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Original Research Article

Chemical characteristics, phytochemicals and cacao shell toxicity changes during the processing of cocoa beans

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ABSTRACT

Cacao shell is one of the waste from cocoa field that still contains nutrition and bioactive compound. The amount of cacao shells produced is abundant and unused. If there is no handling, it will have an effect on environmental pollution. The handling has ever done is to make cacao shell as animals feed. Before using the cacao shell, it must go through testing of toxic substances. Fruit fusion, fermentation, and drying is an important steps as it influences of changing the nutrition and toxicity in seeds and cacao shell. The methodology used in this research is descriptive method. The toxicity test uses *Brine Shrimp Lethality Test* method by counting the value of LC_{50}. There are several changing of nutrition of fresh cacao shell after fermentation and drying. There is an increase of fat, crude fiber, and polyphenols and the decrease of water and tannins. The fresh and after drying cacao shell contain phytochemical compounds such as polyphenols, tannins, and flavonoids. After the fermentation process, cacao shells contain tannins only. Based on toxicity test using *Artemia salina L.*, there is a changing toxicity of cacao shells from its LC_{50} value. Fresh cacao shells LC_{50} value is 57.38 ppm, after fermentation is 127.54 ppm and after drying is 220.15 ppm. The toxicity of fresh cacao shells, after fermentation, and after drying are less than 1000 ppm which indicates that cacao shells contain toxic substance.

Keywords: Cacao shell, Toxicity test, *Artemia salina L.*

Introduction

Cacao is one of field commodities that have a quite important role in the Indonesian economy and became one of the revenue of the country. Indonesia is the third largest cacao producer in the world after Pantai Gading and Ghana country with the total production is 486,000 tons. The amount of Cacao Exports is 365,000 tons and 121,000 tons is processed in the country (Ministry of Industry of Indonesia, 2007).
The increase of cacao production would be balanced by growing waste production from cacao fields. In contrast to the waste of food crops, the waste of field crops can be generated in a place with large quantities to make it possible to be processed as an industrial raw materials. Cacao waste consists of fruit peel, bean shell, and sludge (pulp) cacao. The bean shell is the biggest waste that generated after processing, for food industry that use dry beans as a raw material. Based on data from the Central Bureau of Statistics in 2009, Indonesia has produced 70 919 tons of dry cacao. According to Hugg et al. (2006), the amount of cacao shell is ranged from 2.59 to 2.42% of the total seed. Based on the conditions, with 70919 tons of dry beans production capacity per year, the amount of bean shell

raw materials that can be used ranged from 1836.80 to 1716.23 tons per year.
Cacao shell has been used as a mixture of feed for livestock because it's potential fiber and protein (Schwan and Fleet, 2014). Although it has been used as feed, but the cacao shell are still abundant and untapped for other purposes. If that was untreated, the wastes potentially contaminate the environment, so it needs further treatment (Utama, et al., 2017).
Cacao shell contains fiber, protein and polyphenolic compounds such as tannins and anthocyanins, which the content of tannins in cacao is 2% while in the bean shell is 1.3% (Minifie, 1988). The bean shell also contains an alkaloid compound that became the forerunner flavor called theobromine (3, 7 dimethyl zanthine), which the theobromine in the cacao shell is higher than its pulp (Devendra, 1977).
The processed of fresh cacao shell into dried cacao and ready to be used was involve a series of processes which include: splitting the pulp, fermentation, soaking and washing, drying, sorting and standardization (Ministry of Industry of Indonesia, 2007). Beans and it shells is still a single unit, starting from the beans were still in fresh condition/after cleavage until the separation stage (*Winnowing*), so the control of the stages of indirectly affect the condition of the bean shell. Cleavage stage of pulp when the beans are still fresh, fermentation stage, and drying stage is the important stage to be noted because this stage affect the changes in chemical composition (nutrients and toxic substances) that is contained in the beans and shells of cacao beans.
One of testing methods that we can do to determine the toxic effects of a substance to human is *Brine Shrimp Lethality Test* (BSLT) method or it also called shrimp larvae toxicity test method. The shrimp larvae *Artemia salina* toxicity test method has been used since 1956 for various bioactivity observations of natural compounds material. It makes a possibility for the shrimp larvae toxicity test to be used as a first stage of screening the bioactive compounds of a series of toxicity tests to obtain a safe dose for humans. Some advantages of bioactivity test using *Brine Shrimp Lethality Test* (BSLT) that use shrimp larvae is the rapid time of the test, simple (without aseptic technique), cheap (not require animal serum), obtain plenty numbers of organisms, comply the statistics validation with a slightly sample (Meyer , 1982).
The advisability and safety inspection of materials from toxic substances / hazardous become an important things to do before it is used as a supply or as a food that ready to be consumed. Therefore, it is necessary to do research related to cacao shell toxicity at the stage of bean processing, in this case it is limited to the condition of fresh bean, after passing through the fermentation and drying stage, in order to obtain data about the effectiveness of the process that was done to the content and toxicity characteristic of the cacao shell.

