Chlorophylls Recovery from Tea Dregs of Commercial Green Teas and Its Potency as Sensitizer for Photodynamic Inactivation of *Listeria monocytogenes*

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Abstract

Many studies have been addressed into polyphenolic fractions of green tea, its existence in the fresh tea leaves, in processed leaves, even to in its waste after brewing. Only few investigations are dealt with the chlorophylls content of tea leaves. In fact, the transformation of chlorophylls takes part in the development of flavor and color of green tea. Since the chlorophylls have poor solubility in water; it was supposed that most pigments are remained in the tea dregs after brewing. The present study was aimed to evaluate the possibility of chlorophylls recovery after hot water extraction of commercial green teas and afterwards the potency of its use for photodynamic inactivation of pathogenic bacteria, *Listeria monocytogenes*. HPLC and spectroscopic analyses were performed to predict the type and ratio of chlorophyll derivatives which present in the tea dregs. We found that the most dominant fraction was pheophytin *a* (~50%), followed with its epimers as well as pheophorbide *a*. In addition, the crude pigment extract from tea dregs was able to inactivate up to eighty percent of *L. monocytogenes* cells after photodynamic reaction under illumination of red or blue LED lamp. These results revealed a new sight for the utilization of tea dregs in the eradication of pathogenic bacteria.

Keywords: Chlorophylls; Green tea; Photodynamic inactivation; Tea dregs.

Introduction

Chlorophylls are perhaps the most obvious pigments in the world surface, naturally produced by autotrophic organisms and performing the function to capture light energy

for photosynthesis.[1] Due to their abundance as well as photophysical and photochemical properties, chlorophylls have been one of the most promising natural sensitizers for antimicrobial photodynamic therapy.[2] In addition, several studies have also revealed the potency of chlorophyll derivatives to exhibit similar even enhanced stability in absorbing photon energy and generating the production of singlet oxygen, as is the main requirement of sensitizing material.[3,4]

The manufacturing of green tea, which is often comprised of several heating steps and oxidation, impacts on the structural modification of chlorophylls. Despite of the massive discussions on the green tea polyphenols and its health benefits, some analyses of chlorophylls and their derivatives in green tea were well-reported.[5–7] The established methods for chlorophylls extraction are either using organic solvents or supercritical fluid, due to its low polarity.[8] Hence, the common hot water extraction of green tea was detected to leave the chlorophylls on the tea dregs.[9,10] To our knowledge, the influence of temperature and time of hot water extraction on the residual chlorophylls have not been studied yet.

The present research was aimed to determine the effect of time of hot water extraction on the residual chlorophylls on green tea dregs, and to evaluate the photodynamic activity of those chlorophylls extract against pathogenic bacteria, *Listeria monocytogenes*, compared to purified chlorophyll. This investigation could be one of the attempts to maximize the utilization of tea waste, especially dealing with the continuous increase in demand and production of green tea as well as ready-to-drink tea beverage. In addition, the exploration of chlorophyll derivatives from tea processing might also lead to the development of novel sensitizer.

Materials and Methods

Materials

Two commercial green teas were purchased from local market. Those samples were come from different brands and manufactured in different tea factories, i.e. GT1 (Bogor, West Java, Indonesia) and GT2 (Pekalongan, Central Java, Indonesia).

Chlorophylls Extraction from Green Tea Dregs

Each green tea sample (1.85 g) was brewed separatedly with 200 mL hot water (90±5^{0}C) for 5 and 20 min. The tea extract was then decanted and the tea dregs was collected and dried. A certain amount of tea dregs (0.15 g) was subsequently mixed with 1 mL acetone, vortexed three times in 1 min, and the supernatant was collected through centrifugation (14,000 rpm, 1 min). The extraction procedure was repeated three times until the dregs turned pale in color. The acetone extract was collected in vial, dried, and flowed with Nitrogen gas, kept in freezer (-20^{0}C) until the time of further analysis. The green tea sample without hot water extraction was also extracted with the same procedure as a comparison.

