SHORT REPORT

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Highly Oxygenated Flavonoids from the Leaves of *Nicotiana plumbaginifolia* (Solanaceae)

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Abstract: *Nicotiana plumbaginifolia* Viv. is an annual herb of the family Solanaceae, which grows abundantly in the weedy lands of Bangladesh. This plant possesses analgesic, antibacterial, anti-anxiety and hepatoprotective properties, and produces various phenolic compounds including flavonoids. The present study afforded determination of total phenolic and flavonoid contents, and for the first time, the isolation and characterization of highly oxygenated flavonoids, *e.g.*, 3,3',5,6,7,8-hexamethoxy-4',5'-methylenedioxyflavone (**1**), 3,3',4',5',5,6,7,8-octamethoxyflavone (**2**, exoticin), 6,7,4',5'-dimethylenedioxy-3,5,3'-trimethoxyflavone (**3**) and (3,3',4',5,5',8-hexamethoxy-6,7-methylenedioxyflavone (**4**) from the leaves of *N. plumbaginifolia*. All these flavonoids are rather rare natural products, and only found in a few genera, *e.g.*, *Polygonum* and *Murraya*. The structures of the isolated flavonoids were elucidated by comprehensive spectroscopic analyses, *e.g.*, UV, ^{1}H, ^{13}C NMR, DEPT, HSQC, HMBC and MS.

Keywords: Highly oxygenated flavonoids; *Nicotiana plumbaginifolia*; Solanaceae; Phenolics

1. Plant Source

In continuation of phytochemical and bioactivity studies on Bangladeshi medicinal plants [1-6], we have investigated *Nicotiana plumbaginifolia* Viv. (Solanaceae). Here, we report on the total phenolic and flavonoid contents, and for the first time, the isolation and characterization of four highly oxygenated flavonoids (**1**-**4**) from the leaves of *N. plumbaginifolia* (Figure 1).

The plant was collected from the campus of Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka, in August 2015. Different parts including leaves of *N. plumbaginifolia* were identified by Dr Bushra Khan, Principal Scientific Officer Bangladesh National Herbarium, Mirpur, Dhaka, where a voucher specimen of this collection (DACB: 41889) has been retained.

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2. Previous Studies

Alkaloids, cardiac glycosides, flavonoids, saponin, steroids, tannin and terpenoids have previously been isolated from various parts of *N. plumbaginifolia* [7-9], and analgesic, antibacterial, anti-anxiety and hepatoprotective properties of this plant have also been reported [10, 11].

3. Present Study

The shade-dried and ground leaves of *N. plumbaginifolia* (550 g) were macerated in methanol (MeOH, 2000 mL), with occasional stirring for seven days at 25±2°C. After seven days, the extract was filtered using a Buchner funnel and the solvent was evaporated under reduced pressure using a rotary evaporator at 40°C. After drying, 21.03 g (yield 3.82%) of dried extract was obtained and stored in a dry and cold place.

Determination of total phenolic content (TPC) and flavonoid content (TFC)

The total phenolic content of the MeOH extract of *N. plumbaginifolia* was determined by the Folin–Ciocalteu's reagent [12], and the total flavonoid content was estimated using the method outlined by Selim *et al.* [13]. The total phenolic and flavonoid contents were calculated as 275.45 ± 2.75 mg_{GAE} /g extract and 439.69 ± 0.87 mg_{QE} /g extract from the regression equation y = 0.0056x + 0.0455 (R^2 = 0.9987) and y = 0138x + 0.0455 (R^2 = 0.9981), respectively. The results re-established the fact that *N. plumbaginifolia* leaves contain a considerable amount of flavonoids as well as phenolics.

