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# Isolation, structure elucidation and antioxidant screening of some natural products from *Colebrookia oppositifolia*.

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The phytochemical investigation on the chloroform and ethyl acetate soluble fractions of *Colebrookia oppositifolia* Smith. led to the isolation of four new-source compounds namely 1-pentacosanol (1), stigmast-7-ene-3*β*-ol (2), *p*-hydroxybenzoic acid (3), ursolic acid (4) and seven known compounds namely triacontane (5), triacontanol (6), betulonic acid (7), negletein (8), 5,2′,6′-trihydroxy-7-methoxy flavone (9), quercetin (10) and 5,7,2′-trihydroxyflavone-2′-*O*-*β*-D-glucopyranoside (11). Their structures were established by using UV, IR, mass (EIMS, HREIMS, FABMS, HRFABMS), 1D NMR ($^{1}$H, $^{13}$C) and 2D NMR (HMQC, HMBC, COSY) and by comparison with the published data of the related compounds. All the isolates (1-11) were studied for their antioxidant potential. Among these *p*-hydroxybenzoic acid (3), negletein (8), 5,2′,6′-trihydroxy-7-methoxy flavone (9), quercetin (10) and 5,7,2′-trihydroxyflavone-2′-*O*-*β*-D-glucopyranoside (11) showed good antioxidant potential when compared with the BHT (butylated hydroxyl toluene, reference standard antioxidant)/blank..

**Key words: *Colebrookia oppositifolia* Smith., Phytochemical investigation, DPPH assay, Total antioxidant activity, FRAP assay.**

*Colebrookia oppositifolia* Smith. locally known in Pakistan as “Bhinda”, “Pathan” or “Kala Behakar,” is widespread in Pakistan and India and belongs to the family Lamiaceae/Labiateae, whose flowering period is July–September. *Colebrookia oppositifolia* Smith is also distributed in semi-hilly and hilly areas of Pakistan and India up to an altitude of 49,000 m. Ethno-medicinal uses of *Colebrookia oppositifolia* include the use of their leaves as applied to wounds and ulcers as an antiseptic. Roots’ extract, which is used in epilepsy treatment, contains flavones (Khan et al., 2003). Ansari et al (1982) performed chemical investigation and screening of active compounds from roots of *Colebrookia oppositifolia*, Stem bark of *F. bengalensis*, roots of *Asparagus racemosus*, fruits of *Annona squamata*, and shoots of *Colebrookia oppositifolia* along with stem and leaves are crushed and eaten on an empty stomach as a cure for urinary problems (Paudyal, 2000). *Colebrookia oppositifolia* reportedly contains a number of flavonoids and glycoflavonoids (Yang et al., 1996). Pure acteoside of high hepatoprotection was isolated from aerial parts of *Colebrookia oppositifolia* (Qazi et al., 2006).

Antioxidants have been used as important protective agents for human health. The crude extracts of various parts of plants contain antioxidants (Patnibul et al., 2008). Currently there is much interest in the protection of low density lipoprotein and important cells and organs, as well as food systems, against oxidative damage caused by superoxide, hydroxyl and peroxyl radicals. Obviously, there has been an increasing demand to evaluate the antioxidant properties of direct plant extracts or isolated products from plant origin rather than looking for synthetic ones. It is an established fact that polyphenolic compounds, such as flavonoids, anthraquinones, anthocyanidins and xanthones, possess remarkable antioxidant activities which are present quite commonly in the plant family (Siddhuraju et al., 2002). Numerous studies have shown that aromatic

and medicinal plants are sources of diverse nutrient and non nutrient molecules, many of which display antioxidant and antimicrobial properties which can protect the human body against both cellular oxidation reactions and pathogens. Thus it is important to characterize different types of medicinal plants for their antioxidant potential (Sengul et al., 2009). Antioxidants are used to preserve foods by retarding discoloration, rancidity, or deterioration due to auto-oxidation. However, synthetic antioxidants have been reported to be carcinogenic. Hence, several attempts to replace synthetic antioxidants with natural anti-oxidants have been developed. Antioxidative substances obtained from natural sources, such as oilseed, grains, beans, vegetables, fruits, leaf waxes, bark, roots, spices, hulls and seaweeds, have been investigated (Yen et al., 2000).

## MATERIALS AND METHODS

### General:

All chemicals used in the experiments were of analytical grade and were used without further purification otherwise specified. The solvents used were purified with distillation before use. For column chromatography (CC), silica gel (70-230 mesh) was used. TLC was performed on pre-coated silica gel G-25-$UV_{254}$ plates. Detection was carried out at 254 nm, and by ceric sulphate reagent. Purity was checked on TLC with different solvent systems using methanol, acetone, and $CHCl_3$ giving single spot. $^1$H-NMR and $^{13}$C-NMR spectra were recorded in $CD_3OD$ run on Bruker spectrometers operating at 400 MHz. The chemical shifts are given in $\delta$ in ppm and coupling constants in Hz. DPPH$^{\cdot}$(1,1-Diphenyl-2-picrylhydrazyl radical), TPTZ (2,4,6-Tripyridyl-s-triazine), trolox and BHT (Butylated hydroxytoluene) were obtained from Sigma Chemical Company Ltd. (USA) and organic solvents (*n*-hexane, chloroform, ethyl acetate, *n*-butanol), sulphuric acid, sodium phosphate, ammonium molybdate and ferric chloride from Merck (Pvt.) Ltd. (Germany).

