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Research Article

In Vivo Antimammary Tumor Effects of Soybean Extract with Targeted Lunasin (ET-Lun)

Numlil Khaira Rusdi[1,2], Erni Hernawati Purwaningsih[3,7], Andon Hestiantoro[4], Berna Elya[5], Kusmardi Kusmardi[6-8,*]

ABSTRACT

Background/Objective: Lunasin is a peptide, consist of 44 amino acids which have anti-cancer, antioxidant, and anti-inflammatory activity. The price of commercial Lunasin was very expensive due to the high cost of lunasin synthesis and the lack of methods to obtain the pure lunasin weight from plant sources, involving time-consuming analytical instruments. To overcome these problems, the soybean extract with targeted Lunasin (ET-Lun) was made. The aim of this study was to investigate anti-cancer properties of ET-Lun in breast cancer models *in vivo*. **Methods:** Effect of ET-Lun was evaluated in 7,12-*Dimetilbenz[a]antrasen* (DMBA) induced breast cancer rat model. Tumor Mass, volume, and number were measured. The expression of HER2 and EGFR from each treatment group in DMBA-induced rat was evaluated using immunohistochemistry. **Results:** The results shown that ET-Lun could reduced tumor volume (p=0,021). ET-Lun decreased EGFR expression compared to negative control DMBA (p=0,012). **Conclusions**: These results indicated that the ET-Lun has anti-breast cancer activity *in vivo*.
Key words: *In-vivo*, Soybean, Breast cancer, HER2, EGFR.

Numlil Khaira Rusdi[1,2], Erni Hernawati Purwaningsih[3,7], Andon Hestiantoro[4], Berna Elya[5], Kusmardi Kusmardi[6-8,*]

[1]*Doctoral Program for Biomedical Sciences, Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.*
[2]*Faculty of Pharmacy and Sciences, Universitas Muhammadiyah Prof. DR. Hamka, Jakarta, INDONESIA.*
[3]*Department of Pharmacy, Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.*
[4]*Department Obstetrics and Gynaecology, School of Medicine, Universitas Indonesia, Dr Cipto Mangunkusumo Hospital, Jakarta, INDONESIA.*
[5]*Department of Phytochemistry, Faculty of Pharmacy, Universitas Indonesia, Depok, INDONESIA.*
[6]*Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia, Jakarta, INDONESIA.*
[7]*Drug Development Research Cluster, Indonesian Medical Education and Reseach Institute, Universitas INDONESIA.*
[8]*Human Cancer Research Cluster, Indonesian Medical Education and Research Institute, Universitas INDONESIA.*

Correspondence

Kusmardi Kusmardi

Department of Anatomic Pathology, Faculty of Medicine, Universitas Indonesia, Jakarta; Drug Development Research Cluster; Human Cancer Research Cluster, Indonesian Medical Education and Research Institute, Universitas INDONESIA.

E-mail: kusmardi.ms@ui.ac.id

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INTRODUCTION

Breast cancer (BC) is the most common malignancy occurred in women worldwide.[1] In 2018, Globocan data shown that the BC's prevalence was 11.6 %.[2] Breast cancer is also one of the most frequent cancers in Indonesia.[3] According to the Riskesdas in 2018, breast cancer prevalent was increased from 1.4% in 2013 to 1.8% in 2018.[4]

Cancer therapy is still developing, one way is by natural compound exploration as sources of therapy to reduce side effects which may cause due to undesirable outcomes of chemotherapy. One of the medicinal plants that has anti-cancer activity has and being developed as an anticancer drug is soybeans (Glycine max (L.) Merr.). Consumption of soy products has been known to decrease mortality and incidence of breast cancer[5], prostate cancer[6], colon cancer[7], and lung cancer[8,9]. The active compounds in soybeans were isoflavonoids (genistein, daidzein and glycitein), Bowman-Birk protease inhibitor, Kunitz trypsin inhibitor, sitosterol, saponin, lectins, and lunasin.[9-10] Isoflavones were compound of flavonoid in soybeans has known as a strong antioxidant. Soybean has many advantages to health may be obtained from isoflavonoid. While many research has been extended to understand the anti-cancer potential of isoflavonoid, not every anti-cancer effect related to soybean consumption was from isoflavonoids.[9] Recent studies shown that a significant anti-cancer compound in soybean was a bioactive peptide : lunasin.[10],[11]

Lunasin is a peptide, consist of 44 amino acids,[12] which have anti-cancer,[9,13] antioxidant and anti-inflammatory activity[14]. Galves et al. [15] demonstrated that lunasin can inhibit the mitosis of MCF-7 cancer cells, *murine hepatoma* (Hepa 1c1c7), and embryonic fibroblast murine cells (C3H 10T1/2), resulted cell death due to binding chromatin in the kinetochore area in the centromere and blocking the microtubule attachment. Lunasin can also increase apoptosis by inducing PTEN and demonstrated to inhibit caspase-3 *in vitro* and in *vivo*.[16] Previously, lunasin also found to inhibit metastasis by suppressing cellular migration, invasion, and expression of matrix metalloproteinases (MMP)-2 and MMP-9.[17]

