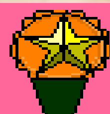
Journal of Medicinal Plant Research

Volume 10 Number 15, 17 April, 2016

ISSN 1996-0875



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Journal of Medicinal Plants Research

Table of Contents: Volume 10 Number 15 17 April, 2016

ARTICLE

Antifungal activity of selected medicinal plants against *Alternaria* species: The pathogen of dirty panicle disease in rice 195
Sawatdikarn Sanit

Identification of alkaloids of Indonesian Cacao beans (*Theobroma cacao* L.) and its effect on tooth enamel hardness 202
Rina Permatasari, Dewi Fatma Suniarti, Ellyza Herda and Zainal Alim Mas'ud

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Vol. 10(15), pp. 195-201, 17 April, 2016
DOI: 10.5897/JMPR2013.5117
Article Number: 196FC4958106
ISSN 1996-0875
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Journal of Medicinal Plants Research

Full Length Research Paper

Antifungal activity of selected medicinal plants against *Alternaria* species: The pathogen of dirty panicle disease in rice

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Received 12 June, 2013; Accepted 8 March, 2016

Antifungal activity of the ethanolic crude extracts from ten plants, namely: *Curcuma xanthorhiza, Curcuma aromatica, Kaempferia parviflora, Syzygium aromaticum, Origanum vulgare, Synedrella nodiflora, Sorghum bicolor, Rosmarinus officinalis, Piper longum* and *Eupatorium odoratum* were tested against *Alternaria* species (the pathogen of dirty panicle disease in rice) by poisoned food technique at 0, 1,000, 2,500, 5,000, 7,500 and 10,000 ppm. The inhibition of mycelial growth and spore germination were evaluated. The results showed *C. aromatica, S. aromaticum* and *O. vulgare* crude extracts at 1,000 ppm, the *R. officinalis* crude extracts at 5,000 ppm, and the *C. xanthorrhiza* crude extracts at 10,000 ppm showed the highest inhibition of mycelial growth at 100%, whereas *S. nodiflora* and *S. bicolor* crude extracts at 10,000 ppm had the inhibition at 56 and 58%, respectively. For inhibition of spore germination, the three plant crude extracts; *C. aromatica, S. aromaticum* and *O. vulgare* at 1,000 ppm and *C. xanthorrhiza, S. nodiflora, S. bicolor, R. officinalis* and *P. longum* crude extracts at 2,500 ppm reached the highest inhibition of spore germination at 100%, whereas *E. odoratum* and *K. parviflora* crude extracts at 10,000 ppm had the inhibition at 20 and 55%, respectively.

Key words: Antifungal activity, dirty panicle disease, *Alternaria* species, medicinal crude extract, rice.

INTRODUCTION

Dirty panicle disease is one of the most serious diseases of rice, affecting rice production in the world and rice production grown in Thailand and tropical locations (Ou, 1985). The causal organisms are several pathogens, *Fusarium* species, *Alternaria* species, *Cercospora* species and *Curvularia* species (Abdelmonem, 2000). The panicle dirty control had several methods, mechanical, cultural, biological and chemical method. Chemical control is the best method for dirty panicle disease, whereas this method is harmful for environmental condition, consumer and user. Nowadays, the farmers use the biological control for dirty panicle control. The medicinal herb crude extracts for the seed borne pathogen control have attracted wide interest, the

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medicinal herbs comprises a rich phytochemicals and biochemicals substances to be used as biopesticide which are more friendly environment than synthetic chemicals.

There are reports that phytochemicals and biochemicals substances of some plants showed fungicidal activity against pathogenic fungi; *Syzygium aromaticum* crude extract at 1,000 ppm showed 100% inhibition on mycelial growth of *F. oxysporum* and *F. equiseti* (Kritzinger et al., 2002) and the *S. aromaticum* crude extract can be used for *Staphylococcus aureus* control (Briozzo et al., 1989). Rosemary (*Rosmarinus officinalis*) crude extracts showed the growth inhibition of three pathagens, namely *Leuconostoc mesenteroides, Listeria monocytogenes* and *S. aureus* (Campo et al., 2000). For the some medicinal crude extracts on mycelial growth of some pathogens control including the oregano (*Origanum vulgares*) crude extract for *Bacillus cereus* control (Paster et al., 1995); *Curcuma xanthorhiza* for three pathogen control, namely *B. cereus, S. aureus* and *Escherichia coli* (Husein et al., 2009); *Curcuma aromatica* for *S. aureus* and *E. coli* control (Saleem et al., 2011) and *Kaempferia parviflora* crude extracts for *B. cereus* and *S. aureus* control (Butkhup and Samappito, 2011).

