Plant Extract Activities as Antioxidant and Antibiofilm against Chicken Gut Bacteria

Gracia E[1], Magdalena S[1], Wina E[2], Sinurat AP[2], Purwadaria T[2]

[1]*Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jakarta 12930, Indonesia*
[2]*Indonesian Research Institute for Animal Production, PO Box 221, Bogor 16002, Indonesia*
E-mail:tpurwadaria@yahoo.co.uk

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ABSTRAK

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Terjadinya resistensi mikroba terhadap antibiotik akibat penggunaan *antibiotic growth promoter* (AGP) dengan dosis subterapeutik pada unggas dapat dicegah dengan senyawa antibiofilm. Senyawa sekunder tanaman memiliki aktivitas seperti antioksidan, antimikroba, maupun antibiofilm. Penelitian ini bertujuan mendapatkan tanaman yang memiliki aktivitas antibiofilm tertinggi dan juga aktivitas antioksidan dan dengan cara menganalisis aktivitas senyawa sekunder beberapa tanaman. Sampel tanaman yang diuji adalah daun cengkeh, tanaman meniran, kulit manggis, cangkang jambu mete, daun jambu, dan daun salam. Tanaman tersebut diekstraksi dengan metanol dan n-heksana menggunakan metode sonikasi. Aktivitas antioksidan ekstrak metanol tanaman diukur dengan mencari nilai IC_{50} pada uji α,α-diphenyl-β-picrylhydrazyl (DPPH). Aktivitas inhibisi pembentukan biofilm diuji terhadap *Escherichia coli*, *Salmonella enteritidis*, dan *Staphylococcus aureus* ATCC® 29213™ menggunakan ekstrak metanol dan n-heksana. Seluruh sampel memiliki aktivitas antioksidan. Sampel daun cengkeh dan tanaman meniran memiliki aktivitas antioksidan tertinggi. Sementara, ekstrak metanol kulit manggis memiliki aktivitas antibiofilm tertinggi terhadap seluruh bakteri uji. Jenis bakteri uji juga mempengaruhi aktivitas antibiofilm. *E. coli* dan *S. enteritidis* lebih resisten terhadap antibiofilm dibandingkan *S. aureus.* Ekstrak kulit manggis memiliki aktivitas antibiofilm dan antioksidan yang tinggi sehingga berpotensi untuk digunakan sebagai pakan imbuhan untuk mengendalikan bakteri patogen dalam saluran pencernaan unggas.

Kata Kunci: Ayam, Tanaman, Senyawa Sekunder, Antioksidan, Antibiofilm

ABSTRACT

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The occurrence of microbial resistance against antibiotic due to the subtherapeutic dosage of antibiotic growth promoter (AGP) in poultry can be prevented by the antibiofilm substance. Plant secondary compounds have some activities like antioxidant, antimicrobial, and antibiofilm. This research was conducted to obtain the plant with the highest activity of antibiofilm and also antioxidant by analyzing several plant secondary compounds as antioxidant and antibiofilm against chicken's gut bacteria. The tested plants were clove leaves, leaffruit plants, mangosteen peel, cashew nut shell, guava leaves, and bay leaves. These plants were extracted with methanol or n-hexane using sonication method. The antioxidant activity as the IC_{50} value of the plant methanol extracts were determined using α,α-diphenyl-β-picrylhydrazyl (DPPH) assay. The biofilm inhibition activity was tested against *Escherichia coli*, *Salmonella enteritidis*, and *Staphylococcus aureus* ATCC® 29213™ using methanol and n-hexane extracts. All of the samples had antioxidant activity. The clove leaves and leaffruit plants had the highest antioxidant activity, while mangosteen peel extract in methanol had the highest antibiofilm activity against all tested bacteria. The species of bacteria also affected the antibiofilm activity. *E. coli* and *S. enteritidis* were more resistant to antibiofilm then *S. aureus*. Mangosteen peel extract which showed high antioxidant and antibiofilm activity is potential to be used as a feed additive to control the pathogenic bacteria.

Key Words: Chicken, Plant, Secondary Compounds, Antioxidant, Antibiofilm

INTRODUCTION

In poultry industries, pathogenic bacteria like *Escherichia coli*, *Salmonella enteritidis* (both Gram negative), and *Staphylococcus aureus* (Gram positive) often cause severe illness. These industries have been using subtherapeutic dosage of antibiotic growth promoter (AGP) in their poultry feed to kill the bacteria and increase poultry feed efficiency for more than 5 decades (Lin et al. 2013). The use of AGP as feed additive will decrease good gut bacteria bile salt hydrolase (BSH) enzyme activity, such as *Lactobacillus* spp. which takes part in poultry lipid metabolism (Lin 2014).

