Cocoa Extract Indicated Has Activity on Selectively Killing Breast Cancer Cells

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ABSTRACT

Effect of the cocoa crude extract on mortality of breast cancer cell lines i.e. MCF-7, T47D and normal cell (Vero), was observed. Crude cocoa extract prepared from a freshly dried cocoa bean that was containing 14% catechin and 0.6% caffeine. Catechin and caffeine content were modulated to 2-folds (28% catechin or 1.2% caffeine) and 3-folds (42% catechin or 1.8% caffeine) by adding pure compounds. Extracts were dissolved in dimethylsulfoxide (DMSO) at concentrations ranging from 200 to 1600 μ g/ml. The positive control was doxorubicin (0.5-16 μ g/ml in DMSO). Cell lines (MCF-7, T47D, and Vero) were incubated in test sample for 24h at 37°, prior to 3-(4,4-dimetylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The absorbance of each well was measured at 550 nm, and lethal concentration (LC50) was calculated. The cocoa extract induced mortality of breast cancer cell lines but not in Vero cells. The effect on MCF-7 was greater than on T47D, given the LC50 was 1236 μ g/ml (MCF-7) and 1893 μ g/ml (T47D). Cytotoxic potential of cocoa extract was much lower than doxorubicin whose LC50 was 0,777 μ g/ml (MCF-7) and 0,082 μ g/ml (T47D). Increasing catechin content to 2-folds did not significantly affect LC50 value, but 3-folds catechin content reduced LC50 to 1021 μ g/ml. Meanwhile increasing caffeine content to 2-folds significantly reduced LC50 to 750 μ g/ml, however, 3-fold content resulted in slightly higher LC50 at 780 μ g/ml. This indicates that cocoa extract have anti-cancer potential, and purification may improve this property.

Keywords: breast cancer, cocoa, cytotoxicity, MCF-7, T47D

INTRODUCTION

Statistics revealed 32.6 million people ever lived with cancer, and 8.2 million died due to cancer in 2012. The numbers are increasing from 2008 whereas 7.6 million cancer-related deaths occurred. It is estimated that 14.1 million cases arose in 2012, compared with 12.7 million cases in 2008. Breast cancers account for 11.9% cases among diagnosed cancers, in which data from 2008-2012 showed increasing 20% incidence and 14% mortality [1]. Smoking, alcohol consumption, and imbalance diet are among main factors [2]. Options for treatment fall into three categories i.e. surgical treatment (breast-conserving surgery and mastectomy), radiation and chemotherapy. Those therapies indeed carry side effects such as lymphedema, chest discomfort, impaired fertility, premature menopause and a higher risk of osteoporosis [3].

To improve the chance of combating cancers, and to reduce the feeling of sickness following therapy, cancer patients seek for complementary and alternative medicines, in which most of them incorporate herbal extract [4]. Green tea, phytoestrogens, and mistletoe are among frequently used herbs [5] while breast cancer survivors also supplement with flaxseed, vitamin C and vitamin E [6]. The anti-cancer potency of plants extract is due to secondary metabolites, particularly terpenoids and phenolic compounds, that promote sensitivity and prevent the resistance of cancerous cells towards conventional therapies [7].

Cocoa is cultivated in southern hemisphere countries, to be consumed mostly by northern hemisphere population. The production is continually enhanced to overcome escalating consumption [8]. Cocoa provides a significant amount of phenolic and methylxanthine compounds that have been employed as an anti-inflammatory and stimulatory agent [9,10]. This study aims

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to observe the activity of cocoa extract in inducing cancer cells death, and to compare with doxorubicin as a chemotherapy agent. The composition of two secondary metabolites (catechin and caffeine) were modulated to determine the putative anti-cancer compound.

MATERIALS AND METHODS

Cocoa extract (CE) powder (Theobroma cacao L.) was obtained from Indonesian Coffee and Cocoa Research Institute. MCF-7, T47D and Vero cell lines cultures were obtained from Gajah Mada University, Yogyakarta. Reagents and solutions were Trypsin-EDTA 1x (Gibco), RPMI 1640 medium (Gibco), FBS (Fetal Bovine Serum) (Gibco), 0.5% fungizone (Gibco), 2% penicillin-streptomycin (Gibco), MTT (3-(4,5))dimetiltiazol-2-yl) -2,5 diphenyl tetrazolium bromide) (Sigma), DMSO (Dimethyl Sulfoxide) (E-Merck), SDS (Sodium Dodecyl Sulphate), HCl (E-Merck), PBS (Phosphate Buffer Saline). Other chemicals are doxorubicin (Kalbe), catechin (Sigma) and caffeine (Merck).

