# Cytotoxic Activity and Apoptosis Induction of Ethanolic Extract of Pericarps of Mangosteen (Garcinia mangostana Linn.) on WiDr Cells and Interaction Study of Alpha-mangosteen to IKK and VEGF Based on Molecular Docking

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# Cytotoxic Activity and Apoptosis Induction of Ethanolic Extract of Pericarps of Mangosteen (*Garcinia mangostana* Linn.) on WiDr Cells and Interaction Study of Alpha-mangosteen to IKK and VEGF Based on Molecular Docking

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## Abstract

One of the compounds found efficacious as an anti-proliferative on colon cancer is alpha-mangosteen, a xanthon compound that is abundant in pericarp of mangosteen. This study focused to evaluate anticancer activity of the ethanolic extract of pericarp of mangosteen (*Garcinia mangostana* Linn.) on WiDr colon cancer cells and to observe the affinity of alpha-mangosteen in inhibiting IKK and VEGF. Cytotoxic effect was tested by MTT assay and apoptosis induction was evaluated by double staining method on WiDr colon cancer cells, while interaction between alpha-mangosteen to the receptors was measured by molecular docking. The ethanolic extract was proven to have cytotoxic activity against WiDr colon cancer cells with $IC_{50}$ of 25 μg/mL. In addition, the observation of apoptosis induction by double-staining method showed that apoptosis associated the cytotoxic effect of ethanolic extract of pericarp of mangosteen (EPM) on WiDr colon cancer cells. Molecular docking showed that active compounds in pericarp of mangosteen could compete with the native ligand of VEGF because the docking score of alpha-mangosteen was almost equal with native ligand. From these results would be concluded that the cytotoxic effect of EPM to WiDr cells was mediated by cell apoptosis. This extract was potential to be developed as chemopreventive agent.

**Keyword** : *Garcinia mangostana* Linn., *cytotoxic effect, apoptosis, WiDr cell, molecular docking*

## INTRODUCTION

Colon cancer is the fourth leading cause of death. Colon cancer incidence rates continue to increase along with population growth in both developing and developed countries (Winawer, 2007). In colon cancer, increased expression of cyclooxygenase-2 (COX-2) induce the formation of prostaglandin (PGE2) from arachidonic acid. It is related to the regulation of cell proliferation, differentiation, and tumorigenesis (Turini and Dubois, 2002). Overexpression of COX-2 associates with the activation of NF-κB. NF-κB is activated by phosphorylation of IκB, an inhibitor of protein bound to NF-κB, by IKK (IκB kinase) and resulted in the outbreak of the bond between NF-κB and IκB. Induction of NF-κB showed the inhibition of apoptosis and increase of proliferation of cancer cells thus inhibition of NF-κB suppressed tumorigenesis and metastasis and decreased VEGF expression in some cancer cells (Fujioka, *et al.*, 2003). Therefore, inhibition of NF-κB activation through suppression of IKK, inhibiting the expression of COX-2 and VEGF may be one specific target in colon cancer chemoprevention.

Until now, the result for cancer treatment is still unsatisfactory. Therefore, the study of cancer drug discovery is still intensively conducted. One of the medicinal plants that become the object of study is the pericarp of the mangosteen fruit.

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Based on *in vitro* assays, DLD-1 cells (human colon cancer cell model) showed that the mangosteen pericarp extract can inhibit cancer cell growth at low doses of 5-20 µm (Matsumoto, *et al.*, 2005). Water extract, 50 and 95% ethanol, ethyl acetate of pericarp of mangosteen has antioxidant activity against free radical 2,2-diphenyl-1-pikrlhidrazil (Weecharangsan, *et al.*, 2006). Alpha-mangosteen as the main compound of mangosteen pericarps extract can reduce the occurrence of focal lesions and rat colonic epithelium induced 1.2-dimetilhidrazin (DMH) (Nabandith, *et al.*, 2004). The findings became the basis for further development of the mangosteen pericarp extracts as chemopreventive agents especially colon cancers.

Mangosteen pericarp that usually discarded can turn into a potential chemopreventive agent. This study was to test the cytotoxicity of ethanolic extract of pericarp of mangosteen to the WiDr cell as another model for colon cancer besides DLD-1. WiDr cell line was chosen because it reportedly safe for use in a variety of cytotoxic tests, easily for cultured and treatment. The cytotoxic effect was determined by MTT assay. Furthermore, the effect of ethanolic extract of mangosteen pericarp (*Garcinia mangostana* Linn.) against WiDr cell on apoptosis induction were observed. The observation of the effect of apoptosis induction was done by double-staining method. This research also studied the inhibitory effect of NF-κB expression through suppression of IKK, as well as inhibition of the expression of VEGF by ethanolic extract of pericarp of mangosteen (*Garcinia mangostana* Linn.) by using molecular docking. The results of this study are expected to provide scientific evidence regarding the cytotoxic activity of ethanolic extract of pericarp of mangosteen (EPM) and apoptosis induction against WiDr colon cancer cells so it can be a basis for the use of natural materials as a chemopreventive agent to prevent the incidence of cancer.

