ISSN: 2088-0197 e-ISSN: 2355-8989



Reveal Cytotoxicity and Antigenotoxicity of *Piper nigrum* L. Ethanolic Extract and its Combination with Doxorubicin on CHO-KI Cells

Nur Fitra Sari¹, Beni Lestari¹, Dian Saputri¹, Anisa Fauzia Ahsani¹, Ragil Anang Santoso¹, Ediati Sasmito², Edy Meiyanto^{1,2*}

¹Cancer Chemoprevention Research Center, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia ²Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Black pepper (Piper nigrum L.), one of the most popular Indonesian spices has been reported to possess various therapeutic effects. The aim of this study is to evaluate the cytotoxicity and antigenotoxicity of black pepper ethanolic extract (BPE) and its combination with doxorubicin (Dox) on CHO-K1 cells. Based on thin layer chromatography analysis, BPE contained piperine. Under MTT assay, BPE showed cytotoxic effect with the IC50 value of 68 µg/mL and performed synergism in combination with Dox. In vitro micronucleus test using Giemsa staining revealed that BPE did not cause morphological changes qualitatively on CHO-KI cells at concentration of 8.5 µg/mL, whereas using flow cytometry analysis showed that BPE could decrease the number of micronucleus (MN) formation induced by doxorubicin. In addition, BPE reduced the ROS level on the CHO-KI cells which observed by reactive oxygen species (ROS) intracellular assay. The decrease in ROS level indicated that the antioxidant activity of BPE contribute to the antigenotoxicity. Furthermore, molecular docking performed that piperine interacted with DNA Topoisomerase II with docking score of -80.68. Overall, BPE performed cytotoxic effect in single treatment, increased the cytotoxicity and reduced the genotoxicity of doxorubicin. Thus, BPE has potential to be developed further as co-chemotherapeutic and antigenotoxic agent.

Keywords: Cytotoxic, genotoxic, Piper nigrum L., CHO-KI, micronucleus

INTRODUCTION

Spices are common food ingredient consumed by Asian people to make several foods taste better. One of the most famous spices with its distinctive spicy flavor and aroma is black pepper (*Piper nigrum* L.) that is widely cultivated in tropical countries such as Indonesia (Damanhouri and Ahmad, 2014). Alongside being a supplementary ingredient in food, the community empirically has utilized black pepper as a body warmer, stamina enhancer, and one of the components in traditional medicine (Ahmad, *et al.*, 2012). Particularly, in the field of drug development, the exploration of the medical benefits of this spice becomes a challenge.

Black pepper is known to contain piperine or 1-peperoyl piperidine (Fig. 1a) as the major compound which the first active compound that isolated from different members of Piperaceae family and was found to possesss diverse pharmacological activities (Damanhouri and Ahmad, 2014). Piperine has been studied to have potentially various therapeutic activities such as antihypertensive and antiplatelets (Taqvi, *et al.*, 2008), antiasthmatics (Parganiha, *et al.*, 2011), antipyretic, analgesic, anti-in ammatory, antidiarrheal, antispasmodic, anxiolytic, antidepressants (Li, *et al.*, 2007), hepatoprotective (Bajad *et al.*, 2001), anti-inflammatory, antioxidant, antitumor (Manoharan, *et al.*, 2009), immunomodulator, immunostimulant, antibacterial, antifungal, and may increase the bioavailability of the drug in the body (Meghwal, *et al.*, 2013).

Although black pepper has been widely used routinely as a spice or herbal medicine, there has not been much evaluation of the genotoxicity of black pepper extract using CHO-K1 cells as a common model for gentoxicity test.

^{*}Corresponding author e-mail: edy_meiyanto@ugm.ac.id



The genotoxicity of piperine has been investigated by several scientists; reverse mutation assays in *Salmonella typhimurium* (Ames Test) showed negative result (Andrews, *et al.*, 1980). An *in vitro* micronucleus test using hepatoma cells (H4IIEC3/G-cells) was negative response (Singh, *et al.*, 1994), while *in vivo* Comet assay in lungs of male mice (Selvendiran, *et al.*, 2005) at concentration levels of up to 75 mg/kg bw performed no genotoxic activities. Piperine has also no genotoxic potential under MNT *in vitro* and *in vivo* evaluation (Thiel, *et al.*, 2014). Therefore, this study convey information regarding to the antigenotoxicity of black pepper extract especially in combination with doxorubicin (Dox).

