Cytotoxicity Testing of Graviola (Annona muricata Linn.) Leaf Extracts In vitro

Koravit Somkid1*, Pornchai Sincharoenpokai1, Sakwichai Ontong1, Somchit Niumsakul1 and Nuchatra Chansuvanich1

ABSTRACT

Graviola (A. muricata) is a medicinal plant that has been widely used for the treatment of various diseases, such as rheumatoid, neuralgia, diabetes, hypertension, insomnia, parasitic infection, and cancer. However, there has been no report whether the extracts from A. muricata leaves have any potential to produce any adverse effect to the normal cells. The present study aims to evaluate the cytotoxicity of three extracts from A. muricata leaves using a cell proliferation assay in normal cell (Chang-Liver) and hepatocarcinoma cell (Hep G2) lines. The first extract (or Amm1) showed the most potent anti-cell proliferation effect on both cell lines with the IC50 value at 3.13 µg/ml in Chang-Liver cells and 11.06 µg/ml in Hep G2 cells. Amm2 extract had no effect on cell proliferation in both cell lines. Amm3 extract inhibited only Hep G2 cell proliferation at IC50 value at 20.56 µg/ml. These results suggest that the leaf extracts from A. muricata from the different processes of extraction exhibit the different effects on cell proliferation. Therefore, a concurrent consumption of A. muricata must be done with caution, particularly on type of extractions or sample preparations.

Key words: Annona Muricata Linn., Graviola, Cytotoxicity

*Corresponding author; e-mail address: koravit.sdmac.mail.go.th

1 Medicinal Plant Institute, Department of Medical Science, Ministry of Public Health, Nonthaburi, 11000
INTRODUCTION

Herbal medicines are products from plants that have been widely used as complementary and alternative medicines for health promotion (Ernst, 1998; Ernst, 2002; Eisenberg et al., 2001; Ernst). The increasing of herbal medicine consumption has raised concern in safety of use. As it has been believed that the herbal medicines are safe and free from adverse effects, because they are of natural origin. Such information is unfortunately not true, and often misleading (Astin, 1998). Natural products, as used by the general population, are usually the complex mixtures of many chemical compounds. Mixture of compounds in the herbal products may produce the toxicity, the pharmacological effects, and is capable of modulating the physiological action through either synergistic or antagonistic effects with concurrent conventional drugs (Pal and Mitra, 2006).

Annona muricata Linn. (A. muricata or Graviola) is a medicinal plant that has been used for the treatment of various diseases. Recently, a number of pharmacological activities of Graviola leaves extracts in animals have been reported, including a lowering of plasma glucose, anti-inflammation, anti-spasmodic, anti-convulsant, vasodilation and cardiodepressant (N’Gouemo et al., 1997; Feng et al., 1962; Wang et al., 2002; Carbajal et al., 1991). A. muricata contains anti-cancer annonaceous acetogenin compounds, therefore, leaf extracts from A. muricata are used in the studies for anti-tumor properties in various types of cancer cells. Nutraceuticals derived from A. muricata leaf, including several extracts, are commercially available in the market and are commonly used. It is possible that, the putative active ingredients and other constituents present in A. muricata may have the potential to cause the adverse affects in consumers. Therefore, the present study aimed to investigate the cytotoxicity of various extracts from different extraction protocols in normal and cancer cell lines for the prediction of its safety.

MATERIALS AND METHODS

1. Plant extraction

Leaves of A. muricata were collected from Phatthalung province and used for three different extraction methods. Fresh leaves of A. muricata were extracted using reflux and non-reflux methods. Leaves were sliced and extracted with sterile water for non-reflux method (% yield=9.97 w/w). The extract from this method is referred to as Amm1. Leaves were sliced and extracted with boiling in sterile water for three times in reflux method (% yield=9.70 w/w), referred to as Amm2. In dried leaves extraction, leaf was sliced, dried, pulverized, and extracted with reflux method (% yield=26.19 w/w), referred to as Amm3. The extracts were dissolved in sterile water and diluted to 20 mg/ml as a stock solution for cytotoxicity study.

