Indian Journal of Chemistry
Vol. 56B, August 2017, pp 880-889

A new flavonoid diglycoside from *Biophytum reinwardtii* (Zucc.) Klotzsch. and evaluation of its antioxidant and anticancer activities

Srinivasan Govindarajapuram Varadarajan[a], Vijayan Kochukarattu Krishnan*[a] & Ravi Subban[b]

[a] Organic Chemistry Research Laboratory, Department of Chemistry, University of Calicut, Thenhipalam 673 635, India
[b] Department of Chemistry, Karpagam University, Coimbatore 641 021, India
E-mail: prof.kkvijayan@gmail.com

Received 8 July 2016; accepted (revised) 18 April 2017

A new flavonoid diglycoside namely apigenin-4′,5-O-β-D-diglucoside **1** has been isolated from the whole plant of *Biophytum reinwardtii* (Zucc.) Klotzsch. and its structure has been elucidated by a combination of spectroscopic (^{1}H, ^{13}C and 2D NMR spectroscopy), mass spectral data and chemical methods. The compound **1** has been evaluated for its antioxidant activities *viz*., DPPH free radical scavenging activity and modified ferric reducing/antioxidant power (FRAP) assay and anticancer activity *viz*., MTT assay. The compound **1** has been found to possess significant antioxidant and anticancer activities.

Keywords: *Biophytum reinwardtii*, oxalidaceae, flavonoid diglycoside, antioxidant activity, MTT assay

Biophytum reinwardtii (Zucc.) Klotzsch. (family: Oxalidaceae) is an annual herb seen during the rainy season throughout the warm parts of India. It is also distributed in Indo-Malaysia and China[1,2]. The leaves and roots of *B. reinwardtii* are used to treat common fever. The plant is reported to possess tonic and stimulant properties, used for chest complaints, convulsions, cramps, inflammatory tumours and its ash for stomachache. The whole plant is dried, powdered and given internally to cattle to stop excess salivation. The leaves are diuretic and possess astringent and antiseptic properties. The pounded and bruised leaves or their juice is used in dressing burns and convulsions. Leaf paste is applied to wounds and cuts and leaf decoction given for asthma and phthisis. The mature leaves contain insulin like principle and are recommended for diabetes. Saline extract of the leaves showed hypoglycemic activity in rabbits[1]. The whole plant is used as a sedative, for removing dandruff and as hair tonic. In Bihar state of India, the leaves and roots are given for insomnia, fever, gonorrhoea and lithiasis[1,3].

Amentoflavone (I3′, II8-biapigenin) was isolated from the roots of *B. sensitivum*, a closely related species and proved to be a selective inhibitor of cyclooxygenase COX-1 catalyzed prostaglandin bio-synthesis when tested *in vitro* with an IC_{50} value of 12.4 μM[4]. Two biflavones, cupressuflavone and amentoflavone, three flavonoids, luteolin 7-methyl ether, isoorientin and 3′-methoxyluteolin 7-O-glucoside, as well as two acids, 4-caffeoylquinic acid and 5-caffeoylquinic acid were isolated from the aerial parts of *B. sensitivum*[5]. To the best of our knowledge, no flavonoid compound has been reported from *B. reinwardtii*. In the present paper, we describe the isolation and structural elucidation of a new flavonoid diglycoside namely apigenin-4′,5-O-β-D-diglucoside (compound **1**) from the whole plant of *B. reinwardtii*. The antioxidant activity of the compound **1** *viz*., DPPH free radical scavenging activity and modified ferric reducing/antioxidant power (FRAP) assay were investigated. The anticancer activity of compound **1** was also investigated by MTT assay.

Results and Discussion

A 80% aqueous methanol extract of *B. reinwardtii* was fractionated with petroleum ether, ethyl acetate and n-butanol in that order. The butanol extract was subjected to column chromatography over silica gel followed by preparative TLC to yield a pure flavonoid diglycoside, **1** (Figure 1). The compound **1** was checked for homogeneity by HPLC which afforded RT of 7.12 min.

The compound **1** was assigned the molecular formula $C_{27}H_{30}O_{15}$ by analysis of ESIMS (+ve and −ve modes). The compound **1** showed a quasi molecular ion peak at *m/z* 595.3274 $[M+H]^+$ and 593.2058 $[M-H]^-$ in the ESIMS. CHN analysis of **1** also agrees with the assigned molecular formula: (Found: C, 54.3%; H, 5.15%. $C_{27}H_{30}O_{15}$ requires: C, 54.55%; H,

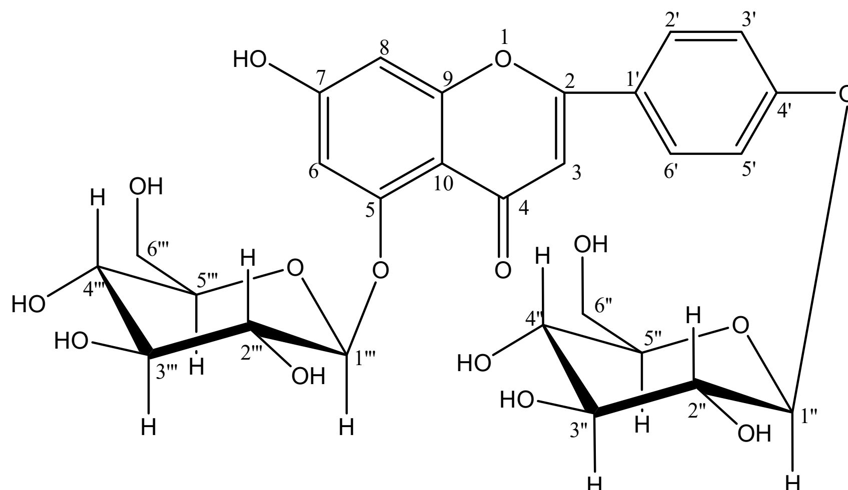


Figure 1 — Structure of apigenin-4′,5-O-β-D-diglucoside **(1)**

5.05%). UV-Visible spectrum of **1** exhibited maximum absorption at 271 and 332 nm with a characteristic flavonoid pattern indicates the presence of a flavonoid nucleus in the molecule. The IR spectrum presents characteristic absorption bands at 3405 cm^{-1} for OH group and 1654 cm^{-1} for α, β-unsaturated carbonyl group and absorption at 1618 cm^{-1} for C=C group in the molecule.

