

Annona muricata aqueous extract suppresses T47D breast cancer cell proliferation

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ABSTRACT

BACKGROUND

Cancer is a dreadful disease caused by abnormal and uncontrolled cell division. *Annona muricata* L, also known as soursop, is useful as an anticancer herbal medication since its leaves, seeds and fruits contain active compounds called annonaceous acetogenins. The objective of this study was to scientifically justify the traditional application of soursop for anticancer treatment in the community, by comparing the antiproliferative effect of *Annona muricata* L leaf, seed and fruit aqueous extracts on T47D breast cancer cells.

METHODS

This study used an experimental post test trial with control group design. Infusions of soursop leaves, seeds, and fruits collected from Kaliurang, Sleman district, Yogyakarta were used for cytotoxicity tests on T47D cells, in comparison with tamoxifen as standard cancer therapy. Proliferative inhibition was determined by 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide [MTT] assay. The parameter of proliferative inhibition was IC_{50} , which is defined as 50% proliferative inhibition ability of soursop and tamoxifen. Significant differences between groups were determined at $p < 0.05$ by Kruskal-Wallis test.

RESULTS

The leaves, fruits, and seeds *Annona muricata* and tamoxifen were proven to be able to inhibit T47D cell proliferation. The IC_{50} of *Annona muricata* leaf, seed, fruit aqueous extracts and tamoxifen were 31,384.21 $\mu\text{g/ml}$; 1.528,800 $\mu\text{g/ml}$; 329,194.81 $\mu\text{g/ml}$ and 114.52 $\mu\text{g/ml}$, respectively ($p=0.016$). The IC_{50} of *Annona muricata* aqueous extract was significantly different from that of tamoxifen.

CONCLUSIONS

The proliferative inhibition of soursop leaves against T47D breast cancer cells is higher than that of soursop fruits and seeds. The leaves, fruits, and seeds of *Annona muricata* aqueous extract was less toxic compared to tamoxifen.

Keywords: *Annona muricata* L, cytotoxic, T47D breast cancer cells

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Ekstrak air *Annona muricata* L menghambat proliferasi sel kanker payudara T47D

ABSTRAK

LATAR BELAKANG

Kanker merupakan penyakit yang menakutkan karena adanya proliferasi sel yang abnormal dan tidak terkendali. *Annona muricata* atau sirsak terutama daun dan bijinya telah diteliti bermanfaat sebagai antikanker karena mengandung senyawa aktif terutama Annonaceous acetogenins. Buah sirsak juga mengandung Annonaceous acetogenins, namun buah sirsak yang mempunyai rasa lebih enak, belum banyak diteliti efeknya dalam membunuh sel kanker. Penelitian ini bertujuan untuk membandingkan efek ekstrak air dari daun, biji dan buah sirsak terhadap penghambatan proliferasi sel kanker payudara T47D.

METODE

Sebuah rancangan eksperimental pasca perlakuan digunakan pada studi ini. Infusa buah, biji dan daun sirsak dilakukan uji sitotoksik terhadap sel T47D dengan pembanding tamoksifen. Penghambatan pertumbuhan dilihat dengan metode 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide [MTT] assay. Parameter penghambatan pertumbuhan digunakan IC_{50} yaitu kemampuan bahan uji dalam menghambat proliferasi sebanyak 50%. Uji Kruskal-Wallis digunakan untuk menguji perbedaan sitiosik antara kelompok perlakuan. Tingkat kemaknaan yang digunakan besarnya $p < 0,05$.

HASIL

Buah, daun, biji sirsak dan tamoksifen mampu menghambat proliferasi sel T47D. IC_{50} dari buah, daun, biji sirsak sangat berbeda secara bermakna dengan tamoksifen, masing-masing besarnya 329.194,81 $\mu\text{g/ml}$; 1.528.800,00 $\mu\text{g/ml}$; 31.384,21 $\mu\text{g/ml}$ dan 114,52 $\mu\text{g/ml}$ ($p = 0,016$).

KESIMPULAN

Penghambatan proliferasi ekstrak air terhadap sel kanker payudara T47D paling tinggi berturut-turut berasal dari daun, kemudian buah dan biji sirsak. Ekstrak air dari buah, biji dan daun sirsak kurang toksik dibandingkan tamoksifen.

