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PHYTOCHEMICAL STUDIES OF *PREMNA SCHIMPERI*, AND ANTIOXIDANT ACTIVITIES OF THE EXTRACT AND AN ISOLATED COMPOUND

Mohammedamin Nasir Isa, Wendimagegn Mammo and Estifanos Ele Yaya*

Department of Chemistry, Addis Ababa University, P.O. Box 33658, Addis Ababa, Ethiopia

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ABSTRACT. In this study, the attempts were made to isolate secondary metabolites from the solvent extract of the dried leaves of *Premna schimperi* (*P. schimperi*). Thus, six compounds, namely, *ent*-8β,12-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid-γ-lactone (**1**), 3,5,5'-trihydroxy-6,7,3',4'-tetramethoxyflavone (**2**) and 3,5,7,5'-tetrahydroxy-6,3',4'-trimethoxyflavone (**3**), *p*-hydroxycinnamic acid (**4**), luteolin (**5**) and 1-nonacosanol (**6**) were isolated and characterized by NMR, UV-Vis, MS and FTIR spectroscopic techniques. All the compounds; except luteolin (**5**) were reported for the first time from the leaves of *P. schimperi.* In addition, the GC-MS analysis of the essential oil of the plant leaves revealed 47 compounds, of which α-curcumene (16.38%), caryophyllene (10.73%), eugenol (6.85%), β-sesquiphellandrene (4.65%), γ-gurjunene (4.55%), terpinen-4-ol (2.9%), γ-eudesmol (4.43%), linalool (2.2%) and caryophyllene oxide (2.67%) were the major compounds. Furthermore, assessment of the antioxidant activities of the MeOH extract and compound **1** using the DPPH assay revealed 95.3% and 62.2% DPPH inhibition, respectively, at concentration of 100 µg/mL.

KEY WORDS: *P. schimperi*, GC-MS, DPPH radical scavenging assay, Antioxidant activity, Acetylation

INTRODUCTION

Premna schimperi belongs to the family of Lamiaceae (Verbenaceae). It is a spreading shrub or small tree up to 7-10 m high and has pale brown to grey branches. This plant is widely distributed in most parts of Ethiopia, Sudan, Kenya and Tanzania [1]. Traditionally, extract of the leaves of this plant are used to treat pyogenic infections, trauma, fracture, dysentery, toothache, skin disease, rheumatic arthritis, hypertension, external injuries and secondary infection in wounds [2, 3]. The plant was reported to exhibit antioxidant, antibacterial, anti-inflammatory, cytotoxic, anticancer, antithrombotic and hepatoprotective [4, 5] activities. Andrographolide, luteolin, quercetin, kaempferide, *ent*-12-oxolabda-8,13(16)-dien-15-oic acid, (5R,8R,9S,10R)-12-oxo-ent-3,13(16)-clerodien-15-oic acid and 16-hydroxy-clerod-3,13(14)-diene-15,16-olide were previously reported from the leaves of *P. schimperi* [6, 7]. However, there is no report on the essential oil components of the plant. In this study, attempts were made to characterize the essential oil components of extract and compound **1** were also tested for their antioxidant activities using the DPPH assay.

EXPERIMENTAL

General experimental procedures

UV-Vis spectra were recorded using a UV-T60 spectrophotometer, while FTIR spectra were determined using a PerkinElmer Spectrum 65 instrument in the range 4000–400 cm^{-1}. ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance 400 NMR spectrometer at 400.13 and 100.6 MHz, respectively. Column chromatography was performed using silica gel 60 (Merck), particle size 0.063–0.200 (70–230 mesh ASTM) and preparative thin-layer chromatography (PTLC) was carried out on acid-washed silica gel with 254 nm fluorescent indicator (Sigma-Aldrich,

*Corresponding author. E-mail: estifanose.ele@aau.edu.et
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Germany) pre-coated on glass (20 × 20 cm). Thin-layer chromatography (TLC) was performed on Merck Kieselgel 60 F254 precoated plates and were visualized under UV light at 254 and 365 nm.

Plant material

The leaves of *P. schimperi* were collected from Yabello city, Borena Zone, Oromia regional state, Ethiopia, in May 2019. The plant specimen (voucher specimen No. M002) was identified and deposited at the National Herbarium, College of Natural and Computational Sciences, Addis Ababa University, Ethiopia.

Solvent extraction of the dried leaves of P. schimperi

The air-dried and ground leaves of *P. schimperi* (300.0 g) was placed in a 3 L Erlenmeyer flask containing 1 L petroleum ether and was shaken on a mechanical shaker at room temperature for 24 h. The mixture was filtered and another 1 L of the solvent was added to the marc and the same procedure was repeated for another 24 h. The solvent was removed from the combined extract by rotary evaporation to afford a dark jelly material (15.0 g). Following the same procedure, the marc left after extraction with petroleum ether was extracted with chloroform (2 x 1 L) to yield a dark jelly extract (20.0 g). Finally, the marc left after chloroform extraction was extracted with methanol (2 x 1 L) to afford a dark jelly extract (20 g). The methanol extract was further partitioned between water and ethyl acetate to give 150 mg of the ethyl acetate fraction.

Essential oil extraction of P. schimperi leaves by hydrodistillation

To a 5 L round bottomed flask containing water (2 L), finely ground leaves of *P. schimperi* (250 g) was added and a Clevenger apparatus, which was connected to a condenser, was attached to the flask. The content of flask was heated to boiling on a heating mantle and hydrodistillation was carried out for 5 h. The essential oil (0.60 g, 0.24%) was collected, dried over anhydrous magnesium sulfate ($MgSO_4$), filtered and stored until analysis by GC-MS.

Characterization of the essential oil

The essential oil of the dried leaves of the *P. schimperi* was analyzed by using Agilent Technology 7820A GC system coupled with an Agilent Technology 5977E MSD equipped with an auto-sampler. The chromatographic separation was performed on a DB-1701 micro-column (30 m long, 0.25 mm internal diameter and 0.25 µm particle size) at a flow rate of 1 mL/min. Ultra-pure helium (99.999%) was used as a carrier gas at constant flow. An Agilent G4567A autosampler was used to inject 1 µL of the sample with a splitless injection mode into the inlet heated to 275 ºC with a total run time of 29.33 min. Oven temperature was programmed at 60 ºC with the hold-time of 2 min and the column temperature was increased at the rate of 10 ºC/min until the temperature reached 200 ºC, and the heating was continued at the rate of 3 ºC/min until the temperature reached 240 ºC. The mass spectra were not collected during the first 4 min of the solvent delay. The transfer line temperature was maintained at 280 ºC and the ion source temperature was kept at 230 ºC. The ionization energy of the EI source was 70 eV. Mass spectral data were collected from 40–650 m/z. Characterization was done by matching the mass spectra with those of reference compounds recorded in NIST 2014 mass spectral library [8]. The retention indices of the essential oil components were calculated by using the van den Dool and Kratz relationship [9] (Equation 1):

$$I = 100n + 100\left(\frac{R_{t(unknown)} - R_{t(n)}}{R_{t(n+1)} - R_{t(n)}}\right) \quad (1)$$

where *I* and *n* represent the retention index of the analyte, and the number of carbon atoms of the *n*-alkane eluting immediately before the analyte, respectively. $R_{t(unknown)}$ is the retention time of the analyte, $R_{t(n)}$ and $R_{t(n+1)}$ represent the retention times of the reference *n*-alkanes eluting immediately before and after the analyte, respectively.

