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Original article

Holothuria scabra Extract Improves Histopathological Features and Inhibits Cancer Growth Through IL-6 and NF-κB Signaling Pathways in Breast Cancer Mice Model

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Abstract

Background and objectives: The high incidence and mortality rate of breast cancer indicates that managing this disease is still problematic. A number of studies have shown that sea cucumbers, marine invertebrates, contain anticancer compounds. This study aimed to determine the effects of *Holothuria scabra* (sea cucumber) methanol extract on histopathological features, interleukin-6 (IL-6) concentration, and nuclear factor kappa B (NF-κB) expression in the breast cancer mice model. **Methods:** The body of *H. scabra* without internal organs was extracted with methanol. Thirty female *Mus musculus* C57BL6 mice were randomly divided into a control group, a breast cancer mice model group, and three treatment groups, which were breast cancer mice models administered with three various doses *H. scabra* methanol extract (0.33 (T1); 0.66 (T2); and 0.99 g/Kg BW (T3)) for 12 weeks. The breast cancer mice model was treated with a high-fat diet and 1 mg/kg BW 7, 12-dimethylbenz[a]anthracene subcutaneous injection into one of the breasts. Examination of IL-6 concentration was conducted by ELISA and NF-κB gene expression by qRT-PCR. **Results**: The results showed an improvement in histopathological features as revealed by the lower Nottingham scores in the T2 (5.17±0.75) and T3 groups (3.00±0.89) compared to positive control group 7.00±1.26 ($p<0.05$). *Holothuria scabra* methanol extract reduced IL-6 concentration in treatment groups dose-dependently and was significantly lower than the positive control group ($p<0.01$). The T2 and T3 groups showed significantly lower NF-κB expression than the positive control group ($p<0.05$). **Conclusion:** The sea cucumber *Holothuria scabra* methanol extract is a therapeutic candidate for breast cancer by suppressing IL-6 concentration and NF-κB gene expression.

Keywords: antineoplastic agent; breast neoplasms; cancer; *Holothuria scabra*; sea cucumbers

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Introduction

Breast cancer is the most common carcinoma in women worldwide [1]. Based on GLOBOCAN data, the International Agency for Research on Cancer in 2018, there were 11.6% of new cases and 6.6% of deaths in breast cancer, while in 2020, there was an increase of 11.7% of new cases and 6.9% of deaths due to breast cancer in the world [2,3]. These data show increased incidence and mortality rates of breast cancer worldwide.

The high mortality rate of breast cancer indicates that managing this disease is still problematic. Cancer therapy has limitations due to various considerations regarding efficacy, safety, and

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side effects. Therefore, it is necessary to develop a treatment for cancer using natural ingredients that contain metabolites as an anticancer [4].

Ocean life has a variety of natural metabolite sources that have anticancer potential. Sea cucumbers are soft-bodied marine animals shaped like a cucumber. These marine animals belong to the class Holothuroidea of the phylum Echinodermata, which are invertebrate animals that live on the seabed [5]. There are 1,716 species of sea cucumbers with the most biodiversity in Asia Pacific waters [6]. Sea cucumbers have long been used as food and medicine in Asia. Some research revealed that these submarine invertebrates have various anticancer mechanisms, such as anti-angiogenesis, cytotoxic effects, inducing apoptosis and inhibition of metastasis, cell cycle, cancer growth; they also prevent treatment resistance [5].

Various studies have revealed that there are various anticancer metabolites in sea cucumbers that can inhibit cancer, namely A1(HA1), 24-dehydroechinoside A (DHEA), colochiroside A, cucumarioside A2-2, ds-echinoside A, echinoside A, frondanol A5, frondoside A, glycosides 1 dan 2, holothurin A, holothurin B, intercedensides A(1), B(2), dan C(3), philinopside A, philinopside E, saponin, scabraside D, sphingoid bases, stichoposide C, and stichoposide D [7].

Our previous in vitro study found that methanol extract of *Holothuria scabra* Jaeger exhibited cytotoxic activity by inhibiting the growth of T47D cancer cells and stimulating 99% of cancer cells to undergo apoptosis [5]. Histopathological features of mammary tissue, levels of proinflammatory cytokine IL-6, and expression of NF-κB as a transcription factor in tumor development play a role in the pathogenesis of breast cancer. Therefore, this study was conducted to examine the effect of *H. scabra* methanol extract on the histopathological features, interleukin-6 (IL-6) concentration, and nuclear factor kappa B (NF-κB) expression in the breast cancer model.

