Anticancer Effects of Polyisoprenoid From *Nypa fruticans* Leaves by Controlling Expression of p53, EGFR, PI3K, AKT1, and mTOR Genes in Colon Cancer (WiDr) Cells

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Abstract

The current research seeks to examine the anticancer effect of polyisoprenoids from mangrove palm, *Nypa fruticans*, leaves in WiDr cells by analyzing the cell cycles of cancer and regulating the expression of p53, epidermal growth factor receptor (EGFR), PI3K, AKT1, and mammalian target of rapamycin (mTOR) genes by using the reverse transcription-polymerase chain reaction (RT-PCR). An inhibited cell cycle analysis was conducted using the flow cytometry, and the upregulation or downregulation of the expression of p53, EGFR, PI3K, AKT1, and mTOR genes was obtained using RT-PCR. The data were then statistically analyzed using one-way analysis of variance by a post hoc test, a parametric statistical analysis using Tukey’s honest significant difference. Polyisoprenoids in *N. fruticans* extracts worked as chemotherapeutic in the G0-G1 cycle is 79.0%, however, with positive control 5-fluorouracil as 88.1% and are carried out by the specific upregulation of the expression of the p53 gene and the downregulation of the expression of the EGFR, PI3K, AKT1, and mTOR genes. This study can also explain the significant pharmacological properties of the leaves of the species *N. fruticans* that work specifically in the G0-G1 phase to upregulate the expression of the p53 gene and downregulate the expression of the EGFR, PI3K, AKT1, and mTOR genes. This study also revealed polyisoprenoid (100% dolichol), which blocked the growth and development of WiDr colon cancer cells.

Keywords

polyisoprenoid, anticancer, *Nypa fruticans*, up and downregulation

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Cancer is the leading cause of death in the world in which colorectal cancer rank third in 2018.¹ Furthermore, more than 70% of deaths due to cancer occur in low-income and middle-income countries, and the percentage of patients diagnosed with cancer is escalating.¹ Risk factors for cancer include tobacco use, alcohol, obesity, environmental hazard viruses, and ionizing radiation.³ Cancer is a disease with a growth phase faster than that of normal cells. Here, uncontrolled cells form lumps known as tumors or occur in blood vessels (leukemia) or lymph nodes (lymphoma).³ 5-Fluorouracil (5-FU) is most commonly used to treat colon cancer; however, in the long term, it can have toxic effects or resistance to effectiveness.⁴

*Nypa fruticans*, a member of mangrove palm, is known to produce a variety of secondary metabolites such as lignin, hemicellulose, phenolic, flavonoid, and polyisoprenoids.⁵-⁷ Polyisoprenoids are long-chain secondary metabolites (>C50) found in almost all living cells, including various plant tissues.⁵-⁷ Furthermore, polyisoprenoid comprise of polyalcohol and dolichol, which are efficacious in anticancer and antibacterial activities.¹⁰-¹² The polyisoprenoid composition in *N. fruticans* has been reported to have 100% dolichol.⁷ Moreover, our previous screening study on cytotoxic and...
antiproliferative activity of polyisoprenoids in 17 mangroves found *N. fruticans* to exhibit the highest anticancer activity, with half-maximal inhibitor concentration (IC$_{50}$) at 180.2 μg/mL, on WiDr cells through the inhibition of cyclooxygenase-2. This study is an extension of our previous work to analyze the anticancer effects against gene expressions that oppose the proliferation of colon cancer cells, namely, p53, epidermal growth factor receptor (EGFR), PI3K, AKT1, and mammalian target of rapamycin (mTOR) pathway in colon cancer cells (WiDr) using reverse transcription-polymerase chain reaction (RT-PCR) in various inhibitions of growth, development of cancer cells, and development of molecular biology for the treatment of toxic chemotherapy.

