

Cytotoxic Effect of Polyisoprenoids from *Rhizophora mucronata* and *Ceriops tagal* Leaves against WiDr Colon Cancer Cell Lines

(Kesan Sitotoksik Poliisoprenoid daripada Daun *Rhizophora mucronata* dan *Ceriops tagal* terhadap Titisan Sel Kanser Kolon WiDr)

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ABSTRACT

The mangrove plant is known to produce secondary metabolite compounds, mainly isoprenoids. Polyisoprenoids (dolichol and polyprenol) are known to have antimicrobial, anticancer and antiviral activity. Therefore, this study aimed to determine the cytotoxic effects of polyisoprenoids from *Rhizophora mucronata* and *Ceriops tagal* leaves by evaluating the induction of apoptosis and cell cycle arrest and the inhibition of the expression of Bcl-2 and cyclin D1 in WiDr colon cancer cells. Cell death was determined based on IC_{50} values in MTT assays. The induction of apoptosis and alterations in the cell cycle were observed by flow cytometry. The expression of Bcl-2 and cyclin D1 proteins, which play a role in apoptosis and cell cycle regulation, was observed by immunocytochemistry. The results showed that polyisoprenoids from *R. mucronata* and *C. tagal* leaves exhibited toxicity against the WiDr cell line, with IC_{50} values of 278 ± 5.77 and 276 ± 9.54 $\mu\text{g/mL}$, respectively. Polyisoprenoids from *R. mucronata* and *C. tagal* leaves significantly induced apoptosis and caused cell cycle arrest in G0/G1 phase, while also decreasing the expression of Bcl-2 and cyclin D1. Our results confirmed that polyisoprenoids from *R. mucronata* and *C. tagal* leaves have the potential to be developed as anticancer agents for colon cancer.

Keywords: Apoptosis; *Ceriops tagal*; cytotoxic; dolichol; *Rhizophora mucronata*

ABSTRAK

Tumbuhan bakau diketahui menghasilkan sebatian metabolit sekunder, terutamanya isoprenoid. Poliisoprenoid (dolichol dan poliprenol) diketahui mempunyai aktiviti antimikrob, antikanser dan antivirus. Oleh itu, kajian ini bertujuan untuk menentukan kesan sitotoksik poliisoprenoid daripada daun *Rhizophora mucronata* dan *Ceriops tagal* dengan menilai induksi apoptosis tangkapan kitaran sel serta kesan ekspresi Bcl-2 dan siklin D1 dalam titisan sel kanser kolon WiDr. Kematian sel ditentukan berdasarkan nilai IC_{50} dalam asai MTT. Induksi apoptosis dan perubahan dalam kitaran sel diperhatikan dengan aliran sitometri. Ekspresi Bcl-2 dan protein siklin D1 yang memainkan peranan dalam apoptosis dan kawal-selia kitaran sel diperhatikan melalui imunositokimia. Keputusan menunjukkan bahawa poliisoprenoid daripada daun *R. mucronata* dan *C. tagal* mengeluarkan ketoksikan terhadap titisan sel WiDr, masing-masing dengan nilai IC_{50} 278 ± 5.77 dan 276 ± 9.54 $\mu\text{g/mL}$. Poliisoprenoid daripada daun *R. mucronata* dan *C. tagal* dengan ketara mengaruh apoptosis dan menyebabkan tangkapan kitaran sel dalam fasa G0/G1, selain menurunkan ekspresi Bcl-2 dan siklin D1. Keputusan kami mengesahkan bahawa poliisoprenoid daripada daun *R. mucronata* dan *C. tagal* mempunyai potensi untuk dibangunkan sebagai agen anti-kanser untuk kanser kolon.

Kata kunci: Apoptosis; *Ceriops tagal*; dolichol; *Rhizophora mucronata*; sitotoksik

INTRODUCTION

Colorectal cancer, also referred to as colon cancer, is a malignant disease that occurs in the colon, rectum and appendix. Colon cancer is a deadly disease around the world that affects both men and women. Colon cancer is a leading cause of cancer deaths and ranking third for both sexes (Siegel et al. 2017). The incidence of colon cancer is about one million cases per year, with approximately 522,000 deaths per year (Ferlay et al. 2015). The pathophysiology of colon cancer involves several causes, such as changes in histopathologically normal colonic cells through molecular events. Another cause is the formation of adenomatous polyps that develop into colon cancer due

to the process of carcinogenesis. Most colon cancers are adenocarcinomas (Corley et al. 2014). Chemotherapy is one of the treatments used for cancer, but it has unpleasant side effects, such as various skin reactions, hypersensitivity reactions, and peripheral neuropathy (Iwamoto 2013). Most of the currently used anticancer drugs, such as doxorubicin, have undesirable side effects, such as cardiotoxicity and tumour drug resistance (Leonard et al. 2009).