Cocoa Extract Preparation

CE was prepared by soaking cocoa powder (fat removed, unroasted, unfermented) in ethanol for overnight. The liquid was filtered and was concentrated by using vacuum evaporator to result in CEE powder. CEE containing 14% catechin (assayed by Folin-ciocalteu's reagent [11]) and 0,6% caffeine (assayed using acid-base chromatographic column [12]).

Cell Culture and Sample Preparation

Cells were grown in RPMI 1640 medium containing 2% Penicillin-streptomycin, 0.5% fungizone, and 10% FBS. Cells were distributed in 96-well microtiter plate at density 2x104 cells/ml and allowed to attach during incubation at 37°C for 24 h under the presence of CO2. The test samples were prepared by dissolving 10 mg CE in 50 ml DMSO and 950 ml culture medium. The solution was gradually diluted in medium, to reach concentration 600, 800, 1000, 1200, 1400, and 1600 µg/ml. Cocoa extract was modified by adding pure catechin, so the final concentration is 28% (CE-cat2) and 42% (CE-cat3). Caffeine was also added to crude extract to concentration 1.2% (CE-caf2) and 1.8% (CE-caf3). The modified extract was prepared in a similar manner with crude extract with concentrations at 600, 1000, 1400, and 1800 µg/ml. Doxorubicin as the positive control was dissolved in DMSO and culture medium with concentration ranges from 0.25-8 μg/ml.

Cell Viability Assay

Cytotoxic activity were evaluated through MTT assay described by Hamedeyazdan et al. [13]. Briefly, cells were separately incubated in the presence of CE, modified extracts, or doxorubicin at 37°C for 24 h. After removing the medium, cells were washed with PBS solution prior receiving 3-(4,5 dimetiltiazol-2-yl) -2,5 diphenyl tetrazolium bromide (MTT) reagent and incubated for another four hours. MTT reaction was stopped by addition of 10% SDS in 0.01 N HCl. The absorbance value was read at 550 nm wavelength using ELISA reader.

Tests were done triplicate. A concentration that result cells death by 50% population (IC50) was estimated from probit analysis by using SPSS 18 statistical software.

RESULTS AND DISCUSSION

Cytotoxicity of ethanolic cocoa extract

CE induced cytotoxicity in MCF-7 cells while demonstrated weak toxicity on T47D cells. The initial concentration of 600 μ g/ml has already resulted in mortality 33±5,2% of MCF-7 cells, while, at the same concentration, T47D cell death was 1.15±5.13%. Increasing CE concentration significantly enhanced MCF-7 cells mortality (p<0,01, α =0,05). The concentration of 1600 μ g/ml resulted in cell death more than 50% population of MCF-7 cells, but for T47D cells the mortality was only 37±2.2% [Figure 1].

Vero cells representing normal cell lines were mildly affected by the presence of CE. At concentration 900 μ g/ml, CE induced 14±6,5% Vero cells death. The



Figure 1. Effect of CE on MCF-7, T47D, and Vero cells mor tality. Cells were incubated for 24h with various concentrations of CE (200-2100 μ g/mL). The figure shows in general CE induced mortality of cells, however, concentration initiating cell death varied depending on cell lines.The mortality rate in each cell lines depends on concentration.

viability of Vero cells exceeded 50% even after incubation in CE at very high concentration (2100 μ g/ml). This data indicates the selectivity of CE towards breast cancer cells, particularly MCF-7, over normal cells.

Anticancer Potential of Cocoa Extract

The concentration that inhibits 50% viable cells (IC50) of CE was 1236 μ g/ml against MCF-7. The value could not be calculated for T47D and Vero cells since CE was unable to induce 50% mortality even at high concentration. The positive control, doxorubicin, exhibited IC50 value 0,07 μ g/ml, which is 1600-fold greater than CE.

Modulation of Catechin and Caffeine Concentration Affected Cytotoxic Potential

Higher catechin and caffeine content in CE increased cytotoxic potential. At concentration 600 μ g/ml, MCF-7 cells mortality was increased from 33±5,2% to 40±2,2% when catechin content was 2-fold, and to 48±1,7% at 3-fold. The same manner observed when caffeine content was increased from the initial concentration (33±5,21%) to 2-fold (47±1,8%) and 3-fold (40±5,7%). Catechin augmentation may improve IC50 value from 1236 μ g/ml to 1208 μ g/ml and 1021 μ g/ml. On the other hand, increasing caffeine content to 2-fold may enhance IC50 to 750 μ g/ml, however, 3-fold caffeine resulted in a slightly higher IC50 780 μ g/ml [Figure 2]. This research suggests caffeine plays a major role in the cytotoxic activity of CE towards MCF-7 cells.



