

Cytotoxic effect of *Annona muricata* Linn leaves extract on Capan-1 cells

Mohamad Norisham Mohamad Rosdi¹, Nik Nurul Najihah Nik Mat Daud¹, Razauden Mohamed Zulkifli², Harisun Ya'akob⁴

¹Department of Bioprocess Engineering, Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310, Skudai, Johor, Malaysia. ²Department of Biosciences and Health Sciences, ³Faculty of Bioscience and Medical Engineering, Universiti Teknologi Malaysia, 81310, Skudai, Johor, Malaysia.

⁴Institute of Bioproduct Development, Universiti Teknologi Malaysia 81310, Skudai, Johor, Malaysia.

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ABSTRACT

Annona muricata Linn has been reported to contain valuable bioactive compounds known as Annonaceous acetogenins. These long chain fatty acids were widely discussed for its potential in promoting anticancer and anti-proliferative activity in various cancer cell lines. However, little study has been done on *A. muricata* effect in pancreatic cancer cells. In this study, the viability of Capan-1 after treatment with *A. muricata* extracts was determined by using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The results displayed that only hexane and commercialized extract inhibited cell proliferation in a concentration-dependent manner with IC₂₅ varied ~7.8-8µg/ml and ~0.9-1.0µg/ml respectively. The data demonstrate that *A. muricata* hexane and commercialized extracts induced mild cytotoxicity in pancreatic cancer cells (Capan-1).

INTRODUCTION

Pancreatic cancer remains as one of the most aggressive cancer in the world, with 280000 and 338000 new cases diagnosed in 2008 and 2012 respectively (Ferlay *et al.*, 2013, 2010). Although these numbers accounts for only 3% of all types of cancer, pancreatic cancer continues topping the chart as a top leading cause of cancer mortality and it also has recorded the highest death rate compared to other cancers (Hidalgo, 2010; Siegel *et al.*, 2012). This circumstance is reflected by the data reported by National Cancer Institute (NCI) indicating that from 2001 until 2007, the dismal 5 year survivability for majority of the patients was 5.5% and this number has not varied for the last few decades despite immense efforts ("SEER Stat Fact Sheet: Pancreas," n.d.). In the recent decades, the cross application of different cancer treatments such as by integrating natural

substances into the established chemotherapy is steadily gaining interest. Plant-derived natural substances have long been targeted to possess capability in controlling cancer cells progression. In fact, majority of the chemo-drug used in clinical today are originated from plants.

For example, *Annona muricata* or Graviola has been vastly postulated to own valuable natural products that purportedly play key role in promoting anticancer activity. *A. muricata* leaves have been subjected to investigation of numerous numbers of human diseases, including cancer (Taylor and Press, 2005). The highly sought effects are most probably induced by its major bioactive components known as annonaceous acetogenins (ACGs).

ACGs are widely reported for their anti-proliferative activity towards numerous numbers of cancer cell lines (Chang and Wu, 2001; Liaw *et al.*, 2002; McLaughlin, 2008; Oberlies *et al.*, 1995; Tormo *et al.*, 2003). ACGs promotes cytotoxicity by affecting important process during cell metabolism, ATP generation and NADH oxidation. For example, Bullatacin, an ACG, has been reported to inhibit the proton pumping function of mitochondrial complex I resulting in short ATP supply (Degli Esposti *et al.*, 1994).

* Corresponding Author

Harisun Ya'akob, Institute of Bioproduct Development, Faculty of Chemical Engineering, Universiti Teknologi Malaysia, 81310, Skudai, Johor, Malaysia. Mail id: harisun@ibd.utm.my

Besides inhibiting the complex I in the electron transport chain, ACGs also block the NADH oxidases on plasma membranes of cancer cells from functioning. The interruption in ATP production and NADH oxidation may contribute to the accumulation of superoxide and reactive oxygen species (ROS) that may encourage disastrous diseases.

In this study, cytotoxicity assay was carried out to provide preliminary evidence on the activity of *A. muricata* leaves extract fractions on pancreatic cancer cell line (Capan-1).