### Plant Material:

The plant *Colebrookia oppositifolia* Smith. was collected from the hills of Kotli, which is a district of Azad Kashmir (Pakistan), in 2009, in the month of June. Mr. Muhammad Ajaib identified the plant who is taxonomist of botany department of the GC University, Lahore. Voucher number of the specimen, GC. Herb. Bot. 622 has been submitted in the herbarium of GC University.

### Extraction, Fractionation and Isolation:

The whole plant material (17 kg) was dried under the shade and then ground to fine powder. The dried powder was soaked in methanol at room temperature and its methanolic extract was obtained (20L × 4). Evaporation of the methanolic extract was done under vacuum on rotavapour to yield the residue (1026 g). This residue was then dissolved in the distilled water (2 L), which was subjected to solvent extraction and partitioned first of all with *n*-hexane (1 L × 4), then with the chloroform (1 L × 4), after this with the ethyl acetate (1 L × 4) and at the end with *n*-butanol (1 L × 4). These four organic fractions, as well as the remaining aqueous fraction were subjected to rotavapour and concentrated separately under vacuum. The yields of *n*-hexane soluble fraction, chloroform soluble fraction, ethyl acetate soluble fraction, *n*-butanol soluble fraction and remaining aqueous fraction were 201g, 148g 124g, 285g and 268g respectively.

The chloroform soluble fraction (148 g) was subjected to the column chromatography (CC) over the silica gel column (70-230 mesh) using solvent system of *n*-hexane with the gradient of $CHCl_3$ (from *n*-hexane:$CHCl_3$, 98:2, 95:5, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30: 70, 20:80, 10:90, 0:100) (5000 ml for each gradient) and followed by acetone up to 100 %. Eight fractions (fraction 1-8) were collected, having the elution volumes as 3500 ml, 4400 ml, 3450 ml, 3650 ml, 3550 ml, 4100 ml, 2700 ml and 3400 ml respectively. Fraction 2 (4400 ml) was subjected to CC and eluted with *n*-hexane:$CHCl_3$ (75:25) (1000ml) to get purified **5** (16 mg). Fraction 4 (3650 ml) was loaded on silica gel and eluted with *n*-hexane:$CHCl_3$ (60:40) with elution volume 950 ml to afford **1** (25 mg). The fraction 5 (3550 ml) showed was loaded on CC and eluted with *n*-hexane:$CHCl_3$ (50:50) (4000 ml) isolate purified **6** (23.5 mg). Fraction 6 (4100 ml) was loaded on CC and eluted with *n*-hexane:$CHCl_3$ (40:60) with elution volume 3000 and as a result purified **2** (22.5 mg) was obtained.

The ethyl acetate soluble fraction (124 g) was loaded on column chromatography over silica gel (70-230 mesh) using *n*-hexane with gradient of $CHCl_3$ (from 50:50, 30: 70, 20:80,

10:90, 0:100) (5000 ml for each gradient) and followed by acetone and then methanol up to 100 %. Twelve fractions (fraction 1-12) were collected, having elution volumes as 3200 ml, 4600 ml, 4250 ml, 3900 ml, 4150 ml, 3700 ml, 3500 ml, 3600 ml, 4000 ml, 4100 ml, 3550 ml, and 4350 ml respectively. CC was performed for fraction 2 (4600 ml) of first column, eluted at*n*-hexane:Acetone (60:40) to isolate **7** (25.5mg). Fraction 3 from the first column (4250 ml) isolated **3** (26 mg) when it was subjected to CC and eluted with *n*-$CHCl_3$:Acetone (90:10) (1100 ml). Fraction 5 (4150 ml) was loaded on the silica gel and eluted with $CHCl_3$:acetone (80:20) (950 ml) to obtain purified **8** (20.5 mg). Fraction 6 (3700 ml) was subjected to CC and eluted with acetone:$CHCl_3$ (30:70) having the elution volume 900 ml and as a result purified **4** (24.5 mg) was obtained. Fraction 8 (3600 ml) was loaded on the silica gel and eluted with $CHCl_3$:acetone (60:40) (1000 ml) to isolate **9** (24 mg). Compound **10** (22 mg) was isolated in purified form from the fraction 9 (4000 ml) when it was subjected to CC and eluted with $CHCl_3$:acetone (40:60) having elution volume 1100 ml. CC was performed on fraction 10 (4100 ml), eluted with $CHCl_3$:acetone:MeOH (20:79:1) having 1000 ml elution volume to isolate **11** (22 mg).

