Number of e-cadherin and VEGF in WiDr cell line post-induced by extract ethanol Annona muricata leaf

Yoni Astuti*, Agus Suharto1, Sabtanti Harimurti3, Helmi Hana Prinanda4, Wahyu Joko Priambodo5

ABSTRACT

**Introduction:** Soursop leaves have benefits including anticancer, antifungal, and antibacterial. Soursop leaves are proven to contain various phytochemicals, namely alkaloids (ALKs), essential oils and acetogenins. Annonaceous acetogenins compounds soursop cytotoxic effect on cancer cells. One of the reasons for the malignancy of cancer cells is their ability to move to other tissues. Migration ability was related with the expression of e-cadherin, in cancer cells the expression of e-cadherin was low. Malignancy is also related to the amount of VEGF, in the type of tumor VEGF expression increases. Therefore, the aim of this study was to measure the amount of e-cadherin and VEGF of the WiDr colon cancer cell model after being induced by soursop leaf ethanol extract.

**Methods:** Ethanol extract of soursop leaves is obtained by maceration. The measurement of the amount of e-cadherin used the elisa kit e-cadherin while the amount of VEGF was used by the elisa kit VEGF. The amount of e-cadherin and VEGF measurement used was an elisa reader at a wavelength of 560 NM.

**Results:** The results showed there were significant differences between various doses of extract ethanol Annona muricata to the number of e-cadherin. There were significant differences between various doses of Extract ethanol Annona muricata to the number of VEGF.

**Conclusions:** Ethanol extract Annona muricata leaf showed the anti migration and anti angiogenesis effect on WiDr cell line.

**Keywords:** Annona muricata, angiogenesis, migration, cell line WiDr.


INTRODUCTION

Cancer is one of the major diseases that could lead to death. The World Health Organization (WHO) in 2020 showed interesting data about this issue, namely in 2020 there are 10 million deaths, 1 in six deaths worldwide are caused by cancer. Among that 10 million, the highest number of deaths is lung cancer, then colon and rectum, liver, stomach, and the last breast cancer.1,2,3,4,5,6,7,8,9,10,11

Colorectal cancer has become the predominant cancer in the past few years, especially in Western countries. Indeed, this kind of cancer is also happening in a large number here, in Indonesia. The main reasons for this increased phenomenon of colorectal cancer could be a bad diet, less physical activity, smoking, alcohol as a habit, and obesity.2,3 To overcome colorectal cancer and cancers in general, scientists have developed some therapeutic strategies such as laparoscopic surgery and chemotherapy, but, despite the advanced technologies used in those strategies, the cure rates are not increasing significantly and still have side effects that could harm the cancer patients. Based on that problem, new methods should be applied to beat colorectal cancer, such as herbal-based therapy which barely gives a negative impact on cancer patients.1,4

Annona muricata is one of the plant species that are widely known as an anticancer.7 The extract of Annona muricata has the highest anticancer potential, even higher than the other nine plants that have been studied, such as Bush mango, J. gossypfolia, N. latifolia, P. staudtii, A. precatorious, A. paniculate, G. kola, and orange peels.1,4 The anticancer activity works well in the lung cancer cell line, with the possible reason is that Annona muricata has high content of flavonoids, because, it is known that flavonoids have many advantages to beat human diseases, including cancer.2,3,4 This huge breakthrough about Annona muricata anticancer activity could be used as the initial knowledge for herbal-based colorectal cancer therapy development. Moreover,
**METHODS**

**Treatment and Cell Culture**

Human WiDr cells were maintained in medium by having 10% (v/v) Fetal Bovine Serum (FBS, Invitrogen), and antibiotics (penicillin 100 U/mL, streptomycin 100 µg/mL, and fungizone 250 µg/mL). The MTS assay (10,000 cells in 100 µL of media) was performed by placing grained cells in each hole of a 96-well plate and hatched for two hours at 37 °C in a humid setting containing 5 percent CO₂. At 492 nm, formazan was measured with a microplate reader.

