RESEARCH ARTICLE

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The Combined Effects of *Eleutherine bulbosa* Ethanol Extract and Tamoxifen On *Cox-2* Levels in a BaLB/c Mouse Breast Cancer Model

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Abstract

Objective: Chronic inflammation and oxidative stress play important roles in breast cancer progression. Cyclooxygenase-2 (*COX-2*) is a major proinflammatory enzyme that is often overexpressed in tumor cells. Eleutherine bulbosa (Dayak onion) is a traditional Indonesian medicinal plant with antioxidant and anti-inflammatory properties. This study aimed to evaluate the effect of a combination of ethanol extract of E. bulbosa and tamoxifen on *COX-2* levels in a 7,12-dimethylbenz[a]anthracene (DMBA)-induced BALB/c mouse model. **Methods:** A total of thirty-six 8–10-week-old female BALB/c mice were randomly divided into six groups: a negative control, a positive control (DMBA alone), and four treatment groups that received E. bulbosa extract (180 mg/kg BW), tamoxifen (10 mg/kg BW), or their combination for 14 days. *COX-2* levels were measured using an enzyme-linked immunosorbent assay (ELISA). Statistical analysis included the Shapiro–Wilk test (normality), Levene's test (homogeneity), Brown–Forsythe test, and Games–Howell post hoc test. **Result:** All treatment groups showed a decrease in *COX-2* levels compared to the positive control. The combination group (tamoxifen + E. bulbosa) exhibited the lowest *COX-2* levels (3.86 ng/mL), close to the value observed in the negative control group (3.07 ng/mL), indicating a synergistic effect between the two agents. **Conclusion:** The combination of tamoxifen and E. bulbosa ethanol extract significantly reduced *COX-2* levels in DMBA-induced breast cancer models. These results suggest the potential of this combination as an effective adjuvant therapy. Further studies are needed to confirm the underlying molecular mechanisms and to evaluate its toxicity profile.

Keywords: *COX-2*- Eleutherine bulbosa- tamoxifen- DMBA- breast cancer

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Introduction

Breast cancer has the second highest prevalence globally and is the leading cause of cancer-related deaths in women [1]. Based on international data, it accounts for approximately 11.7% of all cancer cases, with more than 2.3 million new cases and 685,000 deaths each year [2]. In Indonesia alone, the prevalence of breast cancer will reach 41.8% of all cancer cases in women by 2022, making it a very important public health issue. The high mortality rate is largely due to late detection, limited access to treatment, and the emergence of resistance to standard therapies, such as tamoxifen [3, 4].

Tamoxifen is the main hormonal therapy used to treat breast cancer with estrogen receptor-positive (ER+) expression [5] . It works by inhibiting estrogen receptors, thereby inhibiting cancer cell proliferation [6]. It works by inhibiting estrogen receptors, thereby inhibiting cancer cell proliferation . However, its long-term use is associated with various serious side effects, such as the risk of endometrial cancer and thromboembolism, as well as the emergence of drug resistance, which results in decreased treatment effectiveness [7, 8]. In addition, clinical and molecular studies suggest that chronic inflammation, especially involving increased activity of the enzyme cyclooxygenase-2 (*COX-2*), plays an important role in breast cancer progression through mechanisms such as cell proliferation, formation of new blood vessels (angiogenesis), and inhibition of cell death (apoptosis) [9].

The increasing focus on the role of inflammatory pathways, such as *COX-2*, in cancer has prompted the development of various therapeutic agents targeting these pathways [10]. One promising approach is the use of natural compounds from plants with anti-inflammatory and antioxidant effects, which are considered safer for decreasing COX- 2 expression. One plant that is

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commonly used in Indonesian traditional medicine and has potential as a source of antioxidants and therapeutic agents is *Eleutherine bulbosa*, also known as Dayak onion [11]. This plant is rich in active compounds, such as flavonoids, alkaloids, quinones, and polyphenols, which have been shown to have cytotoxic, antiproliferative, and antioxidant effects based on various in vitro and in silico studies [12, 13]. Active components such as eleutherine and avenasterol have also shown the ability to bind to breast cancer receptors such as PARP1 and HER2 and inhibit the associated inflammatory pathways [14].

However, scientific evidence regarding the effectiveness of Dayak onion extract in animal models (in vivo), especially when combined with tamoxifen, remains limited. However, studies on the synergistic potential of tamoxifen and *Eleutherine bulbosa* in reducing *COX-2* levels have not been conducted. In fact, *COX-2* is also known to have and the estrogen hormonal pathway, which may affect the response to tamoxifen [6]. This study also addresses the challenges of cost and side effects of conventional therapy by presenting local plant-based alternatives that can be used as adjunctive therapy.

This study aimed to assess the effect of administering Dayak onion ethanol extract, either alone or in combination with tamoxifen, on *COX-2* levels in BALB/c mice with breast cancer induced by the carcinogenic substance 7,12- dimethylbenz[a]anthracene (DMBA). This animal model was chosen because it mimics the biological characteristics of human breast cancer, including *COX-2* expression and estrogen sensitivity. We hope that the results of this study can provide a strong preclinical basis for the potential of the combination of the two agents as a more effective, affordable, and local natural resource-based approach to breast cancer therapy.

