OP	E001

MOLECULAR BIOLOGY

CYTOTOXIC ACTIVITY OF POLAR, SEMIPOLAR, AND NON POLAR FRACTION OF ETHANOL EXTRACT OF SALA PLANTS LEAVES (Cynometra ramiflora Linn.) AGAINTS WiDr CELL

Haryoto[1*], Anis Novida Irjayanti[1], Andi Suhendi[1], Muhtadi[1], Tanti Azizah Sujono[1]
[1]Facultyof Pharmacy, Universitas Muhammadiyah Surakarta
Surakarta, Central Java, Indonesia

*E-mail: haryoto@ums.ac.id

***Abstract*—Ethanol extract of the leaves sala plant (*Cynometraramiflora*Linn.) have cytotoxic activity against WiDr cells with IC_{50} of 6.37 µg/mL. This study aims to determine the cytotoxic activity of polar, semipolar, and nonpolar fractions of ethanol extract of leaves sala plant (*Cynometraramiflora*Linn.) against WiDr cells. Fractionation was performed on Vacuum Liquid Chromatography method with silica G 60 as stationary phase and n-hexane: ethylacetate(8:2; 7.5:2.5; 7:3,6:4 and 3:7) and ethanol as mobile phase. Qualitative screening of major compounds used TLC method with spray reagent. Cytotoxicity determination performedin vitro model cell lines with MTTreagent.The result showed that IC_{50} of polar, semi polar and non polar fractions were 231.95 µg/mL and cannot determined. Doxorubicin was used as positive control and obtained IC_{50} of 1.721 µg/mL.**

Keywords*—Cytotoxic; MTT assay; Cynometra ramiflora; WiDr cell; IC_{50}

I. INTRODUCTION

According to the world health organisation, cancer to cause of death fifth largest, in the world especially in developing countries [1].Cancer is term, commonly used to mention cell growth very quick and uncontrolled to infiltrate and pressed the normal cells which influence the function of the body.Colon cancer has increased significantly within a decadein Indonesia and in the world [2].In Indonesia, colon cancer is the fifth cancer in all cancer cases.

Recently, the types of cancer treatment are the dissection, radiation and chemotherapy which the effective and safety are still questioned. Therefore, many researchers who do research to get new drugs that are more selective and safer from natural medicine [3].Indonesia is mega biodiversity country that provide source of materials as natural medicine. Traditionally natural medicine from herbs, and vegetables have been used in the treatment of disease cancer [4].

One of the plants have the potential as an anticancer is sala plant or *Cynometraramiflora* Linn. Previous studies stated that these plant have antimicrobial activity [5], antioxidant [6], antidiabetic drug [7], anticancer against some cells lines, such as human gastric, colon, and breast cancer cell lines [8].Based on research of [9], the leaves of sala planthad anticancer activityonMCF-7 cell lines with IC_{50} of 317 µg/mL and on the WiDr cell lines with IC_{50} of 6.37 µg /mL [10].

The purpose of this research was to determine cytotoxic activity of polar, semi polar, and nonpolar fractions of Sala leaves.

II. MATERIALS AND METHODS

A. Materials

1.The device: rotary evaporators, water bath, glassware, compressor, a microscope inverted (olympus ckx41, an incubator CO_2, elisa readers, tissue culture flask (nunclone, laminar water flow (labconco, conical tube, 96-well plate, hemocytometer, counter, and micropipet (soccorex).

2.Matter: the leaves at the end sala(*Cynometraramiflora* Linn.), cells WiDr, ethanol 96 %, acetone, silica g 60 (merck, silica gel GF 254, plate TLC silica gel GF 254, ethanol pa, n-hexane, ethyl acetate, aquabidest, RPMI 1640, FBS 10 %, penicillin-streptomycin,PBS (phosphate a buffer saline), MTT 5 mg/ mL in PBS, SDS 10 %, DMSO 100%, tripsin-EDTA (trypsin 0.25%), anisaldehyde, $FeCl_3$, Citroboric acid, and Dragendorff.

