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Caesalpinia sappan L. heartwood ethanolic extract exerts genotoxic inhibitory and cytotoxic effects

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Abstract

Brazilin and brazilein, the major compounds of *Caesalpinia sappan* L. (CS) have been reported to possess antioxidant and cytotoxic activities and could potentially be used as an antigenotoxic as well as an anticancer. This study was conducted to investigate the cytotoxic and antigenotoxic effects of CS ethanolic extract (CEE). In vivo mammalian micronucleus test of CEE at the dose of 500 mg/kg BW and 1000 mg/kg BW decreased the number of MNPCE and increased the ratio of PCE to NCE meaning that CEE performed antigenotoxic effect in an in vivo model. In contrast, CEE and doxorubicin (DOXO) performed cytotoxic effect on CHO-K1 cells under MTT assay with IC_{50} values of 67 μg/mL and 6 μM, respectively. Interestingly, treatment of CEE in combination with DOXO and H_2O_2 as genotoxic inducer decreased intracellular ROS levels. In addition, in vitro genotoxicity study by using cytokinesis-block micronucleus (CBMN) assay, both of Giemsa staining and flow cytometric analysis showed that the treatment of CEE increased the number of micronuclei and correlated with apoptotic induction results. Moreover, the combination of CEE and DOXO induced cells accumulation in Sub-G1 and G2/M phase. In conclusion, CEE performed antigenotoxic effect in an in vivo model and cytotoxic effect on CHO-K1 cells.

Keywords *Caesalpinia sappan* L. · CHO-K1 cells · Cytotoxic · Antigenotoxic · ROS

Introduction

In the modern life, people are exposed to the dangerous chemical, physical, and biological agents causing many risks on human health, particularly carcinogenic and genotoxic effects. Genotoxicity is defined by the genetic instability and DNA damage phenomena leading to the alteration of the biological and functional system of an organism (Gray and Collins 2000). In common biological processes, genotoxic agents interact with the macromolecules, especially DNA, that may lead to genetic disruption and cancer development. Hence, the development of the antigenotoxic and chemopreventive agents to prevent the genotoxic effects of the potentially carcinogenic agents is needed.

In general, antigenotoxic agents can be found from plant or natural products. The anti-genotoxicity potency of natural resources is possibly correlated to their antioxidant substances such as phenolic compounds (Do et al. 2014). However, other herbal compounds could also perform prooxidant, as well as both of antioxidant and prooxidant activities at the same time (Sakihama et al. 2002). Nevertheless, some plant substances demonstrate the ability to alter the physiological processes of the cells through some particular mechanisms irrespective to the antioxidant and pro-oxidant mechanism (Lobo et al. 2010). Therefore, understanding the genotoxic properties of natural products through comprehensive studies of the antioxidant-prooxidant capacity and also the cytotoxicity is important to consider the rational use of herbal products.

Some herbal products from Indonesia are well known for their medicinal properties and their antioxidant capacities.

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Caesalpinia sappan (CS) L. (commonly known as sappan wood) is one of Caesalpiniaceae families found in Indonesia, empirically used to cure some diseases such as inflammation and cancer (Wang et al. 2011). Moreover, this plant is commonly consumed as traditional herbal beverages and as a component of food coloring agent (Toegel et al. 2012). Thus, the evaluation of the cytotoxicity and genotoxicity are essential to measure the balance dose between safety and toxicity in consuming of CS extract.

Sappan wood contains flavonoid and phenolic compounds such as 4-O-methylsappanol, protosappanin A, protosappanin B, protosappanin E, brazilin, brazilein, caesalpin, brazilide A, neosappanone A, caesalpin P, sappanchalcone, 3-deoxysappanone,10 7,3′,4′-trihydroxy-3-benzyl-2H-chromene, and others (Batubara et al. 2009). Brazilin (Bi) and brazilein (Be) are the major compounds of CS and have been widely explored to possess potent antioxidant activity (Batubara et al. 2010; Lim et al. 1996). The antioxidant activity of Bi and Be basically due to their activities in the radical scavenging or enzymatic inhibition. These mechanisms involve the phenolic groups on the chemical structure as an electron transfer or an electrostatic interaction with the corresponding receptors (Nirmal and Panichayupakaranant 2015). On the other hand, Bi and Be also have been known to interact with some protein kinases as the basic mechanism for its cytotoxic activities toward several types of cancer cells (You et al. 2005). All the data presented by Bi and Be indicated that both of these CS major compounds performed pleiotropic effect leading to the possibility of some physiological alterations on the cells. In this concern, we have to consider that the cytotoxic activity of substances to the proliferative cells could lead to DNA damage (genotoxic like effect) that seems to be contradictive to its antioxidant activity. Therefore, the aim of this study is to observe the possible correlation between genotoxic and cytotoxic effects of CS ethanolic extract (CEE) under in vitro and in vivo models that is important as a basic knowledge in the use of sappan products.

Materials and methods

Sample preparation

CS heartwood powder was obtained from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT), Tawangmangu, Indonesia and was determined in the Faculty of Pharmacy, Universitas Gadjah Mada. 500 g of CS was extracted by maceration of ethanol 70% in 5 days, then was concentrated by rotary vacuum evaporator to get CEE. The extraction process yielded 79.82 g of CEE (15.96%). The extract was autenticated by Thin Layer Chromatograpgy (TLC) for Brazilein/

Brazilin content. Doxorubicin (DOXO) and cyclophosphamide (CYC) were purchased from Sigma and Kalbe Farma respectively.

