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In-Silico Study Using Prediction of Activity Spectra for Substances (PASS) Method with Structure Activity Relation (SAR) Approach of Bioactive Compounds in Cocoa Beans

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ABSTRACT
Indonesia is one of the countries that cultivates the largest cocoa (Theobroma cacao L) plants in the world. The research on cocoa has received considerable attention, especially for its high flavonoid content as a potential antioxidant. The objective of the study was to identify the types of flavonoid compounds from cocoa beans originating from Kademangan Village, Blitar Regency, both fermented and unfermented, and the skin, and their potential as antioxidants in silico. Identification of compounds was carried out by qualitative phytochemical screening and continued with LCMS examination. The results showed that cocoa beans that were not fermented with n-hexane as a solvent contained alkaloids, tannins, and polyphenols, while those with methanol contained tannins, saponins, polyphenols, and vitamins C and E. Meanwhile, cocoa beans fermented with methanol contained tannins, polyphenols, and vitamins C and E. Through LCMS examination, epicatechin and anthocyanins were found with the highest levels found in unfermented cocoa beans. In silico studies showed that compounds in chocolate function as antioxidants and free radical scavengers where the active compounds were directly related to the expression of catalase (CAT) and glutathione peroxidase (GPx) but not directly related to SOD protein levels. Thus, it is necessary to carry out further research with in vivo studies.
Keywords: catalase; flavonoids; glutathione peroxidase; superoxide dismutase; *Theobroma cacao L*

INTRODUCTION
Cocoa is a plant that is rich in nutrients. Cocoa beans contain fiber, protein, carbohydrates, and fats, vitamins, minerals, and bioactive compounds such as methylxanthines, theobromine, and flavonoids. Flavonoid compounds are reported to prevent diseases caused by oxidative stress that can damage DNA, proteins, and lipids in cell membranes [1-4].

Indonesia is one of the countries that cultivates the largest cocoa crop in the world and is the third largest cocoa producer after the Ivory Coast and Ghana, whose production value reaches 1,315,800 tons per year [5]. In recent years, research on cocoa and cocoa derivatives has received considerable attention, especially for its high flavonoid content and potential as antioxidants. Flavonoids can act as anti-oxidants by capturing free radicals so that they can maintain a balance between oxidant and antioxidant levels in the body [3,6] and inhibit enzymes that act as pro-oxidants [7]. Epidemiological studies report that cocoa consumption has a beneficial effect on preventing and improving health, including cardiovascular function, metabolism, brain function, immune system, and prevention of cancer [8-11].

One of the cocoa plantations in East Java is located in Plosorejo Kademangan Village, Blitar Regency (Figure 1 and Figure 2). Before being processed, cocoa beans are dried, but some are processed by fermentation. The results of the cocoa bean industry's processing in this area have been utilized at home and abroad. However, the active compound content has never been explored. In silico method was carried out to obtain an overview of the mechanism of action of the bioactive as an antioxidant before conducting in vitro studies. This study aims to identify bioactive compounds in cocoa beans that grow in Kademangan Village, Blitar Regency, and determine their potential as antioxidants in-silico.

Figure 1. Kakao tree (*A. cocoa lindak* sub-type *T. cacao sphaerocarpum*; *B. cacaonoble* sub-type *T. cacao cacao*)

Figure 2. Cocoa fruits and beans

METHODS
The research was designed descriptively to identify the types of flavonoids present in cocoa beans and to evaluate the antioxidant potential of cocoa bean flavonoids *in silico*. All findings were presented using graphical data displays. The study used cocoa beans (*Theobroma cacao*) obtained from Kademangan Village, Blitar Regency, including cocoa husks, dry cocoa beans, and fermented cocoa beans.

The research activities were conducted across several locations. Extraction was performed using methanol as the solvent. Morphological identification to determine the cocoa species took place at the UPT Plant Conservation Center, Purwodadi Botanical Gardens. Flavonoid identification was carried out at Balai Materia Medika Batu, while LCMS examination was conducted at the State Polytechnic of Malang. The research was completed between November and December 2017.

