Original Article Open Access

Archives Of Breast Cancer

DOI: 10.32768/abc.2025122203-210

Soybean Extract Rich in Lunasin Enhances p21 Expression in DMBA-Induced Breast Cancer in Rats: A Potential Adjuvant Therapy

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ARTICLE INFO

Received:
20 January 2025
Revised:
9 March 2025
Accepted:
16 March 2025

Keywords:
adjuvant, pharmaceutic, breast neoplasm, cyclin-dependent kinase inhibitor p21, soybean protein

ABSTRACT

Background: Breast cancer treatment in Indonesia faces challenges due to side effects, limited accessibility, and resistance to therapies like tamoxifen, highlighting the need for effective adjuvant strategies. Lunasin, a soybean-derived peptide, exhibits anticancer potential by upregulating p21, a tumor suppressor involved in cell cycle arrest and apoptosis. This study examines the effect of soybean extract rich in lunasin on p21 expression in a DMBA-induced breast cancer rat model.

Methods: Thirty female Sprague-Dawley rats were induced with breast cancer via intragastric injection of 7,12-Dimethylbenz[a]anthracene (DMBA), with 20mg/kgBW given twice weekly for a total of 11 injections. Once the tumor volume reached 1-2cm^3, the rats were given 10mg/kgBW of tamoxifen (positive control), 500 mg/kgBW of soybean extract rich in lunasin (lunasin), and a combination of both (combination) for eight weeks. Rats in the negative control group did not receive any medication. Then, the rats were sacrificed to extract the tumor tissue. This tumor tissue was examined for the expression of p21 protein using immunohistochemistry staining.

Results: The highest average p21 expression was observed in the normal group, followed by the combination, lunasin, positive control, and negative control groups. The combination group showed significantly higher p21 expression than the negative control ($P<0.05$), while no significant difference was observed between the positive control and lunasin groups ($P>0.05$).

Conclusion: Soybean extract rich in lunasin upregulates p21, supporting its role in tumor suppression. While lunasin alone had no significant advantage over tamoxifen, their combination significantly increased p21 expression, suggesting a synergistic effect, making it a promising adjuvant therapy.

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INTRODUCTION

Cancer is the second leading cause of death in the world after cardiovascular disease.[1] The Global Burden of Cancer Study estimated over 2 million new cases of breast cancer.[2] Additionally, according to

Basic Health Research, cancer prevalence in Indonesia increased from 1.4% in 2013 to 1.8%.[3–5]

Breast cancer treatment in Indonesia relies on local and systemic therapies. Local therapies, such as surgery and radiation, can only be given in the early stages of the disease.[6] However, approximately 70% of breast cancers in Indonesia are detected at late stages, needing systemic therapies, including chemo, hormonal, and targeted therapy.[7] While essential, these treatments are associated with severe side effects such as neurotoxicity, bone marrow suppression, and organ toxicity.[8,9] Furthermore, access to these therapies is often limited to specialized hospitals in urban areas, posing significant treatment barriers. Tamoxifen, a widely used estrogen receptor antagonist, plays a crucial role in breast cancer management, but it is associated with treatment resistance and complications in some cases, further highlighting the need for effective adjuvant therapies.[7]

In the last couple of years, many researchers have highlighted the anticancer potential of natural compounds such as soybean plants *(Glycine max)*. Soybean is cheap, widely available, and has low toxicity, making it an ideal candidate for adjuvant therapy.[10] The anticancer activity of soybean is linked to lunasin, a bioactive peptide with 43 amino acids with anti-inflammatory, immunoregulating, and antioxidant properties. This peptide is found abundantly in soybeans. Lunasin works by modulating histone acetylation and regulating protein expression. The C-terminus domain in lunasin can bind with H3 and H4 histone, affecting the formation of the centromere complex, resulting in mitotic arrest and cell death. Lunasin can also upregulate certain proteins, such as p21.[11]

