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The Cytotoxic Effects Of Forest Honey (*Apis dorsata*) On T47D Breast Cancer Cells

Malik Hisyam Adnan[1]*, Yanuarita Tursinawati[2], Maya Dian Rakhmawatie[3]

[1,2,3] Faculty of Medicine, Universitas Muhammadiyah Semarang, Semarang, Central Java, Indonesia

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Correspondence:
malikhisyam.unimus@gmail.com

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Abstract

Background: In 2018, an estimated 2 million people had breast cancer. Forest honey (Apis dorsata) can have antioxidant activity due to the presence of flavonoid saponin, alkaloid, and tannin compounds; therefore, it can be used as an anticancer through the induction of apoptosis.

Objective: To determine the IC50 of forest honey (Apis dorsata) on T47D breast cancer cells and see the morphology of T47D cells after administration of forest honey.

Methods: This study is an in vitro quantitative experimental research design with a post-test-only control group design test. The cytotoxic activity was measured by the value of IC50 from forest honey against T47D breast cancer cells using the MTT assay method. The concentration of forest honey was prepared by the two-fold microdilution method in the range of 1000-31.25 µg/mL. Doxorubicin was used as a control drug with a concentration of 20-0.675 µg/mL. The morphology of T47D cells after treatment was observed with an inverted microscope with 400x magnification.

Results: Forest honey (Apis dorsata) from any concentration did not show any inhibition of the growth of T47D breast cancer cells. Meanwhile, doxorubicin had an IC50 of 3.746 µg/mL. The morphology of T47D cells with honey administration showed many live cells with formazan crystals.

Conclusion: Forest honey has no cytotoxic activity against T47D breast cancer cells.

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INTRODUCTION

Breast cancer is the most common malignancy in women. Breast cancer is a malignancy of glandular cells, glandular ducts, and breast-supporting tissues but does not include breast skin.[1] In 2018, it is estimated that people living with breast cancer worldwide will reach 2 million population, which is the second largest number of all cancers.[2] In Indonesia, the incidence of breast cancer in Indonesia in 2018 was 42.1 per 100,000 population, with an average death rate of 17 per 100,000 population.[3]

Currently, one chemotherapy for cancer can use the drug doxorubicin, which is an anthracycline class of antibiotics. Therapy with doxorubicin cannot cause apoptosis in all cancer cells but generally only in solid cancers such as breast cancer. The mechanism of action of doxorubicin against breast cancer is not yet known with certainty. Still, several mechanisms include intercalating into DNA, inhibiting the topoisomerase enzyme, disrupting mitochondrial function, and increasing the production of free radicals and oxidative damage.[4] In addition, chemotherapy with doxorubicin can cause death in other normal cells because of the inability to act selectively.[5] Long-term therapy will also increase the occurrence of drug resistance.[6]

Forest honey (*Apis dorsata*) contains antioxidants in flavonoids, saponins, tannins, and alkaloids that can potentially be anticancer compounds and antiinflammation and affect estrogen activity.[7] The flavonoid content in forest honey is 65.65 mg/kg, and the phenolic content is 352.73 mg/kg, indicating that forest honey has a high antioxidant potential to act as an anticancer agent. Several previous studies that tested the cytotoxic effect of honey include the impact of Tualang honey on MCF-7 and MDA-MB-231 breast cancer cells through mitochondrial membrane depolarization. In this study, tualang honey has the potential as adjuvant therapy for tamoxifen for cell apoptosis, especially MDA-MB-231.[8] The effectiveness of other honey was reported from manuka honey, which was shown to cause apoptosis in MDA-MB-231 and MCF7[9] cell lines. Tualang honey has a selectivity effect. This honey is quite good against cancer cells because it has been shown not to cause apoptosis in normal breast epithelial cell lines MCF-10A.[8] This study aims to determine the IC50 value through the cytotoxic test of the combination of forest honey (*Apis dorsata*) with doxorubicin against T47D breast cancer cells. Half-maximal inhibitory concentration, or IC50, is a quantitative measure that indicates how much of a particular inhibitory substance is needed to inhibit a biological process or biological component by 50%.

