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Use of Cocoa Ethanolic Extract for Treatment of *Staphylococcal* Infection in Rabbit-Skin Model

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Abstract

In septic condition, the skin normal flora *Staphylococcal* spp. may trigger local and sistemic skin infection. In this study antibacterial activity of cocoa ethanolic extract (CEE) against *Staphylococcus aureus* and *Staphylococcus epidermidis* infections was observed in vitro and in vivo. Ethanolic extract from unfermented cocoa beans was prepared as solution in the in vitro testing, while for in vivo testing the extract was prepared as cream. Agar well diffusion assay showed that CEE ranging from 7.8 mg/mL to 1000 mg/mL demonstrated inhibitory activity against growth of either *S. aureus* and *S. epidermidis*. Inhibitory activity of CEE was in concentration dependent manner, and was less potential than either cephalexin 4 x 10^{-3} mg/mL or cefotaxime 8 x 10^{-3} mg/mL. Linear regression of CEE concentration plotted against inhibition zone values had predicted the minimum inhibitory concentrations (MIC) of CEE towards *S. aureus* and *S. epidermidis* were at 341.9 mg/mL and 359.7 mg/mL, respectively. Topical application of cream containing CEE at several concentrations (2%, 4%, and 8%) demonstrated healing properties towards incision wound infected with *S. aureus* and *S. epidermidis* cultures in rabbit-skin model. CEE cream promoted wound contraction and higher recovery rate than of base cream (negative control) but lower than mupirocin 2% cream. In *S. aureus* and *S. epidermidis* infected wound models, CEE cream 8% improved wound recovery to 72.7% and 86.1% from original rates of 23.5% and 34.7% (base cream application). Catechin and procyanidis are suggested playing roles in alleviation of wound inflammation and stimulation of extracellular matrix accumulation, thus accelerate the wound healing process. This study proposes utilization of cocoa bean as source of active ingredient for skin care products.

Keywords: cocoa, polyphenol, *staphylococcus*, rabbit-skin model, wound

INTRODUCTION

Global burden studies in 2010 noticed that skin health management should earn global concern, since nonfatal diseases were initiated from skin (Hay *et al*., 2013). Skin is the habitat of microorganisms where commensal relationship is developed and there are conditions when normal skin residents shift into pathogenic state and induce local and systemic diseases (Dryden, 2009). *Staphylococcus aureus* and *Staphylococcus epidermidis* are parts of skin normal flora and regularly found in healthy human skin at any age (Oh *et al*., 2012; Otto, 2009). *Staphylococcus aureus* may generate local

infection in skin and soft tissue such as impetigo, folliculitis, boils, carbuncles and myositis. Systemic infection triggers toxic shock and scalded skin syndrome, whose symptoms are rash and desquamation on skin (Dryden, 2009). *Staphylococcus epidermidis* is iniator of most nosocomial infections which transmitted through indwelling medical devices (Otto, 2012). Its was estimated that United State of America spent about USD 2 billion for treatment of bloodstream infection due to *S. epidermidis* (Otto, 2009).

Staphylococci gained more attention since some strains showed decreasing susceptibility toward antibiotics. Ability to form biofilm has protected themselves from penetration of methycillin, thus lead to emergence of methycillin-resistant staphylococci (Singh *et al.*, 2010). Consequently, attempt to treat staphylococcal skin infection demands new antibacterial agents. Studies have observed antibacterial potency of several plant extracts, and reported efficacy of *Vitis vinifera* L. (Al-Habib *et al.*, 2010), *Turnera ulimifolia* L. (Coutinho *et al.*, 2009), and some indigenous plants extracts (Ali *et al.*, 2011; Luciano-Montalvo *et al.*, 2013; Naveed *et al*., 2013) to inhibit growth and virulence of *S. aureus* and *S. epidermidis*.

Cocoa (*Theobroma cacao* L.) is native plant of Amazon basin, which was utilized for ritual servings, currency and medicine (Dillinger *et al*., 2000; Lippi, 2009). The medicinal use is coming along with deposits of nutrients and phenolic compounds. New perspective expands the use of cocoa bean not only for confectioneries but also for functional foods. Recent consumers are motivated to eat chocolate and cocoa products, in order to obtain health benefit of cocoa which consist of macronutrients and micronutrients. Cocoa powder which has undergone fermentation, roasting, fat removal and grinding, is rich of carbohydrate, dietary fiber and protein, and is a good source of potassium, phosphorus and magnesium. Cocoa also provides catechin, a member of plant phenolic compound that is associated with various health impacts, from antioxidant to anticancer. The total polyphenol content in cocoa reaches 48.2 $\pm$ 2.2 mg/g. The phenolic fraction in that cocoa demonstrated inhibitory activity against gram negative bacteria, i.e. *Escherichia coli* and *Shigella dysentriae* (Crozier *et al*., 2011; Sari *et al*., 2014; Misnawi *et al.,* 2015). This study is aimed to investigate antibacterial potency of polyphenol-rich cocoa ethanolic extract against *S. aureus* and *S. epidermidis*. Bacteria growth inhibition was assessed *in vitro* through agar well diffusion assay, while its effect on infected wound was evaluated in rabbit-skin model.