Material and Methods

Ethical considerations

This study followed the institutional and national guide for the care and use of laboratory animals and has been approved by the Research Ethics Commission, Faculty of Medicine, Maranatha Christian University Bandung Indonesia, with the number 064/KEP/IV/2021.

Chemicals

Methanol pro analysis was obtained from Merck, Germany; 7, 12-dimethylbenz[a]anthracene (DMBA) (Sigma-Aldrich D3254, USA) was used to induce breast cancer in mice. The enzyme-linked immunosorbent assay (ELISA) kit (Elabscience E-EL-M0044, USA) was used to measure IL-6 levels. GENEzol^{TM} GZR200 (Geneaid, Taiwan), Thunderbird Probe One-step qRT-PCR kit QRZ101 (Toyobo, Japan), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and NF-κB primer sequences (Genetika Science, Indonesia) were used for NF-κB gene expression measurement.

Holothuria scabra extraction

Fresh *Holothuria scabra* were collected from the sea of Lamongan, East Java, Indonesia, and identification was done based on the Food and Agriculture Organization of The United Nations Species Catalog for Fishery Purposes [8]. The internal organs of the sea cucumbers were removed before the extraction process. The bioactive compound *H. scabra* was obtained by soaking the slices of sea cucumber in methanol with a ratio of 1:1 for 24 hours. The solvent was filtered using Whatman number one filter paper, and the filtrate was concentrated in a rotary evaporator.

Group treatments and breast cancer modeling in C57BL6 mice

Thirty female *Mus musculus* C57BL6 inbred mice aged 10-11 weeks were randomly divided into five groups (n=6). Mice without treatment were the negative control (NC) group, while a breast cancer animal model was a positive control (PC) group. Treatment groups I, II, and III (T1, T2, T3) consisted of breast cancer animal models treated with various doses of *Holothuria scabra* methanol extract.

The breast cancer modeling was performed on PC, T1, T2, and T3 groups, which were fed twice daily with a high-fat diet (57% fat) and water ad libitum for three weeks. According to research by Cranford et al. in 2019, C57BL6 mice fed high-fat had significant effects on tumorigenesis, such as increased tumor weight and tumor number [9].

On the 22nd day, mice were induced with 1 mg/kg BW DMBA in 0.5 mL sesame oil, given every two days, ten times, by subcutaneous injection into the right breast.

Administration of *Holothuria scabra* methanol extract

Three doses of *Holothuria scabra* extract (0.33, 0.66, and 0.99 g/kg BW) were given to T1, T2, and T3 groups simultaneously since the first 7, 12-dimethylbenz[a]anthracene induction, once daily, per oral, for 12 weeks. After 12 weeks of *H. scabra* extract treatment, 0.5 mL of retro-orbital blood was collected. The mice were sacrificed by cervical dislocation preceded by ketamine injection, and the mammary nodules were removed.

Histopathological examination of the mice mammary

Histological grading of the breast cancer was performed on the primary tumor using the Elston/Nottingham modification of the Bloom-Richardson system, based on tumor tubule formation, number of mitotic figures in most active areas and nuclear pleomorphism. This scoring method evaluates three parameters and assigns a score of 1 to 3 for each parameter: tubular formation (> 75% = 1, 10% to 75% = 2, and < 10% = 3), nuclear pleomorphism (absent = 1, moderate = 2, and apparent = 3), and mitotic activity was found in 10 high-power fields (HPF), based on an HPF size of 0.274 mm2 (< 10 mitoses = 1, 10 to 19 mitoses = 2, and > 19 mitoses = 3). The final score was based on the total score of these three parameters (range from 3 to 9): a score of 3, 4, or 5 = grade 1 (well differentiated); a score of 6 or 7 = grade 2 (moderately differentiated); and a score of 8 or 9 = grade 3 (poorly differentiated) [10]. The examination uses a light microscope with 400x magnification.

