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Article

Phytochemical Profile and Cervical Anticancer Activity of an In Vitro n-Hexane Extract of Kunto Dewo Fruit *(Kigelia pinnata)* Peel and Flesh

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Abstract: Introduction: Cervical cancer is the fourth highest cancer occurring and causes death in women. Currently, the treatment has a variety of adverse side effects, so it needs alternative treatments that are supportive and with minimal side effects. Kunto Dewo (*Kigelia pinnata*) is herbal plant which can be used as traditional medicine as an anticancer. This plant has antimicrobial and cytotoxic effects on cancer cells. The aim of this study was to determine the phytochemical profile and evaluate the invitro anticancer activity of the n-hexane extract obtained from both the peel and flesh of Kunto Dewo (*Kigelia pinnata*) fruit against cervical cancer HeLa cells. The peel and flesh of *Kigelia pinnata* fruit are macerated in n-hexane solvent then the resulting filtrate is evaporated to become an extract. The extract is used for phytochemical profile, carry out through phytochemical screening, thin layer chromatography, calculation of total phenol, and total flavonoids. The extract was also tested for cytotoxic activity against cervical cancer HeLa cells using MTT assay. The n-hexane extract of the skin and flesh of the *Kigelia pinnata* fruit contains triterpenoids. In TLC analysis, there were found 4 components in the n-hexane extract of *Kigelia pinnata* fruit skin and 8 components in the n-hexane extract of *Kigelia pinnata* fruit flesh. The cytotoxic activity of the n-hexane extract of the skin and flesh of *Kigelia pinnata* fruit is included in the moderately active category. The n-hexane extract of the skin and flesh of the kigelia pinnata fruit has potential as an anti-cervical cancer.

Keywords: n-Hexane extract of *Kigelia pinnata* fruit; Phytochemical profile; in-vitro test; cervical cancer HeLa cell

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1. Introduction

Cervical cancer is cancer that grows in cells in the cervix. This cancer generally develops slowly and only shows symptoms when it has reached an advanced stage. Therefore, it is important to detect cervical cancer early before serious complications arise. Cervical cancer is the fourth most common cancer that occurs and causes death in women. Therefore, adequate management is needed to prevent its development. Management is done based on the severity of cervical cancer itself. The most common ways to do this are lesion therapy (cryotherapy, electrocautery, electrocoagulation diathermy, and laser), conization, radiation, pharmacotherapy, and surgery [1]. However, from the management carried out, there are several side effects that can occur, such as fatigue, nausea, loss of appetite, skin irritation, irregular menstruation, and vaginal pain [2] so alternative treatments are needed that are supportive and have minimal side effects. For this reason, there are many who are looking for *Complementary and Alternative Methods* (CAM) as a treatment

option after a cancer diagnosis. Complementary medicine is a complement to conventional medicine, and alternative medicine is a substitute for conventional medicine. CAM itself consists of non-conventional medical treatments such as diet, massage, acupuncture, and biological products such as herbs [3].

One of the herbal plants that is an anticancer candidate is *Kigelia pinnata* (Kunto Dewo). According to a study, *Kigelia pinnata* has biological activity that has antimicrobial and cytotoxic effects on cancer cells [4]. This research was conducted to identify and analyze organic compounds by means of phytochemical tests, total phenolic content, and total flavonoids in *Kigelia pinnata.* This study also looked at the anticancer activity of *Kigelia pinnata* in vitro on HeLa cervical cancer cells. *Kigelia pinnata*, or the Kunto Dewo plant, comes from the family Bignoniaceae and the genus *Kigelia. Kigelia pinnata* is an endemic plant from Africa. This plant is also known as the sausage tree or *wormwood* [4]. The trunk of this tree is gray, smooth, and peels off over time. The leaves are fully grown near the ends of the branches, with the young leaves being brownish red (Figure 1). Has red flowers that are slightly yellow on the outside; it usually blooms in spring or summer. The fruit is cylindrical in shape, resembling a hanging gray sausage. The fruits range in size from 30 cm to 1 m in length and up to 18 cm in diameter (Figure 2) [5,6]. This plant is widely used to treat various diseases, for example, treating wounds, abscesses, ulcers, stomach aches, syphilis, and rheumatism. In addition, the leaves and branches can be used to treat wounds, dysentery, snake bites, and rheumatism. The fruit can also be used to treat constipation, hemorrhoids, lumbago, dysentery, and *gynaecological disorders* [6].

