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Isolation, characterization and antioxidant activities of steroidal constituents from the aerial parts of *Didymocarpus pedicellata* R. Br.

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Abstract
Didymocarpus pedicellata R.Br. (Gesneriaceae) is used to treat renal diseases particularly kidney stones, cuts and wounds. As a part of our previous studies in which we have revealed the protective effect of *D. pedicellata* on ferric nitrilotriacetate (Fe-NTA) induced renal oxidative stress and hyper proliferative response, the present study was designed to isolate, characterize and evaluate antioxidant potential of constituents of *D. pedicellata*. Phytochemical investigations of the chloroform extract of aerial parts of *D. pedicellata* resulted in the isolation of three steroidal constituents characterized as 21-hydroxy- β-sitosteryl *n*-octadec-9′,12′,15’-trienoate (1), 21-hydroxy- β-stigmasteryl *n*-octadec-9′,12′,15’-trienoate (2) and stigmasterol 3-O-β-D-glucopyranoside (3) for the first time from this plant. The first two compounds 1 and 2 are the new compounds isolated for the first time from a plant source. The structures of these phytoconstituents were established by analysis of the spectral data. The isolated compounds were evaluated for their *in vitro* antioxidant activities. Among them, compounds 1 and 3 showed significant antioxidant effects.

Keywords: *Didymocarpus pedicellata*, Gesneriaceae, Aerial parts, Steroids, Antioxidant activity.

1. Introduction
Didymocarpus pedicellata R.Br. (Gesneriaceae), known as patharphori, *shilapushpa* and pashanbhed, is a small herb with a reduced stem, bearing 2-3 pairs of opposite, roundly ovate, glabrous, highly folded leaves (Shah *et al.,* 1972) [21]. The herb mostly grows in the subtropical Himalayas at altitudes of 2,500 to 5,500 meters. It is used to treat renal diseases particularly kidney stones. It regulates calcium absorption in the body with its diuretic effect and maintains the healthy urinary tract. A leaf paste is applied as an antiseptic to cure cuts and wounds. Its leaves contain an essential oil whose chief constituent, didymocarpene, is used in indigenous healthcare systems for its well-rounded urinary tract support (Bahl and Seshadri, 1978; Nadkarni, 1992) [4, 14]. The herb is a major constituent of cystone, a drug used to cure renal ailments such as urolithiasis (Rai, 1960) [16], neuro-ureterolithiasis (Misgar, 1982) [12], burning micturition (Garg and Singh, 1985) [7]. and several other renal disorders (Sharma *et al.*, 1983) [22]. In our previous studies, we have documented the protective activity of the crude ethanolic extract against ferric nitrilotriacetate-induced renal oxidative stress and hyperproliferative response (Kaur G *et al.*, 2006) [11]. It also possessed significant antioxidant activity and protected against nephrotoxicity and tumor promotion response (Kaur G *et al.*, 2006) [11]. Chalcones, flavones, flavanones, pedicellic acid and β-sitosterol have been reported from the plant (Agarwal, 1972; Bose 1978a, 1978b; Rao, 1966; Guha and Bhattacharya, 1992; Rathore *et al*. 1981a, 1981b) [1, 5, 6, 17, 8, 17-19]. This manuscript reports the isolation, characterization and antioxidant potential of steroids from the aerial parts of *D. pedicellata*.

2. Experimental
2.1 General Experimental Procedure
The melting points were measured by means of a thermoelectrically operated Perfit apparatus and are uncorrected. UV spectra were recorded on Beckman DU-6 spectrophotometer in either methanol or chloroform. FT-IR spectra were recorded on Jasco FT/ IR-55000 spectrophotometer using KBr pellet; ν_{max} values are given in cm^{-1}. NMR spectra were recorded on Bruker Spectrospin 400-MHz instrument with DMSO-d_6 or $CDCl_3$ as solvent and tetramethylsilane as the internal standard. The ^1H NMR, ^{13}C NMR, DEPT, COSY, HMBC and

HMQC were measured at 27 ^{0}C. Mass spectra were scanned on a JEOL-SX 102/DA-6000 Mass spectrometer/ Data system using Argon/ Xenon (6KV, 10mA) as the FAB gas. The purity of the isolated compounds was checked on precoated TLC plates with Silica gel $60F_{254}$ (Merck, 0.25 mm), which were visualized either under UV-light or by exposure to iodine vapors.

2.2 Plant material

The aerial parts of *D. pedicellata* were purchased from Khari Baoli, New Delhi, India and authenticated by Dr H. B. Singh, Taxonomist at NISCAIR, New Delhi. A voucher specimen (*Ref. NISCAIR/RHMD/Consult/-2007-08/865/49*) has been deposited in the herbarium of NISCAIR, New Delhi.

2.3 Extraction and fractionation

The powdered shade-dried aerial parts of *D. pedicellata* (2.0 Kg) were Soxhlet-extracted exhaustively with 95% ethanol. The extract obtained was concentrated to dryness under reduced pressure at 40 ^{0}C in a rotary evaporator to yield light brownish mass (628.0 g, 31.4 %). A portion of this extract (550.0 g) was suspended in water and fractionated successively with petroleum ether, chloroform and *n*- butanol. These fractions were concentrated to dryness under reduced pressure (at 40 ^{0}C) to yield extracts of petroleum ether (45 g, 2.5 %), chloroform (131 g, 7.4 %) and *n*-butanol (272 g, 15.5 %), respectively. All these extracts were stored at 4 ^{0}C until use.

