Antioxidant Flavonoid Glycoside from Leaves of Cacao Mistletoe (*Scurrula ferruginea* (Jack) Danser)

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Abstract: Scurrula ferruginea *(Jack) Danser is a folk medicine to treat several diseases.* S. ferruginea *is the mistletoe that lives by deriving nutrients from the host. Hence, the host has a significant effect on the biological activities and bioactive components of* S. ferruginea. *In this study, the leaves of* S. ferruginea *were macerated by methanol solvent to extract the chemical components and fractionated by hexane and ethyl acetate solvent, respectively, to separate organic compounds. The biological activity of crude extracts as the antioxidant was investigated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The result showed that the highest antioxidant activity was obtained from the ethyl acetate fraction with the* IC_{50} *of 10.88 ± 0.39 µg/mL. Furthermore, the trituration method used several solvents to isolate secondary metabolites from the initial crude methanol extract. This purification process produced a flavonoid compound of quercitrin. The antioxidant activity of quercitrin was also determined by the DPPH method. The result showed that this compound has stronger activity than the crude extracts with the* IC_{50} *of 5.95 ± 0.11 µg/mL. This study demonstrated that cacao mistletoe of* S. ferruginea *may have the potential activity to treat the diseases caused by the presence of free radicals.*

Keywords: Scurrula ferruginea *leaves; antioxidant; quercitrin*

■ INTRODUCTION

Plants are natural resources that have many benefits to human life. For instance, utilization of the part of the plant as a herbal medicine has been widely used in society since the ancient period. *Scurrula ferruginea* (Jack) Danser, known as "Dedalu Api Merah" in Indonesia, is mistletoe, a hemiparasitic plant from Santalales ordo and belongs to the family of Loranthaceae. The mistletoes have an interesting way to live, in which they grow and attach to the host. The mistletoes obtain nutrition by absorbing the water and mineral from the host plant and live with their photosynthesis [1-2].

S. ferruginea is a mistletoe that lives by deriving nutrients from the host. Hence the influence of the host in specific function as herbal medicine cannot be a negligible factor. The host species significantly affect the chemical constituents and biological activities of *S. ferruginea* [3-5]. The common host plants of the mistletoe of *S. ferruginea* are *Tabebuia pallida* and *Lagerstroemia speciosa* [6]. Traditionally, *S. ferruginea* has been used to treat wounds, snakebite, fever, beriberi, malaria, and postpartum [7]. Some previous studies have scientifically proved that *S. ferruginea* possesses pharmacological activity, such as cytotoxic effect on human cell lines of DU145 and U251 [7], human breast cancer cell MDA-MB-231 [8], antihypertensive activity [9], anti-inflammatory activity with the host of *Tecoma stans* [10], and antimicrobial activity against *Staphylococcus aureus* S261 and *Escherichia coli* E57 [11].

However, the studies about isolated compounds of *S. ferruginea* are not extensively reported. As far as our literature studies, only one exhibited the isolated compounds from the *S. ferruginea* plant: three flavonols, such as quercitrin, quercetin, and 4"-*O*-acetylquercitrin

from an unspecified host [12]. Another literature reported the chemical constituents of the crude extract based on the principal component analysis (PCA) method from ^{1}H-NMR data resulting in some identified compounds, such as quercitrin, 4''-*O*-acetylquercitrin, catechin, alanine, threonine, valine, leucine, isoleucine, histidine, formic acid, malic acid, succinic acid, citric acid, fumaric acid, acetic acid, gallic acid, chlorogenic acid, and choline [5].

Therefore, this study aims to isolate the metabolite compound from a specified host (cacao) of *S. ferruginea* and evaluate the biological activity as an antioxidant from the isolated compound and the crude extract of *S. ferruginea*.

■ EXPERIMENTAL SECTION

Materials

S. ferruginea leaves were obtained from Bayang, Pesisir Selatan Regency, West Sumatera, Indonesia. The sample was identified at the Herbarium Department of Biology, Faculty of Mathematics and Natural Sciences, Andalas University, with the official letter-number 163/K-ID/ANDA/IV/2017.

Instrumentation

Instruments used in this study were spectrophotometers of UV-1700/Shimadzu, FTIR-1600/Perkin Elmer, and NMR of JEOL JNM-ECZR 500 MHz.

