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ORIGINAL RESEARCH

In Vitro Evaluation of NIH/3T3 Fibroblast Cell Viability After Treatment with Reundeu (Staurogyne Elongata) Leaf Extract

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ABSTRACT

Background: Reundeu (Staurogyne elongata) is a plant traditionally used as food and medicine, especially in the Java region. It contains bioactive compounds that have the ability to improve health. Therefore, it may show potential for formulation into standardized herbal medicine.
Objective: The aim of this study was to determine whether an ethanolic extract of reundeu (Staurogyne elongata) leaves exhibits a cytotoxic effect on NIH/3T3 fibroblast cells using a WST-8 assay.
Method: In this in vitro laboratory examination, the NIH/3T3 cell line, cultured in 96-well plates, was treated with reundeu leaves ethanolic extract at 3.125%, 1.56%, 0.78%, 0.39%, and 0.19% concentrations, with DMEM and a cell-control as controls. Cell viability was assessed using the WST-8 assay and measured at 490 nm using a spectrophotometer. Data were analyzed using one-way ANOVA to assess differences among groups, followed by Tukey's post-hoc test for multiple pairwise comparisons.
Outcome: The ethanolic extract at 3.125% concentration had mild toxicity to NIH/3T3 cells, but other concentrations showed high viability. One-way ANOVA demonstrated a significant effect of reundeu leaf extract concentration on NIH/3T3 cell viability ($p < 0.05$). Tukey's post-hoc analysis showed significant differences ($p < 0.05$) between the 3.13%, 1.56%, and 0.19% groups and other concentration groups.
Conclusion: The study showed that reundeu leaf ethanolic extract can be considered safe but mildly cytotoxic to fibroblasts at a concentration of 3.125%.

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INTRODUCTION

The use of medicinal plants as primary constituents of therapeutic agents has been widely practiced in society since they are accessible, relatively low cost, and have an apparent safety profile. Plant-derived compounds continue to provide an important source of bioactive substances for medical or pharmaceutical development.[1,2] To integrate herbal medicine into modern therapeutic treatment, it is necessary to conduct structured and continuous research to prove its effectiveness against disease, its level of safety for humans, and to recognize its cellular and molecular mechanisms.[3] One of the plants that has been discovered for therapeutic purposes is reundeu (Staurogyne elongata).

The reundeu plant, also referred to as keji in certain areas of Central Java, has been traditionally used by society, especially in Java and Sumatra, as a food and medicinal plant. Empirically, the leaves and roots are either eaten directly or decocted in water for various health purposes such as joint pain remedy, kidney conditions, perinatal care, or canker sores.[4-8] This traditional use highlights its potential as a candidate for advanced pharmacological and toxicological evaluation.

Phytochemical screening conducted on the leaves of this plant indicated the presence of secondary metabolites such as flavonoid compounds, saponins, tannins, polyphenols or phenols, and triterpenoids or steroids.[7,8] The content of these phytochemicals can help protect the body from damage with antimicrobial, anti-inflammatory, and antioxidant effects.[9-11] A previous study by Noverita and Sinaga (2021) revealed that 50% reundeu leaf methanol extract has an inhibition zone against Staphylococcus aureus and Bacillus cereus with the agar diffusion method. Moreover, the active substance of reundeu leaves has strong antioxidant ability as shown by Marsiati and Patimah (2023), which indicated that the IC50 of reundeu leaf water extract based on the DPPH test was 17.73 ppm.[12,13]

Based on the observation of empirical uses and previous studies, Reundeu (Staurogyne elongata) leaf has considerable potential for development as medicinal agents; however, substances planned for therapeutic or dietary use must be evaluated for potential toxic or harmful effects. Cytotoxicity testing on cultured cells is commonly performed to assess the safety of bioactive materials prior to further development.[14,15] These assays evaluate cellular responses, including changes in viability, proliferation, and cell damage following exposure to the tested substance.[16]

NIH/3T3 fibroblast cells are frequently used as an in vitro model to evaluate cytotoxic effects on normal cells, as they play a crucial role in tissue maintenance and wound healing and exhibit relatively stable growth characteristics. Therefore, assessment of fibroblast cell

viability provides valuable information regarding the biocompatibility of plant-derived extracts.[17] In this study, cytotoxicity testing was conducted to evaluate the effect of reundeu leaf extract at various concentrations on fibroblast cell viability, aiming to determine a concentration range that is non-cytotoxic and potentially safe for further application.