P21 protein is a tumor suppressor gene that binds cyclin-dependent kinase (CDK) and proliferates cell nuclear antigen (PCNA) during DNA replication. This causes cell cycle arrest that prevents DNA synthesis and replication. Furthermore, p21 protein prevents tumor progression by inducing cell apoptosis.[11] In neoplasm, expression of p21 protein decreases due to high levels of oncogene and is associated with poor prognosis.[12]

Despite extensive in vitro studies on lunasin, its in vivo effects, particularly on p21 expression, remain poorly understood. To our knowledge, this study is the first to examine whether soybean extract rich in lunasin can modulate p21 expression in vivo. Using Sprague-Dawley (SD) rats with DMBA-induced breast cancer, we aim to assess lunasin's ability to upregulate p21 and explore its therapeutic potential as an adjuvant therapy to overcome limitations of conventional breast cancer treatments before advancing to clinical research.

METHODS

Study Design

This true experimental in vivo study was conducted in the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia, from September 2023 to September 2024. The tissue samples we used belonged to a previous study by Numlil Khaira Rusdi, "In Vivo Antimammary Tumor Effects of Soybean Extract with Targeted Lunasin (ET-Lun)."

Lunasin Extraction from Soybean

Soybean of the Grobogan variety was bought from Indonesian Legumes and Tubers Crops Research Institute (Balitkabi), Malang, East Java. Lunasin extraction started by squeezing the seeds for 30 minutes at 100-150atm at 48.89°C. Then, the soybean was crushed to create a powder. The powder was liquefied using Phosphate Buffered Saline (PBS), five times the powder weight, at a pH of 7.4 for 60 minutes, followed by filtration using WhatmanTM 54. A vacuum rotary evaporator was used to create a thick extract from the filtrate. The lunasin concentration was determined using high-performance liquid chromatography (HPLC). A 100 mg extract of soybeans rich in lunasin was dissolved in 8 mL of distilled water. The solution was sonicated for 30 minutes, then distilled water was added until the concentration of the solution reached 10,000 ppm. Then, the solution was centrifuged for 30 minutes at 12,000 rpm, and the supernatant was collected. The filtrate was passed through a 0.22µm Millipore filter, resulting in a clear, colorless solution. The HPLC method was performed using acetonitrile as the mobile phase with a C18 column and a UV-Vis detector. The water had a ratio of 5:95, with a retention time of 35 minutes, a wavelength of 295nm, an injection volume of 20µL, and a mobile phase flow rate of 2mL/min. The extract with the solvent containing the highest solubility of lunasin was used in this study.[13]

Treatments of the Animals

Female SD rats aged 6 weeks were divided into 5 groups of 6 animals each: normal, negative control, positive control, lunasin, and combination. The sample size was determined using Federer's formula, which calculated a minimum requirement of five rats per group, but six rats were used to account for potential mortality.[13] DMBA was given intragastrically with a dose of 20 mg/kgBW twice a week until 11 total injections were administered to induce breast cancer in all groups except the normal group. Once the tumor volume reached 1-2 cm^3, treatments were given for 8 weeks. The positive control group received tamoxifen 10 mg/kgBW, the

lunasin group received soybean extract rich in lunasin, and the combination group received both tamoxifen and soybean extract rich in lunasin. The negative control group did not receive any treatment. Later, the rats were terminated, and tumor tissues were taken, processed, and embedded in paraffin blocks for immunohistochemical staining (IHC) following the protocol from Abcam.[13]

Tumor Tissue Sampling

Tissue sample preparation involved several steps. First, fixation was performed by immersing breast cancer tissue sections in a formalin buffer solution (10% formalin in sodium acetate buffer, pH 7.0), followed by cutting the tissue to a thickness of 3-5 mm with a scalpel. Second, the tissue was dehydrated by soaking it in 96% ethanol for 30 minutes. This process was repeated five times, replacing the ethanol each time. Third, the tissue was cleared by immersing it for 15 minutes in xylene, followed by another 15 minutes in a second xylene solution. Finally, the tissue was placed in solid paraffin (melting point 60–70 °C) for 30minutes and then cooled in a freezer for 10 to 15minutes for impregnation and embedding. The paraffin block was stored at room temperature before being prepared for hematoxylin and eosin staining.[13]

