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Original Research Article

Sea Cucumber Reduces Breast Cancer Tumor Size Through Inhibition of MMP9 Dependent HER2 Expression in Breast Cancer Mice Model

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ABSTRACT

Breast cancer is one of the most common types of cancer affecting Indonesian women, with more than 80% of cases being found at an advanced stage with distant metastases. HER2-positive breast cancer is an aggressive subtype of metastatic breast cancer. HER2 is an oncogene responsible for cell growth but also associated with tumor invasion by activating the MMP9 promoter. This study aims to determine the role of sea cucumber *Holothuria scabra* methanol extract (HSE) in reducing tumor size by inhibiting HER2 and MMP9 overexpression in a breast cancer mice model. Thirty female C57BL6 mice were divided into five groups (n=6), NC (negative control), PC (positive control/breast cancer mice model), and T1, T2, and T3 as treatment groups. The PC, T1, T2, and T3 were given a high-fat diet and DMBA 1 mg/kgBW by subcutaneous injection. Administration of HSE 0.33, 0.66, and 0.99 g/kgBW for 12 weeks to the treatment groups. The results showed a highly significant difference in tumor size between PC compared to T1, T2, and T3 groups ($p<0.001$), the tumor size reduced in a dose-dependent manner by 25.98%, 32.83%, and 43.30% compared to the PC group. Administration of HSE 0.66 and 0.99 g/kgBW significantly decreased HER2 and MMP9 expression, and there is a positive high correlation ($R2=0.7707$) between HER2 and MMP9 expression. It can be concluded that the administration of HSE decreases the overexpression of HER2 and MMP9. Therefore, it can be used as a therapeutic candidate against breast cancer. The MMP9 expression partly depends on HER2 activity.

***Keywords*:** breast cancer, sea cucumber, *Holothuria scabra*, tumor size, HER2, MMP9.

Introduction

Breast cancer is one of the most common types of cancer in Indonesian women, and it is estimated that in 2018, the incidence of breast cancer was 12/100,000 women, with a high mortality rate of 27/100,000. In Indonesia, more than 80% of breast cancer cases are found to be at an advanced stage and have spread to other organs.[1] Nearly 90% of cancer patients die not from their primary cancer but from distant metastases. The metastasis form of the disease remains the leading cause of death in the majority of breast cancer patients. The 5-year survival rate in breast cancer without metastasis is 99%, whereas when distant metastasis has occurred, the rate drops significantly to 26%. The occurrence of cancer metastases is caused by the occurrence of angiogenesis and invasion of cancer cells into surrounding tissues or to other organs far from primary cancer.[2,3] Metastasis is a gradual process, starting with primary cell tumors undergoing clonal expansion, growth, diversification, and angiogenesis. During clonal expansion, tumor cells will adhere and penetrate the basalis membrane, then through the extracellular matrix and undergo intravasation.[4] Several factors play a role in this metastasis, including Matrix Metalloproteinase-9 (MMP-9).

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Overexpression of MMP9 is associated with cancer cell aggressiveness and poor prognosis in breast cancer. It is noticed that MMP-9 has a direct correlation with poor prognosis in breast tumors.[5] Yousef (2014) stated that increased MMP-9 levels were mainly found in triple-negative and HER2-positive breast cancer, the most aggressive subtypes of breast cancer with poor histopathological features and clinically proven in metastatic breast cancer with a poor prognosis. It is also argued that MMP-9 is a prognostic biomarker for high-stage breast cancer.[6]

Human epidermal growth factor receptor-2 (HER2), also known as ErbB2 or neu, a 185-kDa transmembrane glycoprotein receptor tyrosine kinase, located on chromosome 17q21, is part of the epidermal growth factor receptor (EGFR/ErbB/HER1) family.[7] HER2 is an oncogene which is involved in the signal transduction pathways, responsible for cell growth and differentiation. HER2 overexpression occurs in approximately 20-30% of breast cancers and is associated with excessive cell proliferation, apoptosis inhibition and often shows a poorer prognosis, known as HER2-positive breast cancer.[8,9]

