ARTIKEL HASIL PENELITIAN

Antitumor Activity of Soursop (*Annona muricata* L.) Leaves On Prostate Cancer Cell Line

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Abstract

Soursop (*Annona muricata* L.) leaves were used as a traditional drug for many diseases such as cancer. Statistical data from one cancer hospital in Jakarta in the period of 2005 and 2007 showed that prostate cancer has become one of ten common cancer cases in ambulatory patients. This research was conducted to investigate the potency of an herbal drug for prostate cancer. The soursop leaves were extracted by maceration and fractionated by liquid-liquid extraction. The activity of all samples was then tested by Brine Shrimp Lethality Test (BSLT) and MTT assay using prostate cell line, LNCaP. The result showed that soursop leaves extract and all fractions had a cytotoxic activity to *Artemia salina* L. larvae (LC_{50}<1000 μg/ml). The MTT assay showed that soursop leaves extract, n-hexane and ethyl acetate fractions had a proliferative effect to LNCaP cell line (IC_{50}<350 μg/ml). Whereas, water fraction had no effect up to 400 μg/ml. Ethyl acetate fraction showed the best antitumor activity because its LC_{50} and IC_{50} values were the lowest. It can be suggested that soursop leaves showed antitumor activity and had a prospective to be developed as herbal drug for prostate cancer therapy.

Keywords: Soursop leaves, *Annona muricata* L., Brine Shrimp Lethality Test (BSLT), LNCaP

Abstrak

Daun sirsak (*Annona muricata* L.) banyak digunakan dalam pengobatan tradisional untuk berbagai macam penyakit seperti kanker. Berdasarkan data statistik dari salah satu rumah sakit kanker di Jakarta pada periode 2005 hingga 2007, menunjukkan bahwa kanker prostat telah menjadi 1 dari 10 macam kasus kanker yang umum diderita oleh pasien ambulatori. Penelitian ini dilakukan untuk menyelidiki potensi dari obat herbal untuk kanker prostat. Daun sirsak diekstraksi dengan cara maserasi dan fraksinasi menggunakan ekstraksi cair-cair. Aktifitas dari keseluruhan sampel kemudian diuji menggunakan Brine Shrimp Lethality Test (BSLT) dan uji MTT dengan kultur sel kanker, LNCaP. Hasil penelitian menunjukkan bahwa ekstrak daun sirsak dan seluruh fraksinya memiliki aktifitas sitotoksik terhadap larva *Artemia salina* L. dengan LC_{50}<1000 μg/ml. Hasil dari pengujian MTT menunjukkan bahwa ekstrak daun sirsak dari fraksi n-heksana dan etil asetat memiliki efek proliferatif pada kultur sel LNCaP (IC_{50} < 350 μg/ml). Sedangkan fraksi air dari daun sirsak hingga konsentrasi 400 μg/ml tidak menunjukkan adanya efek yang sama. Fraksi etil asetat menunjukkan aktivitas antitumor yang paling baik karena memiliki nilai LC_{50} dan IC_{50} yang paling rendah. Sehingga dapat disimpulkan bahwa daun sirsak menunjukkan adanya aktivitas antitumor dan berpotensi untuk dikembangkan sebagai obat herbal untuk terapi kanker prostat.

Kata kunci : Daun sirsak, *Annona muricata* L., Brine Shrimp Lethality Test (BSLT), LNCaP

Introduction

Cancer was a leading cause of death and accounted for 7.6 million deaths in the world (around 13% of all deaths) in 2008. Lung, breast, colorectal, stomach and prostate cancers cause the majority of cancer deaths (WHO 2013). In Indonesia, cancer was the sixth major cause of death (5.7% of 4,552 death cases) according to health survey period July 2006-August 2008. Heart, lung, cervix, breast, ovary and prostate cancers cause the majority of cancer deaths (Balitbangkes 2008). Statistical data from one cancer hospital in Jakarta in the period of 2005-2007 showed that prostate cancer has become one of ten common cancer cases in ambulatory patients (National Cancer Center 2013).

A lot of anticancer agents were derived from plants such as *podophyllin* from *Podophyllum rhizome, vinca alkaloid* from *Vinca rosea*, *paclitaxel/taxol* from *Taxus brevifolia*, and *camptothecin* from *Camptotheca acuminata* (Nwafor *et al.* 2001). This research was conducted to investigate the potency of soursop leaves (*Annona muricata* L.) for prostate cancer.

