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Identification of alkaloids of Indonesian Cacao beans (*Theobroma cacao* L.) and its effect on tooth enamel hardness

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Dental caries is still a major dental health problem in Indonesia, and preventive measures needs to be done to resolve it. Enamel is the outer layer of the tooth that is important to protect tooth against caries. Until now, fluoride is well known as one of the reinforcing materials which effectively prevent enamel from dental caries, but the side effects of fluorosis remain debatable because the dose that causes it can not be measured. Therefore, the development of alternative reinforcing materials which are relatively safe in an effort to prevent tooth enamel caries is still required. Theobromine, one of three types of alkaloids contained in cocoa (*Theobroma cacao* L.) has been reported to prevent caries by increasing the resistance of tooth enamel tissue. Two types of local clones of Indonesian cocoa beans, Sulawesi 1 (S1) and Sulawesi 2 (S2), were analyzed for its alkaloid's characteristics using HPLC, and its influence on the enamel hardness were determined using Vickers hardness tester machine. The difference in the value of Vickers hardness numbers (VHN) of tooth enamel were analyzed using one-way ANOVA ($p < 0.05$). Optimal concentration and immersion time was obtained by comparing three types of Theobomine solution concentration (0.1%, of 0.05%, and a 0.01%) and three kinds of immersion time (1 h, 30 min, and 15 min). VHN value is highest in the group of single theobromine 0.1% - 1 h ($p = 0.000$). VHN values of mixed group of alkaloids S1 - 1 h showed significant differences with the group buffer (negative control) ($p = 0.028$) and did not differ significantly with single theobromine group of 0.05% - 1 h ($p = 1.000$), 0.1% - 30 min ($p = 1.000$), 0.1% - 15 min ($p = 1.000$) and a 0.01% - 1 h ($p = 0685$). The alkaloids content of Sulawesi 1 with a mixture ratio of alkaloids theobromine: theophyline: Caffeine, 6: 1 : 1, affected tooth enamel hardness.

Key words: Alkaloids, cocoa beans, dental caries, tooth enamel, hardness.

INTRODUCTION

Dental caries is still a prevalent issue in the field of dental and oral health in Indonesia. The percentage of dental caries reached up to 72.1%, where 46.5% of this was active dental caries and has not yet been treated (Ministry of Health Republic of Indonesia, 2008). Such a high percentage reveals the importance of prevention

measures that can be a better and affordable alternative than rehabilitative measures in dealing with dental caries in Indonesia.

The causes of dental caries are virulent bacteria, fermentable carbohydrates, saliva quality, and tooth enamel strength against acid as well as time. Tooth enamel is the outermost layer of the tooth that protects the crown from wear and tear of chewing and is very important in protecting the teeth from acid. Enamel resistance against acid is influenced by the chemical composition of tooth enamel, which is different in each individual, and other factors that can influence teeth structure. The process of tooth decay starts with the demineralization of enamel triggered by the increase in the acidic level of bacterial plaque (Fejerskov and Kidd, 2008). Enamel is the only tooth tissue that has no ability to regenerate or to heal itself after it is fully formed. This emphasizes the importance of preventing demineralization of the enamel (Robinson et al., 1998).

The demineralization of the enamel does not occur unceasingly because physiologically, the remineralization process will follow. The main mineral sources for the natural enamel remineralization process are calcium and phosphate from the saliva in saturated condition (Cury and Tenuta, 2009). Research on remineralization of the enamel has been done for about 100 years and it has been suggested to be the non-invasive treatment for early phase dental caries lesions (Reynolds, 2008).

