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Cytotoxicity of Buah Merah (*Pandanus conoideus Lamk.*) Extract on Breast Cancer Cell Line (T47D)

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Abstract

Buah Merah (*Pandanus conoideus* Lamk.) has been extensively used to treat various diseases including cancer. There are many varieties of *buah merah* and there was no scientific study comparing cytotoxicity of different varieties. The objective of this study was to investigate the cytotoxicity of three varieties of *buah merah* known as Barugum, Maler and Yanggiru on breast cancer cell line (T47D). All samples were collected from Papua, Indonesia. Each sample was extracted consecutively using three solvents chloroform, methanol and water resulted to nine crude extracts. The cytotoxic activities were determined using MTT assay. The crude extract showed the lowest IC_{50} was selected for further bioassay-guided fractionation. Fractionation was done using vacuum liquid chromatography coupled with preparative TLC to find the active compounds. Several detection reagents were applied to TLC for identification of the class of the potent compounds. The result showed that the potent extracts was obtained from Barugum methanol extract followed by Maler chloroform extract with IC_{50} value of 132.83 µg/ml and 139.72 µg/ml, respectively. All Yanggiru extracts did not show activity. The bioassay-guided fractionation of Barugum and Maler extracts showed that the most potent fraction eluted by a mixture of hexane:ethyl acetate (75:25), was in Maler variety with IC_{50} value of 25,7 µg/ml, four times higher than the most potent fraction of Barugum with IC_{50} value of 104,61 µg/ml. TLC analysis of the most potent fraction showed that the active compounds was class of terpene. Result of this study supported the utilization of *buah merah* Maler variety for breast cancer treatment.

Key words: *Buah Merah*, cytotoxicity, T47D, vacuum liquid chromatography, thin-layer chromatography

Introduction

Plants have an almost limitless ability to synthesis bioactive substances. Most are secondary metabolites, of which at least 12.000 have been isolated, a number estimated to be less than 10% of the total. Only a small percentage of the 400.000 to 500.000 species in the plant kingdom has been phytochemically investigated (Wink, 2003). It is well established that secondary metabolites becomes one of the most important sources of pharmacologically active compounds including for cancer treatment (Cragg *et al.*, 1999).

Breast cancer is the second leading cause of death in women today and is the most common cancer among women. Though much less common, breast cancer also occurs in men. More than a half of breast cancer cases are caused by unknown factors (Madigan *et al.*, 1995). Nevertheless, some risk factors which increase someone's probability of having breast cancer are postmenopausal hormone therapy, exposed to ionizing radiation, alcohol consumption,

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and have *BRCA1* and *BRCA2* mutant genes (Longnecker *et al.,* 1995 and Hortobagyi, 1998). The probability of a woman with the mutant genes to has breast cancer is 50-85% (Hedenfalk *et al.,* 2001).

Different techniques can be used for the chemical evaluation of plants. Bioassay-guided fractionation using vacuum column chromatography (VLC) coupled with preparative Thin-layer chromatography (TLC) considers as the simplest and cheapest method. Cytotoxic screening models provide important preliminary data to select plant extracts with potential anti-cancer properties. The most commonly cytotoxic assay used is MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay.

Buah merah (*Pandanus conoideus* Lamk.) grows naturally in Papua, Indonesia at 1,200-2,000 m above sea level (Anonim, 2005). Recently, *buah merah* has been extensively used to treat HIV/AIDS, diabetes mellitus, gout and rheumatism, osteoporosis, and cancer. According to Moeljopawiro *et al.* (2007), extracts of *buah merah* have ability to inhibit breast, cervical and colon cancer cells proliferation. The scientific studies of anti-cancer activity of *buah merah* on the growth of cancer cells are relatively very few. In Papua, several varieties of *buah merah* are observed and so far there were no study comparing the cytotoxicity among these varieties. Therefore, the objective of this research was to study the cytotoxicity of three varieties of *buah merah* known as Barugum, Maler and Yanggiru and determine the group of bioactive compounds.

Materials And Methods

Three varieties of *buah merah* including Barugum, Maler, and Yanggiru, collected from Sentani, Jayapura, Papua, Indonesia were used in this study. The breast cancer cell line used was T47D. This cell line was obtained from a cell culture continually grown at the Laboratorium Penelitian dan Pengujian Terpadu (LPPT) UGM. Doxorubicin (Ebewe), was used as a reference on cytotoxic assay.

