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Determination of the best method for processing gambier liquid by-product [*Uncaria gambir* (hunter) roxb] as natural antioxidant sources

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ABSTRAK

Penelitian ini bertujuan untuk menentukan metode terbaik dalam pengolahan limbah cair gambier (GLB) berdasarkan aktivitas antioksidan sebagai sumber antioksidan alami. Metode yang digunakan adalah A (fraksinasi etil asetat), B (pengeringan menggunakan oven) dan C (pengeringan menggunakan freeze dryer). Variabel yang diamati terdiri dari ekstrak kering (menggunakan metode penimbangan), kadar tannin (menggunakan metode tepung kulit), kadar katekin (menggunakan metode SNI 01-339-2000), total fenol (menggunakan metode folin ciocalteu), dan aktivitas antioksidan (menggunakan metode DPPH) berdasarkan nilai IC₅₀. Pada pengujian aktivitas antioksidan juga dibandingkan dengan vitamin C sebagai kontrol positif. Hasil dari penelitian ini menunjukkan bahwa metode pengolahan berpengaruh secara nyata (P<0,05) terhadap jumlah ekstrak kering, kadar tannin, kadar katekin, total fenol dan aktivitas antioksidan GLB. Jumlah ekstrak kering, kadar tannin, kadar katekin, total fenol dan aktivitas antioksidan tertinggi secara berurutan terdapat pada perlakuan B (10.76%), B (39.69%), A (86.07%), C (80.97 mg GAE/g), and C (IC₅₀ 2.74 µg/mL). Aktivitas antioksidan pada pengeringan dengan freeze dryer ini setara dengan aktivitas antioksidan vitamin C (2.88 µg/mL). Jadi, perlakuan C (pengeringan menggunakan freeze dryer) adalah metode pengolahan GLB terbaik untuk menghasilkan aktivitas antioksidan tertinggi.

Kata Kunci : Aktivitas antioksidan, fraksinasi etil asetat, limbah cair gambier, metode pengeringan, senyawa aktif.

ABSTRACT

The study was conducted to determine the best gambier liquid by-product (GLB) processing methods based on antioxidant activity as natural antioxidant sources. The processing methods were A (ethyl acetate fractionation), B (oven drying) and C (freeze drying) methods. The observed variables were dry extract (using weighing method), tannin (using hide powder method), catechin (using SNI 01-339-2000 method), total phenol (using folin ciocalteu reagents) and antioxidant activities (DPPH method) based on IC₅₀. These antioxidant activities were compared with vitamin C as a positive

control. The results indicated that the different processing methods significantly affected ($P < 0.05$) dry extract, tannin, catechin, total phenol and antioxidant activity of GLB. The highest dry extract, tannin, catechin, total phenol, and antioxidant activity from GLB were at treatment B (10.76%), B (39.69%), A (86.07%), C (80.97 mg GAE/g), and C ($IC_{50} 2.74 \mu\text{g/mL}$), respectively. The antioxidant activity on treatment C was not different from vitamin C antioxidant activity ($2.88 \mu\text{g/mL}$). Thus, the treatment C (freeze drying method) was the best method to process GLB based on antioxidant activity as natural antioxidant sources.

Keywords: antioxidant-activity, chemical content, drying method, ethyl acetate fractionation, gambier liquid by-product.

INTRODUCTION

Gambier is a product from the leaves and twigs water extract of gambier plant (*Uncaria gambier* (Hunter) Roxb). The major compound of gambier is tannin up to 26% (Yeni, *et al.*, 2014) and catechin up to 52.25% (Widiyarti, *et al.*, 2020). In Indonesia, gambier is used as a mixture of chewing herb and as anti-diarrhea (Murdja, *et al.*, 2017), and gambier also has been known that has an antioxidant activity (Widiyarti, *et al.*, 2020). Gambier is produced by boiling the leaves and twigs of gambier plant for one hour, and then pressed, precipitated and drained to remove the watery fraction, and the drain material then molded and dried (Andasuryani, *et al.*, 2014). The watery fraction that obtained from the process of making gambier is called gambier liquid by-product (GLB) or "Kalincuang" as local name in West Sumatra, Indonesia.