The extraction process began with sun-drying the cocoa beans and blending them into powder. The cocoa powder was weighed, placed into an Erlenmeyer flask, and mixed with ethanol solvent. The mixture was shaken and sonicated for 60 minutes, then filtered using filter paper. This

produced cocoa bean extract in ethanol and cocoa bean precipitate. The extract was evaporated using a rotary evaporator and flushed with nitrogen gas (N_2) to remove n-hexane solvent, yielding 13 g of cocoa bean extract. An initial phytochemical screening was then performed, followed by identification of flavonoid compounds using Liquid Chromatography–Mass Spectrometry (LCMS).

Phytochemical screening involved several identification steps. Alkaloids were identified by adding heated distilled water to the extract, filtering it, and dividing the filtrate into three test tubes, each treated with Meyer's, Dragendorff's, or Bouchardat's reagent. Positive results were indicated by a white precipitate (Meyer), an orange precipitate (Dragendorff), and a brown color (Bouchardat). Tannins were identified by adding $FeCl_2$ 1% to the filtrate, producing blackish brown, blackish blue, or blackish green colors. Saponins were detected by vigorous shaking of the filtrate with hot water, producing stable foam lasting at least 10 minutes and remaining intact after adding concentrated HCl. Polyphenols were identified using $FeCl_2$ 1% with similar color indications as tannins. Vitamin C was identified using Benedict's reagent, heated to boiling for two minutes, producing a yellowish-green to red precipitate. Vitamin E was identified by adding absolute alcohol and concentrated HNO_3, then heating at 75°C for 15 minutes, resulting in an orange to red color.

Active compounds in cocoa beans were further identified using LCMS at the State Polytechnic of Malang. Their biological activity was predicted using the Prediction of Activity Spectra for Substances (PASS) server, which applies the Structure Activity Relation (SAR) approach to estimate potential biological activity based on structural similarity to known active compounds. SMILES structures were uploaded to the server, and compounds with Pa > 0.7 were considered highly likely to exhibit biological activity experimentally.

To evaluate the antioxidant potential of cocoa bean compounds, interactions between the active compounds and the enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) were analyzed. Protein structures were obtained from the Protein Data Bank (ID 3SWR, resolution 2.49 Å), while natural compound structures were retrieved from PUBCHEM NCBI. The inhibitory ability of each compound was assessed by examining its binding affinity to the active sites of SOD, CAT, and GPx. Molecular docking was performed using Autodock Vina in PyRx 0.8, with ligands manually prepared as pdbqt files and energy-minimized, and proteins uploaded as macromolecules. Both molecules were protonated prior to docking. Targeted docking was conducted at specific amino acid residues representing active sites, with grid box dimensions adjusted accordingly. Docking results were visualized using PyMOL.

To determine molecular interactions such as hydrogen bonding and hydrophobic interactions mediating ligand–protein binding, inhibition mechanisms were examined by observing active groups of compounds bound to amino acid residues at the active sites. Molecular interaction analysis and visualization were performed using LigandScout V.2.0.

RESULTS

Based on Table 1, it is obtained that for the extraction of active compounds in chocolate, it is better to use a polar solvent, namely methanol, when compared to n-hexane. In addition, the fermentation process in cocoa beans can reduce the content of active compounds, especially alkaloids, or the possibility that the fermentation process causes changes in compounds.

Table 1. Summary of phytochemical screening on cocoa beans and husks (*Theobroma cacao*) originating from Kademangan Village, Blitar

Sample vame	Solvent	Alkaloid			Tannin	Saponin	Polyphenol	Vit C	Vit E
		Meyer reactor	Dragendorf reactor	Bauchardat reactor					
Fermented chocolate bean	Methanol	-	-	-	+	-	+	+	+
	n-Hexane	-	-	-	-	-	-	-	-
Non-fermented chocolate bean	Methanol	-	-	-	+	+	+	+	+

Based on Figure 3, the compounds contained in fermented cocoa beans based on their molecular weight are epicatechin and procyanidin. Catechin compounds, epigallocatechin gallate (EGCG), and anthocyanins were not detected in this examination. It is possible that the levels of these compounds are very small or below the minimum amount that can be detected by LCMS, so that they cannot be measured. According to the peaks that appear on the chromatogram, it is possible that the catechin groups present are gallocatechin, epigallocatechin, epicatechin gallate, catechin gallate, and gallocatechin gallate.