NMR spectral studies of compound 1

The ^{1}H NMR spectrum (Table I) taken in DMSO-d_6, exhibited a characteristic signal at δ 13.52 corresponding to phenolic group. The aromatic protons exhibited at δ 7.96 (2H, d, J = 9.2 Hz) for H-2′ and H-6′ protons and at δ 6.94 (2H, d, J = 8.6 Hz) for H-3′ and H-5′protons. The other AX coupling system at δ 6.73 (1H, d, J = 2.0 Hz) and δ 6.84 (1H, d, J = 2.0 Hz) was assigned to H-6 and H-8 protons respectively. These ^{1}H NMR δ values mostly corresponds with flavonoid proton shifts. The spectrum also supported the presence of two glucose moieties with the anomeric proton signals at δ 5.15 (J = 7 Hz) and at δ 5.03 (J = 7 Hz). The other proton signals of glucose moieties are also present at δ 3.18 (H-2″), 3.50 (H-3″), 3.38 (H-4″), 3.22 (H-5″), 3.60 and 3.80 (H-6″), 3.25 (H-2‴), 3.38 (H-3‴), 4.60 (H-4‴), 3.90 (H-5‴), 3.40 and 3.80 (H-6‴). This confirms the presence of two glucose moieties in the molecule. The ^{1}H NMR δ values mostly corresponds with flavonoid aglycon proton shifts. These data indicates that the molecule is a hydroxy flavonoid diglycoside.

The ^{13}C NMR (Table I) spectrum gave 27 carbon signals which indicated the presence of 15 carbon signals due to the flavonoid skeleton. The DEPT spectrum indicates the presence of two CH_2 carbons, eight quaternary carbons and 17 CH carbon atoms in

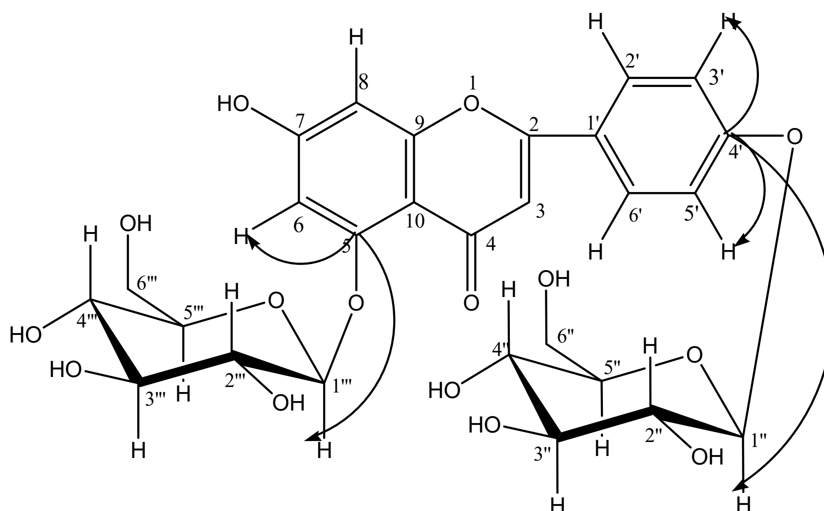
Table I — ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectral data of compound **1** in DMSO-d_6

C	δ_C	δ_H (mult., J in Hz)	HMBC
1			
2	162.5		H-3, 2′, 6′
3	103.3	6.70 (s)	
4	182.2		H-3
5	164.3		H-6, 1‴
6	104.2	6.73 (d, 2.0)	
7	156.5		H-6, 8
8	94.0	6.84 (d, 2.0)	
9	159.4		H-8
10	106.0		H-3, 6, 8
7-OH		13.52 (s)	
1′	121.9		H-3, 2′, 3′, 5′, 6′
2′	128.7	7.96 (d,9.2)	
3′	115.1	6.94 (d, 8.6)	
4′	161.5		H-2′, 3′, 5′, 6′,1″
5′	115.0	6.94 (d, 8.6)	
6′	128.7	7.96 (d, 9.2)	
1″	101.3	5.03 (d, 7.0)	
2″	81.0	3.18 (m)	
3″	77.3	3.50 (m)	
4″	73.9	3.38 (m)	
5″	70.9	3.22 (m)	
6″	60.9	3.60 (dd,10.2, 2); 3.80 (dd, 11.8, 4.2)	
1‴	101.3	5.15 (d, 7.0)	
2‴	79.0	3.25 (m)	
3‴	75.8	3.38 (m)	
4‴	72.7	4.60 (m)	
5‴	69.6	3.90 (m)	
6‴	60.4	3.40 (dd, 10.2, 2); 3.80 (dd, 11.8, 4.2)	

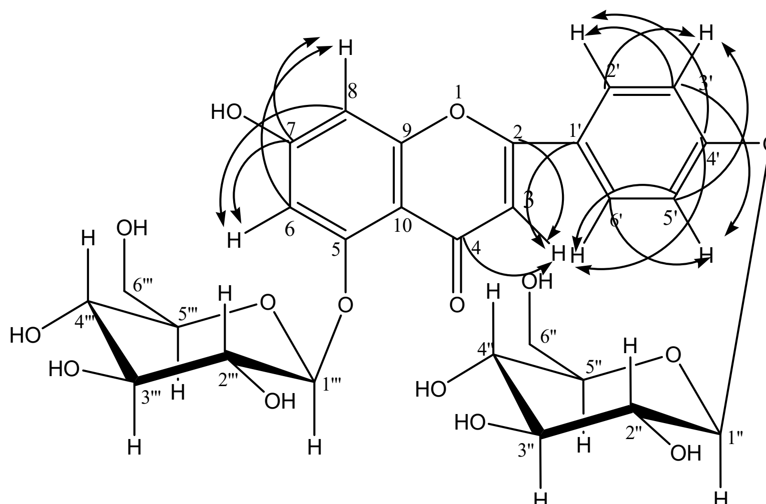
the compound. On comparison, ^{1}H and ^{13}C NMR spectral data of the aglycone moiety matches well with those reported for apigenin in the literature[6].

