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In Vitro Antioxidant and Cytotoxicity Activity of Extract and Fraction Pyrrosia piloselloides (L) M.G Price

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Abstract: The herbs of *Pyrrosia piloselloides* (L) M.G Price are well known as traditional medicine in Indonesia for breast cancer . This research was conducted to evaluate the of cytotoxic effect of *P. piloselloides* (L) M.G Price on MCF-7 Breast cancer cell and in vitro antioxidant activity. *P. piloselloides* (L) M.G Price herbs macerated successively using n-hexane, dichloromethane and metanol for result n-hexane extract (EHP), dichloromethane extract (EDCMP) and methanol extract (EMP). Cytotoxicity test using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Antioxidant activity test using the 1,1 diphenyl-2-picrylhydrazyl (DPPH). The results showed that EDCM has the potential as an antioxidant with the smallest IC_{50} value that is equal to 12.82 µg/mL and best cytotoxity with IC_{50} 39.54 µg/mL. EDCMP fractionated using column chromatography with eluent mixture of n-hexane-ethyl acetate and ethyl acetate-methanol with increasing polarity. The results obtained by fractionation of six fractions (FI-FVI EDCMP). Antioxidant activity assay results showed that FIV EDCMP have the greatest antioxidant activity with IC_{50} value of 28.29 µg/mL. Cytotoxicity activity results that FVIEDCMP have the greatest with IC_{50} value 28.73 µg/mL. *P. piloselloides* (L) M.G Price potent for inhibit breast cancer and antioxidant.
Key words: Antioxidant, DPPH, Cytotoxixity, MTT Assay, MCF-7, *Pyrrosia piloselloides* (L) M.G Price.

1.Introduction

Aging process and degenerative diseases such as cancer, cardiovascular, blood vessel blockage which includes hiperlipidemic, atherosclerosis, stroke, and high blood pressure as well as the disruption of the body's immune system can be caused by oxidative stress. Oxidative stress is a state of imbalance of oxidants and prooxidant amount in the body. In these conditions, the activity of free radical molecules or reactive oxygen species (ROS) can cause cellular and genetic damage. Dietary deficiencies and any compounds or xenobiotics from food polluted environment will worsen the situation. Antioxidants are substances that the body needs to neutralize free radicals and prevent the damage caused by free radicals on normal cells, proteins, and fats. Antioxidants stabilize free radicals by complete deficiency of electrons possessed free radicals and inhibiting the chain reaction of the formation of free radicals that can cause oxidative stress.[1-3] Antioxidants are substances that can capture free electrons released by free radicals without causing instability in molecule itself, thus reducing DNA damage. Therefore it is assumed that oncogenesis in very early phases can be prevented by administering antioxidant.

Plant *P. piloselloides* (L) MG Price is one of the traditional medicinal plants of Polygonaceae familia. *P. piloselloides* (L) MG Price are epiphyt plants that can be found in all regions of Tropical Asia. *P. piloselloides* (L) MG Price not parasitic because it can make their own food. Empirically this plant is used as a breast cancer drug.[4] The results of studies that have been carried out it was found that the water extract of leaves of *P. nummularifolia* (Sw.) Ching has antiproliferation activity against tumor cells MCM-B2 sustainable by 59.09% at a concentration of 1050 ppm. Antiproliferation activity of aqueous extract of leaves of this plant likely due to the active compounds of flavonoids, saponins, steroids and tannins contained in them.[4]

2. Material and Methods

2.1 Plant collections

Fresh herbs of *P. piloseloides* (L) M.G Price collected from Ciawi, West java in March 2012 and were determined by the botanist at Indonesian Institute of sciences, Biologist Research Center, Cibinong. The Fresh herbs sorted, washed, dried in cabinet dryer at 50 ^{o}C. Then the dried herbs homogenize to fine powder and stored in airtight botlles.

