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Content

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REVIEW ARTICLE

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Elephantopus scaber Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34

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Abstract

BACKGROUND: Previous research has demonstrated the effect of *Elephantopus scaber* Linn. leaf extract (ESLE) on various cancer cell lines. However, research on the effects of ESLE on oral squamous cell carcinoma (OSCC), especially tongue cancer, is still lacking. Moreover, the apoptotic mechanisms induced by ESLE are not well understood and require further exploration. Therefore, this study was conducted to investigate the effects of ESLE on cell viability and apoptosis in human squamous cell carcinoma (HSC)-3 tongue cancer cells.

METHODS: HSC-3 cells were treated with varying concentrations of ESLE, doxorubicin, and a combination of both. Cell viability and apoptosis were assessed using MTT and Sub-G1 assays. The expression levels of survivin and its phosphorylated form at threonine (Thr)34 were evaluated using Western blot analysis.

RESULTS: ESLE exhibited a concentration-dependent cytotoxic effect on HSC-3 cells in decreasing cell viability (Kruskal Wallis, p=0.001) and increasing apoptotic cells (ANOVA, p=0.001) significantly. When combined with doxorubicin, ESLE further enhanced the induction of apoptosis compared with doxorubicin alone. The combined treatment resulted in a decrease in the levels of phosphorylated survivin (p-Surv) Thr34, indicating the inhibition of survivin's anti-apoptotic function.

CONCLUSION: ESLE significantly enhances the efficacy of doxorubicin, thereby sensitizing its ability to induce apoptosis in HSC-3 tongue cancer cells. This sensitization occurs through the inhibition of survivin activity, particularly at the Thr34 phosphorylation site. These findings suggest that ESLE could serve as a potential adjuvant to improve the effectiveness of doxorubicin in inducing apoptosis in tongue cancer cells.

KEYWORDS: *Elephantopus scaber*, doxorubicin, tongue cancer, HSC-3 cells, apoptosis, Survivin, Thr34 phosphorylation

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Introduction

The field of natural product research is growing rapidly, especially in the search for effective anticancer agents from plants.(1) Natural products are particularly well-suited for this purpose due to their minimal side effects, ability to target multiple cancer processes, and potential for synergistic effects.(2) Their complex chemical diversity and capacity make them valuable candidates for drug discovery

and development.(3,4) Among many studied plants, *Elephantopus scaber* Linn., which is known as Elephant's Foot and belongs to the Asteraceae family, stands out due to its traditional use in folk medicine and its promising results in modern research.(5)

Previous research has shown that some parts of *E. scaber*, such as the leaves and the roots, possess pharmacological activities due to their rich chemical composition.(6) This plant has gained attention for its potential therapeutic benefits. *E. scaber* contains a range of bioactive metabolites, including flavonoids, triterpenoids and sesquiterpene lactones.(7) The flavonoids in the plant are well-known for their strong antioxidant and anti-inflammatory properties.(8) Triterpenoids further enhance the plant's therapeutic value with their diverse effects, including anti-inflammatory and antimicrobial activities.(9) Additionally, sesquiterpene lactones, due to their complex structures, are noted for their potent biological effects, such as inducing cell death and inhibiting cell growth.(10)

A regulated process of cell death, apoptosis, plays a vital role in removing cancerous cells and inhibiting tumor progression.(11,12) This process can be triggered by either the mitochondrial (intrinsic) pathway, which is mediated by caspase-9, or the death-receptor (extrinsic) pathway, which is mediated by caspase-8. Ultimately, both pathways converge to activate the effector caspases-3 and -7, which execute the cell death program.(13) One of the critical regulators of apoptosis is Survivin, a key member of the inhibitor of apoptosis (IAP) protein family.(14) Survivin is essential in regulating apoptosis by inhibiting caspase activity and promoting cell survival.(15) The phosphorylated variant of Survivin, known as p-Survivin (p-Surv) threonine 34 (Thr34), further modulates this function by altering its interactions with apoptotic machinery.(16) Phosphorylation at Thr34 affects Survivin's stability and its ability to bind to second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), a mitochondrial protein that promotes apoptosis by antagonizing IAPs.(17)

Previous studies have reported the effects of *E. scaber* leaf extract (ESLE) on breast cancer cell lines (18,19) and colorectal cancer cell lines (20). However, research on the effects of ESLE on oral squamous cell carcinoma (OSCC), especially tongue cancer, is lacking. Furthermore, the apoptotic mechanisms triggered by ESLE are not well understood and require further investigation. Consequently, this study aims to examine the effects of ESLE on cell viability and apoptosis in human squamous cell carcinoma (HSC)-3 tongue cancer cells.

Methods

Preparation of ESLE

The leaves of *E. scaber* L. were acquired from Indonesian Institute for Testing Instrument Standard for Spices, Medicinal, and Aromatic Plant, Ministry of Agriculture. The ESLE was obtained using maceration technique. *E. scaber* leaves were finely minced and dried. The dehydrated material was extracted with 70% ethanol solution, followed by filtration and evaporation using rotary evaporator. The crude ESLE obtained was thereafter kept at a temperature of 4°C.

HSC-3 Cell Culture

The HSC-3 cell culture was conducted using a previously reported method (21), with specific modifications. The HSC-3 cell line was acquired from Sigma-Aldrich (St. Louis, MO, USA). HSC-3 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich) complete medium contained 50 U/mL penicillin 50 µg/mL, streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany). The cells were cultured in a humidified incubator at 37°C, 5% CO_2. The HSC-3 cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) once they reached 80% confluence.

Cell Viability Assay

The measurement of viable cells was conducted with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following a previously reported method.(13) In 96-well plates, HSC-3 cells were placed (5×10^3/well) and treated with/without 1, 10, or 100 µg/mL ESLE or 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) for 24 hours. MTT was added in the treated well (100 µL/well) and incubated for 4 hours. Then, the suspension in each well was removed and dissolved in 100 µL dimethylsulfoxide (DMSO). The formazan crystal that was formed was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at OD570. The measurements for each experimental group were conducted in sextuplicate.

Sub-G1 Assay

Apoptotic HSC-3 cells were measured using the sub-G1 assay in order to evaluate the cytotoxic effects of ESLE, based on previously reported method.(13) Treated-HSC-3 cells were collected and incubated in a hypotonic fluorochrome solution (50 µg/mL of propidium iodide (Sigma-Aldrich),

0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan)). Subsequently, the cell suspensions were incubated in darkness for 30 minutes. The fluorescence of individual nuclei was quantified using a FACSCanto II flowcytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and a total of 400 events were recorded.

Western Blotting Assay

HSC-3 cells that were treated with/without various concentrations of ESLE or 25 nM YM155 (Tocris, Bristol, UK) were then harvested and incubated with radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA). YM155, a survivin suppressant, was used as a positive control in this study to demonstrate its ability to reduce or inhibit p-Surv. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk in Tris-buffered saline (TBS, 150 nM NaCl and 50 nM Tris-HCl, pH 7.4), the sheets were incubated with rabbit polyclonal anti-β-Actin (Cat. No. 4967; Cell Signaling, Danvers, MA, USA) and rabbit polyclonal anti-phospho-survivin (Thr34) (Cat. No. 8888; Cell Signaling) antibody. The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit (Cell Signaling) IgG antibody, diluted 1:1000. The bound antibodies were visualized using Clarity Western ECL (Bio-Rad) and captured using Alliance 4.7 (UVItech, Cambridge, UK).

Statistical Analysis

The Shapiro-Wilk normality test was utilized for statistical analysis. Then, one-way ANOVA test was used to analyze the findings of a normally distributed dataset, followed by Tukey's post-hoc test. Subsequently, the results of abnormal data distribution were tested using Kruskal-Wallis test, followed by Mann-Whitney's post-hoc test.

Results

ESLE Decreased HSC-3 Viable Cells

The results in Figure 1 showed that the number of HSC-3 viable cells in 1 µM doxorubicin group (56±11.12) was significantly lower (Mann-Whitney's post-hoc test, p=0.004) than the ones in the sham group (9,212±65.58). The number of HSC-3 viable cells in ESLE-treated groups decreased significantly (Kruskal Wallis, p=0.001) in concentration-dependent manner. The number of HSC-3 viable cells in 1 µg/mL ESLE-treated group (9,268±424.76) did not significantly differ (Mann-Whitney's post-hoc test, p=0.423) than the ones in the sham group, meanwhile the number of HSC-3 viable cells in 10 µg/mL ESLE-treated group (8,173±316.61) and 100 µg/mL ESLE-treated group (6,952±602.94) 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 ESLE in inducing apoptosis of HSC-3 cells was 222.34 µg/mL.

ESLE Increased HSC-3 Apoptotic Cells

The results in Figure 2 showed that the percentage of HSC-3 apoptotic cells in 1 µM doxorubicin group (95.73±0.48%) was significantly higher (Tukey's post-hoc test, p=0.001) than the ones in the sham group (4.62±0.48%). The percentage of HSC-3 apoptotic cells in ESLE-treated groups increased significantly (ANOVA, p=0.001) in concentration-dependent manner. The percentage of HSC-3 apoptotic cells in 1 µg/mL ESLE-treated group (6.08±0.34%) did not significantly differ (Tukey's post-hoc test, p=0.120) than the ones in the sham group, meanwhile percentage of HSC-3 apoptotic cells in 10 µg/mL ESLE-treated group (18.88±0.75%) and 100 µg/mL ESLE-treated group (33.45±2.09%) differed significantly (Tukey's post-hoc test, p=0.001) than the ones in the sham group.

Combination of 100 µg/mL ESLE with 0.25 µM Doxorubicin Increased HSC-3 Apoptotic Cells

The results in Figure 3 showed that the percentage of HSC-3 apoptotic cells in 1 µM doxorubicin group (95.71±0.47%) was significantly higher (Mann-Whitney's post-hoc test, p=0.004) than the ones in the 100 µg/mL ESLE + 0.25 µM

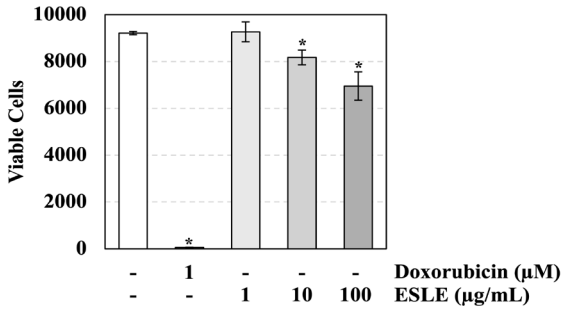

Figure 1. ESLE decreased HSC-3 viable cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different concentrations for 24 h. Viable cells were measured using MTT assay as outlined in methods. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Mann-Whitney's post-hoc test when compared to the sham group.

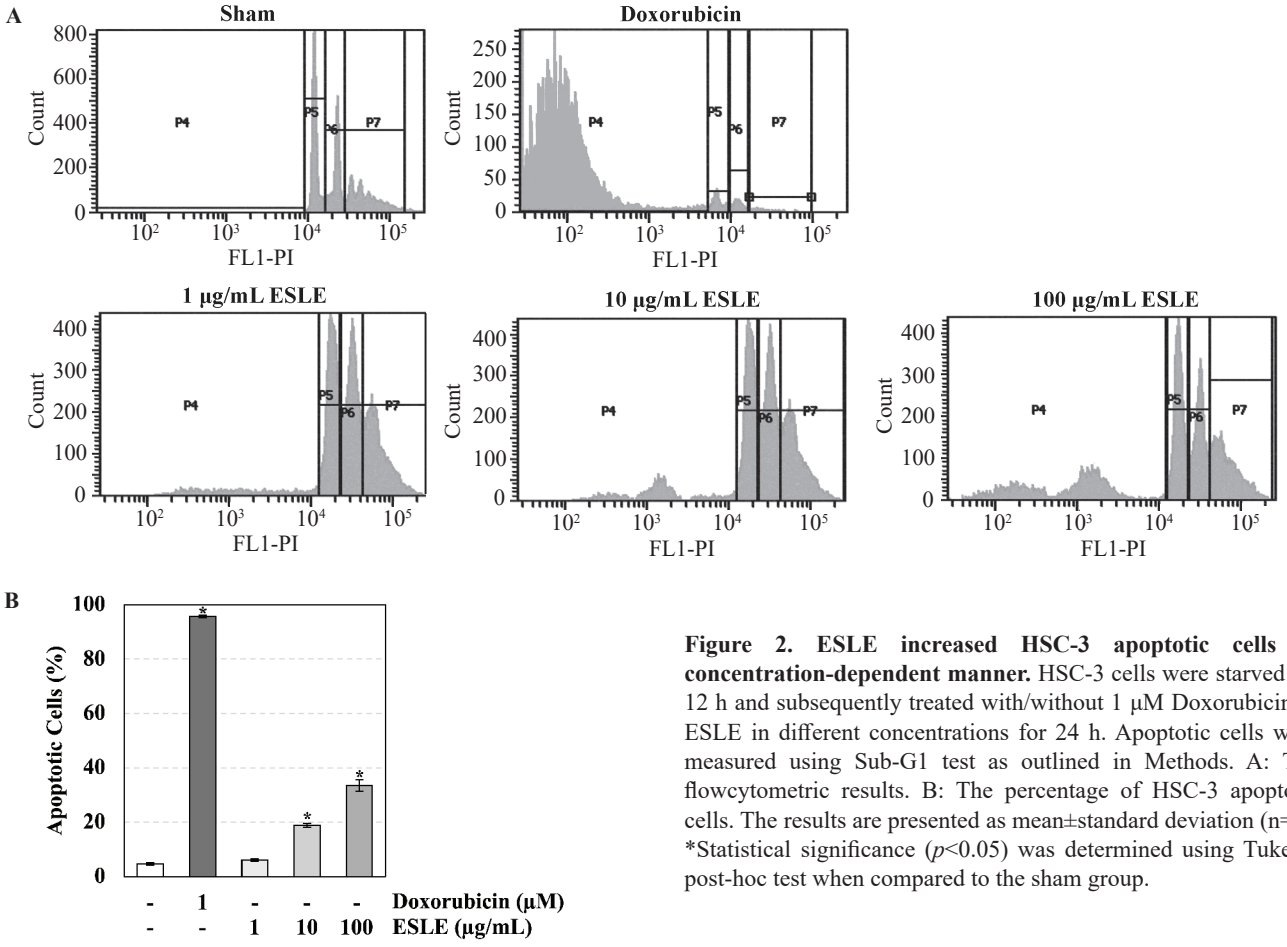

Figure 2. ESLE increased HSC-3 apoptotic cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different concentrations for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. A: The flowcytometric results. B: The percentage of HSC-3 apoptotic cells. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Tukey's post-hoc test when compared to the sham group.

doxorubicin-treated group (65.00±10.05%). The percentage of HSC-3 apoptotic cells in the 0.25 µM Doxorubicin group (22.98±5.98%) and the 100 µg/mL ESLE-treated group (33.45±2.09%) were low. However, the percentage of HSC-3 apoptotic cells in the 100 µg/mL ESLE + 0.25 µM doxorubicin-treated group was significantly higher (Mann-Whitney's post-hoc test, p=0.004) compared to the 0.25 µM Doxorubicin group and the 100 µg/mL ESLE-treated group.

