately 70% of all cases.[6,7] This subtype is particularly managed by hormone therapy to inhibit ER activation.[8] However, ER in luminal A breast cancer can undergo some alterations, making the cancer cells resistant and the therapy no longer efficacious.[9,10] Therefore, some strategies for enhancing luminal A breast cancer treatment efficacy are mandatory.

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Over recent years, many researchers have been trying to explore medicinal plants containing abundant natural compounds to fight cancers. Natural compounds can potentially help to solve cancer therapy problems due to their advantageous properties, including targeting numerous signaling pathways, possessing many pharmacological effects, and diminishing the toxicity effects of drugs.[11] Medicinal plants can be used to treat luminal A breast cancer as a complementary therapy and targeted therapy.[12]

Selaginella doederleinii Hieron is a medicinal plant broadly distributed in some regions of Asia, specifically China, South Korea, and several Southeast Asian countries.[13] Biflavonoids, lignans, terpenes, polysaccharides, and other compounds from this plant possess anticancer, antidiabetic, antioxidant, and antibacterial properties.[14] This plant extract has also been studied for its anticancer activities against CNE nasopharyngeal cancer, HT-29 colorectal cancer, HepG2 liver cancer cells, and PC9 lung cancer cells.[15–17] However, there are very few studies on the anticancer activity of *S. doederleinii* extract against luminal A breast cancer. Anggraini *et al.* (2023) studied the cytotoxicity activity of *S. doederleinii* ethanol extract on luminal A breast cancer (MCF-7) cells (IC_{50}= 215 µg/mL).[18] Nevertheless, the potency and mechanism of actions of S. *doederleinii* extract and fractions against luminal A breast cancer have not been thoroughly investigated.[18] Therefore, this research aimed to comprehensively analyze the anticancer potency and mechanism of actions of *S. doederleinii* extract and fractions on luminal A breast cancer (T47D) cells.

Materials and Methods

Plant material

Aerial parts of *S. doederleinii* were collected, identified, and authenticated by UPT Laboratorium Herbal Materia Medica, Batu, Indonesia, in September 2020 (reference number: 074/695/102.20-A/2022). The dried powder of this plant used for this research was also purchased from the institution.

Preparation of S. doederleinii extract and fractions

S. doederleinii powder (500 grams) was extracted using the methods described by Brad *et al.* (2017) and Djati *et al.* (2021) with modifications. Briefly, the powder was macerated with 95% ethanol (1:10 ratio) for 24 h.[19,20] The mixture was filtrated using Whatman filter papers (Whatman Ltd., England) and evaporated using a rotatory evaporator at 50°C in vacuo. These maceration procedures were conducted twice. The liquid-liquid extraction method used a separatory funnel to obtain fractions. Partitioning was conducted using the method described by Abu *et al*. (2017) with some modifications.[21] Briefly, the ethanol extract *of S. doederleinii* was dissolved in warm distilled water with a ratio of 1: 20 (w/v). The ethanol extract was partitioned using n-hexane and ethyl acetate sequentially. Firstly, the ethanol extract was partitioned using n-hexane with a ratio of 20:20 (v/v), shaken vigorously, and allowed to separate into two layers (the n-hexane and the aqueous layers). This procedure was replicated three times to obtain the total n-hexane layer. Then, the water layer was partitioned using ethyl acetate with a 1:1 (v/v) ratio, shaken vigorously, and separated into the ethyl acetate and water layers. The procedure was repeated thrice to collect the total ethyl acetate layer. Subsequently, each of the total ethyl acetate (EA) fraction, the n-hexane (HEX) fraction, and the distilled water (AQ) fraction was evaporated in vacuo using a rotatory evaporator at 50°C before being properly stored and used for further experiments.[22]

Cell culture

The T47D human breast cancer and the TIG-1 human fetal lung fibroblast cell lines were acquired from the Faculty of Medicine, Brawijaya University, Indonesia, and the Japanese Collection of Research Bioresources (JCRB) Cell Bank, Japan, respectively. The T47D cell line was cultivated in the RPMI complete medium, comprising RPMI-1640 medium, 10% FetalBovine Serum (FBS), and 1% Penicillin-Streptomycin (Gibco™, Thermo Fisher Scientific, USA). Meanwhile, the TIG-1 cell line was seeded in the MEM complete medium, containing MEM medium, 10% FBS, and 1% Penicillin-Streptomycin (Gibco™, Thermo Fisher Scientific, USA). Each cell line was incubated at 37°C in a humidified incubator containing 5% CO_2.[23]