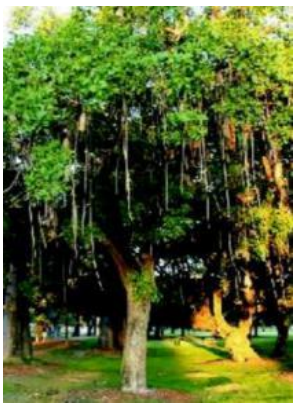

Figure 1. *Kigelia pinnata* tree [5]

The hypothesis in this study is that the n-hexane extract of *Kigelia pinnata* fruit peel and flesh has cervical anticancer activity against HeLa cells. With the general aim of determining the phytochemical profile and in vitro anticancer activity of the n-hexane extract of Kunto Dewo fruit peel and pulp (*Kigelia pinnata*) against HeLa cervical cancer cells. Coupled with the specific objective of conducting a phytochemical profile of the n-hexane extract of the peel and flesh of Kunto Dewo fruit through phytochemical tests, analysis of total phenols and total flavonoids, and to determine the cervical anticancer activity of the n-hexane extract of Kunto Dewo fruit.

Figure 2 *Kigelia pinnata* fruit [5]

In the previous phytochemical study *of Kigelia pinnata,* it was found that many of the secondary metabolites contained were flavonoids, iridoids, limonoids, and steroids. In a study in Bogor, Indonesia, using methanol extract samples from *Kigelia pinnata* fruit, saponins, flavonoids, alkaloids, tannins, triterpenoids, and phenols were found [7]. Differences in test results from each study are influenced by several factors, such as genetic factors, climate, humidity, plant age, temperature, exposure to UV light, and environmental stress [8]. Studies show *Kigelia pinnata* extract seems to have an effect on HeLa cell proliferation. In the methanol extract, inhibition of HeLa cell proliferation reached 81% of the control HeLa cells [6].

HeLa cells are *the cell line* of adenocarcinoma or cervical cancer. HPV-18 DNA was present in HeLa cells after the identification of cervical cancer cases in 1984. There was a BNA HPV 18 genome in HeLa cells with different genome lengths of 7.8 kb and 6.9 kb. HeLa cells are used in cervical cancer research, both in biology research and in diagnosis and treatment. In addition, HeLa cells are also the first human cell line that can grow and divide endlessly in the laboratory. This ability to divide contributes to being the main cell and is commonly used in biomedical and medical research cultures [9].

This research is experimental design to determine the phytochemical screening, total phenols, total flavonoids, and cytotoxic effects of the skin and pulp extract of Kunto Dewo (*Kigelia pinnata*) fruit against cervical cancer cells. The research was conducted at the Chemistry Laboratory, Faculty of Medicine, University of Indonesia. The study was conducted from September 2019 to November 2020. The population for this study was cervical cancer cell cultures found at IMERI FKUI.

The samples used were the n-hexane extract of the skin and flesh of the Kunto Dewo fruit (*Kigelia pinnata*) from Borobudur, Magelang. The sample will be tested for its phytochemical profile, and then an MTT assay test will be performed to test for cytotoxicity at eight different concentrations, with three repetitions for HeLa cells. The concentrations used were 1.56 ppm, 3.12 ppm, 6.25 ppm, 12.5 ppm, 25 ppm, 100 ppm, and 200 ppm, and there was one control group. The number of samples required is 76. Research requires a water heater, vortex, *96-well microplate*, CO_2 incubator, *microplate ELISA reader*, *microplate syringe*, tube, and centrifugation equipment. Using *Kigelia pinnata* extract samples and hexane solvent.

2. Result

2.1 Phytochemical analysis results

In Table 1, the n-hexane extract of *Kigelia pinnata* both peel and flesh contains triterpenoids.

Table 1. Results of the phytochemical analysis of the n-hexane extract of *Kigelia pinnata* peel and flesh

Test Compound	*Kigelia pinnata* peel	*Kigelia pinnata* flesh
Glycoside	-	-
Alcaloid	-	-
Flavonoid	-	-
Tannin	-	-
Saponin	-	-

Triterpenoid/Steroid	Triterpenoid	Triterpenoid

2.2 TLC Test Results

The TLC results of the skin and flesh of the kunto dewo fruit are as shown in Figure 4 and the Rf values can be seen in Table 4. TLC analysis of the n-hexane extract of the peel and pulp of *Kigelia pinnata* used a mixture of Hexane : Ethyl acetate, 10:1 to assess the mobile phase . As seen from table 4, the n-hexane peel extract has 4 components, namely 0.64; 0.70; 0.88; 0.94. Meanwhile, the n-hexane extract of flesh has 8 components, namely 0.48; 0.56; 0.57; 0.64; 0.70; 0.88; 0.91; 0.97.