Breast cancer with MMP9 and HER2 overexpression has verified more aggressive tumor growth and poorer prognosis.[6,7] Fatunmbi (2012) showed that *in vitro* studies on human mammary epithelial cells and several human breast cancer cell lines found that exposure to MMP-9 significantly increased HER2/neu expression, and inhibition of MMP-9 caused a decrease in HER2/neu expression. This study suggests that MMP9 regulates HER2/neu gene expression at the molecular level.[10] On the contrary, Shan's (2015) *in vitro* study in the human gastric cancer cell line found that HER2 overexpression is not only closely related to tumor growth but is also associated with tumor invasion through the activation of the MMP9 promoter. It can be said that MMP9 expression depends on HER2 activity.[11] Given the role of HER2 and MMP9 in the pathogenesis of breast cancer and as a factor that exacerbates prognosis, inhibition of MMP9 and HER2 is a critical therapeutic approach for combating breast cancer progression.

In developing countries, the high cost of cancer treatment makes people look for alternative or complementary treatments. Therefore, research is needed on natural ingredients that have the potential as anti-cancer by utilizing the culture of the local community regarding traditional medicine. Engel (2014) states that medicinal plants can be a promising source of anticancer agents. Her research succeeded in finding endemic plants from southern Nigeria that have potential as anticancer agents in positive estrogen receptor breast cancer.[12] In this present study we intend to raise traditional medicine that comes from natural resources from East Java coastal, Indonesia, namely sea cucumbers.

Sea cucumbers are soft-bodied marine invertebrates from the class Holothuroidea that have long been used as food and traditional medicine, especially in Asian countries. A comprehensive survey of pharmacologic activity by the U.S. National Cancer Institute found that 4% of the marine species (mainly animals) examined contained anti-tumor compounds.[13,14]

The sea cucumber species used in our study is *Holothuria scabra*, collected from South Malang coastal, Indonesia. Based on our previous *in silico* studies, it has been known that the active compound content from *Holothuria scabra* extracts has three main components, namely holothurin A, holothurin B, and holothurin B3 which play an essential role in the process of apoptosis, cell cycle, and suppressing tumor.[15]

Many *in vitro* studies proved that holothurin, as the anticancer compound of sea cucumber glycoside, have cytotoxic activity against human cancer cell line, such as suppressing the cell adhesion, migration, and invasion of human hepatocellular carcinoma cells (HEPG2),[16] suppressed the proliferation of glioma cells,[17] human colon cancer cell, leukemia cell line, human cervical cancer cell line, and many other cancer cell lines.[18,19] The triterpene glycosides, namely holothurin A3 and A4, which were isolated from the methanol extract of the *Holothuria scabra*, were found to be strongly cytotoxic in human epidermoid carcinoma and human hepatocellular carcinoma.[20]

Thus, we are interested in determining the effect of sea cucumbers on decreasing breast cancer tumor size through MMP-9 and HER2 inhibition in a breast cancer mice model. We also analyze the correlation between HER2 and MMP9 expression to prove whether HER2 regulates MMP9 activity or vice versa.

Materials and Methods

Sea cucumber (Holothuria scabra) extraction

Fresh sea cucumber *Holothuria scabra* samples were collected from South Malang coastal, East Java, Indonesia, on May, 2019. The identification of sea cucumber was done based on the Food and Agriculture Organization of The United Nations Species Catalog for Fishery Purposes.[21] The bioactive compound *Holothuria scabra* was obtained by soaking the slices of sea cucumber in methanol overnight. The solvent was filtered using Whatman number one filter paper, and the filtrate was concentrated in a rotary evaporator.[22]

Breast cancer mice model dan group treatments

Thirty female Mus musculus C57BL6, aged 10-11 weeks, adapted for seven days, were randomly divided into five groups (n=6). Mice without treatment and fed with standard diet as a negative control (NC) group; breast cancer mice model as a positive control (PC) group; treatment group I, II, and III (T1, T2, T3) were breast cancer mice models treated with *Holothuria scabra* methanol extract with three various doses.

