Cytotoxic Activity of Soursop "Annona muricata" Leaves Extracts and their Phytochemical Contents

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Abstract

Objective: Annona muricata (soursop) leaves belong to Annonaceae family, has been used as traditional medicine in Indonesia. Several plant polyphenols, such as flavonoid quercetin and flavone (2-phenyl-4H-1-benzopyran-4-one) may have chemoprevention property by reducing the incidence of many types of cancer, especially in colon epithelia. This study aims to determine cytotoxic activity of the extracts and their total flavonoid content of the leaves extract. Methods: Extract used in flavonoid level determination is standardized water extract (Zirzak Orac), ethanol-soluble fraction (ESFAM) and ethanol-insoluble fraction (EIFAM). The level of flavonoid content was determined by spectrophotometer UV-Vis with quercetin and rutin as standard. Antiproliferative effect was evaluated against colorectal cancer cell line (DLD-1 and COLO 205), and normal cell (HEK) using microculture assay based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT). Results: Flavonoid concentrations of Zirzak Orac, ESFAM, EIFAM are 0.15%, 0.83% and 0.07%, respectively. EIFAM shows the lowest IC50 value against DLD 1. The value of IC50 on the normal cells HEK is expected to be as high as possible, EIFAM has the highest. Conclusion. EIFAM showed selective cytotoxic activities in vitro, while it contains the lowest concentration of flavonoid and annonacin.

Keywords: Annona muricata L., Cytotoxicity, Flavonoid.

INTRODUCTION

In several areas in the world, tea leaves or decoction of Annona muricata helps in curing several diseases.1-4 Members of family Annonaceae have been investigated as potential sources of biologically active Annonaceous acetogenins, some of which demonstrated a powerful anti-tumor activities.5 Currently, 34 acetogenins have been identified in the leaves of A. muricata.6 The cytotoxicity of acetogenins has been known to be stronger in tumorous than in normal cells.7 The primary site of action of the acetogenins is complex I of the electron transport chain in mitochondria.8 Caspase-3 activity and deoxynucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL)
assay results showed that the ethanolic extract of A. muricata induced apoptosis in the myelogenous leukemic K562 cell line. A. muricata is considered to be a poten
tial candidate as a natural product source for the development of pro-apoptotic drugs.\[^9\]

Several plant polyphenols, such as flavonoid quercetin and flavone (2-phenyl-4H-1-
benzopyran-4-one) may have chemoprevention property by reducing the inci
dence of many types of cancer, especially in colon epithelia. \[^10\] The leaves of A. muricata has a potent antioxidative activity. \[^11\] The flavonoid found to be a
stronger apoptosis inducer than the clinically established antitumor agent camptothecin. The effects of flavone in HT-29 cells were associated with changed
mRNA levels of cell-cycle- and apoptosis-related genes including cyclooxygenase-2
(COX-2), nuclear transcription factor kB (NF-kB), and bcl-Xl. In addition, flavone
exhibited a high selectivity for the induction of apoptosis and of growth inhibition only in
the transformed colonocytes. \[^12\] People have used A. muricata leaves traditionally as tea
drinks as a water extract and found to be safe. \[^13\] Hence, it needs further
investigation, especially determination of phytochemical content of A. muricata leaves extract and its cytotoxic activity. This study aims to determine cytotoxic activity of the extracts and their total flavonoid content of the leaves extract.

MATERIALS AND METHODS

Preparation of Extracts

A. muricata extract used for this study is a fraction derived from standardized water
extract produced by Javaplant namely Zirzak Orac. The extract was fractionated
using ethanol 95% to produce ethanol-soluble fraction (ESFAM) and ethanol-
insoluble fraction (EIFAM).

EIFAM was assessed for cytotoxicity in in vitro study. The cytotoxicity of others type of
extract (Zirzak Orac and ESFAM) have already published in other journal.\[^14\]

Determination of Flavonoid Content

The extract was dissolved with 10 mL methanol pa. Then 1,0 mL of it added with 3
mL methanol, 0,2 mL of AlCl\textsubscript{3} 10%, 0,2 mL potassium acetate, and 5,6 mL sterile
distilled water, and followed by incubation for 30 minutes in dark place at room
temperature. The absorption was measured with spectrofotometric UV-Vis at
wavelength 415 nm. The level of flavonoid total was calculated as rutin equivalent
(RE). All types of extract (Zirzak Orac, ESFAM and EIFAM) were assessed.

