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SELECTIVE POLARITY-GUIDED EXTRACTION AND PURIFICATION OF ACETOGENINS IN *Annona muricata* L. LEAVES

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ABSTRACT

Herein is described the selective polar-guided extraction and column chromatography separation of annonaceous acetogenins from the leaves of *Annona muricata* L., commonly known as soursop. The objective of this study is to obtain an acetogenin-rich fraction, containing mainly annonacin, known to be present in the highest amount in soursop leaves. Solvents with various polarities (ethyl acetate, hexane, chloroform, ethanol, water) and their mixtures were used in the extraction, fractionation, and isolation steps. The liquid chromatography-mass spectrometry analysis of the isolate sample showed that annonacin, having a molecular mass of 597.23, is the dominant acetogenin present. The amount of acetogenin isolated from the soursop leaf samples was 242 mg/g, based on the total lactone determination using the Kedde reagent. The cytotoxicity activity of the isolate sample was determined using the brine shrimp test. This study shows that there is a positive correlation between lactone content due to acetogenins and the level of cytotoxicity in the fractions obtained. The low LC_{50} value of 1 ppm showed a significant cytotoxicity of the ethyl acetate fraction obtained, higher than cytotoxicity of cancer drug cyclophosphamide. High lactone content and a high cytotoxicity of the ethyl acetate fraction indicate the potential of *A. muricata* leaves as the source of bioactive compounds for anti-cancer treatment.

Keywords: Acetogenin; *Annona muricata*; Annonacin; Extraction

1. INTRODUCTION

Cancer is the leading cause of death in the developed world and the second-leading cause of death in developing countries. As of 2012, more than 60% of the cancer cases comes from the developing countries. That number represents two-thirds of the deaths caused by cancer, which are 8.1 million people. Treatment of cancer using conventional chemotherapy is quite expensive and has negative side effects, such as the decreased function of the healthy organs. Therefore, treatment using bioactive compounds obtained from plants would be a promising alternative approach.

Soursop (*Annona muricata* L.) is a plant in the family *Annonaceae* used as a source of natural remedies. The leaves and stems of soursop show active cytotoxicity against cancer cells, due to bioactive compounds called acetogenins. These acetogenins are non-toxic to normal cells, but are highly toxic to cancer cells (Oberlies et al., 1995; Villo, 2008). Acetogenins from the soursop plant are usually extracted using ethanol as the organic solvent (Zeng et al., 1996). These compounds, collectively, have shown antitumor, parasitical, pesticidal, and antimicrobial

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activities (McLaughlin, 2008; Oberlies et al., 1997). The cytotoxicity of annonaceous acetogenins is due to the depletion of ATP levels via the inhibition of the NADE-ubiquinone oxidoreductase (Complex I) of the mitochondrial electron transport system (Zafra-Polo et al., 1996). The lactone ring present in acetogenin molecules plays an important role in the anticancer mechanism, present (Kreuger et al., 2012). Annonacin is known as the most prevalent acetogenin present in soursop leaves (Champy et al., 2004; Yuan et al., 2003), however, the method for separation of annonacin from soursop leaves is not widely reported. More reports are available on various methods to obtain new acetogenins from soursop leaves (Kim et al., 1998; Zeng & McLaughlin, 1995).

The objective of this study was to obtain a simple and practical separation technique for acetogenins, mainly annonacin, from soursop leaves. In order to isolate annonacin, selected solvents with various polarities (Luna et al., 2006). Kedde reagents were employed to determine the total lactone that represented the amount of acetogenins present in each of the fractions. Andrographolide was used as the standard compound in the determination of the total lactone present in the sample (Aromdee et al., 2005), while Brine Shrimp Test (BST) was used to determine the cytotoxicity of the isolate samples (Kim et al., 1998). A liquid chromatography-mass spectrometry (LC-MS) analysis was used to validate the presence of annonacin in the sample.