2.4 Isolation

The chloroform extract residue (131.0 g) of aerial parts of *D. pedicellata* was dissolved in little quantity of methanol and adsorbed on silica gel (60-120 mesh) for the preparation of slurry. It was dried in air and chromatographed over silica gel column packed in petroleum ether. The column was successively eluted with petroleum ether, petroleum ether-chloroform mixtures; chloroform and chloroform-ethyl acetate mixtures; ethyl acetate and ethyl acetate-methanol mixtures; and methanol in the order of increasing polarity. Different fractions were collected separately and matched by TLC to check homogeneity. Fractions with similar R_f values were pooled together and crystallized. The isolated compounds were recrystallized to get the following pure compounds:

2.4.1 21-Hydroxy-β-sitosteryl linolenate (1)

Elution of the column with petroleum ether- chloroform (1:4) furnished pale yellow crystals of 1, recrystallized from chloroform-methanol (1:1), 61.4 mg (0.0041 % yield), R_f 0.55, (acetone –toluene, 1:1), m.p: 265 - 267°C; UV λ_{max} (MeOH): 224 nm (log ε 3.8); IR λ_{max} (KBr): 3360, 2920, 2855, 1735, 1640, 1470, 1360, 1210, 1035, 725 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 5.36 (1H, dd, J = 7.2, 3.6 Hz, H-6), 5.30 (1H, m, H-9'), 5.28 (1H, m, H-10′), 5.26 (1H, m, H-12′), 5.23 (1H, m, H-13′), 5.21 (1H, m, H-15′), 5.19 (1H, m, H-16′), 4.09 (1H, brm, $w_{1/2}$ 18.2 Hz, H-3α), 3.03 (2H, d, J=6.4 Hz, H_2-21), 2.75 (2H, m, H_2-11′), 2.70 (2H, m, H_2-14′), 2.29 (2H, t, J= 7.6 Hz, H_2-2′), 1.01 (3H, brs, Me-19), 0.86 (3H, d, J= 6.7 Hz Me-26), 0.83 (3H, d, J= 6.6 Hz, Me-27), 0.81 (3H, d, J= 6.3 Hz Me-18′), 0.79 (3H, t, J= 6.8 Hz, Me-29), 0.67 (3H, brs, Me-18), 1.98-1.07 (43H, m, 18 x CH_2, 7 x CH); ^{13}C NMR ($CDCl_3$): δ 37.16 (C-1), 31.54 (C-2), 71.86 (C-3), 42.32 (C-4), 140.71 (C-5), 121.66 (C-6), 31.96 (C-7), 31.92 (C-8), 50.13 (C-9), 37.25 (C-10), 21.09 (C-11), 39.08 (C-12), 42.21 (C-13), 56.77 (C-14), 24.70 (C-15), 28.25 (C-16), 56.06 (C-17), 11.86 (C-18), 19.04 (C-19), 36.16 (C-20), 62.12 (C-21), 34.05 (C-22), 26.07 (C-23), 45.84 (C-24), 29.36 (C-25), 19.40 (C-26), 19.53 (C-27), 23.07 (C-28), 11.31 (C-29), 173.16 (C-1′), 39.78 (C-2′), 29.96 (C-3′), 29.76 (C-4′), 29.71 (C-5′), 29.60 (C-6′), 29.67 (C-7′), 29.26 (C-8′), 130.22 (C-9′), 130.05 (C-10′), 35.58 (C-11′), 130.02 (C-12′), 129.73 (C-13′), 29.16 (C-14′), 128.07 (C-15′), 117.91 (C-16′), 22.67 (C-17′), 17.16 (C-18′); +ve FAB MS m/z (rel. int.): 691 $[M+H]^+$ ($C_{47}H_{71}O_3$) (1.3), 429 (2.2), 412 (23.5), 278 (67.6), 272 (12.5), 261 (18.2), 255 (27.1), 157 (13.3).

2.4.2 21-Hydroxystigmasteryl linolenate (2)

Elution of the column with chloroform yielded yellow crystals of 2, recrystallized from chloroform-methanol (1:1), 101.7 mg (0.0068 % yield), R_f 0.41, (petroleum ether-chloroform, 3:1), m.p: 271 - 273°C; UV λ_{max} (MeOH): 221 nm (log ε 3.2); IR λ_{max} (KBr): 3351, 2921, 2856, 1731, 1638, 1490, 1373, 1225, 1015, 722 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 5.39 (1H, dd, J=6.8, 5.2 Hz, H-6), 5.32 (1H, m, H-22), 5.29 (1H, m, H-23), 5.27 (1H, m, H-9'), 5.25 (1H, m, H-10′), 5.22 (1H, m, H-12′), 5.20 (1H, m, H-13′), 5.18 (1H, m, H-15′), 5.15 (1H, m, H-16′), 4.18 (1H, brm, $w_{1/2}$ 18.5 Hz, H-3α), 3.10 (2H, d, J=6.6 Hz, H_2-21), 2.77 (2H, m, H_2-11′), 2.69 (2H, m, H_2-14′), 2.31 (2H, t, J= 7.6 Hz, H_2-2′), 1.01 (3H, brs, Me-19), 0.86 (3H, d, J= 6.8 Hz, Me-26), 0.82 (3H, d, J= 6.7 Hz, Me-27), 0.80 (3H, d, J= 6.5 Hz, Me-18'), 0.78 (3H, t, J= 6.8 Hz, Me-29), 0.69 (3H, brs, Me-18), 2.28-1.15 (39H, m, 16 x CH_2, 7 x CH); ^{13}C NMR ($CDCl_3$): δ 37.02 (C-1), 31.55 (C-2), 73.51 (C-3), 412.86 (C-4), 139.72 (C-5), 122.58 (C-6), 31.88 (C-7), 32.50 (C-8), 51.42 (C-9), 38.18 (C-10), 21.04 (C-11), 39.74 (C-12), 37.66 (C-13), 56.70 (C-14), 24.48 (C-15), 30.21 (C-16), 55.05 (C-17), 11.98 (C-18), 19.62 (C-19), 36.16 (C-20), 66.13 (C-21), 130.08 (C-22), 129.99 (C-23), 45.85 (C-24), 27.86 (C-25), 19.33 (C-26), 18.76 (C-27), 23.08 (C-28), 11.86 (C-29), 173.26 (C-1′), 41.18 (C-2′), 34.32 (C-3′), 29.95 (C-4′), 29.83 (C-5′), 27.81 (C-6′), 25.06 (C-7′), 31.44 (C-8′), 130.21 (C-9′), 129.76 (C-10′), 37.44 (C-11′), 128.04 (C-12′), 127.72 (C-13′), 34.41 (C-14′), 125.24 (C-15′), 118.19 (C-16′), 22.63 (C-17′), 14.26 (C-18′); +ve FAB MS m/z (rel. int.): 689 $[M+H]^+$ ($C_{47}H_{69}O_3$) (1.1), 427 (2.5), 411 (3.2), 381 (19.8), 277 (11.1), 261 (3.5), 257 (28.1), 255 (28.7), 240 (13.1), 213 (9.8), 155 (5.3).

