

RESEARCH ARTICLE

Editorial Process: Submission:05/25/2018 Acceptance:09/09/2018

Synergistic Action of 1,2-Epoxy-3 (3- (3,4-dimethoxyphenyl)-4H-1-benzopyran-4-on) Propane with Doxorubicin and Cisplatin through Increasing of p53, TIMP-3, and MicroRNA-34a in Cervical Cancer Cell Line (HeLa)

Lelly Yuniarti^{1,2*}, Mustofa Mustofa³, Teguh Aryandono⁴, Sofia Mubarika Haryana⁵

Abstract

Objective: Cervical cancer is the second most common cancer among women worldwide, with a high mortality rate especially in developing countries. Insufficient treatment for cervical cancer, multiple side effects, and high drug prices encourage researchers to look for effective and selective cancer drugs with appropriate molecular targets. This study explored the cytotoxicity of (1,2-epoxy-3(3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-on) propane (EPI) synthesized from clove leaves oil on HeLa cells, its combination with doxorubicin (DOX) and cisplatin (CIS), and also their influence on p53, TIMP-3, and miR-34a as therapeutic targets. **Materials and Methods:** This research was an experimental in vitro study on cervical cancer uteri culture. The cytotoxicity was analyzed by MTT assay. The drug combination synergisms were indicated by the combination index (CI) (using CompuSyn 1.4). HeLa cells in 32 wells were divided into eight groups as negative control, which were given EPI $\frac{1}{2}IC_{50}$, EPI IC_{50} , EPI 2IC₅₀, DOX IC_{50} , combination of EPI+DOX, CIS, and the combination of EPI+CIS. The p53 and TIMP-3 concentrations were measured using ELISA, and expressions of miR-34a with qRT-PCR. One-way ANOVA and post hoc Tukey tests were performed to determine the mean difference of all variables between the study groups. **Results:** IC_{50} for EPI was 33.24 (± 3.01) $\mu\text{g/ml}$, while DOX and CIS were 4.8 $\mu\text{g/ml}$ (± 0.1), and 23.34 $\mu\text{g/ml}$ (± 3.01), respectively, while CI values for EPI-DOX were <0.1 and for EPI-CIS <0.9 . Expression of p53 in group 6 (1.67 \pm 0.31) $\mu\text{g/ml}$ and 8 (1.18 \pm 0.18) $\mu\text{g/ml}$, TIMP-3 6 (3.81 \pm 0.49) $\mu\text{g/ml}$ and 8 (2.93 \pm 0.42) $\mu\text{g/ml}$ were significantly higher compared to the control group ($p < 0.05$). All treatment groups showed significantly increased miR-34a expressions compared to the control group ($p < 0.05$). **Conclusion:** The combinations showed a very strong synergism and a moderate slight synergism for EPI-DOX and EPI-CIS. Both combinations were able to increase the expressions of p53, TIMP-3 proteins, and MiR-34a in the HeLa cells.

Keywords: Cervical cancer- Epoxy- MicroRNA-34a- p53- TIMP-3

Asian Pac J Cancer Prev, 19 (10), 2955-2962

Introduction

Cervical cancer has a high incidence and mortality rate and is the second most common cancer among women worldwide. About 200,000 women in developing countries died of this disease in 2010, and 46,000 of them were aged 15-49 years (Forouzanfar et al., 2014; Nindrea et al., 2018).

Cervical cancer is caused by a long-term infection of Human Papillomavirus (HPV). DNA viruses merge with human DNA resulting in an increased expression of E6 and E7 proteins, and subsequently interact with the oncogenic proteins and interfere with the cell cycle

(Steben and Duarte-Franco, 2007). Protein E6 inhibits the tumor suppressor gene of p53 by increasing its proteasomal degradation. p53 is a tumor suppressor gene that acts as a transcription factor in regulating the transcription process of hundreds of genes encoding proteins for protection and maintaining genome integrity. It induces cell cycle arrest and DNA repair in the event of DNA damage, and triggers apoptosis when DNA repair is unsuccessful (Kumari et al., 2014).

Inactivation of p53 will cause down-regulation of miR-34a expression, since miR-34a is a direct target of transcriptional p53 transcription factor. Transactivation of expression of miR-34a is triggered by the p53 bond at

¹Department of Biochemistry, Faculty of Medicine Universitas Islam Bandung, Bandung, ²Doctorate Program, Faculty of Medicine, ³Department of Pharmacology and Therapy, ⁴Department of Surgery, ⁵Department of Histology Faculty of Medicine, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. *For Correspondence: lely.yuniarti@unisba.ac.id

the binding site of the promoter region miR-34a. Cells with decreased miR-34a expression showed rapid cell growth and avoided apoptosis. The mechanism of this process involves several components in the cell cycle including CDK4, cyclin E2, E2F-1, MET hepatocyte growth factor and Bcl-2 (Bommer et al., 2007; Feng et al., 2011; Hermeking, 2010).

Human Papillomavirus 16/18 E6 oncoproteins are highly expressed in tumors and associated with Tissue Inhibitor of Metalloproteinases-3 (TIMP-3) inactivation. TIMP-3 is important for limiting inflammation, so the loss of TIMP-3 will induce chronic inflammation and may promote the formation of malignancies, resulting in poor survival and relapse in lung cancer patients infected with HPV. Loss of TIMP-3 activity was found to increase IL-6 production via TNF beta pathways, thus increasing tumor malignancy and resulting in relapse and poor survival in HPV-positive lung cancer patients (Wu et al., 2012).