This study aims to evaluate the cytotoxic and antigenotoxic effects of black pepper ethanolic extract (BPE) through *in vitro* modeling system using the Chinese hamster ovary (CHO-K1) cells solely and its combination with Dox. CHO-K1 cells was exerted as a model of proliferative cells and Dox for modelling a genotoxic inducer. Genotoxic effect was analyzed by cytokinesis-block micronucleus (CBMN) assay through staining with Giemsa and counting the micronucleus formation. Moreover, we also measured reactive oxigen species (ROS) level using DCFDA reagent. The findings from this research can be useful for the development of BPE as co-chemotherapeutic agent overcoming the cytotoxicity of Dox.

MATERIALS AND METHODS

Extract Preparation and Identification Chemical Compound of BPE

Black pepper (*Piper nigrum* L.) powder form was obtained from Balai Materia Medika Batu, Malang, East Java and determined at the same place. Black pepper powder was macerated by ethanol p.a. (Merck) for 24 hours and concentrated by evaporating the solven. The identification of the chemical content in BPE was carried out using thin layer chromatography (TLC) with silica gel 60 F_{254} as the stationary phase and dichlorometan:ethyl acetate (3:1 v/v) as the mobile phase. Spots were identified under UV 254 nm and UV 366 nm.

CHO-KI Cell Culture

CHO-K1 cells were obtained from Prof. Masashi Kawaichi, Nara Institute of Science and Technology, Japan. The cells were cultured in Rosewell Park Memorial Institute (RPMI) medium (Gibco) supplemented with 10 % $^{\text{V}}$ / $_{\text{V}}$ Fetal Bovine Serum (FBS) (Sigma), Fungizone 1.25 μ g/mL and 150 U/mL Penicillin - 150 μ g/mL Streptomycin (Gibco) at 37°C and 5 % CO₂. Trypsin-EDTA 0.25% (Gibco) was used to detach cells from tissue culture dish.

Cytotoxicity Assay

Cytotoxic assay was performed using MTT assay. Cells (briefly 8 x 10³ cells/well) were transferred to 96-wellplate and then incubated for 24 hours. Cells were treated with BPE, Dox, and their combination and then incubated for 24 hours. After 24 hours, medium was removed and cells were washed with 100 mL of PBS. Then 100 mL of MTT reagent with final concentration of 0.5 mg/mL in medium was added into each well and incubated again for 4 hours to form formazan crystal. The stopper reagent (10% SDS in 0.01 N HCl) was used to dissolve then the cell were incubated overnight at room temperature and in the dark (covered with alumunium foil). The next day, the absorbance from each well was measured by ELISA reader with 595 nm wavelength then converted to cells viability percent.

ROS Intracellular Assay

CHO-K1 cells (briefly 5x10⁴ cells/well) were transferred into 24-wellplate and incubated for 24 hours. After 24 hours, medium was removed and cells were washed with PBS. Cells were detached using 200 μL trypsin-EDTA per well. Trypsin was inactivated using 450 μL 1X supplemented buffer per well. Cells were collected and transferred into microtube then stained with DCFDA (2',7'-dichlorofluorescin diacetate) as much as 25 μM/well and incubated for 30 minutes in 37°C incubator. After 30 minutes, cells were treated with BPE, doxorubicin and their combination and incubated for 4 hours. ROS analysis performed using flow cytometry at wavelength of Ex485 nm/Em535 nm.