MATERIALS AND METHODS

Cocoa ethanolic extract (CEE) was prepared by soaking cocoa powder (fat removed, unroasted, unfermented) in ethanol for overnight. Liquid was filtered and was concentrated using vacum evaporator resulted in powder of CEE. For *in vitro* assay, the CEE powder was gradually diluted in water to reach concentrations of 1000 mg/mL, 500 mg/mL, 250 mg/mL, 125 mg/mL, 62.5 mg/mL, 31.2 mg/mL, 15.6 mg/mL, and 7.8 mg/mL. Positive control was chosen based on drug of choice for *S. aurens* and *S. epidermis* which were cephalexin (4 x 10^{-3} mg/mL) and cefotaxime (8 x 10^{-3} mg/mL), respectively. For in vivo test, cream of CEE was prepared by homogenizing CEE powder with mixture of stearic acid, cerae albi, vaseline albi, triethanolamine, propilen glycol and distilled water. Cream may contain CEE at concentration 2%, 4% or 8%.

S. aureus and *S.epidermidis* cultures (10^8 CFU/mL) were seeded by swapping onto plated Mueller-Hinton agar medium. Sterile cork borer was used to create 4 wells on each plate and CEE solution at certain concentration was poured into well. Plates were incubated at 37^{O}C for 24 hours. Clear area developed around well indicated bacteria growth inhibition by test sample. Antibacterial activity was evaluated by measuring outer radius of inhibition zone subtracted by outer radius of well. Positive control for in vitro assay were first line antibiotics, cephalexin 4 x 10^{-3} mg/mL and cefotaxime 8 x 10^{-3} mg/mL, while negative control was ssterile distilled water. Minimum inhibitory concentration (MIC) is the concentration of CEE that resulted inhibitory zore $\geq$6 mm.

Ten albino rabbits aged 3 months (2.5–3 kg) were acclimatized for one week prior to treatment. Dorsal fur was clipped and dorsal skin was anesthetized by subcutaneous injection of one mL lidocain. Skin was incised 2.5 cm using sterile surgical blade, and was infected by intracutaneous injection of 0.5 mL *S. aureus* or *S. epidermidis* culture. Wound was covered with sterile gauze and adhesive bandage. After 24 h, test sample was applied on wound three times daily for the next seven days. Gauze was replaced with new ones after application. Development of wound was observed by including criteria of erythema, while wound opening was measured daily by using caliper. Wound recovery level was calculated based on closure wound length (CWL) as follow:

$$\text{Closure rate (\%)} = \frac{\text{CWL (h-1)} - \text{CWL (h-7)}}{\text{CWL (h-1)}}$$

Positive control for in vivo assay was mupirocin 2% cream, while negative control was base cream with no active ingredient. The trial had acquired ethical approval from Faculty of Medicine, University of Jember.

Experiment was done in triplicates and statistical analysis of the data employed t-test, analysis of variance and Mann-Whitney test for post-hoc analysis. The MIC values from in vitro trial were determined by employing linear regresion.

RESULTS AND DISCUSSION

CEE showed ability to inhibit growth of gram positive bacteria which are *S. aureus* and *S. dysenteriae* in Mueller-Hinton agar after 24 h incubation. Inhibitory activity towards *S. aureus* was much greater than that of *S. epidermidis* (t-test, $P < 0.05$) and this occured in concentration-dependent manner. CEE at concentration below 15.6 mg/mL was not effective to inhibit bacteria growth. There was no inhibition zone found under presence of CEE at 7.8 mg/mL where this result was similar to negative control (sterile distilled water). Compared to cephalexin and cefotaxime, CEE has lower antibacterial potency. Cephalexin (4 x 10^{-3} mg/mL) created 8.05 mm inhibition zone against *S. aureus*, which is greater than inhibition zone of CEE at 1000 mg/mL (7.75 mm). The same situation was demostrated by cefotaxime (8 x 10^{-3} mg/mL) against *S. epidermidis* that produced inhibition zone (12.50 mm) greater than CEE at 1000 mg/mL (7.7 mm).

