# Tablet Formulation of The Ethyl Acetate Soluble Extract of Soursop (*Annona muricata* L.) Leaves

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**ABSTRACT---** ***Tablet dosage formulations of the soursop extract containing acetogenins active substances were prepared for adjuvant cancer therapy.This study consisted of the preparation of the extracts and cytotoxic activity evaluation, followed by the tablet formulation. Detection of the acetogenin constituents was judged by LCMS, and the cytotoxicity assays were performed using BSLT, and the MTT assays. BSLT test using larvae shrimp (Artemia salina* Leach*) aged 48 hours and MTT was performed at the Center for Primate Studies, Institute for Research and Community Service Institute of Agriculture (IPB), Bogor, Indonesia. The antioxidant activity and the stability evaluation of the formula were also observed. LCMS test soursop leaf ethyl acetate fraction showed a wide acetogenin obtained. Initial screening toxicity test with BSLT showed cytotoxic response of highly toxic ethyl acetate fraction with $LC_{50}$ = 12.188 ppm, whereas in vitro by MTT assay test showed $IC_{50}$ = 815.922 ppm. $IC_{50}$ determination of antioxidant activity by DPPH $IC_{50}$ results soursop leaf ethyl acetate fraction 94.169 ppm. Tablet F1 - F4 with a dose of 1-20 mg produced tablets with good quality test. Stability test was referring to the determination of total acetogenin which is also a marker compounds by spectrophotometric method and calculated Arrhenius equation shows that the age of the tablet product was 2.06 years. Tablets containing acetogenins of soursop leaves ethyl acetate-soluble fraction can be prepared by wet granulation and expected to have benefit for traditional cancer adjuvant therapy.***

**Keywords**---- ethyl acetate fraction of the leaves of soursop, *Annona muricata* L., antioxidant activity, adjuvant anticancer therapy, tablets.

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## 1. INTRODUCTION

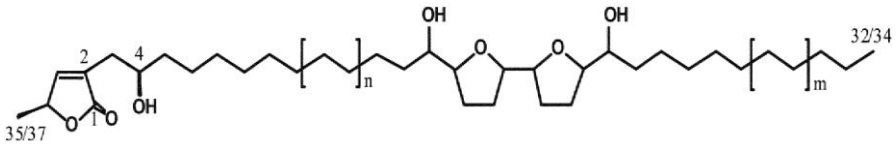
*Annona muricata* L. (Annonaceae) is grown commercially as a fruit crop and is called “guanabana”, “graviola” or “sour sop” throughout the tropical regions of the world. It has reported to have a number of monotetrahydrofuran (mono-THF) acetogenins from the bark, seeds, and leaves. The annohexocin and murihexocins A and B were hexahydroxylated mono-THF ring acetogenins, and the annomuricins A, B, and C, muricatocins A, B, and C, and annopentocins A, B, and C were pentahydroxylated mono-THF acetogenins. Annomutacin and cis- and transannomuricin-D-ones were tetrahydroxylated mono-THF acetogenins, and *cis*- and trans-10-R-annonacin-Aones were trihydroxylated mono-THF compounds (Kim, 1997).

The classic extraction of acetogenins from plants were carried out by successive solvent extractions with increasingly polar solvents, or by liquid/liquid partition from an initial alcoholic extract (Bermejo, 2005). The Annonaceous acetogenins were readily soluble in most organic solvents. Ethanol extraction of the dried plant material followed by solvent partitions, to concentrate the compounds, was the method of choice (Alali, 1999).

