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The Chemical Compounds of *Flacourtia rukam* Leaves and Their Inhibition of Anggiotensin-converting Enzyme (ACE) Activity

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ABSTRACT. *Flacourtia rukam* is a popular plant to treat hypertension, especially the Musi Banyuasin of South Sumatera, Indonesia. Isolation and identification of chemical compounds from *F. rukam* leaves and evaluation of their effects on antihypertensive activity have been conducted. Isolation of chemical compounds using chromatographic methods and identification using spectroscopic methods were compared with the reported data. The drug's effects on antihypertension were determined using the angiotensin-converting enzyme (ACE) inhibitory method. Two compounds have been isolated from the leaves of *F. rukam* and identified as apigenin (**1**, 20 mg) and lupeol (**2**, 17 mg). Lupeol (**2**) was first reported and isolated from the leaves of *F. rukam*. These compounds were demonstrated to be effective in treating antihypertension with 15.12 ± 0.72 μg/mL for apigenin and IC_{50} 656.51 ± 1.55 μg/mL for lupeol. It can be concluded that *F. rukam* leaves are a potential ACE inhibitor that can be explored further as an effective antihypertensive agent.

Keywords: Antihypertension activity, apigenin, *Flacourtia rukam,* lupeol, ACE

INTRODUCTION

The research on bioactive compounds in traditional medicine is moving forward. An ethnobotany survey of traditional drugs in Indonesia identified many medicinal plants traditionally used but not supported by scientific data. One common disease in the community is hypertension, a clinical syndrome defined as systolic blood pressure ≥ 140 mmHg and diastolic blood pressure ≥ 90 mmHg (Oparil et al., 2019). Hypertension can trigger various diseases such as coronary heart disease, stroke, and heart failure. These complications can increase mortality (Fuchs & Whelton, 2020).

One traditional medicine used to treat hypertension is *F. Rukam,* a plant belonging to the family Flacourtiaceae and in Indonesia is known as rukem (Heyne, 1987). This plant is widely used in South Sumatra to treat hypertension (Yustian, Muharni, Sukarmi, Arbi, & Zulaika, 2012). *F. rukam* is a shrub 5-15 m high, with a leaf diameter of about 6 cm, thorny stems up to 10 cm, young leaves measuring 4-8 mm, mature leaves 6-16 × 4-7 cm, yellowish-green flowers, and young green to mature red fruit with a diameter of 2-2.5 cm (Heyne, 1987). Based on previous studies, the leaves of *F. Rukam* are also used to treat eyelid inflammation. Postpartum women use the roots as an antiseptic, and the young leaves can be eaten directly (Ragasa et al., 2016).

In Thailand, the *F. Rukam* wood is used to treat skin infections (Chusri, Chaicoch, Thongza-ard, Limsuwan, & Voravuthikunchai, 2012). Ragasa et al. (2016) reported that the chemical content steroid compounds, chlorophyll and glycerol derivatives of the *F. rukam* fruit. Glycerol derivates are active as anti-inflammatory agents. (Imbs, Ermakova, Fedoreyev, Anastyuk, & Zvyagintseva, 2013) and cytotoxic (Nguyen et al., 2004; Tsai, de Castro-Cruz, Shen, & Ragasa, 2012). Thai et al. (2020) have reported four compounds from *F. rukam* stems led, while three compounds others have reported by Muharni Elfita, Heni, Yasrina, & Miranti (2019) from the stem bark. The similar compound (fliedelin) from the *F. rukam* stem bark also to reported from the root (Muharni et al., 2019). Seventeen phenolic compounds of *F. rukam* leaves and bark led also have been reported and one amoung the compound is apigenin (Afifi et al., 2021). Phytochemical assay of ethanol extracted from the *F. rukam* leaves indicated terpenoid, steroid, flavonoid, and phenol compounds (Muharni, Fitrya, & Nurmaliana, 2016). Two compounds, apigenin (**1**) and lupeol (**2**) were isolated from the leaf extract.

This study evaluated the isolation, structure elucidation, and antihypertensive activity assay of these compounds. The antihypertensive activity of these compounds was determined using the angiotensin converting enzyme (ACE) inhibitory method (Arihara, Nakashima, Mukai, Ishikawa, & Itoh, 2001).

