Indonesian Journal of Cancer Chemoprevention, February 2017
ISSN: 2088–0197
e-ISSN: 2355-8989

Phaleria macrocarpa Fruit Extract Inhibits NF-κB Activation and Apoptosis Induction on HeLa Cells

Meirizky Zulharini S., Amalia Miranda, lina Permatasari, Hilyatul Fadliyah, Riris Istighfari Jenie*

Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

NF-κB is a transcription factor and if activated, it induces apoptosis inhibition. Phalerin from *Phaleria macrocarpa* fruits expected to inhibit NF-κB activation. This research is to investigate anticancer mechanism of *Phaleria macrocarpa* fruits extracts (EBMD) in NF-κB pathway. Molecular docking assay was performed to determine phalerin affinity to IKK. Cytotoxic activity was observed by MTT assay. Double staining was performed to determine the apoptotic cells. Docking score of phalerin to IKK is -60. The IC_{50} value of EBMD is 629 μg/mL. Apoptosis profile shows (shown that) many cells undergoing apoptosis after treatment. Thus, EBMD potentially inhibits activation of NF-κB pathway and triggers apoptosis on HeLa cells.

Keywords : NF-κB, *Phaleria macrocarpa*, sel HeLa, Bcl-2, IKK, molecular docking

INTRODUCTION

NF-κB is a transcription factor that regulates a variety of eukaryotic genes associated with immunity, inflammation and cell survival (Ghosh and Hayden, 2008). NF-κB activation pathway is mutated and caused NF-κB continously activated.

Cervical cancer has quite high incidence in Indonesia. The cause of cervical cancer is HPV (Human Papilloma Virus). This virus can trigger the activation of NF-κB, which then will translocate to the nucleus, resulting cancer cell proliferation. NF-κB activation can increase the expression of Bcl-2 protein. When the protein Bcl-2 increases, the process of cancer cells apoptosis is inhibited. Therefore, we need a chemoprevention agent-based on natural ingredient that is capable on inhibiting proliferative activity of cancer cells in the upstream and downstream NF-κB pathway.

Mahkota dewa (*Phaleria macrocarpa* (Scheff.)) is believed have potential as a chemoprevention agent. *Phaleria macrocarpa* fruit contains phalerin that would increase the regulation of BAX protein (pro-apoptotic) and decrease the regulation of Bcl-2 (anti-apoptotic) at mRNA level (Supreme, *et al*., 2008).

This study was conducted to determine the potential of *Phaleria macrocarpa* fruits extract (EBMD) in inhibiting proliferation of cancer cells by blocking the activation of NF-κB and using HeLa cells as the model of this research (cells of cervical cancer). NF-κB pathway observations is carried out through two parameters: the parameters upstream and downstream. Observations of upstream activation is done through the inhibition of IKK/NF-κB by using molecular docking, while for downstream is done by observing the suppression of Bcl-2 expression profile as a manifestation of apoptosis incidence using double staining method. This study aims to determine the effectiveness of EBMD in inducing apoptosis through the inhibition of NF-κB pathway.

METHODOLOGY

Preparation of Extract

Simplicia of *Phaleria macrocarpa* fruit were collected from Bantul, Yogyakarta, Indonesia. An amount of *Phaleria macrocarpa* fruits were powdered and extracted with methanol for 36 hours on temperature 70ºC-75ºC in 13 cycles soxhlet. Produced filtrate was then dried using rotary evaporator. This simplicia has been extracted in CCRC Faculty of Pharmacy and Biology Faculty of Pharmacy Laboratory UGM. Fractionation processes were monitored using TLC analysis.

*Corresponding author e-mail: ririsjenie@gmail.com

Thin Layer Chromatography Test

The solution stock of the extracts were spotted on a silica gel in Thin Layer Chromatographic (TLC) Plate and developed with a solvent system of chloroform: ethyl acetate: acetate glacial acid (1 : 8 : 0.05). The plate were visualized on UV 254.

