

Anti-cervical Cancer Test of the Ethyl Acetate Fraction of *Terminalia catappa* Linn Fruit Flesh against HeLa Cells**Moch.Chasani*, Senny Widyaningsih, Undri Rastuti, Andhika Ramadhan, Iji Abdul Aziz**Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Jenderal Soedirman
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ABSTRACT. The ethyl acetate fraction from methanol extract of ketapang (*Terminalia catappa* Linn) fruit flesh has anti-cancer potential. Therefore, this study aims to obtain the active anti-cervical cancer agent from the ethyl acetate fraction of ketapang fruit flesh's methanol extract. The first step carried out was fractionation of the ethyl acetate fraction of methanol extract using gravity column chromatography with an eluent of ethyl acetate: chloroform: glacial acetic acid (1:8:1). The second step was a toxicity test on fractions produced from column chromatography. The third step was the test of secondary metabolite and anti-cancer activity of fraction possessing the highest toxicity to *Artemia salina* Leach. Fractionation of the concentrated ethyl acetate fraction by column chromatography, yielded seven subfractions (F1-F7). Furthermore, the cytotoxic test on *A. salina* Leach shrimp larvae generated the following LC_{50} data from the 1st to 7th subfraction in a row, namely 566.2814 ppm, 87.9077 ppm, 216.6232 ppm, 566.2814 ppm, 560, 6647 ppm, 279.9213 ppm, and 194.6674 ppm. The most active subfraction is fraction 2 which have two groups of positive compounds, i.e. phenolics and saponins were obtained from the secondary metabolite test. Data from the infrared spectroscopy performed showed the presence of groups –OH, C-H aromatic, C=O carbonyl, C=C aromatic, C=C alkene, C-H aliphatic, C-H alkane, and C-O. The value of the anti-cancer activity of fraction 2 was LC_{50} = 165.37 ppm, which was included in the fairly active category as an anti-cervical cancer agent.

Keywords: Cervical cancer, cytotoxic, *Terminalia catappa* Linn**INTRODUCTION**

Neoplasm/tumor indicated increased level in the pattern of diseases causing general death in the world. World Health Organization (WHO) reported that in 2018, an estimated 570 000 women were diagnosed with cervical cancer worldwide and about 311,000 women died from the disease (The Global Cancer Observatory, 2020). In Indonesia, cancer affecting the cervix is the second largest after that of the breast (16.6 %), which reaches cases 9.2 % in 2018 (The Global Cancer Observatory, 2020). Therefore, serious immediate efforts are needed to overcome the described condition. One of these includes the obtainment of anti-cervical cancer compounds from biological natural resources. Indonesia is known to have a numerous variety of biological natural resources that can be explored for their medicinal potential, specifically as anti-cancer drugs. Screening and isolation of bioactive compounds, as well as toxicity (LC_{50}) tests on *A. salina* Leach shrimp larvae, are effective steps in the investigation process. A extract of plant species is believed to have anti-cancer potential once the toxicity test results show an LC_{50} value less than 1000 ppm, while the pure sample with an LC_{50} value less than 10 ppm is also declared as an

active anti-cancer material (Reymon et al., 2021). Ketapang (*T. catappa*) is one of the medicinal plants and phytochemical investigations on parts of the plant have been done intensively (Chole & Ravi, 2020). It has been reported that leaf extracts of ketapang play an important role in the treatment of cervical cancer (Lee et al., 2019). However, fruit flesh of ketapang had not been reported as anticancer drugs especially cervical cancer.

This study was conducted to find the anti-cancer active compounds of the flesh of ketapang fruits. In order to obtain active compounds from fractions that have anti-cancer active potential, purification steps are needed. Some of the methods for purification are gravity column chromatography, recrystallization, preparative thin layer chromatography and several other methods. The purification process using column chromatography requires accurate information about the right solvent in order to obtain good separation results from the compound to be purified. Therefore, a preliminary test using thin layer chromatography is needed. Preliminary test that resulted in the best separation for use as a solvent in column chromatography were a mixture of ethyl acetate: chloroform: glacial acetic acid (1:8:1). The obtained

fractions were then grouped based on their TLC pattern. The groups obtained were tested for toxicity using *A. salina* Leach shrimp larvae. The fraction with the highest toxicity was tested for its anti-cancer activity against cervical cancer.

Cervical carcinoma is one of the most common cancers in women. High-risk human papilloma viruses (HPV) such as HPV18 play an important role in the development of essentially all cases of cervical carcinoma (Ekowati et al., 2010). The present study has assessed the effects of toxicity of fraction on HeLa cell.