Based on Figure 4, the compounds contained in fermented cocoa beans based on their molecular weight are epicatechin and procyanidin. Catechin compounds, epigallocatechin gallate (EGCG), and anthocyanins were not detected in this examination. It is possible that the levels of these compounds are under the detection limit of the method. According to the peaks that appear on the chromatogram, it is possible that the catechin groups present are gallocatechin, epigallocatechin, epicatechin gallate, catechin gallate, and gallocatechin gallate.

Based on Figure 5, there is no quercetin compound found in fermented cocoa beans, not fermented cocoa beans. It is possible that this compound is present in small quantities so that it cannot be detected with existing tools.

The purpose of this analysis was to determine the potential of compounds in chocolate as antioxidants and the mechanism of compounds against antioxidant enzymes, namely SOD, CAT, and GPx. Figure 6 below illustrates the results of an in silico study using PASS software on the potential of cocoa as an antioxidant.

Analysis using the way2drug webserver shows that the compounds contained in chocolate are predicted by bioinformatics to have the ability to act as an anti-free radical protein activator. The value of pa (probability to be active) > 0.7 means that the compound has computational and laboratory potential to activate a metabolism. While the value of pa > 0.3 indicates that computationally the compound has potential, in laboratory tests it does not necessarily have that potential. Based on these data, most of the compounds in the computational and laboratory are predicted to act as antioxidants and free radical scavengers. One of the greatest potentials possessed by compounds in chocolate is as a free radical scavenger.

Based on the research results, it is reported that the compounds contained in cocoa include catechins, epicatechins, EGCG, anthocyanins, procyanidins, and quercetin. The potential of these compounds as antioxidants was analysed by using the pathway described in Figure 7.

