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Regulation of IL-10 by Herbal and Hormonal Therapy: The Impact of *Eleutherine Bulbosa* and Tamoxifen in an Experimental Breast Cancer Model

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ABSTRACT

Background: Breast cancer is the most common cancer affecting women worldwide, with the number of cases and deaths continuing to rise. This study evaluated the single and combined effects of *Eleutherine bulbosa* ethanol extract and tamoxifen in reducing interleukin 10 (IL-10) levels in BALB/c mice with breast cancer models.

Methods: This study used a laboratory experimental approach with a posttest-only control group design, using 36 female *Mus musculus* BALB/c strain mice (aged 8–10 weeks), randomly divided into 6 groups: negative and positive control groups and 4 intervention groups. The intervention groups were given *Eleutherine bulbosa* ethanol extract at a dose of 180 mg/kg for 14 days, tamoxifen at a dose of 10 mg/kg every 2 days for 7 days within a 14-day period, a combination of *Eleutherine bulbosa* ethanol extract and tamoxifen, and tamoxifen and *Eleutherine bulbosa* ethanol extract. Effectiveness was evaluated based on IL-10 levels in serum.

Results: The combination of tamoxifen and ethanol extract from *Eleutherine bulbosa* significantly reduced IL-10 levels in BALB/c mice with a breast cancer model. The *Eleutherine bulbosa* ethanol extract reduced IL-10 by 31.4%, while tamoxifen reduced it by 59.1%. The strongest synergistic effect was observed when combined with tamoxifen, reducing IL-10 levels by 64.6%, indicating its effectiveness in suppressing the immunosuppressive response supporting tumor growth.

Conclusion: Tamoxifen and *Eleutherine bulbosa* extract showed synergistic effects in reducing IL-10 levels, suggesting enhanced immunomodulatory action compared with monotherapy. This combination can enhance breast cancer treatment efficacy by diminishing immunosuppression.

INTRODUCTION

Breast cancer is the most common cancer among women worldwide, with incidence rates continuing to rise each year.[1] In 2024, the American Cancer Society estimated 313 510 new cases and 42 780 deaths from breast cancer globally.[2] In Indonesia, breast cancer is a serious health problem, with 66 271 new cases and 22 598 deaths reported in 2020, underscoring the need for effective treatment strategies.[3]

Breast cancer is caused by various complex factors, including genetic mutations, hormonal

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imbalance, and oxidative stress.[4,5] Interleukin 10 (IL-10) plays an important role in breast cancer pathogenesis. Despite its anti-inflammatory properties, IL-10 can support cancer cell proliferation and migration and is associated with a poor prognosis.[6] IL-10 acts through the JAK1-TYK2-STAT3 signaling pathway, which reduces the production of proinflammatory cytokines and T-cell proliferation.[7] In the context of tumors, IL-10 promotes carcinogenesis by affecting neutrophils, making it a biomarker for cancer diagnosis, prognosis, and therapy.[8,9]

Tamoxifen is the primary therapy for estrogen receptor–positive (ER-positive) breast cancer.[10,11] This drug inhibits estrogen action in breast tissue to reduce cancer cell proliferation, although long-term use can cause adverse effects.[12] As an alternative, natural compounds from herbal plants have potential as anticancer agents. *Eleutherine bulbosa*, rich in bioactive compounds such as flavonoids, polyphenols, alkaloids, quinones, and saponins,[13,14] exhibits antioxidant, antitumor, and antimetastatic activities. Its active compounds, such as avenasterol, inhibit the proliferation of MCF-7 breast cancer cells.[15,16] Given the challenges and limitations of conventional therapy, this study investigated the potential of combination therapy with tamoxifen and *Eleutherine bulbosa* ethanol extract to reduce IL-10 levels. An in vivo approach using 7,12-dimethylbenz[a]anthracene (DMBA)–induced BALB/c mice (*Mus musculus*) was chosen to comprehensively evaluate the efficacy and mechanism of action.[17] The main objective was to demonstrate the stronger synergistic effect of combination therapy compared with single therapy in reducing IL-10 levels, thereby potentially improving the efficacy of breast cancer treatment. This study is part of a comprehensive research project that also analyzed TP53 apoptosis, tumor necrosis factor α (TNF-α), cyclooxygenase 2 (COX-2), and interleukin 6 (IL-6).

METHODS

Study design

We employed a completely randomized design (CRD) with a posttest-only control group to minimize selection bias by ensuring equal treatment assignment probability for all subjects. Healthy and energetic female mice (*Mus musculus*), aged 8 to 10 weeks and weighing 18 to 25 g, were included in the study.