MATERIALS AND METHODS

Plant material

A. muricata Linn leaves were collected and deposited in the Herbarium, Biodiversity Unit, Institute of Bioscience, UPM, Serdang, Selangor, Malaysia. Then, the leaves were washed with distilled water and desiccated in the oven until the moisture content decreased to below 10%. The dried leaves were ground.

Preparation of the extract

Maceration process

Ground leaves were macerated in methanol at room temperature for 24 h. The supernatant was filtered using Büchner funnel. Then, the filtrated extract was evaporated using rotary evaporator. The dried extract was dissolved in 10% methanol and sonicated for a few minutes.

The dissolved extract was mixed with hexane followed by chloroform in liquid-liquid portioning method. Therefore, three types of extract namely 10% methanol, hexane and chloroform were tested on cell line.

Commercialized extract

Graviola supplement capsules were purchased from Raintree (Carson City, NV). The capsules consisted of 100% pure Graviola leaf. The capsule contents were suspended in DMSO (100 mg/mL). After incubating for 5 min, the suspension was centrifuged and the supernatant was filtered to remove any remaining particles.

Total phenolic determination

The total phenolic content of each sample was determined by Total Phenolic Content (TPC) assay using Folin-Ciocalteu method based on method described by Lim *et al.* (2006) with some modification. 1 mL of the extracted leaves or a standard solution of gallic acid (0.02, 0.04, 0.06, 0.08, 0.10 mg/mL) were mixed with 1 mL of Folin-Ciocalteu reagent (which was diluted with distilled water) and 1 mL of 1 N sodium carbonate in test tubes. The tubes were then vortexed and incubated in the dark for 60 minutes at room temperature. Thereafter, the absorbance was measured at 725 nm against a blank by using a UV/Vis Spectrophotometer Lambda 35.

Gallic acid standard solution was prepared by dissolving it in ethanol. Standard solution of gallic acid was freshly prepared and all the samples and readings were done in triplicate.

Total flavonoid content determination

The total flavonoid content was measured with an aluminium chloride colorimetric assay according to methodology described by Atanassova *et al.* (2011). 1 mL of extract or a standard solution of catechin (0.05, 0.10, 0.25, 0.50 mg/mL) was added to a 10 mL volumetric flask and 0.3 mL 5% sodium nitrate (NaNO₂) was added to it. After 5 minutes, 0.3 mL 10% aluminium chloride (AlCl₃) was added.

At the sixth minute, 2 mL of 1 M NaOH was added and the total volume was sum up as 3.6 mL. The solution was mixed well and the absorbance was measured against a blank at 510 nm with a UV/Vis Spectrophotometer Lambda 35. Catechin standard solution was freshly prepared by dissolving it in distilled water. All the samples and standard were done in triplicate.

DPPH photometric assay

Sample stock solutions (1.0 mg/mL) were diluted to final concentrations of 500, 250, 125, 50, 25, 10 and 5 µg/mL, in DMSO. An amount of 100 µL of a 0.04% DPPH DMSO solution was added to 100 µL of sample solutions of different concentrations in 96-well plate, and allowed to react at room temperature for 30 min. After that, the absorbance values were measured at 515 nm and converted into the percentage antioxidant activity using the following formula:

$$\% \text{ of inhibition} = \frac{\text{Absorbance}_{\text{control}} - (\text{Absorbance}_{\text{sample}} - \text{Absorbance}_{\text{blank}})}{\text{Absorbance}_{\text{control}}} \times 100$$

DMSO (0.1 mL) plus plant extract solution (0.1 mL) was used as a blank. DPPH solution (0.1 mL; 0.04%) plus DMSO (0.1 mL) was used as a negative control. The positive controls were ascorbic acid. EC₅₀ values were calculated by linear regression of plots where the abscissa represented the concentration of tested plant extracts and the ordinate the average percent of antioxidant activity from three separate tests.