Extraction of Samples

All samples were extracted using three solvents including chloroform (Merck), methanol (Merck), water.Thirty five gram of fresh fruit for each variety was consecutively extracted using Soxhlet apparatus (Pyrex) at 65°C with 250 ml of solvent. Initially, chloroform was used as a solvent and resulted residue was then extracted using methanol. Finally, water was used for the last extraction. Each extract was evaporated to dryness under reduced pressure on rotary evaporator (Rotavapor, Buchi) and was stored at 4°C for further use.

Cytotoxic Assay (Mosmann, 1983)

In preliminary experiments a serial concentration of 0-500 µg/ml of methanol extracts of *buah merah* showed a clear dose response with cell mortality. Therefore this concentration range was used in later experiments.

An aliquot of 100 µl cell suspension (± 1.5 10^4 cells) was loaded into each well of 96-well microtitre plates and incubated for 24 hours at 37˚C, 5% CO_2 to allow for cell attachment. Extracts of *buah merah* at different concentrations ranging from 0-500 µg/ml were added and then incubated for 24 hours at the same condition. For each concentration tested four replicates were used. Two controls were applied, a control consisting of medium and another control consisting of mixture of DMSO and culture cells. At the end of the treatment, the medium and extracts were then removed and 110 µl MTT (Sigma) was added. MTT was prepared as 10% MTT stock solution in culture medium, sterilized by filtration through a 0.2 mm filter and stored at 2-8 °C. The plate was incubated in the dark for 4 hours. After 4 hours, 100 µl stop solution (10% SDS (Sigma) in 0.1 N HCl) was added in each well and again the plates were then incubated in the dark for overnight at room temperature. The absorbance was recorded using ELISA plate reader (Bio-Rad) at 595 nm. The value of IC_{50} was determined by probit analysis.

Bioassay guided Fractionation of Barugum and Maler extracts

Fractionation

The active extracts, methanol extract of Barugum and chloroform extract of Maler, were subjected to fractionation by VLC on Sephadex LH-20 (Sigma) using a mixture of different solvents ranging from non polar to polar including hexane, ethyl acetate, chloroform, methanol and water. TLC was performed to monitor the metabolites contained in each fraction. The fractions with similar TLC profiles were combined. Fractionation of methanol extract of Barugum was done using 15 eluents while the chloroform extract of Maler with 6 eluents.

Preparative TLC

Preparative TLC was performed similarly like the procedure of TLC, except for the stationary phase plates of preparative TLC were silica gel PF_{254}. The plates were developed using a mixture of chloroform : ethyl acetate = 9 : 1 $^{v}/_{v}$. Based on the profile, preparative TLC produced three portions (upper, middle and lower) portions for Barugum and two (upper and lower) portions for Maler. These portions were then subjected to cytotoxic assay to determine the active portion containing the active compounds

Sequence of bioassay-guided fractionation

Due to different results in the first fractionation of Barugum and Maler, therefore different strategies of fractionation were applied.

a. The combined fraction of the methanol extract of Barugum were directly subjected to cytotoxic assay at a single dose of 250 µg/ml representing a middle concentration on the crude extracts cytotoxic assay. The most potent fraction was selected for further separation using preparative TLC then followed by cytotoxic assay.
b. The two fractions of the chloroform extract of Maler showed similar TLC profile therefore these fractions were combined again. Separation was further performed using preparative TLC. The results of the preparative TLC were then subjected to cytotoxic assay. Based on this cytotoxic assay, the most potent portion was further fractionated using 6 eluents. The results of this fractionation were monitored using TLC and the fractions that showed similar TLC profile were combined. This combined fractions were then evaluated the activity using MTT assay.

Identification of Toxic Compounds Classes

The most toxic fraction obtained from preparative TLC was analyzed by TLC and then visualized by spraying reagents including serium (IV) sulfat, Lieberman-Burchard, vanilin sulfat, Sitroborat, Dragendroff , $FeCl_3$, jodium and ammonia to identify the groups of compounds.

RESULTS

Identification of Samples

Based on morphological characteristics, *buah merah* collected from Papua could be classified into three local varieties, Barugum, Maler and Yanggiru with characteristics as described in Table 1. Yanggiru variety has specific yellow color different among the rest varieties. Although both Barugum and Maler have similar color, but the two can be distinguished easiliy by the shape of the fruit. Barugum fruit normally has a bigger fruit and has oblongus triangular in shape while Maler tends to have slimmer oblongus.