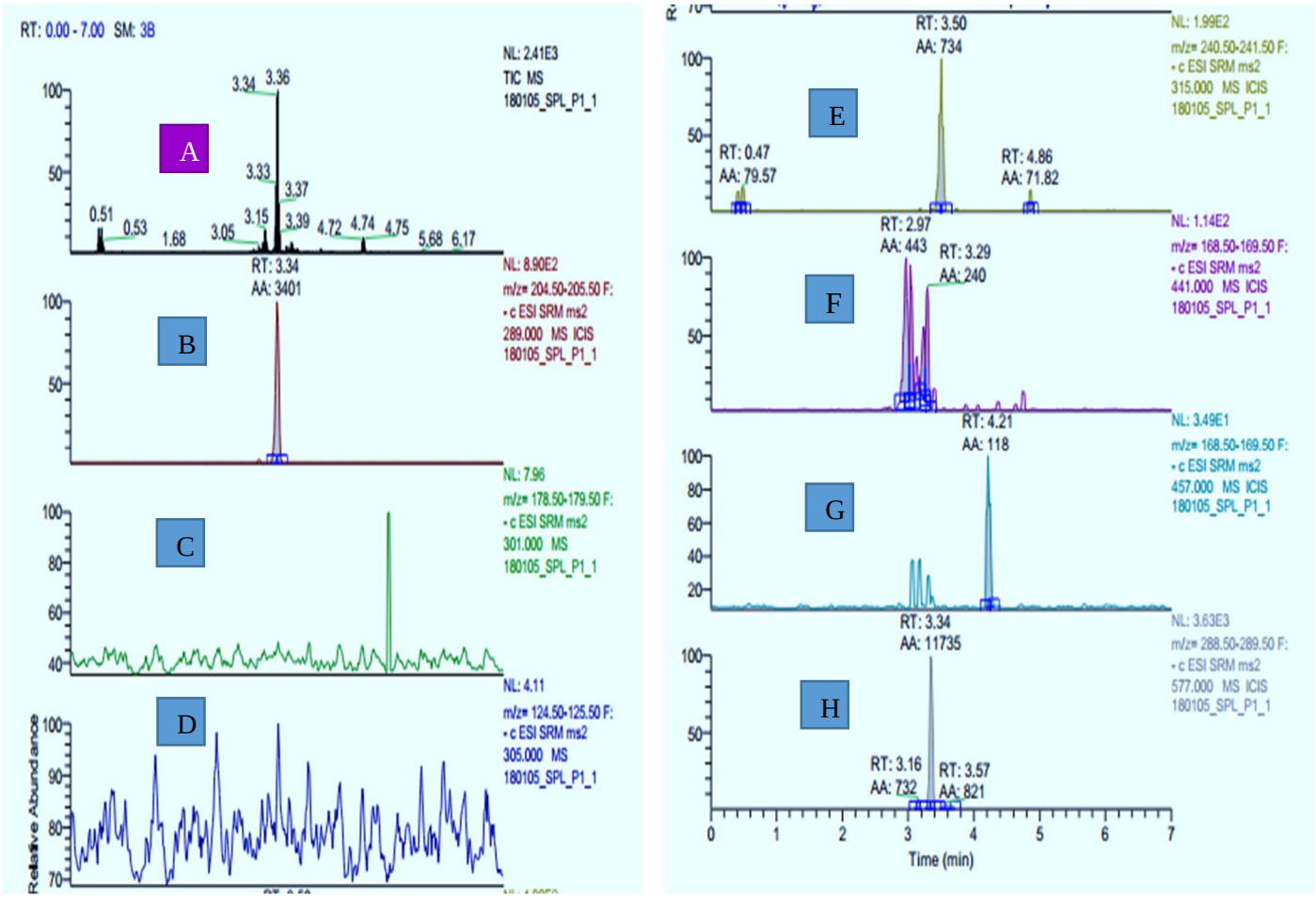

Figure 3. LCMS Chromatogram on Fermented Cocoa (*Theobroma cacao* Linn) Beans. A: total compound, B: epicatechin, C: quercetin, D: gallocatechin and epigallocatechin, E: catechin, F: epicatechin gallate and catechin gallate, G: epigallocatechin gallate, H: procyanidin