Molecular Docking

The Marvinsketch program was used to make 3D phalerin and YASARA software was used for protein target preparation. PLANTS software was used for docking. The target protein choosed was IKK and the experimental ligand was ATP. Test compound used was phalerin. After determining the ligand binding side cribs, ligand-docking test compound in the ligand-binding side. The results of the docking was docking score. Output obtained declare the force of ligand-receptor interaction in the form of docking scores.

Cell Culture

HeLa cervical cancer cell line was kindly provided by Cancer Chemoprevention Research Center (CCRC), Faculty of Pharmacy, Universitas Gadjah Mada. HeLa cells were maintained in DMEM (Dulbecco's modified Eangle's medium) (Gibco) media supplemented with 10% v/v fetal bovine serum (Gibco).

Cytotoxic Test

HeLa cervical cancer cells were added to wells of round-bottom 96-well cell culture plates (10,000 cells/well) in DMEM media. The cells were then incubated for 24 hours at 37°C in a humidified 5% CO_2 incubator. Sample was then dispensed at different concentrations to the experimental wells with 3 replications. Each plate contained the samples, cell control and media control. DMEM was used as the media control and HeLs cervical cancer cell was used as the cell control. At the end of the incubation period, the culture media in the plate was removed, washed with PBS (Sigma), followed by the addition of reagent MTT (3- (4,5-dimetiltiazol-2-yl (-2,5-diphenyl tetrazolium bromide) 10% in PBS (Sigma) of 100 mL each wells. Cells were incubated back for 2-4 hours and then observed under inverted microscope. If the crystal is formed to be formazan, the cells were given stopper reagent and re-incubated overnight at room temperature. After that, the plate is read using an ELISA reader with a wavelength of 595 nm. The results of absorbance is then converted into a percentage of cell viability.

Apoptosis Test with *Double Staining*

Coverslip is added into a 24-well plate and HeLa cells are distributed on it with a cell density of $5x10^4$ cells/well in 1000 mL culture medium. Cells were incubated in a 5% CO_2 incubator (Heraeus) for 24 hours at a temperature of 37ºC in order to re-adapted the cells. Furthermore, it is treated with extract using concentration as IC_{50} resulted before. Then incubated again for 10-24 hours. At the end of incubation, DMEM (Dulbecco's modified Eangle's medium) (Gibco) medium were washed with PBS, and cover slip is lifted from the wells and placed on a glass object and then dropped with acridine orange-ethidium bromide (Sigma) 10 mL. Observation of cell morphology is done with a fluorescence microscope (Zeiss MC 80) at 100x magnification.

Data Analysis

Absorbance data was obtained from cytotoxicity assay then converted to a percent of cell viability and converted into log values. IC_{50} value calculation is performed by the logit method. Molecular docking is done by analyzing the RMSD value to determine the validity of the method and the value of ΔG to evaluate the affinity molecule IKK samples with ATP binding site. RMSD value less than or equal to 2.0 Å is received, indicating that the method is valid. The lower the value of ΔG, the higher the affinity.

RESULTS

Identification of Phalerin in *Phaleria macrocarpa* Fruit Extracts

Identification of phalerin was carried out by using thin layer chromatography (TLC) with chloroform : ethyl acetic : acetic acid (1 : 8 : 0.05) as mobile phase and silica gel as stationary phase (Fig. 1). After elution, the TLC plat was observed under UV254 and there was brownish spot with the Rf value of 0.42. The results showed that PFE propably contains phalerin as its compound.

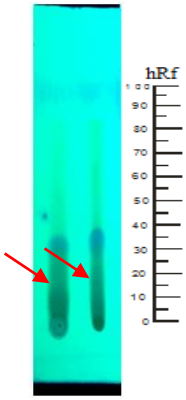

Figure 1. Identification of phalerin using TLC. Plat was observed under UV254. Red arrows showed the possibility spot of phalerin.