METHODS

This research is an in-vitro quantitative experimenttal research design with a post-test-only control group design using the cell culture of breast cancer (T47D) from the cell culture laboratory of Universitas Muhammadiyah Yogyakarta. The forest honey (*Apis dorsata*) is used to produce PT Madu Pramuka, which comes from the Riauforest, and as a comparison using doxorubicin (CKD OTTO). T47D cells ready to be planted on a microplate-96 well with a density of $1x10^4$ cells/well of 100 µL were then incubated for 24 hours using a CO^2 incubator (Thermo Science) at

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37°C. The following process is to replace the RPMI culture media (Gibco) with new RPMI culture media with the addition of forest honey (*Apis dorsata*) solution (1000, 500, 250, 125, 62.5, 31.25 µg/mL) and doxorubicin (20, 10, 5, 2,5, 1.25, and 0.675 µg/mL) with sterile distilled water as much as 10µL/well, furthermore, by re-incubating in a 5% CO^2 incubator for 24 hours at 37°C. The control medium contains RPMI culture media, while the control cell contains only T47D.

The incubation ended with disposing of the culture media, and the intervention was discarded and then washed with PBS (Biogear). Each well-added 100 µL of RPMI culture medium containing MTT solution (Thermo Fisher) with a 5 mg/mL concentration into each well. The microplate was then re-incubated in a 5% CO_2 incubator at 37°C for 4-6 hours. After that, the stopper reagent with trypsin EDTA (Gibco) was given 10% SDS and 0.01 N HCL, which were continued to cover the microplate so that it would not be exposed to light for one night at room temperature. The absorbance can be measured using an ELISA reader (Tecan) with a wavelength of 595 nm.[10] After that, all the wells can observe the morphology of T47D cells using an introverted microscope (Magnus). Calculation of IC50 is obtained from absorbance, which is calculated by the following formula:

$$IC_{50} = \frac{(test\ well\ absorbance - absorbance\ control\ media)}{(control\ cell\ absorbance\ -\ absorbance\ control\ media)}\ X\ 100\%$$

Ethical Clearance was obtained from the Research Ethics Committee Health Faculty of Medicine, University of Muhammadiyah Semarang, with ethical clearance number No. 17/EC/FK/2022.

RESULTS

Based on Figure 1, the calculation of probit regression analysis, the IC50 of doxorubicin against T47D breast cancer cells was 3,746 µg/mL. The linear regression equation in Figure 1 obtained the value of y=-45,981x+76.378 with an R^2 value for the line equation of 0.92, which means that 92% of T47D cells died due to doxorubicin administration. The IC50 value of doxorubicin against T47D cells was highly cytotoxic.

The probit regression analysis of IC_{50} in Forest honey (*Apis dorsata*) against T47D cells could not be calculated. This result is because the linear regression equation in Figure 2 is obtained y=3.144x+97.711. If you look for the value of y=50, it will produce a negative number that cannot be found in the antilog. So, it can be concluded that the IC_{50} of Forest Honey (*Apis dorsata*) against T47D breast cancer cells cannot be calculated or has no cytotoxic potential.

On microscopic observation, differences in cell morphology were seen by giving MTT solution, which would react to form formazan crystals in living cells, causing the color to turn purple. Meanwhile, dead cells did not show formazan crystals and color changes. The color intensity in cells that died with doxorubicin treatment tended to fade and become more assertive in cells that were still alive and were given a solution of forest honey (*Apis dorsata*). The readings on the ELISA reader measure the color intensity of the crystal formation, which is directly proportional to the living cells.

