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Polyisoprenoids from *Avicennia marina* induces on P13k, Akt1, Mammalian target of rapamycin, Egfr, and P53 Gene Expression Using Reverse Transcription-Polymerase Chain Reaction

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Abstract

BACKGROUND: According to the Global Cancer Observatory in 2018, Asia was the first to note the incidence of colon cancer, which was 51.8% of cases of colon cancer which ranked the top three in the number of causes of death in the world. Cancer is a disease characterized by uncontrolled cell growth. Potential natural ingredient developed as chemotherapeutic agents includes from mangrove leaves. Studies reporting on the pharmacological activity of polyisoprenoid from mangrove species are still limited, therefore, it is essential to achieve the prospects, potential, and mechanisms polyisoprenoid in mangroves as a natural ingredient of pharmaceutical and medication.

AIM: The aim of the study was to investigate the inhibition activities of polyisoprenoids in mangrove plant *Avicennia marina* in WiDr cells induces on P13k, Akt1, mammalian target of rapamycin (mTOR), Egfr, and P53 gene expression using reverse transcription-polymerase chain reaction (RT-PCR).

MATERIAL AND METHODS: The leaves of *A. marina* were dried and extracted with n-hexane followed by evaporation and freeze-drying. Polyisoprenoid contents were analyzed with two-dimensional thin-layer chromatography method. Cell viability was assessed with 3-(4,5-dimetiltiazol-2-il)-2,5-diphenyl tetrazolium bromide assay. The cycle cell was tested with flow cytometry method. The apoptotic test was determined with a double-staining method. The gene expression on P13k, Akt1, mTOR, Egfr, and P53 was analyzed by RT-PCR method.

RESULTS: The results showed that 48 h cytotoxic activity of polyisoprenoids against WiDr cells and 5-Fu (positive control) had IC_{50} values, 295.25 μg/mL and 17.43 μg/mL. Cell cycle analysis depicted that the inhibition of polyisoprenoid occurred in the G0-G1 phase and 5-Fu in S phase. Polyisoprenoid and 5-Fu had the same mechanism in the early apoptotic phase. RT-PCR revealed that polyisoprenoids downregulated the P13k, Akt1, mTOR, and Egfr gene expression, and however, upregulated P53 gene expression.

CONCLUSION: The present study confirmed that polyisoprenoids from *A. marina* leaves showing as chemopreventive agents for colon cancer.

Introduction

According to the Global Cancer Observatory in 2018, Asia first in the incidence of colon cancer, which is 51.8% of cases of colon cancer ranked in the top three in the number of causes of death worldwide [1]. One of the risk factors for colon cancer is related to an unhealthy diet (intake). Low fiber intake and high fat will increase the risk of colon cancer [2]. The WHO data showed in 2014 that the number of men with colon cancer was 15,985 cases, while women were 11,787 cases. The high incidence of colorectal cancer occurred in Indonesia, the death rate of men in Indonesia due to colon cancer was 10.2% (103,100 deaths, while women were 8.5% with 92,000 deaths) [3]. Cancer is a disease characterized by uncontrolled cell growth. Cancer cells may avoid apoptosis and signals that suppress growth, the ability to form new blood vessels (angiogenesis), and the ability to invade and metastasize [4].

The use of 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, hair follicles, and lymphocyte tissue [4]. 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 medicines [5].

Potential natural ingredient developed as chemotherapeutic agents includes from mangrove

leaves. Mangrove vegetation defined as a plant or shrub distributed in intertidal zone of tropical and subtropical region [6]. Polyisoprenoid is secondary metabolites found in mangroves, classified as dolichol and polyprenol on mangrove leaves and roots [7], [8]. So far studies reporting pharmacological activity in polyisoprenoid of mangrove species are still limited, so it is important to achieve the prospects, potential, and mechanisms polyisoprenoid in mangroves as a natural ingredient of pharmaceutical and medication [7].

Recently, it has been shown that polyisoprenoid in *Nypa fruticans* induced the cancer cell cycle inhibition of adenocarcinoma of the colon (COLO 320 HSR cells, cell WiDr, and LS174 cells) in G2/M phase and reduce the percentage of Bcl-2 and Bcl-xL [8]. It has been also reported that polyisoprenoid of *Avicennia marina* and *Aerva lanata* leaves has anticancer colon activity. Polyisoprenoid of *A. marina* has 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 method [9].

