One component that has a high chance of being used in cancer therapy is cyclooxygenase 2 (COX-2) against colon cancer cells.

The rapid development of the pharmaceutical industry in creating 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 Sumatra 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.35/4/IPH.1.01/II/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

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].
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% CO₂.

**Cytotoxic test**

The cytotoxic activity of polysoprenoids 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 5×10⁴ cells per well and incubated with the test sample in various concentrations (15.625, 31.25, 62.5, 125, 250, and 500 μg/ml) using DMSO solvent and incubated in incubator (Herneus) for 24 h at 37°C with 5% CO₂. The end of incubation, into each well was added 100 μL MTT in RPMI 1640 medium. Then, the plate was incubated again for 4 h at 37°C with 5% CO₂ 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 polysoprenoids from *N. fruticans* leaves was plotted, and IC₅₀ was calculated through their interpolation through regression equation, IC₅₀ was the concentration of polysoprenoids 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 5×10⁴ cells/well, incubated for 24 h at 37°C with 5% CO₂. Furthermore, the polysoprenoids from *N. fruticans* leaves in various concentrations (90, 180 μg/ml) were added to the cells and incubated for 24 h with 5% CO₂. The cells were washed with PBS. Then, cells were placed in the glass object for 5 min and added hydrogen peroxide 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 polysoprenoids 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 polysoprenoids from *N. fruticans* leaves in various concentrations are shown in Fig 1. Polysoprenoids from *N. fruticans* leaves R²=0.9158 showed cytotoxic activity in colon cancer cells WiDr depending on concentration. The IC₅₀ value of polysoprenoids 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 polysoprenoids from *N. fruticans* leaves obtained linear regression equation Y=-0.4891x+1.3994. From the derived linear regression can be calculated the IC₅₀ value. The value of IC₅₀ obtained from polysoprenoids *N. fruticans* leaves is 180.186 μg/ml from the calculation of concentration to live cells percentage.

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

In this study, observation of protein suppression test of COX-2 has been 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 polysoprenoids 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 polysoprenoids from *N. fruticans* leaves of 90 μg/ml and 180 μg/ml and gives a decrease of COX-2 Expression pressure 36.65±0.1% 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 Polysoprenoids from *N. fruticans* leaves 90 μg/ml inhibits COX-2 protein expression. Similarly, administration of

<table>
<thead>
<tr>
<th>Table 1: Number of cells that expressed COX-2.</th>
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<tbody>
<tr>
<td>Treatment</td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Untreated</td>
</tr>
<tr>
<td>Polysoprenoids from <em>N. fruticans</em> leaves</td>
</tr>
<tr>
<td>90 μg/ml</td>
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<td>Polysoprenoids from <em>N. fruticans</em> leaves</td>
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*Referred to the significant difference to untreated cells (p<0.05). COX-2: Cyclooxygenase-2, *N. fruticans*: Nypa fruticans
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 side effect.

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AUTHOR'S CONTRIBUTION

Collection of *N. fruticans* 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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