Cytotoxicity test

Before being used for the anticancer assays, *S. doederleinii* extract or fraction was dissolved in dimethyl sulfoxide (DMSO) (Supelco, Merck, Germany). Meanwhile, the T47D and TIG-1 cells were cultured in 96-well plates (NEST®, NEST Scientific, USA) at 7.5×10^3 cells/well and 10.0×10^3 cells/well, respectively, and incubated for 24 h. After discarding the culture medium, the different concentrations of extract or fraction dissolved in the new complete medium were immediately given to the cells and incubated for 24 h. Subsequently, the WST-1 reagent (Roche Diagnostics GmbH, Germany) in 100 µL of the new medium was introduced to the cells/well.[24] Then, the plate was incubated for 30 min, and the absorbance was detected at 490 nm. After calculating % cell viability from the absorbance data, the IC_{50} values were determined from the linear regression equation between log (extract concentration) and % cell viability.[25] The IC_{50} value of an extract or a fraction against TIG-1 cells was divided by those of T47D to find the selectivity index (SI).[26]

Apoptosis assay

The T47D cell line was cultured in 24-well plates for 24 h. Cisplatin (PT. Ferron Par Pharmaceuticals, Indonesia) or *S. doederleinii* fraction (IC_{50}, $2xIC_{50}$, and $4xIC_{50}$) in 1 mL of complete RPMI medium was introduced to the cells for 24, 48, or 72 h. Cisplatin, a chemotherapy agent, was used for positive control in this study. The treatment medium was collected into the 15 mL Eppendorf tube (NEST®, NEST Scientific, USA). Meanwhile, cells were immediately harvested using trypsin EDTA (Gibco™, Thermo Fisher Scientific, USA) and transferred to the Eppendorf tube containing their medium. Subsequently, the cell mixture was centrifuged at 2,500 rpm for 5 min at 10°C to remove the medium. After washing with phosphate buffer saline/PBS (Biowest, USA), the cells were incubated with FITC Annexin V/Propidium Iodide (PI) antibody (BioLegend™, San Diego, USA) for 20 min in the dark.[27] Then, the cells that had been dissolved in PBS were used for apoptosis analysis using a flow cytometer (BD FACSCalibur™, CA, USA) and BD CellQuest Pro Software (BD Biosciences, CA, USA). Furthermore, the inverted microscope (Olympus, PA, USA) was utilized to morphologically observe the T47D cells at 200 x magnification.[28]

Cell cycle assay

After harvesting T47D cells that had been introduced by *S. doederleinii* fraction for 24, 48, and 72 h, both cells and their treatment medium were collected into a 15 mL Eppendorf tube and separated by centrifugation. Cell cycle assay was conducted using the method by Pumiputavon *et al*. (2017) with modifications.[29] The cells were fixed using 70% ethanol dropwise, mildly vortexed, and incubated in an ice box for 30 min. After eliminating the ethanol and washing with PBS twice, the cells were incubated with PI for 15 min in the dark. The cells that had been dissolved in the PBS were analyzed with flow cytometry.

Detection of relative glucose consumption and lactate secretion

T47D cell treatment media before and after the introduction of *S. doederleinii* fraction to the cells for 24 and 48 h, were centrifuged and diluted using NaCl to be applied as samples. Glucose Colorimetric Assay Kit E-BC-K234-S (Elabscience, Wuhan, China) was used to measure glucose concentrations that could be detected at 505 nm.[30] Meanwhile, Lactic Acid Colorimetric Assay Kit E-BC-K044-S (Elabscience, Wuhan, China) was utilized to determine lactate concentrations that could be detected at 530 nm.[30] The concentration data were normalized with the number of cells. The relative glucose consumption and lactate secretion of the cells were displayed on the diagrams.

