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Synergistic Effects of Ethanol Extracts of *Eleutherine bulbosa* (Dayak Onion) and Tamoxifen on TNF-α and Breast Tissue Morphology in a Mouse Model of Breast Cancer

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ABSTRACT

Breast cancer is the most common malignancy in women globally, influenced by inflammatory mediators like Tumor Necrosis Factor-alpha (TNF-α). Although, tamoxifen is a standard therapy for breast cancer, its effectiveness is often limited by adverse side effects and development of resistance. This study aimed to investigate the synergistic potential of tamoxifen and *Eleutherine bulbosa* ethanol extract in modulating TNF-α levels and improving breast tissue morphology in mouse model of breast cancer. Breast cancer was induced in five of six BALB/c mouse groups (n = 36) by oral administration of 7,12-dimethylbenz(a)anthracene (DMBA) for 28 days, while the negative control remained uninduced. Following induction, the mice received oral treatments with *E. bulbosa* extract (180 mg/kg) and tamoxifen (10 mg/kg), administered either individually or in sequential combination for 14 days. Serum TNF-α levels were assessed on day 15 using ELISA, and breast tissue morphology was examined histologically. All treatment groups showed a significant TNF-α reduction compared to the positive control ($p < 0.001$). The sequential administration of tamoxifen followed by *E. bulbosa* produced the most pronounced reduction in TNF-α levels (53.32 ± 4.94 ng/mL), approaching that observed in the Negative Control group (40.10 ± 5.10 ng/mL). This effect was significantly greater than that achieved with *E. bulbosa* extract alone (87.48 ± 3.70 ng/mL) or tamoxifen monotherapy (65.68 ± 6.51 ng/mL). Histopathological analysis showed significant improvements in breast tissue morphology, closely resembling normal histological architecture. In conclusion, tamoxifen combined with *E. bulbosa* extract showed superior efficacy in reducing TNF-α and restoring breast tissue.

Keywords: Breast cancer, *Eleutherine bulbosa*, Tamoxifen, Tumor Necrosis Factor-alpha, Histopathology.

Introduction

According to GLOBOCAN 2020 reports, breast cancer is the most commonly diagnosed cancer among women globally, with about 2.3 million new breast cancer cases and 685,000 breast cancer-related deaths recorded globally.[1] This steadily increasing incidence rate is associated with multiple factors such as dietary patterns, ethnic background, socioeconomic disparities, and limited access to healthcare services.[2-5] Tumor Necrosis Factor-alpha (TNF-α) is one of the key pro-inflammatory mediators involved in the pathogenesis of breast cancer. This cytokine plays a pivotal role in driving inflammatory processes, stimulating angiogenesis, and promoting the proliferation of malignant cells.[6] Elevated TNF-α levels have been closely linked to increased tumor aggressiveness and the development of resistance to hormonal therapy. Tamoxifen remains the standard first-line treatment for patients with estrogen receptor– positive (ER+) breast cancer. However, its therapeutic efficacy is often limited by biological resistance mechanisms, including elevated expression of High-Mobility group Box 1 (HMGB1) and genetic variations in Cytochrome P450 2D6 (CYP2D6), as well as an increased risk of adverse effects such as thromboembolism.[7–10] Therefore, developing combination therapy strategies that incorporate natural compounds with potential synergistic effects and a better safety profile is highly needed.[11,12]

Eleutherine bulbosa, a tuberous plant belonging to the Iridaceae family, is an endemic medicinal plant of Kalimantan. It contains various bioactive compounds, including flavonoids, naphthoquinones, and phenolics, that possess antioxidant, anti-inflammatory, and anticancer properties.[13–15] Flavonoids, in particular, are known to suppress the expression of TNF-α and Interleukin-6 (IL-6), suppress tumor cell growth and trigger apoptosis through the regulation of the Phosphoinositide 3-kinase/Akt (PI3K/Akt) and p38 Mitogen-Activated Protein Kinase (p38 MAPK) signaling pathways.[14–16]

BALB/c mice with 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer are widely used as an experimental breast cancer model because their histopathological and molecular profiles closely resemble those of human breast cancer, particularly in terms of estrogen receptor alpha (ERα) activation and the upregulation of *Cyclin D1* and *c-Myc* genes.[17–19] This model is particularly relevant to this study because DMBA induction is known to elicit inflammatory responses and elevate cytokine levels, including TNF-α, which is the primary target of this research.