ESLE Decreased p-Surv (Thr34) of HSC-3 Cells

The results in Figure 4 showed that the p-Surv (Thr34) amount of HSC-3 cells in YM155 group (28±7) was significantly lower (Tukey's post-hoc test, p=0.000) than the ones in the sham group (933±49.14) (Figure 5). The β-Actin was used as a loading control. The p-Surv (Thr34) amount in ESLE-treated group decreased significantly (ANOVA, p=0.000) in concentration-dependent manner. The p-Surv (Thr34) amount in the 1 µg/mL ESLE-treated group (659±125.74), 10 µg/mL ESLE-treated group (408±36.47) and 100 µg/mL ESLE-treated group (179±62.4) differed significantly (Tukey's post-hoc test, p=0.000) than the ones in the sham group.

Discussion

In the current study, ESLE exhibited a concentration-dependent cytotoxic effect on HSC-3 cells. Results from the MTT (Figure 1) and sub-G1 assays (Figure 2) showed a reduction in viable HSC-3 cells, which was attributed to apoptosis induction. These findings align with previous studies that reported the ability of ESLE to induce apoptosis in T47D (breast cancer), MCF-7 (breast cancer), and HCT116 (colorectal cancer) cell lines.(18,19) IC_{50} of ESLE in inducing apoptosis of HSC-3 cells (222.34 µg/mL) was categorized as weak cytotoxicity (201-500 µg/mL).(22) This IC_{50} value was higher than those in inducing apoptosis of T47D cells (132.17±9.69 µg/mL) (18), MCF-7 cells (14.69±0.29 µg/mL) (19) and HCT116 cells (14.69±0.29 µg/mL) (20). However, although having weak cytotoxicity, ESLE could enhance the potency of doxorubicin in inducing apoptosis of HSC-3 cells. Specifically, the combination of 100 µg/mL ESLE with 0.25 µM doxorubicin increased the percentage of HSC-3 apoptotic cells more than treatment with either agent alone (Figure 3). These results suggest a

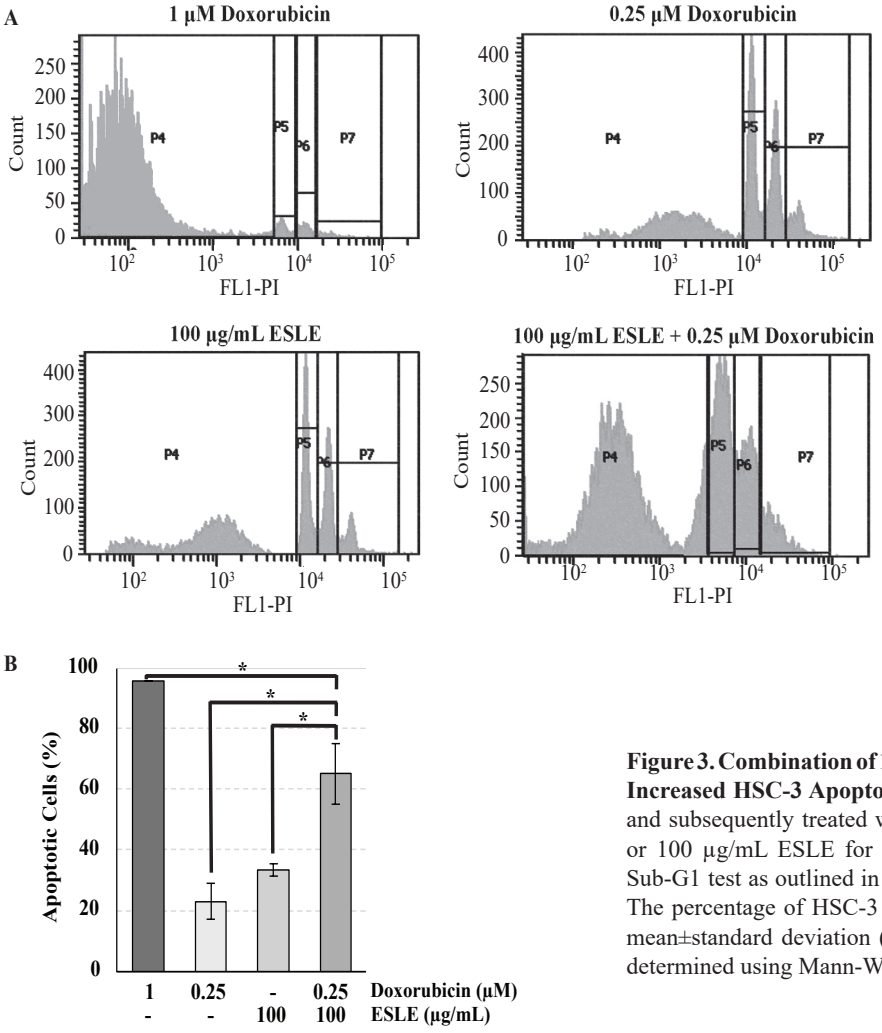

Figure 3. Combination of 100 µg/mL ESLE with 0.25 µM Doxorubicin Increased HSC-3 Apoptotic Cells. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM or 0.25 µM Doxorubicin, or 100 µg/mL ESLE for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. A: The flow cytometric results. B: The percentage of HSC-3 apoptotic cells. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Mann-Whitney's post-hoc test.

synergistic effect of combining 100 µg/mL ESLE and 0.25 µM doxorubicin in inducing apoptosis in HSC-3 cells. This finding indicates that ESLE can sensitize the apoptotic effect of doxorubicin, potentially allowing for lower doses of doxorubicin to be used in cancer therapy, thereby reducing its associated side effects.

The observed synergy between ESLE and doxorubicin is likely due to several interacting mechanisms. This combination seems to enhance doxorubicin-induced DNA damage and inhibit survival pathways in cancer cells, making them more susceptible to doxorubicin. This result aligns with previous studies that examined the effects of ESLE in combination with tamoxifen in MCF-7 breast cancer cells.(23) These studies demonstrated that ESLE enhances the effectiveness of tamoxifen in targeting breast cancer cells. The combination of ESLE and tamoxifen led to cell cycle arrest at the S phase, downregulation of pro-survival genes heat shock protein (HSP)-105, upregulation of the pro-apoptotic genes, implicating both intrinsic and extrinsic apoptotic pathways.(23)

The present study showed that the ESLE-treated group exhibited a decrease in the p-Surv (Thr34) levels in HSC-3 cells (Figure 4) in a concentration-dependent manner. This reduction in p-Surv (Thr34) is comparable to the effect of YM155, a known survivin suppressant. YM155 has been shown to inhibit survivin expression and induce apoptosis in various cancer cell lines.(24,25) In this study, YM155 was used as a positive control to validate the effect of ESLE on survivin phosphorylation. The significant reduction in p-survivin levels in the ESLE-treated cells, similar to that observed with YM155, underscores the potential of ESLE as an effective anti-cancer agent targeting survivin. This result aligns with previous studies that showed ESLE decreased survivin expression at the transcript level.(26) Survivin, which is phosphorylated at Thr34 by the cyclin-dependent kinase (CDK)1 during the G2/M phase of the cell cycle, is crucial for its anti-apoptotic function. A reduction in p-survivin levels could disrupt the function, thereby promoting apoptosis in cancer cells.(27).

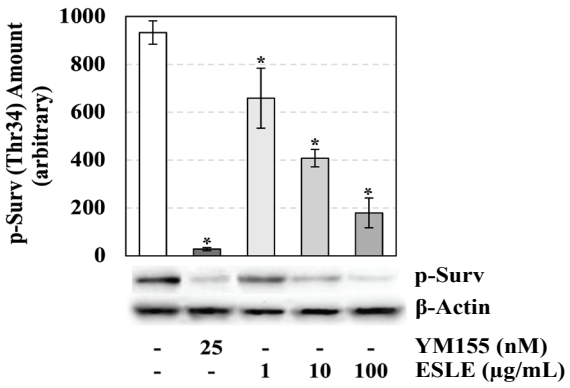

Figure 4. ESLE decreased the amount of p-Surv (Thr34) in HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 25 nM YM155 or ESLE in various concentrations. The amount of p-Surv (Thr34) was measured using Western Blot, as outlined in Methods. *Statistical significance (p<0.05) was determined using Tukey's post-hoc test when compared to the sham group.

Further studies are needed to fully elucidate the mechanism of action of ESLE. Specifically, future research should examine other potential phosphorylation sites on survivin, such as Ser 70 and Ser81, may be crucial to its anti-apoptotic function. Moreover, exploring the effects of ESLE on other signaling pathways involved in cancer cell survival and proliferation could provide a more comprehensive understanding of its therapeutic potential.

Conclusion

Taken together, ESLE significantly enhances the efficacy of doxorubicin, thereby sensitizing its ability to induce apoptosis in HSC-3 tongue cancer cells. This sensitization occurs through the inhibition of survivin activity, particularly at the Thr34 phosphorylation site. These findings suggest that ESLE could serve as a potential adjuvant to improve the effectiveness of doxorubicin in inducing apoptosis in tongue cancer cells.

Authors Contribution

FS and TP were involved in conceptualizing and planning the research, performing data acquisition and collection, as well as conducting data analysis. FS and AES drafted the manuscript and designed the figures. FS, RAH, DR, and KHL aided in interpreting the results and provided critical discussion. FS, RAH, DR, TP, AES, and KHL participated in the critical revision of the manuscript.

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RESEARCH ARTICLE

Elephantopus scaber Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34

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Abstract

BACKGROUND: Previous research has demonstrated the effect of *Elephantopus scaber* Linn. leaf extract (ESLE) on various cancer cell lines. However, research on the effects of ESLE on oral squamous cell carcinoma (OSCC), especially tongue cancer, is still lacking. Moreover, the apoptotic mechanisms induced by ESLE are not well understood and require further exploration. Therefore, this study was conducted to investigate the effects of ESLE on cell viability and apoptosis in human squamous cell carcinoma (HSC)-3 tongue cancer cells.
METHODS: HSC-3 cells were treated with varying concentrations of ESLE, doxorubicin, and a combination of both. Cell viability and apoptosis were assessed using MTT and Sub-G1 assays. The expression levels of survivin and its phosphorylated form at threonine (Thr)34 were evaluated using Western blot analysis.
RESULTS: ESLE exhibited a concentration-dependent cytotoxic effect on HSC-3 cells in decreasing cell viability (Kruskal Wallis, p=0.001) and increasing apoptotic cells (ANOVA, p=0.001) significantly. When combined with doxorubicin, ESLE further enhanced the induction of apoptosis compared with doxorubicin alone. The combined treatment resulted in a decrease in the levels of phosphorylated survivin (p-Surv) Thr34, indicating the inhibition of survivin's anti-apoptotic function.
CONCLUSION: ESLE significantly enhances the efficacy of doxorubicin, thereby sensitizing its ability to induce apoptosis in HSC-3 tongue cancer cells. This sensitization occurs through the inhibition of survivin activity, particularly at the Thr34 phosphorylation site. These findings suggest that ESLE could serve as a potential adjuvant to improve the effectiveness of doxorubicin in inducing apoptosis in tongue cancer cells.
KEYWORDS: *Elephantopus scaber*, doxorubicin, tongue cancer, HSC-3 cells, apoptosis, Survivin, Thr34 phosphorylation

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Introduction

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and development.(3,4) Among many studied plants, *Elephantopus scaber* Linn., which is known as Elephant's Foot and belongs to the Asteraceae family, stands out due to its traditional use in folk medicine and its promising results in modern research.(5)