Animals and dosing

Thirty female Swiss mice aged 6–7 weeks with body weight average 22.5–27.5 g were used in this study. The animals were divided into five groups of treatment such as untreated group, CYC treatment with the dose of 50 mg/kg BW, extract dose 1 (CYC + CEE 250 mg/kg BW), extract dose 2 (CYC + CEE 500 mg/kg BW), extract dose 3 (CYC + CEE 1000 mg/kg BW). Administrations of CEE were conducted every day for 7 days and the treatment of CYC given in the last 2 days of treatment (Krishna and Hayashi 2000). Each animal was maintained in the cage with the temperature of 23–25 °C, humidity of 70–80%, and was fed by pellets and water ad libitum.

Mammalian in vivo micronucleus test

Peripheral blood sampling via tail vein was performed in each group on the last day of treatment, 6 h after the administration of CYC. The peripheral blood smear was made on microscope slides, continued by the methanol fixation for 15 min, and were stained with Giemsa 10% on Phosphate Buffer Saline (PBS) pH 6.8 for an hour. The slides were observed under the microscope with 1000× magnification. The number of micronucleated polychromatic erythrocytes (MNPCE) per 1000 polychromatic erythrocytes (PCE) was counted. In order to avoid the false negative results and to evaluate the toxicity of the substances administered, the ratio of PCE and normochromatic erythrocytes (NCE) were counted in five different microscope field of view.

Cells culture

The Chinese Hamster Ovary cells (CHO-K1) were obtained from Prof. Masashi Kawaichi, Laboratorium of Gene Function Animal, Graduate School of Biological Sciences, Nara Institute of Science and Technology (NAIST), Japan. CHO-K1 cells were grown in the medium consisting of Roswell Park Memorial Institute medium (RPMI) (Gibco), 10% Fetal Bovine Serum (FBS), 1% (w/w) penicillin–streptomycin (Gibco), and 0.5% fungizone (Gibco). Cells were incubated at 37 °C and 5% CO_2.

MTT assay

CHO-K1 cells were grown in the 96-well plate at the concentration of 1×10^4 cells/well and divided into untreated and treated groups. For cell viability assay, cells were treated for 24 h with various concentrations of CEE and doxorubicin

(DOXO). After the treatment, 100 μg/mL of MTT solution (0.5 mg/mL in PBS) was added to each well continued by incubating for 3 h at 37 °C. The reaction was stopped by dilution with 10% (w/v) sodium dodecyl sulfate (SDS) in 0.01 N HCl, and cells were incubated overnight. The absorbance was determined by using ELISA reader (Bio rad) at λ 595 nm.

Reactive oxygen species (ROS) assay (DCFDA staining)

CHO-K1 cells were grown on 24-well plate with RPMI culture medium (Gibco) overnight. Cells were harvested with trypsin–EDTA 0.25% (Gibco), then added 500 μL 1× supplemented buffer for inactivation of trypsin. Cells were stained using a 15 μM 2',7'–dichlorofluorescin diacetate (DCFDA) (Sigma) then were incubated at 37 °C for 30 min. Each cell was treated with CEE and the combination with DOXO or H_2O_2 then were incubated at 37 °C for 4 h. Intracellular ROS was analyzed by BD Accuri C6 flow cytometer (BD Biosciences).

Cytokinesis-block micronucleus (CBMN) assay

Chinese Hamster Ovary cells (CHO-K1) were grown for a complete cell cycle (12 h) before the treatments. The treatments were carried out as follows: (a) untreated, (b) DOXO treated, (c) Treatment of CEE in various concentrations and were incubated based on procedure from Menoli et al. (2001). The criteria utilized for the micronuclei determination in binucleated cells was established by Fenech (2000). After the treatments, the cells were washed with PBS and 5 mL of medium. At the end of the culture period, the cover slips were fixed hypo tonically shocked (0.075 M KCl, 37 °C, 10 min) and fixed in methanol/acetic acid (3:1) and cover slips were stained with 5% Giemsa on PBS pH 6.8 for an hour. After staining, the cover slips were air-dried. The visualization and scoring of the cells were done using an inverted microscope. The reduction percentage of micronuclei was calculated by dividing the number of cells with micronuclei observed.

Flow cytometric scoring of micronucleus

Another genotoxic evaluation for quantitative analysis of micronucleus (MN) frequency was revealed by flow cytometric analysis based on the Microflow assay (Kirsch-Volders et al. 2003; Phelps et al. 2002). Briefly, CHO-K1 cells were first stained with a photoactivated dye 1 (EMA; Molecular probes) and then washed, lysed, and stained with lysis buffer containing RNase, nucleic acid dye 2 (SYTOX Green; Molecular probes). DNA from apoptotic/necrotic cells with compromised cell membranes was labeled with

both EMA and SYTOX Green, which can be distinguished from EMA-negative and SYTOX Green-positive MN. Samples were analyzed with BD Accuri C6 flow cytometer (BD Biosciences). Events were gated as shown in Fig. 3a. The incidence of MN was determined through the acquisition of 10,000 healthy nuclei (EMA-/SYTOX+) per well. In addition, the SYTOX fluorescence histogram showed the cell cycle progression profile and the hypodiploid gate was used to determine whether aneuploidy was induced.