**Materials and Methods**

**Plant and Chemical Materials**

*Nypa fruticans* Wurmb (Arecaaceae) leaves were collected from Lubuk Kertang mangrove forest, Langkat, North Sumatra, Indonesia in February 2019. The mangrove forest is situated at 04°07’39.71” North latitudes and 98°30’97.87” East longitudes. The plant material has been identified in the Indonesia Institute of Science, Research Center for Biology, Bogor, Indonesia. A voucher specimen has been deposited in the herbarium. Chloroform, methanol, hexane, ethanol, HCl and KOH were obtained from Merck (Germany). Prediluted serum blocking, streptavidin horse enzyme peroxidase, 3,3′-diaminobenzidine, Mayer solution-hematoxylin, xylol, Roswell Park Memorial Institute (RPMI) 1640 medium, dimethylsulfoxide (DMSO), [3-(4,5-dimethylimidazole-2-yI)−2,5-diphenyl tetrazolium bromide], phosphate buffer saline (PBS) and sodium dodecyl sulfate were purchased from Sigma-Aldrich (USA). PI3K, AKT1, mTOR, p53, and EGFR genes were used. Regarding the condition of the colon cancer cell culture (WiDr), the human colon cancer cells were extracted from the large intestine of a 78-year-old woman and later isolated. WiDr was cultured in RPMI 1640 and supplemented with 10% (v/v) fetal bovine serum, 1% penicillin and streptomycin, and 0.5% fungizone were inserted inside an incubator at 37°C with 5% CO$_2$.2

**Sample Preparation**

*Nypa fruticans* leaves powder (500 g) was homogenized and macerated with solvent reagent chloroform:methanol (2:1, v/v) for 48 hours, and this treatment procedure was borrowed from the previous work. The lipid extract of the *N. fruticans* leaves was saponified at 65°C for 24 hours in 86% ethanol comprising of 2 M KOH. The unsaponifiable lipids of mangrove palm leaves were extracted with hexane, and the organic solvent evaporated and redissolved in hexane. Leaf extracts (50 μg) were applied to the thin layer chromatography plate to identify the polyisoprenoid composition. The polyisoprenoid compound in *N. fruticans* leaves contained the dolichol family 100% (C75-C90) with quantities of 10.7 mg/g dolichol.

**Preparation of Stock Solution**

Respectively, Fifty mg of polyisoprenoids from *N. fruticans* was dissolved in 10 mL of RPMI 1640 medium with the aid of 1 mL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) to obtain a concentration of 5000 μg/mL.

**Inhibition Analysis of Cell Cycles with Flow Cytometry**

The WiDr cells (5 × 10$^3$ cells/well) were inserted inside a 6-well plate and incubated. Subsequently, the cells were treated with various concentrations of polyisoprenoid in *N. fruticans* and then incubated again. The floated cells found attached were collected by using 0.025% trypsin and then transferred into the cone tube. Then, 1 mL of PBS was added, after which PBS was extracted using a micropipette and centrifuged at 2500 rpm for 5 min. The liquid was released, and the RNase/propidium iodide (PI) dye solution added for 10 min in a dark place (avoiding light) at 37°C and then analyzed using the FACScan flow cytometer so that the percentage of cells obtained in each cell cycle phase (G1/S and G2/M) could be calculated using Modfit Lx. 3.0s.

**Analysis of Gene Expressions In Vitro with RT-PCR**

The total ribonucleic acid (RNA) used (3000 ng) was reverse transcribed with a 1 μg random primer and ReverTra Ace (Toyoobo) to yield complementary deoxyribonucleic acid (cDNA) (20 μL) for 10 min at 30°C, 60 min at 42°C, and 5 min at 99°C as per the manufacturer's protocol. The resulting cDNA mixture was diluted in a TE buffer solution and directly used for the subsequent PCRs. Semiquantitative RT-PCR for p53, EGFR, PI3K, AKT1, and mTOR genes was determined using 1 μL cDNA added to the 25 μL PCR Master Mix (12.5 μL GoTaqGreen, 9.5 μL DNase/RNase free water, 1 μL primer forward and 1 μL primer reverse, as displayed in Table 1). Subsequently, 8 μL of the mixed solution (5x RT-buffer 4 μL, random primary 1 μL, deoxyribonucleoside triphosphate 2 μL and Revers Tra-Ace 1 μL) was added, PCR was performed at 30°C for 10 min, 42°C for 60 min, and 99°C for 5 min. The RT-PCR product was obtained in the form of cDNA and stored at −20°C. The expression of the gene was examined using 1 μL cDNA added to 25 μL PCR Master Mix with 35-40 cycles carried out for 15-30 s at 94°C, 45 s at 94°C, and 10 s at 55-60°C with the final extension phase at 72°C for 5 min; the PCR products were then stored at 4°C.

