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In vitro antibacterial activity of cocoa ethanolic extract against *Escherichia coli*

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Abstract

Cocoa Ethanolic Extract (CEE) was observed for antibacterial activity against *Escherichia coli.* CEE at different concentrations in *E. coli*-seeded Mueller-Hinton agar medium, resulted zone of inhibition after 24 h incubation in 37°C. CEE concentrations at 15.6, 31.25, 62.5, 125, 250, 500 and 1,000 mg/ml, developed annular radius of inhibition 0.4, 1.0, 1.9, 3.3, 4.5, 5.3 and 6.31 mm respectively, while 8 µg/ml Ceftriaxone as positive control resulted zone of inhibition 6.48 mm. Minimum Inhibitory Concentration (MIC) to suppress *E. coli* growth was 835 mg/ml. Scanning Electron Microscopy (SEM) observation showed that CEE and Ceftriaxone caused bacteria cell elongation. CEE concentration above 15.6 mg/ml induced *E. coli* cells fragmentation suggesting permanent damage. This research finding clearly indicates that CEE could be an alternative to be used as antibacterial agent with respect to overcome the bacterial resistance.

Introduction

Escherichia coli (*E. coli*) member of enterobacteriaceae, is characterized as Gram-negative and rod-shaped bacteria. Some types are non-pathogenic and commonly found in intestine tract; however other groups are pathogenic and responsible for various harmful diseases, from diarrhea to urinary tract infection and meningitis (de Sousa, 2008). *E. coli* occurs largely in tropical region, but its outbreaks have been reported in subtropical region (Escobar-Páramo *et al*., 2004). Several outbreaks are reported in Japan (1966-2009); in Denmark (2006) the outbreak was found from salad dressing; in United States (2009) from prepackaged cookie dough, and lately in Germany (2011) was suggested from sprouts (Pakalniskiene *et al*., 2009; Frank *et al*., 2011; Konishi *et al.*, 2011; Neil *et al.*, 2012). Evolution of *E. coli* resulted persistent strains that induce recurrent diseases (Sánchez *et al*., 2009; Skjøt-Rasmussen *et al*., 2011).

Treatments of *E. coli* infectious diseases primarily aim to minimize symptoms and to kill bacteria through antibiotics administration. Antibiotic agents to treat *E. coli* comprise classes of beta-lactams (penicillin, cephalosporine including ceftriaxone), aminoglycoside and fosfomycin. Susceptibility of *E. coli* is considerably high towards ertapenem, imipenem, amikacin, cefepime, cefotaxime, cefoxitin, ceftazidime, ceftriaxone, piperacillin-tazobactam and fosfomycin (Falagas *et al*., 2010; Hoban *et al.*, 2010). However, Vieira *et al*. (2011) observed prevalence's of resistance to ampicillin, aminoglycosides, third-generation cephalosporins, and fluoroquinolones, thus suggesting that alternative antibacteria agents are required.

Attempts to discover non-conventional antimicrobial agents with high effectiveness and lower toxicity, have put great concern to plant extracts. Phytochemical compounds have been extensively studied to perform antibacterial and antifungal activities. Extract of grape, turmeric and tea leaves have been demonstrated their antibacterial potency against various human pathogens (Sebai *et al*., 2010; Zhang *et al*., 2010; Cui *et al*., 2012; Kawamoto *et al*., 2012). Cocoa bean is one of possible source of antibacterial agent, and had been utilized by ancient Aztecs community for treatment of intestinal ailments (Dillinger *et al*., 2000). Cocoa bean is also abundant deposit of polyphenols. Total polyphenol content of cocoa is significantly higher than acai berry, blueberry, cranberry and pomegranate, and consequently antioxidant activity is much higher than others (Crozier *et al.*, 2011).

This study aimed to observe activities of Cocoa Ethanolic Extract (CEE) towards *E. coli*. Experiments used several CEEs concentrations and annular radius of inhibition was observed to elicit potent antibacterial activity with regard to Ceftriaxone. Minimum Inhibitory Concentration (MIC) was determined to find least CEE concentration performing effective inhibition. Eventually, morphological changes in

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bacteria cell due to presence of CEE was assessed by using Scanning Electron Microscopy (SEM).

