Journal of Applied Pharmaceutical Science Vol. 4 (12), pp. 014-019, December, 2014
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2014.41203
ISSN 2231-3354

Combination of Cisplatin and Cinnamon Essential Oil Inhibits HeLa Cells Proliferation through Cell Cycle Arrest

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ARTICLE INFO

Article history:
Received on: 24/07/2014
Revised on: 21/08/2014
Accepted on: 11/09/2014
Available online: 29/12/2014

Key words:
Cinnamon essential oil, cisplatin, HeLa cells, apoptosis, cell cycle.

ABSTRACT

Cisplatin is drug of choice toward cervical cancer despite having many side effects, thus researches are conducted in order to find the effective and synergistic co-chemoterapeutic agent combined with cisplatin. In this study, we observed the potential of the cinnamon essential oil (CEO) isolated from *Cinnamomum burmannii* as co-chemoterapeutic agent of cisplatin on HeLa cells covering cytotoxic effect, cell cycle modulation and induction of apoptosis. Cytotoxic effect was determined by using MTT assay; while induction of apoptosis and cell cycle profile were observed by using flow cytometry. At 24 hours of incubation, CEO showed cytotoxic effect on HeLa cells with IC_{50} value of 250 µg/mL, while cisplatin showed cytotoxic effect with IC_{50} value of 18 µM. Combination of CEO and cisplatin reduced cells viability compared to cisplatin solely. Moreover, flow cytometry using annexin-V and PI showed that CEO and its combination with cisplatin induced apoptosis lower than cisplatin alone at 24 hours of incubation. Further analysis on the cell cycle progression showed that CEO induced S-phase arrest on HeLa cells, cisplatin induced G1 arrest, while combination of CEO and cisplatin induced G2/M arrest. Thus, the inhibition of HeLa cells growth at 24 hours is likely through cell cycle modulation rather than apoptosis.

INTRODUCTION

Cervical cancer shows high incidence in the world even ranked first as deadly canceron women in Indonesia (Lewis, 2004; Aziz, 2009). Chemotherapeutic agent used widely in the treatment of cervical cancer is cisplatin or *cis*-diamminedichloroplatinum (II). Platinum in the compound makes crosslinking with DNA bases, especially guanine, and interfere cell mitosis, leading to apoptosis (Trzaska, 2005). However, cisplatin possessed genotoxicity by inducing DNA damage of recombinational origin (Danesi *et al.*, 2010), and anticipated to be human carcinogen (Waalkes *et al.*, 2006). There are side effects from the use of cisplatin, such as nephrotoxicity, ototoxicity, nausea, vomiting, and resistance of the cancer cells (Cepeda *et al.*, 2007; Fuertes *et al.*, 2003). Combination chemotherapy or co-chemotherapy is an approach to increase the effectiveness of cisplatin as well as suppress its side effects. Combination chemotherapy targets on several physiological and molecular phenomenon of cancer cells. Two or more agents used in combination chemotherapy should perform synergistic effect, either act on the same target or not. An example for clinically accepted combination chemotherapy is combination of trastuzumab and docetaxel for HER2 positive breast cancer (Bullock and Blackwell, 2008). Trastuzumab triggers HER2 internalization and degradation as well as cause antibody-dependent cellular cytotoxicity. The most recognized mechanism of trastuzumab is to inhibit the MAPK and PI3K/Akt pathways, leading to an increase in cell cycle arrest, and the inhibition of cell growth and proliferation (Vu and Claret, 2012).

Docetaxel shows different mechanism of action by stabilizing microtubule assembly, thus preventing physiological microtubule depolymerisation and inhibits mitotic cell division. Docetaxel also has been found to lead the phosphorylation of anti-apoptotic protein bcl-2, leads to cancer cells apoptosis (Lyseng-Williamson and Fenton, 2005). As for cervical cancer, Food and Drug Administration (FDA), USA, proves the combination of cisplatin and topotecan for women with with stage IV B, recurrent, or persistent carcinoma of the cervix (Brave *et al.*, 2006).

