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Cytotoxicity Test of Cocoa Bean Extract (*Theobroma cacao* L.) Fermented and Unfermented Edel Varieties Against Human Gingival Fibroblast Cells

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ABSTRACT

Cocoa beans are one of the most widely developed herbal plants, one of which is edel cocoa beans. Processing of cocoa beans can be through a fermentation process or not. The fermentation process can significantly reduce the concentration of total polyphenols and other active components in cocoa beans through oxidation and exudation processes. Cocoa beans are one of the plants that are rich sources of polyphenols. These polyphenols, especially flavonoids, have various benefits against several pathological disorders, such as cardiovascular disease, inflammatory processes, and cancer. In dentistry, cocoa bean extract gel has been shown to increase the number of fibroblast cells in the socket after tooth extraction of male Wistar rats, so that it has potential as a therapeutic agent. The requirement for a material to be used as therapy is that it must be safe and non-toxic, so it is necessary to carry out a biocompatibility test to ensure safety for the human body.

Keywords: toxicity; fibroblast cells; cocoa bean extract

INTRODUCTION

Background

All Indonesia is one of the third largest cocoa producing countries in the world with a production of 13% of the world's cocoa(1). Cocoa is one of the plants that is rich in polyphenol sources, especially the type of flavanol which is also known as flavan-3-ounce or catechin, where the highest content is monomers (epicatechin and catechin) and flavanol oligomers (procyanindins). These polyphenols, especially the flavonoids contained in cocoa, have various benefits for the health of the human body. One of the benefits of polyphenols for health is that they can act as antioxidants that provide various benefits against several pathological disorders, such as cardiovascular disease, inflammatory processes, and cancer(2).

Cocoa has been extensively researched for treatment in the field of dentistry. Cocoa bean extract gel has been shown to increase the number of fibroblast cells in the socket after tooth extraction of male Wistar rats (3). The use of cocoa bean extract gel was proven to be effective in accelerating the healing process in tooth extraction wounds of male Wistar rats by reducing the number of macrophage cells(4). In addition, cocoa bean husk extract was shown to have an antibacterial effect against Streptococcus mutans (5).

One type of cocoa commonly used in research is type of edel cocoa (*Trinity*) which has the best seed quality. Edel cocoa has characteristics such as a flat oval seed shape, purple, oval fruit shape, rough to smooth skin texture, and has a strong chocolate taste. In addition, this type of cocoa is classified as a cultivar that is resistant to diseases and pests and has high production. The content of edel cocoa active substances such as polyphenols is much higher than other types of cocoa beans(6).

Based on the processing, cocoa beans are processed through a fermentation or non-fermentation process. Fermentation is a process to improve the quality of cocoa beans with good taste. The purpose of fermentation is to remove the mucilage from the cocoa beans which is important for improving the quality of the cocoa. Unfermented cocoa beans do not give off a specific cocoa aroma when roasted and still have a bitter taste and a strong astringent taste. Polyphenols are compounds that contribute to the bitter and astringent taste of cocoa. The fermentation process can significantly reduce the concentration of total polyphenols, the amount of flavonoids, epicatechin, and catechin content of cocoa beans through the oxidation and exudation processes that occur when cocoa beans are fermented(2).

The requirement for a material to be used as therapy is that the material must be safe and non-toxic. The use of plants for the treatment of the human body without an adequate scientific basis does not guarantee its safety for the body and can be potentially harmful to the human body. In addition, the misuse of plants as medicine can cause poisoning to the human body. Fibroblast cells have the ability to grow and adhere to high with rapid regeneration[7]. These cells have a special function in the process of regenerating alveolar bone and cementum attached to the gingiva, besides that they also play a role in the process of cell repair and remodeling or repair of damaged tissue[8].

Cocoa peel extracts at concentrations of 1.56% and 3.125% have been shown to have no toxic effect on BHK-2 fibroblast cells[9]. However research to test the biocompatibility of cocoa bean extract against tissues or cells in the oral cavity has never been done. Therefore, there is a need for research on the toxicity test of fermented and non-fermented edel cocoa bean extracts to ensure the safety of medicinal plants for the human body. Based on the above considerations, a research on the toxicity of fermented and unfermented edel (*Theobroma cacao* L.) cocoa bean extract was conducted on human gingival fibroblast cells in vitro.