Exclusion criteria

Mice that refused food/water, died during the study, or were found to be pregnant at any point were excluded from the analysis. The dropout criteria included mice that died prior to the conclusion of the study. The mice were sourced from the Healthy Animal Laboratory in Malang, Indonesia.

Eleutherine bulbosa extract preparation

Eleutherine bulbosa, a medicinal plant indigenous to Kalimantan, was harvested in amounts totaling 40 kg from the Kalimantan area. The reddish-purple tubers were separated, cleaned, sliced, and dried at 40 °C for 1 week at the Women's Health Laboratory of Hasanuddin University. The dried tubers were pulverized and macerated with 96% ethanol over 3 periods of 24 hours at the Phytopharmaceutical Laboratory. The filtrate was subjected to filtration, evaporation, and subsequent drying to yield a concentrated dark crimson extract for further pharmacological evaluation.

Breast cancer model induction

This study adhered to ethical animal procedures. Breast cancer was induced in 36 BALB/c mice using the carcinogenic substance DMBA. Each mouse weighing 20 g was administered a dose of 1 mg of DMBA per day for 42 days, resulting in a total DMBA dose of 42 mg per mouse (1 mg/d × 42 days = 42 mg). Before administration, DMBA was dissolved in sesame oil to form a 1% solution, and each mouse received 0.1 mL of this solution per day via an oral tube. This procedure aimed to induce breast cancer in vivo as an experimental model and was performed at Satwa Sehat, Malang. The breast cancer model in mice was validated by examining histopathological changes (cell and tissue structure) and using immunohistochemistry (IHC) techniques. In addition, the presence of lumps and tumor masses physically detected in DMBA-induced mice was also an indication of the success of the model.

Experimental procedures

Experimental groups. This study employed a laboratory experiment utilizing a posttest-only control group design within a completely randomized design framework. This approach was implemented to mitigate selection bias and ensure each subject had an equal probability of being assigned to any treatment group. The study included 36 female BALB/c mice (*Mus musculus*), aged 8 to 10 weeks and weighing approximately 18 to 25 g. Female BALB/c strain mice that satisfied the inclusion and exclusion criteria were allocated to 2 control groups and 4 intervention groups, with 6 mice in each group. These mice were randomly allocated to groups. The negative control group consisted of healthy mice that were only fed and given water without any treatment. The positive control group consisted of breast cancer model mice that did not receive *Eleutherine bulbosa* ethanol extract or tamoxifen. Intervention group 1 was given *Eleutherine bulbosa* ethanol extract at 180

mg/kg daily for 14 days,[18] while intervention group 2 was given tamoxifen at 10 mg/kg every 2 days for 7 doses. Intervention group 3 received a combination of *Eleutherine bulbosa* extract and tamoxifen simultaneously, and intervention group 4 received a combination of tamoxifen followed by *Eleutherine bulbosa* extract sequentially for 14 days.

Fourier transform infrared spectroscopy analysis**.** Fourier transform infrared (FTIR) spectroscopy analysis was performed at the Analytical Chemistry and Food Control Laboratory, Faculty of Agriculture, Hasanuddin University, Makassar, to nondestructively identify the functional groups of active chemicals in the concentrated *E bulbosa* ethanol extract. A droplet of the extract was deposited onto the surface of an attenuated total reflectance (ATR) crystal, and the instrument was scanned across the range of 4000 to 400 cm^{-1}. The resultant spectrum was examined to identify the –OH, –C=O, and –C–O functional groups, which are typically present in flavonoids and phenolic compounds. This technique is rapid, precise, and suitable for phytochemical analysis of plant extracts (Figure 1).

