Journal of Medicinal Plants Studies
www.PlantsJournal.com

ISSN (E): 2320-3862
ISSN (P): 2394-0530
NAAS Rating 2017: 3.53
JMPS 2017; 5(4): 166-169

Received: 05-05-2017
Accepted: 06-06-2017

Khushbu Sharma
Department of Medicinal Chemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221 005, India

Mahendra Sahai
Department of Medicinal Chemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221 005, India

Correspondence
Mahendra Sahai
Department of Medicinal Chemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi-221 005, India

Chemical constituents of *Boerhavia diffusa* leaves

Khushbu Sharma and Mahendra Sahai

Abstract
Phytochemical investigation of ethanolic extract of *Boerhavia diffusa* leaves afforded uridine characterized as uridine triacetate (1), quercetin 3-O-α-D-rhamnoside (2), eupalitin 3-*O*-β-*D*-galactopyranoside (3), 3-O-β-D-glucopyranosyl sitosterol (4), boeravinone B(5) β-amyrin (6), β-amyrin acetate (7) and β-sitosterol (8). The compounds 1 and 2 are reported for the first time from this plant. The structures were elucidated by analysis of their spectroscopic data.

Keywords: *Boerhavia diffusa*; Nyctaginaceae; flavonoids; glycosides.

1. Introduction
Boerhavia diffusa Linn. (Nyctaginaceae) commonly known as 'Punarnava' is a significant drug of Ayurvedic system of medicine in India [1]. It occurs abundantly as a weed throughout India. In India, the plant is used as a medicine with multiple actions such as stomachic, antileprosy, diuretic, antiasthmatic, diaphoretic, anthelmintics, febrifuge, antiscabies and antiurethritis [2, 3]. The ethanolic extract of this planthas exhibited antiproliferative and immunomodulatory properties [4, 5]. The major chemical constituents C- methyl flavone characterized as 5, 7-dihydroxy 3′, 4′-dimethoxy-6, 8-dimethyl flavone [6], rotenoid analogs, boeravinone A-F have been reported from the roots of *B. Diffusa* [7-10]. Whole plant of *B. repens* reported eupalitin 3-*O*-β-D-galactopyranosyl-(1‴→2″)- β-D-glucopyranoside, eupalitin 3-*O*-β-D-galactopyranoside and 6- methoxy kaempferol 3-*O*- β-D (1→6) robinoside. The inhibitory activity of these compounds towards bone resorption induced by PTH was evaluated, and found to have significant activity [11, 12].
In the present study, we report the isolation and characterization of three compounds (Figure 1) including the uridine characterized as uridine triacetate (1), quercetin 3-O-α-D-rhamnoside (2), eupalitin 3-*O*-β-*D*-galactopyranoside (3), and 3-O-β-D-glucopyranosyl sitosterol (4) from *n*-butanol soluble fraction and boeravinone B(5), β-amyrin (6), β-amyrin acetate (7) andβ-sitosterol (8) from the chloroform soluble fraction of the ethanol extract of the leaves of *Boerhavia diffusa*. The compounds 1 and 2 are reported for the first time from this plant. The structures were elucidated by analysis of their spectroscopic data.

2. Materials and Methods
2.1 General
Optical rotations were measured on a Perkin Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and 1cm micro cell. UV spectra were obtained from a Shimadzu 1601PC spectrophotometer. IR spectra were taken using a Hitachi 270-30 spectrophotometer. All the NMR spectral data were recorded on Bruker 200 and 300 MHz NMR spectrometer. TLC was carried out using Kieselgel 60 F_{254} (0.25mm thick, Merck, Darmstadt, Germany) and the spots were visualized by spraying with 10% H_2SO_4 in methanol. Sugar analysis by TLC was done using acetone: water (19:1, which separated galactose).

2.2 Plant material
The leaves of *Boerhaavia diffusa* Linn. were collected from Varanasi, India in the month of October 2015. The authentication were made by Prof V.K. Joshi, Department of Dravya Guna, Institute of Medical Sciences, Banaras Hindu University, Varanasi- 221005, Voucher specimen (MC005/2015) is kept in the herbarium of the investigators Department.

2.3 Extraction

Powdered leaves of *Boerhaavia diffusa* (2.0 Kg) were placed in percolator with ethanol (5 L) and allowed to stand at room temperature for about 24 hours. The percolate was collected and this process of extraction was repeated for four times. The combined extract was filtered, concentrated under vacuum using rota vapour at 40 ^{0}C and weighed (300g, 15% yield).

