



## RESEARCH ARTICLE

**REVISED** Effects of polyisoprenoids from *Avicennia lanata* and *Avicennia alba* leaves on the gene expression of PI3K, Akt1, mTOR, P53, and EGFR in human colorectal adenocarcinoma WiDr cells [version 3; peer review: 1 approved, 1 not approved]

Effects of polyisoprenoids from *Avicennia lanata* and *Avicennia alba* leaves on the gene expression of PI3K, Akt1, mTOR, P53, and EGFR in human colorectal adenocarcinoma WiDr cells using reverse transcription PCR

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### Abstract

**Background:** Mangrove plants produce polyisoprenoid compounds. Polyisoprenoids have been proven to have anticancer properties. This study investigated the inhibitory activity of polyisoprenoids derived from the leaves of mangrove plants *Avicennia alba* and *Avicennia lanata* regarding the expression of PI3K, Akt1, mTOR, P53, and EGFR genes against human colorectal adenocarcinoma WiDr cells.

**Methods:** Anticancer activity was tested through the MTT assay method performed on WiDr cells. The cell cycle and apoptosis were analysed by flow cytometry and double staining. Gene expression of PI3K, Akt1, mTOR, P53, and EGFR was observed using the RT-PCR method.

**Results:** Cytotoxic activity against WiDr cells showed that the IC<sub>50</sub> for *A. alba* and *A. lanata* was 258.14 ug/mL and 243.32 ug/mL, respectively. This observation indicated the possibility to develop moderate anticancer agents. The cell cycle showed that inhibition of *A. alba* and *A. lanata* occurred in the late phase of apoptosis S (10.60 and

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1. **Ewa Swiezewska** , Polish Academy of Sciences, Warsaw, Poland

10.51%) and G2-M1 (22.05 and 23.84%), which was higher than negative and positive control cells. Furthermore, the polyisoprenoids derived from *A. alba* and *A. lanata* leaves exhibited anticancer activity in WiDr cells through the downregulated gene expression of PI3K, Akt1, mTOR, and EGFR as well as the upregulated gene expression of P53.


**Conclusion:** This study demonstrated that polyisoprenoids obtained from *A. alba* and *A. lanata* leaves are promising chemopreventive agents for colon cancer.

### Keywords

Avicennia, cytotoxic, polyisoprenoids, colon cancer, mangrove

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**REVISED Amendments from Version 2**

We have revised in the part of Results and Discussion by adding new references no. 19, 33, 34 to incorporated Reviewers' suggestions and to support our results.

**Any further responses from the reviewers can be found at the end of the article**

## Introduction

Cancer is a disease characterised by uncontrolled cell growth. Cancer cells can evade apoptosis and avoid signals that suppress its growth, impede the ability to form new blood vessels (angiogenesis), and spread its invasion and metastasis<sup>1</sup>. According to the Global Cancer Observatory, in 2018, Asia had the highest incidence of colon cancer with 51.8% of the global cases. Colon cancer is one of the top three causes of death in the world<sup>1</sup>. The use of chemotherapeutic agents constitutes a treatment for colon cancer, in addition to surgery and radiation therapy. Chemotherapeutic agents generally suppress the growth or proliferation of cancer cells, simultaneously causing toxicity in the body<sup>2</sup>.

Natural substances developed as potential chemotherapeutic agents include components of mangrove leaves. Mangroves are vegetation formations found in littoral areas in tropics and subtropics<sup>3</sup>. Polyisoprenoids are secondary metabolites found in several mangroves, distributed as dolichol and polyprenol on the leaves and roots of mangrove plants<sup>4</sup>. So far, few studies have reported the pharmacological activity of polyisoprenoids obtained from mangrove species. Thus, it is essential to study the potential and mechanisms of polyisoprenoids in mangroves as a natural ingredient for anticancer pharmaceuticals and medication<sup>4</sup>. For instance, methanol extracts of *Avicennia alba* (bark and leaves) present anti-proliferative activity in human breast cancer cell lines MCF-7 and T47D<sup>5</sup>. Additionally, this extract has cytotoxic effects on a variety of cancer cells, including colon cancer cells – HT-29<sup>6</sup>. Previous research suggests that polyisoprenoids induce cancer cell cycle inhibition in adenocarcinoma of the colon (COLO 320 HSR, WiDr, and LS174 cells) in the G2-M phase and reduce the percentage of Bcl-2 and Bcl-xL<sup>7</sup>. Polyisoprenoids have been previously reported as chemopreventive agents for colon cancer<sup>8</sup>, given that polyisoprenoids in *A. lanata* leaves have displayed anticancer activity for the same<sup>9</sup>. On the other hand, *A. alba* contains polyisoprenoids that induce cell cycle, apoptosis, and gene expression of cyclooxygenase-2 (COX-2) in colon cancer cells WiDr<sup>10</sup>. Polyisoprenoids have a mechanism to inhibit the cell cycle at the G0-G1 phase, and apoptotic analysis occurs in the early phase of apoptosis in WiDr cells<sup>9,10</sup>.

The present study analysed the effect of immune-related genes' expression on WiDr cells *in vitro* using reverse transcription-polymerase chain reaction (RT-PCR). RT-PCR was developed as an *in vitro* test to measure the biological activity of plasmid DNA-based products (pDNA). The said test measures RNA-specific transgenic messengers (mRNA) derived from

transfected cultured cells. Forward and reverse primers have been designed to trigger selective RT-PCR reactions for plasmid mRNAs<sup>11</sup>. Therefore, the present study aims to investigate the inhibitory activity of polyisoprenoids obtained from the leaves of mangrove plants *A. alba* and *A. lanata* concerning the expression of PI3K, Akt1, mTOR, P53, and EGFR genes in human colorectal adenocarcinoma WiDr cells. The selection of the particular pro-apoptotic genes analysed, PI3K, Akt1, mTOR, and EGFR as the target of cancer therapy in the process of proliferation and induction of apoptosis, whereas the prevention of cancer formation involves a mechanism on the tumour suppressor protein p53.

## Methods

### Plant material

The leaves of two mangrove species – *A. alba* and *A. lanata* – were collected from the village of Lubuk Kertang, Brandan Barat, Langkat, North Sumatra, Indonesia. The sample site is situated at 04° 07' 39.71" North latitude and at 98° 30' 97.87" East longitude.

### Preparation of isolation polyisoprenoid alcohols

Five hundreds g of powdered mangrove leaves of *A. alba* and *A. lanata* was macerated with a mixture of chloroform/methanol (2:1, v/v) (CM21) for 48 h. Precipitate insoluble in CM21 was removed by filtration paper (Advantec, Japan) and the extract was partially purified as lipid extract. The lipid extracts of leaves were refluxed at a temperature of 65°C for 24 h in 86% ethanol containing KOH 2 M. The unsaponifiable lipid partitioned into 2 mg/mL n-hexane. The extract in n-hexane was concentrated using rotary evaporator at 40°C. Subsequently, a thick extract was obtained and the concentration was adjusted to 1 mg/mL n-hexane.

The unsonifiable lipid (50–100 mg) leaf extracts were analysed by silica gel 60 thin layer chromatography (TLC) and RP-18 high performance thin layer chromatography (HPTLC) plates (Merck) to identify the polyisoprenoid composition<sup>4,12,13</sup>. The polyisoprenoid standards were generously provided by Dr. Ewa Swiezewska (Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland). The quantity of polyisoprenoids in *A. alba* leaves was 5.5±0.8 mg/g dry weight and *A. lanata* leaves was 14.9±1.2 mg/g dry weight. Data are represented as the means ± SEM ( $n=3$ ). The polyisoprenoid compounds in the *A. alba* (PAA) and *A. lanata* (PAL) leaves were detected to be 100% dolichol family with chain length of C60–C100 and C70–C100 in two dimensional TLC chromatogram (2D TLC), respectively<sup>4</sup>. No polyprenol was found in both mangrove leaves. The 2D TLC of both samples was performed triplicates and showed identical pattern, we confirmed that two mangrove leaves extracts contained 100% dolichols, therefore it is not required to purify the polyisoprenoid samples and used for further investigation.

### WiDr and Vero cell culture

WiDr cells (isolated human colon cancer cells) and normal cells (Vero ATCC® CCL-81™, an immortalized cell line, derived from the kidney of an African green monkey) were kindly

provided by the Laboratory of Parasitology Collection, Faculty of Medicine, Gadjah Mada University (Yogyakarta, Indonesia). The WiDr cell lines were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (RPMI powder sachet contained 2 mM L-glutamine without  $\text{NaHCO}_3$ , 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid),  $\text{NaHCO}_3$ , 1 N HCl, 1 N NaOH, sterile water) (Sigma Aldrich, Singapore) and the Vero cell lines were nurtured in M199 (M199 powder sachet with Earle's salt and 2mM L-glutamine without  $\text{NaHCO}_3$ , 20 mM HEPES, 1 N HCl, 1 N NaOH, sterile water) (Gibco, USA). Both cells were supplemented with 10% (v/v) foetal bovine serum (FBS) (Gibco), 1% penicillin and streptomycin (Gibco), and 0.5% fungizone (amphotericin B) in a 37°C incubator with 5%  $\text{CO}_2$ <sup>11</sup>.

### Administration of the leaf extracts to the cells

Unsaponifiable lipid of PAA and PAL were weighed 5 mg each in a microtube, and 50  $\mu\text{M}$  of 5-fluorouracil (5-FU) were dissolved in a 100  $\mu\text{L}$  dimethyl sulfoxide (DMSO: 0.05 %) co-solvent and vortexed for 10 min. The test solution was serially diluted with a concentration of 1000  $\mu\text{g}/\text{mL}$ , 500  $\mu\text{g}/\text{mL}$ , 250  $\mu\text{g}/\text{mL}$ , 125  $\mu\text{g}/\text{mL}$ , and 62.5  $\mu\text{g}/\text{mL}$ . All sample dilution processes was carried out using the RPMI culture media supplemented with 10% (v/v) FBS, 2% penicillin-streptomycin, 0.5% amphotericin B (fungizone) for the WiDr cell test solution and M199 culture media (M199 medium, 10% FBS, 3% penicillin-streptomycin, and 1% amphotericin B (fungizone)) for Vero cell test.

