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Research Article

n-Hexane Extract of Mangrove Leaves Effect on p21 and Akt 2 Gene Expressions of WiDr Cells

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ABSTRACT

Objectives: The study aims to examine the anticancer effect of polyisoprenoid of *Nypa fruticans, Ceriops tagal,* and *Rhizophora mucronata* leaves in WiDr cells by evaluating the regulation of p21 and Akt 2 gene.
Design: *Nypa fruticans, Ceriops tagal,* and *Rhizophora mucronata* leaves were dried and extracted with n-hexane, analyzed the increase or decrease in regulation of p21 gene and Akt 2 expression which was determined the Reverse Transcription Polymerase Chain Reaction (RT-PCR) method.
Interventions: The variable that was intervened in this study was the 3 sample mangrove leaves.
Main outcome measure: The main measurement results in this study were to study n-hexane extracts of mangrove leaves able to suppress the expression of p21 and Akt 2 genes so that cancer cell growth is inhibited.
Results: n-hexane extract of *Ceriops tagal* leaves was more effective than *Nypa fruticans* and *Rhizophora mucronata*, in which there was up-regulated (p21) of 1.19 and down regulated (Akt 2) of 0.78 on colon cancer cells (WiDr). N-hexane extract of mangrove leaves has cancer chemoprevention activity with up-regulated and down-regulated on WiDr cells, in which the sample was more effective than n-hexane extract of *Ceriops tagal* leaves.
Conclusion: N-hexane extract of mangrove leaves had cancer chemoprevention activity with up-regulated and down-regulated on WiDr cells, in which the sample was more effective than n-hexane extract of *Ceriops tagal* leaves.

Keywords: Rhizophora mucronata, Nypa fruticans, leaves, Ceriops tagal, daun

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INTRODUCTION

Free radicals are highly active and unstable molecules with orbits in which in the outer layer, one electron is unpaired so as it tends to find its electrons by seizing other electrons, so that it is called reactive oxygen species (ROS) [1]. Free radicals tend to look for electrons, causing damage to cells of other molecules that have lost their electrons. Electron uptake causes a free radical chain reaction that is increasing and tends to damage the macro-forming cells, such as proteins, carbohydrates, fats, and DNA. If the gen damage is not too serious, it can still be repaired with the body's immune response system [2]. However, if it cannot be repaired, it can cause cell division to be interrupted. There are even abnormal changes in certain genes in the body to cause cancer. Cancer in Indonesia in 2019 is estimated to increase by 30% [3]. Free radicals are from the rest of the body's metabolism, lack of fiber food, or from the outside environment (UV light, pollutants) [4]. Efforts to overcome cancer is from a source of free radical antioxidant compounds. There are two types of antioxidants made from

synthetic and natural ingredients. Natural antioxidants are obtained from extracts of natural ingredients, while synthetic ingredients are from chemical synthesis [5].

Most genetic changes cause abnormal cell cycles [6]. In normal cells, there is a balance between the proliferation of cell death in regulation through the cell cycle with cellular checkpoints. Before the cell enters the next phase in the cycle, it must go through a checkpoint that decides whether the previous process in this phase has been completed [7]. The cell cycle in the G1 phase is carried out by regulators by cyclin, cyclin-dependent kinase (CDK). Other proteins are CIP/KIP which include p21, p27, and p57 which are bound to the CDK cyclin complex [8].

Mangrove plants with characteristics that generally grow in areas on the coast of coral, coral covered with thin sand, muddy soil, or sandy soil, receive sufficient water supply from the ground, protected from large waves, and strong tidal currents [9]. Mangroves are not only beneficial to marine forest ecosystems and coastal areas, insecticides, and natural pesticides [10], but also been beneficial to health in recent years, known as advances in anticancer treatment technology because of the high polyisoprenoid in each mangrove species. The highest anticancer in the world are breast cancer and colon cancer. Thus, the researcher is interested in the p21 and Akt 2 gene expression in colon cancer (WiDr) in molecules using the RT-PCR method.

MATERIAL AND METHODS

Material

All chemicals and reagents were procured from certified suppliers and were of the highest analytical standard. The colon cancer cell (WiDr) was obtained from Laboratory of Parasitology, Universitas Gadjah Mada, Yogyakarta, Indonesia [11-12].

Sample Preparation and Extraction

Nypa fruticans, *Ceriops tagal* and *Rhizophora mucronata* leaves were collected from Lubuk Kertang mangrove forest, Langkat, North Sumatra Province, Indonesia, in February 2019. The leaves powder (500 g) of *N. fruticans, C. tagal* and *R. mucronata* were macerated with chloroform: methanol (2: 1, v/v) for 48 hours, as previously reported [13]. The lipid extract of the leaves was saponified at 65 ºC for 1 d in 86% ethanol containing 2 M KOH. The non-saponifiable lipids of both mangrove leaves were extracted with hexane, and the organic solvent was evaporated and re-dissolved in hexane [14]. The major polyisoprenoid compounds of both species were dolichol, and polyprenol was not detected.