Statistical analysis

Statistical analysis were done by using SPSS version 17.0 through one-way analysis of variance (ANOVA) followed by Tukey's post hoc test and independent *T* test. p value for each experiment is included in the associated figure legends. Data were expressed as mean ± SEM (Standard Error Mean).
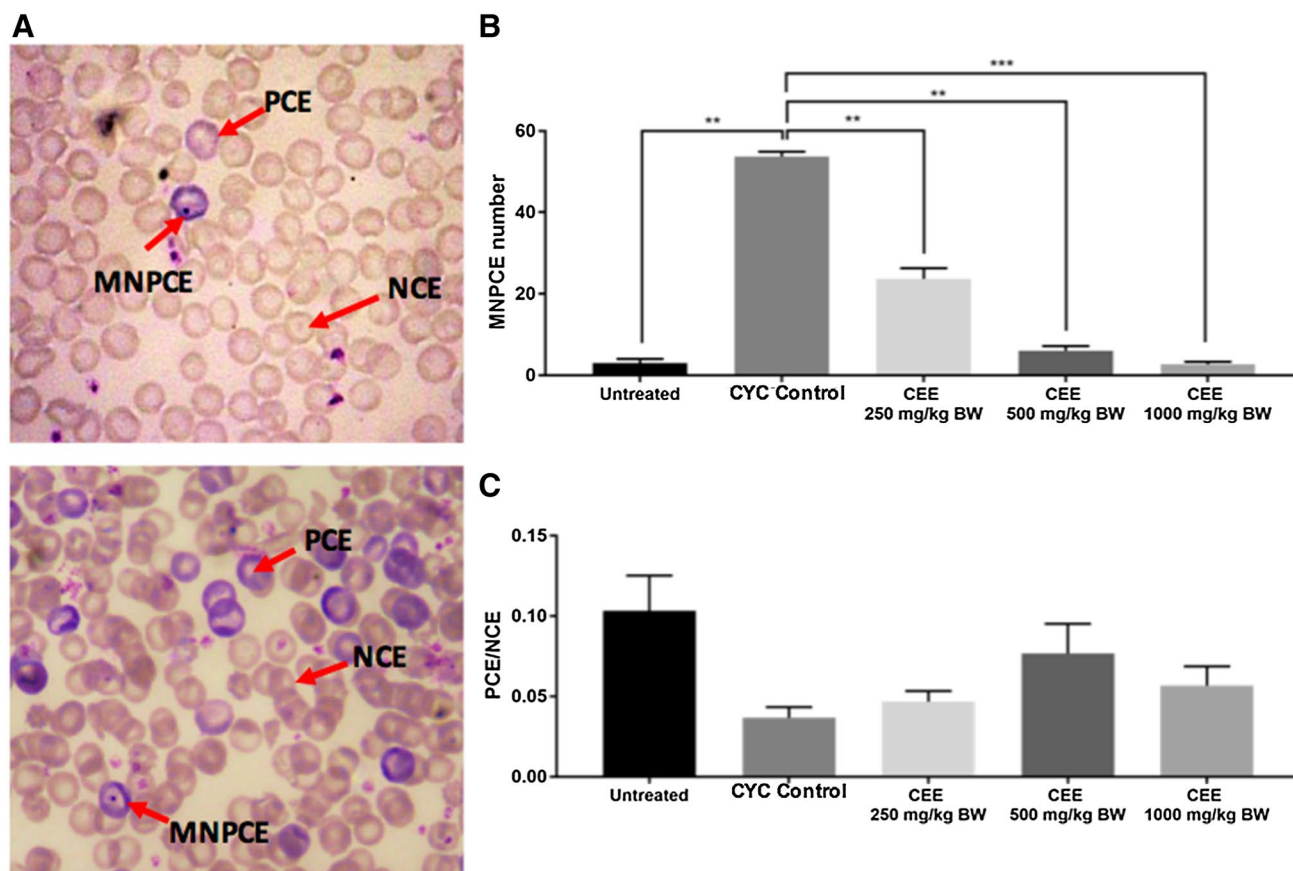
Results

Antigenotoxic activity of CEE by mammalian in vivo micronucleus test in peripheral blood cell

The mammalian in vivo micronucleus test on mice peripheral blood cells is one of the most suitable methods for genotoxic assessment. In vivo assay is also useful for further investigation of genotoxicity following in vitro observation. Genotoxic and antigenotoxic activity were assessed by the frequency of MNPCE per 1000 PCE, whereas cytotoxicity and non-cytotoxicity were evaluated by the ratio of PCE to NCE (Fig. 1a). In this study, we used CYC as a positive control for MNPCE as CYC has been proven to cause DNA damage in normal tissues (Crook et al. 1986). We found that the treatment of CEE at the dose of 500 mg/kg BW and 1000 mg/kg BW significantly reduce the number of MNPCE per 1000 PCE compared to the positive control group of CYC (Fig. 1b). This phenomenon means that CEE showed antigenotoxic activity towards CYC. In addition, the statistical analysis demonstrated that the treatment of CYC caused a significant reduction in the PCE/NCE ratio. Meanwhile, the treatment of CEE increases the ratio of PCE/NCE (Fig. 1c), indicating that CEE had no genotoxic effect under these experimental conditions and showed to block the CYC cytotoxicity.

