Trop J Nat Prod Res, May 2024; 8(6):7368-7373 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Isolation and Characterization of Flavonol glycoside as Antioxidant and Antidiabetic Compounds from Ebony (*Diospyroscelebica*Bakh.)Leaf Ethanol Extract

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ARTICLE INFO

Article history:
Received 01 April 2024
Revised 29 May 2024
Accepted 31 May 2024
Published online 01 July 2024

ABSTRACT

Ebony (*Diospyroscelebica*.Bakh) is an Indonesian medicinal plant used as antidiabetic herbs. A previous study found that the ethanol extract of ebony leaf has strong antioxidant and α-glucosidase inhibitory activity. The aim of this study was to isolate and characterize antioxidant and antidiabetic compounds from the leaves of Ebony. *Powdered e*bony leaves were extracted with 96% ethanol, and then fractionated with ethyl acetate. The ethyl acetate fraction was purified by column chromatography which led to the isolation of compound Fr.36-13a.The structure of compoundFr.36-13awas elucidated through a combination of ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR spectroscopy. The antioxidant activity of compound Fr.36-13awas determined by DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging assay. The antidiabetic activity of the compound was assessed *in vitro* by the α-glucosidase inhibitory assay. Compound Fr.36-13a was identified as kaempferol-3-*O*-*β*-glucoside through the 1D and 2D NMR data, and by comparison of the spectroscopic data with literature values. Compound Fr.36-13a (Kaempferol-3-*O*-β-glucoside) showed strong antioxidant and antidiabetic activities by scavenging DPPH radical and inhibiting α-glucosidase enzyme. Kaempferol-3-*O*-*β*-glucoside could serve as a potential therapeutic agents for the treatment of diabetes mellitus.

***Keywords*:** *Diospyros celebica*, Antioxidant, Antidiabetic, α-glucosidase, Kaempferol-3-*O*-*β*-glucoside.

Introduction

Ebony (*Diospyroscelebica*) is a native plant of Sulawesi island, Indonesia that has been reported to have antioxidant, and antidiabetic activities.[1–3]Empirically, the part of ebony plant that is used as an antidiabetic is the leaves, which are processed by decoction and the extract drunk directly by the patient.[4]in recent times, the processing of herbal medicine is carried out using an extraction process that aims to extract the active ingredients using organic solvents. The important factor that plays a role in the extraction process is the type of solvent used.[5]Kartini*et al*. (2018) reported that ebony leaves ethanol extract significantly reduced blood glucose levels in alloxan-induced diabetic rats at a dose of 500 mg/kgBW.[3]

Some of the antidiabetic assay procedures often used for the screening of natural product drugs is inhibition of α-glucosidase enzyme and antioxidant. Previous studies have reported the antioxidant and α-glucosidase inhibitory activities of the ethanol extract of ebony leaves with IC_{50}values ranging from 8.12 ± 1.04 to 13.74 ± 1.77 ppm, and 5.79 ± 0.32 to 14.90 ± 1.44 ppm, respectively.[6,7]

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Citation: Apriandini L,Hanafi M, Djamil R,Desmiaty Y,Lotulung PDN, Artanti N Isolation and Characterization of Flavonol glycoside as Antioxidant and AntidiabeticCompounds from Ebony (*Diospyroscelebica*Bakh.)Leaf Ethanol Extract. Trop J Nat Prod Res. 2024; 8(6): 7368-7373. https://doi.org/10.26538/tjnpr/v8i6.5

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria

In general, medicinal plant extract contain a mixture of bioactive compounds, and to facilitate the separation of these bioactive compounds from an extract, a fractionation process is carried out. The simplest fractionation procedure is carried out by solvent-solvent partitioning using two immiscible solvents.[5]Studies have shown a strong correlation between the antioxidant and antidiabetic properties of plants and their secondary metabolites content, especially phenolic and flavonoid compounds.[6,8]The phenolic and flavonoid compounds that have been identified from ebony leaves include methyl gallate and epiafzelechin 3-*O*-gallate.[6,9]The present study aims to isolate and characterize other bioactive compounds in the ethyl acetate fraction of ebony leaf extract, and investigate their antioxidant and antidiabetic activities.