Alternative cancer treatments can be developed by utilizing naturally occurring compounds. Therefore, there is a need for research on alternative treatments for colon cancer to identify effective and efficient treatments based on natural ingredients. Various efforts to find alternative

medicines are underway to identify drugs that have high efficacy but little-side effects for patients. One source that can be exploited for anticancer therapies is herbal medicine (Reddy et al. 2003). One group of plants that can be used for cancer treatment is the mangroves (Bandaranayake 1998). Mangroves are well known for producing secondary metabolite compounds, mainly isoprenoid. Polyisoprenoids are not toxic and are known to show some pharmacological activity as anticancer (Kuznecovs et al. 2007), antidyslipidemic (Singh et al. 2007), anti-influenza and antiviral agents (Safatov et al. 2005). In previous studies, polyisoprenoids (> C30) have been isolated and characterized from mangrove forests in Iriomote Island, Japan, and North Sumatra, Indonesia (Basyuni et al. 2018, 2017, 2016).

Rhizophora mucronata and *Ceriops tagal* are two of the most widespread plants in Indonesia. *R. mucronata* has various benefits as an antiviral (Premanathan et al. 1996) and has antibacterial, cytotoxic, analgesic and diuretic activities (Howlader et al. 2013). *C. tagal* also has various benefits such as antiviral (Sudheer et al. 2011), antitumour (He et al. 2007), antimicrobial (Dhas et al. 2013) and antihyperglycaemic activity (Tiwari et al. 2008). These studies suggest that polyisoprenoids from *R. mucronata* and *C. tagal* leaves can act as chemopreventive agents, and they are also some of the most important mangrove species to be investigated.

This research aimed to examine the cytotoxic activity of polyisoprenoids from *R. mucronata* and *C. tagal* leaves against WiDr colon cancer cell lines. These compounds inhibited the cell cycle and induced apoptosis of WiDr cells through the inhibition of Bcl-2 and cyclin D1 proteins. The results of this study are expected to be the basis for the development of polyisoprenoids from *R. mucronata* and *C. tagal* leaves as anticancer agents for colon cancer.

MATERIALS AND METHODS

PLANT MATERIALS AND ISOLATION OF POLYISOPRENOIDS

The leaves of *R. mucronata* and *C. tagal* were obtained from Lubuk Kertang, Langkat, North Sumatera province, Indonesia. The isolation of polyisoprenoids was performed as previously described (Arifiyanto et al. 2017; Basyuni et al. 2017, 2016). The leaves of *R. mucronata* and *C. tagal* were dried at room temperature for a week and ground into powder. The dried leaves powder (500 g) extracted with a mixture of chloroform methanol with a ratio 2:1 (2 chloroform: 1 methanol, v/v) solvent for 48 h. The lipid extract from the leaves was saponified at 65°C for 24 h in 86% ethanol containing 2 M KOH. The nonsaponifiable lipids were extracted with hexane, and the organic solvent was evaporated and re-dissolved in hexane.

CELL LINE AND CULTURE CONDITIONS

WiDr cells (colon adenocarcinoma) were kindly provided by the Parasitology Laboratory, Faculty of Medicine,

Gadjah Mada University, Yogyakarta, Indonesia. The cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% (v/v) foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 2% penicillin-streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 0.5% fungizone (Sigma-Aldrich, St. Louis, MO, USA), in a 37°C incubator with 5% CO₂ (Heraeus).

PREPARATION OF STOCK SOLUTION

Respectively 50 mg polyisoprenoids from *R. mucronata* and 50 mg polyisoprenoids from *C. tagal* was dissolved in 10 mL of RPMI 1640 medium with the help of 1 mL of DMSO (Sigma-Aldrich, St. Louis, MO, USA) to obtain a concentration of 5000 µg/mL. With a multilevel dilution scheme, a range of concentrations was obtained (15.625, 31.25, 62.50, 125, 250 and 500 µg/mL).