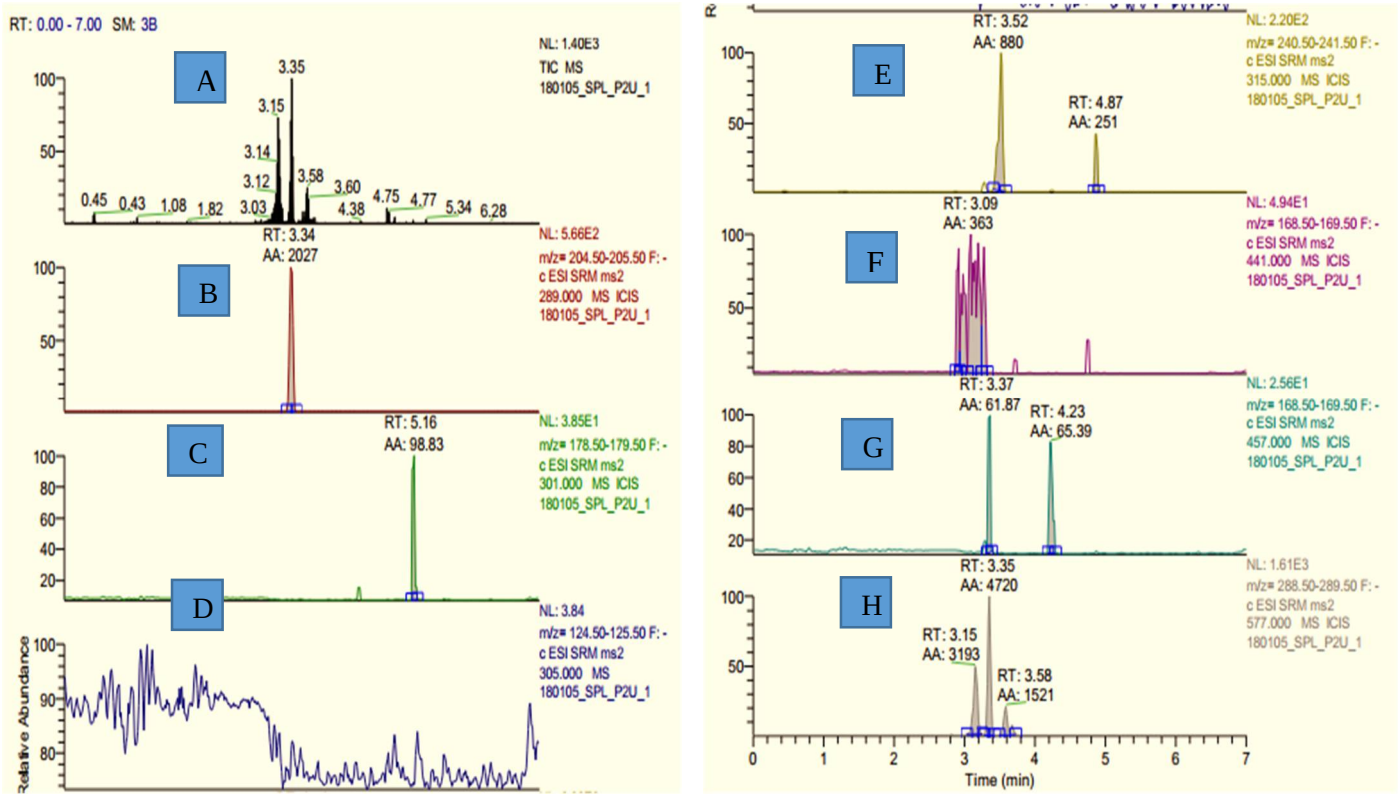

Figure 4. Chromatogram of LCMS on unfermented cocoa (*Theobroma cacao* Linn) beans. A stands for total compound, B stands for epicatechin, C stands for quercetin, D stands for gallocatechin and epigallocatechin, E stands for catechin, F stands for epicatechin gallate and catechin gallate, G stands for epigallocatechin gallate, and H stands for procyanidin.

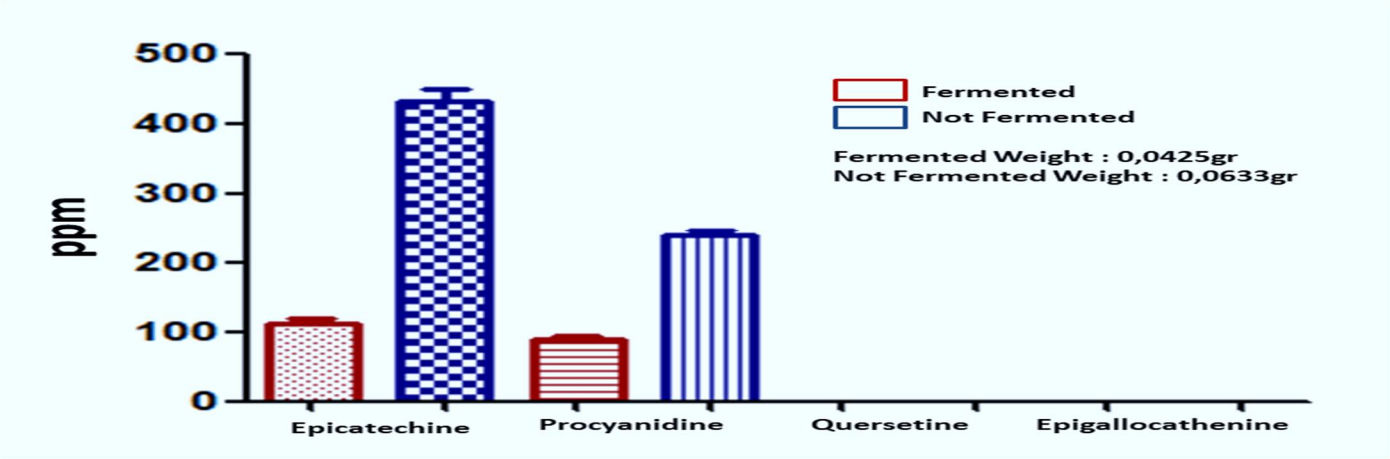

Figure 5. The results of the calculation of epicatechin, quercetin, EGCG, and procyanidin compounds in chocolate