**Protein Extraction**

Phosphate Buffer Saline (PBS) was used to clean the cells twice after being extracted, and MPER (Thermo Scientific) kit was used to generate protein lysates with protease resistor cocktail and stopping the phosphatase resistor cocktail (Thermo Scientific). Fifteen minutes of Centrifugation at 14,000 rpm and 4 °C carried out on the cell extracts. Based on the fabrication's guide, the supernatant was shifted to other tubes, and the protein content assessed using Bradford dye and a microplate reader (Bio Rad Laboratories).

**Gel Electrophoresis**

Protein (60 µg) was dissolved in loading buffer (loading buffer: 2.5 µL of NuPage LDS Buffer sample (4X, Invitrogen), 1 µL of NuPage Sample Decreasing Agent (10) (NP0004, Invitrogen), and 6.5 µL of deionized water. The mixture was heated to 70 °C for 10 minutes, and then it was electrophoresed on NuPage Novex 12 percent (v/v) BisTris Mini Gels. When transferring proteins using the wet transfer approach, an Immobilon-P polyvinylidene difluoride (PVDF) membrane (0.45 µm) is utilized. To block the membrane for one hour at room temperature, 30 mL of 5 percent (w/v) skim milk was used in Tris-Buffered Saline with TBS-T. After blocking, the blots were treated with rabbit polyclonal antibodies against e-cadherin (Thermo Fisher LOT WG 3340922C) and VEGF at a 1:1000 dilution in immunoreaction enhancer solution for the primary antibody for an overnight incubation at 4 °C. Following a TBST wash, the membranes were incubated with 1:5000 horseradish peroxidase (HRP) diluted with goat anti-rabbit IgG antibody conjugated in TBST for 1 hr at room temperature by 5% milk. The membrane was cleaned with TBST three times after 1 mL of Western blotting Enhanced Chemiluminescent (ECL) detection reagent was added. Bands were then identified using an image reader, and band intensity was calculated using ImageJ Software.

**MTS Assay**

Using the MTS test, the proliferation of WiDr cells in culture was evaluated. 96-Cell Proliferation Kit by Cell Titer For this assay, which posit on tetrathiazolium cellular reduction to formazan synthesis, one aqueous solution containing 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethyl oxophenyl-2-(4-sulfonyl)-2H-tetrazolium, salt in (MTS)) and phenazine methosulfate was employed. Number of living cells directly correlates with the amount of formazan synthesis. The fabrication's guide informed the tests are conducted. Following a 24-hour incubation period, 20 µL of MTS reagent was poured into 100 µL of media per well of a 96-well plate, and hatched for two hours at 37 °C in a humid setting containing 5 percent CO₂. At 492 nm, formazan was measured with a microplate reader.

**ELISA Assay**

WiDr cells’ cell culture supernatant was gathered in a tube, and the tube centrifuged at 2000 rpm for 20 min. When the components of the cell were carefully removed from the supernatant, PBS (pH 7.2–7.4) was used to dilute the cell suspension to a cell concentration of 1 million/ml. This experiment utilized the Human VEGF and Human e-cadherin ELISA kits from Bioassay Technology Laboratory (Cat. Nos. E0080Hu and E0209Hu, respectively). The plates have been pre-coated with e-cadherin and VEGF antibodies, and e-cadherin and VEGF that are present in the samples are added to bind to antibodies coated on the wells. The procedures provided by the manufacturer were followed during the testing. Within 10 minutes of the samples’ transition from blue to yellow, the optical density value (OD Value) for each well was calculated using a microplate reader set to 450 nm.

**Statistical analysis**

Data were analyzed statistically by using SPSS. The results were presented as mean ± standard deviation (SD). The analysis was carried out using the ANOVA test followed by post-hoc. When p-value less than 0.05 was indicated any significant differences.

