

## Free radical scavenging and cytotoxic assay of soursop fruit juice (*Annona muricata* Linn.) on cervical cancer cell lines (HeLa)

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### ABSTRAK

**Latar belakang:** Kanker serviks merupakan penyakit yang menduduki posisi kedua penyebab kematian pada wanita. Berbagai terapi pendukung mulai dikembangkan, seperti melalui bahan makanan yang dipercaya memiliki efek anti-kanker. Buah sirsak disebut memiliki kandungan fitokimia seperti Annonaceous acetogenin, flavonoid dan fenol yang bermanfaat sebagai anti-kanker.

**Tujuan:** Penelitian ini dilakukan untuk mengetahui potensi anti-oksidan dan sitotoksitas dari sari buah sirsak pada sel HeLa secara *in vitro*.

**Metode:** Penelitian ini dilakukan dengan membuat sari buah sirsak dengan cara diblender kemudian dikeringkan dengan metode freeze-drying, yang kemudian dilanjutkan dengan uji 2,2-diphenyl-1-picrylhydrazyl (DPPH) untuk mengetahui potensi penghambatan radikal bebas, dan potensi sitotoksitas melalui uji MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difenitetrazolium bromide) assay pada sel kanker serviks HeLa.

**Hasil:** Hasil uji aktivitas anti-oksidan menunjukkan persamaan regresi linier ( $y=0,0149x - 2,8812$ ) dan nilai perhitungan  $IC_{50}$  sari buah sirsak sebesar 3549  $\mu\text{g/mL}$  dan hasil uji sitotoksitas menunjukkan persamaan regresi linier ( $y=0,0197x + 0,3101$ ) dan nilai perhitungan  $IC_{50}$  sari buah sirsak pada sel HeLa sebesar 2522,33  $\mu\text{g/mL}$ .

**Kesimpulan:** Sari buah sirsak memiliki aktivitas anti-oksidan yang sangat rendah dan tidak berpotensi sebagai anti-kanker terhadap sel HeLa secara *in vitro*.

**KATA KUNCI :** *Annona muricata* Linn, anti-oksidan, kanker, sitotoksitas

### ABSTRACT

**Background:** Cervical cancer is the second leading cause of death in women. Various supporting therapies have been developed, such as through food ingredients which are believed to have anti-cancer effects. Soursop is known to be high phytochemical content such as Annonaceous acetogenin, flavonoid and phenols which are useful as anti-cancer.

**Objectives:** This research was conducted to determine the antioxidant and cytotoxic activity of soursop juice on HeLa cell lines.

**Methods:** This study started by making the soursop fruit extract by blending then dried with freeze-drying method, and then proceed with 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay to determine the potential free radical scavenging activity, cytotoxic potential activity *in vitro* through MTT (3-(4,5-dimethylthiazol-2-il)-2,5-difenitetrazolium bromide) assay on HeLa cell lines.

**Results:** The  $IC_{50}$  antioxidant activity of soursop fruit extract is 3549  $\mu\text{g/mL}$  with linear regression equation ( $y=0.0149x - 2.8812$ ) and the  $IC_{50}$  cytotoxicity test of soursop fruit extract on HeLa was 2522,33  $\mu\text{g/mL}$  with linear regression equation ( $y=0.0197x + 0.3101$ ).

**Conclusion:** The conclusion in this study is that soursop fruit extract has very low antioxidant activity and has no *in vitro* potential effect as an anti-cancer on HeLa cell lines.

**KEYWORDS :** *Annona muricata* Linn, antioxidant, cancer, cytotoxic

## INTRODUCTION

Cancer is a condition of changes in cell structure and function, which results in uncontrolled or abnormal cell division processes (1). Cancer is the second largest cause of death in the world. In Indonesia the prevalence of cancer is reported at 1.4 per 1000 population or around 330,000 people (2).

One type of cancer is cervical cancer, this cancer is the second most common type of cancer after breast cancer in women of reproductive age in Indonesia. Since 2000-2012 the number of cervical cancers has continued to increase and has resulted in 28% of deaths in Indonesia (3). Various types of treatment were applied to overcome this, but the results were not effective enough and had several downside (1).

