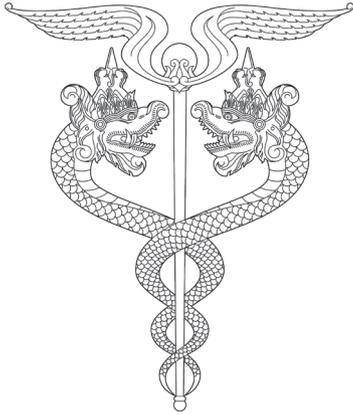


The Indonesian BIOMEDICAL JOURNAL



Volume 16 Number 4
August 2024

Published by:



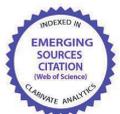
Secretariat: Prodia Tower 9th Floor
Jl. Kramat Raya No.150,
Jakarta 10430, Indonesia
Tel.: +62-21-3144182
Fax.: +62-21-3144181
E-mail: Secretariat@InaBJ.org
Website: www.InaBJ.org



#200/M/KPT/2020

Included in:

Scopus[®] DOAJ DIRECTORY OF
OPEN ACCESS JOURNALS



REVIEW ARTICLES

Sarcopenic Obesity: The Underlying Molecular Pathophysiology and Prospect Therapies

Meiliana A, Dewi NM, Defi IR, Rosdianto AM, Qiantori AA, Wijaya A

RESEARCH ARTICLES

The Regulation of SPRY4 Intronic Transcript 1 (SPRY4-IT1) on KIT Signaling and Imatinib Resistance of Gastrointestinal Stromal Tumor (GIST) Cells

Yu Y, Jiang Z, Zhao S, Liu C, Ma J, Li S

Beetroot (*Beta vulgaris* L.) Extract Gives Superior Effect than Beetroot Juice on Increasing HDL and Decreasing LDL and IL-6 in Dyslipidemic Rats Model

Riri MJ, Harioputro DR, Wardhani LO

Andrographis paniculata Ethanol Extract Alleviates High Glucose-induced Senescence of Human Umbilical Vein Endothelial Cells via the Regulation of mTOR and SIRT1 Pathways

Khatimah NG, Arozal W, Barinda AJ, Antarianto RD, Hardiany NS, Shimizu I, Fadhillah MR

The Injected Plasma of Myasthenia Gravis Patient with A Low T-reg Level Caused Clinical Myasthenic Syndromes in Swiss-Webster Mice

Pasmanasari ED, Purwaningsih EH, Retnaningsih R, Purba JS, Octaviana F

Lactiplantibacillus plantarum IS-10506 Supplementation Improves Clinical Outcome and Immunology Markers in Psoriasis Vulgaris Patients: A Randomized Controlled Trial

Umborowati MA, Hasna IH, Endaryanto A, Surono IS, Prakoeswa CRS

Normal Value of Thrombocytes Indices in Indonesian Adults: Focus on Gender and Ages

Sukorini U, Arjana AZ, Ratnaningsih T, Satria RD

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

Sandra F, Hayuningtyas RA, Ranggaini D, Pang T, Scania AE, Lee KH

Urinary PYD/Creatinine Ratio Has Negative Correlation to Serum 25(OH)D and Positive Correlation to Chronic Lead Exposure Index

Hajar SS, Mudjihartini N, Manikam NRM, Mulyana, Mansyur M

Exosomal miRNAs as Potential Biomarkers for Preeclampsia: miR-1283 Has the Highest Expression, while miR-152-3p Has the Lowest Expression

Sumawan H, Giantari I, Mubarika S, Hadiati DR, Pradjatmo H

The Indonesian BIOMEDICAL JOURNAL

Volume 16 Number 4, August 2024

Editor in Chief

Dewi Muliaty (Prodia Clinical Laboratory, Indonesia)

Board of Editors

Dinath Ratnayake (The University of Western Ontario, Canada)
Geraldine Budomo Dayrit (University of the Philippines, Philippines)
Joseph Bercmans Lopez (MAHSA University College, Malaysia)
Koichi Nakayama (Saga University, Japan)
Rajiv Timothy Erasmus (Stellenbosch University, South Africa)
Rizky Abdulah (Universitas Padjadjaran, Indonesia)
Roberto Volpe (National Research Council of Italy, Italy)
Tar Choon AW (ICON Central Laboratory, Singapore)
Trilis Yulianti (Prodia Clinical Laboratory, Indonesia)

Editorial Team

Gerard Pals (Amsterdam University Medical Center, Netherlands)
Ines Atmosukarto (Australian National University, Australia)
Irawan Satriotomo (University of Florida, United States of America)
Solachuddin Jauhari Arief Ichwan (Universiti Brunei Darussalam, Brunei Darussalam)

Peer Reviewers

Adekunle Bashiru Okesina (University of Ilorin Teaching Hospital, Nigeria)
Antonia Anna Lukito (Universitas Pelita Harapan, Indonesia)
Anwar Santoso (Universitas Indonesia, Indonesia)
Cynthia Retna Sartika (Prodia Stem Cell Laboratory, Indonesia)
Prof. Djangan Sargowo (Universitas Brawijaya, Indonesia)
Elizabeth Henny Herningtyas (Universitas Gadjah Mada, Indonesia)
Indriyanti Rafi Sukmawati (Prodia Clinical Laboratory, Indonesia)
Jajah Fachiroh (Universitas Gadjah Mada, Indonesia)
Khosrow Adeli (University of Toronto, Canada)
Laifa A Hendarmin (Syarif Hidayatullah State Islamic University, Indonesia)
Marita Kaniawati (Universitas Bhakti Kencana, Indonesia)
Melisa Intan Barliana (Universitas Padjadjaran, Indonesia)
Miki Nakajima (Kanazawa University, Japan)
Rahajuningsih Dharma Setiabudy (Universitas Indonesia, Indonesia)
Raj Kumar Yadav (Anderson Cancer Center/University of Texas, USA)
Samuel Vasikaran (Fiona Stanley Hospital, Australia)
Siti Boedina Kresno (Universitas Indonesia, Indonesia)
Sunarno (Ministry of Health of Republic Indonesia, Indonesia)
Yenny Surjawan (Prodia Clinical Laboratory, Indonesia)

Contact Address

Secretariat of The Indonesian Biomedical Journal
Prodia Tower 9th Floor
Jl. Kramat Raya No.150, Jakarta 10430, Indonesia
Tel.: +62-21-3144182, ext. 3872
Fax.: +62-21-3144181
WhatsApp No.: +62 877-3616-3117
E-mail: Secretariat@InaBJ.org
Website: www.InaBJ.org

Focus & Scope

The Indonesian Biomedical Journal (InaBJ) is an open access, peer-reviewed journal that encompasses all fundamental and molecular aspects of basic medical sciences, emphasizing on providing the molecular studies of biomedical problems and molecular mechanisms.

InaBJ is dedicated to publish original research and review articles covering all aspects in biomedical sciences. The editors will carefully select manuscript to present only the most recent findings in basic and clinical sciences. All professionals concerned with biomedical issues will find this journal a most valuable update to keep them abreast of the latest scientific development.

Section Policies

Review Article

Review Article should consist of no more than 10,000 words, not including the words in abstract, references, table, figure, and figure legend. The manuscript should have no more than eight figures and/or tables in total and no more than 250 references. Only invited authors are allowed to submit review article.

Research Article

Research Article should consist of no more than 3,500 words, not including the words in abstract, references, table, figure, and figure legend. The manuscript should have no more than six figures and/or tables in total and no more than 40 references.

Peer Review Process

All manuscripts submitted to InaBJ will be selected and double-blind peer-reviewed by two or more reviewers to present valuable and authentic findings in biomedical sciences. At least, an external reviewer will be included as the reviewer in each manuscript reviewing process.

Author can suggest reviewer/s that not having publication together within five years and should not be member/s of the same research institution. However, reviewers will be selected independently by Section Editor based on their expertise, specialties, and independencies to fit the topic. Section Editor will ensure that the reviewers will be not from the same institution as the author.

Manuscript will be reviewed comprehensively, including appropriate title; content reflecting abstract; concise writing; clear purpose, study method and figures and/or tables; and summary supported by content. Supplementary data will also be sent to reviewer. The reviewing process will take generally 2-3 months depends on sufficiency of information provided.

Decisions are ultimately made by the Section Editor based on the peer-reviewing results. Therefore, Section Editor will consider thoroughly, if necessary Section Editor can invite another one or more reviewer/s to conclude the final decision.

Publication Frequency

InaBJ is published bimonthly (in February, April, June, August, October, and December).

Open Access Policy

InaBJ provides immediate open access to its content on the principle that making research freely available to the public supports a greater global exchange of knowledge.

Content

The Indonesian Biomedical Journal
Volume 16 Number 4, August 2024

REVIEW ARTICLE

Sarcopenic Obesity: The Underlying Molecular Pathophysiology and Prospect Therapies

Meiliana A, Dewi NM, Defi IR, Rosdianto AM, Qiantori AA, Wijaya A
p.292-308

RESEARCH ARTICLE

The Regulation of SPRY4 Intronic Transcript 1 (SPRY4-IT1) on KIT Signaling and Imatinib Resistance of Gastrointestinal Stromal Tumor (GIST) Cells

Yu Y, Jiang Z, Zhao S, Liu C, Ma J, Li S
p.309-23

Beetroot (*Beta vulgaris* L.) Extract Gives Superior Effect than Beetroot Juice on Increasing HDL and Decreasing LDL and IL-6 in Dyslipidemic Rats Model

Riri MJ, Harioputro DR, Wardhani LO
p.324-32

***Andrographis paniculata* Ethanol Extract Alleviates High Glucose-induced Senescence of Human Umbilical Vein Endothelial Cells via the Regulation of mTOR and SIRT1 Pathways**

Khatimah NG, Arozal W, Barinda AJ, Antarianto RD, Hardiany NS, Shimizu I, Fadhillah MR
p.333-42

The Injected Plasma of Myasthenia Gravis Patient with A Low T-reg Level Caused Clinical Myasthenic Syndromes in Swiss-Webster Mice

Pasmanasari ED, Purwaningsih EH, Retnaningsih R, Purba JS, Octaviana F
p.343-52

RESEARCH ARTICLE

***Lactiplantibacillus plantarum* IS-10506 Supplementation Improves Clinical Outcome and Immunology Markers in Psoriasis Vulgaris Patients: A Randomized Controlled Trial**

Umborowati MA, Hasna IH, Endaryanto A, Surono IS, Prakoeswa CRS
p.353-62

Normal Value of Thrombocytes Indices in Indonesian Adults: Focus on Gender and Ages

Sukorini U, Arjana AZ, Ratnaningsih T, Satria RD
p.363-71

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

Sandra F, Hayuningtyas RA, Ranggaini D, Pang T, Scania AE, Lee KH
p.372-8

Urinary PYD/Creatinine Ratio Has Negative Correlation to Serum 25(OH)D and Positive Correlation to Chronic Lead Exposure Index

Hajar SS, Mudjihartini N, Manikam NRM, Mulyana, Mansyur M
p.379-86

Exosomal miRNAs as Potential Biomarkers for Preeclampsia: miR-1283 Has the Highest Expression, while miR-152-3p Has the Lowest Expression

Sumawan H, Giantari I, Mubarika S, Hadiati DR, Pradjatmo H
p.387-96

RESEARCH ARTICLE

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

Ferry Sandra^{1,*}, Ria Aryani Hayuningtyas¹, Dewi Ranggaini², Tiffany Pang³,
Alifah Evi Scania⁴, Kyung Hoon Lee⁵

¹Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia

²Department of Physiology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia

³Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia

⁴The Prodia Education and Research Institute, Jl. Kramat Raya No. 150, Jakarta, 10430, Indonesia

⁵Research Institute, Ballys Co. Ltd, Incheon-22219, Republic of Korea

*Corresponding author. Email: ferry@trisakti.ac.id

Received date: May 20, 2024; Revised date: Aug 22, 2024; Accepted date: Aug 23, 2024

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

Indones Biomed J. 2024; 16(4): 372-8

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% CO₂. 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 (5×10³/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, IC₅₀ 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

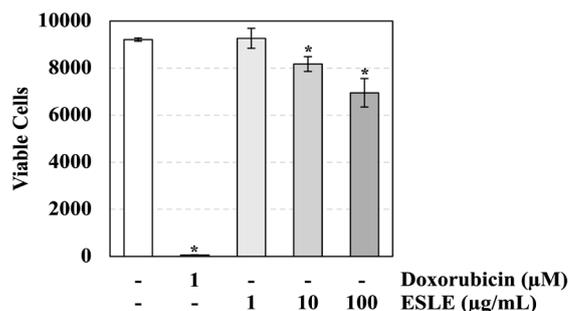


Figure 1. ESLE decreased HSC-3 viable cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 μM Doxorubicin or ESLE in different concentrations for 24 h. Viable cells were measured using MTT assay as outlined in methods. The results are presented 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.