After undergoing primary treatments both surgery and radiation, 40% of cervical cancer patients still have residual tumors, distant metastasis and/or relapse (Minig et al., 2014). Chemotherapeutic drugs such as cisplatin (CIS) have many side effects, including nephrotoxicity, bone marrow suppression, neurotoxicity, and vomiting (Kilic et al., 2014; Kitagawa et al., 2015). Doxorubicin (DOX), an anthracycline antibiotic is one of the chemotherapies for the treatment of some cancers. The antitumor activity of DOX is mainly due to the DNA damage caused by inhibition of DNA topoisomerase II, and its side effects are cardiotoxicity and resistance (Park et al., 2014). Less optimal treatment of cervical cancer and side effects encourage researchers to look for the more effective and selective cancer drugs or for a combination of chemotherapeutic drugs (co-chemotherapy).

The co-chemotherapy approach is one of the alternative methods for solving drug resistance problems, and increases efficacy and allows the use of drug's lower doses to reduce side effects (Chou, 2010). The compound of 1,2-epoxy-3(3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-on) propane (EPI) obtained from clove leaf oil (eugenol) is widely available in Indonesia. Its structure is similar to genistein, an isoflavone found in soybeans (Nurung et al., 2016). According to our knowledge, this is the first research about the anticancer and co-chemotherapy effects of EPI compounds on cultures of cervical cancer. The aim of this study was to observe the anticancer mechanism of EPI and its combination with DOX and CIS, and their effect on miR-34a, p53, and TIMP-3 as molecular targets.

Materials and Methods

This study was a randomized post-test only control group design in vitro experimental research. The HeLa cells were randomly divide into 8 groups, with Group 1 (negative control), 2 (was given EPI $\frac{1}{2}$ IC₅₀), 3 (was given EPI IC₅₀), 4 (was given EPI 2IC₅₀), 5 (was given DOX IC₅₀), G6 (was given EPI $\frac{1}{2}$ IC₅₀ + DOX $\frac{1}{2}$ IC₅₀), 7 (was given IC₅₀ CIS), and 8 (was given EPI $\frac{1}{2}$

IC₅₀ + CIS $\frac{1}{2}$ IC₅₀). The EPI compound was synthesized at the Faculty of Mathematics and Natural Sciences of Universitas Gadjah Mada by Dr. Andi Hairil Alimuddin. HeLa cells obtained from Parasitology Laboratory, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Indonesia. The cells were grown using RPMI (Gibco), supplemented with 10% Fetal Bovine Serum (FBS Qualified, Gibco, Invitrogen USA), 1.5% (w/w) penicillin-streptomycin (Gibco, Invitrogen USA), and 0.5% (w/w) fungizone (Gibco, Invitrogen USA). Cells were incubated at 37°C and 5% CO₂.

Cytotoxic assay

The cytotoxic activity was measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to obtain the value of the inhibitory concentration 50% (IC₅₀) values. HeLa cells were distributed in the 96-well plates and seeded at the density of 10⁴ per well. The cells were grown for 24 hours in a humidified incubator at 37°C and 5% CO₂. After seeding, the cells were treated with various serial concentrations of EPI. The tests were performed in concentrations of 250, 125, 62.5, 31.25, 15.625, 7.8125; and 3.906 µg/ml EPI. The cytotoxicity tests of doxorubicin were done in a series of 100, 50, 25, 12.5, 6.25, 3.125; and 1.56 µg/ml. The cytotoxicity tests of cisplatin were done in a series of 200, 100, 50, 25, 12.5, 6.25, and 3.125 µg/ml. The absorbance of each well was measured using an ELISA reader (Bio-Rad) at λ 595 nm and converted to a percentage of viable cells. The percentage of viable cells was determined according to the following formula:

$$\text{Cell viability (\%)} = \frac{A_{\text{treatment}} - A_{\text{media control}}}{A_{\text{cell control}} - A_{\text{media control}}} \times 100\% \quad A = \text{absorbance}$$

This compound was combined with doxorubicin at concentrations of $\frac{1}{2}$ IC₅₀, $\frac{3}{8}$ IC₅₀, $\frac{1}{4}$ IC₅₀, $\frac{1}{8}$ IC₅₀ to determine the potential use of the EPI compound as a co-chemotherapy agent for doxorubicin in HeLa cervical cancer cells. The same concentrations were used for the combination of EPI and CIS. Interpretations of the Combination Index (CI) values were based on Table 1 (Chou, 2006).

Analysis of drug synergy was performed by calculating the CI to measure the interaction between two drugs. The CI was calculated according to the median-effect principle of the Chou and Talalay method, using the CompuSyn software 1.4 (Chou, 2006).

ELISA

HeLa cells were grown with the density of 10⁶ cells in the 6-well plates for 24 hours before treatment with EPI, DOX, CIS, or their combinations (EPI-DOX and EPI-CIS). Cells were treated for 24 hours and washed twice using PBS. The harvested cells were removed to conical tubes. Cells were harvested and counted, then centrifuged to precipitate cells, after adding a TEGM buffer solution which was a mixture of 10 mM Tris-HCl, five mM EDTA, 10% glycerol, and 3 mM MgCl₂, pH 6.8 with a 1 ml TEGM ratio. Next, the cells were homogenized at a moderate rate with a homogenizer (10 beats, top and

bottom). The homogenate obtained was incubated at 4°C for 18 hours. Next, they were centrifuged at a rate of 1,000 × g for 20 min. The supernatant collected would be used for the measurement of the protein p53 (cat#EH 3898 Fine Test, China) and TIMP3 (cat#EH 0296) using the ELISA technique. The optical density of each well was determined using a microplate reader (Biorad). Concentrations were calculated per 1 million cells by the formula: protein concentration = [concentration] / cell number x 1 million cells.