Cell culture

Pancreatic cancer cell line (Capan-1) was purchased from the American Type Culture Collection (ATCC). In this study, passage 3 cells were used. Cells were cultured in Iscove's Modified Dulbecco's medium (IMDM) supplemented with 20% FBS and antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). The cells were maintained at 37 °C and 5% CO₂ in a humidified atmosphere.

Cytotoxicity assay

To determine the cytotoxicity of *A. muricata* extract on Capan-1 cells, 3 × 10⁴ cells were seeded per well on a 96-well plate in IMDM supplemented with 20% FBS and antibiotics. After cells reach about 80-90% confluence, different ten-fold concentrations (0, 0.1, 1, 10, 100 µg/mL) of the extracts were added into triplicate wells. After 48 h, solution containing thiazolyl blue tetrazolium bromide (MTT) reagent (Sigma Aldrich, St. Louis, MO) was added into each wells. After 3 h incubation at

37 °C in 5% CO₂ in humidified atmosphere, the media was replaced with 100 µL of isopropanol and DMSO solution (1:1) and the corresponding cytotoxicity values were calculated ($\lambda = 540$ nm). The experiment was repeated at least three times.

Statistical analysis

All values were represented as mean \pm SEM of three different experiments. The Graphpad Prism 6 software was used to determine the statistical significance within the treatment replicates in each experiment. The test of significance was applied wherever necessary and values obtained as $p < 0.05$ were considered as statistically significant.

RESULTS

Phenolic composition of *A. muricata*

The results indicated that polyphenols content in the extracts is less varied between extracts. The concentration of polyphenols is slightly higher in 10% methanolic extract. While, the level of flavonoids in extracts varied with hexane showed significantly higher concentration of flavonoids.

Table 1: Phenolic composition of *A. muricata* extract

Extract	Polyphenols (GAE)/g material)	(mg dried)	Flavonoids (CE)/g material)	(mg dried)
Hexane	35.76 \pm 0.000*		966.39 \pm 0.001*	
Chloroform	54.53 \pm 0.001*		681.09 \pm 0.001*	
10% Methanol	67.54 \pm 0.000*		64.86 \pm 0.001*	
Commercialized extract	8.342 \pm 0.001*		17.5 \pm 0.001*	

Values are represented as mean \pm SEM, n = 3. *P < 0.0001 compared among types of extracts. Statistical analysis was done using one-way ANOVA.

DPPH scavenging activity

The DPPH radical scavenging activity was recorded in terms of % Inhibition as shown in Figure 1. It was observed from Figure 1 that 10% methanolic and commercialized extract have minimum DPPH scavenging activity (lesser than 50%) and hexane extract has maximum DPPH scavenging activity (78.652 \pm 2.062%). Chloroform extract also showed significantly higher activity (68.529 \pm 2.709%). The results obtained were statistically significant with $p < 0.05$.

Graviola extract induces cytotoxicity of Capan-1 cells

Pancreatic cancer cell line (Capan-1) was incubated for 48 h with different concentrations of *A. muricata* fractions namely 10% methanol, hexane commercialized extract and chloroform. The results from the MTT cytotoxicity assay demonstrated a progressive increase in cytotoxicity effect with the increase in the concentrations of the hexane and commercialized extract (Figure 2).

No cytotoxicity activity was recorded for 10% methanol and chloroform extract (data not shown). After 48 h of treatment, the resulting IC₂₅ of hexane and commercialized extract on Capan-1 cells was 7.8-8 and 0.9-1.0 µg/ml, respectively.

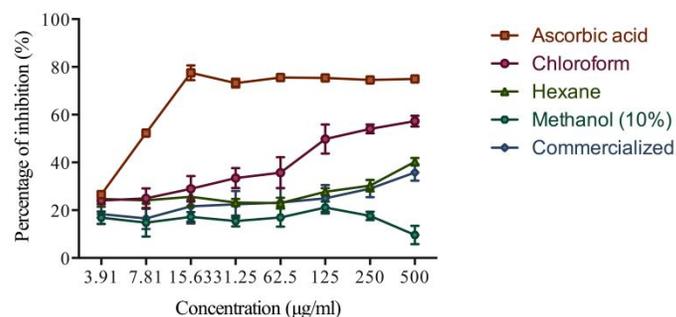


Fig. 1: Percentage of inhibition of extracts in DPPH assay. Values are represented as mean \pm SEM, (n=3).