**1-Pentacosanol (1)**

Colourless powder (25 mg); IR $\nu_{max}$ $cm^{-1}$ (KBr): 3532 (OH), 2824 (C-H str.), 1041 (C-O primary alcohol), 812 (C-C str.); $^1$H-NMR (500MHz) ($CDCl_3$) *δ*: 3.71 (2H, t, *J*=6.5 Hz, H-1), 1.56 (2H, br.s, H-2), 1.25 merged (H-3 – H-24), 0.84 (3H, t, *J*=6.4 Hz, $CH_3$-25); $^{13}$C-NMR (125MHz) ($CDCl_3$) *δ*: 67.51 (C-1), 30.87 (C-2), 29 merged (C-3 – C-23), 21.69 (C-24), 14.56 (C-25); EI-MS: *m/z* 368. The physical and spectral data completely matched with the literature values for 1-pentacosanol (Dipak et al., 2010).

**Stigmast-7-ene-3*β*-ol (2)**

Colourless amorphous powder (22.5); IR $\nu_{max}$ $cm^{-1}$ (KBr): 3574 (OH), 1641 (C=C); $^1$H-NMR (600MHz) ($CDCl_3$) *δ*: 5.35 (1H, br.s, H-7), 3.57 (1H, m, H-3), 0.93 (3H, d, *J*= 6.6Hz, $CH_3$-21), 0.89 (3H, t, *J*= 7.7 Hz, $CH_3$-29), 0.86 (3H, d, *J*= 7.2Hz, $CH_3$-26), 0.82 (3H, s, $CH_3$-19), 0.71 (3H, d, *J*= 6.7Hz, $CH_3$-27) and 0.61 (3H, s, $CH_3$-18); $^{13}$C-NMR (150MHz) ($CDCl_3$) *δ*: 122.75 (C-8), 102.47 (C-7), 71.70 (C-3), 58.16 (C-17), 51.73 (C-14), 49.42 (C-9), 47.29 (C-24), 43.12 (C-13), 41.13 (C-5), 39.73 (C-12), 37.39 (C-4), 37.39 (C-20), 37.39 (C-1), 35.11 (C-10), 34.76 (C-22), 30.71 (C-2), 29.31 (C-6), 28.91 (C-25), 28.95 (C-16), 25.29 (C-23), 24.16 (C-28), 22.16 (C-11), 22.14 (C-15), 20.54 (C-26), 19.83 (C-21), 19.32 (C-19), 18.34 (C-27), 12.33 (C-18), 12.41 (C-29); EI-MS: *m/z* 414. The physical and spectral data completely matched with the literature values for stigmast-7-ene-3*β*-ol (Lee et al., 2005).

***p*-Hydroxybenzoic Acid (3)**

White crystalline solid (26 mg); IR $\nu_{max}$ $cm^{-1}$ (KBr): 3511, 3320-2710, 1705; UV ($CHCl_3$). $\lambda_{max}$ (log *ε*) nm: 311 (3.8), 290 (4.06), 218 (3.9); $^1$H-NMR (400MHz) ($CDCl_3$) *δ*: 8.01 (1H, d, *J*=8.7 Hz, H-2&6), 6.88 (1H, d, *J* =8.7 Hz, H-3&5); $^{13}$C-NMR (100MHz) ($CDCl_3$) *δ*: 180.0 (C-7), 160.3 (C-4), 131.5 (C-2&6), 122.4 (C-1), 115.8 (d, C-3&5); HR-EI-MS: *m/z*:138.0306 (calculated for $C_7H_6O_3$, 138.0309). The physical and spectral data completely matched with the literature values for *p*-hydroxybenzoic acid (Aldrich Library, 1992).

**Ursolic Acid (4)**

Colourless needles (24.5 mg); IR (KBr) $\nu_{max}$ $cm^{-1}$: 3510, 3050, 1697, 1635, 820; $^1$H-NMR (400MHz) ($CDCl_3$) *δ*: 5.11 (1H, m, H-12), 3.19 (1H, dd, *J*= 10.0, 4.5 Hz, H-3), 1.20 (3H, s, $CH_3$-27), 1.07 (3H, s, $CH_3$-23), 0.94 (3H, s, $CH_3$-25), 0.91 (3H, d, *J* = 6.6 Hz, $CH_3$-30), 0.86 (3H, s, $CH_3$-26), 0.81 (3H, s, $CH_3$-24), 0.80 (3H, d, *J* = 6.8 Hz, $CH_3$-29); $^{13}$C-NMR (100MHz) ($CDCl_3$) *δ*: 176.2 (C-28), 138.7 (C-13), 125.8 (C-12), 79.1 (C-3), 55.2 (C-18), 52.4 (C-5), 47.9 (C-17), 47.4 (C-9), 42.0 (C-14), 39.6 (C-8), 38.5 (C-1), 37.0 (C-22), 37.1 (C-10), 33.2 (C-7), 30.5 (C-19), 30.3 (C-20), 29.4 (C-15), 27.5 (C-21), 24.5 (C-27), 27.4 (C-2), 24.0 (C-23, C-30), 23.9 (C-11), 23.5 (C-16), 22.4 (C-29), 18.3 (C-6), 17.2 (C-26), 15.9 (C-25), 14.4 (C-24); HR-EI-MS: *m/z* 456.3599 (calculated for $C_{30}H_{48}O_3$, 456.3603). The physical and spectral data completely matched with the literature values for ursolic acid (Hadaka et al., 1987).