In the last 50 years, fluoride has been claimed to be effective in preventing the caries process through inhibiting demineralization and increasing remineralization by forming fluorapatite and calcium fluoride as well as inhibiting the work of bacterial enzymes through antimicrobial activities (Kirkham et al., 1994; Torgay et al., 1994; Pearce et al., 1995; Cury and Tenuta, 2009). However, the safe dosage of fluoridation and the danger of fluorosis are still debatable. Research by the National Health and Medical Research Center in Melbourne and The International Society for Fluoride Research concluded that more research needs to be done into the use of fluoride in the fluoridation process (National Health and Medical Research Center in Melbourne, 1999; The International Society for Fluoride Research, 2000). Recent consensus claims that fluoride is no longer used systemically but rather used locally through direct application on the teeth (Cury and Tenuta, 2008). Aside from fluoride, there are now three other materials for remineralization, which are casein phosphopeptide stabilized amorphous calcium phosphate (CPP-ACP), unstabilized amorphous calcium phosphate (ACP), and bioactive glass containing calcium sodium phosphosilicate. These three materials rely on calcium and phosphate to increase the remineralization ability of the saliva when the demineralization of enamel occurs. However, there is not yet sufficient clinical evidence to support this mechanism (Cury and Tenuta, 2009).

Research on the development of natural materials that have anti-caries effects and are relatively safe and affordable for use is still ongoing until now. Cacao beans (*Theobrema cacao L.*) contain secondary metabolites in the form of purine alkaloids derived from Xanthine such as theobromine, caffeine and theophylline. For several years until now, theobromine has been explored to find its benefits for dental health. Sadeghpour reported that theobromine in chocolate powder has better anticariogenic effect than fluoride in reducing enamel solubility (Sadeghpour, 2007). Kargul et al. claimed that the hardness of enamel was related to the mineral exchange on the surface of enamel and that Thebromine 200mg/l has positive effects on enamel remineralization (Kargul et al., 2010). This result is supported by research conducted by Grace et al., which claimed that theobromine was a material that can prevent potential dental caries because of its ability to increase the hardness of enamel (Grace et al., 2012). Kargul et al. further claimed that through in vitro the effectiveness of theobromine on the hardness of the enamel and remineralization process was equal to Acidulated Phosphate Fluoride (APF) gel and Casein Phosphopeptide Stabilized Amorphous Calcium Phosphate (CCP-ACP) (Kargul et al., 2012).

Indonesia is one of the biggest cacao beans producers in the world. The province of Central Sulawesi is one of the biggest cacao beans producing areas in Indonesia in the last 10 years (Business Competition Supervisory Commission, 2009). The Sulawesi Island right now has superior locally cloned cacaos, which are Sulawesi 1 and Sulawesi 2. These two clones have shown good adaptation quality to Sulawesi's agroclimatic condition and they have been widely cultivated in central areas for cacao production in Sulawesi Island (Indonesian Coffee and Cacao Research Centre, 2007). The quality of cacao beans is influenced by the two important compounds resulted from secondary metabolites contained within the beans, which are polyphenol and alkaloid (Bravo, 1998; Wollgast and Anklam, 2000). With the abundant availability of good quality cacao beans in Indonesia, it is important to harness its benefits widely in the field of medicine, in particular in dental health.

This research was conducted to identify the characteristics of the alkaloid content and the benefits of these clones of cacao beans from Indonesia, Sulawesi 1 and Sulawesi 2, to tooth enamel hardness. As part of the

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Figure 1. Tooth enamel specimen that is ready to use.

development of cross-disciplinary research especially oral biology, chemistry, and materials science, this study establishes the efficacy and mechanism of action of the alkaloid extract of cocoa beans to the tooth enamel, in order to obtain the scientific basis of the use of alkaloid extract of Indonesian cocoa beans for *in vivo* studies and clinical trials, if the future, the extract applied in dentistry as an alternative material of dental caries prevention. With this research it is hoped that cacao beans can be utilized as medicinal plants to improve dental health and in particular in preventing dental caries.

MATERIALS AND METHODS

The effect of cacao beans alkaloid was evaluated through in vitro tests. This *in vitro* laboratory experimental research comprised of phytochemical test to get the crude extract of alkaloid as well as to identify alkaloid content. Furthermore, simulation of alkaloid extract solution was conducted from the two cacao bean clones from Indonesia, which were Sulawesi 1 (S1) and Sulawesi 2 (S2) from Central Sulawesi. The effects of cacao beans alkaloid were evaluated base on the hardness of the enamel of teeth.

