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The inhibition of *Typhonium flagelliforme* Lodd. Blume leaf extract on COX-2 expression of WiDr colon cancer cells

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ABSTRACT

Objective: To determine the inhibition activity of *Typhonium flagelliforme* Lodd. Blume (*T. flagelliforme*) leaf extract on cyclooxygenase 2 (COX-2) expression of colon cancer cells.

Methods: *T. flagelliforme* leaf extract was prepared to macerate in ethyl acetate. *In vitro* anticancer activity was assayed by MTT method on WiDr colon cancer cells. This study applied apoptosis induction assay to investigate the mechanism of cell death using double staining method. COX-2 expression was stained by immunocytochemistry.

Results: *T. flagelliforme* showed anticancer activity and induced apoptosis on WiDr cells through inhibition of COX-2 expression with IC_{50} 70 μg/mL.

Conclusions: This study showed that *T. flagelliforme* is a promising chemopreventive agent for colon cancer through COX-2 inhibition.

1. Introduction

Colon cancer is one of the leading causes of cancer related death in developed countries, and it is a frequently diagnosed cancer in both of male and female [1]. It was also ranked as the third most commonly diagnosed cancer worldwide, especially in the Southeast Asian Nations [2,3]. Colon cancer is known as a disease in industrialized countries, but the pattern is economically changing nowadays. Since its incidence rapidly increases [4,5], many attempts are employed to prevent and cure colon cancer. There are many possible clinical managements of colon cancer treatment, such as surgery, chemotherapy, radiotherapy and adjuvant chemotherapy [5,6]. Many studies had been designed to develop colon cancer treatment by attacking specific targets which is a key strategy to cure colon cancer without endangering normal cells [7–9].

Colon cancer overexpresses cyclooxygenase 2 (COX-2) in both carcinoma and adenoma [10–12]. COX-2, an enzyme that is responsible for converting arachidonic acid to prostaglandins [13], plays an important role in cell proliferation and apoptosis regulation of colon cancer [14]. Prostaglandin E_2, a type of COX-2 product, promotes angiogenesis and stimulates colon cancer growth by preventing apoptosis [15,16]. Thus, COX-2 inhibitors had shown to successfully prevent colon cancer growth and polyp formation [16–21]. Moreover, COX-2 is a specific molecular target for anticancer screenings on colon cancer.

Many studies investigated a natural product activity on colon cancer [22–25], especially addressing COX-2 as molecular target [10,25]. Curcumin, a yellow pigment isolated from turmeric, successfully inhibited COX-2 on WiDr colon cancer cells [21]. Another promising natural product which potentially exhibits anticancer activity on colon cancer is *Typhonium flagelliforme* Lodd. Blume (*T. flagelliforme*). Its leaves contain glycoside flavonoid, isovitexin, as well as alkaloids [26,27]. Previous studies showed that *T. flagelliforme* revealed cytotoxic activity on MCF-7 breast cancer cells [28], and induced apoptosis on murine leukemia WEHI-3 cells [29] and lymphocyte CEM-SS [30]. However, the cytotoxic activity of *T. flagelliforme* on colon cancer cells with COX-2 as the molecular target remains delusive. Therefore, this study investigated cytotoxic activity of

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T. flagelliforme leaf extract on WiDr colon cancer cells that highly express COX-2.

2. Materials and methods

2.1. Plant materials

The leaves of *T. flagelliforme* were harvested from Malang (East Java, Indonesia) in June 2014, and sun-dried after thorough washing. The plant materials were identified in Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada (Reference No. BF/275/Ident/Det/VI/2014).

2.2. Cytotoxic, apoptosis and immunocytochemistry assay materials

WiDr cells were collected from Parasitology Department, Faculty of Medicine, Universitas Gadjah Mada. The cells were maintained in Roswell Park Memorial Institute medium (Gibco) containing fetal bovine serum 10% (v/v) (Gibco) and penicillin-streptomycin 1% (v/v) (Gibco). Dimethyl sulfoxide was used to dissolve stock solution of extract and celecoxib was purchased from Merck. Celecoxib, as a positive control, was prepared from Celebrex®. Cytotoxic assay was measured by MTT (Sigma) method, while the apoptosis assay was examined by ethidium bromide and acridine orange (Sigma) staining. Immunocytochemistry used COX-2 primary antibody purchased from Thermo Scientific Lab Vision, while the universal secondary antibody was derived from Starr Trek Universal HRP Detection System No.901-STUHRP700-090314. All culture plates used in this study were Iwaki® and all tips and microtubes were supplied by Biologix®.