**Triacontane (5)**

White amorphous solid (16 mg); IR $\nu_{max}$ $cm^{-1}$ (KBr): 2864 (C-H str.), 1412 ($CH_3$), 811 (C-C str.); $^1$H-NMR (400MHz) ($CDCl_3$) *δ*:1.22 (56H, s, H-2 − H-29), 0.83 (6H, t, *J* = 6.9 Hz, $CH_3$-1 & $CH_3$-30); $^{13}$C-NMR (100MHz) ($CDCl_3$) *δ*: 29 (C-3 − C-28), 23.04 (C-2 & C-29), 14.11 (C-1

& C-30); EI-MS: *m/z* 422. The physical and spectral data completely matched with the literature values for triacontane (Nikki, 1990).

**Triacontanol (6)**
Colourless powder (23.5 mg); IR $v_{max}$ $cm^{-1}$ (KBr): 3576 (OH), 2848 (C-H str.), 1054 (C-O primary alcohol), 816 (C-C str.); $^{1}$H-NMR (500MHz) ($CDCl_3$) *δ*: 3.86 (2H, br.s, H-1), 1.52 (2H, br.s, H-2), 1.20 merged (H-3 – H-29), 0.82 (3H, t, *J*=7.1 Hz, $CH_3$-30); $^{13}$C-NMR (125MHz) ($CDCl_3$) *δ*: 63.36 (C-1), 30.78 (C-2), 28 merged (C-3 – C-28), 20.51 (C-30), 14.29 (C-30); .EI-MS: *m/z* 438. The physical and spectral data completely matched with the literature values for triacontanol (Ansari et al., 1982).

**Betulonic Acid (7)**
Colourless powder (25.5 mg); IR $v_{max}$ $cm^{-1}$ (KBr): 1710 (C=O), 2976 (O-H), 1645 (C=C), 813 (C-C str.) $cm^{-1}$ $^{1}$H-NMR (600MHz) ($CDCl_3$) *δ*: 4.54, 4.75 (each 1H, br.s, $CH_2$-29), 2.89 (1H, td, *J* = 10.6, 4.2 Hz, H-19), 2.41 (2H, m, H-2), 1.62 (3H, s, $CH_3$-30), 1.08 (3H, s, $CH_3$-22), 1.01(3H, s, $CH_3$-26), 0.94 (3H, s, $CH_3$-24), 0.92 (3H, s, $CH_3$-25), 0.87 (3H, s, $CH_3$-23); $^{13}$C-NMR (150MHz) ($CDCl_3$) *δ*: 218.03 (C-3), 179.06 (C-28), 145.91 (C-27), 106.47 (C-29), 56.26 (C-17, 53.97 (C-5), 49.62 (C-9), 48.99 (C-18), 47.26 (C-4), 45.97 (C-19), 42.11 (C-14), 40.51 (C-8), 38.74 (C-13), 36.64 (C-21), 36.38 (C-10), 34.09 (C-2), 33.41 (C-7), 32.84 (C-16), 29.86 (C-20), 29.49 (C-1), 29.47 (C-15), 25.23 (C-12), 25.13 (C-22), 23.01 (C-23), 21.98 (C-11), 19.43 (C-6), 18.29 (C-30), 15.97 (C-25), 15.56 (C-24), 13.88 (C-26); EI-MS: *m/z* 454. The physical and spectral data completely matched with the literature values for betulonic acid (Lee et al., 2005).

**Negletein (8)**
Orange pellets (20.5 mg); IR $v_{max}$ $cm^{-1}$ (KBr) 3476 (OH), 1731 (C=O), 1629 (C=C); $^{1}$H-NMR (600MHz) ($CD_3OD$) *δ*: 7.91 (2H, dd, *J* = 1.7, 8.1 Hz, H-2′& H-6′), 7.35 (3H, s, H-3′, H-4′& H-5′), 6.81 (1H, s, H-3), 6.59 (1H, s, H-8), 3.85 (3H, s, $H_3$-1″); $^{13}$C-NMR (150MHz) ($CD_3OD$) *δ*: 182.41 (C-4), 163.14 (C-2), 153.98 (C-7), 148.57 (C-5), 145.66 (C-9), 132.54 (C-6), 131.63 (C-4′), 130.14 (C-1′), 129.02 (C-3′), 128.81 (C-5′), 126.28 (C-2′), 125.99 (C-6′), 105.43 (C-10), 103.68 (C-3), 91.39 (C-8); .EI-MS: *m/z* 284. The physical and spectral data completely matched with the literature values for negletin (Yang et al., 1996).