In response to these conditions, several studies have been conducted to find supportive therapy through food to overcome cancer, because there are several food ingredients believed to be useful as anti-cancer. One example is soursop or *Annona muricata* Linn. (*A. muricata*). In soursop fruit, the main substance is carbohydrates (mainly fructose) and the dominant vitamin is vitamin C (20 mg / 100 grams) which acts as an antioxidant (4).

The soursop plants also consist of Annonaceous acetogenins, which there were more than 100 types of Annonaceous acetogenins were found in the leaves, bark, roots, and fruit. This compound has been scientifically proven to be an anti-cancer and anti-tumor because it has toxic effects on cancer cells without damaging healthy cells (5).

Anti-cancer potential can be identified by conducting cytotoxic tests. The testing of the anti-neoplastic activity of a compound can be detected in vitro as indicated by  $IC_{50}$  values. In previous studies, cytotoxic tests using methanol extract of fruit, leaves and soursop seeds have been carried out on blood cancer cells (CCRF-CEM cell) with  $IC_{50}$  values of (4.58 ± 0.25), (0.57 ± 0.02), and (0.36 ± 0.03) µg / mL, respectively (6).

While the portion of the soursop fruit that is widely consumed is the flesh and is usually processed into soursop juice, there is still no cytotoxic effect test of soursop flesh on cervical

cancer cells. While the portion of the soursop tree that is widely consumed is part of the fruit and is usually processed into soursop juice. Therefore, to determine the presence of anti-cancer potential of soursop juice in HeLa cells, it is necessary to test the antioxidant and cytotoxic activity.

This study aims to determine the value of antioxidant activity and the cytotoxic potential of soursop juice (*A. muricata*) on cervical cancer cells (HeLa).

## MATERIALS AND METHODS

### Preparation of sample

The material used in this study was soursop fruit which was purchased from plantations in the Materia Medika, East Java, Indonesia. The criteria of soursop fruit include ± 12 weeks of fruitage, yellowish-green fruit skin, soft fruit, not sharp and far apart, and fragrant soursop fruit. The fruits were blended then squeezed using a filter cloth, then dried using freeze-drying method. The extracts were stored at 4°C for further experiments (7,8). DPPH Free Radical Scavenging Activity Assay.

One ml of *A. muricata* extract were introduced into a tube then added 3 ml DPPH solution. The mixture then shaken using vortex and measured with spectrophotometer at 517 nm. Free radical scavenging activity of the sample was measured according to <sup>9</sup> :

$$\text{Scavenging \%} = (Ac - As) / Ac \times 100.$$

As: sample absorbance

Ac: negative control absorbance (without sample)

### Cytotoxic Assay

HeLa cells were cultured on RPMI medium which added 10% FBS, and 1% penicillin-streptomycin. Cells were then incubated at 37°C and 5% CO<sub>2</sub>. HeLa cells were harvested when they reached 80% confluent and were planted in the 96-well plate, 5.000 cells for each well<sup>10</sup>. After 24 hours of incubation, the cells are then treated with *A. muricata* extract with various concentrations (62.5; 125; 250; 500; and 1000 µg/mL) for 24 hours.

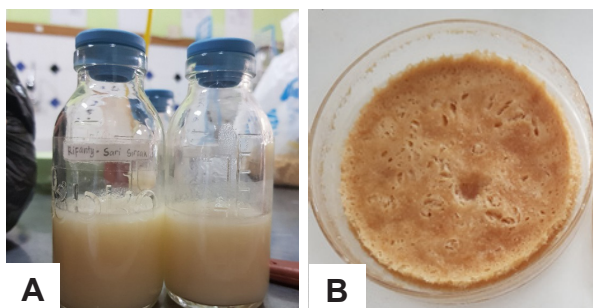
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is used to

determine cell viability. 20  $\mu$ l MTT was added to each well and incubated with temperature 37°C, 5% CO<sub>2</sub> under dark conditions for 4 hours. The reaction was stopped by adding 10% SDS. The absorbance is measured using a microplate reader at 570 nm<sup>11</sup>. The data were analyzed using ANOVA, followed by Turkey post hoc test. To determine the value of inhibitory concentration (IC<sub>50</sub>), a Probit Analysis was used with Excell Microsoft Office 2010.