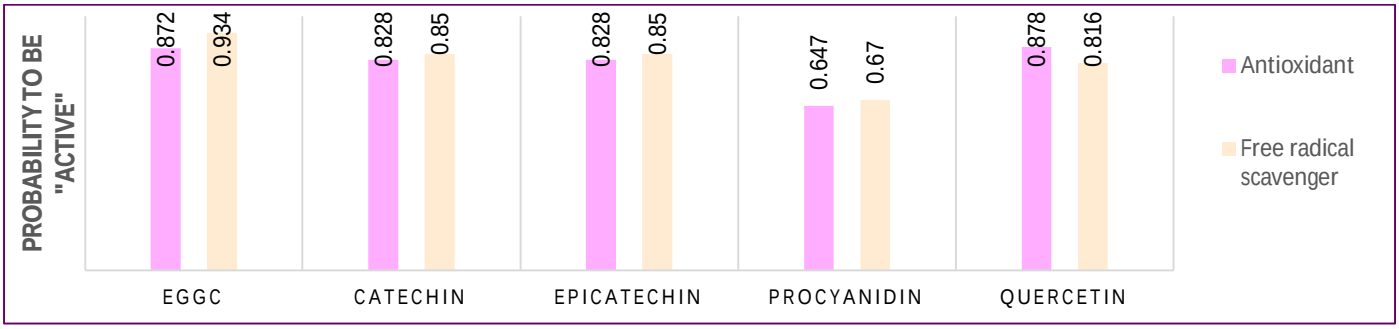

Figure 6: Prediction of active compounds in *Theobroma cacao* Linn as antioxidants. Compounds in cocoa have potential as antioxidants and free radical scavengers, where the potential as free radical scavengers is more dominant.