qRT-PCR

Total RNA was extracted from HeLa Cells samples using Qiagen miR-easy Mini Kit (cat #217004) according to manufacturer’s protocol. Synthesis of cDNA was following the protocol of QiagenmiScript II RT Kit (cat#218161). MicroRNA expression was measured with the qRT-PCR method quantified by an Applied Biosystem 7500 FAST real-time PCR using Qiagen miScript SYBR Green PCR Kit cat#218073). Appropriate internal control used Ctrl_miRTC_1 miScript Primer Assay (MS00000001) and primer for PCR Hsa-miR-34a (MS00003318), Universal primer were from Qiagen and used as directed. U6 was used as the host keeping gene, and the fold change in gene expression was calculated by the 2^{-ΔΔCT} method.

Statistics

The expression of miR-34a, concentration of p53, and TIMP-3 protein were analyzed using one-way ANOVA and Post hoc test with Tukey test. The level of significance was set at p<0.05.

Ethical issues

This research was approved by the ethical committee of the Faculty of Medicine, Public Health and Nursing, UniversitasGadjahMada, Yogyakarta (Ethical Clearance Number: KE/FK/0126/EC/2017).

Results

Cytotoxicity Assessments of EPI, DOX, and CIS

The cytotoxicity test on HeLa cells was performed in a 24-hour incubation time. The MTT result of the EPI compound showed that the IC₅₀ value was 33.24 µg/ml, while the cytotoxicities of DOX and CIS against Hela cells were at the IC₅₀ value of 4.8 µg/ml, and 23.34 µg/ml, respectively. The average IC₅₀ values and the standard

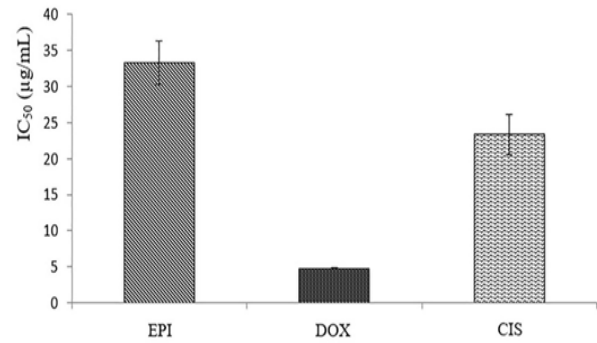


Figure 1. IC₅₀ of EPI, DOX, and CIS

deviations of EPI, DOX, and CIS can be seen in Figure 1.

Combination Test of EPI with DOX using MTT

Synergistic effects of drug combinations were measured by the isobologram method to obtain the CIs. The dose-effect curve of the EPI, DOX, and its combination to the viability of HeLa cells are shown in Figure 2A, while the CI values of EPI and DOX combinations are shown in Figure 2B.

Combination Test of EPI with CIS using MTT

The dose-effect curve of the EPI, CIS and its combination to the viability of HeLa cells are shown in Figure 3A, while the CI value of EPI and CIS combination are shown in Figure 3B.

Measurement of the p53 protein expression with ELISA Effect of EPI, DOX, CIS, and their combinations on p53 protein

The concentrations of p53 in the various groups are

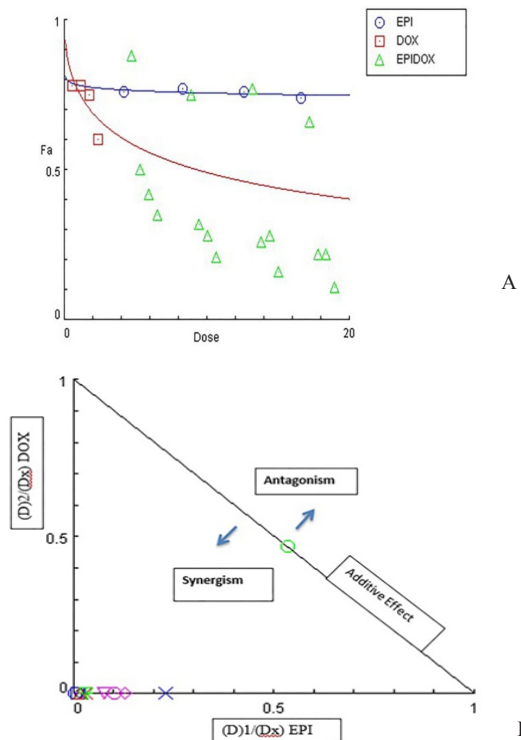


Figure 2. Effect Curve of EPI and DOX on HeLa Cells (A). Normalized Isobologram for Combo, EPI-DOX (dose in µg/ml) (B).