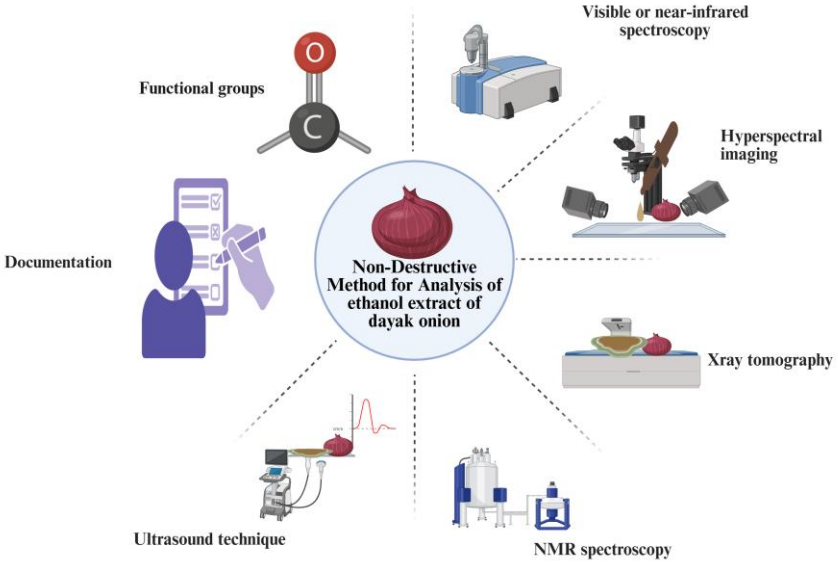

Figure 1. Examination Process of Fourier Transform Infrared Spectroscopy of Ethanol Extract of *Eleutherine bulbosa.* This image shows various nondestructive techniques, such as FTIR, nuclear magnetic resonance (NMR), ultrasound, x-ray tomography, and hyperspectral imaging, which are used to analyze bioactive compounds without damaging the sample. FTIR was used to identify the functional groups of phenol, alcohol, and alkyl from organic compounds, flavonoids, aldehydes, ketones, esters, and glycosides that play a role in pharmacological activity. (BioRender)

Hematoxylin-eosin analysis

Histopathological examination was performed at the Satwa Sehat Laboratory in Malang using hematoxylin-eosin staining as the conventional method for breast cancer diagnosis. Tissue was acquired via biopsy, preserved in 10% formalin, embedded in paraffin blocks, and finely sectioned using a microtome. Hematoxylin staining highlights cell nuclei, whereas eosin imparts color to the cytoplasm. The data were analyzed microscopically to evaluate cell differentiation, mitotic activity, and invasion patterns, and to ascertain the malignancy grade using the Nottingham approach to inform the treatment plan.

Immunohistochemistry

The IHC procedure on the blood plasma of breast cancer mice was performed by collecting blood into an EDTA tube and centrifuging it at 1500*g* for 10 minutes at 4 °C to obtain plasma. A total of 100 µL of plasma was placed on a glass slide using the cytospin method (600 rpm, 4 minutes) and fixed with 4% paraformaldehyde for 10 minutes. After rinsing with phosphate-buffered saline (PBS), the sample was treated with 0.1% Triton X-100 for 10 minutes for permeabilization and blocked with 5% normal serum for 45 minutes. Next, the slides were incubated with p53 primary antibody overnight at 4 °C, followed by incubation with horseradish peroxidase (HRP)–labeled secondary antibody for 1 hour. Staining was performed with DAB substrate until a brown color appeared, followed by staining with hematoxylin contrast dye, gradual dehydration, and permanent mounting. Positive and negative controls, as well as 4

intervention groups, were used to ensure specific results were obtained.

ELISA examination**.** This test was conducted by the Healthy Animal Laboratory in Malang using the enzyme-linked immunosorbent assay (ELISA) method, a quantitative immunological technique used to measure IL-10 levels in biological samples such as serum, plasma, or tissue homogenates. The ELISA method is a sandwich technique, wherein the IL-10 antigen in the sample binds to specific antibodies coated on a microtiter plate, followed by the addition of biotinylated antibodies and streptavidin-HRP enzyme. The enzymatic reaction produces a color change proportional to the concentration of IL-10, which was read using a spectrophotometer at 450 nm (Figure 2). The findings indicated a reduction in IL-10 levels, signifying an increase in the inflammatory response.