EXPERIMENTAL SECTIONS

General Experiment Procedure

The UV spectrum was recorded on single-beam Shimadzu-UVmini-1240. The IR spectrum in KBr was recorded on a Perkin Elmer-FTIR spectrometer. The NMR spectra were recorded on an Agilent DD2 (500 MHz for 1H-NMR and 125 MHz for 13C-NMR). Ultraviolet lamps were the CAMAG UV lamp dual wavelength (λ = 254 and 365 nm). Chemicals and reagents used in this study were purchased from Sigma-Aldrich and Merck.

Plant Materials

Fresh leaves from the *F. rukam* were collected in January 2019 from the Regency of Musi Banyuasin, South Sumatera, Indonesia, and identified by Dr Laila Hanum, Head of the Botany Laboratory, University of Sriwijaya. The voucher specimen was deposited in the laboratory (specimen number VIC 2702).

Preparation of Extracts

Fresh leaves from *F. rukam* were cut into small pieces (600 g) and extracted using *n*-hexane (2.5 L) solvent by the maceration method for 24 hours. The extraction process was repeated three times using a rotary evaporator at about 50^{0}C to dryness to obtain a crude *n*-hexane extract The residue was then re-extracted successively with ethyl acetate and methanol. Each extract was concentrated to yield a crude ethyl acetate and methanol extract (Metasari, Elfita, Muharni, Yohandini, 2020).

Fractionations and Purification

The ethyl acetate extract (15 g) was separated using vacuum column chromatography with silica gel G 60 (120 g) as a stationary phase. The sample was prepared using pre-adsorption with a silica gel 70-230 mesh at a ratio of 1:1. Furthermore, elution was conducted with *n*-hexane and then with a solvent *n*-hexane : ethyl acetate (9:1-5:5). The eluent was collected in 500 mL bottles and evaporated. TLC analysis identified the component UV light and cerium sulfate to the component viewer. The eluent showed the same TLC patterns combined into one fraction, then concentrated to obtain five fractions: F1 (3.202 g), F2 (1.224 g), F3 (2.520 g), F4 (0.742 g), and F5 (5.421 g). Fraction F5 (5g) was fractionated using gravity column chromatography with silica gel 70-230 mesh (60 g) as a stationary phase and a solvent by gradually increasing the polarity with ethyl acetate in *n*-hexane for elution (9:1-5:5). The eluent showed the same TLC patterns combined into one fraction and then concentrated to obtain five fractions: F5.1 (1.152 g), F5.2 (0.675 g), F5.3 (1.554 g), F5.4 (0.543 g), and F5.5 (0.422 g). The F5.5 (0.422) fraction was purified again using gravity column chromatography. The eluent showed the same TLC patterns combined into one fraction, then concentrated to obtain five fractions: F5.5.1 (102 mg), F5.5.2 (51 mg), F5.5.3 (71 mg), F5.5.4 (103 mg), and F5.5.5 (62 mg). In F5.5.1, the fraction was obtained from white crystalline needles (**1**, 20 mg).

The methanol extract (10 g) was separated using vacuum column chromatography. The sample was prepared by pre-adsorption using silica gel 70-230 mesh, and elution was carried out in the mobile phase using a solvent with a mixture of *n*-hexane: ethyl acetate (5:5, 4:6, 3:7, 2:8, and 1:9) and ethyl acetate: methanol (9:1). The filtrate was concentrated and analyzed using TLC. Samples showing the same stain pattern were combined into one fraction (F1-F4). The fraction (F3) was then separated using gravity column chromatography with a diameter of 2 cm (Roge et al., 2011). Pre-adsorption was conducted on the selected fraction (F3) using a solvent similar to the fraction. Samples were eluted using a mixture of *n*-hexane: ethyl acetate (5:5, 4:6; 3:7, 2:8, and 1:9) and ethyl acetate 100%, each 200 mL. The resulting eluate was collected in vials (±10 mL) and identified using TLC. Vials with the same stain were combined into one fraction (F3.1-F3.6). The subfraction F3.4 showed greenish-white crystals, and after purification with *n*-hexane, white crystals (**2**, 17 mg) were well soluble in chloroform.

Antihypertensive Activity by Angiotensin Converting Enzyme (ACE) Inhibition.