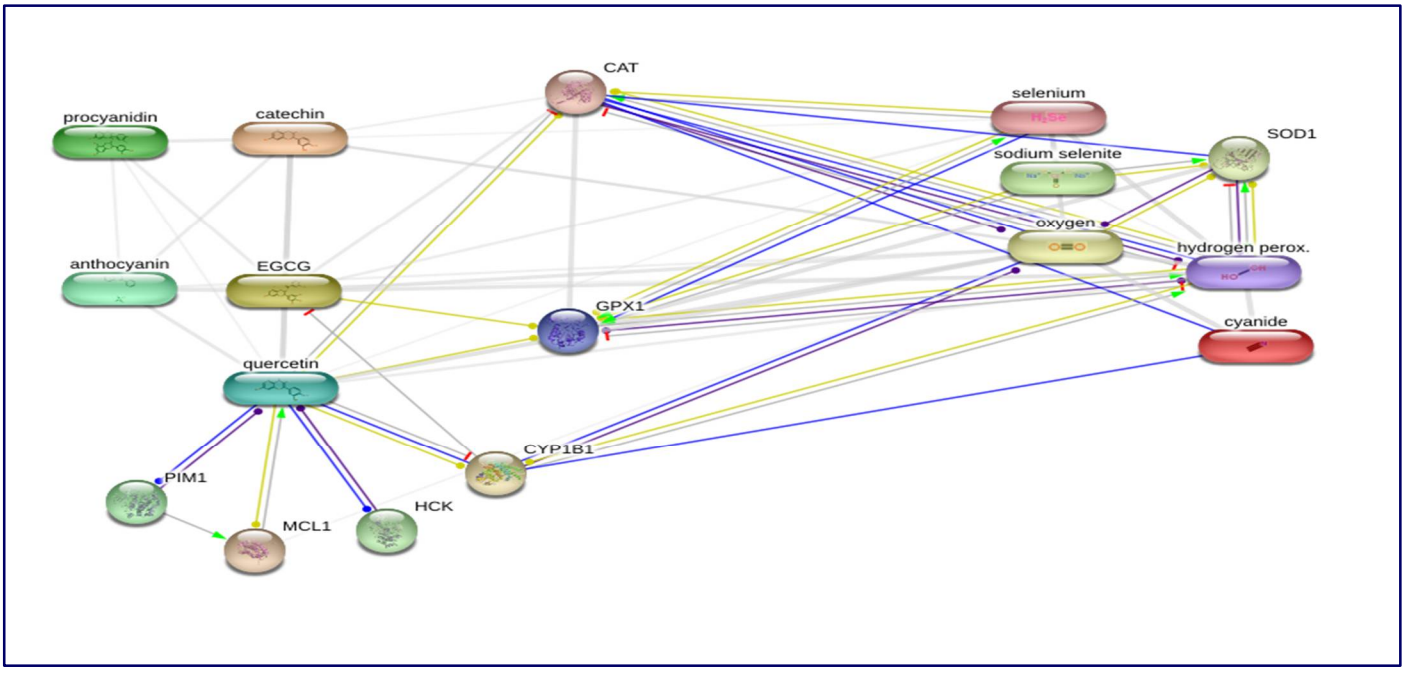

Figure 7. Results of pathway analysis of compounds on *Theobroma cacao* with SOD, CAT, and GPx

Analysis of the relationship of active compounds from cocoa with CAT, GPx, and SOD can be determined by pathway analysis. The results of the analysis showed that the active compound affected the expression of catalase (CAT) and glutathione peroxidase (GPx) but was not directly related to SOD protein levels.

DISCUSSION

Physical description of cocoa fruit extract

Based on observations, both the skin and cocoa beans have almost the same color and turbidity, which is brown. The polyphenol content in cocoa plays a role in providing colour, an astringent taste, and benefits as antioxidants. Fresh cocoa beans contain a purple pigment that indicates

the presence of anthocyanin compounds. The turbidity of the cocoa fruit extract is caused by the tannin compounds contained within. In legume seeds, the darker the extraction colour indicates, the higher the tannin content [12-15].

Active compounds in cocoa seeds and peels

In this study, a qualitative test of phytochemical components was carried out, which aimed to identify the compounds in the tested extracts through the occurrence of colour changes, the formation of foam or the formation of precipitates. One of the components that contribute to the taste and aroma of chocolate is polyphenol compounds. Polyphenols and methylxanthines also contribute to the bitter taste of chocolate, followed by alkaloids, some amino acids, and peptides. One of the steps that affects the quality of cocoa products is the fermentation process. In this process, the chocolate fruit is broken, then put into a wooden box with a hole in the wall, covered with banana leaves, and left for 2 nights. Then for the next 2 days, the cocoa beans are removed, washed, and dried in the sun until the water content is between 8–17%. In the fermentation process, the formation of ethanol and lactic acid will occur. But on the other hand, the fermentation process can cause a decrease in the amount of polyphenols by up to 70% and epicatechins by up to 90% [9]. Cocoa beans that are not fermented with n-hexane as a solvent contain alkaloids, tannins, and polyphenols. Meanwhile, the methanol solvent contains tannins, saponins, polyphenols, and vitamins C and E. It is explained that cocoa beans fermented with n-hexane solvent do not contain all the compounds identified. while the methanol solvent contains tannins, polyphenols, and vitamins C and E. This shows that the fermentation process in cocoa beans can reduce the content of active compounds, especially alkaloids [16-22].

It is obtained that for the extraction of active compounds in chocolate, it is better to use a polar solvent, namely methanol, when compared to n-hexane. Methanol solvent penetrates more easily into cell membranes, making it easier to extract plant active ingredients [23]. According to Baharum et al., solvents such as ethanol, methanol, acetone, ethyl acetate or a combination of these solvents can extract cocoa pods at a faster rate and more types of compounds [8].