Procedure

Extraction

Leaves of *S. ferruginea* were air-dried and milled. The powder of *S. ferruginea* leaves (1.3 kg) was macerated with methanol solvent three times at room temperature for 3 × 24 h for each extraction. The material-solvent ratio was maintained at approximately 1:2 (w/w) for each extraction. The crude methanol extract was concentrated by a rotary evaporator, yielding 55.65 g of crude methanol extract. After 24 h, a yellow solid was formed on the crude methanol extract. The crude methanol extract was re-dissolved with methanol solvent and fractionated with hexane solvent to separate the solid component and obtain the hexane extract. The polar fraction was further fractionated by ethyl acetate solvent. The rotary evaporator concentrated the extracts to evaporate the solvent and produced hexane, ethyl acetate, and methanol extracts.

Phytochemical screening

The extracts of *S. ferruginea* leaves were evaluated quantitatively to identify the chemical constituents in the extracts. The procedure was conducted based on the standard method of Harborne [13].

Purification and characterization

The isolated compound was purified using the trituration method with several solvents, such as hexane and ethyl acetate. The purification process produced 2.77 g of yellow powder from the isolated compound. The isolated compound was identified by ultraviolet (UV), infrared (IR), and nuclear magnetic resonance (NMR) spectra analysis compared to literature.

Antioxidant activity

The antioxidant activity was conducted with 2,2-diphenyl-1-picrylhydrazyl (DPPH) method [14] with slight modification. The tested sample was the isolated compound and the extract of hexane, ethyl acetate, and methanol. Ascorbic acid was used as a positive control. The DPPH solution with the concentration of 0.1 mmol/L was added into the tube containing 2 mL of the tested sample in various concentrations. The concentrations were varied for each tested sample, such as from 1.5625 to 25 μg/mL for the isolated compound, methanol, and ethyl acetate extracts, from 12.5 to 200 μg/mL for hexane extract, and from 0.625 to 10 μg/mL for ascorbic acid. The mixture of DPPH and sample was allowed to stand for 30 min at room temperature and unlighted. The absorbance was measured at the wavelength of 517 nm as the function of the DPPH solution. The varying of the sample was conducted to make the curve inhibition resulting and generate the IC_{50} value by the regression equation. The IC_{50} value means the required concentration to inhibit 50% of free radicals. The inhibition of free radical DPPH (I%) was calculated by the equation as follows:

$$I\% = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100\%$$

where A_{blank} is the absorbance of blank contained DPPH solution and A_{sample} is the absorbance of the tested sample. All of the tests were done in triplicate.

■ RESULTS AND DISCUSSION

Extraction

The fractionation process of the crude methanol extract of *S. ferruginea* leaves with several solvents, such as hexane and ethyl acetate, respectively, produced three different fractions with specific yields for each fraction (Table 1).

Secondary metabolite compounds generally exist in small amounts in plants. The suitable extraction method is a crucial factor to extract the organic compound from the plant material with a high yield and minimal change of the functional properties of the extract. The conventional extraction method is commonly performed through maceration in which the plant material is soaked with the solvent at room temperature to avoid degradation or functional group changing of the organic compounds. Hence, it is required to select the proper solvent based on sample matrix properties or chemical compound properties contained in plant material [15-18].

In this study, the methanol solvent was used initially to maximize the extraction of secondary metabolites from *S. ferruginea* since the methanol has good penetration properties to the cell-matrix of the plant. Then, the methanol crude extract was separated with hexane and ethyl acetate solvents, respectively, to obtain the crude fractions of *S. ferruginea* leave extract. The limitation of the yield percentage (Table 1) of the extract was assumed due to the less repetition in initial methanol extraction, which was only three times of maceration.

As shown in Table 1, there were three fractions with different polarities. Each fraction contained different chemical constituents, such as hexane fraction with non-polar compounds, ethyl acetate fraction with semi-polar compounds, and methanol fraction with polar compounds.

