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MIGRATION INHIBITION ACTIVITY BY METHANOL EXTRACT *Hibiscus tiliaceus* Linn. ON 4T1 BREAST CANCER

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ABSTRACT
The prevalence of breast cancer cases in Indonesia is increasing along with the ability of cancer cells to migrate or move from the primary tumor mass and form new colonies elsewhere. The migration of cancer cells has encouraged the develop anticancer drugs from natural ingredients. Waru leaves have been shown to have cytotoxic activity. This study aims to determine the inhibition activity of migration of breast cancer cells 4T1 from methanol extract of waru leaves. Waru leaves methanol extract was obtained using the maceration method. Cytotoxic test of methanol extract of waru leaves (MEWL) was the migration test used in the scratch wound healing method at concentrations 162.5, 325, and 650 µg/mL at 0, 18, 24, and 42 hours after treatment. Analysis of IC_{50} using linear regression, while large areas were analyzed using Image-J software. The percentage of data closure was analyzed statistically with the Anova Repeated Measure test. All concentrations of Methanol Extract of Waru Leaves had significant inhibition of cell migration ($p<0,05$) compared to control at each observation time at 0, 18, 24, and 42 hours after treatment. So, MEWL is able to inhibit migration in 4T1 cells.

Keywords: Methanol Extract of Waru Leaves (*Hibiscus tiliaceus* Linn.); 4T1 cells; MTT Assay; Scratch wound healing

1. INTRODUCTION

Breast cancer is a crucial and unavoidable health problem for most women. Based on data from the Global Cancer Observatory in 2020, breast cancer cases are ranked first, namely 65.858 cases or 30.8% of the total 213.546 cancer cases that occur in women. The mortality rate for breast cancer is in second rank among all cancer cases (Globocan, 2021). Meanwhile, the incidence of breast cancer is 42.1 per 100.000 population, with an average death rate of 17 per 100.000 population (Kemenkes RI, 2019).

The leading cause of the high death rate in breast cancer is related to the ability of cancer cells to move from their primary tumor mass and form new colonies elsewhere, or cells can metastasize (Sopik & Narod, 2018). The process of cell migration mediates breast cancer cell metastasis (Medeiros & Allan, 2019). The migration of cancer cells has encouraged the development of anticancer drugs from natural ingredients. One part of the potential as an anticancer is waru leaves (*Hibiscus tiliaceus* Linn.), but research on waru leaves is rarely carried out.

Waru leaves (*Hibiscus tiliaceus* Linn.) is a wild plant used in traditional medicine. Waru leaves are widely used in Indonesia as an anti-inflammatory, laxative in urine, sputum, and reduce fever and tonsils (Dalimartha, 2006). Methanol extract from waru leaves contains chemical compounds such as tannins, flavonoids, alkaloids, and saponins (Surahmaida et al., 2020). Based

on studies, methanol extract of waru leaves has a selective cytotoxic effect on MDA-MB-435S breast cancer cells (Uddin et al., 2011). 4T1 cells and MDA-MB-435S cells are categorized into the triple negative Breast Cancer (TNBC) group because they have the same characteristics (Tao et al., 2008; Vuletic et al., 2015). 4T1 cells are cancer cells isolated from the mammary glands of mice (Mus musculus) from the BALB/cfC3H strain, which have characteristics similar to advanced/metastatic breast cancer (BCRJ, 2023). So far, the existing research is only related to cytotoxic activity, so this research was carried out to determine the resulting migration effect. The large number of cases of cancer cell metastasis encourages the importance of research into antimigration. This study aimed to determine the migration inhibition activity of the methanol extract of waru leaves (*Hibiscus tiliaceus* Linn.) against 4T1 cells.

2. METHODS

2.1. Materials

The primary material used is fresh green waru leaves obtained from Kunduran Village, Blora Regency, Central Java. Determination of the waru plant was carried out at the Ecology and Biosystematics Laboratory, Department of Biology, Mathematics and Natural Sciences, Diponegoro University, Semarang. The 4T1 cell test subjects were obtained from the collection of the In Vitro Cell Culture Laboratory, Faculty of Medicine and Health Sciences, Muhammadiyah University, Yogyakarta.