^{1}H-^{1}H COSY spectra showed cross peaks between H-2′ (δ 7.96) and H-3′ (δ 6.94); H-6 (δ 6.73) and H-8 (δ 6.84) indicating that they are adjacent to each other. HMBC spectra showed the long range correlations between signals at δ 161.45 (C-4′) and the protons at δ 5.03 (H-1″), δ 6.94 (H-5′and 3′) and δ 7.96 (H-2′ and 6′) confirming that the glycosylation was at C-4′. Further correlations were showed between the signal at δ 164.25 (C-5) and the anomeric proton at δ 5.15 (H-1‴) and the proton at δ 6.73 (H-6) (Scheme I and Scheme II). If the glycosylation is at C-7 OH, the correlation would have been obtained between C-7 (156.52) with C-8 (6.84, H-8) and C-6 (6.73, H-6). This observation confirms that the second glycosylation is at C-5. The other correlations in HMBC spectrum are given in Table I (Scheme I, Scheme II and Scheme III).

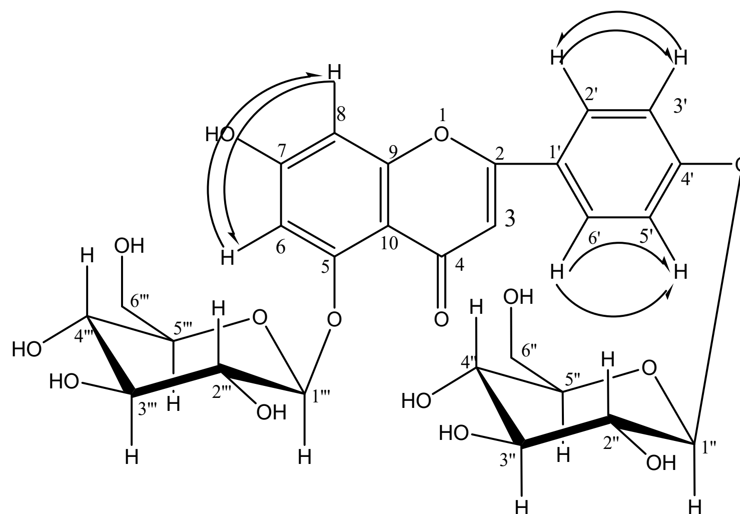
On acid hydrolysis, **1** yielded only D-glucose which was identified by comparison with an authentic sample by TLC and optical rotation $[\alpha]^{22}_{D}$: 51° (H_2O, c 0.5). Its absolute configuration was determined by GC-MS analysis of their TMSi derivative. D-glucose was identified by GC-MS of the pertrimethylsilylated-methylglucoside. Furthermore HMBC spectrum disclosed long range correlations between the anomeric proton H-1″ (δ 5.03, d, J = 7 Hz) and C-4′ (δ 161.45) and also between H-1‴ (δ 5.15, d, J = 7 Hz) and C-5 (δ 164.25) which confirmed the situation of β-glucopyranosyl moiety at C-4′ and C-5 of apigenin. With the interpretation of ^{1}H NMR, ^{13}C NMR and other 2D NMR spectra (Table I), all protons and carbons were assigned.



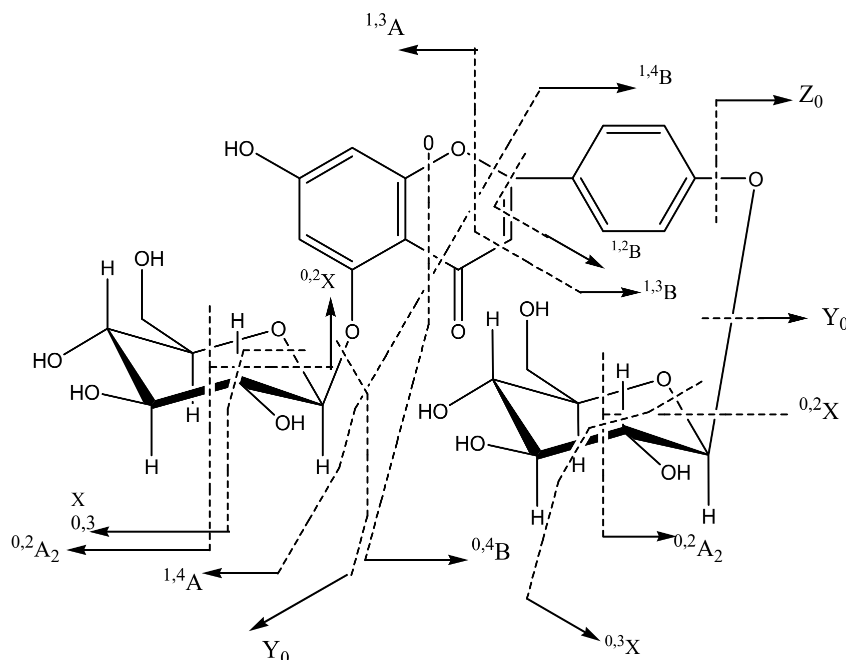
Scheme I — HMBC Correlation showing connectivity of aglycone C with anomeric H of glucose moieties



Scheme II — HMBC Correlation showing Connectivity of aglycone C with H



Scheme III — H-H Correlation between H of aglyocone



Scheme IV—MS degradation pattern of compound**1**

The above information suggests that **1** is 5-((2S,3S,4S,5S)-tetrahydro-3,4,5-trihydroxy-6-(hydroxyl-methyl)-2*H*-pyran-2-yloxy) -2-(4-((2S,3S,4S,5S)-tetrahydro-3,4,5-trihydroxy-6-(hydroxymethyl)-2*H*-pyran-2-yloxy)phenyl)-7-hydroxy-4*H*-chromen-4-one named apigenin-4′,5-O-β-D-diglucoside. This diglucoside is not previously reported from plant sources. The structure of apigenin-4′,5-O-β-D-diglucoside is shown in Figure 1.

Mass spectral studies of compound 1

The mass spectral characterization of **1** was carried out by +ve and –ve ion ESI-MSMS spectral analysis. The molecular ion was observed at *m/z* value of 595 $(M+H)^+$ in +ve mode and *m/z* value of 593 $(M-H)^-$ in –ve mode indicating that the molecular wt is 594. A peak at *m/z* 577 $[M+H-H_2O]^+$ indicates loss of water molecule from the molecular ion, a pattern usually exhibited by flavonoids. The pattern of degradation is represented in the Scheme IV. This is based on the ESI-MS (positive and negative ion) degradation pattern of flavoniods and flavonoid glycosides published earlier[7-9].