Therefore, this study aimed to comprehensively evaluate the synergistic potential of the ethanol extract of *E. bulbosa* tubers and tamoxifen in modulating TNF-α levels and improving breast tissue morphology in a DMBA-induced BALB/c mouse model of breast cancer.

To date, no *in vivo* studies have specifically examined the combined

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effects or sequential administration of *E. bulbosa* and tamoxifen on TNF-α levels and breast tissue morphology. The novelty of this study lies in exploring an integrative therapeutic strategy that combines an Indonesian endemic natural peoduct with standard hormonal therapy, while also evaluating the optimal administration sequence as a potential approach to overcoming tamoxifen resistance.

Materials and Methods

Plant collection and identification

Eleutherine bulbosa bulbs were gathered from the Dayak region of North Kalimantan, Indonesia (GPS coordinates: 2°50'12.8"N 117°24'11.6"E) on November 25, 2024. The plant material was taxonomically identified and authenticated at the Plant Systematics Laboratory, Faculty of Biology, Gadjah Mada University (UGM), Yogyakarta, Indonesia. A voucher specimen was deposited at the UGM Herbarium under the voucher number 00960/S.Tb./IX/2025.

Extract preparation

The bulbs were cleaned, thinly sliced, and air-dried in a well-ventilated shaded area at room temperature, followed by drying in an oven (Kirin, KBO-RAB, Indonesia) at 40°C to minimize moisture content. The dried material was then ground into a fine powder with the ais of an electric blender (Philips, HR2222/30, Indonesia).

The powdered bulbs (1 kg) were extracted by maceration in 10 L of 96% ethanol (Merck, purity ≥96%) in the dark at room temperature for 24 hours with periodic stirring. The process was repeated three times using fresh solvent to maximize the extraction yield. The macerate was filtered using Whatman No. 1 filter paper (Maidstone, UK), and the resulting filtrate was concentrated in a rotary evaporator (Büchi, Rotavapor R-300, Switzerland) at 40°C under low pressure until a thick extract was obtained. The extract was subsequently kept in a dark glass container and stored in the refrigerator at 4°C until it was needed for further use.[20,21]

Phytochemical analysis of extract

Phytochemical analysis of the *Eleutherine bulbosa* ethanol extract was performed to identify its chemical constituents and measure the levels of major bioactive compounds, including flavonoids and polyphenols.

Qualitative tests

Qualitative phytochemical screening was conducted to identify key secondary metabolites such as flavonoids, alkaloids, tannins, saponins, terpenoids, and steroids. The analysis was performed using standard phytochemical screening methods based on specific colour reactions and precipitation.[22]

Quantitative tests

Quantitative phytochemical analysis focused on determining the total flavonoid and polyphenol contents due to their biological relevance to anticancer activity. Measurements were performed by colorimetric methods using a UV-Vis spectrophotometer (Shimadzu UV−1900, Japan).