Cytotoxic and antioxidant effect of CEE and DOXO on CHO-K1 cells

Our previous research noted that CEE containing brazilin and brazilein performed cytotoxic effect on breast cancer cells such as T47D, MCF-7, and 4T1 through cell cycle arrest and apoptosis induction (Handayani et al. 2016;

A

B

C

Fig. 1 Antigenotoxic effect of CEE in mice peripheral blood. **a** Representative figures of Giemsa staining on mice peripheral blood cell. The arrows showed the MNPCE, PCE, and NCE. **b** The bar graph of MNPCE number of PCE/NCE. The results showed that CEE dose 250, 500, and 1000 mg/kg BW could significantly reduce the number of MNPCE in 1000 PCE. The number of MNPCE and PCE/NCE were observed in five microscopic fields and analysis were done by independent T test (**$P < 0.01$; ***$P < 0.005$). **c** The treatment of CYC could reduce the ratio of PCE/NCE compared to the untreated group unsignificantly. The error bars showed SEM from three independent replications

Haryanti et al. 2016; Nurzijah et al. 2012). Thus, the aim of this research is to examine the cytotoxic and genotoxic effect of CEE for evaluating the safety of this compound using in vitro and in vivo model systems. In this report, we used mammalian CHO-K1 cells as a type of proliferative cells commonly used for measuring cytogenetic damage. A single treatment of CEE at the dose range of 5–100 µg/mL and DOXO 0.1–7.5 µM showed cytotoxic effect in dose dependent manner with IC$_{50}$ values of CEE and DOXO, 67 µg/mL (Fig. 2a) and 6 µM (Fig. 2b), respectively. These IC$_{50}$ values were also important for ROS assay, in vitro genotoxicity assessment by using flow cytometric analysis and Giemsa staining.

Since the genotoxicity of some compounds is closely related to the ROS activity, thus the following experiment was done to determine genotoxicity of CEE. Firstly, we measured the intracellular ROS levels. In this study, we used DOXO and H_2O_2 as the inductor of ROS, as previously reported elsewhere (Gao et al. 2013; Damian et al. 2012).

The addition of CEE 30 µg/mL in combination with DOXO and H_2O_2 significantly decreased the number of intracellular levels of ROS compared to the single treatments of DOXO and H_2O_2 on CHO-K1 cells (Fig. 2c). The plots of ROS overlay histogram related to the mean DCF fluorescence intensity were showed in Fig. 2d. This result confirmed that CEE has free radical scavenging capacity and should be explored the antigenotoxic activity through some specific methods such as CBMN assay.

Evaluation of genotoxic effect of CEE by in vitro cytokinesis block micronucleus assay

In general, cytotoxic substances could potentially genotoxic due to its ability to abrogate the molecular mechanism underlying physiological processes. On the other hand, CS has been known to contain antioxidant compounds which are potential to inhibit genotoxicity species. In this concern, we evaluated the genotoxic effect
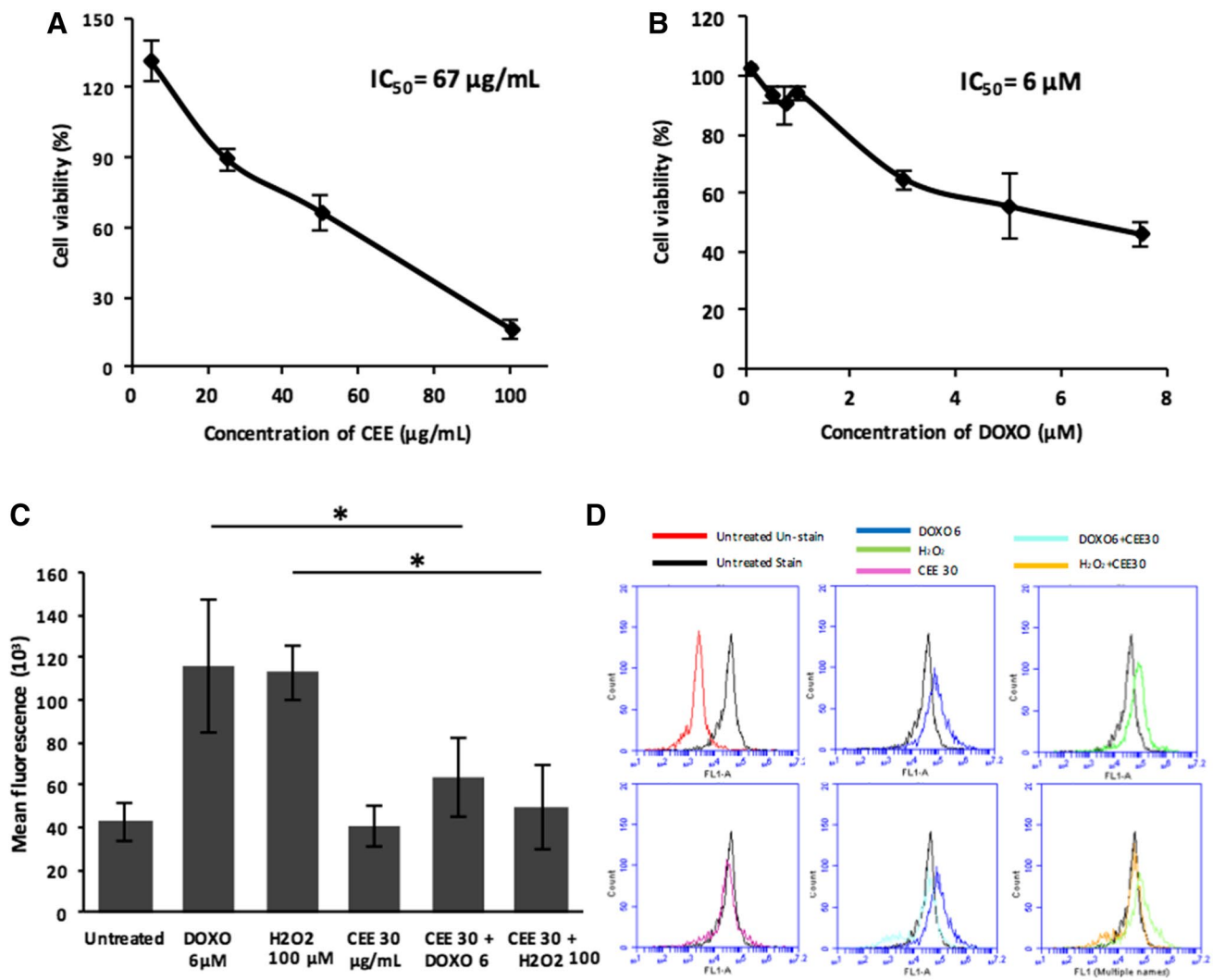
Fig. 2 Effect of CEE on intracellular ROS level of CHO-K1 cells in combination with DOXO and H_2O_2 treatment using DCFDA staining. Cytotoxic effect of CEE was evaluated by MTT assay, CHO-K1 cells (1×10^4 cells/well) were treated with CEE (**a**) and DOXO (**b**) in the concentration as indicated for 24 h. The IC_{50} values were important to the next assays and were calculated by using linear regression in three independent experiments (N = 4; $P < 0.05$ for CEE and N = 7; $P < 0.01$ for DOXO). **c** Total ROS level after single treatment of CEE and combination with DOXO and H_2O_2 in CHO-K1 cells. Statistical analysis was done by using One-way ANOVA with post hoc Tukey HSD (Honestly Significant Difference) Test (N = 3; $P < 0.05$). **d** Histograms of ROS-positive cell percentage by mean DCF fluorescence intensity