Peel Flesh

Figure 3. TLC Analysis Results

2.3 Results of the Total Phenol Test and the Total Flavonoid Test

From the results of the total phenol test and the total flavonoid test for the n-Hexane extract of the skin and flesh of the Kunto Dewo fruit, no results were obtained from the spectrometry readings. This happens because the extract used is a non-polar compound, while the total phenol and phytochemical tests are more suitable for calculating polar compounds.

2.4 MTT Assay against HeLa Cervical Cancer Cells

Kigelia pinnata peel and flesh against HeLa cervical cancer cells using the MTT method. Cytotoxicity activity will be seen from the IC_{50} value , which is the concentration of the extract that can inhibit 50% of HeLa cells. From the results of statistical analysis, it was seen that the data distribution was normal ($p>0.05$), so that the one-way ANOVA test could be used. From the one-way ANOVA test, $P = 0.003$ which indicates a significant difference between the extract and the positive control. Then a post-hoc test was carried out.

IC_{50} value of the peel n-hexane extract was higher, 77.36 μg/mL when compared to the flesh n-hexane extract, which was 64.89 μg/mL. For the positive control, the MTT assay uses doxorubicin (DOX) which has an IC value of 50 5.32 μg/mL.

Table 2. IC_{50} value (μg/mL) of *Kigelia pinnata* n-hexane extract against HeLa cervical cancer cells

	PEEL	**FLESH**	**DOX**
U1	53.83	45.47	7.03
U2	96.61	70.31	4.10
U3	81.66	78.89	4.84
Means	77.36	64.89	5.32

Note: U = repetition

The cytotoxic activity of the IC_{50} value of a n-hexane extract of *Kigelia pinnata* peel and flesh against HeLa cervical cancer cells was obtained by substituting the value of y =

50 in the linear equation of log concentration to percent inhibition. The linear equation of three repetitions for the n-hexane extract of *Kigelia pinnata* rind against HeLa cervical cancer cells is U^1, y = 21.68x + 12.474 with R^2 = 0.9363; U^2, y = 23.818x + 2.7224 with R^2 = 0.9259; and U^3, y = 24.018x + 4.079 with R^2 = 0.9523 as shown in Figure 4.