High performance liquid chromatography (HPLC) analysis

A qualitative analysis for pigment extract from tea dregs was carried out using high performance liquid chromatography (HPLC) according to published method.[11] The crude extract was dissolved in 1 mL acetone and filtered (0.22 μm, Nylon, Whatman, Kent, UK) prior to injection into C_8 column installed on HPLC instrument (LC-20A) equipped with SPD-20MA diode array detector (Shimadzu, Kyoto, Japan). Each separated peak in visible range (350–700 nm) was identified based on its retention time and spectral characteristics, compared to the reference.[9]

Preparation of Sensitizers

Purified chlorophyll *a* and *b* were obtained from acetonic extract of Suji leaves (*Pleomele angustifolia*). The crude extract was dissolved in 2-propanol and acetone, and separated from carotenoid fractions by means of column chromatography with silica gel 60 as stationary phase and hexane-acetone as mobile phase. The purified chlorophylls were collected after elution in HPLC with gradient mobile phase comprised of methanol and acetone.[12] Pheophytin *a* was obtained from the pure chlorophyll *a* after the reaction with acetic acid in acetone, and further elution in HPLC instrument using determined method.[11] Purified pigments and crude extract of tea dregs were suspended in 1% Tween 80 (100 μg/mL) for photodynamic experimentation. The concentration was determined

by calculation of Lambert-Beer equation using determined extinction coefficient for each purified chlorophyll and pheophytin,[13] whereas that of the crude extracts were adopted from its dominant fraction.

LED Light sources

Commercial blue and red LED lamps (Yumiko YL-2550, 50 Watt, 4800 Lumens) were adopted as the source of light in photodynamic treatment. The LED lamps exhibit maximum of emission spectrum at 445 and 640 nm for blue and red lamp, respectively, confirmed by Ocean Optics Spectrometer. Both LED lamps were coupled above the orbital shakers to give 300±15 μmol.m^2.s^{-1} photons upon the well plate during the experiments.

Microorganism experiment

The culture of *Listeria monocytogenes* (FNCC 0156) was obtained from Food and Nutrition Culture Collection Division, Gadjah Mada University, Jogjakarta, Indonesia. In briefly, an inoculum at 10^8 CFU/mL was prepared in Nutrient Broth (NB) and thereafter a volume of 75 μL was transferred to an Eppendorf containing 1425 μL of the sensitizer in Tween 80 1.0% (wt./vol.). The suspension was then aliquoted into individual 400 μL volumes, transferred into the wells of 12-well plate for LED exposure (blue and red) as well as the unilluminated sample. Then, the samples were illuminated for 30 min at 18^0C under agitation (100 rpm). After the illumination, each sample was diluted with 4.6 mL Mullen Hinton Broth (MHB), then 0.5 mL of the suspension was added into 9.5 mL of peptone solution, and after being well-vortexed, 20 μL of inoculum was plated in Trypticase Soy Agar (TSA), incubated for 24 hours at 37^0C, and the CFU number was counted. The same experimentation was conducted with control groups using the same amount of inoculum and 1% aqueous Tween 80 solution without sensitizer, and all experiments were performed in three repetitions.

Statistical analysis

The nested analysis of variance (ANOVA) was designed with the two factors, i.e. the wavelength region of LED lamps and the type of sensitizer, followed with either *t*- or

Dunnett's post-hoc test. Data interpretation was created after calculation using Minitab v.17 at the 5% significance level.

Results and Discussion

The typical appearance of tea dregs after hot water extraction is dark in color, suggesting the presence of some pigments. According to our findings, further extraction of the tea dregs using acetone would leave pale pellet and the absorption spectra of the crude extract were depicted in Fig 1. Some maxima were observed, i.e. 410, 533, 606, and 665 nm. The absorption spectra represent the dominancy of chlorophyll groups, having the obvious Soret, Qx, and Qy bands.[14] However, the Soret bands were blue-shifted from typical 430 nm of that for chlorophyll *a* into 410 nm, indicating the presence of chlorophyll derivatives. A significant blue-shift of Soret band as well as the lowered signal of Qy band might occur as the consequence of reduction in molecular symmetry after the release of central Magnesium.[15] Additionally, the shouldering peak at ~450 nm suggested the presence of some carotenoids. Carotenoids are the accessory pigments which are integrated to pigment-protein complexes in photosynthesis apparatus.[16]