of CEE through in vitro and in vivo studies. In general, many genotoxic agents are not detectable in in vitro genotoxicity assays unless the concentrations tested induce some degrees of cytotoxicity. On the other hand, excessive cytotoxicity can interfere with the evaluation of the genotoxic endpoint, resulting in art factual cytotoxicity related to genotoxicity. In order to balance these considerations, this research evaluated the genotoxic effect of CEE using two independent methods: CBMN assay with Giemsa staining and flow cytometry. For MN detection, the cellular DNA is stained with Giemsa during the fixation process for qualitative analysis. As a result, this method does not indicate the cellular micronuclei but the

treatment of DOXO showed morphological changes of the cells showing the evidence of cell death (Fig. 3c). Interestingly, there was a different phenomenon under flow cytometric scoring for the quantitative analysis of MN. Ethidium monoazide bromide (EMA) was used as a fluorescent nucleic acid stain which detected necrotic and apoptotic cells followed by SYTOX Green which provided pan-DNA labelling. This method provided a suspension of free nuclei and sub-2n particles (Avlasevich et al. 2011). By excluding the EMA-positive cells, the number of nuclei (purple region) and micronuclei (green region) were produced clearly (Fig. 3a). CEE with three different concentrations exhibited MN induction higher
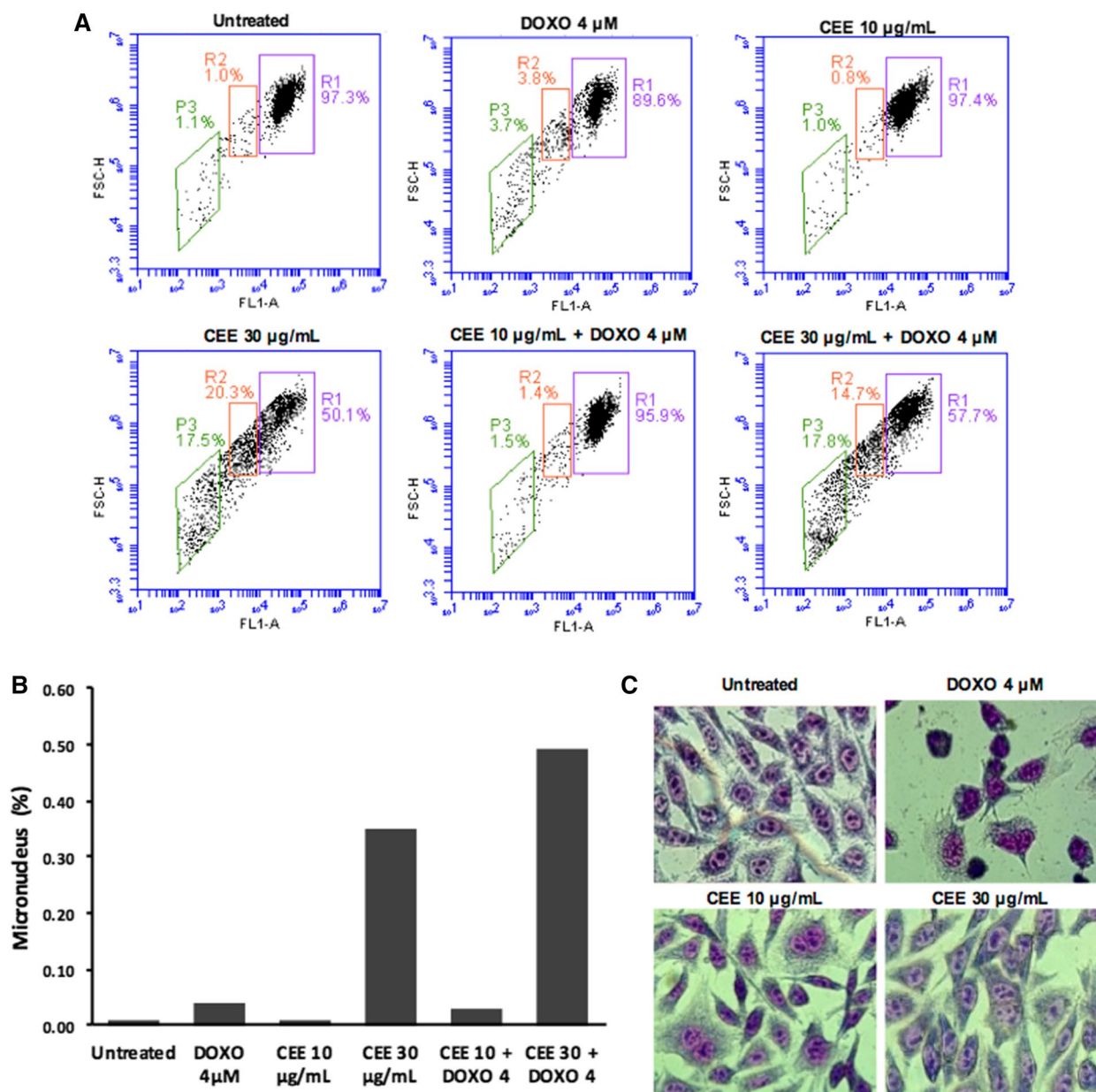
Fig. 3 Genotoxicity evaluation of CEE on the micronuclei formation under DOXO treatment by in vitro micronucleus assay on CHO-K1 cells. For the quantitative analysis, CHO-K1 cells were treated with compounds at various concentrations for 24 h and then processed for flow cytometric analysis of micronucleus (MN) formation. **a** Bivariate graphs illustrate representative FSC vs. Green Fluorescence pro- files of gated (EMA-negative) events resulting from CHO-K1 cells that have been processed for the micronucleus scoring application. **b** Frequencies of micronuclei of six treatments. **c** In addition, for the qualitative analysis, CHO-K1 cells were incubated subsequently for 48 h. At the end of the culture period, the cover slips were fixed by hypotonically shocked, and cover slips were stained with 5% Giemsa