Invitro antihypertensive activity was determined based on ACE inhibitor activity according to the method described by Arihara et al. (2011). Eighty (80) µL of the sample (series concentrations 100, 200, 300, 400, and 500 μg/mL for sample, and 10, 20, 30, 40, and 50 for standard captopril μg/mL) was mixed with 50 µL of hippuryl-L-histidyl-L-leucine (HHL) buffer (2.5 mM HHL in 0.05 M of sodium borate buffer pH 8.3), then 15 µL bovine serum albumin (BSA); the mixture was allowed to stand for five min at 37°C and then added 50 µL ACE (0.05 units/mL). The mixture was allowed to stand for 60 min at 37°C. The reaction was stopped by adding 250 µL of 0.5 M HCl and 1.7 mL of ethyl acetate, forming hippuric acid. The mixture was then centrifuged at a speed of 10,000 rpm for 10 min. An 800 µL supernatant was oven-dried at 95°C for 75 min. The resulting hippuric acid was dissolved into 1,000 µL of aqua dest. Absorbance was measured at a wavelength of 228 nm using a UV-Vis spectrophotometer. As a control positive ACE inhibitor using captopril. The activity of the ACE inhibitors was calculated using the formula:

(%) inhibitor activity ACE = (A-B) - (C-D) / (A-B) x 100%

A = Absorbance control (substrate HHL + enzyme ACE)
B = Absorbance blank control (substrate HHL + aquades)
C = Absorbance sample (substrate HHL + enzyme ACE + sample)
D = Absorbance blank sample (substrate HHL + sample)

Data Analysis

The ACE inhibition activity experiment was carried out in triplicate. Data were expressed as mean ± SD. The percent inhibition and IC_{50} were determined using analysis of variance (ANOVA) alfa 0.05, followed by a Duncan new multiple range test (DNMRT) at alfa 0.05 using the Statistical Package for the Social Science (SPSS 14) software.

RESULTS AND DISCUSSION

The extraction (600 g) of fresh *F. rukam* leaves was accomplished using the maceration method with a step gradient polarity solvent (*n*-hexane, ethyl acetate, and methanol) and after evaporation to generate the crude *n*-hexane extract (28.97 g), ethyl acetate extract (16.49 g), and methanol (28.98 g). We successfully isolated pure compounds in the form of yellow crystalline (20 mg **1**) from ethyl acetate extract with a melting point of 340-343°C and a pure compound of white crystalline needles (17 mg **2**) from the methanol extract with a melting point of 214-216°C.

The UV spectrum compound **1** showed patterns at 268 nm and 336 nm, indicating C = C conjugated to the aromatic ring. IR spectrum showed the absorption at 1610, 1555 and 1496 cm^{-1}. These were characteristic C = C aromatic, and 3093 cm^{-1} was characteristic of the stretching C-H aromatic. Other absorption peaks included 3327 cm^{-1} for bending O-H, 1180 cm^{-1} for C-O phenol, and 1654 cm^{-1} for peak ketone carbonyl. The IR spectra data accordance to the IR data of apigenin available in the literature (Nayaka, Londonkar, Umesh, & Tukappa, 2014). The ^{1}H-NMR spectrum showed the chemical shifts at δ 7.91 (2H, *d*, *J* = 8.5) and 6.93 (2H, d, *J* = 8.5). These were characteristic of the four aromatic protons; ortho a coupled from the aromatic di substitutions. Two other aromatics proton at 6.19 (1H, *d*, *J* = 2) and 8.64 (1H, d, *J* = 2) with meta coupled. The proton NMR spectrum also showed a region of chemical signal at 12.96 (1H, *brs*) was chelated hydroxyl. The signal at δ_H 6.78 (1H, *s*) showed the presence of proton of aromatic not coupled.

