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PHYTOCHEMICAL AND CYTOTOXICITY TESTING OF RAMANIA LEAVES (*Bouea macrophylla* Griffith) ETHANOL EXTRACT TOWARD VERO CELLS USING MTT ASSAY METHOD

(Preliminary study of adjuvant therapy materials to the preparation of the drug)

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ABSTRACT

Background: *Ramania leaves (Bouea macrophylla Griffith) are one of the local medicinal plants of South Kalimantan which is used as adjuvant therapy material. Previous research showed that ramania leaves ethanol extract showed that the extract has potential as adjuvant therapy material for anticancer, but the safety of this material is not yet known so it needs to be tested with cytotoxicity test to know the toxic properties of the material toward normal cells in vitro.* ***Objective:*** *This research aims to determine the cytotoxicity properties of ramania leaves ethanol extract toward Vero cells using MTT assay method.* ***Method:*** *It was a true experimental research using posttest-only with control group design consist of 8 treatment groups: 31.25µg/mL, 62.5µg/mL, 125µg/mL, 250µg/mL, 500µg/mL, 1000µg/mL, 2000µg/mL and 4000µg/mL and 2 control groups which were cell control and media control. Study phases include phytochemical test, tannic acid test, cell culture, harvest and cell calculation and cytotoxicity test. The result test was read by ELISA reader, the absorbance will be calculated by a formula.* ***Results:*** *The test result showed that ramania leaves ethanol extract contained secondary metabolite such as phenol, flavonoid, steroid and terpenoid. The identification test of tannic acid compound using TLC method (thin layer chromatography) showed the presence of tannic acid compound.The probit analysis showed IC_{50} in 35.808µg/mL.* ***Conclusion:*** *Based on the research, it can be concluded that ramania leaves ethanol extract are found cytotoxic toward Vero cells after the analysis using MTT assay method (IC_{50}<100µg/mL).*

Keywords: *ramania leaves ethanol extract, Vero cells, cytotoxicity test, MTT assay, phytochemicals.*

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INTRODUCTION

Cancer is a non-contagious disease which increases every year. WHO data in 2010 stated that cancer occupies second place in the most death, just under cardiovascular disease.[1] According to RISKESDAS (2013), Indonesia placed fifth in the most non-contagious disease prevalence, meanwhile South Kalimantan placed twelfth with 12% percentage. Mouth and throat cancer is also the sixth most found cancer disease with the most common type to be found is squamous cell carcinoma which is 90%.[2,3,4]

Treatment of cancer with conventional therapies such as surgery, chemotherapy and radiotherapy are not effective for metastatic cancer, so that combination therapy is required with adjuvant therapy by medicinal plants.[5,6] One of the local medicinal plants of South Kalimantan that used as adjuvant therapy is ramania (*Bouea macrophylla* Griffith) leaves. Research of Aqilla et al. (2017) showed that ramania leaves ethanol extract contained flavonoids that proved potential as anticancer with LC_{50} value 408.950mg/L.[7]

Medicinal plants used for adjuvant therapy has not been researched scientifically by testing its cytotoxicity to determine the safety of medicine. Cytotoxicity test used was methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay toward Vero cells. Vero cells derived from the kidney of African green monkey (*Cercopithecus aethiops*). These cells are homologous with human cells and easy to bred.[8,9]

Based on the description above, the researcher want to do a research to test the cytotoxicity of ramania leaves ethanol extract toward Vero cells using MTT assay that can be used as adjuvant therapy from natural material. The aim of this research is to determine the cytotoxicity properties of ramania leaves ethanol extract toward Vero cells by using MTT assay method.

MATERIALS AND METHODS

This research began with the making of research permit and ethical clearance issued by Faculty of Dentistry Lambung Mangkurat University no.049/KEPKG-FKGULM/EC/IX/2017.

This research was a true experimental research with posttest-only control group design. Research

1mL Dragendroff reagent (potassium bismuth iodide) was added to 1mL sample. The formation of red-shaped sediment showed positive result of the sample containing alkaloid.