CBMN Assay using Giemsa Staining

CHO-K1 cells (briefly 1.2x10⁴ cells/well) were seeded on coverslips in 24-wellplate and incubated for 24 hours. Cells were treated with BPE, Dox, and their combination then incubated for 24 hours. After 24 hours, medium was removed and washed with 500 μL of PBS per well. Cells were fixed with 500 μL of cold hypotonic solution (KCl 0.075 M) and incubated for 6 minutes. The cold hypotonic solution was discarded then added 500 μL of methanol:acetic acid (3:1) solution and incubated for 30 minutes. Cells were stained using 500 μL of 5% Giemsa and incubated for 1 hour. Coverslips were removed from wells to object glasses. Observation was performed under light microscope with 400x magnifications.

CBMN Assay using Flow cytometry

The genotoxic flow cytometry assay was performed as describe previously with slightly modification (Avlasevich, et al., 2006). CHO-K1 cells (briefly 5x10⁴ cells/well) were seeded in 6wellplate and incubated for 24 hours. Then, cells were treated with various concentrations of BPE, Dox, and their combination and incubated for 24 hours. After 24 hours, cell medium was transferred into conical tube. Cells were washed with PBS of 500 μL and placed in conical tube. As much as 200 μL of trypsin-EDTA was added and incubated for 3 minutes. Tripsin was inactivated by added 1 mL of medium. Cells suspension were transferred into conical tube and centrifuged at 1500 rpm for 5 minutes. As much as 100 µL of Nucleic Acid Dye Working Solution was added then soaked in ice and irradiated with a light source for 20 minutes. As much as 3 mL of cold buffer solution was added and placed in dark room. The cells were centrifuged at 1500 rpm for 5 minutes. The supernatant was removed then the cell pellet was resuspended with 1 mL of Complete Lysis Solution 1 and incubated at

room temperature for 1 hour. Lysis Solution 1 was prepared with aquabides, 0.584 mg/mL NaCl, 1 mg/mL sodium citrate, 0.3 μ L/mL IGEPAL, 1 mg/mL Rnase A (Sigma) and 0.4 μ L/mL SYTOX Green (Life Technologies). Furthermore, 1 mL of Complete Lysis Solution 2 was added and incubated at room temperature for 30 minutes. Lysis Solution 2 was prepared with aquabides, 85.6 mg/mL sucrose (Merck), 15 mg/mL citric acid (Merck), and 0.4 μ L/mL of SYTOX Green. Then, samples were analyzed using flow cytometer.

Molecular Docking

Molecular docking was performed to evaluate the interaction between piperine and DNA Topoisomerase II protein (PDB ID: 4GOV). Piperine structure was prepared using Marvin Sketch while DNA Topoisomerase protein was obtained from protein Data Bank (http://www.pdb.org/). Validation was done with parameter value of RMSD (Root Mean Square Distances) where RMSD value is must be less than 2 Å (Purnomo, 2011). Docking was done using PLANTS software to obtain docking score. Docking score indicates the chemical bonding strength between ligand and receptor.

RESULTS

Extraction and Identification of BPE Phytochemical Content

From maceration, we gained viscous black pepper extract (BPE) of 6.89% (w/w) yield. Qualitative identification of the phytochemical content of BPE was performed by using TLC. The result showed that there was same spot between the piperine and BPE sample at hRf value of 82.5 which indicated similar polarity between chemical component of BPE and piperine standard (Fig. 1b). This phenomenon was predicted that BPE contained piperine as its chemical compound.



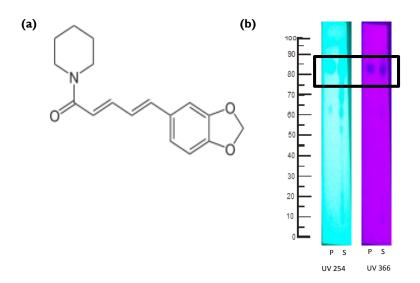


Figure 1. Chemical structure and phytochemical profile of piperine in BPE. (a) Chemical structure of piperine as the major compound of BPE. (b) Chromatogram profile of piperine by thin layer chromatography (TLC). The elution was carried out with a silica gel 60 F₂₅₄ as stationary phase and the dichloromethane:ethyl acetate (3:1 v/v) as mobile phase with an elution distance of 8 cm. Detection was performed under UV254 and UV366. (P: piperine standard; S:BPE sample).