2. EXPERIMENTAL

2.1. Chemicals

Silica gel 60, 3.5-dinitrobenzoic acid (>99%), and HPLC-grade solvents (ethanol, ethyl acetate, n-hexane, dimethylsulfoxide) were purchased from Sigma Aldrich. Standard andrographolide (min. 98%) was purchased from the Aktin Chemical Inc. (China).

2.2. Isolation of Annonacin

The procedure to isolate acetogenins reported by Luna et al. (2006) was used and modified. Figure 1 shows the flow diagram of the fractionation and isolation of acetogenins from soursop leaves. The first stage is the maceration of the soursop leaves using ethanol. Approximately 500 g dried soursop leaves (*Annona muricata* L) were reduced in size, and then soaked in 95% ethanol for 5 days. Using a rotary evaporator, ethanol was then evaporated and the sludge was redissolved in acetone. The solution was passed into a Buchner funnel containing activated carbon to reduce the levels of chlorophyll and then the acetone was evaporated.

The second stage is fractionation of the obtained solid, which was carried out in four stages using a Buchner funnel with silica gel 60 on a filter paper. The solvents used to leach the solid crude extract were water, water-ethanol (7:3 v/v), and water-ethanol (1:1 v/v) to obtain fractions referred to as F1, F2, and F3, respectively. Then, the following solvents were used consecutively, combined, and evaporated using a rotary evaporator to obtain fraction F4: ethanol, ethanol-ethyl acetate (1:1 v/v), and ethyl acetate. The F4 fraction was sent to an open column chromatography with silica gel 60 as the stationary phase. Solvents used as eluents were hexane, hexane-chloroform (8:2 v/v), hexane-chloroform (1:1 v/v) + chloroform, and ethyl acetate. For further testing, the resulting ethyl acetate fraction F4.4 fraction was separated and evaporated to dryness.

2.3. Kedde Reagents and Lactone Concentration Test

Acetogenins were quantified as the total lactone present in the plant extracts, using andrographolide as a standard compound. Both acetogenins and andrographolide form a pink-purple colored complex with the addition of the Kedde reagent (Aromdee et al., 2005). This reagent consists of equal volumes of 2% (w/v) solution of 3.5-dinitrobenzoic acid in ethanol and 5.7% (w/v) solution of KOH in ethanol. The sample solution was mixed with the Kedde

reagent in 2:1 ratio and incubated for 1 min. The absorbance was read using a UV spectrophotometer (UNICO 1100RS) at 536 nm and compared to the standard absorption curve of andrographolide. The quantity of acetogenins present is expressed as total lactone equivalent to andrographolide.

