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Antioxidant and Antidiabetic Compounds from Ebony Leaf Extract (*Diospyros celebica* Bakh.) and Their Correlation with Total Phenolic and Flavonoid Compounds in Various Solvent Extraction

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ABSTRACT: Ebony (*Diospyros celebica*. Bakh) is a native plant of Indonesia that is used as a traditional medicine for diabetes mellitus. Previous studies state that ethanol extract from ebony leaves significantly reduced blood glucose levels of diabetic rats induced by alloxan. This ability is thought to be caused by secondary metabolites which is phenolic and flavonoid compound. However, research related to structure compounds in extract and composition of solvent variations in the extraction process to get the most optimal activity and also their correlation with total phenolic and flavonoid compounds has not been carried out. This study aimed to evaluate the composition of the optimum solvent in the extraction process and also to know the structure compounds contained in the extract. In the present study, we extracted the ebony leaves with three solvents of different polarity (ethanol-water), i.e. ethanol 50% (EtOH), EtOH 70%, and EtOH 96%. Quantitative estimation of total phenolic and flavonoid contents. These extracts were future evaluated for their activity and the content of compounds in the extract was identified using LCMS/MS. The results showed that phenolic compounds that have been identified in ebony leaf extract are methyl gallate and (-)-Epiafzelechin 3-gallate and we found there were significant differences in all test parameters between the three extracts which is EtOH 96% extract gave the best.

KEYWORDS: *Diospyros celebica*, leaf, ethanol-water, antioxidant, antidiabetic, TPC, TFC

1. INTRODUCTION

Indonesia is one of the largest biodiversity countries in the world after Brazil and declared to have a lot of herbal medicine as local wisdom, especially in rural people [1]. *Diospyros L* is a large genus from the Ebenaceae family and consists of more than 500 species spread across various warm-climate countries such as China, Korea, Japan, Brazil, Turkey, Italy India, and Pakistan. Several species of this genus also have been reported as ethnomedicine such as *D.kaki* has been reported as a nerve agent in China [2]. Other studies also reported that several species of this genus showed some pharmacological activities as antimicrobial, hepatoprotective, antioxidant, and antidiabetic [3–5]. One of the medical plants from the *Diospyros* genus that have been ethnobotanically used as an antidiabetic in Indonesia is ebony leaves (*Diospyros celebica* Bakh.) [6]. Kartini et al. (2018) reported that 500 mg/kgBW of ethanolic extract eboni leaves (*D.celebica* Bakh.) was significantly able to reduce blood glucose levels in alloxan-induced diabetic rats. Besides being able to provide as an oral antidiabetic, ethanol extract of ebony leaves in a concentration of 10% for topical use has been reported to accelerate the wound healing of diabetic rats induced with alloxan [7]. All activity of ebony leaf is suspected to be caused by the activity of some secondary metabolites compounds such as phenolic, flavonoids, saponins, tannins, and alkaloids [8].

Some phenolic and flavonoid compounds from plants generally provide antidiabetic activity through mechanisms such as antioxidants. Antioxidants are reported to reduce oxidative stress so have to prevent

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diabetes mellitus [9,10], and it's able to protect the beta pancreatic cells from the toxic effects of free radicals that are produced during chronic hyperglycemia so the insulin levels can be maintained in the blood glucose level in normal condition [11,12]. In addition, flavonoids are also through to reduce blood glucose levels by inhibiting the absorption of glucose in the gastrointestinal tract [13–15].

Extraction optimization aims to find or process development that can produce the best value from the condition used. The optimization process is usually focused on cost-effectively selecting the right conditions so that can provide effective and efficient results from a process perspective [16]. The important factor that plays a role in the extraction process is the selection type of solvent used. The choice of solvent is based on the law of like-dissolve-like where a solvent having the same polarity or close to the desired solute will provide better performance in the extraction process [17,18], so the extraction compounds will indirectly be affected by the type of solvent used and of course will affect to their activity[19,20]. Based on the description, this study was carried out to see the effect of various compositions of ethanol and water solvent in the extraction process of ebony leaf on secondary metabolite profile and biological activities including antioxidant and α-glucosidase inhibition. This study will also evaluate the correlation between biological activities and the total phenolic and flavonoid contained in each extract.