Materials and Methods

Materials

Materials used for the extraction is fresh cacao shell (*Theobroma cacao L.*), after being fermented, and after being dried, ethanol 96%, and distilled water. Cacao shells in this research were Caucasian Forastero cacao shells obtained from PTPN VIII Rajamandala estate, Padalarang. Fresh cacao shell, after being fermented, and after being dried in this research is assumed to have the same conditions and treatment.
Materials used for chemical analysis is simplisia cacao shell, K_2SO_4: $CuSO_4$ (8:1), concentrated H_2SO_4,NaOH 30%, HCl 0,1N, Tashiro indicator, n-hexan, active asbestos, H_2SO_4 1.25%, NaOH 1.25%, Follin Denis reagent, Na_2CO_3 35%, distilled water, Folin-Ciocalteau reagent, Na_2CO_3 7.5%, Ca_2CO_3, ethanol, saturated Pb acetate, d-natrium oxalate, Anthrone reagent, cacao bark extract, ammonia, chloroform, H_2SO_4 2 M, Dragendorf reagent, Meyer, Hager, Wegner, concentrated HCl, amyl alcohol, hot ethanol, diethyl ether, concentrated H_2SO_4, CH_3COOH anhydrous, natriumcarbonate (Na_2CO_3), distilled water, and FeCl, while the material for toxicity testing is using BSLT methods such as eggs shrimp *Artemia salina L*. NaCl-Fis. Distilled water, and the cacao shell extract.

Equipment

The equipment used is analytical balance, porcelain saucer, oven, furnace, desiccator, Kjeldhal flask, measuring pipette, Kjeldhal distillation tools, drop pipette, erlenmeyer flask, burette, measuring cups, tools soxhlet tools, soxhlet flask, spatula, reflux tools, measuring flask, rotary vacuum evaporator, thermometer, hot plate, centrifuge, refrigerator with temperature of-20ºC, UV-vis spectrophotometer, filter paper, knife, and sponge for aluminum scourer.

Methods

The method used in this research is an experimental method which analyzed descriptively by describing

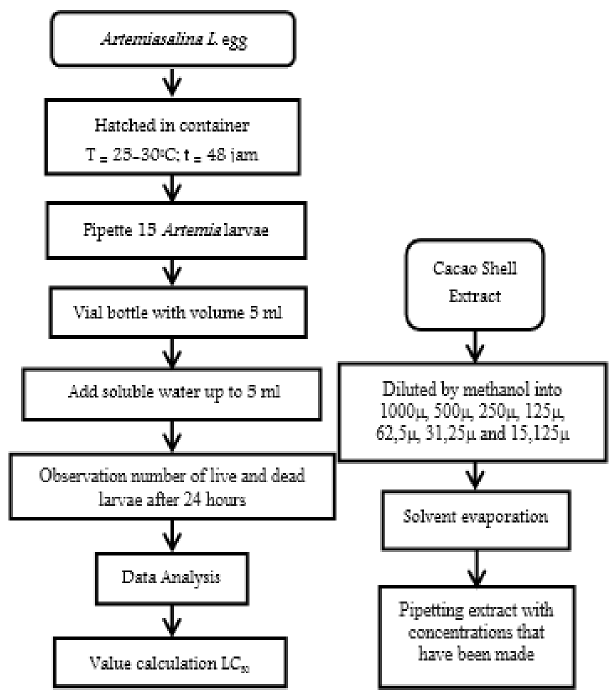
the research data to make a conclusion about the research. The research describe and interpreted about the toxicity of fresh cacao shell extract, after being fermented, and after being dried toward the test animals that is larvae *Artemia salina L*. This research was conducted by observing toxicity of the cacao shell as follows:
A = Fresh cacao shell
B = Fermented cacao shell
C = Dried cacao shell

Experiment Implementation
Implementation of the experiment carried out in two stages, they are preliminary experiments and the main experiments.

1. Preliminary experiments
Preliminary experiments carried out in three stages, that is:
a. Chemical test of fresh cacao shell (AOAC, 1995)
b. Extraction of fresh cacao shell (Juniarti 2009 with modifications)
c. Phytochemical analysis of cacao shell extract (Harborne, 1997)