Measurement of IL-6 by ELISA technique

Mice blood samples were put into well-plates. Then, a biotinylated detection antibody specific for IL-6 and Avidin-Horseradish Peroxidase was added and incubated at 37°C. Furthermore, the addition of substrate solution to each well, in which each well-containing IL-6, will turn blue. This reaction ended with the addition of a stop solution, and the color changed to yellow. Optical density is read by spectrophotometry with a wavelength of 450 nM.

Analysis of NF-κB expression by RT-PCR

Extraction of mRNA from mammary tissue was carried out using the procedure from GENEzolTM. Examination of NF-κB gene expression using qRT-PCR, with One-Step RT-PCR Kit. PCR settings: heating 20 minutes (45 °C), 1 cycle 1 minute (95 °C), 40 cycles 10 seconds (95 °C), 10 seconds (55 °C), and 30 seconds (72°C). The GAPDH as a housekeeping standard gene and NF-κB primer sequences are as follows:

GAPDH	F	TTGATGGCAACAATCTCCAC
	R	CGTCCCGTAGACAAAATGGT
NF-κB	F	GGCCGGAAGACCTATCCTACT
	R	CTACAGACACAGCGCACACT

Statistical analysis

Analysis of histopathological features used the non-parametric Kruskal Wallis statistic with $\alpha = 0.05$ and was followed by the Mann-Whitney test. Statistical analysis for data on IL-6 levels and NF-κB expression used the one-way Analysis of Variance (ANOVA) method with $\alpha = 0.05$, and the Tukey HSD post-hoc test.

Results and Discussion

The histopathological features of the breast cancer were performed on the primary tumor (Figure 1). They were analyzed by calculating the Nottingham score, a scoring system to assess the breast cancer grade based on tubule formation, nuclear polymorphism, and mitotic activity. The Nottingham score 3-5 assigns as well-differentiated, scores 6-7 as moderately differentiated, and scores 8-9 as poorly differentiated [10].

The Nottingham score of the NC group was 0.17±0.41, a highly significant difference (p=0.003) compared to the PC group with a score of 7.00±1.26. The Nottingham score of the PC group is moderately differentiated, while the NC group is assigned as a normal breast. This result means that histopathologically, it was proven that a malignancy was confirmed in the PC group (breast cancer mice model). The histopathological features and the Nottingham score are shown in Figure 2.

The administration of three various doses of *H. scabra* extract reduced the Nottingham Score in the treatment group, T1=6.33±1.03, T2=5.17±0.75, and T3=3.00±0.89 compared to the PC group (7.00±1.26). Even in the T3 group the Nottingham score was reduced by 57% compared to the PC group. The statistical analysis showed that the Nottingham scores of

the T2 (*H. scabra* extract 0.66 g/kg BW) and T3 (0.99 g/kg BW) groups were significantly different compared to the PC group with p=0.026 and p=0.004 but not significantly different in T1 group (*H. scabra* extract 0.33 g/kg BW) with p= 0.238.

Based on the Nottingham score, the PC group was assigned as moderately differentiated, while the T2 and T3 groups were assigned as well differentiated. This histopathological analysis proved that there had been an improvement in the histopathological features of breast cancer tissue due to *H. scabra* extract administration. This study showed that administration of *H. scabra* extract 0.66 and 0.99 g/kg BW improves the histopathological features of breast cancer.

The ELISA method has been used to measure IL-6 concentration levels in blood serum, and the results are shown in Figure 3.

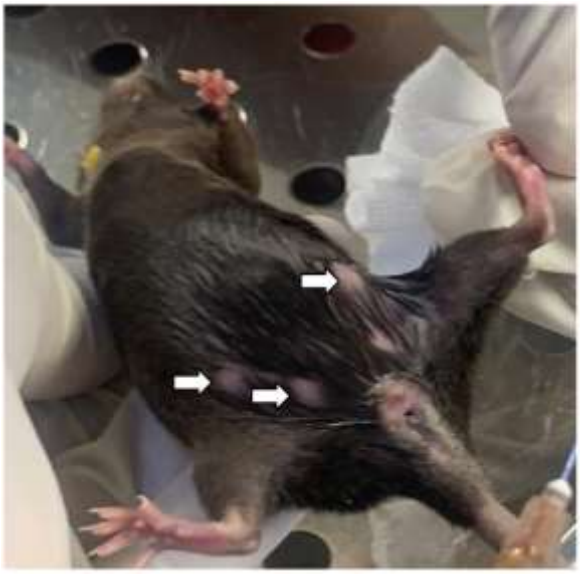

Figure 1. Primary tumor in mammary mice nodules (arrows)