Analysis of oncogenic proteins (p-mTOR, c-Myc, and HK2) expression levels

After isolating the total T47D cells that had been introduced with *S. doederleinii* fraction for 24 and 48 h, Intracellular Fixation Buffer (Invitrogen, Scientific, USA) was given to the cells for 20 min in an icebox. Then, the mixture was resuspended in the Permeabilization Buffer (Invitrogen, Scientific, USA). After centrifugation, the total cells were incubated with antibodies for p-mTOR, c-Myc, or Hexokinase 2 (HK2): PE-Phospho-mTOR, Pierce c-Myc Mouse Monoclonal conjugated with Fluorescein Goat Anti-Mou, or Anti-HK2 monoclonal antibody conjugated with Fluorescein Goat Anti-Mou (Thermo Fisher Scientific, USA), in PBS for 20 min in the dark.[31] The analysis of protein expression levels was accomplished using flow cytometry after dissolving the cells in PBS.

Statistical analysis

The two-way ANOVA test in the IBM SPSS Statistics 26 software (IBM Corp., New York) was applied for data statistical analysis. If $p < 0.05$, the data was indicated as statistically significant and further analyzed using Duncan's Pos-Hoc test.

Results and Discussion

Cytotoxicity and Selectivity of S. doederleinii against T47D Cell Line

The WST-1 cytotoxicity test was used to determine the cytotoxic effect of *S. doederleinii* extract/fraction on T47D breast cancer cells and calculate its selectivity index (SI). The selectivity index was obtained from the ratio of the IC_{50} value of normal cells to those of cancer cells.[26] In this study, human fetal lung fibroblast (TIG-1) cells were used to represent normal cells. An extract/fraction with an SI

value of more than 3 is considered very selective in killing cancer cells without promoting any toxicity to normal cells.[26]

The WST-1 cytotoxicity test results demonstrated that the higher the *S. doederleinii* extract and fraction concentration, the lower the viability of T47D cancer and TIG-1 normal cells (Figure 1). The concentrations of the ethanol extract, n-hexane (HEX) fraction, ethyl acetate (EA) fraction, and aqueous (AQ) fraction in killing 50% of both cell populations (IC_{50}) were displayed in Table 1. EA fraction had the lowest IC_{50} value on the T47D cells (19.31 µg/mL), but the highest IC_{50} value on the TIG-1 cells (365.67 µg/mL). The lower the IC_{50} value, the more robust the cytotoxicity effect of a fraction. The selectivity index (SI) demonstrated that the EA fraction was the only fraction exerting a high selectivity in killing the T47D cells (S>3).[26] Meanwhile, the ethanol extract and other fractions did not selectively kill the T47D cancer cells. The previous study also showed that the EA fraction of *S. doederleinii* demonstrated a strong cytotoxic effect on some cancer cells, including HL60 leukemia cells, A549 lung cancer cells, and CNE2 nasopharyngeal carcinoma cells, as compared to other fractions.[32] Additionally, some studies confirmed that the EA fraction of *S. doederleinii* did not demonstrate any toxicity in healthy mice and Vero normal monkey kidney cells.[16,33] A prior investigation revealed that the EA fraction of *S. doederleinii* contained numerous biflavonoid compounds, the dimers of flavonoids possessing many health benefits, including anticancer and antioxidant.[34] Therefore, bioflavonoids in the EA fraction of *S. doederleinii* might contribute to its selective cytotoxic effect on T47D cells and its protective effect on TIG-1 cells. However, biflavonoids are not the only compounds from the EA fraction of *S. doederleinii* showing anticancer properties. Other bioactive compounds in the EA fraction of *S. doederleinii* also showed anticancer activities, such as ferulic acid and vanillic acid.[19,35,36] Therefore, this study revealed that the EA fraction of *S. doederleinii* could specifically kill the T47D cancer cells without causing any toxicity to TIG-1 normal cells. The EA fraction of *S. doederleinii* was further examined using various assays.