Cell viability assays such as the WST (Water Soluble Tetrazolium)-8 assay are a common test employed to evaluate the cytotoxic effects of bioactive compounds in vitro. The WST-8 assay is a colorimetric method used to assess cell viability and examine whether fibroblast cell proliferation is affected by treatment.[18] This study aims to examine the cytotoxicity of reundeu leaf ethanolic extract by measuring cell viability as an indicator of treatment-induced cell death in vitro on fibroblast cells.

RESEARCH METHODS

Leaf extract

Reundeu leaves were obtained from a reundeu cultivation area in Susuru, Ciamis, West Java, Indonesia. Plant identification was conducted at the Plant Taxonomy Laboratory of the Mathematics and Science Faculty, Padjadjaran University. Ethanol extracts of the leaves were prepared by maceration using 70% ethanol.[19] Phytochemical compounds present in the extract were identified using specific reagents for each compound. The ethanolic extract of the leaves was subsequently formulated by dilution with distilled water to obtain concentrations of 3.13%, 1.56%, 0.78%, 0.39%, and 0.19%.

WST-8 assay materials

The 3T3 fibroblast cell culture used was the NIH 3T3 cell line cultured at the Aretha Cell Culture Laboratory, Bandung, from ATCC CL 183 P9 mouse embryonic cells. Fibroblast cells were seeded at a density of 5000 cells/well. Materials used in the assay were Dulbecco's Modified Eagle's Medium/DMEM (Elabscience) as a culture growth medium; WST-8 reagent (Elabscience); antimitotic antibiotics; nanomycopulitin (Biowest); gentamycin; amphotericin; 0.25% trypsin EDTA (Merck); DMSO (Merck); PBS; a Biological Safety Cabinet (Thermo Scientific 1300 Series); a CO2 incubator (Thermo Scientific); a 96-well plate (Costar); a microplate reader (Thermo Scientific); a micropipette (Finnpipette F2); an analytical balance (AXIS); a water bath; a refrigerator; a liquid nitrogen tank; and a microscope (Olympus CX31).

WST-8 assay

The prepared extracts will be tested using the WST-8 assay. Concentrations of 3.13%, 1.56%, 0.78%, 0.39%, and 0.19% on a 96-well plate were determined by purposive sampling.

This assay is based on the reduction of the WST-8 reagent, a water-soluble tetrazolium salt, by NADPH generated in viable cells. Formazan in WST-8 dissolved in tissue culture media. Subsequent quantitative spectrophotometry measurement was evaluated at a 490 nm wavelength, and the absorbance result will determine the viability of the cells. The cells in the well plate will visibly have an orange color.

The cytotoxicity assay was performed after preparation of the ethanolic extract of reundeu leaves at the previously explained concentrations as treatment. 3T3 fibroblast cells were cultured and prepared, thawed, and maintained under required conditions. Cells were seeded in 96-microplates at 5,000 cells per well, and the volume was 100 μL per well. Afterward, they were incubated at 37°C in a condition of 5% CO2 until the confluence reached 80%. This assay was performed in triplicate.

Cells in the well plate were treated with serial concentrations of the extract, similarly for control treatments, then incubated for 24-48 hours, followed by the addition of WST-8 reagent and further incubation for 3-4 hours. Measurement of absorbance was done with an ELISA reader at a 490 nm wavelength, followed by the calculation of cell viability based on the absorbance values.

RESEARCH FINDINGS

Fibroblast cell viability was assessed using the WST-8 assay, with spectrophotometric measurements of absorbance obtained at a wavelength of 490 nm. Fibroblast cell cultures were exposed to reundeu (Staurogyne elongata) leaf extract at concentrations of 3.13%, 1.56%, 0.78%, 0.39%, and 0.19%. The observed cell viability values at 0.19%, 0.39%, 0.78%, and 1.56% concentrations remained above 90%, which is commonly indicated as non-cytotoxic or safe. In contrast, the lowest cell viability was observed at the highest concentration (3.13%); however, this reduction remained within the range of mild cytotoxicity, according to the International Organization for Standardization (ISO) 10993-5 standard, which divides toxicity levels based on the percentage of cell viability. It interprets 100%-80% viability as non-toxic, 60-80% as mild toxicity, 40-60% as moderate toxicity, and less than 40% as very toxic. The results of the WST-8 assay and cell viability percentages are presented in Table 1 and Figure 2, respectively.