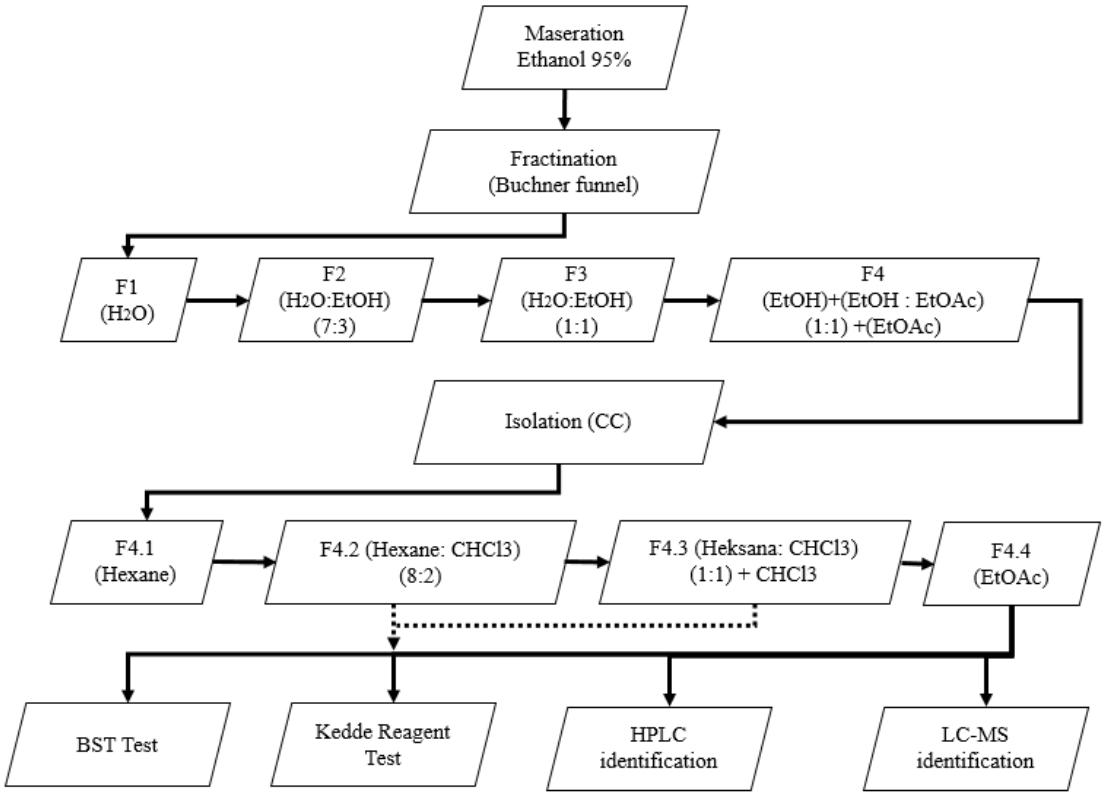

Figure 1 Flow diagram of acetogenin separation and purification

2.4. Brine Shrimp Test (BST) for Cytotoxicity Determination

The procedure used follows that reported by Meyer et al. (1982). Sample solutions with different concentrations were prepared in dimethylsulfoxide (DMSO): 10 mg of the sample in 10 ml of DMSO (1000 ppm solution A), 9 mL of DMSO added into 1 mL of solution A (100 ppm solution B), and 9 mL of DMSO into 1 mL of solution B (10 ppm solution C). Then, 0.05 mL of each sample solution was pipetted into test tubes and 5 mL of seawater and 10 shrimp larvae were added. The control solution for this test was prepared with similar treatment as the sample solution, but with 0.05 mL of DMSO added into the test tube. Sample and control solutions were prepared in triplicate. The test tubes containing shrimp larvae were left for 24 hours under illumination. Using the following equation, the percentage of dead larvae was calculated at the end of the test period.

$$\%\ \text{dead larvae} = \frac{\text{Dead shrimp (test solution)} - \text{dead shrimp (control solution)}}{\text{Total shrimp used}} \times 100\% \quad (1)$$

The lethal concentration for 50% of the population (LC_{50}) values were determined using a linear regression from the plot of the percentage of dead larva and the sample solution concentration.

2.5. HPLC and LC-MS Analysis

A Shimadzu (Japan) High Performance Liquid Chromatography (HPLC) equipped with a C18 reverse phase column type (4 mm diameter and 250 mm long) was used for qualitative analysis of the fractions obtained. The Waters ACQUITY QTOF Xefo G2S FT Liquid Chromatography-Mass Spectrometry (LC-MS) instrument equipped with an LC column (2 mm diameter and 250 mm long) was used to determine the type of acetogenin obtained. The eluent was a mixture of methanol-water (85:15 v/v) used in an isocratic system.

3. RESULTS AND DISCUSSION

3.1. Total Lactone in Acetogenin using Kedde Test

All of the sample solutions and standard solution showed a pink-purple color after addition of the Kedde reagent. The calibration curve of the standard solution showed a good linearity (R^2 = 0.9887). The total lactone of sample/fractions were determined based on this calibration curve and the results are shown in Table 1. The total lactone content of the fractions decreased in the following order: F4.4 > F4.3 > F4.2 The observed trend indicates that fractions containing higher lactone concentrations were obtained using slightly polar solvents, such as ethyl acetate, rather than the less polar solvents. In addition, the amount of lactone in the ethyl acetate (F4.4) fraction was also found to be higher than that in the F005 fraction, obtained without the open column chromatography (Kim, 1998). The results indicate that the extraction-isolation method used in this study improved the extraction yield of lactone-containing compounds.