Effects of BPE and Its Combination With Dox on CHO-KI Cells Viabillity and Intracellular ROS Level

Cytotoxic assay was performed using MTT Assay to determine the effect of BPE on CHO-K1 cells viability. The results indicated that BPE caused reduction of cells viability with IC $_{50}$ value of 68 μ g/mL (Fig. 2). IC $_{50}$ value was used as fundamental to determine concentration used in followed experiments.

Dox was used in combination treatment as one of chemotherapeutic agents that can cause cell toxicity through the mechanism of inhibition of DNA Topoisomerase II, intercalation with DNA causing inhibition of DNA synthesis, as well as the formation of free radicals of semiquinone and oxygen free radicals (Bruton, *et al.*, 2005). Combination treatment of BPE and Dox was revealed by using BPE concentration of 8.5 μg/mL

while the concentrations of doxorubicin used were 1, 2, and 4 μ M. The combination could decrease cell viability greater than single treatment (Fig. 2b). In addition, the combination treatment has combination index (CI) value of <1 which indicated synergistic activity between BPE and Dox (Fig. 2c). These results suggested that BPE could be developed as a co-chemotherapeutic agent against cancer cells in order to improve the effectiveness of Dox.

ROS (Reactive Oxygen Species) intracellular assay was aimed to determine the ability of BPE in lowering ROS levels in CHO-K1 cells. The results showed decreasing in fluorescence intensity which indicated reducing of intracellular ROS in CHO-K1 cells (Fig. 2d). That phenomenon was probably due to the antioxidant activity of BPE. This result strengthened the potency of BPE as antigenotoxic agent by neutralizing radical compound which cause DNA damage.



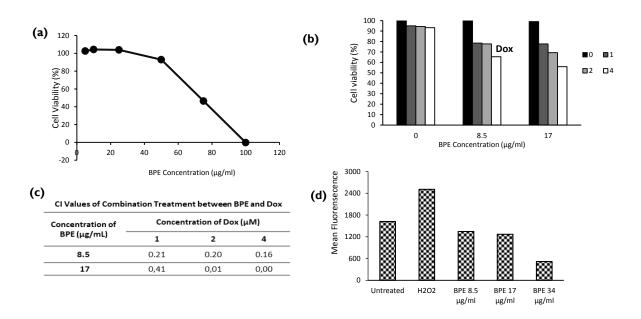


Figure 2. Effect of single treatment of BPE and the combination with Dox on cell viability and intracellular ROS levels on CHO-K1 cells. Cytotoxic effect of BPE (a) and the combination with Dox (b and c) were conducted by MTT assay, CHO-K1 cells (1x10⁴ cells/well) were treated with the compounds in the concentration as indicated for 24-h. The IC₅₀ values were important to the further assays and were calculated by using linear regression in three independent experiments. The intracellular ROS level of BPE was measured using flow cytometry with DCFDA staining. (d) Histograms of ROS-positive cell percentage by mean of DCF fluorescence intensity.

Effects of BPE on Micronucleus Formation

Micronucleus is one of parameter to determine genetic damage. Micronucleus would be expressed on imperfectly divided cells because of DNA damage. Damaged DNA is unable to reach the spindle string during mitosis. Therefore, in telophase stage, cells form nuclear membrane that would cover damaged DNA and form small nucleus called micronucleus (Fenech, 2000).

Based on qualitative observation of CBMN assay, Dox 1 μ M treatment showed micronucleus formation and changed in cell morphology while in BPE 8.5 μ g/mL treatment, it was not found morphological changes. However, in the

combination of BPE and Dox, the micronucleus formation was difficult to observe (Fig. 3a). These results qualitatively showed that low concentrations of BPE did not induce genotoxic in CHO-K1 cells.

Genotoxic assay using flowcytometry revealed that Dox 1 µM did not induce micronucleus formation. However, doxorubicin 2 nM showed micronucleus formation with MN percentage of 94.83% while BPE 17 and 34 µg/mL did not show micronucleus formation. In combination of Dox and found that BPEcould decrease micronucleus percentage (Fig. 3b). This results suggested that BPE is potential as an antigenotoxic agent on CHO-K1 cells.