Aim

The purpose of this research is to find out the toxicity of fermented and unfermented cocoa bean extract (*Theobroma cacao* L.) to fibroblast cells and to determine the difference in toxicity between fermented and unfermented cocoa bean extract (*Theobroma cacao* L.) on fibroblast cells.

METHOD

This research was an experimental in vitro laboratory with the research design used was the post test only control group design. The sample used in this study was edel varetas cocoa bean extract with a total sample of 40 samples. The fermented and unfermented edel varieties of cocoa beans were taken from Kedaton Plantation, PTPN XII Jember and extracted using the Ultrasound Assisted Extraction (UAE) method with 96% ethanol solvent, then made cocoa bean extract with concentrations of 1.56%, 3.125%, 6.25%, and 12.5%. Human gingival fibroblast cells in 96 well microplate were exposed to fermented and unfermented edel variety cocoa bean extract. Cytotoxicity test was carried out by the method of MTT assay (Microculture Tetrazolium Technique Assay)Then the color absorbance was measured using an ELISA *reader* at a wavelength of 570 nm. The percentage of live cells was calculated based on the absorbance value from the ELISA *reader* readings, the live cells were then calculated using the following formula[9]:

$$\% \text{ cell viability} = \frac{(\text{Absorbansi perlakuan} - \text{Absorbansi Media})}{(\text{Absorbansi kontrol sel} - \text{Absorbansi media})}$$

Information :
% cell viability = Percentage of the number of cell life after the test
Treatment absorbance = Formazan Optical Density (OD) value of each sample after the test
Media Absorption = Formazan OD value on the average of each control media
Cell Absorbance = Formazan OD value on mean of control cells

Based on the absorbance value of cells from each group, it can be categorized into two, namely if the live fibroblast cells are greater than 50%, it means that the cocoa bean extract is non-toxic. Meanwhile, if the living fibroblast cells are smaller than 50%, it means that the cocoa bean extract is toxic.

The data were analyzed using the normality test and homogeneity test with the results of the data being normally distributed and the existing data homogeneous. Furthermore, parametric statistical tests were carried out withOne Way Anova.From ANOVA test was obtained the results showed a significant difference in the treatment group. Next to find outdifferent sample groups LSD (Least Significance Different) test is used.

RESULTS

Based on readings with the ELISA *reader*, the absorbance value was obtained and then processed to obtain the viability value of the fermented edel variety cocoa bean extract presented in Table 1 and the unfermented edel variety cocoa bean extract presented in Table 2. Based on the absorbance value, the percentage of fibroblast cells obtained was live after incubation with fermented and unfermented edel cocoa bean extract. The viability value of fibroblast cells after being incubated with fermented edel variety cocoa bean extract at a concentration of 1.56% was 75% which was the highest viability in this study. The value of fibroblast cell viability after being incubated with unfermented edel variety cocoa bean extract at a concentration of 1.56% also showed the highest value of 54%.

Table 1. The average percentage of viable fibroblast cells after incubation with fermented edel variety cocoa bean extract

Concentration	Fermentation		
	Average OD (X ± SD)	Cell Viability (X ± SD)	Category
1.56%	0.565 ± 0.01	75±6%	Non-toxic
3.125%	0.546 ± 0.01	72±5%	Non-toxic
6.25%	0.523 ± 0.01	67±5%	Non-toxic
12.5%	0.503 ± 0.02	64±3%	Non-toxic

Table 2. The average percentage of viable fibroblast cells after incubation with unfermented edel variety cocoa bean extract

Concentration	No fermentation		
	Average OD ($\bar{X} \pm SD$)	Cell Viability ($\bar{X} \pm SD$)	Category
1.56%	0.450 ± 0.06	54±13%	Non-toxic
3.125%	0.445 ± 0.05	53±14%	Non-toxic
6.25%	0.388 ± 0.06	43±16%	Toxic
12.5%	0.375 ± 0.02	41±8%	Toxic