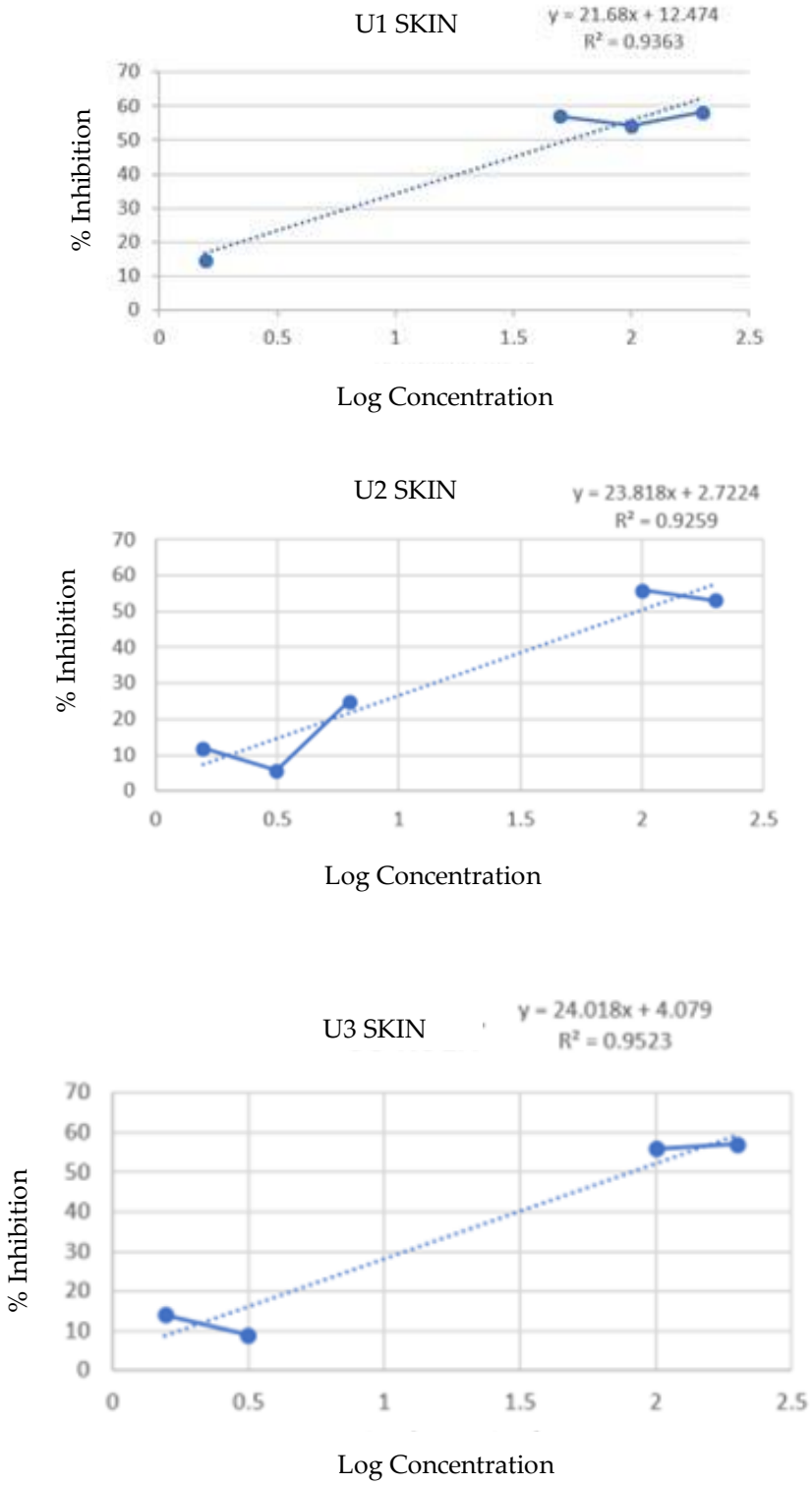

Figure 4. Graph of percent inhibition to log concentration of n-hexane extract of *Kigelia pinnata* peel

The linear equation of three repetitionsfor the n-hexane extract of *Kigelia pinnata* pulp against HeLa cervical cancer cells is U1, y = 18.31x + 19.648 with R^2 = 0.9999; U2, y = 19.92x + 13.214 with R^2 = 0.917; U3 y = 26.243x + 0.2238 with R^2 = 0.9058 as shown in Figure 5.