B. Methods

1. A twenty-five grams of extract diluted in methanol and mixed with silica G60 (30-70 mesh).Sample placed at the top of column VLC and eluted by mobile phase (n-hexane: ethyl acetate (8:2; 7.5: 2.5; 7: 3;6: 4; 3: 7 and ethanol, @ 200 mL). Fraction collected and evaluated by TLC method. Fractions collected and combined based on the separation profile.
2. Qualitative evaluation using TLC method with spray reagents. Elution system used was n-hexane: ethyl acetate (7:3) as mobile phase and silica gel GF254 with UV 254 nm and 366 nm.

3.Cytotoxic test

A) A ten milligrams of extract weighed and diluted with 100µL DMSO to obtain 100 µg/ mL. Sub stock made of a solution of stock and added culturemedia so obtained concentration 500, 250, 125, 62.5, 31.25, 15.625, and 7.8125 µg/mL.All performed in LAF cabinet and sterile condition.

B) Cytotoxictest: Cance cell lines WiDrwith density of 10,000 placed into micro plate 96 well, then incubated over night in CO_2 incubator.After that media culture which contain samples removed then added 110 µL media culture that contain 5 mg/mL of MTT into each well. Micro plate then incubated for 4 hours at 37^0C. Alive cell lines will react with MTT to form crystal formazan. After four hours, add the stopper, 100 µL SDS solution to the well, then incubated in room temperature for a night. The solution obtained then measure by ELISA reader at λ 595 nm.

III. RESULT AND DISCUSSION

A. Fractionation

Fractionation aimed to simplify the profile compounds based on polarity the compounds.The result showed that clear profile to classified the fraction (fig 1).

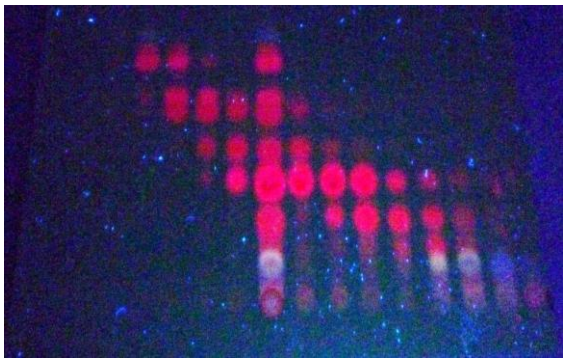

Figure 1.Chromatogram of fractionation (Silica gel GF254, n-hexane: ethyl acetate (7: 3) and observed in uv 366 nm) 13, 14, 15) fractions.

Qualitative evaluation of fractions stated that polar fraction had flavonoids and polyphenol compounds (fig. 2).

C. WiDr Cells Cytotoxic

Cytotoxic test done to see if the polar, semipolar and nonpolar fractions have cytotoxic activity against the polar cell WiDr. The method used is the MTT assay, because it has some advantages such as the sensitivity and the high productivity, the ease in doing so, and speed in performing the method MTT [13]. Absorbtion MTT compounds by living cells and reduced tertazolium reductase system by succinate in the mitochondrial respiratory chain [4]. Mitochondrial enzymes can metabolize the salt tetrazolium tetrazolium ring so that the termination by the enzyme dehydrogenase is causing tetrazolium Crystal turned into the purple formazan which is not soluble in water [14] (figure 5, but soluble in HCl 10 in SDS [15].

Absorbance readings are performed using ELISA reader, because the crystals are soluble formazan SDS 10 in HCl. Absorbance obtained illustrate the number of cells that are still alive. The greater the absorbance obtained, the more cells that are alive and can reduce MTT formazan crystals become.