Most of the gambier is produced in the Province of West Sumatra. In 2018, gambier production was up to 28.739,5 ton (BPS, 2020) and the amount of GLB was approximately 4% of the weight of the gambier product (Sofyan, *et al.*, 2015). So, in 2018, there was about 1.149,56 liter

GLB that have not been used optimally because most of the traditional gambier farmers just reused it by mixing with water in boiling process and some of them were just discarded on the land.

Even though most of the compounds of GLB is water, it is assumed that GLB still contains the active compounds such as in the gambier. This is because tannin and catechin are also water soluble. So, GLB has the potency to be utilized as gambier. As describe above, that gambier has an antioxidant activity, so it is considered that GLB also has an antioxidant activity. It might be used as a non-nutritive feed additive in the future, for example to reduce heat stress on poultry.

The GLB is a by-product that most of its content is water. So, to obtain the optimal benefit

of this GLB as the source of natural antioxidant, it needs a specific processing method. The lack of sufficient information on the processing method of GLB as the source of natural antioxidant, has led us to study the appropriate processing methods to obtain the highest dry extract, tannin, catechin, total phenol, and antioxidant activity of GLB. This proper processing method can be used in the next time to process GLB optimally as natural antioxidant source for application in poultry diets.

MATERIALS AND METHODS

Material

The materials used in this study were gambier liquid by-product (GLB), cotton bud, ethyl acetate, hide powder and DPPH. Gambier liquid by-product was taken from the gambier home industry in Siguntur Muda Village, Koto XI Tarusan District, Pesisir Selatan Regency, West Sumatra Province, Indonesia. The GLB was filtered using cotton buds to separate the insoluble materials, and stored at -4°C before utilized to avoid damage of the active compounds therein contained.

Method

This study was performed in a completely randomized design (CRD) with three treatments and five replicates for each treatment. For the antioxidant activity, vitamin C was used as the positive control. The treatments applied to process GLB were as follows: treatment A was fractionation with ethyl acetate which was then evaporated with rotary evaporation, treatment B was a drying method by using an oven, and treatment C was also a drying method by using a freeze dryer (Torres, *et al.*, 2010). Measured variables were dry extract, tannin, catechin and total phenol contents, and antioxidant activity of GLB.

Data collecting

Dry extract determination: Ethyl acetate fractionation was carried out by using sonicator (Elma S 300 H Elmasonic) to accelerate the extraction process (Luque-Gracia and Castro, 2004). The fractionation process was carried out as much as 3 times until extraction was completed. This ethyl acetate fraction then was evaporated using a rotary evaporator (Buchi Rotavapor R-215, Vacuum Controller V-850, Recirculating Chiller F-114, Bath Heating B-491) at 55°C until the fraction was thickening. The thickened fraction then was dried in an oven at 55°C until the constant weight of dry extract was obtained. The oven drying process was carried out at 60°C until the constant weight of a dry extract was obtained, while the freeze drying was performed with a freeze dryer (Christ Alpha 1-2 LDplu) at a temperature of -55°C and vacuum (vacubrand RZ 2.5) until the constant weight of dry extract was obtained. The dry extracts of the three treatments then were calculated using the formula as follows:

$$\text{Dry extract (\%)} = \frac{\text{Dry extract (gram)}}{\text{Gambier liquid by-product (ml)}} \times 100$$

Tannin determination: Tannin level in the dry extracts was determined by the hide-powder method that had been modified by Seigler, et al. (1986). A number of dry extracts was dissolved in distilled water, then filtered and the obtained filtrate then divided into two parts. The first part was dried to see the level of water soluble extract. The second part was treated with the hide powder to withhold the tannin content. The level of tannin was calculated by the formula:

$$\text{Tannin (\%)} = \text{level of water soluble extract (\%)} - \text{level of water soluble extract without tannin (\%)}$$