2. MATERIALS AND METHODS

2.1. Materials

Dimethyl Sulfoxide (DMSO), NaH_2PO_4 (Merck), Na_2HPO_4 (Merck),α-glucosidase enzyme, 4-nitrophenyl-α-D-glucopyranoside (p-NPG), Na_2CO_3 (Merck), Kreb-Ringer bicarbonate, 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose, $NaNO_2$ (Merck), NaOH (Merck), DPPH (TCI), Folin-Ciocalteureagen (Merck), aluminum chloride (Merck), Methanol (Merck), quercetin standard (Sigma Aldrich), gallic acid standard (Sigma Aldrich), silica gel G60 0,063 – 0,200 mm (Merck)

2.2. Methods of Sample Collection and Preparation

Ebony leaves were collected from the medicinal plant garden National Research and Innovation Agency Serpong - Indonesia. The sample was identified and authenticated in the Research Center for Plant Conservation and Botanic Garden Indonesian Institute of Science – Bogor Indonesia. The sample was dried under sunlight and then ground. The powder was stored in a container for future use.

2.3. Extraction

Twenty g sample was macerated in each various solvents combination which is ethanol 96, 70, and 50% for 24 hours in room temperature conditions, and then, the macerate was filtered and re-extracted two times again. Macerate concentration was carried out using a rotary evaporator and the concentrated extract was dried in an oven at 50^{o}C temperature for 3 days then, it was weighed and stored at room temperature.

2.4. Determination of Total Phenolic Content (TPC) by Spectrophotometric method

The total phenolic content was determined using the Folin-Ciolcalteu (F-N) colorimetric method [21,22]. Briefly, 250 µL Folin-Ciolcalteu reagent (2N) was added to 250 µL sample (concentration 1000 ppm) and 3,75 mL aqua dest, then homogenized using a vortex mixer for 1 min. After homogenizing, 750 µL sodium carbonate (Na_2CO_3) 2% was added to the mixture and incubated for 30 minutes at room temperature. The absorbance of the solution was measured at 760 nm. In this study, gallic acid in concentrations 0 to 25 ppm was prepared and used as a calibration curve. The samples were analyzed in triplicate.

2.5. Determination of Total Flavonoid Content (TFC) by UV-Vis Spectrophotometry

The total flavonoid content was determined according to the aluminum chloride method [22,23]. Briefly, 250 µL of the sample (concentration 1000 ppm); 2,45 mL aquadest, and 150 µL of $NaNO_2$ 5% were added into a test tube and then mixed using a vortex mixer for 10 s. The solution was left to stand at room temperature for 5 minutes. After that, 150 µL of $AlCl_3$ 10% and 2 mL NaOH 1 M were added to the reaction sample. The absorbance of the solution was measured at 510 nm. Quercetin in concentrations 0 to 30 ppm was prepared and used as a standard calibration curve. The samples were analyzed in triplicate.

2.6. Characterization of secondary metabolites by Thin Layer Chromatography and screening antioxidant activity

Each methanol extract stock solution was made in a concentration of 1000 ppm. 1 µL of each extract was transferred on the silica plate F_{254} (Merck) and left for a second to air-dried. After the extract solution in the silica plate was dried, it was eluted with some type of composition mobile phase. After completing elution, the results were observed under UV light at 254 nm and 366 nm. The plate was also sprayed with DPPH solution in methanol. The white band on the TLC plate indicated the active compounds in the extract.

2.7. Identification of secondary metabolites by Liquid Chromatography Mass Spectrophotometry (LC-MS)

The secondary metabolites in each extract were identified using LC-MS/MS Xevo, G2-XS QTof (Waters MS Technologies). Each extract in methanol solution was made in a concentration of 100 ppm. 10 µL of each extract was injected in system chromatography with separation carried out on a Shim-pack XR-ODS III column (150 x 2.0 mm; 2.2 µm). Column temperature was set at 40°C. The mobile phase consisted of aqua dest with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The gradient of elutions was as follows: 0 – 1 min, 5% B; 11 – 14 min, 100 % B and 14 - 17 min, 5% B. The flow rate of the mobile phase was set at 0.3 mL/min.