Table 1 Lactone concentration and LC_{50} in each sample solution

Sample/fraction	Eluent	LC50 (ppm)	Lactone concentration (mg/gr sample)
cyclophosphamide*	-	16.3	-
F4.4	ethyl acetate	1.04	242
F4.3	hexane:CHCl3 (1:1) + CHCl3	90.6	163
F4.2	hexane:CHCl3 (8:2)	182.0	123
F005**	-	9.3	188

* M.J. Moshi, 2010

** Ethanol extract, ethyl acetate fractionation, without column separation

3.2. The Cytotoxicity Test using the Brine Shrimp Test (BST)

BST test results from several sample solutions and control solutions were used to determine LC_{50} values, i.e. the bioactive concentration corresponds to 50% mortality rate of the shrimp larvae tested. Figure 2 shows the comparison of the average percentage of shrimp larvae mortality as a function of the logarithmic concentration of the tested solutions.

Table 1 gives the LC_{50} values of each fraction obtained from the extraction and fractionation of the soursop leaves. As the polarity of the eluent used is greater, F4.4 (ethyl acetate) > F4.3 (mixture of hexane-$CHCl_3$ 1:1 v/v and pure $CHCl_3$) > F4.2 (hexane-$CHCl_3$ 8:2 v/v), their LC_{50} value are lower (1.04 < 90.6 < 182.05 ppm). Comparison between the LC_{50} of the F005 fraction (9.32 ppm), obtained using a different extraction method, and the LC_{50} of the F4.4 fraction (1.04 ppm), indicates that F4.4 fraction has greater cytotoxicity activity. This table also indicated that the lactone concentration is high in the low LC_{50} value fractions. This result

indicates that there is a correlation between the lactone content due to the presence of acetogenins in the samples and its cytotoxicity. Moshi et al. (2010) reported that plant extracts with an LC_{50} value below 20 ppm could be considered having anticancer compounds. They also reported that brine shrimp test is a proven parameter for predicting the presence of anticancer compounds in plant extract (Asif et al., 2013). In addition, the LC_{50} value of the F4.4 fractions is much lower than that of the anticancer drug cyclophosphamide. This result indicated that ethyl acetate fraction (F4.4) has the potential to be used as an active compound in controlled drug release for cancer therapy.

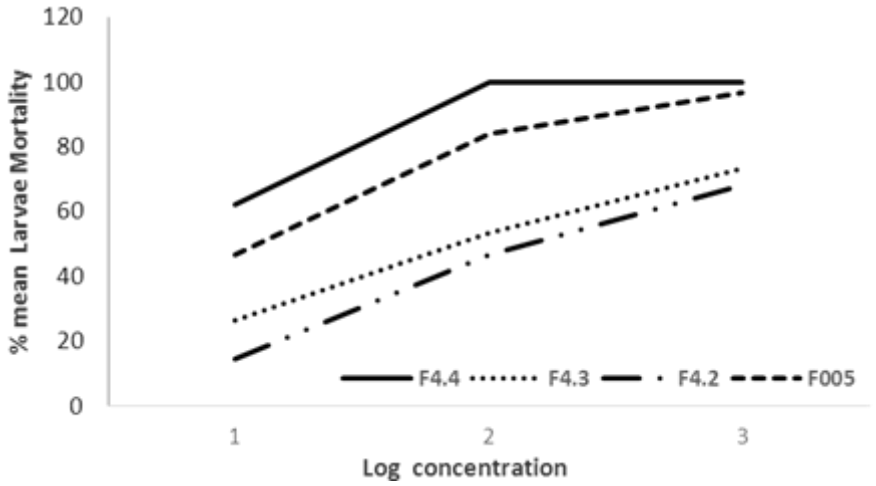

Figure 2 Average percentage of shrimp larvae mortality vs. logarithmic concentration

3.3. HPLC and LC-MS Analysis

The method of extraction and isolation to obtain the fraction F4.4 worked quite effectively since only several peaks appeared in the liquid chromatogram at a retention time longer than 25 min. The compounds separated on HPLC analysis were clarified further using LCMS analysis. The LCMS graph of F4.4 fraction is shown in Figure 3.

