

Cytotoxicity test of binjai leaf (*Mangifera caesia*) ethanol extract in relation to Vero cells

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ABSTRACT

Background: Binjai leaves (*Mangifera caesia*) constitute one part of a medicinal plant from South Borneo that contains potential anticancer and antioxidant flavonoids. Before using medicinal plants as adjuvant therapy material, a cytotoxicity test of a material extract needs to be conducted in order to establish the safety of natural ingredients that will be used in the production of medicinal products. **Purpose:** This research aimed to determine whether the ethanol extract of binjai leaves proved cytotoxic to Vero cells and determine the value of IC_{50} after the administering of ethanol extract of Binjai leaves by means of an MTT assay method. **Methods:** This research incorporated a true experimental method with posttest-only control design that consisted of ten groups. The Binjai leaf ethanol extract of varying concentrations was administered to eight groups, namely; 1.25µg/mL, 62.5µg/mL, 125µg/mL, 250µg/mL, 500µg/mL, 1000µg/mL, 2000µg/mL and 4000µg/mL. The control groups consisted of two groups, one cell control group and one media control group. The cell viability percentage was calculated by an absorbent of ELISA reader. **Results:** The probit analysis result had an IC_{50} value of 2498.48µg/mL ($IC_{50} > 1000µg/mL$ constituted a non-toxic category). **Conclusion:** Ethanol extract of Binjai leaves is not cytotoxic to Vero cells as shown by an assay MTT method which produced an IC_{50} value of 2498.48µg/mL.

Keywords: Cytotoxicity; ethanol extract of binjai (*Mangifera caesia*) leaves; flavonoid; MTT assay; Vero cell

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INTRODUCTION

Cancer is a disease resulting from damage to and abnormal development of body tissue cells inducing genetic mutations with the potential to produce cancer cells.^{1,2} In 2013, according to Riset Kesehatan Dasar (Riskesdas), the prevalence of cancer in Indonesia stood at 1.4 per 1000 people or approximately 347,000 individuals. Globally, Indonesia ranked in fifth position, while South Borneo was the region that placed twelfth within the country in terms of cancer prevalence at 1.6%.³ Cancer of the oral cavity and throat placed sixth with regard to all forms of the disease around the world. In Indonesia, oral cancer represents about 3-4% of all cases of the disease, producing mortality rate of 2-3%.⁴

Cancer can be managed effectively with conventional treatments such as surgery, chemotherapy and radiotherapy.⁵ Surgery is considered ineffective, particularly in those cases where the cancer has metastasized. Chemotherapy and radiotherapy treatments are also less selective in their effect, one side-effect of prolonged use of the former being its toxicity to healthy tissue.⁶ Due to the ineffectiveness and limited selectivity of conventional treatments, medicinal plants are currently being used as adjuvant therapy or secondary treatment for cancer. Medicinal plants are currently in demand within society as an alternative adjuvant therapy to traditional treatment because it is relatively safe and ubiquitous.⁵

One of the medicinal plants utilized by the people of South Borneo is the Binjai plant (*Mangifera caesia*). Binjai

is commonly consumed in South Borneo for mixed sauce, a melange of pickles which is consumed with freshwater fish.⁷ Binjai, including *Mangifera*, has secondary metabolites such as tannins, alkaloids, triterpenoid and flavonoids. Binjai leaves contain flavonoid which has potential as an anticancer agent⁸ since has been proven to inhibit the proliferation of certain cancer cells, while demonstrating low cytotoxicity or even non-toxicity in relation to normal cells.⁶ Previous research has proved that ethanol extract of Binjai leaves is effective in protecting against the mortality of *Artemia salina* Leach larvae within a Brine Shrimp Lethal Test (BSLT) method because its value of $LC_{50} < 1000$ mg/L was 489.059 mg/L.⁸