Table 1. The Measurement of Combination Index (CI)

CI value	Interpretation
<0.1	very strong synergism
0.1-0.3	strong synergism
0.3-0.7	synergism
0.7-0.9	moderate slight synergism
0.9-1.1	nearly additive
1.1-1.45	slight to moderate antagonism
1.45-3.3	antagonism
>3.3	very strong antagonism effect

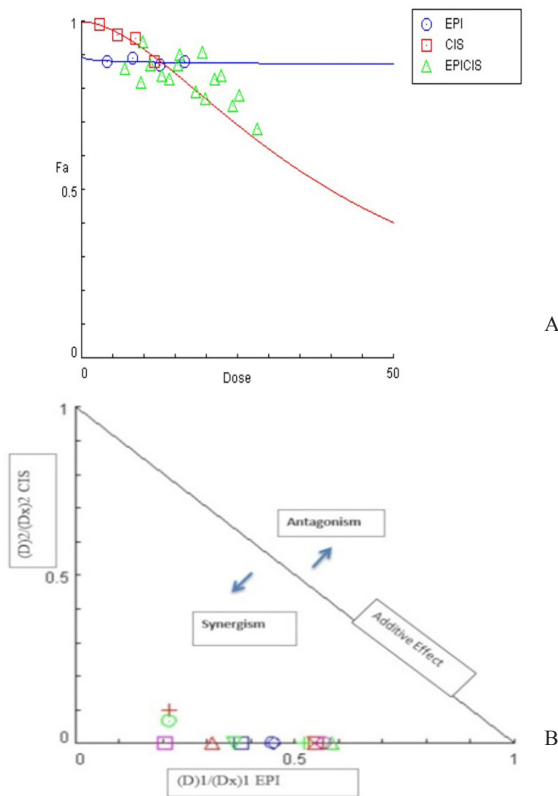


Figure 3. Effect Curve of EPI and CIS on HeLa Cells (A). Normalized Isobologram for Combo, EPI-CIS (dose in µg/ml) (B).

shown in Figure 4A.

The Elisa examination revealed that TIMP-3 levels were higher in groups 6, 7, and 8 compared to group 1, i.e. 1.67 ± 0.31 (6); 0.63 ± 0.11 (7); 1.18 ± 0.18 (8). The result was statistically significant in groups 6, 7, and 8, $p=0.00$.

Effect of EPI, DOX, CIS, and their combinations on TIMP-3 protein

The concentrations of TIMP-3 in the various groups are shown in Figure 4B.

The Elisa examination revealed that TIMP-3 levels were higher in almost all treatment groups compared to group 1, i.e. 0.89 ± 0.09 (2); 0.80 ± 0.13 (4); 0.91 ± 0.37 (5); 3.81 ± 0.50 (6); 1.33 ± 0.14 (7); 2.93 ± 0.42 (8). The result was statistically significant in groups 6 and 8, $p=0.00$.

Effect of EPI, DOX, CIS, and their combinations to miR-34a expression

The expressions of miR-34a on the various groups are shown in Figure 4C.

The qRT-PCR examination revealed that miR-34a had significantly higher expression ($p=0.00$) in all treatment groups compared to group 1, i.e. 1.75 ± 0.01 (2); 3.18 ± 0.40 (3); 2.36 ± 0.43 (4); 8.76 ± 0.92 (5); 2.42 ± 0.40 (6); 1.44 ± 0.27 (7); 1.42 ± 0.09 (8).

Discussion

The results of this study showed that the IC_{50} values of each tested single compound on HeLa cell line were

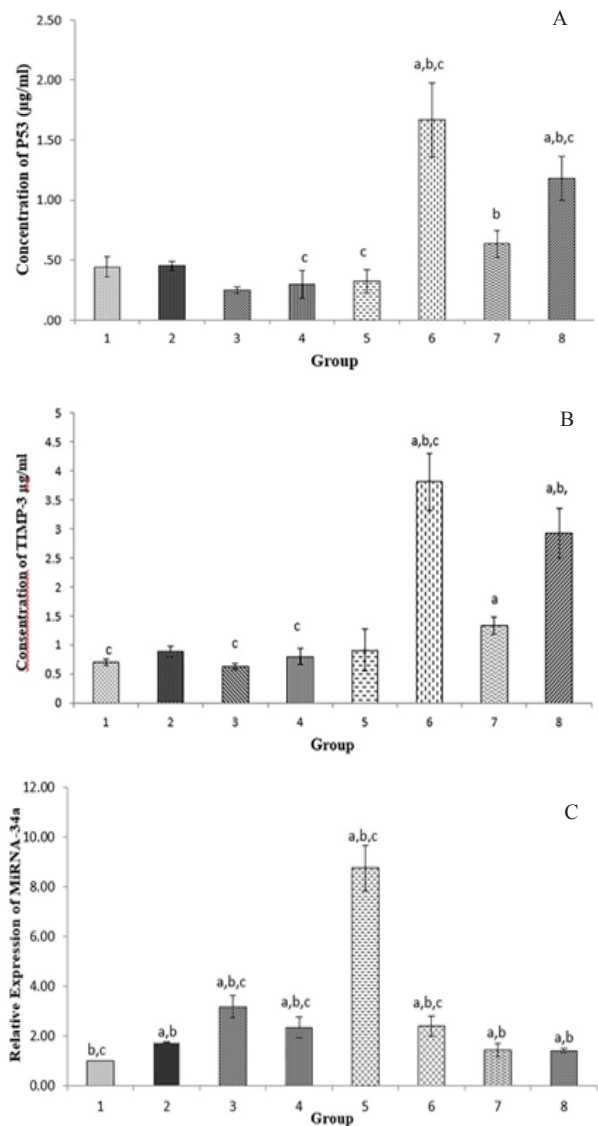


Figure 4. Effect of EPI, DOX, CIS, and Their Combination on p53 of HeLa Cells (A). TIMP-3 of HeLa cells (B). MiR-34a of HeLa cells (C). a, Significantly different from Group 1; b, Significantly different from Group 5; c, Significantly different from Group 7 ($p<0.05$).