The fraction distribution of *S. ferruginea* leave extract is shown in Fig. 1. It revealed that the chemical constituents of the extract were dominated by polar compounds, in which the methanol fraction had the highest

Table 1. Fraction weight and yield of *S. ferruginea* leaves extract

No	Extract	Weight (g)	Yield (% w/w)*
1	Hexane	12.84	0.99
2	Ethyl acetate	3.32	0.26
3	Methanol	36.72	2.82

*Yield was the percentage value of crude extract weight compared to plant material weight

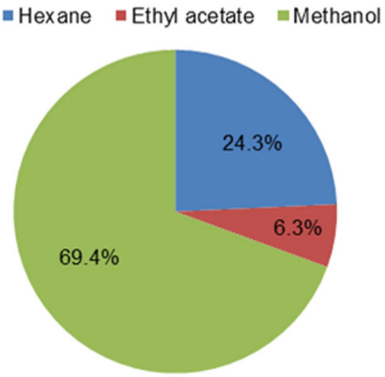

Fig 1. Fraction distribution of *S. ferruginea* extract

distribution percentage among the other fractions, with a value up to 69.4%. The quantity of the fraction is affected by the chemical composition and compound types. The chemical compounds of the extract will be distributed in the solvent according to their suitable polarity properties. Organic compounds with non-polar properties, such as terpenoid, steroid, and lipid, are dissolved properly in a non-polar solvent, such as hexane [19-20]. In contrast, organic compounds with polar properties, such as phenolic compounds, are highly distributed in the polar solvent [17].

Phytochemical Screening

The phytochemical screening of *S. ferruginea* extract exhibited the presence of secondary metabolite compounds such as steroids, flavonoids, and phenols, as shown in Table 2. Alkaloids were not detected in all extracts of *S. ferruginea,* whereas steroids were found in hexane and methanol extracts. The extracts of ethyl acetate and methanol had potential chemical constituents such as flavonoids and phenols. The phytochemical compound detection would be helpful information for future research, especially in pharmacological and medicinal topics. For example, steroids have been reported

Table 2. Phytochemical screening of *S. ferruginea* extract

Chemical constituent	Test type	Extract		
		Hexane	Ethyl acetate	Methanol
Alkaloids	Mayer	-	-	-
Steroids	Liebermann–Burchard	+	-	+
Flavonoids	Shinoda	-	+	+
Phenols	Ferric chloride	-	+	+

(+) indicated the presence of constituents, and (-) indicated the absence of the constituents

as antifungal, antidiabetic, antibacterial, and anti-inflammatory [21]. Flavonoids and phenols are also known to have highly ranging diverse biological activities, such as antioxidant, anti-inflammatory, and anticancer [22].

Purification and Characterization

The isolated compound was obtained from the initial crude methanol extract as a yellow powder. The solid shape of the compound was formed after obtaining the concentrated crude methanol extract. This yellow solid compound was purified with the simple trituration method with a gradient solvent system, such as hexane and ethyl acetate solvent, respectively. This method was carried out since the compound was already in solid form due to the high concentration of the compounds in the extract.

The purity of the isolated compound was determined by thin-layer chromatography (TLC) using various eluent ratios (Fig. 2). The mixture of the eluent with several ratios produces the solvent's different polarity, resulting in variation of the retention factor (Rf) value. The results showed a constant single spot on the TLC plate for all eluent ratios with the Rf value of 0.13, 0.31, 0.51, 0.67, and 0.70, respectively (Fig. 2(a-e)). It confirmed the purity of the isolated compound without the presence of impurities.

The purified compound was identified using UV, IR, and NMR data. All these analyses were combined to conclude the structure of the isolated compound. The compound was measured by a UV instrument combined with shift reagents to investigate the interaction of the compound against several shift reagents (Table 3).

Based on Table 3 shows that the UV spectrum of the compound gives two maximum absorption bands. Band II (240–280 nm) corresponds to A-ring with the benzoyl system and band I (300–380 nm) indicates to

