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Study on Activity of Cocoa Ethanolic Extract Against *Shigella dysenteriae*

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Abstract

Shigella dysenteriae is a gastrointestinal pathogen which shows resistance to antibiotics. A study has been conducted to investigate alternative antibacterial agents, due to the emerging resistance of *S. dysenteriae* to ciprofloxacin and other antibiotic classes. In this study, antibacterial properties of cocoa ethanolic extract (CEE) and its impact on growth and morphology of *S. dysenteriae* were evaluated. The effect of CEE on bacterial growth was assayed by using agar-well diffusion method and by observing morphological changes of bacterial cells through the use of scanning electron microscopy (SEM). Furthermore, CEE was also applied orally to mice infected with *S. dysenteriae*. The intestinal fluids was cultured in selective medium to evaluate growth of *S. dysenteriae* colonies. This study demonstrated that CEE at concentrations of 15.6 mg/mL inhibited *S. dysenteriae* growth, and at concentrations of 500 mg/mL and 1,000 mg/mL exhibited equal activity to 6.5 μg/mL of ciprofloxacin. SEM showed that *S. dysenteriae* cells had formed filaments, indicating that CEE caused cellular stress to *S. dysenteriae*. In *in vivo* assay, CEE showed suppression of *S. dysenteriae* colony in the mice intestine. This research suggests that CEE could potentially be used as antibacterial agent againsts *S. dysenteriae*.

Keywords: antibacterial, dysentery, cocoa, active compound, antibiotic

INTRODUCTION

The World Health Organization (WHO) reported that diarrhea killed 1.9 million people each year, including 760,000 children under five-year of age. In fact, the highest incidence of diarrhea was among infants 6-11 months of age, who typically suffered 4.5 diarrhea incidents per year (Walker *et al.*, 2012). Among other diarrheal pathogens, *Shigella dysenteriae* produces the most severe disease and complications, because its Type-1 species releases toxins.

Instead of vaccines, *Shigella* infection is treated by using antibiotics particularly from the class of β-lactams, quinolones or macrolides (Christopher *et al.*, 2010). Among these, ciprofloxacin from quionolone class is the first line of treatment, while pivmecillinam, ceftriaxone and azithromycin are the most used second options (Christopher *et al.*, 2010; WHO, 2005). However, Shiferaw *et al.* (2012) has reported resistance of *Shigella* towards ampicillin, streptomycin, trimethoprim-sulfamethoxazole, sulfamethoxazole-sulfisoxazole, and tetracycline, and decreasing susceptibility against ciprofloxacin and nalidixic acid. Alternative antimicrobial agents for *Shigella* has been investigated from plant extract, for instance garlic and allicin (Andualem, 2013; Gull *et al.*, 2012). Native medicinal plants, such as *Channa striatus* and *Picralima nitida*

also demonstrated promising antimicrobial activity (Haniffa *et al.*, 2013; Kouitcheu *et al.*, 2013).

Cocoa bean (*Theobroma cacao* L.) was anciently utilized as a medicine, despite the current use for confectioneries. It is a valuable commodity for the Aztecs due to its nutrition, energetic, and aphrodisiac benefits. Cocoa beans were used to treat stomach and intestinal pains including dysenteriae as described in "Florentine Codex," a record of indigenous Aztec medical practices (Dillinger *et al.*, 2000; Lippi, 2009). In recent times, health properties of cocoa beans have been extensively investigated, particularly for the prevention of cardiovascular diseases. We have found antibacterial properties against *Escherichia coli, Staphylococcus aureus* and *Staphylococcus epidermidis*, as well as immunomodulatory and anticancer properties (Sari *et al.*, 2015; Sari *et al.*, 2014; Sari *et al.*, 2016). This study investigated the effects of cocoa ethanolic extract (CEE) on *Shigella dysenteriae* employing *in vitro* assay scanning electron microscopy (SEM) and *in vivo* testing in mice.

MATERIALS AND METHODS

Six kilograms of cocoa beans dried under the sun without prior fermentation, were ground into five-mesh particle size. The paste was pressed at 80–90^{O}C to remove cocoa butter, and was soaked overnight in n-hexane for thorough fat removal. The defatted mass was then filtered to remove liquid and the solid fraction was soaked in ethanol overnight. Finally, the ethanol extract was concentrated and spray dried to produce powder. Total polyphenol content (TPC) was measured using Folin-Ciocalteau method and was expressed as catechin equivalent and gallic acid equivalent.