than that of the treatment of DOXO as a positive control (Fig. 3b). Continuous treatment in the combination of CEE and DOXO increased the number of MN in dose dependent manner. It should be noted that there might be a relationship between MN and apoptotic evidence which is depending on the concentration.

Cell cycle modulation and apoptosis induction in response to CEE and DOXO treatments on CHO-K1 cells

In addition to providing MN frequency data, we found that flow cytometry analysis using SYTOX-green staining

could also provide cell cycle data for qualitative assessment. The information regarding cell cycle effects displayed in the SYTOX-associated signal as fluorescence area (FLA-1). The most interesting one is that the combination of CEE 30 μg/mL and DOXO induced cells accumulation in Sub-G1 as apoptotic population and changed the cell cycle profile to bring into cell cycle arrest at G2/M phase (Fig. 4a). Whereas DOXO alone caused cell cycle arrest at S-G2/M phases. This data indicated that CEE could enhance the cytotoxic effect of DOXO to CHO-K1 through cell cycle modulation.

However, due to the limitation of this detection system to recognize the apoptotic cells, we investigated the cell death mechanism in Sub-G1 population through apoptosis assay using propidium iodide-annexin V staining. As shown in Fig. 4a, apoptotic cells were detected after 24 h treatment. This finding confirmed that the combination of CEE (10 and 30 μg/mL) and DOXO (4 μM) induced dead cells compared to the single treatment of CEE and DOXO as shown in Fig. 4b. In conclusion, apoptosis is the significant reason

behind increasing number of MN under CEE and DOXO combination treatment.

Discussion

Our concern in this study is to evaluate the likely pleiotropic effect of CS, especially in the relation to genotoxicity and cytotoxicity in order to gain the consideration dose in particular usage. The studies were set through in vitro and in vivo experiment to differentiate the effect under different environment. CHO-K1 cells used in in vitro experiment are immortalized ovarian cells characterized by high expression of estrogen receptor (ER-β) (Thomas et al. 2003). In the in vivo study, we used genotoxicity-induced mice and evaluated the status of MNPCE. By cross analysis through these differences setting, we could know the detailed response of CEE concerning cytotoxicity and genotoxicity.