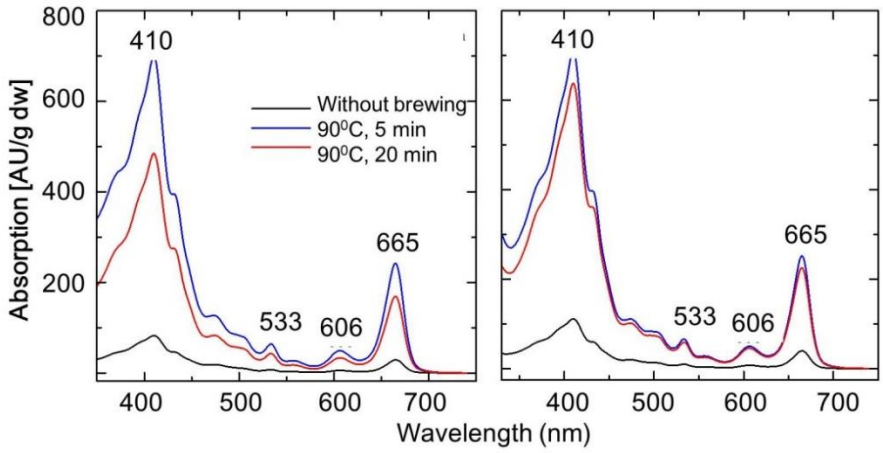

Fig.1: Absorption spectra of the crude chlorophylls extract from green tea dregs: GT1 (left) and GT2 (right) after hot water (90^0C) extraction for 5 and 20 min.

Generally, there is no significant difference between the absorption spectra of the acetonic extract of green tea dregs, GT1 and GT2, except the reduced absorption in GT1 after 20 min of tea brewing time. Indeed, the chlorophylls are heat-labile pigments which

could be degraded continuously during the prolonged heating. Interestingly, the absorption spectra of acetonic extract of green tea without brewing have much lower signal. The reasonable explanation should be addressed into the possibility of cell shrinkage and case hardening after the drying of tea leaves. Hence, the hot water extraction might re-dilate the cell walls and ease the solvent to extract the chlorophylls.

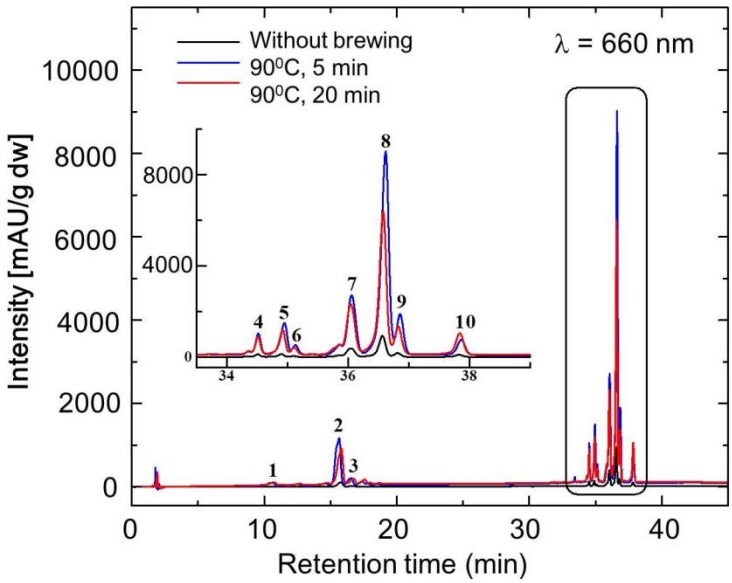

Fig 2: HPLC profile of the crude chlorophylls extract from green tea dregs. The peak number corresponds to the identification at Table 1.

The types of chlorophyll derivatives were confirmed through HPLC analysis , and the chromatograms were shown in Fig. 2. Each distict peak was identified according to the reference.[9] The most dominant derivatives was pheophytin *a*, being eluted at 36.57 min, followed with pheophorbide *a* as well as several epimers of pheophythin *a*. No chlorophyll *a* was detected. This results were consistent with the previous study which revealed the absence of most chlorophyll *a* and *b* in commercial teas marketed in Indonesia, being difference to that of Japanese tea.[7,9] The *in vivo* formation of pheophytin may occur because of the activity of Mg-chlorophyllase, or induction by heat and acid.[17] Pheophytin also occurs naturally in the plant leaves and is important as the first electron carrier intermediate in the electron transfer pathway of photosystem II in plants. It is

known that the manufacturing of green tea involves heating, especially in the drying stages.[18]

Table 1: Detection of chlorophylls in the extract of green tea dregs.