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Both of chemotherapeutic agent cause cancer cell apoptosis by intercalating DNA and disrupts DNA repair mechanism, although each agent targets different base in DNA. Moreover, combination chemotherapy is suggested to target on the several of ten hallmarks of cancer (Mahadevan, 2013). Phytochemical compounds are promising to be combined with the chemotherapeutic agent (Tyagi*et al*., 2004). Plant that could be the object of studies regarding to its potency as a co-chemotherapeuticagent is cinnamon bark (*Cinnamomum burmannii*). The active compounds of cinnamon bark usually taken by distillation or water extraction, resulted in Cinnamon Essential Oil (CEO). CEO contains cinnamaldehyde as the main compound and other volatile compounds, such as aldehyde, alcohol, alkane, and eugenol (Li *et al*., 2013). Lee *et al.,*(1999) reported that cinnamaldehyde performed antiproliferative activity against several cancer cells, including breast cancer, leukemia, ovarian cancer, and lung cancer. There are several mechanisms of CEO inhibiting cancer proliferation; including inhibiting the activation of NFκB and AP1, modulating cell cycle, and inhibiting the angiogenic proteins (Schoene *et al*., 2008; Kwon *et al*., 2010; Lu *et al.,* 2010).

The aims of this study are to observe synergistic effect of CEO combined with cisplatin in cytotoxic effect, induction of apoptosis, and cell cycle modulation on HeLa cervical cancer cells. The result is expected to be a reference for further research in order to utilize cinnamon essential oil as an alternative co-chemotherapeutic agent for cisplatin in cervical cancer therapy.

MATERIALS AND METHODS

Preparation of CEO

Cinnamon bark was obtained from Boyolali, Jawa Tengah, Indonesia and determined at Pharmaceutical Biology Laboratory, Faculty of Pharmacy, Universitas Gadjah Mada. The barks was dried and chopped, then placed in the sample flask. Distillate water was added to the water flask (± 3 L) and boiled at 100°C. Steam was produced under the sample and condensed as distillate oil (CEO).

Cell Culture

HeLa cells (ATCC origin) was obtained from Prof. Tatsuo Takeya (Nara Institute of Science and Technology, Japan) and grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% v/v 10,000 units/mL penicillin-10,000 µg/mL streptomycin (Gibco) at the temperature of 37 °C and with a flow of 5% CO_2 .

Cytotoxic determination with MTT assay

HeLa cells were grown in 96-well plates at 1x10^{4} cells/well and divided into control and treatment groups, and each group made in triplicate. Final concentrations of CEO were 50, 100, 150, 200, 300, 400 and 500 µg/mL while concentration of cisplatin were 5, 10, 20, 30, 40, and 50 µM. Concentration series of CEO and cisplatin then diluted in culture medium up to final concentrations. After 24 h incubation, medium was removed and cells were washed using PBS (Sigma). About 5 mg/mL of MTT on PBS (Sigma) was diluted by DMEM (1:9) and 100 µl of reagent was added into each well. After 3 hours of incubation, reaction was stopped by adding of SDS 10% in HCl 0,01 N. The plate then incubated overnight in room condition at dark place. To make sure the formazan dissolved, the plate was shaked for 10 minutes and the absorbance was measured using ELISA reader at λ 595 nm.

Flow Cytometric Assay

HeLa cells were seeded at 5x10^{5}cells/well on six wells tissue culture plate. After 24h of incubation, cells were treated with CEO (125 µg/mL) alone and its combination with cisplatin (9 µM). After 24h of these treatments, cells were trypsinized and spin at 2000 rpm for 3 minutes, then washed twice with cold PBS. To examine the induction of apoptosis, cells were re-suspended in 500 µl of AnnexinV buffer (Biovision) and then treated with AnnexinV and propidium iodide (PI) for 10 minutes at 37^{0}C. To examine the cell cycle profile, Cells were re-suspended in propidium iodide solution (1 mg/mL in PBS contained 1% triton X-100) and then treated with RNAse (10 µg/mL) for 10 minutes at 37^{0}C. The treated cells then were subjected to FACS flow cytometry.