Practically, the AGP supplementation doesn't kill the pathogenic bacteria effectively due to their ability to form biofilm which is a complex community of microbial cells that are associated with a surface and enclosed in a self-produced biopolymer matrix. The biofilm cannot be easily penetrated by the AGP. Such structured community is a response of bacteria to a signal system named quorum sensing (QS) constituted by inter-cell Therefore, the AGP will only kill the planktonic bacteria (Høiby et al. 2010). The subtherapeutic AGP also leads the bacteria to produce antibiotic resistance gene (Lin et al. 2013). Besides that, the mutation can be occurred due to the oxidative stress of the bacteria colony in the biofilm. When the bacteria had the oxidative stress, the bacteria will be mutated to survive. One solution to control the oxidative stress and decrease the mutation is antioxidant addition (Høiby et al. 2010).

The structure of biofilm matrix caused gradient of oxygen and nutrient occur in biofilm matrix and it is related to the different kind of growth rate of the biofilm active growing and biofilm persistent microbes (del Pozo & Patel 2007). Biofilm active growing microbes grow faster than the biofilm persistent microbes because they are outside of the colony, where the oxygen and nutrition supplies are better. Less oxygen and nutrition supplies in deeper biofilm matrix caused the biofilm persistent microbes have lower metabolism and slower growth rate. To kill the bacteria inside the biofilm or the persistent bacteria need to be noticed. The biofilm structure that cannot be easily penetrated by the antibiotic also protected the bacteria inside from being killed also caused the bacteria inside hard to kill. Another problem is the antibiotic resistance gene that produced by the bacteria easily transferred among the bacteria due to the bacterial communication (QS) inside the biofilm matrix. The communication also can change the non-pathogenic bacteria to pathogenic bacteria. Therefore, antibiofilm substance addition is important to control the pathogenic bacteria (Bjarnsholt et al. 2013).

Naturally, some of higher plants have the ability to produce secondary compounds which have some activities like antioxidant (Bjarnsholt et al. 2013), antimicrobial (Budzyńska et al. 2011), or antibiofilm (Geethashri et al. 2014). Secondary compounds in clove leaves had antimicrobial and quorum quenching (anti QS) activities (Aparna et al. 2014); in leaffruit plants had anticancer, anti-inflammation, and antibacterial activities (Sarin et al. 2014); in mangosteen peel had antioxidant, antimicrobial, antifungal, antiviral, and anti-inflammation activities (Palakawong et al. 2010); in cashew nut had antifungal and antibacterial activities (Harlita et al. 2016); in guava leaves had antimicrobial, quorum quenching, and anti-inflammation activities (Lazar et al. 2013; Biswas et al. 2013); and in bay leaves had antibacterial, antifungal, and antioxidant activities (Kusuma et al. 2011). Secondary compounds might be used simultaneously with AGP as feed supplements. The compounds will inhibit the biofilm formation, while the AGP will kill the pathogen bacteria. This condition will make the subtherapeutic AGP addition become effective and not result in any resistance. Therefore, the benefit of AGP application might be maintained and permitted. This research was conducted to discover the activities of plant secondary compounds as antioxidant and antibiofilm against chicken gut bacteria.

MATERIALS AND METHODS

Plant materials

This research examined the secondary metabolites activity of clove leaves (*Syzygium aromaticum*), leaffruit plants (*Phyllanthus urinaria*), mangosteen peel (*Garcinia mangostana*), cashew nut shell (*Anacardium occidentale*), guava leaves (*Psidium guajava*), and bay leaves (*Syzygium polyanthum*). These samples were collected, dried and powdered by Indonesian Research Institute for Animal Production at Ciawi, West Java.

Source of test microorganisms

Pathogenic cultures used in this research are *E. coli*, *S. enteritidis*, and *S. aureus*. Pure cultures of *E. coli* and *S. enteritidis* were isolated from the poultry gut and obtained from Sri Laboratory, Bogor, while the pure culture of *S. aureus* ATCC® 29213™ was obtained from Atma Jaya Catholic University of Indonesia, Jakarta.

Preparation of plant extracts

All of the plant samples were extracted with methanol and n-hexane. The methanol extract was used for antioxidant and antibiofilm assays, while the

n-hexane extract only for antibiofilm assay. The stock extract solution for each sample was carried out by soaking 0.5 gram powdered sample in 10 mL methanol or hexane and macerated in sonication water bath for 30 minutes. Then, the extract was filtered with filter paper (Annegowda et al. 2010). The methanol extract was then diluted to appropriate solution for antibiofilm assays.