2.2 .Materials

Breast cancer (*cell line* MCF-7) from research and testing laboratory, Gadjah Mada University, Yogyakarta, Indonesia. Materials for cytotoxicity : Dulbecco's Modified Eagle Medium (DMEM) (Gibco), Penisilin-streptomisin (Sigma), Foetal Bovine Serum (FBS) 10 % v/v (Gibco), Phosphate Buffer Saline (Merck), Trypsin 0,25% (Sigma), MTT (Sigma), Fungizone Amphotericin B (Gibco), Reagen Stopper SDS 10% HCl 0,1 N (Merck), DMSO (Merck) materials for extraction and fractination including n-heksan, methanol, dichloromethane, ethyl acetate, methanol technical (Brataco chemika, Indonesia) that have been distilled, n-hexane p.a, ethyl acetate.p.a (Merck, Germany), deminelarized distilled water (Brataco chemika, Indonesia), hydrochloric acid (Univar, USA), thin layer chromatography plate of silica gel 60 F 254 (Merck,Germany), silica gel 60 GF 254 (Merck), DPPH (Sigma Aldric, Singapore)

2.3.Extraction and isolation

Weighed less than 2 kg dry powder macerated with n-hexane solvent. Maceration repeated again with the same solvent until the filtrate gives clear maceration results. Maceration results obtained filtered and the filtrate was concentrated using a vacuum rotary evaporator at a temperature of approximately 50 ^{o}C to obtain a thick n-hexane extract (EHP). Residu n-hexane maceration that macareted again by solvent dichloromethane then filtered and the filtrate maceration result obtained was concentrated by vacuum rotary evaporator at a temperature of approximately 50 ^{o}C to obtain a viscous dichloromethane extract (EDCMP), residu from this maceration then macerated with methanol then filtered and the filtrate maceration result obtained was concentrated by vacuum rotary evaporator at a temperature of approximately 50 ^{o}C to obtain a viscous methanol extracts (EMP). Then each extract were weighed and calculated for yield of extracts

A total of 30 g of Active extract with IC_{50} smallest was mixed with silica gel G-60 (70-230 mesh), subjected to the colomn (4,5 cm x 75 cm) then eluted with gradient polarity using n-hexane, n-hexane-ethylacetate, ethyl acetate, ethyl acetate-methanol, methanol ranging from (100,0); (98,2); (96,4) to ratio of (2,98); (0,100). Appropiate fractions (100 mL) were collected. Those fractions showing similar TLC profile by n-hexan:ethyl acetate (4:1) were combined and evaporated yielding some fractions . Each fraction evaluated antioxidant and cytotoxicity activity.

2.4. Assay of Cytotoxic activity

The MCF-7 cell line were cultured stock in DMEM with 10% FBS, 100 µg/mL streptomycin and penicillin (100 IU / ml) and 2 mm glutamine. Cell were incubated in humidified atmosphere of 5% CO_2 at 37 ^{o}C. 100 µl cell suspension with 1.5 X 10^4 cells included in microplate 96 well. EHP/EDCMP/EMP/ fractions of active extract with concentration 39, 78, up to 500 µg/mL with triple replications each cell controls and medium controls . Microplate incubated for 24 hours at 37 ^{o}C 5% CO_2, the culture medium removed and washed with PBS. Into each well plate added 10 µL of MTT solution (1 mL MTT in 10 ml culture medium) and microplate incubated at 37 ^{o}C in 5% CO_2. After 4 hours of stopper reagent added 100 mL of 10% SDS in 0.1 N HCl into each well (to dissolve the purple formazan crystals). Absorbance is read using an ELISA reader at a wavelength of 550 nm.The percentage of cell viability and cell death of extract and fractions on MCF-7 cell line was calculated for each assay by using the formula :

$$\% \text{ viability cell} = \frac{Ods - OD\,m}{ODc - OD\,m} \times 100\ \%$$

% death cell = 100 - % viability Cell

Where ODs = optical density cell with extract or fractions, ODc = optical density cell withaout extract or fractions, ODm = optical density media withaout cell .