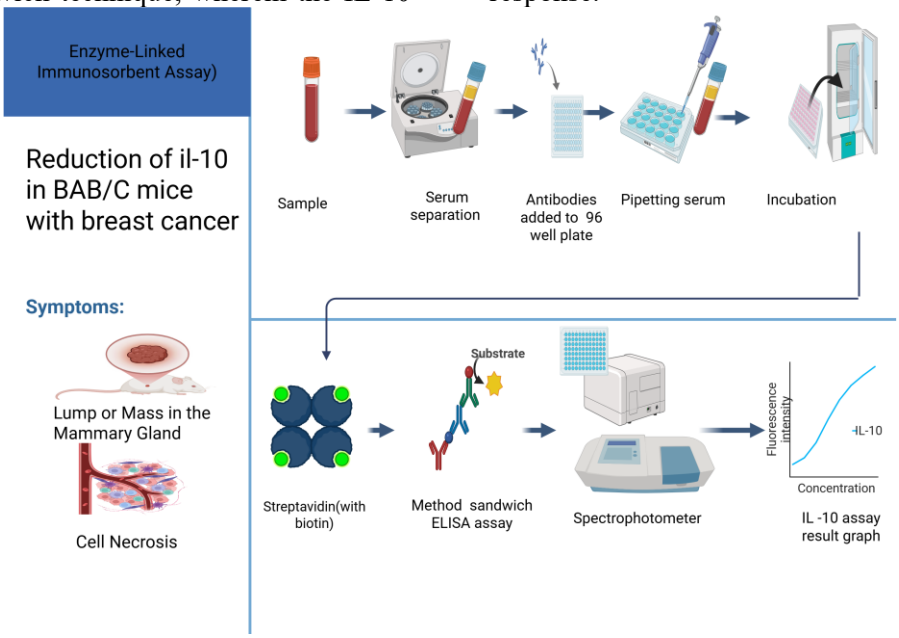

Figure 2. IL-10 ELISA Assessment Procedure in Each Treatment Cohort of Experimental Animals Using Blood Samples.[19] This image depicts the phases of IL-10 level assessment using the sandwich ELISA technique in BALB/c mice with breast cancer, marked by tumors in the mammary glands and cellular necrosis. The findings indicated a reduction in IL-10 levels, signifying an increase in the inflammatory response. This result corresponds with findings in the journal Biomedical Research and Therapy, highlighting the significant function of IL-10 as an anti-inflammatory cytokine in the pathology of chronic illnesses.

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Statistical analysis

In the data analysis using SPSS version 25, the normality test was performed first, as indicated by the results of the Kolmogorov-Smirnov and Shapiro-Wilk tests ($P > 0.05$), indicating that the data were normally distributed. Next, a homogeneity test was performed, and because the results were not homogeneous, a Brown-Forsythe test was performed to determine whether there were significant differences between the intervention groups. Subsequently, to identify the specific intervention groups that had significant differences, a post hoc Games-Howell test was used, which showed significant differences between the intervention groups.

RESULTS

Analysis results of Eleutherine bulbosa ethanol extract

FTIR analysis of the extract revealed the presence of several functional groups that significantly contributed to its biological activity (Table 1).

Table 1. Analysis of *Eleutherine bulbosa* Ethanol Extract

Cluster	Peak	Compound
O–H (hydroxyl)	3348.85 cm^{-1}	Phenol or alcohol
C–H (aliphatic)	2927.28 cm^{-1}	Alkyl of organic compounds
C=O (carbonyl)	1649.65 cm^{-1}	Flavonoids, aldehydes, or ketones
C=C (aromatic)	1451.43 cm^{-1}	Flavonoids
C–O	1055.00 cm^{-1}	Alcohols, esters, or glycosides

The absorption spectra at approximately 3348.85 cm^{-1} signify the presence of hydroxyl (O-H) groups in phenolic chemicals or alcohols, which are known for their antioxidant properties. The absorption peak

at 2927.28 cm^{-1} signifies aliphatic C-H groups from alkyl chains, whereas the peak at 1649.65 cm^{-1} denotes carbonyl groups (C=O), typically present in flavonoids, aldehydes, or ketones, which possess significant anti-inflammatory and anticancer properties. The detection of aromatic C=C bonds at 1451.43 cm^{-1} corroborates the existence of aromatic chemicals such as flavonoids, and the peak at 1055.00 cm^{-1} signifies C-O groups from alcohols, esters, or glycosides. These findings suggest that the extract contains bioactive chemicals that can be developed as pharmacological agents for cancer therapy.

Results of histopathological examination

Histopathological analysis of breast tissue from a murine model of breast cancer using hematoxylin-eosin staining revealed distinct cellular morphological variations among the different treatment groups. The positive control group exhibited predominant and dense proliferation of cancer cells, which displayed distinct malignant characteristics, such as hyperchromatic nuclei, pleomorphism, and an elevated nucleus-to-cytoplasm ratio. The tissue architecture was disordered, exhibiting stromal invasion with few signs of apoptosis. Concurrently, the cohorts administered tamoxifen or *Eleutherine bulbosa* ethanol extract exhibited a reduction in tumor cell density, expansion of intercellular gaps, and indications of apoptosis, including pyknosis, karyorrhexis, and cytoplasmic vacuolization. The combination of tamoxifen and *Eleutherine bulbosa* extract exhibited the most significant therapeutic effect, characterized by an extensive area of focal necrosis and a marked decrease in the number of active cancer cells, suggesting the synergistic potential of these agents in suppressing proliferation and inducing tumor cell apoptosis (Figure 3).

