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Research Article

In Vitro Tracing of Cytotoxic Compounds in Jarak Cina Stem Bark (*Jatropha Multifida Linn.*)

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Abstract: Cancer remains one of the diseases with increasing number of sufferers, but research on compounds that act as anti-cancer is also ongoing. Terpenoids have been known as a compound that can inhibit the proliferation of cancer cells. One of the medical plants that produce terpenoids is Jarak cina (Jatropha multifida Linn.). Therefore, the possibility of Jarak cina (Jatropha multifida Linn.) stem bark to have a cytotoxic activity on cancer cell proliferation is reasonably high, but it has never been tested on cancer cells before. This research was conducted to trace extracts that had the highest cytotoxic activity against cancer cell MCF-7 by comparing the solvents used in extraction of Jarak cina (Jatropha multifida Linn.) stem bark. The extraction was done using the multilevel soxhlet extraction method with n-hexane, ethyl acetate, and ethanol as the solvents. All the three extracts were then tested against MCF-7 cancer cells using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method. Data analysis was performed for IC50 (µg/mL) parameter. The results showed that the IC50 of n-hexane extract was 313.21 µg/mL, while the ethyl acetate extract reached 258.38 µg/mL of IC50, and the IC50 of ethanol extract was 418.51 µg/mL. The highest potential of cytotoxicity was found in the ethyl acetate extract, so further testing would be required to optimize the proliferation inhibitory activity.

Keywords: jarak cina, jatropha multifida linn., MCF7, cytotoxic

Introduction

Cancer is one of the world's seven causes of death, outnumbering the deaths from AIDS, TB, and malaria [1]. It is estimated to increase in number from 14 million in 2012 to 22 million in 20 years to come (WHO, 2015). Lung, liver, and breast cancers remain at the top incidence of cancer worldwide. From the data collected until 2012, breast cancer became the second most suffered from by the world's population with the fastest development among other types [2].

Research on natural compounds as potential anticancer becomes important as this disease is predicted to grow rapidly. The fast growth is partly caused by the mechanism of cancer cells that can develop immunosurveillance to hide and escape from the immune system [3, 4]. Meanwhile, natural compounds have been widely studied for their anticancer agents, and one of them is terpenoids [5, 6].

Terpenoids have the structure similar to hormones in the human body that can reduce the risk of such chronic diseases as cancer. In vitro and in vivo studies have proved that terpenoids are capable of inhibiting the proliferation and growth of human cancer cells [7, 8]. Terpenoids are found in Jarak cina (Jatropha multifida Linn.) together with other major components in the different type of extract such as alkaloids, steroids, tannin, flavonoid, phenols, phloroglucinols, and cyanoglucosides [9-12], leading to an underlying assumption that Jarak cina has anticancer activities. Several studies have been conducted to test the anticancer activity of various species of Jatropha sp, and efficacy of cancer inhibition was discovered [13,

14]. However, research on the effectiveness of Jatropha multifida stem bark powder as an anticancer has yet to be carried out.

The search for cytotoxic compounds can be done by comparing extracts using solvents with different polarities. This can help to find out the presence of active compounds that will facilitate further research for the isolation of cytotoxic active compounds. This method has been widely carried out by previous studies by comparing the activity between extracts and active compounds found in extracts with the highest activity [15, 16].

Meanwhile, human cancer cells can be isolated and cultivated in vitro in the laboratory. In this study, the extract of Jarak cina bark was tested on breast cancer as the leading cancer prevalence in Indonesia. In addition, MCF-7 cell cultures were selected as the test models because they are non-invasive and ERα-positive [17]. The purpose of this study was to investigate the highest cytotoxic activity of n-hexane, ethyl acetate, and ethanol extracts of Jatropha multifida Linn. stem bark powder.

Materials and Methods

Materials

This study used Jarak cina stem bark powder obtained from Merapi Farma Herbal Kaliurang, Sleman, Yogyakarta. The MCF-7 cells were obtained from the Parasitology Laboratory of the Faculty of Medicine, Gadjah Mada University. Some chemicals consist of n-hexane (Merck, USA), ethyl acetate (Merck, USA), ethanol (Merck, USA), Dimethyl Sulfoxide (DMSO; Sigma, USA), MTT reagent, flask (Iwaki, Japan), microplate (Iwaki, Japan), blue tip (Axygen, USA), yellow tip (Axygen, USA), white tip (Axygen, USA), trypan blue (Sigma, USA), Phosphat Buffer Saline (PBS; Sigma, USA), Fetal Bovine Serum (FBS; Gibco, Australia), Fungizone (Gibco, UK), Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Sweden), Trypsin (Gibco, UK), Pen-strep (Gibco, UK), $FeCl_3$ (Merck, USA), $AlCl_3$ (Merck, USA), anisaldehyde – sulfuric acid (Merck, USA), and Dragendorff.