In the in vitro trial annular radius of inhibition zone is representing antibacterial potency of test substance. Test substance that develops inhibition zone ≥6 mm is considered inducing bacteria susceptibility (Bell *et al.*, 2011). Minimum inhibitory concentration (MIC) of CEE is the lowest concentration that resulted inhibition zone ≥6 mm. Regression method showed that MIC of CEE against *S. aureus* and *S. epidermidis* were 341.9 mg/mL ($R^2 = 0.995$) and 359.7 mg/mL ($R^2 = 0.910$), respectively. This assay showed

Table 1. Inhibition zone of test substance in bacteria-seeded agar plate

Test sample	Annular radius of inhibition zone, mm	
	S. aureus	*S. epidermidis*
CEE 1000 mg/mL	15.5 ± 0.5	9.2 ± 0.3
CEE 500 mg/mL	9.6 ± 0.6	7.7 ± 0.8
CEE 250 mg/mL	2.3 ± 0.5	5.0 ± 01
CEE 125 mg/mL	11.0 ± 0.9	2.6 ± 0.7
CEE 62.5 mg/mL	4.1 ± 0.4	1.5 ± 0.0
CEE 31.2 mg/mL	12.7 ± 0.3	1.2 ± 3.5
CEE 15.6 mg/mL	6.5 ± 1.0	0.5 ± 0.1
CEE 7.8 mg/mL	0.0 ± 0.0	0.0 ± 0.0
Sterile distilled water	0.0 ± 0.0	0.0 ± 0.0
Cephalexin 4 x 10^{-3} mg/mL	16.1 ± 0.6	NA
Cefotaxime 8 x 10^{-3} mg/mL	NA	13 ± 0.8

Notes: Data are means of three replications; ± = standard deviation; NA = not applicable; * = data were not significantly different based on Mann-Whitney post-hoc test (a = 0.05).

that MIC against *S. aureus* was lower than against *S. epidermidis,* suggesting that *S. aureus* is more susceptible toward CEE.

CEE cream at several concentrations demonstrated ability to promote wound recovery on rabbit skin model as indicated from length of wound closure (Table 2). In general, CEE promoted recovery of *S. epidermidis* infected wound better than *S. aureus* infected wound (t-test, P <0.01). Wound closure of *S. aureus*-infected wound was originally 23.60% (base cream) and was promoted by topical application of CEE cream into 41.20% (CEE 2%); 57.20% (CEE 4%) and 72.80% (CEE 8%). Towards *S. epidermidis*-infected wound, CEE cream has successfully increased recovery level from 34.72% (base cream) to 50.56% (CEE 2%); 66.40% (CEE 4%) and 86.08% (CEE 8%). Compared to mupirocin 2% cream as positive control, ability of CEE to promote wound recovery was lower. At concentration 4% and 8%, CEE promoted recovery of *S. epidermidis*-infected wound better than *S. aureus*-infected wound (P <0,01). This result is in contrary to *in vitro* assay which indicates *S. aureus* was more susceptible against CEE rather than *S. epidermidis*.

Catechin may polymerize to form procyanidin (proanthocyanin) which consists of at least two binding catechins. Isomers of catechin exist in the form of (+)-catechin, (-)-catechin, (+)-epicatechin and (-)-epicatechin. Research by Misnawi *et al.* (2002) indicated degradation of polyphenol during cocoa fermentation, whereas 5-day fermentation reduces total polyphenol content as much as 58%. Furthermore, a process known as alkalization ('Dutch' process) aimed to increase color intensity of cocoa powder, reduces total phenol content more than 90% (Miller *et al*., 2008). In this experiment, we attempted to conserve phenolic compound in cocoa bean by avoiding fermentation and roasting process. Powdered ethanolic extract of cocoa bean provides concentrated phenols, with total phenol content was 14% (expressed as (+)-catechin equivalent) or 29.7 g (gallic acid equivalent) per 100 g powder. This amount is much higher than 100 g of natural cocoa powder that provides 6.32 g polyphenol (gallic acid equivalent) (Miller *et al.*, 2008).

Oral and topical administrations of cocoa, in the form of butter, dark chocolate or cocoa powder, have shown benefits to skin condition. During inflammatory stage,

Table 2. Length of wound closure with respect to bacteria infection

Treatment	Wound closure, cm			
	S. aureus infection		*S. epidermidis* infection	
	H-7	Closure, %	H-7	Closure, %
Base cream	1.9 ± 0.0	23	1.7 ± 0.3	31
CEE 2% cream	1.5 ± 0.1	41	1.2 ± 0.0	53
CEE 4% cream	1.1 ± 0.1	57	0.8 ± 0.1	68
CEE 8% cream	0.7 ± 0.0	73	0.3 ± 0.1	87
Mupirocin 2% cream	0.3 ± 0.0	89	0.1 ± 0.0	93

Notes: Data are means of three replications, ± = standard deviation; all treatments showed significant differences based on Mann-Whitney post-hoc test (a = 0.05).

immune response is also promoted by cocoa flavanols due to increasing blood flow to dermal area (Neukam *et al.*, 2007). Moreover, studies suggested cocoa flavonoids alleviate inflammation, by modulating immune cells activity and secretion of pro-inflammatory signaling molecules (García-Lafuente *et al.*, 2009). Wound contraction is enhanced by presence of tannin, thus enabling connectivity of dermal tissue. Li *et al*. (2011) reported that enhanced wound contraction after topical application of tannin with comparable result to erythromycin ointment.

