P-ISSN: 2305-6622; E-ISSN: 2306-3599
International Journal of
Agriculture and Biosciences
www.ijagbio.com; editor@ijagbio.com

Research Article

Active Compound Characterization and Cytotoxic Activity of Soursop (*Annona muricata* L) Leaves Extract Against Murine Cells Leukemia P-388

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Article History: Received: April 23, 2016 Revised: July 05, 2016 Accepted: September 02, 2016

ABSTRACT

The Objectives of this study were to characterize active compounds from leaves of soursop and determine cytotoxic activity against murine leukemia P–388 cells. Leaves of soursop were macerated in 96% ethanol then dried with rotary evaporator. The leaves extract was fractionated using Vacuum Liquid Chromatography (VLC) to produce uncomplex fractions. A toxicity screening test was conducted using Brine Shrimp Lethality Test (BSLT). The results showed that fraction A, C and F had LC_{50} values of 275.5, 320.3 and 94.5 µg ml^{-1}, respectively. These fractions were further purified using preparative thin layer chromatography. Based on LC_{50} value and yield then F3, F fraction and EtOAc extract then were analyzed by MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay to determine cytotoxic activity against murine leukemia P-388 cells. LC_{50} value for the EtOAc extract, F fraction and F3 fraction were 0.16, < 0.1, and > 100 µg mL^{-1}, respectively. These showed that activity of EtOAc extract and F fraction in inhibiting growth of murine leukemia P-388 cancer cells were five times better compared to positive control (Artonin E). Characterization of active compound was performed using gas chromatography-mass spectroscopy. Chromatogram showed 20 peaks of F fraction, ten peaks of phenolic compounds and other peaks showed alkaloids, terpenoids and fatty acids, while in F3 fraction, dominant peak was at 28,67 minutes retention time. This peak indicated potential compound, a pyrido [1',2':1,2] imidazo [5,4-c]-cinnoline which was an alkaloid.

Key words: *Annona muricata* L, anticancer, leukemia, murin P-388

INTRODUCTION

Cancer is one of most deadly disease in the world nowaday. Scientistcontinously conductingvarious experiments using natural substances that have anticancer activity to reduce cancer risks.*Annona squamosa*, *Annona montana*, *Annona dioica* and *Annona muricata*are some species of Annona thathad been well reported genus as anticancer (Consolacion *et al*., 2012; Torres *et al*., 2012; Gu *et al*., 1996; Sun *et al*., 2014). However, soursop (*A. muricata*) is more popularlyrecognized as "The Cancer Killer" thanother species in the same genus and empirically has been used as traditional medicine for cancer treatment. Some anticancer research using soursop leaves have been often reported in crude extract. As reported by Hamizah *et al*. (2012), ethanol extract of sourspop leaves could reduce papilomagenesis effect. Another study also reported that the ethanol extract could inhibit EACC carcinoma Erlich cancer cell and both MDA and SKBR3 breast cancer cell (Gavamukulya *et al*., 2014), while the methanol extract has cytotoxic effect on HEp-2 larynx and NCI-H292 cancer cell (Melo *et al*., 2010).

Experiments on anticancer using pure compound, was mainly focused on acetogenin which was a polyketide. More than 400 acetogenins from annonanceae have been succesfully isolated (Aminimoghadamfarouj *et al*., 2011). As reported by Sun *et al*. (2014), Consolacion, *et al*. (2012), Kim *et al*. (1998) and Gu *et al*. (1996) acetogenin compound fromsoursop has cytotoxic activity against some cancer cells such as pancreatic PACA-2, prostate PC-3, lungs A-549, hepatoma Hep G2, MCF-7 breast, kidney A-498 and colon HT-29 cancer cells. Besides acetogenin, alkaloids compounds such as anonaine and annonamine have been isolated from soursopleaves and showed cytotoxic effect on neuroblastoma cells SH-SY5Y (Matsushige *et al*., 2012). Kaempferol extract, a flavonoid also obtained from soursop leaves showed inhibitory