Assessment of antibacterial activity was carried out by performing agar-well diffusion assay (Rojas *et al.*, 2006). The CEE solution was prepared by diluting in sterile distilled water to create concentrations of 1,000; 500; 250; 250; 125; 62.5; 31.3; 15.6 and 7.8 mg/mL. Positive control suspension was prepared from ciprofloxacin 6.5 x 10^{-3} mg/mL, while negative control was prepared from sterile distilled water. Culture of *S. dysenteriae* (1 x 10^{8} CFU/mL) were streaked on plates containing Mueller Hinton agar. The agar was perforated to form well (diameter 10 mm, 4 wells/plate) and the CEE solutions were poured into each well. Plates were incubated at 37^{O}C for 24 hours, and development of clear area surrounding each well was observed. The inhibition zone was visible as a clear area surrounding each well. The inhibition zone diameter (IZD) was the diameter of the visible clear zone subtracted from the well diameter. The relative inhibition zone diameter (RIZD) of CEE was $RIZD_{CEE}(\%) = [(IZD_{CEE} - IZD_{Negative\ control})/IZD_{positive\ control}] \times 100\%$ (Rojas *et al.*, 2006). The assay was done triplicate.

To observe morphological changes of the bacteria cells, 10 µL of *S. dysenteriae* culture was added to vials containing CEE at concentrations of 7.8 mg/mL, 15.6 mg/mL, and 31.2 mg/mL, and was incubated at 35^{O}C for 20 hours. The bacterial cells were fixated in glutaraldehyde 2% at 4^{O}C, and were immersed in osmic acid 1% at 4^{O}C and were subsequently dehydrated with ethanol. Preservation of the cells were made by using absolute amyl acetate solution. The cells then were dried on glass prior to gold glazing and microspic observation process.

Male Swiss-Webster mice, aged 2–3 months with average weight of 25–30 g underwent acclimatization for one week prior to treatment. Mice were infected through intraperitoenal

route with 1.5 mL suspension of *S. dysenteriae* culture (10^5 CFU/mL). Twenty four hours post infection, mice orally received CEE (0.65; 1.3; and 2.6 mg/mL), ciprofloxacin (1.3 mg/mL), or sterile water (1mL). Application was done twice a day for seven consecutive days. On the 8th day, mice were sacrificed and the intestine wash was cultured in SS agar. After incubation for 20 days, colonies of *S. dysenteriae* shown in agar was counted. This trial has acquired ethical approval from committee from Faculty of Medicine, Jember University.

Experiment was done in triplicates and statistical analysis of the data used t-test, Kruskal-Wallis analysis, and Mann-Whitney test for post-hoc analysis.

RESULTS AND DISCUSSION

Secondary metabolites of plants have attracted studies on their antibacterial potency. Among these, polyphenol is considered as the most potent antibacterial agent, since its function in plant defense against predators. Polyphenol content in cocoa beans is higher than in fruits, such as pomegranates, cranberries, blueberries and acai berries (Crozier *et al.*, 2011). Compared to other beverages, cocoa provides more phenolic content per serving than coffee, tea and wine. This studies showed that total phenolics (gallic acid equivalent) and flavonoids (epicatechin equivalents) in cocoa per serving were about two to three fold greater than that of black tea, green tea and red wine (Lee *et al.*, 2003). Polyphenol accumulates in the nonfat solids of cocoa bean, thus the concentration is higher in chocolate products with less added ingredients. Chocolate, for instance, has less polyphenol content than cocoa powder and cocoa liquor (Gu *et al.*, 2006).

Cocoa polyphenol is composed largely of monomers, with a smaller number of trimers and tetramers (Misnawi *et al.*, 2002). Extract of cocoa bean may contain tannin, (-)-epicatechin and (+)-catechin, and some of phenolic acids, flavonoids (quercetin, kaempferol, naringenin, myricetin, luteolin, and apigenin) and alkaloids (caffeine, theophylline and theobromine) (Elwers *et al.*, 2009; Ortega *et al.*, 2008; Sánchez Rabaneda *et al.*, 2003). The alkaloids are distributed in various parts of cocoa (bean, pulp, and shell), where the amount is gradually decreasing during fermentation (Brunetto *et al.*, 2007). Proportion between theobromine and caffeine content is different in each cocoa variety, whereas Criollo bean has lower theobromine/caffeine concentration, while Forastero bean has high theobromine/caffeine concentration (Brunetto *et al.*, 2007).