Previous research has shown that some parts of *E. scaber*, such as the leaves and the roots, possess pharmacological activities due to their rich chemical composition.(6) This plant has gained attention for its potential therapeutic benefits. *E. scaber* contains a range of bioactive metabolites, including flavonoids, triterpenoids and sesquiterpene lactones.(7) The flavonoids in the plant are well-known for their strong antioxidant and anti-inflammatory properties.(8) Triterpenoids further enhance the plant's therapeutic value with their diverse effects, including anti-inflammatory and antimicrobial activities.(9) Additionally, sesquiterpene lactones, due to their complex structures, are noted for their potent biological effects, such as inducing cell death and inhibiting cell growth.(10)

A regulated process of cell death, apoptosis, plays a vital role in removing cancerous cells and inhibiting tumor progression.(11,12) This process can be triggered by either the mitochondrial (intrinsic) pathway, which is mediated by caspase-9, or the death-receptor (extrinsic) pathway, which is mediated by caspase-8. Ultimately, both pathways converge to activate the effector caspases-3 and -7, which execute the cell death program.(13) One of the critical regulators of apoptosis is Survivin, a key member of the inhibitor of apoptosis (IAP) protein family.(14) Survivin is essential in regulating apoptosis by inhibiting caspase activity and promoting cell survival.(15) The phosphorylated variant of Survivin, known as p-Survivin (p-Surv) threonine 34 (Thr34), further modulates this function by altering its interactions with apoptotic machinery.(16) Phosphorylation at Thr34 affects Survivin's stability and its ability to bind to second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), a mitochondrial protein that promotes apoptosis by antagonizing IAPs.(17)

Previous studies have reported the effects of *E. scaber* leaf extract (ESLE) on breast cancer cell lines (18,19) and colorectal cancer cell lines (20). However, research on the effects of ESLE on oral squamous cell carcinoma (OSCC), especially tongue cancer, is lacking. Furthermore, the apoptotic mechanisms triggered by ESLE are not well understood and require further investigation. Consequently, this study aims to examine the effects of ESLE on cell viability and apoptosis in human squamous cell carcinoma (HSC)-3 tongue cancer cells.

Methods

Preparation of ESLE

The leaves of *E. scaber* L. were acquired from Indonesian Institute for Testing Instrument Standard for Spices, Medicinal, and Aromatic Plant, Ministry of Agriculture. The ESLE was obtained using maceration technique. *E. scaber* leaves were finely minced and dried. The dehydrated material was extracted with 70% ethanol solution, followed by filtration and evaporation using rotary evaporator. The crude ESLE obtained was thereafter kept at a temperature of 4°C.

HSC-3 Cell Culture

The HSC-3 cell culture was conducted using a previously reported method (21), with specific modifications. The HSC-3 cell line was acquired from Sigma-Aldrich (St. Louis, MO, USA). HSC-3 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich) complete medium contained 50 U/mL penicillin 50 µg/mL, streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany). The cells were cultured in a humidified incubator at 37°C, 5% CO_2. The HSC-3 cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) once they reached 80% confluence.

Cell Viability Assay

The measurement of viable cells was conducted with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following a previously reported method.(13) In 96-well plates, HSC-3 cells were placed (5×10^3/well) and treated with/without 1, 10, or 100 µg/mL ESLE or 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) for 24 hours. MTT was added in the treated well (100 µL/well) and incubated for 4 hours. Then, the suspension in each well was removed and dissolved in 100 µL dimethylsulfoxide (DMSO). The formazan crystal that was formed was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at OD570. The measurements for each experimental group were conducted in sextuplicate.

Sub-G1 Assay

Apoptotic HSC-3 cells were measured using the sub-G1 assay in order to evaluate the cytotoxic effects of ESLE, based on previously reported method.(13) Treated-HSC-3 cells were collected and incubated in a hypotonic fluorochrome solution (50 µg/mL of propidium iodide (Sigma-Aldrich),

0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan)). Subsequently, the cell suspensions were incubated in darkness for 30 minutes. The fluorescence of individual nuclei was quantified using a FACSCanto II flowcytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and a total of 400 events were recorded.

Western Blotting Assay

HSC-3 cells that were treated with/without various concentrations of ESLE or 25 nM YM155 (Tocris, Bristol, UK) were then harvested and incubated with radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, MA, USA). YM155, a survivin suppressant, was used as a positive control in this study to demonstrate its ability to reduce or inhibit p-Surv. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk in Tris-buffered saline (TBS, 150 nM NaCl and 50 nM Tris-HCl, pH 7.4), the sheets were incubated with rabbit polyclonal anti-β-Actin (Cat. No. 4967; Cell Signaling, Danvers, MA, USA) and rabbit polyclonal anti-phospho-survivin (Thr34) (Cat. No. 8888; Cell Signaling) antibody. The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit (Cell Signaling) IgG antibody, diluted 1:1000. The bound antibodies were visualized using Clarity Western ECL (Bio-Rad) and captured using Alliance 4.7 (UVItech, Cambridge, UK).

Statistical Analysis

The Shapiro-Wilk normality test was utilized for statistical analysis. Then, one-way ANOVA test was used to analyze the findings of a normally distributed dataset, followed by Tukey's post-hoc test. Subsequently, the results of abnormal data distribution were tested using Kruskal-Wallis test, followed by Mann-Whitney's post-hoc test.

Results

ESLE Decreased HSC-3 Viable Cells

The results in Figure 1 showed that the number of HSC-3 viable cells in 1 µM doxorubicin group (56±11.12) was significantly lower (Mann-Whitney's post-hoc test, p=0.004) than the ones in the sham group (9,212±65.58). The number of HSC-3 viable cells in ESLE-treated groups decreased significantly (Kruskal-Wallis, p=0.001) in concentration-dependent manner. The number of HSC-3 viable cells in 1 µg/mL ESLE-treated group (9,268±424.76) did not significantly differ (Mann-Whitney's post-hoc test, p=0.423) than the ones in the sham group, meanwhile the number of HSC-3 viable cells in 10 µg/mL ESLE-treated group (8,173±316.61) and 100 µg/mL ESLE-treated group (6,952±602.94) 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 ESLE in inducing apoptosis of HSC-3 cells was 222.34 µg/mL.

ESLE Increased HSC-3 Apoptotic Cells

The results in Figure 2 showed that the percentage of HSC-3 apoptotic cells in 1 µM doxorubicin group (95.73±0.48%) was significantly higher (Tukey's post-hoc test, p=0.001) than the ones in the sham group (4.62±0.48%). The percentage of HSC-3 apoptotic cells in ESLE-treated groups increased significantly (ANOVA, p=0.001) in concentration-dependent manner. The percentage of HSC-3 apoptotic cells in 1 µg/mL ESLE-treated group (6.08±0.34%) did not significantly differ (Tukey's post-hoc test, p=0.120) than the ones in the sham group, meanwhile percentage of HSC-3 apoptotic cells in 10 µg/mL ESLE-treated group (18.88±0.75%) and 100 µg/mL ESLE-treated group (33.45±2.09%) differed significantly (Tukey's post-hoc test, p=0.001) than the ones in the sham group.

Combination of 100 µg/mL ESLE with 0.25 µM Doxorubicin Increased HSC-3 Apoptotic Cells

The results in Figure 3 showed that the percentage of HSC-3 apoptotic cells in 1 µM doxorubicin group (95.71±0.47%) was significantly higher (Mann-Whitney's post-hoc test, p=0.004) than the ones in the 100 µg/mL ESLE + 0.25 µM

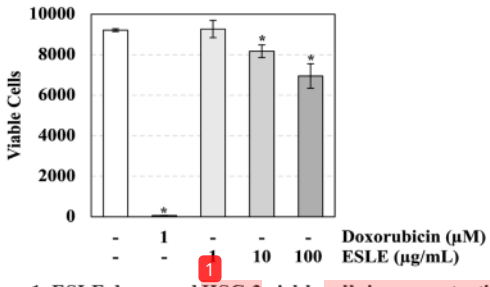

Figure 1. ESLE decreased HSC-3 viable cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different concentrations for 24 h. Viable cells were measured using MTT assay as outlined in methods. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Mann-Whitney's post-hoc test when compared to the sham group.

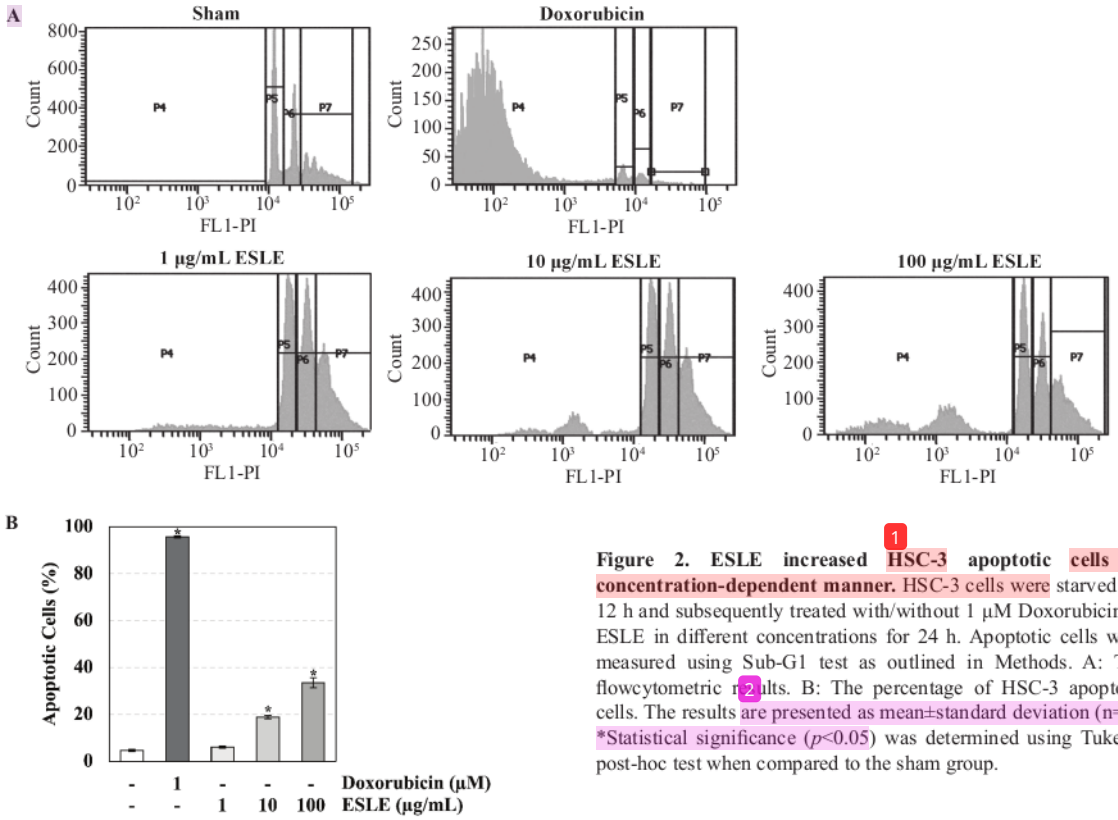

Figure 2. ESLE increased HSC-3 apoptotic cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different concentrations for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. A: The flowcytometric results. B: The percentage of HSC-3 apoptotic cells. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Tukey's post-hoc test when compared to the sham group.

doxorubicin-treated group (65.00±10.05%). The percentage of HSC-3 apoptotic cells in the 0.25 µM Doxorubicin group (22.98±5.98%) and the 100 µg/mL ESLE-treated group (33.45±2.09%) were low. However, the percentage of HSC-3 apoptotic cells in the 100 µg/mL ESLE + 0.25 µM doxorubicin-treated group was significantly higher (Mann-Whitney's post-hoc test, p=0.004) compared to the 0.25 µM Doxorubicin group and the 100 µg/mL ESLE-treated group.

ESLE Decreased p-Surv (Thr34) of HSC-3 Cells

The results in Figure 4 showed that the p-Surv (Thr34) amount of HSC-3 cells in YM155 group (28±7) was significantly lower (Tukey's post-hoc test, p=0.000) than the ones in the sham group (933±49.14) (Figure 5). The β-Actin was used as a loading control. The p-Surv (Thr34) amount in ESLE-treated group decreased significantly (ANOVA, p=0.000) in concentration-dependent manner. The p-Surv (Thr34) amount in the 1 µg/mL ESLE-treated group (659±125.74), 10 µg/mL ESLE-treated group (408±36.47) and 100 µg/mL ESLE-treated group (179±62.4) differed significantly (Tukey's post-hoc test, p=0.000) than the ones in the sham group.