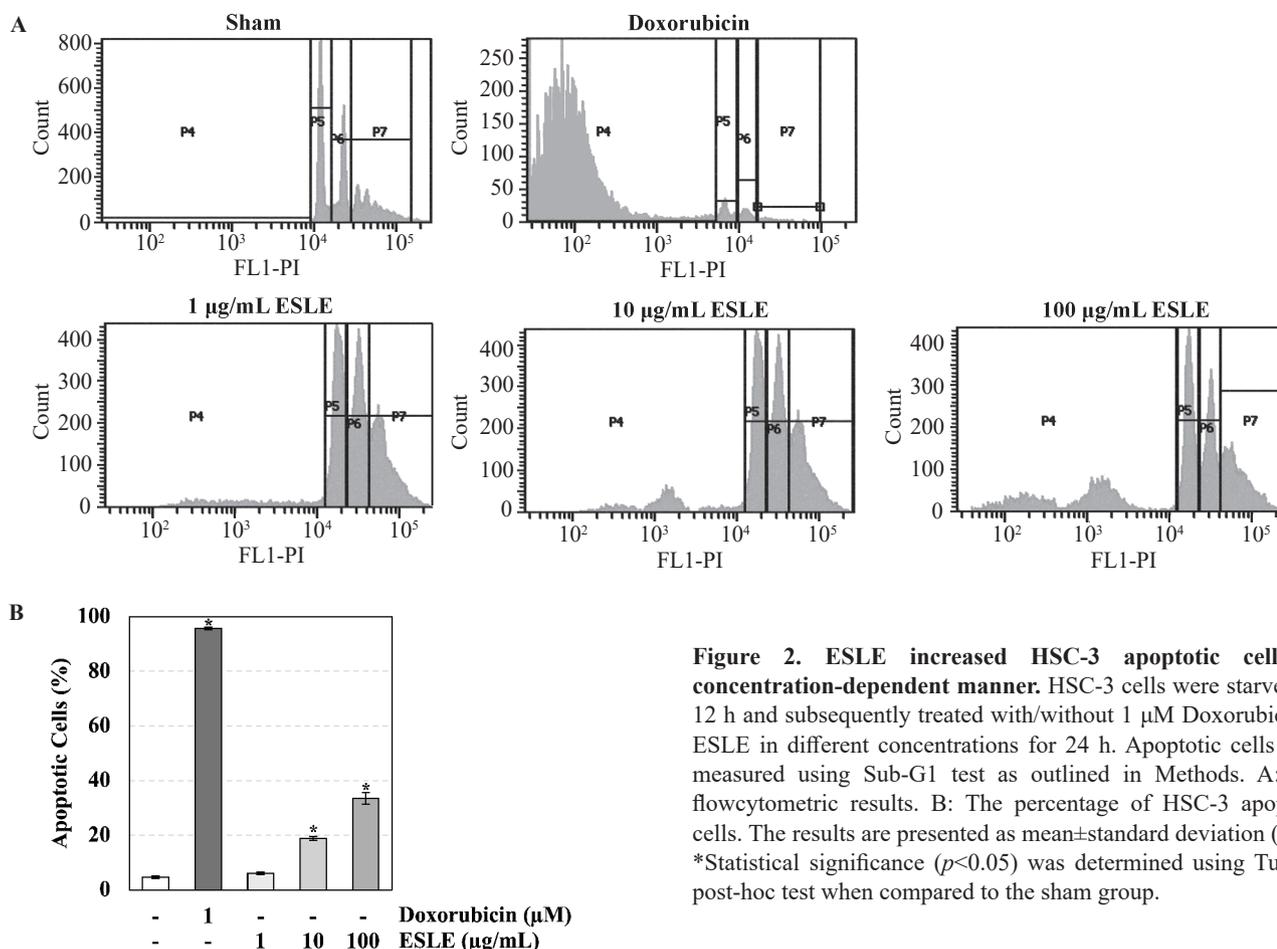


Figure 2. ESLE increased HSC-3 apoptotic cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different concentrations for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. A: The flowcytometric 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 ($65.00\pm10.05\%$). The percentage of HSC-3 apoptotic cells in the $0.25\ \mu\text{M}$ Doxorubicin group ($22.98\pm5.98\%$) and the $100\ \mu\text{g/mL}$ ESLE-treated group ($33.45\pm2.09\%$) were low. However, the percentage of HSC-3 apoptotic cells in the $100\ \mu\text{g/mL}$ ESLE + $0.25\ \mu\text{M}$ doxorubicin-treated group was significantly higher (Mann-Whitney’s post-hoc test, $p=0.004$) compared to the $0.25\ \mu\text{M}$ Doxorubicin group and the $100\ \mu\text{g/mL}$ 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 $1\ \mu\text{g/mL}$ ESLE-treated group (659 ± 125.74), $10\ \mu\text{g/mL}$ ESLE-treated group (408 ± 36.47) and $100\ \mu\text{g/mL}$ 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 ($222.34\ \mu\text{g/mL}$) was categorized as weak cytotoxicity ($201\text{-}500\ \mu\text{g/mL}$).(22) This IC_{50} value was higher than those in inducing apoptosis of T47D cells ($132.17\pm9.69\ \mu\text{g/mL}$) (18), MCF-7 cells ($14.69\pm0.29\ \mu\text{g/mL}$) (19) and HCT116 cells ($14.69\pm0.29\ \mu\text{g/mL}$) (20). However, although having weak cytotoxicity, ESLE could enhance the potency of doxorubicin in inducing apoptosis of HSC-3 cells. Specifically, the combination of $100\ \mu\text{g/mL}$ ESLE with $0.25\ \mu\text{M}$ doxorubicin increased the percentage of HSC-3 apoptotic cells more than treatment with either agent alone (Figure 3). These results suggest a

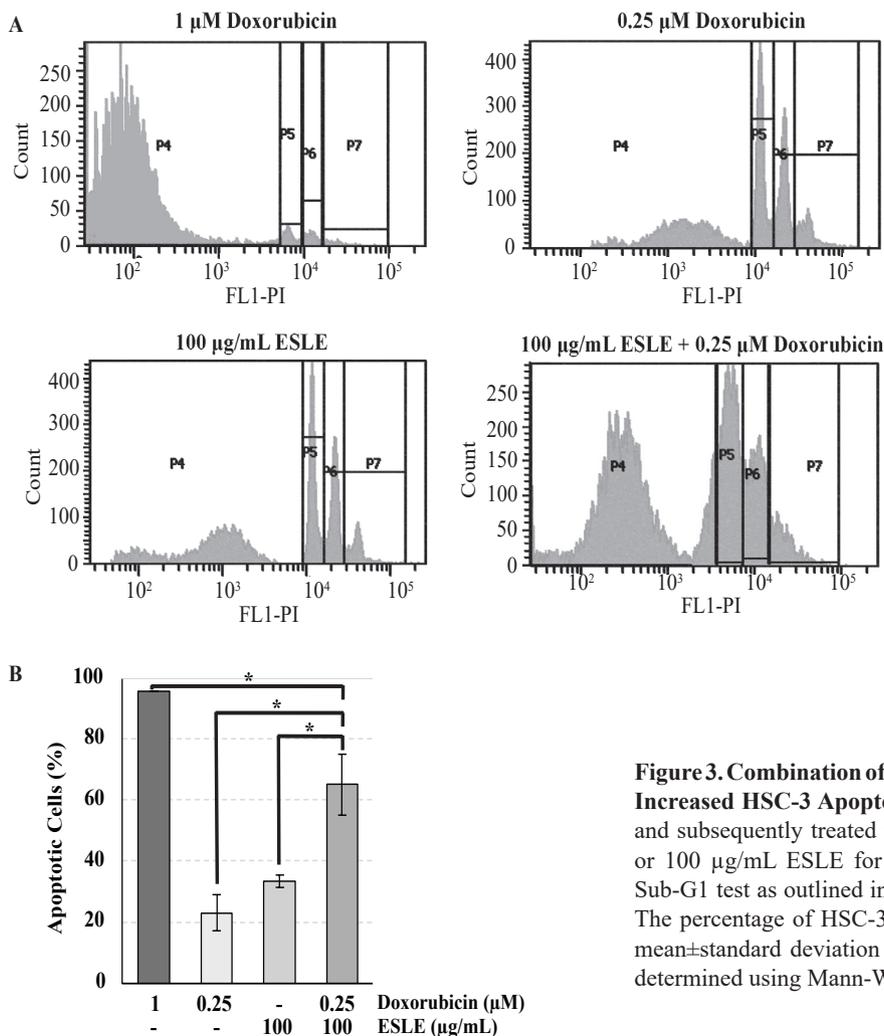


Figure 3. Combination of 100 μg/mL ESLE with 0.25 μM Doxorubicin Increased HSC-3 Apoptotic Cells. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 μM or 0.25 μM Doxorubicin, or 100 μg/mL ESLE for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. A: 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-survival 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-survival levels could disrupt the function, thereby promoting apoptosis in cancer cells.(27).

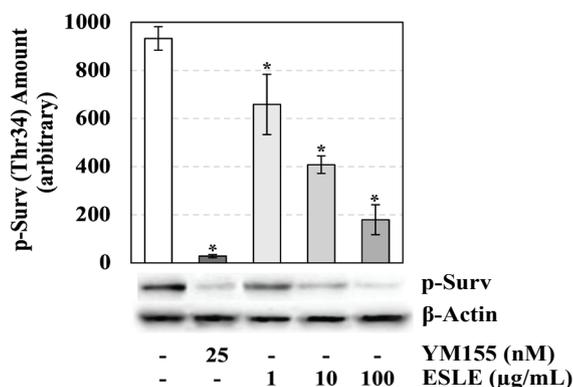


Figure 4. ESLE decreased the amount of p-Surv (Thr34) in HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 25 nM YM155 or ESLE in various concentrations. The amount of p-Surv (Thr34) was measured using Western Blot, as outlined in Methods. *Statistical significance ($p < 0.05$) 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.

References

- Naeem A, Hu P, Yang M, Zhang J, Liu Y, Zhu W, Zheng Q. Natural products as anticancer agents: Current status and future perspectives. *Molecules*. 2022; 27(23): 8367. doi: 10.3390/molecules27238367.
- Castañeda AM, Meléndez CM, Uribe D, Pedroza-Díaz J. Synergistic effects of natural compounds and conventional chemotherapeutic agents: recent insights for the development of cancer treatment strategies. *Heliyon*. 2022; 8(6): e09519. doi: 10.1016/j.heliyon.2022.e09519.
- Eziefulo OM, Arozal W, Wanandi SI, Louisa M, Wuyung PE, Dewi S, *et al.* Andrographis paniculata ethanolic extract improved doxorubicin-induced cardiac inflammation, alterations in liver function parameters and anemia. *Mol Cell Biomed Sci*. 2024; 8(2): 117-26.
- Callixte C, Baptiste NJ, Arwati H. Phytochemical screening and antimicrobial activities of methanolic and aqueous leaf extracts of Carica papaya grown in Rwanda. *Mol Cell Biomed Sci*. 2020; 4(1): 39-44.
- Nguyen PAT, Khang DT, Nguyen PTT, Do HDK. The complete chloroplast genome of Elephantopus scaber L. (Vernoniaceae, asteraceae), a useful ethnomedicinal plant in Asia. *Mitochondrial DNA B Resour*. 2023; 8(9): 936-41.
- Hiradeve SM, Rangari VD. Elephantopus scaber Linn.: A review on its ethnomedical, phytochemical and pharmacological profile. *J Appl Biomed*. 2014; 12(2): 49-61.
- Hiradeve SM, Rangari VD. A review on pharmacology and toxicology of Elephantopus scaber Linn. *Nat Prod Res*. 2014; 28(11): 819-30.
- Andari D, Khan FI, Jakfar SI. Methanol extract of katuk (Sauropus androgynus) leaves as an anti-inflammatory agent: Animal study in carrageenan-induced rat models of inflammation. *Mol Cell Biomed Sci*. 2022; 6(3): 129-34.
- Girsang E, Lister INE, Ginting CN, Khu A, Samin B, Widowati W, *et al.* Chemical constituents of snake fruit (Salacca zalacca (Gaert.) Voss) peel and in silico anti-aging analysis. *Mol Cell Biomed Sci*. 2019; 3(2): 122-8.
- Hjazi A, Alissa M, Alqasem AA, Alghamdi A, Alghamdi SA. Cynaropicrin, a sesquiterpene lactone, triggers apoptotic cell death in triple negative breast cancer cells. *Mol Biol Rep*. 2024; 51(1): 856. doi: 10.1007/s11033-024-09723-y. PMID: 39066893.
- Haifa R, Sartika CR, Faried A, Hadisaputri YE, Chow A, Wijaya A, *et al.* Potency of peripheral blood-and umbilical cord blood-derived dendritic cells and their secretomes as vaccines for cancer. *Mol Cell Biomed Sci*. 2024; 8(1): 31-6.
- Sandra F, Hendarmin L, Nakao Y, Nakamura N, Nakamura S. TRAIL cleaves caspase-8,-9 and-3 of AM-1 cells: A possible pathway for TRAIL to induce apoptosis in ameloblastoma. *Tumor Biol*. 2005; 26(5): 258-64.
- Sandra F, Rizal MI, Dhaniar AY, Scania AE, Lee KH. Cosmos caudatus leaf extract triggers apoptosis of HSC-3 cancer cells by decreasing bcl-2 and increasing bax. *Indones Biomed J*. 2024; 16(3): 285-91.
- Wanandi SI, Syahrani RA, Suraduhita A, Yunita E, Louisa M. Andrographolide reverses doxorubicin resistance in human breast cancer stem cells by regulating apoptotic gene expressions. *Indones Biomed J*. 2023; 15(5): 288-96.
- Rahman MN, Wijaya CR, Novalentina M. Survivin clinical features in cervical cancer. *Mol Cell Biomed Sci*. 2017; 1(1): 6-16.
- Liao J, Qing X, Deng G, Xiao Y, Fu Y, Han S, *et al.* Gastrodin destabilizes survivin and overcomes pemetrexed resistance. *Cell*