Materials and Methods

Materials

Cocoa beans were obtained from Research Station of Indonesian Coffee and Cocoa Research Institute. Bacteria isolate was from Laboratory of Microbiology, Faculty of Medicine, Jember University.

Preparation of cocoa ethanolic extract

Six kilograms of unfermented cocoa bean (moisture content 7%) was ground into particle size of 5 mesh and subsequently hot-pressed at 80-90°C to remove most of cocoa butter. Pressed cocoa cake was then soaked in *n*-hexane (1kg cocoa cake required 2l *n*-hexane). After 24 h of immersion, its liquor was filtered through colorless fabric and excess liquid was pressed out from solid cocoa powder. This treatment was aimed to remove remaining fat from cocoa powder.

Fat-free cocoa powder was immersed in 90% ethanol with volume (ml) three times of cocoa powder weight (g). After 24 h, solid fraction was removed while liquid fraction was transferred into rotary evaporator chamber. Evaporation was conducted at temperature of 60°C for 3.5 h. Ethanolic concentrate was subsequently vaccum dried at 40-60°C to produce solid particles, considered as Cocoa Ethanolic Extract (CEE). Analyte was prepared by diluting 2 g of CEE in 2 ml hot sterile distilled water. After vigorous shaking, obtained solution was gradually diluted with sterile distilled water to obtain concentration of 1000, 500, 250, 250, 125, 62.5, 31.25, 15.6 and 7.8 mg/ml.

Determination of total polyphenol content

The Total Polyphenol Content (TPC) was spectrophotometrically measured as described by Anesini *et al*. (2008) with slight modification. Briefly, 250 mg of CEE powder was diluted in 50 ml acetone 80%. After sonication for 30 min, 1 ml filtered solution was transferred to tubes then added with 70 ml distilled water and 5 ml 2 N Folin-Ciocalteu's reagent and allowed to stand at room temperature for 2 minutes. Afterwards, 15 ml of saturated Na2CO3 solution was added and tubes were allowed to stand at room temperature for 2 h. Absorbance at 765 nm was measured against acetone. Standard curve was produced by using (+)-catechin at concentration range 0-900 mg/L (R^2 = 0.9922). TPC was stated as (+)-catechin equivalent.

Preparation of E. coli inoculum

Isolate of E. coli obtained from Faculty of Medicine, Jember University (East Java) was pre-cultured in nutrient broth overnight at 37°C. Incubated inoculum was adjusted with Mc.Farland reagent (1 x 10^8 CFU/ml) which is mixture of 9.95 ml 1% sulphuric acid solution and 0.05 ml 1% barium chloride solution. Positive control suspension was prepared by gradually diluting Ceftriaxone powder with sterile distilled water to reach concentration of 8 µg/ml.

Antibacterial activity observation

Two grams of Mueller-Hinton agar powder was added with sterile distilled water in an Erlenmeyer flask, boiled and autoclaved at 121°C for 30 min before being poured into plates and incubated at 37°C for 24 h. Spread plate method was used to introduce *E. coli* in Mueller-Hinton medium. Sterile swab was dipped into inoculum and stroked on Mueller-Hinton plates. *E. coli*-seeded plates were perforated (4 wells per plate) and CEE solution was poured into well. After 24 h incubation at 37°C, zone of inhibition was identified from clear area surrounded each well. Annular radius was measured by subtracting outer radius of well and radius of zone of inhibition.

Results and Discussion

Antibacterial activity of cocoa ethanolic extract

Antibacterial potency of test substance is indicated from bacteria growth prevention. On bacteria-seeded agar medium, expansion of bacteria colonies will be blocked by test substance. After incubation, a zone of inhibition is identified from circular transparent area that is free from bacteria colonies. Zone of inhibition was resulted by Ceftriaxone in E. coli-seeded plates while it was not developed under presence of sterile distilled water. This result confirmed the role of ceftriaxone as positive control and sterile distilled water as negative control. Ceftriaxone, inspite of resistance reports, is antibiotic agent recommended for treatment of *E. coli* infection (Hoban *et al.*, 2010; Tan *et al.*, 2010; Park *et al*., 2012).