The DMBA (7,12-Dimethylbenz[a]anthracene)-induced breast cancer mice model was performed on PC, T1, T2, and T3 groups, fed twice daily with a high-fat diet (57% fat) and water *ad libitum* from day 1 until day 21. On the 22nd day, induced with 1 mg/kg BW DMBA in sesame oil, given ten times every two days by subcutaneous injection of 0.5 mL on the right breast.

This study was performed in accordance with all international guidelines and regulations. Ethical approval was obtained from the Research Ethics Committee, Faculty of Medicine, Maranatha Christian University, under reference number 020/KEP/III/2022.

Holothuria scabra methanol extract (HSE) administration and tumor size measurement.

Group T1, T2, and T3 are breast cancer mice models given treatment with three doses of *Holothuria scabra* methanol extract (HSE) 0.33, 0.66, and 0.99 g/kg BW per oral, once daily, for 12 weeks. *Holothuria scabra* methanol extract was given since the 22nd day, simultaneously with the first DMBA injection. All treatment and PC groups were given a high fat diet twice daily and drank water *ad libitum*.

After 12 weeks of treatment, breast area that had been injected with DMBA was measured using a vernier caliper, and the circular area of the tumor was calculated using the formula for the area of a circle (π x r^2). The tumor circular area was compared between treatment groups. Then the mice were sacrificed by ketamine injection, and cervical dislocation and the tumor was removed. Histological preparations of the breast tumor were made to confirmed that breast cancer had occurred.

Analysis of HER2 and MMP-9 expression by qRT-PCR

The qRT-PCR technique was used to detect the relative expression of HER2 and MMP9 with GAPDH used as an internal control. Total RNA extraction was carried out using Genezol^{TM} (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's instructions. Reverse-transcribed 2 uL RNA into cDNA using cDNA synthesis kits (RevertAid first strand cDNA synthesis kit, ThermoScientific^{TM}), then analysis of cDNA using one step qRT-PCR kit (Thunderbird SYBR qPCR Mix, Toyobo). The reactions were carried out with 2 µL cDNA template in a total volume of 25 µL, containing 3 µL cDNA (60 ng) as a template, 0.5 µL SYBR Green Master Mix, 0.6 µL forward primer, 0.6 µL reversed primer and PCR water, then running on the thermocycler machine, with the setting as follows: initial denaturation at 94 °C for 2 min, then 30 cycles of denaturation at 94°C for 10 s and annealing at 60°C for 20 s, followed by extension at 72°C for 1 min 10 s then hold in 4°C. These reactions were carried out in triplicate for each sample. The primer sequences showed in Table 1.

Statistical analysis

All the data were analyzed using the Statistical Package of Social Science (SPSS ver. 25.0). Significant difference between treatment groups were determined by one-way ANOVA test and continued with post-hoc Tukey HSD for normal data and determined bu Kruskal Wallis continued with Mann-Whitney test for abnormal data. Differences between treatment groups at a p value $\leq$ 0.05 were considered to be statistically significant. Pearson correlation coefficient was used to analyze the correlation and the strength of the relationship between HER2 and MMP9 expression and were determined based on the Table 2.

Table 1: Primer sequences

Primer	Forward	Reverse	
MMP9[35]	5-CCTTCCTTATCGCCGACAAG-3	5-TGAACAGCAGCATCTTCCCC-3	225 bp
HER2[36]	5-CCAGCCCTCTGACGTCCAT-3	5-TCCGTTTCCTGCAGCAGTCTCC-3	142 bp
GAPDH[35]	5-GACCTGCCGTCTAGAAAAAC-3	5-TTGAAGTCAGAGGAGACCAC-3	126

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Table 2: The scale of Pearson's Correlation Coefficient[23]

Scale of Correlation Coefficient	Value
$0< r \leq 0.19$	Very low correlation
$0.2 \leq r \leq 0.39$	Low correlation
$0.4 \leq r \leq 0.59$	Moderate correlation
$0.6 \leq r \leq 0.79$	High correlation
$0.8 \leq r \leq 1.0$	Very high correlation

Result and Discussion

As we all know that cancer is a disease when abnormal cells grow uncontrollably beyond their usual boundaries to invade or spread to other organs. The tumor size can reflect the progressivity of cancer growth. In this study, measurements of the circular area of the tumor in DMBA-induced breasts were carried out. This circular area tumor can describe as the tumor size and the result shown in Figure 1.