**RESULTS**

Standard curve for dose serial for cadherin is needed as reference to calculate the number of cadherin samples. Figure 1 shows the linear equation for the standard curve, y=0.0005x+0.0582 with R² = 0.9948. Table 1 showed the average of cadherin for some various doses of Annona on WiDr Colon cancer cells. This table showed the decreasing e-cadherin number around
4.11 % for decreasing 452.85 µg/ml level of extract ethanol *Annona muricata*, but the decreasing extract *Annona* to level 226.425 µg/ml follow with increasing of e-cadherin number 32.7 %.

The significant differences between dose of extract ethanol to number of e-cadherin showed as figure 2.

Standard curve for dose serial for VEGF is needed as reference to calculate the number of cadherin samples. Figure 3 shows the linear equation for the standard curve, $y=0.0002x+0.0146$ with $R^2=0.9912$.

Table 2 showed the average of e-cadherin for some various doses of Annona on WiDr Colon Cancer cell. This table showed the decreasing e-cadherin number around 58.38 % for decreasing 452.85 µg/ml level of extract ethanol *Annona muricata*, but the decreasing extract of Annona to level 226.425 µg/ml followed with an increase of e-cadherin number 49.96 %.

Figure 4 showed the significant differences between doses of extract ethanol *Annona muricata* on the number of VEGF WiDr cell lines.

**DISCUSSION**

E-cadherin is set by the CDH1 gene placed on the human chromosome 16q22. E-cadherin is a calcium-dependent cell-cell adhesion protein that is crucial for forming the structure of the epithelium and preserving cell polarity and differentiation. The loss of cell–cell adhesion and cell junctions mediated by homophilic binding of e-cadherin is believed to allow cells to separate from the primary tumour, penetrate the surrounding tissue, and migrate to distant areas. However, carcinomas and distal metastases often retain e-cadherin expression, and EMT (Epithelial Mesenchymal Transition) is in fact not necessary for metastasis, but it has been proven that tumor cell groups have been found to cause breast metastases. e-cadherin expression is regulated by many factors including genetic mutations, DNA methylation. Low expression of e-cadherin has been linked to histological and clinical characteristics of malignancy, including metastasis, recurrence, poor survival, and tumour differentiation, a “high risk marker of malignancy.” This study using WiDr as model of colon cancer cell that already known expressed e-cadherin. This study showed that the number of cadherin in WiDr colon cancer cell line was decreased during induction with higher dose of extract ethanol *Annona muricata*. But during induced with the low dose extract ethanol *Annona muricata* the number of e-cadherin was increased. This research did not show the expression e-cadherin...
**ORIGINAL ARTICLE**

Simultaneously decreasing vimentin and matrix metalloproteinase (MMP) expression. This suggests a-Solanine prevented EMT and reduced the viability and motility of human PCa cells. Mesenchymal marker inhibition including N-cadherin and vimentin as good as an elevation of e-cadherin were seen in PCa cells after treatment with simvastatin and metformin, which helped to prevent TGF-β1-induced EMT. The highly selective IkB kinase (IKK) inhibitor BMS-345541 also demonstrated impedimental impact in PCa cells by phenotypic return of the EMT, as demonstrated by a surge in e-cadherin and reductions in N-cadherin, snail, slug, and TWIST. How do the extract ethanol of Annona muricata involved in the turnover of e-cadherin, need more experiment. Meanwhile Extract ethanol Annona muricata showed anti migration effect on WiDr Cancer cell line as a model of Colon cancer cell. The powerful angiogenic factor recognized as vascular endothelial growth factor (VEGF) was initially identified as being crucial for the proliferation of vascular endothelial cells. It is generally known that VEGF give an effect in tumour angiogenesis and that it upregulated in many cancers. Many non-endothelial cells like tumour cells, express VEGF and VEGF receptors in addition to endothelial cells. This review addresses the potential mechanisms at play and considers the relevance of VEGF signalling in non-endothelial cells. The discussion of autocrine VEGF motioning paths in tumour cells relates to the development of anti-VEGF anti-tumour therapies. When tumour cells are directly stimulated by VEGF, the cells may be shielded from apoptosis and become more resistant to conventional chemotherapy and radiotherapy. In this research showed that the decreased of dose extract ethanol Annona muricata followed by the decrease of total VEGF in WiDr cancer cell line. How does the mechanism still need more explorations. Some ingredient of extract Annona behave as cytotoxic with killed the apoptosis G2 phase. Previous research using extract hexane of Annona reticulate seed showed the ability to decrease angiogenesis in Rat with lowering VEGF A expression. Colon cancer patients