However, commercial price of Lunasin was very expensive due to the high cost of lunasin synthesis and the lack of methods to obtain the pure lunasin weight from plant sources, involving time-consuming analytical instruments.[18-19] The concentration of Lunasin may be influenced by cultivar, environmental factors, in particular temperature and conditions during processing.[20] To overcome such limitations, the soybean extract with targeted Lunasin (ET-Lun) was made. This extraction method was obtained from several combinations of research that conducted by Vuyyuri, et al[9], and Serra, et al[19]. ET-Lun was lunasin that extracted from soybean seed, which that has been undergoing fat removal, followed by PBS solvent under a pH of 7.4. The lunasin content in the extract was 0.86 mg/g extract of soybean.[21]

Several studies related to ET-Lun activity, including the potential of ET-Lun to reduced COX-2 expression in a dose of 150 mg/kg BW and 200 mg/kg BW of mice ($p < 0.05$). ET-Lun were also shown to decrease iNOS expression in a dose of 150 mg/kg BW of mice.[22] Moreover, ET-Lun can inhibit Goblet cell counts and micro blood density,[23] inhibit colon cancer by increased apoptosis in a dose of 150 mg/kg BW in mice, and reduced dysplasia at a dose of 200 mg/kg BW mice.[24].

The purpose of this study was to analyze the anti-breast cancer activity of ET-Lun in vivo assay. *In vivo* assay was evaluated the expression of HER2, and EGFR of cancer mammae from the treatment group in DMBA-induced rat by immunohistochemistry.

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METHODS

Plant material collection, identification and extraction

Soybean seeds (Glycine max (L.) Merr) of Grobogan variety were purchased and identified from Indonesian Legumens and Tubers Crops Research Institude, (Balitkabi) Malang, East Java. The first extraction process was oil removal of soybean seed by pressing the seeds at 100-150 atm for 30 minutes at a temperature of 120°F (48.89°C). The process was followed by a blending process to produce a powder. Soybean powder was macerated in Phosphate Buffered Saline (PBS) solvent at pH 7.4 by volume as much as 5 times of weight of the powder for 60 minutes, followed by filtration using Whatman™ 54. The filtrate was then evaporated using a vacuum rotary evaporator until thick extract was obtained.

Preliminary phytochemical screening

Preliminary phytochemical screening of ET-Lun was performed on various phytoconstituents such as alkaloids, saponins, tannins, phenolics, flavonoids, triterpenoids, steroids and glycosides, using various reagents.[25]

Quantitative estimation of phytochemicals

Extract's Phytochemical compounds were analyzed using several methods as follows; water value and ash value determined by Thermal Volumetric Analysis (aufhauser) and Gravimetric analysis, the microbial contaminations and were evaluated using direct inoculation, and heavy metal contaminant was examined using Atomic Absorption Spectrophotometer (AAS)

Ethics and Study Design

Female Sprague Dawley (SD) rat aged 6 weeks, divided into 5 groups of four animals in each. The groups were normal control (NOR), rats induced by 7,12-Dimethylbenz[a]anthracene (DMBA) that received corn oil as vehicle as negative control. Rats that induced by DMBA were divided into several treatments such as Tamoxifen (TAM), Lunasin (ET-Lun), and combination of ET-Lun and tamoxifen (Adjuvant group). This experiment has been approved by the Ethics Commission of the Faculty of Medicine, the University of Indonesia (the number certificate was KET-647 / UN2.F1 / ETIK/ PPM.00.02 / 2019

Animal Experimental

All rats in negative control groups were firstly induced by DMBA that has been dissolved in 2 mg/ml of corn oil. The DMBA induction was given intra-gastric at a dose of 20 mg/kg BW, carried out 11 times, twice a week.[26] Treatment was given to rats with a tumor volume of 1-2 cm^3. The ET-Lun group was given ET-Lun at doses 500 mg/kg BW, TAM was treated with Tamoxifen 10 mg/kg BW, and adjuvant group was treated with combination ET-Lun and Tamoxifen. Treatment was conducted in 8 weeks. For negative controls, tumor growth in rats was observed for 8 weeks. After following treatment, rats were terminated and the tumor tissue was removed for analysis. Tumor tissue was processed and embedded in paraffin blocks for Immunohistochemical (IHC) testing.