Major contribution to the detection, isolation, characterization, and dereplication of Annonaceous acetogenins in the last two years has come through the application of liquid chromatography-electrospray ionization mass spectrometry (LC/EIMS) techniques. Using the positive-ion mode and under conditions of atmospheric pressure in-source collision-induced dissociation (APICID), the acetogenins have provided reproducibly characteristic ion patterns and fragment ions. Analyzing the selected ion chromatograms (SIC), the presence of these acetogenins and other derivatives in

crude plant extracts and chromatographic fractions can be readily detected. Utilizing the LC/(+)ESI-APCID-MS technique, acetogenins produce characteristic ion patterns consisting of $[M + Na]^+$ and $[M +H]^+$ molecular ions, as well as ions showing the consecutive losses of $H_2O$ from the $[M + H]^+$ ion. Gu et al. then applied the LC/(+)- ESI-MS method to direct the isolation of two of the new compounds (Alali, 1999). However, HPLC-MS analysis identified acetogenin compounds in the fruit pawpaw extracts, and the relative amount of acetogenin compounds in the extracted materialwas likely reflected in the bioassay. Some hydrophobic substances contained in the extracts could also display bioactivity (Pomper, 2009).

The use of natural materials, especially natural vegetation is mostly in the form of traditional medicine such as herbal medicine class, whereas in pharmaceutical dosage forms such as tablets, caplets and capsules are still very rare. Preparing oral dosage in tablet form of soursop leaves extracts will have more practical application. Tablet dosage proved to be very beneficial, because it easy to store and handy to carry and appropriate dose adjustment. Tablet is a medicinal ingredient in a solid dosage form that is usually made with the addition of appropriate additional pharmaceutical (Ansel, 1989). Tablet additives should be neutral, odorless and tasteless, and colorless as possible. Additional materials used can serve as fillers, binders, lubricants substances, destructive substances or other suitable substance. Substance use or binder is expected to provide compactness and durability tablet. The use of a binder preferably in an amount sufficient was to generate compactness. Compactness and destruction tablet is linked with one another. Polyvinylpyrrolidone (PVP) binder that can be used in wet granulation method (Voight, 1995). Annonaceous acetogenins (polyketides) are a group of extensively investigated natural compounds possessing antitumor, antiparasitic and pesticidal activities. Over 350 acetogenins have been isolated and most of them have one to three tetrahydrofuran (THF) cores, several hydroxyls and a terminal γ-lactone ring. As part of our investigation of the title species, we have reported four new $C_{35}$ acetogenins: muricatalin, muricatalicin, annonacin-B3 and murihexol and four known acetogenins: annonacin, annonacin-A, annonacin-10-one and donhexocin. Among them, murihexol and donhexocin 3 are two non-THF acetogenins (Li,Yu De, 2000, and Spurr, 2010).




Ficture 1. Acetogenin structure (Bermejo, 2005).

The common skeleton is most often characterised by an unbranched $C_{32}$ or $C_{34}$ fatty acid ending in a C-lactone. Several oxygenated functions, such as hydroxyl, ketone, epoxide, tetrahydrofuran (THF) and tetahydropyran (THP), may be present, as well as double and triple bonds. Thus several types of acetogenins were characterised, based on the nature of the functional groups which are present. Acetogenins exhibit a broad range of biological properties such as cytotoxic, antitumoural, antiparasitic, pesticidal, antimicrobial and immunosuppresive activities (Bermejo, 2005).

In another study acetogenin obtained from the process soursop leaf extraction, then analyzed qualitatively (TLC, BST, FTIR) and quantitative (UV-Vis spectrophotometry) (Swari, 2012).

## 2. METHODS

Soursop leaves were collected from Bekasi area, Indonesia. The voucher specimen was identified and deposited at Herbarium Bogoriensis, Indonesian Institute of Sciences. This botanical materials were evaluated and comply the quality of described in Materia Medika Indonesia (Ministry Of Health, 1989).

Samples were extracted using maceration method by weighing a number (10.0 kg) soursop leaves (*Annona muricata* L.) was dried and crushed, then put into containers maceration then added thereto a number of 96% ethanol (80.0 liters) to soak the entire sample. The samples were left in the container for several days maceration (5 days) and then dilute extract obtained was concentrated by using a rotary evaporator, and then insert it into the oven temperature up to 50$^o$C thick extract is obtained. Viscous extract obtained by adding the number of partitioned into solvent extracts (ethanol: n-hexane) with a ratio of 1:1, then added distilled water with the same ratio and mix using an electric mixer until a few moments. After mixing is complete, it is obtained that does not mix the two layers, separated by a layer of water on the solvent and subsequent water fraction do the same process with the new solvent (ethyl acetate). The process is complete when the solvent used was clear. Ethyl acetate fraction obtained followed by concentration using a rotary evaporator, then put into an oven temperature of 50° C to obtain a viscous extract.