In the in vivo study, we used CYC for the positive control as antineoplastic agents that were causing genotoxic
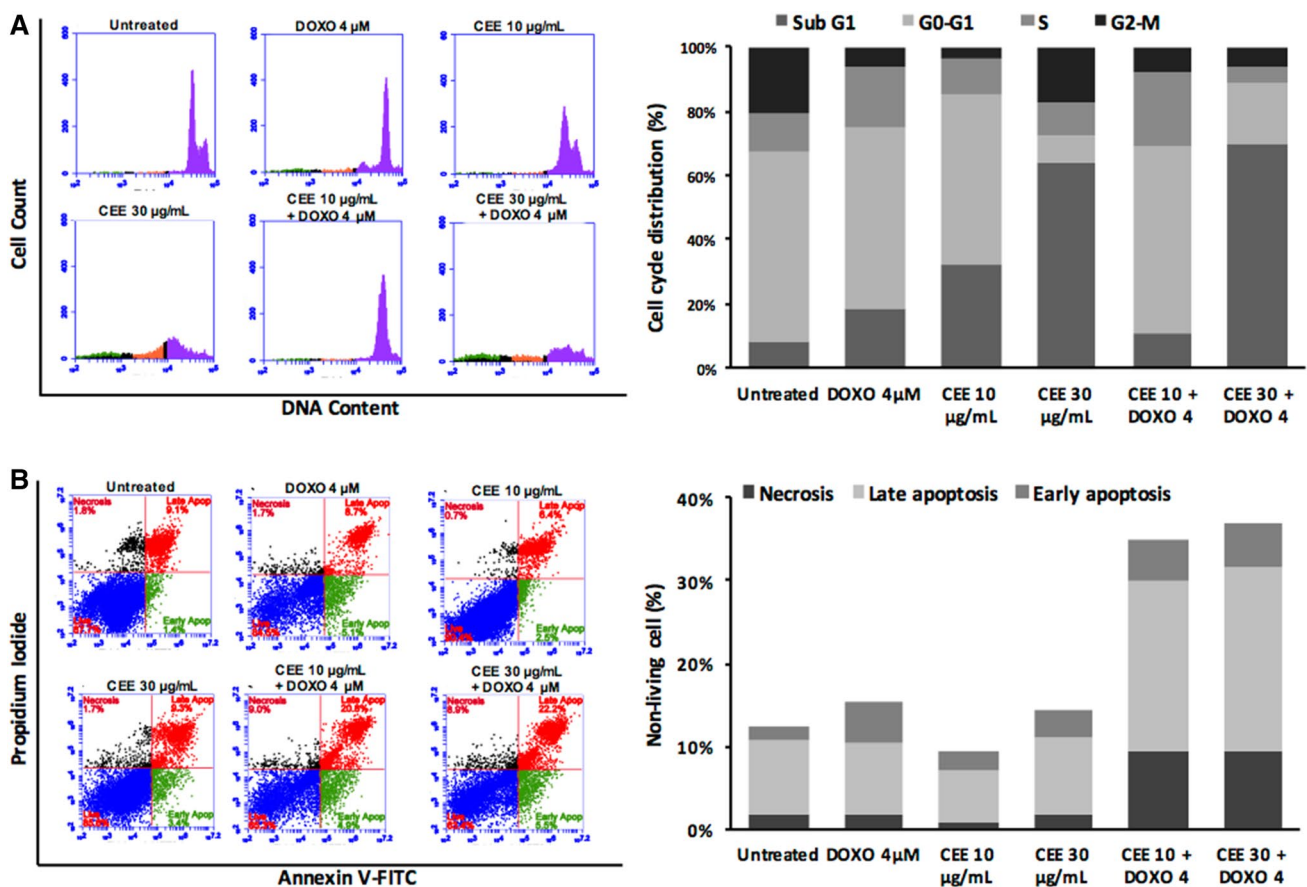
Fig. 4 Combination effects of CEE and DOXO on cell cycle modulation and apoptotic induction of CHO-K1 cells. **a** Cell cycle profiles based on SYTOX-associated fluorescence (FLA-1). These flow cytograms represent cell cycle information that is acquired simultaneously with micronucleus data by the treatment of CEE and DOXO on CHO-K1 cells. **b** Cell apoptosis profiles of different treatment groups by Annexin V-FITC/propidium iodide (PI) staining. Quantitative percentages of cells were measured by flow cytometry

effect from the previous studies. CYC is one of the alkylating agents that are widely used as an anticancer drug in the treatment of different types of neoplasms (Rao et al. 2005). CYC is metabolized in the liver by CYP3A4 and resulting in genotoxic compounds such as acrolein and phosphoramide mustard which can interfere the DNA synthesis and cause the DNA damage (Bryce et al. 2010; Tripathi and Jena 2009). The genotoxicity of CYC has been reported in many in vitro and in vivo studies, and also in several patients (Rehman et al. 2012). Therefore, this study evaluated the antigenotoxic activity of CEE by measuring the inhibitory effect of CYC induced genotoxicity. The oral administration of 250, 500, and 1000 mg/kg BW of CEE prior to CYC exposure decreased the number of MNPCE in all groups studied. The frequency of MNPCE showed the level of genetic damage in the erythropoiesis process (Krishna and Hayashi 2000). In addition, CEE could reduce the toxicity of the CYC exposure by increasing the value of PCE/NCE ratio. The antigenotoxic mechanism of CEE has not been known yet, however, some compounds of CEE might scavenge free radicals or rescue DNA strand breaks or enhance DNA repair. This interesting finding should be confirmed the underlying target mechanism further.

