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Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology
2015 Conference, MCLS 2015

Macrophage Activity and Capacity Following Oral Administration of Cocoa Extract to Mice

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Abstract

The activity of an ethanolic extract from cocoa bean (*Theobroma cacao* L.) towards the non-specific immune response in mice being challenged with *Staphylococcus epidermidis* was studied. Mice (Swiss-Webster, 12 weeks old, 35 ± 1.9 g) received oral administration of cocoa extract (CE), positive control or negative control, every day for seven consecutive days. Cocoa extract (CE) was in three different doses, i.e. CE1 7.14 mg/30 g body weight (BW), CE2 14.28 mg/30 g BW, and CE3 28.57 mg/30 g BW. The positive control was *Phyllanthus niruri* Linn. (PN) extract (Stimuno®) 17.55 mg/30 g BW, while the negative control was sterile water (SW). On day 8, mice were given intraperitoneal injections of *S. epidermidis* suspension (0.5 ml, 105 CFU). After being settled for one hour, mice were sacrificed and peritoneal fluid was withdrawn for staining and microscopy observation. The number of macrophages performing phagocytosis and number of bacterial cells being recruited were counted. CE increased the number of active macrophages as well as enhanced macrophage phagocytic capacity against *S. epidermidis* cells. Various doses of CE increased the number of active macrophages from $46 \pm 5\%$ (SW) to $73 \pm 3\%$ (CE1), $76 \pm 3\%$ (CE2), and $85 \pm 12\%$ (CE3). Phagocytic capacity was elevated more than 2-fold after consumption of CE, from 215 ± 25 cells (SW) to 437 ± 9 cells (CE1), 452 ± 4 cells (CE2), and 511 ± 6 cells (CE3). CE3 with the highest dose had activity equal to that of PN ($p = 0.68$; $\alpha = 0.05$). This research suggests a potential use of CE as an immunostimulant. This study indicates macrophage activity and capacity in mice were enhanced by oral consumption of cocoa extract.

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Peer-review under responsibility of the organizing committee of the Molecular and Cellular Life Sciences: Infectious Diseases, Biochemistry and Structural Biology 2015 (MCLS 2015)

Keywords: cocoa; macrophage; immunomodulator; *S. epidermidis*; *Phyllanthus niruri*

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1. Introduction

Cocoa bean as the raw material of chocolate is a very important ingredient for food and beverage preparation. The indispensable chocolate flavor has made the demand for cocoa bean continuously increase, with 3% increment per year¹. Some people have exceptional appreciation of chocolate that the terms of ‘chocoholics’ and ‘chocolate-craver’ exist^{2,3}. However, with the emerging trend of healthy eating, public perception of chocolate and cocoa products may change from ‘enjoyable’ to ‘threatening’. High fat and sugar content attributed to cocoa products have induced guilty feelings after eating chocolate.

Attempts to enhance public perception of cocoa have been focused on health benefit, and accordingly numbers of cocoa products have entered the market with various claims. Popular health properties introduced to consumers are for cardiovascular protection⁴, antitussive properties⁵ and skin perfection⁶. Two bioactive classes of compounds were documented, namely polyphenols and alkaloids. Cocoa polyphenols mainly come from the flavonoid class among which (+)-catechin and (–)-epicatechin are dominant compounds. In the other hand, cocoa also contains alkaloids (theobromine and caffeine) that have been extensively explored.

Our previous work reported that cocoa extract inhibited growth of *Escherichia coli*⁷ and *Shigella dysenteriae*⁸. In subsequent research, it was observed that effective doses for in vivo testing were much lower than the theoretical dose derived from in vitro evaluation, suggesting cocoa extract may induce the immune response in animals rather than directly counteract bacteria cells. This study aimed to explore immunomodulatory properties of cocoa extract, with a focus on the non-specific immune response exerted by macrophages in a mouse model.

2. Methods

Cococa extract (CE) was prepared from freshly dried cocoa powder (fat removed, unroasted, unfermented) extracted using ethanol overnight. The liquid was filtered and was concentrated by using a vacuum evaporator to result in CE powder containing 14% catechin (assayed by Folin-Ciocalteu’s reagent⁹) and 0.6% caffeine (assayed using acid-base chromatographic column¹⁰). The positive control was syrup of *Phyllanthus niruri* Linn. extract (Stimuno[®], DexaMedica). Male Swiss-Webster mice (12 weeks, 30-35 g) were purchased from a local breeder. A *Staphylococcus epidermidis* culture (5×10^5 CFU/ml), chloroform, immersion oil, phosphate buffered saline, and Giemsa stain were provided by the Microbiology Laboratory, Faculty of Medicine, Jember University.