Catechin determination: The level of catechin in the dry extracts was determined by using the SNI 01-339-2000 method. The dry extract of GLB was extracted with ethyl acetate, then filtered by using whatman filter paper 41 and diluted with ethyl acetate until the absorbance was read. The absorbance was measured by a spectrophotometer (UV-1800 Shimadzu A11455008975) at a wavelength of 279 nm. Standard catechin (Sigma C1251-5G) was used as comparison. The level of catechin was calculated by the formula:

$$\text{Catechin (\%)} = \frac{\text{Et 279 } W_s}{\text{Ec 279}} \times \frac{W}{W_s} \times 100$$

Where Et 279 is the absorbance of sample solution at a wavelength of 279 nm; Ec 279 is the absorbance of standard solution at a wavelength of 279 nm; WS is the standard weight; W is the sample weight.

Total Phenol determination: Total phenol was determined by Folin Ciocalteu reagents (Singleton and Rossi, 1965), as was performed by Marinova et al. (2005). The standard was made with 1 ml gallic acid at four different concentrations (120, 140, 160 and 180 ppm) then added with 9 ml H₂O, and 1 ml of 9% folin ciocalteu and boiled. The mixture was then incubated at room temperature for 5 minutes, and then 10 ml of Na₂CO₃ 7% and 4 ml H₂O were added. The mixture was stirred and incubated at room temperature for 90 minutes. The absorbance was measured using a spectrophotometer (UV-1800 Shimadzu A11455008975) at a wavelength of 750 nm. Gallic acid was used as a standard and the result was expressed as milligram of gallic acid equivalents (GAE) per gram of defatted sample.

Antioxidant activity determination: Antioxidant activity was determined by the DPPH method (Blois, 1958) and expressed in IC₅₀ (Molyneux, 2004). An amount of 2 ml of each extract sample with 6 different concentrations (dry extract dissolved in methanol) added with 1 ml of 2,2-diphenyl-picrylhydrazyl (DPPH) reagent (Sigma-Aldrich). The mixtures were then stirred and incubated for 30 minutes at room temperature that is protected from light. The absorbance was measured by a spectrophotometer (UV-1800 Shimadzu A11455008975) at a wavelength of 517 nm. Antioxidant activity (%) was calculated by:

$$\text{Antioxidant activity (\%)} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100$$

Value of IC₅₀ for each treatment was determined by linear equations of antioxidant activity from 6 different concentrations. As a positive control, vitamin C (Sigma-Aldrich) was used.

Data analyses

All of the data were analyzed by one-way ANOVA of completely randomized design (CRD), and followed by Duncan Multiple Range Test for testing the significant level at 5%. The data obtained were statistically analyzed using SPSS software version 21.0 (SPSS, 2012).

RESULTS AND DISCUSSIONS

The average of dry extract, tannin, catechin and total phenol of the three different methods of processing and analyses of GLB are figured out in Table 1. The results of antioxidant activity are depicted in Figure 1.

Table 1 shows that the different in GLB processing methods significantly affected ($P < 0.05$) the dry extract amount. The average of dry extract content obtained by drying in the oven (treatment B) was higher than those of the other two treatments (treatment C and treatment A) ($P < 0.05$). The dry extract content in treatment C was higher than that of treatment A ($P < 0.05$). So, the highest dry extract content was produced by treatment B.

The highest dry extract content in treatment B was due to that in this treatment, only water