A number of natural ingredients can be utilized within adjuvant therapy and, therefore, need to be subjected to a test of material extract cytotoxicity to establish the safety of those that will become medicinal products. The method employed is that of Methylthiazol-2-yl-2,5-diphenyl tetrazolium (MTT) assay because it is relatively rapid, accurate and sensitive.⁹ MTT assay method constitutes an in vitro cytotoxicity test using a cell culture. The cell culture identical to normal human cells is reported to consist of Vero cells which are normal cells isolated from the kidneys of African green apes (*Cercopithecus aethiops*) which were first extracted by T. Yasamura and T. Kwalata at the University of Chiba, Japan in 1967.^{10–12}

Against this background, a cytotoxicity test of Binjai leaves ethanol extract in relation to Vero cells by MTT assay method had not yet been undertaken. The cytotoxicity of Binjai leaves ethanol extract against Vero cells was, therefore, established to determine the safe concentration of an adjuvant therapy material made from natural ingredients.

MATERIALS AND METHODS

The research process began with a request for the necessary research permit and ethical clearance to be issued the Ethics Committee of the Faculty of Dentistry, University of Lambung Mangkurat No. 034/KEPKG-FKGULM/EC/IX/2017. The research methodology was true experimental with posttest-only in nature with a design control. The research population was divided into ten groups; eight groups administered with Binjai leaves ethanol extract at varying concentrations (31.25 µg/mL, 62.5 µg/mL, 125 µg/mL, 250 µg/mL, 500 µg/mL, 1000 µg/mL, 2000 µg/mL and 4000 µg/mL), one cell control group and one media control group. A minimum number of three repetitions in each group was obtained through application of the Federer formula.¹³

The instruments used for making Binjai leaves ethanol extract comprised a maceration vessel, plastic container, strainer, micro-pipette, volume pipette, glass beaker, test tube, shelf test tube, volumetric flask, stirring bar, blender, digital balance, water bath and vacuum rotary evaporator. The cytotoxicity test of Vero cells in vitro used a 96-well

microplate, CO₂ incubator, Laminary air flow cabinet, pipette, flask, centrifugal, inverted microscope, 15 mL conical tube, yellow tip, ELISA plate reader, electric pipette and a hemacytometer and counter. The production materials for Binjai leaf ethanol extract consisted of Binjai leaves (obtained from Sungai Lutut Village, East Banjarmasin sub-district, Banjarmasin district, South Borneo), 70% ethanol solvent and filter paper. The Vero cells used in the cytotoxicity test, namely: M199 media, Foetal Bovine Serum (FBS) 10% trypsin-EDTA, Dimethyl Sulfoxide (DMSO) and reagent Methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide were grown at the Dengue Laboratory of Airlangga University.

Preparation and production of extract using a maceration method.⁸

The Binjai leaves (Figure 1) used consisted of mature samples (the fourth leaf from the apex to the fifth leaf from the basal)¹⁴ selected by the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Lambung Mangkurat (125/LB.LABDASAR/IX/2017). Leaves were separated from their branches, the dirty specimens being subsequently washed in water. At the extraction stage, 1 kg of wet Binjai leaves were cut into small pieces, dried in the open air and pulverized in a blender to form dried simplicia, 430g of which was quantified by means of an analytical balance. The dried simplicia was inserted into a maceration vessel and soaked in 2.5 L of ethanol solvent 70%. Maceration was conducted over three days, with the liquid protected from sunlight, while being agitated occasionally with a stirring bar to ensure that all the simplicia powder would dissolve in the solvent to produce the required solution concentration. After maceration, the resulting liquid was passed through Whatman number 1 filter paper to remove the filtrate which was subsequently concentrated using a rotary evaporator at 50°C. The filtrate was poured into a vaporizer cup, before being evaporated in a waterbath. The final result of the thick ethanol extract of Binjai leaves (Figure 2) was calculated for yield using the formula below:¹⁵

$$\% \text{ yield} = \frac{\text{the weight of the extraction obtained (g)}}{\text{the weight of the original simplicia (g)}} \times 100\%$$



Figure 1. Binjai leaves.