- Signal. 2023; 110: 110851. doi: 10.1016/j.cellsig.2023.110851.
17. Pandey SK, Paul A, Shteinfein-Kuzmine A, Zalk R, Bunz U, Shoshan-Barmatz V. SMAC/diablo controls proliferation of cancer cells by regulating phosphatidylethanolamine synthesis. *Mol Oncol.* 202; 15(11): 3037-61.
 18. Sulistyani N, Nurkhasanah. The cytotoxic effect of *Elephantopus scaber* Linn extract against breast cancer (T47D) cells. *IOP Conf Ser Mater Sci Eng.* 2017; 259: 012006. doi: 10.1088/1757-899X/259/1/012006
 19. Ho WY, Yeap SK, Ho CL, Raha AR, Suraini AA, Alitheen NB. *Elephantopus scaber* induces cytotoxicity in MCF-7 human breast cancer cells via p53-induced apoptosis. *J Med Plants Res.* 2011; 5(24): 5741-9.
 20. Chan CK, Supriady H, Goh BH, Kadir HA. *Elephantopus scaber* induces apoptosis through ROS-dependent mitochondrial signaling pathway in HCT116 human colorectal carcinoma cells. *J Ethnopharmacol.* 2015; 168: 291-304.
 21. Wicaksono BD, Tangkearung E, Sandra F. *Brucea javanica* leaf extract induced apoptosis in human oral squamous cell carcinoma (HSC-2) cells by attenuation of mitochondrial membrane permeability. *Indones Biomed J.* 2015; 7(2): 107-10.
 22. Sajjadi SE, Ghanadian M, Haghghi M, Mouhebat L. Cytotoxic effect of *Cousinia verbascifolia* Bunge against OVCAR-3 and HT-29 cancer cells. *J Herbmec Pharmacol.* 2015; 4(1): 15-19.
 23. Ho WY, Liew SS, Yeap SK, Alitheen, NB. Synergistic cytotoxicity between *Elephantopus scaber* and tamoxifen on MCF-7-derived multicellular tumor spheroid. *Evid Based Complement Alternat Med.* 2021; 2021: 6355236. doi: 10.1155/2021/6355236.
 24. Mackay RP, Weinberger PM, Copland JA, Mahdavian E, Xu Q. YM155 induces DNA damage and cell death in anaplastic thyroid cancer cells by inhibiting DNA topoisomerase α at the ATP-binding site. *Mol Cancer Ther.* 2022; 21(6): 925-35.
 25. Premkumar DR, Jane EP, Foster KA, Pollack IF. Survivin inhibitor YM-155 sensitizes tumor necrosis factor- related apoptosis-inducing ligand-resistant glioma cells to apoptosis through Mcl-1 downregulation and by engaging the mitochondrial death pathway. *J Pharmacol Exp Ther.* 2013; 346(2): 201-10.
 26. Kabeer FA, Rajalekshmi DS, Nair MS, Prathapan R. Molecular mechanisms of anticancer activity of deoxyelephantopin in cancer cells. *Integr Med Res.* 2017; 6(2): 190-206.
 27. Hu F, Pan D, Zheng W, Yan T, He X, Ren F, *et al.* Elucidating respective functions of two domains BIR and C-helix of human IAP survivin for precise targeted regulating mitotic cycle, apoptosis and autophagy of cancer cells. *Oncotarget.* 2017; 8(69): 113687-700.



Digital Receipt

This receipt acknowledges that Turnitin received your paper. Below you will find the receipt information regarding your submission.

The first page of your submissions is displayed below.

Submission author: Ferry Sandra
Assignment title: SIJALI 1
Submission title: Elephantopus scaber Linn. Leaf Extract Sensitizes Doxorubic...
File name: 3096-8750-2-PB.pdf
File size: 1.13M
Page count: 7
Word count: 4,238
Character count: 23,315
Submission date: 25-Sep-2024 09:10AM (UTC+0700)
Submission ID: 2307765485

DOI: 10.15585/iahy.v1i04.3096 *Elephantopus scaber* Sensitizes Doxorubicin in Inducing Apoptosis of HSC-3 Cells (Sandra F, et al.) *Indonesian Biomed J* 2024; 16(4): 372-8

RESEARCH ARTICLE

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

Ferry Sandra^{1*}, Ria Aryani Hayuningtyas¹, Dewi Ranggaini², Tiffany Pang³, Alifiah Evi Scania⁴, Kyang Hoon Lee⁵

¹Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11460, Indonesia
²Department of Physiology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia
³Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11460, Indonesia
⁴The Prodia Education and Research Institute, Jl. Kramat Raya No. 150, Jakarta, 10430, Indonesia
⁵Research Institute, Bafly Co. Ltd, Incheon-22219, Republic of Korea

*Corresponding author. Email: ferry@trisakti.ac.id
Received date: May 20, 2024; Revised date: Aug 22, 2024; Accepted date: Aug 23, 2024

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 (Thr34) 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

Indonesian Biomed J 2024; 16(4): 372-8

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

Copyright © 2024 The Prodia Education and Research Institute.
This work is licensed under a Creative Commons Attribution-NonCommercial 4.0 International (CC BY-NC) License.

372

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

by Ferry Sandra

Submission date: 25-Sep-2024 09:10AM (UTC+0700)

Submission ID: 2307765485

File name: 3096-8750-2-PB.pdf (1.13M)

Word count: 4238

Character count: 23315

RESEARCH ARTICLE

***Elephantopus scaber* Linn. Leaf Extract Sensitizes Doxorubicin in Inducing Apoptosis in HSC-3 Tongue Cancer Cells through Inhibiting Survivin Activity at Thr34**Ferry Sandra^{1,*}, Ria Aryani Hayuningtyas¹, Dewi Ranggaini², Tiffany Pang³,
Alifah Evi Scania⁴, Kyung Hoon Lee⁵¹Department of Biochemistry and Molecular Biology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia²Department of Physiology, Division of Oral Biology, Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia³Faculty of Dentistry, Universitas Trisakti, Jl. Kyai Tapa No. 260, Jakarta 11440, Indonesia⁴The Prodia Education and Research Institute, Jl. Kramat Raya No. 150, Jakarta, 10430, Indonesia⁵Research Institute, Ballys Co. Ltd, Incheon-22219, Republic of Korea

*Corresponding author. Email: ferry@trisakti.ac.id

Received date: May 20, 2024; Revised date: Aug 22, 2024; Accepted date: Aug 23, 2024

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

Indones Biomed J. 2024; 16(4): 372-8

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% CO₂. 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 (5×10³/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.(12) 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 (Toctris, 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, Tris-buffered saline (TBS, 150 nM NaCl and 50 nM Tris-HCl, 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 μ M doxorubicin group (56 ± 11.12) was significantly lower (Mann-Whitney's post-hoc test, $p=0.004$) than the ones in the sham group ($9,212 \pm 65.58$). 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 μ M ESLE-treated group ($9,268 \pm 424.76$) 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 μ M ESLE-treated group ($8,173 \pm 316.61$) and 100 μ M ESLE-treated group ($6,952 \pm 602.94$) 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 μ g/mL.

ESLE Increased HSC-3 Apoptotic Cells

The results in Figure 2 showed that the percentage of HSC-3 apoptotic cells in 1 μ M doxorubicin group ($95.73 \pm 0.48\%$) was significantly higher (Tukey's post-hoc test, $p=0.001$) than the ones in the sham group ($4.62 \pm 0.48\%$). 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 μ M ESLE-treated group ($6.08 \pm 0.34\%$) 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 μ M ESLE-treated group ($18.88 \pm 0.75\%$) and 100 μ M ESLE-treated group ($33.45 \pm 2.09\%$) differed significantly (Tukey's post-hoc test, $p=0.001$) than the ones in the sham group.

Combination of 100 μ g/mL ESLE with 0.25 μ M Doxorubicin Increased HSC-3 Apoptotic Cells

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

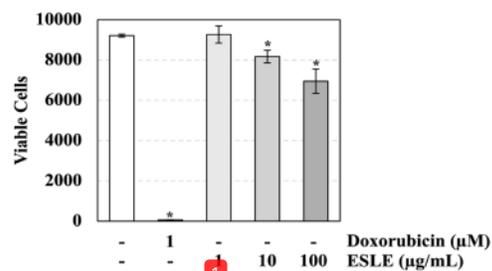


Figure 1. ESLE decreased HSC-3 viable cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 μ M Doxorubicin or ESLE in different concentrations for 24 h. Viable cells were measured using MTT assay as outlined in methods. The results are presented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

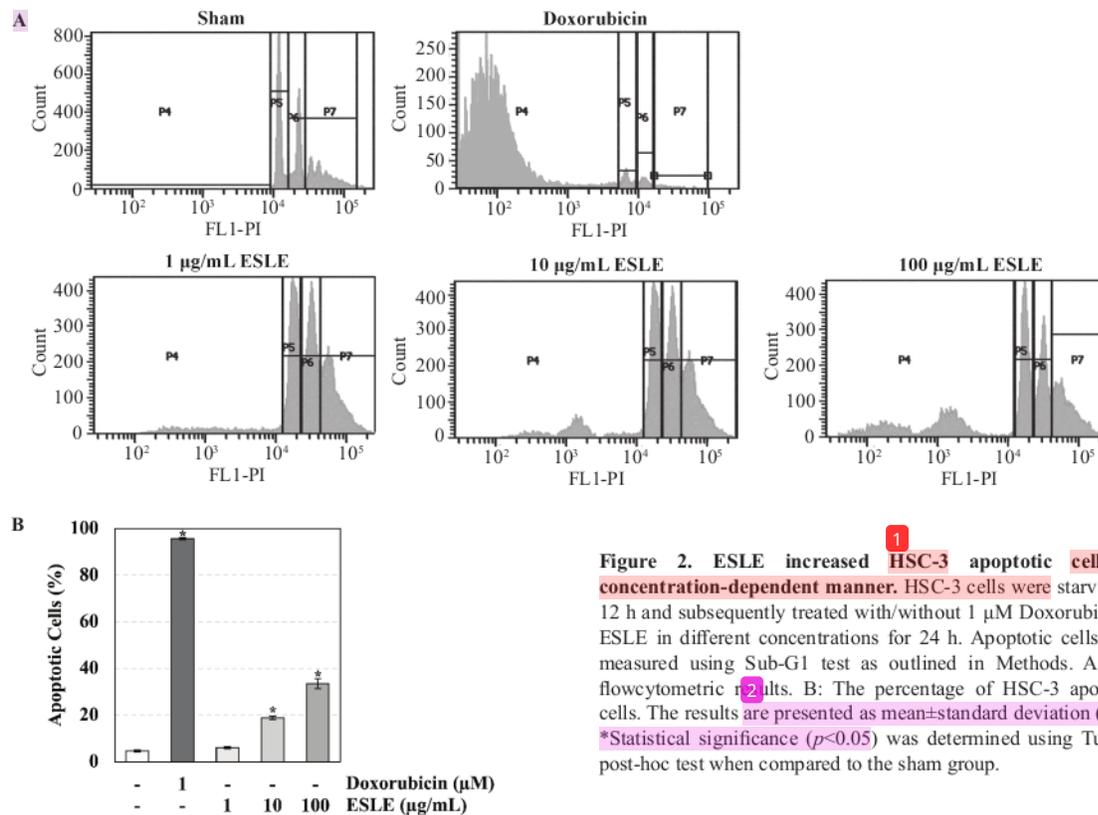


Figure 2. ESLE increased HSC-3 apoptotic cells in concentration-dependent manner. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different concentrations for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. A: The flow cytometry 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 ($65.00\pm 10.05\%$). The percentage of HSC-3 apoptotic cells in the $0.25\ \mu\text{M}$ Doxorubicin group ($22.98\pm 5.98\%$) and the $100\ \mu\text{g/mL}$ ESLE-treated group ($33.45\pm 2.09\%$) were low. However, the percentage of HSC-3 apoptotic cells in the $100\ \mu\text{g/mL}$ ESLE + $0.25\ \mu\text{M}$ doxorubicin-treated group was significantly higher (Mann-Whitney's post-hoc test, $p=0.004$) compared to the $0.25\ \mu\text{M}$ Doxorubicin group and the $100\ \mu\text{g/mL}$ 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 $1\ \mu\text{g/mL}$ ESLE-treated group (659 ± 125.74), $10\ \mu\text{g/mL}$ ESLE-treated group (408 ± 36.47) and $100\ \mu\text{g/mL}$ 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 ($222.34\ \mu\text{g/mL}$) was categorized as weak cytotoxicity ($201\text{-}500\ \mu\text{g/mL}$). (22) This IC_{50} value was higher than those in inducing apoptosis of T47D cells ($132.17\pm 9.69\ \mu\text{g/mL}$) (18), MCF-7 cells ($14.69\pm 0.29\ \mu\text{g/mL}$) (19) and HCT116 cells ($14.69\pm 0.29\ \mu\text{g/mL}$) (20). However, although having weak cytotoxicity, ESLE could enhance the potency of doxorubicin in inducing apoptosis of HSC-3 cells. Specifically, the combination of $100\ \mu\text{g/mL}$ ESLE with $0.25\ \mu\text{M}$ doxorubicin increased the percentage of HSC-3 apoptotic cells more than treatment with either agent alone (Figure 3). These results suggest a

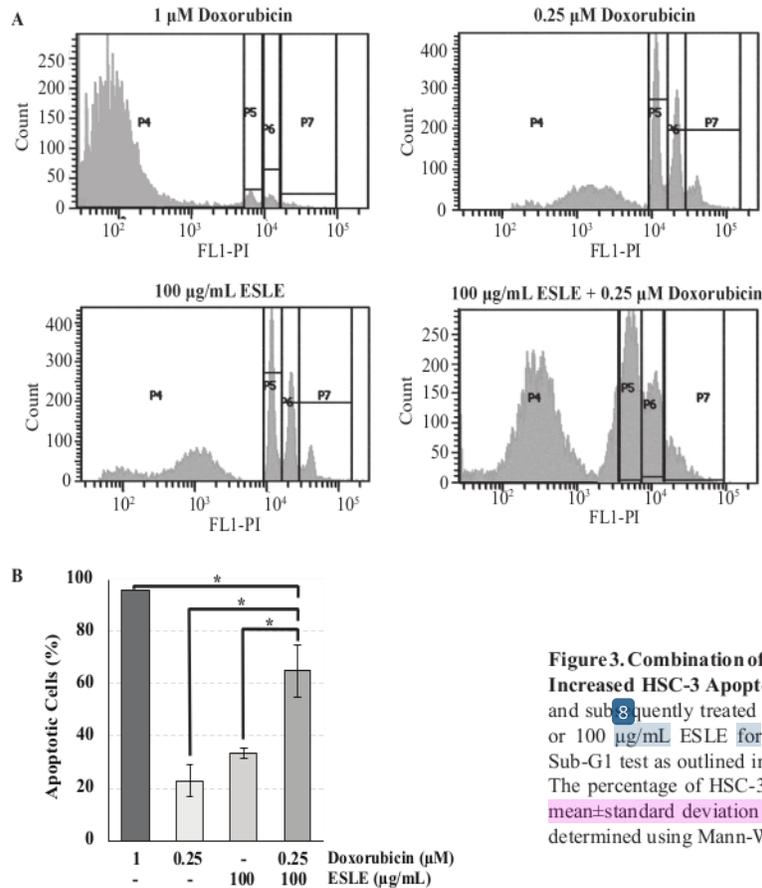


Figure 3. Combination of 100 μg/mL ESLE with 0.25 μM Doxorubicin Increased HSC-3 Apoptotic Cells. HSC-3 cells were starved for 12 h and subsequently treated with/without 1 μM or 0.25 μM Doxorubicin, or 100 μg/mL ESLE for 24 h. Apoptotic cells were measured using Sub-G1 test as outlined in Methods. A: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

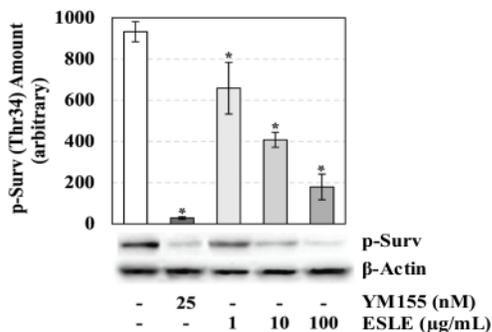


Figure 4. ESLE decreased the amount of p-Surv (Thr34) in HSC-3 cells in concentration-dependent manner. HSC-3 cells were treated with/without 25 nM YM155 or ESLE in various concentrations. The amount of p-Surv (Thr34) was measured using Western Blot, as outlined in Methods. *Statistical significance ($p < 0.05$) 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.