2.4.3 Stigmasterol 3-O-β-D-glucopyranoside (3)

Elution of the column with chloroform-methanol (19:1) afforded colourless crystals of 3, recrystallized from acetone-methanol (1:1), (106 mg, 0.00605% yield), *R_f*: 0.9 (chloroform); m.p.: 278- 279 ^{0}C; UV *λ_{max}* (MeOH): 210 nm (log ε 2.8); IR ν_{max} (KBr): 3478, 3361, 2936, 2849, 1647, 1471, 1343, 1219; 1169, 1113, 1059, 1027 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ 5.31 (1H, m, H-6), 5.24 (1H, m, H-22), 5.16 (1H, m, H-23), 5.10 (1H, J=7.2 Hz, H-1′), 4.39 (1H, m, H-5′), 4.20 (1H, m, H-2′), 3.71 (1H, m, H-3′), 3.46 (1H, brm, $w_{1/2}$=18.2 Hz, H-3α), 3.39 (1H, m, H-4′), 3.04 (2H, d, J=7.6 Hz, H_2-6′), 1.01 (3H, brs, Me-19), 0.93 (3H, d, J= 6.3 Hz Me-26), 0.87 (3H, d, J= 6.1 Hz Me-26), 0.83 (3H, d, J= 6.3 Hz, Me-27), 0.77 (3H, t, J= 6.5 Hz, Me-29), 0.67 (3H, brs, Me-18); ^{13}C NMR ($CDCl_3$): δ 37.11 (C-1), 31.48 (C-2), 72.39 (C-3), 42.76 (C-4), 139.97 (C-5), 122.31 (C-6), 31.77 (C-7), 32.58 (C-8), 51.47 (C-9), 37.89 (C-10), 21.36 (C-11), 41.05 (C-12), 37.28 (C-13), 56.73 (C-14), 24.81 (C-15), 29.86 (C-16), 55.14 (C-17), 11.81 (C-18), 19.16 (C-19), 35.66 (C-20), 23.11 (C-21), 133.19 (C-22), 129.73 (C-23), 45.41 (C-24), 27.51 (C-25), 19.31 (C-26), 19.08 (C-27), 23.26 (C-28), 11.65 (C-29),

100.54 (C-1′), 75.96 (C-2′), 73.39 (C-3′), 67.76 (C-4′), 76.87 (C-5′), 60.96 (C-6′); +ve FAB-MS *m/z* (rel. int.): 575 [M +H]+ ($C_{35}H_{59}O_6$) (1.9), 412 (82.4), 395 (34.1), 179 (12.5), 163 (19.3).

2.5 *In vitro* antioxidant activity

2.5.1 Reducing Power

Reducing power of the extract was determined by the method of Oyaizu *et al.* (1986). Briefly, different concentrations of the extract solutions (1 ml) were incubated with 2.5 ml of 0.2 M phosphate buffer (pH 6.6), and 2.5 ml of 1% $K_3Fe(CN)_6$ at 50 °C for 20 min. 2.5 ml of 10 % TCA was added to the mixture and centrifuged at 3000 rpm for 10 min. 2.5 ml of upper layer of the solution was mixed with 2.5 ml distilled water and 0.5 ml of 0.1 % $FeCl_3$, and the absorbance was detected at 700 nm.

2.5.2 Radical Scavenging Activity of Extract

2.5.2.1 Scavenging of DPPH radicals

To 1 ml of *D. pedicellata* extract solution (in methanol), 0.5 ml of 0.15 mM DPPH solution (in methanol) was added and mixed vigorously. After incubation at 20 °C for 30 minutes, the absorbance was measured at 517 nm.

2.5.2.2 Scavenging of reactive oxygen species (ROS)

H_2O_2 scavenging was assessed by following the alteration in absorbance of H_2O_2 solution (at 230 nm) upon co-incubation with *D. pedicellata* extract (Ruch *et al.*, 1989) [20]. Four millimolar H_2O_2 (0.6 ml) solution (prepared in PBS) was added to 4 ml solution of extract and incubated for 10 min. The absorbance of the solution was measured at 230 nm against a blank solution containing the extract without H_2O_2.

The ˙OH scavenging was determined by the method of Aruoma and Halliwell based on deoxyribose degradation by ˙OH (Aroma and Halliwell, 1987) [3]. The ˙OH radicals were generated by incubating the following reagents in a final volume of 1.2 ml 10 mM KH_2PO_4–KOH buffer (pH 7.4) at 37 °C for 60 min: 1.4 m M H_2O_2, $FeCl_3$ (20 M) and 2.8 mM deoxyribose, 100 µM EDTA, and ascorbic acid 100 µM in presence or absence (control) of the extract. Ascorbic acid was added in the end to start the reaction. Degradation of deoxyribose sugar induced by ˙OH radicals was determined by addition of 1 ml TBA (1% w/v) and 1 ml TCA (5.0% w/v) and heating at 100 °C for 20 min. The pink chromogen formed was determined by measuring its absorbance at 535 nm.

2.5.2.3 NO Scavenging

To determine NO scavenging, 5 mM sodium nitroprusside (SNP) was incubated with *D. pedicellata* extract at 25 ^{0}C for 120 min. 0.5 ml of incubation solution was thereafter withdrawn and mixed with 0.5 ml of Griess reagent. The absorbance was measured at 550 nm. Calculation of the amount of nitrite was based on standard curved constructed using sodium nitrite.

