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Research Article

THE INHIBITION OF POLYISOPRENOIDS FROM *NYPA FRUTICANS* LEAVES ON CYCLOOXYGENASE 2 EXPRESSION OF WIDR COLON CANCER CELLS

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ABSTRACT

Objective: The objective of the study was to investigate the inhibitory activity of polyisoprenoids from *Nypa fruticans* leaves on the expression of cyclooxygenase 2 (COX-2) against colon cancer cells.

Methods: Anticancer activity performed was tested by dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method on colon cancer cell WiDr. The expression of COX-2 was observed by the immunocytochemistry method.

Result: Polyisoprenoids from *N. fruticans* leaves exhibit anticancer activity on WiDr cells through inhibition of COX-2 expression with IC_{50} 180.186±7.16 µg/ml.

Conclusions: This study showed that polyisoprenoids from *N. fruticans* leaves promise chemopreventive agents for colon cancer through COX-2 inhibition.

Keywords: Cytotoxic, Polyisoprenoids, *Nypa fruticans*, Cyclooxygenase-2.

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INTRODUCTION

Cancer is one of the primary causes of death worldwide. Lung cancer, liver, stomach, colon, and breast cancer are the biggest causes of cancer deaths annually [1]. Colon cancer is the third most frequently diagnosed cancer-causing health problems and the fourth leading cause of cancer death worldwide. There is an increasing number of colon cancer patients each year. The incidence of colon cancer mainly occurs because it is influenced by socioeconomic level, behavior, and lifestyle [2]. Low physical activity and high fat consumption cause easy absorption of carcinogen compounds in the body and slow the transport time to the intestine which can lead to increased risk of colon cancer [3]. Increased incidence of colon cancer or colon cancer mortality was found in countries with low Human Development Index (HDI) levels, especially in Eastern Europe, Asia, and South America. On the contrary, the incidence of colon cancer and death from colon cancer has stabilized or decreased in a number of high HDI, such as the United States, Australia, New Zealand, and some countries in Western Europe [4].

Arachidonic acid metabolism is thought to play a significant role in the occurrence of carcinogenesis [5]. This metabolic pathway is associated with the formation of prostanoids. Prostanoids belong to the subclass of eicosanoids that convert to prostaglandins, thromboxane, and prostacyclin [6]. Cyclooxygenase (COX) is a critical enzyme in the conversion of arachidonic acid into prostaglandins [7].

The rapid development of the pharmaceutical industry in creating synthetic drugs currently aims to prevent and treat cancer, but most are toxic. Treatment technologies such as surgery, radiation, and chemotherapy are performed by administering anticancer drugs. This type of therapy makes patients experience nausea, vomiting, and hair loss [8]. Recommended companion therapy is the use of compounds that can reduce the effects of growth factors that may stimulate the rapid growth of cancer. Therapies aimed at enhancing the immune response to cancer also began to be widely tested in clinical studies. One component that has a high chance of being used in cancer therapy is medicinal plants [9]. Medicinal plants can be used for various functions and one of them as an inhibitor of COX-2 protein [10].

Mangrove has activity as a medicinal plant, and only a few have been explored [11]. Mangrove is famous for producing secondary metabolite compounds mainly from isoprenoid compound groups. The polyisoprenoids compound consists of two families, namely polyprenol and dolichol, polyprenol is known to have some pharmacological activity such as anticancer [12], antidyslipidemic [13], anti-influenza, and antiviral activity [14].

The distribution and diversity of polyisoprenoids compound in mangrove forests of Iriomote Island, Japan and North Sumatra, Indonesia have been reported by Basyuni *et al.* [15,16]. The promising mangrove species that potentially exhibit anticancer activity are *Nypa fruticans* [17]. However, the cytotoxic activity of polyisoprenoids from *N. fruticans* leaves on colon cancer cells with COX-2 as a target molecule is unclear. Therefore, this study aims to examine the cytotoxic activity of polyisoprenoids from *N. fruticans* leaves on high-frequency colon cancer cells expressing COX-2.

METHODS

Plants and isolation of polyisoprenoids

The sample used in this research is the leaves of *N. fruticans* obtained from the Lubuk Kertang Village, North Sumatera Province in February 2017. This plant is determined in Indonesia Institute of Science Research Center for Biology Bogor. A specimen voucher has been deposited there (No.354/IPH.1.01/If.07/IV/2017).

Preparation of polyisoprenoids from *N. fruticans* leaves was performed as described previously [15,16,18]. The leaves of *N. fruticans* were dried for 1–2 days at 60°C and then crushed to a powder. The leaves powder was extracted with mixture chloroform:methanol (2:1, v/v) for 48 h, then filtered and the remaining is a lipid extract in chloroform. The lipid extract in the chloroform of the leaves was saponified at 65°C

for 24 h in 86% ethanol containing KOH 2 M. The unsaponifiable lipids were further dissolved with n-hexane, and the solvent was evaporated.