Mass spectra (MS) or molecular weight were obtained with liquid chromatography-mass spectroscopy (LCMS), mariner biospectrometry spectrometer using electrospray ionization (ESI), system and positive ion mode, mobile phase (MeOH-water 9:1), flow rate 1 mL/minute.

The sample was dissolved using DMSO to achieve a concentrated of 200 ppm. The sample was diluted with a different concentration from 10-200 ppm in microtiter plate. Each well received an additional of 5 µl diphenylpicrylhydrazyl (DPPH) (1 mg/ml in methanol). The microtiter plate was then vortexed and incubated for 30 minutes in the dark room. The

adsorbance was measured on 517 nm wavelength, and the total percentage of radical scavenging activity was calculated based on the following formula : [(A control – A sample) / A control] x 100%.

The BSLT was conducted following the established procedure (Mc Laughlin, 1998). The BST (*Artemia salina* Leach) was routinely employed for evaluating the crude extracts, fractions, and isolated compounds from the leaves of *A.muricata*. In order to determine $LC_{50}$, data was derived from calculating the death of *Artemia salina* in different concentrations of acetogenin within 24 hours. The data was analyzed by using SPSS program.

The cytotoxicity evaluation was performed using MTT based on previous method (Gerlier, 1986). In brief, cells that had been grown on a sustainable $T_{25}$ flask were subcultured, and then cells were grown in 96 wells tissue culture plate with the number 5000 cells / well and incubated for 24 h in growth media at 37°C and 5% $CO_2$. Bioactive compounds at each concentration was added as 100µL/well, included untreated cells as control cells incubated further back for 48 hours. The compound 3 - (4,5-Dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) was added and incubated for 4 h at 37°C and 5% $CO_2$. Cell supernatant was discarded, formazan crystals formed were dissolved in 70% ethanol. Reading of the optical density (OD) performed using the microplate reader at a wavelength of 565 nm. Counting cells with a hemocytometer : Cells that had been grown on a sustainable $T_{25}$ flask were subcultured, and then cells were grown in 96 wells tissue culture plate with a number of 10000 cells / well and incubated for 24 h in medium grower at 37°C and 5% $CO_2$. Bioactive compounds at each concentration was added as 2 mL / well, cells without treatment was included as a further control cells incubated again for 72 hours. Cell supernatant was discarded, cells are added with 2 mL of PBS (Phosphate Buffer Saline) to clean off the rest of the media, as much as 1 mL trypsin was added and incubated for 5 min at 37°C and 5% $CO_2$. Supernatant was discarded, the cell pellet was added 1 mL of PBS. Cell count was done by using Spencer Bright-Line hemocytometer (Improved Neubauer). In this study, the MTT test conducted at the Center for Primate Studies, Institute for Research and Community Service Institute of Agriculture (IPB), Bogor, Indonesia.