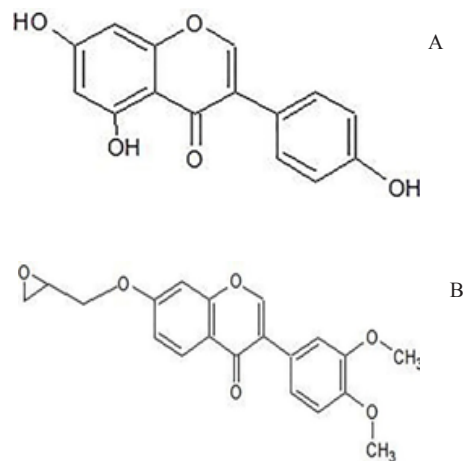


Figure 5. Structure of Genistein (A), 1,2-Epoxy-3-(3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-on) Propane

33.24 ± 3.01 µg/ml, 4.8 ± 0.10 µg/ml, 23.34 ± 2.76 µg/ml for EPI, DOX, and CIS, respectively (Figure 1). Based on the IC₅₀ values, these results showed that EPI and CIS compounds were in a moderate category of anticancer activities, whereas DOX was categorized as having a potent anticancer activity against the HeLa cell line (Baharum et al., 2014; Caamal-Fuentes et al., 2015).

The EPI is an isoflavone compound synthesized from clove leaf oil. Its structure is like genistein and resembles 17β-Estradiol, which selectively binds to estrogen receptors (ER) with different affinity, and modulates recruitment of co-receptors and co-activators to influence the signaling of estrogen receptors (Hervouet et al., 2013). The chemical structure of the EPI compound is shown in Figure 5B, while the genistein's structure is shown in Figure 5A.

Genistein was reported to have many anticancer activities through several mechanisms such as antioxidant action, inhibiting tyrosine-specific protein kinases, and suppressing cell growth by stopping cell cycles. It is also known to induce apoptosis through multiple signaling pathways including Akt, NF-Kappa β, and Wnt signaling or by inhibition of angiogenesis (Huang et al., 2005; Li et al., 2011).

The structure of EPI in Figure 5B shows the molecular structure of 3 rings with double bonds. The C2-C3 double bond significantly contributes to the molecular planarity and conjugation between the potent C and A/B rings as an anticancer agent (Wang et al., 2017). Previous studies demonstrated that the presence of C2-C3 double bonds was an important feature for inducing cytotoxicity in different cancer cells and for other pharmacological activities, such as colon adenocarcinoma (Murthy et al., 2012) and MDA-MB-231 breast cancer cells (Amrutha et al., 2014).

The addition of epoxy groups to the flavonoid skeletal framework of the EPI structure was thought to enhance the anticancer action, as in 4a, 5b-epoxy-germacra-1-(10), 11-(13)-dien-12,6a-olide which possesses anticancer properties through intrinsic pathway induction and extrinsic apoptosis in variable tumor cells through NF-Kappa β inhibition, p53 signaling activation, and regulation of Bcl-2 and ROS formation (Muhtasib et al., 2015).

The CI measurements of EPI-DOX were mostly below 0.1, indicating that the EPI compound has very strong synergism with DOX. The best combination with the 11% cancer cell viability was achieved at the combinations of ½IC₅₀ of EPI and ½IC₅₀ DOX. The CI test of the combination of EPI and CIS was between 0.7 and 0.9. This CI value indicates that the EPI-CIS combination has moderate slight synergism (Chou, 2006). The best combination with the cancer cell viability of 68% was achieved at the combinations of ½IC₅₀ of EPI and ½IC₅₀ CIS. Normalized isobologram for Combo EPI-DOX and EPI-CIS (Figure 2B and 3B) showed that almost all combinations of EPI-DOX and EPI-CIS were located to the left of the curve. This result indicates that EPI had a synergistic effect with DOX and CIS. The findings of this study were similar to other studies showing that a combination of genistein with cisplatin could increase HeLa cell death by activating the NF-κB, Akt, and mTOR

pathways (Sahin et al., 2012). Previous studies have shown that isoflavones such as genistein and daidzein synergized with many standard cancer drugs in many cancer cells such as pancreatic cancer and MCF-7 (Banerjee et al., 2007; Farjadian et al., 2015). Other studies shown that genistein is synergic with several standard drugs in several cancer cell cultures such as: prostate cancer cell culture (PC-3), pancreatic cancer (Bx PC-3), lung cancer cells (H460), and breast cancer cells (MDA-MB-231) (Li et al., 2005). Overexpression and activation of NF-κB could inhibit apoptotic response, and thereby reduce chemotherapeutic efficacy. The polyphenolic structure in genistein could alter this action, since genistein could decrease antiapoptotic protein Bcl-2 and Bcl-xL and down regulation NF-κB in pancreatic carcinoma cells (Lewandowska et al., 2014).

Figure 4A shows that the concentrations of p53 in the groups treated with the combination of EPI-DOX and EPI-CIS were higher than the control group and the group treated with DOX and CIS alone (p<0.05), while the group treated with EPI was not significantly different from the control group. This result contradicted a previous study which shown that genistein may trigger apoptosis by inhibiting APE1 redox activity, increasing p53 or inhibiting p53 degradation in A549 lung cancer cell culture (Zhu et al., 2015), and also inhibiting signal transduction of growth factors and other factors that can induce cell growth. The hydroxyl group-enriched structure of the flavonoid makes it more nucleophilic which could inhibit carcinogenesis (Cai and Han, 2015). Antitumor activity of genistein was associated with the down regulation of Akt, which serves as the major anti-apoptosis main pathway activated by EGFR. Akt pathways activate NF-κB through EBFR-independent resulting in the transcription of several genes, such as survive cyclooxygenase-2, Bcl-xL, and Bcl-2. These genes play a significant role at some stage in the cell cycle (cell growth, invasion, angiogenesis, and apoptosis). Genistein also activates p53, suppresses the expression of Bcl-2, increases the expression of Bax and Bak, and then activates the release of cytochrome c from mitochondria. The release of cytochrome c from mitochondria activates caspase 9, and this caspase activates caspase 3, 6, and 7, which in turn will induce apoptosis (Yeh et al., 2015).