For antibiofilm assay samples of sonicated extracts in methanol or n-hexane were evaporated using rotary evaporator at 40 ˚C (Annegowda et al. 2010) until dry and diluted with 2 mL of 10% dimethyl sulfoxide (DMSO) into extract concentration of 50 mg/mL. After that, all filtrates were filtered using 0.2 µm non-polar filters (Selim et al. 2014).

Preparation of bacterial cultures

Stock cultures were stored in the mixture of tryptic soy broth (TSB) and 87% glycerol (4:1) at -80 ˚C. While working cultures were maintained in nutrient agar (NA) and kept at 4 ˚C. In each experiment, the cultures were refreshed in NA overnight at 37 ˚C. After that, the bacteria were prepared in broth medium. *E. coli* and *S. enteritidis* were inoculated in TSB, while *S. aureus* in TSB with 2% glucose and 2% sucrose overnight at 37 ˚C (Djordjevic et al. 2002).

Antioxidant activity assay with α,α-diphenyl-β-picrylhydrazyl (DPPH)

DPPH solution was diluted in methanol (0.2 mM). A series of different concentrations of samples was prepared from stock extracts to obtain 50% inhibition depending on the kind of plant extracts. To determine the antioxidant activity, 1 mL of sample was reacted with 2 mL DPPH solution. As control, 2 mL DPPH solution was reacted with 1 mL methanol. Ascorbic acid (5-20 ug/ml) was also used as the reference. All mixtures were incubated at room temperature in the dark condition for 30 minutes. The absorbance of each sample was measured at 517 nm wavelength. The blank used in this measurement was methanol. As control, 1 ml of methanol was reacted with 2 ml of DPPH solution. The DPPH inhibition activity of each sample was calculated towards control absorbance, while IC_{50} value was determined in the curve of DPPH inhibition percentage towards plant extract concentration (Shekhar & Anju 2014).

$$\% \text{ DPPH Inhibition} = \frac{X - Y}{X} \times 100\%$$

X = Control absorbance
Y = Sample Absorbance

Biofilm inhibition activity assay

The absorbance of each bacterial culture prepared in the broth medium was measured at 600 nm wavelength to reach 0.132 (0.5 McFarland). The bacterial cultures were inoculated as much as 180 µL together with 20 µL plant extracts in 96-wells polystyrene microplates (Iwaki) overnight at 37˚C. As positive control, 180 µL cultures were inoculated together with 20 µL sterile 10% DMSO. However, the negative control only used 200 µL broth medium without bacterial culture. For biofilm inhibition activity against *S. aureus*, the methanol extract's concentrations were diluted 10 times.

After the incubation time, medium and planktonic cells were removed and the plate was washed with distilled water twice with flow through method. Then, the plate was air dried for the staining for 15 minutes. Each well was stained with 200 µL 0.4% crystal violet in ethanol for 30 minutes. Crystal violet was then removed and the plate was washed with distilled water three times with flow through method. The plate was air dried again and added with 200 µL ethanol for 30 minutes. The blue solution appeared as the biofilm expression and the solution was transferred to the new microplate and the absorbance of each well was measured with BIO-RAD Model 680 Microplate Reader at 595 nm wavelength. Biofilm inhibition activity was measured with this equation below (Djordjevic et al. 2002).

$$\text{Biofilm inhibition} = \frac{A - B}{C} \times 100\%$$

A = Positive Control
B = Sampel absorbance
C = Positive Control absorbance

RESULTS AND DISCUSSION

Antioxidant activity

The samples concentration for the most suitable IC_{50} value was varied depending on the sample source (Table 1). The highest IC_{50} value means the lowest antioxidant activity. Based on the IC_{50} value data, it seems that there were two groups of antioxidant activity. The first group with the high antioxidant activity (275-360 µg/mL) were cashew nut shell, mangosteen peel, leaffruit plants, and clove leaves, while the second with low antioxidant activity (> 600 µg/mL) were bay leaves and guava leaves. However, the samples with high antioxidant activity had significantly lower antioxidant activity compared to the ascorbic acid which had 14 µg/mL IC_{50} value. It was

Table 1. Antioxidant sample concentration and IC_{50} value

Samples	Concentration range (µg/mL)	IC50 Value (µg/mL)
Bay leaves	500-800	760
Guava leaves	400-800	626
Cashew nut shell	100-500	360
Mangosteen peel	200-350	338
Leaffruit plants	100-500	292
Clove leaves	100-400	275
Ascorbic acid*	5-20	14

* Ascorbic acid was used as a reference.
**These antioxidant activities were also correlated with their anti bacteria and antifungi activities in Sinurat et al. (2017).

possible since ascorbic acid is a pure compound having very high antioxidant activity, while the plant extracts contained mixture of secondary compounds. Purification of the compound in plant extract might increase the activity.