Fermentation of cocoa beans may reduce bitterness and astringency caused by polyphenol, hence its concentration. Misnawi *et al.* (2003) demonstrated that fermentation not only reduced polyphenol concentration but also altered the composition of anthocyanin, epicatechin and catechin. Alkalization step also degrades polyphenols in cocoa. By mixing cocoa powder with an alkaline solution (a practice known as "Dutch process"), one will produce a dark, yet mild tasting, cocoa powder. Degree of alkalization may be determined based on cocoa appearance, as light alkalization converts natural cocoa powder colors from light brown to red/brown and heavy alkalization results in dark red/black colors (Miller *et al.*, 2008).

In this experiment, cocoa ethanolic extract (CEE) was produced from unfermented, unroasted, natural cocoa powder. The CEE contained TPC 140 mg/g (+)-catechin equivalent and 397 mg/g (gallic acid equivalent),

amounts that are almost 10-fold greater than in natural roasted cocoa powder (TPC 40.59–63.20 mg/g) and 40-fold greater than in heavily alkalized powder (TPC 9.54 mg/g) (Miller *et al.*, 2008).

In *in vitro* assay, the inhibition zone diameter (IZD) of CEE was ranging from 0.4 to 11.1 mm. After applying regression on IZD towards CEE concentration, a logarithmic response was observed ($R^2 = 0.9675$). This experiment indicated that antibacterial activity was concentration-dependent, however the concentration higher than 500 mg/mL may bring less significant effect. A comparable effect was shown from CEE at 500 mg/mL, 100 mg/mL and ciprofloxacin 6.5×10^{-3} mg/mL ($P>0.05$). On the other hand, there was no significant difference between the effect of CEE at 7.8 mg/mL and sterile water, indicating that in this concentration CEE was not effective as an antibacterial agent. RIZD, the ratio of IZD between the extract and positive control, is expressing the antimicrobial activity of CEE relative to ciprofloxacin (Rojas *et al.*, 2006). A strong antibacterial activity was shown by CEE at concentrations between 500–1,000 mg/mL, which featured a RIZD of 97–106% (Table 1).

The polyphenol in CEE may perform bacteriostatic and bactericidal activities through several actions, including disturbance of bacterial cell metabolism, inhibition of cell wall synthesis, disruption of cell membrane permeability, and interference with nucleic acid and gene expression (Cui *et al.*, 2012; Denyer & Maillard, 2002). Tannin works primarily on the bacterial outer membrane, since its protein-precipitation ability may interrupt membrane continuity (Liu *et al.*, 2013). Research on green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCG), demonstrated destructive activity toward *E. coli*, by inducing leakage and providing gateways to the cell interior (Cui *et al.*, 2012). In addition to polyphenols, antibacterial activity of cocoa extract may be due to the presence of methylxanthines. Caffeine and derivate of theophylline were reported to decrease growth of gram-negative bacteria (Al-Janabi, 2011). Particularly, caffeine stimulates bacteria cell to undergo morphological changes and lysis (Dash & Gummadi, 2008).

Susceptibility of gram-negative or gram-positive bacteria is different, depending on characteristics of the cell wall. Gram-negative cells are equipped with a lipopolysaccharide

Table 1. Inhibition zone diameter (IZD) of CEE in various concentrations (mg/mL)

Treatment	Concentration (mg/mL)	IZD*, mm	RIZD*, %
CEE	7.8	0.0 ± 0.0^{a}	0
	15.6	0.4 ± 0.3^{c}	3
	31.2	2.3 ± 0.4^{d}	22
	62.5	5.1 ± 0.2^{e}	48
	125	8.1 ± 0.6^{f}	77
	250	9.0 ± 0.3^{g}	86
	500	10.1 ± 0.6^{b}	97
	1,000	11.1 ± 0.3^{b}	106
Ciprofloxacin	6.5×10^{-3}	10.5 ± 0.5^{b}	100
Sterile water		0 ± 0^{a}	0

Notes: Data are means of three replications. Data in the same column followed by the same letter are not significantly different based on Mann-Whitney post hoc ($\alpha = 0.05$).

outer membrane which determines virulence and resistance. Studies on tea (+)-catechin reported low bactericidal activity against gram-negative bacteria due to the negative charge of lipopolysaccharides (Kajiya *et al.*, 2004). Nevertheless, the contact between proanthocyanidins and lipopolysaccharides may disrupt cellular homeostasis (Johnson *et al.*, 2008).