References

- Nacem A, Hu P, Yang M, Zhang J, Liu Y, Zhu W, Zheng Q. Natural products as anticancer agents: Current status and future perspectives. *Molecules*. 2022; 27(23): 8367. doi: 10.3390/molecules27238367.
- Castañeda AM, Meléndez CM, Uribe D, Pedroza-Díaz J. Synergistic effects of natural compounds and conventional chemotherapeutic agents: recent insights for the development of cancer treatment strategies. *Heliyon*. 2022; 8(6): e09519. doi: 10.1016/j.heliyon.2022.e09519.
- Eziefulo OM, Arozal W, Wanandi SI, Louisa M, Wuyung PE, Dewi S, *et al*. *Andrographis paniculata* ethanolic extract improved doxorubicin-induced cardiac inflammation, alterations in liver function parameters and anemia. *Mol Cell Biomed Sci*. 2024; 8(2): 117-26.
- Callixte C, Baptiste NJ, Arwati H. Phytochemical screening and antimicrobial activities of methanolic and aqueous leaf extracts of *Carica papaya* grown in Rwanda. *Mol Cell Biomed Sci*. 2020; 4(1): 39-44.
- Nguyen PAT, Khang DT, Nguyen PTT, Do HDK. The complete chloroplast genome of *Elephantopus scaber* L. (Vernoniaceae, asteraceae), a useful ethnomedicinal plant in Asia. *Mitochondrial DNA B Resour*. 2023; 8(9): 936-41.
- Hiradeve SM, Rangari VD. *Elephantopus scaber* Linn.: A review on its ethnomedical, phytochemical and pharmacological profile. *J Appl Biomed*. 2014; 12(2): 49-61.
- Hiradeve SM, Rangari VD. A review on pharmacology and toxicology of *Elephantopus scaber* Linn. *Nat Prod Res*. 2014; 28(11): 819-30.
- Andari D, Khan FI, Jakfar SI. Methanol extract of katuk (*Sauropus androgynus*) leaves as an anti-inflammatory agent: Animal study in carrageenan-induced rat models of inflammation. *Mol Cell Biomed Sci*. 2022; 6(3): 129-34.
- Gisang E, Lister INE, Ginting CN, Khu A, Samin B, Widowati W, *et al*. Chemical constituents of snake fruit (*Salacca zalacca* (Gaert.) Voss) peel and in silico anti-aging analysis. *Mol Cell Biomed Sci*. 2019; 3(2): 122-8.
- Hjazi A, Alissa M, Alqasem AA, Alghamdi A, Alghamdi SA. Cynaropicrin, a sesquiterpene lactone, triggers apoptotic cell death in triple negative breast cancer cells. *Mol Biol Rep*. 2024; 51(1): 856. doi: 10.1007/s11033-024-09723-y. PMID: 39066893.
- Haifa R, Sartika CR, Faried A, Hadisaputri YE, Chouw A, Wijaya A, *et al*. Potency of peripheral blood-and umbilical cord blood-derived dendritic cells and their secretomes as vaccines for cancer. *Mol Cell Biomed Sci*. 2024; 8(1): 31-6.
- Sandra F, Hendamin L, Nakao Y, Nakamura N, Nakamura S. TRAIL cleaves caspase-8,-9 and-3 of AM-1 cells: A possible pathway for TRAIL to induce apoptosis in ameloblastoma. *Tumor Biol*. 2005; 26(5): 258-64.
- Sandra F, Rizal MI, Dhaniar AY, Scania AE, Lee KH. *Cosmos caudatus* leaf extract triggers apoptosis of HSC-3 cancer cells by decreasing bcl-2 and increasing bax. *Indones Biomed J*. 2024; 16(3): 285-91.
- Wanandi SI, Syahrani RA, Suraduhita A, Yunita E, Louisa M. *Andrographolide* reverses doxorubicin resistance in human breast cancer stem cells by regulating apoptotic gene expressions. *Indones Biomed J*. 2023; 15(5): 288-96.
- Rahman MN, Wijaya CR, Novalentina M. Survivin clinical features in cervical cancer. *Mol Cell Biomed Sci*. 2017; 1(1): 6-16.
- Liao J, Qing X, Deng G, Xiao Y, Fu Y, Han S, *et al*. *Gastrodin* destabilizes survivin and overcomes pemetrexed resistance. *Cell*

- Signal. 2023; 110: 110851. doi: 10.1016/j.cellsig.2023.110851.
17. Pandey SK, Paul A, Shteinfer-Kuzmine A, Zalk R, Bunz U, Shoshan-Barmatz V. SMAC/diablo controls proliferation of cancer cells by regulating phosphatidylethanolamine synthesis. *Mol Oncol.* 202; 15(11): 3037-61.
 18. Sulistyani N, Nurkhasanah. The cytotoxic effect of *Elephantopus scaber* Linn extract against breast cancer (T47D) cells. *IOP Conf Ser Mater Sci Eng.* 2017; 259: 012006. doi: 10.1088/1757-899X/259/1/012006
 19. Ho WY, Yeap SK, Ho CL, Raha AR, Suraini AA, Alitheen NB. *Elephantopus scaber* induces cytotoxicity in MCF-7 human breast cancer cells via p53-induced apoptosis. *J Med Plants Res.* 2011; 5(24): 5741-9.
 20. Chan CK, Supriady H, Goh BH, Kadir HA. *Elephantopus scaber* induces apoptosis through ROS-dependent mitochondrial signaling pathway in HCT116 human colorectal carcinoma cells. *J Ethnopharmacol.* 2015; 168: 291-304.
 21. Wicaksono BD, Tangkearung E, Sandra F. *Brucea javanica* leaf extract induced apoptosis in human oral squamous cell carcinoma (HSC-2) cells by attenuation of mitochondrial membrane permeability. *Indones Biomed J.* 2015; 7(2): 107-10.
 22. Sajjadi SE, Ghanadian M, Haghighi M, Mouhebat L. Cytotoxic effect of *Cousinia verbascifolia* Bunge against OVCAR-3 and HT-29 cancer cells. *J Herbm Pharmcol.* 2015; 4(1): 15-19.
 23. Ho WY, Liew SS, Yeap SK, Alitheen, NB. Synergistic cytotoxicity between *Elephantopus scaber* and tamoxifen on MCF-7-derived multicellular tumor spheroid. *Evid Based Complement Alternat Med.* 2021; 2021: 6355236. doi: 10.1155/2021/6355236.
 24. Mackay RP, Weinberger PM, Copland JA, Mahdavian E, Xu Q. YM155 induces DNA damage and cell death in anaplastic thyroid cancer cells by inhibiting DNA topoisomerase α at the ATP-binding site. *Mol Cancer Ther.* 2022; 21(6): 925-35.
 25. Premkumar DR, Jane EP, Foster KA, Pollack IF. Survivin inhibitor YM-155 sensitizes tumor necrosis factor- related apoptosis-inducing ligand-resistant glioma cells to apoptosis through Mcl-1 downregulation and by engaging the mitochondrial death pathway. *J Pharmacol Exp Ther.* 2013; 346(2): 201-10.
 26. Kabeer FA, Rajalekshmi DS, Nair MS, Prathapan R. Molecular mechanisms of anticancer activity of deoxyelephantopin in cancer cells. *Integr Med Res.* 2017; 6(2): 190-206.
 27. Hu F, Pan D, Zheng W, Yan T, He X, Ren F, *et al.* Elucidating respective functions of two domains BIR and C-helix of human IAP survivin for precise targeted regulating mitotic cycle, apoptosis and autophagy of cancer cells. *Oncotarget.* 2017; 8(69): 113687-700.

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

ORIGINALITY REPORT

12%

SIMILARITY INDEX

10%

INTERNET SOURCES

9%

PUBLICATIONS

1%

STUDENT PAPERS

PRIMARY SOURCES

| | | |
|---|--|----|
| 1 | hirosaki.repo.nii.ac.jp Internet Source | 1% |
| 2 | www.ukaazpublications.com Internet Source | 1% |
| 3 | academic.oup.com Internet Source | 1% |
| 4 | Ferry Sandra, Yudi Her Oktaviono, Mohammad Aris Widodo, Yanni Dirgantara, Angliana Chouw, Djanggan Sargowo. "Endothelial progenitor cells proliferated via MEK-dependent p42 MAPK signaling pathway", Molecular and Cellular Biochemistry, 2014 Publication | 1% |
| 5 | assets.researchsquare.com Internet Source | 1% |
| 6 | bioone.org Internet Source | 1% |

| | | |
|-----------------|--|-----|
| 7 | Liang, Wei-Zhe, Chiang-Ting Chou, Ti Lu, Chao-Chuan Chi, Li-Ling Tseng, Chih-Chuan Pan, Ko-Long Lin, Chun-Chi Kuo, and Chung-Ren Jan. "The mechanism of carvacrol-evoked $[Ca^{2+}]_i$ rises and non- Ca^{2+} -triggered cell death in OC2 human oral cancer cells", <i>Toxicology</i> , 2013. | 1 % |
| Publication | | |
| 8 | Limei Wang, Dongjie Yin, Yanhui Fan, Ting Min, Yang Yi, Hongxun Wang. "Molecular mechanism of the anti-gastric cancer activity of 1,2,3,6-tetra-O-galloyl- β -D-glucose isolated from <i>Trapa bispinosa</i> Roxb. shell in vitro", <i>PLOS ONE</i> , 2022 | 1 % |
| Publication | | |
| 9 | www.grafiati.com | 1 % |
| Internet Source | | |
| 10 | Submitted to Jawaharlal Nehru Technological University | 1 % |
| Student Paper | | |
| 11 | iovs.arvojournals.org | 1 % |
| Internet Source | | |
| 12 | rupress.org | 1 % |
| Internet Source | | |
| 13 | daneshyari.com | 1 % |
| Internet Source | | |

14

Abid Naeem, Pengyi Hu, Ming Yang, Jing Zhang, Yali Liu, Weifeng Zhu, Qin Zheng. "Natural Products as Anticancer Agents: Current Status and Future Perspectives", *Molecules*, 2022

Publication

<1 %

15

Hong Zhu, Wan-Jing Ding, Rui Wu, Qin-Jie Weng, Jian-Shu Lou, Rong-Jia Jin, Wei Lu, Bo Yang, Qiao-Jun He. "Synergistic Anti-Cancer Activity by the Combination of TRAIL/APO-2L and Celastrol", *Cancer Investigation*, 2009

Publication

<1 %

16

www.ejournal.utp.ac.id

Internet Source

<1 %

Exclude quotes On

Exclude matches < 15 words

Exclude bibliography On



Ferry Sandra <ferry@trisakti.ac.id>

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

Secretariat of InaBJ <secretariat@inabj@gmail.com>
To: ferry@trisakti.ac.id

Wed, Aug 21, 2024 at 2:33 PM

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.**

This manuscript is interesting, however based on the peer-reviewers and editors review results, this manuscript needs some revisions before it can be published. Please find the manuscript attached to see detailed comments.

Please make sure you read all the comments and revise the manuscript based on the suggestions given. Besides the comments our reviewers have given, please also pay attention to the use of English language, make sure you avoid any grammatical and diction errors.

Revise this manuscript thoroughly and according to the suggestions before **August 26, 2024**. Mark/highlighted the revised part of the manuscript, so that the editor will notice the changes. You are also obligated to provide a response letter with your response or the answer to reviewers' questions/comments. For an example on how to write a response letter, we also attach a response form template. Hopefully you find it well.

When you are done, you can upload it in: <https://inabj.org/index.php/ibj/author/submissionReview/3096>, or simply send us an email of your revised manuscript and response letter.

Please reply/notify us when you have received this email. If you have any questions, do not hesitate to contact us. Thank you for your attention. We wish you a nice day.