Cytotoxicity Test against Vero Cell

Vero cells from the Dengue Laboratory of Universitas Airlangga, were grown in an M199 medium and incubated in a CO₂ 5% incubator at a temperature of 37°C. The Vero cell condition while in the CO₂ incubator was closely observed. If the cell is 80% confluent, it is washed twice with Phosphate Buffer Saline (PBS) and added to trypsin-EDTA 0.2% in order to release cells from the flask. Cell density was calculated using a hemasitometer.¹⁶

Vero cells were transferred to a 96-well microplate at a density of 2×10^4 cell in 100µL. Binjai leaf ethanol extract at varying concentrations: 31.25µg/mL, 62.5µg/mL, 125µg/mL, 250µg/mL, 500µg/mL, 1000µg/mL, 2000µg/mL and 4000µg/mL was then added – a process repeated for three times. 25µL reagent MTT was inserted in each microplate, including the control medium and incubated for four hours. 240µL stopper solution DMSO 0.01% was inserted in each microplate after four hours of incubation. The 96-well microplate was absorbed by the ELISA reader at a wave length 595nm with the resulting data being used to calculate the viability percentage of Vero cells with the formulation below:¹⁷

$$\% \text{ viability} = \frac{(\text{Abs. treatment} - \text{Abs. media control})}{(\text{Abs. cell control} - \text{Abs. media control})} \times 100\%$$

Note: Abs = absorbent

The percentage value of Vero cell viability is analysed and an Inhibitory Concentration 50 (IC₅₀) value obtained. The IC₅₀ value was arrived at by means of probit analysis using SPSS 23.0 for Windows.

RESULTS

The extraction of simplicia from Binjai leaves used a maceration method with ethanol 70% as solvent with 430g pf simplicia being obtained from 73.43g of leaves. The resulting thick extract was dark brown in color. Using the formula above, the calculation of the yield produced a figure of 17.076%. A cytotoxicity test of Binjai leaf ethanol extract in relation to Vero cells produced a viability percentage as shown in Figure 3.



Figure 2. Ethanol extract of Binjai leaves

In Figure 3 shows that the life percentage of Vero cell a concentration of 31.25µg/mL was 98%; at 62.5µg/mL - 85%; 125µg/mL - 83%; 250µg/mL - 77%; 500µg/mL - 66%; 1000µg/mL - 61%; 2000µg/mL - 56% and 4000µg/mL - 45.7%. The Vero cell was viewed through an inverted microscope at 100x magnification in order to observe the morphological changes in cell death caused by the ethanol extract of Binjai leaves.¹⁷ Normal Vero cell observed in Figure 4 and 5. The morphological changes in cell death can see Figure 6A and Figure 7.

The result data of percentage cell viability was analysed and the Inhibitory Concentration 50 (IC₅₀) value calculated on the basis of the data obtained from three repetitions. The IC₅₀ value, based on probit analysis using SPSS 23.0 for Windows, was 2498.48 µg/mL with a minimum level of 1715.843µg/mL and an upper level of 4131.846µg/mL.

DISCUSSION

This research aimed to establish whether Binjai leaf ethanol extract proved cytotoxic against Vero cells and to determine the presence of IC₅₀ after its administering by means of MTT assay method. According to the cytotoxicity

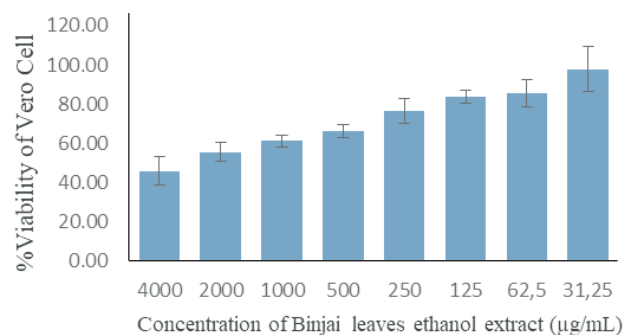


Figure 3. Binjai leaves ethanol extract and the average percentage of Vero cell viability.