This study presents scientific novelty through a combination approach between the administration of *Eleutherine bulbosa* (Bawang Dayak) ethanol extract and tamoxifen therapy in reducing *COX-2* enzyme levels in breast cancer animal models using DMBA-induced BALB/c mice. Although the anticancer potential of E. bulbosa and the effectiveness of tamoxifen have been studied separately, exploration of their synergistic effects in regulating inflammatory pathways, particularly *COX-2* expression, remains limited. Another specialty of this study is the integration of in silico and in vivo approaches to evaluate molecular interactions and biological responses more comprehensively. Thus, this study is expected to contribute significantly to the development of safer and more effective natural ingredient-based combination therapies and open up opportunities for the utilization of pharmacoinformatics approaches in the clinical practice of breast cancer treatment.

This study is part of an academic collaboration that uses *Eleutherine bulbosa* ethanol extract as the main intervention. However, this study specifically focused on *COX-2* biomarkers, which distinguishes it from other peer studies that emphasize inflammatory biomarkers such as apoptosis, *P53, TNF-α, IL-6,* and *IL-10.*

Materials and Methods

Study Design

This study used a randomized controlled experimental design with BALB/c female mice as an animal model for breast cancer induced using the compound 7,12-dimethylbenz[a]anthracene (DMBA). This study aimed to assess the effects of the ethanol extract of *Eleutherine bulbosa* (EBE), tamoxifen, and their combination on the expression of cyclooxygenase-2 (*COX-2*) enzyme.

Study Subject

A total of 36 female BALB/c mice aged 8-10 weeks with a body weight of 18-25 grams were obtained from a certified animal facility (Satwa Sehat Laboratory, Malang). Animals were adapted for 7 days under standard laboratory conditions (temperature 22±2°C, light/dark cycle 12 hours, relative humidity 55±10%), with free access to standard feed and drinking water.

Breast cancer was induced by oral administration of DMBA once per week for four consecutive weeks at a dose of 20 mg/kg body weight, dissolved in corn oil, and administered through a sonde. Tumor development was monitored weekly through palpation, and only mice that showed tumor lumps after 4 weeks were included in further treatment stages.

Division of Treatment Groups

Mice were randomly divided into six groups, with each group consisting of six mice, except for one group that consisted of five mice. The groups were divided as follows:

Negative control

Without DMBA induction, without additional treatment

Positive control

Mice induced with DMBA but not treated.

Intervention 1

DMBA + ethanol extract of Dayak Onion (180 mg/kg BW, daily for 14 days)

Intervention 2

DMBA + tamoxifen (10 mg/kg BW, every other day, seven doses in total).

Intervention 3

DMBA+ combination of dayak onion extract and tamoxifen (given a combination of Dayak onion ethanol extract at a dose of 180 mg/kg BW daily for 14 days and tamoxifen therapy at a dose of 10 mg/kg BW every 2 days for 7 times over 14 days)

Intervention 4

DMBA+ tamoxifen followed by administration of dayak onion extract (given a combination therapy of tamoxifen at a dose of 10 mg/kg BW every 2 days for 7 times over 14 days and a combination of Dayak onion ethanol extract at a dose of 180 mg/kg BW daily for 14

days).

The doses used were based on previous data on the safety and biological activity of EBE in experimental animals. All materials were freshly prepared before administration using a calibrated oral sonde.

Eleutherine bulbosa Extraction Procedure

To obtain active compounds from *Eleutherine bulbosa*, an extraction process was performed using 70% ethanol solvent based on a standardized method. The extraction stages include drying, pulverizing the material into a powder, and maceration. Ethanol was chosen as the solvent because of its high ability to dissolve polar phenolic and flavonoid compound, which are known to play an important role in the pharmacological activity of this plant.

Based on the figure above, the preparation of the ethanol extract from *Eleutherine bulbosa* begins with the collection of fresh bulbs that have been botanically identified and stored as reference specimens in the herbarium. The cleaned bulbs were then thinly cut and dried in an oven at 40-50°C for two to three days. After the drying process was complete, the bulbs were pulverized into a coarse powder using a special grinder.

The extraction stage was carried out through the maceration method with 70% ethanol solvent, using a ratio of 1:10 between the material and solvent. This process was performed for 72 h at room temperature, accompanied by occasional stirring. After maceration, the solution was filtered to separate the remaining solids and evaporated using a rotary evaporator at 40-45°C under low pressure conditions. The resulting thick extract was re-dried if necessary and stored in a tightly closed container at 4°C until use.

COX-2 Measurement and Sample Collection

At the end of the treatment period, all mice were anesthetized with ketamine-xylazine and euthanized. Tumor tissues were removed, homogenized, and total protein was extracted. *COX-2* levels were measured using a mouse-specific ELISA kit (Catalog Number: MBS175946, MyBioSource, USA) according to the manufacturer's instructions. Absorbance was measured at 450 nm using a microtiter reader (BioTek Epoch, USA). All measurements were performed in duplicate to ensure their reliability. The sensitivity of the method reached 10 pg/mL, with an intra-assay coefficient of variation of < 8%.