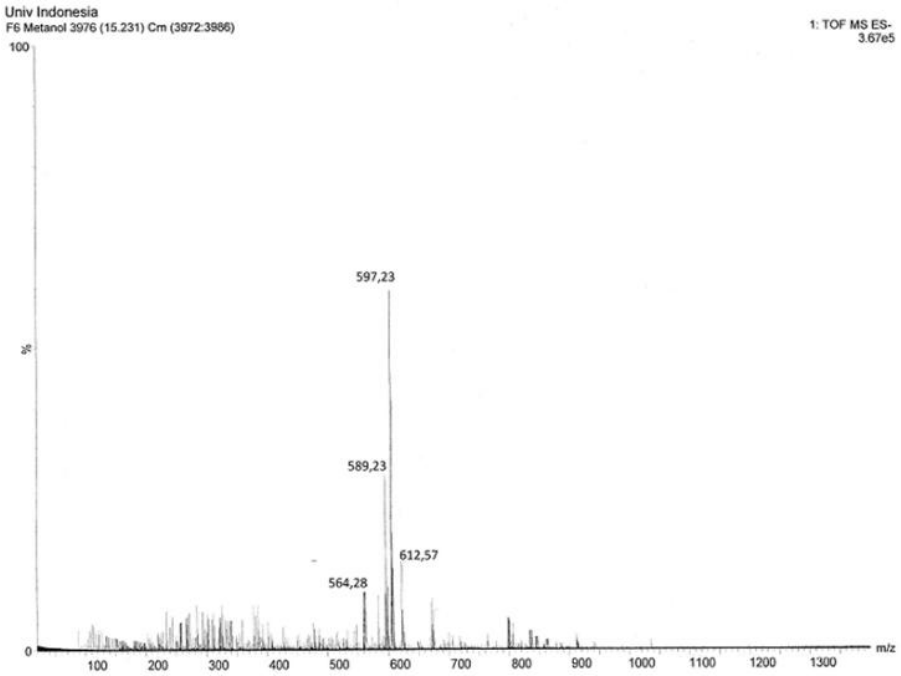

Figure 3 Mass spectrometry graph from the ethyl acetate fraction

The four significant lines that appeared in this graph were considered as the majority of the compounds present in this fraction. Dominant ions at m/z of 597.23 had the largest percentage, as much as 60%, of the total ions present. Annonacin, as a suspected compound in the sample, is known to have a molecular weight of 596.88. The line at m/z 597.23 indicated the protonated molecular ion $(M+H)^+$ of annonacin.

The measured molecular mass of annonacin reported by Luna et al. (2006) from mass spectroscopy analysis is quite similar. The slight difference between the two data might come from the different flow rate of the mobile phase used in this study, 0.5 ml/min compared to 1 ml/min used by Luna et al. (2006). The F4.4 fraction contained annonacin and showed a high lactone content, as shown in Table 1. The presence of annonacin in the F4.4 fraction indicated that the separation procedure worked effectively to separate annonacin from the other compounds existing in the soursop extract.

4. CONCLUSION

Acetogenins in the soursop leaves could be fractionated and enriched using the present open column chromatography approach, resulting in a higher lactone yield in the ethyl acetate fraction (242 mg/g) compared to the previous method of extraction without open column chromatography (188 mg/g). The HPLC and LC-MS analysis of the ethyl acetate fraction showed that annonacin was present and it could be separated. The line in the LCMS graph with m/z of 597.23 seems to be the $(M+H)^+$ ion of annonacin. The presence of annonacin in the ethyl acetate fraction had an important role in the high cytotoxicity activity and the high lactone contents. This study shows that there is a positive correlation between the lactone content due to acetogenins and the level of cytotoxicity of the fractions obtained. The cytotoxicity assay using the brine shrimp test gave an LC_{50} of 1 ppm, higher than the reported LC_{50} value of an anticancer drug cyclophosphamide; an indication that this fraction could be applied in a formulation for cancer therapy.

5. ACKNOWLEDGMENT

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