Immunohistochemistry of HER2 and EGFR

The IHC stain were performed according to the IHC-Paraffin Protocol from Abcam®. Assessment of HER2, and EGFR expression was performed using weighted histoscore/ H-Score, which was based on the percentage of stained cells and the intensity of the streaks. The intensity measurement was given a score of 0 - 3 (0 = none; 1 = weak; 2 = moderate; and 3 = strong). Cell calculations were performed using ImageJ and Image Profiler software, using a histoscore (H-score). The H-score was calculated by multiplying the percentage value of the intensity score. HER2 and EGFR H-Score[27,28] = 1 x (cell membrane and cytoplasm of tumor cells that stained with weak intensity, 1+) + 2 x (cell membrane and cytoplasm of tumor cells were stained with moderate intensity, 2+) + 3 x (cell membrane and cytoplasm of tumor cells that stained with with strong intensity, 3+).

RESULTS

Plant material collection, identification and extraction

Soybean plant certification was issued by the Indonesian Legumens and Tubers Crops Research Institute, (Balitkabi) Malang, East Java. The variety of soybean seed was Grobogan. (Certificate number is 0310/009. KD. Gro. BS. Kp. 19/08.19-LA).

Physicochemical evaluation

The standardization of the extract obtained was a water value of 29.82%, ash value of 2.75% and the percentage of extraction yield was 12.34%, heavy metals; lead (Pb) was 1,05 ppm and cadmium (Cd) was negative. Phytochemical screening found that soybean extracts contained flavonoids, alkaloids, saponins, triterpenoids, and glycosides.

Effect on mammary tumors

During treatment, tumor diameter and volume were evaluated once a week. The results of tumor volume for 8 weeks of treatment shown that treatment with tamoxifen, ET-Lun, and a combination of ET-Lun and tamoxifen/ adjuvant group, could reduce tumor volume when compared to negative controls (DMBA). The reduction in tumor volume was shown by the adjuvant group (Figure 1). There were differences in the tumor volume of the DMBA group and the ET-Lun and Adjuvant group starting at week 3 to 8 of treatment ($p < 0.05$). At week 3, tumor volume of ET-Lun and Adjuvan group were different compared to the negative control group, while but tumor volume of the tamoxifen group did not differ with the negative control group ($p = 0.149$). The tumor volume in the tamoxifen group was different from the DMBA group at week 4 to week 7 ($p < 0.05$). At week 8, the tumor volume in the tamoxifen group did not differ from the tumor volume in the DMBA group, whereas the tumor volume in the adjuvant group and the group given ET-Lun shown differences in the DMBA group.

The results of tumor weight (g/kg BW) after treatment, the group given the ET-Lun, tamoxifen, and the adjuvant group, could reduce tumor weight when compared to negative controls DMBA (Figure 2A). There was no difference in the weight of the DMBA tumor group with the tamoxifen, ET-Lun, and Adjuvant groups ($p > 0.05$). The tumor volume in the tamoxifen group, the ET-Lun group, and the adjuvant group was shown decreased when compared to DMBA negative control group (Figure 2B). Statistical analysis has shown that tumor volume in the DMBA group were differed from the tumor volume in the ET-Lun group ($p = 0.021$), while no difference in tumor volume of tamoxifen and adjuvant groups ($p > 0.05$).

Immunohistochemistry analysis for EGFR and HER2

The expression of EGFR (Figure 3A) showed a significant difference between the negative control DMBA and the group given ET-Lun (p=0,012) and adjuvant (p=0,021). The results of microscopic observation of EGFR expression also showed that the ET-Lun and adjuvant group was able to reduce EGFR expression compared to the DMBA group (Figure 4). EGFR expression is indicated by the presence of brown-stained cells (red arrow) on the cell membrane and cytoplasm. HER2 expression is indicated by the presence of brown-stained cells (red arrow) on the cell cytoplasm (Figure 5). In the DMBA group, almost all cell membranes were brown with moderate to strong intensity covering the membrane and cytoplasm. In contrast, in the ET-Lun group, the presence of brown-stained cells were not as much as in the DMBA group with weak intensity. Some epithelial cells were negative stained (blue), with the nucleus still clearly visible.