Hematoxylin and Eosin Staining

Hematoxylin and eosin (HE) staining was performed to assess the breast cancer incidence in rats after DMBA induction. Tissue preparation of 4 µm was cut from the paraffin block and attached to slides. The samples were then deparaffinized in xylene I, II, and III for 5 minutes each. Next, the samples were rehydrated with 96% and 70% ethanol for 5 minutes each, followed by a rinse under running water for 5 minutes. The samples were stained in hematoxylin for 7 minutes, rinsed in running water for 10 minutes, and then dipped in saturated lithium carbonate for 1-2 minutes followed by another 5-minute rinse. If the blue color was insufficient, the samples were returned to hematoxylin for 2 minutes, rinsed, and then stained in eosin for 1-2 minutes. Dehydration was performed with 70%, 80%, and 96% ethanol, followed by clearing with xylene I, II, and III. Finally, a drop of Entellan, Sigma-Aldrich was added, and the samples were covered with a cover slip. The stained tissue samples were examined under a light microscope at 400x magnification. Positive HE staining was followed by immunohistochemical staining.[13]

Immunohistochemistry Staining

Breast cancer tissue in paraffin blocks was cut transversely at 3µm thickness using a microtome. The samples were placed on glass slides with a poly-L-lysine coating and heated in an oven at 60°C overnight. To remove paraffin, the samples were dipped in xylene three times for 3 minutes each, followed by dehydration in 100%, 95%, and 70% ethanol for 3minutes, 2minutes, and 1minute, respectively. The samples were then immersed in 0.01 M sodium citrate (pH 6.0) in a microwave for 5 minutes. After that, 3% hydrogen peroxide was applied for 5minutes at room temperature to eliminate endogenous peroxidase.[14] The samples were incubated with p21 cyclin antibody for 2hours at room temperature in PBS using a humidity chamber, followed by overnight incubation at 4°C. A negative control, N-Universal, was used. The samples were then incubated with a secondary antibody at room temperature for 1 hour, followed by 30 minutes with HRP-conjugated streptavidin. To visualize the protein, 3.3'-diaminobenzidine (DAB) was applied for 10minutes at room temperature. Finally, the samples were counterstained with Harris hematoxylin, dehydrated, and mounted.[15]

Interpretation of P21 Protein Expression

P21 protein expression was assessed using a weighted histoscore (H-Score) based on the intensity of the streaks and the percentage of stained cells. P21 protein intensity was gathered using ImageJ and IHC Profiler. The intensity score measurement ranged from 0–3 (0=no protein expression; 1=weak expression; 2=moderate expression; 3=strong expression). The H-score was obtained by multiplying the percentage value of the intensity score = (% of cell with 0 x 1) + (% of cell with 1 x 2) + (% of cell with 2x3) + (% of cell with 3x4).[16]

Statistical Analysis

H-Score data in percentages were analyzed using SPSS 27 with a confidence interval of 95%. Normality testing was carried out using a Shapiro-Wilk test, and homogeneity was assessed using Levene's test. Normal and homogeneous data (P>0.05) were subsequently processed using one-way parametric ANOVA. Post hoc Tukey's HSD test was conducted to examine possible differences between treatment groups.