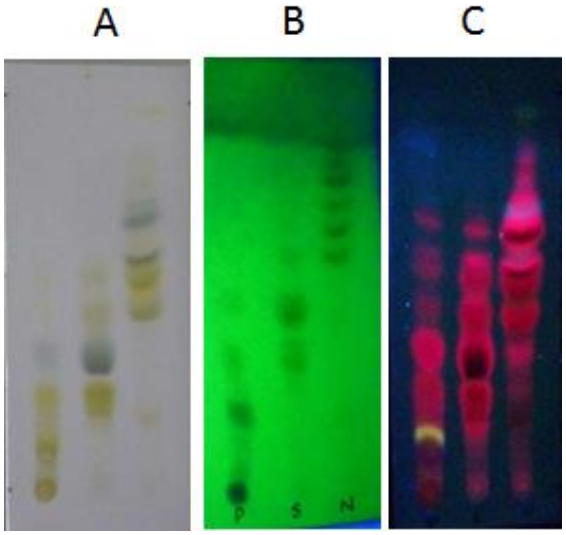

Figure 2. Profile Chromatogram Fractions-fraction before sprayed and observed in (a) the Beam Looked, (b) UV 254 nm, and (c) UV 365 nm with a Silica Plate Silent phase of GF 254 and N-Motion Phase n-Hexane: Ethyl acetate (7:3).

Based on the profile, the fraction grouped into 3 categories, namely polar (fraction number 4, 5 and 6), semi polar (8, 9, 10, and 11) and nonpolar (12,

Fractions' yield showed that polar fraction had highest percentage with 2.94 %, the lowest percentage was semi polar fraction with 2.20%.

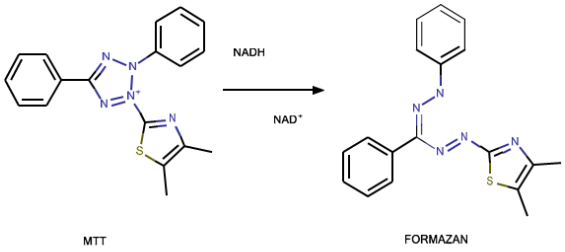

Figure 3: Formazan Reaction

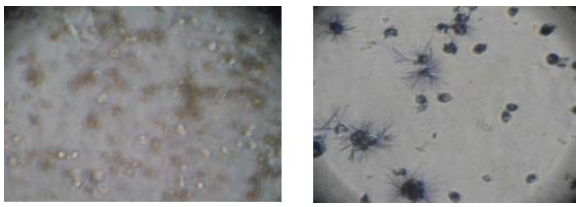

Figure 4. (A) a cell that has received the treatment (a: living cells, b cell: dead), (B) the formation of crystals of Formazan with MTT after treatment (c: crystals of Formazan)

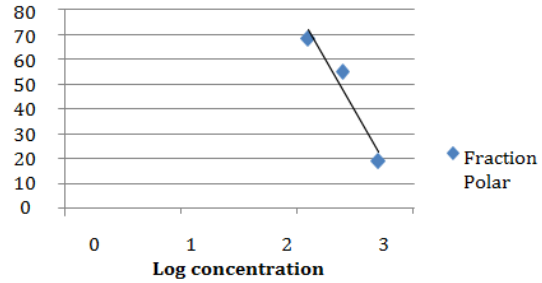

Figure 5. Relationship graph Log Concentration Vs living cells Fraction of ethanol extracts of Leaves of plants Polar Sala *(Cynometraramiflora* Linn).

Based on the image above, it can be seen if the dead cells undergo changes in morphology and look dimmer (figure 4A. b.), whereas the cells that are still life looks light (image. 4A. a). Results of the ELISA readings in the polar fraction.

The test results of cytotoxic fraction polar only taken three point IE at concentrations of 500 µg/mL, 250 µg/mL, and 125 µg/mL because of 3 times the replication done this three-point which gives a profile of live cells are similar. A linear regression equation obtained i.e. Y -81. 349x 242.423 with value R2 of 0.932. IC50 values are obtained for the polar fraction of 231.953 µg/mL. The fraction of ethanol extracts of leaves of plants polar sala have cytotoxic activity in inhibiting cell growth WiDr. The presence of flavonoids content in the polar fraction allegedly capable of inhibiting cell growth WiDr. Flavonoid compounds able to obstruct the process of carcinogenic either in vitro or in vivo. The inhibition occurs at the initiation stage or progression through molecular mechanism of inactivation of carcinogenic compounds, among others, effect tumor cells, inhibition of angiogenesis and cell cycle, apoptosis and induction of antioxidant activity [16].