Molecular Docking

Molecular docking was done to predict the interaction between active compound (ligand) and IKK. ATP was used as native ligand to compare the bonding ability of phalerin with IKK. The bonding possition between active compound and protein also could be identified through molecular docking. Docking score was used as a parameter of energy needed to make bonding. Lower docking score then increase the strength or affinity of bonding. Docking score of ligand and IKK was shown (Table 1).

The results showed that phalerin had higher docking score to IKK than ATP. But, from the visualization, the bonding between phalerin and IKK had some similar binding site on amino acid residue with the bonding between ATP and IKK. Thus, phalerin could compete with ATP to interact with IKK (Fig. 2).

Cytotoxic Test

Cytotoxic test was conducted through MTT assay to determine the IC_{50} value of PFE on HeLa cells. The toxicity of PFE on HeLa cells could be identified through the IC_{50} value and cells morphology. The IC_{50} value of PFE on HeLa cells was 629 µg/mL. The curved showed that PFE could decrease the cells viability with dose dependent manner (Fig. 3 and 4).

Table 1. Docking Score

Ligand	Docking score to IKK
RMSD	5.5035
ATP	-74.6874
Phalerin	-60.2977

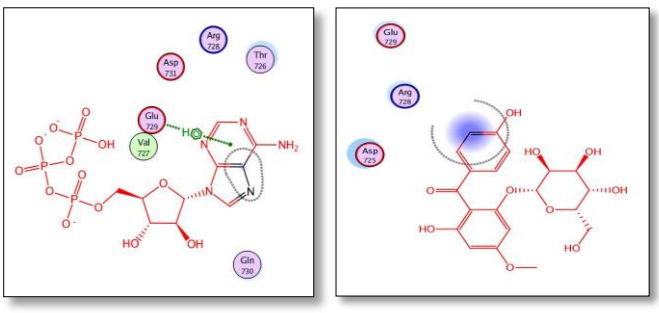

Figure 2. 2D visualization of molecular docking. 3BRV-ATP (left); 3BRV-phalerin (right)

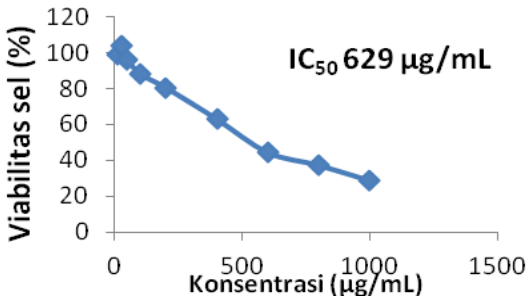

Figure 3. Correlation between the percentage of cells vialbility and PFE concentration. Cytotoxic test was determined with MTT assay. Cells were incubated for 24 h and the cells viablitity was determined using linear regression. The value of IC_{50} was 629 µg/mL.

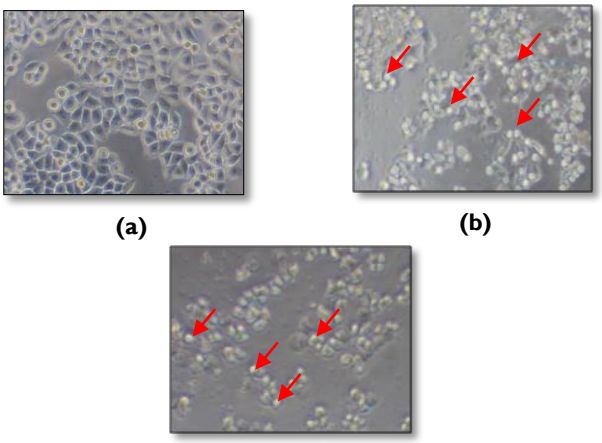

Figure 4. Effect of PFE on HeLa cells morphology. HeLa cells (a) negative control (b) treated with 600 µg/mL PFE; (c) treated with 1000 µg/mL PFE. Cells were observed after being incubated for 24 hours. Red arrows indicate cells undergoing morphological changes.