RESULTS

Expression of P21 Protein on Immunohistochemical Staining

Cytoplasm expresses p21 protein in brown, with the color intensity aligning with the level of protein expression. A strong brown color in the cytoplasm indicates a higher p21 expression, while cells with a lower p21 expression have a light brown to blue color. As can be seen in Figure 1, the normal group (A) has a high expression of p21 marked by a high intensity

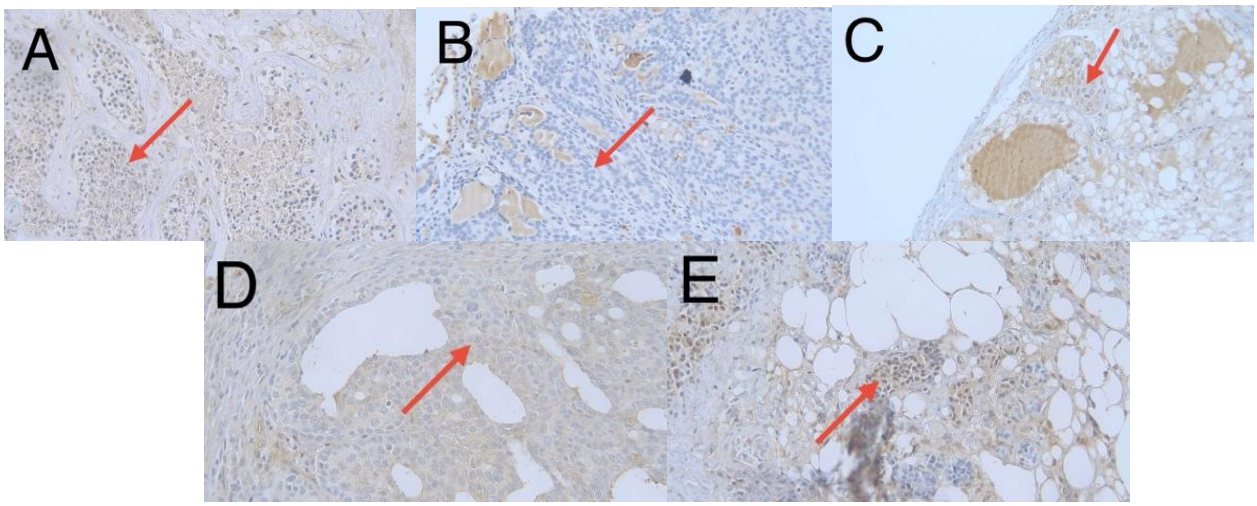

Figure 1. A, normal group; B, negative control; C, positive control; D, lunasin group; E, combination group. Immunohistochemical staining of p21 in rat breast cancer tissue at 400× magnification. The red arrows indicate p21 expression, shown as brown staining in the cytoplasm. The intensity of the brown staining corresponds to p21 expression levels. (A) The normal group exhibits the highest p21 expression, with strong brown staining. (B) The negative control group shows the lowest p21 expression, with weak or absent brown staining. (C) The positive control group displays moderate p21 expression, with visible but less intense staining. (D) The lunasin group shows slightly higher p21 expression compared to the positive control. (E) The combination group exhibits stronger brown staining than both the positive control and lunasin groups.

of brown coloring. The negative control group (B) has a low p21 protein expression, shown by blue coloring in the cytoplasm.

P21 Protein Expression in Different Groups

The average p21 protein expression, written as an H-Score from highest to lowest, was found between the normal, combination, lunasin, positive control, and negative control groups. The lunasin group, which received only soybean extract rich in lunasin, and the combination group, given both soybean extract rich in lunasin and tamoxifen, saw an increase in p21 protein expression compared to the negative control group ($P<0.05$). There is no statistically significant difference in p21 protein expression between the positive control and negative control groups ($P>0.05$). (Table 1)

Table 1. Comparison of p21 protein expression between the groups

Group	n	P21 expression (mean±SD)	P (ANOVA)
Normal	6	200.96±14.72	<0.01
Negative	6	149.58±8.71	
Positive	6	162.85±18.98	
Combination	6	185.05±7.12	
Lunasin	6	172.36±13.48	

Table 2. P-values between the groups in post-Hoc Tukey analysis

Group	Normal	Negative	Positive	Combination
Normal				
Negative	<0.01			
Positive	<0.01	0.437		
Combination	0.264	0.001	0.055	
Lunasin	<0.01	<0.01	0.730	0.481

DISCUSSION

Role of DMBA in Breast Cancer Induction

In this study, the normal group, which did not receive any treatment, showed significantly ($P<0.05$) higher p21 protein expression compared to all other treatment groups, except for the combination group. The activation of nuclear factor kappa beta (NF-κβ) by DMBA caused a reduction in protein expression in the negative and positive controls, as well as the adjuvant groups.