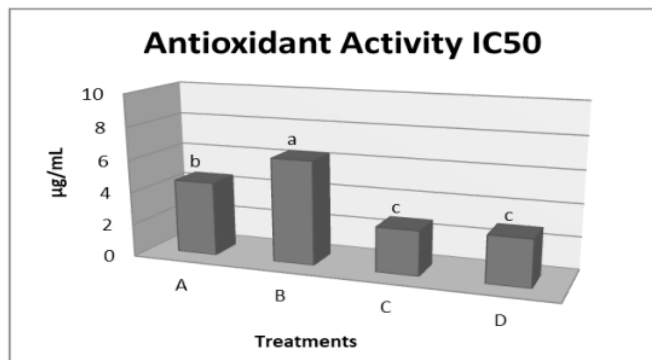
portion that was evaporated, while all of the dissolved chemical compounds remained as residues (dry extract) (Azwanida, 2015). On the other hand, freeze drying method (treatment C) has the same principle as drying a sample, in which only water that will be eliminated. Even though, the results of dry extract content produced by treatment B was higher than that in treatment C, this difference was very small (only 0.4%). This was due to that the freeze drying method by using a vacuum removed the gas from sublimation process of GLB, so that the volatile compound could be easier to be eliminated (Nireesha, *et al.*, 2013). The same case was also reported by Azwanida (2015), who found that the use of freeze dryer could remove some of the sample by splattering out into the freeze-flask.

The dry extract content produced by treatment A was the lowest among the treatments.

Table 1. The average of dry extract amount and their tannin, catechin and total phenol contents of GLB.

Treatments	Dry extract amount (%)	Tannin Content (%)	Catechin Content (%)	Total Phenol mg GAE/g
(A) fractionation with ethyl acetate	4.37 + 0.10 ^c	13.44 + 1.28 ^c	86.07 + 1.70 ^a	54.67 + 4.80 ^b
(B) drying using an oven	10.76 + 0.05 ^a	39.69 + 0.32 ^a	4.53 + 2.61 ^c	16.25 + 3.01 ^c
(C) drying using a freeze dryer	10.36 + 0.14 ^b	37.29 + 2.06 ^b	16.84 + 3.10 ^b	80.97 + 6.73 ^a

a,b,c The treatment mean with different superscripts in the same column indicated significant different ($P < 0.05$).



A: Dry extract was produced by fractionation with ethyl acetate

B: Dry extract was produced by oven drying

C: Dry extract was produced by freezer drying

D: Vitamin C as positive control

The different a, b, c indicated significant difference among treatments ($P < 0.05$).

Figure 1: Antioxidant activities of dry extracts compare to vitamin C

This was due to the fact that the ethyl acetate was a semi-polar organic solvent with the polarity value of 4.4 (Vuong, *et al.*, 2010), in which this solvent was only dissolved in the semi-polar compounds of the GLB (Yeni *et al.*, 2014), while the polar and non-polar active compounds were mostly left in the GLB. That was why the dry extract content obtained in this treatment was the smallest.

The average of tannin content in each of dry extract produced from different GLB treatments was significantly different ($P < 0.05$) (Table 1). The tannin content in dry extract produced by treatment B was significantly higher than those of the other treatments (C and A) ($P < 0.05$). The tannin content of dry extract of treatment C was also significantly higher than that of the treatment A ($P < 0.05$). Thus, the highest tannin content of dry extract was obtained from treatment B.

The highest tannin content of dry extract from treatment B was due to the presence of direct heat for a long time in oven drying which resulted in a polymerization reaction. This polymerization reaction changed the catechin compound to condensed tannin. As a result, the tannin level in this treatment B was the highest among the other treatments (C and A). Consequently, level of catechin compound was lower in the dry extract of this treatment B. This result was supported by Shi *et al.* (2020) who explained that catechin/epicatechin, epicalocatechin, epicatechin gallate, dimer catechin, fisetinidin, chebulic acid are the main monomer unit structures of condensed tannin. The tannin content in this dry extract of treatment B was also higher than that in the raw gambier from Siguntur village (26%) found by Yeni *et al.* (2014), and in tea leaves (9.25%) reported by Tugiyanti *et al.* (2019).

The average of catechin content in each dry extract was significantly influenced ($P < 0.05$) by processing methods GLB. The catechin content in dry extract of treatment A was higher ($P < 0.05$) than those of treatments C and B. The catechin content of treatment C was higher ($P < 0.05$) than that of the treatment B. So, the highest catechin content of dry extract of GLB was produced by treatment A.