Table 1: IC_{50}values and selectivity indexes of *S. doederleinii* ethanol extract and fractions

Sample	IC_{50} in T47D (µg/mL)	IC_{50} in TIG-1 (µg/mL)	Selectivity Index (SI)
Ethanol extract	170.87 ± 2.85	218.20 ± 2.96	1.28
Ethyl acetate (EA) fraction	19.31 ± 0.95	365.67 ± 2.85	18.94
n-Hexane (HEX) fraction	93.46 ± 0.59	157.33 ± 0.22	1.68
Aqueous (AQ) fraction	347.42 ± 0.95	148.06 ± 1.60	0.43

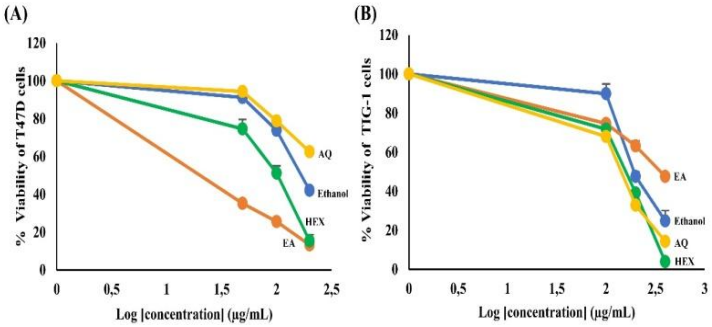

Figure 1: The ethanol extract, ethyl acetate (EA), n-hexane (HEX), and aqueous (AQ) fractions of *S. doederleinii* suppressed the (A) T47D and (B) TIG-1 cells viability in 24 h. Values shown are mean ± standard deviation (SD) (*n* = 3).

Apoptosis induction in EA fraction-treated T47D cells

Early apoptosis is generally identified from the presence of phosphatidylserine on the cell surface, which can only interact with Annexin V, whereas late apoptosis is distinguished by the occurrence of DNA fragmentation, which can interact with Annexin V and PI. The treatment with $2xIC_{50}$ and $4xIC_{50}$ of EA fraction for 48 h significantly enhanced early apoptotic cells, with percentages of 20.78% and 24.48%, respectively (p<0.05). Both of them also markedly reduced live cells after 48 h, with percentages of 50.59% and 52.93%, respectively (Figures 2A and 2B). Interestingly, both treatment groups did not demonstrate any significant difference from each other. After that, the early apoptotic cell percentages rose, while the live cell percentages dropped at 72 h. Furthermore, both treatment groups significantly enhanced late apoptosis (26.05% and 20.31%), but did not markedly promote necrosis/cell debris (2.58% and 2.28%) in T47D cells. Both treatment groups did not exhibit any significant difference from each other and did not show any notable change at 72 h. Both treatment groups exhibited lower early apoptotic cell percentages than cisplatin but higher late apoptotic cell percentages. Meanwhile, the microscopic observation revealed that untreated T47D cells demonstrated normal morphological characteristics and excessive confluency (Figure 2C).