Discussion

In the current study, ESLE exhibited a concentration-dependent cytotoxic effect on HSC-3 cells. Results from the MTT (Figure 1) and sub-G1 assays (Figure 2) showed a reduction in viable HSC-3 cells, which was attributed to apoptosis induction. These findings align with previous studies that reported the ability of ESLE to induce apoptosis in T47D (breast cancer), MCF-7 (breast cancer), and HCT116 (colorectal cancer) cell lines.(18,19) IC_{50} of ESLE in inducing apoptosis of HSC-3 cells (222.34 µg/mL) was categorized as weak cytotoxicity (201-500 µg/mL).(22) This IC_{50} value was higher than those in inducing apoptosis of T47D cells (132.17±9.69 µg/mL) (18), MCF-7 cells (14.69±0.29 µg/mL) (19) and HCT116 cells (14.69±0.29 µg/mL) (20). However, although having weak cytotoxicity, ESLE could enhance the potency of doxorubicin in inducing apoptosis of HSC-3 cells. Specifically, the combination of 100 µg/mL ESLE with 0.25 µM doxorubicin increased the percentage of HSC-3 apoptotic cells more than treatment with either agent alone (Figure 3). These results suggest a

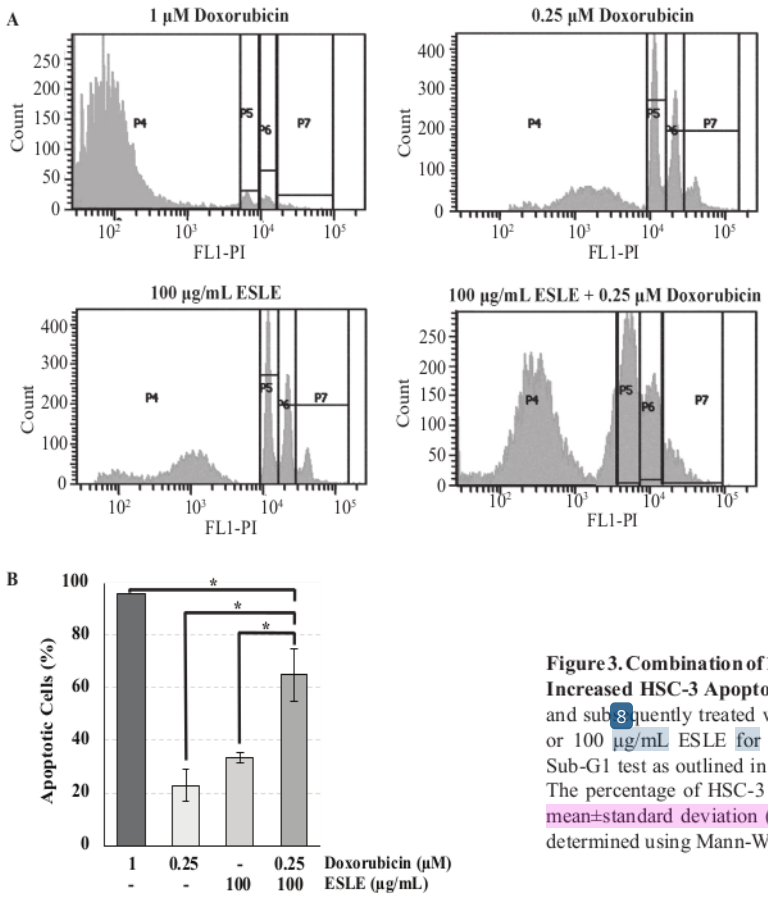

Figure 3. Combination of 100 µg/mL ESLE with 0.25 µM Doxorubicin Increased HSC-3 Apoptotic Cells. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM or 0.25 µM Doxorubicin, or 100 µg/mL ESLE for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. A: The flow cytometric results. B: The percentage of HSC-3 apoptotic cells. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Mann-Whitney's post-hoc test.

synergistic effect of combining 100 µg/mL ESLE and 0.25 µM doxorubicin in inducing apoptosis in HSC-3 cells. This finding indicates that ESLE can sensitize the apoptotic effect of doxorubicin, potentially allowing for lower doses of doxorubicin to be used in cancer therapy, thereby reducing its associated side effects.

The observed synergy between ESLE and doxorubicin is likely due to several interacting mechanisms. This combination seems to enhance doxorubicin-induced DNA damage and inhibit survival pathways in cancer cells, making them more susceptible to doxorubicin. This result aligns with previous studies that examined the effects of ESLE in combination with tamoxifen in MCF-7 breast cancer cells.(23) These studies demonstrated that ESLE enhances the effectiveness of tamoxifen in targeting breast cancer cells. The combination of ESLE and tamoxifen led to cell cycle arrest at the S phase, downregulation of pro-survival genes heat shock protein (HSP)-105, upregulation of the pro-apoptotic genes, implicating both intrinsic and extrinsic apoptotic pathways.(23)

The present study showed that the ESLE-treated group exhibited a decrease in the p-Surv (Thr34) levels in HSC-3 cells (Figure 4) in a concentration-dependent manner. This reduction in p-Surv (Thr34) is comparable to the effect of YM155, a known survivin suppressant. YM155 has been shown to inhibit survivin expression and induce apoptosis in various cancer cell lines.(24,25) In this study, YM155 was used as a positive control to validate the effect of ESLE on survivin phosphorylation. The significant reduction in p-survivin levels in the ESLE-treated cells, similar to that observed with YM155, underscores the potential of ESLE as an effective anti-cancer agent targeting survivin. This result aligns with previous studies that showed ESLE decreased survivin expression at the transcript level.(26) Survivin, which is phosphorylated at Thr34 by the cyclin-dependent kinase (CDK)1 during the G2/M phase of the cell cycle, is crucial for its anti-apoptotic function. A reduction in p-survivin levels could disrupt the function, thereby promoting apoptosis in cancer cells.(27).

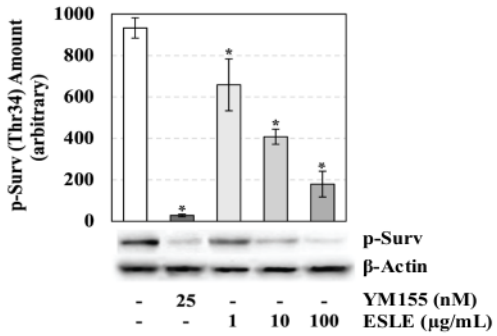

Figure 4. ESLE decreased the amount of p-Surv (Thr34) in HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 25 nM YM155 or ESLE in various concentrations. The amount of p-Surv (Thr34) was measured using Western Blot, as outlined in Methods. *Statistical significance (p<0.05) was determined using Tukey's post-hoc test when compared to the sham group.

Further studies are needed to fully elucidate the mechanism of action of ESLE. Specifically, future research should examine other potential phosphorylation sites on survivin, such as Ser 70 and Ser81, may be crucial to its anti-apoptotic function. Moreover, exploring the effects of ESLE on other signaling pathways involved in cancer cell survival and proliferation could provide a more comprehensive understanding of its therapeutic potential.

Conclusion

Taken together, ESLE significantly enhances the efficacy of doxorubicin, thereby sensitizing its ability to induce apoptosis in HSC-3 tongue cancer cells. This sensitization occurs through the inhibition of survivin activity, particularly at the Thr34 phosphorylation site. These findings suggest that ESLE could serve as a potential adjuvant to improve the effectiveness of doxorubicin in inducing apoptosis in tongue cancer cells.

Authors Contribution

FS and TP were involved in conceptualizing and planning the research, performing data acquisition and collection, as well as conducting data analysis. FS and AES drafted the manuscript and designed the figures. FS, RAH, DR, and KHL aided in interpreting the results and provided critical discussion. FS, RAH, DR, TP, AES, and KHL participated in the critical revision of the manuscript.

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Secretariat of InaBJ <secretariatinabj@gmail.com> Wed, Aug 21, 2024 at 2:33 PM
To: ferry@trisakti.ac.id

Dear Dr. Ferry Sandra,

Good day. We have reached a decision regarding your submission to The Indonesian Biomedical Journal, "***Elephantopus scaber* Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34**."

Our decision is to: **Resubmit for Review.**

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Elephantopus scaber Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34

ABSTRACT

BACKGROUND: Previous research has demonstrated the effect of *Elephantopus scaber* (*E. scaber*) Linn. leaf extract (ESLE) on various cancer cell lines. However, research on the effects of ESLE on oral squamous cell carcinoma (OSCC), especially tongue cancer, is still lacking. Moreover, the apoptotic mechanisms induced by ESLE are not well understood and require further exploration. Therefore, this study was conducted to investigate the effects of ESLE on cell viability and apoptosis in human oral squamous carcinoma (HSC)-3 cells.

METHODS: HSC-3 cells were treated with varying concentrations of ESLE, doxorubicin, and a combination of both. Cell viability and apoptosis were assessed using MTT and Sub-G1 assays. The expression levels of survivin and its phosphorylated form at threonine (Thr)34 were evaluated using Western blot analysis.

RESULTS: ESLE exhibited a concentration-dependent cytotoxic effect on HSC-3 cells in decreasing cell viability (Kruskal Wallis, p=0.001) and increasing apoptotic cells (ANOVA, p=0.001) significantly. When combined with doxorubicin, ESLE further enhanced the induction of apoptosis compared with doxorubicin alone. The combined treatment resulted in a decrease in the levels of phosphorylated survivin (p-Surv) Thr34, indicating the inhibition of survivin's anti-apoptotic function.

CONCLUSION: ESLE significantly enhances the efficacy of doxorubicin, thereby sensitizing its ability to induce apoptosis in HSC-3 tongue cancer cells. This sensitization occurs through the inhibition of survivin activity, particularly at the Thr34 phosphorylation site. These findings suggest that ESLE could serve as a potential adjuvant to improve the effectiveness of doxorubicin in inducing apoptosis in tongue cancer cells.

KEYWORDS: *Elephantopus scaber*, doxorubicin, tongue cancer, HSC-3 cells, apoptosis, Survivin, Thr34 phosphorylation

INTRODUCTION

The field of natural product research is growing rapidly, especially in the search for effective anticancer agents from plants.(1) Natural products are particularly well-suited for this purpose due to their minimal side effects, ability to target multiple cancer processes, and potential for synergistic effects.(2) Their complex chemical diversity and capacity make them valuable candidates for drug discovery and development.(3,4) Among many studied plants, *Elephantopus scaber* (*E. scaber*) Linn., which is known as Elephant's Foot and belongs to the Asteraceae family, stands out due to its traditional use in folk medicine and its promising results in modern research.(5)

Previous research has shown that some parts of *E. scaber*, such as the leaves and the roots, possess pharmacological activities due to their rich chemical composition.(6) This plant has gained attention for its potential therapeutic benefits. *E. scaber* contains a range of bioactive metabolites, including flavonoids, triterpenoids and sesquiterpene lactones.(7) The flavonoids in the plant are well-known for their strong antioxidant and anti-inflammatory properties.(8) Triterpenoids further enhance the plant's therapeutic value with their diverse effects, including anti-inflammatory and antimicrobial activities.(9) Additionally, sesquiterpene lactones, due to their complex structures, are noted for their potent biological effects, such as inducing cell death and inhibiting cell growth.(10)

A regulated process of cell death, apoptosis, plays a vital role in removing cancerous cells and inhibiting tumor progression.(11,12) This process can be triggered by either the mitochondrial (intrinsic) pathway, which is mediated by caspase-9, or the death-receptor (extrinsic) pathway, which is mediated by caspase-8. Ultimately, both pathways converge to activate the effector caspases-3 and -7, which execute the cell death program.(13) One of the critical regulators of apoptosis is Survivin, a key member of the inhibitor of apoptosis (IAP) protein family.(14) Survivin is essential in regulating apoptosis by inhibiting caspase activity and promoting cell survival.(15) The phosphorylated variant

of Survivin, known as p-Survivin threonine 34 (Thr34), further modulates this function by altering its interactions with apoptotic machinery.(16) Phosphorylation at Thr34 affects Survivin's stability and its ability to bind to second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), a mitochondrial protein that promotes apoptosis by antagonizing IAPs.(17)

Previous studies have reported the effects of *E. scaber* leaf extract (ESLE) on breast cancer cell lines (18,19) and colorectal cancer cell lines (20). However, research on the effects of ESLE on oral squamous cell carcinoma (OSCC), especially tongue cancer, is lacking. Furthermore, the apoptotic mechanisms triggered by ESLE are not well understood and require further investigation. Consequently, this study aims to examine the effects of ESLE on cell viability and apoptosis in human oral squamous carcinoma (HSC)-3 cells.

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MATERIAL AND METHODS

Preparation of *Elephantopus scaber* Linn. Leaf Extract (ESLE)

The leaves of *E. scaber* L. were acquired from Indonesian Institute for Testing Instrument Standard for Spices, Medicinal, and Aromatic Plant, Ministry of Agriculture. The ESLE was obtained using maceration technique. *E. scaber* leaves were finely minced and dried. The dehydrated material was extracted with 70% ethanol solution, followed by filtration and evaporation using rotary evaporator. The crude ESLE obtained was thereafter kept at a temperature of 4°C.

HSC-3 Cell Culture

The HSC-3 cell culture was conducted using a previously reported method (21), with specific modifications. The HSC-3 cell line was acquired from Sigma-Aldrich (St. Louis, MO, USA). HSC-3 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich) complete medium contained 50 U/mL penicillin 50 µg/mL, streptomycin (Sigma-Aldrich) and 10% fetal bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany). The cells were cultured in a humidified

incubator at 37°C, 5% CO_2. The HSC-3 cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) once they reached 80% confluence.

Cell Viability Assay

The measurement of viable cells was conducted with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following a previously reported method.(13) In 96-well plates, HSC-3 cells were placed ($5x10^3$/well) and treated with/without 1, 10, or 100 μg/mL ESLE or 1 μM Doxorubicin (Dankos Farma, Jakarta, Indonesia) for 24 hours. MTT was added in the treated well (100 μL/well) and incubated for 4 hours. Then, the suspension in each well was removed and dissolved in 100 μL dimethylsulfoxide (DMSO). The formazan crystal that was formed was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at OD_{570}. The measurements for each experimental group were conducted in sextuplicate.