Soursop leaves were empirically used as a traditional drug. In America, this is use for treatment of hypertension, colon parasite, tumor, and cancer; in Peru it is use for treatment of digestion, inflammation, parasite and tumor (Zuhud 2011) and in Indonesia it is used for treatment of Cyst, hypertension, diabetic, tumor, and cancer (prostate, pancreatic and lung) (Radi 1998).

Scientific research has been showed that soursop leaves were able to kill various cancer cells (McLaughlin and Sastrodihardjo 1995), inhibited the growth of bile cancer cell line (Rieser *et al*. 1996), and cytotoxic agent of tumor cells (Chang and Wu

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2001). From this data, we assumed that soursoup leaves have the potency for prostate cancer therapy.

Materials and Methods

Materials

Soursop leaves, shrimp egg (*Artemia salina* Leach), aquadest, ethanol 95%, ethyl acetate, n-hexane, toluene, chloroform, ether, amyl alcohol, hydrochloric acid, sulfuric acid, acetic acid glacial, ammonia, bismuth-subnitric, mercury(II)chloride, Ferri (III) chloride, chloral hydrate, sodium hydroxide, potassium Iodide, sodium chloride, sodium tiosulphate anhydrate, gelatin powder, vanillin powder, magnesium powder. LNCaP cell line, RPMI-1640 media, antibiotic Penicillin-Streptomycin, Fetal Bovine Serum.

Tools

Oven, grinder, macerator, rotavapor (Buchi Rotava-por R-3000), waterbath, electric dryer (MITSEDA HD 350), separation funnel, picnometer, crucible porcelain, aerator (RESUN air-pump), neon lamp, micropipette, volume pipette, 96-well plate, distillation apparatus, analytical balance, common glassware in laboratory, microscope (OLYMPUS CX31), furnace, spectrophotometry UV-Vis, micro-plate reader.

Sample preparation

Soursop leaves were collected from Rancaekek, West Java-Indonesia in February 2012. The plant was identified in Plant Taxonomy Laboratory, Departement of Biology, Faculty of Mathematic and Natural Science Universitas Padjadjaran that belong to *Annonaceae,* species *Annona muricata* L. Powdered crude drug was extracted by maceration using 96% ethanol. The filtrate was condensed using vacuum rotavaporator then electric dryer. Concentrated extract was then fractionated by liquid-liquid extraction (LLE) using n-hexane and ethyl acetate, respectively, where the extract was dissolved in aquadest previously. Macroscopic and microscopic analysis, extract quality characterization and phytochemical screening have been done using standard procedures (Ditjen POM 1987; 1979).

Bioactivity screening

Bioactivity of extract and all LLE fractions was monitored by Brine Shrimp Lethality Test (BSLT) using nauplii *Artemia salina* Leach. Procedures were developed by McLaughlin *et al.* (1998) and Meyer *et al.* (1982) where natural product extract and fractions are tested at initial concentrations of 10, 100, and 1000 ppm or (μg/mL) in vial containing of 5 mL artificial sea water (38 g sea salt per liter of water) and 10 brine shrimp in each of three replicates. Alternatively, material may be dissolved in dimethyl sulfoxide up to 50 μL. Survivors of *Artemia salina* Leach are counted after 24 hours. These data are processed in simple program for probit analysis on personal computer to estimate LC_{50} values with 95% confidence intervals for statistically significant comparison of potencies. In cases where data were insufficient for this technique, the dose-response data were transformed into a straight line by means of log transformation; the LD_{50} was derived from the best fit line obtained by linear regression analysis.

Antitumor activity

Antitumor activity was performed using LNCaP human prostate cancer cell lines in the presence of various concentrations of extract or fractions by a colorimetric tetrazolium salts (3(4,5-dimethyl-thiazol-2-yl)-2,5 diphenyltetrazolium bromide) or MTT assay. This methods has described previously in Octaviani *et al*. (2013). Briefly, LNCaP cell lines (2 × 104 in 50 μl/well) were plated in 96-well plates. After the initial cell seeding, different concentrations of extract or fractions were added and incubated for 24 h. Ten microliters of water-soluble tetrazolium (WST)-8 assay. Cell-counting solution was added to each well and incubated at 37°C for 3h. After the addition of 100 μl/well of 1 NHCl, the cell proliferation rate was then determined by measuring the absorbance at 450 nm, with a reference wavelength of 650 nm. The absorbance was read using a microtiter plate reader. Results were derived from triplicate experiments.