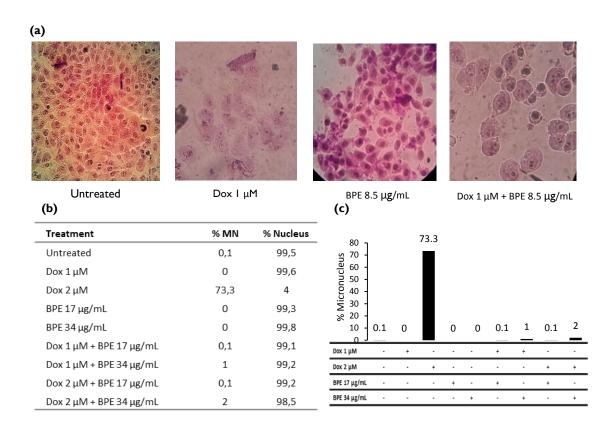


Figure 3. Effects of BPE administration on micronucleus formation in CHO-K1 cells. CHO-K1 cells of 12 x 10³ cells/plants were seeded on coverslip within 24-wellplate for CBMN test and 5 x 10⁴ cells/wells in 6-wellplate for genotoxic test with flowcytometry. Cells were treated with BPE, Dox, and their combination. (a) Cell morphology of CHO-K1 after being stained with Giemsa 5%. The observation was done using light microscope with 400x magnification. (b) Percentage of micronucleus formation on CHO-K1 cells.

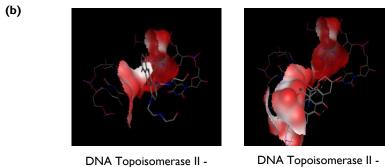
Molecular Interaction between Piperine and DNA Topoisomerase II

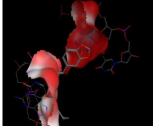
Molecular docking was done to predict interaction of active compound in BPE toward DNA Topoisomerase II protein as the molecular target of Dox. Native ligand mitoxantron was used to compare the interaction of piperine on DNA Topoisomerase II protein. The lower docking score then easier to form bonding between ligand and

protein target. Docking score of ligand and DNA Topoisomerase II was showed in Fig. 4a. The docking results showed piperine had lower affinity than mitoxantron on DNA Topoisomerase II protein because it had higher docking score than mitoxantron. Therefore, piperine could compete with mitoxantron in binding with DNA Topoisomerase II protein and might decrease the possibility of DNA damage.



(a)	Ligand	Docking score
	Native ligand (Mitoxantron)	-109,51
	Doxorubicin	-86,9733
	Piperin	-80,6793





mitoxantron

doxorubicin

DNA Topoisomerase II -

Figure 4. Interaction between mitoxantron (native ligand), Dox, and piperine on DNA Topoisomerase II. Mitoxantron (native ligand), Dox, and piperine structures were prepared using MarvinSketch while DNA Topoisomerase II was prepared using YASARA. Docking simulation was performend using PLANTS software. (a) Docking score and (b) 3D visualization of interaction.

DISCUSSION

Genetic instability as an indicator of exposure to genotoxic agents is the common phenomena leading to the disease, included cancer. Dox is well known as the most common chemotherapeutic agent compared to another agent due to the strong cytotoxic activity (Cardoso, et al., 2009). However, Dox reported to possess genotoxicity that is caused by the produce of oxidative stress which reduces the activity of antioxidant, increase the lipid peroxidase and increase intracellular ROS level such as superoxide radical anion, hydroxyl radical, and singlet oxygen. This mechanism leads to the cell death and the micronucleus formation (Bryce, et al., 2010). Hence, an agent that possess strong cytotoxic activity as well as reduce the genotoxicity of doxorubicin are considered to develop a new cochemotherapeutic agent.