### Cytotoxicity analysis

Cytotoxicity tests were conducted on the WiDr and Vero cells using the MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) method. Cells were grown in 96 well microplates to obtain a density of  $1 \times 10^4$  cells/well and incubated in a 5%  $\text{CO}_2$  incubator at a temperature of 37°C for 48 h to ensure good growth. Once the new medium had been replaced, the cells were exposed with serially diluted concentrations of PAA and PAL (1000, 500, 250, 125, and 62.5  $\mu\text{g}/\text{mL}$ ) in the cell cycle analysis as previously reported<sup>10</sup>. 5-FU (Sigma Aldrich) was used as a positive control with the same concentration of PAA and PAL and incubated in 5%  $\text{CO}_2$  at 37°C for 48 h. At the end of the incubation, the culture media was removed, and the cells were washed with PBS. In each of the wells, 100  $\mu\text{L}$  of culture medium (RPMI) and 10  $\mu\text{L}$  MTT (Sigma Aldrich) were added. The cells were incubated again for 3–6 h in 5%  $\text{CO}_2$  at 37°C. The reaction was stopped with 10% SDS reagent (Sigma Aldrich) in 0.01 N HCl (Merck). The plate was wrapped to protect it from the light so that the wells were opaque, and it was left overnight at room temperature<sup>14</sup>. Absorption was measured by the ELISA reader (Benchmark 10431, BioRad) at a wavelength of 595 nm.

Selectivity index (SI) was determined from the  $\text{IC}_{50}$  of the polyisoprenoid extract from PAA and PAL leaves in Vero cells versus WiDr cells to exhibit the cytotoxic selectivity of the polyisoprenoid extract, as previously reported<sup>9</sup>.  $\text{IC}_{50}$  was calculated from concentrations that caused death among 50% of the cell population analysed using probit analysis in SPSS version 23 with a significance of 0.05<sup>8</sup>.

### Cell cycle analysis

The WiDr cells ( $5 \times 10^3$  cells/well) were added to a 6-well plate which was incubated for 24 h for optimal growth. Subsequently, the cells were exposed to selected concentrations of PAL and PAA (1/5  $\text{IC}_{50}$ ) and incubated again<sup>10</sup>. Floating as well as attached cells were collected by adding 0.025% trypsin. The cells from each well were transferred to a separate eppendorf tube. 1 mL PBS was added and the PBS was removed with a micropipette and centrifuged at 2500 rpm for 5 min. The supernatant was removed and 1  $\mu\text{L}$  RNase/PI staining solution (Thermo Fisher Scientific) was added and kept for 10 min a dark place (avoiding light) at 37°C. The cell cycle distribution was analysed using the FAC Scan Flow Cytometer (BD Biosciences) and the percentage of cells obtained in each cell cycle phase (G1-S and G2-M) was calculated using the software ModFit LT. 3.0 s for Windows (Verity Software House).

### Apoptosis analysis

The double staining method was used to determine the level of apoptosis. WiDr cells were grown in a 6-well microplate at density of  $5 \times 10^5$  cells/well and incubated for 24 h. The following day, the cells were treated with concentrations of PAA and PAL (1/5  $\text{IC}_{50}$ ) and incubated for 24 h. The procedure for apoptosis with double staining was conducted by taking cells from the  $\text{CO}_2$  incubator and observing the conditions. Then, the calculated cells were used to prepare 24-well plates and slipcovers. 200  $\mu\text{L}$  of cell suspension was evenly and slowly transferred just above the coverslip. The cell was kept in the incubator for 3–30 min to attach to the coverslip with 800  $\mu\text{L}$  of culture media which was added for 48 h of incubation. The culture media was slowly disposed, and the cells were washed with PBS (500  $\mu\text{L}$ ). The sample and media were added into the well for control cells and then incubated. All media from the well was slowly removed using a Pasteur pipette. Cells in the wells were washed with PBS. The coverslip was removed using tweezers, placed on a glass slide, and labelled. 10  $\mu\text{L}$  of the reagent mixture of ethidium bromide acridine orange (Sigma Aldrich) was added over the slipcover. The mixture was flattened and gently rocked. Apoptosis was observed under a fluorescence microscope (Olympus CKX41)<sup>9</sup>. ImageRaster 4.0.5 (Miconos, Yogyakarta, Indonesia) was used to count green and red fluorescence signals of three microscopic snapshots of individual experiments. The fluorescent green cells were alive and the fluorescent red cells were dead<sup>15</sup>. Red fluorescent intact cells indicated necrotic cells while fragmented cells indicated apoptotic cells.

### Gene expression analysis

Total RNA was extracted from the WiDr cells treated with PAA and PAL ( $7.5 \times 10^8$  cells/well) using the Total RNA Mini Kit (Geneaid), according to the manufacturer's protocol. The total RNA (0.3  $\mu\text{g}$  each) was reverse-transcribed with 1  $\mu\text{g}$  random primer to produce cDNA in a total volume of 20  $\mu\text{L}$  using ReverTra Ace kit (Toyobo) with 10 mM dNTP to incubate for 10 min at 30°C, for 60 min at 42°C, and for 5 min at 99°C according to manufacturer's procedure. The resulting cDNA mixture was diluted using 100  $\mu\text{L}$  TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0) and directly used for the subsequent PCR.

Semi-quantitative RT-PCR for genes p53, EGFR, PI3K, Akt1, and mTOR<sup>16-21</sup> were assessed using 1  $\mu$ L cDNA added to 25  $\mu$ L PCR Master Mix which contained 12.5  $\mu$ L GoTaqGreen, 1  $\mu$ L primer forward, and 1  $\mu$ L primer reverse (as listed in Table 1), and 9.5  $\mu$ L DNase/RNase free water. 35–40 cycles of semi-quantitative RT-PCR (ProFlex PCR system, Thermo Fisher Scientific) were conducted under the following cycling conditions: 15–30 sec at 94°C, 45 sec at 94°C, and 10 sec at 55–60°C, with the final extension phase at 72°C for 5 min and then storage at -20°C<sup>22</sup>. Semi-quantitative RT-PCR products were observed using 2% agarose gel and stained with ethidium bromide. The bands were documented using the image scanner Doc XR Gel (Bio-Rad)<sup>23</sup>. Each data represents the average of three independent RT-PCR measurements with standard errors of individual experiments. To quantify the PCR product, Quantity One® 1-D analysis software (Bio-Rad) used to assess bands intensity of genes analysed.  $\beta$ -actin was reference gene to normalize the PCR efficiency.

### Statistical analysis

All the data were analysed using SPSS version 23, followed by Duncan's multiple range test for treatment comparisons. The data are presented as mean  $\pm$  standard error of the mean (SEM).

One-way variance analysis (ANOVA) was used to compare the results for different conditions.  $P < 0.05$  was considered significantly different.

## Results and discussion

### Cell cytotoxicity of PAA and PAL

The cytotoxicity test is a preliminary parameter to determine the potential toxicity of a test substance, particularly cancer cells. The toxicity is expressed by  $IC_{50}$  parameters. However, the cytotoxic test can also be performed to assess the toxicity of a test substance on normal cells. So, it can be used to demonstrate selective cytotoxic effects against a cancer line. In this study, the cytotoxic test material was derived from polyisoprenoids of mangrove leaves (PAA and PAL) against colon cancer cells (WiDr) with a concentration series of 1000, 500, 250, 125, and 62.5  $\mu$ g/mL. The purpose of testing the extract was obtaining the smallest  $IC_{50}$  value for subsequent use as an advanced test for anticancer activity. The results showed that the smallest  $IC_{50}$  value obtained from the polyisoprenoids of leaves from PAL was 243.32  $\mu$ g/mL and from PAA was 258.14  $\mu$ g/mL. Therefore, these concentrations were used in the remaining experiments of the study. The cytotoxic effects were indicated by absorbance values and analysed using a probit analysis to obtain  $IC_{50}$ , as shown in Table 2.

**Table 1. Primer sequences used in the present study.**

Gene		Sequences	Amplicon (bp)
Akt1	R	5'-GAG GCC AGC CAC GTC AGT CTG GAT G-3'	240
	F	5'-ATG ATT GCT AGC GTG GTG GAC AAT-3'	
PI3K	R	5'-TGC TGG TGG TTT CTG GAT-3'	349
	F	5'-CCA GGA ATT TCG CAG CAA-3'	
mTOR	R	5'-AAC AAA CTC GTT GCT TCC ATG G-3'	110
	F	5'-CCA ATT CAG ATC CGC ATT TCC-3'	
P53	R	5'-GCT TTG CTG CTG AGG CCA CCA GTA TCC ACT-3'	360
	F	5'-ATT CAG CTC CTC CTC CAT GAA GAA TCG GCG-3'	
EGFR	R	5'-CGC CGA AAC TTT CTA GGG T-3'	320
	F	5'-CGA CAA CAT AAG CTC CCA-3'	
$\beta$ -actin	R	5'-TCA TAC TCG CTG TCC TGC AT TTG-3'	100
	F	5'- GCT CCT CCT AAG CGC GAG T- 3'	

**Table 2.  $IC_{50}$  values of WiDr cells treated with two n-hexane extracts of mangrove leaves for 48 h. SI: selectivity index.**

	Mangrove species	WiDr cells ( $\mu$ g /mL)	Vero cells ( $\mu$ g /mL)	SI
$IC_{50}$ ( $\mu$ g/mL)	<i>A. lanata</i>	243.32 $\pm$ 2.64 <sup>b</sup>	147.36 $\pm$ 1.67 <sup>b</sup>	0.61 $\pm$ 0.00 <sup>b</sup>
	<i>A. alba</i>	258.14 $\pm$ 3.84 <sup>a</sup>	176.24 $\pm$ 2.05 <sup>a</sup>	0.68 $\pm$ 0.02 <sup>b</sup>
	5-Fu (positive control)	17.43 $\pm$ 0.19 <sup>c</sup>	45.15 $\pm$ 1.71 <sup>c</sup>	2.59 $\pm$ 0.11 <sup>a</sup>

Data are represented as the mean  $\pm$  SEM. Means with the same superscript are not significantly different for each other ( $P < 0.005$  followed by Duncan's test)

The greatest cytotoxic activity against WiDr cells, shown by the smallest  $IC_{50}$  value, was obtained from PAL. Therefore, PAL has the most active anticancer activity because the  $IC_{50}$  value showed that PAL could block 50% of the WiDr cell growth. Some extracts are only considered active if they have  $IC_{50}$  values  $\leq 100 \mu\text{g/mL}$ <sup>16</sup>. However, it has been demonstrated that an extract value of  $IC_{50}$  of 100–500  $\mu\text{g/mL}$  can be classified as moderate and, therefore, can potentially be developed as an anticancer agent<sup>17</sup>. Even though a study has reported that an extract is considered active if  $IC_{50} > 500 \mu\text{g/mL}$ <sup>18</sup>. As shown in Table 2, the SI of PAL and PAA were lower than that of the positive control. The cytotoxicity of PAL and PAA in the present study included an interesting SI against WiDr cells in a dose-dependent manner<sup>17</sup>, suggesting the higher cytotoxic selectivity (i.e. safety) of polyisoprenoid extracts against normal than cancer cells. The previous study has reported that mangrove genus of *Avicennia* have cytotoxicity effects against several cancer cell lines (HL-60, HepG2, NCI-H23) utilize MTT assay method<sup>19</sup>.