2.3. Extract preparation

The extract was prepared by using maceration method. Dried leaves of *T. flagelliforme* were ground and soaked in ethyl acetate (1:10) for 24 h until all substances were extracted. The liquid extract was slowly evaporated to discard the residual solvent, until viscous extract was obtained.

2.4. MTT cytotoxic assay

Cytotoxic assay was designed based on several previous studies [31–33]. WiDr colon cancer cells were cultured in culture tissue flask until 70%–80% confluent, and then 5×10^3 cells were seeded into 96-well plate. The plate was incubated at 37 °C and under 5% CO_2 for 24 h. The medium was removed and the cells were rinsed twice by using phosphate buffer solution (PBS). The stock solution of extract and celecoxib were prepared to dissolve them into dimethyl sulfoxide and dilute them with the medium into various concentrations. Each concentration (100 μL/well) was added into 96-well plate and measured in triplicates. Later, the plate was incubated at 37 °C and under 5% CO_2 for 24 h. The medium was then removed and 10% MTT containing medium was added into each well. The reaction between MTT and succinate hydrogenase of cells to form formazan needed 4 h. At the end of incubation time, 100 μL sodium dodecyl sulfate was added to each well to dissolve formazan crystals. The plate was incubated in dark room for 12–24 h and formazan crystals were measured by using ELISA reader at a wavelength of 595 nm.

2.5. Apoptosis induction assay

WiDr cells were cultured into coverslips in 24-well plate (5×10^4/well). The cells were adapted at 37 °C and 5% CO_2 for 24 h. The medium was removed and rinsed twice by using PBS. The extract (100 μg/mL) and celecoxib at IC_{50} concentration (68 μmol/L) were added into the plate and incubated in the same condition as previously described. The medium was removed from the cells and rinsed by using PBS. The coverslips were transferred to object glasses, then acridine orange/ethidium bromide was dropped into the coverslips. The cells were immediately observed under fluorescence microscope.

2.6. Immunocytochemistry assay

WiDr cells were seeded in 6-well plate and incubated under 5% CO_2 and 37°C for 24 h. The extract at 100 μg/mL and celecoxib at IC_{50} 68 μmol/L were added to the cells and incubated for further 24 h. At the end of incubation time, cells were harvested and washed by PBS. The cells were suspended in medium, placed and fixed in object glass for 5 min. Hydrogen peroxidase was dropped into the object glass and incubated at room temperature for 10–15 min. The cells were washed twice with PBS and monoclonal antibody of COX-2 was added into the cells and incubated at least for 1 h at room temperature. The cells were washed three times with PBS and added with secondary antibody, incubated at room temperature for 10 min, and washed four times with PBS. The solution of 3,3′-diaminobenzidine, as chromogen, was added to the cells and incubated for 3–8 min. Finally, the cells were washed with distilled water and added with hematoxylin solution followed by 3–4 min incubation. The expression of COX-2 was observed under inverted microscope.

2.7. Data analysis

Cell viability was calculated from MTT data using equation:

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

Cell viability data were analyzed by using linear regressions at four linier points to calculate IC_{50} of extract and celecoxib. Apoptosis induction semiquantitatively counted the number of apoptotic, necrotic and living cells in three different areas of an object glass. Each treatment was performed in triplicate. The number of COX-2 expressing cells was analyzed by *t*-test using Microsoft Excel 2013.

3. Results

This study investigated the cytotoxic effect of *T. flagelliforme* leaf extract on WiDr colon cancer cells by targeting COX-2. Celecoxib, a selective COX-2 inhibitor, was used as a positive control. Dixon *et al.* reported celecoxib was a prospective anticancer on colon cancer [10]. Cytotoxic effect, assessed with

MTT assays, measured the absorbance of formazan complex at 595 nm that equaled to the number of living cells. As presented in Figures 1 and 2, *T. flagelliforme* leaf extract (R^2 = 0.995) and celecoxib (R^2 = 0.954) showed dose dependent cytotoxic activity on WiDr colon cancer cells with IC_{50} 70 µg/mL and 68 µmol/L, respectively.