In vitro cytotoxicity by MTT assay

Cytotoxicity of the extracts in in vitro study measured using inhibitory concentration (IC
50). IC50 value indicates the concentration to inhibit proliferation of cancer cells by
50%. The lower the
IC 50, the lower its value the greater its
potency to inhibit cell proliferation.

EIFAM is tested against colorectal cancer
cells (DLD-1 and COLO 205) and normal
human cell (HEK). DLD-1 was derived from
Dukes' type C human colorectal adenocarcinoma tissues that metastasized
until regional lymph node. COLO 205 was
derived from Dukes' type D human
colorectal adenocarcinoma tissue that had
distant metastases.\[^15\]

The human colorectal cell line types used in
this study were COLO 205 and DLD-1 and
were purchased from ATCC\textsuperscript{8} (Catalog No.
CCL-222 and Catalog No. CCL-221, respectively); they were maintained
according to supplier guidelines (American
Type Culture Collection, ATCC, Manassas,
VA). In addition, the human embryonic
kidney (HEK) cell line was obtained from
the Institute of Human Virology and Cancer
Biology, University of Indonesia and used as
the normal control cell line.

Cells were incubated with 95% air and 5%
CO\textsubscript{2} at 37°C; all cells were maintained below
passage 20 and used in experiments during
the linear phase of growth. The human
colorectal cancer cell lines DLD-1 and COLO
205 were maintained in RPMI 1640 medium
and HEK was maintained in DMEM, both
media were supplemented with 10% FCS,
2 mM L-glutamine, 100 units/mL penicillin,
and 100 mg/mL streptomycin and
maintained in a humidified environment
containing 5% CO\textsubscript{2} at 37 °C.
Antiproliferative effect of, dose range 62.5-2000 ppm, was evaluated against cell lines with micro culture assay based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); compared with 5-Fluorouracil. The cytotoxic activity of extract was assessed using an MTT assay; cells were cultured in 96-well micro titer plates, where each well contained 2×10^4 cells, and treated for 48 h. Cytotoxicity was assessed using the MTT test (Trevigen’s TACS® MTT Cell Proliferation Assay), in triplicate. \[16, 17\]

RESULTS

Extract used in flavonoid level determination are standardized water extract (Zirzak Orac), ethanol-soluble fraction (ESFAM) and ethanol-insoluble fraction (EIFAM). Zirzak Orac contains 0.018% w/w annonacin, while ESFAM contains 0.36% w/w annonacin or 3.6 mg/g. \[14\] Acetogenin spot of EIFAM is undetectable. Flavonoid content of A. muricata leaves extract showed on Table 1.

Extract with higher cytotoxicity shows lower IC 50 values. IC 50 values of EIFAM against DLD-1 is 148.4 μg/mL. Citotoxicity of EIFAM against COLO is not better than against DLD-1 which is 1492.2 μg/mL (Fig. 2). Extracts are not expected to have cytotoxicity against normal human cell (HEK), and EIFAM has less cytotoxic activity than ESFAM and Zirzak Orac (Fig. 3). IC50 values of ESFAM and Zirzak Orac are 353.4 μg/mL and 1043.5 μg/mL, respectively. All that values are lower than the IC50 values of EIFAM, 5-Fluorouracil >2000 μg/mL. In summary, EIFAM shows the lowest IC50 value against DLD 1. IC50 on the normal cells HEK is expected to be as high as possible. The bioactive compound in EIFAM found to inhibit the cancer cell, resulted in low IC50 value in both cell lines, DLD-1 and COLO 205) but does not inhibit the normal cell (high IC50 value in HEK cell line).