Cacao beans collection

The cacao beans were taken from the Sidondo experimental plantation in Palu, Central Sulawesi, between September to November 2013. The head of Sidondo experimental plantation ensured its type, where the specimen number of KW162 was identified as clones Sulawesi 1 (S1) and the specimen number of KW163 identified as clones Sulawesi 2 (S2). The cacao beans were extracted from ripened cacao fruits, and then the pith attached to the seeds was removed. The beans were dried in the sun for 6 to 7 days. After they were dried, the shell attached to the seeds was removed. The seeds were then ground using a dry grinder (Phillips HR-2071).

Preparation and analysis of crude extracts

The cocoa beans S1 and S2 were defatted though the sohxlet extraction method using reflux and Chiller instrument (Eyela CA-1111). The next phase was the alkaloid extraction using the method from the Association of Analytical communities (AOAC) 2006. After this, the identification of the components of crude alkaloid extract was done quantitatively using High Performance Liquid Chromatography (HPLC) (Shimadzu LC-6), C18 column, UV detector, methanol: CH_3COOH: aquadest = 20: 1: 79 (Horwitz and Latimer, 2006). The analysis result was stated in the form of type and percentage of natural ratio of mixed groups alkaloids of theobromine, theophylline, and caffeine contained within S1 and S2 cacao beans.

Preparation of buffer solutions

Preparation of carbonate buffer solution (pH 10, 0.1 M) was done by mixing sodium bicarbonate ($NaHCO_3$) with sodium carbonate (NA_2CO_3) (Merck) in 100 ml aquadest. The pH was adjusted by Sodium hydroxide NaOH (0.1 M) added inside the solution until it reached pH 10.The pH was measured with a pH meter (DKK-TOA HM 20J).

Preparation of tooth enamel samples

Caries free human premolar teeth that had just been extracted were cut using a carborundum disc at low speed, in order to obtain part of the tooth crown. Each tooth crown was then cut from the coronal direction to the apical, right in the middle of mesial and distal side until the two enamel surfaces, the buccal and lingual surfaces, were acquired. All crowns were cleaned using 2.5% Sodium hypochlorite (NaOCI) and followed by 70% alcohol using an ultrasonic cleansing tool (Cole-Palmer 8891), then immersed in saline solution until the time of the experiment.

Thirthy crowns were placed in an epoxy resin. Stickers were attached to the flattest surface of enamel, put on glass pads, and then a ring mould was placed as the printing tool. A liquid epoxy resin mixed with catalyst was immediately poured into the ring mould. After it hardened, the stickers and ring mould were removed from the resin, and then the surface of the enamel that was not covered by resin was ready to be sharpened and polished with a grinding and polishing machine (Steuers laboPol-21) using 2000 grit sandpaper, polishing paste (DiaPro), and polishing cloth. Grinding and polishing should not be more than 0.5 mm of the enamel surfaces thickness. After the polishing was done, the 30 enamel surfaces that were sufficiently wide, flat, and ready to be analyzed were acquired (Figure 1).

Immersion of tooth enamel samples in alkaloid solutions

Alkaloid powder was measured using an analytic scale (Satorus BS 124S) and then diluted in buffer solution with the help of an ultrasonic tool (Power sonic 510) for 10 to 60 min at temperatures between 30 and 37°C, until 0.01, 0.05 and 0.1% concentration were acquired within single or mixed theobromine concentration that were in accordance with natural ratio of alkaloid solution contained within S1 and S2 cacao beans.

Eight kinds of solutions were acquired, namely Buffer solution (B - 0); single theobromine 1000 mg/L (T - 0.1%), 500 mg/L (T - 0.05%) and 100 mg/L (T - 0.01%); single theophylline 1000 mg/L (TF - 0.1%); single caffeine 1000 mg/L (C - 0.1%); mixed Alkaloid with S1 natural ratio (T : TF : C = 6 : 1 : 1) and S2 natural ratio (T : TF : C = 4 : 1 : 1).

Next, thrity specimens of tooth enamel were divided randomly

Table 1. HPLC analysis of alkaloid extracts of cacao beans S1 and S2 in the form of their natural ratios.