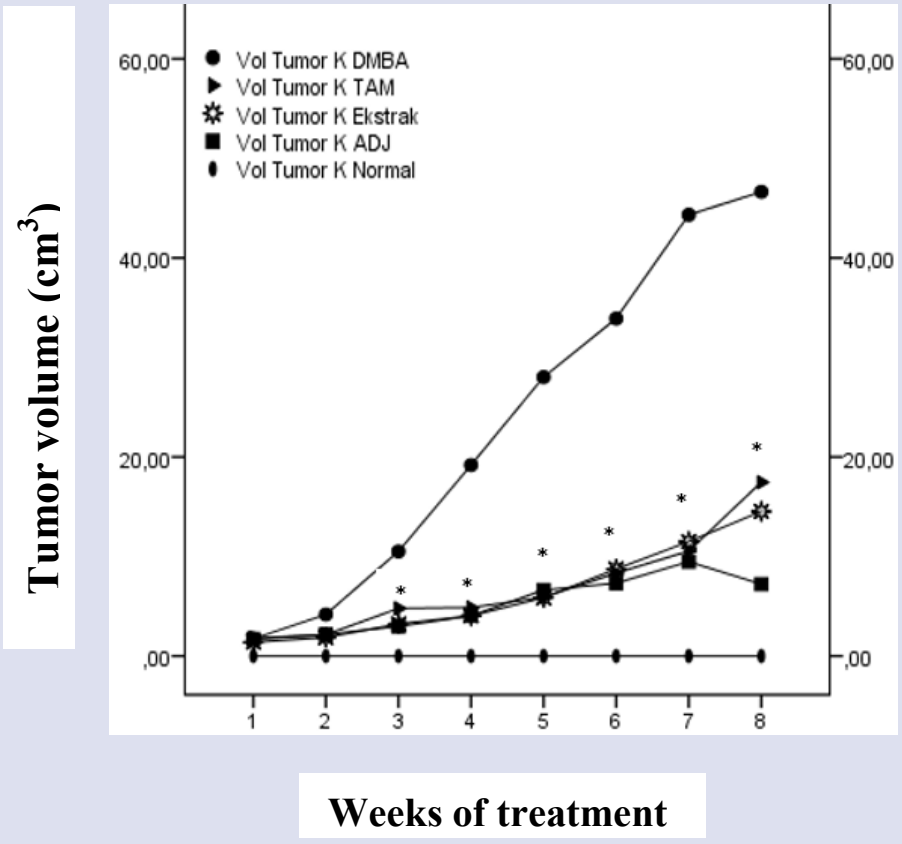
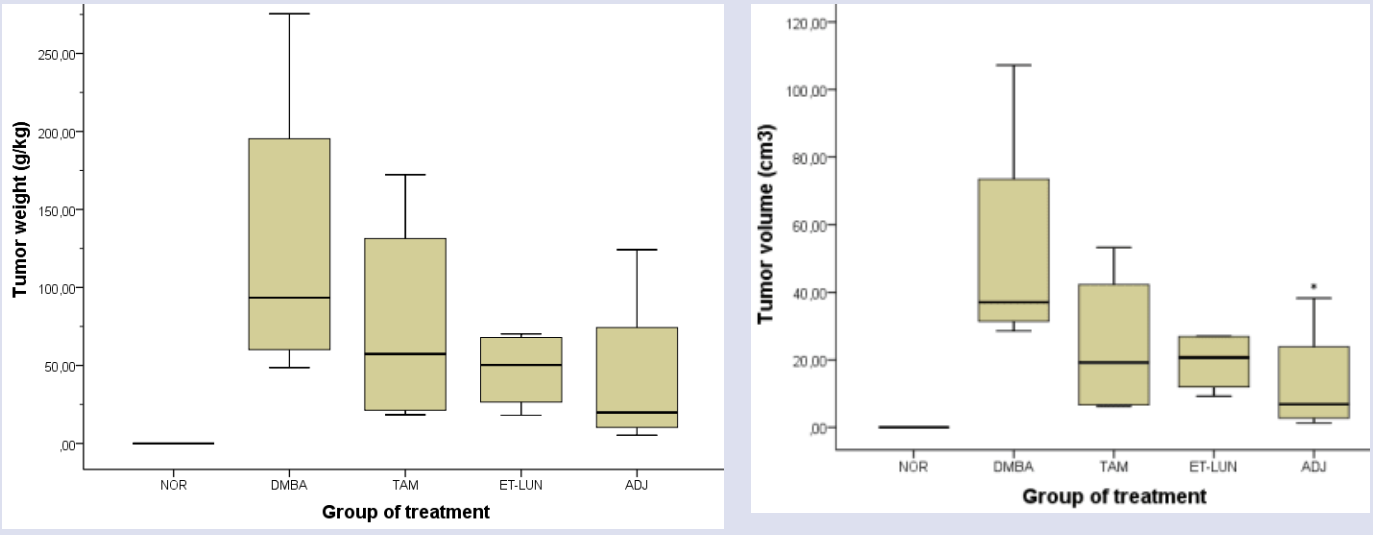


Figure 1: The tumor volume (cm3) for 8 weeks of each group treatment. Normal = normal control treated with corn oil as vehicle. DMBA = negative control treated with DMBA dissolved in corn oil; Tam = positive control treated with tamoxifen 10 mg/kg BW. K extract = treated with ET-Lun 500 mg/kg BW. Adj = treated with tamoxifen and ET-Lun. *p<0,05 compare negative group (DMBA).



(A) **(B)**

Figure 2: (A) The weight of tumor after treatment (g/kg). (B) The volume of tumor after treatment (cm^3). Nor = normal control treated with corn oil as vehicle. DMBA = negative control treated with DMBA dissolved in corn oil; Tam = positive control treated with tamoxifen 10 mg/kg BW. ET-Lun = treated with ET-Lun 500 mg/kg BW. Adj = treated with tamoxifen and ET-Lun. *p<0,05 compare negative group (DMBA).