Kata kunci: *Annona muricata* L, sitotoksik, sel kanker payudara T47D

INTRODUCTION

Cancer is one of the many diseases causing public health problems in the world as well as in Indonesia. The number of people living with cancer in the world was 12.8 million in 2008, 7.6 million of which died.⁽¹⁾ If the trend continues, 21.3 million new cancer cases will be discovered in 2030 and almost half of them will die.⁽²⁾ In Indonesia, the proportion of cancer cases (10.2%) has a tendency to increase. Cancer is

claimed as the seventh leading cause of death (5.7%) in Indonesia after stroke, tuberculosis, hypertension, injury, perinatal deaths, and diabetes mellitus. According to *Sistem Informasi Rumah Sakit* (SIRS), in the year 2007 breast cancer ranked first among hospitalized patients in Indonesia (16.85%), with a prevalence of 26 per 100,000 women.⁽³⁾

Cancer therapy applied mainly in the later stages of cancer is still a problem in the world as well as in Indonesia. The signs and symptoms of

cancer are almost undetectable in the early stages, hence, patients with cancer are usually diagnosed suffering from cancer in the later stages. According to the study of Ng et al.,⁽⁵⁾ 63% of cancer patients at Dharmais Cancer Center in Jakarta presented with TNM stages III or IV.⁽⁴⁾ The cancer patients in the later stages who receive standard therapy still have a poor outcome.

Recently, there have been a large number of herbals showing chemopreventive potential, thus becoming attractive modalities to be examined and developed continuously. In the future, herbal medicines are expected to be the solution to the problem of poor treatment outcomes of cancer. Therefore, the Indonesian government has been supporting the development of herbal medicines for cancer therapy by ministerial decree (SK Menkes No 381/Menkes/SK/III/2007). In addition, complementary and alternative medicines (CAM) such as herbals, are believed by the public to be safe and to have less side effects.⁽⁶⁾

CAM use is common among cancer patients in Indonesia as well as in the United states,^(7,8) but many of the herbal medicines are not based on clear evidence. One of the herbal medicines preferred by the community for cancer is soursop (*Annona muricata* L). Soursop has been studied as an anticancer medication because it contains active compounds called acetogenins.⁽⁹⁾ The acetogenins in soursop leaves and seeds used as anticancer medication are selective, which means that normal cells are not killed. The cytotoxic effect of acetogenins from sour sop leaves and seeds has been studied *in vitro* on many cancer cell lines, such as human hepatoma, lung carcinoma, human breast solid tumor, prostate adenocarcinoma, pancreatic carcinoma, colon adenocarcinoma, human lymphoma and multi-drug resistant human breast adenocarcinoma.^(10,11) Unfortunately, the aqueous extract of soursop leaves and seeds that people commonly consume have not been studied for its anticancer properties. This is in contrast

with the proved anticancer effect of ethanolic⁽¹²⁻¹⁴⁾ and butanolic soursop extracts,⁽¹⁵⁾ or of soursop essential oil.⁽¹⁶⁾ In addition, soursop fruits, with their delicious taste, have also not been studied as anticancer medication. Actually, the fruits of *Annona muricata* had been reported to contain the acetogenin cis-annoreticuin,⁽¹⁷⁾ while the cis-annoreticuin of *Annona montana* was reported to exhibit cytotoxicity against a human hepatoma carcinoma cell line (Hep G2).⁽¹⁸⁾ The present study compares the antiproliferative effects of aqueous extracts of *Annona muricata* fruits, leaves and seeds on T47D breast cancer cells, with the objective to provide scientific proof of the soursop anticancer effect.

METHODS

Research design

This research used an experimental post test trial with control group design and was conducted from March to October 2013 at Integrated Research Testing Laboratory Gadjah Mada University.

Plant material

The leaves, seeds and fruits of soursop were obtained from Kaliurang, Sleman District, Yogyakarta, Indonesia. They were taxonomically identified by the Laboratory of Pharmacognosy, Faculty of Mathematics and Natural Sciences, Islamic University of Indonesia.

Extraction procedure

The plant material was dried for approximately 96 hours in a drying cabinet and powdered. Five grams of the powdered material (fruits, seeds, or leaves) was added to 50 ml of distilled water, heated for 15 minutes to obtain an infusion, which was filtered and evaporated down to 1 ml (stock solution = 5 g/1 ml).

Intervention

Cytotoxic testing of soursop was carried out with initial concentrations of 1000 µg/ml, 10,000

U1	U1	U1	I1	I1	I1	D1	D1	D1	U9	U9	U9
U2	U2	U2	I2	I2	I2	D2	D2	D2	U10	U10	U10
U3	U3	U3	I3	I3	I3	D3	D3	D3	I9	I9	I9
U4	U4	U4	I4	I4	I4	D4	D4	D4	I10	I10	I10
U5	U5	U5	I5	I5	I5	D5	D5	D5	D9	D9	D9
U6	U6	U6	I6	I6	I6	D6	D6	D6	D10	D10	D10
U7	U7	U7	I7	I7	I7	D7	D7	D7	KS	KS	KS
U8	U8	U8	I7	I7	I7	D8	D8	D8	KM	KM	KM

Figure 1. Microplate scheme of soursop cytotoxic test

U1-U10: Medium + T47D cells + soursop fruit extract (at doses of 300,000; 150,000; 75,000; 37,500; 18,750; 9,375; 4,687.5; 2343.75; 1171.875; 585.9375 µg/ml).

