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Cytotoxic Selectivity of *Ganoderma lucidum* in Colon Cancer through Cyclooxygenase 2 (COX-2) as Its Molecular Target

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ABSTRACT

Many studies were designed explore chemopreventive activity of natural products on colon cancer especially addressing COX-2 as molecular target. Another promising source of natural product that potentially exhibit anticancer activity on colon cancer is *Ganoderma lucidum*. This study assessed selectivity of cytotoxic effect of *G. lucidum* extract on WiDr to Vero cells and investigated molecular mechanism on COX-2. *G. lucidum* extract was prepared by reflux extraction method; in vitro anticancer was assayed by MTT method on WiDr and Vero cell line. This study applied apoptosis induction assay to observe cell death mechanism using double staining method; further COX-2 expression was stained by immunocytochemistry method. *G. lucidum* extract has cytotoxic effect on WiDr cells with IC50 135 µg/mL. However, the cytotoxic effect had low selectivity towards Vero cells with Selectivity Index (SI) 3.66. The extract induced apoptosis and suppressed COX-2 expression in WiDr cells. *G. lucidum* extract was potential to be developed as anticancer agent towards colon cancer.

Keywords: *Ganoderma lucidum, selectivity, colon cancer, COX-2*

INTRODUCTION

Colon cancer has high mortality case in developing countries [1] and it was ranked as the third most frequently diagnosed cancer in both of male and female in all of the countries [1, 2, 3], as well as in the South East Asian Nations (ASEAN) countries [2, 3]. Since its incidence rapidly increases [4, 5], many studies are employed to prevent and cure colon cancer. There are many possible clinical treatments of colon cancer; such as surgery, chemotherapy, radiotherapy, and adjuvant chemotherapy [5, 6]. However, painful, nausea, vomiting, skin changes, diarrhea, hair loss, bleeding and fatigue are severe side effects of the treatment [7]. The side effects arise because of non-selective and specific target of the treatments. Therefore, more studies set to discover specific and selective colon cancer treatment [8, 9, 10] to kill colon cancer cells without endangering normal cells.

Colon cancer cells excessively produce cyclooxygenase 2 (COX-2) in both of carcinoma and adenoma [11,12, 13]. COX-2, an enzyme that is responsible for converting arachidonic acid to prostaglandins (PGs) [14], plays an important role in inflammation, cell proliferation and apoptosis regulation of colon cancer [15]. One of COX-2 product, prostaglandin E2 (PGE2), promotes angiogenesis and stimulates colon cancer growth by preventing apoptosis [16, 17]. Thus, the inhibition activity of COX-2 is an alternative to suppress colon cancer. Previous studies concluded that COX-2 inhibitors successfully prevented colon cancer growth and polyp formation [17, 18, 19, 20, 21, 22]. Therefore, COX-2 is an important molecular target for anticancer chemopreventive screenings on colon cancer.

Chemopreventive agents are compounds that are able to reverse cancer initiated cells to be normal cells. Chemopreventive agent is a strategy to specifically and selectively eliminate cancer. It should be able to block carcinogenesis process in promotion and progression stages [23]. Many studies investigated chemopreventive activity of natural products on colon cancer [24, 25, 26, 27], especially addressing COX-2 as molecular target

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[12, 27]. Among them, there was curcumin, a yellow pigment isolated from turmeric that successfully inhibited COX-2 on WiDr, a model of COX-2 overexpressing cancer cells [24].