Other compounds detected in fractions-fraction of ethanol extracts of leaves of plants is chlorophyll sala. The existence of compounds

suspected chlorophyll may inhibit the growth of cells WiDr. Biological activities of chlorophyll that is associated with the prevention of growth of cancer cells. Include antioxidantand animutagenik activities, involve trapping, metabolic modulation of apoptosis induction and xenobiotin [17].

Table 1. Result of Cytotoxicity Semipolar Fraction

Cons.	Log Conc	% Life Cell 1	% Life Cell 2	Conc	Log. Conc.	%Life cell
500	2.69897	3.3308	41.9211	400	2.60206	11.5618
250	2.39794	73.8	80.3447	200	2.30103	74.2104
125	2.09691	74.1961	87.3891	100	2	108.6931
62.5	1.79588	68.9028	91,33	50	1,69897	98.0295
31.25	1.49485	78.2651	87.6354	25	1,39794	99.5943
15.625	1.19382	89.7879	94.9753	12.5	1,09691	97.2181
7.8125	0.89279	102.2469	95.5664	6.25	0.79588	116.8646

Table 2. Result of Cytotoxicity nonpolar Fraction

Conc	Log Conc	% Life cell 1	% Life cell 2	Conc	Log Conc	%Life cell
500	2.69897	32.5339	56.3546	400	2.60206	23.8481
250	2.39794	69.6950	64.7783	200	2.30103	49.3480
125	2.09691	83.9549	79.1625	100	2	67.1979
62.5	1.79588	94.2890	94.8768	50	1.69897	80.9910
31.25	1.49485	83.8825	91.33	25	1.39794	87.3659
15.625	1.19382	100.7346	99.5073	12,5	1.09691	97.8557
7.8125	0.89279	104.5515	104.0886	6.25	0.79588	111.5908

Therapeutic properties which belonged to the chlorophyll that is able to stimulate the immune system, colon cleanse, detox and the ability to prevent the occurrence of cancer and can be used in cancer therapy [18]. Based on research conducted by [19], the presence of chlorophyll in the extract of Conyzatriloba may inhibit the growth of cancer cells HeLa and H4IIE1, A549, HT29, and PC3 cell lines with IC_{50} values of 0.07-0.87 µg. Other studies mention that the concentration of chlorophyll derivative 138 times lower than the MTX (methotrexate) can kill 50 MCF-7 cells which is derived from breast cancer cells [20].

Compared to other studies on leaf extract sala plants WiDr cells against the IC_{50} value of 6.37 µg/mL [10], the IC_{50} value for polar fraction with different extracts. Sala leaf extract has a high activity in inhibiting cell growth WiDr whereas polar fraction has a low activity in inhibiting cell growth WiDr. The difference between the activity of extracts and fractions occurred due to the effects of synergism compounds in the extract so that the possibility of therapeutic activity total extract greater than individual therapeutic activities [21].

The results of testing of semi polar and nonpolar fraction not obtained values of IC_{50}, however, for the fraction of semipolar with 15.625 µg/mL can inhibit cell growth of 7.618, while for non polar fraction with 250 µg/mL can inhibit cell growth of 32.763. The IC_{50} values on both the fraction is not obtained because of the three times the replication has been done not obtained profile of live cells are similar. But only on the concentration of 15.625 µg/mL on a fraction of semipolar and 250 µg/mL in nonpolar fraction that gives a profile of live cells are similar.

Cytotoxic test using Doksorubisin as a positive control. Based on the results of the readings obtained by ELISA, calculation of living cells are listed in table 3.