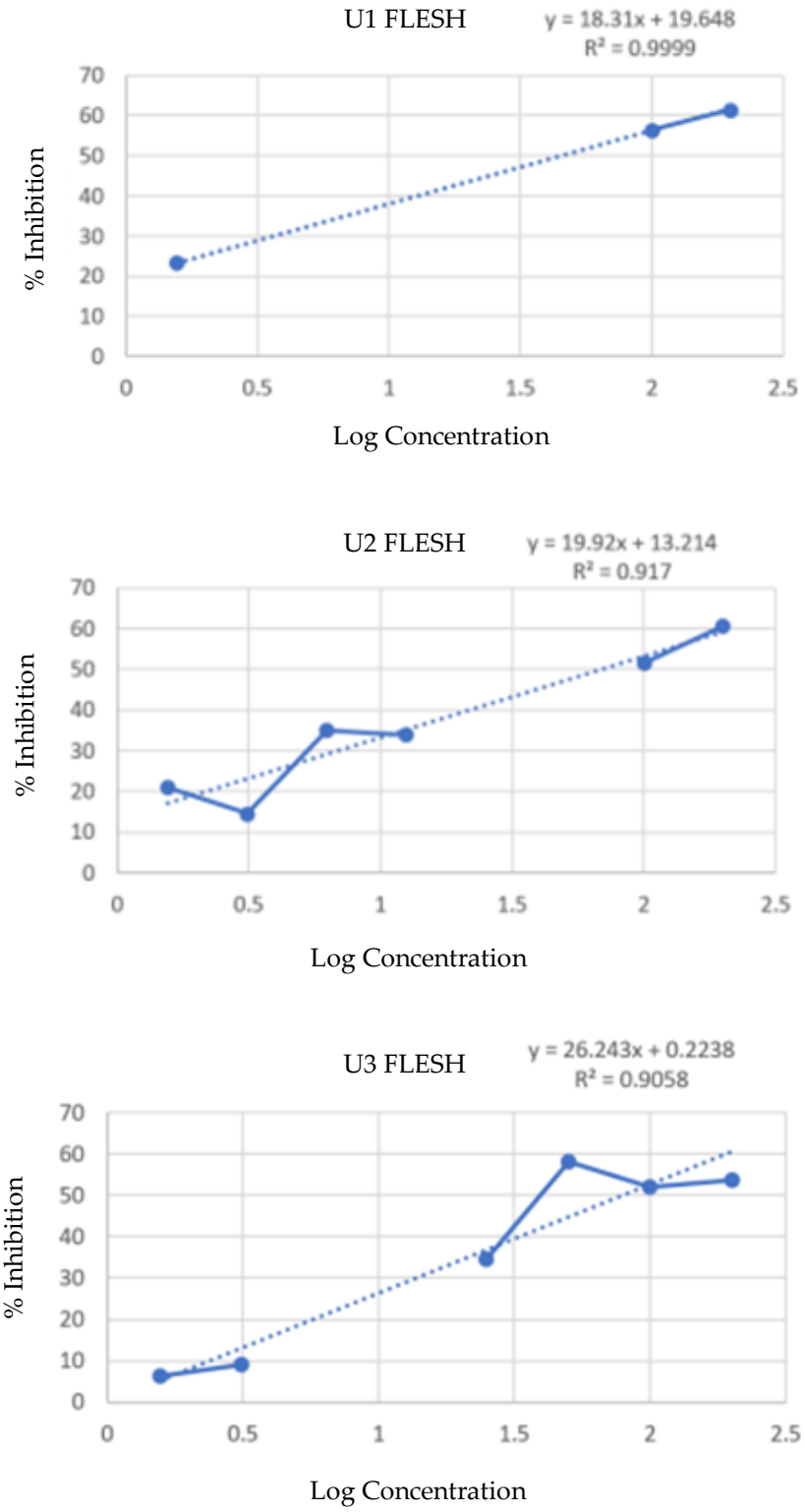

Figure 5. Graph of percent inhibition to log concentration of n-hexane extract of *Kigelia pinnata* flesh.

The linear equation of three repetitions for the positive control of doxorubicin against HeLa cervical cancer cells is U^1, y = 28.688x + 25.711 with R^2 = 0.9409; U^2, y = 16.229x + 40.058 with R^2 = 0.9871; U^3 y = 17.168x + 38.24 with R^2 = 0.9355 as shown in figure 6.

