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The Inhibition of Ethanol Extract of *Phaleria macrocarpa* Stem Bark on iNOS Expression of HCT116 Colorectal Cancer Cell Line

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Abstract:
To determine the effect of ethanol extract from *Phaleria macrocarpa* stem bark on the expression of inducible Nitric Oxide Synthase (iNOS) in colorectal cancer cell line HCT116 and determined chemical substance on the extract. This experiment is done in in-vitro setting using colorectal cancer cell line HCT 116 which are given ethanolic extract of *Phaleria macrocarpa* bark in 3 different dosages which are 50 ppm; 100 ppm; and 200 ppm. The specimen is stained with immunocytochemistry staining with anti-iNOS antibody. The expression of iNOS is measured using H-Score and compared with control negative which are not given any extract. This study also examined the chemical compound found in the ethanol extract of *Phaleria macrocarpa* using qualitative phytochemistry screening test. Highest expression of iNOS is seen in the control negative group (234,88). Administration of ethanol extract of mahkota dewa bark (*Phaleria macrocarpa*) in 200 ppm dose significantly decrease the H-score by 23,5% to 179,67 with p=0,00. In Post-Hoc test, significantly different H-Score is only seen in 200 ppm group. Administration of ethanol extract of mahkota dewa (*Phaleria macrocarpa*) bark can decrease the expression of iNOS in colorectal cancer HCT 116 cell line.
Keywords: *Phaleria macrocarpa*, colorectal cancer, immunocytochemistry, iNOS, HCT 116 colorectal cancer cell line

INTRODUCTION

Colorectal cancer is one of the highest morality cause in the world. Today, colorectal cancer is the third most common malignancy found in the world.[1] This number is increasing especially in Asia Pacific.[2] In Indonesia, 19,1 of 100.000 men and 15,6 of 100.000 women have colorectal cancer. Most cases of colorectal cancer is found in productive age usually younger than 40 years old.[3]

The characteristic of colorectal cancer found in Indonesia is different from colorectal cancer found in western countries. Colorectal cancer in Indonesia grows sporadically. Colorectal cancer in Indonesia usually found more distally located compared to those found in western countries. Clinically, colorectal cancer in Indonesia is more severe and have lower survival rate compared to western countries.[3] This shows on going threat which may threat public productivity.

One of study found that the level of nitrogen monoxide (NO) produced by inducible-Nitric Oxide Synthase (iNOS) can contribute to tissue damaged and inflammation which may cause the formation of colorectal cancer.[4] Other study stated that iNOS is also one of the key factor in the formation of colorectal cancer caused by mutation of adenomatous polyposis coli (APC) gene, believed to be underlying mechanism of sporadic colorectal cancer.[5] On the other side, study on colorectal cancer is intensely growing. One of study found that ethanol extract of Phaleria macrocarpa fruit may induce tissue repair in mouse with colorectal cancer.[6]

The ability of Phaleria macrocarpa fruit to induce tissue repair in colorectal cancer, opens the potential of other part of Phaleria macrocarpa to be used in treating colorectal cancer. Based on these possibilities we are interested to see the effect of ethanol extract of Phaleria macrocarpa bark on expression of iNOS in HCT 116 colorectal cancer.

MATERIALS AND METHODS:

Plant and Cell Line Materials

The stem and bark of Phaleria macrocarpa was harvested from Kuningan region in West Java, Indonesia on June 2018. The harvested stem and bark was dried before extracted. HCT 116 Colorectal cancer cell line was obtained from Pathological Anatomy Department of Universitas Indonesia. The HCT 116 cells were sustained in Dulbecco's Modified Eagle Medium /DMEM (Gibco) which consists of penicllin-streptomycin 1% and fetal bovine serum medium 10% (Gibco).

Extract Preparation

Extraction process was done in Department of Chemistry of Universitas Indonesia. 500 g of dried stem and bark was grinded and macerated in 1.500 mL of ethanol solvent for 24 hours in a container while stirred 2-3 times a day. The maceration process was repeated three times to get more filtrate. The filtrate was dried by using rotatory evaporator to get crude ethanolic extract of Phaleria macrocarpa stem and bark.

Immunochemistry Assay

HCT 116 cells were prepared in a petri dish with sterile object glass inside. The cells were incubated in 50% CO2 in temperature 370C for 24 hours. Ethanol extract of Phaleria macrocarpa with concentration of 50; 100; and

200 ppm was introduced into the cell and re-incubated for 24 hours. As negative control, the cells were placed in DMEM medium without adding the ethanol extract of Phaleria macrocarpa.

After incubation, the cells were washed by using Phosphate Buffer Saline (PBS) solution. Cells were fixated on an object glass by dropping absolute methanol on the object glass and left for 10 minutes. Hydrogen peroxide (H2O2) 3% were dropped on the surface of object glass to remove endogen peroxide. The cells were then dipped in 0.01 M citrate buffer (pH = 6) and are dropped with normal serum.