Figure 3. Doses serial of VEGF number as reference standard equation.

Figure 4. Significant differences between various dose of Extract ethanol Annona muricata to number of VEGF (**), dose 905.7 ug/ml and 226.425 ug/ml, dose 905.7 ug/ml and dose 452.85 ug/ml, also dose 452.85 ug/ml and 226.425 ug/ml.

in certain parts of the cell, but was using extract protein of culture cell, so it assumes that total e-cadherin was available. The increasing soluble e-cadherin related with the malignancies of cell, as reported that soluble e-cadherin in patient with gastric cancer was higher than normal subject. Extract ethanol Annona muricata is known as anti cytotoxic WiDr cell line with IC50 on dose 908 mg/ml which meant that a half population was death on this dose, so total of e-cadherin also low. But when the dose of extract ethanol Annona muricata was low, there was higher population of WiDr Cell line still alive, so total of e-cadherin also higher. On the gastric tumor patient showed higher e-cadherin than no tumour subject. On this condition assumes that e-cadherin was retained on tumour gastric. The rates of protein internalization, degradation, and recycling dictate the amount of e-cadherin that is present at cellular junction sites. There were estimates for the half-life of e-cadherin ranging from a few minutes to hours. Also, due to trafficking between the cell surface and the cytoplasm, where it can be stored, quickly rerouted to the membrane, or destroyed, e-cadherin experiences continual turnover. Meanwhile a-Solanine, a certainly happening glycoalkaloid extracted from nightshade (Solanum nigrum Linn), was discovered by one group to significantly increase e-cadherin expression though
showed high levels of VEGF which was significant in rapid metastasis. Post-resection causes difficulty in recovery and survival of colon cancer patients. It was reported that changes in EMT did not result in a significant reduction in cell exodus and assault in VEGF-depleted CRC cells. Annotations made by SW620 and HT29 cells. miR-1 hinders VEGF expression directly at posttranscriptional level over targeting its 3 OS tumours was confirmed from a reasonable analysis of miRNA expression profiles compared with paired normal tissues. The expression levels of miR-1, miR-133b, and miR-378 in tumours were significantly decreased compared with normal bone from noncancerous patients based on RT-PCR in 56 patients with OS. Meanwhile VEGF expression increasing in comparing normal to oral epithelial dysplasia to oral squamous cell carcinoma.

CONCLUSION
This research reveals that the decreasing dose of extract ethanol of Annona muricata is followed by the increasing of e-cadherin and on the other hand is followed by decreasing of VEGF number. To prove the real mechanism of both results requires more experimentation. Further research with a different study design and a larger sample size is needed, as well as a more in-depth analysis to determine the factors that affect number of e-cadherin and VEGF in WiDr cell line post-induced by extract ethanol Annona muricata leaf.

CONFLICT OF INTEREST
There is no conflict of interest.

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ETHICAL CONSIDERATION
The research was carried out following the research protocol that was approved by the Health Research Ethics Committee of Universitas Muhammadiyah Yogyakarta.

AUTHOR CONTRIBUTION
All authors contributed to this study’s conception and design, data analysis and interpretation, article drafting, critical revision of the article, final approval of the article, and data collection.

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