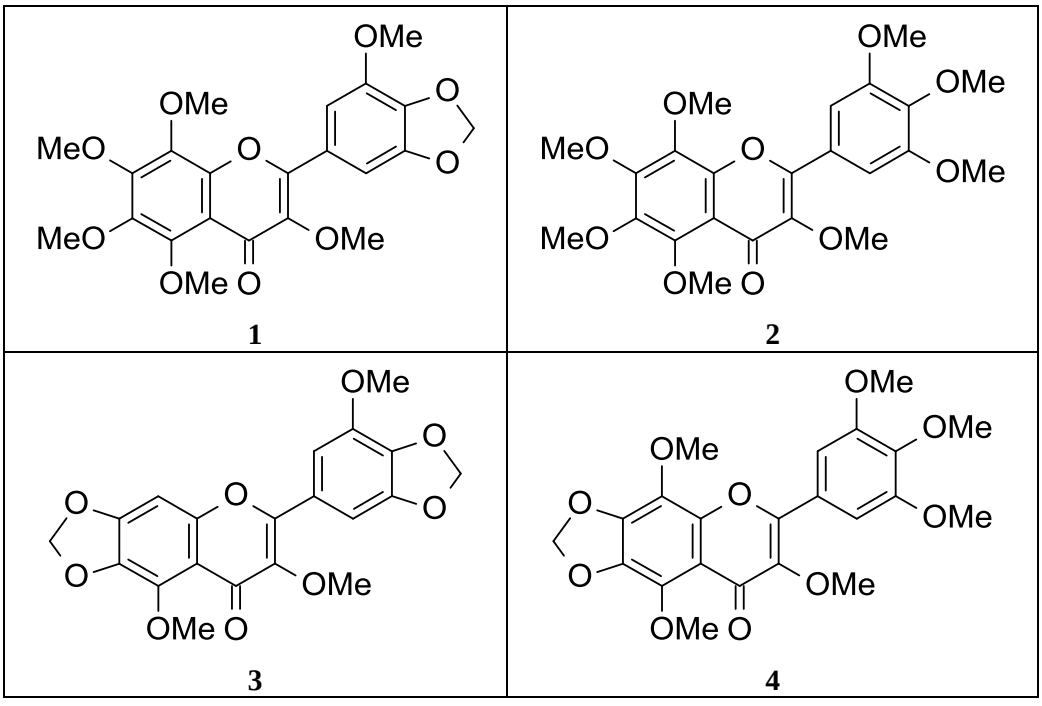

Figure 1: Structures of highly oxygenated flavonoids **1-4**

Isolation and identification of highly oxygenated flavonoids

A portion of the MeOH extract (11.25 g) was chromatographed using Kieselgel 60 (200 g) column (81 cm × 40 mm × 45 mm) packed in *n*-hexane, and eluted with petroleum ether (100 %) followed by an increasing gradient of chloroform from 5% up-to 100% as eluent. This was followed, in turn, by an increasing gradient of MeOH from 0.5% to 100%. A total of 47 fractions (100 mL each) were collected and the solvents were evaporated using a rotary evaporator. The fractions were

analyzed by TLC using the mobile phase, toluene:ethyl acetate (4.5: 0.5 to 3.5: 1.5). Based on TLC analyses, similar fractions were pooled together and 12 main fractions (F1-F12) were obtained. The fraction 12 (2% MeOH in chloroform, 4.58 g) was subjected to PTLC analysis. Fraction F 12 (25 mg) was dissolved in a small amount of chloroform-MeOH (5:1) and the PTLC (60 F_{254}, 20 × 20 cm) plates were developed in toluene:ethyl acetate (3.5: 1.5). Multiple developments were used to improve separation. The developed plates were observed under 254 and 356 nm wavelength of UV lamp and four bands were identified having R_f values of 0.17, 0.25, 0.28 and 0.41. The identified bands were scraped off the plates and eluted three times with chloroform (100%), chloroform:ethyl acetate (1:1), chloroform:methanol (9:1). The isolates were allowed to dry and further eluted by *n*-hexane to obtain pure compounds, 3,3',5,6,7,8-hexamethoxy-4',5'-methylenedioxyflavone (**1**, 2.41 mg, R_f 0.17), 3,3',4',5',5,6,7,8-octamethoxyflavone (**2**, exoticin, 3.19 mg, R_f 0.25), 6,7,4',5'-dimethylenedioxy-3,5,3'-trimethoxyflavone (**3**, 2.38 mg, R_f 0.28), and (3,3',4',5,5',8-hexamethoxy-6,7-methylenedioxyflavone (**4**, 2.56 mg, R_f 0.41) (Figure 1). The PTLC process was repeated to increase the mass of the compounds and finally, 361.51, 478.72, 357.48 and 384.35 mg of compound **1**, **2**, **3** and **4** were obtained, respectively. All these flavonoids are rather rare natural products, and only found in a few genera, *e.g.*, *Polygonum* and *Murraya*. The structures of the isolated flavonoids were elucidated by comprehensive spectroscopic analyses, *e.g.*, UV, ^{1}H, ^{13}C NMR, DEPT, HSQC, HMBC and MS, and by comparison with the respective literature data [14-15].