Table 3.DataLog Concentration and% Life Cell Doksorubisin

Conc	LogConc	%Life
100	2	9.8844
50	1.69897	13.4493
25	1.39794	16.9061
12.5	1.09691	22.8476
6.25	0.79588	29.8693
3.125	0.49485	40.5639
1.5625	0.19382	54.1032

Doxorubisin treatment data using only a 5 poin concentration series, this is because the fifth point gives good value for R2 or linear. Based on the dataliving cells are obtained by linear regression equation, i.e. Y= -30.59E17 X+57.21 with R^2 of 0.970. From the equation obtained values of IC_{50} of 1.721 µg/mL. Doxorubisin used as positive controls because these drugs have been shown to have good potential in inhibiting the growth of cancer cells. In addition, doxorubisin is used as a comparison to see the activity of cytotoxic WiDr cells against from each fraction [22].

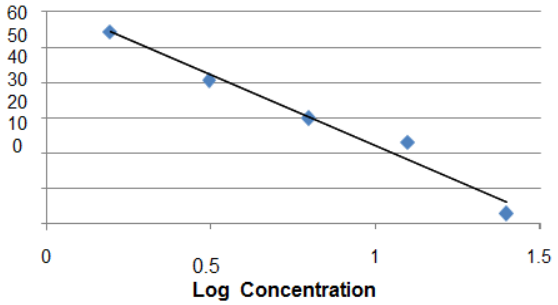

Figure 7. Relationship graph Log Concentration Vs living cells with the treatment Doksorubisirin

IV. CONCLUSION

1. Extract the polar fraction of ethanol plant leaves sala plant (*Cynometra ramiflora* Linn.) cytotoxicity against cell WiDr, semipolar and nonpolar fraction have no cytotoxic activity against WiDr cells.
2. Polar fraction of IC_{50} values 231.953 µg/mL, whereas for the fraction of semipolar and nonpolar IC_{50} value is unavailable.
3. Compounds contained in the fraction of ethanol extracts of leaves of plants polar sala is a phenolic, flavonoids, and alkaloids, and at a fraction of semipolar and nonpolar compounds contain phenolic and alkaloids.

Acknowledgment

We would like to thank to Rector UMS and Dean of Faculty of Pharmacy Universitas Muhammadiyah Surakarta for support this research.

References

[1]. WHO, 2008, *Global Cancer Control; Worldwide Cancer Burden*, 42-55, Geneva, Switzerland,WHO Press.

[2]. Mardihusodo, H. R., Wahyuningsih, M. S. R., Ardian, O. & Empel, G. V., 2011, Sitotoksik Campuran Ekstrak Etano lDaun Kembang Bulan (*Tithonia diversifolia* (Hemsley) A. Gray.) dan Rimpang Kencur (*Kaempferia galangal* L.) pada Sel WiDr, *Majalah Obat Tradisional,* 16,3,174-181.

[3]. Mangan,Y., 2003, *Cara Bijak Menaklukkan Kanker*, 1, 29, 30, Jakarta, Argomedia Pustaka.

