Correlation Phenolic Concentration to Antioxidant and Antibacterial Activities of Several Ethanolic extracts from Indonesia

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Abstract. Increasing concerns on the adverse effect of synthetic antioxidants and the emergence of antibiotic-resistant Staphylococcus aureus have become two essential problems to be addressed. To tackle them, exploration of natural resources to discover novel antioxidants and/or antibacterial agents is urgently required. The aim of this research was to investigate the correlation of phenolic and flavonoid contents of extracts to their antioxidant and antibacterial activities. Green tea, green coffee, cocoa pod husks, bee pollen, and rosella calyces were processed and subjected to 80% ethanol-based maceration procedure to obtain extracts with appropriate condition. Each extract was examined for its phenolic and flavonoid concentrations using the Folin–Ciocalteau method and the aluminum chloride colorimetric assay, respectively. Further analysis on the free-radical scavenging potential and antibacterial/antibiofilm activity against S. aureus were carried out. Samples were found to contain total phenolics (TP) and total flavonoids (TF) at different concentrations. The highest level of TP and TF was identified in green tea extract and corresponded to the lowest IC50 against DPPH and the lowest MIC against S. aureus colonies or to their respective biofilm. In contrast, low amounts of TP and TF were found in cocoa pod husks and bee pollen which were further demonstrated high IC50 and high MIC. Collectively, our results suggested the linear correlation of phenolic- and flavonoid contents to the antioxidant and antibacterial/antibiofilm activities of plant extracts. The higher the phenolics and flavonoids level, the better the antioxidant and antibacterial/antibiofilm activities obtained from the corresponding extracts.

1. Introduction
Polyphenols and other phenolic compounds in plants and natural products are known to have antioxidant and antimicrobial activity [1,2]. These compounds range structurally from simple to complex phenolic molecules including high molecular-weight polymers. Some phenolic-containing compounds present in plants and plant products such as roselle calyces [3,4], cocoa husks [5,6], green tea ([7,8], and bee pollen [9,10]. Except for cocoa pod husks, these are commonly used in Indonesia community as drinks (green tea, green coffee, and roselle calyx) and supplements (bee pollen).

Green tea contains mainly epigallocatechin gallate (EGCG), epigallocatechin (EGC), epicatechin gallate (ECG). The compounds have strong antioxidant properties and also antimicrobial activity[11]. According to Chan et al. ([12]), dried water extract of green tea has antibacterial and antioxidant activity and the fraction of non-polymeric phenolics were major contributors to the antioxidant and
antibacterial properties. However, a 2% infusion tea beverage did not have antioxidant and antibacterial activity [13].

Green coffee, in addition to containing caffeine and derivatives thereof also contains phenolic compound, especially chlorogenic acid. Chlorogenic acid (CGA) and related compounds are the main components of the phenolic fraction of green coffee beans, reaching levels up to 14% (dry matter basis). Chlorogenic acid was found to be an antibacterial with MICs ranging from 20 to 80 μg/mL against the tested bacterial pathogens [14,15]. Chlorogenic acid and caffeic acid were shown to have antioxidant activity in vitro and in vivo [16].

Roselle calyces have phenolic compounds, especially anthocyanins [17,18]. Three roselle varieties exhibited antioxidant activity, inhibiting lipid peroxidation between 69 and 79% [19]. The antibacterial activity of roselle extracts against Escherichia coli, Staphylococcus aureus, Streptococcus mutans and Pseudomonas aeruginosa, showed varying degrees of inhibition on the tested organisms [3].

Cocoa pod husks, waste from the processing of cocoa was presented to have antibacterial activity against Streptococcus mutans and antioxidant activity as shown by DPPH method [6]. Bee pollen ethanolic extract contains phenolic compounds that have antioxidant activity, as shown by the carotene bleaching method, as well as antibacterial activity against Bacillus subtilis, P. aeruginosa, and Klebsiella sp. [20].

The content of phenolic components in extracts depends on extraction methods, solvent polarity, the ratio of the sample/solvent and variety of the plant [21]. This study aimed to determine the antioxidant and antibacterial activities of several phenolic-containing extracts, extracted by maceration methods using 80% ethanol with a ratio of sample: solvent 1:10. Extracts with the best activity may in the future be investigated as antibacteria or antibiotic modulators against antibiotic-resistant pathogenic bacteria, especially S. aureus.

2. Material and Methods

2.1 Collection of Samples
Cocoa pod husks, green coffee, and roselle calyces were collected from a local farm and thoroughly washed with distilled water to remove dirt and dried in a fruit dehydrator. Green tea and bee pollen products were collected from the market. All of the samples were ground into a coarse powder.