No information of ten medicinal herb crude extracts on inhibition of mycelial growth and spore germination of *Alternaria* spp. (pathogen of dirty panicle disease in rice). The objective of this research was to evaluate ten medicinal herb crude extracts on the mycelial growth and spore germination of *Alternaria* spp. in Central area, Thailand.

MATERIALS AND METHODS

This work was conducted at the Department of Applied Science, Faculty of Science and Technology, Phranakhon Si Ayutthaya Rajabhat University, Phranakhon Si Ayutthaya for 2010 to 2011 to test the antifungal activity of *C. xanthorhiza, C. aromatica, K. parviflora, S. aromaticum, O. vulgare, Synedrella nodiflora, Sorghum bicolor, R. officinalis, Piper longum* and *Eupatorium odoratum* against *Alternaria* spp. (pathogen of dirty panicle disease in rice) in distilled water and ethanol by using food poisoning technique (Prasad et al., 2010)**.**

Preparation of rice seeds and isolation of pathogen

Rice seeds were obtained from three location in Central area, Phranakhon Si Ayutthaya, Aungthong and Prathumthani province. *Alternaria* spp. from the rice seeds were isolated and maintained on petri dishs containing in Potato dextrose agar (PDA) and incubated at 25°C for 3 days before the tests. The preparation of rice seeds and the isolation of pathogen followed by the methods of Sawatdikarn (2011).

Collection of and preparation of plants samples

Ten medicinal crude extracts used in this study were obtained from three location in Phranakhon Si Ayutthaya province, Bangban, Wangnoi and Bangpa-in area. Fresh rhizomes of *C. xanthorhiza, C. aromatica* and *K. parviflora* were collected from Bangban, fresh leaves of *O. vulgare, S. nodiflora, S. bicolor, R. officinalis* and *E. odoratum* were collected from Wangnoi and fresh bud and fruit parts of *S. aromaticum* and *P.longum*, respectively were collected from Bangpa-in. They were washed with tap water and air dried for three days to eliminate surface moisture. Then each part of the medicinal plants was packed into envelop and kept in oven at 105°C temperature until it is dried. Dried parts were ground separately in an electic grinder to obtain powder which was than kept in plastic bags before the tests.

Preparation of crude extracts

100 grams of the dried powdered plant were soaked in 1,000 ml of 90% ethanol. These mixtures were refluxed followed by agitation at 200 rpm for 1 h. The ethanolic extracts were squeezed and filtered by muslin cloth. The crude extracts were placed into a wide tray to evaporate ethanol and added with water plant extracts (Prasad et al., 2010).

Antifungal activity test

Mycelial growth test

Food poisoning technique: Diffusates were added in PDA and poured into petri dishes. PDA medium added only with ethanol and water served as control treatment. Each petri dishes was inoculated with 5 mm plug of pure isolate taken from margins of actively growing culture of pathogen. All petri dishes were incubated at 25°C. The screening of crude extracts for antifungal activity was conducted using the agar dilution method. Different crude extracts were tested using food poisoning technique. Each tested crude extracts was used at different concentrations; 0 (control treament), 1,000, 2,500, 5,000, 7,500 and 10,000 ppm. The petri dishes were incubated in room temperature for 7 days. The efficacy of treament was assessed from all the four plate by mesuring fungal colony development (cm). The mycelail growth inhibition (M) with respect to the control treament was calculated from the formula (Sheng-Yang et al., 2005).

$$M = [(A-B) / A] \times 100$$

where A is the colony diameter of the control treament and B is the colony diameter of the treated crude extracts.