The prominent daughter ions formed in the MSMS spectra of **1** gave major ions with the *m/z* values 355, 312, 310, 297 and 282 apart from the molecular ion. The possible mechanism of formation of the fragment ions and the structures of the ions are depicted in the Scheme V.

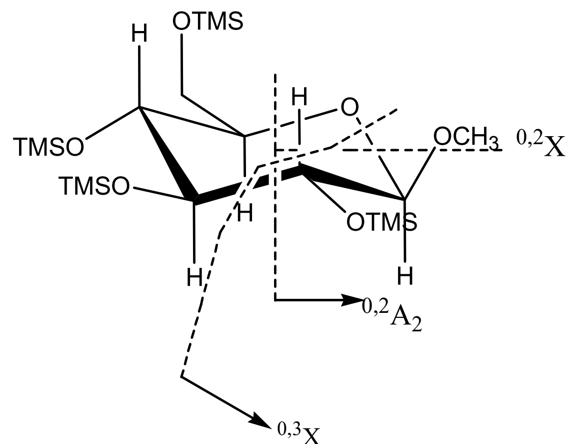
These are characteristic of flavonoid diglucoside and thus the compound **1** can be assigned the structure apigenin-4′,5-O-β-D-diglucoside.

Mass spectral studies of trimethylsilylated glucose

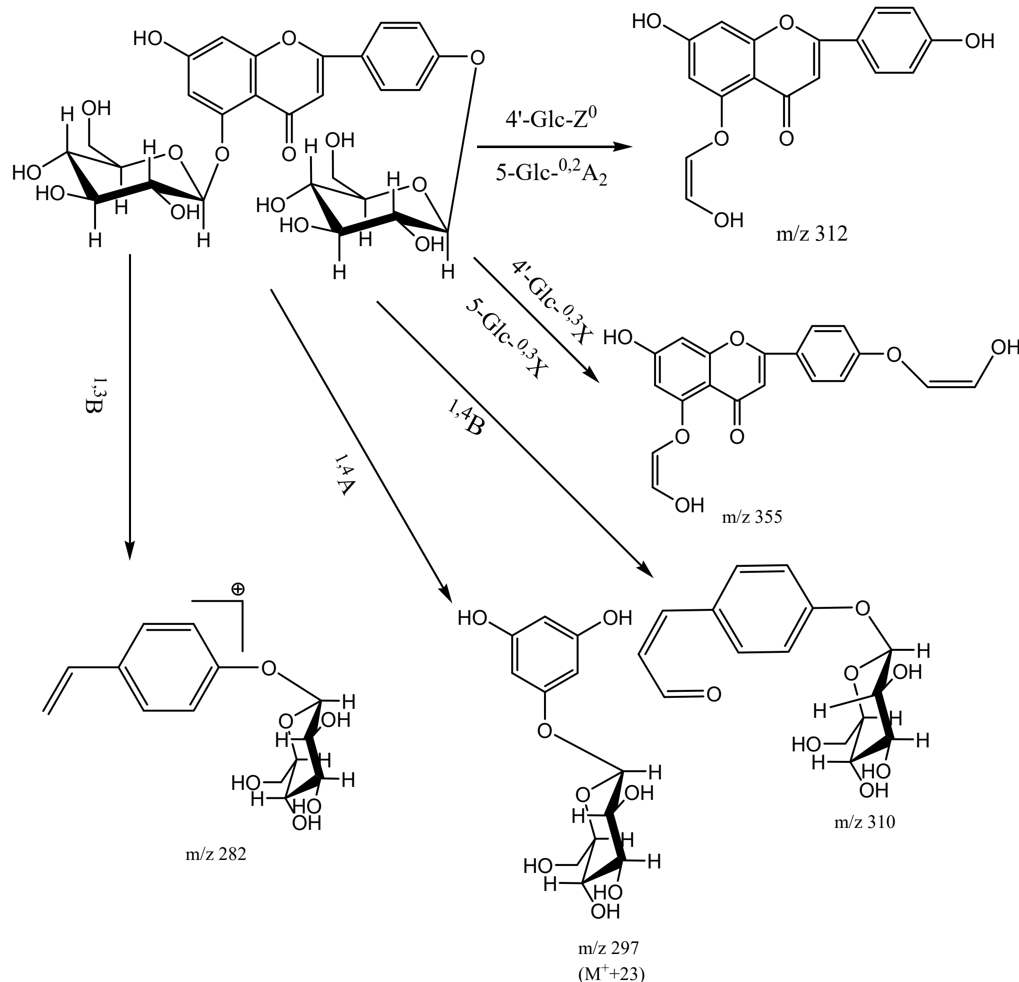
The compound **1** on hydrolysis with methanolic hydrochloric acid and subsequent trimethylsilylation and MS analysis gave a degradation pattern with ions of *m/z* values characteristic of methyl-2,3,4,6-tetra-O-trimethylsilyl-β-D-glucopyranoside (M^+ ion 435). Other peaks at *m/z* 129, 147, 191, 204, 305 and 345 can be observed in the spectrum establishing that carbohydrate moiety is β-D-glucopyranoside. This observation corresponds with that of the published data on the studies of trimethylsilylated monosaccharides published[10]. The pattern of degradation is represented in the Scheme VI.

Antioxidant studies

The antioxidant activity of compound **1** was evaluated by *in vitro* DPPH free radical scavenging activity and FRAP assay. The results obtained in the present study demonstrate that **1** showed good antioxidant properties in DPPH and FRAP assays. The compound **1** showed a concentration dependent antiradical activity by inhibiting DPPH radical with an EC_{50} value of 7.85 ± 0.32 μg/mL. The compound **1**



Scheme VI — MS degradation pattern of trimethylsilylated glucose



Scheme V — MS degradation pattern of compound **1** and formation of daughter ions

Table II — DPPH free radical scavenging activity of std BHA and Compound **1**

Conc. (μg/mL)	BHA % Inhibition	$^aEC_{50}$ (μg/mL)	Conc. (μg/mL)	Compound **1** % Inhibition	$^aEC_{50}$ (μg/mL)
5	15.65 ± 0.11		2	13.29 ± 0.18	
15	48.23 ± 0.18		4	25.06 ± 0.23	
25	71.65 ± 0.14	16.30± 0.20	6	38.51 ± 0.44	7.85± 0.32
30	78.35 ± 0.23		8	51.01 ± 0.32	
40	82.55 ± 0.35		10	62.49 ± 0.46	
50	88.69 ± 0.42		12	76.63 ± 0.58	
			14	85.36 ±0.61	