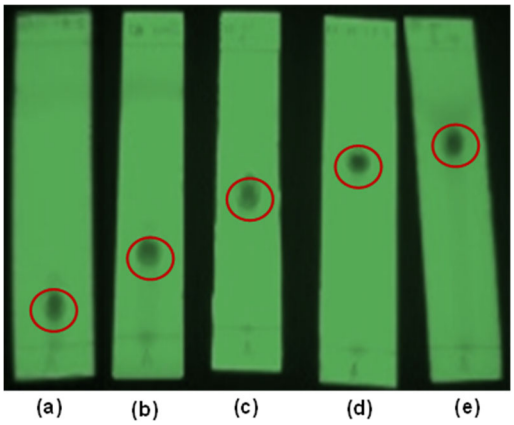

Fig 2. TLC of the isolated compound with various eluent ratios; (a) hexane:EtOAc (10:90), (b) EtOAc 100%, (c) EtOAc:MeOH (90:10), (d) EtOAc:MeOH (80:20), (e) EtOAc:MeOH (70:30)

Table 3. UV Analysis of Isolated Compound of *S. ferruginea*

Solvent/shift reagent	Band II (λ_{max}, nm)	Band I (λ_{max}, nm)	Shift Observed and Interpretation [23]
MeOH	256	350	Flavonol with 3-OR
MeOH+NaOH	272	398	Band I (+ 48 nm) → 4'-OH
		(Additional band, 326)	Additional band between 320-335 nm → 7-OH
MeOH+$AlCl_3$	275	429	Band I (+79 nm) → ortho position of OH at B-ring
MeOH+$AlCl_3$+HCl	272	401 (Additional band, 346)	Band I (+51 nm) → 5-OH
MeOH+NaOAc	266	393	Band II (+10 nm) → 7-OH
MeOH+NaOAc+H_3BO_3	263	374	Band I (+24 nm) → ortho position of OH at B-ring

B-ring with the cinnamoyl system [24]. This spectrum is affected significantly by the oxygenation pattern in the flavonoid structure. Generally, an increase in the oxygenation level will contribute to the shift of the absorption bands to the bathochromic system (longer wavelength). Shift reagents also give useful information to identify the flavonoid structure since each reagent will give a specific reaction with certain oxygenation positions [23]. Shift reagents used in this UV analysis were NaOH, $AlCl_3$, $AlCl_3$/HCl, NaOAc, and NaOAc/H_3BO_3. The result of the interpretation from UV data indicated that the isolated compound was a flavonol which had substituent "R" at oxygen atom in atom C-3 (C-OR) and hydroxyl (OH) substituent at atom carbon of 5, 7, 3', and 4' (Fig. 3).

The predicted structure from UV analysis was supported by IR data that confirmed the presence of the functional group of hydroxyl (OH, ν_{max} at 3237 cm^{-1}), C=C aromatic (ν_{max} at 1465 cm^{-1}), C-O ether/alcohol (ν_{max} at 1258 cm^{-1}), and an aliphatic C-H (ν_{max} at 2930 cm^{-1}).

The ^{13}C-NMR chemical shift of the isolated compound (Table 4) indicated the presence of 21 carbon atoms consisting of 12 aromatic carbon signals at δ 95.17–166.87 ppm, 1 carbonyl signal at δ 179.88 ppm, 2 ether alkene signals at δ 136.48 and 158.72 ppm, and 6 specific signals for glycoside with 4 signals at δ 72.22–74.84 ppm, 1 signal of C-1" of *O*-glycoside at δ 103.83 ppm, and 1 signal of C-6" of *C*-methylation at δ 17.96 ppm. Whereas the chemical shift of ^{1}H-NMR (Table 4) exhibited the appearance of 11 proton signals consisting of 5 aromatic proton signals at δ 6.19–7.34 ppm,