Cite This Article as: Hidayat S, P Sugita and IH Suparto, 2016. Active compound characterization and cytotoxic activity of soursop (*Annona muricata* l) leaves extract againts murine cells leukemia P-388. Inter J Agri Biosci, 5(6): 347-351. www.ijagbio.com

activity against Raji cells (Mustariani, 2011). Even though the activity in various cancer cells have been reported, secondary metabolites of soursopand its activity against murine leukemia P-388 cells remains unknown. Because an anticancer should be able to demonstrate its inhibitory activity on various types of cancer cells, research on anticancer activity of soursop leaves still needed. Therefore, this study aimed to detect active compounds from soursopleaves that have cytotoxic activity against murine leukemia P-388cells.

MATERIALS AND METHODS

Sample preparation

Samples were collected from Conservation and Cultivation unit of Biopharmaca Research Center, Bogor Agricultural University, Indonesia. Samples were washed and dried until the leaves turned dry then powdered, the water content of powder was determined (AOAC 2005).

Sample extraction

Approximately 2 kg of dried leaves powder of *Annona muricata* Lwere macerated with 96% ethanol for 3x24 hours. Then, macerate was concentrated to determine the amount of yield. Ethanol extract was separated from the chlorophyll and tannins using MeOH: H_2O (1: 1) solvent for 72 hours. The precipitate obtained was chlorophyll, while filtrate was liquid-liquid partitioned using EtOAc to obtain tannin and EtOAcextract. Bothphasewere concentrated and the yield were determined (modification from Minarni and Okeke 2014). Secondary metabolites of all extracts then weretested (Harborne, 1987).

Fractionization and characterization of active fraction

EtOAc extract was fractionated by Vacuum Liquid Chromatography (VLC) using best eluent. Staining pattern of eluates were analyzed by Thin Layer Chromatography (TLC), and the eluates with the same staining pattern were combined as well as the toxicity was tested using Brine Shrimp Lethal Test (BSLT). The best TLC profile and BSLT result of fractions purified using Preparative Thin Layer Chromatography (PTLC). Staining pattern from PTLC was observed. Furthermore, single stain profile were tested using BSLT and characterized using GC-MS analysis (modification from Moghadamtousi *et al*. 2014).

Toxicity test using brine shrimp lethality test (BSLT) methods

Amount 20 mg of *Artemia salina* eggs were hatched and incubated for 48 hours. The mother solution of fraction that prepared with a concentration of 1000 ppm then diluted based on concentrations used. Ten larvae along with tested sample solution were entered in each well, then incubated for 24 hours. Percentage of dead larvae was determined and LC_{50} values was determined by probit analysis (Meyer *et al*., 1982).

Cytotoxicity assay against murin leukemia P – 388 Cell

Cell suspension was firstly made of 3 x 10^3 cells mL^{-1} of RPMI 1640 media. The cells were inoculated in microplate96 and cultivated in a CO_2 incubator for 24 hours. Samples were diluted by adding a phosphate buffer solution (PBS) with pH of 7.30 to 7.65 with different concentrations (0, 0.1, 0.3, 1, 3, 10, 30 and 100 μg mL^{-1}) then added into microplate, while Artonin E used as positive control with concentrations 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1 μg mL^{-1}. Cells were incubated for 48 hours, then MTT (3- (4,5-dimethyl thiazole-2-yl) -2,5-diphenyl tetrazolium bromide) reagent was added. The cells that was shaken by a microplate mixer then incubated for four hours until stop solution (SDS) was added. After that, the microplate was re-incubated for 24 hours.

Measurement of the optical density (OD) carried out by microplate reader at 540 nm wave length, 24 hours after the addition of stop solution (Alley *et al*., 1988). OD data then inserted in the Origin 8 software to calculate the IC_{50} value. Compound content of best isolates were identified using Gas Chromatography-Mass Spectroscopy analysis.

RESULTS

Secondary metabolites

Extract of ethanol obtained from 2000 g macerated sample was 285.91 g (15.71%). Three extracts: chlorophyll, tannins and EtOAc were yielded by separation process of each weight were 124.4, 95.5 and 27.54 g, respectively. The secondary metabolite results for each extract were shown in Table 1.