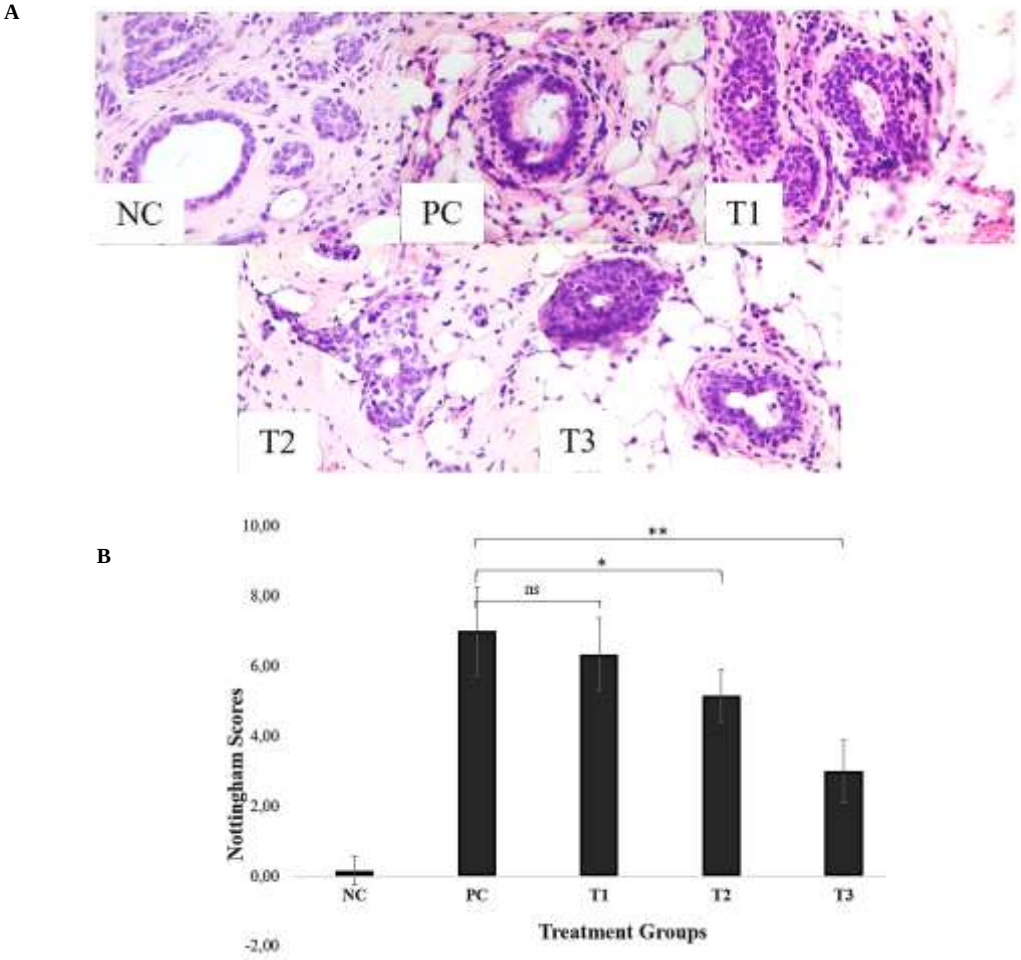

Figure 2. Histopathological features (A) and the Nottingham score of the treatment groups; ns: non-significant; *: p<0.05; **: p<0.01) (B) NC: negative control, normal diet; PC: positive control, high-fat diet + DMBA (7, 12-dimethylbenz[a]anthracene); T1: high fat diet + DMBA + HME (*Holothuria scabra* methanol extract) 0.33 g/kg BW; T2: high fat diet + DMBA + HME 0.66 g/kg BW; T3: high fat diet + DMBA + HME 0.99 g/kg BW

This study showed that IL-6 concentration in the T1 (207.44±26.94 pg/mL), T2 (70.07±38.09 pg/mL), and T3 (68.52±16.07 pg/mL) were significantly lower compared to the PC group (268.01±15.76 pg/mL) with $p<0.001$. *Holothuria scabra* methanol extract in three various doses decreased the IL-6 concentration in a dose-dependent manner compared to the PC group or breast cancer mice model.