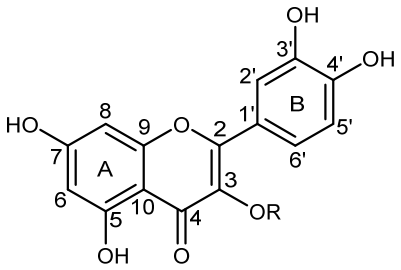

Fig 3. Predicted structure from UV analysis

Table 4. NMR chemical shift of isolated compound compared to literature

No	Carbon type	Isolated compound*			Literature [27]	
		δ_C (ppm)	δ_H (ppm) (multiplicity, *J*)	HMBC	δ_C (ppm)	δ_H (ppm) (multiplicity, *J*)
2	C	158.72			158.73	
3	C	136.48			136.08	
4	C	179.88			179.46	
5	C-OH	163.48			163.08	
6	CH	100.32	6.19 (d, *J* = 1.5 Hz)	C-8, C-10, C-5, C-7	100.63	6.19 (d, *J* = 1.4 Hz)
7	C-OH	166.87			168.26	
8	CH	95.17	6.36 (d, *J* = 2.5 Hz)	C-6, C-10, C-9, C-7	95.32	6.35 (d, *J* = 1.6 Hz)
9	C	159.39			159.09	
10	C	105.99			105.27	
1'	C	123.15			123.00	
2'	CH	117.21	7.34 (d, *J* = 2.0 Hz)	C-1', C-3', C-4', C-2	116.19	7.35 (d, *J* = 1.8 Hz)
3'	C-OH	146.60			146.52	
4'	C-OH	149.99			149.97	
5'	CH	116.67	6.92 (d, *J* = 8.5 Hz)	C-1', C-6', C-3', C-4'	116.42	6.93 (d, *J* = 8.3 Hz)
6'	CH	122.99	7.32 (dd, *J* = 2.0 and 8.5 Hz)	C-2', C-4', C-2	122.85	7.32 (dd, *J* = 1.9 and 8.3 Hz)
1"	CH	103.83	5.35 (d, *J* = 1.5 Hz)	C-2", C-3	103.55	5.36 (d, *J* = 1.2 Hz)
2"	CH	72.22	4.22 (brdd, *J* = 1.5 and 3.0 Hz)	C-4"	72.14	4.25 (d, *J* = 1.1 Hz)
3"	CH	72.71	3.76 (dd, *J* = 3.0 and 9.5 Hz)	C-4"	72.05	3.78 (dd, *J* = 3.3 and 3.3 Hz)
4"	CH	73.55	3.35 (d, *J* = 9.5 Hz)	C-6", C-2"	73.30	3.35 (d, *J* = 2.3 Hz)
5"	CH	74.84	3.44 (m)		71.94	3.43 (d, *J* = 6.0 Hz)
6"	CH3	17.96	0.95 (d, *J* = 6.0 Hz)	C-3"	17.67	0.96 (d, *J* = 6.1 Hz)

*The solvent was CD_3OD at 500 MHz for ^{1}H-NMR and 125 MHz for ^{13}C-NMR

1 proton signal of H-1" of 3-*O*-rhamnoside at δ 5.35 ppm, 1 proton signal of methyl (H-6") at δ 0.95 ppm, and 4 proton signals for sugar at δ 3.35–4.22 ppm.

The coupling constant (*J*) interpretation showed the presence of three signals of aromatic proton in the ABX spin system, in which the proton at δ 7.32 ppm (H-6') had the doublet of doublets multiplicity coupled at meta position with H-2' (δ 7.34 ppm) confirmed by the *J* value of 2.0 Hz and at ortho position with H-5' (δ 6.92 ppm) indicated by the J value of 8.5 Hz. This observation confirmed the presence of the substituent at C-3' and C-4'. An anomeric proton of the isolated compound was at δ 5.35 ppm (d, *J* = 1.5 Hz, H-1") correlated with carbon signal at δ 103.83 ppm in the HSQC spectrum. This coupling constant of 1.5 Hz confirmed the α-orientation of L-rhamnosyl moiety [25-27]. The constant coupling value of methyl proton of H-6" was 6.0 Hz, which this coupling constant was an identical value to Zhang et al. [26] and Gopi et al. [27].

HMBC analysis presented the correlation between protons and carbons separated by two or three and sometimes up to four bonds in the conjugated system. In the HMBC data (Table 4), the anomeric proton (δ 5.35 ppm, H-1") of the rhamnose was connected to the carbon signal at δ 136.48 ppm (C-3). It revealed that the rhamnose was attached at C-3 of the aglycone.

All spectroscopy data confirmed that the isolated compound was assigned as a flavonol of quercetin 3-*O*-α-L-rhamnoside, known as quercitrin, with the molecular formula of $C_{21}H_{20}O_{11}$ (Fig. 4). As additional information, this study was the first report that successfully isolated a quercitrin compound from *S. ferruginea* with the specified host of cacao. The isolated compound of *S. ferruginea* has not been extensively explored. Only three isolated compounds have been reported, such as 4"-*O*-acetylquercitrin, quercetin, and quercitrin [12]. Although quercitrin had been previously isolated from *S. ferruginea*, it was from an unspecified host. Quercitrin was also found from *Zanthoxylum bungeanum* [26], *Euphorbia hirta* [27], *Pistacia lentiscus* [28], and *Euphorbia characias* subsp. *wulfenii* [29].