The bands were documented using the image scanner. Testing of activity avoidance from the gene p53, EGFR, PI3K/AKT1/mTOR was carried out...
by amplifying cDNA in the WiDr cells with a test solution, after which the amplified cDNA results were added using electrophoresis on agarose 2% with medium 10% TBE.23

Statistical Analysis
The data comprised density base pairs of p53, EGFR, PI3K, AKT1, and mTOR genes that were first analyzed statistically using one-way analysis of variance and then with a post hoc test consisting of the Tukey’s honest significant difference (HSD) test, significant at $P < 0.05$ as the threshold.

Results

Inhibition Analysis of Cell Cycles with Flow Cytometry
The results of the cell cycle inhibition analysis using flow cytometry testing were carried out at 488 nm and medium speed (500 cells/s) light emission with PI reagents.24 Figure 1 and Table 2 show that polyisoprenoids in the n-hexane extraction of N. fruticans worked as a chemotherapeutic in the G0/G1 phase of the cell cycle is 79.0%. Furthermore, cell accumulation in G0/G1 phase in N. fruticans was found to be greater with the control cell at 76.6% but higher in 5-FU (88.1%) as displayed in Table 2.25 A higher percentage of positive controls indicated the presence of accelerated cells in the preparation of DNA material to be synthesized.

The cell cycles were examined using flow cytometry on WiDr cells using the staining method.26 PI was used to dye the DNA so that it could be detected by the fluorescence-activated cell sorting detector.

Gene Expression with RT-PCR
The measurement of expression of the genes p53, EGFR, PI3K/AKT1/mTOR was carried out by amplifying the cDNA WiDr cells given a test solution. Subsequently, the amplified cDNA results were separated using electrophoresis on agarose 2% with medium tris-borate-ethylenediaminetetraacetic acid (TBE) 10% and an electric current of 70 mv. The results of WiDr cell electrophoresis are displayed in Table 3. The bands of EGFR and PI3K are more expressed than the control group (Table 3).

The electrophoresis results obtained were analyzed using the Quantity One software (Bio-Rad) to acquire the density of bands in the order in which they appeared, after which the average and standard deviations were calculated. The results of the thickness of the density of the gene expression p53, EGFR, PI3K/AKT1/mTOR are shown in Table 4, where N. fruticans polyisoprenoid upregulated the p53 expression and, in contrast, polyisoprenoid downregulated the expression of EGFR, PI3K, AKT1, and mTOR genes. It is notable that EGFR and PI3K gene expressions showed significant difference either with control cell or 5-FU as depicted in Table 4.26 Figure 2 shows the ratio of p53, EGFR, PI3K, Akt1, and mTOR genes to the internal standard ($\beta$-actin) evaluated using a one-way post hoc test and Tukey’s HSD test with significant difference with the control cell and 5-FU as depicted in Table 4.26

Figure 2 shows the ratio of p53, EGFR, PI3K, Akt1, and mTOR genes to the internal standard ($\beta$-actin) evaluated using a one-way post hoc test and Tukey’s HSD test with significant difference with the control cell and 5-FU as depicted in Table 4.26

Discussion
This article expresses the ability of N. fruticans polyisoprenoids to inhibit the expression of EGFR, PI3K, AKT1, and mTOR genes in WiDr cells as a therapeutic option in the treatment of colon cancer.27 Moreover, p53 can be utilized in the apoptosis process and cell cycle of WiDr. In this context, PI3K/AKT1/mTOR is a pathway in the process of developing cancer cells. This pathway facilitates the process of cell division and deactivates the apoptosis process.28 Furthermore, the activity of the PI3K/AKT1/mTOR genes that decrease indirectly can cause cell cycle inhibition events and trigger the occurrence of apoptosis so that the development of cancer cells is inhibited. Our
previous study showed that polyisoprenoids (from *N. fruticans* leaves) worked in the WiDr colon cancer cell line. It has been compared with more than 60% of apoptosis at 90 µg/mL; G0-G1 cell cycle arrest did not appear in this study. We used different normal cells (control cells) and positive control compared with the present study. In the present work, no difference percentage accumulated in the G0-G1 cell cycle arrest as shown in Table 2.