Parameters of the ESI source were operated in positive ion mode. The capillary voltage was 2 kV with a source temperature of 120°C. The cone voltage was 30 V with a desolvation temperature of 500°C, a desolvation gas flow of 1000 L/h, and a cone gas flow of 50 L/h. The range of collision energy was 10.00 to 40.00 eV. The precursor ions were selected to perform the analysis.

2.8. Measuring the IC_{50} value for Antioxidant Activity

Antioxidant activity was measured by the DPPH scavenging assay method. This method was carried out according to the procedures of Sasmita et al. and Djamil et al.[24-26], with minor modifications. In this study, quercetin was used as a control. The first step is to prepare a control solution with ranging concentrations of 1 to 20 ppm and a sample solution of 5 – 25 ppm. Briefly, 2 mL sample solution and 500 µL DPPH 0,1 M solution were transferred to a test tube and were mixed with a vortex mixer for 10 s. The solution was allowed to stand for 30 minutes in a dark place to react and then the absorbance was measured at a wavelength of 517 nm. The free radical scavenging activity was expressed as IC_{50} obtained from the regression equation between the percentage inhibition and concentration of each sample. For the % inhibition obtained through calculation with the formula:

$$\%inhibition = \frac{A.blank - A.Sample}{A.blank} x\ 100\%$$

Where $A._{blank}$ is the absorbance of the DPPH solution without sample and $A._{sample}$ is the absorbance of the samples. Each concentration of sample and control was analyzed in duplicate.

2.9. Measuring the IC_{50} value for α-glucosidase Activity

α-glucosidase activity was carried out according to Sajid et al. and Djamil et al procedure [27,28] with minor modification. Sample solutions of each extract were prepared at different concentrations (10 – 50 ppm) in phosphate buffer pH 6,8 with 5% dimethyl sulfoxide (DMSO) as a solvent. Briefly, 50 µL of each sample was mixed with 50 µL of α-glucosidase enzyme (0,35 U/mL) in a 96-well microplate and incubated at 37°C for 10 min. After that, 50 µL of 1.5 mM p-NPG was added, and the samples were further incubated at 37°C for 20 min. To terminate the reaction, 100 µL of Na_2CO_3 (1 M) was added and the absorbance of the solution was measured at 450 nm using a microplate reader. In this test, quercetin was used as a positive control. The process was repeated in triplicate, and the percent inhibition was calculated with the following equation:

$$Inhibition = \frac{(A - B) - (C - D)}{(A - B)} x100$$

where A is the absorbance of blank reaction containing only 5% DMSO in phosphate buffer, B is the absorbance of control reaction containing 5% DMSO in phosphate buffer and α-glucosidase enzyme, C is the absorbance of sample reaction containing sample solution and α-glucosidase enzyme, D is the absorbance of control sample containing only sample solution.

The concentration of samples that inhibited α-glucosidase activity by 50% was defined as the IC_{50} value and in this test, quercetin was used as a control solution with a ranging concentration of 1 to 20 ppm and 4-16 ppm. Each concentration of sample and control was analyzed in duplicate.

2.10.Data analysis

The total phenolic, flavonoid, and activity test results were analyzed with SPSS 25.0 using the One-way ANOVA method (sig.<0,05). The results of the analysis were expressed as mean ± standard deviation and significance difference between samples. For analysis of the correlation between total phenolic and flavonoid content with activity, two-tailed Spearman's rank correlation was used.

3. RESULTS

3.1. Extraction yield

Ebony leaves were extracted sequentially by maceration method using several solvent variations including ethanol 96%, 70%, and 50%. Yield extract can be seen in Table 1.