DISCUSSION

The major bioactive nutraceutical compounds in plants are flavonoids as phenolic compounds with potent antioxidants and metal chelating properties. Other properties such as anti-inflammatory, anti-allergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities have also been reported. \[18\] Tea flavonoids are potent antioxidants that are absorbed in the gut after consumption and leads to a significant increase in the antioxidant capacity of the blood. \[19\] A study characterizes a positive, significant linear relationship between antioxidant activity and total phenolic content of traditional Chinese medicinal plants associated with anti-cancer. \[20\]

This study is part of a study that used several compounds tested against human colorectal cancer cell lines COLO 205 and DLD-1 and non-cancer human embryonic kidney (HEK) cell lines. In previous paper there was a concentration dependent in decreasing viable cells after treatment with the Zirzak Orac (standardized water extract) and ESFAM (Ethanol Soluble Fraction of water extract) (0.0625 – 2mg/ml) for 48 hour in both cancer cell lines. \[14\] In this paper EIFAM also showed similar results. These findings are consistent with previous studies in demonstrating the cytotoxic activity of extracts as well as its fractions against HeLa cell culture and T47D breast cancer cell lines, pancreatic carcinoma cell (PACA-2), prostate adenocarcinoma (PC-3), hepatoma cell lines Hep G(2) and 2,2,15, and colon adenocarcinoma (HT-29) cell lines. \[21, 22\] Other study in mice showed that annonacin inhibited the normal growth of the lung tumors during two-week period, it did not eradicate the tumors nor stop their growth in mice. Whereas ethanolic extract of the leaves from Colombia, Indonesia and Taiwan were active against Cells-MDBK, CA-Mammary-MCF-7, and Hep G 2,2,15 respectively. Ethanol extract of the leaves from Borneo, Costa Rica, USA were active against cell culture CA-9KB. \[1\]

The difference between previous types of A. muricata extracts (Zirzak Orac and ESFAM) and EIFAM laid on cytotoxicity against normal human cell line (HEK). IC50 against this type of cell is expected to be as high as possible and EIFAM had the highest. It demonstrated that cytotoxicity of ESFAM and Zirzak Orac are not selective against colorectal cancer cell lines because they also inhibited normal cell line. \[14\] In this in vitro study EIFAM is considered to be more potential than the others as shown by the lowest IC50 value against DLD-1 colorectal...
cancer cell lines, and comparable with standard treatment 5 FU against COLO 205, with the highest IC\textsubscript{50} value against HEK.

The cytotoxicity of acetogenins has been shown to be stronger in tumorous than in normal cells.\textsuperscript{7} In this study only EIFAM showed consistent result with the previous study despite its low (even undetected) annonacin content, indicating that \textit{A. muricata} leaves should be further investigated for other bioactive compounds exist in EIFAM especially the unknown ones with anticarcinogenic properties to the cells but protecting normal cells in vitro. Moreover, obtaining EIFAM is simpler and cheaper as compared to ESFAM. The water extract of \textit{A. muricata} leaves represent traditional used. The extract fraction containing the highest acetogenin concentration was assessed to find out the acetogenin concentration-functional corelation of fractional extracts in inducing cytotoxicity. The results show that unknown compound(s) may contribute to cytotoxic activity as well as even selectivity showed by ethanolic insoluble fraction of \textit{A. muricata} water extract (EIFAM). Taken together, EIFAM showed more potential cytotoxic activities \textit{in vitro}. However this result needs further investigation to ensure its effect in human body.

Table 1: Flavonoid content of \textit{a.muricata} leaves extract

<table>
<thead>
<tr>
<th>Samples</th>
<th>Flavonoid concentration (% w/w)</th>
</tr>
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<tbody>
<tr>
<td>\textit{A. muricata} water extract (Zirza Orac)</td>
<td>0,15</td>
</tr>
<tr>
<td>Ethanol-soluble fraction (ESFAM)</td>
<td>0,83</td>
</tr>
<tr>
<td>Ethanol-insoluble fraction (EIFAM)</td>
<td>0,07</td>
</tr>
</tbody>
</table>

Figure 1. Procedure of Fractionation on Standardized Water Extract of \textit{A. muricata} Leaves
CONCLUSION

EIFAM contains the lowest concentration of flavonoid and annonacin but showed the most potential cytotoxic activities in vitro. Because it fulfills the ideal criteria’s of cancer chemotherapy which found to inhibit the cancer cell, but does not inhibit the normal cell, it warrants further investigation.

References

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