Instruments

The instruments consisted of soxhlet extractor (Iwaki Pyrex, Japan), rotary evaporator (Heidolph, Germany), analytical scales (Mettler Toledo, USA), a set of glassware, Laminar Air Flow Purifier Class II Biosafety Cabinet (LAF; LABCONCO, USA)), centrifuges (Hettich, Germany), autoclave (Hirayama, Jepang), water bath (Memmert, Germany), CO_2 incubator (Memmert, Germany), oven (Memmert, Germany), inverted microscope (Olympus CKX41, Japan), vortex (Genie, USA), semi analytical scales (Mettler Toledo, USA), micropipette (Transferpette, Germany), refrigerator (4 ^oC, -20 ^oC, -80 ^oC), haemocytometer (Neubauer), microplate reader (BioTek, USA) and thin layer chromatography (TLC).

Extraction

A total of 100 grams of Jatropha multifida Linn. bark powder was extracted using the soxhlet extraction method. The solvents were from non-polar to polar, consisting of n-hexane, ethyl acetate, and ethanol to obtain three different extracts that were concentrated by a rotary evaporator. Testing of residual solvents was then performed using GC-MS (Shimadzu QP 2010 SE, Japan) with the stationary phase was RTX-5S and the mobile phase was helium gasses.

Identification of Compound Jarak cina stem bark

The qualitative test for the content of all extracts was done using TLC-Densitometry. The n-hexane, ethyl acetate and ethanol extracts were each prepared in 50.000-µg/mL solution. The stationary phase was Silica Gel 60 GF254 plates, and the mobile phase was chloroform. TLC plates were then sprayed with $FeCl_3$, $AlCl_3$, anisaldehyde – sulfuric acid, and Dragendorff.

Cytotoxic Activity

The cells were proliferated using MEM medium containing 10% FBS, 0.5% Fungizone, and 1.5% Pen-strep. Cytotoxicity testing was performed with MCF-7 cells grown in 96-well plates for 24 hours (confluent). Extracts of *Jatropha multifida* Linn. bark were added to MCF-7 cells with 5 different concentrations. The dose starting from 100 µg/ml was added with four other smaller doses: 50, 25, 12.5, and 6.25 µg/ml. These five doses were used for identification of the optimum dose for the cytotoxicity test of *Jarak cina* bark extract. Then, 50 µL of MTT solution was added to each plate and incubated for 4 hours. Purple formazan crystals were formed and then dissolved with 80 µL of DMSO. Finally, the solution was tested for its absorbance using a microplate reader at a wavelength of 570 nm.

Similar tests were performed on Vero cells used as a comparator to determine the effect of extracts on normal cells. An effective anticancer compound is capable of inhibiting the proliferation of cancer cells without preventing normal cell proliferation. The test was performed by comparing the cytotoxic activity on MCF-7 cells with that on Vero cells.

Data Analysis

Elution spot of n-hexane extract, ethyl acetate extract, and ethanol extract were tested by densitometer to determine the value of Rf and the area under curve that formed. Further testing was done by spraying the reagent at the elution site for determining the possibility of the compound affecting the cytotoxic activity of each extract.

The absorbance value from the experiment was processed to obtain the IC_{50} value. The value of IC_{50} was obtained from the linear regression equation between the number of living cell in percent (y) and the number of extracts exposed on cells (x). Percent of living cell is calculated by equation 1. The IC_{50} value is expressed as the required extract rate killing 50% cells. The extracts said to be cytotoxic active if IC_{50} value was <20 µg/mL; moderate is on when> 20 µg/mL; and is inactive if IC_{50}> 100 µg/mL.

$$\text{The percentage of living cells} = \frac{\text{Absorbance of cells by treatment- absorbance of media control}}{\text{absorbance of control cell- absorbance of media control}} \times 100\% \ldots\ldots. (1)$$

Results and Discussions

Extraction

The extracts had relatively varied characteristics (Table 1). The three extracts of *J. multifida* were further tested to determine the presence / absence of the extracting solvent still remaining after the evaporation process (Figure 1). The test was performed using the GCMS instrument. Solvent residual testing is necessary to ensure that the results obtained in the cytotoxic test are correct due to the activity of each extract, not the effect of the solvent extract. The results showed in Figure 1 that all of n-hexane, ethyl acetate, and ethanol extracts of *J. multifide* had no solvent peak, so all of them were not contain any solvents left.