2.6 Stastical analysis

The levels of significance between different groups are based on Students t-test followed by analysis of variance test. The level of statistical significance was chosen $p<0.05$.

3. Results and discussion

3.1 Structure elucidation of compounds

Compound 1, named 21-hydroxy-β-sitosteryl linolenate, was obtained as pale yellow crystals from petroleum ether-chloroform (1:4) eluants. Its IR spectrum exhibited characteristic absorption bands for hydroxyl function (3360 cm^{-1}), ester group (1735 cm^{-1}), unsaturation (1640 cm^{-1}) and long aliphatic chain (725 cm^{-1}). Its molecular ion peak was determined at *m/z* 691 $[M+H]^+$ on the basis of mass and ^{13}C NMR spectra consistent with the molecular formula of a sterol ester $C_{47}H_{71}O_3$. The ion peaks arising at *m/z* 429 [CO-O fission, $C_{29}H_{49}O_2]^+$, 261 [M – 429, $CH_3(CH_2)_7(CH{=}CHCH_2)_3CO]^+$, 412 [M - $CH_3(CH_2)_7(CH{=}CHCH_2)_3COOH]^+$ and 278 $[CH_3(CH_2)_7(CH{=}CHCH_2)_3COOH]^+$ suggested that linolenic acid was esterified with the sterol. The ion fragments generating at m/z 157 [$C_{10}H_{21}O$, side chain]$^+$, 272 [429 – side chain]$^+$ and 255 [412 – side chain]$^+$ suggested the presence of the hydroxyl group linked to the side chain. The ^{1}H NMR spectrum of 1 displayed a one-proton double doublet at δ 5.36 (J=7.2, 3.6 Hz) and six one-proton multiplets from δ 5.30 to 5.19 assigned to vinylic H-6, H-9′, H-10′, H-12′, H-13′, H-15′ and H-16′ protons. A one-proton broad multiplet at δ 4.09 with half-width of 18.2 Hz was ascribed to α-oriented oxygenated methine H-3 proton. A two-proton doublet at δ 3.03 (J=6.4 Hz) was accounted to hydroxymethylene H_2-21 proton. A two-proton triplet at δ 2.29 (J=7.6 Hz) was due to methylene H_2-2′ protons adjacent to the ester function. Two three-proton broad singlets at δ 1.01 and 0.67, two three- protons doublets at δ 0.86 (J=6.7 Hz) and 0.83 (J=6.6 Hz) and two three-proton triplets at δ 0.81 (J=6.3 Hz) and 0.79 (J= 6.8 Hz) were associated with tertiary C-19 and C-18, secondary C-26 and C-27 and primary C-18′ and C-29 methyl protons, respectively. The remaining methylene and methine protons resonated from δ 2.75- 1.07. The ^{13}C NMR spectrum of 1 displayed signals for ester carbon at δ 173.16 (C-1′), vinylic carbons at δ 140.76 (C-5), 121.70 (C-6), 130.22 (C-9′), 130.05 (C-10′), 130.02 (C-12′), 129.73 (C-13′), 128.07 (C-15”) and 117.91 (C-16’), oxygenated methine carbon at δ 71.86 (C-3), and hydroxymethylene carbon at δ 62.12 (C-21). The ^{1}H-^{1}H COSY spectrum of 1 showed correlations of H-3 with H_2-1, H_2-2 and H_2-4; H-6 with H_2-7 and H-8; H_2-21 with H-20 and H-17; H-9′ with H-10′, H_2-8′ and H_2-11′; and H-13′ with H_2-11′, H-12’ and H_2-14′. The HMBC of 1 exhibited interactions of H_2-1, H_2-2 and H_2-4 with C-3; H_2-4, H-6 and H_2-7 with C-5; H-17, H_2-21 and H_2-22 with C-20; H-3 and H-$_2$-2’ with C-1’; and H-9’, H-10’, H_2-11’ and H-13’ with C-12’. The HSQC spectrum of 1 showed interactions of H-3 at δ 4.09 with C-3 at δ 71.86; H-6 at δ 5.36 with C-6 at 121.66; H_2-21 at δ 3.03 with C-21 at δ 62.12; H-9’ at δ 5.30 with C-9’ at δ 130.22; and H-15’ at δ 5.21 with C-15’ at δ 128.07. The ^{1}H and ^{13}C NMR spectral data of steroidal nucleus were compared with the reported spectral values of steroids (Jung *et al.,* 2012; Mustafa and Ali, 2001; Akhtar *et al.,* 2001) [9]. On the basis these evidences the structure of 1 has been elucidated as 21-hydroxy- β-sitosteryl *n*-octadec-9′,12′,15’-trienoate. This is a new steroidal ester.

Compound 2, named 21-hydroxy-β-stigmasteryl linolenate, was procured as yellow crystals from chloroform and had IR distinctive absorption bands for hydroxyl function