2. Main experiments
The second stage in the main research is:
a. Chemical test of the after fermented cacao shell and after dried cacao shell (AOAC, 1995).
The test of chemical content of cacao shell is moisture, fat, crude fiber, polyphenols, and tannins. The obtained result is expressed as wet basis percentage (% wb).
b. Cacao shell extraction after fermented and after dried (Juniarti 2009 with modifications).
After cacao shell being fermented and dried, it is extracted using maceration method with 96% ethanol solvent for 2 x 24 hours at room temperature (25ºC). After that, the solvent of extras evaporation using a vacuum rotary evaporator at temperature of 35-40ºC to produce the cacao shell extracts.
c. Phytochemical analysis of cacao shell extract after fermented and after dried (Harborne, 1997).
The extract obtained from the result of solvent evaporation, its phytochemical content is being analysed such as alkaloids and flavonoids.
d. Toxicity test of the cacao shell extract after fermented and after dried, using Brine Shrimp Lethality Test method (Meyer et al., 1982).
Artemia salina L. will be used for this test, its being cultured in seawater medium at first. The seawater medium that is used is 3.8% salt solution (19 g sea salt in 500 mL water). *Artemia salina L*. breeding carried out for 48 hours. The time was the optimum time to breed *Artemia salina L*. Each extract was weighed and dissolved in ethanol to obtain various concentrations. The variations of the concentration is used in this BSLT methods are 1000 ppm, 500 ppm, 250 ppm, 125 ppm, 62.5 ppm, 31.25 ppm and 15.125 ppm.
BSLT result data obtained is being processed using the formula of Meyer et al., (1982), the LC_{50} value of fresh extract, after fermented, and after dried cacao shells could be known. LC_{50} (Median Lethal Concentration) is a measure of the compound concentration, at these concentrations the compound can kill 50% of the animal tests population. A compound can be said as toxic if the value of LC_{50} less than 1000 ppm and it can be said as very toxic if the value of LC_{50} less than 30 ppm (Meyer et al., 1982).

Fig - 1. Diagram of toxicity test process of cacao



shell using BSLT method.
(Source: Meyer, 1982 modified by Hardianto, 2009)

Result

1. Chemical compounds of cacao shell
a. Cacao shell moisture
Based on the results, fresh cacao shell have a high moisture and increasing after fermentation, then

plummeted after drying process (Figure 2). The Increasing moisture during the fermentation process was due to a number of water that is produced by mold respiration at fermentation (Fardiaz, 1992). Before the fermentation process, some water molecules form a hydrate with other molecules which contain oxygen atoms, hydrogen and nitrogen such as carbohydrates, proteins, salts and other organic compounds. During the fermentation, microbial enzymes break the carbohydrates and the compounds, so that water become free (Meyer et al., 1982). Sasongko (2009) explain that the longer fermentation time, the hydrolysis activity of the components become higher, which impact the amount of liberated bound water become plentiful. Therefore, the moisture of the skin increased after the fresh cacao shells are fermented.

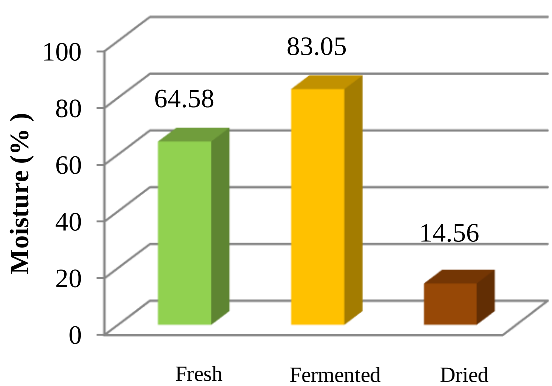


Fig - 2. Changes moisture charts of fresh, after fermented, and after dried cacao shell

A drastic moisture reduction occurs when the cacao shell is being dried. Drying is one of the stages of processing cacao shells while play an important role in determining the quality. At PTPN VIII Rajamandala area, drying process of cacao is carried out at 45-60ºC for 30-36 hours. During the drying process, mailard reaction occurs so the color of the cacao become more brown and typical aroma of cacao is increasingly formed. In addition to formation of color and flavor, during drying process occur moisture reduction due to long and continuous thermal granting. According to Winarno (2008), when the temperature increases, the average number of water molecules in the group will be decreased and the hydrogen bonds will break and formed again quickly. But, if heated continuously, water molecules will move quickly and water vapor pressure will exceed the atmospheric pressure, so water molecules become free.

b. Fat Content of Cacao Shell

The test results obtained that fat content of fresh cacao shells is 0.24%, 0.24% after being fermented, and 4.52% after being dried (Figure 3). Fat content of dried cacao shell amounted to 8.82% according to Sutardi (1991). This amount is larger than the research sample of cacao shell that only contain 4.52%. Zulfahri (2012), fat composition of an animal or plant materials are closely related to weather conditions, soil type, season, ripeness, fertility, plant, microbial, plant flowering and genetic variation.

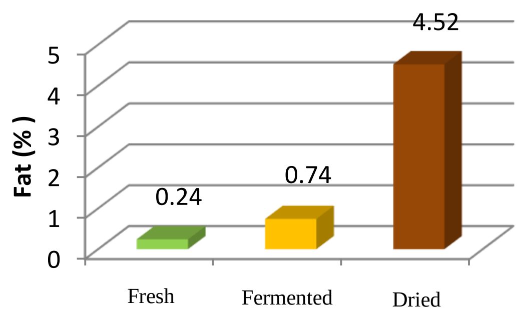


Fig - 3. Charts of Fat Content Changes of fresh, after fermented, after dried cacao shell

Fat content of fresh cacao shell increased after the cacao shell being fermented. Increasing fat content of cacao shell after fermentation likely due to the formation of fatty acids from the cacao beans fermentation. Yeast is a group of fungi that have a strong reducing properties, which in an active fermentation condition, medium that contain sugar or other compounds that is added, there will be aldehyde reduction into alcohol, by one of a type there will formed glycerol (Judoamidjojo, 1992; Roostita and Fleet, 1999; Utama, et al., 2016).

