RESEARCH ARTICLE

Stenochlaena palustris Ethanol Extract Decreases Viability and Induces G1-Phase Cell Cycle Arrest in HSC-3 Tongue Cancer Cells via p21 and p27

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Abstract

BACKGROUND: Oral squamous cell carcinoma (OSCC) of the tongue is an aggressive cancer with a poor prognosis due to its resistance to standard treatments. *Stenochlaena palustris*, a medicinal fern containing bioactive compounds, has shown potential anticancer properties. However, there is a lack of studies addressing the effects of *S. palustris* ethanol extract (SPEE) on tongue cancer. This study examined the effects of SPEE on the cell viability and cell cycle of human squamous cell carcinoma (HSC)-3 tongue cancer cells.

METHODS: SPEE was prepared with the maceration method. HSC-3 cells were treated with SPEE at concentrations of 100, 500, and 1000 µg/mL for 24 and 48 hours. Cell viability was measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cell cycle analysis was performed using flow cytometer. Immunoblotting was used to measure amount of cell cycle regulators, protein 21 (p21) and protein 27 (p27).

RESULTS: SPEE treatment led to a significant decrease in HSC-3 viable cells in a concentration- and time-dependent manner, with the most pronounced effect at higher concentration and prolonged treatment time. There was a slightly increase in the percentage of cells in the Sub-G1 phase in SPEE-treated group, meanwhile there was a significant increase in the percentage of cells in the G1-phase. Increased amount of p21 and p27 were observed in SPEE-treated group.

CONCLUSION: SPEE significantly inhibited HSC-3 cell proliferation in a concentration- and time-dependent manner, primarily by inducing G1-phase cell cycle arrest through the upregulation of p21 and p27. Taken together, SPEE could be a potential anti-cancer agent for tongue cancer cell.

KEYWORDS: *Stenochlaena palustris*, tongue cancer, cytotoxic, cell cycle arrest, HSC-3 cells, p21, p27

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Introduction

Oral squamous cell carcinoma (OSCC), specifically when it occurs as tongue cancer, is a highly aggressive malignancy with a poor prognosis due to its invasive characteristics and resistance to standard treatments.(1) Despite advancements in therapeutic approaches, recurrence rates remain high, highlighting the need for alternative treatments, particularly those derived from natural sources.(2) Natural compounds

have gained attention in cancer therapy for their selective cytotoxicity toward cancer cells and reduced toxicity to normal tissues.(3,4) Among the promising natural sources, several plants have demonstrated potential anticancer properties in various cancer cells, including *Elephantopus scaber* (3), *Curcuma xanthorrhiza* (5), *Physalis angulata* (6), and *Mangifera indica* (7) offering hope for more effective treatment options.

Stenochlaena palustris, a fern with a long history in Southeast Asian medicine, is emerging as a notable candidate due to its extensive range of medicinal properties. (8) Research indicates that various parts of *S. palustris*, including the leaves, stems, and roots, are rich in bioactive compounds such as flavonoids, phenolic acids, and terpenoids, which exhibit significant anticancer activities. (9) *S. palustris* is recognized for containing various di- and mono-acylated kaempferol glycosides, which are strong derivatives of kaempferol in the flavonoids group, and these flavonoids, the primary bioactive compounds in this plant, have also demonstrated anti-inflammatory effects.(4) These effects can contribute to overall health by reducing chronic inflammation, which is often linked to various diseases, including cancer. Thus, the bioactive compounds in *S. palustris* offer a range of therapeutic benefits, particularly for cancer treatment.(10)

The principal anticancer mechanism of flavonoids is related with the cell viability, a process that is often disrupted in cancer, leading to uncontrolled cell proliferation. (11,12) The disruption of uncontrolled cell proliferation is frequently linked to the dysregulation of cyclin-dependent kinase inhibitors (CDKIs) such as protein 21 (p21) and protein 27 (p27). p21 normally functions to arrest the cell cycle, allowing for DNA repair or initiating apoptosis if damage is irreparable. In cancer cells, however, p21 is often downregulated, which impairs its ability to regulate cell proliferation effectively.(13) Similarly, p27, which helps control cell cycle progression by inhibiting CDK complexes, is frequently reduced or dysfunctional in cancer, leading to unchecked cell division and resistance to apoptosis. Therefore, proper regulation of p21 and p27 is essential for maintaining cellular homeostasis and preventing cancer development.(14,15)