Cell culture and conditions

The WiDr cell used in this study is a collection of the Parasitology Laboratory of Gadjah Mada University, Jogjakarta, Indonesia. WiDr cells were grown in Roswell Park Memorial Institute 1640 (RPMI 1640) was purchased from Gibco (Carlsbad, CA, USA) containing FBS 10% (v/v) was purchased from Sigma-Aldrich (St. Louis, MO, USA), penicillin antibiotics 100 units/ml and streptomycin 100 µg/ml were purchased from Gibco (Carlsbad, CA, USA), phosphate buffer saline (PBS) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and incubated in an incubator at 37°C, 5% CO_2.

Cytotoxic test

The cytotoxic activity of polyisoprenoids from *N. fruticans* leaves was performed according to the method of Mosmann [19] using a tetrazolium microculture (MTT) test method with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. The MTT powder was purchased from Sigma-Aldrich (St. Louis, MO, USA). The WiDr cells were distributed into 96 wells plate (Nunc) with a total of 5×10^3 cells per well and incubated with the test sample in various concentrations (15.625, 31.25, 62.525, 125, 250, and 500 µg/ml) using DMSO solvent and incubated in incubator (Heraeus) for 24 h at 37°C with 5% CO_2. At the end of incubation, into each well was added 100 µL MTT in RPMI 1640 medium. Then, the plate was incubated again for four h at 37°C with 5% CO_2 until formed formazan crystals, see under an inverted microscope (Olympus, Tokyo, Japan). The living cells react with the MTT to form a purple color. After 4 h, the MTT reaction was discontinued by adding 10% SDS reagent (Sigma-Aldrich, St. Louis, MO, USA) 100 µL at each well then incubated overnight at room temperature covered by aluminum foil. Absorption is read by ELISA reader (Bio-Rad) at 595 nm wavelength.

Percent of inhibition is calculated based on the following equation:

$$\%\text{inhibition} = \frac{\text{Sample treatment absorbance} - \text{Medium absorbance}}{\text{Untreated cells absorbance} - \text{Medium absorbance}} \times 100$$

The correlation between the percentage of inhibition and the concentration of polyisoprenoids from *N. fruticans* leaves was plotted, and IC_{50} was calculated through their interpolation through regression equation, IC_{50} was the concentration of polyisoprenoids from *N. fruticans* leaves inhibiting the growth of 50% treated cells and cell morphology becomes abnormal.

Observation of COX-2 protein expression with immuno cytochemistry

Analysis of inhibition of COX-2 protein expression using immunocytochemistry methods was performed as described previously by Galgano with slight modifications [20]. The WiDr cells were seeded on 24-wells plate, first included coverslip on each well. Cells were seeded with a density of 5×10^4 cells/well, incubated for 24 h at 37°C with 5% CO_2. Furthermore, the polyisoprenoids from *N. fruticans* leaves in various concentrations (90, 180 µg/ml) were added to the cells and incubated for 24 h with 5% CO_2. The cells were washed with PBS. Then, cells were placed in the glass object for 5 min and added hydrogen peroxidase to the glass object and incubated at room temperature for 10–15 min. The cells washed twice with PBS and onto the glass object then added COX-2 monoclonal antibody (human anti-COX-2) (Santa Cruz Biotechnology, Dallas, TX, USA), and incubated 1 h at room temperature. The cells were washed 3 times with PBS, then added with secondary antibody (Biotinylated universal secondary antibody), and incubated at room temperature for 10 min, and washed twice with PBS. As chromogen, added 3,3-diaminobenzidine, then incubated for 3–8 min. The cells were washed with distilled water and added hematoxylin solution and incubated for 5 min at room temperature. The COX-2 expression was observed under light microscope and documented. Data were expressed as a percentage of cells expressing the COX-2 in 10 fields of view in each treatment group. The COX-2 expression appears brown in the cell nucleus and cytoplasm whereas cells without protein expression appeared purple.

STATISTIC ANALYSIS

Data were expressed as the mean±standard deviation of triplicate experimental value (n=3). The analysis was performed using one-way ANOVA followed by Duncan's test differences for comparison between control and treatment groups. All statistical analyzes were performed using SPSS for Windows Version 23.