Spore germination test

Effect of ten medicinal crude extracts on spore germination of *Alternaria* spp. was carried out by the spore germination method (Ting-Ting et al., 2011). A spore suspension of *Alternaria* spp. was prepared by adding 10 ml of sterile water to 15 days old petri plate culture of *Alternaria* spp. grown at 22°C on PDA. The surface of agar was washed through a double layer of gauze with sterile water. The inoculum was adjusted to a concentration of 1×10^6 ml with haemacytometer. One milliliter of ten crude extract in 20 and 40 mg/ml was mixed with the same amount of *Alternaria* spp. spore suspension. The mixture was pipetted onto clean concave slips and incubated at room temperature for 24 h. In the control, the sterile water was mixed with spore suspension. Germination rate of spores was observed with microscope after incubation of 12 h.

Each tested crude extracts was used at different concentrations; 0 (control treament), 1,000, 2,500, 5,000, 7,500 and 10,000 ppm. The efficacy of treament was assessed from four slide after treatment by measuring number of spore germination. The spore

Table 1. Efficacy of different concentration of some medicinal plant crude extracts on mycelial growth inhibition of *Alternaria* spp.

Medicinal plant crude extracts	**Mycelial growth inhibition degree** (%)				
	1,000 ppm	**2,500 ppm**	**5,000 ppm**	**7,500 ppm**	**10,000 ppm**
Curcuma xanthorrhiza	84[b]	87[b]	89[b]	92[b]	100[a]
Curcuma aromatica	100[a]	100[a]	100[a]	100[a]	100[a]
Kaempferia parviflora	49[c]	57[c]	70[c]	72[c]	75[b]
Sygygium aromaticum	100[a]	100[a]	100[a]	100[a]	100[a]
Origanum vulgare	100[a]	100[a]	100[a]	100[a]	100[a]
Synedrella nodiflora	25[d]	42[d]	53[d]	55[d]	56[c]
Sorghum bicolor	40[c]	43[d]	50[d]	53[d]	58[c]
Rosmarinus officinalis	88[b]	90[b]	100[a]	100[a]	100[a]
Piper longum	47[c]	52[c]	68[c]	70[c]	75[b]
Eupatrium odoratum	40[c]	46[d]	60[c]	69[c]	71[b]
CV (%)	10.28	11.26	9.86	12.64	8.54

In each column, mean followed by a common letter are not significantly different at the 5% level by DMRT.

germination inhibition (S) with respect to the control treament was calculated from the formula (Sheng-Yang et al., 2005).

$$S = [(A-B) / A] \times 100$$

where A is the spore germinarion number of the control treament and B is the spore germination number of the treated crude extracts.

Statistical analysis

For statistical analysis, Duncan Multiple Range Test was used to compare the average.

RESULTS AND DISCUSSION

The ten medicinal herb crude extracts showed inhibition on mycelial growth of *Alternaria* spp. different concentrations (Table 1). The crude extracts of *C. aromatica* (Figure 1), *S. aromaticum* and *O. vulgare* at all concentrations, the *R. officinalis* crude extracts at 5,000 ppm and the *Curcuma xanthorrhiza* crude extracts at 10,000 ppm (Figure 2) showed 100% inhibition on mycelial growth, whereas the *S. nodiflora* (Figure 3) and *S. bicolor* (Figure 4) crude extracts at 10,000 ppm gave the highest inhibition of 56 and 58%, respectively.

C. aromatica, *S. aromaticum* and *O. vulgare* crude extracts showed that 100% inhibition on spore germination (Table 2) at all concentrations can use crude extracts of these species for *Alternaria* spp. control (the pathogen of dirty panicle disease in rice) at all concentration (1,000 to 10,000 ppm).

The *C. aromatica* crude extracts showed 100% inhibition on mycelial growth and spore germination at all concentrations (1,000-10,000 ppm); this result agrees with *C. aromatica* for *S. aureus* and *E. coli* control (Saleem et al., 2011). For *S. aromaticum*, the crude extracts showed 100% inhibition on mycelial growth and spore germination at all concentration (1,000-10,000 ppm) ; this result agrees with the clove oils for antimicrobial activity on some pathogen; *S. aureus* (Briozzo et al., 1989; Tranter et al., 1993), *Fusarium oxysporum* and *F. equiseti* (Kritzinger et al., 2002) and *Phomopsis azadirachtae* (Prasad et al., 2010) showed the mycelial growth inhibition.