According to Ambardini (2010), glucose catabolism produces is carbon dioxide, water, energy and acetyl-Co-A. Acetyl Co-A is the result of fat metabolism and it is a substrate of fatty acid synthesis. The relationship between fatty acids synthesis and glucose catabolism due to the availability of glycerol to synthesis fatty acids. While glycerol itself is a byproduct of glycolysis and it is the base material of fat formation. During glycolysis, there is formed dihydroxyacetone compound which can be transformed into glycerol. Therefore, the presence of glycerol as a result of the glucose recast by yeast will be able to influence lipid levels at the end of fermentation. In addition, during fermentation, there occur fat formation of glycolysis byproducts performed by the group of natural lactic acid bacteria in the pulp bean. With the synergic cooperation between yeasts during fermentation and natural bacterial activity, which is contained in the pulp bean pulp will further improve the lipid levels. The increased fat percentage in the cacao shell after drying may be due to the reduction of moisture levels. This is consistent with Yusianto et al., (1997) statement that thermal granting can cause water evaporation and degradation of other nutrients such as protein, carbohydrates and bioactive substances that increase the percentage of fat when it is tested.

c. Crude Fiber Content of Cacao Shell

Based on the test results, it showed that the crude fiber of cacao shell contained 59.6%, after fermented decrease to 45.1%, but after dried increased to 56.79% (Figure 4).

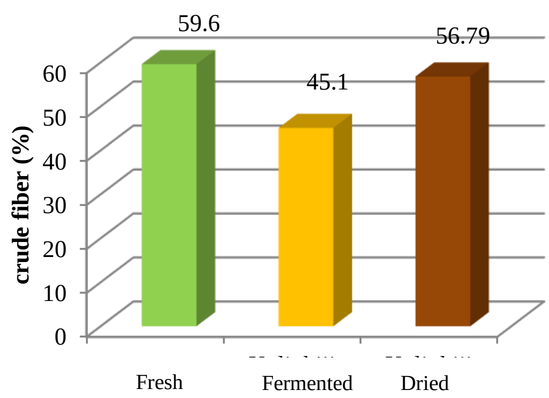


Fig - 4. Charts of Crude Fiber Content Changes of fresh, after fermented, and after dried cacao shell

The reduction of crude fiber content is parallel with the mold growth during the fermentation process. As reported by Sasongko (2009) that the mold is able to degrade cellulose, and in line with the growth of mold, the fungi activities are increasing, so the ability to produce cellulolytic enzymes are also increasing. Crude fibers will decrease if the fermentation which is conducted to the material is longer. This reduction can also be attributed to the activity of a probiotic microorganism group that break down the crude fiber (prebiotic).

Besides caused by fungi cellulolytic enzyme and probiotic microorganism activity, reduced levels of cacao shell fiber after being fermented can also be caused by rising moisture. As mentioned before, there are increased the breaking components of material during fermentation that cause the amount of liberated bound water become much more. These moisture and mucus resulting little amount of fiber which it is a solid fraction. After being dried, cocoa beans have an increase in crude fiber content. Naturally, fibers composed of several types of carbohydrates such as cellulose, hemicellulose, and pectin. Cellulose dissolves well in acidic conditions while pectin is soluble in water (Winarno, 2008). Crude fibers can also be composed of such materials and the existence can be influenced by drying. Cellulose for example, in a state of fermentation when the amount of acid is increasing, there will be more dissolved substances. When the cacao shell is being dried, the vapor is evaporated and the crude fibers in the form of solids are left in large quantities.

d. Polyphenol Content of Cacao Shell

The test results obtained that the polyphenol content of fresh cacao shell is 0.00057% and the increased after fermentation is 0.016% and after drying, it increases sharply to 0.34% (Figure 5).

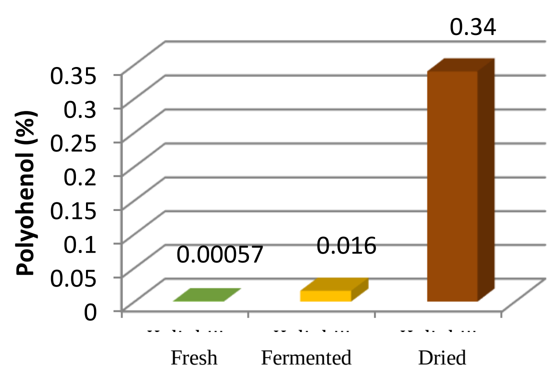


Fig - 5. Charts of Polyphenols Content Changes of fresh, after fermented, and after dried cacao shell