An Analysis of Gene Expression p21 and Akt 2 in Vitro by RT-PCR

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 Rever Tra Ace (Toyobo) to produce a cDNA in a total volume of 20 µl for 10 min at 30 ºC, 60 min at 42 ºC, and 5 min at 99ºC according to manufacturer's procedure. The resulting cDNA mixture was diluted TE buffer and directly used for the subsequent PCRs.

Semi-quantitative reverse transcription-PCR (RT-PCR) for p21 and Akt 2 genes [15-19] was examined using 1 µL cDNA added to 25 µL PCR Master Mix (12.5 µL GoTax Green, 1 µL primer forward and 1 µL primer reverse as listed in Table 1, 9.5 µL DNase/RNase free water). Semi-quantitative RT-PCR was carried out with 35-40 cycles for 15-30 sec at 94ºC, 45 sec 94 ºC and for 10 sec at 55-60 ºC, with the final extension phase at 72 ºC for 5 min then stored at -20^{0}C [20]. 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) [20-21].

Table 1. Description for the RT-PCR primers

Genes		Sequence (5'-3')	Length (bp)
p21	F :	GCCAGATTTGTGGCTCACTTCG	300
	R :	ACGCTTGGCTCGGCTCTGG	
Akt 2	F :	ATGAATGAGGTGTCTGTCATCAAAGAAGGC	315
	R :	TGCTTGAGGCTGTTGGCGACC	
β-actin	F :	GCTCCTCCTAAGCGCGAGT	100
	R :	TCATACTCGCTGTCCTGCATTTG	

Statistical analysis

Data were statistically analyzed using one-way ANOVA for density base pair of p21 and Akt 2 genes, then completed with a Post Hoc Test consisting of the Tukey HSD test. The value of P <0.05 was chosen as the threshold of statistical significance.

RESULT AND DISCUSSION

The results of testing 3 samples of mangrove leaves n-hexane extract using RT-PCR method can be seen on the Figure 1

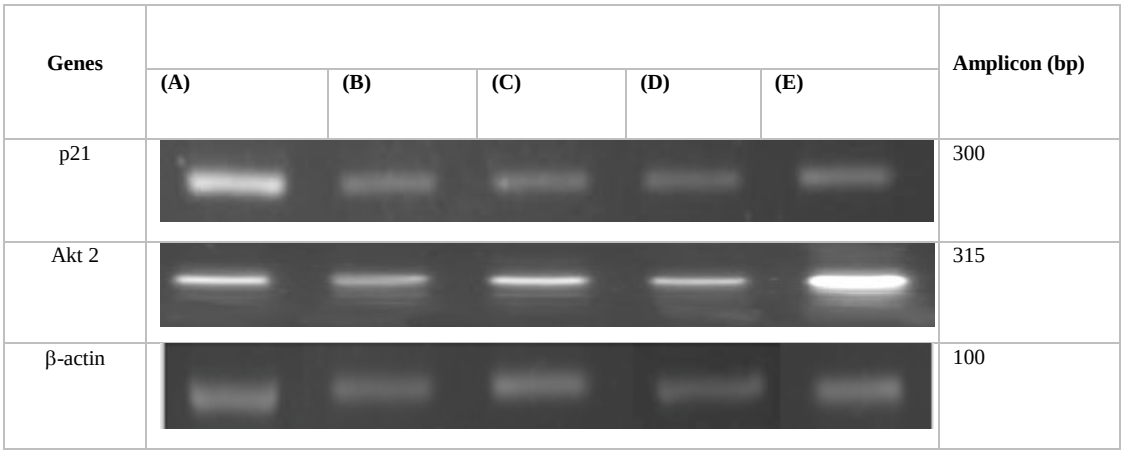

Figure 1. Gene expression of p21, Akt 2 and β-actin analyzed by RT-PCR method to the treatments of, A: Control cell, B: 5-FU, C: *Ceriops tagal*, D: *Rhizophora mucronata*, E: *Nypa fruticans*

Figure 1 shows the semi-quantitative expression of the p21, Akt 2, and β-actin genes of *Nypa fruticans*, *Ceriops tagal* and *Rhizophora mucronata* which were analyzed based on base pairs of each gene with RT-PCR. β-actin as housekeeping gene showed stability expression in cell control, 5-FU as positive control of *Nypa fruticans, Ceriops tagal*, and *Rhizophora mucronata* (Figure 1A-E). β-actin produced an amplification of 100 bp. Figure 1A illustrates the p21 expression band with 300 bp of PCR product (300 bp), in which 5-FU showed a clear band. On the other hand, Akt 2 was expressed (315 bp) in all the samples tested where the cell control accumulated small band intensities (Figure 1). The p21 gene as a tumor suppressor gene occurred in all samples with the highest band intensity in the *Ceriops tagal*.