EXPERIMENTAL SECTION

Materials

The materials used were ketapang fruit obtained from the Purwokerto area, distilled water, glucose, Dragendorff reagent, vanillin-HCl reagent, silica gel G_{60} , TLC plate G_{254} , *A. salina* Leach shrimp larvae, Dulbecco's modified eagle medium (DMEM), FBS, methanol, *n*-hexane, ethyl acetate, ammonia, I_2 , chloroform (Merck), and acetic acid (Merck).

Fractionation of Ketapang Fruit Flesh Extract by Gravity Column Chromatography

The ethyl acetate fraction of methanol extract of ketapang fruit flesh was mixed with silica gel G_{60} and ground until homogeneous, then air-dried. Afterward, the homogeneous mixture was put into a chromatographic column that has been prepared with silica gel G_{60} as a stationary phase and a ketapang mobile phase with an eluent of ethyl acetate: chloroform: glacial acetic acid (1:8:1). The following step was elution, carried out using a more polar eluent, namely a mixture of ethyl acetate: chloroform: glacial acetic acid at 2:8:1, 3:8:1, 4:8:1, and 5:8:1. The last was a mixture of ethyl acetate: chloroform: glacial acetic acid: acetone at 2:8:1:1, while each eluent mixture used was 250 mL. These fractions were accommodated in test tubes with an eluate amount of 30 mL per tube, each was identified using TLC with ethyl acetate: chloroform: glacial acetic acid at 1:8:1 and UV light at 254 nm and 366 nm. Fractions with the same R_f as identified by TLC were combined into one, followed by evaporation of the eluent using a vacuum rotary evaporator. The combined fractions evaporated with solvents were tested for anti-cancer activity against *A. salina* Leach shrimp larvae.

Cytotoxic Activity Test of Column Chromatography Samples Against Shrimp Larvae *A. salina* Leach (Umri et al., 2019)

The toxicity test was carried out by entering 10 of 48-hour *A. salina* Leach shrimp larvae. Shrimp eggs were placed in a beaker filled with seawater, then illuminated and allowed to hatch for 48 hours. Samples were made of each group of fractions from the results of column chromatography with a concentration of 2000, 200, and 20 ppm. The solvent used was seawater and for extracts that are difficult to dissolve in seawater, 10 μ L of DMSO was added. After

48 hours, 10-15 shrimp larvae and 100 μ L of seawater were put into the test vial, followed by 100 μ L of the extract to ensure final concentrations in the vial were 1000, 100, and 10 ppm. Each concentration was repeated thrice, while the control was prepared without extract addition. After 24 hours, live and dead shrimp larvae were counted and the combined fraction sample from column chromatography which has the highest anti-cancer activity (smallest LC_{50} value) was tested *in vitro* on cervical cancer cells. Cervical cancer tested at the Faculty of Medicine, University of Indonesia.

Anticancer Activity Test Against HeLa Cells (Diani et al., 2015)

HeLa cells were cultured using DMEM media, 10% FBS, 100 g/mL and 100 g/mL streptomycin, then count the initial cell count under microscope. Cells were incubated in an incubator at 37 °C and 5% CO_2 . After that, cells were harvested with the addition of trypsin. The sample of the ethyl acetate fraction that the most active were centrifuged until two layers were formed, namely the precipitate and supernatant. The precipitate obtained will be formed into pellets and added to the media new growth, while the supernatant was discarded. The number of cells that have been fulfilled, the cells were grown on 96 multi-well plates. Every well filled with 5×10^3 cells in 100 μ L of DMEM medium. Cell incubation carried out for 1-2 hours until the cells adhere. A total of 100 μ L sample extract the most active ethyl acetate fraction with various concentrations was added at each wells so that each well contains 200 μ L. Media control is made with a well filled with DMEM media, while control cells were made with wells containing 23 DMEM media and complete cells without extract. Incubation is carried out in an incubator CO_2 5% for 24 hours at 37° C. Cells seen again after 24 hours below microscope. MTT of 10 μ L with a concentration of 5 mg/mL was added to every well. Incubation was carried out again for 4 hours until the formation of formazan. The MTT reaction was stopped by adding 50 μ L of SDS as stop solution, then incubated in a dark room for 12 hours. Formazan was eluted from cells with 150 μ L of DMSO solution. Absorbance read using an ELISA microplate reader at a wavelength of 595 nm. Anticancer activity was determined by the IC_{50} value obtained from the x. value by substituting $y = 50$. This value indicates a decrease in absorbance as much as 50% on the equation of the line $y = mx + b$. The equation of the line obtained a graph of the relationship between % inhibition and sample concentration will be made.