## MATERIALS AND METHODS

### Molecular Docking

Molecular docking method were done to know the interaction between alpha-mangosteen as the main compound of mangosteen pericarps extract to binding site of IKK and VEGF. The structure of alpha-mangosteen was drawn by Marvin Sketch Software. The structure of protein complex was taken from the Protein Data Bank (PDB) sites (http://www.pdb.org/pdb/home/home.do). The PDB ID for IKK and VEGF were 2GNG and 2P2I respectively. The analysis was done to get the docking score to show the interaction affinity between the ligand and the protein receptor. The affinity increases as the docking score value reduces.

### Extraction

Mangosteen (*Garinia mangostana* Linn from Purworejo, Central Java, Indonesia) fruit pericarp was collected, dried and powdered at room temperature naturally. The determination of the powdered material was done at laboratory of Pharmacognocy, Faculty of Pharmacy, Universitas Gadjah Mada. As many as 500 g of powdered material was extracted with 5 L of 70% ethanol for five days at room temperature, blended continuously. The extracts were filtered by sentrifugation and concentrated to remove the solvent using vacuum rotary evaporator, and more than 240.0 g of crude ethanolic extract was yielded.

### Cell Line and Culture

WiDr colon cancer cells collected by Cancer Chemoprevention Research Center, Faculty of Pharmacy Universitas Gadjah Mada obtained from Prof. Masashi Kawaichi (Nara Institute of Science and Technology, Japan). The cells were maintained in RPMI-1640 (Gibco) supplemented with 10% FBS (Gibco), penicillin (10,000 U/mL), streptomycin (10 mg/mL) (Gibco) and Fungizone (0.5 mL) in a humidified atmosphere of 50 µg/mL $CO_2$ at 37°C.

### *In Vitro* Assay for Cytotoxic Activity (MTT Assay)

The cytotoxicity of ethanolic extract of pericarps of mangosteen (EPM) on WiDr cells was determined by the MTT assay. Cells ($5 \times 10^3$/well) were plated in 100 µL of medium/well in 96-well plates. After incubation overnight in a humidified atmosphere 5% $CO_2$ at 37°C, EPM was added in various concentrations (10, 15, 20, 25 and 30 µg/mL). After treatment with EPM, 100 µL of 0.5 mg/mL MTT (pH 4.7) was added per well and cultivated for another 4 hours, the reaction was stopped by 100 µL SDS 10% in HCl 0.01 N per well. After incubation overnight, the

absorbance at 595 nm was measured with ELISA reader (Bio-Rad), using wells without cells as blanks. All experiments were performed in triplicate. The effect of EPM on the proliferation of colon cancer cells was expressed as the % cytoviability, using the following formula:

$$\% \text{ Cell viability} = (\text{absorbance of cell treated} - \text{absorbance of control medium}) \,/\, (\text{absorbance of controll cell} - \text{absorbance of control medium})$$

The concentration of the extract that caused the death of 50% of the population of cells ($IC_{50}$) was calculated from the linearity of the relationship between the concentration versus percent cell viability.

### Observation of Apoptosis Induction by Double Staining Method

WiDr cells (50,000 cells/well) were cultured on coverslips in the 24 well-plate in 1000 mL of culture media. Then cells were incubated for 24 hours in a $CO_2$ incubator to re-adapted cells. After incubation, EPM was added in concentration of $IC_{50}$, the other wells were the control cells and the solvent DMSO (Sigma) control. At the end of incubation, culture medium (RPMI 1640) was washed with PBS (Sigma), and the cover slip was removed from the wells and placed on the object glass and then poured with acridine orange-ethidium bromide (Sigma) as much as 10 μL. Observation of cell morphology was done by using a fluorescent microscope magnification 10x10. Living cells will fluorescence green with the nucleus intact cells, early apoptosis would be bright green with fragmented or condensed chromatin, late apoptotic will look orange with fragmented or condensed chromatin, and cells that die because of necrosis will be colored orange with a normal structure of the nucleus (Ribble, *et al.,* 2005).