Apoptosis Test using Double Staining

Observation of apoptosis on HeLa cells was conducted to identify apoptosis effect of PFE on HeLa cells. The concentration of PFE which was in apoptosis test was 630 µg/mL for 20 h of incubation. The results showed, the negative control cells were had green fluorescence (Fig. 5) which indicated living cells. While, the treated cells were undergone apoptosis characterized by orange fluorescence and DNA fragmentation of cells. However, apoptosis on HeLa cells could indicate reduction of Bcl-2 (anti-apoptosis protein) which is downsteam of NF-κB pathway.

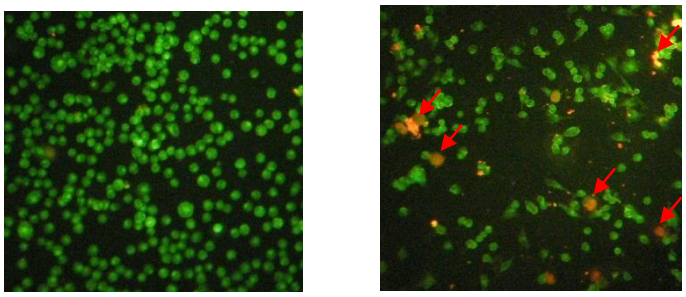

Figure 5. Effect of PFE on apoptosis on HeLa cells. (a) negative control; (b) treatment with PFE's IC_{50}. Incubation was conducted for 20 hours, observation was done under the fluoresence microscope with 100x magnification. Red arrows showed the cells with apoptosis.

DISCUSSION

The aim of this study is to develop one of Indonesian natural potential, mahkota dewa (*Phaleria macrocarpa (Scheff.))* as natural resource of anticancer agent. Previous research suggested that *Phaleria macrocarpa (Scheff.)* fruit has phalerin as its major compound which has anticancer activity. This fruit had been traditionally used as medicine on cancer diseases especially breast cancer and brain tumor (Astuti, *et al.*, 2007).

Analysis of phalerin in PFE was identified by thin layer chromatography. Then, molecular docking was conducted to know the mechanism of NF-κB pathway inhibition through IKK protein by phalerin in PFE. Results showed that phalerin could interact on IKK with docking score of -60.2977 and docking score of ATP on IKK was 74.6874. These showed that affinity between phalerin and IKK was lower than ATP and IKK. But, from 2D visualization, ATP and phalerin had similar binding site on three amino acid residue which is Glu 729, Arg 728, dan Asp 725. So, phalerin is potentially to interact with IKK and inhibit the activation of NF-κB.

From the cytotoxic test, IC_{50} of PFE on HeLa cells was 629 µg/mL. The curve of PFE concentration versus cell viability showed dose dependent manner, where the higher concentration of PFE would lower cell viability. Morphological changes of cells was observed under inverted microscope. The results showed that there was morphological change of HeLa cells after being treated with PFE.

NF-κB has vital role in various physiological and pathological process of cell such as the regulation of gene which produce apoptosis protein. On normal cell, NF-κB is a dimer of p65 and p50. As an inactive form of NF-κB, these dimer would form complex with IκB-α. When IKK phosphorilate IκB-α, the complex is being separated. After that, NF-κB would translocated into the nucleus and induce expression of anti apoptosis protein, such as Bcl-2. If Bcl-2 is overexpress, it would inhibit the apoptosis process on cancer cell.

Apoptosis is programmed cell dead which cause distinct on morphology and biochemical process of cells. Induction of apoptosis by anticancer therapeutic agent is expected effect in cancer therapy. In general, manifestation of apototic is reduction of cell proliferation. Apoptosis observation of PFE on HeLa cells was conducted through double staining using etidium bromide-acrydine orange. When apoptosis happen, cell membrane became more permeable so the ethidium bromide could intercalate with DNA in nucleus and give orange fluorescence.