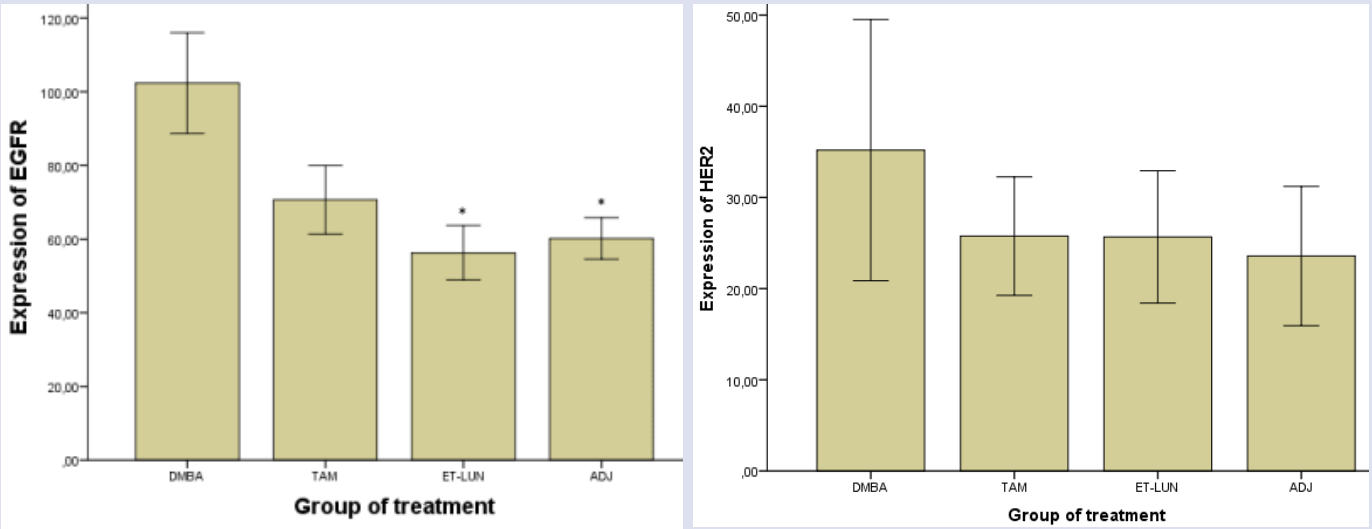


Figure 3: (A) EGFR Expression Score of the treatment group. (B) HER2 Expression Score of the treatment group. DMBA = negative control treated with DMBA dissolved in corn oil; Tam = positive control treated with tamoxifen 10 mg/kg BW. ET-Lun = treated with ET-Lun 500 mg/kg BW. Adj = treated with tamoxifen and ET-Lun. Data are presented as means±SEM (n=4). ** $p < 0,05$ compare DMBA.