SEM observation found that the *S. dysenteriae* cell in sterile water (negative control) was measured 2.04 µm in length. After CEE exposure for 20 hours, the bacterial cells extended and formed filaments. Filamentation began at the lowest concentration of CEE, 7.8 mg/mL (4.89 µm), and continued at concentrations of 15.6 mg/mL (6.39 µm) and 31.2 mg/mL (10.61 µm). The extended length of the cells indicates that the bacteria is receiving stress factors. Bacterial stress stimuli may include an unfavorable environment, nutritional imbalance, overpopulation, host immunity, and presence of toxins and antibiotics (Justice *et al.*, 2008; Laureti *et al.*, 2013; Poole, 2012). In response to stimuli, the stress response system (SOS response) is activated. This response system leads to cell shape modification, biofilm formation, mutation, and shifted virulence (Poole, 2012). When a DNA damage occurs, bacteria will delay mitosis to prevent transfer of defective DNA to the new cells. This condition leaves cells extend without cleaving, thus manifest in form of filaments (Justice *et al.*, 2008).

In the in vivo assay, antibacterial potency was indicated from the ability of test sample to suppress the growth of the *S. dysenteriae* colony in the mice intestine. There were significant differences in number of *S. dysenteriae* colonies between sterile water and CEE treatments, except for CEE at the lowest dose (0.56 mg/mL). A similar effect was shown after application of CEE in equal dose with ciprofloxacin (1.3 mg/mL, $p>0.05$). CEE at the highest dose (2.6 mg/mL) had significantly reduced the colony number (Figure 1).

Interestingly, while in the in vitro assay the effective dose of CEE was $7x10^4$-fold from ciprofloxacin (CEE 500 mg/mL vs. ciprofloxacin 6.5 x 10^{-3} mg/mL), in the in vivo assay the equal dose between CEE and ciprofloxacin had produced comparable effects. Doubling the dose of CEE from that

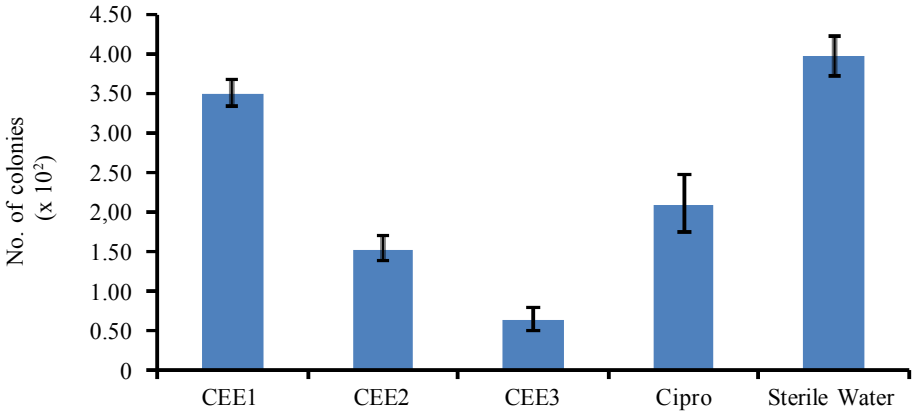

Figure 1. Number of *S. dysenteriae* colonies from mice intestine. CEE 1 = 0.56 mg/mL, CEE 2 = 1.3 mg/mL, CEE 3 = 2.6 mg/mL, and Cipro = ciprofloxacin 1.3 mg/mL as positive control. Data are means of three replications (bars indicated standard deviation of means)

of ciprofloxacin had significantly suppressed the colony growth. This indicates that CEE is not only attributed with antibacterial activity, but also with immuno-stimulation properties. Some studies revealed that cocoa polyphenols may induce changes composition and activity of immune cells as well as immune response. Ramiro-Puig *et al.* (2008) reported that a 10% cocoa diet had changed lymphosite composition in peyer's patches and mesenteric lymph nodes. In addition, Vázquez-Agell *et al.* (2011) demonstrated through a clinical study that cocoa consumption had acutely decreased the concentration of NF-kB, the signaling molecule mediating inflammation.

In an attempt of replacing conventional treatments, we could try to apply both CEE and an antibiotic, to utilize the benefits of both treatments. Similarly, studies have shown that a combination of (-)-epigallocatechin-3-gallate (EGCG) and cefotaxime induced permanent damage in an *E. coli* cells, though those results have not been achieved by EGCG alone (Cui *et al.*, 2012).

CONCLUSIONS

Antibacterial activity against *S. dysenteriae* was demonstrated by CEE, which had inhibited growth and triggered cellular stress. The effective dose in the in vivo assay was 1.3 mg/mL which equals to ciprofloxacin. This indicates that CEE may also stimulate immune system. This research suggests that CEE could potentially be used as a treatment of *S. dysenteriae* infection, though further investigation is necessary.

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