During fermentation, cacao shell polyphenols has increased due to the diffusion possibilities for polyphenol compounds out of the pieces bean that accumulates on the cacao shells. In addition, the increasing compounds of polyphenol can also be caused by the work of microorganisms (bacteria and yeasts) during fermentation. Plant cell walls contain hydroxycinnamic acids which are esterified into polysaccharides cell wals. The increasing of phenolic compounds during fermentation caused by microorganisms have an ability to change a decarboxylatecinnamic acid component such as trans-4-hydroxy-3-methoxycinnamic acid (ferulic acid [FA]) and trans-4-hydroxycinnamic acid (p-coumaric acid [PCA]) forming a phenol compound which is 4-vinylguaiacol [4-VG] and 4-vinylphenol [4-VP] (Supriyono, 2008). Decarboxylation of cinnamic acid into vinyl phenol by yeasts occurs because vinyl phenol reductase enzyme activity (Beek and Priest, 2000). This is in accordance with the opinion of Bisson (2001) that yeast has an ability to produce phenol compounds. In addition, *Lactobacillus* also has a reductase enzyme of ferulic acid and vinyl phenol reductase to degrade ferulic acid and cinnamic acid which it is a component of polysaccharides cell wall into 4-vinyl phenol and 4-vinyl guaiacol (Rodriguez et al, 2008).

After being dried, polyphenol content of cacao shells is increased again due to a heating process that makes the activity of polyphenol oxidase enzyme is stopped. One of the factors that affect the polyphenol content is polyphenol oxidase enzyme (Taranto et al., 2017). The purpose of heating is to inactivate polyphenol oxidase enzyme. The higher of heating temperature, leading to higher inactivation of polyphenol oxidase enzyme, so the enzyme activity would be lower and the damage of polyphenol would be smaller. The higher of heating temperature, polyphenol oxidase enzyme become lower, and the polyphenol compounds will be increased from 0.016% to 0.34%.

Temperature and drying duration can also cause degradation of cacao shell cell walls due to the destruction of carbohydrates (including fiber) and protein (insoluble component) by thermal, so it will make easier the release of polyphenols from material (Chu and Juneja, 1997). Most of the dried cacao shell component, based on the preliminary research is fiber (59.60%) and protein (7.81%). Therefore, the heating temperature increases and heat exposure in a long time, then the protein complexes with polyphenols or polyphenol fiber be disconnected and freeing the polyphenols until the amount is increasing.

e. Tannin Content of Cacao Shell

Some food experts argue that tannin is consist of catechin, leukoanthocyanin, and hydroxy acids which each of them can show color when it reacts with metals (Winarno, 2008). The presence of tannins in food can also determine the taste of foods. Astringent taste in food is usually caused by tannins. Tannins have a function as antibacterial, antiviral, and antiparasitic (Doss et al., 2009). Figure 6 shows the tannin content of fresh, after fermented, and after dried cacao shell.

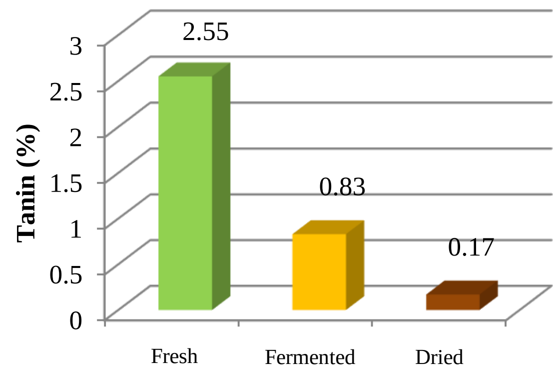


Fig - 6. Charts of tannin contents changes of fresh, after fermented, and after dried cacao shell.

Fresh cacao shell tannins is decreased after fermentation. It is expected that during cacao fermentation, there is hydrolysed of tannins because of the characteristic of tannins which very sensitive to acidic conditions. During the fermentation, there are certain amount of acid produced by lactic acid bacteria which can lowing the pH and causing acidic conditions. Therefore, tannin contents is decreased after fermentation.

In addition, according to Martinez-Pinilla et al., (2015), the existence of tannin is inversely proportional to the amount of theobromine. In the event of reduction of tannins, then after fermentation and drying theobromine amount as flavor precursors will increase. Fermentation causing the reduction of dissolved tannin and at this stage, reduction / expenditure of theobromine is occurring and caffeine also with the volatile components (alcohols, esters, and aldehydes).

The complex bond formed between tannins and protein during heating. In general, heating of tannin-containing substances is conducted to disable tannin, thus increasing their nutritional value as a protein source. As reported by Yulistiani et al., (2010), that tannin-containing Acacia saligna that were dried under the sun for 3-4 days shown the decreased of tannin content, so that increase the digestibility of the protein compared when it is given freshly. Heating effect which causes reduction of tannin content is also occurs on the leaves of lamtoro plants (Yulistiani et al., 2010). Heating can be done by using temperature and duration variations with the choice of high temperatures in a short duration or low temperature in a long duration. Albanu (2002) states that boiling with high temperatures in a short time can solve the phenol bonds so the amount of tannins is reduced. The same thing is thought to occur on the cacao shell where there was tannin reduction after being dried at 45-60ºC for 36 hours.

2. Phytochemical contents of cacao shell

a. Alkaloid contents of cacao shell

The alkaloid tests result showed that the alkaloid content of fresh and dried cacao shell extract is positive (Table 1).

Table - 1. The alkaloid tests result of fresh, fermented, and dried cacao shell.