Validation and Reproducibility

To ensure the validity and consistency of the results, this study applied the principle of replication, both biologically and technically. Each treatment group consisted of at least six mice, ensuring adequate statistical power and anticipating the possibility of sample loss during the study. In contrast, *COX-2* levels were measured using ELISA method was performed in technical duplicates to increase the accuracy and reliability of the results.

The accuracy of the analytical method was guaranteed using ELISA kits verified by the manufacturer for *COX-2* specificity in mice without cross-reactivity with other proteins. In addition, the assay performance was validated using a standard curve with a coefficient of determination (R^2) of at least 0.98, indicating high linearity and consistency in the reading of the results.

Data analysis

Data analysis of *COX-2* expression levels were analyzed using parametric statistical methods. *COX-2* levels are expressed as mean ± standard deviation (SD). Error bars in all graphs represent SD from six independent biological replicates. Preliminary testing of data distribution was performed using the Shapiro-Wilk and Levene's tests to assess normality and homogeneity of variance. The results of both tests showed that the data met the assumptions of parametric analysis ($p > 0.05$). Next, a one-way ANOVA was conducted to evaluate the differences in *COX-2* levels between the treatment groups. This analysis showed a statistically significant difference ($p < 0.05$), so it was followed by the Brown-Forsythe test. All statistical analyses were performed using SPSS software version 25, and a p-value < 0.05 was considered statistically significant and was performed using GraphPad Prism version 10.0 (GraphPad Software, USA).

Results

Antioxidant Activity Test Results of Dayak Onion Extract with DPPH method

The antioxidant activity of Dayak onion extract can be quantitatively measured using the DPPH free radical capture method for compounds with antioxidant potential. This measurement was performed by observing the change in absorbance using a UV-Vis spectrophotometer at a wavelength of 517 nm. From the results of these measurements, the IC50 value was obtained, which is the concentration of the compound required to neutralize 50% of the free radicals. The lower the IC50 value, the higher is the ability of the compound to bind to free radicals. Based on the IC50 value, the antioxidant activity is categorized as very strong if IC50 <50 ppm, strong in the range of 50-100 ppm, moderate at 100-150 ppm, and weak if it is between 150-200 ppm. The results of the antioxidant activity analysis using the DPPH method are shown below Figure 1.

Based on the test results using the DPPH method, the ethanol extract of Dayak Onion showed a significant increase in the percent inhibition, which reached 44.25% in the sample test of Extract 3 with a concentration of 100 mg/L. The activity of M. indica as a free radical antidote was also classified as strong, with an IC_5 value of 82.66 mg/L. The high antioxidant activity is thought to be due to the content of bioactive compounds in Dayak onion bulbs, especially naphthoquinone and flavonoid compounds, which are known to have strong antioxidant abilities. The results of the percent inhibition of the sample are shown in the form of a graph in Figure 2.

The Figure 3 above shows data on the percentage inhibition of three types of plant extracts (Extract 1, Extract 2, and Extract 3) tested at various concentrations between 60 and 100 ppm. The results showed that all

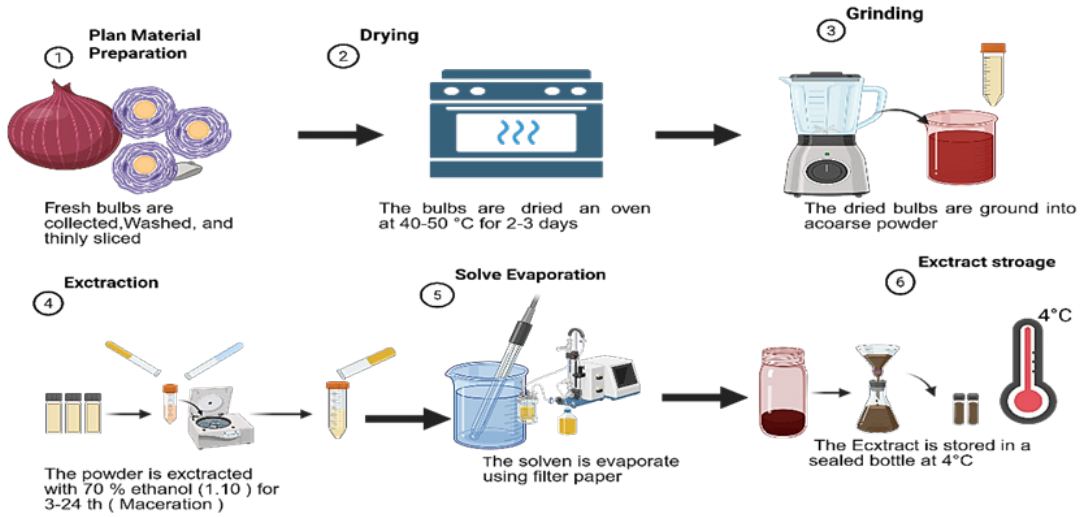

Figure 1. Procedure for Obtaining Ethanol-based Extract from *Eleutherine bulbosa*

extracts exhibited increased inhibitory activity with increasing concentration, indicating a dose-response pattern. Extract 2 showed the highest inhibitory effect, with the inhibition value increasing from about 39% at the lowest concentration to almost 57% at the highest concentration. Extract 1 also showed a fairly good ability, with an increase from about 39% to 52%. Meanwhile, Extract 3 had the lowest inhibition activity, which increased from 30% to 45%. Overall, these results indicate that all three extracts have potential as natural antioxidants, with Extract 2 being a prime candidate for further investigation as a free radical-inhibiting agent.