## RESULTS AND DISCUSSION

### Interaction of alpha-mangosteen with IKK and VEGF

The structure optimization of alpha-mangosteen was done by using Marvin Sketch, while the 3D form of its protein receptor was created by using YASARA. After that, we docked the compound with binding site of IKK (PDB ID: 2GNG) and VEGF (PDB ID: 2P2I). Based on the molecular docking of alpha-mangosteen with VEGF, the docking score was lower than its native ligand. This result showed us that interaction of alpha-mangosteen with VEGF was stronger, so alpha-mangosteen could inhibit VEGF in cytotoxic mechanism. In another hand, the docking score of alpha-mangosteen with IKK was bigger than its native ligand. This result showed that alpha-mangosteen could not inhibit the stimulation of NFkB by supressing the activity of IKK.

**Table I. Score of molecular docking**

| Protein | Compound | Score Docking |
|---|---|---|
| IKK | Alpha-mangosteen | -11.4116 |
| | Ligand Native SEP | -17.827 |
| | ATP | -77.4527 |
| VEGF | Alpha-mangosteen | -83.7684 |
| | Native Ligand 608 | -17.7012 |
| | ATP | -87.4485 |

**Figure 1. 2D visualization of molecular interaction.** Alpha-mangosteen and VEGF (A); alpha-mangosteen and IKK (B); Amino acid of VEGF do more interaction with alpha mangosteen than the IKK.

### Cytotoxicity on WiDr Cells Using MTT Assay

Cytotoxic activity was tested by *in vitro* to determine the potential of a compound such as cytotoxic anticancer drugs. Toxicity of a compound was expressed in $IC_{50}$ value that can inhibit cell proliferation by 50% of the population. This study used MTT assay to determine the cytotoxic potential of ethanolic extract of pericarps of mangosteen (EPM). The test is based on the measurement of color intensity that occurs as a result of metabolism of a substrate by living cells into colored products. In this test, tetrazolium salt MTT [3-(4,5-dimetiltiazol-2-yl)- 2.5-diphenyl tetrazolium bromide] was used. MTT reduced to formazan by the succinate tetrazolium reductase system, which is included in the mitochondria of living cells. Formazan intensity was measured using ELISA reader at a wavelength of 595 nm. Color intensity is proportional to the number of living cells.

Before being treated with MTT reagent, cell morphology was observed after treatment with EPM for 24 hours. EPM was supplied with various concentrations of 10, 15, 20, 25 and 30 µg/mL. Observations showed that the higher concentration of the extract may result in more cell death (Fig. 2). Living cells have leaf morphology and remained attached to the bottom wells, whereas cells undergoing death looked round and not strongly attached to the bottom wells. Black arrows indicate dead cell.

By using ELISA Reader, absorbance values were obtained. Then, the absorbance values were converted into the percentage of living cells. After that, the $IC_{50}$ value was calculated using the linear regression between extract concentration and percentage of living cells, and obtained $IC_{50}$ value of 25 µg/mL. It showed that EPM with a concentration of 25 µg/mL could inhibit the proliferation 50% of WiDr colon cancer cell population. In other words, EPM has cytotoxic effect against WiDr colon cancer cells. In addition, dose-dependent phenomenon which is higher concentration of EPM, lower cell viability dose was also shown by EPM in WiDr colon cancer cells (Fig. 3).

### Apoptosis Induction of Ethanolic Extract of Mangosteen Pericarps on WiDr Cells with Double Staining Method

Apoptosis induction on WiDr cells was performed to determine that cell was dead due to an apoptotic mechanism. Apoptosis characterized by chromatin condensation, nucleus fragmentation and changes in cell morphology (Gewies, 2003; Ricci and Zong, 2006). The assay was done by observing a fluorescent compound that can bind to DNA/RNA and providing a distinctive color in the cell. The compounds used are acridine orange-ethidium bromide. Acridine orange will permeate into the cell and all cells colored green.

Besides, ethidium bromide will only permeate into the cell if the cell loses its membrane integrity and provided red. Color was caused by ethidium bromide on dead cell is more dominant when compared with acridine orange so that the nucleus of dead cells will be orange (Nishioka, *et al.*, 1995). Living cells with intact nucleus of cells will appear green, early apoptosis would be bright green with fragmented or condensed chromatin, late apoptotic will look orange with fragmented or condensed chromatin, and dead cells have necrosis will be colored orange with the normal structure of nucleus (Renvoize, *et al.*, 1998).

EPM showed apoptosis activity (Fig. 4b), where the nucleus appeared orange with fragmented or condensed chromatin. While, it was not visible in the control group with an intact cell nucleus and fluoresces green (Fig. 4a). The result of this staining proves that EPM was able to induce apoptosis on WiDr cells.

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