Table 1. Characterization of each extract

Extract	Color
N-Hexane	RAL 6020 *Chrome green*
Ethyl Acetate	RAL 6007 *Bottle green*
Ethanol	RAL 8023 *Orange brown*

* Color assignment refers to RAL *Classic Colour Chart*

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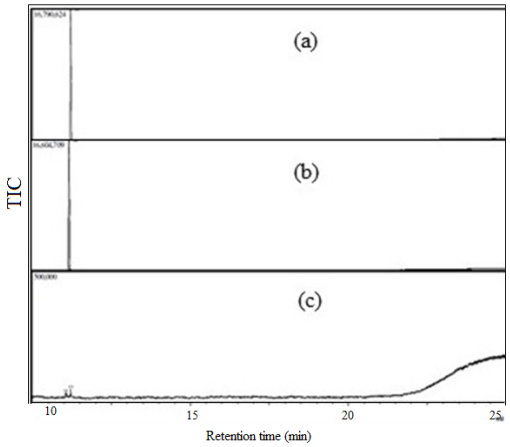
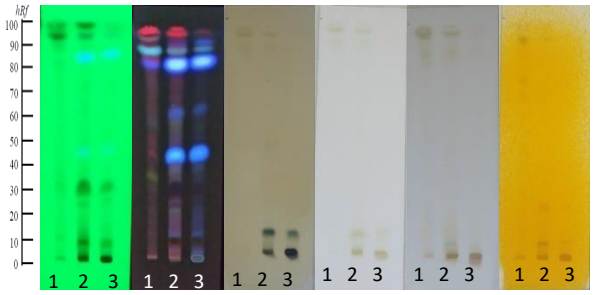


Figure 1. Solvent residue test on n-hexane extract (a), ethyl acetate extract (b), and ethanol extract (c) using GC-MS

Component Identification

The identification test qualitatively showed the differences in the components of each extract (Figure 2). We were used four different spray reagents, namely $FeCl_3$, $AlCl_3$, anisaldehyde-sulfuric acid, and Dragendorff with different purpose for each spray reagent. $FeCl_3$ were used to detect phenols compound, $AlCl_3$ to detect flavonoids, anisaldehyde-sulfuric acid to detect terpenoids, and Dragendorff to detect alkaloids.



(a) (b) (c) (d) (e) (f)

Figure 2. TLC results (a) under UV_{254} ; (b) under UV_{366}; (c) phenol identification ($FeCl_3$); (d) flavonoid identification ($AlCl_3$); (e) terpenoid identification (anisaldehyde-sulfuric acid); (f) alkaloid identification (Dragendorff). Mobile phase = chloroform p.a., stationary phase = silica gel 60 F_{254}, number 1: n-hexane extract, 2: ethyl acetate extract, 3: ethanol extract

Figure 2 shows that n-hexane extract and ethyl acetate extract of *Jatropha multifida* bark had more component varieties than ethanol extract. It was also based on the number of chromatographic peaks from the densitometer. This occurred because the extraction process at each stage of the solvent was not perfectly completed, leaving some remaining compound and sour on the following solvent.

The three extracts of *J. multifida* contained phenols, flavonoids, terpenoids, and alkaloids with various hRf, color intensities, and areas. This indicated that the class of compounds– including phenols, flavonoids, terpenoids, and alkaloids – tested using spray reagents was present in each extract though with different polarity properties. In Figure 2, for example, the area of phenols and flavonoids was the largest in n-hexane extract since phenols and flavonoids can normally be extracted in nonpolar solvents.

The phenols and flavonoids in n-hexane and ethyl acetate extracts were located on hRf 94 (Showed in Table 2 - 4) with smaller area in ethyl acetate extract than in n-hexane extract because phenols and flavonoids are usually extracted better in n-hexane solvents, resulting in larger area. In addition, terpenoid compounds in n-hexane and ethyl acetate extracts were found in hRf 94 with larger area in ethyl acetate extract because terpenoids are commonly found in semi-polar solvents [1].