DPPH radical scavenging assay

DPPH radical scavenging assay is a simple method to determine antioxidant activity by measuring absorbance at 517 nm due to the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [10]. The DPPH radical scavenging assay of the MeOH extract of the dried leaves of *P. schimperi* was assessed according to the procedure described by Hoque *et al.* [11]. Thus, the MeOH extract (10.0 mg) was first dissolved in MeOH (10.0 mL). Seven different concentrations, i.e., 500, 250, 125, 62.50, 31.25, 15.63 and 7.81 µg/mL, of the MeOH extract and compound **1** were prepared by diluting the stock solution (1.0 mg/mL) with MeOH. To 1 mL of each solution, 0.004% DPPH in MeOH (4.0 mL) was added to make 100, 50.00, 25.00, 12.50, 6.25, 3.13 and 1.56 µg/mL solutions. The mixtures were shaken and kept at room temperature for 30 min. The absorbances of the solutions were then recorded at 517 nm by using a UV-Vis spectrophotometer. All measurements were performed in triplicates and the same procedure was used to determine the radical scavenging activity of ascorbic acid standards. The percentage DPPH inhibition was then calculated using (Equation 2):

$$\%\ \text{DPPH inhibition} = \frac{A_{control} - A_{extract}}{A_{control}} \times 100 \quad (2)$$

where, $A_{control}$ is the absorbance of DPPH solution in MeOH and $A_{extract}$ is the absorbance of the test sample in MeOH plus DPPH solution.

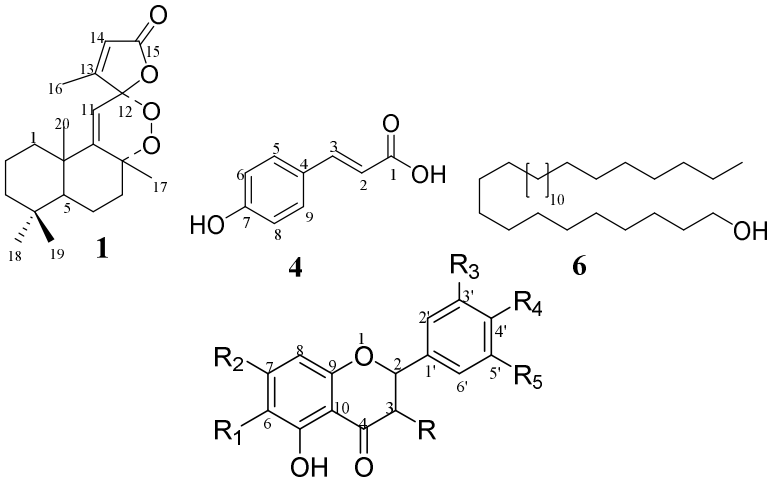
Isolation of compounds from the petroleum ether, chloroform and ethyl acetate-soluble portion of methanol extracts

The chloroform extract (14.0 g) was dissolved in chloroform, adsorbed on silica gel (14.0 g) and fractionated over the column of silica gel using a gradient of petroleum ether (PE) in chloroform ($CHCl_3$):(PE:$CHCl_3$, 100:0→0:100) and then chloroform:methanol ($CHCl_3$:MeOH, 100:0→70:30, v/v). A total of 120 fractions were collected and combined in to 4 groups and designated as N_1 (2.0 g), N_2 (2.6 g), N_3 (3.6 g) and N_4 (3.5 g). Sub-fraction N_1 and N_3 were subjected to column chromatography and eluted using a gradient of chloroform in petroleum ether (0:100→100:0) and ethyl acetate in chloroform (0:100→100:0, v/v), respectively. The PE:$CHCl_3$ eluents with the ratio of 4:1 and 7:3 were recrystallized from hexane and methanol, respectively, to yield compound **1** (30.0 mg). The $CHCl_3$:EtOAc (80:20, v/v) eluent was purified over silica gel to afford compound **2** (30.0 mg) and the $CHCl_3$:EtOAc (60:40, v/v) eluent was recrystallized from methanol to afford compound **3** (5.0 mg). The methanol extract (10.0 g) was partitioned between H_2O and EtOAc using a separatory funnel to obtain ethyl acetate-soluble fraction (150.0 mg). The ethyl acetate-soluble portion (150.0 mg) was chromatographed on a column of silica gel and eluted with $CHCl_3$:EtOAc (1:1), and crystallization of Fr.13-20 and Fr. 32-40 from acetonitrile afforded compound **4** (12.0 mg) and **5** (15.0 mg), respectively. The petroleum ether extract (15.0 mg) was fractionated over a column of silica gel using PE:$CHCl_3$ (0:100→100:0) as eluent to give compound **6**.

*Acetylation of compound **2***

To a 10 mL round bottomed flask, compound **2** (27.0 mg), pyridine (1.35 mL) and dried acetic anhydride (5 mL) were added. The mixture was heated over an oil bath at 70 ºC for overnight.

The progress of the reaction was monitored by TLC (chloroform:ethyl acetate 5:1). After completion of the reaction, the mixture was allowed to cool to room temperature and was poured in to a beaker containing crushed ice. The solid material was collected by filtration using a sintered glass funnel and was washed with distilled water exhaustively and dried to yield the acetylated product (**2-Ac**) as yellowish powder (34.0 mg, 97%).



Compound	R	R_1	R_2	R_3	R_4	R_5
2	OH	OMe	OMe	OMe	OMe	OH
3	OH	OMe	OH	OMe	OMe	OH
5	H	H	OH	OH	OH	H

Figure 1. The structure of isolated compounds from the leaves of *P. schimperi*

Physical and spectroscopic data of compounds isolated from the dried leaves of P. schimperi

*ent-8β,12-Epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid-γ-lactone (**1**)*. White crystals; m. p. 214-215 ºC, lit [11] m. p. 216 ºC. $[\alpha]_D$ -131 ($CHCl_3$, c 0.1), Lit. [6] $[\alpha]_D$ -128 ($CHCl_3$, c 0.1); UV (EtOH, λ_{max}, nm): 227; IR_v (KBr, cm^{-1}): 2996, 2955, 2926, 2906, 1764, 1659, 1457, 1438, 1241, 1239; HRESIMS *m/z* 333.2066 $[M+H]^+$ (calculated for $C_{20}H_{28}O_4$, 333.206585, Δ -0.05 ppm); ^1H- and ^{13}C-NMR data (Table 2 and 3) [6].