Circular area tumor or tumor size in the PC group (breast cancer mice model) was the most prominent (194.64±17.07 mm^2), whereas, in the NC group (normal mice), there was no tumor. Administration of HSE in all doses (0.33, 0.66, and 0.99 g/kg BW) reduced tumor size; in the T1 (144.08±23.45 mm^2), T2 group (130.75±28.01 mm^2), and T3 110.36±11.29 mm^2.

One-way ANOVA statistical analysis was performed, preceded by the Shapiro Wilk normality test. The results showed a highly significant difference in tumor size between PC and T1, T2, and T3 ($p<0.001$). This result proves that the administration of HSE succeeded in reducing tumor size. There were non-significant differences in tumor size between T1 and T2 groups and between T2 and T3 groups ($p>0.05$). If the tumor size in the PC group is considered 100%, Figure 2 shows the percentage regression of tumor size.

HSE administration reduced the tumor size in a dose-dependent manner. In the T1 group (HSE 0.33 g/kg BW), tumor size was reduced by 25.98%; in the T2 group (HSE 0.66 g/kg BW) reduced by 32.83%, and in the T3 group (HSE 0.99 g/kg BW) it was reduced by 43.30% compared to the PC group.

Based on our previous *in silico* study, it is known that the anticancer compound from sea cucumber *Holothuria scabra* isolated from South Malang, Indonesian coastal, contains three types of anticancer compounds, namely holothurin A, holothurin B, and holothurin B3, which play an essential role in the process of apoptosis, cell cycle regulation and, suppressing tumors.[15]

The study by Sajwani (2019) found that Frondoside A, a glycoside from Cucumaria frondosa sea cucumber, inhibits tumor growth and reduces tumor volume by 87% in an athymic mouse model using MDA-MB-23 triple-negative breast cancer cells.[24]

In this study, the tumor size regression could be the result of secondary metabolites activity, holothurin derived from *Holothuria scabra*, which have cytotoxic activity, cell cycle arrest and reduction of tumor growth, and increase the apoptosis, remove the unwanted, old, or injured cells including cancer cells, therefore, an essential mechanism for tumor suppression.[3,25]

To determine the mechanism of tumor growth inhibition in this study, we analyzed the effect of holothurin on the HER2 oncogene, a receptor tyrosine kinase responsible for cell growth and differentiation. HER2 amplification or overexpression can disrupt homeostatic mechanisms, thus potentially causing the formation of aggressive tumor cells.[8,9] The HER2 expression showed in Figure 3.

Figure 3 shows that the NC group (normal mice) also express HER2 at low levels (0.03 ± 0.08) compared to the PC group (breast cancer mice model) with a HER2 expression 1.00±0.08. In normal cells, HER2 regulates critical biological processes, including cell proliferation, differentiation, metabolism, and cell survival, by activating an array of downstream signaling pathways.[8]

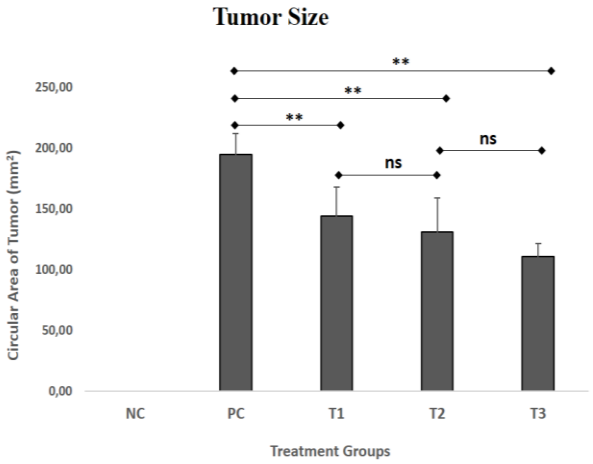

Figure 1: Circular area tumor (mm^2) or tumor size in 5 treatment groups (NC: normal mice; PC: breast cancer mice model; T1, T2, T3: breast cancer mice model treated with various *Holothuria scabra* methanol extract; **: highly significant; ns: non-significant).