The results align with many studies showing that sea cucumber compounds reduce IL-6 concentration. Previous research states that sea cucumber extract has an anti-inflammatory effect by reducing IL-6 concentration [11]. Methanol extract of sea cucumber *Holothuria edulis* inhibits the production of proinflammatory cytokines such as IL-6. Another in vitro study found that *Holothuria polii* decreased the production of IL-6 in SCp2 cells (mid-pregnancy mouse mammary gland cell line) [12].

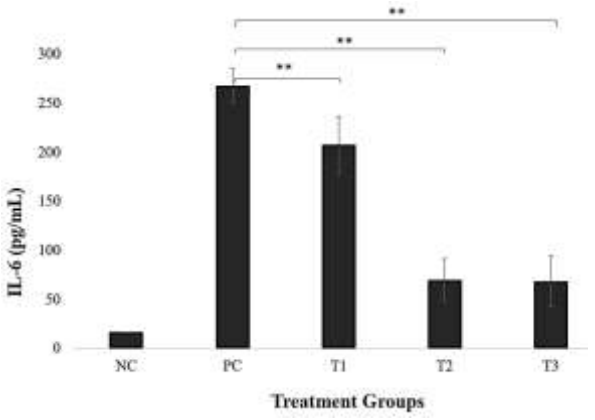

Figure 3. IL-6 concentration in blood of treatment groups (pg/mL); **: $p<0.01$; NC: negative control, normal diet; PC: positive control, high-fat diet + DMBA (7, 12-dimethylbenz[a]anthracene); T1: high fat diet + DMBA + HME (*Holothuria scabra* metanol extract) 0.33 g/kg BW; T2: high fat diet + DMBA + HME 0.66 g/kg BW; T3: high fat diet + DMBA + HME 0.99 g/kg BW

Interleukin 6 (IL-6) is a cytokine formed by glycopeptides (185 amino acids, 25 kDa) and plays a role in breast cancer pathogenesis, which can induce cancer cell proliferation and contribute to tumor invasion and development [13,14]. Increased concentrations of IL-6 are associated with a poor prognosis [15]. The mechanism of IL-6 in the pathogenesis of breast cancer is by activating the Janus-activated kinase (JAK) and signal transducer and activator of transcription (STAT) pathways [16].

The metabolites in sea cucumbers can inhibit cancer cell development and induce cell apoptosis by inhibiting the Janus-activated kinase (JAK) pathway [17]. In breast cancer, IL-6 will activate the Janus kinase pathway through IL-6 binding to the mIL-6Ra receptor; this will cause the phosphorylation of the signal transducer and activator of transcription 3 (STAT3) activation, which causes the entry of STAT3 into the nucleus and so begins the proliferation, invasion, migration, and angiogenesis of cancer cells [18].

Excessive activation of the NF-κB signaling pathway has been recognized in various solid tumors, including breast cancer [19]. That is why we also analyzed the role of *H. scabra* extract on NF-κB expression. In this study, the expression of the NF-κB gene in the PC group (2.82±0.71) was significantly higher ($p<0.05$) compared to the NC group (1.45±0.27); this proved that NF-κB plays a vital role in cancer pathogenesis. Administration of *Holothuria scabra* methanol extract in the treatment groups decreased the expression of NF-κB (T1=2.33±0.24; T2=2.06±0.33; and T3=1.66±0.45) compared to the PC group (2.82±0.71). Statistical analysis revealed that the T2 group (*H. scabra* methanol extract 0.66 g/kg BW) showed significantly lower ($p<0.05$) compared to the PC group and the T3 group (*H. scabra* methanol extract 0.99 g/kg BW) showed highly significant lower ($p<0.001$) compare to the PC group. However, administering *H. scabra* methanol extract 0.33 g/kg BW did not decrease the NF-κB expression compared to the positive control group ($p>0.05$). It means administering *H.scabra* methanol extract 0.66 g/kg BW and 0.99 g/kg BW decreased the NF-κB gene expression in the breast cancer mice model. The expression of NF-κB is shown in Figure 4.