*3,5,5′-Trihydroxy-6,7,3′,4′-tetramethoxyflavone (**2**)*. Yellowish solid; m. p. 210-211 ºC, lit [7] m. p. 211 ºC; UV (MeOH, λ_{max}, nm): 255, 267, 340, 358; IR_v (KBr, cm^{-1}): 3537, 3436, 3256, 2993, 2922, 1655, 1587, 1493, 1434, 1279, 1215; ^1H- and ^{13}C-NMR data (Table 2 and 3) [7].

3,5,5′-Triacetoxy-6,7,3′,4′-tetramethoxyflavone (2-Ac). Yellowish solid; m. p. > 300 ºC: UV (MeOH, λ_{max}, nm): 262, 318, IR_v (KBr, cm^{-1}): 2937, 1776, 1648, 1461, 1200; HRESIMS *m/z* 517.1344 $[M+H]^+$ (calculated for $C_{25}H_{24}O_{12}$, 517.1346, Δ 0.40 ppm); ^1H- and ^{13}C-NMR data (Tables 2 and 3) [7].

*3,5,7,5′-Tetrahydroxy-6,3′,4′ trimethoxyflavone (**3**)*. Yellowish solid; m. p. 152-153 ºC, UV (MeOH, λ_{max}, nm): 254, 262, 301, 339, 362; IR_v (KBr, cm^{-1}): 3469, 3412, 3332, 2994, 2923, 1656, 1493, 1445, 1228, 1200; ^1H- and ^{13}C-NMR data (Tables 2 and 3) [7].

*p-Hydroxy cinnamic acid (**4**)*. Light-yellow solid; m. p. 210-212 ºC, lit [12] m. p. 211.5 ºC; UV (EtOH, λ_{max}, nm): 211, 224, 292, 299, 309; IR_v (KBr, cm^{-1}): 3374, 2928, 1672, 1601, 1450, 1245, 1212; HRESIMS *m/z* 165.0551; $[M+H]^+$ (calculated for $C_9H_8O_3$, 165.0551, Δ 0.42 ppm); ^{1}H-and ^{13}C-NMR data (Table 2 and 3) [13].

*Luteolin (**5**)*. Yellow amorphous powder; m. p. > 300 ºC; lit [14] m. p. 325 ºC, UV (EtOH, λ_{max}, nm): 255, 268, 292, 352; IR_v (KBr, cm^{-1}): 3426, 2923, 1655, 1615, 1455, 1265, 1246; HRESIMS *m/z* 287.0558 $[M+H]^+$ (calculated for $C_{15}H_{10}O_6$, 287.0556, Δ -0.82 ppm); ^{1}H- and ^{13}C-NMR data (Table 2 and 3) [15].

*1-Nonacosanol (**6**)*. White solid; m. p. 76.5-78 ºC; IR_v (KBr, cm^{-1}): 3405, 2922, 2853, 1644, 1461,1245, 719; HRESIMS, *m/z* 425.4 $[M+H]^+$; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.8 (3H, *t*, *J* = 6.8Hz), 1.3 (50H, *bs*), 1.6 (4H, *m*, *J* = 6.8), 3.68 (2H, *t*, *J* = 6.6Hz); ^{13}C-NMR (100MHz, $CDCl_3$, δ, ppm): 63.12, 32.82, 31.93, 29.71, 29.67, 29.62, 29.44, 25.37, 22.74, 14.12.

RESULTS AND DISCUSSION

DPPH assay

Radical scavenging activity was quantified by the decrease in absorbance of seven different concentrations of the MeOH extract and compound **1** in DPPH solution (0.004%). The antioxidant activity of each concentration was measured in relation to ascorbic acid (a known antioxidant) standards. All determinations were performed in triplicates and percent DPPH.

Both the MeOH extract and the isolated compound **1** were able to reduce the stable DPPH radical indicating their potential as radical scavenger. The extract and compound **1** showed 95.3% and 62.2% DPPH inhibition, respectively, at concentration of 100 µg/mL (Table 1).

Table 1. Radical scavenging activities of the methanol extract and compound **1** of the leaves of *P. schimperi*.

Concentration (µg/mL)	% DPPH inhibition		
	MeOH extract of the dried leaves of *P. schimperi*.	Compound **1**	Ascorbic acid standard
100.00	95.3 ± 0.88	62.2 ± 0.57	96.09 ± 0.16
50.00	91.3 ±1.24	59.7 ± 0.47	96.26 ± 0.06
25.00	88.6 ± 0.45	45.0 ± 0.81	96.06 ± 0.12
12.50	80.5 ± 0.41	35.5 ± 0.40	96.06 ± 0.12
6.25	61.3 ± 1.24	25.9 ± 0.77	91.30 ± 0.31
3.13	34.9 ± 0.89	21.6 ± 0.62	54.06 ± 1.27
1.56	27.1 ± 0.49	17.4 ± 0.81	35.00 ± 0.52

The results are reported as mean ± SD of triplicates.

Characterization of compounds isolated from the MeOH extract of the leaves of P. schimperi

Compound **1** was obtained as white crystals and had a melting point of 206-208 ºC. The molecular formula of this compound was determined to be $C_{20}H_{28}O_4$ based on its positive ion HRESIMS, which showed a molecular ion peak ($[M+H]^+$) at *m/z* 333.2066. The UV spectrum (EtOH) showed absorption maxima at 227 nm which indicated the presence a conjugated carbonyl group in the molecule. The IR spectrum (KBr) indicated the presence of a conjugated carbonyl (1764 cm^{-1}) and a double bond (1659 cm^{-1}). The ^{1}H-NMR spectrum of **1** (Table 2) showed five methyl proton signals at δ_H 0.91 (3H, *s*, H-18), 0.94 (3H, *s*, H-19), 1.23 (3H, *s*, H-20), 1.69 (3H, *s*, H-17) and 1.98 (3H, d, *J* = 2 Hz, H-16). Two olefinic proton signals appeared at δ_H 5.23 (1H, *s*, H-11) and