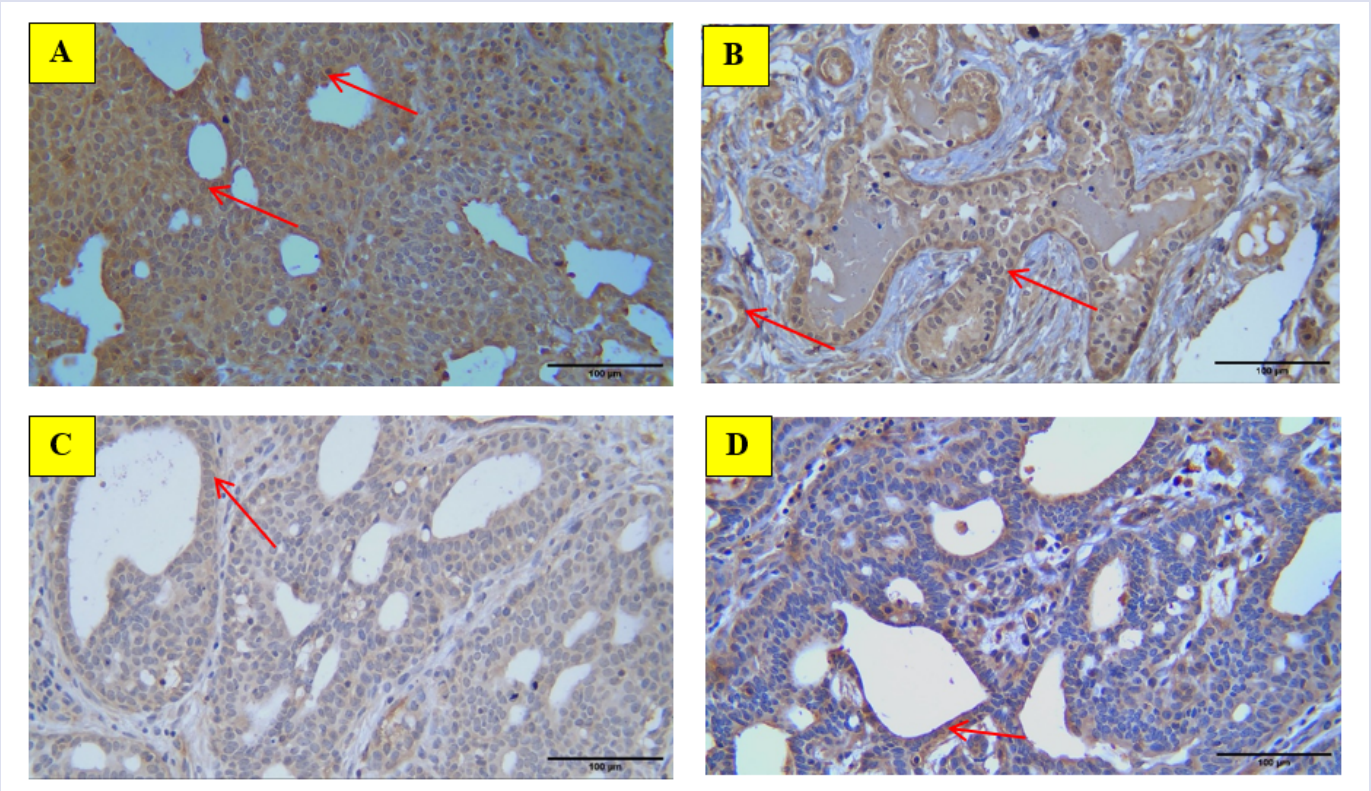


Figure 4: EGFR expression from breast cancer tissue with immunohistochemical staining in the treatment group (400X). (A) DMBA group, (B) Tamoxifen group, (C) ET-Lun, and (D) Adjuvan group.The red arrow indicates the expression of EGFR in membrane and cytoplasm of tumor cells.

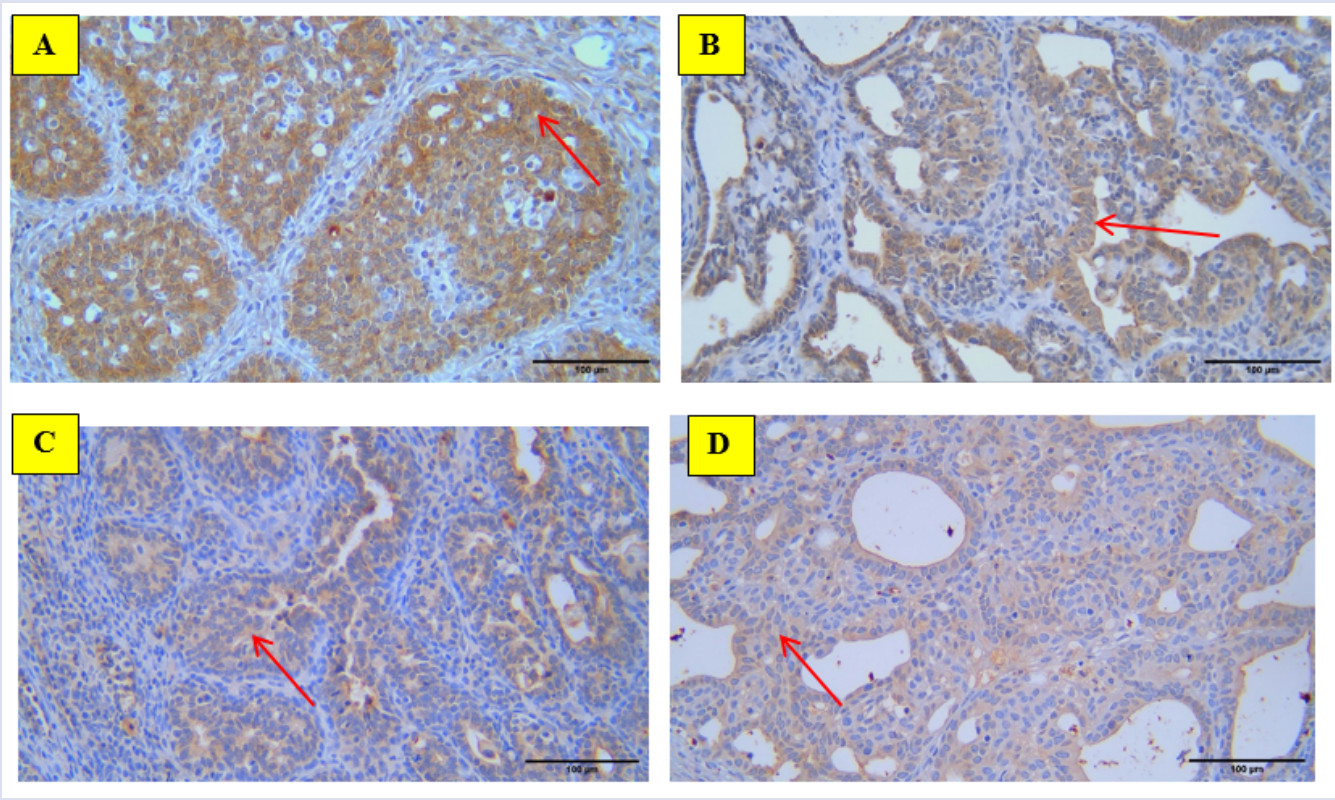


Figure 5: HER2 expression from breast cancer tissue with immunohistochemical staining in the treatment group (400x). (A) DMBA group, (B) Tamoxifen group, (C) ET-Lun, and (D) Adjuvan group. The red arrow indicates the expression of HER2 in cytoplasm of tumor cells.