O. vulgare crude extracts showed 100% inhibition on mycelial growth and spore germination of *Alternaria* spp. at all concentration (1,000 to 10,000 ppm); this result agrees with the *O. vulgare* crude extracts inhibited the growth of *Bacillus cereus* (Paster et al., 1995). For the three crude extracts showed the inhibited mycelial growth in *Alternaria* spp., because phytochemical effects for antimicrobial activity, curcumin from the rhizome of *C. aromatica* (Saleem et al., 2011), phenolic compound and eugenol from the bud or fruit of *S. aromaticum* (Briozzo et al., 1989; Maarse, 1991) and carvacrol from the leaves of *O. vulgare* (Ultee et al., 2000; Chaudhry et al., 2007).

The two crude extracts, *R. officinalis* (5,000 to 10,000 ppm) and *C. xanthorrhiza* (10,000 ppm) showed 100% of inhibition on mycelial growth of *Alternaria* spp. (Table 1). This result agrees with the *R. officinalis* crude extracts showed the growth inhibition of *Leuconostoc mesenteroides, Listeria monocytogenes* and *S. aureus* (Campo et al., 2000) and the result indicated that the *C. xanthorrhiza* crude extracts showed the inhibition for three pathogen control, namely *B. cereus, S. aureus* and *E. coli* (Husein et al., 2009).

The three crude extracts, namely *C. xanthorrhiza*, *R. officinalis* and *P. longum* at 2,500 ppm reached the highest inhibition of spore germination at 100% (Table 2). For the *R. officinalis* and *C. xanthorrhiza* crude extracts showed the inhibition of mycelial growth and spore germination of *Alternaria* spp., because of phytochemical potential for antimicrobial activity, carnosic acid and

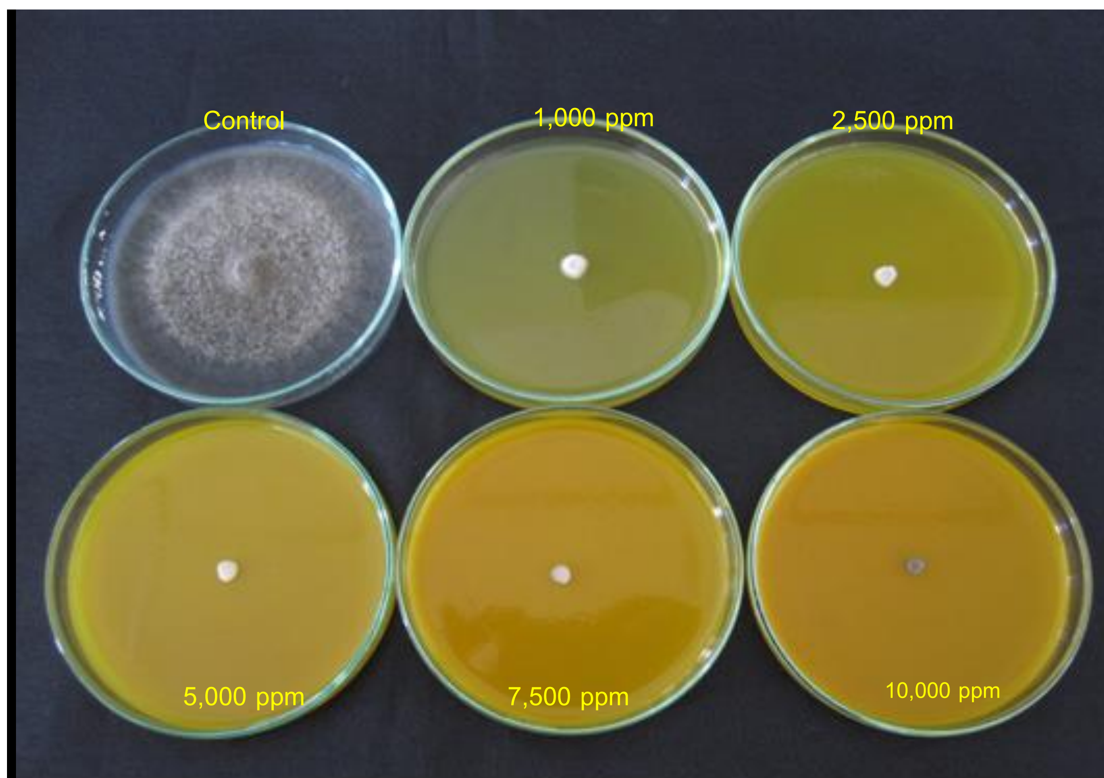

Figure 1. Effect of *Curcuma aromatica* crude extract on the mycelial growth of *Alternaria* sp. at different concentrations (Control treatment, 1,000 2,500 5,000 7,500 and 10,000 ppm).