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Table 2. Results of UV366 TLC-densitometer test of n-hexane extract of *J. multifida* bark

Spot	hRf	Qualitative test of spray reagent*			
		$FeCl_3$	$AlCl_3$	anisaldehyde-sulfuric acid	*Dragendroff*
1	8	-	-	-	-
2	16	-	-	-	-
3	20	-	-	-	-
4	28	-	-	-	-
5	32	-	-	-	-
6	38	-	-	-	-
7	42	-	-	-	-
8	50	-	-	-	-
9	61	-	-	-	-
10	67	-	-	-	-
11	73	-	-	-	-
12	81	-	-	-	-
13	86	Beige grey	Zinc yellow	-	Clay brown
14	94	Beige grey	Lemon yellow	Oxide red	-

* Color assignment refers to RAL *Classic Colour Chart*

By comparing the area of chromatogram and the spots detected from the spray reagent in the three extracts, it was revealed that the highest content of phenols and flavonoids was found in the n-hexane extract, while terpenoids and highest alkaloid contents were located in the ethyl acetate extract (showed in Table 2 - 4). Although the spray reactor detected phenols, flavonoids, terpenoids, and alkaloids in the ethanol extract, this content was not as much as that in the other two extracts.

Table 3. Results of UV366 TLC-densitometer test of ethyl acetate extract of *J. multifida* bark

Spot	hRf	Qualitative test of spray reagent*			
		$FeCl_3$	$AlCl_3$	anisaldehyde-sulfuric acid	*Dragendroff*
1	5	Grey brown	Brown beige	Heather violet	Brown beige
2	7	Traffic black	Saffron yellow	Rose	Brown beige
3	17	-	-	-	-
4	27	-	-	-	Pastel yellow
5	33	-	-	-	-
6	36	-	-	-	-
7	46	-	-	-	-
8	55	-	-	-	-
9	61	-	-	-	-
10	74	-	-	-	-
11	80	-	-	-	-
12	84	Beige grey	-	-	Clay brown
13	94	Beige grey	Lemon yellow	Oxide red	-

* Color assignment refers to RAL *Classic Colour Chart*

Cytotoxic Activity

Cytotoxicity can be compared by IC_{50} parameter, which indicates the dose of extract that can inhibit cell proliferation by 50 percent. The lower the value is, the better the inhibitory activity against cell proliferation is. In the case of MCF-7 cells, the extract is expected to have a low IC_{50} value that can kill

cancer cells. Extracts are expected to have low IC_{50} values in Vero cells because good extracts do not inhibit normal cell proliferation. The value of IC_{50} can be seen in Table 5.

Table 4. Results of UV366 TLC-densitometer test of ethanol extract of *J. multifida* bark

Spot	hRf	Qualitative test of spray reagent*			
		$FeCl_3$	$AlCl_3$	anisaldehyde-sulfuric acid	*Dragendroff*
1	4	*Graphite black*	*Ochre yellow*	*Heather violet*	-
2	7	-	-	-	*Brown beige*
3	14	*Traffic black*	*Saffron yellow*	-	-
4	17	-	-	-	-
5	25	-	-	-	*Pastel yellow*
6	32	-	-	-	-
7	59	-	-	-	-
8	71	-	-	-	-
9	79	-	-	-	-
10	83	-	-	-	-

* Color assignment refers to RAL *Classic Colour Chart*

Table 5. Comparison of IC_{50} of *J. multifida* bark extract on MCF-7 cell and Vero cell lines

Extract	IC_{50} (µg/mL)	
	MCF-7	Vero
N-Hexane	313.21	152.16
Ethyl acetate	258.38	84.74
Ethanol	418.51	397.06

Metabolite compounds of natural substances are expressed as having cytotoxic capability if IC_{50} <100 µg/mL, moderate when IC_{50} > 20 µg/mL, and cytotoxically active if IC_{50} <20 µg/mL . The extracts of n-hexane, ethyl acetate, and ethanol all caused death in both MCF-7 and Vero cells, though at different levels. None of the three extracts had a cytotoxic activity against MCF-7 cells based on IC_{50} values. However, the IC_{50} of ethyl acetate extract against MCF-7 cells and Vero cell lines was lower than that of n-hexane and ethanol extracts. The active cytotoxic ability of ethyl acetate extract could be sourced from the activity of terpenoid compounds and alkaloids, which were more qualitatively predominant in ethyl acetate extract than in hexane extract and ethanol extract based on the previous TLC-densitometer test results. This proves that the extract of *Jatropha multifida* had no better cytotoxic activity against MCF-7 cells than their isolates.

Conclusion

The result of cytotoxicity test showed that IC50 of n-hexane extract, ethyl acetate extract, and ethanol extract reached more than 100 µg/mL, indicating that the three extracts did not have the cytotoxic potential against MCF-7 cells. However, the IC50 value of ethyl acetate extract (258.38 µg/mL) was the lowest against MCF-7 cells as opposed to that of n-hexane extract (313.21 µg/mL) and ethanol extract (418.51 µg/mL).

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