Sample	Sampel consentration (% b/b)		
	Theobromine	Theophylline	Caffeine
S1	1.35 (6)	0.13 (1)	0.21 (1)
S2	1.17 (4)	0.37 (4)	0.27 (4)

Table 2. Tooth enamel hardness value (VHN) after all specimens were immersed in multiple-dose trial solutions in accordance with the duration of immersion, n = 3.

Sample	n	Mean ± SD	Min-Max	*p*
Buffer - 0 - 1 h	3	370.33 ± 8.622	361 - 378	0.000
T - 0.1% - 1 h	**3**	**541.33 ± 31.005**	**510 - 572**	
T - 0.1% - 30 min	3	435.00 ± 28.618	417 - 468	
T - 0.1% - 15 min	3	431.00 ± 24.249	405 - 453	
T - 0.05% - 1 h	3	431.00 ± 12.124	417 - 438	
T - 0.01% - 1 h	3	401.33 ± 11.240	389 - 411	
C - 0.1% - 1 h	3	388.00 ± 22.716	372 - 414	
TF - 0.1% - 1 h	3	350.33 ± 23.116	336 - 377	
S1 - 1 h	**3**	**434.67 ± 18.475**	**424 - 456**	
S2 - 1 h	3	390.33 ± 18.475	369 - 401	

into 10 groups, that is, B - 0 - 1 h (Control), T - 0.1% - 1 h, T - 0.1% - 30 min, T - 0.1% - 15 min, T - 0.05% - 1 h, T - 0.01% - 1 h, TF - 0.1% 1 h, C - 0.1% - 1 h, S1 - 1 h and S2 - 1 h. Every group was comprised of three specimens. All specimens were then immersed in experiment material solution in accordance with the immersion duration.

Tooth enamel hardness test

The hardness test was conducted using an indenter Vickers (Shimadzu HMV2) with 100 g load for 10 s with three indentations to each specimen. The test result was stated in VHN (Vickers Hardness Number).

RESULTS

The results of component identification in the crude alkaloid extract using HPLC in quantitative terms can be seen in Table 1. The proportion natural ratio (% b/b) of the mixed group of alkaloid theobromine, theophylline, and caffeine contained in the S1 cacao beans was 6 : 1: 1, and in the S2 cacao beans was 4: 1: 1.

The results of tooth enamel hardness test can be seen in Table 2. The normality of data tested with One-Sample Kolmogorov Smirrnov shows that all the data acquired had normal distribution (p=0.362, p>0.05), and the data from the homogenity test using one way ANOVA shows that all data are homogeneous (p=0.399, p>0.05). Furthermore, the statistical analysis with one-way ANOVA test shows that there are significant differences among and within research groups (p=0.000, p<0.05).

From post hoc test with Tuckey HSD, the difference among groups was clearly seen. There was a significant difference among T - 0.1% - 1 h group (with the highest VHN value) with the rest of experiment groups (p=0.000). There was a significant difference among Buffer - 1 h group (the lowest VHN value) and the T - 0.1% - 1 h (p=0.000), T - 0.1% - 30 min (p=0.027), S1 - 1 h (p=0.028), T - 0.05% - 1 h (p=0.046) and T - 0.1% - 15 min (p=0.046) groups. Although the VHN value of S1 - 1 h group was significantly different from T - 0.1% - 1 h (p=0.000), it did not have significant difference with T - 0.05% - 1 h (p=1.000), T - 0.1 % - 30 min (p=1.000), and T - 0.1% - 15 min (p=1.000).

Aside from theobromine, the two other single alkaloids, which were caffeine and theophylline that were contained within C - 0.1% - 1 h and TF - 0.1% - 1 h, apparently had VHN values that were not significantly different from Buffer – 1 h (p=0.990) and (p=0.977) groups. The T - 0.01% - 1 h (p=0.741) group was the only group of single theobromine that had similar VHN value with Buffer - 1 h group.

DISCUSSION

This research was aimed to identify the alkaloid content of Indonesian cacao beans (*T. cacao* L.) and its effect on the tooth enamel hardness through in vitro measures. The trend of doing minimal intervention has been a much-discussed topic in the field of dentistry. Preventing

demineralization of tooth enamel is the most important measure in the attempt to avoid dental caries.