Cells were incubated overnight with anti-iNOS antibody inside buffer phosphate saline solution. The cells were re-incubated for 10 minutes after introducing the secondary antibody and for 3-8 minutes after introducing HRP-conjugated streptavidin. Cells were washed by running distilled water followed by introduction of hematoxylin-harris solution.

The expression of iNOS was observed under light microscope with 400x magnification by counting H-Score based on cell color intensity. The intensity ranges from 0-2. Score 0 is given to cells which are not stained; Score 1 is given to cells stained less than 50% of the whole surface and Score 2 is given to cells stained more than 50% of the whole surface. H-Score is counted by using the following equation:

H-score = (% of cells with color intensity 0 x 1) + (% of cells with color intensity 1 x 2) + (% of cells with color intensity 2 x 3)

Phytochemistry Qualitative Screening Test

In this study we assessed 6 groups of chemical substance which are saponin, flavonoid, triterpenoid / steroid, alkaloid, tannin, and glycoside. For assessing the presence of saponin we look for the presence of positive foam test result. The presence of triterpenoid / steroid is assessed by using Liebermann Burchard reaction. Presence of alkaloid is tested by using Dragendorf's and Mayer's test. Tannin is positive when the tested solution become blackish green after introduction of FeCl3. Presence of flavonoid is examined by boiling solution until it is dried followed by addition of pure acetone and pure boric acid and oxalate acid powder. The mixture is then added with pure ether and examined under UV light 366 nm. Glycoside is positive if the solution form blue or green color after boiling and introduction of pure acetic anhydrate acid followed by pure sulfate acid.

Data Analysis

Expression of iNOS was measured using H-score from immunocytochemistry staining and was analyzed by One-Way ANOVA using IBM SPSS Statistic Version 20 Software.

RESULTS AND DISCUSSION

This study evaluates whether ethanol extract of Phaleria macrocarpa stem and bark may decrease the expression of iNOS on colorectal cancer cell line HCT 116. This study also examined the chemical groups present in the ethanol extract of Phaleria macrocarpa stem and bark by using qualitative phytochemical screening test.

Immunochemistry Assay for iNOS

The effect of ethanol extract of Phaleria macrocarpa stem and bark on the expression of iNOS by colorectal cancer cell line HCT 116 is examined by comparing means of control and treated groups. There was a statistically significant mean difference between groups as tested by one-way ANOVA (p=0.000). Bonferroni post hoc test revealed that the expression of iNOS was lower after treated with 200 ppm concentration of ethanol extract of Phaleria macrocarpa stem and bark. The effect of treatment with 200 ppm concentration of ethanol extract of Phaleria macrocarpa stem and bark reduce 23,5% of H-Score compared to negative control groups.

From previous studies, iNOS plays as one of factors which contribute to growth of colorectal cancer by formation of NO which can interact with ROS intracellularly to cause Reactive Oxidative Nitrogen Species, one of free radicals. RONS may cause genetic mutation and cellular damage which may contribute to the formation of colorectal cancer. [5] This theory suggests the use of iNOS inhibitor to inhibit the development of colorectal cancer. Previous studies shows that treatment of S,S′-1,4-phenylene-bis(1,2- ethanediyl)bisisothiourea (PBIT) dan N6-iminoethyl-lysine tetrazoleamide (NILT), an iNOS inhibitor, is effective in inhibiting the formation of colonic aberrant crypt foci found in colon cancer in in vivo setting.[4] Another study shows that administration of curcumin may inhibit the formation of chemically induced colon cancer by suppressing the expression of iNOS.[4]

Table 1. H-score from the observation on iNOS expression of HCT116 cells

Variable	Take	H-score	Average H-score
Negative control	1	216,867	220,259
	2	221,875	
	3	222,034	
Extract 50 ppm	1	207,468	212,298
	2	212,500	
	3	216,928	
Extract 100 ppm	1	187,072	192,077
	2	178,814	
	3	210,345	
Extract 200 ppm	1	177,922	181,541
	2	179,679	
	3	187,023	

Table 2. One-way ANOVA test result

Treatment	Mean ± SD	Sig.
Negative control	234,88 + 5,320	0,000
Extract 50 ppm	235,76 + 5,365	
Extract 100 ppm	223,58 + 3,966	
Extract 200 ppm	179,67 + 1,568	

Table 3. Post hoc Bonferroni test result

Group 1	Group 2	Sig.
Negative control	Extract 50 ppm	1,000
	Extract 100 ppm	0,615
	Extract 200 ppm	0,000
Extract 50 ppm	Negative control	1,000
	Extract 100 ppm	0,493
	Extract 200 ppm	0,000
Extract 100 ppm	Negative control	0,615
	Extract 100 ppm	0,493
	Extract 200 ppm	0,001
Extract 200 ppm	Negative control	0,000
	Extract 50 ppm	0,000
	Extract 100 ppm	0,001