Peak Number	t_R (min)	Fractions[9]	λmax (nm)					GT1	GT2
1	10,16	Feoforbid *b* sp.	437	524			650	+	-
2	15,77	Feoforbid *a*	409	508	538	609	665	+	+
3	16,57	Feoforbid *a* sp.	409				665	-	+
4	34,5	Feofitin *b* sp.	434	524		598	652	+	+
5	34,9	Feofitin *b*	434	527		598	652	+	+
6	35,08	Feofitin *b* sp.	434				652	+	-
7	36,04	Feofitin *a* sp.	409	503	533	609	664	+	+
8	36,57	Feofitin *a*	409	506	536	610	665	+	+
9	36,82	Feofitin *a* epimer	409	506	538	610	666	+	+
10	37,82	Feofitin *a* sp.	409	508	538		665	+	+

Furthermore, the photodynamic experiments revealed the reduction of living cells after treatment with the sensitizer and LED illumination (Fig. 3). The percentage of cell death reflected the difference of the CFU counting in dark and illuminated plates. We also observed that the number of living *L. monocytogenes* cells was reduced in the control group after illumination, though there is no addition of sensitizers. This finding is consistent with the previous investigation concerning the potential presence of endogenous sensitizers in bacterial cells, which generated radical oxygen upon illumination, and triggered the cell death.[19,20] The percentage of cell death was increased when the sensitizer was incorporated to the cells suspension, even being significantly greater in the use of chlorophylls extract from the green tea dregs ($p<0.05$). The previous study revealed the potency of synergystic effect when more than one sensitizers are used in photodynamic treatment.[21] In this case, the photodynamic activity of the chlorophylls extract from green tea dregs was compared to that of purified chlorophyll *a* and *b*, as well as pheophytin *a*. there is no significant difference ($p>0.05$) among the antimicrobial photodynamic activity of GT1 and GT2.

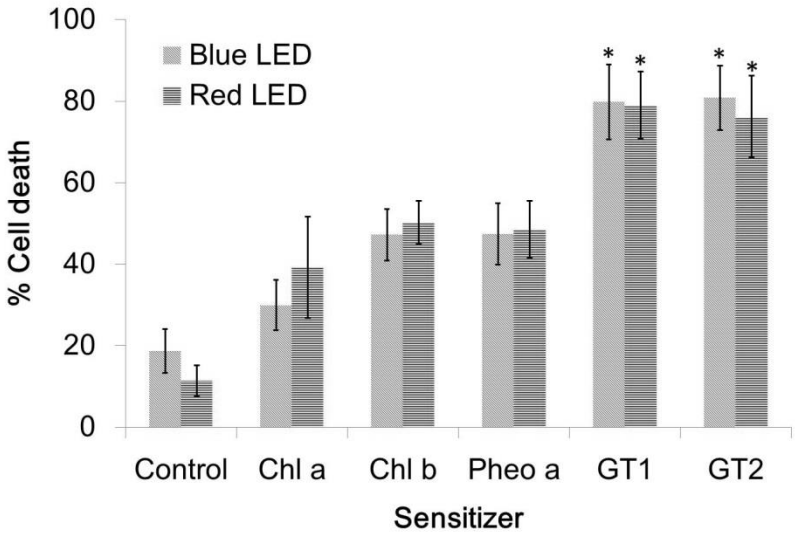

Fig.3: Percentage of *L. monocytogenes* cells death for different types of sensitizer, after illumination with blue and red LED lamps. Values are expressed as means±SEM, and the star represents the treatment with significant difference (p<0.05).

Overall, our findings evidenced the feasibility of chlorophylls recovery from green tea dregs and its photodynamic activity for eradication of *Listeria monocytogenes*. The current use of tea waste is mostly limited to the blend of animal feed and fertilizers.[22] Besides the potential green tea dregs, the self-assembled instrumentation using commecial LED lamps was proven to be able to reduce up to 80% of *Listeria monocytogenes* cells in concentration of 100 μg sensitizer/mL, being worthy for further development. The antimicrobial photodynamic treatment is now promising not only for medical purpose but also in field of food and agriculture,[23] especially to combat the antibiotic-resistant microorganism.[24]

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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