Materials and Methods

Plant collection and Identification

Ebony (*Diospyroscelebica*.Bakh) leaves were collected from the medicinal plant garden of the National Research and Innovation Agency Serpong, Indonesia, from mid-September to the end of October, 2019. The sample was identified and authenticated at the Research Center for Plant Conservation and Botanical Garden, Indonesian Institute of Science, Bogor, Indonesia. A voucher number B-3990/IPH.3/KS/XI/2019 was assigned to the plant. The plant sample was dried under sunlight and then ground into powder. The powdered sample was stored in an air-tight container until ready for use.

Extraction and purification

Ebony leaf extract was prepared by macerating 400 g of the powdered sample in ethanol (96% analytical grade) at room temperature. The extract was filtered, and concentrated in a rotary evaporator (BUCHI Rotavapor^{TM} R-100, Canada) at reduced pressure. The concentrated extract was dried in an oven (Binder Drying and Heating chamber ED56, Germany) at 50ºC for 3 days. The dried extract (100 g) was

fractionated with ethyl acetate (analytical grade) and then concentrated in a rotary evaporator at reduced pressure. The concentrated ethyl acetate fraction was dried in an oven at 50ºC for 3 days. The ethyl acetate fraction (10 g) was then purified using column chromatography with silica gel G60 (0.063 – 0.200 mm, Merck 1.07734.1000). The sample was eluted with a solvent mixture of n-hexane, ethyl acetate, and methanol in a step-wise gradient of increasing polarity. The eluates were monitored by TLC (silica gel 254 aluminum sheets, Merck, 1.05554.0001) using appropriate eluents, the TLC plates were viewed under UV 254 and 366 nm (CAMAG® UV Cabinet 4), 67 fractions were collected, and 582.72 mg of fraction 36was further purified with column chromatography using n-hexane and ethyle acetate (1: =3) as the mobile phase, which led to 24 sub-fractions. Sub-fraction 13 (38.7 mg) was dissolved three times using 10 mL dichloromethane, and this led to the isolation of compound Fr. 36-13a as a precipitate. The compound was dried, and the structure was elucidated by a combination of ^{1}H-, ^{13}C-, and 2D-NMR (Jeol Resonance NMR ECZ00R, 500 MHz, USA).
The Flow chart for the extraction and purification process is shown in Figure 1.

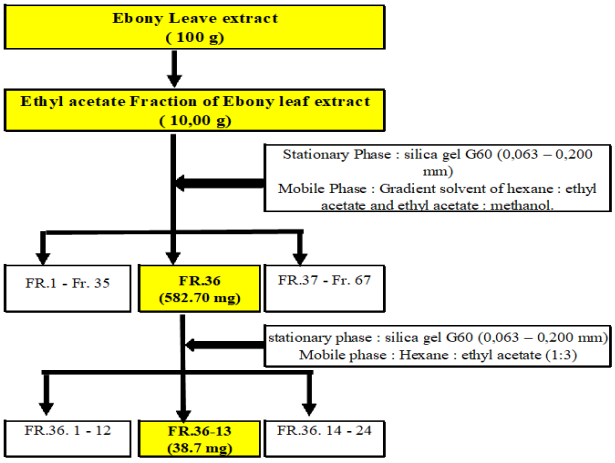

Figure 1:Flow chart of the extraction and isolation of Fr.36-13a

Determination of antioxidant activity
Antioxidant activity of the isolated compound was assessed by the1,1-Diphenyl-2-picryl hydrazyl (DPPH)radical scavenging method. The assay was done according to the procedure reported by Djamil *et al* (2021),[10] with minor modifications. Quercetin (Sigma Aldrich – USA, Purity ≥ 95 %) at concentrations of0.5 – 10 ppm was used as control. Briefly, 2 mL of sample solution and 500 µL of 0.1 M DPPH (TCI - Japan, Purity ≥ 97.0 %) solution were transferred into a test tube and mixed with a vortex mixer for 10 s. The solution was allowed to stand for 30 min in a dark place, and then the absorbance was measured at a wavelength of 517 nm with a spectrophotometer (Shimadzu UV-1800, Japan). The free radical scavenging activity was expressed as percentage inhibition of DPPH radical using the formula below:

$$\%\ Inhibition = \frac{A_{blank} - A_{sample}}{A_{blank}}\ x\ 100$$

Where A_{blank} is the absorbance of the DPPH solution without the sample and A_{sample} is the absorbance of the DPPH solution with the sample.