5.98 (1H, q, J = 2 Hz, H-14 with allylic coupling to the methyl proton signal at H-16. In addition, the ^{13}C-NMR together with the DEPT-135 spectrum (Table 3) suggested the presence of carbonyl, five methyl, five methylene and two olefinic carbon atoms. The HMBC spectrum of **1** revealed correlations of H-18 (δ 0.91) to C-19 (δ 21.24), C-4 (δ 33.60), C-3 (δ 41.87) and C-5 (δ 43.69) and H-19 (δ 0.94) to C-18 (δ 32.99), C-4 (δ 33.60), C-3 (δ 41.87) and C-5 (δ 43.69) which suggested that C-18 and C-19 are attached to C-4 (δ 33.60). Correlations of H-20 (δ 1.23) to C-10 (δ 38.47), C-1 (δ 40.39), C-5 (δ 43.69) and C-9 (δ 163.13) suggested that C-20 is attached to C-10 (δ 38.47). Correlations of H-17 (δ 1.69) to C-7 (δ 26.39), C-8 (δ 78.94) and C-9 (δ 163.13) was used to confirm that the signal at δ 1.69 is due to the proton at C-17 which is attached to C-8 (δ 78.94). Correlations of H-16 (δ 1.98) to C-12 (δ 107.19), C-14 (δ 121.05) and C-13 (δ 162.43) suggest that the signal at δ 1.98 is due to the proton at C-16 which is attached to C-13 (δ 162.43). Based on the above spectroscopic data, compound **1** was proposed as ent-8β,12-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oic acid-γ-lactone. This is the first report of compound **1** from *P. schimperi*. Compound 1 was first isolated from *P. oligotricha* and was reported to exhibit weak antibacterial activity [6].

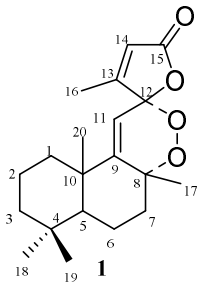


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Table 2. The ^{1}H-NMR data.

H	**1**	**2**	**2-Ac**	**3**	**4**	**5**
H-1	1.20 and 1.85 (2H, *m*)	---	---	---	9.20 (COOH, *bs*)	---
H-2	1.50-1.60 (2H, *m*)	---	---	---	6.35 (1H, *d*, *J* = 16 Hz)	6.60 (1H, *s*)
H-3	1.18 and 1.48 (2H, *m*)	---	---	---	7.63 (1H, *d*, *J* = 16 Hz)	---
H-4	---	---	---	---	---	13.04 (OH, *s*)
H-5	1.57 (1H, *m*)	---	---	---	7.56 (2H, *d*, *J* = 8.8 Hz)	6.26 (1H, *d*, *J* = 2.0 Hz)
H-6	1.50-1.60 (2H, *m*)	---	---	---	6.91 (2H, *d*, *J* = 8.8 Hz)	---
H-7	1.65 (2H, *m*)	---	---	---	---	6.54 (1H, *d*, *J* = 2.0 Hz)
H-8	---	6.55 (1H, *s*)	6.89 (1H, *s*)	6.69 (1H, s)	6.91 (2H, *d*, *J* = 8.8 Hz)	---
H-9	---	---	---	---	7.56 (2H, *d*, *J* = 8.8 Hz)	---
H-10	---	---	---	---	---	---
H-11	5.23 (1H, *s*)	---	---	---	---	---
H-12	---	---	---	---	---	---
H-13	---	---	---	---	---	---

H-14	5.98 (1H, *q*, *J* = 2 Hz)	---	---	---	---	---
H-15	---	---	---	---	---	---
H-16	1.98 (3H, d, *J* = 2 Hz)	---	---	---	---	---
H-17	1.69 (3H, *s*)	---	---	---	---	---
H-18	0.91 (3H, *s*)	---	---	---	---	---
H-19	0.94 (3H, *s*)	---	---	---	---	---
H-20	1.23 (3H, *s*)	---			---	---
H-2′	---	7.45 (1H, *d*, *J* = 2 Hz)	7.18 (1H, *s*)	7.54 (1H, *d*, *J* = 2 Hz)	---	7.52 (1H, *d*, *J* = 2.0 Hz)
H-5’	---	---	---	---	---	7.00 (1H, *d*, *J* = 8.4 Hz)
H-6′	---	7.50 (1H, *d*, *J* = 2 Hz)	7.28 (1H, *s*)	7.49 (1H, *d* *J*=2 Hz)	---	7.48 (1H, *dd*, *J* = 8.4 and *J* = 2.0 Hz)
OMe-6	---	4.01 *s*	3.88 *s*	3.89 *s*	---	---
OMe-7	---	3.99 *s*	4.00 *s*	---	---	---
OMe-3′	---	3.95 *s*	3.94 *s*	3.95 *s*	---	---
OMe-4′	---	3.98 *s*	3.95 *s*	3.98 *s*	---	---
OH-5	---	11.63 (3H, *s*)	---	12.27 (1H, *s*)	---	---
Ac	---	---	2.35 (3H, *s*) 2.38 (3H, *s*) 2.50 (3H, *s*) 2.35 (3H, *s*)	---	---	---

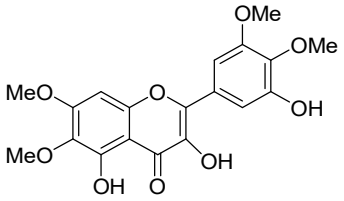
^{1}H-NMR data of compounds **1**, **2**, **2-Ac** and **3** in $CDCl_3$ and the compounds **4** and **5** in Acetone-d_6 at 400 MHz.

Table 3. The ^{13}C-NMR data.

C	**1**	**2**	**2-Ac**	**3**	**4**	**5**
1	40.39	---	---	---	167.57	---
2	19.04	144.96	153.77	145.45	114.82	164.25
3	41.87	136.23	133.35	136.35	144.82	103.30
4	33.60	175.40	170.10	176.18	126.16	182.16
5	43.69	151.37	142.06	151.64	130.05	162.48
6	16.56	132.23	139.86	130.87	115.81	98.81
7	26.39	159.42	158.11	157.21	159.64	164.00
8	78.94	90.75	98.18	93.80	115.81	93.79
9	163.13	152.19	153.39	152.26	130.05	157.89
10	38.47	104.38	111.30	103.79	---	104.44
11	111.24	---	---	---	---	---
12	107.19	---	---	---	---	---
13	162.43	---	---	---	---	---
14	121.05	---	---	---	---	---
15	169.52	---	---	---	---	---
16	13.08	---	---	---	---	---
17	25.38	---	---	---	---	---
18	32.99	---	---	---	---	---
19	21.24	---	---	---	---	---
20	25.66	---	---	---	---	---
1′	---	126.31	124.66	126.45	---	122.84
2′	---	104.51	110.06	103.76	---	115.75
3′	---	152.45	153.77	153.11	---	145.62

4′	---	137.30	142.57	138.26	---	149.24
5′	---	149.26	143.97	150.40	---	113.23
6′	---	107.55	115.80	108.99	---	119.25
6-OMe	---	60.95	61.58	59.89	---	---
7-OMe	---	56.41	56.55	---		---
3′-OMe	---	56.08	56.34	55.54		---
4′-OMe	---	61.12	60.90	59.98		---
Ac	---	---	169.37, 168.83 168.00 20.99, 20.73 20.59	---	---	---

^{13}C-NMR data of compounds **1**, **2**, **2-Ac** and **3** in $CDCl_3$ and the compounds **4** and **5** in Acetone-d_6 at 100 MHz.