2.4 Fractionation of ethanolic extract

Ethanolic extract (300g) was triturated with hexane (200 ml x 5), hexane soluble fraction concentrated under reduced pressure; weight of extract obtained was 85g (4.25%). Residue obtained after triturating with hexane was triturated with chloroform (200L x 5), chloroform soluble fraction was concentrated under reduced pressure; weight of extract obtained 75g (3.75% yield). Residue obtained after triturating with chloroform, suspended in distilled water (300 ml) and then extracted in a separating funnel with *n*-BuOH saturated with water (200ml x 4). *n*-BuOH soluble fraction concentrated under vacuum using rotavapor at 40 ^{0}C, weight of fraction obtained 65g, (3.25% yield). Water-soluble fraction was concentrated under vacuum using rota vapour at 40 ^{0}C, weight of fraction obtained 80g, (4% yield).

2.5 Gross column chromatography of *n*-butanol soluble fraction

n-butanol soluble fraction was taken up for the isolation of compounds. The *n*-butanol fraction 65 g was dissolved in methanol, adsorbed over silica gel (60-120 mesh, 90 g) and subjected to column chromatography over silica gel (60-120 mesh, 700 g). The column was eluted with a mixture of chloroform: methanol to afford 5 fractions.

2.6 Isolation of compound 1

The fraction eluted with chloroform : methanol (95:5) were pooled together, concentrated and kept for 3 days, a solid separated out which was subjected to further chromatography over silica gel (230-400 mesh). Elution of column with mixture ofchloroform: methanol (90:5) but was again obtained impure thus it was acetylated using acetic anhydride and pyridine and kept for 24 hours at RT. This acetylated product was then chromatographed by isocratic elution with hexane: chloroform (40:60) pure compound 1a was obtained in form of acetate (12 mg), mp: 128-130ºC. [α] $^{26}_D$: +3.2º (c, 0.25, $CHCl_3$). IR (KBr)ν_{max}: 3453, 2084, 1638, 1465, 1380, 1249, 1050, 671.cm^{-1}. UV λ_{max} nm: 222 (sh), 277, 287 (sh), 294. FAB-MS: m/z 371[M+H] $^+$, 331, 313, 259, 217, 178, 169, 154.

2.7 Isolation of compound 2

The fraction eluted with chloroform:methanol (90:10) were rechromatographed over over silica gel (230-400 mesh) using chloroform: methanol (93:7) as the initial eluent together were pooled together and dissolved in methanol and slightly warmed. The solution was kept under refrigeration for 2 days; yellow solid separated out which was recrystallized to obtain the pure compound 2 (10 mg), mp: 182-185°C. [α] $^{15}_D$: -158º (c, 0.61, MeOH). IR (KBr)ν_{max}: 3430, 1720, 1543, 1418, 1260, 1132 cm^{-1}. UV □$_{max}$ nm: MeOH 256, 265(sh), 301(sh) and 350 nm. EI-MS: m/z 449 [M+H] $^+$, 302, 147, 129 and 111.

2.7.1 Preparation of acetate derivative of 2

Compound 2 (5mg) was dissolved in pyridine (1ml), to this was added acetic anhydride (1ml); the reaction mixture was kept at room temperature for 16 hours. The acetate derivative thus obtained was recrystallized from methanol. FAB-MS: m/z 743 [M+H] $^+$.

2.8 Isolation of compound 3

The fraction eluted with chloroform: methanol (85:15) was rechromatographed over silica gel (230-400 mesh) using chloroform: methanol (95:5) as the initial eluent. The solvent polarity was increased to chloroform: methanol (85:15) and then finally eluted with chloroform: methanol (80:20). Fractions of 25 ml each were collected, reduced to 5 ml and TLC was checked for each fraction. As per TLC profile, sub fraction chloroform: methanol (90:10) was made. Fractions showing single spot on TLC were pooled together and evaporated to dryness afforded compound 3 (12 mg).