*3,3',5,6,7,8-Hexamethoxy-4',5'-methylenedioxyflavone (**1**):* Pale yellow needles; MP. 148-149°C; UV (MeOH): λ_{max}: 220, 252, 273 (sh) and 345 nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.54 (1H, s, H-2'), 7.42 (1H, s, H-6'), 4.01 (3H, s, 7-OMe), 4.00 (3H, s, 8-OMe), 3.99 (3H, s, 3'-OMe), 3.98 (3H, s, 5-OMe), 3.95 (3H, s, 6-OMe), 3.89 (3H, s, 3-OMe), 6.10 (2H, s, 4′,5′-O-CH_2-O-); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 173.8 (C-4), 152.7 (C-2), 151.4 (C-7), 149.1 (C-5′), 148.2 (C-5), 146.7 (C-9), 143.9 (C-6), 143.5 (C-3′), 140.9 (C-3), 137.8 (C-8), 137.5 (C-4′), 125.0 (C-1′), 114.1 (C-10), 108.9 (C-2′), 102.7 (C-6′), 102.0 (4′,5′-O-CH_2-O-), 62.3 (5-OMe), 61.9 (8-OMe), 61.8 (6-OMe), 61.6 (7-OMe), 59.9 (3-OMe), 56.6 (3′-OMe); ESI-MS *m/z* 447.98 $[M+H]^+$ and 915.22 $[2M + Na]^+$, corresponding to the molecular formula $C_{22}H_{22}O_{10}$. All data were comparable to the published data [14].

*3,3',4',5',5,6,7,8-Octamethoxyflavone (**2**, Exoticin):* Pale yellow needles; MP. 126-127°C; UV (MeOH): λ_{max}: 220, 253, 267 (sh) and 332 nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.53 (2H, s, H-2'/ H-6'), 4.11 (3H, s, 7-OMe), 4.01 (3H, 8-OMe), 3.99 (3H, s, 5-OMe), 3.96 (12H, s, 6-OMe, 3′-OMe, 4′-OMe, 5′-OMe), 3.91 (3H, s, 3-OMe); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 173.9 (C-4), 153.2 (C-2), 152.7 (C-3′ and C-5′), 151.4 (C-7), 148.2 (C-5), 146.7 (C-9), 143.9 (C-6), 140.1 (C-4′), 137.8 (C-8), 126.0 (C-1′), 115.1 (C-10), 105.9 (C-2′ and C-6′), 62.3 (5-OMe), 61.8 (6-OMe), 61.9 (8-OMe), 61.7 (7-OMe), 61.0 (4′-OMe), 60.0 (3-OMe), 56.2 (3′-OMe and 5′-OMe); ESI-MS *m/z* 463.99 $[M+H]^+$ and 947.27 $[2M + Na]^+$, corresponding to the molecular formula $C_{23}H_{26}O_{10}$. All data were comparable to the published data [14].