Identification of Saponin

2mL sample was added with 2 mL water and shaken. If the foam was sable ± 7 minutes, the sample was positively containing saponin.

Identification of Phenol

3-4 drops of $FeCl_3$ 10% was added to 1mL sample. Positive samples containing phenol would produce a dark green, blue or black color in result.

Identification of Tannin

3-4 drops of 1% gelatin solution containing NaCl were added to 1mL sample. White sediment formation was shown positive result of the sample containing tannin.

Identification of Flavonoid

1mL of sample were added with NaOH solution. If the faded yellow formed when the weak acid solution was added, it shows positive result which means that the sample contains flavonoid.

Identification of Steroid

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added. If brown ring was formed, it shows the presence of steroid.

Identification of Terpenoid

Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow colour, it shows the presence of terpenoid.

Identification of Anthraquinone

samples were 27 Vero culture cells consisted of 8 treatment groups which were 31.25µg/mL, 62.5µg/mL, 125µg/mL, 250µg/mL, 500µg/mL, 1000µg/mL, 2000µg/mL and 4000µg/mL, and two control groups which were 1 cell control and 1 media control without Vero cells with three replicas.

The Extraction of Ramania Leaves Ethanol Extract

The extraction of ramania leaves ethanol extract began by cleaning, drying and blending the samples until it smooth, so that 500g simplicia powder of ramania leaves could be obtained. Simplicia was extracted by the maceration method for 3 days using 70% ethanol solvent as much as 8 liters with 3 times maceration. The liquid extract obtained was filtered with filter paper, then evaporated with a rotary evaporator at 50˚C, then concentrated on the waterbath to obtain a concentrated extract weight 66.25g.

Phytochemical Test of Ramania Leaves Ethanol Extract

Identification of Alkaloid

100mg samples dissolved in 10mL of aquadest. The mixture was filtered, the filtrate (2mL) was added with 5mL of benzene. The extract was then added with ammonia and then shaken. The formation of red color indicates positive containing of anthraquinone.

Identification Test of Tannic Acid Compound with TLC Method

The tannic acid and the ramania leaves ethanol extract were each dissolved in ethanol solvent proanalysis. Then bottled onto KLT plate, elated with prepared eluent. The plate was then seen for its spotted on UV rays λ = 254nm and 366nm.

Vero Cells Culture Manufacture

The manufacture of Vero cells with confluent M119 media was discarded and then the cells were rinsed with 10mL of M199. 5mL of tripsin-EDTA is added and incubated at 37˚C for 2 -3 min until the cell sign starts to detach from the flask. 5mL of M119 culture media supplemented with 10% fetal bovine serum (FBS) was added to tripsin-EDTA to be inactive. Cells were rinsed in M199 by using a pipette slowly to decompose cell clumps. The cells suspension was transferred from the flask into a sterile 15mL conical tube and then centrifuged for 5 minutes at room temperature. The supernatant was discarded and cells resuspended using 10mL M119 with 10% FBS. The desired cell dilution was prepared for a total of 12-20mL M119 with 10% FBS and added to the cell culture flask.

Harvest and Calculation of Vero Cells

The cells were taken from the CO_2 incubator, observed the cells condition. Harvesting cells were

done after cell 80% confluent. Removed media by using pasteur sterile pipette. Cells were washed with PBS. Tripsin-EDTA was added evenly and incubated in incubator for 3 minutes. Add tripsin-EDTA evenly and incubated in the incubator for 3 minutes. ± 5mL medium was added to activate trypsin. Observed the cells condition in the microscope. Resuspense if there were still cells that cluster. Cells that have been released were transferred into the new sterile conical. Resuspense the cells in the conical tube of the collected cells. 10µl of the collected cell was taken and moved it with pipette to the hemasitometer. Cells were counted under an inverted microscope with a counter.