Table 1. Viability of fibroblast cells with treatment from each group

Treatment	Viability (%)	Inhibition (%)	p-value
Negative control	100.00 ± 1.56	0.00 ± 1.56	
1% DMSO	98.42 ± 3.00	1.58 ± 3.00	
SE 3.125%	79.62 ± 2.07	20.38 ± 2.07	
SE 1.56%	90.72 ± 0.88	9.28 ± 0.88	0.000*
SE 0.78%	97.03 ± 1.32	2.97 ± 1.32	
SE 0.39%	100.57 ± 0.82	-0.57 ± 0.82	
SE 0.19%	106.13 ± 2.92	-6.13 ± 2.92	

Explanation:
* : ANOVA test shows p<0.05 considered significant;
SE : *Staurogyne elongata* extract

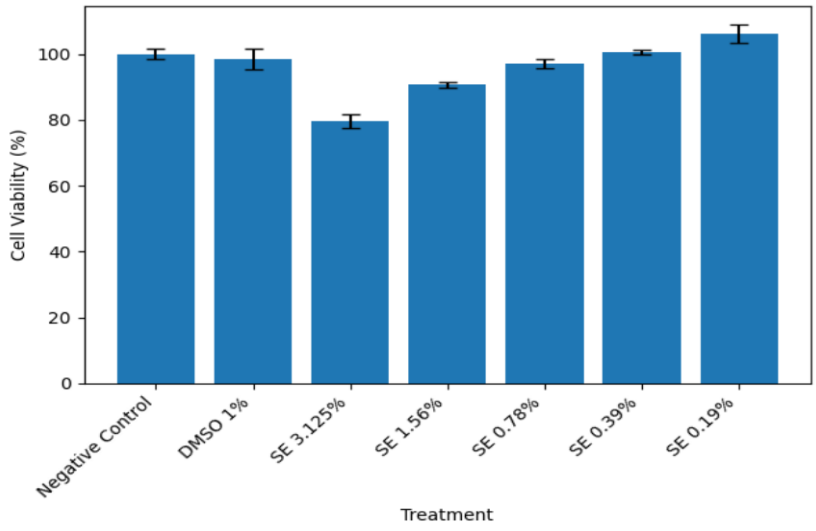


Figure 1. Viability of 3T3 fibroblast cells after treatment with Negative control, DMSO, and S. elongata leaves (SE) at various concentration as calculated using the WST-8 assay. Data are presented as mean ± SD.

The results of the Shapiro-Wilk normality test and Levene's test showed that the data were normally distributed and homogeneous (p>0.05). Statistical analysis using a One-Way ANOVA test showed a significant effect of reundeu leaf extract concentration on NIH/3T3 fibroblast cell line viability (p<0.0001). The differences between treatment groups at various concentrations were further analyzed with a Post Hoc Tukey HSD test. The analysis showed that several treatment groups differed significantly, while no significant differences were observed among the negative control group, DMSO, and lower extract concentrations, as presented in Table 2. Furthermore, microscopic examination confirmed these findings, revealing that the viable cells retained a light or shiny appearance across the control and treatment groups, with no prominent morphological degradation observed at lower concentrations (Figure 1).

Table 2. NIH/3T3 fibroblast cell viability difference among all groups

	NC	1% DMSO	SE 3.125%	SE 1.56%	SE 0.78%	SE 0.39%	SE 0.19%
NC	-	0,911	0,000*	0,000*	0,377	1,000	0,004*
1% DMSO		-	0,000*	0,000*	0,951	0,721	0,000*
SE 3.125%			-	0,000*	0,000*	0,000*	0,000*
SE 1.56%				-	0,003*	0,000*	0,000*
SE 0.78%					-	0,200	0,000*
SE 0.39%						-	0,010*
SE 0.19%							-

Explanation:
* : Tukey HSD post-hoc test with p<0,05 showed that the difference considered significant.
NC: negative control; SE: *Staurogyne elongata* extract.