Sub-G1 Assay

Apoptotic HSC-3 cells were measured using the sub-G1 assay in order to evaluate the cytotoxic effects of ESLE, based on previously reported method.(13) Treated-HSC-3 cells were collected and incubated in a hypotonic fluorochrome solution (50 μg/mL of propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan)). Subsequently, the cell suspensions were incubated in darkness for 30 minutes. The fluorescence of individual nuclei was quantified using a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and a total of 400 events were recorded.

Western Blotting Assay

HSC-3 cells that were treated with/without various concentrations of ESLE or 25 nM YM155 (Tocris, Bristol, UK) were then harvested and incubated with radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Fisher Scientific, Waltham, USA). Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk in Tris-buffered saline (TBS, 150nM NaCl and 50 nM Tris-HCl, pH 7.4), the sheets were incubated with rabbit polyclonal anti-β-Actin

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(Cat. No. 4967; Cell Signaling, Danvers, USA) and rabbit polyclonal anti-phospho-survivin (Thr34) (Cat. No. 8888; Cell Signaling) antibody. The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit (Cell Signaling) IgG antibody, diluted 1:1000. The bound antibodies were visualized using Clarity Western ECL (Bio-Rad, Hercules, CA, USA) and captured using Alliance 4.7 (UVItech, Cambridge, UK).

Statistical Analysis

The Shapiro-Wilk normality test was utilized for statistical analysis. Then, one-way ANOVA test was used to analyze the findings of a normally distributed dataset, followed by Tukey's *post hoc* test. Subsequently, the results of abnormal data distribution were tested using Kruskal-Wallis test, followed by Mann-Whitney's *post hoc* test.

RESULTS

ESLE Decreased HSC-3 Viable Cells

The results in Figure 1 showed that the number of HSC-3 viable cells in 1 μM doxorubicin group (56±11.12) was significantly lower (Mann-Whitney's *post hoc* test, p=0.004) than the ones in the sham group (9,212±65.58). The number of HSC-3 viable cells in ESLE-treated groups decreased significantly (Kruskal Wallis, p=0.001) in concentration-dependent manner. The number of HSC-3 viable cells in 1 μg/mL ESLE-treated group (9,268±424.76) did not significantly differ (Mann-Whitney's *post hoc* test, p=0.423) than the ones in the sham group, meanwhile the number of HSC-3 viable cells in 10 μg/mL ESLE-treated group (8,173±316.61) and 100 μg/mL ESLE-treated group (6,952±602.94) 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 ESLE-induced apoptotic HSC-3 cells was 222.34 μg/mL.

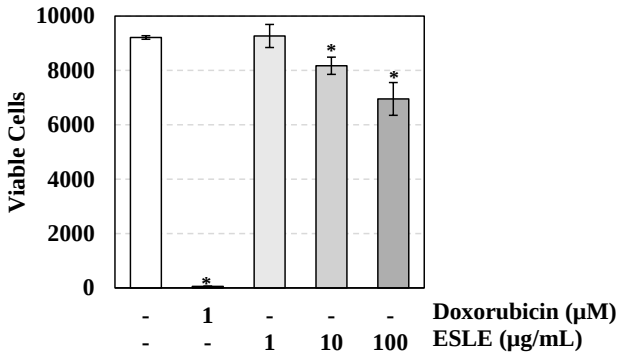

Figure 1. ESLE decreased HSC-3 viable cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different concentrations for 24 h. Viable cells were measured using MTT assay as outlined in methods. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Mann-Whitney's *post hoc* test when compared to the sham group.

ESLE Increased HSC-3 Apoptotic Cells

The results in Figure 2 showed that the percentage of HSC-3 apoptotic cells in 1 µM doxorubicin group (95.73±0.48%) was significantly higher (Tukey's *post hoc* test, p=0.001) than the ones in the sham group (4.62±0.48%) (Figure 2). The percentage of HSC-3 apoptotic cells in ESLE-treated groups increased significantly (ANOVA, p=0.001) in concentration-dependent manner. The percentage of HSC-3 apoptotic cells in 1 µg/mL ESLE-treated group (6.08±0.34%) did not significantly differ (Tukey's *post hoc* test, p=0.120) than the ones in the sham group, meanwhile percentage of HSC-3 apoptotic cells in 10 µg/mL ESLE-treated group (18.88±0.75%) and 100 µg/mL ESLE-treated group (33.45±2.09%) differed significantly (Tukey's *post hoc* test, p=0.001) than the ones in the sham group.

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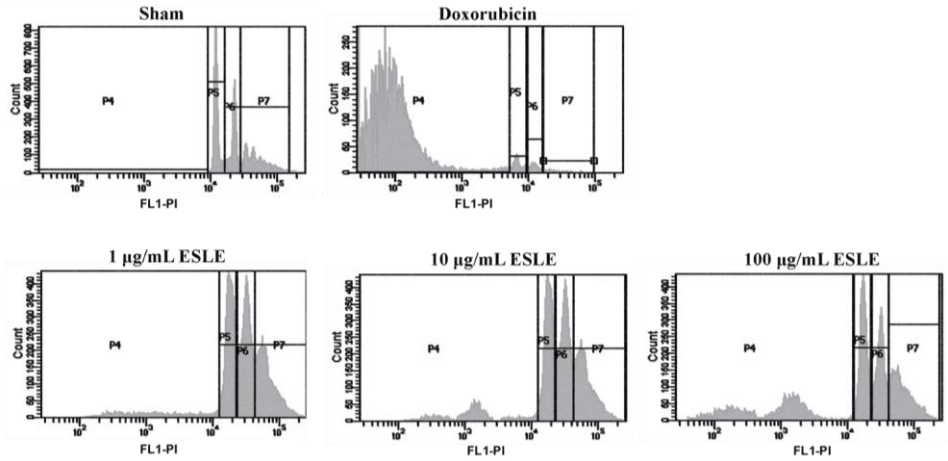

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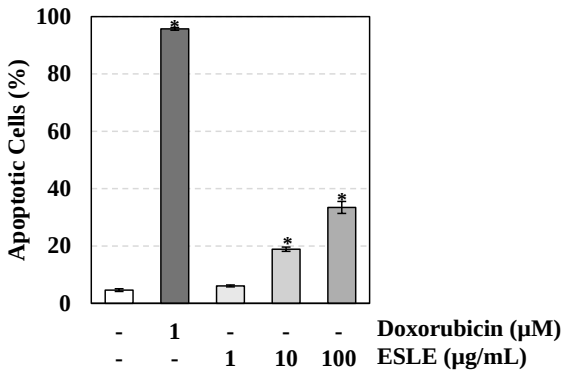

Figure 2. ESLE increased HSC-3 apoptotic cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different concentrations for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. (A) The flow cytometric results. (B) The percentage of HSC-3 apoptotic cells. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Tukey's *post hoc* test when compared to the sham group.

Combination of 100 µg/mL ESLE with 0.25 µM Doxorubicin Increased HSC-3 Apoptotic Cells

The results in Figure 3 showed that the percentage of HSC-3 apoptotic cells in 1 µM doxorubicin group (95.71±0.47%) was significantly higher (Mann-Whitney's *post hoc* test, p=0.004) than the ones in the 100 µg/mL ESLE + 0.25 µM doxorubicin-treated group (65.00±10.05%). The percentage of

HSC-3 apoptotic cells in the 0.25 µM Doxorubicin group (22.98±5.98%) and the 100 µg/mL ESLE-treated group (33.45±2.09%) were low. However, the percentage of HSC-3 apoptotic cells in the 100 µg/mL ESLE + 0.25 µM doxorubicin-treated group was significantly higher (Mann-Whitney's *post hoc* test, p=0.004) compared to the 0.25 µM Doxorubicin group and the 100 µg/mL ESLE-treated group.

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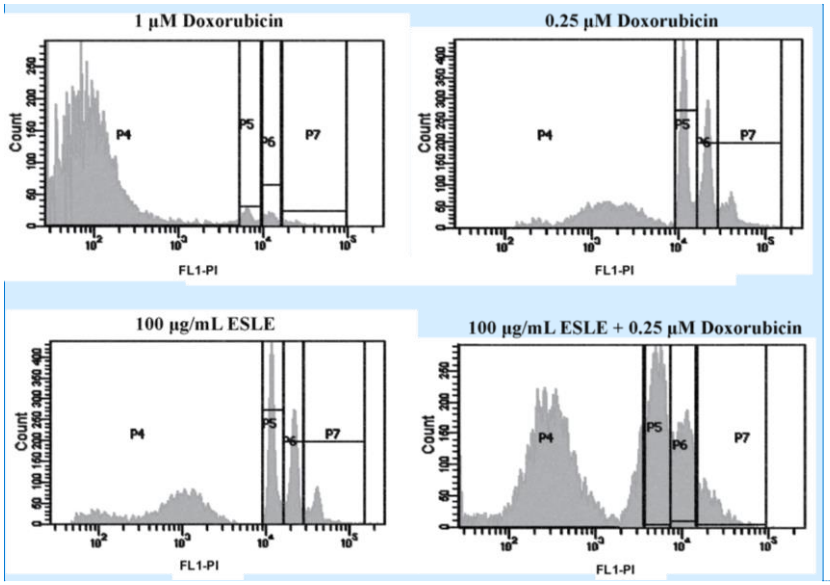

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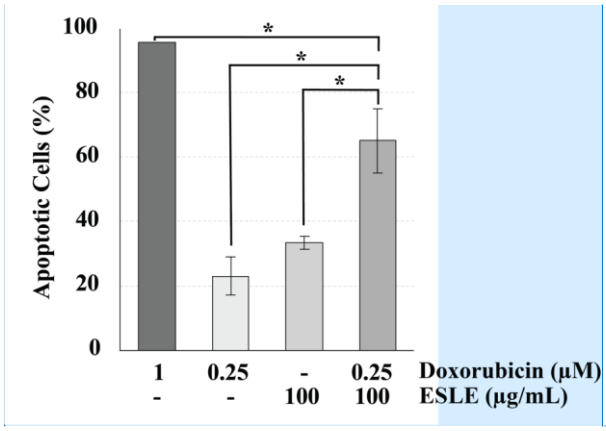

Figure 3. Combination of 100 µg/mL ESLE with 0.25 µM Doxorubicin Increased HSC-3 Apoptotic Cells. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM or 0.25 µM Doxorubicin, or 100 µg/mL ESLE for 24 h. Apoptotic cells were measured using Sub-G1

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test as outlined in Methods. (A) The flow cytometric results. (B) The percentage of HSC-3 apoptotic cells. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Mann-Whitney's *post hoc* test.

ESLE Decreased p-Surv (Thr34) of HSC-3 Cells

The results in Figure 4 showed that the p-Surv (Thr34) amount of HSC-3 cells in YM155 group (28±7) was significantly lower (Tukey's *post hoc* test, p=0.000) than the ones in the sham group (933±49.14) (Figure 5). The β-Actin was used as a loading control. The p-Surv (Thr34) amount in ESLE-treated group decreased significantly (ANOVA, p=0.000) in concentration-dependent manner. The p-Surv (Thr34) amount in the 1 µg/mL ESLE-treated group (659±125.74), 10 µg/mL ESLE-treated group (408±36.47) and 100 µg/mL ESLE-treated group (179±62.4) differed significantly (Tukey's *post hoc* test, p=0.000) than the ones in the sham group.

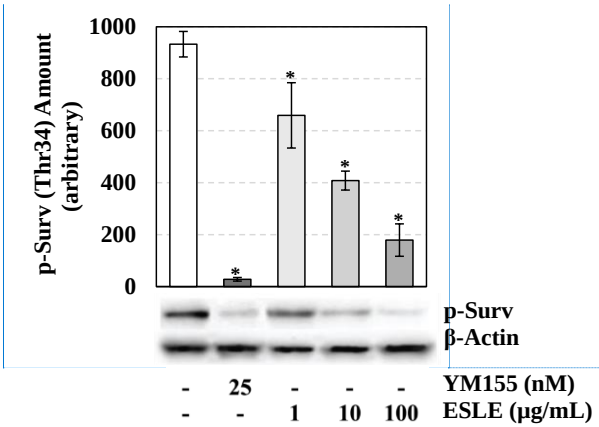

Figure 4. ESLE decreased the amount of p-Surv (Thr34) in HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 25 nM YM155 or ESLE in various concentrations. The amount of p-Surv (Thr34) was measured using Western Blot, as outlined in Methods. *Statistical significance (p<0.05) was determined using Tukey's *post hoc* test when compared to the sham group.