Secondary Metabolite Test of The Fraction with The Highest Anticancer Activity (Patil et al., 2013)

The combined fraction which had the highest anticancer activity was tested for its secondary metabolites was qualitatively tested with color reagents. The secondary metabolite test on the isolates of the highest anticancer active compounds was carried out using a color test on the chromatogram of

the elution results using eluent ethyl acetate: chloroform: glacial acetic acid (1:8:1), except for the saponin test, which was carried out with shaking.

The test procedure for each secondary metabolite is as follows: (1) Alkaloid test: The eluted spots are sprayed with Dragendorff's Reagent. A positive test is indicated by a change in the color of the spot to orange. (2) Terpenoid Test (Lieberman Burchard Test): The eluted spots were detected with I_2 . A positive test is indicated by the formation of a brown color by I_2 vapor. (3) Steroid Test: The eluted spots were sprayed with Liebermann Burchard reagent. positive test marked by a change in the color of the spots to green or blue. (4) Flavonoid Test (Shinoda Test): The eluted spots were detected with ammonia vapor and vanillin-HCl reagent. The presence of flavonoids was indicated by visible spots or changes in the fluorescence of the spots after being given ammonia vapor and purple when sprayed with vanillin-HCl reagent. (5) Saponin Test: A total of 1 mL of the most active fraction was dissolved with 10 mL of distilled water and then shaken vigorously. If a stable foam is formed for not less than 10 minutes as high as 1-10 cm, it indicates the presence of saponin compounds.

RESULTS AND DISCUSSION

Ethyl Acetate Fraction Column Chromatography

The fractionation results of the ketapang fruit extract showed 146 fractions with a TLC profile that

can be seen in **Figure 1**. Fractions 1 to 146 were eluted using a mixture of ethyl acetate: chloroform: glacial acetic acid (1:8:1). Based on **Figure 1**, the obtained fractions were grouped based on the similarity of their TLC profile. Based on the TLC test results, fractions were grouped according to the similarity of the spot patterns formed and up to 7 groups were obtained. Each group was then evaporated with solvent and the respective weight data presented in Table 1 was determined. Based on the yield of data **Table 1**, fraction 3 has the highest yield, followed by fractions 2, 1, 6, 4, 7, 5. **Figure 1** shows that fraction 1 (right side) is the lowest polarity, then followed by fraction 2 to fraction 7, which has the highest polarity.

Anti-cancer Activity Test

The results of the toxicity test with Brine Shrimp Lethality Test (BSLT) method from seven fraction groups showed that the higher the tested extract's concentration, the higher the mortality percentage of *A. salina* Leach shrimp larvae. It is because the concentration of the test extract shows the number of toxic compounds. The mortality rate of larvae was caused by the extract that given against *A. salina* larvae (Rasyid et al., 2020). No dead larvae were found in the control, meaning there is no toxic sample extract in its vial while the tested fraction is toxic, hence, the complete test results can be seen in **Table 2**.

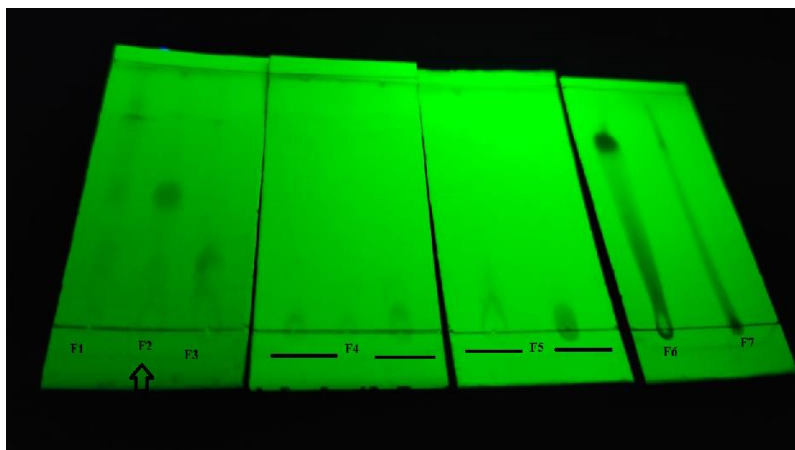


Figure 1. TLC profile from column chromatography (a mixture of ethyl acetate: chloroform: glacial Acetic Acid = 2:8:1 (F1-F3), 3:8:1 (F4), 4:8:1(F5), 5:8:1(F6) and a mixture of ethyl acetate: chloroform: glacial acetic acid: acetone = 2:8:1:1(F7))