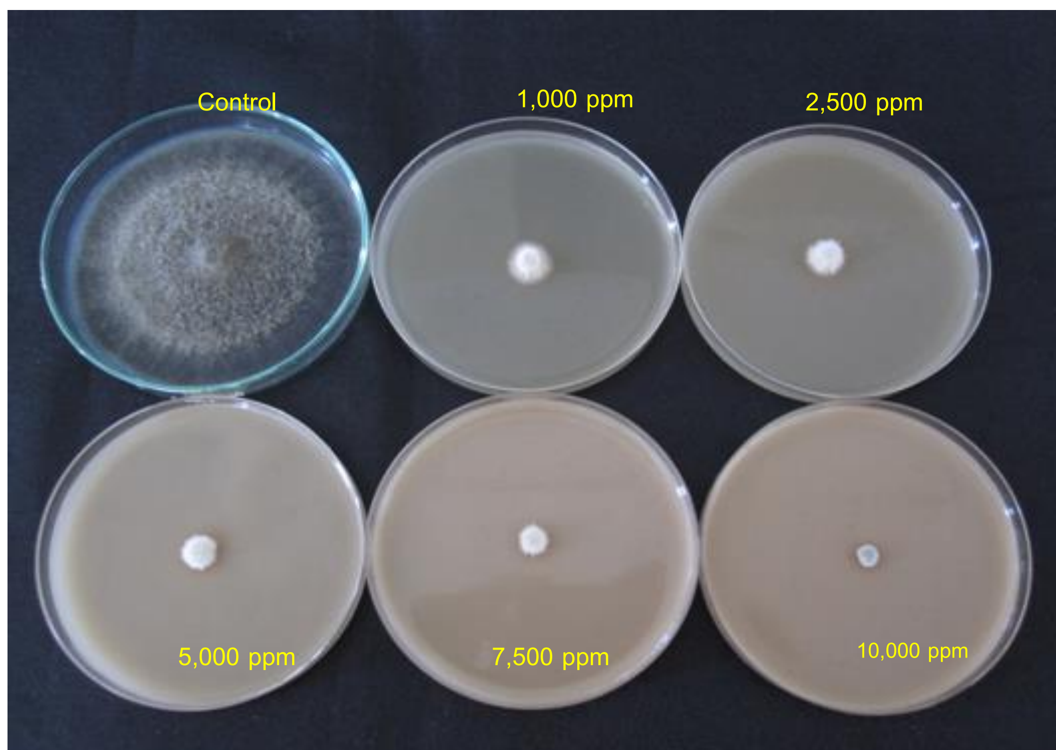

Figure 2. Effect of *Curcuma xanthorrhiza* crude extract on the mycelial growth of *Alternaria* sp. at different concentrations (Control treatment, 1,000 2,500 5,000 7,500 and 10,000 ppm).