## RESULTS

### Soursop Fruit Extract

Soursop fruit juice made from one fruit of soursop weighing 910 g. Furthermore, the fruit flesh is separated from the skin and seeds, and the net weight of fruit flesh is 691 grams. Followed by the process of blending and then filtered with a filter cloth, and obtained as much as 450 ml of soursop juice. 100 ml of soursop juice were taken for the drying process freeze-drying. Where from 100 ml of soursop fruit juice the dry yield is 19 grams.



**Figure 1.** Sample soursop juice. Soursop fruit that has been blended and filtered (A) and the sample results of the freeze-dry soursop extract (B)

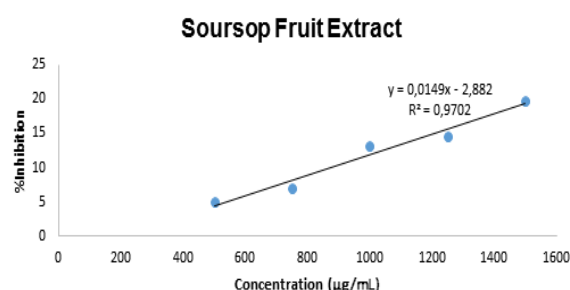
### DPPH Free Radical Scavenging Activity Assay

The antioxidant activity test on soursop juice was carried out with 5 concentrations, namely 500; 750; 1000; 1250; and 1500  $\mu$ g / mL. The percentage of inhibition is higher in proportion to the higher concentration of soursop juice, this is presented in **Table 1**. The highest percentage of inhibition is at a concentration of 1500  $\mu$ g / mL, which is equal to 19.83%. A linear regression curve was made from the data concentration and the percentage of inhibition (**Figure 2**), which is used to calculate the

IC<sub>50</sub> value. The IC<sub>50</sub> value obtained was 3549.074  $\mu$ g/mL.

**Table 1.** Percentage of Soursop Fruit Extract in DPPH Test

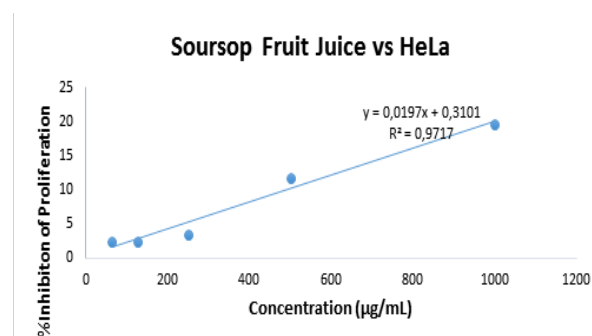
Concentration ( $\mu$ g/mL)	Absorbance	% Inhibition	IC <sub>50</sub> ( $\mu$ g/mL)
Blank	1.069	0.00	
500	1.015	5.05	
750	0.993	7.11	
1000	0.928	13.19	3549
1250	0.912	14.69	
1500	0.857	19.83	



**Figure 2.** Linear Regression Curve of Soursop Fruit Extract DPPH Test

### Cytotoxicity Assay

This test was carried out with 5 concentrations namely, 62.5; 125; 250; 500; and 1000  $\mu$ g/mL, and incubated for 24 hours. The results then were used to read the absorbance using an ELISA reader with a wavelength of 570 nm. The proliferation percentage is presented in **Table 2**. The standard curve used to calculate the IC<sub>50</sub> value is shown in **Figure 3**. Furthermore, the calculation of the IC<sub>50</sub> value is obtained at 2522.33  $\mu$ g / mL.



**Figure 3.** Linear Regression Curve of MTT Assay Cytotoxicity Test

**Table 2. Inhibition of Proliferation of Soursop Fruit Juice in HeLa Cells**

Sample	Concentration (µg/mL)	% Inhibition Concentration 1	% Inhibition Concentration 2	% Inhibition Concentration 3	Mean Value	SD
Soursop Fruit Extract	62.5	3.737	1.823	1.823	2.461	1.105
	125	5.652	0.729	0.729	2.370	2.842
	250	1.459	3.191	5.925	3.525	2.251
	500	14.950	8.660	11.668	11.759	3.146
	1000	16.864	22.607	19.599	19.690	2.873

## DISCUSSION

In general, extraction can be done with polar solvents (water, ethanol, methanol, etc.) or non-polar solvents (petroleum ether, chloroform, etc.), depending on the polarity of the compounds to be extracted, commonly called the principle of like dissolves like<sup>12</sup>. Commonly, to test the cytotoxicity, the extraction process of soursop plants was done with polar solvents. Like the cytotoxic test on soursop seeds, the extraction process on soursop seeds was carried out by maceration method using 96% ethanol to extract the chemical compounds contained in soursop seeds<sup>13</sup>. Likewise in other studies extraction of leaves, seeds and soursop fruit using methanol solvent to obtain crude extract<sup>6</sup>. This is because the chemical compounds that want to be extracted from the soursop plant for cytotoxicity tests have polar properties, including alkaloids, acetogenin, flavonoids, phenols, and other compounds such as vitamin C7.