Several studies using animal models have documented that flavonoids or phenolic compounds such as quercetin, act as monofunctional inducers in the liver, kidney, and colon, which allows for metabolism of carcinogens by decreasing the activity of cytochrome enzymes and increasing phase II metabolism to promote excretion of most xenobiotics (Siess et al. 2000). The major component of CEE, Bi and Be belong to phenolic compounds, are famous as antioxidant agents through their capability to scavenge free radicals and inhibit responsible enzymes for radical production such as CYP 3A4 (Ho et al. 2001). Our data give insight that the possible mechanism of antigenotoxic effects of CEE might be caused by the interaction of Bi and Be to the binding site of CYP 3A4. Further investigations should be conducted through molecular docking, receptor binding assay and enzymatic activity.

The interesting results were discovered from in vitro studies through cytotoxic and genotoxic assessment. In the cytotoxicity study, we used DOXO, a DNA breaking agent as the positive control. DOXO is well known to induce ROS production in various tissues causing to enhance the cytotoxic and genotoxic effects. Our experiment found that the IC_{50} value of CEE on CHO-K1 cells was below 100 µg/mL meaning that CEE has strong cytotoxic activity (Prayong et al. 2008). This result was consistently comparable to the previous studies which were reported that sappan wood extract gave IC_{50} approximately 26 µg/mL on HeLa cells (Hung et al. 2014), 37 µg/mL on MCF-7 cells (Khamsita et al. 2012), 25 µg/mL on MCF-7/HER2 cells (Rachmady et al. 2016), and 20 µg/mL on 4T1 cells (Haryanti et al. 2017).

Therefore, these findings supported that CEE was potential to be developed as co-chemotherapeutic and chemopreventive agents. The molecular mechanism of these findings and also the exploration of its combination with DOXO need to be elucidated further.

On the other hand, it is not surprising that CEE is good antioxidant by decreasing the ROS levels in CHO-K1 cells compared to the DOXO or H_2O_2 treatments. This result agreed with those previous reports (Badami et al. 2003; Palasuwan et al. 2005). However, this result suggested that antioxidant action of CEE might be a major mechanism of their antigenotoxic activity even, we still have a question, how CEE exhibited cytotoxic to the cells? This phenomenon could be mediated through some other mechanism rather than oxidative signaling. In other experiment (studies) we found that Be and Bi (and CEE) performed cytotoxic effect to breast cancer cells through cell cycle arrest and apoptosis induction (Haryanti 2016). This phenomenon suggests that CEE at the particular concentration exhibits a pleiotropic effect to the cells that we should consider in using this herb.

The more interesting result in this study is that in the in vitro experiment by flow cytometry showing that CEE tended to increase the number of MN that might be due to increasing of apoptosis evidence. This method seems to be suitable for measuring nucleus fragmentation in correlation with cell cycle distribution rather than MN only. In this regards, we revealed that the combination treatment between CEE and DOXO increased the number of apoptotic cells which leading to cells death. This effect might be caused by the antioxidant mechanism of Bi and Be as the enormous compounds of CEE. On the other hand, the enhancing of cytotoxic effect in this synergistic effect could still be mediated through the interaction of DNA topoisomerase II as the primary mechanism of anticancer by DOXO and disruption of some kinases activation by sappan compounds. Further investigation through apoptosis induction and another molecular pathway is needed to acquire a comprehensive knowledge of the possible mechanism of the cytotoxic and antigenotoxic effect of single treatment of CEE and the combination with DOXO.

In general, our study indicated that CEE in particular doses demonstrated two beneficial effects, such as antioxidant and cytotoxic effect. These both effects are interesting to overcome some health problems such as ageing syndromes induced by ROS and cancer which is mostly activated by MAPK signaling activation. The usage of CS would be beneficial for our health, but the consumption of CS should be paid special attention due to the nonspecific effects to the normal cells as well. The critical point in these both effects may be correlated to its dose. With regards to the common use of CS among people as tonic herbal beverages, therefore we should give more attention to divine the suitable dose of CS for daily consumption. In this regard,

we found that CEE performed antigenotoxic effect at the dose of 500 mg/kg BW of mice and equivalent to the dose approximately of 25–100 mg as daily human consumption. This dose is also similar or under to the amount of CS use as the daily beverages among people. Since the dose of the in vitro experiment for ROS activity is the same with the dose of cytotoxic activity, we can predict that the application of CS as the herbal beverages (assuming at the dose of 200 mg) would give the benefit for the health. However, we should consider that such at the consumption dose may affect to disrupt to the ovarian cells or other normal cells. We have to concern in this possible phenomenon through further study.

Conclusion

We conclude that *Caesalpinia sappan* L. shows antigenotoxic effect through the antioxidant mechanism and reducing MNPCE. At the same concentration by in vitro experiment, *Caesalpinia sappan* L. performs cytotoxic effect on CHO-K1 cells to induce apoptosis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement on the welfare of animals All procedures performed in studies involving animals were in accordance with the ethical standards from Universitas Gadjah Mada with the approval number 462/KEC-LPPT/IV/2016 at which the studies were conducted.

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