RESULTS

This study examined the cytotoxic effects of polyisoprenoids from *N. fruticans* leaves on colon cancer cell WiDr with an inhibitory observation on COX-2 protein. Cytotoxic effects were tested with MTT test, then measured the absorbance of formazan complex at a wavelength of 595 nm equivalent to the number of living cells. The results of the viability of colon cancer cells after administration of polyisoprenoids from *N. fruticans* leaves in various concentrations are shown in Fig 1. Polyisoprenoids from *N. fruticans* leaves (R^2=0.9158) showed cytotoxic activity in colon cancer cells WiDr depending on concentration. The IC_{50} value of polyisoprenoids from *N. fruticans* leaves was 180,186±7.16 µg/ml.

The linear regression equation can be seen in the comparison graph with live cell percentage. From the results obtained, there is a decrease in the number of living cells based on the increased concentration given. Concentration 250 µg/ml has the best inhibition of cancer cell WiDr with a small percent of living cells. The polyisoprenoids from *N. fruticans* leaves obtained linear regression equation Y=−0.4891x+139.94. From the derived linear regression can be calculated the IC_{50} value. The value of IC_{50} obtained from polyisoprenoids *N. fruticans* leaves is 180.186 µg/ml from the calculation of concentration to live cells percentage.

Effect of polyisoprenoids from *N. fruticans* on COX-2 expression suppression

In this study, observation of protein suppression test of COX-2 has done by immunocytochemistry which then analyzed qualitatively and quantitatively. From the qualitative analysis, it shows that with increasing test extract levels, the positive COX-2 expressing cells are less, indicating that there has been a decrease in COX-2 expression in WiDr cells. Immunocytochemistry coloring results can be seen in Fig. 2. Furthermore, quantitative analysis was done to find out the percentage emphasis of COX-2 protein expression by polyisoprenoids from *N. fruticans* leaves. Quantitative suppression of COX-2 expression is done by calculating the percentage of COX-2 expression. The results of the calculations can be seen in Table 1. In Table 1, it can be seen that the increasing concentration of polyisoprenoid from *N. fruticans* leaves of 90 µg/ml and 180 µg/ml and gives a decrease of COX-2. Expression pressure 36.83±2.01% and 16.42±2.86%, respectively. While the average control of COX-2 suppression was 68.13%±3.07. It has been shown that administration of Polyisoprenoids from N. fruticans leaves 90 µg/ml inhibits Cox-2 protein expression. Similarly, administration of

Table 1: Number of cells that expressed COX-2.

Treatment	% Expression of COX-2
Untreated	68.13±3.07
Polyisoprenoids from *N. fruticans* leaves 90 µg/ml	36.83±2.01*
Polyisoprenoids from *N. fruticans* leaves 180 µg/ml	16.42±2.86*

*Referred to the significant difference to untreated cells ($p<0.05$).
COX-2: Cyclooxygenase-2, *N. fruticans: Nypa fruticans*

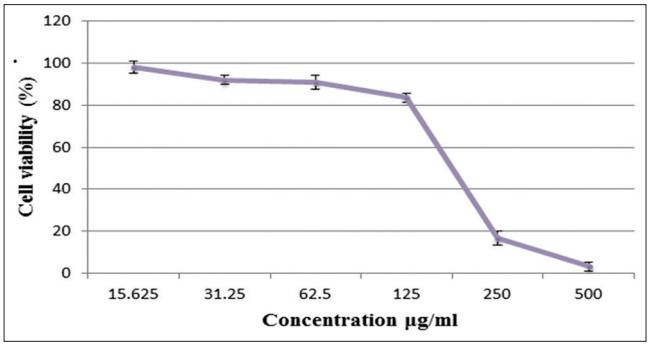

Fig. 1: Effect treatment of polyisoprenoids from Nypa fruticans leaves on colon cancer cells WiDr

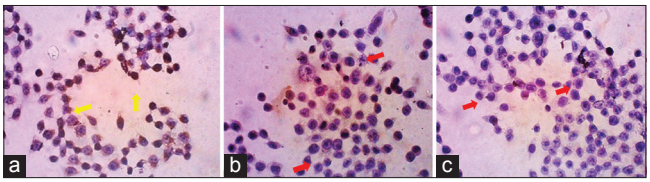

Fig. 2: Immunocytochemistry results of cyclooxygenase-2 (COX-2) on WiDr cells qualitatively. (a). The negative control, the group untreated with COX-2 specific antibody coloring. Visible all cells are brown, showing a positive result against COX-2. (b). Treatment of polyisoprenoids from Nypa fruticans leaves 90 µg/ml. (c). Treatment of polyisoprenoids from *N. fruticans* leaves 180 µg/ml. The yellow arrows showed the positive result of COX-2, the red arrow shows the negative result of COX-2. Observations under a light microscope with the magnification of ×40

Polyisoprenoids from N. fruticans leaves 180 µg/ml, shows that purple cells are clearer than controls. Furthermore, the percentage of COX-2 expression suppression data in each treatment and control group was statistically analyzed using one-way ANOVA parametric statistical analysis followed by Duncan's test. Statistical analysis showed that COX-2 protein expression suppression in various treatment and control groups gave significant difference ($p<0.05$). The increase in the level of the given test extract was able to provide significantly increased percentage COX-2 expression when compared with the control.