DISCUSSION

Authentication and standardization are prerequisite steps while considering the source materials for herbal formulation in any system of medicine. The standardization of medical plant was necessary to ensure the effectiveness, safety, stability, and quality of phytoconstituents in medicinal plant.[29] The soybean plant is one of the plants whose their activity is being developed as a medical plant.[30] In this study, the extraction of soybean seed powder followed the previous research procedure.[22,24]

The variation of natural product composition in extract may have the pharmacological effects synergically, so that the characterization of the extract is needed for quality assurance.[29] The water value is needed to maintain the extract stability. The results of this study shown that the water value of thick extract was 29,82%. This result was in accordance with the requirements for thick extract content to that 5-30%[31] , while the ash value was 2,75%. The determination of the ash value is an indication of certain medicinal plant species because each plant has specific remains. In additions, the results of microbial contamination and heavy metal contamination are in accordance with the requirements and safety to be used.

Epidermal Growth Factor Receptor (EGFR) overexpression is phenotypes of an aggressive subtype of breast cancer and generally has a poor clinical prognosis. Sprague Dawley rat induced by DMBA, increased expression of ER, PR, EGFR, IGF1R, and activation of MAPK, JNK, and Akt signals, resulted in the development of carcinoma in breast tissue.[32,33] In this study there increased of EGFR expression was found in DMBA-induced.

Giving ET-Lun can reduce EGFR expression compared to DMBA negative control. This was also supported by a preliminary in silico test explained that lunasin can bind EGFR (data not shown). The binding of ET-Lun to EGFR resulted in decrease of EGFR expression and affects the signal propagations which are responsible for cell growth and apoptosis.

On the other hand, the EGFR expression of the tamoxifen group was not significantly different to the DMBA control group. Tamoxifen is a *selective estrogen receptor modulator* (SERM), suppress breast cancer growth by acting as an ER antagonist, by binding to the ER and inducing conformational changes that support the recruitment of corepressor proteins *nuclear receptor co-repressor* (NcoR) and *silencing mediator for retinoids and thyroid receptors* (SMRT) through the activity of histone deacetylation that plays a role in transcriptional repression.[34] In this case, tamoxifen acts only as an antiestrogen, but cannot inhibit the integration of signal transduction *growth factors*, such as EGFR.

The result of HER2 expression showed no difference between the DMBA group and ET-Lun, and Adjuvant groups. There was no difference in HER2 expression in the negative control group with the normal control group, indicated that DMBA induction in SD rats was not affected HER2 signaling. In this study, SD rats induced by DMBA increased the expression of the EGFR growth factor but did not alter HER2 expression.

CONCLUSIONS

This study demonstrated that ET-Lun could decrease EGFR expression compared to the negative control (DMBA) and might be used as a candidate for anti-breast cancer.

COMPETING OF INTEREST

None to declare.