Figure 4, Data are presented as mean ± SD (n=6 per group). Error bars represent standard deviation (SD) from six independent biological replicates. Statistical significance was determined using Games-Howell post-hoc test: *p<0.05, **p<0.01, ***p<0.001 compared to positive control (KP). The combination group I4 (Tamoxifen+E.bulbosa) showed significantly lower *COX-2* levels compared to positive control (***p<0.001), tamoxifen monotherapy I2 (*p<0.05), and E.bulbosa monotherapy I1 (**p<0.01).

Results of Histopathology Analysis of Mammary Tissue

Histopathological analysis was performed on the mammary tissue of mice from each treatment group to observe the morphological changes that occurred due to DMBA induction and to evaluate the effect of the given intervention. The examination was performed in the Pathology Laboratory using a Nikon Eclipse type Ei light microscope equipped with an Optilab camera integrated with a computer. Observations were made directly on five different fields of view (LP) with 400x magnification, including the measurement of mammary tissue diameter and number of alveoli. The data from each sample were averaged for further analysis, as shown in Table 1.

Based on the results of mammary diameter measurements presented in Table 1, variations were observed between the treatment groups. The negative control group (KN) showed an average mammary diameter of 110.40 µm. In contrast, the positive control group (KP) experienced a significant decrease to 83.01

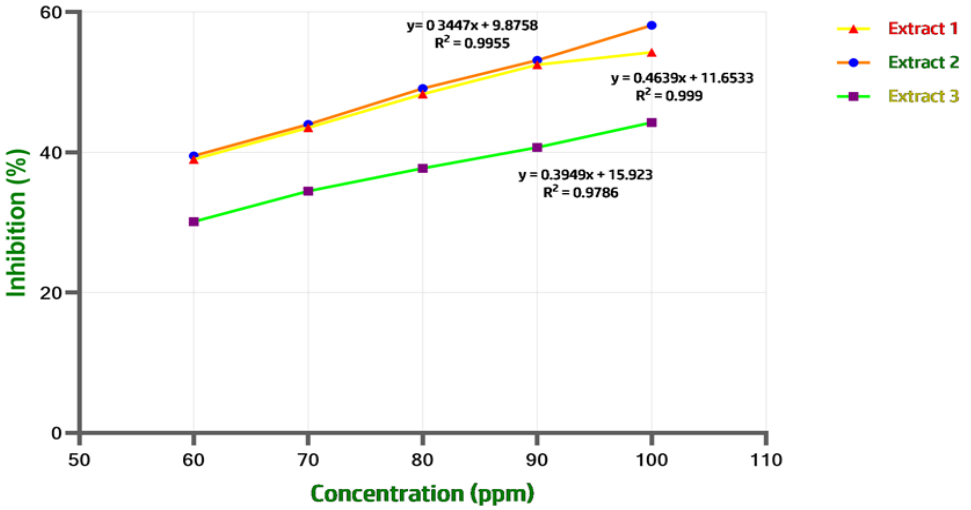

Figure 2. Graph of Free Radical Scavenging Activity of Ethanol Extract of Dayak Onion

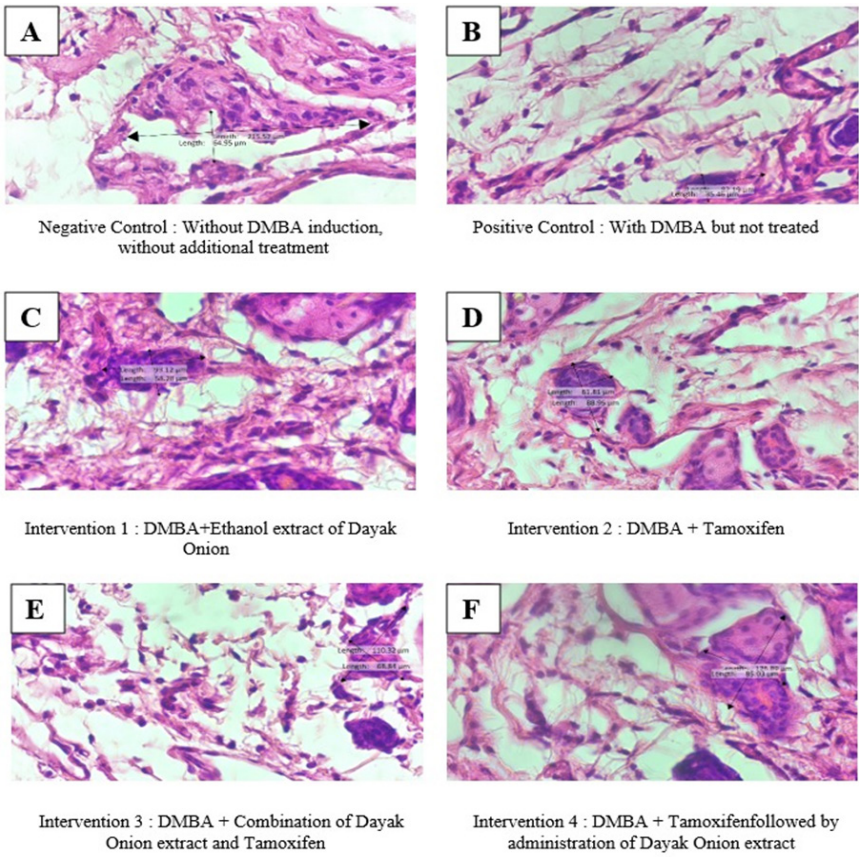

Figure 3. Histological Visualization of Mammary Tissue of Mice in Various Treatment Groups, Stained with Hematoxylin-Eosin and Observed at 400 × Magnification