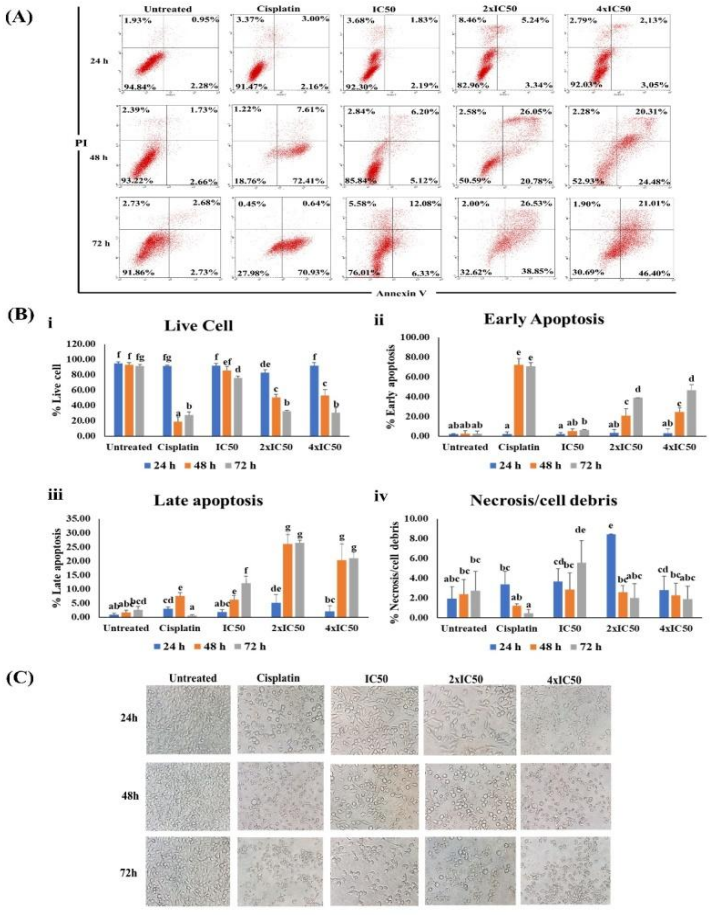

Figure 2: The EA fraction of *S. doederleinii* (IC_{50}, $2xIC_{50}$, $4xIC_{50}$) could stimulate apoptosis in the T47D cells after 24, 48, and 72h. (A) Dot plots and (B) diagrams presented the cell populations and percentages of four classifications, respectively: live cells (bottom-left quadrant), early apoptosis (bottom-right quadrant), late apoptosis (top-right quadrant), and necrosis/debris cell (top-left quadrant). The displayed values are mean ± SD (n = 3). (C) Morphological appearance of *S. doederleinii* - induced T47D cells observed using an inverted microscope.

EA fraction and cisplatin could promote some characteristics of apoptotic cells, such as rounded shape, swelled, low confluency level, membrane blebbing, apoptotic bodies, and floating cells, which could be observed more clearly as concentration and time increased. Therefore, these findings indicated that EA fraction could markedly trigger total apoptosis in T47D cells, starting at the dose of $2xIC_{50}$ in 48 h. The previous investigation reported that the EA fraction of *S. doederleinii* could also stimulate apoptosis in the colorectal cancer cells by impeding the PI3K/Akt/mTOR.[37] Additionally, the EA fraction of *S. doederleinii* could trigger apoptosis in A549 lung cancer cells by increasing the caspase expression level and decreasing the Bcl2 expression level.[33]

Cell cycle inhibition in EA fraction-treated T47D cells

Many anticancer drugs can block the cancer cell cycle at the sub-G1, G0/G1, S, or G2/M stage to hinder proliferation.[38] This research revealed that the treatment with $2xIC_{50}$ and $4xIC_{50}$ of EA fraction for 48 and 72 h significantly enhanced the sub-G1 cells ($p<0.05$) (Figure 3). The percentages of sub-G1 cells after treatment with $2xIC_{50}$ of EA fraction for 48 and 72 h were 29.45% and 66.64%, while the percentages after treatment with $4xIC_{50}$ of EA fraction for 48 and 72 h were 39.08% and 68.63%, respectively. This finding was consistent with the apoptosis assay result, in which these treatment groups demonstrated a high number of total apoptotic cells. Therefore, these treatment groups promoted sub-G1 arrest in T47D cells. Sub-G1 arrest often indicates that the cells have undergone apoptosis.[39] Furthermore, the induction of EA fraction to the cells for 24 h could significantly stimulate G2/M arrest as the concentrations increased ($p<0.05$).