2.9 Isolation of compound 4

One of the fractions eluted with chloroform: methanol (90:10) concentrated and kept for few days, a compound 4 separated out as white solid. This was recrystallized from methanol to afford compound 4 (4.5g). Physicochemical data of compound 4, mp: 280-282^0. [α] $^{26}_D$: – 40.2º (c, 0.42, Pyridine). IR (KBr) ν_{max}: 3426, 2942, 2326, 1644, 1556, 1442, 1044 cm^{-1} FAB-MS: m/z 577 [M+H] $^+$, 414 [M–Glc+H]. EI-MS: m/z 414, 399 [M-Me] $^+$, 396 [M–H_2O] $^+$, 384, 354, 273, 255, 83, 69. ^{1}H NMR: (Pyridine-d_5, 200MHz) 5.42 (1H, distorted triplet, H-6), 1.00 (3H, *s*, H-18), 0.70 (3H, *s*, H-19), 0.94 (3H, *d*, *J*=6.44 Hz, H-26), 0.86 (3H, *d*, *J*=6.4 Hz, H-27), 0.82 (3H, *d*, *J*=7.4 Hz), H-21), 0.69 (3H, *t*, *J*=7.2 Hz, H-29), 0.90-1.35 (*m*, aliphatic protons), 3.00-5.00 (broad hump, sugar protons). ^{13}C NMR : (Pyridine-d_5, 50 MHz) 37.0 (1), 30.8 (2), 78.9 (3), 40.9 (4), 142.9 (5),121.8 (6), 32.3 (7), 32.9 (8), 50.2 (9), 36.0 (10), 21.9 (11), 39.0 (12), 43.0 (13), 56.2 (14), 25.2 (15), 29.2 (16), 56.2 (17), 12.9 (18), 20.0 (19), 37.2 (20), 19.2 (21), 34.2 (22), 26.2 (23), 45.9 (24), 29.2 (25), 19.6 (26), 20.1 (27), 23.2 (28), 12.4 (29), 102.1 (1′), 75.8 (2′), 78.4 (3′), 71.6 (4′), 78.3 (5′), 62.1 (6′).

2.10 Isolation of compounds 5-8 from chloroform soluble fraction

The $CHCl_3$ soluble fraction (74g) on repeated chromatography over silica gel using mixture $CHCl_3$: MeOH eluent, afforded boeravinone B (5, 5mg) β-amyrin(6, 25mg), β-amyrin acetate (7,4mg) andβ-sitosterol (8,7mg).

3. Result and Discussion

The compound 1 was isolated as an acetate derivative (1a). One of the *n*-butanol fractions, repeated efforts to obtain pure compounds from these mixtures was unsuccessful. Acetylation using pyridine and acetic anhydride of this fraction, followed by column chromatography led to the isolation of acetate derivative (1a). IR spectrum showed a strong absorption at 3453 cm^{-1} for =NH function and 1638 cm^{-1} for carbonyl function. The UV spectrum exhibited absorption maxima at 222 (sh), 277, 287 (sh), 294 nm in methanol. FAB-MS spectrum showed molecular ion peak [M+H] $^+$ at m/z 371 and other peaks at m/z 331, 259, 169, corresponding to molecular formula, $C_{15}H_{18}N_2O_9$.

The ^{1}H NMR spectrum of compound 1a (Table 1) showed a doublet at δ 7.34 (1H, *d*, *J*= 8.1 Hz) assigned to proton at C-6, a doublet at δ 5.73 (1H, *d*, *J*=8.1 Hz) assigned to proton at C-5, a singlet at δ 9.41 assigned to exchangeable NH proton at position 3. The anomeric proton appeared as doublet at δ 5.97 (*J*= 4.8 Hz). The other protons of ribose sugar appeared at δ 5.28 assigned to two protons at C-2', 3' and at δ 4.28 assigned

to three protons at C-4', 5'. The presence of six sugar protons indicated the absence of hexose sugar and probability of a pentose sugar. The three singlets appearing at δ 2.03, 2.06 and 2.07 indicated the three acetyl groups suggesting the parent compound of 1 to be a trihydroxy compound which again indicated presence of a pentose sugar. ^{13}C NMR spectrum of 1a (Table 1) showed five quaternary carbons at δ 150.1, 162.7 assigned to C-2, C-4 carbonyl carbons and at δ 170.1, 169.6 (two carbons) assigned to three carbonyl carbons of acetyl group. Two methines at δ 103.4 and 139.3 were assigned to C-5, C-6 respectively. Five hydroxylated methines at δ 87.5, 72.7, 70.1, 79.9 and 63.1 were assigned to C-1', 2', 3', 4', 5' respectively indicating the attachment of ribose sugar at position 1. The methyl carbons of acetyl function appeared at δ 20.8, 20.5 and 20.4. These spectral studies suggested the structure of compound 1a to be uridine triacetate. The structure was also confirmed by comparing the TLC of 1 with the triacetate derivative prepared from the authentic sample. Finally the structure was confirmed by comparing the spectral and physicochemical data of 1 with that reported in literature [13, 14]. This compound is reported for the first time from this plant.