(3351 cm^{-1}), ester group (1731 cm^{-1}), unsaturation (1638 cm^{-1}) and long aliphatic chain (722 cm^{-1}). Its molecular ion peak was established at *m/z* 689 $[M+H]^+$ on the basis of mass and ^{13}C NMR spectra corresponding to the molecular formula of a sterol ester $C_{47}H_{69}O_3$. The ion peaks arising at *m/z* 427 [CO-O fission, $C_{29}H_{47}O_2]^+$, 261 [M – 429, $CH_3(CH_2)_7(CH{=}CHCH_2)_3CO]^+$, 411 [M - $CH_3(CH_2)_7(CH{=}CHCH_2)_3COOH]^+$ and 277 $[CH_3(CH_2)_7(CH{=}CHCH_2)_3COO]^+$ suggested that linolenic acid was esterified with the sterol. The ion fragments generating at m/z 155 $[C_{10}H_{19}O$, side chain $]^+$, 272 [427 – side chain]$^+$ and 256 [411 – side chain]$^+$, 240 [255 – Me]$^+$, 213 [256 –ring D fission]$^+$ suggested the location of the hydroxyl group in the side chain of a β-sitosterol-type molecule. The ^1H NMR spectrum of 2 exhibited a one-proton double doublet at δ 5.39 (J=6.8, 5.2 Hz) and eight one-proton multiplets from δ 5.32 to 5.15 ascribed to vinylic H-6, H-22, H-23, H-9′, H-10′, H-12′, H-13′, H-15′ and H-16′ protons. A one-proton broad multiplet at δ 4.18 with half-width of 18.5 Hz was ascribed to α-oriented oxygenated methine H-3 proton. A two-proton doublet at δ 3.10 (J=6.6 Hz) was accounted to hydroxymethylene H_2-21 proton. A two-proton triplet at δ 2.31 (J=7.6 Hz) was due to methylene H_2-2′ protons adjacent to the ester function. Two three-proton broad singlets at δ 1.01 and 0.69, two three-protons doublets at δ 0.86 (J=6.8 Hz) and 0.82 (J=6.6 Hz) and two three-proton triplets at δ 0.80 (J=6.5 Hz) and 0.78 (J= 6.8 Hz) were associated with tertiary C-19 and C-18, secondary C-26 and C-27 and primary C-18′ and C-29 methyl protons, respectively. The remaining methylene and methine protons resonated from δ 2.77- 1.15. The ^{13}C NMR spectrum of 2 displayed signals for ester carbon at δ 173.26 (C-1′), vinylic carbons from δ 140.76 to 118.19, oxygenated methine carbon at δ 73.51 (C-3), and hydroxymethylene carbon at δ 60.13 (C-21). The ^1H-^1H COSY spectrum of 2 showed correlations of H-3 with H_2-1, H_2-2 and H_2-4; H-6 with H_2-4, H_2-7 and H-8;H-22 with H-20 and H-24; H_2-21 with H-20 and H-17; H-9′ with H-10′, H_2-8′ and H_2-11′; and H-13′ with H_2-11′, H-12’ and H_2-14′. The HMBC of 2 exhibited interactions of H_2-1, H_2-2 and H_2-4 with C-3; H_2-4, H-6 and H_2-7 with C-5; H-17, H_2-21 and H-22 with C-20; H-3 and H_2-2’ with C-1’; and H-9’, H-10’, H_2-11’ and H-13’ with C-12’. The HSQC spectrum of 2 showed interactions of H-3 at δ 4.18 with C-3 at δ 73.51; H-6 at δ 5.39 with C-6 at 122.50; H_2-21 at δ 3.10 with C-21 at δ 60.13; H-22 at δ 5.32 with C-22 at δ 130.08, H-9’ at δ 5.27 with C-9’ at δ 128.04; and H-15’ at δ 5.18 with C-15’ at δ 125.24. The ^1H and ^{13}C NMR spectral data of steroidal nucleus were compared with the reported spectral values of steroids (Jung *et al.,* 2012; Mustafa and Ali, 2001; Akhtar *et al.,* 2001) [9]. On the basis these evidences the structure of 2 has been elucidated as 21-hydroxy- β-stigmasteryl *n*-octadec-9′, 12′, 15’-trienoate. This is a new steroidal ester.

Compound 3 was a known phytoconstituent characterized as stigmasterol 3-O-β-D-glucopyranoside (Ridhay *et al.*, 2012; Chaturvedula and Prakash, 2013).

3.2. Antioxidant Activity

The ability of *D. pedicellata* compounds to reduce various free radicals was evaluated. Fig. 3 corresponds to the reducing power of *D. pedicellata* compounds (3, 2 and 1) as compared to gallic acid and ascorbic acid. The reducing power of *D. pedicellata* compounds were found to be effective and dose dependent, with the compounds 3 and 2 having highest reducing power (Fig. 3).

The capacity of *D. pedicellata* compounds to scavenge DPPH, H_2O_2, $^{\bullet}OH$ and NO were measured and the results are shown in Table 1 to 3. The *D. pedicellata* compounds effectively and dose dependently quenched DPPH radicals. (Table 1 to 3). Highest DPPH radical scavenging activity was observed by compounds 1 and 2 (IC_{50} being 44.1 and 38.5 μg/ml respectively). 100 μg/ml of 1 and 2 scavenged DPPH radicals by 84.7 and 90.9 %, respectively. Compounds 3 scavenged as much as 60.2 at the same concentration (IC_{50} values being about 79.0) (Table 1 to 3).

D. pedicellata compounds effectively and dose dependently scavenged ROS such as H_2O_2 and $^{\bullet}OH$ (Table 1 to 3). BHA, a positive standard antioxidant, scavenged 71.3% of $O_2^{\bullet-}$ at a concentration of 200 μM. Ability of *D. pedicellata* compounds to scavenge H_2O_2 was found to be dose dependent (Table 1 to 3). Compound 2 scavenged as much as 24.8% H_2O_2 at a concentration of 100 μg/ml and 1 scavenged as much as 12.1% H_2O_2 at this concentration. Compound 3 scavenged 12.5% H_2O_2 at the same concentration.

D. pedicellata compounds also scavenged dose dependently $^{\bullet}OH$ radicals generated by Fenton type reaction. At 100 μg/ml, 28.2% of $^{\bullet}OH$ radicals were quenched by compound, 2 (IC_{50} = 215 μg/ml) and 1 quenched as much as 26.7% at the same concentration (IC_{50} = 211 μg/ml). A dose-dependent response in $^{\bullet}OH$ scavenging was observed by other compound as well (IC_{50} being about 218 μg/ml for 3). (Table 1 to 3).