Data Analysis

Single Cytotoxicity assay

Linear regression between concentration and % cells viability giving the equation y = Bx + A were used to calculate IC_{50} value, which is the concentration inhibiting 50% of cell growth. The IC_{50} value was determined using Excell MS Office 2007.

Combinational Cytotoxicity Assay

Combinational treatment was evaluated by calculating the Combination Index (CI) value (Reynolds and Maurer, 2005), which has the formula as follows.

$$CI = D1/Dx1 + D2/Dx2$$

D1 and D2 represent the concentrations used in combinational treatment, while Dx1 and Dx2 are single treatment concentration giving the same response as D1 and D2, respectively. CI value acquired will allow the evaluation of CEO's potency in combinatorial treatment with cisplatin on HeLa cells. Interpretation was done based on the classification listed in Table 1.

Cell Cycle

The data obtained from flow cytometry was analyzed by using ModFit LT 3.0 program.

RESULTS AND DISCUSSION

Results

Cytotoxic Effect of CEO and Cisplatin on HeLa Cells

Cytotoxicity assay of CEO and cisplatin were done on HeLa cells in various concentration. Both CEO and cisplatin

showed cytotoxic effect in dose dependent manner with IC50 value of CEO was 250 µg/mL (Fig.1A) and IC50 value of cisplatin was 18 µM (Fig.1B). This cytotoxic effect was also observed by the morphological changes of the cells under microscope, in which the cells forming irregular and smaller round cell shape (Fig.1D).

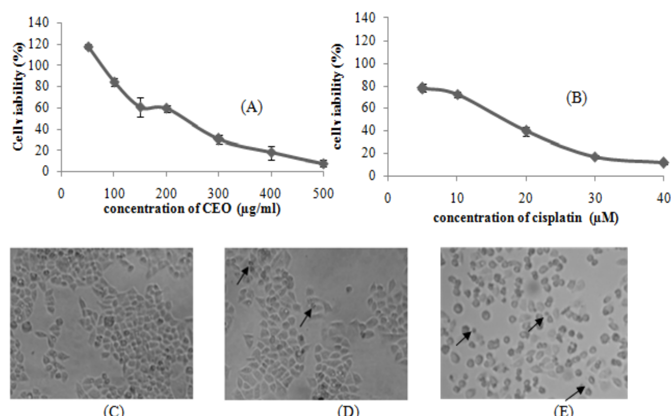

Fig. 1: CEO and cisplatin cytotoxic effects on HeLa cells.
HeLa cells (1x10^{4} cells/well) were seeded in 96 wellplate and incubated for 24h, then treated with CEO (50-500 µg/mL) or cisplatin (5-50 µM).Cells viability was determined by using MTT assay as described in methods. Graph between concentration and cells viability of (A) CEO and (B)Cisplatin. CEO and cisplatin cytotoxicity expressed by percent cell viability is shown as the mean + SE of 3 experiments. IC_{50} values obtained from the calculation of linear regression of concentration vs % cells viability with $p < 0.05$. CEO and cisplatin altered HeLa cells morphology. Morphology of cells treated with (C) none (control), (D) CEO 200µg/mL, and (E) cisplatin 20 µM. Arrows (→) indicates cells undergoing morphological changes.Cells were observed under light microscope with 400x magnification.

Cytotoxic Effect of Combination of CEO and Cisplatin on HeLa Cells

Combinational treatment of CEO and cisplatin on HeLa cells was conducted to observe the ability of CEO to increase cisplatin's cytotoxicity on HeLa cells. Combination assay was done by combined CEO and cisplatin on HeLa cells with serial concentrations of 1/20, 1/8, 1/4, and 1/2 IC_{50} of CEO and cisplatin. Combination of CEO and cisplatin decreased cells viability rather than cisplatin solely (Fig.2A). Combination index (CI) was calculated to evaluate the synergistic effect of the combination. According to the CI values as described by Reynolds and Maurer (2005) (Table 1), all of the tested combination gave synergistic effect, and some of them are strong synergist (Table 2). However, at higher dose of CEO (125 µg/mL) combined with 0.9 µM cisplatin, the cells viability was not different from the single treatment of CEO (Fig. 2). This phenomenon indicates that the cytotoxic effect of CEO is more dominant compared to cisplatin in combination of them.