**5,2′,6′-Trihydroxy-7-Methoxy Flavone (9)**
Pale yellow needles (24 mg); IR $v_{max}$ $cm^{-1}$ (KBr): 416 (OH), 1691 (C=O), 1609 (C=C); $^{1}$H-NMR (600MHz) ($CD_3OD$) *δ*: 7.18 (1H, t, *J* = 8.1 Hz, H-4′), 6.71 (1H, d, *J* = 8.1 Hz, H-3′), 6.68 (1H, d, *J* = 8.1 Hz, H-5′), 6.59 (1H, d, *J* = 1.9 Hz, H-8), 6.38 (1H, d, *J* = 1.9 Hz, H-6), 6.23 (1H, s, H-3), 3.71 (3H, s, $H_3$-1″) $^{13}$C-NMR (150MHz) ($CD_3OD$) *δ*: 179.90 (C-4), 162.51 (C-2), 164.71 (C-7), 161.04 (C-5), 158.37 (C-5′), 157.98 (C-9), 156.52 (C-2′), 155.99 (C-6′), 134.74 (C-4′), 113.46 (C-3), 109.84 (C-1′), 105.62 (C-3′), 104.70 (C-10), 96.41 (C-6), 91.66 (C-8); .EI-MS: *m/z* 300. The physical and spectral data completely matched with the literature values for 5,2′,6′-trihydroxy-7-methoxy flavone (Jayakrishna et al., 2001).

**Quercetin (10)**
Yellow amorphous powder (22 mg); IR $v_{max}$ $cm^{-1}$ (KBr): 3541(O-H), 1742 (C=O), 1637 (C=C); $^{1}$H-NMR (400MHz) ($CD_3OD$) *δ*: 7.81 (1H, d, *J* = 2.2 Hz, H-2′), 7.67 (1H, dd, *J* = 2.2, 7.6 Hz, H-6′), 6.75 (1H, d, *J* = 8.1 Hz, H-5′), 6.34 (1H, d, *J* = 2.3 Hz, H-8), 6.19 (1H, d, *J* = 2.3 Hz, H-6); $^{13}$C-NMR (100MHz) ($CD_3OD$) *δ*: 177.43 (C-4), 166.12 (C-7), 161.51 (C-5), 154.65 (C-9), 149.27 (C-4′), 144.96 (C-2), 141.16 (C-3′), 134.33 (C-3), 123.71 (C-1′), 121.65 (C-6′), 116.27 (C-5′), 115.94 (C-2′), 101.16 (C-10), 97.53 (C-6), 92.64 (C-8); EI-MS: *m/z* 302. The physical and spectral data completely matched with the literature values for quercetin (Guvenalp and Demirezer, 2005).

**5,7,2′-Trihydroxyflavone-2′-*O*-*β*-D-Glucoside (11)**
Yellowish powder (22 mg); IR $v_{max}$ $cm^{-1}$ (KBr): 3462-3261 (OH), 1726 (C=O), 1610 (C=C); $^{1}$H-NMR (600MHz) ($CD_3OD$) *δ*: 7.79 (1H, d, *J* = 8.2 Hz, H-6′), 7.49 (1H, dd, *J* = 7.3, 8.1 Hz, H-4′), 7.31, (1H, d, *J* = 8.3 Hz, H-3′), 7.16 (1H, dd, *J* = 7.2, 8.3 Hz, H-5′), 7.01 (1H, s, H-3), 6.39 (1H, d, *J* = 2.1 Hz, H-6), 6.15 (1H, d, *J* = 2.1 Hz, H-8), 5.12 (1H, d, *J* = 7.1 Hz, H-1″), 4.53 (2H, m, H-6″), 3.72 (1H, m, H-5″), 3.39 (1H, m, H-3″), 3.28 (1H, m, H-2″), 3.15 (1H, m, H-4″); $^{13}$C-NMR (150MHz) ($CD_3OD$) *δ*: 183.04 (C-4), 165.91 (C-2), 163.28 (C-7), 158.56 (C-5), 156.45 (C-2′), 152.77 (C-9), 131.11 (C-4′), 128.92 (C-6′), 124.80 (C-1′), 121.32 (C-3), 119.37 (C-5′), 116.65 (C-3′), 103.52 (C-10), 101.33 (C-1″), 97.49 (C-6),

93.84 (C-8), 77.50 (C-5″), 74.59 (C-3″), 71.08 (C-2″), 69.22 (C-4″), 61.76 (C-6″); EI-MS: *m/z* 302. The physical and spectral data completely matched with the literature values for 5,7,2′-Trihydroxyflavone-2′-*O*-*β*-D-Glucoside (Yang et al., 1996).