2.2. Extract Preparation of War Leaves

Methanol extract of waru leaves was prepared by maceration method, using methanol solvent with a ratio 1 : 10. As much as 500 gram of waru leaves powder is soaked in 3.750 mL of methanol for three days, stirring occasionally twice a day. After three day of maceration, the extract is filtered, and the dregs are filtered to obtain macerate (filtrate I). and then remaceration is carried out by adding 1.250 mL of methanol to the dregs. The extract is filtered to obtain filtrate II. The macerate was mixed until homogeneous and pure, then filtered to separate the filtrate from the remaining powder. Furthermore, the extraction results were concentrated using a rotary vacuum evaporator at 50 °C.

2.3. Scratch Wound Healing Assay

Cell concentration required for the scratch wound healing method is $7.5x10^4$ cells/well-distributed into 24-well (500 µL/well). Cells were incubated for 24 hours in a CO_2 incubator at 37 °C. Then, scratches are made on the bottom surface of the well using a sterile yellow tip. Media was discarded, and the cell culture was washed with 500 µL PBS (Phosphate Buffer Saline) each well. It is necessary to ensure that the cells are washed completely clean so that there are no cells attached to the scratches and no floating cells. The cells were then given EMDW solution with a concentration of 162.5, 325, and 650 µg/mL as much as 500 µL/well, then incubated in a CO_2 incubator. Control media used empty wells without cells with the addition of DMSO (Dimethyl Sulfoxide) solvent and culture media. Observations were made at 0, 18, 24, and 42 hours after treatment. The search results were documented every observation time with the same magnification microscope and camera. Image-J software measured the initial scratch area with the empty area (CCRC, 2015).

2.4. Data Analysis

The area of cell closure in each treatment group was analyzed using the Microsoft Excel 2013 program to obtain data on the percentage closure ratio for each treatment. The formula for calculating percent closure:

$$\%\ Closure : \frac{(\text{Area t0} - \text{Area tn})}{\text{Area t0}} \times 100\% \quad (1)$$

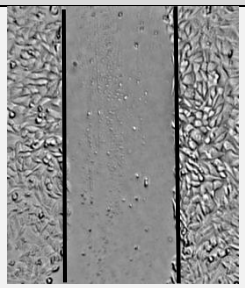
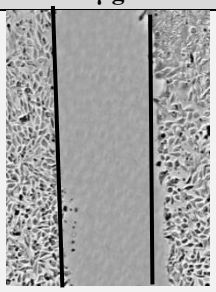
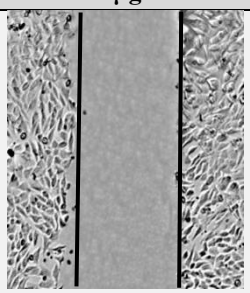
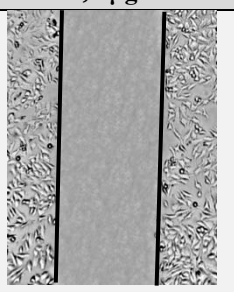
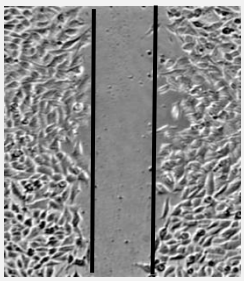
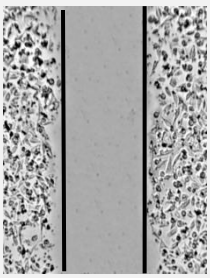
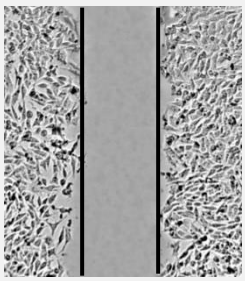
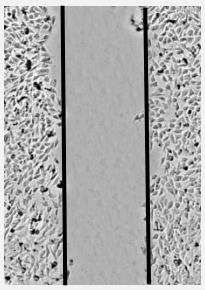
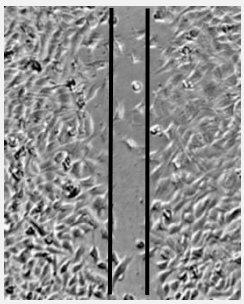
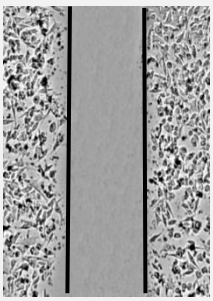
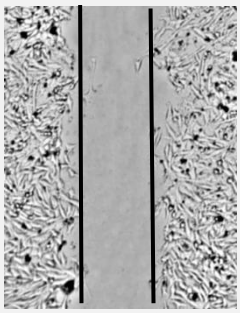
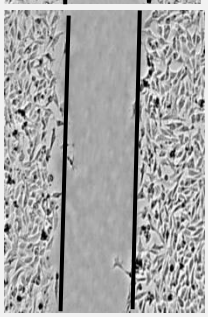
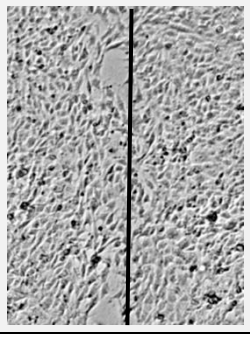
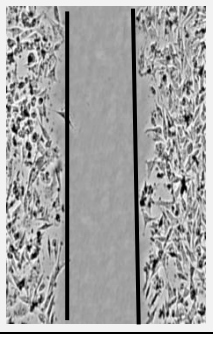
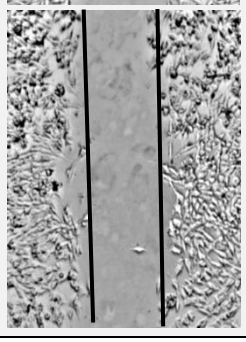
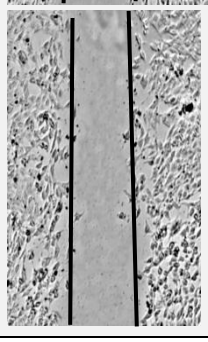
Data on the results of % closure at the 0, 18, 24, and 42 hours were analyzed using the Anova Repeated Measure test. Percent closure is said to have a difference at each observation