Table 1: Interpretation of CI value representing potency of combinational application.

CI	Interpretation	CI	Interpretation
< 0.1	Very strong synergist	0.9-1.1	Closely additive
0.1-0.3	Strongly synergist	1.1-1.45	Middle antagonist
0.3-0.7	Synergist	1.45-3.3	Antagonist
0.7-0.9	Middle synergist	>3.3	Strongly antagonist

Table 2: Combination Index combination of CEO and cisplatin.

		Cis (µM)			
		0.9	2.25	4.5	9
CEO	12.5	0.14	0.31	0.33	0.51
(µg/mL)	31.25	0.30	0.36	0.77	0.84
	62.5	0.26	0.28	0.38	0.55
	125	0.31	0.34	0.40	0.92

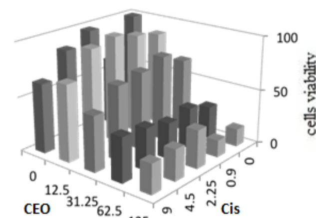

Fig. 2: Effect of CEO-cisplatin combination treatment on cells viability of HeLa cells.HeLacells (1x10^{4} cells/well) were seeded in 96 wellplate and incubated for 24h, then treated with combination of CEO and cisplatin (1/2,1/4,1/8, and 1/20 of each IC_{50}). Cells viability was determined by using MTT assay as described in methods. Graph is means from 3 replications.Data were shown as mean ± SE by three independent experiments.

Effect of CEO and Cisplatin on Induction of Apoptosis in HeLa Cells

The decrease of cells viability caused by combination of CEO and cisplatin could be related with induction of apoptosis. Thus, flow cytometric assay using AnnexinV-PI reagent was used to observe the induction of apoptosis caused by CEO and cisplatin quantitatively. Both of CEO and cisplatin were capable to induce apoptosis at concentration of ½IC50. However, combination of CEO and cisplatin gave less apoptotic cells rather than cisplatin alone (Fig. 3; Table 3). This data seemed unrelated with the decrease of cells viability in combinational treatment..

Table 3: Cells population (%) in various phases of cell death.

	Viable cell	*early apoptosis*	*late apoptosis*	necrosis	total apoptosis
Control	96.85%	1.52%	1.05%	0.58%	2.57%
Cisplatin	18.04%	29.09%	42.5%	10.38%	71.59%
CEO	50.55%	19.55%	23.34%	6.55%	42.89%
Combination	56.01%	11.01%	26.77%	6.21%	37.78%

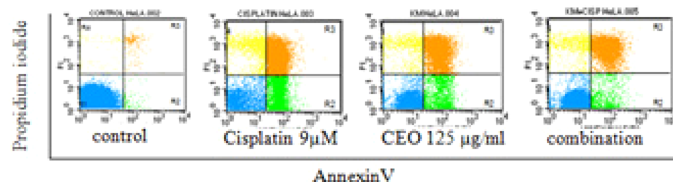

Fig. 3: Effect of CEO-cisplatin in HeLa cell apoptosis.HeLa cells were seeded at 5x10^{5} cells/well on six wells tissue culture plate, then treated with CEO (125 µg/mL) alone and its combination with cisplatin (9 µM). After 24 hours of incubation, cells were harvested as described in methods, added with AnnexinV and PI reagent, then subjected to FACS flow cytometry. (A) Combination of CEO and cisplatin decreased apoptotic cells compared to cisplatin solely. (B) Quantitative graph of apoptosis phenomenon caused by CEO and cisplatin.

Effect of CEO and Cisplatin on Cell Cycle Profile

The decrease of cell viability is usually related with two physiology phenomenon, cell death and/or inhibition of cell division. In this regard, flow cytometric assay was used to observe the distribution of cell population on cell cycle progression caused

by CEO and cisplatin. As shown in Fig.4 and Table 4, there was an accumulation at S phase on HeLa cells treated CEO, cisplatin induced accumulation at G1 phase, while combination of CEO and cisplatin caused G2/M arrest.