Each concentration of sample and control was analyzed in duplicate. The IC_{50}value was obtained from the regression equation between the percentage inhibition and concentration of each sample.

Determination of α-glucosidase inhibitory activity
Alpha-glucosidase inhibitory activity was carried out according to the method described by Djamil *et al*. (2017),[11] with minor modifications. Sample solutions of each fraction and isolated compound were prepared at different concentrations of1.25 – 50 ppm in phosphate buffer pH 6.8 with 5% dimethyl sulfoxide(EMSURE® ACS – Merck, Purity ≥ 99.9 %)as a solvent. Briefly, 50 µL of each sample was mixed with 50 µL of α-glucosidase enzyme (Wako 465100, concentration: 0.35 U/mL) in a 96-well microplate and incubated at 37ºC for 10 min. Thereafter, 50 µL of 1.5 mM p-NPG (Sigma-Aldrich 487506) was added, and the samples were incubated at 37ºC for 20 min. To terminate the reaction, 100 µL of 1 M Na_2CO_3 (EMSURE® ISO – Merck, Purity ≥ 99.9 %) was added and the absorbance of the solution was measured at 450 nm using a microplate reader. Quercetin (Sigma Aldrich – USA, Purity ≥ 95 %) was used as a positive control at concentrations of 4-16 ppm. The experiment was done in triplicate, and the percentage inhibition was calculated using the formula:

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

Where; A is the absorbance of the blank reaction containing only 5% DMSO in phosphate buffer, B is the absorbance of the control reaction containing 5% DMSO in phosphate buffer and α-glucosidase enzyme, C is the absorbance of the sample reaction containing sample solution and α-glucosidase enzyme, and D is the absorbance of the control sample containing only sample solution.
The concentration of samples that inhibited α-glucosidase activity by 50% was defined as the IC_{50} value.

Results and Discussion

Yields of extract and fraction
Ebony leaves were extracted with ethanol (96%) solvent. The choice of ethanol as the extracting solvent in this study was based on a report that found that96% ethanol was the most optimal solvent for extracting phenolic and flavonoid compounds. Phenolic and flavonoid compounds were also reported have a strong correlation with the antioxidant and α-glucosidase inhibitory activities of the extract.[6]Subsequently, the ethanol extract was fractionated with ethyl acetate. The choice of ethyl acetate as the solvent for fractionation was based on a previous report that the ethyl acetate fraction of ebony leaf extract had a strong antioxidant and α-glucosidase inhibitory activities with IC_{50} values of 8.80 ± 0.21 ppm and 4.58 ± 0.14 ppm, respectively.[7]The percentage yields of the ethanol extract and ethyl acetate fraction of ebony leaf extract were30.80%, and 41.73%, respectively (Table 1).

Table 1:Yields of the Extract and fraction of Ebony leaves

Sample weight (g)	Extraction Solvent	Weight (g)	Yield (%)
Powdered Ebony leaves (400 g)	Ethanol	123.20	30.80
Ethanol extract of Ebony Leaves (100 g)	Ethyl acetate	41.73	41.73

Isolation and characterization of compound Fr. 36-13a
Compound Fr. 36-13a was isolated by column chromatography, and the structure was elucidated by a combination of ^{1}H-, ^{13}C-, and 2D-NMR.
^{1}H-NMR spectrum of compound Fr. 36-13a (Figure 2) indicates the presence of four aromatic groups and one glucopyranoside group. The chemical shifts δ_H= 6.36 (*d*, 1.5 Hz) and 6.18 (*d*, 1.5 Hz) indicate the presence of aromatic protons with a "meta" position characteristic of ring A in flavonol. The chemical shift δ_H= 6.88 (s, 2H) indicates the presence of an aromatic group with a symmetrical proton position in gallic acid, while the chemical shifts δ_H= 6.73 (*d*,7Hz , 2H) and 7.93 (*d*, 7 Hz, 2H)indicate the presence of 1,4-disubtituted aromatic ring as seen in Figure 2.The chemical shifts δ_H= 5.04 (*d*, 8 Hz), 3.84 (*t*, 6.5 Hz), 3.78 (*t*, 6 Hz), 3.84 (*dd*, 3.5 Hz, 11.5 Hz), 3.82 (*t*, 7.5 Hz;*t*, 5.5 Hz), 4.34 (*dd*, 7.5 Hz, 15.5 Hz) and 4.14 (*dd*, 5.5 Hz, 15.5 Hz) indicated the presence of glucopyranoside moiety.The^{13}C-NMR

spectrum shows the presence of carboxylate and carbonyl groups at chemical shifts of 168.09and 179.67 ppm, respectively (Figure 3).