Determination of total flavonoid content (TFC): TFC was determined using the aluminum chloride ($AlCl_3$) colorimetric method. The results were estimated based on a quercetin standard curve and expressed as milligrams of quercetin equivalent per gram of dry extract (mg QE/g extract). [23]

Determination of total polyphenol content (TPC): TPC was decided using the Folin-Ciocalteu colorimetric method. The results were quantified based on a gallic acid standard curve and expressed as milligrams of gallic acid equivalent per gram of dry extract (mg GAE/g extract).[23]

Experimental animals

Thirty-six BALB/c mice (6–8 weeks old, weighing 18–20 g) were obtained from the Healthy Animal Laboratory, Malang, Indonesia. The animals were kept in well-ventilated plastic cages with wood shavings as bedding. The animals were acclimatized to the laboratory conditions for seven days; a controlled temperature of 22–25°C, humidity of 50–60%, and a 12:12-hour light/dark cycle. Standard feed (Comfeed Indonesia, PT Charoen Pokphand) and drinking water were provided *ad libitum*.[24–26]

The inclusion criteria were healthy, active female mice within the specified age and weight range. Mice that were sick, pregnant, or died during the acclimatization or study period were excluded.

Ethical consideration

All experimental procedures were carried out following the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines and were approved by the Health Research Ethics Committee, Faculty of Public Health, Hasanuddin University, Makassar, Indonesia (approval No. 046/UN4.14.1/TP.01.02/2024).

To ensure data validity while adhering to animal ethics principles, particularly the 3R principle (Replacement, Refinement and Reduction), the sample size was set at n = 6 per group. This minimum number was selected based on literature and preliminary studies, that revealed that a sample size of six animals per group provides a statistical power of ≥80% to detect significant biological differences ($p < 0.05$) among groups.[27,28]

Study design

This experiment employed a Completely Randomized Design with a post-test–only control group model. A total of 36 mice were randomly divided into six groups of six mice per group (n = 6). Random allocation was performed utilizing sealed envelopes prior to treatment initiation. No blinding was conducted during the treatment or analysis stage due to resource limitations; however, all procedures were carried out in strict compliance with standardized protocols to minimize potential bias.[29,30]

The six experimental groups were assigned as follows:

Negative Control (A/NC): DMBA-induced mice administered 0.5% sodium carboxymethyl cellulose (Na-CMC) orally.

Positive Control (B/PC): DMBA-induced mice devoid of treatment.

Treatment 1 (C/T1): DMBA-induced mice administered ethanol extract of *Eleutherine bulbosa* (180 mg/kg BW) daily for 14 days.

Treatment 2 (D/T2): DMBA-induced mice administered tamoxifen (10 mg/kg BW) every 2 days for 14 days.

Treatment 3 (E/T3): DMBA-induced mice treated with *E. bulbosa* extract at a dose of 180 mg/kg BW daily, followed by tamoxifen at 10 mg/kg BW every two days for 14 days.

Treatment 4 (F/T4): DMBA-induced mice treated with tamoxifen at a dose of 10 mg/kg BW every two days, followed by *E. bulbosa* extract at 180 mg/kg BW once daily for 14 days.

All treatments were administered orally using a gavage (sonde) needle according to the specific dosing schedule for each group throughout the designated treatment period.

Cancer induction and treatment

Breast cancer was induced with 1% DMBA (Sigma-Aldrich, St. Louis, USA; purity ≥99%) dissolved in corn oil (Merck; purity ≥98%).[31] The solution was administered orally at a dose of 1 mg/mouse/day for 28 consecutive days.[19]

During the 28-day induction period, the mice were routinely monitored and examined weekly to confirm tumor development in the breast tissue through palpation and measurement. Once tumours were detected, their size was assessed weekly by measuring the two longest dimensions (length and width) with the aid of a digital caliper (Mitutoyo, Japan).

Treatment commenced on day 29, immediately after the induction period ended. On this day, baseline tumor measurements were recorded. The success of DMBA induction was confirmed when at least 80% of the mice in the induction group developed palpable tumors with a minimum diameter of 3 mm.