Research on the benefits of cacao plants also show the rising tendency of people all over the world to consume more safe and healthy food products. With the slogan “back to nature,” Indonesia as a country that has rich natural resources and is the second largest biodiversity in the world after Brazil as well as the third biggest cacao bean producer in the world has the chance to participate in the development of medicinal plants, in particular in the development of cacao as a highly beneficial medicinal plant. Indonesia is rich with biodiversity and organic chemical compounds resulting from metabolism process contained within them in the form of primary metabolites such as protein, carbohydrates, and fat which used by the plants to grow, and in the form of secondary metabolites such as terpenoids, steroids, coumarin, flavonoid, and alkaloid. Secondary metabolites compounds are organic chemical compounds that generally have bioactivity ability and synthetised by an organism not only to fulfill its basic needs but also to maintain its existence in interacting with other organisms in an ecosystem. Secondary metabolites within medicinal plants can be utilized as benefical materials for human beings (Ministry of Agriculture Republic of Indonesia, 2007).

Indonesia has a wide variety of medicinal plants that contain beneficial alkaloid compounds, including those contained in the two cacao beans clones from Central Sulawesi. Currently, Sulawesi has its own cacao clones, which are Sulawesi 1 and Sulawesi 2. They are local and hybrid cacao clones resulted from the crossbreeding between Forastero and Criollo types. These two types of cacao clones have been known and used by the cacao farmers for more than 30 years in Central, South, Southeast, and West Sulawesi. The province of Central Sulawesi is one of the biggest cacao producing areas in Indonesia in the past 10 years (Indonesian Coffee and Cacao Research Centre, 2007).

The resilient nature of cacao plants against pests is influenced by, among others, the level of secondary metabolites, particulary alkaloid that is contained within the plant. Theobromine, one of the alkaloids compounds in secondary metabolites contained within cacao beans aside from caffeine and theophylline is reported to have the ability to strengthen tooth enamel even though its present in chocolate processed products that have been mixed with sugar and thus contradicting with its ability to strengthen the tooth enamel. The benefits of natural materials, as well as chocolate, are much-discussed topics and have become the target of many researches.

In this research, alkaloid was directly applied to the premolar tooth enamel that was free from caries and was extracted for orthodontic treatment purposes. By doing this, it was expected that the effectiveness of alkaloid compounds in strengthening tooth enamel could be analyzed. Variations theobromine concentrations used in the research were 1000 mg/L (0.1%), 500 mg/L (0.05%), and 100 mg/L (0.01%) in both single and mixed theobromine concentrates that were adjusted to the natural ratio of alkaloid theobromine, theophylline, and caffeine mixtures contained within cacao beans S1 (6: 1: 1) and S2 (4: 1: 1).

Alkaloid was dissolved in carbonate buffer solution with pH 10, 0.1 M, and 100 ml aquadest. Among several kinds of buffer, it was found that carbonate buffer with pH 10 was the most effective to dissolves alkaloid. The time required for alkaloid to dissolve was between 30 and 60 min. The dosage variation in actuality could still be multiplied between the range of 100 to 1000 mg/L. In concentrations above 1000 mg/L, carbonate buffer could no longer dissolved alkaloid perfectly (no sediment). Different durations of immersion also could still be propagated between the range of 15 to 60 min, leading to the use of alkaloid as a material to be applied topically by dental professionals as a preventive measure for dental caries.

The hardness of tooth enamel could be analyzed with a Vickers hardness tester. This method was chosen because it is often used to determine the hardness of tooth enamel by many researchers. The higher the VHN value produced, the harder the tooth enamel. The dosage and duration of immersion were equally influential to the differences in VHN values. The dosage of single theobromine 0.1% (1000 mg/L) and 0.05% (500 mg/L) could increased the VHN value significantly. The level of theobromine 100 mg/L apparently did not have any impact on the hardness of tooth enamel. Immersing tooth enamel for one hour could increased the VHN value significantly compared to immersing tooth enamel for only 30 and 15 min. The optimal dosage of single theobromine was 0.1% (1000 mg/L) and the optimal immersion duration was 1 h.