NO liberated by NO donor, SNP was also scavenged by *D. pedicellata* compounds. Incubation of SNP solution at 25 °C for 2 h released significant amount of NO (Table 1 to 3). *D. pedicellata* compounds dose dependently scavenged NO released (Table 1 to 3). 37.9% and 29.9% of NO were quenched by compounds 2 and 1 at 100 μg/ml, respectively (IC_{50} being about 208 and 160 μg/ml respectively). NO quenching by other compound at the same concentration showed 31.9% for compound 3 (Table 1 to 3). Further, studies on the isolation of phytochemicals from *D. pedicellata* revealed several compounds with potent antioxidant activity in the activity order GA > AA > 2 > 1 > 3. These compounds comprised mainly of phytosterols. Although steroidal compounds (1-3) were previously isolated from other plant source; however, the these compounds are reported for the first time from *D. pedicellata*. Phytochemical studies have also revealed compounds 1 and 2 as novel compounds to be reported for the first time from a plant source. Their structures have been fully elucidated by 1D and 2D NMR. The content of phytosterols was highly related to reducing power and free radical scavenging activities, suggesting that these constituents contribute to the potent antioxidant activity of the plant possibly due to their synergistic effects. Thus, intake of these compounds (1 and 2) may have a potential function in reducing the occurrence of numerous diseases including cancer. Further, these compounds can serve as an important lead for novel drug discovery. The plant needs further phytochemical investigation so as to isolate the potent bioactive compounds responsible for the potent activity of the extract.

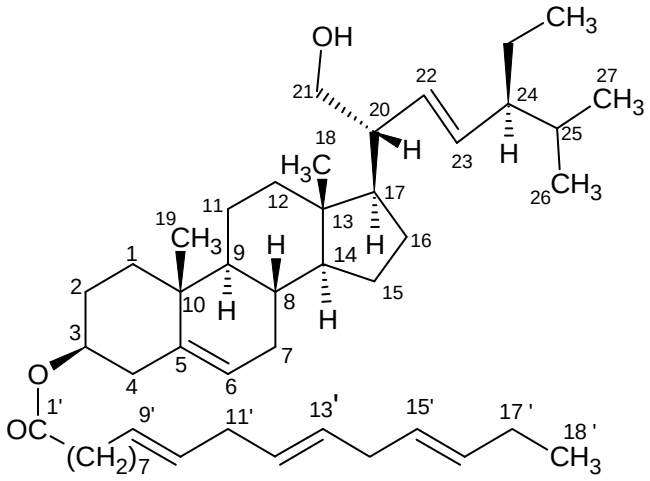

Fig 1 (a): Structure of 21-hydroxy-β-sitosteryl linolenate (1)

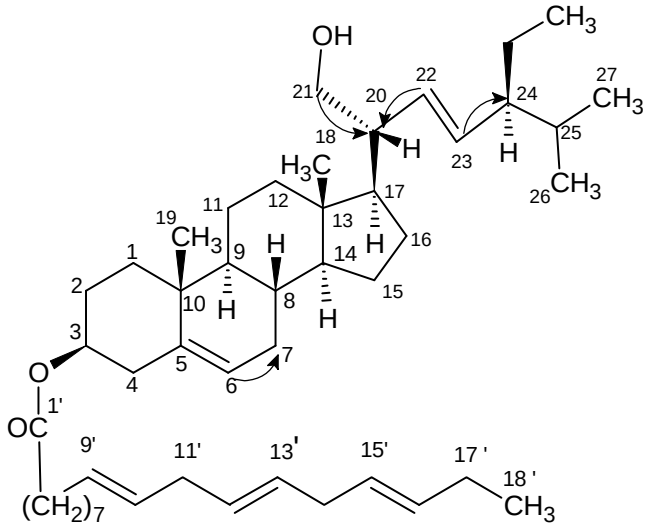

Fig 1 (b): Key COSY correlations of 21-hydroxy-β-sitosteryl linolenate (1)

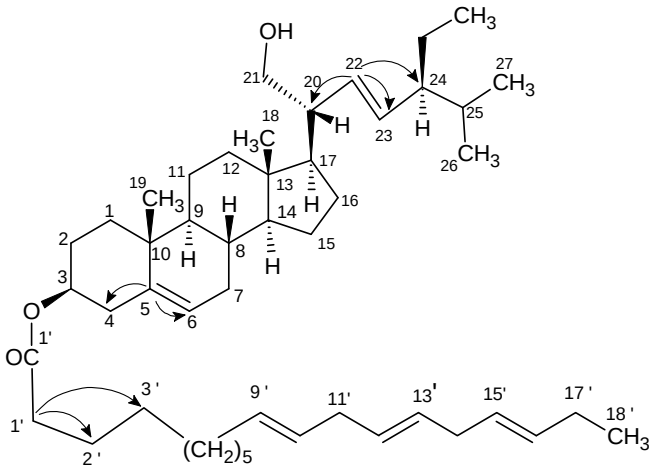

Fig 1 (c): Key HMBC correlations of 21-hydroxy-β-sitosteryl linolenate (1)

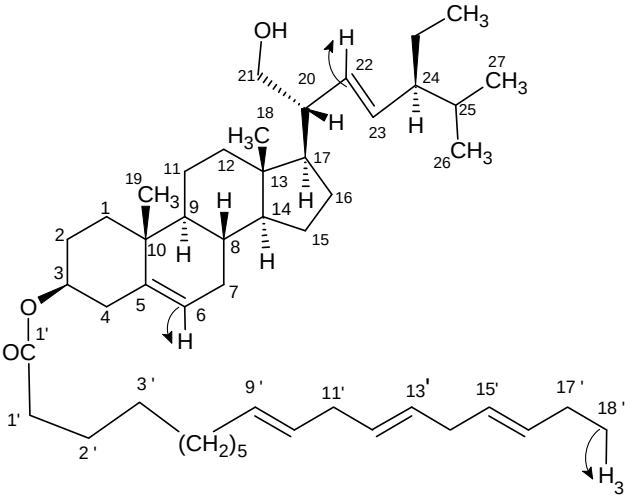

Fig 1 (d): Key HMQC correlations of 21-hydroxy-β-sitosteryl linolenate (1)