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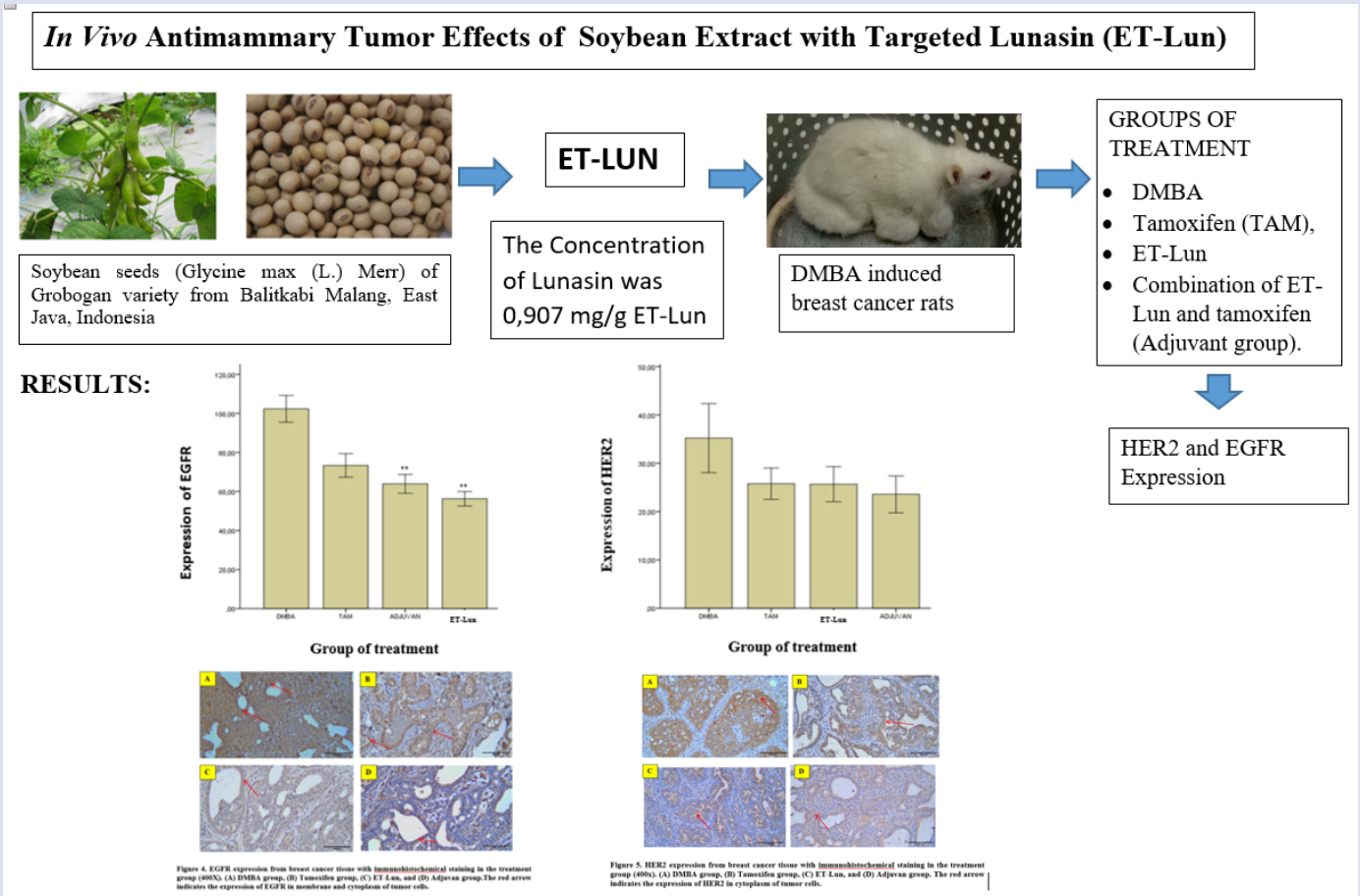
GRAPHICAL ABSTRACT



ABOUT AUTHORS

Numlil Khaira Rusdi. Lecturer in the Department of Pharmacy, University of Muhammadiyah Prof. Dr. HAMKA Jakarta. Focuses on Pharmacology and Community Pharmacy, and Doctoral student at Biomedical Sciences, Faculty of Medicine, Universitas Indonesia. She is currently conducting research on anti-breast cancer in medical plants.

Berna Elya, is Professor at the Faculty of Pharmacy, Universitas Indonesia. She is Head of Laboratory of Phy-tochemistry and Pharmacognosy. Has expertise in the area of Pharmacognosy and Phytochemistry of Natural Products, working mainly in: Natural Product Isolation and Bioassay.

Andon Hestiantoro is a Professor in obstetrics and gynecology, and works as consultant in reproductive endocrinology and infertility. He achieved his MD and PhD degrees from University of Indonesia, Jakarta, and obtained his MPH degree from University of Gajah Mada, Yogyakarta. He is the head of Human Reproduction, Infertility, and Family Planning Cluster, Indonesia Medical and Research Center, Universitas Indonesia. He conducts research activities at the Yasmin Fertility Clinic, Cipto Mangunkusumo Hospital, Jakarta, Indonesia. He is interested in research in female endocrinology.

Erni H. Purwaningsih is a Professor in Medical Pharmacy, Faculty of Medicine Universitas Indonesia, and as a vice coordinator of Drug Development Cluster, Institute of Medical Education and Research in Indonesia (IMERI) Universitas Indonesia. She achieved her MD, Magister and Doctor degrees from Faculty of Medicine, Universitas Indonesia. She conducts research activities especially on Herbal Medicine, and as a supervisor for several candidates on Biomedical Magister and/or Doctorate Program Faculty of Medicine, Universitas Indonesia. She is consultant and/or reviewer on Traditional Herbal Medicine that conducted by the Director of Traditional Health Services, the Ministry of Health and for the National Agency of Drug and Food Control of Indonesia.

Kusmardi is Associate professor at Anatomic Pathology, Faculty of Medicine, Universitas Indonesia. The major research focus on colorectal and breast cancer, include the potential inhibition of some Indonesian natural medicine on the both carcinogenesis, the indentification of normal tissue *vs* cancer development using some molecular marker and computational model. He wrote the mouse model for breast cancer book, the mouse model for colorectal adjuvant chemopreventive book, and Lunasin: a soybean polypeptide as chemopreventive adjuvant for colon cancer.

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