Experiment using CEE solution at 15.6, 31.25, 62.5, 125, 250, 500 and 1,000 mg/ml resulted zone of inhibition on *E. coli*-seeded plates, where annular radius of inhibition was expanding on dose-dependent manner (Figure 1). CEE concentration at 7.8 mg/ml did not perform inhibition. Antibacterial potency was much lower to that of ceftriaxone. Bacteria inhibition required ceftriaxone at very low concentration (8

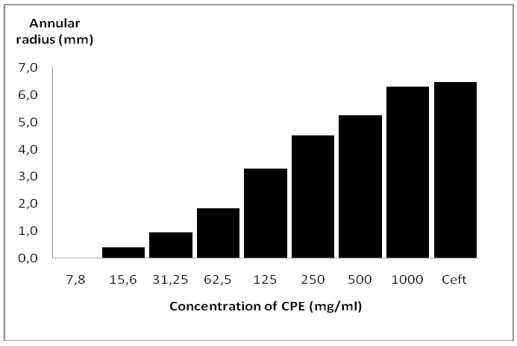

Figure 1. Annular radius of zone of inhibition (mm) developed by various CEE concentrations in *E. coli*-seeded plates

µg/ml), while it required CEE more than 15.6 mg/ml (1.95 x 10^3 fold greater than ceftriaxone). Annual radius developed by ceftriaxone (8 µg/ml) was comparable to 1,000 mg/ml CEE.

Minimum Inhibitory Concentration (MIC) of antibacterial agent refers to least concentration that affects bacteria resistance and surpresses colonies growth. Calculation of MIC requires annular radius measurement, which is acquired from difference of zone-well outer radius. MIC is considered from concentration that construct zone of inhibition with annular radius ≥6 mm (Elgayyar *et al*., 2001). Following test of normality (Kolmogorov-Smirnov test, p = 1.00) and significance (p = 0), calculation of MIC requires logarithm of CEE concentrations towards annular radius numbers. Linear regression resulted equation of Y = -3.415 + 3.200X, where X is independent variable acquired from CEE concentration and Y is dependent variable denotes annular radius of zone of inhibition. By entering annular radius (Y) = 6 into the above equation, it was found that the breakpoint value of CEE concentration (X) = 835.21 mg/ml. This value indicates onset of CEE concentration that performs bacterial inhibitory. The MIC of CEE was concluded at above 835 mg/ml.

The use of ethanol as extracting solvent was intended to obtain polyphenolic compounds from cocoa fatty matrix. Evaporation removed most of ethanol and concentrate extract into powder with of total polyphenol content ((+)-catechin equivalent) was 21.9%. Studies reported antibacterial activity of various polyphenols and their derivates. Mechanism of bacteriostatic and bactericidal activities was carried out in several mode of actions, including disturbance on bacterial cell metabolism, inhibition on cell wall synthesis, disruption on cell membrane permeability, and interference on nucleic acid and gene expression (Percival *et al*., 2006; Cui *et al*., 2012). Green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCG), demonstrated destructive activity towards *E. coli*, by inducing leakage and debilitating cell wall particles. Although this condition was reported reversible, EGCG resulted synergistic effect with cefotaxime by inducing permanent cellular destruction (Cui *et al.*, 2012). Cocoa polyphenol itself is reported to reduce biofilm formation by *S. mutans* and *S. sanguinis*, and inhibited acid production by *S. mutans* (Percival *et al.*, 2006).

Polyphenols were also reported to weaken toxicity effect on Gram-negative bacteria by mediating cell response under lipopolysaccharide induction. Resveratrol of red grape found to attenuate lipopolysaccharide effect following controlled induction in target cells (Sebai *et al*., 2010; Zhang *et al*., 2010). Major polyphenol component of turmeric, curcumin, was reported to modulate inflammatory response of brain cells due to lipopolisaccharide-induction, while ameliorating memory impairment and preventing adverse damage on central nervous system (Kawamoto *et al*., 2012).