The ^{13}C-NMR spectrum (in CD_3OD) showed 13 carbon signals, all for carbon C sp^2. The signal at δ_C 181.7 was characteristic for C carbonyl ketones, which supported the absorption for a peak of a carbonyl group in the IR spectrum at 1654 cm^{-1}. Fourteen other signals carbon signals for aromatic carbon. The HSQC spectra correlation δH 7.91 to carbon at 128.5 and 6.93 to carbon at 115.9 demonstrated that each represented two carbon signals. Other signals at the HSQC spectra showed 6.78 to 102.6 carbon, 6.48 to 98.8 carbon, and 6.19 to 93.9 carbon. Based on the HSQC spectra, there were 15 carbon signals attributed to seven methine and eight quaternary carbons. The HMBC spectra showed a correlation proton of δ_H 6.78 (1H, *s*) to carbon at δc 163.7, δc 181.7 , δc 103.6, and δc 121.1. Proton at δ_H 6.48 to carbon at δc 164.1, δc 157.3, and δc 103.8. Others correlations at the HMBC spectra proton included δ_H 6.19 to carbon at δc 102.6, δc 161.4, and δc 93.9. Proton at δ_H 7.91 to carbon δc 161.1 and δc 181.7 and proton δ_H 6.93 to carbon δc 121.1 and δc 161.1 NMR data of ^{1}H, ^{13}C, and HMBC NMR for compounds **1** are shown in **Table 1**. The correlations at HMBC and HSQC for the structure of compounds **1** are shown in **Figure 2**. Based on these data, compound **1** was determined to be apigenin (**Figure 1**).

The IR spectrum compound **2** showed an absorption peak of 3377 cm^{-1} were characteristic of the hydroxyl group (-OH). The hydroxyl group (-OH) was supported by the absorption peak of 1020 cm^{-1} for stretching of the C-O. In addition, the absorption bands of 2959 cm^{-1} and 2868 cm^{-1} (CH aliphatic asymmetric and symmetric). An additional two peaks at 1481 cm^{-1} and 1366 cm^{-1} represented bending frequencies for the CH_2 and CH_3 cycloaliphatic. The ^{1}H NMR spectrum (500 MHz, $CDCl_3$) compound **2** (**Table 2**) showed a signal at 3.20 ppm (1H, *m*), typical for H-3 of a triterpene type carbon skeleton. Signals at 4.56 and 4.68 (1H, each) indicated a vinylic proton at C-29. Besides these, the spectrum displayed a signal multiplet of one proton at 2.36 ppm assigned to H-19. The spectrum also showed 2.00 and 1.98 masing (2H, m) as signals for two methylene groups in the cycloaliphatic form and seven signal singlets of methyl groups at 0.75 (3H, *s*), 0.78 (3H, *s*), 0.82 (3H, *s*), 0.94 (3H, *s*), 0.96 (3H, *s*), 1.02 (3H, *s*), and 1.24 ppm (3H, *s*). The ^{13}C-NMR spectrum showed 30 carbon signals. Signals at 151.1 and 109.5 were assigned to the olefinic carbon and signal at δ_c 79.2 ppm; these are characteristic of the C-3 in triterpenoid that binds the OH groups. Other signals that accumulate in areas below δc 60 ppm are signals for sp^3 carbon in a cycloaliphatic form for terpenoid compounds. The 1H and ^{13}C NMR values for all the protons and carbons (**Table 2**) were compared with previous values (Shwe, Win, Moe, Myint & Win, 2019). These data confirmed that compound **2** is lupeol (**Figure 1**).

Apigenin (**1**) compound based on the literature study have been reported from *F. rukam* (Afifi et al., 2021), but there are no studies on the presence of compounds **2**. This study is the first on the isolation of compounds **2** from *F. rukam*. The apigenin compound have been reported of F. rukam leaves and bark led (Afifi et al., 2021). The flavonoid compounds have been reported in other species in the genus *Flacourtia*.

Table 1. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz) data compound **1** (CD_3OD)

Carbon Position	δ_C	δ_H [∑H, mult., *J* (Hz)]	HMBC H → C
1	-	-	-
2	163.7	-	-
3	102.6	6.78 (1H, *s*)	163.7; 181.7; 103.8; 121.1
4	181.7	-	-
5	161.4	-	-
6	98.8	6.19 (1H, *d*, *J* = 2 Hz)	161.4; 93.9
7	164.1	-	-
8	93.9	6.48 (1H, *d*, *J* = 2 Hz)	161.4; 157.3; 103.8
9	157.3	-	-
10	103.8	-	-
1′	121.1	-	-
2′/6′	128.4	7.91 (2H, *d*, *J* = 8.5 Hz)	128.4; 161.1
3′/5′	115.9	6.93 (2H, *d*, *J* = 8.5 Hz)	121.1
4′	161.1	-	-

Table 2. ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), and DEPT data compound **2**[b] ($CDCl_3$), lupeol[a] ($CDCl_3$), (Shwe et al., 2019)