In the 2D-NMR (HMQC) spectrum, the aromatic proton in ring A with chemical shift δ_H= 6.36 (*d*, 1.5 Hz) was bonded to the carbon at δ_c= 95.06 ppm, and proton 6.18 (*d*, 1.5 Hz) to the carbon at δ_c= 100.17 ppm .Furthermore, two symmetric protons with chemical shift δ_H= 6.88 ppm (2H, *s*) in the gallic acid moiety were bonded to carbon at at δ_c= 110.21 ppm. In the third aromatic ring, there were 4 protons that form symmetrical bonds with chemical shift δ_H= 7.93 (*d*, 7 Hz) bonded to 132.47 ppm,and δ_H= 6.82 (*d*, 7 Hz) bonded to 116.20 ppm carbon. For proton with chemical shift δ_H= 5.21 (*d*, 3.5 Hz); 3.56 (*dd*, 3.5 Hz, 6 Hz); 3.78 (*t*, 6 Hz); 3.84 (*t*, 3.5 Hz, 11.5 Hz); 3.82 (*t*,7.5 Hz); 4.14 (*dd*, 5.5 Hz, 15.5 Hz) and 4.34 (*dd*, 7.5 Hz, 15.5 Hz) were respectively bonded to carbons 105.24, 73.01, 74.64, 70.16, 75.34, and 64.11 ppm in the α-glucoside ring. While proton sδ_H= 5.04 (*d*, 8 Hz), 3.84 (*t*, 6.5 Hz), 3.78 (*t*, 6 Hz), 3.84 (*t*, 3.5 Hz, 11.5 Hz), 3.82 (*t*, 5.5 Hz), 4.14 (*dd*, 5.5 Hz, 15.5 Hz), and 4.34 (*dd*, 7.5 Hz, 15.5 Hz) constituted the*β*-glucoside residue (Figure4).To confirm the position of the carboxylate functional group, HMBC experiment was done to determine long-range coupling for up to 3 bonds (Figure 5). On the basis of the 1D and 2D NMR data, compound Fr.35-13a was identified as derivatives of Kaempferol-3-*O*-glucoside, namely; Kaempferol-3-*O*-*β*-glucoside (6"-gallate) and Kaempferol-3-*α*-glucoside (6"-gallate).

Kaempferol-3-*O*-glucoside is a flavonol group of flavonoid compounds with a white needle-like appearance. This compound has been found in several plants, for example soursop leaves, and was reported to have very strong antioxidant activity withIC_{50}value of 1.25 ± 0.09 µg/mL.[12]The comparison of^{1}H-NMR and^{13}C-NMR data ofFr.36-13aandKaempferol-3-*O*-*β*-glucoside isolated from soursop leaf extract is presented in Tables 2 and 3. The comparison showed that the ^{1}H-NMR and ^{13}C-NMR data for Fr,36-13a are in agreement with the chemical shift of Kaempferol-3-*O*-*β*-glucoside. The striking difference was the presence of a galloyl group in the C6" glucoside of Fr.36-13a.