Information :
Viability: Percentage of live cells after the test
OD : *Optical density*
$\bar{X}$: Average value
SD : Standard deviation

Based on the percentage of cell viability of fermented edel variety cocoa bean extract, all concentrations and unfermented edel variety cocoa bean extract concentrations of 1.56% and 3.125% were non-toxic because the cell viability value was greater than 50%, while the concentration of unfermented edel variety cocoa bean extract 6.25% and 12.5% are toxic because the value of cell viability is less than 50%.

The percentage of viable fibroblast cells after incubation with fermented edel cocoa bean extract showed a higher viability value than unfermented cocoa bean extract at all concentrations.

IC_{50} was measured using linear regression analysis in the form of the x-axis being the log concentration of fermented and unfermented edel cocoa beans extract and the y-axis being the average viability of human gingival fibroblast cells. The results of the calculation of the IC_{50} value can be seen in Figure 1 and Figure 2.

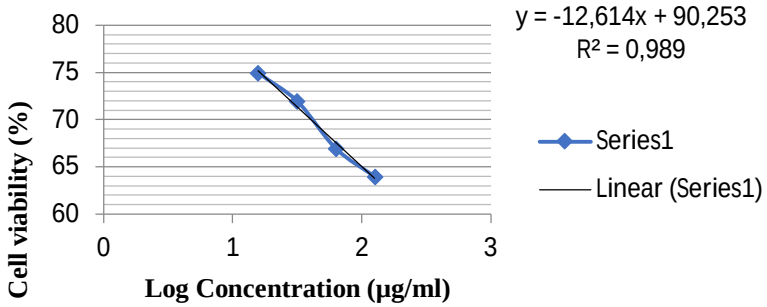

Figure 1. Linear regression of the effect of cocoa bean extract concentration of fermented edel variety on the percentage of viability of human gingival fibroblast cells.

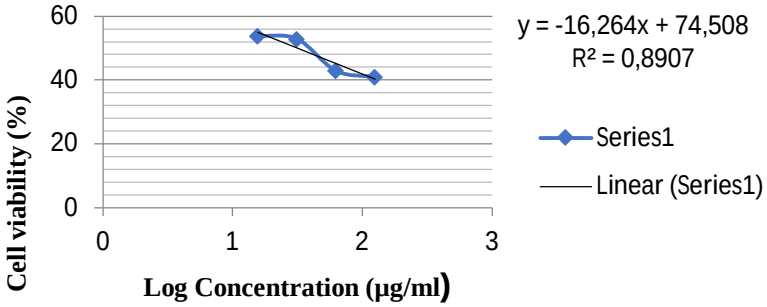

Figure 2. Linear regression of the effect of the concentration of unfermented edel cocoa bean extract concentration on the percentage of viability of human gingival fibroblast cells.

Based on Figure 1 shows that the regression equation obtained is y = -16,264x + 74.508 with R^2 = 0.8907. The negative value of the x coefficient indicates that the concentration is inversely related to the viability of human gingival fibroblast cells. Linear regression analysis gave the IC_{50} number of fermented edel variety cocoa bean extract of1552.877 g/ml. According to the USA National Cancer Institute explains that vulnerable values of IC_{50} ≥ 500 g/ml is non-toxic[10]. Based on these criteria, the cytotoxicity treatment group on human gingival fibroblast cells showed that the fermented edel variety cocoa bean extract was included in the non-toxic category. Meanwhile, based on Figure 2shows that the regression equation obtained is y = -16,264x + 74.508 with R^2 = 0.8907.Linear regression analysis gave the IC_{50} number of fermented edel variety cocoa bean extract of32.1282 g/ml. According to the USA National Cancer Institute explains that vulnerable values of $IC_{50} \geq 500$ g/ml is non-

toxic[10]. Based on these criteria, the cytotoxicity treatment group on human gingival fibroblast cells showed that the non-fermented edel cocoa bean extract treatment was included in the toxic category.