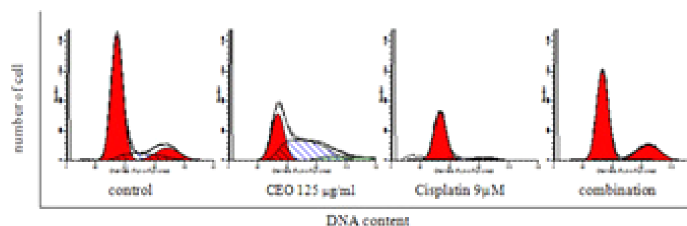

Fig. 4: Cell cycle analysis.HeLa cells were seeded at $5x10^5$ cells/well on six wells tissue culture plate, then treated with CEO (125 µg/mL) alone and its combination with cisplatin (9 µM). After 24 hours of incubation, cells were harvested as described in methods, added with PI reagent, then subjected to FACS flow cytometry.

(A) Treatment with CEO, cisplatin, and combination of them resulted in change of cell cycle progression.

(B) Quantitative graph of cell cycle modulation caused by CEO and cisplatin

Table 4. Cells distribution (%) in various phases of cell cycle.

	G1	S	G2
Control	71.95%	13.53%	14.25%
CEO	42.25%	58.36%	3.90%
Cisplatin	84.44%	10.25%	5.31%
Combination	71.68%	3.98%	24.34%

DISCUSSION

This study demonstrated the potency of CEO as an anticancer which has the potency of inhibiting cell proliferation, as well as the co-chemotherapeutic agent of cisplatin. Cytotoxic assay showed that CEO inhibited the growth of HeLa cells with IC_{50} value of 250µg/mL in dose-dependent manner. Previous study by Koppikar *et al.,* (2010) on SiHa cells, other type of cervical cancer cells, showed that cinnamon aqueous extract up to the concentration 320 µg/mL did not perform cytotoxic effect. These results suggest that cinnamon solely perform low cytotoxicity on cervical cancer cells. However, CEO increased the cytotoxic effects of cisplatin on HeLa cells, which was characterized by a decrease in cells viability compared to cisplatin treatment alone. Synergistic combination was shown with CI <0.9 and almost all of the combination showed synergistic effect. Combination with the lowest CI was reached by the lowest concentration of both CEO and cisplatin; CEO 12.5 µg/mL ($^1/_{20}$ IC_{50}) and cisplatin 0.9 µM ($^1/_{20}$ IC_{50}). While higher dose of CEO combined with constant concentration of cisplatin did not increase cytotoxicity. This finding suggests that CEO might perform dominant effect on the combination rather than cisplatin. Thus, dose of cisplatin as a chemotherapeutic agent treatment of cervical cancer could be reduced by administration of CEO, but still provide the same effect in inhibiting the growth of cancer cells. In clinical applications, lowering the dose of cisplatin therapy may reduce side effects and resistance. Thus, CEO has potency to be developed as co-chemotherapeutic agent for cisplatin in cervical cancer. Generally, the cytotoxic activity could be attributed to the ability of compounds to induce apoptosis and/or cause cell cycle arrest. Based on this study, both CEO and cisplatin induced apoptosis on HeLa cells. HeLa cells used as a model of cervical cancer cells in this experiment shows low level of p53 (Macville *et al*., 1999) and overexpression of antiapoptotic protein Bcl-2 (Darma, 2011). However, induction of apoptosis on HeLa cells could be occur via upregulation of pro-apoptotic proteins and inhibition of NFκB, AP1, and their target genes (Kwon *et al*., 2010), decreased expression of proteins involved in the G0/G1 and anti apoptotic proteins, and a decrease in membrane potential causing the release of mitochondrial cytochrome c and activation of caspase-3 (Chuang *et al.,* 2007). Previous researches show that cinnamaldehyde in cinnamon bark induced apoptosis through multiple mechanisms. Kwon *et al.,*(2010) reported that the induction of apoptosis in several cells by cinnamaldehyde is through the decrease of levels and activation of NFκB and AP1, which leads to down regulation of proteins play a role in cell survival. Cinnamaldehyde also induces transitions mediated by mitochondrial Reactive Oxygen Species (ROS) and causes the release of cytochrome c from mitochondria (Ka *et al.,* 2003). Furthermore, the release of cytochrome c and SMAC/ DIABLO to the cytosol due cinnamaldehyde causes caspase-3 and PARP change in its active form. Cinnamaldehyde also reduces levels of anti-apoptotic proteins XIAP and Bcl-2 along with the accumulation of pro-apoptotic protein Bax (Lin *et al.,* 2013). Meanwhile, the cytotoxic mechanism of cisplatin is by forming a crosslink in DNA, so that DNA could not be open and cause the attraction of other proteins to DNA, causing cell cycle arrest and apoptosis (Jordan and Carmo-Fonseca, 2000). Interestingly, although there was a decrease of cells viability in combinational cytotoxicity assay, combination of CEO-cisplatin caused lower percentage of cells undergoing apoptosis compared with single cisplatin administration on 24-hour of incubation. It is an interesting phenomenon in which the decrease of cells viability in caused by combination for 24 hours was not due to apoptosis. It was also confirmed that combination of CEO and cisplatin lower the number of necrotic cells compared to cisplatin treatment alone, which will give benefit in the clinical application later. We found that CEO treatment alone caused S-phase arrest. Cinnamaldehyde also caused S-phase arrest in human PLC/PRF/5 cells (Wu *et al*., 2005). In present study, cisplatin induced HeLa cell cycle arrestat G1 phase; the same results showed by Koprinarova *et al.,*(2010),while combination of CEO and cisplatin caused G2/M-phase arrest. Previous studies mentioned that trans-cinnamaldehyde of *Cinnamomum osmophloeum* shows cytotoxic effect on cancer cells Jurkat and U937 (myeloid cells) and causes G2/M arrest in both cell types (Fang *et al*., 2004). Study by Schoene *et al.,*(2008) also proves that aqueous extract of cinnamon interacts with signal transduction activity of phosphorylation/dephosphorylation that reduces cell proliferation, as well as causing G2/M arrest of the cell cycle.This cell cycle arrest was the reason of HeLa cells growth inhibition becaused of