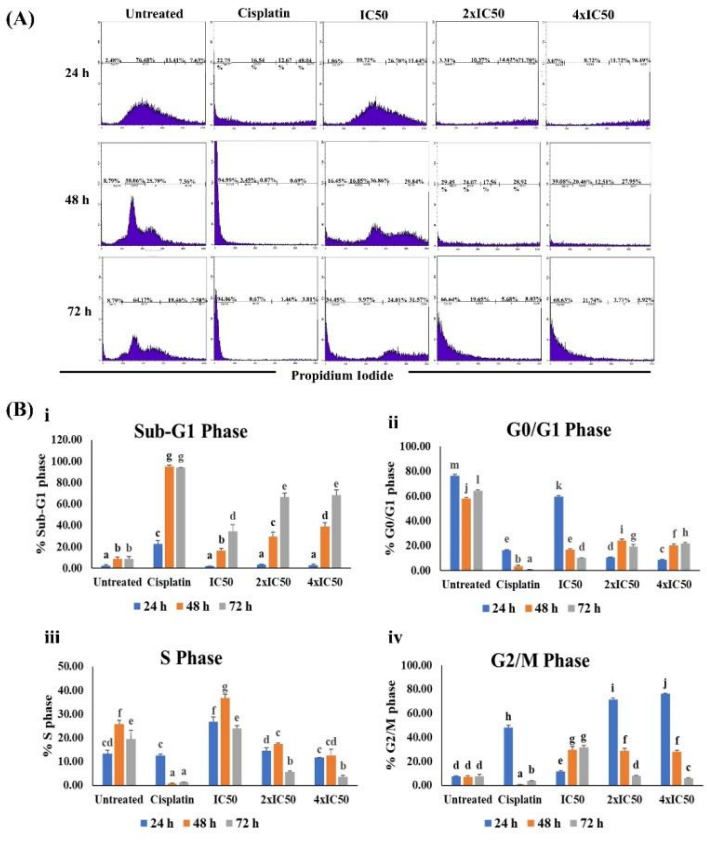

Figure 3: The EA fraction of *S. doederleinii* (IC_{50}, $2xIC_{50}$, $4xIC_{50}$) halted the cell cycle of T47D cells. (A) Histograms and (B) graphs displayed the cell cycle stage distributions and percentages, respectively: sub-G1, G0/G1, S, and G2/M (from left to right) after 24, 48, and 72 h. The values are provided as mean ± SD (n = 3).

The treatment with $4xIC_{50}$ of EA fraction for 24 h promoted the highest percentage of G2/M cells (76.49%). The G2/M arrest hinders the cells containing DNA breaks from entering the mitotic phase. The G2/M arrest also indicates that DNA break repair is hard to achieve.[39] Many anticancer drugs were reported to suppress the cancer cell cycle by targeting the G2/M and S phases.[33] Meanwhile, cisplatin also exhibited the G2/M arrest after 24 h (48.04%), followed by sub-G1 arrest after 48 and 72h (94.99% and 94.06%). These results concluded that the EA fraction halted the cell cycle at sub-G1 and G2/M in T47D cells, depending on its concentration and incubation time. A prior study also showed that the EA fraction of *S. doederleinii* could arrest colorectal cancer (HCT116) cells at the G2/M phase.[37]

Relative glucose consumption and lactate secretion of EA fraction-treated T47D cells

Besides identifying apoptosis promotion and cell cycle inhibition activities, we also studied the anti-aerobic glycolysis effect of *S. doederleinii* fraction. Aerobic glycolysis, frequently referred to as the Warburg effect, is a metabolic reprogramming applied by cancer cells to fulfill their major nutrient needs, i.e. glucose, for supporting their unlimited growth and proliferation through elevating glucose consumption, glycolysis rate, and lactate secretion levels under both aerobic and anaerobic conditions.[40] Additionally, aerobic glycolysis can induce apoptosis and suppress the cell cycle.[41] Hindering aerobic glycolysis also potentially lowers cancer cells' resistance to conventional cancer treatments.[42] Therefore, aerobic glycolysis is an attractive therapeutic target in cancer. The aerobic glycolysis inhibition of the EA fraction was identified by measuring relative glucose consumption and lactate secretion levels using colorimetric assays. EA fraction markedly reduced the relative glucose consumption of T47D cells as the time and concentration increased ($p<0.05$) (Figure 4A). The $2xIC_{50}$ and $4xIC_{50}$ of fraction A for 48 h promoted the lowest relative glucose consumption (0.15 and 0.17). Both treatment groups did not exert any significant difference from each other. Moreover, the EA fraction notably decreased the relative lactate secretion of T47D cells ($p<0.05$) (Figure 4B). The variety of incubation times did not significantly affect the relative lactate secretion. Treatments with $4xIC_{50}$ and $2xIC_{50}$ of EA fraction induced the lowest relative lactate secretion of T47D cells in 24 and 48 h. Therefore, this research concluded that EA fraction could inhibit cancer aerobic glycolysis in T47D cells by decreasing relative glucose consumption and lactate secretion. The previous molecular docking study confirmed that the activity of some aerobic glycolysis proteins (HK2, GLUT1, and LDHA) could potentially be blocked by several compounds in EA fraction of *S. doederleinii*, particularly diosgenin, frutinone A, vitexin, 5-hydroxyferulic acid methyl ester, and valeroidine.[43]

Surprisingly, this study revealed that cisplatin could increase relative glucose consumption levels after 48 h. The promotion of glucose consumption and aerobic glycolysis rate can induce intrinsic and innate cisplatin resistance in some cancer cells.[44]