DISCUSSION

This study evaluated the anticancer activity of polyisoprenoids from *N. fruticans* leaves on colon cancer cells WiDr. Polyisoprenoids from *N. fruticans* leaves tested against colon cancer cell WiDr have IC_{50} value 180.186±7.16 µg/ml. The IC_{50} value in ranging from 100 to 300 is considered as a weak anticancer activity, whereas the IC_{50} value over than 300 is regarded as inactive compounds [21]. This suggests that polyisoprenoids from *N. fruticans* leaves have a potent cytotoxic effect on WiDr cells.

This study evaluated the anticancer activity of polyisoprenoids from *N. fruticans* leaves on colon cancer cells WiDr through inhibition of COX-2 expression. Many studies have been conducted both *in vitro* and *in vivo* on the activity of COX-2 inhibitors as cancer therapy [22,23]. The inhibition of COX-2 protein is an effective strategy to screen for chemopreventive agents in colon cancer [24]. Our results conclude that polyisoprenoids from *N. fruticans* leaves have anticancer activity in WiDr cells by inhibiting COX-2 expression. The results of this study support previous studies on the anticancer activity of *N. fruticans* leaves have cytotoxic effects on breast cells (MCF-7) and hepatocellular carcinoma (HepG2) [25]. *N. fruticans* leaves have been reported to contain polyisoprenoids, with majority dolichol compounds, no polyprenol detected [16]. Secondary metabolite compounds mainly from the group of isoprenoid also known as terpenoids, affect anticancer [26]. Terpenoids are compounds that have a high antitumor activity that has been tested through the ability to block nuclear factor-kappa B, induce apoptosis, activate transcription, and angiogenesis. Although the mechanism of anticancer in colon cancer cells is unclear, terpenoids can be useful in the treatment of various types of cancer [27]. COX-2 inhibitory activity by polyisoprenoids from *N. fruticans* leaves with immunocytochemistry. The observed expression of COX-2 (Fig. 2) showed COX-2 expression due to polyisoprenoids treatment from *N. fruticans* leaves decreased compared to control. The inhibitory activity of COX-2 expression by polyisoprenoids from *N. fruticans* leaves may be due to inhibition of nuclear factor-kappa B. Terpenoids contained in *N. fruticans* leaves may inhibit NF-κB and Iκ-Bα [28]. This present results in a decrease of COX-2 expression.

COX-2 is an enzyme responsible for inflammatory response and prostaglandin production [29] and high expression in tumor cells [30]. Prostaglandins are reported to play a role in vascular endothelial growth factor upregulation and induce angiogenesis in tumor cells [31]. Thus, it is suspected that the activity polyisoprenoids from *N. fruticans* leave as anticancer is mediated by COX-2 inhibition as one of its mechanisms. The active compounds are primarily responsible for all these effects have not been further investigated, but it is suspected that the active compound was terpenoids. Terpenoids play a role in the regulation of the COX-2 expression [32]. Previous studies have shown similar relationships between terpenoids and anticancer effects [33,34]. The triterpenes and sterols were reported to exhibit antioxidant and anticancer properties [35]. The COX-2 expression assessment provides information on the prognosis and determines the treatment modalities. Treatment using COX-2 inhibitors can be done when in part encountered excessive COX-2 expression. The angiogenesis process as an indicator of the aggressiveness of some neoplasms is also essential in scores on the growth of colon cancer. Further research is needed to investigate the effects of polyisoprenoids from *N. fruticans* leaves in suppressing COX-2 expression. This experiment is expected to enrich the scientific evidence of polyisoprenoids activity from *N. fruticans* leaves as an anticancer, specifically to colon cancer.

CONCLUSIONS

Polyisoprenoids from *N. fruticans* leaves promise as a chemopreventive agent in colon cancer. Our data showed that polyisoprenoids from *N. fruticans* leaves inhibit expression of COX-2. Therefore, inhibition of COX-2 is one of the targeted therapy options developed for the treatment and prevention of cancer. Studies relating to the discovery of COX-2 inhibitor compounds still need to be developed to achieve maximum inhibitory effect but with minimal skill effect.

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AUTHOR'S CONTRIBUTION

Collection of *N. frutican* leaves: DPS, MB, and RW. Performed the experiments and analyzed the data: DPS and RW. Draft preparation: DPS. Paper writing: DPS, MB, RW, and PAZH. All of the authors read and approved the final manuscript.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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