DISCUSSION

In the current study, ESLE exhibited a concentration-dependent cytotoxic effect on HSC-3 cells. Results from the MTT (Figure 1) and sub-G1 assays (Figure 2) showed a reduction in viable HSC-3 cells, which was attributed to apoptosis induction. These findings align with previous studies that

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reported the ability of ESLE to induce apoptosis in T47D (breast cancer), MCF-7 (breast cancer), and HCT116 (colorectal cancer) cell lines (18,19). IC_{50} of ESLE-induced apoptotic HSC-3 cells (222.34 µg/mL) was categorized as weak cytotoxicity (201-500 µg/mL) (22). This IC_{50} value was higher than those observed for ESLE-induced apoptotic T47D cells (132.17±9.69 µg/mL) (18), MCF-7 cells (14.69 ± 0.29 µg/mL) (19) and HCT116 cells (14.69 ± 0.29 µg/mL) (20). However, ….ketika Else dikombinasi doxo konsentrasi rendah…. bisa meningkatkan doxo). The combination of 100 µg/mL ESLE with 0.25 µM doxorubicin increased the percentage of HSC-3 apoptotic cells more than treatment with either agent alone (Figure 3). These results suggest a synergistic effect of combining 100 µg/mL ESLE and 0.25 µM doxorubicin in inducing apoptosis in HSC-3 cells. This finding indicates that ESLE can sensitize the apoptotic effect of doxorubicin, potentially allowing for lower doses of doxorubicin to be used in cancer therapy, thereby reducing its associated side effects.

The observed synergy between ESLE and doxorubicin is likely due to several interacting mechanisms. This combination seems to enhance doxorubicin-induced DNA damage and inhibit survival pathways in cancer cells, making them more susceptible to doxorubicin. This result aligns with previous studies that examined the effects of ESLE in combination with tamoxifen in MCF-7 breast cancer cells.(23) These studies demonstrated that ESLE enhances the effectiveness of tamoxifen in targeting breast cancer cells. The combination of ELSE and tamoxifen led to cell cycle arrest at the S phase, downregulation of pro-survival genes HSP 105, upregulation of the pro-apoptotic genes (ICAM1, VEGF, and c-Jun,), implicating both intrinsic and extrinsic apoptotic pathways.(23)

The present study showed that the ESLE-treated group exhibited a decrease in the p-Surv (Thr34) levels in HSC-3 cells (Figure 4) in a concentration-dependent manner. This reduction in p-Surv (Thr34) is comparable to the effect of YM155, a known survivin suppressant. YM155 has been shown to inhibit survivin expression and induce apoptosis in various cancer cell lines.(24,25) In this study, YM155 was used as a positive control to validate the effect of ESLE on survivin phosphorylation. The significant reduction in p-survivin levels in ESLE-treated cells, similar to that

observed with YM155, underscores the potential of ESLE as an effective anti-cancer agent targeting survivin. This result aligns with previous studies that showed ESLE decreased survivin expression at the transcript level.(26) Survivin, which is phosphorylated at Thr34 by the cyclin-dependent kinase CDK1 during the G2/M phase of the cell cycle, is crucial for its anti-apoptotic function. A reduction in p-survivin levels could disrupt the function, thereby promoting apoptosis in cancer cells.(27).

Further studies are needed to fully elucidate the mechanism of action of ESLE. Specifically, future research should examine other potential phosphorylation sites on survivin, such as Ser 70 and Ser81, may be crucial to its anti-apoptotic function. Moreover, exploring the effects of ESLE on other signaling pathways involved in cancer cell survival and proliferation could provide a more comprehensive understanding of its therapeutic potential.

CONCLUSION

Taken together, ESLE significantly enhances the efficacy of doxorubicin, thereby sensitizing its ability to induce apoptosis in HSC-3 tongue cancer cells. This sensitization occurs through the inhibition of survivin activity, particularly at the Thr34 phosphorylation site. These findings suggest that ESLE could serve as a potential adjuvant to improve the effectiveness of doxorubicin in inducing apoptosis in tongue cancer cells.

AUTHORS CONTRIBUTION

FS and TP were involved in conceptualizing and planning the research, performing data acquisition and collection, and conducting data analysis. FS and AES drafted the manuscript and designed the figures. FS, RAH, DR, and KHL aided in interpreting the results and provided critical discussion. FS, RAH, DR, TP, AES, and KHL participated in the critical revision of the manuscript.

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Manuscript Review Form

Reviewer	:	R2
Manuscript #	:	M2024239
Manuscript Title	:	*Elephantopus scaber* Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34

No.	Manuscript Components	Yes	No
1.	Does this manuscript present new ideas or results that have not been previously published?	✓	
	Notes:		
2.	Are the title and abstract of the manuscript appropriate?	✓	
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4.	Is the significance of the study well explained at the Background?	✓	
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	enough for publication?		
	Notes:		
7.	Are all figures and tables necessarily presented?	✓	
	Notes:		
8.	Is there a logical flow of argument in the Discussion which elucidate all the presented/obtained data?	✓	
	Notes:		
9.	Are the conclusions and interpretations valid and supported by the data?	✓	
	Notes:		
10.	Is the manuscript clear, comprehensible, and written in a good English structure?	✓	
	Notes: The manuscript has a few grammatical errors and non-English terms that may affect its readability.		

Specific Reviewer's Comments and Suggestions:

In this *in vitro* study, the authors present interesting data on the effects of Elephant's Foot or *Elephantopus scaber* Linn. leaf extract (ESLE) in combination with doxorubicin in inducing apoptosis in human oral squamous carcinoma (HSC)-3 cells. The authors demonstrate that ESLE enhances the efficacy of doxorubicin in promoting apoptosis in HSC-3 cells. Moreover, they report that this enhancement is attributed to the inhibitory effect of ESLE on survivin activity at the Thr34 phosphorylation site. This finding holds significant promise for the therapeutic potential of ESLE in oral cancer.

Sufficient information about previous studies is provided, allowing readers to understand the rationale and procedures of the current study. The experimental methods are robust, and the statistical analyses are appropriately conducted.

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Nevertheless, there are a few concerns that need to be addressed:

- Since apoptotic activity was investigated by assessing the Sub-G1 population using flow cytometry, it would be preferable to use the more familiar term "flow cytometry" rather than "Sub-G1 assay."
- A few grammatical errors were found in the manuscript. Additionally, there are non-English phrases in the Discussion section. The authors are requested to carefully review and revise the manuscript.

Reviewer's Recommendation (Please tick only one option)	√
Accept Submission (No significant alterations suggested)	
Revisions Required (Suggest changes to the manuscript as specified in this review)	✓
Resubmit for Review (Major revisions should be made and suggestions as specified in this review must be addressed. Revised manuscript should be resubmitted to the reviewer for further review)	
Decline Submission (Do not encourage a rewrite, manuscript is totally rejected)	

Further Reviewer's Comments Regarding Disposition of the Manuscript:

UNIVERSITAS TRISAKTI

Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2024239 Editor Decision Round 1 - Resubmit for Review

Ferry Sandra <ferry@trisakti.ac.id> Thu, Aug 22, 2024 at 4:58 PM
To: Secretariat of InaBJ <secretariatinabj@gmail.com>

Dear Secretariat of The Indonesian Biomedical Journal,

Thank you for your email and for the detailed feedback on our manuscript. We have carefully reviewed the comments and suggestions provided by the reviewers and editors. We have revised the manuscript accordingly and made sure to address all the points raised. Additionally, we have improved the English language to enhance clarity and readability.

Please find attached:

1. The revised manuscript with highlighted changes.
2. The response form detailing our answers to the reviewers' comments.

We have uploaded the revised manuscript and response form via the provided link. Please let us know if there are any additional steps required or if further revisions are needed. Thank you for your consideration and the opportunity to improve our work. We look forward to your feedback.

Best Regards,
Dr. Ferry Sandra

[Quoted text hidden]

--
Ferry Sandra, D.D.S., Ph.D.
Head of Medical Research Center
Universitas Trisakti

2 attachments

M2024239 Manuscript - Round 1 (Resubmit for Review)- Revised.docx
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Elephantopus scaber Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34

ABSTRACT

BACKGROUND: Previous research has demonstrated the effect of *Elephantopus scaber* (*E. scaber*) Linn. leaf extract (ESLE) on various cancer cell lines. However, research on the effects of ESLE on oral squamous cell carcinoma (OSCC), especially tongue cancer, is still lacking. Moreover, the apoptotic mechanisms induced by ESLE are not well understood and require further exploration. Therefore, this study was conducted to investigate the effects of ESLE on cell viability and apoptosis in human squamous cell carcinoma (HSC)-3 tongue cancer cells..

METHODS: HSC-3 cells were treated with varying concentrations of ESLE, doxorubicin, and a combination of both. Cell viability and apoptosis were assessed using MTT and Sub-G1 assays. The expression levels of survivin and its phosphorylated form at threonine (Thr)34 were evaluated using Western blot analysis.

RESULTS: ESLE exhibited a concentration-dependent cytotoxic effect on HSC-3 cells in decreasing cell viability (Kruskal Wallis, p=0.001) and increasing apoptotic cells (ANOVA, p=0.001) significantly. When combined with doxorubicin, ESLE further enhanced the induction of apoptosis compared with doxorubicin alone. The combined treatment resulted in a decrease in the levels of phosphorylated survivin (p-Surv) Thr34, indicating the inhibition of survivin's anti-apoptotic function.

CONCLUSION:

ESLE significantly enhances the efficacy of doxorubicin, thereby sensitizing its ability to induce apoptosis in HSC-3 tongue cancer cells. This sensitization occurs through the inhibition of survivin activity, particularly at the Thr34 phosphorylation site. These findings suggest that ESLE could serve as a potential adjuvant to improve the effectiveness of doxorubicin in inducing apoptosis in tongue cancer cells.

KEYWORDS: *Elephantopus scaber*, doxorubicin, tongue cancer, HSC-3 cells, apoptosis, Survivin, Thr34 phosphorylation

INTRODUCTION

The field of natural product research is growing rapidly, especially in the search for effective anticancer agents from plants.(1) Natural products are particularly well-suited for this purpose due to their minimal side effects, ability to target multiple cancer processes, and potential for synergistic effects.(2) Their complex chemical diversity and capacity make them valuable candidates for drug discovery and development.(3,4) Among many studied plants, *Elephantopus scaber* (*E. scaber*) Linn., which is known as Elephant's Foot and belongs to the Asteraceae family, stands out due to its traditional use in folk medicine and its promising results in modern research.(5)

Previous research has shown that some parts of *E. scaber*, such as the leaves and the roots, possess pharmacological activities due to their rich chemical composition.(6) This plant has gained attention for its potential therapeutic benefits. *E. scaber* contains a range of bioactive metabolites, including flavonoids, triterpenoids and sesquiterpene lactones.(7) The flavonoids in the plant are well-known for their strong antioxidant and anti-inflammatory properties.(8) Triterpenoids further enhance the plant's therapeutic value with their diverse effects, including anti-inflammatory and antimicrobial activities.(9) Additionally, sesquiterpene lactones, due to their complex structures, are noted for their potent biological effects, such as inducing cell death and inhibiting cell growth.(10)

A regulated process of cell death, apoptosis, plays a vital role in removing cancerous cells and inhibiting tumor progression.(11,12) This process can be triggered by either the mitochondrial (intrinsic) pathway, which is mediated by caspase-9, or the death-receptor (extrinsic) pathway, which is mediated by caspase-8. Ultimately, both pathways converge to activate the effector caspases-3 and -7, which execute the cell death program.(13) One of the critical regulators of apoptosis is Survivin, a key member of the inhibitor of apoptosis (IAP) protein family.(14) Survivin is essential in regulating

apoptosis by inhibiting caspase activity and promoting cell survival.(15) The phosphorylated variant of Survivin, known as p-Survivin (p-Surv) threonine 34 (Thr34), further modulates this function by altering its interactions with apoptotic machinery.(16) Phosphorylation at Thr34 affects Survivin's stability and its ability to bind to second mitochondria-derived activator of caspase/direct inhibitor of apoptosis-binding protein with low pI (Smac/DIABLO), a mitochondrial protein that promotes apoptosis by antagonizing IAPs.(17)

Previous studies have reported the effects of *E. scaber* leaf extract (ESLE) on breast cancer cell lines (18,19) and colorectal cancer cell lines (20). However, research on the effects of ESLE on oral squamous cell carcinoma (OSCC), especially tongue cancer, is lacking. Furthermore, the apoptotic mechanisms triggered by ESLE are not well understood and require further investigation. Consequently, this study aims to examine the effects of ESLE on cell viability and apoptosis in human squamous cell carcinoma (HSC)-3 tongue cancer cells.

MATERIAL AND METHODS

Preparation of *Elephantopus scaber* Linn. Leaf Extract (ESLE)

The leaves of *E. scaber* L. were acquired from Indonesian Institute for Testing Instrument Standard for Spices, Medicinal, and Aromatic Plant, Ministry of Agriculture. The ESLE was obtained using maceration technique. *E. scaber* leaves were finely minced and dried. The dehydrated material was extracted with 70% ethanol solution, followed by filtration and evaporation using rotary evaporator. The crude ESLE obtained was thereafter kept at a temperature of 4°C.

HSC-3 Cell Culture

The HSC-3 cell culture was conducted using a previously reported method (21), with specific modifications. The HSC-3 cell line was acquired from Sigma-Aldrich (St. Louis, MO, USA). HSC-3 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich) complete medium contained 50 U/mL penicillin 50 μg/mL, streptomycin (Sigma-Aldrich) and 10% fetal

bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany). The cells were cultured in a humidified incubator at 37°C, 5% CO_2. The HSC-3 cells were detached with trypsin-ethylenediamine tetraacetic acid (EDTA) solution (Sigma-Aldrich) once they reached 80% confluence.