time across groups if the value is ($p < 0.05$) (Costantini et al., 2022). The smaller the percentage of cell closure (% closure), the methanol extract of waru leaves has better 4T1 breast cancer cell migration inhibition activity (Seppatria, 2019; Zulharini et al., 2018).

3. RESULTS AND DISCUSSION

Extraction of the active substance from war leaves Simplicia powder using the cold method of extraction, namely maceration. The choice of the maceration method aims to reduce the risk of damage to the active compound content, especially flavonoid compounds, because these compounds are not resistant to high temperatures. Methanol extract of waru leaves (MEWL) obtained a yield of 12.98%. Research on hibiscus leaves extracted using 96% ethanol solvent showed a yield of 10.2% (Hidayati et al., 2022). This shows that a greater yield was obtained using methanol solvent. The concentration of MEWL used for research on inhibiting cell migration with the scratch wound healing method was 162.5, 325, and 650 µg/mL. The observation time was repeated at 0, 18, 24, and 42 hours after treatment. Treatment of 4T1 breast cancer cells with MEWL showed activity in inhibiting cell migration (**Table 1**).

Table 1. Microscopic activity of inhibition cell migration after treatment with MEWL

Time	Control cell	MEWL 650 µg/mL	MEWL 325 µg/mL	MEWL 162,5 µg/mL
0 hours				
18 hours				
24 hours				
42 hours				

Based on the results of migration inhibition (**Table 1**) it can be seen that MEWL had better 4T1 cancer cell migration inhibition activity than control cells at 18, 24 and 42 hours of observation. The activity of inhibiting MEWL cell migration is thought to be due to the presence of flavonoids, alkaloids, tannins, and terpenoids contained in MEWL. Another study stated that the methanol extract of red betel leaves containing flavonoid compounds effectively inhibited the migration of 4T1 cells with 58% cell closure results and no increase in the percentage of cell closure at 18, 24, and 42 hours (Zulharini et al., 2018).