2. Human cell lines

Hep G2 and Chang-liver cell lines were obtained from American Type culture Collection (ATCC, USA) and CLS cell line service (Germany), respectively. Cell lines were cultured in appropriate medium and conditions. Briefly, cells were grown in minimum essential medium eagle supplemented (MEM), supplemented with L-glutamine (2 mM), non-essential amino acids (1%), sodium pyruvate (1 mM), and 10% fetal bovine serum (Sigma-Aldrich, UK).
3. Cytotoxicity study

Sulforhodamine B (SRB) cell cytotoxicity assay is the quantitative of cellular proteins staining that has been used for various drug and chemical compound screenings (Perez et al., 1993). Cells were grown in 96 well plates at a density of $5 \times 10^4$ cells/ml. The cells were treated with various doses of ellipticine (positive control) and A. muricata extracts. For the positive control group, cells were treated with four doses of ellipticine (0.032, 0.016, 0.16, and 4 µg/ml). For the treatment groups, cells were treated with the extracts from A. muricata at the doses of 0.0062, 0.032, 0.16, 0.8, 4, and 20 µg/ml. The SRB assay was performed as described by Houghton et al. (2007). The percentage of cell survival was measured as the absorbance of treatment compared with the control (non-treated cells). The IC50 values were calculated from the Prism program obtained by plotting the percentage of surviving cells versus the concentrations.

RESULTS AND DISCUSSION

To determine the cytotoxicity activity of the extracts from A. muricata, the inhibition concentration (IC50) in cell proliferation was evaluated using SRB essay. Ellipticine, a positive control, was able to inhibit cell proliferation activity (Figure 1, 2 and Table 1) at the IC50 of 2.32 µg/ml and 0.59 µg/ml in Hep G2 and Chang-Liver cells, respectively. Amm1 inhibited cell proliferation at a concentration of 11.06 µg/ml in Hep G2 cells and 3.13 µg/ml in Chang-Liver cells. In contrast, Amm2 treatment did not have an effect on cell proliferation in both cell lines. Anm3 inhibited only Hep G2 cell proliferation at a concentration of 20.56 µg/ml, with no effect on cell proliferation at any doses in Chang-Liver cells. The results showed that the treatment of fresh leaf extract (Amm1) affected both cancer and normal cell proliferation. Additionally, IC50 of Amm1 was lower in the normal cells compared to those of cancer cells (Figure 1, 2 and Table 1). However, Amm3 only inhibit the cancer cell proliferation, not the normal cells. Therefore, the method used for plant extraction is very important for its specific activities. In sum, A. muricata using as herbal supplement should be study more in its safety.

From various properties of Graviola, all parts of this plant including bark, leaf, root, fruit, and seed are used as an alternative herbal medicine. Leaves of this plant are used for the treatment in various kinds of diseases such as inflammation conditions, rheumatism, neuralgia, diabetes, hypertension, insomnia, cystitis, parasitic infections, and cancer (Wang et al., 2002; Guadano et al., 2000; Torres et al., 2012). Graviola has been sold wildly in the market as a natural supplement for cancer therapy. Products of Graviola are in forms of powder, capsule, and also juice and has been claimed to kill the cancer cells. However, side effects of this plant also reported in various animal models, such as hypertension, vasodilation, activities, and herb-drug interaction (Lannuzel et al., 2002; Carbajal et al., 1991). Therefore, a consumption of A. muricata must be done with caution. Concern should also be made on the side effect from using the plants and the interference with the therapeutic efficacy of modern medicines.
Table 1: IC₅₀ values of ellipticine and A. muricata extracts in Hep G2 and Chang-liver cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IC₅₀ (µg/ml)</th>
<th>Ellipticine</th>
<th>Amm1</th>
<th>Amm2</th>
<th>Amm3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepG2</td>
<td>2.32</td>
<td>11.06</td>
<td>Neg*</td>
<td>20.56</td>
<td></td>
</tr>
<tr>
<td>Chang-Liver</td>
<td>0.59</td>
<td>3.13</td>
<td>Neg*</td>
<td>Neg*</td>
<td>Neg*</td>
</tr>
</tbody>
</table>

* Neg = Negative result; IC₅₀ > 20 µg/ml
Amm1= fresh leaves water extract
Amm2= fresh leaves hot water extract
Amm3= dried leaves hot water extract

**Figure 1.** The proliferation of Chang-Liver cells after the treatment of various doses of ellipticine, fresh leaves water extract (Amm1), fresh leaves hot water extract (Amm2), and dried leaves hot water extract (Amm3).
**Figure 2.** The proliferation of Hep G2 cells after the treatment of various doses of ellipticine, fresh leaves water extract (Amm1), fresh leaves hot water extract (Amm2), and dried leaves hot water extract (Amm3).

**CONCLUSION**

In conclusion, the modulation of cytotoxicity activity of fresh leaf extract is important in terms of safety in using as *A. muricata* produces adverse effects on cell proliferation. Additionally, *A. muricata* may produce side effects and also interact with concomitant drugs used in treatment either via the induction or the reduction of their properties. However, it was still unclear whether the adverse effects exhibited by *A. muricata* appear to occur in human, the further study should be conducted.

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