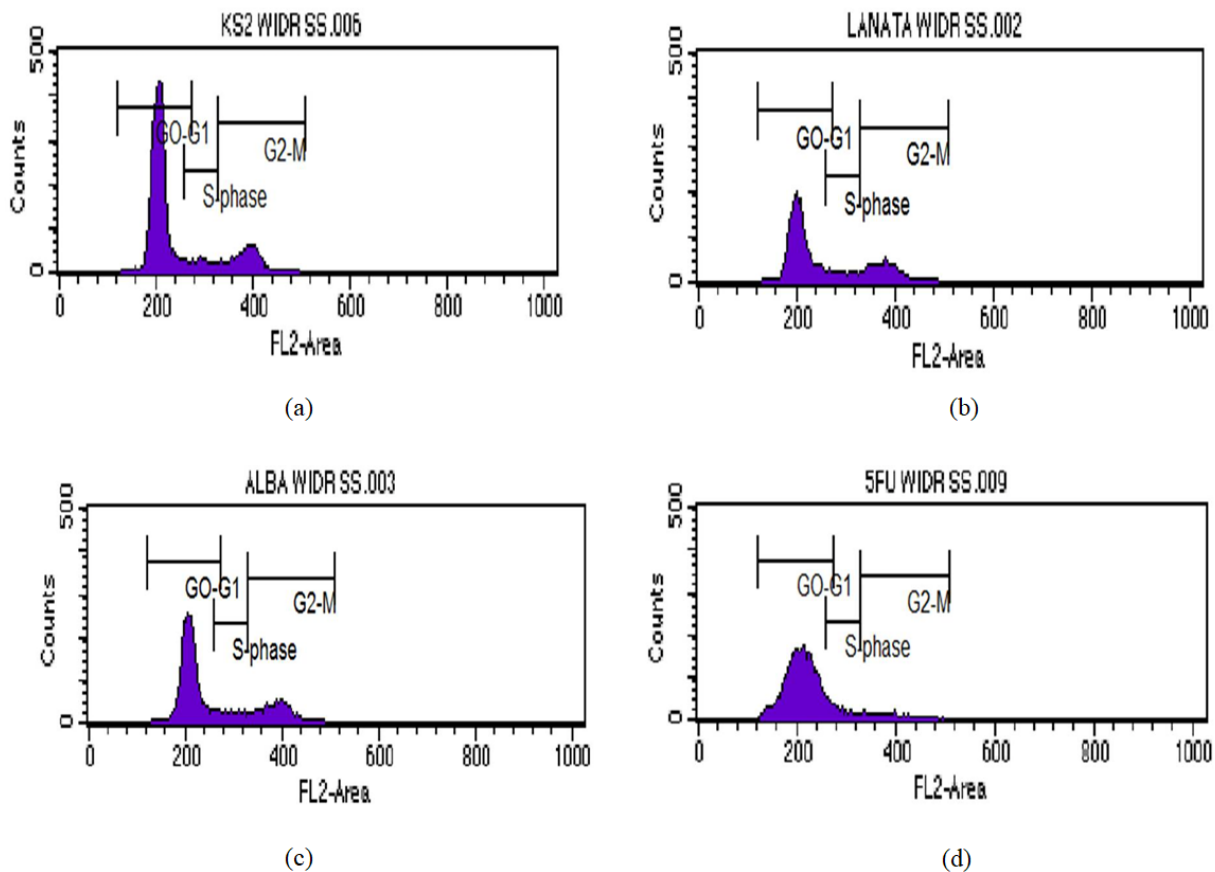
### Cell cycle analysis with PAA and PAL

A previous study showed that methanol and water extracts of *A. alba* leaves have distinctive properties in regulators and

mediators of cancer<sup>20</sup>. This study tested the cell cycle using flow cytometry to determine the distribution of cells in each phase of the cell cycle at sub G1, S, and G2-M after treatment and obtained predictable pathway inhibition using PAA and PAL to inhibit the cycle cell<sup>21</sup>.

The inhibition of the cell cycle in this study is shown in Figure 1 and Table 3. Table 3 shows the control group's WiDr cell accumulation in the G0-G1, S, and G2-M phase as 76.63%, 7.22%, and 17.93%, respectively. The accumulation of cells in the S phase and G2-M cells increased by 10.60%, 10.51% and 23.84%, and 22.05%, respectively after being administered with a concentration of PAL  $1/5 IC_{50}$  and PAA with the concentration of  $1/5 IC_{50}$ . The phase change is considered related to the concentration. However, the overall mechanism of inhibition of the cell cycle for PAA and PAL occurred at S and G2-M phases.

As Table 3 illustrates, the administration of 5-FU with  $1/5$  concentration  $IC_{50}$  decreases the accumulation of WiDr cells in the G2-M phase at 6.42%. The increase in cell accumulation occurred in the G0-G1 phase – S was 88.12 and 9.52%. However, it can be confirmed that the overall mechanisms of



**Figure 1.** Cell cycle analysis showing the percentage accumulated in each phase of the cell cycle WiDr after the administration of the extract of mangrove leaves and 5-FU as a positive control: (a) Control cell; (b) PAL  $1/5 IC_{50}$ ; (c) PAA  $1/5 IC_{50}$ ; and (d) 5-FU  $1/5 IC_{50}$ .

cell cycle inhibition of 5-FU (in the G0-G1 and S phases) had a different mechanism compared with PAL and PAA. Treatment of cancer cells with 5-FU can accumulate cells at the G1 phase and at the beginning of the synthesis phase (G1-S arrest)<sup>22</sup>. However, the cell cycle's inhibitory activity using 5-FU depends on the type of cancer cell. In colon cancer cells HCT-15 and HT-29, 5-FU inhibited at the G2-M phase. 5-FU increases the expression of cyclin A, cyclin B, and CDC2, which

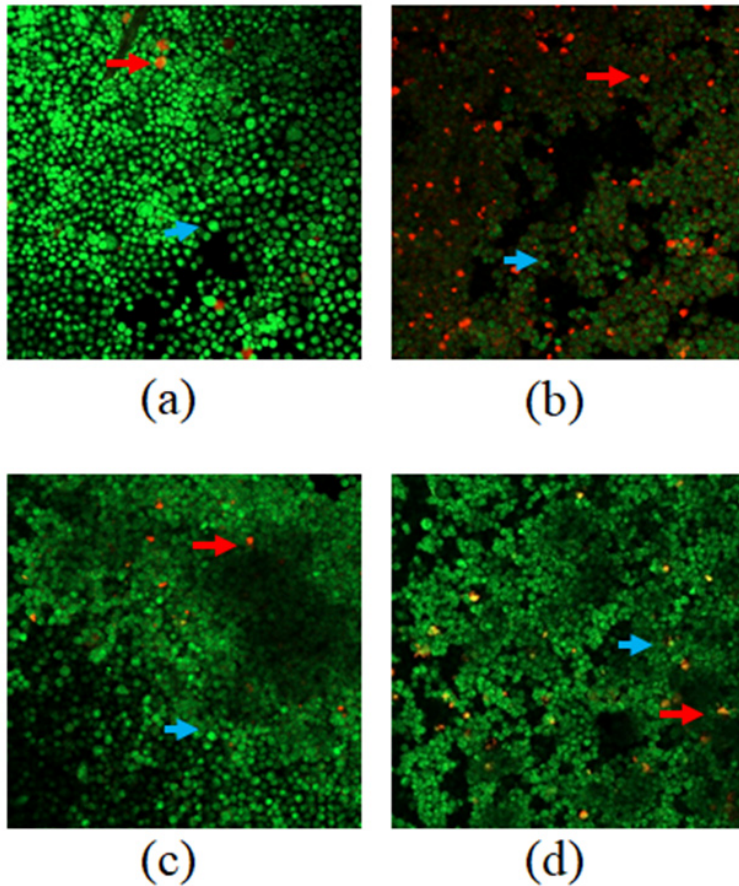
is a regulatory protein in the G2-M phase<sup>23</sup>. The mechanism that mediates the activity in this phase needs to be explored further. In Lovo and WiDr cells, 5-FU inhibits the cell cycle in the S phase<sup>18</sup>. This suggests that the activity of 5-FU is not always associated with thymidylate synthase inhibitory activity, and the activity of 5-FU in the cell cycle if used in a different cell needs to be researched further.

**Apoptosis analysis with PAA and PAL**

In the present study, increased apoptosis (seen using the reagent acridine orange-ethidium bromide through the fluorescence microscope) was obtained by a percentage increase in each of the phases. Apoptosis is generally characterised by different morphological characteristics and biochemical mechanisms that depend on energy<sup>24</sup>. Apoptosis usually occurs during development and aging and as a homeostatic mechanism to maintain the population of cells in the network. It also is a defence mechanism when cells are damaged by disease or harmful agents or during immune reaction<sup>25</sup>. Cytotoxic data test samples indicate the presence of a cytotoxic effect as shown in Figure 2. The green cells are the live ones while the red ones are dead. The range of red fluorescent cells represents the necrotic cells.

**Table 3. Accumulated values in each phase of the cell cycle of WiDr cells treated with of mangrove leaves *Avicennia alba* (PAA) and *Avicennia lanata* (PAL)**

Treatment	Concentrations (µg/mL)	Phase of the cell cycle (%)		
		G0-G1	S	G2-M
Control cell	-	76.63	7.22	17.93
PAL 1/5 IC <sub>50</sub>	50	68.70	10.60	23.84
PAA 1/5 IC <sub>50</sub>	52	70.39	10.51	22.05
5-FU 1/5 IC <sub>50</sub>	3.6	88.12	9.52	6.42



**Figure 2. Observation of apoptotic cells in a fluorescence microscope with a magnification of 40x and 3x. (a) Control cell; (b) PAL1/5 IC<sub>50</sub>; (c) PAA1/5 IC<sub>50</sub>; and (d) 5-FU1/5 IC<sub>50</sub>. death cells (apoptotic) → living cells →**

The present analysis used ImageRaster to count the dead cells and control cells after observing them under a fluorescence microscope. In average, 96.21% green colour (living cells) and 3.79% red colour (dead cells) were observed in control cells with three independent experiments. With the PAL 1/5 IC<sub>50</sub> treatment, the cells produce 65.66% green colour (living cells) and 34.34% red (dead cells). With PAA 1/5 IC<sub>50</sub> treatment, cells produce 72.42% green colour (living cells) and nearly 27.58% red colour (dead cell). With 5-FU 1/5 IC<sub>50</sub> treatment, the cells produce 82.46% green (live cells) and 17.53% red colour (dead cells). There was no statistically significant difference among the treatments (Figure 2).