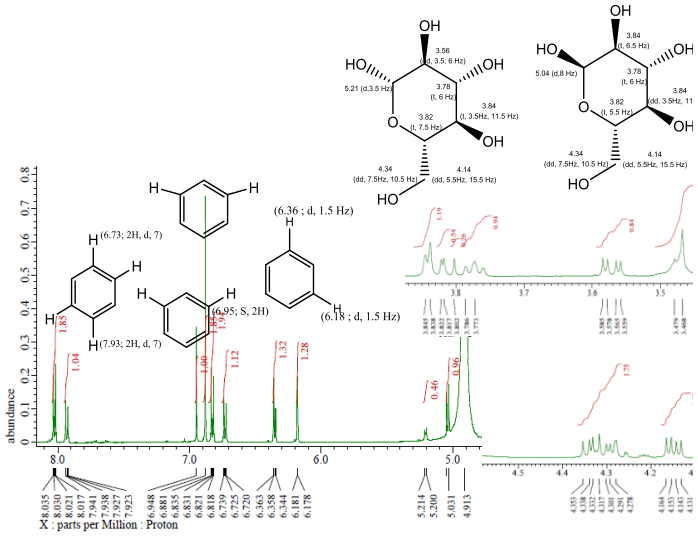

Figure 2:^{1}H-NMR Spectrum of compound Fr.36-13a

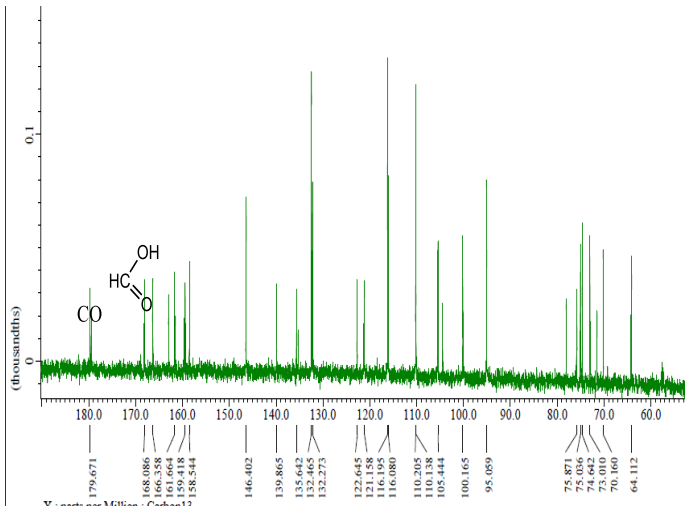

Figure 3:^{13}C-NMR Spectrum of compound Fr.36-13a

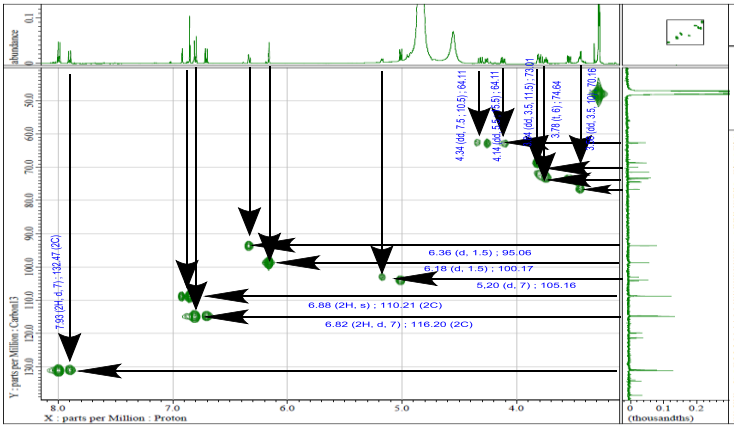

Figure 4: HMQC Spectrum of compound Fr.36-13a

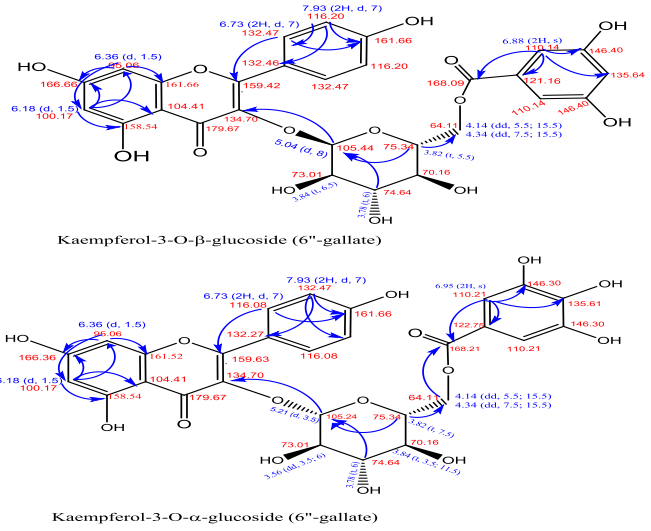

Figure 5:HMBC Correlation of Compound Fr.36-13a

Antioxidant activity of compound Fr.36-13a

The antioxidant activity of compound Fr.36-13awas assessed by the DPPH radical scavenging activity. Compound Fr.36-13a was shown to have strong antioxidant activity with IC_{50} value of 4.49 ± 0.53 µg/mL (Figure 6). A previous study reported that kaempferol-3-*O*-glucoside has a very strong antioxidant activity withIC_{50} value of 1.25 ± 0.09