Table 1: Secondary metabolites of ethanolic, EtOAc, tannin and chlorophyll extract

Secondary metabollites group	Phytochemical test result of extract			
	Ethanol	EtOAc	Tannin	Chlorophyll
Flavonoid	+++	+++	-	-
Phenol	+++	+++	+++	+++
Terpenoid	-	-	-	-
Steroid	+++	-	-	+++
Tannin	+	-	++	+
Saponin	-	-	-	-
Alkaloid	+++	+++	-	-

Note: + = detected, - = not detected, number of + = colour intensity

Fractionization and toxicity

Twenty grams of EtOAc extract was fractioned by VLC using *n*-hexane: EtOAc eluent gradient and methanol was used in the final step. Eighteen fractions were obtained from this process. Staining pattern was observed in all fractions by TLC, and same staining pattern then merged so that nine fractions were obtained: A-I fraction (Figure 1). Toxicity of all fractions (A-I) obtained from BSLT is shown in Table 2.

Based on VLC analysis, activity of each fraction arranged in following order: G > E > I > F > B > A > C > H > D, while availability was G > H > E > F > A > D > C > B. A, C and F fraction were selected to be analyzed in next purification step using PTLC. Stain profile of all fractions obtained from PTLC analysis were observed by VLC. *n*-hexane: EtOAc eluent was used for A and C fraction (Figure 2) and DCM: MeOH (19.5:0.5) eluent was used for F fraction (Figure 3). Toxicity of fraction showing single stain: A_1, A_2, A_3, A_4 and F_3, were measured by BSLT method and shown in Tabel 3. Based on PTLC analysis, activity of each fraction arranged in following order: A_2> F_3> A_4> A_1> A_3, while availability was F_3> A_3> A_4> A_2> A_1.

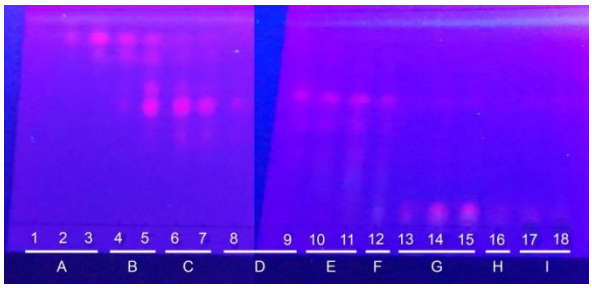


Fig. 1: TLC profile of 1-18 eluates from VLC analysis by *n*-hexane:EtOAc (7:3) eluent under UV light (λ_{366nm}).

Table 2: Yield and toxicity value of fractions obtained from EtOAc extract by VLC

Fraction	weight (g)	Yield (%)	LC_{50} (µg ml^{-1})
A	0.16	0.79	275.53
B	0.08	0.42	158.98
C	0.08	0.42	320.35
D	0.15	0.77	941.71
E	0.25	1.27	70.09
F	0.21	1.05	94.51
G	0.61	3.05	64.09
H	0.42	2.10	334.71
I	17.88	89.44	83.62

Table 3: Yield and toxicity value of fractions obtained from PTLC analysis

Fraction	weight (mg)	yield (%)	LC_{50} (µg ml^{-1})
A_1	32.1	20.11	259.23
A_2	38.7	24.23	73.42
A_3	50.8	31.83	272.12
A_4	49.5	31.01	128.30
F_3	146.7	69.86	79.23

Table 4: IC_{50} value of sample againts growth of murin leukemia P-388 cell

Sample	IC_{50} (µg. mL^{-1})
EtOAc extract	0.16
Fraction F	< 0.1
Fraction F_3	> 100
Artonin E	0.76