Once tumor formation was confirmed, treatments were administered orally once daily for 14 days. The ethanol extract of *E. bulbosa* was given at a dose of 180 mg/kg BW, and tamoxifen (Sigma-Aldrich, St. Louis, USA; purity ≥98%) at 10 mg/kg BW. The combination group received both agents at the same respective doses. These dosages were chosen based on previous studies demonstrating their effectiveness and safety in animal cancer models.[32,33]

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Determination of TNF-α expression
At the end of the treatment period, the mice were humanely sacrificed by cervical dislocation under mild anesthesia using ketamine (50 mg/kg BW) and xylazine (5 mg/kg BW) to minimize pain and stress, in accordance with ethical procedures for animal research. Blood samples were collected via cardiac puncture, and serum was separated to measure TNF-α levels using a mouse-specific ELISA kit (BT Lab, Shanghai, China; Code E0764Ra) following the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (iMark Model, Bio-Rad, USA), and TNF-α concentrations were calculated based on the corresponding standard curve. This procedure is a validated and widely used method for cytokine and hormone analysis in DMBA-induced breast cancer models. [19]

Histopathological examination of breast tissue
Breast tissue samples were collected immediately after euthanasia and fixed in 10% buffered formalin for at least 24 hours to preserve cellular architecture. Following fixation, the tissues were processed using the paraffin embedding method, cut into 5 µm sections with a microtome, and stained with hematoxylin and eosin (HE) to examine cellular and tissue morphology.
The stained sections were examined under a light microscope (Olympus CX23, Japan) to assess histological alterations in the breast tissue, including epithelial proliferation, necrotic areas, and lymphocyte infiltration, that indicate the pathological response to DMBA induction and subsequent treatment.
Histopathological evaluation was then performed on one representative mouse in each treatment group. For each tissue section, observations were conducted in five microscopic fields of view at 400× magnification using a light microscope. The mean values obtained by these five fields of view were used as descriptive data. Since each group consisted of only one animal (n = 1) for histopathological evaluation, inferential statistical analysis could not be performed. Therefore, the findings were presented descriptively to illustrate trends and morphological differences among treatment groups.
All stages of the histopathological analysis were conducted in accordance with standard procedures commonly applied in animal research. To ensure objectivity and minimize potential assessment bias, the evaluation and interpretation of tissue sections were employed systematically by an independent pathologist. This approach is consistent with the recommendations of Bolon,[34] who emphasized the importance of disguised evaluation in improving the validity and reliability of histopathological results in toxicology and biomedical studies.

Statistical analysis
Statistical analysis *w*as performed using IBM SPSS Statistics (version 26). Data normality was examined using the Kolmogorov–Smirnov and Shapiro–Wilk tests, while the homogeneity of variances was assessed using Levene's test. Differences among groups were determined using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was set at $p < 0.05$. All data were expressed as mean ± standard deviation (SD).

Results and Discussion

Phytochemical constituents of ethanol extract of Eleutherine bulbosa
Phytochemical analysis showed that the ethanol extract of *Eleutherine bulbosa* contained flavonoids (0.2785%) and polyphenols (0.7332%), with positive results in the qualitative tests for both compounds (Table 1).

Table 1: Quantitative and qualitative phytochemical composition of ethanol extract *of Eleutherine bulbosa* bulb

Constituent	Quantitative (% w/w)	Qualitative
Flavonoids	0.2785	Detected (+)
Polyphenols	0.7332	Detected (+)

The flavonoid and polyphenol constituents of the extract are thought to be crucial in modulating inflammatory pathways. Specifically, flavonoids may inhibit the production of pro-inflammatory cytokines, such as TNF-α and IL-6 by regulating the PI3K/Akt and p38 MAPK signaling pathways, while also inducing apoptosis in cancer cells.[35,36] Polyphenols act as potent antioxidants that help reduce oxidative stress, suppress lipid peroxidation, and inhibit abnormal cell proliferation.[13,37]