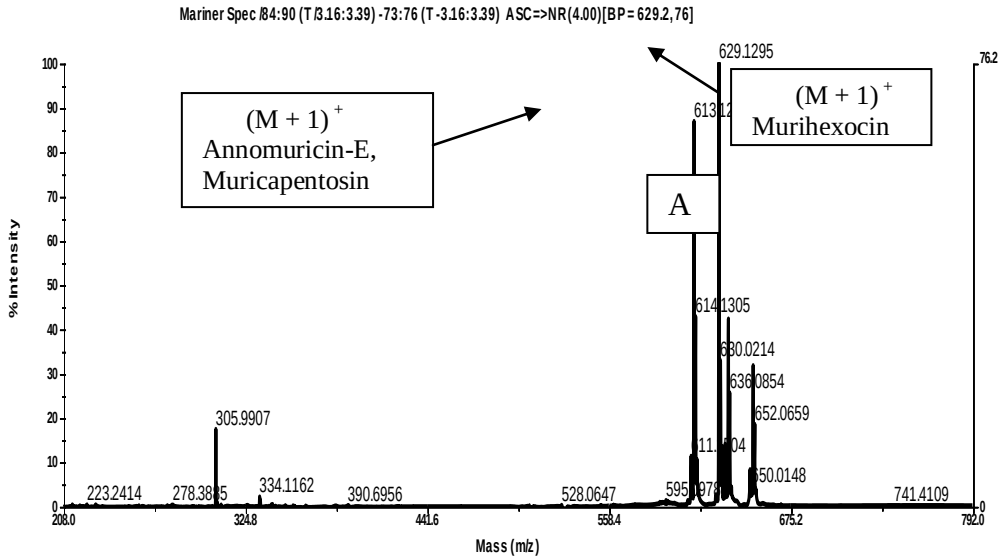
**Tablet Formulation** :

Soursop leaf fraction : ( 1 – 160 mg); Avicel pH 101: Lactose (1: 1) : qs; Corn starch : 10%; PVP 4%; LHPC-LH11: 5%; Magnesium stearate: 1%; Talc 1%; Aerosol : 0,5%

## 3. RESULTS and DISCUSSION

Testing the identification of soursop leaf monograph based on Materia Medika Indonesia, where all the samples tested gave positive results.

Results of analysis of compounds asetogenin soursop leaf ethyl acetate fraction on chromatography LCMS provides a peak ("peak") which states that in the ethyl acetate fraction contains many phytochemical compounds such as flavonoids, polyphenols and asetogenin (Cos, 2001). Identification of the LC chromatogram with MS based (Mass Spectroscopy) to LCMS showed that some LC chromatogram giving the same weight to the molecular weight compounds in soursop asetogenin (Bermejo, 2005 and Dictionary (software, 2005). Chromatographic separation of the less polar fractions of a petroleum extract from A. ***muricata*** : $CH_4$,: *m/z* 565, $[MH]^+$) (Saw, 1991). LC-MS identification results (*m/z*) for ethyl acetate fraction soursop leaves can be seen acetogenin containing compounds (murihexocin-A, annomuricin-E, muricapentocin, gigantetrocin A, corossolon).