**Antioxidant Assays DPPH Radical Scavenging Activity**

The DPPH radical scavenging activities of pure compounds isolated from *Rhynchosia pseudo-cajan* were examined by comparison with that of known antioxidant, butylated hydroxytoluene (BHT) using the method of Lee and Shibamoto (2001). Briefly, various amounts of the compounds (500 µg/mL, 250 µg/mL, 125 µg/mL, 60 µg/mL, 30 µg/mL, 15 µg/mL) were mixed with 3 ml of methanolic solution of DPPH (0.1mM). The mixture was shaken vigorously and allowed to stand at room temperature for one an hour. Then absorbance was measured at 517 nm against methanol as a blank in the spectrophotometer. Lower absorbance of spectrophotometer indicated higher free radical scavenging activity.

The percent of DPPH decoloration of the samples was calculated according to the formula: Antiradical activity = $A_{control}$ - $A_{sample}$/ $A_{control}$ ×100

Each sample was assayed in triplicate and mean values were calculated.

**Total Antioxidant Activity by Phosphomolybdenum Complex Method**

The total antioxidant activities of the pure compounds isolated from *Rhynchosia pseudo-cajan* were evaluated by phosphomolybdenum complex formation method (Prieto et al., 1999). Briefly, 500 µg/mL of each sample was mixed with 4 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) in sample vials. The blank solution contained 4 mL of reagent solution. The vials were capped and incubated in water bath at 95$^{o}$C for 90 minutes. After the samples had been cooled to room temperature, the absorbance of mixture was measured at 695 nm against blank. The antioxidant activity was expressed relative to that of butylated hydroxytoluene (BHT). All determinations were assayed in triplicate and mean values were calculated.

**Ferric Reducing Antioxidant Power (FRAP) Assay**

The FRAP assay was done according to Benzie and Strain (Benzie and Strain, 1996) with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $CH_3COONa.3H_2O$ and 16 mL $CH_3COOH$), pH 3.6, 10 mM TPTZ (2, 4, 6-Tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution and 2.5 mL $FeCl_3.6H_2O$ solution and then warmed at 37$^{o}$C before using. The solutions of isolated compounds and that of trolox were formed in methanol (250 µg/mL). 10 µL of each of compound solution and BHT solution were taken in separate test tubes and 2990 µL of FRAP solution was added in each to make total volume up to 3 mL. The compounds were allowed to react with FRAP solution in the dark for 30 minutes. Readings of the coloured product (ferrous tripyridyltriazine complex) were then taken at 593 nm. The FRAP values were determined as micromoles of trolox equivalents per mL of sample by computing with standard calibration curve constructed for different concentrations of trolox. Results were expressed in TE µM/mL.

**Statistical Analysis**

All the measurements were done in triplicate and statistical analysis was performed by Statistical software. All the data were expressed as ± S.E.M.

**RESULTS AND DISCUSSION**

The chloroform and ethyl acetate soluble fractions of *Colebrookia oppositifolia* yielded eleven compounds, 1-pentacosanol (1) (Dipak et al., 2010), stigmast-7-ene-3*β*-ol (2) (Lee et al., 2005), triacontane (5) (Nikki, 1990), triacontanol (6) (Ansari et al., 1982) from the chloroform soluble fraction while *p*-hydroxybenzoic acid (3) (Aldrich Library, 1992), ursolic acid (4) (Hadaka et al., 1987), betulonic acid (7) (Lee et al., 2005), negletein (8) (Yang et al., 1996), 5,2′,6′-trihydroxy-7-methoxy flavone (9) (Jayakrishna et al., 2001), quercetin (10) (Guvenalp and Demirezer, 2005) and 5,7,2′-trihydroxyflavone-2′-*O*-*β*-D-glucopyranoside(11) (Yang et al., 1996) were purified from ethyl acetate soluble fraction. Among these isolated compounds, four were new-source compounds namely

**Table 1: DPPH radical scavenging activity, total antioxidant activity and FRAP values of various compounds isolated from *Colebrookia oppositifolia.***