*6,7,4',5'-Dimethylenedioxy-3,5,3'-trimethoxyflavone (**3**):* Colourless needles; MP. 150-151°C; UV (MeOH): λ_{max}: 220, 239, 272 (sh) and 336 nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.41 (1H, d, H-2', J = 1.6 Hz), 7.29 (1H, d, H-6', J = 1.6 Hz), 6.08 (2H, s, 4′,5′-O-CH_2-O-), 6.07 (2H, s, 6,7-O-CH_2-O-), 4.15 (3H, s, 5-OMe), 3.98 (3H, s, 3′-OMe), 3.88 (3H, s, 3-OMe); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 174.1 (C-4), 153.3 (C-9), 152.7 (C-2), 149.1 (C-5′), 143.3 (C-3′), 140.9 (C-3), 139.3 (C-7), 137.1 (C-4′), 134.9 (C-6), 134.3 (C-5), 125.9 (C-1′), 114.1 (C-10), 108.9 (C-2′), 102.5 (6,7-O-CH_2-O-), 102.1 (C-6′), 102.0 (4′,5′-O-CH_2-O-), 93.0 (C-8), 61.2 (5-OMe), 59.9 (3-OMe), 56.8 (3′-OMe); ESI-MS *m/z* 823.09 $[2M + Na]^+$, corresponding to the molecular formula $C_{20}H_{16}O_9$. All data were comparable to the published data [15].

*3,3',4',5,5',8-Hexamethoxy-6,7-methylenedioxyflavone (**4**):* Pale yellow needles; MP. 167-168°C; UV (MeOH): λ_{max}: 221, 279 and 332 nm; ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 7.50 (2H, s, H-2'/ H-6'), 6.10 (2H, s, 4′,5′-O-CH_2-O-), 4.08 (3H, s, 8-OMe), 4.07 (3H, s, 5-OMe), 3.95 (9H, s, 3'-OMe, 4′-OMe, 5′-OMe), 3.91 (3H, s, 3-OMe); ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) = 174.0 (C-4), 153.1 (C-2), 152.3 (C-3′ and C-5′), 146.2 (C-9), 142.8 (C-7), 141.2 (C-3), 140.2 (C-4′), 136.3 (C-6), 136.0 (C-5), 128.1 (C-8), 126.0 (C-1′), 113.8 (C-10), 105.8 (C-2′), 105.8 (C-6′), 102.5 (6,7-O-CH_2-O-), 61.6 (5-OMe), 61.1 (8-OMe), 61.0 (4′-OMe), 59.9 (3-OMe), 56.2

(3′-OMe and 5′-OMe); ESI-MS *m/z* 447.96 $[M+H]^+$ and 915.21 $[2M + Na]^+$, corresponding to the molecular formula $C_{22}H_{22}O_{10}$. All data were comparable to the published data [14].

Whilst 3,3',5,6,7,8-hexamethoxy-4',5'-methylenedioxyflavone (**1**), exoticin (**2**) and 3,3',4',5,5',8-hexamethoxy-6,7-methylenedioxyflavone (**4**) were previously reported from *Polygonum orientale* [14], 6,7,4',5'-dimethylenedioxy-3,5,3'-trimethoxyflavone (**3**) was isolated from *Polygonum minus* before [15]. Exoticin (**2**) was also reported from *Murraya exotica* [16] and *M. paniculata* [17]. To the best of our knowledge, this is the first report on the isolation of these highly oxygenated flavonoids from the leaves of *N. plumbaginifolia*. Also, unambiguous assignment of all NMR data of these compounds is not available in the literature to date. Therefore, unequivocal assignment of all NMR data, based on comprehensive 1D and 2D NMR data analyses, has been presented here for the first time. Flavonoids, previously reported from *N. plumbaginifolia*, almost exclussively possess a quercetin skeleton, and a high degree of methyl ether formation (methoxy group, OMe), ranging from just one OMe to four OMe, can also be observed [9]. These structural features exist in the majority of falvonoids isolated from the genus *Nicotiana*, that comprises about 80 species. Thus, quercetin-type flavonol skeleton and high degree of methoxylation might be used as plausible chemotaxonomic markers in the genus *Nicotiana*, and possibly, to establish phylogenetic relationships among the genera within the family Solanaceae.

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