Best Regards,

--

Secretariat of The Indonesian Biomedical Journal

Prodia Tower 9th Floor

Jl. Kramat Raya No.150, Jakarta 10430, Indonesia

Phone. +62-21-3144182 ext. 3872

Fax. +62-21-3144181

<https://www.inabj.org>

2 attachments

 **M2024239 Manuscript - Round 1-ETA Comments.docx**
1149K

 **Response Form for Reviewer's Comments.xlsx**
11K

***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.

Commented [EA1]: HSC-3 like previous page

1 MATERIAL AND METHODS

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

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

8 HSC-3 Cell Culture

9 The HSC-3 cell culture was conducted using a previously reported method (21), with specific
10 modifications. The HSC-3 cell line was acquired from Sigma-Aldrich (St. Louis, MO, USA). HSC-
11 3 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich) complete
12 medium contained 50 U/mL penicillin 50 µg/mL, streptomycin (Sigma-Aldrich) and 10% fetal
13 bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany). The cells were cultured in a humidified

14 incubator at 37°C, 5% CO₂. The HSC-3 cells were detached with trypsin-ethylenediamine tetraacetic
15 acid (EDTA) solution (Sigma-Aldrich) once they reached 80% confluence.

16 **Cell Viability Assay**

17 The measurement of viable cells was conducted with 3-(4,5-dimethylthiazol-2-yl)-2,5-
18 diphenyltetrazolium bromide (MTT) assay, following a previously reported method.(13) In 96-well
19 plates, HSC-3 cells were placed (5×10^3 /well) and treated with/without 1, 10, or 100 µg/mL ESLE or
20 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) for 24 hours. MTT was added in the treated
21 well (100 µL/well) and incubated for 4 hours. Then, the suspension in each well was removed and
22 dissolved in 100 µL dimethylsulfoxide (DMSO). The formazan crystal that was formed was measured
23 using a microplate reader (Bio-Rad, Hercules, CA, USA) at OD₅₇₀. The measurements for each
24 experimental group were conducted in sextuplicate.

25 **Sub-G1 Assay**

26 Apoptotic HSC-3 cells were measured using the sub-G1 assay in order to evaluate the cytotoxic
27 effects of ESLE, based on previously reported method.(13) Treated-HSC-3 cells were collected and
28 incubated in a hypotonic fluorochrome solution (50 µg/mL of propidium iodide (Sigma-Aldrich),
29 0.1% Triton X-100 (Sigma-Aldrich), and 0.1% sodium citrate (Wako, Osaka, Japan)). Subsequently,
30 the cell suspensions were incubated in darkness for 30 minutes. The fluorescence of individual nuclei
31 was quantified using a FACSCanto II flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA),
32 and a total of 400 events were recorded.

33 **Western Blotting Assay**

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

Commented [EA2]: What is it ?

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

45 **Statistical Analysis**

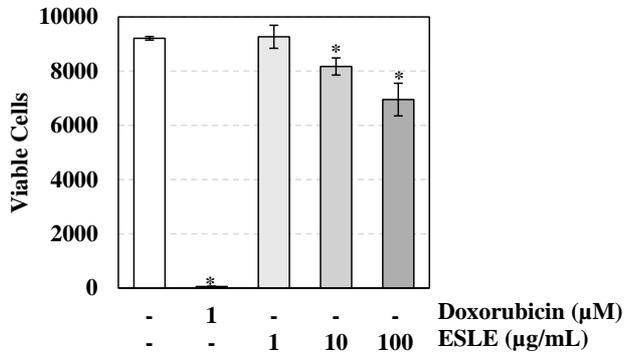
46 The Shapiro-Wilk normality test was utilized for statistical analysis. Then, one-way ANOVA test
47 was used to analyze the findings of a normally distributed dataset, followed by Tukey's *post hoc* test.
48 Subsequently, the results of abnormal data distribution were tested using Kruskal-Wallis test,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

50

51 **RESULTS**

52 **ESLE Decreased HSC-3 Viable Cells**

53 The results in Figure 1 showed that the number of HSC-3 viable cells in 1 μ M doxorubicin group
54 (56 ± 11.12) was significantly lower (Mann-Whitney's *post hoc* test, $p=0.004$) than the ones in the
55 sham group ($9,212 \pm 65.58$). The number of HSC-3 viable cells in ESLE-treated groups decreased
56 significantly (Kruskal Wallis, $p=0.001$) in concentration-dependent manner. The number of HSC-3
57 viable cells in 1 μ g/mL ESLE-treated group ($9,268 \pm 424.76$) did not significantly differ (Mann-
58 Whitney's *post hoc* test, $p=0.423$) than the ones in the sham group, meanwhile the number of HSC-
59 3 viable cells in 10 μ g/mL ESLE-treated group ($8,173 \pm 316.61$) and 100 μ g/mL ESLE-treated group
60 ($6,952 \pm 602.94$) differed significantly (Mann-Whitney's *post hoc* test, $p=0.004$) than the ones in the
61 sham group. In this MTT assay, IC_{50} concentration of ESLE-induced apoptotic HSC-3 cells was
62 222.34 μ g/mL.



63

64 **Figure 1. ESLE decreased HSC-3 viable cells in concentration-dependent manner.** HSC-3 cells
 65 were starved for 12 h and subsequently treated with/without 1 µM Doxorubicin or ESLE in different
 66 concentrations for 24 h. Viable cells were measured using MTT assay as outlined in methods. The
 67 results are presented as mean±standard deviation (n=6). *Statistical significance ($p < 0.05$) was
 68 determined using Mann-Whitney's *post hoc* test when compared to the sham group.
 69

70 ESLE Increased HSC-3 Apoptotic Cells

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

80

81

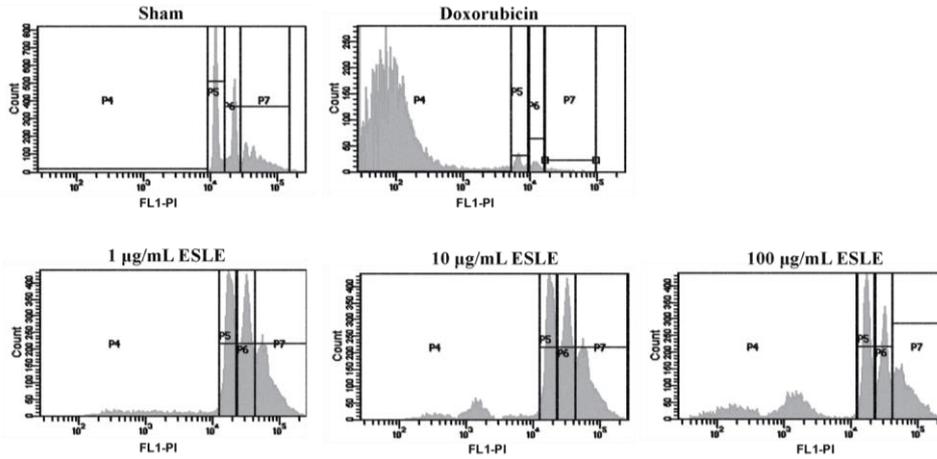
82

83

84

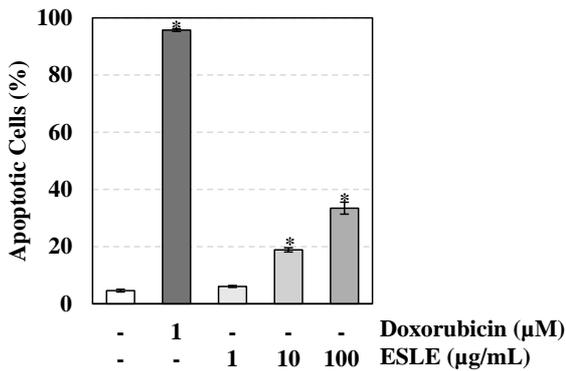
85

86 A



87

88 B



89

90

91

92

93

94

95

96

97

98

99

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.

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

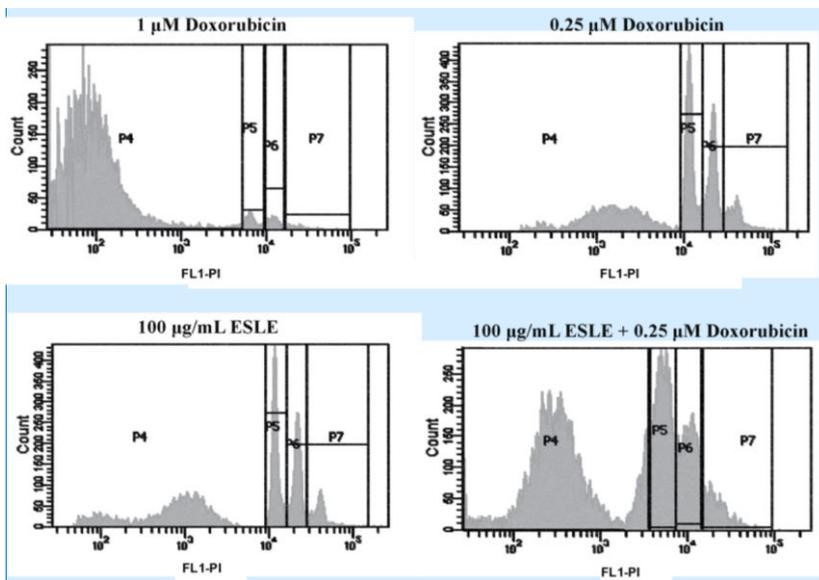
100 The results in Figure 3 showed that the percentage of HSC-3 apoptotic cells in 1 µM doxorubicin

101 group ($95.71 \pm 0.47\%$) was significantly higher (Mann-Whitney's *post hoc* test, $p = 0.004$) than the ones

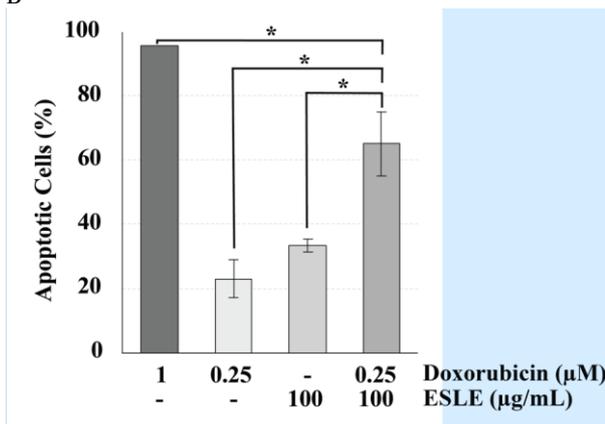
102 in the 100 µg/mL ESLE + 0.25 µM doxorubicin-treated group ($65.00 \pm 10.05\%$). The percentage of

103 HSC-3 apoptotic cells in the 0.25 μM Doxorubicin group ($22.98 \pm 5.98\%$) and the 100 $\mu\text{g/mL}$ ESLE-
 104 treated group ($33.45 \pm 2.09\%$) were low. However, the percentage of HSC-3 apoptotic cells in the 100
 105 $\mu\text{g/mL}$ ESLE + 0.25 μM doxorubicin-treated group was significantly higher (Mann-Whitney's *post*
 106 *hoc* test, $p=0.004$) compared to the 0.25 μM Doxorubicin group and the 100 $\mu\text{g/mL}$ ESLE-treated
 107 group.

108 A
 109



110 B
 111



112 **Figure 3. Combination of 100 $\mu\text{g/mL}$ ESLE with 0.25 μM Doxorubicin Increased HSC-3**
 113 **Apoptotic Cells.** HSC-3 cells were starved for 12 h and subsequently treated with/without 1 μM or
 114 0.25 μM Doxorubicin, or 100 $\mu\text{g/mL}$ ESLE for 24 h. Apoptotic cells were measured using Sub-G1
 115

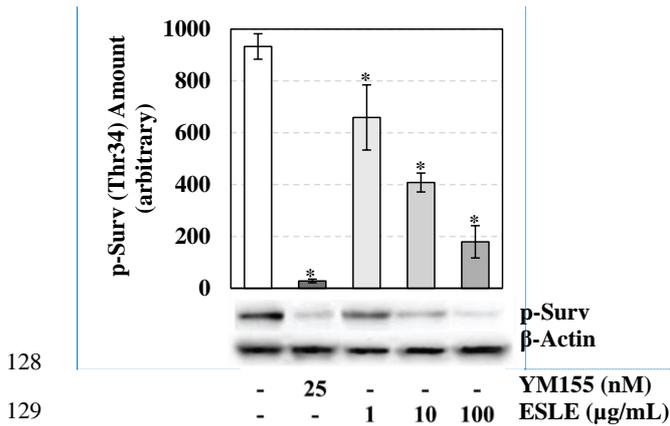
Commented [EA3]: Please put control figure to compare the result with doxo and ESLE

Commented [EA4]: Check the legend of Fig 3B, there are two of 0,25 in doxorubicin, and 100 in ELSE, is it typo ?

116 test as outlined in Methods. (A) The flow cytometric results. (B) The percentage of HSC-3 apoptotic
 117 cells. The results are presented as mean±standard deviation (n=6). *Statistical significance ($p<0.05$)
 118 was determined using Mann-Whitney's *post hoc* test.
 119

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

121 The results in Figure 4 showed that the p-Surv (Thr34) amount of HSC-3 cells in YM155 group
 122 (28 ± 7) was significantly lower (Tukey's *post hoc* test, $p=0.000$) than the ones in the sham group
 123 (933 ± 49.14) (Figure 5). The β -Actin was used as a loading control. The p-Surv (Thr34) amount in
 124 ESLE-treated group decreased significantly (ANOVA, $p=0.000$) in concentration-dependent manner.
 125 The p-Surv (Thr34) amount in the 1 $\mu\text{g}/\text{mL}$ ESLE-treated group (659 ± 125.74), 10 $\mu\text{g}/\text{mL}$ ESLE-
 126 treated group (408 ± 36.47) and 100 $\mu\text{g}/\text{mL}$ ESLE-treated group (179 ± 62.4) differed significantly
 127 (Tukey's *post hoc* test, $p=0.000$) than the ones in the sham group.



