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Bioactive Potential of Edel Cocoa Bean (Theobroma Cacao L) from Kedaton Jember: Cytotoxicity and Antioxidants Evaluation

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Abstract

Objective: Kedaton, a small village located in Jember is well known as one of the largest edel cocoa producers in the world. The cocoa beans contain nutrients and numerous beneficial properties, one of them is flavonoids, that work as antioxidants that can be utilized in dentistry, particularly orthodontics. The use of plants for the treatment must be ensured as safe without any toxic properties.

Material and Method: This study use DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method to evaluate antioxidant properties and MTT 3-(4.5-Dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide assay to assess the fibroblast cell activity.

Results: The fermented edel cocoa bean extract exhibited the IC50 value of 33.97 ppm and the unfermented edel cocoa bean extract that had the IC50 value of 9.56 ppm. Both of them have very strong antioxidant activity. Fermented edel cocoa bean extract in concentrations of 1.56%, 3.125%, 6.25%, and 12.5%; as well as unfermented edel cocoa bean extract in concentrations of 1.56% and 3.125% did not have cytotoxic effects to fibroblasts.

Conclusion: The unfermented edel cocoa bean extracts have higher antioxidant activity compared to fermented ones. The fermented edel cocoa bean extracts did not have cytotoxic effect, meanwhile the unfermented edel cocoa bean extracts in concentrations of 6.25% and 12.5% have cytotoxic effect to fibroblasts.

Keywords: Aantioxidant, Cocoa bean extracts, Cytotoxicity, Edel cocoa variety, Kedaton
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Introduction

Indonesia is one of the largest countries with the most strategic potential to cultivate and produce cocoa, after Ivory Coast and Ghana.[1] Kedaton village, a small area located in Jember is one of the largest producing area of edel cocoa.[2] The active ingredient of edel cocoa such as polyphenols are higher compared to other types of cocoa beans.[3] The characteristics of cocoa beans including its active properties are highly dependent on its geographical origin, varieties, soil condition, climate, and the processing of the beans.[3,4]

The process of producing a distinctive aroma and quality taste of cocoa usually carried out firstly by fermenting the beans. The end-products of the its fermentation is the change of color into a shiny brownish color and the aroma of cocoa beans become stronger.[5,6] Cocoa beans contained more nutrients and bioactive properties than the rind and the pulp of the fruit itself. It is consist of 30% - 32% of fat, 32% - 39% of water, 8% - 10% of protein, 2% - 3% of sucrose, 4% - 6% of starch, 2% - 3% of cellulose, 4% - 6% of pentose, 5% - 6% of polyphenols, 1% of acids, 1% –2% of theobromine, 1% of caffeine, vitamins A1, B1, C, D, E; iron, magnesium, potassium, as well as calcium.[5-8] The largest derivatives of polyphenols are flavonoids, which account for 12 – 18%, that divided three main groups; proanthocyanins (58%), catechins (37%), and anthocyanins (4%). These groups are widely popular have significant benefits on phytopharmacy for its antioxidant, anti-inflammatory, and antibacterial properties.[9,10] However, the level polyphenol of fermented cocoa beans decrease from 16.11% to 6.01% six days after its fermentation.[11] This condition is due to oxidation and polymerization which induce the degradation of the compound.[1]

Antioxidants are molecules that work to scavenge free radicals so preventing oxidative stress in organisms.[12] The principal concept of most diseases of human body is the formation of free radicals as result of excessive oxidation from numerous mechanisms occurring inside the body.[13] In dentistry, flavonoids are widely known to have the ability to promote differentiation of osteoblast and compress osteoclast.[14] Moreover, cocoa rind extract has proven no toxic effect, particularly on BHK-21 fibroblasts in concentrations of 1.56% and 3.125%.[15] Fibroblasts play a major role in the process of cell self-repair and alveolar bone remodeling, as well as damaged tissue repair. This study is based on the needs and importance of safety of plants as treatment of diseases, especially in dentistry. Misuse of medicinal plants will cause harm by virtue of their

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constituents as toxins.[16]

Because of those reasons, this study aimed to assess the antioxidant and cytotoxic activity of cocoa bean (*Theobroma Cacao* L) extracts. Fermented and unfermented edel cocoa varieties from PTPN Kedaton, Jember used as preliminary study in vitro through experimental model. The purposes of this study were to determine the differences in antioxidant activity, and toxicity of fermented and unfermented cocoa bean extracts.

Material and Methods

The study used 2 kg of fermented and unfermented cocoa beans that were extracted using the Ultrasound Assisted Extraction (UAE) method of 96% ethanol solution with a ratio of 1:4. Extracts were filtered using a funnel that had been coated with filter paper prior to the process, to obtain a clear maceration. These filtrates were then transferred into evaporating flasks, in the purpose of making them more concentrated with a rotary evaporator. At the end of the process, the fermented edel cocoa bean extracts weighed 6.9 gr, meanwhile the unfermented group weighed 8.61 gr.