µg/mL.[12]In polyphenolic and flavonoid compounds, antioxidant activity has been found to be strongly correlated with the number of hydroxyl groups in the compound structure. Compounds with hydroxyl groups are good hydrogen atom donors, which are capable of neutralizing free radical species.[8,13]However, the configuration of the hydroxyl group has been reported to have a major contribution to the antioxidant activity of flavonoids. Some studies reported that the presence of an orthohydroxyl structure in B ring of flavonoids significantly increases the antioxidant activity.[14]

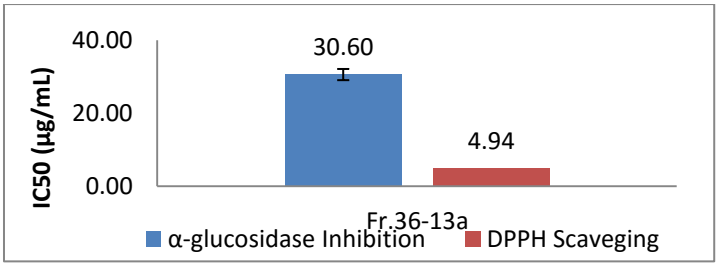

Figure 6:Antioxidant and antidiabetic activities of compound Fr.36-13a

Table 2: Comparison of ^{1}H-NMR data of compound Fr.36-13a with Kaempferol-3-*O*-*β*-glucoside

	Proton	Fr. 36-13a Kaempferol-3-*O*- *β* -glucoside (6”-gallate)	Fr. 36-13a Kaempferol-3-*O*- *β* -glucoside (6”-gallate)	Literature[12, 19]
A-Ring	C_6-H	6.18 (d, *J*= 1.5 Hz)	6.18 (d,*J*= 1.5 Hz)	6.2
	C_8-H	6.36 (d,*J*= 2.5 Hz)	6.36 (d,*J*= 2.5 Hz)	6.4
B-Ring	$C_{2'}$-H	6.73 (d, *J*= 7 Hz)	6.73 (d, *J*= 7 Hz)	8.06
	$C_{3'}$-H	7.93 (d, *J*= 7 Hz)	7.93 (d, *J*= 7 Hz)	6.9
	$C_{5'}$-H	7.93 (d, *J*= 7 Hz)	7.93 (d, *J*= 7 Hz)	6.9
	$C_{6'}$-H	6.73 (d, *J*= 7 Hz)	6.73 (d, *J*= 7 Hz)	8.06
Glucoside	$C_{1''}$-H	5.04 (d, *J*= 8 Hz)	5.21 (d,*J*= 3.5 Hz)	3.37
	$C_{2''}$-H	3.84 (t, *J*= 6,5 Hz)	3.56 (dd,*J*= 3.5 Hz; 6 Hz)	3.34
	$C_{3''}$-H	3.78 (t, *J*= 6 Hz)	3.78 (t, *J*= 6 Hz)	3.34
	$C_{4''}$-H	3.84 (t, *J*= 6.5 Hz)	3.84 (t, *J*= 3.5 Hz; 11.5 Hz)	3.33
	$C_{5''}$-H	3.82 (t, *J*= 5.5 Hz)	3.82 (t, *J*= 7.5 Hz)	3.33
	$C_{6''}$-H	4.14 (dd,*J*= 5.5 Hz; 15.5 Hz)	4.14 (dd,*J*= 5.5 Hz; 15.5 Hz)	3.32
		4.34 (dd,*J*= 7.5 Hz; 15.5 Hz)	4.34 (dd,*J*= 7.5 Hz; 15.5 Hz)	
Galloyl	$C_{2'',6''}$-H	6.88 (s)	6.95 (s)	-

Table 3: Comparison of ^{13}C-NMR data of compound Fr.36-13a with Kaempferol-3-*O*-*β*-glucoside