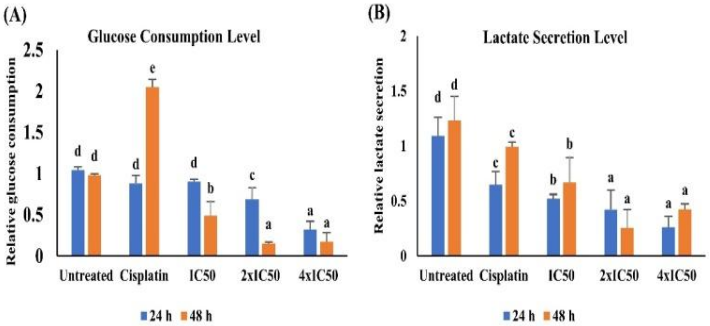

Figure 4: The EA fraction of *S. doederleinii* (IC_{50}, $2xIC_{50}$, $4xIC_{50}$) reduced (A) relative glucose consumption and (B) relative lactate secretion of T47D cells after 24 and 48 h. The values are depicted as mean ± SD (n = 3).

Many studies reported that natural products could overcome cancer resistance through different mechanisms of action, including by suppressing the aerobic glycolysis pathway.[45] Therefore, EA fraction is predicted to diminish cisplatin resistance in T47D cells via the aerobic glycolysis pathway due to its ability to hinder glucose consumption and lactate secretion of T47D cells in this study. More studies are required to verify this hypothesis.

The aerobic glycolysis inhibition by EA fraction might associated with apoptosis induction and cell cycle suppression in this study. Modulating glucose metabolism in cancer using drugs or biological compounds was found to stimulate intrinsic and extrinsic apoptosis by inducing ATP depletion and sensitivity to the death receptor.[46] Additionally, aerobic glycolysis suppression leads cancer cells to depend on oxidative phosphorylation (OXPHOS), producing excessive amounts of Reactive Oxygen Species (ROS), which can activate protumor actions.[47] However, a much greater concentration of ROS could promote anti-tumor activities by triggering mitochondrial pores oxidation and oxidative stress-induced cell death.[47] Additionally, GADPH and PKM2 aerobic glycolysis enzymes may stimulate the cancer cell cycle by inducing cyclin B/CDK1 complex and mediating cyclin D1 expression, respectively.[48]

Oncogenic protein expression levels in EA fraction-treated T47D cells

In this study, we investigated the expression of several oncogenic proteins regulating apoptosis, cell cycle, and aerobic glycolysis i.e., mTOR, c-Myc, and Hexokinase 2 (HK2). mTOR is the central regulator of the growth and metabolism in cancer cells, which can activate c-Myc and HIF1α transcription factors through its effector proteins, such as 4EBP1 and P70S6 kinase.[49] Activated c-Myc interacts with Max to form a dimer that can bind to the promoter of genes responsible for several cancer mechanisms, including apoptosis, cell cycle, and aerobic glycolysis.[49,50] In cancer, the mTOR/c-Myc pathway regulates apoptosis and cell cycle by triggering the Bcl2 anti-apoptotic protein expression and the CDKs/cyclins activation, respectively.[51,52] Additionally, the pathway also modulates HK2, a glycolytic enzyme that not only contributes to aerobic glycolysis but also apoptosis inhibition in cancer cells.[53] The expression levels of these oncogenic proteins in T47D cells after the EA fraction treatment were studied using antibody staining and flow cytometry.

The EA fraction significantly hindered the p-mTOR expression level as the concentration increased ($p<0.05$) (Figure 5A). The p-mTOR expression levels at 48 h tended to be higher than at 24 h, possibly because the majority of mTOR in T47D cells was activated at 48 h. However, the treatment with $4xIC_{50}$ of EA fraction exhibited the lowest p-mTOR expression level at 24 h (0.46%) and 48 h (2.84%). Meanwhile, cisplatin increased p-mTOR expression level in 48 h, indicating that this chemotherapy agent might trigger initial resistance mechanisms in cancer.[54]

This study also revealed that a significant reduction in c-Myc expression level was only observed in the T47D treated with $2xIC_{50}$ of EA fraction for 48 h (28.22%) ($p<0.05$) (Figure 5B). This result indicated that another transcription factor, i.e., HIF-1α, might also contribute to the anticancer activities of the EA fractions.[49] Further study should investigate the effect of the EA fraction on HIF-1α expression in T47D cells. Meanwhile, cisplatin did not significantly affect the c-Myc expression level.