The highest catechin content of dry extract of GLB in treatment A was due to the high solubility of catechin in ethyl acetate solvents. The same result was reported by Yeni *et al.* (2014) who found that the ethyl acetate was a solvent that was able to attract catechin compounds optimally

in gambier compared to the other organic solvents. Catechin content in this dry extract was less than that of dry extract in gambier purification or repeat extraction with ethyl acetate that was found by Yunarto and Aini (2015) (more than 90%), but this catechin content was higher than that of the catechin content in gambier product from Siguntur village (45.73%) (Kasim *et al.*, 2015).

The different GLB treatments (A, B and C) significantly affected ($P < 0.05$) the average of total phenol in each dry extract (Table 1). The total phenol from treatment C was higher than those of treatments A and B, and the total phenol from treatment A was also higher than that of the treatment B. Thus, the highest total phenol of dry extract of GLB was obtained from treatment C.

The highest total phenol of dry extract of GLB from treatment C was consistent with the research results of Torres *et al.* (2010) who found that the freeze drying was the best method for drying samples containing phenol active compounds. With the use of low temperatures, the phenol compounds contained in GLB were stable, so that they could withstand to oxidation or other damage (Kalt *et al.* 1999). Moreover, the GLB was known to contain 27 chemical contents, in which there are phenol group including catechin and tannin (Ismail *et al.* 2021). That was why in this study, the highest total phenol in the dry extract was produced from the treatment C.

Figure 1 shows that the antioxidant activity in each dry extract of GLB was significantly influenced ($P < 0.05$) by processing methods. The antioxidant activity in the dry extract produced by treatment C was higher ($P < 0.05$) than those of treatments A and B, and the antioxidant activity of treatment A was also higher ($P < 0.05$) than that of treatment B. The antioxidant activity of dry extract produced by treatment C was not significantly different ($P > 0.05$) from treatment D (vitamin C as a positive control). So, the highest antioxidant activity of dry extract of GLB was produced by freeze drying (treatment C), and it was equal to vitamin C antioxidant activity.

The dry extracts had high antioxidant activities because the values of IC_{50} of these dry extracts less than 50 $\mu\text{g/mL}$. The IC_{50} values of these dry extract were 2.74, 4.55, and 6.34 $\mu\text{g/mL}$ for treatments C, A and B respectively. The antioxidant activity of these dry extract was higher than that of the gambier product from Halaban village which had IC_{50} value of only 303.4 $\mu\text{g/mL}$, when it was extracted

with ethyl acetate, its IC₅₀ value raised up to 87.52 µg/mL (Yeni *et al.*, 2014). Meanwhile, the antioxidant activities of these GLB dry extracts were also higher than that of the methanol extract of *Uncaria gambier* which had IC₅₀ value of 18.27 µg/mL (Amir *et al.*, 2012)

In the dry extract, the highest antioxidant activity was found in the dry extract produced from treatment C (Figure 1). As mentioned above, this dry extract contained more total phenol compare to other dry extracts obtained from other treatments (A and B). This was due to the use of low temperature (-55°C) in extraction using freeze dryer to remove water content, so the active compounds become more stable (Torres *et al.*, 2010). The high antioxidant activity of treatment C was also due to the high in total phenol of dry extract from treatment C, in which this phenol compounds functioned as an antioxidant by utilizing OH⁺ groups (Cao *et al.* 1997). The total phenol in this treatment reached 80.97 ± 6.73 mg GAE/g and the IC₅₀ reached 2.74 ± 0.39 µg/mL. This was consistent with the result of study by Kour *et al.* (2014) and Otmani *et al.* (2019) who obtained that the total phenol content had a positive correlation with antioxidant activity.

CONCLUSION

The best method for processing GLB based on its antioxidant activity was treatment C (Freeze dryer). This treatment had antioxidant activity with IC₅₀ of 2.74 µg/mL, and it was equal to vitamin C antioxidant activity with IC₅₀ of 2.78 µg/mL as positive control.

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