Previous studies have explored effects of *S. palustris* ethanol extract (SPEE) on cancer cell lines such as those from breast (16), prostate, and liver cancers (17). However, there is a lack of studies addressing the effects of SPEE on oral squamous cell carcinoma (OSCC), particularly tongue cancer. While apoptosis-related mechanisms have been studied to some extent, the effects of SPEE on cell viability and proliferation are not fully understood. Therefore, this study was conducted to investigate the effects of SPEE on the viability and cell cycle of human squamous cell carcinoma (HSC)-3 tongue cancer cells.

Methods

Preparation of *S. palustris* Ethanol Extract

S. palustris were obtained from Indonesian Institute for Testing Instrument Standard for Spices, Medicinal, and Aromatic Plant, Ministry of Agriculture (https://rempahobat.bsip.pertanian.go.id/). The SPEE was prepared with a maceration method. The plant material was finely chopped, dried, and extracted with 96% ethanol. The extract was filtered and concentrated using a rotary evaporator. The resulting SPEE was kept at 4°C.

HSC-3 Cell Culture

HSC-3 cell culture was performed as previously reported.(3) The HSC-3 cell line was maintained in Dulbecco's modified eagle medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 50 U/mL penicillin, 50 µg/mL streptomycin (Sigma-Aldrich), and 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany). The cells were incubated in a humidified environment at 37°C with 5% CO_2. When the HSC-3 cells reached approximately 80% confluence, the cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich).

Cell Viability Assay

Cell viability was performed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described method.(3) HSC-3 cells were seeded into 96-well plates ($5x10^3$ cells/well) and treated with/without 1, 500, or 1000 µg/mL SPEE or 1 µM Doxorubicin (Global Onkolab Farma, Jakarta, Indonesia) for 24 hours. After treatment, MTT solution was added to reach a final concentration of 0.5 mg/mL and incubated for 4 hours. The solution was then removed, and the formazan crystals were dissolved in 100 µL of dimethyl sulfoxide (DMSO). The absorbance of the dissolved formazan was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at OD_{570}. Each experimental group was analyzed in sextuplicate.

Cell Cycle Analysis

Cell cycle distribution and proliferation were evaluated using a flow cytometer, as previously reported.(3) Treated-

HSC-3 cells were collected and incubated in a hypotonic fluorochrome solution consisting of 50 µg/mL propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan). The cell suspensions were then incubated in the dark for 30 minutes. Fluorescence of individual nuclei was quantified using a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with a total of 400 events recorded per sample. Each sample was analyzed for cell cycle phase distribution, focusing on the sub-G1 and G1 phases.

Immunoblotting

HSC-3 cells were lysed and mixed with Laemmli sample buffer (Bio-Rad). The proteins from each experimental condition were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with a 5% skim milk solution, the membrane was incubated with a rabbit monoclonal anti-p21 Waf1/Cip1 (12D1) antibody (Cat. No. 2947; Cell Signaling, Danvers, MA, USA) as the first antibodies. The membrane was then washed and subsequently probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Cat. No. 7074; Cell Signaling Technology). Immunoblot results of p21 Waf1/Cip1 were visualized with Clarity Western ECL (Bio-Rad), captured, analyzed and quantified with Alliance 4.7 (UVItech, Cambridge, UK). For p27 Kip1 detection, the previously quantified membrane for p21 Waf1/Cip1, was stripped, washed, re-blocked, re-probed with rabbit polyclonal anti-p27 Kip1 primary antibody (Cat. No. 2552; Cell Signaling) and HRP-conjugated goat anti-rabbit secondary antibody, visualized with Clarity Western ECL, captured, analyzed and quantified with Alliance 4.7. For β-Actin detection, the previously quantified membrane for p21 Waf1/Cip1 and p27 Kip1, was again reused with the same procedure as for the p27 Kip1 detection. A rabbit polyclonal anti-β-Actin antibody (Cat. No. 4967; Cell Signaling) was used as the primary antibody.