2.2 Preparation of the Extracts
One hundred grams of the coarse sample were extracted in 1 L of 80% ethanol for 5 days by maceration method. The resulting liquid extract was subjected to a vacuum evaporator and freeze-dried to evaporate remaining solvent. From such procedure, a dried crude extract was obtained and considered as the final form of the sample that was used in further analysis. Each sample was processed in a similar fashion and the percentage of yield was determined based on the following equation:

\[ \text{Yield percentage (\%) = } \frac{a}{b} \times 100 \% \]

Where (a) is the dry weight of extract and (b) is the soaked samples material.

2.3. Determination of total phenolic content (TPC)
Determination of TPC was carried out using the Folin–Ciocalteu method [22] with slight modifications. Briefly, stock solutions of plant extracts were prepared in methanol at different concentrations at a range between 1,000 and 10,000 ppm. A total of 0.1 ml of each plant extract solution was transferred to a test tube and subsequently mixed with 2.5 ml of 7.5 % Folin–Ciocalteu phenol reagent. Next, 2 ml of 1% sodium hydroxide solution was added to the mixture and finalized to a final volume of 5 ml. The mixture was thoroughly homogenized and stored in the dark for 60 min. Absorbance spectra of each sample was recorded at 725 nm using glass cuvettes.

The reaction was performed in triplicate and the results were expressed as milligram of gallic acid equivalent (mg GAE) per gram of extract.
2.4. Determination of total flavonoid content
The aluminum chloride (AlCl$_3$) colorimetric method [23] was used to estimate total flavonoid content (TFC) in all tested samples. Briefly, stock solutions of plant extracts in methanol were prepared in different concentrations (1000–10000 ppm). For each sample, a total of 0.1 mL of each solution were transferred to test tube and mixed with 0.1 ml of 10 % aluminum chloride and 0.1 mL of 1 M sodium acetate. The mixture was vortexed thoroughly and then ethanol was added to a total volume of 5 mL. The test tube was incubated for 60 min. Absorbance spectra were recorded at 400–800 nm using glass cuvettes. All samples were processed in triplicate and the results were expressed as milligram of quercetin equivalents (mg QE/ gram of extract).

2.5. Determination of antioxidant activity by DPPH method
Free radical scavenging ability was determined based on the DPPH method described in [24–26] with slight modifications. Briefly, the stock solution of the DPPH radical was prepared by dissolving 24 mg DPPH in 100 mL methanol and was kept in a refrigerator until further use. As a working solution, DPPH stock solution was diluted with methanol to obtain an absorbance of about 0.98 (±0.02) at 517 nm. Next, all crude extract samples were processed for the test. To this end, 3 mL DPPH working solution was mixed with 100 μL plant extract (1 mg/mL) or the standard solution in a test tube and incubated at room temperature for a 30 min period. The mixture was then subjected to absorbance measurement at 517 nm and the calculation of radical scavenging activity was carried out using the following formula:

\[
\% \text{ Antioxidant activity} = \left( \frac{Ac - As}{Ac} \right) \times 100 \%
\]

where, Ac and As are the absorbance of control and sample, respectively. In this test, 100 μL methanol was used as a control in place of the tested sample.

2.6. Determination of minimum inhibitory concentration (MIC) against S. aureus
The MIC of each sample was determined by a two-fold serial microdilution method [27] with slight modification. Each extract was dissolved in dimethyl sulfoxide (DMSO) and seeded into the Mueller Hinton Broth (MHB) media to a final concentration ranging from 10 to 0.015 mg/ml. An overnight culture of S. aureus was prepared and its OD$_{600}$ was determined using spectrophotometer prior to seeding in the MHB media or mixed with extract in the corresponding wells. The well plates were then incubated at 37 °C for 24 h. MIC was determined as the lowest concentration of the plant extract that inhibited the growth of the tested bacteria.

2.7. Determination of biofilm inhibition against S. Aureus
Assessment on S. aureus biofilm formation was carried out according to procedure described previously [28] with slight modifications. Briefly, 300 μL of S. aureus suspension (0.5 × 10$^8$ CFU/ml) was cultured in 30 ml of 2% sucrose-Trypticase Soy Broth (TSB) and a total of 2 ml of the mixture of bacteria and media was transferred to a sterile 24 well-plate and incubated for 24 hours at 37 °C. For the negative control, a two ml of medium in the absence of bacteria suspension was transferred to the assigned well. After 24 hours, the wells were washed with sterile distilled water, stained with a 0.1% crystal violet solution and incubated at room temperature for 15-30 minutes. The residual staining liquid was washed thrice from each well using sterile distilled water and air-dried prior to observation. A positive result of biofilm formation was determined based on the display of purple color attached at the bottom or the peripheral side of the well after washing procedure.