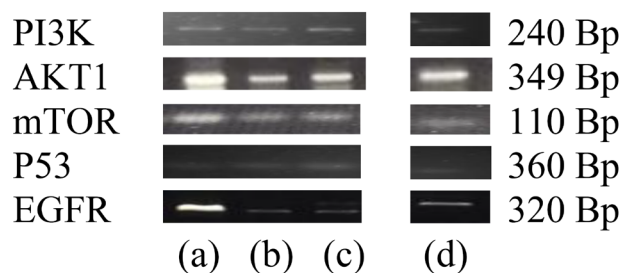
The results of the control cells were observed to be green/alive cells. The green colour comes from the orange acridine penetrating the entire living cell with intact membranes and nuclei. In cells treated with PAL and PAA, there was a predominantly red colour which illustrates that the WiDr cells were dead. The orange colour is produced by ethidium bromide interacting with damaged cell membranes and nuclei<sup>26</sup>. The test results showed that the extract can inhibit the growth of cancer cells, especially in WiDr cancer cells. The inhibition capability through the mechanism of apoptosis can also be evidenced through testing and analysis of double staining flow cytometry. The results of both analyses can illustrate the mechanism of cell death caused by apoptosis both quantitatively and qualitatively. Thus, the induction of apoptosis shows that these treatments are a promising treatment for cancer. The cancer cells undergo apoptosis and lose their ability to proliferate rapidly. This way of treatment may induce usual apoptotic signalling, thereby, potentially eliminating the cancer cells<sup>27</sup>.

The potential working mechanism of PAL and PAA are in the late phase of apoptosis. The potency of PAL and PAA in triggering apoptosis may be caused by compound isoprenoids (based on the results of phytochemical screening of PAL and PAA). Steroids/triterpenoids are compounds that have high anticancer activity, by blocking nuclear factor-kappa B, inducing apoptosis, and activating transcription and angiogenesis, which can be useful in the treatment of various types of cancer<sup>28</sup>.

#### Expression of PI3K, Akt1, mTOR, P53, and EGFR genes

The measurement of the expression of PI3K, Akt1, mTOR, P53, and EGFR genes using RT-PCR produces the band illustrated in Figure 3. Gene expression level was quantified using a computerised system (Table 4). Table 4 shows significant differences between the treatment groups. Gene expression results on PI3K, Akt1, mTOR, P53, and EGFR differed significantly ( $p < 0.05$ ).

In this study, RT-PCR showed that the anti-apoptotic gene expression of P53 increases compared to control, whereas the expression of pro-apoptotic genes (PI3K, Akt1, mTOR, and EGFR) tend to decrease. P53 is a tumour-suppressing protein that can affect the permeability of the mitochondrial membrane and directly induce apoptosis without inducing the transcription of the target gene associated with apoptosis in advance<sup>29</sup>. This condition causes apoptosis when the gene Bax mRNA



**Figure 3. Gene expression of PI3K, AKT, mTOR, P53 and EGFR.** (a) control cell; (b) PAL; (c) PAA; (d) 5-FU.

**Table 4. Gene expression of n-hexane extract of mangrove leaves *Avicennia alba* (PAA) and *Avicennia lanata* (PAL) in WiDr cells.**

No	Gene	Treatment group	Expression level average value $\pm$ SEM
1	PI3K	Control cell	1 $\pm$ 0.00 <sup>bcd</sup>
		PAL	0.76 $\pm$ 0.01 <sup>acd</sup>
		PAA	0.85 $\pm$ 0.01 <sup>abd</sup>
		5-FU	0.95 $\pm$ 0.01 <sup>abc</sup>
2	Akt1	Control cell	1 $\pm$ 0.00 <sup>bcd</sup>
		PAL	0.54 $\pm$ 0.01 <sup>acd</sup>
		PAA	0.86 $\pm$ 0.01 <sup>abd</sup>
		5-FU	0.95 $\pm$ 0.01 <sup>abc</sup>
3	mTOR	Control cell	1 $\pm$ 0.00 <sup>bcd</sup>
		PAL	0.84 $\pm$ 0.01 <sup>acd</sup>
		PAA	0.76 $\pm$ 0.01 <sup>abd</sup>
		5-FU	0.72 $\pm$ 0.01 <sup>abc</sup>
4	P53	Control cell	1 $\pm$ 0.00 <sup>bcd</sup>
		PAL	3.03 $\pm$ 0.01 <sup>acd</sup>
		PAA	2.94 $\pm$ 0.01 <sup>abd</sup>
		5-FU	2.27 $\pm$ 0.01 <sup>abc</sup>
5	EGFR	Control cell	1 $\pm$ 0.00 <sup>bcd</sup>
		PAL	0.19 $\pm$ 0.01 <sup>acd</sup>
		PAA	0.11 $\pm$ 0.01 <sup>abd</sup>
		5-FU	0.04 $\pm$ 0.01 <sup>abc</sup>

A statistically significant followed by Duncan's test

a. (P) < 0.05: There is a significant difference with the normal group (control cell).

b. Sig (P) < 0.05: There is a significant difference with the group PAL.

c. Sig (P) < 0.05: There is a significant difference with the group PAA.

d. Sig (P) < 0.05: There was no significant difference in the positive control group (5-FU).

expression does not increase and, thus, the pathway of apoptosis by P53 has two paths to the mitochondria – directly and indirectly through the activation of the transcription of genes under it. P53 molecule is a tumour-suppressing protein found at a low level under normal conditions and has a short life span. P53 is activated when the cells are exposed to stimuli such as agents that cause DNA damage, hypoxia, lack of nucleotide, or tumour cell activation. As a tumour suppressor, P53 protects the genome and regulates the growth and proliferation of the critical points in response to stress. The P53 molecule is an upstream regulator of the cell cycle as well as the intrinsic apoptotic pathway mediated by the Bcl-2 protein<sup>30</sup>.

Besides from being a tumour suppressor protein, P53 also acts as a transcription factor for the activation of the expression of multiple target genes involved in various biological functions, such as apoptosis and cell cycle arrest<sup>31</sup>. The results of the present study show that PAL and PAA significantly increase the gene expression of P53 comparing to control cell and 5-FU. These results are consistent with previous studies stating that the expression of P53 protein increases apoptosis<sup>32</sup>.

The level of PI3K, Akt1, EGFR and mTOR gene expression was significantly downregulated in treatment cells compared to the control cell in the present study. PAL administration demonstrated a more significant reduction in PI3K, Akt1 and EGFR gene expression than PAA and 5-FU, while mTOR was downregulated more with 5-FU than PAL and PAA. The level of the P53 gene expression was significantly upregulated in the treatment cells compared to the control cell, and this was more significant with PAL than PAA and 5-FU. In this circumstance, the level transcripts may be directly correlated to the activity of protein kinases such as polyisoprenoids decreased the level expression of PI3K, Akt1, and mTOR genes to its function in the cell.

In the present study, polyisoprenoids showed anticancer activity in WiDr cells through the effect of the tumour suppressor and the pro-apoptotic gene expression. This study also indicated that mangrove polyisoprenoids blocked the development of WiDr colon cancer. Furthermore, our previous studies have shown that polyisoprenoids from mangrove leaves induced apoptosis, decreased cell proliferation, and exhibited anticancer activity<sup>8-10</sup>. We on the basis of present and previous studies proposed mechanism of inhibition of cell cycle at G0-G1 phase and enabled the suppression of COX-2 and p53 against WiDr colon cells. Based on the previous investigation results showed that isolated compound from mangrove genus of *Avicennia*

have activities for induce apoptosis by the regulation of apoptotic-related genes (p53 and Bcl-2 pathways) on cancer cell line (i.e. MDA-MB 231 cells) utilize two-step RT-real time PCR method<sup>33</sup>. This postulate represents a new mechanism of mangrove polyisoprenoids to exhibit anticancer activity and appeared to merit further investigation.

5-fluorouracil (5-FU) has been widely used to treat colon cancer since 1957; however in the long term, it can have toxic effects or resistance to effectiveness. Cell cycle perturbation has been reported as one of the causes to lead 5-FU resistance<sup>34</sup>. Therefore, need to seeking the better therapeutic strategies for potential anticancer drugs identified from alternative sources, including natural products e.g. mangrove.

## Conclusions

Overall, the present study confirmed that PAL and PAA can affect anti-apoptotic P53 gene expression by upregulating this gene than the controls, while the expression of pro-apoptotic genes PI3K, Akt1, mTOR, and EGFR were downregulated compared to the controls. In addition, PAL and PAA inhibited the WiDr cell cycle in later apoptosis (S and G2-M1). Therefore, this study confirms that the polyisoprenoids derived from *A. alba* and *A. lanata* leaves are promising chemopreventive agents and facilitate the potential usefulness of polyisoprenoids as new drugs for colon cancer.

## Data availability

### Underlying data

Figshare: Dataset for manuscript: Effects of polyisoprenoids from *Avicennia lanata* and *Avicennia alba* leaves on the gene expression of P13K, Akt1, mTOR, P53, and EGFR in human colorectal adenocarcinoma WiDr cells using reverse transcription-PCR, <https://doi.org/10.6084/m9.figshare.11839350.v4><sup>35</sup>.

This project contains the following data:

- Underlying data for Table 2–Table 4.

Figshare: Dataset for manuscript: Effects of polyisoprenoids from *Avicennia lanata* and *Avicennia alba* leaves on the gene expression of P13K, Akt1, mTOR, P53, and EGFR in human colorectal adenocarcinoma WiDr cells using reverse transcription-PCR, <https://doi.org/10.6084/m9.figshare.11856039.v4><sup>36</sup>.

- Uncropped, unedited images for Figure 2 and Figure 3.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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<http://www.doi.org/10.6084/m9.figshare.11856039.v2>

# Open Peer Review

Current Peer Review Status:  

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## Version 3

Reviewer Report 28 October 2020

<https://doi.org/10.5256/f1000research.30357.r73685>

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### Aldes Lesbani

Department of Chemistry, Faculty of Mathematic and Natural Sciences, Sriwijaya University, Palembang, Indonesia

Approved

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Genetic Natural Product.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.**

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## Version 2

Reviewer Report 09 October 2020

<https://doi.org/10.5256/f1000research.25996.r71835>

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### Aldes Lesbani

Department of Chemistry, Faculty of Mathematic and Natural Sciences, Sriwijaya University, Palembang, Indonesia

This paper describes the effects of polyisoprenoids from *Avicennia lanata* and *Avicennia alba* leaves on the gene expression of PI3K, Akt1, mTOR, P53, and EGFR in human colorectal

adenocarcinoma WiDr cells.

