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Research Article

INHIBITORY AKT2 AND VEGFR2 GENES EXPRESSION OF *Avicennia marina* USING RT-PCR ON WiDr CELLS

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ABSTRACT

Objectives: To evaluate the activities the anticancer of n-hexane extract of *Avicennia marina* leaves on WiDr cells in down-regulated of expression of Akt2 and VEGFR2 genes.

Methods: *Avicennia marina* leaves were dried and extracted with n-hexane, analyzed the down-regulated Akt 2 and VEGFR2 expression which was determined the Reverse Transcription Polymerase Chain Reaction (RT-PCR) method.

Results: N-hexane extract of *Avicennia marina*, in which there were down regulated expression Akt 2 and VEGFR2 of 0.43 and 0.50 WiDr cells. N-hexane extract of mangrove leaves has cancer chemoprevention activity with down-regulated on WiDr cells.

Conclusions: N-hexane extract of *Avicennia marina* leaves had anticancer activity with down-regulated on WiDr cells, suggest that significantly inhibit the expression of Akt2 and VEGFR2 genes.

Keywords: Akt2, VEGFR2, WiDr cells, *Avicennia marina*, RT-PCR.

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INTRODUCTION

Cancer is a disease characterized by uncontrolled cell growth. Cancer cells may avoid apoptotic and signals that suppress growth, the ability to form new blood vessels (angiogenesis), and the ability to invade and metastasize¹. Chemotherapy agents is one of the treatments for colon cancer in addition to surgery and radiation therapy. Chemotherapy agents used today generally not only to suppress the growth or proliferation of cancer cells while causing toxicity to the body but also inhibit the proliferation of normal cell division, including the bone marrow, gastrointestinal mucosa, hairfall, and lymphocyte tissue. This condition raises concerns about various side effects caused by the use of conventional chemotherapeutic agents, such as heart (cardiotoxic) disorders, nausea, diarrhea and suppression of the immune system and the occurrence of resistance, thus increasing people's interest in using traditional medicines².

Potential natural ingredient developed as chemotherapeutic agents includes from mangrove leaves. Mangrove vegetation defined as a plant or shrub distribution in intertidal zone of tropical and sub-tropical regional³. Polyisoprenoid is secondary metabolites found in mangroves, classified as dolichol and polyprenol on mangrove leaves and roots. So far studies reporting pharmacological activity in polyisoprenoid of mangrove species is still limited, so it is important to achieve the prospects, potential and mechanisms polyisoprenoid in mangroves as a natural ingredient of pharmaceutical and medication⁴.

Studies that polyisoprenoid in mangrove leaves *Nypa fruticans* induced the cancer cell cycle inhibition of adenocarcinoma of the colon WiDr cell in G2/M phase and reduce the percentage of Bcl-2 and Bcl-xL⁵. It has been also reported that polyisoprenoid of *A. marina* and *A. lanata* leaves have anticancer colon activity. Polyisoprenoid of *A. marina* have IS value of 5.195 (> 3) that is highly selective.

This polyisoprenoid extract has a mechanism of inhibition of cell cycle at G0-G1 phase, and Apoptotic phase analysis occurs in the early apoptotic phase on the WiDr cells with flow cytometry method⁶.

Activation of Akt, the major downstream effector of PI3K, is frequently observed in human cancers⁷. The Akt kinase family is composed of three members, Akt1, Akt2 and Akt3. Akt1 and Akt2 are ubiquitously expressed, whereas Akt3 has a more limited tissue distribution [8]. Elevated Akt2 expression positively correlates with aggressiveness of cancer and poor survival rates [9]. Amplification and over expression of Akt2 is frequently detected in a number of human tumors. The distribution of PI3K/Akt pathway component expression in human colorectal adenocarcinomas. Regulatory and p110, catalytic subunits of PI3K in colon cancer cell growth using an RNAi approach¹⁰.

VEGF binding to the extracellular domain of the VEGFR results in dimerization and autophosphorylation of the intracellular tyrosine kinases. This activates multiple downstream proteins that play functional roles in cell survival, proliferation vascular permeability and stabilization of new blood vessels. For example, VEGF induces endothelial cell proliferation by activating the protein kinase Ras-MEK-ERK pathway. The pro-survival effects of VEGF/VEGFR-2 are mediated by the PI3K/AKT pathway¹¹. Recent studies indicate that VEGFR are also expressed by some tumor cells and may represent an additional target¹².