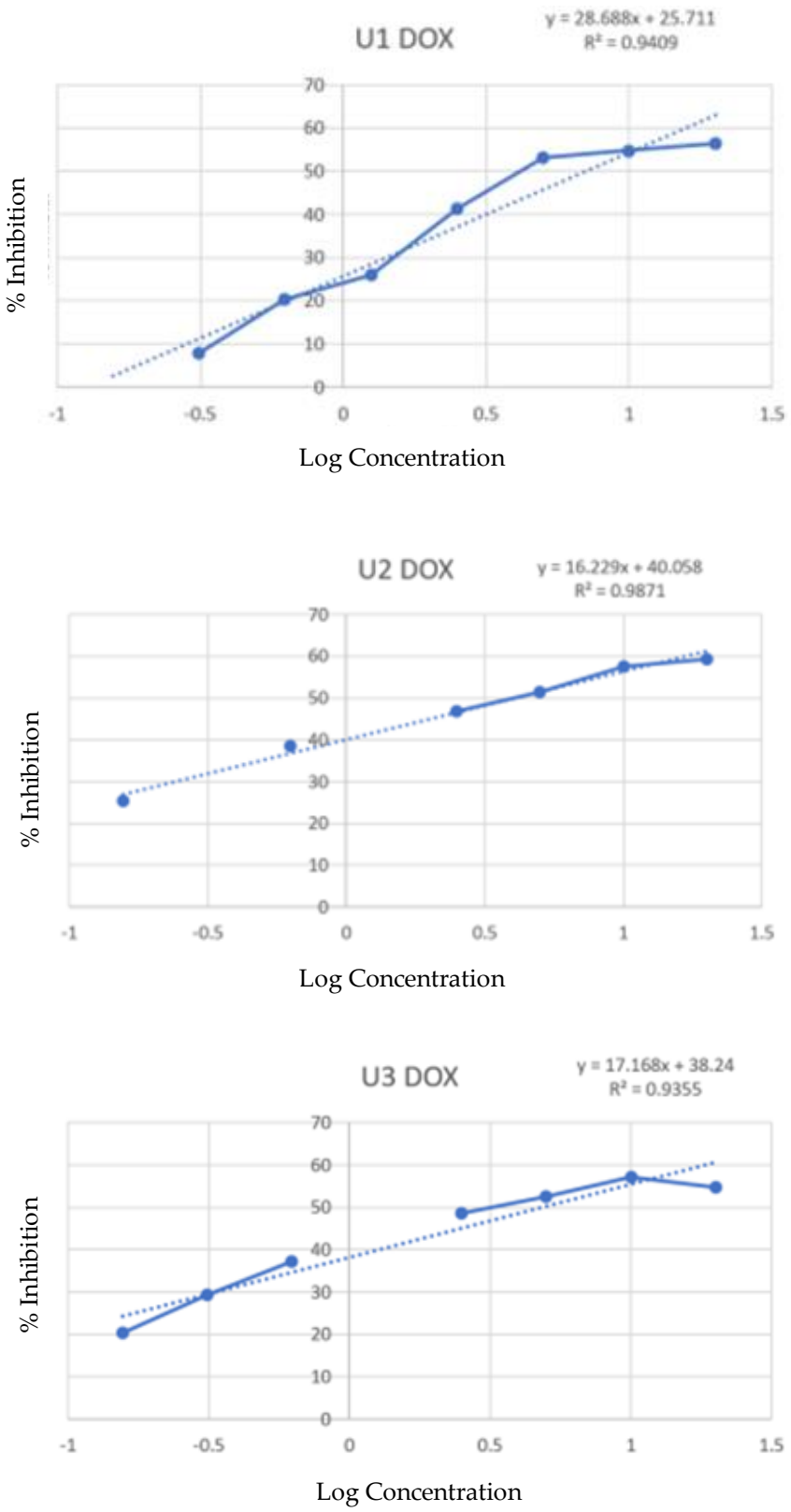

Figure 6. Graph of percent inhibition to log concentration in positive control (dox).

3. Discussion

Phytochemical tests carried out on the n-hexane extract of *Kigelia pinnata* showed the presence of secondary metabolites, as shown in Table 3. The triterpenoids present in the n-hexane extract of *Kigelia pinnata* peel and flesh are metabolites that are common in var-

ious types of plants. In previous studies, triterpenoids have been shown to have anti-inflammatory, hepatoprotective, anti-hypertensive, anti-tumor, and anti-ulcerogenic activities. Mechanisms such as cytotoxicity, inhibition of DNA polymerases, regulation of apoptosis, altered signal transduction, antiproliferative activity, and inhibition of metastases are the potential of extract as an anticancer [10]. In addition, other studies also mention that triterpenoids have activity as immunomodulators, proapoptotic agents, antioxidants, and antibacterials [11], [12], [13].

The percentage inhibition value of the n-hexane extract of *Kigelia pinnata* fruit against HeLa cancer cells can show the ability of inhibition. The n-hexane extract of *Kigelia pinnata* peel had an average percent inhibition of 56.12% at a concentration of 200 μg/ml. The n-hexane extract of *Kigelia pinnata* fruit flesh had an inhibition percentage of 58.57% at a concentration of 200 μg/ml. From the percent inhibition and the log concentration, it forms a linear graph where the R^2 value will be obtained. The R^2 value of n-hexane peel extract was obtained in the range of $R^2 = 0.9259$ to 0.9523. The R^2 value of fruit n-hexane extracts was obtained in the range of $R^2 = 0.9058$ to 0.999. The value of R^2 indicates that there is a directly proportional relationship between the concentration of the extract and the percent inhibition because it is close to 1. *Kigelia pinnata* peel and flesh, as presented in Table 4 and Figure 4, have some of the same components, namely Rf values of 0.64, 0.70, and 0.88.

The cytotoxic activity of the n-hexane extract of *Kigelia pinnata* peel and pulp on HeLa cells was obtained by the MTT method. Cytotoxic activity was obtained from the IC_{50} value, which is the concentration of the extract capable of inhibiting 50% of the growth of HeLa cervical cancer cells. The IC_{50} value of the n-hexane extract of *Kigelia pinnata* peel and flesh was compared to the IC_{50} value of the positive control, namely doxorubicin.

Cytotoxic activity was grouped from the IC_{50} value according to Srisawat et al which modified the categorization from NCI and Geran et al. IC_{50} values are divided into IC_{50} ≤20 μg/ml = very active, IC_{50} 21-200 μg/ml = moderately active, IC_{50} 201-500 μg/ml = less active, and $IC_{50} \geq 501$ μg/ml = not active [14]. Based on this categorization, the n-hexane extract of *Kigelia pinnata* fruit peel and pulp is included in the medium active category.