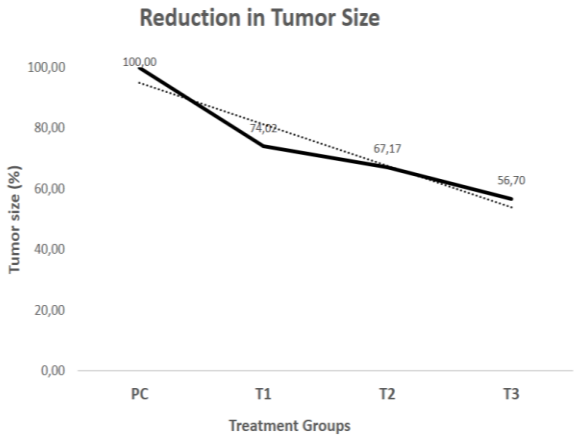

Figure 2: The HSE treatment decrease the tumor size in dose-dependent manner.

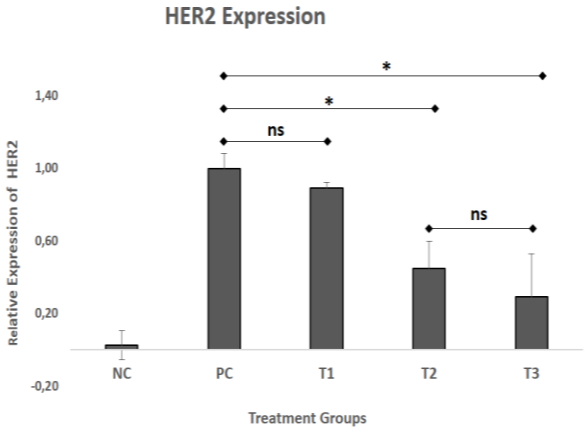

Figure 3: HER2 Expression (ns: non-significant; *: significant)

Administration of HSE reduced the HER2 expression in a dose-dependent manner (T1: 0.89±0.03, T2: 0.45±0.15, and T3: 0.29±0.24). The statistical analysis shows a non-significant difference (p=0.690) in HER2 expression between the PC and T1 groups. It means administration of HSE 0.33 g/kg BW did not decrease the HER2 expression, whereas, the administration of HSE 0.66 and 0.99 g/kg BW, significantly reduced the HER2 expression ($p<0.05$) compared to the PC group.

Based on our previous study regarding the analysis of the active compound of *Holothuria scabra*, using liquid chromatography-mass spectrometry, it was found that the methanol extract of *Holothuria scabra* contains three types of anticancer compounds which are *holothurin* A, *holothurin* B, and *holothurin* B3. Based on our *in silico* study, it was found that those *holothurin* compounds target the BCL2, HDAC1, and PTPN2 protein which play an important roles in the process of apoptosis, cell cycle, and tumor suppression.[15] This present *in vivo* study, revealed that HSE with its active compounds was shown to inhibit HER2, an oncogene that plays a role in cell proliferation and apoptosis inhibition.

In our previous *in silico* study with molecular docking it was also revealed that the methanol extract of *Holothuria scabra* has strong antioxidant activity against NO radicals.[22] Given that free radicals can also initiate carcinogenesis, therefor sea cucumber has the potential to prevent the initiation of carcinogenesis through its role as an antioxidant. Dokunmu study (2021), found that administration of caffeine and artemisin to DMBA-treated albino rat breast cancer induces apoptosis, inhibits angiogenesis and stops the cell cycle. The combined anticancer mechanism of caffeine and artemisinin is due to its role as an antioxidant, thereby inhibiting DNA damage and preventing the initiation of carcinogenesis.[26]