NF-κβ is a transcription factor for the cellular myelocytomatosis oncogene (c-Myc), which acts as a transcriptional repressor of p21 protein.[17] As a CDK inhibitor, p21 functions in cell cycle arrest, and its reduced expression results in uncontrolled proliferation and failure of apoptosis, leading to breast cancer in DMBA-induced rats.[18]

Tamoxifen as Standard Therapy

Data analysis showed that the average p21 protein expression in the tamoxifen-treated group is higher

than in the negative control group, but the difference is not statistically significant (P>0.05). Tamoxifen has been a central part of endocrine therapy for breast cancer since the 1970s.

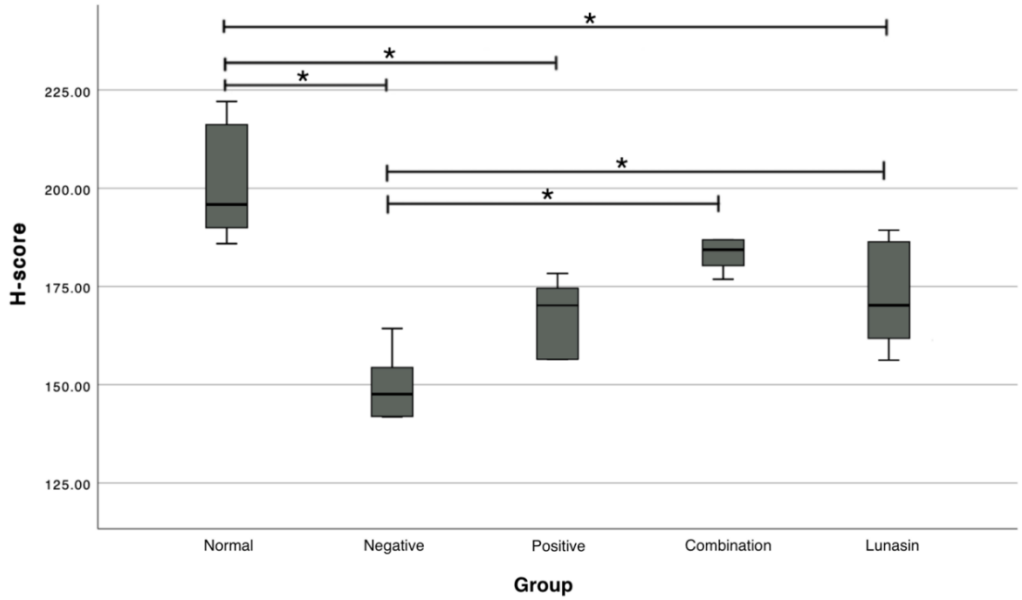

Figure 2. H-score on each group (* shows significant differences between groups)

This drug, a triphenylethylene derivative classified as a selective estrogen receptor modulator (SERM), is used to treat ER-positive breast cancer, which accounts for 70% of breast cancer cases. Tamoxifen acts as a partial estrogen antagonist, interfering with estrogen receptor (ER) function by competing with estrogen for receptor binding. The binding of tamoxifen to ER prevents oncogene activation by estrogen, inhibiting the estrogenic effects involved in cancer cell proliferation and growth.[13,19] Tamoxifen's anti-proliferative effect is believed to occur through the activation of the JNK and p38 MAP kinase signalling pathways, which then activate Egr-1 via Elk-1. The transactivation of Egr-1 has been shown to induce p21 protein expression, promoting cell cycle arrest in the G0/G1 phase.[20,21] However, various studies report resistance of ER-positive breast cancer to tamoxifen due to overexpression of receptor tyrosine kinases (RTK), which activates the PI3K-PTEN/Akt/mTOR pathway, reducing tamoxifen's effectiveness.[22] This may explain the lack of a statistically significant difference in p21 protein expression between the positive and negative control groups. To address the resistance problem, combination therapy with tamoxifen and other drugs should be considered in breast cancer treatment.