The goals of our current study were to extend the analysis of Akt isoforms in colorectal carcinoma as well as metastatic tumors and to identify which specific steps in the metastatic process are Akt2 dependent. Therefore, Akt2 appears to play a critical role in the establishment of metastases in colorectal cancer. Furthermore, concurrent PTEN down-regulation Akt2 expression led to metastatic phenotype acquisition in colon cancer cells that are non-metastatic. This study, therefore, aimed to test of biological and pharmacological activities for the treatment of colon cancer from *Avicennia marina* polyisoprenoid in terms of the cycle and gene expression of Akt2 and VEGFR2 using the Reverse Transcription-Polymerase Chain Reaction (RT-PCR) method.

METHODS AND MATERIALS

Plant material

Mangrove leaves of *Avicennia marina* were collected the village of Lubuk Kertang, District West Brandan, Langkat, North Sumatra. Indonesia.

Preparation of isolation polyisoprenoid alcohols

Powder simplicia mangrove leaves of *Avicennia marina* (500 g) was macerated with a mixture of chloroform: methanol (2:1, v/v) for 48 h. Non-saponified lipid extracts of leaves incubated of 65°C for 24h in 86% ethanol containing KOH 2 M. Non-saponified lipid parts were further diluted with n-hexane, and the solvent was evaporated. Then redissolved in n-hexane, a concentrated dried extract was obtained¹³.

Isolation WiDr Cells

Cell lines and cell culture conditions (WiDr cells), isolated human colon cancer cells from the large intestine of 78-year-old women was provided by the Laboratory of Parasitology collection, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. WiDr cell lines was cultured in RPMI 1640 medium, and supplement with 10% (v/v) fetal bovine serum (FBS), 1% penicillin and streptomycin, fungizone 0.5%, and in a 37°C incubator with 5% CO₂¹⁴.

Analysis of genes expression in vitro with RT-PCR

The expression of the genes was examined Reverse Transcription-Polymerase Chain Reaction (RT-PCR) method. Total RNA was extracted from the control and cultured cells using the Total RNA Mini Kit (Geneaid) according to the manufacturer’s protocol. Total RNA was reverse-transcribed with 1 µg random primer and ReverTraAce (Toyobo) to produce a cDNA in a total volume of 20 µl for 10 min at 30°C, 60 min at 42°C, and 5 min at 99°C according to manufacturer’s procedure. The resulting cDNA mixture was diluted TE buffer and directly used for the subsequent PCRs.

PCR consisted of 35 amplification cycles, and each cycle was carried out for 30s at 95°C, 1 min at annealing temperature (58°C for beta-actin), (55°C for Akt-2) and (60°C for VEGFR2), and 1min at 72°C in a thermal cycle (ProFlex™ 3x 32-well PCR System, Applied Biosystems). The beta-actin housekeeping gene was used as an internal control to standardise the relative expression levels for all bio markers¹⁵. The semi-quantitative RT-PCR product was observed using 2% agarose gel and stained with ethidium bromide. The bands were documented using the image scanner Doc XR Gel (Bio-Rad)¹⁶. The oligonucleotide primers for Akt2, VEGFR2 and beta-actin were shown in Table1.

Table 1: Mouse oligonucleotide primers sequences used for RT-PCR (5-3’) and annealing temperature.

Gene		Primer Sequences	Size (bp)	Temp (°C)
Beta-actin	R	5'- TCGTCATACTCCTGCTTGCTG AT -3'	105	58
	F	5'-GCTCCTCTGAGCGCAAG T-3'		
Akt2	R	5'-TGCTTGAGGCTGTTGGCGACC-3'	315	55
	F	5'-ATGAATGAGGTGTCTGTCTGTGCATCAAAGAAGGC-3'		
VEGFR2	R	5'-GAAATGGGATTGGTAAGGATG-3'	87	60
	F	5'-GTGTCAGAATCCCTGCGAAGTA-3'		

Statistical analysis

Data were statistically analyzed Duncan Test for density base pair of Akt 2 and VEGFR2 genes, then completed with a Duncan test. The value of $P < 0.05$ was chosen as the threshold of statistical significance.