128
 129
 130 **Figure 4. ESLE decreased the amount of p-Surv (Thr34) in HSC-3 cells in concentration-**
 131 **dependent manner.** HSC-3 cells were treated with/without 25 nM YM155 or ESLE in various
 132 concentrations. The amount of p-Surv (Thr34) was measured using Western Blot, as outlined in
 133 Methods. *Statistical significance ($p<0.05$) was determined using Tukey's *post hoc* test when
 134 compared to the sham group.
 135

136 DISCUSSION

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

Commented [EA5]: Do you have combination of doxo 0,25 and 100 ELSE ? Please put it to compare the result with others

140 reported the ability of ESLE to induce apoptosis in T47D (breast cancer), MCF-7 (breast cancer), and
141 HCT116 (colorectal cancer) cell lines (18,19). IC₅₀ of ESLE-induced apoptotic HSC-3 cells (222.34
142 µg/mL) was categorized as weak cytotoxicity (201-500 µg/mL) (22). This IC₅₀ value was higher than
143 those observed for ESLE-induced apoptotic T47D cells (132.17±9.69 µg/mL) (18), MCF-7 cells
144 (14.69 ± 0.29 µg/mL) (19) and HCT116 cells (14.69 ± 0.29 µg/mL) (20). However, ...ketika Else
145 dikombinasi doxo konsentrasi rendah.... bisa meningkatkan doxo). The combination of 100 µg/mL
146 ESLE with 0.25 µM doxorubicin increased the percentage of HSC-3 apoptotic cells more than
147 treatment with either agent alone (Figure 3). These results suggest a synergistic effect of combining
148 100 µg/mL ESLE and 0.25 µM doxorubicin in inducing apoptosis in HSC-3 cells. This finding
149 indicates that ESLE can sensitize the apoptotic effect of doxorubicin, potentially allowing for lower
150 doses of doxorubicin to be used in cancer therapy, thereby reducing its associated side effects.

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

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

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

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

176

177 **CONCLUSION**

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

183

184 **AUTHORS CONTRIBUTION**

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

189

190

191

192 **REFERENCES**

- 193 1. Naeem A, Hu P, Yang M, Zhang J, Liu Y, Zhu W, Zheng Q. Natural products as anticancer
194 agents: Current status and future perspectives. *Molecules*. 2022; 27(23): 8367. doi:
195 10.3390/molecules27238367.
- 196 2. Castañeda AM, Meléndez CM, Uribe D, Pedroza-Díaz J. Synergistic effects of natural
197 compounds and conventional chemotherapeutic agents: recent insights for the development of
198 cancer treatment strategies. *Heliyon*. 2022; 8(6): e09519. doi: 10.1016/j.heliyon.2022.e09519.
- 199 3. Eziefule OM, Arozal W, Wanandi SI, Louisa M, Wuyung PE, Dewi S, *et al.* *Andrographis*
200 *paniculata* ethanolic extract improved doxorubicin-induced cardiac inflammation, alterations in
201 liver function parameters and anemia. *Mol Cell Biomed Sci*. 2024; 8(2): 117-26.
- 202 4. Callixte C, Baptiste NJ, Arwati H. Phytochemical screening and antimicrobial activities of
203 methanolic and aqueous leaf extracts of *Carica papaya* grown in Rwanda. *Mol Cell Biomed Sci*.
204 2020; 4(1): 39-44.
- 205 5. Nguyen PAT, Khang DT, Nguyen PTT, Do HDK. The complete chloroplast genome of
206 *Elephantopus scaber* L. (Vernonioideae, asteraceae), a useful ethnomedicinal plant in Asia.
207 *Mitochondrial DNA B Resour*. 2023; 8(9): 936-41.
- 208 6. Hiradeve SM, Rangari VD. *Elephantopus scaber* Linn.: A review on its ethnomedical,
209 phytochemical and pharmacological profile. *J Appl Biomed*. 2014; 12(2): 49-61.
- 210 7. Hiradeve SM, Rangari VD. A review on pharmacology and toxicology of *Elephantopus scaber*
211 Linn. *Nat Prod Res*. 2014; 28(11): 819-30.
- 212 8. Andari D, Khan FI, Jakfar SI. Methanol extract of katuk (*Sauropus androgynus*) leaves as an anti-
213 inflammatory agent: Animal study in carrageenan-induced rat models of inflammation. *Mol Cell*
214 *Biomed Sci*. 2022; 6(3): 129-34.
- 215 9. Girsang E, Lister INE, Ginting CN, Khu A, Samin B, Widowati W, *et al.* Chemical constituents
216 of snake fruit (*Salacca zalacca* (Gaert.) Voss) peel and in silico anti-aging analysis. *Mol Cell*
217 *Biomed Sci*. 2019; 3(2): 122-8.

- 218 10. Hjazi A, Alissa M, Alqasem AA, Alghamdi A, Alghamdi SA. Cynaropicrin, a sesquiterpene
219 lactone, triggers apoptotic cell death in triple negative breast cancer cells. *Mol Biol Rep.* 2024;
220 51(1): 856. doi: 10.1007/s11033-024-09723-y. PMID: 39066893.
- 221 11. Haifa R, Sartika CR, Faried A, Hadisaputri YE, Chouw A, Wijaya A, *et al.* Potency of peripheral
222 blood-and umbilical cord blood-derived dendritic cells and their secretomes as vaccines for
223 cancer. *Mol Cell Biomed Sci.* 2024; 8(1): 31-6.
- 224 12. Sandra F, Hendarmin L, Nakao Y, Nakamura N, Nakamura S. (2005). TRAIL cleaves caspase-
225 8,-9 and-3 of AM-1 cells: A possible pathway for TRAIL to induce apoptosis in ameloblastoma.
226 *Tumor Biol.* 2005; 26(5): 258-64.
- 227 13. Sandra F, Rizal MI, Dhaniar AY, Scania AE, Lee KH. Cosmos caudatus leaf extract triggers
228 apoptosis of HSC-3 cancer cells by decreasing bcl-2 and increasing bax. *Indones Biomed J.* 2024;
229 16(3): 285-91.
- 230 14. Wanandi SI, Syahrani RA, Suraduhita A, Yunita E, Louisa M. Andrographolide reverses
231 doxorubicin resistance in human breast cancer stem cells by regulating apoptotic gene
232 expressions. *Indones Biomed J.* 2023; 15(5): 288-96.
- 233 15. Rahman MN, Wijaya CR, Novalentina M. Survivin clinical features in cervical cancer. *Mol Cell*
234 *Biomed Sci.* 2017; 1(1): 6-16.
- 235 16. Liao J, Qing X, Deng G, Xiao Y, Fu Y, Han S, *et al.* Gastrodin destabilizes survivin and
236 overcomes pemetrexed resistance. *Cell Signal.* 2023; 110: 110851. doi:
237 10.1016/j.cellsig.2023.110851.
- 238 17. Pandey SK, Paul A, Shteinfer-Kuzmine A, Zalk R, Bunz U, Shoshan-Barmatz V. SMAC/diablo
239 controls proliferation of cancer cells by regulating phosphatidylethanolamine synthesis. *Mol*
240 *Oncol.* 202; 15(11): 3037-61.
- 241 18. Sulistyani N, Nurkhasanah. The cytotoxic effect of *Elephantopus scaber* Linn extract against
242 breast cancer (T47D) cells. *IOP Conf Ser Mater Sci Eng.* 2017; 259: 012006. doi: 10.1088/1757-
243 899X/259/1/012006

- 244 19. Ho WY, Yeap SK, Ho CL, Raha AR, Suraini AA, Alitheen NB. Elephantopus scaber induces
245 cytotoxicity in MCF-7 human breast cancer cells via p53-induced apoptosis. J Med Plants Res.
246 2011; 5(24): 5741-9.
- 247 20. Chan CK, Supriady H, Goh BH, Kadir HA. Elephantopus scaber induces apoptosis through ROS-
248 dependent mitochondrial signaling pathway in HCT116 human colorectal carcinoma cells. J
249 Ethnopharmacol. 2015; 168: 291-304.
- 250 21. Wicaksono BD, Tangkearung E, Sandra F. Brucea javanica leaf extract induced apoptosis in
251 human oral squamous cell carcinoma (HSC-2) cells by attenuation of mitochondrial membrane
252 permeability. Indones Biomed J. 2015; 7(2): 107-10.
- 253 22. Sajjadi SE, Ghanadian M, Haghighi M, Mouhebat L. Cytotoxic effect of Cousinia verbascifolia
254 Bunge against OVCAR-3 and HT-29 cancer cells. J Herbmed Pharmacol. 2015; 4(1): 15-19.
- 255 23. Ho WY, Liew SS, Yeap SK, Alitheen, NB. Synergistic cytotoxicity between Elephantopus scaber
256 and tamoxifen on MCF-7-derived multicellular tumor spheroid. Evid Based Complement Alternat
257 Med. 2021; 2021: 6355236. doi: 10.1155/2021/6355236.
- 258 24. Mackay RP, Weinberger PM, Copland JA, Mahdavian E, Xu Q. YM155 induces DNA damage
259 and cell death in anaplastic thyroid cancer cells by inhibiting DNA topoisomerase α at the ATP-
260 binding site. Mol Cancer Ther. 2022; 21(6): 925-35.
- 261 25. Premkumar DR, Jane EP, Foster KA, Pollack IF. Survivin inhibitor YM-155 sensitizes tumor
262 necrosis factor- related apoptosis-inducing ligand-resistant glioma cells to apoptosis through Mcl-
263 1 downregulation and by engaging the mitochondrial death pathway. J Pharmacol Exp Ther.
264 2013; 346(2): 201-10.
- 265 26. Kabeer FA, Rajalekshmi DS, Nair MS, Prathapan R. Molecular mechanisms of anticancer activity
266 of deoxyelephantopin in cancer cells. Integr Med Res. 2017; 6(2): 190-206.
- 267 27. Hu F, Pan D, Zheng W, Yan T, He X, Ren F, *et al.* Elucidating respective functions of two
268 domains BIR and C-helix of human IAP survivin for precise targeted regulating mitotic cycle,
269 apoptosis and autophagy of cancer cells. Oncotarget. 2017; 8(69): 113687-700.



The Indonesian Biomedical Journal

Prodia Tower 9th Floor, Jl. Kramat Raya No. 150, Jakarta 10430 - Indonesia

Tel.: +62-21-3144182 ext.872, Fax.: +62-21-3144181

Email: Secretariat@InaBJ.org, Website: www.InaBJ.org

Manuscript Review Form

| | |
|------------------|---|
| Reviewer | : R2 |
| Manuscript # | : M2024239 |
| Manuscript Title | : <i>Elephantopus scaber</i> 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? | ✓ | |
| | Notes: | | |
| 3 | Do the title and abstract reflect the study result/content? | ✓ | |
| | Notes: | | |
| 4. | Is the significance of the study well explained at the Background? | ✓ | |
| | Notes: | | |
| 5. | Are the research study methods technically correct, accurate, and complete enough to be reproduced/cited by other scientists? | ✓ | |
| | Notes: | | |
| 6. | Are the results, ideas, and data presented in this manuscript important | ✓ | |



The Indonesian Biomedical Journal

Prodia Tower 9th Floor, Jl. Kramat Raya No. 150, Jakarta 10430 - Indonesia

Tel.: +62-21-3144182 ext.872, Fax.: +62-21-3144181

Email: Secretariat@InaBJ.org, Website: www.InaBJ.org

| | | | |
|-----|--|---|--|
| | 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.



The Indonesian Biomedical Journal

Prodia Tower 9th Floor, Jl. Kramat Raya No. 150, Jakarta 10430 - Indonesia

Tel.: +62-21-3144182 ext.872, Fax.: +62-21-3144181

Email: Secretariat@InaBJ.org, Website: www.InaBJ.org

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:



Ferry Sandra <ferry@trisakti.ac.id>

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

Ferry Sandra <ferry@trisakti.ac.id>
To: Secretariat of InaBJ <secretariatinabj@gmail.com>

Thu, Aug 22, 2024 at 4:58 PM

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
1145K



Response Form for Reviewer's Comments.xlsx
13K

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

3
4 **ABSTRACT**

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

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

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

21 **CONCLUSION:**

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

27

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

30

31 **INTRODUCTION**

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

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

47 A regulated process of cell death, apoptosis, plays a vital role in removing cancerous cells and
48 inhibiting tumor progression.(11,12) This process can be triggered by either the mitochondrial
49 (intrinsic) pathway, which is mediated by caspase-9, or the death-receptor (extrinsic) pathway, which
50 is mediated by caspase-8. Ultimately, both pathways converge to activate the effector caspases-3 and
51 -7, which execute the cell death program.(13) One of the critical regulators of apoptosis is Survivin,
52 a key member of the inhibitor of apoptosis (IAP) protein family.(14) Survivin is essential in regulating

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

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

65

66 **MATERIAL AND METHODS**

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

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

73

74 **HSC-3 Cell Culture**

75 The HSC-3 cell culture was conducted using a previously reported method (21), with specific
76 modifications. The HSC-3 cell line was acquired from Sigma-Aldrich (St. Louis, MO, USA). HSC-
77 3 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich) complete
78 medium contained 50 U/mL penicillin 50 µg/mL, streptomycin (Sigma-Aldrich) and 10% fetal

79 bovine serum (FBS) (PAN-Biotech, Aidenbach, Germany). The cells were cultured in a humidified
80 incubator at 37°C, 5% CO₂. The HSC-3 cells were detached with trypsin-ethylenediamine tetraacetic
81 acid (EDTA) solution (Sigma-Aldrich) once they reached 80% confluence.