The nuclear factor of kappa B (NF-κB) is a family of heterodimeric transcription factors that play a role in cancer initiation, development, metastasis, and resistance. The NF-κB activity promotes tumor cell proliferation, suppresses apoptosis, and increases angiogenesis, but also induces epithelial-mesenchymal transition, which facilitates distant metastases, and can even stimulate the immune system to support tumor growth. Concerning its vital role in cancer pathogenesis, the NF-κB pathway is an essential target for designing cancer therapy [20,21]. That is why it is necessary to develop therapies targeted against NF-κB.

NF-κB plays a role in cell inflammation, and this is often the beginning of the oncogenesis process, where cells undergo differentiation and proliferation [22]. As in this study, IL-6 concentrations in the breast cancer mice model group differed significantly from the standard mice group ($p<0.001$), and *Holothuria scabra* methanol extract showed reduction in IL-6 concentrations. Inhibition of this IL-6 receptor can be followed by inhibition of NF-κB expression.

Maslikah's study found that the active compound of red betel leaves acts as an inhibitor of the NF-κB activation pathway. It starts with the inhibition of TNFR as a surface receptor, followed by IκK with its kinase activity, and then NF-κB. In this study, *H. scabra* methanol extract inhibited NF-κB expression through IL-6 inhibition and subsequently inhibited IkK phosphorylation, thus inhibiting NF-κB gene expression [23]. *Holothuria scabra* methanol extract could be a promising agent for inhibiting tumor progression.

The results of this study align with several in vitro studies showing that compounds in sea cucumbers reduced NF-κB expression. It has been reported that ds-echinoside A of sea cucumber *Peasonothuria graeffei* decreased NF-κB and VEGF expression in HepG-2 human hepatocellular carcinoma cells analyzed by Western Blot [24].

Frondoside A isolated from *Cucumaria frondosa* and *Cucumaria okhotensis*, psolusoside from *Psolus fabricii*, and stichoposide D from *Stichopus chloronotus* were also reported to inhibit the transcriptional activity of NF-κB induced by the epidermal growth factor in epidermal cancer [25,26]. Park et al. reported that frondoside A has antimetastatic potential for treating breast cancer. They revealed that frondoside A inhibited TPA-induced activation of NF-κB and AP-1 and reduced TPA-induced activation of PI3K/Akt, ERK1/2, and p38 MAPK signals, resulting in reduced expression of MMP-9 [27]. Zhao et al., found that holothurin A1 from *Pearsonothuria graeffei* decreased the NF-κB level in human hepatocellular liver carcinoma (HepG-2) cells, which could be associated with the antimetastatic activity of holothurin A1 [28].

This study examined the effect of the methanol extract of *H. scabra* on the breast cancer mice model, which had not been done in previous studies. In vivo study by Assawasuparek et al., found that methanol extract of *H. scabra* can induce apoptosis and reduce tumor size by 41.12% compared to the control in the cholangiosarcoma rat model [29].

Several in vitro studies have revealed the potential of *H. scabra* as an anticancer agent for various cancers. The holothurin compounds A3 and A4 isolated from the methanol extract of the sea cucumber *H. scabra* are highly toxic to human epidermoid carcinoma (KB) and human hepatocellular carcinoma (HepG-2) cell lines [30].