Table 5: Identified compund in F fraction based on library index

Peak (Pk)	Retention time (Rt)	Library/ID
1	5.87	2-methoxy-4 vinylphenol
2	6.84	cinnamic acid
3	7.48	p hydroxybenzoic acid
4	7.97	4-hydroxy-3-methoxybenzoic acid
5	8.74	3-metoxy-4-hydroxybenzoic acid
6	9.4	(3,3-dimethylbicyclo (2.2.1) hept-2-ylidene-2-propanone
7	9.61	(-)-loliolide
8	9.75	3-butylindolizidine
9	9.9	1-propanone,3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)
10	10.18	phenyl thiolacetate
11	10.61	7,9-di-tert--butyl-1-oxaspiro [4.5] deca-6,9-diene-2,8-dione
12	10.8	palmitic acid
13	11.07	4-ethoxy-2,5-dimethoxybenzaldehyde
14	11.93	oleic acid
15	12.07	stearic acid
16	12.26	1,5-diphenyl-1,5-pentanedione
17	16.48	4-(2H-1,4-benzothiazin-3-yl)-3-phenylsydnone
18	17.28	pyrido[1',2':1,2] imidazo [5,4-c]-cinnoline
19	17.7	4-hydroxy-9-vinyl-adamantane-2,6-dione
20	18.34	methyl E-3--(4'-formyl)-2',5'-dihydroxyphenyl)-2-methylpropenoate

Anticancer activity against murin leukemia P-388 Cell

Cytoctoxic test against murin leukemia P-388 cell was carried out for EtOAc, F and F_3fraction extract, while positive control was Artonin E. Cytotoxic activity of all extract tested was shown in Table 4.

Active compound characterization

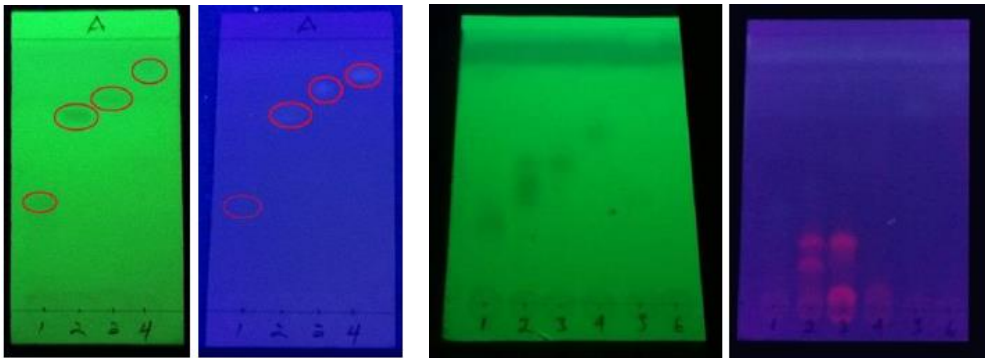
F fraction consisted of 20 compounds according to mass spectroscopy library index, while fraction F_3 consisted of nine compounds, with a dominant compound at 28.67 minutes retention time (Table 5). This compound was detected as pyrido [1',2':1,2] imidazo[5,4-c]-cinnoline based on Willey09TH library index with 83% similarity level. Mass spectra was shown in Figure 4.

DISCUSSION

Ethanol extract contained various secondary metabolites such as flavonoids, phenolics, steroids, tannins and alkaloids. After the separation process of chlorophyll and tannins, secondary metabolites from EtOAc extract detected were only flavonoid, phenol and alkaloid. Some compounds of this class had been isolated from soursop leaves that showed anticancer activity (Matsushige *et al*., 2012; Mustariani 2011; George *et al*. 2014). Therefore, the EtOAc extract in our study further proceed to separation step.