Test of Cytotoxicity by MTT Assay Method

Cells were being transferred into their respective wells of 100µL, incubated in a CO_2 incubator with a temperature of 37°C for a minimum of 24 hours. Cells treatment with the sample was performed after the cells returned in normal circumstances. Took the already contains cell plate from the CO_2 incubator. The cells medium was removed, then gently pressed the plate over the food paper to drain the remaining liquid. 100µL PBS into all filled wells of cells, then discard the PBS. The EEDR concentration was entered series into the wall (Tripolo). Incubation in CO_2 incubator for 24 hours. 25µL of MTT reagents were added to each well, including media controls. Incubate cells in CO_2 incubator for 4 hours. 0.1% DMSO stopper solution was added and then the absorbance of each well was read with an ELISA reader λ= 595nm. The absorbance percentage of cells viability and IC_{50} analysis with SPSS (Probit) were calculated.[10]

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

RESULT

The depiction of Vero Cells after MTT Assay Treatment

The depiction of vero cells after treatment of ramania leaves (*Bouea macrophylla* Griffith) ethanol extract with MTT assay method using inverted microscope with 100x magnification seen as follow:

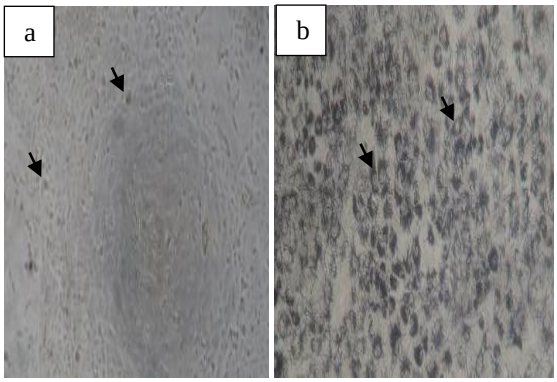

Figure 1 a. Vero cells control before MTT assay
b. Vero cells control after MTT assay

Figure 1 a. showed control of Vero cells before treatment of MTT assay in the form of spherical core, in figure 1 b. showed control of live Vero cells after the treatment of MTT assay formed formazan crystals, cells appear curved and interlocked with other cells.

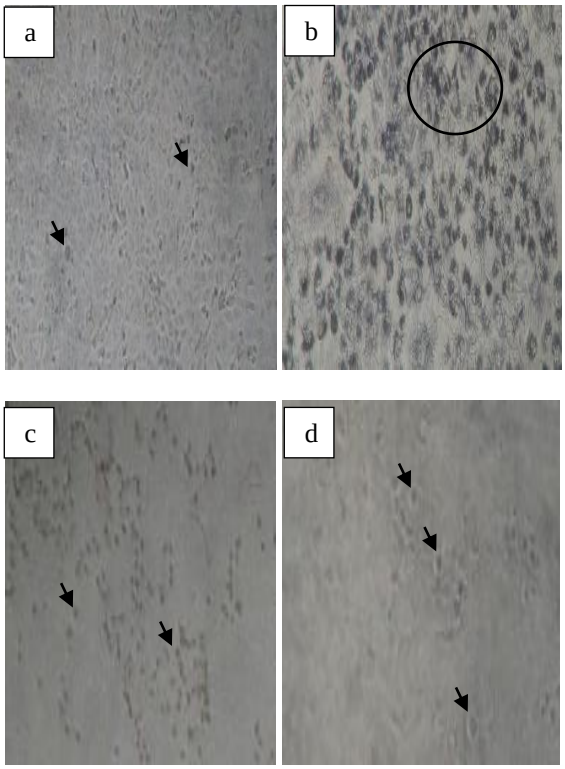

Figure 2. the depiction of Vero cells of ramania leaves (*Bouea macrophylla* Griffith) ethanol extract):

a. Before MTT assay at the lowest concentration of 31.25µg/mL.
b. After MTT assay at the lowest concentration of 31.25µg/mL.
c. Before MTT assay at the highest concentration of 4000µg/mL.
d. After MTT assay at the highest concentration of 4000µg/mL.

Vero cells observation results in figure 2a. and 2c. ramania leaves ethanol extract before treatment of MTT assay showed the Vero cells in the form of a spherical core. Figure 2b. is a formazan crystalline after administration of an MTT assay. The dead Vero cells was shown in figure 2d. because the treatment of giving extract in highest concentration appeared to change from initial form and core becoming black, tend to spread and float.