82

83 **Cell Viability Assay**

84 The measurement of viable cells was conducted with 3-(4,5-dimethylthiazol-2-yl)-2,5-
85 diphenyltetrazolium bromide (MTT) assay, following a previously reported method.(13) In 96-well
86 plates, HSC-3 cells were placed (5×10^3 /well) and treated with/without 1, 10, or 100 µg/mL ESLE or
87 1 µM Doxorubicin (Dankos Farma, Jakarta, Indonesia) for 24 hours. MTT was added in the treated
88 well (100 µL/well) and incubated for 4 hours. Then, the suspension in each well was removed and
89 dissolved in 100 µL dimethylsulfoxide (DMSO). The formazan crystal that was formed was measured
90 using a microplate reader (Bio-Rad, Hercules, CA, USA) at OD₅₇₀. The measurements for each
91 experimental group were conducted in sextuplicate.

92

93 **Sub-G1 Assay**

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

101

102 **Western Blotting Assay**

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

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

115

116 **Statistical Analysis**

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

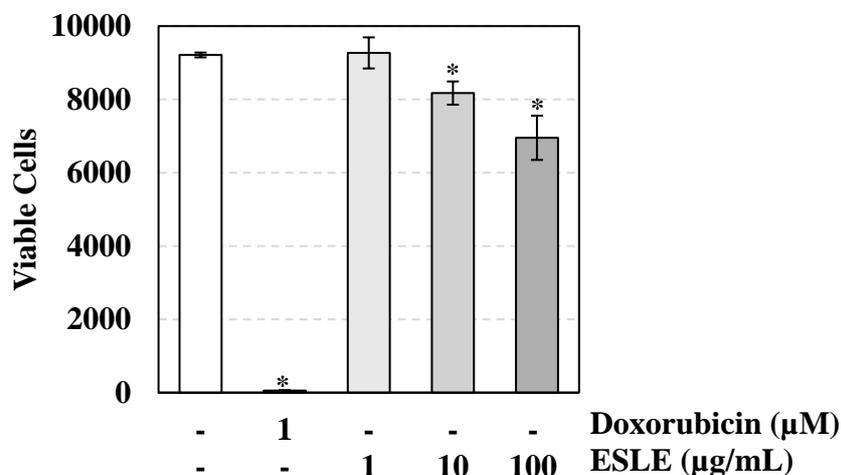
121

122 **RESULTS**

123 **ESLE Decreased HSC-3 Viable Cells**

124 The results in Figure 1 showed that the number of HSC-3 viable cells in 1 μ M doxorubicin
125 group (56 ± 11.12) was significantly lower (Mann-Whitney's *post hoc* test, $p=0.004$) than the ones in
126 the sham group ($9,212 \pm 65.58$). The number of HSC-3 viable cells in ESLE-treated groups decreased
127 significantly (Kruskal Wallis, $p=0.001$) in concentration-dependent manner. The number of HSC-3
128 viable cells in 1 μ g/mL ESLE-treated group ($9,268 \pm 424.76$) did not significantly differ (Mann-
129 Whitney's *post hoc* test, $p=0.423$) than the ones in the sham group, meanwhile the number of HSC-
130 3 viable cells in 10 μ g/mL ESLE-treated group ($8,173 \pm 316.61$) and 100 μ g/mL ESLE-treated group

131 (6,952±602.94) differed significantly (Mann-Whitney's *post hoc* test, $p=0.004$) than the ones in the
132 sham group. In this MTT assay, IC_{50} concentration of ESLE in inducing apoptosis of HSC-3 cells
133 was 222.34 $\mu\text{g/mL}$.



134

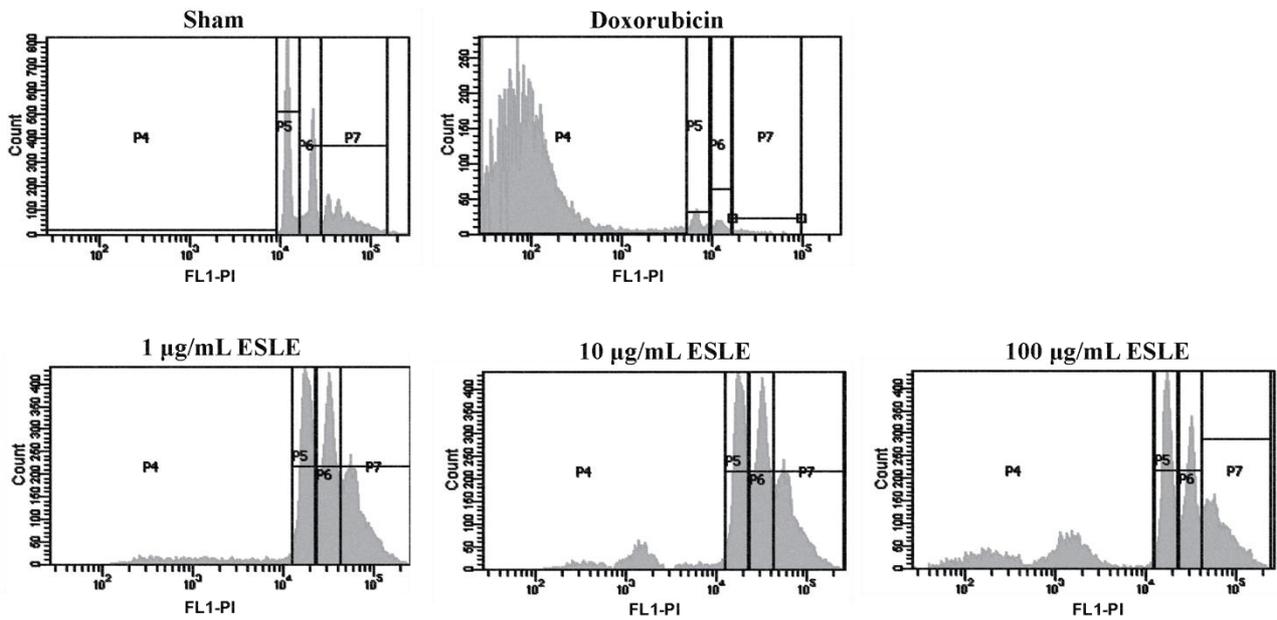
135 **Figure 1. ESLE decreased HSC-3 viable cells in concentration-dependent manner.** HSC-3 cells
136 were starved for 12 h and subsequently treated with/without 1 μM Doxorubicin or ESLE in different
137 concentrations for 24 h. Viable cells were measured using MTT assay as outlined in methods. The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

140

141 **ESLE Increased HSC-3 Apoptotic Cells**

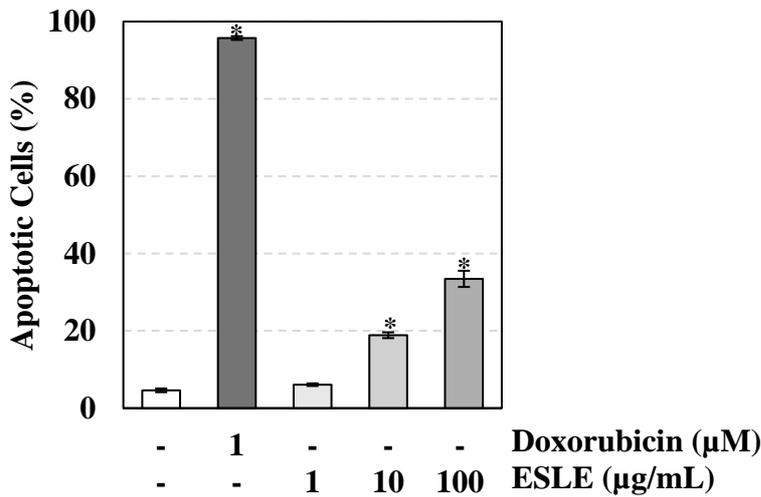
142 The results in Figure 2 showed that the percentage of HSC-3 apoptotic cells in 1 μM
143 doxorubicin group ($95.73\pm 0.48\%$) was significantly higher (Tukey's *post hoc* test, $p=0.001$) than the
144 ones in the sham group ($4.62\pm 0.48\%$) (Figure 2). The percentage of HSC-3 apoptotic cells in ESLE-
145 treated groups increased significantly (ANOVA, $p=0.001$) in concentration-dependent manner. The
146 percentage of HSC-3 apoptotic cells in 1 $\mu\text{g/mL}$ ESLE-treated group ($6.08\pm 0.34\%$) did not
147 significantly differ (Tukey's *post hoc* test, $p=0.120$) than the ones in the sham group, meanwhile
148 percentage of HSC-3 apoptotic cells in 10 $\mu\text{g/mL}$ ESLE-treated group ($18.88\pm 0.75\%$) and 100 $\mu\text{g/mL}$
149 ESLE-treated group ($33.45\pm 2.09\%$) differed significantly (Tukey's *post hoc* test, $p=0.001$) than the
150 ones in the sham group.

151 A



152

153 B



154

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

161

162

163

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

165 The results in Figure 3 showed that the percentage of HSC-3 apoptotic cells in 1 µM doxorubicin

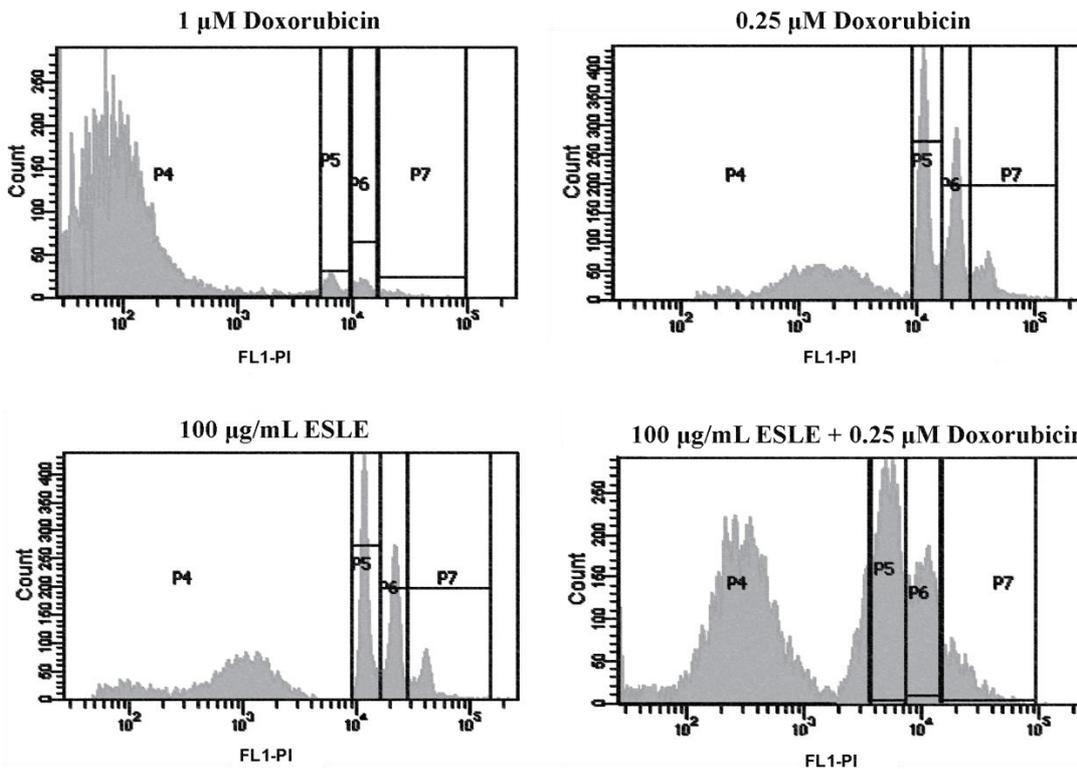
166 group ($95.71\pm0.47\%$) was significantly higher (Mann-Whitney's *post hoc* test, $p=0.004$) than the ones

167 in the 100 µg/mL ESLE + 0.25 µM doxorubicin-treated group ($65.00\pm10.05\%$). The percentage of

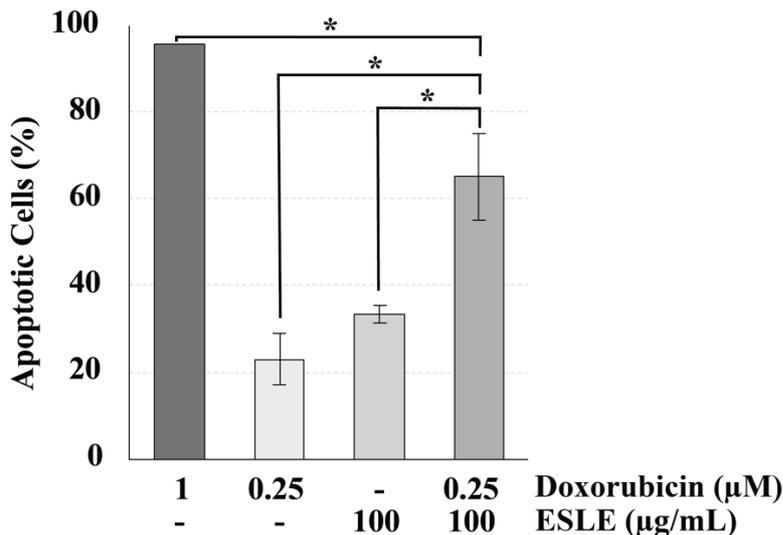
168 HSC-3 apoptotic cells in the 0.25 µM Doxorubicin group ($22.98\pm5.98\%$) and the 100 µg/mL ESLE-

169 treated group ($33.45 \pm 2.09\%$) were low. However, the percentage of HSC-3 apoptotic cells in the 100
 170 $\mu\text{g/mL}$ ESLE + 0.25 μM doxorubicin-treated group was significantly higher (Mann-Whitney's *post*
 171 *hoc* test, $p=0.004$) compared to the 0.25 μM Doxorubicin group and the 100 $\mu\text{g/mL}$ ESLE-treated
 172 group.