^{a} EC_{50} = Concentration of antioxidant required to reduce by 50% the initial conc. of DPPH. n = 4, Mean ± SD

Table III — FRAP assay of std antioxidants and compound **1**

	Gallic acid	Ascorbic acid	Compound **1**
$^aEC_1$ (μmol/L)	183 ± 5	396 ± 7	410 ±8

^{a} EC_1 = Concentration of antioxidant having a ferric reducing ability equivalent to that of 1 mmol/L $FeSO_4.7H_2O$.
n = 4, Mean ± SD

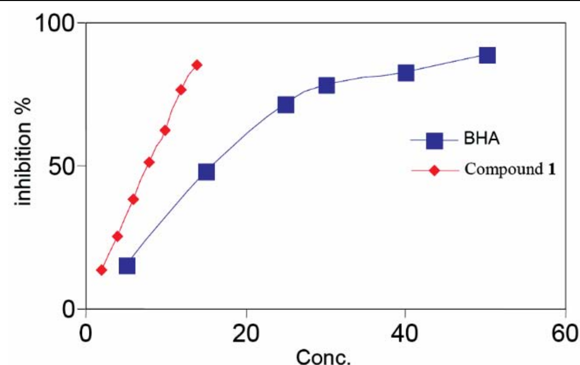


Figure 2 — Plot of DPPH free radical scavenging activity of BHA and compound **1**

showed a better activity than BHA (EC_{50}: 16.30 ± 0.20 μg/mL), which was used as the positive control (Table II, Figure 2). The lower the EC_{50}, the higher the antioxidant level.

Similarly in FRAP assay, compound **1** exhibited good ferric reducing ability comparable with ascorbic acid but lower than gallic acid (Table III, Figure 3). EC_1 values of these antioxidants are in the order compound **1**>ascorbic acid>gallic acid, the higher the EC_1 values, expressed as μM/L, the lower the antioxidant activity[11].

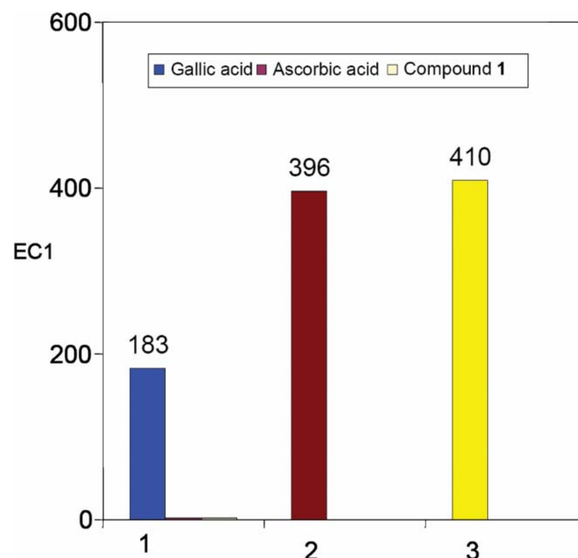


Figure 3 — Plot of FRAP assay of gallic acid, ascorbic acid and compound **1**

Anticancer studies

The anticancer activity of compound **1** was evaluated in two cancer cell lines *viz*., HeLa, human cervical cancer cell lines and HT 29, human colon cancer cell lines. Different concentrations of **1** such as 6.25, 12.5, 25, 50 and 100 μg/mL were tested for cytotoxicity by MTT assay. DMSO was used as the control. Based on our previous research findings that compound **1** possesses good antioxidant activity, the present anticancer study was carried out in **1**.

The present study suggests significant toxicity of **1** against the HeLa cancer cell lines and moderate toxicity against HT 29 cell lines. MTT, a pale yellow substrate that produced a dark blue formazan product when incubated with live cells. From MTT assay, after treatment with various concentrations of **1**, cell viability and percentage toxicity were compared with untreated cells (DMSO).

Cell counting and cell viability tests confirmed anticancer activity in both cell lines. The decrease in cell viability and increase in cytotoxicity by **1** was observed on HeLa and HT 29 cell lines in a dose dependent manner. The photographs of anticancer activity of **1** at various concentrations against HeLa and HT 29 cell lines are given in Figure 4 and Figure 5.

Concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically (Figure 6). Graphs were plotted using % of cell viability against

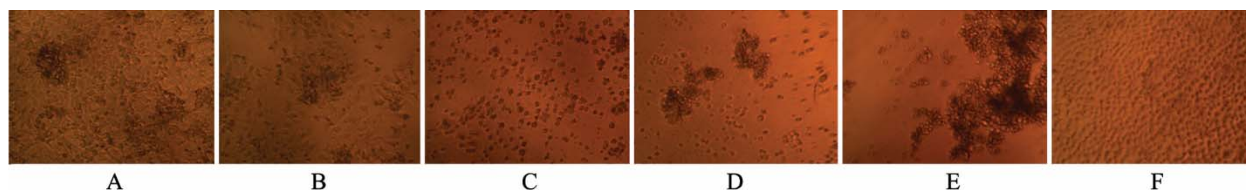


Figure 4 — Cytotoxicity effect of compound **1** on HeLa cell lines at different concs. A) 6.25 µg/mL, B) 12.5 µg/mL, C) 25 µg/mL, D) 50 µg/mL, E) 100 µg/mL and F) Control

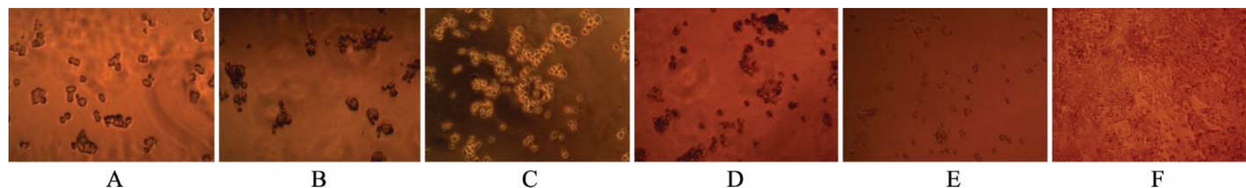


Figure 5 — Cytotoxicity effect of compound **1** on HT 29 cell lines at different concs. A) 6.25 µg/mL, B) 12.5 µg/mL, C) 25 µg/mL, D) 50 µg/mL, E) 100 µg/mL and F) Control