Graph percentage of viability cell against logarithm concentration was plotted. The IC_{50} value were calculated by using curve in linier equations.

2.5. DPPH Radical Scavenging Activity

Free radical scavenging ability of the extracts was tested by DPPH as described by Blois [5] that was modified. The mixture contained 1.0 mL sample at concentration ranging from 0.25, 1.25, to 50 µg/mL, and 1.0 mL DPPH solution of 100 ppm, and 2.0 mL of methanol p.a than homogenized, absorbance of solution was measured at 515 nm after incubation for 30 minutes at 37 ^{o}C in a dark tube. The same procedure was also done to quercetin as positive controls at concentrtion of 0.25 to 1.5 µg/mL. The percentage of the inhibition can be calculated by formula:

$$\% \text{ inhibition} = \frac{Ac - As}{Ac} \times 100\ \%$$

Where Ac = Absorbance control
As = Absorbance sample

The IC_{50} value were calculated by using inhibition curve in linier equations.

3. Results and Discussion

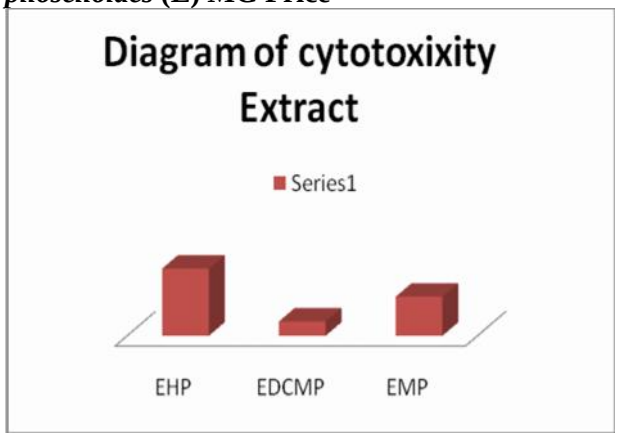
3.1.Cytotoxicity assay

Cytotoxicity assay with MTT is based on tha capacity of mitochondrial dehydrogenase enzym to convert the yellow water soluble substrate MTT into dark blue formazan product which is insoluble in water. The amount of formazan product is directly proportional to the viable cell number in variety of cell types.[6-9] Cytotoxicity testing of plant extracts *P. piloselloides* (L) MG Price using MTT assay after 24 h incubation obtained the following results :

Table I : Cytotoxocity Measurement of extract Pyrrosia *Pyrrosia piloselloides* (L) M.G Price

Sample	Concentration	% Viability cell	IC_{50}	% Death cell	IC_{50}
EH	5000	3,618	185,77	96,381	185,74
	2500	27,855		72,144	
	313	40,802		59,198	
	78	63,295		36,704	
	39	66,608		33,391	
EDCMP	625	4,839	39,54	95,161	39,54
	313	26,373		73,627	
	156	34,656		65,345	
	78	35,266		64,734	
EMP	1.250	5,275	107,89	94,725	107,91
	625	16,870		83,130	
	313	39,756		60,244	
	156	43,461		56,539	
	78	52,223		47,777	

Figure 1. Cytotoxicity activity extract of *Pyrrosia piloselloides* (L) MG Price



Based on the result in table I, each extract show increase viability cell from higher concentrations to small concentration opposite death cell show decrease from higher concentrations to small concentrations. IC_{50} are concentration where 50% death cell or viability cell. Value IC50 each extract can be described in figure 1.