Table 1. Yield extract of ebony leaf

Extracts	Weight of Simplicia (g)	Weight of extract (g)	% Yield
Ethanol 96%	20.00	6.23	31.14
Ethanol 70%	20.00	5.71	28.57
Ethanol 50%	20.00	6.04	30.19

3.2. Total phenolic content and total flavonoid content

The total phenol content was carried out using the Folin-Ciocalteu colorimetry method and measured by UV-Vis Spectrophotometer at 765 nm wavelength. The reaction that occurs in this method is an oxidation-reduction reaction in which phenolic compounds will reduce phosphomolybdate, phosphotungstate in Folin-Ciocalteu become molybdenum blue form as shown in figure 1.[29,30]

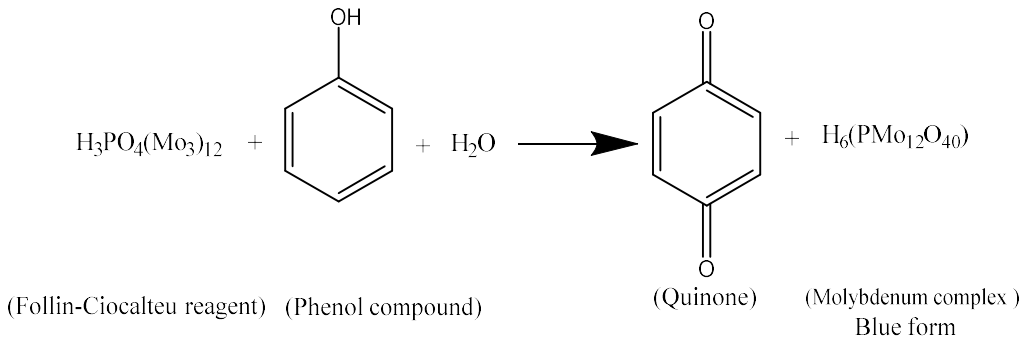


Figure 1. Folin-Ciocalteu and phenolic compound reaction

The increase of blue color intensity in the test solution will be proportional to the compound in the sample. The whole reaction will take place in alkaline conditions obtained by the addition of sodium carbobate[29]. In this study, the regression equation of standard gallic acid is y = 0.1008x + 0.004 with linearity (r^2 = 0.9994). The absorbance of each extracted sample is then extrapolated into a standard curve equation and the result is shown in Table 2.

Table 2. Result of TPC & TFC extracts

Extracts (%)	Total phenolic contains (ppm GAE)	Total Flavonoid contains (ppm QE)
Ethanol 96	**21.26** ± 0.02[a]	**7.14** ± 0.18[a]
Ethanol 70	19.22 ± 0.02[b]	5.55 ± 0.20[b]
Ethanol 50	15.16 ± 0.20[c]	4.75 ± 0.48[c]

Note: GAE: Gallic Acid Equivalent, QE: Quercetin Equivalent
a, b, c: The mean difference is significant at the 0.05 level

In addition to phenolic compounds, flavonoids are also reported to have antidiabetic effects through antioxidant activity and α-glucosidase enzyme inhibition [31]. Determination of total flavonoid content using the $AlCl_3$ colorimetric method was measured at UV-Vis 510 wavelength. The principle of this method is a

complex form reaction between aluminum chloride with a keto group on the C-4 atom and hydroxyl group on the neighboring C-3 or C-5 atom of the flavonol group compound as shown in Fig.2 [23,32].