This study, therefore, aimed to test of biological and pharmacological activities for the treatment of colon cancer from *A. marina* polyisoprenoid in terms of the cycle and gene expression of P53, Egfr, P13k, Akt1, and mammalian target of rapamycin (mTOR) using the reverse transcription-polymerase chain reaction (RT-PCR) method.

Materials and Methods

Plant material

Mangrove leaves of *A. marina* were collected the village of Lubuk Kertang, District West Brandan, Langkat, North Sumatra. The identification of *A. marina* has been confirmed by Herbarium Medanense and the voucher has been deposited.

Preparation of isolation polyisoprenoid alcohols

Powder simplicia mangrove leaves of *A. marina* (500 g) were macerated with a mixture of chloroform:methanol (2:1, v/v) for 48 h. Non-saponified lipid (NSL) extracts of leaves incubated of 65°C for 24 h in 86% ethanol containing KOH 2 M. NSL parts were further diluted with n-hexane and the solvent was evaporated. Then redissolved in n-hexane, a concentrated dried extract was obtained as previously reported [10].

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 were provided by the Laboratory of Parasitology collection, Faculty of Medicine, Gadjah Mada University, Yogyakarta, Indonesia. WiDr cell lines were cultured in RPMI 1640 medium, and supplement with 10% (v/v) fetal bovine serum, 1% penicillin and streptomycin, fungizone 0.5%, and in a 37°C incubator with 5% CO_2 [11].

Cytotoxic test

Cytotoxic test conducted on colon cancer cells WiDr in this study using the 3-(4,5-dimetiltiazol-2-il)-2,5-diphenyl tetrazolium bromide (MTT) method as previously reported [12]. WiDr colon cancer cells grown in microplate 96 wells to obtain a density of 1×10^4 cells/wells. The cell was incubated in a 5% CO_2 incubator temperature at 37°C for 48 h to obtain good growth. Once it replaced with a new medium was then added extract of the test sample of each series of concentration of 5-Fu as a positive control and incubated at 37°C in 5% CO_2 incubator at 37°C for 48 h. At the end of incubation, culture media, and the sample dumped then cells were washed with phosphate buffered saline (PBS). In each of the wells was added 100 mL of culture medium (RPMI) and 10 mL MTT concentration of 5 mg/mL. The cells were incubated back for 3–6 h in a 5% CO_2 incubator at 37°C. The reaction was stopped with 10% sodium dodecyl sulphate (SDS) reagent in HCl 0.01 N. Then, the disc of 96 wells was wrapped tightly and keeps one night at room temperature. ELISA reader was used to measure the absorption at a wavelength of 595 nm [13].

Cell cycle test by flow cytometry

Testing of the cell cycle was used the flow cytometry. Flow cytometry is a technique used to calculate and analyze the microscopic particles suspended in a fluid stream. The basic principle of this method is based on fluorescence. The particles were analyzed to be aspirated or discharged. The cell passed one by one through a focused laser beam surrounded by fluid stream. The laser beam attacked those cells [14]. Cells corresponding to the laser light and the right wavelength were emitted as fluorescence if the cell contained a natural substance one or more fluorochrome fluorescent-labeled antibody attached to the surface or internal structure of the cell. The light absorption depended on the internal structure, size, and shape of cells. Fluorescence light was detected by a series of diodes. The optical filter served to block unwanted light. Flow cytometry was used to analyze the DNA content of cells through the cell staining dye propidium iodide (PI) or 4',6'-diamino-2-phenylindole

(DAPI). With the fluorochrome that had the ability to interact DNA strand with bases such as PI, each cell had a different number of sets of chromosomes to give different fluorescence intensity. The more sets of chromosomes and the fluorescence intensity were higher. Apoptotic was tested by adding annexin V and PI while testing the cell cycle, PI was added. The extract was measured with a flow cytometer [14].

Apoptotic test by double staining

Apoptotic was detected by a double-staining method using acridine orange and ethidium bromide reagents. This method was based on DNA differences fluorescence in cells that live and die bonding acridine orange and ethidium bromide [14]. Acridine orange permeated all parts of the cell and the nucleus looked green. While ethidium bromide was intercalated with the cell membrane and the damaged nucleus was red. The color stained by ethidium bromide on dead cells more dominant compared to acridine orange so that the nucleus of dead cells was colored orange. Living cells with intact membranes had a nucleus with a uniform green color. During the process, the cells underwent apoptotic, and membrane blebbing started happening, ethidium bromide entered the cells and provided the orange color. Green fluorescence cells indicated the living cells and red fluorescence cells indicated the dead cells. Red fluorescence intact showed necrotic cells and cells that were fragmented indicated cells undergoing apoptotic. Apoptotic is programmed cell death that resulted in changes in the morphological and biochemical characteristics of cells. Stimulation of apoptotic included DNA damage, the presence of tumor necrosis factor, or absence of growth factors. Apoptotic was characterized by membrane blebbing presence without loss of membrane integrity, chromatin condensation and fragmentation, cytoplasmic compaction organelles, dilation of endoplasmic reticulum, cell volume reduction, and the formation of apoptotic bodies [15].