Results

SPEE Decreased Numbers of HSC-3 Viable Cells

SPEE concentrations (p=0.000) and treatment times (p=0.000) were significant on the numbers of HSC-3 viable cells, as determined by two-way ANOVA (Figure 1). The numbers of HSC-3 viable cells in the Doxorubicin group at both 24 hours (2,679±1,140; Tukey's post hoc test, p=0.000) and 48 hours (1,980±173; Mann-Whitney's post hoc test, p=0.004) were significantly lower than the ones of each Sham group at both 24 hours (11,500±1,290) and 48 hours (23,000±833). The numbers of HSC-3 viable cells in the 100 µg/mL (10,857±896; Tukey's post hoc test, p=0.878) and 500 µg/mL (9,929±1,155; Tukey's post hoc test, p=0.178) SPEE-treated groups at 24 hours did not significantly differ than the ones in the Sham group, meanwhile the numbers of HSC-3 viable cells in the 1000 µg/mL SPEE-treated group at 24 hours (7,429±926) differed significantly (Tukey's post hoc test, p=0.000) than the ones in the Sham group. In contrast, the numbers of HSC-3 viable cells in the 100 µg/mL (15,822±828), 500 µg/mL (9,799±134), and 1000 µg/mL (6,327±139) SPEE-treated groups at 48 hours, differed significantly (Mann-Whitney's post hoc test, p=0.004) than the ones in the Sham group. In this MTT assay, IC_{50} concentration of SPEE in inducing apoptosis of HSC-3 cells was 2,941 µg/mL.

SPEE Induced G1-phase Cell Cycle Arrest

From the histogram (Figure 2A), an increase in the percentage of cells in the Sub-G1 phase was observed in the

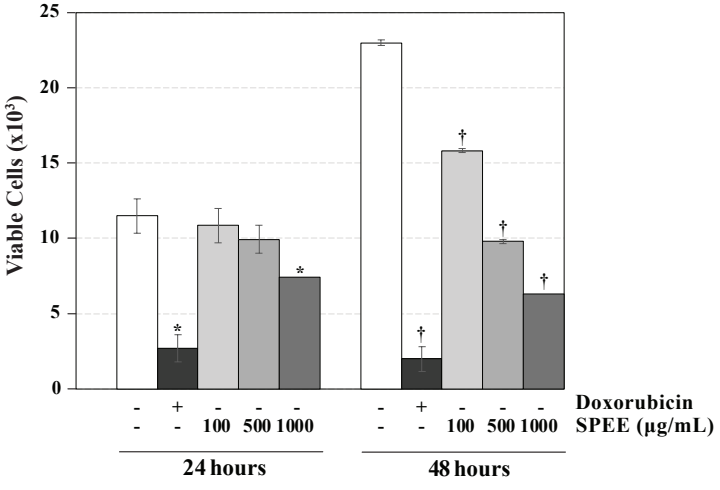

Figure 1. SPEE decreased number of HSC-3 viable cells. HSC-3 cells were starved for 12 hours, then treated with/without 1 µM Doxorubicin or SPEE at various concentrations for 24 and 48 hours. Number of viable cells were assessed with the MTT assay, as described in the Methods. The results are presented as mean±standard deviation (n=6). *significant (Tukey's post hoc test, p<0.05) compared with each Sham group. †significant (Mann-Whitney's post hoc test, p<0.05) compared with each Sham group.

Doxorubicin group, while an increase in the percentage of cells in the G1 phase was evident with rising concentration and prolonged treatment time (48 hours). The calculated percentage data were presented in Figure 2B and Figure 2C.

In the sub-G1 phase (Figure 2B), the percentage of cells in the Doxorubicin group at both 24 hours (79.83±1.72%) and 48 hours (86.17±2.14%) were significantly higher (Tukey's post hoc test, p=0.000) than the ones of each Sham group at both 24 hours (3.33±1.03%) and 48 hours (5.17±1.72%). The 100 µg/mL (4.33±1.21%), 500 µg/mL (6.17±0.75%), and 1000 µg/mL (8.33±1.51%) SPEE-treated groups at 24 hours, showed a slight increase of the cells' percentage in the sub-G1 phase than the ones in the Sham group. Similarly, at 48 hours, there was a slight increase in the percentage of cells the sub-G1 phase for the 100 µg/mL (5.33±1.51%), 500 µg/mL (8.67±1.86%), and 1000 µg/mL (10.17±1.94%) SPEE-treated groups than the ones in the Sham group as well.

