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

*Ade Zuhrotun, Rizky Abdullah, Meliana Thamrin, Resti Febriliza
Faculty of Pharmacy, Universitas Padjadjaran,
Jalan Raya Bandung-Sumedang KM 21.5 Jatinangor 45363 West Java, Indonesia

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

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 Cryst, 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

*Corresponding author. e-mail: airkusaja@yahoo.com
From this data, we assumed that soursop 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 Rotavapor 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, microplate 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 LC50 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 LD50 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.
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 specific odor. Table 1 referred to phytochemical screening results, showed that both crude drug and extract consisted of flavonoid, poliphenolic, mono and sesquiterpénoïd, 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>
<thead>
<tr>
<th>Secondary Metabolites</th>
<th>Crude Drug</th>
<th>Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Tannin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Poliphenolic</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Mono &amp; sesquiterpénoïd</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Steroid</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Triterpenoid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quinon</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Saponin</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1. Result of Phytochemical Screening

<table>
<thead>
<tr>
<th>Sample</th>
<th>Initial Mass (g)</th>
<th>Extractive Matters (g)</th>
<th>Rendement (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soursop leaves</td>
<td>1349.93 (powdered crude drug)</td>
<td>225.25</td>
<td>16.68</td>
</tr>
<tr>
<td>n-hexane fraction</td>
<td>60.00 (concentrated extract)</td>
<td>25.42</td>
<td>42.36</td>
</tr>
<tr>
<td>Ethyl acetate fraction</td>
<td></td>
<td>11.99</td>
<td>19.98</td>
</tr>
<tr>
<td>Water fraction</td>
<td></td>
<td>20.15</td>
<td>33.58</td>
</tr>
</tbody>
</table>

Table 2. Results of extraction and fractionation

Figure 1. First stage % mortality values at different concentration.
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. 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 LC50 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 (LC50 < 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 LC50 value of each fractions. Ethyl acetate fraction have the best cytotoxic activity because its LC50 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. IC50 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 LC50 value 6.08 µg/mL, in an *in vitro* study against LNCaP cancer cell lines also showed the best antitumor activity with IC50 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.

### Table 3. Characteristic of quality

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specific weight</td>
<td>0.766</td>
</tr>
<tr>
<td>Water content (% v/b)</td>
<td>18.000</td>
</tr>
<tr>
<td>Total ash content (% b/b)</td>
<td>5.778</td>
</tr>
<tr>
<td>Acid-insoluble ash content (% b/b)</td>
<td>0.822</td>
</tr>
<tr>
<td>Water soluble extractive matters (% b/b)</td>
<td>27.667</td>
</tr>
<tr>
<td>Ethanol soluble extractive matters (% b/b)</td>
<td>82.333</td>
</tr>
</tbody>
</table>

Conclusions

The BSLT result showed that soursop leaves extract and all fractions demonstrated cytotoxic activity (LC50<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 (IC50<350 µg/ml). Whereas, water fraction had no effect up to 400 µg/ml. Ethyl acetate fraction showed the best anticancer activity.
because its LC50 and IC50 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.

Acknowledgment

We thank to Direktorat Jenderal Perguruan Tinggi (DIKTI) that funded research by Program Hibah Bersaing Desentralisasi Universitas Padjadjaran 2012.

References