Analysis of gene expressions in vitro with RT-PCR

The expression of the genes was examined RT-PCR method [16]. The PCR component in 25 µL PCR Master Mix total contained 1 µL cDNA, GoTaxGreen 12.5 µL, 1 µL forward primer, 1 µL primer reverse, and 9.5 µL DNase/RNase free water. The RT-PCR was performed using iCycler (Bio-Rad) with 35 times cycle as follows: 30 s denaturation at 94°C, 30 s annealing at 48–65°C, and 30 s elongation at 72°C. The primers used for this RT-PCR was displayed in Table 1. RT-PCR product subjected to 2% agarose gel electrophoresis showed a single discrete-sized band as predicted [17].

Results and Discussion

Cytotoxic test

IC_{50} value obtained from polyisoprenoid in the leaves of mangrove species *A. marina* was 295.25 µg/mL with control positive of 5-Fu was 17.43 µg/mL (Table 2). Therefore, based on the flow and previous results on the IC_{50} value, n-hexane extract was used as samples to test anticancer. The relatively high IC_{50} value might be less active as anticancer because some extracts are considered to be active agent as of IC_{50} values ≤100 µg/mL [16]. However, an extract value of IC_{50} was 100–500 µg/mL which can be developed as anticancer with moderate classification [18]. It has been described that an extract had IC_{50} >500 µg/mL, also was considered active [19]. The major polyisoprenoids in *A. marina* leaves (95.8%) and roots (100%) were dolichol, few polyprenols in the leaves (4.2%) [10].

Table 1: Sequences of primer used in this study

Gene		Sequences	Amplicon
β-actin	R	5'-TCGTCATACTCCTGCTTGCTG AT -3'	105 Bp
	F	5'-GCT CCT CCT GAG CGC AAG T-3'	
Akt	R	5'-GAGGCCGTCAGCCACAGTCTGGATG-3'	240 Bp
	F	5'-ATGAGCGACGTGGCTATTGTGAAT- 3'	
P13k	R	5'-TGGTGGTGCTTTGATCTG-3'	349 Bp
	F	5'-GGACAATCGCCAATTCAG-3'	
mTOR	R	5'- AACAAACTCATGTCCGTTGCT G-3'	110 Bp
	F	5'- CCAATCATTCGCATTCAGTCC-3'	
P53	R	5'-CTGAGGTTGGCTCTGACTGTACCACCATCC-3'	360 Bp
	F	5'-CTCATTCAGCTCTCGGAACATCTCGAAGCG-3'	
Egfr	R	5'- CGCAACTTTGGGCGACTA T-3'	320 Bp
	F	5'-CAACATCTCCGAAAGCCA -3'	

mTOR: Mammalian target of rapamycin.

Apoptotic test

Based on the inhibition of cell cycle using PI reagent, the percentage of inhibition at each phase was obtained as shown in Figure 1 and Table 3. Table 3 shows the control group of WiDr cell accumulation in G0-G1 phase, S, and G2-M. Polyisoprenoid with concentration of 1/5 IC_{50} decreased the accumulation of cells in S phase and G2-M to 6.64% and 4.98%, respectively, but increased the G0-G1 phase (Table 3). The change phase might be related to the concentration. Polyisoprenoid in *A. alba* inhibited the WiDr cell cycle in the S phase [20]. However, it can be suggested that polyisoprenoid mechanism of inhibition of cell cycle occurred at G0-G1 phase. Table 3 shows 5-Fu with 1/5 concentration of IC_{50} decreased in the accumulation of WiDr cells in the G2-M phase 6.42%. By contrast, the increase in the cell accumulation occurred in the G0-G1 phase, and S is 88.12 and 9.52%, respectively. However, mechanisms of cell cycle inhibition of 5-Fu indicated in the G0-G1 phase and S. Treatment with 5-Fu in cancer cells led to the accumulation of cells at the G1 phase and the beginning of the synthesis phase