	Carbon	Fr. 36-13a Kaempferol-3-*O*- *β* -glucoside (6”-gallate)	Fr. 36-13a Kaempferol-3-*O*- *β* -glucoside (6”-gallate)	Literature [12,19]
C-Ring	C_2	159.42	159.63	157.73
	C_3	134.70	134.70	134.10
	C_4	179.67	179.67	178.1
A-Ring	C_5	158.54	158.54	157.1
	C_6	100.17	100.17	98.52
	C_7	166.66	166.36	164.58
	C_8	95.06	95.06	93.39
	C_9	161.66	161.52	160.17
	C_{10}	104.41	104.41	104.36
B-Ring	$C_{1'}$	132.46	132.27	121.41
	$C_{2'}$	116.20	116.08	130.89
	$C_{3'}$	132.47	132.47	114.70
	$C_{4'}$	161.66	161.66	157.70
	$C_{5'}$	132.47	132.47	114.70
	$C_{6'}$	116.20	116.08	130.89

	Carbon	Fr. 36-13a Kaempferol-3-*O*- *β* -glucoside (6”-gallate)	Fr. 36-13a Kaempferol-3-*O*- *β* -glucoside (6”-gallate)	Literature [12,19]
Glucoside	C_{1}''	105.44	105.24	102.76
	C_{2}''	73.01	73.01	76.66
	C_{3}''	74.64	74.64	74.35
	C_{4}''	70.16	70.16	73.65
	C_{5}''	75.34	75.34	69.98
	C_{6}''	64.11	64.11	61.26
Galloyl	-COO-	168.09	168.21	-
	C_{1}''	121.16	122.75	-
	C_{2}''	110.14	110.21	-
	C_{3}''	146.40	146.30	-
	C_{4}''	135.64	135.61	-
	C_{5}''	146.40	146.30	-
	C_{6}''	110.14	110.21	-

Alpha glucosidase inhibitory activity of compound Fr.36-13a

Currently, the prevalence of metabolic diseases such as diabetes mellitus continues to increase throughout the world. The use of herbal medicines derived from plants is an alternative that is more popular because of its perceived safety compared to synthetic medicines. Ebony leaves are one of the herbs that have been widely used by Indonesians for the treatment of diabetes mellitus. The interest in this research arises from the lack of scientific information about the content of active compounds in ebony leaves, which may play a role in the blood sugar lowering effect of the plant.

In the present study ,compound Fr.36-13a [kaempferol-3-*O*-*β*-glucoside(6”-gallate) and kaempferol-3-*O*-α-glucoside (6”-gallate)]isolated from ebony leaves exhibited α-glucosidase inhibitory activity with IC_{50} value of 30.60 ± 8.84 µg/mL (Figure 6).Alpha-glucosidase is a digestive enzyme that breaks down oligosaccharides into monosaccharides, so the inhibition of this enzyme can reduce glucose absorption in the lumen of the gastrointestinal tract and also prevent postprandial hyperglycemia.[15,16]In flavonoids, inhibition of α-glucosidase has been shown to be correlated with the number of hydroxyl groups present in the B ring of the flavonoid structure, which is thought to be the binding site of the enzyme.[17]The interaction between flavonoids and α-glucosidase has also been reported to be by the formation of a complex structure through hydrogen bonding or an hydrophobic interaction between the hydroxyl and carbonyl groups in flavonoid structure and the active amino acid residue of the enzyme.[18]

Conclusion

Chromatographic separation of the ethyl acetate fraction of ebony leaf extract led to the isolation of kaempferol-3-*O*-*β*-glucoside (6"-gallate) and kaempferol-3-*O*-α-glucoside (6"-gallate). The isolated compound exhibited strong antioxidant activity and antidiabetic activity through DPPH radical scavenging and α-glucosidase inhibitory activities *in vitro*. These findings show that kaempferol-3-*O*-*β*-glucoside (6"-gallate) and kaempferol-3-*O*-α-glucoside(6"-gallate) have the potential as alternative therapeutic agents for the treatment of diabetes mellitus. However, further research is still needed to provide more robust information regarding its safety and benefits to consumers.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

Acknowledgments

This research was funded by Internal Fund Grant (UP In-House Fund), Pancasila University 2023 and National Research and Innovation Agency, Republic of Indonesia. The authors would also like to thank the Master of Pharmacy Department, Pancasila University for their support and cooperation.

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