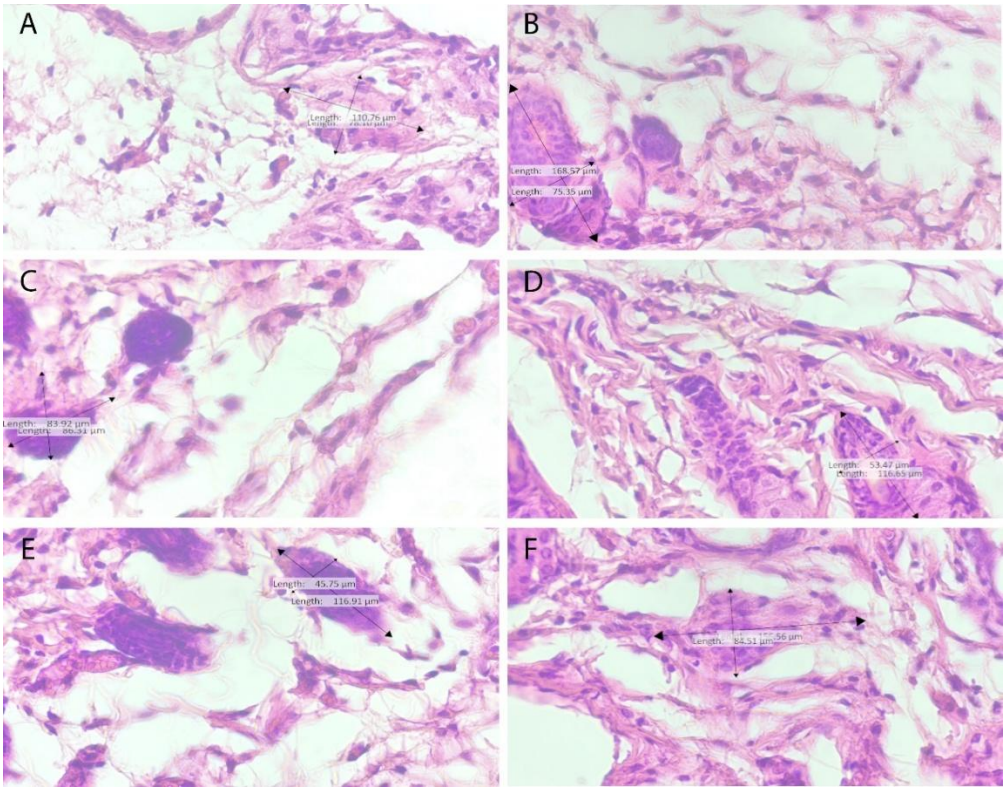

Figure 3. Histopathological Examination of Mammary Tissue in Each Treatment Group. A, Negative control. B, Positive control. C, Intervention 1 (*Eleutherine bulbosa* treatment group). D, Intervention 2 (tamoxifen treatment group). E, Intervention 3 (combination treatment group of *Eleutherine bulbosa* and tamoxifen). F, Intervention 4 (combination treatment group of tamoxifen and *Eleutherine bulbosa*).

Immunohistochemistry

Immunohistochemical examination of breast tissue in experimental animals was performed to evaluate the expression of markers associated with breast cancer growth and responses. The tissue examination results showed differences in the responses of each group. In the control group (Figure 4A), there were no signs of cancer; therefore, the staining of cancer markers was almost invisible. In contrast, in the cancer-induced group (Figure 4B), cancer markers were clearly visible, indicating rapid cancer cell growth. After treatment, *Eleutherine bulbosa* (Figure 4C), tamoxifen (Figure 4D), and *Eleutherine bulbosa* and tamoxifen (Figure 4E) suppressed cancer growth, although tamoxifen showed a stronger reduction. The best results were

observed in the group treated with a combination of tamoxifen and *Eleutherine bulbosa* (Figure 4F), where cancer marker expression decreased drastically, approaching normal conditions. This indicates that the combination of both is far more effective in combating cancer than when they are used separately.