Quercitrin was assumed to be the main chemical

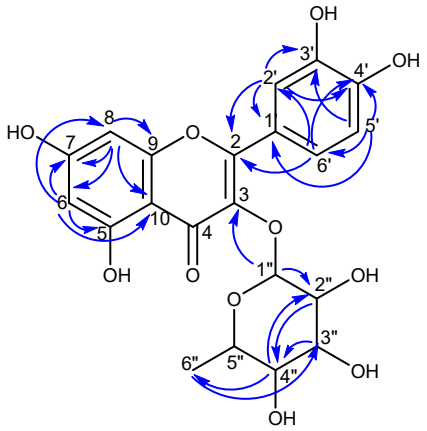

Fig 4. Quercitrin structure with key HMBC (H→C)

constituent in *S. ferruginea* from the cacao host. It was related to the amount of this isolated compound in which the percentage of the quercitrin was about 5.0% of the crude extract. Therefore, the quercitrin compound of *S. ferruginea* from the cacao host was simply isolated by the trituration method.

Antioxidant Activity

Plants are known to possess many bioactive compounds that can act as an antioxidant. Therefore, the evaluation of the antioxidant activity of several medicinal plants, including crude extract and isolated compounds, has attracted the interest of many researchers. The radical scavenging activity of the *S. ferruginea* extract and the isolated compound of quercitrin (Table 5) was investigated by the DPPH method. The principle of this method is based on the color-changing due to stabilizing of DPPH radical by the presence of an antioxidant agent.

As shown in Table 5, the extract of *S. ferruginea* leaves revealed intense antioxidant activity, in which the ethyl acetate extract had the highest activity with the IC_{50} of 10.88 ± 0.39 μg/mL. In contrast, hexane extract had the lowest antioxidant activity. This result was correlated to the active compound contained in each extract. The phytochemical screening of *S. ferruginea* extract (Table 2) had revealed that the ethyl acetate and methanol extracts were dominated by flavonoid and phenolic compounds. These compounds have been known to have potential antioxidant activity [30].

Table 5. Antioxidant activity of *S. ferruginea* extract and quercitrin

No	Sample	IC_{50} (μg/mL) ± SD
1	Hexane extract	102.72 ± 2.06
2	Ethyl acetate extract	10.88 ± 0.39
3	Methanol extract	18.36 ± 0.58
4	Quercitrin	5.95 ± 0.11
5	Ascorbic acid	2.83 ± 0.11

The values were presented as average ± standard deviation (n = 3)

This result was supported by the other works of literature that reported the antioxidant activity from the unspecified host of *S. ferruginea* with IC_{50} 27.81 μg/mL obtained from methanol extract of stem part [8] and IC_{50} higher than 30 μg/mL from acetone extract of the stem, leaves, and flower parts [31]. It revealed that *S. ferruginea* leaves extract from the cacao host had the strongest antioxidant activity among the others.

The isolated compound of *S. ferruginea* leaves, the quercitrin, also demonstrated strong antioxidant activity with the best IC_{50} of 5.95 ± 0.11 μg/mL compared to the crude extract. Quercitrin compound belongs to a phenolic compound that has an aromatic ring. The presence of an aromatic ring can stabilize the radical through the electron resonance effect in the ring system [32].

■ CONCLUSION

This study exhibited that the leaf extract of *S. ferruginea* growing on the cacao plant has the potential to be used as an antioxidant agent. This mistletoe also exhibited a high content of quercitrin compound that has stronger radical scavenging activity than the crude extract. Therefore, the cacao mistletoe of *S. ferruginea* may have the potential activity to treat the diseases caused by oxidative stress generated from free radicals.

■ AUTHOR CONTRIBUTIONS

Dara Pratama conducted the experiment, Afrizal supervised the experiment, Mai Efdi supervised and reviewed the experiment, Tia Okselni interpreted the data, wrote, and revised the manuscript. The authors agreed to the final version of this manuscript.

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