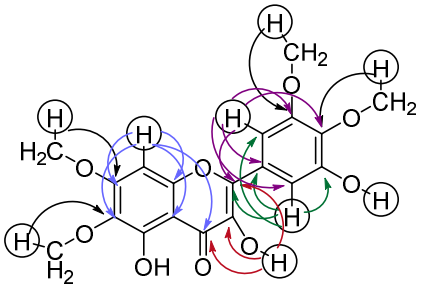
2

Compound **2** was isolated as a yellowish solid and had a melting point of 210-211 ºC. The UV spectrum (MeOH) showed absorption maxima at 255, 267, 340 and 358 nm, which indicated the presence of a flavone moiety [16]. The IR spectrum revealed the presence of hydroxyl stretching bands (3537, 3436 and 3256 cm^{-1}), C-H stretching bands (2993 and 2922 cm^{-1}), α,β-unsaturated carbonyl stretching band (1655 cm^{-1}), C-H bending bands (1493, 1465 and 1434 cm^{-1}) and C-O bending bands (1215 and 1279 cm^{-1}). The aromatic region of the ^{1}H-NMR spectrum (Table 2) of compound **2** showed an ABX spin system [17] indicating a tri-substituted aromatic ring. Four methoxy proton singlets appeared at δ_H 3.95, 3.98, 3.99 and 4.01, which showed the HMBC correlations of H-8 (δ_H 6.55) to the carbons resonances at δ 159.42 and 132.23, and the correlations of these two carbons to the two methoxy proton resonances (δ_H 3.99, δ_C 56.41 and δ_H 4.01, δ_C 60.95, respectively) suggest that the methoxy groups are at C-6 and C-7, but not at C-3. The correlations of H-2′ (δ_H 7.45) to the carbon resonances at δ137.30 and 152.45, and the correlations of these two carbons to the two remaining methoxy groups at δ_H 3.98 (δ_C 61.12) and 3.95 (δ_C 56.08) can occur only if the methoxy groups are placed at C-3′ and C-4′, but not at C-3, could be assigned to the methoxy groups at C-3′, C-4', C-7 and C-6, respectively. The most downfield proton singlet at δ 11.63 (1H) is assignable to the chelated hydroxy group at C-5. The proton signal at δ 6.55 (1H, *s*) can be assigned to H-8 of the 5,6,7-trisubstituted ring-A. The two unsymmetrical *meta*-coupled doublets (J = 2 Hz) at δ 7.45 (1H) and 7.50 (1H) are attributable to H-2′ and H-6′, respectively, of the 3′,4′,5′-trisubstituted ring-B.

The ^{13}C-NMR spectrum of **2** in conjugation with its DEPT-135 spectrum revealed 19 carbon resonances, of which four appeared at δ 56.08, 56.41, 60.95 and 61.12 and are assignable to the methoxy groups at C-3′, C-7, C-6 and C-4′, respectively. The presence of twelve quaternary carbon signals, of which four are due to methoxylated carbon atoms, three due to hydroxylated carbon atoms and one due to a conjugated carbonyl carbon suggest that compound is a trihydroxytetramethoxyflavone.

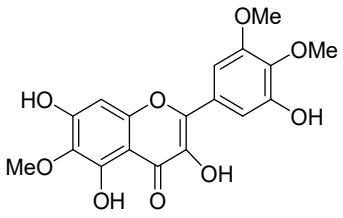
The HMBC correlations of OH proton signal at δ 6.76 to C-3 (δ 136.23), C-2 (δ 144.96) and C-4 (δ 175.40) indicated that this OH is directly attached to C-3 (δ 136.23). In addition, the carbon resonances of the two methoxy groups appeared at 56.08 and 56.41, whereas the signals due to the other two methoxy groups appeared at 60.95 and 61.12 suggesting that only one *ortho*-position

of the former two methoxy-containing carbons is substituted, while both of the *ortho*-positions of the latter two methoxy-containing carbons are substituted [18]. Thus, the former two methoxy groups should be attached to C-7 (δ 159.42) and C-3′ (δ 152.45), but not to C-6 and C-4′ while the two latter methoxy groups could be attached to C-6 (δ 132.23) and C-4′ (δ 137.30), but not at C-7 and C-3′.



Furthermore, acetylation of free hydroxyl groups produces marked changes in the ^{13}C-NMR of flavonoids and it can be used to detect the position of hydroxyl groups on the aromatic ring [19]. After acetylation of compound **2**, the signals of C-5, C-5′ and C-3 were shifted upfield from δ 151.37 to 142.06, from 149.26 to 143.97 and from 136.23 to 133.35, respectively, indicating that C-5, C-5′ and C-3 are the hydroxylated carbon atoms. The signals of C-6′, C-6, C-10, C-4′ and C-2 were moved downfield from δ 107.55 to 115.80, from 132.23 to 139.86, from 104.38 to 111.30, from 137.30 to 142.57 and from 144.96 to 153.77, respectively, which suggested that C-6′, C-6, C-10, C-4′ and C-2 are *ortho* to hydroxylated carbon atoms. The signals due to C-8, and C-2′ were moved downfield from δ 90.75 to 98.18, from 152.19 to 153.39 and from 104.51 to 110.06, respectively, indicating that C-8 and C-2′ are *para* to hydroxylated carbon atoms. The signals due to C-1′, C-7, C-9, and C-3′ showed slight changes from δ 126.31 to 124.66, from 159.42 to 158.11, from 152.19 to 153.39 and from 152.45 to 153.77, respectively, which clearly showed that C-1′, C-7, C-9, and C-3′ are *meta* to hydroxylated carbon atoms [19]. The HRESIMS of compound **2-Ac** showed a molecular ion peak ($[M+H]^+$) at *m/z* 517.1344, corresponding to the molecular formula of $C_{25}H_{12}O_8$.

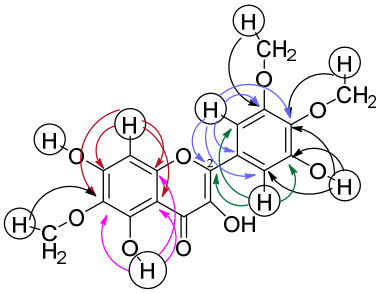
The above spectroscopic data in conjunction with the data reported in the literature allowed for the identification of compound **2** as 3,5,5'-trihydroxy-6,7,3',4'-tetramethoxyflavone. This is the first report of compound **2** from *P. schimperi* and this compound was previously reported from *P. oligotricha* [7]. The biological activity of the compound is not reported yet.