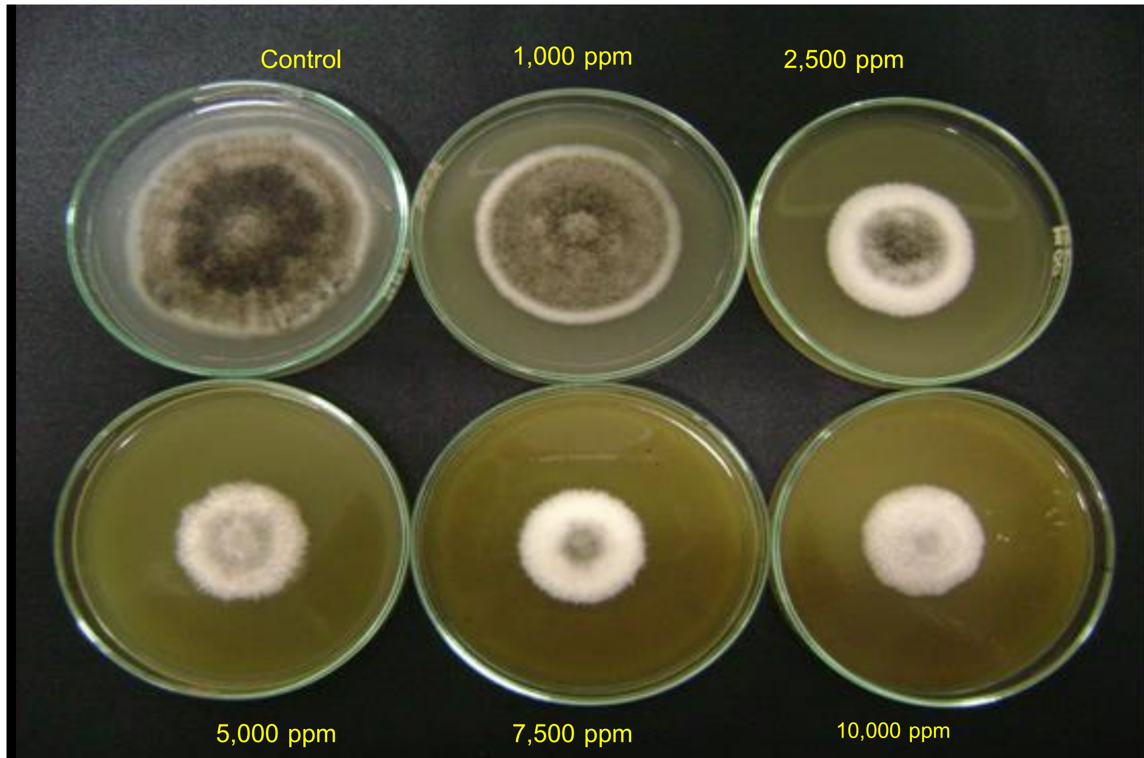

Figure 3. Effect of *Synedrella nodiflora* crude extract on the mycelial growth of *Alternaria* sp. at different concentrations (Control treatment, 1,000 2,500 5,000 7,500 and 10,000 ppm).