Table 1. ^{1}H and ^{13}C data of compound **1** in $CDCl_3$

Position	δ_H (*J* in Hz)	δ_C
2	-	150.1
3 (-NH)	9.41 (*s*)	-
4	-	162.7
5	5.73 (1H, *d*, *J*=8.1)	103.4
6	7.34 (*d*, *J*= 8.1)	139.3
1'	5.97 (*d*, *J*=4.8)	87.5
2'	5.28 (*m*)	72.7
3'	5.28 (*m*)	70.1
4'	4.28 (3H, *s*)	79.9
5'	4.28 (3H, *s*)	63.1
2'-O<u>C</u>OCH$_3$	-	169.6
3'- O<u>C</u>OCH$_3$	-	169.6
5'- O<u>C</u>OCH$_3$	-	170.1
OCO<u>CH$_3$</u>	2.03, 2.06, 2.07 (3H,*s*, each)	20.4, 20.5, 20.8

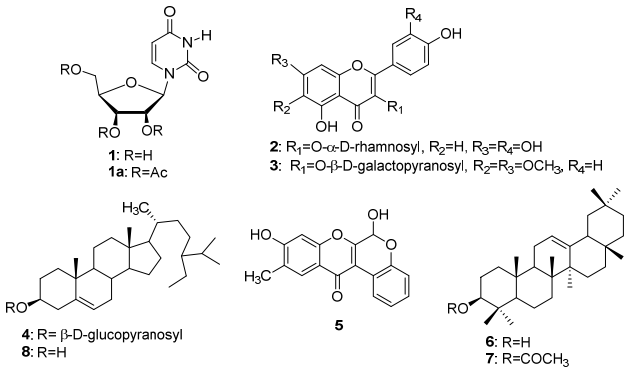

Fig 1: Structure of isolated compounds

The compound 2 was obtained as yellow crystals, mp 182-185°C. It confirmed to Shinoda and Fiegel colour tests for a flavonoid glycoside [15]. Further, it showed strong absorption bands at 3430 cm^{-1} for hydroxyl functions and at 1720 $cm^{-1}$for a carbonyl function. The UV spectra of 2 showed absorption maxima at λ_{max} (MeOH): 256, 265 (sh), 301 (sh), 350. Addition of sodium acetate resulted in bathochromic shift of band II by 16 nm showed the presence of free 7-hydroxy group in the compound. Addition of boric acid to the solution of compound in methanol and sodium acetate showed bathochromic shift of band I by 17 nm relative to methanol spectrum indicated the presence of ortho dihydroxyl groups in the compound. Similarly addition of $AlCl_3$ to the methanolic solution resulted in 29 nm bathochromic shift of band I relative to $AlCl_3$/HCl spectrum. The addition of NaOMe caused bathochromic shift of band I by 43 nm. All these observations suggested the presence of hydroxyl groups at 3', 4', 5 and 7 positions. The EI-MS exhibited molecular ion peak at $[M+H]^+$ at m/z 449 with other fragment peaks at m/z 302, 147, 129 and 111 corresponding to the molecular formula $C_{21}H_{20}O_{11}$.

The ^{1}H NMR spectrum (Table 2) of compound 2 revealed the presence of AX and ABX systems in the molecule due to the appearance of signals pattern showing two *meta* coupled doublets at δ 6.23 (1H) and 6.39 (1H) for protons at C-6 and C-8 position respectively. Another *meta* coupled doublet of one proton at δ 6.89 was assigned to protonate C-2'. The multiplet of two protons at δ 7.65 was attributed to protons at C-5', *6*' indicating substitution at position C-3', 4'. The chelated hydroxyl group at C-5 was identified due to the presence of a singlet at δ 12.65 and a broad hump at δ 9.51 was attributed to hydroxyl group at position C-3. The absence of singlet around δ 6.30 indicated that the C-3 position is substituted. The sugar was identified as rhamnose since the anomeric proton and methyl group of rhamnose were easily recognized; first at δ 5.11 (1H, *d, J*=1.5 Hz) and latter as a doublet at δ 0.90 (3H, *d*, *J*= 6.0 Hz). On acid hydrolysis compound 2 afforded a sugar, identified as rhamnose by co-TLC with authentic samples. Signal H-2" of rhamnose appeared at δ 3.93 (1H, *dd*, J = 1.8, 3.3 Hz), whereas other sugar proton signals appeared in the δ 3.30 - 3.69 region as multiplets. The coupling constants of anomeric proton indicated α-configuration of rhamnopyranosyl moiety [16].