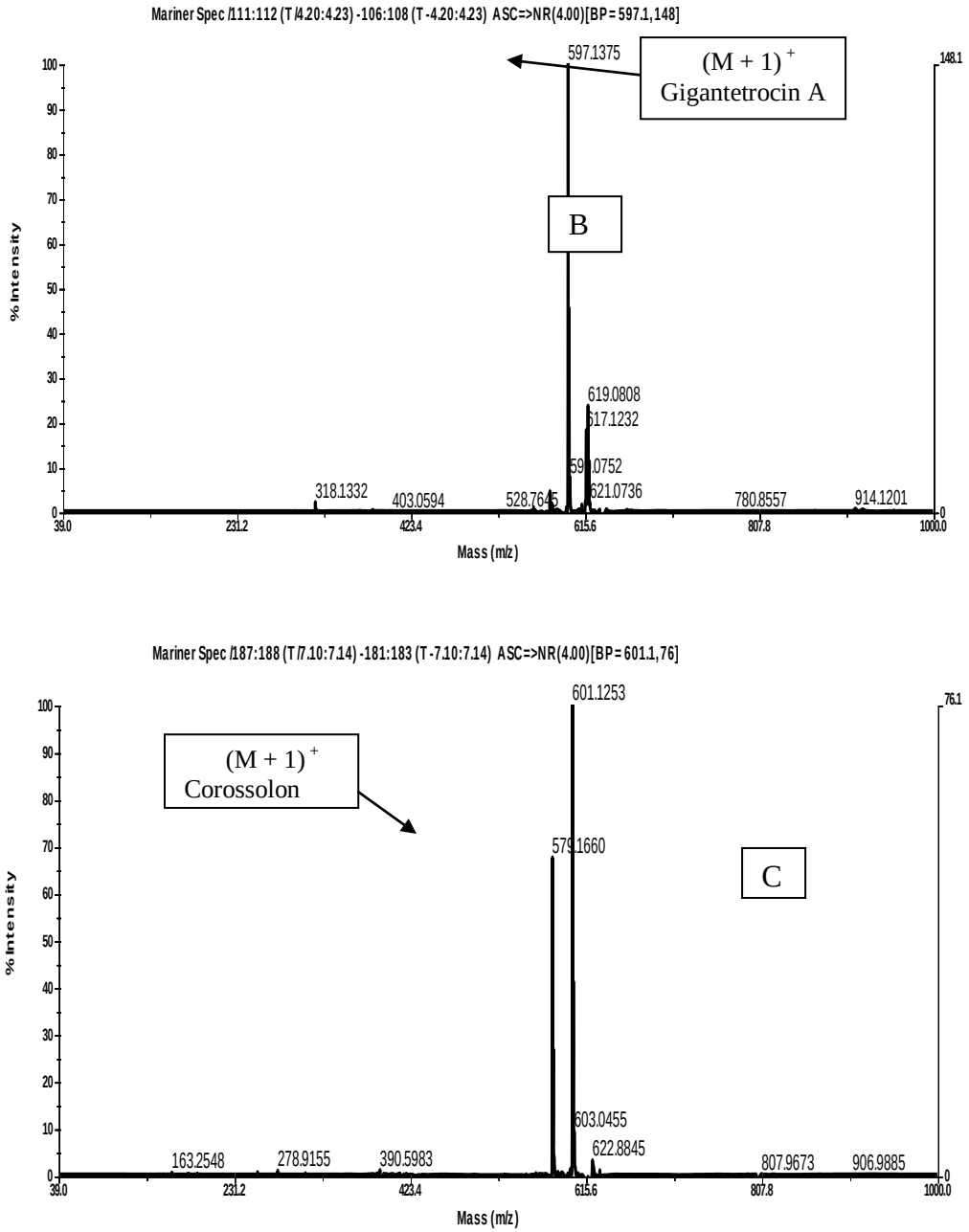



Figure 2. LCMS chromatograms of ethyl acetate fraction of soursop leaf ; Murihexoxin-A, Annomuricin-E, Muricapentosin (A), Gigantrocin A (B), Corossolon (C).

Table 1. The test results of antioxidant activity of ethyl acetate fraction soursop leaves.

| Sample | Concentration (ppm) | Absorbance | % Inhibition | $IC_{50}$ |
|---|---|---|---|---|
| Soursop leaf ethyl | 10 | 1.633 | 19.278 | 94.169 |
| | 50 | 1.481 | 26.792 | |
| | 100 | 1.011 | 50.025 | |
| | 200 | 0.176 | 91.300 | |
| St quercetin | 1 | 1.976 | -4.247 | 11.288 |
| | 10 | 0.887 | 53.205 | |
| | 15 | 0.727 | 61.646 | |
| | 20 | 0.223 | 88.235 | |

Pattern of antioxidant activity of the tested material is declared active when free radicals inhibit more than 80%, when it declared its activity being inhibited 50-80%, and declared inactive when inhibited less than 50% (Yen, 1995).

Based on the data above shows that the ethyl acetate fraction soursop leaves have potential as antioxidants and found that the fraction of soursop with concentrations of 200 ppm has the highest antioxidant activity with inhibition of 91.30%. However quersetin as a positive control has antioxidant activity at concentrations of 20 ppm already have a power resistor of 88.235%. According Armala,2009, the antioxidant power activity ($IC_{50}$) = 100 ppm including stronger in intensity, while activity ($IC_{50}$) = <50 ppm including very strong intensity, so that the ethyl acetate fraction soursop leaves with $IC_{50}$ = 94.169 ppm results are included in the strong intensity compared to standard quersetin which has a very strong intensity ($IC_{50}$ = 11.288 ppm).