Soybean Extract Rich in Lunasin as a Therapy for Breast Cancer

P21 protein expression in the lunasin group was significantly higher than in the negative control group. This is consistent with the findings of a study by Szymczyk *et al.* which found upregulation of p21 protein in breast cancer cells exposed to lunasin.[23,24] The increase in p21 protein occurs through the disruption of integrin signalling.[23,25] Integrins are receptors found on various cells that play a key role in signaling between the cytoskeleton and the extracellular matrix.[26] The RGD domain of lunasin competes with the extracellular matrix for binding to integrins, thereby disrupting signaling pathways involved in cell adhesion, invasion, and interaction with extracellular matrix components.[27] This mechanism contributes to lunasin's recently observed ability to inhibit tumor progression and metastasis.[23,28] The lunasin-integrin binding also leads to downregulation of FAK/Akt/ERK phosphorylation, preventing MDM2-p53 interaction and suppressing c-Myc expression.[25,27] As a result, ubiquitination of p21 protein does not occur, preventing mRNA destabilization and negative regulation of p21. The increase in p21 protein expression induces cell apoptosis and cell cycle arrest in the G1/S phase, allowing time for DNA repair before DNA synthesis.[29,30] The increased p21 protein expression following the administration of soy extract rich in lunasin is also thought to occur through modulation of histone acetylation and deacetylation dynamics, leading to increased expression of the p21 gene.[23,31]

Additionally, lunasin can inhibit nuclear factor kappa β (NF-κβ) activation by increasing the expression of inhibitor of nuclear factor kappa β alpha

(Iκβ-α), a protein that binds NF-κβ in the cytoplasm, preventing its translocation to the nucleus.[25,32,33] This results in reduced cell proliferation and the potential inhibition of cancer cell migration and invasion.[32]

When comparing p21 protein expression between the positive control group and the lunasin group, we found that the latter showed higher expression, though the difference was not statistically significant. This may be due to the relatively small sample size, making it harder to detect true differences. Additionally, the high standard deviation observed suggests biological variability.[34] A larger sample size may be required to better distinguish lunasin from tamoxifen and confirm its impact on p21 expression.

Tamoxifen Combined With Soybean Extract Rich in Lunasin

A commonly used strategy in cancer therapy is the combination of two or more drugs to achieve a synergistic effect in cancer prevention and treatment.[35] In this study, data analysis showed that p21 protein expression in the combination group was significantly higher than in the negative control group. Additionally, the average p21 protein expression was also higher in the combination group compared to the positive control and lunasin groups, although the differences were not statistically significant. This may occur because tamoxifen and lunasin act through distinct signaling pathways to regulate p21 expression. Tamoxifen exerts its effect via Egr-1 activation, while lunasin functions through integrin signalling.[22] Given that tamoxifen resistance is often driven by PI3K/Akt/mTOR pathway activation, lunasin's ability to downregulate Akt suggests a role in overcoming this resistance.[22,25,27] By disrupting integrin signaling and inhibiting FAK/Akt/ERK phosphorylation, lunasin may help sustain p21 expression and counteract resistance mechanisms in breast cancer.[31]

Additionally, reduced p21 and p27 expression has been linked to increased tamoxifen resistance.[36] Since lunasin upregulates both proteins, its co-administration with tamoxifen may further help mitigate resistance and prolong treatment efficacy. Through these complementary mechanisms, ie, Akt downregulation and p21/p27 upregulation, lunasin may enhance tamoxifen's therapeutic effects, potentially improving treatment outcomes.[20,25,36]