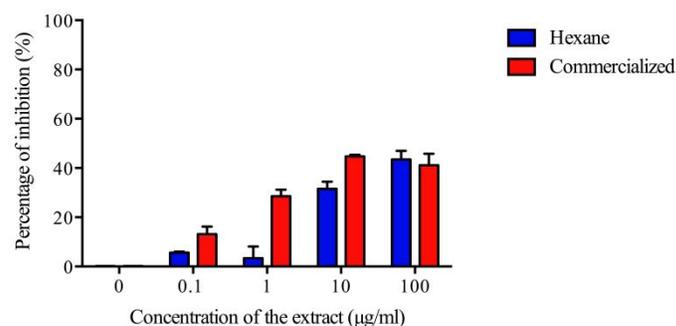


Fig. 2: Cytotoxicity effects of extracts on Capan-1 cells after 48 h treatment. Values are represented as mean \pm SEM, (n = 3). All values are statistically no difference.

DISCUSSION

A. muricata have the ability to treat wide-ranges of human diseases including cancer (Taylor and Press, 2005). There are numerous studies providing evidences to support this claim. Extracts from various parts of *A. muricata* were reported to display selective cytotoxic effects against various cancer cell lines such as pancreatic cancer, cervical cancer, lung cancer, and breast cancer (George and Kumar, 2012; Gomes de Melo *et al.*, 2010; Paul *et al.*, 2013; Torres *et al.*, 2012). These cell proliferation assay results indicate the probability of *A. muricata* to own valuable type of phytochemicals that have significantly contribution to the anti-proliferative effect of *A. muricata*.

Following the vast number of *A. muricata* preliminary anticancer research, it was discovered that the major bioactive components known as annonaceous acetogenins (ACGs) are the major contributor to anticancer effect. ACGs are derivatives of long chain (C35 to C37) fatty acids derived from the polyketide pathway. These fatty acids are found connected to a variable number of tetrahydrofuran (THF) or tetrahydropyran (THP) rings and a terminal lactone moiety (Russo *et al.*, 2010). ACGs are fascinating compounds due to their well-recognized cytotoxicity against various types of cancer cell lines. This type of compound has gathered wide attention since it was first isolated. Since then, ACGs have become a potential candidate for anticancer drug.

However, only few studies were done to study the effect of this plant on pancreatic cancer line. Torres *et al* reported that the commercialized *A. muricata* leaves extract demonstrated

mechanistic anticancer activity in pancreatic cancer cell lines (Torres *et al.*, 2012). In another study, muricoveacin and murihexocin C isolated from the leaves for *A. muricata* displayed cytotoxicity against pancreatic carcinoma (PACA-2) (Kim *et al.*, 1998).

Therefore, in this study, we used fractions prepared through methanolic maceration and liquid-liquid extraction. Non-polar solvent such as methanol, hexane and chloroform are suitable to extract out ACGs from the leaves. As expected, hexane extract which has recorded highest flavonoids content inhibited cell proliferation in MTT assay.

The hexane extract can trigger cell death in Capan-1 cells either through necrosis or apoptosis. Torres *et al.* reported that *A. muricata* promoted its activity through necrosis contrary to evidences which displayed that apoptosis is a mechanism of action (Torres *et al.*, 2012). To further evaluate, flow cytometry analysis, gene expression and protein expression will be carried out in the future experiment.

CONCLUSION

In conclusion, we found that *A. muricata* leaves hexane fraction, which primarily contains flavonoids, reduced cell viability in human pancreatic cancer cells (Capan-1) *in vitro*. This hexane extract, therefore, has the potential of an anticancer agent. However, further study may still be necessary to elucidate the mechanism induced in Capan-1 cells by this extract.

COMPETING INTERESTS

The authors declared no potential conflicts of interest.

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