The results of our study showed that the single treatment of BPE reduced the viability of CHO-K1 cells in dose dependent manner and gave IC₅₀ value of 68 μg/mL. Based on Prayong, et al. (2008), the IC₅₀ value of BPE on CHO-K1 cells was below 100 µg/mL meaning that BPE has strong cytotoxic activity. The result was in accordance with the previous studies, in which BPE was found to have cytotoxic activity on HeLa cell (61.94±0.054 μg/mL) (Paarakh, et al., 2015), 4T1 cells, and K562 cells (Lu, et al., 2012). The combination of BPE and Dox could also reduce cell viability combination index (CI) value of <1 which indicated synergistic activity between BPE and Dox (Fig. 2c). This data supported that BPE was potential to be developed as co-chemotheraetic agent in order to improve the effectiveness of Dox. Thus, continued this research to investigate genotoxicity of BPE in combination with Dox.

In the genotoxicity study, BPE showed antigenotoxic effect and provided protection against toxic genotoxic effects induced chemotherapeutic agent, Dox in in vitro system using CHO-K1 cell lines. There were no increases in the micronucleus-frequencies of the BPE treated group at doses of 17 and 34 µg/mL. The reduction of micronucleus also seen in the combination with Dox compared to the positive control Dox itself (Fig. 3c). The results in the absence of micronucleus formation are in line with the previous literature data of piperine genotoxicity (Thiel, et al., 2014). To conclude on the cytotoxicity and genotoxicity potential of piperine, we considered all relevant data in several literatures: Piperine was tested under the Ames Test (Karekar, et al., 1996) and in vitro MNTs



(Singh, et al., 1994), and showed negative results. Moreover, several studies at doses up to 75 mg/kg bw in different species showed the absence of micronucleus, DNA-strandbreaks, or dominant lethal mutation (Karekar, et al., 1996; Muralidhara and Narasimhamurthy, 1990; Selvendiran, et al., 2005; Balakrishnan, et al., 2008). In addition, several research strongly presented evidence for a chemopreventive activity of piperine rather than for a genotoxic activity (Vellaichamy, et al., 2009; Sehgal, et al., 2013). Furthermore, our result indicated that BPE performed antioxidant activity based on the reducing of intracellular ROS level on CHO-K1 cells compared to H₂O₂ treatment (Fig. 2d). This result enhanced the potency of BPE as antigenotoxic agent might be caused by neutralizing free radical compound which cause DNA damage.

These data suggested that BPE was not genotoxic, regardless of the concentration treatment. From the combination with Dox, BPE increased doxorubicin effectiveness and decreased the frequency of micronucleus, indicated that BPE reduced any toxic effects of Dox. Different mechanism of Dox and piperine might be contributed to the combinational cytotoxic effect of both. Dox interacts with DNA by intercalation and inhibits DNA Topoisomerase II. Therefore, we studied about the interaction between piperine and DNA Topoisomerase II using molecular docking. Molecular docking analysis demonstrated that piperine potentially inhibits the target protein, DNA Topoisomerase II. Although the docking score of piperine was lower than the native ligand, piperine could compete with Dox to interact with DNA Topoisomerase resulting in the increasing of doxorubicin effectiveness therapy. To confirm the anti-genotoxic effect of BPE should be conducted by mammalian in vivo micronucleus test and the others in vitro genotoxicity evaluation such as comet assay and DNA fragmentation assay to detect DNA damage. In addition, studies should be conducted to determine the limit dose in human consumption and provide rational concentration for implementing cochemotherapeutic and antigenotoxic agent.

CONCLUSION

We summarized that BPE was not induce micronucleus formation and could reduce the frequency of micronucleus induced by doxorubicin.

These results suggested that BPE has potency to be developed as an agent for preventing genetic damage. In addition, cytotoxic combination assay indicated that BPE might increase cytotoxic effect of doxorubicin on CHO-K1 cells. Therefore, BPE could be developed as co-chemotherapeutic agent to improve the effectiveness of doxorubicin therapy.

ACKNOWLEDGMENT

We express our gratitute to Directorate General of Learning and Student Affairs, Ministry of Research, Technology and Higher Education for the grant research under Student Creativity Program in 2016. We also thank to Prof. Masashi Kawaichi (NAIST Japan) for the CHO-K1 cells used in this research.

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