The antioxidant activity of ethyl acetate fraction of guessed soursop leaf related to the content of flavonoid compounds and polyphenols, which this compound has a hydroxyl group that can donate hydrogen atoms to neutralize free radicals (Cos, 2001).

Table 2. Artemisia larval mortality assay results.

| No. | Consentration (ppm) | number of deaths | | | Total | % mortality | $LC_{50}$ |
|---|---|---|---|---|---|---|---|
| | | I | II | II | (30) | | |
| 1 | 5 | 2 | 3 | 3 | 8 | 27% | 12,188 |
| 2 | 10 | 7 | 3 | 4 | 14 | 47% | |
| 3 | 20 | 7 | 7 | 8 | 22 | 73% | |
| 4 | 40 | 8 | 9 | 9 | 26 | 87% | |
| 5 | 80 | 10 | 10 | 8 | 28 | 93% | |

The brine shrimp (*Artemia salina* Leach) lethality test (BST) was routinely employed for evaluating the crude extracts, fractions, and isolated compounds (Kim, 1998). Based on the calculation and probit analysis using SPSS 17.0 to find the $LC_{50}$ value of each fraction were tested concentration. BSLT test results can be seen in the price is equal to 12.188 ppm $LC_{50}$. According to Meyer et. Al, 1982, the level of toxicity of plant extracts can be determined by looking at its price $LC_{50}$. An extract is considered highly toxic if had $LC_{50}$ values below 30 ppm, is considered toxic when 30-1000 ppm $LC_{50}$ value and is not considered toxic when $LC_{50}$ values above 1000 ppm. The toxicity level will give meaning to the potential antitumor activity. The smaller the $LC_{50}$ more toxic compound. Thus, based on $LC_{50}$ values obtained from soursop fractions tested, it is declared to be highly toxic. These results are consistent with the results of the study Kim, et.all, 1998, the leaves of A. muricata were extracted with 95% EtOH, and the extracted residue was partitioned by a standard extraction was the most bioactive fraction in the shrimp lethality test with BST $LC_{50}$ = 1,6 ppm.

Table 3. MTT assay results soursop leaf ethyl acetate fraction.

| Fraction (ppm) | ODI | ODII | ODIII | Average | % inhibition | $IC_{50}$ |
|---|---|---|---|---|---|---|
| 5 | 0.076 | 0.091 | 0.075 | 0.081 | 85.663 | 815.922 |
| 10 | 0.230 | 0.142 | 0.100 | 0.157 | 72.212 | |
| 20 | 0.167 | 0.226 | 0.236 | 0.210 | 62.832 | |
| 40 | 0.053 | 0.055 | 0.059 | 0.056 | 90.088 | |
| 80 | 0.055 | 0.060 | 0.059 | 0.058 | 89.734 | |
| 160 | 0.079 | 0.081 | 0.075 | 0.078 | 86.195 | |
| 320 | 0.116 | 0.116 | 0.155 | 0.129 | 77.168 | |
| 640 | 0.232 | 0.291 | 0.297 | 0.273 | 51.681 | |
| Cell Control | 0.566 | 0.565 | 0.565 | 0.565 | 0.000 | |

MTT test data results can be seen hela cells was inhibited by ethyl acetate fraction containing acetogenin soursop leaves at 40 ppm of 90.088%. $IC_{50}$ limit to be declared an extract has potential as an anticancer was 1000 ug / ml or 1000 ppm. Based on this research $IC_{50}$ price is equal to 815.922 ppm, it can be said to be toxic to hela cells.