This research is very interesting. Research has fulfilled the principles of good scientific research. There is a negative control group and a test group. The parameters observed are also quite complete. The results showed that polyisoprenoids obtained from *A. alba* and *A. lanata* leaves can inhibit colon cancer. The major comments as follows:

1. Is there percentage data (%) of DMSO concentration that used in the present study? Will the use of DMSO with a concentration of more than 1% have a cytotoxic effect on cells line too?
2. If you have additional information regarding cytotoxic effect of this plant against whatever cells line, please add evidence that this plant has anticancer activity or any related biological activity to enhance your discussion at Cell Cytotoxicity of PAA and PAL section.
3. If any, please add more review regarding the activity of extract or compound that have activity in the tumour suppressor and the pro-apoptotic gene expression to support your result and discussion at Expression of PI3K, Akt1, mTOR, P53, and EGFR genes section).

Minor comments:

1. The phase of cell cycle should be written consistent between Table 3 and in the text throughout the manuscript.
2. Add this sentence 5-FU is largely use to the treat colon cancer; however in the long term, it can have toxic effects or resistance to effectiveness in the Discussion with specific references suggested by authors.

Overall this research is recommended for indexing.

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**

Partly

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** Genetic Natural Product.

**I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.**

Author Response 20 Oct 2020

**Mohammad Basyuni**, Universitas Sumatera Utara, Medan, Indonesia

This paper describes the effects of polyisoprenoids from *Avicennialanata* and *Avicenniaalba* leaves on the gene expression of PI3K, Akt1, mTOR, P53, and EGFR in human colorectal adenocarcinoma WiDr cells.

This research is very interesting. Research has fulfilled the principles of good scientific research. There is a negative control group and a test group. The parameters observed are also quite complete. The results showed that polyisoprenoids obtained from *A. alba* and *A. lanata* leaves can inhibit colon cancer. The major comments as follows:

*We thank the reviewer for his critical reading on our manuscript and for providing us valuables suggestions. We have revised our manuscript to incorporate following the reviewer's suggestions. We believe that the manuscript is now more attractive and suitable for publication.*

1. Is there percentage data (%) of DMSO concentration that used in the present study? Will the use of DMSO with a concentration of more than 1% have a cytotoxic effect on cells line too?

*Response: We used 0.05% concentration of DMSO in this study. The more DMSO concentration may give a cytotoxic effect on the cell line. This study did not test more than 1% DMSO concentration.*

2. If you have additional information regarding cytotoxic effect of this plant against whatever cells line, please add evidence that this plant has anticancer activity or any related biological activity to enhance your discussion at Cell Cytotoxicity of PAA and PAL section.

*Response: New sentence has been added to incorporate Reviewer's suggestion in the Result and Discussion with additional Reference no. 19. Please refer to revised Results and Discussion.*

3. If any, please add more review regarding the activity of extract or compound that have activity in the tumour suppressor and the pro-apoptotic gene expression to support your result and discussion at Expression of PI3K, Akt1, mTOR, P53, and EGFR genes section.

*Response: New sentence has been added to meet Reviewer's suggestion in the Result and Discussion with additional Reference no. 33. Please refer to revised Results and Discussion.*

Minor comments:

1. The phase of cell cycle should be written consistent between Table 3 and in the text throughout the manuscript.

*Response: The phase of cell cycle has been corrected throughout the manuscript and Table 3.*

2. Add this sentence 5-FU is largely use to the treat colon cancer; however in the long term, it can have toxic effects or resistance to effectiveness in the Discussion with specific references suggested by authors.

*Response: 5-FU has been widely used to treat colon cancer since 1957; however in the long term, it can have toxic effects or resistance to effectiveness. Cell cycle perturbation has been reported as one of the causes to lead 5-FU resistance. To incorporate the Reviewer's comment, a new sentence has been included in Result and Discussion with additional Reference No. 34. Please refer to the revised Results and Discussion.*

**Competing Interests:** No competing interests were disclosed.

Reviewer Report 07 May 2020

<https://doi.org/10.5256/f1000research.25996.r62671>

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**Ewa Swiezewska** 

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

**Liliana Surmacz**

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

We recommend to refuse indexing of the revised manuscript entitled 'Effects of polyisoprenoids from *Avicennia lanata* and *Avicennia alba* leaves on the gene expression of PI3K, Akt1, mTOR, P53, and EGFR in human colorectal adenocarcinoma WiDr cells'.

Although the Authors have introduced some corrections they did not address any of the main critical comments listed in the previous opinion.

The methodological errors (a poorly characterized mixture of lipids used for biological experiments, lack of a negative control in numerous experiments) and a limited number of original data described in the evaluated manuscript (in fact only data of PCR experiment are novel but unfortunately they are unreliable) prompted us to sustain our critical opinion on this report. Since the authors see no need to improve their work, it should be definitely rejected.

A detailed list of comments follows.

#### **Comments for Authors:**

Authors have invested some effort to improve the revised manuscript, in particular, English grammar has been verified and minor corrections indicated in the first review have been introduced.

At the same time, new spelling mistakes appeared in the revised manuscript: 'unsonifiable' instead

of 'unsaponifiable' and 'polysoprenoids instead of 'polyisoprenoids'.

Unfortunately, numerous critical comments, including those concerning the purity of the extracts and the methodology of PCR experiment have not been addressed. Please see below for details.

### Comments:

1. The authors did not consider the first critical comment seriously. No additional purification step of the polyisoprenoids was employed in the revised manuscript and 100% chemical purity of the polyisoprenoid mixtures is postulated based on 2D TLC (data not shown in the revised version of the manuscript although the authors in response to reviewers indicate that new Figure 1 contains these data). Several aspects of these statements are commented on below:

Firstly, it is absolutely impossible to achieve 100% purity of the natural compound even though an extensive multistep purification procedure is employed. In this report, only saponification was used without any subsequent purification.

Secondly, analyses of numerous extracts of plant tissues published in the literature so far revealed the presence of higher or lower amounts of various unsanifiable lipids, including steroids (phytosterols, triterpenes). Their tissue concentration is usually considerably higher than that of polyisoprenoids.

Thirdly, the occurrence of triterpenoids and phytosterols together with dolichols has recently been documented in the leaves and roots of several mangrove species, including *A. alba* and *A. lanata* by the same group of authors (Basyuni et al., Open Access Maced J Med Sci. 2019 Nov 14;7(22):3765-3768).

In summary, with no doubts, the crude unsaponifiable lipid fractions used in this manuscript contained steroid compounds and for this reason, results of all the experiments do not support the suggested role of dolichols. The application of such crude mixture seriously diminishes the value of all the biological experiments performed in this report.

2. Data presented in Table 2 of the revised manuscript have been corrected by including the values of SEM. Unfortunately, no there is no information on the number of biological replicates – it should be indicated in the Table legend, e.g. n=?  
The statement 'The cytotoxicity of PAL and PAA in the present study included an interesting SI against WiDr cells in a dose-dependent manner' is not correct since Table 2 presents data for only one single concentration of PAL and PAA.  
Furthermore, the Authors did not respond to the comment on the selectivity of PAA and PAL raised in the first review. In our opinion, conclusions related to data in Table 2 are misleading. Data presented in Table 2 do not provide any argument supporting the postulated selectivity of PAA and PAL.
3. According to the revised manuscript, three independent experiments were performed to obtain data documenting the apoptotic effect of extracts (Fig. 2). While it is correct to show only one set of data in the main text (Fig. 2), two additional sets have to be shown in the Supplement. Why are the results of the quantitative analysis presented without SEM?
4. Corrections related to this section of the manuscript are very limited – SEM values are now included in Table 3; however, the number of the biological replicates of PCR experiments is not indicated. The method of quantitative analysis of PCR data is described.  
Still, the reference gene,  $\beta$ -actin has to be described in the Methods section and included in

the main figure.

As reviewers we appreciate Authors' efforts to document the PCR results, thank you for sharing with us the raw data for Figure 3 (pictures of gels showing expression of PI3K, AKT, mTOR, P53, EGFR and  $\beta$ -actin genes) and Table 4 (intensity of appropriate bands).

Unfortunately, careful inspection of the gels does not lead to the conclusions presented in the manuscript. The criticism/objections concerns:

- 1) expression of P53 gene – all three gel images representing 3 replicates are in fact copies of only one experiment
- 2) the same applies to  $\beta$ -actin expression used as the reference gene
- 3) one of the images is used twice - as 'Gene expression of EGFR' (repetition 2) and simultaneously as 'Gene expression of Akt1 (repetition 2)'
- 4) the results of mTOR expression are shown in an unacceptable manner. The original gel described as 'Gene expression of mTOR (repetition 3) is turned upside down in Figure 3. Annotation of the lanes in Fig. 3 is not in accordance with the original gel. Original 5-FU (line 5 in gel) is shown as control cells (line a in Figure 3), PAM (line 4 on gel) is presented as PAL (b in Fig.3) and finally line presenting control cells in the gel is shown as 5-FU in Fig. 3. Additionally, how the control cells were obtained? It is not described anywhere in the manuscript.

Moreover, the data presenting a quantitative analysis of PCR product shown in Table 4 are inconsistent with the respective raw data in the gels (raw data for Figure 3).

### **Methodology:**

Information for how long the plant material was stored prior to extraction is missing.

The concentration of 5-Fu used in this study in the context of literature. Were the values used in this study within the range of those used in similar types of assays? This information is still missing in the revised manuscript.

In the revised manuscript the type of solvent used to prepare PAA and PAL solutions for biological tests is not described. According to the Methods 5-FU was dissolved in DMSO but which solvent was used to dissolve Dol mix? Was it DMSO?

Another related question is the negative control for all biological experiments. This critically important issue was not addressed in the revised manuscript since the lack of data for cells treated solely with solvent makes all the biological results questionable.

The dose PAL and PAA and time of exposure of cells for the cell cycle analysis is still missing. The journal referred here *Iran J Pharm Res* is not easily accessible.

To justify the novelty of the manuscript, Authors refer to three papers (ref. No 8-10) previously published by their own team: 'Furthermore, our previous studies have shown that polyisoprenoids from mangrove leaves induced apoptosis, decreased cell proliferation, and exhibited anticancer activity [8– 10].' Consequently, Authors declare that original data are presented solely in Figure 3 and Table 4. Moreover, Dols has already been described in their own previously published reports, e.g. Basyuni *et al.*, Open Access Maced J Med Sci. 2019 – why this publication is not included in the reference list?

Last but not least despite the fact that the Authors discuss the purity of the polyisoprenoid mixture based on 2D TLC chromatogram (Figure 1 mentioned in the Authors' response) it is not included in the revised manuscript.