CYTOTOXICITY TEST

WiDr cells were grown in 96-well microplates to obtain a density of 5×10^3 cells/well and incubated at 5% CO₂ and 37°C for 24 h to achieve confluent growth. Afterwards, the diluted test samples with the co-solvent DMSO were added to the medium and the cells were incubated at 37°C in 5% CO₂ for 24 h. At the end of the incubation, the medium containing the extracts was discarded and the cells were washed with PBS. Next, 100 µL of RPMI culture medium and 10 µL of MTT (Sigma-Aldrich, St. Louis, MO, USA) at a concentration of 5 mg/mL were added to each well. The cells were incubated for 4-6 h in 5% CO₂ at 37°C. The MTT reaction was stopped with 10% sodium dodecyl sulphate (Sigma-Aldrich, St. Louis, MO, USA) in 0.01 N HCl (Merck, Darmstadt, Germany) and the plate was then wrapped and left for overnight at room temperature. The absorption was read on an ELISA reader (Benchmark Bio-Rad, Hercules, CA, USA) at 595 nm wavelength. The data obtained in the form of the absorbance of each well were converted into the percent cell viability. Then, to recover linearity between the log concentration and cell viability, the IC₅₀ value was calculated using probit analysis in SPSS.

MEASUREMENT OF APOPTOTIC CELLS AND CELL CYCLE DISTRIBUTION USING FLOW CYTOMETRY

WiDr cells were grown on 6-well plates with each well containing 1×10^6 cells in a 5% CO₂ incubator at 37°C for 24 h. After incubation for 24 h, extracts at concentrations of 1 IC₅₀, ½ IC₅₀, 1/5 IC₅₀ and 1/10 IC₅₀ were added to each well and the plates were incubated under the same conditions as previously described. Then, the medium was removed from each well and placed in 15 mL conical tubes and the cells were washed with PBS. Trypsin (250 µL) was added to each well and the cells were incubated for 3 min in the CO₂ incubator; then, the liquid was placed in an Eppendorf tube and centrifuged at 2500 rpm for 5 min. The cells in each Eppendorf tube were washed with cold PBS by centrifugation at 1500 rpm. For apoptotic testing,

annexin V (BioLegend, San Diego, CA, USA) and propidium iodide (BioLegend, San Diego, CA, USA) were added, while for cell cycle testing, added only propidium iodide and incubated the cells for 10 min in the dark. The flow cytometry analysis was performed by using FACSVerse (BD Biosciences) after less than 1 h. The results were analysed using CellQuest Pro software.

IMMUNOCYTOCHEMISTRY ASSAY

WiDr cells were grown in a 24 well-plate with a coverslip whereby in each well 5×10^4 cells/well were added and then grown in a 5% CO₂ incubator at 37°C for 24 h. Then, added an extract at a concentration of 1 IC₅₀ and incubated the cells further. After 24 h, the cells were washed with PBS and cold methanol was added. Then, added 3% H₂O₂ in PBS, pH 7.4, to each of the wells, followed by blocking buffer and incubated the cells for 1 h. Then, each well was incubated for at least 1 h with the relevant primary antibody (human anti-Bcl-2/anti-cyclin D1) (Santa Cruz Biotechnology, Dallas, TX, USA) at a 1:100 dilution in PBS with 1% FBS. Secondary antibody a biotinylated anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA, USA) was then added to each well and the plates were incubated at room temperature for 10 min and then washed with PBS. A 3,3'-diaminobenzidine solution, as the chromogen, was added to the cells for 5 min. Next, washed the plates with distilled water, stained the cells with haematoxylin and visualized the colour reaction. Bcl-2 and cyclin D1 protein expression was observed using an inverted light microscope (Olympus, Tokyo, Japan) and documented.

DATA ANALYSIS

Data were presented as the mean \pm SD of at least three independent experiments. Data were analysed by one-way analysis of variance (ANOVA) followed by Duncan's test for comparisons of all treatments. All statistical analyses were performed using SPSS version 23. Differences were considered significant at $p < 0.05$.