| Sr. No. | Compounds | DPPH radical scavenging activity | | Total antioxidant activity ± S.E.M[a)] | FRAP values (TE µM/mL) ± S.E.M[a)] |
|---|---|---|---|---|---|
| | | (% Inh.) 500 µg/mL ± S.E.M[a)] | $IC_{50}$ (µg/mL) ± S.E.M[a)] | | |
| 1 | 1-Pentacosanol (**1**) | 27.09±0.54 | Nil | 0.19±0.02 | 23.70±0.43 |
| 2 | Stigmast-7-ene-3*β*-ol (**2**) | 31.45±0.75 | Nil | 0.24±0.03 | 37.59±1.49 |
| 3 | *p*-Hydroxybenzoic acid (**3**) | 75.18±1.92* | 55.14±0.76* | 0.60±0.08* | 97.07±1.86* |
| 4 | Ursolic acid (**4**) | 27.12±0.54 | Nil | 0.28±0.15 | 29.98±0.72 |
| 5 | Triacontane (**5**) | 32.04±0.56 | Nil | 0.34±0.13 | 32.59±1.82 |
| 6 | 1-Triacontanol (**6**) | 26.11±0.75 | Nil | 0.23±0.04 | 33.10±0.65 |
| 7 | Betulonic acid (**7**) | 21.72±1.96 | Nil | 0.37±0.08 | 30.43±0.39 |
| 8 | Negletein (**8**) | 80.11±0.97* | 43.12±0.58* | 0.93±0.06* | 124.18±0.58* |
| 9 | 5,2′,6′-Trihydroxy-7-methoxy flavones (**9**) | 84.13±0.74* | 29.91±0.46* | 1.37±0.08* | 146.48±0.90* |
| 10 | 3,3′,4′,5,7-Pentahydroxy flavone (quercetin) (**10**) | 89.61±0.85* | 16.99±0.69* | 1.66±0.09* | 214.62±1.75* |
| 11 | 5,7,2′-Trihydroxyflavone-2′-*O*-*β*-D-glucopyranoside (**11**) | 81.05±0.83* | 41.70±0.96* | 0.95±0.04* | 131.58±0.93* |
| 12 | BHT[b)] | 91.74±0.52 | 12.58±0.60 | 0.92±0.94 | - |

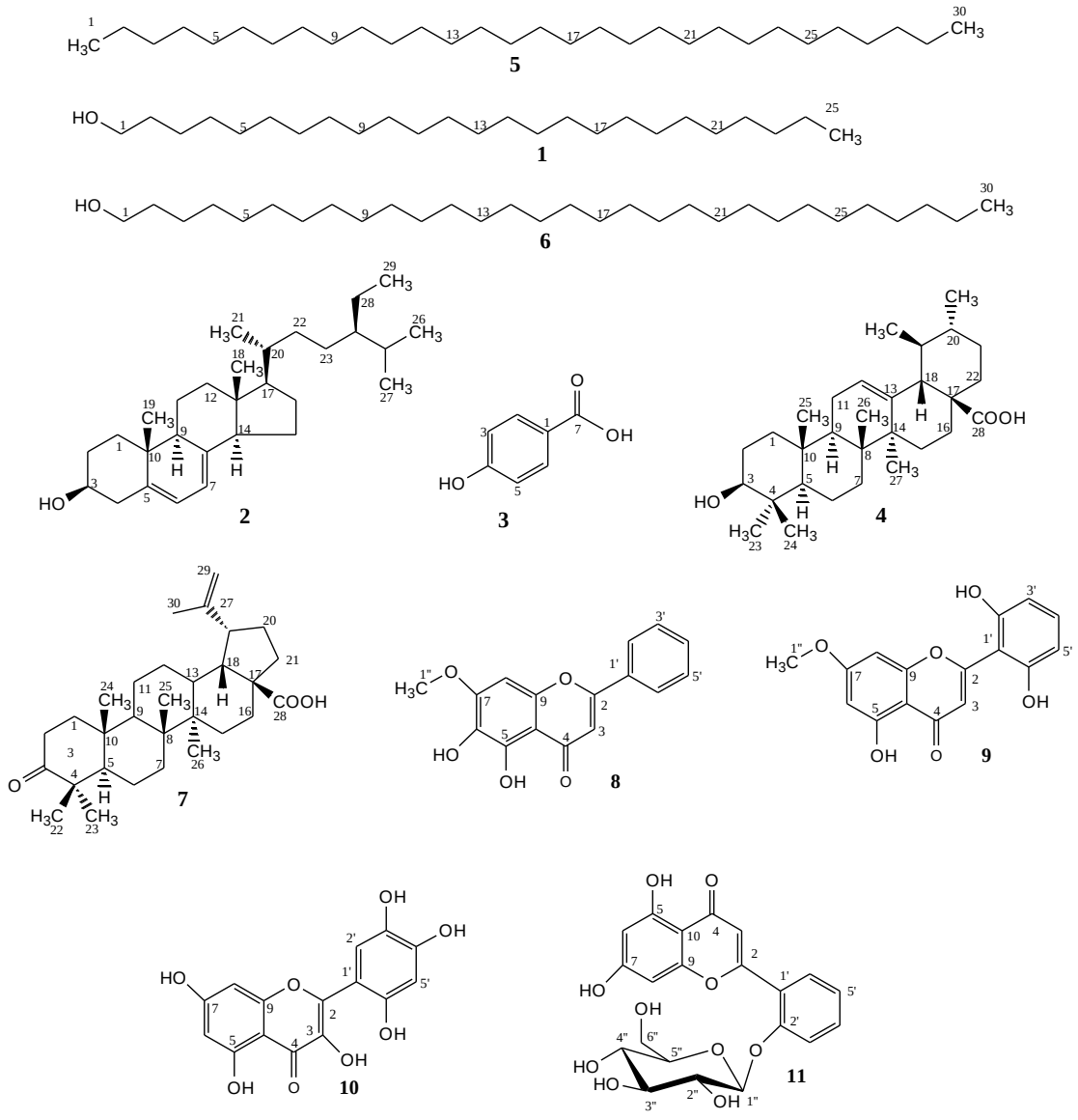
[a)] Standard mean error of three assays.
[b)]Reference standard
*p< 0.05 when comparative to blank/BHT (p<0.05 is taken as significant).