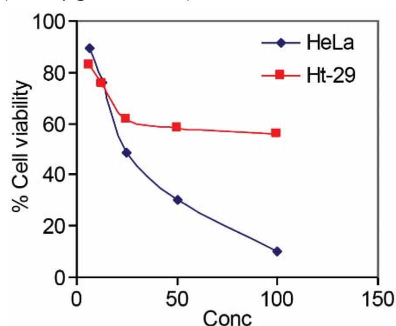


Figure 6 — MTT assay for cytotoxicity and cell viability Insert diagram here.

the conc. of the sample. The compound **1** exhibited cytotoxicity towards HeLa and HT 29 cell lines with IC_{50} values of 24.77 µg/mL and 106.76 µg/mL respectively (Table IV).

In the present study, **1** is effective against both cervical and colon cancer cell lines. This is in support with its good antioxidant properties. The compound **1** did not show cytotoxic effect towards normal cell lines. Further antiproliferative activity of total flavonoids and alkaloids isolated from different plants were reported earlier[12]. The association between flavonoids and reduced cancer risk has been reported which showed that a decrease in cancer risk with consumption of vegetables and fruits rich in flavonoids[13].

The aglycone, apigenin, a common dietary flavonoid abundantly present in fruits and vegetables is reported to have promising biological effects such as prevention and therapy of prostate cancer, suppression of tumorigenesis and angiogenesis in melanoma[14] and breast, skin and colon carcinomas[15]. It has been shown to exhibit antitumoral effects in leukaemia cells by induction of apoptosis through activation of caspases, inhibition of fatty acid synthase and topoisomerase and modulation of Bax and Bcl-2 expression[16] and reported to have antileukaemic effects[17].

Experimental Section

Plant material

The whole plant of *Biophytum reinwardtii* was collected from Kozhikode, Kerala, India during September 2009 and was authenticated by Dr. P. S. Udayan, Professor, Dept. of Botany, Sri Krishna College, Guruvayoor. A voucher specimen was deposited at the herbarium of the University of Calicut for future references. The collected material was then dried in the shade and coarsely powdered in the mixer grinder.

Chemicals and instrumentation

The solvents used were of analytical/ HPLC grade (Merck). DPPH (2,2′-diphenyl-1-picrylhydrazyl), silylating agents–hexamethyldisilazane and trimethyl-chlorosilane and D-glucose were purchased from Sigma Aldrich. BHA (3-t-butyl-4-hydroxyanisole), ascorbic acid, gallic acid and TPTZ (2,4,6-tripyridyl-s-triazine) were purchased from Merck. All other reagents were of analytical grade. Silica gel (Merck, 60-120 mesh) was used for column chromatography. Precoated silica gel 60 F_{254} plates (Merck) of uniform thickness 0.2 mm were used for TLC analysis.

The melting point was determined using a GUNF melting point apparatus and are uncorrected. Optical rotation was measured on a Perkin-Elmer 241 Polarimeter. HPLC system (Shimadzu, LC-20AD) was equipped with diode array detector (SPD-M 20A) and SIL-20A HT autosampler. UV and IR spectra were measured on a Shimadzu UV-1700 and on a Shimadzu

Table IV — MTT assay for cytotoxicity and cell viability

Sample Conc. (µg/mL)	HeLa Cell lines			HT 29 Cell lines		
	A_{540}	% cell viability	$^{a}IC_{50}$ (µg/mL)	A_{540}	% cell viability	$^{a}IC_{50}$ (µg/mL)
Control	0.9995			0.7012		
6.25	0.8907	89.11456		0.5806	82.80091	
12.5	0.7615	76.18809		0.5272	75.1854	
25	0.4864	48.66433	24.77	0.4321	61.62293	106.76
50	0.2988	29.89495		0.4081	58.20023	
100	0.102	10.2051		0.3922	55.93269	

$^{a}IC_{50}$ = Concentration of the sample required for 50% inhibition of cell viability

FTIR-8400S spectrophotometers respectively. ^{1}H and ^{13}C NMR spectra were obtained on a Supercon, Bruker NMR spectrometer operating at 300 MHz for δ_H and 75 MHz for δ_C in DMSO-d_6 using TMS as internal standard. 2D NMR experiments were carried out on a Bruker NMR spectrometer operating at 500 MHz in DMSO-d_6. ESIMS spectrum was recorded in Waters TQD LC MSMS system using the following parameters: Capillary voltage: 3 kV, cone: 30 V, extractor: 3 V, source temp: 150°C, dissolvation temp: 350°C, dissolvation gas (N_2) flow: 650 L h^{-1}, collision gas (Ar) flow: 0.1 mL min^{-1}. The elemental analysis was performed in Thermo Finnigan FLASH EA 1112 series CHNS analyzer. GC-MS analysis was carried out in Perkin Elmer Clarus 680 GC and Clarus 600 T MS system. EIMS spectrum was recorded at 70 eV and NIST library was used for the library search.

Cell lines and cultures used

The two cancer cell lines *viz*., HeLa, human cervical cancer cell lines and HT 29, human colon cancer cell lines were purchased from National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagles Media (Hi Media) supplemented with 10% Foetal Bovine Serum (FBS-Invitrogen) and grown to confluency at 37°C in 5% CO_2 in a humidified atmosphere in a CO_2 incubator (NBS, EPPENDORF, GERMANY). The cells were trypsinized [500 µL of 0.025% Trypsin in PBS/ 0.5 mM EDTA solution (Hi Media] for 2 min and passaged to T flasks in complete aseptic conditions. Extracts were added to grown cells at a final concentration of 6.25, 12.5, 25, 50 and 100 µg/mL from a stock of 20 mg/mL and incubated for 24 h. The untreated cell lines (DMSO) were used as the control. The % difference in viability was determined by standard MTT assay after 24 h of incubation. MTT and DMSO were purchased from S.D. Fine Chemicals, Mumbai.