Fillers used in the preparation of lactose and avicel. This substance showed good stability in combination with the most active substance hydrate or anhydrous. Lactose is an excellent excipient used in the tablets containing the active substance concentrate small, easy to carry out because of the homogeneous mixing. Therefore the formula F1 - F5 using lactose as a filler. Avicel can also be used for a tablet that has a relatively small dose. Aside from being a filler also acts as a binder helpers to control the uniformity of the active substances, to prevent migration of water-soluble dyes, and the increased evaporation is rapid and uniform fluid from wet granulation. Avicel is usually not used in a single tablet as a main filler, except special formulations require bonding properties avicel like the formula F6 - F7. Avicel able to hold (hold) more than 50% active ingredient. Avicel is a charger that is suitable for wet granulation compression to help improve the minimal characteristics of a compression. Avicel suitable for moist sensitive active substances or materials that are closely or hygroscopic (Siregar, C, 2010). Tablet F1 - F4 with a dose of 1-20 mg produced tablets with good quality test, while the tablet F5 - F7 at a dose of 40-160 mg should be repeated in order to get a tablet formulation to meet the standard

quality test.

MTT assay results can be seen in hela cells is inhibited by the acetogenin tablet containing 40 ppm of 92.212%. $IC_{50}$ limit to be declared an extract has potential as an anticancer was 1000 ug / ml or 1000 ppm. Based on this research $IC_{50}$ price is equal to 998.014 ppm, it can be said to be toxic to hela cells.

Over 160 acetogenins, usually having mono-tetrahydrofuran (THF), adjacent or nonadjacent bis-THF, or tri-THF rings, are found in several genera of the Annonaceae (Zeng, 1995, and Mc Laughlin, Jerry, 2008). Therefore, in this study, using a standard solution of Tetrahydrofuran (THF). Results obtained data shows total acetogenin levels ranged from 5.570 to 5.941%. According to the results of Kim et al study, 1997, total acetogenin result obtained was 5.6%.

In other studies, Quantitative analysis was performed by calculating the content of the lactone group acetogenin, using standard compounds andrographolida. The use of standard compounds due andrographolida as pure compounds acetogenin hard to come by, and andrographolida having lactone groups similar to acetogenin, other than that the soursop leaves only acetogenin who had lactone group by group so that the determination of the lactone can be done. The amount of content on the acetogenin is the result of isolation ranged between 7% - 12%.. (Swari, 2012).

The result of research obtained amount of acetogenin showed a decrease at every increase of temperature every hour into 0, ½, 1, 2 and 4. At temperatures 29$^{o}$C shows the time half-life experiencing hike, and at temperature of 40$^{o}$C experiencing a decrease, as well as at a temperature of 70$^{o}$C occurs a decrease back. To calculate the circulation periods, based equation Arrhenius, were made plot log levels of acetogenin total against time, so that is obtained equation linear regression levels of acetogenin with rate constants degradation k. To calculate the rate of degradation at room temperature, then the was made plot log rate constants (log k) against temperature (1 / T), so that is obtained equation linear regression, and rate constants degradation at room temperature (log $K_{25}$) can be calculated. Based on these data in above, then the on temperature of 25$^{o}$C, log $K_{25}$ = -3.884908, so that $K_{25}$ = 0.000130344. With insert the value $K_{25}$ into inside the equation which in can be, with Co = 100% and C = 90%, then the obtained value of t 90% = 18082.292 hour or 2.064 years old. With Thus, tablet acetogenin which made stable during the 2.064 years.

## 4. CONCLUSIONS

The results showed that the of ethyl acetate soluble fraction of the leaves of the soursop was toxic to BSLT with its $LC_{50}$ = 12.188 ppm. Toxicity test of ethyl acetate fraction of the leaves of the soursop against Hela cancer cells using *in vitro* assays (MTT) showed the $IC_{50}$ = 815.922 ppm, while the antioxidant activity of ethyl acetate fraction of the fraction showed the $IC_{50}$ = 94.169 ppm. Tablets prepared from soursop leaves ethyl acetate fraction containing acetogenins in our laboratory met all the requirements for the quality test tablet. Tablet soursop leaf ethyl acetate fraction at a dose of 1-20 mg tablet dosage formulations may be well, while the dose of 40-160 mg formulation should be re-adjusted with additional material. Acetogenin total levels on tablet obtained ranged from 5.570 to 5.941%. The tablets prepapred from ethyl acetate fraction containing acetogenin soursop leaves showed a fairly good stability with shelf life periods for 2.06 years.

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