RESULTS

EFFECT OF POLYISOPRENOIDS FROM *R. MUCRONATA* AND *C. TAGAL* LEAVES ON CELL VIABILITY

A cytotoxic test was performed to determine the toxicity potential of polyisoprenoids from *R. mucronata* and *C. tagal* leaves expressed as the IC₅₀. Treatment of WiDr cells with polyisoprenoids from *R. mucronata* and *C. tagal* leaves extract over a concentration range of 15.625, 31.25, 62.525, 125, 250 and 500 $\mu\text{g/mL}$ showed the IC₅₀ values of polyisoprenoids from *R. mucronata* and *C. tagal* leaves to be $278 \pm 5.77 \mu\text{g/mL}$ and $276 \pm 9.54 \mu\text{g/mL}$, respectively. Polyisoprenoids from *R. mucronata* and *C. tagal* leaves showed a dose-dependent inhibitory effect on WiDr cells after 24 h of treatment (Figure 1).

EFFECTS OF POLYISOPRENOIDS FROM MANGROVE LEAVES ON APOPTOSIS AND CELL CYCLE ARREST OF WIDR CELLS

Staining with annexin V and propidium iodide to detect apoptosis using an Annexin V-FITC Apoptotic Kit showed increased apoptosis in WiDr cells exposed to polyisoprenoids from *R. mucronata* and *C. tagal* leaves for 24 h compared with control cells (Figure 2). The percentage of dead cells and the rate of apoptosis increased in a dose-dependent fashion.

To investigate the cytotoxic mechanisms of polyisoprenoids from *R. mucronata* and *C. tagal* leaves, the cell cycle distribution of WiDr cells treated with various concentrations of polyisoprenoids from *R. mucronata* and *C. tagal* leaves was examined by a FACScan flow cytometer. Our results showed that the number of WiDr cells arrested in G0/G1 increased after exposure to polyisoprenoids from *R. mucronata* and *C. tagal* leaves compared with that of control cells (Table 1).

INHIBITION OF BCL-2 AND CYCLIN D1 PROTEIN EXPRESSION

As shown in Figure 3, immunocytochemistry analysis of Bcl-2 and cyclin D1 protein expression in WiDr cells (5×10^4 cells/mL) treated with polyisoprenoids from *R. mucronata* and *C. tagal* leaves resulted in down-regulation of Bcl-2 and cyclin D1 compared with control cells.

Based on the immunocytochemical test results, we confirmed the semi-quantitative analysis. Polyisoprenoids from *R. mucronata* and *C. tagal* leaves significantly suppressed Bcl-2 and cyclin D1 expression in WiDr cells ($p < 0.05$) (Table 2). These data suggested that polyisoprenoids from *R. mucronata* and *C. tagal* leaves inhibited the expression of Bcl-2 and cyclin D1 in WiDr cells.

DISCUSSION

The cytotoxicity test in this study was conducted to determine the potential toxicity of polyisoprenoids from *R. mucronata* and *C. tagal* leaves against the colon cancer WiDr cell line. The cytotoxic activity of polyisoprenoids from *R. mucronata* and *C. tagal* leaves was determined by MTT method by looking at cell viability which one of anticancer activity test based on cell ability to survive exposure to toxic compounds (Ulukaya et al. 2008). The cytotoxic nature is a major step in the discovery of new anticancer drugs derived from nature (Harvey et al. 2015). In this study as shown in Figure 1, administration of polyisoprenoids from *R. mucronata* and *C. tagal* leaves can cause death of colon cancer cell WiDr at highest dose is $278 \pm 5.77 \mu\text{g/mL}$ and $276 \mu\text{g/mL} \pm 9.54$, respectively. This suggests that the higher concentration of the test samples given the smaller percentage of cancer cell life and the greater the toxicity.

Polyisoprenoids contained in the leaves of *R. mucronata* and *C. Tagal* were shown cell cycle arrest and induced apoptosis as shown through flow cytometry.