Extraction and isolation

1.2 Kg of the dried and coarsely powdered plant material was extracted with 80% aqueous methanol (10 L) in a Soxhlet extractor for 12 h. It was filtered and concentrated under suction in a rotary flash evaporator to obtain about 400 g of the crude green coloured extract. It was then fractionated with petroleum ether (b.p. 60-80°C) followed by ethyl acetate. The red coloured aqueous methanol extract thus obtained was again fractionated with n-butanol (2 L). The butanol was removed from the extract under suction in a rotary flash evaporator to obtain 150 g of the solid mass. It was subjected to column chromatography on a column (100 cm x 4.5 cm) filled with silica gel (60-120 mesh). Before filling up into the column, silica gel was activated at 110 °C for 1 h in a hot air oven. The column was eluted with acetone and different combinations of acetone and methanol. Every 20 mL fractions were collected in different test tubes and monitored by TLC (7.5 cm x 2.5 cm). The fractions eluted with 10:90 acetone-MeOH mixture, yielded a single spot in TLC (acetone-$CHCl_3$-H_2O-MeOH-2-propanol, 5:0.6:2.5:2:1, R_f 0.34). The fractions 33-61 (580 mL) were combined, concentrated in rotary flash evaporator and subjected to preparative TLC on precoated silica gel 60 F_{254} plates using the above mentioned solvent system to afford yellow coloured compound. It was then recrystallized from MeOH-water 1:1 (v/v) to obtain pure yellow amorphous compound **1** (110 mg).

High performance liquid chromatography

The compound **1** was dissolved in 1:1 DMSO-MeOH solvent system and 20 µL was injected into HPLC system. Reversed Phase Enable C18 column (100 × 4.6 mm, 5 µm) was kept at 40°C in a column oven. The compound was eluted with the isocratic mobile phase 45:55 MeOH-H_2O (v/v). The flow rate was set at

1 mL/min. The diode array detector was set at a wavelength of 332 nm. The compound **1** was eluted at RT of 7.12 min.

Compound 1-apigenin-4′,5-O-β-D-diglucoside

Yellow amorphous powder (MeOH); melting at 232°C with charring. $[\alpha]^{22}_{D}$: 82° (DMSO, c 0.01); UV-Vis λ_{max} (DMSO): 271, 332 nm; IR (KBr) ν_{max}: 3405, 2925, 2854, 1654, 1618, 1457, 1189, 1093, 923 cm^{-1}; ^{1}H NMR (300 MHz, DMSO-d_6): 6.70 (1H, s, H-3), 6.73 (1H, d, *J* = 2.0 Hz, H-6), 6.84 (1H, d, *J* = 2.0 Hz, H-8), 13.52 (1H, s, 7-OH), 6.94 (2H, d, *J* = 8.6 Hz, H-3′,5′), 7.96 (2H, d, *J* = 9.2 Hz, H-2′,6′), 5.03 (1H, d, *J* = 7.0 Hz, H-1″), 3.18 (1H, m, H-2″), 3.50 (1H, m, H-3″), 3.38 (1H, m, H-4″), 3.22 (1H, m, H-5″), 3.6 (1H, dd, *J* = 10.2, 2.0 Hz, H-6″), 3.80 (1H, dd, *J* = 11.8, 4.2 Hz, H-6″), 5.15 (1H, d, *J* = 7.0 Hz, H-1‴), 3.25 (1H, m, H-2‴), 3.38 (1H, m, H-3‴), 4.60 (1H, m, H-4‴), 3.90 (1H, m, H-5‴), 3.40 (1H, dd, *J* = 10.2, 2.0 Hz, H-6‴), 3.80 (1H, dd, *J* = 11.8, 4.2 Hz, H-6‴); ^{13}C NMR (75 MHz, DMSO-d_6): 60.4 (CH_2), 60.9 (CH_2), 69.6 (CH), 70.9 (CH), 72.7 (CH), 73.9 (CH), 75.8 (CH), 77.3 (CH), 79.0 (CH), 81.0 (CH), 101.3 (CH), 94.0 (CH), 103.3 (CH), 104.2 (CH), 106.0 (C), 115.0 (CH), 115.1 (CH), 121.9 (C), 128.7 (CH), 156.5 (C), 159.4 (C), 161.5 (C), 162.5 (C), 164.3 (C), 182.2 (C); ESI-MS (−ve mode): *m/z* 593 $[M-H]^-$; ESI-MS (+ ve mode): *m/z* 595 $[M+H]^+$. CHNS analysis: $C_{27}H_{30}O_{15}$ requires: C, 54.55; H, 5.05. Found: C, 54.3; H, 5.15%.

The compound **1** was subjected to the acid hydrolysis by refluxing 25 mg of it with 5 mL of 2N HCl for 3 h. It was then subjected to TLC on pre-coated silica gel 60 F_{254} plates in CH_2Cl_2-CH_3OH-CH_3COOH-H_2O (5:5:2.5:1) solvent system. After spraying the developed plate with 10% ethanolic H_2SO_4 and heating at 110°C in the hot air oven for 5 min, only a dark spot corresponding to glucose at R_f 0.87 was obtained.

Configuration of glucose

Glucose was extracted from the hydrolysate by silica gel TLC in the above described solvent system. It was dissolved in pyridine and analyzed as their TMSi methylglucoside obtained after methanolysis (0.5 N HCl in MeOH for 24 h, 70°C) and trimethyl-silylation[18]. The configuration of the glucose was established by capillary GC and GC-MS[19].

Antioxidant study – DPPH free radical scavenging activity

Stock solutions containing 25 µg/mL of compound **1** and 50 µg/mL of BHA was prepared in methanol. A stock solution of DPPH (1.3 mg/mL methanol) was prepared such that 75 µL of it in 3 mL methanol gave an initial absorbance of 0.9. Antioxidant activity was measured by a decrease in the absorbance at 516 nm of methanolic solution of coloured DPPH brought about by **1** [20,21]. Decrease in the absorbance of **1** at different concentrations was noted after 15 min. EC_{50} was calculated from % inhibition. BHA was used as the positive control.