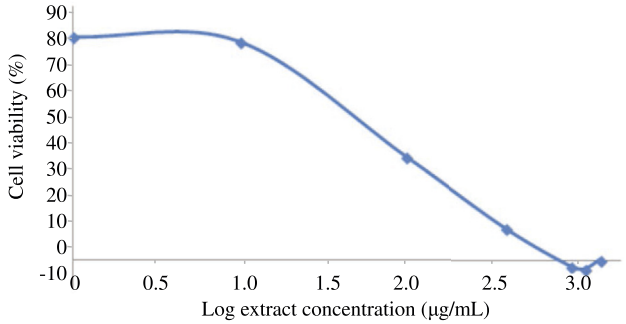

Figure 1. Effect of *T. flagelliforme* leaf extract in various concentrations on WiDr cells viability.

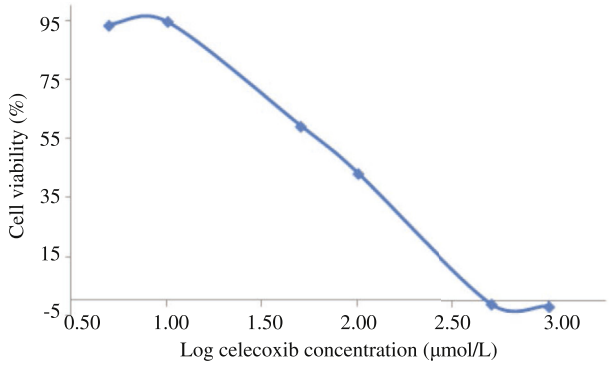

Figure 2. Effect of celecoxib in various concentrations on WiDr cells viability.

Double staining method was employed to determine the mechanism of cells death. *T. flagelliforme* leaf extract induced apoptosis [(65.27 ± 1.27)%] on WiDr cells as well as celecoxib [(88.67 ± 1.23)%] (Table 1). This method also successfully identified apoptosis stage and resulted in insignificant difference to flow cytometry method [34]. In early apoptotic cells, the nuclei showed condensed yellow-green fluorescence by acridine orange. On the other hand, the nuclei of late apoptotic cells showed condensed orange fluorescence by ethidium bromide (Figure 3).

Molecular mechanism of *T. flagelliforme* leaf extract was specifically observed by immunocytochemistry method. COX-2 expressing cells showed brown color while non-expressing cells showed purple color (Figure 4). WiDr cells were stained as intense brown color, but *T. flagelliforme* leaf extract and celecoxib treated cells were stained as purple color. This result indicated that WiDr highly expressed COX-2 but *T. flagelliforme* leaf extract and celecoxib inhibited COX-2 expression. Semi-quantitative analysis of this result confirmed that *T. flagelliforme* leaf extract and celecoxib significantly suppressed COX-2 expression on WiDr cells ($P < 0.05$) (Table 2). This data suggested that cytotoxic activity of *T. flagelliforme* leaf extract on WiDr cells was through COX-2 downregulation.

Table 1
Cell distribution after double staining. %.

Treatment	Apoptosis	Necrosis	Living
TLE	65.27 ± 1.27	14.16 ± 0.48	20.94 ± 1.42
Celecoxib	88.67 ± 1.23	2.23 ± 2.59	9.10 ± 0.00
Untreated cells	1.08 ± 0.00	0.00 ± 0.00	98.90 ± 0.00

Data were expressed as mean ± SD. TEL: *T. flagelliforme* leaf extract.