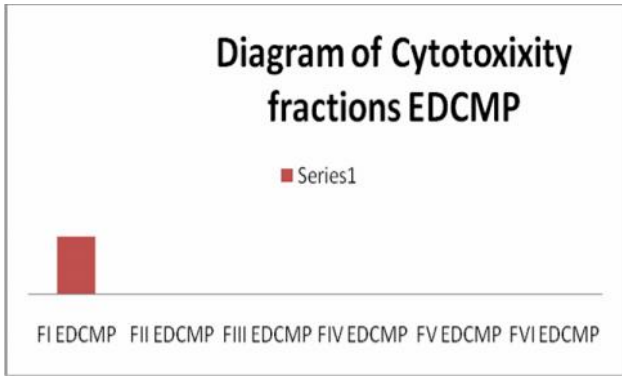
Figure 1 shows that EDCMP have high cytotoxicity because small concentrations of extract (39.54 µg/mL) can reduce death cell by 50% . EDMP are active extract then fractionated with chromatography colomn to obtain fractions. Each fractions collect building on similarity chromatogram on TLC, result six fractions, namely FI EDMP, FII EDMP, FIII EDCMP, FIV EDMP, FVEDMP and FVIEDCMP. Each fractions EDCMP evaluated cytotoxicity by MTT method to obtain the following results.

Table II : Cytotoxicity Measurement of Fraction Pyrrosia *Pyrrosia piloselloides* (L) M.G Price

Fraction	Concentration	% viability cell	IC50	% inhibition cell	IC_{50}
FIEDCMP	2.500	62,506	83.039,93	37,494	83.402,87
	1.250	63,877		36,123	
	625	65,653		34,347	
	156	72,0718		27,928	
	78	73,225		26,775	
	39	76,029		23,971	
FIIEDCMP	1.250	4,518119	151,33	95,481	151,31
	625	9,877543		90,122	
	313	31,59568		68,404	
	156	48,42177		51,578	
	78	68,48846		31,511	
FIIIEDCMP	1.250	1,776	133,11	98,224	133,14
	625	7,104		92,896	
	313	32,125		67,875	
	156	50,291		49,709	
	78	60,200		39,799	
FIVEDCMP	625	14,95	59,34	85,05	59,35
	313	16,3		83,7	
	156	18,05		81,95	
	78	51,8		48,2	
	39	60,5		39,5	
FVEDCMP	625	8,75	37,27	91,25	37,26
	313	9,85		90,15	
	156	13,35		86,65	
	39	55,7		44,3	
FVIEDCMP	313	1,65	28,73	98,35	28,73
	156	8,7		91,3	
	78	27,85		72,15	
	39	45,25		54,75	

Each fraction have various result % viability cell and death cell but every high concentration causing high % death cell and litlle % viability cell. Value IC_{50} each fraction can be shown in figure 2.

Figure 2. Cytotoxicity assay fraction of EDCMP



The results of figure 2 shows that FIEDCMP non cytotoxic because value IC_{50} is very high > 100ug/ml. FVIEDCMP has smallest IC_{50} with value is 28.73 µg/mL, this fraction is very cytotoxic. FVIEDCMP can be developed as an alternative treatment for breast-cancer.

3.2. Antioxidant assay

Cancer can be treated with antioxidant compounds to see if the probability *P. piloselloides* (L) M.G Price also is an antioxidant, each extract from this plant analyzed the antioxidants tested in vitro by DPPH method, the test results can be shown in table III.

Table III: Antioxidant Measurement of extract Pyrrosia *Pyrrosia piloselloides* (L) M.G Price and Standards Quersetin

Sample	Concentration (µg/ml)	% inhibition	IC_{50} (µg/mL)
n-hexan extract	0,25	2,94	41,16
	5	6,34	
	7,5	8,96	
	12,5	18,08	
	50	60,28	
Dicloromethan extract	0,25	8,58	12,82
	1,25	9,41	
	2,5	10,79	
	5	19,92	
	12,5	50,21	
Methanol extract	1,25	3,4	38,94
	2,5	7,62	
	12,5	18,23	
	25	34,97	
	50	62,45	
Quersetin	0,25	3,86	1,57
	0,5	6,98	
	0,75	19,61	
	1	20,51	
	1,25	35,81	
	1,5	54,38	

Based on the results in Table III, we obtained that the EHP, EDCMP and EMP are moderately active antioxidant because they have IC_{50} values below 50 µg/mL.[10] IC_{50} is concentrated when extract can inhibit radical ions equal 50%. Value IC_{50} among three extracts can be described in figure 3.