![Cell cycle with flow cytometry](image)

**Figure 1.** Cell cycle with flow cytometry. (a) Control cell, (b) *Nypa fruticans*, and (c) 5-fluorouracil (5-FU, positive control).

| Sample         | Concentration (µg/mL) | G0-G1 (%) | S (%) | G2-M (%) |
|----------------|-----------------------|-----------|-------|----------|
| Control cell   | -                     | 76.6      | 7.2   | 16.2     |
| *N. fruticans* | 1/5 IC₅₀              | 79.0      | 7.0   | 14.0     |
| 5-Fluorouracil | 1/5 IC₅₀              | 88.1      | 9.1   | 2.8      |

IC₅₀, half-maximal inhibitory concentration.
Additionally, EGFR has become an important target for cancer therapy. Some reports indicate that an increase in the number of copies of EGFR or gene mutations responsible for stream signaling is important to determine the response or resistance to anti-EGFR antibodies. Furthermore, the excessive activation of the PI3K pathway causes an increase in PIP2 to PIP3. This results in the formation of phosphoinositide-dependent protein kinase-1 phosphorylation and protein kinase B (PKB) phosphorylation so that PKB can become active. This protein is a central mediator in the signal transduction from the PI3K pathway and can also phosphorylate other intracellular proteins that play a role in cell cycle regulation, cell proliferation, DNA repair systems, and apoptosis. To avoid geroconversion, cancer cells need to lose the expression of cell inhibitor cycles, such as p53. Thus, between p53 and the mTOR signaling pathway, cells running into apoptosis can be determined. Further, inhibitors from mTOR can suppress geroconversion and protect stem cells from undergoing temporary premature cell aging while preventing oncogenic transformation.

Moreover, 5-FU can inhibit cell cycles and apoptotic triggering without involving p53; however, it entails the increased expression of p21 and pRb, both of which play an important role in the checkpoint system in the G1 phase. Furthermore, the overexpression of pRb will inhibit E2F activity so that it causes the inhibition of cells to exceed R. The p21 expression will also inhibit the activity of cyclin E/CDK2 and cyclin A/CDK2 and can thus inhibit the cell cycle in the G1 and S phase. Cells that are in the G1 phase will stop there, and cells in the S phase will be stopped at that phase. Resistance caused by 5-FU can occur through the mediation of cell cycle inhibition. Cancer cells with mutant p21 cannot spur the cessation of cell cycle so that it can directly accelerate apoptosis; however, cells with normal p21 that spur the cessation of the cell cycle can trigger the emergence of resistant cells. 5-FU activity in apoptotic tracking can be through the p53 pathway (dependent or independent p53). Additionally, it has also been proven that 5-FU can induce apoptosis in cancer cells with p53 deficiency or have mutant p53.

The messenger RNA (mRNA) can be used to describe the expression of a gene because the amount can vary according to the environmental conditions compared with DNA, which is relatively stable. RNA is a biological macromolecule with a number of different functions, and mRNA transcribed from DNA serves as a template for protein synthesis, which is carried out by ribosomes consisting of ribosomal RNA (rRNA) and protein. Amino acids for protein synthesis are sent to ribosomes by transferring the RNA (tRNA) molecules. Most of these RNA molecules consist of tRNA (15%-20%) and rRNA (80%-85%).

| Table 3. Gene Expression of P53, EGFR, PI3K, AKT1, mTOR, β-Actin on the Treatments of WiDr Cell Line (A) Control Cell, (B) 5-FU, and (C) Nypa fruticans. |
|-----------------|------------------|------------------|------------------|
| Gene | Treatment | Amplicon (bp) |
|------|-----------|----------------|
| p53  | (A) 390   |                |
| EGFR | (B) 495   |                |
| PI3K | (C) 195   |                |
| AKT1 |           |                |
| mTOR |           |                |
| β-actin |        |                |