Cytotoxicity of Extracts

Cytotoxicity of 9 crude extracts showed that the water extracts of all samples did not have cytotoxic effect on breast cancer cells. The most toxic extracts with the lowest IC_{50} value were found on methanol extract of Barugum with IC_{50} value of 132,83 µg/ml, followed by chloroform extract of Maler with IC_{50} value of 139,72 µg/ml (Table 2). These two crude extracts were chosen for further bioassay guided fractionation. The three

Table 1. Morphological characteristics of fruits of three local varieties of *buah merah*

Parameters	Varieties		
	Barugum	**Yanggiru**	**Maler**
Size	Big	Small	Medium
Shape	Oblongus triangular	Oblongus	Oblongus
Length (cm)	63	50	65
Base diameter (cm)	19	4	12
Tip diameter (cm)	7	4	3
Weight (kg)	11.25	3.95	5.10
Colour	Red	Yellow	Red
Seed position	Form irregular rows on the axis	Form irregular rows on the axis	Form irregular rows on the axis

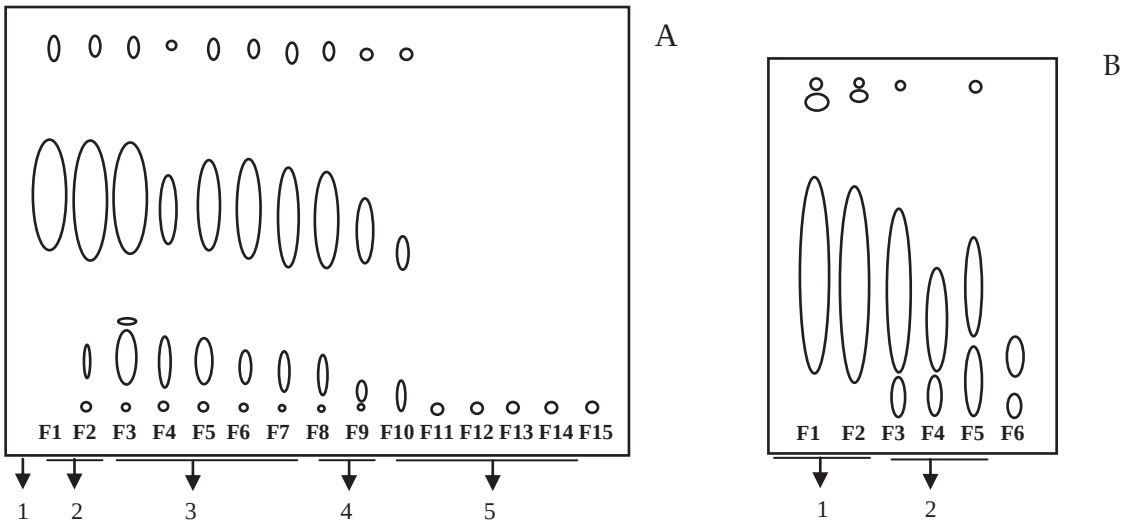

Figure 1. Representative diagram of TLC profiles obtained from methanol extract of Barugum (A) and chlorform extract of Maler (B). The numbers designated with arrows represent the combined fractions.

extracts of Yanggiru variety with yellow coloured did not show any anti-cancer activity indicating by IC_{50} values were higher than 500 µg/ml.

Table 2. The IC_{50} value of chloroform and methanol extracts of *buah merah* on breast cancer cells (T47D)

IC_{50} (µg/ml)			
Chloroform Extract		**Methanol Extract**	
Maler	**Barugum**	**Maler**	**Barugum**
139.72	174.88	352.72	132.83

Isolation of active portions from Barugum and Maler extracts

The fractionation of methanol extract of Barugum yielded five combined fractions (Figure 1A) and directly subjected to cytotoxic assay. Whereas the fractionation of the chloroform extract of Maler resulted two combined fractions (Figure 1B). But due to the high similarity between the two combined fractions on the TLC profile therefore these fractions were again combined. Further separation was done using preparative TLC.

Bioassay guided fractionation of Barugum. Cytotoxic assay of the combined fractions of Barugum showed that the most toxic sample was the first combined fraction having an activity to allow viability of 16.43% of breast cancer cells at 250 µg/ml. Result of the cytotoxic assay could be seen in Table 3.

The potent combined fraction of Barugum was subjected for preparative

TLC. The TLC profile showed that the fraction could be divided into two portions; upper and lower portions. Each portion was dissolved in chloroform : methanol = 9 : 1 $^{v}/_{v}$. These two portions were used for further analysis together with other portion resulted from Maler extract.

Table 3. Viability of breast cancer cells (T47D) after treated with combined fractions of Barugum at single dose 250 µg/ml

Combined Fractions	Cell Viability (%)
1	16.43
2	121.76
3	109.13
4	88.95
5	108.64

Bioassay guided fractionation of Maler. The result of preparative TLC of Maler extract showed that the profile can be divided into 3 portions; upper, middle, and lower portions. Based on the bioassay monitoring of these three portions, the middle portion was confirmed as the potent portion (Figure 2). This was shown by the lower IC_{50} value of the middle portion compared to the upper and the lower portions (Table 4).