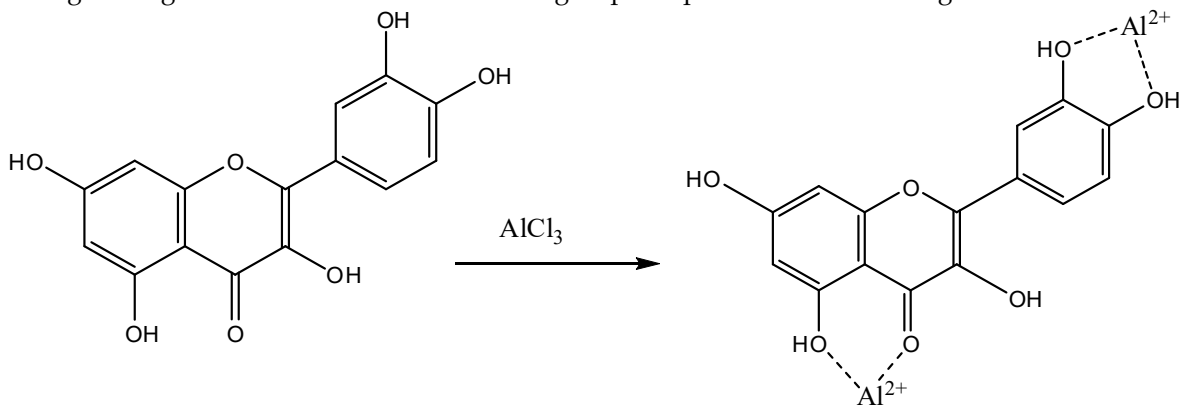


Figure 2. $AlCl_3$ and flavonoid compound reaction

In this study, the linear regression equation of the quercetin standard curve is y = 0.0128x + 0.0669 with linearity (r^2 = 0.9973), and the total flavonoid content of the sample is shown in Table 1.

3.3. Thin layer chromatography of sample

Characterization and screening of antioxidant activity of secondary metabolites in the extract was carried out using thin layer chromatography (TLC). TLC was chosen because it was an effective, fast, and cheaper method for screening compounds in extract and also very simple to determine their inhibitory activities. The result of TLC showed that all ebony leaf extracts have several active antioxidant compounds. White spots or bands indicated antioxidant activity.

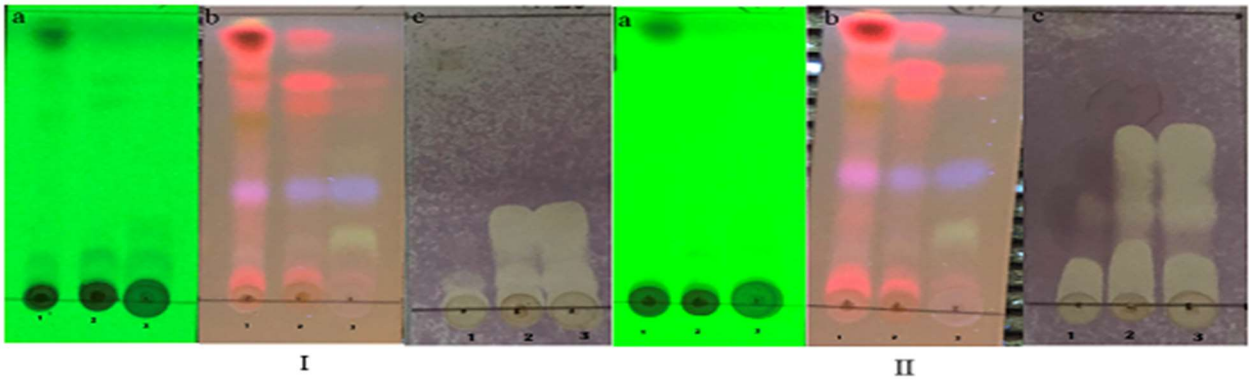


Figure 3. Chromatogram TLC of Diospyros celebica extract: spot 1. Extract ethanol 96%, 2. Extract ethanol 70%, 3. Extract ethanol 50%.
Eluent: I. Hexane : Ethyl Acetate (1:1), II. Hexane : Ethyl Acetate (1:3). Observed in a. UV-Vis 254 nm, b. UV-Vis 366 nm, c. sprayed with 2% DPPH solution in methanol.

3.4. Identification of secondary metabolites by Liquid Chromatography Mass Spectrophotometry (LC-MS)

The results of the identification of secondary metabolite in each extract are shown in Figure 4 and Table 3.