| Table 4. Density Expression of Genes P53, EGFR, PI3K, AKT1, and mTOR in WiDr Cells. |
| No | Genes | Treatment | Density pair ± standard deviation |
|----|-------|-----------|----------------------------------|
| 1  | p53   | Control cell | 1.0 ± 0.0<sup>a</sup> |
|    |       | N. fruticans 1/5 IC<sub>50</sub> | 1.2 ± 0.0<sup>a</sup> |
|    |       | 5-FU 1/5 IC<sub>50</sub> | 1.3 ± 0.0<sup>a</sup> |
| 2  | EGFR  | Control cell | 1.0 ± 0.0<sup>b</sup> |
|    |       | N. fruticans 1/5 IC<sub>50</sub> | 0.4 ± 0.0<sup>ab</sup> |
|    |       | 5-FU 1/5 IC<sub>50</sub> | 0.7 ± 0.0<sup>a</sup> |
| 3  | PI3K  | Control cell | 1.0 ± 0.0<sup>b</sup> |
|    |       | N. fruticans 1/5 IC<sub>50</sub> | 0.4 ± 0.0<sup>ab</sup> |
|    |       | 5-FU 1/5 IC<sub>50</sub> | 0.1 ± 0.0<sup>a</sup> |
| 4  | Akt1  | Control cell | 1.0 ± 0.0<sup>b</sup> |
|    |       | N. fruticans 1/5 IC<sub>50</sub> | 0.3 ± 0.0<sup>a</sup> |
|    |       | 5-FU 1/5 IC<sub>50</sub> | 0.2 ± 0.0<sup>a</sup> |
| 5  | mTOR  | Control cell | 1.0 ± 0.0<sup>b</sup> |
|    |       | N. fruticans 1/5 IC<sub>50</sub> | 0.3 ± 0.0<sup>a</sup> |
|    |       | 5-FU 1/5 IC<sub>50</sub> | 0.2 ± 0.0<sup>a</sup> |
| 6  | β-actin | Control cell | 1.0 ± 0.0<sup>b</sup> |
|    |       | N. fruticans 1/5 IC<sub>50</sub> | 1.1 ± 0.0<sup>a</sup> |
|    |       | 5-FU 1/5 IC<sub>50</sub> | 1.1 ± 0.0<sup>a</sup> |

IC<sub>50</sub>, half-maximal inhibitory concentration; 5-FU, 5-fluorouracil.
<sup>a</sup>Sig (P) < 0.05; there is a significant difference with control cell.
<sup>b</sup>Sig (P) < 0.05; there is a significant difference with 5-FU.
However, mRNA constitutes only 1%-5% of the total RNA despite the actual amount depending on the type and physiological condition of the cell.38

The phase in the normal cycle in the number of chromosome sets, namely G1, and the number of sets of chromosomes is 2n.39 The S phase is 2n and 4n while the G2 and M phases are 4n.40 The number of the sets of chromosomes determines the intensity of the fluorescence measured in the flow cytometry tool.41 The more sets of chromosomes, the greater the intensity of fluorescence.42,43 PI3K/AKT1/mTOR is the pathway process for developing cancer cells and facilitates the processes of cell division and apoptosis.44 The activity of the PI3K/AKT1/mTOR gene, which decreases directly, does not facilitate cell cycle inhibition and improvement of apoptosis so that the development of cancer cells is hampered.45 The inhibition of displaying the PI3K/AKT1/mTOR gene by \textit{N. fruticans} is inseparable from the composition of the secondary metabolites (polyisoprenoid) in the extract. The mixture of sesquiterpene lactones, such as vernodalol, vernodalinol, and vernomygdin, has been shown to inhibit phosphorylation of PI3K/AKT1 in breast cancer cells.46,47 To support these results, in vivo experiments on polyisoprenoids compounds as anticancer agents are needed to gain a complete understanding of the colon cancer mechanism in mangrove plants.

**Supplemental Material**

All data analyzed in this study are included in this published article.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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**Figure 2.** The density value of gene expression p53, EGFR, PI3K, Akt1, and mTOR ratio to internal standard (β-actin). (a) Sig (P) <0.05, a statistically significant difference with the control cell; (b) sig (P) < 0.05, a statistically significant difference with 5-fluorouracil (5-FU).
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