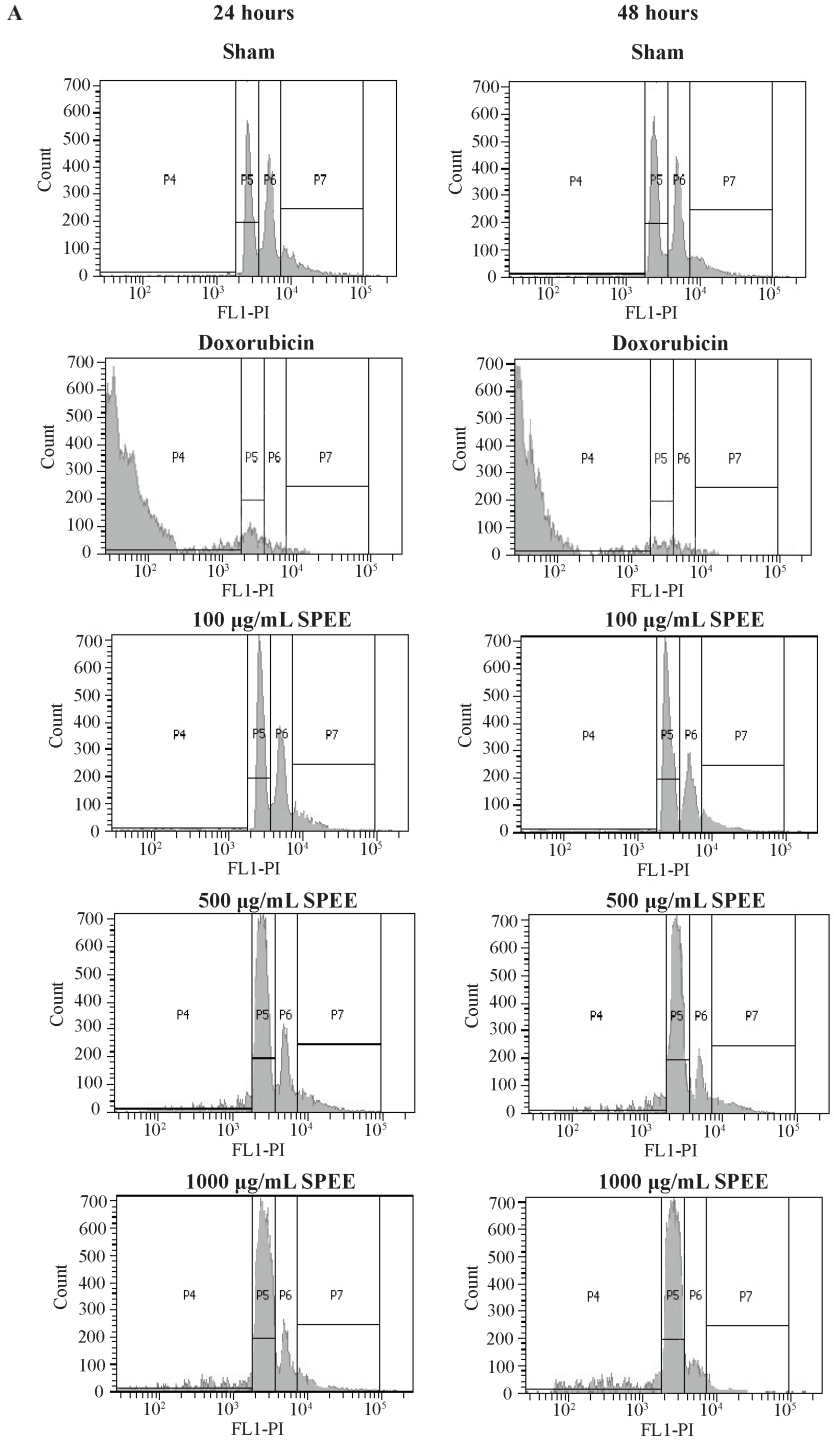

Figure 2. SPEE induced G1-phase arrest. HSC-3 cells were starved for 12 hours and then treated with/without 1 µM Doxorubicin or SPEE at various concentrations for 24 and 48 hours. The cell cycle distribution was analyzed using a flow cytometer, as described in the Methods. A: Histograms of treated HSC-3 cells in Sub-G1, G1, S, G2/M phases. P4: Sub-G phase; P5: G1 phase, P6: S phase; P7: G2/M phase.(continue)