Antioxidant study – FRAP assay

The antioxidant capacity of **1** was estimated using standard protocol[22,23] with some modifications. Briefly 900 µL of FRAP reagent, prepared freshly and warmed at 37°C was mixed with 90 µL of distilled water and 30 µL of **1**. The final dilution of the test sample in the reaction mixture was 1/34. The FRAP reagent contained 2.5 mL of 10 mM/L of TPTZ solution in 40 mM/L HCl, 2.5 mL of 20 mM/L $FeCl_3.6H_2O$ and 25 mL of 300 mM/L acetate buffer having *p*H 3.6. The absorbance was measured at 4 min at 595 nm using a UV-Vis spectrophotometer. The aqueous solution of known Fe (II) concentrations in the range of 100-2000 µM/L ($FeSO_4.7H_2O$) was used for calibration. The parameter equivalent concentration 1 or EC_1 was defined as the concentration of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mM/L $FeSO_4.7H_2O$. EC_1 was calculated as the concentration of the antioxidant giving an absorbance increase in the FRAP assay equivalent to theoretical absorbance value of a 1 mM/L concentration of Fe (II) solution. Water is used as reagent blank and gallic and ascorbic acids were used as positive controls.

Statistical analysis

Statistical results are expressed as the mean ± SD of 4 independent experiments.

Anticancer studies

The anticancer activity of **1** was determined by MTT assay[24,25]. MTT is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble dark purple coloured formazan product. The cells are then solubilised with an organic solvent DMSO and the released, solubilised formazan product was measured at 540 nm. Since the reduction of MTT can occur only in metabolically active cells the level of activity is a measure of the viability of the cells.

MTT was dissolved in phosphate buffered saline (PBS) at 5 mg/mL. The cells (1x105/well) were plated

in 1 mL of medium/well in 24- well plates. After 48 h incubation, the cell reached the confluence. Then the cells were incubated in the presence of various concentrations of **1** in DMSO for 24 h at 37 °C. After removal of the sample solution and washing with PBS 200 µL/well (*p*H 7.4), stock MTT solution (10 µL per 100 µL medium) was added to all wells of an assay and plates were incubated at 37 °C for 4 h. After 4 h, MTT was removed by washing with PBS and 200 µL of DMSO was added to the culture. Incubation was done at room temperature for 30 min until the cell got lysed and the colour was obtained. The solution was transferred to centrifuge tubes and centrifuged at top speed for 2 min to precipitate cell debris. Optical density was read at 540 nm using DMSO as blank in a micro plate reader (ELISASCAN, ERBA).

The concentrations of **1** showing complete cytotoxic effect were recorded. Viable cells were determined by the absorbance at 540 nm. Measurements were performed and concentration required for 50% inhibition of viability (IC_{50}) was determined graphically. The minimum concentration non toxic in Vero but completely inhibiting the cancer cells (HeLa or HT 29) is considered as the effective drug concentration. The effect of the samples on the proliferation of the cell lines HeLa and HT 29 were expressed as the % cell viability using the following formula:

$$\%\text{ Cell Viability} = \frac{\text{Absorbance at 540 } (A_{540}) \text{ of treated cells} \times 100}{A_{540} \text{ of control cells}}$$

Acknowledgments

The authors are grateful to the Department of Science and Technology (DST), Govt. of India and SAIF- IIT Bombay and IIT Madras for recording the spectra.

References

1 Anonymous, *The Wealth of India* (*Raw Materials*), Vol. 2B (Publication and Information Directorate, Council of Scientific and Industrial Research, New Delhi, India) 151 (1988).
2 Hajra P K, Nair V J & Daniel P, *Flora of India* (Botanical Survey of India, Calcutta), 4 (1998) 230.
3 Yoganarasimhan S N, *Medicinal Plants of India*, Vol. I (Interline Publishing Pvt. Ltd., Bangalore, India) 68 (1996).
4 Bucar F, Jachak S M, Noreem Y, Kartnig T, Perera P, Bohlin L & Zsilavecz M S, *Planta Med*, 64 (1998) 373.
5 Lin Y L & Wang W Y, *Chin J Pharm Sci*, 55 (2003) 71.
6 Ying T, Xi-Qiao L & Jun-Xing D, *Acta Pharm Sin*, 44 (2009) 496.
7 Cuyckens F & Claeys M, *J Mass Spectrom*, 39 (2004) 1.
8 Silva D B, Twatte I C C & Gouveia D R, *Sci Rep*, 4 (2004) 4309.
9 Paul G & Lopes N P, *Int J Anal Chem*, 2012 (2012) 259217.
10 Dejongh D C, Radford T, Hribar J D, Hanessia S & Bieber M, *J Am Chem Soc*, 91 (1969) 1728.
11 Pulido R, Bravo L & Saura-Calixto F, *J Agric Food Chem*, 48 (2000) 3396.
12 Vijayan P, Vijayaraj P, Setty P, Hariharpura C, Godavarthi A, Badami S, Arumugam D & Bhojraj S, *Biol Pharm Bull*, 24 (2004) 528.
13 Park H J, Kim M J, Ha E & Chung J H, *Phytomedicine*, 15 (2008) 147.
14 Caltagirone S, Rossi C, Poggi A, Ranelletti F, Natali P, Brunetti M, Aiello F B & Piantelli M, *Int J Cancer*, 87 (2000) 595.
15 Wang W, Heideman L, Chung C, Pelling J, Koehler K & Birt D, *Mol Carcinog*, 28 (2000) 102.
16 Wang I, Lin-Shiau S & Lin J K, *Eur J Cancer*, 35 (1999) 1517.
17 De-Sousa R R R, Fuhler G M, Blom N, Ferreira C V, Aoyama H & Peppelenbosch M P, *Cell Death Dis*, 1 (2010) 1.
18 Kamerling J P, Gerwig G J, Vliegenthart J F G & Clamp J R, *Biochem J*, 151 (1975) 491.
19 Gerwig G J, Kamerling J P & Vliegenthart J F G, *Carbohydr Res*, 62 (1978) 349.
20 Vani T, Rajani M, Shankar S & Shishoo C J, *Int J Pharmacogn*, 35 (1997) 313.
21 Ravishankara M N, Shrivastava N, Padh H & Rajani M, *Phytomedicine*, 9 (2002) 153.
22 Benzie I F F & Strain J J, *Anal Biochem*, 239 (1996) 70.
23 Benzie I F F & Strain J J, *Methods Enzymol*, 299 (1999) 15.
24 Mosmann T, *J Immunol Methods*, 65 (1983) 55.
25Arung E T, Shimizu K & Kondo R, *Biol Pharm Bull*, 29 (2006) 1966.