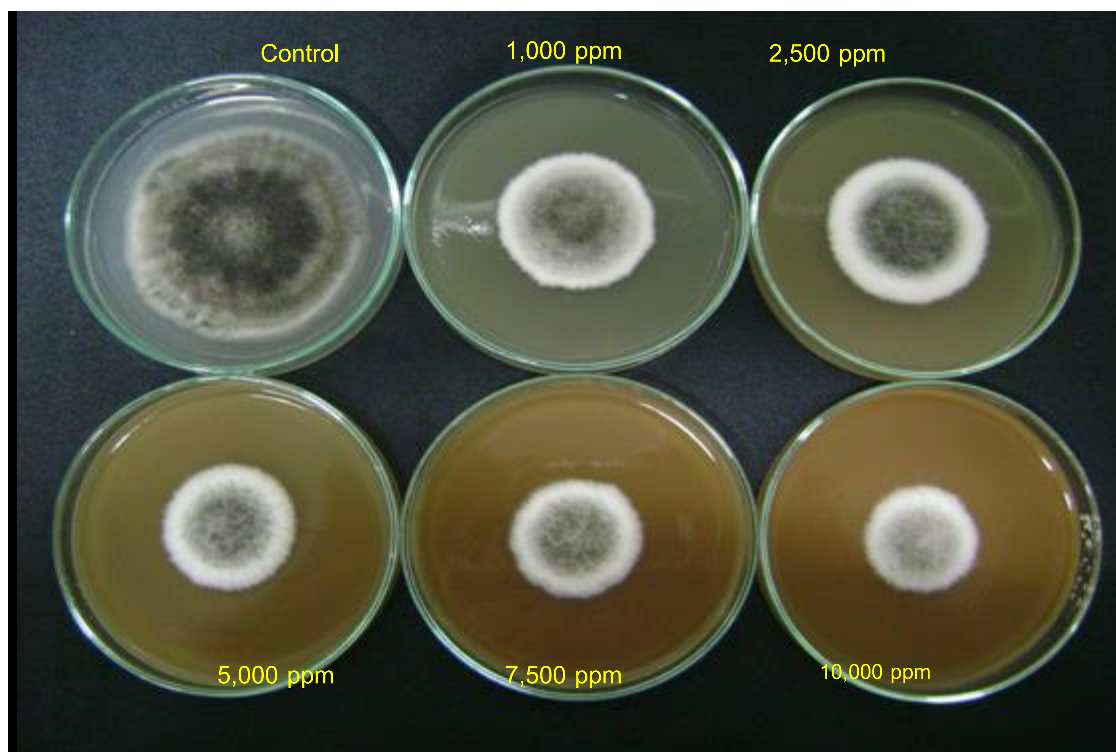

Figure 4. Effect of *Sorghum bicolor* crude extract on the mycelial growth of *Alternaria* sp. at different concentrations (Control treatment, 1,000 2,500 5,000 7,500 and 10,000 ppm).

Table 2. Efficacy of different concentration of some medicinal plants crude extracts on spore germination inhibition of *Alternaria* spp.

Medicinal plant crude extracts	Spore germination inhibition degree (%)				
	1,000 ppm	2,500 ppm	5,000 ppm	7,500 ppm	10,000 ppm
Curcuma xanthorrhiza	90[b]	100[a]	100[a]	100[a]	100[a]
Curcuma aromatica	100[a]	100[a]	100[a]	100[a]	100[a]
Kaempferia parviflora	0[d]	0[b]	25[b]	30[b]	55[b]
Sygygium aromaticum	100[a]	100[a]	100[a]	100[a]	100[a]
Origanum vulgare	100[a]	100[a]	100[a]	100[a]	100[a]
Synedrella nodiflora	90[b]	100[a]	100[a]	100[a]	100[a]
Sorghum bicolor	80[c]	100[a]	100[a]	100[a]	100[a]
Rosmarinus officinalis	85[b]	100[a]	100[a]	100[a]	100[a]
Piper longum	85[b]	100[a]	100[a]	100[a]	100[a]
Eupatrium odoratum	0[d]	0[b]	0[c]	10[c]	20[c]
C. V. (%)	9.24	3.65	6.54	8.78	6.85

In each column, mean followed by a common letter are not significantly different at the 5% level by DMRT.

rosmarinic acid from the leaves of *R. officinalis* (Frankel et al., 1996) and curcumin from the rhizome of *C. xanthorrhiza* (Husein et al., 2009).

The three crude extracts, *K. parviflora, P. longum* and *E. odoratum* at 10,000 ppm had the inhibition on mycelial growth at 75, 75 and 71%, respectively (Table 1). This result agrees with the *K. parviflora*, *P. longum* and *E. odoratum* crude extracts inhibited the growth of some pathogens; *K. parviflora* crude extracts for *B. cereus* and *S. aureus* control (Butkhup and Samappito, 2011) *P. longum* crude extracts for *Bacillus subtilis* control (Kumar et al., 2010) and *E. odoratum* showed the growth inhibition of two pathogen, namely *B. cereus* and *Aspergillus niger* (Owalabi et al., 2010). The phytochemical compounds in each species showed the inhibition on growth of pathogen; methoxyflavone from the rhizome of *K. parviflora* (Kummee et al., 2008), pipenonaline from the fruit of *P. longum* (Lee et al., 2001) and alpha-pinene and beta-pinene from the leaves *of E. odoratum* (Owalabi et al., 2010).

S. nodiflora and *S. bicolor* crude extracts at 10,000 ppm gave the highest inhibition of mycelail growth at 56 and 58%, respectively (Table 1). *S. nodiflora* and *S. bicolor* crude extracts at 2,500 ppm showed the highest inhibition of spore germination at 100% (Table 2). This result agrees with the *S. nodiflora* crude extracts inhibited on growth in five pathogens, namely; *B. subtilis*, *S. aureus*, *E. coli, Candida albicans* and *Aspergillus flavus* (Bhogaonkar et al., 2010) and *S. bicolor* crude extracts inhibited on growth in three pathogens, namely; *Fusarium moniliforme, Curvularia lunata* and *A. flavus* (Seetharaman et al., 1997). The phytochemical compounds of *S. nodiflora* and *S. bicolor* crude extracts showed the inhibition on growth of some pathogens; alkaloids and flavanoids from the leaves of *S. nodiflora* (Bhogaonkar et al., 2010) and saponins and tannins from the leaves of *S. bicolor* (Soetan et al., 2006).