Because hydroxyl groups interfere with the signal pattern, hence its acetate was prepared to obtain the clear spectra. It formed hepta-acetate derivative with $[M+H]^+$ at m/z 743. A downfield shift of each signal was observed in ^{1}HNMR of **2** (experimental section). A doublet of doublet at δ 7.83 (1H, *dd*, *J*= 8.4, 2.2 Hz) was assigned to H-6', another *ortho* coupled doublet at δ 7.40 was due to H-5' proton (1H, *d*, *J*= 8.4 Hz). The protons H-6 and H-8 appeared at δ 7.29 (1H, *d*, *J*= 2.2 Hz) and δ 6.84 (1H, *d*, *J*= 2.2 Hz) as *meta* coupled doublet peaks. Another *meta* coupled doublet at δ 7.73 corresponded to the H-2'. Total of seven hydroxyl groups were identified as seven acetoxyl protons appeared; four corresponding to aglycone aromatic hydroxyls at δ 2.43, 2.34, 2.33 and 2.31 and three to sugar hydroxy protons at δ 2.13, 1.99 and 1.98. Sugar protons appeared at δ 5.66 (H-1″, s), 5.25-4.87 (H-2″, 3″, 4″, 5″; *m*) and 0.90 (3H, *d*, *J*=6.0 Hz).

^{13}C NMR spectrum (Table 2) of 2 showed ten quaternary carbons at δ 178.1 for C-4 carbonyl group, at δ 121.2, 104.0 assigned to C-1' and C-10**,** at δ 156.7, 134.4, 161.3, 164.3, 156.4, 144.9 and 148.5 for hydroxylated carbons assigned to C-2, C-3, C-5, C-7, C-9, C-3' and C-4' respectively. The values at δ144.9 and 148.5 for hydroxylated carbons indicated presence of ortho dihydroxy system at position C-3', 4'. Five aromatic CH at δ 98.8, 93.6, 115.3, 116.2 and 121.7 were assigned to C-6, C-8, C-2', C-5' and C-6'. The rhamnosyl carbons appeared at 102.2 (C-1″), 71.7 (C-4″), 70.8 (C-2″, 3″), 70.4 (C-5″) and 17.8 (C-6″).The glycosylation at position C-3 was established by UV spectroscopy since the compound 2 showed bathochromic shift with sodium acetate indicating free C-7 hydroxyl group [17]. Finally the structure of 2 was confirmed as quercetin 3-O-α-D-rhamnoside commonly known as quercitrin by comparing the spectral and physicochemical data with that reported in literature [18]. This

compound is reported for the first time from this plant.

Table 2: ^{1}H and ^{13}C data of compound **2** in DMSO-d_6

Position	δH(*J* in Hz)	δC
2	-	156.7
3	-	134.4
4	-	178.1
5	-	161.3
6	6.23 (1H, *d*, *J*=1.8)	98.8
7	-	164.3
8	6.39 (1H, *d*, *J*=1.8)	93.6
9	-	156.4
10	-	104.0
1'	-	121.2
2'	6.89 (1H, *d*, *J*= 1.9)	115.3
3'	-	144.9
4'	-	148.5
5'	7.65 (*m*)	116.2
6'	7.65 (*m*)	121.7
1''	5.11 (1H, *d*, *J*= 1.5)	102.2
2''	3.93 (1H, *dd*, J = 1.8, 3.3)	70.8
3''	3.30 - 3.69 (*m*)	70.8
4''	3.30 - 3.69 (*m*)	71.7
5''	3.30 - 3.69 (*m*)	70.4
6''	0.90 (3H, *d*, *J*=6.0)	17.8
5-OH	12.65 (1H, *s*)	-
3-OH	9.51 (*br* hump)	-

The structures of other known compounds from leaves of *Boerhavia diffusa* were identified by comparison of their spectroscopic data with literature values as eupalitin-3-*O*-*β*-D-galactopyranoside [12] (3), 3-O-β-D-glucopyranosyl sitosterol [19, 20] (4), boeravinone B [9], (5) *β*-amyrin [21], (6), *β*-amyrin acetate [22], (7) and*β*-sitosterol [23] (8).

4. Conclusion

In conclusion, the present study involves chemical investigation of *Boerhavia diffusa* leaves. Six compounds were isolated and characterized as uridine triacetate (1a), quercetin 3-O-α-D-rhamnoside (2), eupalitin 3-*O*-β-*D*-galactopyranoside (3), 3-O-β-D-glucopyranosyl sitosterol (4), boeravinone B (5), β-amyrin (6), β-amyrin acetate (7) and β-sitosterol (8) out of which compounds 1 and 2 are reported for the first time from this plant. The structures were elucidated by analysis of their spectroscopic data.

5. Acknowledgements

The authors are thankful to Prof V.K. Joshi, Department of Dravya Guna, Institute of Medical Sciences, Banaras Hindu University, Varanasi- 221005, India, for identification of plant. Khushbu Sharma is thankful to Banaras Hindu University, Varanasi, for providing the Research Fellowship.

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