RESULT AND DISCUSSION

Effect extract *Avicennia marina* on the genes expression Akt2 and VEGFR2 showed that *Avicennia marina* have

anticancer activity inhibition down-regulated expression of Akt2 and VEGFR2. As shown in Figure 1 and table 2 also treatment 5-Fu inhibits gene expression of Akt2. Beta-actin was used as an internal control in the analysis of gene expression because it is a housekeeping gene, the gene that continuously expressed for a living organism. Housekeeping genes have stable expression levels in various tissues during development stage¹⁷.



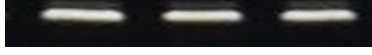
Gene	Expression genes			Base pair (bp)
	(a)	(b)	(c)	
B-actin				105
Akt2				315
VEGFR2				87

Figure 1. Effect *Avicennia marina* on Expression gene in WiDr cells. The Total RNA were isolated and RT-PCR was performed using the indicate primers material and method (a) Control cell, (b) *Avicennia marina* (c) 5-Fu

Figure 1 shows the semi-quantitative expression of the Akt 2, VEGFR2 and Beta-actin genes of *Avicennia marina* which were analyzed based on base pairs of each gene with RT-PCR. Beta actin as housekeeping gene showed stability expression in cell control, 5-Fu as positive control of

Avicennia marina. Beta-actin produced an amplification of 105 bp. Illustrates the Akt2 expression band with 315 bp of PCR product, VEGFR2 expression band with 87 bp of PCR product, in which 5-FU showed a clear band.

Table 2:The value density of genes expression after treatment with WiDr cells.

No	Gene	Control cell	<i>A. marina</i>	5-Fu
1	Beta-actin	1.00± 0.00	0.72 ±0.02	0.75 ±0.03
2	Akt2	1.00±0.00	0.43±0.01	0.50± 0.02
3	VEGFR2	1.00±0.00	0,50 ± 0,03	0,58 ±0.02

Table 2 showed that down-regulation values Akt 2 and VEGFR2 gene expression. The dolichol content of *Avicennia marina* showed the most down-regulated results in the Akt 2 and VEGFR2 genes. The value of gene expression in *Avicennia marina* on control cells had a significant difference with $P < 0.05$. Akt is well-known major regulatory signaling cascade that control cell proliferation, metabolism and survival of cancer cells¹⁸. Therefore, several inhibitors, have been developed and used for treatment to induced apoptotic in cancer cells¹⁹⁻²⁰. Our results indicated that the expression levels of Akt-2 were down regulated as evident from the RT-PCR assay.

The oncogenic potential of Akt2 identified in this study suggests that the frequent amplification and up-regulation of this kinase in colorectal cancer plays an important role during metastatic colonization. Results from our study suggest that colorectal cancer cells required Akt2 over expression and PTEN inactivation during separate steps of the metastatic process. Activated Akt2 appears to influence the metastatic phenotype by promoting extravasation at the secondary metastatic sites, whereas PTEN deficiency

preferentially favors persistence and growth of metastases. PI3K/Akt inhibitors are under development as anti-cancer therapy or have been approved for treatment of certain human cancers²¹.

VEGFR2 was largely and strongly expressed in cancer cells in 203 colon cancer tissues, suggesting that it may have another role in cancer cell biology aside from being a vasculature-restricted receptor. In addition, in accordance with a previous study, which demonstrated significant association of VEGFR2 expression with poor tumor histological differentiation in 128 colorectal adenocarcinoma tissues, the present study showed that VEGFR2 expression correlated significantly with differentiation, metastasis, and poor diagnosis²². In this regard, with its capacity to enhance of tumor cells into endothelial cells, VEGFR2 may be advantageous for tumor progression. In summary, colon cancer cells can trans differentiation along endothelial lineages, both morphologically and functionally. More importantly, this study indicated critical role of VEGFR2 in promoting endothelial differentiation of colon cancer cells.

Results pose several implications for underlying importance of VEGFR2 as useful therapy target.

CONCLUSION

This study confirms that The dolichol content of *Avicennia marina* inhibits down-regulated of Akt 2 and VEGFR2. This study shows that dolichol in *Avicennia marina* blocked the growth factor of the WiDr cells.

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