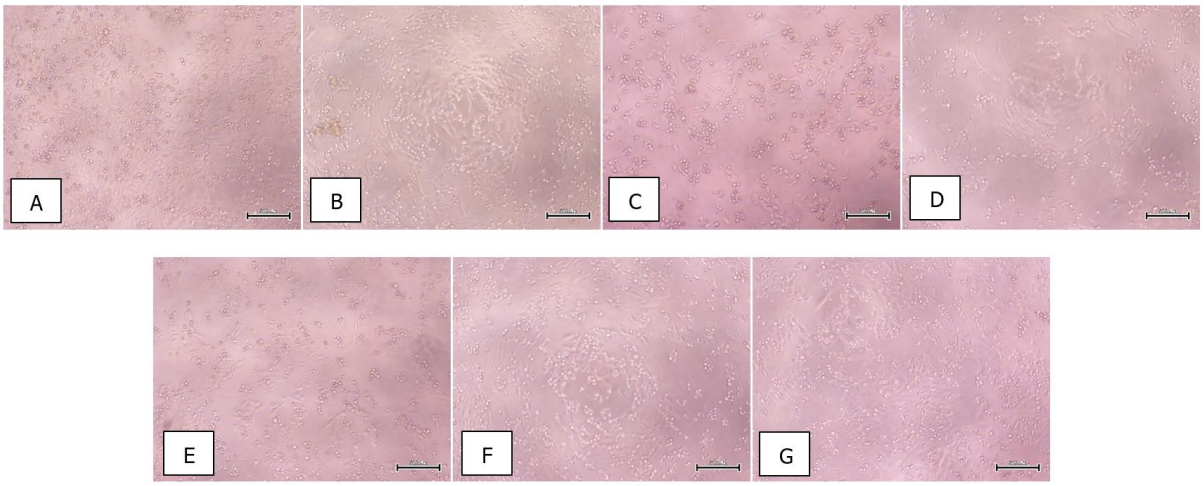


Figure 2. NIH/3T3 fibroblast cells examination with light microscope of 100x magnification with description as follows: A. Negative control; B. DMSO group; C. Application of 3.125% of reundeu leaf extract; D. Group with application of 1.56% extract; E. Group with application of 0.78% extract; F. Group with application of 0.39% extract; G. Group with application of 0.19% extract. The viable cells showed a light or shiny appearance, while unalive cells would appear dark.

DISCUSSION

Reundeu (Staurogyne elongata) is a plant widely used as a medicinal plant in Indonesia. Based on phytochemical tests, its leaves contain various bioactive compounds, such as alkaloids, flavonoids, saponins, polyphenols, and steroids or triterpenoids. Although the phytochemical compounds of reundeu leaves have various therapeutic potentials, evaluating their cytotoxic effect is essential to support their safe usage as herbal medicine.[7]

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Based on the study results, reundeu leaves affected fibroblast cell viability in a concentration-dependent manner, as assessed using the WST-8 assay. The viability of fibroblast cells at the highest concentration (3.13%) exhibited mild cytotoxicity with reduced cell viability compared to other groups. In contrast, lower concentrations showed viability comparable to the negative control and DMSO solvent groups, indicating that lower doses had minimal cytotoxic effects. Statistical tests at concentrations of 0.78% and 0.39% did not differ significantly from the control cell group and the DMSO solvent group. These findings suggest that reundeu leaf extract is concentration-dependent and relatively safe for fibroblast cells at lower concentrations.