Another promising source of natural product that potentially exhibit anticancer activity on colon cancer is *Ganoderma lucidum*. *G. lucidum* is well known as traditional medicine use against cancer, viral and bacterial infection, diabetes, and liver injury. Among its activities, its anticancer properties have been the most interesting studies [28]. It revealed cytotoxic activity of suppressed inflammatory breast cancer [28, 29, 30], ovarian cancer [31], gastric cancer [32] and liver cancer [33]. A large number of chemical compounds can be extracted from the body or spores. There are two major compounds that had anticancer activity in G. lucidum; polysaccharides and triterpenoids. Polysaccharides of *G. lucidum* boosted cytotoxic immune cells so that it could induce apoptosis in cancer cells [34]. Other compounds in G. lucidum, terpenoid compounds revealed cytotoxic, antioxidant as well as antiinflammation activity on cancer cells through immune modulation [35-37]. Terpenoids isolated from *G. lucidum* activated NF-ΚB, a transcription factor that regulated COX-2 expression in cancer cells [38, 39]. They excessively induced cell cycle arrest on G1 phase through immune boosting in colon cancer xenograft model [40]. Another study underlined that ethanolic extract of *G. lucidum* decreased COX-2 expression and increased nitric oxide synthesis in colon HT-29 cells [41]. Therefore, *G. lucidum* is a potential to be developed as chemopreventive agent on colon cancer with COX-2 as the target. Moreover, the molecular pathway involving COX-2 of *G. lucidum* will give a positive contribution to assess its specificity on colon cancer cells.

However, the selectivity of *G. lucidum* as chemopreventive agent is still remaining questioned. Besides the specificity, the selectivity of a chemopreventive agent is also important to investigate. Valadan et al [42] reported *G. lucidum* had cytotoxicity on Vero cells, a model of normal cells. Selectivity Index (SI) (IC50 Vero cells/IC50 cancer cells) indicated cytotoxicity selectivity of a compound or extract to kill normal cells versus in cancer cells [42, 43]. Furthermore, investigating selectivity of cytotoxic effect of *G. lucidum* is an interesting to be further studied.

This study assessed cytotoxic effect of ethanolic extract of *G. lucidum* on WiDr as well as Vero cells and determined SI of the extract. Furthermore, the molecular mechanism of death cells was investigated with apoptosis assay using double staining method and immunocytochemistry of COX-2.

MATERIALS AND METHODS

Plant material

G. lucidum was harvested in June, 2015 from Pandowoharjo, Sleman, Yogyakarta, Indonesia.

Chemicals

Celecoxib was purchased from Sigma-Aldrich with $\geq$ 98% purity. Vero and WiDr cells were cultivated in Fetal Bovine Serum (FBS) 10% (v/v) (Gibco) and 1% (v/v) streptomycin and penicillin (Gibco) in Roswell Park Memorial Institute Medium (RPMI) (Gibco). To dissolve the extract and celecoxib, this study used dimethyl sulfoxide (DMSO) (Merck).

Immunocytochemistry used COX-2 primary antibody from Thermo Scientific Lab Vision and secondary antibody was diluted from Starr Trek HRP universal detection system No.901-STUHRP700-090314. All plates and micro plate in this study were Iwaki®, moreover blue, yellow and white tips were supplied by Axygen®.

Extraction

G. lucidum was dried using oven and homogenously powdered. 10 g of its powder was extracted in 90% ethanol using reflux method for 2 hours. Liquid extract was evaporated under 80°C until reached constant weight.

Cytotoxic assay

Cytotoxic assay towards Vero cells as well as WiDr cells were designed based on our previous studies [45-46]. Cells were seeded in a culture tissue flask until 80% confluent, then 5×10^3 cells were planted into a 96-well micro plate. The cells cultivated in an incubator under 37°C and 5% CO2. The medium was discarded and the cells were gently washed two times using PBS. The initial solution of extract was provided by dissolving them into DMSO and diluted them in DMEM into several concentrations. Each of extract concentration was added 100 µL into each well of the 96-wells micro plate and tested in three times in a set. Moreover, the cells were incubated in the same condition as the previous step. The medium was then discarded and the medium contained 10% MTT was spilled into each well. The reaction between succinate hydrogenase of cell mitochondrion and MTT to form formazan took 3 until 4 hours. After 4 hours incubation, 100 µL of 10% SDS solution was poured to each well to dissolve formazan crystals.

The plate was wrapped in dark paper to avoid light exposure and incubated 12-24 hours, then formazan complex was measured in 595 nm visible wavelength using ELISA reader.