Based on the results of the same phytochemical test but the large number of different compound components in the TLC test, the effect of cytotoxic activity can be seen from the many components of the TLC test compound. The number of components of the TLC test compound from the n-hexane extract of *Kigelia pinnata* flesh was greater than the n-hexane extract from *Kigelia pinnata* peel, which had an effect on cytotoxic activity. The cytotoxic activity of the n-hexane extract of the flesh was better than the n-hexane extract of *Kigelia pinnata* fruit peel.

From the statistical test results, when the normalization test was carried out with *Shapiro-Wilk,* it was found that $p > 0.05$ was obtained for the peel and flesh extract of *Kigelia Pinnata* fruit and doxorubicin. When *Levene's test* or variance similarity test was carried out, the value of $p = 0.097$ was obtained, and homogeneity was fulfilled ($p > 0.05$). Then proceed with the *one-way* ANOVA test ($P = 0.003$), which indicates a significant difference between the extract and the positive control. Because H0 is rejected or there is a difference, the post hoc test is continued using the Bonferroni test. In the post hoc results, flesh and doxorubicin pbonf = 0.012, while the skin with doxorubicin pbonf = 0.005.

4. Materials and Methods

4.1 Sample Preparation

Kunto Dewo (*Kigelia pinnata) fruit* extract is made by grinding the skin and flesh of the fruit using a blender into powder. Next, maceration is carried out. Powder from the skin and flesh of Kunto Dewo fruit (*Kigelia pinnata)* as much as 500 grams is soaked in 1000mL n-hexane and stirred for 2×24 hours. After soaking it is then filtered with filter paper then the dregs are soaked again. Soaking was done three times so that the powder was filtered perfectly and formed maserate. The maserate was then evaporated at a temperature of 69 °C with a *rotary evaporator* and a speed of 50 rpm. The extract was dried in

an oven at 50 °C. The result of this process was called the n-hexane extract of Kunto Dewo fruit skin and flesh, which would be used for the next test as follows:

4.2 Phytochemical Test, including :

4.2.1 Saponin Test

The extract solution was put into a test tube and then shaken vigorously vertically for 10 seconds. Wait 10 minutes if there is foam, and add 1 drop of 2N HCl into the solution. The presence of froth or foam indicates the presence of saponins.

4.2.2 Flavonoid Test

The extract solution is added to a few drops of concentrated HCl. Then add magnesium powder and amyl alcohol solution and shake. If a reddish, orange, or green color appears in the amyl alcohol fraction, it indicates the presence of flavonoids.

4.2.3 Tannin Test

Extract solution added to 1 mL of 1% $FeCl_3$. If a dark blue or greenish-black color is formed, it indicates a positive tannin.

4.2.4 Triterpenoid Test

The extract solution was evaporated, and then 0.5 ml of anhydrous acetic acid and 0.5 mL of $CHCl_3$ were added. Then give a few drops of H_2SO_4 from the test tube wall. If a purplish-red ring is formed, it indicates the presence of positive triterpenoids, whereas if a greenish ring is formed, it indicates positive steroids.

4.2.5 Glycoside Test

The extract solution is evaporated with a water heater. Then dissolved in 5 ml of anhydrous acetic acid and concentrated H_2SO_4. Next dissolved in 5 ml of anhydrous acetic acid and concentrated H_2SO_4. If there is a greenish-blue ring, then the result is positive.

4.2.5 Alkaloid Test

2 mL of extract was evaporated, then 2 mL of 2N HCl was added. Add 3 drops of Mayer's reagent. If an orange or yellow precipitate is formed, it indicates a positive test for alkaloids.

4.3 Flavonoid Total Test

Total flavonoid content was calculated using the standard curve of quercetin solution. A quercetin solution was prepared by dissolving 10 mg of quercetin in 10 mL of 96% ethanol. The solution was divided into several concentrations, namely 1.56 ppm, 3.12 ppm, 6.25 ppm, 12.5 ppm, 25 ppm, 100 ppm, and 200 ppm. Make an extract solution with 10 mg of extract added to 10 ml of 96% ethanol. Quercetin solution and extract solution were added to 1 mL of 10% AlCl3 and 1M KCH3 COO. Incubate the solution in the dark at room temperature for 30 minutes. Each concentration was measured by absorbance spectrophotometry at a wavelength of 425 nm. The measurement was repeated three times, and the average absorbance value for each concentration was taken, and then a standard curve for quercetin was made as well as a standard curve for total flavonoids. The average value of the absorbance of the extract solution is then entered into the curve equation to assess the total flavonoid content in units of mg QE/mL.