Cacao Shell Condition	After Reagen Addition			
	Mayer	Wagner	Dragendrof	Hager
Fresh	+	-	-	+
Fermented	-	-	-	-
Dried	-	+	+	+

Notes:
(+) : detected alkaloid contents
(-) : undetected alkaloid contents

Naturally, alkaloid is an alkali compounds and contains a nitrogen that naturally present in plants or animals (Nassel, 2008). Nonetheless, fermented cacao shell did not reveal any alkaloids. After the fermentation of free alkaloid, it may change partially or completely into alkaloid salts due to the acidic conditions, so it was in an inactive state. This is expected to cause the amount of alkaloid so small that it was undetectable during the test.
The existence of weak alkaloid is also suspected due to the extract used in the testing process is still a crude extract. This situation is supported by the rising moisture because there was amount of water produced by the fruit pulp and fungi in the fermentation process. According to Harborne (1987) alkaloid in base form is insoluble in water so it is not presented in the fermented cacao shell.
Drying process can cause materials containing organic and bioactive compounds to be damaged if done at high temperature or long duration. At the dried stage, the water in cacao shells will be evaporated and the nutrients will be degraded such as carbohydrates and proteins, in addition to mailard reactions that contribute a role in shaping the taste and color. The heating process causes alkaloids that have formed complexes with organic materials having the same N groups as amino acids or in combination with acidic alkaloid salts, are not expected to be bound. Therefore, an alkaloid that was undetected on the fermented cacao shells are found in the dried cacao shell.
Dalunigrum (2009) states that the alkaloids have pharmacological properties, one of them is to widen the airways of shortness breath patients. The alkaloid compounds such as caffeine, theobromine and theophylline (Sirait, 2007). According to Minifie (1989), alkaloids that is identified in the cacao shell in the form of theobromine compound (3,7 dimethyl xanthine) that is alkaloid compound derived from xanthine group.

b. Flavonoid Contents of Cacao Shell

The test results indicate the presence of the flavonoid contents in fresh cacao shell after being dried (Table - 2)

Table - 2. The flavonoid test result of fresh, fermented, and dried cacao shell.

Cacao Shell Condition	After Reagen Addition		
	H_2SO_4 2N	NaOH 10%	HCl 2N + Mg
Fresh	+	+	+
Fermented	-	-	-
Dried	-	-	+

Notes:
(+) : detected flavonoid contents
(-) : undetected flavonoid contents

As in the alkaloid tests, there was no flavonoids founded in the cacao shell after being fermented. This is sustain the causing of the small amount of flavonoid so it was not detected during testing. The existence of such weak flavonoid due to the extract that is used in the testing process are still a crude. This crude extract is obtained by the extraction method with type of solvent extraction or extraction that using solvent with maceration method using ethanol. This crude extract still contains a component that is not a flavonoid compound that is also extracted at the time of fermented cacao shell maceration.
At the time of cacao fermentation, the damage of cell walls causing the entire contents of the cells outed and mixed. According to Harborne (1987), if the cell content of plant is being mixed and damaging the membrane, phenolic compounds including flavonoids will rapidly form a complex with protein. It is also

expected causing the flavonoid that is not presented in the fermented cacao shell. The dried cacao shell contains flavonoid compound. It is expected that there was protein denaturation during the drying process that caused by thermal, so the protein complex and flavonoid is broken. Therefore, flavonoids that was bound become free and the solubility is increasing. The existence of these flavonoids may persist the dried cacao shell. It is expected due to the given thermal is inactivate the enzyme so there was no flavonoid oxidation (Harborne, 1987).
The flavonoid group has the ability to transform the forming compound, so it has a higher biological activity such as antioxidant activity (Sucipto, 2008). Flavonoids are known as good antioxidants because it have at least two hydroxyl groups in ortho and para position (Winarno, 2008). In vitro and in vivo research indicate the biological and pharmacological activity of flavonoids which very diverse, one of them is having antibacterial activity (Sabir, 2005).

3. Cacao Shell Toxicity

The relationship between the log concentrations with mortality of larvae for each bean shell conditions (Figure 7) showed a high determination coefficient of that is above 90%. Regression relationship of both parameters for fresh cacao shell and dried cacao shell is manifested in the form of a linear regression model, while for the fermented cacao shell showed a quadratic relationship. In Figure 7, we can see that there is a close relationship between the concentration addition of fresh cacao shell extract with the dead shrimp larvae. This close relationship is described by a linear equation. Selection of the linear regression model is based on the coefficient value of determination (R^2) obtained is greater than 0.75, that is 0,981. Linear models between the concentration of fresh cacao shell extract with the percentage of the dead shrimp larvae that are expressed in the equation:

$$y = 45,55x + 30,09 \text{ ; } R^2 = 0,981 \text{ and } r = 0,991$$

The slope value is worth 45.55 which means that every 2 times increase of fresh cacao shell from the initial amount, the number of dead shrimp larvae will increase by 45.55%. The regression coefficient or intercept is worth 30.09 which means the initial value calculation Y (x = 0).
The coefficient value of determination (R^2) of 0.981 states that 98.1% of the *Artemia salina L.* larvae mortality is affected by fresh cacao shell extract and the remaining 1.9% is influenced by other factors, such as durability larvae. The correlation coefficient value (r) of 0.991 means a very strong relationship between the concentration of cacao shell extract with the mortality of *Artemia salina L.* larvae. Based on toxicity test using BSLT method is known that the fresh cacao shell have toxic properties. This toxic properties is known by the LC_{50} values which 57.38 ppm. A substance could be said as an active substance or toxic if it has value less than 1000 ppm (Meyer et al., 1982). This is related to phytochemical compounds found in the fresh cacao shell such as alkaloids, tannins and flavonoids, which in a certain levels, it has the potention of an acute toxicity, and it also can cause death *Artemia salina L.* larvae.
Fermentation is a stage of the cacao bean processing series. After fermentation, the cacao shell sustain a change of nutrients bioactive substances. Based on the test results of the regression analysis about the relationship between the concentration of fermented cacao shell extract towards the mortality percentage of *Artemia salina L.* larvae is in close conformity to the quadratic regression models that is presented in Figure 7. Quadratic regression model selection is based on the determination coefficient (R^2) obtained greater than 0.75 is equal to 0.958. Quadratic model between concentration of cacao shell extract after being fermented towards the mortality percentage of *Artemia salina* L. larvae is expressed in the equation:

$$y = 56,487x2 – 186,8x + 148, 79;$$
$$R^2 = 0.940 \text{ and } r = 0,979$$

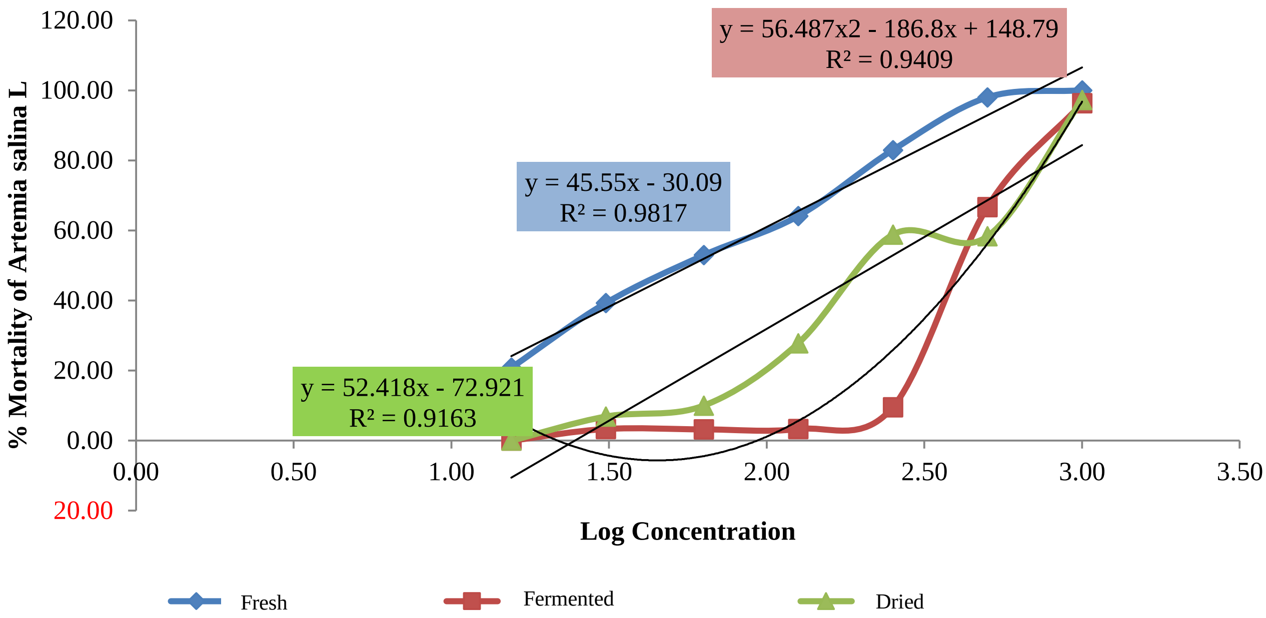


Fig - 7. Charts of the *Artemia salina L.* mortality percentage at some concentrations of Log Cacao Shell Extract.

The value of a (slope) in the equation is positive so the curve opens upward with a single maximum point where it is the highest number of dead larvae. Intercept is worth 186.8 which means if it unextracted (X = 0), then the number of dead larvae is 148.79%. Quadratic regression equation can be used to predict the maximum or minimum response of the extract concentration (X). The results of quadratic regression equation showed that the optimum increasing point of the extract to make the number of dead larvae become maximum is at a concentration of 44.66 ppm with the percentage of dead larvae of 5.64%.