µm, reflecting tissue damage owing to DMBA exposure. In the intervention group, there were differences in the effects depending on the type of treatment administered. Groups I1, I2, and I3 had mean diameters of 86.25 µm, 101.29 µm, and 93.35 µm, respectively. Interestingly, group I4 showed an increase in diameter to 106.71 µm, close to the normal value as in the KN group, indicating a protective effect of the combination of tamoxifen and *Eleutherine bulbosa* extract. In addition, the number of alveoli in mammary tissue was analyzed as an indicator of glandular activity. The Negative Control (KN) group showed a mean number of alveoli of 1.6, while the KP group showed an increase to 2.6, which can be attributed to hyperplasia due to carcinogenic induction. In intervention groups I1 to I4, the number of alveoli was recorded at 2.4, 1.8, 2.0, and 1.8, respectively. The decrease in the number

Table 1. Results of Antioxidant Activity Testing with DPPH Method

Sample	Concentration (ppm)	Absorbance	Control	% inhibition	IC_{50} (mg/L)
Dayak Onion Extract 1	60	0,38	0,62	39,00	86,29
	70	0,35	0,62	43,49	
	80	0,32	0,62	48,31	
	90	0,29	0,62	52,48	
	100	0,28	0,62	54,25	
Dayak Onion Extract 2	60	0,37	0,62	39,48	82,66
	70	0,34	0,62	43,98	
	80	0,31	0,62	49,11	
	90	0,29	0,62	53,13	
	100	0,26	0,62	58,10	
Dayak Onion Extract 3	60	0,45	0,64	30,12	82,66
	70	0,42	0,64	34,47	
	80	0,40	0,64	37,73	
	90	0,38	0,64	40,68	
	100	0,35	0,64	44,25	

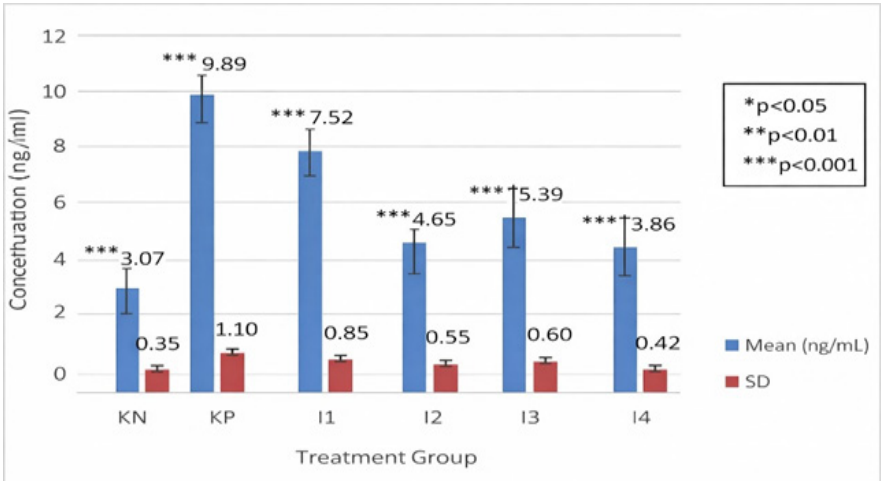

Figure 4. *COX-2* Concentration (ng/mL) in Negative Control, Positive Control, and Intervention Groups.

of alveoli in group I4 indicates a suppressive effect on hyperproliferative activity,This finding is consistent with the morphological improvement in tissue structure.

To evaluate the impact of DMBA induction and the response to the given treatment, histopathological analysis was performed on the mammary tissues of mice from all treatment groups. The tissues were stained with hematoxylin and eosin (HE) and observed under a light microscope at 400 × magnification. Each microscopic image reflects the morphological characteristics of the tissue from each group, that is, negative control (NC), positive control (PC),

and intervention (I1-I4) groups. Alveolar diameter was measured as a quantitative indicator to assess the degree of tissue damage or recovery, with five fields of view randomly selected from each sample for analysis.

Histological examination showed that the negative control group (A) had intact mammary alveolar structures with a large diameter and normal tissue arrangement. In contrast, the positive control (B) group exhibited significant tissue damage due to DMBA induction, characterized by reduced alveolar diameter and morphological disorganization.

In the treatment group, *Eleutherine bulbosa* extract (C) provided mild morphological improvement, whereas tamoxifen (D) showed more pronounced tissue recovery. The low-dose combination (E) began to show structural normalization. The most significant effect was observed with the combination of tamoxifen and E. bulbosa (F), which resulted in alveolar structures resembling normal conditions, indicating a strong protective effect against mammary tissue damage, mammary tissue.