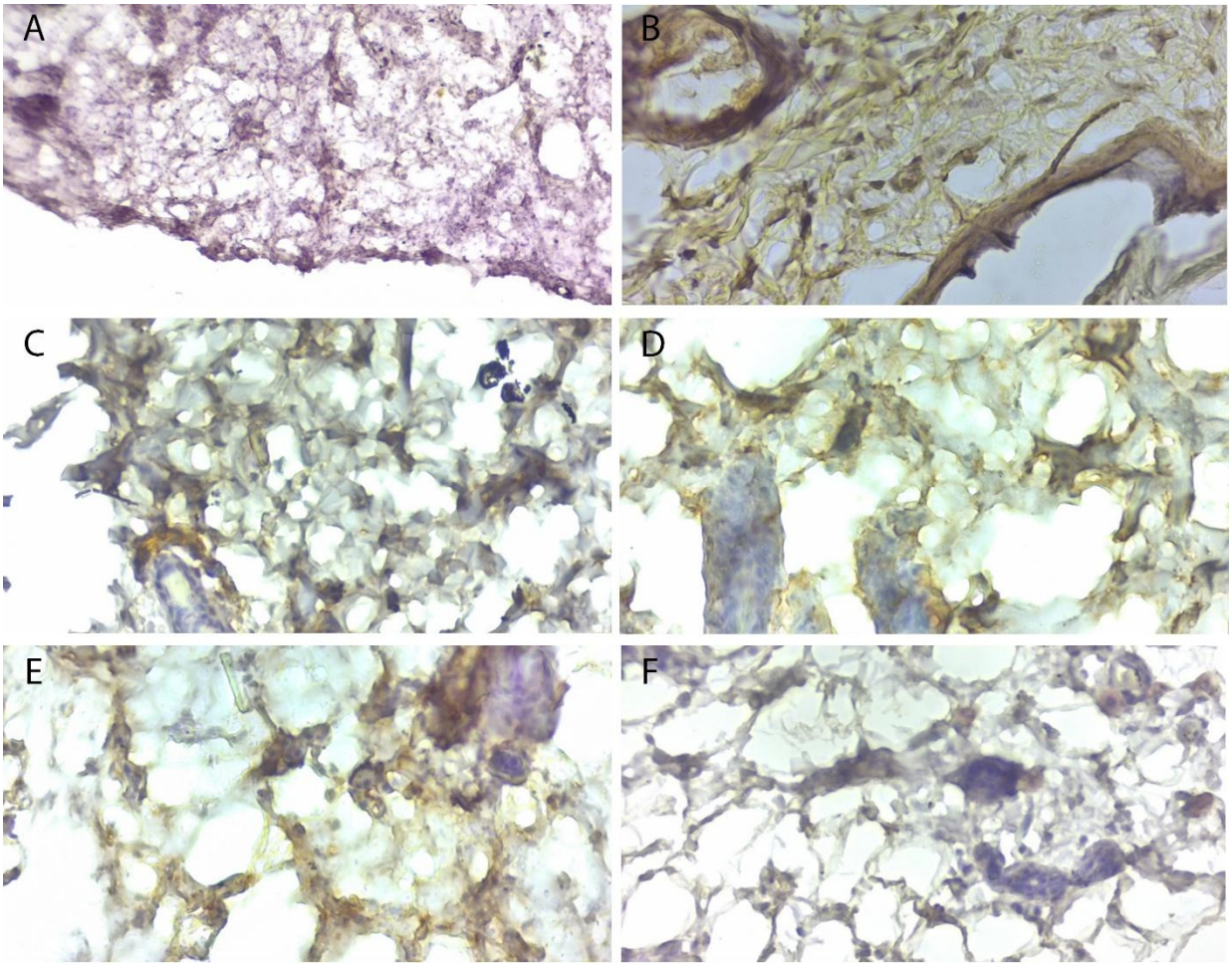

Figure 4. Immunohistochemical Examination of Mammary Tissue in Each Treatment Group. A, Negative control. B, Positive control. C, Intervention 1 (*Eleutherine bulbosa* treatment group). D, Intervention 2 (tamoxifen treatment group). E, Intervention 3 (combination treatment group of *Eleutherine bulbosa* and tamoxifen). F, Intervention 4 (combination treatment group of tamoxifen and *Eleutherine bulbosa*).

ELISA analysis

Results from the ELISA analysis of IL-10 concentrations are presented below.

Normality test results

The normality test results showed that all treatment groups had a normal data distribution, as indicated by a *P* value greater than 0.05. Thus, the data meet the requirements to proceed to the homogeneity test stage to assess variance uniformity, so that the analysis of IL-10 levels between groups can be carried out more accurately and validly.

Homogeneity test result

Levene test for homogeneity of variance on the IL-10 variable yielded a significance value of 0.014 (based on the mean), which is below the threshold α (0.05). This finding indicates that the variances between groups are not homogeneous ($P < 0.05$); therefore, the assumption of variance homogeneity as a key prerequisite for the use of standard parametric tests, such as analysis of variance (ANOVA), is not met. Therefore, for further comparative analysis, it is recommended to use alternative statistical tests that are more robust to variance inequality, such as the Brown-Forsythe test.