Based on the results of the LCMS examination, the compounds found in cocoa beans and skins were epicatechin and procyanidin, with the highest levels found in unfermented cocoa beans. Fermentation may cause the levels of compounds to be reduced or the fermented compounds to turn into other compounds [24-30]. While the catechin and EGCG compounds were not found, it is possible that the cocoa seeds and skin contained other groups of catechin compounds, namely gallocatechin, epigallocatechin, epicatechin gallate, catechin gallate, and gallocatechin gallate according to the peaks that appeared on the chromatogram.

Theobroma cacao potency as an anti-oxidant

Flavonoids are polyphenolic compounds found in plant parts such as leaves, roots, wood, bark, flowers, fruits, and seeds. Sources of polyphenols in nature are vegetables, fruits, and herbal plants [31-35]. More than 8000 flavonoid compounds have been identified [36]. Flavonoids obtained from dietary intake are a group of natural antioxidants. This group of compounds is thought to be able to play a role in fighting cancer through the process of limiting damage due to oxidation reactions in cells. Damage due to the oxidation process in these cells can cause cancer [37-40]. Oxidation of DNA is one of the causes of mutations that can be suppressed by the presence of compounds that are antioxidants [41].

Flavonoids are compounds whose chemical structure allows them to function as electron donors. Flavonoids have a ring structure and a hydroxyl group that allows them to function as antioxidants by searching for partners in the form of superoxide anions, singlet oxygen, lipid peroxy-radicals or stabilizing free radicals originating from oxidation processes in cells through hydrogenation or forming complexes with oxidized species. Several in vitro studies show that flavonols, flavones, and anthocyanins are compounds that have antioxidative activity due to their properties as free oxygen partners [42].

Based on the research results, it is reported that the compounds contained in cocoa include catechins, epicatechins, EGCG, anthocyanins, procyanidins, and quercetin. Based on the results of the in silico study, it is predicted that this can act as an antioxidant and free radical scavenger. One of the greatest potentials possessed by compounds in chocolate is as a free radical scavenger. Meanwhile, based on the analysis of the relationship of active compounds from chocolate with CAT, GPx, and SOD. The active compounds affect the expression of catalase (CAT) and glutathione peroxidase (GPx), but are not directly related to SOD protein levels. This shows that the compounds in chocolate act as transcriptional regulators of CAT and GPx so that the production or levels of CAT and GPx increase. This requires further research in vivo to prove the effect of this active compound on SOD, CAT, and GPx.

The relationship between catalase, SOD, and GPx with flavonoids has the possibility that flavonoids will bind to metal ions. The ligands of each enzyme will significantly increase the enzyme's performance in detoxifying or scavenging free radicals from cells. Flavonoids are compounds whose chemical structure allows them to function as electron donors [43]. Some flavonoids have a ring structure and a hydroxyl group that enable them to function as antioxidants by searching for partners in the form of superoxide anions, singlet oxygen, lipid peroxy-radicals, or stabilizing free radicals [44,45].

Several in vitro studies have shown that flavonols, flavones, and anthocyanins are compounds that have antioxidant activity. because of its nature as a free oxygen partner (Andarwulan et al., 2010; Formagio et al., 2013). Flavonoids can act as antioxidants by inhibiting prostaglandins, LOX, and COX, as well as inducing enzymatic systems that play a role in the detoxification process (Mohamed, 2015). Based on the results of the study, to further the potential of cocoa as an antioxidant, it is necessary to continue with in vivo research.

CONCLUSION

In silico studies showed that compounds in chocolate function as antioxidants and free radical scavengers where the active compounds were directly related to the expression of catalase (CAT) and glutathione peroxidase (GPx) but not directly related to SOD protein levels. Thus, it is necessary to carry out further research with in vivo studies.

Ethical consideration, competing interest and source of funding

-This study was conducted in accordance with established ethical principles, ensuring proper handling of biological materials, responsible data management, and adherence to institutional guidelines throughout all research procedures.
-There is no conflict of interest related to this publication.
-Source of funding is authors.

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