DISCUSSION

One of the requirements for the material to be used as a therapy is that it is biocompatible, namely: if the material does not cause irritation to living tissue, does not cause a toxic response, is free from substances that can trigger allergic reactions, and has no carcinogenic potential[11].

The results of the study show Concentrated fermented edel variety cocoa bean extract 1.56%, 3.125%, 6.25%, and 12.5% obtained fibroblast cell viability greater than 50%. According[9][12], explained that an extract is classified as non-toxic if the average cell viability is more than 50%, so it can be said that the concentration used in this study is non-toxic. The effect on the viability of fibroblast cells can be caused by the content of cocoa bean extract in the form of flavonoid active compounds. Based on research[13] showed that flavonoids from garlic proved to be non-toxic to fibroblast cells. The content of flavonoids acts as a natural antioxidant that provides a safe effect on fibroblast cells, in addition to the fermented edel variety cocoa bean extract significantly during the fermentation process there is a decrease in the total concentration of flavonoids, alkaloids, terpenoids, tannins, and saponins through oxidation and exudation processes[2].

The fermented edel variety cocoa bean extract showed that the lower the concentration of fermented edel variety cocoa bean extract, the higher the absorbance value (optical density) obtained, which means an increase in the number of fibroblast cell viability along with a decrease in the cocoa bean extract concentration. Based on the IC_{50} value of fermented edel variety cocoa bean extract of 1552.877 µg/ml which indicates that fermented edel variety cocoa bean extract is included in the non-toxic category.

In the unfermented edel variety cocoa bean extract concentrations of 6.25% and 12.5% Fibroblast cell viability was found to be less than 50%. According[9][12], explained that an extract if the average cell viability is less than 50% then the extract is toxic. Cocoa beans that do not undergo a fermentation process also do not undergo oxidation and exudation processes that cause the content offlavonoids, alkaloids, terpenoids, tannins, and saponins in cocoa beans remain high. This indicates that the antioxidant effect of flavonoid compounds turns into pro-oxidants that can affect fibroblast cells by causing apoptosis and necrosis in these cells. This is in line with research[14] that the total flavonoid content of parasite leaves from the glodokan tree with a concentration of 2.49% has a toxic effect.against shrimp larvae *Artemia salina.*

Unfermented edel variety cocoa bean extract concentrations of 1.56% and 3.125% showed increased fibroblast cell viability, but at these concentrations did not differ significantly with concentrations of 6.25% and 12.5%, which means all concentrations of edel variety cocoa bean extract were not fermented. toxic to fibroblasts. Based on the IC_{50} value of the unfermented edel variety cocoa bean extract of 32.1282 µg/ml which indicates that the unfermented edel variety cocoa bean extract is included in the toxic category.

The viability of fibroblast cells after incubation with fermented edel varietal cocoa bean extract was significantly higher than that of unfermented edel varietal cocoa bean extract. This is because the flavonoid content in fermented cocoa bean extract is lower than the flavonoid content in unfermented cocoa beans.At low concentrations, flavonoids act as antioxidants, the mechanism of action of flavonoids is by capturing free radicals so that the process of cell oxidation and protein denaturation is inhibited. The content of flavonoids has a toxic effect on cells when at high concentrations because they are pro-oxidants that can induce the formation of Reactive Oxygen Species (ROS) causing oxidative stress. besides that it can also inhibit cell proliferation, inactivation of DNA oxidation and free radicals that will cause cells to lyse[15], but in this study the concentration of flavonoids contained in fermented and unfermented edel varieties of cocoa bean extract was unknown.

The content of other compounds in cocoa beans is thought to cause toxicity to cells. Alkaloids are organic compounds found in natural products such as cocoa (*Theobroma cacao* L.). The mechanism of alkaloid toxicity can affect the insertion of intercalating agents into the DNA structure which will result in a shift in the base framework, disruption of repair, replication, and repair processes.topoimerasewhich causes the cell membrane to leak and eventually undergo apoptosis[16]. According to research[17] it was proven that the toxic alkaloid compounds isolated from the bark of the plant *Polyalthia rumphii (B) merr* against *Artemia salina* shrimp larvae.