The low reactivity of DPPH to hydrophobic antioxidant and pH dependent were the limitations in this assay. Different solvent would cause different results because the reactions of the DDPH were different in every solvent (Sultana et al. 2009). The use of methanol as solvent in this experiment also affected the results. Reactions of phenolic compounds were faster in methanol compared to ethanol or acetone. Since the major secondary compounds of the samples were phenolic compounds, the results showed high antioxidant activity (Xie & Schaich 2014).

Biofilm inhibition activity

All methanol extract samples showed biofilm inhibition to all tested bacteria, except cashew nut shell which is negatively against *E. coli* (Table 2). This condition might occur because the oily compound in the extract adhered to the surface and made the absorbance measurement invalid. Among all methanol extract samples, mangosteen peel had the highest biofilm inhibition activity against all tested bacteria.

Methanol extracts for *S. aureus* were diluted 10 times with sterile 10% DMSO because the antibiofilm activity against *S. aureus* already detected at low concentrations. The 10 times dilution of the filtrates for *S. aureus* showed that *E. coli* and *S. enteritidis* were more resistant compared to *S. aureus*. The data showed that all methanol extract samples had antibiofilm activities and mangosteen peel extract had the highest antibiofilm activity (Table 2). The ability of mangosteen peel as antibiofilm was related to the α-mangostin (xanthone) contained in it. As reported by Nguyen et al. (2015), its antibiofilm activity related to the inhibition of glycosyl-transferases which associated with the formation of the EPS matrix.

Among all methanol extract samples, cashew nut shell liquid had no antibiofilm activity against *E. coli*. The negative result occurred could not be easily described, since the extract containing anacardic acid known as antibacterial compound. The possible explanation is the acid or the oily compound might be used by *E. coli* to form biofilm matrix (Rodrigues 2014). However, it was still unclear whether the oily compound which interfere the absorbance measurement was biofilm matrix or not.

All the n-hexane extracts did not show antibiofilm activity as high as the methanol extract (Table 2). The n-hexane extract samples did not show any biofilm inhibition activity against *S. aureus*. The n-hexane extract samples only showed antibiofilm activity against *E. coli* (leaffruit plants) and *S. enteritidis* (cashew nut shell). This condition might occur due to the sterol and triterpene contained in leaffruit leaves, such as β-sitosterol, β-amyrin, methyl gallate, and trimethyl 1-3,4 dehydrochebulate (Sarin et al. 2014). The ability of cashew nut shell as antibiofilm agent might occur due to the secondary metabolites contained in it which could be extracted in n-hexane. Those secondary metabolites were monounsaturated anacardic acid, β-sitosterol, monounsaturated cardol, saturated cardol, di-unsaturated cardol, and triacontene (Taiwo 2015). The previous study on anaerobic gas production method showed that cashew nut shell extract in n-hexane had antimicrobial activity against poultry gut bacteria (Sinurat et al. 2018). Biofilm inhibition assay method used in the research had some limitations. One of them was the single culture used may not behave or react like the mixed population found in natural environment. Another limitation was microtiter plate itself had different surface compared to the natural environment. Regardless, this method is a widely used tool in the study of biofilm. In this research, the composition of the

Table 2. Biofilm inhibition percentage against tested bacteria

Sample	Inhibition Percentage (%)		
	E. coli	*S. enteritidis*	*S. aureus**
	Methanol extract		
Bay leaves	44.34 ± 3.89	60.22 ± 2.86	85.87 ± 2.15
Guava leaves	49.94 ± 2.38	74.32 ± 1.02	88.47 ± 0.89
Cashew nut shell	**	72.05 ± 1.64	94.71 ± 0.48
Mangosteen peel	62.86 ± 2.30	81.63 ± 1.66	95.28 ± 0.54
Leaffruit plants	47.68 ± 2.86	76.49 ± 3.90	88.48 ± 0.93
Clove leaves	53.24 ± 3.73	79.40 ± 1.72	73.30 ± 2.07
	n-Hexane extract		
Bay leaves	**	**	**
Guava leaves	**	**	**
Cashew nut shell	**	11.80 ± 1.12	**
Mangosteen peel	**	**	**
Leaffruit plants	28.65 ± 6.76	**	**
Clove leaves	**	**	**

* The concentration of methanol extracts for *E. coli* and *S. enteritidis* was 10 times higher than for *S. aureus*.
** Negative results.

media was an important thing because it affected the biofilm formation. The composition of the media was the leading role in forming the biofilm matrix. For *S. aureus* biofilm formation, as reported by (Skyberg et al. 2007), the addition of sugar caused higher biofilm formation (data not shown). However, the addition of sugar for *E. coli* and *S. enteritidis* made the bacteria not forming the biofilm matrix. To get comparable antibiofilm data for each bacterium, optimisation of the media and its microenvironment should be carried out (Skyberg et al. 2007).