Table 2: IC_{50} calculation from polyisoprenoid against WiDr cells

IC_{50} (ug/mL)	
Species	WiDr cells (µg/mL)
Polyisoprenoid	295.25
5-Fu	17.43

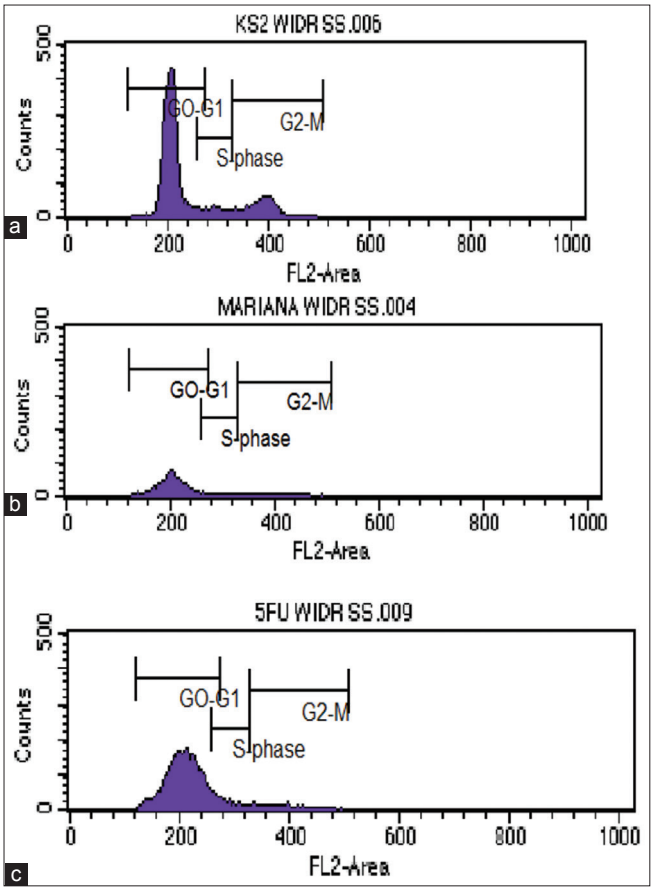

Figure 1: Percentage accumulated in each phase of the cell cycle WiDr after polyisoprenoid extract and 5-Fu as a positive control: (a) Control cell, (b) polyisoprenoid 1/5 IC_{50}, (c) 5-Fu 1/5 IC_{50}

(G1/S arrest)[21]. However, the cell cycle inhibitory activity by 5-Fu depended on the type of cancer cell [21].

Table 3: Percentage accumulated in each phase of the cell cycle

Treatment	Concentrations (µg/mL)	This phase of the cell cycle (%)		
		G1-G0	S	G2-M
Control cell	-	76.63	7.22	17.93
Polyisoprenoid	60	90.49	6.64	4.98
5-Fu	3.6	88.12	9.52	6.42

Cell control was observed in a fluorescence microscope; it appeared that cells produced 89% green fluorescence (Figure 1). In contrast to this observation, polyisoprenoid only occupied 45% fluorescence orange (dead cells), compared to the positive control (5-Fu). Polyisoprenoid produced 40% orange fluorescence indicated that polyisoprenoid had a position similar to the first line therapy cancer colon (5-Fu) (Figure 2).

Gene expression

RT-PCR is a single DNA synthesis method of using mRNA as template. DNA strand that has been formed to cDNA was then amplified by PCR [20]. In this test, WiDr cells treated with the test compounds polyisoprenoid with a concentration of 10 µM. Measurement of the expression of P13k, Akt1, mTOR, P53, and Egfr using RT-PCR method. Furthermore, the electrophoresis of the gene test results to produce band was depicted in Figure 4.

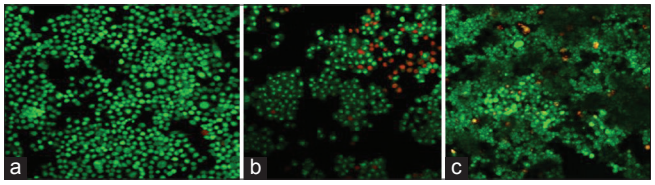

Figure 2: Observation of apoptotic in a fluorescence microscope with a magnification of 40× and 3× (a) control cell; (b) polyisoprenoid 1/5 IC_{50}; (c) 5-Fu 1/5 IC_{50}