The Result of Maceration Rendement of Ramania Leaves Ethanol Extract

Ramania leaves ethanol extract after doing maceration was calculated the rendement with the formulation as follow:[11]

$$\% \text{ rendement} = \frac{\text{the weight of extract (g)}}{\text{the weight of initial simplicia (g)}} \times 100\%$$

$$= \frac{66.25}{500}$$

$$= 13.25\%$$

The result of rendement ramania leaves ethanol extract was showed in table 1 below:

Tabel 1. Rendement of ramania leaves (*Bouea macrophylla* Griffith) ethanol extract

Extract	Simplicia weight (g)	Viscous extract weight (g)	Rendement (%)(b/b)
Etanol 70%	500	66.25	**13.25**

The table above shows the percentage of ramania leaves ethanol extract at 13.25%. The resulted maceration was an amount of compound extract by using dissolved.

Phytochemical Test Result of Ramania Leaves Ethanol Extract

Phytochemical test of ramania leaves ethanol extract are as follow:

Table 2. Phytochemical test result of ramania leaves (*Bouea macrophylla* Griffith) ethanol extract

Compound Group	Ramania leaves ethanol extract	Note
Alkaloid	-	No precipitate formed
Saponin	-	No foam formed
Phenol	+	Blackish-colored formed
Tanin	-	No white precipitate formed
Flavonoid	+	Yellowish color formed
Steroid	+	A faded yellow ring emerges
Terpenoid	+	Yellowish color formed
Anthraquinone	-	Red color is not formed

Based on the following table, the ramania leaves ethanol extract containing secondary metabolites of phenol, flavonoids, steroids and terpenoids. Result of identification test of compound of tannic acid with thin layer chromatography method found that ramania leaves ethanol extract positive contains tannic acid compound.

Results of Cytotoxicity Test of Ramania Leaves Ethanol Extract

The result of cytotoxicity test was calculated the absorbance of live cell number forming the purple colored formazan in each sample by using an ELISA reader obtained resulting in table 3 below:

Table 3. Mean result of vero cells absorbance, Vero cells viability and standard deviation of Vero cells viability

Concentration of Ramania Leaves Extract Group	Mean Absorbance of Vero Cells	%Viability ± SD
31.25µg/mL	0.372	50 ± 2.24
62.5µg/mL	0.374	46 ± 1.66
125µg/mL	0.331	43 ± 1.81
250µg/mL	0.278	34 ± 0.57
500µg/mL	0.236	27 ± 2.36
1000µg/mL	0.215	23 ± 1.76
2000µg/mL	0.194	20 ± 1.01
4000µg/mL	0.184	18 ± 0.75

The table above shows that there was difference of the mean value in each treatment group with Vero cells viability. Based on the above table, the greater the ethanol extract concentration of ramania leaves then the viability of Vero cells decreases.

Results from the study were analyzed using Probit Analysis in Statistical Package for the Social Science (SPSS) 24.0 for windows software. The effects of ramania leaves ethanol extract toward Vero cells were considered based on the Inhibitory Concentration (IC_{50}) value with Confidence Limit (CL) 95%. The result of probit test was obtained IC_{50} 35.808µg/mL, according to Balantye in Mardja et al. (2016) IC_{50} ranged from 10µg/mL up to 100µg/mL including toxic category, so it can be concluded that ramania leaves ethanol extract were toxic toward Vero cells.

DISCUSSION

The results of cytotoxicity test of ramania leaves ethanol extract toward Vero cells showed a change in Vero cells morphology after MTT assay. This is due to the proteins that play a role in the attachment of cells not undergoing polymerization so that the cell bonds are released, the lipid membrane will be rounded, the cytoskeleton is cut off and the apoptosis that occurs in the cells.[12,13] The apoptotic mechanism resulting in cell morphology changes will be inhibited as induced by ramania leaves ethanol extract. Changes in morphology of Vero cells undergoing apoptosis include changes such as shrinkage, membrane blebbing, chromatin condensation, apoptotic body formation and cell nuclear fragmentation.[14]