Figure 3. Result DPPH assay extract *Pyrrosia piloselloides* (L) M.G Price

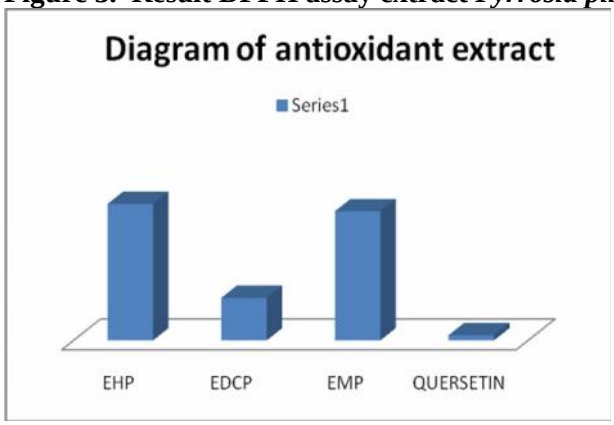


Table IV: Antioxidant Measurement of extract Pyrrosia *Pyrrosia piloselloides* (L) M.G Price and Standards Quersetin

Sample	Concentration (µg/ml)	% inhibition	IC50 (µg/ml)
FIEDCMP	10	14,36	86,47
	15	16,73	
	20	17,65	
	25	21,87	
FIIEDCMP	5	16,86	59,27
	10	23,32	
	15	23,45	
	20	26,61	
	25	29,91	
FIIIEDCMP	5	22,93	109,88
	15	23,98	
	20	24,70	
	50	34,39	
FIVEDCMP	5	15,55	28,296
	10	17,66	
	15	25,96	
	20	39,52	
FVEDCMP	5	15,02	32,997
	10	21,34	
	15	26,22	
	20	37,68	
	25	37,95	
FVIEDCMP	5	15,81	45,68
	10	23,26	
	15	25,96	
	20	28,33	

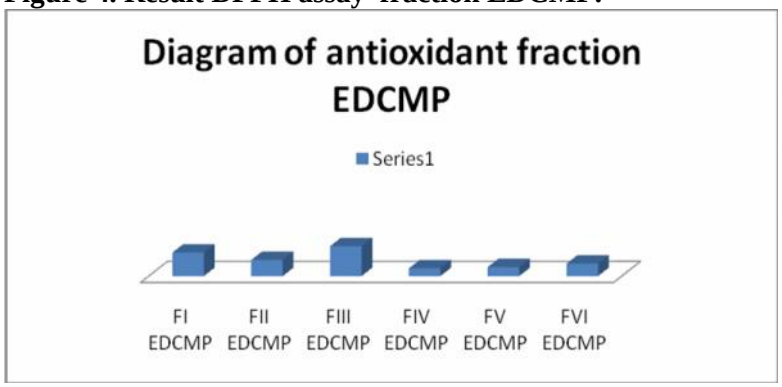
Based on the results in Table IV, the active fractions is FIVEDCMP because it has the smallest IC_{50}. More detail value IC_{50} on each fractions can described in figure 4.

Fraction I, II, IV, V and V have activity antioxidants because value IC50 < 100 µg/ml. Fractions V less potent antioxidant opposite another fractions. FIVEEDCMP are active fraction because they have the smalest IC_{50} (28.29 g/mL). Fractions FIVEDCMP can be developed as antioxidant drug.

4. Conclusion

. Plants *P. piloselloides* (L) MG Price can be developed as antioxidant and anticancer , especially breast cancer because the test results in vitro antioxidant with DPPH method obtain FIVEDCMP as active fraction with IC_{50} 28.29 µg/mL and cytotoxicity assay use MTT obtain FVIEDCMP as active fractions with IC_{50} 28.73 µg/mL.

Figure 4. Result DPPH assay fraction EDCMP.



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