Apoptosis induction assay

Apoptosis induction assay was conducted only in WiDr cells. The cells were cultured 5×10^4 on coverslips in 24-well plate. WiDr cells were adapted in 37°C and 5% CO2 for 24 hours. Then, the medium was discarded and twice soaked in PBS. The extract and celecoxib at IC50 concentration (135 µg/mL and 68 µM) were poured into the plate then it was incubated in the same as previous condition. IC50 concentration of celecoxib was calculated from our previous study [47]. The medium was removed from WiDr cells and they were twice soaked in PBS. The coverslips were taken from the plate and moved to glass objects, and then acridine orange-ethidium bromide (AE) was added into the coverslips. The glass objects were immediately investigated under fluorescence microscope.

Immucytochemistry assay

WiDr cells were spread in 6-well plate and placed in incubator with 5% CO2 and 37°C for 24 hours. The extract at 135 µg/mL and celecoxib at 68 µM were treated on cells and incubated for 24 hours. At the end of the incubation time, cells were collected and washed in PBS three times. The cells were suspended in medium, placed and fixed in object glass for 5 minutes. Hydrogen peroxidase was added into the object glass and placed in room temperature for 10-15 minutes. Then, it was gently washed by PBS twice and monoclonal antibody of COX-2 was dropped into the object glass at least for an hour. The object glass was washed in PBS and secondary antibody was dropped into the glass. It was incubated in room temperature for ten minutes, later it was washed four times in PBS. The solution of 3, 3'-diaminobenzidine (DAB) as chromogen was poured into the cells and incubated for 3- 8 minutes. Finally, the object glass was washed by aquadest and hematoxylin solution was added. It was incubated for 3-4 minutes and dried. The expression of COX-2 was observed under inverted microscope.

Data analysis

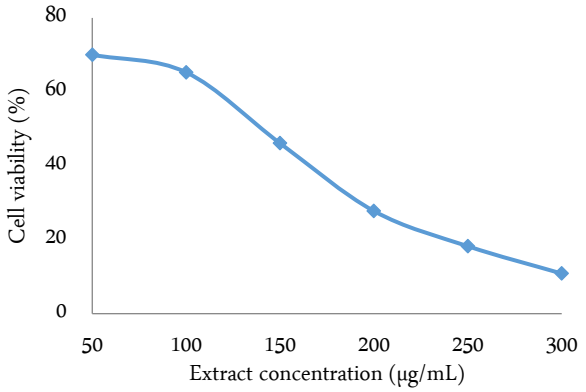
Cell viability was calculated from MTT data using equation:

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

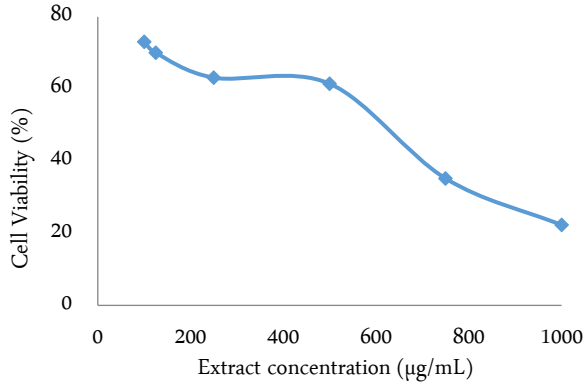
Cell viability data was analyzed using linier regressions to calculate IC50 of extract and celecoxib. This SI was calculated by dividing IC50 towards Vero cells/IC50 towards Vero cells [19]. The number of apoptosis, necrosis and living cells was semi-quantitative analyzed by blind observed by blind counting cell distribution in three areas of object glass.

RESULTS AND DISCUSSION

G. lucidum extract demonstrated dose-depend cytotoxic activity both in WiDr cells (Figure 1a) and Vero cells (Figure 1b). It had IC50 135 µg/mL towards WiDr cells and 494 µg/mL towards Vero cells. Cell morphology observation revealed *G. lucidum* extract IC50 concentration on slightly effected cell population otherwise celecoxib dramatically effected cell population than the extract (Figure 2). By comparing IC50 in Vero cells to IC50 in WiDr cells, the SI was determined 3.66 respectively.