The cytotoxic of soursop juice against cervical cancer cells (HeLa) was analyzed by the MTT Assay method. The results obtained were the highest proliferation inhibition occurred at a concentration of 1000 µg/mL and IC<sub>50</sub> values of 2522.33 µg/mL. Based on U.S National Cancer Institute (NCI) a crude extract is considered to have cytotoxicity activity *in vitro* if it has an IC<sub>50</sub> value of less than 20 µg/mL and less than 4 µg/mL for pure compounds<sup>14</sup>. However, another study said that have active anti-cancer properties with an IC<sub>50</sub> value of 23,649 µg/mL in HeLa cells, 110,403 µg/mL in HepG2 cells, and anti-proliferative with IC<sub>50</sub> values of 220 µg/mL in PC3 prostate cancer cells<sup>15, 10</sup>.

Based on the references above, it shows that anti-cancer activity still does not have a definite cut-off. However, when compared to the results of the

cytotoxic test of soursop juice, the results obtained were too high, so it could be concluded that the soursop juice in this study did not have cytotoxic activity and had no potential as an anti-cancer agent *in vitro* against cervical cancer cells (HeLa).

It caused by several factors, one of which is the phytochemical content in soursop fruit. In general, cytotoxic studies more often use soursop leaves than fruit, this is due to the more phytochemical content in it. The phytochemical content found in soursop fruit between alkaloids, Annonaceous acetogenin and phenol, in which if compared with its content in soursop leaves shows that each of the phytochemical classes has a smaller variation in fruit<sup>5</sup>. In addition it has been proven from cytotoxic studies using samples of leaves and soursop fruit with water solvents on Raji cells, showing IC<sub>50</sub> values of 73.1 ± 1.4 µg/mL and 385.2 ± 1.7 µg/mL<sup>16</sup>. Although the content in soursop leaves has proven to be more varied and has the potential as an anti-cancer, soursop leaves are not a normal part of daily consumption as food.

Based on the results of similar studies using samples in the form of food, IC<sub>50</sub> values of the ethanol extract of melon fruit were 23,649 µg / mL in HeLa cells and 110,403 µg/mL in HepG2 cells<sup>10</sup>. This shows that melon fruit extract has much better anti-cancer properties compared to soursop juice. In other studies it has also been proven that melons have the potential as anti-cancer in PC3 prostate cancer cells and RCM-1 colon cancer cells, because they contain MTPE which functions as an anti-cancer<sup>15, 17</sup>.

The extraction process can also be influential in extracting compounds that play a role in the cytotoxic test. This is because the polarity of the selected solvent can determine what solute will be obtained<sup>18</sup>. In a similar study namely cytotoxic test of



white blood cancer cells using fruit samples, leaves and soursop seeds were extracted with methanol solvents and each had an  $IC_{50}$  value of  $4.58 \pm 0.25$   $\mu\text{g/mL}$ ,  $0.57 \pm 0.02$   $\mu\text{g/mL}$ , and  $0.36 \pm 0.03$   $\mu\text{g/mL}$ <sup>6</sup>. It shows that the methanol solvent is able to extract phytochemical compounds in the fruit, leaves and soursop seeds well so that it has good cytotoxic activity also on white blood cancer cells.

Another study using samples of soursop leaves with ethanol and water solvents showed  $IC_{50}$  values of 88.79  $\mu\text{g/mL}$  and 682.88  $\mu\text{g/mL}$  for 24 hours incubation, and at 14.68  $\mu\text{g/mL}$  and 538.22  $\mu\text{g/mL}$  for 48 hours incubation<sup>19</sup>. In this study showed that ethanol solvents were better at extracting phytochemical compounds in soursop leaves. Then it can be concluded that there is no one type of solvent that is the best, this is influenced by each level of polarity of the substance you want to extract.