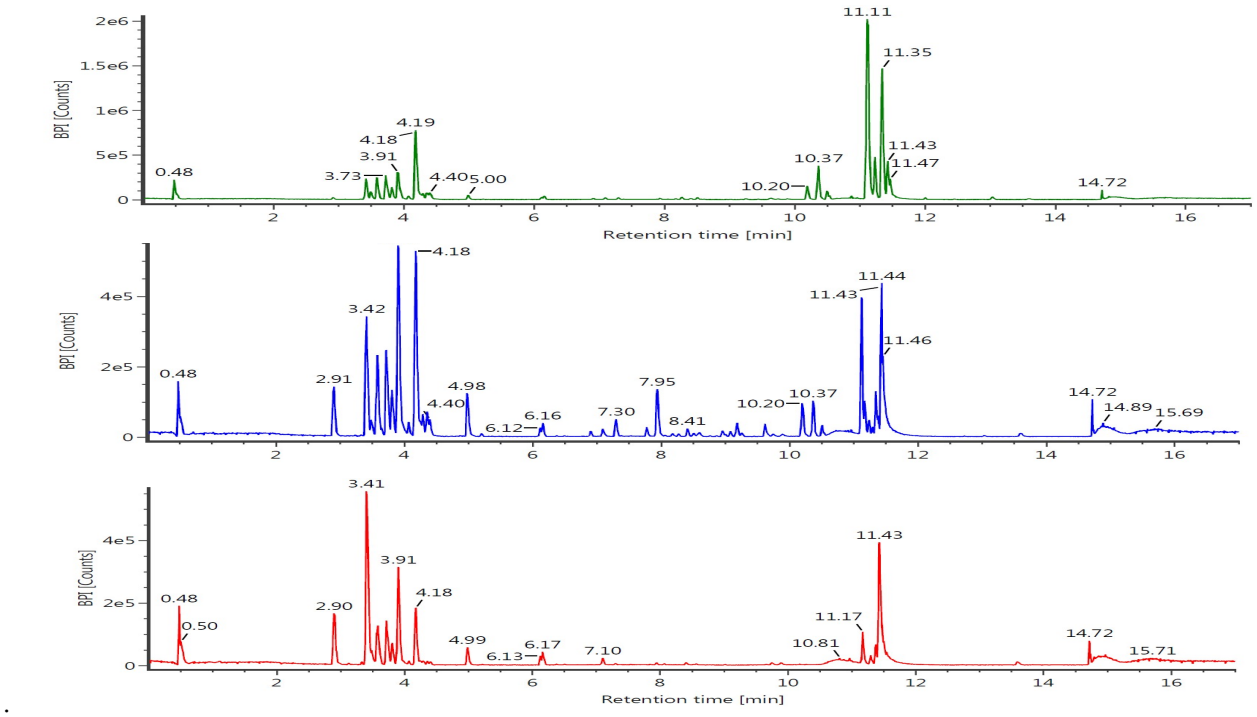
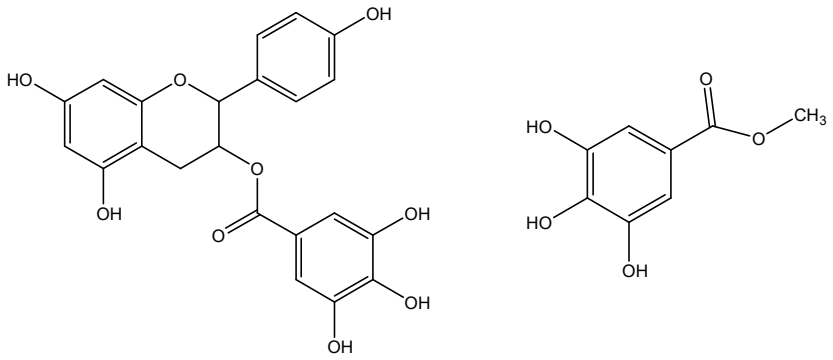


Figure 4. Chromatogram LCMS of *Diospyros celebica extract*
Green: Extract ethanol 96%, Blue: Extract ethanol 70%, Red: Extract ethanol 50%.

Table 3. The result of secondary metabolites compound in ebony leaf extract determined by LCMS