The PCR amplification of selected genes along with internal standard of β-action was displayed in Figures 3-4. Polyisoprenoid and 5-Fu downregulated the expression of P13k. Activation of PI3k has been described in human cancer. This condition occurred due to amplification, overexpression, or of mutations in regulatory subunit P110 or P85. Amplification of 3q26 chromosomal region, which contained the gene which encodes the PIK3CA p110α catalytic subunit of PI3k, occurs in 40% of ovarian [22] and 50% of cervical carcinoma [23]. Somatic mutations of this gene have also been detected in several types of cancer and resulted in increased activity of PI3k kinase PI3k mutant relative to the wild type. P85 mutations in the regulatory subunit have also been detected [24], [25]. Since any changes in individual components resulted in the activation pathway, this study therefore suggested that activation pathway is one of the most common molecular changes in cancer.

The test results of gene expression were shown in Figure 3, polyisoprenoid and 5-Fu decreased the expression of Akt1. Akt1 gene provided instructions for making a protein called kinase Akt1. This protein is found in many types of cells throughout the body, where it plays an important role in many signaling pathways. For example, Akt1 kinase regulates the growth and cell division (proliferation), a process in which cells mature to perform specific functions (differentiation), and cell survival. Kinase Akt1 also help control apoptotic, which is the self-destruction of cells when to be damaged or no longer needed. Signaling involving kinase Akt1 appears to be important for the normal development and function of the nervous system. The present study supported the role of the kinase Akt1 to cell communication in the cell between nerve cells (neurons), neuron survival, and memory formation. Akt1 gene belongs to a class known as oncogenes. When mutated, oncogenes potentially cause normal cells to be cancerous [24].

Figure 4 shows that polyisoprenoid and 5-Fu decreased the expression mTOR. mTOR pathway is involved in the synthesis of proteins by regulating the intake of amino acids, tRNA, and translation initiation. 21 amino acids play a role in mTOR that affects the rate of protein synthesis. mTOR pathway is divided into two distinct protein complexes, namely, TORC1 and TORC2. TORC1 plays a critical role in determining cell size while TORC2 involves in regulating cell shape and actin cytoskeleton [26], [27].

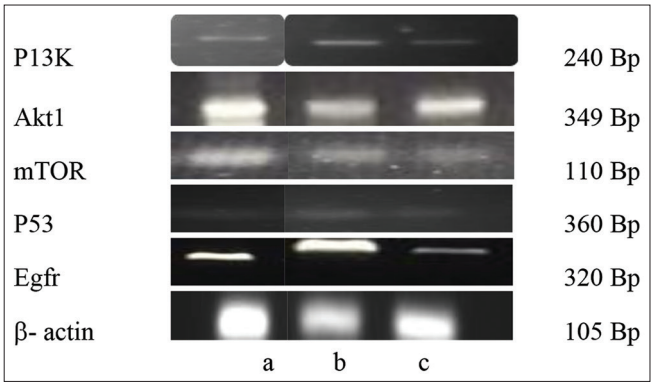

Figure 3: The amplicon of the genes of P13k, Akt1, mammalian target of rapamycin, p53, and Egfr (a) control cell; (b) polyisoprenoid (c) 5-Fu

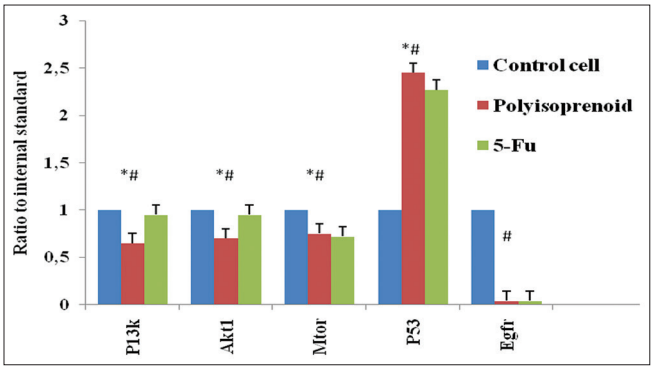

*Figure 4: Expression of the P13k, Akt1, mammalian target of rapamycin, p53, and Egfr genes against an internal standard of β-actin. Value are mean ± SE (n = 3) at p < 0.05 using Dunnett's test.*Significantly different from the normal control group (control cell). #Significantly different from the positive control group (5-fu)*