Taken together, these histopathological findings support the biochemical data related to decreased *COX-2* levels and strengthen the notion that the combination of tamoxifen and dayak onion extract has potential as a protective therapeutic agent against DMBA-induced mammary tissue damage.

Statistical Analysis Results

Intergroup Comparison Test

The Games-Howell post hoc test was applied to assess differences in *COX-2* levels between treatment groups, considering the results of the homogeneity test showed inhomogeneous variances. This analysis revealed significant differences, with the lowest *COX-2* levels in the negative control group and the highest in the positive control group. The combination intervention of tamoxifen and Dayak onion extract (Intervention 4) significantly reduced *COX-2* levels to near-normal levels, indicating a possible synergistic effect. The detailed results of the intergroup comparisons are presented in Table 2.

The data in Table 3 show a significant difference in *COX-2* levels between the treatment groups. The negative control group showed the lowest *COX-2* levels, whereas the positive control group showed the highest values. The combination intervention of tamoxifen and Dayak onion extract (I4) significantly reduced *COX-2* levels, approaching that of the negative control. This finding supports the potential superiority of combination therapy over single-agent therapy. The distribution of *COX-2* levels between groups is shown in the following bar graph.

Based on the analysis of *COX-2* levels using ELISA, there was a significant variation between the treatment groups. The negative control group (KN) showed the lowest *COX-2* levels, reflecting normal physiological conditions without the induction of inflammation. In contrast, the positive control group (KP) showed a significant increase in *COX-2* levels compared to the negative control () ($p < 0.01$), indicating the activation

Table 2. Diameter of the Mammary Gland Network and Alveoli after Treatment in DMBA-Induced Mice

Intervention Group	Tissue Diameter	Average Number of Alveoli
Mammae (μm)		
Negative Control	110.40	1.6
Positive Control	83.01	2.6
Intervention 1	86.25	2.4
Intervention 2	101.29	1.8
Intervention 3	93.35	2.0
Intervention 4	106.71	1.8

Table 3. Gomes Howell Post-Hoc Test Results for *COX-2* Levels across Intervention Groups

Treatment Group	N	Mean ± SD	vs Positive Control	vs I4 (Tamoxifen→E.bulbosa)
KN (Negative Control)	6	3.07 ± 0.35[a]	p < 0.001***	p > 0.05
I4 (Tamoxifen→E.bulbosa)	6	3.86 ± 0.42[ab]	p < 0.001***	-
I2 (Tamoxifen alone)	6	4.65 ± 0.55[bc]	p < 0.001***	p > 0.05
I3 (E.bulbosa+Tamoxifen)	6	5.39 ± 0.60[c]	p < 0.001***	p < 0.05*
I1 (E.bulbosa alone)	6	7.52 ± 0.85[d]	p < 0.01**	p < 0.01**
KP (Positive Control)	6	9.89 ± 1.10[e]	-	p < 0.001***

Note: Values are presented as mean ± standard deviation. Different superscript letters (a-e) indicate significant differences between groups based on Games-Howell post-hoc test (p < 0.05). Significance levels: *p < 0.05, **p < 0.01, ***p < 0.001.

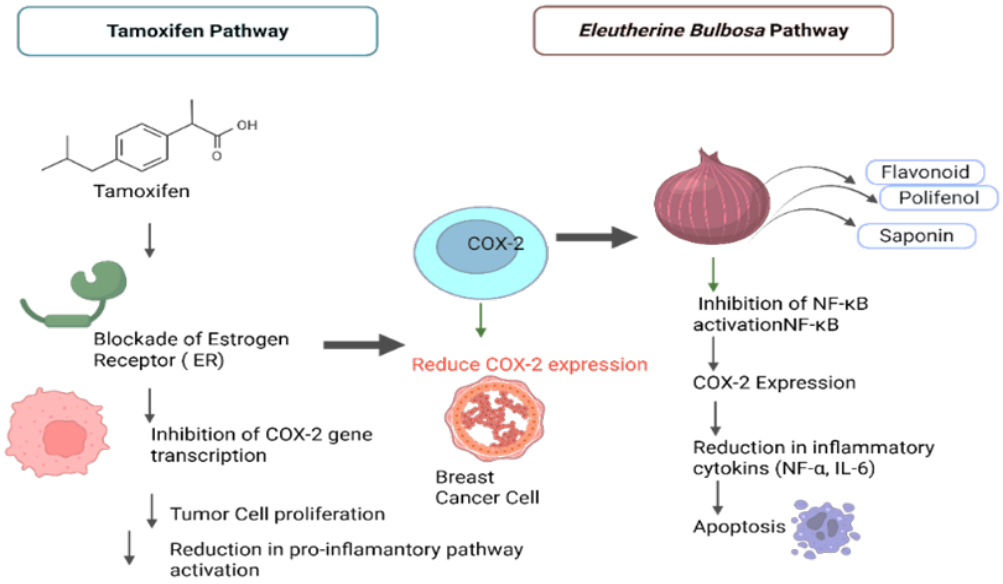

Figure 5. Mechanism of Tamoxifen and *Eleutherine Bulbosa* Extract ini Reducing *COX-2* Ekspression.

of the inflammatory process. The treatment administered to groups I1, I2, and I3 resulted in a gradual decrease in *COX-2* levels compared to the KP group. The decrease was significant in groups I2 and I3 ($p < 0.05$ and $p < 0.01$, respectively) compared to that in the KP group, indicating the potential anti-inflammatory effect of the tested treatments. Overall, the difference in mean *COX-2* levels between the treatment groups was statistically significant (one-way ANOVA, $p < 0.001$), with the presentation of a bar graph accompanied by error bars (standard deviation) showing the distribution of data between replicates. This finding supports the hypothesis that the treatment suppresses *COX-2* expression, which is an indicator of inflammation.