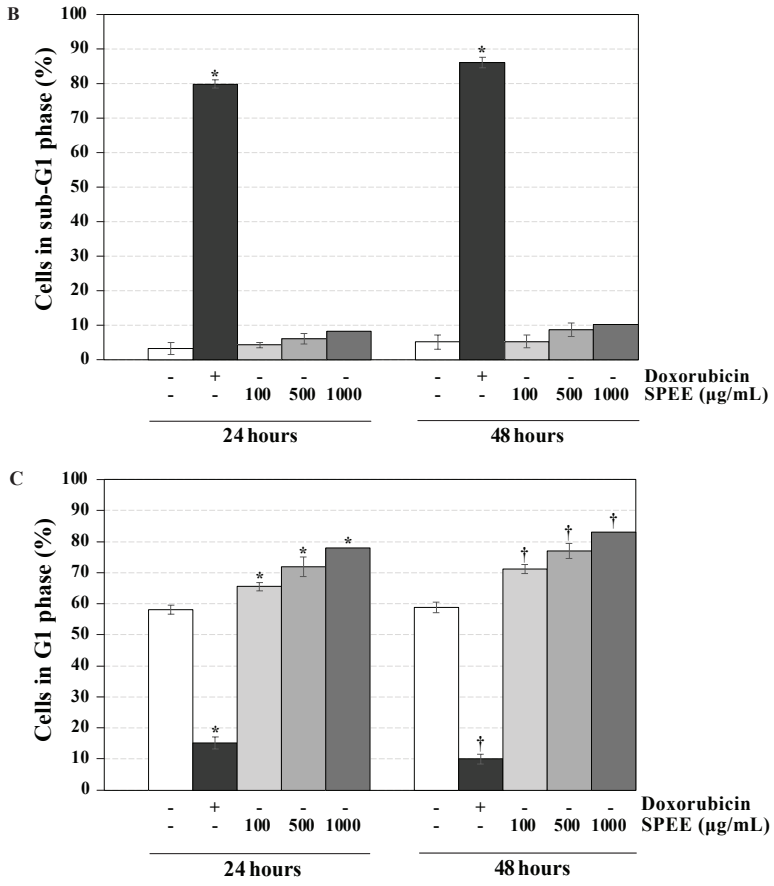

Figure 2. SPEE induced G1-phase arrest. (continue) B: Percentages of treated HSC-3 cells in the Sub-G1 phase. C: Percentages of treated HSC-3 cells in the G1 phase. The results are presented as mean±standard deviation (n=6). *significant (Tukey's post hoc test, $p<0.05$) compared with each Sham group. †significant (Mann-Whitney's post hoc test, $p<0.05$) compared with each Sham group.

In the G1 phase (Figure 2C), the percentage of cells in the Doxorubicin group at both 24 hours (15.17±1.47%; Tukey's post hoc test, $p=0.000$) and 48 hours (10.00±1.79%; Mann Whitney's post hoc test, $p=0.004$) was significantly lower than the ones in the Sham group at both 24 hours (58.00±6.48%) and 48 hours (58.83±2.32%). The 100 µg/mL (65.50±1.87%; Tukey's post hoc test, $p=0.007$), 500 µg/mL (72.00±1.41; Tukey's post hoc test, $p=0.000$), and 1000 µg/mL (78.00±3.16%; Tukey's post hoc test, $p=0.000$) SPEE-treated groups at 24 hours, differed significantly than the ones in the Sham group. Similarly, at 48 hours, the results of the study showed that there was a significant difference (Mann Whitney's post hoc test, $p=0.004$) in the percentage of cells in the G1 phase for the 100 µg/mL (71.17±1.60%), 500 µg/mL (77.00±1.41%), and 1000 µg/mL (83.00±2.35%) SPEE-treated groups than the ones in the Sham group as well.