The cytotoxic effect observed at higher concentrations may be attributed to the increased levels of bioactive compounds. While these compounds present antioxidant properties at low doses, they can induce cellular stress and interfere with mitochondrial metabolic activity at higher concentrations. This is particularly relevant to the WST-8 assay, which reflects the activity of mitochondrial dehydrogenase as a parameter.[19,20]

Reundeu leaves contain phytochemical constituents such as alkaloids, flavonoids, saponins, polyphenols, and steroids or triterpenoids. These have recognized health benefits due to their antioxidant, antibacterial, and anti-inflammatory properties, as well as their protective properties against free radicals.[10,21-23] Several phytochemicals, including flavonoids, saponins, and alkaloids, have toxic potential and can contribute to reduced viability through mechanisms like inducing oxidative stress, cell membrane damage, and cell cycle inhibition.[9,23-27] These compounds have the potential to cause adverse biological effects or toxicity reactions leading to cell death when applied in high concentrations. Therefore, it is important to comprehend the safe dosage if it is aimed to be developed in the field of treatment.[28-30]

The dose-dependent cytotoxicity observed in this study is coherent with findings from an acute oral toxicity study of reundeu leaves. This study reported no significant toxic effects in terms of changes in weight, autonomic system, behavior, sensory, neuromuscular, cardiovascular, respiratory, eye, gastrointestinal, urinary, and skin of mice, and no mice died during the study, indicating an LD50 value greater than 5 g/kg body weight. However, in vivo toxicity outcomes cannot be directly compared with in vitro cytotoxicity results. These findings suggest that differences in metabolic processing and exposure conditions may account for the mild cytotoxicity at higher concentrations in fibroblast cell cultures in this study.[31]

The relationship between the cytotoxicity of herbal extracts, their concentration, and their phytochemical content has been established through studies on cell viability after exposure to various concentrations of plant extracts. A study by Zain (2020) on the cytotoxicity of oil palm leaf on the fibroblast 3T3 cell line using an MTT assay showed that cell line viability at all concentrations was above 70% and also increased viability at lower concentrations.15 Tyatana (2021) demonstrated that the seed coat extract of B. flabellifer L. exhibited a concentration-dependent cytotoxic effect on fibroblasts with an MTT assay; concentrations from 1.56% to 12.5% were classified as moderately cytotoxic, while higher concentrations displayed severe toxicity.[32] Based on the study by Laksmitawati (2016) regarding the effect of G. vulgaris and A. muricata extracts on the viability of macrophage cell line RAW264.7 with an MTS assay, higher concentrations of extracts were cytotoxic, while the application of lower extract concentrations indicated non-toxic levels and higher cell viability.[33] The results of these studies are in line with those of Gothai (2016), who also showed that the toxicity rate of a material is directly proportional to its concentration.[34] However, Harjadi et al. (2022) found that administration of tamarind ethanol extract on 3T3-L1 cells with a WST-8 assay at concentrations of 0.001, 0.004, 0.016, 0.063, 0.25, and 1.00 µg/mL exhibited low toxicity, with a viability level of 85%. These study results indicated that a minimal cytotoxic effect occurred at lower concentrations, but cell viability reduced with increased plant extract concentration. However, the limitations of this study were that the concentration range was less varied, the solvent used was only ethanol, and only a single method of viability assay was performed. In addition, in vitro cytotoxicity testing has another limitation since it cannot completely represent complex biological processes such as systemic effects, metabolism, and cell interactions in tissues that can be examined in vivo.[20,34]

CONCLUSION

This research indicated that the ethanol extract of reundeu (Staurogyne elongata) leaf exhibits mild to minimal cytotoxicity at the tested concentrations. The implication of this study suggests the potential of reundeu leaves for further development as a safe natural ingredient. These findings may give preliminary support for the use of reundeu leaf extract for alternative therapeutic applications, such as in wound healing. Therefore, from these results, further investigations, such as in vivo testing and clinical evaluations, are required to assess the comprehensive level of safety and efficacy of reundeu leaf extract for therapeutic use.

DECLARATION

Authors Contributions

[IS]: Conceptualization, Methodology, Formal Analysis, Writing – Original Draft; **[FN]:** Investigation, Software, Data Curation; **[EY]:** Supervision, Writing – Review & Editing, **[RF]:** Writing – Review & Editing. All authors have read and approved the published version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of Interest

authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethics Approval and Consent to Participate

The study protocol was reviewed and approved by the Ethics Committee of Faculty of Medicine, Universitas Jenderal Achmad Yani (Protocol No. 006/UH.3/02/2025).

Consent for Publication

Not applicable.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Generative AI In Scientific Writing

The authors declare that no generative AI or AI-assisted technologies were used in the writing or preparation of this manuscript.

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