Cell Viability Assay

The measurement of viable cells was conducted with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following a previously reported method.(13) In 96-well plates, HSC-3 cells were placed ($5x10^3$/well) and treated with/without 1, 10, or 100 μg/mL ESLE or 1 μM Doxorubicin (Dankos Farma, Jakarta, Indonesia) for 24 hours. MTT was added in the treated well (100 μL/well) and incubated for 4 hours. Then, the suspension in each well was removed and dissolved in 100 μL dimethylsulfoxide (DMSO). The formazan crystal that was formed was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at OD_{570}. The measurements for each experimental group were conducted in sextuplicate.

Sub-G1 Assay

Apoptotic HSC-3 cells were measured using the sub-G1 assay in order to evaluate the cytotoxic effects of ESLE, based on previously reported method.(13) Treated-HSC-3 cells were collected and incubated in a hypotonic fluorochrome solution (50 μg/mL of propidium iodide (Sigma-Aldrich), 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan)). Subsequently, the cell suspensions were incubated in darkness for 30 minutes. The fluorescence of individual nuclei was quantified using a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA), and a total of 400 events were recorded.

Western Blotting Assay

HSC-3 cells that were treated with/without various concentrations of ESLE or 25 nM YM155 (Tocris, Bristol, UK) were then harvested and incubated with radioimmunoprecipitation assay (RIPA) lysis

buffer (Thermo Fisher Scientific, Waltham, USA). YM155, a survivin suppressant, was used as a positive control in this study to demonstrate its ability to reduce or inhibit p-Surv. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) sheet. After blocking with 5% skim milk in Tris-buffered saline (TBS, 150nM NaCl and 50 nM Tris-HCl, pH 7.4), the sheets were incubated with rabbit polyclonal anti-β-Actin (Cat. No. 4967; Cell Signaling, Danvers, USA) and rabbit polyclonal anti-phospho-survivin (Thr34) (Cat. No. 8888; Cell Signaling) antibody. The secondary antibody was horseradish peroxidase-conjugated donkey anti-rabbit (Cell Signaling) IgG antibody, diluted 1:1000. The bound antibodies were visualized using Clarity Western ECL (Bio-Rad, Hercules, CA, USA) and captured using Alliance 4.7 (UVItech, Cambridge, UK).

Statistical Analysis

The Shapiro-Wilk normality test was utilized for statistical analysis. Then, one-way ANOVA test was used to analyze the findings of a normally distributed dataset, followed by Tukey's *post hoc* test. Subsequently, the results of abnormal data distribution were tested using Kruskal-Wallis test, followed by Mann-Whitney's *post hoc* test.

RESULTS

ESLE Decreased HSC-3 Viable Cells

The results in Figure 1 showed that the number of HSC-3 viable cells in 1 μM doxorubicin group (56±11.12) was significantly lower (Mann-Whitney's *post hoc* test, p=0.004) than the ones in the sham group (9,212±65.58). The number of HSC-3 viable cells in ESLE-treated groups decreased significantly (Kruskal Wallis, p=0.001) in concentration-dependent manner. The number of HSC-3 viable cells in 1 μg/mL ESLE-treated group (9,268±424.76) did not significantly differ (Mann-Whitney's *post hoc* test, p=0.423) than the ones in the sham group, meanwhile the number of HSC-3 viable cells in 10 μg/mL ESLE-treated group (8,173±316.61) and 100 μg/mL ESLE-treated group

(6,952±602.94) 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 ESLE in inducing apoptosis of HSC-3 cells was 222.34 μg/mL.

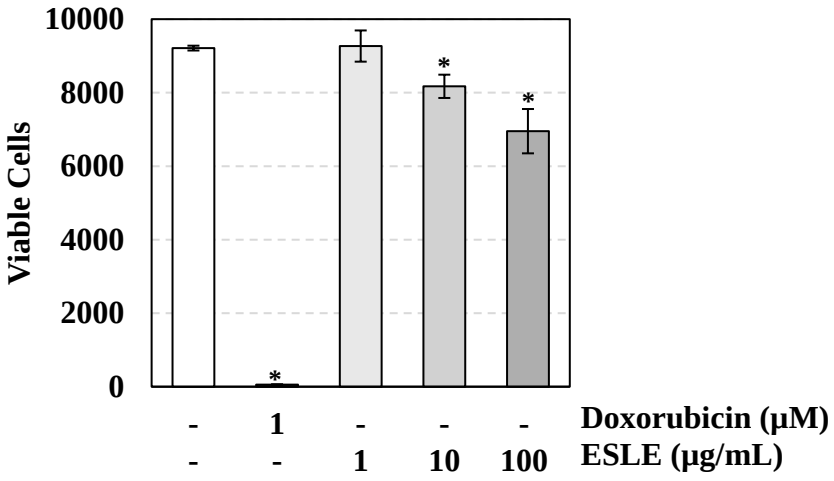
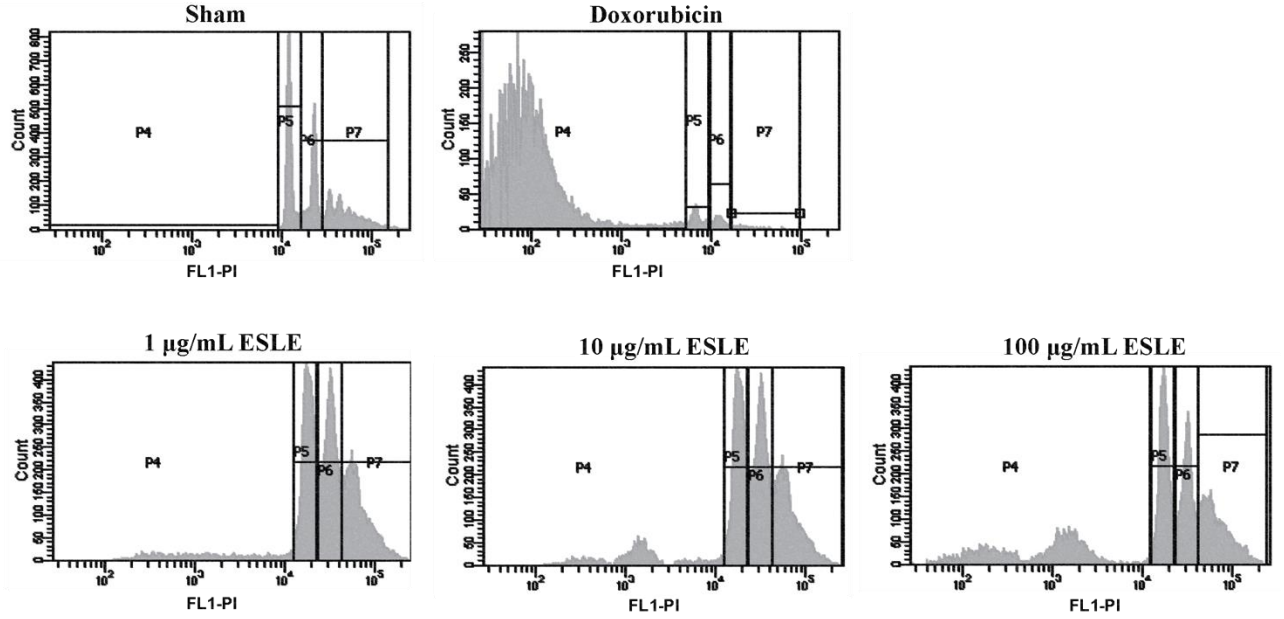


Figure 1. ESLE decreased HSC-3 viable cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 μM Doxorubicin or ESLE in different concentrations for 24 h. Viable cells were measured using MTT assay as outlined in methods. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Mann-Whitney's *post hoc* test when compared to the sham group.

ESLE Increased HSC-3 Apoptotic Cells

The results in Figure 2 showed that the percentage of HSC-3 apoptotic cells in 1 μM doxorubicin group (95.73±0.48%) was significantly higher (Tukey's *post hoc* test, p=0.001) than the ones in the sham group (4.62±0.48%) (Figure 2). The percentage of HSC-3 apoptotic cells in ESLE-treated groups increased significantly (ANOVA, p=0.001) in concentration-dependent manner. The percentage of HSC-3 apoptotic cells in 1 μg/mL ESLE-treated group (6.08±0.34%) did not significantly differ (Tukey's *post hoc* test, p=0.120) than the ones in the sham group, meanwhile percentage of HSC-3 apoptotic cells in 10 μg/mL ESLE-treated group (18.88±0.75%) and 100 μg/mL ESLE-treated group (33.45±2.09%) differed significantly (Tukey's *post hoc* test, p=0.001) than the ones in the sham group.

A



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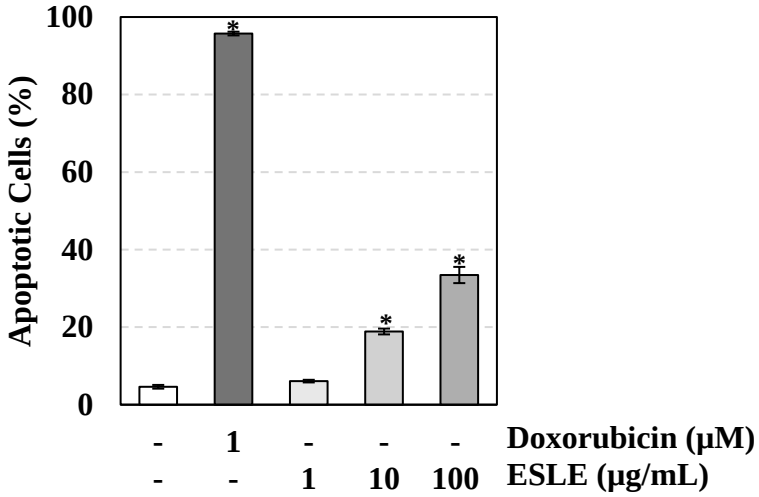

Figure 2. ESLE increased HSC-3 apoptotic cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different concentrations for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. (A) The flow cytometric results. (B) The percentage of HSC-3 apoptotic cells. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Tukey's *post hoc* test when compared to the sham group.

Combination of 100 µg/mL ESLE with 0.25 µM Doxorubicin Increased HSC-3 Apoptotic Cells

The results in Figure 3 showed that the percentage of HSC-3 apoptotic cells in 1 µM doxorubicin group (95.71±0.47%) was significantly higher (Mann-Whitney's *post hoc* test, p=0.004) than the ones in the 100 µg/mL ESLE + 0.25 µM doxorubicin-treated group (65.00±10.05%). The percentage of HSC-3 apoptotic cells in the 0.25 µM Doxorubicin group (22.98±5.98%) and the 100 µg/mL ESLE-

treated group (33.45±2.09%) were low. However, the percentage of HSC-3 apoptotic cells in the 100 µg/mL ESLE + 0.25 µM doxorubicin-treated group was significantly higher (Mann-Whitney's *post hoc* test, p=0.004) compared to the 0.25 µM Doxorubicin group and the 100 µg/mL ESLE-treated group.

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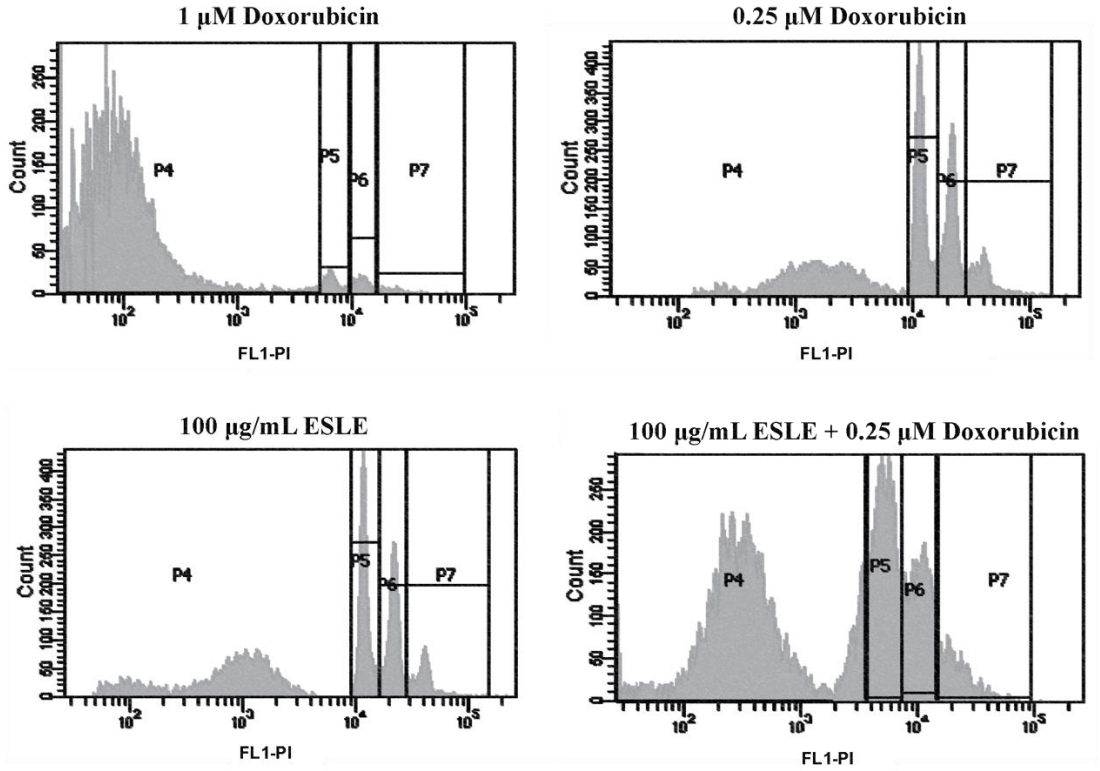

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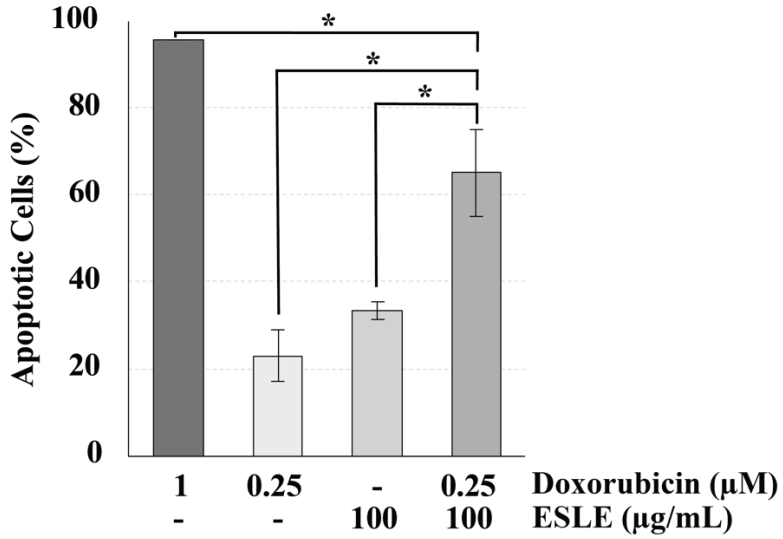

Figure 3. Combination of 100 µg/mL ESLE with 0.25 µM Doxorubicin Increased HSC-3 Apoptotic Cells. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM or 0.25 µM Doxorubicin, or 100 µg/mL ESLE for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. (A) The flow cytometric results. (B) The percentage of HSC-3 apoptotic

cells. The results are presented as mean±standard deviation (n=6). *Statistical significance (p<0.05) was determined using Mann-Whitney's *post hoc* test.