To determine the biofilm inhibition activity, each extract was prepared in the TSB media and subsequently subjected to the procedure outlined above in the assessment of biofilm formation. In brief, each extract was prepared at several different concentrations starting from the one that corresponds to its MIC to the one with 4-fold of the MIC of the extract. Each of these extract-containing media was then transferred to the well plate, separately, and mixed with 100 μL of the overnight S. aureus culture and incubated for 24 hours at 37 °C. At the following day, the well plate
was subjected to biofilm staining procedure, as described above. Excessive dye was dissolved using 96% ethanol and the well plate was left for 30 minutes. The absorbance of samples in each well was measured at 590 nm and percentage of inhibition for each extract concentration was calculated. The results were then used in a linear regression to determine the IC\textsubscript{50} of extract against biofilm.

3. Results and Discussion

3.1. Percentage of yield, TPC, TFC, and IC\textsubscript{50} against DPPH in all tested extracts

The yield of extract results from each sample, total phenolic concentration (TPC) calculated as gallic acid equivalents (GAEs), total flavonoid concentration (TFC) calculated as quercetin equivalents (QE), and antioxidant activity expressed as IC\textsubscript{50} to DPPH free radical can be seen in Table 1.

| Name of ethanolic Extract | Yield of Extract (%) | TPC (mg GAE/g) | TFC (mg QE/g) | IC\textsubscript{50} DPPH (µg/ml) |
|---------------------------|----------------------|----------------|---------------|------------------|
| Green tea                 | 23.41                | 219.6          | 13.2          | 14.37            |
| Green coffee              | 10.51                | 76.4           | 0.59          | 53.41            |
| Cocoa podhusk             | 26.25                | 5.5            | 0.52          | 409.65           |
| Bee pollen                | 11.91                | 12.8           | 1.52          | 370.21           |
| Roselle calyx             | 29.21                | 8.2            | 1.83          | 385.79           |

As shown in Table 1, more than 20 % yields were obtained from the extraction of green tea, cocoa pod husks, and roselle calyces. On the other hand, yields recovered from two other samples, green coffee and bee pollen, were lesser by at least two-folds than the first three samples. Of five samples, the highest yield (around 29 %) was obtained from the extraction of roselle calyces and the highest TPC and TFC was obtained from green tea extract. It is possible chemical compounds in roselle calyces higher solubility in 80 % ethanol, but phenolic compound in green tea higher solubility in 80% ethanol. Phenolic compound in green tea are flavan-3 ol (EGCG, ECG, EGC, Gallocatechin, Catechin, epicatechin), gallic acid derivates, hydroxycinnamate quinic esters, quercetin derivates [29].

3.2 Augmentation of free radical scavenging activity in phenolic-rich extracts

Concentration of phenolic compounds in certain samples have been shown to be highly correlated with their antioxidant potentials [30]. To examine whether such antioxidant potential is also present in our samples, we carried out a free radical scavenging experiment using DPPH method. This is a straightforward, inexpensive, and widely used assay examine antioxidant activity of samples of interests. The sensitiveness of the reaction relies on the scavenging of DPPH by compounds with antioxidant properties [31].

The percentage inhibition of the DPPH radical by the ethanolic extracts used in this study, expressed as the half maximal inhibitory concentration (IC\textsubscript{50}) to DPPH free radical, is shown in Fig. 1. Of all samples, green tea extract demonstrated the lowest IC\textsubscript{50} against DPPH, suggesting its high antioxidant activity. This is probably due to the high concentration of phenolics (Fig. 1A) and flavonoids (Fig. 1B) found in the green tea extract.

Overall, we observed that samples with high content of phenolic compounds yielded lower IC\textsubscript{50} against DPPH than samples with low phenolic concentration, suggesting that antioxidant potential was linearly correlated to the phenolic contents of those samples. The more the phenolic contents, the higher the antioxidant potential. This can be better demonstrated by the results shown in the experiments using green tea and green coffee extracts. Indeed, phenolic contents seem to play a major role in the scavenging ability of certain samples to DPPH thus implying the antioxidant potential of
such samples. This notion has been reported by others [7,32,33] and supported by our results in this study.