Carbon No	^{13}C NMR[a]	^{13}C NMR[b]	^{1}H NMR [δ_H, mult., *J* (Hz)]	^{1}H NMR[b]
1	38.85	38.22	0.90; 1.65	0.99
2	27.44	27.60	1.52; 1.67	1.98 (*m*)
3	78.99	79.19	3.20 (*dd*)	3.19 (*m*)
4	38.70	38.88		
5	55.29	55.46	0.67	0.68
6	18.31	18.50	1.37; 1.52	1.40 (*m*)
7	34.27	34.44	1.39	1.38; (*m*)
8	40.82	40.18		
9	50.43	50.61	1.25	
10	37.16	37.34		
11	20.92	21.10	1.20; 1.40	1.15 (*m*)
12	25,13	25.30	1.06; 1.62	2.00 (*m*)
13	38.04	38.88	1,66	
14	42.82	43.00		
15	27.41	27.46	1,05; 1,60	
16	35.57	35.76	1.35; 1.45	1.40 (*m*)
17	42.99	43,00		
18	48.29	48.47	1.36; 1.37	
19	47.97	48.17	2.40 (*m*) : 1.45	2.36 (m)
20	150.96	151.19		
21	29.84	29.88	1.3 ; 1.91 (*m*)	1.25
22	40.00	39.84	1.18; 1.37	
23	27.98	28.17	0.90 (*s*)	0.96 (*s*)
24	15.36	15.57	0.76 (*s*)	0.75 (*s*)
25	16.11	16.30	0.83 (*s*)	0.82 (*s*)
26	15.96	16.15	1.03 (*s*)	1.02 (*s*)
27	14.60	14.73	0.94 (*s*)	0.94 (*s*)
28	17.99	18.18	0.79 (*s*)	0.78 (*s*)
29	109.31	109.50	4.57 (*s*) ; 4.69 (*s*)	4.56 (*s*) : 4.68
30	19.29	19.48	1.67 (*s*)	1.67 (*s*)

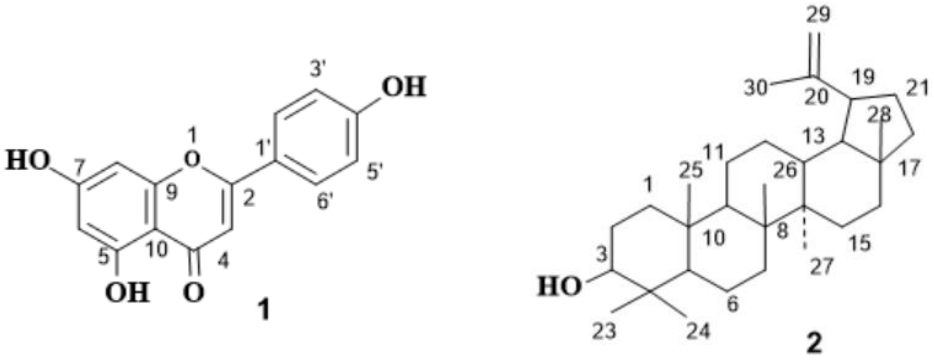

Figure 1. Chemical structures of compounds **1** and **2**

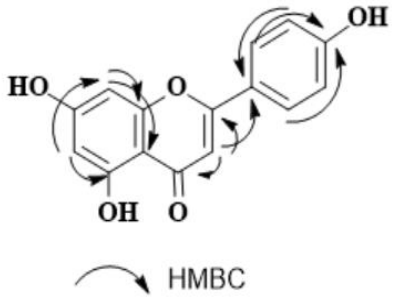

Figure 2. HMBC correlations compound **1**

They include krisoeriol-7-O-β-D-glucopyranoside, catechin-[5,6-e]-4b-(3,4-dihydroxy phenyl), dihydro-2(3H)-pyranone, mururin A (Sashidhara et al., 2013), fisetinidol-(4α-8)-catechin (Zhang et al., 2012), and (+)-catechin, quercetin, luteolin, and rutin (Talukder et al., 2012). Triterpenoid has been reported from *F. rukam,* i.e., friedelin (Muharni et al., 2019).