Sample	Formula Component	Observed m/z	Neutral mass (Da)	Observed RT (min)	Mass error (mDa)	Detector counts	Compound Structure Prediction
Extract Ethanol 96 %	$C_{15}H_{14}O_5$	275.0913	247.0841	3.43	-0.1	257963	Unidentified
	$C_{36}H_{38}N_4O_5$	**607.2918**	**606.2842**	**11.12**	**0.3**	**3823144**	**Unidentified**
	$C_{29}H_{22}O_{13}$	579.1138	578.1060	4.19	0.5	1598148	Unidentified
	$C_{28}H_{24}O_{15}$	601.1193	600.1115	3.74	0.5	653109	Unidentified
	$C_{30}H_{26}O_{10}$	547.1504	546.1526	3.41	0.5	102031	Unidentified
	$C_{22}H_{18}O_9$	427.1017	426.0950	3.92	-0.7	562230	(-)-Epiafzelechin 3-gallate
Extract Ethanol 70 %	$C_{15}H_{14}O_5$	275.0919	247.0841	3.42	0.5	381998	Unidentified
	$C_{36}H_{38}N_4O_5$	**607.2921**	**606.2842**	**11.13**	**0.6**	**783882**	**Unidentified**
	$C_{29}H_{22}O_{13}$	579.1144	578.1060	4.18	1.1	1257255	Unidentified
	$C_8H_8O_5$	185.0443	184.0318	2.91	-0.2	125704	methyl gallate
	$C_{30}H_{26}O_{10}$	547.1614	546.1526	3.40	1.6	112516	Unidentified
	$C_{22}H_{18}O_9$	427.1029	426.0951	3.91	0.6	905926	(-)-Epiafzelechin 3-gallate
Extract Ethanol 50 %	$C_{15}H_{14}O_5$	275.0915	247.0841	3.42	0.1	641660	Unidentified
	$C_{28}H_{24}O_{15}$	**601.1192**	**600.1115**	**3.72**	**0.4**	**388952**	**Unidentified**
	$C_{29}H_{22}O_{13}$	579.1146	578.1060	4.18	1.3	516326	Unidentified
	$C_8H_8O_5$	185.0441	184.0318	2.91	-0.3	148690	methyl gallate
	$C_{30}H_{26}O_{10}$	547.1610	546.1526	3.40	1.1	154057	Unidentified
	$C_{22}H_{18}O_9$	427.1016	426.0951	3.75	-0.7	528635	(-)-Epiafzelechin 3-gallate



(-)-Epiafzelechin 3-gallate Methyl gallate

Figure 5. Chemical Structure of (-)-Epiafzelechin 3-gallate and methyl gallate

3.5. Antioxidant and α-glucosidase inhibitor activity

The result of variance shows that the interaction between the type of solvent has a significant effect (p<0,05) on the antioxidant and α-glucosidase inhibitor activity of ebony leaf extract. The average value of IC_{50} antioxidant and α-glucosidase inhibitor activity can be seen in Table 2.

Table 4. IC_{50} value Antioxidant and α-glucosidase inhibitor of ebony leaf extract

Extract and Control	IC_{50} antioxidant (ppm)	IC_{50} α-glucosidase inhibitor (ppm)
Quercetin (control)	**3.97** ± 0.08[a]	**1.93** ± 0.27[a]
Ethanol 96%	**8.2** ± 1.04[a]	**14.90** ± 1.44[b]
Ethanol 70%	9.85 ± 0.49[b]	15.91 ± 0.60[b]
Ethanol 50%	11.33 ± 2.25[c]	20.27 ± 2.68[c]

Note: The same notation shows there is no significant difference (p<0,05) between samples.

4. DISCUSSION

In this study, we aimed to analyze the impact of different compositions of ethanol and water as extraction solvents on the profile of secondary metabolites compound and also biological activities including antioxidant and inhibitory α-glucosidase enzyme of the ebony leaf which is suspected to correlate with their total contents of phenolic and flavonoid in the extract. EtOH 96%, EtOH 70%, and EtOH 50% were used to extract and 3 extracts were obtained. In Table 1. The result showed that extraction with EtOH 96% had a higher yield compared to other solvents. It's the TLC chromatogram showed non-polar compounds, as red spots.

Phenolic and flavonoid compounds are ubiquitous in most medicinal plants and constitute an essential part of the human diet due to their antioxidant and other beneficial properties including antidiabetic [14,27,33]. Table 2. shows that the highest TPC and TFC were obtained in the ethanol 96% extract and also showed a significant mean difference between all sample extracts (p<0,05). It was indicated that the phenolic and flavonoid compounds contained in ebony leaves tend to be semi-polar, so an increase in the amount of water as the solvent composition can increase the polarity of ethanol, and its ability to attract semi-polar compounds will decrease.