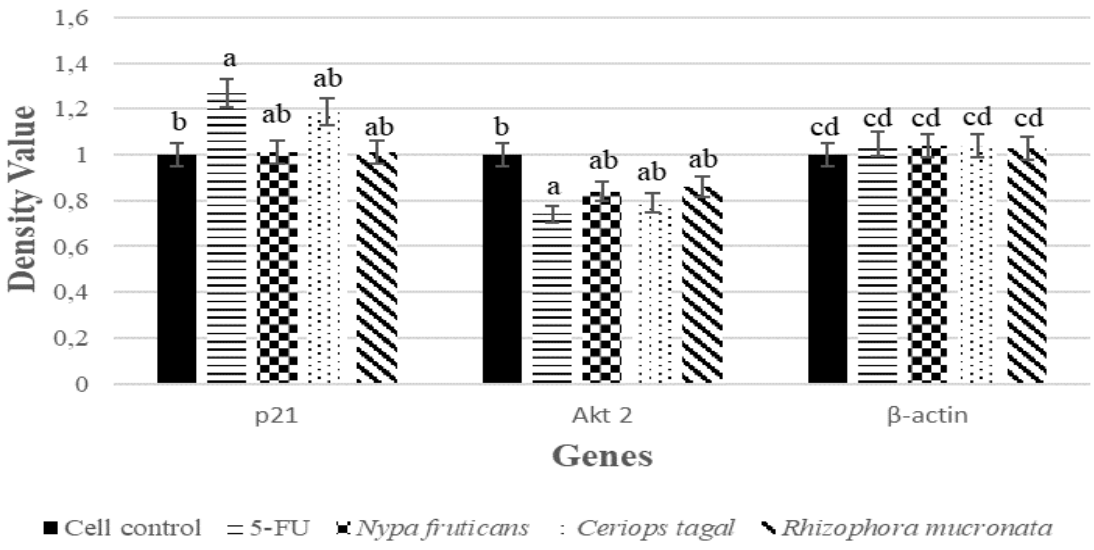

Figure 2. The density value of gene expression evaluated by one Way–Post Hoc Test, Tukey HSD.

a = Sig (*P*) <0.05, a statistically significant difference with control cell, b= Sig (*P*) <0.05 a statistically significant difference with 5-FU, c = Sig (*P*) >0.05 not a significant difference with cell control,
d = Sig (*P*) >0.05 not giving a significant with 5-FU

Figure 2 illustrates the up-regulation and down-regulation values of p21 and Akt 2 gene expression. As p21 is a tumor suppressor gene [22], it can do an action to prevent the growth of cancer cells and inhibit the development of colon cancer cells. The dolichol content of *Ceriops tagal* (100%) showed the most down-regulated results in the Akt 2 gene. In the case of p21, *Ceriops tagal* was the most effective of *Nypa fruticans* and *Rhizophora mucronata*. The value of gene

expression in Nypa fruticans, *Ceriops tagal* and *Rhizophora mucronata* on control cells had a significant difference with P <0.05.

An inverse correlation between p21 and p53 expression in colorectal adenocarcinoma has been introduced in a different series, in addition to the direct correlation between advanced disease stage and shorter patient survival with low or absent expression of p21 [23]. Many recent studies in colorectal carcinoma correlate with high levels of p27 protein with better results [24]. It has been shown that in colorectal carcinoma, loss of p27 protein is associated with shorter survival and development of metastasis [25]. In other large-scale studies, it was found that p21, p27, and p53 are tumor suppressor genes for cell growth [26].

p21 gene expression has been evaluated as a sign for the efficiency of wild-type of p53 transduction. p21 is a CDK inhibitor, which is the p53 downstream target. In transcriptional, it induces high p53 for DNA decay [27]. p21 acts as a negative regulator of growth during the G1 cell cycle checkpoint which is bound and inhibits cyclin / CDK [28]. The hypothesis that p21 is a better mark jet for p53 transduction for tumor cells expressing p53 mutant [29]. As a result, gene 21 expression induced is high both in vitro and in vivo [30].

CONCLUSION

This study confirms that the characteristics of *Ceriops tagal* leaves are more effective than *Nypa fruticans* and *Rhizophora mucronata* leaves. The dolichol content of *Ceriops tagal* provides up-regulation of p21 and down regulation of Akt 2. This study shows that dolichol in *Nypa fruticans*, *Ceriops tagal* and *Rhizophora mucronata* has blocked the growth factor of the colon cancer cell cycle (WiDr).

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