Table 4. TheIC_{50} value of three portions obtained from TLC preparative of chloroform extract of Maler on breast cancer cells (T47D)

TLC Preparative Portions	IC_{50} (µg/ml)
upper	170,61
middle	117,15
lower	> 500

The middle portion of the Maler was further fractionated by VLC using 9 eluents. The TLC profile of these 9 fractions were documented. The fractions with the similar profiles were combined giving 6 combined fractions (Figure 3). Based on the result of TLC monitoring there were only 4 combined fractions including 2,4,5 and 6 used for further bioassay.

Comparison of cytotoxicity between Barugum and Maler. The two portions of preparative TLC of Barugum and these four

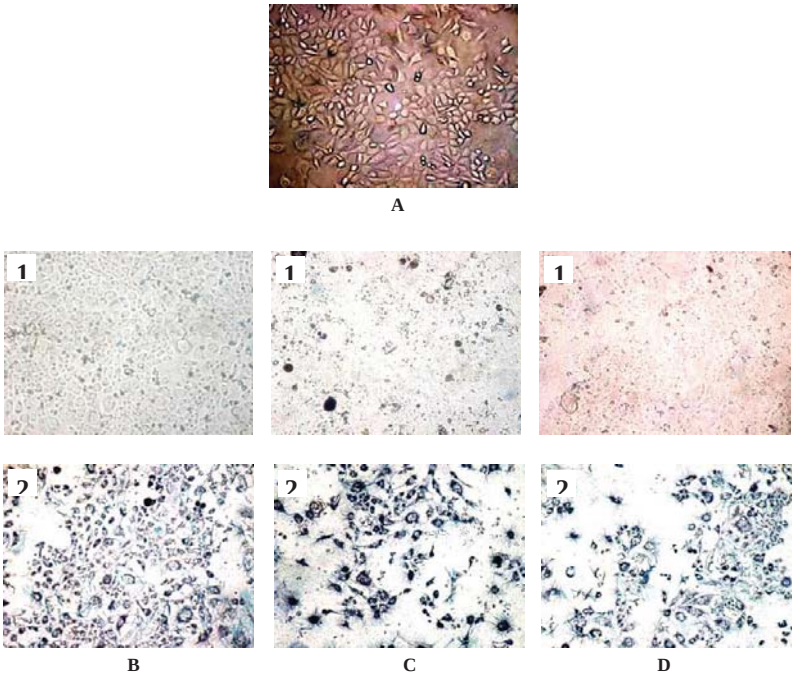

Figure 2. Breast cancer cells (T47D) morphology treated using three portions of chloroform extract of Maler (10 × 10 magnification) A: control, B, C, D: treated with portion solution. (B: upper, C: middle, D: lower).1: before MTT treatment, 2: after MTT treatment.

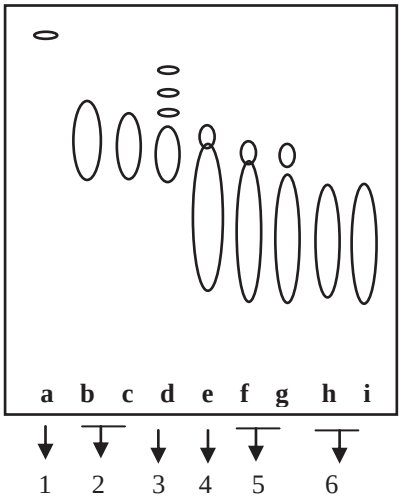

Figure 3. Representative diagram of TLC profile of 9 toxic fractions of chloroform extract of Maler. The numbers designated with arrow represent the combined fractions.

combined fractions of Maler as well as the commercial medicine, doxorubicine were evaluated their activities using MTT assay on breast cancer cells (T47D) (Table 5).

Table 5. The IC_{50} value of two portions derived from TLC preparative of Barugum, five combined fractions of Maler on breast cancer cells (T47D) and Doxorubicin

Varieties/ Sample	Combined Fractions / portions	IC_{50} (µg/ml)
Maler	2	> 500
	4	37,15
	5	38,54
	6	25,70
Barugum	upper	> 500
	lower	104,61
Doxorubicin		41,68

The result of this cytotoxicity showed that Maler variety possesses potent anti-cancer activity on breast cancer cell line (T47D) having lowest IC_{50} value of 25,7 µg/ml. This IC_{50} was lower than Doxorubicin with IC_{50} value of 41,68 µg/ml. The potent combined fraction was further subjected to TLC.