Antibacterial activity of plant phenols has been attributed with its high affinity to bacterial cell wall (Johnson *et al.*, 2008; Matsumoto *et al*., 2012). Attachment of phenolic compound to bacterial cell causes membrane destabilization and induces cell leakage (Bernal *et al.*, 2010; Puupponen-Pimiä *et al.*, 2005). Penetration of phenolic compounds into bacterial cell may reach genetic material and disrupt nucleic acid synthesis, particularly by binding with topoisomerases (Bandele *et al.*, 2008; Suriyanarayanan *et al.,* 2013). Disruption of cell function occures when phenolic compounds interact with proteins and cytoplasmic consituents (Radulovic *et al*, 2013). Gram negative bacteria is more resistant than gram positive bacteria, due to its outer membrane providing barrier towards antimicrobial agent. However, gram positive bacteria is equipped with ability to form biofilm. Biofilm is not only protecting cells from antibacterial agents but also contributing to bacteria virulence (Hall-Stoodley *et al.*, 2004).

Rabbit skin infection model demonstrated wound infection triggered by *S. aureus* or *S. epidermidis*. Recovery of wound requires three stages which are inflammatory, proliferative and remodelling stages. Inflammatory stage is characterized with redness-swelling-heat-pain, indicating immune response to prevent further infection. Proliverative stage is attempted to repair damaged tissue, visually recognized as scar development. Formation of new epithel layer also occurs at this stage. The last remodelling stage requires longer time to resume skin function and appearance (Demidova-Rice *et al.*, 2012). Infection of *S. aureus* in wound is reported to inhibit formation of new epithelial layer. Schierle *et al.* (2009) reported that *S. aureus* develops slimy matrix in between wound gap, thus delaying progress of proliverative stage.

Proliferative stage was promoted by enhancement of skin regeneration. Study on *ex vivo* skin model indicates potency of cocoa polyphenol on generating skin extracellular matrix i.e. glycosaminoglycans and collagen type I, III and IV (Gasser *et al*., 2008). It was reported by Kapoor *et al.* (2004) that catechin gallate induces upregulation of vascular endothelial growth factor (VEGF), where VEGF is important in development in wound healing by stimulating accumulation and proliferation of

endothelial cells (Bao *et al.*, 2009). Interestingly, catechin maintains homeostasis of collagen production and prevents keloid formation due to overly accumulated collagen (Zhang *et al.*, 2006).

Phenolic compound is also promoting stability extracellular matrix, namely collagen, towards environmental and enzymatic degradation. The number of galloyl group in phenolic compound contributes improvement of thermal stability of collagen (Madhan *et al.*, 2007; Tang *et al.*, 2003). Towards enzymatic degradation, polyphenol protects proteolysis of collagen by Matrix-Metalloproteinase (MMP). MMP is enzyme that degrades extracellular matrix such as collagen and gelatin. In inflammatory stage, MMPs is responsible for cleaning wound debridement and decaying proteins of pathogen. However, in proliferative stage, MMP inhibits establishment of collagens and delaying wound recovery even inducing wound chronicity (Muller *et al.*, 2008; Mwaura *et al.*, 2006). Procyanidins improves collagen structure by providing itself as connecting molecules between collagen helixes (He *et al.*, 2011). The crosslinked formation protects collagen from MMP proteolysis (Zhai *et al.*, 2011).

This study also implied potency of cocoa as source of active ingredients to be incorporated in skin care products. Activity of CEE to inhibit growth of *S. aureus* and *S. epidermidis* suggests its function to control population of skin normal flora. Furthermore, antiinflammatory and proliferative effect of CEE may contribute in skin regeneration and wound healing cosmetics.

CONCLUSION

Cocoa ethanolic extract demonstrated antibacterial activity against *S. aureus* and *S. epidermidis* through inhibition of bacteria growth. Eventhough the inhibitory activity was much lower than conventional first line antibiotics. Topical application of the cream containing cocoa ethanolic extract was effective in promoting contraction of cutaneous wound infected by both type of bacteria.

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