Effect of ethanol extract of Eleutherine bulbosa on serum TNF-α levels
This study investigated the potential of *Eleutherine bulbosa* extract to improve the anti-inflammatory effects of tamoxifen in a mouse model of breast cancer by measuring TNF-α levels. The results of serum TNF-α level measurements (Figure 1) demonstrated significant differences among the groups ($P < 0.001$). Tukey's post hoc analysis revealed a significant reduction in TNF-α levels in all treatment groups compared to the positive control (B/PC) ($p < 0.05$). The mean TNF-α concentrations (pg/mL) for each group were as follows: A/NC = 40.10 ± 5.10, B/PC = 118.07 ± 6.62, C/T1 = 87.48 ± 3.70, D/T2 = 65.68 ± 6.51, E/T3 = 75.95 ± 3.50, and F/T4 = 53.32 ± 4.94.

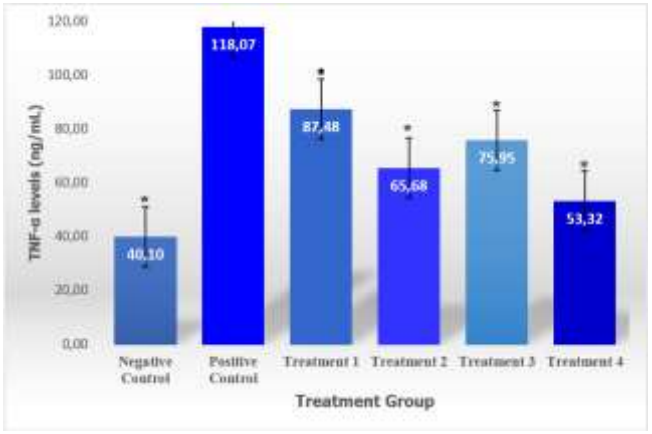

Figure 1: Serum TNF-α levels in both the control and treatment groups.
Each bar chart represents the average serum TNF-α levels ± standard deviation (SD) for each treatment Group, (n = 6). (*) indicates a statistically significant difference ($p < 0.05$) contrasted to the positive control. **NC:** Negative Control (healthy mice devoid of DMBA induction and devoid of treatment), **PC:** Positive Control (DMBA-induced mice devoid of treatment), **T1:** DMBA + *E. bulbosa* ethanol extract, **T2:** DMBA + tamoxifen, **T3:** DMBA + *E. bulbosa* ethanol extract followed by tamoxifen, **T4:** DMBA + tamoxifen followed *by E. bulbosa*

The treatment outcomes demonstrated that T4 (tamoxifen followed by *E. bulbosa* extract) produced the most pronounced and statistically significant reduction in TNF-α levels compared to both the extract (T1) and tamoxifen (T2) monotherapy ($p < 0.05$). The superiority of the combination treatment suggests that dual intervention offers stronger anti-inflammatory and antiproliferative effects than monotherapy. The reduction in TNF-α levels was closely associated with improvements in alveolar morphology, that more closely resembled that of the negative control group, highlighting the treatment's impact on suppressing inflammatory and proliferative activity in breast tissue.
The superiority of the T4 group is likely supported by the synergistic interaction between the two agents. Tamoxifen, as a Selective Estrogen Receptor Modulator (SERM), not only inhibits the proliferation of estrogen-dependent cells but also suppresses the activation of the NF-κB pathway, thereby reducing the release of proinflammatory cytokines.[38–40] Active compounds in *E. bulbosa*, such as flavonoids and polyphenols, enhance this effect by inhibiting TNF-α expression, reducing ROS formation, and suppressing the activity of proinflammatory macrophages.[35,36] Thus, this dual therapy shows stronger anti-inflammatory and antiproliferative effects than a single

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treatment, consistent with the report of Saleh *et al*. (2020)[41] which stated that control of inflammation can increase the effectiveness of hormonal therapy.