Figure 4B shows that in the CIS-treated group and in the combinations of EPI-DOX and EPI-CIS, the concentrations of TIMP-3 were higher than the control group. TIMP-3 concentrations in the groups treated with EPI-DOX and EPI-CIS were significantly higher than those treated with CIS alone (p<0.05). Other studies also showed a combination of genistein and cisplatin were synergistic in human hepatocellular carcinoma (HCC) cells with a CI of 0.41. The anticancer mechanism of this combination involves inhibition of the increase in MMP-2 so as to inhibit metastasis (Chen et al., 2013). The expression of MMP-2 was inhibited by TIMP-3, allowing for a possible decrease in MMP-2 in the study through enhancement of TIMP-3. A recent study showed that 24 hour-treatment of genistein 100µM on HaCaT human cell lines increased the TIMP-3 concentration 2.6 times more

than the negative control (Smolinska et al., 2018). All of these results suggest that the combinations of EPI-DOX and EPI-CIS might increase p53 and TIMP3 concentrations.

Figure 4C shows the miR-34a expressions of all treatment groups were significantly higher than in the control group ($p < 0.05$), indicating that EPI, DOX, CIS, and its combinations might increase miR-34a expressions. While the highest miR-34a expression was in the DOX-treated group, this finding was consistent with a research on cancer animal model indicating that the expression of miR-34a increased in all concentrations of doxorubicin associated with cardiotoxicity (Ruggeri et al., 2018).

The miR-34a gene is located on chromosome 1p36.22 which is a region of frequent deletion and downregulation in many cancers (Henrich et al., 2012). There are two basic mechanisms that effect the regulation of expression of miR-34a which are activation by p53 and CpG island methylation. Oncoprotein E6 from HPV in uterine cervical cancer causes p53 deactivation which can cause downregulation of miR-34a. which then leads to an increase in proliferation and invasion (Zhang et al., 2016). Several previous studies have shown that polyphenols could increase the expression of miR-34a in breast cancer by down regulating Bcl-2 and Bmi-1 as targets (Guo et al., 2013). Luteolin which belongs to the flavonoid family could decrease Bcl-2 expression and induce apoptosis partly through upregulation of miR-34a expression in gastric cancer cells (Wu et al., 2015). The molecular mechanisms leading to miR-34a up regulation by polyphenol are poorly understood, but several lines of evidence suggest that epigenetic modification of promoters may be involved. In cancer, mutations or inactivation of the p53 and CpG methylation genes of miR-34a promoters will result in decreased expression of miRNA-34a (Chim et al., 2010; Hermeking, 2010; Siemens et al., 2013; Nindrea et al., 2017).

The highest p53 concentrations occurred in groups 6, 8, and 7, whereas the highest miR-34a expression occurred in groups 4, 5, and 6. This result did not correspond with the assumption above that high p53 concentrations activated miR-34a and thus increased the expression of miR-34a. This finding might be due to the complexity of the p53 relationship with miR-34a. In addition to p53 activating miR-34a, p53 is both an indirect and direct target of miR-34a. MiRNA-34a has several targets that influence the expression of p53, i.e., Silent Regulator Information 1 (SIRT1), a gene which functions for the deacetylation process of p53, so that the expression of miR-34a will inhibit the process of p53 deacetylation. SIRT1, p53, and miR-34a form feedback loops inducing tumor suppression. SIRT1 can also inhibit miR-34a through histone deacetylation (Navarro and Lieberman, 2015; Okada et al., 2014; Yamakuchi and Lowenstein, 2009). Over expression of mouse double minute 2 homolog (MDM2), (Navarro and Lieberman, 2015; Pollutri et al., 2016), histone deacetylase 1 (HDAC1) (Bittremieux et al., 2016; Kaller et al., 2011), and the p53 transcription factor, Yin Yang 1, can induce downregulation of p53-signaling pathways. (Gronroos et al., 2004; Sui et al., 2004), resulting in

increased expression of miR-34a. Inhibition of these genes will increase p53 expression, while p53 is a direct miR-34a target, and p53 mRNA was found to be highly enriched in the Bi-miR-34 pull-down (PD). According to one recent study, there was a decrease of p53 in 7 cell lines with high miR-34 expression (Navarro and Lieberman, 2015).

In conclusion, isoflavones from clove leaf oil (1,2-epoxy-3(3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-on) propane (EPI) induced cell death in HeLa uterine cervix cancer cells and had a very synergistic co-chemotherapy effect with doxorubicin and was moderately synergistic with cisplatin. Combinations of (1,2-epoxy-3(3-(3,4-dimethoxyphenyl)-4H-1-benzopyran-4-on) propane with doxorubicin and cisplatin increased p53, TIMP-3, and miR-34a expressions in HeLa uterine cervix cancer cells.