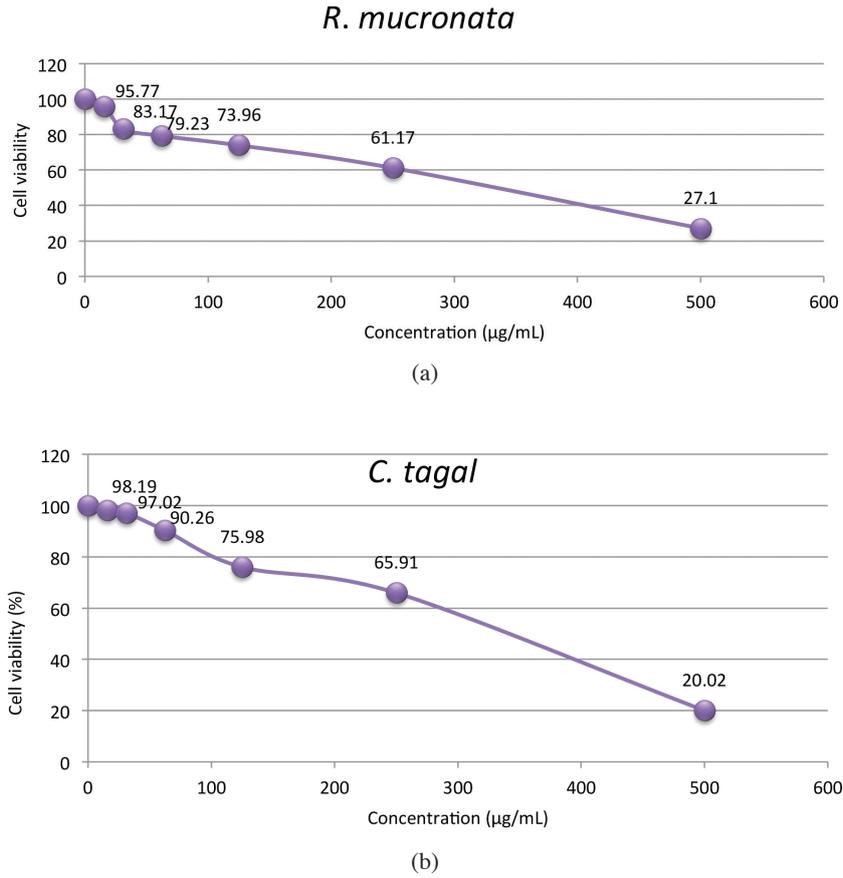
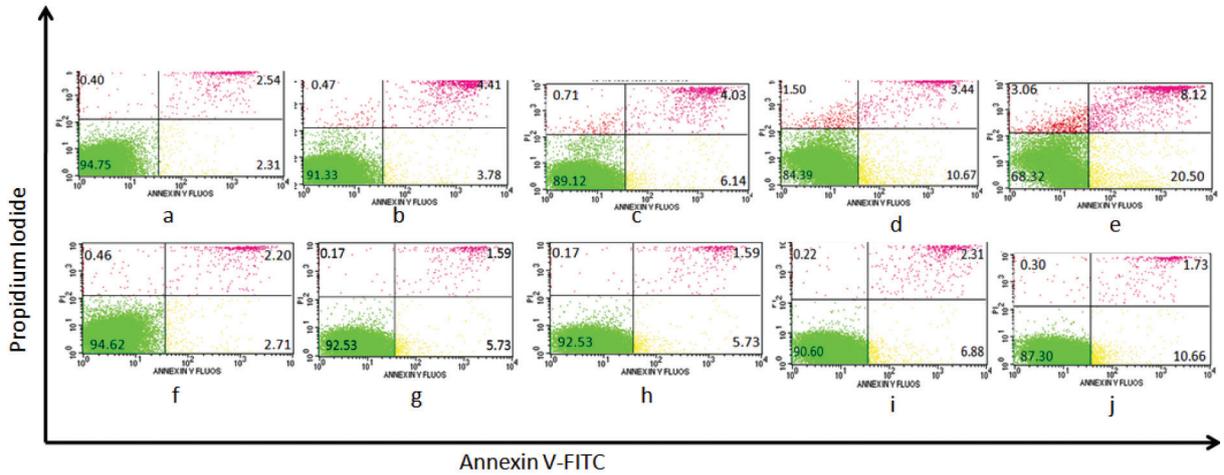


FIGURE 1. Percentage of growth inhibition of WiDr cells after treatment with polyisoprenoids from *R. mucronata* and *C. tagal* leaves (a) Polyisoprenoids from *R. mucronata* leaves (b) Polyisoprenoids from *C. tagal* leaves



PRM= Polyisoprenoids from *R. mucronata*; PCT= Polyisoprenoids from *C. tagal*. Each value represents the mean of three independent experiments