English grammar has been corrected and indicated minor corrections have been introduced; however, some spelling mistakes have to be corrected:

- replace 'unsonifiable' with 'unsaponifiable'
- replace 'polysioprenoids' with 'polyisoprenoids'

**Is the work clearly and accurately presented and does it cite the current literature?**

Yes

**Is the study design appropriate and is the work technically sound?**

Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**

Yes

**If applicable, is the statistical analysis and its interpretation appropriate?**

Yes

**Are all the source data underlying the results available to ensure full reproducibility?**

Yes

**Are the conclusions drawn adequately supported by the results?**

Yes

**Competing Interests:** No competing interests were disclosed.

**We confirm that we have read this submission and believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.**

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Ewa Swiezewska 

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**Liliana Surmacz**

Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

We, Liliana Surmacz and Ewa Swiezewska, found the manuscript entitled 'Effects of polyisoprenoids from *Avicennia lanata* and *Avicennia alba* leaves on the gene expression of PI3K, Akt1, mTOR, P53, and EGFR in human colorectal adenocarcinoma WiDr cells using reverse transcription-PCR' prepared by Dr T. Qurrohman and coworkers not acceptable for publication in the journal F1000Research in its current form.

This manuscript describes elucidation of the biological activity of two polyisoprenoid-containing extracts isolated from leaves of two mangrove plant species *Avicennia lanata* and *Avicennia alba*. To meet the generally acceptable standard of the manuscript quality this preliminary study requires extremely extensive revision.

Firstly, all the biological data were obtained using crude and poorly characterized extracts and consequently all these experiments should be performed once again with highly purified polyisoprenoid mixture (polyisoprenoid content at least 90%). Such purification procedure is doable within a reasonable time period and is not very difficult.

Secondly, several methodological issues require clarification, e.g. selection of the drug concentration points, appropriate control experiments (composition of the cell growth media), number of replicates, methods of quantification of PCR results and statistical analysis of the data. Thirdly, interpretation of the obtained results seems not really clear, in particular results of the analysis of the toxicity should be reconsidered (see below).

Finally, the Authors should carefully discuss the available literature data to document the novelty of their study.

A detailed list of comments follows.

This manuscript describes study on the biological effects of the polyisoprenoid-containing extracts isolated from leaves *Avicennia lanata* and *Avicennia alba*. All tests were performed for two cell lines, WiDr and Vero. Shown are data on the effect of PAA and PAL on the cytotoxic activity, cell-cycle progression and expression of the selected genes: PI3K, Akt1, mTOR, and EGFR.

#### **General comments:**

All the biological tests described in this study were performed using the crude mixture of lipids obtained after alkaline hydrolysis of the leaf extracts. The Authors did not provide the approximate polyisoprenoid content in the mixtures used. Such complex mixture of natural compounds could be used just for preliminary tests of their potential biological activity.

What is more, data presented in this study in fact confirm the weakness of the strategy used – e.g. different effects of pro-apoptotic activity of PAA and PAL on the level of expression of analyzed genes might suggest that this is NOT the effect of polyisoprenoids (very similar profile for both extracts) but rather some other components of these mixtures.

In order to present any reasonable conclusion on the biological effect of polyisoprenoids highly purified polyisoprenoid samples (purity higher than 90% based on the HPLC/UV assay) should be used. For this reason the experiments have to be reproduced with purified polyisoprenoid samples.

In this context the title of the manuscript does not describe the real content of the work.

Data presented in Table 2 suggest that analyzed extracts exert higher toxicity against Vero than

WiDr cells. Consequently the Selectivity Index is below 1. It raises the question whether analyzed extracts could be considered as candidates for anticancer drugs since they possess higher cytotoxic activity against normal than cancer cells. Authors should carefully comment on this observation. Keeping in mind that analyzed extracts were not pure these data do not preclude the potential usefulness of polyisoprenoids as drugs although this conclusion cannot be made on the basis of results shown in Table 2.

Furthermore, interpretation of the quantitative results of cytotoxicity against WiDr cells is questionable – the difference between both IC50 values obtained for PAA and PAL is approx. 6% (Table 2); thus the conclusion presented by Authors seems highly exaggerated. Finally, why are no SD values are presented here? Have these experiments been performed only once?

Experiments on the effect of plant extracts on the cell-cycle progression are summarized as follows: 'The antineoplastic activity of PAL and PAA occur in the S phase of the cell cycle, which involves the possibility of bonding with the DNA through intercalation between the base pairs as well as the inhibition of the DNA and RNA synthesis.' Such conclusion definitely requires additional data supporting the suggested mechanism(s).

Moreover, presented data were not analyzed using statistical method. Fig.2 presents representative data but how many biological replica were performed? How many microscopic snapshots were used for quantitative analysis?

Expression of selected genes - Authors should add a general comment on the biological relevance of the transcriptional data in the context of transcript – translated protein – its function in the cell, e.g. is the level of transcript directly related to the level and/or activity of the appropriate protein? What concentration of PAL or PAA was used to obtain data presented in Fig.3 and Table 4? Since Fig.3 and Table 4 present data for just one concentration point thus the statement is not justified: 'The results of the present study show that PAL and PAA significantly increase the gene expression of P53 at high concentrations, and the increase in P53 gene expression is concentration dependent; the higher the concentration, the higher the level of gene expression.' Furthermore, how quantification of the PCR product, i.e. bands intensity was performed? What software was used for this analysis? Why no reference gene was used as a control for reaction efficiency? How many RT-PCR repetitions were used for quantitative analysis? The term 'Gene expression density' should be replaced with 'Gene expression level' or 'Transcript expression level'

Finally, a general comment concerning the RT-PCR technique - currently a qPCR method is considered an acceptable standard for transcriptomic analysis. It is recommended by these reviewers to repeat analyses using this approach.

In the Introduction the rationale behind the choice of the particular genes to be analyzed (PI3K, Akt1, mTOR, P53, and EGFR) has to be presented together with a brief (one sentence) description of their function in the cancer cell.

Additionally, rewrite the sentence and explain the meaning of the expression 'and differentiate between the level of individual plasmid expression in multivalent pDNA'. How this expression is relevant to the method used in this study?

Methodology requires clarification:

1. Delete the description of the cleaning of the leaves from 'The simplicia procedure ...' to '...stored in tightly closed plastic containers.' Instead, describe the date of the collection of

the leaves, for how long the plant material was stored prior to extraction, the temperature of the extraction procedure.

2. What is meant in the sentence: 'The portions were further saponified and .....?' All the ester have already been saponified upon KOH treatment.
3. Was a crude unsaponifiable lipid fraction used for further tests? It has to be clearly mentioned in the text.
4. If so what was the approximate polyisoprenoid content in this fraction?
5. Data obtained in the biological test are not fully justified keeping in mind the complexity of the lipid fraction used.
6. A type of the TLC plates should be mentioned in the text, moreover what is the meaning of the expression: '... were confirmed to belong to the dolichol family 100%...'.
7. What type of cell line are Vero cells? Which organ is it derived from? Is it an immortalized cell line?
8. Concentration of 5-Fu used in this study – please provide literature data on the concentration of 5-FU used. Were the values used in this study within the range of those used in similar type of assays?
9. The method of administration of the leaf extracts to the cells is not described at all. Polyisoprenoids are not soluble in water so what type of solvent was used to prepare PAA and PAL solutions for all biological tests? And how the control cells were treated – there is no information, neither in the Methods nor in the Figure legends, on the supplementation of the growth media of the control cell cultures with the appropriate amount of the same solvent.
10. For how long the cells were exposed to PAL in the cell cycle analysis? What concentrations of PAL and PAA were used?
11. The description of the apoptosis analysis is unclear and requires rewriting. What type of software was used to count green and red fluorescence signals?
12. The amount of total RNA used in this study in the reverse transcription reaction (according to the manuscript 3 mg in the 20  $\mu$ l reaction mixture) seems unrealistic. Manufacturer's protocols usually recommend to use  $\leq 1$   $\mu$ g RNA. Please correct.

The novelty of the data presented in this study has to be indicated by Authors in the section Discussion or Conclusions – novel data presented in the current manuscript in comparison to the previous already published ones should be clearly depicted.

The manuscript requires an English language edit. Some sentences are misleading, some are just awkward, e.g.

'Cytotoxic activity against WiDr cells showed that the IC50 for *A. alba* and *A. lanata* was 258.14

ug/mL and 243.32 ug/mL, respectively. This indicated that their classification as anticancer agents was moderate.'

'Natural ingredients developed as potential chemotherapeutic agents include mangrove leaves.' should rather read as follows: 'Natural substances developed as potential chemotherapeutic agents include components of mangrove leaves.'

'This extract has a mechanism for inhibiting the cell cycle at the G0-G1 phase...'

'In this study, the test material is cytotoxic for the polyisoprenoids of leaves derived from...'

The word 'simplicia' is used in the text several times– please replace with any typical expression.

#### **Minor remarks:**

- Title – 'using reverse transcription-PCR' - in my opinion these last four words are not necessary and should be deleted; it is nothing special in this RT-PCR technique.
- p.1 – 'Mangrove plants produce a polyisoprenoid compound. Polyisoprenoids have been proven to have anticancer properties.' – these two sentences have to be rewritten. As it is now they do not provide enough information about the subject of study. Moreover, why the term 'polyisoprenoid compound' is used here in a singular form? It is not consistent either with the natural composition of the leaf extract or with the text below.
- p.1 – delete 'inhibited' in the sentence 'The inhibited cell cycle and apoptosis...'
- p.3 – rewrite the second sentence of Introduction – what is meant by the expression 'halt its invasion and metastasis' in the context of this sentence?
- p.3 – replace '...dolichol and polyprenol on the leaves...' with '...dolichol and polyprenol in the leaves...'
- p.3 – 'MCF-7 and T47D' – a brief description of the cell line type is missing.
- p.3 – 'gene expression of COX-2 in colon cancer cells' spell out the name of the enzyme when first used here and throughout the entire manuscript.
- p.3 – delete the word 'Every' in the sentence 'Every 500 g of powdered simplicia mangrove leaves...'. Explain the word 'simplicia' in the text or delete.
- p.3 – 'The cell wall debris insoluble in CM21...' – how about other cellular components, e.g. proteins? – replace 'cell wall debris' with 'Precipitate'.
- p.3 – delete the description of the cleaning of the leaves from 'The simplicia procedure ...' to '...stored in tightly closed plastic containers.' Instead, describe the date of the collection of the leaves, for how long the plant material was stored prior to extraction, the temperature of the extraction procedure.