Table 1. Yield data for the groups of fractions 1 to 7

Fractions	Weight (g)	Yield (% w/w)
F1	3.1481	0.210
F2	3.9067	0.260
F3	11.6822	0.779
F4	1.1322	0.075
F5	0.6288	0.042
F6	1.5935	0.106
F7	0.6322	0.042

Toxicity test used *A. salina* Leach larvae as sample because *A. salina* Leach larvae has high sensitivity to the changes in environmental condition and contamination of chemicals in the environment. LC_{50} is the value that shows the concentration of toxic compounds causing organism mortality up to 50%. LC_{50} focuses on the total mortality of tested animal, therefore, the LC_{50} is used in the short-term test, because *A. salina* Leach larvae has simple digestion system (Rasyid et al., 2020). The toxicity level of the extract was categorized as follows: $LC_{50} \leq 30$ mg/L = Very toxic; $LC_{50} \leq 1.000$ mg/L = Toxic; $LC_{50} > 1.000$ mg/L = Non-toxic (Meyer et al., 1982). The LC_{50} value in **Table 2** showed that the 7 fractions are in the toxic category and indicated have anti-cancer potential. Fraction 2 has the highest toxicity to *A. salina* Leach shrimp larvae, followed by 7 and 3. Based on the level of polarity, the group of fraction 2 has a lower polarity than the group of fractions 4, 5, 6 and 7 but still has a higher level of polarity than the group of fraction 1 and is estimated to be a semi-polar compound

Secondary Metabolite Test for Fractions 2

Based on the TLC data, F2 contains one dominant component (**Figure 2**). Based on the secondary metabolite test results, fraction 2 (F2) consisted of phenolic compounds which were indicated by a positive test result with a change in the spot color to purple. The saponin test also showed a positive result, indicated by the production of a very small foam, once shaking is done. This is probably another group of compounds in fraction 2 with an R_f below the value for phenolics. A thin spot under the phenolic spot indicates that the quantity of saponin contained in fraction 2 is small and this causes the test results to produce relatively small foam after shaking. Moreover, the TLC and secondary metabolite test results from fraction 2 showed that additional purification processes were still needed using recrystallization techniques or solid-liquid chromatography to obtain one pure compound for further identification of its molecular structure. The test results data are shown in **Table 3**.

Table 2. Regression equations along with r and LC_{50} values of the 7 combined fractions

Fractions	Line Equation	r	LC_{50} (ppm)
F1	$y = 0.0938x - 3.1172$	0.8191	566.2814
F2	$y = 0.4831x + 7.5318$	0.8909	87.9077
F3	$y = 0.2277x + 0.6749$	0.9712	216.6232
F4	$y = 0.0938x - 3.1172$	0.8191	566.2814
F5	$y = 0.0862x + 1.6707$	0.8172	560.6647
F6	$y = 0.1728x + 1.6296$	0.9048	279.9213
F7	$y = 0.2204x + 7.0953$	0.994	194.6674

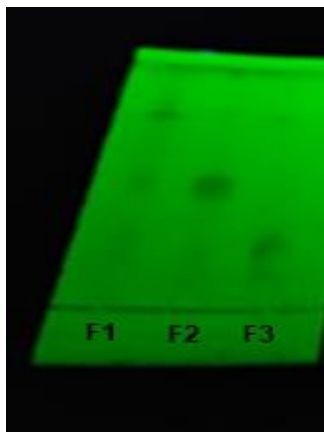


Figure 2. Chromatogram profile of F2 TLC results with eluent ethyl acetate: chloroform: glacial acetic acid (1:8:1)

Table 3. Secondary metabolite test result of F2

Test parameters	Test reagent	Result
Flavonoid	Vanilin-HCl	-
Alkaloid	Dragendorff	-
Terpenoid	I_2 vapor	-
Steroid	Liebermann-Burchard	-
Fenolat	$FeCl_3$	+
Saponin	Aquadest	+

Identification using Infrared Spectroscopy

Fraction 2 was identified using infrared spectroscopy to obtain information about the functional groups present in the molecules of compounds with the best toxicity that were successfully separated by column chromatography. This identification was to also strengthen the provisional assumption of the compounds' class contained in the fraction group detected from the secondary metabolite test data. The FTIR spectroscopy test results can be seen in **Table 4**, where peaks in the wavenumber confirm phenolic compounds' presence in fraction 2. The presence of hydroxyl and aromatic groups strengthens the suspicion of the positive test results of phenolic compounds (Pavia et al., 2013). To increase its activity as anti-cancer, it is necessary to purify the phenolic compounds from impurities.