DPPH powder 2,5 mg dissolved in 50 ml of 96% ethanol to achieve 50 ppm DPPH solution.[17] Preparation of blanko solution used 1 ml of DPPH solution and ethanol up to 5 ml. We used vitamin C as positive control 10 ppm, 20 ppm, and 40 ppm concentration. 1 ml of each extract added of 1 ml DPPH solution by pipette and then mixed into cuvettes with 3 ml of ethanol. After that, the samples were incubated for 30 minutes and the absorbance were measured using a UV-Vis at 517 nm wavelength.[18] The result of the absorbance value was then calculated into percentage of inhibition using the formula[19]:

$$\frac{\text{Absorbance Blanko - Absorbance Sample}}{\text{Absorbance Blanko}} \times 100\ \%$$

The curve was obtained from the % line of inhibition on the y-axis and the x-axis, which is the concentration. After that, a linear regression test was carried out so that the equation was obtained[18]:

Remarks:
y = percentage of absorbed radicals 50%
x = IC_{50} value
a = regression constant
b = regression constant

IC_{50} value was calculated using the same equation above by finding the x value as the effective concentration after substituting 50 for y value.[20]

The main compound for cytotoxicity assay is tetrazolium salt. 1 ml of MTT solution in PBS (5 mg/ml) diluted with culture media of 10 ml with a ratio of 1:10. The media containing cells then discarded, and washed with PBS 1x then added with 50 µl of MTT solution to each well. After that, the sample cells were incubated in the dark for 24 hours using CO_2 incubator. Condition of the tested cells were assessed under the inverted microscope. When the formazan product is seen properly formed under the microscope, a stopper should be added. In this study, we used 50 µl dimethyl sulfoxide (DMSO) as the stopper, into each well. Subsequently, the plate was stirred mechanically with the plate shaker until the formazan crystals dissolve, for approximately 20 minutes. The last step of this assay was to measure the absorbance used spectrophotometry combined with ELISA reader at 630 nm wavelength.[21] The darker of the color, the higher the absorbance value, means the higher of amount of the living fibroblasts. The dead cells were checked and its percentage was calculated with the following formula[15]:

$$\frac{\text{(Absorbance sample-Absorbance Media)}}{\text{(Absorbance cells-Absorbance media)}}$$

$$\text{\% death cells} = 100\% - \text{\% living cells}$$

Remarks:

% cell viability	: Percentage of living fibroblasts after the assay
Absorbance of samples	: Formazan OD value of each post-tested samples
Absorbance of media	: Formazan OD value at average of each control media
Absorbance of cells	: Formazan OD value at average of control cells

The results of this calculation were then converted into the IC_{50} value. If the dead cells of fibroblasts are more than 50%, it implies to the study that the cocoa bean extract can described as toxic. Meanwhile, if dead cells of fibroblasts reach less than 50%, it means that the cocoa bean extract is not toxic.

Results

Based on the observation and measurement using the UV-Vis spectrophotometer, we obtained the mean absorbance value of antioxidant and calculated the inhibition percentage of each sample as tabulated in Table 1.

The linear equation of vitamin C it was y = 1.143x + 44.45, so that the IC_{50} value was 4.86 ppm. Fermented cocoa bean extract has the linear equation of y = 0.763x + 24.081, so that the IC_{50} value

Table 1. Absorbance value and inhibition percentage of vitamin C, fermented and unfermented cocoa bean extracts

Sample	Concentration	N	Average Absorbance	% Inhibition
K -		4	0.28625	
Vitamin C	10 ppm	4	0.144	49.69
	20 ppm	4	0.067	76.59
	40 ppm	4	0.037	87.07
Unfermented	10 ppm	4	0.1355	52.66
	25 ppm	4	0.12925	54.85
	50 ppm	4	0.07375	74.23
Fermented	10 ppm	4	0.19525	31.8
	25 ppm	4	0.16325	43
	50 ppm	4	0.108	62.27

Table 2. Mean Value of Optical Density and Percentage of Living Cells On Groups

Sample	Concentration	N	Average Absorbance	% Inhibition
Control Media		4	0.147	
Control Sel		4	0.702	
Unfermented	1.56%	4	0.450	54.60
	3.125%	4	0.445	53.70
	6.25%	4	0.388	43.42
	12.5%	4	0.375	41.16
Fermented	1.56%	4	0.565	75.38
	3.125%	4	0.546	72
	6.25%	4	0.523	67.76
	12.5%	4	0.503	64.20

is 33.97 ppm, and as for linear equation for unfermented cocoa bean extract it is y = 0.563x + 44.619, so that the IC_{50} value is 9.56 ppm.

As proceeded from the observation and the measurement using the ELISA reader, we obtained the mean value of optical density and the calculated percentage of the living cells, as compiled for cytotoxicity on Table 2.