In addition, the comparison among T4 (tamoxifen followed by extract) and T3 (extract followed by tamoxifen) highlighted the critical role of treatment sequence. The T4 group produced the most pronounced reduction in TNF-α levels, that was significantly lower than that observed in the T3 group. These findings suggest that using tamoxifen as a pre-treatment may effectively occupy estrogen receptors (ER) or modulate NF pathways, thereby creating a more responsive cellular environment for the anti-inflammatory and antioxidant compounds present in *E. bulbosa* extract during the subsequent treatment phase.[42–44] These findings strengthen the hypothesis that the sequential combination of tamoxifen and *E. bulbosa* extract offers enhanced tissue protection through complementary antioxidant and anti-inflammatory mechanisms.

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Effect of ethanol extract of Eleutherine bulbosa on morphology of breast tissue

Histological examination of breast tissue was conducted, and the findings were presented descriptively, as the number of representative samples for histological evaluation was limited to one per group.

Histological observations of breast tissue revealed variations in alveolar diameter and alveolar count across the different treatment groups (Table 2, Figure 2) .The negative control group (A/NC) exhibited normal alveolar morphology, characterized by the largest average alveolar diameter (110.40 µm) and the lowest alveolar count (1.6). The histological structure appeared well organized, with uniform nuclei and a sparse, evenly distributed alveolar pattern.

The positive control group (B/PC) showed the smallest average alveolar diameter (83.01 µm) and the highest alveolar count (2.6). Histological examination revealed dense cellular proliferation, enlarged nuclei, and irregular tissue organization.

Table 2: Mammary alveolar diameter (µm) and number of alveoli per field of view in the treatment group

Treatment Group	Alveolar Diameter (µm)	Number of Alveoli
Negative Control (NC)	110.4	1.6
Positive Control (PC)	83.01	2.6
Treatment 1 (T1)	86.25	2.4
Treatment 2 (T2)	101.29	1.8
Treatment 3 (T3)	93.35	2
Treatment 4 (T4)	106.71	1.8

Data represents the mean of five fields of view. Data presented are descriptive to show morphological predispositions among groups. Inferential statistical analysis was not performed due to the limitations of the sample size (n = 1); therefore, standard deviations (SD) were not presented. Measurements were taken at 400x magnification

NC: Negative Control (healthy mice devoid of DMBA induction and devoid of treatment), **PC:** Positive Control (DMBA-induced mice devoid of treatment), **T1:** DMBA + *E. bulbosa* ethanol extract, **T2:** DMBA + tamoxifen, **T3:** DMBA + *E. bulbosa* ethanol extract followed by tamoxifen, **T4:** DMBA + tamoxifen followed *by E. bulbosa*

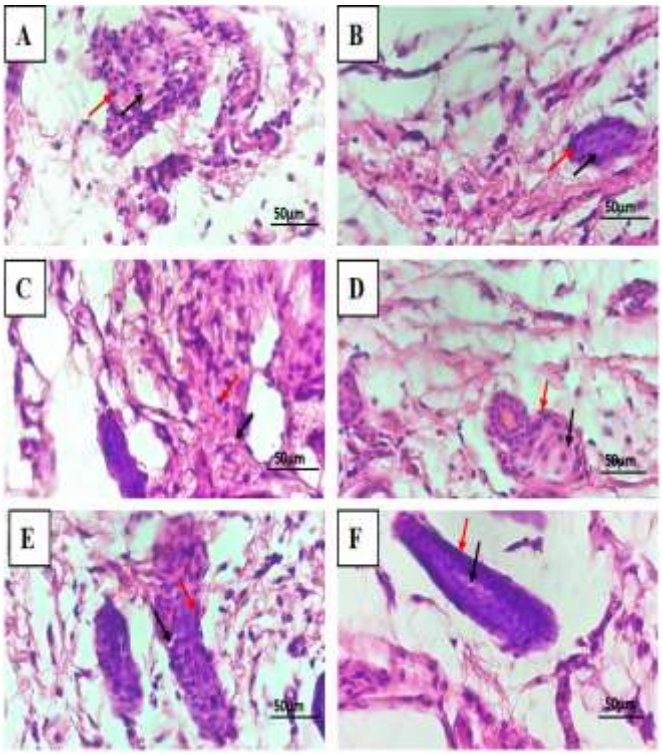

Figure 2: Photomicrograph of histopathological fragments of breast tissue in the treatment groups (H&E Staining, 400× mag.)