Identification of Toxic Compounds Classes

The most toxic combined fraction was analyzed by TLC followed by spraying using many detector reagents to identify the class of the toxic compounds. According to the spots obtained, it showed that the toxic compounds were terpene (Table 6).

Discussion

Secondary metabolites are known for their diversity. This can be observed between species or even within species. This diversity of secondary metabolites within species was well presented in this study. Three different local varieties of *buah merah* showed different cytotoxicity on breast cancer cell lines. The order of varieties of *buah merah* containing the most potent compounds was Maler, Barugum and the last was Yanggiru which have no anti-cancer activity. As Thorne (1975) stated that secondary metabolite could be used as chemotaxonomic characters and had a considerable impact on plant systematics. It could be used for identification and classification of flowering plants into a taxon because of the distribution of secondary metabolites is specific in certain species.

Table 6. The TLC result of the potent combined fraction of Maler

Detector Reagents	Bioactive Compounds	Spot Colour	Compounds Analyzed
UV λ 254 nm	Conjugated bonds	black	Positive (+)
UV λ 366 nm	Conjugated bonds	green fluorescence	Negative (–)
Dragendroff	Alkaloids	orange	Negative (–)
Sitroborat	Flavonoids	yellow	Negative (–)
Ammonium vapour	Phenolic compounds	yellow	Negative (–)
Vanilin sulphate	Terpenes	purple-brown	Positive (+)
Cerium (IV) sulphate	Terpenes	brown, purple, purple-brown	Positive (+)
Lieberman-Burchard	Triterpenes	blue dark	Positive (+)

Most of bioactive compounds isolated from plants do not have cytotoxic activity, so called chemopreventive compounds. Chemoprevention could be described as a compound that prevents, inhibits, and normalizes carcinogenesis or prevents invasive cancer development (Meiyanto *et al.,* 2005). Even though, according to Moeljopawiro *et al.* (2007), extract of *buah merah* have cytotoxic effects on breast cancer cells (T47D) and colon cancer cells (HT-29). Chemopreventive compounds are more selective than cytotoxic compounds on cancer cells because chemopreventive compounds have specific molecular targets (Meiyanto *et al.,* 2005). Their targets are to inhibit carcinogenesis, cell cycle and angiogenesis.

Chemopreventive compounds could not be applied solely for cancer therapy. They can only be used as a supplement to prevent cancer development (Middleton and Kandaswami, 1993). These compounds are very useful in increasing immunity, reducing toxic risk, and lowering cytostatic therapy dose, thus lowering side effect of cytostatic therapy. In addition, the chemopreventive compounds also lowering cancer incidence and risk caused by cancer. Therefore, therapy using combination of cytostatic and chemopreventive compounds gives more effective and efficient results.

Results in this research indicated that extract of *buah merah* might not only as chemopreventive compounds but also have cytostatic activity. It's showed by the lower IC_{50} value of the combined fraction of Maler compared to the medical cancer drug, Doxorubicin. Futher study to understand how bioactive mechanism of the inhibition of the buah merah is needed.

The class of bioactive compounds obtained that have cytotoxic activity on breast cancer cells were terpenes. Terpenoids are the most abundant secondary metabolites that have various molecule structures. Most of the terpenoids are of plant origin; however, they are also synthesized by other organisms, such as bacteria and yeast as part of primary or secondary metabolism. They function as phytoalexins in plant direct defense, or as signals in indirect defense responses which involves herbivores and their natural enemies. Therefore, most of scientists think that terpenes have more ecological than physiological functions. Whereas diterpenes have a physiological function as a plant hormone, gibberellins (Wink, 2003)

Many reported studies have demonstrated the cytotoxic effects of various terpenoids against proliferation, growth and invasion of a variety of liver cancer cell lines. The mechanisms of anti-cancer activities varied including the increase of the apoptotic cell death process and reactive oxygen species (ROS) generation, increase in the tumour suppressor gene *p53*, apoptotic proteins, such as caspase-3, and -9, cytochrome *c* (cyt. *c*), and p38 protein expression (Huang *et al.,* 2008), so that in India and China, terpenoids become the largest group of phytochemicals that are currently being explored as anticancer agents in clinical trials (Thoppil and Bishayee, 2011).

Conclusions

The result of this study showed among 3 varieties of *buah merah*, Maler variety possesses the most potent anti-cancer activity on breast cancer cell line followed by the Barugum variety. The Yanggiru variety having yellow color, did now show any anticancer activity. The class of the toxic compounds on breast cancer cells (T47D) were terpene.

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