Brown-Forsythe test result

The Brown-Forsythe test results for the IL-10 variable showed a statistical value of $F_{5,18.607} = 527.534$, with a significance value of $P < 0.001$. A minimal significance value ($P < 0.05$) indicated a statistically significant difference in the mean IL-10 levels between the treatment groups. Thus, it can be concluded that the treatment given to

each group affects the IL-10 level. The observed variation in IL-10 levels between the groups was statistically significant and not due to chance. These findings provide the basis for continuing the Games-Howell post hoc test analysis to identify which pairs of groups specifically show significant differences.

Games-Howell test result

Based on the results of the Games-Howell test (Table 2), which was used as a post hoc test after it was found that the data did not meet the assumption of variance homogeneity, most of the comparisons between treatment groups showed statistically significant differences in IL-10 levels ($P < 0.05$). This test was chosen because it accommodates conditions of nonhomogeneous variance and unequal sample sizes, making it suitable for continuing the analysis after the Brown-Forsythe test.

Table 2. Results of Games-Howell Test of IL-10 Levels in Mice (*Mus musculus*) Induced with DMBA by Administration of Tamoxifen and *Eleutherine bulbosa* Ethanol Extract

Treatment group	n	IL-10, mean
Negative control	6	0.4140
Intervention 4	6	0.4834
Intervention 2	6	0.5595
Intervention 3	6	0.7543
Intervention 1	6	0.9377
Positive control	6	1.3668

Figure 5 displays the results of IL-10 level measurements (pg/mL) using the ELISA method in various treatment groups. The highest IL-10 level was found in the positive control group at 1.37 pg/mL, followed by intervention group 1 at 0.94 pg/mL, intervention group 3 at 0.75 pg/mL, intervention group 2 at 0.56 pg/mL, and intervention group 4 at 0.48 pg/mL, and the lowest level in the negative control group at 0.41 pg/mL. These results indicate that the treatment in intervention group 1 significantly increased IL-10 levels compared with the negative control, although it was still lower than that of the positive control.

DISCUSSION

The results showed that the combination of tamoxifen therapy and *Eleutherine bulbosa* extract had the most significant effect on reducing IL-10 levels in mice with breast cancer. Group I4 showed a 64.6% decrease in IL-10 levels compared with the positive control (100%) receiving a combination of tamoxifen and *Eleutherine bulbosa*, and only a slight difference was observed from the negative control group, which experienced a 69.7% decrease.

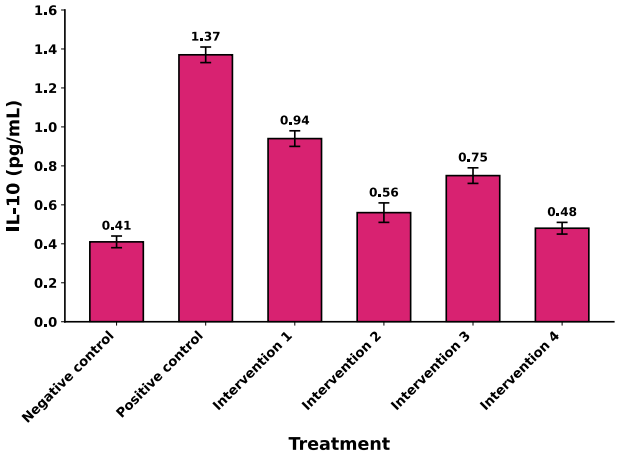

Figure 5. IL-10 Levels in BALB/c Strain Mice (*Mus musculus*) Induced with Breast Cancer Using DMBA After Administration of Tamoxifen and *Eleutherine bulbosa*. NC indicates negative control; PC, positive control; I1, Eleutherine bulbosa treatment group; I2, tamoxifen treatment group; I3, combined Eleutherine bulbosa and tamoxifen treatment group; I4, combined tamoxifen and Eleutherine bulbosa treatment group.

This is likely due to the action of tamoxifen as an antiestrogenic agent that inhibits cancer cell proliferation and reduces excessive immune activation, as well as the contribution of *Eleutherine bulbosa*, which contains active compounds such as flavonoids, polyphenols, alkaloids, quinones, and saponins, which are known to have antioxidant and anti-inflammatory activities. The combination of these 2 agents works synergistically by suppressing the expression of proinflammatory cytokines, such as IL-10, and by enhancing the regulation of the immune system, resulting in a more optimal anti-inflammatory effect than the administration of *Eleutherine bulbosa* and tamoxifen alone or in combination. Therefore, the combination of tamoxifen and *Eleutherine bulbosa* has the potential to be more effective in controlling inflammatory biomarkers such as IL-10 in animal models of breast cancer.[14,20,21]