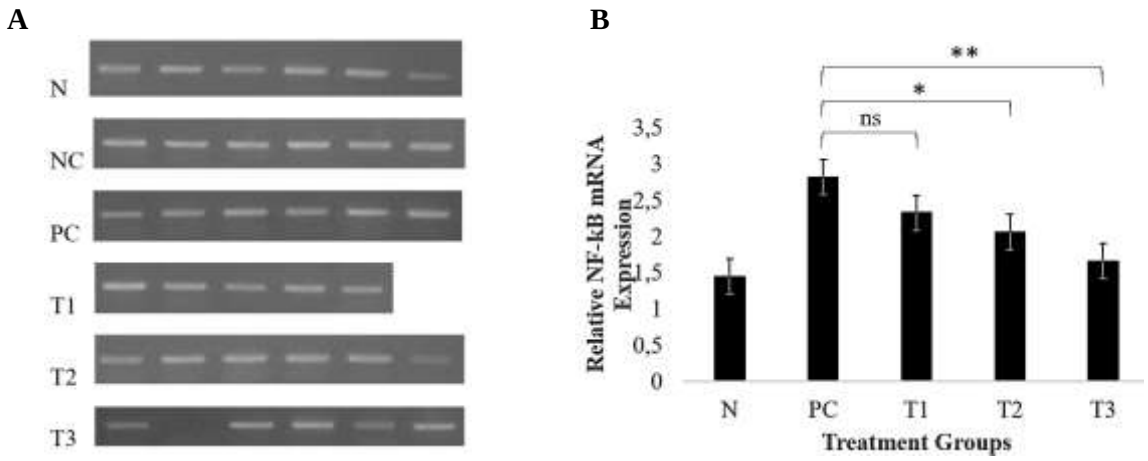

Figure 4. NF-κB gene expression electrophoresis results (A) and NF-κB gene expression measurement result (B) ns:non-significant; *: $p<0.05$; **: $p<0.01$; NC: negative control, normal diet; PC: positive control, high-fat diet + DMBA (7, 12-dimethylbenz[a]anthracene); T1 = high fat diet + DMBA + HME (*Holothuria scabra* metanol extract) 0.33 g/kg BW; T2 = high fat diet + DMBA + HME 0.66 g/kg BW; T3 = high fat diet + DMBA + HME 0.99 g/kg BW

A previous study showed that triterpene glycosides, namely scabraside A and B in *H. scabra* had antiproliferative and cytotoxic effects on several carcinoma cells, such as leukemic cells (P-388), lung carcinoma cells (A-549), colorectal carcinoma cells (HCT-116), gastric carcinoma cells (MKN28), and human mammary carcinoma cells (MCF-7) [31]. Another study found that ethyl acetate fraction of *H. scabra* extract inhibited the production of nitric oxide and pro-inflammatory cytokines via NF-κB and JNK pathways.In their subsequent research publication, they stated that *H. scabra* methanol extract induced apoptosis and suppressed metastasis of human prostate cancer cells by accumulating intracellular ROS resulting in the upregulation of JNK and p38 signaling pathways [32]. Another study predicted that the scabracide and holothurinoside G compounds contained in *H. scabra* have the highest potential as antioxidants and anti-inflammatory agents [33].

An in silico study using garlic compounds as potential anticancer agents showed that tiogenin from the saponin group and lupeol from the terpenoid group were predicted to inhibit the proliferation, migration, and survival of cancer cells by inhibiting the signaling pathways CCR5 and CXCR4 [34]. Therefore, the bioactive compounds from *Holothuria scabra* (holothurin A and B), which belong to the class of saponins or triterpene glycosides, might also act as potential anticancer agents through inhibition of IL-6 and NF-κB.

This research is expected to complement previous studies to reveal the effect of methanol extract of sea cucumber *Holothuria scabra* as an anticancer, especially against breast cancer.

Conclusion

The study concludes that the sea cucumber *Holothuria scabra* methanol extract of 0.33 g/kg BW, 0.66 g/kg BW, and 0.99 g/kg BW reduced the IL-6 concentration in the breast cancer mice model. In comparison, the doses of 0.66 g/kg BW and 0.99 g/kg BW improved the histopathological features of mice's mammary glands and decreased the NF-κB gene expressions in mice models of breast cancer. It can be considered that the sea cucumber *Holothuria scabra* methanol extract is a therapeutic candidate for breast cancer through suppression of IL-6 concentration and NF-κB gene expression.

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Author contributions

Hana Ratnawati designed and assisted in conducting the experiments, performed the statistical analysis and data visualization and wrote the manuscript; Teresa Liliana Wargasetia assisted and conducted all of the experiments and wrote the manuscript; Laella Kinghua Liana, Larissa, and Erica Valencia assisted in some parts of the experiments. All authors have read and approved of the final manuscript.

Declaration of interest

The authors declare that there is no conflict of interest. The authors alone are responsible for the accuracy and integrity of the paper content.

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Abbreviations

ANOVA: analysis of variance; DHEA: 24-dehydroechinoside A; DMBA: 7,12-dimethylbenz[a]anthracene; ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HME: *Holothuria scabra* methanol extract; HPF: high-power fields; IL-6: interleukin-6; JAK: janus-activated kinase; NF-κB: nuclear factor kappa B; qRT-PCR: quantitative reverse transcription polymerase chain reaction; STAT3: signal transducer and activator of transcription 3