There were two major limitations in this study. First, the study was conducted only on one type of cervical cancer cell culture (HeLa). Another limitation was the measurements of each parameter were only done once (posttest only) and not serialized, which led to this study not being able to describe the sequence or cascade of molecular changes in cancer cells due to the treatments. Future studies should focus on the other cervical cancer cell lines such as CaSki, Siha, and C33A and use in vivo assay tests in an animal model.

Acknowledgments

This work was funded by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia through Beasiswa Pendidikan Pascasarjana Dalam Negeri (BPPDN) for the Ph.D. scholarship program and by the Ph.D. Dissertation Grant (number 1598/K4/KM/2017).

References

- Amrutha K, Nanjan P, Shaji SK, et al (2014). Discovery of lesser known flavones as inhibitors of NF- κ B signaling in MDA-MB-231 breast cancer cells - A SAR study. *Bioorganic Med Chem Lett*, **24**, 4735-42.
- Baharum Z, Akim AM, Taufiq-Yap YH, Hamid RA, Kasran R (2014). In vitro antioxidant and antiproliferative activities of methanolic plant part extracts of theobroma cacao. *Molecules*, **19**, 18317-31.
- Banerjee S, Zhang Y, Wang Z, et al (2007). In vitro and in vivo molecular evidence of genistein action in augmenting the efficacy of cisplatin in pancreatic cancer. *Int J Cancer*, **120**, 906-17.
- Bittremieux M, Parys JB, Pinton P, Bultynck G (2016). ER functions of oncogenes and tumor suppressors: modulators of intracellular Ca²⁺ signaling. *BBA Mol Cell Res*, **1863**, 1364-78.
- Bommer GT, Gerin I, Feng Y, et al (2007). p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol*, **17**, 1298-307.
- Caamal-Fuentes EE, Peraza-Sánchez SR, Torres-Tapia LW, Moo-Puc RE (2015). Isolation and identification of cytotoxic compounds from *aeschnomene fascicularis*, a mayan medicinal plant. *Molecules*, **20**, 13563-74.
- Cai S, Han K (2015). Research on expression and importance of p53, p16 and VEGF-C in cervical cancer Étude de l'expression et de l'importance des protéines p53. *J Gynecol*