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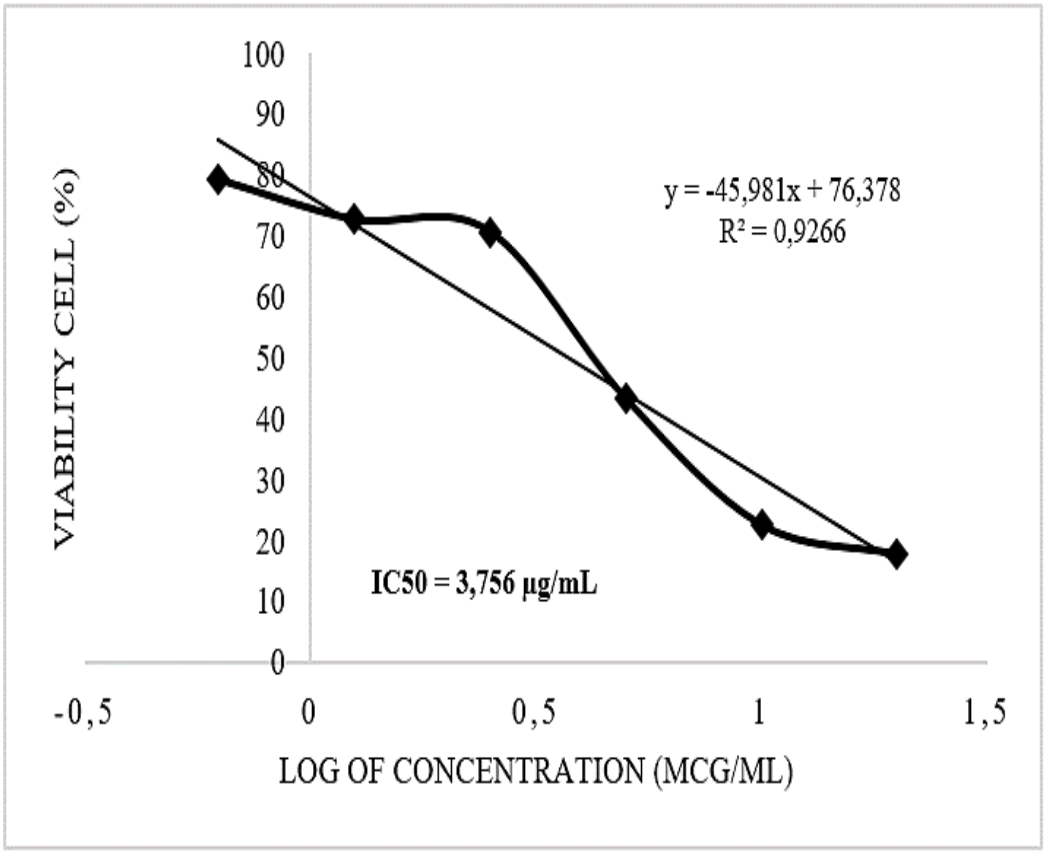

Figure 1. Graph of % viability of T47D cells against the log concentration of Doxorubicin

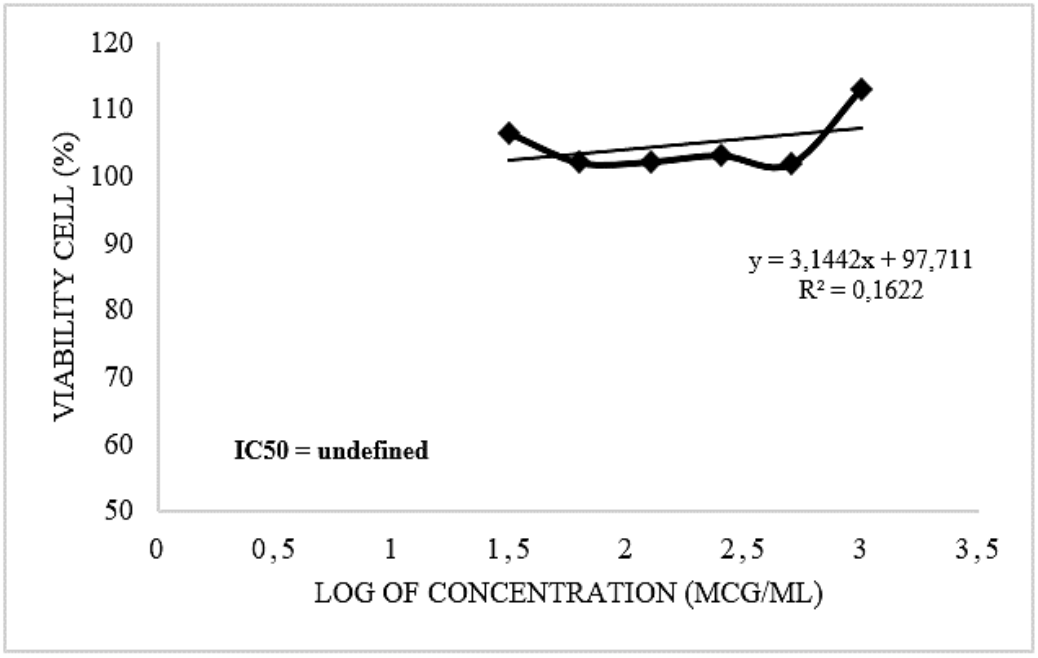

Figure 2. Graph of viability % of T47D cells against the concentration log of forest honey (*Apis dorsata*)