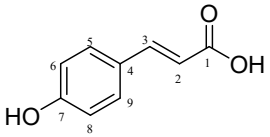
3

Compound **3** was obtained as a yellowish solid and had a melting point of 152-153 ºC. The UV spectrum (MeOH) showed absorption maxima at 254, 262, 301, 339 and 362 nm, which are characteristic of a flavonoid skeleton [16]. The IR spectrum of **3** showed hydroxyl stretching bands (3469, 3412 and 3332 cm^{-1}), C-H stretching bands (2994 and 2923 cm^{-1}) and a conjugated carbonyl stretching band (1656 cm^{-1}). The ^{13}C-NMR together with DEPT-135 spectrum revealed

eighteen carbon resonances corresponding to three methoxy groups, three methine carbons and twelve quaternary carbon atoms, of which three of them are methoxylated, four are hydroxylated and one is a conjugated carbonyl carbon. The ^{1}H-NMR spectrum (Table 2) of **3** showed the presence of three-proton singlets at δ 3.95, 3.98 and 3.89 which can be assigned to the methoxy groups at C-3′, C-4′ and C-6, respectively. The aromatic proton singlet at δ 6.69 (1H, *s*) is assignable to H-8 of the 5, 6, 7-trisubstituted ring-A. The *meta*-coupled doublets (J = 2 Hz) at δ 7.55 (1H) and 7.54 (1H) are attributable to H-2′ and H-6′, respectively, which suggests that ring-B is 3′,4′,5′-trisubstituted. The most deshielded proton singlet at δ 12.27 (1H) can be attributed to the strongly chelated OH group at C-5.



The HMBC spectrum of **3** showed correlations of H-8 (δ 6.69) to the carbon resonance at δ 30.87, and the correlation of this carbon atom to the methoxy proton resonated at δ_H 3.89 (δ_C 59.89) which can occur only if the methoxy is placed at C-6, but not at C-3 or C-7. The correlations of H-2′ (δ 7.54) to the carbon resonances at (δ 153.11 and 138.26), and the correlations of these two carbons to the two methoxy proton signals at δ_H 3.95 (δ_C 55.54) and δ_H 3.98 (δ_C 59.98) can occur only if the methoxy groups are placed at C-3′ and C-4′, but not at C-3. The C-5 OH resonance shows correlations to C-10 (δ 103.79), C-6 (δ 130.87), C-5 (δ 151.64) and C-9 (δ 152.26), and the C-5′ OH signal correlates to C-6′ (δ 108.99), C-4′ (δ 138.26) and C-5′ (δ 150.40). The OMe resonance at δ 55.54 suggests that this OMe should be attached to C-3′/C-5′, but not to C-4′. On the other hand, the OMe resonances at δ 59.89 and 59.98 suggest that these two OMe groups should be attached to C-6 and C-4′, but not at C-7 and C-3′ [18]. In addition, the HMBC correlations of H-8 (δ 6.69) to C-6 (δ 130.87), C-9 (δ 152.26) and C-7 (δ 157.21) and the correlations of H-2′ (δ 7.54) to C-6′ (δ 108.99), C-1′ (δ 126.45), C-4′ (δ 138.26), C-2 (δ 145.45) and C-7 (δ 157.21) were used to deduce the structure of **3**. The above spectroscopic data allowed for the identification of compound **3** as 3,5,7,5'-tetrahydroxy-6, 3', 4'-trimethoxyflavone. This is the first report of compound **3** from *P. schimperi* and this compound was previously reported from *P. oligotricha* [7]. The biological activity of the compound is not reported yet.

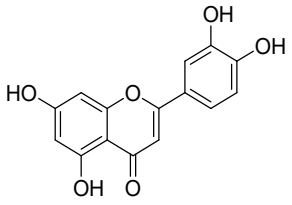


4

Compound **4** was obtained as light-yellow solid and had melting point of 210-212 ºC. The molecular formula of this compound was determined to be $C_9H_8O_3$ based on its positive ion HRESIMS, which showed a molecular ion peak ($[M+H]^+$) at *m/z* 165.0551. The UV spectrum (EtOH) showed absorption maxima at 211, 224, 292, 299 and 309 nm, which is characteristic of a phenolic moiety. The IR spectrum showed a band at 3327 cm^{-1} which can be attributed to the

hydroxy group. The band at 1672 cm^{-1} indicated the presence of carbonyl carbon and the band at 1601 cm^{-1} suggests the presence of C-C double bond.

The ^{1}H-NMR spectrum (Table 2) revealed four aromatic proton resonances, of which the doublet (J = 8.8 Hz) at δ 6.91 (2H) can be attributed to H-6 and H-8. The two-proton doublet at δ 7.56 (J = 8.8 Hz) is assignable to H-5 and H-9 while the doublets (J = 16 Hz) at δ 6.35 (1H) and 7.63 (1H) are assigned to H-2 and H-3, respectively. The ^{13}C-NMR and the DEPT-135 spectrum of **4** revealed nine carbon resonances corresponding to six methine and three quaternary carbon resonances. Two the three quaternary carbon resonances, are due to an oxygenated carbon and a conjugated carbonyl carbon. The spectroscopic data generated for the isolated compound and comparison of these with those reported in the literature allowed for the identification of compound **4** as *p*-hydroxy cinnamic acid. This is the first report of compound **4** from *P. schimperi*. Compound **4** is widely distributed in fruits, vegetables, cereals and mushrooms and it exhibits antioxidant, antitumor, anti-inflammatory, antimicrobial and antidiabetic activities [20].