Discussion

Fermented and unfermented cocoa bean extracts with different concentrations of 10 ppm, 25 ppm, and 50 ppm have different activities of antioxidants. Antioxidant properties increases with the concentration of the extract. This fact is depending on the differences of flavonoid compound levels in each concentration. In higher extract concentration there is higher flavonoid concentration.[22] The higher flavonoid compounds contained in the extract; the stronger antioxidant activity will effectively scavenge free radicals.[23] Antioxidant activity are scientifically converted into IC_{50} value as parameter. If the IC_{50} value is < 50 ppm, the antioxidant activity is considered as very strong or very active. With IC_{50} value of 50 – 100 ppm, antioxidant activity is considered as strong. If the IC50 value falls between 101 – 150 ppm, antioxidant activity can be categorized as moderate. Weak antioxidant activity possesses the IC_{50} value of more than 150 ppm.[17]

In our study, fermented edel cocoa bean extract obtained the IC_{50} value of 33.97 ppm, and as converted into the category, it can be considered that the fermented cocoa bean has a very strong antioxidant activity. Concurrently, the obtained IC_{50} value of unfermented edel cocoa bean extract was 9.56 ppm, in which we categorize to also have very strong antioxidant activity. Both products of edel cocoa bean extracts are have very active antioxidant activity due to its great amount of flavonoid compound

that contributes directly to the significant antioxidant properties.[25] Futhermore, edel cocoa bean extract contains bioactive ingredients theobromine (1 – 2%) and caffeine (1%) that have bioactive ingredients methylxanthines. Methylxanthines is an alkaloid that have antioxidant properties to scavenge Reactive Oxygen Species (ROS).[5-25] However, effective concentration of unfermented edel cocoa bean extract is lower than the fermented ones. This is due to the fermentation process degrades the active ingredients of cocoa beans as they diffuse out of the cotyledons.[11]

Vitamin C solution in 10 ppm, 20 ppm, and 40 ppm concentrations as positive control value of % inhibition possess higher antioxidant activities along with the increase of concentration. The biggest percent inhibition value out of all groups is acquired by vitamin C that performs as positive control in this study. Vitamin C solution is categorized as very strong antioxidant, it contains IC_{50} value of 4.86 ppm. The reason behind this ability is its molecular formula, $CH_6H_8O_6$, that acts as a reductor agent. Vitamin C will donate its electrons, which are its hydrogen atoms, to the hydroxyl group on the C2 and C3 double bonds. This will result in forms that will be easier to capture for free radicals, thus will lead to the formation of reduced free radicals which are already stable and neutralized.[26]

Effects on the viability of fibroblasts are possibly caused by active flavonoid compounds in edel cocoa bean extracts. The flavonoids have toxic effect on cells when it is on high concentration, because it is a pro-oxidant which has ability to induce the formation of ROS result in oxidative stress. Moreover, it also induces cell proliferation, inactivate DNA oxidation as well as free radicals that have ability to cause the lysis of the cells.[27] The alkaloid toxicity mechanism may affect the insertion of intercalating agents into the DNA, which will affect the base building block of DNA, disrupt the repair, replication, and topoisomerase of cells that will lead to cell membrane apoptosis.[28]

Terpenoids are natural chemicals contained inside cocoa beans. Cytotoxic effect of terpenoids exerts employs under insertion of other secondary metabolites into the cell membranes. This metabolite inhibits the growth of cells by interacting with porins (transmembrane barrel protein) on the outer membrane walls that in turn will form strong polymer bonds. This process results in the damage of porins, which cause uncontrolled entry and exit of substances, and in turn will lead to nutrient deficiency and decreased wall permeability of the cells. This mechanism will stunt the growth of the cells and lead to cell death.[29]

The results this study are concentrations of 1.56%, 3.125%, 6.25%, and 12.5% of fermented edel cocoa bean extracts are categorized as having no cytotoxic activity against fibroblasts; with living cells percentage of 75.383%, 72.002%, 67.764%, and 64.202%. This condition can be due the fermentation of cocoa bean reduced concentration of total polyphenols, flavonoid levels, epicatechins, and catechins contents in cocoa beans. Cocoa beans underwent oxidation and exudation process during the fermentation process.[30] The antioxidant properties of flavonoid provided safety and protection on fibroblasts so preventing the death of fibroblasts. The 1.56% and 3.125% unfermented edel cocoa bean extracts also exhibited no cytotoxic activity with percentage of living cells on 54.599% dan 53.697%. However, on concentrations 6.25% and 12.5%, the cytotoxic activity was found to affect the fibroblasts, decreased the living cells percentage to 43.417% and 41.163%, respectively. Due to the increase of concentrations of edel cocoa bean extracts, in the higher concentration flavonoids will change its role into pro-oxidants. Active ingredients of flavonoids, alkaloids, and terpenoids will then cooperate to affect the fibroblasts by causing apoptosis and necrosis to these cells.[28]

Conclusion

Fermented and unfermented edel cocoa bean extracts (*Theobroma cacao* L.) contain very active antioxidant properties. Unfermented edel cocoa bean extracts contained higher antioxidant activity compared to fermented ones. The fermented edel cocoa bean extracts did not have cytotoxic effect, meanwhile the unfermented edel cocoa bean extracts in concentrations of 6.25% and 12.5% have cytotoxic effect to fibroblasts.

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Conflict of Interest

The authors report no conflict of interest.

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