Activation of HER2 receptor begins with the binding of growth factors and hormones, followed by oligomerization and autophosphorylation in the intracellular domain of HER2, subsequently initiating a cascade of phosphorylation and signal transduction to transcription of specific genes, such as the mitogen activated protein kinase (MAPK) and the phosphatidyl inositol 4,5-bisphosphate 3-kinase (PI3K) pathways which are associated with cell cycle progression and survival proliferation. The PI3K/AKT/mTOR pathway is the most frequently enhanced oncogenic pathway in breast cancer, along with the inhibition of PTEN.[27,28] Following ligand activation, cell surface receptors are sent to the lysosome for degradation, recycled to the cell surface, or translocated into the nucleus.[27] Amplification and overexpression of this HER2 oncogene can result in dysregulated cell proliferation of the breast and has been associated with a more aggressive cancer with a poor prognosis.[9,28]

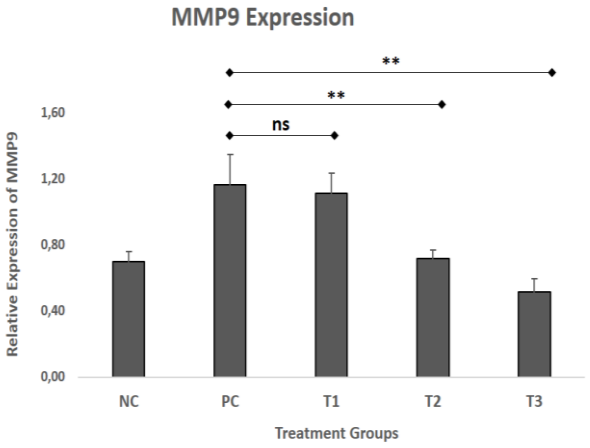

Figure 4: Expression of MMP9 gene

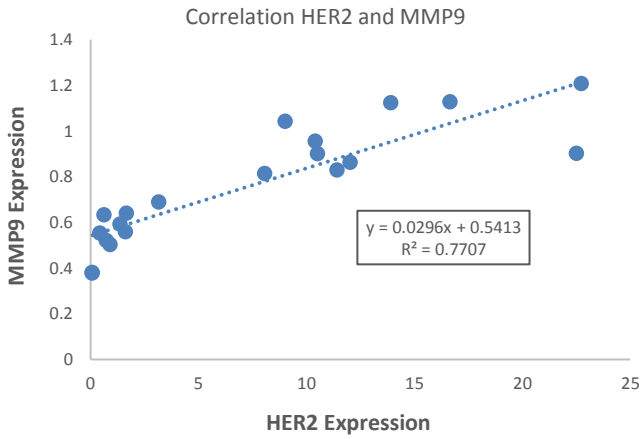

Figure 5: A positive and high correlation between HER2 and MMP9 expression (R^2=0.7707).

Targeted therapies against the tyrosine kinase receptors have been developed, such as trastuzumab, a monoclonal antibody that interacts with the extracellular domain of ErbB2/HER2, causing its downregulation. Moreover, lapatinib and neratinib, a small molecule as antagonists of ErbB2/HER2, are used to treat selected breast cancer patients.[29] However, some HER2 overexpression tumors do not respond or resist the treatment. The factors that cause resistance are, alteration of HER2 structure which can prevents trastuzumab from binding to HER2, dysregulation of downstream signaling effectors such as PIK3CA mutation or PTEN inactivation, and interactions of HER2 with other membrane receptors.[30] Therefore, several studies are needed to identify substances or molecules that can inhibit HER2 amplification. This tyrosine kinase receptor is a promising potential treatment for targeted therapy in HER2 positive breast cancer.

Breast cancer cells that express HER2 are more likely to progress to metastasis.[31] HER2's tumorigenic actions are not limited to its potential proliferative effects. In fact, HER2 has been shown to be a metastatic inducing factor. Overexpression of HER2 and its activation by the ligands has been associated with increased invasion and more metastases. The main mechanism by which HER2 enhances metastatic potential lies in their ability to secrete basement membrane degradative enzymes, such as matrix metalloproteases (MMPs), and changes in tissue structure, facilitating interaction between tumor cells, that are frequently observed in tumor cells with HER2 overexpression.[28] This study also analyzed the effect of HSE on MMP9 expression, the result is shown in Figure 4.