Result and Discussion

Macroscopic and Microscopic analysis

Macroscopic analysis of soursop leaves showed that soursop leaves appear as simple mild brown leaves, obtuse apices, attenuate or obtuse bases, ciliate margin, laevis texture, and ovale or obovate shape with long 6-18 cm wide 2-6 cm. By microscopic analysis fragments identified were epidermis with anomositic stomates, vascular trace with trachea, and filament. These result has appropriate with characteristic of soursop leaves in reference standard.

Extract quality characterization

Extract quality characterization is beneficial to indicate identity and quality of soursop leaves extract that was done by determination of specific and non-specific parameters. This was one of requirement of natural product (extract or fractions) that will

developed as Obat Herbal Terstandar (Indonesian Standardized Herbal Drug).

Specific parameters are describe extract efficacy that associates with secondary metabolite content or active substance in extract. This was reflected by organoleptic analysis, total of extractive matters and phytochemical screening (Depkes RI 2000). According to organoleptic analysis, soursop leaves extract appeared as viscous mass, sticky, dark green with spesific odor. Table 1 referred to phytochemical screening results, showed that both crude drug and extract consisted of flavonoid, poliphenolic, mono and sesquiterpenoid, steroid and quinon. This also pointed out that extraction solvent was appropriated as it was able carried all metabolites from initial sample (crude drug). Extractive matters of maceration process and LLE are shown in Table 2.

Non-specific parameters i.e specific weight, water content, total ash content and acid-insoluble ash content are describes safety and stability of soursop leaves extract (Table 3). Specific weight associates with purity. Concentrated extract must have water content under 30% that kept from fungi growth (Voight 1984). Ash contents are figure out the amount of external and internal mineral (Depkes RI 2000).

Table 1. Result of Phytochemical Screening

Secondary Metabolites	Crude Drug	Extract
Alkaloid	-	-
Flavonoid	+	+
Tannin	-	-
Poliphenolic	+	+
Mono & seskuiterpenoid	+	+
Steroid	+	+
Triterpenoid	-	-
Quinon	+	+
Saponin	+	+

Table 2. Results of extraction and fractionation

Sample	Initial Mass(g)	Extractive Matters (g)	Rendement (%)
Soursop leaves	1349.93 (powdered crude drug)	225.25	16.68
n-hexane fraction	60.00 (concentrated extract)	25.42	42.36
Ethyl acetate fraction		11.99	19.98
Water fraction		20.15	33.58

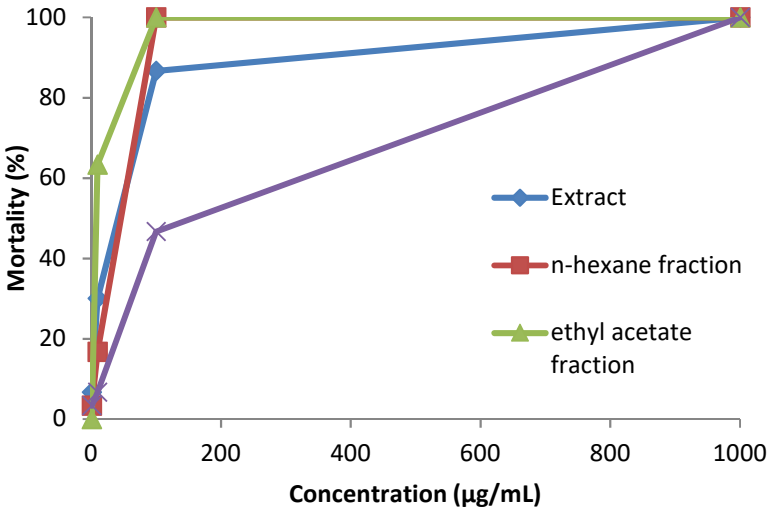

Figure 1. First stage % mortality values at different concentration.