- Obstet Biol Reprod*, **44**, 639-45.
- Chen P, Hu M-D, Deng X-F, Li B (2013). Genistein reinforces the inhibitory effect of cisplatin on liver cancer recurrence and metastasis after curative hepatectomy. *Asian Pac J Cancer Prev*, **14**, 759-64.
- Chim CS, Wong KY, Qi Y, et al (2010). Epigenetic inactivation of the miR-34a in hematological malignancies. *Carcinogenesis*, **31**, 745-50.
- Chou TC (2006). Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol Rev*, **58**, 623-74.
- Chou TC (2010). Drug combination studies and their synergy quantification using the Chou-Talalay method. *Cancer Res*, **70**, 440-7.
- Farjadian SN, Khajoei M, Fazeli F, Askari S, Zaeri (2015). Doxorubicin cytotoxicity in combination with diadzein on MCF-7 breast cancer cells.pdf. *Mal J Nutr*, **21**, 67-73.
- Feng Z, Zhang C, Wu R, Hu W (2011). Tumor suppressor p53 meets microRNAs. *J Mol Cell Biol*, **3**, 44-50.
- Forouzanfar MH, Foreman KJ, Delossantos AM, et al (2014). Breast and cervical cancer in 187 countries between 1980 and 2010: a systematic analysis. *Lancet*, **378**, 1461-84.
- Gronroos E, Terentiev AA, Punga T, Ericsson J (2004). YY1 inhibits the activation of the p53 tumor suppressor in response to genotoxic stress. *Proc Natl Acad Sci U S A*, **101**, 12165-70.
- Guo J, Li W, Shi H, et al (2013). Synergistic effects of curcumin with emodin against the proliferation and invasion of breast cancer cells through upregulation of miR-34a. *Mol Cell Biochem*, **382**, 103-11.
- Henrich K-O, Schwab M, Westermann F (2012). 1p36 tumor suppression-a matter of dosage?. *Cancer Res*, **72**, 6079-88.
- Hermeking H (2010). The miR-34 family in cancer and apoptosis. *Cell Death Differ*, **17**, 193-9.
- Hervouet E, Cartron P-f, Jouvenot M, Delage-mourroux R (2013). Epigenetic regulation of estrogen signaling in breast cancer. *Epigenetic*, **3**, 237-45.
- Huang X, Chen S, Xu L, et al (2005). Genistein inhibits p38 Map kinase activation, matrix metalloproteinase type 2, and cell invasion in human prostate epithelial cells. *Cancer Res*, **65**, 3470-8.
- Kaller M, Liffers ST, Oeljeklaus S, et al (2011). Genome-wide characterization of miR-34a induced changes in protein and mRNA expression by a combined pulsed SILAC and microarray analysis. *MCP*, **10**, M111-010462.
- Kilic U, Sahin K, Tuzcu M, et al (2014). Enhancement of cisplatin sensitivity in human cervical cancer: epigallocatechin-3-gallate. *Front Nutr*, **1**, 28.
- Kitagawa R, Katsumata N, Shibata T, et al (2015). Paclitaxel plus carboplatin versus paclitaxel plus cisplatin in metastatic or recurrent cervical cancer: the open-label randomized phase III trial JCOG0505. *JCO*, **33**, 2129-35.
- Kumari R, Sen N, Das S (2014). Tumour suppressor p53: understanding the molecular mechanisms inherent to cancer. *Curr Sci*, **5**, 786-94.
- Lewandowska U, Grolach S, Owczarek K, Hrabec E, Szewczyk K (2014). Synergistic interactions between anticancer chemotherapeutics and phenolic compounds and anticancer synergy between polyphenols. *Postepy Hig Med Dosw*, **68**, 528-40.
- Li Y, Ahmed F, Ali S, et al (2005). Inactivation of nuclear factor κB by soy isoflavone genistein contributes to increased apoptosis induced by chemotherapeutic agents in human cancer cells. *Cancer Res*, **65**, 6934-42.
- Li Y, Kong D, Bao B, Ahmad A, Sarkar FH (2011). Induction of cancer cell death by isoflavone: the role of multiple signaling pathways. *Nutrients*, **3**, 877-96.
- Minig L, Patrono MG, Romero N, Rodriguez Moreno JF, Garcia-Donas J (2014). Different strategies of treatment for uterine cervical carcinoma stage IB2-IIB. *World J Clin Oncol*, **5**, 86-92.
- Muhtasib GH, Hmadi R, Kareh M, Tohme R, Darwiche N (2015). Cell death mechanisms of plant-derived anticancer drugs: beyond apoptosis. *Apoptosis*, **20**, 1531-62.
- Murthy CN, Kim J, Vikram A, Patil BS (2012). Differential inhibition of human colon cancer cells by structurally similar flavonoids of citrus. *Food Chem*, **132**, 27-34.
- Navarro F, Lieberman J (2015). miR-34 and p53: new insights into a complex functional relationship. *PLoS One*, **10**, 1-23.
- Nindrea RD, Aryandono T, Lazuardi L (2017). Breast cancer risk from modifiable and non-modifiable risk factors among women in Southeast Asia: a meta-analysis. *Asian Pac J Cancer Prev*, **18**, 3201-6.
- Nindrea RD, Harahap WA, Aryandono T, Lazuardi L (2018). Association of BRCA1 Promoter methylation with breast cancer in Asia: a meta-analysis. *Asian Pac J Cancer Prev*, **19**, 885-9.
- Nuring AH, Herwiyanti S, Paramita DK (2016). Effect of 1,2-epoxy-3[3-3[3,4-dimethoxyphenyl]-4h-1-benzopiran-4-on] Propane (epi) on Sirtuin-1 and nuclear factor-κB expression of DMBA induced mammary tumors in sprague dawley rats. *J Med Sci*, **48**, 142-53.
- Okada N, Lin CP, Ribeiro MC, et al (2014). A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. *Genes Dev*, **28**, 438-50.
- Park H-k, Lee J-e, Lim J, et al (2014). Combination treatment with doxorubicin and gamitrinib synergistically augments anticancer activity through enhanced activation of Bim. *BMC Cancer*, **14**, 1-9.
- Pollutri D, Gramantieri L, Bolondi L, Fornari F (2016). TP53/microRNA interplay in hepatocellular carcinoma. *Int J Mol Sci*, **17**, 2029.
- Ruggeri C, Gioffrè S, Achilli F, Colombo GI, D'Alessandra Y (2018). Role of microRNAs in doxorubicin-induced cardiotoxicity: an overview of preclinical models and cancer patients. *Heart Fail Rev*, **23**, 109-22.
- Siemens H, Neumann J, Jackstadt R, et al (2013). Detection of miR-34a promoter methylation in combination with elevated expression of c-Met and beta-catenin predicts distant metastasis of colon cancer. *Clin Cancer Res*, **19**, 710-20.
- Smolinska E, Moskot M, Jako J, et al (2018). Molecular action of isoflavone genistein in the human epithelial cell line HaCaT. *PLoS One*, **13**, e0192297.
- Steben M, Duarte-Franco E (2007). Human papillomavirus infection: epidemiology and pathophysiology. *Gynecol Oncol*, **107**, 2-5.
- Sui G, Affar el B, Shi Y, et al (2004). Yin Yang 1 is a negative regulator of p53. *Cell*, **117**, 859-72.
- Wang Ty, Li Q, Bi Ks (2017). Bioactive flavonoids in medicinal plants: Structure, activity and biological fate. *Asian J Pharm Sci*, **13**, 12-23.
- Wu D-W, Tsai L-H, Chen P-M, et al (2012). Loss of TIMP-3 promotes tumor invasion via elevated IL-6 production and predicts poor survival and relapse in HPV-infected non-small cell lung cancer. *Am J Pathol*, **181**, 1796-806.
- Wu H, Huang M, Liu Y, Shu Y, Liu P (2015). Luteolin induces apoptosis by up-regulating miR-34a in human gastric cancer cells. *Technol Cancer Res*, **14**, 747-55.
- Yamakuchi M, Lowenstein CJ (2009). MiR-34, SIRT1 and p53: the feedback loop. *Cell Cycle*, **8**, 712-5.
- Yeh C-C, Fan Y, Jiang L, et al (2015). Genistein suppresses growth of human uterine sarcoma cell lines via multiple mechanisms. *Anticancer Res*, **35**, 3167-73.
- Zhang R, Su J, Xue S-L, et al (2016). HPV E6/p53 mediated

down-regulation of miR-34a inhibits Warburg effect through targeting LDHA in cervical cancer. *Am J Cancer Res*, **6**, 312.
Zhu J, Zhang C, Qing Y, et al (2015). Genistein induces apoptosis by stabilizing intracellular p53 protein through an APE1-mediated pathway. *Free Radic Biol Med*, **86**, 209-18.



This work is licensed under a Creative Commons Attribution-Non Commercial 4.0 International License.