Mice were acclimatized for at least one week while receiving feed and water *ad libitum*. Twenty mice were distributed into 5 groups, 3 groups of treatment, positive control and negative control group. Treatment groups receiving different doses of CE, i.e. 7.14 mg/30 g body weight (BW) (CE1), 14.28 mg/30 g BW (CE2), and 28.56/30 g BW (CE3). The positive control group received 17.55 mg/30 g BW of *Phyllanthus niruri* Linn. extract (PN) and negative control consumed sterile water (SW). Test samples were delivered through oral administration once a day for 7 days. On day 8, *S. epidermidis* culture was injected intraperitoneally, and after one hour of settlement mice were sacrificed. Intraperitoneal fluid was obtained and processed for fixation and staining prior to microscopic observation. This study has been approved for ethical clearance by the Committee from Faculty of Medicine, Jember University.

Data analysis employed *Minitab 14* and *SPSS 22 for Windows* statistical software for testing normal distribution, homogeneity and one-way analysis of variance (ANOVA). Significant difference between groups was evaluated using *post-hoc* *Duncan’s* test to determine the *p* value with $\alpha = 0, 05$.

3. Results and discussion

The effect of oral administration of SW, PN and CE in Swiss-Webster male mice for seven consecutive days was evaluated from the number of active macrophages per hundred total macrophages (%). The negative control group consuming sterile water (SW) had $46 \pm 5\%$ active macrophages. In the other hand, the PN group had a greater number of active macrophages, $80 \pm 4\%$. PN extract has been studied for immunomodulatory properties, particularly in stimulating macrophage activity and T-lymphocyte proliferation¹¹.

After consumption of CE1, the active macrophage number was improved to $73 \pm 3\%$. Higher concentration in CE2 and CE3 resulted in proportions of active macrophages of $76 \pm 3\%$ and $85 \pm 12\%$, respectively, which were not significantly different compared to CE1. This indicates improvement of CE in macrophage activation did not occur in a dose-dependent manner at these (high) doses. However, since CE1 did not show a significantly different result

than PN, it was suggested that a comparable effect may be exerted by CE at the lowest dose of 7.14 mg/30 g BW (Fig. 1).

In addition to determining active macrophage numbers, the number of bacterial cells being recruited by macrophages during phagocytosis was evaluated as the phagocytic capacity of macrophages. Baseline phagocytic capacity in the untreated group (SW) was 216 ± 25 bacteria/50 macrophages. This was improved dramatically in the PN group to 506 ± 12 bacteria/50 macrophages. Improvement was also observed after administration of CE, which in the lowest dose produced high phagocytic capacity (437 ± 9). The effects of CE1 (437 ± 9) and CE2 (452 ± 4) were not significantly different ($p = 0.199$, $\alpha = 0.05$), but CE3 (511 ± 6) produced a significantly higher effect. Administration of CE3 resulted in an effect comparable to that of PN ($p = 0.68$, $\alpha = 0.05$) (Fig. 2).

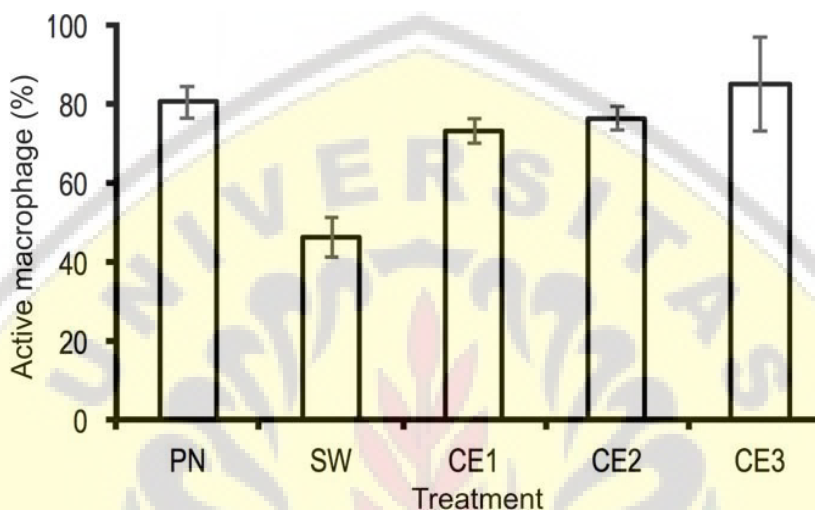


Fig. 1. Percentage of active macrophage in intraperitoneal fluid following administration of sterile water (SW), CE dose 1 (CE1), dose 2 (CE2), dose 3 (CE3), and *Phyllanthus niruri* Linn. extract (PN).