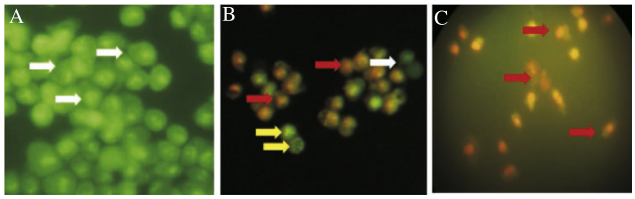

Figure 3. The observation of apoptotic cells on double staining method under fluorescence microscope using 400× magnification.
A: Untreated cells; B: Cells treated 100 µg/mL *T. flagelliforme* leaf extract; C: Cells treated 68 µmol/L celecoxib; White arrow: Living cell; Yellow arrow: Early apoptotic cell; Red arrow: Late apoptotic cell.

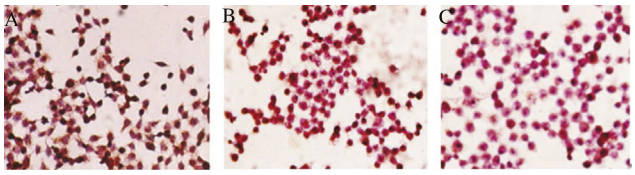

Figure 4. The observation of COX-2 expression cells on immunocytochemistry method under light microscope using 400× magnification.
A: Untreated cells; B: Cells treated 100 µg/mL *T. flagelliforme* leaf extract; C: Cells treated 68 µmol/L celecoxib; Brown color: COX-2 expression.

Table 2
Number of cells that expressed COX-2.

Treatment	Mean ± SD
Untreated cells	69.36 ± 10.20
Extract	39.22 ± 3.63[a]
Celecoxib	14.32 ± 1.24[a]

[a]: Referred to significant difference to untreated cells ($P < 0.05$).

4. Discussion

This study evaluated the anticancer activity of *T. flagelliforme* leaf extract on WiDr colon cancer cells through COX-2 expression. COX-2 produced prostaglandin E_2 that increased proliferation and prevented cells from apoptosis [14,15]. Addressing COX-2 is an effective strategy to screen chemopreventive agents on colon cancer.

Our results concluded that *T. flagelliforme* leaf extract, as well as celecoxib, performed anticancer activity on WiDr cells by inhibiting COX-2 expression. These results have also opened interesting question on whether the inhibition of COX-2 expression of *T. flagelliforme* leaf extract was possible due to the presence of antioxidant compounds. It is well known that the colon is prone to oxidative condition and antioxidant deficiency [35]. Under oxidative stress, COX-2 was highly produced to modulate inflammation and induce carcinogenesis [36]. Pro-oxidant stress factor such as cigarette smoking, a diet high in

n-6 polyunsaturated fatty acid, and alcohol consumption may increase genotoxic damage of intestine [37].

On the other hand, the presence of antioxidant suppresses reactive oxygen species (ROS) formation and inhibits cancer cell proliferation [38,39]. A study by La Vecchia *et al.* showed an inverse correlation between antioxidant intakes and the risk of colorectal cancer [40]. Another study reported that low antioxidant status could support cancer development and therefore antioxidant supplement may be beneficial for cancer patient [41].

Isovitexin, isolated compound from ethyl acetate extract of *T. flagelliforme* leaves [26], showed antioxidant activity protecting cells from ROS [42]. This compound was reported to be responsible for the cytotoxic activity and COX-2 inhibition activity of *T. flagelliforme* leaf extract on WiDr cells. Previous studies established that antioxidants reduced the risk of colorectal cancer by COX-2 downregulation [43,44]. However, the mechanism of anticancer activity through antioxidant activity still needs to be further investigated since the role of ROS on carcinogenesis has dual role in either induction or inhibition of carcinogenesis [40,45]. Carcinogenesis can be induced by ROS formation in higher concentration, while ROS could also be possible to induce apoptosis on cells [40,46].

To conclude, *T. flagelliforme* leaf extract offers a new promising chemopreventive agent on colon cancer. Our data showed that *T. flagelliforme* leaf extract inhibits COX-2 expression. Nevertheless, details on molecular mechanism of these benefits remain to be established in the future study.

Anticancer activity of *T. flagelliforme* leaf extract and celecoxib on WiDr colon cancer cells was mediated by COX-2 inhibition, however the molecular mechanism needs to be further investigated.

Conflict of interest statement

We declare that we have no conflict of interest.

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