Phaleria macrocarpa is known for its various positive effect on human health including anti hyperglycemic, anti-hyperlipidemia, anti, inflammation, antibacterial, antiparasitic, and antioxidant effect.[7,8] Phaleria macrocarpa is also known for its anti-cancer activity and has been used in treatment of bone cancer, breast cancer, and cervical cancer. [8]

We choose ethanol as solvent in this study because it is relatively safe to be consumed by human. Ethanol has been approved by Food and Drug Administration (FDA) and classified as generally recognized as safe additive in food.[9] In addition, ethanol has been known for its good ability to extract polar substance from tested herbs, the same characteristic of substance that we would like to extract from Phaleria macrocarpa stem and bark. This safe profile of solvent and positive result of this study may suggest the potential of ethanol extract of Phaleria macrocarpa stem and bark to reduce iNOS expression to act as an iNOS inhibitor and indirectly reduce the development of colon cancer.

Qualitative Phytochemistry Screening Test

The phytochemistry screening test done in this study is a qualitative test for examining the presence of saponin, tannin, flavonoid, glycoside, triterpenoid, steroid, and alkaloid. From this screening test, the ethanol extract of Phaleria macrocarpa stem and bark is positive for the presence of tannin, flavonoid, glycoside, and triterpenoid.

Tannin is one of complex organic compound which does not consist of nitrogen. One of the significant physical properties of tannin is good solubility with polar solvent. [10] Presence of tannin compound (gallic acid) has already been found in pericarp extract of Phaleria macrocarpa. [11] Flavonoid can also be found in the ethanol extract of Phaleria macrocarpa stem and bark. Kaempferol, one of flavonoid compound, has also been find positive on pericarp extract of Phaleria macrocarpa. [12] Glycoside compound called phalerin is found positive in pericarp extract of Phaleria macrocarpa.[13] The pericarp extract is also positive for presence of phenolic compounds, terpenoid, and alkaloid. [7]

The ability of ethanol extract of Phaleria macrocarpa stem and bark may be associated with the presence of these chemical compounds. Tannin is known for its antioxidant and proapoptotic activity on cell in in-vitro setting.[11] Flavonoid has polyphenol structure and its antioxidant, anti-inflammatory, antimutagenic, and anti-carcinogenic activity.[12] Triterpenoid takes a part in induction of apoptosis and inhibiting angiogenesis on tumor. [14] Glycoside is also known for its antiapoptotic ability by increasing expression of BAX protein and decreasing expression of Bcl-2 protein which will interact and activate intracellular molecular pathway resulting proapoptotic effect. [7] Although its richness of beneficial compounds, further molecular assessment on which chemical compound plays major role in decreasing expression of iNOS by the ethanol extract of Phaleria macrocarpa stem and bark is still needed.

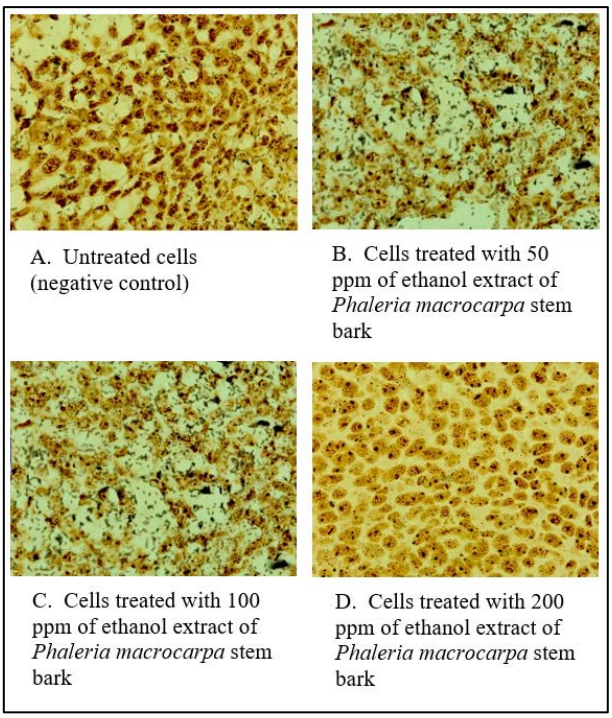

Figure 1. The observation of COX-2 expression on HCT116 cells on immunocytochemistry method under light microscope using 400x magnification.

CONCLUSION

Ethanol extract of Phaleria macrocarpa bark may reduce expression of iNOS on colorectal cancer cell line HCT 116. This ability allows this extract to act as iNOS inhibitor and offers a new promising potential of chemo preventive agent on colorectal cancer. The ability of this extract to inhibit expression of iNOS on colorectal cancer HCT 116 may be associated with the presence of tannin, glycoside, triterpenoid, and flavonoid in it. However, the molecular mechanism of this extract wasn't assessed on this study. Therefore, further research on exact molecular mechanism and biosafety of this extract in the future are still needed.

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Conflict Of Interest

The Authors declare no conflict of interest.

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