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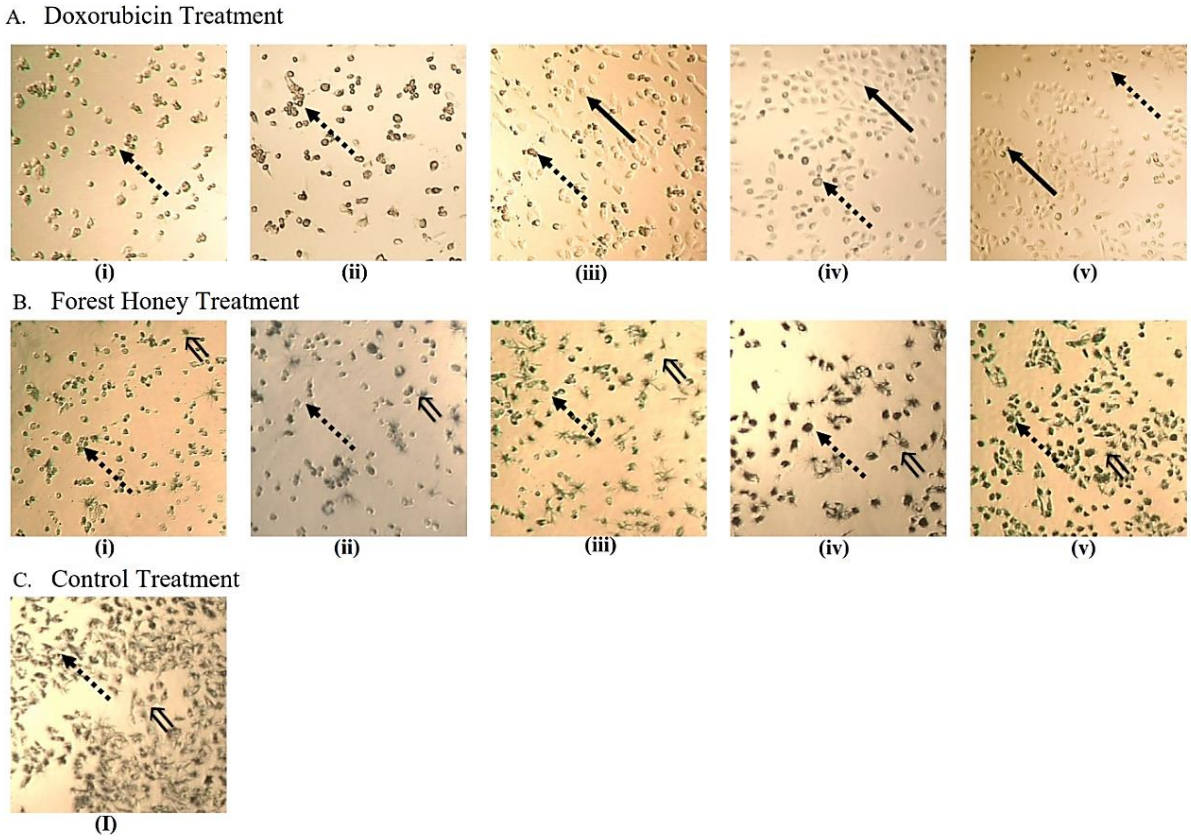

Figure 3. Morphology of T47D cells after the cytotoxic test was performed on an inverted microscope with 400x magnification. **Note:** (A) doxorubicin treatment (i) 20 µg/ml; (ii) 10 µg/ml; (iii) 5 µg/ml; (iv) 2.5 µg/ml; (v) 1.25 µg/ml; (B) forest honey treatment (i) 1000 µg/ml; (ii) 500 µg/ml; (iii) 250 µg/ml; (iv) 125 µg/ml (v) 62.5 µg/ml; (C) control T47D cells. Living cells are marked (→), dead cells (⇢), and formazan crystals are marked (⇒)

DISCUSSION

Doxorubicin, as one of the therapeutic modalities in breast cancer, has been shown to have a cytotoxic effect. This study proved this drug by looking at the impact on T47D breast cancer cells. The IC_{50} value reached 3.746 g/mL or is highly toxic.[11] The mechanism of doxorubicin on T47D breast cancer cell death is related to the induction of apoptosis through two pathways, namely DNA-mediated intercalation by topoisomerase II as well as through the formation of free radicals that will damage cellular membranes, DNA, and proteins.[12] Doxorubicin will affect the DNA replication and transcription process through DNA intercalation. The mediating from topoisomerase II will separate the double strands in DNA to briefly cause cells to stop in the G1 and G2 phases, which causes apoptosis induction. Mitochondrial membrane potential results in the activation of caspase-9, which triggers the process of apoptosis.[13]