[4]. Maryati & Sutrisna, EM., 2007, Potensi Sitotoksik Tanaman Ceplukan (*Physalis angulata* L.) terhadap Sel HeLa, *PHARMACON*, 8, 1, 1-6.
[5]. Khan, M.A.A., Prasanta, P. & Mohammed, T.I., 2006, *Phytochemical and Pharmacological Screening of Shingra (Cynometra ramiflora* Linn*. Family: Leguminosae) Bark Based on Its Traditional Uses*, Departmentof Pharmacy Southern University.
[6]. Bunyapraphatsara, N., Jutiviboonsuk, A., Sornlek, P., Therathanathrn, W., Aksornkaew, S., Fong, H. H. S., *et al*., 2003, Pharmacological Studies of Plants in the Mangrove Forest, *Thai Journal of Phytopharmacy*, 10, 2, 1-11.
[7]. Tiwari, P., Rahuja, N., Kumar, R., Lakshmi, V., Srivastava, M.N., Agarwal, S.C., Raghubir, R., *et al*., 2008, Search for Antihyperglycemic Activity in Few Marine Flora and Fauna. *Indian Journal of Science and Technology*, 1(5),1-5.
[8]. Uddin, S. J., Grice, D. I. &Tiralongo, E., 2009, Cytotoxic Effects of Bangladeshi Medicinal Plant Extracts. Original Article. *eCAM Advance Access published* August, 25, 1-6.
[9]. Subarnas, A., Diantini, A., Abdulah, R., Zuhrotun, A., Yamazaki, C., Nakazawa, M. & Koyama, H., 2012, Antiproliferative Activity of Primates-Consumed Plants against MCF-7 Human Breast Cance rCell Lines,*Journal of Medicina lResearch*, 1, 4.
[10]. Haryoto, Indrayudha, P., Muhtadi, Suhendi, A. & Sujono, T. A., 2013, *Cytotoxic Effects of Ethanol Extract from Cynometra ramiflora Linn leaves on T47D, HeLa and WiDr Cancer Cell Lines*, International Conference of Natural Product (ICNP2013).
[11]. Rohman, A., 2009, *Kromatografi untuk Analisis Obat*, Yogyakarta, Graha Ilmu.
[12]. Wagner, H. & Bladt, S., 1996, *Plant Drug Analysis : A Thin Layer Chromatography Atlas*, second edition, Springer-Verlag, Berlin.
[13]. Ferrari, M., Fornasiero M. C. & Isetta A. M., 1990, MTT Coloricmetric Assay For Testing Macrophage Cytotoxic Activity in Vitro, *Journal of Immunological Methods,* 131, 165-172.
[14]. Mosmann,T.,1983, Rapid Colorimetric Assay for Cellular Growth, and Surviral: Aplication to Proliveration and Cytotoxicity Assay, *Journal of Immunological Methods*.
[15]. Doyle, A. & Grifith, S. J. B., 2000, *Cell and Tissue Culture for Medical Research*, John Willey and Sons, Ltd, NewYork.
[16]. Ren, W., Qiao, Z., Wang ,H., Zhu ,L. & Zhang, L., 2003, Flavonoids: Promosing Anticancer Agents, *Medicinal Research Review*, 23, 4, 519-534.
[17]. Ferruzzi, M. G. & Blakeslee, J., 2007, Digestion, Absorption, and Cancer Preventative Activity of Dietary Chlorophyll Derivatives, *Nutrition Research*, 27, 1, 1-12.
[18]. Inanc, A.L., 2011, Chlorophyll: Structural Properties, Health Benefits and Its Occurrencein Virgin Olive Oils, *Academic Food Journal*, 9, 2, 26-32.
[19]. El-Sayed, W. M., Hussin, W. A., Mahmoud, A. A. &Alfredan, M. A., 2013, The *Conyzatriloba* Extracts with High Chlorophyll Content and Free Radical Scavenging Activity had Anticancer Activity in Cell Lines, *Biomed Research International*, 2013 (2013), 11.
[20]. Gomaa, I., Ali, S. E., El-Tayeb T. A. & Abdel-kader, M. H., 2012, Chlorophyll Derivative Mediated PDT Versus Methotrexate: an in Vitro Study Using MCF-7 cells, *Phatodiagnosis and Photodynamic Therapy*, 9(4), 362-368.
[21]. Dewick,P.M., 1987, *Tumor Inhibitor From Plants in Pharmacognosy 12th Edition,* Edited by George Eduward Trease & William Charles Evans, ELBS published Bailliere Tindal, 33th Avenue, Eastbourne (631).
[22]. Herwinda, S., 2011, Efek Ekstrak *Andrographis panicula* Ness. Terhadap Colon Cancer Modelling dengan Induksi DMBA, *Tesis*, Program Pascasarjana Faklutas Farmasi, Universitas Airlangga.