(a)

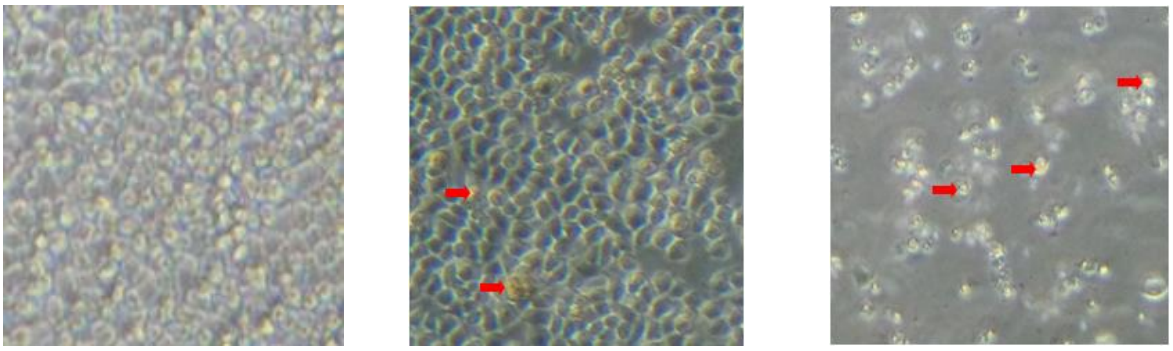


(b)

Figure 1. The effect of *G. lucidum* extract on WiDr cells (a) and Vero cells (b)

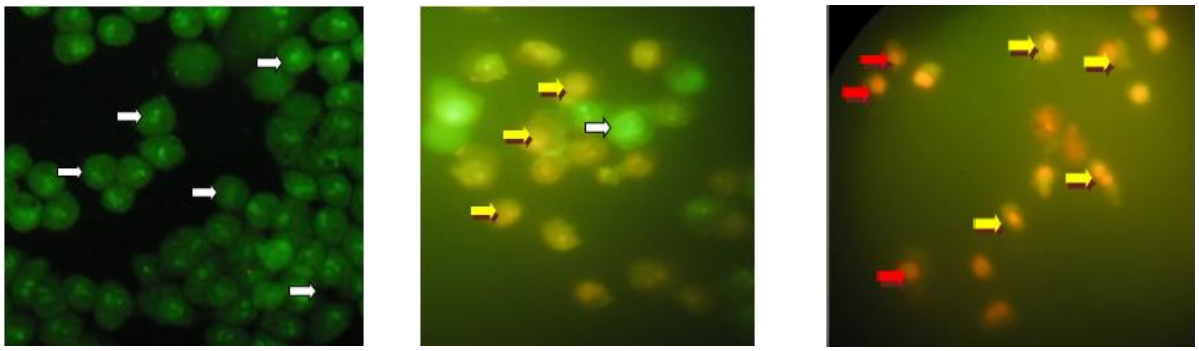
Tabel 1. Cell distribution in apoptosis assay

Treatment	Apoptosis (%) ± SD	Necrosis (%) ±SD	Living (%)± SD
G. lucidum extract	46.4 ± 0.56	5.21 ± 1.12	48.40 ± 5.95
Celecoxib	81.80 ± 0.88	3.48 ± 1.43	14.68 ± 0.65
Untreated cells	1.42 ± 0.65	0.72 ± 0.04	97.86 ± 0.61



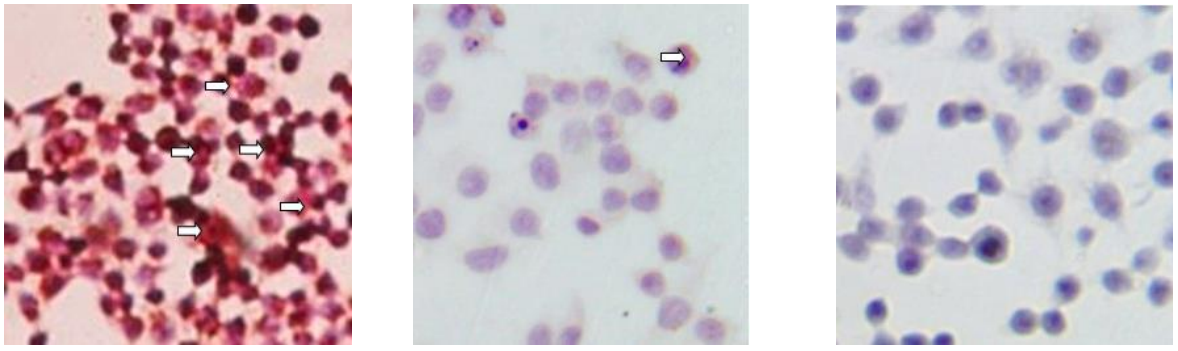
(a) (b) (c)