SPEE Increased p21 of HSC-3 Cells

The p21 amount of HSC-3 cells treated with Doxorubicin (6,164±227.83) did not significantly differ (Tukey's post hoc test, $p=0.999$) than the ones in the sham group (6,436±382.42) (Figure 3). In contrast, p21 amount in the SPEE-treated groups increased significantly (One-way ANOVA, $p=0.000$) in a concentration-dependent manner. The p21 amount in the 100 µg/mL SPEE-treated group (6,408±395.74) did not significantly differ (Tukey's post hoc test, $p=1.000$) than the ones in the sham group, meanwhile p21 amount in the 500 µg/mL (17,303.33±485.33) and the 1000 µg/mL (23,586±2,877.33) SPEE-treated groups differed significantly (Tukey's post hoc test, $p=0.000$) than the ones in the sham group.

SPEE Increased p27 of HSC-3 Cells

The p27 amount of HSC-3 cells treated with Doxorubicin (7,140.67±145.51) did not significantly differ (Tukey's post hoc test, $p=1.000$) than the ones in the sham group (7,107.67±99.72) (Figure 4). Similar to the p21 amount, the p27 amount in the SPEE-treated groups increased significantly (One-way ANOVA, $p=0.000$) in a concentration-dependent manner. The p27 amount in the 100 µg/mL SPEE-treated group (9,783±387.4) did not significantly differ (Tukey's post hoc test, $p=0.070$) than the ones in the sham group, meanwhile p27 amount in the 500 µg/mL (18,282.33±514.02) and the 1000 µg/mL (22,821.33±2,285.85) SPEE-treated groups differed significantly (Tukey's post hoc test, $p=0.000$) than the ones in the sham group.

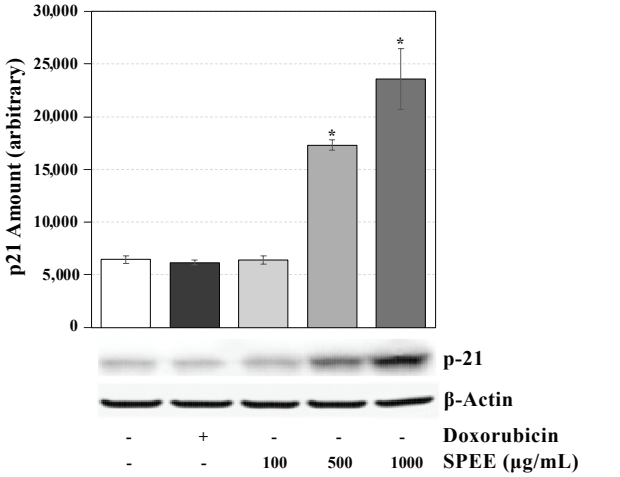

Figure 3. SPEE increased p21 amount of HSC-3 cells. HSC-3 cells were treated with/without 1 µM Doxorubicin or SPEE in various concentrations. The p21 amount was assessed with Immunoblotting assay, as described in the Methods. *significant (Tukey's post hoc test, $p<0.05$) compared with Sham group.

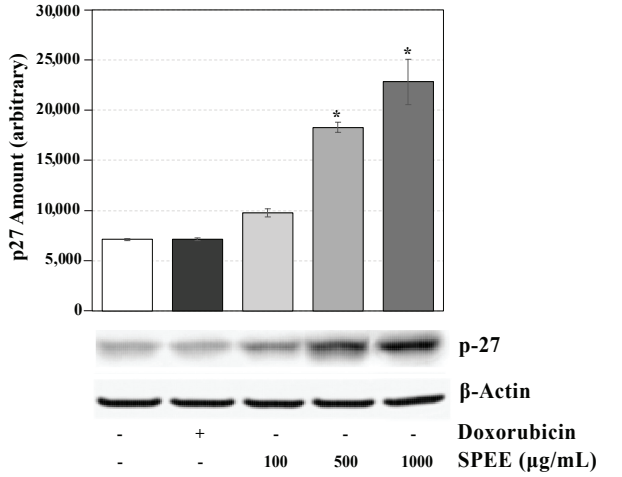

Figure 4. SPEE increased p27 amount of HSC-3 cells. HSC-3 cells were treated with/without 1 µM Doxorubicin or SPEE in various concentrations. The p27 amount was assessed with Immunoblotting assay, as described in the Methods. *significant (Tukey's post hoc test, $p<0.05$) compared with Sham group.