Test for Anti-cervical Cancer Activity

Activity test from group 2 was carried out *in vitro* on HeLa cells which are cervical cancer cells produced from infection by Human Papilloma Virus (Goodwin & DiMaio, 2000) and the results are presented in **Table 5**. This cell line model is widely used in the biomolecular field with more than 70,000 publications (Landry et al., 2013).

The data showed that fraction 2 which has the highest toxicity value from the BSLT results, namely LC_{50} of 87.9077 ppm, actually possesses high anti-cervical cancer activity, namely $IC_{50} = 165.37$ ppm. The IC_{50} is the sample concentration value i.e the fraction in this case, which shows cell proliferation inhibition by 50% of the population. The cytotoxic extracts/fractions against cancer cells are classified as very active when the IC_{50} value is < 10 ppm, 10-100 ppm is in the active category and 100-500 ppm is quite active (Diani et al., 2015) while for pure

compounds the level of cytotoxic compounds can be grouped into three categories, namely the IC_{50} value < 10 -100 $\mu\text{g/mL}$ toxic and $IC_{50} < 100$ -500 less toxic (Weerapreeyakul et al., 2012). Based on these categories, fraction 2 has a quite active anti-cervical cancer compound. It is known that phenolic compounds constitute one of the largest and most ubiquitous group of plant metabolites (Derenne et al., 2013) and in general that phenolic compounds have antioxidant activity and many of them have anti-cancer (Cai et al., 2004) and antimicrobial activity (Patle et al., 2020)(Cai et al., 2004; Patle et al., 2020). Based on the results of the cervix test, the fraction 2 showed high activity. The main spot of fraction 2 based on secondary metabolite assays is phenolic compounds, strengthening the suspicion that anticancer activity is caused by it. To increase its activity as anti-cancer, it is necessary to purify the phenolic compounds from impurities. Based on the secondary metabolite test results, there were 2 positive compounds, namely phenolics and saponins. Phenolic as the main spot that is clearly visible, while the impurities that are still in fraction 2 are saponin compound.

Moreover, the test results showed a match between the toxicity estimated in BSLT and the fraction 2 anti-cancer activity, specifically against cervical cancer. These data also inform that fraction 2 has the potential to be investigated further by the purification process and anti-cervical cancer activity test as well as through identification of its molecular structure. Therefore, the information generated on the pure compound obtained tends to contribute majorly to the treatment of diseases, particularly cervical cancer, which is currently ranked second after breast cancer in Indonesia.

Table 4. The absorption peaks of the FTIR spectrum from fraction 2

Wavenumber	Intensity	Functional Group
3433.29	wide, strong	-OH
2924.09	sharp, medium	C-H aromatic
2854.65	sharp, weak	C-H aliphatic
1735.93	sharp, strong	C=O carbonyl
1635.64	sharp, medium	C=C alkene
1442	sharp, weak	C=C aromatic
1381.03	sharp, weak	C-H alkane
1257.59	wide, weak	C-O

Table 5. Data of anti-cervical cancer activity test against HeLa cells from faction group 2

Concentration	% inhibition 1	% inhibition 2	% inhibition 3	IC_{50} 1	IC_{50} 2	IC_{50} 3	IC_{50} average
3.125	4.616724739	3.571428571	8.275261324				
6.25	14.02439024	15.85365854	15.06968641				
12.5	21.34146341	22.12543554	21.8641115	164.87	154.64	176.6	165.37
25	27.87456446	31.01045296	31.53310105				
50	36.75958188	35.19163763	35.97560976				

CONCLUSION

The results of column chromatography of the ethyl acetate fraction of methanol extract ketapang fruit flesh obtained seven groups of fractions which as a whole have potential as anti-cancer compounds. This can be seen from the cytotoxic test results on *Artemia salina* Leach shrimp larvae, where all fractions produced LC₅₀ values below 1000 ppm.

The most active subfraction is fraction 2 which have two groups of positive compounds, i.e. phenolics and saponins were obtained from the secondary metabolite test. The value of the anti-cancer activity of fraction 2 was IC₅₀ = 165.37 ppm, which was included in the fairly active category as an anti-cervical cancer agent.

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