5

Compound **5** was obtained as yellow solid. Its molecular formula was determined to be $C_{15}H_{10}O_6$ based on its positive ion HRESIMS, which showed a molecular ion peaks ($[M+H]^+$) at *m/z* 287.0558. The UV spectrum (EtOH) showed absorption maxima at 255, 268, 292 and 352 nm, which revealed the presence of a flavonoid moiety [16]. The IR spectrum showed the presence of hydroxyl (3426 cm^{-1}), carbonyl (1655 cm^{-1}) and C-C double bond (1615 cm^{-1}). The ^{13}C-NMR together with the DEPT-135 spectrum of **5** revealed fifteen carbon resonances corresponding to six methine and nine quaternary carbon resonances. The most downfield quaternary carbon signal at δ 182.16 was assigned to the carbonyl carbon at C-4. The ^{1}H-NMR spectrum (Table 2) revealed six aromatic proton resonances, of which two are *meta*-coupled doublets (J=2.0 Hz) at δ 6.26 (1H) and 6.54 (1H) which can be attributable to H-6 and H-8, respectively, of the 5,7-disubstituted ring-A. The singlet at δ 6.60 (1H) is assignable to H-3 on ring-C, and the remaining three aromatic protons which appeared at δ 7.00 (1H, J = 8.4 Hz), 7.52 (1H, J = 2.0 Hz) and 7.48 (1H, J = 8.4, 2.0 Hz) are assigned to H-5′, H-2′ and H-6′, respectively, of the 3′,4′-disubstituted ring-B. The most deshielded proton singlet at δ 12.87 (1H) is assignable to the strongly chelated OH group at C-5. Based on the analysis of the spectroscopic data and comparison with the literature, compound **5** was identified to be 3′,4′,5,7-tetrahydroxyflavone (*luteolin*) which was previously reported from the leaves of *P. schimperi*. *Luteolin* was also reported from many plant species such as *Achillea millefolium*, *Aiphanes acueleata* and *Ajuga reptans* and it showed antioxidant, anti-inflammatory, antimicrobial, anticancer and anti-allergic activities [21].

Compound **6** was obtained as white solid from the petroleum ether extract of the leaves of *P. schimperi* and had a melting point of 76.5-78 ºC. The IR spectrum showed a stretching band at 3405 cm^{-1} due to the presence of an OH group. The spectrum also revealed aliphatic C-H stretching bands at 2922 and 2853 cm^{-1}, C-O stretching band at 1644 cm^{-1}, C-H bending bands at 1240 and 1461 cm^{-1} and C-C stretching band at 719 cm^{-1}. The molecular formula of this compound was determined to be $C_{29}H_{60}O$ based on its positive ion HRESIMS, which showed a molecular ion peak ($[M+H]^+$) at *m/z* 425.4 The ^{1}H-NMR spectrum showed a triplet at δ 0.80 (3H, *t*, J = 6.8 Hz) due to protons of a terminal methyl group. The unresolved broad signal centered at δ 1.30

integrated for 50 protons and suggested the presence of 25 methylene groups. The multiplet at δ 1.60 integrated for four protons and corresponds to two methylene groups. The most deshielded protons signal appeared as a triplet at δ 3.68 (2H, J = 6.6 Hz) and suggested the presence of a hydroxymethylene group. It was evident from the ^{1}H-NMR spectrum that this compound is 1-nonacosanol. The molecular formula of $CH_3(CH_2)_{27}OH$ can be deduced for **6** from the ^{1}H-NMR spectrum.

The ^{13}C-NMR spectrum of **6** showed signals at δ 14.12, 22.70, 25.74, 29.37, 29.44, 29.62, 29.67, 29.71, 31.93, 32.82 and 63.10. The most downfield signal at δ 63.10 is due to the oxymethylene group while the most upfield carbon resonance at δ 14.1 is due to the terminal methyl group. Although the total number of carbon atoms could not be discerned from the ^{13}C-NMR spectrum due to signal overlapping, structure **6** was tentatively assigned to this compound based on the above spectroscopic data. This is the first report of compound **6** from *P. schimperi*. The biological activity of 1-nonacosanol was reported to exhibit antibacterial and antituberculosis activities [22].

Characterization of the essential oil of the dried leaves of P. schimperi.

The GC-MS analysis of the essential oil obtained from the dried leaves of *P. schimperi* revealed 47 compounds (Figure 2, Table 4). Characterization of compounds was done by matching the mass spectra with those of reference compounds recorded in NIST 2014 mass spectral library and confirmed by the retention indices obtained from a series of *n*-alkanes. The major compounds identified were α-curcumene (16.38%), caryophyllene (10.73%), eugenol (6.85%), β-sesquiphellandrene (4.65%), γ-gurjunene (4.55%), terpinen-4-ol (2.9%), γ-eudesmol (4.43%), linalool (2.2%) and caryophyllene oxide (2.67%).

Table 4. Compounds identified from the essential oil of the dried leaves of *P. schimperi.*

Pk	RT	Area %	Compound name	Q
1	6.7121	1.8257	D-limonene	99
2	7.0554	1.171	euclaptol	76
3	8.8797	2.2042	linalool	88
4	9.9151	2.8912	terpinen-4-ol	93
5	10.3527	0.4586	α-terpineol	93
6	11.5939	0.3212	1a,2,5,5-tetramethyl-trans-1a,4a,5,6,7,8-hexahydro-γ-chromene	96
7	11.7505	1.1214	α-copaene	99
8	11.8928	0.5251	7-epi-sesquithujene	98
9	11.9759	0.3533	(-)-β-bourbonene	99
10	12.0976	0.3223	*trans*-α-bergamotene	93
11	12.1684	0.2975	γ-elemene	97
12	12.4623	1.1165	β-ylangene	99
13	12.536	1.6386	*trans*-β-bergamotene	99
14	12.6319	10.7252	caryophyllene	99
15	12.7942	0.8584	2-methoxy-4-vinylphenol	91
16	12.8662	0.7358	germacrene D	89
17	12.9961	0.5243	alloaromadendrene	90
18	13.0643	0.3465	6,6-dimethylhepta-2,4-diene	76
19	13.1224	6.8519	eugenol	93
20	13.2075	3.0656	bicyclo[4.4.0]dec-1-ene,2-isopropyl-5-methyl-9-methylene-	86
21	13.3155	0.9663	sesquisabinene	68
22	13.4505	16.38	α-curcumene	93
24	13.5722	1.684	β-curcumene	97
25	13.6675	0.8699	isoaromadendrene epoxide	70

26	13.7793	4.5476	γ-gurgujene	89
27	13.8651	4.6537	β-sesquiphellandrene	95
28	13.9342	0.9455	2-methyl-6-(*p*-tolyl) hept-2-en-4-ol	78
29	14.8977	0.3015	β-bisabolene	87
30	15.0786	0.8902	bicyclo[10.1.0]tridec-1-ene	64
31	15.2699	1.2701	10α-eremophilane	49
32	15.3778	1.1401	*trans*-octahydro-4a,7,7-trimethyl-2(1H)-naphthalenone,	53
33	15.4265	0.4726	4,5-diethyl-3,5-octadiene,	35
34	15.5386	4.2929	(5E,7E)-dodeca-1,5,7-triene	50
35	15.5935	2.6743	caryophyllene oxide	87
36	15.7124	0.5065	*trans*-γ-bergamotene	76
37	15.823	0.7124	aromandendrene	98
38	15.8954	1.0336	(Z,Z)-α-farnesene	86
39	15.9939	1.1192	2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene,	96
40	16.15	1.7195	*trans*-α-bergamotene	84
41	16.2857	1.3812	7-(1-methylethylidene)-bicyclo[4.1.0]heptane,	74
42	16.342	4.4341	γ-eudesmol	96
43	16.4361	1.1611	γ-bisabolol	90
44	16.5653	4.022	1-methyl-6-methylenebicyclo[3.2.0] heptane	50
45	16.7884	1.0183	α-costol	84
46	17.743	1.0253	aristolene epoxide	81
47	18.1549	0.6325	amberial	84

Pk = peak number, RT = retention time (min), Q=quality, *I*=retention index.