The value of determination coefficient (R^2) of 0.940 states that 94.0% larvae mortality is affected by concentration of fermented cacao shell extract and the remaining 6% is influenced by other factors such as durability larvae, the testing place environment, and artificial sea water. The value of the correlation coefficient (r) of 0.979 means a very strong relationship between the concentration of the extract with the mortality percentage of *Artemia salina L.* larvae. Based on toxicity tests that have been carried out by BSLT method obtained the LC_{50} values of 124.75 ppm. LC_{50} <1000 ppm means the cacao shell is having toxic properties that can kill *Artemia salina*. Shrimp larvae.

LC_{50} value of the fermented cacao shell is greater than the fresh one, which indicates that the cacao shell decreased the toxicity after fermentation process. The test results showed that only detected tannins in a fermented cacao shell. This is consistent with the statement (Rasulu et al., 2012) that the fermentation of a material will decrease toxic substances including bioactive that can affect the mortality of larvae *Artemia salina L.* larvae.

The addition a concentration of dried cacao shell extract with the dead shrimp larvae showed a close relationship as shown in Figure 7. This close relationship is described by a linear equation. Selection of the linear regression model based on the determination coefficient (R^2) obtained greater than 0.75 is equal to 0.916. Linear models between the concentration of dried cacao shell extract with dead shrimp larvae is expressed in the equation:

$$y = 52{,}41x – 72{,}92;\ R^2 = 0{,}916 \text{ and } r = 0{,}957$$

The slope value is worth 52.41 which means that every 2 times increase of fresh cacao shell fresh from the initial amount, the number of the dead shrimp larvae will increase by 52.41%. The regression coefficient or intercept is worth 72.92 which means the initial value calculation Y (x = 0).

The value of determination coefficient (R^2) of 0.916 states that 91.6% mortality of *Artemia salina L.* larvae is affected by fresh cacao shell and the remaining 8.4%

influenced by other factors, such as durability larvae. The value of the correlation coefficient (r) of 0.957 means a very strong relationship between the concentration of fresh cacao shell extract with the mortality of *Artemia salina L.* larvae. Based on toxicity tests that have been carried out by BSLT method, there is obtained the LC_{50} values of 220.75 ppm. LC_{50} <1000 ppm means that the cacao shell are toxic that can kill *Artemia salina L.* larvae shrimp (Meyer et al., 1982).

Reviewed from the concentration similarity that is used during the test, most of the shrimp larvae died because of the fresh cacao shell extract. These results indicate that the fresh cacao shell is more toxic than the fermented and dried cacao shell. The dried cacao shell had the biggest toxicity compared to the fermented cacao shell except at concentration of 500 ppm. Phytochemical testing of the existence of alkaloids, tannins and flavonoids showed that the fresh and dried cacao shell contain those three substances. But, the fermented cacao shell only contain tannins. It is expected that this is what causes the fresh and dried cacao shell had a higher killing power against *Artemia salina L.* larvae at the same concentration.

Phytochemical compounds that is found in cacao shell can affect the mechanism of *Artemia salina L.* larvae mortality, so it obstruct the eating of its larvae (antifeedant). The workings of these compounds are to act as a stomach poisoning, therefore if these compounds enter the larvae body, the digestive apparatus will be disturbed. In addition, these compounds inhibit the receptors of taste in the mouth of the larvae. It makes the larvae failed to get the taste stimulus until it cannot recognize the food and the larvae will die (Nguyen and Widodo, 1999).

Sabir (2005) in the research found that flavonoids are able to release transduction energy towards the cytoplasmic membrane of bacteria, but it also inhibits the motility of bacteria. However, the hydroxyl group is contained in the structure of flavonoid compounds cause changes in organic components and transports nutrients that eventually will lead to the toxic effects on bacteria.

The presence of flavonoids within the cell, causing the OH groups in flavonoids bind to the integral protein cell membrane. It causes obstruction of the active transport of Na^{+} - K^{+}. The cessation of active transport causes uncontrolled Na^{+} ion implantation into cell and causing rupture of the cell membrane. (Scheuer, 1994). This rupture of cell membranes that causes the cell death.

Based on the LC_{50} value found that the fresh cacao shells are very toxic and the toxicity decrease after fermented and after dried. This is consistent with the statement of Rasulu et al., 2012, that the fermentation process which followed by a drying process can assist in the reduction or elimination of toxic compounds.

The toxicity level of the cacao shell extract cannot be said that it is toxic to humans. The toxicity values can be applied to living organisms (larvae and microorganisms) that very sensitive to chemical or bioactive components that exist in the fresh cacao shell. If the BSLT test results obtained LC_{50} value <1000 ppm, it is necessary to do further research to find out the toxic substances from the material using the more specific method.

Conclusion

There were changes of the nutrition content of fresh, fermented, and dried cacao shell. Cacao shell have increasing the fat compounds, crude fiber, and polyphenol. But the moisture and tannins are decreasing. Dried cacao shell contains phytochemical compounds such as alkaloids, tannins and flavonoids, but after being fermented its only contains tannins. Cacao shell toxicity is having LC_{50} values of 57.38 ppm, 124.75 ppm after being fermented, and 220.15 ppm after being dried. The fresh, fermented, and dried cacao shell toxicity <1000 ppm indicate that cacao shell containing toxic compounds to the *Artemia salina L.* larvae.

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