Figure 2. Morphology observation of WiDr cells under light microscope using 400 × magnification in untreated cells (a), cells treated by 135 µg/mL extract (b), and cells treated by 68 µM celecoxib (c). Living and dead cells are indicated by white arrow and red arrow.



(a) (b) (c)

Figure 3. Apoptotic observation in WiDr cells using double staining method under flourenscence microscopy using 400 × magnification in untreated cells (a), cells treated by 135 µg/mL extract (b), and cells treated by 68 µM celecoxib (c). Living, dead, and apoptotic cells are indicated by white arrow, red arrow, and yellow arrow.



(a) (b) (c)

Figure 4. COX-2 expression in WiDr cells under light microscope using 400 × magnification in untreated cells (a), cells treated by 135 µg/mL extract (b), and cells treated by 68 µM celecoxib (c). Brown colour shows COX-2 expression in cells.

Furthermore, this study investigated apoptosis induction in WiDr cells using double staining method. The nuclei showed condensed yellow-green fluorescence by acridine orange in early stage of apoptosis. On the other hand, nuclei of cells showed condensed orange fluorescence by ethidium bromide in late apoptosis stage [47]. Apoptosis cells were showed in orange colour with condensed nuclei while necrosis cells was stained in orange colour with uncondensed nuclei (Figure 3). *G. lucidum* extract as well as celecoxib induced apoptosis in WiDr cell (Table 1). It confirmed from our previous research that celecoxib strong induced apoptosis in WiDr cells [47]. Molecular mechanism of extract in WiDr cells was further investigated through COX-2 expression analysis. COX-2 suppressing cells were stained in brown color while non-suppressing cells were stained in purple color. Immunocytochemistry staining revealed extract suppressed COX-2 expression. This suppression of the extract was lower than celecoxib (Figure 4).

Overall, this study evaluated cytotoxic activity of *G. lucidum* extract on WiDr colon cancer cells and its selectivity on normal cells. This study was designed to discover new therapeutic agent of colon cancer that has less side effects to cancer patient. This study used Vero cells, as model of normal mammalian cell line that derived from the kidney of the African green monkey (Cercopithecus aethiops). This cell line was recommended for screening chemical toxicity in vitro [48, 49].

Selectivity Index (SI) is parameter that indicates selectivity of a compound or an extract. Based on Moustafa et al. (2014) [43], the SI value of *G. lucidum* extract (SI value < 10) was consider as unselective extract towards normal cells. However, other studies mentioned that SI value < 2 indicating high selectivity [50, 51]. *G. lucidum* extract induced apoptosis in WiDr cells.

Instead of its selectivity and apotosis induction, molecular mechanism of *G. lucidum* extract on WiDr cells must be further investigated. This study focused on COX-2 protein that produced PGE2 that increased cell proliferation and escaped from apoptosis [16, 17]. Therefore, COX-2 is important target to screen chemopreventive agent on colon cancer. The result of this study showed that *G. lucidum* extract suppressed COX-2 expression in WiDr. The suppression effect was lower than celecoxib, a selective COX-2 inhibitor, as positive control. Celecoxib is one of prospective drug against colon cancer [52] and our previous research showed it suppressed COX-2 expression in WiDr cells [47]. The suppression effect of the extract may be activated by terpenoids in *G. lucidum* to NF-ΚB, a transcription factor that regulated COX-2 expression in cancer cells [38, 39].

G. lucidum extract offers a new promising for selective chemopreventive agent on colon cancer. Our data revealed it inhibited COX-2 expression, even though detail molecular mechanism of these benefit remain to be established in the future study

CONCLUSION

G. lucidum extract has selective cytotoxic effect towards WiDr cells than Vero cells. It molecular mechanism in WiDr cells involved COX-2 suppression.

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