Terpenoids are hydrocarbon compounds present in cocoa beans. Cytotoxic effects resulting from terpenoids withinserting another secondary metabolite into the membrane so that it will inhibits growth by reacting with porins (transmembrane proteins) on the outer membrane of the wall which can form strong polymer bonds, resulting in damage to the porin which results in the entry and exit of substances, will result in nutritional deficiencies and reduce cell wall permeability so that growth is inhibited or dead[18].

Tannins are one of the active compounds of secondary metabolites and derivatives of polyphenols. At high concentrations, tannins can cause genotoxicity. Genotoxicity itself is the ability of chemicals that can damage genetic information in cells so that they can cause mutations in these cells[19]. This is in line with research[20] that the tannin content of mangosteen rind at a concentration of 6.25% was proven to be toxic to BHK-21 fibroblast cells. The mechanism of action of tannins, namely by binding to polar compounds and cell lipoproteins, will result in fat accumulation so that cell permeability is disrupted and cells will undergo necrosis [21].

Saponins are a group of glycosides that can be found in cocoa beans. The toxic effect produced by saponins is caused by amphipathic molecules (containing hydrophilic and hydrophobic regions) in saponin compounds that can dissolve membrane proteins. At the hydrophobic end of alane saponins bind to hydrophilic regions on cell

membrane proteins causing a shift in lipid constituents in most of the bound regions. On the other hand, the hydrophilic end of saponins, namely the free end, will take up protein in solution as a protein-detergent so that it will cause cell membranes to rupture and lysis, which in turn will result in cell necrosis[21]. In addition, saponins also have the ability to induce apoptosis in fibroblast cell cultures[22].

CONCLUSION

Based on the results of the research that has been carried out, it can be concluded that the extract of cocoa beans (*Theobroma cacao* L.) fermented edel variety did not show cytotoxic effects on fibroblast cells and cocoa bean extract (*Theobroma cacao* L.) unfermented edel variety showed a cytotoxic effect on fibroblast cells.