In addition to phenolic compounds, some flavonoids have been reported to yield antioxidant activity [34]. In agreement to the available literatures, we also found that extracts with high content of flavonoids yielded low IC$_{50}$ against DPPH, indicating their superior antioxidant potential. In contrast, extracts with low amount of flavonoid produced appropriate reaction with DPPH at a higher IC$_{50}$, further suggesting their weak antioxidant potential (Fig 1B). However, to be noted, different result was observed in the experiment to assess the IC$_{50}$ of green coffee extract against DPPH. As shown in Fig 1B, green coffee extract with a relatively low concentration of flavonoids could scavenge DPPH at a low value of IC$_{50}$. The cause of this remains to be elucidated. Nevertheless, based on the results shown in Fig 1, it is apparent that antioxidant potential of samples examined in this study was occurred in a manner dependent on the concentration of phenolic and flavonoid compounds contained in the samples.

3.3 High correlation of antibacterial and antibiofilm activity with concentration of phenolic and flavonoid in tested samples

Certain phenolic and flavonoid compounds have been demonstrated to yield antibacterial activity against several pathogenic microorganisms [35], including Staphylococcus aureus. S. aureus is a Gram-positive bacterium that has been shown to exert negative impacts on the health of human population worldwide [36]. To make it worst, the emergence of methicillin-resistant S. aureus (MRSA) and other antibiotic-resistant strains of S. aureus has increased the level of threat to an alarming cataclysm. Based on that sense, antibacterial compounds with high efficacy against S. aureus and its large selection of antibiotic-resistant strains are urgently required. To this end, such needs could be fulfilled by phenolic-type and/or flavonoid compounds isolated from natural products.

In this study, we analyzed antibacterial properties of five different extracts against S. aureus. As shown in table 2 and Fig. 2.

Low concentration of phenolic- and flavonoid-rich green tea extract (MIC = 0.3 mg/ml) could inhibit the growth of S. aureus, suggesting its excellent anti-staphylococcal activity in vitro. On the other hand, 10- to 20-fold concentration of other extracts with low content of either phenolics or flavonoids were required to inhibit the growth of S. aureus (Fig. 2A and Fig. 2B). Based on these results, we concluded that anti-staphylococcal activities of extracts examined in this study was generated in a manner dependent on the concentration of phenolic-type and flavonoid-type compounds contained in the samples.
Table 2. Antibacterial activities of samples expressed by the MIC and IC$_{50}$ biofilm

| Name of the ethanolic Extract | Minimum Inhibitory Concentration (mg/ml) | IC$_{50}$ Biofilm (mg/ml) |
|-------------------------------|------------------------------------------|--------------------------|
| Green tea                     | 0.3                                      | 0.3                      |
| Green coffee                  | 5.0                                      | 14.6                     |
| Cocoa pod husk                | 5.0                                      | 9.2                      |
| Bee pollen                    | 5.0                                      | 15.5                     |
| Roselle calyces               | 2.5                                      | 3.9                      |

![Figure 2](image-url)  
**Figure 2.** Relationship of total phenolic (A) and total flavonoid (B) contents of five different extracts with minimum inhibitory concentration (MIC) against *S. aureus*.

In recent years it has been shown that some bacteria are able to live freely in the environment despite the existence of antibiotic pressure. This can be achieved by the help of a generic bacterial defense mechanism named as biofilm. Biofilm is a built structure in which bacteria cells attached to each other (frequently also to a surface) and encapsulated by an extracellular matrix composed by extracellular polymeric substance [37]. Biofilm has been regarded as one of bacteria’s powerful defense mechanism to overcome the threat of antibacterial agents [38], thus the presence of novel antibacterial compounds equipped with antibiofilm activity is highly preferred. Based on such notion, we next examined the antibiofilm activity of all extracts with a special attention was given to green tea. As shown in Fig. 3, we found that all extracts demonstrated good activity against the formation of *S. aureus* biofilm. However, it is important to note that the best result on the inhibition of biofilm formation was demonstrated by green tea extract (IC$_{50}$ = 0.3 mg/ml).
A & B

**Figure 3.** Relationship of total phenolic (A) and total flavonoid (B) contents of five different extracts with inhibition of *S. aureus* biofilm formation.

4. Conclusion
In this research, we examined the total phenolic and the total flavonoid contents of five different extracts and assessed their antioxidant and antibacterial activities against *S. aureus*. In addition to that, we further analyzed the correlation of phenolic and flavonoid concentration to the antioxidant and antibacterial activities demonstrated by each extract. Collectively, our data showed that the superiority of green tea extract (in comparison to other extracts) in producing its antioxidant and anti-staphylococcal activities with an excellent performance in the inhibition of *S. aureus* biofilm formation was highly correlated to the high concentration of phenolic and flavonoid compounds detected in this sample.

Conflict of Interest
We declare that we have no conflict of interest.

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