The freeze-drying process in various types of conditions can significantly reduce ascorbic acid content<sup>20</sup>. This is evidenced by the reduction in ascorbic acid content by 17% in dried blueberries after 14 days of storage when compared to ascorbic acid content in fresh blueberries. This condition is caused by ascorbic acid is one component of nutrients in food ingredients that are classified as unstable with the influence of light, temperature, osmotic pressure and others. Where it is known that one of the important ingredients in soursop fruit is vitamin C of 20 mg/100 grams which is also useful as an antioxidant<sup>2</sup>. So that in this cytotoxic test it can be said that the vitamin C content in dried soursop juice is reduced or less than in fresh condition, so it also affects its cytotoxic properties.

Other processing is also thought to affect the phytochemical content in soursop fruit. In a study, blending and juicing on several fruits were carried out to determine the difference in phytochemical content in the fruit<sup>21</sup>. In the final product blending process obtained is the form of pulp from all ingredients that are blended, while juicing produces the final product in the form of juice only. After phytochemical content testing showed that the fruit through the blending process has better flavonoid and polyphenol content, which is related to the final result which is in the form of fruit pulp<sup>21</sup>.

In addition to the factors described above, the low cytotoxic of soursop juice is supported by its antioxidant activity which is also classified as very weak, which is 3549  $\mu\text{g/mL}$ . Based on existing research it is stated that there is a relationship between antioxidant activity and anti-cancer potential, where samples that have high anti-cancer potential also have high antioxidant activity<sup>22</sup>.

In this study, the results of the antioxidant activity test showed a very high value of 3549  $\mu\text{g/mL}$ . So it can be concluded that the antioxidant activity in soursop juice is very weak. According to Blois (1985) the strength of antioxidant activity is said to be very strong if the  $IC_{50}$  value  $<50$   $\mu\text{g/mL}$  is strong if the  $IC_{50}$  value is 50-100  $\mu\text{g/mL}$ , moderate if the  $IC_{50}$  value is 101-150  $\mu\text{g/mL}$ , and weak if the  $IC_{50}$  value is  $> 150$   $\mu\text{g/mL}$ . The lower the  $IC_{50}$  value, the greater the power to inhibit free radicals<sup>23</sup>.

The factors that influence antioxidant activity are the same as factors affecting cytotoxicity testing. Antioxidant activity can be influenced by the type of solvent used<sup>24</sup>. In his research, the antioxidant activity of matoa leaf extract showed that matoa leaf extract with acetone solvent was the most powerful compared to methanol, ethanol, water and isopropanol solvents. This shows that phytochemical compounds in matoa leaves which play a role in inhibiting base radicals can be extracted well in acetone solvents<sup>24</sup>.

Likewise in other studies, comparing antioxidant activity in *Annona squamosa* fruit extracts with water solvents and also methanol showed  $IC_{50}$  results of 157.2  $\mu\text{g/mL}$  and 135.2  $\mu\text{g/mL}$  respectively<sup>25</sup>. This shows that antioxidant activity obtained from methanol extract is slightly stronger than water extract. Which means that methanol solvents can extract well the total content of phenols and flavonoids contained in it.

Freeze-drying and processing that affect the final product also play a role in the low phytochemical content in the sample used. Where freeze-drying in various conditions proved to be able to reduce ascorbic acid content in a sample<sup>20</sup>. While the blending process that produces fruit pulp will produce better flavonoids and polyphenols compared to the processing of juicing, which results in fruit juice alone<sup>21</sup>.

The antioxidant activity of soursop juice is much lower when compared to ascorbic acid as a positive control. This is because soursop juice is a crude extract, while ascorbic acid is a standard control in the form of very pure compounds<sup>26</sup>.

## CONCLUSION AND RECOMMENDATION

From the results of data analysis in this study, it can be concluded that soursop juice has very low antioxidant activity and is not proven to have anti-cancer potential in HeLa cervical cancer cells in vitro.

### Recommendation

1. Adding a phytochemical content test from soursop juice, including the content of vitamin C, flavonoids and phenol
2. Test the soursop juice on other cancer cells
3. Using samples in the form of whole fruit or in the results of the blender without squeezing

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