Lunasin's Therapeutic Challenges and Safety Considerations

The bioavailability of food-derived bioactive compounds depends on their stability during absorption, distribution, and metabolism.[37] However, therapeutic peptides, including lunasin, face challenges due to enzymatic degradation and limited gastrointestinal permeability.[38]

In vitro studies show that lunasin undergoes significant degradation, although soybean-derived protease inhibitors, such as Bowman-Birk and Kunitz trypsin inhibitors, may enhance its stability and bioavailability.[37] Lunasin crosses the Caco-2 cell monolayer via paracellular diffusion, with 4.5% detected in human plasma and 30% absorbed in mice.[39,40] Once in circulation, it is distributed to tissues, crosses the blood-brain barrier, and enters cells despite its hydrophilic nature.[40] Its bioactivity depends on evading proteolysis, necessitating further stability assessments for therapeutic applications.[41]

Animal studies suggest that lunasin selectively targets cancer cells without adverse effects.[40] Treated mice showed no significant physiological or biochemical changes, including liver enzymes and creatinine levels.[42] Despite crossing the blood-brain barrier, lunasin did not affect mouse behavior, although further neurological evaluations are needed.[41,43] Hsieh *et al*. found that lunasin inhibited breast cancer cell proliferation in a dose-dependent manner without affecting normal cell growth.[32]

Rusdi *et al*. assessed the acute and sub-chronic toxicity of soybean seed extract and lunasin-targeted extract (ET-Lun). No mortality was observed at doses of 500, 2000, and 5000 mg/kgBW over 14 days. Sub-chronic toxicity testing showed no significant differences in serum glutamate oxaloacetate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) levels between control and ET-Lun-treated groups (250–750mg/kgBW). Histopathological analysis confirmed normal liver architecture, with no signs of hepatotoxicity.[10] While lunasin shows promising results, large-scale human trials are needed to confirm its safety and efficacy in cancer treatment.

Limitations

While this study provides valuable preclinical insights, the relatively small sample size may have limited statistical power, contributing to high variability in p21 expression, especially in the lunasin group. Additionally, only a single lunasin dose (500mg/kgBW) was used, preventing the assessment of dose-dependent effects. The long-term impact of tamoxifen-lunasin combination therapy remains unknown, as this study focused on short-term tumor responses without assessing potential adverse effects or resistance mechanisms. Lastly, the use of the DMBA-induced breast cancer rat model may not fully replicate human breast cancer, requiring validation in human cell lines and clinical studies. Future studies should address these limitations.

CONCLUSION

Breast cancer treatment faces challenges due to side effects and therapy resistance, highlighting the need for effective adjuvant strategies. This study found that soybean extract rich in lunasin upregulates p21, supporting its role in tumor suppression. Combination therapy showed the highest p21 levels, suggesting a potential synergistic effect. Given its distinct mechanism of action, lunasin may help overcome tamoxifen resistance, making it a promising adjuvant therapy.

ETHICAL CONSIDERATIONS

The study was approved by the Ethics Committee of Faculty of Medicine, Universitas Indonesia (KET/647/UN2.F1/ETIK/PPM.00.02.2019).

ACKNOWLEDGMENTS

The authors wish to express sincere gratitude to Anne L. Lashbrook from the Department of Physics, University of Washington, for her invaluable assistance in proofreading this article.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

FUNDING

Directorate of Research and Development, Ministry of Research, Technology, and Higher Education, Republic of Indonesia/ National Agency for Research (PTUPT contract number NKB-318/UN2.RST/HKP.05.00/2020) funded this study.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author or LE upon reasonable request.

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How to Cite This Article

Evita L, Kusmardi K, Rusdi NK. Soybean Extract Rich in Lunasin Enhances p21 Expression in DMBA-Induced Breast Cancer in Rats: A Potential Adjuvant Therapy. Arch Breast Cancer. 2025; 12(2):203-10.
Available from: https://www.archbreastcancer.com/index.php/abc/article/view/1077