A comparison of IL-10 levels between intervention 3 (*Eleutherine bulbosa* and tamoxifen) and intervention 4 (tamoxifen and *Eleutherine bulbosa*) showed a significant difference. The mean IL-10 level in intervention 3 was 0.7543 pg/mL, while in intervention 4, it was 0.4834 pg/mL, representing a decrease of 0.2709 pg/mL or 35.9% when the sequence of administration began with tamoxifen followed by *Eleutherine bulbosa*. Conversely, the IL-10 level in intervention 3 was 56.0% higher than that in intervention 4, indicating that the sequence of administration potentially affects the combined immunomodulatory effect. Intervention 4 (tamoxifen and *Eleutherine bulbosa*) was more effective in reducing IL-10 than intervention 3

(*Eleutherine bulbosa* and tamoxifen) due to a combination of pharmacodynamic and pharmacokinetic factors influenced by the sequence of administration. First, tamoxifen has an "off-target" immunomodulatory effect on macrophages that tends to reset the tumor microenvironment toward a proinflammatory response (eg, increased M1 macrophage activation, reduced IL-10), so that administering tamoxifen first can reduce the initial immunosuppressive pressure and open a "window" in which the immunostimulatory action of *Eleutherine bulbosa* compounds can work more effectively.[22,23] Second, the phytochemical compounds in *Eleutherine bulbosa* (flavonoids, polyphenols, alkaloids, quinones, and saponins) are known to suppress the nuclear factor κB (NF-κB) pathway and increase the expression of proimmune cytokines that support T-cell activity when administered after tamoxifen; the inhibitory effect of IL-10 by both agents can be synergistic, resulting in a greater reduction in IL-10.[24,25] Third, from a pharmacokinetic perspective, administering *Eleutherine bulbosa* before tamoxifen risks reducing tamoxifen availability or altering its metabolism through enzymatic (CYP) interactions or protein binding mechanisms, as reviewed in studies on the interaction between *Eleutherine bulbosa* and tamoxifen, thereby reducing tamoxifen efficacy if *Eleutherine bulbosa* is administered first. Conversely, administering tamoxifen first reduces this risk and allows *Eleutherine bulbosa* to act as an adjuvant.[26] Overall, modern literature supports the notion that the sequence of administration of systemic drugs and natural agents influences immunological and therapeutic outcomes, such that protocols in which tamoxifen is administered before *Eleutherine bulbosa* biologically result in a greater reduction in IL-10.[24,26]

The challenges, safety, and clinical implications of combining *Eleutherine bulbosa* ethanol extract and tamoxifen should be considered before use in patients. Although animal studies have shown a decrease in IL-10 levels, human studies are still needed to confirm safe and effective dosages that do not cause organ damage. The active compound content of *Eleutherine bulbosa* may also vary depending on the growing location and extraction method, necessitating the standardization of products. Additionally, interactions with tamoxifen via metabolic enzymes may affect efficacy or increase adverse effects; therefore, the order of administration must be considered. Clinically, these findings support more personalized cancer treatment by combining conventional therapy with natural compounds to enhance therapeutic outcomes and improve the immune response.[27–29]

CONCLUSION

This study revealed that the combination of tamoxifen and ethanol extract from *Eleutherine bulbosa* significantly reduced IL-10 levels and improved breast tissue architecture in a mouse model of breast cancer. The combination group had the most favorable outcomes, with IL-10 levels nearing normalcy and tissue architecture similar to that of healthy tissue, suggesting a synergistic effect on immune modulation and tissue preservation. These findings support the possibility of this combination as adjunctive therapy; nevertheless, additional research is required for clinical implementation.

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CONFLICT OF INTEREST

The writers assert that they possess no conflicts of interest to reveal.

ETHICAL CONSIDERATIONS

This study was conducted following the acquisition of ethical approval (number 049/UN4.14.1/TP.01.02/2024) from the Health Research Ethics Committee (KEPK) of the Faculty of Public Health, Hasanuddin University.

FUNDING

None.

DATA AVAILABILITY

Data can be obtained upon request from the corresponding authors.

AUTHOR CONTRIBUTIONS

R: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. ANU: Conceptualization, Methodology, Data curation, Project administration, Writing – review & editing. RY: Data curation, Formal analysis, Supervision, Writing – review & editing. S: Data curation, Formal analysis, Writing – review & editing. AA: Investigation, Writing – review & editing.

AI DISCLOSURE

Artificial intelligence–assisted tools were used during manuscript preparation to improve language clarity and grammar. These tools were not involved in study design, experimental procedures, data analysis,

or interpretation of results. The authors reviewed and approved the final manuscript and take full responsibility for its content.

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