Discussion

This study confirmed that the combined administration of *Eleutherine bulbosa* ethanol extract and tamoxifen significantly reduced *COX-2* levels in DMBA-induced BALB/c mice [15]. This animal model is widely used to replicate the biological characteristics of human breast cancer; therefore, these findings strengthen the hypothesis that dual intervention in inflammatory and hormonal pathways can provide more effective therapeutic effects [14, 16].

COX-2 is an important enzyme in the inflammatory process and cancer progression [17]. Increased expression of this gene has been shown to be closely related to high cell proliferation rates, increased formation of new blood vessels (angiogenesis), and increased metastatic ability in various cancers, including breast cancer [18]. Tamoxifen, widely known as a selective estrogen receptor modulator (SERM), not only inhibits estrogen-dependent tumor growth but also exerts anti-inflammatory effects by suppressing NF-κB activation and proinflammatory cytokine production [5, 6, 19].

The administration of *Eleutherine bulbosa* ethanol extract at a dose of 180 mg/kg body weight to intervention group I1 caused a decrease in *COX-2* levels to 7.5220 ng/L. This decrease indicates the anti-inflammatory activity of the active compounds in Bawang Dayak, such as flavonoids, alkaloids, and saponins, which are believed to work by inhibiting the NF-κB pathway, a major regulator of *COX-2* expression [20]. The administration of *Eleutherine bulbosa* ethanol extract at a dose of 180 mg/kg body weight to intervention group I1 caused a decrease in *COX-2* levels to 7.5220 ng/L. This decrease indicates the anti-inflammatory activity of the active compounds in Bawang Dayak, such as flavonoids, alkaloids, and

saponins, which are believed to work by inhibiting the NF-κB pathway, a major regulator of *COX-2* expression . Nonetheless, *COX-2* levels in this group were still higher than those in the other intervention groups, suggesting the need for dose adjustment or duration of administration to achieve the maximum effect [21-23].

In coxifen, a selective estrogen receptor modulator (SERM), has a dual mechanism of action, blocking estrogen receptor activity and suppressing the expression of inflammatory genes, including *COX-2* [19]. These findings strengthen the role of tamoxifen as a hormonal therapy agent and an anti-inflammatory agent in the management of breast cancer [7].

Furthermore, the combination of Tamoxifen and Bawang Dayak extract showed a synergistic effect in reducing *COX-2* levels. Group I4, which received the highest dose of the combination, showed *COX-2* levels of 3.8615 ng/L, which was close to the levels observed in the negative control group (KN) of 3.0693 ng/L. This indicates that the combination therapy is more efficient than monotherapy in inhibiting *COX-2* expression. This synergistic effect most likely arises from the combined mechanism of action, namely the inhibition of hormonal pathways by tamoxifen and the reduction of inflammatory response by the active compounds of Bawang Dayak.

To provide a clearer understanding of the molecular mechanism underlying the synergistic effect between tamoxifen and *Eleutherine bulbosa* extract, a biological pathway diagram depicting the point of action of each compound and its effect on *COX-2* regulation was prepared. Tamoxifen, as an estrogen receptor modulatory agent, inhibits the transcription of estrogen-induced genes including *COX-2* through the estrogen receptor (ER)-dependent pathway. Meanwhile, bioactive compounds in E. bulbosa, such as flavonoids and saponins, suppress the activation of NF-κB, a major transcription factor that regulates the expression of *COX-2* and various pro-inflammatory cytokines. These two agents reduce *COX-2* levels through different mechanisms, namely hormonal and inflammatory pathways, which complement each other and produce synergistic effects by suppressing cell proliferation and promoting apoptosis. This series of mechanisms is illustrated in the following Figure (Figure 5).

Tamoxifen acts through the estrogen receptor (ER) pathway by inhibiting estrogen signaling, thereby suppressing the proliferation of hormone-dependent breast cancer cells. Simultaneously, *Eleutherine bulbosa* exerts anti-inflammatory effects through the bioactive compounds, such as flavonoids, polyphenols, and saponins, which play a role in reducing *COX-2* expression, most likely through the inhibition of the NF-κB transcriptional pathway. The synergy of these two mechanisms results in a more effective decrease in *COX-2* expression, thereby inhibiting the inflammatory process that drives tumor growth and improving the overall therapeutic response [24].