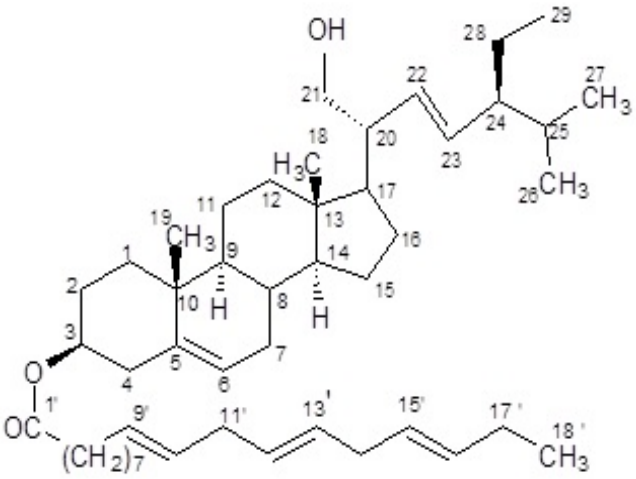

Fig 2 (a): Structure of 21-hydroxystigmasteryl linolenate (2)

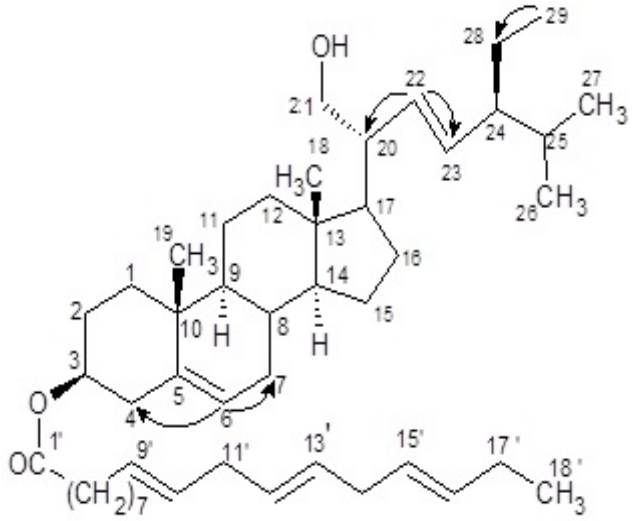

Fig 2 (b): Key COSY correlations of 21-hydroxystigmasteryl linolenate (2)

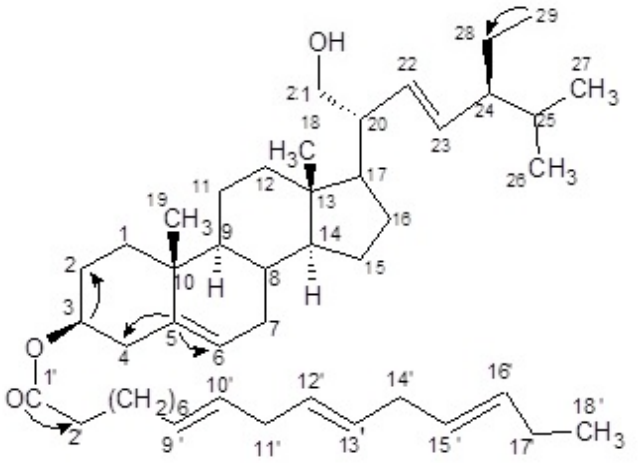

Fig 2 (c): Key HMBC correlations of 21-hydroxystigmasteryl linolenate (2)

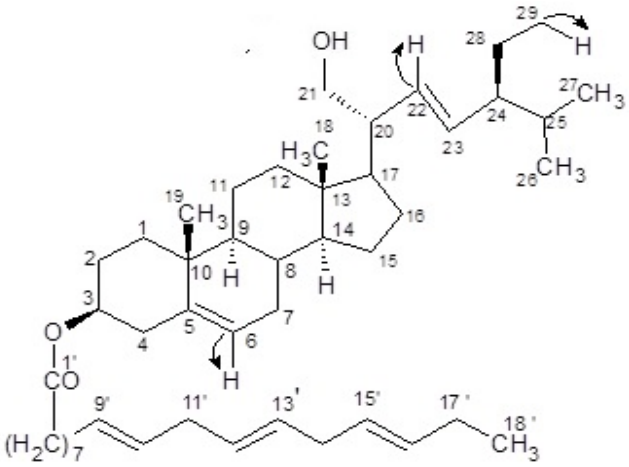

Fig 2(d): Key HMQC correlations of 21-hydroxystigmasteryl linolenate (2)

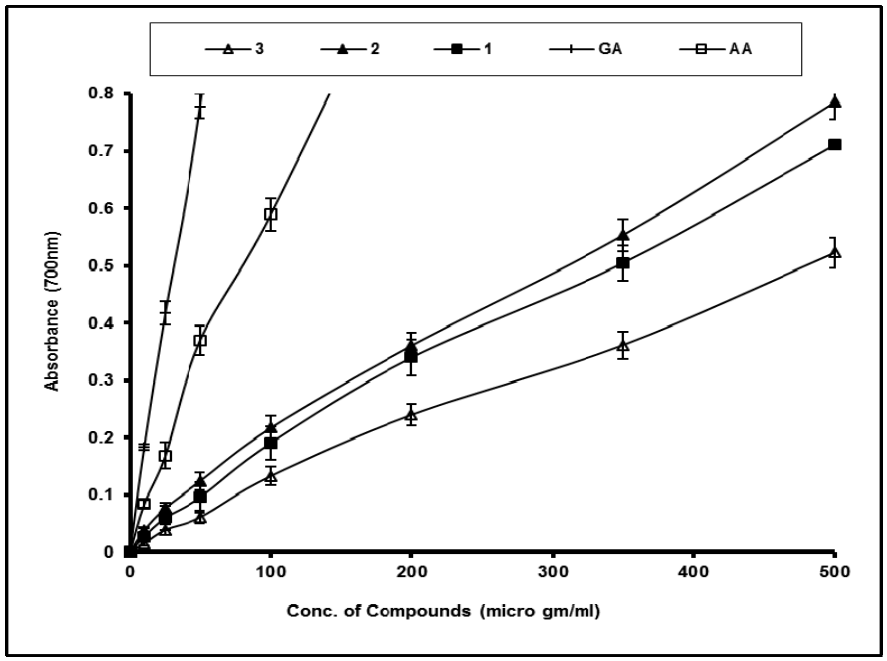

Fig 3: Reducing power of compounds of *Didymocarpus pedicellata* in comparison to that of gallic acid ascorbic acid. Reducing power was evaluated by spectrophotometric detection of Fe^{2+} to Fe^{3+} transformation. Each value is mean ± S.E. (*n=5*).