ESLE Decreased p-Surv (Thr34) of HSC-3 Cells

The results in Figure 4 showed that the p-Surv (Thr34) amount of HSC-3 cells in YM155 group (28±7) was significantly lower (Tukey's *post hoc* test, p=0.000) than the ones in the sham group (933±49.14) (Figure 5). The β-Actin was used as a loading control. The p-Surv (Thr34) amount in ESLE-treated group decreased significantly (ANOVA, p=0.000) in concentration-dependent manner. The p-Surv (Thr34) amount in the 1 μg/mL ESLE-treated group (659±125.74), 10 μg/mL ESLE-treated group (408±36.47) and 100 μg/mL ESLE-treated group (179±62.4) differed significantly (Tukey's *post hoc* test, p=0.000) than the ones in the sham group.

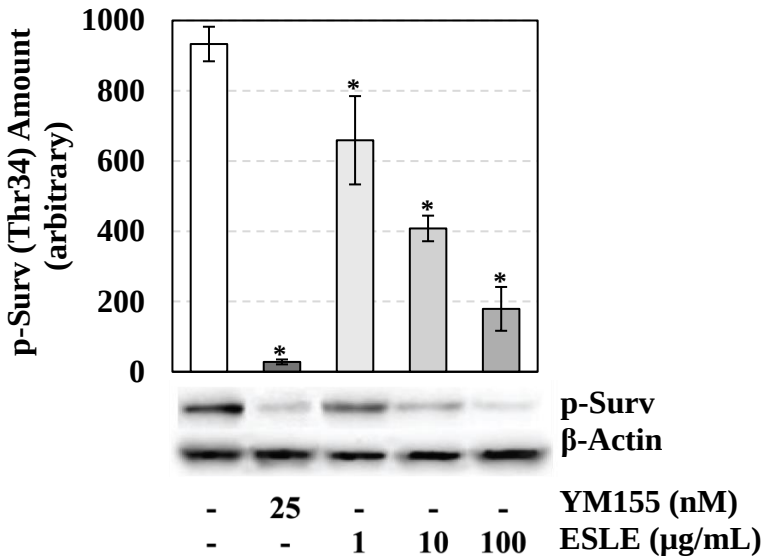

Figure 4. ESLE decreased the amount of p-Surv (Thr34) in HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 25 nM YM155 or ESLE in various concentrations. The amount of p-Surv (Thr34) was measured using Western Blot, as outlined in Methods. *Statistical significance (p<0.05) was determined using Tukey's *post hoc* test when compared to the sham group.

DISCUSSION

In the current study, ESLE exhibited a concentration-dependent cytotoxic effect on HSC-3 cells. Results from the MTT (Figure 1) and sub-G1 assays (Figure 2) showed a reduction in viable HSC-3 cells, which was attributed to apoptosis induction. These findings align with previous studies that reported the ability of ESLE to induce apoptosis in T47D (breast cancer), MCF-7 (breast cancer), and

HCT116 (colorectal cancer) cell lines (18,19). IC_{50} of ESLE in inducing apoptosis of HSC-3 cells (222.34 µg/mL) was categorized as weak cytotoxicity (201-500 µg/mL) (22). This IC_{50} value was higher than those in inducing apoptosis of T47D cells (132.17±9.69 µg/mL) (18), MCF-7 cells (14.69±0.29 µg/mL) (19) and HCT116 cells (14.69±0.29 µg/mL) (20). However, although having weak cytotoxicity, ESLE could enhance the potency of doxorubicin in inducing apoptosis of HSC-3 cells. Specifically, the combination of 100 µg/mL ESLE with 0.25 µM doxorubicin increased the percentage of HSC-3 apoptotic cells more than treatment with either agent alone (Figure 3). These results suggest a synergistic effect of combining 100 µg/mL ESLE and 0.25 µM doxorubicin in inducing apoptosis in HSC-3 cells. This finding indicates that ESLE can sensitize the apoptotic effect of doxorubicin, potentially allowing for lower doses of doxorubicin to be used in cancer therapy, thereby reducing its associated side effects.

The observed synergy between ESLE and doxorubicin is likely due to several interacting mechanisms. This combination seems to enhance doxorubicin-induced DNA damage and inhibit survival pathways in cancer cells, making them more susceptible to doxorubicin. This result aligns with previous studies that examined the effects of ESLE in combination with tamoxifen in MCF-7 breast cancer cells.(23) These studies demonstrated that ESLE enhances the effectiveness of tamoxifen in targeting breast cancer cells. The combination of ESLE and tamoxifen led to cell cycle arrest at the S phase, downregulation of pro-survival genes heat shock protein (HSP) 105, upregulation of the pro-apoptotic genes, implicating both intrinsic and extrinsic apoptotic pathways.(23)

The present study showed that the ESLE-treated group exhibited a decrease in the p-Surv (Thr34) levels in HSC-3 cells (Figure 4) in a concentration-dependent manner. This reduction in p-Surv (Thr34) is comparable to the effect of YM155, a known survivin suppressant. YM155 has been shown to inhibit survivin expression and induce apoptosis in various cancer cell lines.(24,25) In this study, YM155 was used as a positive control to validate the effect of ESLE on survivin phosphorylation. The significant reduction in p-survivin levels in ESLE-treated cells, similar to that

observed with YM155, underscores the potential of ESLE as an effective anti-cancer agent targeting survivin. This result aligns with previous studies that showed ESLE decreased survivin expression at the transcript level.(26) Survivin, which is phosphorylated at Thr34 by the cyclin-dependent kinase CDK1 during the G2/M phase of the cell cycle, is crucial for its anti-apoptotic function. A reduction in p-survivin levels could disrupt the function, thereby promoting apoptosis in cancer cells.(27).

Further studies are needed to fully elucidate the mechanism of action of ESLE. Specifically, future research should examine other potential phosphorylation sites on survivin, such as Ser 70 and Ser81, may be crucial to its anti-apoptotic function. Moreover, exploring the effects of ESLE on other signaling pathways involved in cancer cell survival and proliferation could provide a more comprehensive understanding of its therapeutic potential.

CONCLUSION

Taken together, ESLE significantly enhances the efficacy of doxorubicin, thereby sensitizing its ability to induce apoptosis in HSC-3 tongue cancer cells. This sensitization occurs through the inhibition of survivin activity, particularly at the Thr34 phosphorylation site. These findings suggest that ESLE could serve as a potential adjuvant to improve the effectiveness of doxorubicin in inducing apoptosis in tongue cancer cells.

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Response Form for Reviewer's Comments

Correspondin : Ferry Sandra
Manuscript C : M2024239
Manuscript T : Elephantopus scaber Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34

Reviewer	**Comments** (Comments/question from reviewer or editor)	**Author's Response** (Please write your response regarding the comment here)	**Line Number** (Please write the line number of the said revision)
R1 #1	The name of the cells better the same like title Tongue Cancer Cells	Thank you for your suggestion. We have revised to "human squamous cell (HSC)-3 carcinoma tongue cancer cells" throughout the manuscript. This revision ensures that the terminology is consistent with the title and accurately reflects the specific cell line used in our study.	10 and 64
R1 #2	Highlighted: "However, ….ketika Else dikombinasi doxo konsentrasi rendah…. bisa meningkatkan doxo)."	We apologize for the oversight. The highlighted section has been corrected for clarity and proper scientific language.	209-212
R2 #1	Since apoptotic was investigated by assessing the Sub-G1 population using flow cytometry, it would be preferable to use the more familiar term "flow cytometry" rather than "Sub-G1 assay"	Thank you for your insightful feedback. We understand your suggestion to use "flow cytometry" for clarity. However, we will continue using the term "sub-G1 assay" in our manuscript, as it specifically refers to the measurement of the sub-G1 population which is central to our analysis of apoptosis. We appreciate your understanding and will ensure that the context and methodology are clearly explained to avoid any confusion.	
R2 #2	A few grammatical errors were found in the manuscript. Additionally, there are non English phrases in the Discussion section. The authors are requested to carefully review and revise the manuscript.	We appreciate your careful review. The manuscript has been revised to correct the grammatical errors, and the non-English phrases in the Discussion section have been translated to ensure clarity and consistency.	209-212

R3 #1	YM155, what is it?	Thank you for your question. YM155 has been clearly mentioned and defined in the manuscript to ensure that readers are familiar with its role and significance in our study.	105-106
R3 #2	Please put control in figure 3A to compare the result with doxo and ESLE	Thank you for your suggestion. We would like to clarify that the control data you are requesting to be included in Figure 3A has already been presented in Figure 2A of the manuscript (sham group). Including the sahm group again in Figure 3A would result in redundancy, as the purpose of Figure 3A is to focus specifically on the effects of doxorubicin and ESLE, building upon the data already in Figure 2A. We hope this clarification helps in understanding our presentation strategy.	
R3 #3	Check the legend of Fig 3B, there are two of 0,25 in doxorubicin, and 100 in ELSE, is it typo ?	Thank you for your observation. There seems to be a misunderstanding. The figure legend for Figure 3B correctly represents the experimental conditions. Specifically: - The concentration of 0.25 refers to doxorubicin alone. - The concentration of 100 refers to ESLE alone. - Additionally, there is a combination of 0.25 doxorubicin and 100 ESLE. These are distinct experimental groups designed to evaluate the individual and combined effects of doxorubicin and ESLE on the cells. Therefore, the legend accurately reflects the different conditions and is not a typographical error. We hope this explanation clarifies the information presented in Figure 3B.	

R3 #4	Do you have combination of doxo 0,25 and 100 ELSE ? Please put it (in figure 4) to compare the result with others	Thank you for your suggestion. Since we are specifically examining the effect of ESLE on cellular defense mechanisms, including cell viability and apoptosis, doxorubicin was not effective in this context due to its lack of impact on apoptotic cells. Therefore, YM155, which works through a different mechanism by targeting survivin, was used instead of doxorubicin. We believe	

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[InaBJ] M2024239 Letter of Acceptance

Secretariat of InaBJ <secretariatinabj@gmail.com> Wed, Sep 4, 2024 at 10:17 AM
To: Ferry Sandra <ferry@trisakti.ac.id>

Dear Dr Ferry Sandra,

Please have the certificate of acceptance for manuscript "E**lephantopus scaber Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34**", which has been published in Indonesian Biomedical Journal Vol.16 No.4.

Congratulations on your interesting research, and thank you for allowing us to publish this valuable material. Please let us know once you have read this email. We wish you a nice day.

Best Regards,

--
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NOMOR : 479/BKD/FKG-USAKTI/VIII/2024

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Dasar : Sehubungan dengan kegiatan publikasi penelitian dan penulisan Jurnal Ilmiah yang dilaksanakan oleh Para Dosen/Staf Pengajar Fakultas Kedokteran Gigi Universitas Trisakti.

M E N U G A S K A N :

Kepada : drg. Ferry Sandra, Ph.D
drg. Ria Aryani Hayuningtyas, M.Sc.
drg. Dewi Ranggaini, M.K.G

Untuk : Melakukan penulisan pada jurnal ilmiah dengan judul : *Elephantopus scaber Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34* yang dipublikasikan di bulan Agustus 2024 pada The Indonesian Biomedical Journal.

Demikian agar tugas tersebut dilaksanakan dengan penuh rasa tanggung jawab.

Ditetapkan di : Jakarta
Pada tanggal : 1 Agustus 2024
Dekan,

drg. Wiwiek Poedjiastoeti, M.Kes., Sp.BMM., Ph.D.