Discussion

In the present study, SPEE showed significant cytotoxic effects on HSC-3 cells. The results demonstrated a concentration- and time-dependent manner in decreasing HSC-3 viable cells (Figure 1). At 48 hours, SPEE-treated groups (100, 500, and 1000 µg/mL) showed a significant decrease in the numbers of HSC-3 viable cells. These findings align with prior research showing SPEE-induced apoptosis in various cancer cell lines, including MCF-7 (breast cancer) (16), DU-145 (prostate cancer), and HepG2 (liver cancer) (17). IC_{50} of SPEE in inducing apoptosis of HSC-3 cells (2,941 µg/mL) was categorized as non-toxic (>501µg/mL) µg/mL).(18) Although the IC_{50} value was higher than those in inducing apoptosis of MCF-7 cells (493.57 µg/mL) (16) and HepG2 (224.12 µg/mL) (17), the treatment at 48 hours still showed significant cytotoxic effects. This suggested that the effectiveness of SPEE might extend beyond its cytotoxic effects, particularly in terms of its capacity to inhibit cell proliferation. *S. palustris* contained several di- and mono-acylated kaempferol glycosides, which were highly effective kaempferol derivatives within the flavonoid group in inducing cytotoxic effect.(19) These findings indicate that the anticancer properties of SPEE are likely attributable to its capacity to suppress cell proliferation, a mechanism commonly observed in natural compounds such as flavonoids, phenolic acids, and terpenoids, which interfere with cell cycle progression.(20,21)

Treatment of SPEE could cause only a slight increase of cells' percentage in the Sub-G1 phase, suggesting that SPEE did not play an important role in inducing apoptosis (Figure 2B). However, the SPEE could induce significant percentage of cells in the G1-phase in concentration- and time-dependent manner, suggesting that the SPEE-treated cells were arrested in G1 phase cell cycle (Figure 2C). The G1-phase arrest induced by SPEE could be mediated by key cell cycle regulators, such as p21 and p27. The p21 and p27 have been widely reported to have a capacity in inhibiting cyclin-dependent kinases (CDKs) and preventing the phosphorylation of retinoblastoma protein (Rb), thereby halting cell cycle progression.(22)

SPEE induced a slower yet more enduring reduction in cell viability by promoting G1-phase arrest and inhibiting cell cycle progression. This sustained inhibition of cell proliferation indicated that SPEE may be a more favorable candidate for long-term cancer treatment (23), as it might cause less damage to surrounding healthy cells compared with doxorubicin, which is known for its broader cytotoxicity (24). The mechanism of SPEE facilitates a gradual reduction in cell viability, along with other natural resources, which could help limit tumor growth while minimizing the aggressive side effects commonly associated with conventional chemotherapy.(15,25)

Present results were similar with previous reports due to the capacity of natural resources in inducing G1 phase. *Ferulago angulata* leaf extract significantly arrested MCF-7 cells in the G1 phase of the cell cycle, leading to an upregulation of p21 and p27 proteins, as well as promoting

apoptosis by increasing Bax levels while decreasing Bcl-2. (26) Similarly, flavonoids found in citrus fruits was shown to inhibit MCF-7 cell proliferation in a concentration-dependent manner over 48 and 72 hours by inducing G1 phase cell cycle arrest, downregulation of CDKs, and upregulation of p21Cip1 and p27Kip1.(27)

Further research is required to fully explore the potential of SPEE in cancer treatment. Animal studies could provide valuable insights into its efficacy, safety, and pharmacokinetics. Additionally, investigating its detailed effects on cell cycle regulation and its potential synergy with other anti-cancer agents could lead to more effective and less toxic treatment options.

Conclusion

SPEE significantly decreased HSC-3 cell proliferation in a concentration- and time-dependent manner. This inhibition was achieved by inducing G1-phase cell cycle arrest, which is associated with the upregulation of cell cycle regulators p21 and p27. Taken together, SPEE could be a potential anti-cancer agent for tongue cancer cell.

Authors Contribution

FS and EYT contributed to the conceptualization and design of the research, data acquisition, and analysis. FS and AES were responsible for drafting the manuscript and creating the figures. FS, BOR, DR, JH, and KHL assisted in interpreting the results and engaged in critical discussions. FS, BOR, DR, JH, EYT, AES, and KHL took part in the critical revision of the manuscript.

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