As depicted in Figure 4, polyisoprenoid and 5-Fu upregulated the expression P53. P53 is a tumor suppressor protein that acts as a cell cycle regulator. P53 protein plays an important role in response to cellular stress, such as exposure to carcinogens [28]. This protein would inhibit the proliferation of abnormal cells that have been initiated carcinogens to prevent the development of neoplasms. Protein inactivity can lead to cancer malignancy is malignant [29]. Besides functioning to regulate cell proliferation, p53 also regulates apoptosis, inhibits angiogenesis, and regulates DNA repair. In cancer commonly mutated p53 activity [30]. Most of the p53 mutation is the case which is missense mutation. Mutations can be in the form of degradation of p53, the loss of the ability of p53 to induce cell cycle arrest or apoptosis, and lose affinity binding of p53 to DNA damaged [31]. Saponins, flavonoids, polyphenols, and physalin [32] play a role in the inhibition of cancer cells. Saponin compounds inhibit the formation of Bcl-2 expressed too high, induces caspase-3 protein expressed is too low, increase the expression of p53, and may also trigger the G1 cell cycle arrest [33].

Figure 4 shows that polyisoprenoid and 5-Fu downregulated the expression of Egfr. Some reports indicate that an increase in copy number of Egfr genes or gene mutations that are responsible for signaling streams is an important determinant of response or resistance to anti-Egfr antibody [34]. This study analyzed the impact of KRAS mutations on the clinical activity of anti-Egfr targeted treatment. The underlying hypothesis is that most of KRAS mutations cause a gain of function activates Ras pathways/MAPK. As the activated signal transduction at the level of KRAS proteins, inhibition upstream by the Egfr-targeted agents becomes ineffective [34].

β-actin was widely used as an internal control in the analysis of gene expression because it is a housekeeping gene, the gene continuously expressed for a living organism. Housekeeping genes have stable expression levels in various tissues during development stage [35].

Table 4 summarizes the gene expression ratio to internal standard from polyisoprenoid extract. Polyisoprenoid suppressed the expression of P13k, Akt1, mTOR, P53, and Egfr with the lowest P13k gene expression ratio value. This value shows that the effect was significantly different from cell control and 5-Fu, while Akt1 gene expression ratio in polyisoprenoid which showed significantly different from cell control and 5-Fu. Furthermore, the smallest on mTOR gene expression ratio value in polyisoprenoid (0.75 ± 0.008) which showed significantly different from cell control and 5-Fu. The P53 gene expression ratio value was in polyisoprenoid (2.45 ± 0.01) which showed that the effect was significantly different from cell control and 5-Fu (Table 4).

Table 4: Gene expression ratio of polyisoprenoid extract

Gene	Treatment groups	Ratio standard ± SE
P13k	Control cell	1 ± 0.00^{bc}
	Polyisoprenoid	0.65 ± 0.057^{ac}
	5-Fu	0.95 ± 0.008^{ab}
Akt1	Control cell	1 ± 0.00^{bc}
	Polyisoprenoid	0.70 ± 0.011^{ac}
	5-Fu	0.95 ± 0.005^{ab}
mTOR	Control cell	1 ± 0.00^{bc}
	Polyisoprenoid	0.75 ± 0.008^{ac}
	5-Fu	0.72 ± 0.005^{bc}
P53	Control cell	1 ± 0.00^{bc}
	Polyisoprenoid	2.45 ± 0.01^{ac}
	5-Fu	2.27 ± 0.01^{ab}
Egfr	Control cell	1 ± 0.00^{bc}
	Polyisoprenoid	0.04 ± 0.01^{c}
	5-Fu	0.04 ± 0.01^{b}

[a]Significantly different with the normal group (control cell) p<0.05, [b]p<0.05, there is a significant difference with the polyisoprenoid group, [c]p<0.05 was no significant difference in 5-Fu group. mTOR: Mammalian target of rapamycin.

The lowest expression of Egfr was in polyisoprenoid which showed that the effect was not significantly different from that of 5-fu but significantly was different from cell control (Table 4). The polyisoprenoid and 5-Fu downregulated gene expression of P13k, Akt, mTOR, and Egfr, by contrast upregulated P53 gene expression.

Conclusion

Polyisoprenoid from *A. marina* inhibited the cell cycle cancer phase G0-G1 phase and phase S. Polyisoprenoid and 5-Fu improved WiDr cell apoptotic in the early phase of apoptotic with a double-staining method. Polyisoprenoids downregulated the P13k,

Akt1, mTOR, and Egfr gene expression, and however, upregulated P53 gene expression. The present study confirmed that polyisoprenoids from *A. marina* leaves showing as chemopreventive agents for colon cancer.

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