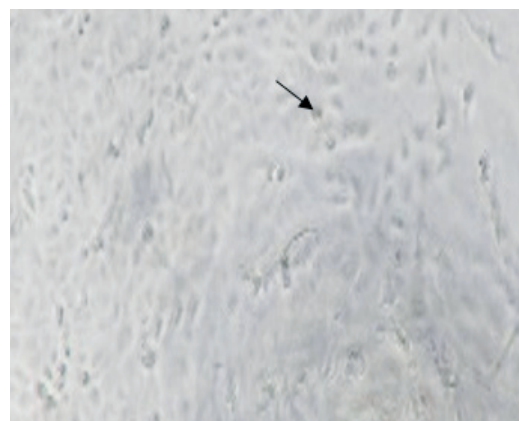


Figure 4. Morphological observation of normal Vero cells before being induced with extract and being administered with MTT (indicated by the arrow).

test result based on this method, the ethanol extract of the Binjai leaves had an IC_{50} value of $2.498.48\mu\text{g/mL}$. IC_{50} can show the cytotoxic potential of the compound. With regard to the cytotoxicity of natural ingredients, in cases where $IC_{50} > 1000\mu\text{g/mL}$, they are classified as non-toxic.¹⁸ The final cytotoxicity test result provided information about the percentage of cells which had been able to survive.¹⁵ This data proved that ethanol extract of Binjai leaves administered by MTT assay method was not cytotoxic to Vero cells. In Figure 6B, vero cell had been able to survive by ability $>50\%$.

The screening results of the phytochemicals contained in ethanol extract of *Mangifera foetida* confirmed the presence of secondary metabolites, namely: phenols, tannins, alkaloids, saponins, triterpenoids and flavonoids.¹⁹ *Mangifera caesia* which included genus *Mangifera* was considered to have the same compound content as *Mangifera foetida*. Phenol promoted antioxidant activity and played the role of maintaining free radical attack potentially harmful to DNA.²⁰ Tannin is a phenol compound promoting antioxidant activity that is able to protect itself against oxidative damage.²¹ At high concentrations it can prove toxic to cells²² by damaging the cell membrane by

shrinking the cell wall, thereby reducing its permeability. Consequently, the continued viability of the cell is so severely compromised that it dies.²³ In Figure 3, $4000\mu\text{g/mL}$ constituted the highest concentration of ethanol extract of

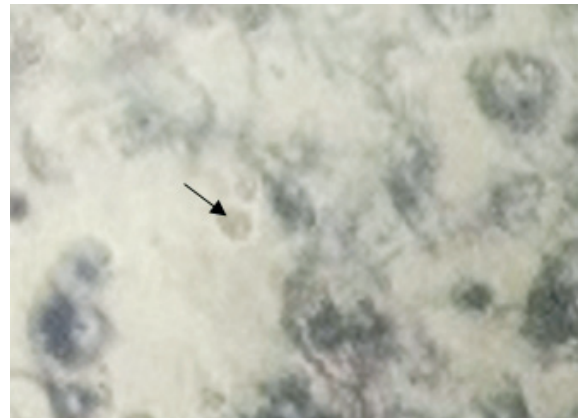


Figure 7. Death of Vero cell at a concentration of $4000\mu\text{g/mL}$. Dead Vero cells resulted in a loss of cytoplasmic fluid because of cell membrane damage which produces black and dark colors (indicated by the arrow).¹⁰

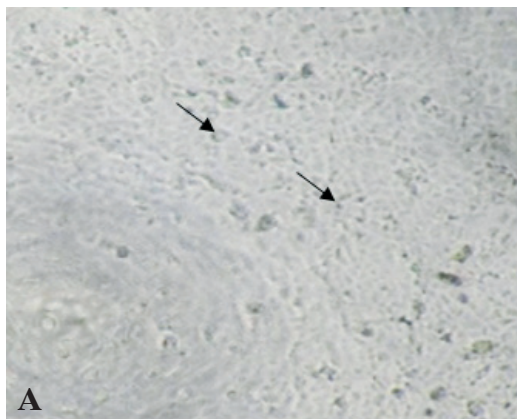


Figure 5. (A) Vero cell after being induced with concentration extract of $4000\mu\text{g/mL}$ (indicated by arrow). (B) Vero cell after being induced with concentrated extract of $2000\mu\text{g/mL}$ and before the administering of MTT (indicated by the arrows).