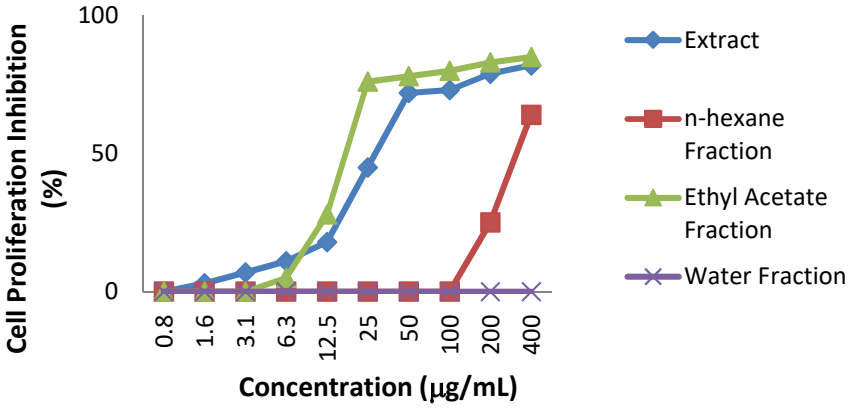

Figure 2. Activity of samples against LNCaP human prostate cancer cell line

Activity test:

a. BSLT results

BSLT was done in two stages. First stage applied with initiated doses 1000 µg/mL, 100 µg/mL, 10 µg/mL, and 1 µg/mL. Figure 1 showed % mortality of each extract, n-hexane fraction, ethyl acetate fraction and water fraction at different concentration.

Table 3. Characteristic of quality

Parameter	Results
Specific weight	0.766
Water content (% v/b)	18.000
Total ash content (% b/b)	5.778
Acid-insoluble ash content (% b/b)	0.822
Water soluble extractive matters (% b/b)	27.667
Ethanol soluble extractive matters (% b/b)	82.333

At the second stages, variation of doses were made in narrow range approached to 50% mortality value of each extract, n-hexane fraction, ethyl acetate fraction and water fraction. After that LC_{50} value of each samples were calculated, that respectively was 70 µg/mL; 64.62 µg/mL; 6.08 µg/mL and 124.98 µg/mL. These results showed that extract and all fractions demonstrated cytotoxic activity (LC_{50} < 1000 µg/ml) to *Artemia salina* L. larvae (Meyer *et al*. 1982). Cytotoxic activity of soursop leaves extract was a synergistic effect of its metabolite constituents, reflected by LC_{50} value of each fractions. Ethyl acetate fraction have the best cytotoxic activity because its LC_{50} values were the lowest.

b. MTT assay result

Figure 2 displayed the activity of samples against LNCaP human prostate cancer cell line. The figure showed that extract, n-hexane fraction, and ethyl acetate fraction inhibited the LNCaP cell and reach 50% cell proliferation inhibition at different concentration. IC_{50} value of each samples were 29.6 µg/mL; 328.2 µg/mL; and 18.2 µg/mL respectively. On the contrary, water fraction until the highest concentration tested failed to reach 50% cell proliferation inhibition, indicated low anticancer activity against LNCaP human prostate cancer cell line.

In this research, correlation between BSLT study with its *in vitro* study (MTT assay) were proven to be true (Meyer *et al.* 1982). In our BSLT results, ethyl acetate fraction that showed the most toxic agent with LC_{50} value 6.08 µg/mL, in an *in vitro* study against LNCaP cancer cell lines also showed the best antitumor activity with IC_{50} value 18.2 µg/mL.

Important finding of our research to support and verify empiric or Indonesian traditional usage of soursop leaves (*Annona muricata* L.) for treatment of prostate cancer. So soursop leaves can be developed as herbal anticancer for prostate cancer therapy, where extract and ethyl acetate fractions showed strong activity.

Conclusions

The BSLT result showed that soursop leaves extract and all fractions demonstrated cytotoxic activity (LC_{50}<1000 µg/ml) to *Artemia salina* L. larvae. The MTT assay showed that soursop leaves extract, n-hexane and ethyl acetate fractions had proliferative effect to LNCaP cell line (IC_{50}<350 µg/ml). Whereas, water fraction had no effect up to 400 µg/ml. Ethyl acetate fraction showed the best anticancer activity

because its LC_{50} and IC_{50} values were the lowest. It can be suggested that soursop leaves showed antitumor activity and prospective to be developed as herbal drug for prostate cancer therapy.

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