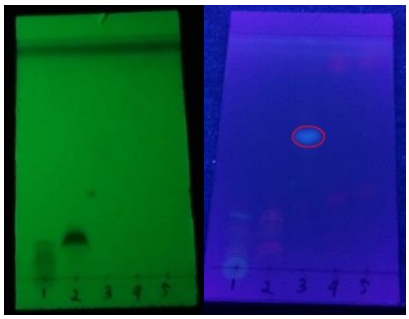
The fractionization using VLC aimed to obtain a more simple fraction. Searchof functionally isolates as anticancer was performed by BSLT. LC_{50} value as toxicity parameters provides information about the concentration of active substance required to kill 50% of the population of experiment animals. Toxicity of a substance grouped into three categories, which were highly toxic when LC_{50} value <30 µg mL^{-1}, toxic when the LC_{50} value ≤ 30 ≤ 1000 µg mL^{-1} and classified as not toxic when LC_{50}> 1000 µgml^{-1} (Meyer *et al*. 1982). A-I fraction were classified as toxic referring to the categorization, because LC_{50} value were in the range of 30-1000 µg mL^{-1}. Fractions of A, C and F were selected to be proceed into separation step using PTLC based on toxicity value of the fraction, yield number, stain separation pattern on TLC, and representation of polarity attribute. In this case, A, C and F fractions represented non-polar, semi-polar and polar fraction, respectively. Compared to LC_{50} value before purification, LC_{50}value after purified was lower. It showed the effect of higher toxicity after purification. LC_{50} value and availability of fractions then were considered to determine which fraction selected to test the cytotoxicity activity against murine leukemia P-388cell.

Base on cytotoxicity assay, the extract of EtOAc, F and F3fractionsshowed very low IC_{50} values. According to Alley *et al* (1988) stated that compounds activity grouping can be based on IC_{50} values. It will be very active whenIC_{50}value between 0 and 2 µg mL^{-1}, active if value is between2 and 4 µgmL^{-1} and inactive when value obtained more than 4 µgmL^{-1}. Based on these categories, then the EtOAc extracts and F fractions had an activity which were classified very active in inhibiting the growth of murine leukemia P-388 cancer cells. Compared to positive control, IC_{50} value obtained by extracts of EtOAc and F fraction were even five times better.



(a) A (b) (a) B (b)

Fig. 2: TLC profile resulted by separation process. (A) A fraction; (B) C fraction by PTLC analysis using *n*-hexane: EtOAc (7:3) eluent. (a)under λ_{254nm} and (b) λ_{366nm} UV light.



(a) (b)

Fig. 3: TLC profile resulted by separation process of F fraction by PTLC analysis using DCM: MeOH (19.5:0.5) eluent. (a) under λ_{254nm} and (b) λ_{366nm} UV light.

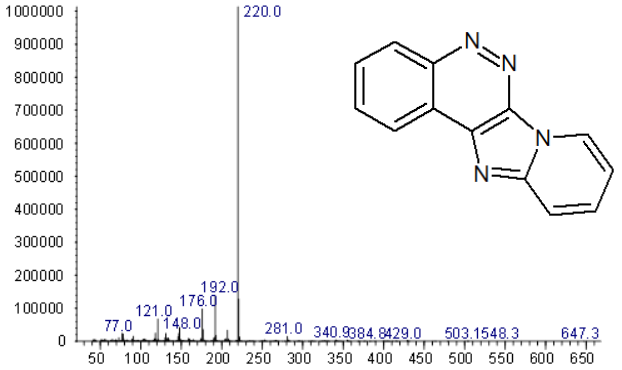


Fig. 4: Mass spectra of F_3 fraction and compound estimation based on WILLEY09TH library index at 28.67 minutes retention time.

However, F_3fraction was classified as inactive because of content difference of each fraction. There were potential compound groups on EtOAc and F fractions such as phenols, flavonoids and alkaloids, while dominant compounds in F_3 fraction that found were pyrido [1 ', 2': 1,2] imidazo [5,4-c] -cinnoline (Figure 7) which was an alkaloid with the ring nitrogen. Absence of hydroxyl groups on the ring made the compound inactive. It is because hydroxyl groups that attached to the aromatic ring is an important factor for cytotoxic activity of compound (Ito *et al*. 2003).

Conclusion

EtOAc extract of the soursop leaves has potential as an agent of leukemia anticancer. Purification of the extract also showed increased inhibitory activity against murine leukemia P-388cancer cells. This was indicated by increasing the IC_{50}value of F fraction, <0.1 μg mL^{-1}. This activity was presumably caused by phenolic compound based on characterization result.

Acknowledgements

The authors thank the Directorate General of Higher Education for education and research grant, and to all Organic Chemistry Laboratory assistants in Bogor Agricultural University (IPB), which has assisted the authors in providing tools and materials during the research.

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