#### **Is the work clearly and accurately presented and does it cite the current literature?**

Partly

#### **Is the study design appropriate and is the work technically sound?**

Partly

**Are sufficient details of methods and analysis provided to allow replication by others?**

No

**If applicable, is the statistical analysis and its interpretation appropriate?**

No

**Are all the source data underlying the results available to ensure full reproducibility?**

Partly

**Are the conclusions drawn adequately supported by the results?**

Partly

**Competing Interests:** No competing interests were disclosed.

**We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.**

Author Response 19 Apr 2020

**Mohammad Basyuni**, Universitas Sumatera Utara, Medan, Indonesia

We, Liliana Surmacz and Ewa Swiezewska, found the manuscript entitled 'Effects of polyisoprenoids from *Avicennia lanata* and *Avicennia alba* leaves on the gene expression of PI3K, Akt1, mTOR, P53, and EGFR in human colorectal adenocarcinoma WiDr cells using reverse transcription-PCR' prepared by Dr T. Qurrohman and coworkers not acceptable for publication in the journal F1000Research in its current form.

This manuscript describes elucidation of the biological activity of two polyisoprenoid-containing extracts isolated from leaves of two mangrove plant species *Avicennia lanata* and *Avicennia alba*. To meet the generally acceptable standard of the manuscript quality this preliminary study requires extremely extensive revision.

Response: We would like to thank the Reviewers' comments and suggestions which significantly improve the manuscript and enrich the content.

Firstly, all the biological data were obtained using crude and poorly characterized extracts and consequently all these experiments should be performed once again with highly purified polyisoprenoid mixture (polyisoprenoid content at least 90%). Such purification procedure is doable within a reasonable time period and is not very difficult.

Response: We agreed with Reviewers' suggestions to revise the procedures. The polyisoprenoid extraction was performed using the established procedures. The lipid extracts of *Avicennia alba* and *A. lanata* was concentrated to dryness and saponified at 65°C for 24 h in 86% ethanol containing KOH 2 M. The unsaponifiable lipid partitioned into hexane by vigorous mixing was analyzed by silica gel 60 TLC and RP-18 high performance thin layer chromatography (HPTLC) plates. The unsanifiable lipid basically denotes simple

lipid fractions except for fatty acids (saponifiable lipid). The leaf extracts (50-100 mg) were applied to TLC plate. The quantity of polyisoprenoids in *A. alba* leaves was  $5.5 \pm 0.8$  mg/g dry weight and *A. lanata* leaves was  $14.9 \pm 1.2$  mg/g dry weight. Data are represented as the means  $\pm$  SEM ( $n=3$ ). We enclose two-dimensional TLC (2D TLC) chromatograms. Figure 1 shows 2D TLC chromatograms of polyisoprenoids from leaves of *A. alba* and *A. lanata*. Dolichols with the chain length of C60-C100 and C70-C100 were detected as major polyisoprenoids alcohols in *A. alba* leaves (A) and *A. lanata* leaves (B), respectively. No polyprenol was found in both mangrove leaves. The 2D TLC has been performed triplicates and showed an identical pattern. We confirmed that leaves extracts contained 100% dolichols as highly purified polyisoprenoid mixture to meet Reviewers' requirement. Therefore it is not needed to purify the polyisoprenoid samples and used for further investigation.

New and revised sentences have added to the revised manuscript to incorporate Reviewers' suggestions. Please refer to the revised version of Preparation of isolation polyisoprenoid alcohols.

Figure 1. Two-dimensional TLC chromatograms of polyisoprenoids from *Avicennia alba* leaves (PAA) (A) and *A. lanata* leaves (PAL) (B)

Secondly, several methodological issues require clarification, e.g. selection of the drug concentration points, appropriate control experiments (composition of the cell growth media), number of replicates, methods of quantification of PCR results and statistical analysis of the data.

Response: To meet Reviewers' suggestions, methodological issues, e.g. selection of the drug concentration points, appropriate control experiments (composition of the cell growth media), number of replicates, methods of quantification of PCR results and statistical analysis of the data have been revised. The raw data of experiments with three independent repetitions has been deposited in <https://doi.org/10.6084/m9.figshare.11839350.v4> and <https://doi.org/10.6084/m9.figshare.11856039.v4>  
Please refer to revised Methods.

Thirdly, interpretation of the obtained results seems not really clear, in particular results of the analysis of the toxicity should be reconsidered (see below).

Response: To incorporate Reviewers' the results of the analysis of the toxicity has been revised by adding the standard errors of the means and statistical analysis. Please refer to revised Table 2.

Finally, the Authors should carefully discuss the available literature data to document the novelty of their study.

Response: We agreed with Reviewers' suggestions, new sentences have been added to meet Reviewers' comments, please refer to revised Discussion.

A detailed list of comments follows.

This manuscript describes study on the biological effects of the polyisoprenoid-containing extracts isolated from leaves *Avicennia lanata* and *Avicennia alba*. All tests were performed for two cell lines, WiDr and Vero. Shown are data on the effect of PAA and PAL on the cytotoxic activity, cell-cycle progression and expression of the selected genes: PI3K, Akt1, mTOR, and EGFR.

General comments:

All the biological tests described in this study were performed using the crude mixture of lipids obtained after alkaline hydrolysis of the leaf extracts. The Authors did not provide the approximate polyisoprenoid content in the mixtures used. Such complex mixture of natural compounds could be used just for preliminary tests of their potential biological activity.

Response: Unsaponifiable lipid was analysed using TLC and 2D-TLC plates to identify polyisoprenoids that contained 100% dolichols with the chain length of C60-C100 and C70-C100 detected as main polyisoprenoids alcohols in *A. alba* leaves and *A. lanata* leaves. New sentences have been added to the revised manuscript. Please refer to revised Preparation of isolation polyisoprenoid alcohols.

What is more, data presented in this study in fact confirm the weakness of the strategy used – e.g. different effects of pro-apoptotic activity of PAA and PAL on the level of expression of analyzed genes might suggest that this is NOT the effect of polyisoprenoids (very similar profile for both extracts) but rather some other components of these mixtures.

Response: We clarified that leaves extracts of PAA and PAL contained 100% dolichols based on the HPTLC chromatograms (Figure 1), we believe that different effects of pro-apoptotic activity of PAL and PAL on the level of expression of analysed from the effect of polyisoprenoids (dolichols). We examined several pro-apoptotic genes as questioned by Reviewers as the weakness of the strategy used, we agreed to this point, however, we also examined an anti-apoptotic gene, p53 to prevent the cancer formation involving a mechanism on the tumour suppressor protein p53.

In order to present any reasonable conclusion on the biological effect of polyisoprenoids highly purified polyisoprenoid samples (purity higher than 90% based on the HPLC/UV assay) should be used. For this reason the experiments have to be reproduced with purified polyisoprenoid samples.

Response: We clarified that leaves extracts of both mangrove contained 100% dolichols based on 2D-TLC chromatograms, no other compounds found. These samples met the criteria as Reviewers' suggestion for purity samples was higher than 90%. Therefore it is not required to purify the polyisoprenoid samples.

In this context the title of the manuscript does not describe the real content of the work.

Response: To incorporate Reviewers's suggestion, the title has been revised to be "Effects of polyisoprenoids from *Avicennia lanata* and *Avicennia alba* leaves on the gene expression

of PI3K, Akt1, MTOR, P53 and EGFR in human colorectal adenocarcinoma WiDr cells"

Data presented in Table 2 suggest that analyzed extracts exert higher toxicity against Vero than WiDr cells. Consequently the Selectivity Index is below 1. It raises the question whether analyzed extracts could be considered as candidates for anticancer drugs since they possess higher cytotoxic activity against normal than cancer cells. Authors should carefully comment on this observation. Keeping in mind that analyzed extracts were not pure these data do not preclude the potential usefulness of polyisoprenoids as drugs although this conclusion cannot be made on the basis of results shown in Table 2.

Response: We agreed with Reviewers' comments on the results of Selectivity Index of PAA and PAL is below 1, showing higher cytotoxic selectivity. We also clarified that the analysed extracts of both samples were pure, contained 100% dolichols. However, as Reviewers also mentioned that PAA and PAL have potential usefulness as drugs.

Furthermore, interpretation of the quantitative results of cytotoxicity against WiDr cells is questionable – the difference between both IC50 values obtained for PAA and PAL is approx. 6% (Table 2); thus the conclusion presented by Authors seems highly exaggerated.

Response: We agreed with Reviewers' suggestion on small difference between both IC50 values obtained for PAA and PAL, we revised Table 2 and the conclusion to incorporate Reviewers' comments.

Finally, why are no SD values are presented here? Have these experiments been performed only once?

Response: SEM values have been added to revised Table 2 to incorporate Reviewers' suggestion. These experiments have been performed triplicate analyses. The raw data on Table 2 has been deposited on this link: <https://doi.org/10.6084/m9.figshare.11839350.v4> Experiments on the effect of plant extracts on the cell-cycle progression are summarized as follows: 'The antineoplastic activity of PAL and PAA occur in the S phase of the cell cycle, which involves the possibility of bonding with the DNA through intercalation between the base pairs as well as the inhibition of the DNA and RNA synthesis.' Such conclusion definitely requires additional data supporting the suggested mechanism(s).

Response: We do not have additional data supporting the suggested mechanism, to incorporate Reviewers' suggestion the sentence 'The antineoplastic activity of PAL and PAA occur in the S phase of the cell cycle, which involves the possibility of bonding with the DNA through intercalation between the base pairs as well as the inhibition of the DNA and RNA synthesis,' has been deleted from the revised manuscript.

Moreover, presented data were not analyzed using statistical method. Fig.2 presents representative data but how many biological replica were performed? How many microscopic snapshots were used for quantitative analysis?

Response: Observation of apoptotic cells in a fluorescence microscope with a magnification of 40x and 3x in cell control, PAA, PAL, and 5-Fu treatments was analysed using SPSS version 23, followed by Duncan's multiple range test for treatment comparisons. ImageRaster 4.0.5 was used to count green and red fluorescence signals of three microscopic snapshots of individual experiments. Please refer to revised raw data of Fig. 2 and revised Apoptosis analysis.

Expression of selected genes - Authors should add a general comment on the biological

relevance of the transcriptional data in the context of transcript – translated protein – its function in the cell, e.g. is the level of transcript directly related to the level and/or activity of the appropriate protein?

Response: New sentence has been added to revised Discussion to incorporate Reviewers' suggestions.

What concentration of PAL or PAA was used to obtain data presented in Fig.3 and Table 4? Since Fig.3 and Table 4 present data for just one concentration point thus the statement is not justified: 'The results of the present study show that PAL and PAA significantly increase the gene expression of P53 at high concentrations, and the increase in P53 gene expression is concentration dependent; the higher the concentration, the higher the level of gene expression.'

Response: We used 5µL PCR product to obtain data presented in Fig. 3 and Table 4. We agreed with Reviewers' comments to correct the sentence to read 'The results of the present study show that PAL and PAA significantly increase the gene expression of P53 comparing to control cell and 5-Fu'.

Furthermore, how quantification of the PCR product, i.e. bands intensity was performed? What software was used for this analysis? Why no reference gene was used as a control for reaction efficiency? How many RT-PCR repetitions were used for quantitative analysis?