All samples had antioxidant and antibiofilm activities. The antioxidant compounds should take the role in inhibition process in all pathogenic bacteria, however, in this experiment not all high antioxidant activities were followed with high antibiofilm activities. When we correlated both activities statistically, there was only one significantly correlation between antioxidant activities of all plant methanol extracts and antibiofilm activities towards *S enteridis* with R^2 = 67%; those from other bacteria and extracts did not significantly correlated. It was reported that the antibiofilm compound of plant secondary metabolites is preventing the communication among the bacteria to build the biofilm (Koh et al. 2013) and it would decrease the mutability of the bacterial cells inside the matrix, especially the ability to form the antibiotic resistance gene. The antibiofilm activity expressed differently in each bacterium might occur due to a specific correlation between each bacterium and the antibiofilm activity of each plant extract. The antioxidant activity of mangosteen peel was quite high to take the role in antibiofilm activity.

In the test of anti-microorganism growth (antibacteria) by *in vitro* fermentation method or by measuring the total gas production, all plant extracts using methanol and hexane inhibited the gas production or the growth of bacteria. However, using clearing zone test only methanol extracts of guava leaves, cashew nut shell, mangosteen peel, and clove leaves showed inhibition activity to *E. coli* but no inhibition to *S. enteritidis*, while the extracts of leaffruit plants and bayleaf did not inhibit the growth of both bacteria. In the hexane extracts, only *S. enteritidis* was inhibited by guava leaves, mangosteen peel, and leaffruit plants, the extracts of cashew nut shell, bay and clove leaves did not form any clearing zones (Sinurat et al. 2017). The antibacteria activity of the plant extracts might affect the antibiofilm activity, however, the gas formation test mimics the digestion system which contain enormous kind of bacteria. Therefore, the data cannot be compared to the antibiofilm activity towards specific pathogen bacteria like in our experiment. Results of clearing zone formations which used same pathogenic bacteria and same concentration of plant extracts may indicate the false conclusion of antibiofilm activity. However, these data were observed from different method. In the top of that a lot of extracts which showed antibiofilm activity did not show antibacterial activity except for methanol extracts of mangosteen

peel, guava and clove leaves for *E.coli*. All other positive antibiofilm activities were not influenced by antibacterial activity. Determinations of antibacterial together with antibiofilm activities were suggested by Er et al. (2014). They reported that some food or feed preservatives such as ciprofloxacin, sodium nitrite and potassium sorbate had more antibacterial than those antibiofilm activities which should be considered for the possibility the cause of resistency.

The antibiofilm activity of the plant extracts will be useful as feed supplement that preventing the quorum sensing of bacterial pathogenic bacteria to form the colonies inside the biofilm or only in the planktonic cell condition. This condition may result the effectivity of the AGP as bacterial killer. At the end no resistant cells will be formed. For future study, the optimum concentration as feed supplement needs to be calculated in relation to replace AGP so the supplementation becomes effective. It would be more interesting if the extracts could replace all the AGP supplementation to reduce the supplementation cost and also to prevent the occurrence of antibiotic resistance genes. For more effective supplementation, probably it needs to purify the extract to get the specific antioxidant and antibiofilm compounds with high activity. Furthermore, it is important to test the effectivity of the pure antioxidant compound as antibiofilm which will show more certain relation of the activities.

CONCLUSION

In conclusion, all of the samples had antioxidant activity. The clove leaves and leaffruit plants had the highest antioxidant activities, while the bay leaves had the lowest antioxidant activity. For the antibiofilm assay, all of the methanol extracts had antibiofilm activity, except cashew nut shell extract against *E. coli*. Mangosteen peel extract in methanol had the highest antibiofilm activity against all bacteria. However, for the n-hexane extract, the antibiofilm activity only showed in leaffruit plants against *E. coli* and cashew nut shell against *S. enteritidis*. The plant extracts which showed high antioxidant and antibiofilm activities such as leaffruit plants, mangosteen peel and clove leaves might be applied as feed supplement for controlling pathogenic bacteria.

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