A = NC (Negative Control) healthy mice devoid of DMBA induction and devoid of treatment, **B** = PC (Positive Control) DMBA-induced mice devoid of treatment, **C** = T1 (DMBA + ethanol extract *E. bulbosa*, **D** = T2 (DMBA + tamoxifen), **E** = T3 (DMBA + ethanol extract of *E. bulbosa* followed by tamoxifen), **F** = T4 (DMBA + tamoxifen followed by *E. bulbosa)* Black arrow indicates the alveolar diameter, and red arrow indicates the proliferative nuclei. Bar scale = 50 µm in each panel.

Treatment with the ethanol extract of *E. bulbosa* (C/T1) resulted in an average alveolar diameter of 86.25 μm and a reduced alveolar count of 2.4, with histological features showing relatively high cellular density. Treatment with tamoxifen (D/T2) resulted in an average alveolar diameter of 101.29 μm and an alveolar count of 1.8. The histological structure showed slight irregularities but appeared more organized compared to the positive control. The combined treatment with *E. bulbosa* ethanol extract followed by tamoxifen (E/T3) produced an average alveolar diameter of 93.35 μm, and an alveolar count of 2.0. Histological observations revealed that some proliferative activity persisted. The combined treatment with tamoxifen followed by *E. bulbosa* ethanol extract (F/T4) resulted in an average alveolar diameter of 106.71 μm and a low alveolar count of 1.8. Histological analysis revealed a well-organized alveolar structure with minimal cellular density, closely resembling normal tissue architecture.

These findings further support the biochemical results. The larger alveolar diameter and more organized tissue architecture observed in the T4 group suggest that the combination treatment may help alleviate tissue damage caused by DMBA-induced chronic inflammation. The reduction in TNF-α levels likely contributed to the observed improvements in alveolar morphology, as TNF-α is known to trigger apoptosis and tissue damage through increased oxidative stress. This study, however, has several limitations. Tumour burden or volume parameters were not measured, so the antiproliferative effects were inferred indirectly by TNF-α levels and histological changes in breast tissue. Moreover, the limited sample size, particularly the use of only one specimen per group for histopathological analysis and the absence of blinding procedures may have introduced potential bias. Nevertheless, these findings offer an important scientific foundation for developing integrative therapies based on natural compounds that act through the regulation of inflammatory and hormonal pathways.[11,12]

Follow-up studies utilizing a double-blind randomized design, accompanied by toxicity assessments, pharmacokinetic profiling, and tumor burden analysis, are necessary to confirm the long-term effectiveness and safety of this approach. The integration of natural compounds such as *E. bulbosa* as an adjuvant in breast cancer therapy holds significant promise for enhancing treatment effectiveness and minimize the adverse effects associated with conventional hormonal therapies.

Conclusion

The combination of tamoxifen and *Eleutherine bulbosa* bulb extract demonstrated greater efficacy than single-agent treatments in modulating TNF-α levels and improving breast tissue histology in a DMBA-induced mouse model of breast cancer. This is the first study to investigate the therapeutic potential of this combination through both hormonal and inflammatory mechanisms within the tumour microenvironment. Although, the histopathological observations in this study were descriptive, as each treatment group consisted of only one mouse (n = 1), the findings still demonstrated notable morphological improvements in breast tissue following the administration of *E. bulbosa* extract, either alone or in combination with tamoxifen. These results offer a promising scientific foundation for the development of natural combination therapy strategies. However, further studies are needed to comprehensively evaluate their safety profile and therapeutic effectiveness in humans.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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