Morphological changes of E. coli cell induced with cocoa ethanolic extract

Observation by using Scanning Electron Microscopy (SEM) showed the size of *E. coli* cell in sterile water was 2.000 µm. After 24 h exposure in 8 µg/ml ceftriaxone, *E. coli* elongated 1.142 µm from its original size. As elicited from agar well diffusion test, *E. coli* growth was inhibited by respective concentration of ceftriaxone, however it was indicated that *E. coli* attempted to survive under antibiotic exposure. Morphological plasticity is a responsive method built by bacteria toward environmental changes. Typically, when sensing insecure circumstances that lead to DNA damage, bacteria will extend their cell to form long strand (filament). By the time DNA is being repaired, filamentation occurs to avoid cell division and to prevent replication of impaired genes to sister cells. Filamentous bacteria exhibits survival to kill of host immune system, predators and antibiotics (Justice *et al.*, 2006).

DNA restoration and filamentation is part of SOS response, an emergency action induced by threatening conditions. Protein playing key role in SOS response is recA which induces gene transcriptions. In normal condition gene transcription is repressed by lexA, and its innactivation at SOS response allows recA operation (Michel, 2005). Complementing the recA and lexA functions, there is sulA that inhibits cell division, thus be responsible for filamentation. Study on Uropathogenic *Escherichia coli* (UPEC), villain of urinary tract infection, showed innactivation of sulA prevented filamentation. SulA is an essential

in UPEC pathogenicity, since filamentous UPEC has ability to evade from phagocytosis killing and to expand infection (Justice *et al*., 2006).

β-lactam antibiotics block bacteria cell wall synthesis, through binding of Penicillin-Binding-Protein (PBP), a vital protein required for peptidoglycan cross-linking. Binding of β-lactam ring to PBP type 3 (PBP-3) impedes septum formation, thus cell growth would never devide and remains filamentous, where its length is correlated to antibiotic concentration (Buijs *et al.*, 2008). Yao *et al.* (2012) suggested that cell elongation by the presence of β-lactam antibiotics, should continue to swell and inactivate prior to lysis death. Hanberger *et al.* (1990) reported that filamentous bacteria were commonly found under presence of β-lactam antibiotics (aztreonam, ceftazidime, cefuroxime, imipenem and piperacillin). Moreover, when antibiotics were below minimum inhibitory concentration, filamentous cell was viable and recover its function following diminishing effect of antibiotics.

Elongation was also exhibited by *E. coli* cells under exposure of CEE for 24 h. At lowest concentration of 7.8 mg/ml, *E. coli* elongated to 4.286 μm. Cells extension was following CEE concentration, showed by extension to 6.859 μm at 15.6 mg/ml and 6.286 μm at 31.25 mg/ml, however at CEE concentration of 15.6 and 31.25 mg/ml, their elongation lead to broken cells, indicating permanent disruption and failure to maintain filamentation.

This study confirmed that polyphenol causes *E. coli* cell elongation as found by Hemaiswarya *et al.* (2011); Cui *et al.* (2012); Wojnicz *et al.* (2012); Liu *et al.* (2013). It is proposed that polyphenols in CEE suppress polymerization of ftsZ, a key protein roles in septum formation. Inhibition of ftsZ activity restrains cell division, thus preserves filamentous form. Among numerous polyphenolic substances, viriditoxin, dichamanetin, and 2'''-hydroxy-5-benzylisovarionol-B had been investigated for their impact on ftsZ, suggesting their ability to hinder bacteria propagation (Foss *et al.*, 2011).

Eventhough promising antibacterial activity is being demonstrated, CEE needs further investigation, such as 1) detailed effect on bacteria cell wall. This could be achieved through transmission electron microscopy (TEM) observation aimed to identify potential cell wall lysis induced by CEE. 2) Post-treatment condition, since some studies found reversibility of cell to its bacillary form following several hours after contact with antibacterial agent (Hanberger *et al.*, 1990; Cui *et al.*, 2012). This condition leads to restoration of growth, multiplication and virulence. 3) Interaction with conventional antibiotic is also should be taken into consideration, due to possible synergistic or antagonistic effect between natural and synthetic substances.

Conclusion

In conclusion, CEE showed antibacterial activity by inhibiting growth of *E. coli* in agar diffusion method, whereas high concentration at 1,000 mg/ml performed comparability to ceftriaxone. Changes of bacteria cell morphology indicates that polyphenol in CEE was playing role as stress stimuli that induced damage in bacteria DNA. This research suggests the potency of cocoa extract as an alternative source to control *E. coli.*

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