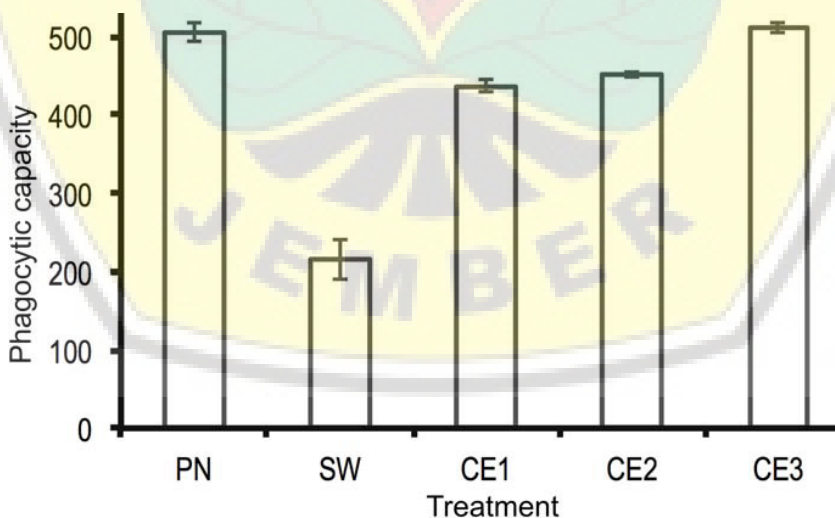


Fig. 2. Phagocytic capacity of macrophages expressed as number of *S. epidermidis* cells recruited per fifty active macrophages, following administration of sterile water (SW), CE dose 1 (CE1), dose 2 (CE2), dose 3 (CE3), and *Phyllanthus niruri* Linn. extract (PN).

The dose variations used in this study can be considered relative to the catechin content in CE. The lowest dose in CE1 (7.14 mg) was estimated to contain 1.0 mg catechin, while CE2 and CE3 delivered 2.0 mg and 4.0 mg catechin, respectively. When the catechin content in CE is extrapolated to a human dose, consumption of CE1 will represent 187 mg catechin intake by a human with body weight 70 kg, hence CE2 and CE3 will deliver 376 mg and 755 mg of catechin, respectively. The catechin amount is consistent with human studies that reported the effective catechin intake for health improvement.

A study by Grassi *et al.*⁴ reported consumption of dark chocolate containing 500 mg flavanol lowered the blood glucose level and enhanced insulin sensitivity of human subjects. This condition was observed after a relatively short term administration for 7 days. In another study, Desideri *et al.*¹² suggested that cocoa flavanol intake of 990 mg/day for 8 weeks contributed to cognitive improvement in 90 elderly individuals with mild cognitive impairment. A moderate intake of cocoa flavanol (550 mg/day) brought a similar effect to that of high flavanol treatment. However, a low intake (45 mg/day) resulted in no significant improvement in trail making and verbal fluency tests. The trail making test is used to evaluate visual-conceptual and visual-tracking ability while indicating visual processing impairment due to brain damage. These two studies indicated the effective cocoa flavanol intake (represented by catechin) is around 500 mg/day.

With regards to the immune system, Ramiro-Puig and Castell¹³ have proposed cocoa polyphenols to be the immunomodulators. Cocoa consumption may affect the immune system in three aspects, i.e. composition, activity and response of immune cells. Mice receiving a cocoa-rich diet underwent alteration of lymphocyte composition in the intestine, particularly in Peyer's patches and mesenteric lymph nodes. In addition, both tissues had increasing amounts of T-cell-antigen receptor¹⁴. Activity of immune cells was also affected by cocoa consumption, whereas lymphocytes exhibited reduced immunoglobulin-A secretion¹⁴. Incubation of EL4.BU.OU6 cell lines with cocoa catechin resulted in a decrease in IL-2 receptor α (CD25) expression and IL-2 secretions, while IL-4 production was enhanced¹⁵.

Macrophage activity and phagocytic capacity following CE administration showed that a low catechin intake may bring significant improvement in the non-specific immune response. The lowest dose of CE administration (7.14 mg/30 g BW, representing 1.0 mg/30 g BW catechin) was sufficient to successfully stimulate macrophage activity as well as phagocytic capacity. Moreover, the higher catechin intake to 4.0 mg/30 g BW had comparable effects to that of PN as the positive control. This research agreed with previous studies on immunomodulatory properties of cocoa.

4. Conclusion

Oral consumption of CE may stimulate non-specific immune responses in adult mice challenged by *S. epidermidis*. This effect was observed after daily administration for seven days. The highest CE dose, delivering 4 mg/30 g BW catechin demonstrated desirable stimulation in terms of active macrophage number and phagocytic capacity.

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