173 A
 174



175 B
 176

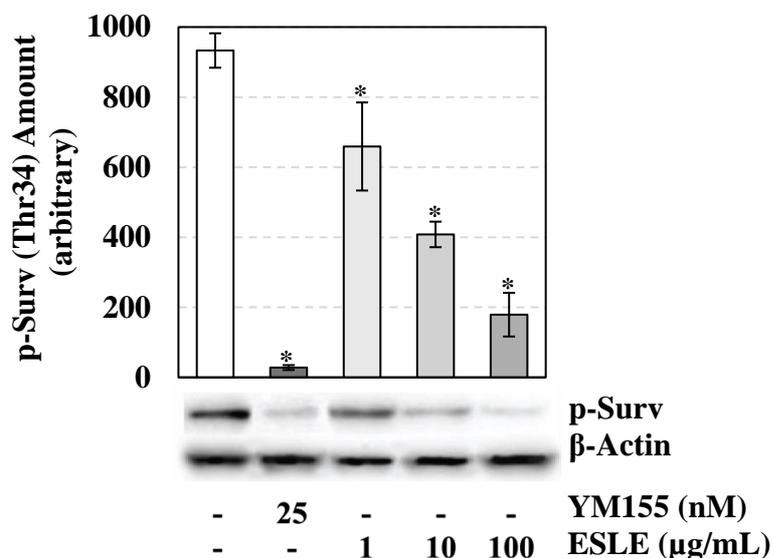


177 **Figure 3. Combination of 100 $\mu\text{g/mL}$ ESLE with 0.25 μM Doxorubicin Increased HSC-3**
 178 **Apoptotic Cells.** HSC-3 cells were starved for 12 h and subsequently treated with/without 1 μM or
 179 0.25 μM Doxorubicin, or 100 $\mu\text{g/mL}$ ESLE for 24 h. Apoptotic cells were measured using Sub-G1
 180 test as outlined in Methods. (A) The flow cytometric results. (B) The percentage of HSC-3 apoptotic
 181

182 cells. The results are presented as mean±standard deviation (n=6). *Statistical significance ($p<0.05$)
183 was determined using Mann-Whitney's *post hoc* test.
184

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

186 The results in Figure 4 showed that the p-Surv (Thr34) amount of HSC-3 cells in YM155 group
187 (28 ± 7) was significantly lower (Tukey's *post hoc* test, $p=0.000$) than the ones in the sham group
188 (933 ± 49.14) (Figure 5). The β -Actin was used as a loading control. The p-Surv (Thr34) amount in
189 ESLE-treated group decreased significantly (ANOVA, $p=0.000$) in concentration-dependent manner.
190 The p-Surv (Thr34) amount in the 1 $\mu\text{g}/\text{mL}$ ESLE-treated group (659 ± 125.74), 10 $\mu\text{g}/\text{mL}$ ESLE-
191 treated group (408 ± 36.47) and 100 $\mu\text{g}/\text{mL}$ ESLE-treated group (179 ± 62.4) differed significantly
192 (Tukey's *post hoc* test, $p=0.000$) than the ones in the sham group.



193

194

195 **Figure 4. ESLE decreased the amount of p-Surv (Thr34) in HSC-3 cells in concentration-**
196 **dependent manner.** HSC-3 cells were treated with/without 25 nM YM155 or ESLE in various
197 concentrations. The amount of p-Surv (Thr34) was measured using Western Blot, as outlined in
198 Methods. *Statistical significance ($p<0.05$) was determined using Tukey's *post hoc* test when
199 compared to the sham group.
200

201 DISCUSSION

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

206 HCT116 (colorectal cancer) cell lines (18,19). IC₅₀ of ESLE in inducing apoptosis of HSC-3 cells
207 (222.34 µg/mL) was categorized as weak cytotoxicity (201-500 µg/mL) (22). This IC₅₀ value was
208 higher than those in inducing apoptosis of T47D cells (132.17±9.69 µg/mL) (18), MCF-7 cells
209 (14.69±0.29 µg/mL) (19) and HCT116 cells (14.69±0.29 µg/mL) (20). However, although having
210 weak cytotoxicity, ESLE could enhance the potency of doxorubicin in inducing apoptosis of HSC-3
211 cells. Specifically, the combination of 100 µg/mL ESLE with 0.25 µM doxorubicin increased the
212 percentage of HSC-3 apoptotic cells more than treatment with either agent alone (Figure 3). These
213 results suggest a synergistic effect of combining 100 µg/mL ESLE and 0.25 µM doxorubicin in
214 inducing apoptosis in HSC-3 cells. This finding indicates that ESLE can sensitize the apoptotic effect
215 of doxorubicin, potentially allowing for lower doses of doxorubicin to be used in cancer therapy,
216 thereby reducing its associated side effects.

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

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

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

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

242

243 **CONCLUSION**

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

249

250

251

252

253

254

255

256

258 **REFERENCES**

- 259 1. Naeem A, Hu P, Yang M, Zhang J, Liu Y, Zhu W, Zheng Q. Natural products as anticancer
260 agents: Current status and future perspectives. *Molecules*. 2022; 27(23): 8367. doi:
261 10.3390/molecules27238367.
- 262 2. Castañeda AM, Meléndez CM, Uribe D, Pedroza-Díaz J. Synergistic effects of natural
263 compounds and conventional chemotherapeutic agents: recent insights for the development of
264 cancer treatment strategies. *Heliyon*. 2022; 8(6): e09519. doi: 10.1016/j.heliyon.2022.e09519.
- 265 3. Eziefule OM, Arozal W, Wanandi SI, Louisa M, Wuyung PE, Dewi S, *et al.* *Andrographis*
266 *paniculata* ethanolic extract improved doxorubicin-induced cardiac inflammation, alterations in
267 liver function parameters and anemia. *Mol Cell Biomed Sci*. 2024; 8(2): 117-26.
- 268 4. Callixte C, Baptiste NJ, Arwati H. Phytochemical screening and antimicrobial activities of
269 methanolic and aqueous leaf extracts of *Carica papaya* grown in Rwanda. *Mol Cell Biomed Sci*.
270 2020; 4(1): 39-44.
- 271 5. Nguyen PAT, Khang DT, Nguyen PTT, Do HDK. The complete chloroplast genome of
272 *Elephantopus scaber* L. (Vernonioideae, asteraceae), a useful ethnomedicinal plant in Asia.
273 *Mitochondrial DNA B Resour*. 2023; 8(9): 936-41.
- 274 6. Hiradeve SM, Rangari VD. *Elephantopus scaber* Linn.: A review on its ethnomedical,
275 phytochemical and pharmacological profile. *J Appl Biomed*. 2014; 12(2): 49-61.
- 276 7. Hiradeve SM, Rangari VD. A review on pharmacology and toxicology of *Elephantopus scaber*
277 Linn. *Nat Prod Res*. 2014; 28(11): 819-30.
- 278 8. Andari D, Khan FI, Jakfar SI. Methanol extract of katuk (*Sauropus androgynus*) leaves as an anti-
279 inflammatory agent: Animal study in carrageenan-induced rat models of inflammation. *Mol Cell*
280 *Biomed Sci*. 2022; 6(3): 129-34.

- 281 9. Girsang E, Lister INE, Ginting CN, Khu A, Samin B, Widowati W, *et al.* Chemical constituents
282 of snake fruit (*Salacca zalacca* (Gaert.) Voss) peel and in silico anti-aging analysis. *Mol Cell*
283 *Biomed Sci.* 2019; 3(2): 122-8.
- 284 10. Hjazzi A, Alissa M, Alqasem AA, Alghamdi A, Alghamdi SA. Cynaropicrin, a sesquiterpene
285 lactone, triggers apoptotic cell death in triple negative breast cancer cells. *Mol Biol Rep.* 2024;
286 51(1): 856. doi: 10.1007/s11033-024-09723-y. PMID: 39066893.
- 287 11. Haifa R, Sartika CR, Faried A, Hadisaputri YE, Chouw A, Wijaya A, *et al.* Potency of peripheral
288 blood-and umbilical cord blood-derived dendritic cells and their secretomes as vaccines for
289 cancer. *Mol Cell Biomed Sci.* 2024; 8(1): 31-6.
- 290 12. Sandra F, Hendarmin L, Nakao Y, Nakamura N, Nakamura S. (2005). TRAIL cleaves caspase-
291 8,-9 and-3 of AM-1 cells: A possible pathway for TRAIL to induce apoptosis in ameloblastoma.
292 *Tumor Biol.* 2005; 26(5): 258-64.
- 293 13. Sandra F, Rizal MI, Dhaniar AY, Scania AE, Lee KH. *Cosmos caudatus* leaf extract triggers
294 apoptosis of HSC-3 cancer cells by decreasing bcl-2 and increasing bax. *Indones Biomed J.* 2024;
295 16(3): 285-91.
- 296 14. Wanandi SI, Syahrani RA, Suraduhita A, Yunita E, Louisa M. Andrographolide reverses
297 doxorubicin resistance in human breast cancer stem cells by regulating apoptotic gene
298 expressions. *Indones Biomed J.* 2023; 15(5): 288-96.
- 299 15. Rahman MN, Wijaya CR, Novalentina M. Survivin clinical features in cervical cancer. *Mol Cell*
300 *Biomed Sci.* 2017; 1(1): 6-16.
- 301 16. Liao J, Qing X, Deng G, Xiao Y, Fu Y, Han S, *et al.* Gastrodin destabilizes survivin and
302 overcomes pemetrexed resistance. *Cell Signal.* 2023; 110: 110851. doi:
303 10.1016/j.cellsig.2023.110851.
- 304 17. Pandey SK, Paul A, Shteinfer-Kuzmine A, Zalk R, Bunz U, Shoshan-Barmatz V. SMAC/diablo
305 controls proliferation of cancer cells by regulating phosphatidylethanolamine synthesis. *Mol*
306 *Oncol.* 202; 15(11): 3037-61.

- 307 18. Sulistyani N, Nurkhasanah. The cytotoxic effect of *Elephantopus scaber* Linn extract against
308 breast cancer (T47D) cells. IOP Conf Ser Mater Sci Eng. 2017; 259: 012006. doi: 10.1088/1757-
309 899X/259/1/012006
- 310 19. Ho WY, Yeap SK, Ho CL, Raha AR, Suraini AA, Alitheen NB. *Elephantopus scaber* induces
311 cytotoxicity in MCF-7 human breast cancer cells via p53-induced apoptosis. J Med Plants Res.
312 2011; 5(24): 5741-9.
- 313 20. Chan CK, Supriady H, Goh BH, Kadir HA. *Elephantopus scaber* induces apoptosis through ROS-
314 dependent mitochondrial signaling pathway in HCT116 human colorectal carcinoma cells. J
315 Ethnopharmacol. 2015; 168: 291-304.
- 316 21. Wicaksono BD, Tangkearung E, Sandra F. *Brucea javanica* leaf extract induced apoptosis in
317 human oral squamous cell carcinoma (HSC-2) cells by attenuation of mitochondrial membrane
318 permeability. Indones Biomed J. 2015; 7(2): 107-10.
- 319 22. Sajjadi SE, Ghanadian M, Haghighi M, Mouhebat L. Cytotoxic effect of *Cousinia verbascifolia*
320 Bunge against OVCAR-3 and HT-29 cancer cells. J Herbmed Pharmacol. 2015; 4(1): 15-19.
- 321 23. Ho WY, Liew SS, Yeap SK, Alitheen, NB. Synergistic cytotoxicity between *Elephantopus scaber*
322 and tamoxifen on MCF-7-derived multicellular tumor spheroid. Evid Based Complement Alternat
323 Med. 2021; 2021: 6355236. doi: 10.1155/2021/6355236.
- 324 24. Mackay RP, Weinberger PM, Copland JA, Mahdavian E, Xu Q. YM155 induces DNA damage
325 and cell death in anaplastic thyroid cancer cells by inhibiting DNA topoisomerase α at the ATP-
326 binding site. Mol Cancer Ther. 2022; 21(6): 925-35.
- 327 25. Premkumar DR, Jane EP, Foster KA, Pollack IF. Survivin inhibitor YM-155 sensitizes tumor
328 necrosis factor- related apoptosis-inducing ligand-resistant glioma cells to apoptosis through Mcl-
329 1 downregulation and by engaging the mitochondrial death pathway. J Pharmacol Exp Ther.
330 2013; 346(2): 201-10.
- 331 26. Kabeer FA, Rajalekshmi DS, Nair MS, Prathapan R. Molecular mechanisms of anticancer activity
332 of deoxyelephantopin in cancer cells. Integr Med Res. 2017; 6(2): 190-206.

333 27. Hu F, Pan D, Zheng W, Yan T, He X, Ren F, *et al.* Elucidating respective functions of two
334 domains BIR and C-helix of human IAP survivin for precise targeted regulating mitotic cycle,
335 apoptosis and autophagy of cancer cells. *Oncotarget*. 2017; 8(69): 113687-700.

336

337

Response Form for Reviewer's Comments

Corresponding: Ferry Sandra

Manuscript C: M2024239

Manuscript T1: 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 sham 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 ? | <p>Thank you for your observation. There seems to be a misunderstanding. The figure legend for Figure 3B correctly represents the experimental conditions. Specifically:</p> <ul style="list-style-type: none"> - 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. <p>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.</p> | |

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



Ferry Sandra <ferry@trisakti.ac.id>

[InaBJ] M2024239 Letter of Acceptance

Secretariat of InaBJ <secretariat@inabj@gmail.com>
To: Ferry Sandra <ferry@trisakti.ac.id>

Wed, Sep 4, 2024 at 10:17 AM

Dear Dr Ferry Sandra,

Please have the certificate of acceptance for manuscript "**Elephantopus 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,

--

Secretariat of The Indonesian Biomedical Journal

Prodia Tower 9th Floor

[Jl. Kramat Raya No.150, Jakarta 10430, Indonesia](#)

Phone. +62-21-3144182 ext. 3872

Fax. +62-21-3144181

<https://www.inabj.org>**Certificate for Author M2024239 - Ferry Sandra [signed].pdf**

154K

SURAT TUGAS

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

Dekan Fakultas Kedokteran Gigi Universitas Trisakti

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.