REFERENCE

1. International Cocoa Organization (ICCO). International Cocoa Organization Quarterly Bulletin of Cocoa Statistics. 2012;8(5): 331-346
2. Dwijatmoko MI, Nurtama B, Yuliana ND, Misnawi M. Characterization of Polyphenols from Various Cocoa (Theobroma cacao L.) Clones During Fermentation. Pelita Perkebunan (a Coffee and Cocoa Research Journal): 2018;34(2): 104-112.
3. Al-Fa'izah Z. Efektivitas Gel Ekstrak Biji Kakao (Theobroma cacao L.) Terhadap Jumlah Sel Fibroblas Pada Soket Pasca Pencabutan Gigi Tikus Wistar Jantan. Digital Repository Universitas Jember: 2018;1- 50
4. Cholid Z, Pertiwi MH. Pengaruh Pemberian Gel Ekstrak Biji Kakao (Theobroma cacao L) 8% terhadap Jumlah Sel Makrofag pada Soket Pasca Pencabutan Gigi Tikus Wistar Jantan. DENTA Jurnal Kedokteran Gigi: 2020;13(2):49-57.
5. Yumas M. Pemanfaatan Limbah Kulit Ari Biji Kakao (Theobroma cacao L) Sebagai Sumber Antibakteri Streptococcus mutans.(Utilization of Cocoa). Jurnal Industri Hasil Perkebunan: 2017;12(2):7-20.
6. Haliza W, Purwani EY, Fardiaz D, Suhartono MT. Kakao Fermentasi: Pelepasan Peptida Bioaktif Dan Manfaatnya Bagi Kesehatan. Perspektif, 2020;18(2): 104-119.
7. Khumairoh I, Puspitasari IM. Kultur Sel. Farmaka, 2016;14(2):98-110.
8. Aprilia V, Meizarini A, Agustantina TH. Sitotoksisitas Kombinasi Seng-oksida dan Ekstrak Curcuma longa Terhadap Fibroblas Gingiva Manusia. Jurnal Material Kedokteran Gigi: 2019;8(1):1-6.
9. Fitriani F, Soetojo A, Subiwahjudi A. Sitotoksisitas Ekstrak Kulit Kakao (Theobroma cacao L.) terhadap Kultur Sel Fibroblas BHK-21. Conservative Dentistry Journal: 2019;9(1):54-65.
10. Hameed-Abdel ESS, Bazaid SA, Shohayeb MM, El-Sayed MM, El Walkil EA. Phytochemical Studies and Evaluation of Antioxidant, Anticancer and Antimicrobial Properties of Conocarpus erectus L. Growing in Taif, Saudi Arabia. European Journal of Medicinal Plants, 2012;2(2):93-112.
11. Al-Afifi NA, Alabsi AM, Bakri MM, Ramanathan A. Acute and sub-acute oral toxicity of Dracaena cinnabari resin methanol extract in rats. BMC complementary and alternative medicine: 2018;18(1):1-14.
12. Nalbantsoy A, Karabay YNU, Sayim F, Deliloglu G, Gocmen B, Arikan H, Yildiz M. Determination of in vivo Toxicity and in vitro Cytotoxicity of Venom from the Cypriot Blunt-Nosed Viper Macrovipera lebetina lebetina and Antivenom Production. The Journal of Venomous Animals and Toxins including Tropical Diseases: 2012;18(2):208-216.
13. Kuntari S, Budipramana E, Emilda Y. Uji toksisitas ekstrak bawang putih (Allium Sativum) terhadap kultur sel fibroblast. Dental Jurnal Kedokteran Gigi: 2014;47(4):215-219.
14. Fahmi A, Bulan R. Uji Aktivitas Toksisitas Dan Antimikroba Flavonoid Total Daun Benalu (Dendrophthoe pentandra (L) Miq) Dari Pohon Glodokan (Polyalthia longifolia). Chempublish Journal: 2018;3(1):32-43.
15. Saxena M, Nema R, Gupta A. Phytochemistry of Medicinal Plants, Joural of Pharmacognosy and Phytochemistry: 2013;12(1):168-169
16. Nararya SA. Uji Toksisitas Daun Kelor (Moringa oleifera) Terhadap Sel Fibroblas Gingiva Menggunakan Uji MTT assay. Jurnal Biosains Pascasarjana: 2015;17(1):52-58.
17. Hakim DR, Teruna HY, Yuharmen Y. Isolasi dan Uji toksisitas Senyawa Alkaloid dari Kulit Batang Tumbuhan Polyalthia rumphii (B) Merr. Jurnal Online Mahasiswa Fakultas Matematika dan Ilmu Pengetahuan Alam Universitas Riau: 2014;1(2):1-6
18. Nugroho SW, Rukmo M, Prasetyo EA, Yuanita T. Antibakteri Ekstrak Kulit Buah Kakao (Theobroma cacao L.) 6, 25% dan NaOCl 2, 5% Terhadap Bakteri Streptococcus sanguinis. Conservative Dentistry Journal: 2019;9(1):19-21.
19. Radak MS, Andjelkovic M. Studying Genotoxic and Anti Mutagenic Effect of Plants Extracts in Drosophila Test System. Botanica Serbica, 2016;40(1):22.
20. Fikarini Hadi P, Wahjuningrum DA, Cahyani F. Uji toksisitas tanin dari kulit manggis (Garcinia mangostana L.) terhadap sel fibroblas BHK-21. Jurnal Conservative Dentistry: 2015;5(1):6-11.
21. Farkhan A, Arijani E, Yuliati. Toksisitas Kandungan Tanin dan Saponin pada Ekstrak Daun Mimba (Azadirachta indica) dengan Menggunakan MTT Assay. Oral Biology Dental Journal: 2012;4 (2):28-32.
22. Gunawan C, Mulawarmanti D, Laihad F. Sitotoksisitas Ekstrak Daun Avicennia marina terhadap Sel Fibroblas. Dental Jurnal Kedokteran Gigi: 2014;8(2):69.