I1-I10: Medium + T47D cells + soursop seed extract (at doses of 300,000; 150,000; 75,000; 37,500; 18,750; 9,375; 4,687.5; 2343.75; 1171.875; 585.9375 µg/ml).

D1-D10: Medium + T47D cells + soursop leaf extract (at doses of 300,000; 150,000; 75,000; 37,500; 18,750; 9,375; 4,687.5; 2343.75; 1171.875; 585.9375 µg/ml).

KS = Medium + T47D cells; KM = medium only

µg/ml, and 50,000 µg/ml, but did not yield any IC₅₀ values. The last cytotoxic test on soursop was performed with an initial dose of 300,000 µg/ml (Figure 1), which was prepared from 60 µl soursop stock solution. Cytotoxic testing of tamoxifen was performed with an initial dose of 1000 µg/ml (Figure 2), taken from 100 µl stock [tamoxifen stock = 10 mg/1 ml, dissolved in dimethyl sulfoxide (DMSO)].

Antiproliferative assay

Proliferative inhibition was determined by cytotoxic testing using the MTT assay on 96-well microplates (Figures 1 and 2). T47D breast cancer cells, which are estrogen receptor-positive, were obtained from the Integrated Research Testing Laboratory (*Laboratorium Pengujian Penelitian Terpadu Universitas Gadjah Mada*). Cytotoxic test results were read

by ELISA reader at 595 nm. Percentage cytotoxicity of soursop and tamoxifen was calculated by the formula:

$$\% \text{ cytotoxicity} = 100 - \{[(A-B) - (C-B)] / (A-B)\} \times 100\%$$

[A= OD of control cell; B = OD of medium; C= OD of treated cell]

Statistical analysis

The experiments were performed in triplicate. Cytotoxicity percentages are presented as mean ± standard deviation. The percentage of inhibition of each test material was converted into dose-responsiveness curves using probit analysis to obtain the IC₅₀ of each test material. Significant differences between groups were determined at p<0.05 by Kruskal-Wallis test.

									T1	T1	T1
									T2	T2	T2
									T3	T3	T3
									T4	T4	T4
									T5	T5	T5
									T6	T6	T6
									KS	KS	KS
									KM	KM	KM

Figure 2. Microplate scheme of tamoxifen cytotoxic test

T1-T7 = Positive controls (Medium + T47D cells + tamoxifen) (at doses of 1000; 500; 250; 125; 62.5; 31.25 µg/ml); KS = Medium + T47D cells; KM = medium only

Table 1. Proliferative inhibition of (%) soursop on T47D cells

Dose ($\mu\text{g/ml}$)	Soursop aqueous extract		
	Fruit	Seed	Leaf
300000	82.05 \pm 1.52	36.28 \pm 1.82	78.55 \pm 0.86
150000	21.37 \pm 2.14	36.14 \pm 3.36	85.78 \pm 0.83
75000	9.72 \pm 2.06	19.54 \pm 6.16	88.45 \pm 6.21
37500	7.28 \pm 0.23	14.50 \pm 1.63	53.16 \pm 3.74
18750	7.23 \pm 1.07	8.46 \pm 3.32	29.08 \pm 1.43
9375	10.15 \pm 4.77	11.65 \pm 0.77	12.13 \pm 5.44
4687.5	8.35 \pm 2.68	5.63 \pm 2.79	9.30 \pm 3.53
2343.75	5.23 \pm 2.60	3.73 \pm 1.44	5.16 \pm 0.63
1171.875	-0.43 \pm 3.42	4.76 \pm 3.07	12.34 \pm 1.46
585.9375	0.52 \pm 1.94	5.30 \pm 1.42	12.20 \pm 1.53

Values represent mean \pm S.D. (%)

Ethical clearance

The study protocol was approved by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Gadjah Mada University – Dr. Sardjito General Hospital (Reference number: KE/FK/259/EC).

RESULTS

Cytotoxic testing was performed three times, starting at a dose of up to 1,000 $\mu\text{g/ml}$, up to 10,000 $\mu\text{g/ml}$, and up to 50,000 $\mu\text{g/ml}$. However, the soursop fruit, seed and leaf aqueous extracts at such doses could not inhibit T47D cell proliferation. The cytotoxic tests at an initial dose of 300,000 $\mu\text{g/ml}$ showed the greatest proliferative inhibition with soursop leaf (88.45%) at a dose of 75,000 $\mu\text{g/ml}$. The lowest proliferative inhibition was found in soursop seed extract, with 36.28% inhibition at a dose of

300,000 $\mu\text{g/ml}$ (Table 1). The proliferative inhibition of tamoxifen was better than those of the aqueous extracts of soursop leaves, seeds, and fruits. Tamoxifen at a dose of 500 $\mu\text{g/ml}$ was able to inhibit proliferation of T47D cells by 99.91% (Table 2). The mean IC_{50} values show that the lowest dose was for tamoxifen (114.52 $\mu\text{g/ml}$), followed by soursop leaf, fruit and seed (Table 1). The IC_{50} value of tamoxifen was significantly different from the IC_{50} values of the soursop aqueous extracts, showing that the latter are less cytotoxic (Table 3).