Graphical analysis of *COX-2* levels supported this conclusion. The KP group showed statistically significant differences compared to all treatment groups, confirming that DMBA induction effectively increased *COX-2* expression [25, 26]. In contrast, the I4 group showed no significant difference from the KN group, indicating the success of combination therapy in normalizing *COX-2* levels. This reinforces the hypothesis that a multi-target approach, humankind, combining inflammation inhibition and cell proliferation control, is more effective in suppressing tumor progression [27].

The combination group (I4: tamoxifen followed by E. bulbosa extract) showed the most significant reduction in *COX-2* levels (3.86 ± [SD] ng/mL, $p<0.001$ vs positive control), approaching levels observed in the negative control group (3.07 ± [SD] ng/mL, $p>0.05$). This effect was superior to tamoxifen monotherapy (I2: 4.65 ± [SD] ng/mL, $p<0.01$ vs I4) and significantly better than E. bulbosa extract alone (I1: 7.52 ± [SD] ng/mL, $p<0.001$ vs I4).

From the perspective of established theory, these results reinforce the understanding that chronic inflammation and elevated *COX-2* levels are important factors in breast cancer progression and therapy resistance [28]. *COX-2* is not only related to inflammation but also has a functional relationship with the estrogen hormonal pathway, which may affect the effectiveness of tamoxifen. Decreasing *COX-2* levels through adjunctive agents such as EBE may improve sensitivity to hormone therapy [29].

This finding is in line with previous studies, both in vitro and in vivo, which showed that the combined use of phytochemicals with conventional therapy can improve treatment effectiveness and reduce the risk of developing drug resistance [30]. The low levels of *COX-2* in the combination group suggest that the inflammatory environment that supports tumor growth was suppressed, which could potentially improve tumor control and reduce recurrence rates [31] .

Overall, the results of this study provide evidence that Bawang Dayak extract has potential as a complementary therapy for breast cancer, especially when combined with tamoxifen. The combination of these two methods provides a more comprehensive approach with diverse molecular targets. However, further research is needed to evaluate the most effective dose, more in-depth mechanism of action, and long-term safety and toxicity aspects before it can be applied in clinical practice.

However, this study has some limitations. The main focus was on measuring *COX-2* levels as an inflammatory marker. Involving other inflammatory markers, such as TNF-α, IL-6, and oxidative stress indicators, can provide a more complete picture. In addition, long-term follow-up studies are needed to evaluate the safety, pharmacokinetics, and optimal dosage of both agents. Although the results are promising, their application in humans requires verification through clinical trials.

In contrast to previous studies that generally assessed *Eleutherine bulbosa* and tamoxifen separately, the present study combined the two using a thorough in vivo approach. The findings suggest that bioactive compounds in E. bulbosa may amplify the effects of tamoxifen by suppressing the NF-κB and *COX-2* pathways, thereby creating a more controllable tumor microenvironment.

Clinically, this combination is promising as a safer, economical, and local resource-based alternative to

conventional complementary therapy. However, further studies are needed to confirm the molecular mechanisms, determine the ideal dose, and assess long-term safety.

The results showed that the combination of *Eleutherine bulbosa* (Dayak onion) ethanol extract and tamoxifen significantly reduced *COX-2* levels in DMBA-induced BALB/c mice, which were used as a breast cancer model. The highest reduction in *COX-2* levels was observed in the group with the largest combination dose, which was close to that of the negative control group. This finding suggests a synergistic interaction between the anti-inflammatory and antioxidant activities of *Eleutherine bulbosa* and the hormonal effects of tamoxifen. Although a single administration of each agent showed a reduction in *COX-2* levels, the most effective results were obtained through their combination. To support these findings, further studies are needed to explore other molecular biomarkers, determine the optimal dose, and conduct clinical trials to assess the safety and effectiveness of this combination therapy in breast cancer.

Author Contribution Statement

DA: Conceptualization, Methodology, Investigation, Data Curation, Writing, Original Draft. ANU: Supervision, Validation, Writing, Review & Editing, Project Administration. RY: Resources, Formal Analysis, Laboratory Support, Visualization. S: Statistical Analysis, Data Interpretation, Review & Editing. AA: Methodology, Histopathology Analysis, Writing, Review & Editing

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General

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Approval

This manuscript is part of the thesis of Dian Andriani entitled "The Effect of Eleutherine Bulbosa Ethanol Extract and Tamoxifen Therapy on the Reduction of *COX-2* Levels in Balb/c Strain Mice (Mus Musculus) in a Breast Cancer Model Induced by DMBA," which has been approved by Hasanuddin University as part of the Master's program in the Department of Midwifery. This thesis was approved on July 15, 2025.

Ethical Declaration

The study was conducted in accordance with the ARRIVE 2.0 guidelines and was approved by the Animal Ethics Committee of Hasanuddin University (Ethics Number: 070/UN4.14.1/TP.01.02/2025).

Data Availability

Data will be available upon request from the respective author.

Study Registration

A study titled "The Combined Effect of Eleutherine Bulbosa Ethanol Extract and Tamoxifen on Cox-2 Levels in a Balb/C Mouse Breast Cancer Model (Balb/C)" has been registered at OSF with link https://doi.org/10.17605/OSF.IO/M9XCS

Conflict of Interest

The authors declare that they have no financial, personal, or professional conflicts of interest that could influence the content of this article

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