The result of cytotoxicity test of ramania leaves ethanol extract toward Vero cells showed a

tendency to decrease Vero cells viability on addition of ramania leaves ethanol extract concentration. According to the theory of Cotran et al. (2010) stimulus from toxic material can cause lesion in cells by damaging cell membrane, mitochondria and disturbing endogenous substrate.[15] Effect toward Vero cells viability was changes in cell membrane permeability. Cytotoxicity effect from cytotoxin can cause changes in cell membrane permeability or damage in cell membrane integrity causing it to be non-viable and lead to the death of the cell. The higher the effect will cause higher cell death, which means that cell death percentage will increase.[16]

Cell death is related to the cytotoxicity of a substance. This may occur because of the biochemical mechanisms of Adenosine Triphosphate (ATP) thinning and defects in cell membranes. Enzyme dehydrogenase is one of the enzymes that play a role in the formation of ATP. An inactive dehydrogenase enzyme may result in the cytotoxic cytotoxicity effect. This results in reduced ATP so that cells functional activity is impaired and cells death occurs.[16]

The probit test analysis obtained from ramania leaves ethanol extract which are toxic toward Vero cells. Toxic properties of ramania leaves ethanol extract influenced by secondary metabolite content contained in ramania leaves. In this research, phytochemical test obtained secondary metabolite compound on ramania leaves which is phenol, flavonoid, steroid and terpenoid. Phenol compounds and their derivatives work by denaturing cells proteins in cell membranes. Denaturation of proteins in the cells membrane causes a change in cells permeability. This resulted in cells membrane inability to retain the components inside the cells and inhibiting the flow of incoming material into the cells resulting in cell death.[15] In this study, the phenol compound test was tannic acid. The tannic acid belongs to the hydrolyzed tannat class. The results of the study of Vejayan et al. (2016) showed that tannic acid gives toxic effect to Vero cells.Tannic acid can induce apoptosis by involving 3/7 Caspase activation and Caspase 9.[17] Caspases are endoproteases that hydrolyzepeptide bonds in a reaction that depends oncatalytic cysteine residues in the caspase activesite and occurs only after certain aspartic acidresidues in the substrate. Caspase plays an important role in maintaining hemeostatis to regulate and execute cells death by apoptosis.[18]

The content of flavonoids is cytotoxic at high concentrations toward normal human cells. Flavonoids can act as antioxidants and prooxidants depending on the concentration of flavonoids given.[19,20] Skibola and Smith in Ke et al. (2013) has found potential toxic effects of excessive flavonoid administration. At high doses, flavonoids act as prooxidant that produce free radicals.[21] Free radicals can trigger the formation of oxidative stress. Oxidative stress occurs when the natural production of ROS (reactive oxygen species) cannot be balanced by the antioxidant capacity of the tissues. Excessive reactive oxygen species (ROS) can induce cellular damage irreversibly and cause cells death through intrinsic apoptotic pathways in mitochondria, resulting in mitochondrial DNA damage, dysfunction and increased cells apoptosis.[22,23] Damaged deoxyribo nucleic acid (DNA), cause accumulation of p53 protein in the cells. This situation will stop the cell cycle (in phase G1) in order to repair the DNA before replication occurs, but if the damage is too severe to repair, especially with the stimulation of the sensor that activates the apoptotic triggering proteins bax and bak by increasing the procloptotic synthesis of bcl-2 groups, p53 protein will trigger apoptosis.[24]

Terpenoid content based on its constituent isomers is classified into monoterpenoids, sesquiterpenoids, diterpenoids, triterpenoids, and tetrapenoids.[25] According Usman (2014), it is reported four halogenated monoterpenoids, which are aplisiapiranoid A-D, showed very high cytotoxicity toward Vero cells. Compound 1, 3 and 6 in diterpenoids showed strong cytotoxicity activity toward Vero cells.[26] Based on the research, it can be concluded that ramania leaves ethanol extract are cytotoxic toward Vero cells after the analysis using MTT assay method (IC_{50}<100µg/mL).

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