4.4 Total Phenol Test

Gallic acid solution was prepared with 10 mg of gallic acid plus 10 mL of 50% ethanol. The solution was made into eight concentrations, namely 1.56 ppm, 3.12 ppm, 6.25 ppm, 12.5 ppm, 25 ppm, 100 ppm, and 200 ppm. Extract the solution plus 96% ethanol, then take 1 ml of the solution and put it in a test tube. Then, to each concentration of gallic

acid solution and extract solution, 5 mL of *2% Folin-Ciocalteu reagent and* 4 mL of 20% Na_2CO_3 solution were added. Let stand for 40 minutes in a dark room, then read at an absorbance wavelength of 745 nm using a spectrophotometer three times. Take the average value of absorbance for each concentration and form a standard curve for total phenol. The average absorbance of the extract solution is then entered into the curve equation to determine the total phenolic content in mg *gallic acid equivalent* (GAE)/ml.

4.5 TLC test

The purpose of the TLC test was to determine the number of components present in the extract. The materials are the extract, TLC plate, the eluent, and the eluent bottle. Lines are drawn 0.5 cm from each end of the top and bottom sides of the 1 cm-wide TLC plate. The extract that has been dissolved in n-hexane is dripped on the bottom line of the plate with a capillary tube and allowed to dry. Then the plate is put into the eluent bottle containing the eluent, which is a mixture of n-Hexane and ethyl acetate with a ratio of 10:1. The eluent will push the extract drops on the TLC plate to the upper limit. Then the TLC plate was lifted with tweezers, dried, and looked at for the measuring distance (Rf), which was then observed under a UV lamp with a 366 nm wavelength. The value of the retention factor (Rf) is calculated by dividing the distance travelled by the extract to the distance travelled by the eluent (mobile phase).

4.6 In vitro cytotoxicity test against HeLa cells

Cytotoxic test using the MTT assay method. Beginning with knowing the number of HeLa cells in the growth medium. Transfer the HeLa cell and 100 µl of the medium to the micro plate well. Incubate the cells for 24 hours. Then take the plate and wash it with 100 µl of *phosphate buffered saline (PBS).* The sample extract solution was divided into eight concentrations: 1.56 ppm, 3.12 ppm, 6.25 ppm, 12.5 ppm, 25 ppm, 100 ppm, and 200 ppm. The plate filled with cells and then filled with 3 wells of extract solution for each concentration. The plates were then incubated for 24 hours. MTT reagent was added in amounts up to 100 µl with a concentration of 5 mg/mL into each test well, and the control was then incubated for 4 hours. After incubation, the cells were observed under a microscope. If there are purple formazan crystals, add 100 µl of *dimethyl sulfoxide (DMSO)* as a *stopper*. Calculate the absorbance with a spectrophotometer at a wavelength of 540 nm. The absorbance value is used as the optical density (OD) value. OD data is used to calculate the percent inhibition with the following formula:

$$\%\ \boldsymbol{inhibition} = \frac{\boldsymbol{OD\ Control - OD\ Concentrates}}{\boldsymbol{OD\ Control}} \times \mathbf{100}\%$$

After obtaining the percent inhibition, a regression curve was made between the log concentration and the inhibition percentage. Then, an analysis was carried out to obtain an IC value of 50. The IC_{50} value is obtained from 50% inhibition in the linear equation. The IC_{50} value of the extract will be compared with the IC_{50} value of the positive control (doxorubicin).

The data obtained will be processed using the JASP application and Microsoft Excel. Normality test using Shapiro-Wilk. If the results of the data distribution are normal, then use the one-way ANOVA test (a parametric test) using JASP. If the test results are not normal, then proceed with the Kruskal-Wallis test (a nonparametric test). The interpretation of $IC_{50,}$ which is the concentration of the *Kigelia pinnata* extract that inhibits HeLa cells by half or 50%, used linear regression analysis with the Microsoft Excel application.

5. Conclusions

In this study, no quantitative tests were detected, namely total phenols and total flavonoids. Meanwhile the n-hexane extract of ***Kigelia pinnata*** peel and flesh are include in the

medium active categories with an IC value each IC_{50} 77.36 μg/mL (peel) and IC_{50} 64.89 μg/mL (flesh) in HeLa cervical cancer cells.

6. Suggestion

Kigelia pinnata peel and flesh used to observe cytotoxic activity against HeLa cervical cancer cells can be further purified so that other pure compounds can be obtained and for the next tester, recommended to do repeated test to get better results.

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