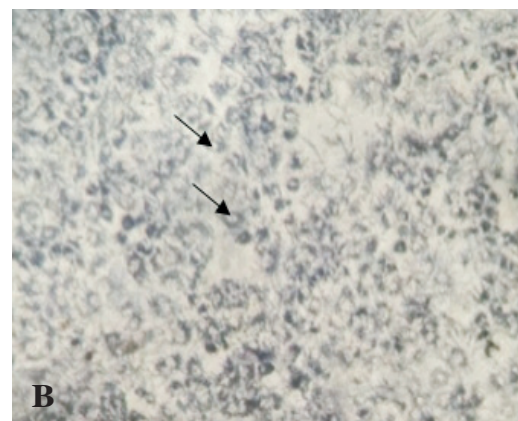


Figure 6. Morphological change of a Vero cell after being induced with Binjai leaves ethanol extract and MTT resulting in a formazan crystal (indicated by arrows). (A) Inhibition Vero cell $<50\%$ at a concentration of $4000\mu\text{g/mL}$ and (B) Inhibition of a Vero cell $>50\%$ at a concentration of $2000\mu\text{g/mL}$.

Binjai leaves. Therefore, it was estimated that tannin played a role in the concentration and promoted a percentage cell viability <50%. Alkaloids, saponins and triterpenoids all have chemoprotective potential and in non-enzymatic conditions can inhibit lipid peroxide.²⁴

The previous result confirmed that Binjai leaves contain flavonoid⁸ which is reported to promote antioxidant activity capable of preventing injury because of the activity of free radical scavengers.²⁵ These work by donating hydrogen ions to hydroxyl radicals and peroxy radicals in the B ring hydroxyl flavonoid group. The flavonoid hydroxyl group renders free radicals inactive with the result that they become more stable and acts as antioxidant radicals.^{25,26} Antioxidant radicals are formed through a resonance process within the structure of an aromatic ring hindering their involvement in a reaction with other radicals with the result that they detoxify free radicals, prevent cell damage and promote cell viability.²⁵

Flavonoid could have an effect through interaction with metabolite enzyme phase I (cytochrome P450) within the cell. Phase I metabolite enzymes activate numbers of procarcinogens to reactivate intermediates and substances which interact with cellular nucleophiles thereby nullifying their capacity to initiate carcinogenesis. Other mechanisms include flavonoid stimulated phase II metabolism enzymes such as Glutathione-S-Transferase, Quinone Reductase, and UDP-Glucuronyl Transferase in which carcinogens was detoxified.²⁷ Phase II detoxification substances can catalyze the reaction which increases excretion of toxic compounds or carcinogenic chemical material in the body.²⁸ Therefore, the flavonoid in ethanol extract of Binjai leaves is able to contribute to the concentration of the extract militating against the viability of Vero cells.

In this research, the method of Binjai leaf extraction employed was that of meseration using ethanol solvent 70%. Ethanol 70% was selected as the solvent in extraction because it pulled the compound in simplicia of Binjai leaves. The compound capable of being pulled by ethanol is flavanoid which has antioxidant, chemoprotective and cytotoxicity potential through a mechanism of cycle arrest or apoptosis.²⁹ Extraction with ethanol 70% involved cytotoxic activity caused by compound varieties such as polar, semi polar or non-polar results in toxins affecting each other.³⁰ Non-polar compounds impede the pulling process of flavonoids because the defatting process did not occur and they caused a decrease in flavonoid activity. Based on the research findings, it can be concluded that ethanol extract of Binjai leaves is not cytotoxic in relation to Vero cells as proved by an MTT assay method with an IC₅₀ value of 2498.48 µg/mL.

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