CEO and cisplatin at 24h treatment. Pucci*et al.,*(2000) reported that cell cycle arrest could lead to apoptosis. Thus, apoptosis analysis of combination of CEO and cisplatin at longer incubation time needs to be conducted. An interesting characteristic of HeLa cells is the occurrence of HPV E6 protein, which degrades p53 through binding complex HPV E6-E6AP, enables E6AP to ubiquitinate p53, leading to p53 degradation. Furthermore, on HeLa cells, p53 stabilization is known to be a cause of cell cycle arrest and/or apoptosis (Reddy, 2001). Stabilization in p53 is able to induce apoptosis through activation of apoptotic Bax/Mitochondrial/Caspase-9 Intrinsic Pathway. In previous study, a histone deacetylase inhibitor stabilizes p53 and induces cell cycle arrest and apoptosis. Moreover, PPM1D activity inhibition leads to p53 activation and induces DNA repair and cell cyclearrest (Roy *et al*., 2005). CEO possibly stabilize p53 to execute G2/M arrest and apoptosis on HeLa cells. Thus, several studies on protein expressed at each cell cycle phase and p53 stabilization caused by combination of CEO and cisplatin are interesting to be conducted.

CONCLUSION

In summary, our data reveal that CEO increases the cytotoxic activity of cisplatin at 24h of incubation. However, the decrease of cells viability is more likely through cell cycle arrest rather than apoptosis. Further confirmation such as determination on induction of apoptosis at longer incubation time as well as observation on proteins regulating cell cycle and apoptosis are worth to be done.

ACKNOWLEDGEMENTS

Our thanks are to Ministry of Education, Republic of Indonesia, which has funded this research.

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How to cite this article:

Yonika Arum Larasati, Dyaningtyas Dewi Pamungkas Putri, Rohmad Yudi Utomo, Adam Hermawan, Edy Meiyanto. Combination of Cisplatin and Cinnamon Essential Oil Inhibits HeLa Cells Proliferation through Cell Cycle Arrest. J App Pharm Sci, 2014; 4 (12): 014-019.