The presence of apoptosis after being treated with PFE, is caused by intercalation of ethidium bromide on DNA because of membrane damage on non-viable cells. Whereas the untreated cell, the membrane is still intact so ethidium bromide cannot enter the cell.

Further studies regarding the isolation of compounds phalerin of PFE and *in vivo* tests need to be conducted to determine the direct effect of phalerin compound in inhibiting cancer development in living bodies.

CONCLUSION

Phaleria macrocarpa (Scheff.) fruit extract potentially inhibits the activation of NF-κB and could trigger apoptosis on HeLa cervical cancer cells.

REFERENCES

Agung, B.S., Faried, A., Arifin, M.Z., Wiriadisastra, K. and Ohta, T., 2008, Herbal Medicine Isolation, Phaleria macrocarpa for Primary Glioblastoma Multiforme Cells, *Ann. Epidemiol.*, **18**(9), 718.

Anonymous, 2012, *Yayasan Kanker Indonesia-Jakarta Race*, http://yayasankankerindonesia.org/2012/yki-jakarta-race/, Cited July 14, 2014.

Backer, C. and van den Brink, R., 1965, *Flora of Java (Spermatophytes Only)*, Volume II, Groningen: Noordhoff.

Baldwin, A.S., Jr., 2001, The Transcription Factor NF-kB and Human Disease, *J. Clin. Investig*., **107**(1), 3–6.

Ghosh, S. and Hayden, M.S., 2008, New Regulators of NF-κB in Inflammation, *Nat. Rev. Immunol*., **8**(11),837-848.

Giardina, C. and Hubbard, A.K., 2002, Growing Old With NF-kappaB, *Cell Stress Chaperones*, **7**(2), 207-212.

Gilmore T.D., 2006, Introduction to NF-B: players, pathways, perspectives, *Oncogene*, **25**(51), 6680–6684.

Hou, S. Y., Wu, S. and Chiang, C., 2002, Transcriptional Activitty among High an Low Risk human Papillomavirus proteins E2 Proteins correlates of E2 DNA binding, *J. Biol. Chemy*., **227**(47), 45619-29.

Kim, H., Kim H., Ashik, M., Rajendra, G., Kwang, S., Somi, K., 2012, Induction of Apoptosis by Ethanolic Extracts of Mango Peel and Comparative Analysis of the Chemical Consists of Mango Peel and Flesh, *Food Chem*., **133**(2), 416-422.

Lodish, H., Berk, A. and Zipursky, S.L., 2000, *Molecular Cell Biology*, 4th edition, New York: W. H. Freeman.

Li, R., Cheng, Y. and Gao, C., 2006, Enhancing Radiosensitivity of Hela Cells by Combining Transfection of bcl-2 c-myc ASODNs, *Bull. Cancer*, **93**(11), E119-25.

Taraphdar, A.K., Roy, M. and Bhattacharya R.K., 2001, Natural Products as Inducers of Apoptosis: Implication for Cancer Therapy and Prevention, *Curr. Sci.,* **80**(11), 1387-1396.

Thu, Y.M. and Richmond, A., 2010, NF-κB Inducing Kinase: A Key Regulator in the Immune System and Cancer, *Cytokine Growth Factor Rev*., **21**(4), 213-226.

Timothy, S., Canavan, M.D., Nipa, R. and Doshi, M.D., 2000, *Cervical Cancer*, http://www.aafp.org/afp/2000/0301/p1369.html Cited December 25, 2011.

Verma, I.M., 2004, Nuclear Factor (NF)-kappa B Proteins: Therapeutic Targets, *Ann. Rheum. Dis*., **62**(2), 1157-1161.

Voorhees, P.M., Dees, E.C., O'Neil, B. and Orlowski, R.Z., 2003, The proteasome as a target for cancer therapy, *Clin. Cancer Res*., **9**(17), 6316-25.

WHO/ICO, 2010, *Information Centre on HPV and Cervical Cancer. Human Papilloma Virus and Cancers in World*, http://www.who.int/hpvcentre, Cited December 20, 2012.