1-pentacosanol (1), stigmast-7-ene-3*β*-ol (2), *p*-hydroxybenzoic acid (3), ursolic acid (4) while all other compounds were known. Their structures (Fig. 1) were established by using UV, IR, mass (EIMS, HREIMS, FABMS, HRFABMS), 1D ($^{1}H$, $^{13}C$) and 2D NMR (HMQC, HMBC, COSY) and by comparison with the published data of the related compounds. All the isolates (1-11) were studied for their antioxidant potential by the methods of 1,1-Diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity, total antioxidant activity and ferric reducing antioxidant power (FRAP) assay and results are shown in Table-1.

Amongst the pure compounds, 4-hydroxybenzoic acid (3), negletein (8), 5,2′,6′-trihydroxy-7-methoxy flavones (9), 3,3′,4′,5,7-pentahydroxy flavone (quercetin) (10) and 5,7,2′-trihydroxyflavone-2′-*O*-*β*-D-glucopyranoside (11) showed 75.18 % 80.11%, 84.13%, 89.61 % and 81.05 % DPPH radical inhibition with $IC_{50}$ value 55.14, 43.12, 29.91, 16.99 and 41.70 respectively, relative to butylated hydroxytoluene (BHT), ($IC_{50}$ 12.58), a reference standard.

Total Antioxidant Activity of the studied fractions was determined by phosphomolybdenum method. The results indicated that 4-hydroxybenzoic acid (3), negletein (8), 5,2′,6′-trihydroxy-7-methoxy flavones (9), 3,3′,4′,5,7-pentahydroxy flavone (quercetin) (10) and 5,7,2′-trihydroxyflavone-2′-*O*-*β*-D-glucopyranoside (11) showed good

**Figure 1.** Structures of isolated compounds from *Colebrookia oppositifolia* Smith.




antioxidant values i.e. 0.60±0.08, 0.93±0.06, 1.37±0.08, 1.66±0.09 and 0.95±0.04 respectively when compared with BHT having total antioxidant activity 0.92±0.94.

FRAP values of all the isolated compounds were calculated. 4-hydroxybenzoic acid (3), negletein (8), 5,2′,6′-trihydroxy-7-methoxy flavones (9), 3,3′,4′,5,7-pentahydroxy flavone (quercetin) (10) and 5,7,2′-trihydroxyflavone-2′-*O*-*β*-D-glucopyranoside (11) showed good FRAP values i.e. 97.07±1.86, 124.18±0.58, 146.48±0.90, 214.62±1.75 and 131.58±0.93 TE µM/mL respectively.

Among these compounds quercetin exhibited highest activity because it contains five active hydroxyl groups.

The antioxidant potential of 4-hydroxybenzoic acid (21), negletein (26), 5,2′,6′-trihydroxy-7-methoxy flavones (27), 3,3′,4′,5,7-pentahydroxy flavone (quercetin) (28) and 5,7,2′-trihydroxyflavone-2′-*O*-*β*-D-

glucopyranoside (29) was found significant ($p<0.05$) when compared with the BHT/blank.

## CONCLUSIONS

The phytochemical investigation on the chloroform and ethyl acetate soluble fractions of *Colebrookia oppositifolia* led to the isolation of four new-source compounds namely 1-pentacosanol (1), stigmast-7-ene-3*β*-ol (2), *p*-hydroxybenzoic acid (3), ursolic acid (4) and seven known compounds namely triacontane (5), triacontanol (6), betulonic acid (7), negletein (8), 5,2′,6′-trihydroxy-7-methoxy flavone (9), quercetin (10) and 5,7,2′-trihydroxyflavone-2′-*O*-*β*-D-glucopyranoside (11). All the isolates (1-11) were studied for their antioxidant potential as summarized above. Among these *p*-hydroxybenzoic acid (3), negletein (8), 5,2′,6′-trihydroxy-7-methoxy flavone (9), quercetin (10) and 5,7,2′-trihydroxyflavone-2′-*O*-*β*-D-glucopyranoside (11) showed good antioxidant potential when compared with the BHT (butylated hydroxyl toluene, reference standard antioxidant)/blank. These compounds comprise phenolic groups which have ability to donate hydrogen so act as antioxidants. Quercetin showed highest antioxidant activity because it contains five phenolic groups..

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