Table 5. Classes of compounds identified from the essential oil.

No	Class of compounds	Compounds	Total % area
1	Sesquiterpenoids	bisabolol, (Z,Z)-α-farnesene, aromandendrene, β-bisabolene, β-sesquiphellandrene, γ-gurgujene, α-curcumene, 2-isopropyl-5-methyl-9-methylene-bicyclo[4.4.0]dec-1-ene, alloaromadendrene, germacrene D, caryophyllene, β-ylangene, γ-elemene, (-)-β-bourbonene, 7-epi-sesquithujene, α-copaene and β-curcumene;	40.65%
2	Oxygenated sesquiterpenoids	isoaromadendrene epoxide, caryophyllene oxide, γ-eudesmol, α-costol, 2-Methyl-6-(p-tolyl) hept-2-en-4-ol and isoaromadendrene epoxide	10.8%
3	Monoterpenoids	limonene and 7-(1-methylethylidene)-bicyclo[4.1.0]heptane	5.73%
4	Oxygenated monoterpenoids	eucalyptol, Terpinen-4-ol and α-terpineol	4.50%
5	Phenolic	eugenol	6.85%
6	Others		32.67%

Eventually it was concluded that the essential oil of the leaves of the plant is largely dominated by sesquiterpenoids.

A total of forty-seven compounds were identified from the essential oil of the dried leaves of the *P. schimperi* that accounts 96.7% of the overall constituents as given in Table 4 with percent yield of 0.25% (v/w) of the total amount of leaves that was hydrodistilled. This value is comparable to most of the investigated *Premna* species [23]. Germacrene D, alloaromadendrene,

β-bisabolene, β-ylangene, γ-eudesmol, *trans*-γ-bergamotene, caryophyllene, eugenol, linalool, limonene, terpinen-4-ol, α-terpineol, α-copaene, *trans*-β-bergamotene, aromandendrene, caryophyllene oxide and eremophilane were previously characterized and reported compounds from different species in the genus *Premna* [23-25]. The biological activities of the caryophyllene oxide and eugenol were previously reported to show antibacterial activities [24]. α-Copaene, β-bisabolene, caryophyllene, eugenol, germacrene D, caryophyllene oxide and limonene showed antioxidant activities [26]. Caryophyllene and caryophyllene oxide reported to exhibit anticancer and analgesic activities [27]. The compounds such as; α-terpineol, terpinen-4-ol, D-limonene, linalool, caryophyllene, α-curcumene, β-sesquiphellandrene and β-bisabolene showed antibacterial activities and nontoxic effects; in addition to that β-sesquiphellandrene showed anticancer activities [28].

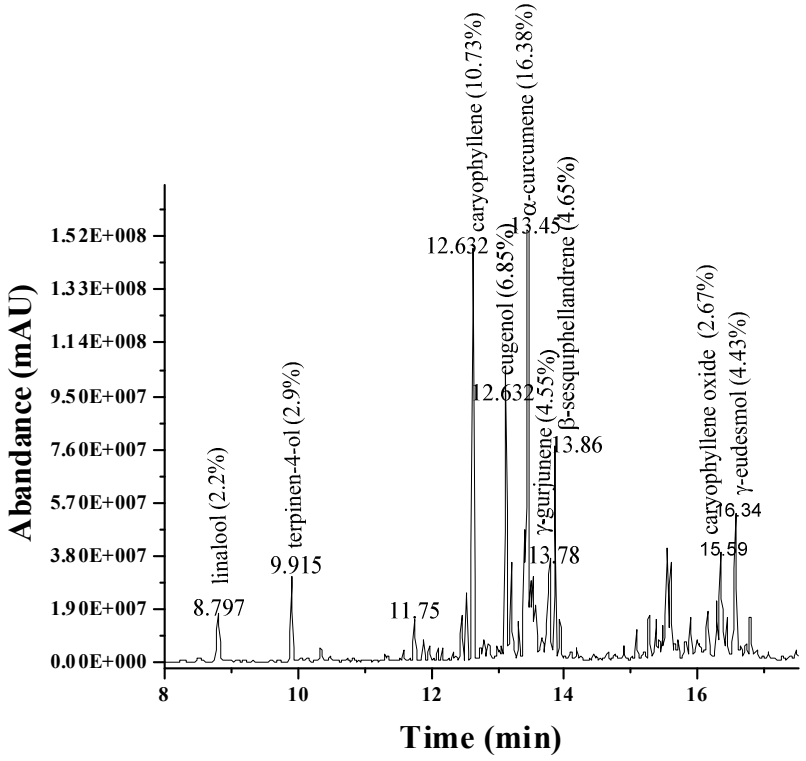


Figure 2. The GC-MS chromatogram of the essential oil from the leaves of *P. schimperi*.

CONCLUSION

The phytochemical investigation of the dried leaves of *P. schimperi* resulted in the isolation of ent-8β,12-epidioxy-12β-hydroxylabda-9(11),13-dien-15-oicacid-γ-lactone (**1**), 3,5,5'-trihydroxy-6,7,3',4'-tetramethoxyflavone (**2**), 3,5,7,5'-tetrahydroxy-6,3',4'-trimethoxyflavone (**3**), *p*-hydroxy cinnamic acid (**4**), luteolin (**5**) and 1-nonacosanol (**6**) using silica gel column chromatography and the isolated compounds were characterized based on spectroscopic techniques such as NMR, MS, FTIR and UV-Vis. All of the isolated compounds except luteolin (**5**) were the first report from

the plant. The antioxidant activities of the crude MeOH extract and compound **1** were 95.3% and 62.2% DPPH inhibition at 100 μg/mL concentrations, respectively. The GC-MS analysis of the essential oil of the leaves of *P. schimperi* showed the presence of major compounds, namely α-curcumene (16.38%), caryophyllene (10.73%), eugenol (6.85%), β-sesquiphellandrene (4.65%), γ-gurjunene (4.55%), terpinen-4-ol (2.9%), γ-eudesmol (4.43%), linalool (2.2%) and caryophyllene oxide (2.67%). The investigation showed that the plant is largely dominated by sesquiterpenoids. The presence of antioxidant compounds supports the traditional use of the plant extract as anti-inflammatory agent.

ACKNOWLEDGMENT

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