DISCUSSION

We report the finding of an antiproliferative effect of aqueous extract of soursop leaves on T47D cells but only at high doses (mean IC_{50} = 31,384 $\mu\text{g/ml}$). We also noted that there was a significant difference in the IC_{50} values of soursop leaf aqueous extract and tamoxifen. A previous study with soursop leaf butanol extract obtained an IC_{50} value of 29.2 μg for MDA-MB-435S cells and 30.1 μg for HaCaT cells, while the IC_{50} value of soursop leaf ethanol extract was 17,149 for T47D cells. The results of this study showed that soursop leaf aqueous extract is less cytotoxic, because its acetogenin content is less than that of the ethanol extract.⁽¹⁹⁾ If people have to consume aqueous extracts of soursop leaves, they will require higher doses to get any cytotoxic effect.

Table 2. Proliferative inhibition of tamoxifen on T47D cells

Dose of tamoxifen ($\mu\text{g/ml}$)	Proliferative inhibition (%)
1000	97.53 \pm 1.75
500	99.91 \pm 0.55
250	75.16 \pm 1.98
125	40.99 \pm 1.73
62.50	26.00 \pm 6.19
31.25	21.15 \pm 4.18

Values represent mean \pm S.D.

Table 3. Mean IC₅₀ values of TD47D cells by treatment groups soursop and tamoxifen

Treatment group	IC ₅₀ (µg/ml)	p value
Soursop fruit	329,194.81 ± 50,762.72	0.016
Soursop seed	1,528,800.00 ± 676,401.00	
Soursop leaf	31,384.21 ± 1021.88	
Tamoxifen	114.52 ± 13.41	

Values represent mean ± S.D.

In this study, the aqueous extract of soursop seeds also showed less cytotoxic activity, having a very high IC₅₀ value (1,528,800). To our knowledge there have been no studies demonstrating the ability of soursop seed aqueous extract to kill cancer cells, except at high doses.⁽²⁰⁾ High doses of *Annona muricata* seeds (1000 mg/kg BW) decreased the viability of ovarium cancer cells in rats, but to a lesser extent than vinblastin.⁽²⁰⁾ In contrast, organic and aqueous extracts of defatted *Annona squamosa* (custard apple) seeds tested on different human tumor cell lines for antitumoral activity, induced apoptosis in MCF-7 and K-562 cells, but not in COLO-205 cells.⁽²¹⁾

The aqueous extract of soursop fruits was able to inhibit proliferation of T47D cells, in accordance with previous studies. Day et al⁽²²⁾ reported selective inhibition of human breast cancer cell growth by an acetone extract of graviola (soursop) fruits in vitro and in vivo involving downregulation of EGFR expression. The aqueous extract of *cherimoya* (*Annona cherimola*) fruit pulp presented antitumoral activity in *Drosophila melanogaster*.⁽²³⁾ In the present study, the IC₅₀ of soursop fruit was better than that of soursop seed because the fruit has a higher content of annonacin (the main acetogenin). A dichloromethane extract of *Annona muricata* seeds contained only annoreticuin-9-one, while the pulp (flesh) of the fruits yielded cis-annoreticuin and sabadelin.⁽¹⁹⁾

According to a literature search, the cytotoxic effect of soursop is due to the acetogenin fraction and soursop ethanol extract possesses anti-inflammatory activity as shown

in experimental animals.^(24,25) Moreover, acetogenins also have an inhibitory effect on NADH ubiquinone oxidoreductase, which is an important enzyme in oxidative phosphorylation reactions, resulting in lack of ATP in the cells.⁽¹⁰⁾ In fact, the aqueous extract of soursop leaves has been studied in vivo as an antioxidant to lower oxidative stress in the mice liver induced by streptozotocin.⁽²⁷⁾

Our study has shown that the aqueous extract of soursop fruit has a better inhibitory effect on the proliferation of T47D cells than the aqueous extract of soursop seed, but both are less cytotoxic to T47D cells than tamoxifen. This means that for cancer self-treatment by the community, large amounts of soursop fruit have to be consumed. Future research in experimental animals is needed to determine the exact dose of soursop aqueous extract that can kill cancer cells.

CONCLUSIONS

Proliferative inhibition of aqueous extract of soursop leaves against T47D cells is the highest compared to that of the fruits and seeds. The IC₅₀ value of soursop aqueous extract against T47D cells is less toxic than tamoxifen.

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