Table 1: Scavenging of Superoxide ($O_2^{\bullet-}$), Hydrogen peroxide (H_2O_2), Hydroxyl radicals ($^{\bullet}OH$) and Nitric oxide (NO) by *Didymocarpus pedicellata* compound (1). Butylated hydroxy anisole (BHA) was taken as a standard. Results are expressed as % of control.

Group	DPPH	H_2O_2 (Percent of Control)	$^{\bullet}OH$	NO
Control	100.0 ± 1.9	100.0 ± 2.8	100.0 ± 3.0	100.0 ± 3.1
Compound 1 (µg/ml)				
10	86.3 ± 3.6	98.3 ± 3.9	94.9 ± 4.8	97.4 ± 4.3
25	65.6 ± 3.0	96.7 ± 3.4	93.3 ± 3.7	93.1 ± 4.1
50	40.1 ± 2.5[b]	91.2 ± 3.1	85.7 ± 3.0	79.1 ± 3.3
100	14.2 ± 1.4[c]	87.6 ± 3.0	73.3 ± 2.8[b]	62.9 ± 3.2[b]
200	-	79.5 ± 2.8[b]	54.2 ± 2.3[b]	41.2 ± 2.3[c]
300	-	43.0 ± 2.1[b]	19.7 ± 1.1[c]	21.2 ± 1.2[c]
BHA				
200 µM	-	36.4 ± 3.2[c]	N.D.*	61.5 ± 2.5[c]

Each value is mean ± S.E. (n=5). [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ Vs control group. * Not determined

Table 2: Scavenging of Hydrogen peroxide (H_2O_2), Hydroxyl radicals ($^{\bullet}OH$) and Nitric oxide (NO) by *Didymocarpus pedicellata* compound (2). Butylated hydroxy anisole (BHA) was taken as a standard. Results are expressed as % of control.

Group	DPPH	H_2O_2 (Per cent of control)	$^{\bullet}OH$	NO
Control	100.0 ± 1.9	100.0 ± 2.8	100.0 ± 3.0	100.0 ± 3.1
Compound 2 (µg/ml)				
10	85.5 ± 3.6	97.1 ± 3.9	93.7 ± 4.2	96.3 ± 3.8

25	62.7 ± 3.4[b]	90.8 ± 3.3	91.4 ± 3.2	91.4 ± 3.1
50	36.1 ± 2.3[c]	87.9 ± 2.8	80.6 ± 3.7	83.8 ± 3.0
100	09.1 ± 0.3[c]	75.3 ± 2.3[b]	71.5 ± 3.3[b]	70.1 ± 2.8[b]
200	-	61.7 ± 2.1[b]	55.3 ± 2.1[b]	52.2 ± 2.1[b]
300	-	34.2 ± 1.4[c]	21.7 ± 1.1[c]	25.3 ± 1.2[c]
BHA				
200 μM	-	36.4 ± 3.2[c]	N.D.*	61.5 ± 2.5[c]

Each value is mean ± S.E. (n=5). [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ Vs control group. * Not determined

Table 3: Scavenging of Superoxide ($O_2^{\bullet-}$), Hydrogen peroxide (H_2O_2), Hydroxyl radicals ($^{\bullet}OH$) and Nitric oxide (NO) by *Didymocarpus pedicellata* compound (3). Butylated hydroxy anisole (BHA) was taken as a standard. Results are expressed as % of control.

Group (Per cent of control)	DPPH	H_2O_2	$^{\bullet}OH$	NO
Control	100.0 ± 1.9	100.0 ± 2.8	100.0 ± 3.0	100.0 ± 3.1
Compound 3 (μg/ml)				
10	93.1 ± 4.6	99.1 ± 4.4	95.9 ± 4.1	96.2 ± 4.0
25	81.9 ± 3.8	98.3 ± 4.2	94.1 ± 4.3	93.1 ± 3.8
50	64.5 ± 2.9[b]	95.4 ± 3.3	89.6 ± 4.1	84.5 ± 3.9
100	39.8 ± 1.3[b]	87.1 ± 2.5	77.6 ± 3.7	68.1 ± 2.9
200	14.7 ± 0.4[c]	71.4 ± 1.4[b]	56.3 ± 2.3[b]	41.1 ± 2.3[b]
300	-	38.2 ± 1.2[b]	22.1 ± 1.3[c]	23.2 ± 1.3[c]
BHA				
200 μM	-	36.4 ± 3.2[c]	N.D.*	61.5 ± 2.5[c]

Each value is mean ± S.E. (n=5). [a]$p<0.05$, [b]$p<0.01$, [c]$p<0.001$ Vs control group. * Not determined

4. Conclusions

The present work characterized steroid constituents isolated from the aerial parts of *D. pedicellata*. The results shown in the work demonstrate that the compounds derived from the extract exhibited strong reducing powers and extremely strong capacity to scavenge free radicals. Such antioxidant constituents may contribute to the effective medicinal properties of the drug.

4.1 Conflict of interest statement

There is no conflict of interest among the authors. The funding agencies had no role in the review framework, concepts, interpretation of literature and the final conclusions.

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