Flavonoid compounds can inhibit cell migration and invasion through the mechanism of significantly suppressing the activity of Matrix Metalloproteinase 9 (MMP-9) by blocking the signaling pathways Protein Kinase C (PKC-α), Extracellular Signal Regulated Kinase (ERK), and Mitogen-Activated Protein Kinase (MAPK), as well as reducing the expression of RhoA, Rac1, and Cdc42 which play an important role in regulating migration activity in cancer cells (Uddin et al., 2011). Other studies have shown that hibiscus leaves contain a class of flavonoid compounds (Vuletic et al., 2015). The quercetin compound is able to suppress cell migration by reducing the expression of Focal Adhesion Kinase (FAK) which is mediated by quercetin compounds (Huang et al., 2018). Quercetin also inhibits TNF-α-induced apoptosis (Chen et al., 2020). Based on the results of calculating the average % closure of the treatment and control groups, there was a significant difference (**Figure 1**).

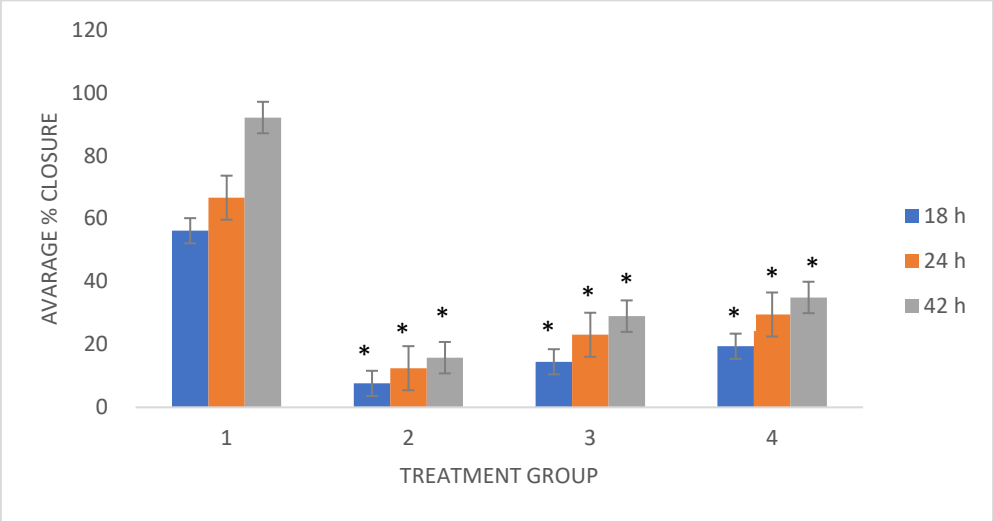


Figure 1. % closure average of migratory cells every after treatment using repeated measures ANOVA statistical test (*p<0,05 has as significant difference to control cells).

The statistical test results for each treatment group using the repeated measurement ANOVA method showed significant differences ($p<0.05$) in the treatment group in inhibiting cell migration compared to control cells at each observation time. While the data on the percentage of cell closure at the 18, 24, and 42 observations of all treatment groups, there was a significant difference ($p <0.05$). So it can be said that MEWL has good cell migration inhibition activity, which is indicated by a small percentage of closure. The smallest percentage of closure was shown at a concentration of 650 µg/mL, which at 42 hours was still able to inhibit cell migration as indicated by the percentage of closure of 16%, while the percentage of closure in the control group was 92%. Similar research regarding antimigration from hibiscus leaf extract using ethanol as a solvent showed that it was able to inhibit the migration of 4T1 cancer cells. Percentage cell closure at 18, 24, and 42 hours with a concentration of 892 µg/mL (Hidayati et al., 2022). These results show that the use of methanol solvent has a smaller concentration in inhibiting 4T1 cell migration compared to the use of ethanol solvent. So, the use of methanol solvent is recommended. In this study, only cell migration was tested, then western blot testing can be carried out to determine the

specific proteins involved in cell migration. But, the IC_{50} in this study still has a fairly large concentration, it may be necessary to isolate the compound from waru leaves to produce maximum effects.

4. CONCLUSION

Methanol extract of waru leaves at concentrations 162,5; 325 and 650 µg/mL showed activity in inhibiting the migration of 4T1 cancer cells so that it could be further developed as an anti-migratory agent.

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6. AUTHOR DECLARATION

Authors' Contributions and Responsibilities

The authors made substantial contributions to the conception and design of the study. The authors took responsibility for data analysis, interpretation, and discussion of results. The authors read and approved the final manuscript.

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Availability of Data and Materials

All data are available from the authors.

Competing Interests

The authors declare no competing interest.

Additional Information

No additional information from the authors.

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