FIGURE 2. Apoptosis assay using flow cytometry after annexin V-FITC and propidium iodide (PI) staining (a) Untreated cells, (b) PRM at 27.8 µg/mL, (c) PRM at 55.6 µg/mL, (d) PRM at 139 µg/mL, (e) PRM at 278 µg/mL, (f) Untreated cells, (g) PCT 27.6 µg/mL, (h) PCT 55.2 µg/mL, (i) PCT 138 µg/mL, (j) PCT 276 µg/mL

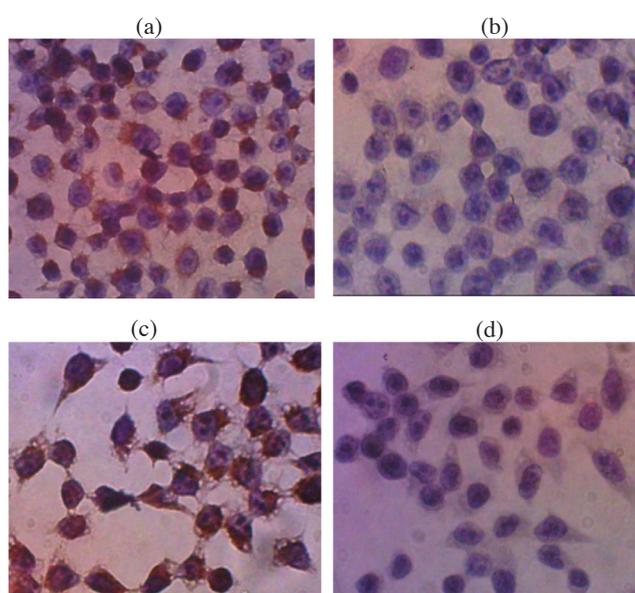
The inhibition of cell cycle is an important strategy for controlling the growth of cancer cells (Qui et al. 2011). From the results of this study, cell accumulation at the G0/G1 phase was observed in WiDr cells treated with

polyisoprenoids from *R. mucronata* and *C. tagal* leaves (Table 1). The inhibition of cell cycle at phase G0/G1 may be contributed by the presence of polyisoprenoids in leaves of *R. mucronata* and *C. tagal* (Elson et al. 1999). Mo

TABLE 1. Cell cycle distribution of WiDr cells after treatment with polyisoprenoids from *R. mucronata* and *C. tagal* leaves

Treatment	Concentration µg/mL	% Cells in each phase		
		G0/G1	S	G2/M
Control	0	64.53	18.20	17.00
Polyisoprenoids from <i>R. mucronata</i> leaves	27.8 (1/10 IC ₅₀)	64.93 ± 0.53	13.73 ± 0.36	19.19 ± 0.64
	55.6 (1/5 IC ₅₀)	67.42 ± 0.66	13.32 ± 0.52	20.77 ± 0.61
	139 (1/2 IC ₅₀)	66.23 ± 0.42	27.35 ± 0.39	8.27 ± 0.41
	278 (1 IC ₅₀)	66.38 ± 0.71	18.87 ± 0.42	14.93 ± 0.53
Polyisoprenoids from <i>C. tagal</i> leaves	27.6 (1/10 IC ₅₀)	69.11 ± 0.59	14.73 ± 0.38	19.19 ± 0.61
	55.2 (1/5 IC ₅₀)	73.64 ± 0.35	12.15 ± 0.54	20.77 ± 0.46
	138 (1/2 IC ₅₀)	70.60 ± 0.51	16.10 ± 0.44	8.27 ± 0.69
	276 (1 IC ₅₀)	68.51 ± 0.38	17.40 ± 0.61	14.93 ± 0.57