Figure 2. MCF-7 cells mortality (%) after incubation in CE for 24h at various catechin and caffeine content; innitial CE (catechin 14%, caffeine 0,6%), 2-fold catechin, 3-fold catechin, 2-fold caffeine, and 3-fold caffeine content. The figure shows cells mortality under effect of secondary metabolite, whereas cytotoxicity was enhanced by increasing catechin and caffeine content to 2 and 3-fold. However IC50 values were mostly affected by caffeine rather than by catechin.

Cytotoxic potential of CE is lower than other plant extracts such as Mangifera indica kernel (15 µg/ml), Elephantopus scaber (14,69 µg/ml) and Dillenia suffruticosa (76 µg/ml) [14-16]. However CE is stronger than Argyreia nervosa leaf (>2500 µg/ml), Dypterocarpus turbinatus leaf (>2500 µl/ml), and Saraca asoca leaf $(>2500 \ \mu g/ml)$ [17]. There are reports from other studies that MCF-7 cells is more sensitive than T47D cells. Both cancer cells are attributed to estrogen-sensitive proliferation; cancer treatments target thus estrogen receptor. Resistant cells alters estrogen receptor expressions [18] while the expressions were increased in resistant MCF-7 cells, it is found to be negative or unchanged in resistant T47D cells. Estrogen receptor expression affects the sensitivity of cells towards tamoxifen or fulvestrant, whereas MCF-7 was more sensitive than T47D cells [18]. Further investigation by Brandie et al. [19] indicates that T47D cells tolerate cellular stress better than MCF-7 cells.

Cancer therapies attempt to induce programmed death in cancer cells. It is approached by either intrinsic or extrinsic apoptotic pathway. As an important drug for breast cancer therapy, doxorubicin mode of action is promoting cellular stress that leads to death through several mechanisms i.e. inhibiting topoisomerase II enzyme, forming doxorubicin-DNA adduct, stimulating oxidative stress, and increasing ceramide production that sensitizes cancer cells [20-22]. Furthermore, Yang et al. [23] suggested that doxorubicin is more likely altering DNA topography, by enhancing nucleosome turnover around promoter gene and triggering DNA instability.

Anti-cancer properties of catechin are associated with inhibition of proliferation and induction of apoptosis. Studies showed catechin regulates apoptosis through downregulation of anti-apoptotic protein Bcl-2 and survivin, while upregulating pro-apoptotic protein Bax [24]. In the other hand, catechin suppresses cell proliferation by inhibiting nuclear factor KB (NF-KB) activation, vascular endothelial growth factor (VEGF) expression, and protecting from reactive oxygen species (ROS) stimulation [25-27]. Schlachterman et al. [28] reported that catechin along with resveratrol and quercetin, could be incorporated in a diet, to reduce breast tumor growth.

Caffeine has long history as an agent to sensitize cancer cells against ionizing radiation [29,30]. It has been investigated for cell cycle modulation and DNA damage signals over-riding [31,32]. Caffeine was also reported to stimulate apoptotic cells either through p53-dependent or p53-independent pathways since it enhanced UV-induced cell death in wild-type mice as well as in p53-knockout mice [33,34]. Besides, caffeine also induces autophagy, when it is found to increase autophagic vacuoles in SH-SY5Y cells at 10 or 25 mM. The level of microtubule-associated protein 1-light chain-3 (LC3) II as the autophagosomal marker was elevated, and this was associated with PI3K/Akt /mTOR/p70S6K pathway inhibition [35].

Greater cytotoxic potential exhibited in CE containing a higher concentration of phytoalexin compound suggests that purification may improve its anti-cancer properties. Lower IC50 value resulted from higher caffeine content instead of catechin indicates the purification method should consider preserving methylxanthine as a polar constituent, rather than semi-polar flavonoid. Even though IC50 of CE is much lower than doxorubicin and other plant extract, CE might be utilized as an anti-cancer agent by using it as a preventive or co-treatment agent. To validate anticancer property of CE, an investigation of immunomodulatory properties, cell sensitization and co-administration with doxorubicin are required.

CONCLUSIONS

This research confirmed the cytotoxic potential of the cocoa extract against breast cancer cells. The effect is more pronounced in MCF-7 cells instead of T47D cells. High concentration of CE induced very low Vero cells mortality, indicating the selectivity of CE on cancerous cells rather than normal cells. Augmentation of phytoalexin constituent lowered IC50 value of CE, whereas caffeine gave greater effect than catechin.

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