The antihypertension activity associated with apigenin (**1**) and lupeol (**2**) were determined based on ACE (Arihara et al., 2001). The high inhibitory activity of ACE corresponds to the low concentration of hippuric acid formation (Ahmad, Yanuar, Mulia, & Mun'im, 2017; Ozarowski et al., 2019). The activity expressed as a percentage of inhibition ACE (% I). As standard using captopril. The higher concentration of the sample, the percentage of ACE inhibition also increases (**Table 3** and **4**). The statistical analysis showed the percentage of ACE inhibition was significantly different for each concentration ($P<0.05$) of apigenin (**1**). For standard captopril at concentrations of 30 and 40 μg/mL, the statistical analysis showed no significant difference ($p>0.05$) in the level of inhibition. For concentration 40 and 50 μg/mL also no significant difference ($p>0.05$). However, there was a significant difference ($P<0.05$) for concentrations 10 and 20 μg/mL. For lupeol (**2**) at concentrations 100 and 200 μg/mL, the statistical analysis showed no significant differences.

Table 3. ACE inhibitory activity of apigenin (**1**) and captopril.

Concentration (μg/mL)	% Inhibition of ACE activity ± SD	
	Apigenin (**1**)	Captopril
10	41.38 ± 0.48^{e}	45.67 ± 2.80^{e}
20	52.49 ± 1.22^{d}	63.24 ± 0.65^{c}
30	63.52 ± 0.56^{c}	85.54 ± 0.57^{a}
40	71.00 ± 0.42^{b}	88.07 ± 0.77^{af}
50	88.16± 1.72^{a}	90.33 ± 0.75^{f}

Note: Values are presented as means ± SD, n=3. According to Duncan New Multiple Range Test (DNMRT), 5%. Numbers followed by the same subscript indicate no significant difference.

Table 4. ACE inhibitory activity of lupeol (**2**).

Concentration (μg/mL)	% Inhibition ± SD
100	6.45 ± 1.15[a]
200	8.31 ± 0.55[a]
300	13.39 ± 0.72[b]
400	26.08 ± 2.24[c]
500	41.34 ± 2.51[d]

Note: Values are presented as means ± SD, n=3, Numbers follow by the same subscript indicate no significant difference according to Duncan New Multiple Range Test (DNMRT) 5%.

Table 5. The IC_{50} value of ACE inhibition

Sample	IC_{50} (mg/L)
Apigenin (**1**)	15.12 ± 0.72[a]
Lupeol (**2**)	656.51 ± 1.55[b]
Captopril	12.55 ± 0.42[a]

Note: Values are presented as mean ± SD, n=3; Numbers follow by the same subscript indicate no significant difference.

IC_{50} values for apigenin (**1**) and lupeol (**2**) were determined using linear regression for curve percentage inhibition versus the concentrations (**Table 5**). The results showed that apigenin (**1**) and lupeol (**2**) yielded IC_{50} values 15.12 ± 0.72 μg/mL and 656.51 ± 1.55 μg/mL, while captopril had an antihypertensive standard IC_{50} value of 12.55 ± 0.42 mg/L. The statistical analysis showed that apigenin (**1**) had no significant IC_{50} value for ACE inhibition compared to standard captopril ($p>0.05$). This study identified antihypertensive compounds from the leaf extract of *F. Rukam* and the potential to develop natural antihypertensive drugs. The ACE inhibitory activity of apigenin from *Adinandra nitida* leaves at 500 µg/mL is 30.27% (Liu, Yang, Ma, Yuan & Chen, 2010). In comparison, in this study, we obtained inhibition more highest (41.38 %) and even at lower test concentrations (10 µg/mL). The difference in the ACE inhibitory activity of apigenin with that reported in the literature because of the difference in the methods was used. Liu et al. (2010) using colourimetry method, and measurement at a wavelength of 420 nm and using kit test and human serum as blanko, while in this research, absorbance was measured at a wavelength of 228 nm.

CONCLUSIONS

Two compounds were isolated from the extract of *F. rukam* leaves, identified as apigenin (**1**) and lupeol (**2**). Compound **2** were the first reported from *F. rukam*. Invitro tests of apigenin (**1**) compound showed antihypertensive activity with an IC_{50} value of 15.12 ± 0.72 μg/mL. It can be concluded that *F. rukam* leaves are a potential ACE inhibitor that can be explored further as an effective antihypertensive agent.

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