Data are presented as the mean ± SD, n=3



Brown colour: Bcl-2 and cyclin D1 expression

FIGURE 3. The observation of Bcl-2 and cyclin D1 expression in cells by immunocytochemistry under a light microscope at 400× magnification (a) Untreated cells (Bcl-2 negative control), (b) cells treated with 278 µg/mL polyisoprenoids from *R. mucronata* leaves, (c) untreated cells (cyclin D1 negative control), and (d) cells treated with 276 µg/mL polyisoprenoids from *C. tagal* leaves

and elson (2004) demonstrated isoprenoid synergistically inhibits human DU145 and LNCaP prostate carcinoma and murine B16 melanoma cells. This isoprenoid induces G1 arrest and initiates apoptosis and differentiation, an effect associated with modulating the cellular signal pathway by either modulating gene expression, suppressing translational signal processing and post-translational growth factor receptors, or altering diacylglycerol signals (Mo & Elson 2004). Isoprenoid also induces G1 growth capture in GBM cells (A172, U87 and U251) and breast cancer cells (MDA-MB-231, 432, 435) (Jiang et al. 2014). Polyisoprenoids from *R. mucronata* and *C. tagal* proven caused cancer cells die. Polyisoprenoids from *R. mucronata* and *C. tagal* leaves has been reported to contain 100% dolichol (C75-C90) but no polyprenol detected (Basyuni et al. 2017). Treatment with dolichol significantly

interferes with the function of Ras and Rho GTPase family, with inhibition of cyclin-dependent kinases (CDKs) and activation of CDK inhibitors (Jakobisiak & Golab 2003). Dolichol is one of the derivatives of mevalonic acid. Mevalonic acid is a precursor in the biosynthesis of isoprenoid compounds, enabling proper functional and cellular refinement of many proteins such as Ras and Rho (Wiemer et al. 2009). In several previous studies dolichol significantly delayed growth arrest in human breast cancer cells Hs578T and MDA231 (Larsson 1994).

In this study, treatment of cancer cells (the colon cancer cell line WiDr) with polyisoprenoids from *R. mucronata* and *C. tagal* leaves suppressed the expression of Bcl-2 and cyclin D1 protein (Table 2; Figure 2) and induced apoptosis (Figure 1). Previous studies of isoprenoid have been shown to induce apoptosis (Holstein & Hohl 2004;

TABLE 2. Number of cells that expressed Bcl-2 and cyclin D1

Treatment	% Expression of Bcl-2	% Expression of cyclin D1
Untreated	63.18 ± 10.63	72.16 ± 8.51
Polyisoprenoids from <i>R. mucronata</i> leaves	32.96 ± 5.48*	20.83 ± 4.83*
Polyisoprenoids from <i>C. tagal</i> leaves	37.16 ± 4.29*	32.93 ± 5.17*

Data are presented as the mean ± SD, $n=3$. * significant difference compared with untreated cells ($p < 0.05$)

Sakagami et al. 2000). One such group of phytochemicals the terpenoids is found in nature. Terpenoids, also referred to as terpenes or isoprenoid, are phytochemicals present in *R. mucronata* and *C. tagal* leaves including lupanes, dammaranes, oleans and dolabranes (Nebula et al. 2013; Zhang et al. 2005). Some of these compounds are known to provide an overview of the antitumor potential of these agents against various cancers (Bishayee et al. 2011; Laszczyk & Pentacyclic 2009; Liby et al. 2007; Petronelli 2009; Setzer & Setzer 2003). One of the derivatives lupanes is found in the leaves of *R. mucronata* and *C. tagal* that causing simultaneous apoptotic induction, decreased Bcl-2 and cyclin D1 and increased Bax gene expression (Bishayee et al. 2011). The transition of cell cycle from one phase to another depends on the complex regulation of cyclin-dependent kinases (CDKs), the active agent that phosphorylates substrate involved in the cell cycle. The G1 phase to S phase of the cell cycle is controlled by a number of cyclin members, including cyclin D1 (Otto & Sicinski 2017). Previous research has shown that excessive expression of cyclone D is seen in many human cancers (Vermuelen et al. 2003). Inhibition of cyclin D1 expression is considered an excellent target in searching for possible chemotherapy and chemo preventative agents.

CONCLUSION

In summary, the results obtained from this study showed that polyisoprenoids from *R. mucronata* and *C. tagal* leaves have cytotoxic effects via apoptosis and arresting the human colon cancer cell line WiDr at the G0/G1 phase. Polyisoprenoids from *R. mucronata* and *C. tagal* leaves appear to be apoptosis-inducing agents and to cause arrest in G0/G1 phase by down-regulating the protein expression of Bcl-2 and cyclin D1, which both play an essential role in apoptosis and cell cycle arrest. These present data can facilitate the development of new treatments for colon cancer and other malignancies that are difficult to treat and are life-threatening.

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