In total, this study indicated that the ten crude extracts can be used for *Alternaria* spp. control and three plant can be used for crude extracts of dirty panicle control. The crude extracts of three species, namely, *C. aromatica, S. aromaticum* and *O. vulgare* showed 100% inhibition on mycelial growth and spore germination of *Alternaria* spp. at all concentrations (1,000 to 10,000 ppm).

Conclusion

C. aromatica, S. aromaticum and *O. vulgare* crude extracts at 1,000 ppm, the *R. officinalis* crude extracts at 5,000 ppm and the *C. xanthorrhiza* crude extracts at 10,000 ppm showed the highest inhibition of mycelial growth at 100%, whereas the *S. nodiflora* and *S. bicolor* crude extracts at 10,000 ppm had the inhibition at 56 and 58%, respectively. For inhibition of spore germination, the three plant crude extracts, *C. aromatica, S. aromaticum* and *O. vulgare* at 1,000 ppm and the *C. xanthorrhiza, Synedrella nodiflora, S. bicolor, R. officinalis* and *P. longum* crude extracts at 2,500 ppm reached the highest inhibition of spore germination at 100%, whereas the *E. odoratum* and *K. parviflora* crude extracts at 10,000 ppm had the inhibition at 20 and 55%, respectively.

Conflict of Interests

The author has not declared any conflict of interests.

ACKNOWLEDGEMENTS

The author thanks the Faculty of Science and Technology, Phranakhon Si Ayutthaya Rajabhat University,

which supported this research and the Office of Higher Education Commission (OHEC) under Identity-Based Research Group (CHE-RES-IG) in 2010-2011, and Office of Higher Education Commission (OHEC) under Research Group of Good Agricultural Practices Procedures on Rice Production in 2011, which supported this research.

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Vol. 10(15), pp. 202-208, 17 April, 2016
DOI: 10.5897/JMPR2016.6052
Article Number: B8613E558108
ISSN 1996-0875
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http://www.academicjournals.org/JMPR

Journal of Medicinal Plants Research

Full Length Research Paper

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

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Received 19 January, 2015; Accepted 21 March, 2016

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 (NaOCl) 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	
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	0.000
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%:

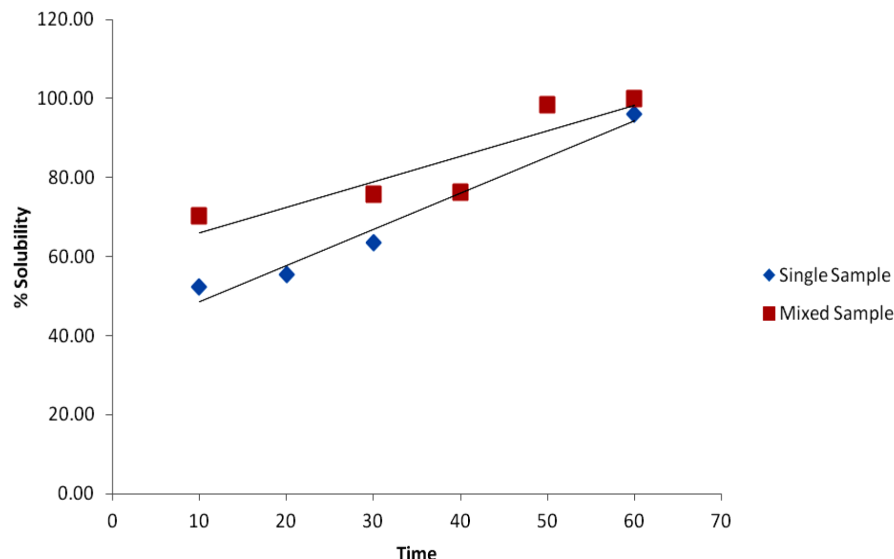

Figure 2. Solubility test graphic of single alkaloid and mixed group alkaloids solution in Carbonate buffer with pH 10.

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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