Figure 4 showed that the NC group (normal mice) also expressed MMP9 (0.51±0.04). Cui's (2017) states that MMPs are also expressed in normal cells due to their essential role in tissue remodeling during various physiological processes, such as embryogenesis, morphogenesis, angiogenesis, and wound **repair.**[32]

The expression of the MMP9 gene in the PC group (breast cancer mice model) showed the highest expression (1.000±0.16) and a significant difference compared to the NC group (p=0.009). In the T2 group (HSE 0.66 g/kg BW) and the T3 group (HSE 0.99 g/kg BW), HSE succeeded in reducing MMP9 expression to 0.615±0.05 and 0.475±0.08 and was significantly different (p=0.008) compared to the PC group. Whereas MMP9 expression between PC and T1 group (0.953±0.11) showed no significant difference with p=0.841, only HSE 0.66 and 0.99 g/kg BW reduce the MMP9 expression.

MMP-9 is an endopeptidase enzyme that can increase the degradation of the extracellular matrix and the degradation of type IV collagen, which is the main component of the basement membrane, making it easier for tumor cells to expand into surrounding tissues. Cancer cells, in a paracrine manner, will secrete interleukins, interferons, and growth factors that will trigger the secretion of MMP-9, thereby increasing the metastasis of cancer cells to surrounding tissues and other organs. In addition, HER2 overexpression also increases cell migration, and matrix proteolysis, mainly by inducing MMP and increasing angiogenesis and vascular invasion.[10,33]

That is why elevated MMP-9 expression is associated with metastasis and decreased survival rates of cancer patients.[5]

The holothurin as an active metabolite from sea cucumbers *Holothuria scabra* has been known in several *in vitro* studies on human cancer cell lines to have a cytotoxic effect. It can inhibit migration and invasion of cancer cells, and in this *in vivo* study, it was shown that HSE inhibit MMP9 expression. Therefore, HSE from *Holothuria scabra* can be a suggested cancer therapeutic candidate through the inhibition of MMP9.

According to an *in vitro* study by Shan (2015) on gastric cancer cells, overexpression of the HER2 is not only related to tumor growth but also to tumor invasion. Therefore this study analyzed the correlation between HER2 and MMP9 expression and the result shown in Figure 5.

Pearson correlation analysis showed a positive and high correlation between HER2 and MMP9 expression, with the coefficient correlation (R^2) 0.7707. It means the higher the HER2 expression, the higher the MMP9 expression will be. The regression model is Y=0.0296X+0.5413, which means that the HER2 influenced the MMP9 expression 54,13%, or it can be said that MMP9 expression partly depends on HER2 activity.

Shan *et al.* analyzed the molecular relationship between HER2 and MMP9 at the gene and protein level in relation to the pathogenesis of gastric cancer. The results showed that HER2 knockdown resulted in a downregulation of MMP-9 expression, while HER2 overexpression increased MMP-9 transcription by activating the MMP-9 promoter. Cell invasion activity was almost completely inhibited when MMP-9 was destroyed. In contrast, MMP-9 overexpression partially rescued the invading ability of the cell strain by knockdown of HER2.[11] Other studies have found that MMP-2 and MMP-9 expression in breast cancer appears to be partly related to AP-2 and HER2 expression. Aberrant expression of AP-2 and the HER2 oncogene is associated with disease progression and invasion in breast cancer, which may be partly due to increased MMP activity.[34]

Taken together, our study determined that the *Holothuria scabra* methanol extract reduced the tumor size by inhibiting HER2 and MMP9 overexpression, reflecting its ability as a therapeutic candidate for combating breast cancer progression.

Conclusion

It can be concluded that inhibition of the HER2 and MMP9 overexpression is one of the crucial ways to prevent the progression of breast cancer, and this ability has been proven by the administration of *Holothuria scabra* methanol extract in breast cancer mice model. Therefore, it can be used as a suggested therapeutic candidate against breast cancer by inhibiting HER2 and MMP9 overexpression. This study also revealed a positive and high correlation between HER2 and MMP9 expression, and the MMP9 expression partly depends on the HER2 activity.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

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

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