Additionally, the EA fraction could significantly diminish the HK2 expression level as the concentration increased ($p<0.05$) (Figure 5C). The treatment with $4xIC_{50}$ of EA fraction exhibited a notable decrease in HK2 expression level at 48 h (22.82%). Similar to mTOR, the HK2 expression levels at 48 h were higher than at 24 h, probably due to the most of HK2 in T47D cells being activated at 48h. Meanwhile, treatment with cisplatin for 48 h inhibited the HK2 expression level (43.21%). Therefore, these results implied that the EA fraction could negatively affect the expression of these oncogenic proteins in regulating apoptosis, cell cycle, and aerobic glycolysis in T47D cells.

Developing EA fraction of *S. doederleinii* to treat luminal A breast cancer through triggering apoptosis, suppressing the cell cycle, and hindering aerobic glycolysis should be directed to become an anticancer agent in complementary therapy, commonly used in combination with chemotherapy agents to enhance the anticancer activities and reduce adverse effects.[42] Further studies should identify the potency of the EA fraction of *S. doederleinii* combined with chemotherapy agents to fight luminal A breast cancer. Moreover, carrying out comprehensive studies to isolate and identify the most promising compounds from the EA fraction of *S. doederleinii* to develop a new targeted therapy agent that can inhibit specific oncogenic proteins in luminal A breast cancer is also necessary.

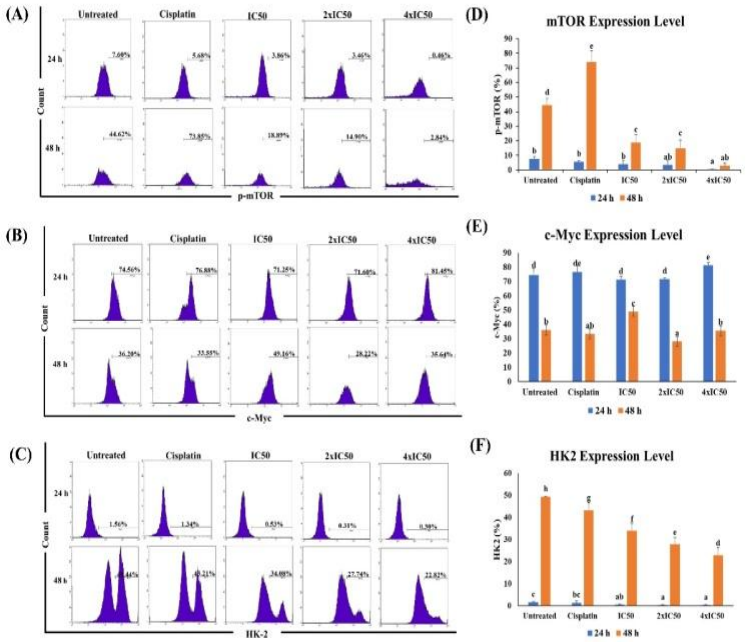

Figure 5: The EA fraction of *S. doederleinii* (IC_{50}, $2xIC_{50}$, $4xIC_{50}$) reduced p-mTOR, c-Myc, and HK2 expression in the T47D cells. The histograms showed the relative amounts of (A) p-mTOR, (B) c-Myc, and (C) HK2 after induction with the EA fraction for 24 and 48 h. The diagrams exhibited the percentages of (D) p-mTOR, (E) c-Myc, and (F) HK2 expression in T47D cells. The values shown are mean ± SD (n = 3).

Conclusion

S. doederleinii ethyl acetate (EA) fraction exerted potent anticancer effects on luminal A breast cancer (T47D) cells by inducing apoptosis, inhibiting the cell cycle, suppressing aerobic glycolysis, and diminishing several oncogenic protein expression levels without causing any cytotoxicity on normal cells. However, further investigations are still required to develop the EA fraction of *S. doederleinii* to become a complementary therapy agent or a targeted therapy agent for luminal A breast cancer.

Conflict of Interest

All authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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