The presence of phenolic and flavonoid compounds in plants generally provides antidiabetic activity with antioxidants and inhibition of the α-glucosidase enzyme mechanism [13,14,34]. In chronic diabetic conditions, supplementation of antioxidants is necessary because in chronic hyperglycemia it will result in increased formation of ROS in plasma and decreased antioxidant defenses which cause stress oxidative that can lead to insulin resistance and cardiovascular disease [35]. Zuhra et al. categorized an antioxidant compound that is known to have very strong activity if it gives an IC_{50} value less than 50 ppm, a strong category (50 – 100 ppm), moderate (100 – 150 ppm) and weak if the IC_{50} value 151 – 200 ppm [36,37], while for α-glucosidase inhibitor an extract categorized to be very active if the IC_{50} value less than 10 ppm, active is 10 – 100 ppm and inactive if IC_{50} more than 100 ppm [38,39]. In this study, all extracts showed very strong antioxidant activities and were active as α-glucosidase inhibitors. Nevertheless, ethanol 96% extract showed higher antioxidant and α-glucosidase inhibitor activities with the IC_{50} 8.12 ± 1.04 as an antioxidant and 14.90 ± 1.44 as an α-glucosidase inhibitor. Many studies reported that the total phenolic content or total flavonoid content in some extracts has a significant correlation with antioxidant and α-glucosidase inhibitor activities

[27,36,40–42]. In our study, Spearman's rank correlation coefficient for total phenolic contain (ppm of Gallic acid equivalent) to antioxidant and α-glucosidase inhibitor activity (IC_{50}) was found to be -1.00 ($p<0,001$), which suggested the perfect correlation between total phenolic contain and both the bioactivities. Similarly, Spearman's rank correlation coefficient for total flavonoid contain (ppm of quercetin equivalent) to antioxidant and α-glucosidase inhibitor activity (IC_{50}) was also found to be -1.00 ($p<0,001$), so total flavonoid contain also have a perfect correlation with antioxidant and α-glucosidase inhibitor activity. The mechanism of flavonoid and phenolic compounds to scavenge DPPH radicals is by donating the hydrogen atom from the hydroxyl group. Hydroxyl radical is the major active oxygen-centered radical formed from the reaction of various hydroperoxides with transition metal ions causing lipid peroxidation and biological damage[43], moreover the antioxidant activity not only depends on the quantity of the hydroxyl but also the structure of the compound [22,44].

In our study, the result of identification using LCMS showed that extract ethanol 50% contains a compound with molecular formula $C_{28}H_{24}O_{15}$ (molecular weight 600.11 Da) while extract ethanol 70% and 96% contains a compound with molecular formula $C_{36}H_{38}N_4O_5$ (molecular weight 606.28 Da). But overall, all formula compounds contained in the three extracts were similar, the most significant difference was seen in the concentration of each compound which was described as a detector count. This is strongly suspected to be one of the factors that caused the activities of all extracts to be similar. Table 3. shows that the identification compound in the ebony leaf is (-)-Epiafzelechin 3-gallate and methyl gallate. This result was similar to a study reported by Rosmani and Sembiring that found methyl gallate as a phenolic compound from ebony leaves [45].

Methyl gallate is one of the gallic acid derivates which is found in many plants and has some activities such as antioxidant, anticancer, and also as antidiabetic for α-glucose inhibitions [46–48]. Meanwhile, Epiafzelechin 3-gallate is a polyphenolic compound that belongs to the flavan-3-ol group which is commonly found in tea leaves (*Camellia sinensis* var. *assamica*) [49]. Hashimoto et al, reported that epiafzelechin 3-gallate showed the highest activity against lipid peroxidation than other flavan-3-ol in tea leaves [50].

5. CONCLUSION

In conclusion, the most optimal solvent to provide the best antioxidant and α-glucosidase inhibitor activity in ebony leaf extract is ethanol 96% and those activities have a perfect correlation with total phenolic and flavonoid compounds. Several phenolic compounds that have been identified in ebony leaf extract are methyl gallate and (-)-Epiafzelechin 3-gallate. However, further research involving the purification process for the determination of other structures of active compounds which still unidentified is still necessary to get better activity.

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