Forest honey (*Apis dorsata*) in the cytotoxic test against T47D breast cancer cells could not determine the IC_{50} value, so it does not have toxic properties. The viability of T47D cells at various concentrations was also more than 100%, which indicates the growth of T47D cancer cells in the administration of forest honey (Apis dorsata) compared to the control variable. In addition, morphological observa-

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tions were evidenced by the large amount of formazan crystal formation in T47D cells given forest honey (*Apis dorsata*), which means that many cells were still alive even though the intervention was in the form of forest honey solution (*Apis dorsata*) was given. The influencing factor of this research is the glucose and fructose content in forest honey (*Apis dorsata*). Several studies have shown that the presence of glucose and fructose can trigger the proliferation of various types of cancer, including breast cancer, so that the expected toxic effect is not achieved.[14–16]

The glucose content in forest honey (*Apis dorsata*) is around 28.79mg/dL, while the fructose content is 36.54 mg/dL.7 In a study conducted by Yilin (2017), we compared several breast cancer cells (MDA-MB231, SKBR3, and MCF-7) with 25mmol (450mg/dL) and 5mmol (90mg/dL) glucose, and the difference in cell viability was higher in the 25 mmol glucose treatment compared to 5 mmol glucose. This condition is related to the activation of Epidermal Growth Factor Receptor (EGFR) through GTPases Rac1 and Cdc42, which causes proliferation by accelerating the cell cycle. Cdc42 also plays a role in preventing EGFR degradation mediated by Cbl protein.[14] This result was also shown in a study by Han (2015) using endometrial cancer cells (ECC-1 and Ishikawa cells), which were given an intervention using a low glucose solution of 1 mmol (18 mg/dL), normal glucose five mmol (90 mg/dL), and high glucose 25 mmol (450 mg/dL). The results of this study indicate that cell proliferation will be directly proportional to the level of intervention given.[17] Xiajing research showed that BALB/c mice injected with 4T1/Luciferase cells with fructose administration showed cell progression to metastasize.[16] This result is in line with research by Nakagawa, which showed that MDA-MB-468 breast cancer cells with fructose were more adhesive to endothelial cells and more aggressive in cell migration, thereby accelerating the metastatic process.[15,16]

Morphological observations of T47D breast cancer cells showed differences between the intervention of doxorubicin and forest honey (*Apis dorsata*). At various doses of doxorubicin, many cells were seen dead, indicated by detachment from the cup, and were round and paler in color. In contrast to forest honey, the morphology of breast cancer cells looks more purple with the formation of formazan crystals. Formazan crystals are formed when metabolizing cells produce mitochondrial reductase enzymes, which react with the MTT solution to form purplish formazan crystals. The intensity of the purple color formed is directly proportional to the proportion of living cells. In other words, the number of living cells increases.[18,19]

Even so, it cannot be ruled out that research on honey triggers the process of apoptosis. Tualang honey combined with tamoxifen can induce apoptosis in MCF-7 and MDA-MB-231 breast cancer cells. The mechanism is through the depolarization of the mitochondrial membrane and the activation of caspase-8 and caspase-9, which induces apoptosis.[8] The phenolic and flavonoid content of tualang honey can also be toxic to HeLa cervical cancer cells. And MDA-MB-231 by increasing Reactive Oxygen Species (ROS), which triggers apoptosis. A low dose of manuka honey (0.15-1.25%) in the cell migration test showed significant results in preventing the invasive ability of MDA-MB-231 cells. Manuka honey also

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performs STAT3 and IL-6 blocking mechanisms, which have roles in proliferation, migration, angiogenesis, and invasion.[9]

Apart from the role of glucose and fructose that can increase the proliferation of cancer cells, the antioxidant content in honey also has a role in suppressing the activity of cancer cell growth. Differences in the content of antioxidant compounds and other compositions also affect the benefits of honey. The source of nectar consumed by honey bees, climate, and geography will affect the diversity of composition and antioxidant compounds in honey.[20]

CONCLUSION

Forest honey (*Apis dorsata*) is not toxic to T47D cancer cells, so it is impossible to determine the combination index of the combination of forest honey (*Apis dorsata*) and doxorubicin against T47D breast cancer cells.

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