The dosage of single caffeine and theophylline which equal to the optimal dosage and duration of single theobromine of 0.1% (1000 mg/L) for 1 h could not increased the VHN value significantly and even tended to reduce the VHN value. This was proved when theobromine with optimal dosage and duration, which was 0.1% (1000 mg/L) for 1 h, was combined with caffeine and theophylline in the simulation of natural alkaloid ratio of the two cacao clones, its ability to increase VHN value tended to decrease. In the ratio contained within cacao beans clone Sulawesi 1, which was the mixed group ratio of theobromine : theophylline : caffeine = 6 : 1 : 1 = 75% : 12.5 % : 12.5%, its ability in increasing VHN value did not decrease significantly and it tended to have a VHN value that did not differ significantly from single theobromine of 0.05% (500 mg/L) for 1 hour and 0.1% (1000 mg/L) for 30 and 15 min, and it had a significant difference from the buffer. Meanwhile, in the ratio contained within cacao beans clone Sulawesi 2, which was the mixed group ratio of theobromine: theophylline: caffeine = 4: 1: 1 = 66.67%: 16.67%:

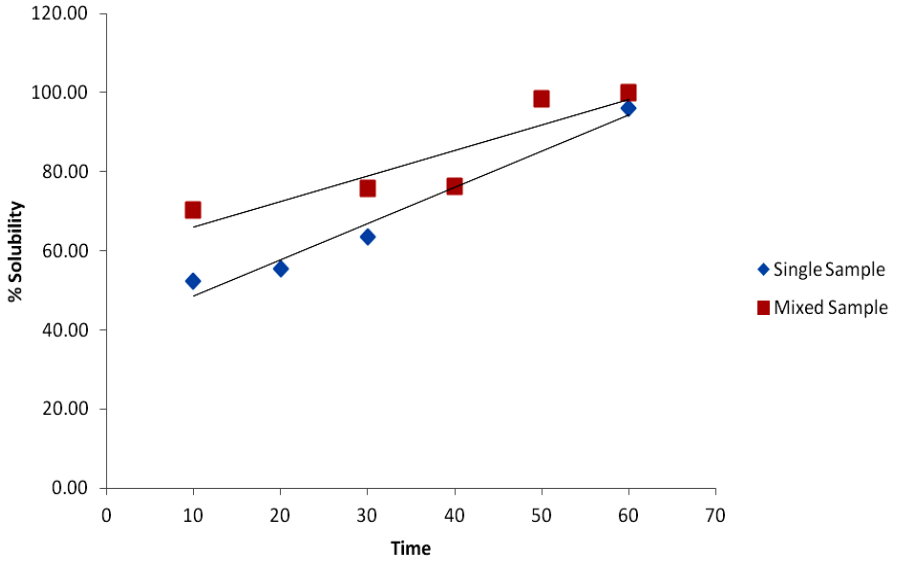

Figure 2. Solubility test graphic of single alkaloid and mixed group alkaloids solution in Carbonate buffer with pH 10.

16.67%, its ability to increased the VHN value decreased significantly, with the VHN value that did not differ significantly from the buffer only.

Besides its effect on VHN value, it appeared that mixed group alkaloids also had some impact on the speed of the dilution time of alkaloid in a solution. This was proven by the result of solution identification by using HPLC in 1 hour. The speed of dilution time of mixed alkaloid was faster than single alkaloid of theobromine only (Figure 2).

Conclusion

This *in vitro* laboratory research showed that the alkaloid content in cacao beans clone from Indonesia, Sulawesi 1, with the natural ratio of mixed groups alkaloids theobromine, theophylline, and caffeine of 6 : 1 : 1, could increased the hardness of tooth enamel. This result was almost equal to the effect of single theobromine in certain concentration and duration. Another benefit of mixed group alkaloids in cacao beans, that is, its effect on the solubility and stability of alkaloid within a buffer solution still needs further research. The working mechanism of mixed group alkaloids and single theobromine in increasing tooth enamel hardness, both on the surface and subsurface, has to be explored further. It is also equally important to involve the role of saliva and pH decrease due to acid so that demineralization and remineralization processes can be simulated as closely as possible to the clinical conditions in the mouth.

Conflict of interest

The authors have not declared any conflict of interest.

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