Response: New sentences and revised Figure 3 have been included to incorporate Reviewers' comments. Each data represents the average of three independent RT-PCR measurements with standard errors of individual experiments. To quantify the PCR product, Quantity One® 1-D analysis software (Bio-Rad) used to assess bands intensity of genes analysed. b-actin was reference gene to normalize the PCR efficiency.

The term 'Gene expression density' should be replaced with 'Gene expression level' or 'Transcript expression level'

Response: 'Gene expression density' has been replaced with 'Gene expression level'. Please refer to revised part Results of Discussion of Expression of PI3K, Akt1, mTOR, P53, and EGFR genes

Finally, a general comment concerning the RT-PCR technique - currently a qPCR method is considered an acceptable standard for transcriptomic analysis. It is recommended by these Reviewers to repeat analyses using this approach.

Response: We agreed with Reviewers' suggestion, however, in the present situation, the authors are unable to repeat analysis using transcriptomic approach.

In the Introduction the rationale behind the choice of the particular genes to be analyzed (PI3K, Akt1, mTOR, P53, and EGFR) has to be presented together with a brief (one sentence) description of their function in the cancer cell.

Response: To incorporate Reviewers' suggestion, a new sentences relating to description function of PI3K, Akt1, mTOR, p53, and EGFR in the cancer cell has been added to Introduction. Please refer to last sentence of Introduction.

Additionally, rewrite the sentence and explain the meaning of the expression 'and differentiate between the level of individual plasmid expression in multivalent pDNA'. How this expression is relevant to the method used in this study?

Response: The sentence 'and differentiate between the level of individual plasmid expression in multivalent pDNA' has been deleted from revised manuscript.

Methodology requires clarification:

1. Delete the description of the cleaning of the leaves from 'The simplicia procedure ...' to '...stored in tightly closed plastic containers.' Instead, describe the date of the collection of the leaves, for how long the plant material was stored prior to extraction, the temperature of the extraction procedure.

Response: The description of the cleaning of the leaves from 'The simplicia procedure ...' to '...stored in tightly closed plastic containers.' has been deleted from revised manuscript.

1. What is meant in the sentence: 'The portions were further saponified and .....'. All the ester have already been saponified upon KOH treatment.

Response: The sentence: 'The portions were further saponified and .....' has been deleted and changed to new sentence to read 'The unsaponifiable lipid partitioned into 2 mg/mL n-hexane'.

1. Was a crude unsaponifiable lipid fraction used for further tests? It has to be clearly mentioned in the text.

Response: A crude unsaponifiable lipid used for further test and has been clearly mentioned in the text of revised manuscript.

1. If so what was the approximate polyisoprenoid content in this fraction?

The quantity of polyisoprenoids in *A. alba* leaves was  $5.5 \pm 0.8$  mg/g dry weight and *A. lanata* leaves was  $14.9 \pm 1.2$  mg/g dry weight. Data are represented as the means  $\pm$  SEM ( $n=3$ ).

1. Data obtained in the biological test are not fully justified keeping in mind the complexity of the lipid fraction used.

Response: We agreed with Reviewers' comments on the complexity of the lipid fraction, however, the biological tests have been clarified to be 100% dolichols.

1. A type of the TLC plates should be mentioned in the text, moreover what is the meaning of the expression: '... were confirmed to belong to the dolichol family 100%...'.

Response: Types of TLC plates were Silica gel 60 thin layer chromatography (TLC) and RP-18 HPTLC plates have been added to revised Methods, the expression: '...were confirmed to belong to the dolichol family 100%...' has been revised to read, '...were detected to be 100% dolichol family...'

1. What type of cell line are Vero cells? Which organ is it derived from? Is it an immortalized cell line?

Response: Vero ATCC® CCL-81™, an immortalized cell line, derived from the kidney of an African green monkey

1. Concentration of 5-Fu used in this study – please provide literature data on the concentration of 5-FU used. Were the values used in this study within the range of those used in similar type of assays?

Response: New sentence has been added to meet Reviewers' comment. 50  $\mu$ M of 5-fluorouracil (5-Fu) were dissolved in a 100  $\mu$ L dimethyl sulfoxide (DMSO) co-solvent. The concentration used in this study within the range of those used in colon cancer cells.

1. The method of administration of the leaf extracts to the cells is not described at all. Polyisoprenoids are not soluble in water so what type of solvent was used to prepare PAA and PAL solutions for all biological tests? And how the control cells were treated – there is no information, neither in the Methods nor in the Figure legends, on the

supplementation of the growth media of the control cell cultures with the appropriate amount of the same solvent.

Response: To incorporate Reviewers' comments, new sub title in the Methods have been added. Please refer to Administration of the leaf extracts to the cells of Methods.

1. For how long the cells were exposed to PAL in the cell cycle analysis? What concentrations of PAL and PAA were used?

Response: The cell was exposed to serially diluted concentrations of PAA and PAL (1000, 500, 250, 125, and 62.5 µg/mL) in the cell cycle analysis for 48 h. Please refer to revised sentence in the Cytotoxicity analysis of Methods.

1. The description of the apoptosis analysis is unclear and requires rewriting. What type of software was used to count green and red fluorescence signals?

Response: New sentence has been added to meet Reviewers' comments. ImageRaster 4.05. (Micronos, Yogyakarta, Indonesia) was used to count green and red fluorescence signals. Please refer to revised Apoptosis analysis method.

1. The amount of total RNA used in this study in the reverse transcription reaction (according to the manuscript 3 mg in the 20 µl reaction mixture) seems unrealistic. Manufacturer's protocols usually recommend to use ≤1 µg RNA. Please correct.

Response: The total RNA (0.3 µg each) has been corrected.

The novelty of the data presented in this study has to be indicated by Authors in the section Discussion or Conclusions – novel data presented in the current manuscript in comparison to the previous already published ones should be clearly depicted.

Response: We agreed with Reviewers' suggestion, new and revised sentences have added to revised Conclusion.

The manuscript requires an English language edit. Some sentences are misleading, some are just awkward, e.g.

'Cytotoxic activity against WiDr cells showed that the IC<sub>50</sub> for *A. alba* and *A. lanata* was 258.14 µg/mL and 243.32 µg/mL, respectively. This indicated that their classification as anticancer agents was moderate.'

Response: To incorporate Reviewers' comments, the sentences have been revised to read 'Cytotoxic activity against WiDr cells showed that the IC<sub>50</sub> for *A. alba* and *A. lanata* was 258.14 µg/mL and 243.32 µg/mL, respectively. This observation indicated the possibility to develop moderate anticancer agents.'

'Natural ingredients developed as potential chemotherapeutic agents include mangrove leaves.' should rather read as follows: 'Natural substances developed as potential chemotherapeutic agents include components of mangrove leaves.'

Response: Natural ingredients developed as potential chemotherapeutic agents include mangrove leaves.' has been corrected to 'Natural substances developed as potential chemotherapeutic agents include components of mangrove leaves.'

'This extract has a mechanism for inhibiting the cell cycle at the G<sub>0</sub>-G<sub>1</sub> phase...'

Response: 'This extract has a mechanism for inhibiting the cell cycle at the G<sub>0</sub>-G<sub>1</sub> phase...' has been revised to 'Polyisoprenoids have a mechanism to inhibit the cell cycle at the G<sub>0</sub>-G<sub>1</sub> phase...'

'In this study, the test material is cytotoxic for the polyisoprenoids of leaves derived from...'

Response: 'In this study, the test material is cytotoxic for the polyisoprenoids of leaves derived from...' has been corrected to 'In this study, the cytotoxic test material was derived

from polyisoprenoids of mangrove leaves (PAA and PAL)..'

The word 'simplicia' is used in the text several times– please replace with any typical expression.

The word 'simplicia' has been deleted to incorporate Reviewers' suggestion throughout the revised manuscript.

Minor remarks:

- Title – 'using reverse transcription-PCR' - in my opinion these last four words are not necessary and should be deleted; it is nothing special in this RT-PCR technique.

Response: 'using reverse transcription-PCR' has been deleted from the title.

- p.1 – 'Mangrove plants produce a polyisoprenoid compound. Polyisoprenoids have been proven to have anticancer properties.' – these two sentences have to be rewritten. As it is now they do not provide enough information about the subject of study. Moreover, why the term 'polyisoprenoid compound' is used here in a singular form? It is not consistent either with the natural composition of the leaf extract or with the text below.

'Mangrove plants produce polyisoprenoid compounds. Polyisoprenoids have been proven to have anticancer properties.'

- p.1 – delete 'inhibited' in the sentence 'The inhibited cell cycle and apoptosis...'

Response: 'inhibited' has been deleted.

- p.3 – rewrite the second sentence of Introduction – what is meant by the expression 'halt its invasion and metastasis' in the context of this sentence?

Response: The second sentence of Introduction 'halt its invasion and metastasis' has been corrected to 'spread its invasion and metastasis' in the context of this sentence.

- p.3 – replace '...dolichol and polyprenol on the leaves...' with '...dolichol and polyprenol in the leaves...'

Response: '...dolichol and polyprenol on the leaves...' has been replaced with '...dolichol and polyprenol in the leaves...'

- p.3 – 'MCF-7 and T47D' – a brief description of the cell line type is missing.

Response: human breast cancer cell lines has been added before MCF-7 and T47D

- p.3 – 'gene expression of COX-2 in colon cancer cells' spell out the name of the enzyme when first used here and throughout the entire manuscript.

Response: 'gene expression of COX-2 in colon cancer cells' has been corrected to 'gene expression of cyclooxygenase-2 (COX-2) in colon cancer cells'

- p.3 – delete the word 'Every' in the sentence 'Every 500 g of powdered simplicia mangrove leaves...'. Explain the word 'simplicia' in the text or delete.

Response: The words 'Every' and 'simplicia' in the sentence 'Every 500 g of powdered simplicia mangrove leaves...' has been deleted to read 'Five hundreds g of powdered mangrove leaves...'

- p.3 – 'The cell wall debris insouble in CM21...' – how about other cellular components, e.g. proteins? – replace 'cell wall debris' with 'Precipitate'.

Response: 'The cell wall debris insouble in CM21...' has been corrected to 'Precipitate insoluble in CM21...'

- p.3 – delete the description of the cleaning of the leaves from 'The simplicia procedure ...' to '...stored in tightly closed plastic containers.' Instead, describe the date of the collection of the leaves, for how long the plant material was stored prior

to extraction, the temperature of the extraction procedure.

Response: The simplicia procedure ...' to '...stored in tightly closed plastic containers.' Has been deleted from the revised manuscript.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

No

If applicable, is the statistical analysis and its interpretation appropriate?

No

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests

No competing interests were disclosed.

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

**Competing Interests:** No competing interests were disclosed.

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